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Role of paraoxonase-1 in bone anabolic effects of parathyroid hormone in hyperlipidemic mice

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ABSTRACT

Hyperlipidemia blunts anabolic effects of intermittent parathyroid hormone (PTH) on cortical bone, and the responsiveness to PTH are restored in part by oral administration of the antioxidant ApoA-I mimetic peptide, D-4F. To evaluate the mechanism of this rescue, hyperlipidemic mice overexpressing the high-density lipoprotein-associated antioxidant enzyme, paraoxonase 1 ($Ldlr^{-/-}PON1^{tg}$) were generated, and daily PTH injections were administered to $Ldlr^{-/-}PON1^{tg}$ and to littermate $Ldlr^{-/-}$ mice. Expression of bone regulatory genes was determined by realtime RT-qPCR, and cortical bone parameters of the femoral bones by micro-computed tomographic analyses. PTH-treated $Ldlr^{-/-}PON1^{tg}$ mice had significantly greater expression of PTH receptor (PTH1R), activating transcription factor-4 (ATF4), and osteoprotegerin (OPG) in femoral cortical bone, as well as significantly greater cortical bone mineral content, thickness, and area in femoral diaphyses compared with untreated $Ldlr^{-/-}PON1^{tg}$ mice. In contrast, in control mice ($Ldlr^{-/-}$) without PON1 overexpression, PTH treatment did not induce these markers. Calvarial bone of PTH-treated $Ldlr^{-/-}PON1^{tg}$ mice also had significantly greater expression of osteoblastic differentiation marker genes as well as BMP-2-target and Wnt-target genes. Untreated $Ldlr^{-/-}PON1^{tg}$ mice had significantly greater expression of PTHR1 than untreated $Ldlr^{-/-}$ mice, whereas sclerostin expression was reduced. In femoral cortical bones, expression levels of transcription factors, FoxO1 and ATF4, were also elevated in the untreated, control $Ldlr^{-/-}PON1^{tg}$ mice, suggesting enhancement of cellular protection against oxidants. These findings suggest that PON1 restores responsiveness to PTH through effects on oxidant stress, PTH receptor expression, and/or Wnt signaling.

1. Introduction

Paraoxonase-1 (PON1), a protein component of high-density lipoprotein (HDL), is synthesized mainly in the liver, with lower expression found in lung, heart, brain, kidney and small intestine [1]. It functions as an antioxidant by hydrolyzing substrates, including lipid peroxides, which are atherogenic agents [2]. In vivo studies suggest that it retards the development of atherosclerosis [3,4]. Epidemiologically, serum levels of PON1 activity correlate inversely with levels of oxidized fatty acids [5]. PON1-deficient mice are also more prone to oxidant stress [6]. We and others have previously shown that transgenic mice overexpressing human PON1 have lower levels of oxidant stress and oxidized lipoprotein particles [3,7].

Lipid oxidation products trigger a cascade of inflammatory reactions responsible for the pathogenesis of cardiovascular diseases, particularly atherosclerosis [8–10]. Accumulating evidence, including the finding that metabolites released from osteoblasts induce nonenzymatic oxidation of lipoproteins [11], now suggests that hyperlipidemia and oxidant stress may also contribute to similar pathological events resulting in bone loss. Levels of oxidized lipids were increased in the bone marrow of hyperlipidemic mice fed a high-fat diet [12]. Such diets also induce bone loss in hyperlipidemic mice [12–14]. Histochemical evidence demonstrates accumulation of lipids in the subendothelial space of Haversian canals in human osteoporotic bone [15] and in osteocytic canaliculae [16,17]. Modulation of oxidative stress in mature osteoblasts in mice also affects bone mass homeostasis [18,19]. In vitro studies have shown that oxidized lipids inhibit osteoblastic differentiation of preosteoblasts [20,21] and promote osteoclastic potential in bone marrow-derived preosteoclasts

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Table 1
Serum analysis of lipid oxidation products and PON1 activity.

