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RESEARCH ARTICLE

Grape seed proanthocyanidins repress the hepatic lipid regulators miR-33 and miR-122 in rats

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Scope: One major health problem in westernized countries is dysregulated fatty acid and cholesterol metabolism that causes pathologies such as metabolic syndrome. Previous studies from our group have shown that proanthocyanidins, which are the most abundant polyphenols in the human diet, regulate lipid metabolism and are potent hypolipidemic agents. The noncoding RNAs, miR-33 and miR-122, regulate genes that are involved in lipid metabolism.

Methods and results: Here, we show that grape seed proanthocyanidins rapidly and transiently repressed the expression of miR-33 and miR-122 in rat hepatocytes in vivo and in vitro. Furthermore, the miR-33 target gene ATP-binding cassette A1 and the miR-122 target gene fatty acid synthase were also modulated by proanthocyanidins. Specifically, ATP-binding cassette A1 mRNA and protein levels were increased, and fatty acid synthase mRNA and protein levels were reduced after the miRNA levels were altered.

Conclusion: These results suggest that proanthocyanidin treatment increased hepatic cholesterol efflux to produce new HDL particles by repressing miR-33, and it reduced lipogenesis by repressing miR-122. These results highlight a new mechanism by which grape seed proanthocyanidins produce hypolipidemia through their effects on miRNA modulators of lipid metabolism.

Keywords:
Abca1 / Fas / Flavonoids / Hepatocytes / MicroRNAs

1 Introduction

The most abundant polyphenols in the human diet are the proanthocyanidins, a subclass of flavonoids [1, 2]. These compounds are mainly present in apples, grapes, nuts, red wine, tea, and cocoa [3]. Proanthocyanidins improve human health with cardioprotectant [4], antigenotoxic [5], anti-inflammatory [6, 7], antioxidant [4], and anticarcinogenic [8] activities. Moreover, proanthocyanidins improve several risk factors for cardiovascular disease (CVD), such as dyslipidemia [9] and insulin resistance [10]. More specifically, grape seed proanthocyanidins are potent hypolipidemic agents. A grape seed proanthocyanidin extract (GSPE) was shown to reduce plasma triglycerides (TGs) levels, apo B and LDL cholesterol, as well as to increase the percentage of HDL cholesterol in healthy rats given an acute oral dose of GSPE [11]. The hypolipidemic effects of GSPE were even more obvious in a lipid tolerance test model [12]. Additionally, chronic treatment with GSPE corrects the dyslipidemia associated with dietary obesity in rats [13]. Several mechanisms by which GSPE induces hypolipidemia have already been described [9]. For example, GSPE activates genes that control fatty acid oxidation and represses genes that control lipogenesis and VLDL assembly in the liver [12–14], thus inducing hypolipidemia. However, it is becoming clear that microRNAs (miRNAs) play key roles in the regulation of genes involved in lipid metabolism in the liver, and the effects of GSPE on these miRNAs is unknown [15–17].

miRNAs are a novel class of noncoding RNAs that are 20–25 nucleotides long. miRNAs regulate the expression of specific target genes at the posttranscriptional level, mainly by triggering mRNA cleavage or inhibiting translation [18].

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Abbreviations: **Abca1**, ATP-binding cassette A1; **CPT1α**, carnitine palmitoyltransferase 1α; **Fas**, fatty acid synthase; **GSPE**, grape seed proanthocyanidin extract; **miRNAs**, microRNAs; **QqQ**, triple-quadrupole; **TGs**, triglycerides

Their effects are mostly mediated by their binding to the 3′ untranslated region of target mRNAs [19], but miRNAs can also bind to other regions, including 5′ untranslated regions and protein-coding exons [20]. miRNAs play important regulatory roles in a variety of biological processes. Specifically, several miRNAs have been correlated with obesity and metabolic syndrome [21] and are proposed to regulate glucose metabolism [22, 23], adipocyte differentiation and adipogenesis [24], and lipid metabolism [15, 16]. Two of the best-studied miRNAs involved in the regulation of lipid metabolism are miR-122 and miR-33 [15]. miR-122 is liver specific and represents 70% of all miRNA expression in liver [25]. The dysregulation of this miRNA has been associated with the dysregulation of genes with key roles in the control of liver lipid metabolism. miR-122 regulates several genes that control fatty acid and TG biosynthesis, such as fatty acid synthase (Fas), acetyl-CoA carboxylase 1, acetyl-CoA carboxylase 2, stearoyl-CoA desaturase 1, diacylglycerol O-acyltransferase 2, ATP citrate lyase, and sterol regulatory element-binding protein 1c, as well as genes that regulate fatty acid β-oxidation, such as carnitine palmitoyltransferase 1α (CPT1α) [15]. A second miRNA, miR-33, plays an important role in the regulation of cholesterol homeostasis, regulating the ATP-binding cassette transporters (ABC transporters), Abca1 and ABCG1, in addition to its role in fatty acid β-oxidation, where it regulates carnitine *O*-octanoyltransferase (CROT), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase β-subunit (HADHB), and CPT1α [15].

Recently, it has been reported that dietary polyphenols such as curcumin [26], resveratrol [27], epigallocatechin gallate [28], ellagitannin [29], isoflavones [30], and proanthocyanidins from grape seed and cocoa [31] modulate miRNA expression. Thus, we hypothesized that miRNAs could mediate the hypolipidemic effects of proanthocyanidins. Here, we test this hypothesis by examining the effects of GSPE on miR-122 and miR-33 levels in hepatic cells, using both in vivo and in vitro models. The results show that GSPE rapidly reduced miR-122 and miR-33 levels in both models. Furthermore, the effects of GSPE on these miRNAs occurred upstream of the identified effects of GSPE on the expression of the miR-33 and miR-122 target genes Fas and Abca1.

