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Lopinavir Impairs Protein Synthesis and Induces eEF2 Phosphorylation Via the Activation of AMP-Activated Protein Kinase

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ABSTRACT

HIV anti-retroviral drugs decrease protein synthesis, although the underlying regulatory mechanisms of this process are not fully established. Therefore, we investigated the effects of the HIV protease inhibitor lopinavir (LPV) on protein metabolism. We also characterized the mechanisms that mediate the effects of this drug on elongation factor-2 (eEF2), a key component of the translational machinery. Treatment of C2C12 myocytes with LPV produced a dose-dependent inhibitory effect on protein synthesis. This effect was observed at 15 min and was maintained for at least 4 h. Mechanistically, LPV increased the phosphorylation of eEF2 and thereby decreased the activity of this protein. Increased phosphorylation of eEF2 was associated with increased activity of its upstream regulators AMP-activated protein kinase (AMPK) and eEF2 kinase (eEF2K). Both AMPK and eEF2K directly phosphorylated eEF2 in an in vitro kinase assay suggesting two distinct paths lead to eEF2 phosphorylation. To verify this connection, myocytes were treated with the AMPK inhibitor compound C. Compound C blocked eEF2K and eEF2 phosphorylation, demonstrating that LPV affects eEF2 activity via an AMPK-eEF2K dependent pathway. In contrast, incubation of myocytes with rottlerin suppressed eEF2K, but not eEF2 phosphorylation, suggesting that eEF2 can be regulated independent of eEF2K. Finally, LPV did not affect PP2A activity when either eEF2 or peptide was used as the substrate. Collectively, these results indicate that LPV decreases protein synthesis, at least in part, via inhibition of eEF2. This appears regulated by AMPK which can act directly on eEF2 or indirectly via the action of eEF2K. J. Cell. Biochem. 105: 814–823, 2008.

KEY WORDS: AMPK; eEF2K; HIV ANTI-RETROVIRAL DRUGS

The regulation of protein synthesis is a complex process involving alterations in the phosphorylation state of many components of the translational machinery. One group of these components consists of the peptide-chain elongation factors (eEFs). Among the various eEFs, phosphorylation of eEF2 by the eEF2 kinase (eEF2K) is the best characterized mechanism controlling the rate of elongation [Riis et al., 1990; Ryazanov et al., 1991; Dorovkov et al., 2002]. eEF2K, also known as Ca^{2+}/calmodulin kinase III, is a protein kinase which phosphorylates eEF2 on Thr−56 and −58 [Price et al., 1991; Mitsui et al., 1993; Redpath et al., 1993]. The phosphorylation of eEF2 is inversely related to the rate of elongation, thereby contributing to the overall decrease of protein synthesis. Likewise, the activity of eEF2K is regulated through single or multisite phosphorylation when cells are exposed to various stimuli. For example, phosphorylation of eEF2K at the Ser 366 residue following exposure to neurotrophic factor or hormones [e.g., insulin or insulin-like growth factor (IGF)-I] decreases the activity of this kinase, while phosphorylation at other sites in response to stress conditions increases its activity [Browne and Proud, 2002; Inamura et al., 2005]. Hence, the phosphorylation of eEF2K can regulate eEF2 either positively or negatively, depending on the stimuli and the particular residue that is phosphorylated.

A number of signaling pathways are involved in the regulation of eEF2 and eEF2K. For instance, AMPK, mTOR and MEK/ERK signaling mediate increased phosphorylation of eEF2 and eEF2K in response to various stimuli such as low cellular energy level, hypoxia, electrical stimulation [McLeod and Proud, 2002; Atherton et al., 2005; Terai et al., 2005], hormones (e.g., serotonin, phenylephrine) and growth factors [insulin, IGF-I, Wang and Proud, 2002; Carroll et al., 2004; Proud, 2004]. Interestingly, some data suggest that these pathways are involved in regulating either eEF2 or eEF2K, but not both. For example, incubation of myocytes with alcohol suppresses eEF2K activity, while increasing eEF2 phosphorylation [Hong-Brown et al., 2007]. In contrast, treatment

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with the mTOR inhibitor rapamycin and the MEK1 inhibitor PD98059 did not affect eEF2K phosphorylation in the presence of cholecystokinin, even though eEF2 phosphorylation was sensitive to these inhibitors [Sans et al., 2004]. The role that AMPK plays in regulating eEF2 is controversial. Published studies report that AMPK is not involved in eEF2 phosphorylation in response to exercise in skeletal muscle cells [Rose et al., 2005]. In contrast, our recent studies suggest that eEF2 can be directly regulated by AMPK in myocytes following alcohol treatment [Hong-Brown et al., 2007]. These latter findings are in agreement with others reporting that stimulation of AMPK by low cellular energy increased eEF2 phosphorylation [McLeod and Proud, 2002].

Lopinavir (LPV) is a human immunodeficiency virus (HIV)-1 protease inhibitor commonly used in combination with other agents as part of the highly active anti-retroviral therapy (HAART). Anti-retroviral therapy helps control HIV infection by suppressing plasma viral levels and enhancing the immunological status of patients, thereby leading to a decline in morbidity and mortality [Palella et al., 1998; Schwarcz et al., 2000]. Unfortunately, the benefits of this therapy are limited, owing to certain inherent adverse effects that anti-retroviral drugs have on bone, lipid, carbohydrate, and protein metabolism [Jain and Lenhard, 2002; Ben-Romano et al., 2006; Ergun-Longmire et al., 2006]. For example, LPV has been reported to contribute to insulin resistance and the development of type 2 diabetes mellitus by inhibiting insulin sensitive glucose transporters in adipocytes and muscle cells [Yan and Hruz, 2005; Noor et al., 2006]. Likewise, LPV impairs lipid metabolism causing hyperlipidemia and lipodystrophy [Montes et al., 2005; Valerio et al., 2005; Prot et al., 2006]. At present, the effects of LPV on protein metabolism have not been thoroughly investigated. However, there is evidence that HIV-related wasting still occurs in patients treated with these drugs [Mangili et al., 2006].

