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Research Paper

Vitis vinifera L. grape skin extract activates the insulin-signalling cascade and reduces hyperglycaemia in alloxan-induced diabetic mice

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Keywords
diabetic mice; grape skin extract; hyperglycaemia; insulin cascade; insulin resistance

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Abstract

Objectives This study examined the effect of *Vitis vinifera* grape skin extract (ACH09) on hyperglycaemia and the insulin-signalling cascade in alloxan-treated mice.

Methods Glycaemia, serum insulin and Western blot analysis of insulin cascade proteins were evaluated in the gastrocnemius muscles of four groups of adult mice: control, ACH09 (200 mg/kg per day, p.o.), alloxan (300 mg/kg, i.p.) and alloxan + ACH09. Insulin secretion in isolated pancreatic islets was also studied.

Key findings Glycaemia values in the alloxan + ACH09 and ACH09 groups were significantly lower than in the alloxan-treated and control groups, respectively. Increased insulin resistance (HOMA index) was observed in the alloxan-treated group but not in the alloxan + ACH09 group. Insulin receptor content and Akt phosphorylation were significantly greater in the alloxan + ACH09 group compared with the alloxan-treated group. The glucose transporter (GLUT-4) content was reduced in alloxan-treated mice compared with the control group, while alloxan + ACH09 and ACH09-treated mice showed a significant increase in GLUT-4 content. ACH09 treatment did not change glucose-induced insulin secretion in isolated pancreatic islets.

Conclusions The results suggest that ACH09 has hypoglycaemic and antihyperglycaemic effects that are independent of an increase in insulin release but are probably dependent on an increase in insulin sensitivity resulting from an activation of the insulin-signalling cascade in skeletal muscle.

Introduction

Diabetes is a major cause of death in developed countries. Type 2 diabetes constitutes about 85–95% of all diabetes cases in these countries and accounts for an even higher percentage of cases in developing nations. Complications from diabetes, such as coronary artery and peripheral vascular diseases, stroke, diabetic neuropathy, amputations, renal failure and blindness result in increasing disability, reduced life expectancy and enormous health costs for society.[1] Therefore, studies targeting the prevention and treatment of type 2 diabetes are important.

Under normal physiological conditions, blood glucose levels are tightly regulated by the secretion of insulin and glucagon by specialized cells in the islets of Langerhans of the pancreas. Type 2 diabetes is characterized by hyperglycaemia, peripheral resistance to the action of insulin, and eventual destruction of insulin producing β-cells in the pancreas. Insulin-stimulated uptake of glucose is mediated by the action of glucose transporters on the cell surface, skeletal muscle being the major site of glucose uptake stimulated by insulin. Glucose transporter 4 (GLUT-4) is expressed by muscle, adipose and kidney cells.[2]

A large body of evidence indicates that moderate, prolonged red wine consumption is associated with decreased cardiovascular mortality.[3–6] The mechanisms underlying the cardioprotective effects of wine and grape products are not completely understood, but pharmacological properties

likely play important roles in the inhibition of platelet aggregation,[7] antihypertensive action,[8] antioxidant capacity,[9] vasodilatation,[10] and increased endothelial-type nitric oxide (NO) synthase expression and activity.[11]

Clinical studies suggest that wine consumption may be salutary for patients with type 2 diabetes. Men and women who ingest small amounts of wine are reported to have a reduced risk of developing diabetes,[12] and diabetic patients who consume a moderate daily amount of red wine have reduced cardiovascular complications after myocardial infarction.[13] Furthermore, red wine consumption during meals significantly preserves plasma antioxidant defences and reduces both low-density lipoprotein oxidation and thrombotic activation in patients with type 2 diabetes.[14] The mechanisms involved in the beneficial action of wine in patients with type 2 diabetes are not completely understood, but it has been suggested that wine has an important influence on reducing insulin resistance.[15] This beneficial cardiovascular effect of wine could be due to its alcohol content or to the presence of polyphenol compounds that have important pharmacological actions.[16]

