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Age-related effects of fenofibrate on the hepatic expression of sirtuin 1, sirtuin 3, and lipid metabolism-related genes

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Background: Sirtuin 1 (Sirt1) and sirtuin 3 (Sirt3) participate in the regulation of lipid metabolism. Our aim was to investigate the effects of the hypolipemic drug fenofibrate (FN) on hepatic Sirt1 and Sirt3 expression, in relation to the expression of lipid metabolism-related genes and in the context of aging. Methods and Results: Young and old male Wistar rats were fed standard chow or supplemented with 0.1% or 0.5% FN for 30 days (*n*=7–10 in each group). In young rats, 0.1% FN did not affect Sirt1 expression, however, 0.5% FN decreased Sirt1 and both doses reduced Sirt3 protein levels. In old rats, 0.5% FN decreased hepatic *Sirt1* mRNA and both doses reduced Sirt1 protein levels, but not Sirt3 expression. Although hepatic Pparα protein levels did not change, FN treatment of young rats induced *Cpt1b* expression, whereas *Lcad*, *Acox1*, *Pmp70*, and *Hmgcs2* expression increased only after 0.1% FN, and *Fas2* expression decreased after 0.5% FN. In the liver of old rats, both doses increased *Cpt1b* and *Lcad* expression. Only 0.1% FN increased *Pmp70* and *Hmgcs2* expression, and only 0.5% FN increased *Acox1* and *Fas2* mRNA levels. Conclusions: Treatment with fenofibrate at low or high doses may downregulate the expression of Sirt1 and Sirt3 proteins in the rat liver. The dosage of FN affects molecular changes, and aging alters the response to 0.5% FN.

Key words: aging; liver; fenofibrate; sirtuin; lipid metabolism

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Abbreviations: Acox1, acyl-coenzyme A oxidase; Acl, ATP-citrate lyase; Cpt1b, carnitine palmitoyltransferase 1b; Fas2, fatty acid synthase 2; FGF21, fibroblast growth factor 21; FN, fenofibrate; HFD, high-fat diet; Hmgcs2, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; Lcad, long-chain fatty acid dehydrogenase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Pmp70, peroxisomal membrane protein 70; PPARα, peroxisome proliferator-activated receptor alpha; Sirt1, sirtuin 1; Sirt3, sirtuin 3; SREBP, sterol regulatory element-binding protein

INTRODUCTION

Aging decreases the ability to respond to stress and leads to the accumulation of molecular damage (Pyo *et al.*, 2020). Aging-associated changes in energy metabolism and nutrient sensing pathways may lead to disorders in lipid homeostasis, which manifest as elevated concentrations of triglycerides, total cholesterol, and LDL cholesterol in blood plasma (Liu & Li, 2015). The hypolipemic drug fenofibrate (FN) is used to counteract disturbances in lipid metabolism. FN decreases serum triglyceride and VLDL- and LDL-cholesterol levels, while it increases serum HDL-cholesterol (Sahebkar *et al.*, 2017). In the liver, FN stimulates fatty acid oxidation in mitochondria and peroxisomes through the induction of peroxisome proliferator-activated receptor alpha (PPARα) (Gebel *et al.*, 1992). FN exerts additional beneficial effects through PPARα-dependent and independent mechanisms, e.g. in cardiac, renal, and vascular disorders (Balakumar *et al.*, 2019). FN may thus offer new treatment venues for conditions besides hyperlipidaemia. However, it has not been well investigated whether FN affects the expression of sirtuins in the liver.

Sirtuins (SIRT1-SIRT7) are a family of NAD^+-dependent deacetylases, which target histones and non-histone proteins (Zhao *et al.*, 2020). Sirtuins can protect cells against metabolic and oxidative stresses, and may therefore safeguard against some aging-associated alterations, as evidenced by genetic studies in mice (Rodgers & Puigserver, 2007; Pfluger *et al.*, 2008; Zhao *et al.*, 2020). The relationships between the activity of sirtuins and energy metabolism make them a potential therapeutic target for the treatment of metabolic diseases. Two members of the sirtuin family are particularly important for lipid metabolism. SIRT1, the best-studied member of the sirtuin family, decreases blood plasma cholesterol and triglyceride concentrations by inhibitory deacetylation of sterol regulatory element-binding proteins (SREBP) in the liver (Walker *et al.*, 2010). The SREBP belong to transcription factors that control lipid homeostasis by promoting the expression of lipogenic (e.g. fatty acid synthase, FAS) and cholesterogenic genes in the fed state (Walker *et al.*, 2010). Moreover, SIRT1 deacetylates peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) in the liver (Rodgers *et al.*, 2005; Rodgers & Puigserver, 2007), skeletal muscle and adipose tissue (Feige *et al.*, 2008). This post-translational regulation of PGC-1α leads to the induction of gluconeogenic genes and glucose output in the liver (Rodgers *et al.*, 2005) and enhances oxidative metabolism in muscles and brown adipose tissue (Feige *et al.*, 2008). SIRT1 also interacts with the transcription factor PPARα at the PPAR response element (PPRE) sequence of target genes (e.g. fibroblast growth factor type 21, FGF21). Upon this interaction with SIRT1, PPARα mediates the adaptive response to fasting and starvation by transcriptional activation of genes involved in fatty acid oxidation (Purushotham *et al.*, 2009).

