

Metformin and Resveratrol Induce Beneficial Metabolic Adaptations in L6 Muscle Cells Treated with HIV Protease Inhibitors: Evidence for Inhibition of JNK1/2 Signaling

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Abstract

C-Jun-N-terminal kinase (JNK1/2) pro-inflammatory signaling has been shown to play a role in highly active antiretroviral therapy (HAART)-induced insulin resistance in skeletal muscle cells. To determine the effectiveness of metformin and resveratrol in blunting HAART-induced insulin resistance, L6 skeletal muscle cells were treated ± (with or without) HIV protease inhibitors (ritonavir+atazanavir sulfate; RA) and either metformin, resveratrol, or the JNK1/2 inhibitor SP600125 and incubated ± insulin (100 nM). SP600125 as well as metformin and resveratrol reduced ($P<0.05$) JNK1/2 phosphorylation in RA-treated cells but not in healthy cells. In basal and insulin-stimulated cells, RA treatment significantly increased JNK1/2 phosphorylation ($P<0.05$). Additionally, when RA-stimulated cells were treated with metformin, resveratrol or SP600125, JNK1/2 phosphorylation was reduced ($P<0.05$) indicating reduced inflammation. Basal and insulin-stimulated glucose uptake was increased in RA-treated cells ($P<0.05$). The presence of insulin resistance in RA-treated cells was partially abrogated ($P>0.05$) by metformin and SP600125 treatment. RA-treatment increased FA (fatty acid) uptake and oxidation in both the basal and insulin-stimulated state. Increased percent change of AKT^{Ser473} phosphorylation indicated increased insulin sensitivity in RA+metformin ($P=0.08$) and RA+resveratrol ($P<0.05$) treated cells. Treatment with metformin, resveratrol or SP600125 had very little impact on p38 phosphorylation in basal and insulin-stimulated cells made insulin-resistant by incubation with RA. Our data indicate that metformin and resveratrol induce beneficial metabolic changes in cells made insulin resistant by treatment with protease inhibitors in part via inhibition of JNK1/2 pro-inflammatory signaling and stimulation of insulin-mediated AKT^{Ser473} phosphorylation.

Keywords: SP600125; p³⁸ MAPK (Mitogen Activated Protein Kinase); Inflammation; AKT2; Glucose uptake

Introduction

Highly active antiretroviral therapy (HAART) is prescribed to human immunodeficiency virus (HIV)-infected patients to slow down the progression of HIV and the development of acquired immunodeficiency syndrome (AIDS) [1]. While HAART has many positive effects in HIV-infected patients, it also induces insulin resistance in a significant number of patients [2,3]. Insulin resistance in skeletal muscle is a multifactorial pathology which is characterized with reduced insulin action and aberrant fatty acid (FA) and glucose metabolism and whose development has been linked to the presence of inflammation [4-7]. In line with the notion that tissue inflammation plays a significant role in the induction of insulin resistance [4,6,8,9] we have shown that C-Jun-N-terminal Kinase (JNK1/2) pro-inflammatory signaling, but not p38 Mitogen Activated Protein Kinase (MAPK) signaling, is upregulated in HAART-induced insulin resistance in skeletal muscle cells [10]. Because insulin resistance is accompanied by significant impairment of metabolic regulation, development of clinical strategies to offset the detrimental consequences of HAART therapy should be investigated.

Clinically, insulin resistance is often managed with the biguanide metformin, a pharmacological agent, which effectively increases insulin sensitivity with chronic use [11-14]. However, in spite of its clinical popularity, the effects of metformin on HAART-induced insulin resistance are not clearly defined [15-17]. Furthermore, although metformin has been shown to affect metabolic regulation in skeletal muscle cells and liver via activation of the AMP-regulated protein kinase 1 (AMPK1) [18,19], it is not clear whether metformin could restore metabolic function in HAART-treated cells via a

reduction in pro-inflammatory JNK1/2 signaling. Additionally, due to its potent anti-oxidative and anti-inflammatory effects, the plant-derived polyphenol resveratrol (3,5,4'-trihydroxystilbene) might be another potential agent for the treatment of HAART-induced insulin resistance [20,21]. There may be strong clinical potential for resveratrol in the treatment of inflammatory diseases because it has the ability to downregulate the inflammatory response via a number of biological activities including but not limited to inhibition of NF- κ B signaling and NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome [22,23]. Resveratrol has also been shown to improve mitochondrial function and protect against metabolic disease via activation of SIRT1 and PGC-1 α [24]. Thus, resveratrol may be capable of blunting HAART-induced insulin resistance.

Insulin sensitivity is regulated by the insulin signaling pathway and within this pathway AKT is a molecule of particular interest. We chose to measure AKT2 because it is expressed in skeletal muscle of mammalian cells and studies involving AKT2 knockout mice demonstrated the necessity of AKT2 for glucose homeostasis since

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AKT2 knockout mice could not effectively regulate their glucose levels and developed a type 2 diabetes-like phenotype.

Given this information, the purpose of this study was to determine in L6 skeletal muscle cells 1) whether short-term treatment with metformin or resveratrol would reverse/reduce insulin resistance induced by HAART treatment and 2) whether reversal of insulin resistance would occur via an inhibition of the JNK1/2 pro-inflammatory pathway. To accomplish these aims, we used L6 skeletal muscle cells made insulin resistant via treatment with the protease inhibitors ritonavir+atazanavir sulfate [10]. To specifically ascertain the role of JNK1/2 signaling, the effects of metformin and resveratrol on muscle metabolism and inflammation signaling were compared to those obtained following treatment with the JNK1/2 inhibitor, SP600125 [25-28]. We hypothesized that both metformin and resveratrol would reverse/reduce HAART-induced insulin resistance and JNK1/2 pro-inflammatory signaling and that these results would be comparable to the data obtained with JNK1/2 inhibition (SP600125).

Methods

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 μ M L-carnitine (Sigma Aldrich, St. Louis, MO) in a humidified incubator at 37° C (95% O₂, 5% CO₂). The α -MEM+ and FCS were purchased from the Cell Culture Facility (University of Southern California, Los Angeles, CA). Cells were grown in 75 cm² 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.

Cell treatments

Atazanavir Sulfate and Ritonavir were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. HAART treatment was induced by incubating the cells with atazanavir sulfate (100 μ M) and ritonavir (25 μ M) for 48 hrs. This regimen was used based on our previous studies of the dose- and time-dependent effects of ritonavir and atazanavir sulfate treatment on glucose uptake [10]. For metformin treatment, we used 800 μ M metformin for 24 hrs because we and others have used this concentration and/or time condition previously [18,29,30]. For resveratrol treatment, we used 100 μ M resveratrol for 24 hrs for comparative purposes and because this concentration had previously been used [31,32]. For the JNK inhibitor treatment option, we used 5 μ M of the inhibitor SP600125 for 48 hrs because previous studies using this treatment option have observed an induction of JNK1/2 inhibition that is similar to that observed with metformin or resveratrol in insulin-resistant cells [26-28,33].

