JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Effects of Pomegranate Seed Oil on Glucose and Lipid Metabolism-Related Organs in Rats Fed an Obesogenic Diet

Jonatan Miranda,[†,‡] Leixuri Aguirre,[†] Alfredo Fernández-Quintela,[†,‡] M. Teresa Macarulla,[†,‡] M. Guadalupe Martínez-Castaño,[§] Josune Ayo,[∥] Elisabette Bilbao,[∥] and María P. Portillo*[,†,‡]

[†]Nutrition and Obesity Group, Department of Nutrition and Food Science, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad, 7. 01006 Vitoria, Spain

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

[§]Clinic Laboratory, University Hospital of Alava, Vitoria, Spain

[∥]Food Research Division, AZTI-Tecnalia, Astondo bidea, 609, Parque Tecnológico de Bizkaia, 48160 Derio, Spain

**ABSTRACT:** Studies conducted in mice have revealed positive effects of punicic acid (PUA). The aim of this study was to analyze the effects of PUA on fat accumulation and glycemic control in rats fed an obesogenic diet. Rats were randomly divided into two groups: control group and PUA group (diet supplemented with 0.5% PUA). No changes were observed in adipose tissue weights. The glucose tolerance test showed that the glycemic value in the PUA group had decreased significantly at the final time (120 min) (−19.3%), as had fructosamine levels (−11.1%). However, homeostasis model assessment (HOMA-IR) showed that insulin resistance did not improve. No changes were observed in the liver, skeletal muscle composition, or peroxisome proliferator-activated receptors (PPARs) activation. Low levels (mg/g tissue) of PUA (0.04 ± 0.01 in both tissues) and higher levels of *cis*-9,*trans*-11 conjugated linoleic acid (0.31 ± 0.08 in liver, 0.52 ± 0.11 in muscle) were found. PUA supplementation induced hypoplasia (−16.1%) due to the antiproliferative effect on hepatocytes. In conclusion, dietary supplementation of 0.5% PUA did not lead to decreased fat accumulation in adipose tissue, liver, or skeletal muscle, or to improved glycemic control. The hypoplasia induced in liver is a negative effect that should be considered before proposing PUA as a functional ingredient.

**KEYWORDS:** *punicic acid, liver, skeletal muscle, rat, glucose tolerance*

## INTRODUCTION

An extensive investigation carried out in recent years has demonstrated that the *trans*-10,*cis*-12 conjugated linoleic acid (CLA, 18:2) isomer has a clear body fat-lowering effect in rodents.[1−3] However, its effects on humans are controversial, and its role as an antiobesity molecule is therefore still a matter for debate.[4,5] In October 2010, the European Food Safety Authority (EFSA) provided a scientific opinion addressing the substantiation of health claims relating to CLA. On the basis of the data presented, the panel concluded that no causal relationship had been established between the consumption of CLA and attaining or maintaining normal body weight.[6] The panel has not yet provided any scientific opinion addressing the relationship between the consumption of CLA and body fat reduction.

Consequently, the scientific community is looking for alternative biomolecules that could be used to manage obesity. Several conjugated linolenic acid (CLNA, 18:3) isomers have been described as potential antiobesity molecules. One advantage of this group of conjugated fatty acids over CLA is that CLNA isomers are present at much higher levels in foodstuffs such as certain seed oils, including bitter gourd (*Momordica charantia*) seed oil, tung (*Vernicia fordii*) seed oil, and pomegranate (*Punica granatum*) seed oil (PSO).

The present study focuses on punicic acid (PUA; *cis*-9,*trans*-11,*cis*-13 18:3), one of the CLNA isomers present in PSO and *Trichosanthes kirilowii*.[7] PUA constitutes 64−83% of PSO.[8] PUA has been nutritionally evaluated in several studies. Arao et al.[9] showed that feeding obese OLETF rats a diet supplemented with 1% PSO did not affect abdominal adipose tissue weight or serum lipids when compared to the control diet. Nevertheless, levels of hepatic triglycerides (TGs) decreased significantly. In another study carried out on db/db mice using a well-established model of obesity and type 2 diabetes, 1% dietary supplementation of PSO improved glucose tolerance and decreased inflammation.[10]

Furthermore, in vitro studies have reported that CLNA isomers act as agonists for peroxisome proliferator-activated receptors (PPARs),[10−12] a family of nuclear receptors that regulate the transcription of a large number of genes involved in numerous metabolic pathways. However, it is important to emphasize that, although a range of compounds, including fatty acids, have been shown to bind to and activate PPARs in vitro, it is still not fully clear which of these compounds serve as ligands in the living organism.[13] It is therefore essential to verify the activating effect of molecules in vivo that have been reported to be PPAR agonists by in vitro studies.

The main aim of the present study was therefore to analyze the effect of PUA on glucose homeostasis impairment induced

Received: November 28, 2012
Revised: May 6, 2013
Accepted: May 7, 2013
Published: May 7, 2013

by an obesogenic diet in rats. We also assessed the preventive action of PUA on other alterations induced by such a diet, such as fat accumulation in adipose tissue, liver, and skeletal muscle. Finally, we examined the action of PUA on PPAR$\alpha$ and PPAR$\delta$ in liver and skeletal muscle, respectively, and the consequences of these effects on fatty acid oxidation.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Kits used to measure total cholesterol, HDL cholesterol, and glucose were obtained from BioSystems (Barcelona, Spain), while kits for measuring TGs, phospholipids, alanine transaminase (ALT), aspartate transaminase (AST), and fructosamine were obtained from Spinreact (Sant Esteve de Bas, Spain). Insulin kit (EZRMI 13K) came from Linco (St. Charles, MO). Apoptosis was measured using the Cell Death Detection ELISA assay from ROCHE Diagnostics (Mannhein, Germany). Sodium methoxide for fatty acid analysis and standards for gas chromatography, including nonadecanoic acid (internal standard), were purchased from Sigma Chemical Co. (St. Louis, MO), and pure CLA and CLNA isomers were from Larodan Fine Chemicals AB (Malmö, Sweden). The SYBR Green Real-time RT-PCR Master Mixes for RT-PCR were purchased from Applied Biosystems (Foster City, CA), while specific primers were commercially synthesized by Eurogentec (Leuven, Belgium). Nuclear Extraction and PPAR$\alpha$ Transcription Factor Assay Kits were obtained from Cayman Chemical Co. (Ann Arbor, MI), and PPAR$\alpha$ (H-98) rabbit antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

