K6PC-5, a novel sphingosine kinase 1 (SphK1) activator, alleviates dexamethasone-induced damages to osteoblasts through activating SphK1-Akt signaling

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Abstract

Long-term glucocorticoid usage is a common cause of non-traumatic femoral head osteonecrosis. Glucocorticoids (i.e. dexamethasone (Dex)) could directly induce damages to osteoblasts. In the current study, we investigated the potential activity of K6PC-5 [N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide], a novel sphingosine kinase 1 (SphK1) activator, against this process. Our data revealed that both osteoblastic-like MC3T3-E1 cells and primary murine osteoblasts were responsible to K6PC-5. K6PC-5 activated SphK1, increased sphingosine-1-phosphate (S1P) production and induced Akt phosphorylation in cultured osteoblasts. Functionally, K6PC-5 protected osteoblasts from Dex-induced apoptosis and necrosis. Such signaling and functional effects by K6PC-5 were prevented by the SphK1 inhibitor N,N-dimethylsphingosine (DMS), and by SphK1-siRNAs. On the other hand, exogenously-added S1P activated Akt and reduced Dex-induced osteoblast damages. LY294002 and MK-2206, two established Akt inhibitors, alleviated K6PC-5- or S1P-mediated osteoblast protection against Dex. Together, our results suggest that K6PC-5 alleviates Dex-induced osteoblast injuries through activating SphK1-Akt signaling. K6PC-5 might be further investigated in animal or clinical studies for its anti-glucocorticoids-associated osteonecrosis potential.

1. Introduction

It is estimated that more than 30 million of Americans require glucocorticoids as part of their treatment regime [1]. Prolonged and/or over-dose glucocorticoids (i.e. dexamethasone (Dex)), however, could cause non-trauma osteoporosis and osteonecrosis, which will eventually lead to bone fractures if not treated properly [2–4]. As a matter of fact, 30–40% or more patients receiving long-term glucocorticoid usage are suffering non-trauma femoral head osteonecrosis [2]. Unfortunately, most of these patients with need surgery, usually total hip arthroplasty (THA), within a few years of onset [5].

Despite the strong association of glucocorticoid usage with osteonecrosis, the underlying mechanisms of glucocorticoid-induced osteonecrosis have been unclear. Suppression of osteoblast and osteoclast precursor production, increased apoptosis of osteoblasts, prolongation of the lifespan of osteoclasts are all direct effects of glucocorticoids [4]. Osteoblasts play vital roles in regulating bone growth and formation, and are the main target cell of glucocorticoids [6,7]. Glucocorticoids could directly induce osteoblast cell death through activating glucocorticoid receptors (GRs) [8–11]. As a matter of fact, glucocorticoids (i.e. Dex) were added directly to cultured osteoblasts to simulate a cellular model of glucocorticoid-induced femoral head osteonecrosis [8–11].

Sphingolipids are ubiquitous membrane constituents of all eukaryotic cells. These sphingolipids, besides being structural components of plasma membranes, are also important signaling molecules, regulating a number of key cellular functions [12–14]. In particular, three of these sphingolipid metabolites, including ceramide, sphingosine, and sphingosine-1-phosphate (S1P), are
proven to be a new class of lipid messengers [12–14]. Interestingly, the balance of these three lipid-signaling molecules is tightly controlled by a key enzyme of sphingosine kinase 1 (SphK1), which catalyzes the formation of S1P by phosphorylating sphingosine [12–14].

Recent studies have characterized a novel and potent activator of SphK1, named K6PC-5 \([N-(1,3-dihydroxyisopropyl)-2-hexyl-3-o xo-decanamide]\) [15,16]. This hydrophobic SphK1 activator contains a ketone group two hydroxy groups, two short alkyl groups, and an amide linkage [15,16]. In the current study, we found that K6PC-5 could alleviate Dex-induced osteoblast damages through activating SphK1-Akt signaling.

