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PHARMACOLOGICAL EFFECTS OF PALMITOYLETHANOLAMIDE (PEA) IN DIFFERENT ANIMAL MODELS OF NEUROPATHIC PAIN

by

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Nothing in life is to be feared,
it is only to be understood.

Marie Curie

Ringraziamenti

Eccomi qui. Giunta a conclusione di una nuova fase della mia vita professionale. Tante le esperienze, le emozioni vissute e tante le persone che con me hanno partecipato a scrivere una parte di questo importante percorso.

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List of abbreviations

2-AG	2-arachidonoyl glycerol
AEA	anandamide
AGE	advanced glycation end-products
ALIAmides	Autacoid Local Injury Antagonism Amides
ATP	adenosine triphosphate
BIPED	burden of disease investigative prognostic efficacy of intervention and diagnostic
BKCa	calcium-activated big-conductance potassium channel
CB_1	cannabinoid receptor, subtype 1
CB_2	cannabinoid receptor, subtype 2
CD86	cluster of differentiation 86
CINP	chemotherapy-induced neuropathic pain
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EA	ethanolamine
ELISA	enzyme-linked immunosorbent assay
FAAH	fatty acid amide hydrolase
$GABA_A$	gamma-aminobutyric acid receptor A
GAGA-A	gamma-aminobutyric acid receptor A
HCl	hydrogen chloride
IENF	intraepidermal nerve fibers
IKCa	calcium-activated intermediate-conductance potassium channel
Kv1.5	potassium voltage-gated channel, shaker-related subfamily, member 5
Kv4.3	potassium voltage-gated channel, Shal-related subfamily, member 3
MAPK	mitogen-activated protein kinase
MIA	monosodium iodoacetate
MMP-3	matrix metalloproteinase-3
mPTP	mitochondrial permeability transition pore
NAAA	N-acylethanolamine-hydrolyzing acid amidase
NAEs	N-acylethanolamides
NaOH	sodium hydroxide
NF-jB	nuclear factor-jB
NF-κB	nuclear factor-κB

NGF	nerve growth factor
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
P450scc	cytochrome P450 side-chain cleavage
PA	palmitic acid
PARP	poly-ADP-ribose polymerase
PBS	phosphate buffered saline
PEA	palmitoylethanolamide
PKC	protein kinase C
PNS	peripheral nervus system
PPAR-α	peroxisome proliferator-activated receptor alpha
PPAR-γ	peroxisome proliferator-activated receptor gamma
ROS	reactive oxygen species
SFI	sciatic functiona index
StaR	steroidogenic acute regulatory protein
STZ	streptozoticin
TRPA1	transient receptor potential cation channel, subfamily A, member 1
TRPV1	transient receptor potential cation channel, subfamily V, member 1
TRPV4	transient receptor potential cation channel, subfamily V, member 4
TTX-Na^+	tetrodotoxin-sensitive sodium channel

Abstract

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as pain that arises as a direct consequence of a lesion or disease affecting the somatosensory system. Neuropathic pain is often poorly alleviated by first-, and second-line medications recommended by the Neuropathic Pain Special Interest Group of the IASP due to lack of efficacy and/or dose-limiting side-effects. Hence, there is an urgent need to develop novel mechanism-based therapeutic agents that are highly efficacious and well tolerated to improve relief of neuropathic pain. Palmitoylethanolamide (PEA) is the parent molecule of ALIAmides (Autacoid Local Injury Antagonism Amides), a group of endogenous fatty acid derivatives sharing anti-inflammatory and antinociceptive effects with the endocannabinoid family mainly through the down-modulation of local mast cell degranulation. Several evidences in literature show the antinociceptive effect of PEA in different animal models of pain, such as spinal cord injury, chronic constriction injury of the sciatic nerve, carrageenan-induced acute inflammation, and complete Freund's adjuvant-induced chronic inflammation. Based on these findings, the aim of this study is to further explore the therapeutic potentiality of PEA in resolving painful states in three very common forms of neuropathic pain in human associated to osteoarthritis, diabetes, and chemotherapy.

Osteoarthritis (OA) is the most common chronic joint disease characterized by a progressive destruction of cartilage, resulting in pain, and loss of articular function. The monosodium iodoacetate (MIA) rat model of osteoarthritis (OA) was used to investigate the effects of PEA. Under a chronic treatment regiment, PEA was able to completely abolish knee swelling and thermal hyperalgesia, that are two important index of inflammation. Moreover, treatment of MIA-treated rats with PEA resulted in a significant relief of mechanical allodynia, as index of neuropathic pain. As expected, intra-articular injection of MIA resulted in a significant increase of joint discomfort. PEA treatment completely restored locomotor functionality, and is also able to preserve cartilage from damage.

Diabetes mellitus is a metabolic syndrome today affecting 382 million people. One of the major and most disabling long-term complications of diabetes is diabetic neuropathy. The well established streptozotocin (STZ)-induced mice model of type 1 diabetes was emploied to explore the antinociceptive effect of PEA in diabetic neurophaty. PEA relieved mechanical allodynia, counteracted

nerve growth factor deficit, improved insulin level, preserved Langherans islet morphology reducing the development of insulitis in diabetic mice.

Chemotherapy-induced neuropathic pain (CINP) is another common and very interesting type of neurophatic pain, affecting up to 90% of patients. The effect of PEA in paclitaxel model of CINP, one of the most common used antineoplastic drugs in clinic, was investigated. Preliminary results show that PEA is able to evoke a total antiallodynic effect in CINP model, after acute administration.

The results of this thesis show the pharmacological effect of PEA to relieve neuropathic pain associated to osteoarthritis, diabetes, and chemotherapy, three very common diseases in human, that lack a resolutive, and effective treatment. These findings allow us to suggest a therapeutic use of PEA in clinic.

Keywords: PEA, neuropathic pain, osteoarthritis, diabetes, CINP

Introduction

Pain

Pain is a complex biological phenomenon that encompasses intricate neurophysiological, behavioural, psychosocial and affective components (McDougall et al., 2011). It is commonly described as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. (Merskey et al., 1991). Pain can be classified in function of persistence, and different components that are involved. he sensory afferent nerves carry sensations from the skin, joints, and viscera via large and small fibres. Large fibres, such as A-alpha, are responsible for limb proprioception and A-beta fibres carry sensations of limb proprioception, pressure, and vibration. Large A-delta myelinated fibres and small C unmyelinated fibres are mainly responsible for carrying nociceptive sensations. Superficial pain is often a sharp or pricking sensation and is transmitted by A-delta fibres. A deep-seated, burning, itching, aching type of pain is often accompanied with hyperalgesia and allodynia and is transmitted via slow, unmyelinated C fibres. Tissue damage results in the release of inflammatory chemicals, such as prostaglandins, bradykinins, and histamines, at the site of inflammation, which triggers the depolarization of nociceptors, thereby generating an action potential. The action potential transmits the nociceptive sensation, via the dorsal root ganglion (DRG), to the dorsal horn of the spinal cord. The release of glutamate and substance P results in the relay of nociceptive sensations to the spinothalamic tract, thalamus, and, subsequently, the cortex, where pain is interpreted and perceived (Willis et al., 1997).

Nociceptive pain is that kind of pain that appears after a damage, such as injury or surgery. It can be somatic if it is caused by tissue lesion, such as skin and muscle, or visceral, if it is associated to internal organs damage. Pain intensity is correlated to severity of damage, and it usually disappears when the cause is resolved (Ueda et al., 2006).

In acute pain, stimulus is associated to many defense reactions that counterbalance or remove pain onset (McDougall et al., 2011). Instead, chronic pain can persist

long after the initial injury is healed, and becomes a disease by itself (Kuner et al., 2010).
On the other hand, neuropathic pain is a type of chronic pain, which occurs as a consequence of a lesion or disease to the somatosensory nervous system (Jensen et al., 2011). Neuropathic pain is very common, affecting 6–8% of the population. Pharmacological management of neuropathic pain includes medications such as NSAIDs, opioid analgesics, anticonvulsants, antidepressants, serotonin noradrenaline reuptake inhibitors, and cannabinoids (Moulin et al., 2014). However, in many cases these treatments are associated with suboptimal therapeutic efficacies and/or side effect.

Palmitoylethanolamide (PEA)

Palmitoylethanolamide (PEA) belongs to the class of fatty acid ethanolamides (or N-acylethanolamides, NAEs), formed *on demand* from membrane phospholipids (Cadas et al., 1996). In tissues, PEA levels depend on enzymatic formation mainly from N-palmitoylethanolamine-phospholipids and on its degradation by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) or N-acylethanolamine-hydrolyzing acid amidase (NAAA) (Ueda et al., 2013). The presence of NAEs and their cognate precursors in various tissues and their pharmacological properties suggest that these molecules play a role as paracrine or autocrine regulators of peripheral functions, therefore AEs were initially called Autacoid Local Injury Antagonism Amides or ALIAmides (Aloe et al., 1993). In particular, PEA has been studied extensively for its anti-inflammatory (Costa et al., 2002; D'Agostino et al., 2007), anti-convulsant (Lambert at al., 2001, and antiproliferative (Di Marzo et al., 2001) effectiveness. The exogenous PEA administration has been reported to evoke antinociceptive effects in different animal models of pain such as spinal cord injury (Genovese et al., 2008), carrageenan-induced acute inflammation (D'Agostino et al., 2009), and complete Freund's adjuvant-induced chronic inflammation (Lo Verme et al., 2006). It is also showed that PEA relieved thermal hyperalgesia and mechanical allodynia in a mouse model of neuropathic pain due to the chronic constriction injury of the sciatic nerve (Costa et al., 2008).

In the past it was proposed the idea that PEA was a cannabinoid receptor CB_2 agonist (Facci et al., 1995), conversely, Lo Verme and coleagues (Lo Verme et al., 2005) showed that PEA had no effect in CB2 knockout mice. Initially, it was found that in some cases PEA could potentiate the effect of anandamide (AEA) on CB or vanilloid receptor 1 (De Petrocellis et al., 2001; Smart et al., 2002). This so-called *entourage effect* could be mediated by PEA competitive inhibition of AEA hydrolysis on FAAH (Jonsson et al., 2001) and/or direct allosteric effect of PEA on transient receptor potential channel type V1 (TRPV1). However, PEA is not a 'classical' endocannabinoid, according to the current pharmacological classification rules (Pertwee et al., 2010).

To date, it is widely recognized that the main PEA pharmacological effects are mediated by activation of peroxisome proliferator-activated receptor (PPAR)-α (Di Marzo et al., 2001). PPARs are regulators of gene networks, which control pain and inflammation, by switching off the nuclear factor-κB (NF-κB) signaling cascade, a key element in the transcription of genes, leading to the synthesis of proinflammatory, and proalgogen mediators (Lambert et al., 2001). Beside PPAR-α, PEA can activate several different receptors and inhibit some ion channels involved in rapid response to neuronal firing, e.g., vanilloid receptor and K^+ channels (Kv4.3, Kv1.5) (Hansen, 2010). Recently, the discovery that PEA, through activation of PPAR-α, stimulates *de novo* neurosteroid synthesis (Sasso et al., 2010), suggests that two separate but converging mechanisms could contribute to the central effect of PEA, an early molecular control through calcium-activated intermediate- and/or big-conductance potassium channels (IKCa and BKCa) opening, silencing neuronal firing (Lo Verme et al., 2006), and thereafter a reinforcing effect mediated by gene transcription and hence neurosteroid synthesis (Sasso et al., 2012; Mattace Raso et al., 2011). It is noteworthy that neuronal hyperpolarization by K^+ efflux is also reinforced by inward chloride currents, sustained by the positive modulation of gamma-aminobutyric acid ($GABA_A$) receptors (Sasso et al., 2012) (**Fig. 1**).

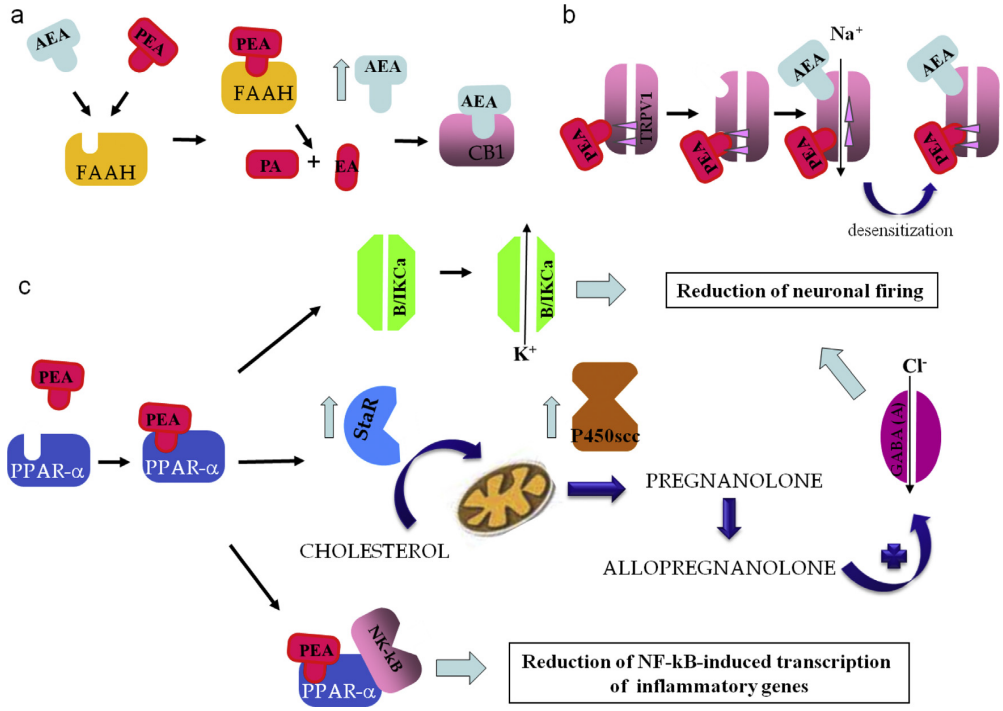

Fig. 1. Direct and indirect mechanisms of action of PEA. The indirect mechanism would involve PEA potentiation of AEA effects through (a) a competitive inhibition of AEA metabolism by FAAH, leading to an increase in AEA levels and its binding to CB_1 (b); an allosteric activity on TRPV1, increasing AEA affinity to this receptor, and inducing later TRPV1 desensitization. (c) Through a PPAR- dependent non-genomic mechanism, PEA increases the gating properties of IKCa and BKCa channels, resulting in a fast reduction of neuronal firing. Moreover, PPAR-α activation, through a genomic mechanism, increases the expression of StaR and P450scc, 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 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 cytoplasmatic complex, that reduces NF-κB transcription activity, dampening the transcription of pro-inflammatory gene (Mattace Raso et al., 2014).

The anti-inflammatory actions of PEA, leading to a reduction of peripheral and central sensitization, are mediated by neuronal and non-neuronal cells. The latter comprise glia (in particular, astrocytes and microglia) as well as peripheral and central mast cells. In particular, mast cells in the CNS have been shown to play a pivotal role in inflammatory and neurodegenerative diseases. Emerging evidence suggests that the cross-talk between mast cells and glia has an important role in neuroinflammation, exacerbating the acute inflammatory response, accelerating neurodegenerative disease progression and promoting pain perception. In this context, PEA can function in maintaining cellular homeostasis, not only by inhibiting mast cell activation in the CNS and regulating microglial cell activity,

but also by blocking peripheral mast cell activation and hence signaling pathways from the periphery to the brain (Skaper et al., 2014). PEA is also able to attenuate the degree of peripheral inflammation in another animal model of peripheral nerve injury, the chronic constriction injury, which is associated to a profound local inflammatory response that involves T cells and macrophages (Uçeyler et al., 2010). After nervous system trauma, PEA reduces edema and macrophage infiltration (Kigerl et al., 2009), evaluated as CD86 positive cells (Di Cesare et al., 2013), responsible to produce high levels of oxidative metabolites (e.g., nitric oxide and superoxide) and proinflammatory cytokines (Ding at al., 1988). In addition to its known anti-inflammatory activity, PEA elicited analgesia in acute and inflammatory pain (Calignano et al., 1998; De Novellis et al., 2012). Evidence indicates that, in animal models of neuropathic pain, hyperalgesia and allodynia were characterized by an increase in classical endocannabinoids (AEA and 2-AG) in nuclei involved in descending nociceptive pathways, as well as other brainstem regions more involved in the emotional components of chronic pain, while PEA levels were significantly decreased (Petrosino et al., 2007). These results indicate not only a role for endocannabinoid elevation in lessening pain perception, but also an involvement of PEA, whose decrease may modulate pain threshold. The complex and generous profile of PEA activity may thus explain its broad potential in treating different disorders related to pain and inflammation.

Osteorthritis

Osteoarthritis (OA) is a chronic joint disease characterized by a progressive destruction of cartilage, resulting in pain and loss of articular function. The human population suffering from OA is approximately 15% (Johnson et al., 2014), with its prevalence projected to double by the year 2020 due largely to an ageing population and an ever-increasing prevalence of obesity (Lawrence et al., 2008). In Europe, 20% of chronic pain is related to OA (O'Brien et al., 2012) and pain is the main symptom. Furthermore, pain related to OA is considered as the prototypical chronic nociceptive pain condition, and this is used as a major clinical model for the development of new analgesics dedicated to treating chronic pain. Pain is a ubiquitous symptom in osteoarticular diseases, especially in OA,

much more prevalent than stiffness and disability. OA frequency is increasing, mostly related to age and obesity. OA pain has been considered as a prototypical nociceptive pain condition, and clinicians have expected that pain can be an alarm signal, correlating with the intensity of joint degradation (Claessens et al., 1990). It can be seen that OA pain is a complex phenomenon, involving peripheral and central mechanisms, modulated by many factors, including psychological (Edwards et al., 2006) and genetic factors (Thakur et al., 2013). The capsule, ligaments, meniscus, periosteum, and subchondral bone are largely innervated by a dense network of myelinic and amyelinic fibers. The synovium is mostly innervated by amyelinic fibers, although cartilage has no innervation. In the joint, there are four types of sensory organs, including type I and type II receptors are localized in the capsule, ligaments, and meniscus, but not in the synovium. These are mechanoreceptors, sensitive to pressure and traction, transmitting the message by myelinic fibers. Type III receptors, formed by thin Aδ myelinic fibers, are located on the ligament surface and they act as mechanoreceptors of high threshold, answering to strong mechanical stimuli and to a lower degree to thermal stimuli. Type IV receptors, also called polymodals, are formed by free terminals of unmyelinated C fibers, and they represent the most important type of joint receptors, in all structures except in the cartilage. They are normally not activated and are called polymodals as they are activated by mechanical, thermal, and chemical stimuli, in pathological conditions such as inflammation. Type III and IV receptors are involved in pain sensation induced by joint lesions (Mapp, 1995). They are also sensitized by increased intra-articular pressure and by chemical local changes. In OA, there are probably both peripheral and central mechanisms at different stages. In particular, peripheral mechanisms more in the early stage and central mechanisms more in the late and chronic stages (Arendt-Nielsen et al., 2010). Interactions between the central and peripheral systems suggest a general plasticity of the nociceptive system in OA pain (Imamura et al., 2008). According to that, the major goal of treatment is pain control with minimal adverse effects, maintenance or improvement of joint mobility and function, and improved health related quality of life. No single therapy is adequate, so the major clinical guidelines for disease management generally agree that therapy should involve a combination of non-pharmacologic and pharmacologic therapies. Non-pharmacologic modalities for osteoarthritis are quite diverse but broadly divided into educational and physical approaches. Educational approaches are based on lifestyle patterns changes (including diet and exercise) and joint protection

techniques as physical tools. Pharmacologic modalities recommended for the initial management of patients with osteoarthritis include acetaminophen (paracetamol), oral and topical non-steroidal anti-inflammatory drugs (NSAIDs), tramadol, and intra-articular corticosteroid injections, glucosamine, chondroitin sulphate and other nutritional supplements. Intra-articular hyaluronate injections, duloxetine, and opioids are conditionally recommended in patients with an inadequate response to initial therapy. Surgical procedures are advised in patients with a long course of disease and/ or untreatable pain or disability with non-surgical methods (Hochberg et al., 2012). However, these treatments still are insufficient to relieve pain and have severe side effects. Thus, there is still a need to investigate new effective drugs as non-surgical treatment of OA.

