Research

Oxidized Phospholipids Regulate Interleukin-6 Expression via the Protein Kinase A and Extracellular Signal-regulated Pathways in Osteoblasts

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Epidemiological evidence suggests a relationship between cardiovascular disease and osteoporosis, a disease where bones become fragile due to imbalance between bone resorption and formation. We previously found that differentiation of bone-forming osteoblasts is inhibited by oxidized lipoproteins, which trigger the pathogenesis of atherosclerosis. Since differentiation of bone-resorbing osteoclasts depends on cytokines produced by osteoblasts, we hypothesize that oxidized phospholipids may also regulate bone resorption by altering osteoblastic expression of such cytokines, including interleukin-6 (IL-6). This study investigated the regulatory mechanism of IL-6 expression by oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (ox-PAPC), an active component of oxidized lipoproteins. In murine calvarial preosteoblasts, ox-PAPC increased IL-6 mRNA expression and activated IL-6 promoter luciferase reporter constructs. Protein kinase A (PKA) inhibitor H89 reduced ox-PAPC induction of IL-6, whereas mutations in the binding sites for nuclear factor κB (NF-κB) or CCAAT/enhancer binding protein (C/EBP) transcription factors did not attenuate ox-PAPC-induced IL-6 promoter activity. These results suggest that PKA, but not NF-κB or C/EBP, mediates the ox-PAPC effect. Western blot analysis showed that ox-PAPC also activated extracellular signal-regulated kinase (ERK). However, ERK inhibition further increased ox-PAPC induction of IL-6, while ERK activation decreased PKA activation of IL-6, suggesting that the ERK pathway antagonizes the PKA pathway to inhibit IL-6 expression. Together, the data suggest oxidized phospholipids increase IL-6 expression in osteoblasts by activating the PKA and ERK pathways, implicating the osteoclast-regulating cytokine IL-6 as a possible link between cardiovascular disease and osteoporosis.

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INTRODUCTION

Although commonly viewed as having distinct etiologies, cardiovascular disease has been associated with osteoporosis, often coexisting in patients independent of age (Hamerman, 2005). High serum lipid levels, one of the risk factors for atherosclerosis, may contribute to osteoporosis, a disease where patients suffer from decreased bone mineral density and subsequently have an increased risk for developing fractures. An estimated 63 percent of osteoporotic patients exhibit hyperlipidemia (cholesterol > 130 mg/dL), according to the 1988 to 1994 National Health and Nutrition Examination Survey III. More specifically, epidemiological studies show that plasma low-density lipoprotein (LDL) levels inversely correlate with bone mineral density in post-menopausal women (Yamaguchi et al., 2002). Likewise, post-menopausal women with high plasma LDL levels are more susceptible to osteopenia, a less severe form of osteoporosis (Poli et al., 2002). As suggested in humans, in vivo studies indicate that mice fed with an atherogenic high-fat diet have lower bone density compared to those fed a normal chow-diet (Parhami et al., 2001). Together these studies indicate a relationship between serum lipid levels and low bone mineral density.

In the vasculature, high circulating plasma LDL particles have an increased susceptibility to become inflammatory lipids when they accumulate in the subendothelial matrix of arteries and subsequently undergo oxidative modification. These oxidized lipoproteins and phospholipids induce the production of inflammatory cytokines and chemokines, triggering the pathogenesis of atherosclerosis. One active component of biologically active LDL that is known to cause inflammatory responses and atherosclerosis both in vitro and in vivo is oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (ox-PAPC) (Watson et al., 1997; Furnkranz et al., 2005).

Not only are oxidized lipids levels elevated in the vasculature, studies also suggest their presence in the bone. Previously, we found lipid deposits within the perivascular space of Haversian canals in osteoporotic patients' cortical bone (Tintut et al., 2004). In addition to identifying lipids in osteoporotic bone, oxidized lipids were detected in the bone marrow of hyperlipidemic mice (Tintut et al., 2004). Interestingly, osteoblasts, the cell type that produces bone, were recently shown to have the capability of oxidizing LDL particles and thereby possibly increasing the local concentration of oxidized LDL (Brodeur et al., 2008). Together these studies suggest the presence of oxidized lipids within the bone.

