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Research Article

Role of PPARα and PPARγ in Mediating the Analgesic Properties of Ibuprofen *in vivo* and the Effects of Dual PPARα/γ Activation in Inflammatory Pain Model in the Rat

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Abstract

Background and Objective: Ibuprofen is commonly used to treat various inflammatory pain disorders. Its analgesic properties are attributed mainly to inhibiting cyclooxygenases enzymes and blocking the production of prostaglandins. The aim of this study is to evaluate the role of PPARα and PPARγ in mediating the anti-nociceptive effects of ibuprofen *in vivo*. The anti-nociceptive effects of PPARα and PPARγ dual activation are also investigated. **Methodology:** Hot plate analgesia meter and the von Frey filament test were used to evaluate the anti-hyperalgesic effects of ibuprofen on CFA inflammatory pain model in rat and the role played by PPARα and PPARγ in mediating these effects. Behavioral tests were performed at two different time points (1 and 6 h) representing the slow and rapid-onset effects of ibuprofen. **Results:** Ibuprofen (100 μg) significantly restored both the Paw Withdrawal Latency (PWL) and Paw Withdrawal Thresholds (PWT) after CFA injection at the 2 time points selected. Co-administration of the PPARα antagonist GW6471 (50 μg) or the PPARγ antagonist GW9662 (50 μg) significantly reduced the anti-nociceptive effects of ibuprofen on PWT 1 h post-drug administration. However, co-administration of GW6471 but not GW9662 significantly reduced the inhibitory effects of ibuprofen on PWL 1 h post-drug administration. Co-administration of GW6471 or GW9662 significantly reduced the inhibitory effects of ibuprofen on both PWL and PWT at 6 h post-drug administration. The dual PPARα/γ agonist tesaglitazar (5 μg) only reversed thermal PWL at 6 h post-drug administration. **Conclusion:** The PPARα and PPARγ at least partially mediate the analgesic properties of ibuprofen. The dual activation of PPARα/γ receptors could provide a promising strategy to control chronic pain conditions characterized by thermal hyperalgesia.

Key words: Peroxisome proliferator-activated receptor α, peroxisome proliferator-activated receptor γ, ibuprofen, tesaglitazar, pain

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The three peroxisome proliferator-activated receptors (PPARs) subtypes, PPARα, PPARβ and PPARγ[1,2] are nuclear receptors which act as transcriptional regulators of large number of genes to modulate many metabolic events such as lipid metabolism[3], inflammation[4] and insulin sensitivity[5]. The PPARα is highly expressed in the liver, skeletal muscle and dorsal root ganglia neurons[6,7]. It can be activated by large number of molecules such as natural fatty acids and fibrate class of hypolipidemic drugs[8] and its activation produces profound anti-nociceptive effects in animal models of inflammatory and neuropathic pain, which are consistent with the PPARα expression in the pain pathway[7,9]. Similarly, PPARγ is widely expressed in areas involved in pain processing including neurons of dorsal root ganglion and dorsal horn of spinal cord[10]. Activation of the PPARγ has a well-documented anti-inflammatory activity[11]. Rosiglitazone (PPARγ agonist) inhibited protein expression of iNOS, cyclooxygenase-2 and nitrotyrosine formation in RAW 264 cells induced by TNF[12]. In whole animal studies, PPARγ agonists showed significant anti-nociceptive effects, for example, pioglitazone reduces mechanical allodynia and thermal hyperalgesia in peripheral nerve injury model in mice[13].

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) widely used to relieve mild to moderate pain and inflammation in many disorders including: Arthritis, primary dysmenorrhea and headache. Yet its use is accompanied by unwanted side effects including gastrointestinal and cardiovascular side effects[14,15]. Ibuprofen possesses anti-inflammatory, antipyretic and analgesic activity and its therapeutic effects are attributed to inhibition of cyclooxygenases (COX-1 and COX-2) enzymes that blocks the production of prostaglandins (PGs) from the membrane-bound phospholipid Arachidonic Acid (AA)[16]. Although, COX enzymes are the major targets of NSAID[16], they are not the only pharmacologically pertinent site of action[17], it is also suggested that some NSAIDs effects are mediated by PPARs[18].

