UNIVERSITY OF NAPLES "FEDERICO II"
DEPARTMENT OF PHARMACY

Ph.D. PROGRAM IN
"PHARMACEUTICAL SCIENCES"

Cycle XXIX

Ph. D. thesis

Pharmacological effects of palmitoylethanolamide on hypertension, insulin-resistance and obesity in murine models

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2017

SUMMARY

Abstract

N-Palmitoylethanolamide (PEA) is an endogenous N-acylethanolamine, first identified in lipid extracts from brain, liver, and muscle of rat and guinea pig. PEA is formed *"on demand"* from membrane phospholipids and it is gaining ever-increasing interest not only for its anti-inflammatory and analgesic effects mediated by peroxisome-proliferator activated receptor (PPAR)-α, but also for its novel metabolic effects. Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. Main consequence of obesity is cardiovascular disease (CVD). The sum of the risk factors that predisposes to CVD goes by the name of "metabolic syndrome" (MetS). Hypertension is an important hallmark of MetS and a common cause of kidney disease.

In the first part of this thesis, we investigated the mechanisms underpinning PEA blood pressure lowering effect, exploring the contribution of epoxyeicosatrienoic acids (EETs), CYP-dependent arachidonic acid (AA) metabolites, as endothelium derived hyperpolarizing factors (EDHF), and renin-angiotensin system (RAS) modulation. To achieve this aim, SHR and Wistar-Kyoto normotensive (WKY) rats were treated with PEA (30 mg/kg/day, s.c.) for five weeks. Functional evaluations on mesenteric bed were performed to analyze EDHF mediated vasodilation. Moreover, mesenteric bed and carotid were harvested to measure the soluble epoxide hydrolase (sEH), which is the enzyme responsible for EETs degradation in their corresponding inactive diols. Effect of PEA on RAS modulation was investigated by analyzing angiotensin converting enzyme (ACE) and angiotensin receptor (AT)1

expression. We showed that EDHF-mediated dilation in response to acetylcholine (Ach) was increased in mesenteric beds of PEA-treated SHR. Interestingly, in both vascular tissues, PEA significantly decreased the sEH protein level, accompanied by a reduced serum concentration of its metabolite 14-15 dihydroxyeicosatrienoic acid (DHET), implying a reduction in EET hydrolysis. Moreover, PEA treatment down-regulated AT1 and ACE expression, indicating a reduction in Ang II-mediated effects. Our data clearly demonstrate the involvement of EETs and RAS in the blood pressure lowering effect of PEA.

The relationship between obesity, insulin-resistance (IR) type 2 diabetes mellitus (T2DM) and MetS is well known. IR is defined as an inefficient glucose uptake and utilization in peripheral tissues in response to insulin stimulation. IR in the prediabetes stage is a feature of glucose intolerance, which includes impaired fasting glucose and/or impaired glucose tolerance. When insulin binds to its transmembrane receptor (InsR), promotes its autophosphorylation (pInsR). Activated pInsR recruits insulin receptor substrate (IRS), leading to insulin signaling cascade. A potential link between inflammation and IR has been shown. Indeed, obesity is characterized by chronic low grade inflammation, where the release of adipose tissue-derived cytokines can block insulin action and cause systemic IR. In fact, serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 are significantly increased in serum from obese patients. All cytokines induce IRS1 protein degradation, which suppresses insulin signaling pathway and subsequently suppresses glucose transporter (GLUT) translocation and glycogen synthesis, contributing to IR and hyperglycemia. Our study was focused on the pharmacological effect of PEA in an animal model of diet-induced

obesity (DIO), feeding mice with a high-fat diet (HFD), and on the mechanisms by which this lipid mediator could modulate the storage and availability of energy sources, restoring lipid/glucose homeostasis. To achieve this aim, mice were fed a standard chow diet (STD group) or HFD (DIO group). After twelve weeks, both STD or HFD mice were treated with PEA (30 mg/kg/day, o.s.) for ten weeks. At the end of the experimental period, body parameters were determined, and serum and tissues collected for following determinations. Interestingly, PEA caused a reduction in body weight and fat mass, improved glucose tolerance and prevented IR, induced by HFD feeding. Moreover, PEA restored the alterations of serum biochemical and inflammatory parameters, inducing a marked reduction of ALT, AST, cholesterol, and pro-inflammatory cytokines, such as TNF-α, IL-1 and monocyte chemoattractant protein (MCP)-1. PEA also normalized metabolic hormone levels and restored insulin sensitivity. At hepatic level, PEA treatment significantly induced an increase in the activation AMPK/ACC pathway, stimulating fatty acid oxidation, compromised in obese mice. To evaluate tissue insulin-sensitivity, we determined the hepatic expression of the InsR, whose expression decreased in liver of DIO mice compared to that of STD animals, and increased in PEA-treated mice. Then, we evaluated the effectiveness of hepatic insulin signaling through the evaluation of InsR and Akt phosphorylated state and the expression of GLUT-2. PEA treatment restored insulin signaling. The protective effect of PEA was strengthened by the evaluation of hepatic IL-6 and TNF-α, whose transcription, upregulated by HFD feeding, was reduced. To address the direct effect of PEA on hepatic insulin-sensitivity, we evaluated the restoration of insulin signaling, altered by the induction of IR, in HepG2

cells, a human hepatocarcinoma cell line. Therefore, we demonstrated in vitro that PEA increased the phosphorylation of Akt in insulin resistant cells, following insulin stimulation. PEA was also able to modulate glucose homeostasis at hypothalamic level. Therefore, we examined neuronal activation at the arcuate (ARC) and ventromedial (VMH) nuclei, evaluating c-fos immunostaining. In the ARC nucleus of DIO mice, a decrease in c-fos labeling was found. Interestingly, in the PEA-treated DIO group, a trend of c-fos labeling increase was evidenced. Consistently, in the VHM of DIO mice a significant decrease in the neuronal activation was shown compared to STD mice, although, no differences were found between DIO and PEA-treated DIO mice. The involvement of the hypothalamic control of glucose homeostasis by PEA was confirmed in in vitro experiments, using human SH-SY5Y neuroblastoma cell line. When insulin-resistant cells were treated with PEA, the re-stimulation with insulin showed a restoration of Akt phosphorylation, and therefore of insulin-sensitivity. These findings show that this acylethanolamide also displays a central effect on glucose homeostasis, reducing neuronal IR. Our data strengthened evidence on the metabolic activity of PEA, through the involvement of central and peripheral mechanisms. PEA clearly ameliorates glucose-tolerance and insulin-sensitivity, indicating its therapeutic potential for the treatment of metabolic dysfunctions associated to obesity, such as IR and T2DM.

Introduction

Fatty acylethanolamides (FAEs), generally referred as N-acylethanolamines (NAEs), are a group of endogenous lipid mediators, derived from different long-chain fatty acids, and synthesized in various mammalian tissues **[1, 2]**. NAEs have increasingly received attention due to their numerous biological activities **[3]**. NAEs include arachidonoylethanolamide (N-arachidonoylethanolamine, anandamide, AEA), stearoylethanolamide (N-stearoylethanolamine, SEA), oleoylethanolamide (N-oleoylethanolamine, OEA), and palmitoylethanolamide (N-palmitoylethanolamine, PEA) (figure 1) **[4, 5]**.

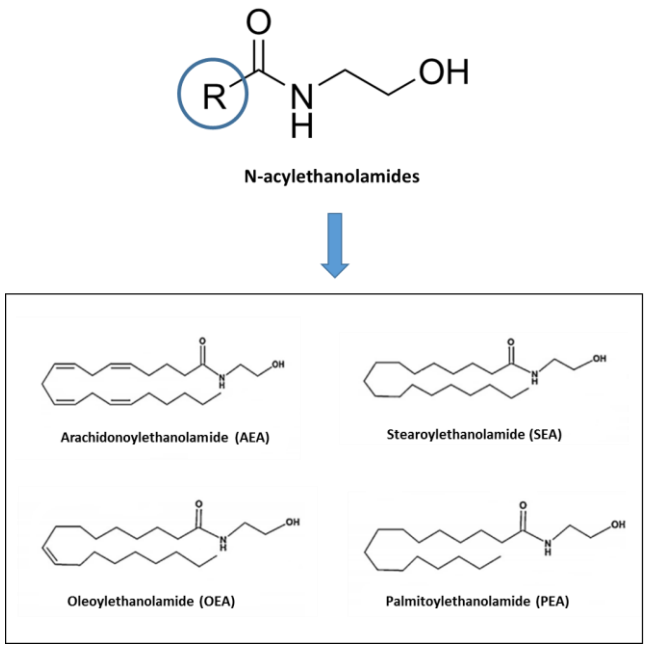

Figure 1. Chemical structures of representative NAEs.

The most known NAE is AEA, an endogenous eicosanoid derivative that was the first endocannabinoid identified, as ligand of cannabinoid receptors CB1 and CB2 **[6]** It appears as a relevant modulator of several physiological functions not only in the central and autonomic nervous system, but also in immune system, endocrine network, gastrointestinal tract and reproductive system **[7]**. It is well known that AEA is able to produce analgesia, control motor activity, to reduce emesis, to stimulate appetite, to induce hypothermia and also to present anti-proliferative effects **[8, 9]**. Its activities and effects seems also mediated by the activation of the endovanilloid receptor (transient receptor potential vanilloid type1, TRPV1) **[10]**. However, AEA is a minor component in most animal tissues compared with other NAEs, such as SEA, OEA and PEA **[6, 11]**. These NAEs, except AEA, do not bind to cannabinoid receptors, but exert their biological actions through several other receptors. Among these, PEA is endogenously produced in every mammalian cells at low levels. Their production is "*on demand"* from membrane phospholipids when there is cellular injury and inflammation **[12]**.

PEA has been studied extensively for its anti-inflammatory, analgesic, and neuroprotective actions, especially in models of pain **[13, 14]**. In the past, PEA was considered a CB2 agonist **[15]**, but other study carried out by Lo Verme et al. **[16]** showed no effect of PEA in CB2 knockout mice. To date, it is well known that PEA actions are mediated by peroxisome proliferator-activated receptor (PPAR)-α **[16, 17]** and GPR55 **[18]**. PPAR-α is a ubiquitous transcription factor, activated by endogenous fatty acid derivatives, including PEA and OEA **[16]**, affecting transcription of numerous genes involved in lipid

metabolism and inflammation. PEA efficacy in inducing anti-inflammatory and anti-nociceptive activities, are mediated by PPAR-α **[13, 16]**.

Overweight and obesity result from an imbalance of food intake, basal metabolism, and energy expenditure. Main consequence of obesity is cardiovascular disease (CVD) **[19]**. A great portion of the CVD resulting from obesity is mediated by type 2 diabetes mellitus (T2DM). However, obesity is accompanied by several other risk factors for CVD. The sum of the risk factors that predisposes to CVD goes by the name of "metabolic syndrome" (MetS).

Since hypertension and obesity are two of five risk factors associated with the onset of MetS, we determined the pharmacological effect of PEA in a genetic animal model of hypertension, i.e. spontaneously hypertensive rats (SHR), and in an animal model of diet-induced obesity (DIO), feeding mice with a high-fat diet (HFD). These preclinical models have allowed us to investigate the mechanisms by which this lipid mediator could modulate functions involved in the storage and availability of energy sources, restoring lipid/glucose homeostasis and its capability in limiting many pathological features of MetS.

1. PEA: origin, structure and activity

PEA is a fully saturated, bioactive, endogenous NAE, identified a century ago in lipid extracts from brain, liver, and muscle of rat and guinea pig **[20]**. Subsequently, it was shown to be present in other organs in higher levels than AEA **[21, 22]** where is synthesized "*on demand"* from membrane phospholipids **[12]**. PEA has long been known to have immunosuppressive **[23]**, analgesic **[24]**, neuroprotective **[25]** and antioxidant **[26]** properties. The anti-inflammatory effect of PEA and interaction upon PPAR-α is well documented. This receptor is distributed at peripheral and central level and functions as a regulator of fatty acid oxidation, lipid metabolism, and inflammation affecting transcription of numerous genes **[27, 28]**.

It is generally accepted that PEA is principally biosynthesized in animal tissues from phosphatidylethanolamine (PE) by two sequential enzyme reactions **[1-3]**. As depicted in figure 2, the first reaction is the transfer of an acyl-group (palmitic acid) from the sn-1 position of phosphatidylcholine (PC) to PE by an N-acyltransferase (NAT), resulting in the generation of N-acyl- PE (NAPE) (1) **[29]**. Subsequently, PEA is released from NAPE by a phosphodiesterase of phospholipase D (NAPE-PLD) (2) **[30]**. PEA is rapidly catabolized by fatty acid amide hydrolase (FAAH) to palmitic acid and ethanolamine (3) **[31, 32]** or N-acylethanolamine-hydrolyzing acid amidase (NAAA) in inflammatory status **[33]**.

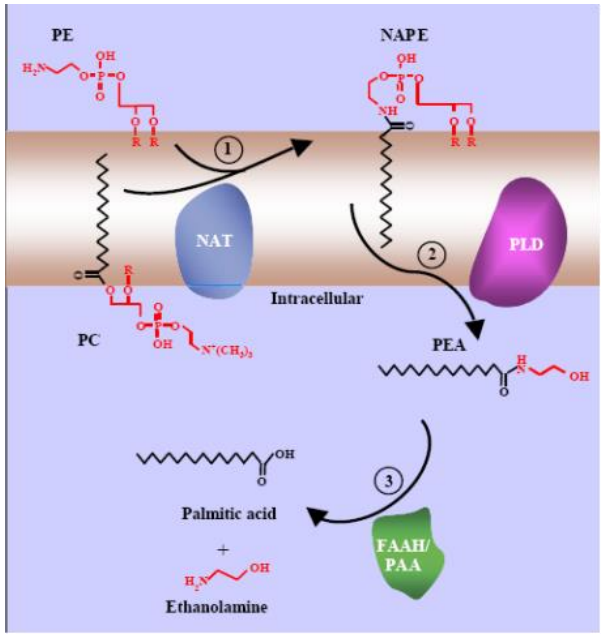

Figure 2. PEA: biosynthetic and degradative pathways of PEA.

In particular, PEA, being a lipophilic molecule, can flip throughout the phospholipid bilayer of the plasma membrane **[34]** and then transported by fatty acid binding proteins or heat shock proteins to degrading enzymes or to target molecules such as PPAR-α **[35]** and vanilloid receptor **[36]**.

1.1. Peroxisome proliferator-activated receptors (PPARs): PPAR-α and PEA

PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily. Three different isoforms (α, β/δ, and γ) (figure 3) have been described, all of which display distinct physiological functions dependent on their differential ligand activation profiles and tissue distribution **[37]**.

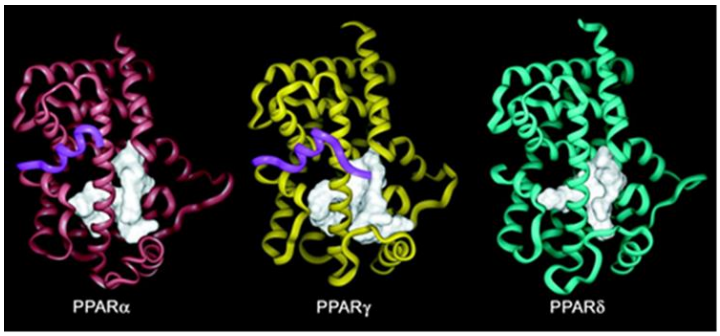

Figure 3. Tridimensional structure of different isoforms of PPARs.

PPARs are regulators of gene networks, which mainly control pain and inflammation, by switching off the nuclear factor-κB (NF-κB) signaling cascade, leading to the synthesis modulation of pro-inflammatory mediators **[38]**. In particular, PPAR-α and PPAR-γ receptor subtypes have been reported to regulate *in vivo* and *in vitro* inflammatory responses **[39, 40]**.

Different type of fatty acids (FAs) binding PPAR induce a conformational change of nuclear receptors, triggering the transcription of specific genes including those encoding for various metabolic and cellular processes such as FA β-oxidation and adipogenesis, thus to be considered key mediators of lipid homeostasis **[41]**. Drugs able to activate these receptors, modulating the lipid and glucose metabolism, constitute a viable therapy for patients with dyslipidemia, T2DM, atherosclerosis and cardiovascular diseases **[42, 43]**.

The tissue distribution of PPAR-α varies: PPAR-α expression has been demonstrated in brain **[44]** and spinal cord **[44-46]** liver, kidney heart and brown adipose tissue **[47]**. Evidence has shown its importance in

the regulation of lipid metabolism, obesity **[48]** behind its capability to modulate inflammation.

The first indication for a role of PPAR-α in modulating inflammation was demonstrated by that the ability of leukotriene B4, a potent chemotactic inflammatory eicosanoid, to bind PPAR-α and induce gene transcription of enzymes involved in ω- and β-oxidation pathways that metabolize leukotriene B4. Moreover, mutant mice lacking PPAR-α (PPAR-$\alpha^{-/-}$) showed a prolonged inflammatory response when challenged with leukotriene B4 or arachidonic acid (AA) **[49]**. The inhibition of pro-inflammatory factors, such as interleukin (IL)-6 and prostaglandins, also participate in the PPAR-α-mediated control of inflammation, probably through the suppression of nuclear factor kappa beta (NF-κB) activity **[50]**.