Previous in vitro and in vivo studies showed that various HIV protease inhibitors impaired protein synthesis, and this response was associated with defects in translation initiation and/or elongation. However, the effect that LPV has on these processes has not been reported. The aim of the present study was to determine whether LPV influences protein synthesis in C2C12 myocytes. In addition, we studied how proteins synthesis-related signaling events were regulated by this drug. LPV decreased protein synthesis in a dose-and time-dependent manner, and this impairment was associated with an increase in eEF2 phosphorylation. LPV also increased eEF2K phosphorylation and activity. Phosphorylation of eEF2K by LPV was mediated via activation of the AMPK pathway. On the other hand, in vitro kinase assays and studies using chemical inhibitors suggested that AMPK can regulate eEF2 independent of eEF2K.

MATERIALS AND METHODS

LPV was provided by the NIH AIDS Research and Reference Reagent Program (Rockville, MD). The majority of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). These included polyclonal antibodies specific for the phosphorylated (p) form of AMPK-α (Thr 172), p-eEF2 (Thr 56), p-eEF2K (Ser 366), p-acetyl CoA carboxylase (ACC; Ser 79). Antibodies to total AMPK-α, eEF2, eEF2K, and ACC were also obtained from the same source. The AMPK and eEF2K inhibitors compound C and rottlerin, respectively, were purchased from CalBiochem (EMD Biosciences, San Diego, CA). ^{35}S-methionine/cysteine (>1,000 Ci/mol) was obtained from MP Biomedicals (Aurora, OH). Cell culture media and fetal bovine serum (FBS) were from Gibco, Invitrogen Corporation (Carlsbad, CA).

CELL CULTURE

C2C12 mouse myoblasts were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin (25 μg/ml). The effect of LPV on protein synthesis was determined as previously described [Hong-Brown et al., 2005] with minor modifications. Briefly, for metabolic labeling, cells were incubated in the presence of LPV and radioisotope for various periods of time with 10 μCi [^{35}S] in methionine/cysteine- free DMEM media. In preliminary studies, the rate of radiolabel incorporation into protein was linear between 15 min and 24 h (data not shown) indicating there was no significant change in the specific activity of the precursor pool. At the conclusion of experiments, cells were collected and precipitated in 10% TCA, and the incorporation of ^{35}S- methionine/cysteine into TCA-precipitable protein was determined via liquid scintillation counting. The results were then compared with those of the appropriate time-matched control group and data were expressed as a percentage of the control value.

WESTERN IMMUNOBLOT ANALYSIS

C2C12 myocytes were sub-cultured in 6-well plates. Cells were incubated in the presence or absence of LPV for 15 min and collected in 2× Laemmli sample buffer (LSB). 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 TBS containing 0.05% Tween-20 (ICI 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, Bickinghamshire, England) and exposed to Kodak X-ray film (Rochester, NY). The film was scanned (ScanMaker 4, Microtek, Los Angeles, CA) and analyzed with NIH Image 1.6 software.

ASSAY FOR eEF2K AND AMPK ACTIVITY

For kinase activity measurements, cells were lysed in 1% NP-40 containing 20 mM Hepes, 150 mM NaCl, and a cocktail of protease and phosphatase inhibitors as described previously [Hong-Brown et al., 2007]. In brief, cell extracts (100–120 μg of protein) were immunoprecipitated overnight with 4–6 μg of specific antibodies against AMPK, eEF2K or eEF2. The antibody–antigen complex was then captured by incubation for 1 h with 40 μl protein A Sepharose (Amersham Biosciences, Piscataway, NJ). Immune complexes were washed with lysis buffer and then incubated with 50 μl reaction

buffer A (40 mM Hepes, 0.2 mM AMP, 80 mM NaCl, 0.8 mM DTT, 5 mM $MgCl_2$, and 0.2 mM [gamma-^{32}P] ATP) or buffer B (50 mM Hepes, 10 mM magnesium-acetate, 100 μM $CaCl_2$, 5 mM DTT, 0.6 μg Ca/calmodulin, and 50 μM [gamma-^{32}P] ATP) for measuring AMPK or eEF2K activity, respectively. The reaction was allowed to proceed for 10–14 min at 30°C, and terminated by addition of 2 × LSB with heating for 5 min. Samples were run on SDS-PAGE gels, dried at 80°C and quantitated using a phosphoimager. The results were standardized with total protein, as determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL).

PHOSPHATASE ASSAY

Myocytes were incubated with LPV as described above and phosphatase activity was measured using a Ser/Thr phosphatase assay kit from Upstate Biotechnology. Cells were lysed with buffer containing 20 mM imidazole–HCl (pH 7.0), 2 mM EDTA, 2 mM EGTA, and a cocktail of protease inhibitors. For detection of PP2A activity, lysates were incubated for 25 min at room temperature with a phosphopeptide (R-K-pT-I-R-R) according to the manufacturer's protocol. The reaction was then terminated following the addition of malachite green reagent and free phosphatase was quantified by measuring the absorbance at 620 nm. To examine the ability of PP2A to dephosphorylate eEF2, an in vitro phosphatase assay was conducted. For these experiments, eEF2 was immunoprecipitated from untreated cells and used as the substrate, while PP2A was isolated from control and LPV treated cells. Substrates and phosphatase were incubated together at room temperature for 25 min, and PP2A activity was quantitated as described above.

STATISTICAL ANALYSIS

For experimental protocols with more than two groups, statistical significance was determined using one-way ANOVA 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 mean ± SE. Mean values were considered significantly different at $P < 0.05$.