Besides triggering the development of atherosclerosis in the vasculature, oxidized lipids also regulate the differentiation of both osteoblasts and osteoclasts, which are cells responsible for bone resorption. Oxidized phospholipids decrease the expression of osteoblastic differentiation markers and the formation of mineralized nodules, which are characteristics of bone formation in vitro (Parhami et al., 1997). Oxidized phospholipids, specifically ox-PAPC, attenuate osteoblastic differentiation induced by anabolic agents, such as parathyroid hormone and bone morphogenetic protein-2 both in vitro and in vivo (Huang et al., 2007; Huang et al., 2008). On the other hand, both oxidized phospholipids and hyperlipidemia promote osteoclastic potential in bone marrow preosteoclasts, although the mechanism underlying this effect remains elusive (Tintut et al., 2004; Tintut et al., 2002).

In bone, osteoblasts not only synthesize bone matrix, but also produce cytokines required for the differentiation and maturation of osteoclasts (Rodan, 1992). However,

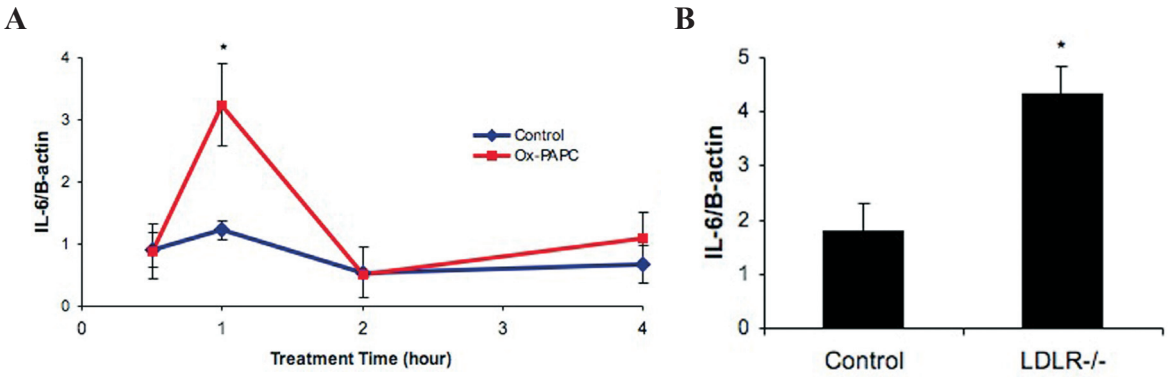

Figure 1. Ox-PAPC upregulates IL-6 mRNA expression. (A) RT-qPCR analysis of total RNA isolated from cells treated with control vehicle or ox-PAPC (40 µg/ml). Beta-actin expression was used as a normalization gene (n = 4). **(B)** RT-qPCR analysis of total RNA isolated from calvarial tissues of control wildtype (C57BL/6) and hyperlipidemic (LDLR-/-) mice (n=4 control mice; n=5 LDLR-/- mice). *, $p<0.05$ vs. control. This Fig. 1B is a part of Fig. 1A in an article by Huang et al. published in J Bone Miner Res (2008).

elevated levels of osteoclast-regulating cytokines have been linked to post-menopausal bone loss (Pfeilschifter et al., 2002). One such cytokine, interleukin-6 (IL-6), has been shown to stimulate osteoclastic differentiation and activity in bone marrow cells (Ishimi et al., 1990). Further, IL-6 has been shown to mediate parathyroid hormone-induced bone resorption in vivo (Grey et al., 2002). It has also been implicated in contributing to the increased sensitivity to bone loss that is often observed in peri-menopausal or estrogen-deficient women who concurrently suffer from primary hyperthyroidism (Masiukiewicz et al., 2002; Insogna et al., 2002). These studies suggest that higher productions of cytokines that promote osteoclast differentiation and activation may play a role in osteoporosis.

As a result, we hypothesize that oxidized phospholipids contribute to osteoporosis not only by inhibiting differentiation of osteoblasts, but also by promoting osteoblast production of osteoclast-regulating cytokines. This current study explores the effect of ox-PAPC on IL-6 expression in pre-osteoblasts. Data indicate that ox-PAPC induces IL-6 mRNA levels in MC3T3-E1, a cell line of murine calvarial pre-osteoblasts, as assessed via RT-qPCR. Importantly, this increase in mRNA expression was found to be due to an increase in IL-6 transcriptional activity as assessed through promoter analysis. Finally, we investigate the regulatory mechanism behind ox-PAPC induction of IL-6. Our results provide understanding of the molecular basis in which oxidized phospholipids may contribute to osteoporosis in addition to cardiovascular disease.

