JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Grape Seed-Derived Procyanidins Decrease Dipeptidyl-peptidase 4 Activity and Expression

Noemi González-Abuín, Neus Martínez-Micaelo, Mayte Blay, Gerard Pujadas, Santiago Garcia-Vallvé, Montserrat Pinent,* and Anna Ardévol

Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain

Supporting Information

ABSTRACT: Dipeptidyl-peptidase 4 (DPP4) inhibitors are among the newest treatments against type 2 diabetes. Since some flavonoids modulate DPP4 activity, we evaluated whether grape seed-derived procyanidins (GSPEs), which are antihyperglycemic, modulate DPP4 activity and/or expression. In vitro inhibition assays showed that GSPEs inhibit pure DPP4. Chronic GSPE treatments in intestinal human cells (Caco-2) showed a decrease of DPP4 activity and gene expression. GSPE was also assayed in vivo. Intestinal but not plasmatic DPP4 activity and gene expression were decreased by GSPE in healthy and diet-induced obese animals. Healthy rats also showed glycemia improvement after oral glucose consumption but not after an intraperitoneal glucose challenge. In genetically obese rats, only DPP4 gene expression was down-regulated. Thus, procyanidin inhibition of intestinal DPP4 activity, either directly and/or via gene expression down-regulation, could be responsible for some of their effects in glucose homeostasis.

KEYWORDS: *diabetes, DPP4 inhibitors, intestinal DPP4, procyanidins*

INTRODUCTION

The high prevalence in developed societies of obesity and the related-associated pathologies, such as insulin resistance and diabetes, highlights the relevance of finding bioactive food components capable of ameliorating these situations. Dipeptidyl-peptidase IV (DPP4) inhibitors are among the newest treatments against type 2 diabetes.[1] DPP4 is a prolyl peptidase that cleaves proteins and peptides with proline or alanine as the penultimate residue and is widely distributed in almost all human tissues and fluids.[2] The gut-derived peptide hormones GLP-1 and GIP, also known as incretins, are among DPP4's targets. Incretins are released in response to glucose ingestion, enhancing insulin secretion by the pancreas. Evidence suggests that incretins might also act to increase β-cell mass and to protect the pancreas from apoptosis (reviewed in refs 3 and 4). Cleavage of incretins by DPP4 starts almost immediately after their secretion due to the presence of DPP4 at their site of production, which results in a short half-life that is less than two minutes.[5] Preventing the degradation of endogenous incretin hormones by inhibiting DPP4 therefore has emerged as a strategy for the control of glucose homeostasis.

Procyanidins are among the bioactive compounds that have been shown to modulate glucose homeostasis. Procyanidins are phenolic structures, abundant in fruits and vegetables, with a wide variety of beneficial effects, and they can act as cardioprotectants, antioxidants, and hypolipidemic agents (reviewed in refs 6 and 7). To exert their effects, procyanidins might use different mechanisms, such as directly interacting and modulating the activity of signaling proteins and/or prevent oxidation.[8] Procyanidins can also interact with transcription factors[9] and enzymes.[10] Several studies have shown an antihyperglycemic effect of procyanidins extracted from grape seed in insulin resistant animals.[11−13] Given the emerging role of DPP4 as a target for glucose-homeostasis regulation, it could be hypothesized that the effects of procyanidins might also be mediated by the modulation of DPP4. There are only a few studies on the effects of phenolic compounds on DPP4 activity, and published results show differential effects. The potential beneficial effects of the phenolic compounds curcumin have been studied to determine if they resulted from the inhibition of DPP4; however, no such inhibitory effect was observed.[14] Similarly, synthetic derivatives of flavone-8-acetic acid, which act as inhibitors of APN/CD13 ectopeptidase, were shown to be unable to inhibit DPP4 peptidase.[15] However, a recent paper has shown that a plant (*P. microphylla*) extract enriched in monomeric flavonoids can modulate in vivo glucose homeostasis and inhibit plasma DPP4 activity when administered intraperitoneally.[16] One of the monomers that this DPP4-inhibitory extract contains is apigenin-7-*O*-glucoside, but its nonglycosylated form, apigenin (as well as genistein but not kaempferol), up-regulates cell-surface CD26 (another name for DPP4) and increases DPP4 activity in human colorectal cancer cells (HT-29) after a 2-day treatment.[17] Finally, a 3-day treatment with grape seed procyanidins in both healthy rats and rats with acute renal failure showed an increase in DPP4 activity in the kidney.[18] Thus, particular flavonoids can modulate the DPP4 activity, although these effects are highly dependent on the flavonoid structure, the experimental conditions, and models used. The present study was therefore developed in order to evaluate whether grape seed-derived procyanidins modulate DPP4 activity.

Received: March 8, 2012
Revised: June 28, 2012
Accepted: August 14, 2012
Published: August 14, 2012

dx.doi.org/10.1021/jf3010349 | *J. Agric. Food Chem.* 2012, 60, 9055−9061

Table 1. In Vitro DPP4 Inhibition[a]

		mg GSPE/L				
		0	10	50	100	200
human	recombinant commercial DPP4	0 ± *0*	13.20 ± *4.1*	37.16 ± *0.0**	54.67 ± *0.7**	69.45 ± *0.7**
	intestinal CaCo2 cells	0 ± *0*	11.68 ± *5.6*		7.45 ± *1.5**	17.33 ± *8.6*
	saliva	0 ± *0*	11.18 ± *4.5**		12.35 ± *4.0**	21.25 ± *6.4**
rat	gut	0 ± *0*		19.68 ± *0.1**	36.88 ± *0.1**	39.60 ± *0.1**
	plasma	0 ± *0*		2.89 ± *0.1**	12.99 ± *0.0**	14.96 ± *0.1**

[a]DPP4 extracted from different sources was incubated with different GSPE concentrations. The data are displayed as % inhibition of the DPP4 activity mean ± SEM. *, significant differences vs 0 mg GSPE/L at $P < 0.05$.

