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Resveratrol Prevents Insulin Resistance Caused by Short-Term Elevation of Free Fatty Acids In Vivo

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ABSTRACT

Elevated levels of plasma free fatty acids (FFA), which are commonly found in obesity, induce insulin resistance. FFA activate protein kinases including the proinflammatory IκBα kinase β (IKKβ), leading to serine phosphorylation of insulin receptor substrate 1 (IRS-1) and impaired insulin signaling. In order to test whether resveratrol, a polyphenol found in red wine, prevents FFA-induced insulin resistance, we used a hyperinsulinemic-euglycemic clamp with tracer to assess hepatic and peripheral insulin sensitivity in overnight-fasted Wistar rats infused for 7 hours with either saline, Intralipid plus 20 U/ml heparin (IH, triglyceride emulsion that elevates FFA levels *in vivo*; 5.5 μl/min) with or without resveratrol (3mg kg^{-1} h^{-1}), or resveratrol alone. Infusion of IH significantly decreased glucose infusion rate (GIR; $P<0.05$), peripheral glucose utilization ($P<0.05$), and increased endogenous glucose production (EGP; $P<0.05$) during the clamp compared with saline infusion. Resveratrol co-infusion, however, completely prevented all these effects induced by IH infusion; it prevented the decreases in GIR ($P<0.05$ vs IH), peripheral glucose utilization ($P<0.05$ vs IH), and insulin-induced suppression of EGP ($P<0.05$ vs IH). Resveratrol alone had no effect. Furthermore, IH infusion increased serine (307) phosphorylation of IRS-1 in soleus muscle (~30 fold, $P<0.001$), decreased total IRS-1 levels, and decreased IκBα content consistent with activation of IKKβ. Importantly, all of these effects were abolished by resveratrol ($P<0.05$ vs IH). These results suggest that resveratrol prevents FFA-induced hepatic and peripheral insulin resistance and therefore, may help mitigate the health consequences of obesity.

Keywords: insulin resistance, dyslipidemia, obesity, skeletal muscle, metabolism

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66 **INTRODUCTION**

67 A close relationship between obesity, insulin resistance, and type 2 diabetes
68 mellitus has been shown by numerous studies. Fasting plasma FFAs are often increased
69 in obesity (Lewis *et al*. 2002).The expanded adipose tissue of obese individuals releases
70 products such as adipokines/cytokines and free fatty acids (FFA), contributing to insulin
71 resistance. Elevated levels of FFA have been shown to cause insulin resistance (Boden *et*
72 *al.* 2001; Kim *et al.* 2004; Lam *et al.* 2002; Roden *et al.* 1996; Yuan *et al.* 2001). Insulin
73 action leads to phosphorylation of insulin receptor substrates (IRS) and downstream
74 activation of Akt. FFA cause insulin resistance in skeletal muscle mainly via increased
75 serine (Copps & White 2012; Guo 2014) and reduced tyrosine phosphorylation of IRS-1
76 (Kim *et al.* 2001; Kim *et al.* 2004; Yu *et al.* 2002). Serine kinases, such as inhibitor of
77 κBα (IκBα) kinase β (IKKβ), protein kinase C (PKC), mammalian target of rapamycin
78 (mTOR), p70S6 kinase, and c-jun NH_2-terminal kinase (JNK) have been shown to
79 mediate this process (Copps & White 2012; Guo 2014).

80 IKKβ is a member of the nuclear factor kappa B (NF-κB) pathway involved in
81 inflammatory responses, activated by cytokines such as TNF-α and also is implicated in
82 insulin resistance. The transcription factor NF-κB is kept inactive in the cytoplasm by an
83 inhibitory protein known as IκBα. Activated IKKβ phosphorylates IκBα, leading to its
84 degradation by the ubiquitin proteasome pathway. This allows NF-κB to translocate to
85 the nucleus and modify transcription (Gilmore 2006). FFA activate IKKβ and thus NF-
86 κB mediated transcription of cytokines (Lee & Lee 2014; Sinha *et al.* 2004). In addition,
87 FFA-activated IKKβ can directly phosphorylate rat IRS-1 on ser-307, and thus impair
88 insulin signaling (De Alvaro *et al.* 2004). *In vivo* studies showed that diet- and obesity-

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89 induced insulin resistance was reversed by targeted disruption of IKKβ or treatment with
90 salicylate, an inhibitor of IKKβ (Yuan *et al.* 2001). Similarly, we and other authors have
91 found that salicylate prevented hepatic and peripheral insulin resistance induced by lipid
92 infusion (Park *et al*. 2007b; Kim *et al*. 2001) and high dose aspirin (salicylate) treatment
93 improved insulin signaling and action also in diabetic patients (Hundal *et al.* 2002).

94 Resveratrol (trans3,4,5-trihydroxystilbene) is a naturally occurring polyphenol
95 compound, found in the skin of grapes and in high concentration in red wine, shown to
96 have antioxidant, anticancer, and anti-ageing properties and to protect against
97 cardiovascular disease (Baur & Sinclair 2006). Importantly, resveratrol has been shown
98 to have antidiabetic properties in vitro and in vivo (Park *et al.* 2007a; Breen *et al.* 2008;
99 Zygmunt *et al.* 2010; Baur *et al.* 2006; Lagouge *et al.* 2006; Su *et al.* 2006; Do *et al.*
100 2012). In skeletal muscle cells *in vitro*, resveratrol increased glucose uptake (Breen *et al.*
101 2008; Zygmunt *et al.* 2010; Park *et al.* 2007a) and abolished the palmitate-induced
102 decline in insulin-stimulated glucose uptake via inhibition of PTP1B expression (Sun *et*
103 *al.* 2007). In *in vivo* studies, resveratrol was shown to prevent high-fat-diet-induced
104 insulin resistance in mice (Lagouge *et al.* 2006; Sun *et al.* 2007), however the effect of
105 oral resveratrol supplements on insulin sensitivity in obese individuals is controversial
106 (Timmers *et al.* 2011; Poulsen *et al.* 2013). Resveratrol activates the NAD-dependent
107 deacetylase SIRT1 (Lagouge *et al.* 2006; Sun *et al.* 2007) and the energy sensor AMP-
108 dependent kinase (AMPK) (Breen *et al.* 2008; Park *et al.* 2007a; Um *et al.* 2010) and
109 these molecules have been proposed to play a significant role in resveratrol's action. In
110 the present study, we determined whether resveratrol has a protective effect against

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111 insulin resistance caused by acute elevation of plasma FFA, which had not been
112 examined previously.

