The role of the small intestine in maintaining blood glucose levels - species-specific differences

PhD thesis

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Introduction

Keeping the blood sugar at a physiological level is extremely important for maintaining a good health and any disturbance may lead to carbohydrate metabolism disorders. These changes are often associated with obesity, what is commonly observed in metabolic syndrome (MetS) and type 2 diabetes (T2DM). It is now a well-known fact that excessive sugar consumption plays a decisive role in the development of MetS and T2DM, with the emphasis of sucrose (table sugar) and high fructose corn syrup. During my PhD I have studied the pathways of carbohydrate metabolism that are localized to the endoplasmic reticulum.

Role of endoplasmic reticulum in carbohydrate metabolism

The endoplasmic reticulum is surrounded by a continuous membrane that is also associated with the nuclear membrane. It is an independent cell organelle with its own proteome, metabolome, and it has a distinct redox environment. Recent research highlights the role of ER as a nutrient sensor based on its redox state, supporting the cellular response to extreme nutritional conditions.

The ER membrane separates the ER lumen from the cytosol, allowing the independent regulation of its redox reactions. The ER lumen is significantly more oxidized than the cytoplasm, and it has its own pyridine nucleotide pool.

Several ER enzymes have their active centers intraluminally, of which I was particulary focused on studying the glucose-6-phosphatase (G6Pase) and the hexose-6-phosphate dehydrogenase (H6PDH). Both enzymes utilize intraluminal G6P; at high luminal G6P levels, H6PDH is more active, while at low G6P concentrations (such as during starvation) G6Pase is more active.

The G6Pase system

This enzyme system is responsible for the last common rate-limiting step of gluconeogenesis and glycogenolysis. The active center of the enzyme is faced to the lumen, so the transport of its substrate (G6P) and products (glucose, phosphate) are required across the ER membrane. The specific glucose-6-phosphate transporter (G6PT) is responsible for the entry of G6P, but there are several theories for the exit of the products. The enzyme glucose-6-phosphatase is not specific for glucose-6-phosphate; it can hydrolyze several types of hexose-phosphates, e.g. mannose-6-phosphate (M6P). The high specificity of the system is based on the function of G6PT, which provides the G6P substrate and this enzyme is also located in the ER membrane.

To date, three different types of G6Pase isoforms have been identified in humans; they are encoded by different genes and have their unique roles, tissue distribution, and kinetic properties. Firstly, G6Pase-α protein was discovered, encoded by G6PC1, the second was the so-called islet-specific glucose-6-phosphatase-related protein (IGRP) (encoded by the

G6PC2 gene) and the last was the G6Pase-β (encoded by the G6PC3 gene). Although the three isoforms show only moderate homology, their membrane topology and catalytic centers are very similar. It is generally accepted that only G6Pase-α (expressed in the liver, kidneys and small intestine) is significantly responsible for maintaining blood glucose levels.

The glucose-6-phosphate transporter was first identified by Gerin et al. in 1997; since that the transport of glucose-6-phosphate in liver microsomes (a rich cell fraction in ER) has been well characterized.

Function of the G6PT-H6PDH-11βHSD1 triad

11β-hydroxysteroid dehydrogenase (11βHSD1) is expressed in the glucocorticoid target organs, such as the liver, skeletal muscle and adipose tissue, and catalyzes the reversible interconversion of cortisone and cortisol.

It is located in the lumen of the endoplasmic reticulum bound to its membrane, and uses NADP+ or NADPH as a coenzyme, depending on the direction of the reaction. Important to note that the ER membrane is impermeable to pyridine nucleotides, therefore only the luminal NADPH pool is available to catalyze the reaction. The regeneration of NADPH used by this enzyme, i.e. the reduction of NADP+, is carried out by another enzyme called hexose-6-phosphate dehydrogenase (H6PD), which is also loacted to the ER lumen and physically linked to 11βHSD1. The NADPH is generated during the transformation of glucose-6-

phosphate (G6P) into 6-phosphogluconate (6PG). G6P enters the ER lumen via the glucose-6-phosphate transporter (G6PT) as already mentioned. In case when H6PD does not reduce the NADP+ to NADPH, the 11βHSD1 enzyme oxidizes, i.e., catalyzes cortisol-cortisone conversion. So ultimately the proper function of 11βHSD1 requires not only the presence and integrity of the enzyme itself, but also the overall functioning of the so called "catalytic triad" of these three proteins.

