



Original Article

Age-related hepatic glucose-dependent insulintropic polypeptide expression is modified by ongoing thiamine supplementation in obese diabetic rats

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ABSTRACT — Obesity and type 2 diabetes mellitus have become worldwide epidemics. Evidence indicates that glucose-dependent insulintropic polypeptide (GIP) secreted by the intestines may partially underlie these conditions, considering that GIP levels are associated with lipid deposition and fat mass expansion. However, recent studies have found that GIP is also present in other tissues, such as the liver. Notably, one study discovered through microarray analyses of livers from obese diabetic rats that the transcriptional modulation of GIP also occurred in the liver. Otsuka Long-Evans Tokushima Fatty (OLETF) rats were chosen for this experiment because previous studies have shown that thiamine (vitamin B1) could successfully decrease the tendency of the animal toward obesity and mitigate the complications of diabetes. Here, the rats were randomly assigned to either the control (non-supplemented) or thiamine-supplemented (2 g thiamine/L in drinking water) groups. For this investigation, unlike that for young rats, OLETF rats were chosen for the experimental period at 93 weeks of age. Ageing is also a risk factor for diabetes and its complications. In this study, hepatic GIP expression was analysed using western blotting, suggesting that GIP was present in the livers of both obese diabetic OLETF rats and obese diabetic rats that received ongoing thiamine supplementation. Results showed that hepatic GIP expression had occurred and that liver-derived GIP may exist. Moreover, results showed that ongoing thiamine supplementation modified the hepatic GIP expression and prevented additional weight gain and complications arising from obesity and diabetes.

Key words: Ongoing thiamine supplementation, Hepatic GIP expression, Glucose toxicity, Obesity, Aged obese diabetic rat

INTRODUCTION

Glucose-dependent insulintropic polypeptide, also known as gastric inhibitory polypeptide (GIP), is a hormone that is secreted in response to nutrient ingestion that triggers insulin release from the pancreatic β -cells (Gautier *et al.*, 2005; Baggio and Drucker, 2007). During the past few decades, the epidemics of obesity and type 2 diabetes have skyrocketed worldwide (Ng *et al.*, 2014; WHO,

2016, 2017; Afshin *et al.*, 2017; Kohda, 2018). It has been conventionally believed that the synthesis of GIP occurs in the gastrointestinal tract (Gautier *et al.*, 2005; Baggio and Drucker, 2007) and that increased signaling contributes to the pathogenesis of obesity and type 2 diabetes (Getty-Kaushik *et al.*, 2006; Marks, 2006; Yip and Wolfe, 2000). However, recent discoveries have challenged this view because GIP and its receptor have been found in the retinas of diabetic rats (Cho *et al.*, 2002). Expression of

GIP in pancreatic β cells has been reported by *Fukami et al.* (2013). Notably, one study discovered through microarray analyses of livers from obese diabetic rats that the transcriptional modulation of GIP also occurred in the liver (Kohda *et al.*, 2012a; Tanaka *et al.*, 2010). These data collectively suggest that GIP is expressed in tissues beyond the gut and that the enhanced hepatic expression of GIP, in particular, appears to be involved in the modification of obesity-related diabetic complications (Kohda *et al.*, 2012a; Tanaka *et al.*, 2010).

Glucose toxicity is important in obesity and diabetes (Rossetti *et al.*, 1990; Giri *et al.*, 2018). Impaired glucose metabolism, decreased levels of thiamine (vitamin B1), and reduced activity of thiamine-dependent enzymes occur in obesity and diabetes (Lonsdale, 2018; Kerns and Gutierrez, 2017; Kerns *et al.*, 2015; Page *et al.*, 2011; Yui *et al.*, 1980; Seligmann *et al.*, 1991). In this study, it has been hypothesised that obesity and diabetes occur because the glucose that is absorbed by the cells is not completely metabolised; specifically, the metabolic syndrome should not develop if the cells completely metabolise glucose. Thiamine acts as a lubricant for carbohydrate metabolism, and the amount of catalytic thiamine that is absorbed must increase for glucose to be metabolised in large quantities. The current results showed that thiamine supplementation prevented excess weight gain and diabetes-related complications.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats were chosen for this experiment because previous studies have shown that thiamine could successfully decrease the tendency of the animal toward obesity and mitigate the complications of diabetes (Kohda *et al.*, 2012a; Tanaka *et al.*, 2010). Here, the rats were randomly assigned to either the control (non-supplemented) group or the thiamine-supplemented (2 g thiamine/L in drinking water) group. In this study, a long-term acclimation experiment was conducted for 1 year and 8 months, from the time the OLETF rats were 4 weeks old till they were 93 weeks old. The impact of ageing on the incidence of diabetes and the mechanisms by which older individuals are affected by diabetes were examined. In general, the risk of diabetes increases with age, with a rise in the risk in middle-aged and older adults (Mokdad *et al.*, 2000; GBD, 2017). Ageing is also a risk factor for the development of complications in people with diabetes (Lascar *et al.*, 2018). Type 2 diabetes is characterised by an individual's inability to use the insulin produced in the body, insulin resistance, and inadequate insulin to normalise the blood glucose level, with pancreatic β -cell dysfunction. Both abnormalities worsen with age and are influenced by environmental factors, such as body weight,

