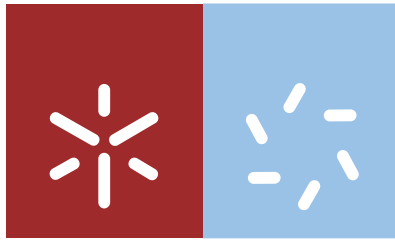**Universidade do Minho**
Escola de Ciências

Vu Anh Tran

Evaluation of dietary carbohydrate and rosmarinic acid on intestinal expression of glucose transporters and digestive enzymes

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Evaluation of dietary carbohydrate and rosmarinic acid on intestinal expression of glucose transporters and digestive enzymes

Dissertation Master
Master in Biotechnology and Bioentrepreneurship in
Medicinal and Aromatic Plant (MBBPAM)

Main Supervisor:
Prof. Doctor Cristina Pereira-Wilson
and Co-supervisor:
Prof. Doctor Cristovão Lima

July 2012

É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

Universidade do Minho, ____/____/_____

Assinatura: _____

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ABSTRACT

The impact of dietary habits on human health is well known and diet plays a significant role in chronic and age related diseases. This study had the following objectives: (1) to characterize the effects of different types of dietary carbohydrate on the expression of the digestive enzyme sucrose-isomaltase (SI) and of the glucose transporters SGLT1 and GLUT2 (sodium dependent transporter and facilitated diffusion, respectively) in intestinal epithelial cells, and (2) describe the effects of the phytochemical agent RA on the modulation of these processes.

Carbohydrate digestion and absorption by the intestine determine the rise in glucose levels of the blood after a meal and in diabetic patients these processes are increased which contributes to the hyperglycemia that characterizes the disease. Other dietary constituents, namely phytochemicals, may have a beneficial effect in this scenario by decreasing carbohydrate digestion and/or intestinal glucose uptake. This study shows that when carbohydrates are removed from the diet there is a marked decrease in SI, SGLT1 and GLUT2 not only at the apical membrane of enterocytes but also in enterocyte whole cell homogenates. Upon reintroduction of diets rich in carbohydrate (glucose or amylopectin) for 7 or 14 days, there is a marked increase in the enterocyte expression of the three proteins. The effects are more pronounced in the glucose diet which suggests a decisive role for luminal glucose on the adaptive response to increasing dietary carbohydrates.

The phytochemical RA demonstrated to decrease the extent of the adaptive increase in the digestive enzyme as well as in the glucose transporters revealing its potential as a regulator of intestinal glucose absorption.

This work is collaboration with Dr. Simon Lee of the University of Macau, China

RESUMO

Os efeitos da alimentação têm grande impacto sobre a saúde humana. Este trabalho tinha como objectivos (1) caracterizar os efeitos de diferentes fontes de hidratos de carbono na dieta de ratos sobre a expressão de enzimas digestivas e transportadores de glucose no epitélio intestinal (2) descrever os efeitos do agente fitoquímico RA na modulação destes processos.

Como é sabido, a digestão e absorção de hidratos de carbono pelo intestino são processos que determinam a subida de glucose no plasma associado à dieta e podem, em doentes diabéticos por exemplo, estar aumentados o que agrava a hiperglicemia presente nestes doentes. Outros constituintes da dieta, nomeadamente agentes fitoquímicos, podem intervir beneficamente neste processo diminuindo a velocidade de digestão dos hidratos de carbono complexos e/ou eficiência do transporte de glucose para a circulação através de efeitos não só na actividade de enzimas digestivas mas também na expressão de transportadores de glucose na membrana apical de enterócitos. Assim, este estudo mostra que quando se retiram os hidratos de carbono da dieta de ratos previamente alimentados com uma dieta normal (contendo amido) os níveis da enzima sacarose-isomaltase e dos transportadores de glucose SGLT1 (sodium dependent glucose transporter 1) e do GLUT2 (difusão facilitada) baixam muito significativamente não só na membrana apical de enterócitos (BMM) como também nos extractos celulares totais de mucosa intestinal. Ao reintroduzir dietas isocalóricas ricas em hidratos de carbono de diferentes tipos – glucose ou amilopectina – por 7 ou 14 dias produzem adaptações ao nível da enzima e das proteínas transportadoras cuja expressão aumenta. Estes efeitos são mais pronunciados no caso da dieta com glucose (do que na dieta contendo amilopectina) o que indica um papel determinante da glucose lumenal livre na estimulaçãp destes mecanismos adaptativos. Os resultados confirmam ainda que a regulação do transporte da glucose e da sacarose-isomaltase parecem estar associados.

O agente fitoquímico testado (RA) demonstrou ter potencial para conter estas respostas adaptativas ao aumento de glucose livre no lúmen do intestino o que revela que poderá ser útil no controle da glicemia.

Este trabalho é o resultado de uma colaboração com o Doutor Simon Lee da Universidade de Macau, China.

TABLE OF CONTENTS

x

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Diabetes is increasing worldwide mainly due to non-healthy diets and lifestyles and dietary habits. According to WHO:

-More than 346 million people worldwide have diabetes.

-Type 2 accounts for around 90% of all diabetes worldwide.

-Diabetes is predicted to become the seventh leading cause of death in the world by the year 2030.

So, what is diabetes? It is an increase in glucose concentration in the blood (plasma glucose), as a result of either lacking insulin produced by the pancreatic beta cells (type 1) or the body's ineffective response to insulin (type 2). While type 1 cannot be prevented, type 2 can be.

1.1. Glucose homeostasis

Glucose homeostasis is the result of the balanced action of two pancreatic hormones, insulin and glucagon, to maintain blood glucose concentration in the range of 3.9 to 5.6 mM. Intestinal glucose absorption, endogenous glucose production by the liver (gluconeogenesis) and glucose utilization by the tissues (glucose clearance) are responsible for glucose concentration in blood. Although insulin, which removes glucose, and glucagon, which produces glucose, are consider as main factors for glucose concentration regulation, there are several other enzymes, substrates, and transporters that help to regulate the plasma glucose levels also (Aronoff et al., 2004).

Recent studies on diabetes focus on the small intestine where the absorption of glucose occurs. This means that we can control the plasma glucose concentration within appropriate limits for diabetic patients by reducing the glucose absorption ability of the intestine. There are several methods to do that such as inhibiting the glycosidase enzymes' activity (carbohydrate digestion), or decreasing the expression of glucose transporters (SGLT1, GLUT2), which help to deliver glucose from food through the brush border membrane (BBM) of intestinal epithelial cells – enterocytes to the blood, and enhancing the incretion hormones secretion, which increase the insulin response to glucose and thereby helping to bring down plasma glucose after meal (Avezdo Mariza F. et al., 2008).

1

1.2.   The small intestine

The small intestine is not only a digestive organ but also the place where amino acids, sugar, fatty acid particles, vitamins, electrolytes and water are absorbed. In human beings, the small intestine is about 2.8m long (Rhoades & Pflanzer, 2003) while it is 100cm long in rat (Miller, 1971). It includes three sections: duodenum (small portion), jejunum and ileum (both have similar length).
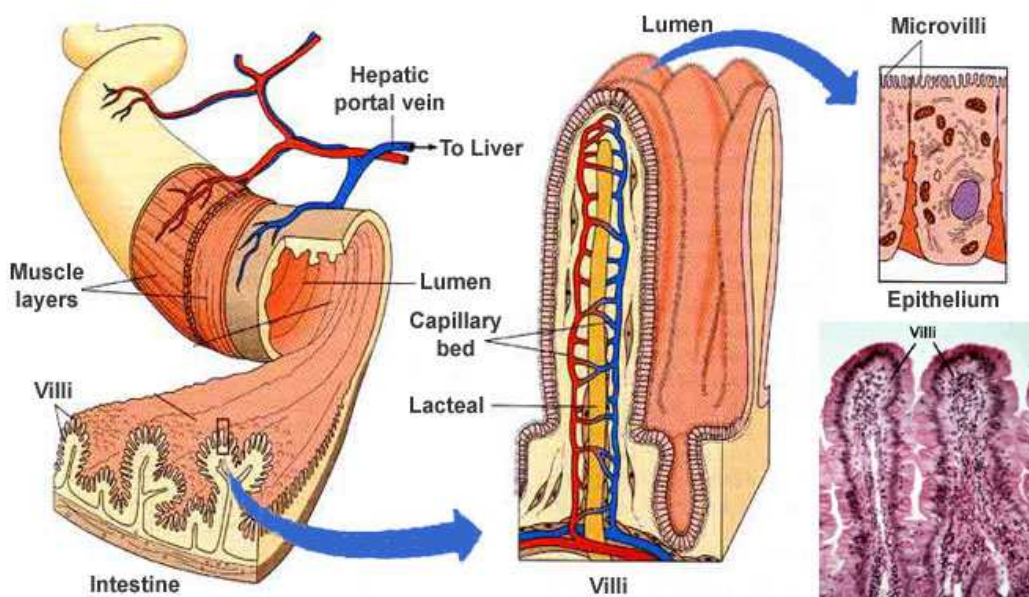
Figure 1. **Cross section and small structure of small intestine**

(http://www.daviddarling.info/encyclopedia/S/small_intestine.html)

1.3.   Carbohydrate digestion, absorption and glucose transporters (SGLT1, GLUT2)

1.3.1.   Carbohydrate digestion

Plants (rice, potato, wheat, etc…) are major supply sources of carbohydrates in the human diet. Their main composition is starch which can be digested to glucose by salivary and pancreatic amylases, as well as by disaccharides of the enterocyte brush border membrane (lactase, isomaltase, and maltase) (Rhoades & Pflanzer, 2003; Drozdowski & Thomson, 2006b).

