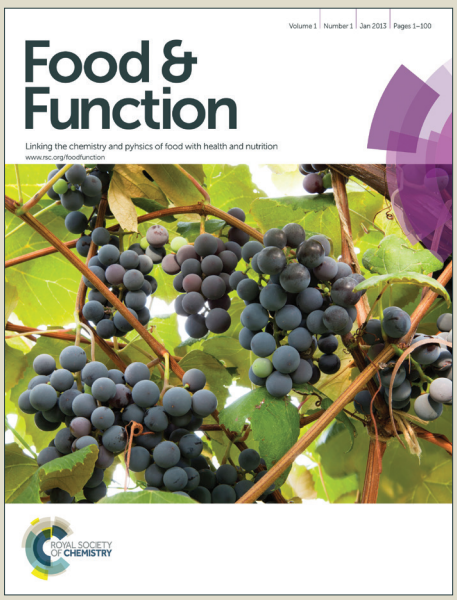
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Pterostilbene improves glycaemic control in rats fed an obesogenic diet: involvement of skeletal muscle and liver

S. Gómez-Zorita[1,2], A. Fernández-Quintela[1,2], L. Aguirre[1,2], M.T. Macarulla[1,2], A.M. Rimando[3], M.P. Portillo[1,2]

[1]Nutrition and Obesity Group. Department of Nutrition and Food Science, University of the Basque Country (UPV/EHU), Vitoria, Spain.

[2]CIBERobn Physiopathology of Obesity and Nutrition, Institute of Health Carlos III (ISCIII), Spain

[3]United States Department of Agriculture, Agricultural Research Service Natural Products Utilization Research Unit, University, MS, USA

Corresponding author/Request for reprints

Prof. María P. Portillo, Department of Nutrition and Food Science, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad, 7, 01006 Vitoria (Spain). Telephone number: +34-945-013067/ Fax number: +34-945-013014

ABSTRACT

This study aimed to determine whether pterostilbene improved glycaemic control in rats showing insulin resistance induced by an obesogenic diet. Rats were divided into 3 groups: control group and two groups treated with either 15 mg/kg/d (PT15) or 30 mg/kg/d of pterostilbene (PT30). HOMA-IR was decreased in both pterostilbene-treated groups, but this reduction was greater in PT15 group (-45% and -22% respectively *vs* control group). The improvement of glycaemic control was not due to a delipidating effect of pterostilbene on skeletal muscle. By contrast, GLUT4 protein expression was increased (+58% and +52% *vs* control group), suggesting an improved glucose uptake. The phosphorylated-Akt/total Akt ratio was significantly enhanced in PT30 group (+25%), and therefore a more efficient translocation of GLUT4 is likely. Additionally, in this group the amount of cardiotrophin-1 was significantly increased (+65%). These data suggest that likely the effect of pterostilbene on Akt is mediated by this cytokine. In liver, glucokinase activity was significantly increased only in PT15 group (+34%), and no changes were observed in glucose-6-phosphatase activity. The beneficial effect of pterostilbene on glycaemic control was more evident with the lower dose, probably because in PT15 group both muscle and liver were contributing to this effect, but in PT30 group only skeletal muscle was responsible. In conclusion, pterostilbene improves glycaemic control in rats showing insulin resistance induced by an obesogenic diet. An increase in hepatic glucokinase activity, as well as in skeletal muscle glucose uptake, seems to be involved in the anti-diabetic effect of this phenolic compound.

Key words: Pterostilbene, insulin resistance, skeletal muscle, liver, rat

INTRODUCTION

Insulin resistance and type 2 diabetes mellitus are two common metabolic conditions, characterized by impaired glycaemic control.[1] They are frequently co-morbilities of a primary disease, obesity, which is increasingly prevalent in our society.[2] Scientific research is interested in useful new biomolecules, such as dietary functional ingredients, in the fight against glycaemic control alterations. In this context, phenolic compounds make up one of the group of molecules that have been most frequently studied in recent years. [3-5]

Resveratrol, a polyphenol in the stilbene group, has been reported to induce beneficial effects in type 1 diabetes,[6-8] type 2 diabetes [9,10] and insulin resistance associated to high-fat feeding.[11,12] Due to its low bioavailability as a consequence of its rapid metabolism in gut and liver,[13,14] the scientific community is looking for other resveratrol-related molecules whose bioavailability is greater. This is the case of pterostilbene, a dimethylether derivative. The substitution of hydroxy with methoxy groups increases the transport of the molecule into cells and reduces its metabolization in gut and liver.[15]

Pterostilbene is part of a plant's defence system and is synthesized, like resveratrol, in response to pathogen infection and to excessive ultraviolet exposure. [16] Its diverse benefits for the prevention and treatment of wide variety of diseases, including cancer,[17-20] dyslipidemia[21,22] and cognitive function degeneration[20,23] have been reported. However, the potential effects of pterostilbene on glucose homeostasis have not been studied in depth to date.

In this scenario, the aim of the present study was to determine whether pterostilbene improves glycaemic control in rats showing insulin resistance induced by feeding an

obesogenic diet. The analysis of several potential mechanisms of action underlying this effect was also undertaken.

MATERIAL AND METHODS

Animals, diets and experimental design

The experiment was conducted with twenty-seven male Wistar rats with an initial body weight of 180 ± 2 grams purchased from Harlan Ibérica (Barcelona, Spain), and took place in accordance with the University of the Basque Country's guide for the care and use of laboratory animals (Reference protocol approval CUEID CEBA/30/2010). The rats were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2ºC) with a 12 h light-dark cycle (light off at 9:00 a.m.). After a 6-day adaptation period, rats were randomly divided in 3 experimental groups of nine animal each, and fed on a commercial obesogenic diet, high in sucrose (20.0%) and fat (22.5%) (Harlan Iberica, TD.06415). Pterostilbene (99.9 % purity) was synthesized according to published procedures.[23] This latter was added to the diet in amounts that assured doses of 15 mg/kg body weight/d (PT15 group) or 30 mg/kg body weight/d (PT30 group). All animals had free access to food and water. Food intake and body weight were measured daily.

At the end of the experimental period (6 weeks) animals were sacrificed, after a 12-hour overnight fasting period, by cardiac exsanguination under anaesthesia (chloral hydrate). Liver and skeletal gastrocnemius muscles were dissected. Serum was obtained from blood samples after centrifugation (1000*g* for 10 min at 4ºC). All samples were stored at -80ºC until analysis.

