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Rosiglitazone and Metformin Have Opposite Effects on Intestinal Absorption of Oligopeptides via the Proton-Dependent PepT1 Transporter

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ABSTRACT

The intestinal H(+)/peptide cotransporter 1 (PepT1) plays a major role in nitrogen supply to the body by mediating intestinal absorption of di- and tripeptides. Previous studies have reported that in animal models of type 2 diabetes/obesity, PepT1 activity and expression were markedly reduced. This prompted us to investigate the effects of two antidiabetic drugs, rosiglitazone and metformin, on PepT1 activity/expression in a murine diet-induced obesity model. C57BL/6J male mice were fed a high-fat diet (HFD) or a standard chow for 6 weeks and then were treated for 7 days with metformin (250 mg/kg/day) and/or rosiglitazone (8 mg/kg/day). For in vitro studies, Caco-2 enterocyte-like cells were treated for 7 days with metformin (10 mM) and/or rosiglitazone (10 μM). A 7-day rosiglitazone treatment increased PepT1 activity and prevented the 2-fold HFD-induced reduction in PepT1 transport. Metformin alone did not modify PepT1 activity but counteracted rosiglitazone-induced PepT1-mediated transport. As with the in vivo studies, rosiglitazone treatment up-regulated PepT1 transport activity with concomitant induction of S6 ribosomal protein activation in vitro. Furthermore, metformin decreased PepT1 expression (mRNA and protein) and its transport activity. The effect of metformin was linked to a reduction of phosphorylated S6 ribosomal protein (active form) and of phosphorylated 4E-BP1 (inactive form), a translation repressor. These data demonstrate that two antidiabetic drugs exert opposite effects on the PepT1 transport function probably through direct action on enterocytes. In our type 2 diabetes/obesity model, rosiglitazone, a peroxisome proliferator-activated receptor-γ agonist compensated for the HFD-induced PepT1 down-regulation, whereas metformin reversed rosiglitazone activity at the translational level.

Introduction

PepT1 is the major intestinal oligopeptide transporter, which accounts for the absorption of 70% of the total nitrogen supplied to the body (Rubio-Aliaga and Daniel, 2008). Furthermore, the transporter is highly regulated by hormones in physiological and physiopathological conditions such as diabetes and obesity. Thus, it has already been shown that insulin up-regulates PepT1 at the post-transcriptional level. Animal models of type 1 and type 2 diabetes mellitus do indeed display significant modifications in the protein expression and activity of PepT1 (Thamotharan et al., 1999; Watanabe et al., 2003). In addition, hormones that are secreted by the gastrointestinal glands into the gut lumen such as epidermal growth factor (Nielsen et al., 2001) and leptin (Buyse et al., 2001) can regulate PepT1 function. We previously established that leptin increases PepT1 transport activity over a short period, and it appears that leptin and insulin share a common mechanism for stimulating dipeptide transport in Caco-2 cells by increasing the trafficking of PepT1 from the intracellular pool to the apical membrane (Buyse et al., 2001). Thereafter, leptin could reconstitute the pool of PepT1 by activating translation of PepT1 mRNA.

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ABBREVIATIONS: PepT1, intestinal H(+)/peptide cotransporter 1; AMPK, 5′ AMP-activated protein kinase; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response elements; mTOR, mammalian target of rapamycin; AICAR, 5-aminoimidazole-4-carboxamide ribotide; SC, standard laboratory chow; HFD, high-fat diet; Gly-Sar, glycyl-sarcosine; KRB, Krebs-Ringer bicarbonate buffer; Gly-Gly, glycyl-glycine; Gly-Pro, glycyl-proline; ELISA, enzyme-linked immunosorbent assay; TEER, transepithelial electric resistance.

TABLE 1
Characteristics of the primers used for the reverse transcription-polymerase chain reaction studies

Gene	Accession No.	Primers		Size
		Direction	Sequences (5′-3′)	
				bp
hPepT1	NM_005073	Forward	GTTGGCAACATCATTGTGCT	149
		Reverse	TCCGCTGGGTTGATGTAAGT	
h18S	X03205	Forward	AGGAATTGACGGAAGGGCAC	320
		Reverse	GGACATCTAAGGGCATCACA	
hβ actin	NM_001101	Forward	GGGTCAGAAGGATTCCTATG	238
		Reverse	GGTCTCAAACATGATCTGGG	
hTBP	NM_003194	Forward	GAGAGCCACGAACCACGG	178
		Reverse	ACATCACAGCTCCCCACCAT	

bp, base pairs.

Moreover, in a mouse model of type 2 diabetes mellitus and obesity characterized by high levels of both circulating insulin and leptin levels, we reported a significant decrease in PepT1 function (expression and activity) that was concomitant with a marked decrease in the expression of intestinal *ob* receptors (Hindlet et al., 2009). The down-regulation of PepT1 has to be carefully examined in a context of glucose intolerance and obesity. Spanier et al. (2009) recently showed that the extinction of PepT1 in *Caenorhabditis elegans* did result in fat accumulation that led to an obesity phenotype in the worm. More importantly, some PepT1 substrates can diminish sodium-dependent glucose cotransporter 1 expression at the brush-border membrane of the enterocyte, and glucose transporter 2 and PepT1 are inversely regulated by the activation of protein kinase CβII (Vernaleken et al., 2007; Mace et al., 2009) in favor of a cross-talk between PepT1 and glucose transporters. Therefore, one would expect that a reduction of PepT1 activity could lead to a worsening in the obesity phenotype on the one hand and to the absence of regulation in intestinal absorption of glucose on the other hand.

Metformin, the first-line treatment for type 2 diabetes, acts through the AMPK signaling pathways to inhibit hepatic gluconeogenesis and enhance glucose use in the muscle (Zhou et al., 2001). Moreover, metformin is also known to reduce protein synthesis by inhibiting the S6 ribosomal protein and by activating the translation inhibitor 4E-BP1 (Dowling et al., 2007). Not only liver and muscle but also intestine are targets for metformin because it has now been established that enterocytes express AMPK and that metformin can regulate AMPK in rat enterocytes (Pieri et al., 2010; Sakar et al., 2010). PPARγ agonists are also used in type 2 diabetes; these compounds form heterodimers with retinoid X receptors, and the complex binds to PPAR response elements (PPREs) within the promoter domains of target genes (Olefsky, 2000). In vivo, PPARγ agonists act on insulin target tissues (fat, muscle, and liver) to improve insulin sensitivity (Olefsky, 2000). Of interest, rosiglitazone, a potent PPARγ agonist, has been shown to also enhance protein synthesis via the mTOR pathway by activating the ribosomal machinery contrary to metformin (Festuccia et al., 2009). The first-line treatment for type 2 diabetes is metformin alone; however, this monotherapy often leads to inefficient glycemic control, and glitazones may be prescribed with metformin.

