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Acute alcohol prevents the refeeding-induced decrease in autophagy but does not alter the increased protein synthetic response in heart

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Abstract

Ethanol produces a state of anabolic resistance in skeletal muscle; however, whether the heart displays a similar defect is unknown. Hence, the purpose of this study was to determine the impact of acute ethanol administration on the major signal transduction pathways in the heart that are responsible for regulating the protein synthetic and degradative response to refeeding. Adult male C57Bl/6 mice were fasted for 12 h. Mice were then either refed normal rodent chow for 30 min or a separate group of mice remained food deprived prior to administration of 3-g/kg ethanol. Cardiac tissue and blood were collected 1 h thereafter and analyzed. Acute ethanol prevented the nutrient-induced stimulation of S6K1 phosphorylation in heart, but did not alter the phosphorylation of S6, eIF4B, and eEF2, known downstream substrates for this kinase. The refeeding-induced redistribution of eIF4E into the active eIF4F complex was also not changed by acute ethanol. Consistent with the above-mentioned changes in signaling proteins, ethanol did not impair the refeeding-induced increase in cardiac protein synthesis. Proteasome activity was not altered by alcohol and/or refeeding. In contrast, ethanol antagonized the refeeding-induced increase in ULK1 phosphorylation and p62 as well as the reduction in LC3B-II and Atg5/12 complex proteins. These data indicate that acute ethanol prevents the normally observed inhibition of autophagy seen after refeeding, while the mTOR-dependent increase in protein synthesis remains largely unaltered by alcohol.

Keywords

Ethanol; Protein synthesis; mTOR; Ubiquitin; Proteasome; Autophagy; Insulin; Heart; Signal transduction

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Conflict of interest statement
None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.alcohol.2018.04.005>.

Introduction

Ethanol, both acute intoxication and chronic consumption, has profound effects on a variety of organs, including the heart, adversely affecting morbidity and mortality. Although overt alcoholic cardiomyopathy is predominantly observed in response to heavy prolonged ethanol consumption, the acute ingestion of ethanol has also been reported to impair various aspects of cardiac contractile function and metabolism (Piano & Phillips, 2014; Steiner & Lang, 2017). For example, in preclinical models, the detrimental effects of acute high dose alcohol intake *in vivo* often include decreased cardiac output, left ventricular (LV) ejection fraction and fractional shortening, LV end-diastolic and end-systolic diameters, and positive and negative dP/dt (El-Mas & Abdel-Rahman, 2014; Ma, Li, Gao, & Ren, 2009; Umoh, Walker, Al-Rubaiee, Jeffress, & Haddad, 2014; Zhu et al., 2018). Contractile abnormalities are also present in humans after acute alcohol intoxication (Delgado, Gortuin, & Ross, 1975; Reant et al., 2012). As a result, these changes that first manifest during acute ethanol exposure may, if sustained over time, contribute to the development of alcoholic cardiomyopathy.

Acute alcohol also impairs various aspects of myocardial glucose, lipid, and protein metabolism. Regarding the latter, changes in tissue protein content are regulated by the dynamic interaction between protein synthetic and degradative pathways (Steiner & Lang, 2017). Hence, alterations in protein homeostasis may compromise the myofibrillar architecture and/or alter the abundance or activity of other cellular proteins that would in part provide an explanation for ethanol-induced defects in cardiac contractility. There are independent reports that ethanol acutely decreases the rate of total (global), myofibrillar (contractile), and sarcoplasmic (non-contractile) protein synthesis and translational efficiency in heart (Lang, Frost, Kumar, & Vary, 2000; Preedy & Peters, 1990; Siddiq, Richardson, Mitchell, Teare, & Preedy, 1993; Siddiq, Richardson, Morton, et al., 1993). This response is present in both atrial and ventricular tissue (Siddiq, Richardson, Morton, et al., 1993), and manifests within 30 min of ethanol administration and persists for up to 4 h (Vary & Lang, 2008). This decrement in the basal rate of protein synthesis is mediated by mTOR (mechanistic target of rapamycin), as evidenced by the reduced phosphorylation of both downstream substrates, eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) and ribosomal protein S6 kinase-1 (S6K1) (Lang et al., 2000). In contrast, ethanol does not acutely alter the eIF2/2B system that is essential in the formation of the ternary complex, the first step in translation initiation (Lang et al., 2000). Moreover, there is little or no change detected in the myocardial content of high-energy phosphates or the NADH redox state (Auffermann, Camacho, et al., 1988; Auffermann, Wu, et al., 1988). As all cellular proteins experience continuous turnover, alterations in protein degradation can also potentially impact protein homeostasis; however, there is a paucity of information pertaining to the acute effects of ethanol on this side of the protein balance equation.