113 MATERIALS AND METHODS

114 Animal care and surgery

115 For all experiments female Wistar rats (Charles River, Quebec, Canada) weighing
116 250-300g were used. The rats were exposed to a 12h light-dark cycle and were fed rat
117 chow (Teklad 2018, 18% fat, Harland Teklad Global Diets, Madison, WI, USA) and
118 water *ad libitum*. Animals were housed in the University of Toronto's Department of
119 Comparative Medicine and were cared for in accordance to the Animal for Research Act
120 of the Government of Canada. The Animal Care Committee of the University of Toronto
121 approved all procedures.

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122 Rats were allowed 3-5 days to adapt to the facility. Thereafter, they underwent
123 vessel cannulation under isofluorane anesthesia as previously described (Park *et al.*
124 2007b). Polyethylene catheters (PE-50; Cay Adams, Boston, MA), each extended with a
125 segment of silastic tubing (internal diameter of 0.58 mm, length of 3 cm; Dow Corning,
126 Midland, MI), were inserted into the right atrium via the jugular vein for infusion and
127 into the aortic arch via the carotid artery for blood sampling. Both catheters were
128 tunneled subcutaneously, exteriorized, filled with heparin (1,000 U/ml) in 60%
129 polyvinylpyrrolidone to maintain patency and finally closed with a metal pin. The rats
130 were allowed a minimum of 3 day recovery from surgery before experiments were
131 carried out.

132 Experimental Design

133 Following an overnight fasting, the animals (n=6-8 rats/group) received a 7 hour
134 i.v. infusion (5.5 μl/min) of either saline (SAL), Intralipid plus Heparin (IH) (20%
135 Intralipid + 20 U/ml heparin), IH plus resveratrol (RSV; 3 mg kg^{-1} h^{-1}), or RSV alone.
136 Just prior to onset of resveratrol infusion a bolus of resveratrol (6mg/kg) was given. At 3-
137 hour point of the infusion period, i.v. infusion of [3-^{3}H] glucose was initiated (8 μCi,
138 bolus + 0.15 μCi/min infusion). To assess hepatic and peripheral insulin sensitivity, a
139 hyperinsulinemic-euglycemic clamp was performed with tracer infusion during the last 2
140 hours of the 7-hour infusion period. Preceding the clamp ("basal period") and for a period
141 of 30 minutes, blood samples were taken every 10 minutes for measurements of plasma
142 glucose, insulin, FFA, and [3-^{3}H] glucose specific activity. The same was done during the
143 last 30 min of the hyperinsulinemic clamp ("clamp period"). At the 5-hour point of the
144 infusion period, an i.v. infusion of porcine insulin (5 mU kg^{-1} min^{-1}) resulting in plasma
145 insulin levels in the postprandial range was initiated. To maintain euglycemia during
146 insulin infusion, an i.v. infusion of 20% glucose was given i.v. and adjusted according to
147 frequent glycemic determinations (every 5 min). The glucose infusate was radiolabelled
148 with 48 μCi/g [3-^{3}H] glucose to maintain plasma glucose specific activity constant. Total
149 blood withdrawal was ~3.8 ml. After plasma separation, the red blood cells were diluted
150 1:1 in heparinized saline (4 U/ml) and re-infused into the rats. Upon completion of the
151 experiments, the rats were anesthetized with i.v. administration of an anesthetic cocktail
152 (ketamine: xylazine: acepromazine (87: 1.7: 0.4 mg/ml) and soleus skeletal muscle was
153 collected.
154 **Plasma Assays**

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155 Plasma insulin levels were determined by radioimmunoassays (RIAs) using kits
156 specific for rodent insulin (but with 100% cross reactivity with porcine insulin used for
157 infusion) as previously described (Park *et al.* 2007b). Plasma glucose levels were
158 measured using a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma
159 radioactivity from the [3-^{3}H] glucose tracer was measured after deproteinization with
160 $Ba(OH)_2$ and $ZnSO_4$ and evaporation to dryness. Aliquots of the [3-^{3}H] glucose tracer
161 and of the radiolabeled glucose infusate were assayed together with the plasma samples
162 (Lam *et al.* 2002). Plasma FFA levels were assayed using a colorimetric kit from Wako
163 Industrials (Osaka, Japan) as previously described (Park *et al.* 2007b; Lam *et al.* 2002).
164 **Immunoblot Analysis**
165 Soleus muscle samples (40 mg) were ground in a glass-on-glass tissue grinder
166 containing ice-cold lysis buffer (50 mM Tris pH 7.5, 1% Nonidet P-40, 150 mM NaCl,
167 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM
168 $Na_4P_2O_4$, 1 μM okadaic acid, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin).
169 Insoluble materials were removed by centrifugation at 2000 rpm for 10 min at 4 °C. The
170 protein concentration in all samples was determined by the detergent-compatible
171 modified Lowry method, using bovine serum albumin as standard. Fifty μg of protein in
172 all samples were mixed with equal volumes of 3X sample-loading buffer (6.86 M urea,
173 4.29% sodium dodecyl sulphate (SDS), 300 mM dithiothreitol, 43 mM Tris-HCl pH 6.8)
174 and left at room temperature for 30 min. The mixture was vortexed and proteins were
175 separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
176 Proteins were then transferred to polyvinylidene fluoride membranes (Biorad) followed
177 by incubation for 1h at room temperature in Tris–Tween normal saline (TTNS) buffer

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178 (pH 7.4) containing 0.1% Tween-20 (Sigma) mixed with 7.5% non-fat dried milk for
179 blocking. Thereafter, membranes were incubated overnight with an affinity-purified
180 polyclonal antibody specific for IRS-1 (1:500 dilution; Upstate Cell Signaling Solutions),
181 phospho (ser307) IRS-1 (1:1000 dilution; Biosource), IκBα (1:2000 dilution; Santa Cruz
182 Biotechnology), phospho (ser32/36) IκBα (1:500 dilution; Santa Cruz), β-actin (Santa
183 Cruz), or the following antibodies (1:1000 dilution; Cell Signaling): phospho (ser 473)
184 Akt, Akt, phospho(thr172) AMPK, AMPK, phospho (ser2448) mTOR, mTOR, phospho
185 (thr389) p70S6K, p70S6K , phospho(thr183/tyr185) JNK and JNK. After washing with
186 TTNS buffer three times for 20 min each, membranes were incubated with horseradish
187 peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for one hour at
188 room temperature. The membranes were then washed three times with Millipore water
189 and developed using enhanced chemiluminescence reagent (Amersham). The bands
190 obtained from immunoblotting were quantified by densitometry.