**Animals, Diets, and Experimental Design.** The experiment used 24 male Wistar rats (132 ± 2 g) purchased from Harlan Ibérica (Barcelona, Spain) and was conducted according to the institution's guide for the care and use of laboratory animals (CUEID CEBA/30/2010). The rats were housed individually in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy), and placed in an air-conditioned room (22 ± 2 °C) with a 12-h day−night rhythm. After a 6-day adaptation period, the rats were randomly divided into two groups of 12 animals: a control group and a PUA group. Both groups were fed for 6 weeks on a high-sucrose and moderately high-fat diet (Table 1). The composition of this diet was based on reports from previous studies by other researchers.[14,15] PUA derived from PSO and it was added to diet at a dose of 0.5 g/100 g in the case of PUA group. We chose this dose (0.5%) because it is commonly used in studies on functional molecules such as CLA and other CLNA isomers.[16−19] Moreover, a promising new investigation recently published by Saha et al. has demonstrated beneficial properties of PUA at this dose.[20,21]

**Table 1. Diet Composition**

| ingredients | control group (g/kg) | PUA group (g/kg) |
|---|---|---|
| sucrose[a] | 411 | 411 |
| casein[b] | 200 | 200 |
| wheat starch[c] | 150 | 150 |
| fat | 150 | 150 |
| soybean oil[a] | 85 | 78.4 |
| lard[a] | 65 | 65 |
| pomegranate seed oil[e] | 0 | 6.6 |
| mineral mix[d] | 40 | 40 |
| cellulose[c] | 30 | 30 |
| vitamin mix[d] | 11 | 11 |
| L-methionine[b] | 4 | 4 |
| choline chlorhydrate[b] | 4 | 4 |

[a]Local market. [b]Sigma St. Louis, MO. [c]Vencasser, Bilbao, Spain. [d]ICN Pharmaceuticals, Costa Mesa, CA. [e]Moleva S.A.-Dallant Group, Lleida, Spain.

**Serum Sampling and Tissue Removal.** At the end of the experimental period, rats were fasted overnight and blood samples were collected under anesthesia (chloral hydrate) by cardiac puncture. Serum was obtained from blood samples after centrifugation (1000$g$ for 10 min at 4 °C). White adipose tissue (WAT) from different anatomical locations (epididymal, perirenal, mesenteric, and subcutaneous), interscapular brown adipose tissue (IBAT), liver, and gastrocnemius muscles were dissected, weighed, and immediately frozen. All samples were stored at −80 °C until analysis.

**Serum Analysis.** Commercial kits were used to measure serum parameters: total cholesterol, HDL cholesterol, glucose, TGs, ALT, AST, fructosamine, and insulin. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated from insulin and glucose values using Matthews' formula (shown below).[22]

$$\text{HOMA-IR} = [\text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)}]/22.5$$

**Glucose Tolerance Test.** During the week prior to the sacrifice, the rats were deprived of food (but not water) for 12 h before the start of the glucose tolerance test. A glucose load at a dose of 2 g/kg body weight was injected intraperitoneally. Blood glucose was determined at 0, 30, 60, 90, and 120 min using blood from the tail vein. The area under the curve (AUC) was calculated using the trapezoidal method.[23]

**Composition of the Liver and the Gastrocnemius Muscle.** Total lipids were extracted from the liver and skeletal muscle as previously described.[24] The lipid extract was then dissolved in isopropanol. TG content in muscle and phospholipids and TGs in the liver were measured using commercial kits. Protein content was analyzed using the Lowry method,[25] and water content was measured gravimetrically by drying samples at 105 °C until a constant weight was reached.

**Fatty Acid Analysis.** In the case of the fatty acid profile of the tissues, nonadecanoic acid was added as internal standard to the samples previously to the extract preparation. Lipids were extracted in duplicate according to the Bligh and Dyer procedure.[26] Sodium methoxide in 0.5 M methanol was added to the lipid extracts to prepare the fatty acid methyl esters (FAME). FAMEs were analyzed using a Hewlett-Packard HP6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector, and split/splitless injection port, an HP Chemstation software data system, and an HP7673 autosampler (Hewlett-Packard) as previously described.[27] Peak identifications were determined by comparing retention times with their respective standards.

**Hepatocyte Size and Number.** DNA content was quantified as previously described.[24] Hepatocyte size and number were then calculated using this data. Apoptosis in the liver was measured using the Cell Death Detection ELISA protocol (ROCHE Diagnostics, Mannhein, Germany) for homogenates of liver samples, following the method described by Selman et al.[28]

**Oxidative Enzyme Activities.** Carnitine palmitoyltransferase-1 activities (CPT-1a in the liver and CTP-1b in skeletal muscle) were assessed in the mitochondrial/peroxisomal fraction following the method outlined by Bieber et al.[29] Liver and muscle samples (0.5 g) were homogenized in 3 mL of buffer solution (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 4 °C), and the supernatant fluid was centrifuged again (12 000$g$ for 15 min at 4 °C). Pellets were resuspended in a buffer solution (pH 7.4) containing 70 mmol/L sucrose, 220 mmol/L mannitol, 1 mmol/L EDTA, and 2 mmol/L HEPES. CPT-1a and CPT-1b activities were expressed as nmol CoA formed/min/mg protein.

**Extraction and Analysis of RNA and Quantification by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA isolation from 100 mg of liver or gastrocnemius muscle and relative quantification of ACO, CPT-1a, CTP-1b, PPAR$\alpha$, PPAR$\delta$, retinoid X receptor-$\alpha$ (RXR $\alpha$), and peroxisome proliferator-activated receptor gamma coactivator (PGC-1$\alpha$) mRNA abundance was performed as previously described using SYBR Green RT-PCR technology.[24] Specific primers were synthesized commercially; the sequences have been previously described.[24,30−33] In addition, the following primers were designed: RXR$\alpha$ 5′-GCC ATC TTT GAC

AGG GTG CTA-3′ (forward); 5′-CTC CGT CTT GTC CAT CTG CAT-3′ (reverse); and PGC-1$\alpha$ 5′-CCA AAG CTGA AGC CCT CTT GC-3′ (forward); 5′-GTT TAG TCT TCC TTT CCT CGT GTC C-3′ (reverse).

The PCR parameters were as follows for CPT-1a, CPT-1b, ACO, PPAR$\alpha$, PPAR$\delta$, RXR-$\alpha$, and ß-actin: initial 2 min at 50 °C, denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s each, and finally by combined annealing and extension at 60 °C for 1 min. In the case of PGC-1$\alpha$, annealing was performed at 68.3 °C for 30 s. Levels of mRNA were normalized to the values of ß-actin levels, and the results were expressed as fold changes of threshold cycle ($Ct$) value relative to controls, using the $2^{-\Delta\Delta Ct}$ method.[34]

**Western Blot Analysis of Hepatic PPAR$\alpha$.** Liver nuclear proteins were extracted according to the manufacturer's instructions (Nuclear Extraction Kit, Cayman Chemical Company, Ann Arbor, MI). Immunoblot analyses were performed using 50 $\mu$g of liver nuclear extracts following the protocol reported by Kelley et al.[35] Blots were exposed to film for differing lengths of time, and the best exposures (405 s) were subjected to the Chemic Doc TM image system (Bio-Rad, Hercules, CA). Equal loading of proteins was confirmed by staining the membranes with Comassie Blue.