MATERIALS AND METHODS

Materials. The grape seed procyanidin extract (GSPE) was purchased from Les Dérivés Résiniques et Terpéniques (Dax, France). The extract contains essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 U; 31.7%) procyanidins.[19,20]

Cell culture reagents were obtained from BioWhittaker (Vervier, Belgium), and the Bradford reagent was from Sigma-Aldrich (Madrid, Spain).

Glucose plasma concentrations were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). Insulin plasma concentrations were determined using Rat Insulin ELISA and Ultrasensitive Rat Insulin ELISA (Mercodia, Uppsala, Sweden). DPP4 activity was measured using the DPP4 Drug Discovery Kit-AK499 (Enzo Life Sciences International, Inc.).

Animal Experimental Procedures. Several groups of animals (detailed below) were purchased from Charles River Laboratories (Barcelona, Spain), housed in animal quarters at 22 °C with a 12-h light/12-h dark cycle, maintained for 1 week in quarantine, and then used for the experiments described below (and summarized in Table 1, Supporting Information). Females were used for the experiments due to their higher sensitivity to the cafeteria diet.[21] After the different GSPE treatments, the animals (except those of the cafeteria treatment) were anesthetized with 50 mg of pentobarbital/kg body weight (bw) and sacrificed by bleeding. The blood was collected, and animal tissues were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. All of the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (permission number: 4250).

Acute GSPE Treatment on Healthy Rats. Overnight-fasted Female Wistar rats were divided into two groups (6 animals/group): (i) the control group, treated with the vehicle (water), and (ii) the GSPE group, treated with 1 g GSPE/kg of bw dissolved in water. Treatment of both groups was administered by an oral gavage after overnight fasting. Tail blood samples were taken at 0 and 60 min of treatment, after which the animals were sacrificed.

Chronic GSPE Treatment on Healthy Rats. Wistar female rats were divided into two groups (11 animals/group): (i) the control group, treated with the vehicle (sweetened condensed milk, diluted 1:10 with water), and (ii) the GSPE group, treated with 25 mg GSPE/kg bw per day. Food was withdrawn daily at 8 a.m. and at 8 p.m., at which time the rats were fed either the vehicle or GSPE dissolved in the vehicle by controlled oral intake with a syringe, and immediately afterward, the food was renewed. At day 19, the rats were fasted overnight, and two glucose tolerance tests were carried out: (i) an oral glucose tolerance test (OGTT), 2 g glucose/kg bw dissolved in water (6 animals/group), and (ii) and an intraperitoneal glucose tolerance test (IPGTT), 1 g glucose/kg bw dissolved in water (the remaining 5 animals/group). Tail blood samples were taken at 0, 15, 30, and 120 min after the glucose load, and plasma glucose and insulin were measured. After 45 days of GSPE treatment, the animals were sacrificed after an overnight fast.

Chronic GSPE Treatment on Cafeteria-Fed Rats. To study the effects of a chronic GSPE treatment on obese animals, obesity was induced by a cafeteria diet (bacon, sweets, biscuits with pâté, cheese, muffins, carrots, and milk with sugar), as previously described.[11] Briefly, female Wistar rats were divided into two groups: a control group (6 animals) fed with standard diet and a cafeteria group (12 animals) fed with standard diet plus a cafeteria diet. After 13 weeks, the cafeteria group was divided into two subgroups (6 animals/group): a control cafeteria group, treated with the vehicle (sweetened condensed milk), and a cafeteria + 25 group, treated daily with 25 mg of GSPE/kg bw (dissolved in the same vehicle). The diet and GSPE dose were daily administered at 9.a.m. After 30 days of GSPE treatment, rats were sacrificed by beheading after three hours of fasting.

Chronic GSPE Treatment on Zucker fa/fa Rats. To work with a genetic model of obesity, we used Zucker animals. This strain has a genetic defect in the leptin receptor causing obesity under homozygosis. Female Zucker rats were divided into three groups (10 animals/group): (i) the control group (Lean), (ii) the fa/fa group, treated with the vehicle (sweetened condensed milk, diluted 1:6 with water), and (iii) the fa/fa + GSPE group, treated daily with 35 mg GSPE/kg bw. Animals were fasted at 9 a.m. every day; at 4 p.m., vehicle or GSPE dissolved in the vehicle was administered by controlled oral intake with a syringe, and 1 h later the food was renewed. At day 60 from the beginning of the GSPE treatment, the animals were sacrificed after an overnight fast.

Cell Culture and Treatments. Caco-2 cells were obtained from the ATCC (American Tissue Culture Collection) and cultured as previously described.[22] The cells were seeded into 12-well culture plates at a cell density of 3.5−4 × 10^4 cells/cm^2 and were used for the experiments after 10 days once the confluent monolayer had formed (3−4 days after seeding). To study the effect of GSPE on DPP4, Caco-2 cells were incubated at 500 mg of GSPE/L for 3 h, 750 mg of GSPE/L for 24 h, and 1, 10, or 100 mg of GSPE/L for 3 days in culture medium. GSPE was diluted in 100% ethanol to a 0.5% final concentration of ethanol on culture medium. Treated cells were used for DPP4 analysis, and cells incubated with the vehicle were designated as the control. At least three independent experiments were performed for each treatment.

Measurement of DPP4 Activity and in Vitro Inhibition Assay. DPP4 was extracted from the Caco-2 cells monolayer and rat intestine as previously described with some changes.[23,24] Caco-2 cells were washed twice with PBS and incubated for 5 min in lysis buffer (PBS containing 100 KIU/mL aprotinin and 1% Triton X-100); intestines were homogenized with the same lysis buffer. The obtained samples were first centrifuged at 1000*g* at 4 °C for 10 min to eliminate cellular remainders and then centrifuged twice at 20000*g* at 4 °C for 10 min. Supernatants were stored at −80 °C until analyses.

