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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
INTRODUCTION

A close relationship between obesity, insulin resistance, and type 2 diabetes mellitus has been shown by numerous studies. Fasting plasma FFAs are often increased in obesity (Lewis et al. 2002). The expanded adipose tissue of obese individuals releases products such as adipokines/cytokines and free fatty acids (FFA), contributing to insulin resistance. Elevated levels of FFA have been shown to cause insulin resistance (Boden et al. 2001; Kim et al. 2004; Lam et al. 2002; Roden et al. 1996; Yuan et al. 2001). Insulin action leads to phosphorylation of insulin receptor substrates (IRS) and downstream activation of Akt. FFA cause insulin resistance in skeletal muscle mainly via increased serine (Copps & White 2012; Guo 2014) and reduced tyrosine phosphorylation of IRS-1 (Kim et al. 2001; Kim et al. 2004; Yu et al. 2002). Serine kinases, such as inhibitor of κBα (IκBα) kinase β (IKKβ), protein kinase C (PKC), mammalian target of rapamycin (mTOR), p70S6 kinase, and c-jun NH$_2$-terminal kinase (JNK) have been shown to mediate this process (Copps & White 2012; Guo 2014).

IKKβ is a member of the nuclear factor kappa B (NF-κB) pathway involved in inflammatory responses, activated by cytokines such as TNF-α and also is implicated in insulin resistance. The transcription factor NF-κB is kept inactive in the cytoplasm by an inhibitory protein known as IκBα. Activated IKKβ phosphorylates IκBα, leading to its degradation by the ubiquitin proteasome pathway. This allows NF-κB to translocate to the nucleus and modify transcription (Gilmore 2006). FFA activate IKKβ and thus NF-κB mediated transcription of cytokines (Lee & Lee 2014; Sinha et al. 2004). In addition, FFA-activated IKKβ can directly phosphorylate rat IRS-1 on ser-307, and thus impair insulin signaling (De Alvaro et al. 2004). In vivo studies showed that diet- and obesity-
induced insulin resistance was reversed by targeted disruption of IKKβ or treatment with salicylate, an inhibitor of IKKβ (Yuan et al. 2001). Similarly, we and other authors have found that salicylate prevented hepatic and peripheral insulin resistance induced by lipid infusion (Park et al. 2007b; Kim et al. 2001) and high dose aspirin (salicylate) treatment improved insulin signaling and action also in diabetic patients (Hundal et al. 2002).

Resveratrol (trans3,4,5-trihydroxystilbene) is a naturally occurring polyphenol compound, found in the skin of grapes and in high concentration in red wine, shown to have antioxidant, anticancer, and anti-ageing properties and to protect against cardiovascular disease (Baur & Sinclair 2006). Importantly, resveratrol has been shown to have antidiabetic properties in vitro and in vivo (Park et al. 2007a; Breen et al. 2008; Zygmunt et al. 2010; Baur et al. 2006; Lagouge et al. 2006; Su et al. 2006; Do et al. 2012). In skeletal muscle cells in vitro, resveratrol increased glucose uptake (Breen et al. 2008; Zygmunt et al. 2010; Park et al. 2007a) and abolished the palmitate-induced decline in insulin-stimulated glucose uptake via inhibition of PTP1B expression (Sun et al. 2007). In in vivo studies, resveratrol was shown to prevent high-fat-diet-induced insulin resistance in mice (Lagouge et al. 2006; Sun et al. 2007), however the effect of oral resveratrol supplements on insulin sensitivity in obese individuals is controversial (Timmers et al. 2011; Poulsen et al. 2013). Resveratrol activates the NAD-dependent deacetylase SIRT1 (Lagouge et al. 2006; Sun et al. 2007) and the energy sensor AMP-dependent kinase (AMPK) (Breen et al. 2008; Park et al. 2007a; Um et al. 2010) and these molecules have been proposed to play a significant role in resveratrol’s action. In the present study, we determined whether resveratrol has a protective effect against
insulin resistance caused by acute elevation of plasma FFA, which had not been
examined previously.

