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Palmitoylethanolamide inhibits rMCP-5 expression by regulating MITF activation in rat chronic granulomatous inflammation

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ABSTRACT

Chronic inflammation, a condition frequently associated with several pathologies, is characterized by angiogenic and fibrogenic responses that may account for the development of granulomatous tissue. We previously demonstrated that the chymase, rat mast cell protease-5 (rMCP-5), exhibits pro-inflammatory and pro-angiogenic properties in a model of chronic inflammation sustained by mast cells (MCs), granuloma induced by the subcutaneous carrageenan-soaked sponge implant in rat. In this study, we investigated the effects of palmitoylethanolamide (PEA), an anti-inflammatory and analgesic endogenous compound, on rMCP-5 mRNA expression and Microphtalmia-associated Transcription Factor (MITF) activation in the same model of chronic inflammation. The levels of rMCP-5 mRNA were detected using semi-quantitative RT-PCR; the protein expression of chymase and extracellular signal-regulated kinases (ERK) were analyzed by western blot; MITF/DNA binding activity and MITF phosphorylation were assessed by electrophoretic mobility shift assay (EMSA) and immunoprecipitation, respectively. The administration of PEA (200, 400 and 800 μg/ml) significantly decreased rMCP-5 mRNA and chymase protein expression induced by λ-carrageenan. These effects were associated with a significant decrease of MITF/DNA binding activity and phosphorylated MITF as well as phosphorylated ERK levels.

In conclusion, our results, showing the ability of PEA to inhibit MITF activation and chymase expression in granulomatous tissue, may yield new insights into the understanding of the signaling pathways leading to MITF activation controlled by PEA.

1. Introduction

Rat mast cell protease-5 (rMCP-5) belongs to a family of serine proteases classified as chymases (Sanker et al., 1997). Among rodent chymases, rMCP-5 is predominantly expressed in connective tissue type mast cells (MCs) and also in early phase of MC development (Sanker et al., 1997). Like other MC proteases (MCPs), rMCP-5 is packed in the MC secretory granules intimately bound to proteoglycans and is released, together with several other stored mediators, following degranulation (Forsberg et al., 1999). Several evidences show that MCPs play a crucial role in the inflammatory/immune process in mammals (Badertscher et al., 2005). It is known that MCPs play an important role in the allergen-induced biphasic skin reaction (Tomimori et al., 2002) and in eliciting or maintaining cutaneous inflammation in atopic dermatitis (Badertscher et al., 2005). Moreover, chymases have been proposed to increase vascular permeability both in skin disease (He and Walls, 1998) and brain edema during intracerebral hemorrhage (Strbian, et al., 2009). Chymases have been shown to induce the release of neutrophil chemoattractants by eosinophils (Terakawa et al., 2006) and to mediate interaction between MCs and eosinophils in allergic diseases (Wong et al., 2009).

We have previously demonstrated that rMCP-5 chymase exhibits pro-inflammatory and pro-angiogenic effects in rat λ-carrageenan-induced granuloma, i.e., a model of chronic inflammation actively sustained by MC activation (Russo et al., 2005). It has been demonstrated that Microphthalmia-associated Transcription Factor (MITF) controls the transcription of a spectrum of genes in MCs, including several MCPs, adhesion molecules, metabolic enzyme and growth factor receptors (Kitamura et al., 2006; Razin et al., 1999; Morii et al., 2001). MITF belongs to Myc supergene family of basic helix–loop–helix leucine zipper (bHLH-Zip) DNA-binding protein which is predominantly expressed in MCs, melanocytes, heart and skeletal muscles (Hershey and Fish, 2004).