**Detection of PPAR$\alpha$ Transcriptional Factor DNA Binding Activity in Hepatic Nuclear Extracts.** Liver nuclear proteins were extracted and quantified using the same protocol as for immunoblot analysis. PPAR$\alpha$ DNA binding to its response element (PPRE, peroxisome proliferator response element) was measured using a commercial kit. The kit specificity for PPAR$\alpha$ was tested (lack of cross-reaction with PPAR$\gamma$ or PPAR$\delta$ transcription factors). The PPAR$\alpha$ DNA binding activity was expressed per gram of nuclear protein.

**Statistical Analysis.** Results are presented as mean ± SEM. Statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL). Data were analyzed using Student's $t$-test. Statistical significance was set at the level of $P < 0.05$.

## RESULTS

**Food Intake, Body Weight, and Tissue Weights.** No significant differences were found between the two experimental groups in final body weight, food intake, gastrocnemius muscle weight, IBAT weight, or WAT weight from epididymal, perirenal, mesenteric, or subcutaneous anatomical locations. Animals fed the PUA-supplemented diet showed a decreased liver weight ($P < 0.001$) (Table 2).

**Lipid Profile of the Extracted Pomegranate Seed Oil (PSO).** The lipid profile of the PSO showed that PUA represented 74.6% of the total FAMES (Table 3).

**Table 2. Food Intake, Final Body Weight, and Weights of Adipose Tissues, Liver, and Muscles in Rats Fed a Moderate High-Fat, High-Sucrose Diet Supplemented with 0.5% Punicic Acid for 6 weeks[a]**

| | control group | PUA group |
|---|---|---|
| food intake (g/d) | 16.5 ± 0.3 | 16.3 ± 0.4 |
| final body weight (g) | 340 ± 5 | 328 ± 4 |
| IBAT (g) | 0.81 ± 0.06 | 0.79 ± 0.02 |
| white adipose tissue weights (g) | | |
| perirenal | 7.76 ± 0.57 | 8.68 ± 0.33 |
| epididymal | 8.24 ± 0.55 | 8.41 ± 0.60 |
| mesenteric | 3.77 ± 0.20 | 4.14 ± 0.37 |
| subcutaneous | 11.9 ± 1.12 | 12.0 ± 0.77 |
| ∑adipose tissues (g) | 31.7 ± 2.10 | 33.2 ± 1.96 |
| liver weight (g) | 9.38 ± 0.17 | 8.28 ± 0.16[b] |
| muscle weights (g) | 2.09 ± 0.07 | 2.19 ± 0.15 |

[a]Values are means ± SEM. Comparisons between control and PUA groups were made by Student's $t$-test. [b]$P < 0.001$.

**Table 3. Lipid Profile of the Extracted Oil from *Punica granatum*[a]**

| fatty acids | % |
|---|---|
| C16:0 | 4.8 ± 0.1 |
| C18:0 | 2.71 ± 0.1 |
| *cis*-9 C18:1 | 5.6 ± 0.2 |
| *cis*-7 C18:1 | 0.9 ± 0.1 |
| *cis*-9,*cis*-12 C18:2 | 7.6 ± 0.4 |
| *cis*-9,*cis*-12,*cis*-15 C18:3 | 0.4 ± 0.1 |
| C20:0 | 0.8 ± 0.1 |
| *cis*-9,*trans*-11,*cis*-13 CLNA | 74.6 ± 0.1 |
| other isomer of CLNA | 2.5 ± 0.1 |

[a]% FAMEs of the total fatty acids identified. Values are means ± SEM.

**Serum Analysis.** There were no significant differences between the experimental groups in serum TGs, HDL cholesterol, or non-HDL cholesterol. Statistical analysis showed a tendency toward increased values of ALT (+53.3%; $P = 0.08$) and AST (+39.5%; $P = 0.09$) for serum concentrations from the PUA-supplemented group (Table 4).

**Table 4. Serum Parameters in Rats Fed a Moderate High-Fat, High-Sucrose Diet Supplemented with 0.5% Punicic Acid for 6 weeks[a]**

| | control group | PUA group |
|---|---|---|
| triglycerides (mmol/L) | 0.63 ± 0.05 | 0.80 ± 0.12 |
| total cholesterol (mmol/L) | 2.01 ± 0.07 | 1.84 ± 0.08 |
| HDL-cholesterol (mmol/L) | 0.72 ± 0.02 | 0.65 ± 0.02 |
| non-HDL cholesterol (mmol/L) | 1.29 ± 0.07 | 1.18 ± 0.08 |
| ALT (U/L) | 11.8 ± 1.1 | 18.1 ± 3.1 |
| AST (U/L) | 67.8 ± 8.0 | 94.6 ± 12.2 |
| fructosamine ($\mu$mol/L) | 297 ± 12 | 264 ± 9[b] |
| glucose (mmol/L) | 11.7 ± 0.5 | 10.2 ± 0.4 |
| insulin (mU/L) | 15.5 ± 1.41 | 17.4 ± 3.0 |
| HOMA-IR | 7.64 ± 1.00 | 7.88 ± 1.45 |

[a]Values are means ± SEM. Comparisons between control and PUA groups were made by Student's $t$-test. ALT: alanine transaminase. AST: aspartate transaminase. [b]$P < 0.05$.

With respect to glycemic control, PUA supplementation did not modify fasting glucose and insulin levels. Consequently, no significant differences were found in HOMA-IR (an indirect index of insulin resistance) (Table 4). The glucose tolerance test showed no significant differences between experimental groups in AUC or in glycemic values for experimental times up until 90 min. However, glycemia in the PUA group was significantly lower at the final time (120 min) (Figure 1A and B). As expected from these data, fructosamine concentration (an indicator of average blood glucose level over short time periods) was significantly reduced by adding PUA to the obesogenic diet (Table 4).

**Composition of Liver and Muscle.** The addition of 0.5% PUA to the diet did not modify the composition of the liver or the skeletal muscle (TG, phospholipid, water, and protein content) (Table 5).

The fatty acid profile, expressed as milligram per gram tissue, shows that low levels of PUA were present in both tissues. The levels of *cis*-9,*trans*-11 CLA found in these tissues were higher than the PUA levels (Table 5).

