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Prostaglandin E₂: From clinical applications to its potential role in bone-muscle crosstalk and myogenic differentiation

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Abstract

Prostaglandin E₂ (PGE₂), a prostanoid synthesized from arachidonic acid via the cyclooxygenase pathway, is a modulator of physiological responses including inflammation, fever, and muscle regeneration. Several patents have been filed that are related to PGE₂, one of them being directly related to skeletal muscles. In this report, we first summarize the key patents describing inventions for the utilization of PGE₂ for either diagnostic or therapeutic purposes, including skeletal muscle. In the second part of our work we present new and exciting data that demonstrates that PGE₂ accelerates skeletal muscle myogenic differentiation. Our discovery resulted from our recent and novel concept of bone-muscle crosstalk. Bone and muscle are anatomically intimate endocrine organs and we aimed to determine whether this anatomical intimacy also translates into a biochemical communication from bone cells to muscle cells at the *in vitro* level. The effects of MLO-Y4 osteocyte-like cell conditioned medium (CM) and three osteocyte-secreted factors, PGE₂, sclerostin and monocyte chemotactic protein (MCP-3), on C2C12 myogenic differentiation were evaluated using morphological analyses, a customized 96-PCR gene array, and measurements of intracellular calcium levels. MLO-Y4 CM and PGE₂, but not sclerostin and MCP-3, induced acceleration of myogenesis of C2C12 myoblasts that was linked with significant modifications in intracellular calcium homeostasis. This finding should further stimulate the pursuit of new patents to explore the use of PGE₂ and the new concept of bone-muscle crosstalk for the development and application of inventions designed to treat muscle diseases characterized by enhanced muscle wasting, such as sarcopenia.

Keywords

Bone-muscle crosstalk; PGE₂; inventions; prostaglandins; prostanoid; EP Receptors; gene expression; MyoD; muscle regulatory factors; myogenic acceleration; calcium release; excitation-contraction coupling; bone cells; osteocytes; MLO-Y4 osteocytes cells like; MLO-Y4 conditioned media; bone secreted factors; muscle secreted factors; myokines; calcium-induced calcium release (CICR); patents

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Introduction

PGE₂ is one of the major products of arachidonic acid through cyclooxygenase-1/2 pathways. In the past decades, it has been related to inflammation, pain and cancer development. On the other hand, PGE₂ is important in regulation of physiological functions, such as sodium and chloride transport in the kidney and smooth muscle tones, suggesting itself or its derivatives would be used in the treatment of diseases. Here we will summarize some patents associated with the application of PGE₂ in diagnosis and therapeutics (Table 1) [1-9].

To date, only one patent has been filed concerning with the effect of PGs on skeletal muscle[1]. Given the extensive usage of PGE₂ in disorders in other tissues, there is a great potential for patenting the employment of PGE₂ for the functional regulation of skeletal muscle and treatment of muscle diseases, such as muscle dystrophy. It is interesting to observe that patent US 8168428 describes an invention aimed at using PGE₂ for the acceleration of growth of hematopoietic stem cells. Since skeletal muscle can use both resident satellite cells and hematopoietic stem cells for regeneration and repair, it would be interesting to test such approach in skeletal muscle cells. To a great extent, we shall present new and exciting data below that will present PGE₂ as a potent signaling molecule in skeletal muscle cells.

In recent years, muscle work as endocrine organs has come to light. Skeletal muscles are the source of “myokines” with known effects on fat and body metabolism[10]. On the other hand, bone cells, and in particularly osteocytes are known to produce ATP, nitric oxide, prostaglandins (major one being PGE₂), sclerostin, fibroblast growth factor 23, MCP-3, and many other factors [11-15]. Therefore, osteocytes can be viewed as a source and potential reservoir of factors in bone with the intrinsic potential of influencing other tissues. Given the intimate relationship between bone and muscle during development and life span[16], it is reasonable to assume that bone secreted factors might influence muscle behavior and function, but this hypothesis has not yet been investigated in a detailed and systematic manner.

To begin to address the potential biochemical relationship between bone and muscle, well established and recognized *in vitro* models of bone and muscle research were employed. C2C12 myoblasts, a well-accepted cell model for myogenic differentiation[17,18], and the osteocyte-like MLO-Y4 cells, a widely used cell model for osteocyte research, were used to investigate the potential effects of osteocyte signaling to muscle cells. As such, we focused our studies on the effects of MLO-Y4 CM and three potential factors derived from MLO-Y4 (PGE₂, sclerostin, and MCP-3) on C2C12 myoblast differentiation into myotubes, gene PCR profile of 96 genes, and intracellular calcium homeostasis.