Cells were first pre-exposed (48 hrs) to atazanavir sulfate (100 μ M) and ritonavir (25 μ M) or vehicle (modified α -MEM+, see above) and, when dictated by the protocol, metformin (800 μ M, Sigma-Aldrich Ltd, St. Louis, MO) or resveratrol (100 μ M, Sigma-Aldrich Ltd, St. Louis, MO) was included in the medium for the final 24 hours. In experiments with the JNK1/2 inhibitor, SP600125, cells were solely or concurrently exposed to 5 μ M SP600125 (EMB Biosciences, Gibbstown, NJ) during the atazanavir sulfate/ritonavir exposure (48 hrs). Following these procedures, cells were incubated with serum-free medium (5 hrs; for

the metabolic measurements) and Krebs Ringer Hepes buffer (KRB) (30 min: 1.47 mM K₂HPO₄/140 mM NaCl/1.7 mM KCl/0.9 mM CaCl₂/0.9 mM MgSO₄/20 mM Hepes; pH 7.4) and 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.

Substrate uptake and oxidation

Following each treatment \pm (with or without) insulin, the experimental medium was replaced with transport medium (100 μ M albumin-bound palmitate, 1:1, 30 min) containing [1-¹⁴C]palmitic acid (4 μ Ci/mL, Perkin Elmer, Boston, MA) to measure palmitate uptake and oxidation [18,34]. Incubations were terminated by removing the media which was used to assay for ¹⁴C-labeled oxidation products (see below). Wells were washed twice with KRB and cells were lysed by mixing with SDS (23°C). Following lysis, one set of duplicate aliquots of the lysate was used to measure protein content using the Bradford method (BioRad, Hercules, CA). Another set of aliquots was mixed with scintillation fluid (BudgetSolve, Research Product International Corp., Mount Prospect, IL) to count radioactivity (Tri-carb 2100TR analyzer, Packard, Downers Grove, IL) and the counts were used to calculate palmitate uptake. For the measurement of oxidation products, ¹⁴CO₂ 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 ¹⁴CO₂ radioactivity. To correct for carbon loss, additional experiments were conducted with 4 μ Ci of (1-¹⁴C) acetic acid (Perkin Elmer, Boston, MA) instead of (1-¹⁴C) palmitic acid [18,34].

For glucose uptake measurements, following each treatment \pm (with or without) insulin, the experimental medium was replaced with transport medium (200 μ M, 5 min) containing [2-³H] deoxyglucose (2-DG) (0.5 μ Ci/mL, MP Biochemicals, LLC, Solon, OH) to measure glucose uptake [34]. Incubations were terminated via removal of the media and lysis with SDS. As for palmitate uptake, duplicate aliquots of lysate were taken for scintillation counting and for protein determination.

Western blot analysis

After the experimental treatments, cells were washed with ice cold KRB and prepared for Western blotting as described [10,18,35]. Lysis buffer was added (20 mM Tris/1% NP-40/137 mM NaCl/1 mM CaCl₂/1 mM MgCl₂/10% (v/v) glycerol/1 mM DTT/1 mM PMSF/2 mM Na₃VO₄) and cells were gently pelleted via centrifugation. Aliquots of the supernatants were assayed for protein content (Bradford method; Bio-Rad, Hercules, CA) or subjected to 12% SDS polyacrylamide gel electrophoresis. The separated proteins were transferred onto Immobilon-P-polyvinylidene difluoride (PVDF) membranes, blocked and incubated overnight with primary antibodies (1:1,000) to measure either the total protein content for AKT2, or SAPK/JNK, or p³⁸ MAP Kinase (Cell Signaling, Danvers, MA), or the phosphorylation state of AKT^{Ser473}, or SAPK/JNK^{Thr183/Tyr185}, or p38 MAP Kinase^{Thr180/Tyr182} (Cell Signaling, Danvers, MA). After exposure to the secondary antibody horseradish peroxidase goat anti-rabbit (1:10,000; Pierce, Rockford, IL), the membranes were developed via enhanced chemiluminescence (Pierce, Rockford, IL) followed by exposure to CL-XPosure film (Pierce, Rockford, IL). Where appropriate, membranes were stripped and re-probed with the GAPDH antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). The films were scanned using a Hewlett Packard ScanJet 6200C and quantified using Scion Image (Scion, Frederick, MD). In

all cases, multiple gels were analyzed and compared to results obtained for control cells that had not been treated with ritonavir+atazanavir sulfate, metformin, resveratrol, SP600125 and/or insulin. Protein content was normalized to GAPDH.

Calculations and statistics

The rates of glucose and palmitate uptake and of palmitate oxidation were calculated as described in details [10,18,36]. All presented data are expressed as mean \pm SE and are expressed as percent of control where control refers to cells that were not treated with any agent (see figure legends for specific details). The percent control was calculated using measured rates (nmol/g/min) for all experimental treatments. Change (Δ) glucose uptake (Figure 2C) represents the percent change in glucose uptake with insulin treatment for each group and was calculated as the rate of insulin-mediated glucose uptake for each well minus the average rate of basal glucose uptake divided by the average rate of basal glucose uptake. Change (Δ) AKT^{Ser473} phosphorylation (Figure 4E) represents the percent change in AKT^{Ser473} phosphorylation with insulin treatment for each group and was calculated as the insulin-mediated AKT^{Ser473} phosphorylation for each gel minus the average basal AKT^{Ser473} phosphorylation divided by the average basal AKT^{Ser473} phosphorylation. In insulin-treated and non-treated (basal) cells, data were analyzed using analysis of variance followed by Fisher LSD *post-hoc* test when appropriate. In all instances, an α of 0.05 was used to determine significance.

Results

Metformin, resveratrol and SP600125 reduce JNK1/2 phosphorylation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate

The effect of RA treatment on JNK1/2 phosphorylation is shown in Figures 1A-D. As shown previously, treatment with RA increased JNK1/2 phosphorylation when compared to respective non-RA treated cells under both basal and insulin-stimulated conditions ($P < 0.05$, Figure 1C & D) [10]. In cells treated with RA, treatment with metformin, resveratrol or SP600125 decreased ($P < 0.05$) basal and insulin-stimulated JNK1/2 phosphorylation such that JNK1/2 phosphorylation was not different ($P > 0.05$) between non-RA treated and RA treated cells (Figure 1C & D). This indicates that the pro-inflammatory JNK1/2 signaling cascade was reduced. Total JNK protein content did not change with any of the treatment conditions ($P > 0.05$, Figure 1A & B).

