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Rosiglitazone and Metformin have opposite effects on intestinal absorption of oligopeptides via the Proton-dependant PepT1 transporter

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Running title : Opposite effects of rosiglitazone and metformin on PepT1

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Non standard abbreviations: PepT1: intestinal H(+)/peptide cotransporter 1, DIO: diet induced obesity, HFD: high-fat diet, 4E-BP1: Eukaryotic translation initiation factor 4E-binding protein 1, PPAR: peroxisome proliferator-activated receptor, EGF: Epidermal Growth Factor, SGLT1: Sodium-dependent glucose cotransporter 1, GLUT2: Glucose transporter 2, PKC: Protein kinase C, AMPK: 5' AMP-activated protein kinase, RXR: retinoid X receptor, PPRE: PPAR response elements, mTOR: mammalian target of rapamycin, DMEM: Dulbecco's Modified Eagle Medium, AICAR: 5-Aminoimidazole-4-carboxamide ribotide, Gly-Sar: Glycyl-Sarcosine, Gly-Gly: Glycyl-Glycine, Gly-Pro: Glycyl-Proline, KRB: Krebs–Ringer bicarbonate buffer, ELISA: Enzyme-linked immunosorbent assay, PCR: polymerase chained reaction, TBP: TATA Box Binding Protein, TEER: Trans Epithelial Electric Resistance, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, CCK:

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cholecystokinin, eIF4E: Eukaryotic translation initiation factor 4E. Papp: apparent permeability. IPGTT and OGTT: intraperitoneal and oral glucose tolerance tests

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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 DIO model. C57Bl/6J male mice were fed high-fat diet (HFD) or a standard chow for 6 weeks and, then were treated for 7 days with metformin (250 mg/kg/d) and/or rosiglitazone (8 mg/kg/d). 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 a concomitant induction of the S6 ribosomal protein activation *in vitro*. Furthermore, metformin decreased PepT1 expression (mRNA, 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 anti-diabetic drugs exert opposite effects on the PepT1 transport function probably through direct action on enterocytes. In our type 2 diabetes/obesity model, rosiglitazone, a PPARγ agonist compensated the HFD-induced PepT1 downregulation whereas metformin reversed rosiglitazone activity at the translational level.

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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. So, 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 in the 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 the Epidermal Growth Factor (EGF) (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. 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) which was concomitant with a marked decrease in the expression of intestinal of *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. recently showed that the extinction of PepT1 in *C. elegans* did result in a fat accumulation which lead to an obesity phenotype in the worm (Spanier et al., 2009). More importantly, some PepT1 substrates can diminish SGLT1 expression at the brush

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border membrane of the enterocyte and GLUT2 and PepT1 are inversely regulated by the activation of PKC βII (Mace et al., 2009; Vernaleken et al., 2007) in favour 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 of type 2 diabetes, acts through the AMPK signalling 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 as it is now 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 (RXRs) 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). Interestingly, 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 glycaemic control, and glitazones may be prescribed with metformin.

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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.

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Materials and methods

Cell culture. Caco2 cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco-Invitrogen, Cergy Pontoise, France) supplemented with 20% fetal bovine serum (Gibco-Invitrogen), 1% non essential 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, NY, NY) at a density of 5 x 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 (Cayman chemicals, SPI-BIO, Montigny-le-Bretonneux, France), 300 µM SR-202 (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 a 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 8-10 weeks. Animals were housed in a room maintained at 21°C with 12:12-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); purchased from SAFE, Augy, France). The SC diet provided 2,820 kcal/kg of food and contains 3% fat (270 kcal/kg), 48% complex carbohydrates (1,910 kcal/kg, primarily starch), and 16%

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protein (640 kcal/kg). The HFD provided 5,320 kcal/kg and includes 36% fat (primarily lard, 3,220 kcal/kg), 35% simple carbohydrates (1,400 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, 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 *per os*, a rosiglitazone group receiving 8 mg/kg/day of rosiglitazone *per os*, 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. The measurement of glycaemia was performed with the ACCU-CHEK Compact Plus System (Roche Diagnostics, Meylan, France). Insulin and leptin plasma concentrations were determined using mice radioimmunoassay kits from Linco® according to the manufacturer's instructions (Linco Research Inc., St. Charles, MO).

PepT1 activity in Caco2 cells. All procedures were conducted as previously described (Hindlet et al., 2007). Briefly, PepT1 activity was measured following the transport of ^{3}H-Gly-Sar (Isobio, Fleurus, Belgium, specific activity: 0.5 Ci/mM) a non-hydrolysable PepT1 substrate, across the Caco2 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

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calculated after measuring radioactivity using a β 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 (PerkinElmer, Courtaboeuf, France, specific activity: 80.4 µCi/mM) or ^{14}C-mannitol (PerkinElmer, Courtaboeuf, France, specific activity: 0.25 Ci/mM) respectively.

Transport of ^{3}H-Gly-Sar in mice. All procedures were conducted as previously described (Hindlet et al., 2007). Briefly, 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, 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 measuring radioactivity using a β counter (LS 6000 TA liquid scintillation counter) and phenol red concentration was evaluated after the addition of NaOH 1N 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 previously described (Hindlet et al., 2007). Proteins (20-25 µg) were separated by electrophoresis on SDS-PAGE gels (8-12%). Proteins were then transferred to nitrocellulose membranes and subjected to immunoblotting. Dilutions of primary antibodies were: 1:1,000 for PepT1 (gift from Dr Merlin (Emory University-Atlanta-

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GA) for *in vitro* or from Pr Kapel (Paris Descartes University-Paris-France) for *in vivo* studies), 1:5,000 for β-actin (clone AC74, Sigma-Aldrich), 1:1,000 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 chimioluminescence system (Perkin Elmer, Courtaboeuf, France). The intensity of the bands was quantified using Scion image (NIH, Scion Corporation, 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 Signalling Technology, Saint-Quentin-en-Yvelines, France). Briefly, 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 2h at 37°C. The absorbance was measured at 405 nm in a microplate ELISA reader (Multiskan FC, Thermo Fisher Scientific, Saint Herblain, France)

Real time PCR analysis. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized by reverse transcription from 5 µg of total RNA using the SuperScript II reverse transcriptase (Invitrogen). Quantification of the cDNA was carried out with the Light Cycler system (Roche diagnostics) according to the manufacturer's instructions. Three housekeeping genes (β-actin, TBP, 18S) were used according to the GeNorm

strategy (Vandesompele et al., 2002). Primers were designed with the Primer3 software (table 1).

