# Chronic, in vivo, PPARα activation prevents lipid overload in rat liver induced by high fat feeding

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## ABSTRACT

**Purpose:** Peroxisome proliferator-activated receptors (PPAR`s) are lipid sensors and when activated they modify gene expression of proteins regulating fatty acid (FA) metabolism in liver cells. The aim of the present study was to examine the in vivo effects of PPAR α and γ activation combined with high fat diet (HFD) feeding on the lipid content and FA profile in the liver.

**Material/Methods:** We assessed whether in vivo activation of PPARs (α or γ) affects lipid accumulation in the liver induced by HFD feeding. Furthermore, as PPAR activity may be a key factor regulating long chain fatty acids (LCFA) flux and subsequent LCFA utilization in the liver, we prompted to investigate also the FA profile in different lipid fractions in this tissue.

**Results:** PPARα agonist (WY 14,643) treatment reduced the accumulation of liver lipids free fatty acids (FFA:-30%, diacylglycerols DAG: -27% and triacylglycerols TAG: –60%, $p<0.05$) evoked by HFD feeding. Interestingly, with PPARγ stimulation liver lipid content was further elevated comparing to the effects of HFD (phospholipids PL: +48%, DAG: +231%, TAG: +346%, $p<0.05$).

**Conclusions:** These findings suggest that in vivo PPARα and PPARγ activation combined with HFD feeding exert different effects on lipid content in rat's liver and in vivo PPARα activation may prevent lipid overload in the liver cells provoked by HFD feeding.

**Key words:** Peroxisome proliferator-activated receptors (PPAR`s), lipids, liver

## INTRODUCTION

Obesity and obesity related fatty liver disease (FLD) are common features of diabetes type II and metabolic syndrome, which are global health problems [1,2]. The pathogenesis of FLD is complex and likely involves a dysregulation of the tight balance between hepatic FA uptake, lipogenesis, lipolysis and FA oxidation [3,4]. It is widely accepted that the first step in the development of FLD includes an excess accumulation of TAG in hepatocytes, mainly because of amplified hepatic lipogenesis [5,6]. However, sources for the excess accumulation of lipids in liver are far more versatile and likely to include: 1) excess of the dietary lipids that reach the liver as chylomicrones, 2) increased TAG synthesis in the liver from FA influx from lypolysis of adipose tissue in obese and/or insulin resistant states, 3) diminished export of TAG`s

from the liver, 4) reduced oxidation of FA in hepatocytes [2,7]. Based on the processes mentioned above, the liver is thought to be a central player in the lipid homeostasis. Accordingly (from transcriptional point of view) lipid metabolism is regulated mainly by the activity of PPARs. Currently, three distinct PPAR isoforms have been identified (PPARα, β/δ and γ). PPARs, in general, act as specific lipid sensors and transcription factors for a number of genes participating in regulating either FA transport or FA oxidation or lipogenesis [8,9]. Specifically, PPARα had been shown to be present in the liver (at relatively high amounts compared to PPARγ) and several studies indicated that increased activity of this nuclear receptor is associated with enhanced expression of genes involved in hepatic mitochondrial biogenesis and FA oxidation [8,10]. In contrast, the transcription factor, PPARγ is expressed in the liver at very low level and it's overexpression leads to

increased activity of several adipogenic genes and subsequent development of hepatic steatosis [11,12]. For both, it has been shown that HFD affects their expression and activity [13], but the reports are still controversial regarding the effects on PPAR activation, especially during simultaneous excess of dietary fat provision.

This seems a bit surprising since HFD results in the increase in serum content of FFA, which are known ligands of PPAR`s and one could expect that the activity of PPAR is highly modulated by the changes in serum FFA levels. Given that, it is of particular interest to examine whether in vivo activation of PPARs (α or γ) affects lipid accumulation in the liver induced by HFD feeding and whether these effects are related to the serum FFA content and composition. Furthermore, as PPAR activity may be a key factor regulating LCFA flux and subsequent LCFA utilization in the liver, we prompted to investigate also the FA profile in different lipid fractions in this tissue.