DPP4 activity from cell and intestine lysates, as well as from rat plasma, was measured following the manufacturer's instructions provided by DPP4 Drug Discovery Kit-AK499 with a few modifications based on the volume of the DPP4-containing sample, adjusting the final volume with Tris-HCl buffer. Protein levels from intestine and Caco-2 cell extracts were determined as previously reported.[25] DPP4 Drug Discovery Kit-AK499 was also used to test the in vitro effects of different concentrations of GSPE on a commercial recombinant human enzyme (provided by the kit). Each assay was performed 3−6 times.

Quantitative RT-PCR. The total RNA from Caco-2 cells was extracted using an RNeasy kit (Qiagen, Hilden, Germany), and the total RNA from intestine was extracted using the TRIzol reactive

following the manufacturer's instructions. cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR amplification and detection were done using TaqMan assay-on-demand probes (Applied Biosystems): Rn00562910_m1 and Hs00175210_m1 for DPP4 and rat and Caco-2 cells, respectively. The results were referenced to β-actin Rn00667869_m1 for rat and cyclophiline Hs99999904_m1 (Ppia) for Caco-2 cells.

Data Analyses. Results are expressed as the mean ± SEM. Effects were assessed by Student's *t* test. All calculations were made with SPSS software.

RESULTS

GSPE Inhibits DPP4 Activity. To assess whether procyanidins inhibit DPP4 activity, we incubated a commercial recombinant human enzyme with different GSPE concentrations. As shown in Table 1, GSPE is able to inhibit DPP4 activity, achieving around 70% inhibition at 200 mg/L of GSPE.

To check if the same inhibition occurs when DPP4 is derived from other sources, we reproduced the same assay using human salivary and intestinal (from Caco-2 cells) DPP4, as well as rat plasmatic and intestinal DPP4. The results showed that the inhibitory activity varies depending on the DPP4 source, always being lower than in the assay with the commercial enzyme. At 200 mg of GSPE/L, the inhibition of the human Caco-2 enzyme and human salivary enzyme is around 20% and that of the rat intestinal DPP4 is around 40%, but there is only around 15% inhibition in rat plasmatic DPP4 (Table 1).

Finally, we compared the human and rat DPP4 protein sequences to evaluate if they are structurally different enough to explain the observed differential GSPE effects. The two sequences show an 84.8% similarity, and only one amino acid of the active center is different (Figure 1, Supporting Information).

GSPE Inhibits DPP4 Activity and Expression in Human Intestinal Caco-2 Cells after a Chronic Treatment. We next assessed whether procyanidins exert inhibitory effects in living cells. To do so, we performed several GSPE treatments in human intestinal Caco2 cells. We found that an acute treatment of 3 h with a high concentration of GSPE (500 mg/L) did not inhibit DPP4 activity (1.00 ± *0.1* and 1.10 ± *0.1*, control and GSPE-treated, respectively). A 24-h treatment with a higher dose of GSPE (750 mg/L) also did not modify DPP4 activity (1.00 ± *0.1* and 1.01 ± *0.1*, control and GSPE-treated, respectively). However, a longer (3-day) treatment with 10 or 100 mg of GSPE/L led to an inhibition of DPP4 activity of around 20%, as shown in Figure 1A. These results suggested that the DPP4 inhibition could be due to a decrease in the DPP4 gene expression; therefore, mRNA levels were studied. As shown in Figure 1B, the higher dose of GSPE (100 mg/L) down-regulates DPP4 gene expression, while 10 mg of GSPE/L does not. All of the doses assayed were nontoxic (Figure 2, Supporting Information).

Oral Intake of GSPE Modulates DPP4 Activity and Gene Expression in Rats. We next assessed whether the DPP4 inhibitory effects of GSPE could also be found in vivo. To do so, we used several animal models. An acute (1 h) treatment with 1 g of GSPE/kg bw resulted in a 34.3% decrease in the intestinal DPP4 activity ($P < 0.05$) and tended to down-regulate its gene expression 21.93% ($P < 0.1$). A slight inhibition (10%) of DPP4 activity was also observed in healthy rats after a chronic (45 days) treatment of 25 mg of GSPE/kg bw. In this case, a stronger effect was observed at the gene expression level, which was down-regulated around 40% (Figure 2A and B). DPP4 did not modify plasma DPP4 activity in any of these experiments (Table 2).

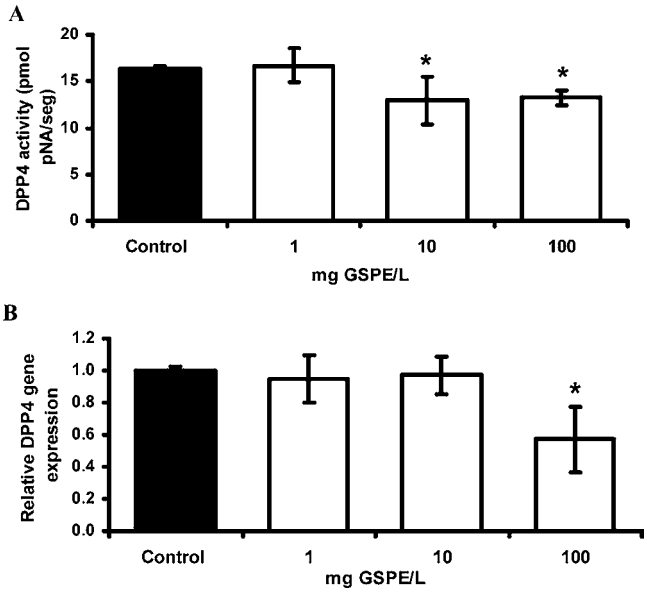

Figure 1. Dose–response effect on DPP4 activity (A) and gene expression (B) in Caco-2 cells treated with GSPE during 3 days. Cells treated with 0 mg GSPE/L were used as the control. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$.

