9-PAHPA long term intake in DIO and db/db mice ameliorates insulin sensitivity but has few effects on obesity and associated metabolic disorders

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Abbreviations: HFHS, high fat high sucrose; DIO, diet- induced obesity; db/db, mouse model of obesity, diabetes, and dyslipidemia wherein leptin receptor activity is deficient; FAHFA, fatty acid esters of hydroxy fatty acids; PAHSA, palmitic acid esters of hydroxystearic acid; 9-PAHPA, 9-palmitic acid esters of hydroxypalmitic acid; 9-OAHPA, 9-oleic acid esters of hydroxypalmitic acid; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; CS, citrate synthase; β-HAD, 3-hydroxyacyl CoA dehydrogenase activity; GLUT2, glucose transporter 2; PPAR, peroxisome proliferator-activated receptors; Nrf2, nuclear factor erythroid-2-related factor 2; ChREBP, carbohydrate-responsive element-binding protein; ALAT, alanine aminotransferase; SOD, superoxide dismutase; GPx, glutathion peroxidase.

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ABSTRACT

Branched fatty acid esters of hydroxy fatty acids are endogenous lipids reported to have antidiabetic and anti-inflammatory effects. Recently, we showed that 9-palmitic acid esters of hydroxypalmitic acid (9-PAHPA) and 9-oleic acid esters of hydroxypalmitic acid increased insulin sensitivity in mice when incorporated to a chow diet or to a high fat and high sucrose diet. However, preventive supplementation with 9-PAHPA and 9-oleic acid esters of hydroxy-palmitic acid in high fat and high sucrose diet mice did not impair significant weight gain or the development of hyperglycemia. The aim of this work was therefore to study whether in two animal models of obesity, namely the classical diet-induced obesity (DIO) and the db/db mice, 9-PAHPA may have beneficial effects against obesity and liver and skeletal muscle metabolic dysfunction. In DIO mice, we observed that 9-PAHPA increased body weight and fat mass. In line with this observation, we found that 9-PAHPA supplementation decreased energy expenditure. In liver and in skeletal muscle, mitochondrial activities and oxidative stress parameters were not modified by 9-PAHPA supplementation. In db/db mice, 9-PAHPA had no effect on the dramatic weight gain and hyperglycemia. In addition, 9-PAHPA supplementation did not correct either the hepatomegaly and hepatic steatosis or the severe muscle atrophy recorded compared with db/ + animals. Likewise, supplementation with 9-PAHPA did not impact the different metabolic parameters analyzed, either in the liver or in the skeletal muscles. However, it decreased insulin resistance in DIO and db/db mice. In conclusion, our study indicated that a long-term intake of 9-PAHPA in DIO and db/db mice improved insulin sensitivity but had only few effects on obesity and associated metabolic disorders.

1.Introduction

Obesity is a worldwide major public health issue [1] . It generally results from an imbalance between body energy intake and expenditure from a metabolic point of view and is associated with numerous metabolic dysfunctions including insulin resistance and inflammation [2] . Obesity affects key metabolic organs, including liver and skeletal muscle. While liver is a key organ that performs a wide range of biochemical functions necessary for the metabolic homeostasis of the entire body, skeletal muscle is essential for energy metabolism and is the predominant site of insulin-mediated glucose uptake in the post prandial state [3] .

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a vast family of endogenous lipids [4–6]. Some of them, in particular PAHSAs (palmitic acid esters of hydroxystearic acid), were proven to favorably modulate insulin sensitivity and glucose metabolism [4 , 7–11]. The anti-inflammatory ability of some FAHFAs, notably from omega-3 fatty acid but also from the PAHSA family, has also been demonstrated [4 , 12–14]. In our laboratory we showed that 9-PAHPA (9-palmitic acid esters of hydroxypalmitic acid) and 9- OAHPA (9-oleic acid esters of hydroxypalmitic acid) increased insulin sensitivity in mice when incorporated to a

chow diet [15] or lowered insulin resistance in mice when incorporated to a high fat high sucrose (HFHS) diet [16] . Moreover, 9-PAHPA and 9-OAHPA induced hepatic steatosis and fibrosis in some healthy mice but not in HFHS mice [15 , 16], with few effects on inflammation. How- ever, preventive supplementation with 9-PAHPA and 9-OAHPA in HFHS mice did not prevent significant weight gain or the development of hyperglycemia. Our assays performed on C2C12 muscle cells suggested a muscular origin of the increase in insulin sensitivity, as 9-PAHPA and 9-OAHPA induced a switch toward a more oxidative contractile phenotype of skeletal muscle [17] . However, the ability of FAHFAs to lower obesity and improved major associated metabolic dysfunction has not been addressed yet

The purpose of this work was therefore to study whether curative supplementation with 9-PAHPA may have beneficial effects in liver and skeletal muscle associated metabolic dysfunction in two animal models of obesity, namely the classical diet-induced obesity (DIO) and the db/db mice [18 , 19]. For this purpose, C57Bl6/J mice were fed for 8 weeks with HFHS diet and then were supplemented with 9-PAHPA for 8 weeks. Moreover, db/db mice were fed for 7 weeks with a chow diet supplemented with 9-PAHPA. Whole- body metabolism was explored with a particular focus on the liver and skeletal muscle functions.

2.Materials and methods

2.1. FAHFAs synthesis

The synthesis of 9-PAHPA was performed using our previously reported procedure [20] .

2.2. Animals and diets

DIO mice: Thirty 6-weeks old male C57BL/6J mice (Charles River, L'Arbresle, France), weighing about 22 g, were housed (five per cage) under conditions of constant temperature (23–24 °C), humidity (35–50%) and a standard dark cycle (19.30–07.30 h). The mice were randomly separated into three groups of 10 animals; a control group (control) was fed for 16 weeks with a control diet, a HFHS group was fed for 16 weeks with a HFHS diet and a 9-PAHPA group (HFHS + 9-PAHPA) was fed for 8 weeks with a HFHS diet and subsequently for eight other weeks with a HFHS diet enriched with 9-PAHPA.

Db/db mice: Ten 5-weeks old male db/ + mice weighing about 22 g and twenty 5-weeks old male db/db mice weighing about 30 g (Janvier, Saint-Berthevin Cedex, France), were housed (two per cage) as previously mentioned. The db/ + mice were fed for 7 weeks a control diet (db/ + group). The db/db mice were randomly separated into two groups of 10 animals; a group (db/db) was fed for 7 week a control diet and a 9-PAHPA group (db/db + 9-PAHPA) was fed for 7 weeks a control diet plus 9-PAHPA.

The detailed composition has been published previously [15 , 16]. The control diet contains 5% lipids as a mixture of rapeseed oil, high oleic sunflower oil, sun- flower oil, and linseed oil (oil mixture of Carrefour) and the HFHS diet contains 25% lipids (5% of a mixture of rapeseed oil, high oleic sunflower oil, sunflower oil, and linseed oil and 20% of lard) and 30% sucrose. The lipid fraction of the control diet was composed of 12.2% saturated fatty acids, 60.6% monounsaturated fatty acids and 27.3% polyunsaturated fatty acids and that of the HFHS diet was com- posed of 30.4% saturated fatty acids, 53.4% monounsaturated fatty acids, and 16.3% polyunsaturated fatty acids. The 9-PAHPA was incorporated into the diet after dis- solution in the oil mixture of Carrefour and the final content of PAHPA was set at 300 μmol/kg diet. Throughout the study, mice were given free access to food and tap water. Mice body weight was assessed weekly and food consumption was determined every 2 d (week) or 3 d (week-end). A detailed scheme of the study design is provided in Figure 1 .