Statistical analysis. All values were expressed as mean ± S.E.M. Mann-Whitney test (or Student t-test when possible) was performed to compare 2 means and Kruskal-Wallis test was performed to compare more than 2 means. Statistical analysis used Graph Pad Prism Software (Graph Pad software, San Diego, CA). The level of significance was set at $P < 0.05$ for all analyses.

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Results

Rosiglitazone prevents HFD-induced PepT1 downregulation but metformin does not.

As expected, mice fed HF-diet had a greater caloric intake than mice fed SC despite a decrease in total food intake (Table 2). This caloric intake resulted, 6-week after, in a significant increase in weight gain, in a marked hyperleptinemia and hyperinsulinemia (Table 2) associated with glucose intolerance in HFD mice monitored by OGTT and IPGTT tests (Fig. 1A, B, C & D). As PPARγ agonists and metformin can be used separately or as a combination in type 2 diabetes patients, we tested the action of each drug alone or in combination. The 7-day treatment with rosiglitazone or metformin or both reversed the fasting hyperglycaemia and the fasting hyperinsulinemia according to their lowering glycaemic properties (Table 3). Additionally, both intraperitoneal and oral glucose tolerance tests showed that the treatment reversed glucose intolerance (Fig. 1E, F & G). Finally, the metformin treatment reduced plasma leptin levels in HFD mice compared to their vehicle-treated HFD litermates with no reduction in the animal weight (Table3).

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- as well as in HFD-fed mice (Fig 2).

RSG and metformin regulate in an opposite manner PepT1directly on the enterocyte.

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To determine whether the effects of rosiglitazone and metformin are direct or not, we used Caco2 cells in culture. The kinetic studies revealed that rosiglitazone (10 µM) and metformin (10 mM) regulated the 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 72h 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 4A). To evaluate whether metformin and/or rosiglitazone act specifically on the modification of PepT1-mediated Gly-Sar transport, we measured paracellular and transcellular transport following the apparent permeability of mannitol and testosterone respectively on Caco2 treated cells. TEER and cell viability were also investigated to establish cell monolayer integrity after the treatment with rosiglitazone and/or metformine. Neither rosiglitazone nor metformin or the combination of the two drugs altered testosterone or mannitol transport across the Caco2 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 as determined by MTT test upon treatments of the cells with the two drugs (Table 4).

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

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

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 5A & 5B).

Moreover, since rosiglitazone regulates PepT1 at a post-transcriptional level we explored the translational machinery in Caco2 cells. In Figure 6A and 6B, we showed that rapamycin treatment reversed the rosiglitazone stimulation of PepT1 activity and protein expression, indicating that it was mTOR-dependant. The downstream targets of mTOR, i.e. S6 ribosomal protein, 4E-BP1 and c-Myc were then analyzed . As shown in Figure 7A , 10 µmol/L 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

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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 dependant on AMPK signalling pathway. As shown in Fig. 8A, activation 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-dependant pathways are involved in the action of metformin (Fig. 8B).

Metformin overcomes the rosiglitazone stimulation of PepT1 function.

However, bearing in mind that rosiglitazone regulated PepT1 at the translationnal level, we attempted to clarify the action of metformin at this particular level by studying the downstream signalling pathway implicated in the translation. As shown in Figure 8A and by contrast to the action of rosiglitazone, metformin inhibited 63% of the phosphorylation of S6 ribosomal protein, and reduced 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 7A and 7B).

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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 mice but both improve their glucose intolerance. In these HFD mice, characterized by a strong reduced PepT1 activity, treatment with rosiglitazone but not 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. The treatment of the enterocyte-like Caco2 cells with rosiglitazone does indeed enhances PepT1 mRNA translation in a PPARγ-dependant manner whereas metformin inhibits PepT1 through both transcriptional and translational mechanisms.

As expected, the oral antidiabetic medications improve glucose intolerance as well as insulin plasma levels. As no modification in the body weight of mice fed with the HF diet was measured during the 1-week oral antidiabetic medications, leptin plasma levels were logically unchanged in rosiglitazone treated animals. However, metformin induced a reduction in leptin levels in HFD mice compared to the HFD mice receiving the vehicle only. This result could be explained by the ability of metformin to reduce leptin levels as it was already reported *in vitro* and in humans (Klein et al., 2004; Morin-Papunen et al., 1998)

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 as PepT1 is also involved in the feed back regulation of gastrointestinal functions and food intake. It was reported that activation of vagal afferents are indeed associated with induction of satiety through a Ca^{2+}-dependant CCK secretion that is

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at least partially dependant on PepT1 activity (Darcel et al., 2005; Nemoz-Gaillard et al., 1998). Moreover, it has been shown that PepT1 substrates could cause a reduction in SGLT1 activity and that the same signalling pathway acted reciprocally on sugar and peptide transporters (Mace et al., 2009; Vernaleken et al., 2007). Finally, PepT1 extinction in *C. Elegans* leads to free fatty acid increased uptake and an obese phenotype in the worm (Spanier et al., 2009).

Interestingly, we found that rosiglitazone restores the HF inhibition in PepT1 activity and even increases its transport activity *in vivo*. It is possible that in our current study, the regulation PepT1 by rosiglitazone, could be due to its ability to improve leptin and insulin sensitivity which in turn tightly controlled activity of the transporter (Buyse et al., 2001; Hindlet et al., 2007; Thamotharan et al., 1999; Yamauchi et al., 2001). However, our *in vitro* data clearly establish that rosiglitazone could directly act on enterocytes by enhancing mRNA translation. In order to decipher the mechanism of such an effect of rosiglitazone, the translation machinery in Caco2 cells was analysed. 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, exploring 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

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different types of PPAR agonists whether they are PPARα or PPARγ agonists (Frohnert et al., 1999; Shimizu et al., 2004). The promoter of PepT1 gene was reported to contain several proposed PPREs and none of these elements were responsive to 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.

