

Short Communication

Identification of New Targets Used by 15-deoxy-delta^{12,14}-Prostaglandin J₂ to Stimulate Skeletal Muscle Cell Proliferation

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Abstract

Introduction: Clinical conduct can influence the healing of injured tissue. Eradication of inflammation seemed a promising strategy to promote musculoskeletal healing until studies showed a delayed/incomplete recovery from partial or complete elimination of inflammation. Endogenous lipid mediators biosynthesized from omega-3 and some from ω-6 fatty acids are molecules potentially playing important roles in the resolution of inflammation. Using such lipid mediators to treat injuries represents an attractive approach due to their anti-inflammatory and pro-resolving roles. Our goal was to identify the intracellular and/or extracellular targets used by 15-deoxy-delta^{12,14}-Prostaglandin J₂ (15Δ-PGJ₂) to stimulate myoblast proliferation.

Methods: Expression of D prostanoid (DP) 1 and 2 receptors was evaluated by western blotting. Proliferation of L6 myoblasts incubated with agonists and antagonists of prostaglandin (PG) D₂ receptors DP₁ and DP₂ and of the peroxisome proliferator-activated receptor (PPAR) δ was assessed. Intracellular and extracellular concentrations of 15Δ-PGJ₂ following L6 cell activation with protease-activated receptor (PAR)-2 agonist were measured by liquid chromatography coupled to tandem mass spectrometry.

Results: Both DP₁ and DP₂ receptors are present in myoblasts. DP₁ agonist did not modulate L6 myoblast proliferation, but DP₂ and PPARδ agonists induced an increase. DP₁ and DP₂ antagonists both significantly inhibited 15Δ-PGJ₂-induced stimulating effect of L6 cell proliferation (60% and 75%, respectively). 15Δ-PGJ₂ was present in the intracellular and extracellular compartments under basal conditions, but was not modulated by PAR-2 receptor activation.

Conclusion: L6 muscle cell can produce 15Δ-PGJ₂ and its effect on cell proliferation likely relies on both DP₁ and DP₂ receptor activation.

ABBREVIATIONS

15Δ-PGJ₂: 15-deoxy-delta^{12,14}-Prostaglandin J₂; α-MEM: α-Minimum Essential Medium; BHT: Butylated hydroxytoluene; CRTH₂: Chemoattractant receptor of Th2 cells; COX-2: Cyclooxygenase-2; DP: D Prostanoid; FBS: Fetal bovine serum; LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry; NF-κB: Nuclear factor-κB; PPAR: Peroxisome proliferator-activated receptor; PG: Prostaglandin; PAR: Protease-activated receptor; PUFA: Polyunsaturated fatty acids

INTRODUCTION

The proper management of tissue damage-induced

inflammation represents an important element in the treatment of sport related acute injuries. While many efforts were devoted to the development of anti-inflammatory strategies, an abundant literature subsequently demonstrated that the inflammatory response is essential for regeneration and its partial or complete elimination can induce delayed and/or inappropriate healing [1-6]. Clearly, new treatment strategies to be developed should aim at promoting regeneration without targeting the inflammatory process.

Formerly regarded as a passive mechanism, the resolution of inflammation is now recognized as an active process modulated by the endogenous and timely production of pro-resolution

and anti-inflammatory molecules [7,8]. Resolvins, protectins and marseins are lipid mediators biosynthesized from omega-3 fatty acids that can potentially play important roles in the anti-inflammatory and pro-resolving processes [9]. In addition, some lipid mediators derived from omega-6 arachidonic acid, such as lipoxins and the cyclopentanone 15-deoxy-delta^{12,14}-Prostaglandin J₂ (15Δ-PGJ₂), also have anti-inflammatory and pro-resolving effects [10].

The metabolite 15Δ-PGJ₂ is formed by a double spontaneous dehydration of prostaglandin (PG) D₂ into PGJ₂ and PGJ₂ into 15Δ-PGJ₂ and acts on different targets to accomplish a wide array of effects [11]. 15Δ-PGJ₂ can promote the resolution of inflammation by inhibiting the activity of the transcription factor nuclear factor-κB (NF-κB) [11]. 15Δ-PGJ₂ can also bind the PGD₂ receptors: D prostanoid (DP) 1 and 2 receptors (DP₂ receptor is also designed chemoattractant receptor of Th2 cells (CRTH₂)), whose activation can lead to various inflammatory and anti-inflammatory effects such as vasodilatation, suppression of cytokine production, activation of immune cells and inhibition of platelet aggregation and chemotaxis [11,12]. Moreover, 15Δ-PGJ₂ can respectively have mitogenic and differentiation-promoting effects in several cell types such as fibroblasts and adipocytes following its interaction with one of its natural receptor, the peroxisome proliferator-activated receptor (PPAR) γ [13,14]. We also recently found that 15Δ-PGJ₂ can also stimulate skeletal muscle cell proliferation *in vitro* [15].