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Palmitoylethanolamide (PEA) is an endogenous lipid signaling molecule produced locally "on demand" and exhibits potent anti-inflammatory properties, which define PEA as ALIAmide (autacoid local inflammation antagonist amide) (Aloe et al., 1993). The ALIA mechanism is based on the important role played by PEA on MCs during inflammation; PEA is able to naturally control MC hyperactivity, which occurs not only in inflammation, but also in hyperalgesia and allodynia (Skaper et al., 2013; De Filippis et al., 2013). Although it has been suggested that PEA can activate different receptors, a specific receptor responsible for PEA effects is still under debate. In fact, PEA exhibits low affinity for cannabinoid CB_1 (Ki $>5\,\mu M$) and CB_2 (Ki $>5\,\mu M$) receptors, and the activation of a CB_2-like receptor has only been hypothesized (Calignano et al., 1998). It has also been described that PEA is able to activate and desensitize the transient receptor potential cation channel V type1 (TRPV1) and K(+) channels (Kv4.3 and Kv1.5) (Ambrosino et al., 2013; De Novellis et al., 2012). Moreover, some anti-inflammatory and anti-nociceptive effects of PEA have been ascribed to a PPAR-α (peroxisome proliferator-activated receptor) direct mechanism (Lo Verme et al., 2005). In addition, the ability of PEA to potentiate anandamide tone (Lambert and Di Marzo, 1999), known as "entourage effect", has been used to explain its pleiotropic effects (Scuderi et al., 2011; D'Agostino et al., 2012). Finally, a so-called orphan receptor GPR-55 has been evoked as responsible for some other PEA-mediated actions (Cantarella et al., 2011).

We have previously demonstrated that PEA was able to reduce granuloma formation in a model of chronic inflammation, the subcutaneous implant of carrageenan-soaked sponge in rat (De Filippis et al., 2010). On the basis of these observations, the aim of the present study was to investigate the effect of PEA on MITF activation and rMCP-5 expression in the same model of chronic inflammation.

2. Material and methods

2.1. Sponge implantation

Male Wistar rats (Harlan, Italy), weighing 200–220 g, were used in all experiments. Animals were provided with food and water ad libitum. Sponge implant in the rat was performed as previously described (De Filippis et al., 2010). λ-Carrageenan (1% w/v) (Sigma) was dissolved in pyrogen-free saline (0.5 ml/sponge), in the presence or absence of 100 μl of micronized synthetic PEA (kindly provided by Epitech Group; purity > 98%) at different concentrations (200, 400, and 800 μg/ml) in final volume of 0.5 ml/sponge; saline (0.5 ml/sponge) was used as control. 96 h after sponge implant rats were sacrificed in an atmosphere of CO_2. The granulomatous tissue around the sponge was dissected by using a surgical blade, weighed, quickly frozen in liquid nitrogen, and stored at −80 °C. Animal care as well as all experiments was in accordance with European Community Council Directive 86/609/EEC and efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts from granulomatous tissues were performed as previously described (De Filippis et al., 2010). Protein concentration was determined by Bio-Rad protein assay kit.

2.3. mRNA analysis

The mRNA level of rMCP-5 in granulomatous tissue was determined using the semi-quantitative RT-PCR method as previously described (Russo et al., 2006, 2008). The PCR-primers were selected according to the rat rMCP-5 cDNA sequence (forward primer 5′-TCCTGCAAACACTTCACCAG-3′, and reverse primer 5′-CGAGATCCAGAGTTAATTCT-3′); and rat β-actin cDNA (forward