It has been postulated that activation of PPAR-α by nonsteroidal anti-inflammatory agents contributes to the anti-inflammatory, anti-pyretic, and analgesic properties of these drugs through stimulation of oxidative pathways involved in the catabolism of eicosanoids **[42]**.

Numerous data suggest that PEA can be considered an endogenous activator of PPAR-α. PEA interacts with this receptor to inhibit inflammatory response with a potency comparable with that of the synthetic PPAR-α agonist GW7647, without activating PPAR-β/δ or PPAR-γ **[16, 51]**. More recently, we have identified the involvement of PPAR-α in PEA protective effect against neurotoxicity and endoplasmic reticulum stress both *in vivo* and *in vitro* models **[52]**.

1.2. Mechanisms of action of PEA

Previously, it was suggested that PEA was a CB2 agonist **[15]**. Afterwards, Lo Verme et al. **[16]** showed that PEA had no effect in CB2 knockout mice. Now, it is well-known that the main PEA pharmacological effects are mediated by PPAR-α activation **[13, 46, 51]**. Initially identified as a receptor for peroxisome-stimulating plasticizers in the liver, PPAR-α is a ubiquitous transcription factor, activated by various endogenous fatty acid derivatives, including PEA and its analog OEA.

It was found that PEA could potentiate the effect of AEA on CB or TRPV1 **[53, 54]**. This so-called "entourage effect" could be mediated by PEA competitive inhibition of AEA hydrolysis on FAAH, leading to an increase in AEA levels and its binding to CB1 **[55]** (figure 4A) and/or direct allosteric effect of PEA on TRPV1, increasing AEA affinity to this receptor, and inducing later TRPV1 desensitization **[56, 57]** (figure 4B). Through a PPAR-α dependent non-genomic mechanism, PEA increases the gating properties of calcium-activated intermediate (IKCa) and big-conductance potassium (BKCa) channels, resulting in a fast reduction of neuronal firing **[51]** Moreover, PPAR-α activation, through a genomic mechanism, increases the expression of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage (CYP450scc), involved in cholesterol transfer into the mitochondria and its metabolism in pregnanolone, respectively. The resulting increase in allopregnanolone levels leads to a positive allosteric activation of γ-aminobutyric acid (GABA) (A) receptors, an increase in Cl^-currents and a reinforcing effect on the reduction of neuronal firing. PEA anti-inflammatory effect appears to be related to

a cytoplasmic complex, that reduces NF-κB transcription activity, dampening the transcription of pro-inflammatory gene (figure 4C) **[13]**.

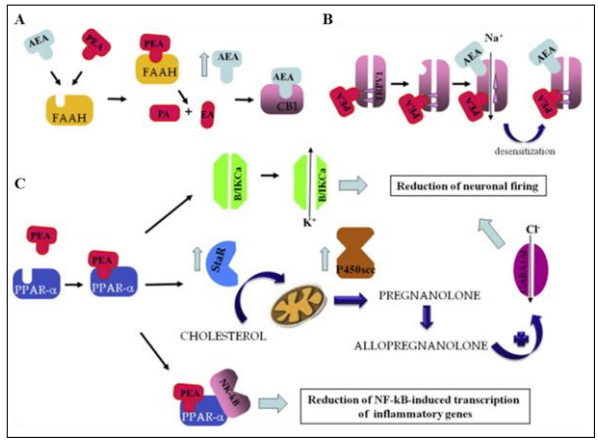

Figure 4. Mechanisms of action of PEA: effect entourage through competitive inhibition of the metabolism of AEA by FAAH (A); allosteric activity on TRPV1 and its desensitization (B); Genomic and not genomic mechanisms dependent on PPAR-α (C).

Furthermore, PEA is reported to have affinity for orphan receptors such as GPR55, a G-protein coupled receptor (GPCR) **[58]**. It has been described as a type 3 cannabinoid receptor (CB3) due to its ability to detect endocannabinoids and may play a role in the endocannabinoid lipid sensing system **[6]**. Indeed, GPR55 activation has been suggested to account for some of the non-CB1, non-CB2 effects reported for certain cannabinoid ligands **[59]**. This receptor has been associated with several physiological roles in inflammation **[60]**, osteogenesis **[61]**, insulin secretion and glucose homeostasis **[62]**. Moreover, it has been demonstrated that PEA could also bind GPR119 **[63]**. This receptor is expressed at particularly high levels in the gut and also found in immune cells. Initially considered a “third” endocannabinoid

receptor, despite sharing little homology with CBs, it has been linked to inflammatory processes as well **[64]**. Previously, Overton and colleagues **[63]** have shown that OEA could be an agonist for GPR119, while PEA and AEA displayed very weak affinity. This receptor is particularly known for its involvement in glucagon-like-protein (GLP)-1 receptor secretion from entero-endocrine cells in the gut and hence glucose homeostasis **[65]**.

1.3. Pharmacological activity of PEA

In addition to the anti-inflammatory activity, PEA inhibits food intake **[66]**, reduces the gastrointestinal motility **[67]**, cell proliferation **[68]** and protects the vascular endothelium in case of myocardial ischemia **[69]**. First experiments led in the 50s and recent studies have demonstrated that PEA inhibits the degranulation of mast cells **[70]** and pulmonary inflammation in the mouse **[71]**. Ross and coll. **[72]** have shown the ability of PEA to reduce the production of nitric oxide (NO) in macrophages.

Several mechanisms of action of PEA have been proposed in a model of acute inflammation (carrageenan-induced paw edema). Animals treated for three days with the PEA (10 mg/kg/day os) showed a significant reduction in the levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, suggesting a genomic mechanism similar to that of the steroid anti-inflammatory drugs **[73]**; however, various studies show that PEA quickly reduces the acute inflammation as well **[16, 73]**, suggesting the involvement of a not genomic mechanism.

Since the analgesia produced by this acylethanolamide is rapid and precedes the action of anti-inflammatory substances, on the basis of the studies performed by Calignano and coll. **[24]**, it was assumed that the endogenous PEA in basal conditions would be involved in the regulation of pain sensitivity. The analgesic effect of endogenous acylethanolamines may be mediated by the modulation of the conductance of specific ion channels **[74]**.

Sasso et al. **[75]** have also shown that the analgesic effects of PEA are in part due to the induction of *de novo* synthesis of neurosteroids exploiting an acute and a chronic animal model of pain: the assay of formalin and carrageenan-induced paw edema, respectively. The administration of formalin or carrageenan causes a decrease at spinal level of two proteins involved in the genesis of neurosteroids: CYP450scc and StAR.

The reduction of the expression of CYP450scc, StAR and the consequent reduction of the levels of allopregnanolone can be prevented through treatment with PEA. This effect is also blocked by inhibitors of *de novo* synthesis of neurosteroids, such as for example aminoglutethimide and finasteride. These results demonstrate that antinociceptive effects of PEA, are not only related to the quick action of damping of neuronal firing, but also to the prolonged long lasting action supported by neurosteroid synthesis.

A role for PPAR-α has been addressed in cardiovascular system and its ligands have been shown to protect against inflammatory damage especially resulting from angiotensin II hypertension. Our recent study demonstrates that PEA significantly reduced blood pressure in SHR and limited kidney damage to high perfusion pressure **[76]**. Recently,

the metabolic activity of PEA has been reviewed **[13]**, highlighting its strong link to PPAR-α, in addition to its capability to induce profound anti-inflammatory and anti-nociceptive effects **[16]**. In fact, our recent study demonstrates anorexic and fat-losing effects of a chronic treatment with PEA in ovariectomized (OVX) obese rats **[77]**.

It has been shown that the activation of PPAR-α mediated the anorexic effect of AEs, and in particular of OEA, on feeding behavior and weight loss **[78, 79]**. PEA, as OEA, binds PPAR-α, but is unable to exert on food intake and lipolysis, effects similar to those of OEA, as shown by Rodriguez de Fonseca **[78]**, possibly because of its lower potency. Administration of PEA in rats and mice induced a rapid decrease in food intake **[80]**. The anorectic action of exogenous PEA is mediated by the activation of the transcription factor PPAR-α in the small intestine **[80, 81]**. PEA, as OEA, has an endogenous anorectic role. In rats, at small intestine level, transient overexpression of NAPE-PDL resulted in decreased food intake in the same time window where increased intestinal levels of OEA and PEA were also observed **[82]**. The activity of NAPE-PLD changes: being low during fasting and high after re-feeding as are the NAPE precursors for OEA and PEA. This data suggests that these intestinal changes in tissue levels are regulated through formation of NAPE precursors and NAPE-PLD activity, but not through changes in NAE degradation **[83]**. Therefore, the activity of the NAPE-synthesizing enzyme NAT may play a central role in regulating the intestinal levels of the anorectic NAEs. Furthermore, in mouse, intestinal level of OEA and PEA seems to decrease in response to high-fat feeding. It was speculated that PEA and other chain fatty acids, are biosensors of

dietary fat. The decreased intestinal level of OEA and PEA may contribute to hyperphagic effect of prolonged intake of dietary fat **[11]**. Dietary fat is digested by hydrolysis of triacylglycerol to 2-monoacylglycerol (2-MAG) and free fatty acids, which are absorbed and used for triacylglycerol synthesis and chylomicron **[84, 85]**. The HFD provides oleic, palmitic and linoleic acids, which are taken up into the enterocyte probably via the membrane-protein CD36 and incorporated in NAPE (figure 5). These fatty acids increase intestinal levels of OEA and PEA. On the other hand, the prolonged intake of fat, which leads to increases in fatty acids and 2-MAG in the lumen, via unknown receptor, results in down regulation of the activity of NAT and thereby decrease in the amount of NAPE species in the enterocyte. So, diet rich in palmitic acid induces a decrease of OEA and PEA, but the decrease in PEA levels is less profound than that of OEA because palmitic acid from diet enriches the NAPE precursor pool with N-palmitoyl-phosphatidylethanolamine and consequently increases PEA. OEA and PEA can activate PPAR-α and bring the signal further to the vagus nerve leading to the brain **[86]**.

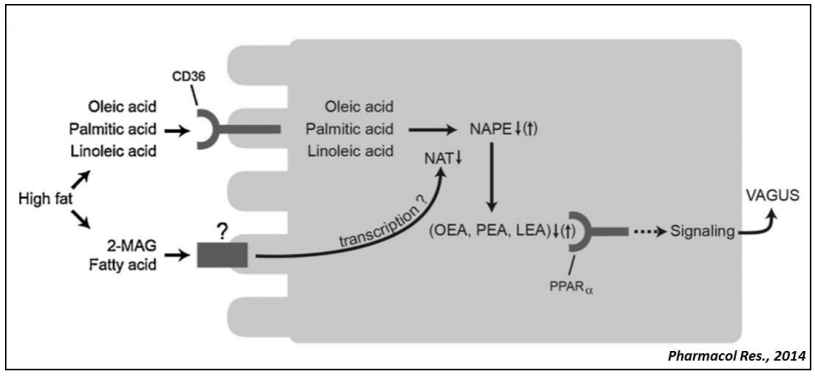

Figure 5. PEA and OEA as biosensors of dietary fat.

1.4. Role of PEA on peripheral or central metabolism

PPAR-α plays an important metabolic role regulating fatty acid oxidation in tissue with a high rate of fatty acid oxidation, such as liver, heart and skeletal muscle **[27, 87]**. All isoforms of PPAR-α were shown to reveal specific patterns of localization in several areas of the brain **[45]** and injection of PPAR-α agonist into the hypothalamus was shown to induce expression of PPAR-α target genes and reduce food intake **[88]**. Thus PEA, binding and activating PPAR-α, acts both at peripheral and central level. As mentioned before, PEA effects have been demonstrated to be consistent with the recovery of the impairment of leptin receptor signaling at hypothalamic level, restoring leptin sensitivity in OVX rats **[77]**. Furthermore, body weight reduction following PEA treatment, together with the reversion in leptin resistance, was related to the suppression of food intake by hypothalamic modulation of AMP-activated protein kinase (AMPK) activity and neuropeptide synthesis. Further the central hypothalamic effect of PEA, was supported by a peripheral effect of this ethanolamide on white adipose tissue. Indeed PEA, increasing AMPK activation in adipose tissue, inhibits pro-inflammatory cytokine synthesis. Consequently, with pAMPK increase in adipose tissue, PEA restores carnitine palmitoyl transferase (CPT)-1 transcription, confirming its role in the lipid catabolic pathways **[77]**.

2. Obesity and related diseases

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health, becoming common worldwide pathologies.

A marker of obesity, related to body fat content, is the body mass index (BMI) which is a simple index of weight-for-height commonly used to classify overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his/her height in meters (kg/m^2). For adults, World Health Organization (WHO) defines overweight when BMI is greater than or equal to 25 and obesity when BMI is greater than or equal to 30. BMI provides the most useful population-level measure of overweight and obesity as it is the same for both sexes and for all ages of adults. The best way to estimate obesity in clinical practice is to measure waist circumference; indeed, an excess of abdominal fat is most closely associated with the metabolic risk factors.

The increase in obesity prevalence is due to two major factors, abundant reserve of inexpensive food and sedentary life. Obesity must also be recognized as a product of free society in which a multitude of food choices and job opportunities are available. Moreover, obesity is not a significant public health problem in world area in which technological progress is less developed.

Main consequence of obesity is CVD **[19]**. A great portion of the CVD resulting from obesity is mediated by T2DM, even if several other risk factors accompanied CVD. Furthermore, obesity is accompanied by other medical complications other than CVD and diabetes; these include fatty liver, cholesterol gallstones, sleep apnea, osteoarthritis,

kidney disease and polycystic ovary disease **[89]**. The sum of the risk factors that predisposes to CVD goes by the name of MetS. The syndrome is a clustering of at least three of the five following pathological conditions:

1) abdominal or central obesity (waist circumference ≥ 102 cm (40 in) in men or ≥ 88 cm (35 in) in women);
2) hypertension (≥ 130/85 mg/kg);
3) elevated fasting plasma glucose (≥ 100 mg/dl);
4) hypertriglyceridemia (≥ 150 mg/dl);
5) low high-density lipoprotein (HDL) levels (< 40 mg/dl in men or < 50 mg/dl in women).

In order to better understand the relationship between obesity, diabetes and CVD as well as for the development of novel therapeutic strategies, animal models that reproduce the etiology, course and outcomes of these conditions are required **[90]**. To reproduce metabolic human diseases in animal models, different diet compositions have been tested **[91]**. It has been shown that a high-sucrose diet exacerbates IR **[92]** a high-salt or high-fructose diet exacerbates hypertension **[93]**, a high-cholesterol diet exacerbates atherosclerosis **[94]** and a short-term HFD results in discrete systolic dysfunction **[95]**. In the scientific literature, it was shown that animals consuming diets containing high proportions of fat gained weight faster than those on diets containing minimal amounts of fat, because more palatable **[96]**. Foods that are rich in fats have been shown to produce increased body weight and diabetes in various strains of mice and rats. Some animals show profound increases in their body fat content while some are resistant to weight gain with HFD **[97]**. One of

these models that are gaining increasing attention is the DIO model in mouse. C57BL/6J mouse is a particularly good model mimicking human metabolic derangements that are observed in obesity because when fed ad libitum with a HFD, these mice develop obesity, hyperinsulinemia, hyperglycemia, and hypertension, but when fed ad libitum to chow diet, they remain lean without metabolic abnormalities **[98]**.

Obesity and its co-morbidities, including T2DM and CVD, are associated with a state of chronic low-grade inflammation that can be detected both systemically and within specific tissues. An increased accumulation of macrophages occurring in obese adipose tissue has emerged as a key process in 'metabolic' inflammation **[99]**. Obesity and the accompanying MetS are strongly associated with an increase of morbidity and mortality in older adults. In the last years, the prevalence of obesity has enhanced dramatically, and it has become the most common metabolic disease worldwide, leading to a global epidemic **[100]**. The etiology of obesity is complex and multifactorial and results from the interaction of genes with the environment, lifestyle, and emotional factors. The modern lifestyle is a potent risk factor for obesity. Declining physical activity levels and increased caloric intake are important environmental factors contributing to obesity **[101]**. The increasing number of overweight and obese individuals is a serious public health problem that has implications for society and healthcare systems on a global scale. The economic consequences of obesity and associated diseases are not limited to high medical costs but also include indirect or social costs, such as decreased quality of life, problems in social adjustment, lost

productivity, disability associated with early retirement, and death **[102]**.

