Phenolic acids suppress adipocyte lipolysis via activation of the nicotinic acid receptor GPR109A (HM74a/PUMA-G)

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Abstract Phenolic acids are found in abundance throughout the plant kingdom. Consumption of wine or other rich sources of phenolic acids, such as the "Mediterranean diet," has been associated with a lower risk of cardiovascular disease. The underlying mechanism(s), however, has remained unclear. Here, we show that many phenolic acids, including those from the hydroxybenzoic and hydroxycinnamic acid classes, can bind and activate GPR109A (HM74a/PUMA-G), the receptor for the antidyslipidemic agent nicotinic acid. In keeping with this activity, treatment with a number of phenolic acids, including cinnamic acid, reduces lipolysis in cultured human adipocytes and in fat pats isolated from wild-type mice but not from mice deficient of GPR109A. Oral administration of cinnamic acid significantly reduces plasma levels of FFA in the wild type but not in mice deficient of GPR109A. Activation of GPR109A by phenolic acids may thus contribute to a cardiovascular benefit of these plant-derived products.— Ren, N., R. Kaplan, M. Hernandez, K. Cheng, L. Jin, A. K. P. Taggart, A. Y. Zhu, X. Gan, S. D. Wright, and T-Q. Cai. **Phenolic acids suppress adipocyte lipolysis via activation of the nicotinic acid receptor GPR109A (HM74a/PUMA-G).** *J. Lipid Res.* 2009. **50:** 908–914.

Supplementary key words GPCR • niacin • plant-derived product

Nicotinic acid has been used in the clinic for more than 40 years in the treatment of dyslipidemia. It produces a prompt reduction in circulating FFAs and a desirable modification of circulating atherogenic lipids and has been shown to reduce cardiac mortality (1–3). The primary actions of nicotinic acid include *a*) reduction of lipolysis in adipose tissue and *b*) induction of a characteristic cutaneous flushing response. Reduction of lipolysis is triggered by binding of nicotinic acid to GPR109A (also named HM74a or PUMA-G), a Gi-G protein-coupled receptor highly expressed in adipocytes. Activation of GPR109A resulted in a Gi-protein-mediated inhibition of adenylyl cyclase, leading to reduction of cAMP accumulation, hormone-sensitive lipase activation, and adipocyte lipolysis (4–7). Flushing is triggered by ligation of GPR109A on Langerhans that in turn induces production of prostaglandin D_2 synthesis and cutaneous vasodilation (8–11).

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. They are essential for the growth and reproduction of plants (12). Phenolic acids form a diverse class that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids [see (12, 13) for details]. Consumption of products rich in phenolic acids, such as wine and the "Mediterranean diet," correlates with a reduced risk of cardiovascular disease (14–16). Since phenolic acids may exhibit antioxidant properties, a role in protecting LDL from oxidative modification has been proposed (17, 18). We note that both phenolic acids and nicotinic acid are small carboxylic acids with close structural similarity. We also note that application of certain phenolic acids, such as benzoic acid, also induces a flushing response and prostaglandin D_2 release in a manner similar to that of nicotinic acid treatment (19, 20). We thus asked whether phenolic acids could act as GPR109A agonists.

MATERIALS AND METHODS

Materials

Nicotinic acid, 3-isobutyl-1-methylxanthine, and all of the phenolic compounds used in this study were purchased from Sigma Chemical (St. Louis, MO). Acifran was synthesized by chemists at Arena Pharmaceuticals (San Diego, CA). ^{35}SGTPγS (1160 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). The 5,6-^{3}H-nicotinic acid (50 Ci/mmol) was from American Radiolabeled Chemical (St. Louis, MO).

Molecular cloning and establishment of stable cell lines

Molecular cloning and establishment of stable cell lines for human GPR109A, GPR109B, GPR81, or mouse GPR109A/PUMA-G were as previously described (21).