2.3. Body composition and metabolic analyses

Mice whole-body composition (fat and lean mass) was measured every 2 weeks throughout the study using an EchoMRI-700 whole-body composition analyzer (Echo Medical Systems, Houston, TX, USA), according to the manufacturer's instructions. Oxygen consumption and carbon dioxide production were measured using a Comprehensive Lab Animal Monitoring System (Oxymax-CLAMS) (Columbus Instruments, Columbus, OH, USA). Mice were housed in individual cage inside a con- trolled cabinet. The environmental enclosure allows precise control over the temperature and light/dark cycle. Mice were acclimatized individually in metabolic cages at 24 °C with ad libitum access to food and water for 24-h, prior to a 24- h period of automated recordings. Sampled air from individual cages was passed through sensors to determine O 2 and CO 2 content. Sensors were calibrated before each experiment with commercial gas mixtures of accurately determined composition (20.5% O2, 0.5% CO2, and 79% N2). For each mouse, volume of O 2 (VO 2) and VCO 2 were measured 111 times in 24 h. The respiratory exchange ratio (RER) was calculated as the volume of CO 2 versus volume of O 2 (VCO 2 /VO 2) ratio.

2.4. Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)

OGTT was performed only in DIO mice and ITT was performed in DIO and db/db mice. Following an overnight fasting (16 h), mice were administrated glucose solution (2 g/kg) by oral gavage, and blood were collected from the tail vein at the indicated times for glucose and insulin determination. Insulin tolerance was assessed after 2 h of fasting by administration of human insulin (0.75 U/kg for DIO mice and 1.5 U/kg for db/db mice) and blood glucose monitoring. Glycemia was measured using a OneTouch Verio glucometer (Lifescan).

2.5. Sampling and routine biochemical analyses

Four to five days after the OGTT or the ITT, blood from 12 h-fasted mice was collected from the retro-orbital sinus and distributed into heparinized tubes. Blood tubes were centrifuged at 1,0 0 0 g for 10 min at 4 °C, plasma was collected and stored at -80 °C until analysis. After cervical dislocation of mice, liver and muscles were removed, rinsed with 0.9% NaCl, weighed, cut into several parts, when necessary, plunged into liquid nitrogen and then kept at

-80 °C until analysis. In addition, liver and muscle fresh samples were frozen in Tissue-Tek (Microm Microtech, Brignais, France) for histological analysis and stored at -80 °C. Plasma levels of glucose, total cholesterol, triglycerides and free fatty acids as well as enzymatic activity of alanine aminotransferase were measured at the ANEX- PLO/CREFRE analysis platform (CHU RANGUEIL-BP 84225, France). Plasma levels of insulin, leptin and IL-6 were quantified with ELISA kits (Merck Millipore, Darmstadt, Germany; Crystal Chem, Zaandam, Netherlands; Abcam, Paris, France, respectively).

2.6. Tissue neutral lipids measurement

Liver and muscle samples were homogenized in aqueous NaCl solution (9 g/L) and Triton X-100 (0.1%) and free fatty acids, triglycerides and total cholesterol levels were quantified on the tissue homogenate by enzymatic methods (Wako-NEFA-C kit, Oxoid, Dardilly, France; Cholesterol CHOD-PAP SOBIODA kit and triglycerides LQ SOBIODA kit, Sobioda 38330 Montbonnot-Saint-Martin, France) [21] .

2.7. Histological study

After euthanasia by cervical dislocation, liver samples and the tibialis anterior muscles were collected. Liver were immediately fixed in 4% Paraformaldehyde (PFA) at 4 °C for 24 h then embedded in paraffin. Tissue sections were cut at a 4 μm thick- ness and stained with Hematoxylin & Eosin. Tibialis anterior muscle were freshly frozen in Tissue-Tek (Microm Microtech, Brignais, France), and then stored at -80 °C. For immunostaining, the sections were fixed in Phosphate Buffered Saline (PBS) (Euromedex, Souffelweyersheim, France), 4% PFA at room temperature for 5 min, permeabilized 30 min in PBS, 20% horse serum, and 0.1% triton at room temperature, and incubated with the anti-laminin (1/200, rabbit polyclonal, Sigma L9393) in a solution of PBS, 20% goat serum, and 1% Bovine Serum Albumin (BSA) for 24 h at 4 °C. Sections were washed in PBS 3X for 10 min and incubated with the secondary antibody in PBS for 1 h at 37 °C. Sections were washed in PBS 2X for 10 min, incubated 30 s with DAPI, and washed once in PBS for 10 min and mounted. For the morphometric analysis, muscle sections were scanned using a NanoZoomer (Hamamatsu Photonics, Massy, France) with a 20X objective. Image J free software was used to analyze and quantify the pictures for each entire area.

2.8. Tissue oxidative stress status and mitochondrial enzymatic activities

Long-established oxidative stress parameters (TBARS, -SH, GSH, catalase, SOD and MnSOD, GPX), citrate synthase (CS) and different mitochondrial respiratory complex (CI, CII, CII + III and COX) activities were measured in liver and muscle as previously reported [15 , 16].

2.9. Western blotting

Protein levels were assessed by Western blotting. Total proteins were measured using the Bio-Rad protein assay (Hercules, CA, USA) according to the manufacturer's instructions. Total proteins were lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, pH 7.5 + complete cocktail protease inhibitors + Pierce phosphatase inhibitors tablet). 40 μg of

cell extracts were loaded and blotted onto a nitrocellulose membrane. The membranes were washed, blocked with 5% nonfat milk and incubated in primary antibody overnight at 4 °C. The membranes were then washed in TBS-Tween (10 mM Tris, 140 mM NaCl, and 0.2% Tween-20), incubated with the appropriate secondary antibody coupled to horseradish peroxidase, and washed again in TBS-Tween. Signals were revealed using a Clarity Western ECL Substrate Kit (Hercules, CA, USA), and proteins were visualized by enhanced chemiluminescence using the ChemiDoc Touch Imaging System (Hercules, CA, USA) and quantified with Image Lab. Touch Software (version 5.2.1) (Hercules, CA, USA). Primary antibodies used include: anti-PPARalpha (1/1,0 0 0, Braissant et al, 1996); anti- chREBP (1/1,0 0 0, ABCam ab92809) and anti-Nrf2 (1/1,0 0 0, ABCam ab137550).

2.10. Statistical analysis

Results were expressed as means ±SD. DIO mice: HFHS diet *versus* control diet and HFHS + 9-PAHPA *versus* HFHS diet were tested by a student t test. Db/db mice: db/db *versus* db/ + and db/db + 9-PAHPA *versus* db/db were tested by a student t test. Statistical analyses were performed using the StatView program (SAS Institute, Cary, NC, USA).

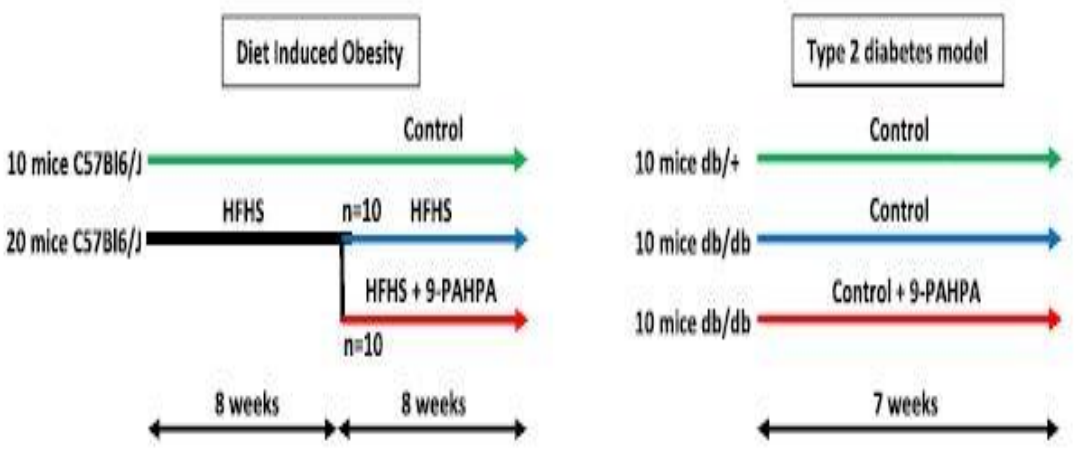

Fig. 1. Design of the study.

3.