By contrast to rosiglitazone, *in vitro* metformin potently reduced PepT1 activity, protein and mRNA levels. We did not observe this inhibitory effect *in vivo* in the mice. This discrepancy could be tentatively explained by the enhanced insulin sensitivity by metformin. Metformin was first described as an AMPK activator by Zhou et al. in hepatocytes (Zhou et al., 2001) and more recently, AICAR, a selective AMPK activator, was shown to reduce PepT1 protein expression and activity in Caco2 cells (Pieri et al., 2010). Our findings are consistent with these data and bring further knowledge of the phenomenon by showing that the activation of AMPK firstly inhibits the transcriptional regulation. Finally, we explored the mechanisms underlying the reversion of the rosiglitazone action on PepT1 by metformin by analysing the translation machinery since 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 eIF4E. Thus, it seems that metformin, not only counteracts the effect of rosiglitazone on translation of PepT1 mRNA, but exerts an additional inhibitory effect on the translation machinery.

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We have demonstrated in the current report that drugs used as a first line treatment of 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 in order to evaluate their potential benefits and disadvantages on intestinal physiology.

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Authorship contribution

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

References

Buyse M, Berlioz F, Guilmeau S, Tsocas A, Voisin T, Peranzi G, Merlin D, Laburthe M, Lewin MJ, Roze C and Bado A (2001) PepT1-mediated epithelial transport of dipeptides and cephalexin is enhanced by luminal leptin in the small intestine. *J Clin Invest* **108**(10):1483-1494.

Darcel NP, Liou AP, Tome D and Raybould HE (2005) Activation of vagal afferents in the rat duodenum by protein digests requires PepT1. *J Nutr* **135**(6):1491-1495.

Dowling RJ, Zakikhani M, Fantus IG, Pollak M and Sonenberg N (2007) Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res* **67**(22):10804-10812.

Festuccia WT, Laplante M, Brule S, Houde VP, Achouba A, Lachance D, Pedrosa ML, Silva ME, Guerra-Sa R, Couet J, Arsenault M, Marette A and Deshaies Y (2009) Rosiglitazone-induced heart remodelling is associated with enhanced turnover of myofibrillar protein and mTOR activation. *J Mol Cell Cardiol* **47**(1):85-95.

Frohnert BI, Hui TY and Bernlohr DA (1999) Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *J Biol Chem* **274**(7):3970-3977.

Hindlet P, Bado A, Farinotti R and Buyse M (2007) Long-term effect of leptin on h+-coupled Peptide cotransporter 1 activity and expression in vivo: evidence in leptin-deficient mice. *J Pharmacol Exp Ther* **323**(1):192-201.

Hindlet P, Bado A, Kamenicky P, Delomenie C, Bourasset F, Nazaret C, Farinotti R and Buyse M (2009) Reduced intestinal absorption of dipeptides via PepT1 in mice with diet-induced obesity is associated with leptin receptor down-regulation. *J Biol Chem* **284**(11):6801-6808.

Klein J, Westphal S, Kraus D, Meier B, Perwitz N, Ott V, Fasshauer M and Klein HH (2004) Metformin inhibits leptin secretion via a mitogen-activated protein kinase signalling pathway in brown adipocytes. *J Endocrinol* **183**(2):299-307.

Mace OJ, Lister N, Morgan E, Shepherd E, Affleck J, Helliwell P, Bronk JR, Kellett GL, Meredith D, Boyd R, Pieri M, Bailey PD, Pettcrew R and Foley D (2009) An energy supply network of nutrient absorption coordinated by calcium and T1R taste receptors in rat small intestine. *J Physiol* **587**(Pt 1):195-210.

Morin-Papunen LC, Koivunen RM, Tomas C, Ruokonen A and Martikainen HK (1998) Decreased serum leptin concentrations during metformin therapy in obese women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **83**(7):2566-2568.

Nemoz-Gaillard E, Bernard C, Abello J, Cordier-Bussat M, Chayvialle JA and Cuber JC (1998) Regulation of cholecystokinin secretion by peptones and peptidomimetic antibiotics in STC-1 cells. *Endocrinology* **139**(3):932-938.

Nielsen CU, Amstrup J, Steffansen B, Frokjaer S and Brodin B (2001) Epidermal growth factor inhibits glycylsarcosine transport and hPepT1 expression in a human intestinal cell line. *Am J Physiol Gastrointest Liver Physiol* **281**(1):G191-199.

Olefsky JM (2000) Treatment of insulin resistance with peroxisome proliferator-activated receptor gamma agonists. *J Clin Invest* **106**(4):467-472.

Pieri M, Christian HC, Wilkins RJ, Boyd CA and Meredith D (2010) The apical (hPepT1) and basolateral peptide transport systems of Caco-2 cells are regulated by AMP-activated protein kinase. *Am J Physiol Gastrointest Liver Physiol* **299**(1):G136-143.

Rubio-Aliaga I and Daniel H (2008) Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* **38**(7-8):1022-1042.

Sakar Y, Meddah B, Faouzi MA, Cherrah Y, Bado A and Ducroc R (2010) Metformin-induced regulation of the intestinal D-glucose transporters. *J Physiol Pharmacol* **61**(3):301-307.

Shimakura J, Terada T, Saito H, Katsura T and Inui K (2006) Induction of intestinal peptide transporter 1 expression during fasting is mediated via peroxisome proliferator-activated receptor alpha. *Am J Physiol Gastrointest Liver Physiol* **291**(5):G851-856.

Shimizu M, Takeshita A, Tsukamoto T, Gonzalez FJ and Osumi T (2004) Tissue-selective, bidirectional regulation of PEX11 alpha and perilipin genes through a common peroxisome proliferator response element. *Mol Cell Biol* **24**(3):1313-1323.

Spanier B, Lasch K, Marsch S, Benner J, Liao W, Hu H, Kienberger H, Eisenreich W and Daniel H (2009) How the intestinal peptide transporter PEPT-1 contributes to an obesity phenotype in Caenorhabditits elegans. *PLoS One* **4**(7):e6279.

Thamotharan M, Bawani SZ, Zhou X and Adibi SA (1999) Hormonal regulation of oligopeptide transporter pept-1 in a human intestinal cell line. *Am J Physiol* **276**(4 Pt 1):C821-826.

van Riggelen J, Yetil A and Felsher DW (2010) MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat Rev Cancer* **10**(4):301-309.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**(7):RESEARCH0034.