2 Materials and methods

2.1 Proanthocyanidin extract

The GSPE used contained: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g), and hexameric procyanidins (0.38 μmol/g) [32]. The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France).

2.2 Analysis of flavanols and their metabolites in plasma

2.2.1 Chemicals and reagents

Methanol (ME03151000, Scharlab S.L., Barcelona, Spain), acetone (34850, Sigma-Aldrich, Madrid, Spain), and glacial acetic acid (131008.1611, Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain).

Stock standard solutions of 2000 mg/L in methanol of (+)-catechin, (–)-epicatechin and pyrocatechol (Fluka/Sigma-Aldrich, Madrid, Spain), and a standard solution of 1000 mg/L in methanol of procyanidin B_2 (Fluka/Sigma-Aldrich) were stored in a dark-glass flask at –20°C. A 100 mg/L stock standard mixture in methanol of (+)-catechin, (–)-epicatechin, and procyanidin B_2 were prepared weekly and stored at –20°C. This stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v/v/v) solution.

2.2.2 Micro-solid flavanol extraction

Prior to chromatographic analysis, the rat plasma samples were pretreated by off-line μSPE following the methodology previously described by Martí et al. [33] using OASIS HLB μElution Plates of 30 μm (186001828BA, Waters, Barcelona, Spain). Briefly, the micro-cartridges were conditioned sequentially with 250 μL of methanol and 250 μL of 0.2% acetic acid. Plasma or amniotic fluid of 350 μL was mixed with 300 μL of 4% phosphoric acid and 50 μL of pyrocatechol (2000 ppb), and then this mixture was loaded onto the plate. The loaded plates were washed with 200 μL of Milli-Q water and 200 μL of 0.2% acetic acid. The retained procyanidins were eluted with 2 × 50 μL of acetone/Milli-Q water/acetic acid solution (70:29.5:0.5, v/v/v). The eluted solution was directly injected in the LC-QqQ-MS^2 (where QqQ is triple-quadrupole), and the sample volume was 2.5 μL.

2.2.3 Instrumental conditions

A 1200 LC Series coupled to a 6410 QqQ-MS/MS (Agilent Technologies, Palo Alto, USA) was used for the metabolites procyanidins and metabolites quantification. The chromatographic method used was, with a Zorbax C18 (100 mm × 2.1 mm id, 1.8 μm particle size) as chromatographic column, from Agilent Technologies. Mobile phases were 0.2% acetic acid (solvent A) and acetonitrile (solvent B). Flow rate was 0.4 mL/min. Elution gradient was 0–10 min, 5–55% B, 10–12 min, 55–80% B, 12–15 min, 80% B isocratic, 15–16 min 80–5% B. A post run of 10 min was applied.

ESI conditions were at 350°C and 12 L/min of drying gas temperature and flow, respectively, 45 psi of nebulizer gas pressure, and 4000 V of capillary voltage. QqQ operated in

Table 1. Quality parameters of the quantitative method by LC-ESI-QqQ/MS^2

Procyanidin	Determination coefficient (R^2)	Linearity (μM)	Recovery (%)	Precision (%RSD, $n = 3$)	LOD (μM)	LOQ (μM)	MDL[a)] (μM)	MQL[a)] (μM)
Acid gallic	0.9994	0.84–59	49	9	0.25	0.84	0.072	0.240
Catequin	0.9995	0.13–35	97	11	0.04	0.13	0.011	0.038
Epicat	0.9995	0.11–35	95	7	0.03	0.11	0.009	0.031
B2	0.9998	0.03–17	90	15	0.01	0.03	0.003	0.010

a) Method detection and quantification limits in μmol/L of fresh sample, calculated for the analysis of 350 μL of plasma sample.

negative mode. QqQ acquisition was done in MRM mode for procyanidins and their metabolites.

2.2.4 Method validation and samples quantification

For the quantitative method validation, calibration curves, linearity, extraction recovery, precision, sensitivity, and method detection and quantification limits were studied by analysis of standard solutions and blank plasma samples spiked with the standard procyanidins. Calibration curves were obtained by plotting analyte/IS peak abundance ratio and the corresponding analyte/IS concentration ratio. Extraction recovery was evaluated by comparison of the spiked samples response with standard solutions calibration curve. Method precision was determined from RSD in a triplicate analysis of a spiked sample. Sensitivity was evaluated by determining the LOD, defined as the concentration corresponding to three times the signal/noise rate, and the LOQ, defined as the concentration corresponding to ten times the signal/noise rate. Method detection and quantification limits (MDL and MQL, respectively) were calculated for the analysis of 350 μL of sample, following the procedure described in previous paragraphs. Table 1 shows the obtained values for each quality parameter.

In the quantification of samples, spiked blank samples at six different levels of concentration were used to obtain calibration curves, and standard compounds in the samples were quantified by interpoling the analyte/IS peak abundance ratio in these curves. In the case of catechin and epicatechin metabolites, due to the lack of standards, they were tentatively quantified using the standard catechin and epicatechin calibration curves, respectively. In the same way, dimer procyanidins B1 were quantified using the calibration curve of dimer procyanidin B2.