Results

3.1. In DIO mice 9-PAHPA increased body weight and fat mass and decreased energy expenditure

As expected, energy intake, final body weight and fat mass were increased with 16 weeks of HFHS diet by comparison to con- trols (Fig. 2 A). Surprisingly, 9-PAHPA supplementation (HFHS + 9- PAHPA) expanded weight gain and fat mass (Fig. 2 A). However, no specific

effect of 9-PAHPA was found on energy intake or on IL6 plasma levels (Fig 2 A). In addition, while plasma cholesterol levels were significantly increased with HFHS diet compared to controls, no specific effect of 9-PAHPA was observed on plasma triglycerides, cholesterol and free fatty acids levels (Fig. 2 A).

We next investigate the basal metabolism, oxygen consumption and carbon dioxide production using Oxymax-CLAMS system. We found that oxygen consumption reflecting metabolic expenditure was decreased in animals fed a HFHS diet compared to controls (Fig. 2 B). In addition, we observed that 9-PAHPA supplementation worsened this decrease of energy expenditure (Fig. 2 B). This result probably explains the increase in body weight and adipose tissue induced by 9-PAHPA (Fig. 2 A). Moreover, analysis of RER revealed that HFHS diet favored the oxidation of lipids, which was reflected by a lower RER value compared to controls (Fig. 2 C). 9-PAHPA supplementation further significantly decreased RER com- pared to HFHS group (Fig. 2 C).

3.2. In DIO mice 9-PAHPA improved insulin sensitivity

Fasted plasma glucose was increase with HFHS diet compared to controls (Fig. 2 D). 9-PAHPA supplementation had no effect on glycemia compared to HFHS group (Fig. 2 D). We found that 9- PAHPA supplementation increased insulin sensitivity in comparison to HFHS diet alone as attested by ITT experiments (Fig. 2 E). However, OGGT indicated that 9-PAHPA had no effect on glucose tolerance compared to HFHS group (Fig. 2 F).

3.3. In DIO mice 9-PAHPA had no effect on liver phenotype nor on mitochondrial function and oxidative stress

Liver weight and the measure of liver triglycerides, total cholesterol and free fatty acid revealed no difference whatever the group of diets (Fig. 3 A-B). We also showed that 9-PAHPA was able to abolish the induction of TNF αinduced by HFHS diet (Fig. 3 C). Furthermore, histologic analysis indicated the presence of some lipid micro droplets in liver of DIO mice compared to controls (Fig. 3 D). Finally, we did not observe any evidence of fibrosis (Fig. 3 D). In line with this observation, plasma ALAT activity (Alanine aminotransferase), a biochemical marker of hepatic fibrosis, was not modified regardless of the diet (Fig. 3 E).

In addition, liver gene expression of peroxisome proliferator- activated receptors alpha (a major metabolic regulator of lipids homeostasis), ChREBP (a transcription factor coupling hepatic glucose utilization and lipid synthesis) and Nrf2 (a master regulator of cellular redox homeostasis) was studied by western-blot. We found that none of these proteins were modified by the diet (Fig. 3 E). However, we observed that 9-PAHPA induced a slight increase in ChREBP and a slight decrease in Nrf2. Moreover, we observed that liver 3-hydroxyacyl CoA dehydrogenase activity (β-HAD), involved in β-oxidation was increase by HFHS diet (Fig. 3 G).

We next investigated the mitochondrial function and oxidative stress parameters in liver. We found a slight increase of the activities of mitochondrial respiratory chain complexes II + III

and IV with the HFHS diet compared to control whereas the activities of the complexes activity I and II remained unchanged (Fig. 4 A). However, 9-PAHPA supplementation had no impact (Fig. 4 A). CS, a marker of mitochondrial activity, was not modified whatever the diet (Fig. 4 B). Moreover, we observed that 9- PAHPA induced a slight decrease level of TBARS (a lipid peroxidation marker) compared to HFHS diet whereas the other biochemical markers of oxidized proteins remained unchanged (SH groups, GSH and GS = SG) (Fig. 4 C-D). In addition, we found that the activities of the SOD (superoxide dismutase) antioxidants enzymes were slightly increased by HFHS diet whereas no change was ob- served for catalase and GPx (glutathion peroxidase) (Fig. 4 E). Last, 9-PAHPA supplementation induced an increase of catalase activity compared to HFHS group (Fig. 4 E).

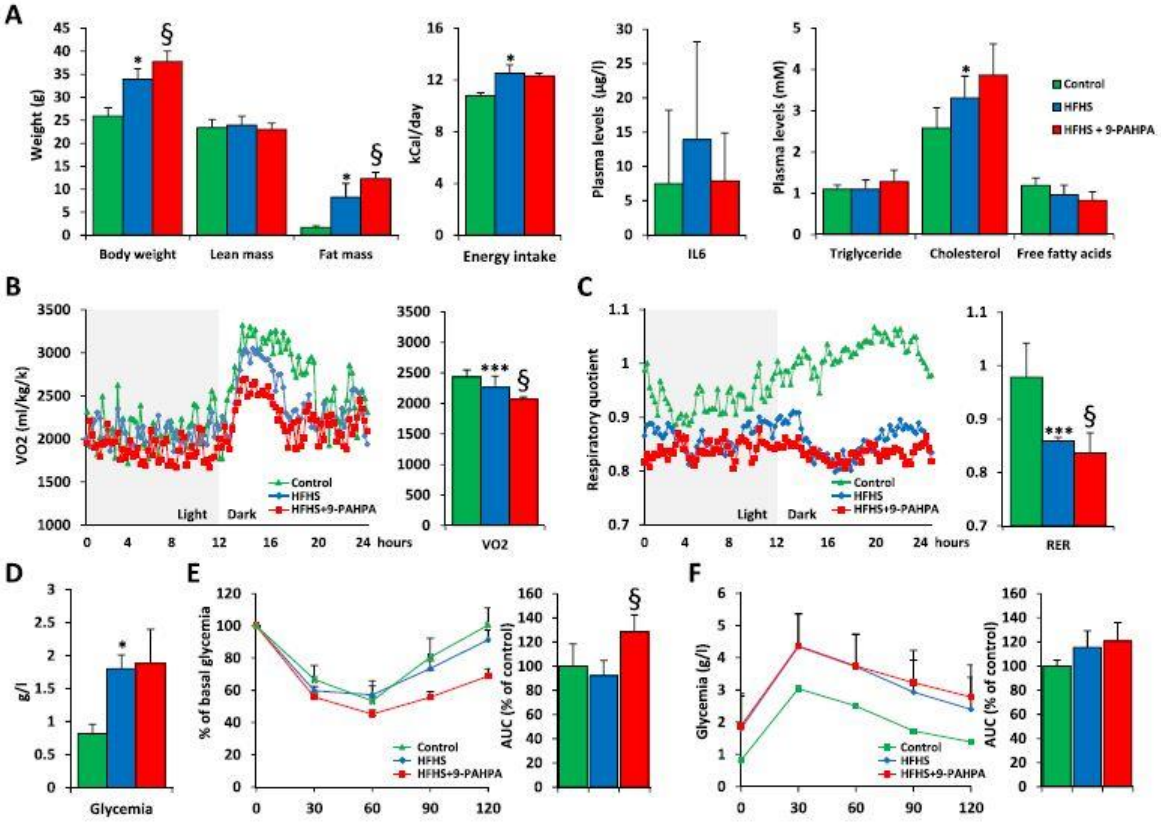

Fig. 2. DIO mice characteristics. (A) Body weight, lean and fat mass, energy intake, plasma IL6, and plasma lipids. (B) Oxygen consumption in mice (VO2). (C) Respiratory exchange ratio (RER) in mice. (D) fasting blood glucose. (E) Insulin tolerance test (ITT). (F) Glucose tolerance test (OGTT). For each mouse, VO2 and VCO2 were measured 111 times in about 24 h. The respiratory exchange ratio (RER) was calculated as the volume of CO2 *versus* volume of oxygen (VCO2/VO2) ratio. Mean VO2, VCO2 and RER values are mean of 8 mice for each group considering all the cycles. For all other parameters, n = 10 animals per group. Results were expressed as means ±SD, Control diet *versus* HFHS diet and HFHS diet *versus* HFHS + 9-PAHPA diet were tested by a student t test; $*P < .05$ HFHS *versus* Control and §$P < .05$ HFHS + 9-PAHPA *versus* HFHS.