Vernaleken A, Veyhl M, Gorboulev V, Kottra G, Palm D, Burckhardt BC, Burckhardt G, Pipkorn R, Beier N, van Amsterdam C and Koepsell H (2007) Tripeptides of RS1 (RSC1A1) inhibit a monosaccharide-dependent exocytotic pathway of Na+-D-glucose cotransporter SGLT1 with high affinity. *J Biol Chem* **282**(39):28501-28513.

Watanabe K, Terada K and Sato J (2003) Intestinal absorption of cephalexin in diabetes mellitus model rats. *Eur J Pharm Sci* **19**(2-3):91-98.

Yamauchi T, Waki H, Kamon J, Murakami K, Motojima K, Komeda K, Miki H, Kubota N, Terauchi Y, Tsuchida A, Tsuboyama-Kasaoka N, Yamauchi N, Ide T, Hori W, Kato S, Fukayama M, Akanuma Y, Ezaki O, Itai A, Nagai R, Kimura S, Tobe K, Kagechika H, Shudo K and Kadowaki T (2001) Inhibition of RXR and PPARgamma ameliorates diet-induced obesity and type 2 diabetes. *J Clin Invest* **108**(7):1001-1013.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ and Moller DE (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**(8):1167-1174.

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Fig 1. Glucose tolerance tests. Glucose (2g/kg) was injected intraperitoneally or given by gavage to the 6-hour fasting animals at t=0 and glycaemia was measured at indicated time points at the tail vein. (A & B) Oral glucose tolerance test at the end of the diet-only period. Area under the curve was calculated using the trapezoidal rule (N=16 in each group)*** p<0.001 ** p<0.01 vs SC group. All data are means ± SEM. (C & 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 vs SC group. All data are means ± SEM. (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 ± SEM. (F & G) Intraperitoneal glucose tolerance test at the end of the 7-day treatment period. Area under the curve was calculated using the trapezoidal rule (n=8-12). *** p<0.001 ** p<0.01 * p<0.05 vs SC group in the time course representation and *** p<0.001 * p<0.05 vs indicated groups in the AUC representation. All data are means ± SEM. (H) Plasma leptin levels at the end of the 7-day treatment period (n=6-9) *p<0.05 vs respective SC group and #f p<0.05 vs HF vehicle treated group for leptin.

Fig 2. Papp of Gly-Sar in mice. Apparent permeability coefficient 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 vs vehicle SC, *** p<0.001 vs vehicle SC, # p<0.05 vs vehicle HF. All data are means ± SEM.

Fig 3. Action of rosiglitazone and metformin in Caco2 cells. Dose-dependant apparent permeability coefficient of Gly-Sar across Transwell® membranes was measured for 30 min. Cells were treated with designated concentrations of

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rosiglitazone (Rosi) or metformin (Met) or both for 7 days. (n=3-6). *** *P*<0.001. All data are means ± SEM.

Fig 4. Effect of rosiglitazone and/or metformin on PepT1 activity, protein and mRNA expression in Caco2 cells. Cells were treated with rosiglitazone (Rosi, 10 µM) and/or metformin (Met, 10 mM) for 7 days. (A) Apparent permeability coefficient of Gly-Sar across Transwell® membranes was measured for 30 minutes. (n=6-12). ** *P*<0.01. All data are means ± SEM. (B) Densitometric analysis of PepT1 protein expression normalised to β actin expression. (n=11-12). * P<0.05. ** P<0.01. All data are means ± SEM. (C) Relative quantification of PepT1 mRNA normalised to housekeeping genes mRNA expression. (n=6). ** P<0.01. All data are means ± SEM.

Fig 5. Activation of PepT1 by rosiglitazone through the PPARγ pathway. Cells were treated for 7 days with rosiglitazone (Rosi, 10 µM) and/or SR-202 (300 µM). (A) Apparent permeability coefficient of Gly-Sar across Transwell® membranes. (n=5-10). ** P<0.01. All data are means ± SEM. (B) Densitometric analysis of PepT1 protein expression normalised to β-actin expression. (n=6). * P<0.05 ** P<0.01. All data are means ± SEM.

Fig 6. Effect of rosiglitazone on PepT1 activity through mTOR. Cells were treated for 7 days with rosiglitazone (Rosi, 10 µM) and/or rapamycin (Rapa, 0.2 nM). (A) Apparent permeability coefficient of Gly-Sar across Transwell® membranes. (n=4). * P<0.05. All data are means ± SEM. (B) Densitometric analysis of PepT1 protein expression normalised to β-actin expression. (n=6). * P<0.05. All data are means ± SEM.

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Fig 7. Effect of metformin on the translation machinery. (A) ELISA analysis of PhosphoS6 protein expression after a 7-day treatment with metformin (Met, 10 mM) and/or rosiglitazone (Rosi, 10 µM) (n=6-12). ** P<0.01 *** P<0.001. All data are means ± SEM. (B) ELISA analysis of Phospho4E-BP1 protein expression after a 7-day treatment with metformin (Met,10 mM) and/or rosiglitazone (Rosi, 10 µM) (n=8). ** P<0.01 *** P<0.001. All data are means ± SEM.

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 (Met, 10 mM) and apparent permeability coefficient of Gly-Sar across Transwell® membranes was measured for 30 min. (n=3). * P<0.05. All data are means ± SEM. (B) Cells were treated for 3 days with metformin (Met, 10 mM) and/or compound C (CC, 5 µM) and apparent permeability coefficient of Gly-Sar across Transwell® membranes for 30 min. (n=5-10). * P<0.05. All data are means ± SEM.

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gene	Access number	Primer sequences (5'-3')	Size (pb)
hPepT1	NM_005073	F-GTTGGCAACATCATTGTGCT R-TCCGCTGGGTTGATGTAAGT	149
h18S	X03205	F-AGGAATTGACGGAAGGGCAC R-GGACATCTAAGGGCATCACA	320
hβ actin	NM_001101	F-GGGTCAGAAGGATTCCTATG R-GGTCTCAAACATGATCTGGG	238
hTBP	NM_003194	F-GAGAGCCACGAACCACGG R-ACATCACAGCTCCCCACCAT	178

Table 1. Characteristics of the primers used for the RT-PCR studies.

