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Oxidized Low-Density Lipoprotein Acts Synergistically With β-Glycerophosphate to Induce Osteoblast Differentiation in Primary Cultures of Vascular Smooth Muscle Cells

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ABSTRACT

Previous studies have localized osteoblast specific markers to sites of calcified atherosclerotic lesions. We therefore decided to use an established in vitro model of vascular calcification in order to confirm earlier reports of oxidized low-density lipoprotein (oxLDL) promoting the osteogenic differentiation of vascular smooth muscle cells. Treatment of primary bovine aortic smooth muscle cells (BASMCs) with β-glycerophosphate was found to induce a time-dependent increase in osteoblast differentiation. In contrast, no effect was seen when BASMCs were cultured in the presence of oxLDL alone. However, when the BASMCs were cultured in the presence of both β-glycerophosphate and oxLDL, β-glycerophosphate's ability to induce osteoblast differentiation was significantly enhanced. In an attempt to resolve the mechanism by which this effect was occurring, we examined the effect of β-glycerophosphate and oxLDL on several pathways known to be critical to the differentiation of osteoblasts. Surprisingly, β-glycerophosphate alone was found to enhance Osterix (Osx) expression by inducing both Smad 1/5/8 activation and Runx2 expression. In contrast, oxLDL did not affect either Smad 1/5/8 activation or Runx2 activation but rather, it enhanced both β-glycerophosphate-induced Osx expression and osteoblast differentiation in an extracellular signal-regulated kinase 1 and 2 (Erk 1 and 2) -dependent manner. When taken together, these findings suggest a plausible mechanism by which oxLDL may promote osteogenic differentiation and vascular calcification in vivo. J. Cell. Biochem. 105: 185–193, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: SMOOTH MUSCLE CELLS; OSTEOBLAST DIFFERENTIATION; OXIDIZED LOW-DENSITY LIPOPROTEIN; OSTERIX

Vascular calcification is a key feature of atherosclerosis. However, until recently it was believed that vascular calcification was a passive process in which calcium was deposited non-specifically onto the vessel wall. Now vascular calcification is thought to be a highly complex process that shares many similarities with the mineralization of bone. In support of this concept, we and others have demonstrated that vascular smooth muscle cells can undergo a phenotypic transition into cells which demonstrate all of the hallmarks of "true" osteoblasts [Shioi et al., 1995; Parhami et al., 1997; Wada et al., 1999; Yang et al., 2005]. In addition, cells expressing osteoblast-specific markers have been identified within the calcified atherosclerotic lesions of human vessels [Ikeda et al., 1993; Shanahan et al., 1994; Dhore et al., 2001; Engelese et al., 2001; Tyson et al., 2003]. When taken together, these findings suggest that osteoblasts may be responsible for the ectopic mineralization that is often seen in atherosclerotic lesions.

Under normal circumstances, osteoblasts originate from a mesenchymal stem cell population that is located within the bone marrow space [Poliard et al., 1995; Hicok et al., 1998; Nuttal et al., 1998]. To differentiate into mature osteoblasts, this population of cells is thought to first differentiate into osteoprogenitor cells, then into preosteoblasts, and finally into mature osteoblasts which promote matrix mineralization. Several key transcription factors are thought to regulate this process. These include Runx2 (previously known as Cbfa1), the homeobox transcription factors (Msx2, Dlx3, Dlx2, Dlx5) and Osterix (Osx) [Gori et al., 1999; Banerjee et al., 2001; Nakashima et al., 2002; Lee et al., 2003; Kim et al., 2004]. Of these, Runx2 and Osx are the two factors believed to be the most critical to

Abbreviations used: BASMCs, bovine aortic smooth muscle cells; oxLDL, oxidized low-density lipoprotein; BGP, β-glycerophosphate; Osx, Osterix; MEM, minimal essential medium; FCS, fetal calf serum; Erk, extracellular signal-regulated kinase; siRNA, small interference RNA.

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osteoblast differentiation. Thus, both Runx2 and Osx are known to bind to the promoter of several osteoblast specific genes including osteocalcin, osteopontin, collagen I and alkaline phosphatase [Ducy et al., 1997; Acampora et al., 1999; Satokata et al., 2000; Robledo et al., 2002; Kim et al., 2004]. In addition, mice deficient in either Runx2 or Osx are also known to be deficient in mature osteoblasts and to therefore have unmineralized cartilaginous skeletons [Nakashima et al., 2002; Komori et al., 1997]. Interestingly, Osx deficient mice express Runx2 while Runx2 deficient mice do not express Osx, suggesting that Osx acts downstream of Runx2.

Numerous factors have been proposed to regulate the process of vascular calcification. For example, oxidized low-density lipoproteins (oxLDLs) have been proposed to promote both the formation of atherosclerotic plaques as well as their calcification [Parhami et al., 1997; Tsimikas and Witztum, 2000; Stocker and Keaney, 2003]. In addition, high phosphate levels are believed to promote vascular calcification in individuals with chronic kidney disease (CKD) [Jono et al., 2000; Giachelli et al., 2005; Jono et al., 2006]. Finally, factors which normally regulate osteoblast differentiation and activity in bone (i.e., bone morphogenetic proteins (BMPs)), have also been localized to calcified atherosclerotic lesions and are thus, likely playing a key role in the calcification process [Gori et al., 1999; Banerjee et al., 2001; Bostrom et al., 2001; Zebboudj et al., 2002, 2003].