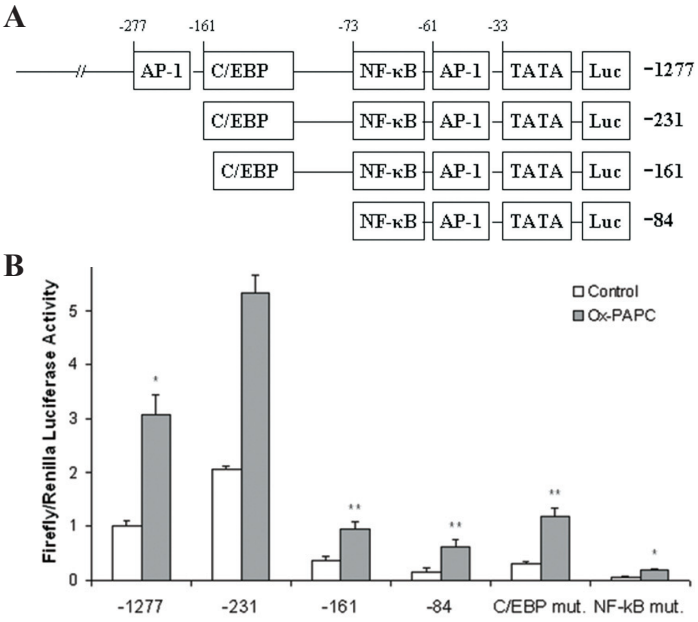

Figure 2. Ox-PAPC induces IL-6 promoter activity. (A) A schematic diagram of IL-6 full-length (-1277) and 5' deletion constructs (-231, -161, -84), generated by Baccam et al. (2003) and provided by Dr. Gail Bishop, University of Iowa. Transcriptional response elements are indicated: AP-1, activator protein-1; C/BEP, CCAAT/enhancer binding protein; NF-κB, nuclear-factor kappa-B. A CRE or cAMP-response element lies between the 231-bp and 161-bp region. **(B)** IL-6 promoter analysis of MC3T3-E1 cells transiently transfected with the indicated constructs and treated with vehicle or ox-PAPC (40 μg/ml) for 7 hr. (n = 3); *, p<0.01, **, p<0.05 to respective vehicle treatment.

METHODS

Materials MC3T3-E1 cells were obtained from Riken Cell Bank (Japan). Ox-PAPC was prepared from auto-oxidizing PAPC from Avanti-Polar Lipids, and its oxidation state was verified by Liquid Chromatography/Mass Spectroscopy. H89 (PKA inhibitor), PD98059 (ERK inhibitor), and forskolin (PKA agonist) were obtained from Calbiochem, while Y-27632 (ROCK II inhibitor) was from Cayman Chemical.

Cell Culture MC3T3-E1 is a murine calvarial preosteoblastic cell line. MC3T3-E1 cells were plated at 3 x 105 cells/well in 6-well plates for RNA analysis or 5 x 104 cells/well in 24-well plates for transfection. Cells were maintained in α-minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and sodium pyruvate. Media was replenished every 3 to 4 days. Treatment media consisted of α-minimum essential medium without ascorbic acid (Gibco) supplemented with 1% fetal bovine serum.

Animals 15-week-old wild-type and LDLR-/- mice (all C57BL/6 background) were obtained from the Jackson Laboratory (Bar Harbor, ME). Tissue from the calvaria, the skull, was isolated for RNA isolation and real time RT-qPCR. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of California at Los Angeles. Animals were given a standard Purina Chow diet.

RNA Isolation and Real-Time RT-qPCR Total RNA was isolated from MC3T3-E1 cells using TRIzol reagent (Invitrogen). DNase-treated RNA was reverse-transcribed using Omniscript RT Kit (Qiagen), and real-time PCR was performed using the Mx3005P Real-Time PCR System (Stratagene). In animal studies, total RNA was isolated from the calvaria and tibia of C57BL/6 and LDLR-/- mice. Real-Time RT-qPCR was performed using the One-Step qRT-PCR SuperMix Kit (BioChain, Inc.) and Mx3005P Real-Time PCR System. Sequences for the primers are as follows: β-actin (sense)

