METABOLIC EFFECTS

Procysteine Increases Alcohol-depleted Glutathione Stores in Rat Plantaris Following a Period of Abstinence

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Abstract — Aims: To assess the effectiveness of procysteine (PRO) supplementation provided during a period of abstinence (ABS) on alcohol-induced skeletal muscle atrophy and oxidant stress. **Methods:** Age- and gender-matched Sprague–Dawley rats were fed the Lieber–DeCarli liquid diet containing either alcohol or an isocaloric substitution (control diet) for 12 week. Next, subgroups of alcohol-fed rats were fed the control diet for 2 week (ABS) supplemented with either PRO (0.35%, w/v) or vehicle. Plantaris morphology was assessed by hematoxylin and eosin staining. Total, reduced and oxidized glutathione (GSH) levels and total antioxidant potential were determined by commercially available assay kits. Antibody arrays were used to determine cytokine levels. Real-time polymerase chain reaction was used to determine gene expressions of two E3 ubiquitin ligases, atrogin-1 and muscle ring finger protein-1 (MuRF-1). **Results:** Plantaris muscles from alcohol-fed rats displayed extensive atrophy, as well as decreased GSH levels, a trend for decreased total antioxidant potential and elevated atrogin-1 and MuRF-1 mRNA levels. GSH levels and total antioxidant potential continued to decrease during 2 weeks of ABS from alcohol, which were normalized in abstinent rats provided PRO. Gene levels of both E3 ligases returned to baseline during ABS. In parallel, plantaris cross-sectional area increased in both groups during ABS. **Conclusions:** PRO supplementation during ABS significantly attenuated alcohol-induced redox stress compared with untreated abstinent rats. Thus, our data may suggest that GSH restoration therapy may provide therapeutic benefits to the overall antioxidant state of skeletal muscle when prescribed in conjunction with an established detoxification program for recovering alcoholics.

INTRODUCTION

Chronic alcohol abuse may lead to a host of skeletal muscle complications, including soreness and atrophy with concomitant losses in strength, altered gait and impaired mobility. These derangements, clinically classified as alcoholic myopathy, are likely caused by severe metabolic, physiological and structural alterations to skeletal muscle (Fernandez-Sola *et al*., 2007; Preedy *et al*., 2003). For example, development of alcoholic myopathy has been attributed in part to altered redox states and antioxidant imbalance (Adachi *et al*., 2000; Fernandez-Sola *et al*., 2007; Otis and Guidot, 2009; Otis *et al*., 2007), disparities between protein catabolism and protein synthesis (Kumar *et al*., 2002; Lang *et al*., 1999; Otis and Guidot, 2009) and acetaldehyde–protein adduct formation (Worrall *et al*., 2001).

In general, most research has suggested that chronic alcoholic myopathy is reversible with abstinence (ABS; Martin and Peters, 1985; Vary *et al*., 2004). For example, Vary *et al*. (2004) have shown that alcohol-induced deficits in protein synthesis were normalized following 72 h of alcohol withdrawal due, in part, to restoration of initiation and elongation factors. However, some case reports have suggested that muscle weakness and atrophy may endure for years (Ekbom *et al*., 1964; Rossouw *et al*., 1976), suggesting that long-term alcohol abuse may produce irreversible molecular or structural changes (Fernandez-Sola *et al*., 2007).

Intriguingly, a recent report has suggested that supplementing the diets of abstinent rats with *N*-acetylcysteine (NAC), a glutathione (GSH) precursor, significantly increased myocardial GSH peroxidase and citrate synthase activities compared with ABS alone (Seiva *et al*., 2009). These data suggested that dietary GSH precursors may improve antioxidant defense systems and energy metabolism

in abstinent rats; however, it is unclear whether similar benefits of GSH restoration extend to skeletal muscles. Encouragingly, when the GSH precursor procysteine (PRO) is provided concurrently with alcohol, alcohol-induced oxidant stress was abated and components of several anabolic pathways were induced, thereby mitigating alcoholic myopathy (Otis and Guidot, 2009). On the basis of this work, we hypothesized that muscle dysfunction reported by a subset of abstinent patients (Ekbom *et al*., 1964; Rossouw *et al*., 1976) may be due in part to alterations in the GSH antioxidant system and persistent oxidant stress. We further hypothesized that PRO supplementation provided concurrently with 2 weeks of ABS would alleviate these derangements to a greater degree than ABS alone.