Plant polyphenols, organic compounds found in numerous plant species and their fruits, are being actively studied as potential treatments for components of metabolic syndrome.[17] *Vitis vinifera* L. has been used as a food and a beverage, as well as a remedy for various complaints in traditional medicine since ancient times.[18] Recently, the benefits of grapes or their components have been studied using animal models of diabetes for analysis of metabolic disturbances. A small number of studies have suggested that grapes or their components may have some protective effects against metabolic disturbances observed in type 2 diabetics.[2]

We recently reported that the insulin resistance observed in rats with experimental pre-eclampsia was significantly reduced by an ethanol-free extract obtained from the skin of the vinifera grape.[19] However, the mechanism underling the effect of the grape skin extract on insulin resistance remains to be elucidated. Thus, the aim of this study was to investigate if *V. vinifera* grape skin extract (ACH09) has antihyperglycaemic effects in a mouse model of diabetes induced by alloxan. We also investigated the mechanisms underlying the effects of ACH09 on insulin secretion and the insulin-signalling cascade in skeletal muscle.

Materials and Methods

Preparation of the grape skin ACH09 extract

The dried and powdered skin fruits of *V. vinifera* L. (Vitaceae) were extracted in an aqueous solution at 100°C with occasional shaking for about 120 min. The solution was then concentrated under vacuum. The concentrated aqueous extract was introduced into an ion-exchange resin column (cationic) and washed sequentially with ethanol, ethanol/H_2O (1 : 1), and H_2O. The H_2O fraction was discarded. The ethanolic and hydroalcoholic fractions were placed together and evaporated under vacuum at 60°C, followed by spray drying of the concentrated solution (inlet temperature 190°C; outlet temperature 85°C). The extract obtained in the process was a fine powder, soluble in H_2O, with about 30% total polyphenols according to the Folin-Ciocalteu assay,[20] and 3% malvidin-3-*O*-glucoside.

Analysis of grape skin ACH09 extract

To identify the active principles in grape skin, the extract was analysed by LC/UV/MS with an atmospheric pressure chemical ionization interface.[21] LC/UV analysis of the dried hydroalcoholic grape skin extract was performed on a Hewlett-Packard series 1100 photodiode array detector (DAD) liquid chromatography system (Hewlett-Packard, Waldbronn, Germany). HPLC/UV/DAD analysis was performed with a Symmetry RP-18 column (4 μm; 250 × 3.9 mm i.d.; Waters, Milford, MA, USA), solvent system: A: MeOH with 0.5% formic acid; B: H_2O with 0.5% formic acid; gradient mode 20% of A to 100% of A in 25 min; flow rate 1 ml/min; injection volume 10 μl; sample concentration 10 mg/ml in MeOH. DAD conditions: 210, 254 and 540 nm; UV data were recorded at 190–600 nm (step 2 nm).

LC/MS^n was performed directly after UV-DAD measurements. A Finningan LCQ ion trap (Finningan MAT, San Jose, CA, USA) was used with an atmospheric pressure chemical ionization interface. MS^n experiments were completed by programming dependent scan events. The first event was a full MS scan Mr (150.0–1500.0) (MS^1); during the second event, the main ion recorded was isolated and selectively fragmented in the ion trap (MS^2). The collision energy was set to 15 eV.

HPLC analysis of the dried hydroalcoholic grape skin extract involved dissolving 10 mg of the extract in 1 ml methanol/H_2O (1 : 1) with 0.5% formic acid (HPLC quality). A total of 20 μl was analysed by HPLC.

Standards of peonidin-3-*O*-glucoside,[1] petunidin-3-*O*-glucoside,[2] malvidin-3-*O*-glucoside[3] and malvidin-3-(6-*O*-*trans*-*p*-coumaryl)-5-*O*-diglicoside[4] were purchased from Polyphenols Laboratories (Sandnes, Norway).

Animals and experimental design

Male Swiss mice (25–30 g) were kept in a room at a controlled temperature (23–25°C) and fed standard chow *ad libitum*. Mice were divided into four groups ($n \geq 6$ per group) according to treatment: control group, ACH09-treated group, alloxan-treated group, and alloxan + ACH09-treated group.