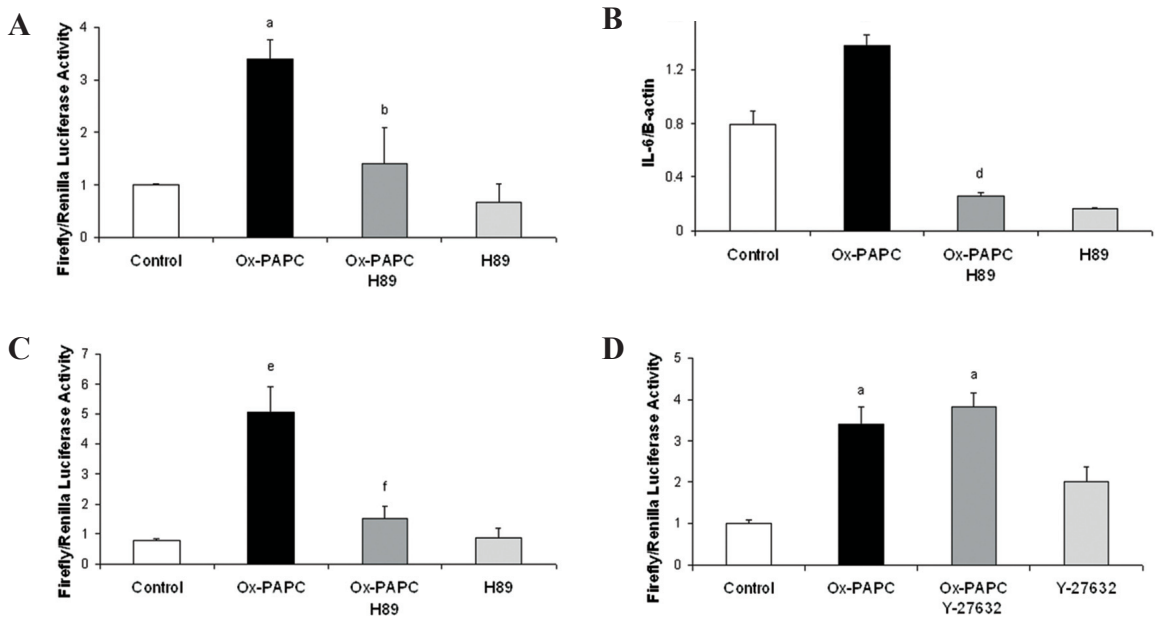

Figure 3. PKA mediates ox-PAPC induction of IL6. (A) IL-6 promoter analysis of cells transfected with full-length (-1277) construct and treated for 7 hr with vehicle, ox-PAPC (40 µg/mL) and/or H89 (10 µM), as indicated. (n=3); a, $p<0.01$ vs. control; b, $p<0.05$ vs. ox-PAPC. **(B)** RT-qPCR analysis of total RNA isolated from cells treated for 1 hr with vehicle, ox-PAPC (40 µg/mL) and/or H89 (10 µM), as indicated. (n = 3); c, $p<0.05$ vs. control; d, $p<0.0001$ vs. ox-PAPC. **(C)** IL-6 promoter analysis of cells transfected with the -84 promoter construct and treated for 7 hr with vehicle, ox-PAPC (40 µg/ml) and/or H89 (10 µM), as indicated. (n = 2); e, $p<0.05$ vs. control; f, $p<0.05$ vs. ox-PAPC. **(D)** IL-6 promoter analysis of cells transfected with the -1277 promoter construct and treated for 7hr with vehicle, ox-PAPC (40 µg/mL) and/or Y-27632 (10 µM), as indicated. Y-27632 was used to rule out the nonspecific inhibition of H89 on ROCK-II. (n = 1); a, $p<0.01$ vs. control. The results suggest that inhibition of PKA but not ROCK-II attenuated ox-PAPC induction of IL-6.

5'-AGAGGGAAATCGT-GCGTGAC-3',(antisense) 5'-CAATAGTGAT-GACCTGGCCGT-3'; IL-6 (sense) 5'-TGTATGAAC-AACGATGATGCACTT-3', (antisense) 5'-GGTACTCCAGAAGACCAGAGGAAAT-3'.

DNA Construct The pmIL-6.luc(-1277) plasmid, a promoter-reporter construct containing the full-length (1277bp) promoter region of murine IL-6 driving the expression of the Firefly luciferase gene as a reporter, as well as three 5' truncated IL-6 promoter-reporter constructs, pmIL-6.luc(-231), pmIL-6.luc(-161), pmIL-6.luc(-84), were gifts from Dr. Gail Bishop from the University of Iowa (Baccam et al., 2003). In addition, two constructs containing mutations in the putative binding site of C/EBP (-161 to -147) or NF-κB (-73 to -64) of the full-length murine IL-6 promoter were also gifts from Dr. Bishop. Sequences of the plasmids were verified upon receipt. The phRL.TK plasmid, containing a Thymidine-Kinase promoter to encode Renilla luciferase, was utilized for the normalization of transfection efficiency.