In the present study, we examine the mechanism by which oxLDL promotes osteoblast differentiation in primary cultures of BASMCs. By culturing BASMCs in the presence or absence of β-glycerophosphate and oxLDL, we demonstrate that oxLDL synergistically enhances β-glycerophosphate-induced osteoblast differentiation. In addition, we demonstrate that oxLDL has no effect on Runx2 expression but rather, it enhances β-glycerophosphate-induced osteoblast differentiation by upregulating Osx expression in an Erk-dependent manner. When taken together, these findings suggest a plausible mechanism by which oxLDL may promote osteogenic differentiation and thus vascular calcification in vivo.

EXPERIMENTAL PROCEDURES

MATERIALS

Human LDL was purchased from Sigma Chemical Co. (St. Louis, MO) while Phospho-Smad 1/5/8 antibody was obtained from Cell Signaling Technology (Beverly, MA). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

OXIDIZATION OF LOW-DENSITY LIPOPROTEINS

To obtain oxLDL, native human LDL was first dialyzed against phosphate-buffered saline (PBS), pH 7.2, for 48 h at room temperature to remove any contaminating EDTA. The native LDL was then oxidized by dialyzing it against 9 μM $FeSO_4$ for 48 h. Following oxidation, a final 24 h dialysis step was performed in PBS containing 100 μM EDTA, pH 7.2, in order to remove any oxidizing agents. The samples were then assayed for protein concentration (BioRad DC), and the extent of LDL oxidation determined by estimating the amount of diene conjugation in each preparation by reading the absorbance at 234 nm as described previously [Velarde et al., 2001].

VASCULAR SMOOTH MUSCLE CELL ISOLATION

Bovine aortic smooth muscle cells (BASMCs) were obtained from explant cultures as described previously [Yang et al., 2005]. Briefly, fresh bovine aorta was obtained under sterile conditions from a local slaughter house (Highland Packers LTD, Hamilton, ON). The aortas were then denuded of endothelium and adventitia and the remaining medial layer (containing SMC) cut into 1–2 mm^2 pieces. The pieces were then cultured in Dulbecco's modified Eagle's media (DMEM, Invitrogen, Burlington, ON) containing 20% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin until smooth muscle cell outgrowth was observed. Once confluent, the cells were harvested and either used immediately or stored in liquid nitrogen. In some cases, cells up to and including passage 2 were used. To confirm that all isolated cells were indeed smooth muscle cells, all preparations were stained for α-actin using a smooth muscle cell specific α-actin antibody (Sigma Chemical Co.)

MEASUREMENTS OF ALKALINE PHOSPHATASE (ALP) ACTIVITY

The extent of osteoblast differentiation in the presence or absence of β-glycerophosphate and oxLDL was determined by quantifying the amount of ALP activity in our vascular smooth muscle cell cultures [Yang et al., 2005]. Briefly, BASMCs were seeded into 24-well plates, at a concentration of 5×10^4 cells/well, and then cultured for up to 9 days in DMEM containing 10% FCS, 0.5 mM ascorbic acid, and either 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or a combination of the two. In some experiments, the cells were also cultured in the presence of the MEK inhibitor, PD098059, or transfected with Erk 1 and 2 specific siRNA (pKD-MAPK1/Erk 2-v1; Upstate, Lake Placid, NY) prior to treating the cells with either β-glycerophosphate or β-glycerophosphate plus oxLDL. At various times thereafter, the cells were harvested, lysed with 1% Triton X-100, and ALP activity assessed at 405 nm using a *p*-nitrophenol phosphate substrate kit (Sigma Chemical Co.). ALP values (U/mg) were normalized to protein using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA).

PREPARATION OF CELL-FREE LYSATES

Vascular smooth muscle cells were cultured to 70% confluency in DMEM containing 10% FCS and 0.5 mM ascorbic acid before being stimulated with either 10 mM β-glycerophosphate (BGP), 100 μg/ml oxLDL, or a combination of the two. At various times thereafter, the cells were rinsed once with ice-cold PBS, isolated using a cell scraper, and then pelleted by centrifugation. The cells were then lysed by resuspending them in a hypotonic buffer (1% deoxycholic acid, 1% Triton X-100, 50 mM Tris–HCl, pH 7.2, 0.25 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, 0.1 mM Na_3VO_4) for 1 h at 4°C. Protein concentrations of the cell lysates were then determined using a Bio-Rad DC protein assay (Bio-Rad).

IMMUNOPRECIPITATION

Immunoprecipitations were performed as follows. Briefly, cell-free lysates were prepared as described above before being incubated overnight, at 4°C, with 2 μg of anti-Smad antibody (Santa Cruz Biotechnology). The cell free lysates were then incubated with Protein G Sepharose (Amersham Pharmacia Biotech, Quebec) for 1 h

in order to precipitate antigen–antibody complexes, and run on 7.5% SDS–PAGE before being transferred to nitrocellulose membranes. Blots were blocked at 4°C with Blotto buffer (50 mM Tris, pH 8, 2 mM $CaCl_2$, 5% skim milk powder, 0.05% Tween-20), and then incubated with an anti-P-Smad antibody (Cell Signaling Technologies, Danvers, MA) before being incubated with a horseradish peroxidase-conjugated secondary antibody and visualized by an enhanced chemiluminescent system.