The aim of this study was thus to investigate the potential effect of the two antidiabetic drugs, rosiglitazone and metformin, on PepT1 in a model of type 2 diabetes and obesity.

TABLE 2
Weight gain and food intake of mice during the diet-only period
Mice were fed the high-fat diet or standard chow for 6 weeks. Food consumption and weight gain were monitored twice weekly. Plasma leptin and insulin were determined by radioimmunoassay at the end of the 6 weeks (n = 35 in each group). All data are means ± S.E.M.

	SC	HFD
Weight gain, g	4.14 ± 0.20	7.52 ± 0.51***
Plasma insulin, ng/ml	0.61 ± 0.13	1.31 ± 0.29*
Plasma leptin, ng/ml	4.94 ± 0.90	10.78 ± 0.77***
Plasma glucose, mg/dl	167 ± 6	201 ± 5**
Food intake, g/day	3.86 ± 0.05	2.72 ± 0.14***
Caloric intake, cal/day	10,896 ± 149	14,450 ± 771***
Lipids, g/day	0.12 ± 0.002	0.98 ± 0.052***
Proteins, g/day	0.62 ± 0.01	0.49 ± 0.03***
Carbohydrates, g/day	1.85 ± 0.03	0.95 ± 0.05***

* $P < 0.05$ versus SC group.
** $P < 0.01$ versus SC group.
*** $P < 0.001$ versus SC group.

Materials and Methods

Cell Culture. Caco-2 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Cergy Pontoise, France) supplemented with 20% fetal bovine serum (Gibco-Invitrogen), 1% nonessential amino acids, and 1% penicillin/streptomycin in a 5% CO_2-95% humidity environment at 37°C. Cells were seeded on Costar Transwell membrane inserts with 0.4-μm pores (Corning Life Sciences, New York, NY) at a density of 5×10^4 cells/cm^2 for transport experiments or plated into 12-well plates for protein and mRNA expression analysis.

Cell monolayers were treated with 10 mM metformin (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and/or 10 μM rosiglitazone (SPI-BIO; Cayman Chemicals, Montigny-le-Bretonneux, France), 300 μM SR-202 (mifobate; Sigma-Aldrich), 10 μM compound C (Sigma-Aldrich), 0.5 mM AICAR (Calbiochem, Darmstadt, Germany), or 0.2 nM rapamycin (Sigma-Aldrich) for a designated period of time. Experiments were conducted on the 17th day of culture. Transepithelial resistance was monitored using an EVOM system (WPI, Saratosa, FL).

Animals. All experiments were performed in accordance with the European Committee Standards concerning the care and use of laboratory animals. Experiments were conducted in C57BL/6J male mice aged 8 to 10 weeks. Animals were housed in a room maintained at 21°C with a 12-h light/dark schedule and fed ad libitum with free access to water. They were fed for 6 weeks with standard laboratory chow (SC) (control mice, A04 biscuits; UAR, Villemoisson, France) or a high-fat, high-sucrose diet [referred to as high-fat diet (HFD); SAFE, Augy, France]. The SC diet provided 2820 kcal/kg food and contains 3% fat (270 kcal/kg), 48% complex carbohydrates (1910 kcal/kg, primarily starch), and 16% protein (640 kcal/kg). The HFD provided 5320 kcal/kg and includes 36% fat (primarily lard, 3220 kcal/kg), 35% simple carbohydrates (1400 kcal/kg, mainly saccharose), and 18% protein (700 kcal/kg). Food consumption and weight were measured with both diets. After the 6-week diet-only period,

