Indinavir impairs protein synthesis and phosphorylations of MAPKs in mouse C2C12 myocytes

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Indinavir impairs protein synthesis and phosphorylations of MAPKs in mouse C2C12 myocytes. Am J Physiol Cell Physiol 287: C1482–C1492, 2004. First published June 30, 2004; doi:10.1152/ajpcell. 00038.2004.—Anti-retroviral therapy promotes clinical, immunologic, and virologic improvement in human immunodeficiency virus-infected patients. Whereas this therapy adversely affects carbohydrate and lipid metabolism, the effects of anti-retroviral drugs on muscle protein synthesis and degradation have not been reported. To examine these processes, we treated C2C12 myocytes with increasing concentrations of the protease inhibitor indinavir for 1 or 2 days. Treatment of myocytes with a therapeutic concentration of indinavir (20 μM) for 24 h decreased basal protein synthesis by 18%, whereas a 42% decline was observed after 48 h. A similar decrement, albeit quantitatively smaller, was detected with other protease inhibitors. Indinavir did not alter the rate of proteolysis. Likewise, indinavir did not impair the anabolic effect of insulin-like growth factor-I on protein synthesis. Mechanistically, indinavir decreased the phosphorylation of the S6 ribosomal protein (rpS6), and this reduction was associated with a decreased phosphorylation of p70S6 kinase and p90rsk as well as the upstream regulators ERK1/2 and MEK1/2. Indinavir also decreased the phosphorylation of Mnk1 and its upstream effectors, p38 MAPK and ERK1/2. Indinavir did not affect the phosphorylation of mTOR or 4E-BP1, but it did decrease the amount of the active eukaryotic initiation factor eIF4G-eIF4E complex. In conclusion, indinavir decreased protein synthesis in myocytes. This decrease was associated with the disruption of the ERK1/2 and p38 MAPK pathways and a reduction in both the level of functional eIF4F complex and rpS6 phosphorylation.

Various anti-retroviral drugs are currently available for the treatment of human immunodeficiency virus (HIV) infection. These drugs are classified according to their mechanism of action as HIV-1 protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), and non-nucleoside reverse transcriptase inhibitors (NNRTIs). The current standard of care for HIV infection involves a combination of anti-retroviral drug regimens that include two or more classes of drugs that block different steps in the viral replication cycle (21). Hence, treatment of patients with a PI-based combination therapy as part of this highly active anti-retroviral therapy (HAART) helps to either restore or maintain immune function via suppression of HIV replication and a reduction of the viral load. Thus the introduction of HAART has led to a dramatic decline of the morbidity and mortality associated with HIV infection (35, 42).

Despite HAART being the cornerstone of current AIDS therapy, it is becoming increasingly evident that HAART is associated with metabolic toxicity (16). The toxic effects of these treatments have commanded little attention in the past, because of the dramatic increase in patient survival. However, concern regarding the adverse effects of these drugs is mounting, because this ultimately impacts the quality of life and long-term survival in patients. The use of HAART regimens is correlated with a wide range of alterations in glucose homeostasis, and these often produce a diabetes-like condition. Of particular interest are those in vivo and in vitro studies demonstrating that the PI indinavir markedly alters the ability of insulin to stimulate glucose uptake in adipose tissue and muscle via alterations in GLUT-4 activity (33, 34). Likewise, there is now extensive literature pertaining to disturbances in lipid metabolism caused by PIs. These derangements include elevated plasma concentrations of triglycerides and disrupted cellular cholesterol homeostasis, as well as fat redistribution (3, 19, 48). In contrast, the ability of PIs to regulate protein metabolism has not been as thoroughly investigated, although there is evidence that HIV-related wasting still occurs in patients treated with these drugs (32).