Muscle protein synthesis, even under steady-state conditions, experiences diurnal fluctuations in response to fasting and refeeding (Garlick, Millward, & James, 1973). Previous attempts to mimic isolated components of the refeeding cycle have included bolus administration of the branched-chain amino acid leucine or insulin-like growth factor (IGF)-I. Acute alcohol has been shown to blunt the IGF-I induced increase in mTOR-dependent signal transduction in heart (Lang, Kumar, Liu, Frost, & Vary, 2003), but not the protein

synthetic effect of leucine (Vary, 2009). While these previous studies generated valuable data, they are nonetheless limited by the non-physiological and pharmacological nature of the stimuli used that does not fully mimic consumption of a complete nutrient load and the resulting endogenous hormonal response. Hence, the purpose of this study was to determine the impact of acute ethanol administration on the major signal transduction pathways in the heart that are responsible for regulating the protein synthetic and degradative response to refeeding.

Materials and methods

Acute alcohol and refeeding protocols

The experimental protocols described below were performed in accordance with the National Institutes of Health (NIH) guidelines for the use of animals and were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine (#46587). Briefly, male 12-week-old C57BL/6 mice (Charles River Breeding Laboratories; Cambridge, Massachusetts) were acclimated for at least 1 week prior to the start of the experiment. Throughout, mice were individually housed in shoebox cages with corn cob bedding under environmentally controlled conditions (22 ± 1 °C; 12-h/12-h light/dark cycle), and mice were provided standard rodent chow (Envigo Global no. 8604 diet; percent calories from protein 32%, from fat 14%, and from carbohydrates 54%; Envigo Teklad; Boston, Massachusetts) and water *ad libitum*. The night before the start of the study, all mice were fasted beginning at 2000 h, but continued to have free access to water throughout the remainder of the experiment. The following morning, mice were randomly assigned to either a) the refed group and were provided access to food for 30 min or b) to the fasted group that remained food-deprived. For the refed group, chow (described above) was placed in a container within the confines of the cage for easy access. After 30 min, food intake for the refed group was measured gravimetrically. Thereafter, mice in the refed and fasted groups were randomly assigned to receive either an intraperitoneal (i.p.) injection of ethanol (3 g/kg) or an equal volume of 0.9% saline (e.g., “control” group). Mice were euthanized 60 min after injection of ethanol or saline. Body weight at the time of euthanasia did not differ among experimental groups: fasted control (23.2 ± 0.5 g), refed control (22.4 ± 0.4 g), fasted ethanol (23.1 ± 0.5 g), and refed ethanol (22.6 ± 0.4 g).

In vivo protein synthesis

The rate of *in vivo* protein synthesis was determined by injecting mice with puromycin (i.p.; 0.04 $\mu\text{mol/g}$) 30 min prior to sacrifice, as previously described (Steiner, Kimball, & Lang, 2016). Mice were anesthetized using isoflurane (3–5% in O_2). The heart from each mouse was excised, frozen between clamps that had been precooled to the temperature of liquid nitrogen and stored at -70 °C, and then weighed. Hearts were homogenized in ice-cold homogenization buffer consisting of (in mM) 48.3 HEPES (pH 7.4), 4 EGTA, 10 EDTA, 15 sodium pyrophosphate, 100 β -glycerophosphate, 25 sodium fluoride, 5 sodium vanadate, 0.1% Triton X-100, and 1 $\mu\text{L/mL}$ protease inhibitor. The amount of protein in each sample was determined using a BioRad protein assay kit (Bio-Rad; Hercules, California). Western blotting was performed, on equal amounts of total protein per sample, using an anti-

puromycin antibody for immunological detection of puromycin-labeled peptides (Kerafast; Boston, Massachusetts).