### 2. Materials and methods

#### 2.1. Chemicals and reagents

K6PC-5 was synthesized by Min-de Biological Company (Suzhou, China) based on the structure from the published literature [15,16]. Sphingosine-1-phosphate (S1P), dexamethasone (Dex), N,N-dimethylsphingosine (DMS) and LY294002 were purchased from Sigma Chemicals (St. Louis, MO). Anti-Akt, and SphK1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing p-Akt (Ser-473 and Thr-308) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies recognizing N,N-dimethylsphingosine (DMS) and LY294002 were purchased from Cell Signaling Technology (Danvers, MA). MK-2206 was obtained from Selleck (Shanghai, China).

#### 2.2. MC3T3-E1 cell culture

The murine calvaria-derived osteoblastic-like MC3T3-E1 cells, purchased from the Cell Bank of Shanghai Institute of Biological Science (Shanghai, China), were maintained in α-MEM supplemented with 12% FBS and 1% penicillin/streptomycin. This basic medium was replenished every three days. The cultures were then induced to differentiate by transferring cells into the medium supplemented with l-ascorbic acid (50 μg/mL) and β-glycerol phosphate (5 mM) (Sigma).

#### 2.3. Culture of primary murine osteoblasts

As reported [11,17], the trimmed calvariae of murine pups at 30–36 h old were isolated and washed, which were then subjected to a series of collagenase digestions. Digests 3–5 were neutralized with α-MEM, pooled, and filtered. The filtrate was centrifuged, cells were resuspended in 3–5 ml α-MEM containing 12% FBS with antibiotics. Cells were further cultured until reaching confluence at 7–9 days, medium was switched every 2–3 days. All animals were maintained in accordance with the guidelines of the NIH and the European Communities Council. The protocol is approved by Animal Care and Use Committee of all authors’ institutions.

#### 2.4. Cell survival assay

Survival of osteoblasts was evaluated with the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Osteoblasts were seeded in 96-well plates at a density of 2 × 10^4 cells/well. After applied treatment, 10 μl of the solution of CCK-8 was added to each well, and the plate was incubated for an additional 2 h. The absorbance of each well was measured at 450 nm with a reference at 655 nm using a microplate reader (Bio–Rad Laboratories, Shanghai, China).

#### 2.5. Cell apoptosis assay

The cell apoptosis histone-DNA ELISA plus kit (Roche, Palo Alto, CA) was utilized to quantify apoptosis in osteoblasts with applied treatment according to the manufacturer’s protocol. Briefly, the cytoplasmic Histone/DNA fragments from osteoblasts were extracted and bound to immobilized anti-Histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was applied for the detection of immobilized Histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the each sample was determined by using a plate reader at 405 nm.

#### 2.6. Cell necrosis assay

The osteoblast necrosis was determined by lactate dehydrogenase (LDH) release measured by a commercially available two-step enzymatic reaction LDH assay kit (Takara, Tokyo, Japan). The percentage of LDH release was calculated by the following formula: % LDH release = LDH released in conditional medium/LDH released in conditional medium + LDH in cell lysates. Osteoblasts were lysed by Triton-X100 (1%).

#### 2.7. Western blots

Cells were lysed in protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL). Lysates were centrifuged at 12,000 g for 15 min and supernatants were collected for Western blots. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Equal protein amounts (20–40 μg) were separated by 10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were subsequently blocked with 5% skimmed milk in TBS-T (20 mM Tris–HCl, pH 7.6; 137 mM NaCl; 0.1% Tween 20) and incubated sequentially with primary antibody (3 h), followed by horseradish peroxidase-conjugated secondary antibody (2 h), at room temperature. Blotting proteins were visualized by enhanced chemiluminescence (Pierce). Bands were quantified through ImageJ software.

#### 2.8. Measurement of SphK1 activity

Cells were scraped in SphK1 assay buffer (20 mM Tris, pH 7.4, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxyxypyrudoxine) and disrupted by freeze-thawing. An 100-μg portion of cell extracts in a 185-μl volume was mixed with 5 μL of \[^1\text{H} - \text{SphK1]-ATP} (5 μCi, Sigma) containing 0.2 M MgCl2 and 10 μL of 1 mM sphenosine (Sigma, dissolved in 5% Triton X-100) and then incubated for 30 min at 37 °C. The reaction was terminated with 10 μL of 1 N HCl. A 400-μl portion of chloroform/methanol/HCl [100:200:1 \((\text{v/v/v})\)] mixture was added and mixed. Then, 120 μL of chloroform and 120 μL of 2 M KCl were added, and phases were separated by centrifugation. The organic phase was dried and resolved by thin-layer chromatography on silica gel G60 with SphK1-butanol/ methanol/acetic acid/water [80:20:10:2] (w/v/w/v). The radioactive spots corresponding to S1P were detected using filmless autoradiographic analysis (NAS-1500; Fujifilm Co. Ltd, Tokyo, Japan).

