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Authors
Pontis, S
Ribeiro, A
Sasso, O
et al.

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Macrophage-derived lipid agonists of PPAR-α as intrinsic controllers of inflammation

Silvia Pontis¹, Alison Ribeiro¹, Oscar Sasso¹, and Daniele Piomelli¹,²

¹Drug Discovery and Development, Istituto Italiano Di Tecnologia, Genoa, Italy and ²Departments of Anatomy and Neurobiology, Pharmacology and Biological Chemistry, University of California, Irvine, CA, USA

Abstract

Macrophages are multi-faceted phagocytic effector cells that derive from circulating monocytes and undergo differentiation in target tissues to regulate key aspects of the inflammatory process. Macrophages produce and degrade a variety of lipid mediators that stimulate or suppress pain and inflammation. Among the analgesic and anti-inflammatory lipids released from these cells are the fatty acid ethanolamides (FAEs), which produce their effects by engaging nuclear peroxisome proliferator activated receptor-α (PPAR-α). Two members of this lipid family, palmitoylethanolamidine (PEA) and oleoylethanolamidine (OEA), have recently emerged as important intrinsic regulators of nociception and inflammation. These substances are released from the membrane precursor, N-acylphosphatidylethanolamine (NAPE), by the action of a NAPE-specific phospholipase D (NAPE-PLD), and in macrophage are primarily deactivated by the lysosomal cysteine amidase, N-acylthanolamine acid amidase (NAAA). NAPE-PLD and NAAA regulate FAE levels, exerting a tight control over the ability of these lipid mediators to recruit PPAR-α and attenuate the inflammatory response. This review summarizes recent findings on the contribution of the FAE-PPAR-α signaling complex in inflammation, and on NAAA inhibition as a novel mechanistic approach to treat chronic inflammatory disorders.

Introduction

Host-defense cells generate a diversity of lipid-derived mediators that are involved in the response to tissue damage and inflammation: for example, they are a major source of eicosanoids such as prostaglandin E₂, which cause local vasodilation and nociceptor sensitization, and act as signals to recruit blood-borne immune cells to injury sites (Laskin et al., 2011). However, a growing body of evidence indicates that host-defense cells can also release bioactive lipids that attenuate rather than instigate pain and inflammation. This expanding group of analgesic and anti-inflammatory lipids includes the fatty acid ethanolamides (FAEs) – palmitoylethanolamidine (PEA) and oleoylethanolamidine (OEA) – which are endogenous agonists for the nuclear receptor, peroxisome proliferator-activated receptor-α (PPAR-α). Macrophages and other cells express a substrate-selective phospholipase D (PLD) that releases PEA and OEA from the membrane phospholipid precursor, N-acylphosphatidylethanolamine (NAPE) (Magotti et al., 2015; Okamoto et al., 2004; Wang et al., 2006). This enzymatic reaction occurs constitutively in intact cells, but is substantially slowed down by a variety of inflammatory insults, including tissue damage or administration of microbial toxins or cytokines (Alhouayek et al., 2015; Bishay et al., 2010; Liu et al., 2006). In macrophages, FAE biosynthesis is inhibited and FAEs levels are reduced mainly through suppression of NAPE-PLD transcription (Sasso et al., 2013; Solorzano et al., 2009; Zhu et al., 2011). Pharmacological blockade of N-acylthanolamine acid amidase (NAAA) – a lysosomal enzyme that is highly expressed in macrophages and B-lymphocytes and is responsible for FAE hydrolysis (Ribeiro et al., 2015; Tsuboi et al., 2007) – restores PEA and OEA levels in inflamed tissues, suggesting that NAAA inhibition might represent a novel mechanistic approach to control inflammation. In this article, we discuss the role of endogenous lipid agonists of PPAR-α as intrinsic controllers of inflammation. Furthermore, we outline recent advances on the discovery of NAAA inhibitors and their effects on the inflammatory process.