MATERIALS AND METHODS

Animal care and surgery

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

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

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

**Plasma Assays**
Plasma insulin levels were determined by radioimmunoassays (RIAs) using kits specific for rodent insulin (but with 100% cross reactivity with porcine insulin used for infusion) as previously described (Park et al. 2007b). Plasma glucose levels were measured using a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma radioactivity from the [3-3H] glucose tracer was measured after deproteinization with Ba(OH)₂ and ZnSO₄ and evaporation to dryness. Aliquots of the [3-3H] glucose tracer and of the radiolabeled glucose infusate were assayed together with the plasma samples (Lam et al. 2002). Plasma FFA levels were assayed using a colorimetric kit from Wako Industrials (Osaka, Japan) as previously described (Park et al. 2007b; Lam et al. 2002).

Immunoblot Analysis

Soleus muscle samples (40 mg) were ground in a glass-on-glass tissue grinder containing ice-cold lysis buffer (50 mM Tris pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM EGTA, 1 mM Na₃VO₄, 100 mM NaF, 10 mM Na₄P₂O₄, 1 μM okadaic acid, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Insoluble materials were removed by centrifugation at 2000 rpm for 10 min at 4°C. The protein concentration in all samples was determined by the detergent-compatible modified Lowry method, using bovine serum albumin as standard. Fifty μg of protein in all samples were mixed with equal volumes of 3X sample-loading buffer (6.86 M urea, 4.29% sodium dodecyl sulphate (SDS), 300 mM dithiothreitol, 43 mM Tris-HCl pH 6.8) and left at room temperature for 30 min. The mixture was vortexed and proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene fluoride membranes (Biorad) followed by incubation for 1h at room temperature in Tris–Tween normal saline (TTNS) buffer.
(pH 7.4) containing 0.1% Tween-20 (Sigma) mixed with 7.5% non-fat dried milk for blocking. Thereafter, membranes were incubated overnight with an affinity-purified polyclonal antibody specific for IRS-1 (1:500 dilution; Upstate Cell Signaling Solutions), phospho (ser307) IRS-1 (1:1000 dilution; Biosource), IκBα (1:2000 dilution; Santa Cruz Biotechnology), phospho (ser32/36) IκBα (1:500 dilution; Santa Cruz), β-actin (Santa Cruz), or the following antibodies (1:1000 dilution; Cell Signaling): phospho (ser 473) Akt, Akt, phospho(thr172) AMPK, AMPK, phospho (ser2448) mTOR, mTOR, phospho (thr389) p70S6K, p70S6K, phospho(thr183/tyr185) JNK and JNK. After washing with TTNS buffer three times for 20 min each, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for one hour at room temperature. The membranes were then washed three times with Millipore water and developed using enhanced chemiluminescence reagent (Amersham). The bands obtained from immunoblotting were quantified by densitometry.

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

**Plasma resveratrol measurements**

Resveratrol was extracted from rat plasma samples using ethyl acetate and centrifuging at 8000 rpm for 1 minute at 4°C. Supernatant was collected and dried using a speed vacuum. The dried product was then dissolved in 200µL of 100% methanol, filtered with 0.2µm syringe for LC-MS analysis. Waters Acquity ultra-performance liquid chromatography (UPLC) consisting of binary solvent manager, sample manager, photodiode array detector, mass spectrometer and MassLynx 4.1 software were used. The injection volume was 3 µL for all samples. The UPLC profiling was performed on a 50
mm X 2.1 mm BEH C18 column packed with 1.7 µm particles (Waters) following a
gradient elution profile. The mobile phase consisted of 0.1% formic acid in water (solvent
A) and 100% acetonitrile (solvent B). The shape of the gradient used was as follows: 0
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
min, 92% A; 8.0 min, 100% A. The column temperature was maintained at 40 °C with a
constant flow rate of 0.3 mL/min. Mass spectrometry was done as ESI spray in the
negative ion mode for trans-resveratrol, at a capillary voltage 3.50 kV, cone voltage 50 V,
source temperature 150 °C with a gas flow rate of 600 L/hr.

