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Oxidized Low-Density Lipoprotein Promotes Osteoblast Differentiation in Primary Cultures of Vascular Smooth Muscle Cells by Up-regulating *Osterix* Expression in an Msx2-Dependent Manner

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ABSTRACT

We have previously shown that oxidized low-density lipoproteins (oxLDLs) act synergistically with β-glycerophosphate to induce the osteogenic differentiation of primary bovine aortic smooth muscle cells (BASMCs). In the present study, we attempt to resolve the mechanism responsible for this effect by examining the expression of several osteoblast-specific transcription factors. Thus, by culturing BASMCs in the absence or presence of β-glycerophosphate and/or oxLDL, we demonstrate that β-glycerophosphate induces both *Runx2* and *Osterix* (*Osx*) expression. In contrast, oxLDL has no effect on *Runx2* expression but rather it enhances β-glycerophosphate-induced osteoblast differentiation by further up-regulating *Osx* expression. In an attempt to elucidate the mechanism responsible for this latter effect, we examined the ability of oxLDL to affect *Msh homeobox 2* (*Msx2*) expression. Similar to its effect on *Osx* expression, oxLDL was found to synergistically enhance β-glycerophosphate-induced *Msx2* expression in an extracellular signal-regulated kinase 1 and 2 (Erk 1 and 2)-dependent manner. Furthermore, oxLDL's ability to enhance both β-glycerophosphate-induced *Osx* expression and alkaline phosphatase activity was prevented when the BASMCs were first transfected with *Msx2*-specific siRNA. Taken together, these findings suggest a plausible mechanism by which oxLDL may promote osteoblast differentiation and vascular calcification in vivo. J. Cell. Biochem. 112: 581–588, 2011.

KEY WORDS: SMOOTH MUSCLE CELLS; OSTEOBLAST DIFFERENTIATION; OXIDIZED LOW DENSITY LIPOPROTEIN; OSTERIX; Msx2

The calcification of vascular tissue is a key feature of the atherosclerotic process. However, despite its importance, little is known about the mechanism(s) by which it occurs. Recent evidence suggests that vascular calcification is a highly complex process that shares many similarities with the mineralization of bone. Thus, the calcification of atherosclerotic lesions appears to coincide with the presence of bone-forming osteoblast-like cells [Watson et al., 1994; Shanahan and Weissberg, 1999; Zeadin et al., 2009]. In support of this, we and others have shown that vascular smooth muscle cells (VSMCs) can be induced to undergo osteogenic differentiation when cultured in vitro [Yang et al., 2005; Chen et al., 2006; Bear et al., 2008]. Together, these findings suggest that bone-forming osteoblast-like cells may be responsible for the vascular calcification that is often seen in association with atherosclerotic lesions.

Under normal conditions, osteoblasts are formed from a mesenchymal stem cell (MSC) population, which is found within the bone marrow. The first step in this differentiation process involves a commitment phase in which MSCs differentiate into osteoprogenitors. Following the initial commitment phase, further regulation by key transcription factors induces osteoprogenitor cells to differentiate into pre-osteoblasts and finally into mature osteoblasts which are capable of mineralization [Manolagas et al., 1981; Kim et al., 2004; Aubin et al., 2006]. Several transcription factors have been identified as being key to this process. In particular, both Runx2 and Osterix (Osx) are transcription factors thought to be critical in the osteogenic differentiation of MSCs [Komori et al., 1997; Otto et al., 1997; Nakashima et al., 2002; Schroeder et al., 2005]. Runx2 appears to act earlier in osteoblast differentiation and is thought to be essential for the establishment of

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a pre-osteoblast phenotype [Komori et al., 1997; Otto et al., 1997; Schroeder et al., 2005; Komori, 2006]. While the more recently discovered Osx is thought to play a role in the later transition from the pre-osteoblast to mature osteoblast [Nakashima et al., 2002]. Thus, *Runx2* is expressed in *Osx*-deficient mice while *Osx* is not expressed in *Runx2*-deficient mice.

Other transcription factors are thought to be critical to the osteogenic process. These include the homeobox transcription factors Dl×2, Dl×3, Dl×5, and Msh homeobox 2 (Msx2). Of these, perhaps the best known is Msx2. Mice deficient in *Msx2* display a defect in craniofacial mineralization during development and a marked reduction in bone formation resulting from decreased osteoblast cell numbers. How *Msx2* expression relates to either *Runx2* or *Osx* expression is not clearly defined. Recent studies have shown that bone morphogenic proteins (BMPs) can induce *Msx2* expression in MSCs derived from both normal and *Runx2*-deficient mice and that this results in enhanced *Osx* expression [Ichida et al., 2004; Matsubara et al., 2008]. Thus, these findings suggest that the expression of *Osx* can be controlled by both Runx2-dependent and *Runx2*-independent pathway and that the Runx2-independent pathway is mediated by Msx2.