Peroxisome proliferator-activated receptor-α

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor superfamily (Issemann & Green, 1990). Three different PPAR subtypes have been identified: PPAR-α, PPAR-γ and PPAR-δ. The PPARs are involved in the modulation of multiple physiological functions, including glucose homeostasis, lipid and lipoprotein metabolism, cell
proliferation and differentiation, apoptosis and control of the inflammatory response (for review, see Kota et al., 2005). Localization studies have shown that PPAR-α is expressed in many mammalian cells and tissues (Bishop-Bailey, 2000; Braissant et al., 1996). These include liver hepatocytes, heart myocytes, intestinal mucosa epithelium and various host-defense cells (including macrophages). Consistent with this broad distribution, PPAR-α plays multiple regulatory functions, including the control of macrophage activity and inflammation (Crisafulli & Cuzzocrea, 2009; Rigamonti et al., 2008).

Upon ligand binding, PPAR-α dimerizes with an obligatory partner, the retinoid X receptor (RXR), and both form a multiprotein complex with a variable set of protein coactivators. In its active form, PPAR-α binds to responsive elements on DNA, enhancing the transcription of various anti-inflammatory proteins, such as inhibitor of κB-α (IkB-α) (Delerive et al., 2000). PPAR-α negatively regulates gene expression of pro-inflammatory proteins by antagonizing the activities of other transcription factors, including members of the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) families, through direct protein-protein interactions (a process referred to as transrepression). Moreover PPAR-α promotes the de novo synthesis of neurosteroids in astrocytes increasing expression of steri-oidogenic acute regulatory protein (StAR) and cytochrome P450 (Mattace-Raso et al., 2009), and potassium-channel regulation (LoVerme et al., 2008).

Endogenous PPAR-α agonists

A variety of naturally occurring fatty-acid derivatives display agonist activity for PPAR-α, including low-potency ligands, such as free fatty acids (Kliwer et al., 1997), and high-potency ligands, such as PEA and OEA. In heterologous expression systems, these FAEs engage PPAR-α with median effective concentration (EC₅₀) values of 0.12 μM for OEA and 3 μM for PEA (Fu et al., 2003; LoVerme et al., 2005). Like other FAEs, PEA and OEA are generated through an enzyme-mediated route that involves two coordinated steps. First, a Ca²⁺-dependent N-acyltransferase activity (Ca-NAT), which remains molecularly uncharacterized, transfers a fatty acyl chain from the sn-1 position of phosphatidylcholine (PC) to the amino group of phosphatidylethanolamine (PE), producing a diverse family of NAPE species. In the second step, the newly formed NAPEs are hydrolyzed to FAEs by NAPE-PLD, a member of the metallo-β-lactamase superfamily of enzymes (Okamoto et al., 2004; Wang et al., 2006) whose three-dimensional structure has been recently elucidated (Magotti et al., 2015). Alternative pathways of FAE biosynthesis that are independent of NAPE-PLD have also been described (Tsmbou et al., 2013). These include: first, the O-deacylation of NAPE to form N-acyl-lyso-PE (lyso-NAPE) followed by lysophospholipase-D (lyso-PLD)-mediated hydrolysis to release FAEs (Simon & Cravatt, 2006, 2008; Sun et al., 2004); second, double O-deacylation of glycerylphospho-FAE (GP-FAE), followed by phosphodiesterase reaction to release FAE (Leung et al., 2006); and, finally, a PLC-type hydrolysis to form FAE-phosphate, followed by phosphatase-mediated hydrolysis to release FAEs (Arreaza et al., 1997; Liu et al., 2006).

