30-Day spexin treatment of mice with diet-induced obesity (DIO) and type 2 diabetes (T2DM) increases insulin sensitivity, improves liver functions and metabolic status

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ABSTRACT

Spexin (SPX) is a 14 aa peptide discovered in 2007 using bioinformatics methods. SPX inhibits food intake and regulates lipid, and carbohydrate metabolism. Here, we evaluate the ability of SPX at improving metabolic control and liver function in obese and type 2 diabetic animals. The effects of 30 days SPX treatment of mice with experimentally induced obesity (DIO) or type 2 diabetes (T2DM) on serum glucose and lipid levels, insulin sensitivity and hormonal profile (insulin, glucagon, adiponectin, leptin, TNF alpha, IL-6 and IL-1β) are characterized. In addition, alterations of hepatic lipid and glycogen contents are evaluated. We report that SPX decreases body weight in healthy and DIO mice, and reduces lipid content in all three animal groups. SPX improves insulin sensitivity in DIO and T2DM animals. In addition, SPX modulates hormonal and metabolic profile by regulating the concentration of adiponectin (concentration increase) and leptin (concentration decrease) in the serum blood of DIO and T2DM mice. Lastly, SPX decreases lipid content as well as IL-6 and TNF-α protein levels in liver of DIO and T2DM mice, and reduces IL-6 and TNF-alpha concentrations in the serum derived from T2DM mice. Based on our results, we conclude that SPX could be involved in the development of obesity and type 2 diabetes mellitus and it can be further evaluated as a potential target for therapy of DIO and T2DM.

1. Introduction

Spexin (SPX), a 14-amino-acid (aa) peptide, was discovered in 2007 using bioinformatics methods (Mirabeau et al., 2007). SPX, also known as Neuropeptide Q (NPQ), results from posttranslational cleavage of a prepropeptide, which is composed of 116-aa residues. The sequence of SPX has high similarity in many mammalian species such as rats, mice, and humans (Liu et al., 2013). In rats, SPX mRNA is widely distributed in numerous tissues (Porzionato et al., 2010). SPX is also present in non-mammals such as fishes. Studies conducted on goldfish demonstrated that SPX inhibits basal, neuropeptide Y-, and orexin-stimulated food consumption (Wong et al., 2013) as well as regulates the reproductive system (Liu et al., 2013). So far, relatively few SPX functions have been described. SPX can affect food intake (Walewski et al., 2014), regulate insulin secretion (Sassek et al., 2018), stimulate lipolysis, and inhibit lipogenesis in vitro (Kolodziejski et al., 2018; Pruszynska-Oszmalek...
et al., 2020). Furthermore, SPX downregulates fatty acid uptake into the hepatocytes (Jasmine et al., 2016). Walewski et al. showed that the level of SPX was lower in obese humans and its concentration was negatively correlated with leptin (Walewski et al., 2014). Our previous research also showed that SPX level was lower in obese women and negatively correlated with their body mass index (BMI), homeostatic model assessment of insulin resistance (HOMA-IR) index, and serum levels of insulin and glucagon (Kołodziejski et al., 2018), while it was positively correlated with the quantitative insulin sensitivity check index (QUICKI) and the levels of hormones such as obestatin, and adiponectin. In addition, we showed that SPX regulates insulin secretion from isolated rat pancreatic islets as well as from insulin-producing murine INS-1E cells (Sassek et al., 2018) and that the mutual interactions between insulin and SPX play a role in regulating the physiology of porcine pancreatic islets (Sassek et al., 2019). Furthermore, other studies have shown a relationship between serum SPX levels and obesity or diabetes - incl. positive correlation between SPX and adiponectin as well as negative correlation between SPX and leptin, triglycerides (Behroz et al., 2020; Bitarafan et al., 2019; Chen et al., 2019; Gu et al., 2019; Karaca et al., 2019). However, it should be emphasized that there is still no clear understanding of the function of SPX in the pathophysiology of these metabolic diseases. The possible role of SPX in treatment of metabolic abnormalities has not yet been tested.

This study aims to investigate the effects of a long-term SPX treatment on carbohydrate and lipid metabolism in the murine model of obesity and type 2 diabetes (T2DM). We mainly focused on the effects of SPX on the hormonal (pancreatic hormones, adipokines and cytokines) and metabolic profiles in healthy, obese, and T2DM animals.

2. Materials and methods

2.1. Ethics

The animal protocol was approved by the Local Ethical Commission for Investigation on Animals, Poznan University of Life Sciences (Permission No. 36/2017).

