Palmitoylethanolamide counteracts hepatic metabolic inflexibility modulating mitochondrial function and efficiency in diet-induced obese mice

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Abstract
Peroxisome proliferator-activated receptor (PPAR)-α activation controls hepatic lipid homeostasis, stimulating fatty acid oxidation, and adapting the metabolic response to lipid overload and storage. Here, we investigate the effect of palmitoylethanolamide (PEA), an endogenous PPAR-α ligand, in counteracting hepatic metabolic inflexibility and mitochondrial dysfunction induced by high-fat diet (HFD) in mice. Long-term PEA administration (30 mg/kg/die per os) in HFD mice limited hepatic lipid accumulation, increased energy expenditure, and markedly reduced insulin resistance. In isolated liver mitochondria, we have demonstrated PEA capability to modulate mitochondrial oxidative capacity and energy efficiency, leading to the reduction of intracellular lipid accumulation and oxidative stress. Moreover, we have evaluated the effect of PEA on mitochondrial bioenergetics of palmitate-challenged HepG2 cells, using Seahorse analyzer. In vitro data showed that PEA recovered mitochondrial dysfunction and reduced lipid accumulation in insulin-resistant HepG2 cells, increasing fatty acid oxidation. Mechanistic studies showed that PEA effect on lipid metabolism was limited by AMP-activated protein kinase (AMPK) inhibition, providing evidence for a pivotal role of AMPK in PEA-induced adaptive metabolic setting. All these findings identify PEA as a modulator of hepatic lipid and glucose homeostasis, limiting metabolic inflexibility induced by nutrient overload.

Abbreviations: ACC, acetyl-CoA carboxylase; AKT, protein kinase B; AMPK, adenosine monophosphate kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; CPT, carnitine-palmitoyltransferase; CSV, Institutional Animal Care and Use Committee; FAO, fatty acid oxidation; FCCP, p-trifluoromethoxy phenylhydrazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; H&E, hematoxylin & eosin; HFD, high-fat diet; HOMA, Homeostasis Model Assessment; IL, interleukin; IR, insulin resistance; InsR, insulin receptor; ITT, insulin tolerance test; MCP1, monocyte chemoattractant protein-1; NAEs, N-acylethanolamines; NAFLD, non-alcoholic fatty liver disease; OCR, oxygen consumption rate; OGTT, oral glucose tolerance test; Pal, sodium palmitate; PEA, palmitoylethanolamide; PPARs, peroxisome proliferator-activated receptors; PTT, pyruvate tolerance test; ROS, reactive oxygen species; RQ, respiratory quotient; SOD, superoxide dismutase; STD, standard diet; TNF, tumor necrosis factor.

Chiara Annunziata, Adriano Lama, and Claudio Pirozzi contributed equally to the study as first authors.
Giuseppina Mattace Raso and Rosaria Meli contributed equally to the study as senior authors.
1 INTRODUCTION

Metabolic flexibility is the ability of several tissues (i.e., liver, adipose tissue, skeletal muscle, and heart) to adapt metabolism and manage nutrient sensing, uptake, transport, storage, and expenditure, switching from fatty acid oxidation (FAO) during fasting to glucose metabolism during the fed state. Impaired metabolic flexibility has been reported to be associated with obesity and type 2 diabetes, but it is still unknown whether this detrimental condition is a cause or a consequence of these pathologies. Upon consumption of a high-fat diet (HFD), lean individuals with adequate metabolic flexibility are able to induce FAO compared to obese ones, increasing the expression of genes involved in fatty acid transport and oxidation. The inflexibility, implicated in the ectopic accumulation of lipid and in the development of insulin resistance (IR), is improved by weight loss. The pathogenesis of IR is based on an imbalance between energy consumption and expenditure that leads to lipid accumulation. IR is crucial for the pathogenesis of type 2 diabetes; therefore, the improvement of insulin signaling pathway leads to the restoration of energy homeostasis and insulin sensitivity, as an effective strategy for the treatment of type 2 diabetes.

In the liver, impaired FAO produces toxic lipid intermediates altering insulin signaling pathway and triggering oxidative stress and subsequent mitochondrial dysfunction. Recently, mitochondria emerged as key regulators of metabolic flexibility selecting fuel in response to nutritional changes and substrate disposal. In non-alcoholic fatty liver disease (NAFLD), chronic toxic lipid overload causes the activation of inflammatory pathways, resulting in increased pro-inflammatory adipokines and reduced insulin-sensitizing adipokines. Besides collecting nutrients, portal blood drains different mediators released from gut and visceral adipose tissue. Therefore, hepatic metabolism is directly affected not only by nutrients assumed from the diet but also by the secretory profile of visceral fat, highlighting the endocrine and immune interactions in NAFLD-related diseases.

Peroxisome proliferator-activated receptors (PPARs) are lipid sensors that modulate metabolic pathways in response to substrate disposal. Indeed, PPAR-α, as a ligand-activated transcription factor, exerts positive and/or negative control over the expression of a range of metabolic and inflammatory genes and regulates cognitive flexibility in mice. The ability of this nuclear receptor to integrate metabolic and inflammatory pathways makes it an attractive target for intervention in metabolic diseases, such as NAFLD and IR.