Calculations

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

Statistical analysis

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

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

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

During the basal period, no differences in endogenous glucose production (EGP) (Figure 2A) between treatment groups were observed. Hepatic insulin sensitivity is measured as the ability of insulin to suppress EGP from basal. In the SAL group, EGP was suppressed by 40% during the last 30 min of hyperinsulinemic clamp (Figure 2A and 2B). In contrast, in the IH group, the suppression of EGP during the clamp was only 2%, i.e. significantly less than the EGP suppression in the SAL group (P<0.05; Figure 2B). Co-infusion of resveratrol with IH resulted in insulin-induced suppression of hepatic
glucose production (43.5%) to similar levels seen in the SAL group, clearly indicating an
action of resveratrol (P<0.05 vs IH) to prevent the IH effect. Resveratrol infusion alone
did not have any effect (Figure 2B).

As expected, peripheral glucose utilization increased during the clamp (186±15
µmol kg⁻¹ min⁻¹) compared to basal period (47±6 µmol kg⁻¹ min⁻¹) (Figure 3). IH infusion
significantly decreased peripheral glucose utilization during the clamp (131±12 µmol kg⁻¹
min⁻¹) when compared with SAL infusion (186±15 µmol kg⁻¹ min⁻¹) (P<0.05; Figure 3)
an indication of peripheral insulin resistance. This decrease was completely abolished in
the group receiving infusion of both IH and resveratrol (176±6 µmol kg⁻¹ min⁻¹). These
data indicate an ability of resveratrol to prevent fat-induced peripheral insulin resistance.
Resveratrol infusion alone had no effect.

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

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

Different kinases have been suggested to increase ser 307 phosphorylation of IRS-
1, including IKKβ. Since we previously found that the IKKβ inhibitor salicylate
prevented insulin resistance due to lipid infusion (Park et al. 2007b) we examined the levels of IκBα, a marker of IKKβ activation. IH infusion increased phosphorylation and decreased muscle content of total IκBα, suggesting activation of IKKβ. Interestingly, resveratrol co-infusion with IH prevented this effect (P<0.05 for IH vs other groups; Figure 5). We also examined mTOR, p70S6 kinase, and JNK all of which have been shown to increase serine phosphorylation of IRS-1 and are implicated in insulin resistance. Total and phosphorylated levels of mTOR, p70S6 kinase, and JNK were not changed by any treatment (Figure 6). In addition, total and phosphorylated levels of AMPK, the upstream regulator of mTOR and p70S6 kinase, were not changed by any treatment (Figure 6).

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

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

**DISCUSSION**

Although the precise mechanism of FFA-induced insulin resistance remains elusive, a consensus exists that impaired post-receptor signaling is involved with serine phosphorylation of IRS-1 being a key event (Le Marchand-Brustel et al. 2003; Copps & White 2012; Guo 2014; Gao et al. 2002). The present study was performed to investigate
the effect of the polyphenolic compound resveratrol on insulin resistance caused by acute elevation of circulating FFA in vivo. We have shown that resveratrol stimulates glucose uptake in L6 myotubes (Breen et al. 2008). Based on this, we hypothesized that resveratrol may prevent insulin resistance caused by our model of short-term (7h) fat infusion. The results of the present study show that resveratrol is effective in preventing fat-induced hepatic and peripheral insulin resistance and suggest that a part of the mechanism may involve restoration of insulin signaling in skeletal muscle.