RESULTS

EFFECTS Of LOPINAVIR ON BASAL PROTEIN SYNTHESIS

Several HIV anti-retroviral drugs have been shown to adversely affect protein synthesis and metabolism. However, little is known regarding the effect that the protease inhibitor LPV has on these processes. In our initial experiments, we treated cells with 10 μM LPV which is similar to those used previously by others and within the range of concentrations observed in the plasma of patients receiving this drug [Guiard-Schmid et al., 2003; Gutierrez et al., 2003; Hsu et al., 2003]. At this dose, there was no apparent toxic effect, because cell numbers were similar following incubation for 24 h in the presence ($50 \pm 5 \times 10^4$) or absence ($52 \pm 7 \times 10^4$) of LPV.

To determine whether LPV altered the basal rate of protein synthesis, myocytes were labeled for various periods of time in media containing 10 μM of the drug. When protein synthesis was assessed, a significant 30% decrease was observed as early as 15 min following treatment, when compared to values from time-matched control cells (Fig. 1A). The inhibitory effect of this drug on protein synthesis was maintained for at least 4 h. However, the level of inhibition was diminished with longer treatment periods, and no change in the overall rate of protein synthesis was detected when cells were incubated in the presence of LPV for 24 h. The lack of an effect at the 24 h time point was most likely due to the relatively short half-life of LPV. Furthermore, this result is consistent with our findings examining the reversible nature of the drug effect. For these experiments, cells were incubated in the presence or absence of LPV for various periods of time. The drug was then removed and cells were allowed to recover for 1 day prior to labeling. As expected, the inhibitory effect of LPV on basal protein synthesis was not sustained when the drug was removed from the media (data not shown).

To investigate the dose-dependent effect of LPV on protein synthesis, C2C12 myocytes were labeled for 15 min in the absence or presence of increasing concentrations of the drug. Treatment of cells with 0.1 or 1 μM LPV did not alter the rate of protein synthesis. However, at a concentration of 10 μM, LPV significantly decreased

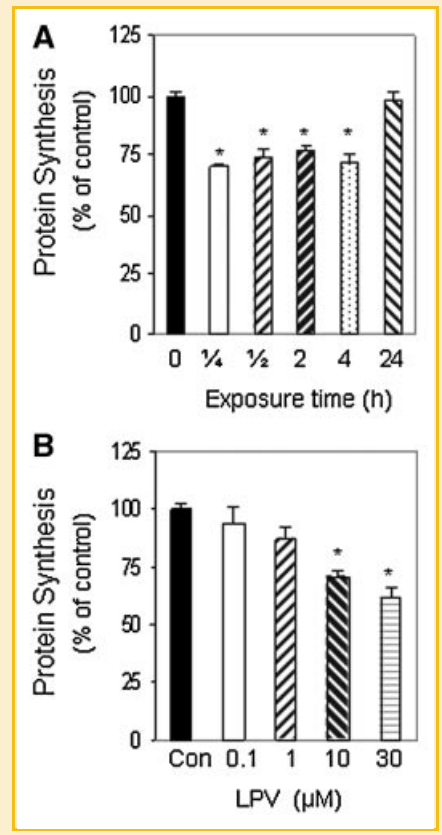

Fig. 1. Time- and dose-dependent effects of lopinavir (LPV) on protein synthesis. C2C12 myocytes were labeled with [^{35}S] methionine/cysteine in 24 well plates. Cells were labeled in the presence of 10 μM LPV for various periods of time (A). Cells were then collected, and the amount of trichloroacetic acid (TCA) -precipitable radioactivity was determined as described under "Materials and Methods Section." C2C12 myocytes were labeled with increasing concentrations of LPV for 15 min and the amount of TCA-precipitable radioactivity was determined (B). Values are compared to time-matched controls that were labeled in the absence of LPV. Each bar represents the mean ± SE of 3–4 independent experiments consisting of 3–6 replicate samples per experiment $^*P < 0.05$ versus matched control value.

protein synthesis by 28%, when compared to untreated control cells (Fig. 1B). Incubation of myocytes with higher concentrations of LPV did not result in a further decline in protein synthesis. For example, 30 μM LPV produced a comparable decrease in protein synthesis when compared to cells treated with 10 μM. Based upon the above results, 10 μM of LPV was chosen for use in all subsequent experiments.

LOPINAVIR AFFECTS THE PHOSPHORYLATION STATE OF eEF2

The activity of eEF2 is critical for the elongation step of translation, and this activity is negatively regulated by its phosphorylation. To determine the effect of LPV on eEF2 phosphorylation, cells were incubated in the presence or absence of 10 μM LPV for 15 min. This time point was chosen because it was sufficient to significantly decrease protein synthesis (see Fig. 1A). As illustrated in Figure 2, LPV increased phosphorylation of eEF2 by twofold at the 15 min time point relative to the control group. In contrast, this drug had no affect on total eEF2 protein content. Therefore, the observed increase in eEF2 phosphorylation is consistent with the ability of LPV to decrease protein synthesis.

LOPINAVIR-INDUCED PHOSPHORYLATION OF eEF2 IS CONTROLLED BY eEF2K

eEF2 is regulated by the calcium and calmodulin-dependent kinase eEF2K, and the activity of this kinase is mediated by phosphorylation events [Browne et al., 2004]. To examine whether eEF2K plays a role in regulating eEF2 phosphorylation in our system, we first examined the effect of LPV on eEF2K phosphorylation. Figure 3A shows that LPV increased eEF2K phosphorylation on the Ser 366 residue by 48%, as compared to control values. This increase

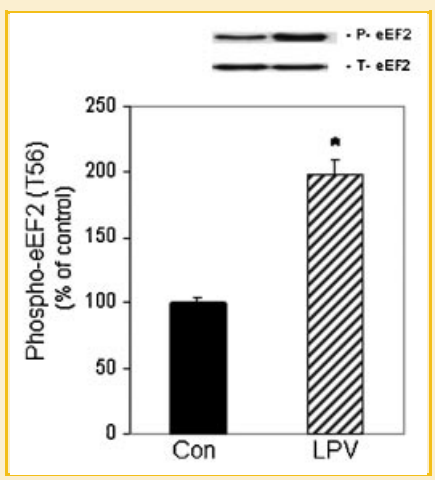

Fig. 2. Effects of lopinavir (LPV) on eEF2 phosphorylation. C2C12 myocytes were incubated in the presence or absence of LPV (10 μM) for 15 min. Cell extracts were collected and analyzed via Western blotting using anti-phospho-eEF2 (T56) and total eEF2 antibodies. Results for phosphor-eEF2 are normalized to total eEF2 and are expressed as a percentage of basal control levels. Each bar graph represents mean ± SE of 6 independent experiments consisting of 3–4 replicate samples per experiment. $^{*}P < 0.05$ versus control values.