Palmitoylethanolamide (PEA, C16:0), an endogenous PPAR-α agonist, has shown to have a bi-faced pharmacological profile. Indeed, beyond the well-known analgesic and anti-inflammatory effects, PEA shows a metabolic activity, modulating energy balance in animals and humans.

Here, we have investigated PEA metabolic activities in liver, focusing on glucose and lipid homeostasis in a mouse model of diet-induced obesity. Moreover, our mechanistic study provides evidence for a central role of AMP kinase (AMPK) in PEA-induced adaptive metabolic setting.

2 MATERIALS AND METHODS

2.1 Ethics statement

All procedures involving the animals were carried out in accordance with the international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration, including the “3R” concept). All animal procedures reported herein were approved by the Institutional Animal Care and Use Committee (CSV) of the University of Naples Federico II under protocol no. 982/2017-PR.

2.2 Animals and in vivo experimental procedures

Male C57Bl/6J mice (Harlan, Italy) at 6 weeks of ages were housed in stainless steel cages in a room kept at 22 ± 1°C with a 12:12 hours light-dark cycle. Standard chow diet (Mucedola srl, Milan, Italy) had 17% fat, without sucrose while HFD (Research Diets Inc, NJ, USA) had 45% of energy derived from fat, 7% of sucrose. Standard and HFD contained 15.8 and 21.9 kJ/g, respectively, determined by bomb calorimeter. Moreover, the diet composition formula, the detailed fatty acid profile, and the relative percentage of monounsaturated and saturated fatty acid of STD diet and HFD are reported in Table S1. The total energy intake was calculated considering the weekly food intake and the energy content of the diets.

After weaning, young mice were randomly divided into three groups (n = 30 animals) as follows: (i) control group...
(STD) receiving chow diet and vehicle per os by gavage; (ii) HFD group receiving vehicle; (iii) HFD group treated with PEA (HFD+PEA, 30 mg/kg/die per os, by gavage). The treatments started after 12 weeks of HFD feeding and lasted 8 weeks. Ultra-micronized PEA was provided by Epitech Group Research Labs (Padova, Italy). It was suspended in carboxymethyl cellulose (1.5%) for oral gavage. During the experimental period, body weight was weekly assessed. At the end of the experimental protocol, before sacrifice, bioelectrical impedance analysis was performed to determine fat body composition assessment using BIA 101 analyzer, modified for the mouse (Akern, Florence, Italy). The animals were anesthetized by enflurane followed by cervical dislocation and serum and tissues were collected. Liver from all mice was removed and the samples not immediately used for mitochondria preparation were frozen and stored at −80°C for subsequent biochemical determinations.

2.3 Measurement of oxygen consumption, carbon dioxide production, and respiratory quotient

Following an adaption period to the experimental environment, oxygen consumption (VO2) and carbon dioxide production (VCO2) were recorded by a monitoring system (Panlab s.r.l., Cornellà, Barcelona, Spain) that is composed of a four-chambered indirect open-circuit calorimeter, designed for continuous and simultaneous monitoring. VO2 and VCO2 were measured every 15 minutes (for 3 minutes) in each chamber for a total of 6 hours. The mean VO2, VCO2, respiratory quotient (RQ) values, and energy expenditure were calculated by the “Metabolism H” software.18

2.4 Glucose, insulin, and pyruvate tolerance tests

At the beginning of 8th week of PEA treatment, oral glucose tolerance test (OGTT), insulin tolerance test, (ITT) and pyruvate tolerance test (PTT) were performed on different subgroups of mice (10 animals/each group).

OGTT was performed in overnight fasted animals receiving glucose (1 g/kg, Sigma-Aldrich, St Louis, MO, USA), whereas insulin (0.75 IU/kg, Humulin, Lilly, Indianapolis, IN, USA) was intraperitoneally injected in fed mice to assess ITT. Blood glucose levels were measured at selected time points after injection. The area under the curve (AUC) was calculated from time 0, as an integrated and cumulative measure of glycemia up to 120 minutes for all animals. For PTT, fasted animals were i.p. injected with pyruvate (2 g/kg, Sigma-Aldrich, St Louis, MO, USA) and glycemia was measured at 15, 30, 60, and 120 minutes after the administration. Glucose levels were measured by the glucometer One Touch Ultrasmart (Lifescan, Milpitas, CA).

