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RESEARCH ARTICLE

Effects of salicylic acid-induced wine rich in anthocyanins on metabolic parameters and adipose insulin signaling in high-fructose fed rats

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ABSTRACT

We evaluated the effects of Syrah red wine treated with salicylic acid (RW SA) and its control red wine (RW) on metabolic parameters, systolic blood pressure and adipose tissue insulin signaling in high-fructose (F) fed rats. Grape treated with SA increased the anthocyanin (ANTs) levels in RW. F induced increased systolic blood pressure, dislipidemia and insulin resistance (HOMA:IR). F rats treated with RW significantly prevented these alterations while RW SA partially attenuated triglycerides levels and HOMA:IR without modifications in HDL cholesterol levels. F impaired the adipose tissue response to insulin. Supplementation with RW and RW SA partially attenuated these alterations. Rats supplemented with RW SA had lesser beneficial effects on metabolic alterations than control RW, while both RW and RW SA attenuated altered adipose response to insulin. More studies are necessary to deeply evaluate the effect on SA-induced RW rich in ANTs levels on metabolic alterations associated to MetS.

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Introduction

Polyphenols are secondary plant metabolites present in fruits and vegetables, which are associated with reduced risk of chronic disease (Woodside et al. 2013). Recent studies have shown the potential benefits of polyphenolic compounds and polyphenol-rich foods or beverage consumption in attenuating adipose inflammation and insulin resistance in experimental models of metabolic syndrome (MetS) and in inflamed adipocytes (Rivera et al. 2008; Vazquez-Prieto et al. 2011b; Vazquez-Prieto et al. 2012; Bettaieb et al. 2014; Vazquez Prieto et al. 2015). MetS is an aggregation of multiple risk factors including impaired glucose tolerance, dyslipidemia, abdominal obesity and high blood pressure (Ford et al. 2002). Accordingly, there is increased evidence that supports that diet-induced increment of adipose tissue, especially visceral adipose tissue (VAT) plays a central role in the development of these alterations. Hypertrophic adipocytes secrete cytokines promoting a state of chronic low-grade adipose tissue inflammation and systemic insulin resistance (Wellen & Hotamisligil 2003; Matsuzawa 2006). In this context, we have previously shown that supplementation with polyphenols or food containing high amount of polyphenols such as red wine (RW) was able to ameliorate complications associated with MetS in high-fructose (F) fed rats (Vazquez-Prieto et al. 2011a, 2015).

Anthocyanins (ANTs) are a sub-family of polyphenols that are well known for their beneficial effects (Bhattacharya et al. 2010). Wine is the world's most consumed beverage and its composition presents a large amount of phenolic compounds which are beneficial to health (Pazzini et al. 2015). During winemaking, grape berries' phenolic compounds suffer transformations such as polymerization and co-pigmentation, among others (Ribareau-Gayon et al. 2006). Achieving an increment of these secondary metabolites on fresh grape as well as on daily consumed products like wine could be interesting for a healthier nutrition quality. Salicylic acid (SA) has demonstrated to be a possible elicitor of phenolic compounds on grape (Li et al. 2008; Wen et al. 2008; Riedel et al. 2012). However, it is still a challenge to go through winemaking process

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preserving the phenol overproduction of the grape berries elicited with SA.

This study was carried out to evaluate if berries treated with SA increased the ANTs content of RW and if ANTs enriched wine is able to improve the metabolic alterations associated to F diet in rats.

Materials and methods

Grape elicitation and winemaking

For this assay, plants of *Vitis vinifera* cv. Syrah from EEA INTA-Luján, Luján de Cuyo, Mendoza conducted on overhead trellis system and weekly irrigated, were used. Plants having a variation coefficient less than 5% in regard to its trunk diameter and good phytosanitary condition were selected. Bunches of five plants were sprayed with salicylic acid (SA) of 8 mM, three times, from veraison, with 15 days interval. Five plants were sprayed with water, as a control.

