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Original Article

Oxidized phospholipids inhibit canonical Wnt signaling and osteoblast differentiation via ERK activation

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Abstract: Accumulating lines of evidence have indicated that hyperlipidemia not only is a well-established etiological factor for cardiovascular diseases (CVD), but also plays an active role in regulating bone metabolism. As a mixture of oxidized phospholipids derived from the oxidation of low density lipoprotein (LDL), oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC) is reported to be one of the major bioactive mediators in hyperlipidemia-associated pathological events. In this study, the effects of ox-PAPC on osteoblast differentiation and the underlying molecular mechanisms were investigated. Briefly, MC3T3-E1 murine pre-osteoblast cells were treated with Wnt-3a and/or ox-PAPC, and changes in canonical Wnt signaling pathway, ERK pathway, p38 MAPK pathway and the expression levels of bone-related genes were evaluated using real-time RT-PCR, alkaline phosphatase (ALP) activity assay, Western blot and immunofluorescent staining. We found that ox-PAPC inhibited canonical Wnt signaling, which resulted in decreased expressions of bone-related genes. Although ox-PAPC treatment activated both ERK and p38 MAPK pathways, the inhibitory effects of ox-PAPC on canonical Wnt signaling and the expressions of bone-related genes were successfully reversed only by ERK inhibitor PD98059. In conclusion, oxidized phospholipids inhibit canonical Wnt signaling pathway and osteoblast differentiation, which is mediated by the activation of the ERK pathway.

Keywords: Oxidized phospholipids, ox-PAPC, osteoblast differentiation, canonical Wnt signaling, ERK pathway

Introduction

As an established etiological factor for cardiovascular diseases (CVD), hyperlipidemia is also reported to play an active role in regulating bone metabolism. For example, patients with atherogenic lipid profiles defined as higher total cholesterol levels or higher low density lipoprotein-cholesteral (LDL-c) levels have decreased bone mineral density (BMD) and an increased risk of osteopenia compared to those with normal lipid profiles [1, 2]. Various hyperlipidemic animal models have also been developed *via* high fat diet and/or gene modification to investigate the effects of increased serum lipids on bone metabolism. These hyperlipidemic animal models show significantly reduced BMD, inhibited bone formation, and resistance to parathyroid hormone (PTH)-induced bone anabolism [3-6]. By blocking lipid oxidation products in these animal models, the inhibitory effects are significantly attenuated [6, 7], supporting that oxidized lipids are the major mediators in hyperlipidemia-associated bone loss [8].

Oxidative modification of low density lipoprotein (LDL) generates mixtures of bioactive oxidized derivatives, which induce a series of biological or pathological events including modifying protein structures, regulating protein activities, disturbing intracellular signal transduction [9]. Oxidized low density lipoprotein (ox-LDL) has shown a variety of effects on osteogenesis and osteoclastogenesis. Ox-LDL is reported to impair proliferation and induce apoptosis of rat bone marrow mesenchymal stem cells (BM-MSCs) in a time- and dose-dependent manner [10, 11]. Many studies also support that minimally modified LDL by oxidation (MM-LDL), rather than the native LDL, inhibits osteogenic differentiation of BM-MSCs and osteoblasts [12-15]. In addition, oxidized lipoproteins and phospholipids enhance the production of interleukin-6 (IL-6), tumor necrotic factor-α (TNF-α) and receptor activator of nuclear factor kappa-B ligand (RANKL) by osteoblast-like cells and T cells, which may promote osteoclast differentiation [16-18]. The impaired cell survival and disturbed osteogenic differentiation of osteo-

Table 1. Primer sequences for real-time PCR analysis of gene expression

Gene	Genebank Accession No.	Forward primer	Reverse primer
GAPDH	NM_001289726	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'
Osterix	NM_130458	5'-CGCATCTGAAAGCCCACTTG-3'	5'-CAGCTCGTCAGAGCGAGTGAA-3'
Runx2	NM_001146038	5'-CCCAGCCACCTTTACCTACA-3'	5'-TATGGAGTGCTGCTGGTCTG-3'
ALP	NM_007431	5'-CTGATGTGGAATACGAACTGGATG-3'	5'-AGTGGGAATGCTTGTGTCTGG-3'
OC	NM_007541	5'-CCACACAGCAGCTTGGCCCA-3'	5'-ACCCAAGGTAGCGCCGGAGT-3'

genic precursor cells, together with the enhanced osteoclastogenesis, contribute to the hyperlipidemia-induced imbalance between bone formation and bone resorption.

Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC) is a mixture of oxidized phospholipids produced by the oxidation of LDL. It is reported to be responsible for some of the biologic activities induced by MM-LDL [19, 20]. Similar to MM-LDL, ox-PAPC inhibits osteogenic differentiation as evidenced by decreased alkaline phosphatase (ALP) activity and calcium uptake in pre-osteoblasts [13]. However, the effects of ox-PAPC on osteoblast gene expression and the underlying molecular mechanisms have not been fully elucidated.