## MATERIAL AND METHODS

Male Wistar rats (200-250g) were housed in approved animal holding facilities (at 22°C±2, on a 12h/12h light-dark cycle, with unrestricted access to water and to a commercial chow). Animal maintenance and treatment were approved by the Ethical Committee for Animal Experiments at the Medical University of Białystok.

Rats were randomly divided into 2 groups: 1) control (receiving standard chow diet, (Agropol, Motycz, Poland)), 2) HFD, receiving chow containing 33.9 % of fat (sunflower oil) by weight for 3 weeks as described previously [14]. HFD group was further subdivided into 3 subgroups: a) control (HFD), b) treated daily for two weeks with a selective PPARα agonist, WY 14,643 (Cayman Chemicals) in a dose of 3 mg/kg of body weight (HFD+WY14,643), and c) treated daily for two weeks with a selective PPARγ agonist, pioglitazone ("Actos", Lilly) in a dose of 3 mg/kg of body weight (HFD+pioglitazone). The HFD was routinely prepared in our laboratory as described previously [14] and was administrated daily in the same amount of calories as in control rats fed on a standard chow (82 kcal per animal per day). The drugs (WY 14,643 and pioglitazone) were suspended in 0.5 % methylcellulose and administrated daily by an oral gavage. After 3 weeks of treatment, rats were anaesthetized by intraperitoneal injection of pentobarbital in a dose of 80 mg/kg of body weight. Samples of the liver were excised, cleaned of blood and immediately freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen and then stored at -80 °C until further analysis.

### Lipid analyses

The liver was pulverized in an aluminum mortar precooled in liquid nitrogen. The powder was transferred to a glass tube and lipids were extracted using the Bligh and Dyer method [15].

The fractions of total phospholipids (PL), triacylglycerols (TAG), diacylglycerols (DAG) and FFA were separated by thin-layer chromatography (TLC) according to van der Vusse et al [16]. Individual FA methyl esters were identified and quantified according to the retention times of standards by gas liquid chromatography (Hewlett-Packard 5890 Series II gas chromatograph, HP-INNOWax capillary column). Total FFA, DAG, PL and TAG content was estimated as the sum of the particular FA species of the assessed fraction and it was expressed in nanomoles per gram of the tissue. We have also calculated the following indices of FA profile of each lipid fractions examined: saturated fatty acids (SAT), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) and presented as the percentage in each fraction.

### Protein analyses

Routine Western blotting procedure was used to detect PPARα and PPARγ proteins as described previously [17,18], with commercially available antibodies (sc 1982, sc 7196, Santa Cruz, CA, respectively). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The total protein expression of FA transporters was determined in crude membranes of the liver. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis. Membranes were immunoblotted with primary antibodies. Protein content was determined with bicinchonic acid method with BSA serving as a protein standard. Signals obtained by Western blotting were quantified by densitometry (Biorad, Poland) and expressed in Optical Density Arbitrary Units. Equal protein concentrations were loaded in each lane as also confirmed by Ponceau S staining.

All data are expressed as mean ± SEM. Statistical difference between groups was tested with analyses of variance and appropriate post-hoc tests, or with a Student t-test (for clarity of the presentation the FA species change in the specific lipid pools were shown as a percentage change). Statistical significance was set at P≤0.05.

## RESULTS

### Effects of HFD feeding and subsequent PPARα or PPARγ activation on FFA serum content

There was a small trend for the increase in the FFA serum levels (+12%, p>0.05, *Fig. 1a*) in HFD fed rats compared to control, and the administration of only pioglitazone (PPARγ activator) in HFD rats resulted in significant decrease in serum FFA (-27%, p<0.05, *Fig. 1a*).

Each treatment lowered the percentage of saturated FA species in FFA serum fraction compared to control with concomitant increase in the percentage of mono-and polyunsaturated fatty acids in this fraction (*Fig. 1b*).