MATERIALS AND METHODS

Animals and diet

Male Sprague–Dawley rats (200–250 g) were purchased from Charles River (Wilmington, MA, USA) and housed in pairs under a 12:12 light–dark cycle. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (protocol 043-2010). Rats were randomly organized into one of four groups (six per group): (a) isocaloric-fed, alcohol-naïve controls, (b) alcohol-fed for 12 week (EtOH), (c) alcohol-fed for 12 week followed by a 2-week ABS period and (d) alcohol-fed for 12 week followed by a 2-week ABS period in which animals were provided PRO (0.35% w/v, Sigma, St. Louis, MO, USA; ABS + PRO) as described previously (Bechara *et al*., 2005; Otis and Guidot, 2009; Otis *et al*., 2007).

Rats were fed the Lieber–DeCarli liquid diet (Research Diets, New Brunswick, NJ, USA) containing either alcohol

or an isocaloric substitution with Maltin–Dextrin (control diet) for 12 weeks as described previously (Otis and Guidot, 2009; Otis et al., 2007). Alcohol was added gradually to acclimatize the rats to the diet. Alcohol was added as 18% of total calories for 1 week, then 27% of total calories for 1 week and finally as 36% of total calories for 10 weeks. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). All plantaris muscles were removed in the morning, blotted dry, weighed and mounted in HistoPrep for histochemical analysis or flash frozen in liquid nitrogen for other analyses as described subsequently. Animals were sacrificed by removal of the diaphragm muscle.

Cross-sectional area measurements

Plantaris muscles were embedded in OCT and immediately frozen in isopentane cooled in liquid nitrogen as described previously (Otis and Guidot, 2009; Otis et al., 2007, 2008). Serial sections from the mid-belly of the plantaris muscle were cut at 14 μm and adhered to superfrost slides. Plantaris sections were processed for hematoxylin and eosin staining, dehydrated, mounted and visualized with a Leica microscope. Approximately 125 fibers per muscle were analyzed and cross-sectional areas (CSAs) were determined using ImageJ software (NIH, Bethesda, MD, USA).

GSH and GSH disulfide levels

GSH and GSH disulfide levels were quantified in fresh plantaris homogenates via commercially available kits according to the manufacturer's instructions (Arbor Assay Systems, Ann Arbor, MI, USA). Briefly, total and oxidized GSH levels were determined using a colorimetric substrate that reacted with the free thiol group on GSH. Absorbance at 405 nm was measured on a Multilabel counter (PerkinElmer, Waltham, MA, USA). Free (reduced) GSH was then calculated as the difference between total and oxidized GSH.

Total antioxidant potential

Total antioxidant potential (e.g. GSH, albumin, ascorbic acid and α-tocopherol) was determined in fresh plantaris homogenates via commercially available kits (OxisResearch, Foster City, CA, USA). Briefly, the reduction potential of the homogenates converted Cu^{2+} to Cu^{1+}. This reduced form of copper creates a stable 2:1 complex with the chromogenic reagent with a maximum absorption at 450 nm. Absorbance was measured on a Multilabel counter (PerkinElmer), and antioxidant potentials were calculated according to the manufacturer's instructions.

Real-time polymerase chain reaction

Plantaris muscles were collected, immediately frozen in liquid nitrogen and stored at −80°C until processed for real-time polymerase chain reaction (PCR) analyses as describedpreviously (Otis and Guidot, 2009; Otis et al., 2007, 2008). Trizol was added to the tissues (1 ml/100 mg tissue) that were then homogenized using an electric tissue homogenizer. Total RNA (2.5 μg) was reverse transcribed in a 40-μl final reaction volume, using random primers and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reverse transcription reaction was incubated at 65°C for 10 min, 80°C for 3 min and 42°C for 60 min,