Canonical Wnt signaling pathway plays a crucial role in the control of bone formation [21]. Wnts activate canonical Wnt signaling pathway through cell surface receptors and eventually cause translocation of β-catenin into the nucleus, where β-catenin binds to members of the T-cell factor (TCF) family transcription factors, and activates the transcription of a variety of osteogenic genes. In this study, we performed a series of *in vitro* studies to further investigate the effects of ox-PAPC on canonical Wnt signaling and osteogenic differentiation in MC3T3-E1 pre-osteoblasts. Moreover, the involvement of mitogen-activated protein kinase (MAPK) pathways in the effects of ox-PAPC was also evaluated.

Materials and methods

Materials and reagents

MC3T3-E1, a murine calvaria-derived pre-osteoblast cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Wnt-3a was purchased from R&D Systems (Minneapolis, MN, USA). Ox-PAPC was from Hycult Biotech (Uden, The Netherlands). SB203580 and PD98059 were from Cell Signaling Technology (Danvers, USA). β-glycerophosphate, ascorbic acid and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

MC3T3-E1 cells were cultured with α-minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Fresh culture medium was replenished every 3 days and confluent monolayers were dissociated with 0.25% (w/v) Trypsin and 0.02% (w/v) EDTA solution for subculturing. Osteogenic medium was regular culture medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid, and was only used in the experiment for PCR analysis to determine the mRNA levels of bone-related genes after ox-PAPC treatment during osteogenic differentiation. Before usage, ox-PAPC was dissolved in chloroform, transferred into glass tubes, dried under pure nitrogen gas, and resolved with culture medium according to the manufacturer's instructions. Ox-PAPC resuspending was performed within 20 minutes before treatment of cells, and the medium was changed every 24 hours. The MAPK inhibitors PD98059 and SB203580 were dissolved in DMSO at the concentration of 20 mM, and diluted to final concentration with culture medium before usage. In experiments with MAPK inhibitors, the cells were pretreated with PD98059 or SB203580 for 1 h, followed by co-treatment with Wnt-3a, ox-PAPC and/or the MAPK inhibitors. And an equivalent amount of DMSO was added to the control cells.

Real-time RT-PCR

MC3T3-E1 cells were treated with 15 μg/ml ox-PAPC in osteogenic medium for 3 h, 1 d, 3 d or 5 d, and total RNA were isolated with RNA iso

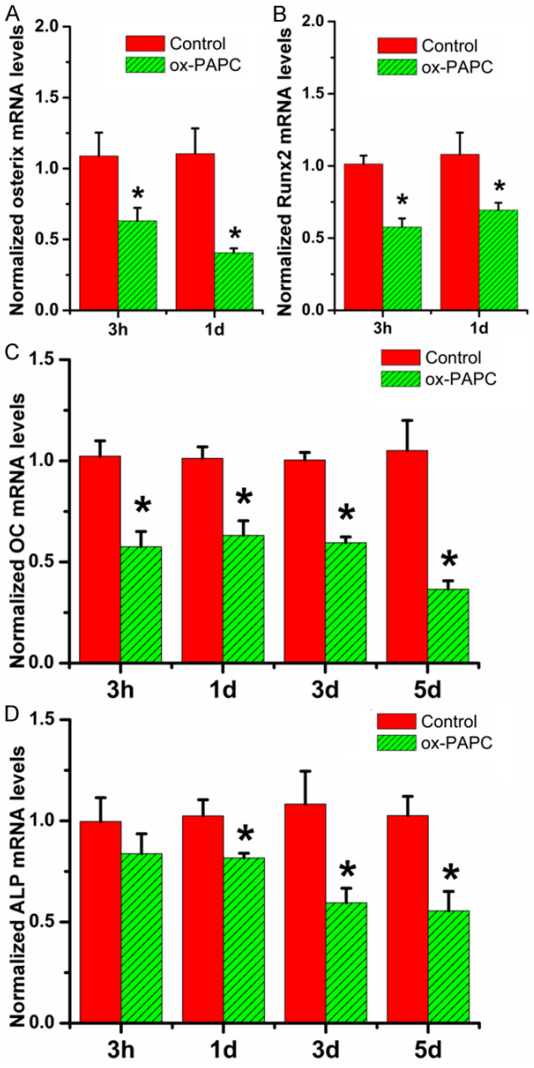

Figure 1. Ox-PAPC decreased mRNA expression levels of bone-related genes in MC3T3-E1 cells. (A-D) Changes in mRNA expression levels of osterix (A), Runx2 (B), OC (C) and ALP (D) in MC3T3-E1 cells treated with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and with or without ox-PAPC (15 μg/ml) for 3 h, 1 d, 3 d or 5 d. *P<0.05 *vs.* control cells.

Plus (Takara, Dalian, China). The first-strand cDNA was reverse-transcribed from 2 μg total RNA using the Primescript RT Reagent Kit (Takara, Dalian, China), and real-time PCR was performed using Light Cycler 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN, USA) on a Light Cycler II 480 (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Sequences of the primers for murine osterix, Runx2, ALP, osteocalcin (OC) and GAPDH were listed in **Table 1**. The evaluation of relative differences of PCR product amounts was carried out by the comparative cycle threshold (CT) method, using GAPDH as an internal control.