activity level, medications, and intercurrent illnesses. It is believed that the increase in type 2 diabetes incidence could be attributed to an increased incidence of obesity among the affected individuals. Thiamine supplementation has been studied as an important element that protects the body against the metabolic syndrome, age-related obesity and diabetes factors and diabetic complications (Kohda *et al.*, 2008, 2010, 2012a, 2012b; Tanaka *et al.*, 2010).

This study is performed to evaluate the GIP protein expression in the liver of obese and diabetic OLETF rats and explore its influence on the progression of these diseases. The liver is involved in glucose production; thus, maintaining a healthy liver is critical for individuals with diabetes or those at risk of developing diabetes. Moreover, obesity and diabetes are risk factors for non-alcoholic fatty liver disease (NAFLD) (Adams and Angulo, 2005; Adams, 2007; Moscatiello *et al.*, 2007; Lazo and Clark, 2008; Argo and Caldwell, 2009; Bellentani and Marino, 2009; Cusi, 2009; Arrese, 2010; Porepa *et al.*, 2010; Bellentani *et al.*, 2010). This study is expected to provide a better understanding of the role of GIP in the liver, as well as its contribution to the obese diabetic state in the liver.

MATERIALS AND METHODS

Chemicals

Thiamine hydrochloride was supplied by Kishida Chemical Co., Ltd. (Osaka, Japan). The following antibodies were used: anti-GIP, anti- β -actin, horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mammalian tissue lysis and extraction reagents were purchased from Sigma (St Louis, MO, USA). Protease inhibitor and phosphatase inhibitor cocktails were supplied by Nacalai Tesque (Kyoto, Japan). All the other chemicals used were of the highest purity and were procured from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Experimental design and blood pressure measurement

The animals were handled according to the institutional guidelines for animal research and the experimental work was approved by the Experimental Animal Research Committee of Osaka University of Pharmaceutical Sciences. OLETF rats that exhibited progressive obesity and metabolic disorders similar to those in the human metabolic syndrome were chosen for this study. Male OLETF rats (Japan SLC, Inc., Shizuoka, Japan) aged 4 weeks ($n = 8$) at the time of study initiation were used. The

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OLETF rats were housed in the animal facility in cages, received a standard diet, and had ad libitum access to water. They were kept under temperature- and humidity-controlled conditions with 12 hr light/dark cycles. OLETF rats were randomly allocated to each of the following drinking water groups: drinking thiamine water ($n = 4$) and drinking tap water ($n = 4$). The thiamine water-drinking group was administered drinking water supplemented with 2 g thiamine/L. Obese diabetic OLETF rats were supplemented with thiamine water and tap water from the time they were 4 weeks old till they were 93 weeks old.

The body weights of the OLETF rats, as an assessment of obesity, were measured throughout the duration of the study. Dermal lesions were macroscopically evaluated with each body weight measurement in obese diabetic rats. Systolic blood pressure, a non-invasive parameter of blood pressure, was monitored using the tail-cuff method. The caudal blood pressure of obese diabetic OLETF rats was measured using a tail-cuff blood pressure apparatus (BP-98A; Softron, Tokyo, Japan). Glucose levels were measured, as an assessment of diabetes, using the blood samples collected from the tail vein of the OLETF rats. Glucose levels were determined using a blood glucose test strip (Aventir Biotech, Carlsbad, CA, USA).