**Hepatocyte Analysis.** Hepatic DNA content, per gram of liver or per milligram of protein, remained unchanged (data not

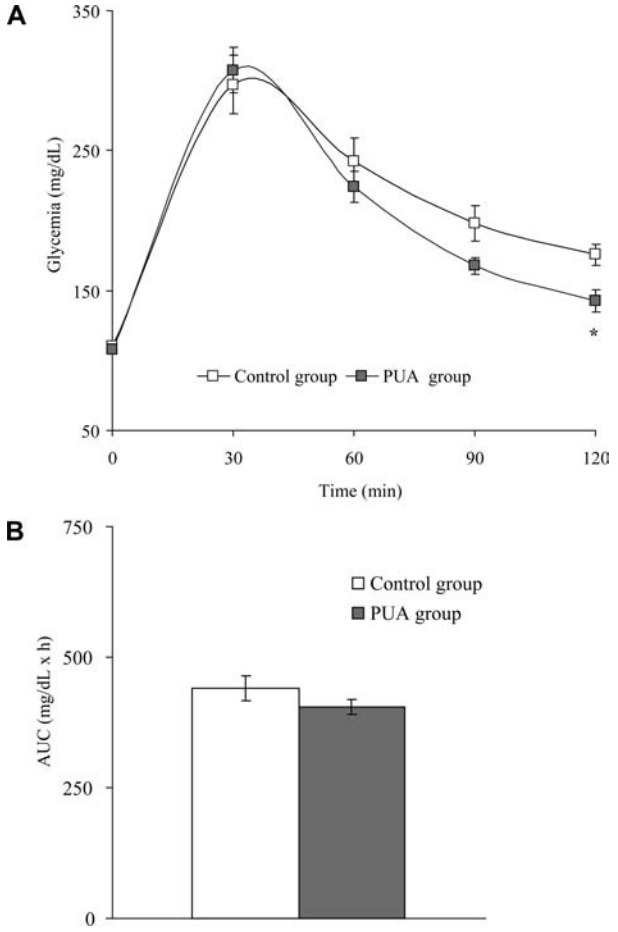


**Figure 1.** Glycemic response in glucose tolerance test (A) and area under the curve (AUC) (B) in rats from control and PUA groups ($n$ = 12). Values are means ± SEM. Differences between groups were determined using Student's $t$-test. *$P$ < 0.05.

shown), as did hepatocyte weight, which indicates that cell size was not modified. However, when the total number of hepatocytes was calculated, a lower value was observed for animals from the PUA group (−16.1% vs the control group; $P$ < 0.05) (Figure 2A). Contrary to expectations, a reduced level of apoptosis was found in this group (−56%; $P$ < 0.01) (Figure 2B).

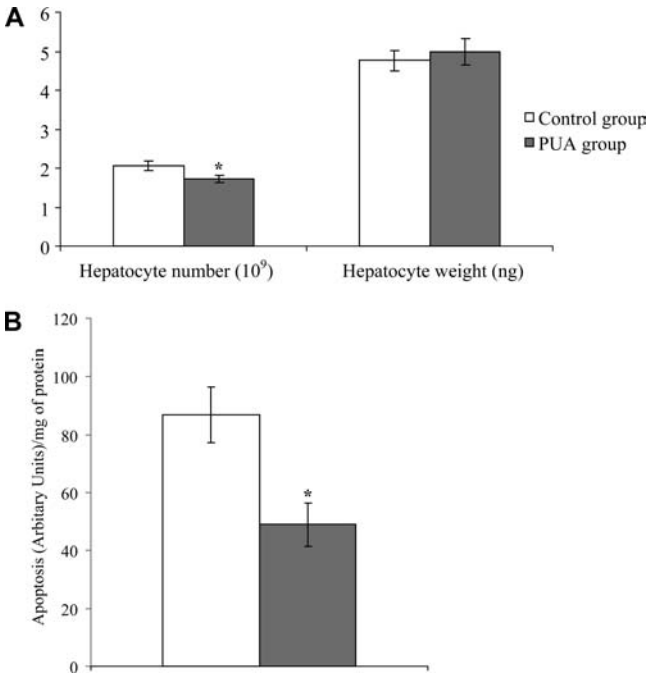


**Figure 2.** Hepatocyte number, hepatocyte weight (A), and apoptosis (B) in rats from control and PUA groups ($n$ = 12). Values are means ± SEM. Differences between groups were determined using Student's $t$-test. *$P$ < 0.05.

**Table 5. Liver and Muscle Tissue Composition of Rats Fed a Diet Supplemented with 0.5% Punicic Acid for 6 weeks[a]**

| | liver | | gastrocnemious muscle | |
|---|---|---|---|---|
| | control group | PUA group | control group | PUA group |
| proteins (mg/g) | 190 ± 8 | 176 ± 6 | 149 ± 8 | 150 ± 10 |
| water content (mg/g) | 667 ± 18 | 704 ± 5 | 759 ± 3 | 764 ± 4 |
| TGs (mg/g) | 19.6 ± 1.36 | 19.4 ± 1.12 | 26.9 ± 1.33 | 31.0 ± 1.53 |
| phospholipids (mg/g) | 13.6 ± 0.97 | 15.7 ± 4.39 | 18.9 ± 1.22 | 17.9 ± 1.23 |
| major fatty acid profile (mg/g tissue) | | | | |
| 14:0 | 0.25 ± 0.13 | 0.14 ± 0.03 | 0.69 ± 0.21 | 0.66 ± 0.11 |
| 16:0 | 7.94 ± 1.82 | 3.14 ± 1.19 | 10.84 ± 2.08 | 10.81 ± 1.25 |
| 16:1 *cis*-7 | 0.89 ± 0.55 | 0.34 ± 0.06 | 1.19 ± 0.30 | 1.73 ± 0.50 |
| 18:0 | 5.24 ± 0.28 | 6.22 ± 0.66 | 4.48 ± 0.66 | 3.66 ± 0.10 |
| 18:1 *cis*-9 | 3.27 ± 0.85 | 3.02 ± 0.56 | 7.46 ± 2.53 | 6.90 ± 1.22 |
| 18:1 *cis*-11 | 2.10 ± 0.76 | 1.57 ± 0.34 | 2.32 ± 0.55 | 2.72 ± 0.42 |
| 18:2 *cis*-9,12 | 7.79 ± 1.48 | 8.54 ± 1.56 | 13.69 ± 3.07 | 12.67 ± 1.57 |
| 18:3 *cis*-9,12,15 | 0.27 ± 0.08 | 0.28 ± 0.07 | 0.53 ± 0.12 | 0.59 ± 0.12 |
| 20:4 *cis*-5,8,11,14 | 7.04 ± 0.21 | 8.42 ± 0.99 | 4.85 ± 0.87 | 3.63 ± 0.43 |
| 20:5 *cis*-5,8,11,14,17 | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.09 ± 0.02 | 0.04 ± 0.01[b] |
| 20:6 *cis*-4,7,10,13,16,19 | 1.77 ± 0.09 | 2.02 ± 0.22 | 4.84 ± 0.95 | 3.52 ± 0.46 |
| 18:2 *cis* -9,*trans*-11 | ND | 0.31 ± 0.08 | ND | 0.52 ± 0.11 |
| 18:3 *cis*-9,*trans*-11,*cis*-13 | ND | 0.04 ± 0.01 | ND | 0.04 ± 0.01 |