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^{35}S-GTPγS binding assay

Membranes prepared from Chinese Hamster Ovary (CHO)-K1 cells stably expressing GPR109A, GPR109B, GPR81, PUMA-G, or vector control (7 μg/assay) were diluted in assay buffer (100 mM HEPES, 100 mM NaCl, and 10 mM MgCl$_2$, pH 7.4) in Wallac Scintistrip plates (PerkinElmer, Shelton, CT) and preincubated with test compounds diluted in assay buffer containing 40 μM GDP (final [GDP] was 10 μM) for ~10 min before addition of ^{35}S-GTPγS to 0.3 nM. To avoiding potential compound precipitation, all compounds were first prepared in 100% DMSO and then diluted with assay buffer, resulting in a final concentration of 3% DMSO in the assay. Binding was allowed to proceed for 1 h before centrifuging the plates at 4,000 rpm for 15 min at room temperature and subsequent counting in a TopCount scintillation counter. Nonlinear regression analysis of the binding curves was performed in GraphPad Prism.

Radioligand competition binding assay

Assays were performed with the same preparations of membrane used for the ^{35}S-GTPγS assay. Equilibrium binding of ^{3}H-nicotinic acid was done with membranes (30 μg/assay) and test compounds diluted in assay buffer (20 mM HEPES, pH 7.4, 1 mM MgCl$_2$, and 0.01% CHAPS) in a total volume of 200 μl. After 4 h at room temperature, reactions were filtered through Packard Unifilter GF/C plates using a Packard Harvester and washed with 8 × 200 μl ice-cold binding buffer. Nonspecific binding was determined in the presence of 250 μM unlabeled nicotinic acid. Competitive binding assays were performed in the presence of 50 nM ^{3}H-nicotinic acid.

Human adipocytes lipolysis assay

Primary subcutaneous human preadipocytes were purchased from Cambrex Bio Science (Walkersville, MD). To prepare the cells suitable for the lipolysis assay, preadipocytes were plated at 3,000 cells/well of a 96-well plate and allowed to grow to confluence in preadipocyte basal medium containing 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. Twenty-four hours after reaching 100% confluence, the medium was removed from the plates and replaced with complete medium containing 10 μg/ml insulin, 1 μM dexamethasone, 200 μM indomethacin, and 500 μM IBMX. The lipid vacuoles began to appear by day 4 and continued to grow for 14 d. When cells are fully differentiated, the medium was removed from the wells, and the plates were washed twice with PBS. Testing compounds (100 μl/well) diluted in Krebs buffer (120 mM NaCl, 4 mM KH$_2$PO4, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 1 mM NaHCO$_3$, 3 mM HEPES, 20 nM Adenosine and 1% BSA) with 0.4% final concentration of DMSO was added to each well. After 10 min incubation at 37°C, 50 μl/well of the nonspecific phosphodiesterase inhibitor, IBMX, was added at a working concentration of 1 mM. After 5 h of incubation at 37°C, a 100μl of culture medium was removed from each well and assayed for glycerol content using the Sigma free glycerol determination kit.