2.1. Components of energy balance system and obesity impairment

The prime function of the physiological system is to maintain homeostasis of energy stores in response to variable access to nutrition and demands for energy expenditure. This system has both afferent sensing components, and efferent effectors. The afferent arm of this system includes several kinds of signals. Some of these reflects short-term events, such as those related to onset or termination of the meals; other ones sense the long-term status of body energy stores. Although these long and short-term signals have often been viewed as operating independently, it now appears that they functionally overlap. Both converge on brain centers, most importantly within the hypothalamus, where the signals are integrated, and the direction and intensity of efferent responses are determined. The efferent effectors of these physiologic systems include those regulating the intensity of hunger and subsequent food intake behavior, the energy expenditure, both in basal condition and during physical activity, the levels of metabolic pivotal circulating hormones, such as insulin and glucocorticoids, and other factors that influence the amount of lean and fat mass in the body. Some of these signals also regulate processes, such as reproduction and growth that are linked to nutritional status (figure 6) **[103]**.

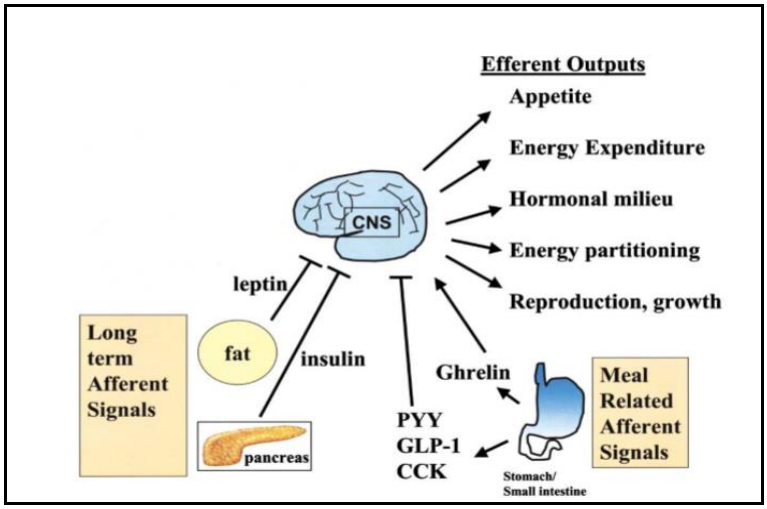

Figure 6. Components of the energy balance system. The energy balance system involves long-term afferent signals from fat (leptin) and pancreatic cells (insulin) and short-term, meal-related afferent signals from the gut, including inhibitors of feeding (PYY, GLP-1, and CCK), and the stimulator of feeding (ghrelin). These inputs are integrated within the brain. Efferent outputs regulate appetite, energy expenditure, hormonal milieu, energy partitioning, and the status of reproduction and growth (Flier, 2004).

The hypothalamus is subjected to a continuous flow of signals coming either from brain superior areas or periphery, with orexigenic or anorexigenic activity, which inform it about energy nutritional conditions of the organism **[104]** and control energy metabolism, producing neuropeptides or hormones which can modulate either feeding behavior or energy expenditure. The energy expenditure and food intake alteration induced by endogenous factors was studied, in obese patients. For example, gut hormones play a pivotal role in communicating information on nutritional status to hypothalamus **[105]** and comprise pancreatic polypeptide-fold peptides, as GLP-1, glucagon, peptide YY (PYY), and pancreatic polypeptide (PP), ghrelin, and cholecystokinin (CCK). PYY is an appetite suppressing hormone. The PYY secretion pattern has a role in satiety. Circulating PYY concentrations are low in fasted state and increase rapidly

following a meal with a peak at 1-2 hours and remain elevated for several hours. PYY release is increased in proportion to calorie intake **[106]**. PYY has been reported to regulate energy expenditure, delay gastric emptying, reduce acid secretion, and inhibit gallbladder contraction and pancreatic exocrine secretions **[107, 108]**. Circulating PYY levels are low in obese subjects **[109, 110]**, and higher in patients with anorexia nervosa than those of control subjects **[111]**.

PP is secreted from PP cells in the pancreatic islets of Langerhans in response to a meal. Anorectic effects of PP are thought to be mediated directly through the Y4 receptor in the brainstem and hypothalamus **[112]**. Plasma PP levels show diurnal variations: lowest levels are observed in the early morning and highest in the evening. The release of postprandial PP is biphasic. Circulating PP concentrations increase after a meal in proportion to the caloric intake, and the increased levels remain high up to 6 hours post-prandially **[113]**.

GLP-1 is co-secreted with PYY from the L cells in the intestine in response to nutrient ingestion. GLP-1 exerts its effect by activation of the GLP-1R to stimulate adenylyl cyclase activity and thereby cAMP production **[114]**. GLP-1R is widely distributed particularly in the brain, gastrointestinal tract, and pancreas. Circulating GLP-1 levels rise post-prandially and fall in the fasted state. Recent evidence also suggests that GLP1 levels rise in anticipation of a meal. **[115]**. GLP-1 not only reduces food intake, but also suppresses glucagon secretion and delays gastric emptying **[116]**. The role of glucagon in glucose homeostasis is well established; glucagon is produced by alpha cells of the pancreatic islets and increases glucose level in response to

hypoglycemia. Glucagon enhances the body's physiological response to stress, by increasing energy expenditure **[117]**.

Ghrelin was identified originally as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) in rat stomach **[118]**. Serum ghrelin levels are increased by fasting and decreased by refeeding or oral glucose administration, but they are not decreased by water ingestion **[119]**. In rats, ghrelin levels show diurnal pattern, with the bimodal peaks occurring before dark and light periods **[120]**. Levels of circulating ghrelin rise pre-prandially and fall rapidly in the postprandial period **[121]**.

CCK was the first gut hormone found to be implicated in appetite control. CCK is secreted post-prandially by the I cell of the small intestine into circulation with a short plasma half-life of few minutes. Plasma CCK levels rise within 15 minutes after meal ingestion **[122]**.

Leptin, a 16 kDa adipocyte-derived hormone, is very important in due to its role in the regulation of energy balance. Leptin, secreted from adipose tissues in proportion to the fat store **[123]**, acts in the hypothalamus to regulate feeding behavior **[124, 125]**. Leptin receptors (ObR) exist as six different isoforms (ObRa–ObRf), classified as short (ObRa, ObRc, OBRd, and ObRf), long (ObRb), and secreted (ObRe) isoforms **[126]**. All isoforms, except ObRe, are transmembrane receptors that share the characteristic of possessing a box 1 motif-binding Janus kinase (JAK)2 **[127]**. ObRb is considered the main functional receptor of leptin **[128, 129]**. By binding to ObRb, leptin activates multiple signaling cascades, such as JAK2–STAT3 (signal transducer and activator of transcription) **[130]**, mitogen activated protein kinase/extracellular signal-regulated kinase

(MAPK/ERK) **[131]**, and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathways **[132]**. The major anti-obesity effects of leptin are initiated by the phosphorylation of JAK2. The activation of JAK2 permits STAT3 phosphorylation and nuclear translocation. Nuclear phospho-STAT3 acts as a transcription factor for STAT3-targeted genes **[133, 134]**.

The energy balance system involves long-term afferent signals from fat (leptin) and pancreatic β cells (insulin). Leptin regulates glucose, lipid and energy homeostasis as well as feeding behavior, serving as a bridge between peripheral metabolically active tissues and the CNS, promoting fat mass loss and body weight reduction. Indeed, this hormone, whose circulating levels mirror fat mass, not only exerts its anti-obesity effects mainly modulating the activity of specific hypothalamic neurons expressing the long form of the leptin receptor (Ob-Rb), but it also shows pleiotropic functions due to the activation of Ob-Rb in peripheral tissues **[135]**. This adipokine exerts metabolic effects in peripheral tissues directly and indirectly through the CNS **[136, 137]**. In fact, leptin modulates AMPK activity and orexigenic/anorexigenic neuropeptides in specific hypothalamic regions **[136, 138, 139]**, leading to a reduction of appetite, and to an overall negative energy balance. However, obesity is often accompanied by leptin resistance. This latter phenomenon is defined as decreased sensitivity to the anorexigenic or weight loss effects of leptin accompanied by hyperleptinemia **[77]**. Nevertheless, several mechanisms have been suggested to mediate leptin resistance, including obesity-associated hyperleptinemia, impairment of leptin access to CNS and the reduction in Ob-Rb signal transduction

effectiveness. During the onset and progression of obesity, the dampening of leptin sensitivity often occurs, preventing the efficacy of leptin replacement therapy from overcoming obesity and/or its co-morbidities **[135]**. Identifying the mechanisms by which leptin resistance develops is critical. Impairment of leptin signaling is thought to be one mechanism: suppressor of cytokine signaling (SOCS)3, a mediator of negative feedback to STAT3, is known to attenuate leptin-induced signaling, hence SOCS3-deficient mice displayed increased leptin-induced STAT3 phosphorylation in the hypothalamus **[140]**. Similarly, protein tyrosine phosphatase (PTP)1B dephosphorylates JAK2, thereby inhibiting leptin activity. Consistent with this action, deactivation of PTP1B results in a decrease in body weight and adiposity, and an increase in energy expenditure in mice **[141]**. Thus, SOCS3 and PTP1B are molecular mediators of leptin resistance, and involves the phosphorylation of STAT3, which is associated to the modulation of orexigenic/anorexigenic neuropeptides in the hypothalamus **[77]**. PTP1B inhibits leptin activities via dephosphorylation of JAK2 **[142]**. In fact, PTP1B knockout mice have increased sensitivity to leptin and are resistant to a HFD **[143]**. Therefore, development of potent and specific inhibitors of PTP1B has become interesting for the treatment of T2DM and obesity **[144]**. Over the last decades, several PTP1B inhibitors have been developed **[145, 146]**. SOCS3 inhibits leptin-induced signal transduction **[147]**. Deletion of SOCS3 in hypothalamic neurons enhances leptin sensitivity, reduces appetite, and protects from HFD **[148]**; while overexpression of SOCS3 in proopiomelanocortin (POMC) neurons leads to hyperphagia and obesity **[149]**. Leptin receptor expressed in

the CNS mediate the anorexigenic effects of this hormone **[150, 151]**. Leptin suppress the activity of the orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons, while it stimulates the anorexigenic POMC/cocaine- and amphetamine-related transcript (CART) neurons **[104, 152]**. Therefore, the restoration of leptin sensitivity at hypothalamic level is consistent with the increase in POMC and the reduction of AgRP **[77]**.

2.1.1. Glucose metabolism and insulin signaling pathways

Many findings show the association among obesity, insulin-resistance (IR) and T2DM. IR is defined as inefficient glucose uptake and utilization in peripheral tissues in response to insulin stimulation. IR in the prediabetes stage is a feature of glucose intolerance, which includes impaired fasting glucose and/or impaired glycemic after bolus of glucose **[153]**.

Insulin is a 51-residue anabolic peptide hormone secreted by the β-cells of the pancreas that plays a critical role in the regulation of human metabolism. Containing two chains (A and B) connected by disulfide bonds, the mature hormone is the post-translational product of a single-chain precursor, designated proinsulin **[154]**. This hormone is widely known to reduce blood glucose levels via stimulation of glucose uptake by peripheral tissues, such as fat, liver, and skeletal muscle. Insulin also inhibits glucose production in liver, kidney and small intestine controlling blood glucose. Long-term exposure of tissues to elevated glucose concentrations is associated with the development of complications, including macro- and microvascular disease, and the typical retinopathy, nephropathy, neuropathy,

coronary heart disease, and cerebrovascular disease. Insulin signaling is triggered by the binding with its receptor and is mediated by protein kinase activity in the beta subunit of the insulin receptor (InsR) **[155]**. The InsR belongs to a superfamily of receptor tyrosine kinase whose activation modulates multiple post-receptor signaling pathways. This stimulation permits phosphorylation of the insulin receptor substrate (IRS) to promote the activation of the PI3K/Akt pathway, which is a major metabolic pathway of insulin **[156]**. In addition to its peripheral actions, insulin enters the brain from the circulation **[157]**, and affects feeding behavior and energy homeostasis **[158]**. Insulin is synthesized by the β cells of the pancreas and is secreted rapidly after a meal, with a well characterized hypoglycemic effect **[159]**. However, insulin also acts as an anorectic signal within the CNS.

Glucose is a carbohydrate, and is the most important simple sugar in human metabolism. Blood glucose is firstly transported into cells by the plasma membrane protein glucose transporter (GLUT). The GLUT family has been divided in several isoforms, GLUT-1, GLUT-2, GLUT-3 and GLUT-4. GLUT-1 is widely distributed in fetal tissues; GLUT-2 is expressed by renal tubular cells, liver cells and pancreatic β cells and it is also present in the basolateral membrane of the small intestine epithelium; GLUT-3 is expressed mostly in neurons and in the placenta; GLUT-4 is expressed in adipose tissues and striated muscle **[160]**. The insulin signaling pathway plays an important role in regulating its translocation (figure 7).

When insulin binds to its transmembrane receptor, promotes its auto-phosphorylation at tyrosine residues (pInsR). Activated pInsR recruits IRS and enhanced its activation by mediating its phosphorylation (p-

IRS); p-IRS subsequently binds to the regulatory subunit, p85, of the PI3K and activates its catalytic subunit p110, which is responsible for stimulating the phosphoinositide-dependent kinase (PDK) **[161]** and its activation promotes the phosphorylation of Akt (pAkt) at Thr308 and Ser473 residues **[162]**.

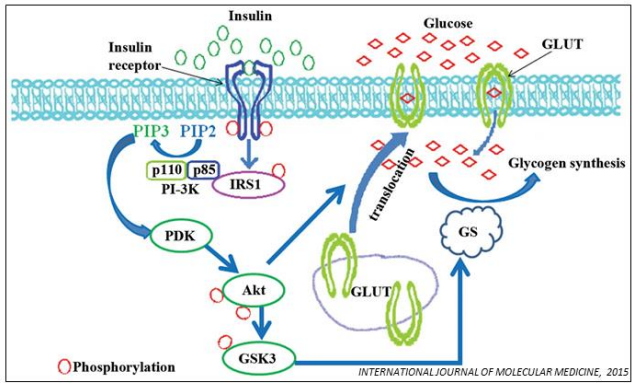

Figure 7. Involvement of the insulin signaling pathway in the regulation of glucose transport.

Phosphorylated Akt mediates the translocation of GLUT from the cytoplasm to the membrane mediating glucose uptake **[163]**. Akt has also been implicated in regulating glycogen synthesis. Glycogen synthase (GS) is a key enzyme involved in converting glucose into glycogen **[164]** as energetic reserve.

2.1.2. Role of low grade inflammation

Obesity is also characterized by chronic low grade inflammation. Systemic inflammation may play a crucial role in the pathogenesis of various obesity-related complications, including metabolic syndrome, T2DM, cardiac disease, liver dysfunction, and cancer. There is accumulating interest in the role of adipose tissue macrophages (ATM)

as inflammatory players in obesity **[165]**. ATMs, accumulated in adipose tissue, consist of at least two different phenotypes: classically activated M1 macrophages and alternatively activated M2 macrophages **[166]**. ATM isolated from lean animals expressed hallmarks of polarization toward M2 macrophages with IL-10 and arginase expression, whereas in obese animals with IR a shift to M1-polarized ATM with increased tumor necrosis factor (TNF)-α and IL-6 **[167]**. These cytokines can block insulin action via autocrine/paracrine signaling and cause systemic IR, providing a potential link between inflammation and IR **[168, 169]**. Therefore, in the blood of obese subjects, TNF-α and IL-6 are significantly increased. TNF-α binds to its receptor and induces iNOS production. ILs, IL-6 in particular, bind to corresponding receptors and promote the expression of SOCS3. All cytokines induce IRS1 protein degradation, which suppresses the PI3K/Akt signaling pathway, and subsequently suppresses GLUT translocation and glycogen synthesis, which results in IR and hyperglycemia (figure 8).

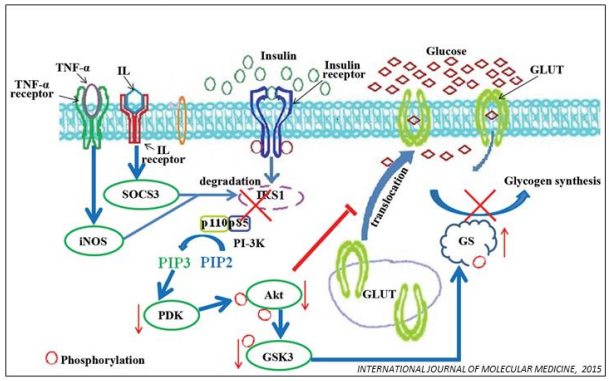

Figure 8. Suppression of the PI3K/Akt signaling pathway mediated by insulin IRS1 degradation.