Diabetic neuropathy

Diabetes mellitus is a metabolic syndrome today affecting 382 million people (8.3% of adults). Recently, the International Diabetes Federation estimates that the number of people with the disease is set to rise beyond 592 million in less than 25 years (International Diabetes Federation, 2013). One of the major and most disabling long-term complications of diabetes is diabetic neuropathy, that is estimated to affect about 50% of patients. Diabetic neuropathy usually appears as distal symmetrical polyneuropathy, characterized by allodynia, paresthesia and hyperalgesia (Obrosova et al., 2009). The exact pathophysiological mechanisms of neuropathic pain in diabetes remain elusive although several mechanisms have been postulated (Tesfaye et al., 2005). Other potential mechanisms include the association of increased blood glucose instability in the genesis of neuropathic pain (Oyibo et al., 2002), an increase in peripheral nerve epineurial blood flow (Eaton et al., 2003), altered foot skin microcirculation (Quattrini et al., 2007), reduced intraepidermal nerve fiber density in the context of early neuropathy (Sorensen et al., 2006), increased thalamic vascularity (Selvarajah et al., 2011), and autonomic dysfunction (Gandhi et al., 2010). Chronic hyperglycemia seems to be the major culprit in the initiation of various metabolic events underlying diabetic neuropathy. Several studies suggested that insulin or C peptide deficiencies or both as such contribute to severe diabetic neuropathy.

Long-term hyperglycemia causes downstream metabolic cascades of polyol pathway hyperactivity, advanced glycation end-products (AGE)/receptor for AGE (RAGE) reactions and increased reactive oxygen species (ROS). They compromise both endoneurial microvessels and neural tissues themselves through activation of poly-ADP-ribose polymerase (PARP), alterations of protein kinase C (PKC) and an increase in mitogen-activated protein kinase (MAPK), as well as activation of nuclear factor-(NF)-jB, resulting in functional and structural changes of peripheral neuropathy. Metabolic aberrations in the nerve elicit pro-inflammatory reactions, inducing release of cytokines, suppression of neurotrophins and migration of macrophages, and promote the development of neuropathy. Recently, cellular factors derived from the bone marrow were found to produce chimeric cells in peripheral nerves of diabetic animals to elicit nerve injury. There is also the possibility that other cellular components from the bone marrow have an influence on the nerve pathology in diabetes. In addition, ischemia/reperfusion might also accelerate nerve injury, in part mediated by inflammatory reactions. Risk factors represented by hypertension, hyperlipidemia, smoking and insulin resistance are also important contributors to the development of neuropathy (Yagihashi et al., 2011) (**Fig. 2**).

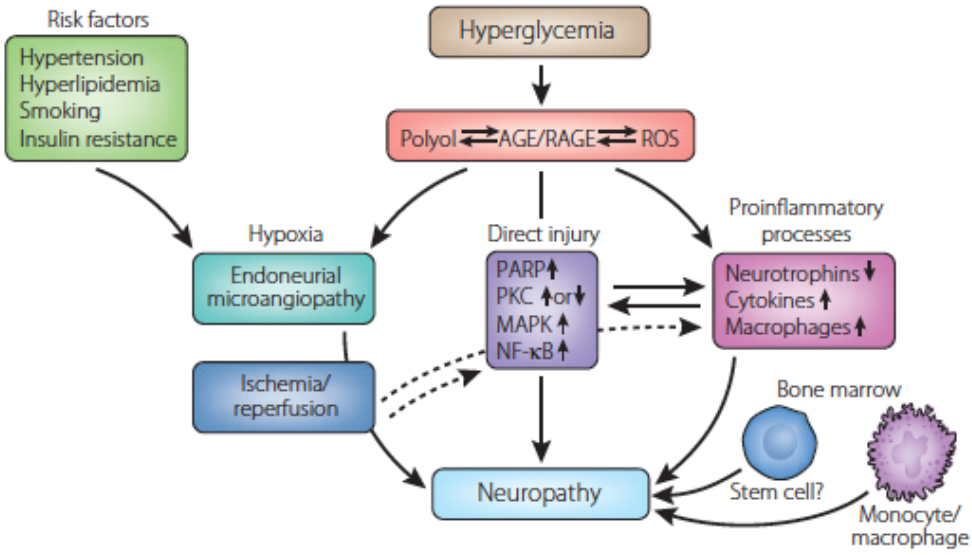

Fig. 2. Summry of pathogenic mechanism of diabetic neuropathy (Yagihashi et al., 2011).

Several pharmacological treatments have proven efficacy in the management of painful diabetic neuropathy, such as antidepressants, opioids, anticonvulsants and antioxidants, although only duloxetine and pregabalin are approved for the treatment of neuropathic pain in diabetes by both the Food and Drugs Administration of the U.S. and the European Medicines Agency Pharmacological treatment of painful diabetic neuropathy is not entirely satisfactory because currently available drugs are often ineffective and complicated by adverse events (Tesfaye et al., 2013).

Chemotherapy-induced neuropathic pain

Chemotherapy-induced neuropathic pain (CINP) is a very common type of neuropathic pain that represents a huge therapeutic problem. Platinum (Pt) analogues (i.e. cisplatin, oxaliplatin), taxanes (i.e. paclitaxel) vinca alkaloids (i.e. vincristine) and proteasome inhibitors (i.e. bortezomib) are the most common antineoplastic drugs successfully employed as first line treatment for several solid and blood cancers, including breast, lung, colorectal, gastric cancers and multiple myeloma. However, a common complication of chemotherapy is neurotoxicity that often manifests itself as peripheral neuropathy. Many cancer drugs can cause chemotherapy-induced peripheral neuropathy, and the incidence can be up to 90%. CINP is often the main reason for reduction or discontinuation of therapy, it may limit the employment of life-saving agents: symptoms are frequently disabling, they may affect patients' daily activities and severely impact on their quality of life (Carozzi et al., 2015). Paclitaxel is a microtubule-binding antineoplastic drug hat is able to bind the lumen of microtubules stabilizing the microtubule lattice and suppressing dynamic instability and depolymerisation. However, the treatment with paclitaxel affects the PNS and leads to neuropathic pain reducing intraepidermal nerve fibers (IENF), dorsal root ganglion neurons, sensory axons and myelin (Fehrenbacher, 2015). One of the most consistent findings for CIPN is reduced IENF density the latter being marked by axonal degeneration, mitochondrial alterations and vacuolar degeneration. It has been suggested that the mechanism of paclitaxel-induced neurotoxicity involves mitochondria. In fact, in several neurodegenerative conditions mitochondrial permeability changes have been observed. The opening of the mitochondrial permeability transition pore (mPTP) is followed by a loss of mitochondrial

membrane potential, increased generation of reactive ROS, a reduction in ATP level, Ca^{2+} release and mitochondrial swelling (Bernardi et al., 2006). Moreover, it has been demonstrated that TTX-sensitive Na^{+} channels play a very significant role in generating and maintaining paclitaxel-induced neuropathic pain (Nieto et al., 2008). In 2012, Materazzi and collaborators demonstrated that members of the TRP family of ion channels, such as TRPV4, TRPV1 and TRPA1 contribute to paclitaxel-induced mechanical and cold hypersensitivity and target at the production of oxidative stress (Materazzi et al.; 2012). Growing evidence suggests that various inflammation phenomena, including regulation of proinflammatory cytokines, macrophage accumulation, microglia activation, are involved in the development of neuropathic pain due to chronic treatment with paclitaxel (Cozzi et al.; 2015) (**Fig. 3**).

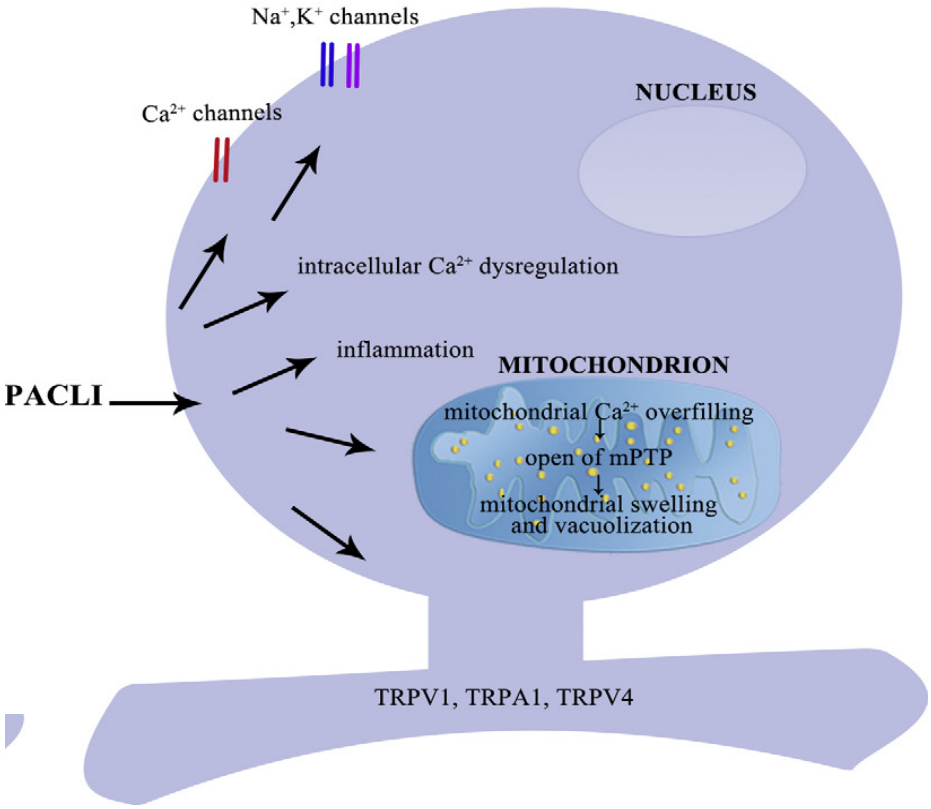

Fig. 3: Paclitaxel (PAC)-induced mechanisms of neurotoxicity (Cozzi et al., 2015).

Currently, no treatment options are available for the prevention of CINP, and only few pharmacological strategies exist for its treatment. Most analgesic drugs that

are already in use for the treatment of neuropathic pain, such as amitriptyline or gabapentin, have failed to alleviate CINP in randomized, placebo-controlled clinical trials (Rao et al., 2007; Kautio et al., 2009). According to this evidence, there is an urgent need to develop new effective treatment for this pathology.

Aims of the thesis

Neuropathic pain is a very common type of chronic pain affecting 6-8% of the population which occurs as a consequence of a lesion or disease to the somatosensory nervous system. Pharmacological management of neuropathic pain includes different medications such as NSAIDs, opioid analgesics, anticonvulsants, antidepressants, serotonin noradrenaline reuptake inhibitors, and cannabinoids. However, in many cases these treatments are associated with suboptimal therapeutic efficacies and/or side effect. Thus, there is an urgent need to develop new effective and safe treatments. Many evidences in the literature show the anti-inflammatory and antinociceptive effects of PEA, the parent molecule of ALIAmides (Autacoid Local Injury Antagonism Amides), a group of endogenous fatty acid derivatives sharing their pharmacological effects with the endocannabinoid family mainly through the down-modulation of local mast cell degranulation.

The general aim of this thesis is to further explore the therapeutic effects of PEA in three animal models of neuropathic pain associated to human diseases. Particularly, the specific aims are discussed as following:

Chapter 1: Pharmacological effects of PEA in osteoarthritis.

Chapter 2: Pharmacological effects of PEA in diabetic neuropathy.

Chapter 3: Pharmacological effects of PEA in paclitaxel model of chemotherapy-induced neuropathic pain.

Chapter 1. Pharmacological effects of PEA in osteoarthritis

Introduction

Osteoarthritis (OA) is a chronic joint disease characterized by a progressive destruction of cartilage, resulting in pain and loss of articular function. The human population suffering from OA is approximately 15% (Johnson et al., 2014), with its prevalence projected to double by the year 2020 due largely to an ageing population and an ever-increasing prevalence of obesity (Lawrence et al., 2008). Currently, acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) are still the class of medication most commonly used in OA treatment, but they are insufficient to relieve pain and have severe side effects. Thus, there is still a need to investigate new effective drugs as non-surgical treatment of OA. For this purpose, animal models are useful to effectively mimic the human pathology, such as the model obtained by single intra-articular injection of monosodium iodoacetate (MIA) in the infrapatellar ligament of the knee of rats. MIA injection, inhibiting glyceraldehyde-3-phosphate dehydrogenase activity in chondrocytes, results in disruption of glycolysis and eventual death of chondrocytes (Kalbhen et al., 1987; Guingamp et al., 1997). This process usually accompanies the initial inflammatory response, histologically known as expansion of synovial membrane, infiltration of macrophages, neutrophils, and lymphocytes. In the later phase, MIA injection into rat knee joint evokes not only inflammation and degenerative change, but also possible localized neuropathic component (Orita et al., 2011). Many evidence in the literature show the pharmacological effects of PEA, in different animal models of inflammation and pain. Considering the dual action of PEA both as anti-inflammatory and antinociceptive, we considered interesting to investigate the pharmacological effects of PEA also in MIA-model of OA. We demonstrated that the chronic oral administration of PEA reduced knee swelling, mechanical allodynia, thermal hyperalgesia, motor impairment and slowed the degradation of cartilage interposition. PEA efficacy was superimposable and in some cases greater than that evoked by nimesulide, and acetaminophen, two of the most widley prescribed drugs used for OA treatment, suggesting a therapeutic use of PEA in clinic.

Materials and Methods

Animals

All experiments performed were in accordance with Italian State and European regulations governing the care and treatment of laboratory animals. Experiments were conducted using male Wistar rats weighing 200-210 g (Harlan, Italy). Rats were housed in standard cages (3 rats/cages) in a climate- controlled environment (room temperature 22±1° C, humidity 60%). The rats were maintained on a 12-hour light/dark cycle and provided with food and water *ad libitum*.

Induction of OA

Knee osteoarthritis was induced by a single intrarticular injection of monosodium iodoacetate (MIA, Sigma-Aldrich, Italy) into the right knee joint of rats, previously anesthetized with an intraperitoneal administration of sodium pentobarbital (60 mg/kg). Briefly, each rat was positioned on their back and the right leg was flexed 90° at the knee. The patellar ligament was palpated below the patella and the injection was made into this region. Each rat received 2mg/25μl of MIA dissolved in sterile saline (0.9%). The control group received the same amount of saline in the right knee.

Drugs and treatments

PEA (Epitech Group s.r.l.), nimesulide and acetaminophen (Sigma-Aldrich, Italy) were dissolved in Pluronic® F-68 (Sigma-Aldrich, Italy), and used at doses of 50, 10, and 300 mg/kg respectively. OA rats were randomly divided in four groups receiving oraly drugs or vehicle once a day for three weeks, starting from the day after OA induction. The dose of PEA, nimesulide, and acetaminophen employed in this study was selected on the basis of our previous data, and in agreement of other studies demonstrating the ability of these compounds to attenuate established weight bearing deficities and allodynia (Sagar et al., 2011), and reduce both hyperalgesia and allodynia in the same animal model (Fernihough et al., 2004).

Assessment of knee swelling

Knee swelling was measured using a digital caliper (ROHS, Compliant Electronic Digital Caliper) on day 1, 3, and 6 at 60 min after drugs administration. Particularly, the knee swelling (edema) was evaluated as a difference (expressed in mm) between the ipsilateral and controlateral knee volume of each rats.

Assessment of thermal hyperalgesia

Thermal hyperalgesia was monitored on day 1, 3, and 6, 60 minutes after the drug treatment. Thermal hyperalgesia was tested according to the Hargreaves procedure (Haergraves et al., 1988) using the plantar test (Ugo Basile, Italy). Briefly, animals were placed in a clear plexiglass box and allowed to acclimatize. A constant intensity radiant heat source was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation until paw withdrawal was recorded.

Assessment of mechanical allodynia

Mechanical allodynia was monitored on day 1, 3, 6, 8, 15, and 21, 60 minutes after the drug administration. Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Particularly, animals were placed in a test cage with a wire mesh floor, and the tip of von Frey-type filament was applied to the middle of the plantar surface of the hind paw. The filament exerted an increasing force starting below the threshold of detection, and increased until the animal removed its paw. Withdrawal threshold was expressed as tolerance level in g. The cut off was set at 50 g in 20 s.

Assessment of Sciatic Functional Index (SFI)

The evaluation of functional nerve recovery was monitored on day 1(60 minutes after the drug treatment), 3, 6, 8, 15, 21, and 22, 24 h after the last administration, using the walking track analysis. Briefly, animal footprints were recorded in a wooden walking alley, an 8.2x42 cm corridor open. Hind limbs were stained with not toxic colours and the rat was allowed to walk down the track, leaving its footprints on normal paper. Recordings continued until five measurable footprints were collected. From the footprints, the following parameters were obtained: print length (PL, distance from the heel to the third toe); toe spread (TS, distance from the first to the fifth toe) and intermediate toe spread (ITS, distance from the second to the fourth toe). These parameters were fed into the equation developed

by de Medinaceli et al. (1982), and adapted by Bain et al. (1989) and Hare et al. (1992) to calculate the sciatic function index (SFI):

$$SFI=(-38.3xPLF)+(109.5xTSF)+(13.3xITF)-8.8$$

in which PLF=(EPL-NPL)/NPL; TSF=(ETS-NTS)/NTS; and ITF=(EIT-NIT)/NIT. Values close to zero indicate a normal function while values toward -100 are associated to total impairment (Dijkstra et al. 2000).

Tissue collection

Animals were sacrificed on day 22 (24 h after the last drug administration), and the synovial fluids from each knee were collected. Briefly, a small incision was made above the patella of right knees of all rat groups and synovial fluid lavages were obtained by intra-articular injection of 1 ml phosphate buffered saline (PBS) into the MIA-injected knees after repeated joint flexing (4x). Synovial lavages were centrifuged at 2,000 rpm for 10 min (4°C) to remove non specific residues. Supernatants were frozen at -80°C until assayed for the evaluation of nerve growth factor (NGF), and matrix metalloproteinase-3 (MMP-3) levels. After the synovial fluid collection, the right joint was immediately disarticulated and fixed in 10% neutral buffered formalin for 24 h. After fixation, an image of the tibial cartilage plateau was captured using an image analysis system (Motic Images Plus 2.0 ML) in order to evaluate the articular cartilage condition.

NGF assay

Nerve growth factor (NGF) levels were determined as following described. Synovial fluids were diluted in 5-fold with Dulbecco's PBS buffer. Samples were acidified to pH < 3.0 by adding 1 N HCl, and then neutralized with 1 N NaOH to pH 7.6. Samples were then centrifuged 10000 g at 4°C for 15 min and the resulting supernatants used to determine NGF protein levels using ELISA kit according to the manufacturer's instructions (Promega, USA). The absorbance at 450 nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). NGF levels were determined by interpolation with standard curves assayed on individual plates, and expressed as pg NGF/mL synovial fluid.

Assessment of articular cartilage damage

In order to evaluate the articular cartilage condition, the tibial plateau was used for image analysis because it provided a relatively flat surface compared with the femoral condyles, allowing the image analysis camera to focus on the entire cartilage surface. Three independent observers assessed cartilage damage in a blinded manner using a scale of 0–4 of increasing severity. Particularly, macroscopic lesions were graded as follows: 0 = normal appearance; 1 = slight yellowish discoloration of the chondral surface; 2 = little cartilage erosions in load-bearing areas; 3 = large erosions extending down to the subchondral bone; and 4 = large erosions with large areas of subchondral bone exposure (Guincamp et al., 1997; Janusz et al., 2001).

MMP-3 assay

Matrix metalloproteinase-3 (MMP-3) levels were measured in the synovial fluid using ELISA kit according to the manufacturer's instructions (Cusabio, USA). The absorbance at 450 nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). MMP-3 levels were determined by interpolation with standard curves assayed on individual plates, normalized to protein content in each tissue sample and expressed as difference of pg MMP-3/mg total protein in the synovial fluid collected from the ipsilateral and contralateral knee.

Data Analysis and Statistical Procedures

All data are expressed as the mean ± S.E.M. and analyzed using one-way ANOVA followed by Tukey's or Dunnett's posthoc test for multiple comparison. Cartilage damage scores were compared using the Kruskal-Wallis post-hoc test. Differences were considered significant at $P<0.05$. All statistical analyses were done using the statistical GraphPad Software package (San Diego, CA, USA).

