Alcohol Regulates Eukaryotic Elongation Factor 2 Phosphorylation via an AMP-activated Protein Kinase-dependent Mechanism in C2C12 Skeletal Myocytes*

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Ethanol decreases protein synthesis in cells, although the underlying regulatory mechanisms of this process are not fully established. In the present study incubation of C2C12 myocytes with 100 mM EtOH decreased protein synthesis while markedly increasing the phosphorylation of eukaryotic elongation factor 2 (eEF2), a key component of the translation machinery. Both mTOR and MEK pathways were found to play a role in regulating the effect of EtOH on eEF2 phosphorylation. Rapamycin, an inhibitor of mammalian target of rapamycin, and the MEK inhibitor PD98059 blocked the EtOH-induced phosphorylation of eEF2, whereas the p38 MAPK inhibitor SB202190 had no effect. Unexpectedly, EtOH decreased the phosphorylation and activity of the eEF2 upstream regulator eEF2 kinase. Likewise, treatment of cells with the inhibitor rotterlin did not block the stimulatory effect of EtOH on eEF2, suggesting that eEF2 kinase (eEF2K) does not play a role in regulating eEF2. In contrast, increased eEF2 phosphorylation was correlated with an increase in AMP-activated protein kinase (AMPK) phosphorylation and activity. Compound C, an inhibitor of AMPK, suppressed the effects of EtOH on eEF2 phosphorylation but had no effect on eEF2K, indicating that AMPK regulates eEF2 independent of eEF2K. Finally, EtOH decreased protein phosphatase 2A activity when either eEF2 or AMPK was used as the substrate. Thus, this later action may partially account for the increased phosphorylation of eEF2 in response to EtOH and the observed sensitivity of AMPK to rapamycin and PD98059 treatments. Collectively, the induction of eEF2 phosphorylation by EtOH is controlled by an increase in AMPK and a decrease in protein phosphatase 2A activity.

Eukaryotic elongation factors (eEFs)2 such as eEF1A, eEF1B, and eEF2 are essential elements of the translational machinery in cells. In this regard, eEF1A promotes the GTP-dependent binding of aminoacyl-tRNAs to ribosomes, and eEF2, a monomeric protein of ~100 kDa, is responsible for the GTP-dependent translocation step (1). Among the various elongation factors, phosphorylation of eEF2 by the Ca2+/calmodulin-dependent eEF2 kinase (eEF2K) is the most studied mechanism controlling the elongation rate (2–4). eEF2 kinase, also known as Ca2+/calmodulin kinase III, is a protein kinase that phosphorylates eEF2 on Thr-56 and -58 in cell extracts (5–7). The phosphorylation of eEF2 decreases its activity, thereby impairing the rate of elongation and contributing to an overall inhibition of protein synthesis (8–10). Similar to eEF2, the activity of eEF2 kinase is regulated through multisite phosphorylation in response to various stimuli. Interestingly, phosphorylation of this kinase can regulate eEF2 either negatively or positively, depending on the stimuli and the particular residue that is phosphorylated (10).

A number of signaling pathways are involved in the regulation of eEF2K and eEF2. For instance, the induced phosphorylation of eEF2K and eEF2 by various growth factors (e.g. insulin and insulin-like growth factor-I) and hormones (e.g. serotonin, phenylephrine) is mediated by mTOR and MEK/ERK signaling (11–15). Likewise, these phosphorylation events can be regulated via p38 MAPK, although the involvement of this pathway is stimuli- and phosphorylation site-specific (16, 17). In contrast, other data suggest that the mTOR, MEK, and p38 pathways are only involved in regulating eEF2 but not eEF2K. For example, treatment with the mTOR inhibitor rapamycin, the MEK1 inhibitor PD98059, or the p38 MAPK inhibitor SB202190 did not affect eEF2 phosphorylation in the presence of cholecystokinin (18) even though eEF2 phosphorylation was sensitive to these inhibitors.

Protein synthesis in general and peptide-chain elongation in particular are energetically expensive processes. One cellular mechanism regulating energy homeostasis is the 5′-AMP-activated protein kinase (AMPK). Activation of AMPK in response to various stimuli including low cellular energy levels, hypoxia, ischemia, hormones, and electrical stimulation is associated with increased phosphorylation of eEF2 and inhibition of protein synthesis (19–23). This phosphorylation may be due to increased eEF2K activity, because it has been suggested that eEF2K activity is enhanced by phosphorylation events mediated by the AMPK pathway (24, 25). In contrast, studies using an eEF2K inhibitor suggested that eEF2K might not be involved in this process (26). Furthermore, recent studies showed that...
activation of AMPK is not correlated with increased phosphorylation of eEF2 in skeletal and heart muscle cells in response to exercise (24, 27).