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191 **MDA assay:** The MDA assay was carried out as previously described (Pereira *et al*.
192 2015)

193 **Plasma resveratrol measurements**

194 Resveratrol was extracted from rat plasma samples using ethyl acetate and
195 centrifuging at 8000 rpm for 1 minute at 4ºC. Supernatant was collected and dried using a
196 speed vacuum. The dried product was then dissolved in 200µL of 100% methanol,
197 filtered with 0.2µm syringe for LC-MS analysis. Waters Acquity ultra-performance liquid
198 chromatography (UPLC) consisting of binary solvent manager, sample manager,
199 photodiode array detector, mass spectrometer and MassLynx 4.1 software were used. The
200 injection volume was 3 µL for all samples. The UPLC profiling was performed on a 50

201 mm X 2.1 mm BEH C18 column packed with 1.7 µm particles (Waters) following a
202 gradient elution profile. The mobile phase consisted of 0.1% formic acid in water (solvent
203 A) and 100% acetonitrile (solvent B). The shape of the gradient used was as follows: 0
204 min, 100% A; 0.6 min, 92% A; 6.0 min, 70% A; 6.50 min, 50% A; 7.0 min, 70% A; 7.50
205 min, 92% A; 8.0 min, 100% A. The column temperature was maintained at 40 °C with a
206 constant flow rate of 0.3 mL/min. Mass spectrometry was done as ESI spray in the
207 negative ion mode for *trans*-resveratrol, at a capillary voltage 3.50 kV, cone voltage 50 V,
208 source temperature 150 °C with a gas flow rate of 600 L/hr.

209 **Calculations**

210 Steele's equation (Steele *et al*. 1956) as modified by Finegood (Finegood *et al*.
211 1987) to take into account the extra tracer infused with the glucose infusate, was used to
212 calculate the glucose turnover (rate of appearance of glucose). In the basal state, the rate
213 of appearance of glucose corresponds to the endogenous glucose production (EGP).
214 During the clamps, EGP was obtained by subtracting the infusion rate of exogenous
215 glucose from the total rate of glucose appearance (endogenous + exogenous). At steady
216 state, glucose disappearance is equal to glucose appearance. Data are average values of
217 the basal period and the last 30 min of the clamp.

218 **Statistical analysis**

219 One-way analysis of variance (ANOVA) followed by Tukey's t test was used to
220 compare differences between treatments groups. Significance was accepted at $P<0.05$.
221 Statistical calculations were performed using the statistical program SPSS (IBM
222 Corporation, Armonk, NY, USA).
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224 **RESULTS**

225 Plasma insulin levels, as expected, were markedly elevated from basal during the
226 clamp due to infusion of exogenous insulin (Table 1). There was no difference in plasma
227 insulin levels between groups during the basal period or during the clamp. Plasma
228 glucose levels were not different between groups during the basal period and during the
229 clamp (Table 1). Plasma FFA levels during the basal period were ~ 2-fold higher in the
230 IH and IH plus RSV groups compared to the SAL group. As expected, plasma FFA
231 levels were lower during the hyperinsulinemic clamp than during the basal period in all
232 groups due to plasma FFA-lowering effect of insulin, but remained higher in the IH and
233 IH+RSV groups compared to controls.

234 Steady-state glucose infusion rate (GIR) during the last 30 minutes of the
235 hyperinsulinemic-euglycemic clamp is an indication of whole body insulin sensitivity.
236 Infusion of IH decreased GIR (86 ± 11 μmol kg^{-1} min^{-1}) ($P<0.05$) compared to SAL
237 infusion (160 ± 16 μmol kg^{-1} min^{-1}) (Figure 1). Co-infusion of resveratrol with IH
238 prevented the IH-induced decrease in GIR (151 ± 6 μmol kg^{-1} min^{-1}) ($P<0.05$ vs IH) while
239 resveratrol infusion alone (162 ± 8 μmol kg^{-1} min^{-1}) had no effect.

240 During the basal period, no differences in endogenous glucose production (EGP)
241 (Figure 2A) between treatment groups were observed. Hepatic insulin sensitivity is
242 measured as the ability of insulin to suppress EGP from basal. In the SAL group, EGP
243 was suppressed by 40% during the last 30 min of hyperinsulinemic clamp (Figure 2A and
244 2B). In contrast, in the IH group, the suppression of EGP during the clamp was only 2%,
245 i.e. significantly less than the EGP suppression in the SAL group ($P<0.05$; Figure 2B).
246 Co-infusion of resveratrol with IH resulted in insulin-induced suppression of hepatic

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247 glucose production (43.5%) to similar levels seen in the SAL group, clearly indicating an
248 action of resveratrol ($P<0.05$ vs IH) to prevent the IH effect. Resveratrol infusion alone
249 did not have any effect (Figure 2B).

250 As expected, peripheral glucose utilization increased during the clamp (186 ± 15
251 $\mu mol\ kg^{-1}\ min^{-1}$) compared to basal period ($47\pm6\ \mu mol\ kg^{-1}\ min^{-1}$) (Figure 3). IH infusion
252 significantly decreased peripheral glucose utilization during the clamp ($131\pm12\ \mu mol\ kg^{-1}$
253 min^{-1}) when compared with SAL infusion ($186\pm15\ \mu mol\ kg^{-1}\ min^{-1}$) ($P<0.05$; Figure 3)
254 an indication of peripheral insulin resistance. This decrease was completely abolished in
255 the group receiving infusion of both IH and resveratrol ($176\pm6\ \mu mol\ kg^{-1}\ min^{-1}$). These
256 data indicate an ability of resveratrol to prevent fat-induced peripheral insulin resistance.
257 Resveratrol infusion alone had no effect.

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258 In an attempt to understand the mechanism of resveratrol action to prevent the IH-
259 induced peripheral insulin resistance we examined total and ser 307 phosphorylation
260 levels of IRS-1 in soleus skeletal muscle.

261 IH infusion markedly increased serine (307) phosphorylation of IRS-1 ($P<0.001$)
262 (Figure 4A) and decreased total IRS-1 protein levels ($P<0.05$) (Figure 4B). Co-infusion
263 of resveratrol with IH however, completely prevented these effects ($P<0.001$ and $P<0.05$
264 vs IH, respectively. Resveratrol alone did not have any effect. Next, we examined total
265 and phosphorylated levels of Akt. IH infusion decreased phosphorylation of Akt and
266 resveratrol co-infusion prevented this decline (Figure 4C). Total Akt levels were not
267 changed by any treatment.