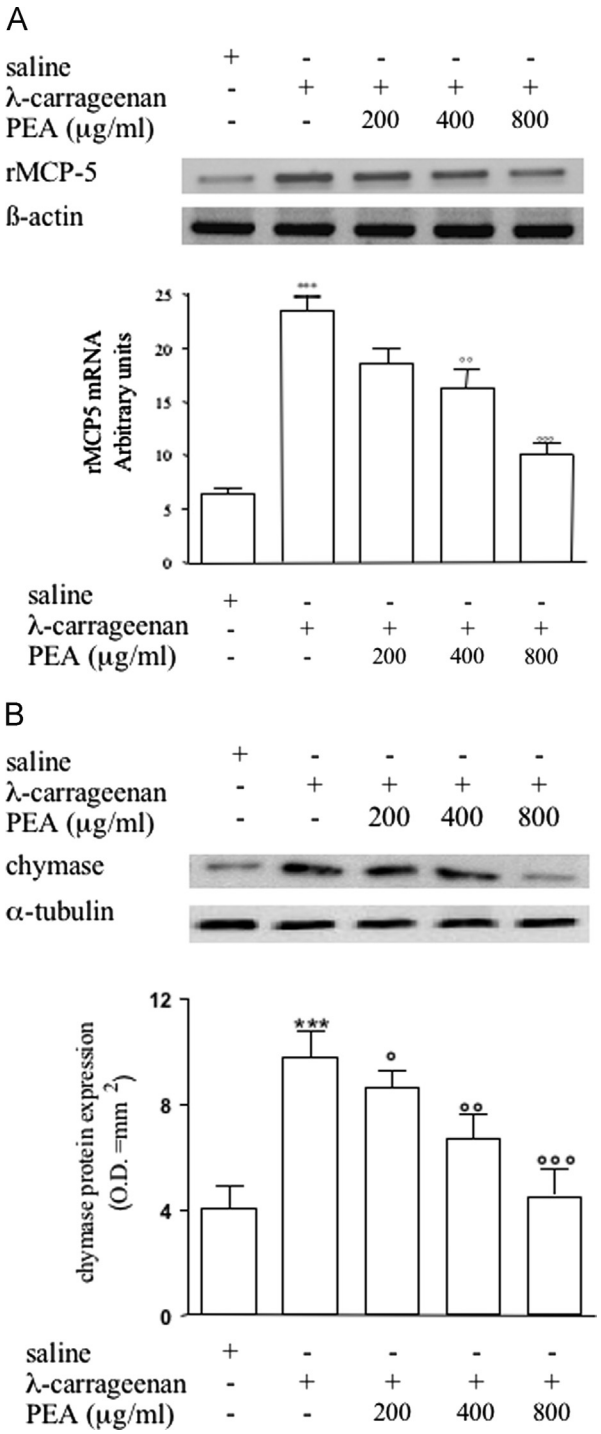

Fig. 1. (A) Effect of PEA on rMCP-5 transcription induced by λ-carrageenan in granulomatous tissue. Representative Vistra green-stained agarose gel of RT-PCR products, corresponding to rMCP-5mRNA in sponges injected with saline, λ-carrageenan (1% w-v), or λ-carrageenan in the presence of increasing amount of PEA (200, 400, and 800 μg/ml). β-actin, a housekeeping gene, was used as control. (B) Effect of PEA on λ-carrageenan-induced chymase expression in granulomatous tissue. Representative western blot analysis and relative densitometric analysis of chymase levels in sponges injected with saline, λ-carrageenan (1% w-v), or λ-carrageenan in the presence of increasing amount of PEA (200, 400, and 800 μg/ml). Tubulin expression is shown as control. Quantification of results is expressed as mean ± S.E.M. of three experiments. ***$P<0.001$ vs. saline.; °$P<0.05$, °°$P<0.01$, and °°°$P<0.001$ vs. λ-carrageenan alone.

primer 5′-GGCACCACACCTTCTACA-3′ nucleotide positions 330–348, and reverse primer 5′-CAGGAGGAGCAATGATCT-3′). 15 μl aliquots of PCR products were electrophoretically fractionated through 1% agarose gel containing the fluorescent Vistra green dye (Amersham Pharmacia Biotech, GE Healthcare; Switzerland). Labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the Molecular Imager FX and Quantity One software (Bio-Rad, Milan, Italy).

2.4. Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides containing the MITF recognition sequence (5′-CCT AGA CAG ACA AAA CCT AGA CAA TCA CGT GGC TGG-3′) were end-labeled with ^{32}P–γ–ATP (Amersham, Milan, Italy), and EMSA was performed as previously described (De Filippis et al., 2010).

2.5. Immunoprecipitation

Immunoprecipitation assay was performed as previously described (Russo et al., 2013) by using anti-mouse MITF (Lifespam; WA, USA). The beads were washed and boiled in the SDS sample buffer. The eluted proteins were loaded on 12% SDS-PAGE and analyzed by western blotting.