The mitochondrial sirtuin SIRT3 regulates multiple mitochondrial enzymes both at the transcriptional level and through their reversible deacetylation in mitochondria. SIRT3 is induced during fasting, and

through deacetylation increases the activity of long-chain acyl-coenzyme A dehydrogenase (LCAD), a key enzyme of mitochondrial fatty acid β-oxidation (Hirschey *et al.*, 2010). Similarly, SIRT3 deacetylates and activates 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) (Shimazu *et al.*, 2010), the rate-limiting enzyme in the ketone body β-hydroxybutyrate synthesis. In addition, SIRT3 activates enzymes of the tricarboxylic acid (TCA) cycle and the urea cycle, and regulates reactive oxygen species levels (reviewed by Zhao *et al.*, 2020). Thus, SIRT3 activity is involved in the regulation of multiple metabolic pathways in mitochondria.

Importantly, both SIRT1 and SIRT3 may mitigate metabolic dysfunctions which are often associated with aging. Transgenic models have shown that increased SIRT1 activity protects against insulin resistance, type 2 diabetes (T2D) and diabetic complications, high-fat diet-induced hepatic steatosis, and adipose tissue inflammation (Rodgers & Puigserver, 2007; Pfluger *et al.*, 2008; Guo *et al.*, 2015). Mice with whole-body SIRT3 knock-out showed peripheral insulin resistance due to increased fatty acid oxidation and reduced glucose uptake in skeletal muscle (Lantier *et al.*, 2015). When SIRT3 KO mice were fed a high-fat diet (HFD) they developed hyperlipidemia, impaired glucose tolerance, insulin resistance, and steatohepatitis (Hirschey *et al.*, 2011).

Despite the well-established roles of SIRT1 and SIRT3 in the control of fuel metabolism and suppression of some mechanisms of aging, there is limited data on the expression of these sirtuins in response to pharmacological challenge with fenofibrate, and in the context of ageing. It was reported that Sirt1 protein levels were upregulated by FN in TNFα-treated adipocytes (Wang *et al.*, 2013), the kidney (Kim *et al.*, 2016), endothelial cells (Wang *et al.*, 2014), and cardiac muscle (Liu *et al.*, 2016) in animal models, and Sirt3 protein abundance decreased in the mouse liver after bezafibrate (100 mg/kg/day for 4 weeks) (Barger *et al.*, 2017). However, it is not clear whether FN affects Sirt1 or Sirt3 levels in the liver. We, therefore, undertook to examine: (1) the effect of aging on hepatic expression of Sirt1 and Sirt3 in the rat; (2) whether FN affects the hepatic expression of Sirt1 and Sirt3 in non-obese, normolipemic rats; and (3) whether this effect of FN is modulated by physiological aging. In our previous work, we have demonstrated that FN had a significant hypolipemic effect in normally-bred old rats (Zubrzycki *et al.*, 2020). Therefore, in the present study, we decided to investigate the molecular changes brought about by FN treatment in rat liver in old as compared to young rats bred in standard, non-obesogenic conditions. We examined the gene expression of factors that play key roles in the regulation of lipid metabolism (Murray *et al.*, 2012): the transcriptional regulators *Ppara*, *Pgc1a*, and *Fgf21*; the mitochondrial enzymes carnitine palmitoyltransferase 1b (*Cpt1b*), long-chain fatty acid dehydrogenase (*Lcad*), as well as the rate-limiting ketogenic enzyme *Hmgcs2*. We also determined the expression of genes encoding the peroxisomal acyl-CoA oxidase (*Acox1*) and the organelle's marker, peroxisomal membrane protein 70 (*Pmp70*). Moreover, we investigated the expression of genes encoding the lipogenic enzymes ATP-citrate lyase (*Acl*) and fatty acid synthase 2 (*Fas2*). In this paper, we demonstrate that depending on the dose, fenofibrate differently affects the expression of genes involved in lipid metabolism and that aging affects the response to high-dose fenofibrate treatment.

MATERIALS AND METHODS

Animals, treatment, and material sampling

Young (4-month-old) and old (24-month-old) male Wistar-Han rats were bred in the Academic Animal Experimental Center in Gdańsk, Poland, and housed one *per* cage in standard breeding conditions (22±2°C, humidity 55±10%, 12 h/12 h light/dark cycle). For 30 days, the animals were given 0.5% FN (Glentham Life Sciences, Corsham, UK) mixed into standard rodent chow (high dose; n=10 young and 9 old animals). Control animals (n=10 young and 7 old) were fed the same chow without supplementation (Labofeed H, Wytwornia Pasz Morawski, Kcynia, Poland). The dose was expected to provide approximately 100 mg/day FF according to the measured baseline food intake (20.7±2.43 g, mean ± S.D., for the young and 23.4±4.97 g for the old rats). With respect to the mean body weight at the onset of the treatment (395 g for the young and 552 g for the old rats), the 0.5% FN dose was equivalent to 260 and 210 mg/kg body weight in young and old rats, respectively. Because the treatment with 0.5% FN had some negative effects on the histology of the liver in both young and old rats (Zubrzycki *et al.*, 2020), in a separate experiment we treated rats with 0.1% FN for 30 days (low dose; n=9–10 animals in each group) in the same breeding conditions as for 0.5% FN. At the end of the experiments, the animals were sacrificed under full isoflurane anesthesia through exsanguination from heart puncture. Liver samples were collected and immediately frozen in liquid nitrogen for molecular analyses. The experimental procedures were approved by the Local Ethics Committee in Bydgoszcz, Poland (protocols No. 41/2017, 58/2017, 40/2018, and 5/2019), and carried out accordingly.

