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Osteogenic Oxysterols Inhibit the Adverse Effects of Oxidative Stress on Osteogenic Differentiation of Marrow Stromal Cells

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Abstract The osteoporosis that occurs with aging is associated with reduced number and activity of osteoblastic cells. Aging, menopause, and osteoporosis are correlated with increased oxidative stress and reduced antioxidant defense mechanisms. We previously demonstrated that oxidative stress induced by a variety of compounds such as xanthine/xanthine oxidase (XXO) and minimally oxidized LDL (MM-LDL) inhibit the osteogenic differentiation of osteoprogenitor cells. Oxysterols are a family of products derived from cholesterol oxidation that have important biological activities. Recently, we reported that a specific oxysterol combination consisting of 22(S)- or 22(R)-hydroxycholesterol and 20(S)-hydroxycholesterol has potent osteogenic properties in vitro when applied to osteoprogenitor cells including M2-10B4 (M2) marrow stromal cells. We now demonstrate that this osteogenic combination of oxysterols prevents the adverse effects of oxidative stress on differentiation of M2 cells into mature osteoblastic cells. XXO and MM-LDL inhibited the osteogenic differentiation of M2 cells, demonstrated by the inhibition of markers of osteogenic differentiation: alkaline phosphatase activity, osteocalcin expression and mineralization. Treatment of M2 cells with osteogenic oxysterol combination 22(S)- and 20(S)-hydroxycholesterol both blocked and reversed the inhibition of osteogenic differentiation produced by XXO and MM-LDL in these cells. The protective effect of the oxysterols against oxidative stress was dependent on cyclooxygenase 1 and was associated with the osteogenic property of the oxysterols. These findings further demonstrate the ability of the osteogenic oxysterols to positively regulate osteogenic differentiation of cells, and suggests that the use of these compounds may be a novel strategy to prevent the adverse effects of oxidative stress on osteogenesis. J. Cell. Biochem. 95: 1276–1283, 2005.

Key words: oxysterols; marrow stromal cells; oxidative stress; osteogenic differentiation

Age-related osteoporotic bone loss remains one of the significant causes of morbidity and mortality in the aging population, resulting in increased fracture incidence at the hip, spine, and other sites [Cummings and Melton, 2002; Ettinger, 2003]. The bone loss in age-related osteoporosis is associated with a marked decrease in osteoblast number and bone forming activity [Quarto et al., 1995; Mullender et al., 1996; Chan and Duque, 2002; Chen et al., 2002; Ichioka et al., 2002]. This decrease in osteoblastic bone formation in parallel with an increase in osteoclastic bone resorption constitute the cellular mechanisms underlying osteoporosis. It is evident that strategies for increasing bone formation by osteoblasts would improve skeletal health and prevent osteoporotic bone loss [Mundy, 2002; Rodan and Martin, 2002].

Although the reason(s) for the decrease in osteoblastic activity and bone formation with age and after menopause is not clearly understood, increased oxidative stress on bone cells may in part explain the reason for this decrease in osteogenic activity. Both aging and menopause are associated with increased oxidative stress and decreased antioxidant defense

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mechanisms [Chang et al., 2002; Sohal et al., 2002]. Increased levels of urinary isoprostane, 8-iso-$PGF_{2\alpha}$ (a biomarker of oxidative stress), is negatively associated with bone mineral density in humans [Basu et al., 2001]. Furthermore, a marked decrease in plasma antioxidants including vitamins C and E, superoxide dismutase, and glutathione peroxidase was reported in aged osteoporotic women compared to controls [Maggio et al., 2003]. In addition, some epidemiological studies have demonstrated the protective effects of increased dietary antioxidants on bone health [Melhus et al., 1999; Schaafsma et al., 2001].

Oxidative stress may negatively impact bone homeostasis by stimulating osteoclastogenesis and bone resorption [Garrett et al., 1990], and by inhibiting osteoblastic differentiation of osteoprogenitor cells [Mody et al., 2001]. We previously demonstrated that oxidative stress induced by xanthine/xanthine oxidase (XXO) or by minimally oxidized LDL (MM-LDL) inhibits osteoblastic differentiation and mineralization in cultures of M2-10B4 (M2) pluripotent marrow stromal cells that can differentiate into osteoblastic cells, and in cultures of MC3T3-E1 calvarial preosteoblasts [Mody et al., 2001]. The adverse effects of oxidative stress on osteoblastic differentiation were inhibited by the antioxidants Trolox and pyrrolidinedithiocarbamate [Mody et al., 2001].