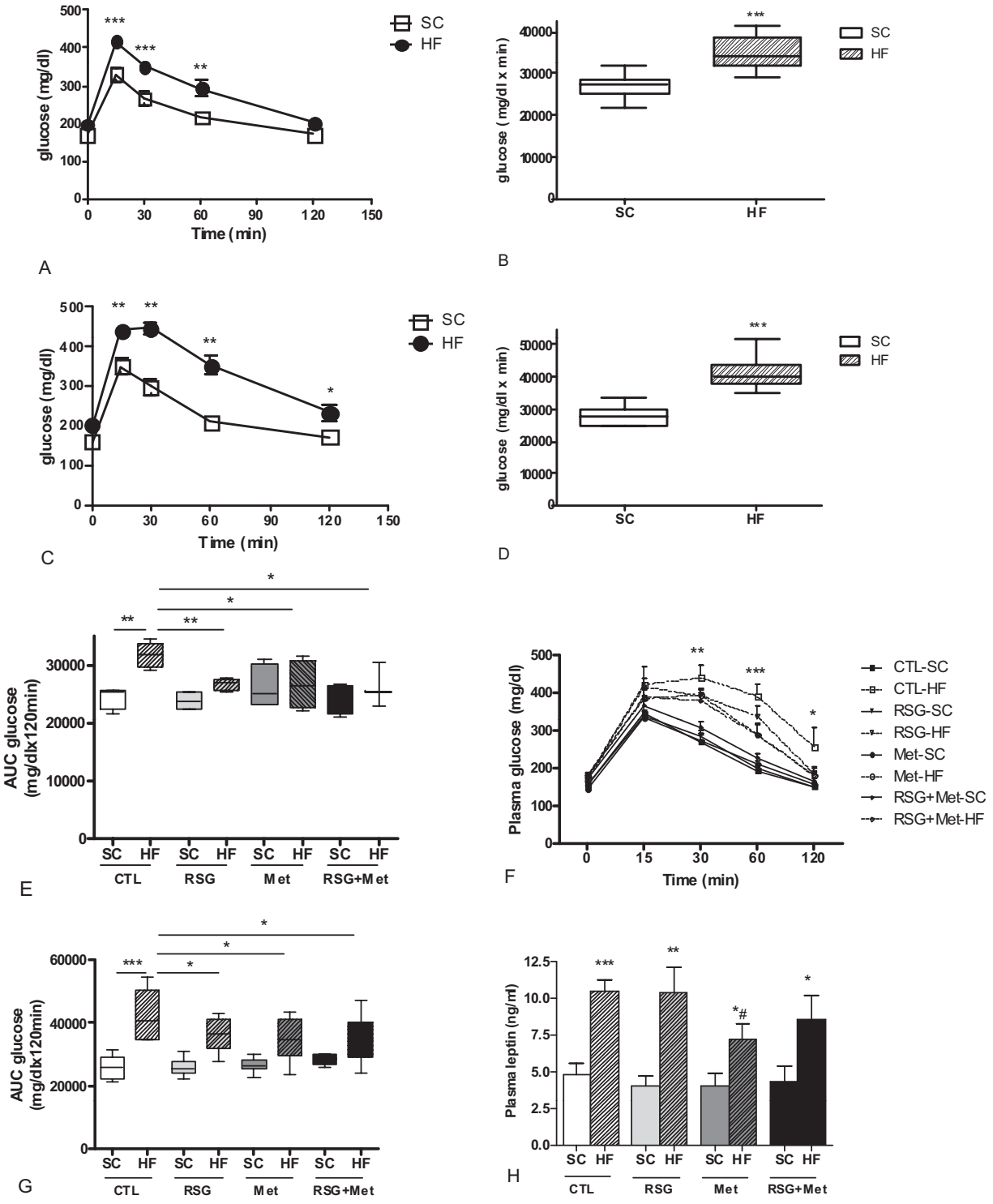

Fig. 1. Glucose tolerance tests. Glucose (2 g/kg) was injected intraperitoneally or given by gavage to the 6-h fasting animals at $t = 0$, and glycemia was measured at indicated time points at the tail vein. A and B, oral glucose tolerance test at the end of the diet-only period. AUC was calculated using the trapezoidal rule ($n = 16$ in each group). ***, $P < 0.001$; **, $P < 0.01$ versus SC group. All data are means ± S.E.M. C and D, intraperitoneal glucose tolerance test at the end of the diet-only period ($n = 16$ in each group). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ versus SC group. All data are means ± S.E.M. E, oral glucose tolerance test at the end of the 7-day treatment period ($n = 4$ in each group). **, $P < 0.01$; *, $P < 0.05$. All data are means ± S.E.M. F and G, intraperitoneal glucose tolerance test at the end of the 7-day treatment period. AUC was calculated using the trapezoidal rule ($n = 8$–12). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ versus SC group in the time course representation and ***, $P < 0.001$; *, $P < 0.05$ versus indicated groups in the AUC representation. All data are means ± S.E.M. H, plasma leptin levels at the end of the 7-day treatment period ($n = 6$–9). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus respective SC group; #, $P < 0.05$ versus high-fat diet vehicle-treated group for leptin. SC, standard chow; HF, high-fat diet; AUC, area under the curve; Rosi, rosiglitazone; Met, metformin; CTL, control; RSC, rosiglitazone-standard chow; RHF, rosiglitazone-high-fat diet; MSC, metformin-standard chow; MHF, metformin-high-fat diet; RMSC, rosiglitazone-metformin-standard chow; RMHF, rosiglitazone-metformin-high-fat diet.

mice were kept on their diet and treated for 1 additional week with antidiabetic drugs. In each diet group, mice were randomly divided into four groups: a metformin group receiving 250 mg/kg/day of metformin orally, a rosiglitazone group receiving 8 mg/kg/day of rosiglitazone orally, a third group receiving both drugs, and a fourth group receiving the vehicle alone.

Glucose Tolerance Tests. Glucose was injected intraperitoneally or given by gavage (2 g/kg), and blood was sampled from the tail vein at $t = 0$ and at 15, 30, 60, and 120 min after the administration of glucose. Glycemia was measured with the ACCU-CHEK Compact Plus System (Roche Diagnostics, Meylan, France). Insulin and leptin plasma concentrations were determined using mice radioimmunoassay kits from Linco Research, Inc. (St. Charles, MO) according to the manufacturer's instructions.

PepT1 Activity in Caco-2 Cells. All procedures were conducted as described previously (Hindlet et al., 2007). In brief, PepT1 activity was measured after the transport of [^{3}H]Gly-Sar (specific activity 0.5 Ci/mmol; Isobio, Fleurus, Belgium), a nonhydrolyzable PepT1 substrate, across the Caco-2 monolayer. The apical compartment of the Transwell was filled with 0.5 ml of a 20 μM Gly-Sar solution in a pH 6.4 KRB containing 0.4 μCi/ml [^{3}H]Gly-Sar. Basolateral compartments (KRB buffer pH 7.4) were sampled at $t = 0$, 5, 10, 15, 20, 25, and 30 min and [^{3}H]Gly-Sar concentration was calculated after radioactivity was measured using a beta counter (LS 6000 TA liquid scintillation counter; Beckman Coulter, Villepinte, France). In experiments testing the transcellular and the paracellular routes, [^{3}H]Gly-Sar was replaced by [^{3}H]testosterone (specific activity 80.4 μCi/mmol; PerkinElmer, Courtaboeuf, France) or [^{14}C]mannitol (specific activity: 0.25 Ci/mmol; PerkinElmer), respectively.

Transport of [^{3}H]Gly-Sar in Mice. All procedures were conducted as described previously (Hindlet et al., 2007). In brief, transport of Gly-Sar was monitored in mice using the ex vivo jejunal loop method. Segments of jejunum were filled with 100 μl/cm of a 20 μM Gly-Sar solution in a pH 6.4 KRB containing 1 μCi/ml [^{3}H]Gly-Sar and 500 mg/l phenol red as a test of paracellular permeability. The PepT1 transport specificity was assessed by the addition of an excess of dipeptide competitors [170 mM (Gly-Gly, Gly-Pro)]. Intestinal segments were ligated at both ends and were incubated at 37°C in a thermostated bath of KRB at pH 7.4. Samples were withdrawn from the bath at $t = 5$, 10, 15, 20, 25, and 30 min. [^{3}H]Gly-Sar concentration was calculated after radioactivity was measured using a beta counter (LS 6000 TA liquid scintillation counter), and phenol red concentration was evaluated after the addition of 1 N NaOH at 570 nm in a microplate reader (Multiskan FC; Thermo Fisher Scientific, Saint Herblain, France).

Western Blot Analysis. For protein extraction, all procedures were conducted as described previously (Hindlet et al., 2007). Proteins (20–25 μg) were separated by electrophoresis on SDS-polyacrylamide gel electrophoresis gels (8–12%). Proteins were then transferred to nitrocellulose membranes and subjected to immunoblotting. Dilutions of primary antibodies were 1:1000 for PepT1 (gift from Dr. D. Merlin, Emory University, Atlanta, GA) for in vitro or from Professor N. Kapel (Paris Descartes University, Paris, France) for in vivo studies), 1:5000 for β-actin (clone AC74; Sigma-Aldrich), 1:1000 for c-Myc phospho (Thr38/Ser62) (clone E203; Millipore, Molsheim, France). Secondary peroxidase-conjugated antibodies (Dako, Glostrup, Denmark) were used at 1:10,000 dilution, and membranes were probed using the ECL chemiluminescence system (PerkinElmer). The intensity of the bands was quantified using Scion Image (National Institutes of Health, Bethesda, MD).