We found that MLO-Y4 CM (down to 10% CM) was a potent stimulant of myogenic differentiation, a phenomenon that was evidenced by morphological, functional, and genetic modifications that clearly indicate a myogenic effect of bone cells on muscle cells. Furthermore, our studies demonstrated that PGE₂, but not sclerostin or MCP-3, induces significant myogenic differentiation that closely mirrors the effects of MLO-Y4 CM, suggesting that this major secreted prostaglandin from osteocytes could influence or modulate muscle function.

Methods

Cell culture

C2C12 myoblasts were maintained in high-glucose Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA, USA) with 10% fetal bovine serum (FBS, Fisher, Pittsburgh, PA, USA), and 100U/ml penicillin and 100µg/ml streptomycin (Fisher) at 37°C in a humidified atmosphere with 5% CO₂ for 24h. This media was removed and then, differentiation medium (2.5% horse serum, no FBS), was added to induce differentiation of myoblasts into contracting myotubes. MLO-Y4 cells were cultured in alpha modified essential medium with 2.5% fetal calf serum and 2.5% calf serum until 70% confluent before the collection of CM. Media without cells and osteoblasts CM were tested and found to have no effect on C2C12 cells (data not shown). The cells were treated with 25% MLO-Y4 osteocyte like cell conditioned media, 50nM PGE₂ (Cayman Chemicals, Ann Arbor, Michigan, USA), 150µg/ml MCP-3 (R&D systems, Minneapolis, MN, USA), or 2µg/ml sclerostin (R&D systems) at the onset of differentiation. Images were taken after differentiation for 3 days using a Leica DMI 4000B microscope (Leica microsystems, Buffalo Grove, IL, USA), and myotube area (µm²) was automatically calculated and measured by the Leica Application Suite Advance Imaging fluorescence software package (Leica microsystems). At least 30 myotubes per well in each experiment were used for determination of cell area comparisons and experiments were repeated 6 times.

Intracellular Ca²⁺ measurements

A Photon Technology International (PTI, Birmingham, NJ, USA) imaging system was used to measure intracellular Ca²⁺ homeostasis. Each myotube imaged was loaded with 2µM Fura-2 AM for 30 min at 37°C, followed by application of 20mM caffeine (Fisher) with a perfusion system (Bioscience Tools, San Diego, CA, USA). Ratiometric analysis (350/375nm excitation ratio; 510nm emission) was performed using PTI's Felix 32 Photometry system. Both intracellular resting Ca²⁺ and the peak responses to caffeine treatment were measured in these studies. These experiments were repeated 3 times and at least 6 cells were tested on each experiment.

Real-time PCR Array

Total RNA was extracted from C2C12 myoblasts after 24h treatment using an RNAqueous®-4PCR kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction, and was quantified in a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) by determining absorbance at 260nm in triplicates. 1µg of each RNA sample with the A260/280 nm absorbance ratio of 1.9-2.1 was reverse transcribed in a 20µl reaction volume using a commercial Quantitec Reverse Transcription Reagents kit (QiagenInc, Valencia, CA). The expression of 91 genes of interest and 5 reference genes was investigated using a customized Mouse RT²Profiler™ PCR Array system (SA Biosciences). Genes were grouped into 5 main groups: heat shock/stress, survival and metabolic, Ca²⁺ signaling, Ca²⁺ release, and hypertrophy genes. qPCR was performed using the Step-One Plus™ Real-Time PCR System (ABI, Foster City, CA, USA) via standard fluorescent methodology and thermal cycling conditions following the manufacture's recommendations, including a threshold of 0.2 and validation of each gene tested by the identification of single peaks in melting curves. The real-time PCR reaction mixture contained 1µl of 50ng of cDNA, 12.5 µl of the RT² Real-Time™ SYBR Green/Rox PCR master mix, 1µl of primer pairs and 10.5 µl of RNase free water to a complete reaction of 25 µl. Data was analyzed using RT² Profiler™ PCR Array software (SA Biosciences), and relative amounts were calculated by the 2^{-ΔΔCT} method as previous described[19]. CT values were normalized to ActB as a reference gene. Gene expression was determined as up/down regulation of the gene of interest compared to the control.