respectively. Real-time PCR products were analyzed using the iCycler iQ system (Bio-Rad, Hercules, CA, USA). cDNA (5 μl of a 1:10 dilution) was amplified in a 25-μl reaction, containing 400 nm gene-specific primer pair and iQ Sybr Green Supermix (Bio-Rad). Primer sequences of superoxide dismutase 1–3 (SOD1–3), catalase, atrogin-1 and muscle ring finger protein-1 (MuRF-1) were previously designed using Primer3 (Otis and Guidot, 2009) and ordered from Sigma-Genosys (The Woodlands, TX, USA). Samples were incubated at 95°C for 15 min, followed by 40 cycles of denaturation, annealing and extension at 95, 60 and 72°C, respectively, with fluorescence recorded at the end of each annealing and extension step. As a control, real-time PCR was also performed on 2 μl of each RNA sample to confirm the absence of contaminating genomic DNA. All reactions were performed in triplicate, and the starting quantities of the genes of interest were normalized to 18S rRNA (primers supplied by Ambion, Austin, TX, USA). The $2^{-\Delta\Delta C_T}$ method was used to analyze alterations in gene expression, and values were expressed as fold changes relative to control (Otis and Guidot, 2009; Otis et al., 2007, 2008).

Statistics

One-way analyses of variance were performed followed by Student–Newman–Keuls post hoc tests using SigmaStat v2.0 software. Significance was accepted at $P \leq 0.05$.

RESULTS

Body weight and plantaris morphology

Rats fed alcohol for 12 weeks had a 29% decrease in body weight compared with control-fed rats (Fig. 1A). In parallel, plantaris fiber CSA from alcohol-fed rats displayed a significant reduction in average CSA compared with controls (Fig. 1B). Although 2 weeks of ABS was insufficient to restore body weight, both groups of abstinent rats had increased plantaris CSAs compared with muscles from alcohol-fed rats ($P \leq 0.05$).

Markers of oxidant stress

We next determined the levels of total GSH, free GSH (reduced form) and GSH disulfide (GSSG, oxidized form). Chronic alcohol ingestion reduced total and free GSH levels (Fig. 2A and B, respectively; $P \leq 0.05$). Interestingly, total and free GSH levels continued to plunge during this 2-week ABS period, but were improved by PRO supplementation ($P \leq 0.05$). GSSG levels were increased in abstinent rats supplemented with PRO (Fig. 2C).

We also determined the total antioxidant potential of plantaris muscles. This assay measures the activities of several antioxidant mechanisms, including enzymatic systems such as GSH peroxidase and catalase, large molecule systems such as albumin and small molecule systems such as uric acid, α-tocopherol and ascorbic acid. Although not statistically significant ($P = 0.06$), there was a trend for a decreased total antioxidant potential in plantaris muscles from alcohol-fed rats compared with controls (Fig. 3). Total antioxidant potential continued to decrease during this 2-week ABS period, which mirrored the decrements in the GSH
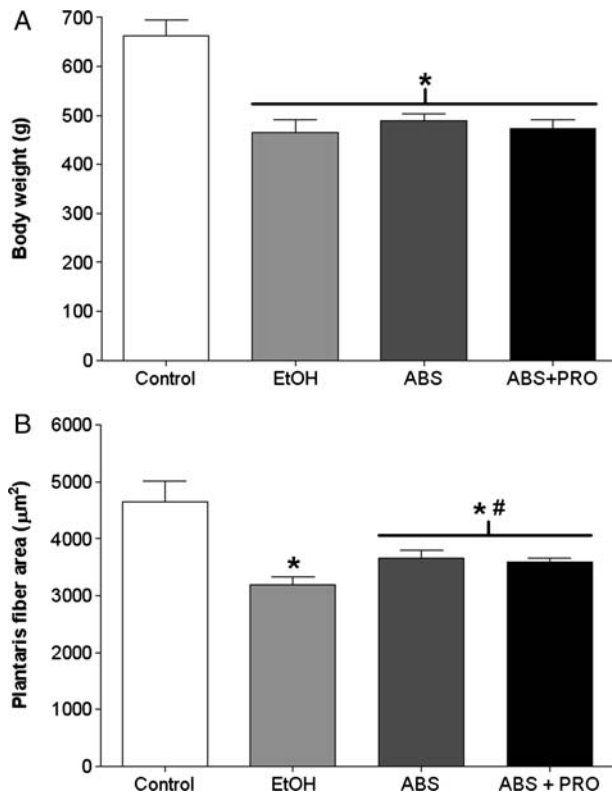
Fig. 1. Body weights and plantaris fiber areas. (A) Body weights were significantly reduced in alcohol-fed, abstinent and abstinent rats provided PRO compared with controls. (B) Plantaris muscles from rats chronically fed alcohol (EtOH) for 12 weeks had a 32% reduction in CSA compared with controls. Two weeks of ABS was sufficient to improve alcohol-induced plantaris CSA with no additional benefits conferred by PRO supplementation. Values are expressed as means + SEM ($n = 6$–7 rats/group). Significance was accepted at $P \leq 0.05$. *, compared with control group. #, compared with EtOH group.

system. PRO supplementation improved total antioxidant potential compared with ABS alone.