To induce diabetes, alloxan monohydrate (Sigma, St Louis, MO, USA) freshly prepared in saline was injected intraperitoneally (three doses of 100 mg/kg every 48 h, beginning on

Day 0) in the alloxan and alloxan + ACH09 groups. This dose of alloxan was previously tested and found to increase blood glucose levels to more than 250 mg/100 ml.

Treatment with ACH09 diluted in drinking water (200 mg/kg per day) of the alloxan + ACH09 group started 7 days (Day −7) before alloxan administration and lasted until the end of the experiments (Day 19). The control group received only water throughout the experiment. The ACH09-treated group received ACH09 (200 mg/kg per day) from Day −7 until Day 19, orally in the drinking water. All animals were killed on Day 19. Mice were weighed once a week, and water or extract intake was recorded three times a week.

The animals were treated in accordance with the Animal Care and Use Committee of the Biology Institute of the Rio de Janeiro State University (protocol no. CEA/023/2010), based on the principles described in the Guide for Care and Use of Laboratory Animals.[22]

Insulin secretion

In-vitro insulin secretion was studied in pancreatic islets isolated from five mice per group. A total of 40 islets per mice were placed on Millipore filters in a perfusion system with Krebs solution and a mixture of CO_2 (5%) and O_2 (95%), with a multichannel peristaltic pump (Amersham, San Francisco, CA, USA) at a flow rate of 1.0 ml/min. Insulin levels were determined every 5 min in the effluent flow over 40 min (basal conditions) under infusion of a 2.8 mmol/l glucose solution. After a steady state was attained, the solution was changed to 16.7 mmol/l glucose (control group) or 16.7 mmol/l glucose plus ACH09 (50 μg/ml) (ACH09 group) and infused for 30 min. Samples from the effluent were collected at different times (15, 20, 25 and 30 min) for determination of insulin concentration.

Blood sampling

Mice were fasted for 16 h and then the blood glucose was assessed in droplets obtained from the tail using a Glucometer (Accu-Chek; Roche, Mannheim, Germany) at Day −7 and 19. At Day 19, after measurement of the fasting glucose levels, all mice were anaesthetized with thiopental (30 mg/kg, i.p.). Blood samples (1.0 ml) were collected and centrifuged at 14 000*g* for 10 min. Plasma samples were separated and kept at −20°C for the insulin assay. Immunoreactive insulin was measured by using a radioimmunoassay kit (MP Biomedicals, Orangeburg, NY, USA). Insulin sensitivity was obtained by the HOMA-IR index calculated by the formula: fasting insulin (μUI/ml) × fasting glucose (mmol/l)/22.5.[23]

Immunoblotting

The gastrocnemius muscle was homogenized in ice-cold HES buffer containing 0.3 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5 mM ethylenediamine-tetraacetic acid, 0.1 mM sodium orthovanadate, Triton X-100, 0.1 M sodium fluoride, 1 M sodium pyrophosphate, 10 μl/ml aprotinin and 10 μg/ml leupeptin. The lysate was centrifuged for 15 min at 42 000*g* at 4°C. Then, the supernatant was centrifuged for 90 min at 220 000*g* at 4°C. The total protein content was determined by the Lowry method. Samples (40 μg of gastrocnemius muscle) were subjected to 10% SDS-PAGE and transferred onto a PVDF membrane for 2 h at 328 mAmp at 4°C (Amersham Bioscience, Piscataway, NJ, USA). The blots were blocked with Tween-TBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.01% Tween-20) containing 2% bovine serum albumin. Primary antibodies used in Western blotting analysis were rabbit anti-GLUT-4, Akt, phospho-Akt Ser-473, IRS-1, IR and PI3-kinase p85 subunit (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). PVDF filters were then incubated with the appropriate secondary antibody conjugated to biotin (1 : 1000; Santa Cruz Biotechnology), followed by 1-h incubation with horseradish peroxidase-conjugated streptavidin (1 : 1000; Caltag Laboratories, Burlingame, CA, USA). Immunoreactive proteins were visualized by 3,3′-diaminobenzidine (Sigma) staining. The bands were quantified by densitometry, using Image J Software (NIH, Bethesda, MD, USA).