#### 2.9. Measurement of S1P formation

S1P formation was measured as described previously [18]. For the radio-labeling of sphingolipids, the osteoblasts (1 × 10^6 cells/100-mm dish) were incubated with regular growth medium containing...
[14C] serine (1 μCi/ml) for 48 h. Then, the medium was changed to serum-free medium containing [14C] serine, and the cells were subjected to applied treatments. Cellular lipids were extracted by the method described above and separated on HPLC plates in the solvent system of 1-butanol/acetic acid/water (60:20:20, v/v) [18,19]. The radioactive S1P spot, identified by comparison with an authentic standard, was scraped off the plate, and the radioactivity was measured as described above. The activity of S1P in the treatment group was normalized to that of untreated control.

2.10. SiRNA downregulation of SphK1

To generate retroviral vectors for siRNA experiments, targeting oligonucleotides were annealed and ligated into the pSuper-retro vectors (Oligoengine, Seattle, WA) using BamHI and HindIII sites. The sequences of used oligonucleotides are as follows: mouse SphK1 siRNAs, 5'-AGCTTAAAAATATGGAACTTGACTGTCCATCTTTGAATGGACAGTCAAGTTCCATAGGG-3' (SphK1 siRNA-1) [20] and 5'-ACCCCGGGTCAGTTGTTATGGATTTCAAGAGAATCCATAACCTCGAC-3' (SphK1 siRNA-2).

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Fig. 1. K6PC-5 activates SphK1, increases S1P production and Akt phosphorylation in cultured osteoblasts. Cultured osteoblastic MC3T3-E1 cells (A–C) or primary murine osteoblasts (D–F) were treated with applied concentration of K6PC-5 for indicated time, SphK1 activity, S1P content as well as expressions of Akt (both regular and phospho-) and SphK1 were tested using corresponding methods described. Effect of S1P (1 μM, 3 h) on Akt phosphorylations was also tested (C and F). Experiments in this and all following figure were repeated three times, consistent results were obtained. Data were presented as mean ± SD. "C" stands for untreated control. Akt phosphorylation (vs. regular Akt) was quantified (C and F). "MW" stands for molecular weight. "p < 0.05 vs. "C" group.
CCGCTTTTTC-3’ (SphK1 siRNA-2). Osteoblasts were transfected with siRNA-SphK1 (1 μg/ml) using Lipofectamine 2000 reagent (Invitrogen), and cultured for 48 h in serum-free medium; Stable cells were selected using puromycin (Invitrogen). Depletion of endogenous SphK1 by siRNA was confirmed by Western blots. Control osteoblasts were transfected with scramble control siRNA containing vector (sc-siRNA).

2.11. Statistical analysis

Experiments were repeated at least three times with consistent results. For each experiment, data from triplicate samples were calculated and expressed as means ± SD. Comparisons across more than two groups involved use of one-way ANOVA and then Student–Newman–Keuls test. P values <0.05 were considered statistically significant. The concentration and duration of drug-treatment were chosen based on literature and results from pre-experiments.