Metformin and SP600125 restore insulin-mediated glucose uptake in L6 muscle cells made insulin resistant by treatment with ritonavir+atazanavir sulfate

As shown previously, basal and insulin-stimulated glucose uptake was higher in cells treated with RA when compared to respective non-RA treated cells and RA treatment completely abolished insulin stimulation of glucose uptake indicating the presence of insulin resistance in RA treated cells ($P < 0.05$, Figure 2A-2C) [10,37-40]. Here we show that incubation with metformin, resveratrol or SP600125 was able to inhibit ($P < 0.05$) basal glucose uptake by 50-55% in RA treated cells (Figure 2A). In these cells, incubation with metformin, resveratrol or SP600125 inhibited ($P < 0.05$) insulin-mediated glucose uptake by 17-36% and insulin-mediated glucose uptake was lower ($P < 0.05$) in the resveratrol group than the metformin group (Figure 2B). We also show that treatment with metformin and SP600125, but not resveratrol, restored insulin action in RA treated cells. This is indicated by the fact

that Δ glucose uptake was 65% ($P < 0.05$) in cells treated with RA and metformin and 62% ($P = 0.07$) in cells treated with RA and SP600125 but only 31% ($P > 0.05$) in cells treated with RA and resveratrol.

As shown previously, metformin increased ($P < 0.05$) basal glucose uptake in non-RA treated cells (Figure 2A) [18]. Resveratrol and SP600125 did not affect ($P > 0.05$) basal glucose uptake in non-RA treated cells. In these cells, metformin increased ($P < 0.05$) insulin-mediated glucose uptake whereas resveratrol and SP600125 had no effect ($P > 0.05$).

Resveratrol and SP600125 modify FA uptake and SP600125 reduces FA oxidation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate

As shown previously, RA treatment increased ($P < 0.05$) basal and insulin-mediated FA uptake and oxidation (Figure 3A-D) [10]. In RA treated cells, incubation with metformin, resveratrol and SP600125 reduced ($P < 0.05$) basal FA uptake by 16-55% (Figure 3A). In these cells, basal FA uptake was lower ($P < 0.05$) in cells incubated with resveratrol (48%) or SP600125 (33%) than in cells incubated with metformin. In contrast to these results, only incubation with SP600125 was associated with a decrease (28%; $P < 0.05$) in basal FA oxidation (Figure 3B). Incubation with metformin or resveratrol had no effect ($P > 0.05$) on basal rates of FA oxidation.

Incubation with resveratrol or SP600125 decreased insulin-mediated FA uptake by 38-60% whereas incubation with metformin had no effect on insulin-mediated FA uptake (Figure 3C). Insulin-mediated FA uptake was 35-57% lower ($P < 0.05$) in cells incubated with resveratrol or SP600125 than in cells incubated with metformin. In line with the data obtained for basal FA oxidation, incubation with SP600125 decreased (20%; $P < 0.05$) insulin-mediated FA oxidation whereas incubation with metformin or resveratrol had no effect ($P > 0.05$) on insulin-mediated FA oxidation (Figure 3D).

As shown previously, metformin decreased ($P < 0.05$) basal and insulin-mediated FA uptake in non-RA treated cells (Figure 3A-D) [18]. Additionally, in these non-RA treated cells, incubation with resveratrol or SP600125 had no effect ($P > 0.05$) on basal or insulin-mediated FA uptake or FA oxidation (Figure 3A-D).

Metformin and resveratrol increase insulin-mediated AKT^{Ser473} phosphorylation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate

As shown previously, RA treatment increased ($P < 0.05$) AKT^{Ser473} phosphorylation in the basal state but did not affect AKT^{Ser473} phosphorylation in the insulin-mediated state (Figure 4C & D) [10]. In RA treated cells, metformin and resveratrol increased ($P < 0.05$) insulin-mediated AKT^{Ser473} phosphorylation when compared to control RA treated cells (Figure 4D). In these cells, SP600125 treatment had no effect on insulin-mediated AKT^{Ser473} phosphorylation. To investigate the effect of drug treatment on insulin stimulation of AKT^{Ser473} phosphorylation, we calculated the change (Δ) in AKT^{Ser473} phosphorylation between the basal and insulin conditions (Figure 4E). Our data show that in cells treated with RA, insulin did not increase ($P > 0.05$) AKT^{Ser473} phosphorylation (Figure 4E). Low insulin-mediated AKT^{Ser473} phosphorylation is commonly seen as an indicator of the presence of insulin resistance [41] and has been observed with this type of protease inhibitor treatment [10]. In these insulin-resistant cells, treatment with metformin ($P = 0.08$) or resveratrol ($P < 0.05$) restored the stimulatory effect of insulin on AKT^{Ser473} phosphorylation. SP600125 treatment had no impact on AKT^{Ser473} phosphorylation in cells treated