WESTERN BLOT ANALYSIS

Western blot analysis was performed as follows. Briefly, cell-free lysates were prepared as described above and then run on a 10% SDS–PAGE before being transferred onto nitrocellulose membranes (Bio-Rad). The membranes were then washed with TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20), blocked with Blotto buffer, and incubated with an anti-P-Erk antibody (Santa Cruz Biotechnology) overnight. P-Erk was then visualized by incubating the blots with a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescent system. Following visualization, the blots were stripped (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) and incubated with an Erk-antibody (Santa Cruz Biotechnology) before visualizing Erk by the same method as P-Erk.

SEMI-QUANTITATIVE RT-PCR ANALYSIS

BASMCs were plated in 60 mm dishes at a cell concentration of 7×10^5 cells/dish and then cultured to 70% confluency in DMEM containing 10% FCS and 0.5 mM ascorbic acid before being stimulated with either 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or a combination of the two. At various times thereafter, the cells were harvested and total RNA isolated using an RNeasy RNA mini kit (Qiagen, Mississauga, ON, Canada). RT-PCR analysis was carried out using 4 μg total RNA which was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). PCR amplification reactions were performed with Supermix (Invitrogen) using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., NV). Specific primers and annealing temperatures are listed in Table I. PCR products were run in triplicate on a non-denaturing 7.5% acrylamide gel and visualized using Kodak BioMax XAR film. Results were quantified using ImageQuant 5.2 software.

siRNA TRANSFECTION

BASMCs were seeded in 24-well plates at a cell density of 5×10^4 cells/well. Twenty-four hours later the cells were transfected with either 2 μg/ml pKD-MAPK1/Erk 2-v1 siRNA expression plasmid (Upstate) or control plasmid (pKD-NegCon-v1, Upstate) for 5 h using Lipofectamine reagent (Invitrogen). Following removal of the Lipofectamine reagent, the cells were allowed to incubate in normal growth media for 48 h before performing an ALP assay as described above.

REAL-TIME PCR

BASMCs were plated in 15 cm dishes at a concentration of 1×10^6 cells/plate and then incubated for 48 h in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were then pretreated with increasing concentrations of PD098059 for 1 h, before being cultured with β-glycerophosphate (10 mM) and oxLDL (100 μg/ml) for 24 h. Total RNA was then isolated using an RNeasy mini kit (Qiagen) and first strand cDNA synthesized from 4 μg of total RNA using random priming and Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR analysis of Osx and GAPDH expression was performed using an ABI PRISM 7300 (Applied Biosystems, USA) and specific primers for Osx and GAPDH (Applied Biosystems). Denaturation took place at 95°C for 15 s, and annealing and extension at 60°C for 1 min, for 40 cycles. Results were expressed as the fold increase, over the respective GAPDH controls.

STATISTICAL ANALYSIS

All experiments were done in triplicate. An analysis of variance was used to compare the results between experimental and control groups. If a significant difference was detected, an unpaired Student's *t*-test was performed for each point. Significance levels were adjusted using a Bonferroni correction for multiple comparisons.

RESULTS

EFFECT OF β-GLYCEROPHOSPHATE AND oxLDL ON OSTEOBLAST DIFFERENTIATION IN PRIMARY CULTURES OF BASMCs

Since hyperlipidemia has been suggested to promote not only plaque development, but also vascular calcification, we were interested in determining if osteoblast differentiation in primary cultures of vascular smooth muscle cells could be induced by oxLDL. We therefore cultured primary BASMCs for up to 8 days in the presence or absence of 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or both. ALP activity was then assessed as an index of osteoblast differentiation at various time intervals. As shown in Figure 1, significantly more ALP activity was detected in the cell lysates of BASMCs when the cells were cultured in the presence of 10 mM β-glycerophosphate (221.0 ± 18.6 U/mg vs. 111.6 ± 14.4 U/mg; $P < 0.05$). In contrast, no increase in ALP activity was seen when the cells were grown in the presence of 100 μg/ml oxLDL. However, when the cells were cultured for 8 days in the presence of

TABLE I. Primers and Protocols for RT-PCR

Genes	Primer sequences (5′–3′)	Annealing temp (°C)	Cycle #	Product length
Runx2	Fwd: CCGCACGACAACCGCACCAT Rev: CTCTAAACACCCGGCCTCGC	58	26	288
Osterix	Fwd: GCTGGCTACGGGAGCAGTGG Rev: ACTTCTTCTCCCGGGTGTG	58	24	288
GAPDH	Fwd: ATGGCCTTCCGCGTCCCCACTCC Rev: AGCCAAATTCATTGTCGTACC	60	18	265