Oxysterols form a large family of oxygenated derivatives of cholesterol that are present in the circulation and in tissues of humans and animals [Edwards and Ericsson, 1999; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002]. They may be formed either by autooxidation, as a secondary byproduct of lipid peroxidation, or by the action of specific monooxygenases, most of which are members of the cytochrome P450 family of enzymes [Russell, 2000]. Oxysterols may also be derived from the diet [Lyons et al., 1999]. A role for specific oxysterols has been implicated in physiologic and pathologic processes including cellular differentiation, inflammation, apoptosis, steroid production, and atherogenesis [Edwards and Ericsson, 1999; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002]. Recently we reported that specific oxysterols, namely a specific combination of 22(R)- or 22(S)- and 20(S)-hydroxycholesterol, have very potent osteogenic activity [Kha et al., 2004]. These oxysterol combinations induce the osteoblastic differentiation of a variety of mesenchymal osteoprogenitor cells including the M2 marrow stromal cells, MC3T3-E1 calvarial cells, C3H10T1/2 embryonic fibroblastic cells, and primary mouse bone marrow stromal cells [Kha et al., 2004]. The osteogenic effects of the oxysterols were mediated via COX/PLA_2- and MAPK-dependent mechanisms [Kha et al., 2004]. In the present study, we examined the ability of these osteogenic oxysterols to inhibit the adverse effects of oxidative stress on osteoblastic differentiation of M2 cells, as well as their ability to protect these cells and rescue them from the effects of oxidative stress induced by the XXO reaction or by MM-LDL.

MATERIALS AND METHODS

Materials

Oxysterols, beta-glycerophosphate, ascorbate, xanthine, and xanthine oxidase were obtained from Sigma (St. Louis, MO), RPMI 1640 from Irvine Scientific (Santa Ana, CA), fetal bovine serum (FBS) from Hyclone (Logan, UT), and SC-560 from Cayman Chemical (Ann Arbor, MI).

Cell Culture

M2-10B4 mouse marrow stromal cell line obtained from American Type Culture Collection (ATCC, Rockville, MD) was derived from bone marrow stromal cells of a (C57BL/6J $\times$ C3H/HeJ) F1 mouse, and supports human and murine myelopoieses in long-term cultures (as per ATCC). These cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, and supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 U/ml streptomycin (all from Irvine Scientific). The osteogenic medium for these studies consisted of RPMI 1640 with all supplements described above to which 5% FBS, 25 μg/ml ascorbate, and 3 mM beta-glycerophosphate were also added.

Primary mouse bone marrow stromal cells were isolated from male 4- to 6-month-old C57BL/6J mice and cultured and propagated as previously described [Kha et al., 2004].

Lipoprotein Preparation and Oxidation

Human LDL was isolated by density-gradient centrifugation of serum and stored in phosphate-buffered 0.15M NaCl containing 0.01% EDTA [Havel et al., 1955]. Minimally oxidized LDL was prepared by iron oxidation of human LDL as previously described [Parhami et al.,

1999]. The concentrations of lipoproteins used in this study are reported in micrograms of protein. The lipoproteins were tested pre- and post-oxidation for lipopolysaccharide levels and found to have <30 pg of lipopolysaccharide/ml of medium.

Alkaline Phosphatase Activity Assay

Colorimetric alkaline phosphatase activity assay on whole cell extracts was performed as previously described [Kha et al., 2004].

^{45}Ca Incorporation Assay

Matrix mineralization in cell monolayers was quantified using the ^{45}Ca incorporation assay as previously described [Kha et al., 2004].

RNA Isolation and Northern Blot Analysis

Total RNA was isolated using the RNA isolation kit from Stratagene (La Jolla, CA). Northern blotting and analysis of osteocalcin mRNA and 18S rRNA expression were performed as previously described [Kha et al., 2004].

Statistical Analyses

Computer-assisted statistical analyses were performed using the StatView 4.5 program. All P values were calculated using ANOVA and Fisher's projected least significant difference (PLSD) significance test. A value of $P < 0.05$ was considered significant.