Blood and tissue sampling

The obese diabetic OLETF rats were anaesthetised with 50 mg/kg pentobarbital and sacrificed at 93 weeks of age. Blood samples were collected from the ventral aorta and deposited in heparin tubes. In addition, plasma was separated from whole blood via centrifugation using a refrigerated bench-top centrifuge (Kubota Corp., Tokyo, Japan), and plasma aliquots were stored at -80°C until further analysis. Plasma triglyceride levels were measured using triglyceride test kits. The obese diabetic OLETF rats were exsanguinated and, afterward, the liver, epididymal fat, and retroperitoneal fat samples were collected, rinsed in ice-cold saline, briefly blotted with paper, and weighed.

Preparation of the protein extraction solution from rat livers and Western blot analysis

A liver tissue weight of 300 mg was homogenised at 4°C in 450 μL tissue lysis and extraction reagent with a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Homogenates were centrifuged at 15,000 rpm for 15 min and the supernatants were used for western blot analysis to examine GIP protein expression.

Protein samples were separated by 10%-20% gradient SDS-polyacrylamide gel electrophoresis (SDS-

PAGE). Proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 0.3% skimmed milk in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (TBST) for 1 hr at room temperature. Thereafter, the membranes were incubated with the specific primary antibodies anti-GIP and anti- β -actin in TBST overnight at 4°C . The membranes were washed three-fold in TBST to remove the unbound antibodies. They were then incubated with HRP-conjugated secondary antibody in TBST for 1 hr at room temperature. Chemiluminescence was detected using a LAS-3000 machine (Fuji Film Corp., Tokyo, Japan) with an enhanced chemiluminescence reagent (Chemi-Lumi One; Nacalai Tesque). The expression of β -actin was used as an internal standard.

Statistical analyses

Data are expressed as mean \pm S.E. Group comparisons were performed using a two-tailed Student's *t*-test. All statistical analyses were performed using the Pharmaco Basic software (Scientist Press Co., Ltd., Tokyo, Japan). A *p*-value < 0.05 was considered to indicate statistical significance.

RESULTS

Effect of ongoing thiamine supplementation on the dermal lesion and lifespan in obese diabetic OLETF rats

Dermal lesions were macroscopically evaluated with each body weight measurement. In obese diabetic OLETF rats, severe dermal lesions were observed at 93 weeks of age (Fig. 1A), while there were no or only mild dermal lesions in the thiamine-supplemented group (Fig. 1B). In this study, a long-term acclimation experiment was conducted on OLETF rats for 1 year and 8 months from the time they were 4 weeks old till they were 93 weeks old. The survival rates at 93 weeks of age in the control ($n = 4$) and thiamine-treated ($n = 4$) groups consisting of obese diabetic OLETF rats were 50% (control group: $n = 2$) and 100% (thiamine-supplemented group: $n = 4$), respectively. Therefore, thiamine supplementation may influence dermal lesions and lifespan.

Effect of ongoing thiamine supplementation on body weight, systolic blood pressure, organ weight levels, and metabolic parameters in obese diabetic OLETF rats

At the start of thiamine supplementation (4 weeks of age), the OLETF rats had similar body weights. At