Transient Transfections and Luciferase Reporter Assay Twenty-four hours after plating, MC3T3-E1 cells were transfected with a total of 0.204µg of DNA (0.2µg of reporter plasmid, 0.004µg of phRL.TK) with Effectene Transfection Reagent (Qiagen) according to the manufacturer instructions. Following transfection, cells were allowed to recover in serum-containing media for 24 hr. Cells were then treated in treatment media for 7 hr and then harvested. Firefly and Renilla luciferase levels were measured using Dual-Luciferase Reporter Assay (Promega).

Western Analysis Five days after plating, cells were treated with ox-PAPC in serum-free media, and whole cell lysates were prepared using lysis buffer containing phosphatase and protease inhibitors. Western analysis was performed using standard protocols. The blots were probed with antibody to phosphorylated ERK or total ERK (Cell Signaling) at a 1:1000 dilution.

Data Analysis Each experimental condition was performed in minimum triplicate wells, and the number of independent experiments (n) is indicated in legend. Data are expressed as mean ± Standard Error of Mean (SEM) of a representative experiment. Results were compared using a two-tailed paired Student's t test. In comparisons across all groups, a two-way ANOVA, followed by Fisher's protected

least significant difference (PLSD), was performed using StatView (v4.5, Abacus, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Ox-PAPC upregulates IL-6 mRNA expression To determine the effect of oxidized phospholipids on IL-6 expression, murine preosteoblastic MC3T3-E1, an established cell culture model to study osteogenesis (Peterson et al., 2004), were treated with ox-PAPC at various time points. Quantitative real-time RT-qPCR showed that ox-PAPC significantly induced IL-6 expression by 2.8-folds at 1 hr (Figure 1A). Consistent with our in vitro finding, IL-6 expression in calvarial tissues of hyperlipidemic mice (Huang et al., 2008; Cyrus et al., 2001), which has increased serum oxidized lipids, was 2.4-fold higher than wild-type mice (Fig. 1B). These findings suggest that oxidized lipids upregulate IL-6 expression in osteoblasts.

Region of the IL-6 promoter required for ox-PAPC activation After demonstrating that ox-PAPC increased IL-6 mRNA expression, we investigated whether this induction was at the level of transcriptional activation using a construct, pmIL-6.luc(-1277), containing the Firefly luciferase reporter gene under the transcriptional control of the IL-6 promoter (1277 bases upstream of the transcriptional start site) (Figure 2A) (Baccam et al., 2003). Promoter-reporter analysis showed that ox-PAPC significantly increased the luciferase activity by 3.5-fold (Figure 2B), suggesting that ox-PAPC regulates IL-6 expression at the transcriptional level.

To delineate the region of the IL-6 promoter that ox-PAPC regulates, three IL-6 promoter-reporter constructs with 5' truncations, pmIL-6.luc(-231), pmIL-6.luc(-161), pmIL-6.luc(-84), were employed. Results showed that ox-PAPC significantly activated all three constructs, suggesting that the ox-PAPC regulatory element is present within 84 bp upstream of the transcriptional start site (Figure 2B).

Upon closer examination, results show that ox-PAPC activated the 231-bp IL-6 promoter fragment to a higher level than the full-length IL-6 promoter. However, basal activity of the 231-bp region was increased 2-fold compared to the basal activity of the full-length promoter, possibly due to the presence of an inhibitory response element between the 1277- and 231-bp regions. As a result, this elevated basal activity in the 231-bp region accounts for the seeming increased ox-PAPC-induced IL-6 promoter activity. In fact, further analysis reveals that ox-PAPC only induces this 231-bp fragment by 2-folds compared to vehicle treatment, as opposed to the 3-fold induction by ox-PAPC in the full-length IL-6 promoter construct compared to control.

Transcription factors NF-κB and C/EBP are not involved in ox-PAPC induction of IL-6 At least four different transcription factors, NF-κB, AP-1, C/EBP, and CREB, have been reported to regulate IL-6 transcription with varying effects, depending on the cell and stimulus (Baccam et al., 2003; Motomura et al., 1998). Here the roles of C/EBP and NF-κB in ox-PAPC induction of IL-6 were evaluated. Promoter reporter analysis showed that mutations in the NF-κB or C/EBP binding sites of the full-length IL-6 promoter did not attenuate the ox-PAPC-induced IL-6 promoter activity, suggesting that nuclear factor κB (NF-κB) and CCAAT/enhancer binding protein (C/EBP) are not involved in the ox-PAPC effect (Fig. 2B). Interestingly, mutations in the NF-κB response element nearly abrogated IL-6 promoter basal activity, consistent with reports that NF-κB is necessary for IL-6 basal