RESULTS

Inhibition of XXO and MM-LDL Effects by Osteogenic Oxysterols

Osteoblastic differentiation of progenitor cells is marked by increased expression of markers including alkaline phosphatase activity, osteocalcin mRNA expression, and mineralization [Rickard et al., 1994; Hicok et al., 1998]. In order to assess the effect of osteogenic oxysterols on inhibition of osteoblastic differentiation by XXO and MM-LDL, the above differentiation markers were examined in cultures of M2 cells treated with XXO or MM-LDL alone, or in combination with osteogenic oxysterols 22S + 20S (SS). Alkaline phosphatase activity was inhibited in M2 cells treated for 6 days with XXO or MM-LDL (Figs. 1A and 2A). Co-treatment with SS at concentrations of 0.1–5 μM inhibited the effects XXO and MM-LDL in a dose-dependent manner (Figs. 1A and 2A).

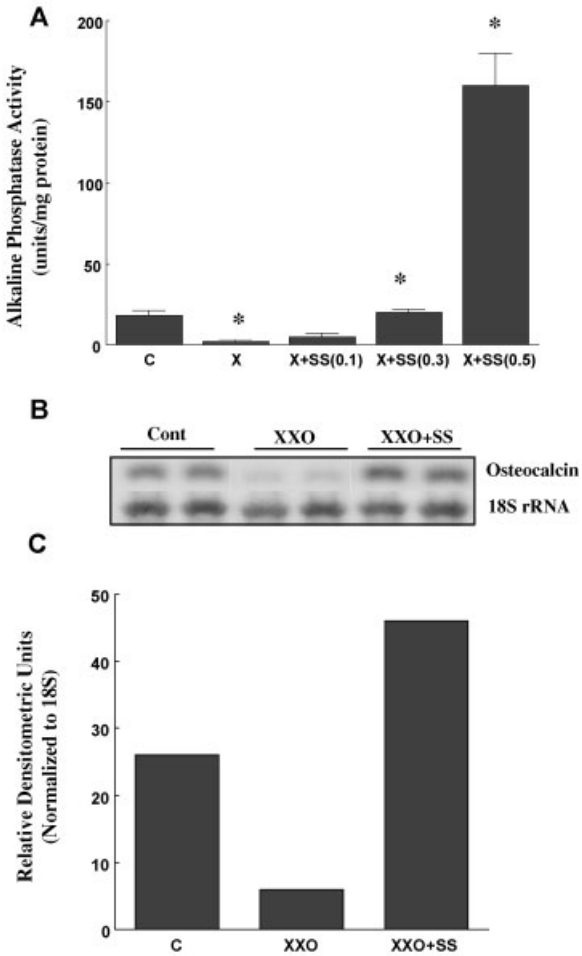

Fig. 1. Effect of oxysterols on xanthine/xanthine oxidase (XXO) inhibition of osteoblast marker expression in marrow stromal cells. **A**: M2 cells at confluence were treated for 6 days in osteogenic medium with control vehicle (C), xanthine/xanthine oxidase (X; 250 μM/40 mU/ml) or the oxysterol combination 22S + 20S (SS; μM), alone or in combination. After 6 days, alkaline phosphatase activity in whole cell extracts was measured. Results from a representative of three separate experiments are reported as the mean ± SD of quadruplicate determinations, normalized to protein concentrations (*$P < 0.01$ for C vs. X, and for X vs. X+SS at 0.3 and 0.5 μM SS). **B**, **C**: M2 cells at confluence were treated for 8 days in osteogenic medium with control vehicle (Cont), xanthine/xanthine oxidase (XXO; 250 μM/40 mU/ml, or SS (5 μM), alone or in combination. After 8 days, total RNA from duplicate samples was isolated and analyzed for osteocalcin or 18S rRNA expression by Northern blotting. Data from densitometric analysis of the Northern blot are shown in C as the average of duplicate samples, normalized to 18S rRNA.