Results and Discussion

Osteoarthritis (OA) is the most common form of joint disease characterized by degeneration of articular cartilage affecting approximately 15% of people around the world. The loss of cartilage may affect the shape of the compromised joint and cause stiffness reduced range of motion. Although, the major symptom of OA is chronic joint pain which has a significant effect on patients' quality of life. Several animal models of OA have been described such as the model obtained by single intra-articular injection of monosodium iodoacetate (MIA) in the infrapatellar ligament of the knee of rats. MIA injection, inhibiting glyceraldehyde-3-phosphate dehydrogenase activity in chondrocytes, results in disruption of glycolysis and eventual death of chondrocytes (Kalbhen et al., 1987; Guingamp et al., 1997). This process usually accompanies the initial inflammatory response, histologically known as expansion of synovial membrane, infiltration of macrophages, neutrophils, and lymphocytes. In the later phase, MIA injection into rat knee joint evokes not only inflammation and degenerative change, but also localized neuropathic component (Orita et al., 2011; Ivanavicius et al., 2007; Thakur et al., 2012). In our study we employed this model, which is highly reproducible and mimics OA pain in humans (Combe et al., 2004) to evaluate the effect of oral chronic administration of PEA (50 mg/kg p.o.) on the development of clinical and pathological manifestations of OA. Furthermore, in order to access the therapeutic impact of PEA, its effect was compared with that induced by the recommended drugs in the management of patients with OA, such as nimesulide (10 mg/kg p.o.), and acetaminophen (300 mg/kg p.o.). During the first week, the knee swelling was evaluated as a index of inflammation. In particular, the knee swelling (edema) was measured as a difference (expressed in mm) between the ipsilateral and controlateral knee volume of each rat. In **Fig. 1A**, the values of edema at 60 min after the first (T1), the third (T3), and the sixth (T6) oral administration of drugs is shown. Already after one oral administration (T1), all drugs reduced edema even if no statistical difference was observed. Particularly, PEA treatment inhibited edema of 26.6%, nimesulide of 40.6% and acetaminophen of 35.2%. Instead, three days after MIA-injection (T3), PEA significantly reduced knee swelling of about 73% (Fig. 1A). The same result was obtained after nimesulide (55% of edema inhibition) and acetaminophen (58% of edema inhibition) administration (**Fig. 1A**). Six days after MIA-injection (T6), all treatments preserved the ability to significantly reduce edema. Particularly, PEA treatment inhibited edema of 72.6%, nimesulide of 57.9% and acetaminophen of

73.1%. According to Bove and colleagues (2003), MIA-model is characterized by an early acute inflammatory phase resulting from a fluid expansion of the synovial membrane, and persisting for about one week after MIA-injection. Starting from this evidence, in order to evaluate the anti-inflammatory efficacy of PEA, nimesulide and acetaminophen treatment in this whole period, we determined for each animal the area under the curve (AUC, edema-time). All drugs significantly reduced AUC edema-time (**Fig. 1B**) with similar degree. Oral treatment with PEA was found to inhibit edema in the OA rats, as well as the most widely prescribed drugs.

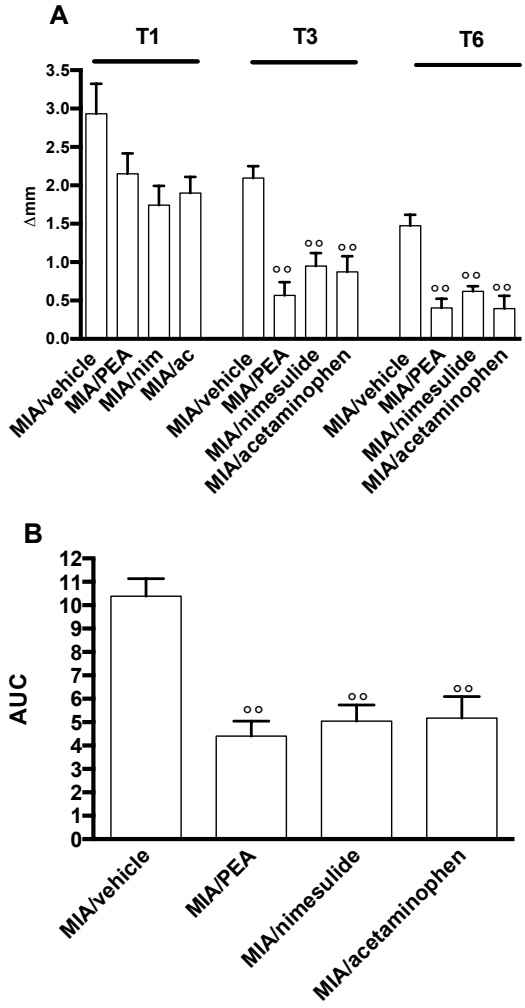

Fig. 1. Effect of palmitoylethanolamide (PEA) (50 mg/kg), nimesulide (10 mg/kg), and acetaminophen (300 mg/kg) administration (p.o.) to OA rats on knee swelling, on day 1, 3, 6, 60 min after the drugs administration

(A). The Area Under Curve (AUC edema-time) shows the anti-inflammatory efficacy of PEA, nimesulide and acetaminophen treatments one week after monosodium iodoacetate (MIA) injection (**B**). Knee swelling (edema) was evaluated as a difference (expressed in mm) between the ipsilateral and controlateral knee volume of each rat and data represent mean±S.E.M. of 6-10 rats. °°P<0.01 vs MIA/vehicle by One-way ANOVA followed by Dunnett's test.

Additionally, the peripheral nerve endings are sensitized by inflammatory mediators resulting in primary hyperalgesia. To evaluate the effect of PEA treatment on this type of pain, we accessed the development of thermal hyperalgesia. The day after MIA-injection, rats developed a significant decrease in thermal withdrawal latency of the ipsilateral paw, as compared to control animals (T1 pre-drug, **Fig. 2A**). Thermal hyperalgesia was stable for one week after MIA-injection and then disappeared in the following days (data not shown). The time course of the effect elicited by the three drugs at 60 minutes after their administration is shown in Fig. 2A. Treatment of MIA rats with a PEA (50 mg/kg, p.o.), nimesulide (10 mg/kg, p.o.), and acetaminophen (300 mg/kg, p.o.) resulted in a significant relief of thermal hyperalgesia. Particularly, PEA abolished thermal hyperalgesia already 60 min after the first single administration, and this effect remained unchanged during the following evaluations. On the other hand, at the same time point, a single administration of nimesulide or acetaminophen evoked only a partial relief of thermal hyperalgesia and this effect persisted during the following time points (Fig. 2A). The prolonged treatments with all three drugs did not affect the response to thermal stimuli of the contralateral paw (data not shown). These data show that PEA treatment was able to counteract inflammatory pain in OA rats slightly better than nimesulide, and acetaminophen.

By day 7, inflammation within the synovium and surrounding tissue has largely resolved and usually a sensitization of dorsal horn of the spinal cord occurs, resulting in persistent pain of neuropathic origin assessable as mechanical allodynia. As expected, after MIA-injection rats developed an exacerbated response to normally innocuous mechanical stimulation with a von Frey filament, that is stable for 21 days after MIA-injection (**Fig. 2B**). The time course of the effect elicited by PEA, nimesulide and acetaminophen at 60 minutes after their administration is shown in Fig. 2B. Treatment of OA rats with PEA (50 mg/kg, p.o.), nimesulide (10 mg/kg, p.o.), and acetaminophen (300 mg/kg p.o.) resulted in a significant, even if partial, relief of mechanical allodynia (Fig. 2B). Particularly, one administration of both PEA and nimesulide evoked a partial anti-allodynic effect (Fig. 2B). On the other hand, the acetaminophen treatment

significantly partially attenuated mechanical allodynia only starting from 60 min after third administration. The prolonged treatments with all three drugs did not affect the response to mechanical stimuli of the contralateral paw (data not shown). These findings show that PEA, nimesulide, and acetaminophen are able to control the neuropathic component of osteoarthritic pain. In fact, all drugs elicited similar effects during the first week after MIA when the contribution of inflammatory mediators was essential. However, during the subsequent two weeks, PEA is able to induce a relief of allodynia better than the other drugs.

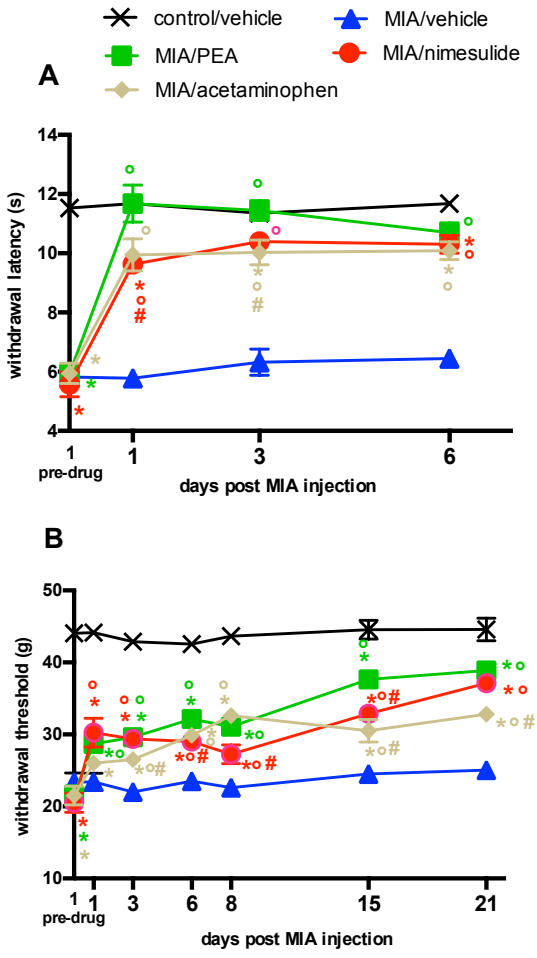

Fig. 2. Effect of palmitoylethanolamide (PEA) (50 mg/kg), nimesulide (10 mg/kg), and acetaminophen (300 mg/kg) administration (p.o.) to OA rats on thermal hyperalgesia,on day 1, 3, 6, pre-drugs administration and 60 min after drugs administration (**A**). Effect of palmitoylethanolamide (PEA), nimesulide, and acetaminophen administration (p.o.) to OA rats on mechanical allodynia, on day 1, 3, 6, 8, 15, and 21, pre-drugs administration and 60 minutes after drugs administration (**B**). Thermal threshold of the paws is

expressed as s and mechanical allodynia is expressed as g. The data represent mean ± S.E.M. of 6-10 rats. *P<0.001 vs non control/vehicle; °P<0.001 vs MIA/vehicle; #P<0.05 vs MIA/PEA by One-way ANOVA followed by Tukey's test.

These results are also supported by the evaluation of the nerve growth factor (NGF) level, a pro-algogen marker, in the synovial fluid of MIA-treated rats. Many studies report that NGF is elevated in a variety of pain conditions, including OA, and it has been implicated in the development of peripheral sensitization (McKelvey et al.; Visser at al.; 1999). Moreover, NGF is overexpressed at the osteochondral junction in individuals with OA, leading to disorganized innervation of previously aneural cartilage and peripheral sensitization (Walsh et al.; 2010). As expected, MIA-injected rats showed higher NGF levels compared to control group, on day 22 post lesion (**Fig. 3**). Repeated administration of PEA restored the physiological NGF level. On the other hand, acetaminophen treatment partially restored NGF levels, while nimesulide treatment had no effect (Fig. 3). These foundings allow us to think that PEA evokes its antiallodynic effect mainly through the control of NGF level that plays an important role in the development, and maintenance OA pain.

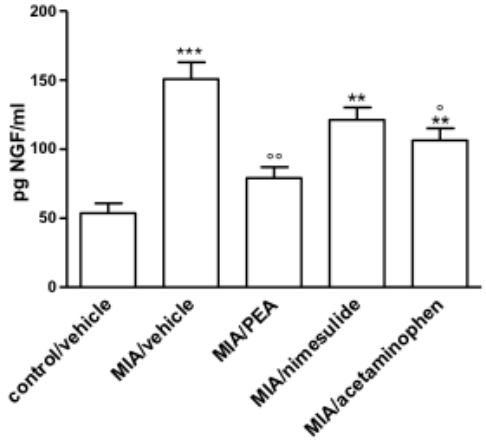

Fig. 3. Effect of palmitoylethanolamide (PEA) (50 mg/kg), nimesulide (10 mg/kg), and acetaminophen (300 mg/kg) p.o. administrated daily to OA rats for 21 days after monosodium iodoacetate (MIA) injection, on NGF levels in the synovial fluids, on day 22 after MIA injection, 24 h after the last drugs administration. NGF levels expressed as pg/ml of synovial fluid. The data represent mean ± S.E.M. of 6-10 rats. ***P<0.001, **P>0.01 vs control/vehicle; °°P<0.01, °P<0.05 vs MIA/vehicle by One-way ANOVA followed by Dunnett's test.

We also verified that local MIA injection produced a compromised walking pattern associated to its toxic effect on chondrocytes. In fact, chondrocytic cell death induces a subchondral bone lesion in MIA model, consistent with the development of subchondral bone lesions, knee pain, and locomotor impairment in human OA (Felson at al., 2001; Wluka et al., 2004). Walking track analysis was used to check locomotor activity in MIA rats.

In **Fig. 4A**, representative footprints of control or MIA-treated rats on day 22 from MIA injection were shown. For each box, on the left there are contralateral footprints , and on the right there are ipsilateral footprints of injected knee (with saline in control rats or with MIA in OA rats). As expected, MIA-injected rats (Fig. 4A, box 2) showed a compromised walking pattern compared to control rats (Fig. 4A, box 1) that is indicative of a locomotor impairment. MIA-injected rats treated with PEA and nimesulide showed right normal footprints, similar to the control group ones (Fig. 4A, box 3 and 4). On the other hand, the right footprint of acetaminophen-treated group was not so clear as well as the MIA group one (Fig. 4A, box 5). Starting from footprints, the so-called SFI, sciatic function index parameter, was calculated (**Fig. 4B**). According to the above footprints, SFI values were approximately around zero in all control rats, indicating a normal locomotor function (Fig. 4B). As expected, intra-articular injection of MIA resulted in a significant increase of joint discomfort already from day 1 post-injection, as shown by a marked drop of the SFI value of walking track analysis towards -100 compared with control group, indicative of a significant impairment of locomotor function. In the following days, such an impairment tended to diminish, even if a statistical difference was preserved at every time point (Fig. 4B). PEA and nimesulide treatment completely restored locomotor impairment already after one administration and this effect remained stable for the following one week, when the contribution of inflammatory mediators was essential. Likewise, acetaminophen treatment induced a total relief of motor impairment starting from day 3 to day 8 after MIA injection. Conversely, during the subsequent two weeks, until day 22 from MIA injection, only PEA treatment preserved such an effect. We can conclude that chronic PEA administration has resulted in a recovery of locomotor functionality not only because of its anti-inflammatory and antinociceptive effect, but also due to its chondroprotective effect.

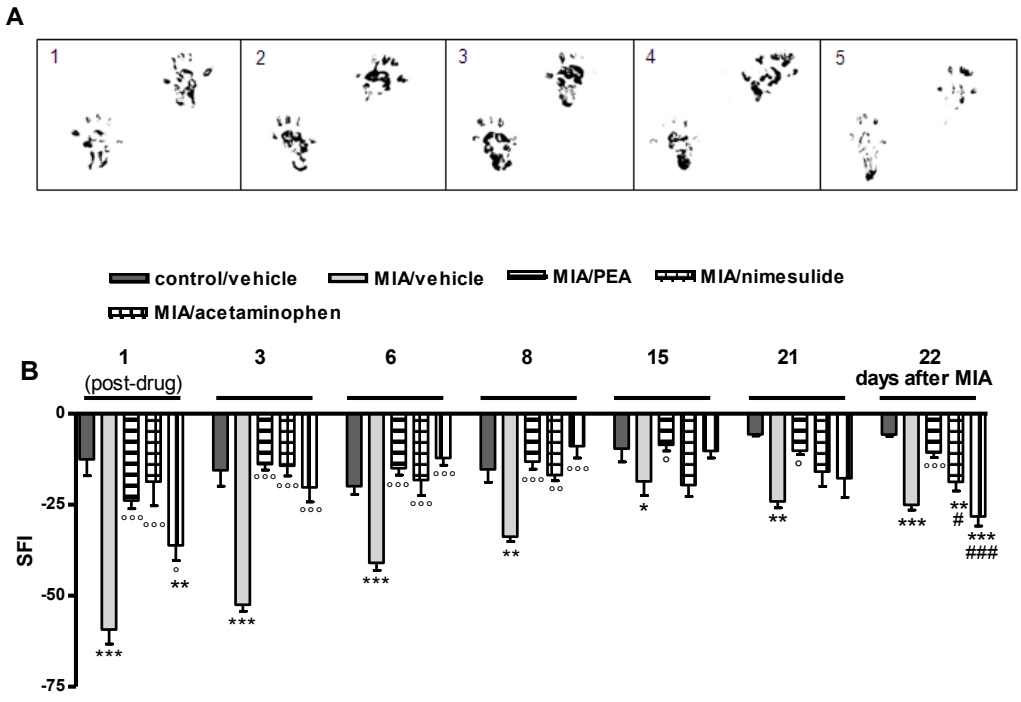

Fig. 4. Rapresentative rats footprints of the following experimental groups: (1) control/vehicle; (2) MIA/vehicle; (3) MIA/PEA; (4) MIA/nimesulide; (5) MIA/acetaminophen, on day 22 after MIA injection, 24 h after the last drugs administration (**A**). Effect of palmitoylethanolamide (PEA) (50 mg/kg), nimesulide (10 mg/kg), and acetaminophen (300 mg/kg) administration (p.o.) to OA rats on functional nerve recovery, on day 1 (60 minutes after the drug treatment), 3, 6, 8, 15, 21, and 22, 24 h after the last drugs administration **(B)**. The locomotor functionality was expressed as Sciatic Funcional Index (SFI). SFI values close to zero indicate a normal function while values toward -100 are associated to total impairment. The data represent mean ± S.E.M. of 6-10 rats. ***P<0.001, **P>0.01, *P<0.05 vs control/vehicle; °°°P<0.001, °P<0.05 vs MIA/vehicle; ###P<0.001, #P<0.05 vs MIA/PEA by One-way ANOVA followed by Tukey's test.

In order to verify whether the chondroprotective effect of PEA can also results in reduction of cartilage damage after MIA injection, a macroscopic analysis of knee joint was performed. Representative images of tibial cartilage (tibial plateau) of control, and treated-MIA rats on day 22 post lesion were shown in **Fig. 5A**. Cartilage damage was assessed from images captured using an image analyser by three independent observers using a scale from 0 to 4 of increasing severity (0=normal; 4=maximum severity). As expected, a mild/moderate cartilage damage was observed after MIA injection in comparison with control rats. Particularly, MIA injection provoked lesions affecting the whole of the articular surface, with large chondral erosions and subchondral bone exposure. Repeated PEA treatment preserves cartilage from damage, conversely to repeated

nimesulide, and acetaminophen treatment that induced severe erosions with large areas of subcondral bone exposure. The grade of cartilage erosion was shown in **Fig. 5B**.