Sucrose-isomaltase (SI) is an apical membrane enzyme of small intestine, which is responsible for starch and sucrose digestion. SI, after being synthesized, is firstly attached to endoplasmic reticulum with N-glycosylation to become a high mannose precursor of 210kDa. Secondly, the precursor is terminal N- and O-glycosylated when moving to the Golgi apparatus. Finally, SI is translocated to the brush border membrane and is cut into sucrose and

isomaltase units by pancreatic proteases in order to digest starch and sucrose to simple sugars (Kazuki Mochizuki et al., 2010).
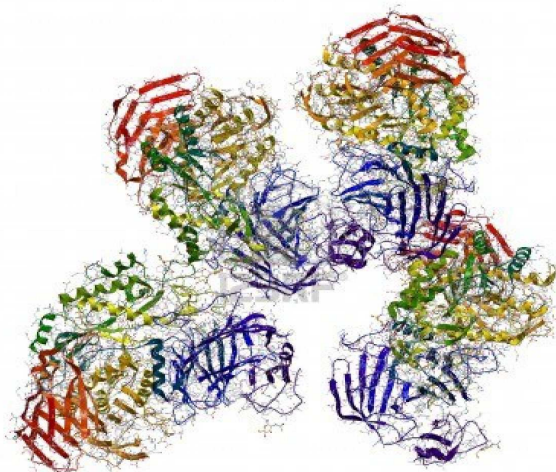
Figure 2. **Sucrose-Isomaltase structure**

(http://es.123rf.com/photo_12771469_sacarasa-isomaltasa-enzima-estructura-es-la-enzima-implicada-en-la-digestion-del-azucar.html)

Due to the importance of carbohydrate digestion and sugar absorption in small intestine, there are many studies on the effect of diets on the SI expression. According to Goda et al., 1994, a diet of high fat/carbohydrate ratio decreased sucrase, isomaltase and maltase activities, which also reduced the sucrase/isomaltase activity ratio (Goda et al., 1993). The cause of the decrease in SI activity ratio is explained by the change in glycosylation of SI, when it is synthesized from a single polypeptide, namely the reduction of unsialylated galactose from the glycosylated chain of the SI complex, (Kazuki et al., 2010).

Kazuhiro et al., 1999 demonstrated that fructose and sucrose diet (40% energy as fructose or sucrose) can enhance the transcription of SI and intestinal hexose transporters (SGLT1, GLUT5 and GLUT2) within short period of time (12h). On the other hand, the mRNA level of SI and GLUT5 couldn't be enhanced by glucose or nonmetabolizable sugar (α-methyglucoside) within 12h. However, within a longer period of time (5 days), a high glucose diet could increase the SGLT1 and GLUT2 mRNA levels or both SI and SGLT1 transcription also were increased with a high-starch diet for 7 days. Researchers therefore suggested two mechanisms to explain gene expression of SI and intestinal hexose transporters due to the carbohydrate diets: the first one is a fast gene transcription stimulated by fructose

or sucrose (12h after meal) and the second one is generated by consumption of starch or glucose within long term period of time(≥5 days) (Kazuhiro et al., 1999).

1.3.1 Carbohydrate absorption

After being digested, these monosaccharides (glucose, fructose and galactose) are absorbed through the enterocytes (George L.Kellet et al., 2008; Figure 3).
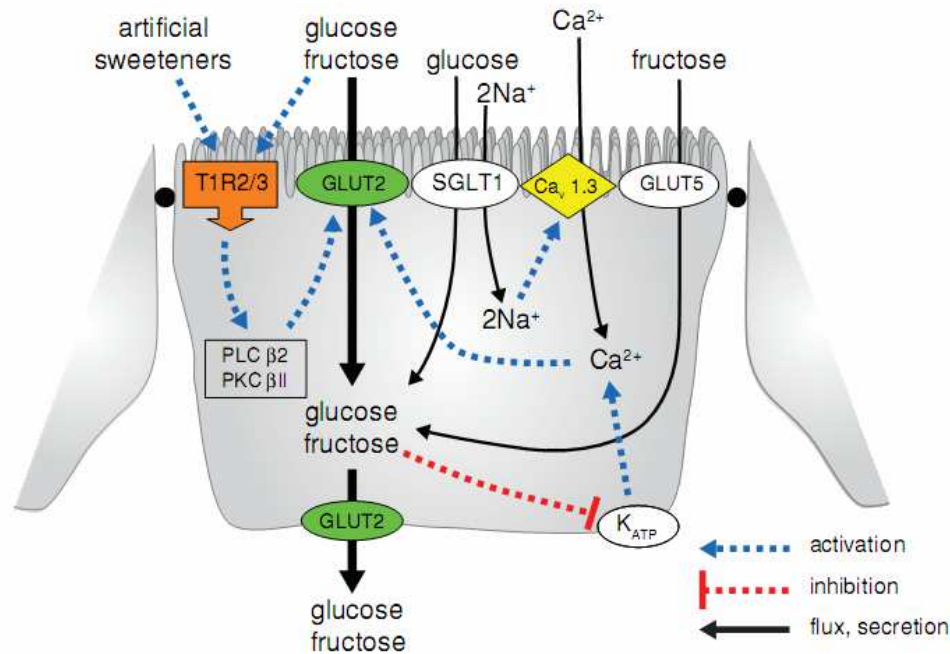
Figure 3. Regulation of apical GLUT2 in enterocytes by calcium and taste receptors. GLUT, glucose transporter; PKC, protein kinase; PLC, phospholipase C; SGLT, sodium-dependent glucose transporter; Ca$_v$1.3, nonclassical neuroendocrine L-type voltage-dependent calcium channel (George L.Kellet et al., 2008)

The tight junctions help to join enterocytes together in order to sustain the polarity of enterocyte with a brush border membrane (or apical membrane) and a basolateral membrane. In addition, these junctions limit molecules to move from the lumen to the blood via a paracellular route (Woudstra & Thomson, 2002).

Specific glucose transporters (SGLT1, GLUT2) help glucose and other monosaccharides diffuse into cells since these molecules are hydrophilic and cannot go through the lipid bilayer by themselves. Besides glucose, galactose and fructose after being absorbed mostly will be converted to glucose in enterocytes (liver will be responsible for the remaining) for later storage or metabolism. (Wright et al., 2003).

In this study, we will focus mainly on two glucose transporters namely SGLT1 and GLUT2 so that firstly let's have a look at them.

SGLT1, sodium-dependent glucose transporter, is the main transporter of glucose and galactose from the lumen across the enterocytes. 2 Na^+ ions and 260 molecules of water are transported through the apical membrane along with the uptake of each glucose molecule (Wright et al., 2004). The majority of glucose after being absorbed into the enterocytes will be transported out of the cell by GLUT2, which is mainly located in the basolateral membrane of enterocytes. Besides the function as glucose transporters, GLUT2 can also transport galactose and fructose from the cytosol to the blood (Wright et al., 2003). In addition, GLUT5 facilitates the fructose transportation across the brush border membrane. These above things are based on the classical model of secondary active intestine glucose absorption. However, there are new evidences that GLUT2 also can play a role in apical glucose absorption like SGLT1, which means that GLUT2 can be translocated to the apical membrane (**Figure 3**).

1.3.2. Glucose transporters (SGLT1 & GLUT2)

SGLT1 (73 Da protein) is a member of the family of sodium dependent glucose transporters, which is found abundantly in the apical brush border membrane of jejunum (it's also located in ileum and duodenum but at lower levels) (Wright et al., 2007; Yoshida et al., 1995). Besides, SGLT1 can also be found in the cardiomyocytes (heart), luminal membrane of intracerebral capillary endothelial cells (brain) and the apical membrane of renal tubule cells (kidneys) (Bouche et al., 2004; Wright, 2001; Zhou et al., 2003; Elfeber et al., 2004). The secondary structure of SGLT1 is a 14 transmembrane α-helice with extracellular N and C terminus (**Figure 4**). It also has two phosphorylation site (between 6 & 7, 8 & 9 transmembrane helices) and one glycosylation site (between 5 & 6 transmembrane helices) (Wright, 1998). SGLT1 can also be divided into two regions, according to their function. The first, called the N-terminal region (from helices 1-9), plays a role in locating of SGLT1 and binding Na^+ whereas the second region, called the C-terminal region (from helices 10-14) is responsible for sugar binding and transport (Wright et al., 2004; Panayotova-Heiermann et al., 1997; Suzuki et al., 2001).