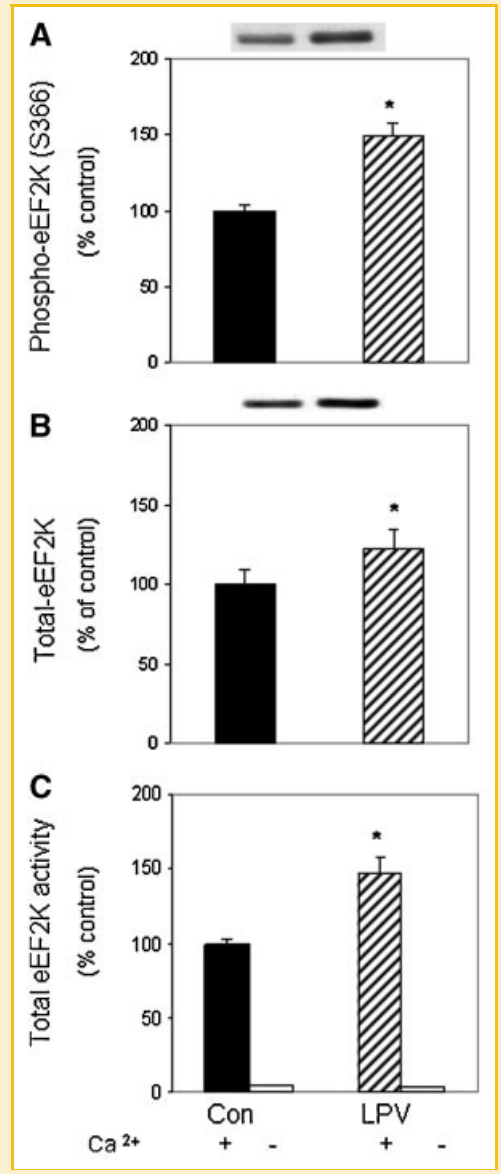

Fig. 3. Lopinavir (LPV) stimulates eEF2K phosphorylation and activity. C2C12 myocytes were treated as described in Figure 2. Cell extracts were analyzed via Western blotting using antibodies that recognize phosphorylated eEF2K at S366 (A) and total eEF2K (B). C: An in vitro eEF2K activity assay was performed in the presence of $CaCl_2$, Ca/calmodulin and ATP, as described in the "Materials and Methods Section." Data are mean ± SE of 3–4 independent experiments consisting of 3–6 replicate samples per experiment. $^{*}P < 0.05$ versus the control value.

was in part due to an unexpected, albeit statistically significant, increase in total eEF2K protein content (Fig. 3B).

The phosphorylation of eEF2K can either inhibit or enhance the activity of its downstream substrate, depending on the site of phosphorylation and the type of stimuli. For example, phosphorylation of Ser 365 following hormone treatment has been shown to decrease the activity of eEF2K [Wang et al., 2001; Browne and Proud, 2002]. In contrast, we observed that LPV increased eEF2K phosphorylation of Ser 366 in conjunction with increased eEF2

phosphorylation. Hence, it is possible that other sites on this kinase, such as Ser 398, may also be phosphorylated following LPV treatment, thereby activating eEF2 phosphorylation [Browne et al., 2004]. To determine whether LPV increased the activity of eEF2K towards eEF2, we performed an in vitro activity assay. For this assay, eEF2 was isolated from control cells and used as a substrate, while eEF2K was immunoprecipitated from cells treated with or without LPV. Incubation of myocytes with LPV increased eEF2K activity by 50% when compared to control values (Fig. 3C). Hence, our results are consistent with a model in which LPV increases eEF2 phosphorylation via the action of eEF2K.

LOPINAVIR INCREASES eEF2 PHOSPHORYLATION IN AN AMPK-DEPENDENT MANNER

The elongation process consumes a considerable amount of cellular energy, thereby linking protein synthesis and signaling pathways that respond to changes in energy levels. For example, the AMPK pathway is activated in response to various stimuli that affect cellular energy levels. Furthermore, AMPK is known to regulate the activity of eEF2K [Horman et al., 2002, 2003; Browne et al., 2004; Crozier et al., 2005]. Thus, AMPK may be an important modulator of the effects of LPV on eEF2 phosphorylation. Figure 4A illustrates that LPV increased the phosphorylation of AMPK by twofold, when compared with control untreated cells. LPV similarly increased the phosphorylation of ACC (Fig. 4B), a known downstream substrate of AMPK. Both of these changes were independent of a change in total AMPK or ACC protein. Thus, these data are indicative of an overall increase in the activity of AMPK in myocytes after exposure to LPV.

To delineate the mode of action of AMPK in regulating eEF2 phosphorylation, we utilized an inhibitor that blocks the activity of this protein. For these experiments, cells were treated with LPV in the presence or absence of the inhibitor compound C. As shown in Figure 5, a combined treatment with compound C and LPV significantly decreased (60%) the level of phosphorylated eEF2K (panel A), relative to cells treated with LPV alone. Likewise, this inhibitor suppressed the increased phosphorylation of eEF2 by 56% when both drugs were present (panel B). Collectively, these data suggest that LPV stimulates AMPK to function as an upstream regulator of both eEF2K and eEF2 phosphorylation.