Quantitative polymerase chain reaction (qPCR)

The mRNA levels of *Acox1*, *Acl*, *Cpt1b*, *Fas2*, *Fgf21*, *Hmgcs2*, *Lcad*, *Pgc1a*, *Pmp70*, *Ppara*, *Sirt1*, and *Sirt3* were assessed in liver samples by qPCR method (StepOne Plus apparatus, Life Technologies-Applied Biosystems, Grand Island, NY, USA). Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland) was used for RNA extraction according to the manufacturer's protocol, and the obtained RNA was stored at –80°C. 1 μg RNA was reverse-transcribed (RT) with M-MuLV RT enzyme (Thermo Fisher Scientific, Fitchburg, WI, USA) and oligo(dT)$_{18}$ primers (Sigma-Aldrich, Munich, Germany). 5× diluted cDNA was used in qPCR reactions with SensiFastSybr™ No-Rox reagent (Bioline, London, UK) and primers at 200 nM final concentration. mRNA levels were quantified relative to the expression of the geometric mean of acidic ribosomal phosphoprotein P0 (*36B4*) and cyclophilin A (*CyclоA*) levels, and analysed using the ΔΔCt method. The primers were designed using Primer3Plus software based on BLAST, Ensembl, and AceView databases (Table 1).

Western blotting (WB)

The semi-quantification of Sirt1, Sirt3, and Pparα proteins in liver samples (6 animals per group) was performed using the WB method. Whole-cell lysates were prepared with Mammalian Cell Extraction Kit (BioVision, Milpitas, CA, USA). Protein samples were separated by 10% SDS-PAGE, transferred to PVDF membranes (Bio-Rad, Warsaw, Poland), and blocked with

Table 1. Primers used in qPCR.

Gene name and symbol	Primer sequence [5′→3′]
Acidic ribosomal phosphoprotein P0 (36B4)	F: CTCAGTGCCTCACTCCATCA R: GGGGCTTAGTCGAAGAGACC
Acyl-CoA oxidase 1 (Acox1)	F: GTCTCTTGTATTCTCTCTATGG R: GTAAGATTCATGGACCTCTG
ATP-citrate lyase (Acl)	F: ATGGCAACACCCTCGTAGAC R: CTCACACGGAAGCTCATCAA
Carnitine palmitoyltransferase 1b (Cpt1b)	F: ATGTTTGACCCAAAGCAGTACCC R: TCGCCTGCGATCATGTAGGAAAC
Cyclophilin A (CycloA)	F: TGTCTCTTTTCGCCGCTTGCTG R: CACCACCCTGGCACATGAATCC
Fatty acid synthase 2 (Fas2)	F: TACGGTCTGCAGTGCACCCA R: GGTCAGCTTTGCCCGGTAGC
Fibroblast growth factor type 21 (Fgf21)	F: AGTTTGGGGGTCAAGTCCGA R: AGGAGACTTTCTGGACTGCCGG
Hydroxy-methylglutaryl-CoA synthase 2 (Hmgcs2)	F: ACCACAAGGTGAACTTCTCTC R: TTTGGGTAACGGCTCTGCTC
Long-chain fatty acid dehydrogenase (Lcad)	F: TCGAGCAGTTTATCCCCCAG R: TGAACACCTTGCTTCCATTGAG
PPAR gamma coactivator-1 alpha (Pgc1α)	F: CACGTTCAAGGTCACCCTACAGC R: TAAATCACACGGCGCTCTTCAAT
70 kDa peroxisomal membrane protein (Pmp70)	F: TGTTCTGCCTGCTCCACAAG R: CACCACAGCTCGCTCTTTCT
Peroxisome proliferator-activated receptor alpha (Ppara)	F: CTATAATTTGCTGTGGAGATCG R: CTACCATCTCAGGAAAAATAG
Sirtuin 1 (Sirt1)	F: CAGAACCACCAAAGCGGAAAAA R: GAAACCCCAGCTCCAGTCAGAA
Sirtuin 3 (Sirt3)	F: AAGCTGGTTGAAGCTCATGGGTC R: TCCAGGGAGGTCCCAAGAATGAG

F, forward primer; R, reverse primer

7% non-fat milk in TBS with 0.1% Tween20 (TBST) for 1 h. The membranes were incubated with the following primary antibodies at 4°C overnight: anti-Sirt1 (Bioss, bs-0921R; Woburn, MA, USA), anti-Sirt3 (Bioss, bs-6105R), or anti-Pparα (Bioss, bs-3614R) rabbit polyclonal antibodies, diluted 1:1 000 in 3% non-fat milk in TBST. After that, the membranes were washed and next incubated with HRP-conjugated anti-rabbit secondary antibodies (1:10 000 in 3% non-fat milk in TBS) (Merck Millipore, A9169; Darmstadt, Germany) for 2 h at room temperature. Bands were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and developed using ImageQuant LAS 500 Chemiluminescence CCD Camera (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Sirt1, Sirt3, and Pparα protein levels were analyzed relative to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) levels (1:50 000 in 3% non-fat milk in TBST, 1 h at room temperature) (Merck Millipore, AB2302) using QuantityOne Software (Bio-Rad).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (v. 6.0; San Diego, CA, USA). Data were analyzed for outliers using the Grubbs' test. Because not all sets of data followed a normal distribution (Shapiro-Wilk test), statistical analysis was performed using the nonparametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons test. Data are presented as mean ±S.D. and normalized to young control animals (expression level=1). Data obtained for control rats on normal diet in the two separately conducted experiments testing 0.1% and 0.5% FF were combined after checking that there were no significant differences between the control groups. Statistical significance was set at $p<0.05$.

RESULTS

Sirt1 and Sirt3 expression at the mRNA and protein level

Young and old control rats did not differ with respect to the hepatic mRNA and protein expression levels of Sirt1 and Sirt3 (Fig. 1).

In young rats, FN treatment at either dose did not affect *Sirt1* and *Sirt3* mRNA levels in the liver (Fig. 1A). Even though *Sirt1* expression was notably upregulated in a subset of rats treated with 0.1% FN, no statistical significance was found. In old rats, 0.1% FN did not cause any changes in *Sirt1* expression, but 0.5% FN decreased *Sirt1* expression 6.25-fold ($p<0.01$; Fig. 1B). FN did not cause any changes in *Sirt3* expression in the liver of old rats (Fig. 1B).