Western blotting

Homogenates were clarified by centrifugation and mixed with 2× Laemmli SDS sample buffer. Equal amounts of protein per sample were subjected to electrophoresis on 4–20% SDS-PAGE Criterion gels for total and phosphorylated S6K1 (Thr389), S6 (Ser235/236), S6 (Ser240/244), and 4E-BP1 (Ser65; Bethyl Laboratories; Montgomery, Texas). In addition, total and phosphorylated p44/42 MAPK ERK (1/2), total and phosphorylated eIF4B (Ser422), total and phosphorylated eIF2a (Ser51), total and phosphorylated eIF4E (Ser209), total and phosphorylated AKT (both Thr308 and Ser473), total and phosphorylated PRAS40 (T246), and total and phosphorylated TSC2 (T1462), as well as REDD1 (regulated in development and DNA damage responses; Millipore; Billerica, Massachusetts) were also determined by Western analysis. To assess autophagy, Western analysis was performed using antibodies against LC3B, p62, and total and phosphorylated ULK (Ser757). The relative amount of 4E-BP1 and eIF4G complexed with eIF4E was determined by immunoprecipitation using an anti-eIF4E monoclonal antibody (Dr. Kimball; Hershey, Pennsylvania). Proteins were transferred onto polyvinylidene fluoride (PVDF; Immobilon P) membranes and incubated with a primary antibody overnight at 4 °C. The blots were developed using enhanced chemiluminescent Western blotting reagents and then exposed to X-ray film in a cassette equipped with a DuPont Lightening Plus intensifying screen. The film was scanned and analyzed using NIH Image 1.6 software.

Protein degradation

Hearts were homogenized in lysis buffer containing (in mM) 25 HEPES, 5 MgCl₂, 5 EDTA, 5 DTT, pH 7.5 at 4 °C followed by centrifugation at 14,000 rpm for 2 min at 4 °C, exactly as described (Steiner et al., 2016). The proteasome enzymatic activity was measured by using a proteasome 20S assay kit (Enzo Life Sciences; Farmingdale, New York) as described (Lang & Korzick, 2014). Proteasome 20S activity was determined by measuring the hydrolysis of a fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC (AMC: 7-amino-4-methylcoumarin), which is cleaved by proteasome activity. The subsequently released free AMC was detected using a fluorometer (excitation wavelength 380 nm; emission wavelength 460 nm). The fluorescence signal was monitored before and 1 h after incubation at 37 °C. The change in fluorescence signal was normalized to the amount of protein determined in the supernatant. Each sample/substrate combination was measured both in the presence and in the absence of a highly specific 20S proteasome inhibitor MG132 (1 μM; Boston Biochem; Cambridge, Massachusetts) to account for any nonproteasomal degradation. Data were recorded for 1 h and chymotrypsin-like activity was calculated as AFU/min/mg protein over the linear range.

RNA extraction and real-time quantitative PCR

To assess the mRNA content for two muscle-specific E3 ligases in heart tissue, total RNA was extracted using Tri-reagent (Molecular Research Center, Inc.; Cincinnati, Ohio) and an RNeasy mini kit (Qiagen; Valencia, California), according to the manufacturer's instructions and exactly as previously described (Steiner et al., 2016). RNA was eluted from the column

with RNase-free water and an aliquot was used for quantitation (NanoDrop 2000, Thermo Fischer Scientific; Waltham, Massachusetts). Quality of the RNA was analyzed on a 1% agarose gel. Total RNA (1 µg) was reverse-transcribed to cDNA using superscript III reverse transcriptase (Invitrogen; Carlsbad, California) in a total reaction volume of 20 µL following instructions from the manufacturer. RT-qPCR was performed on 2 µL of the reversed transcribed reaction mix in a StepOnePlus system using TaqMan gene expression assays (Applied Biosystems; Foster City, California) for the following atrogen-1 (NM_026346.2) and muscle RING-finger 1 (MuRF1; NM_001039048.2). The comparative quantitation method 2^{-Ct} was used in presenting gene expression, normalized to GAPDH mRNA, and all data were referenced to the average value of the fasted saline-injected control (no ethanol) group, which was arbitrarily set at 1.0.

Plasma insulin, alcohol, and branched-chain amino acid concentrations

The plasma concentration of insulin was determined using a mouse-specific ultrasensitive insulin enzyme-linked immunosorbent assay (Alpco; Salem, New Hampshire; catalog #80-INSMSU-E01). The plasma alcohol concentration was determined using a rapid analyzer (Analox Instruments; Lunenburg, Massachusetts). Leucine, isoleucine, and valine were determined using reverse-phase HPLC after precolumn derivatization of amino acids with phenylisothiocyanate.