2.3 Cells and cell culture

FAO cells, a rat hepatoma cell line (ECACC, code 85061112), were grown to 80% confluence in Nutrient Mixture F12 Coon's Modification (F6636–10×1L, Sigma-Aldrich) supplemented with gentamicin (50 μg/mL) (LONZA, Basel, Switzerland), polymyxin B (50 μg/mL) (Sigma-Aldrich), and 10% fetal bovine serum (BioWhittaker, Cologne, Germany) in a 95% air, 5% CO_2 atmosphere at 37°C. At 15 h before GSPE treatment, the media was replaced with serum-depleted media (Coon's modified Ham's F12) supplemented with 100 μM oleic acid (MERCK, Germany) and 40 μM BSA (bovine serum albumin, fatty acid free, Sigma-Aldrich). FAO cells were treated with 10, 25, 50, or 100 mg GSPE per liter of media to select the working dose. For kinetic experiments, cells were treated with 25 mg GSPE per liter of media. GSPE was dissolved in ethanol and added to the culture media; the final concentration of ethanol in the media was 0.05%, a nontoxic percentage. miRNAs and mRNAs were extracted at 0, 0.5, 1, 3, and 5 h after GSPE treatment.

2.4 Animals and experimental design

Male Wistar rats weighing 225 g were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of our university approved all procedures (reference number 4249 by Generalitat de Catalunya). Animals were housed in animal quarters at 22°C with a 12 h light/dark cycle (light from 8:00 to 20:00 h) and fed ad libitum with a standard chow diet (Panlab, Barcelona, Spain).

At 9 a.m. on the day of the experiment, the rats (five animals per group) were orally gavaged with lard oil (2.5 mL/kg body weight) (control group) or GSPE (250 mg/kg body weight) dissolved in lard oil (GSPE group).

At 0, 1, or 3 h after treatment, the rats were sedated using a combination of ketamine (70 mg ketamine/kg body weight, Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg xylazine/kg body weight, Bayer, Barcelona, Spain). After anesthetization, the rats were exsanguinated from the abdominal aorta. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 *g*, 15 min, 4°C) and stored at –80°C until analysis. The liver was excised, frozen immediately in liquid nitrogen, and stored at –80°C until RNA and lipid extraction.

2.5 RNA extraction

Total RNA containing small RNA species was extracted from frozen liver and from FAO cells using the mi/mRNA extraction kit (miRNA kit, E.Z.N.A., Omega Bio-tek, Norcros, USA) according to the manufacturer's protocol. To isolate both total RNA and miRNA, 1.5 volumes of absolute ethanol were added instead of the recommended 0.33 volumes in

step 5. The washing step was performed according to the isolation of large RNAs.

The hepatocytes were washed twice with PBS before extraction. The quality of the purified RNA was checked using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.6 miRNAs quantification by real-time (qRT) PCR

To analyze the expression of each miRNA, reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA-specific reverse transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (Long Gene) was used. The reaction was performed at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The final total RNA concentration used was 2.5 ng/μL. We used 1.33 μL of these diluted cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems) and the associated specific probe provided in the TaqMan® MicroRNA Assay Kit (Applied Biosystems). Specific Taqman probes were used for each gene: microRNA-122 (miR-122: hsa-mir-122), 5′-UGGAGUGUGACAAUGGUGUUUG-3′, and microRNA-33 (miR-33: hsa-mir-33), 5′-GUGCAUUGUAGUUGCAUUG-3′. The results were normalized to the expression of the U6 small nuclear RNA, which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS RT-PCR system (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change in the miRNA level was calculated by the log 2 scale according to the equation $2 - \Delta\Delta Ct$, where $\Delta Ct =$ Ct miRNA – Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples – ΔCt untreated controls.

2.7 mRNA qRT-PCR

mRNA levels were evaluated by reverse transcription performed using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (long gene) was used. The reaction was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. The final total RNA concentration used was 25 ng/μL in 125 μL. We used 5 μL of this diluted cDNA solution for subsequent quantitative RT-PCR amplification using TaqMan Universal PCR master mix (Applied Biosystems). Specific Taqman probes were used for each gene: Abca1 (Rn00710172_m1), Fasn (Rn00569117_m1), CPT1α (Rn00580702_m1). The results were normalized to cyclophilin (PPIA: Rn00690933_m1), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS RT-PCR system (Applied Biosystems) with a protocol of 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The fold change in the mRNA level was calculated by the log 2 scale using the equation $2 - \Delta\Delta Ct$, where $\Delta Ct =$ Ct miRNA – Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples – ΔCt untreated controls.

2.8 Western blot analysis

Proteins were extracted using radio-immunoprecipitation buffer (RIPA: 100 nM Tris-Cl pH 7.4 (300 nM NaCl), Tween 10%, Na-Deoxycholate 10%, H_2O Milli-Q). Equal amounts of proteins, 75 μg for cells, were resolved on 7.5% and 5% Tris-glycine polyacrylamide minigels, for Abca1 and Fas, respectively, and transferred to polyvinylidene fluoride (PVDF) membranes (Immun-Blot PVDF Membrane for Protein Blotting, BR05814503, Bio-Rad Laboratories, UK) using a tank-transfer system. Membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) and incubated with primary antibodies in TBS containing 0.05% Tween-20 overnight at 4°C. Primary antibodies were used at the following dilutions: Abca1 at 1:1000 (ab18180, abcam, Cambridge, UK), Hsp90 at 1:1000 (610419, BD Biosciences, Franklin Lakes, NJ, USA), Fas at 1:5000 (ab128870, abcam). Secondary antibodies were used at the following dilutions: secondary antibody to mouse IgG-H&L (HRP) (ab6728, abcam) was used at 1:5000 and secondary antibody to rabbit (NA934V, Amersham, Buckinghamshire, UK) was used at 1:10 000 in 5% skimmed milk in TBS containing 0.05% Tween-20. Signals were revealed using an enhanced chemiluminescence reagent (ECL Plus Western Blotting Detection System, RPN2132, Amersham), and digital images were taken with a Chemi XL1.4 Camera (Syngene, Cambridge, UK), which permits the semiquantification of the band intensity. Hsp90 was use as an endogenous protein control.