Inhibition of alkaline phosphatase activity by XXO was blocked significantly by as little as 0.3 μM SS, whereas significant inhibition of MM-LDL effect was achieved with 2.5 μM SS. As expected when M2 cells were cultured in an osteogenic medium, osteocalcin mRNA

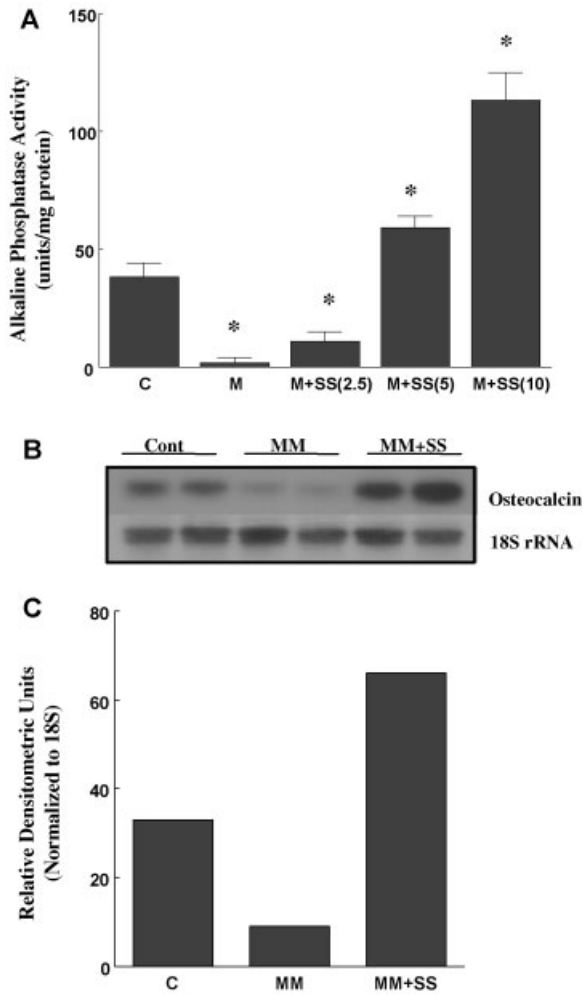

Fig. 2. Effect of oxysterols on minimally oxidized LDL inhibition of osteoblast marker expression in marrow stromal cells. **A**: M2 cells at confluence were treated for 6 days in osteogenic medium with control vehicle (C), minimally oxidized LDL (M; 200 μg/ml) or the oxysterol combination 22S + 20S (SS; μM), alone or in combination. After 6 days, alkaline phosphatase activity in whole cell extracts was measured. Results from a representative of three separate experiments are reported as the mean ± SD of quadruplicate determinations, normalized to protein concentrations (*$P < 0.01$ for C vs. M, and for M vs. M+SS at all SS concentrations). **B**, **C**: M2 cells at confluence were treated for 8 days in osteogenic medium with control vehicle (Cont), minimally oxidized LDL (MM; 200 μg/ml), or SS (5 μM), alone or in combination. After 8 days, total RNA from duplicate samples was isolated and analyzed for osteocalcin or 18S rRNA expression by Northern blotting. Data from densitometric analysis of the Northern blot are shown in C as the average of duplicate samples, normalized to 18S rRNA.

expression increased with time during osteoblastic differentiation of M2 cells (data not shown) [Kha et al., 2004]. XXO and MM-LDL inhibited osteocalcin mRNA expression after 8 days, and this inhibition was completely alleviated in the presence of SS (Figs. 1B and 2B). Furthermore, the inhibitory effect of XXO and MM-LDL on mineralization in cultures of M2 cells was also alleviated in the presence of SS (Fig. 3A). The protective effects of SS against the adverse effects of XXO and MM-LDL were confirmed in cultures of primary mouse bone marrow stromal cells (Fig. 3B). Altogether, these results demonstrate that osteogenic oxysterols inhibit the adverse effects of two factors, XXO and MM-LDL, which cause oxidative stress in M2 cells and inhibit their osteogenic differentiation.