268 Different kinases have been suggested to increase ser 307 phosphorylation of IRS-
269 1, including IKKβ. Since we previously found that the IKKβ inhibitor salicylate

270 prevented insulin resistance due to lipid infusion (Park *et al*. 2007b) we examined the
271 levels of IκBα, a marker of IKKβ activation. IH infusion increased phosphorylation and
272 decreased muscle content of total IκBα, suggesting activation of IKKβ. Interestingly,
273 resveratrol co-infusion with IH prevented this effect ($P<0.05$ for IH vs other groups;
274 Figure 5). We also examined mTOR, p70S6 kinase, and JNK all of which have been
275 shown to increase serine phosphorylation of IRS-1 and are implicated in insulin
276 resistance. Total and phosphorylated levels of mTOR, p70S6 kinase, and JNK were not
277 changed by any treatment (Figure 6). In addition, total and phosphorylated levels of
278 AMPK, the upstream regulator of mTOR and p70S6 kinase, were not changed by any
279 treatment (Figure 6).

280 To investigate whether IH and resveratrol had an effect on oxidative stress, we
281 measured MDA levels in skeletal muscle and found that IH did not affect MDA levels in
282 muscle compared to SAL and that MDA levels were higher in the IH+RSV group
283 compared to the IH group (Figure 7).

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284 Using UPLC we measured resveratrol levels in rat plasma samples. The average
285 resveratrol level in the plasma of 3-rats infused with resveratrol for 7h at the dose of the
286 present study was 1.064 μM while no resveratrol was detected before resveratrol infusion.

287

288 **DISCUSSION**

289 Although the precise mechanism of FFA-induced insulin resistance remains
290 elusive, a consensus exists that impaired post-receptor signaling is involved with serine
291 phosphorylation of IRS-1 being a key event (Le Marchand-Brustel *et al.* 2003; Copps &
292 White 2012; Guo 2014; Gao *et al.* 2002). The present study was performed to investigate

293 the effect of the polyphenolic compound resveratrol on insulin resistance caused by acute
294 elevation of circulating FFA *in vivo*. We have shown that resveratrol stimulates glucose
295 uptake in L6 myotubes (Breen *et al.* 2008). Based on this, we hypothesized that
296 resveratrol may prevent insulin resistance caused by our model of short-term (7h) fat
297 infusion. The results of the present study show that resveratrol is effective in preventing
298 fat-induced hepatic and peripheral insulin resistance and suggest that a part of the
299 mechanism may involve restoration of insulin signaling in skeletal muscle.

300 As expected, infusion of Intralipid + heparin markedly elevated plasma FFA
301 levels, which decreased during the clamp in all groups due to the FFA-lowering effect of
302 insulin. Intralipid is a triglyceride emulsion that is broken down into non-esterified fatty
303 acids and glycerol *in vivo* by lipoprotein lipase, activated by heparin. It is thus possible
304 that glycerol derived from the triglyceride emulsion affects EGP measured in the present
305 study; however, we have previously shown (Lam *et al.* 2002) that glycerol infusion
306 resulting in plasma glycerol levels similar to 7h infusion of IH has no effect on EGP
307 compared with saline infusion.

308 The infusion rate of exogenous glucose is an indication of whole body insulin
309 sensitivity and was reduced by lipid infusion, consistent with previous studies (Boden
310 1997; Boden *et al.* 2001; Boden *et al.* 2005; Kim *et al.* 2001; Kim *et al.* 2004; Lam *et al.*
311 2002; Lam *et al.* 2003; Yu *et al.* 2002). The whole body insulin resistance caused by IH
312 infusion was completely prevented when resveratrol was co-infused. Infusion of [3-^{3}H]
313 glucose enabled us to separately assess hepatic and peripheral insulin resistance. IH
314 infusion decreased insulin-induced suppression of endogenous glucose production (EGP)
315 and insulin-stimulated peripheral glucose utilization, suggesting that lipids caused both

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316 hepatic and peripheral insulin resistance, in accordance with our previous findings (Park
317 *et al.* 2007b). More importantly, resveratrol co-infusion was able to completely prevent
318 the IH-induced insulin resistance at both sites. Our study is in agreement with other in
319 vivo studies showing a prevention of diet-induced insulin resistance in mice (Lagouge *et*
320 *al.* 2006; Um *et al.* 2010) and monkeys (Jimenez-Gomez *et al.* 2013) treated with
321 resveratrol. Plasma levels of resveratrol resulting from consumption of resveratrol in the
322 diet of rodents depend on the dose, and resveratrol concentrations of 10-120ng/ml (44-
323 530nM) in plasma have been reported (Lagouge *et al*. 2006). In humans, dietary
324 supplements of resveratrol have been shown to result in plasma levels of approximately
325 180ng/ml (0.78μM) (Timmers *et al*. 2011). We decided to infuse resveratrol in order to
326 increase the probability of seeing an effect, since the oral bioavailability of resveratrol
327 has been reported to be poor in rats (Kapetanovic *et al.* 2001). Resveratrol levels in the
328 plasma of animals infused with resveratrol was 1.064 μM which is not far from the
329 theoretical concentration of 0.6 μg/ml (2.62 μM) calculated from the infused dose
330 (3mg/kg/h= 0.75mg/h for a 250g rat) and the published clearance (1.24 L/h) of RSV in
331 rats (Colom *et al*. 2011). Colom et al (Colom *et al*. 2011) found that IV bolus
332 administration of 2mg/kg of resveratrol in rats resulted in resveratrol levels of 0.1 μM
333 after 2 h. Similarly, IV bolus administration of 20mg/kg of resveratrol in rats resulted in
334 mean resveratrol concentration of 0.1μg/ml (0.43μM) after 2 h (He *et al*. 2006). Overall
335 the levels of plasma resveratrol in our study although are higher than the levels achieved
336 by oral resveratrol administration in rats, they are not very different from the levels
337 achieved by IV bolus administration of resveratrol in rats in previous studies and
338 importantly are close to the levels seen after oral supplementation in humans.

