ARTICLE
Journal of Cellular Biochemistry 111:1199–1209 (2010)

Journal of Cellular Biochemistry

Hedgehog Signaling and Osteogenic Differentiation in Multipotent Bone Marrow Stromal Cells Are Inhibited by Oxidative Stress

Woo-Kyun Kim,[1] Vicente Meliton,[1] Noam Bourquard,[1] Theodore J. Hahn,[2] and Farhad Parhami[1*]

[1] *Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California*

[2] *VA Greater Los Angeles Healthcare System, Geriatric Research, Education, and Clinical Center, Los Angeles, California*

ABSTRACT

Oxidative stress may play a major role in age-related osteoporosis in part by inhibiting osteoblast generation from osteoprogenitors cells. In the present study, we hypothesized that oxidative stress may inhibit the osteogenic differentiation of bone marrow stromal cells (MSC) in part by inhibiting the Hedgehog (Hh) signaling pathway, which is essential for bone development and maintenance and induces osteogenic differentiation of osteoprogenitor cells. To test this hypothesis, we examined the effects of oxidative stress on Sonic Hh (Shh)-induced osteogenic differentiation and signaling in M2-10B4 (M2) MSC, C3H10T1/2 embryonic fibroblasts, and mouse primary MSC. Treatment of cells with H_2O_2 inhibited Shh-induced osteogenic differentiation determined by the inhibition of Shh-induced expression of osteogenic differentiation markers alkaline phosphatase (ALP), osterix (OSX), and bone sialoprotein (BSP). Similar effects were found when oxidative stress was induced by xanthine/xanthine oxidase (XXO) or minimally oxidized LDL (MM-LDL). H_2O_2, XXO, and MM-LDL treatment inhibited Shh-induced expression of the Hh target genes Gli1 and Patched1 as well as Gli-dependent transcriptional activity in M2 cells. H_2O_2 treatment also inhibited Hh signaling induced by the direct activation of Smoothened by purmorphamine (PM), but not by Gli1 overexpression. This suggests that oxidative stress may inhibit Hh signaling upstream of Gli activation and Gli-induced gene expression. These findings demonstrate for the first time that oxidative stress inhibits Hh signaling associated with osteogenic differentiation. Inhibition of Hh signaling-mediated osteogenic differentiation of osteoprogenitor cells may in part explain the inhibitory effects of oxidative stress on osteoblast development, differentiation, and maintenance in aging. J. Cell. Biochem. 111: 1199–1209, 2010.

KEY WORDS: OXIDATIVE STRESS; MESENCHYMAL STEM CELLS; HEDGEHOG; OSTEOGENESIS

Age-related osteoporotic bone loss is associated with decreased bone formation and increased bone resorption [Cummings and Melton, 2002]. Recent evidence suggests that oxidative stress may play a major role in this process [Almeida et al., 2007, 2009]. Aging is associated with increased oxidative stress and decreased antioxidant defenses [Garrett et al., 1990; Mody et al., 2001; Shouhed et al., 2005]. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radical ($OH^\bullet$), can trigger oxidative damage in lipid, DNA, protein, carbohydrate, and proteoglycan molecules [Valko et al., 2007]. In addition, oxidative stress has been linked to various aging disorders, including atherosclerosis, diabetes, neurodegenerative diseases, and inflammatory arthritis to name a few [Valko et al., 2007]. It has been suggested that oxidative stress may also contribute to age-related osteoporosis in part by decreasing bone formation through inhibition of the osteogenic differentiation of bone marrow stromal cells (MSC) [Garrett et al., 1990]. Oxidative stress induced by H_2O_2, xanthine oxidase, minimally oxidized low-density lipoprotein (MM-LDL), and lipid oxidation products inhibits osteogenic differentiation and bone formation [Mody et al., 2001; Shouhed et al., 2005; Almeida et al., 2009]. A role for MM-LDL in promoting atherosclerosis and regulating osteogenic differentiation has been suggested, with MM-LDL inducing oxidative stress, promoting osteogenic differentiation of vascular mesenchymal cells and inhibiting osteogenic differentiation of bone-derived osteoprogenitor cells [Parhami et al., 1997; Mody et al., 2001; Shouhed et al.,

Grant sponsor: NIAMS; Grant sponsor: National Institutes of Health (NIH); Grant number: RO1AR050426; Grant sponsor: National Institute of Aging; Grant number: 5P30AG028748.

*Correspondence to: Farhad Parhami, PhD, David Geffen School of Medicine at UCLA, Center for the Health Sciences, BH-307, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail: fparhami@mednet.ucla.edu

Received 29 December 2009; Accepted 4 August 2010 • DOI 10.1002/jcb.22846 • © 2010 Wiley-Liss, Inc.
Published online 17 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

2005]. In this regard, we have previously shown that antioxidants and specific osteogenic oxysterols that activate Hedgehog (Hh) signaling in osteoprogenitor cells effectively reverse the inhibitory effects of oxidative stress on osteogenic differentiation [Mody et al., 2001; Shouhed et al., 2005]. Moreover, oxidative stress induced by some forms of oxidized lipids inhibits osteoblastogenesis and increases adipogenesis by inhibiting Wnt signaling that is critical for the regulation of osteogenic and adipogenic differentiation of MSC [Almeida et al., 2009].

Hh signaling is one of the major signaling pathways that regulate osteogenesis and adipogenesis [Spinella-Jaegle et al., 2001; Suh et al., 2006]. The proteins of the Hh family are important signaling molecules that play a central role in embryonic bone development and post-embryonic bone homeostasis [McMahon et al., 2003; Kimura et al., 2008]. In vertebrates, the Hh family consists of three members: Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh (Dhh) [McMahon et al., 2003]. Hh protein binding to the transporter-like receptor Patched (Ptch) releases Ptch inhibition of Smoothened (Smo), a seven-pass transmembrane protein that transduces the Hh signal, which in turn activates members of the Gli family of DNA-binding proteins that mediate the transcription of Hh target genes in cells [McMahon et al., 2003; Beachy et al., 2004]. It has been shown that Hh signaling is involved in post-embryonic bone fracture healing and bone maintenance [Ito et al., 1999; Kimura et al., 2008]. In the initial stages of fracture repair, the expression of molecular components of Hh signaling, including Smo, Indian Hh, and Ptch, is increased [Ito et al., 1999]. Moreover, transient inhibition of Hh signaling in young mice causes severe defects in bone structure [Kimura et al., 2008]. Thus, impaired Hh signaling may be one of the mechanisms that underlie age-related osteoporosis and impaired healing of bone fractures. In the present report, we demonstrate that oxidative stress induced by H_2O_2, XXO, or MM-LDL is associated with inhibition of Hh signaling and reduced osteogenesis in pluripotent MSC that serve as the progenitors of osteogenic cells [D'ippolito et al., 1999], and whose differentiation is shifted in favor of adipogenesis at the expense of osteogenesis in aging and osteoporosis [Meunier et al., 1971; Beresford et al., 1992; Nuttall et al., 1998; Chan and Duque, 2002].

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

M2-10B4 (M2) pluripotent mouse MSC and C3H10T1/2 mouse embryonic fibroblasts were cultured as previously described [Richardson et al., 2007; Kim et al., 2009]. Cell treatment was performed in differentiation medium containing 5% fetal bovine serum (FBS), 50 μg/ml ascorbate, and 3 mM β-glycerophosphate. Primary MSC were isolated from 6 to 8 months old CD1 mice according to our previously published methods [Kha et al., 2004] and maintained in MSC basal medium and mouse mesenchymal stem cell stimulatory supplements from STEMCELL Technology (Vancouver, BC). H_2O_2, xanthine, and xanthine oxidase were purchased from Sigma–Aldrich, Co. (St. Louis, MO), purmorphamine (PM) was from Calbiochem (La Jolla, CA), and recombinant mouse Shh N-terminal peptide was from R&D Systems (Minneapolis, MN). XXO treatment was performed using xanthine (250 μM) and xanthine oxidase (40 mU/ml) in osteogenic medium [Shouhed et al., 2005]. Polyclonal anti-mouse Patched1 antibody was purchased from Novus Biologicals (Littleton, CO), monoclonal anti-mouse Gli1 antibody was purchased from R&D Systems, and monoclonal anti-β-actin antibody was purchased from Sigma–Aldrich, and all antibodies were used according to manufacturer's instructions.