Statistics

Values were presented as means ± SEM for the number of mice per group as indicated in the figures. The data were analyzed using two-way analysis of variance with *post hoc* Student-Newman-Keuls test to determine significant differences among the four experimental groups. Differences were considered significant when $p < 0.05$.

Results

Food intake and blood alcohol concentrations

Food intake during the refeeding period did not differ significantly ($p > 0.05$) between control (420 ± 53 mg) and ethanol-treated mice (408 ± 36 mg). The blood alcohol concentration also did not differ between mice in the refed + ethanol group (37 ± 5 mM) and those in the fasted + ethanol group (34 ± 5 mM).

Signal translation related to protein synthesis

Because of its key regulatory role in protein synthesis under basal conditions and after nutrient stimulation, we examined proximal signaling pathways central to mTOR complex 1 (mTORC1). AKT occupies a key signaling node proximal to mTOR, and AKT phosphorylation at both T308 and S473 increases site-specific phosphorylation of downstream substrates (Manning & Toker, 2017). Fig. 1 illustrates that the extent of T308 and S473 AKT phosphorylation did not differ under fasted conditions in alcohol-treated and time-matched saline-treated control mice. Refeeding increased phosphorylation at both sites in control hearts. However, alcohol selectively prevented the refeeding-induced increase in AKT phosphorylation at T308, but not S473.

As this differential phosphorylation may impact kinase activity, we assessed surrogate markers of AKT activity by examining two downstream substrates – PRAS40 and TSC2 – that have been implicated in regulating protein synthesis (Manning & Toker, 2017). The phosphorylation pattern for PRAS40 (T246) and TSC2 (T1462) was similar to that seen for AKT T308 phosphorylation (Fig. 1). That is, there was no significant effect of alcohol on the basal phosphorylation of these proteins in the fasted state, but acute alcohol prevented the refeeding-induced increase in PRAS40 and TSC2 phosphorylation seen in saline-treated control mice.

Given the discordant results for Akt phosphorylation, we next determined the phosphorylation of 4E-BP1 and S6K1, as these proteins are direct substrates for mTORC1 and are typically used to assess its activity *in vivo* (Frost & Lang, 2011). In saline-treated control mice, refeeding increased 4E-BP1 S65 phosphorylation, compared to time-matched fasted control values (Fig. 2). This increase appeared physiologically important, as it was associated with a reduction in the amount of the translational repressor 4E-BP1 bound to eIF4E as well as an increase in the binding of eIF4G to eIF4E, changes that are consistent with increased cap-dependent translation (Haghighat & Sonenberg, 1997). 4E-BP1 phosphorylation was also increased in alcohol-treated mice under fasted conditions, an increase comparable to that seen in refeed control mice (Fig. 2). However, despite this increase, the relative amount of the inhibitory 4E-BP1 ·eIF4E and stimulatory eIF4G·eIF4E complexes were unchanged in fasted alcohol-treated mice. Additionally, no further increase in 4E-BP1 phosphorylation was observed in alcohol-treated mice after refeeding. Alcohol did not prevent the refeeding-induced increase in eIF4G binding to eIF4E or alter the binding of 4E-BP1 to eIF4E, compared with values from saline-treated control mice. There was no alcohol- or feeding-induced change in the total amount of eIF4E (data not shown).

Refeeding of saline-treated control mice consistently increased T389 phosphorylated S6K1, another mTORC1 substrate (Fig. 3). Furthermore, refeeding coordinately increased the phosphorylation of known S6K1 substrates, ribosome protein S6 and eIF4B, and decreased the phosphorylation of eEF2. In the fasted condition, alcohol-treated mice demonstrated an increased phosphorylation of S6K1 and eIF4G, compared to fasted control values, whereas the phosphorylation for other S6K1 substrates (e.g., S6, eIF4B, and eEF2) did not differ from values in saline-treated control mice (Fig. 3). Similar to control mice, refeeding ethanol-treated mice increased the phosphorylation of S6 and eIF4B while decreasing the phosphorylation of eEF2.

