Oxidative Stress Induces Vascular Calcification through Modulation of the Osteogenic Transcription Factor Runx2 by AKT Signaling*

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Oxidative stress plays a critical role in the pathogenesis of atherosclerosis including the formation of lipid laden macrophages and the development of inflammation. However, oxidative stress-induced molecular signaling that regulates the development of vascular calcification has not been investigated in depth. Osteogenic differentiation of vascular smooth muscle cells (VSMC) is critical in the development of calcification in atherosclerotic lesions. An important contributor to oxidative stress in atherosclerotic lesions is the formation of hydrogen peroxide from diverse sources in vascular cells. In this study we defined molecular signaling that is operative in the H₂O₂-induced VSMC calcification. We found that H₂O₂ promotes a phenotypic switch of VSMC from contractile to osteogenic phenotype. This response was associated with an increased expression and transactivity of Runx2, a key transcription factor for osteogenic differentiation. The essential role of Runx2 in oxidative stress-induced VSMC calcification was further confirmed by Runx2 depletion and overexpression. Inhibition of Runx2 using short hairpin RNA blocked VSMC calcification, and adenovirus-mediated overexpression of Runx2 alone induced VSMC calcification. Inhibition of H₂O₂-activated AKT signaling blocked VSMC calcification and Runx2 induction concurrently. This blockage did not cause VSMC apoptosis. Taken together, our data demonstrate a critical role for AKT-mediated induction of Runx2 in oxidative stress-induced VSMC calcification.

Atherosclerosis is characterized by the presence of atherosclerotic lesions in the arterial intima that leads to narrowing of the vessel lumen. Vascular calcification, the presence of calcium deposits in the vessel wall, is a feature of advanced atherosclerosis and reduces elasticity and compliance of the vessel wall (1). Hence, the extent of calcification is a key risk factor in the pathogenesis of the disease. Several cell types, such as endothelium, monocytes, and vascular smooth muscle cells (VSMC), are involved in different stages of lesion development. VSMC contribute to the development of atherosclerotic lesions through increased migration, proliferation, secretion of matrix components, osteogenic differentiation, and the associated calcification (1). During this process, the differentiated VSMC undergo de-differentiation, and subsequently osteogenic transition that results in vascular calcification (2).

Many factors that have been linked to an increased prevalence of vascular calcification are associated with elevated oxidative stress, including hypercholesterolemia, hypertension, diabetes mellitus, and dialysis-dependent end stage renal disease (3–6). Pro-oxidant events in atherosclerosis include the production of reactive oxygen species (ROS) and nitrogen species by vascular cells (7). Of particular interest is hydrogen peroxide (H₂O₂), which is a cell-permeable ROS that has emerged as a key mediator of intracellular signaling (8–10). H₂O₂ is produced in vascular cells by multiple enzymatic systems including vascular NAD(P)H oxidases, mitochondria, xanthine oxidase, and uncoupled endothelial nitric-oxide synthase (11–13). Under normal conditions constitutive oxidative activities and endogenous scavenger systems, including catalase and glutathione peroxidases, maintain steady-state H₂O₂ levels in vascular tissue (10). Upon stimulation, these oxidases in the endothelium, media, and adventitia can produce H₂O₂ and contribute to increased exposure of VSMC to this oxidant (10, 14).

VSMC exhibit an extraordinary capacity to undergo phenotypic change during development in cultures and in association with diseases (15). Emerging evidence supports the concept that vascular calcification, like mineralization of bones and teeth, is a cell-regulated process (16). Osteogenic differentiation of VSMC is characterized by the expression of multiple