2.9 Plasma and liver lipid analysis

Plasma total cholesterol and TGs were measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). Liver (0.5 g) lipids were extracted using the Folch method [34]. An aliquot of extract was subjected to gravimetry to measure total lipids. The remaining extract was allowed to evaporate under a nitrogen draft and redissolved in a 2:1 mixture of chloroform (Panreac) and methanol (Panreac) and further diluted with NaCl (Panreac). The TG and cholesterol concentrations in the dissolved extract were measured using QCA enzymatic colorimetric kits (QCA) following the manufacturer's protocols.

2.10 Statistical analysis

The results are reported as the mean ± SEM of three independent in vitro experiments or five animals for in vivo experiments. Group means were compared with an independent samples Student's *t*-test ($p \leq 0.05$) using SPSS software.

Table 2. Flavanols and their metabolites quantified in rat plasma over a 1-h period after ingestion of an acute intake of grape seed procyanidin extract (250 mg/kg) with lard oil. The data are given as the mean (μM) ± SEM ($n = 4$–5)

	Total amount (μM)
Compound	
Catechin	0.07 ± 0.01
Epicatechin	0.39 ± 0.02
Procyanidin dimer B2	0.11 ± 0.01
Procyanidin dimer B1 + B3	0.12 ± 0.01
Gallic acid	0.48 ± 0.04
Metabolite	
Catchin-glucuronide	>6.13 ± 0.00
Epicatechin-glucuronide	>6.13 ± 3.21
Methyl-catechin-glucuronide	2.78 ± 0.37
Methyl-epicatechin-glucuronide	1.18 ± 0.14
Catechin-sulfate	nd
Epicatechin-sulfate	nd
3-o-methyl-epicatechin	0.05 ± 0.00
4-o-methyl-epicatechin	0.05 ± 0.01
Methyl-catechin-o-sulfate	0.24 ± 0.01
Methyl-epicatechin-o-sulfate	0.62 ± 0.06

nd: not detected.

3 Results

3.1 Flavanols and their metabolites in plasma

GSPE mostly contains timeric and dimeric proanthocyanidins, but also the monomeric flavon-3-ol catechin, epicatechin, and epicatechin gallate are abundant [32] (see material and methods section). After 1 h of an acute ingestion of GSPE (250 mg/kg body weight) with lard oil, the free forms of catechin, epicatechin, and dimeric procyanidins were determined in rat plasma. The main conjugation forms of catechin and epicatechin were also determined, being the most abundant the glucuronide forms. Moreover, the methyl and sulfate conjugations of catechin and epicatechin were determined in plasma. However, catechin-sulfate and epicatechin-sulfate were not detected (Table 2). These data show that at a concentration of GSPE of 250 mg/kg, the main flavanols metabolites are present in rat plasma after 1 h of ingestion at concentrations in the micromolar range.

3.2 GSPE decreased TG and cholesterol levels in plasma and liver

In control animals, plasma TG levels increased markedly 3 h after the oral administration of 2.5 mL of lard oil/kg of body weight (Table 3). The simultaneous administration of GSPE (250 mg/kg body weight) significantly blocked the increase of plasma TG levels induced by lard oil. However, the simultaneous administration of GSPE had no significant effect on plasma cholesterol level.

TG, cholesterol, and total lipids were quantified to determine the effect of GSPE on liver lipids (Table 3). At 3 h after the administration of lard oil, the TG and cholesterol contents in the liver increased by approximately 40% with respect to the basal condition. As in the plasma, the simultaneous administration of GSPE significantly prevented the accumulation of lipids in the liver after lard oil treatment. These data show that GSPE improves lipid tolerance, both in the plasma and the liver.

3.3 GSPE repressed miR-122 and miR-33 expression and modulated the expression of their target genes in the liver in vivo

Relative changes in the levels of miR-122 and miR-33 in the rat liver were quantified by RT-PCR at 1 and 3 h after the administration of lard oil alone or in combination with GSPE (Fig. 1). At 1 h after administration, GSPE treatment decreased miR-122 and miR-33 levels by 34% and 60%, respectively. At 3 h after GSPE administration, miR-33 levels were similar to those in the livers of rats given lard oil only, whereas miR-122 levels increased by 38%. These results indicate that the repression of miR-122 and miR-33 induced by GSPE was rapid and transient.

To validate the effects of GSPE on miR-122 and miR-33, we quantified the relative changes in the expression levels of several target genes of these miRNAs (Fig. 1). We have chosen Fas for miR-122, Abac1 for miR-33, and CPT1α for both miRNAs. No changes were found in the expression of Fas, CPT1α, or Abca1 after 1 h of GSPE administration. However, Fas was significantly repressed and Abca1 was significantly

Table 3. Triglyceride and cholesterol levels in the plasma and livers of rats fed lard oil with or without proanthocyanidins (grape seed proanthocyanidin extract (GSPE))

	Basal (0 h)	Lard (3 h)	Lard + GSPE (3 h)
Plasma triglycerides (g/100 mL plasma)	71.4 ± 6.0	123.1 ± 6.6	73.4 ± 12.4[a)]
Plasma cholesterol (g/100 mL plasma)	18.5 ± 2.5	31.6 ± 6.1	26.1 ± 3.7
Total liver lipids (g/100 g liver)	6.7 ± 0.5	7.1 ± 0.5	5.5 ± 0.3[b)]
Liver triglycerides (g/100 g liver)	1.7 ± 0.2	2.4 ± 0.2	1.45 ± 0.33[a)]
Liver cholesterol (g/100 g liver)	0.9 ± 0.1	1.3 ± 0.1	0.9 ± 0.01[a)]

Rats fasted for 14 h were orally administered lard oil (2.5 mL/kg) with or without GSPE (250 mg/kg). Lipids were quantified before treatment (0 h) and at 3 h after GSPE administration. The values are the means of five animals per group.
a) Significant difference between the lard group and the lard + GSPE group.
b) Significant difference between the basal group and the lard + GSPE group ($p < 0.05$; Student's t test).