*Figure 1.* **Effects of high fat feeding and PPARα or γ agonists treatment on FFA (free fatty acid) serum content and FA profile (nmol/ml ±SD; n=10). a) TOTAL – Total free fatty acid (FFA) content was estimated as the sum of the particular fatty acid species of the assessed fraction. b) SAT – the relative % of saturated fatty acids; MUFA- the relative % of monounsaturated fatty acids; PUFA- the relative % of polyunsaturated fatty acids; * - p<0,05 vs control; ** - p<0,05 vs HFD**
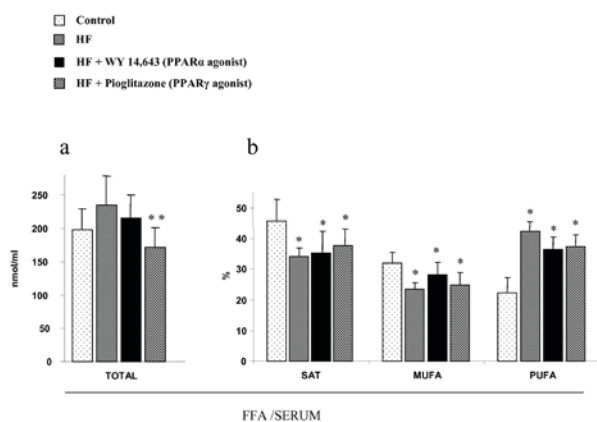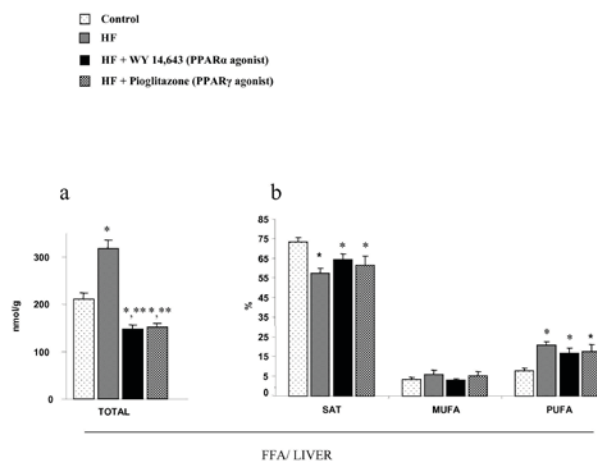


FFA /SERUM

*Figure 3.* **Effects of high fat feeding and PPARα or γ agonists treatment on free fatty acids (FFA) content and composition in rat liver (nmol/g ± SD; n=10). a) TOTAL - Total free fatty acid (FFA) content was estimated as the sum of the particular fatty acid species of the assessed fraction; b) SAT – the relative % of saturated fatty acids; MUFA- the relative % of monounsaturated fatty acids; PUFA- the relative % of polyunsaturated fatty acids; * - p<0,05 vs control; ** - p<0,05 vs HFD**



FFA/ LIVER

### Effects of HFD feeding and subsequent PPARα or PPARγ activation on the phospholipid content in the liver

The liver content of phospholipid fraction was similarly affected by either HFD or HFD and PPARα or PPARγ activation (+35%, +48% and +36%, p<0.05, respectively, *Fig. 2a*).

HFD increased saturation status in liver phospholipid fraction, however both treatments (pioglitazone and WY 14,643) increased the percentage of unsaturated FA species (MUFA and PUFA, *Fig. 2b*).

*Figure 2.* **Effects of high fat feeding and PPARα or γ agonists treatment on phospholipids (PL) content and composition in rat liver (nmol/g ± SD; n=10). a) TOTAL - Total phospholipid (PL) content was estimated as the sum of the particular fatty acid species of the assessed fraction; b) SAT – the % of saturated fatty acids; MUFA- the relative % of monounsaturated fatty acids; PUFA- the relative % of polyunsaturated fatty acids; * - p<0,05 vs control; ** - p<0,05 vs HFD**