In the liver of young rats, 0.1% FN did not affect Sirt1 protein expression; however, 0.5% FN treatment decreased hepatic Sirt1 protein level by 3.33-fold ($p<0.05$; Fig. 1C). Moreover, 0.1% and 0.5% FN decreased Sirt3 protein levels 3.86-fold and 3.33-fold, respectively ($p<0.05$ and $p<0.001$, respectively; Fig. 1C).

In old rats, 0.1% and 0.5% FN decreased hepatic Sirt1 protein level 3.03-fold and 2.86-fold, respectively ($p<0.05$ for both; Fig. 1D). However, neither dose of FN affected Sirt3 protein level in the liver of old rats (Fig. 1D).

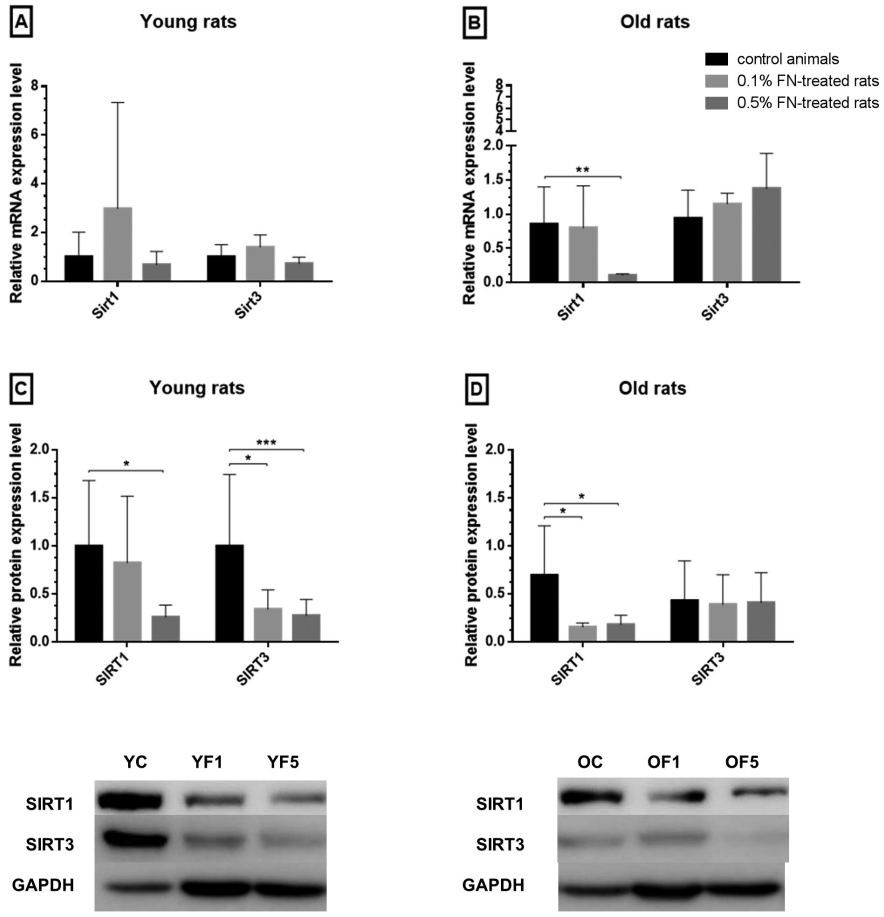

Figure 1. Relative mRNA (A & B) and protein (C & D) expression of Sirt1 and Sirt3 in the liver of control and fenofibrate (FN)-treated young and old rats.
The mRNA and protein expression levels were determined by qPCR and Western blotting, respectively, with representative blots shown. Data are mean ± S.D., presented relative to the level in young control rats (set as 1), n=6–10 animals *per* group. For clarity, data from young and old rats are separated into two graphs, with the scale maintained. *p<0.05, **p<0.01, ***p<0.001 (Kruskal-Wallis ANOVA). Abbreviations: *YC* young (4-month-old) control rats; *YF1* young rats treated with 0.1% FN; *YF5* young rats treated with 0.5% FN; *OC* old (24-month-old) control rats; *OF1* old rats treated with 0.1% FN; *OF5* old rats treated with 0.5% FN

Ppara expression

In old as compared to young rats, hepatic *Ppara* gene expression was 4-fold higher (p<0.001), while Pparα protein levels were comparable in the two groups (Fig. 2).

In the liver of young rats, 0.1% FN increased *Ppara* expression by 3-fold (p<0.05), while 0.5% FN was without effect (Fig. 2A). In the liver of old rats, 0.1% FN did not affect *Ppara* expression, whereas 0.5% FN treatment decreased *Ppara* expression by 5-fold (p<0.01; Fig. 2A).

In both young and old rats, neither dose of FN changed the Pparα protein level in the liver (Fig. 2B).

The effects of fenofibrate on the expression of lipid metabolism-related genes in the liver of young and old rats

In our study, aging was not associated with any changes in the hepatic mRNA levels of *Cpt1b, Lcad, Pgc1a, Hmgcs2*, *Acox1*, *Pmp70*, *Acl*, *Fas2*, and *Fgf21* (Fig. 3).

In young rats, FN treatment increased the hepatic expression of genes encoding enzymes involved in mitochondrial β-oxidation of fatty acids. 0.1% and 0.5% FN increased *Cpt1b* expression levels by 100-fold and 90-fold, respectively (p<0.001 for both; Fig. 3A) and 0.1% FN increased *Lcad* expression by 1.5-fold (p<0.01). However, neither dose of FN affected *Pgc1a* expression (Fig. 3A).