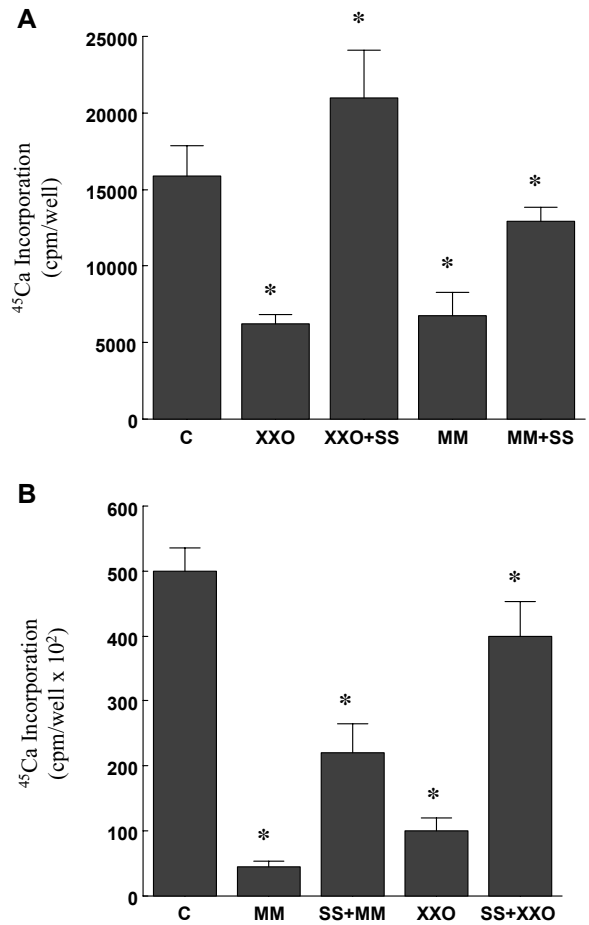

Fig. 3. Effect of oxysterols on inhibition of mineralization in marrow stromal cells. **A**: M2 cells or (**B**) primary mouse MSC were plated at 20,000 cells per cm^2, 4-wells per condition, and treated at confluence for 14 days in osteogenic medium with control vehicle (C), xanthine/xanthine oxidase (XXO; 250 μM/40 mU/ml), minimally oxidized LDL (MM; 100 μg/ml), or SS (5 μM), alone or in combination. After 14 days, matrix mineralization in cultures was quantified using a ^{45}Ca incorporation assay. Results from a representative of three separate experiments are shown, reported as the mean ± SD of quadruplicate determinations (*$P < 0.01$ for C vs. XXO and MM, and for XXO vs. XXO + SS and MM vs. MM + SS).

Osteogenic Oxysterols Protect Against the Effects of XXO and MM-LDL

In order to examine whether in addition to blocking the inhibitory effects of XXO and MM-LDL on the expression of osteogenic differentiation markers in M2 cells, pretreatment of M2 cells with osteogenic oxysterols can protect these cells from oxidative stress, M2 cells were pretreated for 48 h with 2.5 μM SS. After 48 h, SS was removed and XXO or MM-LDL was added to cells that were pretreated with SS or control vehicle. Alkaline phosphatase activity was measured after 6 days. Interestingly, in contrast to cells pretreated with control vehicle, in which alkaline phosphatase activity was inhibited by oxidative stresses, cells pretreated with the oxysterols were completely protected from the inhibitory effects of both XXO and MM-LDL (Fig. 4A). Similarly, protective effects of SS were found on mineralization (Fig. 4B). The protective effects of the osteogenic oxysterols were dependent on COX-1 since cells pretreated with SS and COX-1 inhibitor SC-560 were no longer protected against the adverse effects of XXO and MM-LDL (Fig. 5). Oxysterols and oxidized lipids that do not possess osteogenic effects, including 7-ketocholesterol (an oxysterol) and 4-hydroxynonenal (a product of lipid peroxidation) [Kha et al., 2004], when used at concentrations between 1–10 μM did not protect the M2 cells from the adverse effects of XXO and MM-LDL (data not shown).

Osteogenic Oxysterols Rescue Cells From the Effects of XXO and MM-LDL

Finally, we examined the ability of osteogenic oxysterols to rescue the cells from the inhibitory effects of oxidative stress. M2 cells were pretreated with MM-LDL or XXO for 2 days, followed by their removal and addition of SS or control vehicle for an additional 4 or 12 days, after which alkaline phosphatase activity and mineralization, respectively, were measured. Results showed that alkaline phosphatase activity (Fig. 6A) and mineralization (Fig. 6B) were inhibited in cells treated for 2 days with MM-LDL or XXO, and that the addition of SS rescued the cells from the adverse effects of MM-LDL and XXO.