Glucose and galactose can be transported into the cell against their concentration gradients by SGLT1 due to the electrochemical gradient of Na^+ (Bouche et al., 2004; Wright et al., 2007). In addition, SGLT1 is also considered as a water and urea channel (Wright et al., 2002).
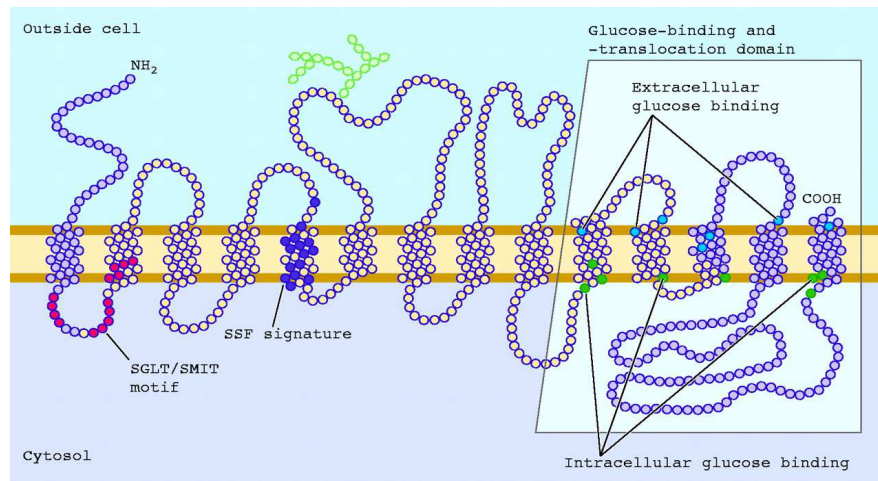
Figure 4. The secondary structure of SGLT1

(http://physiologyonline.physiology.org/content/19/6/370/F1.expansion.html**).**

Another important glucose transporter is **GLUT2** (52-60 kDa in molecular weight), which is found mostly in the basolateral membrane of jejunum of the small intestine, liver, kidney and pancreatic β-cells (Hogan et al., 1991; Bouche et al., 2004). According to **Figure 5**, the secondary structure of GLUT2 consists of 12 transmembrane helices with intrancellular N and C terminus and a larger intracellular loop, which connects 6th & 7th helice (Bouche et al., 2004)
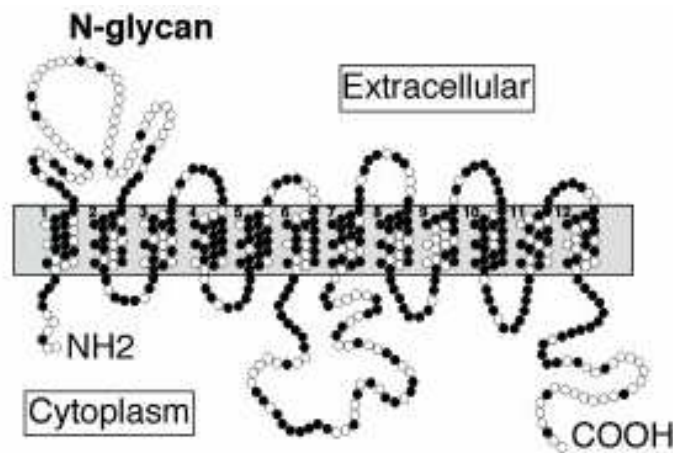
Figure 5. Secondary structure of GLUT2 (Kazuaki et al., 2005)

According to the classical model of glucose absorption, GLUT2 is considered to present only in the basolateral membrane. However, some recent studies showed that GLUT2 can be translocated to the brush border membrane in normal rats with high sugar meal.

GLUT2 can be a permanent resident of brush border membrane due to fructose and fat diet in type 2 diabetic experimental rats, which will enhance the absorption of sugar. (George L.Kellett et al., 2008). According to Oliver J.Mace et al., 2007, GLUT2 translocated rapidly to the brush border membrane due to high concentration of glucose in small intestine (30-200 mM) but low glucose concentration (≤20 mM) did not produce any effects in GLUT2 translocation. However, at low glucose concentration with the present of artificial sweeteners (1-2 mM), GLUT2 can also be trafficked to apical membrane. Sucralose, acesulfame potassium and saccharin (3 common artificial sweeteners) increase the level of apical GLUT2 (Oliver J.Mace et al., 2007).

1.4. Sources of bioactive compounds from medicinal plants

Using traditional medicinal plants as an antidiabetic therapy has been recorded long before the use of insulin in 1922 (Day, 1998). However, until now, scientists can't find any natural source of insulin from plant to apply for T1DM patients. On the other hand, for T2DM, since insulin treatment is not the ideal approach, alternative medicines from herbs might be considered as good complementary treatment (Avezdo F. et al). For example, ginseng (Panax spp.), ivy gourd (Coccinia indicia), garlic and onion (Allium sativum and Allium cepa), holy basil (Ocimun sanctum), milk thistle (Silibum marianum), gurmar (Gymnena sylvestre), bitter melon (Momordica charantia), Aloe vera and Ginkgo biloba are considered as promising antidiabetic plants (Vuksan & Sievenpiper, 2005). Three mechanisms of these antidiabetic plants are the increase of glucose disposal-uptake by muscle and adipose tissues (e.g. ginseng, fenugreek, fig leaf, ivy gourd, bitter melon), the delay of glucose absorption in the gut (e.g. Aloe vera, ginseng, prickly pear cactus, fenugreek), and the glucose stimulated insulin secretion (e.g. ginseng, garlic, holy basil, bitter melon, gurmar) (Vuksan & Sievenpiper, 2005).

Since the hyperglycaemia results in production of more oxidative damage in cells, the antioxidant compounds from herbs (phenolic compounds, alkaloids, flavonoids and glycosides) may support their antidiabetic effects (Roberson & Harmon, 2006). Tanaka et al. also demonstrated in their studies with diabetic animals that antioxidants decrease diabetes progression.

1.5.   Rosmarinic acid

Rosmarinic acid (RA), which is widely found in species of the Boraginaceae and the subfamily Nepetoideae of Lamiaceae, is an interesting biological compound. It can function as an antioxidant, ativiral, antibacterial and anti-inflammatory. RA's chemical structure is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Figure 7)
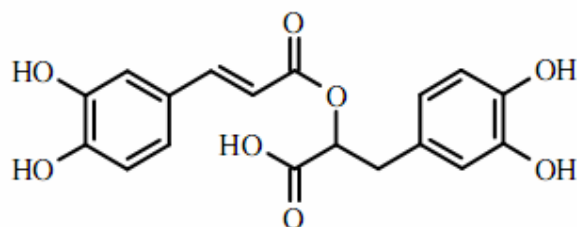
Figure 6. Structure of rosmarinic acid (Toth J. et al., 2003)

Rosmarinic acid has also been found from other plant families (Table 2)

Table 1. RA's occurrence in the plant kingdom (Maike Petersen et al., 2002)

Family	Reference
Dicotyledonae	
Lamiaceae	Scarpati and Oriente, 1958; Litvinenko et al., 1975
Boraginaceae	Kelley et al., 1975
Apiaceae	Hiller, 1965
Araliaceae	Trute and Nahrstedt, 1996
Cucurbitaceae	De Tommasi et al., 1991
Hydrophyllaceae	Petersen, unpublished results
Rubiaceae	Aquino et al., 1990
Plantaginaceae	Kurkin et al., 1988 cited in Holzmannova, 1995
Sterculiaceae	Satake et al., 1999
Tiliaceae	Lasure et al., 1994
Monocotyledonae	
Cannaceae	Simmonds et al., submitted
Zosteraceae	Ravn et al., 1994
Potamogetonaceae	Petersen, unpublished results
Pteridopsida	
Blechnaceae	Häusler et al., 1992
Anthocerotopsida	
Anthocerotaceae	Takeda et al., 1990

Previous studies showed that RA doesn't exist long in the blood circulation (t=81 minute) and its toxicity is low (LD_{50} = 561mg/kg in mice) (Parnham and Kesselring, 1985). Furthermore, RA is also applied in cancer treatment and cosmetic industry due to its anti-oxidant activity (D'Amelio, 1999). In addition, RA, which corresponds to 72% of the total phenolics in herb *S.fruticosa*, could play an important role in controlling the hypeglycaemic

since it decreased SGLT1 levels at the BBM as it decreased the level of heat shock protein-Hsp70, which has been reported to enhance the translocation of SGLT1 to the BBM (Mariza F.Azevedo et al., 2010).

In order to clarify the interrelation of SI and SGLT1 regulated in enterocytes by dietary carbohydrate, an experiment was performed with rats fed with glucose or amyplopectin. Glucose is readily absorbed in the intestine through BBM glucose transporters such as SGLT1 or GLUT2, and amylopectin needs to be digested by BBM enzymes such as SI in order for glucose to be released and absorbed. With the use of two diets above (glucose and amylopectin), we expect to see how independent is the upregulation of glucose transporters and SI in BBM of rats at 4 and 14 days after introduction of these diets to animals which were previously fed for 1 week on CH free diet (that helped to reduce the expression of SGLT1 and SI in BBM).