2.6. Western blotting

Immunoblotting analysis of chymase, ERK, pERK and tubulin proteins was performed on total protein fractions of granulomatous tissue homogenates, as previously described (De Filippis et al., 2010). Membranes were saturated by incubation at 4 °C overnight with 10% non-fat dry milk in 1 X PBS and then incubated with the appropriate antiserum: anti-mouse chymase (1:1000 v-v; Santa-cruz Biotechnology, CA), anti-phosphoserine (1:1000 v-v; Pierce, Rockford, Illinois), anti-pERK (1:2000 v-v, Cell Signaling Technology Inc, MA) and anti-mouse tubulin (1:1000 v-v, Santa Cruz, CA, USA) for 2 h at room temperature. Membranes were washed three times with 1% Triton X-100 in 1 X PBS and then incubated with anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase (1:2000 v-v; Dako, Denmark). The immune complexes were revealed by using enhanced chemiluminescence detection reagents (Amersham, GE Healthcare; Switzerland) according to the manufacturer's instructions in Image quant 800 apparatus. The protein bands were analyzed by densitometric analysis with a GS-800 imaging densitometer.

2.7. Statistical analysis

Results were expressed as the mean ± S.E.M. of n animals where each value is the average of responses in duplicate sites. Statistical comparisons were made by one-way ANOVA followed by Bonferroni's test for multiple comparisons. $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of PEA on rMCP-5 transcription induced by λ-carrageenan in granulomatous tissue

In order to investigate whether PEA was able to influence rMCP-5 levels, we analyzed the amount of rMCP-5 mRNA in