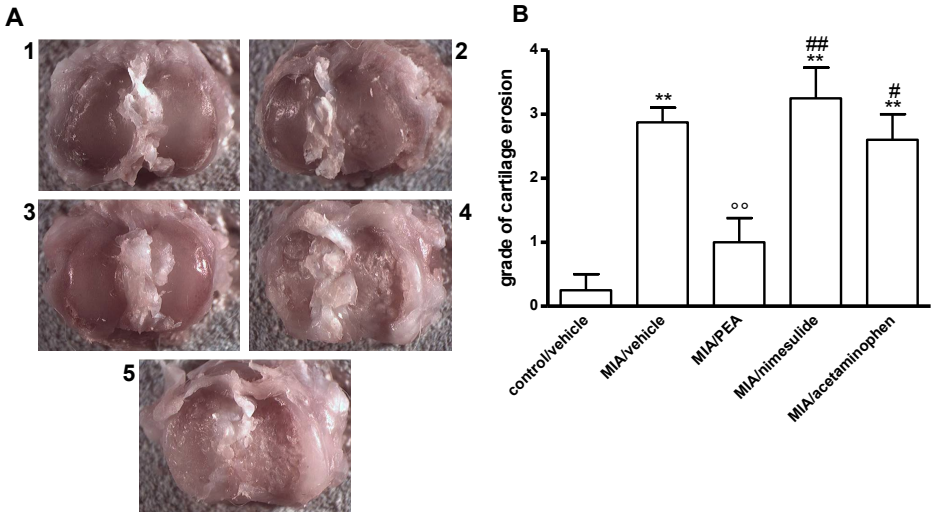

Fig. 5. Rapresentative rats tibial plateau of the following experimental groups: (1) control/vehicle; (2) MIA/vehicle; (3) MIA/PEA; (4) MIA/nimesulide; (5) MIA/acetaminophen, on day 22 after MIA injection, 24 h after the last drugs administration (**A**). Effect of palmitoylethanolamide (PEA) (50 mg/kg), nimesulide (10 mg/kg), and acetaminophen (300 mg/kg) p.o. administrated daily to OA rats for 21 days after monosodium iodoacetate (MIA) injection, on articular cartilage damage, on day 22 after MIA injection **(B)**. Macroscopic lesions were graded as follows: 0 = normal appearance; 1 = slight yellowish discoloration of the chondral surface; 2 = little cartilage erosions in load-bearing areas; 3 = large erosions extending down to the subchondral bone; and 4 = large erosions with large areas of subchondral bone exposure. The data represent mean±S.E.M. of 6-10 rats. **P>0.01, *P<0.05 vs control/vehicle; °°P<0.01 vs MIA/vehicle; ##P<0.01, #P<0.05 vs MIA/PEA by One-way ANOVA followed by Kruskal-Wallis' test.

These results were also confirmed by the evaluation of MMP-3 level in the synovial fluid of MIA-treated rats. MMP-3 is one of catabolic genes that are up-regulated in OA disease through the over-activation of Nf-kB and MAPK pathways in chondrocytes (Goldring et al., 2011b). However, MMPs production, such as MMP-3, is also induced by the activation of macrophages (Blom et al., 2007), fibroblas-like synoviocytes (Sokolove et al., 2013), and fibronectin fragments derived from extracellular matrix degradation (Homandberg G et al. 1996). Accordigly, MIA-injection induced an increase of MMP-3 level in the synovial fluid on day 22 after OA induction (**Fig. 6**). Only chronic treatment with PEA was able to completely restore the physiologic MMP-3 level, while

acetaminophen has had just parial effect. On the other hand, nimesulide administration has elicited no effect on this biomarker. It is important to underline that MMP-3 can be considerated as a prognostic biomarker, according to the BIPED classification system (Burden of disease, Investigative, Prognostic, Efficacy of intervention and Diagnostic), because it is able to take over changes in tissue metabolism and predict benefits from long-term drug treatment (Rousseau JC et al. 2012). Thus, our fundings suggest that a chronic treatment with PEA can exert a protection effect on cartilage damage caused by osteoarthritis.

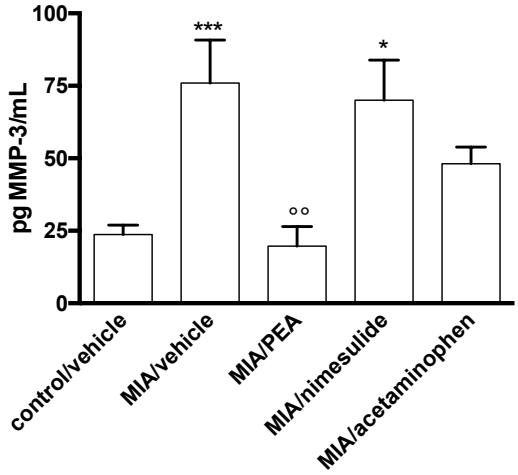

Fig. 6. Effect of palmitoylethanolamide (PEA) (50 mg/kg), nimesulide (10 mg/kg), and acetaminophen (300 mg/kg) p.o. administrated daily to OA rats for 21 days after monosodium iodoacetate (MIA) injection, on MMP-3 levels in the synovial fluids, on day 22 after MIA injection, 24 h after the last drugs administration. MMP-3 levels were expressed as difference of pg MMP-3/mg total protein in the synovial fluid collected from the ipsilateral and contralateral knee. The data represent mean±S.E.M. of 6-10 rats. ***P<0.001, **P>0.01, *P<0.05 vs control/vehicle by One-way ANOVA followed by Dunnett's test.

According to these results, we hypotized a mechanism of action of PEA in OA desease. PEA was shown to act directly on mast cells, via an ALIA (Autacoid Local Injury Antagonism) mechanism (Aloe et al., 1993) and mast cells degranulation triggers deleterious effects in many tissues, where the mast cells reside or are recruited. To date, it is widely recognized that the main PEA pharmacological effects are mediated by activation of peroxisome proliferator-activated receptor (PPAR)-α, an anubiquitous transcription factor (Lo Verme et al., 2006). Thus, PEA could bind PPAR- α receptor espressed by immun cells, neurons and microglia, switching off the nuclear factor-κB (NF-κB) signaling cascade, a key element in the transcription of genes, leading to the synthesis of

pro-inflammatory and pro-algogen mediators (D'Agostino et al., 2009). Moreover, PEA may exert a protection role on cartilage damage reducing the MMP-3 levels in the synovial fluid through the down modulation of NF-κB pathway also in chondrocyes.

In conclusion, for the first time we demonstrated the pharmacological effects of PEA in MIA model of OA. Particularly, orally administration of PEA 50mg/kg for 21 consecutive days had anti-inflammatory and antinociceptive efficacy. Moreover, PEA was able to restore motor function, and slow the erosion of the cartilage interposition. This is an important result considering the absence of a curative therapy for this disease.

These findings highlight the therapeutic potenciality of PEA and allow us to propose PEA as a valid alternative for the treatment of human OA, compared to nimesulide, and acetaminophen, two of the most common drugs used in clinic.

Chapter 2. Pharmacological effects of PEA in diabetic neuropathy

Introduction

Diabetes mellitus is a metabolic syndrome today affecting 382 million people (8.3% of adults). Recently, the International Diabetes Federation estimates that the number of people with the disease is set to rise beyond 592 million in less than 25 years (International Diabetes Federation, 2013). One of the major and most disabling long-term complications of diabetes is diabetic neuropathy, that is estimated to affect about 50% of patients. Diabetic neuropathy usually appears as distal symmetrical polyneuropathy, characterized by allodynia, paresthesia and hyperalgesia (Obrosova et al., 2009). Antidepressants, opioids, anticonvulsants and antioxidants represent the current treatment regimen of this complication, even if these therapies relieve pain only in a very few number of patients and their side effects limit their use (Smith et al., 2011). It has been already showed that PEA relieved thermal hyperalgesia and mechanical allodynia in a mouse model of neuropathic pain due to the chronic constriction injury of the sciatic nerve (Costa et al., 2008). The exogenous PEA administration has been reported to evoke antinociceptive effects in different animal models of pain such as spinal cord injury (Genovese et al., 2008), carrageenan-induced acute inflammation (D'Agostino et al., 2009), and complete Freund's adjuvant-induced chronic inflammation (Lo Verme et al., 2006). The ability of PEA to act as pain killer has also been reported in humans, during chronic lumbosciatalgia (Jack et al., 1996), and chronic pelvic pain conditions (Calabrò et al., 2010; Indraccolo set al., 2010). In addition, it has been already shown that PEA levels increase in the paw skin of mice with streptozotocin-induced diabetic neuropathy (Darmiani et al., 2005), thus suggesting a protective role of PEA in opposing to the severity of disease and its progression. Based on these evidences, we want to further explore the therapeutic potentiality of PEA in resolving painful states through the assessment of PEA antinociceptive effectiveness during diabetic neuropathy, too. The present study shows a strong therapeutic effect of PEA in relieving diabetes-induced neuropathic pain following a three-day repeated treatment, so confirming the beneficial effect of such a molecule for the relief of this type of chronic pain. In

order to evaluate whether the relief of diabetic neuropathy was associated with an improvement of diabetes per se, serum glucose and insulin levels were assessed. Unexpectedly, PEA treatment ameliorated the decrease in insulin preserving pancreatic islet morphology, probably through a mechanism involving mast cell downregulation.

Materials and Methods

Animals

All experiments performed were in accordance with Italian and European regulations governing care and treatment of laboratory animals (Permission # 41/2007B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain (Zimmerman et al., 1983). Experiments were conducted using male C57BL/6J mice 9 weeks years old (25-30 g) (Harlan, Italy), housed under controlled illumination (12h light/12h dark cycle) and standard environmental conditions (room temperature 22±1°C, humidity 60±10%) and allowed to acclimatise for at least one week before experimental use. Standard food and water was available *ad libitum*. All efforts were made to reduce both animal numbers and suffering during the experiments. All behavioural evaluations were performed by experimenters blind to treatments.

Induction of Diabetes

Type 1 diabetes was induced in overnight fasted mice by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma, Italy) at 120 mg/kg, freshly prepared in citrate buffer 0.1 M pH 4.5. This procedure ameliorates the absorption of the drug. Blood glucose concentration (Lifescan One Touch Ultra glucose meter, Milan, Italy) was assessed after the mice were fasted for 4-6 h, one week later on a sample of blood obtained from a tail prick to verify diabetes establishment. Only mice with a blood glucose levels above 250 mg/dL were selected for experiments. Control mice received an i.p. injection of citrate buffer. Blood glucose level was monitored over the whole period of the experimental study (days 14, 17 and 21).

Drugs and Treatments

PEA (Epitech, Saccolongo, Italy) was dissolved in a mixture of 10 % ethanol and 90 % saline, and used at the doses of 0.1, 1, 3 and 10 mg/kg. Diabetic mice were randomly divided in two groups receiving intraperitoneally the compound or its vehicle, once a day for 3 days, starting from the 14th day after the diabetes induction. Control mice received drug vehicle. The treatment with the highest dose (10 mg/kg) was prolonged for other 4 consecutive days. In order to characterize the role of different receptors in the PEA-induced effect, the ability of specific cannabinoid CB_1, CB_2 receptors, transient receptor potential channel of the vanilloid type 1 (TRPV1), peroxisome proliferator-activated receptors (PPAR-α, and PPAR-γ) antagonists to reverse the anti-allodynic effect of PEA was evaluated. Particularly, the CB_1 receptor antagonist SR141716 (1 mg/kg i.p.), the CB_2 receptor antagonist SR144528 (1 mg/kg i.p.), the TRPV1 antagonist capsazepine (5 mg/kg i.p.), the PPAR-α receptor antagonist GW6471 (1 mg/kg i.p.) or the PPAR-γ antagonist GW9662 (1 mg/kg i.p.) were employed. The doses of antagonists employed in this study were selected on the basis of our previous data (Costa et al., 2008) and in agreement of other studies demonstrating the ability of each compound to act in vivo as a selective receptor antagonist: for GW9662 (Collin et al., 2004), for GW6471 (Caprioli et al., 2012), for SR141716 (Hanus et al., 1999), for SR144528 (Hanus et al., 1999) and for capsazepine (Di Marzo et al., 2001). Every day mice received the i.p. injection of the antagonist (or its vehicle) followed 10 min later by PEA 10 mg/kg, as described in Table 1. Non diabetic animals received the vehicles of drugs. SR141716 and SR144528 were kindly supplied by Sanofi-Aventis (Montpellier, France) and were dissolved in a mixture of Tween80: DMSO: distilled water (1:2:7). Capsazepine, GW6471 and GW9662 were purchased from Sigma-Aldrich (Milano, Italy). Capsazepine was dissolved in 1: 9 mixture of DMSO: saline, while GW6471 and GW9662 in a 1:1:8 mixture of ethanol: Tween80: saline.

Assessment of Mechanical Allodynia

The withdrawal latency to von Frey filament was recorded before STZ injection, on day 14 (before starting the treatment and at 30, 60, 90, 120 min after a single PEA administration) and on days 15, 16, 17 and 21, 24 h after the last administration of the compound. In the antagonism studies, mechanical threshold was measured on day 17, 24 h after the last co-administration of compounds. Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) was employed to

asses mechanical allodynia as previously described. Withdrawal threshold was expressed as tolerance level in g. The cut off was set at 5 g in 20 s.

Insulin Assay

Insulin levels were determined in the serum (10 µl) by a sandwich enzyme-linked immunosorbent assay (ELISA) using an ELISA kit according to the manufacturer's instructions (Mercodia, Sweden). The absorbance was recorded at 450 nm on a microplate reader (Multiskan® EX, ThermolabSystem); standard curves were assayed on individual plates and used to interpolate insulin values in order to obtain insulin levels expressed as µg/l.

NGF Assay

Sciatic nerves proximal to the trifurcation were homogenized in a cold lysis buffer (200 µl). The homogenates were centrifuged at 4500 g at 4°C for 10 min, and father processed as previously described. NGF levels were determined by interpolation with standard curves assayed on individual plates, normalized to protein content in each tissue sample and expressed as pg NGF/mg protein.

Histology of Mouse Pancreas

Pancreas was fixed in 4% paraformaldehyde fresh solution (in 0.1 M phosphate buffer, pH 7.4) overnight at 4°C. Following dehydration in serial alcohol concentrations, it was embedded in paraffin wax. Pancreas longitudinal sections (6 µm) were cut with a rotary microtome. Mayer's haematoxylin and eosin (H&E) was employed to stain pancreas longitudinal sections to determine both the total number and the area of Langerhans Islets. About 60 sections were considered to determine the total number of Langerhans islets and about 20 Langerhans islets to evaluate the area (µm2) (n=3 for each experimental group) (Zeiss Axioplan MC 100 microscope plus AxioVision Rel 4.6 software). A colour digital camera (AxioCam MRc 5, Zeiss) captured the images and the analysis were performed by an experimenter blind to pharmacological treatment. The pancreas was also employed for the examination of the insulitis. A total of 20 islets from each mouse were examined and classified as follows: 0, intact islet; I, accumulation of mononuclear cells only at the ductal pole of the islet; II, periinsulitis, infiltration of mononuclear cells only at the periphery of the islet; III, insulitis, lymphocytic infiltrate invading the islets; IV, severe insulitis, massive infiltration of mononuclear cells throughout the islet with small, retracted islets, as reported by others (Baik et al., 1999; Kim et al., 2002). Some sections were assayed for mast

cells determination. Briefly, sections were stained with 0.1% toluidine blue. Intact dark bluestained cells represented resting mast cells. Degranulating mast cells were identified as cells from which some granules have been extruded, but cell outline retained largely intact. Mast cells exhibiting extensive and widespread degranulation, were classified as degranulated.

Data Analysis and Statistical Procedures

All data are expressed as the mean ± S.E.M. and analyzed using one-way ANOVA followed by Tukey's posthoc test for multiple comparison. Student's t-test was used to compare two groups. Differences were considered significant at $P<0.05$. All statistical analyses, including linear regression analysis were done using the statistical GraphPad Software package (San Diego, CA, USA).

Results and Discussion

In diabetes, the chronic hyperglycemia and associated complications affecting peripheral nerves are one of the most commonly occurring complications with an overall prevalence of 50-60%. Among the complications of diabetes, diabetic neuropathy is the most painful and disabling, complication affecting the quality of life in patients. Human diabetic neuropathy is characterized by spontaneous pain, allodynia and alteration in thermal perception. These behavioral signs are shared by mice submitted to streptozotocin-induced diabetic neuropathy a widely employed animal model of diabetic neuropathy (Courteix et al., 1993). For this reason in our study we employed this model, which mimics the autoimmune type 1 diabetes mellitus since STZ destroyed pancreatic β-cells. STZ-injected mice developed mechanical allodynia as showed by significant reductions in paw withdrawal thresholds to von Frey filament two week s after STZ administration (1.998±0.1849 g diabetic mice versus 4.975±0.025 g non diabetic mice). At this time point diabetic mice received a single dose of PEA (10 mg/kg, i.p.) or its vehicle and the behavioural response to von Frey filament was assessed at different time points post-injection (**Fig. 1**). The single dose of PEA evoked a significant anti-allodynic effect that appeared 30 min after the administration, peaked at 60 min and rapidly disappeared (**Fig. 1**). Thus, PEA showed a

pharmacological profile indicative of a significant but short-lasting relief of pain when acutely administered to diabetic mice.

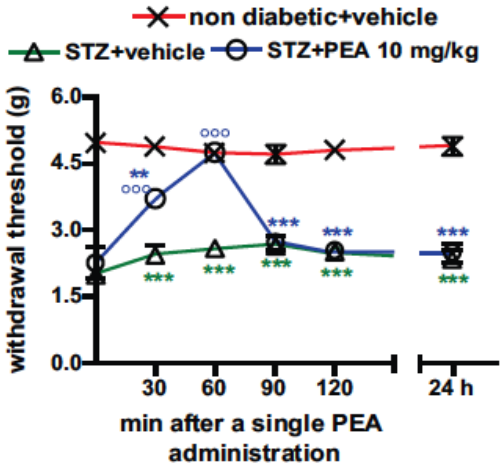

Fig. 1. Effect of a single palmitoylethanolamide (PEA) administration (i.p.) to diabetic mice, at day 14 after streptozotocin (STZ) injection, on mechanical allodynia, at different time points after the treatment. Mechanical threshold of the paws is expressed as g and data represent mean ± S.E.M. of 6-10 mice. ***P<0.001, **P<0.01 vs non diabetic+vehicle; °°°P<0.001 vs STZ+vehicle by one-way ANOVA followed by Tukey's test..

Diabetic mice were submitted to a daily regimen with the same dose of PEA or vehicle and monitored for the nociceptive responses 24 h after the last PEA administration, in order to define whether repeated administration could enhance the relief of pain induced by a single dose of the compound. The results are shown in **Fig.2**. Particularly, panel **A** shows the withdrawal latencies recorded 24 h after the first (T1), the second (T2) and the third (T3) administration of PEA. The data analysis suggests a time-dependent relief of mechanical allodynia characterized by a complete reversal of diabetic neuropathy after three administration of PEA, suggesting a rapid onset of PEA effect. Since the treatment started when the neuropathic pain was evident, the ability of PEA to improve the established disease may have potential therapeutic implications. Thus, we highlighted the great therapeutic significance of repeated PEA treatment. The daily administration of PEA was prolonged for other four days in order to complete a 7-day regimen (**Fig. 2B**) and the data clearly indicate that the PEA-induced relief of mechanical allodynia was still present, indicative of no tolerance development at least following this short repeated treatment

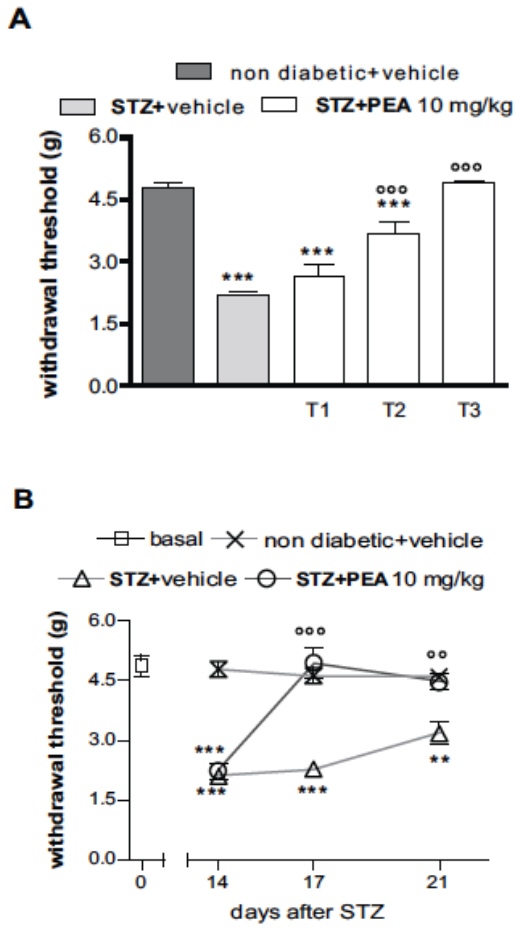

Fig. 2. Effect of palmitoylethanolamide (PEA) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on mechanical allodynia, at day 15 (T1), 16 (T2) and 17 (T3) following the treatment, 24 h after the last administration (**A**). Effect of palmitoylethanolamide (PEA) i.p administered daily to diabetic mice for one week, starting the day 14 after streptozotocin (STZ) injection, on mechanical allodynia (**B**). Mechanical threshold of the paws is expressed as g and data represent mean ± S.E.M. of 6-10 mice. ***P<0.001, **P<0.01 vs non diabetic+vehicle; °°°P<0.001, °°P<0.01 vs STZ+vehicle by one-way ANOVA followed by Tukey's test.