Mouse epididymal fat pad lipolysis assay

Homozygous PUMA-G null (deficient) mice were generated as previously described (5) and were obtained from Taconic Laboratories (Germantown, NY). Epididymal fat pads from wild-type or PUMA-G-deficient male mice (~18 weeks of age, ~10 mice each) were collected into HBSS (Invitrogen). Adipose tissues were sliced into pieces (~20–40 mg/piece). Fat pads (~150 mg per sample) were incubated in 1 ml HBSS buffer with 1% FFA-free BSA (Serologicals) and 0.5 unit/ml adenosine deaminase (Sigma) with or without phenolic acids (Sigma) at 37°C for 60 min. After incubation, medium was removed. Glyerol
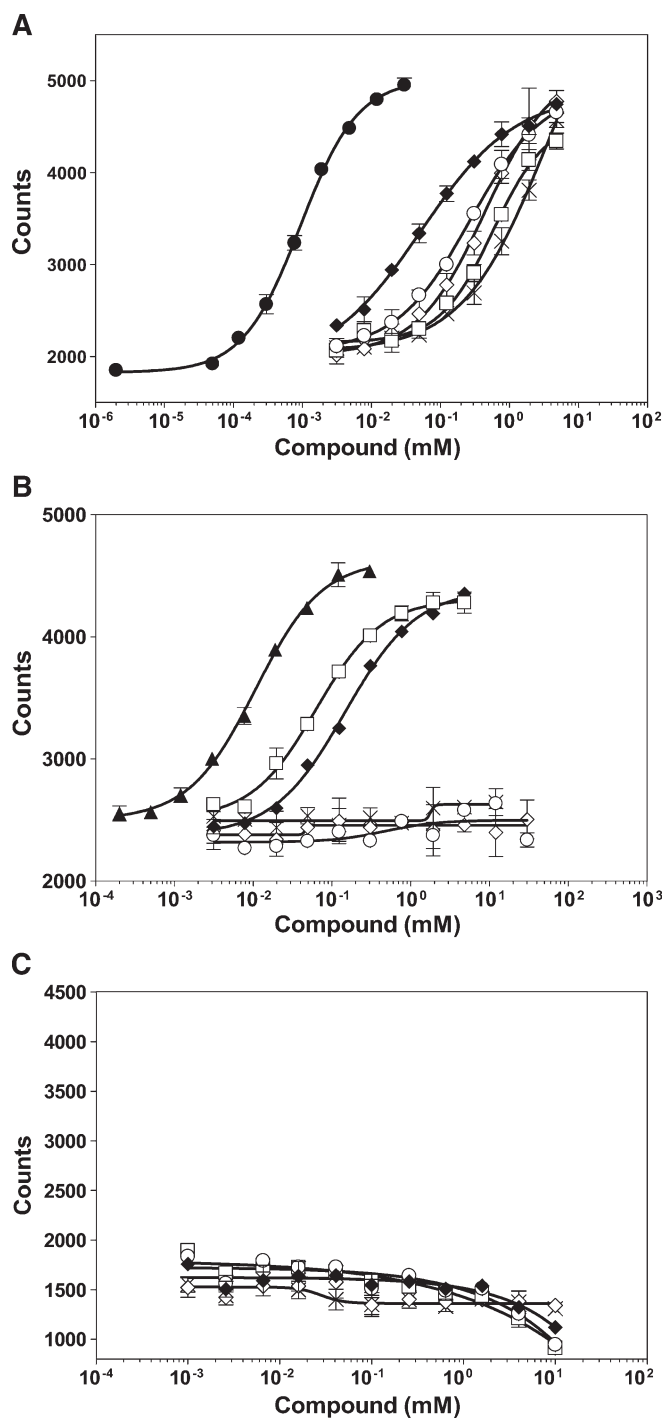
Fig. 1. Phenolic acids stimulate ^{35}S-GTPγS binding to membranes from cells transfected with GPR109A. Ligand-induced ^{35}S-GTPγS binding was studied using membranes prepared from CHO-K1 cells expressing human GPR109A (A), GPR109B (B), or GPR81 (C). Binding of ^{35}S-GTPγS was determined in the presence of increasing concentrations of nicotinic acid (closed circles), acifran (triangles), *trans*-cinnamic acid (closed diamonds), *p*-coumaric acid (open circles), *o*-coumaric acid (squares), benzoic acid (open diamonds), or salicylic acid (crosses). Data are shown as the means ± SD of triplicates of a representative experiment. Three to four independent experiments were performed with similar results.

released from epididymal fat pads were determined by a free glycerol kit (Sigma).

In vivo FFA reduction assay

Homozygous PUMA-G null (deficient) and wild-type littermate control mice (~18 weeks of age, seven mice/group) were fed with a normal rodent chow diet and tap water ad libitum. After 4 h of fasting, mice were orally gavaged with vehicle (0.5% methylcellulose solution), nicotinic acid (100 mg/kg body weight), or cinnamic acid (5 mg/kg body weight). Fifteen minutes after treatment, mice were euthanized by CO_2, and blood samples were collected via cardiac puncture. Serum levels of nonesterified FFAs were determined by an enzymatic colorimetric method (Roche Diagnostics).

TABLE 1.   Activity of phenolic acids in ^{3}H-nicotinic acid binding, ^{35}S-GTPγS binding and human adipocyte lipolysis assays. All compounds listed were inactive on membranes from mock-transfected or GPR81-transfected CHO cells.