2.1.3. Adenosine Monophosphate-Activated Protein Kinase and lipid homeostasis

Adenosine Monophosphate-Activated Protein Kinase (AMPK) is a sensor and regulator of cellular energy metabolism, including obesity **[170]**. AMPK is a serine/threonine protein kinase which physiologically responds to the change in the cellular AMP to ATP ratio. AMPK is composed of 3 subunits (α, β, and γ): a catalytic subunit (α1 or α2) and two regulatory subunits (β1 or β2 and γ1, γ2 or γ3) **[170]** and its activation starts with phosphorylation of threonine-172 in the catalytic domain of the α subunit **[171]**. AMPK activation regulates physiological and pathological responses in different tissues. AMPK activation induces fatty acid oxidation not only in liver and heart, but also in skeletal muscle **[172]** inhibits hepatic lipogenesis and adipocyte differentiation, and stimulates glucose uptake in muscle. Increasing fatty acid utilization appears to be an attractive way to prevent both obesity and IR, which are characterized by intra-muscular and hepatic lipid accumulation **[173]**. In the liver, AMPK enhances fatty acid oxidation by phosphorylating acetyl-CoA-carboxylase (ACC), one of the main enzymes for the synthesis of malonyl-CoA, which is the substrate for fatty acid synthesis and a potent allosteric inhibitor of CPT **[174, 175]**. Under normal conditions, the inhibition of ACC through phosphorylation, leads to a decrease of malonyl-CoA levels, permitting to CPT-1 to transport fatty acids into mitochondria and fatty acid utilization. This event leads to fat burning in liver and muscle. AMPK also represses pyruvate kinase and fatty acid synthase expression, thus contributing to decreased lipogenesis **[176]**.

AMPK can stimulate hepatic PPAR-α activity, as a sensor of the intracellular energy state activated by high AMP-to-ATP ratios, i.e., during fasting **[177]**. Indeed, PPAR-α also controls, directly or indirectly, lipogenic pathways in the liver, besides its ability to orchestrate lipoprotein metabolism **[178]**. As shown in figure 9, lipogenesis is the metabolic pathway allowing fatty acid synthesis when dietary carbohydrates or fats are abundant.

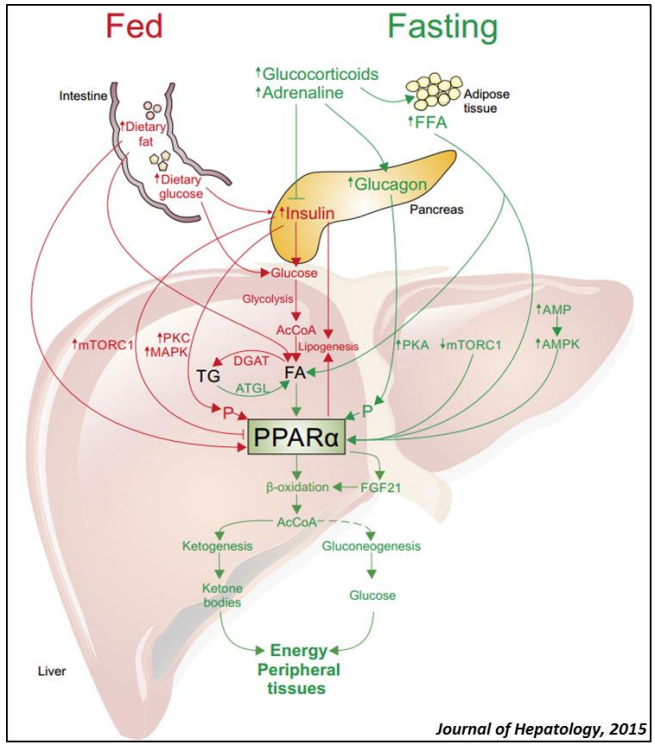

Figure 9. Molecular switch of PPAR-α activity in the fed-to-fasted state.

2.2. Non-alcoholic fatty liver disease (NAFLD)

The liver is the second largest organ in the body. It performs many activities: it processes what we eat and drink into energy and nutrients the body can use. The liver also removes harmful substances from blood. This important organ is subjected to several pathologies. Non-alcoholic fatty liver disease (NAFLD), or steatosis, is one of the most common co-morbidities associated with overweight, metabolic disorders and IR. The liver produces and is exposed to various types of lipids, such as fatty acids, cholesterol and triglycerides via the portal vein from the diet and visceral adipose tissues. The liver and adipose tissue jointly participate in maintaining glucose and lipid homeostasis through the secretion of several humoral factors and/or neural networks **[179]**. NAFLD is one of the most dangerous complications because it can lead to severe liver pathologies, including fibrosis, cirrhosis and hepatic cellular carcinoma (HCC).

A recent meta-analysis raised the question whether NAFLD is a prodromic event of MetS rather than just its hepatic manifestation **[180]**. Evidence indicate that the effects of NAFLD extend beyond the liver and are negatively associated with a range of chronic diseases, most notably CVD, T2DM and chronic kidney disease. A small group of people with NAFLD may progress in a more serious condition, non-alcoholic steatohepatitis (NASH), where fat accumulation is associated with liver cell inflammation. NASH is a potentially serious condition that may lead to severe liver scarring and cirrhosis. Cirrhosis occurs when the liver sustains substantial damage, and the hepatic cells are gradually replaced by scar tissue, which results in the inability of the

liver to work properly **[181]**. NASH is common in patients with obesity, IR and T2DM and NASH increases the risk of HCC **[182]** (figure 10). The mechanisms that promote HCC development in patients with NAFLD or NASH is poorly understood. Several molecular mechanisms have been linked to obesity and its related metabolic diseases, accelerating cancer process. Among these adipose-derived inflammation, lipotoxicity and IR play a pivotal role, as well as mitochondria dysfunction **[183]**.

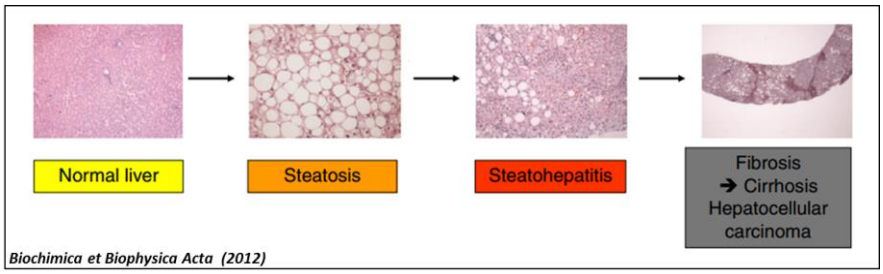

Figure 10. **Sequential steps of NAFLD pathology.** NAFLD develops following a sequence of events from steatosis to NASH, which can progress to additional liver damage. Representative pictures are shown (left to right) of a normal liver, steatosis displaying lipid-filled hepatocytes.

NAFLD is an example of fat accumulation and this lipid accumulation is usually associated with increased secretion of hepatokines **[184]** increased gluconeogenesis, decreased glycogen synthesis and inhibition of insulin signaling **[185]**. Activation of certain metabolic or stress-response pathways, involving NF-κB, phosphatase and tensin homolog (PTEN), occurs in HCC **[186]**. Many of these pathways involved in causing cellular stress also occur within tissues affected by obesity, IR **[187]** which are commonly linked and important in the pathogenesis of T2DM. It has also been suggested that chronic activation of toll-like receptor (TLR)-4, an upstream activator of NF-

κB signaling **[188]**, by gut microbiota-derived ligands such as lipopolysaccharide, may occur. The possibility that alterations in the gut microbiota promote the development of T2DM and NAFLD, makes the gut microbiota an attractive and potentially new therapeutic target in NAFLD (and possibly in T2DM as well, where there is obesity and IR).

Although obesity is strongly associated with hepatic steatosis, excess body fat accumulation is not an essential condition for developing NAFLD. In fact, patients with lipodystrophy have marked IR and commonly develop hepatic steatosis and T2DM, strongly suggesting that adipose tissue dysfunction is the key contributor to the pathogenesis of NAFLD **[189]**. Specifically, increased free fatty acid (FFA) fluxes from the adipose tissue pool increase the availability of long-chain fatty acyl-CoAs for hepatic lipid accumulation, particularly in physically inactive individuals, and evidence is accumulating that hepatic lipid accumulation is capable of causing hepatic/peripheral IR and promotes hepatic inflammation **[190]**.

2.3. Hypertension

Hypertension in MetS differs from other forms of hypertension and recent studies have underlined these differences in many ways **[191]**. Higher cardiometabolic risk profile is characterized by a close relationship between high serum triglycerides and elevated waist circumference, as displayed by risk ratio for future coronary artery disease of 2.40 in men and of 3.84 in women **[192]**.

Excess visceral fat is not only related in the risk for coronary heart disease, but it is also associated with hypertension **[193]**. Among all

factors influencing systolic and diastolic blood pressure, the waist circumference in men and women has showed to be the strongest one **[194]**. It is known that the pathophysiological link between visceral fat and cardiovascular diseases is multifactorial. One mechanism might be the association of visceral and ectopic fat accumulation, e.g. fatty degeneration of cardiac cells. It has been shown that visceral obesity is the best predictor for epicardial and pericardial fat **[195]**. Moreover, weight loss associated with a decrease of visceral fat in severely obese subjects significantly reduces epicardial fat thickness **[196]**. Pericardial fat correlates with adiposity, vascular calcification, and further cardiovascular risk factors **[197]**. An increase of CVD risk, is due to an accumulation of visceral adiposity in contrast to subcutaneous fat, facilitating arteriosclerosis, hypertension, and fat accumulation of the heart.

In hypertensive and obese subjects there is an endothelial dysfunction, characterized by an increased synthesis of angiotensin (Ang) II, the main vasoconstrictor protein, that results from Ang I in a reaction catalyzed by the angiotensin converting enzyme (ACE).

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasoactive substances **[198]**.

In vasodilating pathway, prostacyclin, NO and a family of endothelium-derived hyperpolarizing factors (EDHFs) are the main actors. Endothelium-derived NO mediates vascular relaxation of relatively large, conduit arteries, while EDHF plays an important role in modulating vascular tone in small resistance arteries in rodents **[199, 200]** and in human forearm microcirculation **[201, 202]**.

The alteration of normal endothelial function, therefore, is the cause of many CVDs, such as hypertension, coronary artery disease, myocardial infarction and vascular complications.

2.3.1. Arachidonate metabolites and cardiovascular function

It is well known that cyclooxygenase (COX) and lipoxygenase metabolize AA in 5, 12 and 15- hydroxyeicosatetraenoic acids (HETE), prostaglandins, prostacyclin, leukotrienes and thromboxane. These products modulate the renal and pulmonary functions, vascular tone and inflammatory response **[203]**.

However, many years ago, it was discovered a third pathway of AA metabolism: it was reported that AA is also metabolized by the enzymatic pathway of CYP450, including epoxygenase and hydroxylase enzymes, in the liver and kidney to epoxyeicosatrienoic acids (EET) and HETE (figure 11) **[204, 205]**. Discovery of the ability of CYP to transform AA into products that possess a myriad of potent biological activities has uncovered novel arachidonate metabolites that function as key components in mechanisms that participate in circulatory control and the regulation of renal function **[206, 207]**. 20-HETE, the principal CYP product of renal and cerebral arterioles, mediates the regulation of these vasculatures **[208, 209]**; 20-HETE also modulates electrolytic transport in key nephron segments **[210]**; 20-HETE and a labile epoxide, 5,6-EET, are second messengers, accounting for many of the several actions of peptide hormones as mitogens, secretagogues, vasoactive agents, and regulators of volume and composition of body fluids **[206, 207]**.

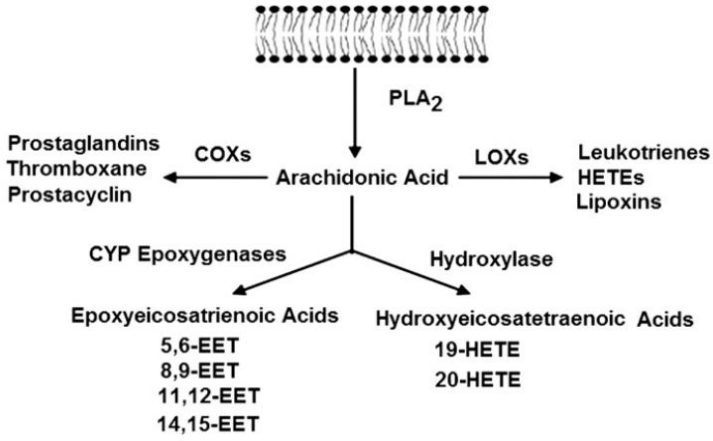

Figure 11. The metabolites of AA from three independent enzymes. Four regioisomeric cis-EETs are primary products of AA metabolism by CYP450 epoxygenases. HETEs are primary products of AA metabolism by CYP450 hydroxylase. COX, cyclooxygenase; LOX, lipoxygenase; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acids.

EETs are CYP epoxygenase metabolites of AA, produced by the vascular endothelium in response to agonists, such as bradykinin and acetylcholine (Ach), or physical stimulus, such as shear stress **[211]**. EETs are hydrolyzed by soluble epoxide hydrolase (sEH) in the corresponding inactive diols, dihydroxyeicosatrienoic acids (DHETs), resulting in attenuation of the vasodilation and anti-inflammatory effect of EETs. EET hydrolysis has been found to be increased in renal fractions of SHR, an animal model of Ang II-mediated hypertension **[212]**. Consistently, sEH was found increased in SHR renal microsomes and cytosol **[212]** and in renal micro-vessels of Ang II-induced hypertensive rats **[213]**. sEH expression has been shown to be also increased in aortas from saline-fed SHR or Ang II-treated normotensive rats. The transcriptional regulation of sEH expression by Ang II has been demonstrated to be mediated by angiotensin II type (AT)1 receptor, since a selective AT1 antagonist reversed this effect

[214]. Therefore, the increased expression of sEH has been interpreted as a result of AT1 and downstream signaling cascade activation, leading to activator protein (AP)-1 transcriptional activity. The enhanced hydrolysis of EETs in DHETs by sEH would unbalance vascular tone and hence increase systemic blood pressure. The administration of an inhibitor of sEH increases the renal formation of EETs and reduces the pressure in the SHR. Further evidence of a function for the sEH and the EET in the blood pressure's control has been reported **[215]**. Furthermore, it was found that sEH gene deletion increases the renal formation of EET and the reduction in blood pressure of 13 mmHg in male mice **[215]**.

EET, DHETs and 20- HETE act mainly as autocrine factors. These compounds are very lipophilic and, in high concentrations was found in tissues rather than, in plasma, urine or interstitial fluid. Moreover, EETs and 20-HETE bind to protein and albumin **[216]**, limiting their distribution in the vascular space when administered systemically.

Molecular and cellular mechanisms, by which EETs regulate different biological functions, have been widely observed. The most frequent perplexity, concerns the possibility of EETs to exert different biological effects.

Campbell et al. **[217]** reported the effect of EET on the relaxation of smooth muscle cells and coronary arteries, through hyperpolarization induction mediated by the activation of calcium-activated potassium channels (K_{Ca}^{2+}), identifying the EET as EDHF.

Following studies showed that hyperpolarizing effects play a role also in peripheral arteries **[218]** as well as, and in airway smooth muscle cells **[219]**. Indeed, Lu et al. **[220]** and Ke et al. **[221]** showed that

EETs had a protective effect at cardiac level, activating the ATP-dependent potassium channels in cardiac sarcolemma, leading myocardial cells hyperpolarization.

2.3.2. Renin-angiotensin system (RAS)

Renin-angiotensin system (RAS) is a major long-term regulator of important physiological processes including the regulation of the pressure of the homeostasis of fluids and electrolytes **[222]**. In fact, pharmacological impairment of RAS has shown that this system can contribute to major cardiovascular diseases. However, recently it has been shown that this system can also play a role in the development of other pathological conditions, such as atherosclerosis, myocardial infarction, heart failure and T2DM **[223, 224]**. Renin converts an inactive peptide, angiotensinogen, in Ang I, that it is converted in Ang II by ACE, mainly expressed at pulmonary capillary level. Ang II is the major bioactive product of RAS and exerts both endocrine and autocrine/paracrine hormonal actions. The circulating Ang II induces vasoconstriction and promotes the release of aldosterone from the adrenal gland. The Ang II may be further split to form Ang III and Ang IV. Actions of RAS include not only endocrine functions but also a paracrine activity in districts, such as brain, heart, vascular tissue, that contain components of the RAS and thus are able locally to generate Ang II **[225]**. The Ang II acts through the activation of two transmembrane receptor subtypes G-protein coupled, AT1 and AT2. Most of actions of Ang II, including blood pressure, fluid and electrolyte regulation, appear mediated by the activation of AT1. It is important to underline that AT1 activation leads to an inflammatory

response by the vascular wall. Two mechanisms are involved in Ang II-induced inflammatory response: generation of reactive oxygen species (ROS) and activation of the NF-κB in vascular tissue **[226]**.

There is evidence for RAS over-activation in obesity; indeed, RAS blockade induced IR, indicating its involvement in the pathogenesis of IR in obesity **[227]**. Pharmacologically, the strongest clinical evidence for an association between systemic RAS and IR originates from clinical trials which have shown that RAS blockade reduces the risk for developing T2DM **[228, 229]**. Pharmacological RAS blockade also improves insulin sensitivity in several rodent models of obesity or IR **[230, 231]**.