The quest to develop new and innovative therapeutic strategies providing faster and more complete recovery from musculoskeletal injury is highly relevant in the field of sports medicine. The study of newly identified endogenous anti-inflammatory/pro-resolving mediators becomes attractive. Our objective was to provide a better understanding of how the metabolite 15Δ-PGJ₂ can influence myoblast proliferation, a key step in muscle healing, by identifying the intracellular and/or extracellular targets of 15Δ-PGJ₂ responsible for the stimulation of L6 myoblast proliferation.

MATERIALS AND METHODS

Cell culture and proliferation assays

L6 rat skeletal myoblasts (ATCC, Manassas, VA, USA) were maintained in α-Minimum Essential Medium (α-MEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) at 37 °C, 5% CO₂.

For measurements of proliferation rate, cells were seeded onto 96-well plate at a density of 3,000 cells/well in 65 μL of α-MEM containing 1% FBS. The number of viable cells after 24 h of incubation was determined by the use of Cell Titer 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA) according the manufacturer's instructions. Various receptors known as potential targets for 15Δ-PGJ₂ were tested: BW 245C (5 nM, 0.01 μM, 2 μM, 5 μM), a selective agonist of DP₁ receptor (Cayman Chemical, Ann Arbor, MI, USA), indomethacin (50 nM, 500 nM, 5000 nM) (Sigma-Aldrich, St. Louis, MO, USA) a DP₂ agonist, GW 0742 (1 nM, 50 nM, 100 nM, 1 μM) a selective PPARδ agonist (Cayman Chemical, Ann Arbor, MI, USA) [16-18]. Since 15Δ-PGJ₂ or trypsin can increase L6 cell proliferation [15], these compounds were used as positive control and to

evaluate the effect of the DP₁ and DP₂ antagonists (BW A868C and BAY-u3405 (Ramatroban), respectively) on L6 myoblast proliferation by pre-incubating myoblasts with the antagonists for 30 min at 37°C before the addition of 15Δ-PGJ₂ or trypsin. DP₁ and DP₂ antagonists were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Western blots of DP₁ and DP₂ receptors in L6 cells

L6 myoblasts were lysed and protein content was assessed according to Duchesne et al. (2013) [19]. DP₁ and DP₂ were immunoprecipitated with Protein A Sepharose 6MB beads (GE Healthcare, Cat # 17-0469-01). To do so, samples were first pre-incubated with goat serum (Sigma-Aldrich, St-Louis, MO, USA). After Protein A Sepharose 6MB beads were washed in lysis buffer, primary antibodies DP₁ (Santa Cruz, Cat. # sc-55815) and DP₂ (Santa Cruz, Cat. # sc-23092) were incubated with samples overnight at 4 °C. Beads were then added to each sample and incubated overnight at 4°C. Samples were centrifuged and the supernatant thrown out. Pellets were suspended in western sample loading buffer (65 mM Tris-base pH 6.8, 26.2% glycerol, 2.1% SDS, 0.52% 2-mercaptoethanol (Sigma-Aldrich, St-Louis, MO, USA) and a few grains of bromophenol blue (Bio-Rad, Hercules, CA, USA). Samples were boiled to release the antibody from the beads and the supernatants were kept for western blots analysis.

Western blots were performed according to Duchesne et al. (2013) [19]. After transfer, membranes were incubated at RT for 2 h with DP₁ and DP₂ antibodies (dilution 1/200). The secondary antibody used was a donkey anti-goat IgG-HRP-conjugated (Santa Cruz, Cat. # sc-2020; dilution 1/10,000).