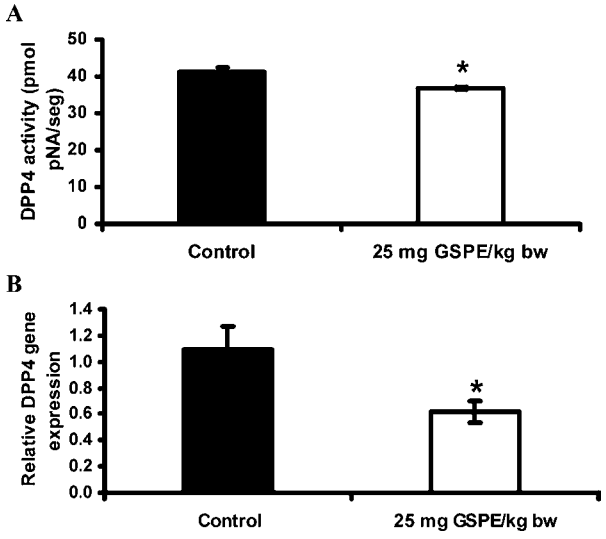

Figure 2. Effect on intestinal DPP4 activity (A) and gene expression (B) on healthy animals treated for 45 days with 25 mg of GSPE/kg bw. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$.

To test whether procyanidins have similar effects under glucose metabolism deregulation, we also tested the GSPE effects on two pathological animal models: one with diet-induced metabolic syndrome and another model of genetically obese rats. Diet-induced metabolic syndrome was achieved by feeding rats with a cafeteria diet. Afterward, these animals were treated with 25 mg of GSPE/kg bw for 30 days. DPP4 plasma activity was not modified due to either the diet or the procyanidins (Table 2). As shown in Figure 3, procyanidin treatment decreased intestinal DPP4 activity by around 40%

Table 2. In Vivo Plasmatic DPP4 Inhibition[a]

	treatment	plasmatic DPP4 activity (pmol pNA/seg)
healthy rats, acute treatment	control	1.54 ± *0.14*
	1 g/kg bw, 1 h	1.48 ± *0.10*
healthy rats, chronic treatment	control	1.53 ± *0.13*
	25 mg GSPE/kg bw, 45 days	1.54 ± *0.09*
Zucker rats	lean	1.54 ± *0.22*
	fa/fa	1.20 ± *0.15*
	fa/fa +35 mg GSPE/kg bw, 60 days	1.13 ± *0.10***
cafeteria rats	control	1.66 ± *0.08*
	cafeteria	1.28 ± *0.28*
	cafeteria +25 mg/kg bw, 30 days	1.52 ± *0.10*

[a]Plasmatic DPP4 activity was determined in different animal models treated with GSPE. The data are displayed as DPP4 activity, mean ± SEM. **, significant differences vs the control group at $P < 0.1$.

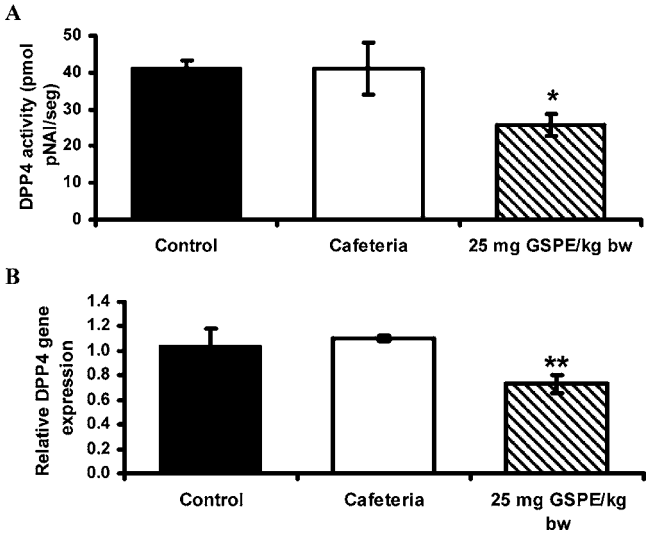

Figure 3. Effect on intestinal DPP4 activity (A) and gene expression (B) on cafeteria rats treated for 30 days with 25 mg of GSPE/kg bw. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$; **, significant differences vs the cafeteria group at $P < 0.1$.

and repressed gene expression by around 30%. The diet itself had no effect on these parameters when compared with with that of normally fed rats (Figure 3). Zucker fa/fa rats, which have genetic obesity, were treated with 35 mg of GSPE/kg bw for 60 days. In this case, only intestinal DPP4 expression was decreased by GSPE; no changes were observed in intestinal (Figure 4) or plasmatic (Table 2) DPP4 activity.

Effects of Chronic GSPE Treatment on the Plasma Insulin/Glucose Ratio after Glucose Tolerance Tests. Finally, we tested whether GSPE had differential effects on insulin secretion after an oral or intraperitoneal glucose tolerance test, which could be linked to a modulation of the incretin effect. To do so, an oral glucose tolerance test (OGTT) and an intraperitoneal glucose tolerance test (IPGTT) were performed in healthy rats that had been treated with 25 mg of GSPE/kg bw for 19 days. The glucose area under the curve (AUC) after an OGTT was lower in the GSPE treated animals than in the controls, an effect not found in the IPGTT (Figure 5A). This could be explained by the fact that the insulin secretion per plasma glucose after oral glucose administration was higher in the GSPE-treated rats (Figure 5B). Instead, the amount of insulin secreted per plasmatic glucose after an intraperitoneal glucose administration was not modified in the GSPE-treated rats when compared to that in control rats. Thus, GSPE-treated rats were more sensitive to orally administered glucose than controls, while the response to intraperitoneal glucose was not changed.

