Metformin normalizes the structural changes in glycogen preceding prediabetes in mice overexpressing neuropeptide Y in noradrenergic neurons

Liisa Ailanen¹,² | Natalia N. Bezborodkina³ | Laura Virtanen¹ | Suvi T. Ruohonen¹ | Anastasia V. Malova² | Sergey V. Okovityi⁴ | Elizaveta Y. Chistyakova⁴ | Eriika Savontaus¹,⁵

¹Institute of Biomedicine, Research Center for Integrative Physiology and Pharmacology and Turku Center for Disease Modelling, University of Turku, Turku, Finland
²Drug Research Doctoral Program, University of Turku, Turku, Finland
³Laboratory of Cellular Pathology, Institute of Cytology of the Russian Academy of Sciences, St. Petersburg, Russia
⁴Department of Pharmacology and Clinical Pharmacology, Saint-Petersburg State Chemical Pharmaceutical Academy, St. Petersburg, Russia
⁵Unit of Clinical Pharmacology, Turku University Hospital, Turku, Finland

Correspondence
Eriika Savontaus, University of Turku, Institute of Biomedicine, Klinamyllynkatu 10, 20520 Turku, Finland.
Email: eriika.savontaus@utu.fi

Abstract
Hepatic insulin resistance and increased gluconeogenesis are known therapeutic targets of metformin, but the role of hepatic glycogen in the pathogenesis of diabetes is less clear. Mouse model of neuropeptide Y (NPY) overexpression in noradrenergic neurons (OE-NPY⁰¹¹H) with a phenotype of late onset obesity, hepatosteatosis, and prediabetes was used to study early changes in glycogen structure and metabolism preceding prediabetes. Furthermore, the effect of the anti-hyperglycemic agent, metformin (300 mg/kg/day/4 weeks in drinking water), was assessed on changes in glycogen metabolism, body weight, fat mass, and glucose tolerance. Glycogen structure was characterized by cytofluorometric analysis in isolated hepatocytes and mRNA expression of key enzymes by qPCR. OE-NPY⁰¹¹H mice displayed decreased labile glycogen fraction relative to stabile fraction (the intermediate form of glycogen) suggesting accelerated glycogen cycling. This was supported by decreased filling of glucose residues in the 10th outer tier of the glycogen molecule, which suggests inhibition of glycogen phosphorylation. Metformin reduced fat mass gain in both genotypes, but glucose tolerance was improved mostly in wild-type mice. However, metformin inhibited glycogen accumulation and normalized the ratio between glycogen structures in OE-NPY⁰¹¹H mice indicating decreased glycogen synthesis. Furthermore, the presence of glucose residues in the 11th tier together with decreased glycogen phosphorylase expression suggested inhibition of glycogen degradation. In conclusion, structural changes in glycogen of OE-NPY⁰¹¹H mice point to increased glycogen metabolism, which may predispose them to prediabetes. Metformin treatment normalizes these changes and suppresses both glycogen synthesis and phosphorylation, which may contribute to its preventive effect on the onset of diabetes.

Abbreviations: IGT, impaired glucose tolerance; IR, insulin resistance; LF, labile glycogen fraction; NPY, neuropeptide Y; SF, stabile glycogen fraction; T2D, type 2 diabetes; TCA, trichloroacetic acid; TGC, total glycogen content; WT, wild type.
1 | INTRODUCTION

Type 2 diabetes (T2D) is a complex disorder of glucose metabolism characterized by insulin resistance (IR), that leads to compensatory hyperinsulinemia and increased hepatic glucose output resulting in decreased glucose uptake in insulin-sensitive tissues (liver, skeletal muscle, and adipose tissue) and increased hepatic glucose production. Glucose is stored in tissues as glycogen, but liver is the only organ capable of producing glucose from glycogen (reviewed by Kowalski et al\(^1\)). The role of hepatic glycogen in the pathogenesis of diabetes is controversial and unclear in many aspects.\(^2\)\(^-\)\(^5\) However, increased glycogenolysis and glycogen cycling have been associated with diabetes in both rodents and humans.\(^6\)\(^,\)\(^7\) Fitting with this, it has been suggested that glycogen degrades more rapidly in T2D, which could explain the uncontrolled hepatic glucose output.\(^8\)

