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ARTICLE

Nutritional strategy to prevent fatty liver and insulin resistance independent of obesity by reducing glucose-dependent insulinotropic polypeptide responses in mice

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Abstract
Aims/hypothesis High intake of carbohydrates, particularly sucrose, in western societies is associated with the development of non-alcoholic fatty liver (NAFL) and diabetes mellitus. It is unclear whether this is related primarily to the carbohydrate quantity or to the hormonal responses, particularly glucose-dependent insulinotropic polypeptide (GIP), which is released in the proximal intestine. Therefore, we investigated the role of GIP by comparing two glucose–fructose dimers, sucrose and Palatinose (isomaltulose), resorbed proximally or distally.
Methods The glycaemic and incretin responses to sucrose and Palatinose were studied by oral gavage and meal tests. We then analysed phenotypic and metabolic diet-induced changes in C57Bl/6J mice exposed to isoenergetic diets differing in carbohydrate type. Studies were repeated in GIP receptor knockout (*Gipr*$^{-/-}$) mice and their wild-type littermates.
Results Compared with sucrose, Palatinose intake resulted in slower glucose absorption and reduced postprandial insulin and GIP levels. After 22 weeks, Palatinose feeding prevented hepatic steatosis (48.5%) compared with sucrose and improved glucose tolerance, without differences in body composition and food intake. Ablation of GIP signalling in *Gipr*$^{-/-}$ mice completely prevented the deleterious metabolic effects of sucrose feeding. Furthermore, our microarray analysis indicated that sucrose increased 2.3-fold the hepatic expression of *Socs2*, which is involved in the growth hormone signalling pathway and participates in the development of NAFL.
Conclusions/interpretation Our results suggest that the site of glucose absorption and the GIP response determine liver fat accumulation and insulin resistance. GIP may play a role in sucrose induced fatty liver by regulating the expression of *Socs2*.

Dr Frank Isken, who contributed to this research, passed away on 4 October 2010 prior to the publication of this work.

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Keywords Fatty liver · GIP response · Palatinose

Abbreviations

ALT	Alanine aminotransferase
BW	Body weight
FI	Food intake
FL	Fatty liver
GH	Growth hormone
GI	Glycaemic index
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide 1
GTT	Glucose tolerance test
HFD	High fat diet
IR	Insulin resistance
NAFL	Non-alcoholic fatty liver
SOCS2	Suppressor of cytokine signalling 2
TG	Triacylglycerol
TEE	Total energy expenditure

Introduction

Non-alcoholic fatty liver (NAFL) affects up to 30% of adults and up to 10% of children in developed countries [1]. The disease is characterised by an excessive accumulation of lipids, mainly triacylglycerol (TG), in the liver [2]. NAFL is considered the hepatic feature of metabolic syndrome and is associated with numerous diseases, including insulin resistance (IR) and type 2 diabetes [3, 4]. The most common cause of NAFL can likely be attributed to an exaggerated intake of dietary energy, especially carbohydrates, inducing a strong insulin response. Therefore, dietary components capable of decreasing postprandial glucose and insulin levels are promising approaches to reduce the development of NAFL. Recently, we observed that acarbose, an α-glucosidase inhibitor, reduced liver fat by attenuating the release of glucose in the gut [5].

The glycaemic response to various carbohydrates is dependent on the rate of their digestion and absorption [6]. Studies indicate that the intake of rapidly digestible sugars such as sucrose, known as high glycaemic index (GI) sugars, as compared with Palatinose (isomaltulose), a slowly and completely resorbed sucrose analogue composed of α-1,6-linked glucose and fructose, has deleterious effects on postprandial glucose, insulin and TG levels, which are associated with the risk of obesity, IR and fatty liver (FL) [7, 8]. We reported previously that mice fed a high-GI diet showed a rapid onset and marked increase in body fat mass, liver fat and hepatic lipogenesis [9].

Delaying carbohydrate absorption in the gut reduces the secretion of the gut hormone glucose-dependent insulinotropic polypeptide (GIP) from intestinal K cells and attenuates glucose appearance in the blood [10]. GIP is an incretin that is synthetised and released from the duodenum and proximal jejunum in response to fat, carbohydrate and protein [11]. Increased postprandial GIP responses are associated with obesity and the severity of liver diseases [12, 13]. Moreover, GIP was recently linked to unfavourable effects of diet-induced FL in animal models [14]. However, previous studies were hampered by the development of obesity in high-GI-fed animals such that the consequences of obesity were not separable from the effects of high-GI foods and the release of GIP [6, 15, 16].

Muscle fatty acid uptake and oxidation are facilitated by fatty acid transporters, which play a role in metabolic flexibility by altering cellular and mitochondrial fat uptake and oxidation and, therefore, are a potential target of incretins.

Suppressor of cytokine signalling 2 (SOCS2) coordinates the complex interplay between inflammation, growth hormone (GH) action and nutritional factors, and integrates the glucose and lipid responses in the liver [17]. Recently, it was shown that *Socs2*$^{-/-}$ mice are protected from diet-induced FL [18]. Therefore, nutrients capable of reducing GH and SOCS2 action could be used to prevent or treat NAFL and its related disorders.