Protein synthesis is a complex process that includes transcription, translation, and signal transduction events. Translation of mRNA on the ribosome involves three stages: initiation, elongation, and termination. Regulation of the initiation step is the primary determinant in controlling the rate of protein synthesis (13, 27). As such, one of the key steps in translation involves the phosphorylation of the S6 ribosomal protein (rpS6), which leads to upregulation of ribosome biogenesis and an increased translational capacity of the cell.

p70S6K and p90rsk are serine/threonine-signaling intermediate kinases that phosphorylate rpS6 (9, 37). Although p70S6K is relatively specific for the phosphorylation of rpS6, the p90rsk kinase has a wide range of substrates (11). Phosphorylation at various sites of p70S6K and p90rsk is important for their kinase activation and function. In the case of p70S6K, this activation is believed to be regulated by several upstream kinases. For example, one pathway is regulated by the mammalian target of rapamycin (mTOR), as evidenced by the use of the immunosuppressant rapamycin. This drug inhibits mTOR and blocks the activation of p70S6K, particularly the phosphorylation at the T389 residue (15, 41, 46). Another possible pathway involves the activation of a series of multisite phosphorylations by proline-directed kinases such as mitogen-activated protein kinases (MAPKs). Recently, a number of studies demonstrated that the MAPK signaling pathway is
important for the activation of both p70S6K1 and p70S6K2 in cardiac myocytes and smooth muscle cells (8, 18, 46). Likewise, p90rsk is a known substrate of MAPKs both in vivo and in vitro (10, 11, 26).

Another key step in the translational control of protein synthesis occurs at the level of the eukaryotic initiation factor 4F (eIF4F) complex. eIF4F is a trimeric complex consisting of eIF4E, eIF4A, and eIF4G. Subsequently, eIF4F binds to the 5' cap structure, and this interaction is required for recruiting the 40S subunit onto the mRNA molecule. eIF4G functions as a scaffold protein that bridges the mRNAs to ribosomes via interaction with eIF4E and eIF3 (29). In addition, eIF4G interacts with other polypeptides involved in translation, such as eIF4A, the poly(A) binding protein, and Mnk1. eIF4E function is regulated in part by its association with the repressor binding protein, referred to as 4E-BP1. Phosphorylation of this protein hinders the formation of the functional complex eIF4F by blocking the binding of eIF4E to eIF4G (14). This regulatory step is altered under catabolic conditions, as evidenced by a decreased eIF4G-eIF4E interaction and increased formation of the inactive 4E-BP1-eIF4E complex (23). Furthermore, this pathway appears to be regulated in part via a MAPK-dependent pathway. Mnk1, a protein kinase, interacts with eIF4G and is responsible for the phosphorylation of eIF4E. Mnk1, in turn, is activated by both ERK1/2 and p38 MAPK (12, 38).

The aim of present study was to determine the effects of various PIs in general and indinavir in particular on protein synthesis in mouse C2C12 myocytes. We also investigated the protein synthesis-related signaling events mediated by indinavir. Various PIs decreased protein synthesis after 1- or 2-day treatments. Indinavir-induced impairment of protein synthesis was associated with alterations in both MEK/ERK and p38 MAPK pathways. The level of ERK1/2 phosphorylation was decreased after indinavir treatment. As expected, this response was associated with a decreased phosphorylation of p70S6K1, p90rsk, and their downstream target, rpS6. The p38 MAPK pathway was also affected by indinavir, and this influenced the phosphorylation of the downstream target Mnk1. Likewise, the interaction of the eIF4G-eIF4E complex was impaired by indinavir.

**MATERIALS AND METHODS**

*Reagents.* Indinavir was a generous gift from Merck (Rahway, NJ). Nelfinavir, saquinavir, amprenavir, and ritonavir were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The majority of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). These included monoclonal antibodies that recognize the phosphorylated form of p38 MAPK (T180/Y182) and ERK1/2 (T202/Y204) as well as polyclonal antibodies specific for phosphorylated (p)-S6 ribosomal protein (S235/236), p-eIF4E (S209), p-eIF4G (S1108), p-p70S6K (T389, T421/S424), p-p90rsk1 (T359/S363), p-Mnk1 (T197/T202), p-mTOR (S2448), and p-MEK1/2 (S217/S221). Antibodies to total mTOR, p90rsk (RSK1-3), p38 MAPK, and ERK1/2 were also obtained from Cell Signaling Technology, whereas p70S6K1, p70S6K2, and Mnk1 were from Santa Cruz Biotechnology (Santa Cruz, CA). The p38 MAPK inhibitor SB-202190 was purchased from EMD Biosciences (San Diego, CA). Propidium iodide was obtained from Sigma (St. Louis, MO), and RNase was obtained from Roche (Indianapolis, IN). Human recombinant insulin-like growth factor (IGF)-I was provided by Genentech (San Francisco, CA). [35S]methionine/cysteine (>1,000 Ci/mol) was obtained from MP Biomedicals (Aurora, OH). Cell culture medium and fetal bovine serum (FBS) were from GIBCO In Vitro (Carlsbad, CA).