The liver glycogen is stored in the form of individual molecules (full-size glycogen molecules, \(\beta\)-particles), which construct larger \(\alpha\)-particle conglomerates consisting of 20-40 \(\beta\)-particles.\(^9\)\(^-\)\(^11\) Individual glycogen molecules are composed of 2 fractions with different solubility in trichloroacetic acid (TCA): a highly soluble labile glycogen fraction (LF; lyoglycogen), which forms the 4 outer tiers of glycogen particles, and a less metabolically active form, stabile glycogen fraction (SF; desmoglycogen).\(^12\) It has been under debate whether the labile glycogen corresponds to larger glycogen particles (with molecular mass of \(\sim 10^7\) Da) and the stabile glycogen to smaller protein (glycogenin)-binding particles (with mass of \(\sim 4 \times 10^5\) Da), and about their actual physiological meanings.\(^13\)\(^-\)\(^16\) However, there is an agreement on the existence of glycogen particles with different solubility in TCA, and that changes occur in the ratio of the labile and stabile structures (LF/SF ratio) in different physiological and pathological conditions.\(^17\) In a physiological glycogen synthesis, when fasted animals are administered with glucose, the stabile fraction is initially quickly increased, but then the labile fraction increases being responsible for the actual glycogen accumulation.\(^18\) In contrast, in a pathologic situation of liver cirrhosis, the stabile fraction is especially increased and the ratio of LF/SF decreased, suggesting a more stable increase in glycogen.\(^19\)\(^-\)\(^22\) It can be hypothesized that similar molecular changes in glycogen structure could also occur in diabetes, as similar disruptions in glycogen metabolism have been shown to occur in cirrhosis and diabetes,\(^23\) and \(\alpha\)-particles of glycogen have been shown to degrade more readily in diabetes.\(^8\) However, it is not known whether the structural changes contribute to the disease pathogenesis, and whether these changes could be targeted by drug therapy.

The aim of this study was to characterize the hepatic glycogen structure and metabolism in a state preceding disruptions in glucose metabolism, and after metformin therapy. The state preceding impaired glucose tolerance (IGT) was modeled by a transgenic mouse overexpressing neuropeptide Y (NPY) in noradrenergic neurons (OE-NPY\(^{DKH}\)). These mice are known to develop prediabetes with obesity, IR and IGT with age, and show increased susceptibility to T2D induced by high caloric diet and low-dose streptozotocin.\(^24\)\(^-\)\(^26\) Hepatic accumulation of triglycerides and glycogen seem to play an important role in the development of prediabetes and streptozotocin-induced diabetes in the model.\(^24\) Our previous findings suggest that increased glycogen cycling precedes glycogen accumulation, but it is not known, whether the changes are detected also in the glycogen structure. Furthermore, we chose metformin as the reference drug, because the main mechanism of its anti-hyperglycemic effect is to decrease hepatic glucose production, and as it has been shown to attenuate the progression of prediabetes to diabetes.\(^27\)\(^-\)\(^29\)

2 | MATERIALS AND METHODS

2.1 | Animals

Homozygous male OE-NPY\(^{DKH}\) mice (\(n = 9\) - 11/group) were used in this study with their wild-type (WT) controls. Generation of the transgenic homozygous OE-NPY\(^{DKH}\) mice, maintained on a C57BL/\(\delta\)N inbred background, and their metabolic phenotype have been described in detail previously.\(^24\)\(^,\)\(^26\) The age of the mice in the current experiment was 4 months at the initiation of metformin treatment, which is the borderline age when the first signs of IR (ie, increased glucose-stimulated insulinenia and IGT) start to emerge eventually leading to clear prediabetic phenotype at the age of 7 months.

2.2 | Study protocol

The study was authorized by the Finnish National Animal Experiment Board (ELLA). Animal care was in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS). Mice were kept in an animal room maintained at 21 ± 3°C with a fixed 12-hour light/dark cycle. A standard rodent chow (SDS, Essex, UK) and tap water were available ad libitum. Mice were housed in individual cages starting from age of 8 weeks. Food intake and body weights were measured once a week throughout the study starting at age of 14 weeks. At 16 weeks, body composition was measured in vivo with quantitative NMR (EchoMRI-700; Echo Medical Systems, Houston, TX, USA) in order to divide OE-NPY\(^{DKH}\) and WT mice into 2 treatment groups matched for weight, fat mass, and food intake. At 17 weeks, mice received drinking water either with or without metformin (300 mg/kg/day; Enzo Life Sciences LTD, Exeter, UK) for 4 weeks. The water bottles were changed and weighed twice a week. The consumption was calculated by subtracting the