Measurement of 15Δ-PGJ₂ concentration

15Δ-PGJ₂ concentration was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). L6 cell culture medium was replaced by α-MEM without FBS but containing 0.005% butylated hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA) as an antioxidant [20]. Cells were stimulated or not with 200 nM trypsin for 3h (Sigma-Aldrich, St. Louis, MO, USA). Culture medium was then removed and cells were detached. A solution deuterated-15Δ-PGJ₂ (15Δ-PGJ₂-D₄; Cayman Chemical, Ann Arbor, MI, USA) was added to culture medium as an internal standard before extraction steps. After acidification of the samples, extraction steps were performed according to Yang et al. (2002) [21]. Analyses were performed in the laboratory of the Bioanalytical Services of the Centre Hospitalier Universitaire de Québec (CHU de Québec), Laval University, Qc, Canada (<http://services.crchuq.ulaval.ca/groups/bioanalytique/wiki/c00fb/Welcome.html>) according to Yang et al. (2002) using a API4000 mass spectrometer (AB Sciex, Concord, On, Canada) [21].

Statistical analysis

All values are expressed as means and standard error. Comparisons between groups were performed by Student's t-test or one-way ANOVA followed by Tukey-Kramer post-hoc test, when appropriate (InStat GraphPad Software Inc., La Jolla, CA, USA). Significance was defined as p < 0.05.

RESULTS AND DISCUSSION

Inflammation has been traditionally considered as harmful and many anti-inflammatory strategies were thus developed to eradicate inflammation in the context of musculoskeletal injuries. However, it has been subsequently demonstrated that elimination of inflammatory actors often leads to delayed tissue healing [2,22,23]. An abundant literature has recently emerged concerning the anti-inflammatory and pro-resolution effects of some omega-6 and omega-3 fatty acids-derived lipid mediators [7-12]. While it is clearly documented that excessive amounts of omega-6 polyunsaturated fatty acids (PUFA) leading to a high omega-6/omega-3 ratio promote the pathogenesis of cardiovascular disease, cancer and inflammatory and autoimmune diseases [24,25], surprisingly some lipids derived from arachidonic acid possess anti-inflammatory properties. Indeed, Gilroy et al. (1999) reported for the first time that cyclooxygenase-2 (COX-2) was not only an inflammatory actor, but can also allow the formation of the anti-inflammatory cyclopentanone 15Δ -PGJ₂ [10]. This lipid mediator can accelerate resolution of inflammation by diverse mechanisms such as providing signals that selectively stop neutrophil infiltration, stimulate non-phlogistic recruitment of monocytes, and activate macrophage phagocytosis of apoptotic cells, to name a few. On the other hand, 15Δ -PGJ₂ can induce mitogenic and differentiation-promoting effects in some cell types [13-15]. However, in skeletal muscle cell, not much is known on the receptors involved and the mechanism of action.

In general, it is assumed that 15Δ -PGJ₂ can induce its effects by interacting with one of its natural receptor, PPAR γ [15], but this nuclear receptor is very weakly expressed in skeletal muscle [26]. It is also possible that 15Δ -PGJ₂ binds the extracellular PGD₂ receptors DP₁ and DP₂ [11,12], which are typically found on bronchial smooth muscle, vascular smooth muscle, dendritic cells and platelets for DP₁ and on Th2 lymphocytes, eosinophils and basophils for DP₂ receptor [27]. The first step of this study was then to assess the presence of DP₁ and DP₂ receptors in L6 skeletal muscle cell. We found that both receptors are present in L6 cells (Figure 1). These results may seem contrary to those reported by Veliça et al. (2010), but it is important to highlight that they evaluated only the mRNA expression of DP₁ and DP₂ receptors in a different cell line (C₂C₁₂) [28].

This observation prompted us to verify whether these extracellular receptors could be implicated in the 15Δ -PGJ₂-induced skeletal muscle cell proliferation that we previously observed [15]. To do so, L6 myoblasts were incubated with different DP agonists to evaluate their impact on skeletal muscle cell proliferation. We found that the DP₁ agonist BW 245C did not modulate L6 cell proliferation at any of the concentrations tested (Figure 2A). We next blocked the DP₁ receptor using the specific antagonist BW A686C to evaluate its implication in 15Δ -PGJ₂-induced L6 cell proliferation; we first verified that incubation of L6 cells with the antagonist alone (1 μ M) did not influence the proliferation rate of L6 cells (data not shown). As previously reported, L6 cell proliferation was increased by 56 % above control when 15Δ -PGJ₂ was added to myoblasts for 24 h (Figure 2A). Interestingly, pre-incubation of L6 cells with DP₁ antagonist caused a significant inhibition of 60% of the stimulating effect

