BzATP induces extracellular matrix proteins synthesis via the PYK2-ERK pathway

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Contents

The following article and new unpublished data (Fig. 2) are a part of this doctoral thesis:

"PYK2 mediates BzATP-induced extracellular matrix proteins synthesis."
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Abstract

Adenosine triphosphate (ATP) is released by mechanical stimulation or inflammation that is mediated by autocrine/paracrine on osteoclasts and osteoblasts in bone remodeling. Mechanical stimuli such as fluid shear and cyclic tension force induce extracellular ATP release in osteoblasts. Extracellular ATP is promoted by many physiological responses through the activation of ATP-binding purinergic (P2) receptors which are divided into G protein-coupled receptors of P2Y family and ligand-gated cation channels of P2X family. In particular, cyclic tension force-induced ATP enhances bone formation through the P2X7 activation. Previous studies showed that mechanical stimuli induced the expression of extracellular matrix protein (ECMP) and osteogenesis through P2X7 activation in MC3T3-E1 cells. These reports indicate that the P2X7 might be a critical factor in osteogenesis.

Proline-rich tyrosine kinase 2 (PYK2)-mediated osteoblast differentiation is induced by mechanical stimuli such as tension force. Furthermore, the activation of PYK2 was a response to integrin through the tension force. Integrins also mediate cell attachment through the activation of many intracellular signaling pathways including tyrosine phosphorylation cascades, calcium influx, inositol lipid turnover, and mitogen-activated protein kinase. A previous study showed that tension force activated PYK2 with the increase of intracellular Ca^{2+} concentration in osteoblasts. However, the effects of the interaction of

2′(3)-*O*-(4-Benzoylbenzoyl) adenosine-5′-triphosphate (BzATP), which is the agonist of the mechanosensitive receptor P2X7, with PYK2 on ECMP productions are poorly understood. Thus, the purpose of this study was to investigate the effects of BzATP on PYK2-mediated signal pathways and ECMP productions in cultured osteoblasts.

Mouse calvaria-derived MC3T3-E1 cells were cultured in the presence or absence of PYK2 inhibitor PF431396 and then stimulated with or without 100 μM BzATP for up to 14 days. The mRNA expression levels of type I collagen (Col I), bone sialoprotein (BSP), osteocalcin (OCN) and osteopontin (OPN) were determined by real-time PCR and the protein expression levels of Col I, BSP, OCN, β-actin, PYK2, phospho-PYK2 (p-PYK2), ERK2 and phospho-ERK2 (p-ERK2) were determined by Western blotting.

BzATP increased p-PYK2 protein expression on 3- and 7-day of culture. The PYK2 inhibitor PF431396 inhibited the stimulatory effect of BzATP on the expression of Col I, BSP and OCN. PF431396 did not inhibit the stimulatory effect of BzATP on OPN mRNA expression. BzATP increased p-ERK2 expression, whereas PF431396 blocked the stimulatory effects of BzATP on the ERK2 activation. These results suggest that OPN expression might be regulated by other signal molecules such as focal adhesion kinase (FAK), and that mechanical stimuli-induced ECMP productions might be linked to extracellular ATP secretion and/or integrin via the PYK2-ERK activation.

In conclusion, a P2X7 specific agonist, BzATP, activated PYK2 and ERK. PYK2 inhibitor PF431396 blocked the stimulatory effect of BzATP on p-ERK2 protein expression and ECMP productions. Therefore, P2X7 might induce ECMP expressions through the PYK2-ERK pathway with the exception of OPN expression. In addition, mechanical stimuli-induced ECMP productions might be implicated in extracellular ATP secretion via the PYK2-ERK activation.

Introduction

The homeostasis of skeletal bone is regulated by various kinds of factors such as hormones, cytokines, and mechanical stimuli. Among them, mechanical stress is a major factor affecting the amount and strength of the bone tissue required for the maintenance of the bone mass in adults [1]. Furthermore, mechanical stimuli can be used to improve the clinical treatments for bone fracture and orthodontic tooth movement.