The effects of RA, previously demonstrated to reduce BBM SGLT1 expression in starch fed animals, is also tested in this study.

Total gene expression (SGLT1, GLUT2 and SI) in whole cell homogenates and the translocation to the BBM will be discussed with the parameters varying with diet, time and RA treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals and solutions

2.1.1 Brush Border Membranes (BBM) isolation solutions

Buffer 1:	**1 l**
100 mM manitol	18.22 g
2 mM Hepes/Tris, pH 7.1	0.477g Hepes (M_{Hepes}=238.5 g/mol)
(pH with 2M Tris)	

Buffer 2:	**500 ml**
100 mM manitol	9.11 g
2 mM Hepes/Tris, pH 7.5	0.238 g Hepes (M_{Hepes}=238.5 g/mol)
0.1 mM $MgSO_4$	0.5 ml stock $MgSO_4$ 100 mM
(pH with 2M Tris)	

Buffer 3:	**100 ml**
300 mM manitol	5.466 g
20 mM Hepes/Tris, pH 7.5	0.477g Hepes (M_{Hepes}=238.5 g/mol)
0.1 mM $MgSO_4$	0.1 ml stock $MgSO_4$ 100 mM
0.02% NaN_3	0.2 ml stock 10% (w/v)
(pH with 2M Tris)	

2.1.2. Western blot solution

2.1.2.1. Gel solutions

Resolving gel (*)

% Acrylamide	Range of separation (KDa)

15	12-43
10	16-68
7.5	36-94
5	57-212

15ml (2 gels)	5%	8%	12%	10%	15%
H₂O UP	8,75ml	7.25ml	5,25ml	6,145ml	3,65ml
4x Tris-HCl/SDS, pH= 8,8	3,75ml	3.75ml	3.75ml	3,750ml	3.75ml
30% Acryl: 0,8%Bis	2,53ml	4.05ml	6ml	5ml	7,5ml
10% APS (100mg/ml)	75µl	75µl	75µl	75µl	75µl
TEMED	30µl	30µl	30µl	30µl	30µl

7,5ml (1 gel)	5%	8%	12%	10%	15%
H₂O UP	4,375ml	3,625ml	2,625ml	3,073ml	1,825ml
4x Tris-HCl/SDS, pH= 8,8	1,875ml	1,875ml	1,875ml	1,875ml	1,875ml
30% Acryl: 0,8%Bis	1,265ml	2,025ml	3ml	2,500ml	3,75ml
10% APS (100mg/ml)	37,5µl	37,5µl	37,5µl	37,5µl	37,5µl
TEMED	15µl	15µl	15µl	15µl	15µl

(*): Resolving gel used in this experiment is an 8% gel.

Stacking gel

3,9% Acrylamide	1 Gel	2 Gels
H$_2$O UP	3,05ml	6,1ml
4x Tris-HCl/SDS, pH= 6,8	1,25ml	2,5ml
30% Acryl: 0,8%Bis	650µl	1,3ml
Bromophenol Blue	25µl (0.5%)	50µl (0.5%) ; 100µl (0,05%)
10% APS (100mg/ml)	25µl	50µl
TEMED	20µl	40µl

- **30% Acrylamide/0,8% Bisacrylamide**

(USING MASK)

		50ml	**100ml**	**200ml**
Acrylamide	(478)	14.6g	29.2g	58.4g
Bisacrylamide	(137)	0.4g	0.8g	1.6g

For 100ml: Add 29.2g de Acrylamide, 0.8g de N,N'-methylenebisacrylamide and H$_2$O UP to 100ml. Filtrate a solution (filter 0.45µm) and keep a 4ºC, in dark.

- **4X Tris-HCl/SDS, pH 6.8** (0.5M Tris-HCl containing 0.4% SDS)

		40ml	**50ml**	**100ml**	
Tris base	(128)	2,42g	3.025g	6.05g	(pH=6.8 with HCl 6N)
SDS	(67)	0,16g	0.2g	0.4g	

For 100ml: Dissolve 6.05g Tris base in 40ml H$_2$O UP and adjust pH to 6.8 with 6N HCl, add H$_2$O UP to 100ml. Filtrate the solution (filter 0.45µm and syringe 20ml) and add 0.4g SDS. Keep at 4ºC.

- **4x Tris-HCl/SDS, pH 8.8**   (1.5M Tris-HCl containing 0.4% SDS)

	150ml	100ml	500ml	
Tris base (128)	27.23g	18.15g	91g	(pH=8.8 with HCl 6N)
SDS (67)	0.6g	0.4g	2g	

For 100ml: Dissolve 18,15g Tris base in 60ml H_2O UP and adjust pH to 8.8 with 6N HCl. Filtrate the solution (filter 0.45μm e syringe 20ml) + 4ml SDS10X, add H_2O UP until 100ml. Keep at 4ºC.

- **10% APS (Ammonium Persulfate)**

0,02g (20mg) APS (powder) + 200μl H_2O UP

- **Bromophenol Blue** (0.05% Bromophenol Blue in Tris-HCl 6.8)

   5mg Bromophenol Blue + 10ml Tris-HCl 6.8 (adjust pH=6.8)

2.1.2.2. Buffers

Towbin buffer 5X

	5X
25mM Tris base	15.1g
192mM Glycine	72g
H_2O UP	1L

- Dissolve Tris base and Glycine in around 800ml H_2O UP

- Add H_2O UP until 1L

- Keep in 4^0C champer

Running buffer

	400ml (for 2 gels)
Towbin buffer 5X	80ml
0.1% SDS	4ml (from the SDS 10% solution)
H_2O UP	316ml

- Stir the solution

- Keep at 4^0C

Transfer buffer 5X (Semi-dry blotting)

	1000ml
48mM Tris base	29.1g
39mM Glycine	14.65g
0.0375% SDS	1.875g
H_2O UP	1L

Transfer buffer

	150ml (1 gel)	**300ml (2 gels)**
Transfer buffer 5X	30ml	60ml
Absolute Metanol	7.5ml	15ml
H_2O UP	112.5ml	225ml

TPBS (0.05% Tween20, PBS pH=7.4)

PBS 10X	100ml
H_2O UP	900ml
Tween20	500 µl

Stripping buffer

	250ml
62.5mM Tris-base	1.893g
2% SDS	5g
100mM 2-Mercaptoetanol	1.748ml

- Dissolve Tris base in 230ml H_2O UP

- Adjust pH to 6.7 with HCl concentrated

- Add SDS and dissolve by gently stirring (to avoid making bubbles)

- Add H_2O UP until 250ml and put all the solution in a bottle

- Add 2-Mercaptoetanol in the hood

- Keep the solution at room temperature in the hood

SDS Sample Buffer 2X (Laemmli sample buffer)

	50ml
-0.125M Tris-HCl, pH 6.8 (stock 0.5M)	12.5ml
-20% Glycerol	12.6g (10ml)
-4% SDS (stock 10%)	20ml
-0,01% Bromophenol Blue (stock 0,5%)	1,0ml
-H_2O UP	45ml

- Dissolve all the components in the solution

- Filtrate the solution with 0.22µm filter

- Make 900µl aliquots

- Keep at -20^0C

Before using, add 100µl DTT 1M in each 900µl aliquot. Reuse the solution remaining (keep at -20^0C)

DTT 1M: 1ml H_2O + 154.3mg DTT (4^0C, MW=154.3mg/ml)

2.1.2.3. Blocking solution

	1 Membrane	**2 Membranes**
TPBS	25ml	50ml
5% Skim milk	1.25	2.5g

2.1.2.4. Antibody solutions

First Antibody

	10ml
1% BSA	100mg
0.05% Sodium Azide (NaN_3)	50µl (Stock 10%)
TPBS	10ml
First antibody	*

* Volume varies depends on the 1st antibody prepared (see the manufacturer's recommendation)

Sodium Azide 10%: 1g azide + 10ml H_2O UP

Secondary antibody (to be freshly made)

	10ml
5% Skim milk	0.5g
TPBS	10ml
Secondary antibody	3µl*

*Volume can varies depends on the type of antibody used

2.1.2.5. Gel staining/distaining solutions

Gel staining solution

	1000ml
0.1% Coomasive Blue	1ml
5% Ethanol	50ml
7% Methanol	70ml
H_2O UP	879ml

-Immerse the gel in staining solution

-Incubate at room temperature in 1h

-The solution can be reused

Gel distaining solution

	1000ml
25% Methanol	70ml
5% Acid Acetic	50ml
H_2O UP	880ml

-Distain the gel during 30 minutes in the hood

-Change the distaining solution and leave it with agitation

- **Ethanol 95%**

95ml ethanol Pure + 5ml H_2O UP

76ml ethanol Pure + 5ml H_2O UP

- **SDS 10X**   10g SDS (67) + 90ml H_2O UP

(Add H_2O UP to 100ml)

- **Membranas PVDF:**   5,9 X 8,8 cm

- **Papel Watman:**   6,3 X 9,1 cm

2.2. Animal

100 small intestine samples collected from 10 groups of rat (10 samples/group).