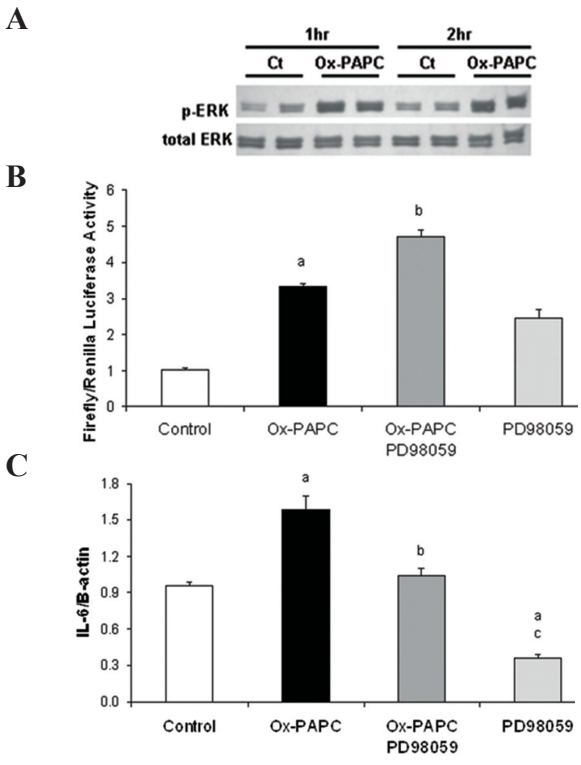

Figure 4. ERK inhibits ox-PAPC induction of IL6. (A) Western analysis of phosphorylated ERK (p-ERK) in MC3T3-E1 cells treated with ox-PAPC (10μg/ml) for 1hr and 2hr. Total ERK was used as a loading control. (n = 3) **(B)** IL-6 promoter analysis of MC3T3-E1 cells transfected with the -1277 construct and treated for 7hr with vehicle, ox-PAPC (40μg/ml) and/or PD98059 (5μM). **(C)** RT-qPCR analysis of total RNA isolated from cells treated for 6 days with vehicle, ox-PAPC (40 μg/mL) and/or PD98059 (5μM). Note that ox-PAPC was able to induce IL-6 in the presence of PD98059 (compare PD98509 vs. ox-PAPC+PD98059. (n = 2); a, $p<0.0001$ vs. control; b, $p<0.0001$ vs. ox-PAPC; c, $p<0.0001$ vs. ox-PAPC+PD98059.

expression in MC3T3-E1 cells (Chen et al., 2004).

Protein kinase A mediates ox-PAPC induction of IL-6 Next, signal transduction pathways employed by ox-PAPC for IL-6 induction were explored. Previously, ox-PAPC has been shown to increase protein kinase A (PKA) activity in human pulmonary artery endothelial cells (Birukov et al., 2004). Furthermore, the cAMP/PKA pathway has been shown to mediate parathyroid hormone activation of IL-6 in murine calvarial preosteoblasts (Huang et al., 2007). Therefore, PKA inhibitor H89 was used to examine whether the PKA pathway is involved in ox-PAPC regulation of IL-6. Results showed that H89 significantly reduced ox-PAPC induction of IL-6 promoter activity by 53% at both 5μM and 10μM, suggesting that the PKA pathway mediates ox-PAPC effects (Figure 3A). Similarly, RT-qPCR showed that H89 attenuated IL-6 mRNA level (Figure 3B). Since the 84-bp promoter region contains ox-PAPC regulatory elements (as shown in Figure 2B), the effect of H89 on this promoter construct was examined. Results showed that H89 also significantly reduced ox-PAPC activation (Fig. 3C). Together, these results suggest that the PKA pathway mediates ox-PAPC induction of IL-6 within the 84-bp region.

Although H89 is marketed as a selective and potent inhibitor of PKA, H89 has been shown to block additional protein kinases (Davies et al., 2000). One such protein kinase is rho-associated kinase II (ROCK-II), one of two