2.2. Animals

C57BJ/6 female mice (n = 60) were purchased from the Mossakowski Medical Research Centre Polish Academy of Sciences. The mice were housed under standard conditions (12/12 h light/dark cycle, 21 ± 1 °C). After the experiment (30 days SPX treatment), the animals were decapitated during the diestrus phase. To determine the phase of the estrous cycle, vaginal smears were obtained daily for 10 consecutive days between 7:00 and 10:00 a.m. and were evaluated using a light microscope (Genetic Pro Bino, Delta Optical, Poland).

2.3. Induction of obesity (DIO) and type 2 diabetes (T2DM) in mice

After 2 weeks of acclimatization, the mice with the body weight of 20 ± 2 g were divided into two groups: one continued to receive the standard laboratory diet (healthy group/mice, n = 20), and for the other (n = 40), the standard diet was replaced by a high-fat diet (HFD) providing 50 % energy from fat (Morawski, Kcynia, Poland). The animals were fed this diet ad libitum for 10 weeks. Body mass and glucose level were monitored once a week.

Subsequently, the mice fed HFD were randomized into two groups: diet-induced obesity mice (DIO mice, n = 20) and T2DM mice (n = 20). DIO mice and T2DM mice were assigned to research groups based on three parameters: blood glucose level, body mass, and fat mass (% of total body weight). To determine the level of glucose, blood was obtained from the animals’ tail and tested using a glucometer (Accu-Chek Active Glucometer, Roche Diagnostics GmbH, Mannheim, Germany). Diagrams illustrating the values (body mass, % of fat, and glucose concentration) determined before the experiment are included in the supplementary data (Suppl. Fig. 2).

Diabetic mice were injected with a single dose of streptozotocin (STZ) (50 mg/kg body weight). After 3 days, their glucose levels were measured, and, if necessary (non-fasting blood glucose level was lower than 10 mmol/l), the injection of STZ (50 mg/kg body weight) was repeated. After the next 4 days, the animals with non-fasting level of blood glucose above 10 mmol/l were considered to be diabetics. Both animal groups (DIO and T2DM) were continuously fed HFD throughout the experiment.

An overview of the experiment is shown in the supplementary data (Suppl. Fig. 1).

2.4. Spexin injections

SPX (25 μg/kg of body weight) was injected intraperitoneally (ip) on all days of the experiment for 30 days. Control groups received an analogous volume of phosphate buffered saline (PBS). The last injection of PBS or SPX was performed approximately 18 h before the end of the experiment.

2.5. Body weight, food intake, and body composition

Body weight was monitored every 3 days during the experiment using an electronic scale. Food intake was monitored at selected time points analogously to body weight measurements. Since the mice were not placed separately in metabolic cages, cumulative food intake was monitored only for the entire group in the cage (Suppl. Fig. 3). The body composition of the mice was analyzed using Minispec LF90II (Bruker, Germany).

2.6. ipGTT and ipITT

Intraperitoneal glucose tolerance (ipGTT) and insulin tolerance (ipITT) tests were performed 5 days before decapitation. In ipGTT, the animals were injected with glucose (2 g/kg of body weight) after 4 h of fasting. Glucose concentration in the blood obtained from the tail was measured before and 5, 15, 30, 45, 60, 90, and 120 min after glucose injection using glucometer as we previously described. In ipITT, the concentration of blood glucose was measured in mice after 2.5 h of fasting. Subsequently, the mice were injected ip with insulin (1 U/kg body weight) (Novolin, Novo Nordisk, Bagsværd, Denmark). Serum glucose level was measured 5, 15, 30, 45, and 60 min after insulin injection. IpGTT (n = 4 from each group) and ipITT (n = 4 from each group) were performed using different cohorts of animals from the same group. To determine the area under curve (AUC), the trapezoidal rule was used. The calculations are shown individually for each mouse as well as for the whole group.

2.7. HOMA-IR and QUICKI

HOMA-IR (Cacho et al., 2008) and QUICKI (Chen et al., 2005) were calculated from the levels of fasting glucose (G₀) and insulin (I₀) and triglycerides (TG₀) using the following formulas: HOMA-IR = (G₀ × I₀)/22.5; QUICKI = 1/[log(G₀) + log(I₀)].