Statistical analysis

The results are expressed as mean ± SEM. Statistical differences were evaluated by a repeated measures analysis of variance to analyse fasting glucose throughout the experiment. 'Treatment' (ACH09 or water) and 'diabetes' (alloxan or control) were used as the between-subjects factor and 'day' was considered the within-subjects factor. Treatment effects were followed by post-hoc analyses using Fisher's protected least significant difference. Two-way analysis of variance, with Bonferroni's post-hoc test was used for the other comparisons. *P* values less than or equal to 0.05 were accepted as statistically significant. For the analyses, we used the SAS statistical package (version 6.12; SAS Institute, Cary, NC, USA).

Results

Isolation and identification of the major constituents of the ACH09 extract

All compounds showed the same UV spectra in the LC/UV/DAD analysis that are characteristic of anthocyanins. Compounds **1**, **2**, **3** and **4** presented molecular ions at m/z 463 $[M]^+$, 479 $[M]^+$, 493 $[M]^+$ and 801 $[M]^+$, respectively (Figure 1). All four showed a similar fragmentation pattern. Compounds **1**, **2** and **3** presented two signals corresponding to the molecular ion $[M^+]$ and the fragment resulting from the loss of a glucose molecule $[M\text{-}162]^+$, corresponding to the aglicon. In the case of compound **4**, the MS spectra

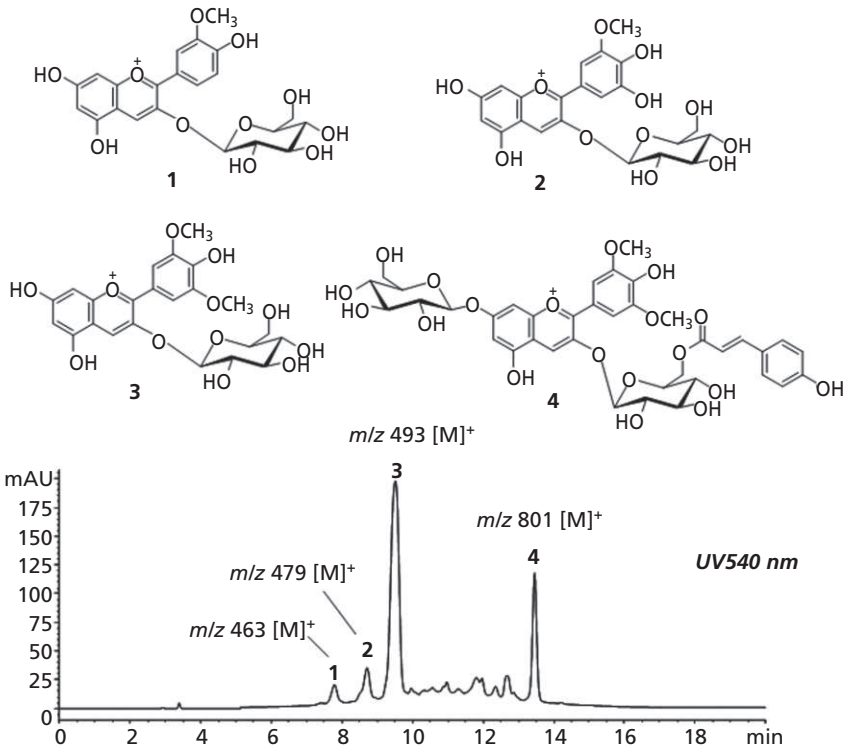

Figure 1 HPLC profile from grape skin ACH09 extract and the structures of the four identified anthocyanins.

Table 1 Body weight, plasma levels of insulin and HOMA index

Day 19	Control	ACH09	Alloxan	Alloxan + ACH09
Body weight (g)	30.46 ± 2.3	30.63 ± 2.3	27.66 ± 0.66	27.75 ± 1.5
Plasma insulin (μIU/ml)	26.12 ± 10.6	21.47 ± 3.4	16.4 ± 3.98*	18.19 ± 3.6
HOMA index	6.44 ± 2.5	3.73 ± 0.8	16.47 ± 3.35*	6.56 ± 2.8#