ELISA Analysis. The analysis was performed according to the manufacturer's instructions with the PathScan Phospho-S6 Ribosomal Protein (Ser235/236) Sandwich ELISA Kit or the PathScan Phospho-4E-BP1 (Thr37/Thr46) Sandwich ELISA Kit (Cell Signaling Technology, Saint-Quentin-en-Yvelines, France). In brief, cell lysates were incubated in coated plates overnight at 4°C and the Phospho-S6 Ribosomal Protein (Ser235/236) or the Phospho-4E-BP1 (Thr37/Thr46) detection antibody was added for 2 h at 37°C. The absorbance was measured at 405 nm in a microplate ELISA reader (Multiskan FC; Thermo Fisher Scientific).

Real-Time Polymerase Chain Reaction Analysis. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantification of the cDNA was performed with the Light Cycler system (Roche Diagnostics) according to the manufacturer's instructions. Three housekeeping genes [β-Actin, TATA box binding protein (*TBP*), and *18S*] were used according to the geNorm strategy (Vandesompele et al., 2002). Primers were designed with Primer3 software (Table 1).

Statistical Analysis. All values are expressed as means ± S.E.M. The Mann-Whitney test (or Student's t test when possible) was performed to compare two means, and the Kruskal-Wallis test was performed to compare more than two means. GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. The level of significance was set at $P < 0.05$ for all analyses.

Results

Rosiglitazone Prevents HFD-Induced PepT1 Down-Regulation but Metformin Does Not. As expected, mice fed a HFD had a greater caloric intake than mice fed SC despite a decrease in total food intake (Table 2). This caloric intake resulted, 6 weeks later, in a significant increase in weight gain and in marked hyperleptinemia and hyperinsulinemia (Table 2) associated with glucose intolerance in HFD-fed mice monitored by oral glucose tolerance and intraperitoneal glucose tolerance tests (Fig. 1, A–D). Because PPARγ agonists and metformin can be used separately or as a combination in patients with type 2 diabetes, we tested the action of each drug alone or in combination. The 7-day treatment with rosiglitazone or metformin or both reversed fasting hyperglycemia and fasting hyperinsulinemia according to their glycemia-lowering properties (Table 3). In addition, both intraperitoneal and oral glucose tolerance tests showed that the treatment reversed glucose intolerance (Fig. 1, E–G). Finally, metformin treatment reduced plasma leptin

TABLE 3

Weight gain in animals during the 7-day treatment with rosiglitazone and/or metformin

Plasma glucose and insulin determined after the 7-day treatment in fasting animals (n = 4–8). All data are means ± S.E.M.

	SC				HFD			
	Vehicle	RSG	Met	RSG + Met	Vehicle	RSG	Met	RSG + Met
Weight gain, g	−0.81 ± 0.37	−0.74 ± 0.34	−1.01 ± 0.43	−0.74 ± 0.47	0.47 ± 0.46	−0.39 ± 0.49	0.07 ± 0.26	0.07 ± 0.46
Fasting plasma insulin, ng/ml	0.27 ± 0.03	0.33 ± 0.02	0.33 ± 0.03	0.32 ± 0.02	0.51 ± 0.13*	0.29 ± 0.02	0.29 ± 0.04	0.34 ± 0.04
Fasting plasma glucose, mg/dl	162 ± 6	151 ± 9	159 ± 3	167 ± 7	246 ± 5***	165 ± 10	187 ± 7	190 ± 10

RSG, rosiglitazone; Met, metformin.

* $P < 0.05$ versus the SC group and HFD-treated groups for insulin.

*** $P < 0.001$ versus SC group and HFD-treated groups for glucose plasma levels.

levels in HFD-fed mice compared with their vehicle-treated HFD littermates with no reduction in the animal weight (Table 3).

Consistent with our previous data, HFD-fed mice exhibited a 2-fold decrease in PepT1 activity. Rosiglitazone induced a similar 1.8-fold increase in PepT1 activity both in SC- and HFD-fed mice. Metformin alone had no effect on PepT1 activity, but when it was combined with rosiglitazone, the rosiglitazone stimulation of PepT1 activity could no longer be observed in SC- or in HFD-fed mice (Fig. 2).

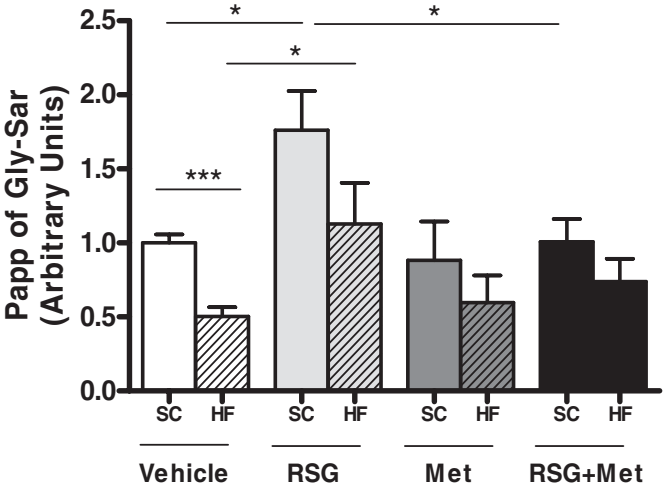

Fig. 2. P_{app} of Gly-Sar in mice. P_{app} of Gly-Sar was monitored using the ex vivo intestinal loop method for 30 min. Gly-Gly and Gly-Pro were used as competitors to assess specific transport via PepT1 (n = 8–11). *, $P < 0.05$; ***, $P < 0.001$. All data are means ± S.E.M. RSG, rosiglitazone; Met, metformin.

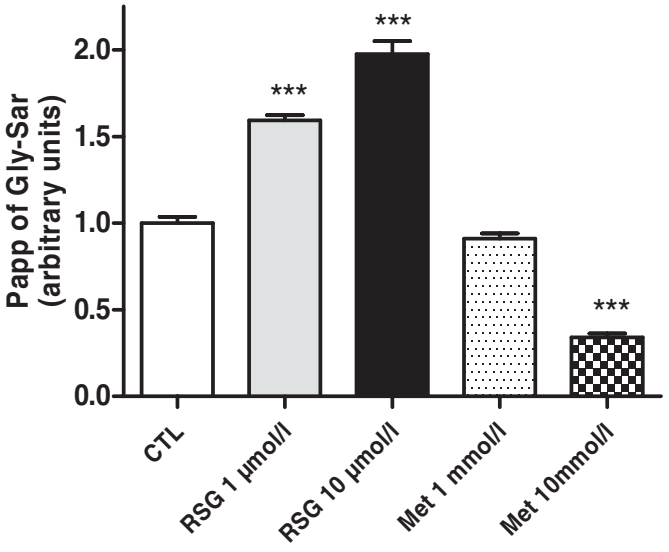

Fig. 3. Action of rosiglitazone (RSG) and metformin (Met) in Caco-2 cells. Dose-dependent P_{app} of Gly-Sar across Transwell membranes was measured for 30 min. Cells were treated with designated concentrations of rosiglitazone or metformin or both for 7 days (n = 3–6). ***, $P < 0.001$. All data are means ± S.E.M. CTL, control.