Alcohol (EtOH) decreases protein synthesis in myocytes, and this response is associated with increased eEF2 phosphorylation (28). However, little is known concerning the mechanisms by which EtOH regulates eEF2. The aim of the present study was to examine the effects of EtOH on signaling events regulating eEF2 phosphorylation in C2C12 myocytes. Increased phosphorylation of eEF2 after EtOH treatment was associated with decreased phosphorylation and activity of eEF2K, suggesting that eEF2K is not involved in the control of eEF2 in C2C12 myocytes. On the other hand, EtOH increased the phosphorylation and activity of AMPK, and this enzyme directly phosphorylated eEF2, thus suggesting a link between changes in AMPK activity and eEF2 phosphorylation. Finally, EtOH resulted in a general decrease in phoshatase activity and a specific decrease in protein phosphatase 2A (PP2A) activity toward eEF2 and AMPK.

MATERIALS AND METHODS

EtOH was purchased from Fisher Scientific Co. The majority of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). These included a rabbit monoclonal antibody recognizing the phosphorylated (p) form of AMPK-α (Thr-172) and polyclonal antibodies specific for p-eEF2 (Thr-56), p-eEF2K (Ser-366), p-acetyl-CoA carboxylase (Ser-79), p-mTOR, p-MEK, and p-ERK1/2. Antibodies to total AMPK-α, eEF2, eEF2K, acetyl-CoA carboxylase, and the MEK1 inhibitor PD98059 were also obtained from the same source. The AMPK inhibitor, compound C, the p38 MAPK inhibitor SB202190, okadaic acid, and rotellerin were purchased from Calbiochem (EMD Biosciences, San Diego, CA). Rapamycin and [35S]methionine/cysteine (>1000 Ci/mol) were obtained from MP Biomedical (Aurora, OH), whereas protein phosphatase inhibitor I-2 was from Sigma. The phosphate assay kit and PP2A antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). AICAR was purchased from Toronto Research Chemicals, Inc. (Northyork, ON, Canada), and cell culture media and fetal bovine serum (FBS) were from Invitrogen.

Cell Culture—C2C12 mouse myoblasts were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (25 μg/ml). The effects of EtOH on protein synthesis were determined as previously described (28, 29). 100 mM EtOH was used unless otherwise indicated because this concentration produces an effect without being cytotoxic to the cells (28). All experiments were conducted using cells at the myoblast stage. For metabolic labeling, cells were incubated in the presence of EtOH and radioisotope for 24 h before harvest. Alternatively, cells were pretreated with EtOH for 24 h and then metabolically labeled for various periods of time in the presence of EtOH. Cells were labeled with 10 μCi of [35S]methionine/cysteine in 2% FBS media because C2C12 myocyte survival is decreased when cells are cultured for extended periods in serum-free media. In preliminary studies the rate of radiolabel incorporation into protein was linear between 1 and 24 h (data not shown) indicating there was no significant change in the specific activity of the precursor pool. Hence, all subsequent studies were conducted using the 24-h labeling protocol. At the end of the experiment, cells were collected and precipitated in 10% trichloroacetic acid, and the incorporation of [35S]methionine/cysteine into trichloroacetic acid-precipitable protein was determined via liquid scintillation counting. The results were then compared with those of the appropriate time-matched control group, and data are expressed as a percentage of the control value.

Immunoblot Analysis—C2C12 myocytes were subcultured in 6-well plates in the presence of EtOH for 24 h as described previously (24). Thereafter, cells were changed to serum-free media in the continued presence of this agent and collected after 15 min in 2× Laemml sample buffer unless otherwise indicated. Equal amounts of protein from cell lysates were electrophoresed on denaturing polyacrylamide gels and transferred to nitrocellulose. The resulting blots were blocked with 5% non-fat dry milk and incubated with the antibodies of interest as described above. Unbound primary antibody was removed by washing with Tris-buffered saline containing 0.05% Tween 20 (ICN Americas, Inc, Wilmington, DE), and blots were incubated with anti-rabbit immunoglobulin conjugated with horseradish peroxidase. Blots were briefly incubated with an enhanced chemiluminescent detection system (Amersham Biosciences) and exposed to Eastman Kodak Co. x-ray film. The film was scanned (ScanMaker 4, Microtek, Los Angeles, CA) and analyzed with NIH Image 1.6 software.