Values represent means ± SEM; $n \geq 6$ mice per group. *$P < 0.05$, alloxan versus control. *$P < 0.001$, alloxan versus control. #$P < 0.001$, alloxan versus alloxan + ACH09).

showed the loss of one glucose molecule and a fragment corresponding to the loss of a *p*-coumaryl glucoside moiety $[M-308]^+$.[24] According to these data, the compounds were identified as peonidin-3-*O*-glucoside,[1] petunidin-3-*O*-glucoside,[2] malvidin-3-*O*-glucoside,[3] and malvidin-3-(6-O-*trans*-*p*-coumaryl)-5-O-diglicoside,[4] all previously described in different *Vitis* spp.[24,25] This conclusion was confirmed by comparison of retention time and UV and MS data in the LC/UV/MS analysis using commercially available standards. Compounds **1–3** are always present in grape extracts, generally in high concentrations, while **4** can be found in low concentrations.[24]

Insulin secretion

The ability of ACH09 to stimulate insulin secretion was investigated in pancreatic islets perfused with 16.7 mmol/l glucose or 16.7 mmol/l glucose plus ACH09. Our data showed that the addition of ACH09 did not significantly change the pattern of insulin secretion by β-cells *in vitro*, as expressed by the area under the curve corresponding to insulin secretion over time.

Effect of ACH09 on body weight and plasma levels of glucose and insulin

There was no significant difference in body weight among the groups throughout the experiment (Table 1). To assess the effect of ACH09 on diabetes, we analysed the plasma levels of glucose and insulin in all groups. As Figure 2 shows, alloxan treatment induced a significant increase in serum glucose level compared with the control group. However, serum glucose levels were significantly lower in the alloxan + ACH09 mice compared with mice treated with alloxan alone. In addition, mice treated with ACH09 alone

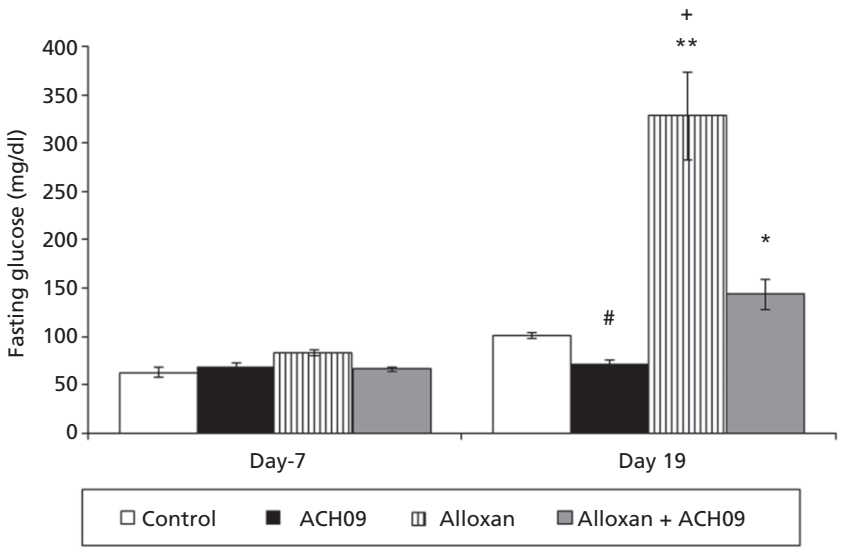

Figure 2 Effects of ACH09 (200 mg/kg per day) on serum glucose levels obtained before the induction of diabetes (Day –7) and at the end of the experiments (Day 19). Values are means ± SEM, $n = 6$ for control and ACH09 groups, and $n = 10$ for alloxan and alloxan + ACH09 groups. $^{+}P < 0.01$, control versus alloxan; $^{*}P < 0.001$, alloxan versus alloxan + ACH09; $^{**}P < 0.05$, ACH09 versus alloxan; $^{\#}P < 0.05$, control versus ACH09.

showed significantly lower serum glucose values compared with untreated control mice.