Rosiglitazone and Metformin Regulate PepT1 Directly on the Enterocyte in an Opposite Manner. To determine whether the effects of rosiglitazone and metformin are direct or not, we used Caco-2 cells in culture (Fig. 3). The kinetic studies revealed that rosiglitazone (10 μM) and metformin (10 mM) regulated PepT1 function in an opposite manner: the rosiglitazone stimulation of Gly-Sar transport became significant after 7 days of treatment, whereas the metformin-induced reduction of PepT1 transport activity was significant after 72 h of treatment (data not shown). The effects of the two drugs were dose-dependent with a maximum 1.8-fold increase in PepT1 activity occurring with 10 μM rosiglitazone and a maximum 60% inhibition of PepT1 activity observed at 10 mM metformin (Fig. 3). To evaluate whether metformin and/or rosiglitazone act specifically on the modification of PepT1-mediated Gly-Sar transport, we measured paracellular and transcellular transport after the apparent permeability (P_{app}) of mannitol and testosterone, respectively, on Caco-2-treated cells. TEER and cell viability were also investigated to establish cell monolayer integrity after the treatment with rosiglitazone and/or metformin. Neither rosiglitazone nor metformin nor the combination of the two drugs altered testosterone or mannitol transport across the Caco-2 cell monolayer, excluding any modification in the transcellular or paracellular permeability of Gly-Sar (Table 4). Furthermore, there was no change in TEER or cell viability on treatments of the cells with the two drugs as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (Table 4).

As shown in Fig. 4A, rosiglitazone stimulated PepT1 activity, whereas metformin decreased it. More importantly, the effects of metformin suppressed the effect of rosiglitazone, resulting in a 36% decrease in the activity of the transporter when rosiglitazone and metformin were combined (Fig. 4A).

We next examined whether the two drugs could modify PepT1 protein and mRNA levels. As shown in Fig. 4, rosiglitazone induced a 39% increase in the amounts of PepT1 protein but did not change PepT1 mRNA levels (Fig. 4, B and C). On the other hand, metformin decreased by 20% the amounts of PepT1 protein and by 43% the levels of PepT1 mRNA (Fig. 4, B and C). As with the PepT1 transport activity, metformin completely overcame rosiglitazone stimulation when the two drugs were combined, leading to a 26 to 46% decrease in PepT1 protein and mRNA levels, respectively (Fig. 4, B and C).

TABLE 4

P_{app} of testosterone (n = 8 in each group) and mannitol (n = 4 in each group), cell viability (n = 8–15), and TEER (n = 10–12) in Caco-2 cells treated for 7 days with rosiglitazone and/or metformin

P_{app} of testosterone and mannitol was monitored for 30 min. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. All data are presented as mean ± S.E.M.

	CTL	RSG	Met	RSG + Met
Testosterone P_{app}, arbitrary units	1.00 ± 0.04	1.08 ± 0.03	1.00 ± 0.05	0.98 ± 0.08
Mannitol P_{app}, arbitrary units	1.00 ± 0.04	1.17 ± 0.17	1.08 ± 0.28	1.46 ± 0.27
Cell viability, %	100 ± 2	97 ± 5	93 ± 6	92 ± 12
TEER, arbitrary units	1.00 ± 0.02	0.94 ± 0.02	0.94 ± 0.05	1.19 ± 0.12

CTL, control; RSG, rosiglitazone; Met, metformin.

Rosiglitazone Activates PepT1 mRNA Translation through the mTOR Pathway. Additional experiments were performed to decipher the action of rosiglitazone on PepT1 expression. First, pharmacological blockade of PPARγ by SR-202, a PPARγ antagonist, abolished the rosiglitazone stimulation of both PepT1 activity and PepT1 protein levels, indicating that rosiglitazone acts through the activation of PPARγ (Fig. 5, A and B).

Moreover, because rosiglitazone regulates PepT1 at a post-transcriptional level, we explored the translational machinery in Caco-2 cells. In Fig. 6, A and B, we show that rapamycin treatment reversed rosiglitazone stimulation of PepT1 activity and protein expression, indicating that it was mTOR-dependent. The downstream targets of mTOR, i.e., S6 ribosomal protein, 4E-BP1, and c-Myc were then analyzed. As shown in Fig. 7A, 10 μM rosiglitazone increased by 30% the amounts of phosphorylated S6 ribosomal protein, and this effect was reversed by SR-202 (data not shown). Finally, rosiglitazone did not affect the levels of phosphorylated 4E-BP1, a translation inhibitor, and of c-Myc, ruling out their implication in the action of the drug on PepT1 regulation (Fig. 7B and data not shown).

Metformin Inhibits PepT1 by the Activation of AMPK. Metformin is described as an AMPK activator; therefore, we tested whether its action on PepT1 is dependent on the AMPK signaling pathway. As shown in Fig. 8A, activa-