LIPOPROTEIN PREPARATION AND OXIDATION

Human LDL was isolated by density-gradient centrifugation of serum, and MM-LDL was prepared by iron oxidation of human LDL

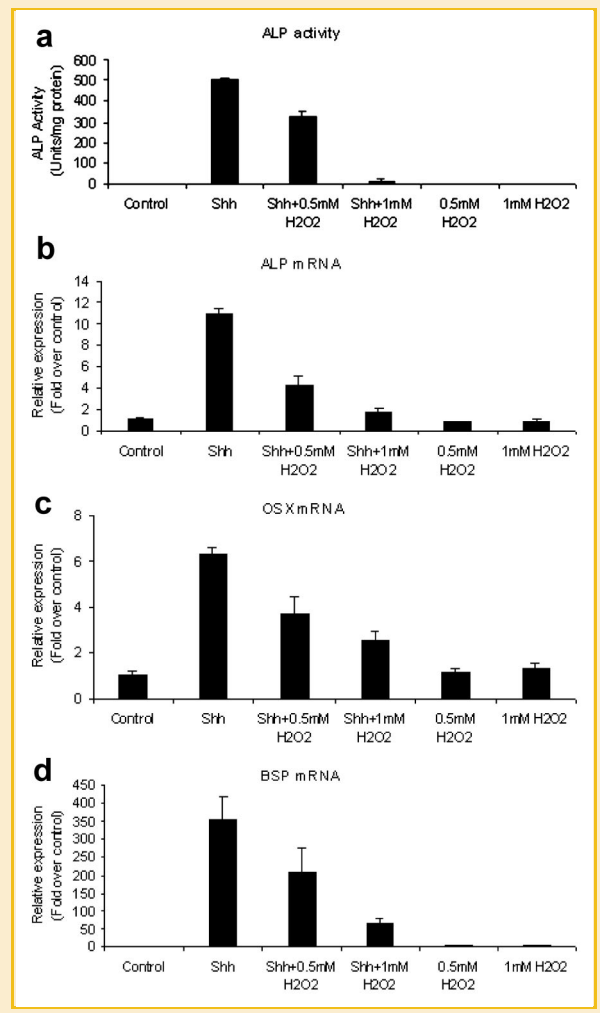

Fig. 1. Oxidative stress induced by H_2O_2 inhibits Shh-induced osteogenic differentiation of bone marrow stromal cells. a–d: M2-10B4 cells were treated at confluence with control vehicle, 200 ng/ml Shh, or 0.5–1 mM H_2O_2, alone or in combination. ALP activity was determined by a colorimetric method, and ALP and OSX mRNA expression were measured by quantitative real-time PCR 3 days after treatment. BSP mRNA expression was measured on day 6 post-treatment. Fold changes in gene expression relative to control are reported as the mean of triplicate determinations ±SD. (a: $P < 0.0001$ for Shh vs. Shh + 1 mM H_2O_2; $P < 0.01$ for Shh vs. Shh + 0.5 mM H_2O_2; b: $P < 0.0001$ for Shh vs. Shh + 0.5 mM H_2O_2 or Shh + 1 mM H_2O_2; c,d: $P < 0.0001$ for Shh vs. Shh + 1 mM H_2O_2, $P < 0.001$ for Shh vs. Shh + 0.5 mM H_2O_2).

as previously described [Parhami et al., 1999]. The levels of lipoproteins used in the present study are reported in micrograms of protein. The lipoproteins were examined pre- and post-oxidation for lipopolysacchride levels and found to have <30 pg of lipopolysaccharide/ml of medium.

QUANTITATIVE RT-PCR

Total RNA was extracted with an RNA isolation kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. RNA was DNase-treated using a DNA-free kit from Ambion (Austin, TX). Three micrograms of RNA was reverse-transcribed using reverse transcriptase from Stratagene to make single-stranded cDNA. The cDNAs were then mixed with Qi SYBR Green Supermix (Bio-Rad, CA) for quantitative RT-PCR assay using a Bio-Rad I-cycler IQ quantitative thermocycler. All PCR samples were prepared in triplicate wells in a 96-well plate. After 40 cycles of PCR, melt curves were examined in order to ensure primer specificity. Fold changes in gene expression were calculated using the $\Delta\Delta C_t$ method. Primers

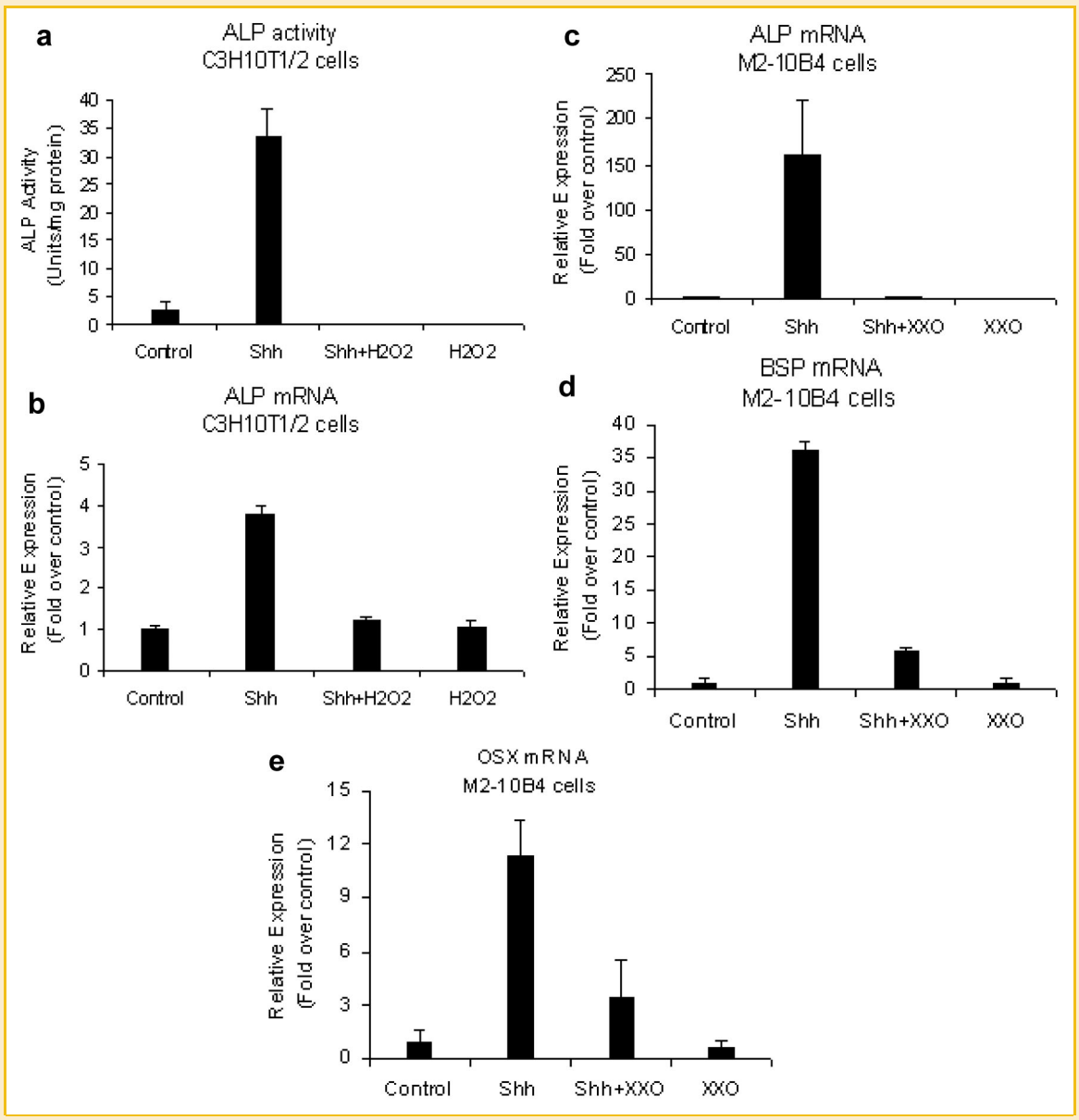

Fig. 2. Oxidative stress induced by H_2O_2 or XXO inhibits Shh-induced osteogenic differentiation in embryonic fibroblasts and bone marrow stromal cells. a: C3H10T1/2 cells at confluence were treated with control vehicle, 200 ng/ml Shh, or 0.75 mM H_2O_2 alone or in combination and ALP activity was measured after 3 days. b: C3H10T1/2 cells at confluence were treated with control vehicle, 200 ng/ml Shh, or 1 mM H_2O_2 alone or in combination for 48 h. ALP mRNA expression was measured by quantitative real-time PCR. Fold changes in gene expression relative to control are reported as the mean of triplicate determinations ±SD. c–e: M2-10B4 cells were treated at confluence with control vehicle, 200 ng/ml recombinant mouse Shh or XXO alone or in combination for 4 days. ALP, BSP, and OSX mRNA expression were measured by quantitative real-time PCR. Fold changes in gene expression relative to control are reported as the mean of triplicate determinations ±SD. (a,b: $P < 0.0001$ for control vs. Shh and for Shh vs. Shh + H_2O_2; c: $P < 0.0001$ for control vs. Shh and for Shh vs. Shh + XXO).

