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Alcohol differentially alters extracellular matrix and adhesion molecule expression in skeletal muscle and heart

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Abstract

Background—The production of fibrosis in response to chronic alcohol abuse is well recognized in liver but has not been fully characterized in striated muscle and may contribute to functional impairment. Therefore, the purpose of this study was to use an unbiased discovery-based approach to determine the effect of chronic alcohol consumption on the expression profile of genes important for cell-cell and cell-extracellular matrix (ECM) interactions in both skeletal and cardiac muscle.

Methods—Adult male rats were pair-fed an alcohol-containing liquid diet or control diet for 24 wks, and skeletal muscle (gastrocnemius) and heart collected in the freely fed state. A pathway-focused gene expression PCR array was performed on these tissues to assess mRNA content for 84 ECM proteins, and selected proteins were confirmed by Western analysis.

Results—In gastrocnemius, alcohol feeding up-regulated expression of 11 genes and down-regulated expression of 1 gene. Alcohol increased fibrosis as indicated by increased mRNA and/or protein for collagen $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(III)$ and $\alpha 2(IV)$ as well as hydroxyproline. Alcohol also increased α -smooth muscle actin protein, an index of myofibroblast activation, but no concomitant change in TGF- β was detected. The mRNA and protein content for other ECM components, such as integrin α -5, L-selectin, PECAM, Sparc and Adamts2 was also increased by alcohol. Only laminin α -3 mRNA was decreased in gastrocnemius from alcohol-fed rats, while 66 ECM- or cell adhesion-related mRNAs were unchanged by alcohol. For heart, expression of 16 genes was up-regulated, expression of 3 genes was down-regulated, and 65 mRNAs were unchanged by alcohol; there were no common alcohol-induced gene expression changes between heart and skeletal muscle. Finally, alcohol increased TNF α and IL-12 mRNA in both skeletal and cardiac muscle, but IL-6 mRNA was increased and IL-10 mRNA decreased only in skeletal muscle.

Conclusions—These data demonstrate a fibrotic response in striated muscle from chronic alcohol-fed rats which is tissue-specific in nature, suggesting different regulatory mechanisms.

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Keywords

ethanol; collagen; fibrosis; myopathy; striated muscle

INTRODUCTION

Chronic excessive alcohol consumption produces a myopathy in both skeletal and cardiac muscle which increases both morbidity and mortality (Lang et al., 2005). While the etiology of this alcoholic muscle disease is not well defined, the preponderance of studies in this area have focused on alcohol-induced changes in myocyte protein balance (synthesis and degradation) (Lang et al., 2007, Hong-Brown et al., 2001). However, one hallmark of chronic alcohol abuse in the liver is increased fibrosis resulting from the increased production and/or a decreased turnover of collagen and other extracellular matrix (ECM) proteins (Siegmund et al., 2005). Studies investigating its etiology are limited, although descriptive studies have reported alcohol-induced remodeling of the ECM in cardiac muscle (El Hajj et al., 2014, Fernandez-Sola et al., 1994, Law et al., 2012), with only nominal observations in skeletal muscle (Wang et al., 2012, Otis et al., 2007). Alcohol-induced fibrosis appears to be a potentially maladaptive response leading to muscle stiffening and dysfunction, especially prominent in the heart (Liu et al., 2011). Whereas the functional significance of alcohol-induced fibrosis in skeletal muscle remains unclear, increased deposition of collagen and ECM remodeling is associated with decreased force of contraction in other pathological conditions (Serrano et al., 2011).

There is a paucity of systematic studies comparing alcohol-induced fibrotic changes specifically in skeletal muscle and none which compare changes in skeletal and cardiac muscle under the same experimental conditions. Therefore, the purpose of the present study was to perform gene expression profiling for genes important for cell-cell and cell-ECM interactions in both types of striated muscle in response to chronic alcohol consumption, with the goal of identifying a conserved set of differentially expressed genes.

MATERIALS AND METHODS**Animal protocol**

Specific-pathogen free adult male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Cambridge, MA). Rats were housed under constant environment conditions (12 h light/12 h dark; 21–22 °C; 30–70% humidity; no environment enrichment provided) and received standard rat chow (LabDiet 5001; PMI Nutrition International, St. Louis, MO) and water ad libitum for 1 wk prior to switching rats to the totally liquid diet. Rats were then randomized to either an alcohol-fed (n = 10) or control group (n = 10). Each group was maintained for 24 wks on the Lieber-DeCarli liquid diet (Bio-Serv, Frenchtown, NJ). Rats consuming the ethanol-containing diet initially received 12% of total calories from ethanol and this percentage was increased weekly by 12% till a maximum of 36% of caloric intake from alcohol was achieved. Time-matched pair-fed control animals received a liquid diet where maltose-dextran was isocalorically substituted for alcohol. Consumption of the liquid diet was assessed daily and animals were weighed weekly. The duration of alcohol

feeding was selected as it has been shown to produce alterations in skeletal and cardiac muscle protein balance (Lang et al., 1999, Vary et al., 2002, Korzick et al., 2013).