The expression of the key ketogenic gene, *Hmgcs2*, in the liver of young rats increased 3.5-fold (p<0.001; Fig. 3C) upon treatment with 0.1% FN; however, 0.5% FN was without effect. With regard to peroxisomal β-oxidation, the hepatic expression of *Acox1* increased 22-fold (p<0.01; Fig. 3C) in rats treated with 0.1% FN, with no effect of 0.5% FN. Similarly, only 0.1% FN highly increased *Pmp70* expression (p<0.05; Fig. 3C).

In young rats, the hepatic expression of the lipogenic gene *Acl* was not affected by FN treatment, while the expression of the key lipogenic gene *Fas2* decreased 4-fold (p<0.05) after treatment with 0.5% FN, with no effect of 0.1% FN (Fig. 3E).

In the liver of old rats, 0.1% and 0.5% FN increased *Cpt1b* expression 15-fold and 50-fold, respectively (p<0.01 for both) and increased *Lcad* expression 6-fold and 2.5-fold (p<0.001 and p<0.05, respectively; Fig. 3B). Similarly as in the liver of young rats, the mRNA levels of *Pgc1a* were not affected by FN treatment (Fig. 3B).

The expression of the ketogenic gene *Hmgcs2* in the liver of old rats increased 3.2-fold (p<0.01; Fig. 3D) upon treatment with 0.1% FN (but not 0.5% FN). In old rats, 0.1% FN did not affect *Acox1* mRNA level, while 0.5% FN increased its expression 17-fold (p<0.001;

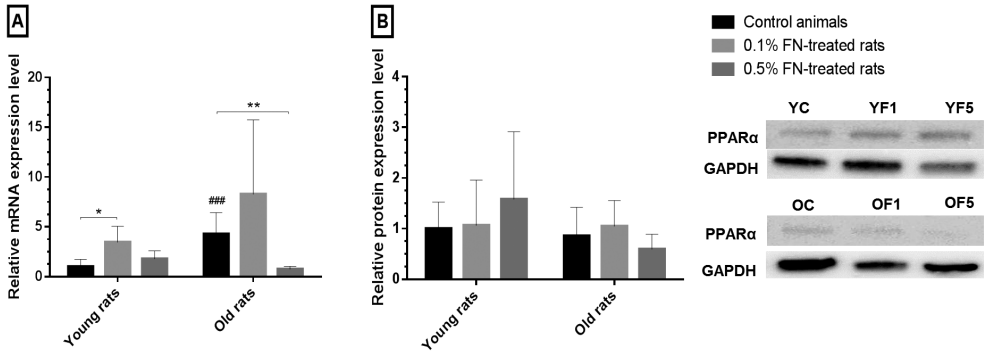

Figure 2. Relative mRNA (A) and protein (B) expression of Pparα in the liver of control and fenofibrate-treated young and old rats.
The mRNA and protein expression levels were determined by qPCR and Western blotting, respectively. Representative blots are shown in (B). Data are mean ± S.D., presented relative to the level in young control rats (set as 1), *n*=6–10 animals *per* group. **p*<0.05; ***p*<0.01; ****p*<0.001 (Kruskal-Wallis ANOVA). Abbreviations as in the description of Fig. 1.