The MEK/ERK/RSK signal transduction pathway can also modulate protein synthesis (Zhang, Liu, Townsend, & Proud, 2013). Whereas there were no refeeding-induced changes in the extent of MEK1/2 (S271/S221), ERK1/2 (T202/Y204) or p90 RSK (S380) phosphorylation in saline-treated control mice, there was a consistent decrease in the phosphorylation state of each of these three proteins in heart in response to alcohol (Fig. 4), but no further change following refeeding.

Because of the unanticipated results in signal transduction described above, we also determined the *in vivo* rate of global protein synthesis. Cardiac protein synthesis did not differ between saline-treated control and alcohol-treated mice in the fasted condition (Table

1). Furthermore, while both saline-treated control and alcohol-treated mice showed a >50% increase in protein synthesis after refeeding, the incremental response was similar in both groups.

Autophagy

Autophagy is a catabolic process that is essential for the normal turnover of cytoplasmic proteins and organelles (Cohen-Kaplan, Livneh, Avni, Cohen-Rosenzweig, & Ciechanover, 2016). Activation of mTORC1 can increase S757 phosphorylation of ULK1, thereby inhibiting autophagy via disruption of the interaction between AMPK and ULK1 (Gallagher, Williamson, & Chan, 2016). Refeeding increased ULK1 S757 phosphorylation more than 2-fold in hearts from saline-treated control mice (Fig. 5). Furthermore, ULK1 phosphorylation was decreased in alcohol-treated mice to the same extent in both the fasted and refed state. Surrogate markers of autophagy were decreased in saline-treated control mice after refeeding as evidenced by the reduction in LC3-II and Atg5-12 protein levels as well as the increase in p62 (Fig. 5). In contrast, LC3-II and Atg5-12 were increased and p62 protein content was decreased in fasted alcohol-treated mice, compared to fasted control values. There were no differences in surrogate markers of autophagy in hearts from alcohol-treated mice in the fasted and refed state. Finally, there was no alcohol- or refeeding-induced change in Beclin1 protein content.

Proteasome activity

The ubiquitin (Ub)-proteasome pathway (UPP) is responsible for the degradation of the majority of intracellular proteins and is activated in many catabolic conditions (Bilodeau, Coyne, & Wing, 2016). We detected no alcohol- and/or refeeding-induced change in the mRNA content for atrogin-1 or MuRF1 (data not shown), two Ub-E3 ligases. Similarly, there was no detectable change in the relative amount of ubiquitinated proteins or the various molecular weight forms of calpastatin in response to alcohol and/or refeeding (data not shown). Lastly, *in vitro* determination of proteasome activity was also not altered by alcohol and/or refeeding (Table 1).

Apoptosis and inflammasome pathway

Surrogate markers of apoptosis as well as the canonical and non-canonical inflammasome pathway were also assessed. There were no statistically significant alcohol or refeeding effects on the protein expression of cleaved caspase 3 and cleaved PARP (e.g., apoptosis), NLRP3 or cleaved caspase 1 (canonical inflammasome), or caspase 11 and Gasdermin D (non-canonical inflammasome) (data not shown).

Plasma insulin and branched-chain amino acid concentrations

There were no differences in the plasma concentrations for the branched-chain amino acids leucine, isoleucine, or valine between saline-treated control and alcohol-treated mice in the fasted condition (Table 1). While refeeding increased the plasma leucine concentration, the increase was comparable in saline-treated control (80%) and alcohol-treated (110%) mice. There were no statistically significant alcohol- or refeeding-induced changes in plasma isoleucine and valine concentrations.

The plasma insulin concentration increased upon refeeding of the saline-treated control mice. Alcohol alone and in combination with refeeding also increased plasma insulin, and these levels did not differ from those observed in the refeed control mice (Table 1).

Discussion

The present study reports a comprehensive analysis of signal transduction pathways central to the regulation of protein synthesis and degradation in cardiac tissue in response to a physiologically relevant refeeding stimulus and the impact of acute alcohol on these multiple pathways. Our data demonstrate that refeeding in saline-treated control mice acutely stimulated the cardiac AKT-mTORC1 pathway, thereby acutely increasing protein synthesis and decreasing protein degradation via autophagy, without apparently altering the activity of the Ub-proteasome. These coordinated changes were consistent with the increased plasma concentrations of insulin and leucine. Acute alcohol did not alter the refeeding-induced increase in cardiac protein synthesis; however, autophagy remained elevated in the alcohol-treated mice after refeeding, despite comparable increases in insulin and leucine. The data suggest that in heart acute alcohol administration has a more profound effect on refeeding-induced changes in protein degradation (i.e., autophagy).