Body composition was determined non-invasively in conscious rats between 0600–0700 hours using a ^1H -NMR analyzer (Bruker LF90 proton-NMR Minispec; Bruker Optics, Woodlands, TX), as previously described (Jiao et al., 2009). Rats were then anesthetized via inhalation of isoflurane (2–3% in O_2 with 1.5% maintenance) and the gastrocnemius + plantaris complex (hereafter referred to as skeletal muscle) and heart (ventricular tissue only) collected for analysis. A blood sample from the abdominal vena cava was collected in a heparinized syringe prior to excision of the heart. All experiments described herein were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine and adhered to the National Institutes of Health (NIH) guidelines for the use of experimental animals.

RNA extraction and real-time PCR

Total RNA was isolated from fresh skeletal muscle and heart using Tri-reagent/RNeasy mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The sample size was $n = 5$ per group per tissue, and samples were randomly selected from the total number ($n = 10$) samples per treatment. Synthesis of first-strand cDNA was performed by reverse transcribing 1 μg of total RNA using the RT² First Strand kit by Qiagen. The cDNA was mixed with the Qiagen RT² SYBR Green master mix and 10 μg was loaded onto rat Extracellular Matrix RT² Profiler PCR array plate (PARN-013ZE) as per instructions (SABiosciences, Foster City, CA) for pathway-focused gene expression analysis. The plate was analyzed on Applied Biosystems 7900HT cycler with the following cycling conditions; 10 min heat activation at 95 °C and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Bioinformatic analysis was performed using the Excel-based RT² Profiler PCR Array Data Analyzer (SABiosciences). The assay was run on individual samples of heart and gastrocnemius from the control and alcohol-fed groups ($n = 5$ per group). Values were obtained for the threshold cycle (C_t) for each gene and normalized using the average of five housekeeping genes (β -actin, LDH, β -2 microglobulin, hypoxanthine phosphoribosyl-transferase 1, and ribosomal protein, large, P1) on the same array which did not differ between groups. Change (C_t) between alcohol and control for each mRNA was reported as fold-change. The comparative quantitation method 2^{-C_t} was used in presenting expression of target genes in reference to the endogenous control. Gene profiling using the PCR array has been reported to yield results which are highly concordant with those of other microarray platforms (Arikawa et al., 2008). A functional grouping of the genes analyzed is provided in Table 1.

Western blotting

Remaining gastrocnemius and heart tissue was clamped in liquid nitrogen-cooled clamps and the tissue subsequently powdered at the temperature of liquid nitrogen. The tissue powder from all rats (10 per group) was homogenized in ice-cold homogenization buffer consisting of (in mmol/L) 20 HEPES (pH 7.4), 2 EGTA, 50 sodium fluoride, 100 potassium chloride, 0.2 EDTA, 50 β -glycerophosphate, 1 DTT, 0.1 phenylmethane-sulphonylfluoride, 1 benzamidine, and 0.5 sodium vanadate and clarified by centrifugation. Equal amounts of

protein per sample were subjected to SDS-PAGE and the relative expression of various proteins was determined by Western blot analysis. Based on the results from the array study, the protein content was determined for: collagen $\alpha 1$ (I), collagen $\alpha 1$ (II) and elastin (Santa Cruz Biotechnology, Santa Cruz, CA); collagen 1 α (III) (LifeSpan Biosciences, Seattle, WA); collagen $\alpha 1$ (II), collagen IV, ADAMTS (ADAM metalloproteinase with thrombospondin type 1 motif)-1, ADAMTS2, CTGF (connective tissue growth factor), integrin- $\alpha 5$, matrix metalloproteinase-3 (MMP3), thrombospondin (THSP)-1 and α -SMA (smooth muscle actin) (Abcam, Cambridge, MA); and TGF (transforming growth factor)- β , tubulin, SPARC (secreted protein acidic and rich in cysteine) and β -actin (Cell Signaling Technology, Beverly, MA). Precision Plus Protein dual color standards were used on all blots to estimate molecular weight of determined proteins (Bio-Rad, Life Technologies Corp, Carlsbad, CA). Blots were developed with enhanced chemiluminescence (ECL) Western blotting reagents and then exposed to X-ray film in a cassette equipped with a DuPont Lightning Plus intensifying screen. The film was scanned (Microtek ScanMaker IV) and band density was analyzed in the linear range using NIH ImageJ 1.6 software.

Hydroxyproline concentration

Powdered skeletal muscle (30 mg) was homogenized in 300 μ l of water and equal volume of 12 N HCl was added and hydrolyzed at 120° C for 3 h according to the manufacturer's instructions (BioVision Incorporation, Milpitas, CA). The hydrolyzed sample was vortexed, centrifuged at 10,000 $\times$ g for 3 min, then 10 μ l was loaded on to a 96-well clear bottom plate and dried in an oven at 60° C for 1h. The hydroxyproline standard (0.2–1 μ g per well) was loaded, 100 μ l chloramine T was added to each well and the mixture incubated at room temperature for 7 min. Then 100 μ l of DMAB concentrate diluted 1:1 in perchloric acid/isopropanol was added, incubated at 60° C for 90 min and absorbance was measured at 560 nm in a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Due to limited tissue availability, hydroxyproline was only determined in skeletal muscle.