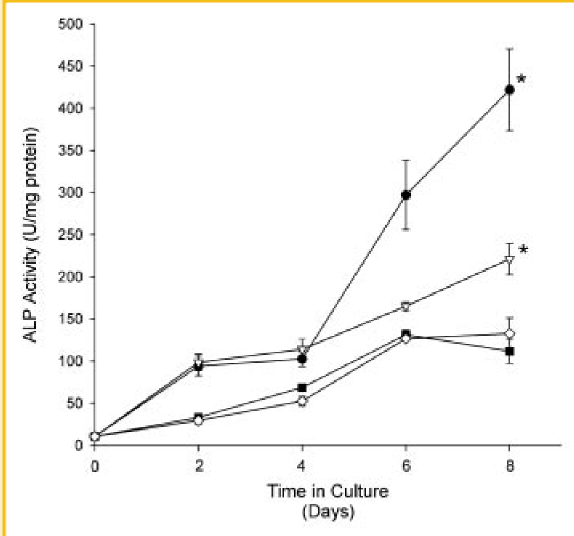

Fig. 1. The effect of β-glycerophosphate and oxLDL on osteoblast differentiation in primary cultures of BASMCs. BASMCs were cultured for 8 days in DMEM alone (■), 100 µg/ml oxLDL (◇), 10 mM β-glycerophosphate (▽) or both β-glycerophosphate (10 mM) and 100 µg/ml oxLDL (●) before assessing ALP activity as an index of osteoblast differentiation. * $P < 0.05$ when ALP activity in the presence of β-glycerophosphate and oxLDL was compared to that obtained in their absence.

both β-glycerophosphate and oxLDL, ALP activity was increased 2.5-fold over that seen with β-glycerophosphate alone (421.8 ± 49.5 U/mg vs. 221.0 ± 18.6 U/mg, respectively; $P < 0.05$; Fig. 1). Taken together, these results suggest that oxLDL acts synergistically with β-glycerophosphate to induce osteoblast differentiation in primary cultures of BASMCs.

EFFECT OF β-GLYCEROPHOSPHATE AND oxLDL ON Smads 1/5/8 ACTIVATION

Since the activation of Smad proteins is known to be a critical step in the differentiation of "true" osteoblasts [Fujii et al., 1999; Afzal et al., 2005], we initially set out to determine if either oxLDL or β-glycerophosphate would have any effect on Smad 1, 5, and 8 phosphorylation. As shown in Figure 2 (inset), Smad 1/5/8 phosphorylation was induced in a time-dependent manner when BASMCs were cultured in the presence of β-glycerophosphate. Smad 1/5/8 activation was detected as early as 10 min with maximal Smad 1/5/8 phosphorylation occurring by 40 min. In contrast, culturing primary BASMCs in the presence of oxLDL alone had no effect on Smad 1/5/8 activation. Surprisingly, repeating these experiments in the presence of noggin had no effect on β-glycerophosphate's ability to induce Smad 1/5/8 activation (data not shown).

To determine if oxLDL was able to synergistically enhance β-glycerophosphate's ability to induce Smad 1/5/8 activation, BASMCs were cultured with β-glycerophosphate in the presence or absence of oxLDL. As shown in Figure 2, a similar response was seen when the cells were treated with either β-glycerophosphate alone or β-glycerophosphate plus oxLDL. Thus, the levels of Smad 1/5/8 phosphorylated protein did not significantly differ ($P > 0.05$) over time when the cell lysates from β-glycerophosphate-treated cells were compared with those treated with β-glycerophosphate and oxLDL (14.3 ± 1.9-fold vs. 13.0 ± 1.4-fold, $P > 0.05$). When taken together, these findings demonstrate that oxLDL does not enhance

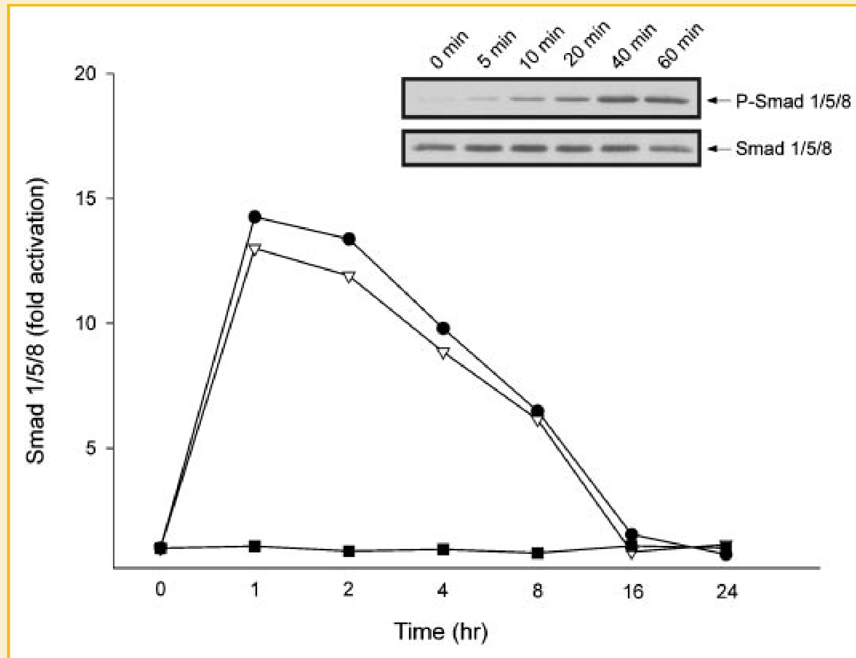

Fig. 2. The effect of β-glycerophosphate and oxLDL on Smad 1, 5, and 8 activation. BASMCs were cultured in DMEM containing 100 µg/ml oxLDL (■), 10 mM β-glycerophosphate (inset; ●), or 10 mM β-glycerophosphate plus 100 µg/ml oxLDL (▽) for various periods of time. Cell lysates were then prepared, immunoprecipitated with a polyclonal anti-Smad 1/5/8 antibody, and immunoblotted for either activated Smad using an anti-P-Smad 1/5/8 antibody or total Smad using an anti-Smad antibody.