Serum analysis

Serum glucose, insulin, non-esterified fatty acids (NEFAs) and cardiotrophin-1 were measured by using commercial kits (BioSystems, Barcelona, Spain; EZRMI-13K, Linco, St. Charles, MO, USA; Roche Diagnostics GmbH, Mannheim, Germany and Uscn Life Science Inc, Huei, PRC, respectively).

The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated from basal insulin and glucose values using Matthews' formula: [24]

HOMA-IR = [Fasting glucose (mmol/L) × fasting insulin (mU/L)]/22.5

Glucose Tolerance Test

The week previous to sacrifice, the rats had been deprived of food, but not of water, 12 hours before the start of the glucose tolerance test. A glucose load at the dose of 2 g/kg body weight was injected intraperitoneally. Blood glucose was determined at 0, 30, 60, 90 and 120 minutes from the tail vein using a glucometer (MediSense, Abingdon, UK) and blood glucose test strips (Optium Xceed, Abbott Diabetes Care). The Area Under the Curve (AUC) was calculated by the trapezoidal rule approach.[25]

Triacylglycerol and cardiotrophin-1 content in skeletal muscle

Total lipids were extracted from muscle samples according to the method described by Folch *et al*. (1957).[26] Lipid extract was dissolved in isopropanol and triacylglycerol content was measured using a commercial kit (Spinreact, Sant Esteve de Bas, Spain). Cardiotrophin-1 amount was determined by enzyme-linked immunosorbent assay (ELISA) (Uscn Life Science Inc, Huei, PRC) in the extract obtained for western-blot analysis described below in this section.

Activities of carnitine palmitoyltransferase-1b and citrate synthase in skeletal muscle

The activity of carnitine palmitoyltransferase-1b (CPT-1b) was assessed in the mitochondrial/peroxisomal fraction. Tissue samples (1 g) were homogenized in 3 mL of buffer pH 7.4 containing 0.25 mol/L sucrose, 1 mmol/L EDTA and 10 mmol/L Tris-HCl. Homogenates were centrifuged (700 *g* for 10 min at 4ºC) and the supernatant fluid was again centrifuged (12,000 *g* for 15 min at 4ºC). Pellets were resuspended in 70 mmol/L sucrose, 220 mmol/L mannitol, 1 mmol/L EDTA, 2 mmol/L HEPES buffer, pH 7.4. CPT-1b activity was assayed by using Bieber method.[27] The pellet protein content was determined by the Bradford method.[28] CPT-1b activity was expressed as nmol CoA formed per minute, per mg protein.

Citrate synthase (CS) activity was determined spectrophotometrically following the Srere method,[29] by measuring the appearance of free CoA. Briefly, muscle samples were homogenized in 10 volumes of 0.1 M Tris-HCl buffer (pH 8.0), and diluted by a factor of 200 in this buffer. Homogenates were incubated for 5 minutes at 30°C with 0.1 M Tris-HCl buffer containing 0.1 mM DTNB, 0.25% Triton X-100, 0.5 mM oxalacetate and 0.31 mM acetyl CoA, and readings were taken at 412 nm. CS activity was expressed as nmol CoA formed per minute, per mg of protein.

Activities of glucose-6-phosphatase and glucokinase in liver

Samples of liver (0.5 g) were homogenized in 5 mL of buffer (pH 7.6) containing 150 mM KCl, 1 mM $MgCl_2$, 10 mM *N*-acetyl-cysteine and 0.5 mM dithiothreitol for glucose metabolism enzyme analysis. After centrifugation at 100,000 *g* for 40 min at 4ºC, the supernatant fraction was used for quantification of enzyme activities.

For glucose-6-phosphatase (G6Pase) the samples were resuspended in a buffer with 0.25 mM $CaCl_2$, 10 mM glucose 6 phosphate, 100 mM Tris HCl, 1.25 mM EDTA, [30]

and the activity of the enzyme was measured by phosphorus production using a commercial kit (Spinreact, Sant Esteve de Bas, Spain) and expressed as μmol of liberated inorganic phosphate per minute per mg of protein. Glucokinase (GK) was measured spectrophotometrically at 340 nm following the method described by Newgard *et al*., (1983)[31] and expressed as nmol NADH per minute, per mg of protein. Both enzyme assays were conducted at 37ºC. Protein concentration was determined by the Bradford method.[28]

Extraction and analysis of RNA and semiquantification by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 100 mg of muscle using Trizol (Invitrogen, Carlsbad, Ca, USA), according to the manufacturer's instructions. RNA samples were then treated with DNA-free kit (Applied Biosystems, Austin, TX, USA) to remove any contamination with genomic DNA. The yield and quality of the RNA were assessed by measuring absorbance at 260, 270, 280 and 310 nm. 1.5 μg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Hormone sensitive lipase (HSL), adipose tissue triglyceride lipase (ATGL), cluster of differentiation 36 (CD36), cytochrome c oxidase subunit II (COXII), mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor β/δ (PPARβ/δ) were quantified, as was 18S, which served as the reference gene.

Real Time PCR Detection System (BioRad, USA) and SYBR Green probes (Applied Biosystems) were used for all the genes with the exception of TFAM. In this case TaqMan probes were used. Specific sequence of sense/antisense primers and the probes are given in Table 1. For HSL, ATGL, CD36, COXII and PPARβ/δ, 0.1 μL of

each cDNA were added to the PCR reagent mixture (SYBR Green Master Mix) with the sense and antisense primers (300 nM for each gene except 900 nM for PPARβ/δ).

The PCR parameters were as follows: start at 50ºC for 2 min, denaturation at 95ºC for 10 min, denaturation at 95ºC for 15s (45 cycles for ATGL and 40 cycles for the other genes), annealing and extension at 60ºC for 30s respectively, except for PPARβ/δ which required a temperature of 58.2ºC.

In the case of TaqMan gene expression assay, 1 µL of each cDNA was added to PCR reagent mixture, Premix Ex Taq (Takara, USA), with sense and antisense primers (300 nM) and the probe (1 µM).

Gene expression analysis was performed using the comparative threshold cycle (Ct) method. Amplification of 18S sequence was performed in parallel and was used to normalize values obtained for target genes. The results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method.[32]

GLUT4 and Akt western blot analysis

For this purpose 100 mg of gastrocnemius muscle were homogenized in 750 µL of cellular PBS (pH 7.4), containing nuclease inhibitors, 100 mM phenylmethylsulfonyl fluoride and 100 mM iodoacetamide. Homogenates were centrifuged at 500 *g* for 10 min at 4ºC. Protein concentrations in homogenates were measured following the Bradford method[28] using bovine serum albumin as standard.