used were as follows: *GAPDH* (5′-ATGGACTGTGGTCATGAGCC-3′ and 5′-ATTGTCAGCAATGCATCCTG-3′), *Gli1* (5′-GAAGCCGAGCC-GAGTATC-3′ and 5′-GGTGAGTAGACAGAGGTTGG-3′), *Ptch1* (5′-CCATCGGCGACAAGAACC-3′ and 5′-CCAGCACAGCAAAGAAA-TACC-3′), *Smoothened* (*Smo*) (5′-AACTATCGGTACCGTGCTGG-3′ and 5′-CATCATGGGAGACAGTGTGC-3′), *c-fos* (5′-TGAGGCTTC-CACCC-3′ and 5′-CTCCAGGTTGCTGA-3′), *Hemoxygenase 1* (*HOX1*) (5-CAGGTGATGCTGACAGAGGA-3′ and 5′-GAGAGTGAGGACC-CACTGGA-3′), *Osterix* (*OSX*) (5′-CCCTTCTCAAGCACCAATGG-3′ and 5′-AGGGTGGGTAGTCATTTGCAT-3′), *Alkaline Phosphatase* (*ALP*) (5′-AAACCCAGAACACAAGCATTCC-3′ and 5′-TCCACCAG-CAAGAAGAAGCC-3′), and *Bone sialoprotein* (*BSP*) (5′-ACGCCA-CACTTTCCACACTCTC-3′ and 5′-TTCCTCTTCCTCTTCTTCTTCTTC-TTCC-3′).

ALKALINE PHOSPHATASE (ALP) ACTIVITY ASSAY

M2 cells at confluence were treated in differentiation medium with control vehicle or test agents. After 72 h, a colorimetric assay for ALP activity was performed on whole cell extracts as previously described [Kha et al., 2004].

MTT ASSAY

The MTT assay was used to measure the potential effects of oxidative stress inducing agents on cell proliferation and/or viability as we have previously reported [Mody et al., 2001].

TRANSIENT TRANSFECTION AND REPORTER ASSAY

M2 cells at 70% confluence were transiently transfected with a Gli response-element reporter construct (pGL3b-8xGli-Luciferase) plasmid, or pGL3b-Luciferase plasmid and pTK-Renilla-Luciferase plasmid as we have previously reported [Dwyer et al., 2007]. After 24 h cells were treated with test agents for 48 h. For Gli1 overexpression studies, M2 cells at 70% confluence were transiently transfected with a Gli1 overexpression vector (kind gift of Dr. William Matsui, Johns Hopkins School of Medicine), pGL3b-8xGli-Luciferase plasmid, or pGL3b-Luciferase plasmid and pTK-Renilla-Luciferase plasmid without Gli1 overexpression vector. Luciferase activity was measured after 48 h and normalized for transfection efficiency using the Renilla luciferase activity. Data are reported as the mean of triplicate determinations ±SD.

WESTERN BLOTTING

Cells were lysed in lysis buffer, and protein concentrations were determined using the Bio-Rad protein assay as previously described [Kha et al., 2004]. Whole cells extracts (30 μg) were separated on SDS–PAGE gels and transferred overnight onto nitrocellulose membrane. Blots were blocked and incubated with specific primary antibodies followed by HRP-linked secondary antibodies, and binding was revealed using the Western Lightning ECL Kit (Perkin Elmer, Boston, MA) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Statistical analyses were performed using the StatView 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

OXIDATIVE STRESS INHIBITS SHH-INDUCED OSTEOGENESIS IN OSTEOPROGENITOR CELLS

We previously reported that oxidative stress induced by XXO or MM-LDL inhibits spontaneous in vitro osteogenic differentiation of MSC cultured in the presence of FBS, ascorbic acid, and β-glycerophosphate (i.e., osteogenic differentiation medium) [Shouhed et al., 2005]. In the present study, we examined whether oxidative stress induced by H_2O_2 inhibits Shh-induced osteogenesis in MSC by assessing its effect on markers of osteogenic differentiation, ALP activity, and ALP, OSX, and BSP mRNA expression, in M2 cells. H_2O_2 significantly reduced Shh-induced ALP activity in a dose-dependent manner (Fig. 1a). Furthermore, both concentrations of H_2O_2 inhibited Shh-induced ALP, OSX, and BSP mRNA expression measured by qRT-PCR (Fig. 1b–d), indicating that H_2O_2-induced oxidative stress inhibits Shh-induced osteogenesis in MSC. H_2O_2 treatment also inhibited Shh-induced ALP activity as well as ALP mRNA expression in C3H10T1/2 embryonic fibroblasts (Fig. 2a,b). Furthermore, consistent with the effects of H_2O_2, XXO, which induces oxidative stress by converting xanthine to uric acid and superoxide anion, significantly inhibited Shh-induced ALP, BSP, and OSX mRNA expression in M2 cells (Fig. 2c–e). We also confirmed that H_2O_2 produces oxidative stress in MSC by measuring its effects on the expression of key oxidative

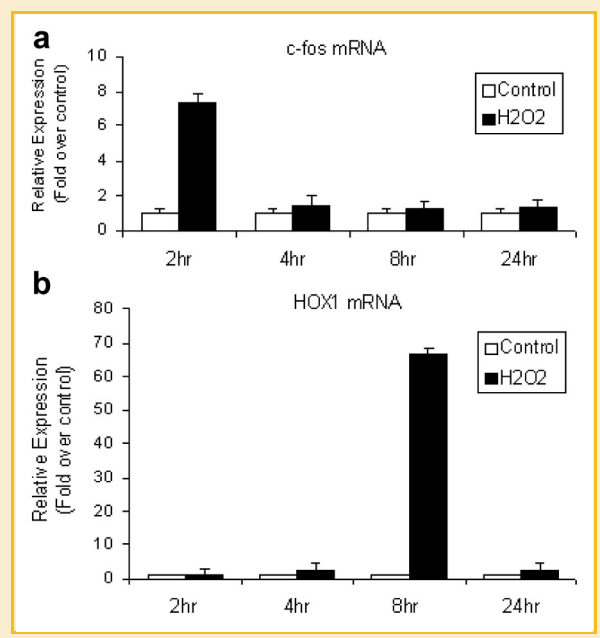

Fig. 3. Effect of H_2O_2 on oxidative stress-related gene expression in bone marrow stromal cells. M2-10B4 cells at confluence were treated with control vehicle or 1 mM H_2O_2. c-fos and HOX1 mRNA expression were measured by quantitative real-time PCR at 2, 4, 8, and 24 h post-treatment. Fold changes in gene expression relative to control cells are reported as the mean of triplicate determinations ±SD. (a: $P < 0.0001$ for control vs. 1 mM H_2O_2 at 8 h and $P < 0.001$ for control vs. 1 mM H_2O_2 at 4 and 24 h; b: $P < 0.0001$ for control vs. 1 mM H_2O_2 at 2 h).

stress-related genes, including c-fos and HOX1 [Orozco et al., 2007] at 2, 4, 8, and 24 h after the addition of H_2O_2. The expression of c-fos mRNA was increased over sevenfold at 2 h in H_2O_2-treated cells ($P < 0.0001$) and returned to baseline levels after 4 h (Fig. 3a). HOX1 mRNA expression was also significantly increased by H_2O_2 treatment showing a robust 60-fold induction at 8 h, with a slight but significant 2.5-fold induction still present at 24 h (Fig. 3b).