eEF2K and AMPK Activity Assay—For kinase activity measurements, cells were lysed in 1% Nonidet P-40 containing 20 mM Hepes, 150 mM NaCl, and a mixture of protease and phosphatase inhibitors. AMPK and eEF2K activities were determined as described previously (30, 31) with minor modifications. Briefly, cell extracts were immunoprecipitated overnight with a specific antibody against eEF2 or with antibodies to the eEF2K or α catalytic subunit of AMPK. The antibody-antigen complex was captured by incubation with a 1 h with protein A-Sepharose (Amersham Biosciences). Immune complexes were washed with lysis buffer and then incubated with reaction buffer A (40 mM Hepes, 0.2 mM AMP, 80 mM NaCl, 0.8 mM dithiothreitol, 5 mM MgCl2, and 0.2 mM [γ-32P]ATP) or buffer B (50 mM Hepes, 10 mM magnesium acetate, 100 μM CaCl2, 5 mM dithiothreitol, 0.6 μg of Ca2+/calmodulin, and 50 μM [γ-32P]ATP) in a total volume of 50 μl for measuring AMPK or eEF2K activity, respectively. The reaction was allowed to proceed for 10–14 min at 30 °C and then terminated by the addition of 2× Laemmi sample buffer and heating for 5 min. Samples were run on SDS-PAGE gels, dried at 80 °C, and quantitated using phosphorimaging. The results were standardized with total protein as determined using a BCA protein assay reagent kit (Pierce).

Phosphatase Assay—Myocytes were incubated with EtOH as above, and phosphatase activity was measured using a Ser/Thr phosphatase assay kit from Upstate Biotechnology. Briefly, cells were lysed with buffer containing 20 mM imidazole-HCl (pH 7.0), 2 mM EDTA, 2 mM EGTA, and a mixture of protease inhibitors. For detection of PP2A or total phosphatase activity,
Lysates were incubated for 15–25 min at room temperature with a phosphopeptide (RKpTIRR; pT is phosphothreonine) or with eEF2 that was immunoprecipitated using anti-eEF2 antibody. The reaction was terminated after the addition of the malachite green reagent. Free phosphatase was then quantified by measuring the absorbance at 620 nm. To examine the ability of PP2A to dephosphorylate eEF2 and AMPK, an in vitro phosphatase assay was conducted. For these experiments, either eEF2 or AMPK was immunoprecipitated from untreated cells and used as the substrate, while PP2A was isolated from control and EtOH-treated cells. Substrates and phosphatase were incubated together at room temperature for 25 min, and PP2A activity was quantified as described above.

**Statistical Analysis**—For experimental protocols with more than two groups, statistical significance was determined using one-way analysis of variance followed by the Dunnett’s test to compare all data to the appropriate time-matched control group. For experiments with only two groups, an unpaired Student’s t test was performed. Data are presented as the mean ± S.E. A value of \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**Effects of EtOH on Basal Protein Synthesis**—EtOH inhibits protein synthesis in various cell and tissue types, including muscle cells (28, 32). Although the mechanisms mediating the effects of EtOH are not fully characterized, there are a number of signaling pathways that regulate the activity of both translation initiation and elongation factors, and these may be adversely affected by EtOH. In the present study we examined the effects of EtOH on protein synthesis in C2C12 myocytes. In addition, we characterized the role that the mTOR, MAPK, and AMPK signaling pathways play in regulating the phosphorylation of elongation factor eEF2 in response to this drug.

Initially, we determined the dose- and time-dependent effects of EtOH on the basal rate of protein synthesis. For these experiments, cells were metabolically labeled for 24 h in the presence or absence of increasing concentrations of EtOH. Alternatively, cells were labeled for various periods of time in the presence or absence of 100 mM EtOH. Treatment of cells with 80, 100, or 120 mM EtOH significantly decreased protein synthesis when results were compared with untreated control cells (Fig. 1A). Interestingly, a 24-h incubation with 100 mM EtOH was required before a significant decline of protein synthesis was observed (Fig. 1B). The absence of an effect after either 2- or 8-h EtOH treatment suggests that there is a lag phase between EtOH exposure and a reduction in protein synthesis. As such, the cumulative 15% decrease observed during the 24-h labeling period most likely underestimates the maximal level of decrease that occurs once EtOH begins to elicit an inhibitory effect. To address these issues, cells were incubated in the presence or absence of 100 mM EtOH for 24 h and then metabolically labeled. EtOH-treated cells were labeled for various periods of time in media containing 100 mM EtOH, whereas time-matched control cells were labeled in media lacking EtOH. A significant 38% decrease in protein synthesis was observed when cells were preincubated for 24 h and then labeled for 30 min in the presence of fresh EtOH (Fig. 1C).