*Cell culture.* C2C12 mouse myocytes were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml penicillin, and 25 µg/ml amphotericin. The effect of various PIs on protein synthesis was determined with minor modification as previously described (17). Briefly, cells were subcultured in 24-well plates to 90% confluence. Most studies were conducted using cells at the myoblast stage, but selected experiments were repeated using differentiated myotubes. Cells were then incubated in 1% FBS medium alone (control) or in media containing one of the anti-retroviral agents described in Reagents. Drugs were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the culture was <0.1%. Preliminary studies indicated that this concentration did not have a significant effect on protein synthesis. For 1-day experiments, cells were incubated in the presence of the drug and radioisotope for 24 h before being harvested. For 2-day experiments, cells were preincubated for 1 day with one of the above agents and then radiolabeled with fresh medium on the second day in the continued presence of this agent. Cells were labeled with 10 µCi [35S]methionine/cysteine in DMEM per well for 24 h unless otherwise indicated. The media contained “cold” methionine and cysteine at concentrations of 30 and 65 µg/ml, respectively. Preliminary studies showed that the rate of radiolabel incorporation into protein was linear between 1 and 24 h (data not shown), indicating that there were no significant changes 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% TCA. The incorporation of [35S]methionine/cysteine into TCA-precipitable protein was determined using liquid scintillation counting. The results were then compared with those of the appropriate time-matched control group, and data were expressed as percentages of the control value. DNA synthesis was determined as described by Rauch et al. (40), with minor modification.

*Protein degradation.* C2C12 myocytes were subcultured in 24-well plates as described previously (17). Cells were pulse labeled with [35S]methionine/cysteine in the absence or presence of indinavir for 1 or 24 h to determine the rate of degradation of short- and long-half-life proteins, respectively. Some cells were collected at this time (pulse), whereas for other cells, the radiolabeled medium was removed and replaced with fresh medium lacking radioactive methionine/cysteine (chase). Myocytes were chased for various times in the absence (control) or presence of this drug. Cells were collected and precipitated in 10% TCA, and the TCA-precipitable counts were determined as described in Cell culture.

*Western blot analysis.* To study the signaling pathways known to regulate protein synthesis, we subcultured C2C12 myocytes in six-well plates in the presence of indinavir for 24 h. Thereafter, cells were changed to serum-free medium in the continued presence of indinavir and collected after 20–25 min in 2X Laemmli sample buffer. Cell lysates were electrophoresed on denaturing polyacrylamide gels and transferred to nitrocellulose. The resulting blots were blocked with 5% nonfat dry milk and incubated with the antibodies of interest described in Reagents. Unbound primary antibody was removed by washing with Tris-buffered saline (TBS) containing 0.05% Tween 20 (ICI Americas, Wilmington, DE), and blots were incubated with anti-rabbit or anti-mouse immunoglobulin conjugated with horseradish peroxidase. Blots were briefly incubated with an enhanced chemiluminescent detection system (Amersham, Amersham, UK) 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.
**Immunoprecipitation of the eIF4E and eIF4G complex.** For quantification of the eIF4G-eIF4E complex, myocytes were grown in 100-mm plates as described and then collected in 20 mM HEPES buffer containing 100 mM KCl, 2 mM EGTA, 0.2 mM EDTA, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM DTT, and protease inhibitor cocktail (Sigma). The supernatants were immunoprecipitated with an anti-eIF4E monoclonal antibody overnight. The antibody-antigen complex was captured by incubation for 1 h with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). The beads were washed in TBS, and proteins were eluted using 2× Laemmli sample buffer. Precipitated material was examined by Western blot analysis as described.