2.8. Metabolic and hormonal profiles in serum

Metabolic profile was determined using commercially available colorimetric assays. The levels of glucose (Cat. No.: G7519), triglycerides (TG; Cat. No.: T7531), total cholesterol (TCh; Cat. No.: C7510), high-density lipoprotein (HDL; Cat. No.: 7545) and low-density lipoprotein (LDL; Cat. No.: 7574-D) fractions, β-hydroxybutyrate (Cat. No.: H7587), and fructosamine (Cat. No.: F7546) (Pointe Scientific, USA). NEFA were measured using kit from WAKO (Cat. No.: 434–91795 and 434–91995; Wako Chemicals, USA). The activities of alanine aminotransferase (ALT; Cat. No.: A7526), aspartate aminotransferase (AST; Cat. No.: A8480), and γ-glutamyltransferase (GGT; Cat. No.: G8480) were assessed using commercially available enzymatic assays (Pointe Scientific, USA). Neutrophils were counted using cytomation kit from Immunotyp (Cat. No.: 702001U) (Immunotyp, Poland).

The animal protocol was approved by the Local Ethical Commission for Investigation on Animals, Poznan University of Life Sciences (Permission No. 36/2017).
2.9. Liver composition and content of IL-6 and TNF-α in liver

The effect of SPX on the content of cholesterol, TG, and glycogen in the liver as well as IL-6 and TNF-α protein was determined as previously described (Kołodziejski et al., 2017) using aforementioned kits. Small pieces of liver tissue were fixed with Bouin’s solution (picric acid-saturated aqueous solution (2.1 %): 40 % formaldehyde: acetic acid glacial, in a 25:5:1 proportion) for 48 h and embedded in paraffin. Sections (5 μm) were cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin to identify empty spaces left by lipids. Since we performed a quantitative analysis of the lipids in the liver, no semi-quantitative analyses of the obtained images were performed.

2.10. Real-time PCR

Total RNA extraction and real-time procedures were performed as described (Kołodziejski et al., 2018). Briefly, for cDNA synthesis, 1 μg of total RNA and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) were used according to the manufacturer’s protocol. Real-time polymerase chain reaction (PCR) was performed using gene-specific primers (Suppl. Table 1), with 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) on QuantStudio 12 K Flex™ real-time PCR system (Life Technologies, Grand Island, NY, USA). The specificity of the reaction products was tested by determining the melting points (0.1 C/s transition rate). Relative gene expression was evaluated by Delta Delta CT (ΔΔCT) with Gapd as a reference.

2.11. Statistical analysis

The results in tables are presented as the arithmetic mean ± standard error mean (SEM) and the data presented of the figures are presented as median with box and whiskers representing the interquartile range and 5th–95th percentiles (GraphPad Prism, GraphPad Software Inc., USA). The effect of the SPX treatment was analyzed using one way ANOVA with Tukey post hoc test. Statistical significance was defined as *P < 0.05 or **P < 0.01.

3. Results

3.1. Effect of SPX administration on body weight and body composition

In Fig. 1A, we showed the changes in body mass during whole experiment. After 30 days of SPX administration, we observed a decrease of changes in body weight compared to initial body weight - day 0 (Δ Body Weight) in healthy (Fig. 1B, P < 0.05) and DIO mice (Fig. 1B, P < 0.01). Moreover, Δ Body Weight was statistically different in obese vehicle mice (Fig. 1B, P < 0.01) compared to control vehicle and SPX mice. Whereas, after SPX injection we mass of DIO mice were compatible with healthy vehicle and SPX mice. We didn’t noted any changes in Δ Body Weight after SPX in T2DM group of mice. However, compared to healthy and DIO mice we noted statistically significant decrease in this parameter in T2DM mice. Moreover, the analysis of the total body composition, including fat (Fig. 1C) and lean (Fig. 1D) tissue mass and body fluid (Fig. 1E), showed a decrease in fat tissue content after SPX treatment in all the investigated animal models (healthy, T2DM, P < 0.05; DIO P < 0.01) compared to the corresponding control groups. Despite this decrease the higher fat content in DIO and T2DM mice compared to control was still observed (Fig. 1C, P < 0.01). We noted also lower fat content in T2DM vehicle (P < 0.05), T2DM SPX (P < 0.01) groups compared to DIO vehicle group. In addition, after SPX treatment, an increase in lean tissue mass was observed in DIO model (Fig. 1D, P < 0.05). This increase meant that lean tissue in this group did not differ statistically significantly from the group of healthy mice in
3.2. SPX improves metabolic abnormalities associated with DIO and T2DM

The first parameter to be investigated is the glucose level in blood serum. We found that SPX treatment decreased glucose level in the T2DM group compared to the T2DM vehicle (Table 1; *P < 0.05*). However, higher glucose concentration in T2DM group compared to DIO and healthy mice we noted (*P < 0.01*). There were no statistically significant differences in the glucose level after SPX treatment in healthy and DIO groups. Moreover, to determine the average glucose concentration over the last 2 weeks of SPX treatment, the fructosamine concentration in serum was measured. Fructosamine levels were lower in the T2DM mice after SPX treatment compared to the corresponding control group. However, similar to glucose, fructosamine levels in the T2DM group were still higher compared to the DIO and healthy mice group (Table 1; *P < 0.05*). Moreover, we noticed the higher fructosamine concentration in DIO vehicle mice compared to healthy group of mice. We didn’t observe similar differences between DIO SPX and healthy mice. This confirms the glucose lowering activity of SPX.