Parameters	*Ldlr*$^{-/-}$		*Ldlr*$^{-/-}$*PON1*^{tg}	
	Control	PTH	Control	PTH
Serum oxidized lipid levels (μg/ml)	4.50 ± 0.26	4.38 ± 0.28	3.73 ± 0.43	3.57 ± 0.17[a]
Serum arylesterase activity (MOD/min/μl serum)	544 ± 28	431 ± 12[b]	1102 ± 100[c]	1472 ± 75[b,c]

[a] $p < 0.05$ *vs.* *Ldlr*$^{-/-}$ control.
[b] $p < 0.01$ *vs.* respective controls.
[c] $p < 0.001$ *vs.* *Ldlr*$^{-/-}$ control.

[15,22]. These findings suggest that oxidized lipids promote bone loss through both decreased formation and increased resorption of mineralized matrix.

Intermittent administration of PTH has robust anabolic effects on bone [23,24]. We previously found that PTH effects, especially on cortical bone, are blunted in mice with hyperlipidemia [25]. In subsequent studies, we demonstrated that administration of an ApoA-I mimetic peptide, D-4F, which reduces plasma levels of oxidized lipids, restores the bone anabolic effects of PTH in cortical bones of hyperlipidemic low-density receptor null (*Ldlr*$^{-/-}$) mice [26]. In this report, we tested PTH anabolism in *Ldlr*$^{-/-}$ mice overexpressing human PON1. Results show that PON1 overexpression rescues PTH resistance in hyperlipidemic mice.

2. Materials and methods

2.1. Animals

Mice overexpressing human PON1 (C57BL/6 background), generated as previously described [7], were crossed with *Ldlr*$^{-/-}$ mice (C57BL/6 background; the Jackson Laboratory, Bar Harbor, ME) to generate *Ldlr*$^{-/-}$*PON1*^{tg} mice. The pups were verified by genotyping for presence of PON1, as described previously [7] and absence of LDL receptor genes, using protocols established by the Jackson Laboratory. Three month-old male littermates of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*PON1*^{tg} mice were divided into four groups: control *Ldlr*$^{-/-}$ ($n = 7$), PTH-treated *Ldlr*$^{-/-}$ ($n = 8$), control *Ldlr*$^{-/-}$*PON1*^{tg} ($n = 5$), and PTH-treated *Ldlr*$^{-/-}$*PON1*^{tg} ($n = 8$). The mice received either control vehicle or hPTH (1–34; 40 μg/kg, s.c.) for 4 weeks, as previously described [25,26]. All the experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California at Los Angeles.

2.2. MicroCT analysis

Right femoral bones were harvested and analyzed for bone mineral content, cortical thickness, and cortical bone area by microCT (Skyscan 1172, Aartselaar, Belgium), as we previously described [12]. For cortical analysis at the mid-diaphysis, the length of each femoral bone was measured, and 40 mid-diaphyseal slices were used. For trabecular analysis, 200 slices at the proximal growth plate were used. The data were collected at 55 kVp and 72 μA at a resolution of 12 μm, and volumetric analysis was performed using Skyscan software.

2.3. Realtime RT-qPCR

Total RNA was isolated from the calvaria and femurs. Real-time RT-qPCR was performed using the One-Step RT-qPCR SuperMix Kit (BioChain, Inc.) and Mx3005P Real-Time PCR System (Stratagene) [27–29].

2.4. Serum analyses

Serum lipid oxidation products were assayed by a fluorescence indicator-based assay [30] using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen), which is sensitive to reactive oxygen species such as lipid oxidation products. Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (ox-PAPC) was used to generate a standard curve. PON1 activity in the serum was assessed by arylesterase activity, as previously described [7].