Is G6Pase expressed in the intestine? Contradicting findings in different species

The presence of G6Pase was confirmed first in the liver, then in the kidneys, and finally in the small intestine. Although small intestinal gluconeogenesis has been thoroughly studied since the 1950s, experiments using different species have often led to conflicting results.

Various methods (using radioactive precursors, measurements on proteoliposomes and microsomes) have been used to provide a quantitative estimate of the extent of the small intestinal gluconeogenesis, leading to several different theories. Some research groups measured higher enzyme activity in the postnatal period and suggested that its major function is to provide the glucose supply to small intestinal muscle tissue in this period of life, while several studies have shown that starvation, diabetes and protein-rich diets have the greatest effect on the enzyme activity.

The most important and most commonly used laboratory animals are the various knockout mouse strains. The first G6Pase knockout (KO) mice were created in 2009. Since then several tissue-specific (liver, small intestine) KO mouse strains have been also developed. Experiments gained from these animals have shown that the small intestine plays a vital role in maintaining fasting blood glucose level, at least in cases where liver gluconeogenesis is not available.

Objectives

As we can see that previous studies for the detection of intestinal gluconeogenesis in different animal models gave conflicting results, we wanted to clarify whether there are species-specific differences. Therefore, we chose 3 species with different feeding behaviours; an omnivore with frugivore ancestors (human), an herbivore–frugivore (guinea pig) and an herbivore (rat) for our investigation. It seemed logical to assume that if there are species-specific differences, they are associated with the diet, mainly with fructose consumption.

It is known that fructose-6-phosphate can be converted to glucose-6-phosphate in the cytoplasm by the enzyme called phospho-glucoisomerase (PGI), however, based on the previous studies by our research group, this transformation can also occur in the ER via phosphohexose isomerase pathway. We were wondering whether this pathway could be observed in the small intestine as well. It was also shown in previous rstudies that glucose is able to enhance 11β-HSD1-

dependent glucocorticoid activation. Therefore we also wish to clarify whether fructose can also affect this activation.

We aimed to answer the following specific questions with our experiments:

1. Can the existance and activity of the G6Pase system be confirmed in the small intestine of 3 different mammals with different feeding behaviours?

2. Does the F6P substrate contribute to the activity of the G6Pase system in the small intestinal microsomes isolated from the same species?

3. How does fructose affect the oxidoreductase and dehydrogenase activity of 11β-HSD1 *in vitro*?

Methods

qPCR

RNA was isolated from rat and guinea pig liver and small intestine with RNeasy Plus Mini Kit (Qiagen). RNAs from human liver and intestinal tissues were obtained from Ambion-Applied Biosystems (First Choice® Human Total RNA Survey Panel) and Biochain. Two micrograms of RNA were transcribed by reverse transcription in a final volume of 20 µl using a Superscript® III First Strand Synthesis System (Invitrogen®) and random hexamers. We compared the expression levels to the liver as our reference, considered as 100%.

Microsome isolation

Isolation of microsomes was performed in male Wistar rats (180-230 g) or guinea pigs (400-450g) by standard differential centrifugation. Human liver and intestinal microsomes were commercially available samples purchesed from Thermo Scientific (liver) and from Xenotech (intestine).

The microsome samples were diluted to a protein concentration of 1 mg / ml and three washing steps were performed to remove cytoplasmic proteins loosely associated with the microsome. For each washing step the samples were shortly centrifuged in MOPS buffer containing 4.5% polyethylene glycol, and the precipitate was resuspended.

Measurement of G6Pase enzyme activity

Measurements of G6Pase enzyme activity were performed by 2 methods, as both endproducts of the reaction, glucose and inorganic phosphate can be measured. We detected the former by the Sigma GO Glusose Kit and the latter by the Molybdate method.

For both measurements, the samples were divided into two groups, and the pH of one group was decreased to pH 5 with 1N HCl and then normalized with 1N $KHCO_3$ after 20 min of incubation at 37ºC. With this step G6Pase was specifically inhibited, so that the remaining activity was attributed entirely to non-specific phosphatases. The activity measured in the other group is the total activity, from which the specific activity can be calculated by subtracting the non-specific activity. Both groups were

further divided into two subgroups; intact and permeabilized microsomes. In the latter case, alamethicin (Sigma-Aldrich, St. Louis, MO, USA) was added to the sample at a concentration of 0.05 mg/mg microsomal protein) to form pores in the membrane in order to make the membrane permeable for small molecules without disrupting the vesicles.