Furthermore, we measured the cholesterol (total, HDL, LDL) levels in serum and found that total cholesterol concentration decreased after SPX treatment in the DIO and T2DM groups (Table 1; *P < 0.01* and *P < 0.05*, respectively). This was accompanied by an increase in the HDL fraction (Table 1; *P < 0.05*). However, as we expected measurement errors due to, for example, food crumbs in the litter, we decided to include only food intake measurement in the supplementary data.

**Table 1**

| Variable                     | Healthy | DIO    | T2DM   |
|------------------------------|---------|--------|--------|
|                              | Control | SPX    | Control| SPX    | Control | SPX    |
| Glucose [mg dl⁻¹]            | 105.1 ± 5.51 | 112.0 ± 7.44 | 129.8 ± 9.14 | 119.2 ± 7.66 | 206.3 ± 12.33 ABCD | 173.0 ± 7.10* ABCD |
| Total Cholesterol [mg dl⁻¹]  | 110.6 ± 3.46 | 105.9 ± 2.69 | 188.1 ± 4.78 AB | 170.2 ± 3.92* AB | 199.5 ± 6.93 ABCD | 181.4 ± 5.00* ABCD |
| HDL cholesterol [mg dl⁻¹]    | 54.4 ± 6.60 | 56.1 ± 4.44 | 87.63 ± 4.35 AB | 102.8 ± 4.14 AB | 92.62 ± 5.08 ABCD | 105.3 ± 5.93 ABCD |
| LDL cholesterol [mg dl⁻¹]    | 48.57 ± 4.11 | 47.51 ± 3.74 | 80.05 ± 2.21 AB | 68.37 ± 1.71 AB | 81.74 ± 4.07 AB | 71.83 ± 6.34 ab |
| Triglycerides [mg dl⁻¹]      | 123.4 ± 6.55 | 124.7 ± 6.15 | 164.2 ± 4.63 AB | 146.1 ± 6.17* AB | 165.0 ± 4.59 AB | 157.8 ± 4.29 AB |
| NEFA [mmol l⁻¹]              | 1.32 ± 0.07 | 1.33 ± 0.09 | 1.943 ± 0.06 AB | 1.817 ± 0.04 AB | 1.683 ± 0.06 AB | 1.675 ± 0.06 ABC |
| Fructosamine [mmol l⁻¹]      | 2.608 ± 0.04 | 2.652 ± 0.04 | 3.055 ± 0.1 AB | 2.881 ± 0.06 | 4.095 ± 0.11 ABCD | 3.757 ± 0.05* ABCD |
| β-hydroxybutyrate [mmol l⁻¹] | 0.632 ± 0.026 | 0.688 ± 0.024 | 0.843 ± 0.074 | 0.716 ± 0.085 | 1.057 ± 0.066 AB | 0.837 ± 0.029* a |
| ALT [IU l⁻¹]                 | 23.61 ± 1.60 | 22.52 ± 1.42 | 33.32 ± 2.08 a | 27.09 ± 1.96* | 53.90 ± 2.14 ABCD | 41.28 ± 4.17** ABCd |
| AST [IU l⁻¹]                 | 45.21 ± 4.35 | 44.68 ± 4.50 | 65.43 ± 3.65 | 54.02 ± 1.97* | 103.5 ± 10.05 ABCD | 70.61 ± 3.70** ab |
| γGT [IU l⁻¹]                | 5.69 ± 0.92 | 5.89 ± 0.97 | 8.09 ± 1.28 | 5.07 ± 0.66* | 13.03 ± 0.96 ABCD | 8.46 ± 0.65** ab |
| Corticosterone [ng ml⁻¹]     | 508 ± 134 | 1360 ± 254** | 1103 ± 211 | 1735 ± 130 A | 1008 ± 170.2 | 1733 ± 278 a |