To examine the potential effects of H_2O_2, XXO, and MM-LDL on proliferation and/or cell viability, we performed MTT assays anticipating that a decrease in proliferation and/or cell viability would be associated with a significant decrease in MTT values. Results showed that in M2 cells after 8, 24, 48, or 96 h, 0.5 mM H_2O_2 caused no significant effects in MTT values. Treatment of M2 cells with 1 mM H_2O_2 caused no significant effects at 8 and 24 h

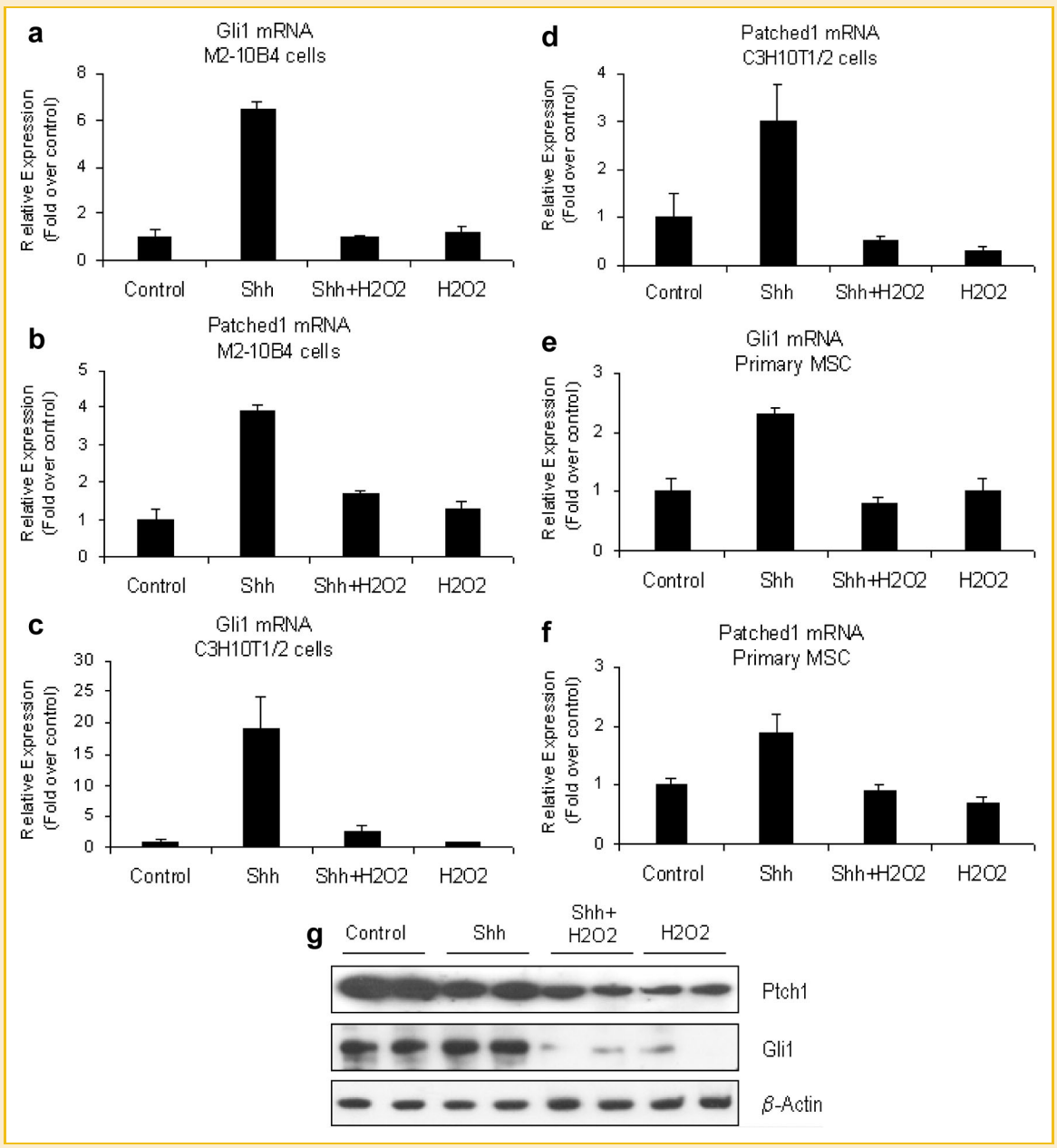

Fig. 4. Oxidative stress induced by H_2O_2 inhibits Hh signaling in osteoprogenitor cells. a,b: M2-10B4 cells at confluence were treated with control vehicle, 200 ng/ml Shh, or 1 mM H_2O_2 alone or in combination for 8 h. Gli1 and Patched1 mRNA expression were measured by quantitative real-time PCR. c,d: C3H10T1/2 cells at confluence were treated with control vehicle, 200 ng/ml Shh, or 1 mM H_2O_2 alone or in combination for 48 h. Gli1 and Patched1 mRNA expression were measured. e,f: Primary MSC at confluence were treated with control vehicle, 600 ng/ml Shh, or 0.5 mM H_2O_2 alone or in combination for 24 h. Gli1 and Patched1 mRNA expression were measured. Fold changes in gene expression relative to control cells are reported as the mean of triplicate determinations ±SD. ($P < 0.001$ for control vs. Shh and for Shh vs. Shh + H_2O_2 in all panels shown). g: M2-10B4 cells were treated with 200 ng/ml Shh or 1 mM H_2O_2 alone or in combination for 24 h. Western blot analysis using whole cell lysates was performed using specific antibodies to Gli1, Ptch1, and β-actin for normalization. Results showing duplicate samples from each condition and representative of two separate experiments are reported.

but did cause a significant 20% decrease after 48 h ($P < 0.01$) and a significant 30% decrease after 96 h ($P < 0.01$) without any evidence of cell rounding or detachment from the monolayer up to 8 days post-treatment. XXO and MM-LDL caused no effects on MTT values up to 96 h post-treatment, and caused no evidence of cell rounding or detachment for the entire duration of the experiments.

OXIDATIVE STRESS INHIBITS HEDGEHOG SIGNALING IN BONE MARROW STROMAL CELLS

Next, we examined the effects of oxidative stress on Shh-induced Hh signaling in M2 cells. Shh increased the mRNA expression of the key Hh signaling target genes, Gli1 and Ptch1 in M2 cells after 8 h of treatment, and these effects were significantly inhibited by H_2O_2 (Fig. 4a,b). These results were confirmed in C3H10T1/2 mouse embryonic fibroblasts (Fig. 4c,d) and in primary murine MSC where H_2O_2 treatment was also found to significantly reduce Shh-induced Gli1 and Ptch1 mRNA expression (Fig. 4e,f). Although previous reports have demonstrated the effect of Hh pathway stimulation on the subcellular distribution and localization of molecular components of the Hh pathway, including Ptch1, Smo, and Gli2, to primary cilia [Rohatgi and Scott, 2007; Milenkovic et al., 2009], we were interested in examining the potential effects of oxidative stress on absolute levels of Ptch1 and Gli1 proteins which were abundantly present at baseline in extracts from confluent cultures of M2 cells (Fig. 4g). In order to examine the effects of H_2O_2 on Gli1 and Ptch1 protein levels, M2 cells were treated with Shh or H_2O_2, separately or in combination, and whole cell extracts were analyzed after 24 h of treatment by Western blotting. In contrast to its previously reported inductive effects on their mRNA expression, Shh had almost no effect on Gli1 protein levels and a slightly inhibitory effect on Ptch1 protein levels (Fig. 4g). However, H_2O_2