Ligand binding assays and x-ray crystallography techniques have shown that many non-steroidal anti-inflammatory drugs including, indomethacin and ibuprofen bind to PPARγ[19,20]. Oral administration of ibuprofen reduced the number of activated microglia and reactive astrocytes in the CNS of APPV717I micein a PPARγ dependent fashion[21]. Although, there is currently no evidence that NSAIDs directly bind to PPARα, it was previously reported that the anti-nociceptive effects of some NSAIDs are mediated through PPARα[22].

The mechanisms by which PPARs produce their effects fall into two categories: First, the genomic or classical pathway, which involves regulation of gene transcription and consequently changes in protein synthesis, responses mediated by this pathway typically have latencies of hours or even days[1,23]. Second, the rapid non-genomic pathway which occurs in minutes and normally peaks at 1 h[24]. Despite the well-documented interaction between NSAIDs and PPARs, the role played by PPARα and PPARγ in mediating the anti-nociceptive effects of ibuprofen *in vivo* is not determined. In addition, the mechanism and the time course by which ibuprofen modulates the activity of PPARα and PPARγ are poorly understood. The aim of this study is to assess the role of PPARα and PPARγ in mediating the anti-nociceptive effects of ibuprofen *in vivo* and further evaluate the time course of these effects by repeating the behavioral tests at different time points, which could reflect different mechanisms of action. Finally, since both receptors have well-documented role in pain modulation, the anti-nociceptive effects of their dual activation *in vivo* will be also evaluated.

MATERIALS AND METHODS

Animals: Behavioral experiments used adult male Sprague Dawley rats (100-250 g, Jordan University of Science and Technology Laboratories). Rats were group housed in the Animal House Unit (The University of Jordan) in a temperature controlled environment 22±1°C at 12 h:12 h light:dark cycle. Procedures were approved by the scientific research committee at the University of Jordan. Experiments were carried out in accordance with the animal (Scientific Procedure) Act 1986 and international association for the study of pain guidelines.

Induction of inflammatory pain model: For inducing inflammatory pain, animals received subcutaneous injections of complete Freund's adjuvant (CFA; 50% in saline, with 5 mg mL^{-1} heat-killed *Mycobacterium tuberculosis*, 0.1 mL) or vehicle into the plantar surface of the left hind paw. Control animals received equal amount of vehicle (0.1 mL of saline). After determining the baseline nociceptive responses, the animals were tested every other day for 21 days after CFA injection.

Assessment of thermal hyperalgesia: Rats were placed individually on a hot plate analgesia meter (Columbus instruments, USA) maintained at a constant temperature of 55±0.1°C after observing them for 5 min in the cage. The Paw Withdrawal Latency (PWL) was recorded as the time taken to exhibit distinct pain behavior either by hind paw licking or hind paw flicks (whichever occurred first). Rats that did not respond within 30 sec were removed from the hot plate to prevent tissue damage[25].

Assessment of mechanical allodynia: The von Frey filament test was used to measure sensitivity to a punctuate pressure stimulus. Rats were placed in a plastic cage with a wire mesh bottom which allowed full access to the paws. Behavioral accommodation was allowed for at least 25 min, until cage exploration and major grooming activities ceased. Subsequently, von Frey filaments (2-15 g with logarithmically incremental stiffness; Bioseb, Vitrolles, France) were applied to the mid-plantar aspect of the left hind paw using the "up-down" method to determine the withdrawal threshold, the von Frey hair was presented perpendicular to the plantar surface and held for approximately 6-8 sec[26]. Data are expressed as Paw Withdrawal Thresholds (PWT) in grams.