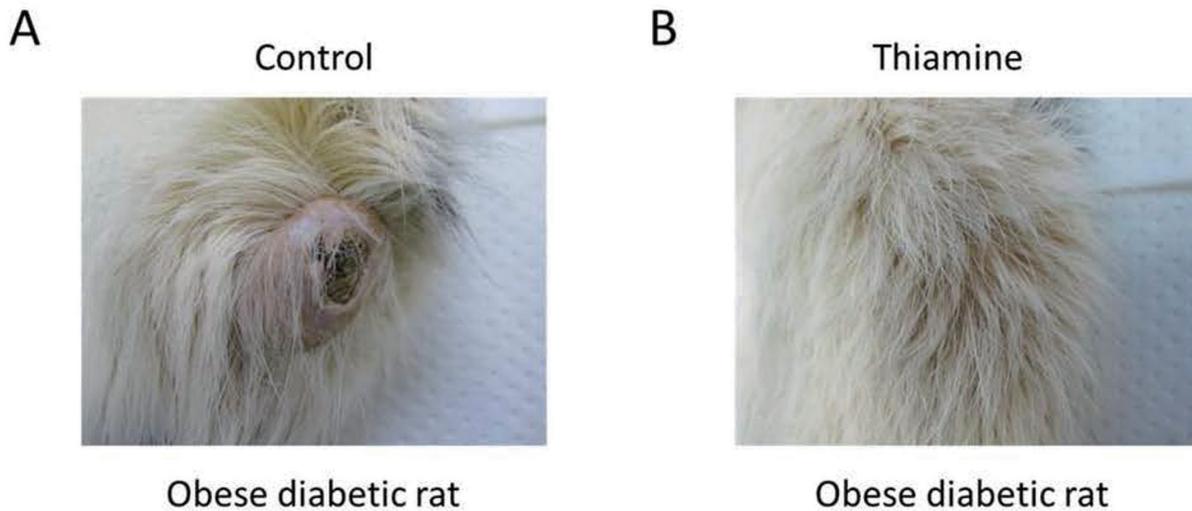


Fig. 1. Effect of ongoing thiamine supplementation on dermal lesion at 93 weeks of age in obese diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. A long-term acclimation experiment was performed on OLETF rats from the time they were 4 weeks old till they were 93 weeks of age. OLETF rats were randomly divided into the following groups: tap water-drinking group (control obese diabetic OLETF rats, $n = 2/4$) and 0.2% thiamine water-drinking group (thiamine-supplemented obese diabetic OLETF rats, $n = 4$). Dermal lesions in obese diabetic control rats (A) and thiamine-supplemented obese diabetic OLETF rats (B) were macroscopically evaluated with each body weight measurement.

93 weeks of age, the body weights were progressing as expected, being increased in the obese diabetic OLETF rats. The body weights of rats in the thiamine-supplemented obese diabetic OLETF rat group were lower than those of rats in the non-supplemented control obese diabetic OLETF rat group (Fig. 2A). Systolic BP in the thiamine-supplemented obese diabetic OLETF rat group was significantly lower than that in the control obese diabetic OLETF rat group (Fig. 2B). Organ weight, epididymal fat weight, retroperitoneal fat, and liver weight in obese diabetic OLETF rats with thiamine supplementation were lower than those in the non-supplemented control obese diabetic OLETF rat group (Fig. 2C, 2D, 2E). Blood glucose levels and plasma triglyceride levels increased in the non-supplemented control obese diabetic OLETF rat group (Fig. 2F and 2G). These levels were significantly lower in the thiamine-supplemented OLETF group than in the non-supplemented control obese diabetic OLETF rat group (Fig. 2F and 2G).

Effect of ongoing thiamine supplementation on the hepatic GIP expression in obese diabetic OLETF rats

The present study clearly showed the localisation of GIP in the livers of obese diabetic OLETF rats (Fig. 3). Hepatic GIP expression was assessed using western blot-

ting analysis. An anti-GIP antibody-positive band indicated that hepatic GIP was detected in both non-supplemented obese diabetic OLETF rats and in obese diabetic OLETF rats that were receiving thiamine supplementation (Fig. 3A). In this study, obese diabetic OLETF rats showed a marked increase in hepatic GIP expression (Fig. 3A). However, at the age of 93 weeks, obese diabetic OLETF rats who were being supplemented with thiamine tended to have lower GIP expression than those who were not supplemented with thiamine (Fig. 3A). The expression of β -actin in the liver was shown as an internal standard (Fig. 3B).

DISCUSSION

The current study reports a new concept related to GIP expression in the liver of obese diabetic rats. Evidence indicates that GIP secreted by the intestines may partially underlie these conditions, considering that GIP levels are associated with lipid deposition and fat mass expansion (Baggio and Drucker, 2007; Marks, 2006; Yip and Wolfe, 2000). However, a recent trial has shown that GIP is also present in other tissues, such as the liver (Kohda *et al.*, 2012a, 2016, 2017). The present study clearly demonstrated the localisation of GIP in the livers of obese diabetic rats. Moreover, a previous trial has shown that the