We have previously shown that oxidized low-density lipoproteins (oxLDLs) act synergistically with β-glycerophosphate to induce the osteogenic differentiation of primary bovine aortic smooth muscle cells (BASMCs). In the present study, we attempt to resolve the mechanism responsible for this effect by examining the expression of several transcription factors thought to be critical to the osteogenic process. Thus, by culturing BASMCs in the absence or presence of β-glycerophosphate and oxLDL, we demonstrate that oxLDL has no effect on *Runx2* expression but rather it enhances β-glycerophosphate-induced *Osx* expression, and thus osteoblast differentiation in an Msx2-dependent manner. When taken together, these findings suggest a plausible mechanism by which oxLDL promotes the calcification of atherosclerotic plaques in vivo.

EXPERIMENTAL PROCEDURES

MATERIALS

Human LDL and the MEK inhibitor, PD098059, were purchased from Sigma Chemical Co. (St Louis, MO.) while primers for RT-PCR were purchased "in house" (Mobix, Hamilton, ON) (See Table I). RNA isolation kits were obtained from Qiagen Inc. (Valencia, CA). Dulbecco's modified eagle media (DMEM) and fetal bovine serum (FBS), were obtained from Invitrogen (Burlington, ON). siRNA duplexes were obtained through Integrated DNA Technologies (Coralville, IA).

CELL ISOLATION

Primary BASMCs were obtained as described previously [Bear et al., 2008]. Briefly, bovine aortas were collected from a local slaughter house (Highland Packers Ltd., Hamilton, ON) and cut open longitudinally. The aortic endothelium and adventitia were then removed and the remaining medial layers cut into small (1–2 mm^2) sections which were cultured in DMEM containing 20% FBS, 100 U/ml penicillin and 100 U/ml streptomycin. After noticeable outgrowth was observed, the cells were harvested and grown to confluency. All cells were then used immediately or subsequently stored in liquid nitrogen. No cells greater than passage three were used. To confirm the identity of the cells as smooth muscle cells, isolated preparations were immunostained with a smooth muscle-specific α-actin antibody (Sigma).

OXIDATION OF LOW-DENSITY LIPOPROTEINS

To obtain oxLDL, native LDL was 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 dialysis against 9 μM $FeSO_4$ for an additional 48 h. Any contaminating Fe^{2+} was then removed by a final dialysis step against PBS (pH 7.2) that contained 100 μM EDTA. Finally, the degree of oxidation was determined by reading the absorbance at 234 nm to detect baseline levels of diene conjugation [Velarde et al., 2001]. Protein levels were also accounted for using a BioRad DC protein assay (Bio-Rad, Hercules, CA) [Bear et al., 2008].

DIFFERENTIATION ASSAY AND MEASUREMENTS OF ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase (ALP) activity was used as an index of osteoblast differentiation [Yang et al., 2005; Bear et al., 2008]. Briefly, BASMCs were first plated in 24-well plates at a density of 5×10^4 cells/well and then cultured for up to 8 days in DMEM containing 10% FBS, 0.5 mM ascorbic acid, and either 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL. At various periods of time thereafter, the cells were harvested and lysed using a 1% Triton X-100/0.9% NaCl solution. ALP activity was assessed using a *p*-nitrophenol

TABLE I. Primer Sequences and RT-PCR Protocols

Genes	Primer sequences (5′–3′)	Annealing temperature (°C)	Cycle	Product length
Osteocalcin	Fwd: CCTGGTGATGCAGAGTCGGG Rev: GACTGGGCCGTAGAAGCGCC	60	28	227
Osteopontin	Fwd: GAATTCTGTGTCCTCTGAGG Rev: CTATGTGTGATGTGAAGTCC	62	22	385
Runx2	Fwd: CCGCACGACAACCGCACCAT Rev: CTCTAAACACCCGGCCTCGC	58	26	288
Osx	Fwd: GCTGGCTACGGGAGCAGTGG Rev: ACTTCTTCTCCCGGGTGTG	58	24	288
Msx2	Fwd: CTCCAGCCTGCCCTTCAGTGTGG Rev: GTTCCTGCATCCACGCGCTCC	60	26	208
GAPDH	Fwd: ATGGCCTTCCGCGTCCCCACTCC Rev: AGCCAAATTCATTGTCGTACC	60	18	265

phosphate substrate kit (Sigma) and measuring absorbance at 405 nm. ALP values (U/mg) were normalized to protein following protein determination with a Bio-Rad DC protein assay.

RT-PCR

BASMCs were plated in 60 mm dishes at a cell density of 7×10^5 cells/dish. Upon reaching 75–85% confluency, the cells were harvested and total RNA isolated using an RNeasy mini kit (Qiagen). In some cases, the cells were stimulated with either 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL prior to isolating total RNA and performing semi-quantitative RT-PCR analysis. RT-PCR was performed using 4 μg of total RNA, which was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Burlington, ON). Amplification of PCR products was performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA). Reverse primers were radiolabeled by ^{32}P isotope end-labeling. All PCR reactions were done in triplicate and run on a non-denaturing 7.5% polyacrylamide gel before being visualized with Kodak BioMax XAR film [Bear et al., 2008]. Results from RT-PCR were quantified using the Image Quant 5.2 Software.