**Cell viability and cell cycle progression.** C2C12 myocytes were subcultured in six-well plates to 90% confluence. Some cells were treated with indinavir, whereas other cells were maintained in media lacking this agent. Time-matched samples were collected, and the cell number was determined using a hemocytometer.

To investigate the effect of indinavir on the cell cycle, we cultured C2C12 cells in six-well plates to 75% confluence in the presence or absence of indinavir for 24 h. Cells were harvested by centrifugation, washed, fixed in 70% cold ethanol, and incubated with 40 μg/ml propidium iodide and 5 μg/ml RNase. Samples were kept in the dark at room temperature for 30 min and analyzed for the cell-cycle profile with the use of a FACScan instrument (Becton Dickinson, San Jose, CA). DNA content was determined using Cell Quest 3.3 data-aquisition flow cytometry software (Becton Dickinson) and analyzed with ModFilt DNA analysis software (Verity Software House, Topsham, ME). Results for the different phases of cells in indinavir-treated cultures or the control group are expressed as percentages of total cycling cells.

**Statistical analysis.** For experimental protocols with more than two groups, statistical significance was determined using one-way ANOVA followed by Dunnett’s test to compare all data with the appropriate time-matched control group. For experiments with only two groups, an unpaired Student’s t-test was performed. Data are presented as means ± SE. Mean values were considered significantly different at P < 0.05.

**RESULTS**

**Effect of protease inhibitors on basal protein synthesis.** To determine whether various protease inhibitors altered the basal rate of protein synthesis, we incubated C2C12 myoblasts with therapeutic concentrations of indinavir, nelfinavir, amprenavir, saquinavir, or ritonavir for 2 days. The drug concentrations utilized for this and subsequent experiments were based on plasma levels reported for patients receiving these treatments (22, 43). Treatment of cells with various protease inhibitors significantly decreased protein synthesis 15–42%, compared with rates in control cells (Fig. 1A). Of the drugs tested, indinavir appeared to have the most pronounced adverse effect on protein synthesis. Therefore, we focused all subsequent studies on the metabolic effects of indinavir. Figure 1B shows that a 24-h incubation with 10 μM indinavir significantly decreased protein synthesis (18%) relative to control values. This decline was more pronounced when cells were exposed to indinavir for 48 h, with protein synthesis being impaired by 42%. Incubation of myocytes with 20 and 40 μM indinavir produced changes in protein synthesis comparable to those seen in cells treated with 10 μM indinavir at both time points.

C2C12 cells consist of myoblasts and myotubes. Therefore, we examined whether indinavir regulates protein synthesis comparably in these cell stages. Cells were allowed to differentiate into multinucleated myotubes, which expressed contractile proteins (data not shown). Incubation of myotubes with 20 μM indinavir for 48 h reduced protein synthesis (85.3 ± 2.8%) compared with time-matched control values (100 ± 3.8%). Because of the comparable response to indinavir, all subsequent studies were conducted using cells at the myoblast stage.

**Effects of indinavir on cell viability, proliferation, and cell cycle progression.** To ensure that the observed effects on protein synthesis were not caused by a decline in cell number, we performed cell counts on plates of myocytes incubated in the presence or absence of 20 μM indinavir for 1 or 2 days. Treatment of cells with indinavir in 1% FBS medium for 2 days did not affect cell viability (76.0 ± 2.3 × 10^3 cells/well) relative to time-matched untreated control cells (74.8 ± 3.4 × 10^3 cells/well), indicating that the reduction in protein synthesis was not due to a decrease in cell number. Results from indinavir-treated cells at day 1 showed a similar lack of effect on cell number (data not shown). The viability of myocytes was further confirmed by the observation that treatment of cells with indinavir in medium containing 10% FBS did not inhibit cell proliferation (Fig. 2A). Likewise, DNA synthesis in indinavir-treated myocytes was not different at 24 h between indinavir-treated and control cells (74.0 ± 2.3 vs. 73.8 ± 3.4%). Therefore, the observed decrease in protein synthesis appeared to be dose dependent and not caused by a decrease in cell number. This decline was more pronounced when cells were exposed to indinavir for 48 h, with protein synthesis being impaired by 42%.