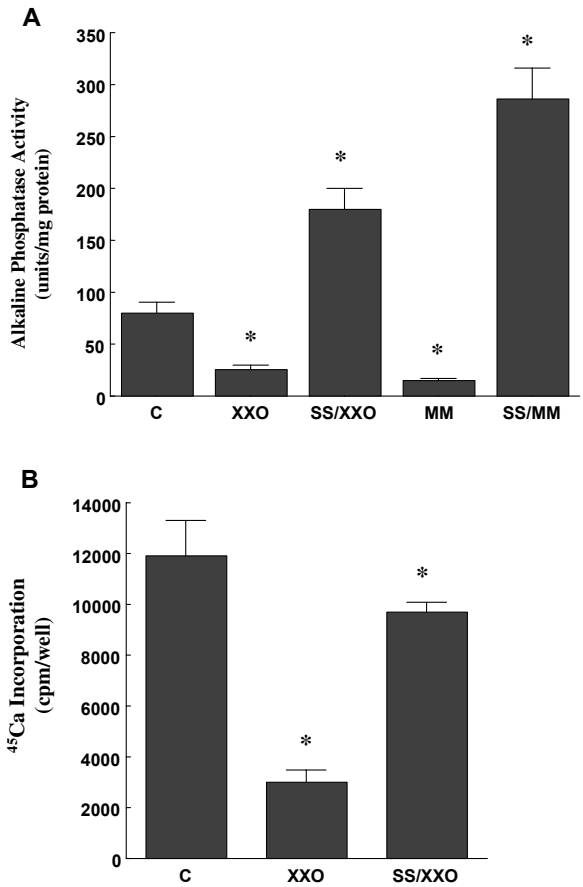

Fig. 4. Protection of marrow stromal cells by oxysterols against the inhibitory effects of xanthine/xanthine oxidase and minimally oxidized LDL on osteoblast marker expression. M2 cells at confluence were pretreated with control vehicle (C) or the oxysterol combination 22S + 20S (SS; 2.5 μM) for 48 h. Next SS was removed, cells rinsed, and xanthine/xanthine oxidase (XXO; 250 μM/40 mU/ml) or minimally oxidized LDL (MM; 200 μg/ml) was added in osteogenic medium. **A**: Alkaline phosphatase activity and (**B**) mineralization were measured after 6 and 14 days, respectively, as previously described. Results from a representative of three separate experiments are reported as the mean ± SD of quadruplicate determinations. (*$P < 0.01$ for C vs. XXO and MM and for XXO vs. SS/XXO and for MM vs. SS/MM in A, and for C vs. XXO and XXO vs. SS/XXO in B).

DISCUSSION

The present study demonstrates the ability of osteogenic oxysterols to block the adverse effects of XXO and MM-LDL, which inhibit osteogenic differentiation of marrow stromal cells through induction of oxidative stress in these cells [Mody et al., 2001]. Furthermore, our data showed that the osteogenic oxysterols are capable of protecting these cells from the adverse effects of the oxidative stress if the cells are pretreated with the oxysterols. Interestingly, although oxysterols were able to protect against the adverse effects of both XXO and MM-LDL on alkaline phosphatase activity, they were not able to protect against the effects of

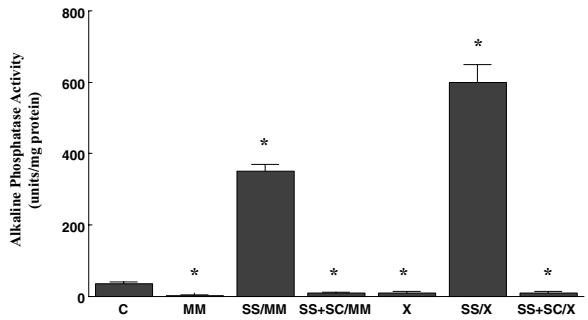

Fig. 5. Effect of cyclooxygenase 1 inhibitor on protection of marrow stromal cells by oxysterols. M2 cells at confluence were pretreated with control vehicle (C) or cyclooxygenase 1 inhibitor, SC-560 (SC; 20 μM) for 2 h. Next, the oxysterol combination 22S + 20S (SS; 2.5 μM) was added. After 48 h of treatment, SS and SC were removed, the cells rinsed and minimally oxidized LDL (MM; 200 μg/ml) or xanthine/xanthine oxidase (X; 250 μM/40 mU/ml) was added. After 6 days of treatment with MM or X, alkaline phosphatase activity in cell extracts was measured. Results from a representative of three separate experiments are reported as the mean ± SD of quadruplicate determinations (*$P < 0.01$ for C vs. MM and X, for MM vs. SS/MM and X vs. SS/X, and for SS/MM vs. SS + SC/MM and SS/X vs. SS + SC/X).

