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Chronic binge alcohol administration accentuates expression of pro-fibrotic and inflammatory genes in the skeletal muscle of simian immunodeficiency virus-infected macaques

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Abstract

Background—Chronic binge alcohol (CBA) administration exacerbates skeletal muscle (SKM) wasting at the terminal stage of simian immunodeficiency virus (SIV) infection in rhesus macaques. This is associated with a pro-inflammatory and oxidative milieu which we have previously shown to be associated with a disrupted balance between anabolic and catabolic mechanisms. In this study, we attempted to characterize the SKM gene expression signature in CBA-administered SIV-infected macaques; using the same animals from the previous study.

Methods—Administration of intragastric alcohol or sucrose to male rhesus macaques began three months prior to SIV infection and continued throughout the duration of study. Gene transcriptomes of SKM excised at necropsy (~10 mo. post-SIV) from healthy naive control (Control), sucrose-administered, SIV-infected (SUC-SIV), and CBA-administered, SIV-infected (CBA-SIV) macaques were evaluated in microarray datasets. The Protein Analysis Through Evolutionary Relationships (PANTHER) classification tool was used to filter differentially regulated genes based on their predicted function into select biological processes relevant to SKM wasting which were: inflammation, extracellular matrix (ECM) remodeling, and metabolism.

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Results—In total, 1124 genes were differentially regulated between SUC-SIV and controls, 2022 genes were differentially expressed between the CBA-SIV and controls and 836 genes were differentially expressed between CBA-SIV and SUC-SIV animals. The relevance of altered gene expression was reflected in the up-regulation of pro-inflammatory *CCL-2*, *CCL-8*, *CX3CL1*, *SELE*, *HP*, and *TNFRS10A* mRNA expression. In addition, ECM remodeling was reflected in the up-regulation of *TIMP-1*, *MMP2* and *MMP9* mRNA expression and TGF- β protein expression. In addition, hydroxyproline content and picrosirius staining reflected increased collagen deposition in the CBA-SIV muscle tissue.

Conclusions—The results of the study demonstrate SKM inflammation as an important underlying mechanism for muscle wasting. In addition, the study provides evidence of SKM fibrotic transformation as a factor in CBA-induced accentuation of SIV-associated muscle wasting.

Keywords

SIV; Alcohol; Microarray; Pro-fibrotic; Skeletal muscle

INTRODUCTION

The use of highly active antiretroviral therapy (HAART) has made Human Immunodeficiency Virus (HIV) infection a chronic disease. The population of individuals with HIV and Acquired Immunodeficiency Syndrome (AIDS) or people living with HIV/AIDS are likely to engage in alcohol and drug abuse at rates higher than, or comparable to, those of the non-infected population (Lefevre et al., 1995). Alcohol use disorders (AUD) have been shown to accelerate the progression of HIV and to contribute to comorbid pathologies associated with alcoholism and HIV infection (Bryant et al., 2010). Among the multisystemic pathophysiological mechanisms known to accelerate disease progression in HIV/AIDS, muscle wasting has been reported to adversely affect survival in people living with HIV/AIDS (Grinspoon et al., 2003).

Skeletal muscle (SKM) pathologies such as muscle wasting, weakness, and dysfunction are evident in the disease states of both alcoholism and HIV (Clary et al., 2011, Lang et al., 2005, Preedy et al., 1994). As supported by our study and other similar studies, catabolic and metabolic dysregulation are central mechanisms underlying the role of alcohol in accentuation of muscle wasting at end-stage disease in simian immunodeficiency virus (SIV)-infected animals (LeCapitaine et al., 2011, Lang et al., 2009, Molina et al., 2008). However, additional mechanisms might also contribute to loss of muscle mass resulting from chronic alcohol abuse and or HIV/AIDS.

Previous studies from our laboratory have demonstrated that chronic binge alcohol (CBA)-administered, SIV-infected (CBA-SIV) macaques had a lower body mass index and limb muscle area when compared to sucrose-administered SIV-infected (SUC-SIV) animals at end-stage SIV disease (SAIDS) (Molina et al., 2008). Furthermore, previous studies using tissues collected from the same animals used in the current study, determined that the SKM milieu of CBA-SIV animals is characterized by up-regulation of inflammatory genes and disruption of anabolic and catabolic processes (LeCapitaine et al., 2011). To further

