Supporting Information

Manuscript title: Rosmarinic acid, major phenolic constituent of greek sage herbal tea, modulates rat intestinal SGLT1 levels with effects on blood glucose

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Supplementary Materials and Methods

Isolation of Brush-Border Membranes

Brush-Border Membranes (BBM) were isolated from frozen jejunal mucosal scrapings using a combination of cation precipitation and differential centrifugation as described previously [1] with few modifications. Briefly, the jejunal mucosa scrapings were thawed on ice and then placed in 50 ml of ice-cold Buffer 1 (100 mM mannitol, 2 mM HEPES, pH 7.1 adjusted with 2 M Tris). The tissue was homogenized with a Waring Blender (Waring Commercial, Torrington, CT, USA), 2 times of 30 s, and then filtered through 100 μ m nylon mesh, and the volume was made up to 100 ml with ice-cold Buffer 1. A solution of 2.5 M MgCl_2 was added to the tissue homogenate to give a final concentration of 10 mM. After stirring on ice for 20 min the solution was centrifuged at $3,000 \times g$ (Eppendorf 5804 R, Hamburg, Germany) at 4°C for 10 min to remove debris. The supernatant was further centrifuged at $30,000 \times g$ (Beckman Avanti J-25I, Beckman, Germany) at 4°C for 30 min and the pellet homogenized in a Buffer 2 (100 mM mannitol, 2 mM HEPES, pH 7.5 with 2 M Tris, and 0.1 mM MgSO_4 added after pH adjustment with Tris) with a glass homogenizer. The homogenate was then centrifuged at $30,000 \times g$ at 4°C for 45 min and the final purified BBM was suspended in a Buffer 3 (300 mM mannitol, 20 mM HEPES, pH 7.5 with 2 M Tris, and 0.1 mM MgSO_4 added after pH adjustment). BBM were then frozen in liquid nitrogen and stored at -80°C until use.

Plasma transaminases activities

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically in rat plasma following the NADH oxidation method, as previously described by Lima *et al.* [2].

Immunohistochemistry

The immunolocalization of GLP-1 in jejunum of rat small intestine and of insulin in pancreatic beta-cells were carried out following the immunohistochemistry method described previously [3] with few modifications. Jejunal tissue was processed and embedded in paraffin, and 5 μ m sections were mounted on 3-aminopropyltriethox-

ysilane (Sigma-Aldrich, St. Louis, MO, USA) coated glass slides, dewaxed in Clear-Rite (Richard-Allan, Kalamazoo, MI, U.S.A.), and rehydrated. For pancreas tissue, sections were subjected to 1% v/v-SDS treatment at room temperature for 5 min. Tissue sections were then incubated for 20 min in blocking reagent (5% normal goat serum/1% bovine serum albumin in TPBS pH 7.4) followed by the respective primary antibody diluted in 1% BSA in TPBS, overnight at room temperature in humidity chambers. Sections were then rinsed in TPBS and incubated with the respective secondary Alexa Fluor 488 conjugated antibody (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C. Negative controls were processed on the same slide in an identical manner (substitution of primary antibody with normal rabbit serum with an equivalent dilution). The antibodies against GLP-1 (Abcam, Cambridge, UK) and insulin (PROGEN Biotechnik GmbH, Heidelberg, Germany) were used diluted 1:1,000 and 1:100, respectively.

The number of GLP-1 expressing L-cells/cm of villus length was assessed using the software LAS AF (version 1.4.1) from Leica Microsystem (Wetzlar, Germany). The average of beta-cell insulin signal and percentage of active beta-cell in islets of Langerhans were quantified using the Sigma Scan Pro (v5) software (SPSS Chicago, IL, USA).

Supplementary references

- [1] Shirazy-Beechey, S. P., Davies, A. G., Tebbutt, K., Dyer, J., *et al.*, Preparation and properties of brush-border membrane vesicles from human small intestine. *Gastroenterology* 1990, 98, 676-685.
- [2] Lima, C. F., Andrade, P. B., Seabra, R. M., Fernandes-Ferreira, M., Pereira-Wilson, C., The drinking of a *Salvia officinalis* infusion improves liver antioxidant status in mice and rats. *J. Ethnopharmacol.*, 2005, 97, 383-389.
- [3] Wilson, J. M., Antunes, J. C., Bouça, P. D., Coimbra, J., Osmoregulatory plasticity of the glass eel (*Anguilla anguilla*): Freshwater entry and changes in branchial ion transport protein expression. *Can. J. Fish Aquat. Sci.*, 2004, 61, 432-442.