It has been reported that adenosine triphosphate (ATP) is released from osteoclasts and osteoblasts in response to mechanical stimulation or inflammation [2]. The release of extracellular ATP is promoted by pressure, stretch, and flow shear, as well as osmotic stress in various cell types including osteoblasts [1-4]. Extracellular ATP is also promoted by many of physiological responses through the activation of ATP-binding purinergic (P2) receptors which are divided into G protein-coupled receptors of P2Y family and ligand-gated cation channels of P2X family [5]. P2X3, P2X4, P2X5 and P2X7 are expressed in mouse osteoblastic MC3T3-E1 cells [6], but P2X4 is not implicated in promoting bone formation by extracellular ATP [7]. Notably, P2X7 is activated by mediated pore formation, which is expressed in response to fluid shearing stress in mouse calvarial cells [8]. $P2X7^{-/-}$ mice also demonstrated decreased osteogenesis in response to mechanical loading in long bones, however, the deficiency of P2X7 did not affect the length of mouse long bones [9]. It is well

known that 2′(3)-*O*-(4-Benzoylbenzoyl) adenosine-5′-triphosphate (BzATP) is a specific agonist of P2X7 receptor [10]. Panupinthu et al., have been reported that BzATP induced bone formation through the production of lysophosphatidic acid and prostaglandin E_2 in mouse calvarial osteoblasts [10]. Previous studies showed that mechanical stimuli induced the expression of extracellular matrix protein (ECMP) and osteogenesis through ATP-mediated P2X7 activation in MC3T3-E1 cells [11, 12]. These reports indicate that BzATP might be a critical factor in osteogenesis.

A intracellular signal molecule, proline-rich tyrosine kinase 2 (PYK2), belongs to focal adhesion kinase subfamily of non-receptor tyrosine kinases. PYK2 is a large multidomain protein containing an N-terminal FERM domain, a central catalytic domain, and a C-terminal segment that includes dual proline rich subdomains and a focal adhesion targeting region [13, 14]. PYK2 interacts with a variety of proteins including p130Crk-associated substrate (CAS) [15], Src [16], Cbl [17], integrins [16], gelsolin [18], and paxillin [19]. Interestingly, integrins are activated by mechanical strain-induced ERK [20] and mediate cell attachment through the activation of many intracellular signaling pathways; tyrosine phosphorylation cascades, calcium influx, inositol lipid turnover, and mitogen-activated protein kinase (MAPK) [21]. A previous study showed that tension force activated PYK2 with the increase of intracellular Ca^{2+} concentration in osteoblasts [22]. However, the interaction of P2X7 with PYK2 on the

expression of ECMPs is elusive. Thus, the purpose of this study was to investigate the effects of P2X7 agonist BzATP on PYK2-mediated signal pathways and ECMP productions in cultured osteoblasts.

Materials and Methods

Cell culuture

The MC3T3-E1 mouse calvarial cell line obtained from Riken Bio Resource Center (Tsukuba, Japan) was used as osteoblast-like cells. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in α-minimal essential medium (MEM; Gibco BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 1% (v/v) penicillin–streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA). Cells were treated with or without 100 μM 2'(3)-O-(4-Benzoylbenzoyl) adenosine-5'-triphosphate (BzATP) (Sigma-Aldrich) and/or PYK2 inhibitor PF431396. The medium was replaced every 3 days.

Real-time polymerase chain reaction (real-time PCR)

Cells were seeded on 6-well plates and cultured for up to 14 days. Total RNA was isolated on 3-, 7-, and 14-day of culture using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and then the RNA concentration was measured by Nano Drop 1000 (ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 0.5 μg of DNase-treated total RNA using the Prime Script RT reagent kit (Takara Bio, Shiga, Japan), and the resultant cDNA was analyzed by real-time PCR using the SYBR® PremixEX

Taq™II (Takara Bio). The primer sequences are shown in Table. 1. The PCR assays were performed with a Smart Cycler II instrument (Cepheid, Sunnyvale, CA, USA) and analyzed using Smart Cycler software. The cycling conditions included 35 cycles at 95°C for 5 sec and 60°C for 20 sec. All real-time PCR experiments were performed in triplicate. The calculated values of the target gene expression were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control [23].