2.5 Serum and hepatic parameters and tissue isolation

At the end of the experimental period (8th week), the sera and livers were collected. In serum, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, and total cholesterol were measured by colorimetric enzymatic method using commercial kits (SGM Italia, Italy and Randox Laboratories ltd., United Kingdom). Serum interleukin (IL)-1, IL-10 (Thermo Scientific, Rockford, IL, USA), tumor necrosis factor (TNF)-α (Biovendor R&D, Brno, Czech Republic), adiponectin and leptin (B-Bridge International Mountain View, CA), and fasting insulin (cat. no. EZRMI-13K; Millipore, Darmstadt, Germany) concentrations were measured using commercially available ELISA kits. As the index of IR, HOmeostasis Model Assessment (HOMA)-IR was calculated, using the formula \[ \text{HOMA} = \frac{\text{fasting glucose (mmol/L)} \times \text{fasting insulin (μU/mL)}}{22.5}. \] Liver samples were homogenized in saline solution and then centrifuged at 5000 rpm for 5 minutes. Supernatants were collected and centrifugated at 14 000 rpm at 4°C for 15 minutes and triglycerides quantified (TGL Flex reagent cartridge, Siemens Healthcare GmbH, Erlangen, Germany). Liver tissues, obtained from all animal groups, were excised for further biochemical and histological analysis.

2.6 Hepatic histological and fatty acids analysis

Livers were collected and either preserved in 10% neutral buffered formalin or snap frozen in Isopentane precooled in liquid nitrogen. Formalin-fixed and paraffin embedded 4 μm sections were stained with hematoxylin and eosin (H&E) for morphology. Cryostat 10 μm liver sections were stained with Oil Red O (# 04-220923, Bio Optica, Milan, Italy) according to the manufacturer’s instruction to measure intracellular lipid droplet accumulation. A double-blinded examination of the sections was made by veterinary pathologists at a magnification ×200 with a concordance rate of 95%. Statistical analysis was performed using SPSS software \( (P < .05) \). The comparison among groups was performed using ANOVA univariate analysis.

Analysis of fatty acids (palmitic, palmitoleic, and oleic acids) and PEA was carried out as described by Piscitelli et al.19 The values (n = 6 each group) are expressed in pmoles or µmoles/g liver tissue.
2.7 Measurements of mitochondrial oxidative capacity and degree of coupling

In another set of animals, the mitochondrial isolation, oxygen consumption, and the degree of coupling measurements were performed as previously reported.20 The degree of coupling was determined in the liver by applying equation by Cairns et al.21: degree of coupling = \( \sqrt{1 - (J_o)_{sh}/(J_o)_{unc}} \) where \((J_o)_{sh}\) represents the oxygen consumption rate in the presence of oligomycin that inhibits ATP synthase, and \((J_o)_{unc}\) is the uncoupled rate of oxygen consumption induced by carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP), which dissipates the mitochondrial proton gradient. \((J_o)_{sh}\) and \((J_o)_{unc}\) were measured as above using succinate (10 mmol/L), rotenone (3.75 μmol/L) in the presence of oligomycin (2 μg/mL) or FCCP (1 μmol/L), respectively.

Carnitine-palmitoyltransferase (CPT) total activity was followed spectrophotometrically as CoA-SH production by the use of 5,5′-dithiobis (nitrobenzoic acid) (DTNB) and as substrate palmitoyl CoA 10 μM. The medium consisted of 50 mM KCl, 10 mM Hepes (pH 7.4), 0.025% Triton X-100, 0.3 mM DTNB, and 10-100 pg of mitochondrial protein in a final volume of 1.0 mL. The reaction was followed at 412 nm with spectrophotometer, and enzyme activity was calculated from volume of 1.0 mL. The reaction was followed spectrophotometrically as CoA-sH production by using 5,5′-dithiobis (nitrobenzoic acid) (DTNB) and as substrate palmitoyl CoA 10 μM. The medium consisted of 50 mM KCl, 10 mM Hepes (pH 7.4), 0.025% Triton X-100, 0.3 mM DTNB, and 10-100 pg of mitochondrial protein in a final volume of 1.0 mL. The reaction was followed at 412 nm with spectrophotometer, and enzyme activity was calculated from volume of 1.0 mL. The temperature was maintained at 25°C.22,23 After 24-hour Pal challenge, cell lipid content was evaluated29 or Western blot analysis on cell lysate was performed.

2.8 Oxidative stress

The levels of reactive oxygen species (ROS) were also determined in liver homogenate as previously reported.20 The specific activity of aconitase and superoxide dismutase (SOD) was spectrophotometrically measured.22,24 Catalase activity was determined based on the decomposition of H₂O₂ at 25°C.25 Reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations in the liver were measured with the dithionitrobenzoic acid-GSSG reductase recycling assay.26

2.9 Cell culture experiments

Human HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 complete medium at 37°C with 5% CO₂. After 16-hour starvation in 5% FBS medium, HepG2 cells were incubated with sodium palmitate (Pal, 100 μM, Sigma-Aldrich, Milan, Italy) or its vehicle, for 24 hours to obtain Pal-induced insulin resistant and control cells, respectively.27,28 2 hours before Pal challenge, cells were pre-treated with PEA (1 μM). When used, compound C (2 μM), an inhibitor of AMPK (Sigma-Aldrich, Milan, Italy), was added 1 hour before PEA stimulation.