**Fig. 1.** Effect of protease inhibitors on protein synthesis. A: C2C12 myocytes were incubated in 24-well plates with medium alone (control, Con) or with therapeutic concentrations of indinavir (Ind; 10 μM), nelfinavir (Nelf; 10 μM), amprenavir (Amp; 24 μM), saquinavir (Saq; 5 μM), or ritonavir (Rit; 5 μM) for 2 days. Cells were labeled and collected as described for A. Data are expressed as percentages of time-matched control values. Each bar graph represents means ± SE of 2–3 independent experiments (n = 4–6 wells/experiment). *P < 0.05 vs. matched control.
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Table 1. Effect of indinavir on cell cycle progression

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<tr>
<td>Control</td>
<td>74.2±3.1</td>
<td>7.8±0.7</td>
<td>18.1±2.8</td>
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<tr>
<td>Indinavir</td>
<td>78.6±1.4</td>
<td>6.4±0.3</td>
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Values are means ± SE of the percentage of cells in different stages; \( n = 7 \) in control group and 9 in the indinavir-treated group. No statistically significant differences were detected between the two groups.

Fig. 2. Effect of indinavir on cell proliferation and viability. A: C2C12 myocytes were incubated in 10% FBS medium in the presence or absence of indinavir (20 \( \mu \)M) for 1 or 2 days, as indicated, and cell counts were determined. Solid bars represent time-matched control cells without indinavir; open bars represent myocytes incubated for the indicated time with indinavir. B: myocytes were treated with indinavir for 2 days in 1% FBS medium. Cells were recovered for 1 day in medium lacking indinavir and then labeled for 24 h. Each bar graph represents means ± SE of 2–3 independent experiments (\( n = 3–5 \) wells/experiment). \( a,b,c \) Groups with different letters are significantly different (\( P < 0.05 \)) from one another; groups with the same letter are not significantly different.

Fig. 3. Indinavir does not inhibit the anabolic effect of insulin-like growth factor (IGF-I) on protein synthesis. C2C12 myocytes were incubated with indinavir (20 \( \mu \)M), IGF-I (12 nM), or a combination of indinavir and IGF-I. The amount of TCA-precipitable radioactivity was determined after 24 or 48 h. Each bar graph represents means ± SE of 2–3 independent experiments (\( n = 5–6 \) wells/experiment). \( a,b,c,d \) Groups with different letters are significantly different (\( P < 0.05 \)) from one another; groups with the same letter are not significantly different.
was not altered by indinavir (data not shown). In a similar manner, incubation of cells with indinavir decreased the phosphorylation of p90rsk by 35% (Fig. 7). This decline was independent of a change in the total p90rsk protein level.

**Indinavir decreases phosphorylation of MEK1/2 and ERK1/2.** To study the upstream signaling pathways known to mediate the phosphorylation of both p70S6K1 and p90rsk, we investigated the influence of indinavir on ERK1/2 and its upstream kinase, MEK1/2. Figure 8A shows that a significant 42% decrease in ERK1/2 phosphorylation was observed after drug treatment. This decline was not due to a decrease in total protein content. Instead, it appears that the decline observed in ERK1/2 phosphorylation resulted from a decreased phosphorylation of MEK1/2 (Fig. 8B).

**Indinavir does not alter mTOR and 4E-BP1 phosphorylation but decreases the association of eIF4G with eIF4E.** Another kinase that plays an important role in the translational control of protein synthesis is mTOR. This protein represents a bifurcation point and may be responsible in part for the phosphorylation of p70S6K and 4E-BP1, which lie on parallel pathways (44). As shown in Fig. 9A, indinavir did not alter mTOR phosphorylation. We also examined the effect of indinavir on 4E-BP1. This protein, when hypophosphorylated, negatively regulates protein synthesis by interfering with the formation of the active eIF4G-eIF4E complex. Treatment with indinavir did not change the phosphorylation state of 4E-BP1 (Fig. 9B). However, indinavir did impair the binding of p-eIF4G with eIF4E (Fig. 9C). Thus the negative effects of indinavir on translation may be due in part to a reduction in the amount of functional eIF4F complex.