The anti-allodynic properties of PEA in diabetic mice were further characterized performing a dose-response pharmacological analysis. Diabetic mice were i.p. injected with different doses of PEA (0.1, 1, 3 and 10 mg/k g) for three days, starting day 14th following STZ and the pain-related behaviour was assayed 24 hours after the last administration of PEA. As shown in **Fig. 3A**, PEA reduced the mechanical allodynia in a dose dependent manner with a maximum effect elicited by 10 mg/k g (r^2 =0.7923 F=83.94, P <0.0001) (**Fig. 3B**).

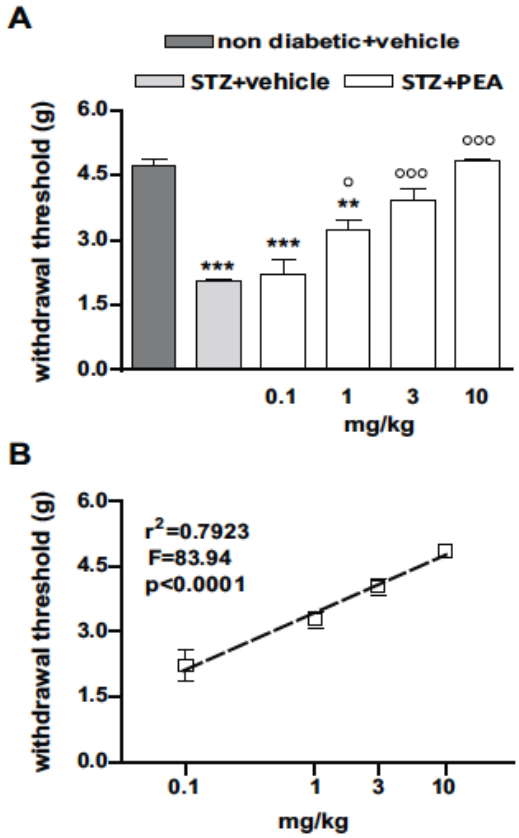

Fig. 3. Effect of palmitoylethanolamide (PEA) (0.1, 1, 3 and 10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on mechanical allodynia, 24 h after the last administration (day 17) (**A**). Linear regression between withdrawal threshold and doses (log scale) (**B**). Mechanical threshold of the paws is expressed as g and data represent mean ± S.E.M. of 6-10 mice. ***P<0.001, **P<0.01 vs non diabetic+vehicle; °°°P<0.001, °P<0.05 vs STZ+vehicle by one-way ANOVA followed by Tukey's test.

In order to understend the mechanism by which PEA induced its effect, the involvement of CB_1, CB_2, PPAR-α, PPAR-γ, and TRPV1 receptors was tested employing specific antagonists administrated i.p. 10 min before each daily PEA 10 mg/kg administration.. All the antagonists employed were able to partially counteract PEA-induced anti-allodynic effect (**Fig. 4**), indicating an involvement of all these receptors in the relief of diabetic neuropathy induced by PEA.

Recently, in literature increasing evidence showed that PEA binds to PPARα receptors that mediates, at least in part, the anti-inflammatory and analgesic effect of PEA (Lo Verme et al., 2005).

In spite of the ability of CB_2 receptor antagonists to reverse many of the

pharmacological action of PEA, including analgesia (Calignano et al., 1998), PEA shows poor affinity for cannabinoid CB_2 or CB_1. However, although the CB_2 antagonist SR144528 prevented the antinoci-ceptive effects of PEA (Calignano et al., 1998), it did not block its anti-inflammatory effects (Costa et al., 2002). One explanation of these discrepancies is the possibility that SR144528 binds to a CB_2-like receptor (Calignano et al., 1998) or, more likely, that PEA could compete with endogenous anandamide (AEA) for fatty acid amide hydrolase-mediated hydrolysis, thus causing an increase in AEA levels, which would then activate the CB_2 receptors. This the so-called *entourage effect* (Ben-Shabat et al., 1998; Lo Verme et al., 2005), strengthens its analgesic action through different molecular mechanisms including the cannabinoid receptor CB1 activation, the desensitization of noxious TRPV1 and the induction of PPAR-γ activity. The capability of receptor antagonists to reverse PEA-induced anti-allodynic effect was employed to assess all the above quoted hypothesis. Our findings showed that the antiallodynia elicited by PEA was partially reversed by the administration of all the antagonists employed, suggesting a role of CB_1, CB_2, TRPV1, PPAR-α, and PPAR-γ receptors in PEA-induced relief of diabetic neuropathy. This result is in agreement to the so-called *entourage hypothesis* so that PEA could indirectly activate CB_1 receptors which are widely expressed in both central and peripheral nervous systems, and CB_2 receptors, primarily present on microglia, and dorsal horn neurons, thus contributing to the modulation of pain perception. Furthermore, PEA could indirectly desensitizes TRPV1 receptors whose role in diabetic neuropathy is now established (Khomula et al., 2013) and activates PPAR-γ receptors with the consequent inhibition of microgliamediated production of inflammatory molecules (Storer at al., 2005).

Furthermore, our data suggest that the well-known PEA receptor, PPAR-α, is involved in the anti-allodynic effect of PEA, confirming recent results revealing a previously unsuspected role of PPAR-α in pain modulation (Lo Verme et al., 2006). In conclusion, exogenous PEA may: a) compete with AEA to fatty acid amide hydrolase-mediated degradation causing an increase in the level of AEA, which in turn activates CB_1, CB_2, TRPV1, and PPAR-γ receptors and b) directly activate PPAR-α receptors, resulting in the relief of diabetic neuropathy.

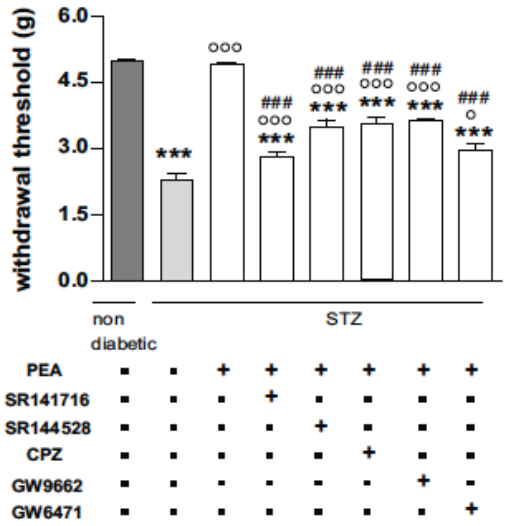

Fig. 4. Effect of daily co-administration of SR141716 (1 mg/kg i.p.), SR144528 (1 mg/kg i.p.), capsazepine (CPZ, 5 mg/kg i.p.), GW9662 (1 mg/kg i.p.) and GW6471 (1 mg/kg i.p.) with PEA (10 mg/kg i.p.) for three days, starting the day 14 after streptozotocin (STZ) injection, on PEA-induced anti-allodynia in diabetic (STZ) mice, 24 h after the last co-administration of compounds (day 17). Mechanical threshold of the paws is expressed as g and data represent mean ± SEM of 8 mice. ***P<0.001 vs non diabetic+vehicle; °°°P<0.001,°P<0.05 vs STZ+vehicle; ###P<0.001 vs STZ+PEA 10 mg kg-1.

Many reports suggested that the development of diabetic neuropathy is accompanied with impaired NGF support to nociceptive neurons (Pitenger et al., 2003). Thus, the putative involvement of this neurotrophic factor in PEAinduced anti-allodynia was assayed. Accordingly, 14 days after STZ, when mechanical allodynia was established, NGF level was significantly decreased (33%) in the sciatic nerve of diabetic mice (**Fig. 5**), and this decrease could be a consequence of glucose-induced oxidative stress (Pitenger et al., 2003) that could influence either production or transport of NGF. The pharmacological treatment with PEA at the dose able to completely relieve mechanical allodynia also resulted in a restoration of NGF. In fact, the level of NGF in the sciatic nerve of diabetic mice treated with PEA for three days or one week was statistically different from that found in the sciatic nerve of diabetic mice and similar to the level present in non diabetic mice (**Fig. 5**).

NGF therapy has been proposed to diabetic patients but, unfortunately, clinical trials have not been successful (Apfel et al., 2000), especially because of the limitation in the exogenous NGF delivery and tolerability.

We have reported here that the repeated treatment with PEA restored normal NGF content in the sciatic nerve of diabetic mice.

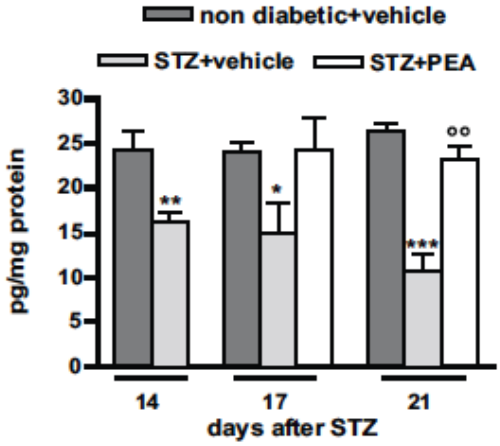

Fig.5. Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for one week, starting the day 14 after streptozotocin (STZ) injection, on NGF levels in the sciatic nerve, at different time points. Data represent mean ± S.E.M. of 5 mice. ***P<0.001, **P<0.01, *P<0.05 vs non diabetic+vehicle; °°P<0.01 vs STZ+vehicle by Student's t-test or by one-way ANOVA followed by Tukey's test

We also found that PEA-induced relief of diabetic neuropathy is not associated with an action upon hyperglycemia. Particularly, hyperglycemia was apparent on day 7 after administration of STZ, and further increased at week two (**Fig. 6**). From this time point PEA was daily administered to diabetic mice for one week . As shown in **Fig. 6**, there is no difference in blood glucose level between PEA-treated mice and vehicle-treated mice, either for three or seven days, showing that the pharmacological treatment did not affect the hyperglicemia induced by STZ.

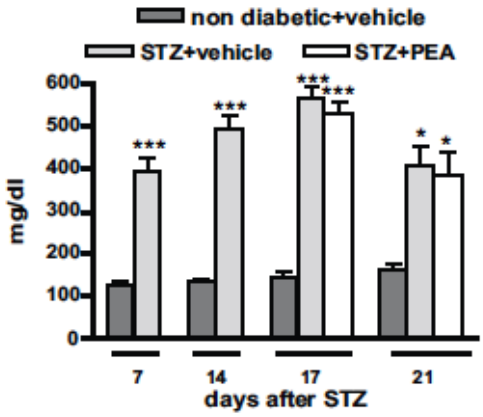

Fig. 6. Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for one week, starting the day 14 after streptozotocin (STZ) injection, on glucose blood level, at different time points. Data represent mean ± S.E.M. of 5 mice. ***P<0.001, *P<0.05 vs non diabetic+vehicle by Student's t-test or by one-way ANOVA followed by Tukey's test.

On the other hand, unexpectedly, PEA treatment induced an increase in insulin level in serum of diabetic mice. To assess the impact of PEA treatment on insulin secretion, serum insulin levels were assayed in normal, diabetic and PEA-treated diabetic mice. As shown in **Fig. 7**, STZ depleted serum insulin levels by up 30% at the evaluation time (17 days after STZ).

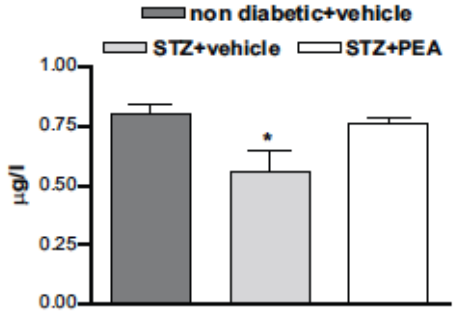

Fig. 7. Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on insulin serum level (day 17). Data represent mean ± S.E.M. of 5 mice. *P<0.05 vs non diabetic+vehicle by one-way ANOVA followed by Tukey's test.

Following PEA treatment, however, serum insulin levels were partially restored and were nearly 1.5-fold higher than those of diabetic mice and were not statistically different from those of non diabetic mice. We hypothesized that such an effect could be ascribed to the anti-inflammatory properties of PEA. To test such hypothesis we evaluated whether PEA treatment could affect the development of insulitis, through the analysis of islet morphology using Haematoxylin and Eosin (H&E) staining. **Fig. 8** shows representative images of pancreas from control (non diabetic) mice (**A**), from STZ-mice treated with vehicle (**B**) and from STZ-mice treated with PEA (**C**). Pancreatic tissue stained with H&E showed a homogeneous distribution of many round-to-elongate and well organized islets of Langerhans in non diabetic mice, whereas following STZ injection a severe decrease in the number of islets as well as in their dimension can be observed. Particularly, the majority of small islets were destroyed and empty spaces, which were previously occupied by the islets, can be seen. The treatment of STZ-mice with PEA induced a mild improvement in the islet density and morphology and all STZ-induced lesions appeared to be significantly alleviated.

About 60 sections for each experimental group were employed to determine the number of islets. The analysis confirmed that the density of islets (expressed as the number of islets/cm^2) was significantly decreased (about 70%) in diabetic mice treated with vehicle. This decrease was significantly lower when diabetic mice were treated with PEA (about 40%) (**Fig. 8D**).

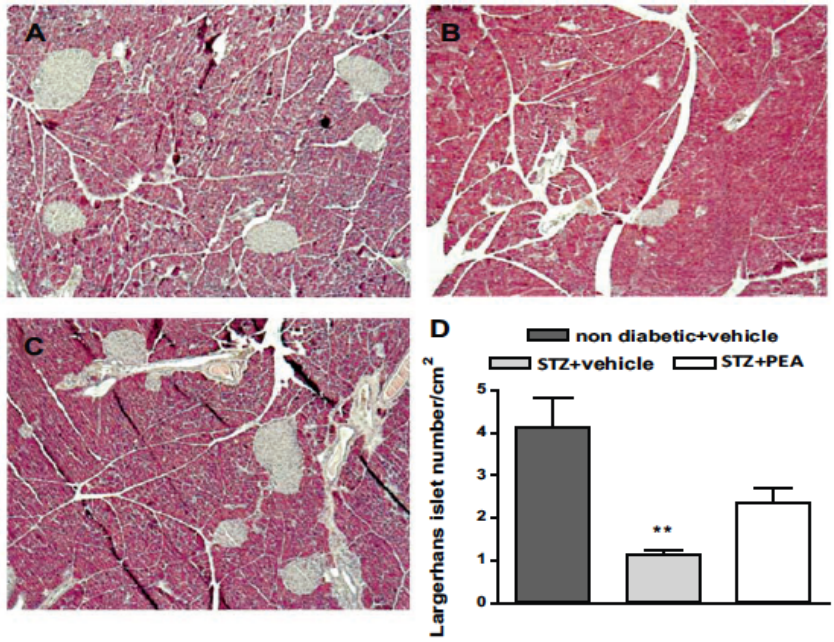

Fig. 8. Light micrograph of pancreas longitudinal section stained with Mayer's haematoxylin and eosin (5x) of the following experimental groups: non diabetic+vehicle (A), STZ+vehicle at day 17 after STZ injection (B), STZ+ palmitoylethanolamide (PEA) (10 mg/kg) at day 17, i.p administered daily to diabetic mice for three days, starting the day 14 after STZ injection (C). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on the density of Langerhans islets (expressed as number of islets/cm2), at day 17. Data represent mean ± S.E.M. of 3-4 mice. **P<0.01 vs non diabetic+vehicle by one-way ANOVA followed by Tukey's test

Fig. 9 shows representative pancreatic islets from non diabetic mice (**A**) and diabetic mice treated with vehicle (**B**) or with PEA (**C**). STZ treatment severely disrupted the islet architecture: the clear round islet boundary was destroyed and islet shrink age was observed (**Fig. 9B**) as compared to normal mice (**Fig. 9A**). Treatment with PEA partially prevented the alteration in islet morphology (**Fig. 9C**). About 20 islets of Langerhans for each experimental group were used to measure the mean area of islets. The results, shown in **Fig. 9D**, highlighted that the mean area of islets from diabetic mice treated with vehicle was significantly reduced than in non diabetic mice (islets were about 50% smaller), and that the repeated treatment with PEA significantly prevented the reduction in islet dimension (**Fig. 9D**).

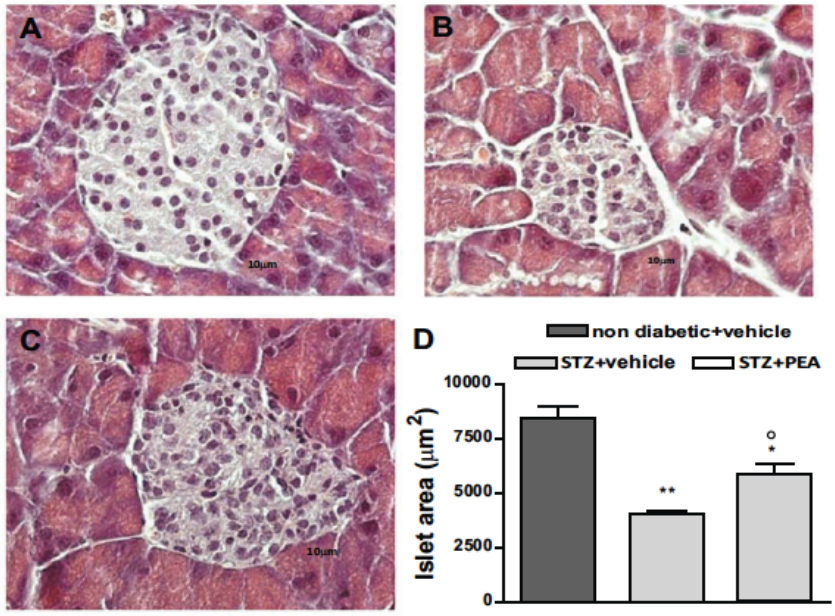

Fig. 9. Light micrograph of Langerhans pancreatic islet stained with Mayer's haematoxylin and eosin (40x) in pancreas longitudinal sections of the following experimental groups: non diabetic+vehicle (A), STZ+vehicle at day 17 after STZ injection (B), STZ+ palmitoylethanolamide (PEA) (10 mg/kg) at day 17, i.p administered daily to diabetic mice for three days, starting the day 14 after STZ injection (C). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on the area of Langerhans islet, at day 17. Data represent mean ± S.E.M. of 3-4 mice. **P<0.01, *P<0.05 vs non diabetic+vehicle, °P<0.05 vs STZ+vehicle by one-way ANOVA followed by Tukey's test.

To assess whether the PEA-induced preservation of islets was associated with an anti-inflammatory effect, the severity of insulitis was evaluated with the insulitis score. The percentage of mice showing grade I-II and III-IV insulitis in the STZ group was 18% and 72%, respectively, indicating that most of animals displayed massive and severe insulitis. Conversely, in PEA-treated group the incidence of grade III-IV insulitis was less than in STZ group with only 28% of mice showing grade III-IV insulitis.These results indicate that PEA is effective in reducing the development of insulitis in STZ-mice. This effect was confirmed by the preservation of the number and dimension of Langherans islets, thus suggesting that the antiinflammatory activity of PEA upon pancreatic tissue exposed to the cytotoxic STZ preserved β-cells damage with a consequent improvement of insulin level. This increase insulin was probably insufficient to modulate hyperglycemia. According to our data, other findings reported that an increase in

plasma insulin similar to that obtained in our hands, is accompanied by a slight improvement of hyperglycemia (Kobori et al., 2009; Kang et al., 2014). Particularly, Kobori et al. reported that the enhancement of plasma insulin level from 0.47 μg/l to 1.02 μg/l (higher than that found by us) only led to a 16% decrease in plasma glucose. Another report suggests that an insulin level of at least 1.4 μg/l is necessary to significantly improve hyperglycemia in STZ model of diabetes (Kang et al., 2014). In addition, in the same model, it has been shown that long-acting insulin treatment at high dose (1IU/kg) exhibited a significant reduction of fasting blood glucose level following 14 days of treatment (Gupta et al., 2014). In conclusion, we cannot exclude that a prolonged treatment with PEA and/or an increased dose could further ameliorate the insulin/glucose level.