Interestingly, the effects of EtOH gradually decreased over
EtOH-induced Phosphorylation of eEF2 Is Dependent on mTOR and MEK Pathways—The activity of eEF2 is critical for the elongation step of translation, and this activity is negatively regulated by phosphorylation (33). To determine the effects of EtOH on eEF2 phosphorylation, cells were incubated in the presence or absence of 100 mM EtOH for 24 h. As illustrated in Fig. 2A, incubation of cells with EtOH increased phosphorylation of eEF2 by ~35%, as compared with untreated controls. Therefore, this increase may account at least partially for the adverse effects of EtOH on protein synthesis. Next, EtOH-treated cells were given an additional 15 min of incubation with

100 mM fresh EtOH to determine whether this would enhance the effects of EtOH on signaling events. Under these circumstances there was a dramatic 3-fold increase in eEF2 phosphorylation levels. These results are consistent with our protein synthesis studies, where there was a greater decrease in synthesis after the re-addition of EtOH as compared with cells treated for 24 h alone. Taken together, these data indicate that C2C12 cells require a period of time to become sensitized to the effects of EtOH. Furthermore, the maximal effects of this drug are most readily apparent immediately after the re-addition of the drug. Based upon these results, for subsequent experiments, cells were routinely pretreated with EtOH for 24 h and then given a 15-min incubation with fresh EtOH before harvest.

Phosphorylation of eEF2 can be regulated through signaling events that are part of the mTOR and MAPK pathways (16, 34). To determine whether EtOH affected eEF2 via these pathways, myocytes were treated with EtOH in the presence or absence of various inhibitors. Incubation with the mTOR inhibitor rapamycin had no effect on the constitutive level of eEF2 phosphorylation, although total eEF2 was decreased ~20% compared with control (Fig. 2B). Treatment of myocytes with a combination of rapamycin and EtOH completely suppressed the EtOH-induced phosphorylation of eEF2, and this effect was partly due to a decrease in total eEF2 content (~24%). These data suggest that there is a requirement for the mTOR-dependent pathway in the regulation of eEF2 phosphorylation. The MAPK pathway also appears to play a role in this process. The observed increase in eEF2 phosphorylation in response to EtOH was greatly repressed in the presence of the MEK inhibitor PD98059 (Fig. 2C). Note, however, that the level of phosphorylated eEF2 in these cells was still slightly, but significantly elevated compared with the control group, which may be due to the requirement for mTOR in this process. Finally, we examined the role of the p38 MAPK pathway in mediating the effects of EtOH. Fig. 2D illustrates that treatment with the p38 MAPK inhibitor SB202190 significantly increased eEF2 phosphorylation in control myocytes (~2-fold). Thus, inhibition of this MAPK pathway activates eEF2 in a manner similar to EtOH. Likewise, the combination of EtOH and SB202190 significantly increased the levels of phosphorylated eEF2 relative to the control value. These data suggest that EtOH does not regulate eEF2 via p38 MAPK signaling.

Because the mTOR and MEK pathways both regulated the effects of EtOH on eEF2 phosphorylation, it is possible that these represent sequential signaling events. To test this idea, we examined whether rapamycin had any effect on MEK phosphorylation in the presence of EtOH or, alternatively, whether PD98059 had an effect on mTOR phosphorylation under these same conditions. EtOH decreased levels of p-MEK1/2 and p-ERK1/2, but this decrease was not influenced by the presence of rapamycin (data not shown). Likewise, PD98059 did not modulate the effect of EtOH on the phosphorylation levels of mTOR. Thus, it appears that these two pathways are not activated in sequence but most likely function in parallel.

EtOH-induced Phosphorylation of eEF2 Is Not Controlled by eEF2K—eEF2 is purportedly regulated by the calcium- and calmodulin-dependent kinase eEF2K, and the activity of this kinase is controlled by phosphorylation events (9). Therefore,
we examined the effect of EtOH on the phosphorylation of eEF2K. Unexpectedly, incubation of myocytes with 100 mM EtOH significantly decreased eEF2K phosphorylation on the Ser-366 residue by ~50% when compared with control values (Fig. 3A). Moreover, this EtOH-induced decrease in phosphorylated eEF2K was independent of a change in the total amount of eEF2K protein. To determine the kinetics of the effects of EtOH on eEF2K phosphorylation, a time course was conducted in which cells were preincubated with EtOH for 24 h and then incubated with fresh EtOH for various periods of time. The phosphorylation of eEF2K decreased at all time points examined between 5 and 30 min (Fig. 3B), although the greatest decline (~50%) was observed when cells were incubated with EtOH for 15 min. Note that decreased levels of eEF2K phosphorylation were inversely correlated with increases in eEF2 phosphorylation (Fig. 3C). Therefore, these data suggest that eEF2K does not regulate the phosphorylation of eEF2 in response to EtOH.