PHOSPHOLIPIDS/ LIVER
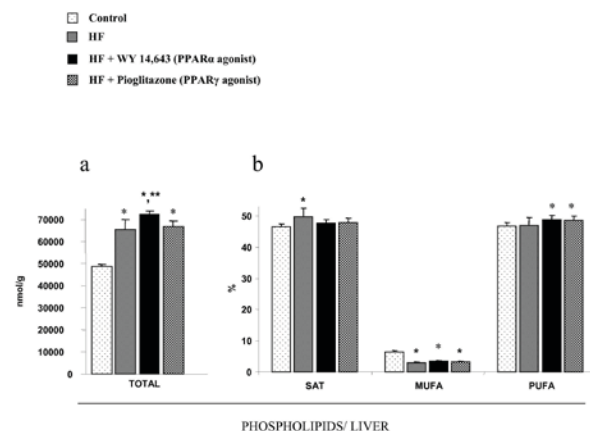
### Effects of HFD feeding and subsequent PPARα or PPARγ activation on the free fatty acids content in the liver

HFD feeding induced a significant increase in liver FFA fraction (+50%, p<0.05, *Fig. 3a*) and interestingly, for both treatments (WY 14,643 or pioglitazone) we observed significant reduction in the content of FFA fraction (-30% and -27%, p<0.05, respectively, *Fig. 3a*) compared to the control, as well as, to the HFD fed rats (-53% and -50%, p<0.05, respectively, *Fig. 3a*).

There was a significant decrease in the percentage of saturated FA species in liver free FA fraction observed in each group compared to control, and concomitantly an increase was noticed only in polyunsaturated fatty acids in this fraction (*Fig. 3b*).

### Effects of HFD feeding and subsequent PPARα or PPARγ activation on the diacylglycerols content in the liver

Similarly, HFD induced the significant elevation in DAG liver content (+229%, p<0.05, *Fig. 4*) but the opposite effects of PPARα or PPARγ activation were observed. Specifically, WY 14,643 (PPARα activator) treatment reduced the DAG lipid content (-27%, p<0.05) compared to HFD, but it was still higher than in the control group (+67%, p<0.05, *Fig. 4a*). However, treatment with pioglitazone (PPARγ agonist) resulted in significant increase in DAG content (+231%, p<0.05, *Fig. 4a*).

Interestingly, with HFD feeding a decrease in saturation of FA species was observed in this lipid fraction, which was followed by an increase in unsaturated FA profile (MUFA and PUFA) and opposite effects were noticed for the treatments with PPARα and γ agonists (*Fig. 4b*).

*Figure 4.* **Effects of high fat feeding and PPARα or γ agonists treatment on diacylglycerols (DAG) content and composition in rat liver (nmol/g ± SD; n=10). a) TOTAL- Total diacyloglycerol (DAG) content was estimated as the sum of the particular fatty acid species of the assessed fraction; b) SAT – the relative % of saturated fatty acids; MUFA- the relative % of monounsaturated fatty acids; PUFA- the relative % of polyunsaturated fatty acids; * - p<0,05 vs control; ** - p<0,05 vs HFD**