3.4. In DIO mice 9-PAHPA had no effect on muscle phenotype nor on mitochondrial function and oxidative stress

We found that the weight of the various muscles analyzed (quadriceps, gastrocnemius and tibialis) was not modified either by the HFHS diet or by the HFHS + 9-PAHPA diet (Fig. 5 A). In addition, muscle triglycerides were increased by HFHS diet compared to controls and 9-PAHPA further increased the level of triglycerides in DIO mice (Fig. 5 B). However, cholesterol and FFA remained un- changed whatever the diet (Fig. 5 B). Next, we examined the cross- sectional area (CSA) of the tibialis muscle from our mice. Quantification of tibialis fiber number and area indicated that neither the HFHS diet nor 9-PAHPA supplementation had any impact on total fiber number and mean CSA (Fig. 5 C-D). Furthermore, analysis of the number of fibers in each range of area also showed no difference with or without 9-PAHPA (Fig. 5 E).

Then, we measured the mitochondrial respiratory chain activities that determine the metabolic properties of skeletal muscle. We found a slight increase of the activities of mitochondrial respiratory chain complexes IV with the HFHS diet compared to control whereas the activities of other complexes remained unchanged (Fig. 6 A). In addition, CS and β-HAD activities were also increased in DIO mice (Fig. 6 B-C). Regarding oxidative stress, HFHS diet in- creased TBARs levels and GSH content but had no impact on –SH content and antioxidant enzyme activities. 9-PAHPA supplementation slightly increased CS compared to HFHS diet but had no im- pact on mitochondrial respiratory chain and β-HAD activities and on oxidative stress markers (Fig. 6 A-C).

3.5. In db/db mice, 9-PAHPA had no influence on body weight and energy expenditure

We next investigate the influence of 9-PAHPA supplementation in db/db mice, a genetical model of obesity and type 2 diabetes. Db/ + mice were used as control. We show that energy intake and final body weight of db/db mice were greatly increased after 7 weeks of diet by comparison to controls (Fig. 7 A). However, no specific effect of 9-PAHPA was found in energy intake and body weight (Fig 7 A). As expected, the lack of leptin receptor leaded to the over-production of leptin compared to db/ + mice (Fig. 7 A). In addition, no changes were observed on IL-6, plasma triglycerides and free fatty acids levels whatever the group (Fig. 7 A).

We next investigate the basal metabolism, oxygen consumption and carbon dioxide production using Oxymax-CLAMS system. We found that oxygen consumption reflecting metabolic expenditure was strongly decreased in db/db mice compared to db/ + (Fig. 7 B). Moreover, analysis of RER revealed that oxidation of carbohydrates is increased in db/db mice, which was reflected by a higher RER value compared to db/ + (Fig. 7 C). This increase in RER reflects the fact that db/db mice were eating during the diurnal phase while db/ + animals were mainly sleeping. 9-PAHPA supplementation had no effect on either VO2 or RER compared with the db/db mice (Fig. 7 B-C).

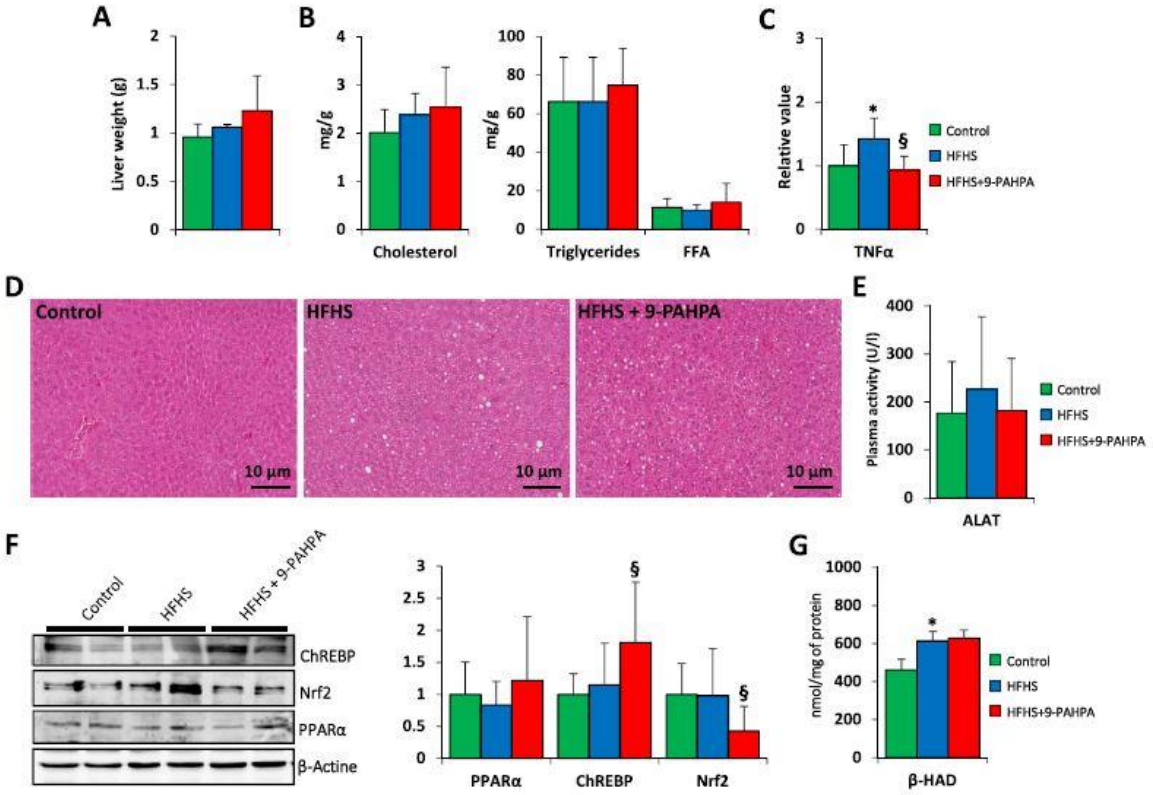

Fig. 3. Liver phenotype. (A) Liver weight after 16 weeks of diet. (B) Hepatic content of cholesterol triglycerides and free fatty acids (FFA). (C) Relative liver gene expression of TNF- α. (D) H&E staining of liver, pictures are representative from each group. Scale bar, 10 µm. (E) Plasma ALAT activity. (F) Relative Liver protein expression of key players in lipogenesis, glucose and cellular redox homeostasis. (G) Liver β-HAD activity. Results were expressed as means ±SD, *n* = 10 animals per group. Control diet *versus* HFHS diet and HFHS diet *versus* HFHS + 9-PAHPA diet were tested by a student t test; ∗*P* < .05 HFHS *versus* Control and §*P* < .05 HFHS + 9-PAHPA *versus* HFHS.