Decreased phosphorylation of eEF2K may not necessarily indicate a decrease in the activity of this kinase. Phosphorylation of eEF2K can inhibit or enhance the activity of its downstream targets, and this appears to depend upon the type of stimuli and the site of phosphorylation (10). To examine whether eEF2 phosphorylation is regulated by eEF2K in our model system, myocytes were incubated with EtOH in the presence or absence of rottlerin, a compound known to inhibit the activity of eEF2K. Initially, we verified the efficacy of rottlerin treatments in myocytes by using this drug in combination with serum or with the AMPK activator AICAR. Treatment of myocytes with AICAR or 10% FBS each increased the levels of eEF2K phosphorylation more than 2-fold. However, the addition of rottlerin blocked the effects of both of these stimulators (Fig. 4A). In our next experiment, we examined the effects of rottlerin on eEF2 phosphorylation after EtOH treatments. The presence of rottlerin did not alter the ability of EtOH to increase the phosphorylation of eEF2 (Fig. 4B). Thus, these data offer further evidence that eEF2K does not play a role in EtOH-mediated signaling events. As a final test of this hypothesis, an in vitro eEF2K activity assay was performed. For this assay eEF2 was used as a substrate, and eEF2K was immunoprecipitated from cells treated with or without EtOH. Incubation of myocytes with EtOH decreased eEF2K activity by 33% relative to control values (Fig. 4C). Taken together, our results indicate that EtOH increases eEF2 phosphorylation, but this response is not mediated by eEF2K.

**EtOH Increases eEF2 Phosphorylation in an AMPK-dependent Manner**—Because EtOH induces eEF2 phosphorylation via an eEF2K-independent pathway, we next attempted to identify another upstream kinase(s) that may be responsible for eEF2 activation. The elongation process consumes a high level of cellular energy, and as such, it can be regulated by factors affecting energy state of the cell. AMPK is a kinase that is activated by various stimuli (e.g. hypoxia, anoxia) that change cellular energy levels, and this leads to increased phosphorylation of eEF2 (19, 35). Therefore, AMPK might also play a role in the phosphorylation of eEF2 in response to EtOH. As is shown in Fig. 5A, the presence of EtOH increased the phosphorylation levels of AMPK greater than 2-fold compared with control untreated cells. Note that similar results were observed when cells were treated with AICAR. Individually, both EtOH and AICAR affected the phosphorylation of a known downstream target of AMPK, acetyl-CoA carboxylase (ACC) (Fig. 5B). Thus,
these data indicate that there is an overall increase in the activity of AMPK in myocytes after exposure to EtOH.

To test whether AMPK modulates the effect of EtOH on eEF2, myocytes were treated with EtOH in the presence or absence of the AMPK inhibitor compound C. When myocytes were incubated with compound C alone, the phosphorylation of eEF2 decreased (Fig. 6A). The extent of phosphorylation remained low after treatment with a combination of EtOH and compound C, and this group also remained significantly below (28%) control values. Thus, these data strongly suggest that EtOH affects eEF2 via the action of AMPK. It should be noted that the mechanism of this action appears to be different from expected. Previous studies showed that AMPK exerts its effect on eEF2 via the action of eEF2K (25). However, our results indicate that eEF2K does not modulate the effects of EtOH on eEF2 phosphorylation. Therefore, AMPK may signal through another kinase, or alternatively, it may directly act on eEF2.