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

The infusion rate of exogenous glucose is an indication of whole body insulin sensitivity and was reduced by lipid infusion, consistent with previous studies (Boden 1997; Boden et al. 2001; Boden et al. 2005; Kim et al. 2001; Kim et al. 2004; Lam et al. 2002; Lam et al. 2003; Yu et al. 2002). The whole body insulin resistance caused by IH infusion was completely prevented when resveratrol was co-infused. Infusion of [3-³H] glucose enabled us to separately assess hepatic and peripheral insulin resistance. IH infusion decreased insulin-induced suppression of endogenous glucose production (EGP) and insulin-stimulated peripheral glucose utilization, suggesting that lipids caused both
hepatic and peripheral insulin resistance, in accordance with our previous findings (Park et al. 2007b). More importantly, resveratrol co-infusion was able to completely prevent the IH-induced insulin resistance at both sites. Our study is in agreement with other in vivo studies showing a prevention of diet-induced insulin resistance in mice (Lagouge et al. 2006; Um et al. 2010) and monkeys (Jimenez-Gomez et al. 2013) treated with resveratrol. Plasma levels of resveratrol resulting from consumption of resveratrol in the diet of rodents depend on the dose, and resveratrol concentrations of 10-120ng/ml (44-530nM) in plasma have been reported (Lagouge et al. 2006). In humans, dietary supplements of resveratrol have been shown to result in plasma levels of approximately 180ng/ml (0.78µM) (Timmers et al. 2011). We decided to infuse resveratrol in order to increase the probability of seeing an effect, since the oral bioavailability of resveratrol has been reported to be poor in rats (Kapetanovic et al. 2001). Resveratrol levels in the plasma of animals infused with resveratrol was 1.064 µM which is not far from the theoretical concentration of 0.6 µg/ml (2.62 µM) calculated from the infused dose (3mg/kg/h = 0.75mg/h for a 250g rat) and the published clearance (1.24 L/h) of RSV in rats (Colom et al. 2011). Colom et al (Colom et al. 2011) found that IV bolus administration of 2mg/kg of resveratrol in rats resulted in resveratrol levels of 0.1 µM after 2 h. Similarly, IV bolus administration of 20mg/kg of resveratrol in rats resulted in mean resveratrol concentration of 0.1µg/ml (0.43µM) after 2 h (He et al. 2006). Overall the levels of plasma resveratrol in our study although are higher than the levels achieved by oral resveratrol administration in rats, they are not very different from the levels achieved by IV bolus administration of resveratrol in rats in previous studies and importantly are close to the levels seen after oral supplementation in humans.
Serine 307 phosphorylation of IRS-1 caused by short-term fat infusion was associated with decreased tyrosine phosphorylation of IRS-1 and impairment of insulin signaling in rat skeletal muscle, although the serine kinase responsible was not identified (Yu et al. 2002). Soleus muscle and gastrocnemius muscle are typically used in the literature to determine the extent of insulin sensitivity in skeletal muscle. It has been reported that i.v. lipid infusions impair insulin-stimulated glucose uptake by the soleus muscle (Kim et al. 2001) and by the gastrocnemius muscle (Kim et al. 2004) in rodents. These two muscles consist of a different proportion of muscle fiber types, with the soleus muscle being considered the more insulin sensitive muscle of the two (Holmang et al. 1992). Therefore, we chose to study soleus muscle to maximize the probability of finding differences in insulin sensitivity.

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

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

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It is possible that resveratrol prevents fat-induced peripheral insulin resistance directly through prevention of IKKβ activity. Alternatively, it is also plausible that IKKβ activation is inhibited via resveratrol-induced amelioration of oxidative stress by the virtue of IKKβ activation occurring downstream of oxidative stress in the mechanism of FFA-induced insulin resistance (Pereira et al. 2014). Support for this comes from a study which showed that oxidative stress can directly activate IKKβ (Kamata et al. 2002). However, we found that IH infusion did not increase levels of MDA, a marker of oxidative stress, in skeletal muscle and that MDA levels were higher in the IH+RSV group compared to the IH group. Based on this marker of oxidative stress, we suggest that oxidative stress is not a key mediator of IH-induced insulin resistance in skeletal muscle and the ability of resveratrol to act as an antioxidant does not play a protective role in our study. We cannot exclude the possibility that the mechanisms through which resveratrol improves insulin sensitivity differ depending on the duration of resveratrol administration. For example, it has been reported that the ability of resveratrol to elevate antioxidant enzyme activity occurs after prolonged exposure (Martins et al. 2014), and therefore, resveratrol’s antioxidant properties may not explain how it improves insulin sensitivity in our acute model. Liver tissue was not collected in the present study and therefore whether resveratrol has similar effects on liver IRS and IκBα remains to be determined.

Although the serine kinases mTOR, p70S6 kinase, and JNK have been shown to increase serine phosphorylation of IRS-1 and are implicated in insulin resistance, they were not affected by IH infusion and do not appear to be involved in our model of insulin resistance induced by short-term lipid infusion.
Some studies suggested that resveratrol provides protection from insulin resistance caused by high-fat diet in mice via activation of SIRT1 (Lagouge et al. 2006; Sun et al. 2007) and SIRT1 was found to inhibit NFκB (Yang et al. 2007), while we (Breen et al. 2008; Zygmunt et al. 2010) and others (Park et al. 2007a) have shown that AMPK is activated by resveratrol and AMPK can inhibit IKKβ (Bess et al. 2011).