2.5. Statistical analysis

The effects of PTH were evaluated by comparing all the groups using ANOVA, followed by Fisher's PLSD. Interaction effects were analyzed by two-way ANOVA, followed by Fisher's PLSD. Values were expressed as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects on levels of serum lipid oxidation products

Levels of lipid oxidation products were assessed in hyperlipidemic (*Ldlr*$^{-/-}$) and hyperlipidemic mice overexpressing PON1 (*Ldlr*$^{-/-}$*PON1*^{tg}), as described previously [30]. Results showed that *Ldlr*$^{-/-}$*PON1*^{tg} mice receiving intermittent PTH had significantly

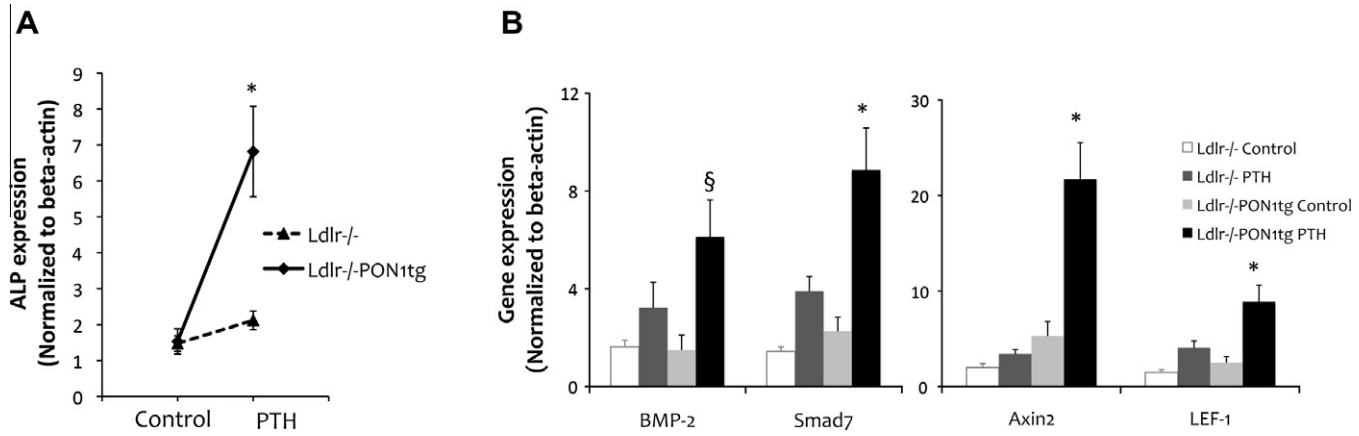

Fig. 1. Effects on osteoblastic gene expression. Realtime RT-qPCR analysis of RNA isolated from calvarial bone for osteoblastic markers and regulators, (A) alkaline phosphatase (ALP), (B) BMP-2 and its target gene, Smad7, and Wnt-target genes, Axin-2 and LEF-1, in *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*PON1*^{tg} mice treated with control vehicle or intermittent PTH for 4 weeks. Values were expressed as mean ± SEM. $^{*}p < 0.005$ *vs.* *Ldlr*$^{-/-}$ (both control and PTH) and *Ldlr*$^{-/-}$*PON1*^{tg} control; $^{\S}p < 0.01$ *vs.* control *Ldlr*$^{-/-}$ and control *Ldlr*$^{-/-}$*PON1*^{tg}.