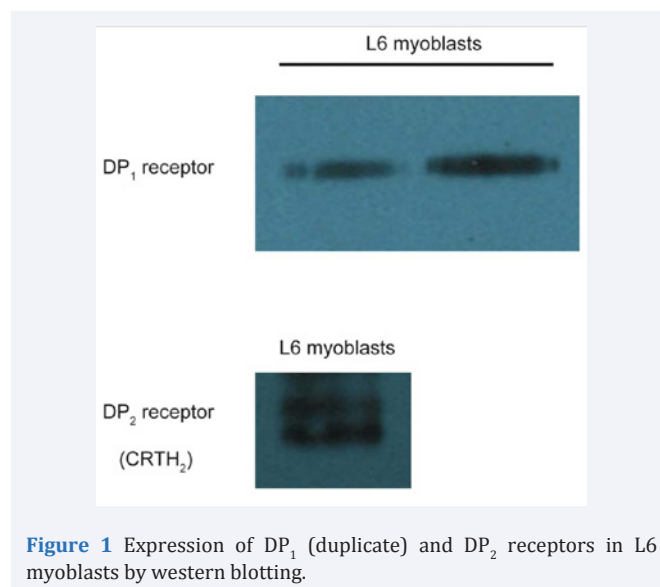


Figure 1 Expression of DP₁ (duplicate) and DP₂ receptors in L6 myoblasts by western blotting.

of 15Δ -PGJ₂ on L6 cell proliferation suggesting that DP₁ receptor is, at least partially, implicated in 15Δ -PGJ₂-induced mitogenic effect (Figure 2C). The absence of effect of the DP₁ agonist on L6 cell proliferation is likely related to the experimental conditions chosen. The 24 h time period selected might have been inappropriate to observe the effect of a compound like BW 245C, which has a different half-life and stability in culture medium compared to 15Δ -PGJ₂. In addition, functional selectivity or biased agonism, a phenomenon by which the activation of the same receptor by different agonists lead to different signaling response and already identified in prostanoid receptors, could also explain such observations [29]. Furthermore, as demonstrated by Petrova et al. (1999), the metabolite 15Δ -PGJ₂ can be more effective than a specific receptor agonist [30].

The culture of L6 cells with indomethacin, an agonist of the DP₂ receptor, slightly increased the proliferation of L6 myoblasts, reaching a significant response of 20 % above control at a concentration of 500 nM (Figure 2B). Pre-incubation of L6 cells with the DP₂ specific antagonist BAY-u3405 decreased by 75% the stimulating effect of 15Δ -PGJ₂ on L6 cell proliferation (Figure 2C); again, incubation of L6 cells with the antagonist alone did not impact on cell proliferation (data not shown). These results strongly suggest that DP₂ receptor is used by the metabolite 15Δ -PGJ₂ to induce its proliferative effect. To the best of our knowledge, this study is the first to identify skeletal muscle cell extracellular targets used by 15Δ -PGJ₂ to accomplish its mitogenic effect, a key step in skeletal muscle repair.

As previously mentioned, PPAR γ is a target of 15Δ -PGJ₂ used to stimulate proliferation in certain cell types, but this cascade is presumably inexistent in skeletal muscle cell in vivo as PPAR γ is very weakly expressed. Since PPAR δ , an ubiquitous member of the PPAR family, is present in skeletal muscle cells, as a preliminary experiment we tested the possibility that this nuclear receptor is involved in the stimulation of L6 cell proliferation by 15Δ -PGJ₂. A 24 h incubation of L6 cells with GW 0742, a specific PPAR δ agonist, significantly stimulates cell proliferation at the concentration of 50 nM (Figure 2D). As previously reported