Expression levels of the candidate reference genes: We also investigated the expression levels and variability for five candidate reference genes (B2m, GAPDH, Hprt1, Rplp1 and Actb), both for control and PGE₂ treatment. CT values from ActB showed an average of 0.3 cycle difference and an efficiency of 94% therefore, this gene was selected for normalization purposes.

Statistical analysis

One-way ANOVA with post hoc Dunnett's test was performed to compare myotube area between control and treatments. Results were expressed as mean $\pm$ SD. Differences were considered significant at $P < 0.05$.

Results

Histological association between bone and muscle

The intimate relationship between bone and muscle is clearly illustrated in Fig. 1. This figure also shows how closely associated these tissues are with the vascular tissue. The close association and functioning of these tissues suggests that they could reciprocally influence each other through secretion and uptake of specific molecules.

MLO-Y4 CM and PGE₂ enhance myogenic differentiation of C2C12 myoblast

To examine the effect of bone-to-muscle signaling on myogenic differentiation, C2C12 cells were treated with MLO-Y4 osteocyte like cell CM, PGE₂, MCP-3, or sclerostin when myoblast media was switched from complete growth medium to differentiation medium. Therefore, under all experimental conditions the effects of treatments were compared to the control effects of myogenic differentiation that are normally produced by removing FBS and replacing it with horse serum (2.5% in our study); therefore the effects of CM and bone specific factors are either seen as enhancing the already potent effects of differentiation media, or reducing, or having no effects. In this study, 25% CM and 50nM PGE₂ were used in all experiments. We also performed dose response curves and found that 100, 50, 25, and 10% MLO-Y4 CM all promoted C2C12 myogenic differentiation, and 50nM PGE₂, produced similar results to those produced by PGE₂ at 0.25, 0.5, 1, and 5 μ M (Data not shown). Both 25% CM and 50nM PGE₂ were selected because of the consistency and small intra-variability between experiments. After 72h of differentiation, C2C12 myotubes were visibly present in all conditions, but the number of developed myotubes was increased by both MLO-Y4 CM and PGE₂ (number of visible myotubes per view: control: 7 ± 2 ; 25% CM: 14 ± 4 , and PGE₂: 13 ± 3), but not by MCP-3 and sclerostin (9 ± 3 and 10 ± 4 , respectively) (Fig. 2A). In addition, significant differences suggestive of cellular hypertrophy were observed in the newly formed myotubes (control cell area $4914 \pm 571 \mu\text{m}^2$; CM cell area $10544 \pm 1466 \mu\text{m}^2$; and PGE₂ cell area $9833 \pm 1033 \mu\text{m}^2$) (Fig. 2B); however, MCP-3 and sclerostin did not have any effect on cell area (4741 ± 831 and $4423 \pm 573 \mu\text{m}^2$, respectively).

Change of gene expression profile after MLO-Y4 and PGE₂ treatment

Next, to explore the mechanism(s) behind the effects of CM and PGE₂, the expression of 91 genes related to cell myogenic differentiation, cell survival, Ca²⁺ signaling and homeostasis, cell metabolism, oxidative stress, and cell growth was measured. MyoD and myogenin, key transcriptional myogenic regulatory factors, were upregulated by 2-3 fold by CM and PGE₂ 24h after the onset of treatment (Fig. 3). Another important change in gene expression profile was the upregulation of Superoxide dismutase (SOD1), and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), the master regulator of mitochondria biogenesis, by more than 3-fold by CM and PGE₂, clearly demonstrating that cells treated with CM and PGE₂ are adapting for higher metabolic demand. Last, the very

high upregulation of troponin (49-fold and 28-fold by CM and PGE₂, respectively) by 24h provided evidence that myoblasts were committed to differentiation into myotubes since they were developing their contractile machinery (Fig. 3).