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final bottle weight from the starting weight and then subtracting the estimated leakage. The value for leakage was based on testing the average leakage of bottles in cages without mice. The water consumption was around 1 mL/10 g body weight, and there were no differences between the treatments and genotypes. Thus, the average daily metformin dose was as planned and similar between the genotypes (WT 300.3 ± 11.8 mg/kg/day, OE-NPY<sup>DIH</sup> 283.1 ± 10.7 mg/kg/day, P = .30). The body composition measurement was repeated prior to the start of drug administration and at sacrifice (at age of 21 weeks). Glucose tolerance test (1 g/kg glucose intraperitoneally) was performed at age of 20 weeks, as described previously. At sacrifice, 4-hour-fasted mice were sedated with CO2, and serum was obtained by cardiac puncture. Liver was perfused and hepatocytes were isolated from 5 mice per group as described below, or dissected and weighed. Samples were stored in −70°C.

2.3 | Hepatocyte isolation and structure analysis

Hepatocyte isolation was performed according to Kudryavtseva et al. Mouse liver (n = 3-7/group) was perfused for 10 minutes in a K,Na-phosphate-sucrose buffer I, pH = 7.8 (475 mL Na<sub>2</sub>HPO<sub>4</sub> × 2H<sub>2</sub>O, 25 mL KH<sub>2</sub>PO<sub>4</sub>, 500 mL sucrose). A piece of liver was placed into buffer I for 20 min, and further a 2 × 2 × 2 mm piece was incubated in a phosphate buffer II, pH = 7.2 (400 mL Na<sub>2</sub>HPO<sub>4</sub> × 2H<sub>2</sub>O, 100 mL KH<sub>2</sub>PO<sub>4</sub>) for 5 minutes. Smears of isolated hepatocytes were prepared by gentle shaking of the pieces in a drop of buffer II using tweezers. The cell suspension obtained was spread over the surface of a slide using quartz glass with a polished edge and fixed with methanol.

The preparations of smears of isolated hepatocytes (n = 3-7/group) were stained with a fluorescent variant of the periodic acid–quartz glass with a polished edge and fixed with methanol. The presence of 2 glycogen fractions in the cells stained in situ with the use of cytochemical techniques corresponds to different solubility of the fractions in TCA. Preparations were placed into sodium periodate solution in diluted HNO<sub>3</sub> (200 mg of sodium periodate, 25 mL of 0.23% HNO<sub>3</sub>) for 1.5 hours. The oxidized preparation was washed under running tap water for 5 minutes and once in distilled water. To detect the labile fraction, the preparations were stained with a fluorescent variant of the periodic acid–ethidium bromide. To excite, Au-SO<sub>2</sub> and EtBr-SO<sub>2</sub> fluorescence interference filters at 450-490 and 546 nm were used, and to record fluorescence interference filters at 515-565 and 590 nm were used, respectively. The intensity of fluorescence of the cells stained with Au-SO<sub>2</sub> and EtBr-SO<sub>2</sub> was measured using ImageJ software (National Institute of Health, USA). The total glycogen content (TGC) in each cell was taken to be equal to the sum of glycogen fractions. The fluorescence of 100 cells was measured for each preparation. The relative number of glucose residues in the outer tiers of the glycogen molecule was calculated using the data on the number of glucose chains that were calculated according to the theoretical formulations of the Whelan’s model.

As labile fraction constitutes the first 8 tiers of the glycogen molecule and consists of 255 glucose chains, it has been accepted that the LF/SF ratio that is ≤ 1 corresponds to the ninth tier, the LF/SF ratio that is ≤ 2 corresponds to the 10th tier, LF/SF ratio that is ≤ 4 corresponds to the 11th tier, and LF/SF ratio that is ≤ 8 corresponds to the 12th end tier of the molecule.

