Sost down-regulation by mechanical strain in human osteoblastic cells involves PGE2 signaling via EP4

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ABSTRACT

Sclerostin is a potent inhibitor of bone formation which is down-regulated by mechanical loading. To investigate the mechanisms involved we subjected Saos2 human osteoblastic cells to short periods of dynamic strain and used quantitative reverse transcriptase polymerase chain reaction to compare their responses to unstrained controls. Strain-induced Sost down-regulation was recapitulated by cyclo-oxygenase-2-mediated PGE2, acting through the EP4 receptor, whereas strain-related up-regulation of osteocalcin was mediated by the EP2 receptor. Strain-related Sost regulation required extracellular signal-regulated kinase signaling, whereas osteocalcin required protein kinase C. These findings indicate early divergence in the signaling pathways stimulated by strain and establish PGE2/EP4 as the pathway used by strain to regulate Sost expression.

1. Introduction

The natural functional regulator of bone architecture is habitual mechanical loading. Age- and estrogen deficiency-related failure of the mechanisms involved is associated with bone loss and increase in fragility fractures. This is characteristic of osteoporosis [1]. Osteoporosis is most commonly treated with anti-resorptives [2]. The only licensed anabolic treatment is intermittent parathyroid hormone (PTH) [3] thought to exert its osteogenic effect, at least in part, through down-regulation of the Wnt/bone morphogenetic protein (BMP) antagonist sclerostin [4]. Neutralizing antibodies against sclerostin are in clinical trials [5].

Like PTH, bone loading down-regulates Sost/sclerostin expression within osteocytes [6–10], whereas unloading increases its production [7,11]. Local control of sclerostin could therefore contribute to the mechanism by which loading regulates bone mass. This is consistent with sclerostin knockout mice having high bone mass and being resistant to unloading-induced bone loss [12].

Abbreviations: PTH, parathyroid hormone; BMP, bone morphogenetic protein; Cox, cyclo-oxygenase; PG, prostaglandin; TCS, TCS2510; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase

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To elucidate the early mechanisms by which loading regulates sclerostin expression we sought to establish the role of cyclo-oxygenase (Cox)-2/prostaglandin (PG) signaling, which is an early component of bone cells’ response to mechanical strain [13–16].

2. Materials and methods

2.1. Choice of cells

Human osteoblastic Saos2 cells (ECACC Cat. No. 89050205) express a differentiated phenotype [17] and have been used to study Sost expression [18].

2.2. Reagents and cell culture

PGE2, AH6809 and AH23848 were from Sigma–Aldrich (Poole, UK). NS398, TCS2510 (TCS), H89, calphostin C, and PD98059 were from Tocris Bioscience (Bristol, UK). Saos2 cells were maintained in phenol red-free DMEM containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin in a 37 °C incubator at 5% CO2, 95% humidity.

2.3. Straining cells in vitro

Cells were seeded on custom-made plastic strips at an initial density of 40 000 cells/cm² in complete medium and allowed to
settle for 72 h before serum-deprivation in charcoal–dextran stripped 2% FCS for 24 h prior to strain or treatment. Strain was applied as previously described [19,20] through 600 cycles of four point bending of the strips with a peak strain of 3400 με.

2.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as previously described [9,19,20]. RNEasy™ Plus Mini Kits (Qiagen, Sussex, UK) were used to eliminate DNA and extract RNA. First strand cDNA synthesis was performed using SuperScriptII™ (Invitrogen, Paisley, UK). Product copy numbers quantified against standard curves were normalized relative to β2-microglobulin. PCR primers were designed using Primer3 Plus [21]. Primer sequences (annealing \(T_m\)) were as follows: Sost (60 °C) sense ACTTCAGAGGAGGCAGAAATGG, antisense CAAGGGGGAATCTATCCACTTTC; B2MG (60 °C) sense AGCAAGGACTGGTCTTATTCTC, antisense CATGTCTCGATCCCACTTAACTATC; EP1 (63 °C) sense CATCCTACTGCGCCAGGCCG, antisense CCAGGCGCTCGGTGTAGGC; EP2 (60 °C) sense TCGGAACGCTCCGGCTCTCA, antisense AAGCCACTGTCGCGTCTCGC; EP3 (65 °C) sense TCCCAGCAGCGGAGTAGGGC, antisense GCATCCCTCCGTAGCCCCG; EP4 (62 °C) sense CCTGCAGCACGTCGGATGCT, antisense GGGCCTCTGCTGTGTGCCAA; osteocalcin (65 °C) sense CTTTGTGTCCAAGCAGGAGG, antisense CTGAAAGCCGATGTGGTCAG.