Pharmacological treatments: For the assessments of the effects of different drugs on the CFA-induced nociceptive behavior, ibuprofen (100 μg), tesaglitazar (5 μg), ibuprofen (100 μg) plus GW6471 (50 μg, a selective PPARα antagonist) or ibuprofen (100 μg) plus GW9662 (50 μg, a selective PPARγ antagonist) were injected into the plantar surface of the left hind paw at day 7 after CFA injection, when inflammation in the left hind limbs was fully developed. The doses of ibuprofen and the PPAR ligands were selected based on previous publications[22,27]. All drugs were diluted in 50 μL of (3% tween 20 in saline). Control animals (CFA without drug treatments) receive (0.1% ethanol or 0.1% DMSO plus 3% tween 20 in saline). Then nociceptive testing was performed at 1 and 6 h post-drug administration. In all behavioral experiments the observer was blinded to the treatments.

Drugs: The CFA was purchased from Sigma-Aldrich, ibuprofen, tesaglitazar, GW6471 and GW9662 were purchased from Tocris Bioscience (UK). Drugs were initially dissolved in ethanol (100%) or DMSO to form a stock solution and then diluted in (3% tween 20 in saline). Ethanol concentrations in the solutions used in the present study did not exceed 0.1%.

Data analysis: For the studies measuring CFA-induced thermal hyperalgesia data are presented as Means±SEM of PWL in seconds and for CFA-induced mechanical allodynia data are presented as Means±SEM of PWT in grams. Two-way ANOVA analysis of variance was used with time and treatment are the main factors. Significant ANOVA ($p \leq 0.05$) was followed by Holm-Sidakpost *post hoc* test using GraphPad statistical program (Prism 6). Also one-way ANOVA test followed by Dunnett's *post hoc* was used as appropriate.

RESULTS

Intraplantar injection of CFA produced a prominent local edema and redness after a few hours. In agreement with previous reports[28], both thermal PWLand mechanical PWT were significantly reduced 1 day after CFA injection. This reduction persisted for about three weeks (Fig. 1).

At day 7 after CFA injection, when PWL and PWT were significantly reduced, ibuprofen (100 μg) was injected into the plantar surface of the left hind paw, anti-nociceptive properties of ibuprofen were evaluated 1 and 6 h post-drug administration. Ibuprofen significantly restored both PWL and PWT (Fig. 2, 3) at the 2 time points selected. Recent studies have implicated a role of PPARα and γ in mediating the anti-inflammatory effects of ibuprofen[28]. This report investigated whether the inhibitory effects of ibuprofen on thermal hyperalgesia or mechanical allodynia were mediated by PPARα or γ. Neither GW6471 nor GW9662 alone altered CFA-induced thermal hyperalgesia or mechanical allodynia. Co-administration of the PPARα antagonist GW6471 significantly reduced the anti-nociceptive effects of ibuprofen on both PWL and PWT 1 h post-drug administration (Fig. 2). However, co-administration of the PPARγ antagonist GW9662 significantly reduced the inhibitory effects of ibuprofen on PWT but not PWL 1 h post-drug administration (Fig. 2).

The PPARα and PPARγ control inflammation by inhibiting the induction of pro-inflammatory cytokines[29] and limiting the recruitment of immune cells to inflammation sites[30]. These effects involve changes in gene expression which are developed over a period of hours or even days. So, the role of PPARα and PPARγ in mediating the analgesic effects of ibuprofen at a longer time point (6 h) was tested. Co-administration of the PPARα antagonist GW6471 significantly reduced the inhibitory effects of ibuprofen on both PWL and PWT at 6 h post-drug administration (Fig. 3). Similarly, co-administration of the PPARγ antagonist GW9662