Furthermore, in AMPK deficient mice fat-induced insulin resistance was not prevented by resveratrol (Um et al. 2010). High-fat diet used in these studies is typically associated with chronically elevated plasma FFA. We found no changes in total and phosphorylated AMPK levels, indicating that AMPK is not activated by resveratrol in our short-term lipid infusion insulin resistance model.

In conclusion, the present study demonstrates that resveratrol prevents hepatic and peripheral insulin resistance caused by acute elevation of circulating FFA in association with prevention of FFA-induced increase in serine (307) phosphorylation of IRS-1 and decrease in total IRS-1 levels in skeletal muscle. Resveratrol prevented the FFA-induced reduction in IκBα levels in skeletal muscle, suggesting inhibition of IKKβ an effect similar to salicylate treatment. Based on the results of the present study, resveratrol represents a potential treatment for FFA-associated insulin resistance.
ACKNOWLEDGEMENTS

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https://mc06.manuscriptcentral.com/apnm-pubs


Table 1. Blood insulin, glucose and FFA levels during the basal period and during the hyperinsulinemic clamp.

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

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.
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.

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:
Phosphorylated (Ser 473) and Total Akt. The immunoblots were scanned and the graph values are arbitrary densitometric units. Data are mean±SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=4-6/group. *P<0.05 vs other groups.

Figure 5. Effect of IH and resveratrol on phosphorylated and total IκBα. Soleus muscle lysates were prepared, resolved by SDS-PAGE and immunoblotted using specific antibodies. Panel A: Representative immunoblots. Panel B: Phosphorylated IκBα. Panel C: Total IκBα. Immunoblots were scanned and the values are arbitrary densitometric units. Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=6-7/group. *P<0.05 vs other groups.

Figure 6: Effect of IH and resveratrol on phosphorylated and total levels of AMPK, mTOR, p70 S6K and JNK. Soleus muscle lysates were prepared, resolved by SDS-PAGE and immunoblotted using specific antibodies. Representative immunoblots are shown. β-actin blot is loading control.

Figure 7. Effect of IH and resveratrol on soleus muscle MDA levels. Data are mean ± SEM. SAL=Saline, IH=Intralipid plus heparin, IH+RSV=IH co-infused with resveratrol, RSV=resveratrol alone. n=7/group. †P<0.05 vs IH.
Figure 1

[Graph showing glucose infusion rate (µmol kg\(^{-1}\) min\(^{-1}\)) for different conditions: SAL, IH, IH+RSV, and RSV. The graph indicates a significant difference (*) between the IH and other conditions at 80 µmol kg\(^{-1}\) min\(^{-1}\).]
Figure 2

A

![Graph showing EGP (µmol kg⁻¹ min⁻¹) with Basal and Clamp conditions for SAL, IH, IH+RSV, and RSV groups.]

B

![Bar chart showing percentage suppression of endogenous glucose production (%) with SAL, IH, IH+RSV, and RSV groups.]
Figure 3

![Graph showing glucose utilization (µmol kg⁻¹ min⁻¹) for different conditions.

- Basal
- Clamp

Conditions:
- SAL
- IH
- IH+RSV
- RSV

Glucose utilization levels are indicated with error bars.
Figure 4

A

Phospho IRS-1
Total IRS-1
β-Actin

B

Phospho(ser307)-IRS1 levels (Arbitrary units)

C

Phospho Akt
Total Akt
β-Actin

Treatment Groups

SAL  IH  IH+RSV  RSV

SAL  IH  IH+RSV  RSV

Phospho Akt
Akt levels (Arbitrary Units)

Total-IRS-1 levels (Arbitrary units)

SAL  IH  IH+RSV  RSV

SAL  IH  IH+RSV  RSV

*
**Figure 5**

A

<table>
<thead>
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<th>Total-IkBα</th>
<th>β-Actin</th>
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B

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<td>RSV</td>
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C

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<td>RSV</td>
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Figure 6

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<tr>
<td>β-Actin</td>
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Figure 7

![Bar chart showing MDA (nmol/g protein) for different conditions: SAL, IH, IH+RSV, RSV. IH+RSV shows a significant increase compared to other conditions.](https://mc06.manuscriptcentral.com/apnm-pubs)