characterize the underlying mechanism responsible for accentuated SKM wasting in CBA-SIV animals, we used high-output microarray analysis to determine differential patterns of SKM gene expression in CBA-SIV and SUC-SIV animals. Our results show significant up-regulation of inflammatory and extracellular matrix (ECM) remodeling genes in the SKM of the CBA-SIV animals. Additionally, measures reflecting ECM remodeling including hydroxyproline content and picrosirius red staining strongly suggest that CBA promotes collagen deposition in SKM of SIV-infected animals, which we predict may impair SKM regeneration and further contribute to SKM wasting.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at both the Tulane National Primate Research Center (TNPRC) in Covington, Louisiana and Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, Louisiana. In addition, all experiments complied with National Institutes of Health (NIH) guidelines for the care and use of experimental animals. The animals studied were healthy male rhesus macaques (*Macaca mulatta*) between four and six years of age, which were obtained from TNPRC breeding colonies (LeCapitaine et al., 2011, Molina et al., 2008). Animals were age- and bodyweight-matched and randomized to the isocaloric sucrose-administered-SIV-infected (SUC-SIV, 9 animals), chronic binge alcohol-administered-SIV-infected (CBA-SIV, 11 animals) and control (SIV-negative, 8 animals) groups. The animals were individually housed in a Biosafety Level 2 containment building at TNPRC.

Experimental Protocol

Animals were administered alcohol or sucrose via a gastric catheter using an infusion pump daily for three months prior to intravenous SIV inoculation, as previously described (Bagby et al., 2006). This protocol, which continued throughout the duration of the study, provided an average dose of 13–14 grams of ethanol (30% w/v water) per kilogram body weight, per week. Intragastric delivery was used to reduce experimental variability and to ensure chronic binge-like intoxicating blood alcohol concentrations of 50–60 mM. The doses of alcohol or sucrose made up 15% the animals' total caloric intake. Animals were fed a commercial primate chow (Lab Fiber Plus Primate diet DT; PMI Nutrition International, St. Louis, MO) at regularly scheduled feeding times (twice daily) and supplemented with fruits and vegetables as part of the feeding enrichment program. The macronutrient composition of the primate chow was made up of 21.3 % protein, 11.1 % fat, 9.5 % fiber, 47.9 % carbohydrate, and 5.9 % mineral. Water was provided *ad libitum* throughout the study. Body weight was obtained weekly.

After three months of alcohol or sucrose administration, animals were inoculated intravenously with 10,000 times the 50% infective dose (ID₅₀) of SIV_{mac251} at the conclusion of an alcohol or sucrose session. This timing of inoculation accommodated elevation of blood alcohol levels to simulate infection during an alcohol binge. The progression of SIV disease was monitored throughout the study period through clinical, biochemical, and immunological parameters (CD4/CD8 lymphocyte ratios) in addition to

plasma viral kinetics (SIV *gag* RNA levels) as described and reported elsewhere (Bagby et al., 2006). In the SUC-SIV group, the mean time of SIV infection was 11.9 ± 3.6 months and in the CBA-SIV group it was 10.8 ± 3.0 months (LeCapitaine et al., 2011). Skeletal muscle (gastrocnemius) samples were obtained at necropsy when animals met criteria for euthanasia based on the following: (1) loss of 25% of body weight from maximum body weight since assignment to protocol; (2) major organ failure or medical conditions unresponsive to treatment; (3) surgical complications unresponsive to immediate intervention; or (4) complete anorexia for 4 days. SKM tissue samples were dissected, snap frozen and stored at -80°C until analyses. SKM samples used for analysis in this study were obtained from animals used in a previously published study (LeCapitaine et al., 2011); in which we reported that SIV infection and CBA administration favored SKM inflammation (*IL-6* mRNA expression) and oxidative stress, decreased antioxidant capacity, disrupted anabolic signaling pathways and increased ubiquitin-proteasome activity.

Microarray

Microarray analysis was performed at the Louisiana Cancer Research Center (LCRC) Translational Genomics Core at LSUHSC in New Orleans, Louisiana. Total RNA was extracted from frozen muscle tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA quantity and quality was assessed by Nano Drop v.3.3.1 (Thermo scientific, Wilmington, DE) and by the Agilent 2100 BioAnalyzer, respectively, prior to hybridizing to Illumina HumanWG6_v3 chips (San Diego, CA), following manufacturer's instructions as described previously (Kim et al., 2012).

Transcriptomes of SKM samples obtained from control, SUC-SIV, and CBA-SIV macaques were normalized using the cubic spline algorithm assuming a similar distribution of transcripts among replicates. The fold change in gene expression of both SUC-SIV and CBA-SIV was obtained by dividing the expression level of each over that of control; the fold change of CBA-SIV/SUC-SIV was obtained by dividing the expression level of each gene between CBA-SIV with SUC-SIV. The rationale for comparing the expression level of the CBA-SIV and SUC-SIV animals to the control was to allow for the determination of the effects of CBA and SIV infection (CBA-SIV) together and the effects of SIV infection alone (SUC-SIV), respectively.