To determine whether the reduced total antioxidant capacity was also due in part to decreased antioxidant enzyme levels, we next quantified gene expressions of catalase, SOD-1, SOD-2 and SOD-3. However, the mRNA levels of these enzymes were unchanged by chronic alcohol ingestion or during the ABS period (Fig. 4).

Atrogin-1 and MuRF-1 gene expressions

Twelve weeks of daily alcohol ingestion induced mRNA expressions of the muscle-specific E3 ubiquitin ligases, atrogin-1 and MuRF-1 (Fig. 5). Regardless of nutritional intervention, a 2-week period of ABS was sufficient to abrogate both gene levels compared with plantaris muscles from alcohol-fed rats ($P \leq 0.05$).

DISCUSSION

In this study, we attempted to enhance muscle recovery during a period of ABS with GSH precursor supplementation. As expected, 12 weeks of daily alcohol ingestion caused significant oxidant stress and plantaris atrophy. In
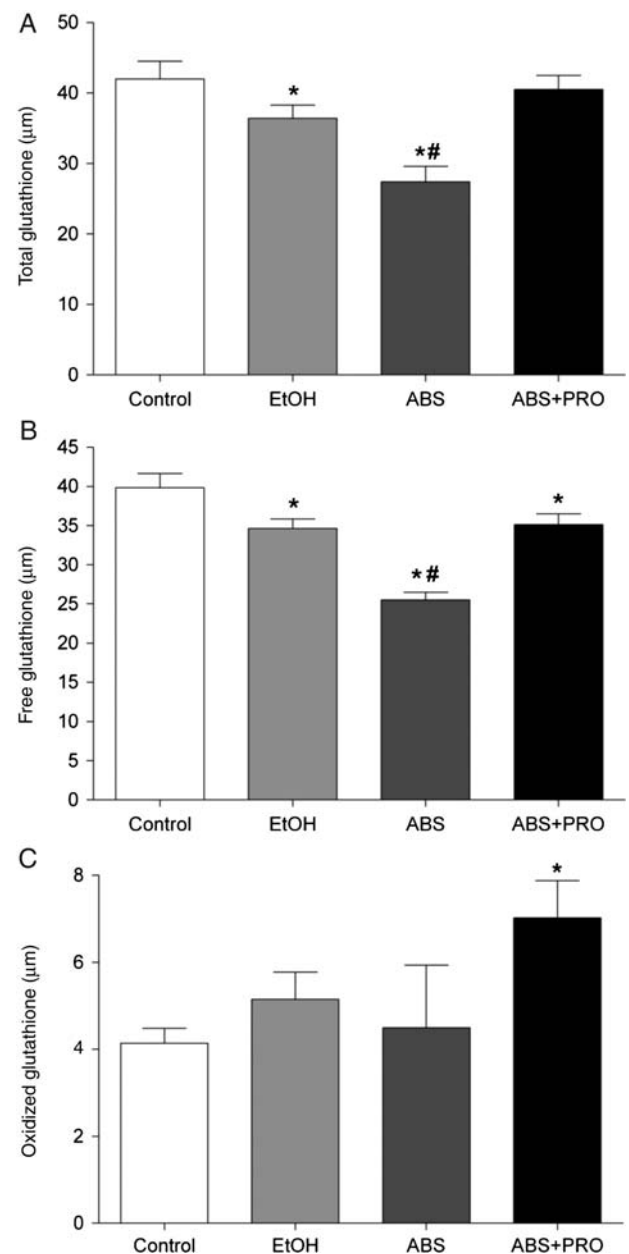

Fig. 2. GSH and GSH disulfide levels in rat plantaris. (A) Total GSH levels (i.e. the sum of free and oxidized GSH pools) were reduced in plantaris muscles from rats chronically fed alcohol (EtOH) for 12 weeks. This trend continued during a 2-week period of ABS that was normalized in rats receiving PRO. (B) The available pool of free (reduced) GSH was decreased in plantaris muscles from alcohol-fed rats and continued to decrease during ABS. PRO supplementation effectively restored the GSH pool. (C) GSH disulfide (oxidized) was increased in plantaris muscles from ABS + PRO rats. Values are expressed as means + SEM ($n = 6$–7 rats/group). Significance was accepted at $P \leq 0.05$. *, compared with control group. #, compared with EtOH group.