Looking for a possible mechanism involved in this PEA-induced effect, we focused on mast cells. In fact PEA was shown to act directly on mast cells, via an ALIA (Autacoid Local Injury Antagonism) mechanism (Aloe et al., 1993) and mast cells degranulation triggers deleterious effects in many tissues, where the mast cells reside or are recruited. Thus, the effect of PEA treatment on mast cell activation in pancreatic tissue was evaluated. The percentage of type 1 mast cells (resting) as well as of degranulating type 2 plus degranulated type 3 mast cells (active) were assessed (**Fig. 10C**). Accordingly to statistical analysis, STZ group showed higher percentage of active mast cells than PEA-treated group, indicative of a downregulation of mast cell activation induced by PEA. Panels **A** and **B** show representative images of pancreatic mast cells in which it is possible to appreciate the intact mast cells in the pancreas of PEA-treated mice (**Fig. 10B**) and degranulated mast cells in STZ animals (**Fig. 10A**). These findings highlight the role of recruited mast cells in the development of type 1 diabetes suggesting that mast cell stabilizer, as PEA, could have beneficial effects.

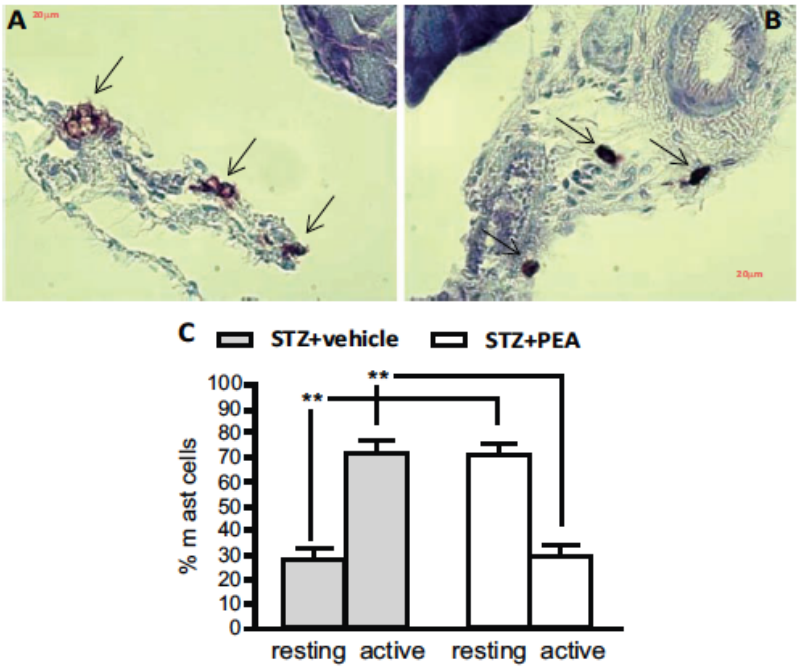

Fig. 10. Light micrograph of pancreas longitudinal sections stained with Toluidine blue (40x) of the following experimental groups: STZ+vehicle on day 17 after STZ injection (A), STZ+palmitoylethanolamide (PEA) (10 mg/kg) at day 17, i.p administered daily to diabetic mice for three days, starting the day 14 after STZ injection (B). Black arrows indicate mast cells. Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on the % of mast cells in the two stage of maturation (resting or degranulated). Data represent mean ± S.E.M. of 3-4 mice. **P<0.01 by Student's t test for non parametric data.

In conclusion, these findings straightened and extended the properties of PEA as pain killer. In fact, diabetic neuropathy was efficiently relieved by PEA treatment as shown previously for other neuropathies. In addition, the *receptor hypothesis* and the *entourage hypothesis* shared the same role in mediating relief of pain induced by PEA, highlighting this molecule as a multi-target compound. The well known ability of PEA to down-regulate mast cell activation was also useful in counteracting pancreas damage, thus suggesting that PEA could be effective in type1- diabetic patients not only as pain reliever but also in controlling the development of pathology.

Chapter 3. Pharmacological effects of PEA in Cheotherapy-induced neuropathic pain

Introduction

Chemotherapy-induced neuropathic pain (CINP) is a severe adverse effect of cytostatic pharmacotherapy, that represents a huge therapeutic problem. Substances that cause CINP include the commonly used platinum analogues, taxanes, vinca alkaloids, and proteasome inhibitors. However, these are the most common antineoplastic drugs successfully employed as first line treatment for several solid and blood cancers, such as breast, lung, colorectal, gastric cancers and multiple myeloma. The incidence of CINP can be up to 90%, it is often the main reason for reduction or discontinuation of therapy, and may limit the employment of life-saving agents. Symptoms are frequently disabling, in fact they may affect patients' daily activities and severely impact on their quality of life (Carozzi et al.; 2015). In general, pharmacological management of neuropathic pain includes drugs such as NSAIDs, opioid analgesics, anticonvulsants, antidepressants, serotonin noradrenaline reuptake inhibitors, and cannabinoids (Moulin et al.; 2014). However, in many cases these treatments are associated with suboptimal therapeutic efficacies and/or side effect. To date, no treatment options are available for the prevention of CINP, and only few pharmacological strategies exist for its treatment. Most analgesic drugs that are commonly in use for the treatment of neuropathic pain, such as amitriptyline or gabapentin, have failed to alleviate CINP in randomized, placebo-controlled clinical trials (Rao et al.; 2007; Kautio et al.; 2009). According to this evidence, there is an urgent need to develop new effective treatment for this pathology.

During my PhD period abroad at Virginia Commonwealth University (Richmond, VA, USA), the aim of my research project was to futher investigate the pharmacological antinociceptive effectiveness of PEA in paclitaxel model of chemotherapy-induced neuropathic pain (CINP), that still lacks a resolutive, and effective treatment. Herein, preliminary results show a very interesting ability of PEA that evokes a total antiallodynic effect in CINP model, after acute administration.

Materials and Methods

Animals

Adult male ICR mice (18-35 gram, Harlan Laboratories) served as subjects in these experiments. Mice were housed four per cage in a temperature (20–22°C), humidity (55 ±10%), and light-controlled (12 hour light/dark; lights on at 0600) AAALAC-approved facility, with standard rodent chow and water available *ad libitum*. All procedures adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University.

Paclitaxel model of chemotherapy-induced neuropathic pain

Paclitaxel was obtained commercially (Tocris, Minneapolis, MN) and dissolved in a veichle solution consisting of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9 % NaCl) in a 1:1:18 ratio. Following baseline behavioral assessment for von Frey thresholds, mice were randomly divided and given an i.p. injection of paclitaxel (8 mg/kg) or vehicle every other day for a total of four injections. This protocol has been well characterized to produce bilateral allodynia (Smith et al., 2004).

Drugs and Treatments

PEA (RTI International, Research Triangle Park, NC) was dissolved in a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9 % NaCl) in a 1:1:18 ratio, and used at the doses of 3, 10, 30 mg/kg. Paclitaxel-treated mice were randomly divided in groups receiving a singular intraperitoneal administration of three different doses of PEA or vehicle. Control mice received drug vehicle.

Assessment of Mechanical Allodynia

Baseline responses to light mechanical touch were assessed using the von Frey test following habituation to the testing environment, as described elsewhere (Murphy et al., 1999). In brief, mice were placed a top a wire mesh screen, with spaces 0.5 mm apart and habituated for approximately 30 min/day for four days. Mice were unrestrained, and were singly placed under an inverted wire mesh basket to allow for unrestricted air flow. The von Frey test utilizes a series of calibrated monofilaments, (2.83 – 4.31 log stimulus intensity; North Coast

Medical, Morgan Hills, CA) applied randomly to the left and right plantar surface of the hind paw for 3 s. Lifting, licking, or shaking the paw was considered a response. For all behavioral testing, threshold assessment was performed in a blinded fashion. Mechanical allodynia was evaluated before injection (0), and 30, 60, 90, 120, 180, 240 min after the acute PEA administration.

Data Analysis and Statistical Procedures

All data are expressed as the mean ± S.E.M. and analyzed using 2-way ANOVA followed by Tukey's posthoc test for multiple comparison. Differences were considered significant at $P<0.05$. All statistical analyses were done using the statistical GraphPad Software package (San Diego, CA, USA).

Results and Discussion

A common complication of chemotherapy is neurotoxicity that often manifests itself as peripheral neuropathy. Many cancer drugs can cause chemotherapy-induced neuropathic pain (CINP), and the incidence can be up to 90%. Although, CINP is a dose-limiting side effect of chemotherapy, and its clinical course is similar to other toxic neuropathies. A significant percentage of patients improve once the offending drug is stopped, but as much as 50% of the affected individuals are left with residual peripheral neuropathy that affects their quality of life. Many drugs that are approved for the treatment of other neuropathic pain states have shown little or no analgesic effect on CINP in large randomized, placebo-controlled clinical trials. Thus, researchers in academia and industry are encouraged to find new effective and safe drugs to treat this pathology. For this reason, in our study we employed paclitaxel-model of CINP, that is commonly used drug in the management of various solid tumour like lung, breast and ovarian cancers. The treatment with paclitaxel affects the PNS and leads to a predominantly sensory axonal peripheral nerve with sensory loss, paresthesia and, pain. Paclitaxel-injected mice developed mechanical allodynia as showed by significant reductions in paw withdrawal thresholds to von Frey filament after four paclitaxel injections. At this time point, paclitaxel-treated mice received a single administration of three doses of PEA (3, 10, and 30 mg/kg) or its vehicle

and the behavioural response to von Frey filament was assessed at different time points post-injection (**Fig. 1A**). Acute administration of PEA 30 mg/kg evoked a peak of total anti-allodynic effect 60 min after the administration, that remained stable at 90 min and then gradually decrease (**Fig. 1A**). At the same time point, PEA 10 mg/kg exerted just a partial effect, while the lowest dose had no effect. Moreover, to underline the antiallodynic effect of PEA treatment, we determined for each animal the area under the curve (AUC). As shown in **Fig. 1B**, PEA evoked its analgesic effect in a dose-dipendent manner. Thus, PEA showed a pharmacological profile indicative of a significant relief of neuropathic pain when acutely administered to paclitaxel-treated mice.

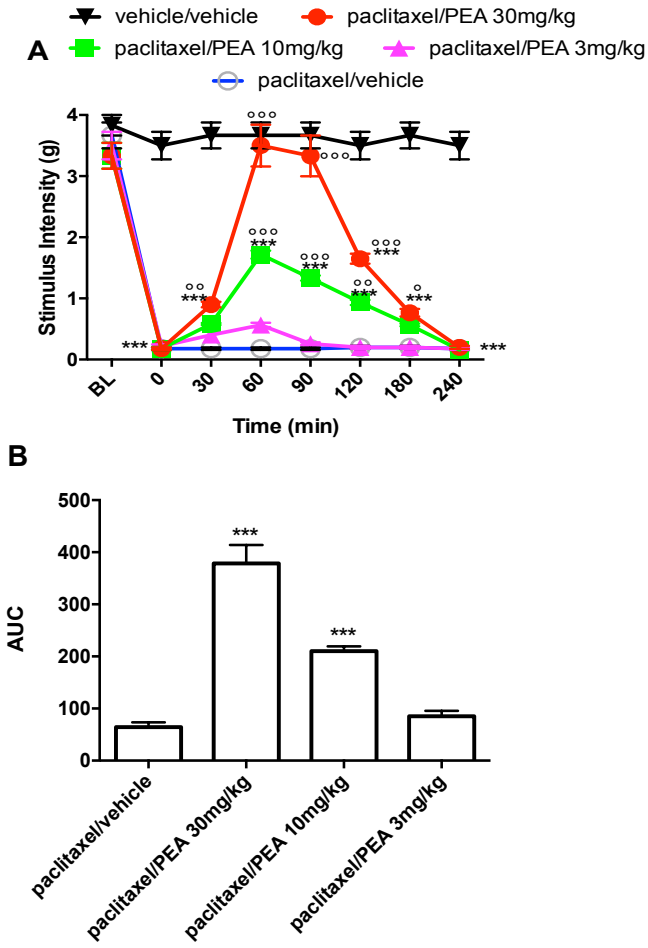

Fig. 1. Effect of a single palmitoylethanolamide (PEA) administration (i.p.) 30, 10, 3 mg/kg to paclitaxel-treated mice (8mg/kg), on mechanical allodynia, before injection (0), and 30, 60, 90, 120, 180, 240 min after treatment (**A**). The Area Under Curve (AUC) shows the antiallodynic efficacy of all three doses of PEA (**B**)

Mechanical threshold of the paws is expressed as g and data represent ± S.E.M. di 6-8 mice. ***P<0.001 vs vehicle+vehicle; °°°P<0.001, °°P<0.01, °P>0.05 vs paclitaxel+vehicle, 2-way ANOVA, test di Tuckey.

For the first time, these data suggest the pharmacological effect of PEA to relive neuropathic pain also in CINP, that still lacks a resolutive, and effective treatment. These enticing preliminary foundings motivates us to go deeper to better understand the molecular mechanism by which PEA exerts its antiallodynic effectiveness in this very common human pathology.

General Discussion and Conclusions

The purpose of the preceding studies was to further investigate the potential effect of PEA to relief pain in very common form of neuropathic pain associated to three human diseases, such as osteoarthirtis, diabetes, and chemotherapy, that still lack of resolutive and safe treatments.

We demonstrated that chronic administration of PEA was able to evoke antinociceptive and anti-inflammatory effects in MIA-induced osteoarthritis (OA) model. Moreover, we found that PEA restored the physiological NGF level, re-established locomotor functionality, preserved cartilage from damage, and reduced MMP-3 level, a cartilage degrading factor, in the synovial fluid of MIA-treated rats. According to these results, we can suggest a mechanism of action of PEA in OA desease. PEA was shown to act directly on mast cells, via ALIA (Autacoid Local Injury Antagonism) mechanism (Aloe et al., 1993), and mast cells degranulation triggers deleterious effects in many tissues, where the mast cells reside or are recruited. To date, it is widely recognized that the main PEA pharmacological effects are mediated by activation of peroxisome proliferator-activated receptor alpha (PPAR-α), an anubiquitous transcription factor (Lo Verme et al., 2006). Thus, PEA could directly bind PPAR- α receptor espressed by immun cells, neurons and microglia, switching off the nuclear factor-κB (NF-κB) signaling cascade, a key element in the transcription of genes, leading to the synthesis of pro-inflammatory and pro-algogen mediators (D'Agostino et al., 2009). Moreover, PEA may exert a protection role on cartilage damage reducing the MMP-3 levels in the synovial fluid through the down regulation of NF-κB pathway as well in chondrocyes. In conclusion, for the first time we demonstrated the pharmacological effects of PEA in MIA-induced OA model. These findings highlight the therapeutic potenciality of PEA and allow us to propose PEA as a valid alternative for the treatment of human OA, compared to nimesulide, and acetaminophen, two of the most widely prescribed drugs in clinic.

In addiction, since diabetic neuropathy is one of the most common long-term complications of diabetes, we aimed to evaluate the ability of PEA to also relief this kind of neuropathic pain, employing the well established STZ-induced animal model of type 1 diabetes. Our findings demonstrated that PEA relieves mechanical allodynia, counteracts NGF deficit, improves insulin level, preserves Langherans islet morphology reducing the development of insulitis in diabetic

mice. Looking for a possible mechanism of action, we found that all CB_1, CB_2, TRPV1, PPAR-α and PPAR-γ receptors are involved in PEA-induced relief in diabetic neuropathy. In perfect congruence with previous studies, our results showed that PEA could indirectly activate CB_1 receptors which are widely expressed in both central and peripheral nervous systems, and CB_2 receptors, primarily present on microglia, and dorsal horn neurons, thus contributing to the modulation of pain perception. Furthermore, PEA could indirectly desensitizes TRPV1 receptors whose role in diabetic neuropathy is now established (Khomula et al., 2013) and activates PPAR-γ receptors with the consequent inhibition of microglia-mediated production of inflammatory molecules (Storer et al., 2005). Moreover, our data suggest also the key role of the well-known PEA receptor, PPAR-α in the antiallodynic effect of PEA (Lo Verme et al., 2006). In addition, we found that in the pancreas of STZ-mice, there was a marked activation of mast cells, with a high percentage of degranulated versus non-active cells. PEA treatment inhibited mast cell degranulation in pancreas of diabetic mice thus preserving islets morphology and function through ALIA mechanism. All these results suggest that PEA could be effective in type1-diabetic patients not only as pain reliever, but also in controlling the development of pathology.

Chemotherapy-induced neurophatic pain is another very frequent form of pain in humans. Starting from all our previous findings, and other studies in the literature, we were also interested in investigate the analgesic effect of PEA in paclitaxel model of CINP, one of the most antineoplastic drugs prescribed in clinic. Preliminary results showed that acute administration of PEA evoked a total reverse of allodynia in paclitaxel-treated mice, suggesting a very captivating ability of PEA to act against this type of neuropathic pain as well. Our intention is to go deeper to better understend the mechanism by which PEA exerts its effect in CINP.

The pattern of findings presented here suggests that the endogenous fatty-acid amide PEA represents a new effective and safe therapeutic approach for the treatment of neurophatic pain associated also to osteoarthritis, diabetes and antineoplastic therapy. In fact, PEA belongs to an entire new class of analgesic products, devoid of addiction potential, without central nervous system side effects, and without clear dose limiting side effects (Esposito et al., 2013). Moreover, drug interations have so far not been documented, and its use has been described together with a number of drugs.

In conclusion, PEA is definitely a very promising analgesic drug for the treatment of neurophatic pain in different human diseases, that still lack a risolutive therapy.

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Appendix

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Palmitoylethanolamide Relieves Pain and Preserves Pancreatic Islet Cells in a Murine Model of Diabetes

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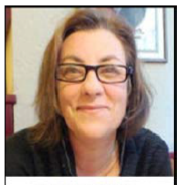

Barbara Costa

Abstract: We previously demonstrated that the intraperitoneal administration of palmitoylethanolamide (PEA) in mice with chronic constriction injury of the sciatic nerve evoked a relief of both thermal hyperalgesia and mechanical allodynia in neuropathic mice. Since diabetic neuropathy is one of the most common long-term complications of diabetes, we explored the ability of PEA to also relief this kind of chronic pain, employing the well established streptozotocin-induced animal model of type 1 diabetes. Our findings demonstrated that PEA relieves mechanical allodynia, counteracts nerve growth factor deficit, improves insulin level, preserves Langherans islet morphology reducing the development of insulitis in diabetic mice. These results suggest that PEA could be effective in type1 -diabetic patients not only as pain reliever but also in controlling the development of pathology.

Keywords Allodynia, diabetes, endocannabinoid, insulin, insulitis, mast cell, nerve growth factor, neuropathy, oxidative stress, Palmitoylethanolamide.

INTRODUCTION

Diabetes mellitus is a metabolic syndrome today affecting 382 million people (8.3% of adults). Recently, the International Diabetes Federation estimates that the number of people with the disease is set to rise beyond 592 million in less than 25 years [1]. One of the major and most disabling long-term complications of diabetes is diabetic neuropathy, that is estimated to affect about 50% of patients. Diabetic neuropathy usually appears as distal symmetrical polyneuropathy, characterized by allodynia, paresthesia and hyperalgesia [2]. Antidepressants, opioids, anticonvulsants and antioxidants represent the current treatment regimen of this complication, even if these therapies relieve pain only in a very few number of patients and their side effects limit their use [3].

We previously showed that palmitoylethanolamide (PEA) relieved thermal hyperalgesia and mechanical allodynia in a mouse model of neuropathic pain due to the chronic constriction injury of the sciatic nerve [4]. PEA is an endogenous lipid, that behaves as an autocoid local injury antagonist (ALIA) amide. PEA was shown to exert anti-inflammatory [5, 6], anti-convulsant [7] and anti-proliferative [8] effectiveness. The exogenous PEA administration has been reported to evoke antinociceptive effects in different animal models of pain such as spinal cord injury [9], carrageenan-induced acute inflammation [10], and complete Freund's adjuvant-induced chronic inflammation [11]. The ability of PEA to act as pain killer has also been reported in humans, during chronic lumbosciatalgia [12], and chronic pelvic pain conditions [13, 14].