Western blotting

Cells were cultured for up to 7 days. Subsequently, the medium were changed serum-free medium and harvested 24 h later. The total protein concentrations in cell lysates were quantified by *DC*™ Protein Assay (Bio-Rad, Hercules, CA, USA), and 20 μg of protein from each sample was resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was then treated with blocking reagent [1 % (v/v) BSA] in Tris-buffered saline (TBS, 10 mM Tris, 145 mM NaCl, pH 7.4) for 18 h at 4°C, washed in TBS containing 0.05% Tween 20, and incubated with rabbit polyclonal IgG antibodies or mouse monoclonal IgG antibody raised against PYK2, phospho-PYK2, ERK1/2, phospho-ERK1/2 (Cell Signaling technology, MA,

USA), type I collagen (Col I), bone sialoprotein (BSP), osteocalcin (OCN), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 or 1:1000 in blocking reagent [1% (v/v) BSA] for 1 h at room temperature. β-actin was used as an internal standard. Membranes were then washed in TBS-Tween and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies diluted 1:5000 in 1% blocking agent for 2 h at room temperature. Chemiluminescent signals were detected by according to the manufacturer's instructions (ECL Prime Western blotting detection reagent, Amersham-Pharmacia Biotech, NJ, USA). Signals were quantified using Image J provided by the NIH and normalized according to β-actin or ERK2 density.

Statistical analysis

Three independent experiments were performed using samples that were prepared in triplicate. Each value is expressed as the mean ± standard error (SEM). Differences between groups were evaluated with the one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test or two-way ANOVA with Bonferroni's multiple comparisons test. Differences were considered statistically significant at $P < 0.05$.

Results

BzATP affects phospho-PYK2 protein expression

To clarify the effect of PYK2 on BzATP in osteoblasts, the protein expression level of phospho-PYK2 (p-PYK2) on BzATP-stimulated MC3T3-E1 cells was determined. BzATP significantly increased protein expression levels of p-PYK2 on 3- and 7-day of culture compared to untreated controls, respectively (Fig. 1).

BzATP affects phospho-ERK1/2 protein expression

To clarify the response to the downstream pathway of the BzATP-PYK2 in osteoblasts, the protein expression level of phospho-ERK2 (p-ERK2) was next determined. BzATP induced the increases of protein expression level of p-ERK2 on 1- and 3-hour of culture compared to untreated controls, respectively, and PYK2 inhibitor PF431396 significantly decreased BzATP-increased p-ERK2 expression level. However, BzATP did not affect the expression levels of ERK1 (Fig. 2).

Effect of BzATP on ECMP mRNA expressions

This study investigated the effect of BzATP on mRNA expression levels of ECMP. BzATP induced increases of mRNA expression level of Col I on 3- and 7-day of culture (by 2.0 and 1.68-fold, respectively, Fig. 3a), BSP on 3-, 7- and 14-day of culture (by 1.5, 1.7 and

3.8-fold, respectively, Fig. 3b), and OCN on 7- and 14-day of culture (by 5.57 and 6-fold, respectively, Fig. 3d) compared to untreated controls, respectively. The mRNA expression level of OPN also increased on 7-day of culture (by 1.42-fold, Fig. 3c) compared to untreated contol. The effect of PYK2 inhibitor PF431396 on ECMP mRNA expressions was further examined in BzATP-stimulated MC3T3-E1 cells. PF431396 blocked the increases of mRNA expression level of Col I on 3- and 7-day (by 0.66 and 0.53-fold, respectively, Fig. 3a), BSP on 3-day (by 0.85-fold, Fig. 3b) and OCN on 7-day (by 0.08-fold, Fig. 3d) compared to untreated controls, respectively. However, PF431396 did not inhibit BzATP-increased mRNA expression level of OPN (Fig. 3c).

Effect of PYK2 on BzATP-induced ECMP protein expression

This study next determined the effect of PYK2 on BzATP-induced ECMP productions. BzATP induced significant increases of protein expression level of Col I and BSP, while PF431396 inhibited BzATP-induced expression levels of ColI and BSP on 3-day of culture (by 0.58 and 0.86-fold, respectively) compared to untreated controls, respectively (Figs. 4a and b). Similarly, BzATP induced the increase of OCN protein expression level on 7-day of culture, while PF431396 significantly inhibited OCN level (by 0.38-fold, Fig. 4c) compared to untreated control. This study did not determine OPN protein expression level because OPN

mRNA level was unaffected by PF431396 (Fig. 3c).