Refeeding-induced changes in saline-treated control mice

In contrast to skeletal muscle, there is a paucity of data in heart on the normal anabolic actions of refeeding in control (e.g., non-alcohol treated) mice. Our data indicate that in the absence of alcohol, refeeding activated the canonical AKT-mTORC1 pathway, a conclusion consistent with that of a more limited earlier study (Vary & Lynch, 2006). Moreover, activation of AKT-mTORC1 has also been observed in heart in response to leucine alone (Vary, 2009), supporting the view that the majority of the feeding-induced increase is due to the presence of this dietary branched-chain amino acid. Activation of AKT-mTORC1 by refeeding was evidenced by the increased T308 phosphorylation of AKT as well as the phosphorylation of two authentic protein substrates, PRAS40 and TSC2. All of these changes are internally consistent with the activation of mTORC1 that was confirmed by the phosphorylation of S6K1 and 4E-BP1. The functional relevance of the refeeding-induced increase in S6K1 was implied by the increased phosphorylation of downstream substrates S6, eIF4B, and eIF4G, all of which would be anticipated to increase mRNA translation initiation (Magnuson, Ekim, & Fingar, 2012). Additionally, S6K1 phosphorylates eEF2 kinase that in turn inhibits the phosphorylation of eEF2. This later change was observed in response to refeeding and would be expected to enhance the elongation phase of mRNA translation (Kenney, Moore, Wang, & Proud, 2014). The refeeding-induced increase in 4E-BP1 phosphorylation also appears to be physiologically relevant, being associated with a redistribution of eIF4E from the inactive 4E-BP1•eIF4E complex to the active eIF4G•eIF4E complex. These latter changes are consistent with the ability of hyperphosphorylated 4E-BP1 to disrupt the inhibitory 4E-BP1•eIF4E complex, and thereby stimulated cap-dependent translation (Sekiyama et al., 2015). Overall, the activation of the AKT-mTORC1 pathway is consistent with the increase in global protein synthesis observed in hearts of mice not receiving alcohol.

The refeeding-induced activation of mTORC1 was also associated with increased S757 phosphorylation of ULK1, a key regulator of protein degradation via the autophagy pathway. Increased ULK1 phosphorylation has been reported to decrease autophagy (Gallagher et al., 2016), and this change is consistent with the decrease in LC3B-II and Atg 5/12 as well as the increase in p62 seen in hearts of refed saline-treated control mice. These coordinated changes are also consistent with the refeeding-induced increase in plasma concentrations of insulin and leucine. In contrast, refeeding is unlikely to acutely alter protein degradation via the Ub-proteasome pathway, as we detected no change in 20S proteasome activity, Ub content, or the mRNA content for the muscle-specific Ub-E3 ligases atrogin-1 and MuRF1. However, this conclusion is limited because only a single time point was determined and we cannot exclude the possibility that refeeding affects the Ub-proteasome pathway at a later time. Overall, by increasing the circulating levels of both insulin and leucine, the refeeding stimulus was sufficient to fully activate mTORC1, thereby increasing protein synthesis and inhibiting autophagy in hearts from control mice that did not receive alcohol.

Acute alcohol and cardiac protein balance

In the fasted condition, acute alcohol did not alter the plasma concentration of any of the branched-chain amino acids, but did increase plasma insulin to levels seen in saline-injected control mice after refeeding. *In vivo* administration of alcohol has variable effects on insulin secretion, with the plasma insulin concentration having been reported to be increased, decreased, or unchanged depending on the specific experimental context (Steiner, Crowell, & Lang, 2015). The reason for this inconsistent response in plasma insulin, in both the basal condition and in response to secretagogues, is currently unknown and difficult to isolate because of differences in experimental protocols (e.g., animal species and sex as well as the amount and duration of alcohol exposure). Regardless of the mechanism, the alcohol-induced increase in insulin was associated with increased mTORC1 activity, as evidenced by the increased phosphorylation of both S6K1 and 4E-BP1. However, this increase appeared independent of upstream activation of AKT (e.g., no phosphorylation of AKT, PRAS40, or TSC2). Moreover, activation of mTORC1 did not appear to be complete as there was no change in the phosphorylation of eIF4B and eEF2 by S6K1 nor a redistribution of eIF4E between the active and inactive eIF4F complexes as a result of 4E-BP1 phosphorylation after alcohol administration in the fasted state. The reason for the discrepancy between the extent of 4E-BP1 phosphorylation and the redistribution of eIF4E is not known, but has been previously reported and may reflect dynamic temporal differences in these signaling events that are not captured in studies looking at a single time point (Vary & Lynch, 2006).

