Role of Cellular Cholesterol Metabolism in Vascular Cell Calcification*

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Vascular calcification impairs vessel compliance and increases the risk of cardiovascular events. We found previously that liver X receptor agonists, which regulate intracellular cholesterol homeostasis, augment PKA agonist- or high phosphate-induced osteogenic differentiation of vascular smooth muscle cells. Because cholesterol is an integral component of the matrix vesicles that nucleate calcium mineral, we examined the role of cellular cholesterol metabolism in vascular cell mineralization. The results showed that vascular smooth muscle cells isolated from LDL receptor null (Ldlr−/−) mice, which have impaired cholesterol uptake, had lower levels of intracellular cholesterol and less osteogenic differentiation, as indicated by alkaline phosphatase activity and matrix mineralization, compared with WT cells. PKA activation with forskolin acutely induced genes that promote cholesterol uptake (LDL receptor) and biosynthesis (HMG-CoA reductase). In WT cells, inhibition of cholesterol uptake by lipoprotein-deficient serum attenuated forskolin-induced matrix mineralization, which was partially reversed by the addition of cell-permeable cholesterol. Prolonged activation of both uptake and biosynthesis pathways by cotreatment with a liver X receptor agonist further augmented forskolin-induced matrix mineralization. Inhibition of either cholesterol uptake, using Ldlr−/− cells, or of cholesterol biosynthesis, using mevastatin-treated WT cells, failed to inhibit matrix mineralization due to up-regulation of the respective compensatory pathway. Inhibition of both pathways simultaneously using mevastatin-treated Ldlr−/− cells did inhibit forskolin-induced matrix mineralization. Altogether, the results suggest that up-regulation of cholesterol metabolism is essential for matrix mineralization by vascular cells.

Vascular calcification is frequently found in advanced atherosclerotic lesions and is an independent predictor of cardiovascular morbidity and mortality in patients with chronic kidney disease (1–3). Long considered to be a passive process, there now exists much evidence to suggest that vascular calcification is an active cell-mediated phenomenon that is highly regulated through complex mechanisms under active investiga-

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3 The abbreviations used are: VSMC, vascular smooth muscle cell; LXR, liver X receptor; CVC, calcifying vascular cell; LPDS, lipoprotein-deficient serum; ALP, alkaline phosphatase; qPCR, quantitative PCR.

A common complication of chronic kidney disease is hyperparathyroidism. Interestingly, parathyroid hormone, an activator of the PKA pathway, has been shown to have a stimulatory role in vascular calcification both in vitro and in vivo. When parathyroid hormone levels are continuously elevated, they induce vascular calcification in rat models, irrespective of renal function (9). Additionally, parathyroid hormone-related protein, also a PKA activator, has been found in calcified atherosclerotic lesions (10). Accordingly, we and others have demonstrated previously that mineralization of vascular smooth muscle cells (VSMCs)3 is induced by PKA agonists in vitro (11, 12) as well as in vivo (13).

Cholesterol is an integral component of cell membranes and matrix vesicles (14). The latter are secreted by VSMCs, chondrocytes, and osteoblasts, and they are instrumental in biomineralization, including that of skeletal bone, cartilage, and the artery wall (15–17). In a tightly regulated cholesterol homeostatic process, cells obtain essential cholesterol by endogenous synthesis or uptake from the extracellular milieu. For endogenous synthesis, the rate-limiting enzyme is HMG-CoA reductase, which is blocked by the class of drugs known as statins. Alternatively, if circulating cholesterol levels are high or cholesterol synthesis is inhibited by statins, cells take up cholesterol from their extracellular environment in the form of the cholesterol-rich LDL particle via the LDL receptor (18, 19). Thus, statins are highly effective at lowering circulating levels of LDL and are among the most commonly prescribed medications for patients with atherosclerotic cardiovascular disease.

We demonstrated previously that both bovine and murine VSMCs undergo osteogenic differentiation and mineralization spontaneously as well as in the presence of PKA activators or high phosphate concentrations (11, 20–22). Furthermore, we found that activation of liver X receptor (LXR), which up-regulates the expression of genes involved in cholesterol efflux (23, 24), augments PKA- and high phosphate-induced mineralization of VSMCs (21, 25). Consistent with these findings, inhibition of LXR by the dominant-negative form of LXRα and/or LXRβ inhibits mineralization of VSMCs (21). In this study, we investigated the role of cholesterol metabolism in vascular cell calcification and demonstrated that both cellular biosynthesis...
and uptake of cholesterol are essential to the mineralization of vascular cells.

**EXPERIMENTAL PROCEDURES**

Reagents—Forskolin was purchased from Calbiochem, T0901317 from Cayman Chemical (Ann Arbor, MI), and mevastatin from BIOMOL (Plymouth Meeting, PA). Water-soluble cholesterol was purchased from Sigma-Aldrich.