Grapes were harvested at 25°Brix and red wine was made up either with berries under control treatment (RW) or from the berries treated with SA (RW SA). Both wines were filtered with cellulose acetate membranes (Sartorius) of 45 µm pore diameter and phenolic profile was analyzed by HPLC-DAD (SPD-M10 AVP, Shimadzu). Anthocyanins profile analysis was made up according to the compendium of international methods of wine and must analysis (OIV 2014). The measurements were realized by using a Licrosorb RP-18 5 µm, 250 mm ×4.6 mm. Mobile phases were A: 87% water, 3% acetonitrile and 10% formic acid and B: 40% water, 50% acetonitrile and 10% formic acid. The standard curve for the quantification was constructed by using the malvidin HCl standard (Sigma). Elution was carried out by the following gradient: phase A: 94% at min 0, 70% at min 15, 50% at min 30, 40% at min 35, 94% at min 41, being maintained until min 45. The flux was 0.8 ml/min and the injection volume was 20 µl. Absorbance was measured at 518 nm.

Concentration in mg/l of wine was determined for delphinidin-3-glucoside (Df), cianidin-3-glucoside (Cn), petunidin-3-glucoside (Pt), peonidin-3-glucoside (Po), malvidin-3-glucoside (Mv), peonidin-acetyl-glucoside (PoAc), malvidin-acetyl-glucoside (MvAc), peonidin-cumaril-glucoside (PoCu) and malvidin-cumaril-glucoside (MvCu). Total ANT concentration was calculated as the sum of all. In addition, calculations were made for each ANT percentage over the total ANT concentration and the ratios: trisubstituted/bisubstituted ((Df + Pt + Mv)/(Cn + Po)); methylated/no methylated F3′H (Po/Cn), methylated/no methylated F3′5′H ((Mv + Pt)/Df), % acetylated ((PoAc + MvAc)*100/TA) and % of cumarilated ((PoCu + MvCu)*100/TA).

Animal studies

All animal studies were conducted in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. Six-week-old male Sprague–Dawley rats ($n = 26$) were used for this eight-week supplementation study. Rats weighing 160–180 g were housed in cages in a room under conditions of controlled temperature (21–25 °C) and humidity with a 12 h light/dark cycle with access to standard rat chow (Gepsa-Feeds, Buenos Aires, Argentina) and water *ad libitum*. Rats were randomly assigned to the following four groups: Control diet (C), fructose-fed rats (F) (fructose 10% (w/v) added in the drinking water), and F plus red wine (F + RW) or red wine treated with SA in a dose of 20% (v/v) (F + RW SA) administered in the drinking water diluted in a 10% fructose solution ($n = 7$, each group). The dose of red wine was chosen based on a previous study performed by our group using Malbec red wine in the same experimental model of MetS in rats (Vazquez-Prieto et al. 2011a). Body weight was recorded weekly. Food and water intakes were determined three times a week and systolic blood pressure (SBP) was measured at the beginning, middle and at the end of the protocol by tail-cuff method and determined with a plethysmography Koda2® (Kent Scientific Corporation, Torrington, CT). After eight weeks of their respective dietary treatments, and after overnight fast, rats were weighed and then anesthetized with ketamine (50 mg/kg) and acepromazine (1 mg/kg). In order to evaluate the insulin signaling cascade in epididymal adipose tissue, half of animals per group were intraperitoneally injected with insulin (10 mU/g body weight human insulin; Humulin; Eli Lilly) or saline solution and euthanized after 10 min. Blood was collected from the abdominal aorta into EDTA tubes and plasma was obtained after centrifugation at 3000 rpm for 15 min at 4 °C. Epididymal adipose tissue was weighted and flash-frozen in liquid nitrogen and then stored at −80 °C until assayed.

Biochemical determinations

Plasma triglyceride (TG) and HDL cholesterol concentrations were determined by enzymatic colorimetric methods using commercial kits (GTLab, Buenos Aires, Argentina). Glucose was measured in blood collected from the tail using a glucometer (Accu-ChekPerforma, Roche, Buenos Aires, Argentina). Insulin was measured using the Ultra Sensitive Insulin ELISA kit (Crystal Chem, Downers Grove, IL), and insulin resistance was

assessed using the homeostasis model assessment (HOMA-IR) originally described by Matthews et al. (1985). HOMA-IR was calculated using the following formula: HOMA-IR (mg/dl × μU/ml) =fasting glucose (mg/dl) × fasting insulin (μU/ml)/405.