**Indinavir decreases Mnk1 and p38 MAPK phosphorylation.** Mnk1, a kinase that interacts with eIF4G, is thought to be responsible for the phosphorylation of eIF4E. As shown in Fig. 10A, indinavir modestly increased the phosphorylation of eIF4E, without altering total eIF4E levels. However, indinavir significantly decreased the phosphorylation of Mnk1 by 38% (Fig. 10B). This result was unexpected and appears to be in
Therefore, studies were conducted to determine whether selected signal-mediated events also responded in a similar manner following the addition and subsequent removal of the drug. Figure 11 shows that decreased levels of ERK1/2 (A) and p38 MAPK (B) phosphorylation were not sustained when indinavir was removed from the media.

On the basis of the above data, indinavir appears to impair protein synthesis via the p38 MAPK and ERK1/2 pathways. To further verify the involvement of these pathways in regulating protein synthesis, we treated C2C12 cells with the p38 MAPK inhibitor SB-202190 in either the presence or absence of indinavir. Figure 12 shows that SB-202190 alone decreased protein synthesis 40% compared with the control group. A greater decline was observed when cells were cultures in the combination of SB-202190 and indinavir, indicating that there was an additive effect of these drugs.

**DISCUSSION**

In this study we investigated the effect of the anti-retroviral drug indinavir on protein metabolism and signal transduction in cultured myocytes. Our results demonstrate that indinavir decreased the basal rate of protein synthesis in cultured myocytes after a 24- or 48-h exposure. However, this treatment did not alter proteolysis. Myocytes treated with other PIs such as nelfinavir, saquinavir, amprenavir, and ritonavir also inhibited basal protein synthesis. This comparable response indicates that as a class, these drugs can adversely influence muscle protein metabolism under in vitro conditions.

The effects of indinavir have been tested in a number of cell lines. In the present study we used C2C12 myoblasts, which are a well-characterized model system. These cells embody a muscle precursor phenotype, much like satellite cells that are resident in mature muscle. In addition, we found that indinavir also decreased protein synthesis in myotubes, although the response was less dramatic. Likewise, treatment of adipocytes with indinavir has been shown to have a negative effect on

**Fig. 6.** Indinavir decreases p70S6K phosphorylation in C2C12 myocytes. Cells were treated as described in Fig. 5 legend. The cell extracts were analyzed via SDS-PAGE and Western blotting, using antisera that recognize p70S6K phosphorylated at T389 (A) or T421/S424 (B). Total protein levels were determined by blotting with an antibody that recognizes the carboxy terminus of p70S6K (C). Data are means ± SE of 4–6 experiments (n = 4–5 wells/experiment). *P < 0.05 vs. control.

Contrast to the findings that indinavir increased the phosphorylation of eIF4E, therefore, alternative mechanisms may be responsible for regulating the phosphorylation of eIF4E in this cell type. As expected, the indinavir-induced reduction in Mnk1 phosphorylation was associated with the downregulation of the upstream kinase p38 MAPK. We observed a 33% decrease in the phosphorylated form of p38 MAPK in response to indinavir, whereas no change was detected in the content of total p38 MAPK (Fig. 10C).

We previously demonstrated that the indinavir-induced depression of protein synthesis is a reversible effect (see Fig. 2B).
protein synthesis (19). On the other hand, past studies reported that treatment of nonmuscle cells for 2 h with the PI saquinavir did not adversely affect protein synthesis or protein content (39). This apparent discrepancy may be due to the difference in cell type or the much shorter duration of the experimental protocol. It is noteworthy that the indinavir-induced decrease in protein synthesis did not result from a change in cell viability. These data are in agreement with previous reports that treatment of nondifferentiated adipocytes with indinavir for several days did not alter cell number (2). Furthermore, our data showed that indinavir did not affect the cell cycle progression. This finding is consistent with the result reported by Chavan et al. (4) that treatment of Jurkat or PM1 T-cell lines with 5–50 μM indinavir did not influence their cell cycle profiles.