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The abbreviations used are: VSMC, vascular smooth muscle cells; ROS, reactive oxygen species; ALP, alkaline phosphatase; Col I, type I collagen; OC, osteocalcin; Runx2, runt-related transcription factor 2; α-SMA, α-smooth muscle actin; SRF, serum response factor; PI3K, phosphatidylinositol 3-kinase; shRNA, short hairpin RNA; Ad-Runx2, adenovirus encoding murine wild-type Runx2; ERK, extracellular-regulated kinase; PLC, phospholipase C; p-, phosphorylated; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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bone-related molecules including alkaline phosphatase (ALP), type I collagen (Col I) and osteocalcin (OC) and the formation of mineralized bone-like structures (17). During osteoblast differentiation these molecules are expressed at different phases and reflect different aspects of osteoblast function and bone formation. ALP and Col I are early markers, and OC is a late marker (18–20). Runx2 is a key transcription factor that regulates osteoblast (21) and chondrocyte differentiation (22). Runx2 has been shown to induce ALP activity and the expression of bone matrix protein genes, including OC, Col I, bone sialoprotein, and osteopontin, as well as mineralization in immature mesenchymal cells and osteoblastic cells in vitro (21, 23). Runx2 expression has been identified in atherosclerotic calcified human vascular tissue specimens (24–26) and in calcifying aortic smooth muscle cells in mice (16) but not in normal vessels. Furthermore, increased expression of Runx2 is associated with VSMC calcification in vitro (16, 27, 28), supporting a role for Runx2 in vascular calcification. However, the potential link between Runx2 regulation and oxidative stress-induced vascular calcification has not been examined.

In the present study we hypothesized that H2O2 regulates VSMC calcification through modulation of the activity and expression of Runx2. Using a cell culture model we found that Runx2 is essential for H2O2-induced VSMC calcification. This oxidative stress-activated Runx2 response is in turn dependent on the activation of AKT. Taken together these studies demonstrate for the first time key steps in the redox cell signaling pathways that lead to VSMC calcification and the essential role of Runx2 in this process.

EXPERIMENTAL PROCEDURES

VSMC Culture—Primary VSMC used in the present studies were isolated from the aortas of C57BL/6 mice (29, 30) and confirmed by flow cytometry with the use of smooth muscle specific α-actin antibody (α-SMA). All experiments were performed with VSMC at passages 3–5.

In Vitro Calcification—Calcification of VSMC was induced by the addition of H2O2 in osteogenic media containing 0.25 mM l-ascorbic acid, 10 mM β-glycerophosphate, and 10^{-8} M dexamethasone (Sigma-Aldrich) for 3 weeks. Alternatively, cells were incubated with glucose oxidase (2 or 5 milliunits/ml) for 12 h with media changes every 2–3 days.

Mineralization was determined by von Kossa staining as previously described (31). Briefly, cells were fixed by 2% paraformaldehyde in phosphate-buffered saline for 15 min and washed with deionized water 3 times. After adding 5% silver nitrate (Sigma-Aldrich) for 12 h with media changes every 2–3 days.

Detection of Runx2 Transactivity—Runx2 transactivity was determined by a Dual-Luciferase Reporter assay with the use of p6xRunxLuc, a luciferase reporter construct containing six Runx binding elements in the promoter region (41). VSMC were seeded in 6-well plates and transiently transfected with 1 μg of p6xRunxLuc using FuGENE 6 (Roche Applied Science). A plasmid encoding Renilla luciferase gene downstream of a minimal SV40 promoter was used to normalize for transfection efficiency (42). 24 h after transfection VSMC were washed and treated with 0.4 mM H2O2 for an additional 24 or 48 h.

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Lentiviral shRNA Transduction for VSMC—Five lentiviral vectors expressing a 21-nucleotide Runx2 short hairpin RNA (shRNA) were purchased from Open Biosystems. The expression of shRNA is driven by a human U6 promoter followed by puromycin driven by human phosphoglycerate kinase promoter. Each vector was packed into lentivirus-like particles pseudotyped with the vesicular stomatitis virus glycoprotein as previously described (43). The position of each of the core 21-nucleotide sequences targeted nucleotides was 4765–4785 (#1), 1664–1684 (#2), 1231–1251 (#3), 1757–1777 (#4), and 131–151 (#5), of the murine Runx2 gene (GenBankTM accession number NM_009820). Blast search was performed using the National Center for Biotechnology Information (NCBI) Expressed Sequence Tags (EST) database base to ensure that the shRNA construct only targeted murine Runx2. Transduction was performed by incubating VSMC with recombinant lentiviral vector in growth media supplemented with 10 μg/ml diethylaminoethyl (DEAE)-dextran. After 24 h cells were washed with phosphate-buffered saline and cultured in growth media containing 5 μg/ml puromycin for 2 weeks to

AKT signaling pathways in oxidative stress-induced VSMC calcification were determined with inhibitors, including PD98059, U73122, LY294002, and AKT Inhibitor IV (Calbiochem).