FIGURE 4. EtOH stimulates eEF2 phosphorylation via an eEF2K-independent pathway. Cells were preincubated for 1 h in the presence or absence of rottlerin (Rott, 2 μM) and then treated for 2 h with 1 mM AICAR or 10% FBS (panel A). Cells were pretreated with rottlerin as described above and then incubated with 100 mM EtOH as described in Fig. 2 (panel B). Cell extracts were analyzed via Western blotting using anti-phospho-eEF2K (panel A) or anti-phospho-eEF2 (Thr-56) antibody (panel B). Panel C, an in vitro eEF2K activity assay was performed in the presence of CaCl₂, Ca²⁺/calmodulin, and ATP as described under "Materials and Methods." Data are the mean ± S.E. of three-four independent experiments consisting of three-five replicate samples per experiment. *, p < 0.05 versus the control value. T, total.
Alcohol and eEF2 Signaling

**FIGURE 6.** AMPK-mediated phosphorylation of eEF2 is stimulated by EtOH. Cells were pre-incubated for 1 h in the presence or absence of compound C (CC, 20 µM) and then treated with EtOH. Cell extracts were analyzed via Western blotting using antiserum that recognizes eEF2 phosphorylated at Thr56 (panel A). Panel B, in vitro AMPK activity was examined in the presence or absence of AMP as described under “Materials and Methods.” Data are the mean ± S.E. of three-five independent experiments consisting of three-five replicate samples per experiment. *p < 0.05 versus the control value. Groups with different letters are significantly different from one another (p < 0.05). Groups with the same letters are not significantly different.

EtOH Decreases PP2A Phosphatase Activity—The activity of kinases in cells is balanced by the action of general or specific phosphatases. Accordingly, the increased phosphorylation of eEF2 after EtOH treatments could be due in part to a decreased phosphatase activity. To address this question, we used an in vitro phosphatase activity assay. Control and EtOH-treated cells were harvested, and cell lysates were examined for phosphatase activity using eEF2 as a substrate. Fig. 7A shows that EtOH alone decreased phosphatase activity in total cell lysates by 27% compared with control values. Thus, these and the above results establish a correlation between decreased phosphatase activity and increased phosphorylation of eEF2 after EtOH treatments. To further characterize and identify the phosphatases involved in this process, we next utilized phosphatase inhibitors. Treatment of cells with the inhibitor okadaic acid (OA) significantly decreased phosphatase activity when eEF2 was used as the substrate (Fig. 7A). This most likely represents a decrease in PP2A activity because low concentrations of OA affect this phosphatase. Note that the combination of EtOH and OA did not decrease the activity further, indicating that there is no additive effect. In contrast to these results, treatment of lysates with the phosphatase inhibitor I-2 did not affect phosphatase activity, and this inhibitor did not modulate the effect of EtOH.

Because OA treatments affected phosphatase activity when eEF2 was used as a substrate, we next examined whether this response was associated with a concomitant in vivo changes in eEF2 phosphorylation. Treatment of C2C12 myocytes with OA significantly increased eEF2 phosphorylation (Fig. 7B). This result was similar to those obtained after EtOH treatments, suggesting that both of these drugs may utilize similar mechanisms. Along these lines, when EtOH and OA were used in combination, they did not exhibit an additive effect. Thus, both of these drugs decreased phosphatase activity in myocytes and increased eEF2 phosphorylation.

Taken together, the above results suggest that PP2A plays a role in eEF2 dephosphorylation. This is in agreement with previous studies in reticulocyte lysates, where PP2A was found to dephosphorylate eEF2 (36). To further verify the role of PP2A in this process, we performed additional in vitro phosphatase assays. In the initial assay, we utilized a synthetic phosphopeptide that acts as a specific substrate for PP2A activity. Here, the activity of PP2A in total cell lysates was observed to decrease ~25% after EtOH exposure (Fig. 7C). Thus, this specific eEF2-targeting phosphatase is adversely affected by EtOH. Finally, we performed an in vitro phosphatase assay to directly test the role that PP2A plays in dephosphorylating eEF2. For these experiments, total and Thr-56-phosphorylated eEF2 were isolated from untreated cells and used as the substrate, whereas PP2A was immunoprecipitated from control and EtOH-treated myocytes. Treatment with EtOH significantly decreased the phosphatase activity of PP2A toward eEF2 (Fig. 7D). Note, however, that there was a greater decrease when total eEF2 was utilized as compared with the phosphorylated form. This finding suggests that PP2A may affect other phosphorylation sites in addition to Thr-56. Therefore, taken together, our results indicate that EtOH decreased overall phosphatase activity while specifically decreasing the ability of PP2A to dephosphorylate eEF2. Fur-
thermore, this decreased PP2A activity is associated with an increase in eEF2 phosphorylation. The effect of EtOH on phosphatase activity may reconcile our earlier findings whereby mTOR and MEK appeared to regulate eEF2 phosphorylation. Because rapamycin and PD98059 both blocked the effects of EtOH on eEF2 phosphorylation (Fig. 2, B and C), mTOR and MEK must affect this process, albeit via an eEF2K-independent branch of this pathway. In this regard, rapamycin is known to increase PP2A activity (37), and it is possible that this activity can counteract the effects of EtOH on eEF2 phosphorylation. To test this idea, we examined the in vitro phosphatase activity of PP2A after EtOH and rapamycin treatments. In myocytes, rapamycin significantly increased PP2A activity when eEF2 was used as the substrate (Fig. 8A). Furthermore, this activity remained high when cells were incubated with a combination of rapamycin and EtOH. Treatment with PD98059 produced similar results (Fig. 8B), indicating that both of these drugs increased PP2A activity toward eEF2. Thus, this increased phosphatase activity may reduce the level of phosphorylated eEF2 that would otherwise be observed in cells exposed to EtOH.

