Short Communication

Identification of New Targets Used by 15-deoxy-delta-\textsubscript{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 -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-\textsubscript{12,14}-Prostaglandin J\textsubscript{2} (15\Delta-PGJ\textsubscript{2}) 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) D\textsubscript{2} receptors DP\textsubscript{1} and DP\textsubscript{2}, and of the peroxisome proliferator-activated receptor (PPAR)\textsubscript{δ} was assessed. Intracellular and extracellular concentrations of 15\Delta-PGJ\textsubscript{2} following L6 cell activation with protease-activated receptor (PAR)-2 agonist were measured by liquid chromatography coupled to tandem mass spectrometry.

Results: Both DP\textsubscript{1} and DP\textsubscript{2} receptors are present in myoblasts. DP\textsubscript{1} agonist did not modulate L6 myoblast proliferation, but DP\textsubscript{2} and PPAR\textsubscript{δ} agonists induced an increase. DP\textsubscript{1} and DP\textsubscript{2} antagonists both significantly inhibited 15\Delta-PGJ\textsubscript{2}-induced stimulating effect of L6 cell proliferation (60\% and 75\%, respectively). 15\Delta-PGJ\textsubscript{2} 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 15\Delta-PGJ\textsubscript{2} and its effect on cell proliferation likely relies on both DP\textsubscript{1} and DP\textsubscript{2} receptor activation.

ABBREVIATIONS

15\Delta-PGJ\textsubscript{2}: 15-deoxy-delta-\textsubscript{12,14}-Prostaglandin J\textsubscript{2}; α-MEM: α-Minimum Essential Medium; BHT: Butylated hydroxytoluene; CRTH\textsubscript{2}:Chemotactic 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 maresins 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 (15-ΔPGJ2), also have anti-inflammatory and pro-resolving effects [10].

The metabolite 15-ΔPGJ1 is formed by a double spontaneous dehydrogenation of prostaglandin (PG) D2 into PGJ2 and PG12 and acts on different targets to accomplish a wide array of effects [11]. 15-ΔPGJ1 can promote the resolution of inflammation by inhibiting the activity of the transcription factor nuclear factor-κB (NF-κB) [11]. 15-ΔPGJ2 can also bind the PGE2 receptors: D prostanoid (DP) 1 and 2 receptors (DP2 receptor is also designed chemotaxtractant receptor of Th2 cells (CRTH2)), whose activation can lead to various inflammatory and anti-inflammatory effects such as vasodilation, suppression of cytokine production, activation of immune cells and inhibition of platelet aggregation and chemotaxis [11,12]. Moreover, 15-ΔPGJ2 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) y [13,14]. We also recently found that 15-ΔPGJ2 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 15-ΔPGJ2 can influence myoblast proliferation, a key step in muscle healing, by identifying the intracellular and/or extracellular targets of 15-ΔPGJ2 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) supplemented with 0.005% butylated hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA) as an antioxidant [20]. Cells were detached. A solution deuterated-15-ΔPGJ2 (15-ΔPGJ2-D2; Cayman Chemical, Ann Arbor, MI, USA) was added to culture medium then incubated for 30 min at 37°C before the addition of BW 245C or trypsin. DP2 and DP3 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]. DP2 and DP3 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 DP3 (Santa Cruz, Cat. # sc-23092) were incubated with samples overnight at 4 °C. Beads were then washed 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) and were transferred to a clean tube. Analyses were performed in the laboratory of the Bioanalytical Services of the Centre Hospitalier Universitaire de Québec (CHU de Québec) using a API4000 mass spectrometer (AB Sciex, Concord, On, Canada) [21]. 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 15-ΔPGJ2 concentration

15-ΔPGJ2 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-15-ΔPGJ2 (15-ΔPGJ2-D2; 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) 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 [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 15∆-PGJ\textsubscript{2} [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, 15∆-PGJ\textsubscript{1} 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 15∆-PGJ\textsubscript{1} can induce its effects by interacting with one of its natural receptor, PPAR\textgamma [15], but this nuclear receptor is very weakly expressed in skeletal muscle [26]. It is also possible that 15∆-PGJ\textsubscript{1} binds the extracellular PGD\textsubscript{2} receptors DP\textsubscript{1} and DP\textsubscript{2} [11,12], which are typically found on bronchial smooth muscle, vascular smooth muscle, dendritic cells and platelets for DP\textsubscript{1}, and on Th2 lymphocytes, eosinophils and basophils for DP\textsubscript{2} receptor [27]. The first step of this study was then to assess the presence of DP\textsubscript{1} and DP\textsubscript{2} 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 DP\textsubscript{1} and DP\textsubscript{2} receptors in a different cell line (C\textsubscript{2}C\textsubscript{14}) [28].

This observation prompted us to verify whether these extracellular receptors could be implicated in the 15∆-PGJ\textsubscript{2} 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 DP\textsubscript{1} agonist BW 245C did not modulate L6 cell proliferation at any of the concentrations tested (Figure 2A). We next blocked the DP\textsubscript{1} receptor using the specific antagonist BW A686C to evaluate its implication in 15∆-PGJ\textsubscript{2} induced L6 cell proliferation; we first verified that incubation of L6 cells with the antagonist alone (1 μM) 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 15∆-PGJ\textsubscript{1} was added to myoblasts for 24 h (Figure 2A). Interestingly, pre-incubation of L6 cells with DP\textsubscript{1} antagonist caused a significant inhibition of 60% of the stimulating effect of 15∆-PGJ\textsubscript{2} on L6 cell proliferation suggesting that DP\textsubscript{1} receptor is, at least partially, implicated in 15∆-PGJ\textsubscript{1}-induced mitogenic effect (Figure 2C). The absence of effect of the DP\textsubscript{1} 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 15∆-PGJ\textsubscript{1}. 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 prostanooid receptors, could also explain such observations [29]. Furthermore, as demonstrated by Petrova et al. (1999), the metabolite 15∆-PGJ\textsubscript{2} can be more effective than a specific receptor agonist [30].

The culture of L6 cells with indomethacin, an agonist of the DP\textsubscript{1} 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 DP\textsubscript{2} specific antagonist BAY-u3405 decreased by 75% the stimulating effect of 15∆-PGJ\textsubscript{1} 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 DP\textsubscript{2} receptor is used by the metabolite 15∆-PGJ\textsubscript{2} 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 15∆-PGJ\textsubscript{2} to accomplish its mitogenic effect, a key step in skeletal muscle repair.

As previously mentioned, PPAR\textgamma is a target of 15∆-PGJ\textsubscript{1} used to stimulate proliferation in certain cell types, but this cascade is presumably inexistent in skeletal muscle cell in vivo as PPAR\textgamma is very weakly expressed. Since PPAR\textdelta, 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 15∆-PGJ\textsubscript{2}. A 24 h incubation of L6 cells with GW 0742, a specific PPAR\textdelta 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.

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 15Δ-PGJ_2 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 15Δ-PGJ_2, 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 15Δ-PGJ_2 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) 15Δ-PGJ_2 is produced by skeletal muscle cell and 2) DP_2 and DP_1 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 15Δ-PGJ_2 production, our results remain very physiologically relevant since 15Δ-PGJ_2 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 15Δ-PGJ_2 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 15Δ-PGJ_2 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 15Δ-PGJ_2 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 15Δ-PGJ_2 on muscle repair since in addition to its anti-inflammatory properties, mitogenic effects have been associated to this metabolite.

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REFERENCES


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