SIRNA TRANSFECTION

Primary cultures of BASMCs were seeded into 24-well plates at a density of 5×10^4 cells/well. Twenty-four hours later, Lipofectamine reagent (Invitrogen) was used to transfect the cells with either a negative control siRNA or an *Msx2*-specific siRNA (Integrated DNA Technologies). After 5 h, the transfection media was removed and the cells were incubated for an additional 24 h in normal growth media before treating the cells with either 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL.

IMMUNOBLOTTING

Primary BASMCs were grown to 50–70% confluency in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin before being treated with either 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL. Twenty-four hours later, the cells were washed with PBS and then lysed with 1% sodium deoxycholate, 1% Nonidet P-40, 0.5% sodium dodecyl sulfate, and 50 mM NaF in 50 mM Tris–HCl (pH 7.4) containing leupeptin (5 μg/ml), aprotinin (5 μg/ml), pepstatin A (1 μg/ml), PMSF (1 mM), 0.1 mM Na_3VO_4, and EDTA (0.25 mM). The cell lysates were then run on 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked overnight with 5% casein at 4°C. The membranes were then incubated with an anti-Osx antibody (Santa Cruz, CA) for 3 h before being incubated with horseradish peroxidase-conjugated secondary antibodies (Abs), and detecting Osx with an enhanced chemiluminescent system. Following visualization of Osx protein, the blots were stripped (62.5 mM Tris–HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and incubated with anti-actin antibody (Santa Cruz). Actin was visualized by the same method as was used for Osx.

STATISTICAL ANALYSIS

All experiments were performed 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. All results are expressed as a mean ± standard error of the mean.

RESULTS

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

To confirm our previous finding that oxLDL acts synergistically with β-glycerophosphate to induce osteoblast differentiation in primary cultures of BASMCs, we cultured BASMCs in the presence or absence of oxLDL, β-glycerophosphate, or both oxLDL and β-glycerophosphate for 8 days. ALP activity was then determined as an index of osteoblast differentiation. As seen in Figure 1A, treatment of BASMCs with 10 mM β-glycerophosphate resulted in a significant increase in ALP activity (168.5 +/− 23.8 U/mg vs. 68.6 +/− 15.7, respectively, $P < 0.05$). In contrast, oxLDL alone had no effect on

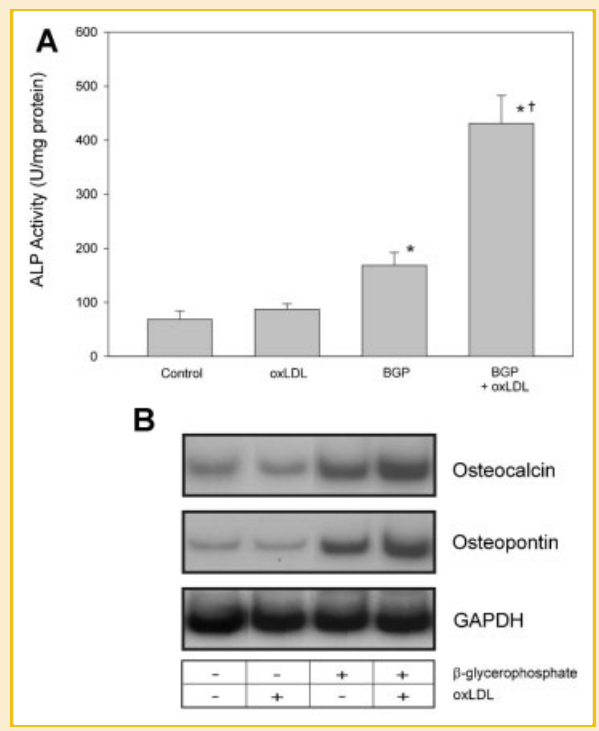

Fig. 1. The effect of oxLDL and β-glycerophosphate on osteoblast differentiation. BASMCs were cultured for 8 days in the absence or presence of 100 μg/ml oxLDL, 10 mM β-glycerophosphate, or both β-glycerophosphate and oxLDL (10 mM and 100 μg/ml, respectively). ALP activity (panel A) or osteocalcin and osteopontin expression (panel B) was then assessed as markers of osteoblast differentiation. Data is expressed as mean +/− SEM. $^*P < 0.05$ when ALP activity is compared to that of control. $^\dagger P < 0.01$ when ALP activity in the presence of both β-glycerophosphate and oxLDL is compared to that with β-glycerophosphate alone.

osteoblast differentiation as measured by ALP activity. However, when the cells were cultured for 8 days in the presence of both oxLDL and β-glycerophosphate, ALP activity was increased 2.5-fold over that seen with β-glycerophosphate alone (431.2 +/− 51.7 U/mg vs. 168.5 +/− 23.8 U/mg, respectively, $P < 0.05$). Similar findings were obtained when the expression of several other osteoblast specific markers was examined by RT-PCR at day 8 (Fig. 1B). Thus, the expression of both osteocalcin and osteopontin was unaffected when primary BASMCs were cultured in the presence of oxLDL for 8 days. However, when the cells were cultured in the presence of 10 mM β-glycerophosphate, both osteocalcin and osteopontin expression was found to be significantly increased (3.3 ± 0.2 and 4.7 ± 0.3, respectively; $P < 0.01$). In addition, the expression of these two osteoblast-specific markers was increased even further when the cells were cultured in the presence of both oxLDL and β-glycerophosphate (5.9 ± 0.2 and 9.2 ± 0.3; $P < 0.01$)(Fig. 1B). Taken together, these data suggest that oxLDL can act synergistically with β-glycerophosphate to induce osteoblast differentiation in primary cultures of BASMCs.