2.4. Metabolic syndrome (MetS)

As mentioned before obesity can be defined as an excess of body fat, accompanied by other several complications defined as MetS.

In particular, the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) **[232]** identified the MetS as a multiplex risk factor for CVD that is deserving of more clinical attention. ATP III identified 6 components of the metabolic syndrome that relate to CVD (figure 12):

- abdominal obesity
- atherogenic dyslipidemia
- raised blood pressure
- IR ± glucose intolerance
- pro-inflammatory state
- pro-thrombotic state

The advantage of measuring waist circumference is that an excess abdominal fat is correlated more closely with the presence of metabolic risk factors than total body fat. Adiposity is associated with a state of low-grade chronic inflammation, with increased TNF-α and IL-6 release, which interfere with adipose cell differentiation, and the action pattern of adiponectin and leptin until the adipose tissue begins to be dysfunctional. In this state, the subject presents IR and hyperinsulinemia, probably the first step of a dysfunctional metabolic system. In subjects with MetS an energy balance is critical to maintain a healthy body weight, mainly limiting the intake of high energy density foods (fat). The first factor to be avoided in the prevention of MetS is obesity, and the percentage of fat in the diet has traditionally been associated with the development of obesity **[233]**.

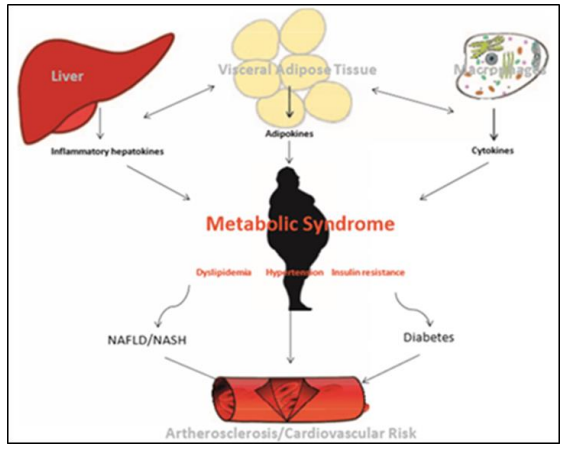

Figure 12. Pathogenesis of elevated cardiovascular risk in the MetS.

EXPERIMENTAL SECTION

Materials and Methods

3. Model of genetic spontaneously hypertensive rat (SHR)

3.1. Animals and treatments

Eight-week-old male SHRs and age-matched Wistar Kyoto normotensive (WKY) rats, obtained from Harlan Italy, were used for this study. Animals were housed in temperature (23±2°C) and light-controlled (12:12-hours light-dark cycle) condition and food and water freely available. The animals were divided into four groups (n = 10): (1) WKY and (2) SHR control; (3) WKY and (4) SHR rats receiving PEA (30 mg/kg/die subcutaneous injection).

PEA (Tocris Cookson Ltd., UK) was dissolved in PEG400 and Tween 80 2:1 (Sigma-Aldrich, Milan, Italy). Control WKY and SHR received PEA vehicle. All animals were treated for 35 days (five weeks). The experimental design is described in figure 13:

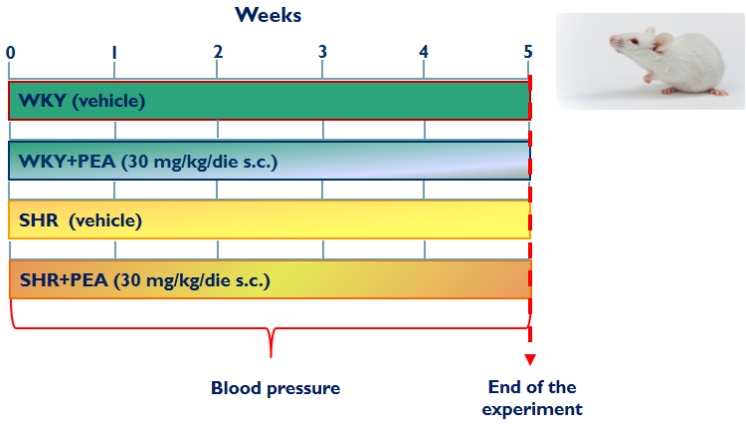

Figure 13. Experimental protocol of genetic spontaneously hypertensive rats.

Throughout the experimental period, systolic blood pressure (SBP) was monitored in conscious rats. At the end of the treatment period, animals were anesthetized, to minimize pain, and euthanized for blood and tissue collection.

3.2. Serum determinations

14,15-DHET was measured by using an enzyme-linked immunosorbent assay (14,15-DHET ELISA kit; Detroit R&D Inc., Detroit, MI, USA). Glucose levels were measured by the glucometer One Touch Ultrasmart (Lifescan, Milpitas, CA). Insulin concentrations were measured by rat/mouse insulin ELISA kit (cat. no. EZRMI-13K; Millipore). As index of IR, HOMA-IR (HOmeostasis Model Assessment) was calculated, using the formula [HOMA=fasting glucose ($mmol \cdot l^{-1}$) x fasting insulin ($\mu U \cdot ml^{-1}$)/22.5].

3.3. Measurement of arterial blood pressure in conscious rats

SBP was measured in conscious rats with a non-invasive common indirect method using a tail-cuff device in combination with blood flow sensor and recorder (Ugo Basile, Biological Research Apparatus). Briefly, rats were housed for 30 minutes in a warmed room (28–30°C), then a tail cuff was placed about 2 cm from the base of the tail for measuring systolic blood pressure. Care was taken in selecting an appropriate cuff size for each animal. Rats were allowed to habituate to this procedure for 2 weeks before the start of treatment. SBP was measured between 09:00 and 12:00 a.m. and values were recorded and were averaged from at least three consecutive readings per rat.

3.4. Isolated and perfused mesenteric bed

These experiments were conducted in collaboration with prof. Sorrentino and prof. d'Emmanuele di Villa Bianca. In brief, rats were euthanized and the superior mesenteric artery was cannulated to perfuse the whole vascular bed with Krebs' buffer containing heparin (10 IU/ml; Sigma-Aldrich, Milan, Italy) for 5 minutes at 2 ml/min. Mesenteric bed was separated from the intestine by cutting along the closed intestinal border and connected to a pressure transducer (Bentley 800 Trantec; Ugo Basile, Comerio, Italy).

It was perfused with Krebs' buffer (2 ml/min) composed of 115.3 mM NaCl, 4.9 mM KCl, 1.46 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 11.1 mM glucose (Carlo Erba Reagents, Milan, Italy), warmed at 37°C, and oxygenated (95% O_2, 5% CO_2). After approximately 20 minutes of equilibration, methoxamine (MTX, 100 μM; Sigma-Aldrich), an adrenergic α1-agonist was added to the Krebs' solution. In order, to visualize NO synthase (NOS) and COX-independent relaxation i.e., EDHF, a concentration-response curve of acetylcholine (Ach bolus injection; 1-10-100-1000 pmoles; Sigma-Aldrich; Milan, Italy) was performed on stable tone of MTX in Krebs' solution medicated with indomethacin (INDO; 10μM; Sigma-Aldrich) and NG-nitro-L-arginine methyl ester (L-NAME, 100μM; Sigma-Aldrich) as COX and NOS inhibitors, respectively.

3.5. Western blot analysis

Mesenteric tissues and carotids were excised, harvested, frozen in liquid nitrogen and stored for protein evaluations. Later tissues were

homogenized on ice in lysis buffer (Tris-HCl, 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml leupeptin and trypsin inhibitor). After 1 h, tissue lysates were obtained by centrifugation at 13000 rpm for 20 minutes at 4°C. The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Milan, Italy), using bovine serum albumin as standard. For Western blot analysis, 35 μg protein of tissue lysate was dissolved in Laemmli's sample buffer, boiled for 5 minutes, and subjected to SDS-PAGE (8% or 12% polyacrylamide). The blot was performed by transferring proteins from a slab gel to nitrocellulose membrane at 240 mA for 40 minutes at room temperature. The filter was then blocked with 1x PBS, 5% non-fat dried milk for 40 minutes at room temperature and probed with rabbit polyclonal antibodies anti-sEH or anti-AT1 or anti-ACE (1:2000; Upstate Biotechnology, Lake Placid, NY, USA) or anti-IκBα (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or phospho-signal transducer and activator of transcription (STAT)3 or STAT3 (1:1000; Cell Signaling Technology, Danvers, MA, USA) or phospho-extracellular signal-regulated-kinases (ERK) 1/2 (1:200; Cell Signaling Technology, Danvers, MA, USA) dissolved in 1x PBS, 5% non-fat dried milk, 0.1% Tween 20 at room temperature, overnight or for 2 hours. The secondary antibody (anti-rabbit IgG-horseradish peroxidase conjugate 1:5000 dilution) was incubated for 1 hour at room temperature. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the immune complex visualized by Image

Quant. The protein bands were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories, Milan, Italy). Western blot for α-tubulin (Sigma; St. Louis, MO, USA) was performed to ensure equal sample loading.

3.6. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test, using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA). Statistical significance was set at $P<0.05$.

4. Mouse model of diet-induced obesity (DIO)

4.1. Animals and treatments

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 lights-dark cycle. All procedures involving the animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of Ministero della Salute and associated guidelines in the European Communities Council Directive of November24, 1986 (86/609/ECC). All animal procedures reported herein were approved by the Institutional Animal Care and Use Committee (CSV) of University of Naples "Federico II" under protocol no. 2011–0129170. Standard chow diet had 17% fat, without sucrose while HFD for diet-induced obesity (DIO), had 45% of energy derived from fat, 7% of sucrose. Standard and HFD contained 3.3 kcal/g and 5.24 kcal/g, respectively. After weaning, young mice were randomly divided into four groups (at least 10 animals for each group) as follows: 1) control group (STD) receiving chow diet and vehicle by gavage (per os); 2) STD group treated with PEA (STD+PEA, 30 mg/kg/die per os); 3) DIO group receiving vehicle; 4) DIO group treated with PEA (DIO+PEA, 30 mg/kg/die per os). The PEA treatments started after 12 weeks of feeding with STD or HFD and continued for 10 weeks. Ultramicronized PEA was provided by Epitech Group Research Labs and was suspended in carboxymethyl cellulose (1.5%, 300 µl per mouse). In figure 14 the scheme of experimental protocol and animal treatments are shown.

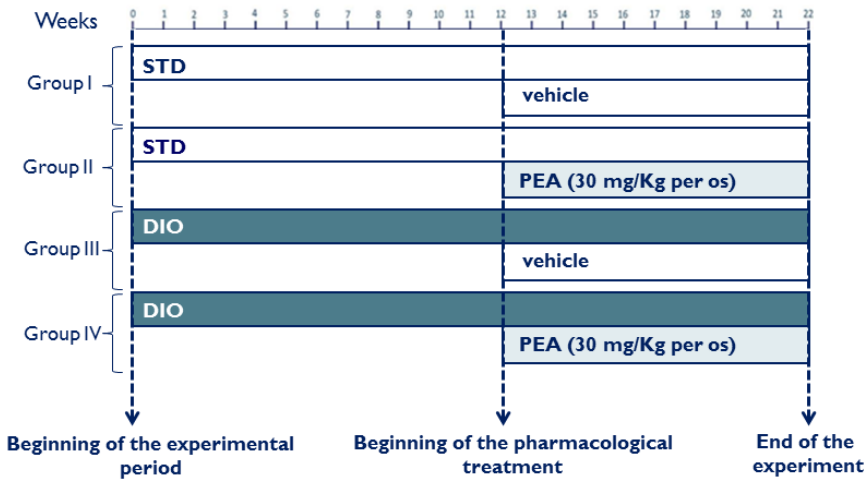

Figure 14. Experimental protocol of DIO model.

4.2. Body weight, food intake, blood pressure and body gain in fat

During the experimental period, body weight was assessed one time each week, while food intake was evaluated every day. Lastly, food intakes were cumulated. The systolic blood pressure (SBP) was recorded in conscious mice by a tail cuff connected to a blood pressure recorder (Blood Pressure Recorder, Ugo Basile Apparatus, Comerio, Italy). After a week of training period, mice were treated as described above and blood pressure was monitored every 2 days. At the end of the experimental protocol, before sacrifice, bioelectrical impedance analysis was applied to determine fat body composition assessment using BIA 101 analyzer, modified for the mouse (Akern, Florence, Italy). Fat free mass was calculated and fat mass content was determined as the difference between body weight and fat-free mass. Fasting glucose levels were measured by the glucometer One Touch Ultrasmart (Lifescan, Milpitas, CA).

4.3. Glucose and insulin tolerance tests

Glucose tolerance test (OGTT) was performed in 12 hours fasted animals, which received oral glucose (1g/kg) and glycaemia was measured at 0, 30, 60, 90 and 120 minutes.

Insulin tolerance test (ITT) was performed in 5 hours fasted mice. After determination of basal blood glucose levels, each animal received an intraperitoneal injection of insulin (Humulin, Lilly), 0.75 U/kg. Blood glucose levels were measured at 15, 30, 60, 90 and 120 minutes after insulin injection. The areas under the curve (AUC) were calculated from time zero, as integrated and cumulative measure of glycaemia up to 120 minutes for all animals. Glucose levels were measured by the glucometer One Touch Ultrasmart (Lifescan, Milpitas, CA).

4.4. Pyruvate tolerance test (PTT)

One week before sacrifice, in overnight fasted mice pyruvate tolerance test (PTT) was carried out. After the serum glucose determination, fasted animals were injected intraperitoneally with pyruvate (Sigma), 2g/kg and glycaemia was measured at 15, 30, 60 and 120 minutes after pyruvate. The areas under the curve (AUC) was calculated from time zero, as integrated and cumulative measure of glycaemia up to 120 minutes for all animals. Glucose levels were measured by the glucometer One Touch Ultrasmart (Lifescan, Milpitas, CA).

4.5. Tissue collection, hematic parameters and triglycerides content

STD and DIO mice, treated or not with PEA for 10 weeks, were sacrificed after overnight fasting. Blood collected by cardiac puncture was centrifuged at 2500 rpm at 4°C for 12 minutes, and sera were

stored at -80°C for later biochemical and hormonal determinations. Alanine amino transferase (ALT), aspartate amino transferase (AST), total cholesterol, low density lipoprotein (LDL) and triglycerides (TGL), leptin, adiponectin, TNF-α, monocyte chemoattractant protein (MCP)-1 and IL-1 were measured by ELISA kits. Fasting insulin concentrations were measured by rat/mouse insulin ELISA kit (cat. no. EZRMI-13K; Millipore). As index of IR, HOMA-IR (HOmeostasis Model Assessment) was calculated, using the formula [HOMA=fasting glucose (mmol l^{-1}) x fasting insulin (μU ml^{-1})/22.5].

About 200 mg of liver sample were added with 2 ml of a solution (0.9% NaCl) and sonicated. The mixture was vortexed for 20 s. Then, was centrifuged at 5.000 rpm for 5 minutes to room temperature. The supernatants were collected and was again centrifuge. The supernatants were collected and stored at −80°C for following liver triglycerides determination.

4.6. Western blotting

Liver was homogenized and total protein lysates were obtained using complete lysis buffer containing 20 mM Tris–HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), leupeptin and trypsin inhibitor (10 μg/ml). After 1 hour, cell lysates were obtained by centrifugation at 13.000×g for 15 minutes at 4°C.

Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amount of protein was subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blot

was performed by transferring proteins from a slab gel to nitrocellulose membrane at 240 mA for 60 minutes at room temperature. The filter was then blocked with 1X PBS and 5% nonfat dried milk for 60 minutes at room temperature and probed with rabbit polyclonal antibody against anti GLUT-2 (dilution 1:1000, Millipore Corporation, Billerica, MA, USA), or anti phospho Akt (pAkt, dilution 1:1500; Cell Signaling Technology, Danvers, MA, USA) or anti Akt (dilution 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or phosphor InsR (pInsR, dilution 1:250; Thermo Scientific, Rockford, USA) or InsR (dilution 1:1000; Cell Signaling Technology, Danvers, MA, USA.), or phosphor AMPK (pAMPK, dilution 1:250, Cell Signaling Technology, Danvers, MA, USA) or AMPK (dilution 1:500, Cell Signaling Technology, Danvers, MA, USA), phospho ACC (pACC, dilution 1:1000, Cell Signaling Technology, Danvers, MA, USA) or ACC (dilution 1:1000, Cell Signaling Technology, Danvers, MA, USA), or PPAR-α (dilution 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Western blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich, Milan, Italy) was performed to ensure equal sample loading.