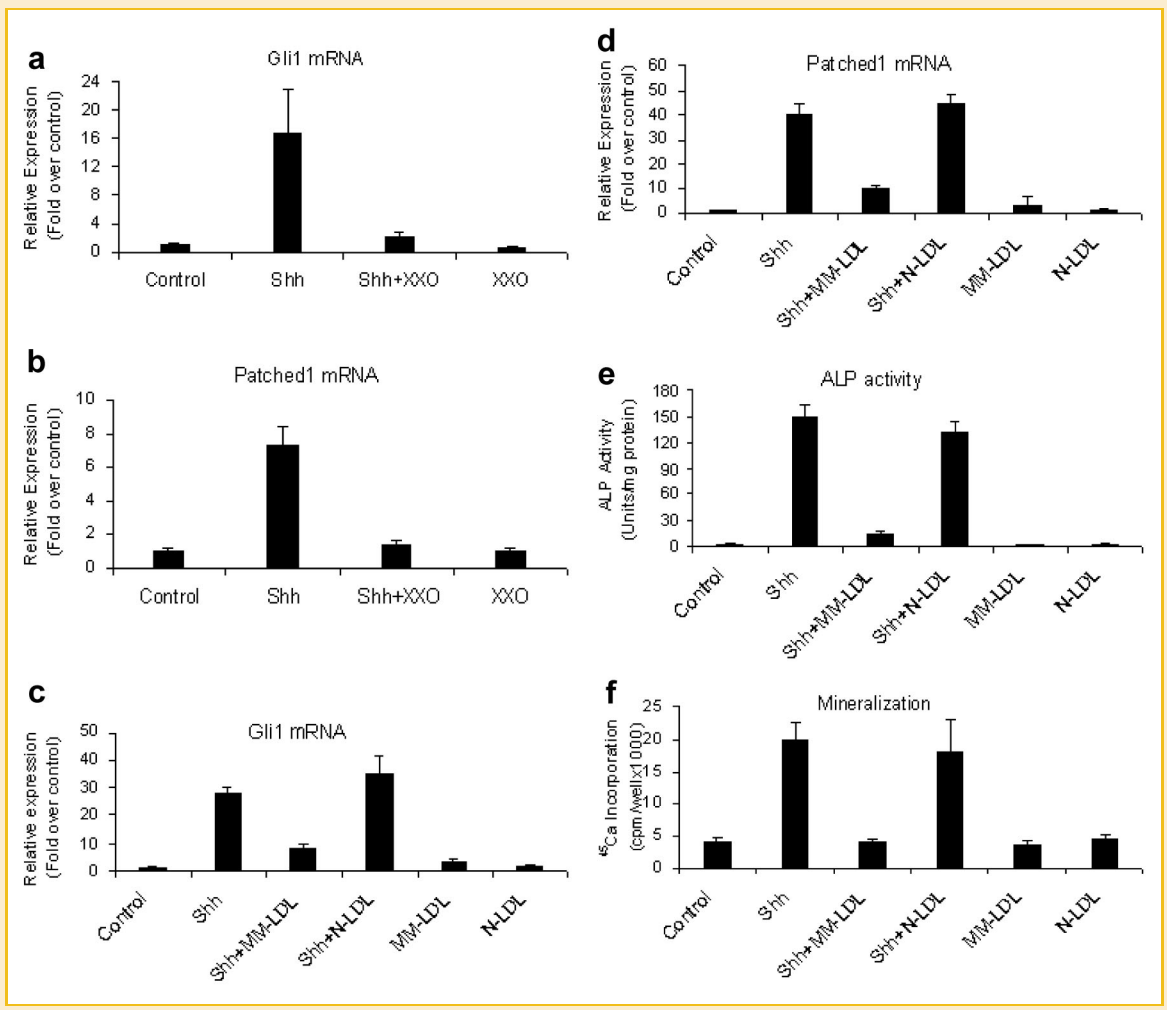

Fig. 5. Oxidative stress induced by XXO or MM-LDL inhibits Hh signaling and osteogenic differentiation of bone marrow stromal cells. a,b: M2-10B4 cells at confluence were treated with control vehicle, 200 ng/ml Shh, or XXO alone or in combination for 24 h. Gli1 and Patched1 mRNA expression were measured by quantitative real-time PCR. c–f: M2-104 cells at confluence were treated with control vehicle, 200 ng/ml Shh, MM-LDL (200 μg/ml), or native LDL (N-LDL; 200 μg/ml) alone or in combination. Gli1 and Patched1 mRNA expression were measured by quantitative real-time PCR at 48 h post-treatment. Fold changes in gene expression relative to control cells are reported as the mean of triplicate determinations ±SD. ALP activity was assayed in whole cell lysates after 3 days (e), and mineralization in parallel cultures was quantified after 14 days of treatments (f). (a,b: $P < 0.001$ for control vs. Shh and for Shh vs. Shh + XXO; c–f: $P < 0.001$ for control vs. Shh and for Shh vs. Shh + MM-LDL).

robustly reduced the protein levels of both Gli1 and Ptch1 in control as well as in Shh-treated cells (Fig. 4g).

In addition, we examined whether oxidative stress induced by XXO and MM-LDL inhibits Hh signaling in M2 cells. XXO and MM-LDL treatment significantly inhibited Gli1 and Ptch1 mRNA expression (Fig. 5a–d), whereas treatment with unoxidized native LDL (N-LDL), which does not induce oxidative stress, did not inhibit the expression of these genes. We also found that MM-LDL significantly inhibited Shh-induced osteogenic differentiation of M2 cells as suggested by the inhibition of ALP activity and mineralization in these cells (Fig. 5e,f).

KINETICS OF OXIDATIVE STRESS–INDUCED INHIBITION OF HEDGEHOG SIGNALING

We examined the kinetics of H_2O_2 effects on Hh signaling in M2 cells by testing the effects of H_2O_2 when added prior to or after treating the cells with Shh. An 8 h pretreatment time point with H_2O_2 was chosen since H_2O_2 induction of oxidative stress occurred as early as 2 h after treatment as evident by the induction of c-fos, and was persistent for at least 8 h as evident by the robust induction of HOX1 at this time point (Fig. 3b). Subsequent to the 8 h pretreatment, H_2O_2 was completely removed from the cells and the cells were then treated with Shh for an additional 24 h in order to examine whether the inhibitory effects of H_2O_2 are persistent or transient and reversible upon its removal. Results showed that the effects of H_2O_2 appear to be transient and therefore the fold changes in the induction of Gli1 and Ptch1 are similar with and without H_2O_2 pretreatment (Fig. 6a,b). Next we examined whether H_2O_2 is capable of inhibiting Hh signaling if the cells have encountered Shh prior to the induction of oxidative stress by H_2O_2. Similar to co-treatment of cells with Shh and H_2O_2, treatment of M2 cells with Shh first for 16 h followed by the addition of H_2O_2 for 8 h in the presence of Shh produced a significant inhibition of Gli1 and Ptch1 mRNA expression (Fig. 6c,d) while significantly inducing HOX1 expression (Fig. 6e).

MOLECULAR MECHANISMS UNDERLYING THE INHIBITORY EFFECTS OF OXIDATIVE STRESS ON HEDGEHOG SIGNALING

In order to begin identifying the molecular mechanisms by which oxidative stress inhibits cellular responses to Hh proteins, we