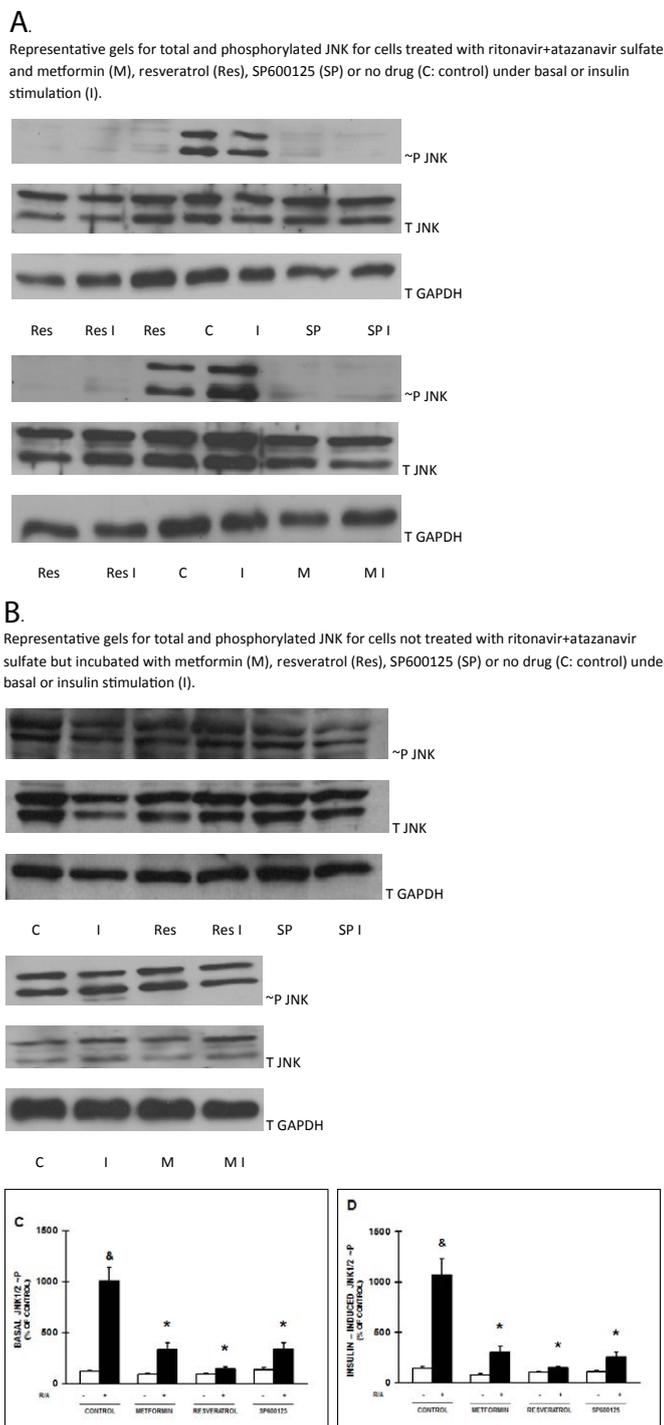


Figure 1: Metformin, resveratrol and SP600125 reduce JNK1/2 phosphorylation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate. (A) Representative gels for total and phosphorylated JNK for cells treated with ritonavir+atazanavir sulfate and metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I). **(B)** Representative gels for total and phosphorylated JNK for cells not treated with ritonavir+atazanavir sulfate but incubated with metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I). **(C)** Basal JNK1/2 phosphorylation was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). **(D)** Insulin-mediated (100 nM) JNK1/2 phosphorylation was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). **(A) & (B):** For each group of blots, the top panel shows a representative gel of JNK1/2 phosphorylation, the middle panel shows a representative gel of total JNK1/2 protein content and the bottom panel shows a representative gel of GAPDH protein content (loading control). **(C) & (D):** Values are mean \pm SE for all treatment groups (n=3-6 per condition) and are expressed as percentage of control, where control refers to cells that are not treated with ritonavir+atazanavir sulfate, metformin, resveratrol or SP600125. **(C):** & $P < 0.05$ vs. respective non-ritonavir+atazanavir sulfate treated state; * $P < 0.05$ vs. control group similarly treated. **(D):** & $P < 0.05$ vs. respective insulin-mediated non-ritonavir+atazanavir sulfate treated state; * $P < 0.05$ vs. insulin-mediated control group similarly treated.

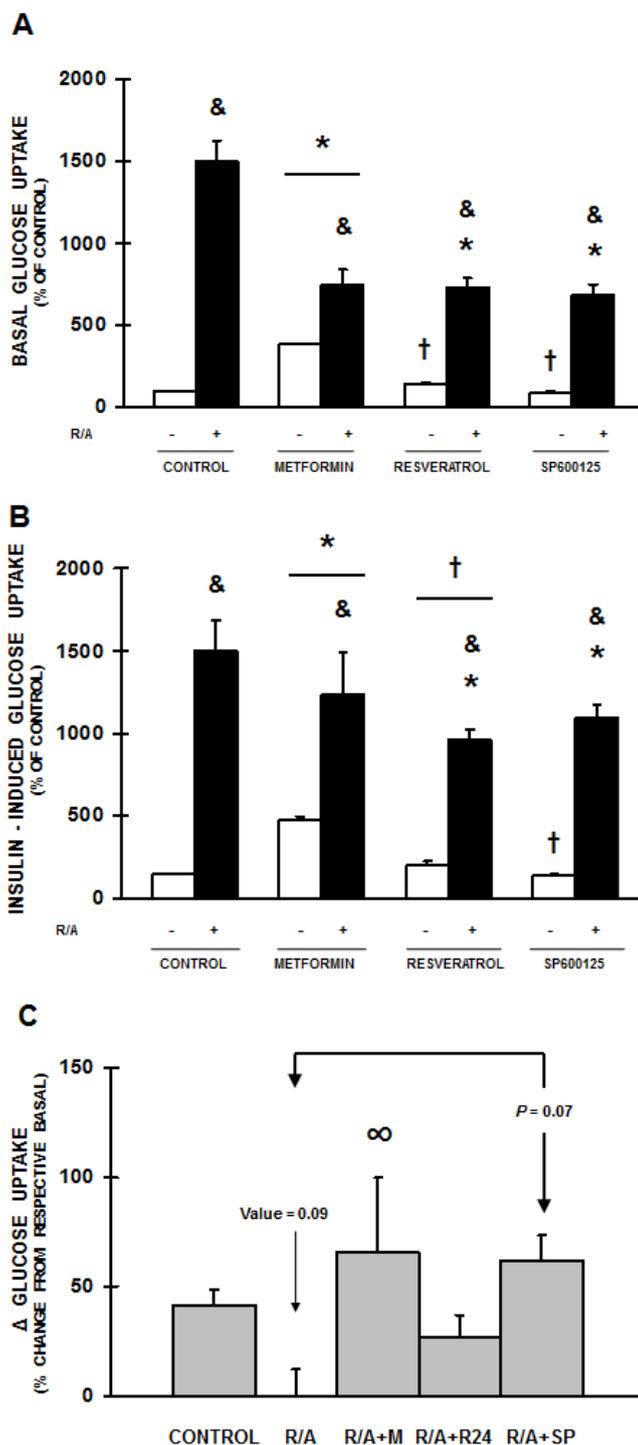


Figure 2: Metformin and SP600125 restore insulin-mediated glucose uptake in L6 muscle cells made insulin resistant by treatment with ritonavir+atazanavir sulfate. (A) Basal glucose uptake was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). (B) Insulin-mediated (100 nM) glucose uptake was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). For (A), (B) and (C), values are mean \pm SE for all treatment groups (n=4-8 per condition) and are expressed as percentage of control, where control refers to cells that are not treated with ritonavir+atazanavir sulfate, metformin, resveratrol or SP600125. (C) Δ glucose uptake represents the percent change in glucose uptake with insulin treatment for each group and was calculated as the rate of insulin-mediated glucose uptake for each well minus the average rate of basal glucose uptake divided by the average rate of basal glucose uptake. RA refers to the group treated with ritonavir+atazanavir sulfate, RA+M to the group treated with ritonavir+atazanavir sulfate and metformin, RA+R to the group treated with ritonavir+atazanavir sulfate and resveratrol and RA+SP to the group treated with ritonavir+atazanavir sulfate and SP600125. (A): & $P < 0.05$ vs. respective non-ritonavir+atazanavir sulfate treated state; * $P < 0.05$ vs. control group similarly treated; † $P < 0.05$ vs. metformin group similarly treated. (B): & $P < 0.05$ vs. respective insulin-mediated non-ritonavir+atazanavir sulfate treated state; * $P < 0.05$ vs. insulin-mediated control group similarly treated; † $P < 0.05$ vs. insulin-mediated metformin group similarly treated. (C): ∞ $P < 0.05$ vs. RA. $P = 0.07$ and Value = 0.09 are indicated for comparisons between R/A and R/A+M, and R/A and R/A+SP, respectively.