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339 Serine 307 phosphorylation of IRS-1 caused by short-term fat infusion was
340 associated with decreased tyrosine phosphorylation of IRS-1 and impairment of insulin
341 signaling in rat skeletal muscle, although the serine kinase responsible was not identified
342 (Yu *et al.* 2002). Soleus muscle and gastrocnemius muscle are typically used in the
343 literature to determine the extent of insulin sensitivity in skeletal muscle. It has been
344 reported that i.v. lipid infusions impair insulin-stimulated glucose uptake by the soleus
345 muscle (Kim *et al*. 2001) and by the gastrocnemius muscle (Kim *et al*. 2004) in rodents.
346 These two muscles consist of a different proportion of muscle fiber types, with the soleus
347 muscle being considered the more insulin sensitive muscle of the two (Holmang *et al*.
348 1992). Therefore, we chose to study soleus muscle to maximize the probability of finding
349 differences in insulin sensitivity.

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350 In the present study, we show that IH infusion causes a marked increase in serine
351 307 phosphorylation of IRS-1 in rat soleus muscle, which was completely abolished by
352 resveratrol co-infusion. Furthermore, resveratrol prevented IH-induced reduction in IRS-
353 1 protein levels, which is observed in various animal models of insulin resistant states
354 (Anai *et al.* 1998; Kerouz *et al.* 1997; Saad *et al.* 1992) and has been associated with
355 serine/threonine phosphorylation of IRS-1 (Pederson *et al.* 2001). Decrease in IRS-1
356 protein levels leading to insulin resistance has also been linked to suppressor of cytokine
357 signaling-mediated ubiquitination and degradation (Rui *et al.* 2002; Ueki *et al.* 2004).
358 Interestingly, phosphorylation of Akt was reduced by IH and restored by resveratrol co-
359 infusion. Together, these findings suggest that the effect of resveratrol to prevent IH-
360 induced peripheral insulin resistance may, at least in part, be due to restoration of insulin-

361 induced tyrosine phosphorylation of IRS-1 and consequent activation of the insulin
362 signaling cascade.

363 In muscle, we show that a marker of IKKβ activation, namely decreased IκBα
364 content, is induced by IH, but resveratrol administration prevents this effect. Numerous
365 studies (Arkan *et al.* 2005; Boden *et al.* 2005; Cai *et al.* 2005; Kim *et al.* 2001) have
366 implicated activation of IKKβ-NFκB- inflammatory pathway in fat-induced insulin
367 resistance, although it is not clear whether insulin resistance is due to the direct effect of
368 IKKβ on insulin signaling or to the indirect effect of NFκB-mediated production of pro-
369 inflammatory cytokines. However, some studies including ours (Park *et al.* 2007b)
370 provide strong evidence implicating IKKβ activation and downstream IRS-1 serine
371 phosphorylation in fat-induced insulin resistance (Kim *et al.* 2001; Itani *et al.* 2002;
372 Boden *et al.* 2005). Despite the fact that a number of studies have examined the anti-
373 diabetic effects of resveratrol, to our knowledge, our study is the first to examine the
374 effect of resveratrol on short-term lipid infusion model of insulin resistance and the first
375 study ever to show an effect of resveratrol on skeletal muscle IκBa levels. Our study is in
376 agreement with other studies which have demonstrated that resveratrol can inhibit IKKβ
377 and/or NFκB activated by cytokines and lipopolysaccharide (LPS) *in vitro* (Birrell *et al.*
378 2005; Estrov *et al.* 2003). Indeed resveratrol was shown to inhibit LPS-induced IκBα
379 phosphorylation in human intestinal (Cianciulli *et al.* 2012), and microglia (Capiralla *et*
380 *al.* 2012) cells. In agreement with our study Do et al (Do *et al.* 2012) recently found
381 decreased phosphorylated IKKβ levels in the liver of db/db mice treated with resveratrol
382 for 6 weeks. Since short-term fat infusion activates IKKβ (Boden *et al.* 2005; Kim *et al.*
383 2001) and IKKβ may directly phosphorylate serine (307) residue of IRS-1 (Gao *et al.*

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384 2002), it is possible that resveratrol prevents fat-induced peripheral insulin resistance
385 directly through prevention of IKKβ activity. Alternatively, it is also plausible that IKKβ
386 activation is inhibited via resveratrol-induced amelioration of oxidative stress by the
387 virtue of IKKβ activation occurring downstream of oxidative stress in the mechanism of
388 FFA-induced insulin resistance (Pereira *et al.* 2014). Support for this comes from a study
389 which showed that oxidative stress can directly activate IKKβ (Kamata *et al.* 2002).
390 However, we found that IH infusion did not increase levels of MDA, a marker of
391 oxidative stress, in skeletal muscle and that MDA levels were higher in the IH+RSV
392 group compared to the IH group. Based on this marker of oxidative stress, we suggest
393 that oxidative stress is not a key mediator of IH-induced insulin resistance in skeletal
394 muscle and the ability of resveratrol to act as an antioxidant does not play a protective
395 role in our study. We cannot exclude the possibility that the mechanisms through which
396 resveratrol improves insulin sensitivity differ depending on the duration of resveratrol
397 administration. For example, it has been reported that the ability of resveratrol to elevate
398 antioxidant enzyme activity occurs after prolonged exposure (Martins *et al*. 2014), and
399 therefore, resveratrol's antioxidant properties may not explain how it improves insulin
400 sensitivity in our acute model. Liver tissue was not collected in the present study and
401 therefore whether resveratrol has similar effects on liver IRS and IκBα remains to be
402 determined.
403 Although the serine kinases mTOR, p70S6 kinase, and JNK have been shown to
404 increase serine phosphorylation of IRS-1 and are implicated in insulin resistance, they
405 were not affected by IH infusion and do not appear to be involved in our model of
406 insulin resistance induced by short-term lipid infusion.

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407 Some studies suggested that resveratrol provides protection from insulin
408 resistance caused by high-fat diet in mice via activation of SIRT1 (Lagouge *et al.* 2006;
409 Sun *et al.* 2007) and SIRT1 was found to inhibit NFκB (Yang *et al.* 2007) , while we
410 (Breen *et al.* 2008; Zygmunt *et al.* 2010) and others (Park *et al.* 2007a) have shown that
411 AMPK is activated by resveratrol and AMPK can inhibit IKKβ (Bess *et al.* 2011).
412 Furthermore, in AMPK deficient mice fat-induced insulin resistance was not prevented
413 by resveratrol (Um *et al.* 2010). High-fat diet used in these studies is typically
414 associated with chronically elevated plasma FFA. We found no changes in total and
415 phosphorylated AMPK levels, indicating that AMPK is not activated by resveratrol in our
416 short-term lipid infusion insulin resistance model.
417 In conclusion, the present study demonstrates that resveratrol prevents hepatic and
418 peripheral insulin resistance caused by acute elevation of circulating FFA in association
419 with prevention of FFA-induced increase in serine (307) phosphorylation of IRS-1 and
420 decrease in total IRS-1 levels in skeletal muscle. Resveratrol prevented the FFA-induced
421 reduction in IκBα levels in skeletal muscle, suggesting inhibition of IKKβ an effect
422 similar to salicylate treatment. Based on the results of the present study, resveratrol
423 represents a potential treatment for FFA-associated insulin resistance.
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Table 1.
Blood insulin, glucose and FFA levels during the basal period and during the hyperinsulinemic clamp.