**EtOH Affects AMPK Phosphorylation via an mTOR- and MEK-dependent Mechanism**—Our results suggest that PP2A may play an important role in directly regulating the phosphorylation level of eEF2. In addition, PP2A could also regulate eEF2 indirectly if this phosphatase affected the phosphorylation of AMPK. To address this question, rapamycin and EtOH were again used as positive and negative effectors of PP2A activity. As shown in Fig. 9A, rapamycin blocked the stimulatory effects of EtOH on AMPK phosphorylation. As expected, similar results were obtained when cells were treated with the MEK inhibitor PD98059 (Fig. 9B). One interpretation of these results is that rapamycin and PD98059 decreased AMPK phosphorylation by increasing PP2A activity. To test this possibility, we performed an in vitro PP2A phosphatase assay using AMPK as the substrate. Treatment of cells with EtOH significantly decreased PP2A activity toward AMPK, as compared with untreated cells (Fig. 9C). In contrast, rapamycin and PD98059 increased phosphatase activity toward AMPK (Fig. 9, C and D).

**DISCUSSION**

EtOH decreases protein synthesis, and this effect is associated with changes in the phosphorylation status of several key components of the translational machinery. At present, however, little is known about the signaling mechanisms regulating this process. In this study EtOH increased the phosphorylation of the elongation factor eEF2, and the change coincided with a reduced activity of this protein. This result appears to account

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**FIGURE 7. Effects of EtOH on phosphatase activity.** Cells were treated with 100 mM EtOH and 5 nM OA, or lysates were incubated with 50 nM protein phosphatase I-2 before the determination of total phosphatase activity using eEF2 as a substrate (panel A). Panel B, phosphorylation of eEF2 in response to OA in the presence or absence of EtOH. Panel C, cell lysates were incubated with synthetic phosphopeptide (RKpTIRR). Panel D, total or p-eEF2 was immunoisolated from cell lysates and used as a substrate for determining PP2A phosphatase activity. For this experiment, PP2A was isolated from control and EtOH-treated cells. Data are means ± S.E. of three independent experiments consisting of three-five replicate samples per experiment. *, p < 0.05 versus the control value. Groups with different letters are significantly different from one another (*, p < 0.05). Groups with the same letters are not significantly different.
for at least a part of the observed decrease in protein synthesis after EtOH exposure (28).

In our study EtOH decreased eEF2K phosphorylation at Ser-366 concurrently with an increase in eEF2 phosphorylation. These findings initially appeared to be contradictory because an increase in eEF2K activity was expected to account for the increase in eEF2 phosphorylation. On the other hand, it has been reported that EtOH and other stimuli decrease S6K1 and p90rsk phosphorylation, and these proteins are upstream activators of eEF2K (28, 34). Therefore, our signaling data are consistent with the idea that EtOH suppresses eEF2K phosphorylation.

Previous studies reported that insulin or insulin-like growth factor-1 increased phosphorylation of eEF2K at Ser-366 and decreased eEF2 activity. In contrast, phosphorylation at other residues in response to stress conditions increased eEF2 activity (10). Therefore, our signaling data are consistent with the idea that EtOH suppresses eEF2K phosphorylation.
assay, there was a significant decrease in activity after EtOH treatments. Taken together, our findings indicate that the effect of EtOH on eEF2 is independent of eEF2K activity. This conclusion is consistent with previous studies where exercise or treatment with a farnesyltransferase induced inactivation of eEF2 in association with inhibition of protein synthesis (24, 26, 27). These effects were also independent of the action of eEF2 kinase.