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	SC	HF
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/d)	3.86 ± 0.05	2.72 ± 0.14***
Caloric intake (cal/d)	10,896 ±149	14,450 ±771***
Lipids (g/d)	0.12 ± 0.002	0.98 ± 0.052***
Proteins (g/d)	0.62 ± 0.01	0.49 ± 0.03***
Carbohydrates (g/d)	1.85 ± 0.03	0.95 ± 0.05***

Table 2. Weight gain and food intake of mice during the diet-only period. Mice were fed the high-fat diet or the standard chow for 6 weeks. Food consumption and weight gain was monitored twice-weekly. Plasma leptin and insulin were determined by RIA at the end of the 6 weeks (n=35 in each group). *p<0.05, ***p<0.001 vs SC group. All data are means ± SEM.

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	SC				HF			
	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

Table 3. Weight gain in animals during the 7-day treatment with rosiglitazone (RSG), and/or metformin (Met). Plasma glucose and insulin determined after the 7-day treatment in fasting animals (n=4-8). *p<0.05 vs SC group and HF treated groups for insulin. ***p<0.001 vs SC group and HF treated groups for glucose plasma levels. All data are means ± SEM.

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	CTL	RSG	Met	RSG+Met
Testosterone Papp (Arbitrary units)	1.00±0.04	1.08±0.03	1.00±0.05	0.98±0.08
Mannitol Papp (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

Table 4. Papp 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 Caco2 cells treated for 7 days with rosiglitazone (RSG) and/or metformin (Met). Apparent permeability coefficient of testosterone and mannitol was monitored for 30 min. Cell viability was determined by the MTT test. All data are means ± SEM.

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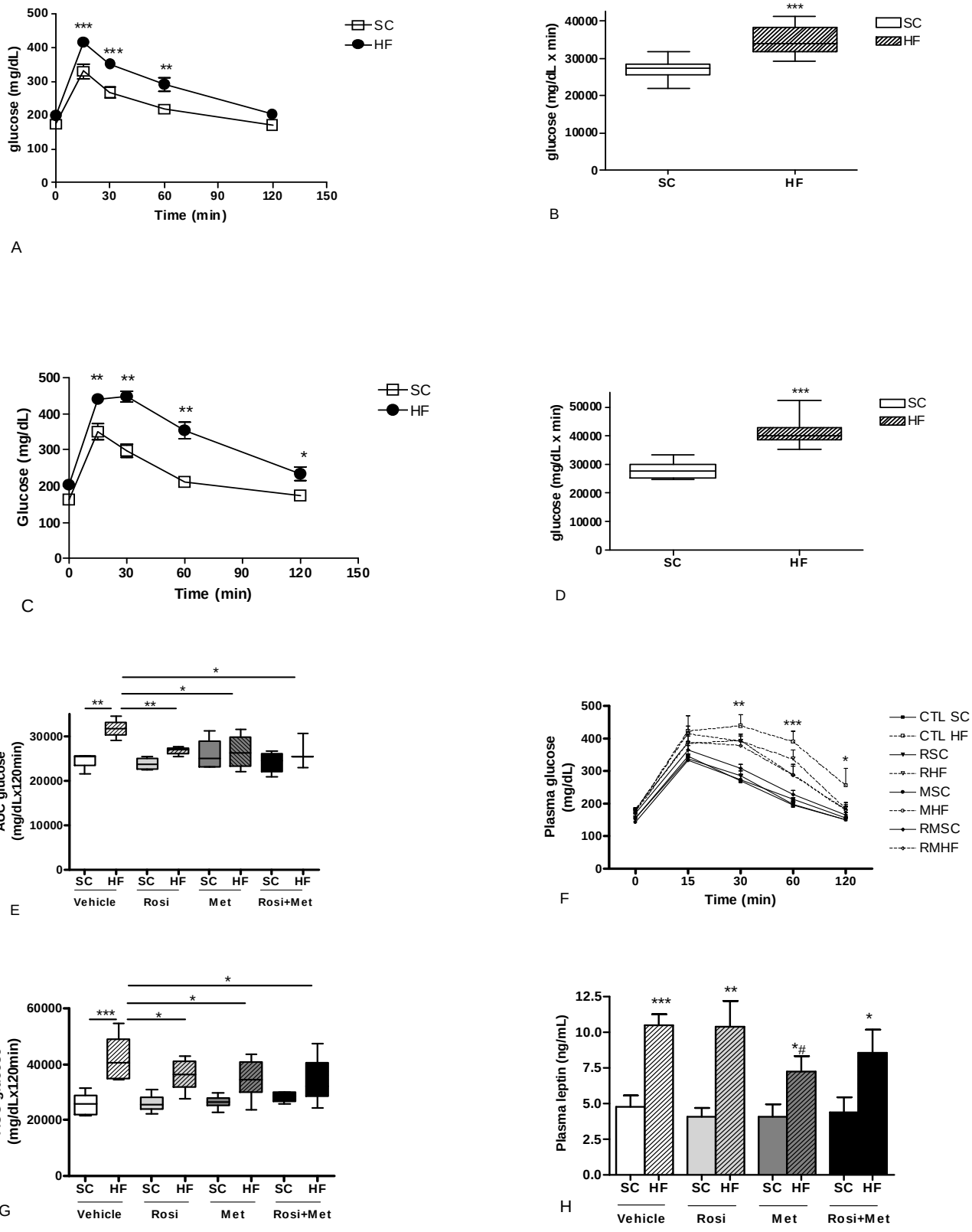

Figure 1

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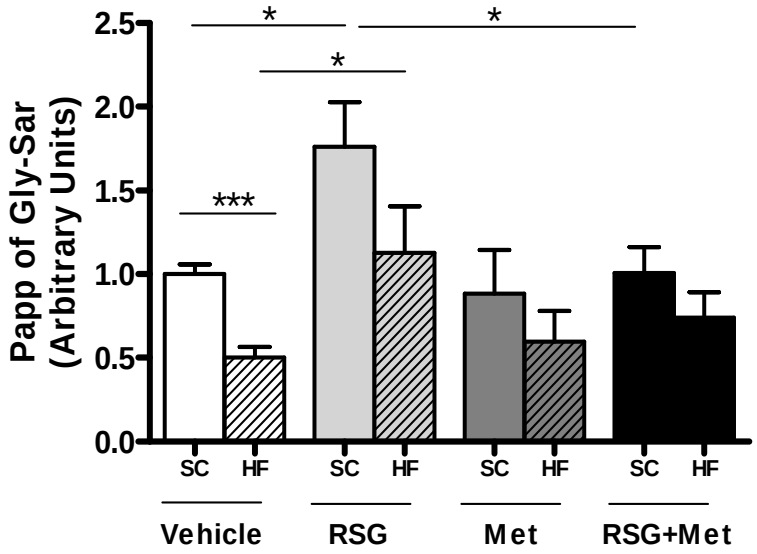