Cell Culture—Bovine calcifying vascular cells (CVCs) were isolated and maintained as described previously (20). Murine aortic cells (passages 6–10) were isolated from the aortas of C57BL6 (WT) and Ldlr−/− mice as described previously (11). Cells were maintained in DMEM containing 20% FBS. 3–4 days after plating, cells were treated with forskolin (10 μM, unless indicated otherwise) in α-Minimum Essential Medium containing 10% FBS and 5 mM β-glycerophosphate. Lipoprotein-deficient serum (LPDS) was kindly provided by the UCLA Atherosclerosis Research Unit and was used at a final concentration of 0.8 mg/ml. LDL receptor deficiency in Ldlr−/− cells was confirmed by RT-PCR following protocols established by The Jackson Laboratory. Construction of adenoviral constructs expressing VP16-LXRα, VP16-LXRβ, and SREBP-1c (a constitutively active form of SREBP-1 (sterol regulatory element-binding protein 1c); Addgene) and transduction to CVCs were carried out as described previously (21).

Matrix Calcium Quantitation—After the indicated periods, matrix calcium levels were analyzed by the o-cresolphthalein complexone method (Teco Diagnostics, Anaheim, CA). Each condition was assayed in quintuplicate and normalized to total protein using the Bradford method (11).

Alkaline Phosphatase Activity—After the indicated periods, alkaline phosphatase (ALP) activity was assayed colorimetrically using Sigma 104 phosphatase substrate. Each condition was assayed in quintuplicate and normalized to total protein using the Bradford method (11).

Intracellular Cholesterol Measurement—Total cellular cholesterol content was measured using the Amplex Red cholesterol assay kit from Invitrogen (Carlsbad, CA). Cell lysate was collected with the reaction buffer provided in the kit.

Gene Expression—Total RNA was isolated from cells using TRIzol reagent (Invitrogen). Real-time RT-quantitative PCR (qPCR) was performed using a One-Step qRT-PCR kit (BioChain Institute, Inc. Hayward, CA) in an Mx3005P system (Stratagene La Jolla, CA). β-Actin was used for normalization.

Data Analysis—Each experiment was performed in at least quadruplicate wells and repeated at least three times (n ≥ 3). Data are expressed as means ± S.E. Student’s t test was used for comparison between two groups. For more than two groups, mean values were compared using one-way analysis of variance, with comparison of different groups by Fisher’s protected least significant difference test. A value of p ≤ 0.05 was considered significant.

**RESULTS**

Effects of Cholesterol Uptake Deficiency on Osteoblastic Differentiation and Matrix Mineralization—To investigate the effects of impaired cholesterol uptake on osteoblastic differentiation and mineralization, aortic smooth muscle cells were iso-

![FIGURE 1](image-url)
lated from C57BL/6 (WT) and Ldlr−/− mice, and osteoblastic differentiation and mineralization were assessed. ALP activity, an early marker of osteoblastic differentiation, was assessed after 4 days in culture. The results showed that WT cells had 2.5–3-fold greater ALP activity than Ldlr−/− cells (Fig. 1A). The level of matrix calcium mineral was also reduced by 4-fold in Ldlr−/− cells (Fig. 1B). The impaired cholesterol uptake in Ldlr−/− cells was evident by the reduced intracellular cholesterol levels (Fig. 1C).

Because murine aortic cells have low base-line levels of matrix calcification, we repeated the effects of reduced cholesterol uptake using a subpopulation of bovine CVCs that have higher base-line levels. These cells have been characterized previously as capable of undergoing spontaneous osteoblastic differentiation and mineralization (20). The results showed that CVCs cultured in LPDS had significantly less matrix calcium than those cultured in normal serum (Fig. 1D). The steady-state intracellular cholesterol levels of CVCs in LPDS culture were also less than those in normal serum (0.96 ± 0.01 versus 1.11 ± 0.04 μg/ml; p < 0.05).

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Effects of Osteogenic Activators on Cholesterol Metabolism and Osteoblastic Differentiation—Real-time RT-qPCR analysis showed that treatment of murine WT cells with forskolin, a PKA agonist, induced expression of LDL receptors and HMG-CoA reductase acutely at 6 h but not after prolonged treatment (7 days) (Fig. 2, A and B). As shown in our previous study (11), forskolin treatment of WT cells increased ALP activity and matrix mineralization (Fig. 2C).

Effects of LPDS on Osteoblastic Differentiation and Mineralization—We also tested the effects of cholesterol uptake on forskolin-induced matrix calcification by culturing murine WT cells in medium containing LPDS versus normal serum. The results showed that forskolin-induced ALP activity and matrix mineralization were attenuated in LPDS (Fig. 3, A and B). This effect was rescued by the addition of cell-permeable cholesterol, a cholesterol-cyclodextrin complex (Fig. 3C), suggesting that cholesterol uptake is necessary for forskolin-induced matrix calcification. Treatment with cyclodextrin alone did not have any significant effect (data not shown).