Experiment with 100 male rats with ~150g

Feeding regimes:

HCD – normal rat chow

LCD – high protein, low carbohydrate diet (7days)

HCD1 – high glucose diet (4 days and 15 days)

HCD2 – high amylopectin diet (4 days and 15 days)

(The different diets are isocaloric)

W – Tap water drinking

RA – rosmarinic acid in tap water drinking (~600ug/ml)

Groups of rats:

Group 1 (G1): 11 days on HCD/W diet

Group 2 (G2): ~7 days on HCD/W + 7 days on LCD/W

Group 3 (G3): 7d on HCD/W + 7d on LCD/W + 4 days on HCD1/W

Group 4 (G4): 7d on HCD/W + 7d on LCD/W + 4 days on HCD1/RA

Group 5 (G5): 7d on HCD/W + 7d on LCD/W + 4 days on HCD2/W

Group 6 (G6): 7d on HCD/W + 7d on LCD/W + 4 days on HCD2/RA

Group 7 (G7): 7d on HCD/W + 7d on LCD/W + 15 days on HCD1/W

Group 8 (G8): 7d on HCD/W + 7d on LCD/W + 15 days on HCD1/RA

Group 9 (G9): 7d on HCD/W + 7d on LCD/W + 15 days on HCD2/W

Group 10 (G10): 7d on HCD/W + 7d on LCD/W + 15 days on HCD2/RA

2.3. Methods

2.3.1. Preparation of brush-border membrane vesicles (BBMV)

This method can be used with fresh and frozen tissues.

All steps should be done at 4ºC → **4ºC Room**.

Procedure	Notes
1. Place all the equipment in the 4ºC room some time in advance to adapt to the correct temperature.	
2. Place 50 ml of *buffer 1* in the homogenizer container. Take the sample (~2g) from the criotube (still frozen) and place in the 50 ml of buffer (wait about 2-3 min to defrost).	
3. Homogenize (in the Waring Blender), at V_{LO}, for 30 sec. Wait about 1 – 2 min to slow down again the temperature of the system.	→ Nylon yellow filters (0.45 µm)
4. Homogenize again at V_{LO} for 30 sec.	→ **Hi** = total volume of 50 ml
5. Filter the homogenate through a nylon filter (yellow) to two 50 ml-tube (25 ml for each). → **Take about 1 ml of crude homogenate (Hi)**	
	→ **Total volume**: 2 x 50 ml = 100 ml **diluted Hi**

6. Add *buffer 1* to each tube until 50 ml.	
7. To each 50 ml-tube, add 200 μl of MgCl$_2$ (2.5 M) to obtain a final concentration of 10 mM MgCl$_2$. Stir on ice during 20 min. (3 in 3 minutes) →Meanwhile switch on the 50 ml-tube centrifuge for cooling at 4ºC.	(the divalent cations will crosslink with all non-BBM membranes to induce precipitation) (At this point all connective tissue, intact-cells, debris, nuclei, mitochondria, basolateral and intracellular membranes will collect in the pellet leaving BBM in the supernatant)
8. Centrifuge all tubes at 3,000 x g (Eppendorf Centrifuge 5804 R) for 10 min. →Meanwhile switch on the Avanti centrifuge for cooling at 4ºC.	Be careful to correctly identify the goblet with the correct sample, and place on ice. Cover goblet with aluminium foil.
9. Reject the pellet and join supernatant from the same sample in a 100 ml goblet. Divide sample for 3 tubes of Avanti centrifuge and centrifuge supernatant at 30,000 x g (15.749 rpm, JA25-50 rotor, Beckman Avanti J-25I) for 30 min.	**CALIBRATE VERY WELL THE TUBES in a balance.** (centrifugation is to pellet the Brush-border membranes.

	The second supernatant is discarded -contains only soluble cytosolic proteins).
10.Resuspend pellet of each tube with 10ml of **buffer 2** (10 strokes – with a 5 ml syringe + a yellow tip). Join all the BBM from the same sample in one tube.	→ **Total volume**: 3 x 11 ml = ~33 ml of **diluted BBM**
11.Centrifuge tubes at 30,000 x g (15.749 rpm, JA25-50 rotor, Beckman Avanti J-25I) for 45 min.	
12.Resuspend pellet (BBM) in 1-2 ml of **buffer 3** (first with a P1000 pipette and then passing through a 27 gauge needle at least six times). → Use a 1 ml or 2 ml syringe.	**CALIBRATE VERY WELL THE TUBES in a balance.** (to "wash" BBM - to remove the last of the $MgCl_2$; BBM are precipitated again)
13. Make BBM granules in liquid nitrogen and keep it at -80ºC (for Western blot), or in liquid nitrogen (if it is to use in transport studies).	(This procedure does not damage the membranes and helps to form the vesicles) • Protein concentration should be higher than ~5 – 7 mg/ml.

MATERIAL   (for isolation of 2 samples - **2 rats**)

- 2 boxes with ice

- Waring Blender + Container 37-110 mL

- 1 50 mL graduated cylinder

- 1 forceps

- 4 50 mL-tube/rat

- 2 nylon filters

- P200 + tips

- 1 plastic Goblet for residues

- 2 glass Goblets >100mL + Aluminium foil

- 6 Avanti centrifuge tubes

- P1000 + tips (for calibration of tubes in the balance)

- 2 5mL-syringe + 2 yellow tips

- 2 2mL-tubes

- Container for liquid N2 to make BBM and Hi granules

- Plastic spoon to recover BBM and Hi granules made in liquid N2

- Criotubes to keep samples at -80ºC (Hi e BBMV)

- Time counter

- Liquid N2 + appropriate container

- Cleaning paper

- Goblet to reject supernatant from centrifugation of BBM (to avoid pellet detachment when coming to the laboratory).

2.3.2. Determination of Protein (BRADFORD Method)

Sample dilution:

Hi – dilute 1:2 or no dilution (20µl sample + 20µl buffer1 from protocol of BBMV isolation).

BBMV – dilute 1:10 (4µl sample + 36µl buffer3 from protocol of BBMV isolation)

Make 2 Standard curves:

+ BSA 5mg/mL in buffer 1 to Hi

+ BSA 5mg/mL in buffer 3 to BBMV

Standards:

5mg/mL   3mg/mL   2mg/mL   1mg/mL   0.5mg/mL   0.25mg/mL

Pipette to a 96-well plate in the following order:

Hi

-First:

5µl blank (buffer 1)

5µl standard (total of 6 standards in 6 wells)

5µl sample

-Second:

250µl Bradford Reagent

⇨ Incubate 5-10 min, room temp.

BBM

-First

5µl blank (buffer 3)

5µl standard (total of 6 standards in 6 wells)

5µl sample

-Second:

250µl Bradford Reagent

⇨ Incubate 5-10 min, room temp.

2.3.2. Western blot

2.3.2.1. Preparing the gel

-Resuspend all the necessary components (APS and TEMED should be added at the end) and pour quickly with p1000 into the two glasses.

-Immediately adding 95% ethanol on the top of the resolving gel and let it polymerize in 40 minutes. (Ethanol helps the gel avoid being contacted with O_2, which inhibits the polymerization).

-While waiting for the solidification of the gel, we can make the stacking gel (without adding APS and TEMED) and prepare the running buffer.

-After the first gel has solidified, pour out the ethanol and wash 2 times with H_2O UP, then dry with filter paper without touching the gel.

-Pour the stacking gel into the remaining empty space of the glasses, put the combs and wait for 15-20 minutes for the stacking gel gets solidified.
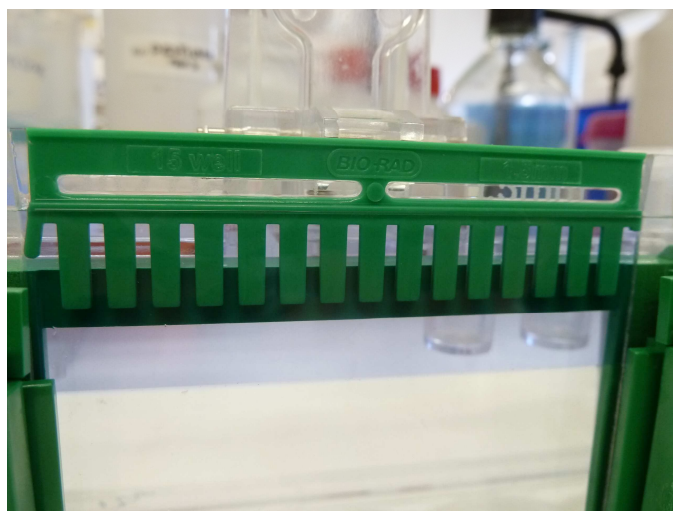

Figure 7. Gel after being prepared.

2.3.2.2. Electrophoresis

-Put the glasses with the gel into the electrophoresis system with the combs facing inward.