Ribonuclease protection assay (RPA)

Total RNA, DNA and protein were extracted from gastrocnemius and heart obtained using a mixture of phenol and guanidine thiocyanate (TRI Reagent, Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNA was separated from protein and DNA by the addition of bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in formamide, RNA samples were quantified by spectrophotometry. Ten micrograms of RNA was used for each assay. Riboprobes were synthesized from a custom multi-probe rat template set containing probes for tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-12, IL-10, nitric oxide synthase (NOS)-2, GAPDH, and L32 mRNA, as previously described (Frost et al., 2002). The labeled riboprobe was hybridized with RNA overnight using an RPA kit and the manufacturer's protocol (Pharminigen). Protected RNAs were separated using a 5% acrylamide gel (19:1 acrylamide/bisacrylamide). Gels were transferred to blotting paper and dried under vacuum on a gel dryer. Dried gels were exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and the resulting data were quantified using ImageQuant™ software and normalized to the mouse ribosomal protein L32 mRNA signal in each lane.

Alcohol concentration

Plasma alcohol concentration was determined by a rapid analyzer (Analox Instruments, Lunenburg, MA).

Statistical analysis

Experimental data are summarized as means $\pm$ standard error of the mean (SEM) where the number of rats per group is provided in the legend to the figure or table. Statistical evaluation of the data was performed using 2-tailed Students' *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

General characteristics of alcohol-induced changes in muscle and heart

The body weight at the start and the conclusion of the study did not differ between control and alcohol-fed rats (Figure 1A); however, the increase in body weight tended ($p = 0.06$) to be lower (9%) in rats consuming alcohol (Figure 1B). The absolute mass of both the gastrocnemius/plantaris complex (Figure 1C) and the cardiac ventricular tissue (Figure 1D) was decreased in alcohol-fed rats compared to pair-fed control values (20% and 14%, respectively). However, tissue weight normalized to body weight did not differ between control and alcohol-fed rats for either skeletal muscle (control = $0.45 \pm 0.05\%$ vs alcohol = $0.37 \pm 0.04\%$) or heart (control = $0.24 \pm 0.03\%$ vs alcohol = $0.22 \pm 0.03\%$). Whole-body composition assessed by ^1H -NMR showed that alcohol-fed rats had a reduced lean body mass when expressed either as absolute mass or normalized to body weight (Figure 1E and 1F, respectively). Conversely, the amount of whole-body fat tissue was increased in alcohol-fed rats only when data were normalized to body weight (Figure 1G and 1H, respectively).

The concentration of ethanol in the blood of alcohol-fed rats averaged 18 ± 3 mmol/L, while no ethanol was detected in control-fed rats.

Gene profiling

The effect of chronic alcohol consumption on ECM and adhesion molecule expression was investigated using the RT² Profiler PCR array. For the gastrocnemius, of the 84 genes in the array, only cadherin 3, type1 (CDH3), hyaluronan and proteoglycan link protein 1 (HAPLN1), extracellular matrix protein 1 (ECM1), laminin, alpha 1 (LAMA1), and synaptotagmin 1 (SYT1) were excluded due to an average Ct > 35 for all samples or more than half of the samples being below the detection limit. For those remaining genes, the average Ct did not differ between skeletal muscle from control and alcohol-fed rats (19.29 ± 0.16 vs 19.21 ± 0.16 ; mean $\pm$ SD). Table 2 shows the 11 genes for which expression was significantly ($P < 0.05$) up-regulated in gastrocnemius from alcohol-fed rats compared to control muscle. Nine of these mRNAs were elevated between ~30–70% above time-matched control values, while collagen $\alpha 1(\text{I})$ and L-selectin were increased more than 2-fold in muscle from alcohol-fed rats. In contrast, alcohol significantly ($P < 0.05$) decreased the mRNA content for only 1 gene, laminin, alpha 3 (LAMA3; 62% decrease) in skeletal muscle (Table 2). Supplemental Table S1 contains all remaining mRNAs in gastrocnemius which did not differ statistically between control and alcohol-fed rats.

For ventricular heart muscle, all 84 genes in the array were detectable and none were excluded because of a Ct > 35. The average Ct did not differ between heart from control and alcohol-fed rats (19.51 ± 0.15 vs 19.88 ± 0.28 ; mean $\pm$ SD). Table 3 shows the 17 genes which were significantly ($P < 0.05$) up-regulated in heart of alcohol-fed compared to control-fed rats. Fifteen of these genes were increased ~20–70%, while matrix metalloproteinase 9 (MMP9) and P-selectin (SELP) were increased ~2-fold in alcohol vs control heart tissue. In contrast, the mRNA content for collagen $\alpha 1$ (II), vitronectin (VTN) and cadherin 4 (CDH4) was significantly ($P < 0.05$) decreased ~40% in alcohol-fed rats (Table 3). None of the mRNAs in heart which were increased or decreased by alcohol were similarly altered in gastrocnemius. Supplemental Table S2 provides a complete list of all mRNAs in heart which did not differ between control and alcohol-fed rats.

Protein content

Based on the array data above, Western blot analysis was performed to determine whether there was a coordinate up-regulation of proteins in skeletal muscle altered by alcohol feeding. Tissue fibrosis is characterized by deposition of various types of collagens (Kjaer, 2004). Monomeric collagen I consists of $\alpha 1$ and $\alpha 2$ chains. Monomeric collagen $\alpha 1$ (I) was increased 9-fold in skeletal muscle from alcohol-fed compared to pair-fed control rats (Figure 2). Likewise, protein content for collagen $\alpha 2$ (I) and $\alpha 1$ (III) was also increased by chronic alcohol consumption (Figure 2). However, collagen $\alpha 1$ (II) protein content in skeletal muscle did not differ between control and alcohol-fed rats despite the observed increase in its mRNA content.