Effects of Activation of Both Cholesterol Uptake and Synthesis on Matrix Mineralization—To investigate the effects of activating cholesterol metabolism, murine WT cells were treated with T0901317, an LXR agonist. Treatment with T0901317 alone induced expression of the LDL receptor and HMG-CoA reductase at 7 days (Fig. 4A). Cotreatment of cells with T0901317 and forskolin sustained LDL receptor expression and HMG-CoA reductase at 7 days (Fig. 4A). This cotreatment also augmented forskolin-induced matrix mineralization (Fig. 4B). T0901317 alone did not induce matrix mineralization (Fig. 4B), suggesting that, in the absence of forskolin, increased cholesterol uptake and synthesis are not sufficient for inducing matrix calcification. Interestingly, the steady-state intracellular cholesterol levels in T0901317-cotreated cells were lower than those in cells treated with forskolin alone (Fig. 4C). This is possibly due to T0901317 induction of cholesterol efflux by up-regulating an ATP-binding cassette transporter, ABCA1 (Fig. 4D). This effect was not observed with forskolin alone (Fig. 4D).

Recently, we found that LXR activation augments phosphate-induced mineralization of CVCs through an SREBP-1-dependent mechanism (21). SREBP-1 is known to be a major regulator of the LDL receptor, particularly in the liver (26). Overexpression of LXRa, LXRB, or SREBP-1c (a constitutively active form of SREBP-1), which increases phosphate-induced mineralization (21), also induced expression of the LDL receptor in CVCs (Fig. 4E).
sustained the induction of LDL receptor expression in murine WT cells (Fig. 5A). The intracellular cholesterol content was also increased by mevastatin treatment (Fig. 5B). Consequently, mevastatin treatment did not attenuate forskolin-induced matrix mineralization (Fig. 5C).

**Effects of Inhibition of Cholesterol Uptake on Matrix Mineralization**—To investigate the effects of inhibition on cholesterol uptake, we used murine aortic cells lacking the LDL receptor. Similar to WT cells, forskolin induced HMG-CoA reductase in Ldlr−/− cells at 6 h but not at 7 days (Fig. 6A), suggesting that the cholesterol biosynthetic pathway was not impaired. Forskolin treatment also increased ALP activity and matrix mineralization (Fig. 6B). Cotreatment with T0901317 and forskolin further induced matrix mineralization in Ldlr−/− cells (Fig. 6C).

**Effects of Inhibition of Both Cholesterol Uptake and Synthesis on Matrix Mineralization**—To test the effects of inhibiting both uptake and synthesis of cholesterol, Ldlr−/− cells were cotreated with mevastatin and forskolin. The results showed that, in contrast to the findings in WT cells, forskolin-induced matrix mineralization was attenuated by mevastatin (Fig. 7).
Intracellular cholesterol levels were also not further induced by mevastatin (data not shown).

**DISCUSSION**

Differentiating chondrocytes and osteoblasts produce a mineralizing matrix by shedding matrix vesicles, which bud off from the plasma membrane and serve as nucleation centers for mineral crystals. Because cholesterol content is greater in matrix vesicles than in the cytoplasm (14), vesicle production may drain the cell of its cholesterol stores, unless cholesterol synthesis is up-regulated.

In this study, we found that cholesterol metabolism plays an important role in vascular cell mineralization. Spontaneous calcification was inhibited when cholesterol metabolism was reduced. The differentiation activator forskolin acutely up-regulated genes involved in both cholesterol uptake and biosynthesis. In the presence of forskolin, it appears that when either pathway (cholesterol uptake or biosynthesis) is inhibited, the other pathway overcompensates, yielding even greater matrix mineralization. Inhibition of both pathways appears to be required to attenuate mineralization. Interestingly, steady-state intracellular cholesterol levels did not correlate well with the propensity for forskolin-induced mineralization. This may be due to a high-throughput state, in which cholesterol is incorporated into matrix vesicles, which are exported as part of the process of extracellular matrix mineralization.

It is interesting to note that statins have been shown to inhibit human VSMC calcification induced by an inflammatory “mixture” containing interferon-γ, tumor necrosis factor-α, and oncostatin M (27). Such inhibitory effects may be due to their known anti-inflammatory effects (28).

We found previously that LXR agonists, which regulate cellular cholesterol homeostasis, accelerate PKA-induced matrix mineralization of murine aortic cells (25) and high phosphate-induced bovine CVCs (21). In this study, we have shown that sustained up-regulation of the LDL receptor and HMG-CoA reductase augments matrix mineralization. This mechanism may work in concert with a parallel mechanism in which lipogenesis is up-regulated by LXR agonists (21).

These findings provide insight into how matrix vesicles form in the process of vascular calcification. They also have broader implications because matrix vesicles are a type of microparticle, which is now recognized as a novel mechanism of intercellular communication, transcellular delivery, and interaction of cells with their local extracellular environment.

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**FIGURE 7. Effects of inhibition of both cholesterol uptake and synthesis on matrix mineralization.** Matrix calcium levels of murine Ldlr−/− cells treated with control vehicle, forskolin (Fsk; 10 μM), and/or mevastatin (1 μM) as indicated for 10 days. #, p < 0.0001.