The hydrolysis of FAEs into fatty acid and ethanolamine is catalyzed by either of two enzymes: the serine hydrolase fatty acid amide hydrolase (FAAH), which is ubiquitously expressed in rodent tissues but is particularly abundant in brain and liver (Cravatt et al., 1996; Desarnaud et al., 1995; McKinney & Cravatt, 2005), and the cysteine amidase NAAA, which is preferentially expressed in macrophages and B-lymphocytes, but is also present in T-lymphocytes (Ribeiro et al., 2015; Tsuboi et al., 2007) (Figure 1). NAAA was first identified in human megakaryoblastic cells (Ueda et al., 1999), and its cDNA was subsequently cloned from human, rat and mouse (Tsuboi et al., 2005). The protein consists of 359 (human) and 362 (rat and mouse) amino acid residues, with 76.5% sequence identity between human and rat, 76.7% identity between human and mouse, and 90.1% identity between rat and mouse. NAAA shows no sequence homology with FAAH, but has a 33–35% amino acid identity to acid ceramidase, a cysteine amidase encoded by the Asah-1 gene, which hydrolyzes ceramides to fatty acid and sphingo- sine (Tsuboi et al., 2005). The catalytic activity of NAAA is optimal at pH 4.5-5, which is consistent with its postulated lysosomal localization (Tsuboi et al., 2007). Human and rat NAAA mRNA are translated into inactive pro-enzymes and are activated by autocatalytic cleavage (Tsuboi et al., 2005). Localization studies have shown that NAAA is present in tissues that contain high numbers of macrophages and macrophage-like cells – including lungs, spleen and thymus in rodents, as well as prostate, liver, spleen, kidney and pancreas in humans. Relatively low expression levels of the enzyme are found in the rodent and human brain (Tsuboi et al., 2005).

Control of inflammation and macrophage activity

Macrophages eliminate pathogens, remove cellular debris during tissue repair and aging, process antigens and exert antigen-presentation functions (Kaufmann, 2008). They originate from bone marrow-derived monocytes and migrate to tissues, where they undergo differentiation and become specialized to different contexts, developing diverse functions, biochemistry and physiology (e.g. alveolar macrophages in the lungs, Kupffer cells in the liver, osteoclasts in the bones) (Nagy et al., 2012). When exposed to environmental signals deriving from microbes, damaged cells or activated lymphocytes, macrophages undergo functional re-programming that generates a continuum of activated functional phenotypes. Two extremes of the polarized phenotype spectrum have been described: classical M1 activated macrophages, stimulated by Toll-like receptor (TLR) ligands and interferon-γ (IFN-γ), and alternative M2 activated macrophages stimulated by interleukin-4 (IL-4) and IL-13 (Sica & Mantovani, 2012). M1 macrophages express high levels of pro-inflammatory cytokines, produce reactive oxygen and nitrogen species and act as effectors of resistance
against intracellular parasites and tumors. On the other hand, M2 macrophages are characterized by the expression of mannose and galactose receptors, and the production of polyamines. M2 cells are inefficient at antigen presentation, but promote wound healing and tissue remodeling, and exert immune-regulatory functions. The M1/M2 polarized states are regulated by a variety of factors including members of the PPAR family of nuclear receptors (Kiss et al., 2013; Mattace-Raso et al., 2014; Rigamonti et al., 2008).

Several studies have documented the anti-inflammatory consequences of PPAR-α activation in human and murine macrophages. PPAR-α is involved in the in vitro polarization of macrophages isolated from mice infected with Trypanosoma cruzi (Penas et al., 2015). T. cruzi infection determines M1-type macrophage activation, inducing the expression of nitric oxide synthase-2 (NOS-2), the release of nitric oxide and the secretion of tumor necrosis factor-α (TNF-α), IL-6 and IL-1β. The synthetic PPAR-α agonist WY14643 promotes the switch from the M1- to the M2-polarized phenotype, inducing a decrease in the expression of NOS-2 and pro-inflammatory cytokines, and increasing expression of M2 markers like arginase 1, Ym1, mannose receptor and transforming growth factor-β (TGF-β). In addition, in lipopolysaccharide (LPS)-stimulated human monocytes, PPAR-α activation inhibits the production of various pro-inflammatory molecules such as metalloproteinase-9 (MMP-9) (Shu et al., 2000) and tissue factor (TF) (Marx et al., 2001; Neve et al., 2001) by negatively interfering with the AP1 and NF-kB signaling pathway (Figure 2). These anti-inflammatory effects are absent in macrophages from PPAR-α-deficient mice (Crisafulli, 2009), which also show enhanced responses to several inflammatory stimuli (Crisafulli, 2009), an important indication that PPAR-α participates in the control of inflammation. Interestingly, exogenous PEA stimulates phagocytosis of Escherichia coli K1 by macrophages and increases the resistance of mice against infections; it is not known, however, whether this effect is mediated by PPAR-α activation (Redlich et al., 2014).