Discussion

BzATP is a specific agonist of P2X7 that induces the opening of P2X7 channels, by causing the elevation of intracellular Ca^{2+} and depolarization of the plasma membrane [24]. P2X7 receptor is a nonselective cation channel permeable to Na^{+}, K^{+}, and Ca^{2+}. Previous studies demonstrated that BzATP or a high concentration of ATP increased cytosolic Ca^{2+} concentration through P2X7 activation [10, 25]. Functional P2X7 receptors can be expressed in osteoblasts both *in situ* and *in vitro* [10]. Furthermore, $P2X7^{-/-}$ mice had reduced osteogenesis in load-bearing bones, which suggests that the activation of P2X7 is responsive to mechanical stress in the skeletal bone [8, 9]. However, Orriss et al., reported that the activation of P2X7 receptor reduced bone formation in primary rat osteoblasts [7]. These different findings between mice and rats suggest that the effect of P2X7 on bone formation may be dependent on the species-specific manner. In fact, this study clearly showed the inductive action of P2X7 on ECMP productions in mouse osteoblastic MC3T3-E1 cells.

Mechanical stimuli which include pressure, stretch, fluid shear and osmotic stress induce extracellular ATP release in osteoblasts [2, 3]. Extracellular ATP is also released during apoptosis of osteoblasts and bone remodeling [26]. It has been demonstrated that the secretion of extracellular ATP promotes osteogenesis in rodent calvarial cells *in vitro* [10, 27]. Other studies have also demonstrated that cyclic tension force and low-intensity pulsed ultrasound

induced extracellular ATP release along with bone formation through the P2X7 in MC3T3-E1 cells [11, 12]. These findings suggest that mechanical stimuli-induced bone formation closely link to the extracellular ATP secretion and P2X7 expression in osteoblasts. In addition, present data give a possibility that mechanical stimuli increase the expression of P2X7 receptor through extracellular ATP secretion in MC3T3-E1 cells.

Studies using antisense depletion of PYK2 indicated that the catalytic activity of PYK2 might be dispensable [28, 29]. A mechanical transducer of integrin was mediated by the PYK2-Ca^{2+} pathway in osteoblasts [20]. Boutahar et al., also reported that tension force activates PYK2 involved in ERK activation [22]. This present study showed that BzATP increased phospho-ERK2 expression, whereas PF431396 blocked the stimulatory effect of BzATP at 1- and 3-hour of culture (Fig. 2). These results suggest that PYK2-activated ERK is closely related to the downstream signaling of the BzATP-PYK2 pathway in osteoblasts.

Periodic mechanical stress induces chondrocyte proliferation and matrix synthesis via the calmodulin-dependent kinase II (CaMKII)-PYK2 pathway [30]. CaMKII is a multifunctional serine/threonine kinase and has been confirmed to be a critical regulator of Ca^{2+} in various signaling pathways including the nuclear factor of activated T-cells (NFAT) c1 pathway. These findings indicate that expressions of PYK2 and CaMKII are involved in the Ca^{2+}-dependent pathway. Moreover, Grol et al., have reported that BzATP induced the

Ca^{2+}-NFATc1 activation, and Panupinthu et al. have also indicated that BzATP increases the gene expressions of ECMP in osteoblasts [10, 25]. These findings suggest that BzATP enhances ECMP expressions through NFATc1-CaMKII pathway. Thus, this study focused on the effects of PYK2 on BzATP-induced ECMP expression. Indeed, BzATP increased p-PYK2 on 3- and 7-day of culture. Furthermore, the PYK2 inhibitor PF431396 suppressed the stimulatory effect of BzATP on the expression levels of Col I, BSP and OCN (Figs. 1, 3 and 4). These results strongly suggest that PYK2 affects BzATP-induced ECMP productions.

Col I acts as a scaffold for the nucleation of hydroxyapatite crystals through the calcification process, and therefore is a major component of ECMP in bone tissue. BSP, OPN, and OCN are non-collagenous matrix proteins that have important roles in the organization of the collagen matrix [31]. BSP has a role of nucleation center for the hydroxyapatite formation in the mineralized nodule formation in bone [32]. OCN has small γ-carboxyglutamate protein, that is selectively expressed by osteoblasts [33]. OCN is the most abundant non-collagenous bone matrix protein [34] and is a major marker of differentiated osteoblasts. The functions of OPN are diverse and directly related to bone formation and remodeling, as with fundamental roles in host defense and tissue repair [35]. A previous study reported that mechanical stimuli induce OPN mRNA expression in osteoblasts, but PYK2 did not mediate it [36]. Similarly, this study showed that BzATP induced OPN expression, whereas PF431396 did not affect its

expression. These results suggest that BzATP-induced OPN might be regulated by other signal molecules such as focal adhesion kinase (FAK), as described in a previous report [36]. Taken together, these results suggest that mechanical stimuli-induced ECMP productions might be linked to extracellular ATP secretion and/or integrin via the PYK2-ERK activation.