Western blots

Adipose tissue was homogenized in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% w/v sodium dodecylsulfate, 1% w/v Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors) as previously described.

Homogenates were centrifuged at 15,000×*g* for 30 min, the supernatant was collected and protein concentration was measured using the Bradford method. Aliquots containing 40 μg protein were denatured with Laemmli buffer, separated by reducing 10–12.5% (w/v) polyacrylamide gel electrophoresis, and electroblotted to nitrocellulose membranes. Membranes were blotted for 2 h in 5% (w/v) nonfat milk, and subsequently incubated in the presence of the corresponding primary antibodies (1:1000 dilution for all the antibodies) overnight at 4 °C. After incubation for 90 min at room temperature in the presence of the corresponding secondary antibody (either HRP or biotinylated antibody followed for 1 h with streptavidin), the conjugates were visualized and quantified by chemiluminescence detection in a Luminescent Analyzer Image Reader (LAS-4000) Fujifilm. The densitometric analysis was performed using the Image J Program.

Statistical analysis

Wine polyphenol data were analyzed by using the DESCO test, Spad version 5.6, where a test-value higher than 2 in absolute value, makes a significant difference.

Data from animal study were expressed as mean ± SEM. The statistical significance was assessed by one-way ANOVA followed by Newman–Keuls Multiple Comparison Test. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) was used for all statistical analysis. Differences were considered significant at $p < 0.05$.

Results

Determinations on wines

The oenological parameters were similar in both wines, RW and RW SA (Table 1). Reduction sugars, alcohol graduation and volatile acidity showed exactly the same values for both of them. Moreover total acidity, pH and free SO_2 values were much close on both wines, making the comparison acceptable between them.

Salicylic acid treatment led to 2.8-fold increase of the total ANT concentration in comparison with the control RW, 197.14 mg/l versus 70.21 mg/l, respectively (Figure 1(A)). When we evaluated the ANT profile of both wines (Figure 1(B)), we observed that SA increases uniformly the different ANTs, suggesting that the general ANT profile of the RW SA compared to the RW was not modified.

Moreover, no differences were observed in the percentages of the glycosylated, acetylated and cumarilated ANT between the RW and RW SA (Figure 2). Overall, SA significantly increased the ANTs of Syrah berries preserving the ANT profile and, thus, the typical characteristics of this cultivar.

Effects of RW and RW SA on metabolic variables in high-fructose fed rats

The effect of RW and RW SA supplementation on metabolic parameters was investigated in F rats as shown in Table 2. Accordingly to our previous studies (Vazquez-Prieto et al. 2011a, 2011b), the water intake was similar between groups. On the other hand, the daily food intake was higher in the C group compared to F groups. There was no difference in body weight between groups. Chronic fructose consumption significantly increased the SBP (Figure 3) and plasma levels of triglycerides and decreased the levels of HDL cholesterol in comparison with C group (Table 2). The addition of RW and RW SA to F rats totally and partially prevented the increased SBP and triglycerides levels, respectively (Figure 3 and Table 2). Furthermore, administration of RW to F rats significantly increased the levels of HDL compared with F rats. No differences were observed in plasma levels of glucose between groups. However, chronic fructose consumption lead to increased insulin levels and the insulin resistance index (HOMA:IR) compared with control group. F rats supplemented with RW and RW SA totally and partially

Table 1. Oenological parameters of Syrah red wine (RW) and Syrah red wine made up with berries treated with salicylic acid (RW SA).

Analysis	RW	RW SA
Reduction sugars[a] (g/l)	– of 1.80	– of 1.80
Alcohol	14.3°	14.3°
Total acidity	4.80	4.87
pH	3.55	3.60
Free SO_2 mg/l	26	23
Volatile acidity	0.33	0.33

[a]At the end of fermentation.

attenuated this increases, respectively. Overall, in terms of metabolic parameters and SBP, supplementation with control RW to F rats had better effect than RW SA.