2.4 | Gene expression analyses

Samples for RNA isolation (n = 9-11/group) were stored in RNA Stabilization Reagent (RNAlater, Qiagen, Hilden, Germany). Liver RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) with DNase treatment (Sigma-Aldrich, St. Louis, MO, USA). RNA was converted to cDNA with High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). mRNA expression was analyzed with SYBR Green (KAPA SYBR<sup>&reg;</sup> FAST ABI Prism<sup>®</sup>; Kapa Biosystems, Woburn, MA) technique using 7300 Real-Time PCR System (Applied Biosystems). The expression of target genes was quantified relative to geometrical mean of reference genes (β-actin and ribosomal protein S29). Primers used for quantification are shown in Table 1.

2.5 | Statistical analysis

Statistical processing of glycogen contents were analyzed with Student’s t test, using SigmaPlot v 11.0 software (Systat Software Inc., San Jose, CA). All the other parameters were analyzed with GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA). Tests with multiple time points were analyzed with repeated measures two-way ANOVA. Parameters with single time points were analyzed with two-way ANOVA taking into account the genotype and drug effects, and their interaction, and were followed by Bonferroni post hoc tests when interaction was significant. The results are expressed as means ± SEM. P < .05 was considered statistically significant.

3 | RESULTS

3.1 | Body composition and food intake

OE-NPY<sup>DIH</sup> mice in this study, in contrast to previous reports, were similar in body weight and adiposity compared to WT mice
FIGURE 2A and B. To note, WT mice were mildly obese for their age, likely due to single housing. Metformin significantly decreased gain in body weight and whole body fat mass over the treatment period in both genotypes (Figure 2C and D). The decrease in adiposity was mainly due to a decrease in visceral fat and was more pronounced in WT mice, as retroperitoneal fat tended to be smaller in metformin-treated groups \((P = .075)\), but mesenteric adipose tissue depot was significantly smaller in metformin-treated WT mice compared to controls, but not in OE-NPY \(^{D\text{PH}}\) mice (Figure 2E). Metformin did not influence the sizes of other WAT depots (epididymal, inguinal subcutaneous) (Figure 2E), interscapular brown fat or the liver in either genotype (data not shown). There were no significant differences in absolute (Figure 2F) or cumulative food intake between treatment groups (Metformin-treated WT 107.2 \(\pm\) 1.2 g/day and OE-NPY \(^{D\text{PH}}\) 109.2 \(\pm\) 1.8 g/day vs control WT 111.8 \(\pm\) 1.5 g/day and OE-NPY \(^{D\text{PH}}\) 111.6 \(\pm\) 2.9 g/day; Treatment effect \(P = .08\)).

### 3.2 | Glucose metabolism

Fasting blood glucose was similar between treatment groups and genotypes (Figure 3A). GTT revealed no difference between genotypes in glucose clearance fitting with similar body weights and adiposity between genotypes. Metformin treatment reduced the increase in glucose (area under the curve, AUC) during the first 20-min period being more pronounced in WT mice without affecting the clearance rate of glucose at 20-90-min period (Figure 3B-D). The more pronounced therapeutic effect in WT mice could be due to decreased adiposity as AUC values of GTT positively correlated with fat mass \((r = .655, P < .05)\), but not lean mass. There were no differences in the mRNA expressions of known targets of metformin: the gluconeogenic enzymes phosphoenolpyruvate carboxykinase 1 \((Pck1)\), glucose-6-phosphatase \((G6pc)\), or peroxisome proliferator-activated receptor gamma, coactivator 1 alpha \((Ppargc1a)\) (Figure 3E-G).

### 3.3 | Glycogen molecule structure in OE-NPY \(^{D\text{PH}}\) mice

In order to detect glycogen structure preceding prediabetes, TGC and the glycogen fractions were analyzed from OE-NPY \(^{D\text{PH}}\) and WT mice receiving drinking water without metformin. TGC in hepatocytes was similar between the genotypes. However, OE-NPY \(^{D\text{PH}}\) mice showed a decreased content of the labile fraction and an increased content of the stable fraction resulting in reduced LF/SF ratio, which suggests enhancement of glycogen synthesis (Table 2). TGC correlated significantly with both the labile \((r = .933, P < .001)\) and stable fraction \((r = .700, P < .001)\) (Figure 3E-G).