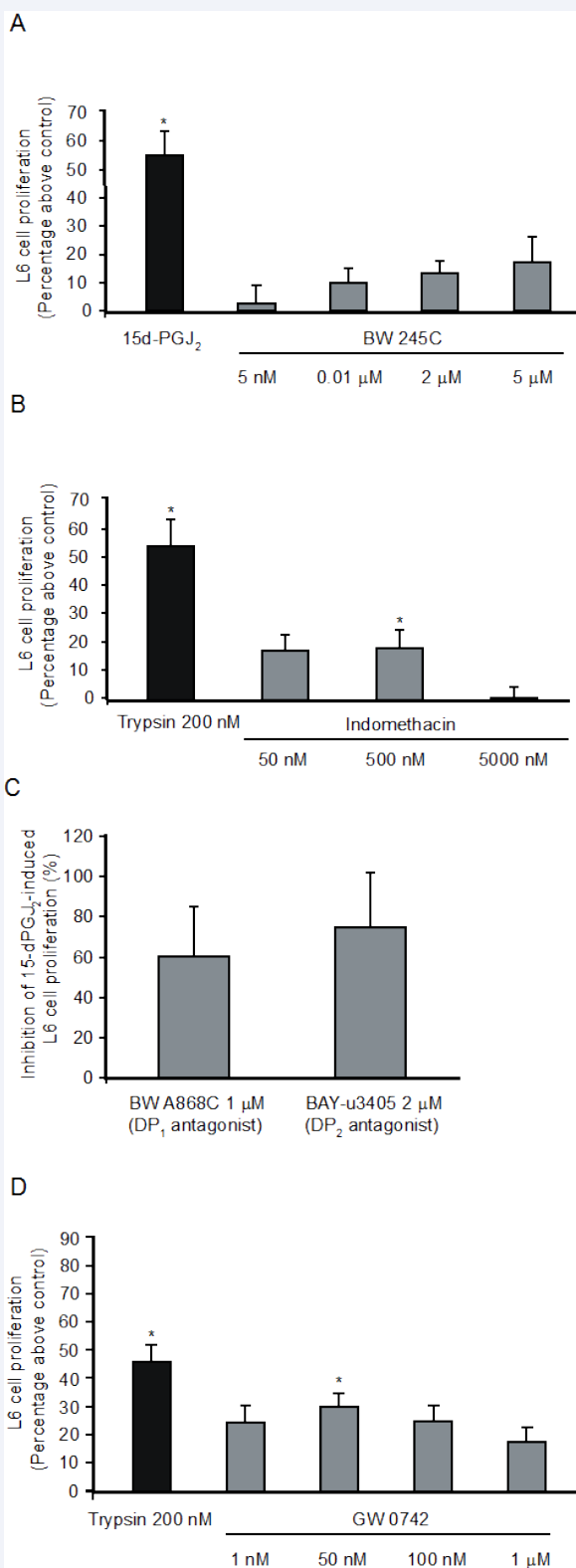


Figure 2 L6 myoblast proliferation following treatment with either (A) - DP₁ agonist BW 245C, (B) - DP₂ agonist indomethacin or (D) - PPARδ agonist GW 0742, while control wells received medium with 1% FBS only and positive control wells received 15Δ-PGJ₂ or trypsin. (C) - L6 myoblasts were pre-incubated either with DP₁ antagonist BW A868C or DP₂ antagonist BAY-u3405 before the addition of 15Δ-PGJ₂. *Significantly different from control, $P < 0.05$.

[31], activation of this nuclear receptor can stimulate myoblast proliferation and it can be assumed that 15Δ-PGJ₂ may target this intracellular nuclear receptor to induce its proliferative effect. However, this field deserves a more complete investigation.

The second objective of the study was to verify if 15Δ-PGJ₂ is produced by L6 muscle cells following protease-activated receptor (PAR)-2 activation, as seen with fibroblasts in culture. 15Δ-PGJ₂ was detected in L6 cell culture media under basal conditions after 3 h in culture (Figure 3A). However, this extracellular concentration of 15Δ-PGJ₂ remained statistically unchanged when L6 cells were stimulated with trypsin 200 nM (Figure 3A). These results prompted us to verify if 15Δ-PGJ₂ is produced intracellularly by L6 cells following PAR-2 activation without being thereafter externalized. Culture media