[a]Values are means ± SEM. Comparisons between control and PUA groups were made by Student's $t$-test. ND: not detected. [b]$P$ < 0.05.


dx.doi.org/10.1021/jf305076v | J. Agric. Food Chem. 2013, 61, 5089−5096

**Enzyme and Transcriptional Factor Expressions.** In the liver, CTP-1a and ACO gene expression was not modified following PUA supplementation (Figure 3A), and the

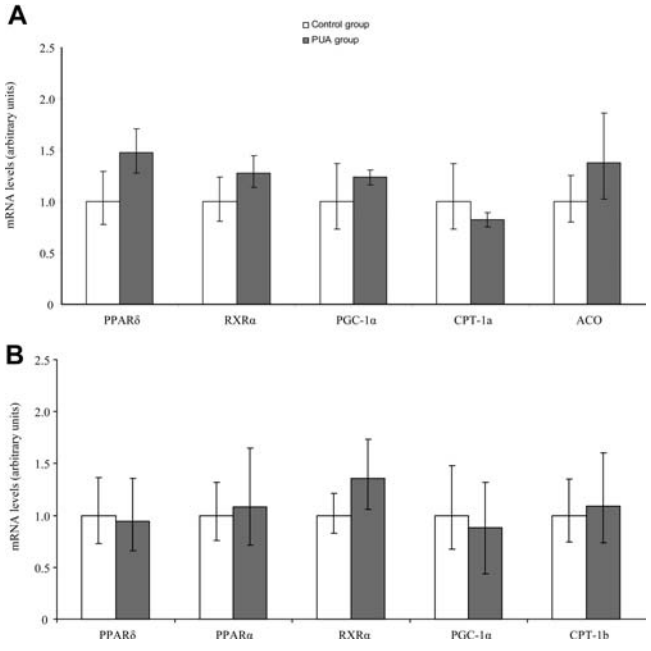


**Figure 3.** Gene expression in liver (A) and skeletal muscle (B) in rats from control and PUA groups ($n$ = 12). Values are means ± SEM. Differences between groups were determined using Student's $t$-test.

expression of PPAR$\delta$, RXR$\alpha$, and PGC1$\alpha$ coactivator remained unchanged (Figure 3A). A significant increase in PPAR$\alpha$ expression was found in the PUA group ($P < 0.05$) (Figure 4A). In the skeletal muscle, no significant changes in the expression of CPT-1b were induced by PUA. Similarly, the mRNA levels of the transcription factors that regulate the activity of this enzyme (PPAR$\delta$, PPAR$\alpha$, and RXR$\alpha$) were not modified, nor were those of the PGC1$\alpha$ coactivator (Figure 3B).

**PPAR$\alpha$ Transcriptional Factor Protein Expression and DNA Binding Activity in Hepatic Nuclear Extracts.** With respect to PPAR$\alpha$ protein expression, no changes were observed between the two experimental groups (Figure 4B and C).

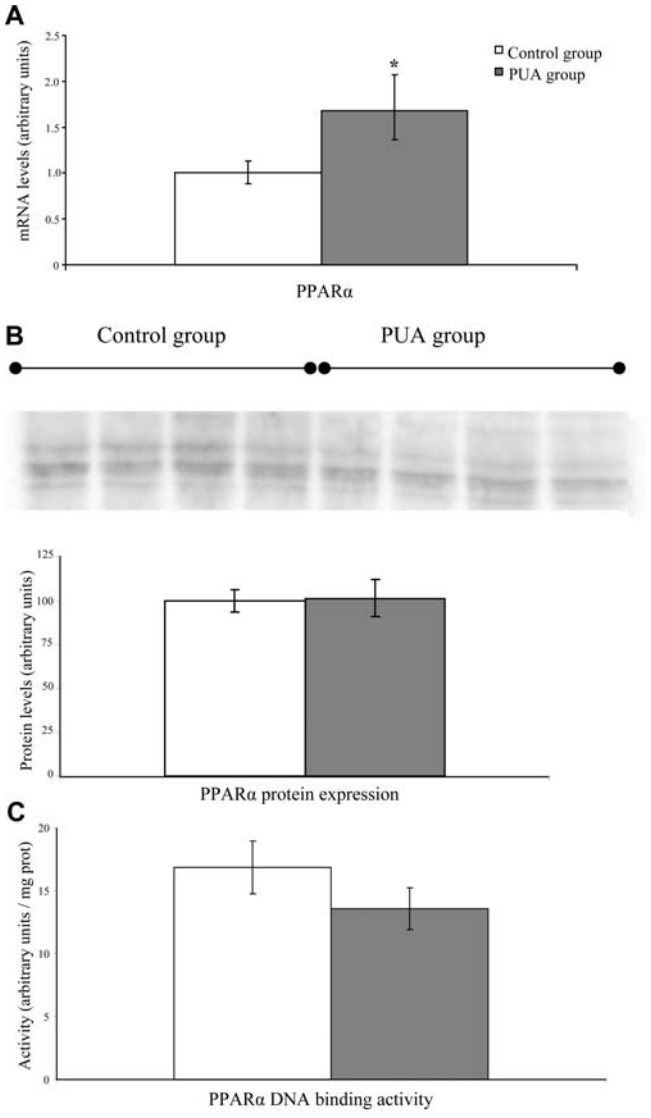


**Figure 4.** PPAR$\alpha$ mRNA levels (A), PPAR$\alpha$ protein expression (B), and PPAR$\alpha$ activation (C) in rats' liver from control and PUA groups ($n$ = 12). In the case of (B), upper panel shows representative data from four rats per group, and bottom panel shows the density of each group band ($n$ = 12). Values are means ± SEM. Differences between groups were determined using Student's $t$-test. *$P < 0.05$.

## DISCUSSION

Studies carried out in mice have revealed that PUA has a preventive effect on hepatic TG accumulation, obesity, and insulin resistance induced by high-fat diets.[9,10,36−38] In rats, however, some of the beneficial effects of PUA observed in mice have not been found. This suggests that the rat could be less responsive to PUA than the mouse. A similar situation had been previously observed with *trans*-10,*cis*-12 CLA. In this context, the present study was designed to gain further insight into the effects of PUA on alterations induced by obesogenic feeding, such as fat accumulation and impairment of glucose homeostasis, in rats.