EFFECT OF β-GLYCEROPHOSPHATE AND oxLDL ON Msx2 EXPRESSION

Since oxLDL was found to enhance ALP activity in the presence of β-glycerophosphate, we next set out to determine the effect of oxLDL and β-glycerophosphate on *Msx2* expression. Msx2 is a transcription factor believed to be critical to the early stages of osteogenic differentiation. Semi-quantitative RT-PCR was utilized to examine the expression of *Msx2* in BASMCs cultured in the presence of either β-glycerophosphate, oxLDL, or both β-glycerophosphate and oxLDL. As seen in Figure 2, oxLDL alone had no effect on *Msx2* expression. However, *Msx2* expression was increased when BASMCs were cultured in the presence of β-glycerophosphate (7.0 ± 0.5-fold, $P < 0.01$) and this expression was enhanced even further when the BASMCs were cultured in the presence of β-glycerophosphate plus oxLDL. (11.3 ± 0.3-fold, $P < 0.01$) (Fig. 2). Together, these findings raise the possibility that oxLDL enhances β-glycerophosphate-induced osteoblast differentiation by up-regulating the expression of *Msx2*.

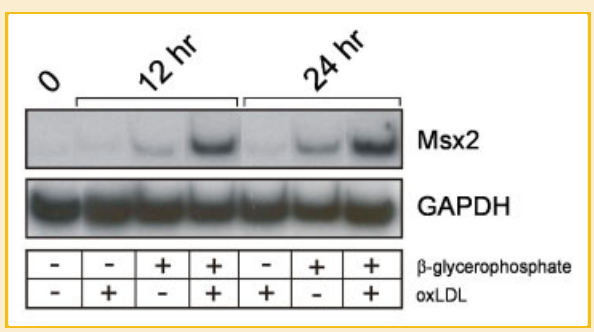

Fig. 2. The effect of β-glycerophosphate and oxLDL on Msx2 expression. BASMCs were cultured in the absence or presence of 10 mM β-glycerophosphate, 100 μg/ml oxLDL, or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL. At various times thereafter, total RNA was isolated and subjected to semi-quantitative RT-PCR in order to assess the effect on *Msx2* expression.

In a previous study, we demonstrated that oxLDL could activate the mitogen-activated protein kinase/extracellular-signal-related kinase (MAPK/Erk) pathway in BASMCs and that this mediated oxLDL's ability to enhance osteoblast differentiation in the presence of β-glycerophosphate [Bear et al., 2008]. Therefore, we next decided to determine if oxLDL was promoting β-glycerophosphate-induced *Msx2* expression in an Erk 1/2-dependent manner. As shown in Figure 3A and B, *Msx2* expression was enhanced by oxLDL when the BASMCs were cultured in the presence of both β-glycerophosphate and oxLDL. However, when the cells were also cultured in the presence of the MEK inhibitor, PD098059, oxLDL was no longer able to synergistically enhance β-glycerophosphate-induced Msx2 expression (Fig. 3). Taken together, these findings suggest that oxLDL enhances β-glycerophosphate-induced *Msx2* expression by activating the MAPK/Erk pathway.

EFFECT OF β-GLYCEROPHOSPHATE AND oxLDL ON Runx2 AND Osx EXPRESSION AFTER siRNA-MEDIATED KNOCKDOWN OF Msx2 EXPRESSION

Since we had previously shown that β-glycerophosphate could induce *Runx2* expression [Bear et al., 2008], we next decided to determine if β-glycerophosphate's effect on *Runx2* expression was