2.10 Cellular oxygen consumption measurement

HepG2 cells were seeded in Seahorse XFp Analyzer (Agilent Technologies, Santa Clara, CA, USA) in mini plates at 30 000 cells/well in RPMI growth medium overnight using Mito Stress Test. Cells were treated with the tested compounds as described above; then, the medium was replaced with 750 μL unbuffered Seahorse XF Base medium supplemented with glucose (10 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), equilibrated at 37°C in a CO₂-free incubator for 1 hour, following manufacturer’s instructions. Respiration was expressed as oxygen consumption rate (OCR, pmol/min). The proton leak was determined after inhibition of mitochondrial ATP production by 1 μM oligomycin (as an inhibitor of the F0/F1 ATPase). Afterward, the mitochondrial electron transport chain was maximally stimulated by the addition of the uncoupler FCCP (2 μM). The extra-mitochondrial respiration was estimated after the injection of rotenone (1 μM) and antimycin A (0.5 μM), inhibitors of the complexes I and III, respectively. Coupling efficiency is the proportion of the consumed oxygen to drive ATP synthesis compared with that driving proton leak (% of ATP-linked OCR/basal OCR).30 OCR was normalized to the protein content of each well for all measurements by Bradford assay.31

2.11 Histological score analysis

For histological examination, three main broad categories of histological features were analyzed: steatosis, inflammation, and necrosis. The grading system was adapted from Kleiner et al.32 as previously described.33 Kleiner's grading system considers the following histological variables: severity of steatosis (quantified by low- to medium-power evaluation of parenchymal involvement by steatosis): score 0, <5%; score 1, 5-33%; score 2, >33-66%, score 3, >66%; location (predominant distribution pattern): zone 3, score 0; zone 1, score 1; azonal, score 2; inflammation: lobular inflammation (overall assessment of all inflammatory foci): score 0, no foci; score 1, <2 foci per ×200 magnification field; score 2, 2-4 foci per ×200 magnification field; score 3, >4 foci per ×200 magnification field; necrosis: score 0, present; score 1, absent.

2.12 Western blotting

Livers, after homogenization, and HepG2 cells were lysed and total protein lysates were underwent to SDS-PAGE. The
filter was probed with a rabbit polyclonal antibody against anti-GLUT2 (Millipore Corporation, Billerica, MA, USA), anti-phospho protein kinase B (AKT) or anti-AKT, anti-phosphoAMPK (pAMPK) or anti-AMPK, anti-phospho acetyl-CoA carboxylase (pACC) (Ser 79) or anti-ACC, anti-insulin receptor (InsR) (Cell Signaling Technology, Danvers, MA, USA), or anti-phospho InsR (pInsR, Thermo Scientific, Rockford, USA), anti-PPAR-α (Sigma-Aldrich, Milan, Italy), anti-PPARGC1-α (PGC1-α, Elabscience, Houston, Texas), and anti-CPT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Western blot for (GAPDH), Tubulin, and β-Actin (Sigma-Aldrich, Milan, Italy) were performed to ensure equal sample loading. Bands were detected by ChemiDoc imaging instrument (Bio-Rad, Segrate, Italy).

2.13 | Real-time semi-quantitative PCR

Total RNA, isolated from liver, was extracted following protocols as previously reported. Each cDNA sample (500 ng) was mixed with 2X QuantiTech SYBRGreen PCR Master Mix and primers, Ppara, Fasn, Tnfa, Il6, and Ucp2 (Qiagen, Hilden, Germany). The relative amount of each studied mRNA was normalized to Actb as a housekeeping gene, and the data were analyzed according to the 2−ΔΔCt method. Real-time PCR was performed by CFX96 instrument (Bio-Rad, Segrate, Italy).

2.14 | Statistical analysis

Data are presented as mean value ± SEM or SD. Statistical analysis was performed by one- or two-way ANOVA followed by Bonferroni’s post-hoc, for multiple comparisons. Differences among groups were considered significant at values of \( P < .05 \); different superscripted letters indicate significant statistical differences.

Analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | PEA reduced lipid accumulation, increased energy expenditure, and resting metabolic rate

The mean body weight of all groups was reported, starting 12 weeks after HFD feeding throughout the 8-week treatment period (Figure 1A). PEA treatment gradually reduced body weight, reaching significance after 7 weeks. Consistently, 8-week treatment with PEA reduced fat mass (Figure 1B). Total energy intake of HFD+PEA group was lower than that of HFD (Figure 1C). Moreover, PEA treatment increased energy metabolism, as shown by the higher \( \text{VO}_2 \) consumption and \( \text{VCO}_2 \) production (Figure 1D) compared to HFD mice. Notably, RQ ratio decreased in PEA-treated HFD mice indicating an increase in FAO (Figure 1E). Consistently, the energy expenditure of HFD + PEA mice was increased compared to HFD ones (Figure 1F).