β-glycerophosphate-induced osteoblast differentiation by upregulating the activation of Smads 1/5/8.

EFFECT OF β-GLYCEROPHOSPHATE AND oxLDL ON Runx2 AND Osx EXPRESSION

Given that oxLDL did not enhance β-glycerophosphate-induced Smad activation, we next set out to determine the effect of β-glycerophosphate and oxLDL on Runx2 expression. Semi-quantitative RT-PCR was used to examine the expression of Runx2 in BASMCs treated with oxLDL alone, β-glycerophosphate alone or β-glycerophosphate plus oxLDL. As seen in Figure 3A, Runx2 expression was increased 3.0 ± 0.7-fold ($P < 0.01$) when BASMCs were cultured in the presence of β-glycerophosphate alone. In contrast, culturing the cells in the presence of oxLDL alone had no effect on Runx2 expression, nor was Runx2 expression in the presence of β-glycerophosphate enhanced by oxLDL (3.0 ± 0.7 vs. 3.0 ± 0.8, respectively; $P > 0.05$). When taken together, these findings suggest that oxLDLs effect on β-glycerophosphate-induced osteoblast differentiation is independent of Runx2 expression.

Next we examined the effect of β-glycerophosphate on Osx expression in the absence or presence of oxLDL. Like Runx2, Osx is a transcription factor which is thought to play a critical role in osteoblast differentiation. It is believed that Osx acts downstream of Runx2 since Runx2 −/− mice do not express Osx, whereas, Osx −/− mice do express Runx2 [Nakashima et al., 2002]. As shown in Figure 3B, treatment of BASMCs with β-glycerophosphate resulted in a modest increase in Osx expression (2.2 ± 0.2-fold, $P < 0.05$). In contrast, treatment with oxLDL alone had no effect on Osx expression. However, Osx expression was significantly increased when the cells were treated for 24 h with oxLDL in the presence of β-glycerophosphate (10.9 ± 0.8 vs. 2.2 ± 0.2-fold, $P < 0.01$). Collectively, these results suggest that oxLDL enhances β-glycerophosphate-induced osteoblast differentiation by promoting the expression of Osx.

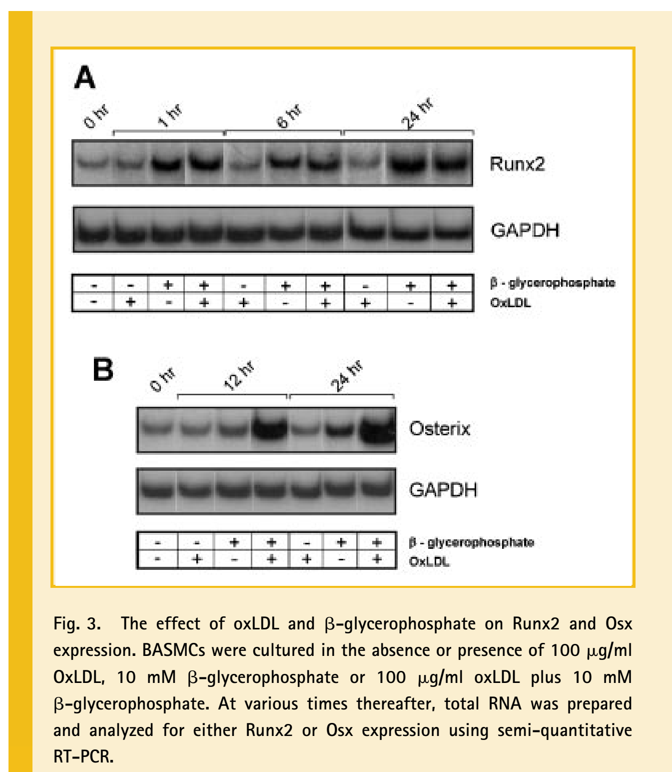

Fig. 3. The effect of oxLDL and β-glycerophosphate on Runx2 and Osx expression. BASMCs were cultured in the absence or presence of 100 µg/ml OxLDL, 10 mM β-glycerophosphate or 100 µg/ml oxLDL plus 10 mM β-glycerophosphate. At various times thereafter, total RNA was prepared and analyzed for either Runx2 or Osx expression using semi-quantitative RT-PCR.

THE ROLE OF ERK 1/2 IN oxLDL-INDUCED OSTERIX EXPRESSION

Because oxLDL has been shown to activate the mitogen-activated protein kinase/extracellular-signal-related kinase (MAPK/Erk) pathway in immortalized endothelial cells [Lupo et al., 2005], we next decided to determine if oxLDL could activate Erk 1 and 2 in our BASMCs and if so, whether this was mediating oxLDLs ability to enhance Osx expression. As shown in Figure 4A, oxLDL stimulated Erk 1 and Erk 2 activation in a time-dependent manner. Maximal