The above data are consistent with a model whereby LPV-induced stimulation of AMPK regulates the phosphorylation of eEF2K and eEF2. Note, however, that compound C may have other non-specific effects. To address this issue, we used a complementary approach in which AMPK activity was determined using an in vitro kinase assay in the presence or absence of compound C. In Fig. 6A, we show that LPV increases the ability of AMPK to phosphorylate eEF2K. However, if compound C was included in the in vitro reaction mixture, there was a significant effect on the ability of LPV to increase AMPK activity. Note that 20 μM compound C had a greater effect on AMPK activity in control versus LPV treated samples. However, higher concentrations of compound C reduced this activity further. Thus, these data verify that compound C can directly attenuate the ability of AMPK to phosphorylate its downstream target. As expected, similar results were obtained when ACC was utilized as a substrate in these experiments (Fig. 6B). LPV stimulated the ability of AMPK to activate ACC, and this response was suppressed in the presence of compound C.

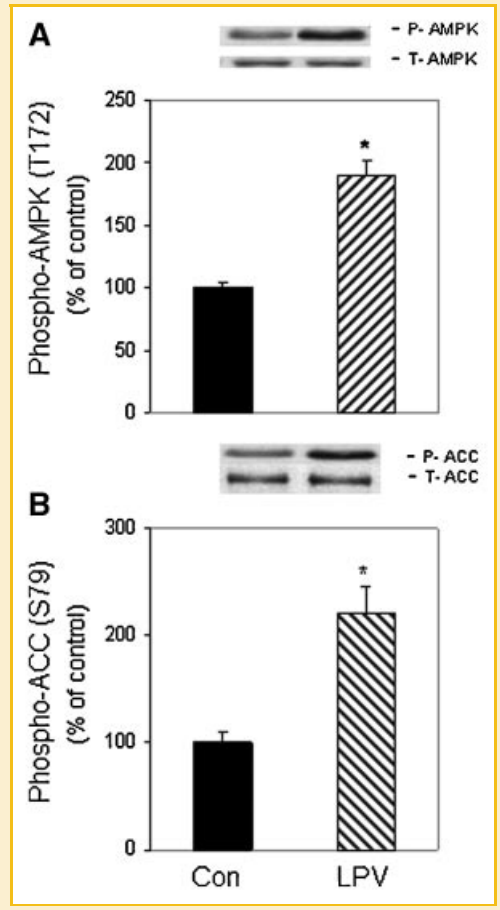

Fig. 4. Lopinavir (LPV) stimulates phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC). C2C12 myocytes were treated as described in Figure 2. Western blots were performed with antibodies that recognize phosphorylated AMPK at T172 (A) and phosphorylated ACC at S79 (B). Data are means ± SE of 3–5 independent experiments consisting of 3–4 replicate samples per experiment. $^{*}P < 0.05$ versus the control value.

Because treatment of cells with compound C blocked the effects of LPV on both eEF2K and eEF2, this suggests that these two proteins are downstream of AMPK signaling. As such, they can be blocked by the action of compound C on AMPK activity. Alternatively, compound C may directly affect these proteins or other kinases that regulate this pathway. To distinguish between these possibilities, we performed an in vitro eEF2K kinase assay using eEF2 as the substrate. LPV increased the activity of eEF2K to phosphorylate eEF2. However, in contrast to our result on AMPK, the addition of compound C did not block this effect (Fig. 6C). Thus, these data suggest that compound C does not directly inhibit the activity of eEF2K, but instead act independent of this protein.

As reported previously [Hong-Brown et al., 2007], eEF2K is not always required for the transmission of signals to eEF2. Therefore, we next determined whether eEF2K was necessary for the phosphorylation of eEF2 under our experimental conditions. For these experiments, myocytes were treated with LPV in the presence or absence of the inhibitor rottlerin, a drug which inhibits eEF2K and

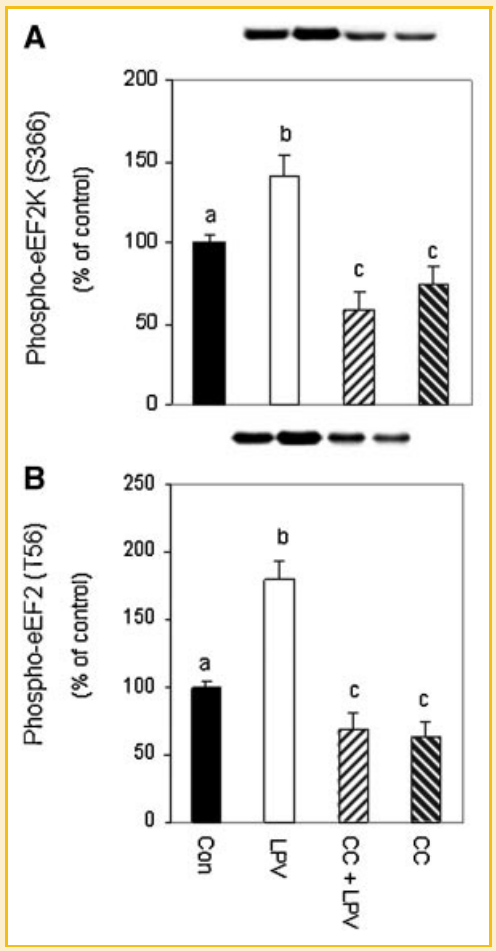

Fig. 5. Lopinavir (LPV) stimulates eEF2 phosphorylation via an AMPK-dependent pathway. Cells were pre-incubated for 1 h in the presence or absence of the AMPK inhibitor compound C (20 μM) and then treated with 10 μM LPV for 15 min. Cell extracts were analyzed via Western blotting using antiserum that recognizes eEF2K phosphorylated at S366 (A) or eEF2 phosphorylated at T56 (B). Data are mean ± SE of 3–4 independent experiments consisting of four 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.