In summary, a BzATP specific agonist, P2X7, activated PYK2 and ERK. PYK2 inhibitor PF431396 blocked the stimulatory effect of BzATP on p-ERK protein expression and ECMP productions.

Conclusion

These results suggest that mechanical stimuli-activated P2X7 might induce ECMP expressions through the PYK2-ERK pathway with the exception of OPN expression. In addition, mechanical stimuli-induced ECMP productions might be implicated by extracellular ATP release via the PYK2-ERK activation.

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Table 1. PCR primers used in the experiments

Target	Primers	GenBank Acc. No.
Col I	5'-TGGGCGCGGCTGGTATGAGTTC-3' 5'-ACCCTGCTACGACAACGTGCC-3'	NM_007743.2
BSP	5'-AATTCTGACCCTCGTAGCCTTCATA-3' 5'-GAGCCTCGTGGCGACACTTA-3'	NM_008318.3
OPN	5'-TACGACCATGAGATTGGCAGTGA-3' 5'-TATAGGATCTGGGTGCAGGCTGTAA-3'	NM_009263.3
OCN	5'-AAGCAGGAGGGCAATAAGGT-3' 5'-ACCCTGCTACGACAACGTGCC-3'	NM_007541.2
GAPDH	5'-AAATGGTGAAGGTCGGTGTG-3' 5'-TGAAGGGGTCGTTGATGG-3'	NM_008084.2

Col I, type I collagen; BSP, bone sialoprotein; OPN; osteopontin, OCN, osteocalcin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

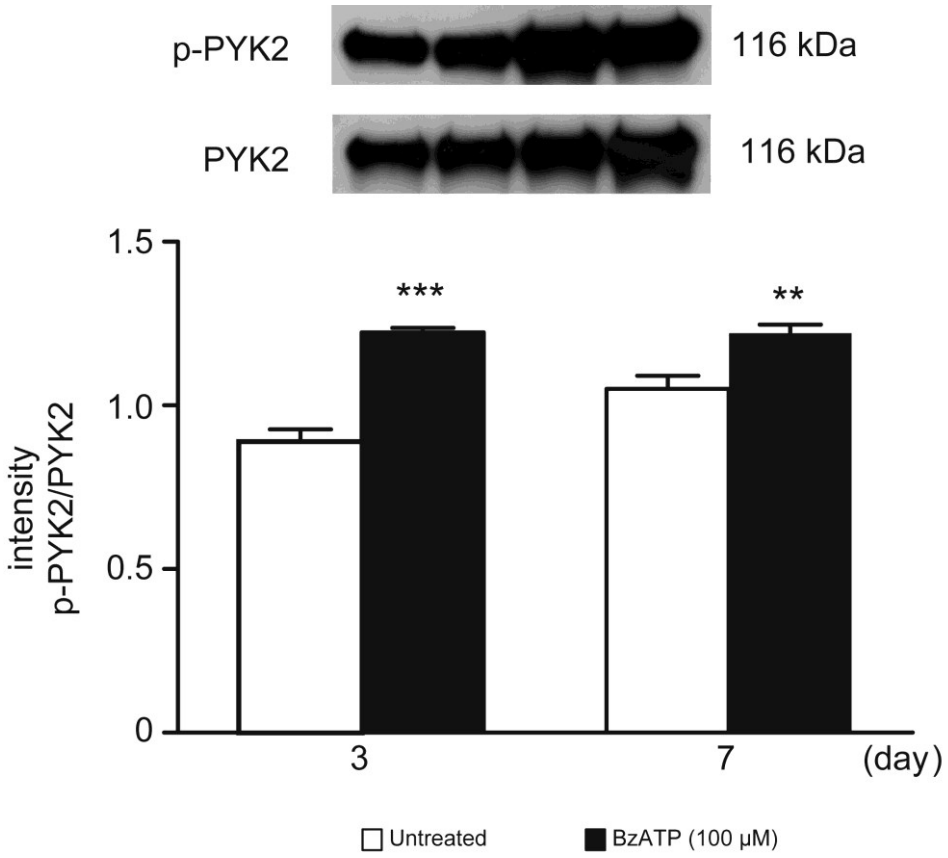

Fig. 1. Effect of BzATP on p-PYK2 protein expression

Cells were stimulated with BzATP (100 μM) or left without stimulation (Untreated) and the protein expression levels of p-PYK2 and PYK2 were determined on 3- and 7-day of culture by Western blotting. Histograms show the intensity of protein bands under each condition. Data are expressed as the mean ± SEM of triplicate; $^{**}p < 0.01$, vs. untreated.