Comparisons between the gene expression levels of CBA-SIV with SUC-SIV animals reflect the impact of CBA on SIV-mediated changes in gene expression. Using PANTHER analysis, genes with a fold change ≥ 3 (at least 50% variation) were categorized into biological processes. Differentially regulated genes were then categorized into biological processes that were relevant to muscle wasting (inflammation, ECM remodeling, and metabolism) were verified by qPCR (Table 1). There were no genes selected in the SUC-SIV animals that had a fold change of ≥ 3 and had functions related to the three categories. For genes with a fold change less than one, the [negative] reciprocal was used (Holstege et al., 1998).

Real-time RT- PCR (qPCR)

Total RNA was extracted from cryopreserved SKM using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions as described above. Reverse transcription with 1µg of RNA was performed using the TaqMan reverse transcriptase kit (Applied Biosystems, Foster city, CA). The amplification primers for chemokine (C-C motif) ligand-8 (*CCL-8*), chemokine (C-X3-C motif) ligand 1 (*CX3CL1*), thrombospondin-1 (*THBS1*), lysyl oxidase (*LOX*), matrix metalloproteinase-11 (*MMP11*), tumor necrosis factor receptor superfamily 10A (*TNFRSF10A*), haptoglobin (*HP*), chemokine (C-C motif) ligand-2 (*CCL-2*), e-selectin (*SELE*), tissue inhibitor of matrix metalloproteinase-1(*TIMP-1*), protein phosphatase regulatory subunit (*PPP1R3C*), estrogen receptor-1(*ESR-1*), apelin (*APLN*), ATPase, Ca⁺⁺ transporting plasma-membrane-2 (*ATP2B2*), tao-kinase 1(*TAOK1*), collagen V(*COL5A2*), and transforming growth factor-beta 1 (*TGF-β1*) (Table 2) were purchased from Integrated DNA Technologies and used at a concentration of 600 nmol/L (IDT, Coralville, IA). In addition, custom-made collagen I (*COL1A1*), collagen III (*COL3A1*), collagen IV (*COL4A1*), *MMP 2*, and *MMP 9* primers were purchased from SA Biosciences (Valencia, CA) and used at a concentration of 600 nmol/L. All reactions were performed on a CFX96 system using SYBR green technology (Bio-Rad Laboratories, Hercules, and CA). qPCR data were analyzed using the delta-delta Ct ($\Delta\Delta C_T$) method. Target genes were compared with the house-keeping control, ribosomal protein S 13 (*RPS13*), and normalized to control values (Eisenberg and Levanon, 2003).

Muscle Protein Expression and Western Blot Analysis

SKM tissue samples were homogenized in RIPA buffer containing 50 mM Tris HCL pH 8, 150 mM NaCL, 1 % NP-40, 0.5 % sodium Deoxycholate, 0.1 % SDS and Halt protease and phosphatase inhibitor cocktail (Pierce Thermo Scientific, Rockford, IL). Samples were centrifuged at 14,000 g for 20 minutes at 4°C, and protein concentrations determined by the BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (30 ug) were separated by SDS page gel electrophoresis and transferred to Immobilon-PSQ transfer membrane (Millipore, Billerica, MA). TIMP-1 (LifeSpan Biosciences, Seattle, WA) and TGF-β1 (Abcam, Cambridge, MA) antibodies were used for Western blot analysis. Bands were visualized using Chemiluminescence Reagent Plus (PerkinElmer Life Science, Boston, MA), and quantified based on density with the Carestream molecular imaging software. β-actin was used as a loading control.

Hydroxyproline Assay

To quantify the amount of total collagen present in the SKM obtained for each animal group, hydroxyproline content was determined. 10 mg of SKM tissue was homogenized in 100 µl of distilled water and 100 µl of concentrated HCL (10N) and incubated at 120° for 24 hours. The sample was centrifuge at 10,000 rpm for 3 minutes to remove any hydrolyzed residue that may have been present in the sample. The supernatant was used for the hydroxyproline assay according to the manufacturer's instructions (Chondrex, Inc., Redmond, VA).

Picrosirius Staining and Fibrotic Index Calculation

Twenty μm representative sections of frozen gastrocnemius muscle from Control (n=3), SUC-SIV (n=6) and CBA-SIV (n=6) animals were stained with picrosirius red (Sigma-Aldrich, St. Louis, MO) to detect collagen deposition. Bright-field images were captured at a 10X magnification using Nikon Eclipse TE2000-U microscope (Nikon instruments Inc., Melville, NY) and NIS Elements Imaging Software (Version 3.22.11). The fibrotic index was calculated as percent area of collagen of the total tissue area using the NIH Image J Software (<http://rsbweb.nih.gov/ij/>). Due to low sample number of specimens available for histological analysis, a different set of animals was used to increase sample size for both the SUC-SIV and CBA-SIV animals. These animals had undergone a similar experimental protocol of CBA or SUC administration and SIV-infection. Tissues for histological analysis were excised at 11 months post-SIV infection.