**TABLE 1** qPCR primers

| Gene name                        | Forward 5’-3’          | Reverse 5’-3’          |
|----------------------------------|------------------------|------------------------|
| Bact, beta actin                 | tccatcatgaagtgacgt     | gagcaagtacgtgtcctcat   |
| Gys2, glycogen synthase 2        | cgtgctgtcagaaaaagctg   | agcctcttccaaagtcac    |
| G6p, glucose-6-phosphatase       | cgactgctgtctccaagtga   | gggctgtgtccaaacacagat |
| Pck1, phosphoenolpyruvate carboxykinase 1, cytosolic | agcctacgcacccagtttc | cagctgtcatgtttaatcaaa |
| Ppargc1a, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha | tatggagtgacataagatgttgtct | ccacccaaacagtaacactgttttc |
| Pygl, liver glycogen phosphorylase | ccagagtgctctaccacaaat | cccaccaaaagtagactctgttttc |
| S29, Ribosomal protein S29       | atgggtcaccaggatccta    | agcctgtcatctccgtctact |
and the stable fraction content ($r = .896, P < .001$) in WT hepatocytes. In OE-NPYD$^{DH}$ mice, correlation between the parameters was similar, but less pronounced ($r = .696, P < .001$ and $r = .712, P < .001$, respectively).

The filling of the tiers of the glycogen molecule was analyzed according to Whelan’s model. The 10th tier of the glycogen in WT mice was filled, on average, by 81.7% indicating constant regulation of blood glucose. Instead in OE-NPYD$^{DH}$ mice, the 10th tier was only filled by 58.4%, suggesting enhanced activity of glycogen phosphorylase (Table 3).

### 3.4 Effects of metformin on glycogen structure

In order to study whether metformin influences the glycogen content or structure, TGC and different glycogen fractions were analyzed from OE-NPYD$^{DH}$ and WT mice receiving metformin for 4 weeks, and compared to control groups. There was no change in glycogen content or structure in WT mice between the treatments. However, metformin treatment markedly decreased the content of the stable fraction and significantly also the content of the labile fraction in OE-NPYD$^{DH}$ resulting in reduced TGC and normalization of the LF/SF ratio to the level of the WT group (Table 1). Furthermore, the composition of glycogen residues to the 11th tier of glycogen molecule was present in metformin-treated OE-NPYD$^{DH}$ mice pointing to a decreased glycogen phosphorylase activity as glycogen phosphorylase is responsible for degradation of upper tiers of glycogen molecules (Table 2). In accordance, mRNA expression of glycogen phosphorylase ($Pygl$) was significantly decreased in metformin-treated OE-NPYD$^{DH}$ mice. No differences were detected in WT mice, between genotypes in control-treated mice, or in glycogen synthase ($Gys$) expression between the treatment groups (Figure 4A-B).

### 4 DISCUSSION

Metformin is the first-line anti-diabetic agent to treat T2D, but multiple studies have shown that metformin is also effective in preventing or delaying the onset of T2D.$^{27-29}$ Metformin improves insulin sensitivity by decreasing hepatic glucose production due to decreased ATP availability,$^{5,34}$ but also several other contributing mechanisms have been reported.$^{35-37}$ The roles of gluconeogenesis as a source of hepatic glucose overproduction and as a target of metformin action are well described, but less is known about the role of changes in glycogen structure and metabolism, especially in prediabetic state. In this study, we show that early phase changes in glycogen structure may contribute to the development of IGT and diabetes, and treatment with metformin in this early phase decreases hepatic glycogen levels and normalizes glycogen structure, which may have therapeutic relevance in prevention of the onset of prediabetes or diabetes.
The glycogen structure was elucidated in a genetically modified mouse model of prediabetes, OE-NPYD\(_b\)H mouse, at an age prior to manifestation of overt IGT in order to evaluate our previous findings of increased hepatic glycogen cycling (ie, increased mRNA expression of glycogen synthase and phosphorylase) preceding glycogen accumulation. Despite equal glycogen contents and similar glucose tolerance between the genotypes, changes in the molecular structure of glycogen in OE-NPYD\(_b\)H hepatocytes, that is, increased content of the stabile fraction and decreased content of the labile fraction supported by decreased filling of the 10th tier in the glycogen molecules, suggest that increased glycogen synthesis and degradation takes place before prediabetes. Using the same cytofluorometric analysis similar changes in glycogen structure were detected in metformin-treated OE-NPYD\(_b\)H vs WT mice.