Selected proteins which are found predominantly in the basement membrane were also assessed (Figure 3). The protein content for collagen IV, laminin-1/2 and SPARC were increased 2- to 4-fold after alcohol. In contrast, elastin protein was reduced more than 50% in gastrocnemius from alcohol-fed rats.

Alcohol feeding increased the content of ADAMTS1 protein in skeletal muscle, whereas the abundance of the ADAMTS2 protein did not differ between control and alcohol-fed rats (Figure 4). Integrin $\alpha 5/\beta 1$ is a receptor for fibronectin and fibrinogen (Suehiro et al., 2000) and alcohol increased protein content for integrin $\alpha 5$ (Figure 4). The protein content for L-selectin in gastrocnemius was increased ~4-fold in response alcohol feeding (Figure 4), but a reliable change in PECAM was not detected (data not shown). Although there was a non-statistically significant 6-fold increase in MMP3 mRNA in alcohol-fed rats compared to control values, MMP3 protein content in skeletal muscle did not differ after alcohol feeding (Figure 4).

Activation of myofibroblasts leads to ECM generation and α -SMA is routinely used as a marker for activated myofibroblasts (Henderson et al., 2013). Chronic alcohol ingestion increased α -SMA in skeletal muscle more than 5-fold. However, activation of the TGF- β signal transduction pathway was not apparent as the protein content for both TGF- β and CTGF (a TGF- β immediate early gene mediating many of the pro-fibrotic actions of this cytokine) (Ding et al., 2013) did not differ in gastrocnemius between control and alcohol-fed rats (Figure 5). Finally, thrombospondin (THBS)-1 is a matrix glycoprotein important in the

activation of latent TGF- β (Daniel et al., 2004), and THBS-1 protein content was decrease ~25% in muscle from alcohol-fed rats, compared to control values (Figure 5).

Western blot analysis was also performed for all of the above mentioned proteins in the heart of control and alcohol-fed rats. Initial analysis of collagen $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(II)$, III and IV revealed no difference in the protein content for these collagen isoforms in heart from the two groups (data not shown). Subsequent analysis of all remaining proteins previously assessed in skeletal muscle also failed to show any alcohol-induced change (data not shown).

Hydroxyproline

This nonproteinogenic amino acid is used as a direct measure of the amount of collagen present in tissue. The hydroxyproline content in skeletal muscle of alcohol-fed rats was ~30% greater than in time-matched control muscle (alcohol = 15.9 ± 1.3 vs control = 12.1 ± 0.9 μg hydroxyproline/mg tissue; $P < 0.05$)

Tissue cytokines

As inflammation can induce fibrosis and may contribute to the etiology of tissue remodeling, we also assessed alcohol-induced changes of select cytokines in both gastrocnemius and heart (Figure 6). The mRNA content for the inflammatory mediators TNF α , IL-6, IL-12 and NOS2 was increased in skeletal muscle from alcohol-fed rats. In contrast, alcohol decreased the mRNA content for the anti-inflammatory IL-10. No alcohol-induced change was detected for IL-1 β in skeletal muscle. Alcohol feeding produced fewer changes in cytokine expression in cardiac muscle, with only TNF α and IL-12 mRNA being increased in heart from alcohol-fed rats.

DISCUSSION

Adhesion molecules constitute an important component of junctional complexes between endothelial cells but also the surface of different types of leukocytes (Ivetic, 2013). These molecules are important determinants of the normal restrictive endothelial barrier function but also lymphocyte-endothelial interactions and leukocyte extravasation. The mRNA content in gastrocnemius of prominent adhesion molecules such as ICAM-1, VCAM-1 and cadherins 1–4 did not differ between alcohol-fed and control rats. In contrast, L-selectin mRNA and protein and PECAM-1 mRNA were increased by alcohol which might possibly aid in the recruitment of leukocytes and platelets to muscle (Ivetic, 2013). The alcohol-induced increase in TNF α , IL-6, IL-12 and NOS2 in gastrocnemius is consistent with the observed increase in L-selectin and PECAM (Ivetic, 2013), but the pro-inflammatory milieu was apparently not sufficient to alter the mRNA expression of other adhesion molecules. To our knowledge, neither L-selectin mRNA nor protein has been reported in myocytes *per se* and the alcohol-induced increase in this adhesion molecule may represent the infiltration of leukocytes in whole muscle tissue. Likewise, alcohol feeding increased CD44 in muscle, a response similar to that seen in liver (Urashima et al., 2000). Although CD44 is ubiquitously expressed and participates in numerous cellular functions, it plays a central role in the trafficking of leukocytes subsets as it binds to various ECM molecules, including

hyaluronan, L-selectin, collagen and laminin (Naor et al., 1997). Therefore, it is possible the alcohol-induced up-regulation of CD44 aids the recruitment of leukocytes to areas of inflammation in muscle, as is evidenced in liver and adipose tissue in response to other inflammatory stimuli (McDonald and Kubes, 2015). Finally, the integrins are ubiquitously expressed transmembrane receptors which are also important for cell-cell and cell-matrix interactions (Henderson et al., 2013). There are 24 different $\alpha\beta$ integrins which bind to tissue- and organ-specific ligands and their ability to regulate diverse cellular functions is defined by non-covalent heterodimeric association between eighteen α - and eight β -subunits. While a relatively specific increase in both mRNA and protein for integrin- $\alpha 5$ (ITGA5) was seen in muscle from alcohol-fed rats, the physiological importance of this change is unclear since there was no concomitant increase in the mRNA for the other assessed integrins (i.e., ITGA2-4, ITGB1-4, ITGAD, ITGAE, ITGAL, ITGAM, and ITGAV).