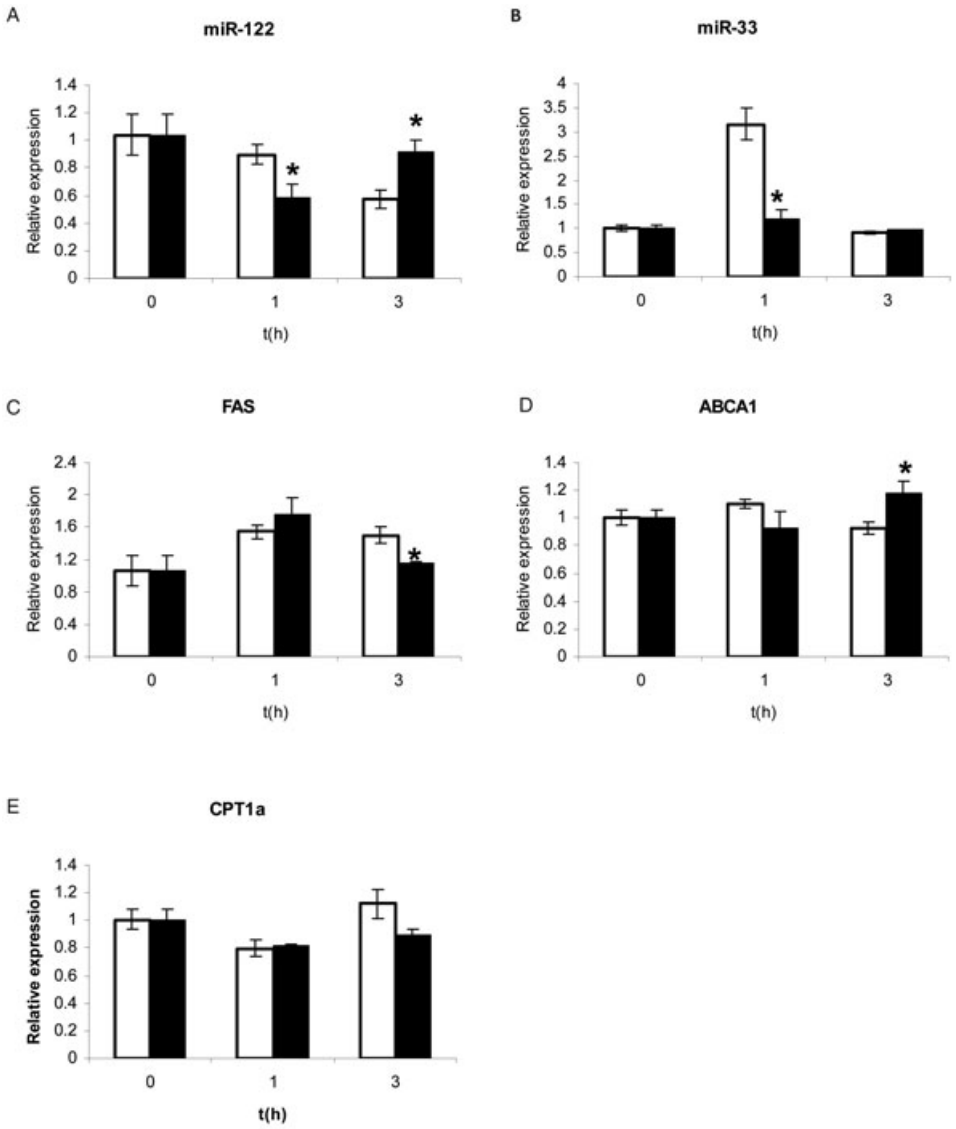

Figure 1. Levels of miR-122 (A), miR-33 (B), and their target mRNAs (C–E) in the livers of rats fed on lard oil with or without proanthocyanidins (grape seed proanthocyanidin extract [GSPE]). Rats were fasted for 14 h and then orally administered lard oil (2.5 mL/kg) with or without GSPE (250 mg/kg). RNAs were quantified prior to treatment (0 h) and at 1 and 3 h after GSPE administration. miRNA levels were normalized to U6 small nuclear RNA. The values shown are the means of five animals per group. White bars, control group; black bars, GSPE treated group. *Significant difference between the lard group and the lard + GSPE group ($p < 0.05$; Student's *t* test).

overexpressed at 3 h after GSPE administration. Additionally, CPT1α tended to be repressed at this time. These results show that GSPE modulated the expression of miR-122 and miR-33 prior to that of their target genes, Fas, CPT1α, and Abca1.

3.4 GSPE repressed miR-122 and miR-33 expression and modulated their target genes at mRNA and protein levels in FAO cells

To assess whether GSPE repressed miR-122 and miR-33 directly, we studied the effect of GSPE treatment in vitro using the rat hepatoma FAO cell line. To select the working dose of GSPE, FAO cells were treated with different doses of GSPE, and changes in miR-122 and miR-33 levels were analyzed after 1 h of treatment (Fig. 2). The expression of both miRNAs was decreased significantly at a dose of 25 mg/L of GSPE. However, higher doses of GSPE (50 and 100 mg/L) did not increase the repression of either miR-122 or miR-33. Consequently, we chose to use 25 mg/L of GSPE in the kinetic experiments.