DISCUSSION

Given the fact that procyanidins affect glucose homeostasis[26] and that the enzyme DPP4 plays an important role in glucose homeostasis regulation, we have explored whether procyanidins modulate the enzyme DPP4. Tebib et al.[27] previously showed that very high concentrations of grape seed tannins are able to inhibit DPP4 intestinal activity. In the present study, we used a different approach; we gave moderate grape seed extract concentrations to mimic human daily food intake. Our results show that GSPE inhibits DPP4 activity and down-regulates its gene expression in the intestine.

The in vitro experiments show that GSPE can directly inhibit DPP4 activity. The level of inhibition depends on the source of the enzyme: maximal inhibition was achieved when purified human enzyme was used; when using cell lysates, animal tissue lysates, or fluids (plasma, saliva), the effects were lower. Such differential effects were not species-related, as expected by the high sequence similarity between the human and rat DPP4 sequences. Procyanidins have affinity for salivary and plasma proteins;[28−30] thus, the low inhibition found in in vitro assays with rat plasma and human saliva suggests that procyanidins could have higher affinity for other plasmatic and salivary proteins than for DPP4. This could also take place in vivo, as none of the studies that we have performed showed plasma DPP4 activity inhibition by GSPE. Another explanation would be that the forms in which GSPE reaches the systemic circulation are not the effective forms that directly inhibit DPP4 activity. The grape seed procyanidin extract is a mixture that contains a variety of structures, most of which have still have not been detected in plasma.[31,32] In fact, to our knowledge, the only previous report on the effects of flavonoids in vivo showed a plasma DPP4-inhibitory effect by an extract enriched in some monomeric structures; however, this extract was administered intraperitoneally, not orally.[16] The in vivo experiments performed in this study cover a wide range of conditions including different GSPE doses (acute high-dose to chronic lower-doses treatments) and different periods of time between GSPE administration and blood sample recovery to assay the DPP4 activity (1, 5, 13, or 17 h after GSPE administration), all of which suggest that although a direct inhibition of plasma DPP4 activity by GSPE cannot be fully discarded, it is not a primary mechanism that would explain the procyanidin effects on glucose homeostasis.

Our previous experience has shown that GSPE effects might depend on the experimental model.[26] Therefore, in this study, we have used several different animal models to analyze the effects of GSPE in vivo, including normal-weight and obese (genetically and diet-induced) rats. In the obese animals, we have not found effects of diet or genetic background on DPP4 plasmatic activity. In mice, a high-fat diet also did not modify plasmatic DPP4 activity.[33] Instead, and in contrast to our results, previous studies have shown that plasma activity is increased in rats due to a high fat diet.[34] In Otsuka Long−Evans Tokushima fatty (OLETF) rats, DPP4 plasma activity

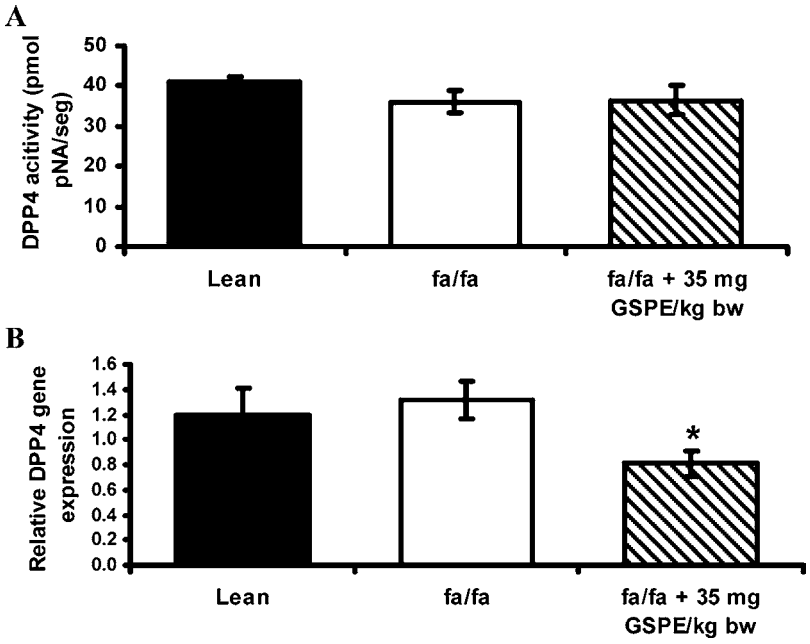

Figure 4. Effect on intestinal DPP4 activity (A) and gene expression (B) on Zucker rats treated for 60 days with 35 mg of GSPE/kg bw. The data are displayed as the mean ± SEM. *, significant differences vs the fa/fa group at $P < 0.05$.

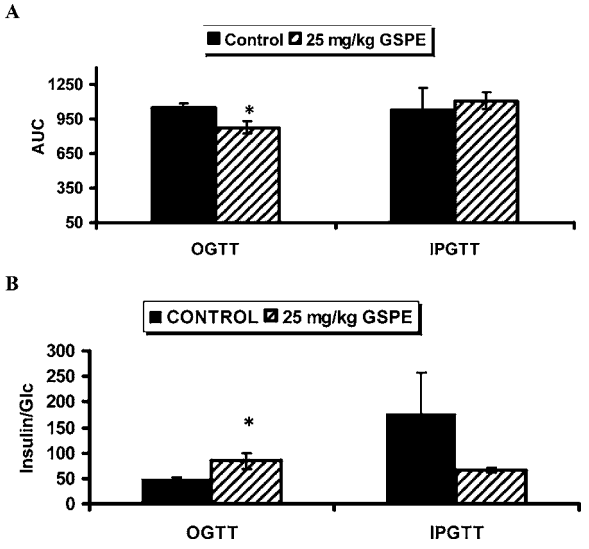

Figure 5. OGTT or IPGTT were performed on healthy animals after 19 days of treatment with 25 mg of GSPE/kg bw. (A) Area under the curve (AUC) of glucose plasma concentrations. (B) Insulin concentration per plasma glucose at $t = 30$ min. The data are displayed as the mean ± SEM. *, significant differences vs the control group at $P < 0.05$.