parallel, we showed that chronic alcohol ingestion strongly induced atrogin-1 and MuRF-1, E3 ubiquitin ligases implicated in skeletal muscle atrophy (Attaix *et al.*, 2005; Bodine *et al.*, 2001; Glass, 2005; Gomes *et al.*, 2001). Two weeks of ABS was sufficient to abate expressions of the E3 ligases and minimize the extent of alcohol-induced muscle atrophy. Despite these improvements, several markers of oxidant

stress continued to decrease during ABS. Importantly, abstinent rats provided PRO displayed marked improvements in GSH availability and total antioxidant potential compared with ABS alone. Together, these data suggest that antioxidant therapy using GSH precursors such as PRO, NAC or S-adenosyl-L-methionine may provide additional therapeutic benefits to muscle oxidant states when prescribed in conjunction with an appropriate detoxification program.

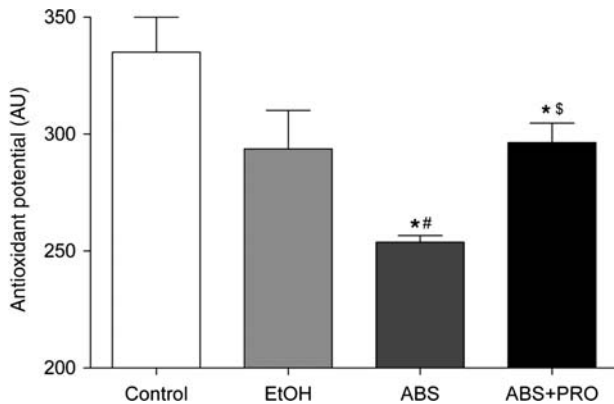Reduced levels of skeletal muscle GSH have been reported in a variety of disease states, including HIV

Fig. 3. Antioxidant potential in rat plantaris. There was a trend ($P = 0.06$) for reduced total antioxidant potential in plantaris muscles from rats fed alcohol (EtOH) for 12 weeks. Total antioxidant potential continued to decrease during ABS, but was increased due to PRO supplementation. Values are expressed as means + SEM ($n = 6$–7 rats/group). Significance was accepted at $P \leq 0.05$. *, compared with control group. #, compared with EtOH group, $, compared with ABS group.

infection (Droge et al., 1994), critical illnesses that may result in prolonged bed rest and reduced muscle activity (Burnham et al., 2005; Servais et al., 2007) and chronic obstructive pulmonary disease (Rabinovich et al., 2006). Similarly, we and others have shown that chronic alcohol ingestion altered normal GSH metabolism in skeletal muscle (Fernandez-Sola et al., 2002; Otis and Guidot, 2009; Otis et al., 2007). We now suggest that the deleterious effects of long-term alcohol ingestion on GSH metabolism and antioxidant capacity continued for at least 2 weeks of ABS. Persistent oxidant stress in muscles from abstinent rats is likely not due to defects in the SOD1–3 or catalase scavenging systems and may suggest oxidant stress may normalize with longer durations of ABS. Nevertheless, oxidant stress and reduced GSH levels did not affect muscle hypertrophy during ABS, but GSH deficiency may have significant impacts on multiple biological mechanisms. For example, GSH is the principal nucleophilic scavenger of free radicals in skeletal muscle, stabilizes other antioxidant systems, reduces proteins and helps to maintain their function, attenuates redox-sensitive catabolic factors and has salutary effects on membranes through the reduction of alcohol-induced lipid peroxidation (Jackson, 2008; Otis et al., 2007; Wu et al., 2004). Thus, this enduring oxidant stress may provide a partial reason why muscle dysfunction persists in small subsets of abstinent patients (Ekbom et al., 1964; Rossouw et al., 1976).