The basement membrane lies adjacent to the endothelium and provides support for both epithelial and endothelial cells (Carmignac and Durbeek, 2012). Collagen IV and laminin can self-assemble to form networks and, along with nidogen and perlecan, comprise the four major components of the basement membrane. Collagen IV is found exclusively in and is the predominant structural element of the basement membrane of all tissues and found primarily in the basal lamina. We determined 3 of the 6 protein-encoding genes for collagen IV (i.e., $\alpha 1-3$). While only collagen $\alpha 2(IV)$ mRNA was significantly increased by alcohol, Western analysis indicated a marked increase in total collagen IV protein in muscle from alcohol-fed rats. Laminins are also an integral part of the basal lamina and interact with a variety of cell surface receptors on adjacent cells (Li et al., 2002). Alcohol increased LAMC1 (formerly $\gamma 1$) which is necessary for laminin heterotrimer assembly (Nomizu et al., 1994). Alcohol did not alter the mRNA content for LAMA1, A2, B2 or B3 in muscle, but LAMA3 was reduced in alcohol-fed rats by 62%. Using an antibody which recognized both laminin 1 and 2, we detected 2-fold increase in skeletal muscle of alcohol-fed rats. This increase in collagen IV and laminin deposition in skeletal muscle is similar to that previously reported in alcohol-induced hepatic fibrosis (Tsutsumi et al., 1993), but is a novel observation in skeletal muscle.

The interstitial matrix is subjacent to the basement membrane and mainly comprised of collagen fibrils of different types. Collagen is the primarily protein present in the ECM and is arranged in bundles of fibers providing structural support for the tissue. The induction of collagen I and III is a hallmark of the acquisition of a fibrotic phenotype (Leask and Abraham, 2004). The most abundant isoform of collagen is type I which is a triple helix composed of two $\alpha 1$ chains and one $\alpha 2$ chain. Our data indicate alcohol increased collagen $\alpha 1(I)$ mRNA and protein as well as collagen $\alpha 2(I)$ protein in skeletal muscle, a response similar to that previously reported (Wang et al., 2012). While the alcohol-induced change in collagen $\alpha 1(III)$ mRNA did not achieve statistical significance, the protein content for this collagen isoform was increased. In contrast, alcohol feeding did not alter the mRNA content of other collagen isoforms, such as $\alpha 1(V)$, $\alpha 1(VI)$ and $\alpha 1(VIII)$ in muscle, and hence their protein content was not determined. Although an alcohol-induced increase in the protein content was detected for many of the collagen isoforms in skeletal muscle, it should be noted that tissue was homogenized in an aqueous buffer (e.g., no acid or enzyme extraction)

and the Western blot profile is primarily indicative of the soluble fraction of collagens as opposed to the more insoluble cross-linked fraction. Collectively, these data extend the limited number of previous studies showing that chronic alcohol feeding either increases the fibrotic index in skeletal muscle (Wang et al., 2012) or conversely produced no change (Preedy et al., 1989) based on histological examination. Lastly, although chronic alcohol intake was sufficient to increase the content of hydroxyproline in skeletal muscle, whether these alcohol-induced changes were sufficient to produce a net deposition of ECM and histological evidence of fibrosis was not directly assessed in the current study as all tissues were freeze-clamp upon collection.

An increase in collagen deposition and development of fibrosis in liver (Wiercinska et al., 2006) and heart (Law and Carver, 2013) is often attributed to TGF- β -dependent activation of myofibroblasts, as evidenced by increased α -SMA. Chronic alcohol consumption has been reported to increase TGF β 1 mRNA and protein in skeletal muscle (Clary et al., 2011). Moreover, *in vitro* studies indicate the alcohol-induced increase in collagen I protein in fibroblast conditioned medium (Law and Carver, 2013) and collagen α 1(I) protein in myoblasts (Hong-Brown et al., 2015) was associated with increased TGF- β . Moreover, pretreatment with a TGF β receptor inhibitor (SB 431542) or a soluble recombinant TGF- β II receptor prevented collagen accumulation suggesting the autocrine/paracrine actions of this cytokine were causally related to the profibrotic effect of alcohol (Law and Carver, 2013). In contrast, we did not detect a significant change in the protein content for either TGF β or its downstream mediator CTGF in skeletal muscle in response to chronic alcohol feeding. There was also no difference in the mRNA content for TGF beta-induced (TGFBI) which is up-regulated by TGF β . A potential reason for the difference between the current *in vivo* study and previous *in vitro* studies is that the increase in TGF β (and collagen I) *in vitro* was only observed with relatively high concentrations of ethanol (100 mM), whereas the prevailing blood alcohol concentration *in vivo* is generally < 25 mM. Also, the current study assessed fibrotic changes in the whole tissue, compared to in cultured fibroblasts. However, high doses of alcohol also increase collagen in myoblasts per se (Hong-Brown et al., 2015). Thrombospondin (THBS)-1 is a matrix glycoprotein important in the activation of latent TGF- β in liver and can be increased by TNF α (Sweetwyne and Murphy-Ullrich, 2012). Although we detected an alcohol-induced increase in THBS-1 mRNA, THBS-1 protein was decreased in muscle from alcohol-fed rats. Although our data cannot exclude the possibility that the alcohol-induced fibrotic response in skeletal muscle was TGF- β independent, a more likely explanation is that TGF- β expression and/or signaling was increased at an earlier time point which was missed because only a single time was examined.