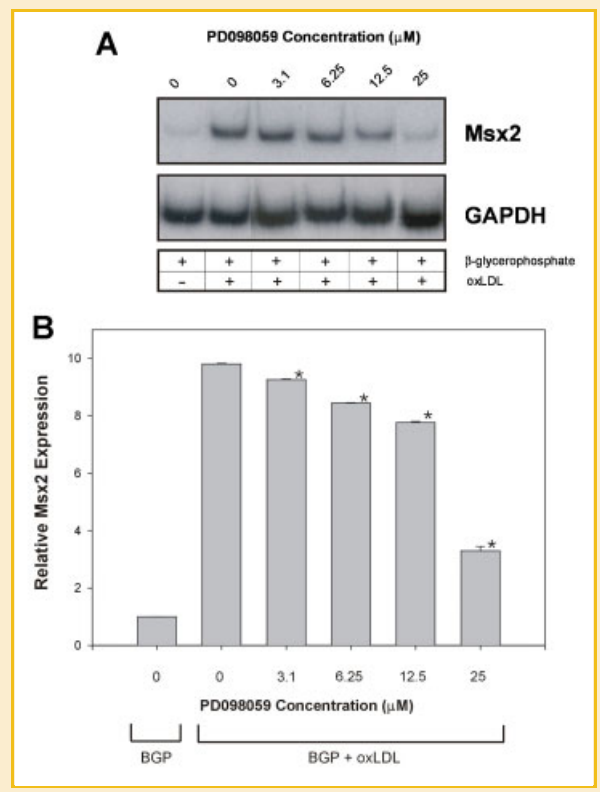

Fig. 3. The effect of MEK inhibitor, PD098059, on the ability of oxLDL to enhance Msx2 expression. BASMCs were pre-treated with PD098059 and then cultured in the absence or presence of both 10 mM β-glycerophosphate and 100 μg/ml oxLDL. Twenty-four hours later, total RNA was prepared and analyzed for Msx2 expression using semi-quantitative RT-PCR. Data is expressed as mean +/− SEM. $^{*}P < 0.05$ when *Msx2* expression in the presence of PD098059 is compared to that obtained in its absence.

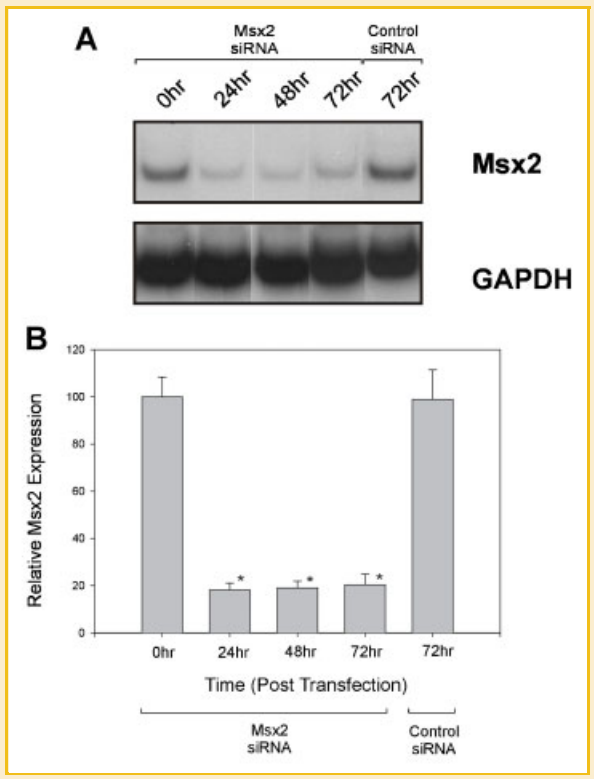

Fig. 4. The effect of Msx2-specific siRNA on Msx2 expression. BASMCs were transfected with either a negative control siRNA or *Msx2*-specific siRNA as described in the Experimental Procedures Section. At various times thereafter, total RNA was isolated and subjected to RT-PCR analysis in order to assess the effect on *Msx2* expression. Data is expressed as mean +/− SEM. $^{*}P < 0.005$ when *Msx2* expression in BASMCs transfected with *Msx2*-specific siRNA is compared to *Msx2* expression in either untransfected BASMCs or BASMCs transfected with negative control siRNA.

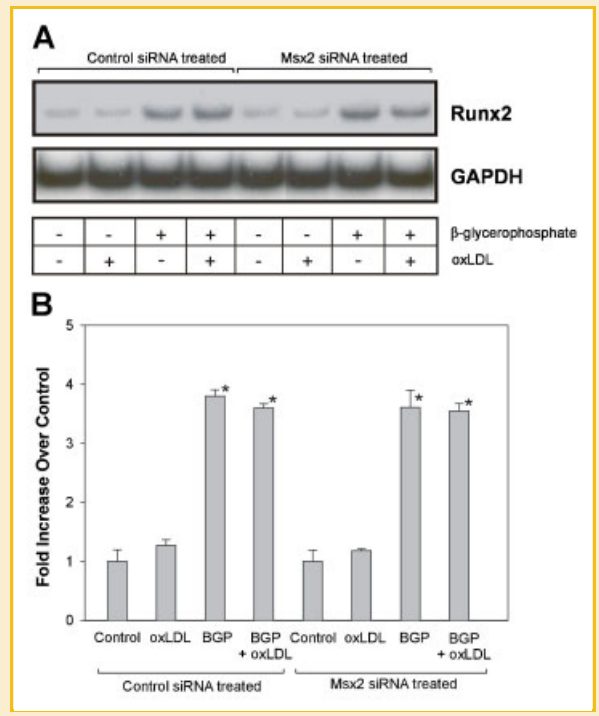

Fig. 5. The effect of Msx2-specific siRNA on Runx2 expression. BASMCs were transfected with either a negative control siRNA or *Msx2*-specific siRNA as described in the Experimental Procedures Section. The transfected cells were then cultured in the absence or presence of 10 mM β-glycerophosphate, 100 μg/ml oxLDL or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL. Twenty-four hours later, total RNA was isolated and subjected to RT-PCR analysis in order to assess the effect on Runx2 expression. Data is expressed as mean +/− SEM. $^{*}P < 0.01$ when *Runx2* expression in the presence of β-glycerophosphate or β-glycerophosphate plus oxLDL is compared to that obtained in their absence.