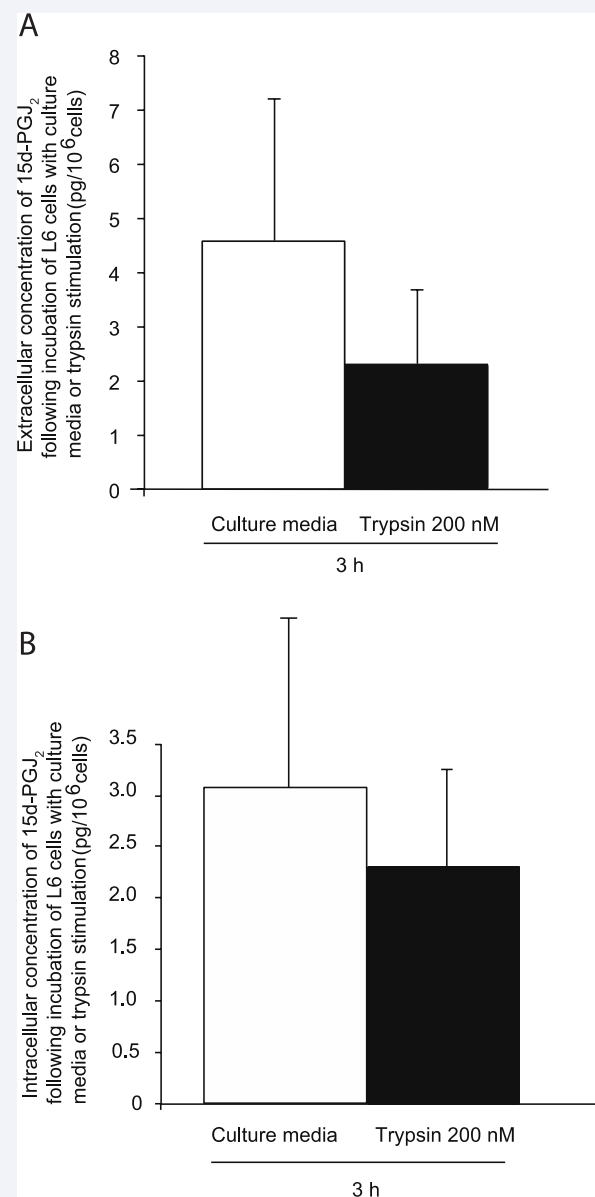


Figure 3 (A) - Extracellular and (B) - intracellular concentration of 15Δ-PGJ₂ following a 3 h incubation of L6 cells with or without trypsin, a PAR-2 agonist.

surrounding L6 cells was thus removed at the end of incubation period and cells were lysed. A level of 15 Δ -PGJ₂ comparable to the one observed in extracellular media was measured in L6 cell homogenates (Figure 3B) and trypsin-induced PAR-2 activation [15] did not influence the intracellular concentration (Figure 3B). Overall, the experimental protocol used clearly allowed to show that L6 cells can produce 15 Δ -PGJ₂, but we were unable to demonstrate that its production was sensitive to PAR-2 receptor activation. The complex and costly procedures involved in the extraction and measurement of this molecule makes it difficult to perform protocols with various time course designs in terms of duration of stimulation and sampling post-stimulation. Further work is needed before one can conclude solidly on the responsiveness of 15 Δ -PGJ₂ production to PAR-2 activation.

Thus, key elements of the signaling cascade leading to skeletal muscle cell proliferation following PAR-2 activation remain to be discovered. We were able to demonstrate that 1) 15 Δ -PGJ₂ is produced by skeletal muscle cell and 2) DP₁ and DP₂ receptors are expressed in skeletal muscle cells. We have thus identified intra- and extracellular receptors potentially used by this metabolite to induce its proliferative effect. Even if we were unable to demonstrate that PAR-2 activation leads to an increased 15 Δ -PGJ₂ production, our results remain very physiologically relevant since 15 Δ -PGJ₂ can also be released by other cell types found in skeletal muscle, such as fibroblasts and mast cells, under basal and inflammatory conditions [13,32]. The discovery of receptors used by the metabolite 15 Δ -PGJ₂ can lead to the development of new therapeutic approaches in the treatment of musculoskeletal injuries relying on endogenous molecules synthesized during the period of resolution of inflammation. Further studies focusing on the interaction of 15 Δ -PGJ₂ with nonreceptor protein following its entry into cells, possibly by an active transport system [11], are needed to complete our observations. Indeed, as demonstrated by Oliva et al. (2003), the direct activation of Ras/Erk MAP kinase pathway by 15 Δ -PGJ₂ at cytosolic level may be a key element in the myogenic process [33].

CONCLUSION

The treatment of musculoskeletal injuries takes an enormous place in the field of sport medicine and there is obviously room to improve clinical conduct which is not always evidence-based. The fine understanding of the processes leading to tissue repair following acute injury is critical for ensuring an optimal treatment and healing. This is particularly true for the athlete population, where complete recovery of original tissue strength must be aimed to avoid re-rupture of structures submitted to high mechanical stress. Lipid mediators have been largely studied in disease models, but their implication in the treatment of acute musculoskeletal injury remains relatively unexplored. It seems promising to study the effect of 15 Δ -PGJ₂ on muscle repair since in addition to its anti-inflammatory properties, mitogenic effects have been associated to this metabolite.

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