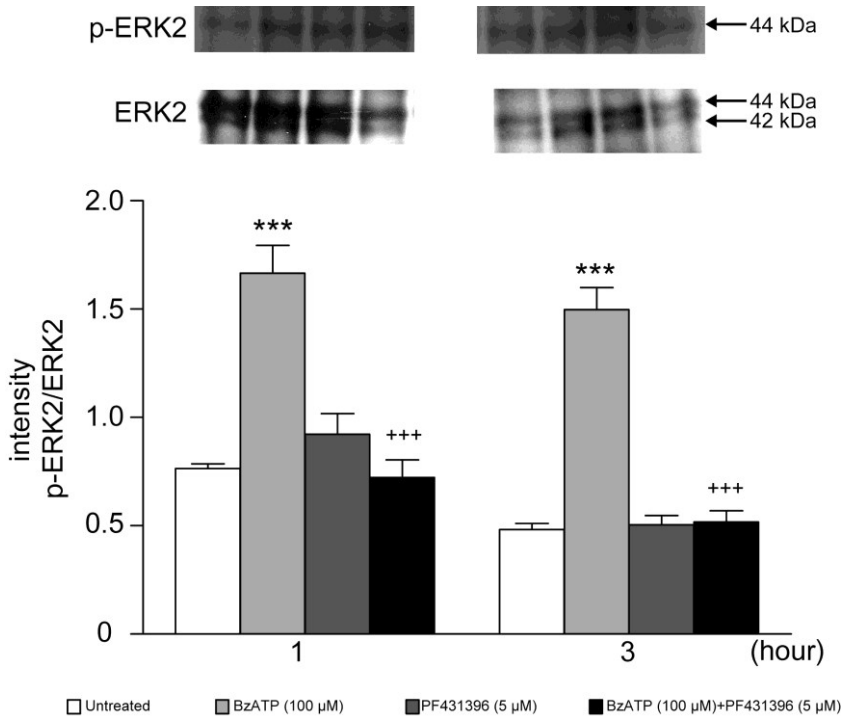

Fig. 2. Effect of PYK2 inhibitor on BzATP-induced p-ERK protein expression

Cells were stimulated with BzATP (100 μM) and/or PYK2 inhibitor PF431396 (5 μM) or left without stimulation (Untreated) and the protein expression levels of ERK2 and p-ERK2 were determined on 1- and 3-hour of culture by Western blotting. Histograms show the intensity of protein bands under each condition. Data are expressed as the mean ± SEM of triplicate; ***$p < 0.001$, vs. untreated, +++$p < 0.001$, vs BzATP.