Nevertheless, muscle atrophy is often reversible for former alcoholics enrolled in a rehabilitation program that focuses on ABS and nutritional support (Andersen et al., 1998; Fernandez-Sola et al., 2000; Peters et al., 1985; Sestoft et al., 1994; Slavin et al., 1983). Our data may suggest that these
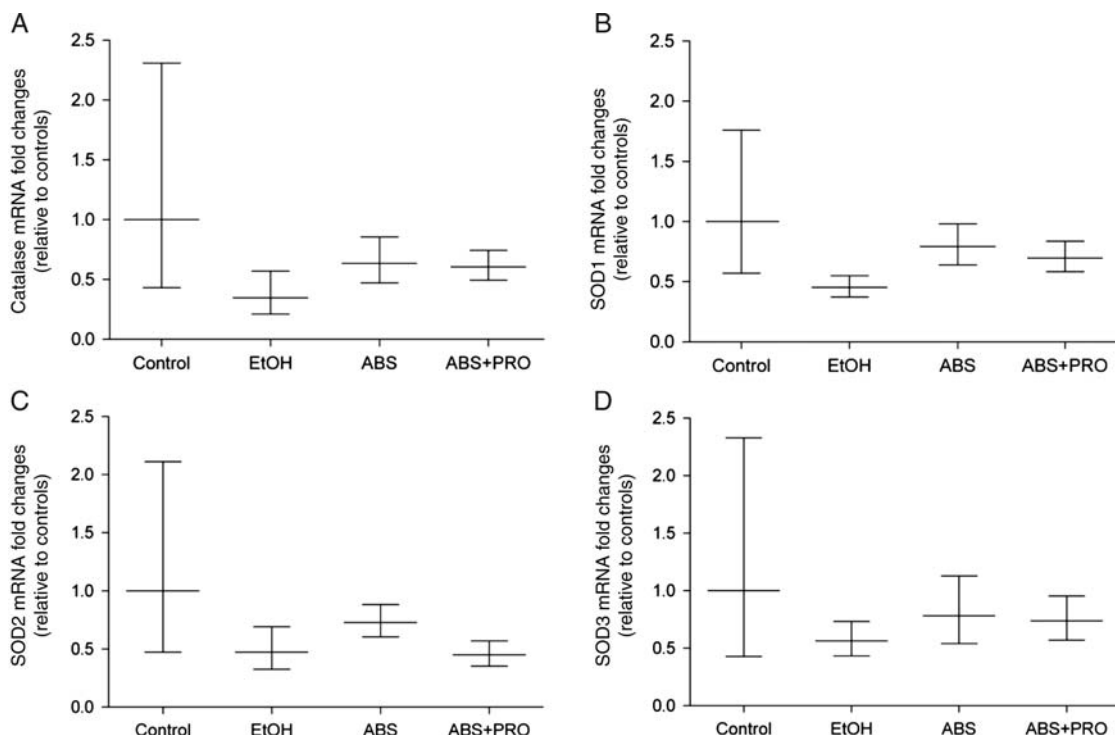

Fig. 4. Catalase and SOD isozymes gene expressions in rat plantaris. Gene expressions of (A) catalase and (B–D) three SOD isozymes (i.e. Cu/Zn-SOD1, Mn-SOD2 and Cu/Zn-SOD3) were unchanged in any group. Data are represented as means ± range of potential values based on the $2^{-\Delta\Delta C_T}$ method (Otis et al., 2007; Otis et al., 2008) and expressed as fold changes relative to controls ($n = 6$–7 rats/group). Significance was accepted at $P \leq 0.05$.
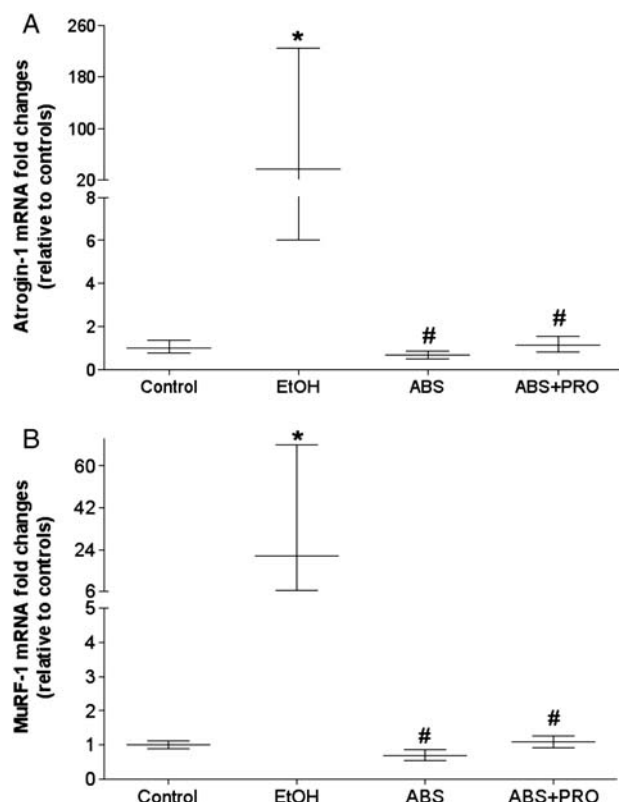
Fig. 5. Atrogin-1 and MuRF-1 gene expressions in rat plantaris. Real-time PCR analyses were performed for atrogin-1 and MuRF-1in plantaris muscles from rats chronically fed alcohol (EtOH) for 12 weeks. Alcohol ingestion dramatically increased mRNA levels of both E3 ligases. Two weeks of ABS was sufficient to normalize atrogin-1 and MuRF-1 inductions due to alcohol ingestion with no additional benefits conferred by PRO supplementation. Data are represented as means ± range of potential values based on the $2^{-\Delta\Delta C_T}$ method (Otis *et al.*, 2007; Otis *et al.*, 2008) and expressed as fold changes relative to controls ($n = 6$–7 rats/group). Significance was accepted at $P \leq 0.05$. *, compared with control group. #, compared with EtOH group.