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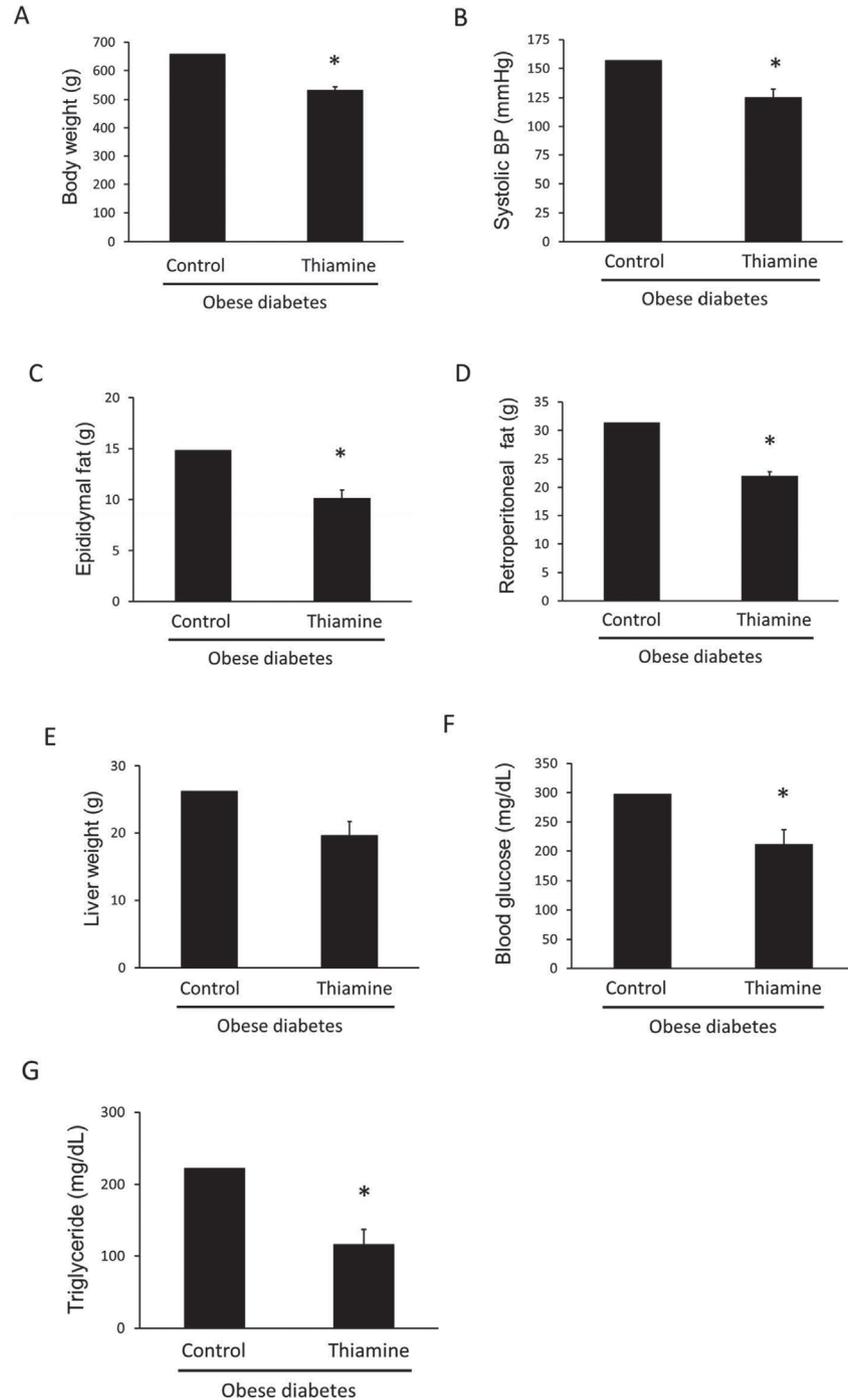


Fig. 2. Effect of ongoing thiamine supplementation on body weight (A), systolic blood pressure (systolic BP) (B), epididymal fat weight (C), retroperitoneal fat weight (D), liver weight (E), blood glucose (F), and plasma triglyceride levels (G) in 93-week-old obese diabetic Otsuka Long–Evans Tokushima Fatty (OLETF) rats. OLETF rats were randomly divided into the following groups: tap water-drinking group (control obese diabetic OLETF rats, $n = 2/4$) and 0.2% thiamine water-drinking group (thiamine-supplemented obese diabetic OLETF rats, $n = 4$). Significant differences were observed between the control and thiamine groups at 93 weeks of age in obese diabetic rats. Each value represents the mean \pm S.E. values. * $p < 0.05$ compared to the obese diabetes control group at 93 weeks of age.