In the present study, we investigated the effects of distinct dietary sugars with different absorption rates in the gut, namely Palatinose and sucrose, on stimulating GIP release and the progression to IR and FL. To determine the role of GIP in diet-induced FL and impaired glucose homeostasis, we performed a long-term diet intervention in GIP receptor knockout (*Gipr*$^{-/-}$) mice and their wild-type (WT) littermates. Finally, molecular mechanisms were investigated by unbiased microarray analysis and confirmed by quantitative PCR.

Methods

Animals Experimental protocols were approved by the local governmental animal ethical committee in the State of Brandenburg, Germany. Experiments were performed in 18-week-old male C57Bl/6J mice (Janvier Labs, Saint Berthevin, France), unless otherwise stated. Mice were housed in individual cages with free access to water and standard rodent chow, with a 12:12 h light–dark cycle and a temperature of 23±2°C. Mice were allowed a 1-week acclimatisation period before starting the experiments. *Gipr*$^{-/-}$ mice on a C57Bl/6J strain background were generated as previously described [19]. In order to explant organs, overnight fasted mice were sedated using isoflurane (Baxter, Unterschleissheim, Germany) and killed by cervical dislocation. Organs were isolated rapidly, snap frozen in liquid nitrogen and kept at −80°C for RNA isolation.

Oral administration of sugars Sugars were administered orally as described in the electronic supplementary material (ESM).

Feeding test In a separate set of animals, mice were trained for 4 days to consume either a Palatinose- or a sucrose-containing diet as detailed previously [9]. Briefly, individually housed mice were given 500 mg of the experimental diet following an overnight fast. Blood samples from the tail vein were drawn at 0 (overnight fasted), 30, 60, 90 and 120 min of consuming the whole portion of test meals within 15 min.

Both interventional diets were isoenergetic and contained 40.5% (wt/wt) carbohydrate, 41.5% (wt/wt) fat and 18% (wt/wt) protein (Table 1).

Dietary intervention Body weight (BW) matched mice were fed the above diets for 22 weeks. To elucidate the role of GIP, another long-term experiment was performed in BW-matched *Gipr*$^{-/-}$ and WT mice fed the aforementioned diets.

Body composition Body fat and lean mass were measured before the experiment and at indicated times using nuclear magnetic resonance spectroscopy (Mini Spect MQ10 NMR Analyser Bruker, Karlsruhe, Germany). Throughout the diet interventions, BW was determined once per week.

Table 1 Macronutrient composition of the experimental diets

Composition	Sucrose	Palatinose
Diet composition, g/kg		
Sucrose[a]	400	–
Palatinose [b]	–	440
Casein[c]	200	200
Safflower oil[d]	10	10
Linseed oil[e]	10	10
Coconut oil[f]	170	170
Cellulose[g]	100	100
Mineral mixture[h]	50	50
Vitamin mixture[h]	50	50
Macronutrient, metabolisable energy, %		
Protein	18	18
Carbohydrate	40.5	40.5
Fats	41.5	41.5
Measured diet energy content, kJ/g (kcal/g)	20.20 (4.83)	20.14 (4.81)

[a] Pfeifer & Langen, Köln, Germany
[b] Beneo Palatinit, Mannheim, Germany
[c] Dauermilchwerk Peiting, Landshut, Germany
[d] Kaufland Warenhandel, Neckarsulm, Germany
[e] Kunella-Feinkost, Cottbus, Germany
[f] Kölln KGaA, Elmshorn, Germany
[g] JRS Pharma, Rosenberg, Germany
[h] Altromin, Lage, Germany

Digestibility of diets Food intake (FI) was measured weekly and expressed as grams per week. The metabolisable energy intake was calculated according to the weekly FI and 1-week faecal samples. After drying, the energy content of diet and faeces samples were determined as previously explained [14].

Hydrogen breath test as a biomarker of colonic fermentation A hydrogen exhalation test was performed as previously described [20] in samples, which were collected in duplicates in the fed state.

Liver TG and glycogen quantification These variables are described in detail in the ESM.

Indirect calorimetry Daily total energy expenditure (TEE) was estimated as described previously [14]. Oxygen consumption and CO_2 production were determined to calculate RQ by dividing $\dot{V}CO_2$ by $\dot{V}O_2$. TEE is expressed per metabolic body mass and, therefore, allows comparison between groups.

Plasma analysis Retro-orbital blood samples were collected for evaluation of plasma glucose, TG, fructoseamine, alanine aminotransferase (ALT) and NEFA using a commercial kit (glucose: HKCP; TG: Fructo Cal; ALT CP: ABX Pentra, Montpellier, France). Each variable was measured by an autoanalyser (Cobas Mira S, Hoffmann-La Roche, Basel, Switzerland). Plasma insulin levels were measured by ELISA as described elsewhere [9]. GH was assessed using a commercial ELISA kit for rat/mouse GH (EMD Millipore, St Charles, MO, USA).

Plasma resistin and leptin levels were measured by a commercial mouse Milliplex kit based on multiplex technology (Millipore, Billerica, MA, USA). Adiponectin levels were measured using the Milliplex$_{MAP}$ mouse adiponectin single-plex panel (Millipore). All measurements were performed on a Luminex 200 system (Luminex, Austin, TX, USA) in 96-well format as previously explained [21].