Effect of RW and RW SA treatment on adipose insulin signaling cascade in F rats

Chronic fructose consumption had been shown to contribute to adipose tissue insulin resistance, so we investigated the molecular basis involved in insulin signaling. As we have explained before C, F, F + RW and F + RW SA groups were injected with saline or insulin, and then basal and insulin-stimulated signaling was determined in adipose tissue (Figure 4(A)). Insulin-stimulated F group increased serine phosphorylation of the IRS-1 at ser307 compared with C rats, which were attenuated upon RW and RW SA supplementation. No significant differences were observed on IRS-1 under basal conditions between groups. On the other hand, basal and insulin-stimulated phosphorylation of AKT, a downstream positive mediator of insulin signaling, was significantly decreased in F rats compared with C rats. While basal p-AKT was significantly higher in F rats treated with both RW and RW SA, no differences were observed in insulin-stimulated rats. Then, we explored PPARγ expression, a chief regulator of adipogenesis, inflammation and insulin resistance (Shen et al. 2014). We observed that PPARγ expression was significantly lower in insulin-stimulated F rats compared with C group. Insulin-stimulated F rats supplemented with both RW and RW SA significantly increased PPARγ protein expression. No significant differences were observed on PPARγ under basal conditions between groups (Figure 4(A)).

Next, we evaluated negative regulators of the insulin signaling such as JNK, which induces a serine phosphorylation of IRS-1, and PTP-1B which dephosphorylates tyrosine residues of the insulin receptor (IR) and IRS-1 (Haj et al. 2012) (Figure 4(B)). Chronic fructose consumption significantly induced higher expression of p-JNK (Thr183/Tyr185) and PTP-1B in insulin-

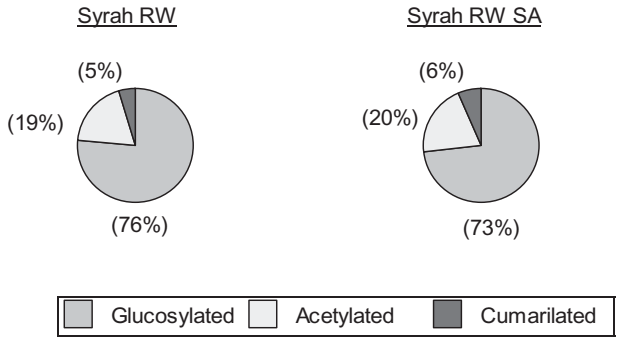

Figure 2. Percentage of glucosylated, acetylated and cumarilated anthocyanins on Syrah red wine (RW) and Syrah red wine made up with berries treated with salicylic acid (RW SA).