The mechanisms by which indinavir alters muscle protein synthesis have not been investigated previously. In general, upregulation of the biosynthetic apparatus is needed to support cell growth and proliferation. One essential component of the protein synthetic machinery is rpS6, a downstream substrate of p70S6K. The phosphorylation of rpS6 by p70S6K has a positive effect on the translation of 5′-TOP mRNAs and hence increases the overall translational capacity of cells (20). Our data clearly demonstrate that indinavir produces a consistent,
A number of in vivo and in vitro studies indicate that the activity of p70S6K1 is regulated by phosphorylation, and this is important for the maintenance of normal rates of protein synthesis. The p70S6K1 activation process relies on the sequential phosphorylation of multiple sites located in different domains of the kinase. One set of phosphorylation sites is within the linker region and catalytic domain (15, 36). The phosphorylation sites of the second set reside within the autoinhibitory domain, and these have been suggested to be important for the subsequent phosphorylation of T389 in the linker region (25). Our results show that indinavir decreased the phosphorylation of the T424/S421 sites located in the autoinhibitory domain as well as the T389 site in the linker region. A portion of this decline was attributed to a decrease in the total p70S6K1 protein level. On the other hand, this inhibitory effect could be due to a decreased upstream kinase activity and/or an increased upstream phosphatase activity. In this regard, we found that the indinavir-induced decrease in phosphorylation of sites within the autoinhibitory domain was more dramatic than that observed in the linker region, indicating that these sites may be regulated by different upstream kinases.

Previous studies (9, 37) have shown that both p70S6K and p90rsk phosphorylate the same serine residue of rpS6, indicating that p90rsk is also an upstream kinase of rpS6. Similar to p70S6K, phosphorylation of various sites in p90rsk is important for its functional activity. In the current study, we dem-

Fig. 10. Indinavir decreases Mnk1 and p38 MAPK phosphorylation. C2C12 myocytes were treated with indinavir as described in Fig. 5 legend. Proteins were subjected to Western blot analysis using antibodies specific for total and phosphorylated form of eIF4E (A), Mnk1 (B), and p38 MAPK (C) as indicated. Data are means ± SE of 3–5 independent experiments (n = 4–6 wells/experiment). *P < 0.05 vs. control.

albeit modest, decrease in the phosphorylation of rpS6. These data suggest that the indinavir-induced impairment in protein synthesis might be due in part to decreases in the level of the ribosomal biosynthetic machinery.

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Fig. 11. Effects of indinavir on ERK1/2 and p38 MAPK phosphorylation are reversible. C2C12 myocytes were incubated in the absence or presence of indinavir (20 μM) for 2 days. Cells were recovered for 2 days in medium lacking indinavir. At this time, fresh medium was added, and cells were lysed after 20 min. Phosphorylation of ERK1/2 and p38 MAPK was determined using specific antibodies that recognize the dual-phosphorylated ERK1/2 (A) and phosphorylated p38 MAPK (B). Each bar graph represents means ± SE of 2–3 independent experiments (n = 3–4 wells/experiment). *P < 0.05 vs. control.
Fig. 12. p38 MAPK inhibitor (SB-202190) suppresses protein synthesis. C2C12 cells were incubated in the absence or presence of indinavir (20 μM) for 24 h. Thereafter, SB-202190 (30 μM) was added to selected wells for 1 h before labeling. Cells were collected, and the amount of TCA-precipitable radioactivity was determined as described in MATERIALS AND METHODS. Each bar graph represents the mean ± SE of two independent experiments (n = 5 wells per experiment). abc-a Groups with different letters are significantly different (P < 0.05) from one another.

onstrated that indinavir decreases phosphorylation of p90rsk at T359 and S363 located in the linker region. This implies that the function of this kinase is diminished and that the inhibition may contribute to the decreased phosphorylation of rpS6. In contrast, one additional phosphorylation site (S381) in the same region of p90rsk was unaffected (data not shown). Previous studies showed that the phosphorylation of S381 is important for activation of the NH2-terminal domain (6). However, the specific role of various phosphorylation sites in the activation of p90rsk is not well established.