Incretin measurement Plasma GIP levels were quantified using a rat/mouse total GIP ELISA kit (EMD Millipore). Blood samples were collected in tubes containing heparin lithium (Sigma-Aldrich, St Louis, MO, USA). Levels of plasma glucagon like peptide-1 (GLP-1) were determined by the GLP-1 (active) ELISA kit (Shibayagi, Gunma, Japan). Recombinant GLP-1 (7-36) was used as the standard. Blood samples were collected in tubes containing EDTA-Na2 (Sigma-Aldrich) and aprotinin (Carl Roth, Karlsruhe, Germany). To avoid the degradation of GLP-1, a DPP-IV inhibitor (EMD Millipore) was added to the plasma samples. All samples were stored at −80°C until assay.

Glucose tolerance test A glucose tolerance test (GTT) was performed by i.p. glucose (2 g/kg BW) injection after overnight fasting. Plasma samples for glucose and insulin measurements were collected before and at 10, 30, 60 and 120 min after glucose challenge.

RNA extraction and quantitative RT-PCR Total RNA was purified and quantified from liver and gastrocnemius muscle tissue samples as described [9]. Quantitative RT-PCR was performed using ABI Prism 7900 HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The quantity of target and the housekeeping gene (*Hprt*) were calculated according to a standard curve. Primer sequences are listed in ESM Table 1.

Microarray analysis The quantity and quality of liver RNA were assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Total RNA (300 ng) was amplified using the Illumina TotalPrep RNA Amplification kit (Ambion, Carlsbad, CA, USA). Amplified cRNA was hybridised to Mouse Ref-8 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA). Staining and scanning were done according to the Illumina expression protocol. Transcriptome analyses were performed by the statistical programming environment R implemented in CARMAweb (https://carmaweb.genome.tugraz.at; version 1.5.13) [22]. Genewise testing for differential expression was done using the Limma *t* test (Linear Models for Microarray Data, www.bioconductor.org/packages/release/bioc/html/limma.html; version 3.4.0) and Benjamini–Hochberg multiple testing corrections. Pathway enrichment analyses were done with the Ingenuity pathway software (Qiagen, Hilden, Germany). Array data were submitted to the Gene Expression Omnibus (GSE54723).

Data analysis Data were analysed using IBM SPSS statistics 20 (SPSS, Chicago, IL, USA). Comparisons between two groups were performed using unpaired Student's *t* test. Multiple comparisons were tested by one-way ANOVA, followed by post hoc Tukey or Games–Howell tests according to the homogeneity of variances (Levene's test). Statistically significant effects of genotype and diet were determined using two-way ANOVA. The AUC was calculated by the trapezoid rule. Statistical significance was defined as $p<0.05$. Results are presented as mean±SEM.

Results

Glucose homeostasis and incretin response to sucrose and Palatinose Oral challenge with the experimental sugars revealed that exposure to sucrose solutions induced a remarkable acute increase in glucose, insulin and GIP levels, without effects on GLP-1 secretion, which were avoided with Palatinose (ESM Fig. 1a–h).

To resemble normal eating we performed food training tests. Acute intake of diets (Table 1) with Palatinose or sucrose showed similar patterns in regulating fasting and postprandial glucose levels (Fig. 1a, b). Fasting insulin levels were significantly higher in sucrose-fed compared with Palatinose-fed mice ($p<0.01$), indicating a compensatory effect of insulin upon sucrose feeding to maintain similar glucose levels. Feeding challenge resulted in increased insulin release in both groups, which was significantly greater at 30 min in sucrose-fed compared with Palatinose-fed animals ($p<0.05$; Fig. 1c). $\text{Insulin}_{\text{AUC}}$ was significantly different between both groups ($p<0.01$; Fig. 1d). Despite comparable fasting values, the GIP response was 12-fold higher after 30 min ingestion of sucrose compared with Palatinose ($p<0.001$), and the response remained significantly elevated over 120 min of test

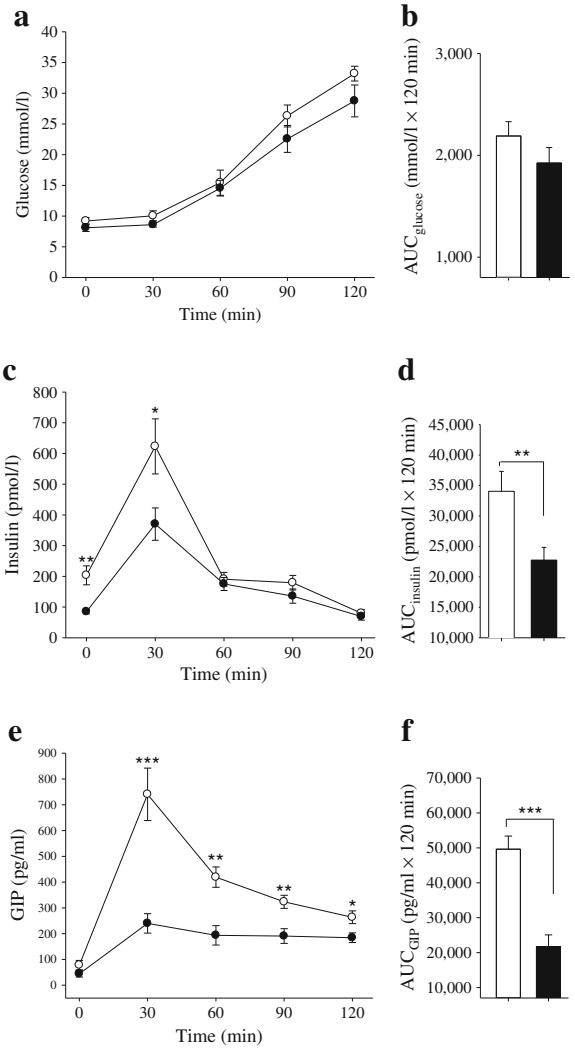

Fig. 1 Effects of sucrose (white circles and bars) and Palatinose (black circles and bars) in meal tests on plasma levels and AUCs of (**a**, **b**) glucose, (**c**, **d**) insulin and (**e**, **f**) GIP. $n=8$ per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$; for parts (**c**) and (**e**), p values are vs Palatinose

(Fig. 1e). The dramatic differences are also evident in the GIP_{AUC} ($p<0.001$; Fig. 1f).