Figure 3 shows the expression kinetics of miR-122 and miR-33 from 0 to 5 h after GSPE treatment in FAO cells. No changes in miRNAs levels were observed after 30 min of treatment. However, after 1 h of treatment, GSPE decreased the levels of miR-122 and miR-33 by 34% and 39%, respectively. miR-33 expression was further decreased (up to 50%) at 3 h and returned to the baseline value after 5 h of treatment. In contrast, miR-122 expression remained low until 5 h of treatment (62% decrease). Thus, GSPE directly modulates the expression of miR-122 and miR-33 in hepatic

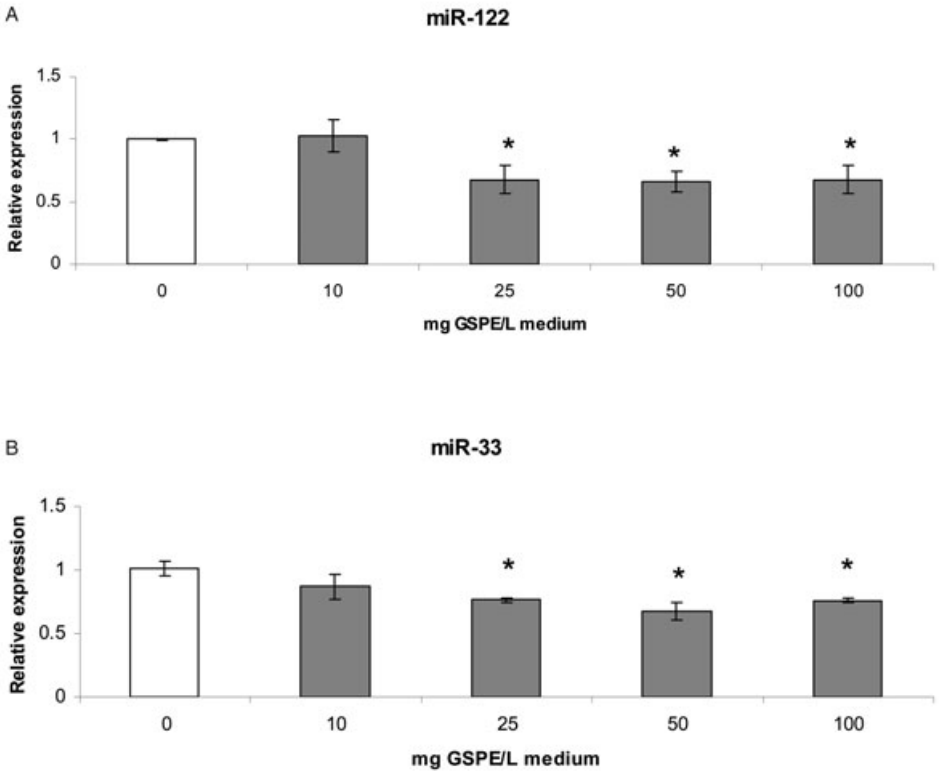

Figure 2. The effect of increasing doses of proanthocyanidin extract on miR-122 (A) and on miR-33 (B) levels in FAO cells. FAO cells were treated with the corresponding concentrations of GSPE for 1 h. miRNA levels were determined by RT-qPCR and normalized to U6 small nuclear RNA levels. All values shown are the means of three independent experiments. *Significant difference between control cells (0 mg/L) and treated cells ($p < 0.05$; Student's *t* test).

cells. GSPE repressed miR-122 and miR-33 in FAO cells with kinetics similar to those observed in vivo (1 h), but compared with the in vivo system, the repression was constant and not transient. Moreover, both in vivo and in vitro systems show that miR-33 was repressed to a greater degree than miR-122.

Abca1 and Fas expression was also analyzed in FAO cells (Fig. 3). Abca1 was overexpressed after 1 and 3 h of GSPE treatment. Fas was repressed only after 5 h of treatment. Moreover, after 5 h of treatment the protein levels of these target genes were analyzed (Fig. 4). According to mRNA levels, the Abca1 protein was increase and Fas was decrease.

4 Discussion

Flavonoid consumption is associated with lower risk of death from CVD. More specifically, five subclasses of flavonoids, including proanthocyanidins, are individually associated with lower risk of fatal CVD [35]. One of the mechanisms by which proanthocyanidins exert their cardiovascular protection is by reducing postprandial hypertriglyceridemia and LDL cholesterol [9]. Previously, we showed that proanthocyanidins repress TG secretion in the liver through an farnesoid X receptor- and small heterodimer partner-dependent pathway [36, 37]. However, lipid metabolism in liver is controlled by diverse signaling pathways, including miR-122 and miR-33, working in concert [15, 17]. We have used microarray analyses to show that proanthocyanidins from grape seed and cacao are able to modulate miRNAs levels in HepG2 cells after 5 h of treatment [31]. However, we did not observe any significant modulation of miRNAs related to lipid metabolism at 5 h. Therefore, because GSPE hypolipidemic action is very fast, we hypothesized whether miRNA regulators of lipid metabolism, such as miR-122 and miR-33, could be modulated by proanthocyanidins at earlier timepoints. Our results show that proanthocyanidin treatment significantly reduced miR-122 and miR-33 levels in rat hepatic cells after 1 h of GSPE treatment, both in vivo and in vitro.

Previously, to determine the effect of GSPE on miRNAs levels in liver in vivo, we performed a lipid tolerance test to confirm the hypolipidemic effects of GSPE administration. The administration of lard oil concomitant with GSPE produced a clearly hypolipidemic effect, reducing plasma TG by a 40%, plasma cholesterol by 18%, and liver TG and cholesterol by 40% relative to the levels in animals given lard oil alone. The hypolipidemic effect of GSPE was similar to those observed previously using the same animal model [12]. Therefore, the livers of these animals were a good model in which to study the roles of miR-122 and miR-33 in the hypolipidemic effects of GSPE.