	Basal period				**Hyperinsulinemic clamp**			
	SAL	IH	IH+RSV	RSV	SAL	IH	IH+RSV	RSV
Insulin (pM)	104±22	159±37	206±53	161±36	1052±233	735±99	933±128	885±83
Glucose (mM)	6.64±0.39	6.78±0.30	7.05±0.43	7.33±0.27	6.82±0.31	6.21±0.51	7.06±0.37	7.23±0.33
FFA (µEq/l)	664±102	1251±215†	993±130	535±50	167±23	748±174†	524±47†	209±53

Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=5-7/group. † P<0.05 vs SAL and RSV.

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FIGURE CAPTIONS

Figure 1. Effect of IH and resveratrol on glucose infusion rate, an indicator of whole body insulin sensitivity, during the last 30 min of the hyperinsulinemic-euglycemic clamp. Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=5-7/group. *$P<0.05$ vs other groups.

Figure 2. Panel A: Effect of IH and resveratrol on endogenous glucose production (EGP) during the basal period and during the last 30 min of the hyperinsulinemic-euglycemic clamp. Panel B: Effect of IH and resveratrol on insulin-induced suppression of hepatic glucose production from the basal period during the last 30 min of the hyperinsulinemic-euglycemic clamp. Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=5-7/group. * $P<0.05$ vs other groups. †$P<0.05$ vs. SAL and RSV.

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Figure 3. Effect of IH and resveratrol on peripheral glucose utilization during the basal period and during the last 30 min of the hyperinsulinemic-euglycemic clamp. Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=5-7/group. *$P<0.05$ vs other groups.

Figure 4. Effect of IH and resveratrol on phosphorylated and total IRS-1 and Akt. Soleus muscle lysates were prepared, resolved by SDS-PAGE and immunoblotted using specific antibodies. Representative immunoblots including β-actin for loading control are shown at the top. Panel A: Phosphorylated (Ser 307) IRS-1. Panel B: Total IRS-1 Panel C:

25 Phosphorylated (Ser 473) and Total Akt. The immunoblots were scanned and the graph
26 values are arbitrary densitometric units. Data are mean±SEM. SAL=Saline,
27 IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol
28 alone. n=4-6/group. *P<0.05 vs other groups.
29
30 **Figure 5.** Effect of IH and resveratrol on phosphorylated and total IκBα. Soleus muscle
31 lysates were prepared, resolved by SDS-PAGE and immunoblotted using specific
32 antibodies. Panel A: Representative immunoblots. Panel B: Phosphorylated IκBα. Panel
33 C: Total IκBα. Immunoblots were scanned and the values are arbitrary densitometric
34 units. Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-
35 infused with resveratrol, RSV=resveratrol alone. n=6-7/group. *P<0.05 vs other groups.
36
37 **Figure 6**: Effect of IH and resveratrol on phosphorylated and total levels of AMPK,
38 mTOR, p70 S6K and JNK. Soleus muscle lysates were prepared, resolved by SDS-PAGE
39 and immunoblotted using specific antibodies. Representative immunoblots are shown.
40 β-actin blot is loading control.
41
42
43 **Figure 7.** Effect of IH and resveratrol on soleus muscle MDA levels. Data are mean ±
44 SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol,
45 RSV=resveratrol alone. n=7/group. †P<0.05 vs IH.

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Figure 1

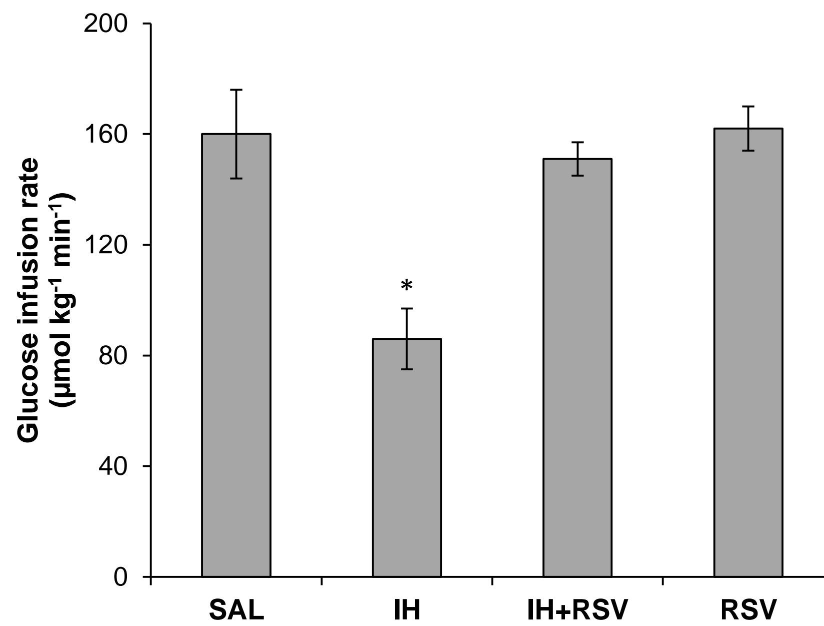
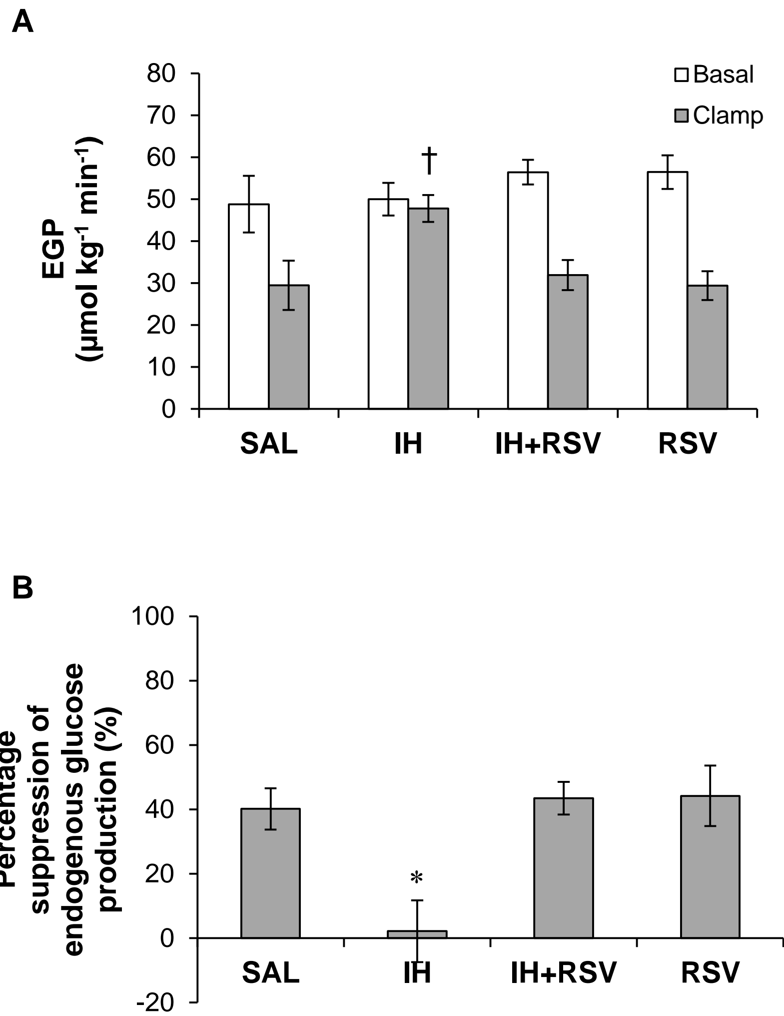