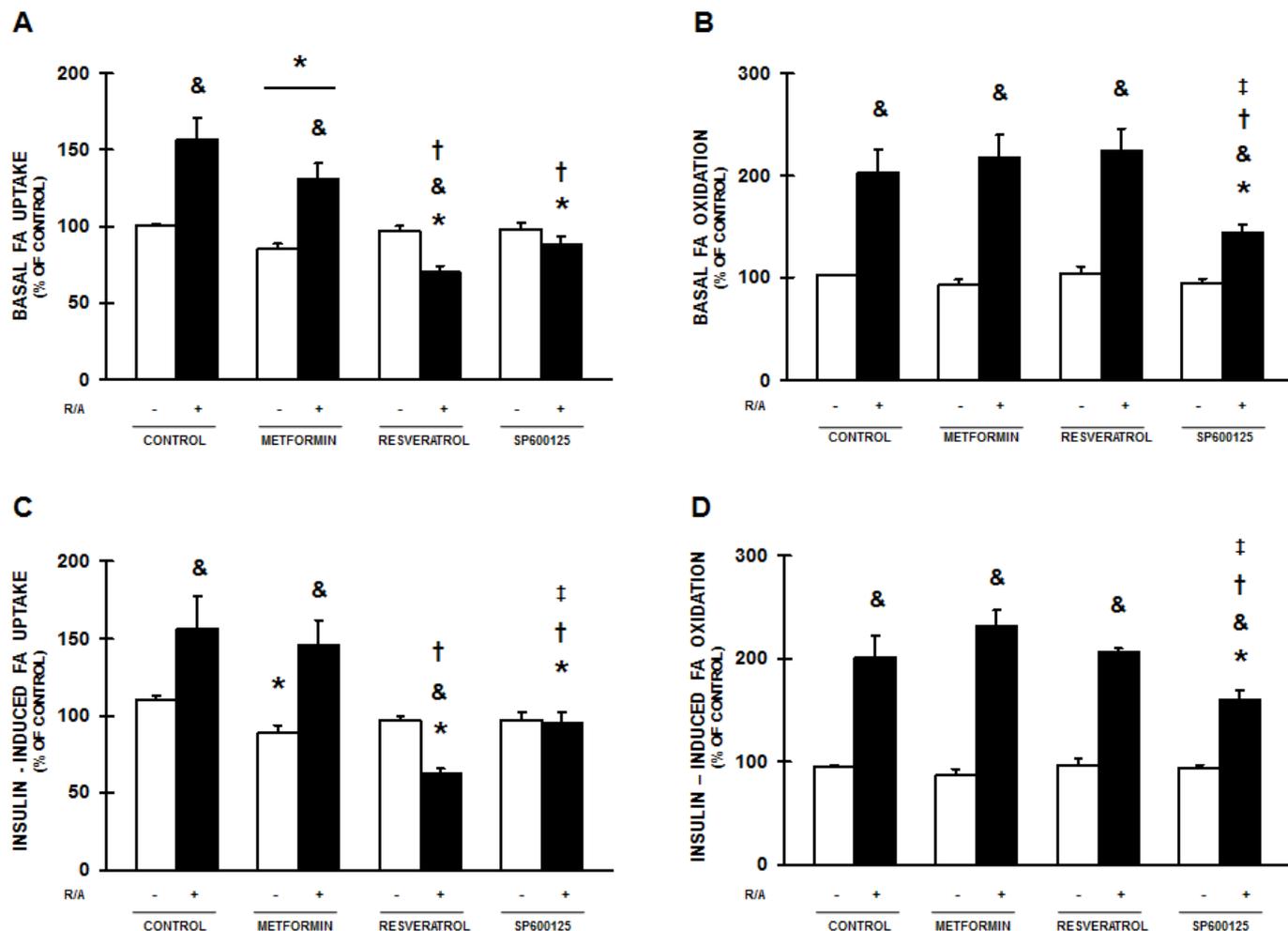


Figure 3: Resveratrol and SP600125 reduce FA uptake and SP600125 reduces FA oxidation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate. Basal FA uptake (A) and oxidation (B) was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). Insulin-mediated (100 nM) FA uptake (C) and oxidation (D) was assessed in cells treated with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). Values are mean \pm SE for all treatment groups (n=4-14 per condition) and are expressed as percentage of control, where control refers to cells that are not treated with ritonavir+atazanavir sulfate, metformin, resveratrol or SP600125. (A) & (B): & P <0.05 vs. respective non-ritonavir+atazanavir sulfate treated state; * P <0.05 vs. control group similarly treated; † P <0.05 vs. metformin group similarly treated; ‡ P <0.05 vs. resveratrol group similarly treated. (C) & (D): & P <0.05 vs. respective insulin-mediated non-ritonavir+atazanavir sulfate treated state; * P <0.05 vs. insulin-mediated control group similarly treated; † P <0.05 vs. insulin-mediated metformin group similarly treated; ‡ P <0.05 vs. insulin-mediated resveratrol group similarly treated.

with RA compared to controls (P >0.05, Figure 4E). Total AKT2 protein content was not altered by any of the treatment conditions (P >0.05, Figure 4A and B).

Metformin, resveratrol and SP600125 did not reduce p³⁸ phosphorylation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate

Treatment with metformin, resveratrol or SP600125 had very little impact on p³⁸ phosphorylation in basal and insulin-stimulated cells made insulin-resistant by incubation with RA (Figure 5A-D). In the insulin-mediated condition, treatment with RA increased p³⁸ phosphorylation (13%) when compared to the respective non-RA treated control cells (P <0.05, Figure 5D). Metformin decreased insulin-mediated p³⁸ phosphorylation by 32% in RA-treated cells (P <0.05, Figure 5D). Total p³⁸ protein content was not affected by any of the treatment options under basal conditions (P >0.05). In the insulin-

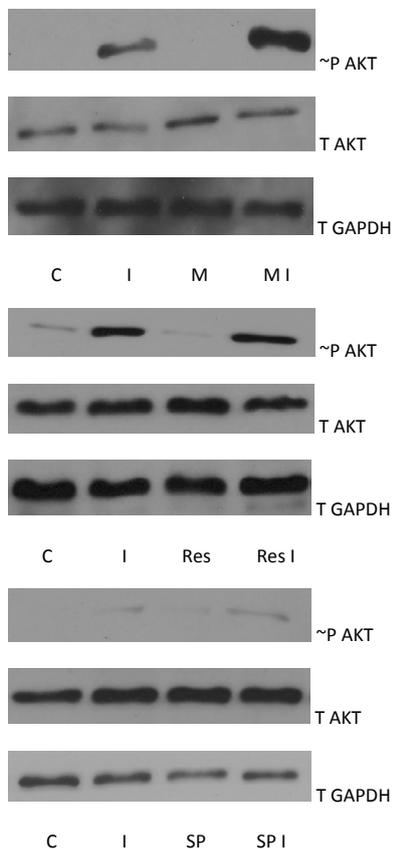
stimulated condition, total p³⁸ protein content was increased (P <0.05) in cells treated with RA (32% vs. insulin alone) or metformin (34% vs. insulin alone).