Palatinose and sucrose diets resulted in comparable body composition and energy intake Ad libitum access to diets containing Palatinose and sucrose resulted in similar BW over the experimental period (Fig. 2a). Body composition analysis revealed that body fat and lean mass were comparable between the two groups (Fig. 2c, d). Weekly food consumption and even cumulative intake were similar in Palatinose- and sucrose-fed mice (Fig. 2b). Importantly, energy intake did not differ between the groups throughout the study (Table 2). Similar colonic fermentation rates, estimated at week 12, indicated complete resorption of both sugars ($p=0.08$; Fig. 2e).

RQ and TEE at week 10 exhibited circadian rhythmicity in energy expenditure, with no significant differences in either sucrose- or Palatinose-fed groups (Fig. 2f, g). Therefore, the metabolic and phenotypic alterations described below are not related to obesity, FI or circadian rhythms.

Similar digestibility of the diets For further analysis of digestibility of the diets, faecal excretion and FI were recorded at week 7. The energy content of the respective diets was comparable between groups (Palatinose, 20.14 kJ/g; sucrose, 20.2 kJ/g [Table 1]). Accordingly, faecal energy excretion was not significantly different between the groups. Digestibility of the diets was 89% with sucrose and 88.5% with Palatinose, indicating similar digested energy (Table 2).

Palatinose feeding reduced hepatic lipid accumulation and improved glucose metabolism After a 22-week dietary intervention, liver TG levels were nearly 2-fold lower in Palatinose-fed vs sucrose-fed mice ($p<0.01$; Fig. 3a). In agreement with this finding, plasma ALT levels were also lower in Palatinose-fed mice ($p<0.05$; Fig. 3c). Liver

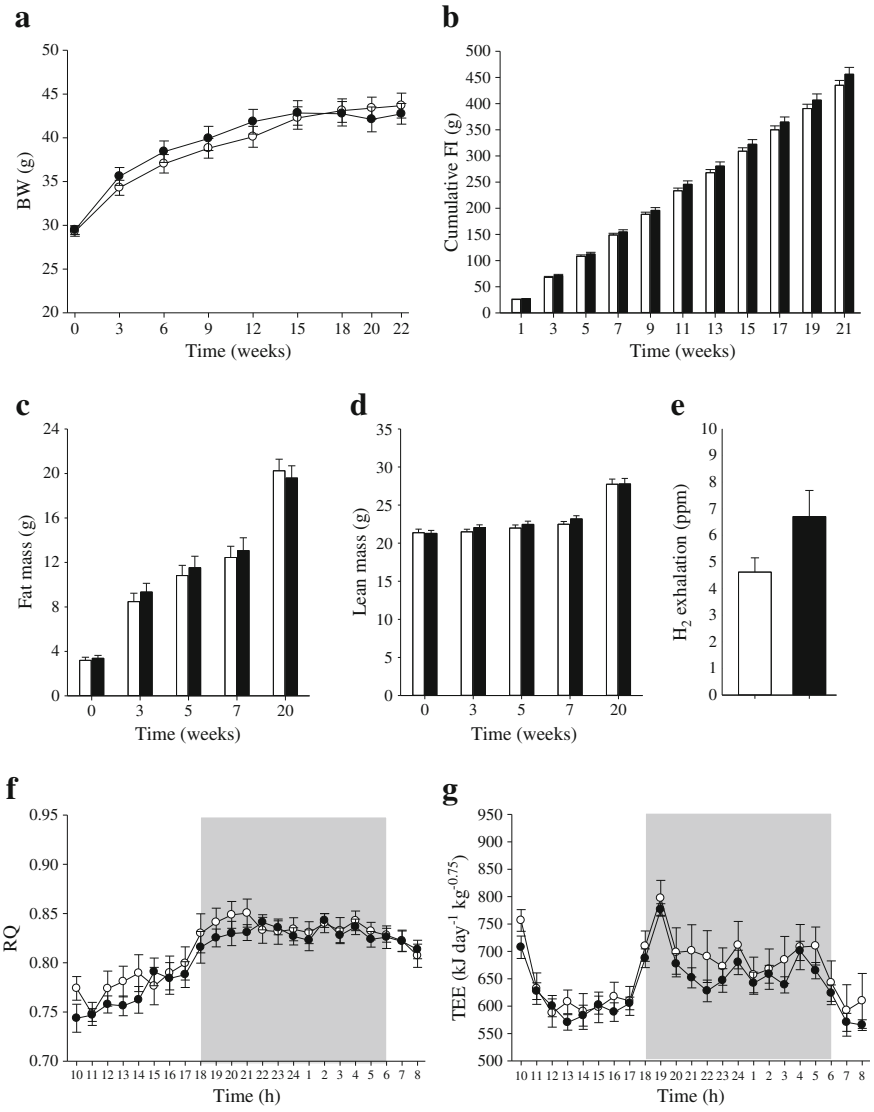

Fig. 2 Isoenergetic diets containing Palatinose (black circles and bars) and sucrose (white circles and bars) reveal no differences in (**a**) BW, (**b**) cumulative FI, (**c**, **d**) body fat and lean mass, (**e**) hydrogen breath test, (**f**) RQ and (**g**) TEE. Shaded area represents dark phase. $n=10$–12 per group