mediated by Msx2. As seen in Figure 4, *Msx2* expression was significantly decreased when BASMCs were transfected with *Msx2*-specific siRNA (82.0 ± 1.1; $P < 0.005$). In contrast, transfection with *Msx2*-specific siRNA did not affect *Runx2* expression either in the absence or presence of either oxLDL or β-glycerophosphate (Fig. 5). Thus, when BASMCs transfected with either negative control siRNA or *Msx2*-specific siRNA were cultured in the presence of oxLDL alone, no effect on *Runx2* expression was observed. In addition, β-glycerophosphate treatment of negative control siRNA or *Msx2*-specific siRNA transfected cells increased *Runx2* expression equally (3.8 +/− 0.1-fold vs. 3.6 +/− 0.3-fold, respectively; $P < 0.01$). Finally, oxLDL did not synergistically enhance β-glycerophosphate-induced *Runx2* expression when transfected BASMCs were cultured in the presence of both oxLDL and β-glycerophosphate (Fig. 5). Taken together, these findings suggest that Msx2 does not mediate β-glycerophosphate's effect on *Runx2* expression and that the effect of oxLDL on β-glycerophosphate-induced osteoblast differentiation is Runx2-independent.

Next we decided to first confirm our previous findings that oxLDL promotes β-glycerophosphate-induced *Osx* expression, and to then determine if this effect was mediated by *Msx2* expression. Therefore, we transfected BASMCs with either negative control siRNA or *Msx2*-specific siRNA and then cultured the cells in the presence of either oxLDL, β-glycerophosphate, or both before determining the effect on *Osx* expression. As shown in Figure 6A and B, no effect on *Osx* expression was seen when cells transfected with either negative control or *Msx2*-specific siRNA were treated with oxLDL alone. In contrast, treatment with β-glycerophosphate did result in a small but significant increase in *Osx* expression (2.2 +/− 0.02-fold, $P < 0.05$). In addition, similar to the effect on *Msx2* expression, *Osx* expression was enhanced even further when the cells transfected with negative control siRNA were cultured in the presence of both β-glycerophosphate and oxLDL (Figure 6A and B) (2.2 +/− 0.02-fold vs. 4.2 +/− 0.1-fold, $P < 0.01$, respectively). However, when BASMCs transfected with *Msx2*-specific siRNA were cultured in the presence of both β-glycerophosphate and oxLDL, oxLDL was unable to enhance β-glycerophosphate-induced *Osx* expression (2.1 +/− 0.05 vs. 1.8 +/− 0.03, $P > 0.05$). Similar findings were obtained when Osx protein expression was detected by Western blotting (Fig. 6C). When taken together, these findings suggest that oxLDL enhances β-glycerophosphate-induced *Osx* expression by first up-regulating the expression of *Msx2*.