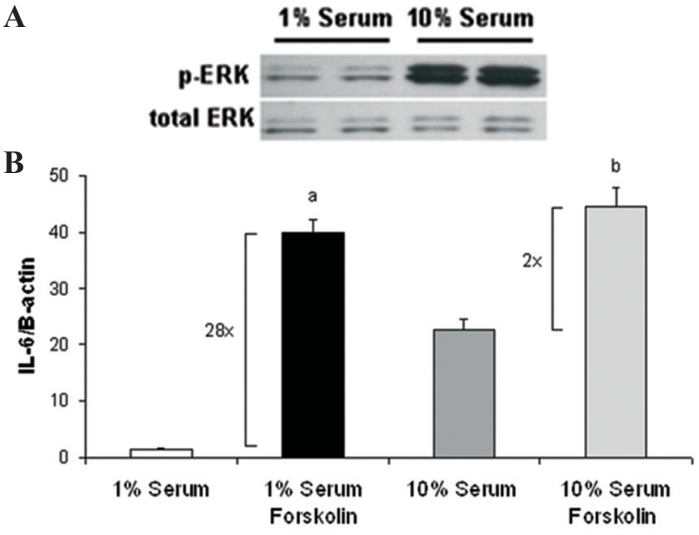

Figure 5. Serum reduces PKA activation of IL-6. (A) Western analysis of phosphorylated ERK in MC3T3-E1 cells treated with 1% or 10% serum for 15min. Total ERK is used as a loading control. (n=1) **(B)** RT-qPCR analysis of total RNA isolated from cells treated for 1hr with 1% serum, 10% serum, and/or forskolin (25uM), as indicated. Note that forskolin induction was lower in 10% serum, suggesting that ERK activation has an inhibitory role. (n = 2); a, $p<0.0001$ vs. 1% serum; b, $p<0.0001$ vs. 10% serum.

isoforms of Rho kinase, which is a serine/threonine protein kinase that is activated when bound to GTPase Rho (Liao, 2006). The ROCK pathway mediates vascular smooth muscle contraction, cell adhesion, and cell motility. More importantly, it has gained importance in the pathogenesis of hypertension and atherosclerosis by mediating abnormal smooth muscle contractions in humans and contributing to inflammations and early lesion formation in mice vasculature (Liao, 2006; Ziad et al., 2003; Akihiro et al., 2001). Therefore, we assessed ROCK-II's role in mediating ox-PAPC activation of the IL-6 promoter. The inhibitor Y-27632, which renders ROCK-II only 13% active but leaves PKA still functional at 91% of full activity, was used to rule out the nonspecific inhibition of H89 on ROCK-II (Davies et al., 2000). Results showed that inhibition of ROCK-II with Y-27632 did not attenuate ox-PAPC induction of IL-6 promoter activity, suggesting that ROCK-II does not mediate ox-PAPC effects on IL-6 expression (Figure 3D).

Extracellular signal-regulated kinase inhibits IL-6 activation Previously, we have shown that oxidized lipoproteins activate ERK in bone marrow stromal cells, which have the potential to differentiate into osteoblasts (Parhami et al., 1999). In addition, ox-PAPC inhibits osteogenic effects via the ERK pathway (Huang et al., 2007). As a result, ERK's involvement in ox-PAPC effects was examined. Western blotting analysis showed that stimulation of MC3T3-E1 cells with ox-PAPC for both 1hr and 2hr increased ERK phosphorylation, demonstrating that ox-PAPC activates ERK (Figure 4A). To assess whether ERK mediates ox-PAPC induction of IL-6, transiently transfected cells with pmIL-6.luc(-1277) were co-treated with ox-PAPC and ERK inhibitor, PD98059. Results showed that ERK inhibition further increased ox-PAPC-induced IL-6 promoter activity, (Figure 4B). In addition, RT-qPCR showed that ox-PAPC induced IL-6 by 2.9-fold when co-treated with PD98059, as opposed to the 1.6-fold increase carried out by ox-PAPC alone (Figure 4C). Together, these results suggest that ERK acts as an inhibitor of ox-PAPC effects.

ERK attenuates PKA activation of IL-6 To determine ERK's mechanism of action, we assessed whether ERK inhibition of IL-6 was due to antagonistic effects on PKA. Cells were co-treated with serum, activating ERK (Figure 5A), and PKA activator forskolin. RT-qPCR showed that increasing serum levels reduced forskolin induction of IL-6 mRNA level from 28-folds to 2-folds (Figure 5C), suggesting that ERK inhibits PKA activation of IL-6

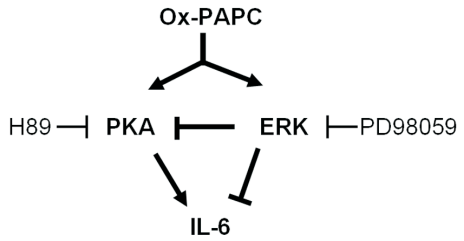

Figure 6. Proposed regulatory mechanisms of ox-PAPC on IL-6 expression. Ox-PAPC activates both PKA and ERK. PKA activation induces while ERK activation inhibits IL-6 expression. Our results suggest that ERK inhibits IL-6 expression by antagonizing PKA. Inhibitors of PKA (H89) and ERK (PD98059) are indicated.

expression. Together, these results suggest that ERK antagonizes PKA.