4.7. Real-time semi-quantitative PCR

Total RNA, isolated from liver was extracted using TRIzol Reagent (Bio-Rad Laboratories), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Nucleospin®, MACHEREY-NAGEL GmbH & Co, Düren, Germany) from 6 μg total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad

Laboratories). The PCR conditions were 15 minutes at 95°C followed by 40 cycles of two-step PCR denaturation at 94°C for 15 s, annealing extension at 55°C for 30 s and extension at 72°C for 30 s. Each sample contained 500 ng cDNA in 2X QuantiTech SYBRGreen PCR Master Mix and primers, TNF-α and IL-6 (Qiagen, Hilden, Germany). The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

4.8. c-fos staining

Overnight fasted male mice were injected (i.p.) with vehicle or PEA (30 mg/kg) at 09:00 a.m. Mice were anaesthetized by isofluorane and killed by perfusion fixation (4% paraformaldehyde in 0.1 M phosphate buffer saline), followed by overnight post-fixation in 4% paraformaldehyde. After 30 minutes washing in phosphate buffer, brains were cut into 50 μm tick sections and the same that containing the ARC were collected. After 15 minutes in buffer, the sections were incubated in blocking solution for 30 minutes at room temperature. The primary antibody goat anti c-fos (Santa Cruz Biotechnology) was then applied overnight at room temperature. The next day, sections were washed 3 times in buffer and incubated with the secondary antibody for 1 hour at room temperature. Finally, the sections were analyzed using fluorescence microscopy.

4.9. Statistical analysis

All data were presented as mean ± SEM. Statistical analysis was performed by ANOVA test followed by Bonferroni's test for multiple comparisons. Statistical significance was set at $P<0.05$.

5. *In vitro* cell models

5.1. Induction of IR in human neuroblastoma cell line

5.1.1. Cell culture

SH-SY5Y human neuroblastoma cells were grown in DMEM (Dulbecco's modified Eagle's medium)-F12 and EMEM (Eagle's modified essential medium) 1:1 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO_2 atmosphere at 37°C. The medium was replaced every two days.

5.1.2. Cell treatments and immunoblotting

SH-SY5Y cells were starved in serum-free DMEM-F12 for 16 hours and stimulated with insulin (100 nM) in order to induce IR. To evaluate PEA effect, during insulin incubation, cells were treated with PEA (1 µM) the last 6 hours. Cells were then washed and re-stimulated with the hormone for 10 minutes. After the stimulation, cells were washed twice with ice cold PBS, harvested, and re-suspended in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), leupeptin and trypsin inhibitor (10 µg/mL). After 1 hour, cell lysates were obtained by centrifugation at 13.000×g for 15 minutes at 4°C.

Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amount of protein was subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blot

was performed by transferring proteins from a slab gel to nitrocellulose membrane at 240 mA for 60 minutes at room temperature. The filter was then blocked with 1X PBS and 5% nonfat dried milk for 60 minutes at room temperature and probed with rabbit polyclonal antibody against anti phosphor Akt (pAkt, dilution 1:1500; Cell Signaling Technology, Danvers, MA, USA) or anti Akt (dilution 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Western blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich, Milan, Italy) was performed to ensure equal sample loading.

5.2. Induction of IR in human hepatocyte cell line

5.2.1. Cell culture

Human HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and streptomicin) at 37°C with 5% CO_2.

5.2.2. Cell treatments and immunoblotting

HepG2 cells, starved in serum-free medium for 16 hours, were incubated with insulin (100 nM) or its vehicle for 24 hours to obtain insulin resistant and control cells, respectively **[234]**. To evaluate PEA effect, both control and insulin resistant cells were simultaneously treated with PEA (0.3, 1 and 3µM). After 24 hours, cells were washed and then challenged with 100 nM insulin for 10 minutes to determine insulin signaling pathway evaluating phospho Akt/Akt ratio.

5.3. *In vitro* statistical analysis

All *in vitro* data are reported as mean ± standard error mean (S.E.M.) values of independent experiments, which were done at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test.

Results

6. PEA prevents endothelial vascular dysfunction and hypertension, acting on EETs pathway and RAS in SHR

6.1. Effect of PEA on IR and blood pressure

Since hypertension, hyperglycemia and IR are important features of MetS, we evaluated fasting glucose and insulin levels in all rat groups (figure 15).

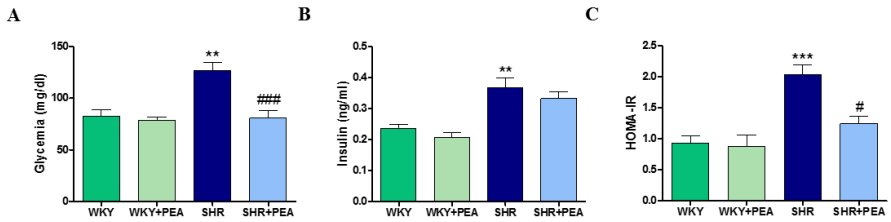

Figure15. Effect of PEA on IR. PEA improve body glucose homeostasis. Fasting glucose (A), insulin levels (B) and HOMA-IR (C) were reported. Data are expressed as mean ± SEM (n=10 for each group). ***P< 0.001, **P<0.01 vs STD; ###P<0.001 and #P< 0.05 vs DIO.

PEA did not modify all these metabolic parameters in control animals. Interestingly, the SHR receiving PEA, showed a decrease in glucose (figure 15A), a trend of reduction in insulin (figure 15B) and significant reduction of HOMA-IR (figure 15C) when compared to untreated SHR group.

Moreover, SHR rats showed a significant increase in SBP (mmHg) values compared to WKY rats. No significant differences in blood pressure were observed in PEA-treated WKY rats compared to WKY, even if a trend of decrease was recorded. Conversely, a lowering effect on blood pressure was shown following PEA treatment of SHR for 5 weeks compared to untreated SHR (figure 16A). In order to determine

the contribution of EDHF in mesenteric bed, as NOS- and COX-independent relaxation, a concentration-response curve to Ach (1–1000 pmoles) in Krebs solution medicated with INDO (10 μM) and L-NAME (100 μM) was performed on MTX stable tone. The EDHF-mediated relaxation resulted significantly reduced in SHR compared with WKY. Interestingly, the treatment with PEA caused a significant increase in EDHF-mediated relaxation in SHR compared with SHR untreated group. No significant effect was observed in WKY rats after PEA treatment (figure 16B).

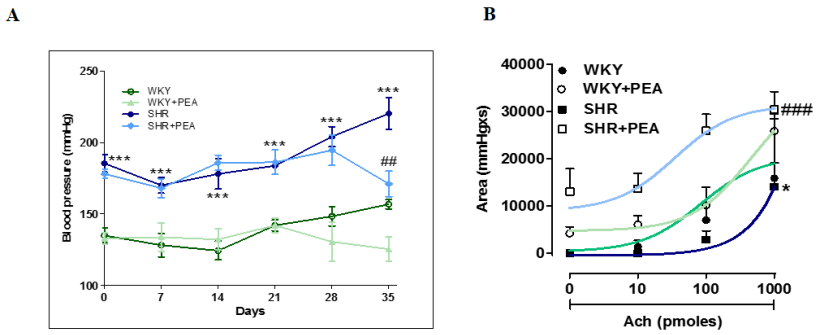

Figure 16. **Effect of PEA on systolic blood pressure and EDHF-mediated relaxation on mesenteric arterial bed on stable tone of MTX** Panel A shows systolic blood pressure (SBP) values of hypertensive (SHR) and normotensive (WKY) rats treated or not with PEA. Panel B shows EDHF contribution in the relaxation of mesenteric bed. ***P< 0.001, *P< 0.05 vs WKY; ###P< 0.001, ##P<0.01 vs SHR.

6.2. PEA reduces sEH expression in vascular tissues and increases serum levels of EETs

To evaluate systemic EET hydrolysis, we analyzed sEH expression, by western blot analysis, of vascular tissues and DHETs concentration in serum of all animals. sEH is implicated in blood pressure control by virtue of its ability to degrade EETs that exert vasodilatory effects. Indeed, it is already known that sEH protein expression is increased in SHR, consistently with its hypertensive role. As shown in figure 17

immunoblots performed on mesenteric bed (figure 17A) and carotid (figure 17B) from SHR animals revealed a marked increase in sEH expression, which was significantly prevented by PEA treatment. Consistently, the increase in serum 14,15-DHETs shown in SHR, was significantly prevented by PEA (figure 17C), suggesting a reduced hydrolysis of EETs in PEA-treated SHR and hence an increase of their concentration.

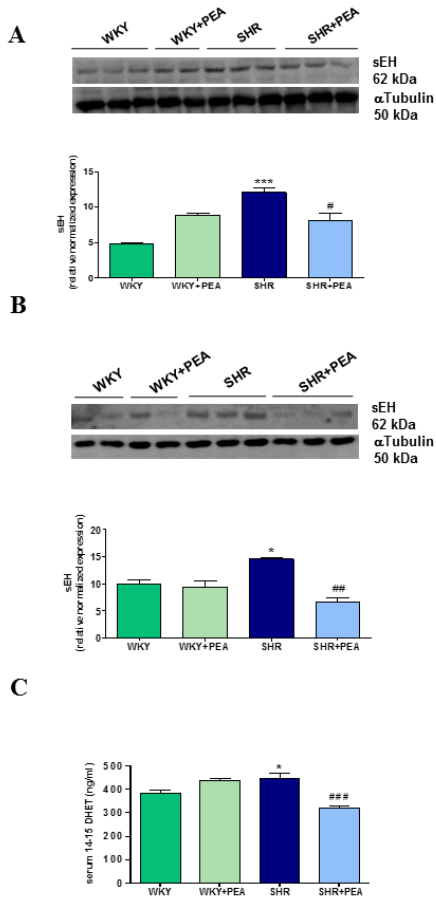

Figure 17. **Effect of PEA on sEH protein expression in mesenteric bed and carotid**. Immunoblot of sEH from mesenteric bed (A) and carotid (B) of SHR and WKY rats are shown. Serum 14-15DHET (ng/ml) is reported in panel C. Data are expressed as means ± SEM. ***P<0.001 and *P<0.05 vs WKY; ###P<0.001, ##P<0.01 and #P<0.05vs SHR.

6.3. PEA modulation of RAS in the vasculature

6.3.1. PEA effect on AT1 and ACE expression

To evaluate whether the hemodynamic effects of PEA bears any relationship to the modulation of RAS, we determined the expression of AT1 receptors and ACE in the vasculature from SHR. As shown in figure 18, AT1 expression has been found increased both in mesenteric bed and carotid in SHR compared with WKY (panel 18A and 18C, respectively), whereas it significantly decreased in SHRs treated with PEA. Thereafter, we also evaluated the vascular source of Ang II, determining ACE protein expression. As shown in figure 18B and figure 18D, the increased expression of ACE in both vascular tissues of SHR, was blunted by PEA treatment in mesenteric bed and carotid, respectively.

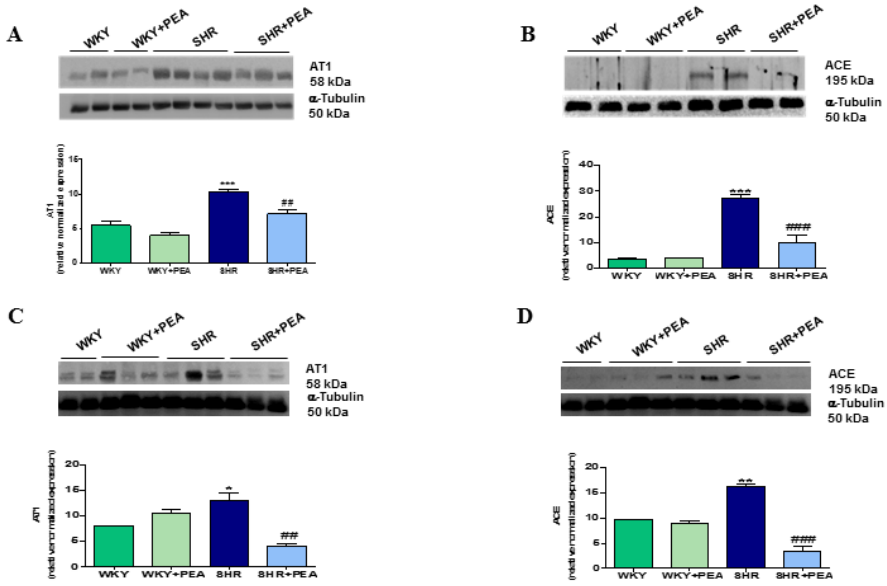

Figure 18. **Effect of PEA on AT1 and ACE protein expression in mesenteric bed and carotid.** Representative Western blots show bands in mesenteric bed (A and B) and carotid (C and D) of SHR and WKY rats. Data are expressed as means ± SEM. ***P<0.001, **P<0.01 and *P<0.05 and vs WKY; ###P<0.001 and ##P<0.01 vs SHR.

6.3.2. PEA reduces the activation of AT1 pathways in mesenteric bed

To evaluate the modulation of AT1 activation following PEA treatment, we analyzed several signaling pathways downstream this receptor, such as NF-κB activation through IκBα degradation, and STAT3 and ERK1/2 phosphorylated state. As shown in figure 19, PEA treatment partially prevented IκBα degradation (figure 19A), reduced the increased phosphorylation of STAT3 (figure 19B) and ERK1/2 (figure 19C) in SHRs, attenuating the increase of AT1-mediated signaling in SHR basal conditions.

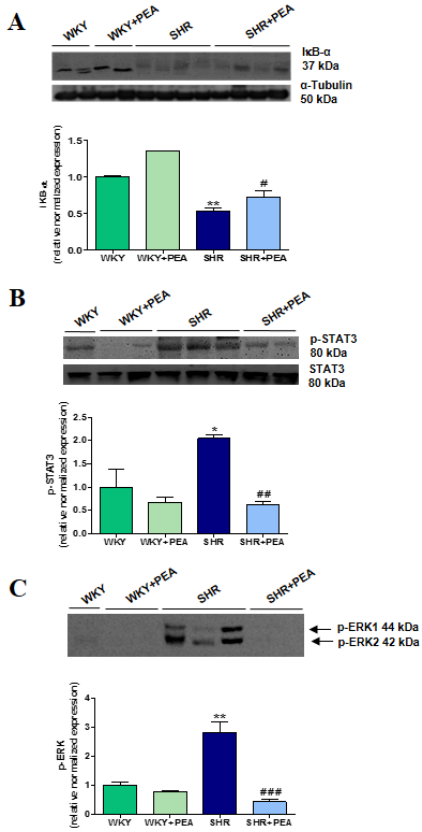

Figure 19. **Effect of PEA on activation of transcription factors in mesenteric bed and its modulation.** IκBα (A), pSTAT3/STAT (B), and pERK1/2 (C) immunoblots are shown. Data are expressed as means ± SEM. **P<0.01 and *P<0.05 vs WKY; ###P<0.001, ##P<0.01 and #P<0.05 vs SHR.

7. PEA improves glucose tolerance, insulin sensitivity and lipid homeostasis in DIO animals reducing hepatic and systemic inflammation

7.2. Effect of PEA on body weight, food intake and fat mass in DIO mice

Consistent with numerous previous studies, the DIO mice gained significantly more body weight compared to STD animals throughout the experimental period. As shown in figure 20A, a slight steady decrease in body weight was evidenced in PEA-treated DIO animals during 10 weeks of treatment. However, AUC of body weight during time was markedly increased by HFD and significantly reduced by PEA treatment (figure 20B). As shown in figure 20C, fat mass, measured at the end of the study, was significantly increased in DIO animals, compared to STD mice, and reduced by PEA treatment of DIO mice. The food intake rates for all animals were measured once a week. As shown in figure 20D no difference in food intake, in the following 10 weeks, was found between DIO and PEA-treated DIO mice. No modification of body weight, fat mass, or food intake were recorded in PEA-treated STD mice compared to control STD.

7.3. Effect of PEA on blood pressure

Since hypertension is an important feature of MetS, we also evaluated blood pressure. As shown in figure 21A, DIO mice showed an increase

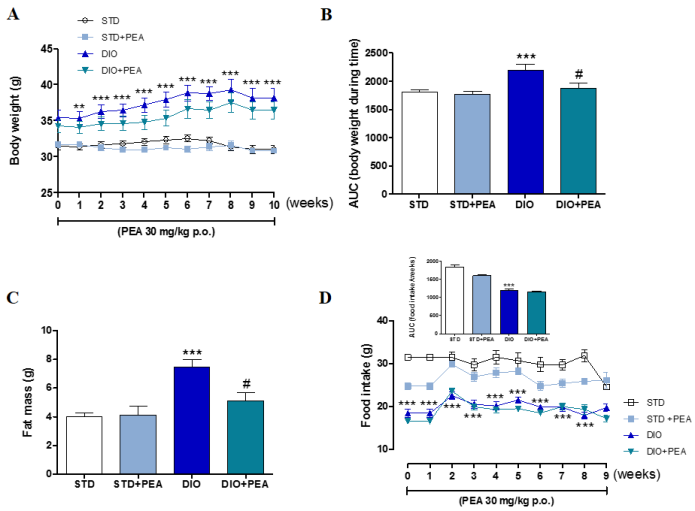

Figure 20. **PEA treatment induced body weight, fat mass and food intake modifications in diet induced obese mice**. Body weight (A) and related AUC (B), fat mass (C) and food intake (D) of STD and DIO mice treated or not with PEA are shown. Data are expressed as mean ± SEM (n=10 for each group). ***P<0.001 and **P<0.01 vs STD; #P<0.05 vs DIO.

of blood pressure values compared to STD animals detected at 8-th week of treatment. PEA-treated mice showed a trend of decrease in blood pressure. Conversely, a strong and significant effect on blood pressure was shown following PEA treatment of DIO at 9-th week compared to DIO mice (figure 21B).