was only modified during the early phase (insulin-resistant diabetes) but not in hypoinsulinemic diabetes.[35] In humans, there is also controversy as to whether the plasma DPP4 activity is increased in pathologic situations. Some studies showed increased DPP4 plasma activity due to obesity,[36,37] although discrepancies between plasma DPP4 activity and the incretin levels have been found.[36] It therefore has been hypothesized that plasma DPP4 activity is of less relevance for incretin metabolism; instead, DPP4 activity in tissues close to the production site for incretins would be of more relevance.[36,38] Our results showed reduced DPP4 activity in the intestine in most of the experiments. Considering that the experimental procedure included washing the lumen intestine at the moment of sample collection, the mechanisms for such DPP4 inhibition could involve a strong (irreversible) inhibition of the enzyme found at the brush border membrane of the absorptive intestinal cells; it could also be due to direct inhibition of inner intestinal DPP4 by metabolyzed forms of procyanidins. Although absorption of flavonoids is not completely characterized, some flavonoid monomeric forms, like catechin and epicatechin, and also flavonoid metabolites have been reported to be absorbed by the intestinal cells and reach the systemic circulation.[32,39,40,41] Also, dimeric and trimeric procyanidins could be detected in plasma of rats after an acute oral load of procyanidins.[42] In addition, we found that procyanidins down-regulated DPP4 intestinal gene expression, and in fact, gene expression was more sensitive to procyanidins than DPP4 activity. The repression of gene expression could also contribute to the decrease in the intestinal DPP4 activity in chronic treatments, both in healthy and cafeteria-treated rats. Instead, in Zucker rats, GSPE-induced down-regulation of DPP4 gene expression was not enough to modify the enzyme activity. There is not much information concerning the modulation of intestinal DPP4 gene expression. Yang et al.[23] have shown that a high-fat diet increases the intestinal activity and expression of DPP4. We did not find differences in DPP4 activity or expression due to the genetic background or diet. The discrepancies between these and our results could be due to the experimental differences, which include the different genders of animals used (they worked with male rats and we with females), different lengths of the diet treatment (theirs is 12 weeks; ours is 17 weeks), different diets used, and the fact that in our study the samples were obtained after an overnight fasting, whereas in the study of Yang et al., rats seem not to be fasted. However, that study pointed out the importance of DPP4 intestinal gene expression and activity in obesity.

In fact, the exact relationship between DPP4 gene expression regulation and activity is not fully resolved. In Caco-2 cells, high

glucose concentrations increase DPP4 activity by up-regulating its gene expression.[43] Our experiments in intestinal human Caco-2 cells reinforce the idea that DPP4 gene expression is a target for procyanidins, although the decrease in DPP4 activity does not seem to be dependent on the effects of expression, as low GSPE concentrations decrease DPP4 activity but not gene expression.

In any case, the reduction in intestinal DPP4 activity could be linked to the effects of procyanidins on glucose homeostasis. We have previously shown that 25 mg of GSPE/kg bw in cafeteria-treated rats ameliorate their HOMA-IR index;[11] therefore, modulation of DPP4 activity could also be involved in GSPE effects in addition to the previously suggested effects on glucose absorption and/or action in peripheral tissues. Furthermore, in the present article, we show that chronic GSPE treatment in healthy rats, which decreased intestinal DPP4 activity, increases the plasma insulin/glucose ratio in an oral glucose tolerance test, while it does not do so when glucose is administered intraperitoneally, suggesting an incretin effect. This would support the hypothesis that intestinal DPP4 could be involved in the modulation of insulin production. However, further experiments on the effects of procyanidins in the pancreas, a target of DPP4 inhibitors, as well as more knowledge on the exact relationship between intestinal DPP4 activity and effects on glucose homeostasis, which remain unresolved, will be required to fully elucidate the mechanism of action of procyanidins.

In conclusion, we have found that a grape seed procyanidin extract is able to inhibit DPP4, directly as well as by other mechanisms, such as down-regulation of DPP4 gene expression. GSPE also increases the plasma insulin/glucose ratio in response to orally administered glucose, suggesting an incretin effect that could be responsible for some of the effects of GSPE in glucose homeostasis.

■ ASSOCIATED CONTENT

Supporting Information

Summary of animal experimental procedures; DPP4 protein sequence comparison; and cytotoxicity of Caco-2 cells treated with 100 of GSPE/L during 3 days. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Departament de Bioquímica i Biotecnologia, C. Marcel·lí Domingo, s/n, 43007, Tarragona, Spain. Phone: 34 977 558778. Fax: 34 977 558232. E-mail: montserrat.pinent@urv.cat.

Funding

This study was supported by a grant (AGL2008-01310) from the Spanish government. N.G.-A. is a recipient of a FPI fellowship from the Spanish Ministry of Science and Innovation (MICINN), and N.M.-M. is the recipient of a fellowship from Universitat Rovira i Virgili.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the technical support by Niurka Llopiz. We also thank members of the Nutrigenomics group who collaborated with us in caring for and sampling the animals.

■ ABBREVIATIONS USED

DPP4 dipeptidyl-peptidase 4; GSPE grape seed procyanidin extract; bw body weight; OGTT oral glucose tolerance test; IPGTT intraperitoneal glucose tolerance test

■ REFERENCES

(1) Nauck, M. A.; Vilsbøll, T.; Gallwitz, B.; Garber, A.; Madsbad, S. Incretin-based therapies: viewpoints on the way to consensus. *Diabetes Care* **2009**, *32* (Suppl 2), S223−S231.