Table 2 Energy variables per week (exemplary week 7)

	Sucrose	Palatinose	*p* value
FI, g	21.14±0.3	21.38±0.5	0.72
Energy intake, KJ	427.06±7.3	430.66±11.4	0.81
Faeces weight, g	3.27±0.08	3.37±0.1	0.43
Faeces energy loss, KJ	46.95±1.1	49.67±1.4	0.152
Digestible energy intake, KJ	380.11±6.4	380.9±10.1	0.975
Digestible diet energy, %	89±0.16	88.5±0.15	0.02

Values are mean±SEM

glycogen content did not differ between Palatinose and sucrose challenge (p=0.4; Fig. 3b). The dietary intervention did not result in significant changes in terms of plasma TG, NEFA, fructoseamine and insulin levels (Table 3). However, postprandial glucose levels were significantly higher in sucrose-fed mice (p<0.01; Table 3).

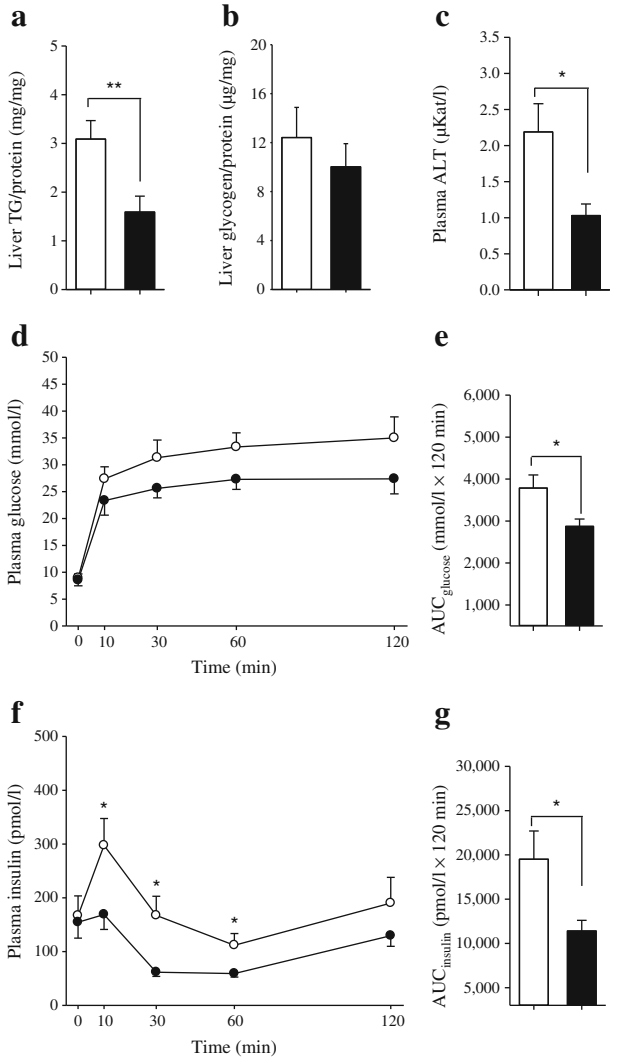

Fig. 3 Effects of long-term diet intervention on (**a**) liver TG, (**b**) liver glycogen and (**c**) plasma ALT and markers of glucose metabolism by i.p. GTT as shown on plasma and AUCs for (**d**, **e**) glucose and (**f**, **g**) insulin. White circles and bars: sucrose; black circles and bars: Palatinose. n=8–10 per group. *p<0.05, **p<0.01; for part (**f**) p values are vs Palatinose

Table 3 Assessment of plasma metabolic variables in sucrose- and Palatinose-fed animals

Variable	Sucrose	Palatinose
Fructoseamine (µmol/l)	203.82±5.9	205.73±5.74
TG (mmol/l)	1.89±0.21	2.09±0.17
NEFA (mmol/l)	1.12±0.03	1.08±0.04
Glucose (mmol/l)	10.79±0.3	9.49±0.27 **
Insulin (pmol/l)	1,024.04±154.13	1,283.98±149.08

Blood samples were taken after 22 weeks of diet intervention in the fed state

Values are mean±SEM. **p<0.01

Additionally, we assessed glucose/insulin homeostasis following a 15-week dietary intervention. The findings revealed a significant impairment of glucose tolerance in sucrose-fed mice (Fig. 3d) as illustrated by the AUC results (p<0.05; Fig. 3e). The significantly reduced levels of insulin in Palatinose-fed compared with sucrose-fed mice (p<0.05; Fig. 3f, g), indicate improved glucose metabolism in the former.

Thus excluding the possibility that FI and BW differences are contributing factors to the reduced liver TG observed in the Palatinose-fed mice, we suspected that these findings might be due to attenuated GIP and insulin secretion.