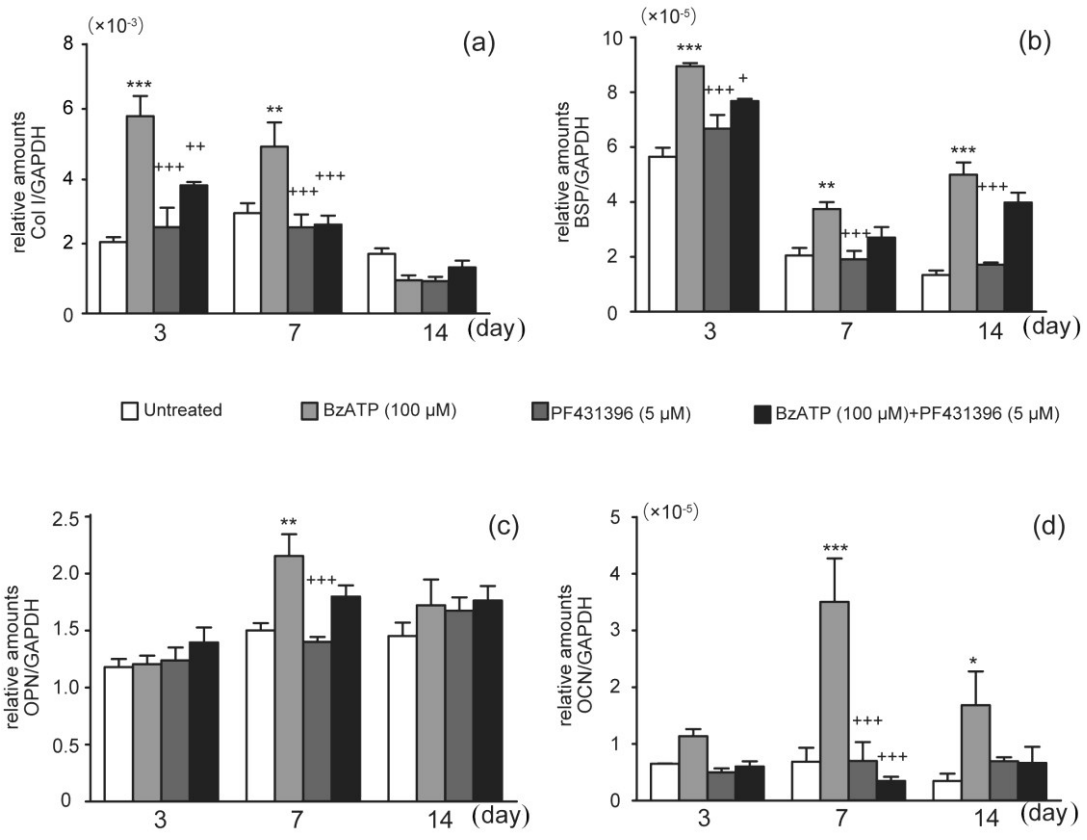

Fig. 3. Effect of PYK2 inhibitor on BzATP-induced ECMP mRNA expression

Cells were stimulated with BzATP (100 μM) and/or PYK2 inhibitor PF431396 (5 μM) or left without stimulation (Untreated) and the mRNA expression levels of Col I (a), BSP (b), OPN (c) and OCN (d) on 3-, 7- and 14-day of culture were determined by real-time PCR. Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. untreated, $^{++}p < 0.01$, $^{+++}p < 0.001$, vs BzATP.

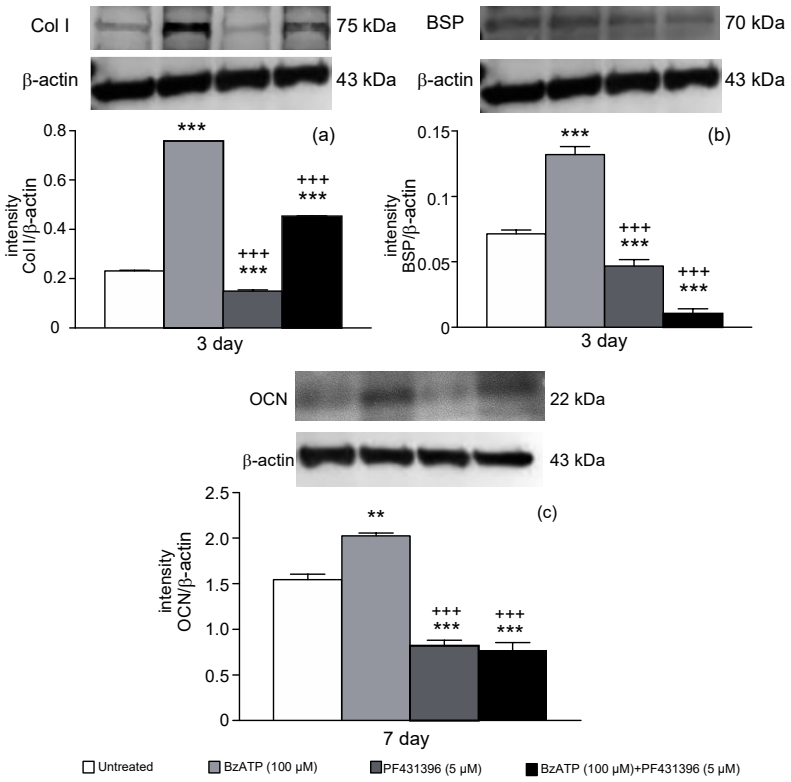

Fig. 4. Effect of PYK2 inhibitor on BzATP-induced ECMP protein expressions

Cells were stimulated with BzATP (100 μM) and/or PYK2 inhibitor PF431396 (5 μM) or left without stimulation (Untreated) and the protein expression levels of Col I (a), BSP (b), and OCN (c) on 3- and 7-day of culture were determined by Western blotting. Histograms show the intensity of protein bands under each condition. Data are expressed as the mean ± SEM of triplicate; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. untreated, $^{+++}p < 0.001$, vs BzATP.