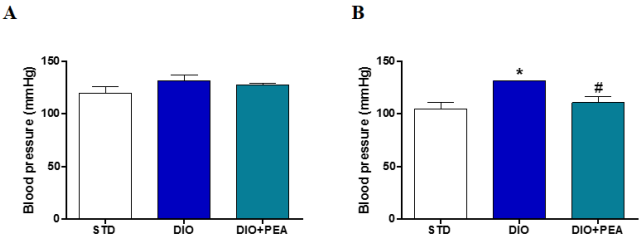

Figure 21. PEA treatment on systolic blood pressure. Panel A shows systolic blood pressure values of STD and DIO mice treated with PEA measured at 8-th week. Panels B show the systolic blood pressure values at 9-th week of PEA treatment. Data are expressed as mean ± SEM (n=10 for each group). *P<0.05 vs STD; #P<0.05 vs DIO.

7.4. Effect of PEA on serum biochemical parameters

The effects of the chronic treatment with PEA on biochemical serum parameters in mice fed STD or HFD are shown in figure 22. As expected, DIO mice showed a significant increase in cholesterol (figure 22A), triglycerides (figure 22B), and ALT (figure 22C) levels compared to STD animals. The chronic administration of PEA to DIO mice led to a strong reduction of all parameters examined.

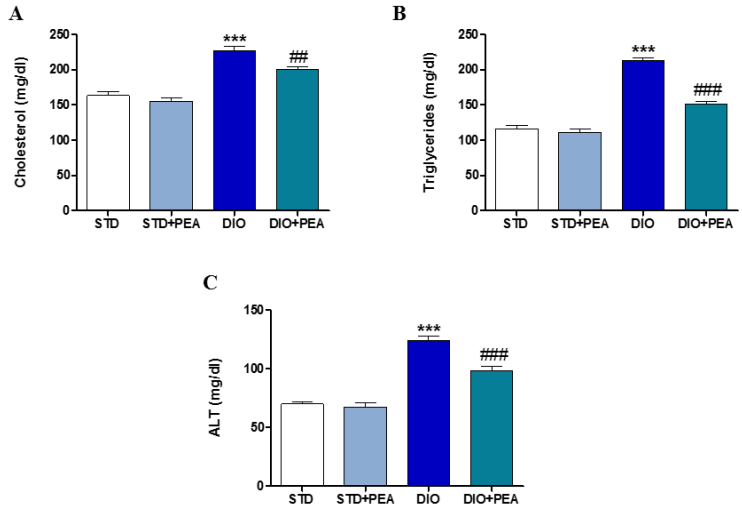

Figure 22. PEA restore serum metabolic parameters. Cholesterol (A), triglycerides (B) and ALT (C) serum levels from STD and DIO treated or not with PEA are shown. Data are expressed as mean ± SEM (n=10 for each group). ***P<0.001 vs STD; ###P<0.001 and ##P<0.01 vs DIO.

7.5. PEA restores serum adipokines levels and systemic glucose homeostasis

PEA treatment improves serum adipokines levels and systemic glucose homeostasis. Indeed, serum leptin levels, significantly increased in DIO mice, were significantly reduced by PEA (figure 23A). Conversely, DIO animals showed lower levels of adiponectin

than STD group, the adipokine levels were normalized by PEA treatment (figure 23B).

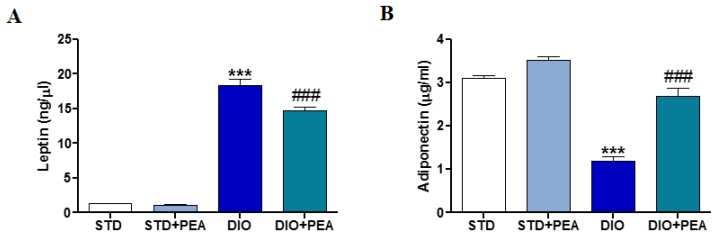

Figure 23. Effect of PEA on circulating leptin and adiponectin levels. Leptin (A) and adiponectin (B) are shown. Adipokine levels were normalized Data are expressed as mean ± SEM (n=10 for each group). ***P<0.001 vs STD; ###P<0.001 vs DIO.

Furthermore, compared with STD, DIO mice showed a marked increase in fasting glucose and insulin level. PEA prevented glucose (figure 24A) and insulin (figure 24B) alterations, improving HOMA-IR (figure 24C).

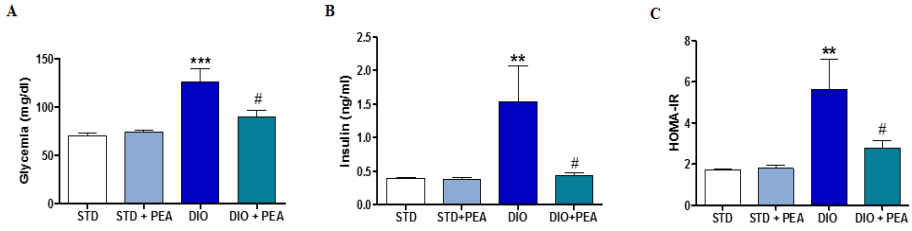

Figure 24. Effect of PEA on IR. PEA improves body glucose homeostasis. Fasting glucose (A), insulin levels (B) and HOMA-IR (C) were reported. Data are expressed as mean ± SEM (n=10 for each group). ***P<0.001 and **P<0.01 vs STD; #P<0.05 vs DIO.

7.6. PEA improves glucose and pyruvate tolerance and insulin sensitivity

One week before sacrifice, oral glucose tolerance (OGTT) and insulin tolerance test (ITT) were performed to evaluated glucose metabolism. PEA, chronically administered in DIO mice, had a clear euglycemic

effect determining a significant decrease in glucose levels at 30 and 60 minutes after glucose administration (figure 25A). Insulin tolerance was also evaluated by measuring glucose levels after systemic injection of insulin in 5 hours fasted animals. Our results clearly show that PEA-treated animals decreased glucose levels at 60, 90 and 120 minutes, restoring insulin sensitivity (figure 25B). Regarding pyruvate tolerance (PTT), also in this case, an increase in glucose levels was observed at 30 and 60 minutes in DIO animals compared to STD mice that showed a trend of decrease in DIO mice treated with the PEA (figure 25C).

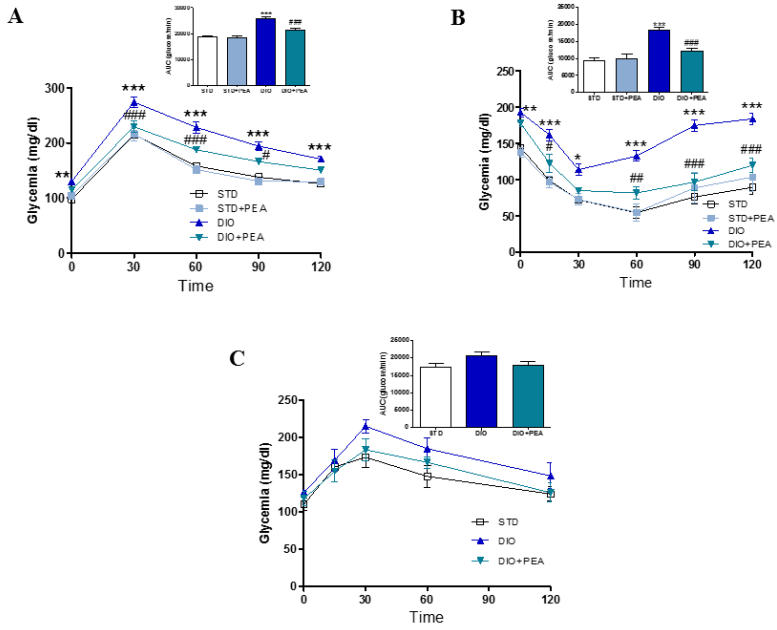

Figure 25. PEA effect on glucose homeostasis. Glucose (A), insulin (B) and pyruvate (C) tolerance tests were performed in STD and DIO mice. PEA improves glucose tolerance, determining a significant decrease in glucose levels at 30 and 60 minutes after glucose administration (A); restores insulin sensitivity decreasing glucose levels at 60, 90 and 120 minutes (B) and shows a trend of decrease in DIO mice treated with the PEA (C). Data are expressed as mean ± SEM (n=10 for each group). ***P<0.001, **P<0.01 and *P<0.05 vs STD; ###P < 0.001, ##P<0.01 and #P <0.05 vs DIO.

7.7. PEA reduces both hepatic and systemic inflammation

An important evidence is that obesity is characterized by chronic low grade inflammation. The inflammatory mediators induce the inhibition of insulin signaling, resulting in IR and hyperglycemia. The strong increase of inflammatory cytokines and proteins, as TNF-α, IL-1 and MCP-1 in serum (figure 26A, 26B and 26C, respectively) and hepatic gene expression of TNF-α and IL-6 (figure 26D and 26E, respectively) of obese mice, was significantly reduced by PEA treatment, according to its well-known anti-inflammatory activity. Therefore, in animals treated with PEA, liver inflammatory damage appears reduced. This effect was also associated with a reduction of hepatic triglycerides content that was significantly enhanced by DIO feeding (figure 26F).

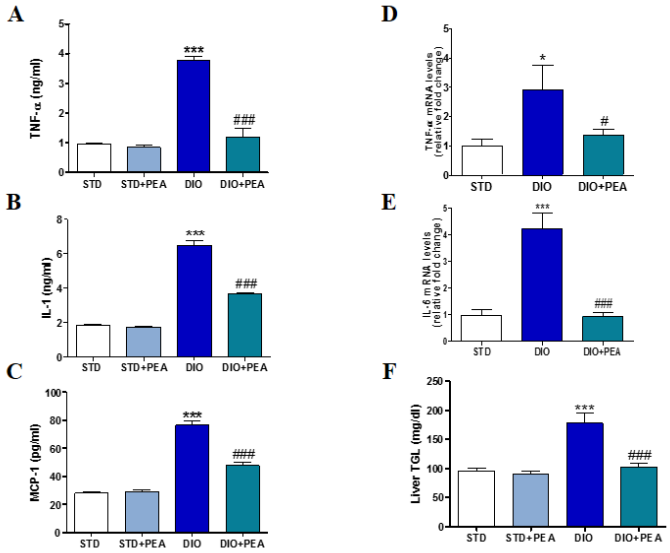

Figure 26. Effect of PEA on hepatic and systemic inflammation. The expression of pro-inflammatory cytokines TNF-α (A), IL-1 (B) and MCP-1 (C) were analyzed by ELISA kit in serum extracts. The mRNA levels of pro-inflammatory cytokines TNF-α (D) and IL-6 (E) were analyzed by real-time PCR in liver extracts. Triglycerides were measured in liver (F). Data are expressed as mean ± SEM (n=10 for each group). ***P< 0.001 and *P<0.05 vs STD; ###P<0.001 and #P< 0.05 vs DIO.

7.8. PEA improves glucose and lipid homeostasis

According with the inflammation, other severe alterations demonstrated metabolic impairment and tissue IR in DIO mice. We examined AMPK and ACC phosphorylation and the expression of its downstream protein, PPAR-α. The evaluation of AMPK/ACC pathway activation in liver revealed a reduction in the phosphorylated AMPK protein in DIO animals and its downstream substrate ACC that were prevented by PEA treatment (figure 27A and 27B), indicating an increase in fatty acid oxidation, compromised in obese mice. The basal level of hepatic PPAR-α, down-regulated in DIO mice, was restored by PEA (figure 27C).

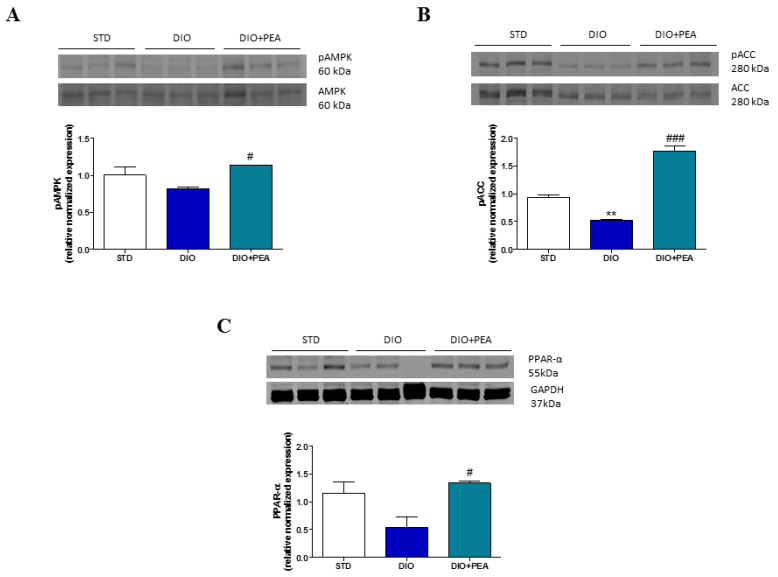

Figure 27. Effect of PEA lipid impairment. The expression of pAMPK/AMPK (A), pACC/ACC (B) ratio together PPAR-α expression (C), were analyzed by western blot in liver extracts. Data are expressed as mean ± SEM (n=10 for each group). **P<0.01 vs STD; ###P<0.001 and #P<0.05 vs DIO.

Insulin signaling was evaluated determining the activation of its pathway and GLUT expression. We evaluated the phosphorylation of

the InsR, that showed a trend of decreased in liver of DIO mice compared to that of STD animals (figure 28A). Then, we evaluated the effectiveness of insulin signaling through the evaluation of Akt phosphorylated state (pAkt) and the expression of GLUT-2. PEA treatment restored the phosphorylation of Akt and GLUT-2 expression markedly reduced in DIO animals (figure 28B and 28C). To address the direct effect of PEA on hepatic insulin-sensitivity, we evaluated the modulation of insulin signaling altered by the induction of IR *in vitro* using human HepG2 cells. Here, we have demonstrated that PEA increases the phosphorylation of Akt, following hormone stimulation in insulin-resistant cells (figure 28D).

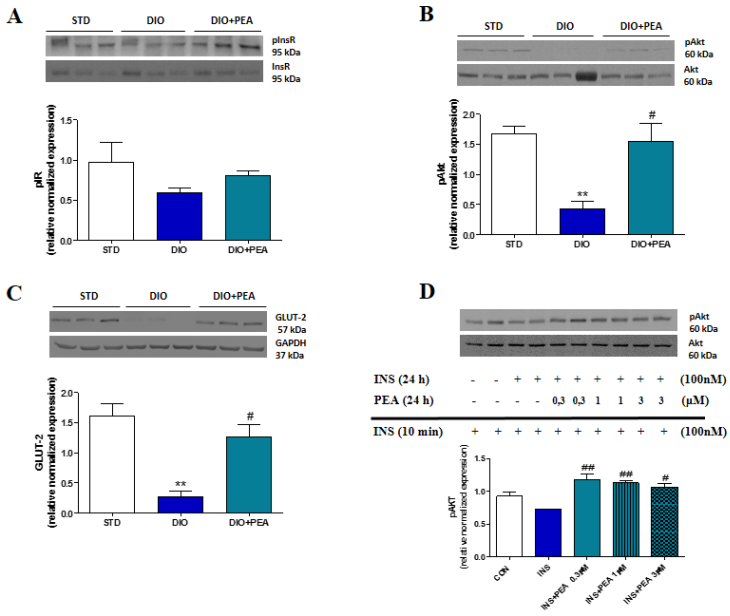

Figure 28. Effect of PEA on metabolic impairment and IR. Liver pInsR/InsR (A), pAkt/Akt (B) ratio and GLUT-2 expression (C), were evaluated. pAkt/Akt (D) ratio was also determined in HepG2 cells after stimulation or not with insulin (100 nM) for 24 hours and with or without PEA (0.3, 1 and 3μM). (CON=control cells, INS=insulin resistant cells, and INS+PEA=insulin resistant cells treated with PEA). After wash out, all cells were stimulated with insulin for 10 minutes. *In vivo* data are expressed as mean ± SEM (n=10 for each group); *in vitro* data are from triplicate experiments. **P<0.01 vs STD; ##P<0.01 and #P<0.05 vs DIO; ##P<0.01 and #P<0.05 vs INS.

7.9. Effect of PEA on neuronal activation in the ARC and VMH hypothalamic nuclei and in human neuroblastoma cells

Finally, we studied whether PEA was also able to modulate central glucose homeostasis at hypothalamic level. Therefore, we examined the ARC and VMH neuronal activation, and c-fos immunostaining was performed. In the ARC nucleus of DIO mice, a decrease in c-fos labeling was found. Interestingly, in the PEA-treated group, a trend of c-fos labeling increase was evidenced (figure 29A). Conversely, in the VHM of DIO mice a significant decrease in the neuronal activation was shown compared to STD mice. In this case, no differences were found between DIO and PEA-treated DIO mice (figure 29B).