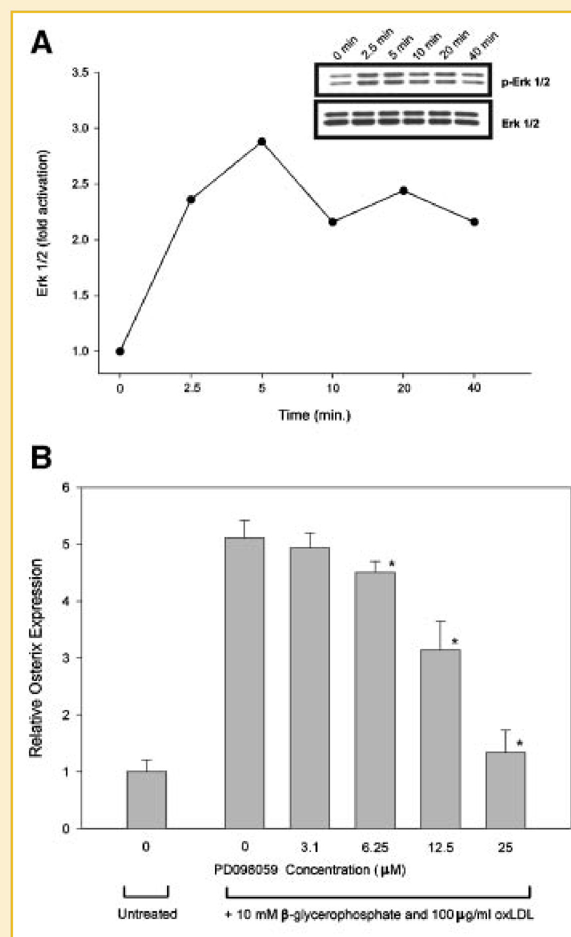

Fig. 4. The role of ERK 1/2 in oxLDL-induced Osx expression. Panel A: BASMCs were cultured in the presence oxLDL (100 µg/ml) for increasing periods of time. Cell lysates were then immunoblotted with a polyclonal anti-phospho Erk 1/2 antibody before stripping the blots and re-probing them with a polyclonal anti-Erk 1/2 antibody. Panel B: BASMCs were maintained in the presence of β-glycerophosphate (10 mM) plus oxLDL (100 µg/ml) and increasing doses of the Erk inhibitor, PD098059. Twenty-four hours later, total RNA was prepared and analyzed by real-time PCR for either Osx or GAPDH expression. Data are expressed as a mean ± SEM. $^{*}P < 0.01$ when Osx expression in the presence PD098059 was compared to Osx expression in its absence.

stimulation of Erk (2.9 ± 0.2-fold, $P < 0.01$) was obtained when BASMCs were treated for 2.5 min and was sustained for at least 20 min before returning to control levels.

Finally, to determine if Erk activation by oxLDL was capable of inducing Osx expression, we cultured BASMCs in the presence or absence of the MEK inhibitor PD098059 and β-glycerophosphate plus oxLDL. As shown in Figure 4B, Osx expression was induced when BASMCs were cultured in the presence of oxLDL and β-glycerophosphate. However, when the cells were also cultured in the presence of PD098059, oxLDL and β-glycerophosphate were no longer able to enhance Osx expression (Fig. 4B), suggesting that oxLDL enhances β-glycerophosphate-induced Osx expression by activating the MAPK/Erk pathway.

ROLE OF ERK 1/2 IN oxLDL-INDUCED OSTEOBLAST DIFFERENTIATION

To prove that oxLDL was inducing osteoblast differentiation in the presence of β-glycerophosphate by activating the MAPK/Erk pathway, we treated primary cultures of BASMCs with β-glycerophosphate in the presence or absence of oxLDL and increasing doses of the MEK inhibitor PD098059. Nine days later, the number of osteoblast-like cells was determined by quantifying the amount of ALP activity in our vascular smooth muscle cell cultures as a measure of osteoblast differentiation. As shown in Figure 5, ALP activity was significantly increased when BASMCs were cultured in the presence of β-glycerophosphate (179 ± 51 U/mg vs. 43.9 ± 6.5 U/mg; $P < 0.05$). This was enhanced even further when the cells were also cultured in the presence of oxLDL (726 ± 219 U/mg vs. 179 ± 51 U/mg; $P < 0.05$). However, oxLDL failed to enhance β-glycerophosphate-induced ALP activity when the cells were cultured in the presence of the MEK inhibitor, PD098059 (Fig. 5).

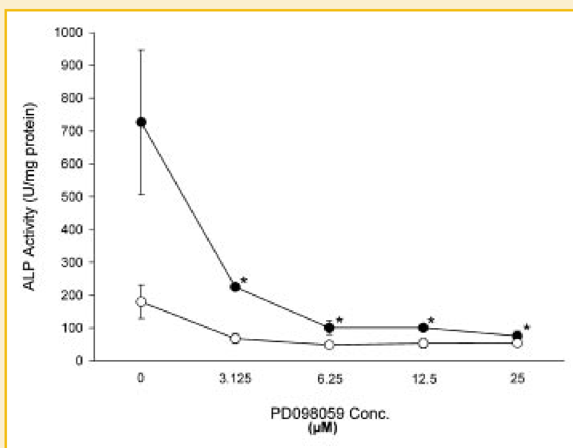

Fig. 5. The effect of PD098059 on oxLDL's ability to enhance β-glycerophosphate-induced osteoblast differentiation. BASMCs were maintained in the absence or presence of 10 mM β-glycerophosphate (○) or 100 μg/ml oxLDL plus 10 mM β-glycerophosphate (●) and increasing doses of the Erk inhibitor, PD098059. Nine days later, the cells were lysed and the amount of ALP activity quantified as an index of osteoblast differentiation. ALP activity in the absence of both β-glycerophosphate and oxLDL or in the presence of oxLDL alone was 43.9 ± 6.5 U/mg and 52.3 ± 5.2 U/mg, respectively. Data are expressed as a mean ± SEM. $^*P < 0.01$ when ALP activity in the presence PD098059 was compared to ALP activity in its absence.