Total Calcium Measurement—Cells were lysed with 0.5 N HCl by shaking overnight at room temperature. Total calcium in the cell lysates was determined by Arsenazo III (Sigma-Aldrich).

Reverse Transcriptase-Polymerase Chain Reaction and Real-time PCR Analysis—The effect of oxidative stress on the expression of bone-related and smooth muscle-specific gene markers was determined by RT-PCR and real-time PCR. Total RNA was isolated from VSMC using Trizol (Invitrogen) and reverse-transcribed into cDNA. PCR were performed using specific primers for murine ALP (33), Col IA1 (34), OC (35), Runx2 (36), α-SMA (37), SM22α (38), myocardin (39), serine response factor (SRF) (39), and GAPDH as control (40). SYBR Green-based real-time PCR was performed in a 96-well plate format using SYBR Premix Ex Taq (TaKaRa) on an iCycler Thermal Cycler (Bio-Rad).

Western Blot Analysis—VSMC cell extracts were prepared, and protein concentration was measured as described previously (29, 30). Western blot analyses were performed with the use of specific antibodies for extracellular-regulated kinase (ERK), p-ERK, PLCγ, p-PLCγ, AKT, p-AKT (Cell Signaling), and Runx2 (Calbiochem) and detected with a Western blot chemiluminescence detection kit (Millipore).

The roles of mitogen-activated protein kinase kinase, phospholipase C (PLC), and phosphatidylinositol 3-kinase (PI3K)
select stable expression transfectants, which were further cultured in osteogenic media for 3 weeks to induce calcification.

**Adenovirus-mediated Transduction of Runx2**—Adenovirus encoding murine wild-type Runx2 (Ad-Runx2) was kindly provided by Dr. Renny Franceschi (University of Michigan) (44), and transduction of adenovirus into VSMC was performed as we previously described (30). Briefly, VSMC grown in six-well plates were infected with Ad-Runx2 at different multiplicities of infection for 2 h in serum-free media and 16 h in serum-containing media. Subsequently, cells were cultured in osteogenic media for 3 weeks.

**Assessment of Apoptosis**—To examine the effect of oxidative stress or AKT inhibition on VSMC apoptosis, annexin V and propidium iodine staining were performed with the annexin V-FITC apoptosis detection kit (BD Biosciences) as we previously described (45). Apoptosis was reported as the percentage of annexin V-positive and propidium iodine-negative cells.

**Statistical Analysis**—Results are expressed as the means ± S.D. Differences between two groups were identified with Student’s *t* tests. Significance was defined as *p* < 0.05.

**RESULTS**

**Hydrogen Peroxide Induces VSMC Calcification**—First, we examined the effect of H$_2$O$_2$ added directly to the cells or generated at a low flux from the enzyme glucose oxidase (46) on *in vitro* calcification of VSMC. We found that glucose oxidase or H$_2$O$_2$ at the non-toxic concentrations of 0.1–0.4 mM induced VSMC calcification in concentration-dependent manners, as indicated by the black granule formation after von Kossa staining (Fig. 1, A and B) or total calcium levels (Fig. 1C). Taken together, these results establish an *in vitro* oxidative stress-induced differentiation and calcification of VSMC model *in vitro*.

**Oxidative Stress Induces the Expression of Bone Markers and Down-regulates Smooth Muscle Cell Markers**—Accumulating evidence supports the concept that vascular calcification resembles the process of osteogenesis (47, 48). Therefore, we determined the effect of oxidative stress on osteogenic transdifferentiation of VSMC and examined the expression of bone and VSMC markers.

