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HIV protease inhibitors induce metabolic dysfunction in part via increased JNK1/2 pro-inflammatory signaling in L6 cells

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ABSTRACT

Protease inhibitors (PIs), such as atazanavir sulfate and ritonavir, are used clinically to prevent the progression of HIV and are known to induce insulin resistance. To determine whether PI-mediated insulin resistance is induced by activation of pro-inflammatory cascades, L6 skeletal muscle cells were treated ±atazanavir sulfate, ritonavir, or atazanavir sulfate + ritonavir, and ±insulin. Treatment with atazanavir sulfate, ritonavir, or atazanavir sulfate + ritonavir for 24 or 48 h significantly increased basal glucose uptake ($P < 0.05$) and atazanavir sulfate + ritonavir treatment increased basal glucose uptake significantly more than ritonavir or atazanavir sulfate treatment alone ($P < 0.05$). Atazanavir sulfate + ritonavir treatment for 48 h completely prevented insulin stimulation of glucose uptake ($P > 0.05$). When compared to untreated cells, basal palmitate uptake and oxidation was found to be significantly higher in cells treated with PIs alone or in combination ($P < 0.05$). Prior PI treatment alone or in combination prevented ($P > 0.05$) the insulin-mediated increase in palmitate uptake and the insulin-mediated decrease in palmitate oxidation observed in the control group. Atazanavir sulfate treatment alone or in combination with ritonavir significantly increased JNK1/2 phosphorylation when compared to the control or ritonavir group ($P < 0.05$) and this was accompanied by a rise ($P < 0.05$) in AKT^{Ser473} phosphorylation in the basal state. Total JNK1/2 and p38 MAPK protein content and p38 MAPK phosphorylation state were not altered in any of the treatment groups ($P > 0.05$). Our data indicate that, in muscle cells, PIs induce metabolic dysfunction that is not limited to insulin-sensitive metabolism and that is potentially mediated by a rise in JNK1/2 pro-inflammatory signaling.

1. Introduction

Highly active antiretroviral therapy (HAART), a combination of three types of drugs comprising non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs), is used to control replication of the immunodeficiency virus (HIV) and the development of acquired immunodeficiency syndrome (AIDS) in HIV-infected patients (Havlir and Lange, 1998). Treatment of HIV infection with HAART has resulted in declines in morbidity and mortality due to AIDS (Palella et al., 1998). However, despite the clinical successes associated with this drug regimen, it is recognized that many patients treated with HAART develop adverse metabolic consequences which are usually characterized by the presence of peripheral insulin resistance as well as dyslipidemia and fat redistribution (Flint et al., 2009). Given that HIV patients started to demonstrate the clinical symptoms associated with these metabolic pathologies when a cocktail of PIs was introduced in the treatment regimen (Carr et al., 1998; Noor, 2007), the specific role of PIs in the development of HAART-induced metabolic dysfunction remains an important research topic. Most studies investigating the cellular mechanisms responsible for HAART-induced insulin resistance have focused on adipose tissue (Adler-Wailes et al., 2010; Murata et al., 2000; Ranganathan and Kern, 2002) while only limited experimental data are available on the cellular mechanisms by which PI cocktails induce insulin resistance in skeletal muscle.

Insulin resistance in skeletal muscle is a multifactorial pathology which is characterized by reduced insulin action and impairment in fatty acid (FA) and glucose metabolism and whose development has been linked to the presence of inflammation (Bastard et al., 2006; DeFronzo et al., 1992; Shoelson et al., 2006; Shulman, 2000). Given that circulating levels of inflammatory markers such as tumor necrosis factor α receptor (TNFαR) have been shown to be elevated in HIV-infected patients receiving PI-inclusive drug regimens (Mynarcik et al., 2000), inflammation

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may play a significant role in HAART-induced insulin resistance in skeletal muscle cells. Two key intracellular markers of inflammation within skeletal muscle cells include p38 MAPK (*M*itogen *A*ctivated *P*rotein *K*inase) and C-Jun-N-terminal kinase (JNK1/2). These markers have been shown to be upregulated in inflammatory conditions such as obesity and to negatively affect the induction of the insulin signaling cascade (Hirosumi et al., 2002). Thus, if inflammation is a significant mechanism by which PI-induced insulin resistance develops, PIs may upregulate p38 MAPK and JNK1/2.

Given this information, the purpose of this study was to determine in skeletal muscle cells (1) whether short-term chronic exposure to a PI cocktail that includes atazanavir sulfate and ritonavir would negatively impact the regulation of glucose and FA metabolism under basal and insulin-mediated conditions and (2) whether these metabolic alterations would be associated with an upregulation of p38 MAPK and JNK1/2 pro-inflammatory signaling. We hypothesized that PI treatment would increase the activity of pro-inflammatory markers and be associated with muscle insulin resistance as it pertains to both glucose uptake and FA uptake and oxidation. To accomplish our aims, we used the L6 skeletal muscle cell line and treated the cells with atazanavir sulfate and/or ritonavir for up to 48 h. We used atazanavir sulfate and ritonavir because the Department of Health and Human Services Panel on Antiretroviral Guidelines for Adults and Adolescents (2008) recommends their use to maintain an acceptable virologic response while minimizing adverse effects on glucose and lipid metabolism (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2008). To ascertain the independent effects of each PI on cell metabolism and signaling, the responses of cells exposed to both PIs were compared to the responses of cells exposed to one PI only.