For this purpose, we fed rats an obesogenic diet supplemented with PUA. As regards obesity, our findings showed that the rats fed the PUA-supplemented diet did not show reduced body weight or adipose tissue weights. These results concur with those previously reported in rats by Arao et al.[9]

The effects of PUA on glucose homeostasis in mice have created a great deal of expectation. The first study on this topic indicated that PUA improved insulin sensitivity, but this conclusion was based only on the basal insulin concentration, with no further determination based on glycemic control.[37] It was subsequently reported that at a dose of 1%, PSO improved insulin sensitivity after 14 weeks[38] and glucose tolerance after 4 weeks in mice fed a high-fat diet.[10]

To our knowledge, the effect of PUA on glycemic control in rats has not been extensively studied in the literature to date. In the present study, glycemia had decreased significantly in the PUA group by the final time (120 min), as had serum fructosamine concentration, an indicator of average blood glucose level over short time periods. However, the HOMA-IR showed no improvement in insulin resistance, nor did other glucose tolerance test values (taken at 30, 60, and 90 min).


dx.doi.org/10.1021/jf305076v | J. Agric. Food Chem. 2013, 61, 5089−5096

These results suggest that the beneficial effect of PUA on glucose homeostasis is weaker in rats than in mice.

We measured liver weights and found that they were lower in rats treated with PUA. There was, however, no change in composition, which means that liver delipidation can be disregarded as a possible cause. DNA content was measured in an attempt to explain this decrease in liver weight. No changes were observed in DNA/g liver or in DNA/mg protein. Hepatocyte weight therefore remained unchanged, which indicates that cell size was not modified. However, when the total number of hepatocytes was calculated, a significant decrease was observed in animals treated with 0.5% PUA. Apoptosis in the liver was therefore measured in an attempt to explain these results. Surprisingly, a negative value was found for apoptosis in PUA-fed animals (−56%; $P < 0.01$). Consequently, it could be suggested that the decrease in liver weight caused by PUA was associated with hypoplasia, probably due to reduced hepatocyte proliferation. It would appear, therefore, that this CLNA isomer has the undesirable effect of hepatocyte antiproliferation. To the best of our knowledge, this effect of PUA has never previously been reported.

Moreover, other side effects related to the liver were also observed. PUA supplementation tended to increase ALT and AST serum levels, two indicators of liver damage ($P = 0.08$ and $P = 0.09$, respectively). This observation is consistent with the values obtained by Meerts et al. in rats after 4 weeks of treatment.[39] This study showed that, in groups supplemented with 5% and 15% PSO, ALT increased by 27% and 291%, and AST by 27% and 497%, respectively.

It has been reported that one of the most important mechanisms underlying the effects of PUA on lipid and glucose homeostasis is the activation of PPAR$\alpha$, a transcription factor that plays an important role in TG metabolism in the liver and skeletal muscle, and in fuel adjustments induced by dietary conditions. In 2006, Chuang et al.[11] showed PUA to be an agonist of PPAR$\alpha$, but pointed out that further studies would be required to demonstrate that this was also the case in vivo. Similar results were reported by Hontecillas et al. in 2009.[10] The same research group subsequently demonstrated that oral PUA administration improved glucose tolerance and that this effect was accompanied by the upregulation of PPAR$\alpha$-related genes.[40]

Taking this into consideration, in the present study we analyzed the expression of PPAR$\alpha$ in the liver, along with other parameters of the metabolic route controlled by this transcription factor. In PUA-treated rats, PPAR$\alpha$ was upregulated. However, this increase in PPAR$\alpha$ did not result in increased PPAR$\alpha$ protein expression. Moreover, PPAR$\alpha$ activation was not modified by PUA treatment. Also unaltered was the expression of other genes related to PPAR$\alpha$, such as RXR$\alpha$, the transcription factor that forms a heterodimer with PPAR$\alpha$ to activate the response-element, and coactivator PGC-1$\alpha$, and the enzymes controlled by PPAR$\alpha$, such as CPT-1 and ACO. These results clearly demonstrate that the metabolic pathway of fatty acid oxidation was unaffected by PUA in rats under our experimental conditions. These results correspond to those reported by Arao et al.,[9] who found no changes to CPT activity in a model of obese (OLETF) rats treated with PUA.

Several of these parameters were also analyzed in skeletal muscle, another important oxidative organ. No changes were observed in the expression of the genes analyzed: PPAR$\delta$, RXR, PGC-1$\alpha$, CPT-1b, and ACO. The discrepancy between our data and that obtained for mice[10] appears to indicate once again that response to PUA is species-specific, with mice being more sensitive than rats.

It could be hypothesized that the differences in response to PPAR agonists are due to qualitative and/or quantitative variations in PPARs. However, if we consider that mice and rats have similar amounts of PPAR$\alpha$,[41,42] it seems likely that the discrepancies between these two species are due to a different activation of PPAR$\alpha$ by PUA. Further research is needed to clarify this.

To gain a deeper understanding of the results obtained in the present study, the fatty acid profile was determined for the liver and the skeletal muscle. Previous studies have reported that CLA isomers have been detected in the serum and tissues of animals fed CLNA isomers,[43,44] which suggests that CLNA isomers may, to some extent, metabolize into CLA isomers. Certainly, in the present study, very low levels of PUA were found in both the liver and the skeletal muscle of rats fed the PUA-supplemented diet. Moreover, in these animals the *cis*-9,*trans*-11 isomer of CLA was also present in the liver and skeletal muscle, at higher levels than PUA. Considering that this CLA isomer was not present in the diet, it could be suggested that its origin was PUA metabolism.

The low levels of PUA found in these tissues may explain why the supposed positive actions of this fatty acid were not observed in the present study. Most of the PUA was metabolized into *cis*-9,*trans*-11 CLA, which has previously been shown to be inactive in reducing body fat or liver fat.[45,46] On the other hand, several of the effects of the CLNA isomers may, at least in part, be the result of the derived CLA isomers.[36,44,47] Therefore, the *cis*-9,*trans*-11 CLA in the liver could be responsible for the decrease in hepatocyte proliferation, as was reported by Hirao et al.[48] for a study carried out in rats.

The transformation of PUA into CLA could also at least partly explain the differences in sensitivity between mice and rats. In the present study, most of the PUA was transformed into CLA: the amount of CLA was 8 times that of PUA in the liver and 11 times that of PUA in skeletal muscle. By contrast, the transformation of PUA into CLA appears to be lower in mice. Koba et al.[36] reported similar levels of both fatty acids in liver TGs of mice fed a diet supplemented with 2.5% PUA, and levels of CLA double that of PUA when the diet was supplemented with 5% PUA. Moreover, Yamasaki et al.[44] found similar levels of PUA and CLA in liver lipids from mice fed a diet supplemented with 0.12% PSO, and a level of CLA double that of PUA when supplemented with 1.2% PSO. It follows that these higher levels of PUA found in mouse tissues are responsible for the beneficial effects of PUA observed in this species.

In conclusion, these results show that dietary supplementation of PUA at a dose of 0.5% does not lead to decreased fat accumulation in adipose tissue, liver, or skeletal muscle or to improved glycemic control in rats. Furthermore, the hypoplasia induced in the liver, together with the tendency toward increased transaminase levels, should be considered before proposing PUA as a functional ingredient. This matter warrants further toxicological study.