The expression of bone markers ALP, Col IA1, and OC was dramatically increased in H$_2$O$_2$-treated cultures (Fig. 2A). In contrast, the expression of VSMC markers α-SMA and SM22α decreased gradually during the osteogenic differentiation of VSMC (Fig. 2A). Real-time PCR was performed, which confirmed increased expression of ALP, OC, and Col IA1 after VSMC were exposed to 0.4 mM H$_2$O$_2$ for 10 days (fold increase: ALP = 8.7 ± 1.2, OC = 5.3 ± 1.4, and Col IA1 = 6.6 ± 0.9, compared with control, Fig. 2B). On the other hand, the expression of α-SMA was decreased by 79% and SM22α by 83% after VSMC were exposed to 0.4 mM H$_2$O$_2$ for 10 days (Fig. 2C). These results demonstrated that induction of bone markers and loss of smooth muscle cell-specific phenotypes is associated with oxidative stress-induced VSMC calcification.

**Oxidative Stress Increases Runx2 Expression and Transactivity in VSMC**—The transcription factor Runx2 is a key regulator of osteoblast differentiation which regulates the expression of many bone markers (21). Thus, we determined whether H$_2$O$_2$ affects the expression and transactivity of Runx2 during VSMC calcification.

We found that H$_2$O$_2$ increased the expression of the Runx2 transcript (Fig. 3A). This result was further confirmed by quantitative real-time PCR, demonstrating a 4.5-fold increase in Runx2 mRNA after VSMC were exposed to H$_2$O$_2$ for 10 days (Fig. 3B). To test if the H$_2$O$_2$-induced increase in Runx2 message was translated to the protein level, Western blot analysis was performed. Expression of Runx2 protein was increased in VSMC exposed to H$_2$O$_2$ for 2 and 3 weeks (Fig. 3C). To deter-
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To determine whether Runx2 knockdown affects H2O2-induced expression of bone-related molecules and down-regulation of VSMC markers, we compared the expression of those marker genes in control and Runx2 knockdown VSMC. Increased expression of ALP and Col IA1 by H2O2 was totally blocked in Runx2 knockdown VSMC (Fig. 4B). Real-time PCR confirmed the down-regulation of bone-related molecules in Runx2 knockdown VSMC exposed to H2O2 (Fig. 4C). We found that the expression of VSMC transcription factors myocardin and SRF was decreased after VSMC were exposed to H2O2 for 2 weeks (Fig. 4B). The inhibitory effect of H2O2 on the expression of transcription factors was not restored in Runx2 knockdown cells, which was confirmed by real-time PCR analysis (Fig. 4D). Furthermore, H2O2-inhibited expression of SMC marker genes was not totally rescued by Runx2 knockdown, suggesting that additional signaling pathways independent of Runx2 may regulate H2O2-inhibited expression VSMC markers.

Overexpression of Runx2 Increases VSMC Calcification—To verify if Runx2 alone is sufficient to induce VSMC calcification, we overexpressed Runx2 in VSMC by adenovirus carrying the Runx2 gene (A4). Western blot analysis revealed that control VSMC expressed barely detectable levels of Runx2 protein. In contrast, Ad-Runx2-infected cells showed a concentration-dependent increase in Runx2 levels (Fig. 5A). VSMC calcification was enhanced by Ad-Runx2 with maximal induction of mineralization observed at a 200 multiplicity of infection (Fig. 5B), whereas VSMC calcification was not affected in VSMC transduced with control virus (Ad-GFP). These results demonstrate that Runx2 is an essential and sufficient player in regulating oxidative stress-induced VSMC calcification.