Figure 2

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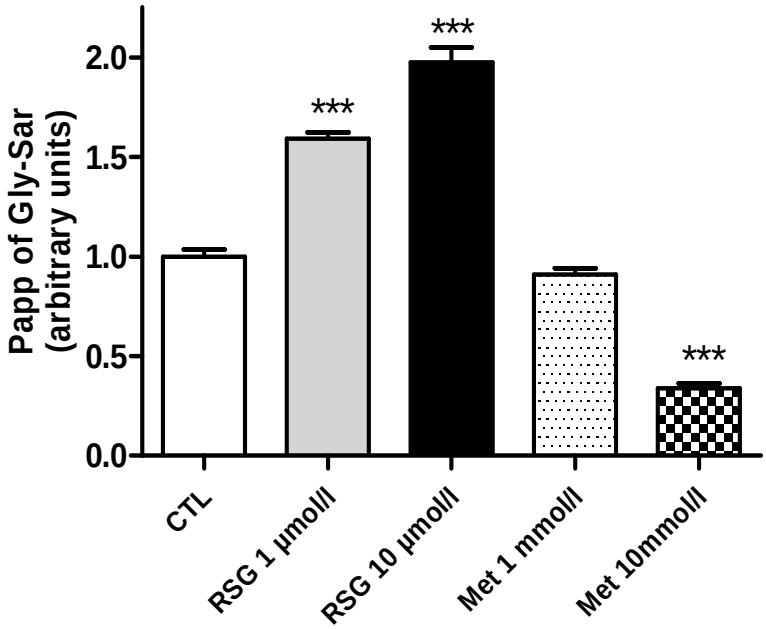

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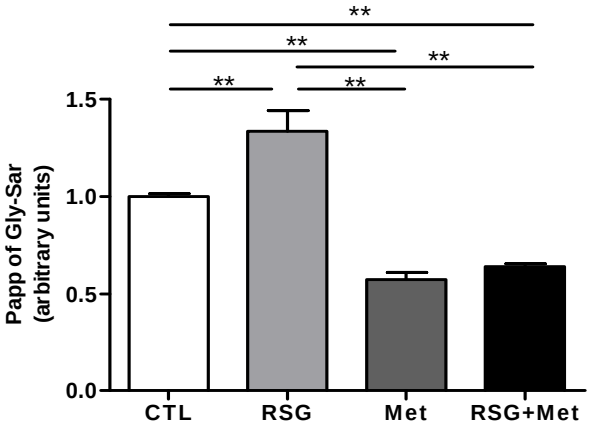

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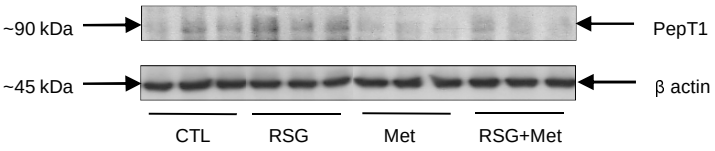

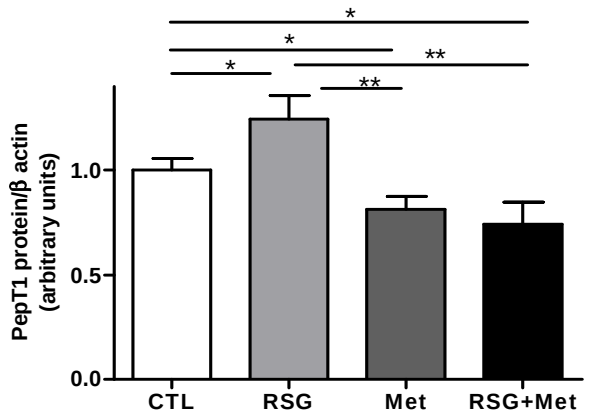

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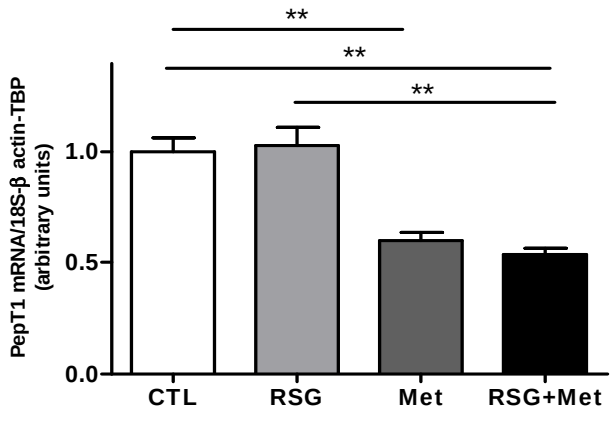

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Figure 4

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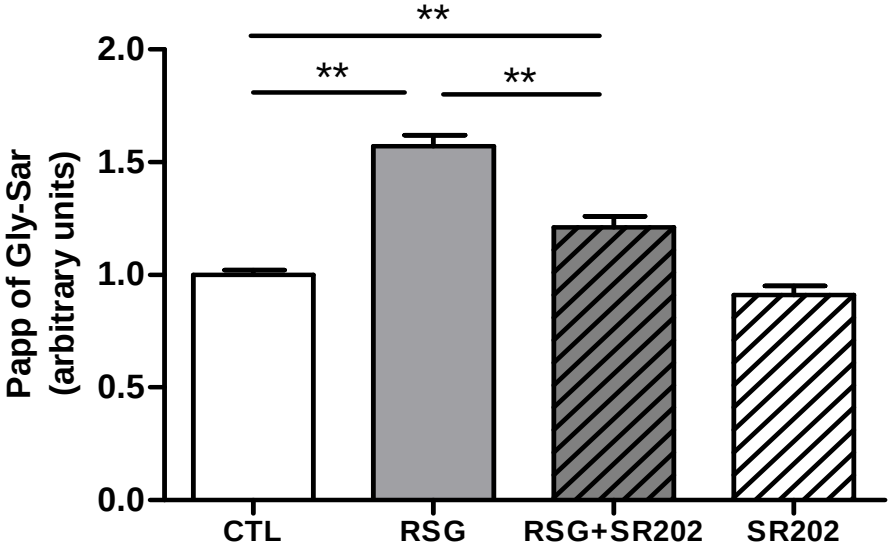