Data Analysis

All data are presented as the mean $\pm$ SEM for each experimental group. Results were analyzed by One-way analysis of variance with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Statistical analyses were performed using Prism 5 software (GraphPad Software, San Diego, CA).

RESULTS

Microarray Analysis of Skeletal Muscle

A scatterplot was generated from the analysis on Genomic Studio v2001.1 (Illumina) to show the distribution of differentially regulated genes (>1.5 -fold) in each animal group. A total of 1124 genes were differentially expressed between the SUC-SIV and control animals, of which, 657 were up-regulated and 467 were down-regulated. A total of 2022 genes were differentially expressed between CBA-SIV and control animals, of which, 1094 genes were up-regulated and 928 were down-regulated. A total of 836 genes were differentially expressed between CBA-SIV and SUC-SIV animals, of which, 431 genes were up-regulated and 405 genes were down-regulated (Figure 1A).

The PANTHER classification tool was used to generate a pie-chart representation of the overall biological processes of the 836 genes differentially expressed in the CBA-SIV/SUC-SIV animals (Figure 1B). Based on a three-fold cut off and functional relevance related to muscle wasting, the differentially regulated genes for each of the comparison groups were further categorized as inflammation, ECM remodeling, and metabolism. Selecting for relevant functional categories associated with muscle wasting, the percentage of genes differentially expressed from control in the SUC-SIV group belonged to: inflammation (69 % up-regulated, 25 % down-regulated), ECM remodeling (6 % up-regulated, 5 % down-regulated) and metabolism (25% up-regulated, 67% down-regulated) categories (Figure 1C). For CBA-SIV, the percentage of genes differentially expressed from control belonged to: inflammation (52 % up-regulated, 12 % down-regulated), ECM remodeling (11 % up-regulated, 4 % down-regulated), and metabolism (37 % up-regulated, 84 % down-regulated) categories. When differentially regulated genes from CBA-SIV vs. SUC-SIV animals were contrasted, they belonged to: inflammation (50 % up-regulated, 17 % down-regulated),

ECM remodeling (20 % up-regulated, 0 % down-regulated) and metabolism (30 % up-regulated, 83 % down-regulated) (Figure 1C). Selected genes that were 3-fold differentially expressed and had functions related to the categories mentioned above were verified by qPCR (Table 1).

qPCR Verification of Differentially Regulated Genes

The pro-inflammatory genes *CCL-8*, *CX3CLI*, *CCL-2*, *SELE*, *HP* and *TNFRS10A* were differentially expressed between control, SUC-SIV and CBA-SIV animals, which correlated with the gene expression changes detected by microarray analysis (Figure 2A). The mRNA expression of *CCL-2* was significantly higher in the SKM of the SUC-SIV animals compared to that of control ($P < 0.05$) (Figure 2A). Moreover, the mRNA expression of *CCL-8*, *CX3CLI*, *CCL-2* and *SELE* was significantly increased in the CBA-SIV animals compared to those of control animals (Figure 2A). In addition, *CCL-8* mRNA expression was significantly higher in CBA-SIV animals compared to SUC-SIV animals (Figure 2A). However, only mRNA expression, of the ECM remodeling gene, *THBS1* was significantly different in the CBA-SIV compared to the SUC-SIV animals (Figure 2B). The mRNA expression of the anabolic genes, *ATP2B2*, *PP1R3C*, *TAOK1*, *APLN*, and *ESR-1* was lower in the CBA-SIV animals than in the controls. However, this difference failed to reach statistical significance (Data not shown).

mRNA Expression of Extracellular Matrix Remodeling Genes

mRNA expression of *MMP 2* was significantly increased in the SKM of SUC-SIV animals relative to the controls (Figure 3A). However, mRNA expression of *MMP 9* was significantly increased in the SKM of the CBA-SIV animals compared to controls (Figure 3B). mRNA expression of *TGF- β 1* was modestly ($p = \text{NS}$) increased in the SKM of the CBA-SIV and SUC-SIV animals as compared to the control animals (Figure 4A). However, Western blot analysis revealed a significant increase in protein expression of TGF- β 1 (Figure 4B) in the SKM of CBA-SIV compared to control animals. Despite the alterations in expression of genes involved in ECM remodeling, no significant differences in the mRNA expression of collagens I, III, IV, and V were detected (Figure 3C–F).