PI3K/AKT Signaling Mediates Oxidative Stress-induced Runx2 Activation and VSMC Calcification—H2O2 has been implicated in the activation of many downstream signaling pathways, including PI3K, ERK, and AKT, in a variety of cells (8, 9). Thus, we determined whether activation of these signaling pathways by H2O2 was required for VSMC calcification. We found that H2O2 stimulated rapid activation of ERK, AKT, and PLCγ, an important mediator for intracellular calcium signaling, with maximal induction at 15 min (Fig. 6A). Inhibition of MAPK kinase by PD98059 (0.1–100 μM) or PLC by U73122 (0.1–10 μM) did not affect H2O2-induced VSMC calcification (data not shown). However, inhibition of AKT signaling by LY294002 and AKT inhibitor IV potently prevented oxidative stress-induced VSMC calcification (Fig. 6B). The effects of these inhibitors on AKT activation was confirmed by Western blot analysis, demonstrating inhibition of p-AKT in VSMC exposed to these inhibitors (Fig. 6B). Furthermore, we found one that knocked down Runx2 protein most effectively was selected for further studies. As expected, VSMC infected with a control virus (lenti-GFP) underwent calcification when treated with H2O2 (Fig. 4A). By contrast, H2O2-induced calcification was inhibited when Runx2 was knocked down (Fig. 4A). We confirmed that the expression of Runx2 was inhibited by shRNA (Fig. 4B), and inhibition of calcification was correlated with reduced levels of Runx2 (Fig. 4, A and B). Thus, our data support an essential direct role of Runx2 in oxidative stress-induced VSMC calcification.

mine whether H2O2-induced Runx2 protein was transcriptionally active, a luciferase reporter assay was performed. A multimerized Runx2 promoter reporter was transiently transfected into VSMC, which was subsequently stimulated with H2O2 (Fig. 3D). A 2-fold increase in Runx2 transactivity was observed at 24 and 48 h after VSMC was exposed to H2O2. These results suggest that H2O2-increased Runx2 expression and transactivity may contribute to oxidative stress-induced VSMC calcification.

Down-regulation of Runx2 Inhibits VSMC Calcification—For a mechanistic understanding of H2O2-induced Runx2 expression and VSMC calcification, we employed shRNA-containing lentiviral vectors to knock down the expression of Runx2. Five lentiviral vectors from Open Biosystems were tested, and the
that increased expression of Runx2, ALP, and Col IA1 under oxidative stress was blocked by AKT inhibition, whereas H$_2$O$_2$-induced down-regulation of α-SMA was not restored by AKT inhibition (Fig. 6C). Inhibition of oxidative stress-induced Runx2 transactivity was also demonstrated with AKT inhibitor IV (Fig. 6D). In addition, the effect of oxidative stress or AKT inhibition on VSMC calcification was not due to increased apoptosis (Fig. 6E). In summary, our data demonstrate that Runx2 is an integral component of oxidative stress-induced calcification of VSMC, and AKT signaling plays an important role in mediating oxidative stress-induced Runx2 up-regulation and VSMC calcification.

**DISCUSSION**

Increased vascular formation of H$_2$O$_2$, a highly diffusible ROS capable of initiating redox cell signaling, is known to play an important role in the pathogenesis of atherosclerosis (14). In the present study we demonstrated that H$_2$O$_2$, at concentrations of 0.1 to 0.4 mM, induces osteogenic differentiation and calcification of VSMC in a concentration-dependent manner. These levels of H$_2$O$_2$ are consistent with the amounts generated by the increased levels of oxidant enzymes associated with atherosclerosis including xanthine oxidase and NAD(P)H oxidases (14). Furthermore, previous reports have shown that the viability of VSMC is not affected by H$_2$O$_2$ concentrations up to 800 μM (49). Here we confirmed that the effect of H$_2$O$_2$ on VSMC calcification is not due to VSMC apoptosis (Fig. 6E), although VSMC apoptosis has been implicated in vascular calcification in atherosclerotic lesions (50) and in the initiation and propagation of the calcification of calcifying vascular cells induced by BMP2 (1).

Our observation that oxidative stress-induced VSMC calcification is consistent with the previous reports that H$_2$O$_2$ induces osteogenic differentiation of calcifying vascular cells (51, 52) and odonto-