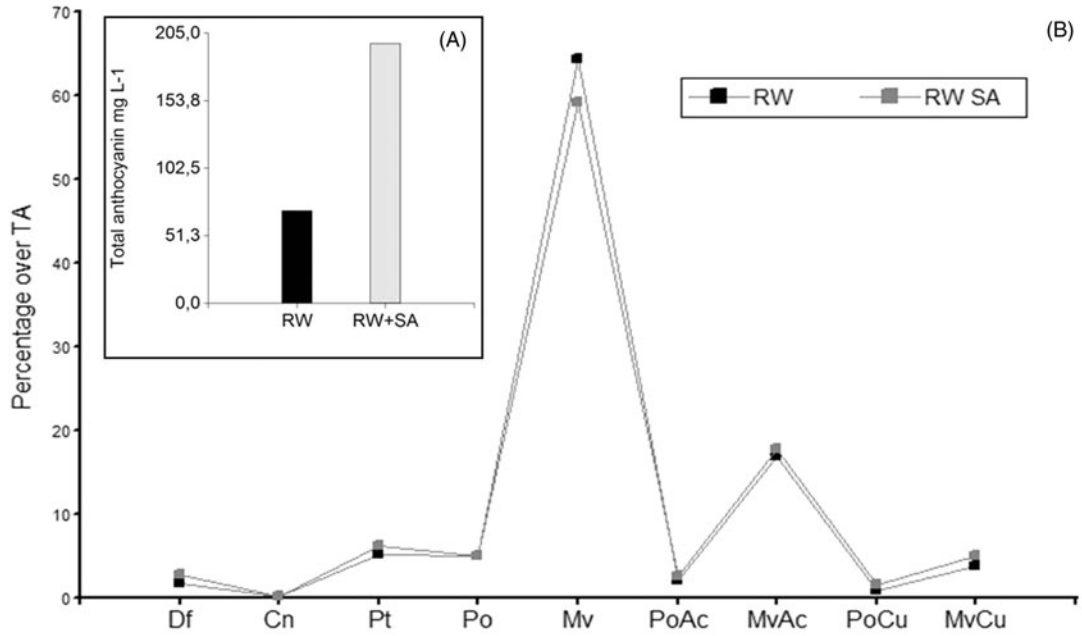

Figure 1. Effects of salicylic acid applied to berries on Syrah wine. (A) Total anthocyanin content (mg L^{-1}) in Syrah red wine (RW) and Syrah red wine made up with berries treated with salicylic acid (RW SA) and (B) anthocyanin profile of RW and RW SA. The multivariate profile shows the percentages of delphinidin-3-glucoside (Df), cianidin-3-glucoside (Cn), petunidin-3-glucoside (Pt), peonidin-3-glucoside (Po), malvidin-3-glucoside (Mv), peonidin-acetyl-glucoside (PoAc), malvidin-acetyl-glucoside (MvAc), peonidin-cumaril-glucoside (PoCu) and malvidin-cumaril-glucoside (MvCu).

stimulated rats compared with C group, which was partially and totally attenuated by RW and RW SA administration to F rats, respectively (Figure 4(B)). No significant differences were observed on JNK under basal conditions among groups while PTP-1B was significantly higher in F rats compared with C and F rats supplemented with RW and RW SA. Together, these results suggest that RW and RW SA attenuated altered adipose insulin response.

Discussion

In the present study, we demonstrated that berries treated with salicylic acid lead to an increment of the ANT concentrations in red wine, maintaining similar proportion of glucosylated, acetylated and cumarilated ANT compounds compared to the control untreated-RW. As far as we know, this is the first study demonstrating that the treatment with salicylic acid increases the ANT concentrations in grape berries, and it is maintained through winemaking process. The increase observed in ANT concentration after salicylic acid spray, may be the result of a re-distribution of the precursor upstream in the phenylpropanoid pathway (Jeandet et al. 1995). The re-distribution may cause a decrease in other phenolics, such as catechins and quercetins, which have important health benefits (Fraga et al. 2010; Galleano et al. 2012).

ANTs are reported to enhance insulin sensitivity in experimental models of obesity (Seymour et al. 2011; Vendrame et al. 2014; Guo & Ling 2015; Park et al. 2015), in human adipocytes (Scazzocchio et al. 2015) and in subjects with type 2 diabetes consuming 160 mg of ANTs (Li et al. 2015). In spite of SA significantly increasing the ANT levels of RW, we did not observe any improvement of metabolic parameters as we did with control not treated with RW, while both control and SA RW attenuated adipose tissue insulin resistance. This leads us to think that even though the SA RW was rich in ANTs, a low concentration of other bioactive compound may ameliorate its beneficial effect. One limitation of our study is that we did not analyze other major polyphenol families to determine if the increase of the ANT concentrations could affect the concentrations of further important polyphenols found in red wine such as tannins. In this matter, besides ANTs, resveratrol content was analyzed, but not quantifiable amounts were detected in both wines.

The beneficial effect of moderated red wine consumption on parameters related with MetS has been well demonstrated (Queipo-Ortuno et al. 2012; Chiva-Blanch et al. 2013; Droste et al. 2013). Moreover, previously we have shown that red wine administration to F rats improved metabolic parameters related with MetS independent to its alcohol content (Vazquez-Prieto et al. 2011a), suggesting that the