Results are expressed as means ± SEM (n = 10 per group). Statistically significant changes between SPX and control groups are marked *P < 0.05* and **P < 0.01. a P < 0.05; b P < 0.01 compared healthy PBS group; c P < 0.05; B P < 0.01 compared healthy SPX group; c P < 0.05; C P < 0.01 compared DIO PBS group; d P < 0.05; D P < 0.01 compared DIO SPX group. NEFA, non-esterified fatty acids.
3.4. SPX modulates pancreatic hormones, adipokines and cytokines profiles

First, we investigated the effect of SPX on insulin and glucagon concentrations and we found no statistically significant differences between the control and respective SPX-treated animals in all the investigated groups. However, we noted a decreasing trend in the insulin level in the T2DM group after SPX treatment (Fig. 3A, \( P = 0.068 \)). After experiment we observed also the higher insulin concentration in T2DM groups compared to healthy mice and in T2DM vehicle compared to DIO group. We did not notice statistically significant changes between T2DM SPX vs. DIO groups of mice. In addition, a decreasing trend in glucagon concentration was observed in the T2DM mice after SPX treatment (Fig. 3B, \( P = 0.061 \)). Moreover, we demonstrated that T2DM vehicle mice had the higher concentration of glucagon compared to healthy and DIO mice. Additionally, we observed also that after SPX the T2DM group did not differ statistically significantly from the other groups of mice. Subsequently, we investigated the effect of SPX on leptin and adiponectin concentrations in serum and we found the lower leptin concentration after SPX treatment in the DIO group compared to the corresponding control (Fig. 3C, \( P < 0.01 \)). Similarly, we observed the increase in adiponectin level in DIO and T2DM animals after SPX treatment compared to the control (Fig. 3C, \( P < 0.01 \)). We noted also statistically significant higher concentration of leptin in DIO groups of mice and T2DM vehicle compared to control. Moreover, we found differences (lower concentration of leptin) between T2DM mice and DIO vehicle group. In case of adiponectin we noted also statistically significant differences (lower adiponectin concentration) between DIO vehicle group and healthy SPX group.

![Figure 2](image1.png)

**Fig. 2.** Effect of SPX administration on insulin sensitivity and glucose utilization rate. (A) Intraperitoneal Glucose Tolerance Test (ipGTT). (B) AUC calculated for individual animals during ipGTT. (C) Intraperitoneal Insulin Tolerance Test (ipITT). (D) AUC calculated for individual animals during ipITT. (E) HOMA-IR and (F) QUICKI determined experimental animals. The data are presented as median with box and whiskers representing the interquartile range and all values (\( n = 4 \) per group for ipGTT, ipITT, and AUC experiments; \( n = \text{min. 8} \) for HOMA-IR and QUICKI determination). Statistically significant changes in groups are marked \({ }^{*}(P < 0.05)\) and \({ }^{**}(P < 0.01)\) compared to the corresponding control receiving PBS, \( a (P < 0.05), A (P < 0.01)\) compared healthy PBS group; \( b (P < 0.05), B (P < 0.01)\) compared healthy SPX group; \( c (P < 0.05), C (P < 0.01)\) compared DIO PBS group; \( d (P < 0.05), D (P < 0.01)\) compared DIO SPX group.

![Figure 3](image2.png)

**Fig. 3.** Terminal changes in hormonal profile in mice after SPX administration. (A) Insulin, (B) glucagon, (C) leptin, (D) adiponectin, (E) TNF-α, (F) IL-6, and (G) IL-1β in experimental animals. The data are presented as median with box and whiskers representing the interquartile range and all values (\( n = \text{min. 8} \) per group). Statistically significant changes in groups are marked \({ }^{*}(P < 0.05)\) and \({ }^{**}(P < 0.01)\) compared to the corresponding control receiving PBS, \( a (P < 0.05), A (P < 0.01)\) compared healthy PBS group; \( b (P < 0.05), B (P < 0.01)\) compared healthy SPX group; \( c (P < 0.05), C (P < 0.01)\) compared DIO PBS group; \( d (P < 0.05), D (P < 0.01)\) compared DIO SPX group.
vs. healthy vehicle group and T2DM vehicle vs. healthy groups and DIO SPX group (Fig. 3D).

In addition, we studied the effect of SPX on serum levels of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β which also serve as surrogate markers of nonalcoholic fatty liver disease (NAFLD). We found the decrease in IL-6 and TNF-α concentrations after SPX treatment in the T2DM group compared to the corresponding control (Fig. 3E, F, P < 0.05). No other differences were found in all the investigated parameters (TNF-α, IL-6, and IL-1β) after SPX in the DIO and healthy mice. What was surprising after SPX treatment we did not observe statistically significant changes in TNF-α and IL-6 in T2DM compared to healthy groups of mice, while in T2DM vehicle these changes were noted (P < 0.01).