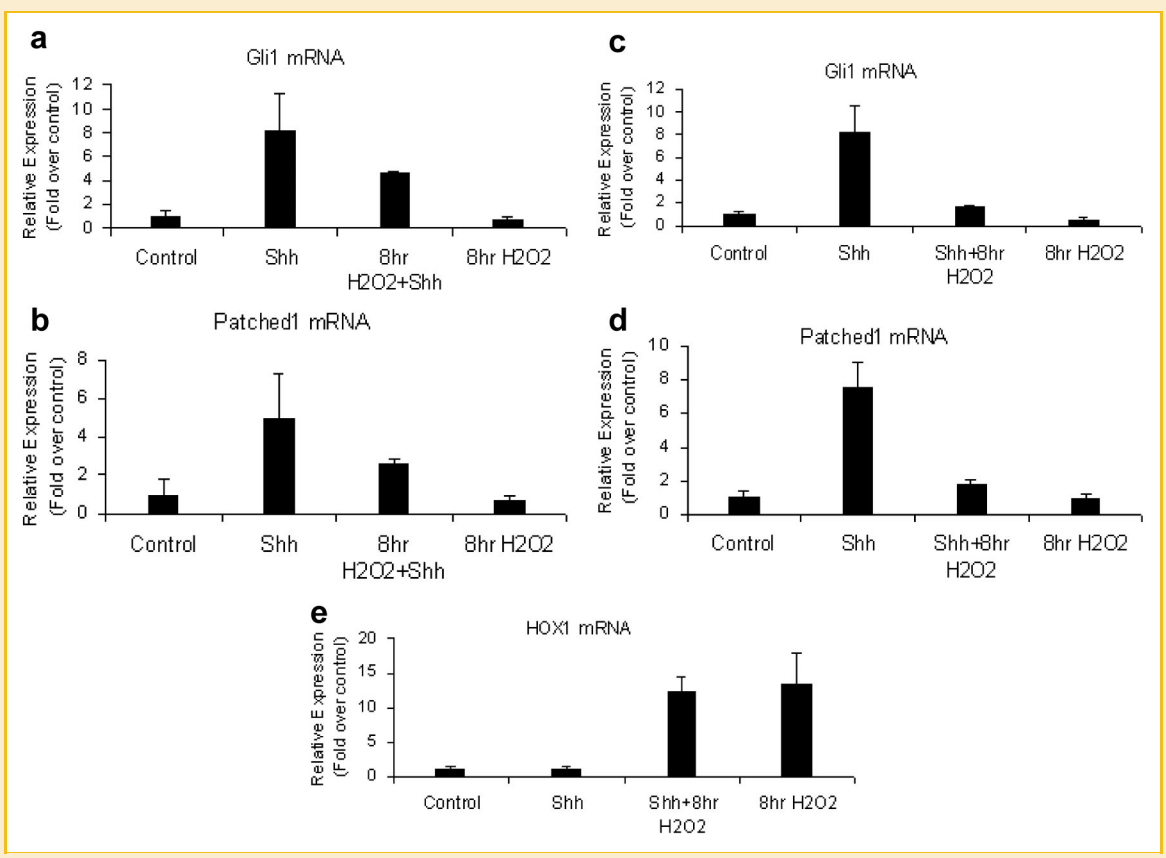

Fig. 6. Kinetics of inhibition of Hh signaling by oxidative stress in bone marrow stromal cells (a,b). M2-10B2 cells at confluence were pretreated with 1 mM H_2O_2 for 8 h. Next, H_2O_2 was removed and the cells were treated with control vehicle or 200 ng/ml Shh for 24 h. Gli1 and Patched1 mRNA expression were measured by quantitative real-time PCR. c–e: M2-10B4 cells at confluence were treated with control vehicle or 200 ng/ml Shh for 24 h. At 16 h post-treatment, 1 mM H_2O_2 was added into Shh + H_2O_2 or H_2O_2 treatment groups for the remaining 8 h. Gli1, Patched1, and HOX1 mRNA expression were measured by quantitative real-time PCR. Fold changes in gene expression relative to control cells are reported as the mean of triplicate determinations ±SD. (a,b: $P < 0.001$ for control vs. Shh and $P < 0.05$ for Shh vs. 8 h H_2O_2 + Shh; c,d: $P < 0.0001$ for control vs. Shh or Shh + 8 h H_2O_2; e: $P < 0.0001$ for control vs. Shh + 8 h H_2O_2 or 8 h H_2O_2).

examined the effects of H_2O_2 on Hh signaling induced by the binding of Shh to Ptch versus that which is induced by Smo agonists that directly bind to Smo and bypass the Ptch regulatory step in activating Hh signaling. Direct activation of Smo was achieved by treating M2 cells with PM, which activates Hh signaling by direct binding to Smo [Sinha and Chen, 2006]. As expected, H_2O_2 treatment significantly inhibited Shh-induced Gli1 and Ptch1 mRNA expression (Fig. 7a,b). Similarly, H_2O_2 treatment also inhibited PM-induced Gli1 and Ptch1 mRNA expression (Fig. 7a,b), indicating that oxidative stress inhibits Hh signaling downstream or at the level of Smo. This is consistent with the robust H_2O_2-induced reduction in Gli1 protein levels which is in part responsible for transducing the transcription regulating signals from Smo to the nucleus. Furthermore, in preliminary unpublished studies we have found that treatment of M2 cells for 24 h with 1 mM H_2O_2 resulted in a significant 70% inhibition of Smo mRNA expression. In addition, H_2O_2 treatment significantly inhibited Shh-induced Gli reporter activity in a dose-dependent manner in M2 cells (Fig. 7c). However, H_2O_2 did not inhibit Gli reporter activity induced by the overexpression of Gli1 (Fig. 7d), suggesting that inhibition of Hh signaling occurs upstream of Gli-induced Hh target gene expression and may involve inhibition of Gli1 expression which is compensated by the overexpression of Gli1.

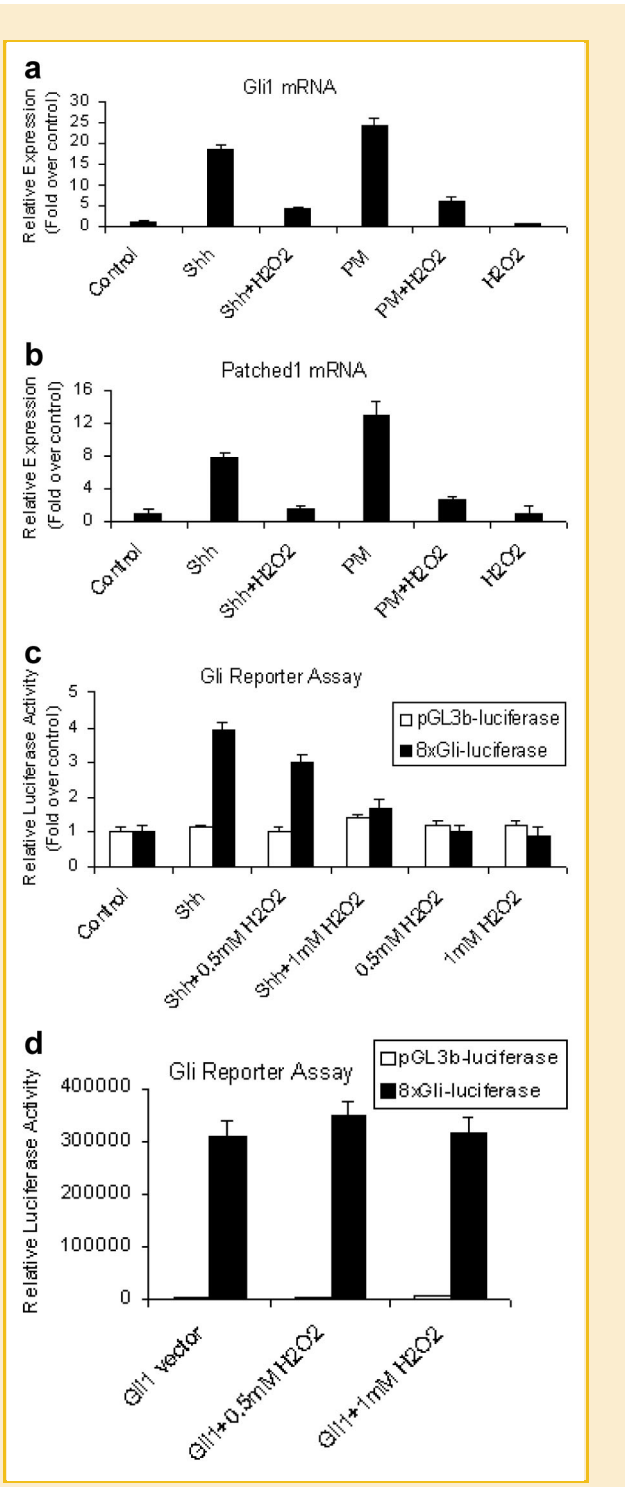

Fig. 7. Mechanism of oxidative stress-induced inhibition of Hh signaling. a,b: M2-10B4 cells at confluence were treated with control vehicle, 200 ng/ml Shh, 1 μM PM, or 1 mM H_2O_2 alone or in combination for 48 h. Gli1 and Patched1 mRNA expression were measured by quantitative real-time PCR. Fold changes in gene expression relative to control cells are reported as the mean of triplicate determinations ±SD. c: M2-10B4 cells at 70% confluence were transiently transfected with a Gli response element reporter construct (pGL3b-8xGli-Luciferase) or pGL3b-Luciferase plasmid in combination with pTK-Renilla-Luciferase plasmid. After 24 h cells were treated with control vehicle, 200 ng/ml Shh, 0.5 or 1 mM H_2O_2 alone or in combination for 48 h. d: M2 cells at 70% confluence were transiently transfected with a Gli1 overexpression vector, a Gli response element reporter construct (pGL3b-8xGli-Luciferase) or pGL3b-Luciferase plasmid in combination with pTK-Renilla-Luciferase plasmid. Luciferase activity was measured after 48 h and normalized for transfection efficiency using the Renilla luciferase activity. Data are reported as the mean of triplicate determinations ±SD. (a,b: $P < 0.0001$ for control vs. Shh or PM, for Shh vs. Shh + H_2O_2 and for PM vs. PM + H_2O_2; c: $P < 0.0001$ for control vs. Shh and for Shh vs. Shh + 1 mM H_2O_2 and $P < 0.01$ for Shh vs. Shh + 0.5 mM H_2O_2).