	Chemical Structure	^{3}H-Nicotinic acid binding IC50 (µM)	^{35}S-GTPγS binding EC50 (µM)			Adipocyte lipolysis IC50 (µM)
		GPR109A	GPR109A	PUMA-G	GPR109B	
Nicotinic acid		0.1	0.1	0.04	inactive	0.2
Acifran		1.1	1.1	0.4	7	2.1
Trans-cinnamic acid		36	240	26	180	160
o-Coumaric acid		190	1270	303	70	470
p-Coumaric acid		58	310	102	>30,000	130
Caffeic acid		490	>30,000	inactive	inactive	14
Ferulic acid		>10,000	>30,000	>30,000	inactive	Inactive
Sinapic acid		>10,000	>30,000	>30,000	inactive	Inactive
Benzoic acid		113	670	133	inactive	710
salicylic acid		790	1800	484	inactive	2070
p-Hydroxybenzoic acid		920	2200	1904	inactive	2520
Protocatechuic acid		410	800	104	inactive	20
Gallic acid		322	>30,000	>30,000	inactive	30
Syringic acid		inactive	>30,000	>30,000	inactive	Inactive

RESULTS

Phenolic acids stimulate ^{35}S-GTPγS binding to membranes from cells transfected with GPR109A

We first studied ligand-stimulated guanine nucleotide exchange using a ^{35}S-GTPγS binding assay with membranes prepared from cells transfected with a control vector or vectors that express GPR109A, GPR109B, PUMA-G, or GPR81. GPR109B and GPR81 are close homologs of GPR109A, with $\sim$95 and 50% identical amino acid sequence to GPR109A, respectively. As expected, nicotinic acid stimulated ^{35}S-GTPγS binding only in membranes from cells expressing GPR109A, with an EC$_{50}$ of 104 $\pm$ 5 nM for human GPR109A and 43 $\pm$ 3 nM for the mouse ortholog PUMA-G. As a further positive control, we used acifran, a nicotinic acid ana-

log known to be active on both GPR109A and GPR109B. We observed an EC$_{50}$ of 1127 $\pm$ 59 nM for human GPR109A and 7037 $\pm$ 598 nM for GPR109B. Based on their commercial availability and potential existence in the plant products, we assembled a set of phenolic acids from the hydroxybenzoic and hydroxycinnamic acid classes and studied them in the same assay. A number of molecules from both classes stimulated ^{35}S-GTPγS binding in membranes from cells expressing GPR109A but not in that of GPR81 (**Fig. 1** and **Table 1**). Two of these phenolic acids, *trans*-cinnamic and *o*-coumaric acids, were also found active in the GPR109B-expressing membrane (Fig. 1B), suggesting that these two compounds may behave like acifran, acting as dual agonists to both GPR109A and GPR109B. Of the phenolic acids that
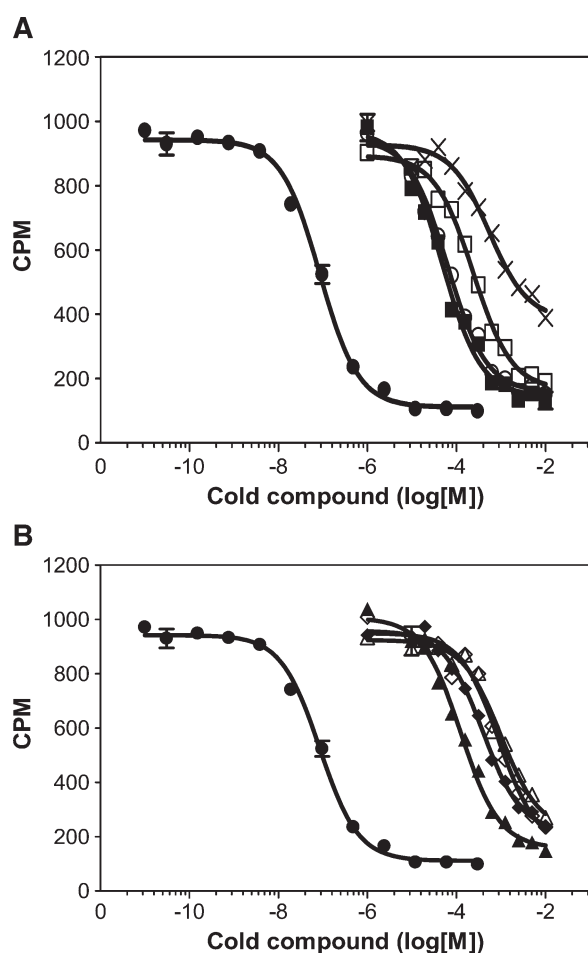