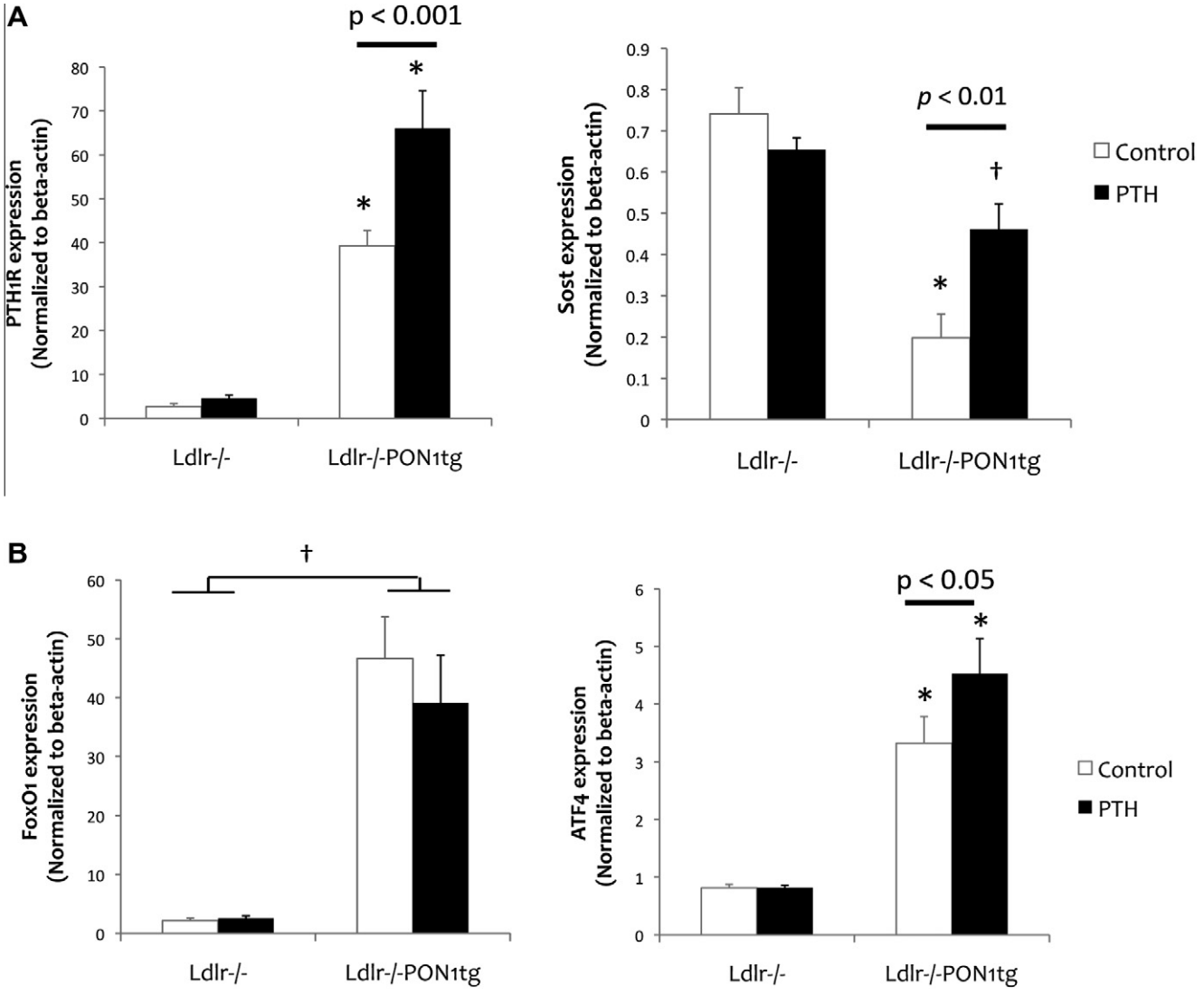

Fig. 2. Effects on osteocytic gene expression. Realtime RT-qPCR analysis of RNA isolated from femoral cortical bone, devoid of bone marrow, for (A) PTH1R and Sost, (B) FoxO1, and ATF4 expression. Values were expressed as mean ± SEM. $^{*}p < 0.001$ *vs.* $Ldlr^{-/-}$ (both control and PTH); $^{\dagger}p < 0.05$ *vs.* $Ldlr^{-/-}$ (both control and PTH).

lower levels of serum lipid oxidation products compared with control $Ldlr^{-/-}$ mice (Table 1). As expected, $Ldlr^{-/-}PON1^{tg}$ had 2-fold higher serum PON1 (arylesterase) activity compared with control $Ldlr^{-/-}$ mice (Table 1). Interestingly, intermittent PTH treatment further increased PON1 activity in $Ldlr^{-/-}PON1^{tg}$, whereas it reduced PON1 activity in control $Ldlr^{-/-}$ mice (Table 1).