3. Results

3.1. K6PC-5 activates SphK1, increases S1P production and Akt phosphorylation in cultured osteoblasts

As discussed early, K6PC-5 is a novel and specific SphK1 activator [15,16]. Thus, we first tested its role on SphK1 activity in cultured osteoblasts. Results in Fig. 1A showed that K6PC-5 dose-dependently increased SphK1 activity in MC3T3-E1 osteoblastic cells. As a consequence, the content of S1P in K6PC-5-treated osteoblastic-like cells was increased (Fig. 1B). Notably, the
expression level of SphK1 was not affected (Fig. 1C). S1P was known to activate Akt through direct or indirect mechanisms [21, 22], we thus tested the role of K6PC-5 on Akt activation in MC3T3-E1 cells. Results showed that K6PC-5 dose-dependently induced phosphorylation of Akt at both Ser-473 and Thr-308 (Fig. 1C). Meanwhile, exogenously-added S1P also activated Akt in MC3T3-E1 cells (Fig. 1C). We also repeated above experiments in primary murine osteoblasts, and results showed that SphK1 activity (Fig. 1D), S1P content (Fig. 1E), and Akt phosphorylation (Fig. 1F) were all increased after applied K6PC-5 treatment in primary osteoblasts. Together, these results indicate that K6PC-5 increases SphK1 activity, S1P production and Akt phosphorylation in cultured osteoblasts.

3.2. K6PC-5 protects osteoblasts from Dex-induced damages

Dex and other glucocorticoids are shown to induce both apoptotic and non-apoptotic death of osteoblasts [10, 17, 23]. Similarly, we found that Dex treatment inhibited MC3T3-E1 cell survival (Fig. 2A, CCK-8 assay). Meanwhile, Dex induced MC3T3-E1 cell

Fig. 3. Activation of SphK1 is required for K6PC-5-mediated Akt activation, S1P production and osteoblast-protection. MC3T3-E1 cells or primary murine osteoblasts were pretreated with SphK1 inhibitor DMS (10 μM, 1 h pre-added), or transfected with vector containing scramble control siRNA ("si-SC") or SphK1 siRNA ("siSphK1")-1/-2, followed by K6PC-5 (10 μM) stimulation for 3 h. Akt activation (A and B) and S1P content (C and D) were tested. Above cells were also incubated in Dex (1 μM) containing medium for 48 h, cell survival was tested by CCK-8 assay (E and F). Data were presented as mean ± SD. *C stands for untreated control. Akt phosphorylation (vs. regular Akt) and SphK1 expression (vs. regular Akt) were quantified (A–B). *p < 0.05.
apoptosis (Fig. 2B, Histone-DNA ELISA assay) and necrosis (Fig. 2C, LDH release assay). Significantly, pre-treatment with K6PC-5 dramatically inhibited Dex-induced viability reduction, apoptosis and LDH release in MC3T3-E1 cells (Fig. 2A-C). The effect of K6PC-5 was again dose-dependent (Fig. 2A-C). Note that K6PC-5 alone at applied concentrations had no significant effect on MC3T3-E1 cell survival (Fig. 2D), apoptosis (Data not shown) nor necrosis (Data not shown). Similar results were also observed in primary murine osteoblasts, where this novel SphK1 activator alleviated Dex-induced viability reduction (Fig. 2D), apoptosis (Fig. 2E) and LDH release (Fig. 2F). Together, these results demonstrate that K6PC-5 protects osteoblasts from Dex-induced damages.

3.3. Activation of SphK1 is required for K6PC-5-mediated Akt activation, S1P production and osteoblast-protection

The above results showed that K6PC-5 activated SphK1 and protected osteoblasts from Dex. Next, we studied the link between the two. Pharmacological and siRNA strategies against SphK1 were applied. In MC3T3-E1 cells and primary murine osteoblasts, results showed that K6PC-5-induced Akt activation (Fig. 3A and B) and S1P production (Fig. 3C and D) were largely inhibited by SphK1 specific inhibitor DMS. Further, siRNA-mediated silencing of SphK1 also dramatically inhibited K6PC-5’s effects on Akt and S1P (Fig. 3A–D). Note that two non-overlapping siRNAs against SphK1 were applied in this study, with consistent results obtained. Significantly, K6PC-5-induced osteoblast-protection against Dex was almost nullified by DMS or siRNA-mediated SphK1 silencing (Fig. 3E and F). Together, these results show that activation of SphK1 is required for K6PC-5-mediated Akt activation, S1P production, and osteoblast-protection.