Collagen Deposition in the Skeletal Muscle Extracellular Matrix

To further examine the functional significance of the changes in gene expression involved in ECM remodeling, SKM tissue sections were analyzed and quantified for collagen content. Hydroxyproline content was significantly greater in SKM of the CBA-SIV compared to control animals (Figure 5A). Moreover, histological analysis showed a greater increase in collagen deposition in SKM of CBA-SIV than in SUC-SIV compared to those of controls, but this difference failed to reach statistical significance.

DISCUSSION

We examined the differential gene expression pattern in SKM of CBA-SIV and SUC-SIV animals using high throughput microarray analysis and demonstrated that the pattern of gene expression in SKM of CBA-SIV animals reflects a pro-inflammatory and a pro-fibrotic milieu, which we predict may contribute to the accentuated loss of SKM mass. Further,

significant dysregulation of expression of genes involved in ECM remodeling, greater hydroxyproline content, and increased picrosirius red staining, in combination, suggests development of a pro-fibrotic milieu in SKM of CBA-SIV animals. These data are also consistent with our previously published findings on the same cohort of animals, which demonstrated that alcohol accentuates inflammation and catabolic changes and blunts anabolic mechanisms in the SKM at the terminal stage of SIV infection (LeCapitaine et al., 2011).

Muscle wasting results from disruption in the balance between protein synthesis and protein degradation (Lecker et al., 2006). Inflammatory cytokines, such as TNF- α increase NF- κ B activation and lead to increased expression of the E3 muscle specific ubiquitin ligase, MuRF-1, and consequently protein degradation (Lecker et al., 2006). Previously we have shown increased expression of pro-inflammatory cytokines (TNF- α , IFN- β , IL-6 and IL-1 β), and atrogene mRNA expression in the SKM of SIV-infected animals, which is exacerbated with CBA administration (Molina et al., 2008, LeCapitaine et al., 2011). The present study reinforces the previous findings by providing evidence of a pro-inflammatory milieu reflected as up-regulation of genes such as *CCL-2*, *CCL-8*, *HP*, *SELE*, *CX3CL1*, and *TNFRS10A* in the SKM of CBA-SIV animals. *CCL-2* gene expression was significantly increased in both SUC-SIV and CBA-SIV, suggesting that SIV infection alone can promote a pro-inflammatory environment. However, there was a greater increase (~2-fold) in the CBA-SIV animals, which supports our previous observations that CBA administration further accentuates the pro-inflammatory milieu. *CCL-2* encodes for the monocyte chemotactic protein-1, a potent monocyte attractant and member of the CC chemokine subfamily, the expression of which is induced by pro-inflammatory mediators such as TNF- α , IL-6, and IL-1 (Strieter and Kunkel, 1993, Proost et al., 1996, Yadav et al., 2010). Likewise, *CCL-8* gene expression was significantly increased in the CBA-SIV compared to control and SUC-SIV animals. Moreover, both CBA and SIV infection increased gene expression of *CX3CL1* and E-selectin (*SELE*), both of which contribute to inflammatory cell recruitment and inflammation. Increased levels of the soluble form of E-selectin has been reported in serum of HIV-infected individuals (Sfikakis et al., 1995, Sipsas et al., 2003) and in chronic alcohol consuming patients (Sacanella et al., 1999).

Thus, the results of our present study provide additional support for the significance of a chronic inflammatory milieu as an underlying mechanism for accentuated SKM wasting in CBA-SIV macaques. It can be speculated that CBA impairs resolution of an early acute inflammatory period associated with the initial phase of SIV infection, promoting a chronic inflammatory milieu. Moreover, we hypothesize this chronic inflammatory state promotes development of a pro-fibrotic phenotype, that likely impairs SKM regeneration (Serrano et al., 2011).

Fibrosis is the aberrant deposition of ECM components leading to loss of tissue architecture and function (Serrano et al., 2011). The results from the microarray analysis support the concept of pro-fibrotic changes as indicated by the up-regulation of *THBS1*, *MMP11*, *TIMP-1* and *LOX* in CBA-SIV animals. *THBS1* codes for an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interaction and activates TGF- β 1 during injury and development (Schultz-Cherry and Murphy-Ullrich, 1993). In our study, TGF- β 1 was also

significantly up-regulated in the SKM of the CBA-SIV animals. Up-regulation of TGF- β 1 has been implicated in conditions and disease processes such as inflammation, cancer, and fibrosis (Taipale et al., 1994). In addition, TIMP-1 protein expression was also greater in the SKM of the CBA-SIV (data not shown). TIMPs are endogenous protein tissue inhibitors of matrix metalloproteinases (MMPs), proteases responsible for ECM remodeling (Murphy, 2011). TIMP-1 controls MMP activity, thereby preventing excess tissue proteolysis (Birkedal-Hansen et al., 1993). To prevent muscle fibrosis, however, some amount of MMP activity is needed to aid in ECM remodeling and muscle repair. Thus, the up-regulation of TIMP would decrease MMP expression and ultimately prevent proteolytic events needed to prevent fibrosis (Birkedal-Hansen et al., 1993). In our study, the expression of both TIMP-1 and *MMP 9* was significantly increased in the SKM of the CBA-SIV animals and the expression of *MMP 2* was increased with SIV infection alone, SUC-SIV. A potential explanation for this confounding observation is the possibility that although MMP gene expression is increased, their activity may have not been altered.