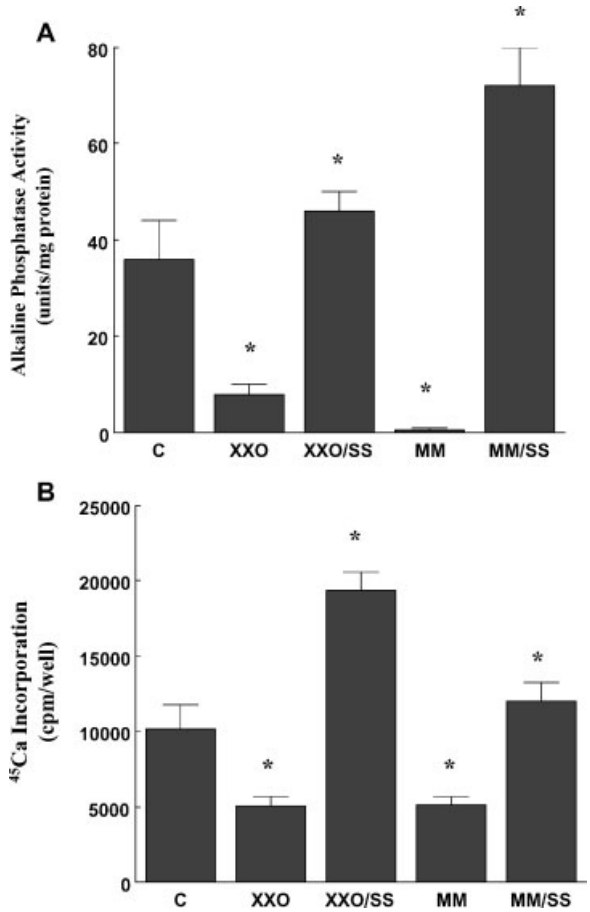

Fig. 6. Oxysterols rescue marrow cells from the inhibitory effects of xanthine/xanthine oxidase and minimally oxidized LDL on osteoblast marker expression. M2 cells at confluence were pretreated for 2 days with control vehicle (C), xanthine/xanthine oxidase (XXO; 250 μM/40 mU/ml) or minimally oxidized LDL (MM; 200 μg/ml) in osteogenic medium. Next, XXO and MM were removed and vehicle or the combination of 22S + 20S oxysterols (SS; 2.5 μM) was added. Alkaline phosphatase activity (**A**) and mineralization (**B**) were measured after 4 and 12 days of treatment with SS, respectively. Results from a representative of three separate experiments are reported as the mean ± SD of quadrupliate determinations (*$P < 0.01$ for C vs. XXO and MM, and for XXO vs. XXO/SS and for MM vs. MM/SS in A and B).

MM-LDL on mineralization unless SS was present through out the duration of treatment with MM-LDL. We speculate that this finding may be due to the greater inhibitory effects of MM-LDL compared to XXO on factors that are important for mineralization to occur. In addition, a much larger dose of oxysterols was required in order to protect against the inhibitory effects of MM-LDL on alkaline phosphatase activity in the M2 cells than the dose necessary for inhibition of XXO effects. We speculate that the underlying reason(s) for these observations may include: (1) the greater inhibitory effects of MM-LDL on osteogenic differentiation of cells as a result of multiple inhibitory signals triggered by MM-LDL compared to XXO; and (2) an inhibitory effect of MM-LDL, but not XXO, on the osteogenic effects of oxysterols. In support of the former reason, MM-LDL [Parhami et al., 1999], but not XXO (Parhami et al., unpublished observations), enhanced the adipogenic differentiation of cells in parallel to inhibiting their osteogenic differentiation. Since adipogenic differentiation is thought to occur at the expense of osteogenic differentiation in mesenchymal cells [Nuttall and Gimble, 2000], this observation suggests that additional molecular signals may be regulated by MM-LDL, which may have inhibitory effects on osteogenic differentiation and require greater doses of oxysterols to reverse. In support of the latter reason, we have observed that MM-LDL, but not XXO, inhibits the osteogenic effects of osteoinductive factors including oxysterols and BMP2 on pluripotent mesenchymal cells (Parhami et al., unpublished observations). The molecular mechanism(s) for this inhibitory effect of MM-LDL is currently under investigation.