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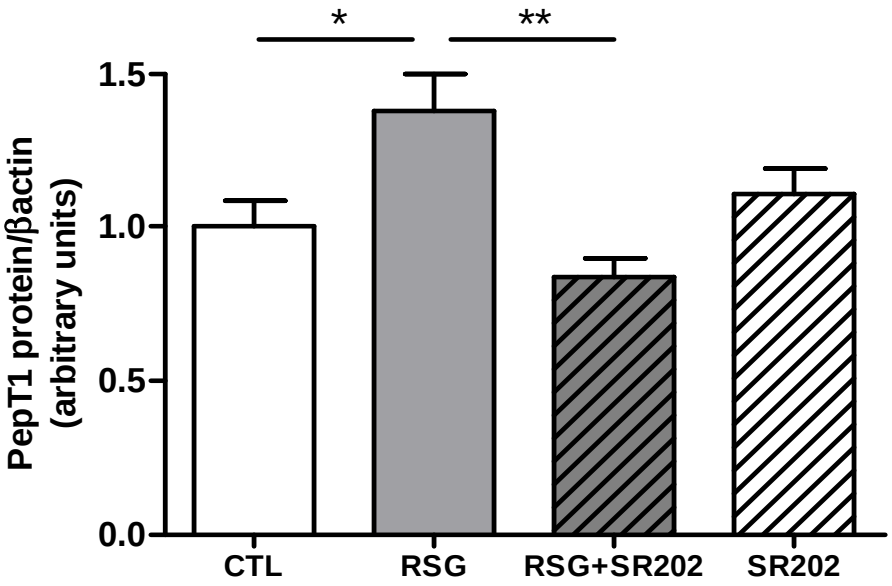

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Figure 5

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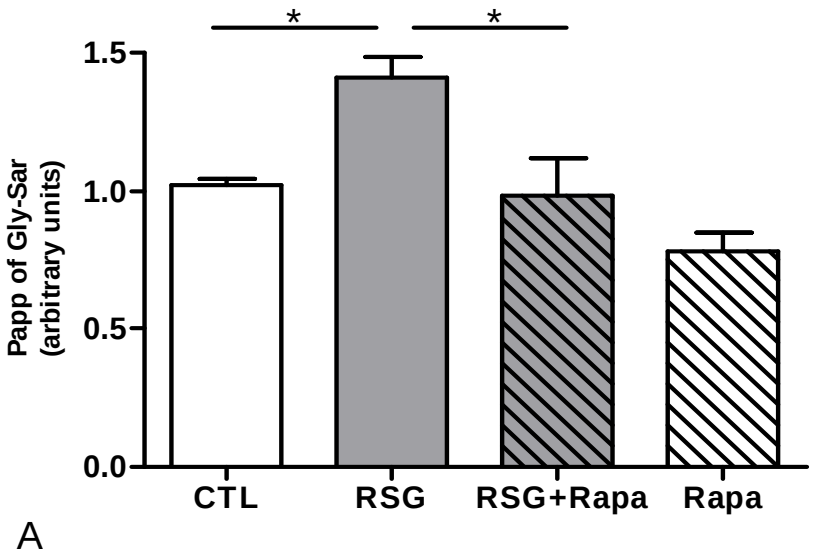

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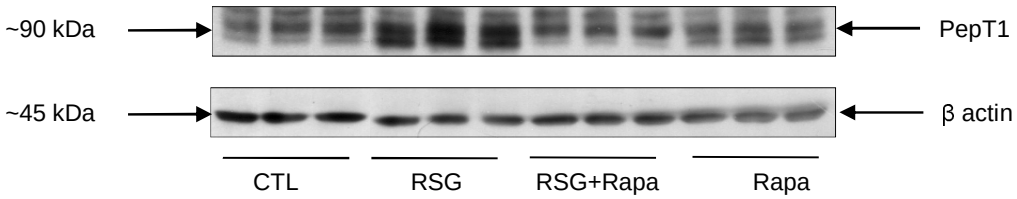