Discussion

Our data provide new evidence for a central role of JNK1/2 pro-inflammatory signaling in the induction of insulin resistance by treatment with protease inhibitors such as ritonavir and atazanavir sulfate. They also provide novel information about the cellular mechanisms by which metformin and resveratrol restore insulin action in skeletal muscle cells made insulin resistant by this type of protease inhibitor treatment. Herein we show that, in skeletal muscle cells made insulin resistant by exposure to protease inhibitors, treatment with either metformin or resveratrol leads to a reduction in JNK1/2 phosphorylation and an increase in insulin-mediated AKT^{Ser473} phosphorylation. Interestingly, we also show that these signaling

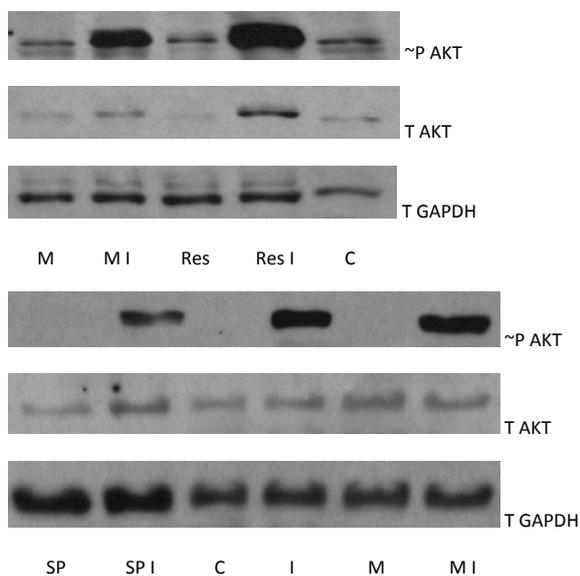
A.

Representative gels for total and phosphorylated AKT for cells treated with ritonavir+atazanavir sulfate and metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I).



B.

Representative gels for total and phosphorylated AKT for cells not treated with ritonavir+atazanavir sulfate but incubated with metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I).



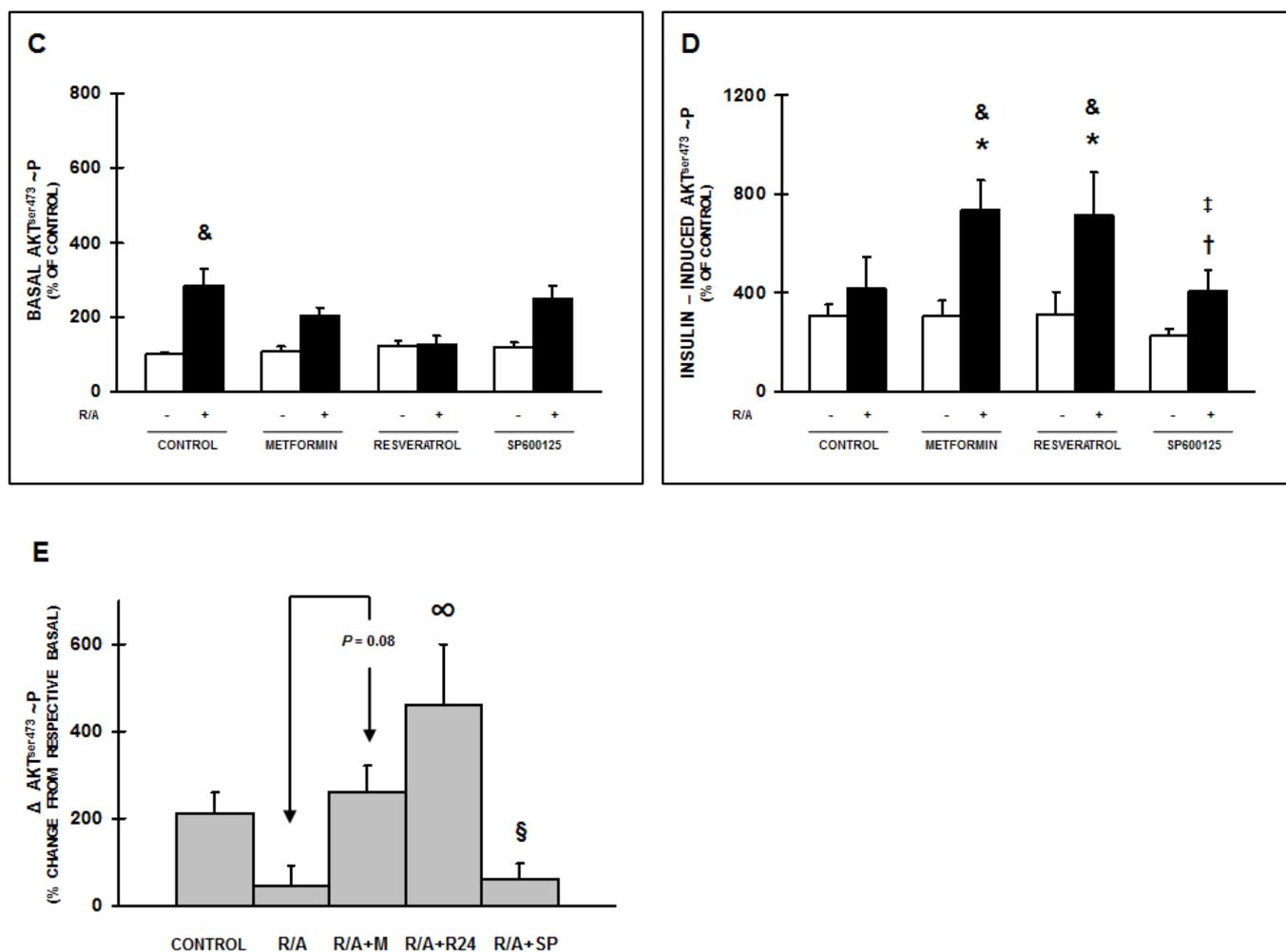


Figure 4: Metformin and resveratrol increase insulin-mediated AKT^{Ser473} phosphorylation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate. (A) Representative gels for total and phosphorylated AKT for cells treated with ritonavir+atazanavir sulfate and metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I). (B) Representative gels for total and phosphorylated AKT for cells not treated with ritonavir+atazanavir sulfate but incubated with metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I). (C) Basal AKT^{Ser473} phosphorylation was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μM and 100 μM, respectively, 48 hr) and metformin (800 μM, 24 hr), resveratrol (100 μM, 24 hr), SP600125 (5 μM, 48 hr) or no drug (Control). (D) Insulin-mediated (100 nM) AKT^{Ser473} phosphorylation was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μM and 100 μM, respectively, 48 hr) and metformin (800 μM, 24 hr), resveratrol (100 μM, 24 hr), SP600125 (5 μM, 48 hr) or no drug (Control). (A) & (B): For each group of blots, the top panel shows a representative gel of AKT^{Ser473} phosphorylation, the middle panel shows a representative gel of total AKT^{Ser473} protein content and the bottom panel shows a representative gel of GAPDH protein content (loading control). (C), (D) and (E): Values are mean ± SE for all treatment groups (n=3-6 per condition) and are expressed as percentage of control, where control refers to cells that are not treated with ritonavir+atazanavir sulfate, metformin, resveratrol or SP600125. (E) Δ AKT^{Ser473} phosphorylation represents the percent change in AKT^{Ser473} phosphorylation with insulin treatment for each group and was calculated as the insulin-mediated AKT^{Ser473} phosphorylation for each gel minus the average basal AKT^{Ser473} phosphorylation divided by the average basal AKT^{Ser473} phosphorylation. RA refers to the group treated with ritonavir+atazanavir sulfate, RA+M to the group treated with ritonavir+atazanavir sulfate and metformin, RA+R to the group treated with ritonavir+atazanavir sulfate and resveratrol and RA+SP to the group treated with ritonavir+atazanavir sulfate and SP600125. (C): & P<0.05 vs. respective non-ritonavir+atazanavir sulfate treated state; * P<0.05 vs. insulin-mediated control group similarly treated; † P<0.05 vs. insulin-mediated metformin group similarly treated; ‡ P<0.05 vs. insulin-mediated resveratrol group similarly treated. (E): ∞ P<0.05 vs. RA; § P<0.05 vs. RA+R24.