PKC delta with equal efficacy [Gschwendt et al., 1994; Parmer et al., 1997, 1999]. Rottlerin suppressed the stimulatory effect of LPV on eEF2K phosphorylation (Fig. 7A). The presence of rottlerin also blocked the ability of LPV to increase eEF2K activity. For example, when rottlerin was added to the in vitro reaction mixture, this drug inhibited the stimulatory effect that was otherwise observed following LPV treatments (Fig. 7B) Interestingly, rottlerin did not block the effects of LPV on eEF2 phosphorylation (Fig. 7C). Thus, these results indicate that LPV can stimulate eEF2 phosphorylation independent of the action of eEF2K, perhaps via the action of AMPK. Along these lines, we observed that rottlerin did not block the activity of AMPK under in vitro circumstances. As such, AMPK activity against eEF2K was increased following LPV treatment, and this activity remained elevated in the presence of the inhibitor (Fig. 7D).

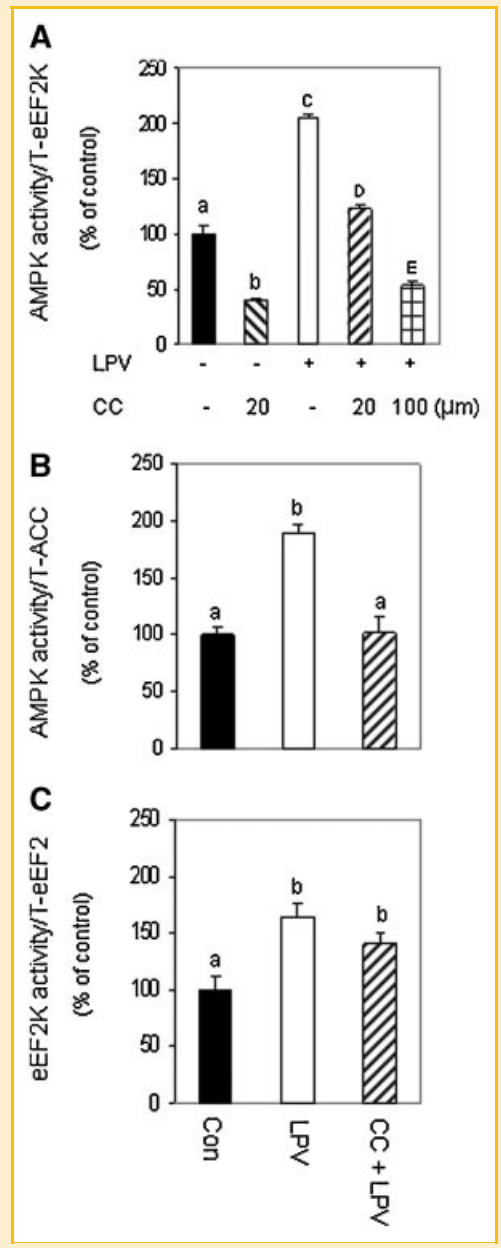

Fig. 6. Compound C inhibits AMPK but not eEF2K activity. C2C12 myocytes were treated as described in Figure 2 and the specificity of compound C (CC) to inhibit AMPK was examined using an in vitro AMPK activity assay where eEF2K (panel A) or ACC (panel B) were utilized as substrates. AMPK was immunoprecipitated from 100 μg of lysates and the activity was determined in the presence or absence of 20 μM CC, $MgCl_2$ and AMP as described under "Materials and Methods Section." Panel C: An in vitro eEF2K activity was determined using eEF2 as the substrate in the presence of CC, $CaCl_2$, Ca/calmodulin, and ATP, as described in the "Materials and Methods Section." Data are mean ± SE of three independent experiments consisting of four replicate samples per experiment. Groups with different letters are significantly different from one another ($^{*}P < 0.05$). Groups with the same letters are not significantly different.

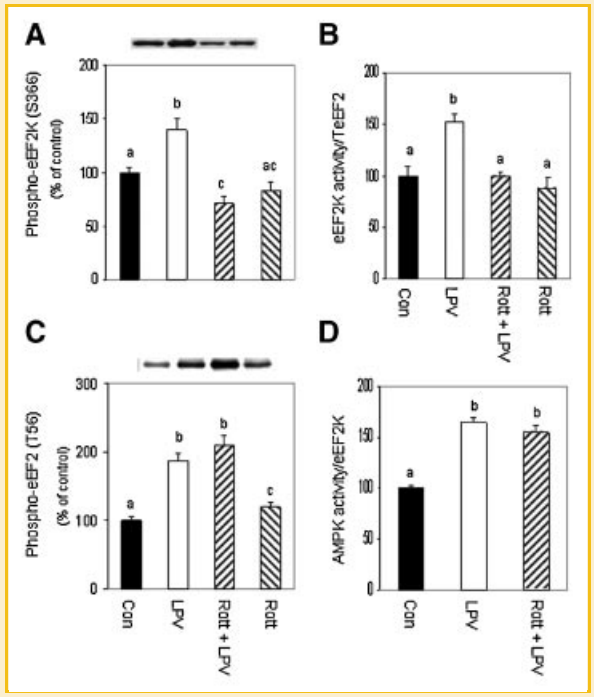

Fig. 7. Lopinavir (LPV) stimulation of eEF2 phosphorylation is eEF2K-independent. C2C12 myocytes were pre-incubated for 1 h in the presence or absence of 2 μM rottlerin (Rott) and then treated with 10 μM LPV for 15 min. Cell extracts were analyzed via Western blotting using anti-phospho-eEF2K (panel A). Panel B: the specificity of rottlerin to inhibit eEF2K was determined using an in vitro eEF2K activity assay with eEF2 as the substrate. eEF2K was immunoprecipitated from 100 μg of lysates and the activity was determined in the presence or absence of Rott, $CaCl_2$, Ca/calmodulin, and ATP as described above. Panel C: C2C12 myocytes were treated as described in panel A. Cell extracts were analyzed via Western blotting using anti-phospho-eEF2 (T56) antibody. Panel D: An in vitro AMPK activity assay was performed using eEF2K as the substrate in the presence or absence of Rott, $MgCl_2$ and AMP as described above. Data are mean ± SE of four independent experiments consisting of 3–4 replicate samples per experiment. Groups with different letters are significantly different from one another ($^*P < 0.05$). Groups with the same letters are not significantly different. $^*P < 0.05$ versus the control value.