3.5. SPX improves liver functions

SPX decreased the activity of liver markers (ALT, AST, and GGT) and liver mass and therefore we examined changes in liver composition and genes expression. Specifically, we measured the expression of key genes related to carbohydrate–lipid metabolism (Pepck, G6pc, Pgc-1α, and Fasn), as well as proinflammatory markers such as IL-6 and TNF-α.

First, we investigated the effect of SPX on liver mass. We noted the lower liver mass in DIO (Fig. 4A, P < 0.05) and decreasing trend in T2DM (Fig. 4A, P = 0.08) after SPX. Despite the apparent effect of SPX, the weight of the livers in the DIO and T2DM groups differed statistically significantly compared to healthy mice. Similar effect was observed analyzing liver TG content. We noted the statistically significant decrease in liver TG after SPX in DIO and T2DM mice compared to corresponding control (Fig. 4B, P < 0.05). In analyzing cholesterol content in liver we noted that SPX treatment decrease liver cholesterol in T2DM mice (Fig. 4C, P < 0.05), whereas in DIO and healthy mice we did not observe any differences after SPX. In the last part of our analysis we investigated the effect of SPX on glycogen content in liver. We found the statistically significant increase of glycogen content in DIO mice after SPX (Fig. 4D, P < 0.01).

These changes corresponded to changes in mRNA expression, while no differences were observed in the investigated genes (Pepck, G6pc, Pgc-1α, and Fasn) in the healthy group. In the DIO mice, the increase in Pgc-1α gene expression (Fig. 4H; P < 0.01) was observed. Additionally investigating differences between experimental models we observed statistically significant changes between healthy vehicle, healthy SPX and DIO SPX vs. T2DM groups (Fig. 4H; P < 0.01) as well as between healthy vehicle vs DIO vehicle (Fig. 4H; P < 0.01). Moreover, the statistically significant differences were observed between healthy group and DIO vehicle as well as between DIO vehicle and T2DM SPX group in

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Fig. 4. Terminal changes in the liver mass (A) and composition: (B) triglycerides, (C) cholesterol and (D) glycogen content in mice. Panel (E) present representative pictures from Liver H&E staining. (F) Pepck, (G) G6pc, (H) Pgc-1α, (I) Fasn mRNA expression in the liver. (J) Hepatic IL-6 and (K) TNF-α protein level. The data are presented as median with box and whiskers representing the interquartile range and all values (Protein expression in liver n = 7 per group; mRNA expression n = 6 per group; liver composition min. n = 8 per group). Statistically significant changes in groups are marked *(P < 0.05) and **(P < 0.01) compared to the corresponding control receiving PBS, a (P < 0.05), A (P < 0.01) compared healthy PBS group; b (P < 0.05), B (P < 0.01) compared healthy SPX group; c (P < 0.05), C (P < 0.01) compared DIO PBS group; d (P < 0.05), D (P < 0.01) compared DIO SPX group.
**Fasn** mRNA expression. There were no other statistical changes in mRNA expression of the studied genes. Furthermore, we investigated the effect of SPX on TNF-α and IL-6 levels in the liver (as surrogate parameters of NASH). We found the decrease in IL-6 in the T2DM group after SPX treatment (**Fig. 4J, P < 0.05**) and TNF-α (**Fig. 4K, P < 0.01**). No statistically significant changes were observed in the healthy mice. Despite this decrease hepatic IL-6 and TNF-α the higher level of these markers was still noticeable in the diabetic group compared to the healthy mice groups (**Fig. 4J and K, P < 0.05**).

4. Discussion

Obesity, insulin resistance, and diabetes are associated with changes in the circulating level of SPX (**Al-Daghri et al., 2018a, 2019a; Karaca et al., 2019; Kołodziejski et al., 2018; Walewski et al., 2014**). Most of the aforementioned studies showed a decrease in SPX concentration accompanying the T2DM and obesity. However, the role of SPX in the context of DIO and T2DM has been poorly documented so far. Here, we report that long-term SPX injections improves the metabolic abnormalities associated with obesity and type 2 diabetes in mice. SPX decreased body mass, body composition, and carbohydrate and lipid metabolism. Most importantly, SPX treatment decreases body fat in the DIO and T2DM groups, and improves insulin sensitivity.