DIACYLGLYCEROLS/ LIVER

*Figure 5.* **Effects of high fat feeding and PPARα or γ agonists treatment on triacylglycerols (TAG) content and composition in rat liver (nmol/g ± SD; n=10). a) TOTAL - Total triacylglycerol (TAG) content was estimated as the sum of the particular fatty acid species of the assessed fraction; b) SAT – the relative % of saturated fatty acids; MUFA- the relative % of monounsaturated fatty acids; PUFA- the relative % of polyunsaturated fatty acids; * - p<0,05 vs control; ** - p<0,05 vs HFD**
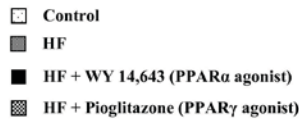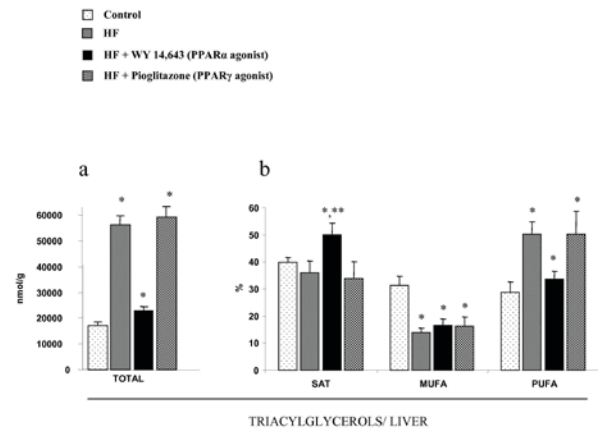
TRIACYLGLYCEROLS/ LIVER
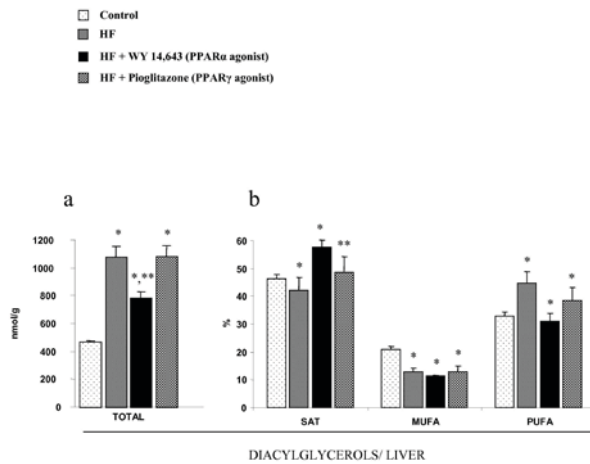
*Figure 6.* **Effects of high fat feeding and PPARα or γ agonists treatment on total expression of liver PPARα and PPARγ protein expression (n=10). * - p<0,05 vs control; ** - p<0,05 vs HFD**

## Effects of HFD feeding and subsequent PPARα or PPARγ activation on PPARα and γ protein expression

There was a small, but not significant, increase in the expression of PPARα in HFD fed rats (+16%, p<0.05, *Fig. 6*) compared to control rats. In this group (HFD), treatment with pioglitazone did not change significantly the expression of PPARα (+15%, p<0.05 *Fig. 6*), however WY 14,643 treatment resulted in a significant increase in PPARα expression (+20%, p>0.05, *Fig. 6*).

Liver PPARγ expression was found at a very low level compared to PPARα content confirming previous reports showing the abundance of PPARα over PPARγ protein content in rat`s liver (44 z JPP). HFD feeding resulted in significant increase in PPARγ expression (+88%,p>0.05, *Fig. 6*). Interestingly, both treatments (Pioglitazone and WY14,643) in HFD group lowered the total expression of PPARγ nearly to the same extent (-63%, and -53%, p>0.05, respectively, *Fig. 6*).

## Effects of HFD feeding and subsequent PPARα or PPARγ activation on the triacylglycerols content in the liver

Liver content of TAG fraction was significantly increased after HFD feeding (+329%, p<0.05, *Fig. 5a*) and after HFD combined with pioglitazone (+346%, p<0.05, *Fig. 5a*) but reduced with respect to HFD after PPARα treatment (-60%, p<0.05, *Fig. 5a*). Interestingly, this reduction in total content of TAG fraction was accompanied by a significant increase in FA saturation status (*Fig. 5b*).

## DISCUSSION

The present study revealed profound effects of chronic, in vivo, HFD feeding as well as HFD combined with PPARα or PPARγ activation on the hepatic lipid content and FA profile in different lipid fractions. Interestingly, HFD feeding in our study resulted only in minor increase in FFA serum content, which might be surprising since most of the studies [19] report rather pronounced amplification in this lipid fraction after HFD, although others show either no changes [20,21] or decrease [22] in FFA serum depending on diet restrictions. Our diet protocol (3 weeks HFD and increased concentration