Immunoblot analyses were performed using 15 µg and 30 µg of gastrocnemius muscle extracts for GLUT4 and protein kinase B (Akt) respectively, separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were then blocked with 5% caseine PBS-Tween buffer for 2 hours at room temperature. Subsequently, they were blotted with the appropriate antibodies

overnight at 4ºC. GLUT4 levels were detected via specific antibodies (1:10000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:5000) (Sigma, St. Louis, MO, USA) and Akt (1:1000) by calculating the phosphorylated Akt to total Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA) ratio. Afterwards, polyclonal anti-mouse (for β-actin and Akt) and anti-goat antibodies (for GLUT4) (1:5000) (Sigma, St. Louis, MO, USA) were incubated for 2 hours at room temperature. The bound antibodies were visualized using a chemiluminescent substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and quantified by a ChemiDoc MP imaging system (BioRad, Hercules, CA, USA). β-actin was used as a loading control to normalize the results.

Statistical analysis

Results are presented as means ± standard error of the means. Statistical analysis was performed using SPSS 17.0 (SPSS Inc. Chicago, Illinois, USA). All the parameters are normally-distributed according to the Shapiro-Wilks test. Then data were analyzed by using one-way ANOVA followed by Newman Keuls *post hoc* test. Statistical significance was set-up at the $P < 0.05$ level.

RESULTS

Energy intake and food efficiency

No significant changes were observed among groups in energy intake (kcal/d): 82.4 ± 2.8 (Control group); 81.5 ± 2.2 (PT15 group) and 81.1 ± 1.4 (PT30 group). We previously reported that body weight was not modified by pterostilbene in this cohort of rats. [33] Thus, food efficiency (g body weight gain/100 kcal) remained unchanged (3.0 ± 0.2; 3.1 ± 0.1 and 3.0 ± 0.1 for Control, PT15 and PT30 groups respectively).

Serum parameters and glucose tolerance test

Rats from PT15 group showed significantly reduced values of basal glucose and insulin. In the case of rats from PT30 group, only glucose was significantly reduced. Consequently, although HOMA-IR was decreased in both pterostilbene-treated groups, this reduction was significant only in PT15 group. No significant changes were observed in cardiotrophin-1 and NEFAs concentrations (Table 2).

In the same way, when glucose tolerance test data were analyzed, glycaemic values and the AUC for each experimental time were significantly lower in PT15 group when compared with the control, and a tendency towards reduced values was observed in PT30 group (Figures 1A and 1B).

Triacylglycerol content, cardiotrophin-1 content and enzyme activities in skeletal muscle

When triacylglycerol content was analyzed no significant differences were observed among the three experimental groups (8.0±0.7; 7.7±0.5 and 9.8±1.2 mg/g tissue for control, PT15 and PT30 groups respectively). In spite of this, the activities of CPT-1b and CS were increased in both pterostilbene-treated groups (Figures 2A and 2B).

Cardiotrophin-1 content was significantly increased in PT30 group, but not in PT15 group ($P<0.05$) (Figure 3).

Hepatic glucose metabolism enzyme activities

No significant changes were observed in the activity of G6Pase in the groups treated with pterostilbene (Figure 4A). Regarding GK, a significant increase was observed in PT15 group, but not in PT30 group ($P<0.05$) (Figure 4).

Gene and protein expressions in skeletal muscle

No significant changes were observed in the expression of both lipases, ATGL and HSL, among the three experimental groups. With regard to TFAM and COXII, two genes related to mitochondriogenesis, an increase in their expression was observed in pterostilbene treated rats, although in the case of TFAM gene the PT15 group showed only a slight tendency (P<0.1). PPARβ/δ, a transcriptional factor controlling mitochondriogenesis and fatty acid oxidation, was not modified by pterostilbene treatment. Finally, CD36, a fatty acid transporter, was significantly up-regulated in both pterostilbene-treated groups (Table 3).

As far as protein expression of the glucose transporter GLUT4 is concerned, rats from both pterostilbene-treated groups showed significantly higher values than those from the control group. No significant differences between the two groups treated with pterostilbene were observed (Figure 5). In PT30 group, but not in PT15 group, the phosphorylated-Akt/total Akt ratio was significantly increased (P<0.05) (Figure 6).

DISCUSSION

As indicated in the Introduction, the scientific community is looking for functional biomolecules showing beneficial effects on health. As far as insulin resistance and diabetes are concerned, resveratrol, a polyphenol belonging to the group of stilbenes, has emerged as a useful compound. Nevertheless, due to its low bioavailability, the search of alternative molecules is a matter of interest. In this context, in the present study, the effects of pterostilbene, a resveratrol-derivative which is metabolized to a lesser extent, [15] have been assessed.

Several authors have analyzed the effects of this phenolic compound in rats showing type 2 diabetes induced by streptozotocin-nicotinamide treatment,[34,35] but there are no

data concerning the effects of this molecule on insulin resistance associated with obesogenic feeding.

It is well known that a high-fat, high-sucrose diet (obesogenic diet), as that we used in the present study, leads not only to increased body fat accumulation, but also to insulin resistance.[12] Basal glucose data in the present study show that, indeed, this alteration in glucose homeostasis was induced by the obesogenic diet. [36] In the present study, pterostilbene, at a dose of 15 mg/kg/d, partially prevented insulin resistance, as shown by both HOMA-IR values and the glucose tolerance test. Indeed, data concerning these two parameters were significantly lower than those found in non-treated rats fed the obesogenic diet. In the case of the higher dose (30 mg/kg/d), although these changes were weaker than those induced by the lower one, glycaemic control was somewhat ameliorated, as shown by the significant reduction in serum glucose levels and the glucose tolerance test.

Although in the present study an experimental group fed a standard diet was not included, taking as a reference data from rats fed a chow diet in previous studies carried out in our laboratory (serum glucose: 75.4 mg/dL; insulin: 0.5 ng/mL), we can state that the improvement in glycaemic control induced by pterostilbene was partial in the obesogenic diet fed animals.