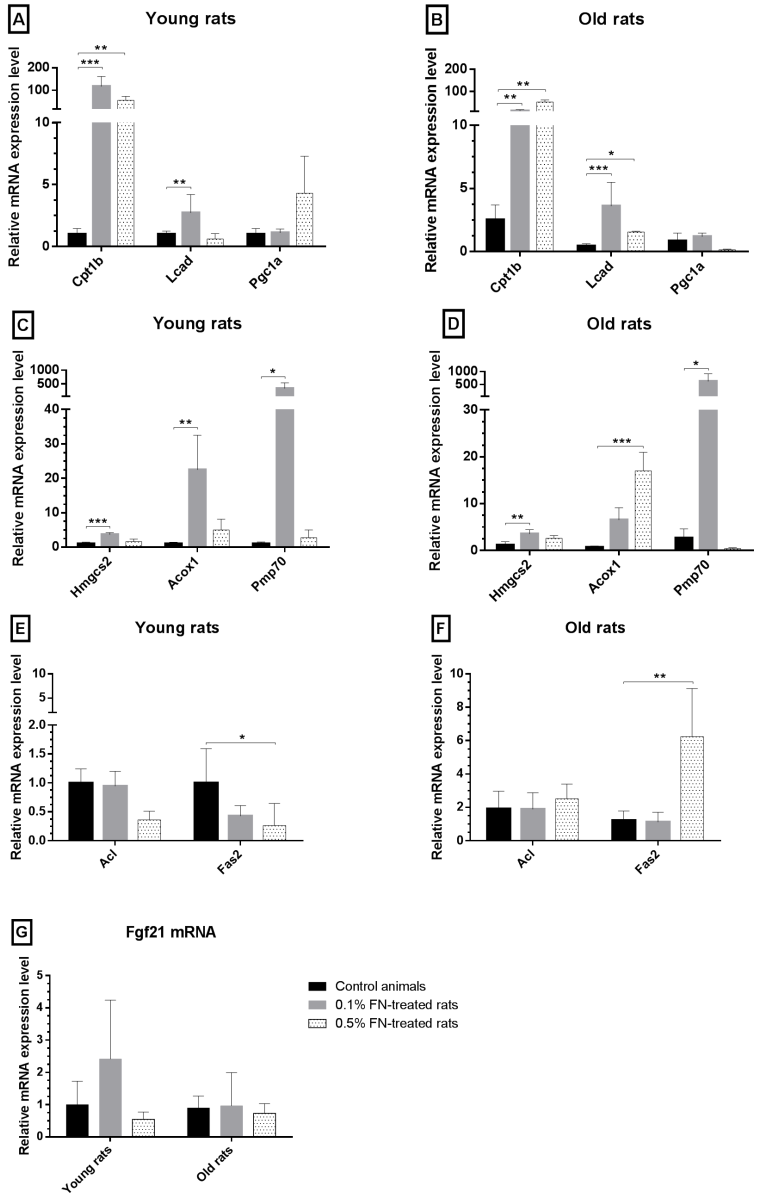

Figure 3. Relative mRNA expression of genes involved in lipid metabolism and Fgf21 in the liver of control and fenofibrate-treated young and old rats.
A & B: *Cptb1*, *Lcad*, and *Pgc1a*. **C & D**: *Hmgcs2*, *Acox1*, and *Pmp70*. **E & F**: *Acl* and *Fas2*. **G**: *Fgf21*. For clarity, data from young and old rats are separated into two graphs (except for G), with the scale maintained. The mRNA expression levels were determined by qPCR and presented as mean ± S.D. relative to young control rats (set as 1), *n*=7–10 animals per group. **p*<0.05, ***p*<0.01, ****p*<0.001 (Kruskal-Wallis ANOVA)

Fig. 3D). Only 0.1% FN highly increased *Pmp70* expression ($p<0.05$; Fig. 3D) in the liver of old rats.

The treatment of old rats with 0.5% FN did not alter hepatic *Acl* expression. However, in contrast to young animals, in old rats treated with 0.5% FN the hepatic expression of *Fas2* increased 6-fold ($p<0.01$; Fig. 3F).

The expression of *Fgf21* was not affected by FN treatment in either young or old rats (Fig. 3G).

DISCUSSION

We present a comprehensive analysis of Sirt1 and Sirt3 expression in the liver, the key organ of lipid metabolism, upon 30-day treatment of young and old rats with two doses of FN, i.e. 0.1% (low dose) and 0.5% (high dose). We demonstrate that these two doses of FN differently affected the expression of genes involved in lipid metabolism, with aging affecting the response to 0.5% FN.

Sirt1 and Sirt3 have both been associated with protection against age-related diseases, including metabolic derangements (reviewed by Zhao *et al.*, 2020). Therefore, we first examined whether aging affects the hepatic expression of these sirtuins. We found no age-related differences in either mRNA or protein levels of Sirt1 and Sirt3. Similarly, no effect of aging on Sirt1 protein level was observed in the liver of 19–26 month-old C57BL/6 mice compared to young, 2–4 month-old animals, even though there were reductions in other tissues (Xu *et al.*, 2020). In contrast, Jin and others (Jin *et al.,* 2011) reported that in the liver of old mice Sirt1 mRNA and protein expression decreased due to repression of the gene promoter by CCAAT/Enhancer Binding Protein/histone deacetylase 1 (C/EBPb-HDAC1) complex. Sirt3 protein level was downregulated in the liver of 20-month-old mice (Kwon *et al.*, 2017) and this reduction was attributed to the decreased level of Sirt1, which deacetylates and stabilizes Sirt3 (Kwon *et al.*, 2017). It is plausible that differences in chow composition and time of probing, together with different genetic backgrounds of rat and mouse strains may account for the discrepancies. Moreover, it is important to consider that aging-related changes differ between organs and tissues. For example, in the same rat strain bred under similar conditions, we have found that the old compared to young rats showed lower *Sirt3* expression in skeletal muscle, but maintained high expression in cardiac muscle (Ławniczak *et al.*, 2022).

Considering the involvement of Sirt1 and Sirt3 in the control of lipid homeostasis, we examined whether the hypolipemic drug FN affects their hepatic expression. Since dyslipidemia is increasingly common with advancing age (Liu & Li, 2015), we chose normal breeding conditions to determine the effect of FN during physiological aging. We found that depending on the dose and the rat's age FN decreased the protein abundance of Sirt1 and Sirt3 in the liver, with little effect on the mRNA levels. It is generally accepted that the protein levels rather than mRNA are more important for the activity of a particular bioactive molecule. Sirt1 activity can be regulated by several mechanisms, including activation through autocatalytic deacetylation (Fang *et al.*, 2017) or cAMP-dependent activating phosphorylation (Gerhart-Hines *et al.*, 2011). With regard to Sirt3, the decrease in protein levels observed in our study upon FN treatment of young rats may be related to the reduction in Sirt1 level, since Sirt1 was shown to deacetylate and stabilize Sirt3 (Kwon *et al.*, 2017).

To our best knowledge, this is the first study examining the expression of Sirt1 and Sirt3 in the liver of young and old FN-treated rats under normal breeding conditions. The FN-induced reduction of Sirt1 abundance is consistent with some other studies conducted in different experimental settings. In young Wistar rats with HFD-induced hyperlipidaemia, the highly elevated hepatic Sirt1 protein and mRNA expression was reduced after treatment with FN for 21 days, although to a level significantly higher than in control animals (Le *et al.*, 2014). In contrast, no effect on hepatic Sirt1 protein level was observed in young mice treated with approx. 400 mg/kg FN for 5 days (Li & Wu, 2018). Reports on hepatic Sirt3 expression upon FN treatment showed either no impact of the drug (approx. 400 mg/kg/day, 5 days) (Li & Wu, 2018) or prevention of iron overload-induced downregulation of Sirt3 (100 mg/kg, i.p., 6 weeks) (Mandala *et al.*, 2021).