-Pour the running buffer into the mini tank, fill the space between the gels and remove the bubbles.
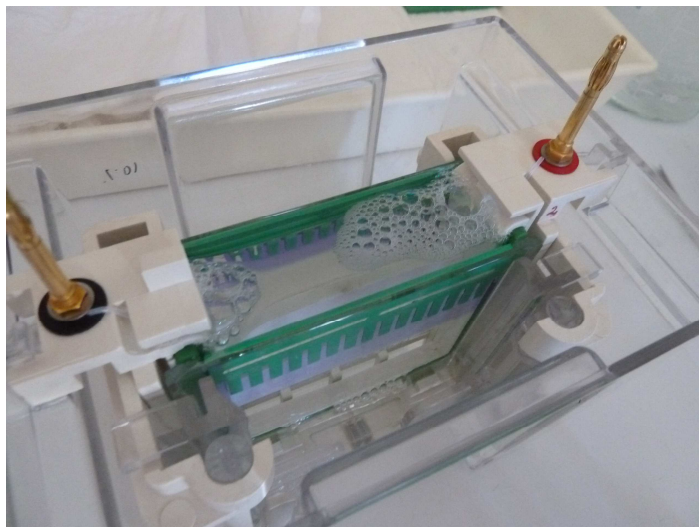
Figure 8. Gels and running buffer inside an electrophoresis system.

-Cover and leave the tank with gel + buffer in 4^0C overnight or continue.

-Carefully remove the combs while the system is immersed in the running buffer and use p100 to wash the wells with running buffer.

-Prepare to run the samples treated with SDS in order to have the protein concentration around 20µg/µl.

-Load the wells (15 wells/gel) with the same volume:

+1st well: 5-10µl Molecular Marker All Blue Bio-Rad + 15-20µl SDS buffer

+ 13 next wells: 20-30µl samples (in order to have 20µg protein/well)

+ last well: SDS buffer

-Cover the tank and connect to the power:

+Run at 100V in 15 minutes to let the samples concentrate at the beginning of the separation gel.

+After that, run at 150V in 1 hour for separating the samples in the resolving gel.

2.3.2.3. Transfer – Semi dry blotting

-Prepare the transfer buffer: 300ml for 2 gels/ 150ml for 1 gel. (1)

-Incubate 3MM paper (2 papers/gel) in transfer buffer for 15 minutes with agitation. (2)

-Incubate the PVDF membrane, which was marked, hydrated 1 min in methanol, and washed 3X in water, in transfer buffer for 15 minutes with agitation. (3)

25

-After the electrophoresis running finished, remove the gel support and the stacking gel, make a mark on the opposite side to the marker on the resolving gel by cutting a square or a triangle; then place it in transfer buffer in 20 minutes with agitation.

Note: (1), (2) and (3) should be prepared during 1 hour electrophoresis running.

-Construct the sandwich in Trans-SD Blotting:

> *Cathode (top plate)*
>
>> -Paper Watman 3MM (4)
>>
>> -Gel (3)
>>
>> -PVDF Membrane (2)
>>
>> -Paper Watman 3MM (1)
>
> *Anode (low plate)*

Roll with the cylinder to remove bubbles. Dry the excess of liquid around the sandwich. The proteins with negatively charged due to the buffer will be transferred from the gel to the membrane in a cathode (-) to anode (+) direction.

-Place the lid, connect to the electric power and turning to 15V for 40 minutes (for gels 1.5mm)

-After 40 minutes transferring, disconnect the equipment:

> +Place the membranes in TPBS for 10 minutes, agitating 2X
>
> +Place the gel in Coomassie for 20 minutes, then water and let it be agitated as long as you wish.

2.3.2.4. Marking with antibody – Immunodetection

-Remove the TPBS and incubate the membranes with blocking buffer in 1-2 hours at room temperature with agitation, or leave overnight at 4^0C. This blocking step helps unoccupied sites of the membrane be saturated and prevent the non-specific bindings of the antibodies.

-Remove the blocking solution and wash the membranes 3X with TPBS for 5-10-15 minutes.

-Incubate the membranes with primary antibody *(*)* at 4^0C overnight in the roller falcon.

-Take the membranes out of the primary antibody falcon (reusable) and wash 3X in TBPS for 5-10-15 minutes.

-Incubate the membranes with conjugate 2nd antibody *(**)* for 1 hour at room temperature. (2nd

antibody can be anti-rabbit, anti-mouse or anti-goat…depends on the primary antibody)

Note:

(*): primary antibodies used are SGLT1 (ab 14686, Abcam), GLUT2 (Anti-Glut2 Millipore, 07-1402) and SI (Sucrase isomaltase L-20, sc-27608, Santa Cruz).

(**): secondary antibodies used are Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling, 7074 and Donkey anti-goat IgG-HRP: sc-2033, Santacruz.

2.3.2.5. Detection

-Turn on the PC and activate Program Quantaty One 4.6>File>ChemidocXRS (*)

-Prepare a solution Amersham ECL TM Kit or Millipore Western Blotting Detection Kit 1A:1B (4^0C) – Adding 400µl solution A + 400µl solution B (for one membrane) and mix well.

-Remove the membrane from the TPBS buffer and put it on a plastic; pour the solution prepared before onto the membrane and leave in 1 minute.

-Dry the membrane by dripping it on the absorbent paper then put it in a plastic membrane. Reveal the membrane inside the ChemidocXRS system.

-In the apparatus:

　　+UV filter (the tab)>button EpiWhite : for marker photo

　　+Chemi filter>disconnect button EpiWhite: for bands photo

-In the PC:

　　+(*)>select>cristovao membrane>zoom>freeze>auto expose: for marker photo

　　+select>chemidoc HS>Iris open>choose exposure time (first: 20 second)>manual expose

-Photo saves: File>Save as and File>Export JPEG>Export

-Quantify bands: Open file you want to quantify>Volume button Quick Guide>Button 4th>circling bands>button 9th>vol Adj>done.

2.3.3. Experimental design

　　The animals were fed a regular rat chow for 1 week before being fast sampled and introduced of the low carbohydrate diet - LCH diet (see composition in Table 3). A week later, the animal were sampled again and assigned (10 per group) to 4 different treatments:

　　+High glucose diet (HGD)-water (W).

+High glucose diet (HGD)-rosmarinic acid (RA).

+High amylopectin diet (HAD)-water (W).

+High amylopectin diet (HAD)-rosmarinic acid (RA).

Table 2. Diets' composition

Composition	Diets		
	LCH	HGD	HAD
Casein, %	53,300	20,500	20,500
Amylopectin corn starch, %	28,000	--------	61,000
Glucose, %	--------	61,000	--------
Cellulose powder (68% CF), %	6,000	6,000	5,000
L-Cysteine, %	0.500	0,300	0,300
Minerals & trace elements, %	6,000	6,000	6,000
Vitamin premix, %	1,000	1,000	1,000
Choline Chloride, %	0,200	0,200	0,200
Soybean oil, %	5,000	5,000	6,000
Crude protein, %	47,3	18,3	18,3
Crude fat, %	5,2	5,1	5,1
Crude firbe, %	4,1	4,1	4,1
Crude ash, %	5,7	5,3	5,3
Starch, %	28,0	-------	60,2
Sugar, % [1]	0,8	61,6	0,8
Energy (Atwater), MJ/kg	15,3	15,8	15,8
kJ% Protein	52	19	20
kJ% carbohydrate	35	69	68

kJ% Fat	13	12	12
Fatty acid, % of diets			
C14:0	0,05	0,03	0,03
C16:0	0,60	0,55	0,55
C18:0	0,26	0,24	0,24
C20:0	0,03	0,03	0,03
C16:1	0,03	0,03	0,03
C18:1	1,37	1,33	1,33
C18:2 *n6*	2,66	2,65	2,65
C18:3 *n3*	0,33	0,33	0,33

CHAPTER 3

RESULTS AND DISCUSSION

In this study, rats fed a normal diet for 7 days (NFD), were fed a low carbohydrate diet (LCH) for another 7 days in order to decrease enterocyte levels of sucrose-isomaltase (SI), sodium dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2), proteins involved in carbohydrate digestion and glucose absorption. After this, the animals were divided in groups and the diets changed to high glucose (HGD) or high amylopectin (HAD) with or without rosmarinic acid in the drinking water. Small intestinal mucosal samples were taken at 4 and 14 days.

After the 7 days on the low carbohydrate diet (LCD) decreases in protein levels of SI, SGLT1 and GLUT2 were observed. These results are in agreement with previous data reported by us and others (Azevedo et al., 2011).

Generally, upon the reintroduction of CH (as glucose or as amylopectin), we observed an increase in expression of SI, SGLT1 and GLUT2 in whole cell homogenates. However, for these proteins to play a role in carbohydrate digestion and absorption from the intestinal lumen, they need to be translocated to the brush border membrane (BBM) of enterocytes. Therefore, the data is presented as BBM levels and whole cell levels of each of the proteins. We interpreted increases in whole cell homogenate levels of the above proteins as the result of increase gene expression and the increase in BBM expression levels as a consequence of protein translocation from the cell's cytosol to the apical membrane. The effects of diet (type of carbohydrate) will be discussed first and the modulation by RA will be presented last.