Obesity and T2DM are caused by metabolic disorders related to nutrition as well as energy management of the body (**Hamilton et al., 2007**). Our data clearly show that 30-day SPX treatment decreased body weight in obese and healthy mice, which confirms the results of a previous study by **Walewski et al. (2014)**. Interestingly, such changes were not observed in the T2DM mice. However, this may be due to the decrease in the body weight of T2DM mice in the first few days, which was related to the progression of this pathological state while in the following days of the experiment, the body mass remained stable (**Kim et al., 2016**).

We and others showed that SPX is one of the most downregulated genes in obesity. The exposition of preadipocytes to SPX caused a decrease in the differentiation of cells to mature adipocytes, stimulation of lipolysis in adipocytes as well as reduced adipose tissue inflammation (**Gambaro et al., 2020; Kołodziejski et al., 2018; Walewski et al., 2014**). In all three experimental animal groups in our current study, the body fat mass declined in the groups receiving SPX, which corresponds directly with the results reported by Gambaro, who showed that adipocyte size was reduced in obese mice after 10 days of SPX treatment (**Gambaro et al., 2020**). Moreover, we also noted an increase in the lean tissue mass in the DIO mice after SPX treatment, which may suggest tissue specific effects of SPX on fat and muscle. However, these are only speculations which need to be proved in a separate study.

It is well known that both body weight and composition affect insulin resistance (**Yki Jarvinen and Koivisto, 1983**). Therefore, we investigated how changes in body weight and body composition can influence glucose-related metabolic changes. In ipGTT and ipITT, SPX improved insulin sensitivity and increased glucose utilization in the DIO and T2DM mice. These effects were also confirmed by the results of HOMA-IR and QUICKI. However, in the healthy group, only differences in ipITT were observed between SPX and control mice. Animals that received SPX were characterized by improved insulin sensitivity. Gu et al. showed previously that the concentration of SPX was lower in T2DM patients and, in contrast to the glucose level, SPX was decreased during GTT (**Gu et al., 2015**). Other studies have also confirmed that the levels of SPX are reduced in type 2 diabetes and obesity (**Al-Daghri et al., 2018b, 2019b; Karaca et al., 2019**). Taken together, our results supported by the results of others suggest that SPX may be one of the factors involved in pathological abnormalities associated with T2DM.

Previous studies indicated that the SPX level is correlated with other hormones. Therefore, we also investigated the effect of SPX on biochemical and hormonal profile of animals. Our results show that SPX injection lowered the level of serum leptin in T2DM and obese mice, and increased adiponectin secretion. These data indicate that SPX improves metabolic abnormalities associated with in obesity and T2DM by directly affecting the endocrine functions of the adipose tissue, however, this requires further research. In 2014, Walewski et al. demonstrated that SPX is one of the most downregulated genes in the fat tissue during obesity in mice and humans (**Walewski et al., 2014**) and indicated that SPX/GALR2 system could be involved in pathophysiological changes in obesity. (**Bitarafan et al., 2019; Gambaro et al., 2020; Kołodziejski et al., 2018; Walewski et al., 2014**) The results of Walewski’s study as well as others confirmed that SPX is negatively correlated with the level of leptin during obesity (**Bitarafan et al., 2019; Gambaro et al., 2020; Kołodziejski et al., 2018; Walewski et al., 2014**). However, these studies involved only short-term administration of SPX, which is different in comparison to our experimental design. Here, we show for the first time that the leptin-lowering effect of SPX is maintained even after a long-term treatment.

Adiponectin is a protective adipokine that reduces inflammation and insulin resistance (**Gokulakrishnan et al., 2015; Radin et al., 2009; Trayhurn and Wood, 2004**), and its concentration is downregulated during obesity and T2DM. Our previous research showed a positive correlation between the concentrations of adiponectin and SPX in obese women (**Kołodziejski et al., 2018**). The results of the present study demonstrate that the decrease of leptin concentration in the continuously SPX-treated groups was accompanied by an increase in adiponectin concentration, which directly indicates that SPX improves the fat endocrine function and metabolism.

T2DM and obesity are associated with various comorbid conditions such as NAFLD or NASH (**Kneeman et al., 2012; Paschos and Paletas, 2009**). In the pathogenesis of NAFLD/NASH, an excessive lipid accumulation, as well as lipotoxicity, due to free fatty acids and high serum level of LDL cholesterol is highly relevant. Therefore, we investigated the effect of SPX on the negative changes associated with NAFLD/NASH development like changes in the expression of genes encoding proteins related to lipid and carbohydrate metabolism (**Min et al., 2012**). Previous studies indicated that SPX decreased total lipids in obese mice (**Jasmine et al., 2016**). On the other hand, other studies did not show any effect of SPX on lipid content in the liver (**Gambaro et al., 2020**). Our results clearly indicated that SPX injection decreased the content of cholesterol and TG in the liver of DIO and T2DM mice, in addition to a decrease in liver weight, which confirms the results reported by **Jasmine et al. (2016)**. The differences in these results are caused by differences in the duration of SPX administration as well as by different animal model.