Similar findings were obtained when our BASMCs were first transfected with Erk-specific siRNA. Thus as shown in Figure 6, ALP activity was significantly increased when BASMCs, transfected with control siRNA (pKD-NegCon-v1), were cultured in the presence of β-glycerophosphate (171.7 ± 7.7 U/mg vs. 106.3 ± 9.3 U/mg, respectively, $P < 0.05$). A significant increase in ALP activity was also seen when the pKD-NegCon-v1-transfected BASMCs were cultured in the presence of both β-glycerophosphate and oxLDL (297.2 ± 18.5 U/mg vs. 106.3 ± 9.3 U/mg, respectively, $P < 0.05$). However, no increase in ALP activity was seen when the BASMCs were first transfected with Erk-specific siRNA (pKD-MAPK1/Erk 2-v1 siRNA) and then cultured in the presence of oxLDL and β-glycerophosphate (97.8 ± 13.9 U/mg vs. 106.3 ± 9.3 U/mg, respectively, $P > 0.05$). When taken together, these findings suggest that Erk 1/2 plays a significant role in oxLDL's ability to synergistically enhance β-glycerophosphate-induced osteoblast differentiation.

DISCUSSION

Previously, we demonstrated that primary cultures of BASMCs can undergo a phenotypic transition into mature osteoblasts and mineralize when cultured in the presence of β-glycerophosphate [Yang et al., 2005]. In the present study, we demonstrate that oxLDL can synergistically enhance this process by upregulating Osx expression. In addition, by culturing primary BASMCs in the presence of the MEK inhibitor, PD098059, or by using cells transfected with Erk 1 and 2 specific siRNA, we also demonstrate that oxLDL's ability to enhance both β-glycerophosphate-induced osteoblast differentiation and Osx expression is Erk 1/2-dependent. When taken together, these findings suggest a plausible mechanism by which oxLDL may promote osteogenic differentiation and vascular calcification in vivo.

We have previously shown that β-glycerophosphate promotes osteoblast differentiation and mineralization in primary cultures of BASMCs [Yang et al., 2005]. Numerous studies have reported a similar requirement for β-glycerophosphate when studying osteoblast differentiation and mineralization in primary cultures of bone marrow stromal cells [Gerstenfeld et al., 1987; Peter et al., 1998]. Why β-glycerophosphate is required for these processes to occur is unknown. Prior to our current findings, we like others, believed that β-glycerophosphate was functioning merely as a source of inorganic phosphate and that it was required in the growth medium of differentiating osteoblasts so that bone nodules of hydroxyapatite could form. However, our current results clearly demonstrate that like BMPs, β-glycerophosphate, can induce Smad 1/5/8 phosphorylation, suggesting that β-glycerophosphate plays a much more complex role in these processes.

The mechanism by which β-glycerophosphate is able to activate Smad 1/5/8 is unclear. It is known that β-glycerophosphate can be hydrolysed into inorganic phosphate by alkaline phosphatase [Chung et al., 1992; Chang et al., 2000] and that inorganic phosphate

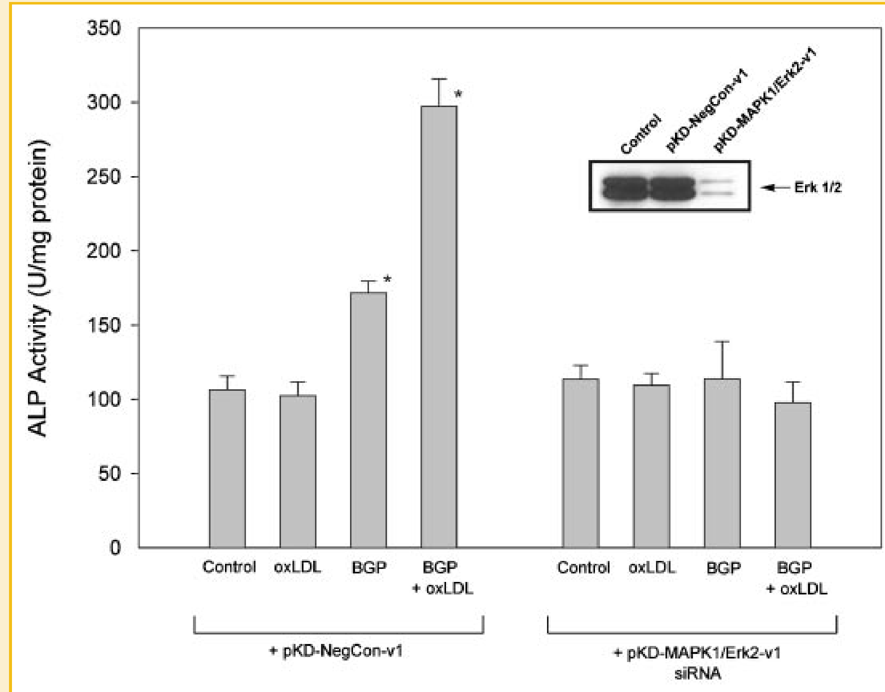

Fig. 6. The effect of Erk-specific siRNA on oxLDL's ability to enhance β-glycerophosphate-induced osteoblast differentiation. BASMCs were transfected with either control siRNA (pKD-NegCon-v1) or Erk-specific siRNA (pKD-MAPK1/Erk 2-v1) and then maintained in the absence or presence of β-glycerophosphate (10 mM), oxLDL (100 μg/ml), or a combination of the two. Nine days later, the cells were lysed and the amount of ALP activity quantified as an index of osteoblast differentiation. Inset: Erk 1/2 expression following transfection of BASMCs with either control siRNA (pKD-NegCon-v1) or Erk-specific siRNA (PKD-MAPK1/ERK 2-v1). Data are expressed as a mean ± SEM. $^{*}P < 0.01$ when ALP activity in cells cultured in the presence of β-glycerophosphate or β-glycerophosphate and oxLDL were compared with cells cultured in their absence.