If we compare the present results with those concerning resveratrol effects previously reported by our group, it can be observed that resveratrol, at a dose of 15 mg/kg bw/day, is less efficient in lowering serum glucose levels than pterostilbene [37]. However, when both phenolic components are supplemented at 30 mg/kg bw/day, resveratrol induced a larger reduction in serum glucose levels. [38]

Skeletal muscle and liver play important roles in glycaemic control. Consequently, several aspects of their metabolism were assessed in the present study. Under overfeeding conditions, which lead to increased body fat accumulation, there is an increased fatty acid influx from adipose tissue to skeletal muscle. This causes increased intramyocellular triacylglycerol levels, which results in insulin resistance by perturbing the insulin signalling pathway.[39-42]

As a consequence, the amount of intramyocellular lipids in physical inactive individuals can be used as a marker of insulin resistance.[43] In the present study no significant differences were observed in gastrocnemius muscle triacylglycerol content among experimental groups, suggesting that the improvement in insulin resistance induced by pterostilbene was not due to the reduction in skeletal muscle lipotoxicity.

Taking into account that resveratrol, a stilbene showing a very similar chemical structure to that of pterostilbene, increases fatty acid oxidation in muscle due to increased number of mitochondria,[44-46] we analyzed the effects of pterostilbene on the activity of CPT-1b and CS. The activity of CPT-1b, a rate-limiting enzyme in mitochondrial fatty acid oxidation, which allows long-chain fatty acids to enter into the mitochondria, was significantly increased in PT15 group, but not in PT30 group. Moreover, CS activity, a marker of mitochondria density, was significantly increased by pterostilbene in both treated groups.

We also measured gene expression of TFAM, a marker of mitochondriogenesis and COXII, a mitochondrion-encoded protein which is a critical component of the oxidative phosphorylation pathway. Both genes were up-regulated in pterostilbene-treated groups, although in the case of TFAM gene this was only a tendency in the PT15 group. Taken

together, these results suggest that pterostilbene increased fatty acid oxidation in gastrocnemius muscle, in all likelihood due to increased mitochondriogenesis.

Gene expression of PPARβ/δ, the transcriptional factor which controls mitochondria biogenesis and thus fatty acid oxidation was also measured. No differences among groups were observed. Previously, Rimando et *al.* (2005)[21] demonstrated that pterostilbene is an agonist of PPARα. Thus, mirroring this effect, the activation of PPARβ/δ by this phenolic compound cannot be discarded.

A decrease in gastrocnemius triacylglycerol content should be expected according to the results concerning fatty acid oxidation and mitochondriogenesis. However, this decrease was not observed in our study. In order to find an explanation for this apparent discordance, we analyzed the expression of CD36, a transporter which allows muscle to uptake fatty acids resulting from circulation or hydrolysis of circulating triacylglycerols. This parameter was significantly increased in both pterostilbene-treated groups, suggesting greater skeletal muscle fatty acid availability in these groups. These fatty acids were oxidized due to the greater mitochondrial oxidative capacity induced by this phenolic compound. This finally resulted in no changes in muscle triacylglycerol content between control and pterostilbene-treated rats. The maintenance of the high amount of triacylglycerols in muscle, induced by obesogenic feeding, without adverse effects on insulin sensitivity in pterostilbene-treated rats, suggests an endurance-training-like effect of pterostilbene, as previously proposed by Timmers *et al*. (2011)[10] for resveratrol in a study conducted in obese humans.

Diacylglycerols, a by-product of lipolysis which shows lipotoxic effects in skeletal muscle, are derived from triacylglycerol hydrolysis by adipose triglyceride lipase ATGL, and are further hydrolyzed by the diacylglycerol hydrolase HSL.[47] Thus, an

imbalance between both lipases is causally linked to insulin resistance.[48,49] In the present study gene expression of these two lipases was not modified by pterostilbene supplementation. These results suggest that changes in diacylglycerol content among groups would not be likely.

Skeletal muscle is the tissue which contributes to a greater extent to glucose uptake. In this tissue GLUT4 mediates insulin-dependent glucose uptake. [50,51] Reduced GLUT4 or a defect in this glucose transporter have been reported in insulin resistance and type 2 diabetes. [52] In the present study, increased GLUT4 protein expression in skeletal muscle from pterostilbene-treated rats suggests enhanced muscle insulin-stimulated glucose uptake in these animals, an effect that may well have contributed to the prevention of insulin resistance induced by obesogenic feeding.

In insulin cascade signalling Akt is a protein required for the insulin-induced translocation of GLUT4 transporter to the plasma membrane, which is activated by phosphorylation. [53] Moreover, cardiotrophin-1, a member of the gp130 family of cytokines expressed in muscle, heart, liver and white adipose tissue, is a key regulator of glucose homeostasis. This molecule activates Akt and enhances insulin-induced Akt activation. [54,55] In the present study the ratio phosphorylated-Akt/total Akt was significantly increased in PT30 group, and consequently it can be supposed that in this group GLUT4 was more efficiently translocated. Additionally, in this group the amount of cardiotrophin-1 was significantly increased. These data suggest that likely the effect of pterostilbene on Akt is mediated by this cytokine.

Insulin resistance and diabetes also lead to alterations in hepatic enzymes of glucose metabolism, such as GK and G6Pase.[56-58] GK regulates glucose uptake and glycogen synthesis and suppresses glucose production. Very often, this enzyme is inhibited when

glycaemic control is impaired.[59] In the present study the administration of pterostilbene to rats resulted in a significant increase in the activity of this enzyme in PT15 group, but not in PT30 group. It should be remembered that only the low dose of pterostilbene reduced insulin resistance. Thus, it may be suggested that increased glucose utilization induced by this phenolic compound is on the basis of its anti-diabetic effect. G6Pase, the enzyme which dephosphorylates glucose phosphate resulting from glycogenolysis and gluconeogenesis, is also crucial in glucose homeostasis.[60] The activity of this enzyme is increased in diabetes.[61-63] The present results show no changes in rats treated with pterostilbene, indicating that this enzyme was not involved in the beneficial effect of pterostilbene on glycaemic control. These results partially agree with those reported by Pari *et al.* (2006) [35] in streptozotozine-nicotinamide-treated rats, orally administered with 40 mg/kg/d of pterostilbene. The discrepancy with this study can be attributed to the fact that rats in Pari's study showed type 2 diabetes, and rats in the present study were insulin resistant.

In the present study the beneficial effect of pterostilbene on glycaemic control was more evident with the lower dose (15 mg/kg/d). A possible explanation could be that while in PT15 group both muscle and liver were contributing to this effect, in PT30 group only skeletal muscle was responsible.