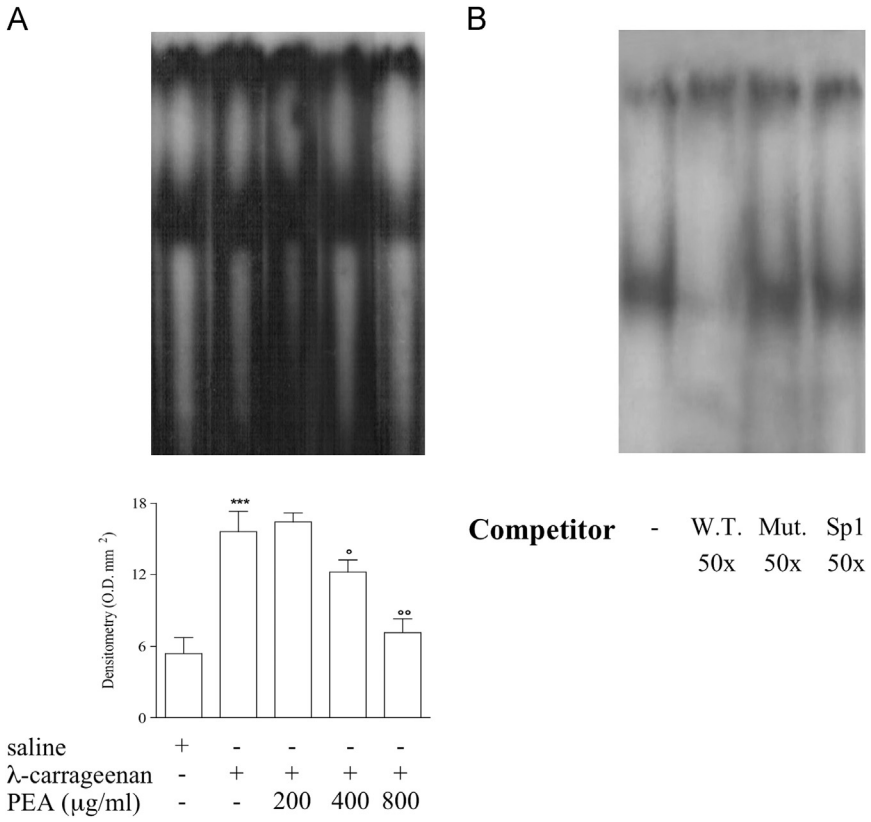

Fig. 2. Effect of PEA on DNA binding activity and characterization of MITF complex in λ-carrageenan-induced granuloma. (A) EMSA showing the MITF binding activity in nuclear extracts from granulomatous tissues. Data are representative of three separate assays. Densitometric data are expressed as mean ± S.E.M. of three sponges from three rats. ***$P < 0.001$ vs. saline; °$P < 0.05$, and °°$P < 0.001$ vs. λ-carrageenan. (B) In competition reaction, nuclear extracts were incubated with radiolabeled MITF probe in the absence or presence of identical but unlabeled oligonucleotide (WT, 50 ×), mutated non-functional MITF probe (Mut., 50 ×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50 ×). Data are representative of three experiments.

λ-carrageenan-induced granulomatous tissue in the presence or absence of PEA. As expected, λ-carrageenan treatment resulted in accumulation of rMCP-5 transcript compared to saline. Co-treatment of λ-carrageenan and PEA (200, 400 and 800 μg/ml), locally injected, caused a dose-dependent reduction of rMCP-5 mRNA levels in tissue homogenates compared to λ-carrageenan alone (Fig. 1A). The inhibition of rMCP-5 expression induced by PEA was also confirmed by western blot analysis. As shown in Fig. 1B, PEA treatment decreased the chymase rMCP-5 protein amounts in a dose dependent manner.

3.2. Effect of PEA on MITF DNA binding activity in λ-carrageenan-induced granuloma

MITF has been reported to regulate the development of MCs and the expression of some MCPs in mouse (Ge et al., 2001). In the attempt to understand the mechanism by which PEA is able to regulate rMCP-5 expression, we investigated whether PEA was able to influence MITF DNA binding activity. To this aim, nuclear extracts of granulomatous tissues from saline-treated, λ-carrageenan-treated or λ-carrageenan and PEA co-treated animals were analyzed by EMSA. Although a basal level of MITF DNA binding activity was detected in nuclear extracts from tissues of saline-treated sponges evaluated 96 h after implant, λ-carrageenan treatment induced a marked increase of MITF DNA binding activity. Intrestingly, the local administration of PEA resulted in a significant and dose-dependent reduction of MITF DNA binding activity (Fig. 2A). The specificity of MITF DNA complex was determined by competition experiments. In fact, 50-fold molar excess of unlabeled MITF probe was able to completely displace MITF from DNA. In contrast, a 50-fold molar excess of unlabeled mutated MITF probe or Sp-1 had no effect on DNA binding activity (Fig. 2B).

3.3. Effect of PEA on MITF phosphorylation in granulomatous tissues induced by λ-carrageenan

Proteins phosphorylation–dephosphorylation is a key step in the regulation of protein–protein interactions. To further analyze MITF-mediated down-regulation of rMCP-5 expression caused by PEA administration, we verified whether PEA was able to control the phosphorylation status of MITF in granulomatous tissue. To this purpose, immunoprecipitation experiment was performed. MITF was specifically immunoprecipitated from protein extracts of tissue from saline-treated, λ-carrageenan-treated or λ-carrageenan and PEA co-treated animals and the amount of phosphorylated form of MITF was detected by western blotting using anti-phosphoserine antibody. Fig. 3B shows that a significant increase (2.6-fold) of the amount of phosphorylated MITF in λ-carrageenan-induced granulomatous tissue compared to saline was observed. Interestingly, PEA treatment caused a decrease in the levels of phosphorylated MITF compared to λ-carrageenan-induced granulomatous tissue. Our data are in agreement with several evidences indicating that phosphorylation of MITF Ser73 increases its transcriptional activity (Price et al., 1998).