We next analyzed the hepatic expression of genes encoding enzymes and transcriptional regulators involved in lipid metabolism, the activity of which is directly or indirectly regulated by SIRT1 and SIRT3. We have previously reported that in our cohort of rats FN exerted a hypolipemic effect, which was more prominent in old than in young animals (Zubrzycki *et al.*, 2020). The major molecular target activated by FN (Gebel *et al.*, 1992), the nuclear receptor PPARα, is regulated by ligand binding (Balanarasimha *et al.*, 2014) and post-translationally by phosphorylation (Nakamura *et al.*, 2019). Similarly to our results, various studies demonstrated FN action without altering PPARα level (e.g. Zhao *et al.*, 2015; Liu *et al.*, 2016). We analyzed several transcriptional targets of PPARα (Rakhshandehroo *et al.*, 2010): *Cpt1b*, an enzyme responsible for the transport of fatty acids across the mitochondrial outer membrane, *Lcad*, involved in mitochondrial β-oxidation of fatty acids, and *Acox1,* the rate-limiting enzyme of peroxisomal β-oxidation. Among the *Cpt1* isoforms, the *Cpt1b* gene is expressed in the liver at lower levels than the *Cpt1a* isoform, which is known to be upregulated by FN (e.g. de la Rosa Rodriguez *et al.,* 2018). The *Cpt1b* expression in our study was strongly upregulated by both doses of FN in either age group. *Lcad* expression was induced by 0.1% FN and to a smaller degree and only in old rats by 0.5% FN. Stimulation of mitochondrial fatty acid oxidation-related genes by FN confirms the results of most other studies (Karahashi *et al.*, 2013; Zhao *et al.*, 2017). While *Acox1* expression was induced by both FN doses in young rats, its induction by only the higher FN dose in old animals may reflect the general rule that in aging higher doses of activators are needed for the induction of many target genes (e.g. Kobilo *et al.*, 2014; Zubrzycki *et al.*, 2020). The expression of *Pmp70*, which has been described as a specific marker for PPARα activation and peroxisome proliferation (Colton *et al.*, 2004), was highly induced by 0.1% FN in both age groups.

SIRT1 has been reported to interact with PPARα at the PPAR response element sequence of target genes (Purushotham *et al.*, 2009). Sirt1 levels in young rats did not change upon 0.1% FN treatment but decreased after 0.5% FN. This may explain the induction of PPARα-regulated target genes for mitochondrial and peroxisomal enzymes primarily after 0.1% FN in young animals. However, old rats showed downregulation of Sirt1 protein levels after both doses, indicating Sirt1-independent modulation of genes for fatty acid oxidation. Indeed, Bonzo and others (Bonzo *et al.,* 2014) showed that hepatic SIRT1 is dispensable for FN-induced PPARα function *in vivo*. In young mice treated with 0.1% FN for

5 days following the HFD, hepatocyte-specific *Sirt1* knockout did not affect the induction of PPARα target genes (Bonzo *et al*., 2014).

PPARα also controls the expression of *Fgf21*, a hepatokine that promotes lipid oxidation, lipolysis, and ketogenesis in the liver during ketotic states (Badman *et al*., 2007) and upon treatment with sodium butyrate (Li *et al.,* 2012). We did not observe any significant changes in *Fgf21* mRNA expression upon treatment with FN, in contrast to the induction of both mRNA and protein levels of FGF21 reported in hepatocytes treated *in vitro* with 500 μmol/L FN (Li *et al.,* 2012). On the other hand, FGF21 has been recently demonstrated to be dispensable for FN action on lipogenesis- or autophagy-related proteins in the murine liver (Jo *et al*., 2017). Furthermore, the ketogenesis-related gene *Hmgcs2*, whose transcription is regulated by both PPARα (Meertens *et al*., 1998; Vilà-Brau *et al*., 2011) and reciprocally by FGF21 (Meertens *et al*., 1998; Badman *et al*., 2007; Vilà-Brau *et al*., 2011), was induced in our study after treatment with 0.1% FN in both young and old rats. In line with our results, a principal transcriptomics analysis of the livers of the FN-treated hepatocyte humanized mice revealed that among many established PPARα targets FN induced *Hmgcs2* (de la Rosa Rodriguez *et al*., 2018). Likewise, dietary obese mice after a single injection of bezafibrate (100 mg/kg) showed elevated hepatic expression and serum concentration of FGF21, elevated expression of *Hmgcs2*, but no change in the serum concentration of the ketone body β-hydroxybutyrate (Li *et al.,* 2012). In our experimental setting, it is unlikely that the modestly increased *Hmgcs2* expression led to any significant change in ketone body production.

SIRT1 activates PGC1α by deacetylation (Rodgers *et al*., 2005), whereas PGC1α stimulates the expression of SIRT3 (Kong *et al*., 2010). PGC-1α and PPARα cooperate to control the transcription of genes encoding mitochondrial fatty acid oxidation enzymes (Vega *et al*., 2000), whereas SIRT3 modulates the activity of these enzymes by deacetylation (e.g. Hirschey *et al*., 2010; Shimazu *et al*., 2010). Even though we noted no significant changes in *Pgc1a* mRNA expression, it should be stressed that this transcriptional coactivator is regulated primarily post-transcriptionally through phosphorylation, acetylation, monomethylation, and O-linked β-N-acetylglucosaminylation (Housley *et al*., 2009). Similarly to *Pgc1a*, FN did not affect the mRNA expression of *Sirt3* in either age group. However, FN caused downregulation of the Sirt3 protein, which suggests decreased stability or increased degradation of Sirt3 upon treatment. Regrettably, in our study, it was not possible to assess Sirt3 function, e.g. by examining the acetylation status of Sirt3 target proteins. In contrast to our results, a study in *Ppara* knockout mice treated with 100 mg/kg FN for 6 weeks showed downregulation of hepatic Sirt3 expression, indicating the dependence of Sirt3 expression on PPARα in mouse liver (Mandala *et al*., 2021). Moreover, the FN treatment prevented iron-induced downregulation of hepatic Sirt3 in wild-type iron-overloaded mice (Mandala *et al*., 2021). Modulation of SIRT3 expression by FN appears to be variable under different experimental conditions.