While the above-mentioned alcohol-induced changes in signaling were capable of stimulating mTORC1, at least in part, they were not sufficient to increase global cardiac protein synthesis. The lack of an alcohol-induced effect on cardiac protein synthesis differs from previous studies that have reported a decrease (Lang et al., 2000; Preedy & Peters, 1990; Vary, 2009). Importantly, the three studies showing an alcohol-induced decrease in cardiac protein synthesis were all performed in rats where there was no compensatory increase in insulin. Such data are suggestive of a species-specific response and emphasizes the importance of assessing known stimulators for the protein synthesis so that studies can be critically compared. Moreover, these data are generally consistent with the ability of

insulin and amino acids to independently stimulate protein synthesis in response to feeding (Suryawan & Davis, 2011).

Likewise, when alcohol was acutely administered in the fasted state, there was no change in the phosphorylation state of ULK1, as might be expected with full activation of mTORC1 (Gallagher et al., 2016). As a result, acute alcohol increased LC3B-II and Atg 5/12 and decreased p62 content, suggesting increased autophagy in hearts of fasted mice. In contrast, alcohol did not appear to acutely alter protein degradation, as the mRNA content for the E3 ligases MuRF1 and atrogin-1 was unchanged, as was the relative amount of ubiquitinated proteins and *in vitro*-determined proteasome activity.

Alcohol modulates refeeding-induced changes

Acute alcohol largely antagonized the refeeding-induced increase in the phosphorylation of AKT (T308), PRAS40, and TSC2. Alcohol also prevented the expected increase in phosphorylation of S6K1 and 4E-BP1. Despite this inhibitory effect of alcohol, refeeding increased the phosphorylation of S6 and eIF4B, decreased the phosphorylation of eEF2, and increased the amount of active eIF4G•eIF4E complex. Together, these signaling changes were associated with an increased rate of myocardial protein synthesis. Similarly, a previous study in rats reported a comparable increment in cardiac protein synthesis in response to a maximally stimulating pharmacological dose of leucine given orally after acute alcohol (Vary, 2009). Collectively, these data demonstrate there is no alcohol-induced anabolic resistance in heart after refeeding. It is noteworthy that these data are in contrast to the marked anabolic resistance observed in skeletal muscle when acute alcohol administration precedes leucine or intragastric refeeding (Lang, Frost, et al., 2003; Sneddon et al., 2003). The underlying mechanism for this differential tissue specificity of alcohol on activating protein synthesis remains to be elucidated. In heart, refeeding was not sufficient to prevent the alcohol-induced increase in autophagy, as evidenced by the reduced phosphorylation of ULK1, increased content of LC3B-II and Atg 5/12, and decrease in p62 protein. Hence, our data demonstrate that while acute alcohol does not alter the protein synthetic anabolic response to refeeding, alcohol did effectively prevent the ability of refeeding to decrease autophagy in the heart. Additional studies will be needed to determine whether such a change persists with chronic alcohol consumption, and whether the resulting imbalance in protein homeostasis is causally related to the development of alcoholic cardiomyopathy.

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Fig. 1.

Refeeding-induced changes in AKT, PRAS40, and TSC2 phosphorylation in hearts from saline-treated control and alcohol-treated mice. A representative Western blot for each protein of interest is shown in the top left panel. Samples from all four experimental groups were run on the same gel and the vertical white line indicates that intervening lanes of the gel have been removed for presentation purposes. Bar graphs represent densitometric analysis of all immunoblots, where the value from fasted saline-treated control mice was set at 100 AU. Values are means $\pm$ SEM; $n = 10, 10, 12$, and 12 , respectively. Means with different letters (a vs. b) are statistically different from each other ($p < 0.05$), whereas means with the same letter are not statistically different.