Supplementary tables

Suppl. Table 1 – Analytical constituents of the different diets (average values in %).

	HC diet	LC diet
Carbohydrate	60.3	28.0
Protein	15.4	47.5
Fibre	4.1	4.2
Fat	2.9	2.0
Ash	5.3	6.3
Water	12	12

Suppl. Table 2 – Effect of 14 days SFT treatment on rat plasma transaminase activities (alanine aminotransferase – ALT and aspartate aminotransferase – AST).

Plasma Transaminases			
	<i>In vivo</i> beverage	ALT (U/l)	AST (U/l)
Healthy	Water	22.6±2.0	31.1±2.6
	Tea	23.4±1.3	32.2±5.2
Diabetic	Water	25.5±1.7	20.3±2.9
	Tea	24.2±1.2	22.7±2.2

Values are means ± SEM (*n*=6)

Mean values were not significantly different when compared with the respective control group (*P*>0.05).

Supplementary Figures

Suppl. Fig. 1 - Effects of *S. fruticosa* tea (SFT) treatment on STZ-induced decrease of insulin-producing β -cells. A – Immunofluorescence images of insulin signal in pancreatic islets β -cells: (1) healthy water group; (2) healthy tea group; (3) STZ-diabetic water group; (4) STZ-diabetic tea group. B – Percentage of active β -cells in rat islets of Langerhans. C – Intensity of insulin signal in immunoreactive β -cells. The % of active β -cells was determined by measuring the total islet area (A_T) observed in phase contrast field and the immunofluorescence insulin signal area (A_{IF}), using the following formula: % active β -cells = $A_T \times 100 / A_{IF}$. Values are means $\pm$ SEM, $n=6$. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) was significant ($^{+++}P < 0.001$). The post-hoc test Student-Newman-Keuls indicates that $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared with the respective healthy control.

Suppl. Fig. 2 - Western blot analysis of GLUT2 levels in brush-border membrane (BBM) of healthy (white bars) and STZ-induced diabetic rats (grey bars) treated with water or SFT. Values are means $\pm$ SEM, $n=5-6$. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) rats was not significant ($P>0.05$). No others significant statistically differences were obtained.

Suppl. Fig. 3 - Effects of *S. fruticosa* tea (SFT) treatment on STZ-induced reduction of GLP-1 producing cells (L-cells) in the jejunal epithelium. The number of GLP-1 expressing enteroendocrine L-cells is expressed per cm of villus length. Values are means $\pm$ SEM, $n=5-6$. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) was significant ($^{++}P<0.01$). The post-hoc test Student-Newman-Keuls indicates that $^{*}P\leq 0.05$ when compared with the respective healthy control.

Supplementary Fig. 4 text

In a preliminary experiment, the effects of carbohydrate content manipulation in the diet on SGLT1 levels were evaluated in order to choose the better time to stop the experiment after the HC diet reintroduction. As shown in Suppl. Fig. 4, 2 days after the reintroduction of the HC diet were not sufficient to significantly recover the normal levels of SGLT1 on the BBM. Therefore, 4 days of reintroduction of HC diet was chosen. Also in this preliminary experiment, the effect of *S. fruticosa* tea for 4 days in reducing the dietary adaptive SGLT1 increase was observed (Suppl. Fig. 4).

Suppl. Fig. 4 - Levels of SGLT1 in rat jejunum brush-border membrane (BBM) from the different feeding groups. Western blot analysis of SGLT1 levels in BBM of rats fed either their regular high carbohydrate (HC-HC), or changed from a HC to low carbohydrate (LC) diet (HC-LC) or returned to a HC diet following LC diet (HC-LC-HC). In this latter feeding regime animals were given either sage tea (T) or water drinking (W) *ad libitum* for 2 days (hatched bars) or 4 days of treatment (white bars). Values were means $\pm$ SEM, $n=4$. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P>0.05$). Effect of drinking (white bars): *** $P\leq 0.001$ when compared with the HC-LC-HC/W group.

Suppl. Fig. 5 - Levels of Hsp70 in whole cell homogenates of rats fed either their regular high carbohydrate (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bars). In this latter feeding regime animals were given either water (W), *S. fruticosa* tea (SFT) or rosmarinic acid (RA) to drink *ad libitum* for 4 days. Values are means $\pm$ SEM, $n = 6$. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$). Effect of drinking (white bars): no significant statistical differences ($P > 0.05$) were obtained when compared with the HC-LC-HC/W group.