The net accumulation of collagen is regulated by processes which enhance production and/or impair the removal of collagen. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases responsible in large part for the degradation and remodeling of ECM and play a central role in maintaining the integrity of myofibers (Carmeli et al., 2004). We detected no significant difference between the mRNA content for any of the MMPs determined between control and alcohol-fed rats (i.e., MMP1-3 and MMP4-16). In contrast, oral gavage of alcohol for 12 wks has been previously reported to increase mRNA, protein and activity for MMP9 in the gastrocnemius and plantaris (Wang et al., 2012). Members of

the tissue inhibitor of metalloproteinases (TIMPs) gene family are found in the basement membrane and can inactivate MMPs by binding to their catalytic zinc cofactor (Carmeli et al., 2004). However, there was no difference in the mRNA expression for TIMP-1, -2 or -3 in skeletal muscle from alcohol-fed compared to pair-fed control rats. This finding differs from the upregulation of TIMP1 and TIMP2 in the fibrotic response of liver to alcohol abuse (Wang et al., 2013) and possibly suggests a different mechanism of action. Finally, SPARC (secreted protein acidic and rich in cysteine) is a major non-structural glycoprotein which binds ECM-associated components, influences remodeling, and accordingly increased expression may reflect increased fibrotic activity (Atorrasagasti et al., 2013). In the current study, alcohol increased SPARC mRNA and protein expression in skeletal muscle. While SPARC has been reported to be of importance in fibrogenesis in liver (Atorrasagasti et al., 2013), it has not been previously reported to be increased in skeletal muscle in response to alcohol.

A family of extracellular peptidases which function in the processing of procollagens and cleavage of select matrix proteoglycans, referred to as A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS), are also important in connective tissue organization (Porter et al., 2005). Alcohol feeding coordinately increased ADAMTS1 mRNA and protein content in skeletal muscle. ADAMTS1 has a number of different substrates, including THBS-1 (Porter et al., 2005). Hence, the inverse relationship between ADAMTS1 protein (e.g., protease) and reduced THBS-1 (e.g., substrate) in muscle from alcohol-fed rats is internally consistent. Other potential substrates for this enzyme, such as aggrecan and versican, were not assessed. ADAMTS2, also known as procollagen I N-proteinase (PCI-NP), cleaves the propeptides of collagen I and II, but not collagen III, prior to fibril assembly (Wang et al., 2003). While alcohol increased ADAMTS2 mRNA, protein content did not differ between groups and the cellular content of its substrates were not decreased.

It is noteworthy that in contrast to the alcohol-induced increases in collagens I, III, IV and several of the ECM proteins described above, gene array data of rats and humans experiencing disuse muscle atrophy showed decreased mRNA and/or protein for collagen $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(III)$ and IV as well as SPARC, LAMC1, fibronectin 1, and fibulin (Lecker et al., 2004, Urso et al., 2006). Hence, despite the superficial similarity between alcohol- and disuse-induced atrophy, there are marked differences in the ECM gene expression profile which accompany the loss of muscle in these two catabolic conditions.

While the characterization and importance of ECM alterations in other types of dilative cardiomyopathies is well recognized (Kapelko, 2001), there is a paucity of data related specifically to fibrotic changes in the heart produced by alcohol. Early reports indicated that chronic alcohol consumption is capable of producing fibrotic changes in the heart of rodents (El Hajj et al., 2014, Liu et al., 2011), dogs (Rajiyah et al., 1996), chickens (Morris et al., 1999), monkeys (Vasdev et al., 1975) and humans (Fernandez-Sola et al., 1994), although there are also reports that the extent of cardiac fibrosis does not differ between control and alcohol-fed animals (Wang et al., 2005, Kita et al., 1996). When present, the alcohol-induced increase in cardiac remodeling was associated with increased chamber stiffness and ventricular dysfunction (Fernandez-Sola et al., 1994, Morris et al., 1999, Rajiyah et al.,

1996). Rats exposed to 2 wks of alcohol vapor experienced an increase in the cardiac protein content of collagen I and III, and α -SMA, but not MMP9 or MMP2 (El Hajj et al., 2014). The opposite results were obtained in the present study where rats were fed an alcohol-containing diet for 24 wks. That is, we did not detect an alcohol-induced change in the protein or mRNA content for collagen I and III or α -SMA, but alcohol feeding did increase the mRNA content for MMP9 and MMP2 (as well as MMP14). However, other MMPs in heart (e.g., MMP1, MMP3, MMP7, MMP8, MP10, MMP11, MMP12, MMP13, MMP15 and MMP16) did not differ between control and alcohol-fed rats. The reason for these discordant results between our study and an earlier work is not known but the route and duration of alcohol exposure differs significantly. Also, as indicated previously, the current study assessed collagen content in the soluble fraction of the tissue homogenate whereas the approach used by El Hajj et al (2014) permitted extraction of insoluble collagens.