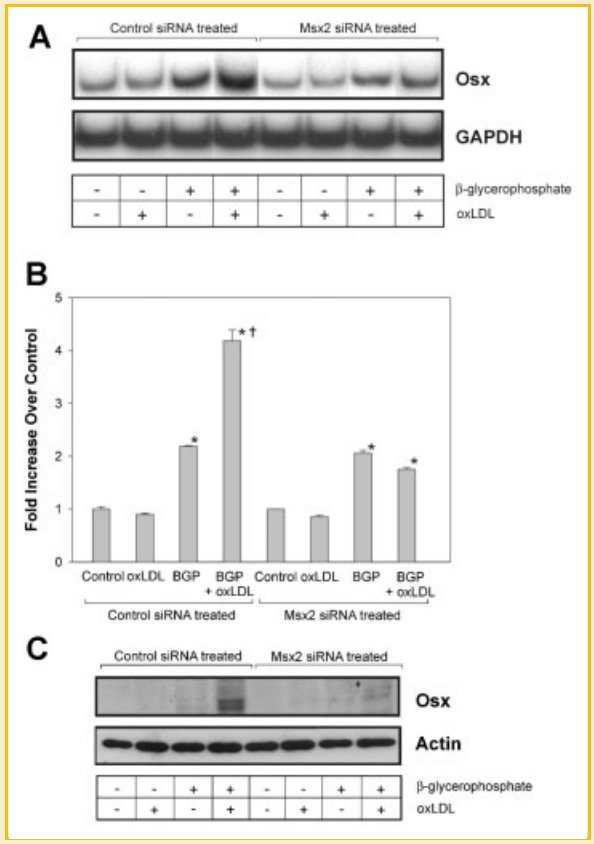

Fig. 6. The effect of Msx2-specific siRNA on Osx expression. BASMCs were transfected with either a negative control siRNA or *Msx2*-specific siRNA as decribed in the Experimental Procedures Section. The transfected cells were then cultured in the absence or presence of 10 mM β-glycerophosphate, 100 μg/ml oxLDL or both 10 mM β-glycerophosphate and 100 μg/ml oxLDL. Twenty-four hours later, total RNA was isolated and subjected to RT-PCR analysis in order to assess the effect on *Osx mRNA* expression (Panels A, B). Alternatively, in some experiments Western blotting was performed in order to assess the effect on Osx protein expression directly (Panel C). $^{*}P < 0.05$ when Osx expression in the presence of β-glycerophosphate or β-glycerophosphate plus oxLDL is compared to that obtained in their absence. $^{\dagger}P < 0.01$ when *Osx* expression in the presence of both β-glycerophosphate and oxLDL is compared to that with β-glycerophosphate alone.

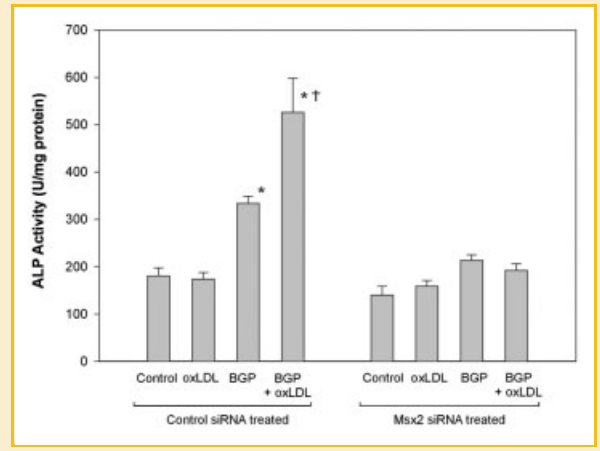

Fig. 7. The effect of Msx2-specific siRNA on osteoblast differentiation of BASMCs. BASMCs were transfected with either *Msx2*-specific siRNA or a negative control siRNA as described in the Experimental Procedures Section. Following transfection, BASMCs were cultured for 8 days in the presence of oxLDL (100 μg/ml), β-glycerophosphate (10 mM), or both β-glycerophosphate (10 mM) and oxLDL (100 μg/ml). ALP activity was then assessed as a marker of osteoblast differentiation. Data is expressed as mean +/− SEM. $^{*}P < 0.05$ when ALP activity is compared to that of control. $^{\dagger}P < 0.01$ when ALP activity in the presence of both β-glycerophosphate and oxLDL was compared to that with β-glycerophosphate alone.

EFFECT OF β-GLYCEROPHOSPHATE AND oxLDL ON OSTEOBLAST DIFFERENTIATION IN PRIMARY CULTURES OF BASMCs FOLLOWING siRNA-MEDIATED KNOCKDOWN OF Msx2 EXPRESSION

Finally, to demonstrate that oxLDL's ability to enhance ALP activity in the presence of β-glycerophosphate was mediated by Msx2, we transfected BASMCs with *Msx2*-specific siRNA before culturing the cells with either oxLDL, β-glycerophosphate, or both. Measurements of ALP activity was then used as an index of osteoblast differentiation. As seen in Figure 7, when BASMCs transfected with negative control siRNA were cultured for 8 days in the presence of both oxLDL and β-glycerophosphate, ALP activity was increased nearly two-fold over that seen with β-glycerophosphate alone (526.1 +/− 72.2-fold increase vs. 333.8 +/− 15.4-fold increase respectively ($P < 0.01$). However, no increase in ALP activity was seen when the BASMCs were first transfected with *Msx2*-specific siRNA and then cultured in the presence of both oxLDL and β-glycerophosphate. This suggests that Msx2 plays a critical role in oxLDL's ability to enhance β-glycerophosphate-induced osteoblast differentiation.

DISCUSSION

In a previous study, we demonstrated that oxLDL was capable of acting synergistically with β-glycerophosphate to promote both *Osx* expression and osteoblast differentiation in primary cultures of BASMCs. In the present study, we confirm our earlier observations and demonstrate that oxLDL's ability to promote β-glycerophosphate-induced *Osx* expression and osteoblast differentiation is Msx2-dependent. When taken together, these findings suggest that Msx2 plays a critical role in mediating oxLDL's ability to promote osteogenic differentiation in vitro and suggests a plausible mechanism by which oxLDL may promote vascular calcification in vivo.