3.4. Effect of PEA on ERK protein expression induced by λ-carrageenan

One signaling module that controls MITF is the RAS–RAF–MEK–ERK signaling cascade. It is known that MITF is phosphorylated on Ser73 by ERK, and this modification enhances its transcriptional activity (Price et al., 1998). In order to evaluate the role of ERK in the phosphorylation of MITF upon PEA treatment, proteins were extracted from tissue of saline-treated, λ-carrageenan-treated or λ-carrageenan and PEA co-treated animals and were analyzed by western blotting. Fig. 4 shows a significant increase in the amount of phosphorylated ERK in λ-carrageenan-induced granulomatous tissue compared to saline. On the contrary, PEA treatment caused a decrease of phosphorylated ERK levels compared to λ-carrageenan-induced granulomatous tissue. The intracellular levels of ERK protein were not affected by λ-carrageenan or PEA treatment (Fig. 4).

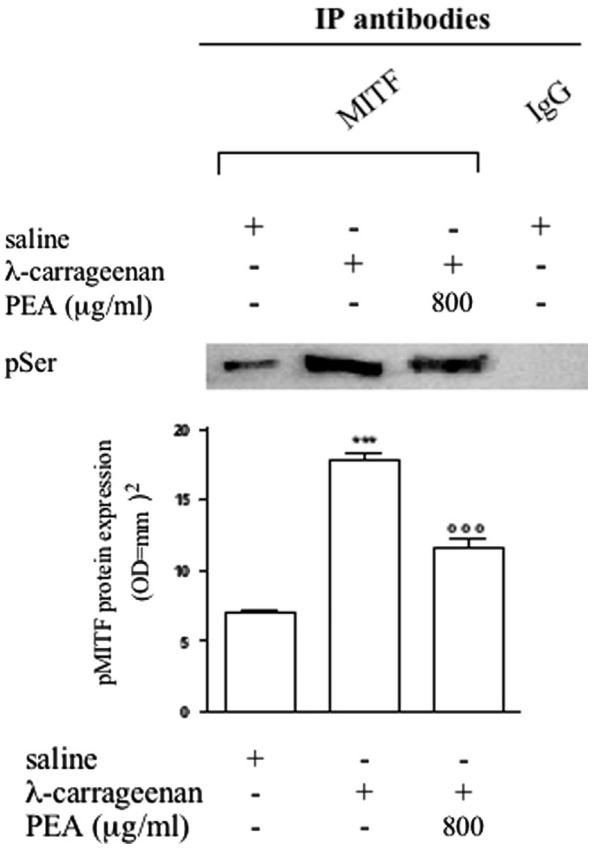

Fig. 3. (A) Effect of PEA on λ-carrageenan-induced MITF protein phosphorylation in granulomatous tissue. Representative western blot analysis and relative densitometric analysis of phosphorylated MITF protein levels. MITF was specifically immunoprecipitated from granulomatous tissues treated with saline, λ-carrageenan (1% w-v), or λ-carrageenan in the presence of PEA 800 μg/ml. After immunoprecipitation, the phosphorylation of MITF was detected by immunoblot analysis by using phospho-serine (pSer) antibody. The levels of proteins were quantified by PhosphorImager (Bio-Rad). Quantification of results is expressed as mean ± S.E.M. of three experiments. $^{***}P < 0.001$ vs. saline.; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, and $^{\circ\circ\circ}P < 0.001$ vs. λ-carrageenan alone.

4. Discussion

Several evidences indicate that PEA is able to control both acute and chronic inflammations, including that mediated by MC (Skaper et al., 2013). We have previously studied rMCP-5 chymase, a protease selectively stored in MC, for its pivotal role in granuloma formation induced by λ-carrageenan in rat (Russo et al., 2005). In the same model of chronic inflammation, we demonstrated that the local administration of PEA, by controlling MC activation, accounted for a significant reduction of both granulomatous tissue and associated angiogenesis (De Filippis et al., 2010, 2011). On the basis of these evidences, in the present study we investigated the effects of PEA on the expression of rMCP-5 chymase, a pro-inflammatory and pro-angiogenic mediator. We found that PEA was able to down-regulate rMCP-5 chymase mRNA and protein expression in the granulomatous tissue induced by λ-carrageenan in rat. It has been reported that MITF is essential in the regulation of