Although this could be surprising, there are data in the literature that show dose-response patterns in the same line by using related stilbenes, such as resveratrol. Thus, Cho *et al*. (2012) [63] showed that a low dose of resveratrol (0.005 % in the diet) was more effective than a higher dose (0.02 % in the diet) in suppressing adiposity. Similarly, in a previous study from our group carried out in genetically obese rats, resveratrol at a dose of 15 mg/kg/d, but not at 30 mg/kg/d, reduced non HDL-

cholesterol and serum transaminases. Moreover, liver triacylglycerol infiltration, measured by histology, was more greatly reduced by the low dose.[64]

In conclusion, the present results show that pterostilbene improves glycaemic control in rats showing insulin resistance induced by an obesogenic diet. An increase in hepatic glucokinase activity, as well as in skeletal muscle glucose uptake, seems to be involved in the anti-diabetic effect of this phenolic compound.

Acknowledgements

This study was supported by grants from the Ministerio de Economía y Competitividad (AGL2011-27406-ALI), Instituto de Salud Carlos III (CIBERobn), Government of the Basque Country (IT-512-13) and University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32). S. Gómez-Zorita is a recipient of a doctoral fellowship from the University of the Basque Country (UPV/EHU)

Figure 1. Glycaemic response (A) and Area Under the Curve (AUC) (B), in the glucose tolerance test performed in rats from control and pterostilbene-treated groups (n=9). Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test. Values not sharing a common letter are significantly different

Figure 2. Carnitine palmitoyltransferase-1b (A) and citrate synthase (B) activities in skeletal muscle from control and pterostilbene-treated groups (n=9). Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test. Values not sharing a common letter are significantly different

Figure 3. Cardiotrophin-1 content in skeletal muscle from control and pterostilbene-treated groups (n=9). Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test. Values not sharing a common letter are significantly different.

Figure 4. Glucose-6-phosphatase and glucokinase activities in liver from control and pterostilbene-treated groups (n=9). Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test. Values not sharing a common letter are significantly different

Figure 5. GLUT4 protein expession in skeletal muscle from control and pterostilbene-treated groups (n=9). Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test.

Values not sharing a common letter are significantly different.

Figure 6. Phosphorylated Akt/total Akt ratio in skeletal muscle from control and pterostilbene-treated groups (n=9). Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test. Values not sharing a common letter are significantly different.

References

1. American diabetes association, *Diabetes Care*, 2013, **36**, S67-S74
2. A. Rodriguez, V. Catalan, J. Gomez-Ambrosi and G. Fruhbeck, *Curr. Pharm. Des*., 2007, **13**, 2169-2175.
3. A. Crozier, I. B. Jaganath and M. N. Clifford. *Nat. Prod. Rep.*, 2009, **26**, 1001-1043.
4. K. Hanhineva, R. Törrönen, I. Bondia-Pons, J. Pekkinen, M. Kolehmainen, H. Mykkänen and K. Poutanen, *Int. J. Mol. Sci.*, 2010, **11**, 1365-1402.
5. D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges and A. Crozier, *Antioxid Redox Signal*, 2013, **18**, 1818-1892.
6. S. M. Lee, H. Yang, D. M. Tartar, B. Gao, X. Luo, S. Q. Ye, H. Zaghouani and D. Fang, *Diabetologia*, 2011; **54**, 1136-1146.
7. F. Akar, M. B. Pektas, C. Tufan, S. Soylemez, A. Sepici, A. T. Ulus, B. Gokalp, K. Ozturk and H. S. Surucu, *Cardiovasc. Drugs Ther*., 2011, **25**, 119-131.
8. C. R. Ku, H. J. Lee, S. K. Kim, E. Y. Lee, M. K. Lee and E. J. Lee, *Endocr. J*., 2012, **59**, 103-109.
9. K. P. Goh, H. Y. Lee, D. P. Lau, W. Supaat, Y. H. Chan and A. F. Y. Koh, *Int. J. Sport Nutr. Exerc. Metab*., 2013, 25, 119-131.
10. S. Timmers, M. K. Hesselink and P. Schrauwen, *Ann. N. Y. Acad. Sci*., 2013, **1290**, 83-89.
11. Y. Jimenez-Gomez, J. A. Mattison, K. J. Pearson, A. Martin-Montalvo, H. H. Palacios, A. M. Sossong, T. M. Ward, C. M. Younts, K. Lewis, J. S. Allard, D.

L. Longo, J. P. Belman, M. M. Malagon, P. Navas, M. Sanghvi, R. Moaddel, E. M. Tilmont, R. L. Herbert, C. H. Morrell, J. M. Egan, J.A. Baur, L. Ferrucci, J. S. Bogan, M. Bernier and R. de Cabo, *Cell Metab*., 2013, **18**, 533-545.

12. B. D. Hegarty, S. M. Furler, J. Ye, G. J. Cooney and E. W. Kraegen, *Acta Physiol. Scand*., 2003, **178**, 373-383.

13. T. Walle, F. Hsieh, M. H. DeLegge, J. E. Oatis and U. K. Walle, *Drug Metab. Dispos.*, 2004, **32**, 1377-1382.

14. E. Wenzel and V. Somoza, *Mol. Nutr. Food Res*., 2005, **49**, 472-481.

15. I. M. Kapetanovic, M. Muzzio, Z. Huang, T. N. Thompson and D. L. McCormick, *Cancer Chemoth. Pharm*., 2011, **68**, 593-601.

16. L. Bavaresco, C. Fregoni, E. Cantù and M. Trevisan, *Drugs Exp. Clin. Res.,* 1999, **25**, 57-63.

17. A. M. Rimando and N. Suh, *Planta. Med*., 2008, **74**, 1635-1643.

18. M. H. Pan, Y. S. Chiou, W. J. Chen, J. M. Wang, V. Badmaev and C. T. Ho. *Carcinogenesis*, 2009, **30**, 1234-1242.

19. S. Paul, A. J. DeCastro, H. J. Lee, A. K. Smolarek, J. Y. So, B. Simi, C. X. Wang, R. Zhou R, A.M. Rimando and N. Suh, *Carcinogenesis*, 2010, **31**, 1272-1278.

20. D. McCormack and D. McFadden, *Oxid. Med. Cell Long*., 2013, DOI: 10.1155/2013/575482.

21. A. M. Rimando, R. Nagmani, D. R. Feller and W. Yokoyama, *J. Agric. Food Chem.*, 2005, **53**, 3403-3407.

22. M. A. Satheesh and L. Pari, *J. Appl. Biomed.*, 2008, **6**, 31-37

23. J. A. Joseph, D. R. Fisher, V. Cheng, A. M. Rimando and B. Shukitt-Hale, *J. Agric. Food Chem.,* 2008, **56**, 10544-10551.