can enter vascular smooth muscle cells via the PIT-1 and PIT-2 (inorganic phosphate transporter-1 and -2) receptors [Jono et al., 2000; Villa-Bellosta et al., 2007]. Therefore, the most likely explanation for β-glycerophosphate's ability to activate Smads 1/5/8 is that one or more signaling pathways are activated by inorganic phosphate upon its transport into BASMCs. In support of this possibility, β-glycerophosphate was found to induce Smad 1/5/8 activation in a manner which was independent of BMPs but which occurred within the first 10 min of treatment (Fig. 2, inset). Interestingly, such a mechanism might also help to explain why high levels of inorganic phosphate promotes vascular calcification in patients with CKD and/or end-stage renal disease (ESRD) [Cozzolino et al., 2005; Mehrotra et al., 2005].

Our findings also suggest that β-glycerophosphate can induce both Runx2 and Osx expression. Thus as shown in Figure 3A,B, treatment of BASMCs with β-glycerophosphate increased the expression of both Runx2 and Osx. How β-glycerophosphate is having these effects is not entirely clear. It is known that Smads, once activated, can act as a co-activator of transcription to increase both Runx2 expression and activity [Fujii et al., 1999; Banerjee et al., 2001; Afzal et al., 2005]. Since we have shown that β-glycerophosphate induces Smad 1/5/8 activation, it is possible that the observed increase in Runx2 expression is a result of β-glycerophosphate activating Smad 1/5/8. In addition, since Runx2 has recently been shown to bind to and transactivate the Osx promoter [Nishio et al., 2006], it is also possible that β-glycerophosphate is inducing the expression of Osx by first inducing Runx2 expression and/or activity and that Runx2 then acts to increase Osx expression.

In this study, oxLDL was found to significantly enhance β-glycerophosphate-induced Osx expression (10.9 ± 0.8 vs. 2.2 ± 0.2-fold, $P < 0.01$; Fig. 3B). However, oxLDL did not significantly enhance Runx2 expression either in the absence or presence of β-glycerophosphate (Fig. 3A). This suggests that oxLDL is enhancing β-glycerophosphate-induced Osx expression by a mechanism that is independent of Runx2 activation. Several transcription factors have been proposed to upregulate Osx expression in a manner that is independent of Runx2. These include Dlx5 and Msx2, both members of the homeobox family of transcription factors. Thus, BMP-2 treatment of Runx2 (−/−) cells has been shown to induce Osx expression in a manner that is Dlx5 dependent [Lee et al., 2003]. In addition, overexpression of the homeobox transcription factor, Msx2, in myofibroblasts has been shown to induce Osx expression 10-fold while leaving Runx2 expression unchanged [Cheng et al., 2003]. Therefore, our findings suggest the possibility that oxLDL is inducing Osx expression by upregulating the expression of one or more members of the homeobox family of transcription factors. Interestingly, expression of these transcription factors along with Runx2 and Osx has been detected in the calcified atherosclerotic lesions of human vessels [Ikeda et al., 1993; Shanahan et al., 1994; Dhore et al., 2001; Engelese et al., 2001; Tyson et al., 2003].

Finally, our findings demonstrate that both oxLDL's ability to induce Osx expression (Fig. 4B) and its ability to induce osteoblast

differentiation (Figs. 5 and 6) are Erk 1- and 2-dependent. Several other authors have also reported Erk 1/2-dependent upregulation of Osx when culturing human mesenchymal stem cells in the presence of IGF-1 [Celil and Campbell, 2005; Celil et al., 2005]. Whether Erk 1/2 is affecting Osx expression directly or whether its effects are on some intermediary component such as members of the homeobox family of transcription factors is not known. However, regardless of how oxLDL is upregulating Osx expression, it is very apparent that oxLDL and β-glycerophosphate are functioning to promote osteoblast differentiation through two distinct pathways. Thus β-glycerophosphate appears to induce osteoblast differentiation primarily through the upregulation of Runx2 expression and to some extent by having a modest effect on Osx expression. In contrast, oxLDL appears to have no effect on Runx2 expression while upregulating Osx expression either directly or indirectly, in an Erk 1/2-dependent manner.

In summary, we have shown that oxLDL is capable of acting synergistically with β-glycerophosphate to promote both Osx expression and osteoblast differentiation in primary cultures of BASMCs. In addition, by culturing primary BASMCs in the presence of the MEK inhibitor, PD098059, or by using cells transfected with Erk 1 and 2 specific siRNA, we have demonstrated that the effects of oxLDL are Erk 1/2-dependent. When taken together, these findings suggest a plausible mechanism by which oxLDL may promote osteogenic differentiation and vascular calcification in vivo.

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