GSPE decreased miR-122 and miR-33 levels in liver very rapidly (within 1 h), but after 1 h of GSPE ingestion the concentration of flavanols and their metabolites were already abundant in plasma in the micromolar range. miR-122 inhibition reduces plasma cholesterol levels in normal mice

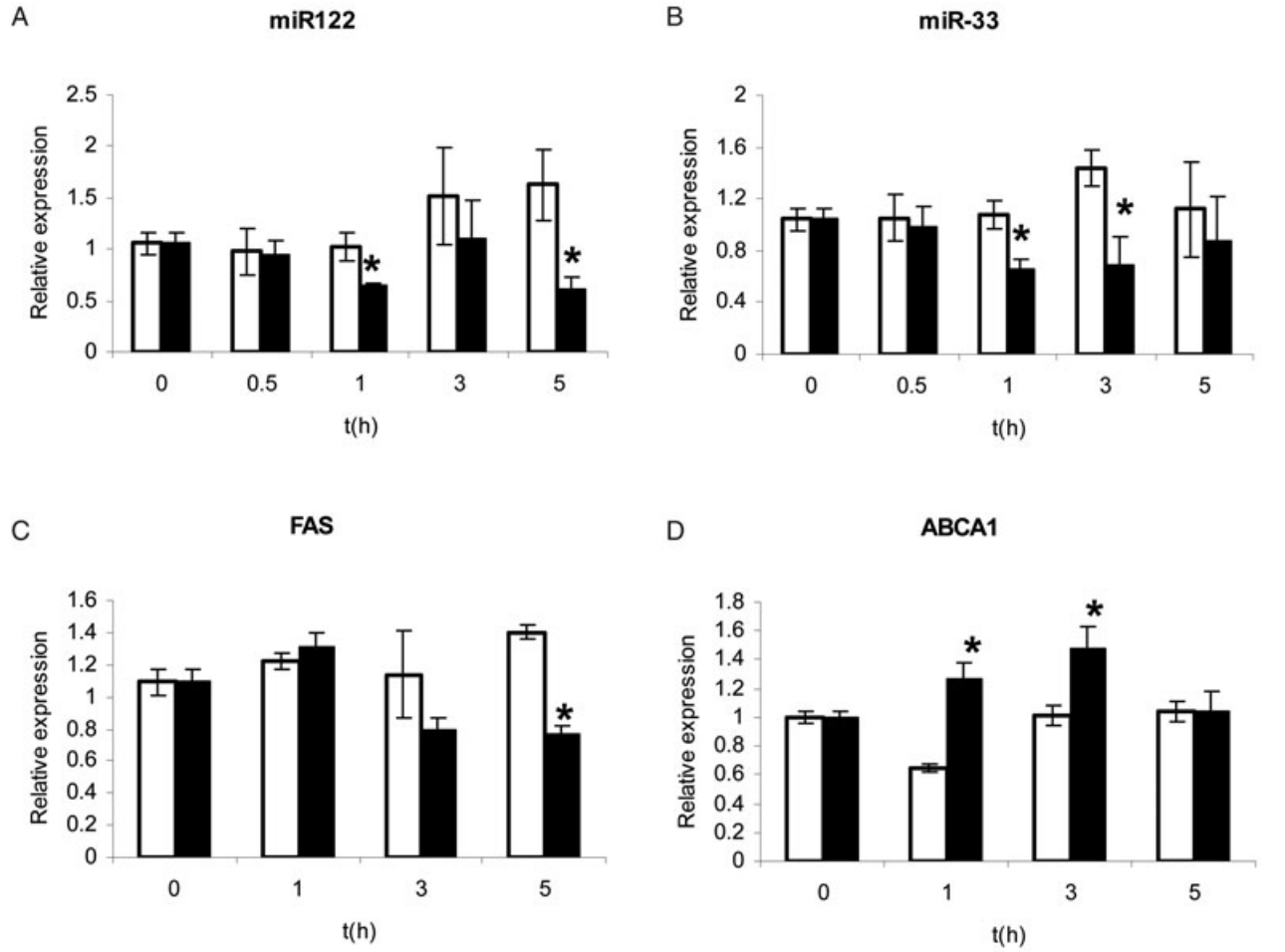

Figure 3. The effects of proanthocyanidin extract on the levels of miR-122 (A), miR-33 (B), and their target mRNAs (C, D) in FAO cells. FAO cells were treated with 25 mg GSPE per liter for 0.5, 1, 3, or 5 h. miRNA levels were determined by RT-qPCR and normalized to U6 small nuclear RNA. All values shown are the means of three independent experiments. White bars, control group; black bars, GSPE treated group. *Significant difference between control cells and treated cells ($p < 0.05$; Student's *t* test).

and decreases plasma cholesterol and hepatic TG levels in a mouse model of diet-induced obesity [38]. Moreover, miR-33 inhibition lowers VLDL-TG in nonhuman primates [39]. Therefore, these miRNAs may be considered putative mediators of the hypolipidemic effect of GSPE.

The repression of miR-122 and miR-33 induced by GSPE in the liver in vivo could be secondary to hormonal changes and/or to variations in the nutrient supply to the liver as a result of the action of GSPE in other organs, such as the intestines. For this reason, we studied the effect of GSPE in hepatic cells in vitro, in which the growth conditions and nutrient composition of the media were the same for control and treated cells and could be strictly controlled. Using this experimental approach, GSPE repressed miR-122 and miR-33 in FAO cells, similar to its effects on the liver in vivo. Therefore, the repression of miR-122 and miR-33 is a direct outcome of GSPE activity in hepatic cells. Moreover, the repression of miR-33 is greater than the repression of miR-122 in both hepatocyte models in vivo and in vitro. This finding suggests that the hypolipidemic effects of GSPE are more strongly mediated by miR-33 than by miR-122.