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**FIGURE 3.** Oxidative stress increases the expression and transactivity of Runx2 in VSMC. A, expression of Runx2 mRNA in VSMC exposed to H$_2$O$_2$ at 0.3 and 0.4 mM in osteogenic media was determined by RT-PCR. B, real-time PCR was performed in VSMC exposed to 0.4 mM H$_2$O$_2$ in osteogenic media for 10 days. Results from three independent experiments performed in duplicate are shown (*, $p < 0.001$ compared with control). C, expression of Runx2 protein in VSMC exposed to H$_2$O$_2$ at 0.3 and 0.4 mM in osteogenic media was determined by Western blot analysis. D, Runx2 transactivity in VSMC under oxidative stress was determined with a luciferase reporter. VSMC were transiently co-transfected with p6xRunxLuc and a plasmid expressing Renilla luciferase as an internal control reporter. Transfected cells were exposed to 0.4 mM H$_2$O$_2$ for 24 or 48 h, and the reporter luciferase activities were determined and normalized to the Renilla luciferase activity using Dual-Luciferase Reporter assay system. Results from three independent experiments performed in duplicate are shown (*, $p < 0.005$ for 48 h compared with control).

**FIGURE 4.** Down-regulation of Runx2 inhibits VSMC calcification but shows no effect on oxidative stress-inhibited smooth muscle markers. A, VSMC were infected with vesicular stomatitis virus glycoprotein pseudotyped lentiviral vector encoding GFP or shRNA against Runx2 (shRunx2) and selected in puromycin for 2 weeks. Subsequently, cells were exposed to 0.4 mM H$_2$O$_2$ in osteogenic media for 3 weeks. In vitro calcification was determined by von Kossa staining. Representative images of two independent experiments are shown: stained dishes (upper) and microscopic views ($× 40$, lower). B, knockdown of Runx2 protein expression in shRunx2-infected VSMC exposed to 0.4 mM H$_2$O$_2$ in osteogenic media for 3 weeks was determined by Western blot analysis (upper two rows). Expression of bone-related molecules ALP and Col IA1 and VSMC markers α-SMA, SM22α, myocardin, and SRF in VSMC infected with shRunx2 exposed to 0.4 mM H$_2$O$_2$ in osteogenic media for 2 weeks was determined by RT-PCR. Representative RT-PCR pictures of two independent experiments are shown. Real-time PCR for bone-related molecules (C) and VSMC markers (D) was performed in shRunx2-infected VSMC exposed to 0.4 mM H$_2$O$_2$ in osteogenic media for 2 weeks.
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(A) Ad-Runx2 (MOI) 0 10 100 200 (MOI)
Runx2
GAPDH

(B) Ad-GFP (MOI) Ad-Runx2 (MOI) 100 0 10 100 200

FIGURE 5. Overexpression of Runx2 increases VSMC calcification. A, VSMC were infected with Ad-Runx2, and the expression of Runx2 protein in Ad-Runx2-transduced cells cultured in osteogenic media for 1 week was determined by Western blot analysis. Representative blot of two independent experiments is shown. MOI, multiplicity of infection. B, VSMC were transduced with Ad-Runx2 or control virus (Ad-GFP) and cultured in osteogenic media for 3 weeks. In vitro calcification was determined by von Kossa staining. Representative images of two independent experiments are shown: stained dishes (upper) and microscopic views (×40, lower).

blasts (53). A recent study demonstrated that increased ROS generation, particularly H$_2$O$_2$, is predominately located around calcifying foci, which potentiates aortic valve calcification progression (54). By contrast, H$_2$O$_2$ has been shown to have adverse effects on osteogenic differentiation of MC3T3-E1 cells, a pre-osteoblastic cell line (53), and primary bone marrow stromal cells and calvarial osteoblast precursors (55). Therefore, the effects of H$_2$O$_2$ on osteogenic differentiation appear to be cell type-dependent. Interestingly, clinical studies have found that increased arterial calcification correlates with an increase in osteoporosis (56), a disease with increased bone loss. It is not known whether the reciprocal effects of oxidative stress on VSMC calcification and osteoblast differentiation are involved in the development of vascular calcification and osteoporosis simultaneously. Thus, further investigations are warranted to elucidate the redox-dependent distinct signaling cascades that regulate VSMC calcification and osteoblast differentiation.

In atherosclerosis, VSMC have the ability to undergo reversible differentiation into cells with features of other mesenchymal lineages, such as osteoblasts, chondrocytes, and adipocytes (15, 57, 58). We found that H$_2$O$_2$-induced VSMC calcification was associated with down-regulation of VSMC-specific markers and induction of bone markers, including ALP, OC, and Col I (Fig. 2), indicating a phenotypic change of VSMC into osteoblast-like cells.