Fig. 2.

Refeeding-induced changes in 4E binding protein-1 (4E-BP1) phosphorylation and the distribution of eukaryotic initiation factor (eIF) 4E in hearts from saline-treated control and alcohol-treated mice. A representative Western blot for each protein of interest is shown in the top right panel. eIF4E was immunoprecipitated from the homogenate and then immunoblotted for 4E-BP1 or eIF4G. Samples from all four experimental groups were run on the same gel and the vertical white line indicates that intervening lanes of the gel have been removed for presentation purposes. Bar graphs represent densitometric analysis of all immunoblots, where the value from fasted saline-treated control mice was set at 100 AU. Values are means $\pm$ SEM; $n = 10, 10, 12$, and 12 , respectively. Means with different letters (a vs. b) are statistically different from each other ($p < 0.05$), whereas means with the same letter are not statistically different.

Fig. 3.

Refeeding-induced changes in the phosphorylation of S6 kinase (S6K)-1, S6, eukaryotic initiation factor (eIF) 4B, and eukaryotic elongation factor (eEF) 2 in hearts from saline-treated control and alcohol-treated mice. A representative Western blot for each protein of interest is shown in the top right panel. Samples from all four experimental groups were run on the same gel and the vertical white line indicates that intervening lanes of the gel have been removed for presentation purposes. Bar graphs represent densitometric analysis of all immunoblots, where the value from fasted saline-treated control mice was set at 100 AU. Values are means $\pm$ SEM; $n = 10, 10, 12$, and 12 , respectively. Means with different letters (a vs. b) are statistically different from each other ($p < 0.05$), whereas means with the same letter are not statistically different.

Fig. 4.

Refeeding-induced changes in the phosphorylation of MEK-1 and -2, ERK-1 and -2, and p90 RSK in hearts from saline-treated control and alcohol-treated mice. A representative Western blot for each protein of interest is shown in the top left panel. Samples from all four experimental groups were run on the same gel and the vertical white line indicates that intervening lanes of the gel have been removed for presentation purposes. Bar graphs represent densitometric analysis of all immunoblots, where the value from fasted saline-treated control mice was set at 100 AU. Values are means $\pm$ SEM; n = 10, 10, 12, and 12, respectively. Means with different letters (a vs. b) are statistically different from each other ($p < 0.05$), whereas means with the same letter are not statistically different.

Fig. 5.

Refeeding-induced changes in ULK1, LC3B-II, p62, Atg5-12, and Beclin1 in hearts from saline-treated control and alcohol-treated mice. A representative Western blot for each protein of interest is shown in the top left panel. Samples from all four experimental groups were run on the same gel and the vertical white line indicates that intervening lanes of the gel have been removed for presentation purposes. Bar graphs represent densitometric analysis of all immunoblots, where the value from fasted saline-treated control mice was set at 100 AU. Values are means $\pm$ SEM; n = 10, 10, 12, and 12, respectively. Means with different letters (a vs. b) are statistically different from each other ($p < 0.05$), whereas means with the same letter are not statistically different.

Myocardial protein synthesis and plasma concentrations of insulin and branched-chain amino acids in saline-treated control and alcohol-treated mice in the fasted condition and after refeeding.

Table 1

	Saline-treated Control		Alcohol-treated	
	Fasted	Refed	Fasted	Refed
Protein synthesis, % control	100 ± 5 ^a	161 ± 11 ^b	89 ± 6 ^a	159 ± 12 ^b
Proteasome activity, nmol/min/μg protein	11.2 ± 1.1	11.7 ± 0.9	12.3 ± 1.5	12.1 ± 1.4
Leucine, μM	63 ± 14 ^a	116 ± 21 ^b	61 ± 9 ^a	128 ± 19 ^b
Isoleucine, μM	77 ± 16	81 ± 17	69 ± 16	75 ± 22
Valine, μM	134 ± 33	154 ± 28	141 ± 26	151 ± 33
Insulin, ng/mL	0.27 ± 0.04 ^a	0.77 ± 0.13 ^b	0.77 ± 0.11 ^b	1.00 ± 0.17 ^b

Values are means ± SEM; n = 10–12 per group. Means with different letters are statistically different from each other ($p < 0.05$).