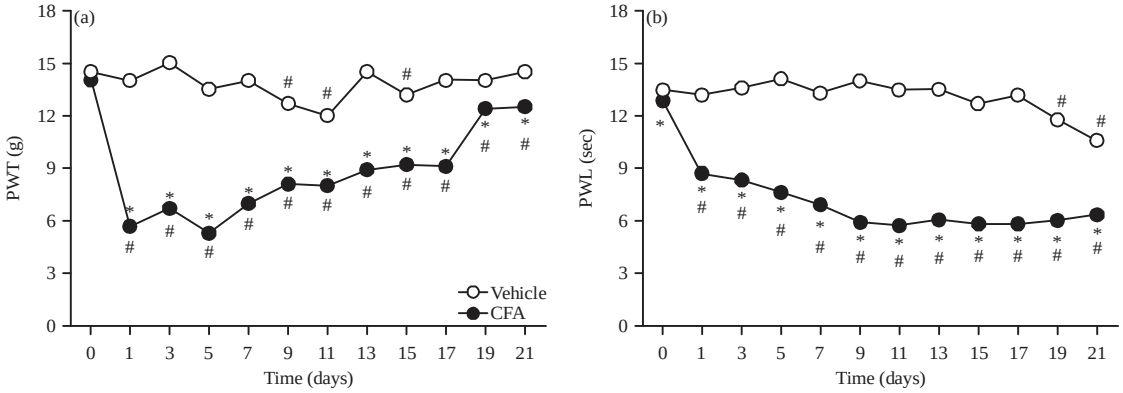

Fig. 1(a-b): Effects of intraplantar injection of Complete Freund's Adjuvant (CFA) on (a), Mechanical PWT and (b) Thermal PWL. Both PWT and PWL were significantly decreased in CFA-treated rats compared with Normal Saline (NS)-treated rats. Two-way ANOVA revealed the following results: (a) Significant main effect of treatment [F (1,120) = 1674; p<0.0001], significant main effect of time [F (11,120) = 55.37; p<0.0001] and significant main treatment×time interaction [F (11,120) = 41.59; p<0.0001], (b) Significant main effect of treatment [F (1,120) = 4047; p<0.0001], significant main effect of time [F (11,120) = 59.04; p<0.0001] and significant main treatment×time interaction [F (11,120) = 40.75; p<0.0001], *Indicate significant difference between groups, #Indicates significant difference vs., day 0 within group. All data represent Mean±SEM of 6 rats, PWT: Paw withdrawal threshold in grams, PWL: Paw withdrawal latency in seconds

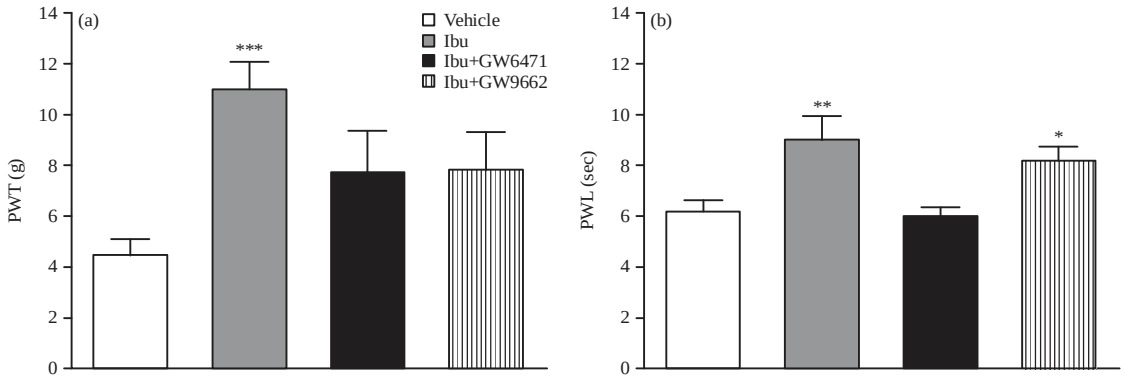

Fig. 2(a-b): (a) Role of the PPARα or PPARγ in mediating the anti-nociceptive effects of ibuprofen on mechanical PWT at 1 h post-drug administration on day 7 after CFA injection and (b) Role of the PPARα or PPARγ in mediating the anti-nociceptive effects of ibuprofen on thermal PWL at 1 h post-drug administration on day 7 after CFA injection. Data are expressed as Mean±SEM and analyzed using one way ANOVA test followed by Dunnett's *post hoc*, all treatments were compared to CFA/vehicle, *p<0.05, **p<0.01, ***p<0.001, n = 7 rats per group