3.2 | PEA modulated serum parameters, improved glucose metabolism, and hepatic insulin sensitivity

In Table 1, biochemical and hormonal parameters are reported. Serum triglycerides, cholesterol, ALT, and TNF-α, increased by HFD, were significantly reduced by PEA. Similarly, the hormonal profile altered by HFD was improved by PEA treatment, as shown by leptin and adiponectin serum levels, whose ratio was consistently reduced. Moreover, PEA treatment reduced serum insulin and glucose levels, resulting in a reduction in the HOMA-IR index.

Glucose metabolism was analyzed (Figure 2); HFD induced a marked increase in glycemia curve in OGTT and AUC value, which were significantly reduced by PEA (Figure 2A). Similarly, peripheral glucose sensitivity was improved following insulin injection in PEA-treated mice, as shown by the increased glucose disposal and AUC (Figure 2B). Moreover, pyruvate administration in PEA-treated animals reduced glucose levels at all examined times compared to HFD mice, indicating the reduction of gluconeogenesis (Figure 2C).

Insulin signaling and GLUT2 expression were reduced in the liver of mice on HFD, revealing IR. PEA treatment increased insulin sensitivity, restoring the phosphorylation of InsR and AKT, and inducing GLUT2 protein expression (Figure 2D-F).

3.3 | PEA reduced liver damage and improved hepatic lipid metabolic impairment

The histological pattern of HFD mice was characterized by micro- and macro-vesicular steatosis with prominent ballooning. Lobular inflammation was characterized by inflammatory cells occasionally arranged in microgranulomas (see arrow) and consisting mostly in lymphocytes, plasma cells, and neutrophils (Figure 3A). Liver sections of HFD+PEA animals showed a mild reduction of zone 1 micro-vesicular steatosis and ballooning, accompanied by a reduction of inflammatory lesions and absence of necrosis (Figure 3B). Moreover, PEA reduced hepatic lipid accumulation caused by HFD feeding, as shown by the slight positivity to ORO staining (Figure 3A). Accordingly, the increase in hepatic...
triglycerides of HFD mice was reduced by PEA treatment (Figure 3C). Notably, HFD decreased significantly hepatic PEA levels that were normalized by PEA oral treatment. Palmitic acid, palmitoleic acid, and oleic acids were increased by HFD and unchanged in HFD+PEA treated mice (Table 2).

We also demonstrated that PEA increased Ppara and reduced Fasn transcription (Figure 3D), together with the increased phosphorylation of AMPK and its downstream target ACC (Figure 3E,F), the main pathway involved in FAO. Moreover, we showed that PEA induced the protein expression of hepatic PPAR-α and its co-activator PGC1α and that of CPT1 (Figure 4A,B).

Based on the extended hepatic inflammation grade of HFD mice, we explored Il6 and Tnfa mRNA expression, two inflammatory mediators implicated in the pathogenesis of steatosis and IR. PEA-treated HFD mice showed a reduction in both cytokine transcription levels, confirming PEA anti-inflammatory profile in HFD-induced steatosis (Figure 3G).

3.4 | Modulation of hepatic mitochondrial efficiency and oxidative stress by PEA treatment

Mitochondrial state 3 respiration, evaluated using succinate as substrate, was decreased in HFD-fed animals compared with the other groups and restored by PEA (Figure 5A). To study FAO, state 3 respiration was evaluated using palmitoyl-carnitine as substrate; PEA increased oxygen consumption compared with STD and HFD groups (Figure 5B). No variation was observed in mitochondrial state 4 respiration among all groups using succinate or palmitoyl-carnitine substrate (Figure 5A,B, respectively). High quality of mitochondrial preparations was indicated by high respiratory control ratio values in all groups (data not shown). CPT activity did not differ between STD- and HFD-fed mice while it was increased by PEA treatment (Figure 5C). To test mitochondrial efficiency, we measured oxygen consumption in the
presence of oligomycin and FCCP (Figure 5D). Oligomycin state 4 respiration showed a significant reduction in HFD animals compared to STD, whereas it was significantly increased in PEA-treated mice (Figure 5D). No variation was found in maximal FCCP-stimulated respiration (Figure 5D). Therefore, hepatic mitochondrial energetic efficiency, assessed as the degree of coupling, was increased in HFD and decreased by PEA treatment (Figure 5E). This reduction in energy efficiency is associated to PEA-induced increase in mRNA expression of uncoupling protein (UCP) 2 (Figure 5F). UCP2 is an anion transporter involved in mitochondrial calcium homeostasis and is related to the increase in proton leak and the reduction of ROS production.35

Hepatic ROS production, increased in HFD mice, was significantly decreased by PEA (Figure 6A). Accordingly, PEA increased antioxidant defense, promoting SOD, aconitase, and catalase activity (Figure 6B-D). Moreover, while the hepatic GSH level was increased by PEA treatment, no difference in GSSG content was shown among groups (Figure 6E). The beneficial effects on liver redox status induced by PEA were clearly indicated by the significant increase of the GSH/GSSG ratio (Figure 6F).