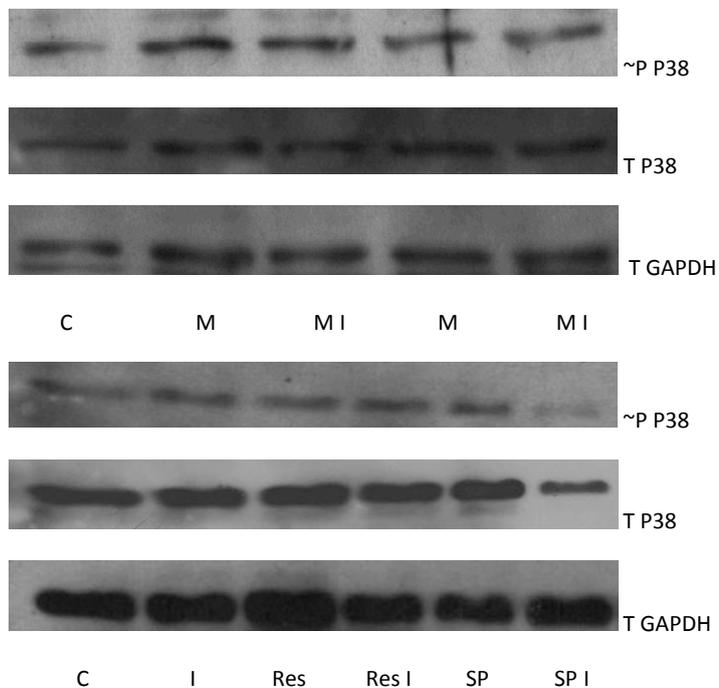
changes are associated with different changes in basal and insulin-mediated metabolism suggesting that the metabolic impact of reduced JNK1/2 phosphorylation is dependent on the therapeutic agent used.

We have previously shown that in skeletal muscle cells treatment with protease inhibitors induces insulin resistance and is accompanied by high levels of JNK1/2 phosphorylation [10]. Herein we show that metformin and resveratrol significantly reduce JNK1/2 phosphorylation in muscle cells made insulin resistant by protease inhibitor treatment and that this is accompanied by an increase

in insulin-mediated AKT^{Ser473} phosphorylation. Our report of an inverse relationship between JNK1/2 phosphorylation and insulin-mediated AKT^{Ser473} phosphorylation confirms previous reports of such an association obtained in different cell types and using different experimental manipulations [42-45]. The results of our study expand on these previous reports by demonstrating that the beneficial metabolic and signaling alterations induced by a reduction in JNK1/2 phosphorylation are dependent on the pharmacological agent or medicinal compound used.

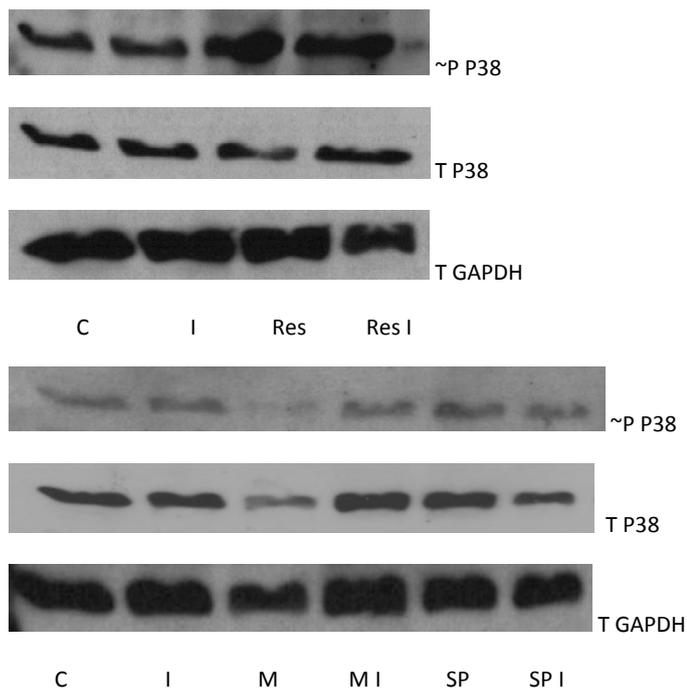
A.

Representative gels for total and phosphorylated p38 for cells treated with ritonavir+atazanavir sulfate and metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation.



B.

Representative gels for total and phosphorylated p38 for cells not treated with ritonavir+atazanavir sulfate but incubated with metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: control) under basal or insulin stimulation (I).