Increased expression of bone markers has been documented in osteogenic differentiation of VSMC induced by acetylated low density lipoprotein (59) and in calcification of calcifying vascular cells induced by tumor necrosis factor-α (60). Simi-
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The amount of H$_2$O$_2$ was not measured, and the direct effect of H$_2$O$_2$ on the expression of VSMC markers was not determined. In our study we determined the direct effect of H$_2$O$_2$ on the expression of VSMC markers with well differentiated VSMC, which express high levels of VSMC markers.

The mechanism by which H$_2$O$_2$ induces down-regulation of VSMC markers is not fully understood. We found that expression of myocardin and SRF, the key regulators for VSMC markers, was inhibited during H$_2$O$_2$-induced VSMC calcification, which may contribute to the down-regulation of VSMC markers (Fig. 4C). A recent study found that overexpression of Runx2 in C3H10T1/2 mesenchymal cells repressed myocardin-induced SMC gene expression, whereas overexpression of myocardin induced a greater level of SMC marker gene expression in the Runx2 knockdown cells (65). Furthermore, a direct interaction of Runx2 with SRF that disrupts the formation of myocardin and SRF complex was shown to mediate the effect of Runx2 on SMC marker gene expression (65). In our system, H$_2$O$_2$-inhibited expression of VSMC markers was not restored by Runx2 knockdown, indicating that additional signaling pathways independent of Runx2 are involved. Apparently, inhibition of the expression of the VSMC transcription factors, myocardin and SRF, contributes to the down-regulation of VSMC markers by H$_2$O$_2$.

We further determined the molecular signals involved in oxidative stress-induced VSMC calcification. H$_2$O$_2$ can induce downstream signaling by both autocrine and paracrine mechanisms. Exogenous...
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Akt1 and Akt2, supporting an important role of AKT signaling in the differentiation of skeletal cells (71). By contrast, activation of PI3K signaling by insulin-like growth factor-I appears to inhibit differentiation and mineralization of calcifying vascular cells (72), indicating that PI3K signaling activated by different stimuli may have different effects on osteogenic differentiation in different cell types. In addition, we have found that H2O2-induced activation of PI3K/AKT signaling regulated Runx2 expression and transactivity as well as the expression of bone markers during VSMC calcification. These results are consistent with observations that PI3K/AKT signaling increases DNA binding of Runx2 and transcriptional activation by Runx2 during osteoblast and chondrocyte differentiation (70).

Taken together, our results reveal a critical role of the PI3K/AKT/Runx2 signaling axis in regulating H2O2-induced VSMC calcification. We found the expression of Runx2 is essential during oxidative stress-induced osteogenic differentiation and calcification of VSMC. Furthermore, enhanced expression of Runx2 is sufficient to induce VSMC calcification. Activation of AKT signaling appears to mediate oxidative stress-induced Runx2 expression and activity during VSMC calcification. Our findings provide important molecular insights into the role of Runx2 and PI3K/AKT signaling in the regulation of vascular calcification and may have important clinical implications in designing successful therapy to prevent and treat diseases associated with vascular calcification.

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9. H2O2-induced activation of PI3K/AKT signaling regulated Runx2 expression and transactivity as well as the expression of bone markers during VSMC calcification. These results are consistent with observations that PI3K/AKT signaling increases DNA binding of Runx2 and transcriptional activation by Runx2 during osteoblast and chondrocyte differentiation.

Taken together, our results reveal a critical role of the PI3K/AKT/Runx2 signaling axis in regulating H2O2-induced VSMC calcification. We found the expression of Runx2 is essential during oxidative stress-induced osteogenic differentiation and calcification of VSMC. Furthermore, enhanced expression of Runx2 is sufficient to induce VSMC calcification. Activation of AKT signaling appears to mediate oxidative stress-induced Runx2 expression and activity during VSMC calcification. Our findings provide important molecular insights into the role of Runx2 and PI3K/AKT signaling in the regulation of vascular calcification and may have important clinical implications in designing successful therapy to prevent and treat diseases associated with vascular calcification.
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