Regarding serum insulin, alloxan-treated mice had significantly decreased plasma insulin levels compared with control mice. In addition, plasma levels of insulin were not significantly different among control, ACH09 and alloxan + ACH09 groups (Table 1). The HOMA index, analysed to investigate insulin sensitivity, showed a decrease in insulin sensitivity in alloxan-treated mice compared with the control group. However, the HOMA index for alloxan + ACH09-treated mice was significantly reduced compared with that of alloxan-treated mice, but did not differ from the control group (Table 1).

Insulin signalling cascade in skeletal muscle

Figure 3 shows the results of the Western blot analysis in gastrocnemius muscle isolated at Day 19 for some proteins present in the insulin-signalling cascade. Our results showed that insulin receptor content was higher in alloxan + ACH09-treated mice compared with mice treated with alloxan alone (Figure 3a). The content of IRS-1, PI3-K, and Akt did not differ among the groups (Figure 3b, 3c and 3d). Akt phosphorylation was reduced in alloxan-treated mice; however, mice previously treated with ACH09 (alloxan + ACH09) displayed a significant increase in Akt phosphorylation relative to alloxan-treated mice (Figure 3e). Mice treated with alloxan had a significant reduction in GLUT-4 content compared with control mice, but GLUT-4 content in alloxan + ACH09-treated mice was higher than in the alloxan group. A significant increase in GLUT-4 content was also observed in ACH09-treated mice relative to the control group (Figure 3f).

Discussion

Epidemiological studies suggest that consumption of wine, grape products and other foods containing polyphenols is associated with a decreased risk for cardiovascular disease and that the benefits of wine consumption appear to be greater than for other alcoholic beverages.[26] Moderate wine consumption has been reported to be beneficial in type 2 diabetes.[13,15] Despite studies showing that wine consumption might have salutary effects, there is no justification for non-drinkers to start consuming wine as a preventive measure[27] because the risk of ethanol addiction is always present. Therefore, a healthier alternative might be to use an extract that preserves the beneficial actions of moderate wine consumption without the harmful effects of ethanol. It is likely that the beneficial effect of wine in type 2 diabetes is attributable to its non-alcoholic components, such as the polyphenols present in wine, because reports show that ethanol-free extracts from vinifera grape produce significant improvement in experimental diabetes models.[28]

The results of the present study are the first to suggest that there is an in-vivo antihyperglycaemic effect of an extract obtained from vinifera grape skin in alloxan-induced diabetic mice: blood glucose levels in mice treated with alloxan + ACH09 were close to those of the control group. It has been suggested that the use of grape seed procyanidin