Effect of inflammation on FAE metabolism

Evidence indicates that inflammatory stimuli can alter the metabolism of bioactive FAEs that, in turn, affect the progression of the inflammatory process. Experiments with cultures of macrophage-like RAW264.7 cells have shown that LPS lowers cellular PEA content by suppressing the transcription of the Napepld gene (Zhu et al., 2011). This effect occurs through an epigenetic mechanism that requires changes in the acetylation state of histone proteins bound to the Napepld promoter. Consistent with those findings, LPS does not lower PEA levels in macrophages isolated from Napepld-deficient (NAPE-PLD−/−) mice, which produce PEA through an alternative enzyme pathway that is not influenced by inflammatory stimuli (Zhu et al., 2011).

Alterations in FAE metabolism have also been observed in various animal models of acute and chronic inflammation. For example, lipid analyses of inflammatory exudates from mice exposed to LPS or the pro-inflammatory polysaccharide carrageenan have shown that PEA levels are substantially
lowered during inflammation (Solorzano et al., 2009). Similarly, the tissue PEA content is reduced in carrageenan-induced granuloma (De Filippis et al., 2010), an established murine model of chronic inflammation (De Filippis et al., 2009), as well as in the small intestine of mice treated with croton oil (Capasso et al., 2001; Izzo et al., 2012). Due to the early discovery that PEA exerts profound anti-inflammatory effects (for review, see LoVerme & Piomelli, 2005), many studies on the impact of inflammation on FAE production have been focused on PEA. However, there is evidence that changes in OEA levels parallel those observed with PEA (Sasso et al., 2013).

Endogenous FAEs might also participate in the modulation of the inflammatory process in humans. This possibility is suggested by studies showing that the concentrations of PEA and OEA in synovial fluid are substantially reduced in subjects with rheumatoid arthritis and osteoarthritis, compared to those measured in healthy controls (Richardson et al., 2008). Moreover, immunohistochemical analysis have shown that PPAR-α expression is decreased in biopsies of colonic epithelium from subjects with active colitis, relative to healthy subjects, and this change is accompanied by a marked local decrease in NAPE-PLD expression and increase in NAAA expression (Suárez et al., 2012). Moreover, NAAA inhibitors reinstate normal FAE levels and restore FAE signaling at PPAR-α, causing an attenuation of the inflammatory response. Interestingly, PPAR-α expression in macrophages is up-regulated by PPAR-α activation (LoVerme et al., 2005), which may contribute to the anti-inflammatory actions of NAAA inhibitors. (see colour version of this figure at www.informahealthcare.com/bmg).