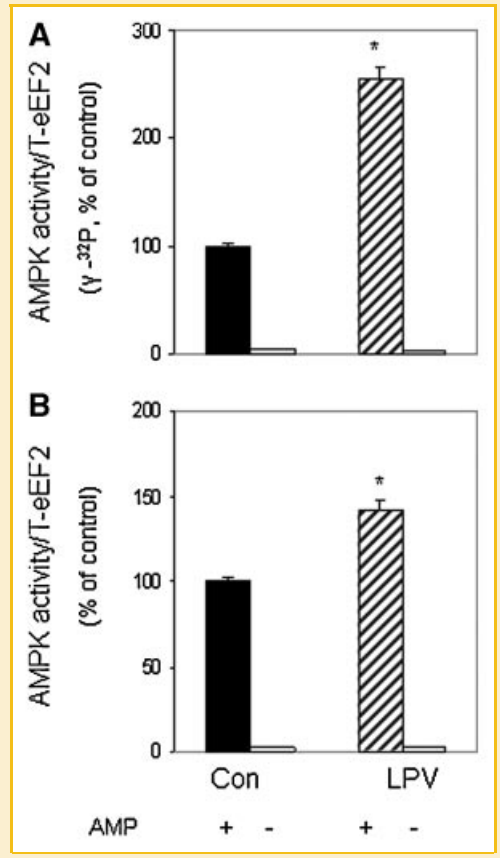

Fig. 8. Lopinavir (LPV) stimulates activity of AMPK. C2C12 myocytes were incubated in the presence or absence of LPV (10 μM) for 15 min. For in vitro kinase activity, AMPK was immunoprecipitated from 100 μg of cell lysates and the activity was assayed using eEF2 as the substrate, while in the presence of $MgCl_2$ and AMP. Reaction mixtures were incubated in the presence (panel A) or absence (panel B) of [γ-^{32}P] ATP as described under "Materials and Methods Section." In panel B, reaction mixtures were examined by Western blot, using the anti-phospho eEF2 (T56) antibody. Results are mean ± SE of three independent experiments consisting of four replicate samples per experiment. $^*P < 0.05$ versus control values.

We previously reported that AMPK can directly phosphorylate eEF2 [Hong-Brown et al., 2007]. Furthermore, the level of activity was shown to increase following treatment with ethanol. Our next experiment examined whether a similar response occurs following treatment of cells with LPV. For these studies, an in vitro kinase assay was utilized to measure AMPK activity. eEF2 was isolated from control cells and used as a substrate, while AMPK was immunoprecipitated from cells treated with or without LPV. Incubation of myocytes with LPV increased AMPK activity towards eEF2 by 2.5-fold, when compared to control values (Fig. 8A). Thus, these data suggest that eEF2 is a direct downstream target of AMPK.

We previously demonstrated the effect of LPV on eEF2 phosphorylation using a T56-specific antibody (Fig. 2). Because our in vitro experiments (Fig. 8A) measured the incorporation of [γ ^{32}P] ATP label into total eEF2 protein, we could not distinguish whether the eEF2 T56 residue was the site that was phosphorylated following treatment with LPV. To address this issue, we performed an in vitro kinase assay as above, albeit using unlabeled ATP. Following completion of the in vitro phosphorylation reaction, the material was subjected to Western blot analysis using the eEF2 antibody that recognized the phosphorylated form of the T56 residue. A significant increase in phosphorylation was observed at the T56 site in cells incubated with LPV (Fig. 8B). Note, however, that this increase appeared less than that observed when we examined AMPK activity using [γ ^{32}P] ATP incorporation (Fig. 8A), suggesting that multiple sites may be targeted for increased phosphorylation by AMPK following exposure to LPV.

LOPINAVIR-INDUCED INCREASES IN eEF2 PHOSPHORYLATION ARE NOT REGULATED BY PP2A PHOSPHATASE

The activity of kinases in cells is balanced by the action of general or specific phosphatases. Accordingly, the increased phosphorylation

of eEF2 following LPV treatment could be due, in part, to a decrease in phosphatase activity. Our final experiments examined whether protein phosphatases play a role in the regulation of eEF2 following incubation with LPV. Control and LPV-treated cells were harvested and total cell lysates or immunoprecipitated PP2A were examined for phosphatase activity. Data in Table I show that LPV caused a small, albeit statistically significant, increase in PP2A activity compared to control values. This increase was observed whether the peptide or eEF2 was used as a substrate. Therefore, these results suggest that a decrease in PP2A is not responsible for the increased phosphorylation of eEF2 following LPV treatments.

DISCUSSION

In this study, we investigated the effects of the anti-retroviral drug LPV on protein synthesis and signaling events related to the control of elongation. Our results demonstrate that the basal rate of protein synthesis declined after a relatively acute exposure of myocytes to LPV. The rapid effect of this drug was in contrast to previous studies, where cells required a significantly longer treatment (24–48 h) to other HIV-protease inhibitors in order to exert an inhibitory effect [Janneh et al., 2003; Hong-Brown et al., 2004, 2005]. Our results are also in contrast to reports where levels of proteins such as P-glycoprotein immunoreactive protein were induced following extended exposure to this drug [Vishnuvardhan et al., 2003], indicating that the synthesis of all proteins is not uniformly suppressed. Although LPV impaired protein synthesis, it is noteworthy that this drug did not appear affect cell viability. This is in agreement with previously reports in which treatment of kidney cells with LPV did not alter cell number, even after several days of drug exposure [Vidal et al., 2006]. Taken together, our findings and published data indicate that protease inhibitors can negatively influence protein metabolism in a variety of cell types.