3.2. Effects on osteoblastic gene expression

We previously found that induction of osteoblastic gene expression was blunted in calvarial tissues of hyperlipidemic compared with WT mice in response to intermittent PTH [25,27]. To test whether PTH induction of these genes is restored by PON1 overexpression, calvarial tissues were harvested, and realtime RT-qPCR was performed. Results showed that expression of osteoblastic differentiation markers, alkaline phosphatase (Fig. 1A) and bone sialoprotein (data not shown), were greater with PTH treatment in $Ldlr^{-/-}PON1^{tg}$ but not in $Ldlr^{-/-}$ mice. PTH and PON1 each had a significant main effect as well as a significant interaction effect on expression of the osteoblastic markers, based on two-way ANOVA.

Since PTH upregulates bone morphogenetic protein 2 (BMP-2) and Wnt signaling in osteoblasts [31,32], we further tested the effects of PON1 overexpression on these potent morphogens and their signaling pathways. Expression of BMP-2 and its downstream target Smad7, as well as the Wnt-target genes, axis inhibition protein 2 (Axin2) and lymphoid enhancer-binding factor 1 (LEF-1), was significantly induced by intermittent PTH treatment in $Ldlr^{-/-}PON1^{tg}$ but not in $Ldlr^{-/-}$ mice (Fig. 1B).

3.3. Effects on osteocytic gene expression

We previously found that gene expression in the osteocyte-rich cortical bones is affected by oxidant stress [12]. Therefore, we tested the effect of PON1 on expression of osteocyte-related genes, PTH receptor 1 (PTH1R) and sclerostin (Sost), using RNA isolated from femoral bones after removal of the marrow. Results showed that, in mice without PTH treatment, PTH1R expression was significantly greater in $Ldlr^{-/-}PON1^{tg}$ mice than in $Ldlr^{-/-}$ mice (Fig. 2A, left), whereas expression of Sost, a major antagonist of Wnt signaling, was significantly less in $Ldlr^{-/-}PON1^{tg}$ mice than in $Ldlr^{-/-}$ mice (Fig. 2A, right). Intermittent PTH treatment significantly induced both PTH1R and Sost expression in cortical bones of $Ldlr^{-/-}PON1^{tg}$ mice, but not of $Ldlr^{-/-}$ mice (Fig. 2A).

To assess effects of PON1 overexpression on cellular resistance to oxidant stress in femoral bone, expression levels of the