Fig. 2. Phenolic acids compete with ^{3}H-nicotinic acid binding to membranes from cells transfected with GPR109A. Binding of 50 nM ^{3}H-nicotinic acid to membranes prepared from CHO-K1 cells expressing human GPR109A was studied in the presence of increasing concentrations of A: nicotinic acid (closed circles) or the cinnamic-acid-related compounds, including *trans*-cinnamic acid (closed squares), *p*-coumaric acid (open circles), *o*-coumaric acid (open squares), and caffeic acid (crosses), or B: nicotinic acid (closed circles) or the benzoic-acid-related compounds, including benzoic acid (closed triangles), *p*-hydroxybenzoic acid (open triangles), salicylic acid (open diamonds), and protocatechuic acid (closed diamonds). Data are shown as the means $\pm$ SD of triplicates of a representative experiment.
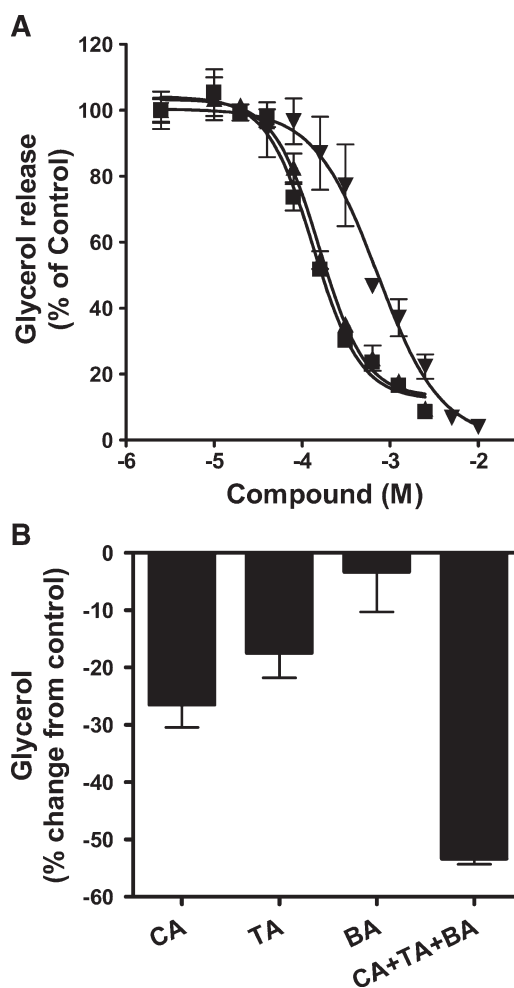

Fig. 3. Phenolic acids inhibit lipolysis in cultured human adipocytes. Differentiated subcutaneous human adipocytes were stimulated with 0.5 mM IBMX, a nonspecific phosphodiesterase inhibitor, in the presence of A: increasing concentrations of *trans*-cinnamic acid (upward-pointing triangles), *p*-coumaric acid (squares), and benzoic acid (downward-pointing triangles), or B: 80 μM coumaric acid (CA), *trans*-cinnamic acid (TA), benzoic acid (BA), or the combination of these three compounds. After 5 h of incubation at 37°C, levels of glycerol released into the medium were determined. Data are shown as the means $\pm$ SD of triplicates of a representative experiment. Three to four independent experiments were performed with similar results.

activate GPR109A, careful dose titration studies enabled us to rank the relative potency among the derivatives. In the cinnamic acid class, we found a potency order of *trans*-cinnamic acid > *p*-coumaric acid > *o*-coumaric acid > sinapic acid > ferulic acid > caffeic acid. In the benzoic class, the order was benzoic acid > protocatechuic acid > salicylic acid > *p*-hydroxybenzoic acid (Fig. 1 and Table 1).

Phenolic acids compete with ³H-nicotinic acid binding to membranes from cells transfected with GPR109A

Next, we asked if aforementioned phenolic compounds would bind to the same binding site as that of nicotinic acid. We performed ligand binding competition assay using ³H-labeled and unlabeled nicotinic acid. Consistent with previous reports (5, 6, 21), ³H-nicotinic acid bound specifically and saturably to membranes from cells expressing GPR109A with dissociation constant (K_d) of 105 nM and an inhibition constant (K_i) of 130 ± 14 nM. Analogous competition assays with the phenolic acids showed similar apparent affinity and potency order with those compounds found active in the aforementioned GTPγS binding assay (**Fig. 2** and Table 1). Overall, it appears that addition of a –CH3 group tends to reduce the binding potency to the receptor, but addition or shifting position of an –OH group did not significantly alter the binding activity. Together, these data demonstrated that cinnamic and benzoic acids, as well as many of their derivatives, may bind to the nicotinic acid receptor directly in a manner that competes with nicotinic acid binding, leading to increased binding of GTPγS.