of sunflower oil) most probably was just sufficient enough to increase FA influx to the tissues with high FA plasma clearance. Under these conditions, it has been proposed that HFD provides more long chain fatty acids for hepatocytes and TAG accumulation occurs in the liver of HFD fed rodents [9,23,24]. However, very limited data exists with respect to the accumulation of other lipid fractions in rodents fed with HFD (or obese Wistar rats). There are reports showing the accumulation of lipids in the liver as the excessive presence of large (macrovesicular) or small (microvesicular) intracytoplasmic fat droplets in hepatocytes [23,24]. Our data indicate that after 3 weeks of HFD there is a significant accumulation of specific lipid fractions, namely: free fatty acids, diacylglycerols, triacylglycerols and phospholipids. Likely explanation for observed lipid accumulation would be that the liver is able to store significant quantities of fat, especially in conditions associated with chronic excess of lipid consumption. Under these circumstances, fatty acids entering hepatocytes (esp. PUFA, present in our HFD) affect nuclear receptors such as: SREBP-1c, ChREBP or PPARγ receptors [7]. In the present study we observed a significant increase in the expression of PPARγ as a result of HFD feeding and only a trend for the increase in liver PPARα content. These findings are similar to the other studies in which stimulation of PPAR`s by a HFD regime increases the activity and/or the expression of PPARs in the liver cells [9,10]. Considerable evidence has now been provided showing a strong correlation between the activation of PPARγ and the regulation of adipogenic genes in the liver [11,12]. Although PPARγ is present in hepatocytes at very low levels, its overexpression leads to hepatic steatosis [11,12]. In accordance with this data, in animal models of insulin resistance associated with liver steatosis the expression of hepatic PPARγ was also found upregulated [11]. These studies suggest that, not only simple excess of dietary lipids leads to hepatic steatosis, but in addition, this process can be amplified by increased expression of PPARγ. More importantly, our study strongly points to the significance of PPARγ activation since pioglitazone treatment in HFD fed rats decreased the total FFA content in serum and persisted liver lipid accumulation was observed. This at first might be a surprising finding since several reports showed an indirect effect of pioglitazone (or other PPARγ agonists) on tissue lipid content as a consequence of lower systemic FFA availability [25,26]. However, since our studies were performed in vivo, it is impossible to determine whether other stimuli (i.e. hormones) participate in observed lipid overload in the liver. Under excessive dietary FFA influx to hepatocytes disturbances in FA oxidation may also occur [27] as liver FA oxidation is roughly proportional to the plasma FFA concentration. Another important consideration is a well known effect of HFD feeding on the induction of the expression of PPARα [27], which in turn activates the PPARα-target genes involved in FA oxidation [28,29]. This HFD effect on PPARα liver expression was also observed in our study (+16%, although it did not reach significance level), as well as in the liver of obese

Wistar rats and in humans NAFLD biopsies [30,31]. However, several studies have dealt with alternations in FA metabolism in liver after PPARα activation showing contradictory results. PPARα knock out mice (PPARα $^{-/-}$) were unable to fully oxidize FA entering hepatocytes and thus developed hepatic steatosis [11,32,33]. On the contrary, it was reported that, WY 14,643 treatment resulted in an accumulation of liver TAG, despite the enhanced rates of LCFA oxidation [34]. In our study additional activation (by WY 14,643) of PPARα in HFD fed rats resulted in a significant decrease in either FFA, DAG or TAG lipid fractions which suggests an increased utilization of the abundant fatty acids in hepatocytes.

To our best knowledge this is the first report showing also particular changes in FA profile after an excess of dietary fat intake in each liver lipid fraction examined. It is widely accepted that in obesity saturated FA residues may accumulate inside the cells and there is a parallel reduction in PUFA [35]. In skeletal muscles a strong correlation has been observed between the saturation of FA species and the severity of insulin resistance [36]. The changes in the FA saturation status were also implicated in the process of downregulation of genes involved in FA oxidation [37]. Furthermore, intramyocellular FA saturation status when changed by pharmacological agents leads to the increase in insulin sensitivity [38]. This implies that, the FA saturation might be an additional parameter involved in the development of insulin resistance [39].

## CONCLUSIONS

The present study revealed pronounced lipid accumulation in the liver in rats fed with a HFD in all lipid fractions examined (FFA, PL, DAG and TAG). Moreover, this effect of HFD was completely abolished by further PPARα activation, but not affected with PPARγ stimulation, which suggests that only, in vivo, PPARα stimulation may prevent lipid accumulation in the liver cells induced by HFD. Interestingly, both treatments (Pioglitazone and WY 14,643) evoked also significant changes in the saturation profile of FA in each liver lipid fraction.

## ACKONWLEDGEMENTS

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