3.4. K6PC-5-mediated osteoblast protection against Dex requires Akt activation

Akt is a major pro-survival and anti-apoptosis signaling [24]. The results above showed that K6PC-5 protected osteoblasts from Dex, which was associated with a significant Akt activation. Next, we studied the role of Akt in K6PC-5-mediated activities. CCK-8 cell viability assay results in MC3T3-E1 cells (Fig. 4A) showed that K6PC-5-mediated protection against Dex was alleviated by the pan PI3K-Akt-mTOR inhibitor LY294002, and by MK-2206, which is a specific Akt inhibitor [25,26]. Further, the anti-apoptosis ability of K6PC-5 against Dex was also suppressed by above Akt inhibitors in MC3T3-E1 cells (Fig. 4B). On the other hand, S1P, which activated Akt in osteoblasts (see Fig. 1), inhibited Dex-mediated cytotoxicity against MC3T3-E1 cells (Fig. 4A,B). Such effects by S1P were also inhibited by LY294002 and MK-2206 (Fig. 4A and B). As expected, both Akt inhibitors blocked Akt phosphorylation by K6PC-5 or S1P in MC3T3-E1 cells (Data not shown). In primary osteoblasts, MK-2206 inhibited K6PC-5- or S1P-mediated osteoblast protection against Dex (Fig. 4C and D). These results indicate that Akt activation is required for K6PC-5- or S1P-mediated protection against Dex in cultured osteoblasts.

4. Discussions

Patients with long-term glucocorticoids usage often suffer osteoporosis [2]. The histological studies analyzing these osteoporosis patients demonstrated fewer osteoblasts in their bone [2,3]. The reduced number of osteoblasts is mainly due to decreased osteoblastogenesis, decreased osteoblast differentiation, and most...
importantly, increased levels of osteoblast apoptosis [2,3]. It is now known that glucocorticoid (i.e. Dex) could directly induce osteoblast injuries [10,11,27,28]. Thus, Dex and other glucocorticoids are applied into cultured osteoblasts to create the cellular model of glucocorticoid-associated osteoporosis [10,11,27,28]. Here we found that K6PC-5, a novel SphK1 activator [15,16], protected osteoblasts from Dex through activating SphK1-Akt signaling.

Although the function of SphK1 activation in promoting cell survival has been well-established. There have been no direct or specific SphK1 activators characterized until recently [15,16]. 12-O-tetradecanoylphorbol-13-acetate showed direct effect on SphK1 in total lysates and fractionated lysates of cultured fibroblasts. However, it is unclear whether this SphK1 activation occurs through direct SphK1 activation by the phorbol ester, or through an enhanced transcription of SphK1 [16]. K6PC-5 was recently identified as a novel, specific and direct activator of SphK1 [15,16]. It has displayed activities in skin keratinocytes, increasing intracellular calcium and promoted cell differentiation through activation of SphK1 [15,16]. Here, we found that cultured osteoblasts (MC3T3-E1 cells and primary osteoblasts) were responsible to K6PC-5. K6PC-5 activated SphK1, increased S1P generation and Akt phosphorylation in above osteoblasts. Significantly, it protected osteoblasts from Dex-induced damages. Such effects by K6PC-5 were largely inhibited by SphK1 silencing or inhibition. Thus, K6PC-5 activates SphK1 to protect osteoblasts from Dex.

S1P is derived from sphingosine, and represents a potent bioactive sphingolipid metabolite [29–32]. Activation of SphK1 leads to S1P generation, which mediates a wide array of cell type-dependent biologic effects [29–32]. It is known S1P could activate Akt to regulate a variety of cellular responses, including survival, motility, and differentiation [30–32]. In the current study, we found that the level of S1P was increased in K6PC-5-treated osteoblasts. Exogenously-added S1P mimicked the actions of K6PC-5, induced Akt activation and protected osteoblasts from Dex. The pro-survival activity by S1P and K6PC-5 in cultured osteoblasts was alleviated by applied Akt inhibitors. These results indicate that S1P is an important mediator following SphK1 activation by K6PC-5 to regulate its osteoblast-protection activity. In summary, the results of this study show that K6PC-5 alleviates Dex-induced osteoblast damages through activating SphK1-Akt signaling. K6PC-5 might be further investigated in animal or clinical studies for its anti-glucocorticoids-associated osteonecrosis potential.

Conflict of interest

The authors declare no conflict of interest.

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