Phenolic acids reduce lipolysis

Activation of the nicotinic acid receptor results in a reduction of hormone-sensitive lipase activation and triglyceride hydrolysis (4–7). We thus asked if phenolic acids would inhibit the hydrolysis of triglycerides in cultured human adipocytes. As expected, treatment with nicotinic acid resulted in a significant reduction of triglyceride hydrolysis, with an apparent IC_{50} of ~0.2 μM. Treatment of the adipocytes with phenolic acids found active in the aforementioned ³H-nicotinic acid competition assay also resulted in a significant reduction of adipocyte lipolysis (**Fig. 3A**). Similar to the results demonstrated by the ³H-nicotinic acid competition assay or ³⁵S-GTPγS binding assay, ferulic, sinapic, and syringic acids were also found to be inactive in this lipolysis assay. The apparent IC_{50} for most of these active compounds matched closely to the respective EC50 found in the ³⁵S-GTPγS binding assay (Table 1).

Interestingly, the apparent potency of caffeic, gallic, and protocatechuic acids observed from the adipocyte lipolysis assay appeared much higher than that found in the ³⁵S-GTPγS binding assay (Table 1), suggesting that a mechanism independent of GPR109A may contribute to an antilipolytic activity of these phenolic compounds as well.

A variety of phenolic acids may be found in a given plant-based product. While considered on an individual basis, the amount of a given phenolic acid may be low, and a combined phenolic acid content could be quite high. For example, in certain red wines, the total phenolic acid
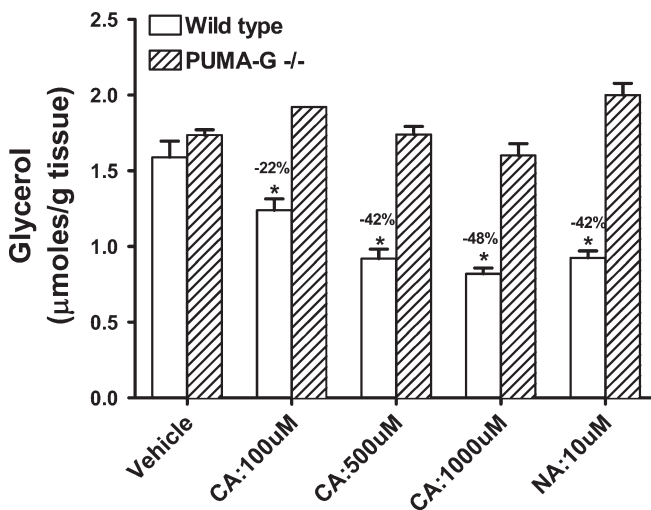

Fig. 4. Cinnamic acid suppresses lipolysis of fat pads isolated from wild-type but not PUMA-G-deficient mice. Epididymal fat pads (~150 mg) isolated from wild-type or homozygous PUMA-G-deficient mice were incubated with 0.5 units/ml adenosine in the presence of increasing concentrations of cinnamic acid (CA) or nicotinic acid (NA; 10 μM). After 60 min of treatment at 37°C, levels of glycerol released into the medium were determined. Data are shown as the means ± SD of quadruplicates of a representative experiment.

content could reach as high as 4 g/l of wine (12). We thus asked if an antilipolytic effect of the phenolic compounds might be additive. As shown in Fig. 3B, we found simultaneous addition of these compounds to adipocytes led to a strong additive suppression of lipolysis. For example, addition of 80 μM of *trans*-cinnamic acid, *p*-coumaric acid, or benzoic acid alone led to a suppression of lipolysis by 17, 26, and 3%, respectively. However, when the same concentrations of these three compounds were combined, a suppression of 53% was observed. These data suggest that while individually an antilipolytic effect of these phenolic compounds may be weak, together they could potentially achieve a functional significance.
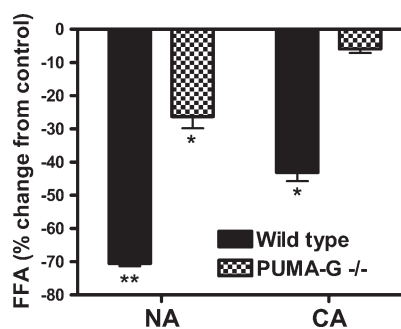