### Table 1

<table>
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<th>STD</th>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>116.1 ± 5.124a</td>
<td>213.6 ± 3.618b</td>
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<td>Total Cholesterol (mg/dL)</td>
<td>163.7 ± 4.479a</td>
<td>227.6 ± 4.694b</td>
<td>190.9 ± 2.489c</td>
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<td>Serum ALT (U/L)</td>
<td>70.14 ± 2.064a</td>
<td>124.1 ± 3.894b</td>
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<td>Serum TNF-α (ng/mL)</td>
<td>0.9514 ± 0.0504a</td>
<td>3.777 ± 0.1205b</td>
<td>1.202 ± 0.2694a</td>
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<td>Leptinemia (ng/μL)</td>
<td>1.29 ± 0.0626a</td>
<td>18.4 ± 0.9042b</td>
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<td>Adiponectinemia (μg/mL)</td>
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<td>Ratio (leptin/adiponectin)</td>
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<td>16.09 ± 1.516b</td>
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<td>Insulin (ng/dL)</td>
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<td>Glucose (mg/dL)</td>
<td>93.5 ± 3.794a</td>
<td>135.4 ± 6.871b</td>
<td>102.5 ± 90.31a</td>
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<td>HOMA index</td>
<td>1.744 ± 0.04303a</td>
<td>5.646 ± 1.467b</td>
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Note: Data are expressed as mean ± SEM (n = 6 for each group). Labeled means without a common letter differ, P < 0.05.

**FIGURE 2** Effect of PEA on glucose homeostasis. A, Oral glucose tolerance test (OGTT), B, Insulin and C, Pyruvate tolerance tests (ITT and PTT, respectively) were performed in all groups of mice. Data are presented as means ± SEM (n = 10 animals each group). D, Liver pInsR-to-InsR, and E, pAKT-to-AKT ratios and F, GLUT2 expression were also evaluated by Western blot and densitometric analysis reported. Data are presented as means ± SEM of animals from different groups (n = 4-6 each group). Labeled means without a common letter differ, P < .05.
FIGURE 3  PEA effect on liver lipid accumulation in HFD-fed mice. A, Paraffin-embedded sections of the liver (n = 4 each group) were stained with H&E and Oil Red O. Micrographs are representative pictures with original magnification x20. B, Effect of PEA treatment on severity of steatosis, hepatic inflammation, and necrosis was also evaluated by Kleiner's grading system. C, Liver triglycerides content, D, Ppara and Fasn mRNA levels, E, Liver pAMPK-to-AMPK, F, pACC-to-ACC were evaluated by Western blot and densitometric analysis shown. G, Il6 and Tnfa mRNA level are reported. Data are presented as means ± SEM of animals from different groups (n = 4-6 each group). Labeled means without a common letter differ, \( P < .05 \)

<table>
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<tr>
<th>TABLE 2</th>
<th>Determination of PEA and long-chain fatty acids (palmitic, palmitoleic, and oleic acids) in mouse liver</th>
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<th>STD</th>
<th>HFD</th>
<th>HFD + PEA</th>
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<tr>
<td>PEA (pmoles/g tissue)</td>
<td>24.824 ± 2.873(^a)</td>
<td>11.432 ± 0.562(^b)</td>
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<td>Palmitic Acid (16:0) (µmoles/g tissue)</td>
<td>35.228 ± 1.911(^a)</td>
<td>44.614 ± 4.326(^b)</td>
<td>44.654 ± 4.448(^b)</td>
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<td>Palmitoleic acid (16:1) (µmoles/g tissue)</td>
<td>2.723 ± 0.496(^a)</td>
<td>6.042 ± 0.810(^b)</td>
<td>4.219 ± 0.528(^b)</td>
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<td>Oleic acid (18:1) (µmoles/g tissue)</td>
<td>12.794 ± 1.577(^a)</td>
<td>39.350 ± 6.395(^b)</td>
<td>32.423 ± 3.720(^b)</td>
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\( \text{Note: Data are expressed in pmoles or µmoles/g liver tissue (n = 6 each group), as mean ± SD. Labeled means without a common letter differ, } P < 0.05. \)
**FIGURE 4**  PEA induced PPAR-α and PGC1α expression in HFD mice. A, In liver, we evaluated protein level of PPAR-α, the co-activator PGC1α, and its downstream target CPT1-L and B, Densitometric analysis was reported. Data are presented as means ± SEM of animals from different groups (n = 4-6 each group). Labeled means without a common letter differ, *P* < .05.