(2) Matteucci, E.; Giampietro, O. Dipeptidyl peptidase-4 (CD26): knowing the function before inhibiting the enzyme. *Curr. Med. Chem.* **2009**, *16*, 2943−2951.

(3) Nauck, M. A. Unraveling the science of incretin biology. *Am. J. Med.* **2009**, *122*, S3−S10.

(4) Baggio, L. L.; Drucker, D. J. Biology of incretins: GLP-1 and GIP. *Gastroenterology* **2007**, *132*, 2131−2157.

(5) Deacon, C. F.; Nauck, M. A.; Meier, J.; Hücking, K.; Holst, J. J. Degradation of endogenous and exogenous gastric inhibitory polypeptide in healthy and in type 2 diabetic subjects as revealed using a new assay for the intact peptide. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 3575−3581.

(6) Serrano, J.; Puupponen-Pimiä, R.; Dauer, A.; Aura, A. M.; Saura-Calixto, F. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res.* **2009**, *53* (Suppl 2), S310−329.

(7) Bladé, C.; Arola, L.; Salvadó, M. J. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol. Nutr. Food Res.* **2010**, *54*, 37−59.

(8) Fraga, C. G.; Oteiza, P. I. Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell signaling. *Free Radical Biol. Med.* **2011**, *51*, 813−823.

(9) Del Bas, J.; Ricketts, M.; Baiges, I.; Quesada, H.; Ardevol, A.; Salvadó, M.; Pujadas, G.; Blay, M.; Arola, L.; Bladé, C.; Moore, D.; Fernandez-Larrea, J. Dietary procyanidins lower triglyceride levels signaling through the nuclear receptor small heterodimer partner. *Mol. Nutr. Food Res.* **2008**, *52*, 1172−1181.

(10) Gu, Y.; Hurst, W. J.; Stuart, D. A.; Lambert, J. D. Inhibition of key digestive enzymes by cocoa extracts and procyanidins. *J. Agric. Food Chem.* **2011**, *59*, 5305−5311.

(11) Montagut, G.; Bladé, C.; Blay, M.; Fernández-Larrea, J.; Pujadas, G.; Salvadó, M.; Arola, L.; Pinent, M.; Ardévol, A. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *J. Nutr. Biochem.* **2009**, *21*, 961−967.

(12) Al-Awwadi, N. A.; Araiz, C.; Bornet, A.; Delbosc, S.; Cristol, J. P.; Linck, N.; Azay, J.; Teissedre, P. L.; Cros, G. Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. *J. Agric. Food Chem.* **2005**, *53*, 151−157.

(13) Pinent, M.; Blay, M.; Blade, M. C.; Salvado, M. J.; Arola, L.; Ardevol, A. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology* **2004**, *145*, 4985−4990.

(14) Kim, T.; Davis, J.; Zhang, A. J.; He, X.; Mathews, S. T. Curcumin activates AMPK and suppresses gluconeogenic gene expression in hepatoma cells. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 377−382.

(15) Bauvois, B.; Puiffe, M. L.; Bongui, J. B.; Paillat, S.; Monneret, C.; Dauzonne, D. Synthesis and biological evaluation of novel flavone-8-acetic acid derivatives as reversible inhibitors of aminopeptidase N/CD13. *J. Med. Chem.* **2003**, *46*, 3900−3913.

(16) Bansal, P.; Paul, P.; Mudgal, J.; G Nayak, P.; Thomas Pannakal, S.; Priyadarsini, K. I.; Unnikrishnan, M. K. Antidiabetic, antihyperlipidemic and antioxidant effects of the flavonoid rich fraction of *Pilea microphylla* (L.) in high fat diet/streptozotocin-induced diabetes in mice. *Exp. Toxicol. Pathol.* **2012**, *64*, 651−658.