24. D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher and R. C. Turner, *Diabetologia*, 1985, **28**, 412-419

25. M. M. Tai, *Diabetes Care*, 1994, **17**, 152-154.

26. J. Folch, M. Lees and G. H. Sloane Stanley, *J. Biol. Chem*., 1957, **226**, 497-509.

27. L. L. Bieber, T. Abraham and T. Helmrath, *Anal. Biochem*., 1972, **50**, 509-518.

28. M. M. Bradford, *Anal. Biochem.,* 1976, **72**, 248-254.

29. P. A. Srere, *Methods Enzymol*., 1969, **13**, 3-5.

30. M. A. Zulet, A. Barber, H. Garcin, P. Higueret and J. A. Martinez, *J. Am. Coll. Nutr.*, 1999, **18**, 36-42.

31. C. B. Newgard, L. J. Hirsch, D. W. Foster, J. D. McGarry, *J. Biol. Chem.,* 1983, **258**, 8046-8052.

32. K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402-408.

33. S. Gómez-Zorita, A. Fernández-Quintela, A. Lasa, L. Aguirre, A. M. Rimando, M. P. Portillo, *J. Agric. Food Chem*., 2014, **62**, 8371-8378.

34. M. A. Satheesh and L. Pari, *J. Phar. Pharmacol*., 2006, **58**, 1483-1490.

35. L. Pari and M. A. Satheesh, *Life Sci*., 2006, **79**, 641-645.

36. Urzúa Z, 2011. *Doctoral Thesis,* Universidad Colima, Mexico.

37. N. Arias, J. Miranda, M. T. Macarulla, L. Aguirre, A. Fernández-Quintela, C. Andres-Lacueva, M. Urpi-Sarda, and M. P. Portillo, *Eur. J. Nutr.*, 2014, 53, 575-582.

38. N. Arias, M. T. Macarulla, L. Aguirre, M. G. Martínez-Castaño, S. Gómez-Zorita, J. Miranda, A. Martínez and M. P. Portillo, *J. Physiol. Biochem.*, 2011, **67**, 471-477.

39. K. Eckardt, A. Taube and J. Eckel, *Rev. Endocr. Metab. Disord*., 2011, **12**, 163-172.

40. J. S. Lee, S. K. Pinnamaneni, J. J. Eo, I. H. Cho, J. H. Pyo, C. K. Kim, A. J. Sinclair, M. A. Febbraio and M. J. Watt, *J. Appl. Physiol*., 2006, **100**, 1467-1474.

41. D. A. Pan, S. Lillioja, A. D. Kriketos, M. R. Milner, L. A. Baur, C. Bogardus, A. B. Jenkins and L. H. Storlien, *Diabetes*, 1997, **46**, 983-988

42. A. S. Greenberg, R. A. Coleman, F. B. Kraemer, J. L. McManaman, M. S. Obin, V. Puri, Q. W. Yan, H. Miyoshi and D. G. Mashek, *J. Clin. Invest*., 2011, **121**, 2102-2110.

43. L. Liu, Y. Zhang, N. Chen, X. Shi, B. Tsang and Y. Yu, *J. Clin. Invest*., 2007, **117**, 1679-1689.

44. M. Lagouge, C. Argmann, Z. Gerhart-Hines, H. Meziane, C. Lerin, F. Daussin, N. Messadeq, J. Milne, P. Lambert P, P. Elliott, B. Geny, M. Laakso, P. Puigserver and J. Auwerx, *Cell*, 2006, **127**, 1109-1122.

45. L. L. Chen, H. H. Zhang, J. Zheng, X. Hu, W. Kong, D. Hu, S. X. Wang and P. Zhang, *Metabolism*, 2011, **60**, 1598-1609.

46. G. Alberdi, V. M. Rodríguez, J. Miranda, M. T. Macarulla, I. Churruca and M. P. Portillo, *Food Chem*., 2013, **141**, 1530-1535.

47. R. Zechner, R. Zimmermann, T.O. Eichmann, S. D. Kohlwein, G. Haemmerle, A. Lass and F. Madeo, *Cell Metab*., 2012, **15**, 279-291.

48. P. M. Badin, K. Louche, A. Mairal, G. Liebisch, G. Schmitz, A. C. Rustan, S. R. Smith , D. Langin and C. Moro, *Diabetes*, 2011, **60**, 1734-1742.

49. P. M. Badin, I. K. Vila, K. Louche, A. Mairal, M. A. Marques, V. Bourlier, G. Tavernier, D. Langin and C. Moro, *Endocrinology*, 2013, **154**, 1444-1453.

50. A. Zorzano, L. Sevilla, E. Tomas, M. Camps, A. Guma and M. Palacin, *Int. J. Mol. Med*., 1998, **2**, 263-271.

51. A. Perez-Martin, E. Raynaud and J. Mercier, *Obes. Rev*., 2001, **2**, 47-59.

52. W. T. Garvey, L. Maianu, J. H. Zhu, G. Brechtel-Hook, P. Wallace and A. D. Baron, *J. Clin. Invest.*, 1998, **101**, 2377-2386.

53. E. M. Van dam, R. Govers and D. E. James, *Mol. Endocrinol.,* 2005, **19**, 1067-1077.

54. M. J. Moreno-Aliaga, N. Pérez-Echarri, B. Marcos-Gómez, E. Larequi, F.J. Gil-Bea, B. Viollet, I. Giménez, J. A. Martínez, J. Prieto and M. Bustos, *Cell Metab.*, 2011, **14**, 242-253.

55. M. J. Moreno-Aliaga, M. A. Romero-Lozano, D. Castaño, J. Prieto and M. Bustos, *Adipocyte*, 2012, **1**, 112-115.

56. M. Latha and L. Pari, *Clin. Exp. Pharmacol. Physiol*., 2003, **30**, 38-43.

57. L. Pari and M. A. Satheesh, *J. Ethnopharmacol*., 2004, **91**, 109-113.

58. T. K. Bera, K. M. Ali, K. Jana, A. Ghosh and D. Ghosh, *Pharmacognosy Res*., 2013, **5**, 277-285.

59. J. M. Gancedo and C. Gancedo, *Arch. Microbiol.,* 1971, **76**, 132-138.

60. G. Mithievre, G. Vidal, C. Zitovn and C. Miriasian, *Diabetes*, 1996, **45**, 891-896.

61. D. Ahmed, M. Sharma, A. Mukerjee, P. W. Ramteke, V. Kumar, 2013, *BMC Complement. Altern. Med.*, 2013, **13**, 10.

62. R. S. Renjith, T. Rajamohan, *Int. J. Diab. Dev. Ctries*., 2012, **32**, 193-198.

63. S. J. Cho, U. J. Jung and M. S. Choi, *Br. J. Nutr.,* 2012, **108**, 2166-2175.

64. S. Gómez-Zorita, A. Fernández-Quintela, M. T. Macarulla, L. Aguirre, E. Hijona, L. Bujanda, F. Milagro, J. A. Martínez and M. P. Portillo, *Br. J. Nutr.* 2012, **107**, 202-210.