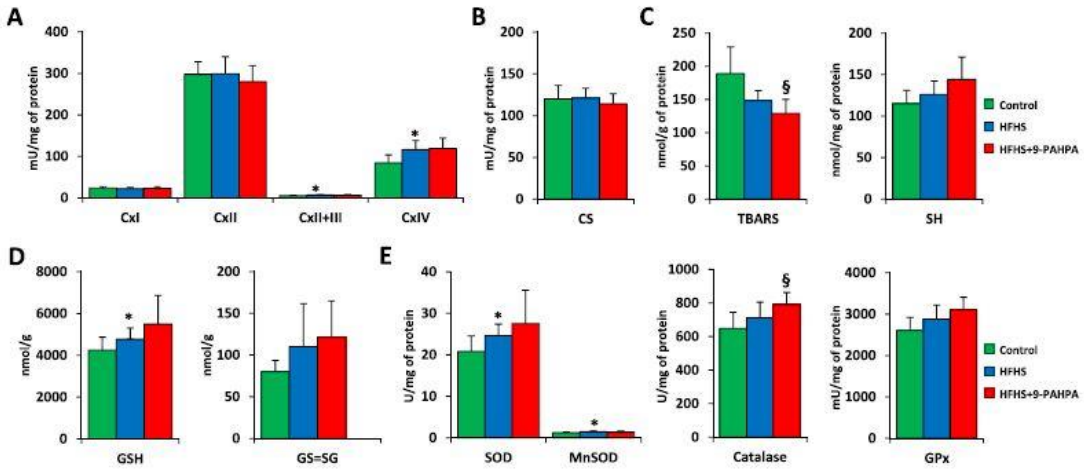

Fig. 4. Mitochondrial activity and hepatic oxidative stress parameters. (A) Mitochondrial respiratory chain activities. (B) Citrate synthase activity. (C) TBARS and –SH values. (D) GSH and GSSG values. (E) SOD, MnSOD, Catalase and GPx activities. Results were expressed as means ±SD, *n* = 10 animals per group. Control diet *versus* HFHS diet and HFHS diet *versus* HFHS + 9-PAHPA diet were tested by a student t test; ∗*P* < .05 HFHS *versus* Control and §*P* < .05 HFHS + 9-PAHPA *versus* HFHS.

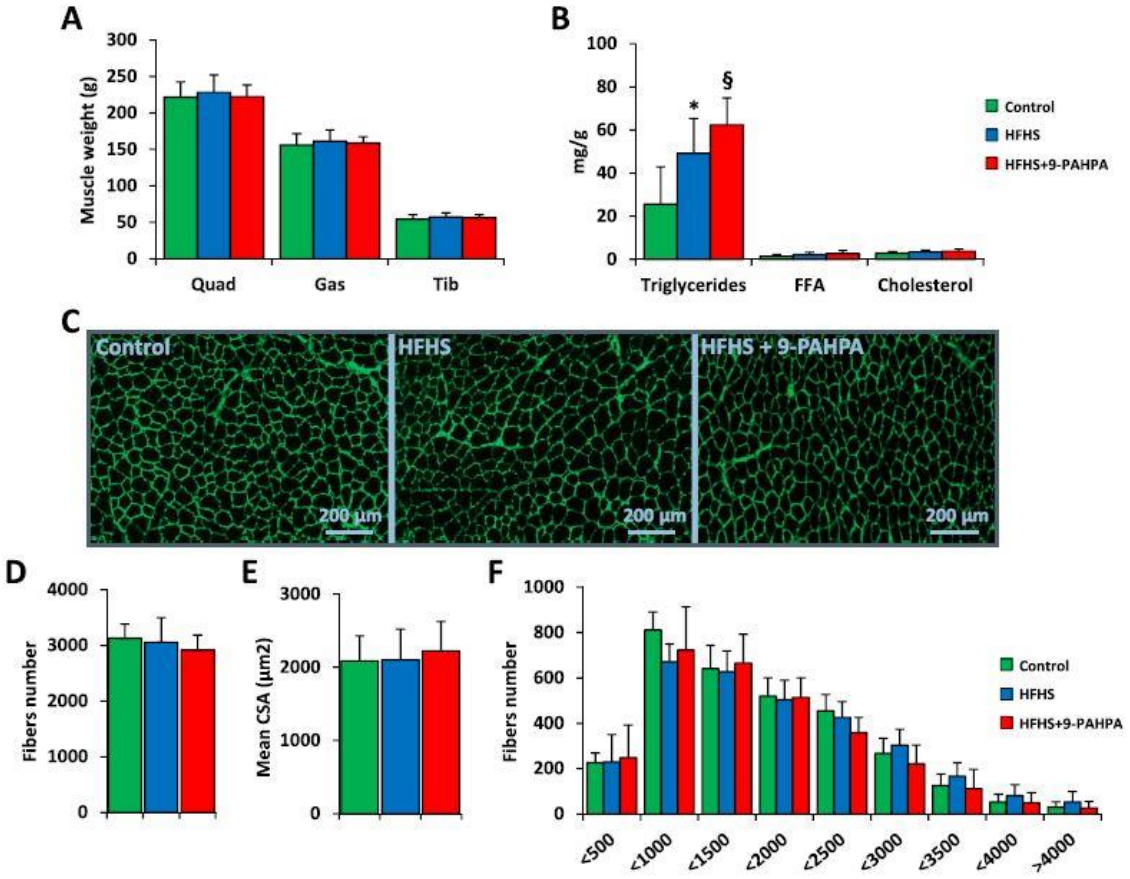

Fig. 5. Muscle phenotype . (A) Quadriceps, gastrocnemius, and tibialis muscles weight. (B) Quadriceps muscle content of cholesterol triglycerides and FFA measured on tissue homogenate by enzymatic method. (C) Representative anti-laminin staining on cryosections of tibialis muscles. Scale bar, 200 µm. (D) Total fibers number. (E) Mean fibers area. (F) Fiber size distribution in tibialis muscles. Results were expressed as means ±SD, *n* = 10 animals per group. Control diet *versus* HFHS diet and HFHS diet *versus* HFHS + 9-PAHPA diet were tested by a student t test; ∗*P* < .05 HFHS *versus* Control and §*P* < .05 HFHS + 9-PAHPA *versus* HFHS.

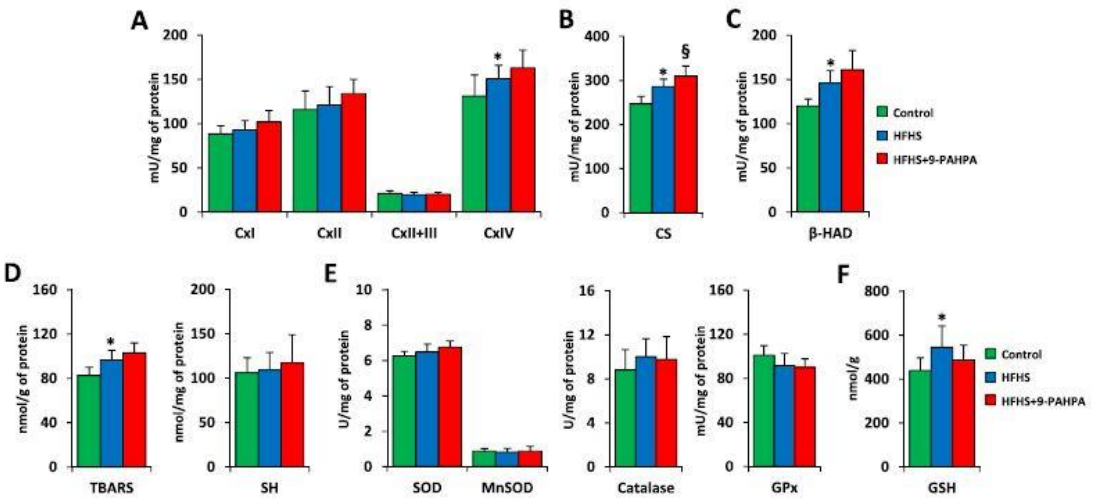

Fig. 6. Mitochondrial activity and muscle oxidative stress parameters. (A) Mitochondrial respiratory chain activities. (B) Citrate synthase activity. (C) β-HAD activity. (D) TBARS and –SH values. (E) SOD, MnSOD, Catalase and GPx activities (F) GSH values. Results were expressed as means ±SD, *n* = 10 animals per group. Control diet *versus* HFHS diet and HFHS diet *versus* HFHS + 9-PAHPA diet were tested by a student t test; ∗*P* < .05 HFHS *versus* Control and §*P* < .05 HFHS + 9-PAHPA *versus* HFHS.