2.5. Statistical analysis

Statistical analysis was carried out on SPSSv17 for Windows. Comparison of two groups was by independent samples \(t\)-tests, more than two groups were by ANOVA with Bonferroni or Games-Howell post hoc adjustments. Data represent pooled results from 2 to 4 independent experiments (each at \(n=4–6\)), unless otherwise stated, and are presented as mean ± S.E.M. \(P<0.05\) was considered significant.

3. Results

3.1. Strain-induced down-regulation of Sost expression involves PG signaling

Saos2 cells were exposed to strain and harvested at set time-points. In each situation their Sost expression was compared to similarly treated control cultures not exposed to strain. Significant Sost down-regulation was observed between 8 h and 24 h (reduced to 52 ± 4% and 50 ± 3% of levels in the respective static controls, \(P<0.001\), Fig. 1A). Blockade of Cox-2 with the selective inhibitor NS398 dose-dependently prevented Sost down-regulation following strain (30 μM NS398; 96 ± 13%, \(P>0.05\), Fig. 1B). Exogenous addition of PGE2 dose-dependently (Fig. 1C) down-regulated Sost expression 6 h following treatment (500 nM PGE; 32 ± 3%, \(P<0.001\)). Levels remained significantly down-regulated 24 h later (46 ± 5%, \(P<0.001\)).

3.2. PGE2/EP4 signaling is involved in strain-induced Sost suppression

RT-PCR established that Saos2 cells express both EP2 and EP4 receptors. EP1 and EP3 were not detected (Fig. 2A). Expression of both EP2 and EP4 was increased by strain (200 ± 16% and 212 ± 13%, respectively, \(P<0.001\), Fig. 2B and C). Blockade of EP2 with 5 μM AH6809 had no significant effect on strain-induced Sost down-regulation (50 ± 8%, \(P<0.001\), Fig. 2A), whereas blockade of EP4 with 5 μM AH23848 prevented its down-regulation by both strain (92 ± 2%, \(P>0.05\), Fig. 3B) and PGE2 (107% ± 19% 24 h following treatment, \(P>0.05\)). Consistent with this result, 2 μM of the selective EP4 agonist TCS [22] down-regulated Sost expression (46 ± 4%, \(P<0.01\), Fig. 3C).

Osteocalcin is another marker of the differentiated phenotype reported to be up-regulated in osteoblastic cells subjected to strain [23] or PGE2 [24]. Strain-related up-regulation of osteocalcin expression (189 ± 16%, \(P<0.001\)) was prevented by blockade of Cox-2 (30 μM NS398; 114 ± 10%, \(P>0.05\)) and EP2 (91 ± 08%, \(P>0.05\), Fig. 3D), but not of EP4 (207% ± 7%, \(P<0.01\), Fig. 3E). PGE2 up-regulation of osteocalcin (0.5 μM PGE2; 326 ± 32%, \(P<0.001\)) was prevented by EP2 blockade (88 ± 15%, \(P>0.05\), Fig. 3F).

Fig. 1. Strain-induced Sost down-regulation involves Cox-2/PG signaling. (A) Saos2 cells were subjected to strain and harvested at the indicated time points with static controls. (B) Cells were pre-treated for 30 min with the indicated doses of NS398 before strain and harvested 8 h later with static controls. (C) Cells were treated with vehicle or the indicated doses of PGE2 and harvested 6 h later. Sost levels were quantified by qRT-PCR. Representative experiments shown, \(n=6\). NS, not significant; */\(P<0.05\); ** \(P<0.01\); *** \(P<0.001\).
3.3. Extracellular signal-regulated kinase (ERK) signaling is involved in strain-induced Sost down-regulation

PGE2 signaling is recognized to proceed through protein kinase C (PKC) and protein kinase A (PKA) [25]. EP4 has been reported to activate ERK in osteoblastic cells [25]. Blockade of PKC with 1 μM photo-activated calphostin C had no significant effect on Sost down-regulation (52 ± 8%, P < 0.001, Fig. 4A), but prevented osteocalcin up-regulation (91 ± 8%, P > 0.05, Fig. 4B). Calphostin C also blocked osteocalcin up-regulation with 0.5 μM PGE2 (91 ± 26%, P < 0.001, Fig. 4C). Blockade of PKA with 5 μM H89 prevented neither strain-related osteocalcin up-regulation (216 ± 17%, P < 0.001) nor down-regulation of Sost by strain or PGE2 (50 ± 7%, P < 0.001 and 42 ± 8%, P < 0.05, respectively) after 24 h.