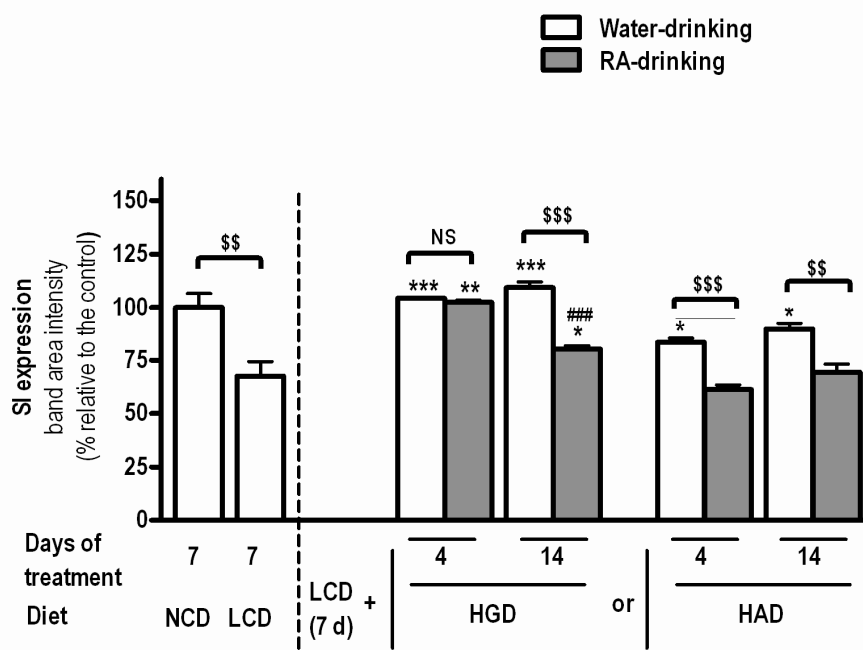*Effect of diets & Rosmarinic Acid (RA) on SI's expression and translocation*

Figure 9. Effects of diets and RA on SI level in jejunal BBM (♦)

(♦) Legend:

d – Days

RA – Rosmarinic Acid

NCH – Normal Carbohydrate Diet

LCH – Low Carbohydrate Diet

HGD – High Glucose Diet

HAD – High Amylopectin Diet

Statistics (unpaired t-tests):

$ - P<0.05; $$ - P<0.01; $$$ - P<0.001 when compared with each other

NS – Not significant (P>0.05) when compared with each other

* - P<0.05; ** - P<0.01; *** - P<0.001 when compared with LCD group

- P<0.05; ## - P<0.01; ### - P<0.001 when compared with the respective treatment group for 4 days.

a - P<0.05; aa - P<0.01; aaa - P<0.001 when compared with NCD group.

32

Relative the LCD, when either glucose or amylopectin were introduced in the diet there is an increase in SI at the BBM which is more significant in the glucose diet than in amylopectin diet (Figure 9). These results are interesting since amylopectin and not glucose is the substrate to this enzyme.

In the whole cell homogenates of HAD animals there is no increase in SI expression relative to the LCD animals at 4 days with a very small increase present at 14 days (Figure 10). Total SI expression levels in the glucose diet were similar to those in the NCD which indicates that in this group less than 4 days were needed to recover normal SI expression. From our data, it seems that free luminal glucose plays an important role in the induction of both translocation (Figure 9) and expression of SI (Figure 10).
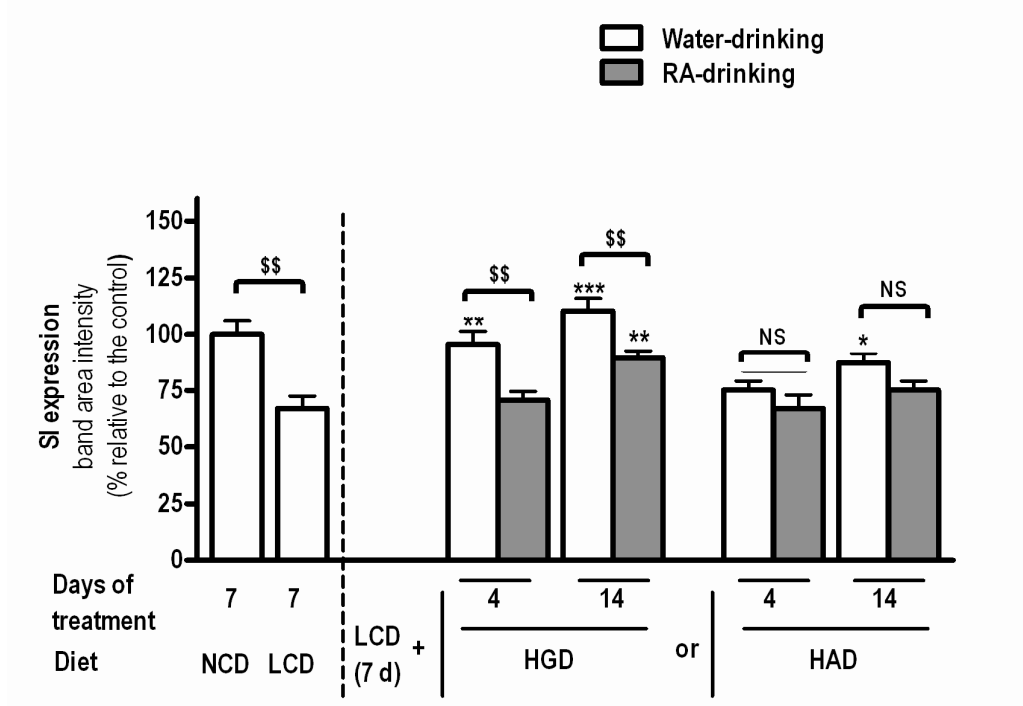

Figure 10. **Effect of diets & Rosmarinic Acid (RA) on SI expression**

in jejunal whole cell homogenates (♦)

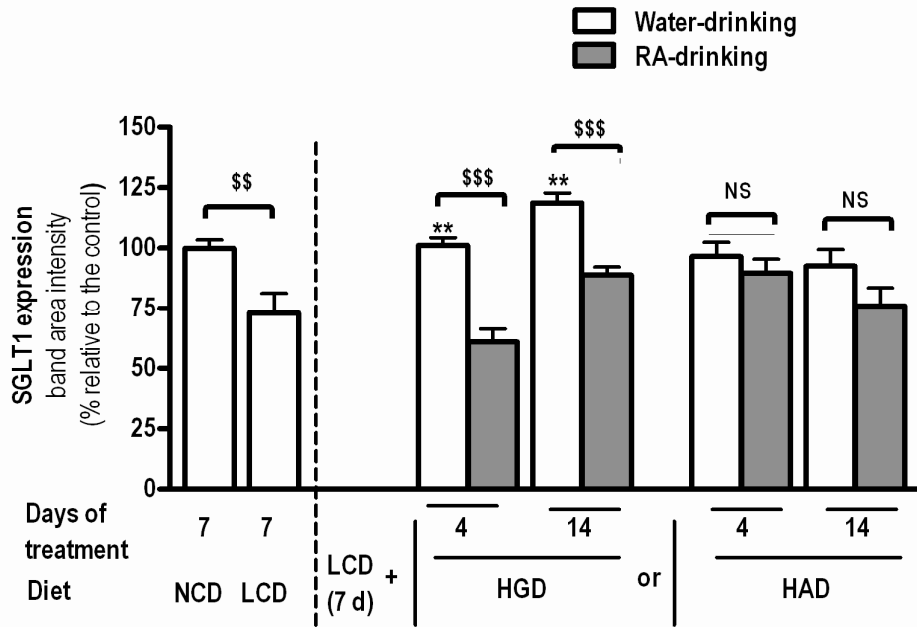*Effect of diets & Rosmarinic Acid (RA) on SGLT1 expression and translocation*

Figure 11. **Effect of diets & Rosmarinic Acid (RA) on SGLT1 expression**

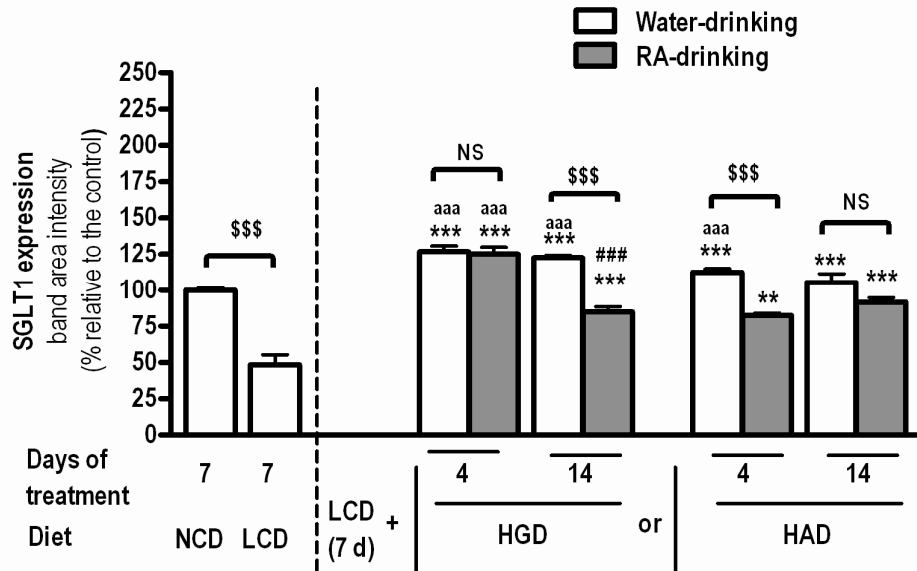**in jejunal whole cell homogenates (♦)**

Figure 12. Effects of diets and RA on SGLT1 level in jejunal BBM (♦)

SGLT1 has been shown to increase in parallel with SI in response to increased CH in the diet. In whole cell homogenate, SGLT1 expression increased after the LCD with glucose but not amyplopectin. (Figure 11). However, BBM SGLT1 increased in both glucose and

34

amylopectin diet (Figure 12). This seems to indicate that the presence of glucose, even in low amounts (HAD) is responsible for the translocation of SGLT1 to the BBM, a process that does not require a *de novo* synthesis of SGLT1. When luminal glucose is high (HGD), in addition to a translocation effect, glucose seems to also have an effect on increasing SGLT1 gene expression.