2. Materials and methods

2.1. Cell culture

L6 myoblasts were cultured in α-minimal essential medium+ (α-MEM+) containing 10% fetal calf serum (FCS), 1% antibiotic–antimycotic solution (Sigma Aldrich Ltd., St-Louis, MO), and 500 μM L-carnitine (Sigma Aldrich, St. Louis, MO) in a humidified incubator at 37 °C (95% O_2, 5% CO_2). The α-MEM+ and FCS were purchased from the Cell Culture Facility (University of Southern California, Los Angeles, CA). Cells were grown in 75 cm^2 sterile culture flasks, sub-cultured at 60–80% confluence and split at a ratio of 1:10 using trypsin–EDTA (Invitrogen, Grand Island, NY). Cells were sub-cultured into 6-well plates and switched to α-MEM+ containing 2% FCS to promote differentiation. By day 4, cells were 100% confluent and spontaneously differentiated into myotubes. L6 myotubes were 10 days post-confluent on the day of the experiment.

2.2. Cell treatments

Atazanavir sulfate and ritonavir were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Cells were first pre-exposed (24, 48, or 72 h) to atazanavir sulfate (100 μM) and/or ritonavir (25 μM) or vehicle (modified α-MEM+, see above) and then incubated with serum-free medium (5 h, for metabolic measurements) and Krebs Ringer Hepes buffer (KRB) (30 min: 1.47 mM K_2HPO_4/140 mM NaCl/1.7 mM KCl/0.9 mM $CaCl_2$/0.9 mM $MgSO_4$/20 mM Hepes; pH 7.4). Cells were then exposed to either insulin (100 nM; Novolin Insulin, University of Southern California Pharmacy) or vehicle (KRB) for 15 min. The cells were then harvested in lysis buffer for Western blot analysis (see below) or subjected to the palmitate uptake and oxidation or glucose uptake assay.

2.3. Palmitate uptake and oxidation

Following each treatment ± insulin, the experimental medium was replaced with transport medium (100 μM albumin-bound palmitate, 1:1, 30 min) containing [1-^{14}C]palmitic acid (4 μCi/mL, Perkin Elmer, Boston, MA) to measure palmitate uptake and oxidation as described previously (Bogachus and Turcotte, 2010; Kelly et al., 2008). Incubations were terminated by removing the media which were used to assay for ^{14}C-labeled oxidation products (see below). Following lysis, one set of duplicate aliquots of the lysate was used to measure protein content using the Bradford method (BioRad, Hercules, CA), while another set was mixed with scintillation fluid (BudgetSolve, Research Product International Corp., Mount Prospect, IL) to count radioactivity (Tri-carb 2100TR analyzer, Packard, Downers Grove, IL). For the measurement of oxidation products, $^{14}CO_2$ was released from duplicate aliquots of experimental media and trapped on filter paper (Whatman). The filter paper was mixed with toluene-based scintillation cocktail and analyzed for $^{14}CO_2$ radioactivity. To correct for carbon loss, additional experiments were conducted with 4 μCi of [1-^{14}C]acetic acid (Perkin Elmer, Boston, MA) instead of [1-^{14}C]palmitic acid (Bogachus and Turcotte, 2010; Kelly et al., 2008).

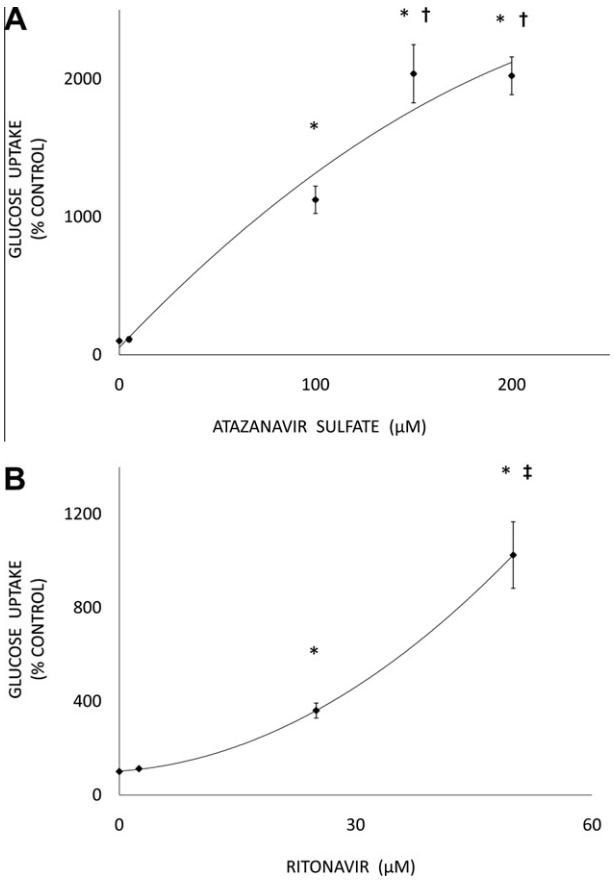

Fig. 1. Dose–response curve for atazanavir sulfate and ritonavir on glucose uptake in L6 cells. Cells were exposed to atazanavir sulfate (0, 5, 100, 150, and 200 μM; A) or ritonavir (0, 2.5, 25, and 50 μM; B) for 48 h. Glucose uptake was measured as described in Methods. Values are mean ± SE for all treatment groups (n = 3–5 per condition) and are expressed as percentage of control, where control refers to cells that are not treated with atazanavir sulfate or ritonavir. $^{*}P < 0.05$ *vs* control and lowest concentration group; $^{\dagger}P < 0.05$ *vs* 100 μM atazanavir sulfate; $^{\ddagger}P < 0.05$ *vs* 25 μM ritonavir.