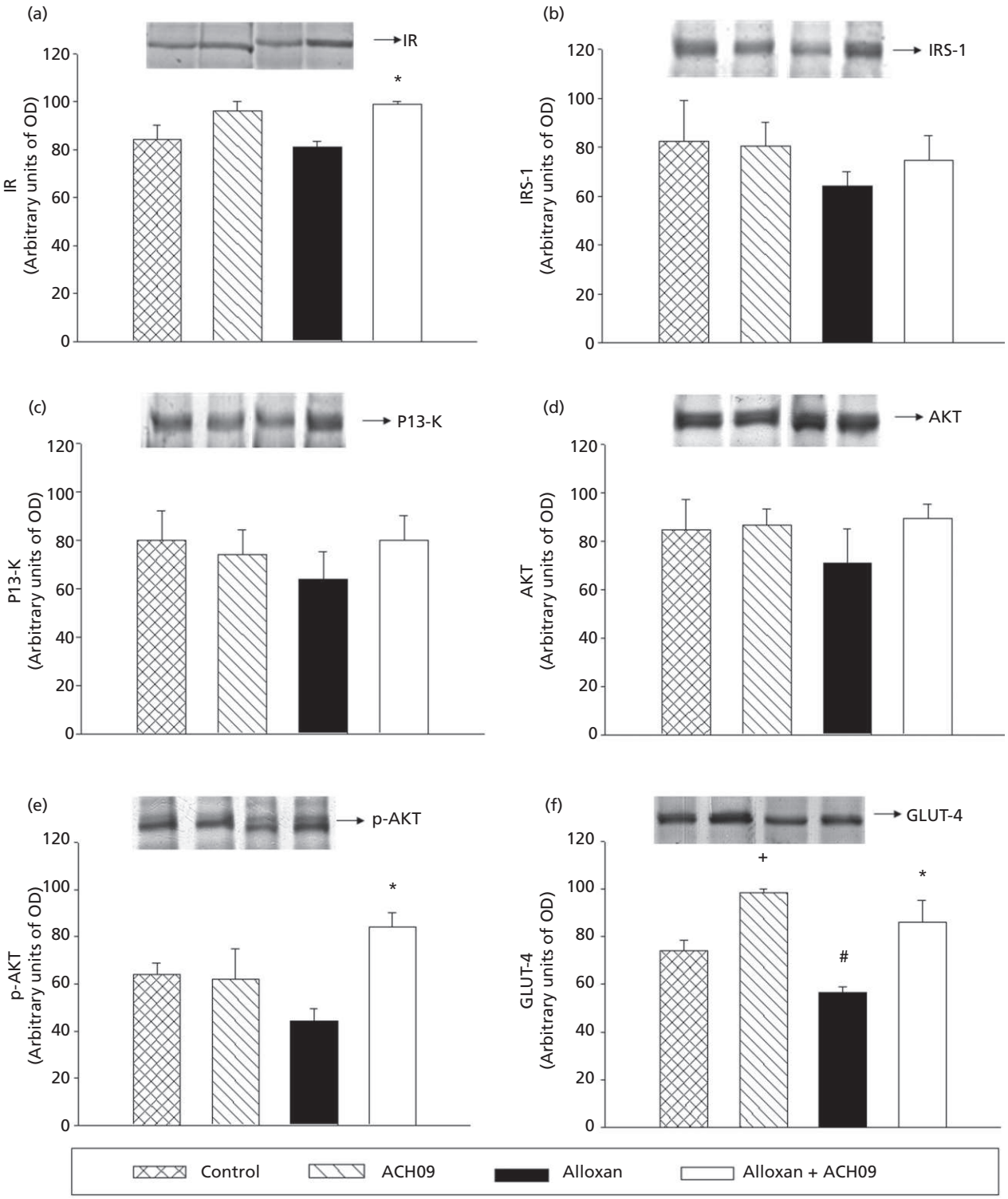

Figure 3 Western blot analysis comparing expression of (a) IR (b) IRS-1 (c) PI3K (d) AKT (e) p-AKT and (f) GLUT-4 in gastrocnemius muscle from alloxan-treated mice and the control group treated or not with ACH09 (200 mg/kg per day). Values are means ± SEM, $n = 5$ per group. $^{*}P < 0.05$, alloxan versus alloxan + ACH09; $^{\#}P < 0.01$, ACH09 versus alloxan; $^{+}P < 0.05$ control versus ACH09.

extract induces a large reduction in blood glucose levels in streptozotocin-induced diabetic rats, but only when administered with low doses of insulin.[29] Therefore, the implication is that the extract obtained from the grape skin seems to be more active than that obtained from the seed.

Alloxan induces diabetes through a deleterious pro-oxidant effect on pancreatic β-cells, leading to reduced pancreatic insulin secretion and a modulator action on insulin sensitivity.[30] In the present study, we showed that alloxan-induced diabetes in mice was accompanied by a significant reduction in plasma insulin levels. We first hypothesized that the antidiabetic effect of ACH09 found in our study could be explained by a protective antioxidant effect of the extract[8] in the pancreas, restoring insulin secretion. However, ACH09 did not increase plasma insulin levels in controls or in alloxan-treated animals, suggesting that the observed antidiabetic effect of ACH09 occurs through a mechanism other than modulation of pancreatic insulin secretion. In accordance with this suggestion, our in-vitro study also showed that ACH09 did not change insulin secretion in isolated pancreatic islets. Thus, the antidiabetic effect of ACH09 does not appear particularly attributable to action on the pancreas, but it could occur through modulation of insulin signalling and regulation of glucose transport.

Many cells take up glucose, although up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle with stimulation of GLUT-4 translocation from intracellular sites to the cell surface.[31] GLUT-4 is the major carrier involved in insulin-stimulated glucose transport, so the amount of this transporter present on the cell surface, at least in part, controls the rate of glucose transport into muscle cells.[2] Pinent *et al*.[29] have shown that extracts from vinifera grape seed have in-vitro insulinomimetic activity and increase the amount of GLUT-4 in insulin-sensitive cell lines, suggesting an extra-pancreatic mechanism for its antidiabetic action.