Table 1. Primers and probe for PCR amplification of each gene studied

	Sense primer	Antisense primer	Probe
HSL	5’-CCCATAAGACCCCATTGCCTG-3’	5’-CTGCCTCAGACACACTCCTG-3’	
ATGL	5’-CACTTTAGCTCCAAGGATGA-3'	5’-TGGTTCAGTAGGCCATTCCT-3’	
CD36	5’-GGTGTGCTCAACAGCCTTATC-3’	5’-TTATGGCAACCTTGCTTATG-3’	
COXII	5’-AACAATTCTCCCAGCTGTCATTC-3’	5’-AGTCAAAGCATAGGTCTTCATAGTC-3’	
TFAM	5’-CACGAGCCCTGGAGTACCC-3’	5’-CCACATTTCCCCGGAACAGC-3’	5’-CGACGACTATAGGCCCGGCGC-3’
PPARβ/δ	5’-GAGGGGTGCAAGGGCTTCTT-3’	5’-CACTTGTTGCGGTTCTTCTTCT-3’	
18S	5’-CATCGAGCAGGTCTGTTCCC-3’	5’-TAGATTGGCTTGACGGACTTGG-3’	5’-CGACGACTATAGGCCCGGCGC-3’

HSL: hormone sensitive lipase; ATGL: adipose triglyceride lipase; CD36: cluster of differentiation 36; COXII: cytochrome c oxidase subunit II; TFAM: mitochondrial transcription factor A; PPARβ/δ: peroxisome proliferator factor receptor β/δ.

Table 2. Serum parameters from rats fed control and pterostilbene-treated diets for 6 weeks

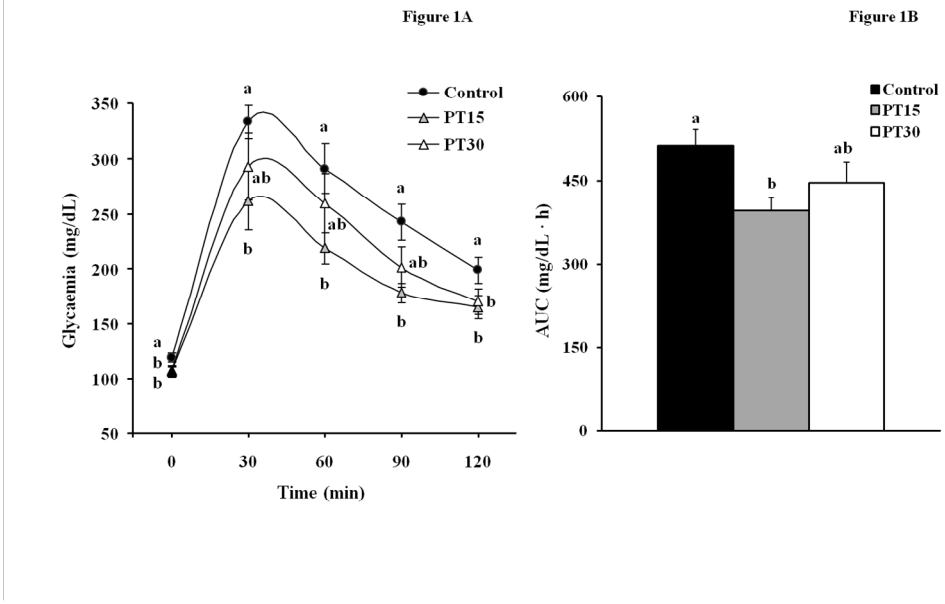
	Control	PT15	PT30	ANOVA
Glucose (mg/dL)	128 ± 2 [a]	111 ± 9 [b]	108 ± 6 [b]	$P<0.01$
Insulin (ng/mL)	3.2 ± 0.4 [a]	2.4 ± 0.1 [b]	3.0 ± 0.2 [a]	$P<0.05$
HOMA-IR	25.3 ± 3.6 [a]	14.0 ± 2.2 [b]	19.8 ± 1.5 [a]	$P<0.05$
NEFAs (ng/dL)	92.9 ± 10.5	82.1 ± 7.1	82.1 ± 3.6	NS
Cardiotrophin-1 (pg/mL)	49.6 ± 12.4	54.3 ± 5.2	57.5 ± 12.3	NS

Values are means ± SEM (n = 9). Values in the same row with different superscript are significantly different at $P<0.05$ as determined by Newman-Keuls test. PT: pterostilbene; NS: not significant; NEFAs: non-esterified fatty acids.

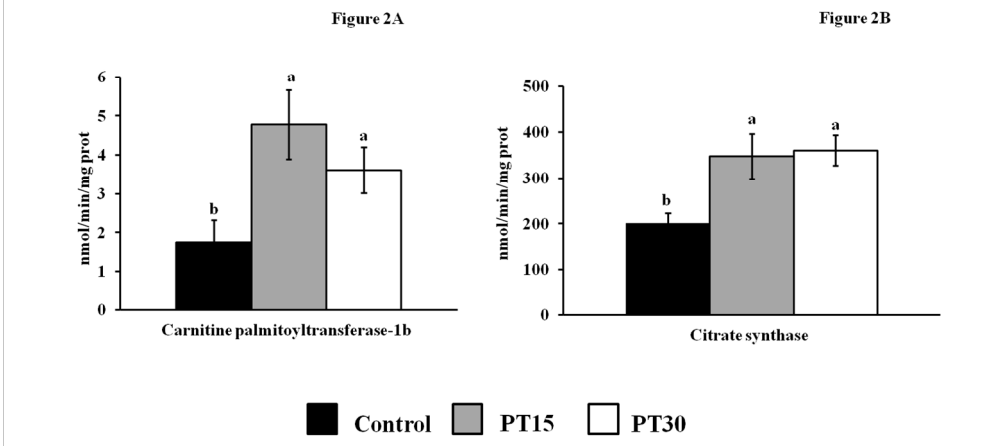
Table 3. Gene expressions in skeletal muscle from rats fed control and pterostilbene-treated diets for 6 weeks