Although observational in nature, our current data are unique as they: 1) use an unbiased discovery-based approach to characterize alcohol-induced changes in ECM and adhesion proteins in both major classes of striated muscle from the same animal model; 2) expand the number of known fibrosis-related mRNA and/or proteins which are up- or down-regulated in striated muscle in response to chronic alcohol feeding; 3) document the non-overlapping and relatively unique alcohol-induced changes in the profibrotic genotype profile between skeletal muscle and heart; and 4) highlight the differential response of ECM changes in skeletal muscle in response to alcohol compared to skeletal muscle disuse, both of which reduce muscle protein synthesis and mass. The differential cytokine response between skeletal and cardiac muscle may, in part, be responsible for lack of a core set of differentially expressed genes driving the pro-fibrotic phenotype. As such, these data expand our understanding of the metabolic pathways regulated by alcohol, and provide targets for future studies which address the mechanistic underpinnings and the functional significance of these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Body weight and composition of control and alcohol-fed rats. Values are means $\pm$ SEM; n = 10 per group. Body composition was determined by ^1H -NMR spectroscopy after 24 weeks of alcohol feeding or in isonitrogenous isocaloric pair-fed control rats. * P < 0.05 compared to values from time-matched pair-fed control rats.

Figure 2.

Collagen isoform content of gastrocnemius from control and alcohol-fed rats. Bar graphs represent quantitation of Western blot images normalized to β -tubulin with the control value set at 100 AU (arbitrary units) for each collagen protein isoform. Each collagen isoform ran slightly below the 150 KD molecular weight (MW) marker which is consistent with the predicted MW of these proteins (~140 KD). Values in bar graph are means $\pm$ SEM; $n = 10$ per group. * $P < 0.05$ compared to values from time-matched pair-fed control rats. Top panel, representative Western blot images from two control and two alcohol-fed rats.

Figure 3.

Basement membrane-related proteins in gastrocnemius from control and alcohol-fed rats. Bar graphs represent quantitation of Western blot images normalized to β -tubulin with the control value set at 100 AU (arbitrary units). COL IV, Laminin, Elastin and Sparc ran at approximately 250, 200, 70 and 40 KD, respectively. Values in bar graph are means $\pm$ SEM; $n = 10$ per group. * $P < 0.05$ compared to values from time-matched pair-fed control rats. Top panel, representative Western blot images from two control and two alcohol-fed rats.

Figure 4.

Protein content for select extracellular matrix (ECM) proteins in gastrocnemius from control and alcohol-fed rats. Bar graphs represent quantitation of Western blot images normalized to β-tubulin with the control value set at 100 AUs. Major bands quantitated were as follows: ADAMTS1 and ADAMTS2 (~100 KD), Integrinα5 (~100 KD), L-selectin (~70 KD) and MMP3 (~60 KD). Values in bar graph are means ± SEM; n = 10 per group. **P* < 0.05 compared to values from time-matched pair-fed control rats. Top panel, representative Western blot images from two control and two alcohol-fed rats.

Figure 5.

Protein content for TGF- β related proteins and myofibroblast activation in gastrocnemius from control and alcohol-fed rats. Bar graphs represent quantitation of Western blot images normalized to β -tubulin with the control value set at 100 AU (arbitrary units) for each protein. Major bands quantitated were as follows: TGF- β (~20 KD), CTGF (40 KD), α SMA (40 KD) and THSP-1 (~170 KD). Other MW isoforms of TGF- β at ~10 and ~70 KD also showed no difference between groups (data not shown). Values in bar graph are means $\pm$ SEM; n = 10 per group. * $P < 0.05$ compared to values from time-matched pair-fed control

rats. Top panel, representative Western blot images from two control and two alcohol-fed rats.

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Figure 6.

Cytokine mRNA content in gastrocnemius and heart from control and alcohol-fed rats. Bar graphs represent quantitation of ribonuclease protection assays (RPA) in which values were normalized to L32 and the control value arbitrarily set at 1.0 AU. Values are means $\pm$ SEM; $n = 7$ rats per group for each tissue. * $P < 0.05$ compared to values from time-matched pair-fed control rats.

Table 1**Functional Gene Groupings**

Extracellular Matrix (ECM) Proteins	
Basement Membrane Constituents	COL4A1, COL4A2, COL4A3, ENTPD1, ITGB4, LAMA1, LAMA2, LAMA3, LAMB2, LAMB3, LAMC1, SPARC, TIMP1, TIMP2, TIMP3
ECM Structural Constituents	COL1A1, COL2A1, COL3A1, COL4A1, COL4A2, COL4A3, COL5A1, COL6A1, COL8A1, HAPLN1, LAMA1
ECM Proteases	ADAMTS1, ADAMTS2, ADAMTS5, ADAMTS8, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP1A, MMP2, MMP3, MMP7, MMP8, MMP9
ECM Protease Inhibitors	COL4A3, TIMP1, TIMP2, TIMP3
Other ECM Molecules	VCAN, CTGF, ECM, EMILIN1, FBLN1, FN1, POSTN, SPOCK1, SPP1, TGFB1, THBS1, TNC
Cell Adhesion Molecules	
Transmembrane Molecules	CD44, CDH1, CDH2, CDH3, CDH4, ENTPD1, ICAM1, ITGA2, ITGA3, ITGA4, ITGA5, ITGAE, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, MMP14, MMP15, MMP16, NCAM1, NCAM2, PECAM1, SELE, SELL, SELP, SGCE, SYT1, VCAM1
Cell-Cell Adhesion	CDH1, ICAM1, VCAM1, PCAM1
Cell-Matrix Adhesion	CTGF, ITGA2, ITGA3, ITGA4, ITGA5, ITGAD, ITGAE, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, SPP1
Other Adhesion Molecules	CATNA1, CTNNA2 (CATNA2), CNTN1, CTNNA1 (CATNA1), COL5A1, COL6A1, COL8A1, VCAN, EMILN1, FN1, HAPLN1, LAMA1, LAMA2, LAMA3, LAMB2, LAMB3, LAMC1, POSTN, TGFB1, THBS1, THBS2, VTN

Abbreviations for gene names provided in Tables 2 and 3. Gene groupings are those identified by SABiosciences (Foster City, CA).