Our data confirm and expand these findings. We found that the antihyperglycaemic effect of ACH09 was accompanied by an in-vivo increase in GLUT-4 and insulin receptor content and in Akt phosphorylation in skeletal muscle, suggesting that the hypoglycaemic and antihyperglycaemic effects of ACH09 might result from activation of insulin signalling and regulation of glucose metabolism. The HOMA index results corroborate this hypothesis: ACH09 treatment significantly prevented the increase in insulin resistance observed in alloxan-treated mice. Because the decreased HOMA index correlated with enhanced GLUT-4 content, we suggest that ACH09 action on insulin sensitivity probably occurs through an increase in GLUT-4 and the resulting increased glucose uptake. The increased Akt phosphorylation in alloxan + ACH09-treated mice indicates that the antihyperglycaemic effect of ACH09 may be the result not only of a higher GLUT-4 content but also of increased sensitivity in the insulin-signalling pathway.

Our data are in accordance with the findings of Napoli *et al*.,[15] who suggested that red wine consumption decreased insulin resistance in patients with type 2 diabetes. Similarly, ingestion of grape seed extract in the diet significantly improved markers of inflammation, glycaemia and oxidative stress in obese patients with type 2 diabetes.[32] On the other hand, in a study of patients with metabolic syndrome receiving grape seed extract for 4 weeks, there were no significant changes in serum lipids or blood glucose measured at the beginning and at the end of the study.[33]

The mechanisms underlying ACH09-induced insulin sensitivity are not yet established. We have shown that the vasodilator effect of grape skin extract is impaired by endothelium disruption, inhibition of endothelial NO synthase and inhibition of guanylyl cyclase.[8] It has been demonstrated that NO seems to modulate insulin sensitivity. Studies on experimental animals have evaluated that the action of insulin to augment glucose uptake by skeletal muscles and other peripheral insulin-sensitive tissues *in vivo* is NO dependent.[34] Erlich and Rosenthal[35] have shown that NO synthase inhibitors decrease glucose transport in skeletal muscle, while NO donors increase it.[36] More recent findings have suggested that NO increases GLUT-4 expression in skeletal muscle by cyclic GMP- and AMPK-dependent mechanisms.[37] Therefore, because grape skin extract seems to stimulate NO synthesis, we suggest that NO could, at least in part, mediate the increased insulin sensitivity induced by ACH09 treatment.

Because ACH09 did not increase insulin secretion by pancreatic islets *in vitro* or serum levels of insulin *in vivo*, the mechanisms behind the antihyperglycaemic effects of ACH09 do not seem to be the same as those of antidiabetic sulfonylureas, which stimulate insulin release by pancreatic β-cells. On the other hand, the antihyperglycaemic effect of ACH09 might be similar to that of compounds such as the thiazolidinediones, which increase insulin sensitivity in peripheral tissues.[38]

Conclusions

This study indicates that ACH09 can exert hypoglycaemic effects in control mice and antihyperglycaemic effects in alloxan-induced diabetic mice. Such effects seem to be independent of an increase in insulin release. The increased Akt phosphorylation and insulin receptor and GLUT-4 content in skeletal muscle suggest that ACH09 may reduce insulin resistance by inducing the activation of insulin-signalling pathways in peripheral tissues. These actions may be due to an antioxidant effect of ACH09. Thus, ACH09 administration and may offer a promising natural and safe new approach for the treatment and prevention of type 2 diabetes.

Declarations

Conflict of interest

Emerson Ferreira Queiroz and Daniele Dal Col Moreira are employees of ACHÉ Laboratórios Farmacêuticos S.A. The company has interest in the development of ACH09 for the treatment of hypertension and metabolic syndrome. Roberto Soares de Moura is the inventor of a patent (PCT/BR02/00038) that supported the development of a new patent application (PI0605693 A2-8). The other authors have no conflicts of interest.

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