(17) Lefort, E. C.; Blay, J. The dietary flavonoid apigenin enhances the activities of the anti-metastatic protein CD26 on human colon carcinoma cells. *Clin. Exp. Metastasis* **2011**, *28*, 337−349.
(18) Stefanovic, V.; Savic, V.; Vlahovic, P.; Cvetkovic, T.; Najman, S.; Mitic-Zlatkovic, M. Reversal of experimental myoglobinuric acute renal failure with bioflavonoids from seeds of grape. *Renal Failure* **2000**, *22*, 255−266.
(19) Montagut, G.; Baiges, I.; Valls, J.; Terra, X.; del Bas, J.; Vitrac, X.; Richard, T.; Mérillon, J.; Arola, L.; Blay, M.; Bladé, C.; Fernández-larrea, J.; Pujadas, G.; Salvadó, J.; Ardévol, A. A trimer plus a dimer-gallate reproduce the bioactivity described for an extract of grape seed procyanidins. *Food Chem.* **2009**, *116*, 265−270.
(20) Terra, X.; Valls, J.; Vitrac, X.; Mérrillon, J. M.; Arola, L.; Ardèvol, A.; Bladé, C.; Fernandez-Larrea, J.; Pujadas, G.; Salvadó, J.; Blay, M. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *J. Agric. Food Chem.* **2007**, *55*, 4357−4365.
(21) Rodríguez, A. M.; Quevedo-Coli, S.; Roca, P.; Palou, A. Sex-dependent dietary obesity, induction of UCPs, and leptin expression in rat adipose tissues. *Obes. Res.* **2001**, *9*, 579−588.
(22) Alvaro, A.; Rosales, R.; Masana, L.; Vallvé, J. C. Polyunsaturated fatty acids down-regulate in vitro expression of the key intestinal cholesterol absorption protein NPC1L1: no effect of monounsaturated nor saturated fatty acids. *J. Nutr. Biochem.* **2010**, *21*, 518−525.
(23) Yang, J.; Campitelli, J.; Hu, G.; Lin, Y.; Luo, J.; Xue, C. Increase in DPP-IV in the intestine, liver and kidney of the rat treated with high fat diet and streptozotocin. *Life Sci.* **2007**, *81*, 272−279.
(24) Thomas, L.; Eckhardt, M.; Langkopf, E.; Tadayyon, M.; Himmelsbach, F.; Mark, M. (R)-8-(3-amino-piperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methyl-quinazolin-2-ylmethyl)-3,7-dihydro-purine-2,6-dione (BI 1356), a novel xanthine-based dipeptidyl peptidase 4 inhibitor, has a superior potency and longer duration of action compared with other dipeptidyl peptidase-4 inhibitors. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 175−182.
(25) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(26) Pinent, M.; Cedó, L.; Montagut, G.; Blay, M.; Ardévol, A. Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. *Crit. Rev. Food. Sci. Nutr.* **2012**, *52*, 569−584.
(27) Tebib, K.; Rouanet, J. M.; Besançon, P. Effect of grape seed tannins on the activity of some rat intestinal enzyme activities. *Enzyme Protein* **1994**, *48*, 51−60.
(28) Hofmann, T.; Glabasnia, A.; Schwarz, B.; Wisman, K. N.; Gangwer, K. A.; Hagerman, A. E. Protein binding and astringent taste of a polymeric procyanidin, 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose, castalagin, and grandinin. *J. Agric. Food Chem.* **2006**, *54*, 9503−9509.
(29) de Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001**, *49*, 940−945.
(30) Brunet, M. J.; Bladé, C.; Salvadó, M. J.; Arola, L. Human apo A-I and rat transferrin are the principal plasma proteins that bind wine catechins. *J. Agric. Food Chem.* **2002**, *50*, 2708−2712.
(31) Serra, A.; Macià, A.; Romero, M.; Valls, J.; Bladé, C.; Arola, L.; Motilva, M. Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. *Br. J. Nutr.* **2010**, *103*, 944−952.
(32) Donovan, J. L.; Manach, C.; Rios, L.; Morand, C.; Scalbert, A.; Rémésy, C. Procyanidins are not bioavailable in rats fed a single meal containing a grapeseed extract or the procyanidin dimer B3. *Br. J. Nutr.* **2002**, *87*, 299−306.
(33) Reimer, M. K.; Holst, J. J.; Ahrén, B. Long-term inhibition of dipeptidyl peptidase IV improves glucose tolerance and preserves islet function in mice. *Eur. J. Endocrinol.* **2002**, *146*, 717−727.
(34) Kirino, Y.; Kamimoto, T.; Sato, Y.; Kawazoe, K.; Minakuchi, K.; Nakahori, Y. Increased plasma dipeptidyl peptidase IV (DPP IV) activity and decreased DPP IV activity of visceral but not subcutaneous adipose tissue in impaired glucose tolerance rats induced by high-fat or high-sucrose diet. *Biol. Pharm. Bull.* **2009**, *32*, 463−467.
(35) Kirino, Y.; Sato, Y.; Kamimoto, T.; Kawazoe, K.; Minakuchi, K. Altered dipeptidyl peptidase-4 activity during the progression of hyperinsulinemic obesity and islet atrophy in spontaneously late-stage type 2 diabetic rats. *Am. J. Physiol. Endocrinol. Metab.* **2011**, *300*, E372−E379.
(36) Carr, R. D.; Larsen, M. O.; Jelic, K.; Lindgren, O.; Vikman, J.; Holst, J. J.; Deacon, C. F.; Ahrén, B. Secretion and dipeptidyl peptidase-4-mediated metabolism of incretin hormones after a mixed meal or glucose ingestion in obese compared to lean, nondiabetic men. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 872−878.
(37) Lugari, R.; Dei Cas, A.; Ugolotti, D.; Barilli, A. L.; Camellini, C.; Ganzerla, G. C.; Luciani, A.; Salerni, B.; Mittenperger, F.; Nodari, S.; Gnudi, A.; Zandomeneghi, R. Glucagon-like peptide 1 (GLP-1) secretion and plasma dipeptidyl peptidase IV (DPP-IV) activity in morbidly obese patients undergoing biliopancreatic diversion. *Horm. Metab. Res.* **2004**, *36*, 111−115.
(38) Burcelin, R.; Dejager, S. GLP-1: what is known, new and controversial in 2010? *Diabetes Metab.* **2010**, *36*, 503−509.
(39) Castell, A.; Motilva, M. J.; Macià, A.; Torrell, H.; Bladé, C.; Pinent, M.; Arola, L.; A., A. Organotypic co-culture system to study plant extract bioactivity on hepatocytes. *Food Chem.* **2010**, *122*, 775−781.
(40) Gardana, C.; Simonetti, P.; Berti, C.; Pietta, P. Evaluation of propolis polyphenols absorption in humans by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3849−3854.
(41) Chow, H.; Cai, Y.; Alberts, D.; Hakim, I.; Dorr, R.; Shahi, F.; Crowell, J.; Yang, C.; Hara, Y. Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol. Biomarkers Prev.* **2001**, *10*, 53−58.
(42) Serra, A.; Macià, A.; Romero, M. P.; Valls, J.; Bladé, C.; Arola, L.; Motilva, M. J. Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. *Br. J. Nutr.* **2010**, *103*, 944−952.
(43) Gu, N.; Tsuda, M.; Matsunaga, T.; Adachi, T.; Yasuda, K.; Ishihara, A.; Tsuda, K. Glucose regulation of dipeptidyl peptidase IV gene expression is mediated by hepatocyte nuclear factor-1alpha in epithelial intestinal cells. *Clin. Exp. Pharmacol. Physiol.* **2008**, *35*, 1433−1439.