Figure 2



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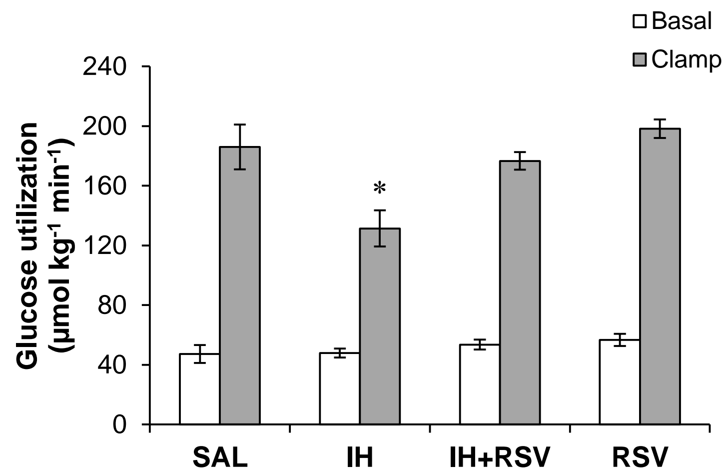

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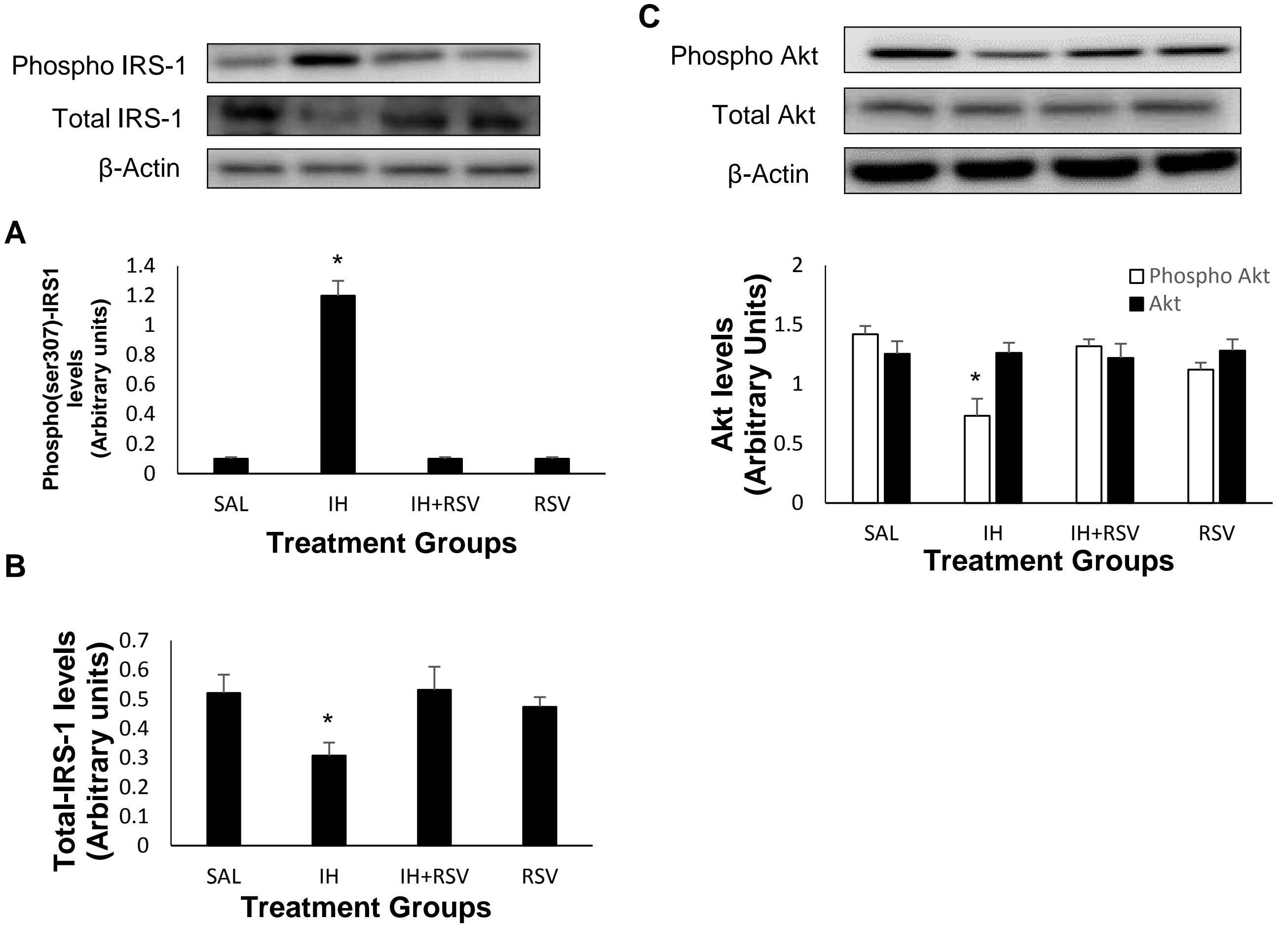