Gipr$^{-/-}$ *mice are protected from diet-induced FL and impaired glucose metabolism* To further delineate the role of GIP in FL, we fed *Gipr*$^{-/-}$ and WT mice with diets containing sucrose or Palatinose. Remarkably, liver TG levels in *Gipr*$^{-/-}$ mice fed sucrose and Palatinose were twofold and threefold less than those of the WT sucrose-fed mice, respectively. The levels of liver TG in WT mice were approximately double those of Palatinose-fed mice (Fig. 4a). Hepatic TG levels were significantly influenced by diet (p=0.003) and genotype (p=0.015). BW gain, fat mass and TEE of *Gipr*$^{-/-}$ and WT mice are presented in ESM Fig. 2.

To determine additional variables contributing to GIP function and nutrient metabolism, we measured adiponectin, leptin and resistin levels in WT and *Gipr*$^{-/-}$ mice. Plasma adiponectin and leptin levels revealed a significant influence of genotype (p=0.034 and p=0.013, respectively), with no effect of diet either for adiponectin or leptin levels (both p>0.05; Fig. 4b). Plasma resistin levels were not significantly different among the groups.

Glucose challenge showed that WT mice fed sucrose had a significantly higher total glucose response relative to Palatinose-fed mice, while plasma glucose levels were not different between Palatinose- and sucrose-fed *Gipr*$^{-/-}$ mice (Fig. 4c). There was no influence of diet (p=0.058), genotype

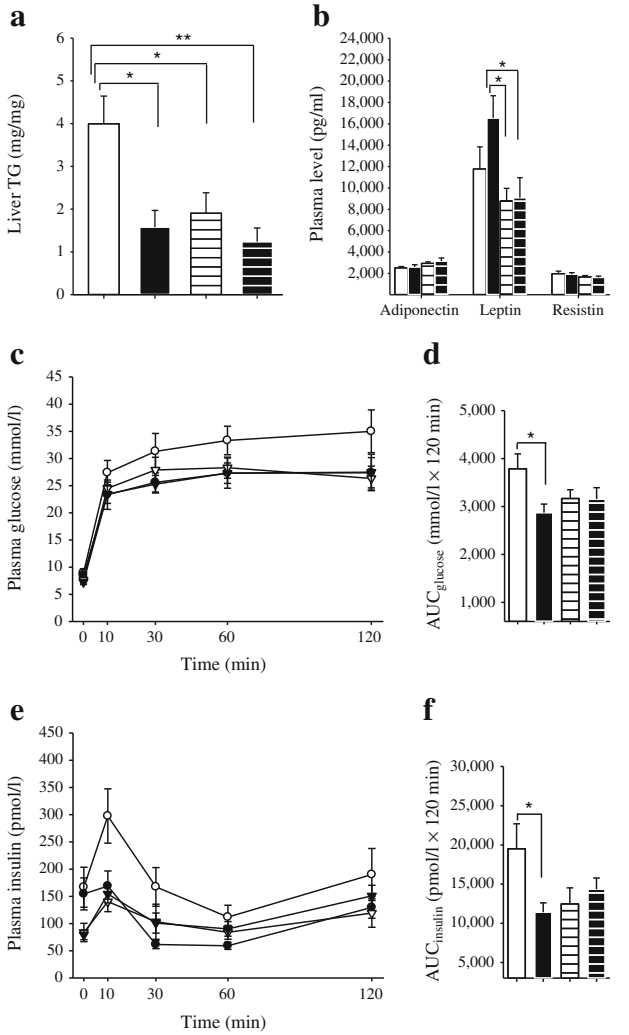

Fig. 4 WT and *Gipr*$^{-/-}$ (knockout [KO]) mice were exposed to diets. (**a**) Sucrose diet in WT mice induced higher levels of hepatic TG compared with WT Palatinose-fed and *Gipr*$^{-/-}$ mice. (**b**) Adiponectin, leptin and resistin plasma levels in the overnight fasted animals. Glucose tolerance, evaluated by i.p. GTT, is presented for plasma and AUCs for (**c**, **d**) glucose and (**e**, **f**) insulin. White circles and bars: WT, sucrose; black circles and bars: WT, Palatinose. White triangles and hatched bars: KO, sucrose; black triangles and hatched bars: KO, Palatinose. n=9 WT and 11 *Gipr*$^{-/-}$ groups. *p<0.05, **p<0.01

(p=0.48) and interaction of diet x genotype (p=0.064) in the glucose$_{AUC}$ (Fig. 4d). Fasting insulin levels in WT mice were significantly higher than *Gipr*$^{-/-}$ mice (p<0.01). After 10 min glucose load, there was a significant divergence in plasma insulin levels of WT mice fed sucrose compared with WT mice fed Palatinose and *Gipr*$^{-/-}$ mice (p=0.01; Fig. 4e). In addition, insulin$_{AUC}$ displayed a significant interaction of genotype × diet (p=0.03; Fig. 4f).