Persistent inflammation promote excess accumulation of ECM components, which lead to replacement of muscle with fibrotic tissue, impairing muscle regeneration (Serrano et al., 2011). The systemic and timely process of breakdown and replacement of the ECM, aided by MMPs, is critical for ensuring full and rapid repair, as well as for avoiding fibrosis (Mann et al., 2011). MMPs are released from both damaged muscle and infiltrating cells in order to disrupt the muscle fiber basement membrane and thus facilitate recruitment of myogenic, inflammatory, and fibroblastic cells to damaged tissue (Serrano et al., 2011). MMPs play a role in development of fibrosis, as collagen accumulation will occur when the rate of collagen synthesis by activated fibroblasts exceeds the rate of breakdown by MMPs. The presence of a pro-fibrotic milieu in our model is also supported by the results from the hydroxyproline assay and histological data suggesting increased collagen deposition in the SKM of CBA-SIV animals. In normal muscle the external lamina is composed of collagen IV, laminin, and heparin sulfate proteoglycans (HSPGS), while the interstitial matrix that surrounds it contains collagens I, III, and V, fibronectin and perlecan (Cornelison, 2008, Grounds et al., 2005, Kääriäinen et al., 2000). We did not observe significant differences in the mRNA expression for the different collagens (*COL I, III, IV, and V*). One possible explanation for this finding is that collagen synthesis may not be changed but collagen degradation and/or ECM remodeling may be impaired.

Disrupted anabolic signaling also plays a role in mechanisms leading to muscle wasting through decreased protein synthesis (LeCapitaine et al., 2011). Several of the down-regulated genes in SKM of CBA-SIV animals belong to functional categories related to anabolic signaling pathways (*PPP1R3C, APLN, ESR, SLC37A4, IRS1, ATP2B2, TAOK1, CRIM1, and RPS6KB*) (data not shown). Future studies will aim to elucidate the role of disrupted skeletal muscle anabolic signaling pathways as a mechanism contributing to muscle wasting.

Microarray analysis is a highly efficient screening tool for detecting differentially expressed genes simultaneously. In our study, although much of the qPCR and Western blot verification data followed the same trend as the microarray analysis, statistical significance was not always reached. Reported in its appropriate sensitivity range, a microarray analysis

can reliably detect the existence and direction of gene expression changes for most genes; although with other verification measures such as qPCR, there can be a difference in magnitude (Draghici et al., 2006). Nevertheless, we consider the biological relevance of our findings merits discussion and consideration in the overall definition of the mechanisms underlying SKM wasting in CBA-SIV macaques.

Taken together, the results of this study reflect up-regulation of gene expression which may promote and facilitate SKM pro-inflammatory and pro-fibrotic pathways in CBA-SIV macaques. The pro-inflammatory milieu present in the SKM of the CBA-SIV animals potentially contributes to the up-regulation of pro-fibrotic gene expression and favors collagen expression, which we predict is likely to impair satellite cell (resident SKM stem cells) function and muscle regeneration. Recent studies from our laboratory have demonstrated that myogenic gene expression is markedly altered in satellite cells isolated from CBA animals and this is associated with impaired myotube formation (Simon et al., 2014). In addition, the down-regulation of genes that code for proteins involved in anabolic signaling suggests that the overall loss in SKM is likely the result of combined enhancement of proteolysis and suppression of anabolic mechanisms. These discoveries provide further support of our previous findings and conclusions, which demonstrated that a pro-inflammatory milieu exists in the SKM of the CBA-SIV animals. Moreover, the study has broadened our understanding of the underlying mechanisms to now include a role for fibrotic changes in SKM wasting in CBA-SIV macaques.