**FIGURE 5**  Effect of PEA on liver mitochondrial function and energy efficiency. A, Mitochondrial respiration in the presence of succinate or B, Palmitoyl-carnitine, as substrates, was determined in presence of ADP (state 3) and in the presence of substrates alone (state 4). C, CPT activity was determined in isolated liver mitochondria. The oxygen consumption rate was evaluated D, In the presence of oligomycin, that inhibits ATP synthase, and FCCP, which dissipates trans-mitochondrial proton gradient. E, The degree of coupling is also shown. F, mRNA levels of UCP2 were also reported. Data are presented as means ± SEM of animals from different groups (n = 4-6 each group). Labeled means without a common letter differ, *P* < .05.
3.5 | PEA counteracts mitochondrial dysfunction in palmitate-challenged HepG2 cells

PEA effect on mitochondrial bioenergetics in Pal-treated cells was assessed using Mito Stress assay by Seahorse analyzer (Figure 7A). PEA treatment recovered Pal-induced mitochondrial dysfunction, increasing basal and maximal respiration (Figure 7B,C), promoting ATP-linked respiration and proton leak (Figure 7D,E), and reducing mitochondrial coupling efficiency (Figure 7F). All these data indicate the direct effect of PEA in counteracting Pal-induced mitochondrial dysfunction.

3.6 | Involvement of AMPK in PEA effect on lipid metabolism in HepG2 cells

The direct lowering effect of PEA on hepatic lipid accumulation induced by Pal was shown on HepG2 cells (Figure 8A). Notably, the PEA effect was blunted by compound C, a potent inhibitor of AMPK (Figure 8A). Indeed, at the mechanistic level, PEA restored AMPK phosphorylation and increased PPAR-α and CPT1 expression in Pal-challenged HepG2 (Figure 8B). All these effects were reversed in the presence of compound C (Figure 8C), clarifying the key role of AMPK in PEA metabolic activity.

4 | DISCUSSION

This study established the modulatory effect of PEA on metabolic inflexibility induced in HFD-fed mice, showing PEA capability to restore glucose and lipid homeostasis. This lipid mediator belongs to bioactive N-acylethanolamines (NAEs) and is involved in many physiopathological processes, revealing pleiotropic activities and carrying out anti-inflammatory, analgesic and neuroprotective effects. Differently from the most known NAE anandamide, that binds cannabinoid receptor, PEA effects are mainly due to its interaction with...
The present study identifies a novel function for PEA, as an insulin sensitizer based on its effects in regulating glucose homeostasis, and managing obesity, liver steatosis, and IR.

We have demonstrated that PEA limits overweight and adiposity induced by HFD, reducing energy intake, increasing energy expenditure, resting metabolic rate, and lipid oxidation, as shown by the reduced RQ. Indeed, the decreased RQ index reflects the increased use of fatty acids, rather than carbohydrate, as the primary fuel, dissipating most part of energy gained from HFD. In obese mice, PEA-induced shift toward lipid oxidation determines an improvement of lipid and inflammatory profile, limiting the progression of hepatic steatosis.

PEA treatment also impacts on glucose homeostasis and IR. Indeed, our data show an improved capacity in glucose disposal and a decrease in HOMA-IR. To assess the intracellular mechanisms, we studied the InsR-AKT pathway in the liver. The insulin-sensitizing effect of PEA is demonstrated by the increase in the phosphorylated state of InsR and AKT, resulting in upregulation of GLUT2 and subsequent glucose clearance.

Recently, the activation of AMPK, a cellular energy sensor, has been identified as a therapeutic target for treating metabolic diseases. It leads to the phosphorylation of key metabolic mediators and transcriptional regulators that are linked to cellular metabolism including PPARs. Indeed, AMPK activation reprograms cells, redirecting metabolism toward inhibited anabolism and increased catabolism, limiting glucose and lipid synthesis and promoting FAO as an energy source. In our experimental conditions, PEA, increasing liver AMPK phosphorylation, reduced lipid synthesis, not only by the inhibitory phosphorylation of ACC but also decreasing fatty acid synthase transcription and inducing PPAR-α expression. Notably, PEA induced PPAR-α both in vivo and in vitro model, increasing also its transcriptional coactivator PGC1α. Consistently, our previous data evidenced PEA capability to increase AMPK activation in adipose tissue, to inhibit cytokine synthesis, and to reduce the activated state of macrophages. Indeed, PEA modulated AMPK phosphorylation both in hypothalamus and white adipose tissue of ovarioectomized rats, leading to a reduction of food intake and fat mass. Previous data demonstrated that activation of AMPK can inhibit the synthesis of proinflammatory cytokines (ie, TNF-α and IL-6) both in macrophages and in adipocytes.

Here, in HFD mice, PEA improves alteration of serum leptin and adiponectin, deeply reducing their ratio. Interestingly, both adipokines inversely modulate glucose and lipid metabolism through AMPK signaling. Metabolic diseases and related comorbidities result from the overproduction of lipids derived by de novo lipogenesis and from the inability to oxidize lipids stored in the liver. These effects are counteracted by AMPK/ACC pathway activation: in fact, the overexpression of AMPK reduces lipogenic gene expression, liver triglycerides, and hepatic steatosis in hyperlipidemic diabetic rats, and reduces intracellular lipid accumulation in hepatocyte cells.