DISCUSSION

Several studies have suggested an association between oxidative stress and impairment of skeletal integrity, and a potentially important role for antioxidants in preventing age-related osteoporosis [Basu et al., 2001; Maggio et al., 2003; Shouhed et al., 2005]. Levels of 8-iso-$PGF_{2\alpha}$, a circulating biomarker of oxidative stress that increases with age were reported to be negatively associated with bone mineral density in both women and men [Basu et al., 2001]. In mice increased oxidative stress with age was associated with reduced osteoblast number and bone formation rate as well as increased osteoblast and osteocyte apoptosis, decreased bone mineral density, and decreased bone strength [Almeida et al., 2007]. Decreased osteogenic differentiation of bone marrow progenitor cells could reduce osteoblast number and bone formation, causing or accelerating age-related osteoporosis [Chan and Duque, 2002; Chen et al., 2002]. Given the importance of Hh signaling in embryonic bone development and post-embryonic maintenance and repair of bone, we focused our attention on the potential adverse effects of oxidative stress on Hh-induced

osteogenesis and Hh signaling in MSC. Indeed, the studies presented here clearly demonstrate the inhibitory effects of oxidative stress on Hh-induced osteogenic differentiation of MSC and support the hypothesis that the contribution of oxidative stress to osteoporosis may be in part due to inhibition of Hh signaling-mediated osteogenesis. In addition, we speculate that oxidative stress may also inhibit Hh-mediated skeletal development, consistent with reports that showed inhibition of Hh-mediated limb patterning and neural plate closure by oxidative stress during development [Dennery, 2007; Aoto et al., 2008]. We propose that impairment of Hh signaling by oxidative stress in adult organisms may have detrimental effects on tissue homeostasis including the skeleton. Oxidative stress may be induced by a variety of physiologically relevant factors that may interfere with Hh signaling. In this report we demonstrate that inflammatory oxidized lipoproteins, such as MM-LDL, which we have shown in the past to impair osteogenic differentiation of osteoprogenitor cells, also disrupt Hh signaling in these cells. This is of particular relevance to the previously suggested role of dyslipidemia and lipid oxidation in bone maintenance and osteoporosis [Parhami et al., 1999, 2001; Parhami, 2003; Almeida et al., 2009]. The importance of this finding, if translated in vivo, will be at least twofold, both in the context of embryonic development and in post-embryonic tissue homeostasis where Hh signaling plays critical roles. Inflammatory oxidized lipoproteins play fundamental roles in the development of atherosclerosis in mice and humans [Rosenfeld, 1991], and in osteopenia/osteoporosis in mice [Parhami et al., 1999, 2001]. Since Hh signaling appears to be important in both these disorders by regulating homeostasis of vessel wall and bone/cartilage [Ehlen et al., 2006; Beckers et al., 2007], respectively, we propose that understanding the molecular interactions between inflammatory oxidized lipoproteins and Hh signaling may provide new insights into the pathogenesis of cardiovascular disease and osteoporosis.

Moreover, we demonstrate here that oxidative stress induced by H_2O_2 inhibits Hh signaling induced by Hh proteins or by ligands such as PM that directly bind to and activate Smo. Together with our finding that activation of Hh signaling by Gli overexpression is not inhibited by oxidative stress, that oxidative stress robustly inhibits Gli1 protein levels, and our preliminary observation that oxidative stress appears to significantly inhibit Smo mRNA expression, we propose that oxidative stress inhibits Hh signaling in part by inhibiting the expression of Gli1 and/or by inhibiting events that occur upstream of Gli activation. Since Smo is the sole transducer of the Hh-mediated signaling and target gene expression, down-regulation of Smo expression may be the main mechanism underlying the inhibition of Hh signaling by oxidative stress. However, since regulation of Smo activity in part involves complex changes in subcellular localization of Smo protein to primary cilia [Milenkovic et al., 2009], in future studies it will be necessary to further examine the effects of oxidative stress on Smo protein localization and trafficking within subcellular compartments. Furthermore, it has been well established that Ptch causes catalytic inhibition of Smo [Taipale et al., 2000]. At present the mechanism for this catalytic inhibition is not clearly understood, although the potential role of intracellular small molecules such as specific oxysterols has been suggested [Beachy et al., 2004; Dwyer et al., 2007; Rohatgi et al., 2007]. It is intriguing to speculate that these small molecule regulators of Ptch–Smo interaction may be targets of oxidative stress that in addition to down regulation of Smo expression may result in impairment of Hh signaling and osteogenic differentiation in cells exposed to oxidative stress.

As noted earlier, recent studies have suggested that primary cilia and the molecular machinery within them are important for the integrity of Hh signaling in mammalian cells [Rohatgi et al., 2007; Wong et al., 2009]. In preliminary studies we have found that in M2 cell oxidative stress induced by H_2O_2 significantly reduces the mRNA expression of Polaris (Parhami et al., unpublished work), a key component of primary cilia whose knockdown has been found to adversely affect primary cilia formation and Hh signaling [Huangfu and Anderson, 2005; May et al., 2005]. Since primary cilia have been reported to also mediate other cell signaling pathways, the previously reported adverse effects of oxidative stress on multiple signaling pathways is consistent with oxidative stress-induced impairment of primary cilia [Simons et al., 2005; Michaud and Yoder, 2006; Alaiwi et al., 2009; Wong et al., 2009]. Disruption of primary cilia and intraflagellar transport within cilia were found to impair normal endochondral bone formation due to disruption of multiple signaling pathways [Haycraft et al., 2007]. Since plasma membrane is highly susceptible to oxidative stress and lipid oxidation, changes in its physical properties and composition often occur with oxidation [Joseph et al., 1998; Stark, 2005; Cuschieri and Maier, 2007]. In fact, microdomains in plasma membrane that centralize signal transduction, such as lipid rafts and caveolae, are targets of oxidative stress and undergo compositional changes that modulate cellular signaling and behavior [Girotti, 2001; Cuschieri and Maier, 2007]. Since primary cilia are plasma membrane microdomains that control signal transduction, we hypothesize that oxidative stress will induce changes in physical properties of ciliary membrane, causing alterations in localization of key membrane-bound members of Hh pathway signaling, Ptch and Smo. This hypothesis remains to be tested in future studies.

In summary, our studies demonstrate for the first time that oxidative stress inhibits Hh signaling and Hh-mediated osteogenic differentiation in osteoprogenitor cells. Inhibition of Hh signaling and osteogenesis by oxidative stress may in part explain the reduced integrity of the skeletal tissue seen with aging, and together with our finding that the inhibitory effects of oxidative stress on Hh signaling appear to be transient, reemphasizes the potential benefits of antioxidants in preventing and/or reversing oxidative stress-induced osteoporosis.

ACKNOWLEDGMENTS

This work was supported in part by NIAMS, National Institutes of Health (NIH) Grant RO1AR050426, and a Pilot Study Award from the UCLA Claude Pepper Older Americans Independence Center funded by the National Institute of Aging Grant 5P30AG028748.

REFERENCES

Alaiwi WAA, Lo ST, Mauli SM. 2009. Primary cilia: Highly sophisticated biological sensors. Sensors 9:7003–7020.

Almeida M, Han L, Martin-Millan M, Plotkin LI, Stewart SA, Roberson PK, Kousteni S, O'Brien CA, Bellido T, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC. 2007. Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. J Biol Chem 282:27285–27297.

Almeida M, Ambrogini E, Han L, Manolagas SC, Jilka RL. 2009. Increased lipid oxidation causes oxidative stress, increased peroxisome proliferator-activated receptor-γ expression, and diminished pro-osteogenic Wnt signaling in the skeleton. J Biol Chem 284:27438–27448.

Aoto K, Shikata Y, Higashiyama D, Shiota K, Motoyama J. 2008. Fetal ethanol exposure activates protein kinase A and impairs Shh expression in prechordal mesendoderm cells in the pathogenesis of holoprosencephaly. Birth Defects Res A Clin Mol Teratol 82:224–231.

Basu S, Michaelsson K, Olofsson H, Johansson S, Melhus H. 2001. Association between oxidative stress and bone mineral density. Biochem Biophy Res Comm 288:275–279.

Beachy PA, Karhadkar SS, Berman DM. 2004. Tissue repair and stem cell renewal in carcinogenesis. Nature 432:324–331.