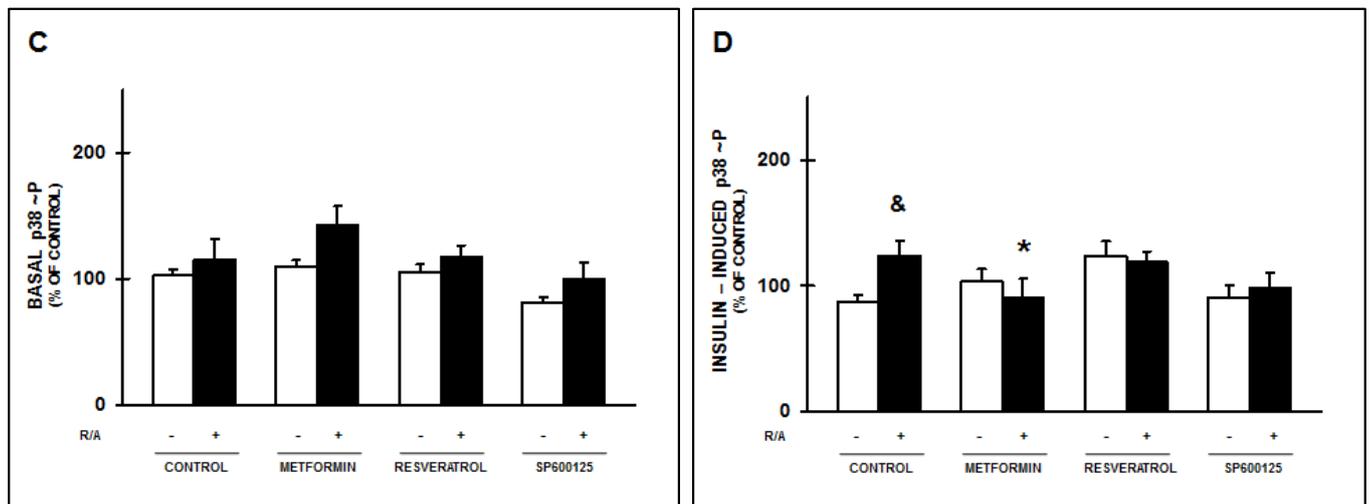


Figure 5: Metformin, resveratrol and SP600125 did not reduce p38 phosphorylation in L6 cells made insulin resistant by treatment with ritonavir+atazanavir sulfate. (A) Representative gels for total and phosphorylated p38 for cells treated with ritonavir+atazanavir sulfate and metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: Control) under basal or insulin stimulation (I). (B) Representative gels for total and phosphorylated p38 for cells not treated with ritonavir+atazanavir sulfate but incubated with metformin (M), resveratrol (Res), SP600125 (SP) or no drug (C: Control) under basal or insulin stimulation (I). (C) Basal p38 MAPK phosphorylation was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). (D) Insulin-mediated (100 nM) p38 MAPK phosphorylation was assessed after treatment with or without ritonavir+atazanavir sulfate (25 μ M and 100 μ M, respectively, 48 hr) and metformin (800 μ M, 24 hr), resveratrol (100 μ M, 24 hr), SP600125 (5 μ M, 48 hr) or no drug (Control). (A) & (B): For each group of blots, the top panel shows a representative gel of p38 MAPK phosphorylation, the middle panel shows a representative gel of total p38 MAPK protein content and the bottom panel shows a representative gel of GAPDH protein content (loading control). (C) & (D): Values are mean \pm SE for all treatment groups (n=3-6 per condition) and are expressed as percentage of control, where control refers to cells that are not treated with ritonavir+atazanavir sulfate, metformin, resveratrol or SP600125. (D): & $P < 0.05$ vs. respective insulin-mediated non-ritonavir+atazanavir sulfate treated state; * $P < 0.05$ vs. insulin-mediated control group similarly treated.

Because it is now well accepted that insulin resistance is characterized by a rise in inflammatory signaling [4,6,46], various pharmacological regimens that use either traditional anti-inflammatory agents or alternative medicinal compounds have been studied to determine whether they might be effective treatment options to defend against the development of insulin resistance. In our study, we used metformin and resveratrol, two insulin-sensitizing agents that have been shown to reduce inflammation [11-13,22,47]. Both metformin and resveratrol have been shown repeatedly to reduce blood levels of inflammatory cytokines such as CRP, IL-1 β , and IL-6 and to decrease the activity of a variety of pro-inflammatory cascades including those associated with NF- κ B, TNF- α or NLRP3 inflammasome [22, 23,47-51]. These anti-inflammatory effects of metformin and resveratrol have been linked to improved metabolic function and neuroprotection and to a reduced incidence of pro-inflammatory disease states such as osteoarthritis and colitis in rodents [47-52]. Metformin has also been shown by some to alleviate some of the negative metabolic effects of HAART therapy [15-17]. Here we show that the beneficial insulin-sensitizing effects of metformin are due to a reduction in the activation state of the pro-inflammatory JNK1/2 signaling cascade and a concomitant restoration of AKT^{Ser473} phosphorylation with insulin stimulation. We also show for the first time that resveratrol protects against the negative effects of protease inhibitor treatment on metabolic function in skeletal muscle cells. As shown in the metformin condition, treatment with resveratrol reduced JNK1/2 phosphorylation and was accompanied by an increase in insulin-mediated AKT^{Ser473}

phosphorylation. Interestingly, metformin and resveratrol treatment were accompanied by different metabolic alterations. As shown previously in healthy skeletal muscle cells, metformin treatment restored insulin-mediated glucose uptake [18]. However, in contrast to its impact in healthy muscle cells, metformin did not alter FA uptake and oxidation in muscle cells made insulin resistant by protease inhibitors. The metabolic changes associated with resveratrol included a reduction in FA uptake but no change in insulin-mediated glucose uptake or FA oxidation. Thus, in insulin-resistant cells treated with resveratrol, insulin-mediated glucose uptake was not restored in spite of high insulin-mediated AKT^{Ser473} phosphorylation. This reinforces the notion that although AKT is an important intermediate of the insulin signaling cascade, activation of other signaling molecules is necessary to restore insulin action as it pertains to glucose uptake [41]. Together, our data confirm that both metformin and resveratrol were capable of reversing the negative impact of the protease inhibitors on JNK1/2 pro-inflammatory signaling and on upstream insulin signaling. Interestingly, our metabolic data also show that restoration of JNK1/2 and insulin signaling does not necessarily provide uniform metabolic adaptations.

Since both metformin and resveratrol reduced JNK1/2 pro-inflammatory signaling in muscle cells treated with protease inhibitors, we also studied the impact of JNK1/2 inhibition per se by using the JNK1/2 inhibitor SP600125. As expected, treatment with SP600125 reduced JNK1/2 phosphorylation in muscle cells made insulin resistant by treatment with protease inhibitors but, interestingly, this

was not accompanied by an increase in insulin-mediated AKT^{Ser473} phosphorylation. Nevertheless, treatment with SP600125 was accompanied by a restoration of insulin-mediated glucose uptake and a reduction in FA uptake and oxidation. Our results are in line with previous data showing that silencing JNK1/2 restored insulin sensitivity and normalized insulin-induced glucose uptake in myotubes [53]. As expected, inhibition of JNK1/2 in a non-inflammatory state did not significantly change the rates of insulin-stimulated glucose uptake in our control skeletal muscle cells and this agrees with previously published data collected in primary neonatal rat myotubes [26]. Together, these data indicate that JNK1/2 inhibition improves insulin action in insulin resistant tissues but it does not promote additional metabolic or anti-inflammatory benefits in insulin sensitive tissues.

To the best of our knowledge, we are the first group to investigate the effectiveness of metformin and resveratrol in blunting or reversing the negative effects of protease inhibitor treatment on metabolic regulation in skeletal muscle cells. More specifically, we provide evidence that both metformin and resveratrol are capable of reducing metabolic dysregulation via a reduction in JNK1/2 pro-inflammatory signaling. We further show that these beneficial metabolic alterations are accompanied by restoration of insulin-mediated AKT^{Ser473} phosphorylation. Further research will need to be completed to determine the cellular mechanisms by which JNK1/2 inhibition induced by metformin or resveratrol mediate different metabolic adaptations. Clinically, a better understanding of the cellular mechanisms by which metformin and resveratrol decrease inflammation and increase insulin action will benefit the treatment of patients with insulin resistance and type II diabetes including those who are HIV-positive and treated with protease inhibitors.

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