Although p70S6K and p90rsk share some amino acid sequence similarity, they appear to be regulated by different kinase cascades. Several lines of evidence indicate that phosphorylation of these intermediate kinases is regulated by various upstream effectors (7). Whereas p90rsk is mainly activated by the MAPK pathway (9, 10), the regulation of p70S6K is complex and not fully understood. For example, at least two major signaling pathways have been described for the phosphorylation and activation of p70S6K1. The first pathway involves phosphatidylinositol 3-kinase 3-kinase and its downstream target, PKD1, and the second essential pathway is mediated by Akt/mTOR (1, 36). Previous studies (5, 24) reported that the MAPK pathway is not required for the phosphorylation of p70S6K1. However, it was demonstrated recently that MAPK family members, such as MEK/ERK, are important for p70S6K1 and p70S6K2 activation (8, 45). In the present study, indinavir decreased the phosphorylation of p70S6K1 (T421 and S424) in the autoinhibitory domain, which contains the consensus serine/threonine-proline motif. Therefore, these sites can be phosphorylated by proline-directed protein kinases such as MAPK kinases (31). Our data demonstrate that the impairment observed in p70S6K1 phosphorylation is associated with a decreased phosphorylation of both MEK1/2 and ERK1/2, suggesting that MAPKs are involved in the regulation of p70S6K. This finding is in agreement with earlier studies in which indinavir inhibited insulin signal transduction at the level of MAPK activation in adipocytes (2). On the other hand, past studies have shown that inhibition of mTOR with rapamycin blocks the T389, but not the T421 and S424, phosphorylation of p70S6K1. These data suggest that mTOR is responsible for the activation of T389 (18). In the present study, mTOR activity was not directly assessed and although indinavir did not alter mTOR phosphorylation, we cannot exclude the possibility that this PI decreased p70S6K phosphorylation via a change in mTOR activity.

The binding of mRNA to the 43S preinitiation complex is mediated by the eIF4F complex. Among the three subunits of eIF4F, eIF4E is the least abundant and is considered to be rate limiting in the binding of mRNA to ribosomes. However, the phosphorylation of eIF4E is not necessarily required for its function because unphosphorylated eIF4E can also stimulate translation. In addition, there is no direct evidence that phosphorylation at S209 has any effect on translation (13, 28). In the present study, indinavir increased the phosphorylation of eIF4E at S209 at the same time that the rate of protein synthesis was decreased. These results are consistent with previous findings that anisomycin or arsenite increased eIF4E phosphorylation, despite decreased rates of translation (30, 47). Collectively, these data indicate that phosphorylated eIF4E is not necessarily correlated with increased rate of translation.

In our study, the indinavir-induced decrease in protein synthesis was also associated with an impaired formation of the active eIF4F complex. Indeed, we found that the protease inhibitor decreased the amount of p-eIF4G associated with eIF4E. As stated above, eIF4E can be regulated by binding to the repressor protein 4E-BP1, which enhances the formation of an inactive 4E-BP1-eIF4E complex by decreasing the γ (hyperphosphorylated)-isoform of 4E-BP1. However, in the present study, there was no detectable alteration in 4E-BP1 phosphorylation. Therefore, the indinavir-induced decrease in protein synthesis does not appear to be mediated through 4E-BP1. It is possible that the action of indinavir targets other proteins, as yet unidentified, that regulate the formation of the eIF4G-eIF4E complex. Furthermore, it is possible that this complex may be regulated by upstream kinases, including Mnk1 and MAPKs. In this regard, it has been demonstrated (12) that Mnk1 is phosphorylated and activated by upstream effectors ERK1/2 and p38 MAP kinases, and this association is supported by our data (see Fig. 8A and 10C). It has been reported (13, 38) that eIF4G binds to Mnk1. Therefore, Mnk1 might regulate the formation of the active eIF4G-eIF4E complex in addition to its role in phosphorylating eIF4E.

In summary, incubation of C2C12 myocytes with indinavir for 24 or 48 h inhibited basal protein synthesis without a concomitant change in protein degradation. Indinavir-impaired protein synthesis was associated with defective MEK1/2, ERK1/2, and p38 MAPK signaling that may account for the reduction in rpS6 phosphorylation. In addition, indinavir decreased the formation of the eIF4F complex, and this change was independent of a change in 4E-BP1 phosphorylation. Hence, in addition to altering carbohydrate and lipid metabolism, indinavir has a pronounced effect on protein synthesis that appears to be mediated by multiple defects in translation initiation. It remains to be determined whether such changes occur under in vivo conditions.
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