In contrast to the transitory effects of GSPE on miRNAs in vivo, the repression of miR-122 and miR-33 by GSPE in FAO cells was persistent. The pharmacokinetics of proanthocyanidins in vivo could be responsible of this discrepancy. In vivo, proanthocyanidin monomers and dimers are absorbed very rapidly, picking at 1–2 h after consumption, and then eliminated from the body [32]. A concentration of 25 mg/L is needed in vitro to reach the significant reduction of miRNAs. A global estimation of the total concentration of catechin and epicatechin (free and conjugated forms) in plasma indicated that in vivo the concentration was five times higher than

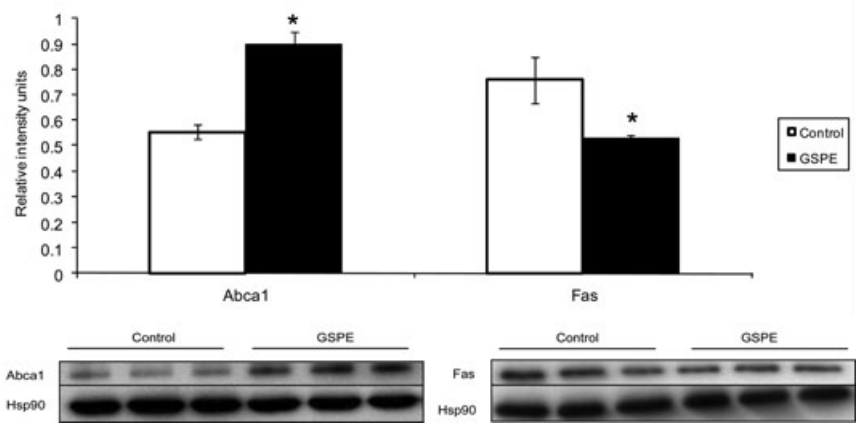

Figure 4. The effects of proanthocyanidin extract on the protein levels of Abca1 and Fas in FAO cells. FAO cells were treated with 25 mg GSPE per liter for 5 h. Proteins were extracted with radioimmunoprecipitation (RIPA) buffer and analyzed with Western blot technique. Proteins were normalized with an endogenous protein, Hsp90. Relative intensity units were obtained dividing the intensity band of the protein problem between the intensity band of the endogenous protein. All values are the means of two independent experiments. *Significant difference between control cells and treated cells ($p < 0.01$; Student's *t* test).

catechin and epicatechin in media at 25 mg GSPE per liter. Although the in vivo and in vitro conditions are not exactly comparable, it will be possible that a lower GSPE concentration in vivo could be also effective repressing miRNAs levels. The used dose of 250 mg of GSPE per kilogram body weight in rats is equivalent to 40.5 mg of GSPE per kilogram body weight in humans [40]. Therefore, for a 70 kg man, this dose corresponds to an intake of 2.8 g of GSPE. Hence, the used dose in this work is six times higher to the estimated proanthocyanidin intake in the high quintile of U.S. population [35]. Therefore, a proanthocyanidin-rich diet could be enough to modulate miRNAs in humans.

The effects of GSPE on the mRNA levels of Fas and Abca1 were consistent with those induced by miR-33 and miR-122. Abca1 mRNA, which is repressed by miR-33 [41], was overexpressed following GSPE treatment. In contrast, Fas mRNA was repressed by GSPE, consistent with the observation that mRNAs involved in lipogenesis tend to be downregulated when miR-122 is inhibited [25, 38]. The Abca1 and Fas protein levels in FAO cells were changed by GSPE correlating with the mRNA levels changes and miRNAs effects. Moreover, GSPE modulated miR-122 and miR-33 expression before effects on Fas and Abca1 were observed, both in vivo and in vitro. Taken together, these data reinforce the hypothesis that miR-122 and miR-33 mediate the hypolipidemic effect of GSPE.

In the liver, Abca1 activity is a rate-limiting step in the formation of HDL and a key determinant of circulating HDL levels [42]. Abca1 mediates cholesterol efflux from hepatic cells for apolipoprotein A-I lipidation, decreasing the hepatic pool of cholesterol [43]. Therefore, the reduction of liver cholesterol induced by GSPE could be explained by an increase of cholesterol transport to form and stabilize nascent HDL via an increase of Abca1 expression levels mediated by miR-33. Moreover, the reduction of plasma and liver TG levels after GSPE administration could be a result of decreased fatty acid synthesis due to Fas repression mediated by miR-122. Also, both miR-33 and miR-122 regulate genes that control fatty acid β-oxidation, and both target CPT1α [44, 45]. But, CPT1α mRNA levels in liver in vivo did not change, even though this gene is modulated by both of these miRNAs.

Little is known about the mechanisms that regulate the expression of miR-122 in the liver. However, the regulation of miR-33 is somewhat better understood. There are two isoforms of miR-33, miR-33a and miR-33b, that are intronic of Srebf2 and Srebf1 genes, respectively [41]. The molecular mechanism by which proanthocyanidins modulate miRNAs levels is unknown. However, there is evidence that polyphenols can bind to mRNAs and proteins [46, 47]. Therefore, it is possible that they also bind to miRNAs or to some component involved in miRNA biogenesis, such as DICER or RISC. More studies will be necessary to identify the mechanism by which GSPE modulate miR-33 and miR-122 levels in liver.

In conclusion, GSPE represses miR-33 and miR-122 in rat hepatic cells, both in vivo and in vitro. The repression of these miRNAs by GSPE is rapid and transient. Moreover, GSPE represses Fas and promotes the expression of Abca1, both of which are target genes of these miRNAs. These data suggest that GSPE increases liver cholesterol efflux to stabilize and promote HDL formation and reduce fatty acid synthesis. Therefore, the repression of miR-122 and miR-33 can be considered a new mechanism of action through which proanthocyanidins exert hypolipidemic effects in the liver.

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