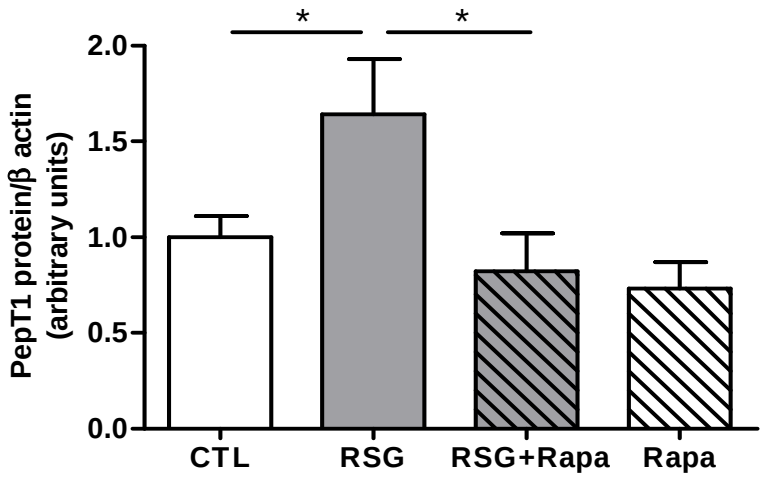

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Figure 6

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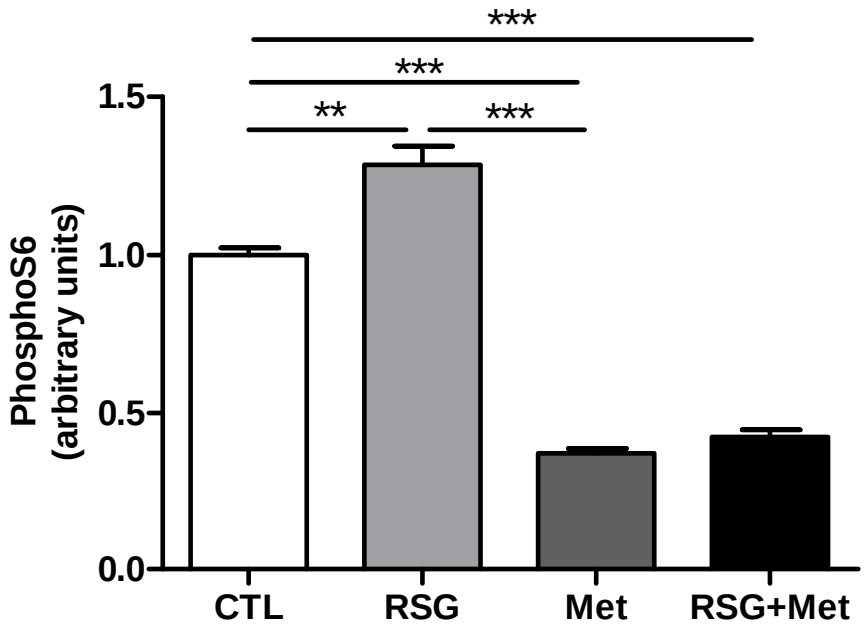

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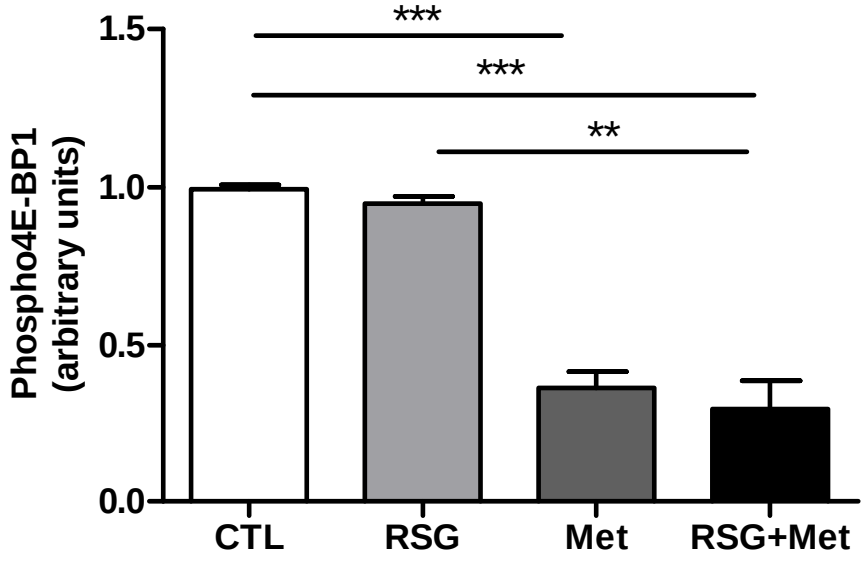

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Figure 7

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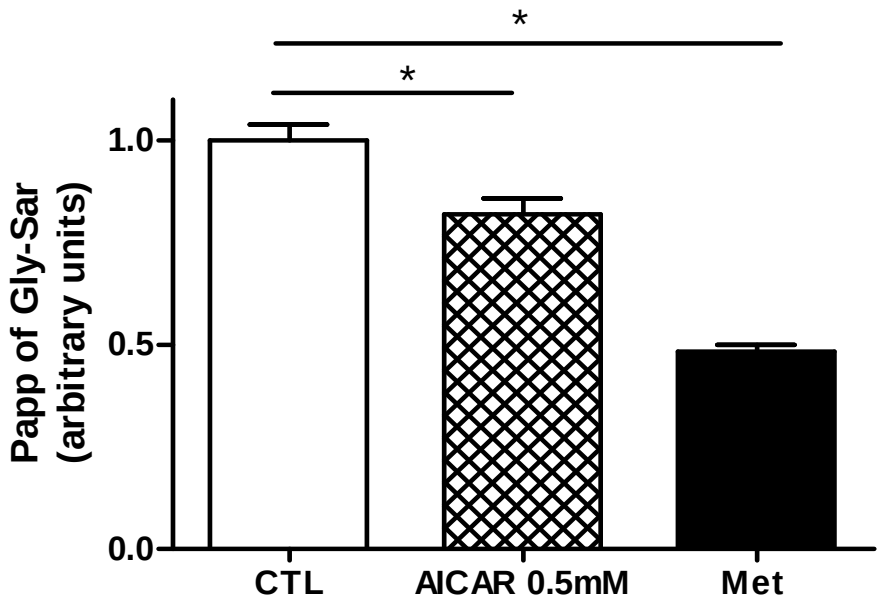

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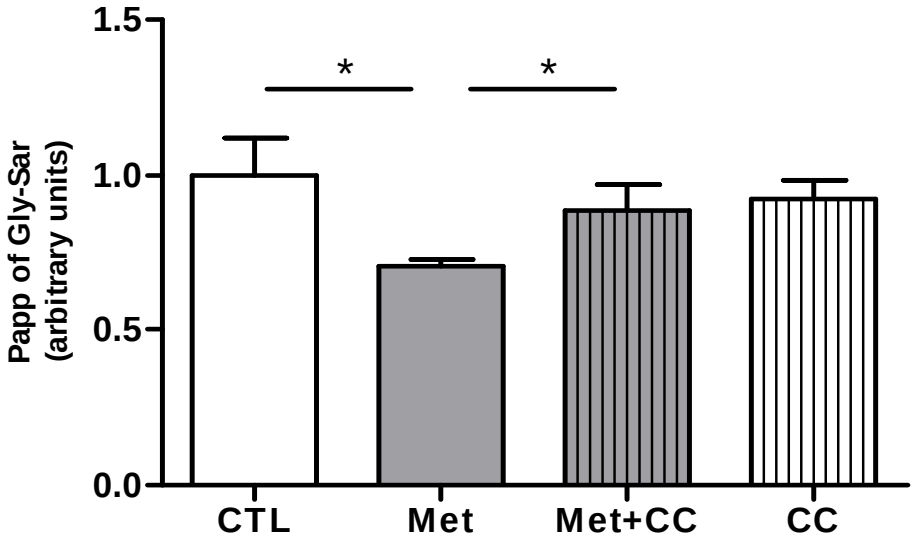

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Figure 8