NAAA inhibitors

Despite the availability of several classes of anti-inflammatory drugs, non-resolving inflammatory pathologies such as rheumatoid arthritis and inflammatory bowel disease remain a medical challenge due to the limited efficacy and frequent side effects of existing therapies. Modulation of FAE levels in inflamed tissues by NAAA inhibition may represent a new therapeutic strategy for the treatment of inflammation (Bandiera et al., 2014; Ribeiro et al., 2015). The first NAAA inhibitors to be identified were obtained by introducing small modifications in the scaffold of the natural substrate, PEA (e.g. replacement of the amide group) (Tsuboi et al., 2004). Among them was the compound...
Table 1. NAAA inhibitors tested on preclinical models of inflammation. Potency, biological effects and administration route are reported.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC50</th>
<th>Biological effects</th>
<th>Administration route</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-OOPP</td>
<td><img src="image" alt="Structure" /></td>
<td>420 nM</td>
<td>Normalizes FAE levels&lt;br&gt;Reduces neutrophil migration into carrageenan-containing sponge.&lt;br&gt;Reduces spinal cord injury.</td>
<td>Topical</td>
<td>Solorzano et al., 2009</td>
</tr>
<tr>
<td>ARN077</td>
<td><img src="image" alt="Structure" /></td>
<td>50 nM on rat NAAA&lt;br&gt;7 nM on human NAAA</td>
<td>Normalizes FAE tissue levels&lt;br&gt;Reduces TPA-induced edema&lt;br&gt;Reduces paw edema and heat hyperalgesia in carrageenan-induced inflammation&lt;br&gt;Reduces heat hyperalgesia and mechanical allodynia induced by chronic nerve constriction&lt;br&gt;Reduces heat hyperalgesia in ultraviolet B-induced inflammation</td>
<td>Topical</td>
<td>Ponzano et al., 2013; Sasso et al., 2013</td>
</tr>
<tr>
<td>AM9053</td>
<td>Unknown structure</td>
<td></td>
<td>Normalizes FAE tissue levels&lt;br&gt;Reduces MPO activity&lt;br&gt;Reduces the expression of proinflammatory cytokines in the colon</td>
<td>i.p.</td>
<td>Alhouayek et al., 2015</td>
</tr>
<tr>
<td>Diacerein</td>
<td><img src="image" alt="Structure" /></td>
<td>7.2 μM</td>
<td>Normalizes FAE tissue levels&lt;br&gt;Reduces paw edema and heat hyperalgesia in carrageenan-induced inflammation</td>
<td>i.p.</td>
<td>Petrosino et al., 2015</td>
</tr>
<tr>
<td>ARN726</td>
<td><img src="image" alt="Structure" /></td>
<td>63 nM on rat NAAA&lt;br&gt;27 nM on human NAAA</td>
<td>Normalizes FAE tissue levels&lt;br&gt;Reduces MPO activity&lt;br&gt;Reduces the expression of proinflammatory cytokines in the colon</td>
<td>Oral</td>
<td>Ribeiro et al., 2015</td>
</tr>
</tbody>
</table>
N-pentadecylcyclohexanecarboxamide, which was reported to be a single-digit micromolar inhibitor of rat lung NAAA activity (median inhibitory concentration, IC50, 4.5 μM (Tsuboi et al., 2004). The compound was also shown to inhibit NAAA activity in rat alveolar macrophages, although incubation with high micromolar concentration was needed to achieve significant enzyme blockade. Subsequent studies failed, however, to confirm the ability of N-pentadecylcyclohexanecarboxamide to affect NAAA activity (Solorzano et al., 2009). Subsequent attempts to modify the structure of palmitic acid to yield NAAA inhibitors were met by limited success (Saturnino et al., 2010).