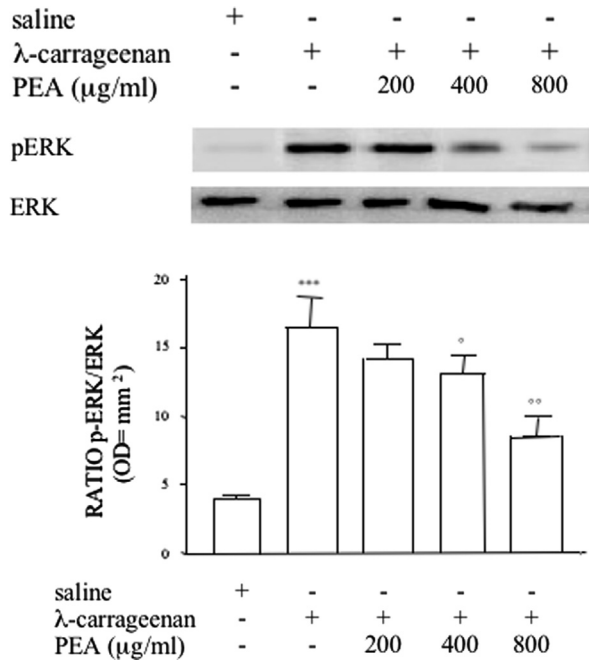

Fig. 4. Effect of PEA on λ-carrageenan-induced pERK protein expression in granulomatous tissue. Representative western blot analysis and relative densitometric analysis of ERK protein levels in sponges injected with saline, λ-carrageenan (1% w-v), or λ-carrageenan in the presence of increasing amount of PEA (200, 400, and 800 μg/ml). The phosphorylation of ERK was detected by immunoblot analysis by using anti-pERK antibody. The ratio of pERK to ERK is shown. Quantification of results is expressed as mean ± S.E.M. of three experiments. $^{***}P < 0.001$ vs. saline; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, and $^{\circ\circ\circ}P < 0.001$ vs. λ-carrageenan alone.

MCP-5 chymase (Morii et al., 1997). Our results, for the first time, show that PEA significantly inhibited MITF/DNA binding activity induced by λ-carrageenan in granuloma.

Changes in protein phosphorylation represent a mechanism to modulate the activity of MITF and three serine sites (Ser73, Ser298 and Ser409) for MITF phosphorylation have been reported (Sonnenblick et al., 2004). The phosphorylation of these serine sites positively correlates with the up-regulation of MITF transcriptional activity (Price et al., 1998; Takeda et al., 2000). In our study, we found that PEA was able to regulate the switch from the phosphorylated MITF (active state) to the unphosphorylated MITF (inactive state), preventing the phosphorylation of MITF triggered by λ-carrageenan in rat granulomatous tissue. Moreover, we demonstrated PEA treatment was able to reduce the amount of ERK phosphorylated form. This evidence suggests that the reduction in the phosphorylated form of MITF may be a consequence of the inhibition of the classical MAP kinase pathway. It has been reported that PEA controls the signal transduction involved in the activation of important pro-inflammatory transcription factors including AP-1 (Scuderi et al., 2011) and NF-κB (D'Agostino et al., 2009). Therefore, it is reasonable to hypothesize that PEA may affect the signaling pathways leading to the activation of MITF and MITF-dependent chymase expressions in granuloma formation. The inhibition of chymase rMCP-5 expression by PEA thus suggests that the control of MC activation, previously demonstrated in the same model of chronic inflammatory (De Filippis et al., 2010), may occur via MITF.

In conclusion, our results put new insights into the long history of experiments connecting PEA and MCs, firstly hypothesized by the Nobel prize winner Rita Levi-Montalcini, indicating the inhibition of MITF a new mode of action for PEA in inflammatory diseases. Otherwise, these data could also be useful to strengthen the previously reported use of PEA in cancers, above all in melanoma (Hamtiaux et al., 2012), where MITF activation plays a crucial role (for review see Koludrovic and Davidson, 2013). Therefore, this study may open the way to a pharmacological approach with PEA for all those chronic-degenerative pathologies which are dependent on MITF activation.

Acknowledgments

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