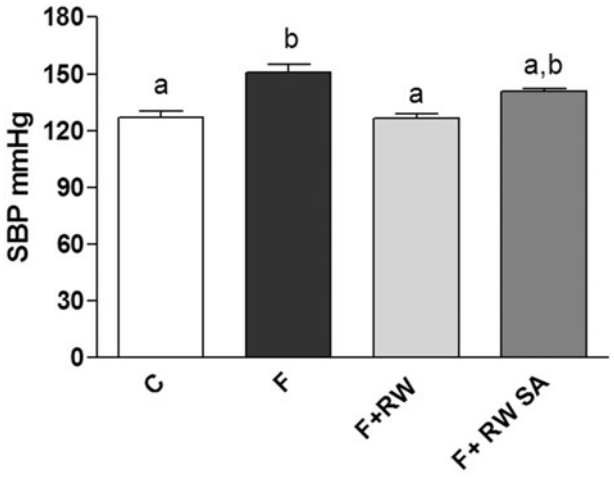

Figure 3. Final systolic blood pressure in fructose fed rats. Values show the systolic blood pressure at the end of the experimental protocol in control (C), F (rats receiving fructose in the drinking water 10% (w/v) during 8 w) and in F rats receiving red wine (F + RW) or red wine treated with SA (F + RW SA) in a dose of 20% (v/v) in drinking water. Values are shown as means ± SEM ($n = 6$). Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Table 2. Metabolic parameters in rats fed for eight weeks without (control) or with 10% (w/v) fructose in the water, in the absence (F) or presence of red wine (F + RW) or red wine plus salicylic acid (F + RW SA) with a dose of 20% (v/v) in drinking water.

	C	F	F + RW	F + RW SA
Food intake (g/d)	28.2 ± 0.9^{a}	19.5 ± 1.2^{b}	18.9 ± 2.1^{b}	18.6 ± 0.8^{b}
Liquid intake (ml/d)	50.8 ± 0.7	70.05 ± 1.2	65.6 ± 5.8	66.1 ± 4.0
Body weight (g)	457 ± 15.0	411 ± 11.0	445 ± 25.0	472 ± 7.0
Triglycerides (mg/dl)	66.4 ± 6.7^{b}	221.0 ± 49.4^{a}	86.7 ± 15.8^{b}	$107.0 \pm 13.5^{a,b}$
HDL (mg/dl)	43.9 ± 1.7^{a}	32.8 ± 2.8^{b}	48.2 ± 2.7^{a}	31.9 ± 3.1^{b}
Plasma glucose (mg/dl)	89.9 ± 3.2	85.9 ± 2.3	87.0 ± 3.2	97.0 ± 3.1
Insulin (μU/ml)	3.2 ± 0.1^{a}	6.4 ± 0.9^{b}	3.4 ± 0.3^{a}	$4.1 \pm 0.5^{a,b}$
HOMA-IR	0.70 ± 0.1^{a}	1.34 ± 0.2^{b}	0.73 ± 0.1^{a}	$0.98 \pm 0.1^{a,b}$

Values are shown as means ± SEM ($n = 6$). Values having different superscripts are significantly different ($p < 0.05$, one way ANOVA). HOMA-IR: homeostatic model assessment of insulin resistance (mg/dl × μU/ml/405).