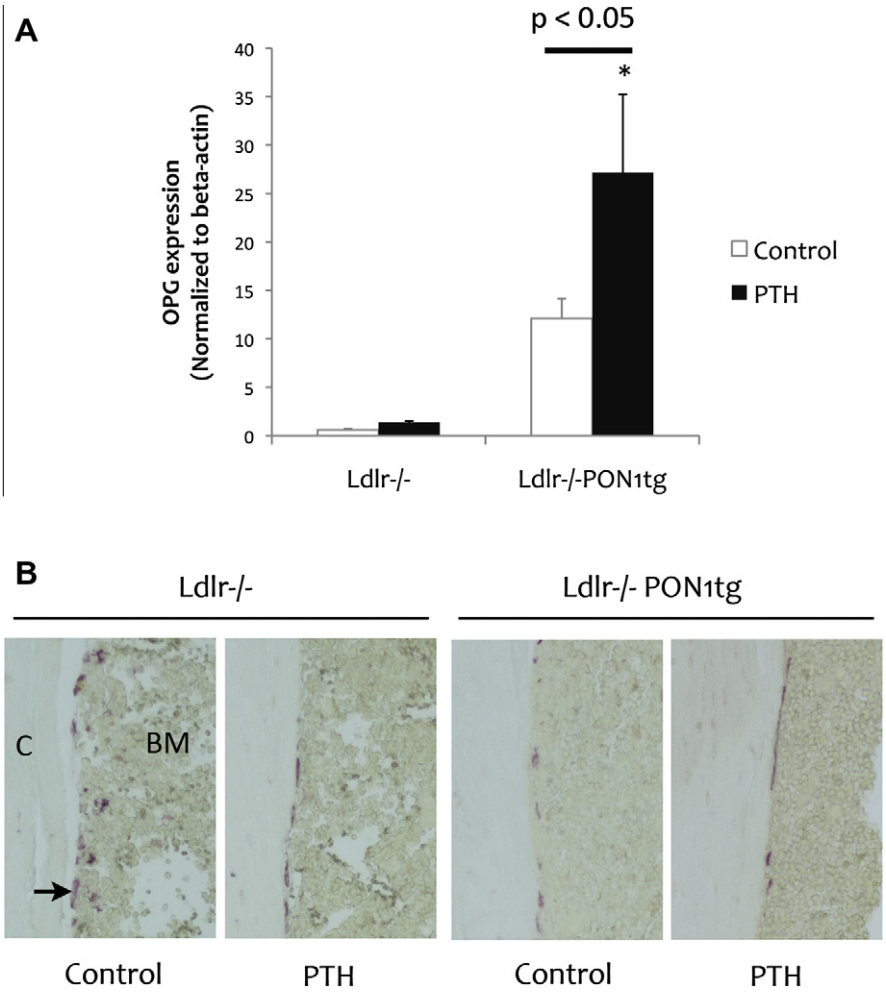

Fig. 3. Effects on osteoclastic activity. (A) Realtime RT-qPCR analysis of RNA isolated from femoral cortical bone for OPG in *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*PON1*^{tg} mice treated with control vehicle or intermittent PTH for 4 weeks. (B) TRAP positive osteoclasts (arrow) in the endocortical envelope of femoral bones. (C, cortex; BM, bone marrow; magnification 20×).

Table 2
Summary of microCT analysis of femoral bone.

Parameters	*Ldlr*$^{-/-}$		*Ldlr*$^{-/-}$*PON1*^{tg}	
	Control	PTH	Control	PTH
Cortical BMC (mg)	0.529 ± 0.023	0.564 ± 0.015	0.469 ± 0.008[c]	0.545 ± 0.014[b]
Cortical thickness (mm)	0.111 ± 0.002	0.116 ± 0.003	0.105 ± 0.002	0.113 ± 0.001[a]
Cortical area (mm^2)	0.893 ± 0.037	0.954 ± 0.024	0.793 ± 0.010[c]	0.913 ± 0.023[a]
Trabecular BMC (mg)	0.102 ± 0.017	0.079 ± 0.011	0.059 ± 0.003	0.063 ± 0.011
Trabecular thickness (mm)	0.055 ± 0.001	0.063 ± 0.002[b]	0.053 ± 0.000	0.061 ± 0.001[b]
Trabecular number (1/mm)	2.573 ± 0.144	2.147 ± 0.104[a]	2.138 ± 0.084	1.963 ± 0.141

BMC, bone mineral content.
[a] $p < 0.05$ *vs.* respective controls.
[b] $p < 0.001$ *vs.* controls.
[c] $p < 0.05$ *vs.* *Ldlr*$^{-/-}$ control. Values are expressed as mean ± SEM.

transcription factor forkhead box O1 (FoxO1) and its binding partner, activating transcription factor 4 (ATF4), were assessed. Results showed that expression levels of both FoxO1 and ATF4 were significantly greater in *Ldlr*$^{-/-}$*PON1*^{tg} than in control *Ldlr*$^{-/-}$ mice (Fig. 2B). PTH treatment further induced ATF4 expression in *Ldlr*$^{-/-}$*PON1*^{tg} mice (Fig. 2B, right).

To assess whether PON1 overexpression has the potential to block bone resorptive activity, we assessed expression of the osteoclast inhibitory cytokine, osteoprotegerin (OPG), which plays a central role in osteocyte-mediated bone remodeling and bone loss [33]. Basal OPG expression trended toward higher levels in cortical bone of *Ldlr*$^{-/-}$*PON1*^{tg} mice compared with that of control *Ldlr*$^{-/-}$ mice ($p = 0.07$; Fig. 3A). Intermittent PTH treatment further induced OPG expression in *Ldlr*$^{-/-}$*PON1*^{tg} mice (Fig. 3A). Histochemical staining for tartrate-resistant acid phosphatase (TRAP), an osteoclast-specific enzyme, showed that, at the endocortical

envelope of femoral bones, bone resorptive activity was significantly reduced by PON1 overexpression as well as by PTH treatment (Fig. 3B).