Finally, the osteogenic oxysterols rescued and reversed the adverse effects of oxidative stress and restored the ability of marrow stromal cells to undergo osteogenic differentiation. There-

fore, the present studies demonstrate for the first time that osteogenic oxysterols may be of potential use in alleviating the adverse effects of oxidative stress on the differentiation of pluripotent mesenchymal cells into osteogenic cells. Furthermore, we speculate that oxysterols may have protective effects against any oxidative stress-mediated adverse effects on the lineage-specific differentiation of mesenchymal cells into other cell types including chondrocytes, myocytes, and fibroblasts, all of which are involved in the formation and maintenance of healthy tissues in adult organisms.

Mesenchymal cell differentiation into osteoblasts is regulated by COX activity [Chikazu et al., 2002; Simon et al., 2002; Zhang et al., 2002], and COX enzymes appear to be involved in bone homeostasis and repair [Simon et al., 2002]. We previously reported that both COX-1 and COX-2 enzymes are present in M2 cells, and that inhibition of COX-1, but not COX-2, inhibited the osteogenic activity of the oxysterols [Kha et al., 2004]. Similarly, the ability of oxysterols to protect cells from the effects of oxidative stress was mediated at least in part by a COX-1 dependent mechanism as demonstrated by the removal of the protection in presence of COX-1 inhibitor, SC-560. This suggests that a product(s) of the COX pathway may be involved in the protective effects of the oxysterols against oxidative stress. Interestingly, in contrast to the ability of PGE_2 to partially reverse the effects of SC-560 on osteoinductive effects of oxysterols [Kha et al., 2004], pretreatment or continuous treatment with PGE_2 at 1–30 μM did not protect against the inhibitory effects of SC-560 on the ability of oxysterols to protect against oxidative stress in M2 cells (data not shown). Moreover, several other known prostanoids that are products of the COX-1/arachidonic acid metabolic pathway, namely PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and Thromboxane B_2 used at 1–30 μM were also unable to reverse the effects of SC-560 (data not shown). The lack of effectiveness of the prostanoids tested may in part be due to the instability of these compounds in culture that would limit their ability to reverse the effects of the COX-1 inhibitor. Alternatively, we speculate that a different byproduct of the COX-1 fatty acid metabolism may mediate the protective effects of the osteogenic oxysterols against the adverse effects of oxidative stress on osteogenic differentiation of M2 cells.

The ability of oxysterols to protect cells against the adverse effects of XXO and MM-LDL on their osteogenic differentiation was associated with the osteogenic property of the oxysterols. This was demonstrated by the fact that oxysterols and oxidized lipids that do not induce osteogenic differentiation also do not protect cells from the adverse effects of oxidative stress on the osteogenic differentiation process. The correlation between protective capacity against oxidative stress and induction of osteogenic differentiation was also demonstrated in the case of rhBMP2. Pretreatment of M2 cells for 48 h with rhBMP2 (250 ng/ml) rendered these cells protected from the adverse effects of oxidative stress on their osteogenic differentiation (data not shown).

A role for oxidative stress in mediating the detrimental effects of aging and menopause has been proposed [Chang et al., 2002; Finkel, 2003]. We previously demonstrated that hyperlipidemia in mice susceptible to oxidative stress results in lowering of bone mineral density (BMD) [Parhami et al., 2001]. We speculate that the inhibitory effect of hyperlipidemia on bone is perhaps in part due to oxidative stress and the generation of oxidized lipids that are similar to those that induce the inflammatory cascade of events in cardiovascular disease [Parhami, 2003]. Preliminary evidence supporting this hypothesis was provided by demonstrating that MM-LDL and other species of bioactive, inflammatory oxidized lipids, such as certain specific isoprostanes and oxidized phospholipids inhibited the osteogenic differentiation of osteoprogenitor cells in vitro, in part through the induction of oxidative stress [Mody et al., 2001]. The present study which demonstrates the ability of osteogenic oxysterols to inhibit the adverse effects of MM-LDL on osteoprogenitor cells in vitro presents the intriguing possibility that these oxysterols may also be protective against the adverse effects of hyperlipidemia and lipid oxidation on bone metabolism in vivo.

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