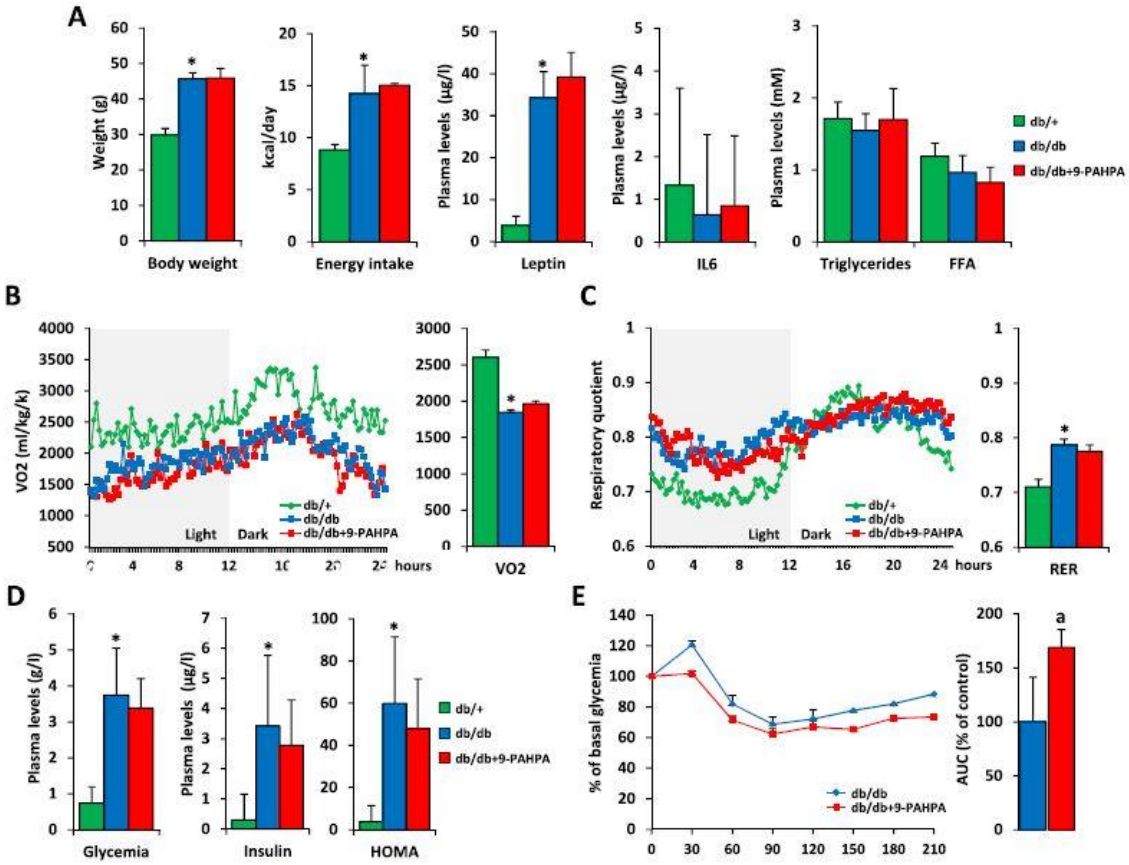

Fig. 7. db/db mice characteristics. (A) Body weight, energy intake, leptin and IL6 plasma levels, and plasma lipids. (B) Oxygen consumption in mice (VO2). (C) Respiratory exchange ratio (RER) in mice. (D) fasting blood glucose, insulin levels and HOMA-IR index. (E) Insulin tolerance test (ITT). For each mouse, VO2 and VCO2 were measured 111 times in about 24 h. The respiratory exchange ratio (RER) was calculated as the volume of CO2 *versus* volume of oxygen (VCO2/VO2) ratio. Mean VO2, VCO2 and RER values are mean of 8 mice for each group considering all the cycles. Results were expressed as means ±SD, $n = 10$ animals per group except for VO2, CO2 and RER data. db/ + *versus* db/db and db/db *versus* db/db + 9-PAHPA were tested by a student t test; $*P < .05$ db/db *versus* db/ + and $ $P < .05$ db/db + 9-PAHPA *versus* db/db.

3.6. In db/db mice, 9-PAHPA improved insulin sensitivity but had no effect on hyperglycemia

In fasted state we showed that plasma glucose, insulin levels and HOMA-IR index were dramatically increased in db/db mice compared to controls (Fig. 7 D). Unfortunately, 9-PAHPA supple- mentation did not improve these parameters compared to db/db mice (Fig. 7 D). However, we found that 9-PAHPA supplementation increased insulin sensitivity in comparison to db/db mice as at- tested by ITT experiments (Fig. 7 E).

3.7. In db/db mice, 9-PAHPA supplementation did not improve liver steatosis nor mitochondrial function and oxidative stress

In db/db mice liver weight and ALAT activity were strongly in- creased compared to db/ + (Fig. 8 A). In line with these observations, in db/db mice, histologic analysis revealed a macrovesicular steatosis with large lipid droplet that filled up the hepatocyte and displaced the nucleus to the periphery (Fig. 8 B). Moreover, we did not observe any evidence of fibrosis. 9-PAHPA supplementation did not correct the hepatomegaly and hepatic steatosis observed in db/db mice (Fig. 8 A-B).

Regarding mitochondrial respiratory chain activities and oxidative stress, we found a slight increase of the activities of mitochondrial respiratory chain complexes I and II + III in db/db mice com- pared to db/ + animals (Fig. 8 C). In contrast, the activities of mitochondrial respiratory chain complexes II and IV were decreased in db/db mice (Fig. 8 C). In addition, CS activity was also decreased in db/db mice (Fig. 8 D). However, 9-PAHPA supplementation had no impact on mitochondrial respiratory chain complexes and CS activities compared to db/db mice alone (Fig. 8 C-D). Moreover, we recorded a modest decrease level of TBARS, a modest increase of SH groups, an increase of GSH and a decrease of GS = SG content in db/db mice compared to controls (Fig. 8 E-F). In addition, we observed an increase of catalase activity and a decrease of Mn-SOD and GPx activities in db/db mice compared to db/ + whereas SOD activity remained unchanged (Fig. 8 G). However, 9-PAHPA supple- mentation had no impact on oxidative stress parameters (Fig. 8 E- G).