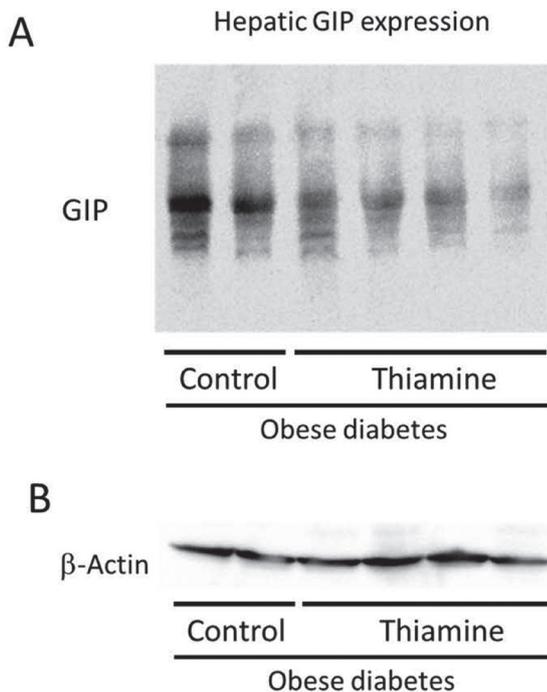


Fig. 3. Glucose-dependent insulintropic polypeptide (GIP) expression of the liver in control Otsuka Long-Evans Tokushima Fatty (OLETF; Obese diabetic) rats ($n = 2$) and thiamine-supplemented Obese diabetic OLETF rats ($n = 4$) at 93 weeks of age. Liver samples were subjected to SDS-PAGE followed by western blot analysis with a specific antibody against GIP (A) and β -actin (B). Thiamine-supplemented groups received 0.2% thiamine in the drinking water from the time they were 4 weeks old till they were 93 weeks old.

transcriptomic profiling data that revealed the modified expression of hepatic GIP after thiamine (vitamin B1) supplementation was correlated with further adiposity in obese diabetic rats (Kohda *et al.*, 2012a; Tanaka *et al.*, 2010). In this study, hepatic GIP expression was analysed by western blotting, suggesting that GIP was detected in both non-supplemented obese diabetic OLETF rat livers and in obese diabetic OLETF rat livers that received ongoing thiamine supplementation.

For this investigation, unlike that for the young rats, OLETF rats were chosen for the experimental period at 93 weeks of age. Ageing is also a risk factor for diabetes and its complications (Mokdad *et al.*, 2000; GBD, 2017; Lascar *et al.*, 2018). The current study advanced these findings with ongoing thiamine supplementation to obese diabetic OLETF rats until they were 93 weeks old in order to characterise the GIP expression in the liver

and assess its effect on obesity and diabetes with ageing. Results showed that ongoing thiamine supplementation modified hepatic GIP expression and prevented additional weight gain and complications arising from obesity and diabetes.

Body weight is a parameter of obesity. In this study, body weight gain was > 20% lower in the ongoing thiamine-supplemented OLETF rat group as compared to that in the control group, which was not supplemented with thiamine. Thiamine supplementation improved the other obesity parameters, including epididymal fat weight, retroperitoneal fat weight, blood glucose levels, and triglyceride levels. Changes in life expectancy of mature rats are reportedly related to the weight level that is eventually achieved, either via gain or loss in body weight (Ross, 1972, 1961). It is suggested that for every 10% reduction in body weight, there was a 13.5% gain in life expectancy (Ross, 1972, 1961). In contrast, for every 10% gain in body weight, there was a 13.5% reduction in life expectancy (Ross, 1972, 1961). As shown in Figs. 1 and 2, thiamine supplementation improved the condition of OLETF rats in terms of dermal lesions, obesity, and diabetes. In this study, the survival rates at 93 weeks of age in the control and thiamine-supplemented groups, consisting of obese diabetic rats, were 50% and 100%, respectively. Thiamine supplementation may influence dermal lesions and lifespan. In fact, as previously reported, it has been shown that benfotiamin, a derivative of thiamine, increased the lifespan in mice (Tapias *et al.*, 2018). Although preliminary data, ongoing thiamine supplementation might tend to increase not only the lifespan but also the healthspan.