A significant step forward was accomplished with the identification of the β-lactone derivative N-[(3S)-2-oxo-3-oxetanyl]-3-phenylpropanamide [(S)-OOPP], which inhibits NAAA activity in vitro with an IC50 of 420 nM (Solorzano et al., 2009) (Table 1). Kinetic analyzes showed that (S)-OOPP affects NAAA activity through a noncompetitive mechanism. Moreover (S)-OOPP was found to be selective for NAAA over acid ceramidase (IC50 = 10.9 ± 3.1 μM) and FAAH (IC50 ≥ 100 μM). When tested on intact RAW264.7 macrophages stimulated with LPS, (S)-OOPP inhibits NAAA activity and blocks LPS-induced reduction of PEA levels (Solorzano et al., 2009). Moreover in vivo studies demonstrated that (S)-OOPP reduces carrageenan-induced leukocytes migration and lowers the expression of inflammatory and apoptotic markers in a mouse model of spinal cord injury (Solorzano et al., 2009). Notably, (S)-OOPP does not decrease inflammatory responses in PPAR-α deficient mice, and its anti-inflammatory effects are mimicked by PPAR-α activation (caused by PEA or the potent synthetic ligand GW7647), providing evidence that NAAA inhibition restores FAE signaling at PPAR-α during inflammation and blocks tissue reactions to inflammatory stimuli. Subsequent investigations lead to the identification of another β-lactone derivative, the compound ARN077 (Table 1), which inhibits NAAA with higher potency (IC50 = 50 nM on rat NAAA and 7 nM human NAAA) and selectivity than (S)-OOPP (Ponzano et al., 2013; Sasso et al., 2013), through a mechanism that requires the formation of a covalent adduct with the catalytic cysteine of NAAA (Armirotti et al., 2012). A similar mechanism of action has been postulated for the compound benzylxoycarbonyl-l-serine β-lactone (West et al., 2012). Topical administration of ARN077 restores tissue PEA and OEA levels, and attenuates hyperalgesic and allodynic states elicited in mice and rats by local inflammation or nerve damage (Sasso et al., 2013).

Due to its metabolic instability, ARN077 can only be used as a soft drug (i.e. by topical administration). This limitation was in part overcome by the introduction of the β-lactam derivative ARN726, the prototype of a new class of NAAA inhibitors with significant systemic activity (Ribeiro et al., 2015). The increased metabolic stability of ARN726, compared to members of the β-lactone series such as ARN077, allowed the compound to be tested in mouse models of pulmonary inflammation. Intratracheal injection of carrageenan lowered FAE levels, stimulated leukocyte migration and increased levels of inflammatory cytokines in pleural exudate. ARN726 dose-dependently prevented each of these responses in wild-type, but not in PPAR-α deficient mice (Ribeiro et al., 2015). Comparable anti-inflammatory effects were seen when inflammation was induced by intranasal instillation of the bacterial endotoxin LPS. Importantly, exposure to ARN726 markedly inhibited TNF-α release from human macrophages stimulated with LPS, suggesting that the β-lactam scaffold might be utilized not only as a probe, but also as a starting point for the discovery of novel anti-inflammatory medicines.

Another systemically active NAAA inhibitor, AM9053, was disclosed by Alhouayek and colleagues, and tested in two mouse models of colitis. AM9053 inhibits NAAA activity with an IC50 of 30 nM while having little or no effect on FAAH activity (IC50 = 100 μM). Administration of this compound increased PEA levels, decreased inflammation and reduced leukocyte infiltration in mouse colon (Alhouayek et al., 2015). Moreover, administration of AM9053 lowered the local expression of inflammatory cytokines (IL-1β, IL-12 and TNF-α) (Alhouayek et al., 2015). At the time of this writing, the chemical structure of AM9053 has not yet been disclosed. Finally, the anti-inflammatory anthraquinone, diacerein (Table 1), was shown to inhibit human NAAA activity with an IC50 of 7.2 μM (Petrosino et al., 2015). Whether NAAA inhibition is involved in mediating the therapeutic effects of this drug remains, however, to be determined.

**Conclusions**

Bioactive lipid mediators have emerged as important regulators of pain and inflammation. In this article, we briefly reviewed evidence supporting the idea that modulating FAE signaling at PPAR-α may contribute to the therapeutic management of painful and inflammatory conditions in which this signaling system is defective. Such conditions might include osteoarthritis, rheumatoid arthritis and inflammatory bowel disease, as evidenced by alterations in the levels of FAEs and NAAA in biopsies from subjects with these disorders (Richardson et al., 2008; Suárez et al., 2012). Targeting NAAA, the enzyme responsible for FAE degradation, appears to be a promising strategy for the treatment of non-resolving inflammatory disorders.

**Declaration of interest**

The authors declare the following financial interest. DP is inventor in patent applications filed by the University of California and the Fondazione Istituto Italiano di Tecnologia, which protect composition and use of chemicals described in this study.

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