**TABLE 2** The contents of different glycogen structures (standard units) in hepatocytes of metformin-treated OE-NPYD\(_b\)H vs WT mice

| Genotype | Treatment | SF     | LF     | TGC    | LF/SF  |
|----------|-----------|--------|--------|--------|--------|
| WT       | Ctr       | 10.59  | 17.63  | 28.53  | 1.63   |
|          | Met       | 9.25   | 15.38  | 25.41  | 1.51   |
| OE-NPY   | Ctr       | 14.25  | 12.90  | 30.87  | 0.62   |
|          | Met       | 5.49   | 10.65  | 16.48  | 1.70   |

The data are represented as mean ± SEM (n = 3 mice/group). SF, stabile fraction; LF, labile fraction; TGC, total glycogen content; Met, metformin-treated group.

**TABLE 3** The filling of outer tiers of glycogen molecules with respect to the content of glucose residues (%) in hepatocytes of metformin-treated OE-NPYD\(_b\)H vs WT mice

| Genotype | Treatment | Outer tiers |
|----------|-----------|-------------|
|          |           | 9th | 10th | 11th |
| WT       | Ctr       | 100 | 81.7 | —    |
|          | Met       | 100 | 83.7 | —    |
| OE-NPY   | Ctr       | 100 | 58.4 | —    |
|          | Met       | 100 | 100  | 52.5 |

The calculations were performed from 100 hepatocytes with Whelan’s model. Ctr, control group receiving drinking water without metformin; Met, metformin-treated group; WT, wild-type.

The glycogen structure was elucidated in a genetically modified mouse model of prediabetes, OE-NPYD\(_b\)H mouse, at an age prior to manifestation of overt IGT in order to evaluate our previous findings of increased hepatic glycogen cycling (ie, increased mRNA expression of glycogen synthase and phosphorylase) preceding glycogen accumulation.
another chronic pathologic condition, liver cirrhosis. This is opposite to the situation of physiological glycogen synthesis, (e.g., in glucose administration after a prolonged fast), where the metabolically active form, labile glycogen, is rapidly increased. Previous data on glycogen structure and metabolism in prediabetes is scarce, but increased glycogen metabolism has also been reported in prediabetic Zucker (fa/fa) rats. In T2D, glycogen metabolism has received more attention, showing increased glycogen metabolism and fragility of the α-particles of glycogen. However, as the glycogen content in diabetes varies depending on the type (1 or 2) and the state (early or established) of diabetes, and the diurnal cycle, accelerated glycogen metabolism and the structure of glycogen molecules seem to be better biomarkers for pathogenic glucose metabolism than the glycogen content itself.

We next studied the effect of metformin on hepatic glycogen structure. In OE-NPYD mice hepatocytes, metformin decreased the contents of total and both structural forms of glycogen. Furthermore, supported by decreased Pygl mRNA expression, increased LF/SF ratio and filling of the 11th tier in the glycogen molecule indicate that glycogen phosphorylase activity was inhibited. When glycogen phosphorylase does not decompose glycogen fast enough and there is no formation of new molecules, glycogen synthase and the branching enzyme attach glucose residues to glycogen molecules that are already formed. As the overall glycogen content was decreased, it seems that also glycogen synthesis was decreased by metformin. Our results reinforce the previous finding of metformin’s ability to reduce glycogen degradation. However, we show that this is the case already in an early phase, before the actual symptoms of IGT. Therefore, this could be a mechanism of action in decreasing hepatic glucose output especially in a situation of prediabetic enhancement of glycogen accumulation and cycling. Inhibition of glycogen phosphorylase activity is known to improve glucose metabolism and has previously been studied as a potential drug target for treating hyperglycemia.

FIGURE 4 mRNA expression of glycogen synthase and phosphorylase in metformin-treated OE-NPYD mice vs WT mice. (A) mRNA expression of glycogen synthase and (B) phosphorylase enzymes in the livers of OE-NPYD mice vs WT mice (n = 9-11/group) after 4 weeks of receiving drinking water with or without metformin. Values are expressed as means ± SEM. Bonferroni post hoc comparison between metformin-treated and control OE-NPYD mice, two-way ANOVA indicated a tendency to treatment x genotype interaction (P = .06). Gys2, glycogen synthase 2; Pygl, liver glycogen phosphorylase.

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