significantly reduced the inhibitory effects of ibuprofen both on PWL and PWT at 6 h post-drug administration (Fig. 3). As both receptors PPARα and PPARγ are involved in mediating the anti-nociceptive properties of ibuprofen, This report investigated the analgesic effects of dual activation of both receptors. Tesaglitazar (5 μg) did not alter mechanical PWT or thermal PWL at 1 h post-drug administration. However, it partially reversed both mechanical PWT and thermal PWL at 6 h post-drug administration, but significant reduction was observed only in thermal PWL (Fig. 4).

DISCUSSION

Employing the CFA-induced inflammatory pain model, this report expectedly highlighted the anti-nociceptive effects of ibuprofen in rat. In the present study, in which this report

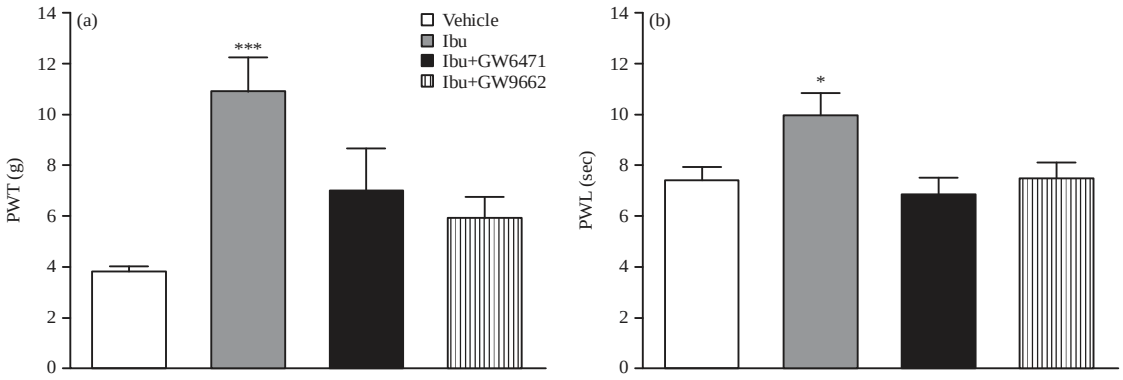

Fig. 3(a-b): (a) Role of the PPARα or PPARγ in mediating the anti-nociceptive effects of ibuprofen on mechanical PWT at 6 h post-drug administration on day 7 after CFA injection and (b) Role of the PPARα or PPARγ in mediating the anti-nociceptive effects of ibuprofen on thermal PWL at 6 h post-drug administration on day 7 after CFA injection. Data are expressed as Mean±SEM and analyzed using one way ANOVA test followed by Dunnett's *post hoc*, all treatments were compared to CFA/vehicle, *p<0.05, ***p<0.001, n = 7 rats per group