As known, AMPK inhibits fatty acid synthesis by inducing the inhibitory phosphorylation of two targets: ACC1, which catalyzes the rate-limiting step in fatty acid synthesis converting acetyl-CoA to malonyl-CoA, and sterol regulatory element-binding protein 1c, a transcription factor that induces the expression of multiple lipogenic enzymes, including ACC1 and fatty acid synthase. Besides the inhibition of lipid anabolism, AMPK also activates lipid catabolism. The AMPK-induced phosphorylation of ACC determines the reduction of malonyl CoA, which, in turn, disinhibits CPT activity, resulting in increased mitochondrial FAO. ACC knock-in mice fed a control diet rapidly develop NAFLD, IR, and liver fibrosis; conversely, the pharmacological inhibition of ACC reduces lipogenesis, increases FAO in isolated hepatocytes, and alleviates NAFLD in rats.

As previously demonstrated, HFD feeding, inducing hepatic fat storage, dysregulated mitochondrial function. Accordingly, our data demonstrate that liver mitochondria from the HFD mice exhibited reduced mitochondrial respiratory capacity and increased oxidative stress. PEA treatment improves mitochondrial respiratory capacity and FAO and stimulated CPT activity. These effects lead to the reduction in hepatic ectopic lipid storage, as shown by triglycerides content and Oil Red O staining. Moreover, a further mechanism converging to fatty oxidation is related to the decrease in mitochondrial efficiency, as shown by the decrease in the degree of coupling in PEA-treated HFD mice. The reduced degree of coupling associated to increased transcription of UCP2 contributed to the decrease in oxidative stress (ie, reduction of ROS production, increase in aconitase activity and GSH/GSSG ratio) and an increase of detoxifying enzymes (ie, catalase and SOD activity) in PEA-treated HFD mice.

In vitro data on the effect of PEA on mitochondrial function obtained in palmitate-challenged HepG2 cells showed similar results, since we observed an increase in basal and maximal respiration and in ATP-linked respiration. Moreover, PEA exposure induced an increase in proton leak, and subsequently a reduction of coupling efficiency.

In our in vitro experiments, we also addressed the direct effect of PEA on hepatic lipid metabolism through AMPK-dependent mechanism using compound C, a potent and selective inhibitor of the enzyme. As known, HepG2 cells treated with palmitate have been commonly used to study the impairment of hepatic lipid metabolism and steatosis related to NAFLD and represent an appropriate tool to determine the involvement of AMPK activation in PEA effects. PEA treatment reduced lipid accumulation induced by palmitate in HepG2 cells, activating the phosphorylation of AMPK, and the expression of PPAR-α and CPT1. Notably, PEA effects
were blunted by AMPK inhibition, evidencing the involvement of this enzyme in PEA-induced improvement of lipid metabolism altered by palmitate.

As known, PPAR-α is an intriguing therapeutic target for fatty liver disease but novel efficient and safe agents stimulating PPAR-α are needed; indeed, the fibrates, regarded available PPAR-α activators, have not reported beneficial clinical effect in the treatment of NAFLD/NASH. Here, we showed that oral administration of PEA induced not only an increase in PEA level but also PPAR-α expression in fatty liver indicating the direct activation of PEA/PPAR-α pathway. Interestingly, it has been already described that PEA levels decreased in rat fatty liver due to overfeeding as well as PEA plasma levels in insulin-resistant obese women; it is likely that PEA levels, due to its biosynthesis/degradation, are modulated by insulin sensitivity.

It has been demonstrated that ultramicronized PEA formulation is promptly absorbed. Even if this exogenous administration increases PEA levels in liver of HFD mice, we did not detect any change either of palmitic or palmitoleic or oleic acid content. In addition, it should be considered that the amount of palmitic acid derived by PEA degradation is negligible respect to whole tissue concentration, which is tightly controlled around a well-defined concentration and depends on exogenous source and endogenous biosynthesis.

Based on our results, PEA can be considered a therapeutic tool to improve metabolic flexibility impaired by...
obesity. The involvement of AMPK in metabolic effects of PEA identifies this molecule as lipid and glucose metabolic regulator, relevant in the modulation of liver insulin sensitivity. The beneficial effects of PEA may be the result of multiple converging mechanisms: the improvement of mitochondrial respiratory capacity and FAO, the decrease in mitochondrial efficiency, the improvement of liver redox status associated to cytoprotective defenses and its well-known anti-inflammatory effect.

ACKNOWLEDGMENTS
We thank Mr. Giovanni Esposito, Mr. Angelo Russo, and Dr. Antonio Baiano for animal care and technical assistance.

AUTHOR CONTRIBUTIONS
R. Meli, G. Mattace Raso, and M.P. Mollica designed the research; C. Annunziata, A. Lama, C. Pirozzi, G. Cavaliere, G. Trinchese, F. Di Guida, A. Nitrato Izzo, F. Cimmino, O. Paciello, D. De Biase, and E. Murru performed experiments; C. Annunziata, A. Lama, C. Pirozzi, G. Cavaliere, G. Trinchese, O. Paciello, and S. Banni analyzed data; R. Meli, G. Mattace Raso, and M.P. Mollica wrote the paper; G. Mattace Raso, M.P. Mollica, and A. Calignano supervised the study.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

REFERENCES


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