## AUTHOR INFORMATION

### Corresponding Author

*Tel.: +34-945-013067. Fax: +34-945-013014. E-mail: mariapuy.portillo@ehu.es.

### Funding

This study was supported by grants from the Basque Country Government (IT-572-13), the Instituto de Salud Carlos III (PREDIMED and CIBERObn), and the University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32).

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The pomegranate (*Punica granatum*) seeds and peels were obtained from byproducts of the processing juice industry, kindly donated by Moleva S.A.-Dallant Group (Lleida, Spain).

## ■ REFERENCES

(1) Wang, Y. W.; Jones, P. J. Conjugated linoleic acid and obesity control: efficacy and mechanisms. *Int. J. Obes. Relat. Metab. Disord.* **2004**, *28*, 941−55.

(2) Bhattacharya, A.; Banu, J.; Rahman, M.; Causey, J.; Fernandes, G. Biological effects of conjugated linoleic acids in health and disease. *J. Nutr. Biochem.* **2006**, *17*, 789−810.

(3) Park, Y.; Pariza, W. Bioactivities and potential mechanisms of action for conjugated fatty acids. *Food Sci. Biotechnol.* **2009**, *18*, 586−593.

(4) Terpstra, A. H. Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. *Am. J. Clin. Nutr.* **2004**, *79*, 352−61.

(5) Salas-Salvadó, J.; Márquez-Sandoval, F.; Bulló, M. Conjugated linoleic acid intake in humans: a systematic review focusing on its effect on body composition, glucose, and lipid metabolism. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 479−88.

(6) Efsa Panel on Dietetic Products, N. a. A. Scientific Opinion on the substantiation of health claims related to conjugated linoleic acid (CLA) isomers and contribution to the maintenance or achievement of a normal body weight (ID 686, 726, 1516, 1518, 2892, 3165), increase in lean body mass (ID 498, 731), increase in insulin sensitivity (ID 1517), protection of DNA, proteins, and lipids from oxidative damage (ID 564, 1937), and contribution to immune defenses by stimulation of production of protective antibodies in response to vaccination (ID 687, 1519) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal 8*, 26.

(7) Joh, Y.-G.; Kim, S.-J.; Christie, W. The structure of the triacylglycerols, containing punicic acid, in the seed oil ofTrichosanthes kirilowii. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1037−1042.

(8) Kaufman, M.; Wiesman, Z. Pomegranate oil analysis with emphasis on MALDI-TOF/MS triacylglycerol fingerprinting. *J. Agric. Food Chem.* **2007**, *55*, 10405−13.

(9) Arao, K.; Wang, Y. M.; Inoue, N.; Hirata, J.; Cha, J. Y.; Nagao, K.; Yanagita, T. Dietary effect of pomegranate seed oil rich in 9cis, 11trans, 13cis conjugated linolenic acid on lipid metabolism in obese, hyperlipidemic OLETF rats. *Lipids Health Dis.* **2004**, *3*, 24.

(10) Hontecillas, R.; O'Shea, M.; Einerhand, A.; Diguardo, M.; Bassaganya-Riera, J. Activation of PPAR gamma and alpha by punicic acid ameliorates glucose tolerance and suppresses obesity-related inflammation. *J. Am. Coll. Nutr.* **2009**, *28*, 184−95.

(11) Chuang, C. Y.; Hsu, C.; Chao, C. Y.; Wein, Y. S.; Kuo, Y. H.; Huang, C. J. Fractionation and identification of 9c, 11t, 13t-conjugated linolenic acid as an activator of PPARalpha in bitter gourd (Momordica charantia L.). *J. Biomed. Sci.* **2006**, *13*, 763−72.

(12) Lewis, S. N.; Brannan, L.; Guri, A. J.; Lu, P.; Hontecillas, R.; Bassaganya-Riera, J.; Bevan, D. R. Dietary $\alpha$-eleostearic acid ameliorates experimental inflammatory bowel disease in mice by activating peroxisome proliferator-activated receptor-$\gamma$. *PLoS One* **2011**, *6*, e24031.

(13) Kersten, S. Peroxisome proliferator activated receptors and obesity. *Eur. J. Pharmacol.* **2002**, *440*, 223−34.

(14) Asai, A.; Miyazawa, T. Dietary curcuminoids prevent high-fat diet-induced lipid accumulation in rat liver and epididymal adipose tissue. *J. Nutr.* **2001**, *131*, 2932−5.

(15) Nishina, P. M.; Verstuyft, J.; Paigen, B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* **1990**, *31*, 859−69.

(16) Dhar, P.; Bhattacharyya, D.; Bhattacharyya, D. K.; Ghosh, S. Dietary comparison of conjugated linolenic acid (9 cis, 11 trans, 13 trans) and alpha-tocopherol effects on blood lipids and lipid peroxidation in alloxan-induced diabetes mellitus in rats. *Lipids* **2006**, *41*, 49−54.

(17) Miranda, J.; Fernandez-Quintela, A.; Macarulla, M. T.; Churruca, I.; Garcia, C.; Rodriguez, V. M.; Simon, E.; Portillo, M. P. A comparison between CLNA and CLA effects on body fat, serum parameters and liver composition. *J. Physiol. Biochem.* **2009**, *65*, 25−32.

(18) Park, Y.; Park, Y. Conjugated nonadecadienoic acid is more potent than conjugated linoleic acid on body fat reduction. *J. Nutr. Biochem.* **2010**, *21*, 764−73.

(19) Sisk, M. B.; Hausman, D. B.; Martin, R. J.; Azain, M. J. Dietary conjugated linoleic acid reduces adiposity in lean but not obese Zucker rats. *J. Nutr.* **2001**, *131*, 1668−74.

(20) Saha, S. S.; Dasgupta, P.; Sengupta Bandyopadhyay, S.; Ghosh, M. Synergistic effect of conjugated linolenic acid isomers against induced oxidative stress, inflammation and erythrocyte membrane disintegrity in rat model. *Biochim. Biophys. Acta* **2012**, *1820*, 1951−70.

(21) Saha, S. S.; Ghosh, M. Antioxidant and anti-inflammatory effect of conjugated linolenic acid isomers against streptozotocin-induced diabetes. *Br. J. Nutr.* **2012**, *108*, 974−83.

(22) Matthews, D. R.; Hosker, J. P.; Rudenski, A. S.; Naylor, B. A.; Treacher, D. F.; Turner, R. C. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **1985**, *28*, 412−9.

(23) Tai, M. M. A mathematical model for the determination of total area under glucose tolerance and other metabolic curves. *Diabetes Care* **1994**, *17*, 152−4.