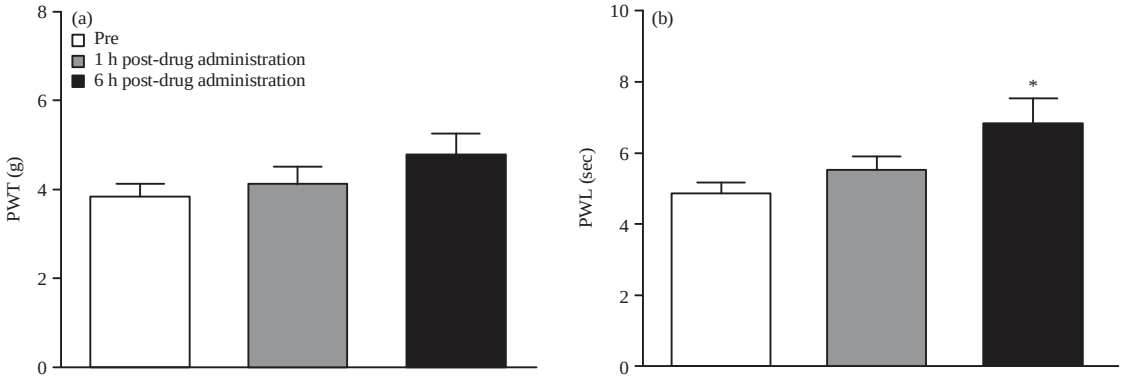

Fig. 4(a-b): (a) Anti-nociceptive effects of tesaglitazar (5 μg) on mechanical PWT at 1 and 6 h post-drug administration on day 7 after CFA injection and (b) Anti-nociceptive effects of tesaglitazar (5 μg) on thermal PWL at 1 and 6 h post-drug administration on day 7 after CFA injection. Data are expressed as Mean±SEM and analyzed using one way ANOVA test followed by Dunnett's *post hoc*, Pre: Anti-nociceptive effects of tesaglitazar (5 μg) measured at the 2 time points were compared to baseline values, *p<0.05, n = 7 rats per group

the anti-hyperalgesic effects of ibuprofen was measured at two different time points reflecting the rapid and slow-onset effects of ibuprofen, it has been revealed that PPARα and PPARγ play substantial role in mediating both the rapid and slow-onset ibuprofen-induced anti-nociceptive effects *in vivo*. Although, it was previously shown that ibuprofen activates both PPARα and PPARγ *in vitro* such as in the CV-1 cell line[19], this data provides the first evidence that the rapid onset effects of ibuprofen are PPAR mediated. These data are in agreement with the previous reports highlighting the apparent analgesic properties of direct PPAR agonists in different pain models both in terms of the magnitude and the time course of the effects[7,9].

The finding that PPARγ mediates the anti-nociceptive effects of ibuprofen is consistent with recently published data reporting that NSAIDs such as celecoxib and diclofenac produces chemo-preventive effects in a rat model of colon cancer in a PPARγ dependent mechanism[31]. Moreover, indomethacin was found to strongly bind to the Activation Function (AF) 2 site in the ligand-binding domain of PPARγ providing the molecular basis for PPARγ-NSAID interaction[32,33].

The ability of ibuprofen to not only inhibit COX but also Fatty Acid Amide Hydrolase (FAAH) enzyme[34,35], the main enzyme responsible for endocannabinoid metabolism, could suggest the mechanism by which ibuprofen produces its anti-nociceptive effects through PPARs, by which

increased levels of AEA and PEA (Anandamide and palmitoylethanolamide), respectively (substrates for FAAH enzyme) produced the anti-nociceptive effects of ibuprofen, both AEA and PEA are endogenous agonists for PPARα[36]. Although, nimesulide (selective COX-2 inhibitor) produces analgesic effects *in vivo* in PPARα dependent fashion, nimesulide failed to significantly displace bodipy FL C16 from the PPARα LBD[22]. Furthermore, the newly developed N-acylethanolamine acid amidase (NAAA) inhibitor 3-(6-phenylhexanoyl) oxazolidin-2-one (F96), an enzyme responsible for PEA metabolism, produces potential analgesic properties in acetic acid-induced visceral pain in a PPARα dependent mechanism[37].