Table 2

Alcohol-induced changes in skeletal muscle mRNA content

Alcohol-induced increases						
Gene Name	Description	MGI #	Alcohol (A)	Control (C)	A/C ratio	P-value
COL1A1	Collagen, type I, alpha 1 [α1(I)]	88467	3.0E-01	1.4E-01	2.19	0.033681
SELL	L-selectin (CD62L)	98279	7.8E-04	3.8E-04	2.07	0.043146 ^A
ADAMTS2	ADAM metalloproteinase with thrombospondin type 1 motif, 2	1347356	2.4E-03	1.4E-03	1.70	0.025278
THBS1	Thrombospondin 1	98737	9.7E-03	5.8E-03	1.67	0.045094
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	96604	3.4E-03	2.1E-03	1.64	0.001368
CD44	CD44 antigen; cell surface glycoprotein CD44	88338	1.6E-04	1.0E-04	1.60	0.009408
SPARC	Secreted protein acidic and rich in cysteine	98373	6.9E-01	4.4E-01	1.56	0.045616
ADAMTS1	Adamts 1	109249	5.2E-02	3.5E-02	1.50	0.017160
LAMC1	Laminin, gamma 1 (formerly Lamb2)	99914	1.1E-01	8.3E-02	1.33	0.005487
COL4A2	Collagen, type IV, alpha 2 [α2(IV)]	88455	2.2E-02	1.7E-02	1.29	0.015277
PECAM1	Platelet/endothelial cell adhesion molecule 1 (CD31)	97537	3.7E-02	2.9E-02	1.28	0.022871
Alcohol-induced decrease						
Gene Name	Description	MGI #	Alcohol (A)	Control (C)	A/C ratio	P-value
LAMA3	Laminin, alpha 3	99909	1.2E-04	3.1E-04	0.38	0.041263

Alcohol (A) and Control (C) values were normalized to the average of five housekeeping genes (β-actin, LDH, β-2 microglobulin, hypoxanthine phosphoribosyltransferase 1, and ribosomal protein, large, P1) which did not differ between groups; n = 5 per group. P-values were calculated based on a 2-tailed Student's t-test of the replicate 2nd Ct values for each gene in the control and alcohol groups. Superscript A: The average Ct for this gene was relatively high (> 30) in the control and was relatively low (< 30) in the alcohol sample, suggesting the actual fold-change value was at least as large as the calculated fold-change (A/C ratio) result. MGI = mouse genome informatics number.

Table 3

Alcohol-induced changes in cardiac muscle mRNA content

Alcohol-induced increases				
Gene Name	Alcohol (A)	Control (C)	A/C ratio	P-value
MMP9	9.8E-05	4.3E-05	2.27	0.010537
SELP	2.4E-04	1.2E-04	2.05	0.018195
MMP14	2.9E-03	1.7E-03	1.69	0.022585
TIMP1	4.5E-02	2.8E-02	1.61	0.050609
ITGB2	4.3E-03	2.8E-03	1.52	0.007478
CTGF	4.0E-02	2.7E-02	1.51	0.057393
ITGA5	1.4E-02	9.7E-03	1.46	0.009640
COL4A3	9.1E-03	6.4E-03	1.41	0.007628
ITGB3	2.6E-03	1.9E-03	1.37	0.005465
FBLN1	2.1E-02	1.5E-02	1.37	0.029988
ITGA4	2.9E-03	2.2E-03	1.36	0.038059
ADAMTS5	2.9E-02	2.2E-02	1.33	0.009260
COL6A1	5.0E-02	4.0E-02	1.24	0.021231
LAMA2	2.1E-01	1.7E-01	1.23	0.013915
LAMB2	2.9E-01	2.4E-01	1.23	0.019462
MMP2	4.1E-02	3.3E-02	1.22	0.045404
CATNA1	2.0E-01	1.7E-01	1.18	0.009431
Alcohol-induced decreases				
Gene Name	Alcohol (A)	Control (C)	A/C ratio	P-value
COL2A1	1.8E-05	2.8E-05	0.66	0.024328
VTN	1.1E-02	1.8E-02	0.60	0.010842
CDH4	3.9E-05	6.6E-05	0.60	0.022446

Alcohol (A) and Control (C) values were normalized to the average of five housekeeping genes (β -actin, LDH, β -2 microglobulin, hypoxanthine phosphoribosyltransferase 1, and ribosomal protein, large, P1) which did not differ between groups; n=5 per group. P-values were calculated based on a 2-tailed Student's t-test of the replicate 2^{-Ct} values for each gene in the control and alcohol groups. All genes have Ct values < 30.