Dietary regulation of Socs2 expression and plasma GH Molecular mechanisms for the observed effects in liver TG without differences in BW and energy metabolism were assessed by mRNA expression of transcription factors and genes involved in metabolic pathways leading to the development of NAFL, including de novo lipogenesis (*Acca*, *Fas*, *Srebp1c* and *Chrebp* [also known as *Acaca*, *Fasn*, *Srebf1* and *Mlxipl*, respectively]), lipid beta-oxidation (*Ppara* and *Cpt1a*) and secretion from liver (*ApoB100* and *Mtp* [also known as *Apob* and *Mttp*, respectively]). However, our quantitative PCR analysis revealed no significant differences in genes involved in lipid metabolism (p>0.05; Fig. 5a). To identify regulated genes in a genome-wide scale we performed a microarray analysis of liver tissue from Palatinose- vs sucrose-fed mice. We identified 608 differentially expressed probes (p<0.02; ESM Table 2), from which 136 had fold changes >1.3. To assess the pathways affected by the altered expression pattern we performed enrichment analyses (ESM Table 3). Among the genes with a higher expression in sucrose- vs Palatinose-treated animals was the *Socs2* gene (2.3-fold), which is known to be transcriptionally regulated by GH signalling [23]. Quantitative PCR analysis validated the microarray data showing augmented expression of the *Socs2* gene (p<0.05; Fig. 5b). In accordance with this finding we observed that plasma GH levels were 55% lower in Palatinose-fed compared with sucrose-fed animals (p=0.07; Fig. 5c). FL is often associated with inflammation. However, we did not find significant regulation of inflammatory marker genes in either the global transcriptome analysis or in the quantitative PCR validation (ESM Table 3; ESM Fig. 3).

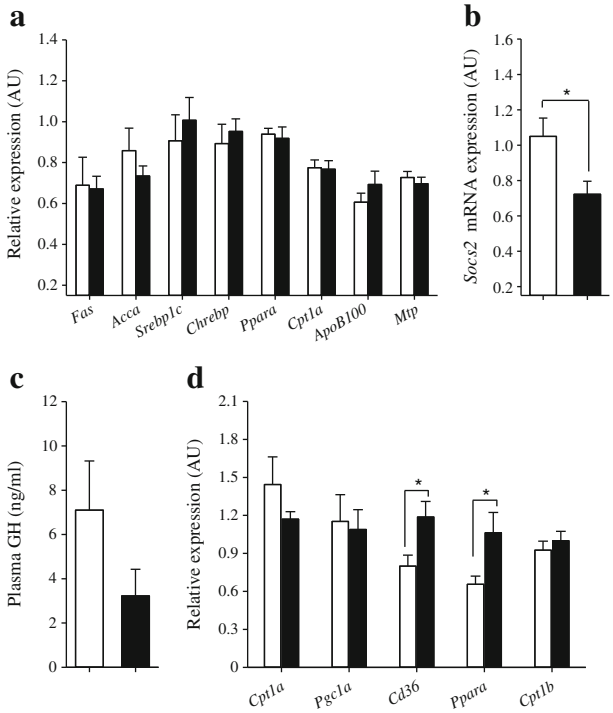

Fig. 5 Hepatic mRNA expression of (**a**) *Fas*, *Acca*, *Srebp1c*, *Chrebp*, *Ppara*, *Cpt1a*, *ApoB100* and *Mtp* and (**b**) *Socs2*. (**c**) Plasma GH. (**d**) Muscle gene expression of *Cpt1a*, *Pgc1a*, *Cd36*, *Ppara* and *Cpt1b*. White bars: sucrose; black bars: Palatinose. n=7 per group. *p<0.05

Palatinose intake enhanced fat uptake and oxidation in muscle Palatinose intake significantly increased gene expressions of *Cd36* (fatty acid transporter) and *Ppara* (beta-oxidation) in muscle (both $p<0.05$), indicating improved fatty acid uptake and oxidation by muscle (Fig. 5d).

Discussion

The main finding of our study was that Palatinose feeding prevented the development of FL and improved glucose metabolism in the setting of a high fat diet (HFD), without differences in energy intake and BW between groups. The highly significant prevention of hepatic fat accumulation was mediated by reduced GIP response, avoiding postprandial hyperinsulinaemia. The results in *Gipr*$^{-/-}$ mice suggest that GIP may mediate the deleterious metabolic effects of sucrose induced IR and FL.

Palatinose, compared with sucrose, is slowly and completely absorbed in the more distal small intestine [24], which can differently influence GIP secretion. Although Palatinose is well-established as a low-GI sugar [25, 26], we provide evidence that its effect on intestinal hormone release is central to its insulin-sparing properties in mice.

We first investigated the acute response of Palatinose and sucrose in glycaemic homeostasis and in the release of intestinal incretins. Sucrose caused an expected rapid increase in glucose accompanied by GIP and insulin release, while GLP-1 levels did not differ between groups. By contrast, Palatinose caused a more delayed increase in glucose, which resulted in little GIP secretion and, accordingly, much lower insulin secretion.

Our next question was whether or not these differences might be maintained by diet. Indeed, the HFD containing sucrose induced a rapid and strong increase of GIP and insulin, whereas Palatinose was not associated with a major increase of GIP and, accordingly, resulted in a smaller increase of insulin. The differences observed in oral and meal tests on plasma glucose are well-known [27] and relate to the content of fat and protein in the whole diet, which slows gastric emptying and thereby delays glucose absorption in the small intestine. The most likely explanation for the differences in GIP release refers to the more distal absorption of Palatinose, which bypasses the proximally located GIP-producing K cells in the small intestine.