The mechanisms by which LPV alters muscle protein synthesis have not been investigated previously. In general, regulation of protein synthesis involves changes in the phosphorylation state of several key components of the translation machinery including the phosphorylation of the elongation factor eEF2. Although we did not directly determine rates of elongation in the current study, these results are consistent with the observed reduction in protein synthesis in response to LPV. The effect of LPV on eEF2 is in agreement with previous studies examining various stressors. For example, treatment with the protease inhibitor indinavir decreased the activity of eEF2 in myocytes [Hong-Brown et al., 2004]. Likewise, alcohol or ATP depletion had a similar effect on the phosphorylation state of this eEF [McLeod and Proud, 2002; Hong-Brown et al., 2007].

In the present study, we provide evidence that eEF2K is an upstream regulator of eEF2. For example, LPV increased eEF2K phosphorylation at Ser 366 and it also increased eEF2K activity. This appears in contrast to others reports where phosphorylation at this same site was correlated with decreased eEF2K activity [Wang et al., 2001; Browne and Proud, 2002; Hong-Brown et al., 2007]. Hence, it is possible that phosphorylation of other eEF2K residues, such as Ser 398, may be responsible for increased kinase activity [Browne et al., 2004]. Nevertheless, our in vivo and in vitro data showed that eEF2K activity increased in response to LPV, regardless of the sites that were phosphorylated.

Our studies also suggest that eEF2K is not necessarily required for the control of eEF2 phosphorylation. As such, treatment with the inhibitor rottlerin did not prevent the LPV-induced increase in eEF2 phosphorylation (Fig. 7C), although it did suppress the stimulatory effect of LPV on eEF2K (Fig. 7A). This idea is further supported by our in vitro kinase assay in which we used eEF2 as a substrate to directly measure LPV-induced changes in eEF2K activity (Fig. 7B). This activity was blocked when rottlerin was included in the reaction mixture, verifying the ability of this drug to inhibit this step. These data are consistent with previous reports where rottlerin failed to block the stimulatory effect of alcohol on eEF2 phosphorylation, even though it did inhibit the increased activity of eEF2K in response to AICAR, FBS or growth factors [Parmer et al., 1997, 1999; Hong-Brown et al., 2007]. Thus, these results indicate that there is an alternative mechanism that can control eEF2 activity, without the involvement of eEF2K. This conclusion is in agreement with published studies whereby exercise or treatment with farnesyltransferase induced inactivation of eEF2 in association with inhibition of protein synthesis [Ren et al., 2005; Rose et al., 2005]. These effects were also independent of the activity of eEF2K.

Previously, AMPK was reported to directly stimulate eEF2 phosphorylation following alcohol treatment [Hong-Brown et al., 2007] and this response did not require the action of eEF2K. In the present study, AMPK was observed to activate eEF2K under in vitro conditions, and this activity increased in the presence of LPV. In addition, the LPV-induced increases in both eEF2K and eEF2 phosphorylation were blocked by the AMPK inhibitor compound C, suggesting that AMPK activates eEF2 via its effects on eEF2K. However, as stated above, data from our rottlerin experiments indicate that eEF2K is not required for this process. Moreover, AMPK was also shown to directly regulate eEF2 following LPV treatment (Fig. 8). Collectively, these data are consistent with the hypothesis that AMPK directly acts on eEF2, even when eEF2K and other upstream kinases pathways such as mTOR/S6K1 and ERK1/2 are inhibited (data not shown). Hence, in response to LPV, AMPK can regulate eEF2 in a manner that is independent of mTOR/eEF2K and ERK/eEF2K pathways.

Finally, we examined the role that phosphatases may play in regulating the effect of LPV on eEF2 phosphorylation. Previously, alcohol has been shown to decrease the PP2A activity against eEF2

TABLE I. Effects of Lopinavir (LPV) on PP2A Activity Using Peptide or Total eEF2 as the Substrate

Substrate	Control	Lopinavir
Peptide	100 ± 2.3	114.9 ± 4.5*
T-eEF2	100 ± 3.8	121.2 ± 6.5*

Control and LPV treated cell lysates were examined for phosphatase activity as described under "Materials and Methods Section." For these experiments, total cell lysates were incubated with a phosphopeptide (R-K-pT-I-R_R) for 20–25 min at room temperature. Alternatively, the ability of PP2A to dephosphorylate eEF2 was assayed using PP2A and eEF2 immunoprecipitates from control and LPV treated cells. Values are mean ± SE of 3–6 experiments consisting of 3–7 replicate samples per experiment.
*$P < 0.05$ versus control values (100%).

[Hong-Brown et al., 2007]. In contrast, LPV did not appear to inhibit PP2A activity. Although a role for other phosphatases cannot be excluded, the observed changes in eEF2 phosphorylation were most likely due to changes in kinase activity.

We propose a model in which LPV increases the phosphorylation and activity of AMPK, thereby leading to an increased phosphorylation and inactivation of eEF2. Based on our in vivo and in vitro inhibitor studies, we suggest that AMPK can act on eEF2 either directly, or indirectly via the action of eEF2K. For example, treatment with the inhibitor compound C blocks the ability of AMPK to phosphorylate either eEF2K or eEF2, although it does not directly inhibit the activity of eEF2K towards eEF2. Likewise, rottlerin treatments block the activity of eEF2K towards eEF2, without affecting AMPK. In summary, these data are in agreement with the decrease in protein synthesis that occurs in myocytes exposed to LPV. As such, this should provide insight into the AMPK signaling mechanism regulating this process.

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