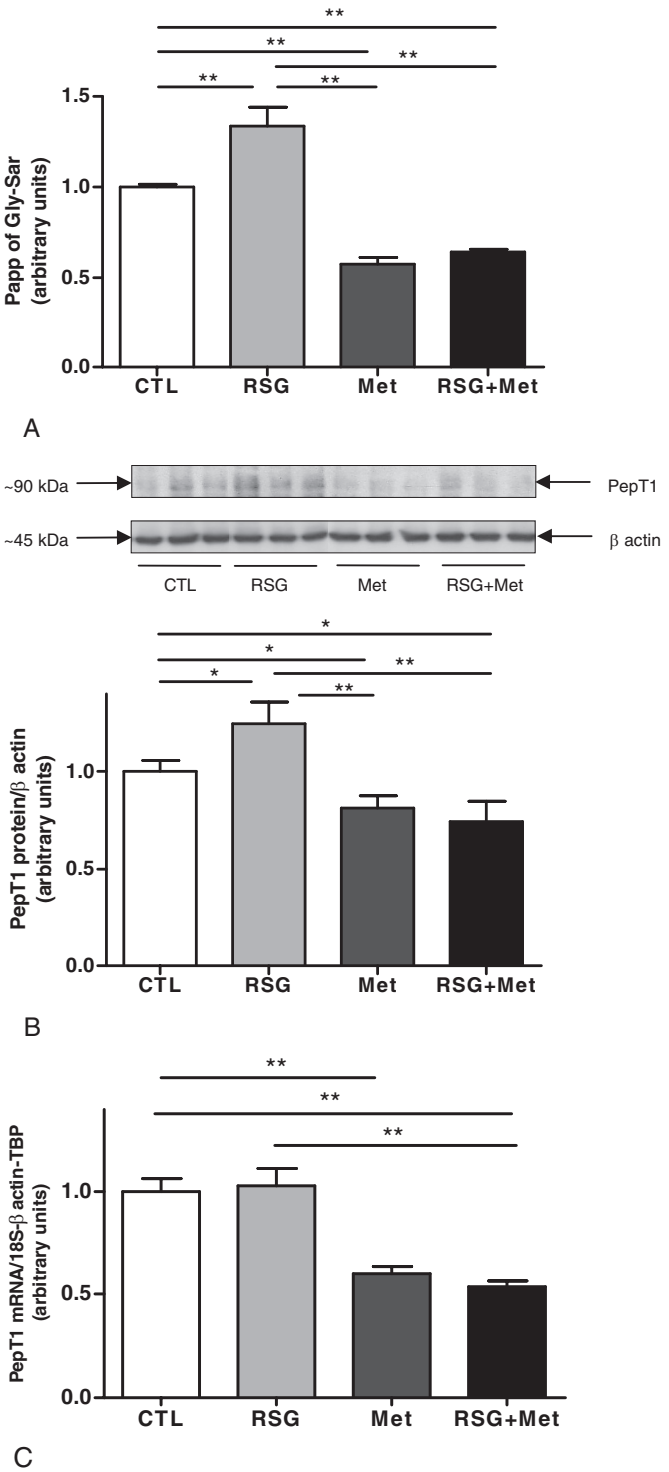

Fig. 4. Effect of rosiglitazone (RSG) and/or metformin (Met) on PepT1 activity, protein, and mRNA expression in Caco-2 cells. Cells were treated with rosiglitazone (10 μM) and/or metformin (10 mM) for 7 days. A, P_{app} of Gly-Sar across Transwell membranes was measured for 30 min (n = 6–12). **, $P < 0.01$. All data are means ± S.E.M. B, densitometric analysis of PepT1 protein expression normalized to β-actin expression (n = 11–12). *, $P < 0.05$; **, $P < 0.01$. All data are means ± S.E.M. C, relative quantification of PepT1 mRNA normalized to housekeeping genes mRNA expression (n = 6). **, $P < 0.01$. All data are means ± S.E.M. CTL, control.

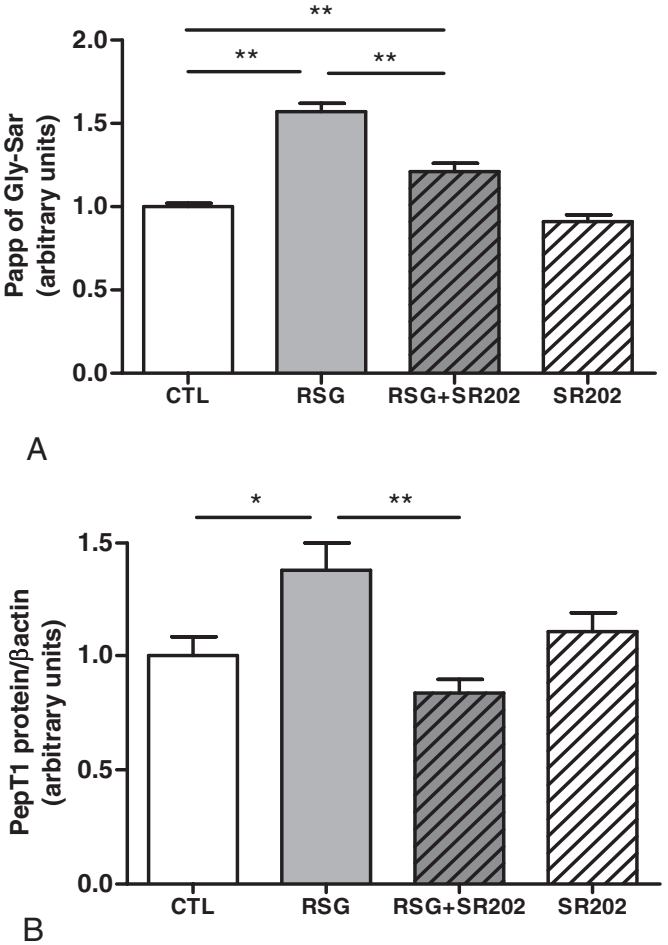

Fig. 5. Activation of PepT1 by rosiglitazone through the PPARγ pathway. Cells were treated for 7 days with rosiglitazone (10 μM) and/or SR-202 (300 μM). A, P_{app} of Gly-Sar across Transwell membranes (n = 5–10). **, $P < 0.01$. All data are means ± S.E.M. B, densitometric analysis of PepT1 protein expression normalized to β-actin expression (n = 6). *, $P < 0.05$; **, $P < 0.01$. All data are means ± S.E.M. CTL, control; RSG, rosiglitazone.

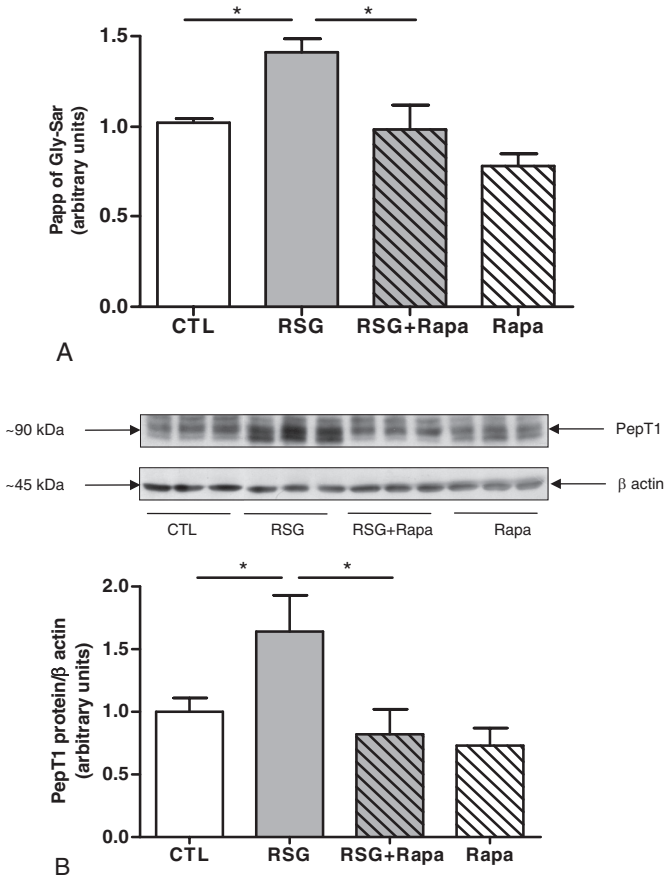

Fig. 6. Effect of rosiglitazone on PepT1 activity through mTOR. Cells were treated for 7 days with rosiglitazone (10 μM) and/or rapamycin (0.2 nM). A, P_{app} of Gly-Sar across Transwell membranes ($n = 4$). *, $P < 0.05$. All data are means ± S.E.M. B, densitometric analysis of PepT1 protein expression normalized to β-actin expression ($n = 6$). *, $P < 0.05$. All data are means ± S.E.M. CTL, control; RSG, rosiglitazone; Rapa, rapamycin.