Monitoring modification of intracellular Ca²⁺ homeostasis caused by MLO-Y4 CM and PGE₂

To physiologically link the morphometric and genetic observations of acceleration of myogenic differentiation produced by both CM and PGE₂, intracellular calcium levels in the newly formed myotubes was measured. Our rationale was based on the fact that intracellular Ca²⁺ homeostasis is critical for muscle myogenesis and contractile function[20-22]. Therefore, intracellular calcium homeostasis could be used as an important functional surrogate between morphological and molecular modifications. The newly developed myotubes were loaded with the fluorescent dye Fura-2 AM, and exposed to 20mM caffeine to quickly and effectively release calcium from the sarcoplasmic reticulum of C2C12 myotubes[23-25]. This manipulation allows for monitoring of resting levels of calcium as well as the typical calcium transient that is produced in muscle cells in response to caffeine stimulation. As shown in Fig. 4, while control myotubes displayed the typical Ca²⁺ transient with a single peak in response to caffeine, myotubes in CM and PGE₂ treated groups exhibited multiple and paced Ca²⁺ transients, suggesting first that more calcium is being release from the sarcoplasmic reticulum (SR), and second that the observed enhancement of calcium release might be related to stimulation of CICR, since caffeine acts in skeletal muscles by sensitizing the SR calcium release channels (Ryanodine receptor 1) to calcium, which is the operational molecular mechanism of CICR[26-28].

Discussion

There are several examples of tissue crosstalk including fat-muscle, bone-kidney, and nerve-muscle[29-32], but a major gap remains in our knowledge regarding the likely important crosstalk between bone and muscle, two of the most intimately related tissues in animals and humans.

Despite the growing evidence of biochemical crosstalk between different body tissues, the traditional view of the muscle-bone relationship is that muscle applies load to the skeleton and that bone provides an attachment site for skeletal muscle – a relatively simple, mechanical relationship[16]. However, it is clear from pathological conditions, that muscle disease cannot fully explain concomitant bone disease. Some classical examples are sarcopenia where muscle atrophy cannot explain the totality of osteoporosis (e.g., muscle atrophy is much severer than bone loss during extended bed rest) and the well documented condition of loss of bone mass in astronauts in space that surpasses the loss in muscle mass and is difficult to recover upon return of astronauts to normal gravitational forces on earth[33-35].

Recently, it has been discovered that muscles, especially contracting muscles, are able to release peptides or proteins termed “myokines”, including interleukin(IL)-6[36], IL-8[37], IL-15[38] that appear to provide a reservoir of muscle derived factors for communication with other organs in the human body via autocrine, paracrine or endocrine mechanisms. The importance of these myokines for human fitness and their roles in diabetes and obesity is beginning to emerge and be recognized by the scientific community[39-41].

On the other hand, bone is also an endocrine organ. Osteocytes are the cells in skeleton that sense and respond to mechanical loads to regulate bone remodeling[42]. Upon sensing load, factors (a major one being PGE₂) are produced and secreted by osteocytes which travel through their lacuna-canalicular system to the circulation, thereby reaching other tissues and

organs[43]. These factors are also thought to be important for regulation of bone mass, resorption as well as bone formation. Intriguingly, the potential role of bone secreted factors on skeletal muscle function has not been considered an important research question. Given the proximity and similarities in developmental processes and their closely related functional aspects, i.e., bone and muscle work as a functional unit, it is important to investigate the possibility that bone and muscle crosstalk through secreted factors.

Obviously, a first level for these new studies is to investigate these potential effects of bone signaling to muscle using *in vitro* models. Our data strongly suggest that at least at the *in vitro* level, bone to muscle crosstalk occurs. Both MLO-Y4 CM and a major factor known to be secreted by osteocytes, PGE₂, exert potent myogenic differentiation effects on C2C12 myoblasts as evidenced by morphological changes, robust modifications in gene expression profile, and very intriguing changes in intracellular calcium homeostasis. Thus, not only more and larger myotubes are formed in the presence of CM and PGE₂, but also relevant functional and biochemical modifications occur within these newly formed myotubes that fully support the concept that osteocytes may induce or maintain myogenesis in muscles. Real-time PCR results provided genetic explanation for these effects. The upregulation of PGC-1 α suggested mitochondria biogenesis was enhanced to match increased energy demand for myogenic differentiation. This finding was further supported by the upregulation of SOD1, likely reflecting adaptation to increased oxidative stress and the demands of larger myotubes. Moreover, the significant higher expression of troponin, a key maker for the organization and maturation of the contractile machinery in skeletal muscle, induced by CM and PGE₂ treatments indicated that the differentiation from myoblasts into myotubes was promoted. Furthermore, our entire conclusion that myogenic differentiation was enhanced by CM and PGE₂ was fully supported by upregulation of the two most important transcriptional regulatory factors, known as muscle regulatory factors, MyoD and myogenin.