Fig. 5. Cinnamic acid reduces serum FFA in wild-type but not PUMA-G-deficient mice. Wild-type or homozygous PUMA-G-deficient mice (n = 7) were orally dosed with vehicle (0.5% methylcellulose solution), nicotinic acid (NA; 100 mpk) or *trans*-cinnamic acid (CA; 5 mpk). Fifteen minutes after treatment, mice were euthanized, and blood samples were collected by cardiac puncture. Serum levels of nonesterified FFAs were measured. Data are shown as means ± SE.

To ask if an antilipolytic effect of the phenolic compounds in adipocytes is mediated through the nicotinic acid receptor GPR109A, fat pad lipolysis assays were performed using epididymal fat tissues isolated from wild-type or homozygous PUMA-G/GPR109A-deficient mice. As with nicotinic acid (**Fig. 4**), treatment of cinnamic acid significantly reduced lipolysis of fat pads prepared from wild-type but not from PUMA-G-deficient mice, indicating that an antilipolytic effect of cinnamic acid is mediated through PUMA-G/GPR109A.

To determine if consumption of a phenolic compound would affect the lipolytic activity in vivo, we studied FFA levels in wild-type and homozygous PUMA-G/GPR109A-deficient mice. We used nicotinic acid at dose of 100 mpk as a reference of maximum effect. As shown in **Fig. 5**, within 15 min of oral administration of 0.15 mg/mouse (~5 mpk) of cinnamic acid resulted in a significant reduction in plasma levels of FFA in the wild-type mice but not in mice deficient of PUMA-G/GPR109A, in a similar manner to mice treated with nicotinic acid.

DISCUSSION

Our data clearly show that many of the phenolic acids could act as ligands of the nicotinic acid receptor GPR109A, and activation of GPR109A by the phenolic compounds leads to a reduction of adipocyte lipolysis. Activation of GPR109A may lead to differential downstream effects on lipolysis and flushing response. For example, a number of GPR109A pyrazole agonists were found capable of inhibiting lipolysis but failed to elicit a flushing response and can antagonize nicotinic-acid-mediated flushing response (22). It is unclear if any of the phenolic acids from plant products, such as wine, could function as pyrazole agonist of GPR109A, thus leading to an escape of a flushing response.

Phenolic substances are present in large quantities in plant-derived products, and they are important components of the human diet. Daily intake of hydroxycinnamic acids alone was estimated at 211 mg/day and can reach up to 800 mg/day (23). However, due to lack of information on concentration, oral bioavailability, pharmacokinetic, or metabolism of phenolic acids at individual or group base in a given food product, it is difficult to make a conclusion on the physiological significance of the current finding. While the potency of a phenolic compound, taken on an individual basis, in activating GPR109A or inhibiting lipolysis is fairly low, we found that an antilipolytic effect of the phenolic compounds is additive. Assuming all of the phenolic compounds in a beverage, such as red wine, or all of the hydroxycinnamic acids in a fruit, such as blueberries, are cinnamic acid, the amount of cinnamic acid orally dosed to the mice (0.15 mg/mouse) would be equivalent to a drink of about two glasses of wine (~350 ml) or 1.5 servings of blueberries (~150 g) for a 70 kg person (12, 23). The Mediterranean diet supplies abundant phenolic compounds in legumes and olive oil. In legumes, total phenolic content can reach >1 mg/g dry beans (24), and >83% of the total phenolic acids were found to be retained during the cooking process (25). As with wine, the Mediterranean diet has long been associated with a lower risk of cardiovascular disease (14–16). While a mechanism(s) independent of GPR109A may contribute to the full benefits of nicotinic acid treatment, in addition to an antilipolysis effect, GPR109A appears to play a role in the anti-inflammatory activities of nicotinic acid as well (26). It is thus tempting to speculate that the basis of the "French paradox" and the benefits of the Mediterranean diet could potentially be attributed in part to the activation of GPR109A by the phenolic acid compounds so richly presented in these products.⬛

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