Western Blot

For western blot measurements, an equal volume of microsomal protein (40 mg) was applied to a 12% polyacrylamide gel from each sample. Samples were blotted overnight with the following primary antibodies at 4 °C: G6Pase catalytic subunit (rabbit, polyclonal) (Abcam, Cambridge, UK) and G6PT (rabbit, polyclonal) (Abcam, Cambridge, UK). Both antibodies were used at a 1: 500 dilution in 5% milk. Blots were analyzed using ImageJ software. The amount of protein in the samples was quantified after normalizing to Ponceau staining.

Microsomal cortisone-cortisol conversion

The activity of the 11β-HSD1 enzyme was determined by the measurement of cortisone and cortisol production. To examine the amount of cortisone reduction, human embryonic kidney cells called HHH7 (which stably coexpress human 11β-HSD1 and H6PDH) were treated with 5 μM cortisone in the presence of various concentrations (0; 0.1; 0.3 1 and 4.5 g/l) of F6P. Oxidation of cortisol was examined under similar experimental conditions in a microsome pretreated with

alamethicin (0.1 mg/mg protein) after the addition of 5 µM cortisone and 50 µM NADP+. The reaction was stopped by adding 150 µl of ice-cold methanol in each case, and the samples were stored at -20 °C until HPLC measurement of cortisone / cortisol.

Statistical analysis

Each data represents the results of three independent measurements. Results are shown as the mean and the standard error of the mean (SEM). GraphPad Prism 8 software was used for the statistical analysis. Statistical differences between groups were analysed by 2-way ANOVA followed by Tukey's post-hoc test. Significance levels were used as follows: $p = 0.05$ -0.01 *; $p = 0.01$–0.001 **; $p \leq 0.001$ ***.

Results

Expression of the glucose-6-phosphatase system in liver and intestinal microsomes

The expression of G6Pase and G6PT mRNAs was determined by real-time PCR, while the expression of proteins was analyzed by Western blot.

Our results showed that G6Pase is expressed in microsomes isolated from the intestine, but the extent of expression is highly dependent of the species under investigation. In guinea pig and human intestinal samples G6Pase mRNA expression is approximately half of that detected in the liver. Contradictory, G6Pase mRNA expression in the rat small intestine is only one-fifth found in the liver. G6PT mRNA levels were also

examined in all three species. We found that the expression pattern of G6PT is very similar to that of G6Pase. In guinea pig and human intestinal microsomes the transporter expression was approximately 50% relative to liver, whereas in rats, intestinal expression was only 17% of liver.

Therefore in the next set of experiments we also examined the presence of G6Pase and G6PT at a protein level in our microsome samples. Positive controls were used to detect both proteins; HepG2 cell lysate for G6Pase and HEK293T cell lysate for G6PT were tested in parallel with microsome samples. The loading of the same amount of proteins was checked by Ponceau staining.

Similar to the results obtained in real-time PCR, both G6Pase and G6PT were expressed in guinea pig and human small intestine microsomes. In guinea pigs the intestinal G6Pase expression was similar to that of the liver, whereas in human samples intestinal G6Pase expression was approximately half of that of liver. G6PT protein levels were also high in guinea pig and human intestinal microsomes. However, when examining rat small intestinal micronomes, we found that the expression of both G6Pase and G6PT proteins were very low either compared to the liver or compared to guinea pig and human intestinal microsome samples.

Enzyme activity of G6Pase

The presence of the G6Pase system in liver tissue is well established in the literature, which is also supported by our previously described

findings of the expression levels. However, we also wanted to set up an accurate measurement of G6Pase enzyme activity in our own experimental system to examine the possible differences between the species.

In order to remove any cytosolic proteins loosely associated to the membrane from the microsomes, they were washed in three consecutive steps prior to the activity measurements.

There was no significant difference in enzyme activities by the addition of G6P substrate in the liver between washed and unwashed samples. In human microsomes, a non-significant decrease in activity was observed upon washing steps, presumably suggesting an extra-microsomal dephosphorylation of G6P.