(24) Miranda, J.; Fernández-Quintela, A.; Churruca, I.; Rodríguez, V. M.; Simón, E.; Portillo, M. P. Hepatomegaly induced by trans-10,cis-12 conjugated linoleic acid in adult hamsters fed an atherogenic diet is not associated with steatosis. *J. Am. Coll. Nutr.* **2009**, *28*, 43−9.

(25) Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−75.

(26) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−7.

(27) Zabala, A.; Portillo, M. P.; Navarro, V.; Macarulla, M. T.; Barron, L. J.; Fernández-Quintela, A. Quantitative gas chromatographic method for the analysis of cis-9, trans-11 and trans-10, cis-12 isomers of the conjugated linoleic acid in liver. *J. Chromatogr., B* **2007**, *855*, 152−8.

(28) Selman, C.; Kendaiah, S.; Gredilla, R.; Leeuwenburgh, C. Increased hepatic apoptosis during short-term caloric restriction is not associated with an enhancement in caspase levels. *Exp. Gerontol.* **2003**, *38*, 897−903.

(29) Bieber, L. L.; Abraham, T.; Helmrath, T. A rapid spectrophotometric assay for carnitine palmitoyltransferase. *Anal. Biochem.* **1972**, *50*, 509−18.

(30) Ferreira, A. V.; Parreira, G. G.; Porto, L. C.; Mario, E. G.; Delpuerto, H. L.; Martins, A. S.; Botion, L. M. Fenofibrate prevents orotic acid–induced hepatic steatosis in rats. *Life Sci.* **2008**, *82*, 876−83.

(31) Rodríguez, A.; Catalán, V.; Becerril, S.; Gil, M. J.; Mugueta, C.; Gómez-Ambrosi, J.; Frühbeck, G. Impaired adiponectin-AMPK signalling in insulin-sensitive tissues of hypertensive rats. *Life Sci.* **2008**, *83*, 540−9.

(32) Lavrentyev, E. N.; Matta, S. G.; Cook, G. A. Expression of three carnitine palmitoyltransferase-I isoforms in 10 regions of the rat brain during feeding, fasting, and diabetes. *Biochem. Biophys. Res. Commun.* **2004**, *315*, 174−8.

(33) Hoekstra, M.; Kruijt, J. K.; Van Eck, M.; Van Berkel, T. J. Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *J. Biol. Chem.* **2003**, *278*, 25448−53.
(34) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402−8.
(35) Kelley, G. L.; Azhar, S. Reversal of high dietary fructose-induced PPARalpha suppression by oral administration of lipoxygenase/cyclooxygenase inhibitors. *Nutr. Metab.* **2005**, *2*, 18.
(36) Koba, K.; Imamura, J.; Akashoshi, A.; Kohno-Murase, J.; Nishizono, S.; Iwabuchi, M.; Tanaka, K.; Sugano, M. Genetically modified rapeseed oil containing cis-9,trans-11,cis-13-octadecatrienoic acid affects body fat mass and lipid metabolism in mice. *J. Agric. Food Chem.* **2007**, *55*, 3741−8.
(37) McFarlin, B. K.; Strohacker, K. A.; Kueht, M. L. Pomegranate seed oil consumption during a period of high-fat feeding reduces weight gain and reduces type 2 diabetes risk in CD-1 mice. *Br. J. Nutr.* **2009**, *102*, 54−9.
(38) Vroegrijk, I. O.; van Diepen, J. A.; van den Berg, S.; Westbroek, I.; Keizer, H.; Gambelli, L.; Hontecillas, R.; Bassaganya-Riera, J.; Zondag, G. C.; Romijn, J. A.; Havekes, L. M.; Voshol, P. J. Pomegranate seed oil, a rich source of punicic acid, prevents diet-induced obesity and insulin resistance in mice. *Food Chem. Toxicol.* **2011**, *49*, 1426−30.
(39) Meerts, I. A.; Verspeek-Rip, C. M.; Buskens, C. A.; Keizer, H. G.; Bassaganya-Riera, J.; Jouni, Z. E.; van Huygevoort, A. H.; van Otterdijk, F. M.; van de Waart, E. J. Toxicological evaluation of pomegranate seed oil. *Food Chem. Toxicol.* **2009**, *47*, 1085−92.
(40) Bassaganya-Riera, J.; Guri, A. J.; Hontecillas, R. Treatment of obesity-related complications with novel classes of naturally occurring PPAR agonists. *J. Obes.* **2011**, *2011*, 897894.
(41) Gonzalez, F. J.; Peters, J. M.; Cattley, R. C. Mechanism of action of the nongenotoxic peroxisome proliferators: role of the peroxisome proliferator-activator receptor alpha. *J. Natl. Cancer Inst.* **1998**, *90*, 1702−9.
(42) Holden, P. R.; Tugwood, J. D. Peroxisome proliferator-activated receptor alpha: role in rodent liver cancer and species differences. *J. Mol. Endocrinol.* **1999**, *22*, 1−8.
(43) Tsuzuki, T.; Kawakami, Y.; Abe, R.; Nakagawa, K.; Koba, K.; Imamura, J.; Iwata, T.; Ikeda, I.; Miyazawa, T. Conjugated linolenic acid is slowly absorbed in rat intestine, but quickly converted to conjugated linoleic acid. *J. Nutr.* **2006**, *136*, 2153−9.
(44) Yamasaki, M.; Kitagawa, T.; Koyanagi, N.; Chujo, H.; Maeda, H.; Kohno-Murase, J.; Imamura, J.; Tachibana, H.; Yamada, K. Dietary effect of pomegranate seed oil on immune function and lipid metabolism in mice. *Nutrition* **2006**, *22*, 54−9.
(45) Martin, J. C.; Valeille, K. Conjugated linoleic acids: all the same or to everyone its own function? *Reprod., Nutr., Dev.* **2002**, *42*, 525−36.
(46) Churruca, I.; Fernández-Quintela, A.; Portillo, M. P. Conjugated linoleic acid isomers: differences in metabolism and biological effects. *Biofactors* **2009**, *35*, 105−11.
(47) Tsuzuki, T.; Tokuyama, Y.; Igarashi, M.; Nakagawa, K.; Ohsaki, Y.; Komai, M.; Miyazawa, T. Alpha-eleostearic acid (9Z11E13E-18:3) is quickly converted to conjugated linoleic acid (9Z11E-18:2) in rats. *J. Nutr.* **2004**, *134*, 2634−9.
(48) Hirao, A.; Yamasaki, M.; Chujo, H.; Koyanagi, N.; Kanouchi, H.; Yasuda, S.; Matsuo, A.; Nishida, E.; Rikimaru, T.; Tsujita, E.; Shimada, M.; Maehara, Y.; Tachibana, H.; Yamada, K. Effect of dietary conjugated linoleic acid on liver regeneration after a partial hepatectomy in rats. *J. Nutr. Sci. Vitaminol.* **2004**, *50*, 9−12.