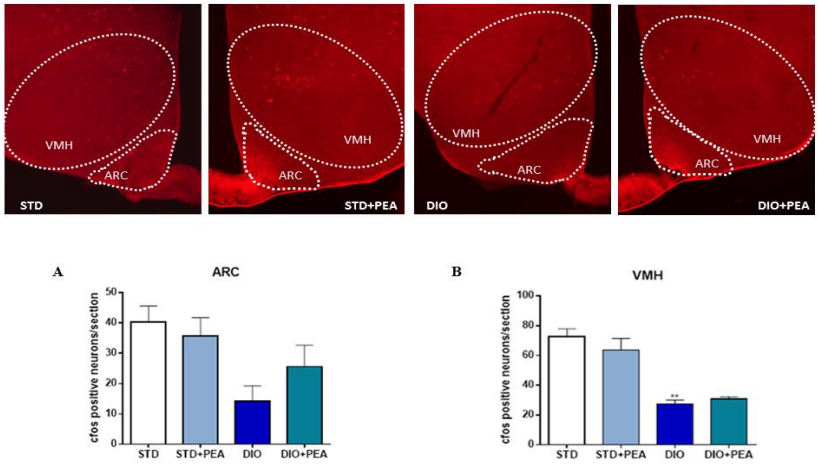

Figure 29. PEA effect on neuronal activation. Immunostaining for c-fos in the hypothalamic arcuate and the ventromedial nuclei from STD and DIO mice, treated with the vehicle or PEA. Data are expressed as mean ± SEM (n=10 for each group). **P<0.01 vs STD.

The involvement of the hypothalamic control of glucose homeostasis by PEA, was confirmed in *in vitro* experiments, using SH-SY5Y neuroblastoma. Indeed, neuronal IR was obtained by a long pre-treatment of SH-SY5Y with insulin (16 hours). When these cells were

re-stimulated with the hormone, they showed a reduction of insulin signaling activation, measured as phosphorylated Akt. When insulin-exposed cells were also treated with PEA (1 μM for 6 hours), the re-stimulation with insulin (10 minutes) showed a restoration of Akt phosphorylation, indicating a recovery of insulin-sensitivity (figure 30). Taken together, these findings underline that this acylethanolamide also displays a central effect on glucose homeostasis, reducing neuronal IR.

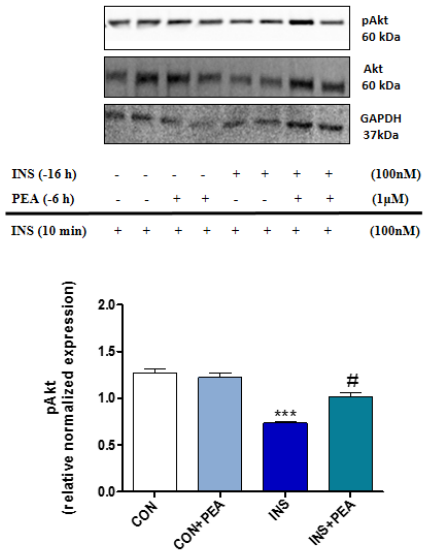

Figure 30. Effects of PEA on Akt phosphorylation in SH-SY5Y cells pAkt/Akt ratio was evaluated in SH-SY5Y by Western blot and densitometric analysis. Data are from triplicate experiments. ***P<0.001 vs CON; #P<0.05 vs INS.

Discussion and conclusions

PEA prevent endothelial vascular dysfunction and hypertension, acting on EETs pathway and RAS in SHR

In this study, we demonstrated the effect of PEA on SHR in reducing hypertension and improving EDHF-mediated vasodilation, by the modulation EET hydrolysis and RAS in the vasculature. Besides, hypertension is an important feature of MetS. PEA ameliorated glucose homeostasis and normalized values of HOMA-IR. In this animal model, the main finding was the capability of PEA in improving EDHF-mediated vasodilator function induced by acetylcholine in pre-constricted mesenteric bed from SHRs. EDHF effect is identified by stimulating resistance vessels with Ach in presence of NOS and COX inhibitors. So, excluding the contribution of NO and prostaglandins, through L-NAME and INDO treatment, we highlighted the EDHF contribution in vasodilatation of mesenteric bed. Interestingly treatment with PEA restored the EDHF impairment observed in SHR. Furthermore, PEA treatment in SHR caused a significant improvement not only in EDHF, but also in the endothelial dysfunction. In fact, evaluating the tone achieved by adding INDO plus L-NAME, the contraction was significantly higher in SHR treated with PEA compared with SHR, implying a major contribution of endothelial-derived relaxing factors in PEA-treated SHRs. A recent clinical study on ocular hypertensive patients showed a decrease in the intraocular pressure following three months PEA treatment, improving the endothelium-dependent flow-mediated vasodilation compared to placebo **[235]**. EDHF plays a main role in the response to vasoactive

substances and regulation of vascular tone at small resistance arteries level. Indeed, the mesenteric vascular bed plays an important role in maintenance of systemic blood pressure producing vascular resistance to develop blood pressure and regulate tissue blood flow.

EDHF is the predominant endothelium-dependent vasodilator in resistance vessels unlike in the large conduit vessels, such as the aorta, endothelium-dependent responses are mediated by NO **[200]**. Thus, the role of EDHF in the resistance arteries is important in cardiovascular diseases, such as hypertension, diabetes mellitus and congestive heart failure **[236]**.

SHR experimental model is characterized by a reduction of EET plasma levels, a reduction of blood pressure in response to sEH inhibition and an increased expression and activity of sEH compared to normotensive animals **[237, 238]**. Consistently, we found an impairment of EDHF vasodilation elicited by acetylcholine in hypertensive rats. This finding agrees with those reported for mesenteric arteries of the SHR **[239]** and in salt-sensitive hypertensive patients **[240]**. These findings suggested us to hypothesize an involvement of EET/DHET metabolites, as EDHF, in blood pressure lowering effect of PEA, and we found a significant decrease in sEH in vascular tissues in SHR after PEA treatment. We showed a significant reduction in sEH expression in the vasculature and in serum 14,15-DHET in PEA-treated SHR, demonstrating, indirectly, an increase in vascular-derived EETs levels. The central role of sEH in the initiation and establishment of hypertension has been confirmed by evidence showing that treatment with sEH-selective inhibitors in Ang II-infused hypertensive rats increases the level of EETs, with related decrease in

systolic blood pressure **[213]**. In the mesenteric artery of aged SHRs, EDHF-mediated hyperpolarization and relaxation are severely attenuated, but completely restored and even augmented after inhibition of the RAS **[241, 242]**. Thus, we also focused on this pathway: in our study, RAS modulation is in agreement with previous findings, since SHRs showed an increase in AT1 and ACE expression, that was prevented by PEA treatment. Consistently with our findings, clinical evidence has demonstrated that the actions of Ang II extend far beyond classical actions of RAS, but are also linked to wide array of cellular pathways including that of inflammatory processes, as well as to endothelial disorders with reduction in endothelium-derived relaxing factors **[222]**. However, in rats, ACE has been shown to be the most important enzyme for Ang II formation **[243]**. Evidence indicate that several tissues, including vasculature, contain all components of the RAS and are thus capable of producing local Ang II **[225]**. Interestingly, vascular inflammatory response has been shown to be more closely related to local than circulating Ang II **[244]**. Therefore, local Ang II appears to be more important in the regulation of Ang II-induced inflammation. Interaction between Ang II and sEH has been established, in fact, the level of this enzyme in the heart and endothelium is upregulated by Ang II *in vitro* in cultured cardiomyocytes and vascular endothelial cells and *in vivo* in rodent models, leading to a reduction of half-life of vasodilating and anti-inflammatory EETs **[245]**. Therefore, the attenuation of RAS activity, shown by AT1 and ACE down-regulation observed in PEA treated rats, could be responsible, at least in part, to the reduced expression of sEH found in PEA-treated SHRs. Consistently, we showed an

attenuation of key downstream factors of Ang II signaling cascade; in particular, PEA treatment increased IκBα content in SHR, demonstrating a reduction of NF-κB activation, and a reduction of the phosphorylated state of STAT3 and ERK1/2. The damping of AT1-mediated signaling cascade by PEA indicates the reduction of transcription factor activation, leading to the modulation of downstream genes, including sEH transcription.

We demonstrate that long-term treatment with PEA alleviates hypertension, by improving EDHF-mediated vasodilatation of arterial vessels compromised in SHRs. PEA increased serum half-life of EETs and reduces AT1 expression and Ang II synthesis in vascular tissues, reducing AT1 downstream pathways. Therefore, this compound, could modulate in a concerted way the interaction among Ang II, sEH, and EETs. In conclusion, PEA could be a useful tool to cure and prevent endothelial vascular dysfunction and hypertension, acting on EETs pathway and RAS.

PEA improves glucose tolerance, insulin sensitivity and lipid homeostasis in DIO animals

Here, we demonstrate, for the first time, that repeated administrations of PEA improve metabolic impairment induced by HFD in an animal model of obesity induced by overnutrition.

As well known, the risk of developing MetS in human depends on synergy of both genetic and environmental factors. Nowadays, this multifactorial condition has an alarming rate of prevalence, since appropriate experimental animal models mimicking the disease in humans are crucial in order to solve the difficulties in evaluating the

pathophysiology of MetS in human and define new pharmacological and multi-target pharmacological therapies.

Many researchers have employed different types of HFDs (from 20 and 60 % of total energy) and the source of the fat component may be either plant-derived oils (e.g. corn, safflower or olive oil) or animal-derived fats (e.g. beef tallow and lard) all used to induce obesity in animals **[91, 246]**. Many studies have also indicated that HFD is effective in promoting hyperglycemia, IR, dyslipidemia and increased free fatty acids in the blood, either independently or concurrently, and assessing the chronic effect of dietary fats with different fat content (10, 32 and 45 %) on body adiposity and metabolism in rats **[247, 248]**.

These findings demonstrated that energy intake, weight gain, fat mass, plasma glucose, cholesterol, triglycerides, free fatty acids, leptin, and insulin levels increased dose-dependently with increased dietary fat. In particular, mice fed with 60 % HFD exhibited increased body weight, total fat pads, plasma triglyceride, high density lipoproteins (HDL) cholesterol, and LDL cholesterol levels **[249]**. It is also well known that the lipid accumulation and consequent inflammation in the liver can cause IR **[250]**. The lipotoxicity hypothesis (overproduction and accumulation of triglycerides in non-adipose tissues, such as liver, muscle, and pancreas) is the common criteria seen in the effect of different diets in the development of MetS **[251, 252]**.

The main finding of this study is that PEA treatment prevented the impairment of glucose homeostasis and the development of IR in a mouse model of MetS. IR, one of the main features of MetS, is characterized by a reduction of insulin signaling activation and its

inability to produce biological effects on metabolically active tissues (liver, fat and skeletal muscle). The insulin receptor, in these tissues, becomes insensitive to the hormone and the body responds increasing its secretion (hyperinsulinemia). The recovery of systemic and liver insulin signaling in “fat over fed mice", following PEA treatment, demonstrates drug efficacy in restoring glucose homeostasis.

Previously, the positive effect of PEA on feeding behavior and body weight gain has been evaluated in a model of mild obesity due to hypoestrogenism induced by ovariectomy in rat **[77]**. Conversely, here, the effects of PEA were studied in a model of severe obesity when the disease and its complications were acclaimed (after 12^{-th} week of HFD feeding). Treatment with PEA (for 10 weeks), induced a constant and slight time-dependent reduction in body weight, that was associated with a significant decrease in fat mass as shown by bioelectrical impedance. A significant effect on reducing blood pressure was also evidenced after a long period of PEA treatment (at 9^{th} weeks) in DIO mice.

The evaluation of glucose tolerance showed that chronic administration of PEA in obese mice, promoted a significant reduction in glycemia in support of its anti-hyperglycemic and euglycemic effect, as also confirmed from ITT. PEA induced an increase of insulin-sensitive tissues after intraperitoneal hormone injection, and a significant reduction in blood glucose levels at different times compared to DIO animals. Pyruvate tolerance supported anti-hyperglycemic action of PEA, suggesting its modulation of hepatic gluconeogenesis.

As expected, the intake of HFD induced a clear change in the lipid profile, characterized by high cholesterol and elevated triglycerides, and fatty liver disease characterized by an increase in transaminase levels. PEA attenuated DIO-induced alteration of lipid (both at hepatic and systemic level, i.e. triglycerides) and hormonal profile, normalized HOMA-IR and liver GLUT-2 expression, reducing IR, liver and systemic inflammation. In fact, PEA counteracts adiponectin and leptin alterations due to obesity: these adipokines are inversely involved in glucose and lipid metabolism, through AMPK activation **[136, 253]**. Our results showed decreased serum leptin levels in PEA-treated mice, consistently with fat mass reduction and restored serum adiponectin levels to those of STD mice, associated to AMPK/ACC pathway activation, and fatty acid metabolism in the liver. The activation of AMPK exhibits multiple effects, including a reduction in inflammation, oxidative stress, IR and an increase in lipid metabolism **[136, 254]**.

In particular, inflammation is known for its contribution in the IR and T2DM **[165, 255]**. In obese patients, an important role is played by the state of systemic inflammation **[256-258]**: in fact, obese individuals show elevated circulating levels of inflammatory markers, both pro-inflammatory cytokines and acute phase proteins **[259, 260]**. There are two types of proteins involved in inflammation: the pro-inflammatory, such as MCP-1, TNF-α, IL-6, IL-8, IL-1, leptin, resistin, and the anti-inflammatory ones, such as IL-10 **[261]**.

In our model, several pro-inflammatory cytokines such as TNF-α, IL-1 and MCP-1 were significantly elevated in serum of DIO animals compared to STD mice, while PEA significantly reduces these levels.

These anti-inflammatory effects were confirmed by the evaluation of gene expression of TNF-α and IL-6 in liver. A reduction, at hepatic level, of inflammatory mediators in animals treated with PEA compared to DIO control was found, thus confirming its anti-inflammatory and hepatoprotective effects.

Physiologically insulin, in target organs involved in glucose metabolism such as liver, adipose tissue and skeletal muscle, binds to its transmembrane receptor and promotes its auto-phosphorylation. The phosphorylated receptor recruits IRS and increases its activation. Phosphorylated IRS activates PI3K and promotes the phosphorylation of Akt. The activation of this kinase regulates glucose metabolism following two ways: first, promoting the translocation of GLUT from the cytoplasm to the membrane mediating the uptake of glucose; second, through glycogen synthesis. In case of inflammation, cytokines inhibit insulin signaling inducing the degradation of IRS1 protein, which suppresses signaling pathway PI3K/Akt and subsequently suppressing the translocation of GLUT and glycogen synthesis, resulting in hyperglycemia and IR.

Here, we demonstrated that PEA restored phosphorylation of insulin receptor, activation of Akt and enhanced GLUT-2 expression. All these effects induced an increase of hepatic glucose uptake and metabolism, contributing to blood glucose reduction and glucose homeostasis. Furthermore, we address the direct effect of PEA on hepatocyte insulin sensitivity, demonstrating the reversal of IR in insulin stimulated HepG2 cells, through the restoration of hormone signaling (pAkt).

Recently a review has drawn a parallel between obesity, adipose tissue changes, and T2DM development **[262]**. Based on literature data the authors show how glucose homeostasis is changed by adipose tissue dysfunction. Adipose tissue dysfunction observed in obesity is characterised by adipocyte hypertrophy, macrophage infiltration, impaired insulin signaling and IR. In addition, there is a release of inflammatory adipokines and an excessive amount of FFA promoting ectopic fat deposition and lipotoxicity in muscle, liver and pancreas. Recent evidence supports the idea that adipose tissue is not a passive energy storage organ but a dynamic endocrine organ, which regulates metabolism through a complex adipocyte communication with the adjacent microenvironment. Further studies are needed to determine how glucose homeostasis is changed by adipose tissue dysfunction and if PEA treatment can modulate this relationship enabling new pharmacological strategies with the goal of preventing obesity and its related disease.

All these findings indicate that PEA could be a new useful therapeutic tool for the treatment of metabolic dysfunctions associated to obesity, such as IR and T2DM. Moreover, its anti-inflammatory activity represents a further advantage in limiting the low-grade hepatic and systemic inflammation often associated to obesity. Future studies are needed to investigate the possible combination of PEA with standard anti-diabetes drugs, allowing their dose reduction and limiting the onset of their adverse effects.

It is certainly alarming that obesity and its complications can be due to fatty acids, as seemingly harmless essential nutrients, but at the same time it is very intriguing that a pharmacological use and dosage of an

endogenous lipid is able to limit or prevent its progression and to restore, at last in part, lipid and glucose homeostasis, showing multi- and conversing mechanisms of action.

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