In addition, it has been already shown that PEA levels increase in the paw skin of mice with streptozotocin-induced diabetic neuropathy [15], thus suggesting a protective role of PEA in opposing to the severity of disease and its progression. Based on these evidences, we want to further explore the therapeutic potentiality of PEA in resolving painful states through the assessment of PEA antinociceptive effectiveness during diabetic neuropathy, too. The present study shows a strong therapeutic effect of PEA in relieving diabetes-induced neuropathic pain following a three-day repeated treatment, so confirming the beneficial effect of such a molecule for the relief of this type of chronic pain. In order to evaluate whether the relief of diabetic neuropathy was associated with an improvement of diabetes *per se*, serum glucose and insulin levels were assessed. Unexpectedly, PEA treatment ameliorated the decrease in insulin preserving pancreatic islet morphology, probably through a mechanism involving mast cell downregulation.

MATERIALS AND METHODS

Animals

All experiments performed were in accordance with Italian and European regulations governing the care and treatment of laboratory animals (Permission n° 41/2007B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain [16]. Experiments were conducted using male C57BL/6J mice 9 weeks years old (25-30 g) (Harlan, Italy), housed under controlled illumination (12h light/12h dark cycle) and standard environmental conditions

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(room temperature 22±1°C, humidity 60±10%) and allowed to acclimatise for at least one week before experimental use. Standard food and water was available *ad libitum*. All efforts were made to reduce both animal numbers and suffering during the experiments. All behavioural evaluations were performed by experimenters blind to treatments.

Induction of Diabetes

Type 1 diabetes was induced in overnight fasted mice by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma, Italy) at 120 mg/kg, freshly prepared in citrate buffer 0.1 M pH 4.5. This procedure ameliorates the absorption of the drug. Blood glucose concentration (Lifescan One Touch Ultra glucose meter, Milan, Italy) was assessed after the mice were fasted for 4-6 h, one week later on a sample of blood obtained from a tail prick to verify diabetes establishment. Only mice with a blood glucose levels above 250 mg/dL were selected for experiments. Control mice received an i.p. injection of citrate buffer. Blood glucose level was monitored over the whole period of the experimental study (days 14, 17 and 21).

Drugs and Treatments

PEA (Epitech, Saccolongo, Italy) was dissolved in a mixture of 10 % ethanol and 90 % saline, and used at the doses of 0.1, 1, 3 and 10 mg/kg. Diabetic mice were randomly divided in two groups receiving intraperitoneally the compound or its vehicle, once a day for 3 days, starting from the 14th day after the diabetes induction. Control mice received drug vehicle. The treatment with the highest dose (10 mg/kg) was prolonged for other 4 consecutive days. In order to characterize the role of different receptors in the PEA-induced effect, the ability of specific cannabinoid CB_1, CB_2 receptors, transient receptor potential channel of the vanilloid type 1 (TRPV1), peroxisome proliferator-activated receptors (PPARα, and PPARγ) antagonists to reverse the anti-allodynic effect of PEA was evaluated. Particularly, the CB_1 receptor antagonist SR141716 (1 mg/kg i.p.), the CB_2 receptor antagonist SR144528 (1 mg/kg i.p.), the TRPV1 antagonist capsazepine (5 mg/kg i.p.), the PPARα receptor antagonist GW6471 (1 mg/kg i.p.) or the PPARγ antagonist GW9662 (1 mg/kg i.p.) were employed. The doses of antagonists employed in this study were selected on the basis of our previous data [4] and in agreement of other studies demonstrating the ability of each compound to act *in vivo* as a selective receptor antagonist: for GW9662 [17], for GW6471 [18], for SR141716 [19], for SR144528 [19] and for capsazepine [20]. Every day mice received the i.p. injection of the antagonist (or its vehicle) followed 10 min later by PEA 10 mg/kg, as described in Table **1**. Non diabetic animals received the vehicles of the drugs (Table **1**). SR141716 and SR144528 were kindly supplied by Sanofi-Aventis (Montpellier, France) and were dissolved in a mixture of Tween80: DMSO: distilled water (1: 2: 7). Capsazepine, GW6471 and GW9662 were purchased from Sigma-Aldrich (Milano, Italy). Capsazepine was dissolved in a 1: 9 mixture of DMSO: saline, while GW6471 and GW9662 in a 1: 1: 8 mixture of ethanol: Tween80: saline.

Table 1. Simplistic representation of the antagonism study protocol.

Group	First i.p. Injection	Second i.p. Injection (10 min Later)
1 (non-diabetic)	Vehicle of antagonist	Vehicle of PEA
2 (diabetic)	Vehicle of antagonist	Vehicle of PEA
3 (diabetic)	Vehicle of antagonist	PEA 10 mg/kg
4 (diabetic)	CB1 antagonist 1 mg/kg	PEA 10 mg/kg
5 (diabetic)	CB2 antagonist 1 mg/kg	PEA 10 mg/kg
6 (diabetic)	TRPV1 antagonist 5 mg/kg	PEA 10 mg/kg
7 (diabetic)	PPARα antagonist 1 mg/kg	PEA 10 mg/kg
8 (diabetic)	PPARγ antagonist 1 mg/kg	PEA 10 mg/kg

Assessment of Mechanical Allodynia

The withdrawal latency to Von Frey filament was recorded before STZ injection, on day 14 (before starting the treatment and at 30, 60, 90, 120 min after a single PEA administration) and on days 15, 16, 17 and 21, 24 h after the last administration of the compound. In the antagonism studies, mechanical threshold was measured on day 17, 24 h after the last co-administration of compounds. Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) was employed to asses mechanical allodynia as previously described [4].

Insulin Assay

Insulin levels were determined in the serum (10 µl) by a sandwich enzyme-linked immunosorbent assay (ELISA) using an ELISA kit according to the manufacturer's instructions (Mercodia, Sweden). The absorbance was recorded at 450 nm on a microplate reader (Multiskan® EX, ThermolabSystem); standard curves were assayed on individual plates and used to interpolate insulin values in order to obtain insulin levels expressed as µg/l.

NGF Assay

Nerve growth factor (NGF) levels were determined as previously described [4]. Particularly, sciatic nerves proximal to the trifurcation were homogenized in a cold lysis buffer (200 µl). The homogenates were centrifuged at 4500 *g* at 4°C for 10 min, and the resulting supernatants diluted in 5-fold with Dulbecco's PBS buffer. Samples were acidified to pH < 3.0 by adding 1 N HCl, and then neutralized with 1 N NaOH to pH 7.6. Samples were then centrifuged 10000 *g* at 4°C for 15 min and the resulting supernatants used to determine NGF protein levels using ELISA kit according to the manufacturer's instructions (Promega, USA). The absorbance at 450 nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). NGF levels were determined by interpolation with standard curves assayed on individual plates, normalized to protein content in each tissue sample and expressed as pg NGF/mg protein.

Histology of Mouse Pancreas

Pancreas was fixed in 4% paraformaldehyde fresh solution (in 0.1 M phosphate buffer, pH 7.4) overnight at 4°C; following dehydration in serial alcohol concentrations, it was embedded in paraffin wax. Pancreas longitudinal sections (6 µm) were cut with a rotary microtome. Mayer's haematoxylin and eosin (H&E) was employed to stain pancreas longitudinal sections to determine both the total number and the area of Langerhans Islets. About 60 sections were considered to determine the total number of Langerhans islets and about 20 Langerhans islets to evaluate the area (μm^2) (n=3 for each experimental group) (Zeiss Axioplan MC 100 microscope plus AxioVision Rel 4.6 software). A colour digital camera (AxioCam MRc 5, Zeiss) captured the images and the analysis were performed by an experimenter blind to pharmacological treatment. The pancreas was also employed for the examination of the insulitis. A total of 20 islets from each mouse were examined and classified as follows: 0, intact islet; I, accumulation of mononuclear cells only at the ductal pole of the islet; II, peri-insulitis, infiltration of mononuclear cells only at the periphery of the islet; III, insulitis, lymphocytic infiltrate invading the islets; IV, severe insulitis, massive infiltration of mononuclear cells throughout the islet with small, retracted islets, as reported by others [21, 22]. Some sections were assayed for mast cells determination. Briefly, sections were stained with 0.1% toluidine blue. Intact dark blue-stained cells represented resting mast cells. Degranulating mast cells were identified as cells from which some granules have been extruded, but cell outline retained largely intact. Mast cells exhibiting extensive and widespread degranulation, were classified as degranulated.

Data Analysis and Statistical Procedures

All data are expressed as the mean ± S.E.M. and analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparison. Student's t-test was used to compare two groups. Differences were considered significant at $P<0.05$. All statistical analyses, including linear regression analysis were done using the statistical GraphPad Software package (San Diego, CA, USA).

RESULTS

PEA Relieves Diabetic Neuropathy

STZ-injected mice developed mechanical allodynia as showed by significant reductions in paw withdrawal thresholds to von Frey filament two weeks after STZ administration (1.998±0.1849 g diabetic mice *versus* 4.975±0.025 g non diabetic mice). At this time point diabetic mice received a single dose of PEA (10 mg/kg, i.p.) or its vehicle and the behavioural response to von Frey filament was assessed at different time points post-injection (Fig. **1**). The single dose of PEA evoked a significant anti-allodynic effect that appeared 30 min after the administration, peaked at 60 min and rapidly disappeared (Fig. **1**). Thus PEA showed a pharmacological profile indicative of a significant but short-lasting relief of pain when acutely administered to diabetic mice. Diabetic mice were submitted to a daily

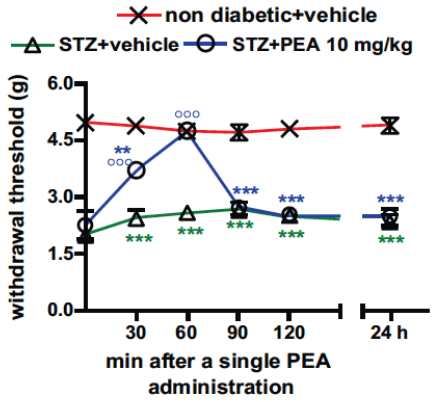

Fig. (1). Effect of a single palmitoylethanolamide (PEA) administration (i.p.) to diabetic mice, at day 14 after streptozotocin (STZ) injection, on mechanical allodynia, at different time points after the treatment. Mechanical threshold of the paws is expressed as g and data represent mean±S.E.M. of 6-10 mice. ***$P<0.001$, **P<0.01 *vs* non diabetic+vehicle; °°°$P<0.001$ *vs* STZ+vehicle by one-way ANOVA followed by Tukey's test.

regimen with the same dose of PEA or vehicle and monitored for the nociceptive responses 24 h after the last PEA administration, in order to define whether repeated administration could enhance the relief of pain induced by a single dose of the compound. The results are shown in Fig. (**2**). Particularly, panel A shows the withdrawal latencies recorded 24 h after the first (T1), the second (T2) and the third (T3) administration of PEA. The data analysis suggests a time-dependent relief of mechanical allodynia characterized by a complete reversal of diabetic neuropathy after three administration of PEA. Since the effect lasted to 24 h, we highlighted the great therapeutic significance of repeated PEA treatment. The daily administration of PEA was prolonged for other four days in order to complete a 7-day regimen (Fig. **2B**) and the data clearly indicate that the PEA-induced relief of mechanical allodynia was still present, indicative of no tolerance development (at least following this short repeated treatment). The anti-allodynic properties of PEA in diabetic mice were further characterized performing a dose-response pharmacological analysis. Diabetic mice were i.p. injected with different doses of PEA (0.1, 1, 3 and 10 mg/kg) for three days, starting day 14^{th} following STZ and the pain-related behaviour was assayed 24 hours after the last administration of PEA. As shown in Fig. (**3A**), PEA reduced the mechanical allodynia in a dose-dependent manner with a maximum effect elicited by 10 mg/kg (r^2=0.7923 F=83.94, $P<0.0001$). Finally, the involvement of CB_1, CB_2, PPARα, PPARγ and TRPV1 receptors in PEA-induced effect was tested employing specific antagonists. These studies were performed administering i.p. the antagonist 10 min before each daily PEA 10 mg/kg administration. All the antagonists employed were able to partially counteract PEA-induced anti-allodynic effect (Fig. **4**), indicating an involvement of all these receptors in the relief of diabetic neuropathy induced by

PEA. The nociceptive response was unaffected by all the antagonists employed, when administered alone (data not shown).

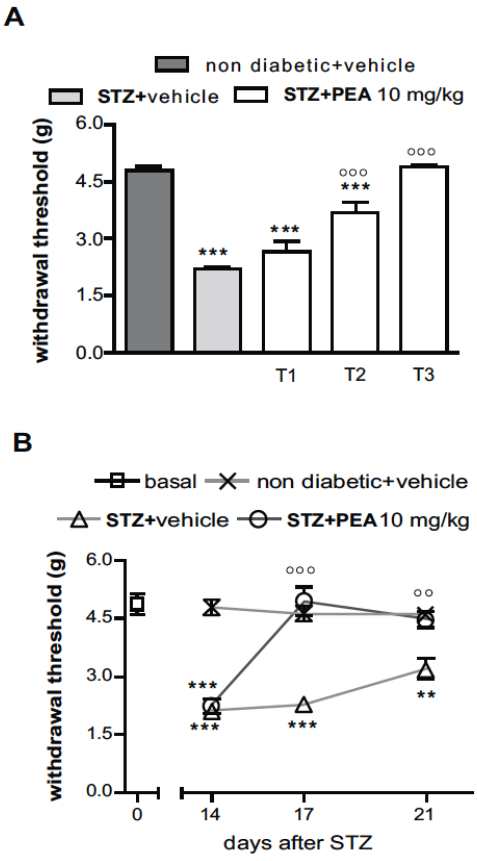

Fig. (2). Effect of palmitoylethanolamide (PEA) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on mechanical allodynia, at day 15 (T1), 16 (T2) and 17 (T3) following the treatment, 24 h after the last administration (**A**). Effect of palmitoylethanolamide (PEA) i.p administered daily to diabetic mice for one week, starting the day 14 after streptozotocin (STZ) injection, on mechanical allodynia (**B**). Mechanical threshold of the paws is expressed as g and data represent mean±S.E.M. of 6-10 mice. ***P<0.001, **P<0.01 *vs* non diabetic+vehicle; °°°P<0.001, °°P<0.01 *vs* STZ+vehicle by one-way ANOVA followed by Tukey's test.

PEA Counteracts NGF Deficit in the Sciatic Nerve of Diabetic Mice

Many reports suggested that the development of diabetic neuropathy is accompanied with impaired NGF support to nociceptive neurons. Accordingly, 14 days after STZ, when mechanical allodynia was established, NGF level was significantly decreased (33%) in the sciatic nerve of diabetic mice (Fig. **5**). The pharmacological treatment with PEA at the dose able to completely relieve mechanical allodynia also resulted in a restoration of NGF; in fact, the level of NGF in the sciatic nerve of diabetic mice treated with PEA for three days or one week was statistically different from that found in the sciatic nerve of diabetic mice and similar to the level present in non diabetic mice (Fig. **5**).

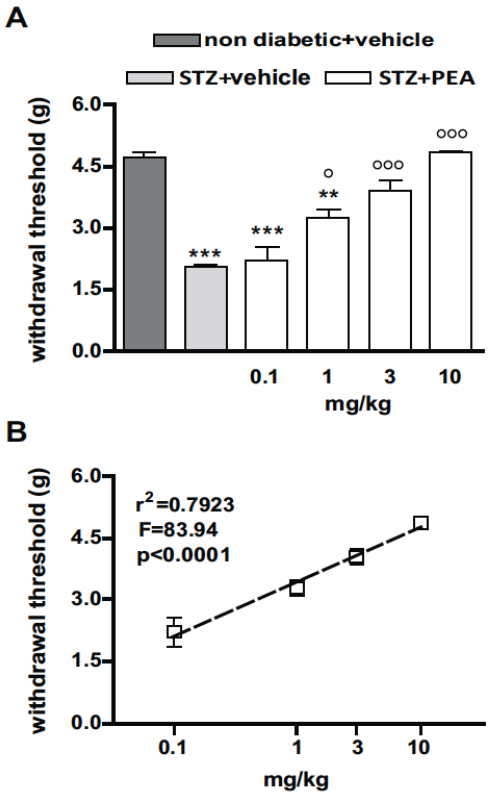

Fig. (3). Effect of palmitoylethanolamide (PEA) (0.1, 1, 3 and 10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on mechanical allodynia, 24 h after the last administration (day 17) (**A**). Linear regression between withdrawal threshold and doses (log scale) (**B**). Mechanical threshold of the paws is expressed as g and data represent mean±S.E.M. of 6-10 mice. ***P<0.001, **P<0.01 *vs* non diabetic+vehicle; °°°P<0.001, °P<0.05 *vs* STZ+vehicle by one-way ANOVA followed by Tukey's test.

PEA Does Not Affect Diabetes-Induced Hyperglycemia

Hyperglycemia was apparent by the 7th day after administration of STZ, and further increased at week two (Fig. **6**). From this time point PEA was daily administered to

diabetic mice for one week. As shown in Fig. (**6**), there is no difference in blood glucose level between PEA-treated mice and vehicle-treated mice, either for three or seven days, showing that the pharmacological treatment did not affect the hyperglicemia induced by STZ.

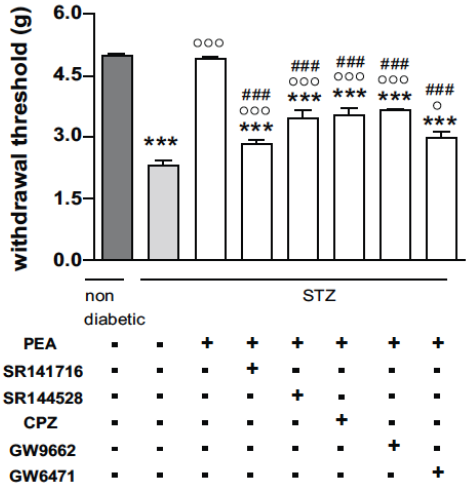

Fig. (4). Effect of daily co-administration of SR141716 (1 mg/kg i.p.), SR144528 (1 mg/kg i.p.), capsazepine (CPZ, 5 mg/kg i.p.), GW9662 (1 mg/kg i.p.) and GW6471 (1 mg/kg i.p.) with PEA (10 mg/kg i.p.) for three days, starting the day 14 after streptozotocin (STZ) injection, on PEA-induced anti-allodynia in diabetic (STZ) mice, 24 h after the last co-administration of compounds (day 17). Mechanical threshold of the paws is expressed as g and data represent mean±SEM of 8 mice. ***P<0.001 *vs* non diabetic+vehicle; °°°P<0.001,°P<0.05 *vs* STZ+vehicle; ###P<0.001 *vs* STZ+PEA 10 mg kg^{-1}.

PEA Improves the Serum Insulin Levels in Diabetic Mice

To assess the impact of PEA treatment on insulin secretion, serum insulin levels were assayed in normal, diabetic and PEA-treated diabetic mice. As shown in Fig. (**7**), STZ depleted serum insulin levels by up 30% at the evaluation time (17 days after STZ). Following PEA treatment, however, serum insulin levels were partially restored and were nearly 1.5-fold higher than that of diabetic mice and were not statistically different from that of non diabetic mice.

PEA Preserves Islet Morphology in Diabetic Mice

To determine the mechanism underlying the beneficial effect of PEA on insulin level, islet morphology was examined using Haematoxylin and Eosin (H&E) staining. Fig. (**8**) shows representative images of pancreas from control (non diabetic) mice (A), from STZ-mice treated with vehicle (B) and from STZ-mice treated with PEA (C).

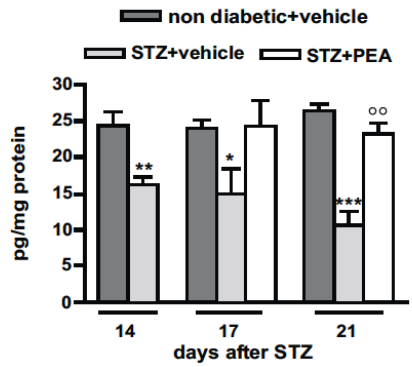

Fig. (5). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for one week, starting the day 14 after streptozotocin (STZ) injection, on NGF levels in the sciatic nerve, at different time points. Data represent mean±S.E.M. of 5 mice. ***P<0.001, **P<0.01, *P<0.05 *vs* non diabetic+vehicle; °°P<0.01 *vs* STZ+vehicle by Student's t-test or by one-way ANOVA followed by Tukey's test.