Previous results from our research group have demonstrated that F6P is transported to the ER lumen, where it is converted to G6P by an unknown enzyme exerting hexose isomerase activity. G6P is then presumably hydrolyzed by luminal G6Pase to glucose and phosphate for the intraluminal reactions. Therefore, we also performed our experiments with the addition of F6P as a substrate.

In case of F6P substrate the washing steps resulted in a more significant decrease in G6Pase activity (about 50%) in microsomes isolated from liver of all three species. This can be explained by the fact that the washing steps may have removed the cytoplasmic

phosphoglucoseisomerase (PGI) enzyme from the outer surface of the microsomes, which is responsible for the F6P - G6P isomerization. By reducing the amount of F6P – G6P conversion resulted less G6P substrate for the G6Pase enzyme.

In both human and guinea pig intestinal microsomes, G6Pase activity was significantly decreased after the washing steps, suggesting that cytosolic non-specific phosphatases contribute more to total activity in this tissue. Therefore, all enzyme activities were measured on three times washed microsomes and are described in detail below.

It is well-known from the literature that a special transporter, G6PT is required for the ER entry of G6P, and in the case of an intact membrane this transport is the rate-limiting step for its hydrolysis to glucose and inorganic phosphate. Upon alamethicin addition tiny pores are formed on the ER membrane, so that G6P can enter freely to the ER by diffusion and utalized by the G6Pase enzyme, making the total activity of the enzyme measurable.

This way we can calculate the latency of the G6Pase enzyme, which was 49.2% in guinea pig liver and 35.7% in rat liver upon G6P addition. The lower latencies measured on human liver and intestinal microsomes (17.4% and 27.1%) may be due to the fact that these samples were purchased from companies, so transport and storage conditions were uncertain. In the case of intestinal microsomes, a lower latency was also observed in guinea pig microsome samples (15%).

Latency values were also measured by the addition of F6P substrate, which was found to be high in all cases, 55.8% in guinea pig liver, 49.3% in rat liver 54.1% in human liver, 89.7% in guinea pig intestine and 54.7% in human intestine. From the high latencies it can be concluded that the substrate transport to the ER greatly influences the degradation rate of G6P.

It has been known for a long time that the G6Pase enzyme is sensitive to pH changes, and while its optimum is around pH 6.5-6.7, it loses its activity at pH 5 or below. In our experiments, we wanted to make a distinction in the substrate hydrolysis due to specific G6Pase activity and cleavage by non-specific phosphohydrolases, so half of the samples were subjected to short-term pH 5 treatment, thus inactivating the enzyme. The activity obtained in these samples was termed as non-specific activity that was fully attributed to the activity of other phosphohydrolases in the sample. Specific activity was calculated as the difference between total activity and non-specific activity measured on control samples.

In all three species, we observed remarkably high specific G6Pase activity in the liver according to our expectations. Enzyme activity was first detected by measuring the glucose production. In general, we measured the highest activity in rat liver, followed by activity in guinea pigs and finally in human liver.

G6P-dependent G6Pase activity was also measured by the malachite green-molybdate method, which detects another end product of G6P

hydrolysis, phosphate. Measurement of phosphate production gave similar results for all three species, however, there was no significant difference in activity between species.

In order to test for the presence of the G6Pase system in the gut, G6P-dependent G6Pase activity was also measured on microsomes isolated from the gut of the same species. All experiments were performed by detecting both of the two end products, glucose and phosphate.

These measurements gave different results depending on the species from which the intestinal microsomes were derived. No specific G6Pase activity was measured in rats, and the hydrolytic cleavage of glucose-6-phosphate was entirely attributed to non-specific phosphatases. Although the specific activity was lower in the intestine compared to the liver in all species, significant G6Pase activity was detected in both human and guinea pig microsomes. The highest activity was measured in guinea pig intestine after the addition of G6P substrate by both methods.

The G6Pase system is intraluminally oriented in the small intestine

The intraluminal orientation of the G6Pase system was examined in guinea pig, rat, and human liver and small intestine microsome samples by the addition of M6P substrate. Under physiological conditions M6P is not a substrate for G6Pase, as the membrane is impermeable to this substrate and M6P is unable to cross native membranes. However, permeabilization of microsomal vesicles with the pore-forming agent

called alamethicin allows M6P to reach the enzyme, thereby making G6Pase enzyme activity measurable.