We further analysed the long-term metabolic response of sugars in a hypercaloric diet. The Palatinose-fed mice exhibited reduced hepatic TG and were protected from diet-induced impaired glucose tolerance. A study in rats has reported that Palatinose-fed animals had higher hepatic insulin sensitivity [25], reduced hepatic TG, and lower postprandial insulin and glucose levels, which is consistent with our results although that study was confounded by differences in BW. Indeed, liver fat is associated with and is likely to be a cause of hepatic IR [4]. The 2-fold increase in liver TG levels with increased levels of ALT, a key indicator of hepatotoxicity, supports the presence of liver damage in sucrose-fed mice. Palatinose intake resulted in a modest reduction in postprandial glucose levels and a 40% reduction in glucose-stimulated insulin response. The latter may also reduce cardiovascular disease risk [28] and the development of diabetes [29].

In our study, comparable BW and FI confirm similar energy intake and digestibility of diets; therefore, the observed metabolic and hormonal differences are related to absorption differences in the gut. In contrast to amylose containing diets, which are not fully digested in the small intestine and fermented in the colon or excreted in the faeces [30, 31], Palatinose and sucrose were fully hydrolysed and absorbed in the small intestine, confirming findings from previous studies [32].

The most compelling evidence in support of the contribution of GIP to FL was the in vivo experiment in *Gipr*$^{-/-}$ and WT mice. *Gipr*$^{-/-}$ mice behaved similarly to WT mice fed Palatinose, and were protected from FL. In addition, there was a tendency for protection from glucose intolerance and hyperinsulinaemia in *Gipr*$^{-/-}$ mice. It is reported that HFD in mice results both in hypersecretion of GIP and extreme visceral fat deposition, while inhibition of GIP signalling protects from obesity, IR and hepatic steatosis [33]. It has also been shown that GIP antagonism is able to reverse liver, muscle and adipose tissue TG infiltration caused by HFD [34].

The peripheral putative mechanism of reduced liver TG levels in *Gipr*$^{-/-}$ mice might be linked to adipokines. The *Gipr*$^{-/-}$ mice exhibited slightly increased adiponectin levels compared with WT mice, whereas the amount of circulating leptin was significantly reduced by 45% in *Gipr*$^{-/-}$ mice. Leptin is secreted by adipocytes in proportion to their size and number [35, 36], which might explain the reduced leptin levels in *Gipr*$^{-/-}$ mice. Although not significant, Palatinose feeding enhanced leptin release, suggesting higher energy expenditure [37]. Leptin exerts crucial metabolic effects by preventing TG storage in peripheral tissues [38]. High leptin levels in the Palatinose-fed mice did occur independently of changes in fat mass and FI most likely as a consequence of central regulation. Our data suggest that GIP may partially affect FL development by regulating adipokine secretion, which needs further attention.

Indeed, Palatinose improved glucose homeostasis and liver fat metabolism in the context of HFD in part by enhancing muscular fatty acid uptake and causing a shift towards fat oxidation instead of fat deposition in the liver, as suggested by increased expression of *Cd36* and *Ppara*.

For additional mechanisms we performed microarray analyses in liver and observed that Palatinose reduced 2.3-fold the mRNA expression of *Socs2*, an inhibitor of cytokine

signalling. Studies have revealed changes in *SOCS2* mRNA levels in human steatotic livers [39, 40]. Recently, it was shown that *Socs2*$^{-/-}$ mice are protected from HFD-induced hepatic steatosis [18]. Moreover, SOCS2 has been implicated in GH signal transduction [23] and the development of NAFL [41]. Because *Socs2* expression was shown to be induced by GH in primary cultures of hepatocytes [23], the reduced expression of *Socs2* mRNA observed in the Palatinose-fed mice could be due to reduced plasma GH levels. It is reported that the GIP receptor mediates an increase in GH after glucose challenge [42, 43]. This suggests that higher plasma GIP levels and GIP receptor expression could lead to GH synthesis and secretion. Our results suggest that Palatinose might indirectly contribute to GH modulation by inhibiting postprandial GIP release and *Socs2* expression in the liver.

The expression of the key lipogenic transcription factors *Srebp1c* and *Chrebp* as well as factors involved in beta-oxidation (*Ppara* and *Cpt1a*) were not altered. This finding is in contrast to other reports in which increased *Ppara* expression and hepatic fat oxidation were observed for the reduced liver fat in Palatinose-fed animals. In these studies, BW differed between Palatinose- and sucrose-fed animals, which may explain the contrasting results [16]. However, pathway analysis of microarray data indicated upregulation of glycogen and NAD biosynthesis pathways (ESM Table 3). Liver NAD biosynthesis is controlled by a salvage pathway using nicotinamide as a precursor and a de novo pathway using tryptophan. Increased NAD in the liver may enhance gluconeogenesis [44]. Although not significant, glycogen content was decreased in fasting mice on Palatinose vs sucrose. Our data indicate higher metabolic flexibility in the Palatinose-fed compared with sucrose-fed mice.

A limitation of this study is the incomplete evaluation of functional and mechanistic behaviour/aspects of *Gipr*$^{-/-}$ mice on diets. Since metabolic responses to nutritional challenges might differ between humans and rodents, controlled interventions are needed in humans to investigate whether or not Palatinose vs sucrose intake can affect hepatic insulin clearance and glucose metabolism.

In conclusion, Palatinose as a food ingredient reduces postprandial GIP secretion by evading upper intestinal absorption. By this mechanism, Palatinose feeding results in reduced glucose and insulin levels. Therefore, nutritional strategies capable of reducing postprandial GIP release seem to represent a promising approach for the prevention and/or treatment of FL and IR in humans.

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FI passed away and was not involved in drafting the paper.

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