Inhibition of mitogen activated protein kinase (MAPK)/ERK1/2 with 10 μM PD98059 significantly reduced Sost levels 8 h after treatment (54 ± 7%, P < 0.001) and prevented further strain-induced down-regulation (95 ± 16%, P > 0.05 versus PD98059-treated static controls). Sost expression in the PD98059-treated static groups was not different from vehicle controls 24 h after treatment (83 ± 8%, P > 0.05) and PD98059 again prevented strain-induced Sost down-regulation (85 ± 6%, P > 0.05, Fig. 4D), but not osteocalcin up-regulation (268 ± 46%, P < 0.05, Fig. 4E). PD8059 also prevented Sost down-regulation by PGE2 24 h following treatment (79 ± 6%, P > 0.05, Fig. 4F).

4. Discussion

We demonstrate here that strain-related Sost down-regulation in cells of the human osteoblastic Saos2 cell line recapitulates...
in vitro, in terms of time, that stimulated in osteocytes by loading of the mouse tibia in vivo [9]. The time course of 

Sost down-regulation by exposure to a short period of cyclic strain in Saos2 cells differs from that in rat UMR-106 cells exposed to 2 h continuous fluid shear [26]. In bone in vivo Sost is osteocyte specific, therefore its synthesis (even at low levels [4]) by Saos2 cells in vitro is likely to reflect these cells’ differentiated phenotype [17,27]. This contrasts with its supra-physiological expression by UMR-106 cells [4].

This study also demonstrates that, in Saos2 cells at least, strain-induced Sost down-regulation proceeds through Cox-2 mediated PGE2 signaling. This is consistent with the recent report that PGE2 down-regulates Sost in UMR-106 cells via an EP2/PKA dependent mechanism [28], whereas here blocking EP2 had no effect on strain-related Sost expression. In contrast blockade of EP4 abrogated strain-related Sost down-regulation and a specific EP4 agonist down-regulates Sost in the absence of either strain or PGE2. This difference between the responses of the Saos2 and UMR-106 cell lines may reflect differences in the cells themselves and/or temporal changes in the mechanism(s) by which Sost is regulated. Although one of cells’ major responses to mechanical strain is PGE2 production, the response to strain involves many other mechanisms [29]. For instance, strain-related PGE2 release occurs through connexin-43 hemi-channels [30] which may result in, and be a response to, activation of many local signaling events in addition to those resulting from a single high dose of PGE2. Nevertheless the involvement of EP4 in strain-related Sost regulation is consistent with reports that in vivo an EP4 selective agonist induces bone formation [31] and enhances loading-related osteogenesis [32,33]. Mice lacking EP4, but not EP1, EP2 or EP3, are unable to form bone in response to local infusion of PGE2 [31]. The effect of EP4 antagonists on loading-related sclerostin down-regulation and osteogenesis now need to be determined in vivo.

Osteocalcin is also up-regulated following strain by a mechanism involving PGE2 acting through the EP2 receptor. This response was dependent on PKC, whereas Sost down-regulation was dependent on ERK. Numerous studies have shown mechanical signals activate ERK [34–36] but whether ERK then targets Runx2 [37,38], a major transcriptional regulator of Sost [18], remains to be determined.

These data suggest that short periods of strain stimulate a number of signaling pathways acting on different targets to regulate osteoblastic cell recruitment, proliferation and differentiation. Relieved antagonism of Wnt signaling through Sost down-regulation would facilitate proliferation, whereas osteocalcin up-regulation suggests promotion of differentiation. Both processes contribute to the actions and interactions inherent in the functional adaptation of bone mass and architecture. Since these events occur practically simultaneously the targets of the diverse signaling pathways may be different cohorts in the heterogeneous osteoblastic population.

The data presented here suggests that EP4-selective agonists in pre-clinical testing [31,39] could complement sclerostin-neutralizing therapies as anabolic agents for the effective treatment of osteoporosis while selectively sparing other effects of PGE2 on osteoblastic cells.

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![Fig. 4. Strain-induced Sost down-regulation involves ERK signaling. (A, B) Saos2 cells were treated with 1 μM calphostin C 30 min before strain and harvested 24 h later. (C) Cells were treated with 1 μM calphostin C 30 min before treatment with 0.5 μM PGE2 and harvested 6 h later. (D, E) Cells were treated with 10 μM PD98059 30 min before strain and harvested 24 h later. (F) Cells were treated with 10 μM PD98059 30 min before treatment with 0.5 μM PGE2 and harvested 24 h later. (A, D, F) Sost and (B, C, E) osteocalcin levels were quantified by qRT-PCR. NS, not significant; *P < 0.05; **P < 0.001.](image-url)