**Pepck, G6pc, Pgc-1α, and Fasn** are the key genes involved in lipid and carbohydrate metabolism of the liver. Increased gluconeogenesis in obesity and T2DM is caused by impaired action mechanisms of insulin and glucagon (**Basu et al., 2005; Magnusson et al., 1992**). Increased glucagon levels, accompanying insulin resistance, cause an increase in gluconeogenesis processes, resulting in pathological increases in blood glucose levels (**Haerdal et al., 2018**). Moreover, previous results showed that glucagon immunoneutralization by antibodies or aptamers improved the metabolic conditions of T2DM mice by, among others, the reduction of gluconeogenesis (**Rinnes et al., 2015; Vater et al., 2013**). **Pepck** and **G6pc** are gluconeogenic genes responsible for hepatic glucose production. Obesity- and T2DM-induced expression of these genes in the liver indicates enhanced gluconeogenesis (**Du et al., 2018**). Our results show that long-term SPX injection caused increased glycogen storage in the liver as well as downregulation of **Pepck** and **G6pc** mRNA expression in the liver. These changes were accompanied by a decreased expression of **Fasn** gene, resulting in a decreased fat content in the liver. Increased hepatic fat content is a hallmark of NAFLD and NASH. Additionally, NASH is characterized by an inflammation, which is associated with a high level of hepatic TNF-α and IL-6. Previously, it was shown that hepatic IL-6 and TNF-α play important roles in the pathophysiology of NASH and that a clear correlation exists between the levels of these cytokines and NAFLD (**Abiru et al., 2006; Ma et al., 2018; Wieckowska et al., 2008; Yamaguchi et al., 2010**). Consistently,
reduction of these cytokines in the liver as well as in serum is correlated with improved liver metabolism. Our results suggest that SPX improves liver functions by affecting the liver carbohydrate and lipid metabolism, as well as by reducing the levels of proinflammatory cytokines such as IL-6 and TNF-α.

Our research has several limitations. First, there is the lack of evidence showing the effect of intracellular mechanisms on the SPX function in the liver; however, we aim to explore this in our next study (in vitro). Another limitation of our study is the use of a single T2DM animal model generated by the combination of HFD feeding and STZ injections. However, HFD as well as HFD/STZ animal models are well established. The number of analyses performed is also limited by the amount of research material that can be obtained from mice, i.e. serum volume or tissue size. Previously it was shown that SPX activity is regulated via two isoforms of galanin receptor – GaIR2 and GaIR3 (Kim et al., 2014). However, based on our results we are not able to identify the receptor through which SPX exerts its positive effects. This is because we did not decide to use specific blockers of this receptors isoform that would allow us identify signaling pathways and determine whether both receptors or only one of them exert this effect, which can also be seen as a limitation in our study. However, research into the physiological role of SPX is very limited and is at an early stage. Despite these limitations, we believe that the obtained results will provide a new perspective on this peptide and indicate new directions for its research.

In summary, we found that 30-day SPX injections decreased the body weight in healthy and DIO mice as well as the body composition in healthy, DIO, and T2DM mice. Moreover, SPX administration increased insulin sensitivity and improved the hormonal and metabolic status of DIO and T2DM animals. Additionally, we found that SPX modulated the liver composition and expression of genes involved in the regulation of carbohydrate–lipid pathways (Pepck, G6pc, Pgc-1a, and Fasn) in the T2DM mice and Pgc-1α and Fasn in the DIO mice.

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Ethics

Study was approved by the Local Ethical Commission for Investigation on Animals, Poznanski University of Life Sciences (Permission No. 36/2017).

CRediT authorship contribution statement

Pawel A. Kolodziejki: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition, Supervision. Natalia Leciejewska: Investigation. Agata Chmurzynska: Investigation. Maciej Sassek: Investigation. Aleksandra Szczepankiewicz: Investigation. Dawid Szczepankiewicz: Investigation. Emilian Malek: Investigation. Mathias Z. Strowski: Supervision. Zuzanna Cecinska-Maciejewska: Investigation. Krzysztof W. Nowak: Supervision. Ewa Prusynska-Oszmolek: Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.