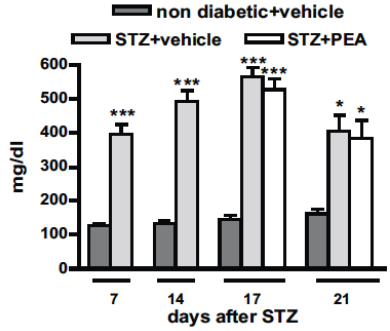

Fig. (6). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for one week, starting the day 14 after streptozotocin (STZ) injection, on glucose blood level, at different time points. Data represent mean±S.E.M. of 5 mice. ***P<0.001, *P<0.05 *vs* non diabetic+vehicle by Student's t-test or by one-way ANOVA followed by Tukey's test.

Pancreatic tissue stained with H&E showed a homogeneous distribution of many round-to-elongate and well organized islets of Langerhans in non diabetic mice, whereas following STZ injection a severe decrease in the number of islets as well as in their dimension can be observed. Particularly, the majority of small islets were destroyed and empty spaces, which were previously occupied by the islets, can be seen. The treatment of STZ-mice with PEA induced a mild improvement in the islet density and morphology and all STZ-induced lesions appeared to be significantly alleviated. About 60 sections for each experimental group were employed to determine the number of islets. The analysis confirmed that the density of islets (expressed as the number of islets/cm^2) was significantly decreased (about 70%) in

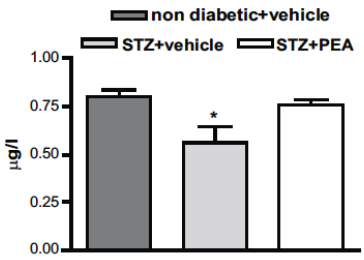

Fig. (7). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on insulin serum level (day 17). Data represent mean±S.E.M. of 5 mice. **P*<0.05 *vs* non diabetic+vehicle by one-way ANOVA followed by Tukey's test.

diabetic mice treated with vehicle. This decrease was significantly lower when diabetic mice were treated with PEA (about 40%) (Fig. **8D**). Fig. (**9**) shows representative pancreatic islets from non diabetic mice (A) and diabetic mice treated with vehicle (B) or with PEA (C). STZ treatment severely disrupted the islet architecture: the clear round islet boundary was destroyed and islet shrinkage was observed (Fig. **9B**) as compared to normal mice (Fig. **9A**). Treatment with PEA partially prevented the alteration in islet morphology (Fig. **9C**). About 20 islets of Langerhans for each experimental group were used to measure the mean area of islets. The results, shown in Fig. (**9D**), highlighted that the mean area of islets from diabetic mice treated with vehicle was significantly reduced than in non diabetic mice (islets were about 50% smaller), and that the repeated treatment with PEA significantly prevented the reduction in islet dimension (Fig. **9D**).

PEA Reduces the Development of Insulitis in Diabetic Mice

To assess whether the PEA-induced preservation of islets was associated with an anti-inflammatory effect, the severity of insulitis was evaluated with the insulitis score. The percentage of mice showing grade I-II and III-IV insulitis in the STZ group was 18% and 72%, respectively, indicating that most of animals displayed massive and severe insulitis.

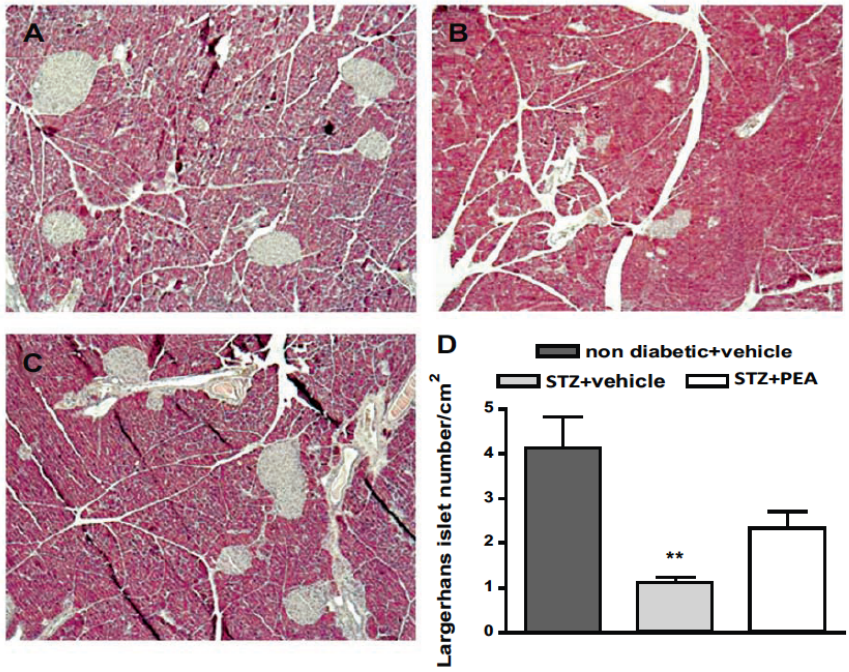

Fig. (8). Light micrograph of pancreas longitudinal section stained with Mayer's haematoxylin and eosin (5x) of the following experimental groups: non diabetic+vehicle (**A**), STZ+vehicle at day 17 after STZ injection (**B**), STZ+ palmitoylethanolamide (PEA) (10 mg/kg) at day 17, i.p administered daily to diabetic mice for three days, starting the day 14 after STZ injection (**C**). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on the density of Langerhans islets (expressed as number of islets/cm^2), at day 17. Data represent mean±S.E.M. of 3-4 mice. ***P*<0.01 *vs* non diabetic+vehicle by one-way ANOVA followed by Tukey's test.

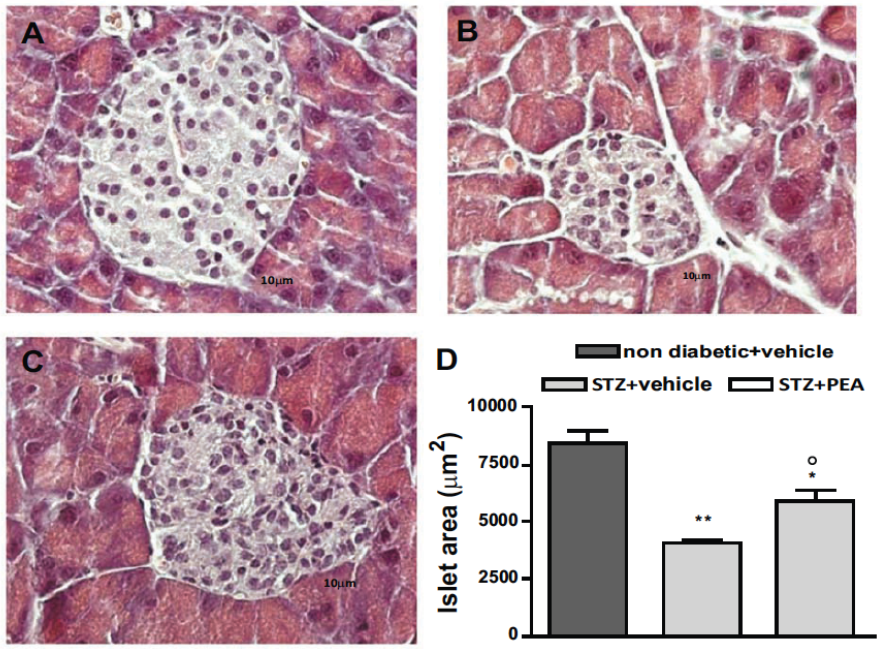

Fig. (9). Light micrograph of Langerhans pancreatic islet stained with Mayer's haematoxylin and eosin (40x) in pancreas longitudinal sections of the following experimental groups: non diabetic+vehicle (**A**), STZ+vehicle at day 17 after STZ injection (**B**), STZ+ palmitoylethanolamide (PEA) (10 mg/kg) at day 17, i.p administered daily to diabetic mice for three days, starting the day 14 after STZ injection (**C**). Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on the area of Langerhans islet, at day 17. Data represent mean±S.E.M. of 3-4 mice. $^{**}P<0.01$, $^{*}P<0.05$ *vs* non diabetic+vehicle, $^{\circ}P<0.05$ *vs* STZ+vehicle by one-way ANOVA followed by Tukey's test.

Conversely, in PEA-treated group the incidence of grade III-IV insulitis was less than in STZ group with only 28% of mice showing grade III-IV insulitis.

PEA Decreases Mast Cell Degranulation in the Pancreas of Diabetic Mice

Because of the established role of mast cells in pancreatic inflammation and considering the well-known capability of PEA to inhibit mast cell degranulation *in vitro*, we evaluated the effect of PEA treatment on mast cell activation in pancreatic tissue. The percentage of type 1 mast cells (resting) as well as of degranulating type 2 plus degranulated type 3 mast cells (active) were assessed (Fig. **10C**). Accordingly to statistical analysis, STZ group showed higher percentage of active mast cells than PEA-treated group, indicative of a downregulation of mast cell activation induced by PEA. Panels A and B show representative images of pancreatic mast cells in which it is possible to appreciate the intact mast cells in the pancreas of PEA-treated mice (Fig. **10B**) and degranulated mast cells in STZ animals (Fig. **10A**).

DISCUSSION

PEA belongs to N-Acylethanolamines family, endogenous bioactive lipids implicated in the regulation of several processes, from the modulation of food intake to pain and inflammation. The ability of PEA to relief chronic pain has been reported in animal models of inflammatory and neuropathic pain due to nerve compression (for review see [23]). Since it's clear from clinical evidence that neuropathic pain from different aetiologies usually responds differently to the pharmacological treatments [24], we aimed to extend the knowledge about the ability of PEA as a pain killer by assessing its effect on diabetic neuropathy. Furthermore, some recent clinical studies have suggested that PEA could also relieve pain in diabetic patients [25, 26] even if no preclinical studies are actually available. Starting from these evidences, as the principal aim of this study, we investigated the therapeutic antinociceptive effect evoked by a repeated treatment with PEA in diabetic mice. Moreover, other aim was to understand the mechanism through which PEA exerted its pharmacological property.

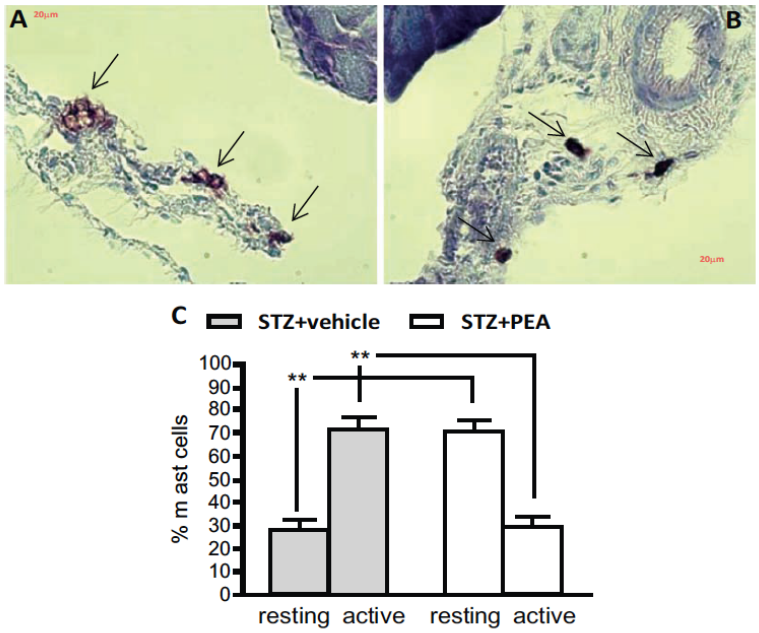

Fig. (10). Light micrograph of pancreas longitudinal sections stained with Toluidine blue (40x) of the following experimental groups: STZ+vehicle at day 17 after STZ injection (**A**), STZ+ palmitoylethanolamide (PEA) (10 mg/kg) at day 17, i.p administered daily to diabetic mice for three days, starting the day 14 after STZ injection (**B**). Black arrows indicate mast cells. Effect of palmitoylethanolamide (PEA) (10 mg/kg) i.p administered daily to diabetic mice for three days, starting the day 14 after streptozotocin (STZ) injection, on the % of mast cells in the two stage of maturation (resting or degranulated). Data represent mean±S.E.M. of 3-4 mice. $^{**}P<0.01$ by Student's t test for non parametric data.

In diabetes, the chronic hyperglycemia and associated complications affecting peripheral nerves are one of the most commonly occurring complications with an overall prevalence of 50-60%. Among the complications of diabetes, diabetic neuropathy is the most painful and disabling, complication affecting the quality of life in patients. Human diabetic neuropathy is characterized by spontaneous pain, allodynia and alteration in thermal perception. These behavioral signs are shared by mice submitted to streptozotocin-induced diabetic neuropathy a widely employed animal model of diabetic neuropathy [27] For this reason in our study we employed this model, which mimics the autoimmune type 1 diabetes mellitus since STZ destroyed pancreatic β-cells. Here we demonstrate that PEA was able to evoke an anti-allodynic effect in a dose-dependent manner. Furthermore, the repeated treatment with PEA, at the dose already found by us to be effective against neuropathic pain evoked by nerve compression [4], also attenuates mechanical allodynia in diabetic mice. In fact, the repeated systemic administration of PEA induced an increase in paw withdrawal thresholds, as compared to corresponding measures in the vehicle control group. By contrast, PEA administration has no effect on the nociceptive response in non-pathological control animals. Particularly, three administrations of PEA already significantly reversed diabetic neuropathy, suggesting a rapid onset of PEA effect. Since the treatment started when the neuropathic pain was evident, the ability of PEA to improve the established disease may have potential therapeutic implications. Interestingly, diabetic mice daily treated with PEA for further four days displayed again a physiological mechanical stimuli perception, suggesting that PEA treatment doesn't induce tolerance development.

The receptors involved in the effects of PEA have been controversial until recently, when increasing evidence showed that PEA binds to PPARα receptors that mediates, at least in part, the anti-inflammatory and analgesic effect of PEA [28, 29]. In spite of the ability of CB_2 receptor antagonists to reverse many of the pharmacological action of PEA, including analgesia [30], PEA shows poor affinity for cannabinoid CB_2 or CB_1. However, although the CB_2 antagonist SR144528 prevented the antinoci-ceptive effects of PEA [30], it did not block its anti-inflammatory effects [5]. One explanation of these discrepancies is the possibility that SR144528 binds to a CB_2-like receptor [30] or, more likely, that PEA could compete with endogenous anandamide (AEA) for fatty acid amide hydrolase-mediated hydrolysis, thus causing an increase in AEA levels, which

would then activate the CB_2 receptors. This the so-called "entourage effect" [31, 32], strengthens its analgesic action through different molecular mechanisms including the cannabinoid receptor CB_1 activation, the desensitization of noxious TRPV1 and the induction of PPARγ activity. The capability of receptor antagonists to reverse PEA-induced anti-allodynic effect was employed to assess all the above quoted hypothesis. Our findings showed that the anti-allodynia elicited by PEA was partially reversed by the administration of all the antagonists employed, suggesting a role of CB_1, CB_2, TRPV1, PPARα and PPARγ receptors in PEA-induced relief of diabetic neuropathy. This result is in agreement to the so-called ''entourage hypothesis": PEA could indirectly activate CB_1 receptors which are widely expressed in both central and peripheral nervous systems, and CB_2 receptors, primarily present on microglia, and dorsal horn neurons, thus contributing to the modulation of pain perception. Furthermore, PEA could indirectly desensitizes TRPV1 receptors whose role in diabetic neuropathy is now established [33] and activates PPARγ receptors with the consequent inhibition of microglia-mediated production of inflammatory molecules [34]. Furthermore, our data suggest that the well-known PEA receptor, PPARα, is involved in the anti-allodynic effect of PEA, confirming recent results revealing a previously unsuspected role of PPARα in pain modulation [11]. In conclusion, exogenous PEA may: a) compete with AEA for fatty acid amide hydrolase-mediated degradation causing an increase in the level of AEA, which in turn activates CB_1, CB_2, TRPV1 and PPARγ receptors and b) directly activate PPARα receptors, resulting in the relief of diabetic neuropathy. On the basis of the pivotal role exerted by NGF in the pathogenesis of diabetic neuropathy [35], we assayed the putative involvement of this neurotrophic factor in PEA-induced anti-allodynia. In fact, NGF level in the sciatic nerve of diabetic mice was very low, and this decrease could be a consequence of glucose-induced oxidative stress [35] that could influence either production or transport of NGF. NGF therapy has been proposed to diabetic patients but, unfortunately, clinical trials have not been successful [36], especially because of the limitation in the exogenous NGF delivery and tolerability. We have reported here that the repeated treatment with PEA restored normal NGF content in the sciatic nerve of diabetic mice.

We also found that PEA-induced relief of diabetic neuropathy is not associated with an action upon hyperglycemia. In fact, the results showed that the repeated treatment with PEA did not result in a decrease of blood glucose level. Unexpectedly, PEA treatment induced an increase in insulin level in serum of diabetic mice. We hypothesized that such an effect could be ascribed to the anti-inflammatory properties of PEA To test such hypothesis we evaluated whether PEA treatment could affect the development of insulitis. We found that the mild insulitis (grade I-II) was the commoner outcome in PEA-treated diabetic mice. These results indicate that PEA is effective in reducing the development of insulitis in STZ-mice. This effect was confirmed by the preservation of the number and dimension of Langherans islets, thus suggesting that the anti-inflammatory activity of PEA upon pancreatic tissue exposed to the cytotoxic STZ preserved β-cells damage with a consequent improvement of insulin level. This increase in insulin was probably insufficient to modulate hyperglycemia. According to our data, other findings reported that an increase in plasma insulin similar to that obtained in our hands, is accompanied by a slight improvement of hyperglycemia [37, 38]. Particularly, Kobori *et al*. [37] reported that the enhancement of plasma insulin level from 0.47 µg/l to 1.02 µg/l (higher than that found by us) only led to a 16% decrease in plasma glucose. Another report suggests that an insulin level of at least 1.4 µg/l is necessary to significantly improve hyperglycemia in STZ model of diabetes [38]. In addition, in the same model, it has been shown that long-acting insulin treatment at high dose (1IU/kg) exhibited a significant reduction of fasting blood glucose level following 14 days of treatment [39]. In conclusion, we cannot exclude that a prolonged treatment with PEA and/or an increased dose could further ameliorate the insulin/glucose level. Looking for a possible mechanism involved in this PEA-induced effect, we focused on mast cells. In fact PEA was shown to act directly on mast cells, *via* an ALIA (Autacoid Local Injury Antagonism) mechanism [40] and mast cells degranulation triggers deleterious effects in many tissues, where the mast cells reside or are recruited. Although mast cells function in human type 1 diabetes mellitus has not been studied, several animal models have demonstrated rather complicated roles of mast cells in type 1 diabetes. Only one paper showed some relationship among insulitis, diabetes and mast cells [41]. Here we found that in the pancreas of STZ-mice, there was a marked activation of mast cells, with a high percentage of degranulated *versus* non-active cells. PEA treatment inhibited mast cell degranulation in pancreas of diabetic mice thus preserving islets morphology and function. These findings highlight the role of recruited mast cells in the development of type 1 diabetes suggesting that mast stabilizers, as PEA, could have beneficial effects.

CONCLUSION

In conclusion, these findings straightened and extended the properties of PEA as pain killer. In fact diabetic neuropathy was efficiently relieved by PEA treatment as shown previously for other neuropathies. In addition the "receptor hypothesis" and the "entourage hypothesis" shared the same role in mediating the relief of pain induced by PEA, highlighting this molecule as a multi-target compound. The well known ability of PEA to down-regulate mast cell activation was also useful in counteracting pancreas damage, thus suggesting that PEA could be effective in type1 - diabetic patients not only as pain reliever but also in controlling the development of pathology.

LIST OF ABBREVIATIONS

AEA = Anandamide

NGF = Nerve Growth Factor

PEA = Palmitoylethanolamide

PPAR = Peroxisome Proliferator-Activated Receptor

STZ = Streptozotocin

TRPV1 = Transient Receptor Potential Channel of the Vanilloid Type 1

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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