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Figure 1. Scatterplot and Pie Chart Representation of the Differentially Expressed Genes in the Skeletal Muscle of Each Animal Group

(A) Scatterplot representation of differentially regulated genes in the skeletal muscle (SKM) of the sucrose-administered, SIV-infected (SUC- SIV) animals compared to control (657 genes Up-regulated, 467 genes down-regulated), chronic binge alcohol-administered, SIV-infected (CBA-SIV) animals compared to that of control (1094 genes up-regulated, 928 genes down-regulated), and CBA-SIV compared to SUC-SIV animals (431 genes Up-regulated, 405 genes down-regulated) (1.5-fold cut-off) (B) Pie-chart representation of the overall biological function of the 836 differentially regulated genes in the CBA-SIV animals compared to the SUC-SIV animals as generated by the Protein Analysis Through Evolutionary Relationships (PANTHER) classification tool. (C) The percentage of differentially expressed genes within functional categories relevant to muscle wasting: extracellular matrix (ECM) remodeling, inflammation and metabolism for SUC-SIV/Control, CBA-SIV/Control, and CBA-SIV/SUC-SIV (3-fold cut-off).

Figure 2. Verification of Differentially Expressed Genes by qPCR Analysis

(A) mRNA Expression of Inflammatory Genes: *CCL-2* mRNA expression was significantly increased in the skeletal muscle (SKM) of both the SUC-SIV (n=8) and CBA-SIV animals compared to those of control animals. *CCL-8* mRNA expression was significantly higher in the SKM of the CBA-SIV animals compared to control and significantly higher when compared to SUC-SIV animals. *CX3CL1* and *SELE* expression was significantly higher in CBA-SIV (n=8) animals compared to that of control (B) mRNA Expression of the ECM Remodeling Genes: *THBS1* mRNA expression was statistically significant in the CBA-SIV animals compared to the SUC-SIV animals. mRNA levels were expressed as fold change of values in control animals. mRNA expression of *TNFRSF10A*, *MMP 11*, *TIMP-1* and *LOX* were not significantly different between groups. Bars with * are significantly different (p < 0.05) from its respective control group. Bars with # are significantly different from SUC-SIV. Data are represented as Mean $\pm$ SEM.

Figure 3. mRNA Expression of ECM Remodeling Genes in Skeletal Muscle Tissue

(A) The expression of MMP 2 was significantly increased in the SUC-SIV (n=8) vs. the controls animals. (B) *MMP 9* expression was significantly increased in the SKM of the CBA-SIV (n=8) animals compared to controls. (C-F) mRNA expression of Collagen *I*, *III*, *IV* and *V* in the SKM of control, SUC-SIV, and CBA-SIV animals. mRNA levels were expressed as fold change of values in control animals (n=8). Bars with * are significantly different (p < 0.05) from its respective control group. Data are represented as Mean $\pm$ SEM.

Figure 4. mRNA and Protein Expression of TGF-β1 in Skeletal Muscle Tissue

(A) mRNA expression for *TGF-β1* in the SKM of the control, SUC-SIV and CBA-SIV animals. mRNA levels were expressed as fold change of values in control animals (n=8). (B) There was a significant increase in protein expression of the pro-fibrotic protein, TGF-β1 in the SKM of CBA-SIV animals vs. control animals. Protein expression data was represented as fold change of values in control animals (n=8). Shown are representative Western blot images and the cumulative ($\pm$ SEM) densitometry values. Expression was normalized to β-actin and expressed as fold change of control values. Bars with * are significantly different (p < 0.05) from its respective control group. Data are represented as Mean $\pm$ SEM

Figure 5. Fibrotic Index of Skeletal Muscle Tissue

(A) The hydroxyproline assay showed a significant increase in total collagen in the SKM of the CBA-SIV animals (n=5) compared to the control animals (n=4). (B) There was a visible increase in the fibrotic index of SUC-SIV (n=6) and CBA-SIV (n=6) SKM compared to controls (n=2). The fibrotic index was calculated as percent area of collagen of the total tissue area using the NIH Image J Software (<http://rsbweb.nih.gov/ij/>). (C) Representative images of SKM tissue stained for picrosirius red showing deposition of collagen (10X Magnification). Note: the presence of increased red staining in the SKM of the SUC-SIV and CBA-SIV animals compared to controls.

Table 1

Functions and Fold-change of Genes that were Differentially Expressed (3-fold) in the Chronic Binge Alcohol-administered, SIV-infected (CBA-SIV) vs. control and vs. Sucrose-administered, SIV-infected groups which are in the Inflammatory, ECM remodeling and Metabolism categories