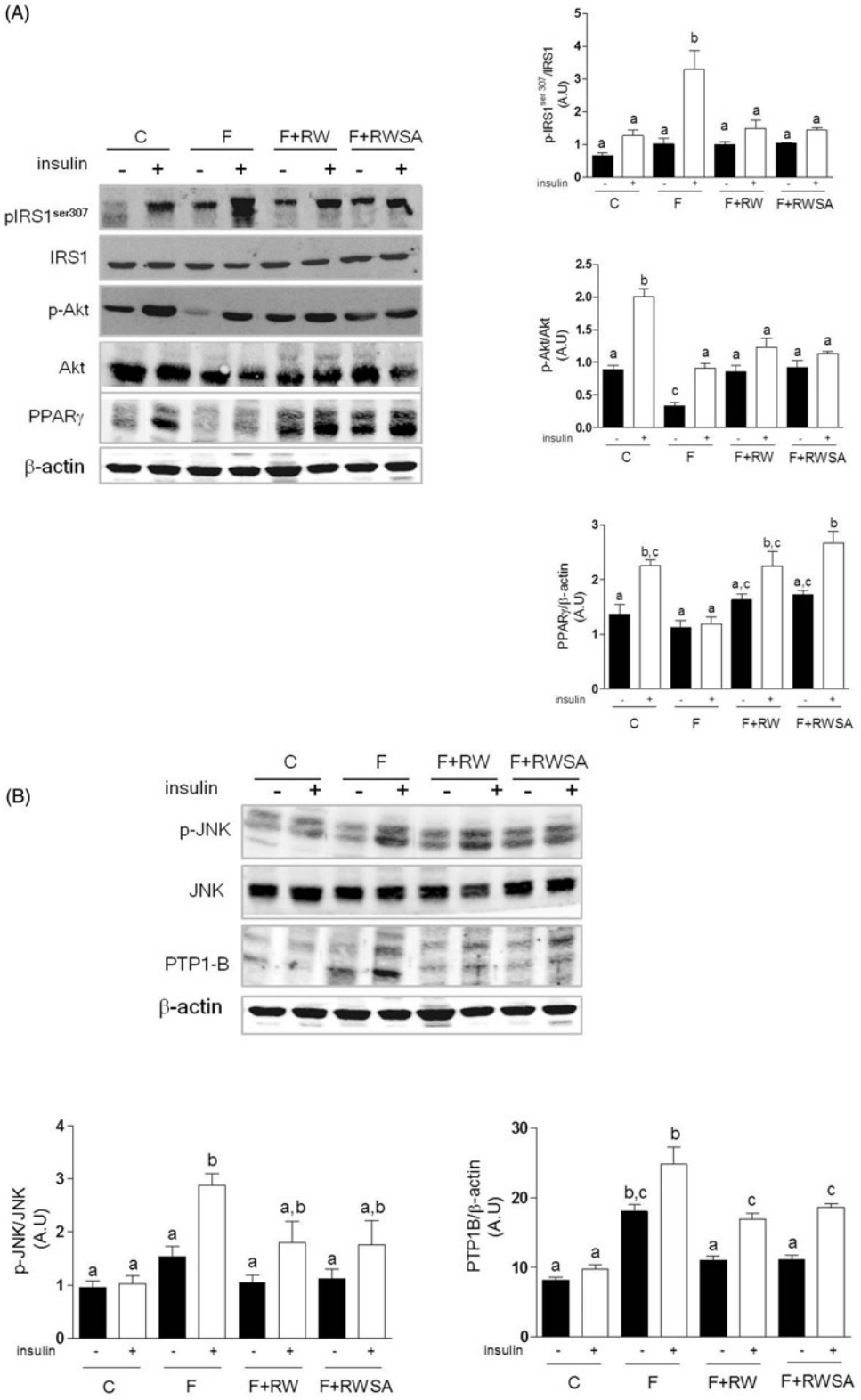

Figure 4. Supplementation with RW and RW SA enhances insulin signaling in epididymal adipose tissues in high F rats. After eight weeks on the corresponding diets, rats were fasted overnight and then injected with saline or insulin (10 mU/g bw) and then sacrificed after 10 min. (A) Protein expression of components of the insulin signaling cascade: IRS1, Akt and PPARγ and (B) negative regulators of insulin signaling cascade PTP-1B and JNK are shown for epididymal adipose tissue. Bands were quantified and results for the F and F plus RW and RW SA were referred to control group values. Results are expressed as the ratio of phosphorylated/total protein level or to β-actin. Results are shown as mean ± SEM of four animals/treatment. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

protective effect of red wine is attributed to the non-alcohol components such as polyphenols (Kuntz et al. 2014; Rebelo et al. 2014; Draijer et al. 2015; Pisano et al. 2015). In this study, RW and RW SA totally and partially prevent the increased SBP, plasma triglycerides, insulin and the index of insulin resistance HOMA-IR in F fed rats. Also, supplementation with untreated RW to F rats significantly increases the levels of HDL cholesterol. Accordingly to previous studies (Vazquez-Prieto et al. 2012; Bettaieb et al. 2014; Vazquez Prieto et al. 2015), chronic fructose consumption leads to adipose dysfunction associated with chronic low grade systemic inflammation and/or insulin resistance. In this study, we evaluated some parameters of insulin signaling in epididymal adipose tissue and we observed that chronic fructose consumption altered the adipose response to insulin while supplementation with both RW and RW SA to F rats attenuated the altered response to insulin in epididymal adipose tissue.

In conclusion, salicylic acid increased the ANT concentration in red wine, however supplementation with RW SA to a F fed rat had lesser beneficial effects on metabolic alterations than control RW. Further studies should be performed to thoroughly assess the main polyphenolic families and the potential use of red wine enriched in ANT to attenuate/mitigate MetS-associated pathologies.

Disclosure statement

The authors have declared that there is no conflict of interest.

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