In the current study, we treated primary BASMCs with β-glycerophosphate, oxLDL, or β-glycerophosphate plus oxLDL, and then examined the effect on several transcription factors, which are known to be critical to the osteogenic process. As seen in Figure 5, β-glycerophosphate appears to act primarily by increasing the expression of *Runx2*. However, a small but significant increase in both *Osx* and *Msx2* expression was also detected in the presence of β-glycerophosphate. How β-glycerophosphate exerts these effects is unknown. Historically, β-glycerophosphate has been thought to act solely as a phosphate donor and as such to be required for the mineralization of primary bone cell cultures in vitro [Bellows et al., 1991; Tenenbaum et al., 1992]. However, we have recently demonstrated that β-glycerophosphate up-regulates Smad 1/5/8

activity in primary BASMCs cultures [Bear et al., 2008]. Smads can act as co-activators of transcription to up-regulate both Runx2 expression and activity [Hanai et al., 1999; Zhang et al., 2000; Afzal et al., 2005]. Therefore, it is possible that the ability of β-glycerophosphate to up-regulate *Runx2* expression is dependent upon Smad activation, and that Runx2 in turn can bind to either the *Osx* or *Msx2* promoter, thereby increasing their expression as well. In support of this theory, Runx2 has been shown to bind to and activate the *Osx* promoter [Nishio et al., 2006]. Thus, β-glycerophosphate appears to up-regulate Runx2 activity in a manner that is analogous to that which occurs upon BMP stimulation [Phimphilai et al., 2006]. If so, it is entirely possible that oxLDL would act synergistically with BMPs to induce the osteogenic differentiation of VSMCs. Interestingly, BMP-2, a BMP which has been shown to promote the osteogenic differentiation of MSCs [Fujii et al., 1999], is known to be present in human calcified atherosclerotic lesions [Bostrom et al., 1993].

In the current study, oxLDL lost its ability to synergistically enhance β-glycerophosphate-induced *Osx* expression when the BASMCs were first transfected with *Msx2*-specific siRNA (Fig. 6). A similar effect on osteoblast differentiation was observed when BASMCs were first transfected with *Msx2*-specific siRNA and then cultured in the presence of both oxLDL and β-glycerophosphate (Fig. 7). This suggests that oxLDL affects *Osx* expression, and thus osteoblast differentiation, by first up-regulating *Msx2* expression. These findings are in keeping with those of Cheng et al. [2003] and Matsubara et al. [2008] who demonstrated that by over-expressing *Msx2*, they could significantly increase *Osx* expression while leaving *Runx2* expression unchanged. Whether any transcription factors other than Msx2 are mediating oxLDL's effect on *Osx* expression is not known. However, BMP-2 treatment of *Runx2* −/− cells has been shown to induce *Osx* expression in a Dlx5-dependent manner [Lee et al., 2003]. Therefore, it is possible that Dlx5 is also mediating oxLDL's effects on Osx expression either prior to, or after, affecting *Msx2* expression. Interestingly, both *Dlx5* and *Msx2* expression along with *Runx2* and *Osx* expression have been detected within the calcified lesions of human arterial vessels [Tyson et al., 2003; Vattikuti and Towler, 2004; Doherty et al., 2004].

Why β-glycerophosphate is required in order to see oxLDL's effect on either Osx expression or osteoblast differentiation is unknown. One explanation could be that the cells which differentiate into osteoblasts in our primary BASMC cultures require β-glycerophosphate to initiate their differentiation, at least partially, down the osteogenic pathway before *Msx2* and/or *Osx* expression can be affected by oxLDL. In keeping with such a theory, *Osx*-deficient mice are known to express *Runx2*, while *Runx2* deficient mice do not express *Osx* [Komori et al., 1997; Nakashima et al., 2002]. Alternatively, it is possible that β-glycerophosphate is acting to increase the expression of a cell surface receptor that is required for oxLDL binding. In any case, our findings suggest that oxLDL can only exert its effects on *Osx* expression in the presence of β-glycerophosphate and that this occurs in a Runx2-independent manner.

In a previous study, we demonstrated that both oxLDL's ability to induce *Osx* expression and its ability to induce osteoblast differentiation were Erk 1/2-dependent [Bear et al., 2008]. In the current study, we demonstrate that oxLDL's effects on both *Osx* expression and osteoblast differentiation are also Msx2-dependent (Figs. 6 and 7) and that oxLDL enhances β-glycerophosphate-induced *Msx2* expression by activating the MAPK/Erk pathway (Fig. 3). Several other authors have reported that *Osx* expression can be up-regulated in an Erk 1/2-dependent manner [Celil and Campbell, 2005; Celil et al., 2005]. Thus, Celil and Campbell [2005] demonstrated that IGF-1 up-regulates *Osx* expression in MSCs and that this occurs in an Erk 1/2-dependent manner. Whether Msx2 mediated these effects as well is unknown. However, based on our findings, it is apparent that oxLDL first up-regulates *Msx2* expression in an Erk 1/2-dependent manner, and that this in turn results in increased Osx expression and osteoblast differentiation in the presence of β-glycerophosphate.

In summary, we have shown that oxLDL acts synergistically with β-glycerophosphate to induce both *Osx* expression and osteoblast differentiation in primary cultures of BASMCs. In addition, by using BASMCs transfected with *Msx2*-specific siRNA, we have demonstrated that these effects are Msx2-dependent. When taken together, these findings suggest a plausible mechanism by which oxLDL may promote the calcification of atherosclerotic plaques in vivo.

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