Although eEF2K does not appear to be involved in regulating the effects of EtOH, this does not exclude the possibility that other cell type-specific isoforms of eEF2K may be involved in this process (40). Alternatively, another kinase(s) may be responsible for the control of eEF2. In this regard, we initially considered that Ca\(^{2+}\)/calmodulin II may be involved in controlling the eEF2 response to EtOH. However, an in vitro kinase assay using eEF2 as a substrate indicated that Ca\(^{2+}\)/calmodulin II does not participate in this process (data not shown). On the other hand, we did observe a role for AMPK, based on two important observations. First, the increase in eEF2 phosphorylation was blocked when the AMPK inhibitor compound C was added in the presence of EtOH. Second, AMPK was found to directly regulate the in vitro activation of eEF2 when this enzyme was isolated from control cells or from cells that were exposed to EtOH. Thus, these data suggest eEF2 is a substrate for the action of AMPK and that AMPK is a key link between energy status and the regulation of protein synthesis in skeletal muscle cells exposed to EtOH.

Different pathways have been implicated in the regulation of AMPK and eEF2. In the current study, the use of the specific inhibitors rapamycin and PD98059 abolished or suppressed the effect of EtOH on eEF2 and AMPK phosphorylation. This suggests that EtOH regulates eEF2 via mTOR- and MEK-dependent pathways, as reported by others (12, 41, 42). The mechanism of this action remains unclear at present, however. Along these lines, previous reports indicated that the activation of AMPK is associated with changes in eEF2K activity. As such, this process is mediated via the mTOR/eEF2K signaling pathways, whereby eEF2K is purported to be the substrate of AMPK (25, 43). In contrast to these findings, our data indicate that eEF2K is not involved in regulating the effects of EtOH. Instead, AMPK directly phosphorylates eEF2 (Fig. 6B). This suggests that AMPK can regulate eEF2 in a manner that is independent of the mTOR/eEF2K pathways, at least in the presence of EtOH. Similar observations were reported previously wherein mild ATP depletion or oxygen deprivation affected eEF2 phosphorylation independently of the mTOR/eEF2K pathway (23, 35).

Finally, we obtained data that support a role for PP2A in regulating the effects of EtOH on protein synthesis. EtOH suppressed PP2A activity, and as such, this could affect a number of components of the protein synthetic signaling pathway. Along these lines, we have demonstrated that eEF2 (Fig. 7D) and AMPK (Fig. 9) are direct substrates for PP2A. Therefore, decreased PP2A activity allows for increased eEF2 phosphorylation as well as increased AMPK phosphorylation. The involvement of PP2A in regulating eEF2 directly (Fig. 8) or indirectly (Fig. 9) via its action on AMPK is supported by experiments in this study using inhibitors such as rapamycin and PD98059. In the present study, rapamycin and PD98059 increased PP2A activity when either eEF2 or AMPK was used as a substrate. Similar results have been obtained previously in other cell types (37, 44, 45). Hence, these inhibitors stimulate PP2A activity, and this in turn can directly dephosphorylate eEF2. Alternatively, increased PP2A activity may decrease AMPK phosphorylation, and this could subsequently decrease eEF2 activity. Indeed, a direct interaction between AMPK and PP2A was observed in our study that is in agreement with previous reports (46).

Two distinct signaling pathways appear to mediate the effect of EtOH on PP2A activity. For example, our results suggest that EtOH affects PP2A activity via the MEK/ERK signaling pathway. This is consistent with a previous study that reported a role for the MEK pathway in regulating PP2A activity (47). We also present data indicating that EtOH affects PP2A via the mTOR pathway. This later result is in agreement with our previous study, where EtOH decreased mTOR and S6K1 phosphorylation (28). At present, it is unclear whether EtOH exerts its effect directly on mTOR or whether this drug initially activates this signaling cascade further upstream. As shown in this study, EtOH affects AMPK phosphorylation, and the AMPK-TSC (tuberous sclerosis complex) pathway (48) may act to regulate mTOR in this process.

In Fig. 10, we illustrate potential roles for PP2A and various kinases in regulating eEF2 phosphorylation after EtOH exposure. In our model, EtOH increases phosphorylation and activation of AMPK, and this leads to increased phosphorylation and inactivation of eEF2. At present, it is unclear whether EtOH affects AMPK phosphorylation directly or if it affects this protein indirectly via the action of an upstream kinase(s). However, EtOH does decrease PP2A activity, which may account for the increase in AMPK and eEF2 phosphorylation. Both the mTOR and MEK signaling pathways appear to play a role in regulating the effects of EtOH on PP2A activity. Thus, taken together, our
results show that EtOH regulates eEF2 by increasing AMPK activity and decreasing PP2A activity.

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