The total phosphohydrolase activity of G6Pase was latent in the experiment with microsomes of all three species after the addition of M6P substrate. Latencies above 90% were measured on guinea pig liver microsomes and rat liver microsomes, while 54% were measured on human microsomes. For intestinal microsomes, latency was 84% and 49% for guinea pig and human samples, respectively. These results further confirm that the microsomal vesicles used in the experiments had an intact membrane. The lower latency observed in the case of human microsomes may be explained by the fact that these samples were not freshly isolated microsomes. Activity measurements with rat intestinal microsomes further support the hypothesis that there is no G6Pase activity (for the addition of M6P substrate), i.e., hydrolysis of M6P by G6Pase was not detectable.

F6P-dependent glucose production in liver and intestine in different species

In the first set of experiments, glucose production from F6P was performed on liver microsomes, confirming previous results - specific, latent G6Pase-dependent glucose production was observed in both guinea pig and human liver microsomes. The same experiments were repeated with intestinal microsome samples. We also found that glucose production was detectable after F6P administration in guinea pig and

human intestinal microsomes, which was specific to G6Pase activity, and that the enzyme was latent on this substrate as well. F6P-dependent glucose production was lower than G6P-dependent, presumably due to the lower capacity of F6P transport or the limited capacity of the isomerization step. Comparing the two species, we can say that we measured higher activity in human liver and intestinal microsomes than in guinea pig microsomes.

Effect of fructose on 11β-HSD1 enzyme activity

It is already known from previous studies that the 11β-HSD1 enzyme activity increases in the presence of extracellular glucose, so we wondered whether similar changes can be observed with fructose. For this reason, we measured 11β-HSD1 oxidoreductase and dehydrogenase activity with the addition of different concentrations of fructose to the medium of HHH7 cells that stably coexpress 11β-HSD1 and H6PDH enzymes.

HHH7 cells were incubated in media containing 0; 0.1; 0.3; 1 and 4.5 g/l fructose. Based on our results, even a fructose concentration of 0.1 g/l proved to be sufficient to maintain the oxidoreductase activity of the enzyme (50% compared to a fructose concentration of 4.5 g/l).

The dehydrogenase activity of the enzyme can be calculated from the measurement of the amount of cortisone formed. This also showed a strong concentration dependence. In the absence of fructose, almost 80%

of the added cortisol was converted to cortisone. This conversion occurred below 20% at a fructose concentration of 4.5 g/l.

These results suggest that fructose contributes even more than glucose to NADPH production in the ER lumen.

Conclusions

1. G6Pase system activity is prominently present on human and guinea pig small intestinal microsomes and cannot be detected on rat intestinal microsomes.

2. Expression of the members of the system (G6PC, G6PT) is perfectly detectable at mRNA and protein levels in human and guinea pig intestines, however, it is significantly lower in rat intestinal samples.

3. Human and guinea pig microsomal G6Pase activity is intraluminal, and not detectable in case of M6P substrate.

4. The human and guinea pig intestinal G6Pase system can also utilize F6P as a substrate.

5. Fructose affects the activity of 11β-HSD1 *in vitro*, which has shifted from dehydrogenase to oxidoreductase activity due to increasing fructose concentration.

Publications

Publications related to the dissertation:

• Legeza B, Marcolongo P, Gamberucci A, **Varga V**, Banhegyi G, Benedetti A, Odermatt A. (2017) Fructose, Glucocorticoids and Adipose Tissue: Implications for the Metabolic Syndrome. Nutrients, 9. (IF: 4,196)

• **Varga V**, Muranyi Z, Kurucz A, Marcolongo P, Benedetti A, Banhegyi G, Margittai E. (2019) Species-Specific Glucose-6-Phosphatase Activity in the Small Intestine-Studies in Three Different Mammalian Models. Int J Mol Sci, 20. (IF: 4,556)

Publications independent of the dissertation:

• Shih CK, Chen CM, **Varga V**, Shih LC, Chen PR, Lo SF, Shyur LF, Li SC. (2020) White sweet potato ameliorates hyperglycemia and regenerates pancreatic islets in diabetic mice. Food Nutr Res, 64. (IF: 3,647)

• Shyur LF, **Varga V**, Chen CM, Mu SC, Chang YC, Li SC. (2021) Extract of white sweet potato tuber against TNF-alpha-induced insulin resistance by activating the PI3K / Akt pathway in C2C12 myotubes. Bot Stud, 62: 7 (IF: 2,163)