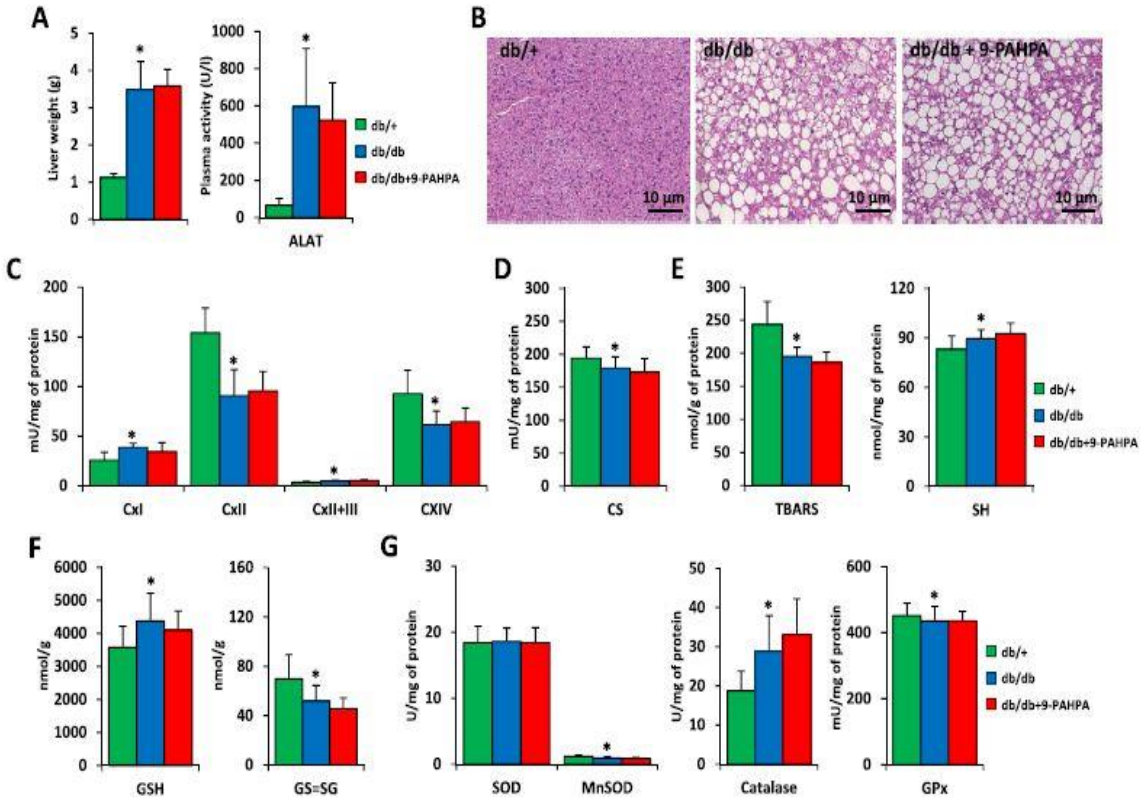

Fig. 8. db/db liver phenotype. (A) Liver weight and plasma ALAT activity after 7 weeks of diet. (B) H&E staining of liver, pictures are representative from each group. Scale bar, 10 µm. (C) Liver mitochondrial activity. (D) Citrate synthase activity. (E) TBARS and –SH values. (F) GSH and GSSG values. (G) SOD, MnSOD, Catalase and GPx activities. Results were expressed as means ±SD, n = 10 animals per group. db/ + *versus* db/db and db/db *versus* db/db + 9-PAHPA diet were tested by a student t test; $*P < .05$ db/db *versus* db/ + .

3.8. In db/db mice, 9-PAHPA had no effect on muscle phenotype nor on mitochondrial function and oxidative stress

In db/db mice, a strong muscle atrophy was recorded compared to db/ + animals as attested by the fall of the muscle weights analyzed (Fig. 9 A). Furthermore, during tissue collection we observed that the muscles of the db/db mice appeared to be very fatty. This observation was confirmed by the measurement of the intramuscular triglyceride content which was strongly increased in db/db mice (Fig. 9 B). As in DIO model, 9-PAHPA supplementation was not able to compensate for this muscle loss or this increase of triglyceride content (Fig. 9 A-B).

Histological analyses of the tibialis muscle (Fig. 9 C) revealed that this drop in muscle mass recorded in db/db mice was not related to a reduction in fibers number but to a significant decrease in mean CSA (Fig. 9 D-E). In addition, analysis of the number of fibers in each range of area also showed that in db/db mice there were more small-sized fibers and fewer large-sized fibers (Fig. 9 F). These analyses confirmed that 9-PAHPA supplementation had no

impact on the total number of fibers, CSAs, and fiber distribution compared with db/db mice alone (Fig. 9 D-F).

Next, we measured the mitochondrial respiratory chain activities that determine the metabolic properties of skeletal muscle. In db/db mice, we found a slight decrease of the activities of mitochondrial respiratory chain complexes IV and II + III compared to db/ + animals (Fig. 10 A). CS and β-HAD activities remained un- changed (Fig. 10 B-C). However, 9-PAHPA supplementation had no impact on mitochondrial respiratory chain, CS and β-HAD activities compared to db/db mice. In addition, analysis of antioxidant enzymes (SOD, Mn-SOD, catalase, and glutathione peroxidase), GSH and markers of oxidative stress (TBARS and SH-group) revealed no difference with or without 9-PAHPA in db/db mice (Fig. 10 D-F).

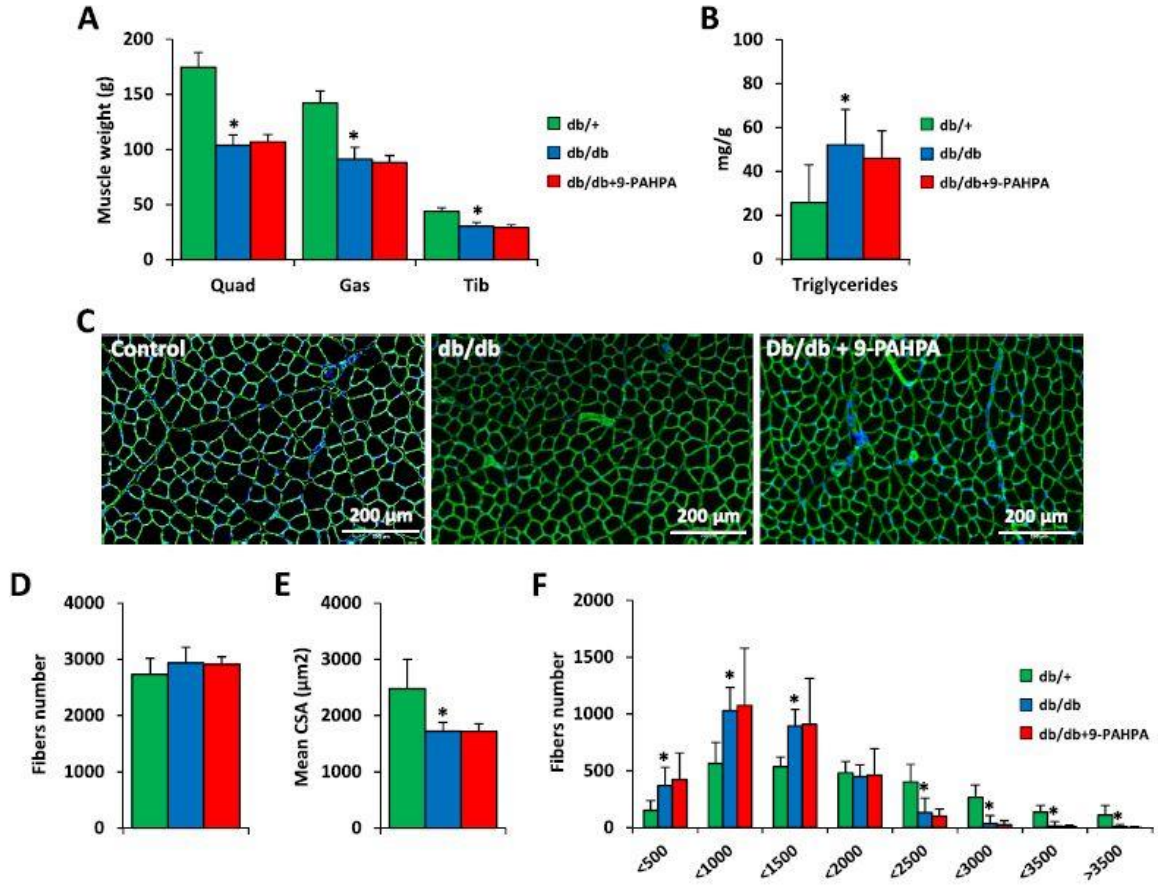

Fig. 9. Muscle phenotype . (A) Quadriceps, gastrocnemius, and tibialis muscles weight. (B) Muscle triglycerides content. (C) Representative anti-laminin staining on cryosections of tibialis muscles. Scale bar, 200 µm. (D) Total fibers number. (E) Mean fibers area. (F) Fiber size distribution in tibialis muscles. Results were expressed as means ±SD, *n* = 10 animals per group. db/ + *versus* db/db and db/db *versus* db/db + 9-PAHPA diet were tested by a student t test; ∗*P* < .05 db/db *versus* db/ + .