3.4. In vivo effects on cortical and trabecular bone parameters

We previously found that bone anabolic effects of PTH were differentially affected in cortical *vs.* trabecular bones in hyperlipidemic mice [25]. In cortical bone, PTH-induced bone mineral content, area, and thickness were significantly blunted, whereas its adverse effects on trabecular bone parameters were less [25]. MicroCT analysis of the femoral bones showed that intermittent PTH significantly induced bone mineral content, cortical thickness, and bone area in $Ldlr^{-/-}PON1^{tg}$ mice (Table 2). Consistent with our previous findings [25], PTH did not significantly increase these parameters in control $Ldlr^{-/-}$ mice (Table 2). Trabecular bone mineral content was not significantly affected, whereas trabecular thickness was significantly induced by PTH in both groups (Table 2).

4. Discussion

We previously showed that oxidized lipids blunt bone anabolic effects of intermittent PTH treatment [25,26]. We further showed that oral administration of the ApoA-I mimetic peptide, D-4F, which has antioxidant and anti-inflammatory properties, rescues at least partially the anabolic effects of intermittent PTH treatment in hyperlipidemic mice [26]. In this report, we found that responsiveness to PTH in cortical bone was restored in hyperlipidemic mice overexpressing PON1, which protects against lipoprotein oxidation through hydrolysis of lipid peroxides. Consistent with our previous findings with oral D-4F [25], cortical bone parameters, bone mineral content, thickness, and area were increased by intermittent PTH treatment in $Ldlr^{-/-}PON1^{tg}$ mice. Our findings are also consistent with those of Jilka and colleagues, who showed that anabolic efficacy of intermittent PTH treatment in older mice is, in part, due to a novel effect of PTH – antagonism of the age-associated increase in oxidative stress [34].

Our findings suggest that one possible mechanism for effects of PON1 is attenuation of oxidant stress in $Ldlr^{-/-}PON1^{tg}$ mice. Redox balance in osteoblasts is regulated in large part by the transcription factor, FoxO1, which is expressed in the endosteum and periosteum, and its interaction with ATF4 [19]. In the present study, we found that basal expression levels of both FoxO1 and ATF4 were increased in cortical bones of $Ldlr^{-/-}PON1^{tg}$ mice, suggesting that cellular defenses against oxidant stress may be retained in these transgenic mice. Consistent with findings of Yu and colleagues, in the present study, intermittent PTH treatment induced ATF4 expression in $Ldlr^{-/-}PON1^{tg}$ but not in $Ldlr^{-/-}$ mice [35].

A second possible mechanism for the effects of PON1 is upregulation of the Wnt signaling pathway in $Ldlr^{-/-}PON1^{tg}$ mice. Sost is a major antagonist of Wnt signaling, and the loss of Sost expression is permissive for endocortical bone formation [36]. In our study, we found that PON1 overexpression led to decreased Sost expression in cortical bone and increased expression of Wnt target genes in calvaria of $Ldlr^{-/-}PON1^{tg}$ mice.

A third possible mechanism for the effects of PON1 is upregulation of PTH1R. Our previous findings showed that oxidized lipids inhibit PTH1R expression in calvarial cells [27] and that oxidant stress reduces basal expression of PTH1R in cortical bones of hyperlipidemic mice [12]. In this study, hyperlipidemic mice expressing the transgene had higher basal expression of PTH1R. Consequently, expression of osteoblastic markers was upregulated by PTH only in $Ldlr^{-/-}PON1^{tg}$ mice. In addition, BMP-2 and its downstream target, Smad7, were upregulated by PTH in calvaria of $Ldlr^{-/-}PON1^{tg}$ mice. This is consistent with our previous findings that PTH enhances BMP-2 in calvarial preosteoblastic MC3T3-E1 cells [37].

In summary, our findings suggest that reducing oxidant stress in hyperlipidemic mice may restore bone anabolic responsiveness to PTH, in part, through one or more of three possible mechanisms, including inhibition of oxidant stress, induction of PTH1R, and induction Wnt signaling pathways.

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