Beckers L, Heeneman S, Wang L, Burkly LC, Rousch MMJ, Davidson NO, Gijbels MJJ, de Winther MPJ, Daemen MJAP, Lutgens E. 2007. Disruption of Hedgehog signaling in ApoE−/− mice reduces plasma lipid levels, but increases atherosclerosis due to enhanced lipid uptake by macrophages. J Pathol 212:420–428.

Beresford JN, Bennett JH, Devin C, Leboy PS, Owen ME. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci 102:341–351.

Chan GK, Duque G. 2002. Age-related bone loss: Old bone, new facts. Gerontology 48:62–71.

Chen XD, Shi S, Xu T, Robey PG, Young MF. 2002. Age-related osteoporosis in biglycan-deficient mice is related to defects in bone marrow stromal cells. J Bone Miner Res 17:331–340.

Cummings SR, Melton LJ III. 2002. Epidemiology and outcomes of osteoporotic fractures. Lancet 359:1761–1767.

Cuschieri J, Maier RV. 2007. Oxidative stress, lipid rafts, and macrophage reprogramming. Antioxid Redox Signal 9:1485–1497.

D'ippolito G, Schiller PC, Ricordi C, Roos BA, Howard G. 1999. Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. J Bone Miner Res 14:1115–1122.

Dennery PA. 2007. Effects of oxidative stress on embryonic development. Birth Defects Res 81:155–162.

Dwyer JR, Sever N, Carlson M, Nelson SF, Beachy PA, Pahami F. 2007. Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. J Biol Chem 282:8959–8968.

Ehlen HWA, Buelens LA, Vortkamp A. 2006. Hedgehog signaling in skeletal development. Birth Defects Res 78:267–279.

Garrett IR, Boyce BF, Oreffo ROC, Bonewald L, Poser J, Mundy GR. 1990. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 85:632–639.

Girotti AW. 2001. Phoosensitized oxidation of membrane lipids: Reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B 63:103–113.

Haycraft CJ, Zhang Q, Song B, Jackson WS, Detloff PJ, Serra R, Yoder BK. 2007. Intraflagellar transport is essential for endochondral bone formation. Development 134:307–316.

Huangfu D, Anderson KV. 2005. Cilia and Hedgehog responsiveness in the mouse. Proc Nat Acad Sci 102:11325–11330.

Ito H, Akiyama H, Shigeno C, Iyama K, Matsuoka H, Nakamura T. 1999. Hedgehog signaling molecules in bone marrow cells at the initial stage of fracture repair. Biochem Biophys Res Comm 262:443–451.

Joseph JA, Denisova N, Fisher D, Shukitt-Hale B, Bickford P, Prior R, Cao G. 1998. Membrane and receptor modifications of oxidative stress vulnerability in aging. Nutritional considerations. Ann NY Acad Sci 854:268–276.

Kha HT, Basseri B, Shouhed D, Richardson J, Tetradis S, Hahn TJ, Parhami F. 2004. Oxysterols regulate differentiation of mesenchymal stem cells: Pro-bone and anti-fat. J Bone Miner Res 19:830–840.

Kim WK, Meliton V, Park KW, Hong C, Tontonoz P, Niewiadomski P, Waschek JA, Tetradis S, Parhami F. 2009. Negative regulation of Hedgehog signaling by liver X receptors. Mol Endocrinol 23:1532–1543.

Kimura H, Ng JMY, Curran T. 2008. Transient inhibition of the hedgehog pathway in young mice cause permanent defects in bone structure. Cancer Cell 13:249–260.

Maggio D, Barabani M, Pierandrei M, Polidori MC, Catani M, Mecocci P, Senin U, Pacifici R, Cherubini A. 2003. Marked decrease in plasma antioxidants in aged osteoporotic women: Results of a cross-sectional study. Clin Endocrinol Metab 88:1523–1527.

May SR, Ashique AM, Karlen M, Wang B, Shen Y, Zarbalis K, Reiter J, Ericson J, Peterson AS. 2005. Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli. Dev Bio 287:378–389.

McMahon AP, Ingham PW, Tabin CJ. 2003. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 53:1–114.

Meunier P, Aaron J, Edouard C, Vignon G. 1971. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. Clin Orthop 80:147–154.

Michaud EJ, Yoder BK. 2006. The primary cilium in cell signaling and cancer. Cancer Res 66:6463–6467.

Milenkovic L, Scott MP, Rohatgi R. 2009. Lateral transport of Smoothened from the plasma membrane to the membrane of the cilum. J Cell Biol 187:365–374.

Mody N, Parhami F, Sarafian TA, Demer LL. 2001. Differential effects of oxidative stress on osteoblastic differentiation of vascular and bone cells. Free Radic Res Med 31:509–519.

Nuttall ME, Patton AJ, Olivera DL, Nadeau DP, Gowen M. 1998. Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: Implications for osteopenic disorders. J Bone Miner Res 13:371–382.

Orozco LD, Kapturczak MH, Barajas B, Wang X, Weinstein MM, Wong J, Deshane J, Biolisetty S, Shaposhnik Z, Shih DM, Agarwal A, Lusis AJ, Araujo JA. 2007. Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. Circ Res 100:1703–1711.

Parhami F. 2003. Possible role of oxidized lipids in osteoporosis: Could hyperlipidemia be a rish factor? Prostaglandins Leukot Essent Fatty Acids 68:373–378.

Parhami F, Morrow AD, Balucan J, Leitinger N, Watson AD, Tintut Y, Berliner JA, Demer LL. 1997. Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation: A possible explanation for the paradox of arterial calcification in osteoporotic patients. Arterioscler Thromb Vasc Biol 17:680–687.

Parhami F, Jackson SM, Tintut Y, Le V, Balucan JP, Territo M, Demer LL. 1999. Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. J Bone Miner Res 14:2067–2078.

Parhami F, Tintut Y, Beamer WG, Gharavi N, Goodman W, Demer LL. 2001. Atherogenic high-fat diet reduces bone mineralization in mice. J Bone Min Res 16:182–188.

Richardson JA, Amantea CM, Kianmahd B, Tetradis S, Lieberman JR, Hahn TJ, Parhami F. 2007. Oxysterol-induced osteoblastic differentiation of pluripotent mesenchymal cells is mediated through a PKC- and PKA-dependent pathway. J Cell Biochem 100:1131–1145.

Rohatgi R, Scott MP. 2007. Patching the gaps in Hedgehog signaling. Nature Cell Bio 9:1005–1009.

Rohatgi R, Milenkovic L, Scott MP. 2007. Patched1 regulates Hedgehog signaling at the primary cilium. Science 317:372–376.

Rosenfeld ME. 1991. Oxidized LDL affects multiple atherogenic cellular responses. Circulation 83:2137–2140.

Shouhed D, Kha HT, Richardson JA, Amantea CM, Hahn TJ, Parhami F. 2005. Osteogenic oxysterols inhibit the adverse effects of oxidative stress on osteogenic differentiation of marrow stromal cells. J Cell Biochem 95:1276–1283.

Simons M, Gloy J, Ganner A, Bullerkotte A, Bashkurov M, Kronig C, Schermer B, Benzing T, Cabello OA, Jenny A, Mlodzik M, Polok B, Driever W, Obara T, Walz G. 2005. Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. Nat Genet 37:537–543.

Sinha S, Chen JK. 2006. Purmorphamine activates the Hedgehog pathway by targeting Smoothened. Nature Chem Biol 2:29–30.

Spinella-Jaegle S, Rawad G, Kawai S, Gallea S, Faucheu C, Mollat P, Courtois B, Bergaud B, Ramez V, Blanche AM, Adelmant G, Baron R, Roman-Roman S. 2001. Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. J Cell Sci 114:2085–2094.

Stark G. 2005. Functional consequences of oxidative membrane damage. J Membr Biol 205:1–16.

Suh JM, Gao X, McKay J, McKay R, Salo Z, Graff JM. 2006. Hedgehog signaling plays a conserved role in inhibiting fat formation. Cell Metab 3:25–34.

Taipale J, Chen JK, Cooper MK, Wang B, Mann RK, Milenkovic L, Scott MP, Beachy PA. 2000. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 406:1005–1009.

Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int J Bio Cell Biol 39:44–84.

Wong SY, Seol AD, So PL, Ermilov AN, Bichakjian CK, Epstein EH, Dlugosz AA, Reiter JF. 2009. Primary cilia can both mediate and suppress Hedgehog pathway-dependent tumorogenesis. Nature Med 15:1055–1061.