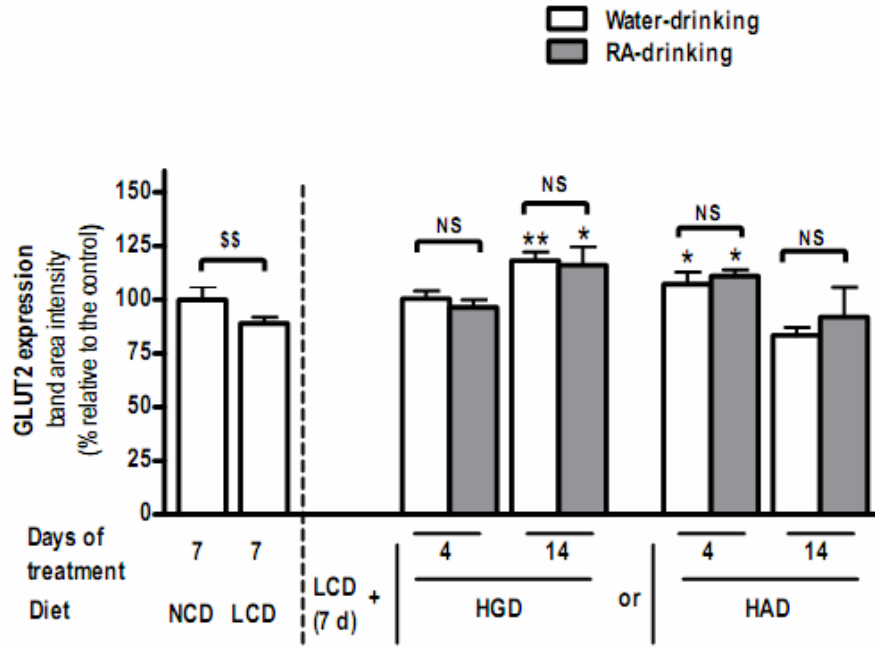*Effect of diets & Rosmarinic Acid (RA) on GLUT2 expression and translocation*

Figure 13. Effect of diets & Rosmarinic Acid (RA) on GLUT2 expression
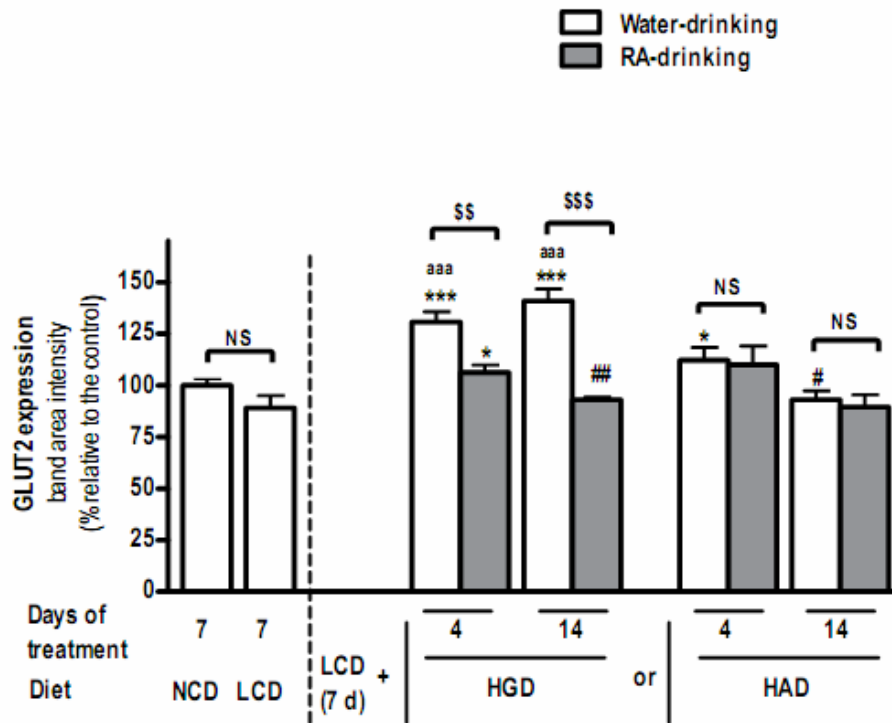
in jejunal whole cell homogenates (♦)

Figure 14. Effects of diets and RA on GLUT2 level in jejunal BBM (♦)

36

With regards to GLUT2, there seems not to be a consistent increase in gene expression after reintroduction of CH (Figure 13). However, there was a very significant effect of luminal glucose on GLUT2 translocation to the BBM, where it is usually present in very low amounts since it is a basolateral protein (Figure 14). This effect of glucose on GLUT2 translocation to the BBM has also been reported by Kellet et al., 2008.

Regarding RA's effect in whole cell homogenate levels of SI and SGLT1, it decreased both SGLT1 and SI expression in HGD (but not HAD). When analyzing BBM levels of the protein, we found a significant decrease of SI in HAD animals and 14 day glucose fed animals (Figure 10). This effect seems to be similar to the one on BBM SGLT1 of HGD animals. However, in HAD animals, RA only decreased BBM SGLT1 level at 4 day (not 14 days like in SI's case). RA had also already been shown to decrease BBM SGLT1 level in rat enterocytes (Azevedo et al., 2011). In that previous report, where a significant effect of RA was demonstrated already at 4 days, the animals had been fed a starch diet (which is composed by amylose and amylopectin), not a glucose diet. The effect of RA on starch and amylopectin fed animals seem to suggest that RA inhibits the digestive process to which SI contributes which is in agreement with SI data. In fact, RA significantly decreased SI BBM levels in HAD group (Figure 9).

In general, we can see that RA effects on BBM SGLT1 and SI levels follow the same pattern and could be explained by an inhibition of both SI and SGLT1 translocation.

In glucose fed animals, at 4 days, RA did not decrease either SGLT1 or SI in the BBM, although it decreased total protein levels (SI and SGLT1 whole cell homogenates). So, RA seems to be inhibiting the expression of these two genes but not translocation.

In case of RA effects on GLUT2, there was no effect on gene expression of GLUT2 (whole cell homogenate). However, in HGD fed animals, RA decreased BBM GLUT2 which may be the result of inhibition of GLUT2 translocation to the BBM. This effect of RA was not apparent in HAD fed animals where GLUT2 expression in BBM was not induced.

Our results show that:

-RA decreases BBM translocation of GLUT2 induced by high luminal glucose

-RA decreases SGLT1 gene expression rather than translocation to the BBM induced by high luminal glucose that is not prevented at 4 days. However at low luminal glucose (4 day

HAD), RA shows a inhibition in SGLT1 translocation to the BBM, but not gene expression inhibition.

-RA decreases SI gene expression and seems also to be effective preventing its BBM translocation.

CHAPTER 4

CONCLUSION

In conclusion, our study shows that HGD and HAD increase the level of BBM protein of SI and SGLT1 whereas only HGD seems to have an effect on increasing the gene expression of these protein in total homogenates. In addition, from our data, it is suggested that free luminal glucose at low amount (HAD) may responsible to enhance mainly the translocation of SI and SGLT1 whereas higher amount of luminal glucose may result in the increase in gene expression of these two protein.

GLUT2, on the other hand, just has the significant increase in translocation to the BBM due to the present of free luminal glucose. HGD and HAD did not affect consistently on the gene expression of GLUT2.

Rosmarinic acid has a down regulation effects to GLUT2, SGLT1, and SI.

The fact that diets (HGD, HAD) and RA affects to SGLT1 and SI in the same pattern, suggests that SGLT1 and SI expression is control by similar mechanism. Since we found no effect of RA on SGLT1 and SI BBM level at 4 day HGD fed animals , it is suggested that RA may interferes to some factors that control the transcription process of these two proteins. One of these factors can be "the binding of histone acetyl-transferase GCN5 and acetylation of histone H3/H4 on these genes" (Seiya Inoue, 2011). So that, we suggest doing further experiments to check whether RA helps decrease the binding of GCN5 and acetylation of histone H3/H4 on the transcription process of these two genes.

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