Therefore, we believe that our results have added a new level of complexity to the bone-muscle loading relationship - the molecular coupling view. This view posits that the intimate association of muscle with bone allows for 1) soluble factors produced by osteocytes within bone to be targeted to muscle, and 2) muscle-derived soluble factors to be targeted to bone. In our study, the myogenic effect of MLO-Y4 CM was mimicked by PGE₂ (but not by MCP-3 and sclerostin), suggesting that PGE₂ could be an important and specific modulator of signaling from bone to muscle.

Conclusions

Using *in vitro* cell models allows insights into the bone-muscle relationship in a broader context. Sarcopenia and osteoporosis are normally related or simultaneously occurring conditions[35,36]. The current interpretation is that sarcopenia contributes to osteoporosis because muscles, through the loading forces of contraction and stretch, are anabolic stimulators of bones[37]. This view essentially negates the potential for a retrograde or backward signaling from bone to muscle. Our findings, from the basic *in vitro* level, demonstrate the potential for biochemical signaling from bone to muscle cells, suggesting that the bone-muscle relationship could be biologically more complex than previously thought and that bones might have much more influence on muscles than previously considered.

Obviously, additional studies in a diverse number of models, including primary cells, *ex vivo* and *in vivo* bone and muscle models of research will be required to provide an indepth view of bone to muscle biochemical crosstalk.

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Figure 1. Histological evidence for the intimate association of bone-circulation-muscle
Mineralized bone is shown in light green. The osteocytes stained in dark blue are the major cell component of mature bone. Vessels can be seen in between the bone and muscle interface. Given the overwhelming proof of tissues' crosstalk it seems feasible to infer that bone and muscle communicate biochemically either through paracrine or endocrine loops.

Figure 2. MLO-Y4 CM and PGE₂ significantly enhance myogenic differentiation of C2C12 myoblasts

(A) Representative pictures of treatments taken 3 days after onset of differentiation. a) control; b) 25% MLO-Y4 CM; c) 50nM PGE₂; d) 2μg/ml sclerostin; e) 150μg/ml MCP-3. (B) myotube area measurement shows that CM and PGE₂, but not sclerostin and MCP-3 treatments significantly increase myotube area. *, significant different from control (P<0.05).

Figure 3. Real time-PCR results of 91-gene array

CM and PGE₂ upregulate the expression of myogenic regulatory factors, and genes related with Ca²⁺ homeostasis and mitochondria biogenesis in C2C12 myoblasts. Two arrays yielded similar results.

Figure 4. Modulation of Ca^{2+} homeostasis in myotubes by osteocyte signaling
18-30 myotubes were tested for each condition. Arrows indicate addition of 20mM caffeine. While a single peak calcium transient is observed in response to caffeine under control conditions, multiple peaks are observed in cells treated with CM and PGE₂.

Table 1
Selected Patents related with usage of PGE₂ in diagnosis and therapeutics

Patent #	Title	Target	Inventor(s)
US 6291528	Prostaglandin E1/F2 in combination with prostaglandin F2.alpha. for enhancing female sexual arousal	Female sexual arousal dysfunction	Nathan Earl Scott
US 6476074	Method and composition for treatment of erectile dysfunction	Impotence	Johan Stjernschantz, Bahram Resul
US 6545045	Prostaglandin E agonists for treatment of glaucoma	Glaucoma and/or ocular hypertension	Peter G. Klimko, Najam A. Sharif, Brenda W. Griffin
US6841573	Use of arachidonic acid as a method of increasing skeletal muscle mass	Skeletal muscle	William Charles Llewellyn
US 6610719	Use of prostaglandin (PGE2) receptor a (EP4) selective agonists for the treatment of acute and chronic renal failure	Renal failure	Vishwas M. Paralkar, David D. Thompson
US 8168428	Method to modulate hematopoietic stem cell growth	Stem cell	Leonard I. Zon, Trista E. North, Wolfram Goessling
US 2010/0267826 A1	Treatment of ischemic episodes and cerebroprotection through Prostaglandin E2 (PGE2) EP2 and/or EP4 agonists	Ischemia	Katrin Andreasson
US 2010/0278875 A1	Prostaglandin E2 (PGE2) as an adjuvant in monoclonal antibody generation	B cell	Jill Giles-Komar, Michael A. Ryczyn
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