Employing surflex computational methods including; ligand similarity, docking and protein pocket similarity, Cleves and Jain[38] predicted that certain PPARα ligands such as gemfibrozil interacts with a new target (the COX enzymes), providing the chemical and structural basis for the crosstalk between PPARα and COX enzymes. However, to exclude the possibility that a direct interaction of ibuprofen with PPARα underlies anti-nociceptive effects of ibuprofen observed *in vivo*, further experiments including the ligand binding assays are required to evaluate the ability of ibuprofen to directly bind to PPARα.

Our pharmacological evidence indicates that PPARγ plays a significant role in mediating the slow-onset anti-nociceptive effect of ibuprofen both in terms of thermal PWL and mechanical PWT. These results are fully consistent with the well-documented anti-inflammatory role of PPARγ ligands[39,40], pioglitazone and rosiglitazone significantly reduced chronic thermal hyperalgesia after Spinal Cord Injury (SCI), these effects were accompanied by inhibition of inflammatory genes including interleukin (IL)-6, IL-1β, monocyte chemo attractant protein-1, intracellular adhesion molecule-1 and early growth response-1[39]. These are genomic transcription-dependent events and fully consistent in terms of the time coarse with the slow-onset effects ibuprofen observed in this study. But when considering the rapid-onset inhibitory effects of ibuprofen on thermal hyperalgesia, it seems that PPARα but not PPARγ is involved in mediating these effects. This finding can be explained by a previous report suggesting that PPARα activation might facilitate the transient receptor potential of vanilloidtype-1 (TRPV1) channel opening[41], a major transduction molecule involved in thermal hyperalgesia[42,43]. The latter group revealed that PEA rapidly inhibited capsaicin-evoked calcium responses in F11 cells[41]. Single injection of rosiglitazone rapidly inhibited neuropathic pain in rat, an effect that peaked 1 h after injection, suggesting a transcription-independent mechanism[44]. Similarly administration of pioglitazone reduces tactile allodynia within 30 min of application in spared nerve injury rats[45].

Keeping in mind that ibuprofen is direct ligand of PPARγ[19], no significant role for PPARγ in mediating the rapid anti-hyperalgesic effects of ibuprofen was recognized in this study, this can be justified by the low potency of ibuprofen in activating PPARγ in comparison to its potency in activating PPARα[46]. The finding that both PPARα and PPARγ are required to mediate the anti-nociceptive effects of ibuprofen indicates that these effects are dependent upon an interaction with more than one cellular target and that the pharmacological effects of NSAID are not only related to their inhibitory profile on COX enzymes, but also on their activation to PPARs.

Tesaglitazar is a dual PPARα/γ agonist[47], previously investigated clinically for its ability to treat type 2 diabetes mellitus[48,49]. It significantly improves insulin sensitivity[50] and attenuates diabetic neuropathy in db/db mice[51]. To the best of our knowledge, this is the first study to report the anti-nociceptive properties of tesaglitazar. We have shown that tesaglitazar reversed the CFA-induced thermal hyperalgesia 6 h post-drug administration, suggesting a slow-onset transcription-dependent mechanism of action. The finding that ibuprofen but not tesaglitazar produced rapid analgesic effects mediated at least in part by PPARs indicates that the downstream effects of PPARs activation depend on the type of the agonist applied, in which ibuprofen but not tesaglitazar can enhance the rapid signaling pathway of PPARα which may include the inhibition of the large and the intermediate conductance K_{ca} channels (BK_{ca} and IK_{ca}), respectively[7] and/or enhance the rapid non-genomic effects of PPARγ activation including suppression of NF-κB, STAT-1 and AP-1 signaling pathways[52,53].

CONCLUSION

In conclusion, ibuprofen produces anti-nociceptive effects that are at least partially mediated via PPARα and PPARγ. In addition, tesaglitazar was effective in reducing CFA-induced thermal hyperalgesia, likely via slow-onset transcription-dependent mechanism of action, this suggests that dual activation of PPARα/γ could provide a promising target to control chronic pain conditions characterized by thermal hyperalgesia.

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