	Control	PT15	PT30	ANOVA
HSL	1.0 ± 0.3	1.1 ± 0.1	0.9 ± 0.3	NS
ATGL	1.0 ± 0.5	1.3 ± 0.2	1.6 ± 0.6	NS
CD36	1.0 ± 0.2 [b]	9.3 ± 2.1 [a]	9.1 ± 2.4 [a]	$P<0.01$
COXII	1.0 ± 0.5 [b]	4.5 ± 0.5 [a]	7.1 ± 0.6 [a]	$P<0.01$
TFAM	1.0 ± 0.4 [b]	2.5 ± 0.8 [ab]	3.7 ± 0.8 [a]	$P<0.01$
PPARβ/δ	1.0 ± 0.5	1.1 ± 0.2	0.9 ± 0.3	NS

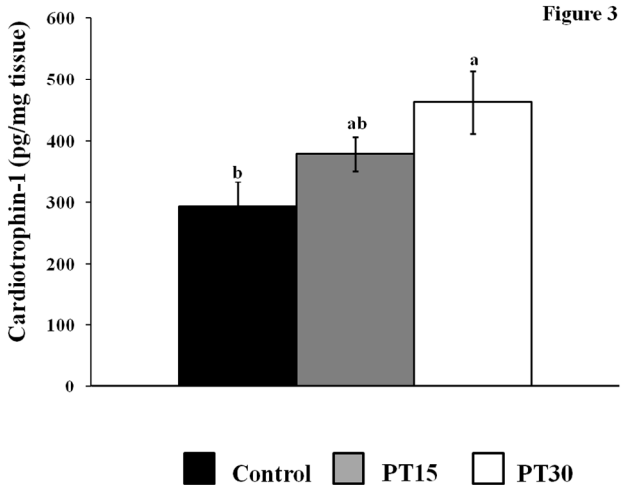
Values are means ± SEM ($n = 9$). Values in the same row with different superscript are significantly different at $P<0.05$ as determined by Newman-Keuls test. PT: pterostilbene; NS: not significant. HSL: hormone sensitive lipase; ATGL: adipose triglyceride lipase; CD36: cluster of differentiation 36; COXII: cytochrome c oxidase subunit II; TFAM: transcription factor A mitochondrial; PPARβ/δ: peroxisome proliferator-activated receptor β/δ.



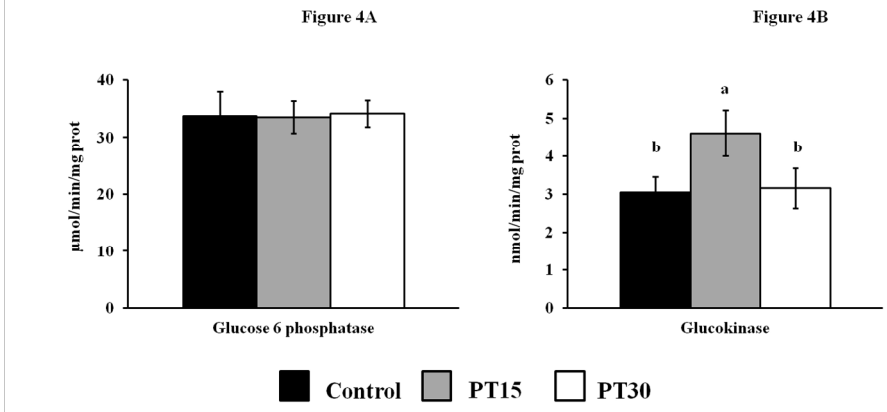
420x297mm (300 x 300 DPI)



420x297mm (300 x 300 DPI)

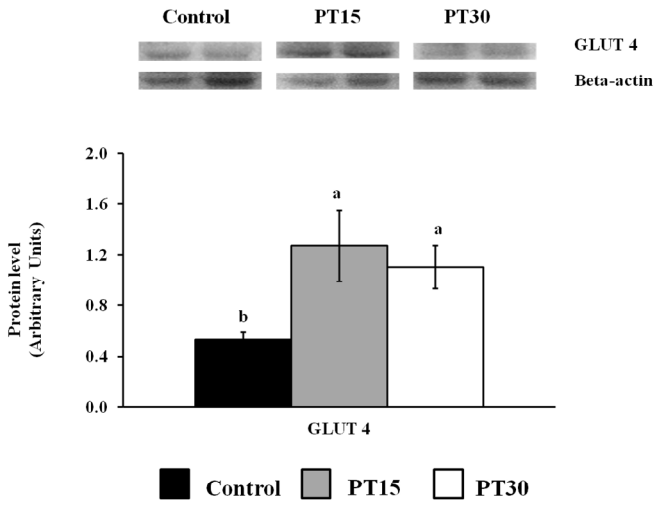


420x297mm (300 x 300 DPI)



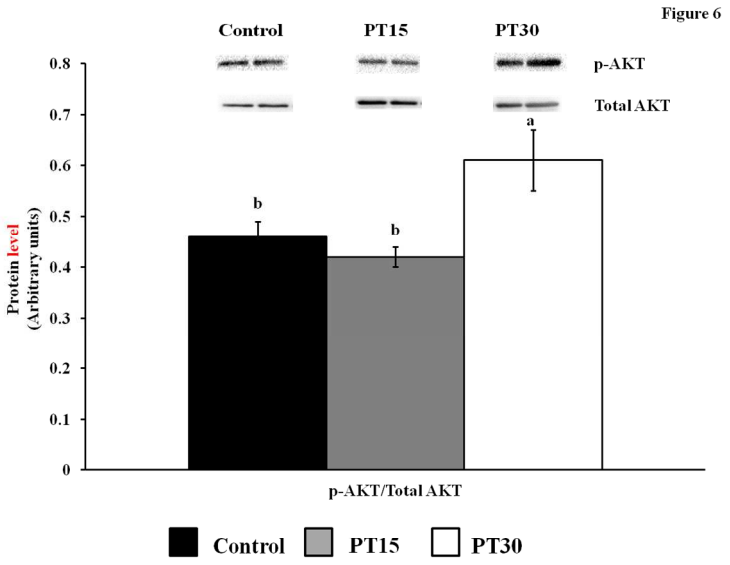
420x297mm (300 x 300 DPI)

Figure 5



420x297mm (300 x 300 DPI)

Figure 6



420x297mm (300 x 300 DPI)