We observed contrasting, dose-dependent effects of FN on the hepatic expression of the rate-limiting lipogenic *Fas2* gene in young and old rats, with *Acl* expression not significantly affected. In young rats, the decrease in *Fas2* expression upon 0.5% FN treatment (though not 0.1% FN) falls in line with similar results reported in adult rats fed a high-fructose diet and treated subsequently with 100 mg/kg FN for 4 weeks (Abd El-Haleim *et al*., 2016). Because FAS activity is mainly regulated at the transcriptional level by nutrients and hormones, this decreased expression could contribute to reduced accumulation of lipid droplets, which we observed under a transmission electron microscope in hepatocytes of young 0.1% FN-treated rats (Zubrzycki *et al*., 2021). In contrast to young rats, in the old ones 0.5% FN increased *Fas2* expression. Similarly, a 2-week treatment with 0.2% FN strongly induced hepatic *de novo* lipogenesis and the expression of *Fas2* in mice (age not specified) (Oosterveer *et al*., 2009). Likewise, an increase in FAS protein expression, concurrently with increased mSREBP-1c level was observed in the liver of 10–12-week-old mice fed 50 mg/kg/day FN for 3 weeks (Jo *et al*., 2017). Further studies are needed to explain the reason for the apparent FN dose- and age-dependent differences with regard to the modulation of lipogenic genes' expression. Nevertheless, it should be noted that the impact on hepatic lipogenesis does not preclude the FN hypolipemic effect through mechanisms including the stimulation of fatty acid β-oxidation and increased lipoprotein lipase activity.

Available evidence suggests that PPARα acts not only as a regulator of fatty acid catabolism but plays a broader role in hepatic lipid metabolism. By using stable isotope techniques, Oosterveer *et al.* demonstrated that the treatment of mice for 2 weeks with 0.2% FN induced hepatic *de novo* lipogenesis and fatty acid elongation *in vivo* in parallel with an increased expression of lipogenic genes (Oosterveer *et al*., 2009). This lipogenic induction was dependent on SREBP-1c which is a target for inhibitory deacetylation by SIRT1 (Walker *et al*., 2010). Thus, in terms of our experiment, FN-associated reduction of Sirt1 expression in the liver of old rats may contribute to the increased *Fas2* expression. However, the striking contrast between young and old rats with respect to the effect of FN on lipogenic gene expression warrants further studies. One factor contributing to this outcome could be food intake. We previously reported that 0.5% FN was associated with markedly reduced food intake during the first few days, followed by increased consumption, more rapid in young than old rats (Zubrzycki *et al*., 2020). A re-feeding effect could contribute to the elevation of lipogenesis in old rats.

Our study provides yet another piece of evidence that at least two concentrations of the studied compound should be applied under both *in vivo* and *in vitro* experimental conditions. We found clear discrepancies in the effects of low dose, i.e. 0.1% FN (equivalent to ca. 52 and 42 mg/kg/day in young and old rats, respectively) *versus* high dose, i.e. 0.5% FN (equivalent to ca. 260 and 210 mg/kg/day) in regard to the expression of Sirt1, *Ppara*, *Lcad*, *Hmgcs2*, *Acox1*, *Pmp70*, and *Fas2*. It should be noted that during long-term treatment in humans, FN is administered once daily typically at a dose of 50-150 mg (preceded by higher doses), whereas the dosage applied to studies in rodents varies widely, from 50 mg/kg/day (Jo *et al.,* 2017) to 400 mg/kg/day (Li & Wu, 2018). Comparison between FN dosage in humans and rodents is difficult because of species differences in the metabolism and elimination patterns of the drug (Caldwell, 1989).

Our study is not without limitations. Firstly, the results of a study examining PPARα agonist effects in rats cannot be directly transferred to human conditions. Upon administration of peroxisome proliferator chemicals, rodents respond with peroxisomes proliferation, hepatomegaly, and increased hepatocyte proliferation, whereas

such a response is typically absent in humans (Klaunig *et al.*, 2003; Peters *et al.,* 2005). Secondly, we measured the mRNA and protein levels of Sirt1 and Sirt3. Considering that activation of sirtuins may be achieved by increasing NAD^+ availability (Zhao *et al.*, 2020), analysis of the hepatic NAD^+/NADH ratio would give more insight into Sirt1 and Sirt3 activity upon treatment with FN. Nevertheless, NAD^+-independent mechanisms of sirtuins' activation also exist, including phosphorylation of SIRT1 at Ser434 *via* the cAMP/PKA pathway (Gerhart-Hines *et al.*, 2011). Thirdly, we measured only the mRNA expression of some genes involved in fuel metabolism, selected as representative for particular metabolic processes and/or under PPARα transcriptional control. Certainly, activity assays, as well as analyses of protein levels and their acetylation status would be more informative with regard to the changes induced by FN. However, these methods were beyond the scope of this project. Lastly, additional studies are needed to examine the mechanisms responsible for the dose-dependent and age-dependent differences in fenofibrate's action in the liver.

CONCLUSIONS

Treatment with fenofibrate at low or high dose affects the hepatic expression of Sirt1 and Sirt3 proteins. The dosage of fenofibrate significantly affects the treatment-induced changes in the expression of lipid metabolism-related genes in the rat liver. Aging influences the response to high-dose fenofibrate. The results of our study emphasize the importance of considering the patient's age before adjusting the fenofibrate dose.

Declarations

Ethical approval: All animal experimental procedures had been authorized by the Local Ethics Committee in Bydgoszcz, Poland (protocols No. 41/2017, 58/2017, 40/2018, and 5/2019), and carried out in compliance with the EU Directive 2010/63/EU for animal experiments.

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