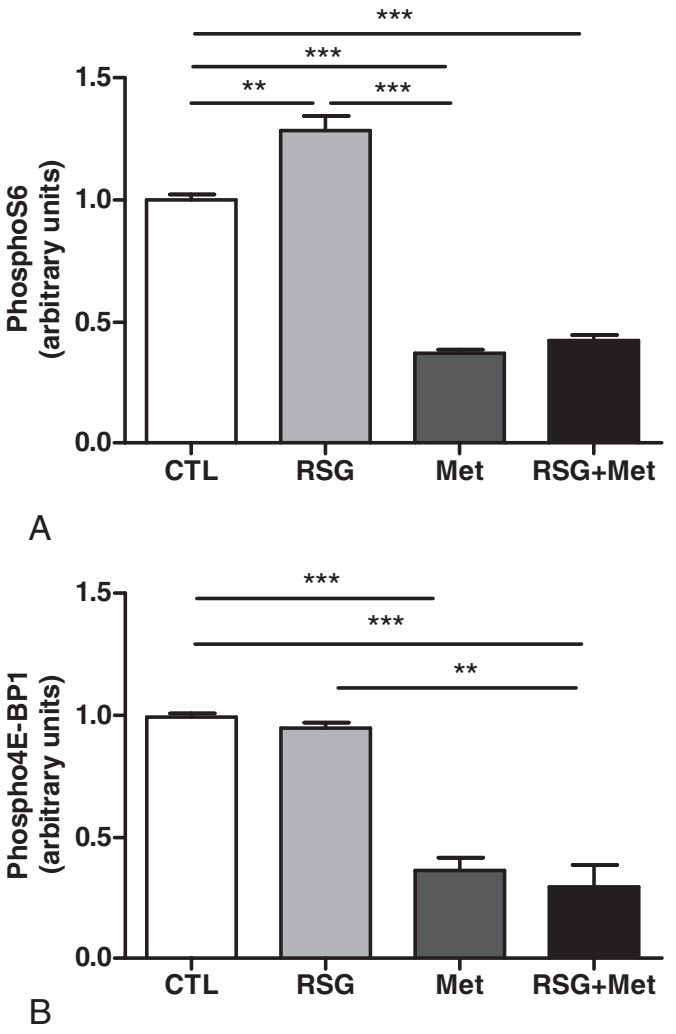

Fig. 7. Effect of rosiglitazone and metformin on the translation machinery. A, ELISA analysis of phospho-S6 protein expression after a 7-day treatment with metformin (10 mM) and/or rosiglitazone (10 μM) (n = 6–12). **, $P < 0.01$; ***, $P < 0.001$. All data are means ± S.E.M. B, ELISA analysis of phospho-4E-BP1 protein expression after a 7-day treatment with metformin (10 mM) and/or rosiglitazone (10 μM) ($n = 8$). **, $P < 0.01$; ***, $P < 0.001$. All data are means ± S.E.M. CTL, control; RSG, rosiglitazone; Met, metformin.

tion of AMPK by AICAR mimicked the effect of metformin on PepT1 activity. Moreover, pharmacological inhibition of AMPK by compound C prevented the metformin inhibition of PepT1-mediated transport, indicating that AMPK-dependent pathways are involved in the action of metformin (Fig. 8B).

Metformin Overcomes Rosiglitazone Stimulation of PepT1 Function. However, bearing in mind that rosiglitazone regulated PepT1 at the translational level, we attempted to clarify the action of metformin at this particular level by studying the downstream signaling pathway implicated in the translation. As shown in Fig. 7A and in contrast to the action of rosiglitazone, metformin inhibited 63% of the phosphorylation of S6 ribosomal protein and reduced by 2.5-fold the phosphorylation of 4E-BP1 (Fig. 7B), indicating activation of this translation inhibitor. When the two drugs were combined, the phosphorylated S6 and 4E-BP1 levels significantly decreased by 58 and 71%, respectively, shutting down the rosiglitazone effects on the PepT1 mRNA translation (Fig. 7, A and B).

Discussion

In the present report, we have clearly shown that rosiglitazone and metformin regulate in an opposite manner the transport function of PepT1 in HFD-fed mice but that both improve their glucose intolerance. In these HFD-fed mice, characterized by strong reduced PepT1 activity, treatment with rosiglitazone but not with metformin restores and stimulates PepT1 transport activity. On the other hand, metformin reversed rosiglitazone activity on PepT1 in vivo. These opposite effects on PepT1 are likely to take place directly on the enterocyte. Treatment of the enterocyte-like Caco-2 cells with rosiglitazone does indeed enhance PepT1 mRNA translation in a PPARγ-dependent manner, whereas metformin inhibits PepT1 through both transcriptional and translational mechanisms.

As expected, the oral antidiabetic medications improved glucose intolerance as well as insulin plasma levels. Because no modification in the body weight of mice fed with the HFD was measured during the 1-week oral antidiabetic medication treatment, leptin plasma levels were logically unchanged in rosiglitazone-treated animals. However, metformin induced a reduction in leptin levels in HFD-fed mice compared with the HFD-fed mice receiving the vehicle only. This result could be explained by the ability of metformin to