gains in muscle fiber size may be due in part to the normal attenuation of catabolic factors such as atrogin-1 and MuRF-1 once the alcohol stimulus has been removed. Unfortunately, complete ABS from alcohol is often difficult to achieve with documented success rates of 33% (Fillmore *et al.*, 1991). Fernandez-Sola *et al.* (2000) have shown that muscle strength can be improved in recovering alcoholics who adhere to a controlled, low-dose alcohol (≤60 g/day) consumption paradigm. Although these data are encouraging for 67% of recovering alcoholics that struggle to completely abstain from alcohol, the impact of low-dose alcohol consumption on oxidant stress was not considered. In light of our current work, alcohol treatment centers may recommend low-dose alcohol consumption in combination with dietary GSH restoration to maximize muscle recovery and more quickly normalize muscle redox states.

Atrogin-1 and MuRF-1 are E3 ubiquitin ligases implicated in skeletal muscle atrophy (Attaix *et al.*, 2005; Bodine *et al.*, 2001; Gomes *et al.*, 2001; Lang *et al.*, 2007; Mastrocola *et al.*, 2008; Otis *et al.*, 2008) and appear to regulate early stages of alcoholic myopathy. Specifically, atrogin-1 was strongly induced in plantaris muscles from rats chronically fed alcohol for 6 weeks, a duration of abuse that preceded

muscle atrophy (Otis *et al.*, 2007). Here, both atrogin-1 and MuRF-1 remained elevated in atrophied plantaris muscles from rats fed alcohol for 12 weeks. Importantly, atrogin-1 was attenuated following GSH restoration during these shorter durations of abuse. Yet, when alcohol abuse continued for longer durations (e.g. 35 weeks), we have shown that atrogin-1 levels become refractory to GSH restoration (Otis and Guidot, 2009), suggesting that early intervention with GSH precursors is integral to combat alcohol-induced catabolic factors. Interestingly, the current work unveiled a cyclical response of atrogin-1 as we have previously shown that 28 weeks of chronic alcohol abuse did not induce expression of this ligase, despite the presence of overt plantaris atrophy (Otis and Guidot, 2009). In support of this notion, several reports suggested that muscle proteolysis or atrophy may occur independently of changes in atrogin-1 mRNA levels (Cai *et al.*, 2004; Fareed *et al.*, 2006; Vary *et al.*, 2008). Together, these data may reveal important temporal associations between alcohol-induced GSH depletion, redox-sensitive induction of E3 ligases and resultant muscle atrophy.

In conclusion, we showed that long-term alcohol ingestion created an overall catabolic state in atrophied rat plantaris muscles, as evidenced by oxidant stress and induction of ubiquitin ligases. Although ABS alone was sufficient to improve skeletal muscle fiber diameter, several markers of oxidant stress persisted. PRO supplementation provided during this abstinent period abrogated oxidant stress, improved muscle antioxidant capacity and attenuated production of the E3 ligase atrogin-1. Thus, our data may suggest that GSH restoration therapy may provide therapeutic benefits to the overall antioxidant state of skeletal muscle when prescribed in conjunction with an established detoxification program for recovering alcoholics.

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