In this study, as shown in Fig. 3, aged obese diabetic rats had a significantly increased hepatic GIP expression. The current results could be suggestive considering the possible involvement of age-related hepatic GIP expression in 93 weeks old obese diabetic rats. It is noteworthy that, as shown in Fig. 3, at the age of 93 weeks, obese diabetic rats with ongoing thiamine supplementation tended to have decreased GIP expression. However, results showed that hepatic GIP expression had occurred and that liver-derived GIP may exist. In fact, as previously reported, it has been shown that GIP expression in the liver of diabetic rats at 55 weeks of age was higher in thiamine-supplemented rats than that in control obese diabetic rats (Kohda *et al.*, 2017). This is in accordance with previous findings that hepatic GIP expression might be prolonged during the ongoing thiamine supplementation period in an obese diabetic state of the liver. Prolonged GIP receptor activation has been reported to improve glucose homeostasis (Kerr *et al.*, 2009). However, it has been

suggested that the absence of GIP secretion alleviates age-related obesity and insulin resistance (Kanemaru *et al.*, 2020). Increased GIP signaling has been suggested to promote fat accumulation (Marks, 2006). Hence, GIP has been described as the obesity hormone and may contribute to the pathogenesis of type 2 diabetes (Yip and Wolfe, 2000; Getty-Kaushik *et al.*, 2006). However, GIP potentiates glucose-dependent insulin secretion and enhances β -cell mass via the regulation of β -cell proliferation, neogenesis and apoptosis (Kim *et al.*, 2005). As previously reported, GIP activates lipoprotein lipase (Kim *et al.*, 2007, 2012), suggesting that GIP expressed in the liver may be involved in glycolipid metabolism. Incretin hormone, such as glucagon-like peptide-1 and GIP, receptor signaling exerts physiologically relevant actions critical for glucose homeostasis and represents a pharmacologically attractive target for the development of therapeutic agents for diabetes (Gault *et al.*, 2003; Cernea, 2011; Irwin and Flatt, 2015). In the current study, at the age of 93 weeks, obese diabetic OLETF rats showed increased age-specific GIP expression that is modified by ongoing thiamine supplementation. These results, contradictory to the suggested role of hepatic GIP in obesity and diabetes, require further study.

Ageing is associated with a progressive worsening of glucose tolerance and a compromised ability of pancreatic β -cells to secrete insulin in response to glucose (Mokdad *et al.*, 2000; Lascar *et al.*, 2018). Obesity is related to a decline in the activities of daily living in elderly people and is related to a high prevalence of medical disorders, such as diabetes, hyperlipidaemia, and hypertension. Thus, it is important for elderly people to prevent excessive fat accumulation. Obesity and diabetes are associated with an increased risk of liver disease (Adams and Angulo, 2005; Adams, 2007; Moscatiello *et al.*, 2007; Lazo and Clark, 2008; Argo and Caldwell, 2009; Bellentani and Marino, 2009; Cusi, 2009; Arrese, 2010; Porepa *et al.*, 2010; Bellentani *et al.*, 2010). It is possible that public health strategies and clinical interventions targeting obesity and diabetes may improve the health of patients with NAFLD and other chronic liver diseases. The current study indicates the concept related to hepatic GIP expression in the liver of obese diabetic rats. Future studies should elucidate the regulatory mechanism of GIP expression in the liver and the effects of GIP signaling on obesity, diabetic complications, and, especially, NAFLD.

Liver health is especially critical for effective diabetes management. The liver is a storage organ for blood-stream components, including glucose. If the liver cells do not respond well to insulin, they will start pumping out glucose, worsening blood glucose control. Maintain-

ing the health of the liver implies avoiding the release of glycolipid-toxic substances. Both diabetes and obesity are metabolic syndrome components and are risk factors for NAFLD (Adams and Angulo, 2005; Adams, 2007; Moscatiello *et al.*, 2007; Lazo and Clark, 2008; Argo and Caldwell, 2009; Bellentani and Marino, 2009; Cusi, 2009; Arrese, 2010; Porepa *et al.*, 2010; Bellentani *et al.*, 2010). In future studies, the assessment of the biomedical role of GIP signaling in the liver of humans will help establish successful diabetes treatments. Moreover, elucidating the mechanisms underlying the activity of not only age-specific hepatic GIP expression, but also ongoing thiamine supplementation-related hepatic GIP expression, is necessary in order to obtain a better understanding of how it influences the obese and diabetic states. Future studies need to set forth the biomedical roles that liver-derived GIP signaling has on the liver in obese diabetic rats.

Conflict of interest---- The authors declare that there is no conflict of interest.

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