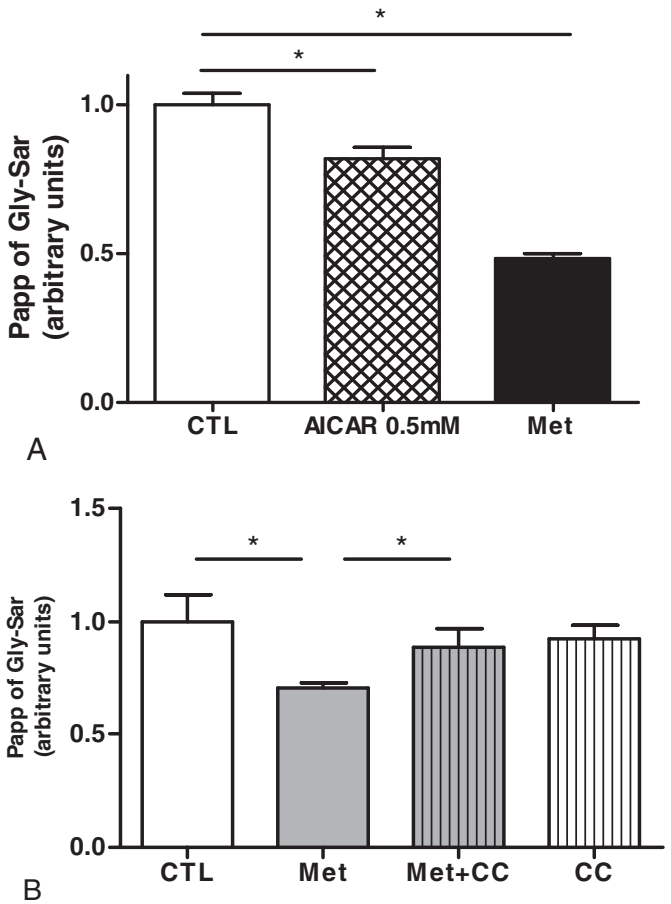

Fig. 8. Inhibition of PepT1 activity by metformin through the activation of the AMPK pathway. A, cells were treated for 3 days with AICAR (0.5 mM) or metformin (10 mM) and the P_{app} of Gly-Sar across Transwell membranes was measured for 30 min ($n = 3$). *, $P < 0.05$. All data are means ± S.E.M. B, cells were treated for 3 days with metformin (10 mM) and/or compound C (5 μM), and the P_{app} of Gly-Sar across Transwell membranes was measured for 30 min ($n = 5$–10). *, $P < 0.05$. All data are means ± S.E.M. CTL, control; RSG, rosiglitazone; CC, compound C.

reduce leptin levels as was already reported in vitro and in humans (Morin-Papunen et al., 1998; Klein et al., 2004).

In addition to the primary function of PepT1 in the supply of nitrogen to the body, the regulation of PepT1 is of importance in the type 2 diabetes mellitus/obesity phenotype because PepT1 is also involved in the feedback regulation of gastrointestinal functions and food intake. It was reported that activation of vagal afferents is indeed associated with induction of satiety through a Ca^{2+}-dependent cholecystokinin secretion that is at least partially dependent on PepT1 activity (Némoz-Gaillard et al., 1998; Darcel et al., 2005). Moreover, it has been shown that PepT1 substrates could cause a reduction in sodium-dependent glucose cotransporter 1 activity and that the same signaling pathway acted reciprocally on sugar and peptide transporters (Vernaleken et al., 2007; Mace et al., 2009). Finally, PepT1 extinction in *C. elegans* leads to increased free fatty acid uptake and an obese phenotype in the worm (Spanier et al., 2009).

Of interest, we found that rosiglitazone restores the HFD inhibition of PepT1 activity and even increases its transport activity in vivo. It is possible that in our current study, regulation of PepT1 by rosiglitazone could be due to its ability to improve leptin and insulin sensitivity, which in turn tightly controlled activity of the transporter (Thamotharan et al., 1999; Buyse et al., 2001; Yamauchi et al., 2001; Hindlet et al., 2007). However, our in vitro data clearly establish that rosiglitazone could directly act on enterocytes by enhancing mRNA translation. To decipher the mechanism of such an effect of rosiglitazone, the translation machinery in Caco-2 cells was analyzed. Apart from activating gene transcription via its nuclear receptors PPARγ, rosiglitazone also activates mTOR (Festuccia et al., 2009). In our model, using the selective mTOR inhibitor rapamycin, we observed that mTOR activation is responsible for the rosiglitazone effect on PepT1 protein expression. Moreover, exploration of the downstream targets of mTOR showed that rosiglitazone increases translation of PepT1 mRNA by activating S6 ribosomal protein with no change in activity of 4E-BP1, an inhibitor of translation. Finally, we also found that another pathway, which has been involved in the activation ribosomal machinery, i.e., c-Myc (van Riggelen et al., 2010), is not activated upon treatment with rosiglitazone.

PPAR agonists are generally reported to act through their transcriptional activity after activation of PPREs, and it is noteworthy that the same PPRE can respond to different types of PPAR agonists whether they are PPARα or PPARγ agonists (Frohnert et al., 1999; Shimizu et al., 2004). The promoter of the PepT1 gene was reported to contain several proposed PPREs, and none of these elements were responsive to the PPARα agonist (Shimakura et al., 2006). Thus, these PPREs may not be functional or the concentration of rosiglitazone used in our study may not have been sufficient for their activation.

In contrast to rosiglitazone, in vitro metformin potently reduced PepT1 activity and protein and mRNA levels. We did not observe this inhibitory effect in vivo in mice. This discrepancy could be tentatively explained by the enhanced insulin sensitivity with metformin. Metformin was first described as an AMPK activator by Zhou et al. (2001) in hepatocytes, and, more recently, AICAR, a selective AMPK activator, was shown to reduce PepT1 protein expression and activity in Caco-2 cells (Pieri et al., 2010). Our findings are consistent with these data and provide further knowledge of the phenomenon by showing that the activation of AMPK first inhibits the transcriptional regulation. Finally, we explored the mechanisms underlying the reversion of the rosiglitazone action on PepT1 by metformin by analyzing the translation machinery because it was reported that metformin can also regulate translation through the inhibition of mTOR (Dowling et al., 2007). We did indeed show that metformin treatment leads to S6 phosphorylation in enterocytes but also results in the dephosphorylation and thus the activation of 4E-BP1, a repressor of translation, allowing the sequestration of the translation initiation factor eukaryotic translation initiation factor 4E. Thus, it seems that metformin not only counteracts the effect of rosiglitazone on translation of PepT1 mRNA but also exerts an additional inhibitory effect on the translation machinery.

We have demonstrated in the current report that drugs used as first-line treatment for type 2 diabetes regulate in an opposite manner PepT1 function, in a context of obesity and glucose intolerance. These effects appear to involve AMPK- and PPARγ-dependent pathways. Given the role of PepT1 as a major determinant of nitrogen absorption and the indirect reciprocal regulation of nutrient transport, our results with

rosiglitazone and metformin require further studies to evaluate their potential benefits and disadvantages on intestinal physiology.

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Authorship Contributions

Participated in research design: Hindlet and Buyse.
Conducted experiments: Hindlet, Barraud, Boschat, and Buyse.
Performed data analysis: Hindlet, Barraud, Boschat, and Buyse.
Wrote or contributed to the writing of the manuscript: Hindlet, Farinotti, Bado, and Buyse.

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