CBA-SIV/Control	Function	Fold Change	CBA-SIV/SUC-SIV	Function	Fold Change
CCL8 (Chemokine (C-C motif) Ligand 8)	Chemokine, activates immune cells and inflammatory cells (monocytes, T cells, NK cells)	16.5	HP (Haptoglobin)	Acute phase protein	20.99
CX3CL1 (Chemokine (C-X3C) motif) Ligand 1)	Chemoattracts T cells and monocytes to site of inflammation	4.65	CCL2 (Chemokine (C-C motif Ligand-2)	Chemoattracts monocytes, memory T cells and dendritic cells to site of inflammation	10.7
THBS1 (Thrombospondin 1)	Mediate cell to cell interactions and cell to matrix interactions	3.57	SELE (E-Selectin)	Leukocyte adhesion	6.37
TNFRSF10A (Tumor Necrosis Factor Receptor Superfamily 10a)	Activates Pro-inflammatory pathways that lead to cell apoptosis	3.47	TIMP1 (Tissue inhibitor of matrix metalloproteinase-1)	Inhibitor of MMPs	3.57
LOX (lysyl oxidase)	Enzyme involved in crosslinking collagen and elastin	3.34	ATP2B2 (ATPase, Ca++ transporting, plasma membrane 2)	Calcium-dependent ATPase activity	-5.0
MMP 11 (Matrix Metalloproteinase-11)	Breakdown of extracellular matrix	3.25	PPP1R3C (protein phosphatase 1, regulatory subunit 3C)	Glycogen-targeting subunit for phosphatase PP1	-4.5
			TAOK-1 (Thousand and one kinase-1)	Regulator of stress-activated MAPK pathway	-3.8
			APLN (Apelin)	Inhibits HIV-1 entry in cells co-expression CD4 and APL	-3.2
			ESR-1 (Estrogen substrate receptor-1)	Steroid hormone receptor	-3.0

Table 2

Rhesus Macaque Primers for Real-time Quantitative PCR Amplification

Target Gene	5' to 3'	Primer Sequences
HP	Forward	5'-TCACGGATATCGCAGATGACGG-3'
	Reverse	5'-CTGGTAGCGAACCGAGTGCT-3'
CCL-2	Forward	5'-GTGTCCCAAAGAAGCTGTGATCTTCAA-3'
	Reverse	5'-TGGAATCCTGAACCCACTTCTGCT-3'
SELE	Forward	5'-ATGAGAAGCCAACGTGTAAAGCCG-3'
	Reverse	5'-AAGGTGAACTCTCCAGCAGGG-3'
TIMP-1	Forward	5'-AGCGAGGAGTTTCTCATTGCTGGA-3'
	Reverse	5'-AAACACTGTGCATTCTCACAGCC-3'
PAPPA	Forward	5'-ATCCTCTCGGAAGTCAAAGAAACGGG-3'
	Reverse	5'-GAACACAAGCTCCCTCCTGCCA-3'
CCL-8	Forward	5'-CTCAGCCAGATTGAGTTTCC-3'
	Reverse	5'-GGT GAT TCT TGT GTA GCT CTG-3'
CX3CL1	Forward	5'-CTAAAGCTGAGGAACCCATC-3'
	Reverse	5'-TCAGGGACAGGAGTGATAAG-3'
LOX	Forward	5'-CCAGCCGACCAAGATATTC-3'
	Reverse	5'-CAGGTCATAGTGGCTAAACTC-3'
MMP11	Forward	5'-CCAGGATGCTGATGGTTATG-3'
	Reverse	5'-TCACCT TCACAGGGTCAA-3'
TNFRS10A	Forward	5'-CGAATCAGGCAATGGACATA-3'
	Reverse	5'-CCAACAGCAACAGGACAA-3'
THBS1	Forward	5'-AGTGACTGAAGAGAACAAAGAG-3'
	Reverse	5'-GTAAGTGAAGTCCGTTGTGATAG-3'
ATP2B2	Forward	5'-CTGGCTGTGCAGATTGGGAAGG-3'
	Reverse	5'-TCCACGGTGAAGTACAGAACCAGGAT-3'
PP1R3C	Forward	5'-GCTGCACCAGAATGATCCAGGTTT-3'
	Reverse	5'-AATGAGCCAAGCAAAGCCTCATGG-3'
TAOK1	Forward	5'-ACATGTATTGAACTAGCGGAAAGGAAGCC-3'
	Reverse	5'-TTTGGGCTATGTGATATAAGGCACTATTG-3'
APLN	Forward	5'-AATGGGCTGGAAGAGGGCAATGT-3'
	Reverse	5'-TCAGAAAGGCATGGGTCCCTTATG-3'
ESR1	Forward	5'-AATGTGTAGAGGGCATGGTGGAGA-3'
	Reverse	5'-AGACTTCAGGGTGCTGGACAGAAA-3'
COL5A2	Forward	5'-GAGGATGAAGGATA GGTGAAG-3'
	Reverse	5'-TCCAAATGTCCCTGTTTAAGT-3'
TGF-B1	Forward	5'-GGGACTATCCACCTGCAAGA-3'
	Reverse	5'-CCTCCTTGGCGTAGTAGTCG-3'