DISCUSSION

Although the impact of oxidized lipids on bone cell differentiation and activity has been shown previously, their underlying mechanism remains elusive. This present study explored the possibility of osteoclast-regulating cytokines, specifically IL-6, as a potential link in mediating these effects. Here we demonstrated that oxidized phospholipids, ox-PAPC, upregulates IL-6 mRNA expression at the transcriptional level in murine calvarial preosteoblasts (Figure 1A). Consistent with these in vitro results, calvarial tissues from the hyperlipemic LDLR-/- mice that exhibit increased levels of oxidized lipids had elevated IL-6 mRNA expression (Figure 1B). Previously we have demonstrated that bone marrow cells from LDLR-/- mice show increased osteoclastic potential, raising a possible role of IL-6 in increased bone resorption under a hyperlipidemic setting (Tintut et al., 2004).

The present findings also elucidated the molecular mechanism by which ox-PAPC increases IL-6 expression in calvarial preosteoblasts. We show that the 84-bp region upstream of IL-6 transcriptional start site contains ox-PAPC regulatory elements (Fig. 2B). Although the transcription factor that may bind in this region has not yet been identified, the 84-bp region contains the NF-κB (-73 to -64) and AP-1 (-61 to -55) response elements. Since a mutation in the NF-κB binding site of the IL-6 promoter did not attenuate ox-PAPC activation of the IL-6 promoter (Fig. 2B), this suggests that members of the AP-1 family of transcription factors, such as c-Jun, c-Fos, ATF2, may mediate ox-PAPC effects. This hypothesis would be consistent with studies that have reported AP-1 as necessary to activate the IL-6 promoter (Baccam et al., 2003; Chen et al., 2004).

In addition to elucidating the transcriptional regulatory mechanism of ox-PAPC on IL-6, we demonstrated that the PKA pathway appears to mediate ox-PAPC induction of IL-6. Although our results show that the ROCK-II pathway is not involved, additional studies demonstrating direct activation of PKA by ox-PAPC are needed. Preliminary data indicates that ox-PAPC did not increase cAMP, a canonical activator of PKA. Two cAMP molecules activate PKA by binding to its regulatory subunits, releasing the catalytic subunits to phosphorylate protein substrates in the cytosol and nucleus (Ma et al., 2005). Additional investigation is required on whether ox-PAPC increases PKA activity and whether higher ox-PAPC concentration at different time points can elevate cAMP levels. If ox-PAPC increases both cAMP levels and PKA activity, this would suggest that ox-PAPC engages the classical cAMP-PKA pathway to induce IL-6 expression. However, if PKA activity rises without any change in cAMP levels, this would suggest that a cAMP-independent PKA pathway mediates ox-PAPC effects. A cAMP-independent PKA pathway would not be unprecedented, as other agents, such as endothelin-1 and sphingosine, have been shown to activate such pathway (Ma et al., 2005; Dulin et al., 2001).

While PKA appears to mediate ox-PAPC activation of IL-6, we demonstrated that the ERK pathway inhibits PKA activation of IL-6 expression. This raises a model of ox-PAPC induction of IL-6, where ox-PAPC activates both PKA and ERK pathways, the latter having antagonistic effect on the former pathway (Figure 6). To confirm the role of ERK in ox-PAPC effects, further experiments will be employed to directly test whether ERK agonists inhibit PKA activity.

In the larger context, the present findings further elucidate the mechanism behind the dichotomic regulation of bone cells by oxidized phospholipids. Our previous studies have well-characterized how oxidized lipids inhibit osteoblast differentiation. While we previously found that atherogenic lipids also increase osteoclast activity, the mechanism remained unclear. Results from this study support the hypothesis that oxidized phospholipids increase osteoblast production of an osteoclast-regulating cytokine, IL-6, thereby possibly increasing increase osteoclast differentiation and activity. These findings indicate that oxidized phospholipids not only lead to the development of atherosclerosis in the vasculature, but also provide a mechanism for increased bone resorption in osteoporotic patients. Better understanding of the molecular basis of oxidized phospholipids may result in therapies to hinder or prevent the commonly associated cardiovascular disease and osteoporosis. The results may pose osteoclast-regulating cytokines as targets of treatment for patients suffering from

these debilitating diseases. In summary, these findings implicate oxidized phospholipids and IL-6 as potential links between cardiovascular disease and osteoporosis.

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