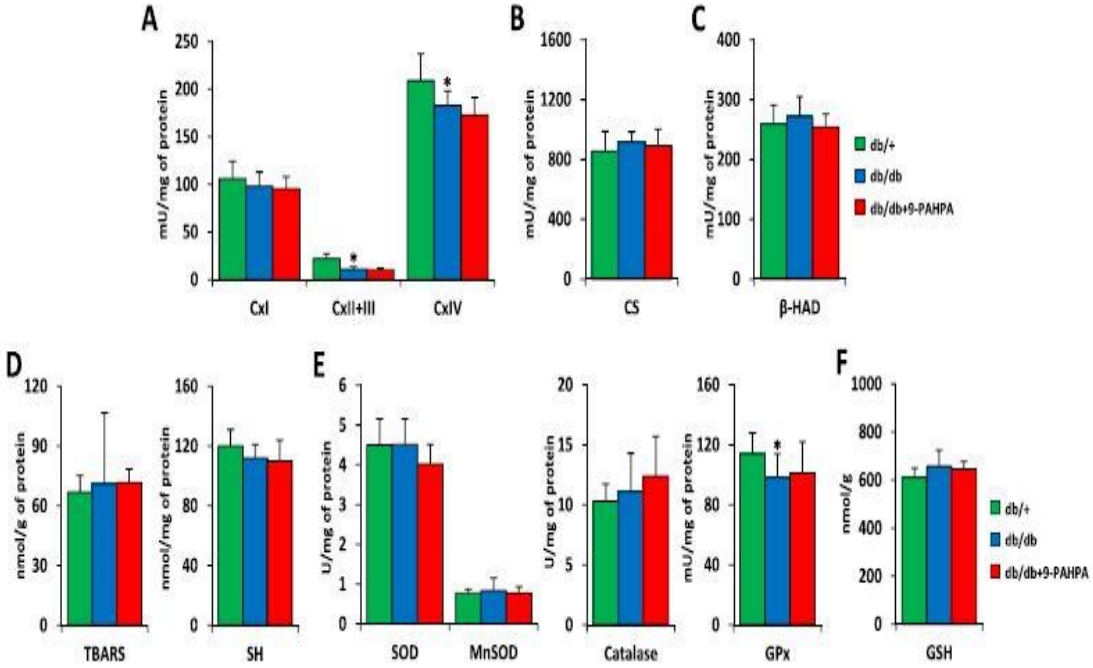

Fig. 10. Mitochondrial activity and muscle oxidative stress parameters. (A) Mitochondrial respiratory chain activities. (B) Citrate synthase activity. (C) β-HAD activity. (D) TBARS and –SH values. (E) SOD, MnSOD, Catalase and GPx activities. (F) GSH values. Results were expressed as means ±SD, *n* = 10 animals per group. db/ + *versus* db/db and db/db *versus* db/db + 9-PAHPA diet were tested by a student t test; ∗*P* < .05.

4. Discussion

We previously showed that 9-PAHPA and 9-OAHPA increased insulin sensitivity in healthy mice when incorporated into a chow diet [15] or lowered insulin resistance in obese mice when incorporated into an HFHS diet [16] . However, in C57Bl6/J mice fed an HFHS diet, preventive supplementation with 9-PAHPA and 9- OAHPA failed to prevent weight gain and hyperglycemia [16] . The aim of this work was therefore to investigate whether curative supplementation with 9-PAHPA may have beneficial effects against obesity and metabolic dysfunction when given curatively in al- ready obese animals. For this purpose, we chose to use two animal models of obesity, the conventional DIO and db/db mice.

Surprisingly, in DIO mice, we observed that 9-PAHPA did not improve the phenotype but rather worsened it, as evidenced by increased body weight and fat mass. In line with this observation, we found that 9-PAHPA supplementation decreased energy expenditure compared to HFHS diet alone. We also recorded a more marked hyperglycemia with 9-PAHPA supplementation. This lack of positive effect on weight gain is in agreement with recent reports indicating that PAHSA [10 , 11] or 12-OAHSA treatment [7] did not reduce body weight gain and fat mass in DIO mice. In the db/db model of obesity, 9-PAHPA supplementation had no effect on the dramatic weight gain and hyperglycemia observed in db/db mice after few weeks on chow diet. In the same db/db mouse model, Wang et al. also

showed that 30 d of treatment with 5-PAHSA was not able to prevent significant weight gain in these mice [22] .

Obesity is a major risk factor for nonalcoholic fatty liver dis- ease (NAFLD) [23] . Although C57BL6/J mice are widely used in obe- sity and diabetes research [24 , 25], their ability to develop NAFLD is quite heterogeneous according to studies [26 , 27]. In this work, de- spite the obesogenic diet, we did not observe any increase in liver weight in HFHS mice and only the histological analysis revealed the presence of some lipid micro-droplets in the liver. In line with this limited impact on the liver, the different metabolic parame- ters analyzed (plasma cholesterol, triglycerides, ALAT, and hepatic mitochondrial activities and oxidative stress parameters) were not modified either by the diet or by 9-PAHPA supplementation. How- ever, we observed that 9-PAHPA increased in the expression of the transcription factor ChREBP. Interestingly, several studies reported that ChREBP acts as a key modulator of hepatic fatty acid com- position and insulin sensitivity [28 , 29]. Our result suggests that 9-PAHPA, by upregulating ChREBP, may participate in the previously described increase in insulin sensitivity. In the db/db obesity model, as expected liver weight and plasma ALAT activity were strongly increase compared to db/ + . In line with these observations, in db/db mice, histologic analysis revealed a macrovesicular steatosis with large lipid droplet. However, 9-PAHPA supplementation did not correct the hepatomegaly and hepatic steatosis ob- served in db/db mice. 9-PAHPA had also no impact on the different metabolic parameters analyzed (plasma cholesterol, triglycerides, ALAT, and hepatic mitochondrial activities and oxidative stress parameters). A study from Wang et al. demonstrated that 5-PAHSA treatment tends to promote promoted fatty liver and inflammation in db/db mice [22] . These set of results indicate no beneficial impact of 9-PAHPA supplementation on liver metabolism on both models of obesity studied.

Skeletal muscle is essential for whole metabolism. It is one of the main regulators of glucose homeostasis, responsible for 80% of postprandial glucose uptake from the circulation [3] . Recently, we showed that 12 weeks of 9-PAHPA or 9-OAHPA supplementation had no effect on the skeletal muscle mass, mitochondrial activity, and oxidative stress parameters of mice fed a chow diet. How- ever, we observed that 9-PAHPA or 9-OAHPA induces a switch to- ward a more oxidative contractile phenotype in these mice [17] . In this work, we found that in DIO mice, 9-PAHPA had no effect on muscle phenotype. In db/db mice, strong muscle atrophy was recorded compared to db/ + animals. However, 9-PAHPA supple- mentation was not able to compensate for this muscle loss, nor did it induce any change in the overall parameters studied.

In this study, the major effect that emerged from curative 9- PAHPA supplementation was its ability to decrease insulin resistance in DIO and db/db mice. This positive result of 9-PAHPA on insulin sensitivity is in agreement with our previous results with healthy or diet-induced obese mice [15 , 16] and those of other groups [4 , 7 , 9 –11]. Furthermore, a possible effect of 9-PAHPA supplementation on hepatic neoglucogenesis was not addressed in this work. However, the observation during insulin sensitivity tests that the return of blood glucose to normal in the second part of the experiment (between 60 and 120 min) was slower in animals

supplemented with 9-PAHPA suggests that it could also decrease hepatic glucose production as it has been shown previously for 9-PAHSA [11] .

In conclusion, we have investigated the impact of curative 9- PAHPA supplementation in two animal models of obesity, the classical DIO and the db/db mice. In these two obesity models, 9- PAHPA was not able to prevent either weight gain or the development of hyperglycemia. Moreover, 9-PAHPA had no impact on the metabolic parameters analyzed at the hepatic and muscular level. In the present study, the only beneficial effect of a curative 9-PAHPA supplementation observed was a decrease in insulin resistance in DIO and db/db mice. These results are comparable with those obtained recently with other FAHFAs and highlight that al- though these molecules alone are able to improve insulin sensitiviity, they have little effects on obesity and associated metabolic dis- orders. Perhaps their action could be more interesting in the longer term or in combination with other molecules or with physical ac- tivity practice.

Authors Contributions

Author contributions were as follows: study design (CC, FC, CF- C), FAHFAs synthesis (LB, MB), data collection (BB, LP, MB, KL, AJCE, CBG), statistical analysis (KL, FC, CF-C), data interpretation (CC, FC, LP, AJCE, MMA, JAR, SG, TD, KL, LB, CF-C), manuscript preparation (FC, CF-C), literature search (CC, FC, CF-C) and funds collection (CC, FC, CF-C). All the authors have read and approved the final version of this manuscript.

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Declaration of competing interests

The authors declare no conflict of interest.

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