Figure 5

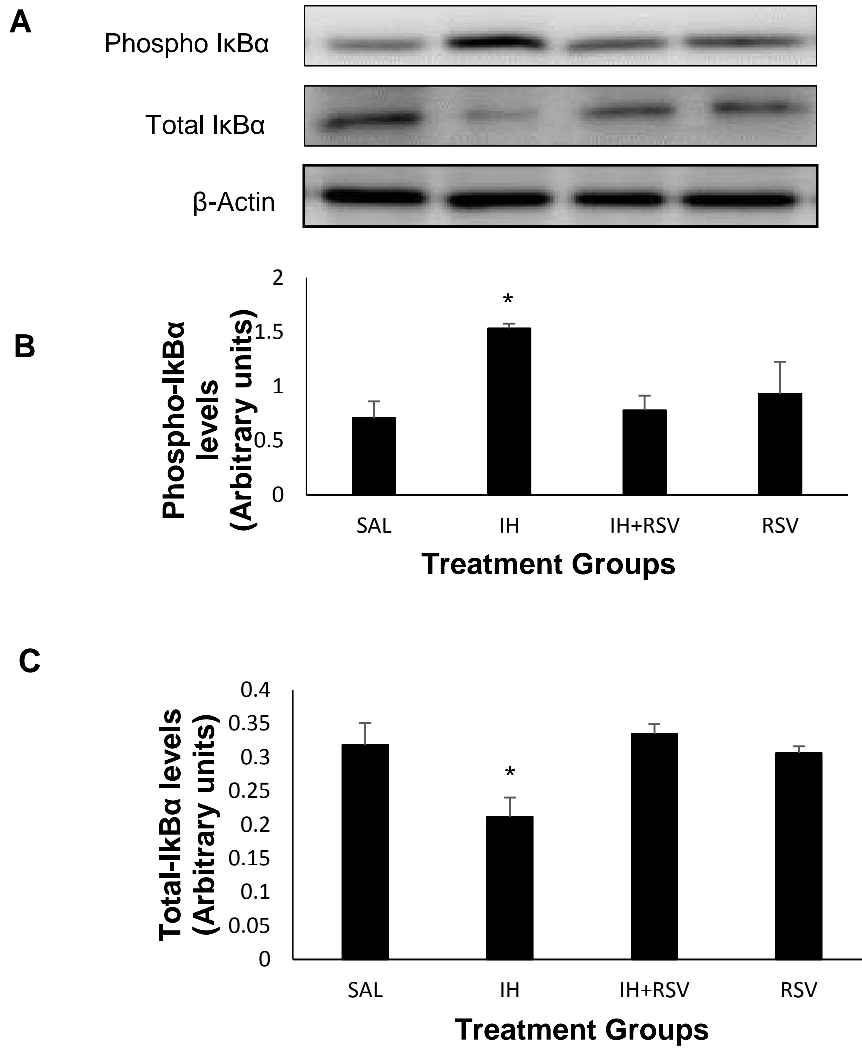

Figure 6

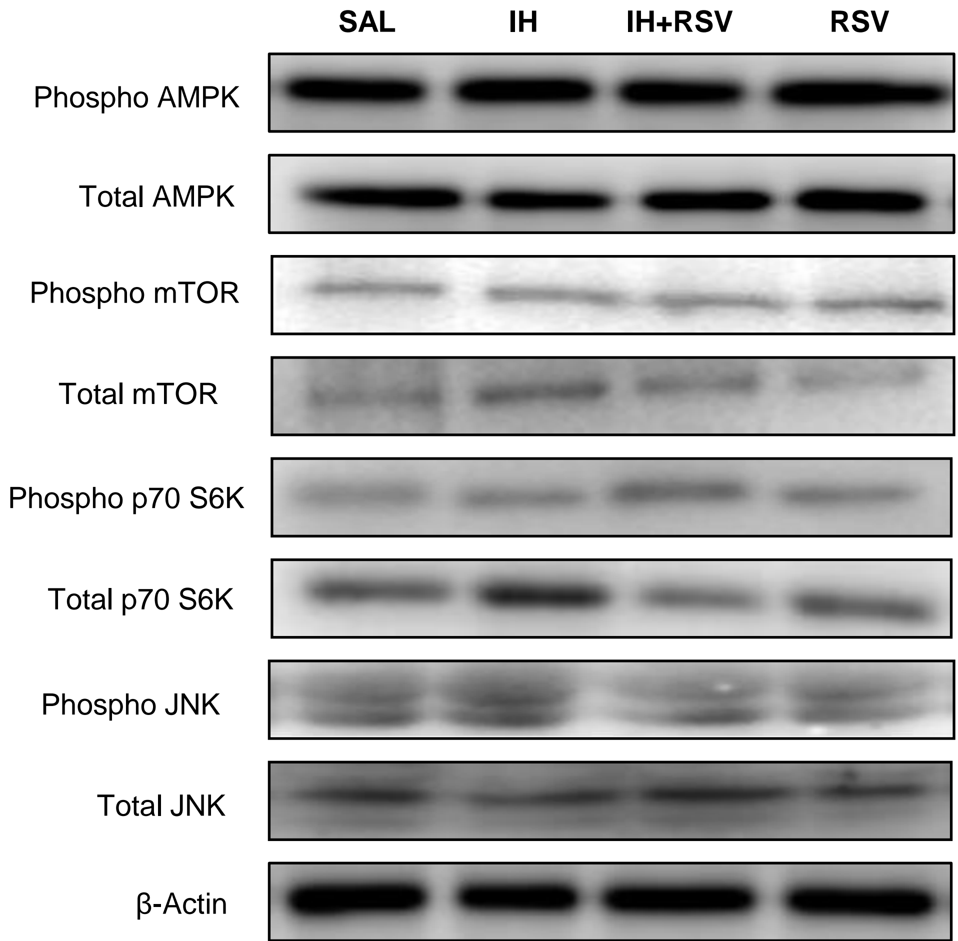

Figure 7