Alkaline phosphatase activity assay

ALP activity was determined using an alkaline phosphatase kit from Beyotime (Shanghai, China). Briefly, cell lysates were mixed with colorimetric substrate para-nitrophenyl phosphate (pNPP) and incubated at 37°C for 30 min. The enzymatic reaction was then stopped, and the optical density of the yellow product para-nitrophenol was determined spectrophotometrically at 405 nm. Protein concentrations of the cell lysates were measured with a BCA Protein Assay Kit (Beyotime, Shanghai, China), and ALP activity was expressed as para-nitrophenol produced in mmol/min/g of protein.

Western blot

Cells were exposed to RIPA lysis buffer (Beyotime, Shanghai, China) containing a cocktail of protease and phosphatase inhibitors. After incubation for 30 min on ice and vortexing at intervals of 10 min, the homogenized lysates were centrifuged at 13,000 g for 15 min at 4°C to remove insoluble material. Nuclear and cytoplasmic extracts were purified using an NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, Rockford, USA) according to the manual. Protein samples were quantified by the BCA Protein Assay Kit, separated using 10-12% Bis-Tris gels (Beyotime, Shanghai, China) and subsequently transferred onto 0.45 μm Immobilon-FL polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). Antibodies for p-ERK (1:3000), ERK (1:2000), p-p38 (1:1000), p38 (1:2000) and β-catenin (1:1000) were obtained from Cell Signaling Technology (Danvers, USA). Antibodies for BSP (1:300), histone H1 (1:200) were purchased from Santa Cruz Biotechnologies (Santa Cruz, USA). Antibodies for Runx2 (1:250) and GAPDH (1:1000) were obtained from Boster Biological Technology (Wuhan, China) and Zhongshan Golden Bridge (Beijing, China), respectively. The secondary antibodies were horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) (Zhongshan Golden Bridge, Beijing, China). Blots were visualized using ECL chemiluminescence reagents from Merck Millipore (Darmstadt, Germany).

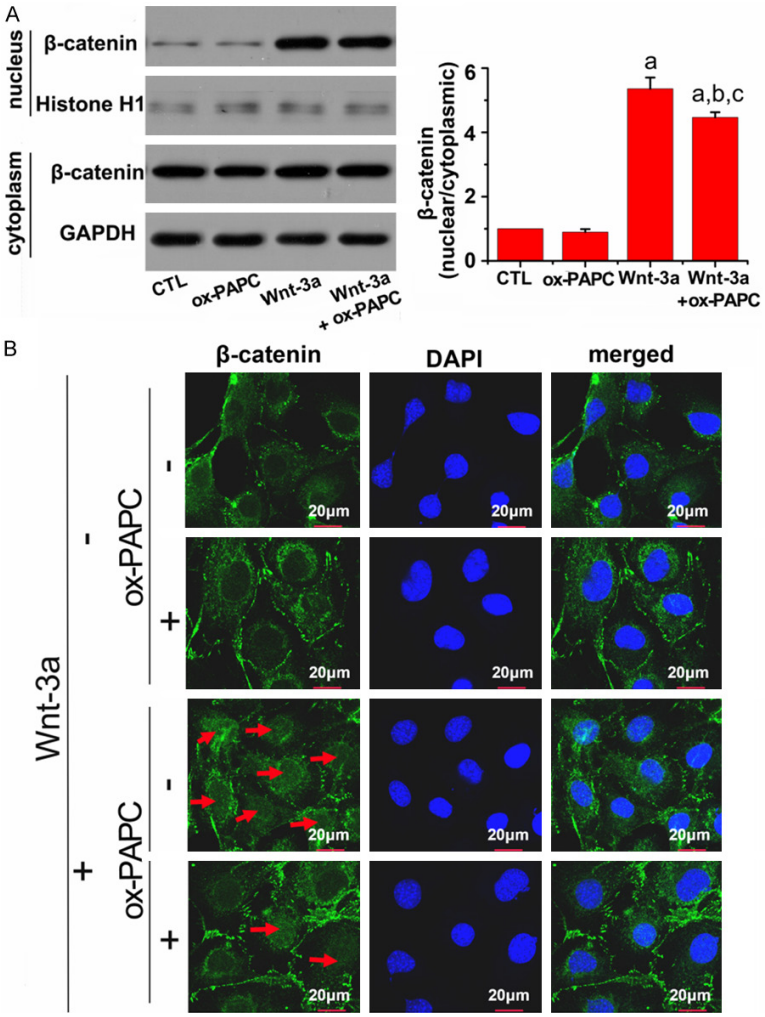

Figure 2. Ox-PAPC treatment inhibited the canonical Wnt signaling pathway *via* disturbance of the nuclear translocation of β-catenin. A. Western blot analysis of β-catenin distribution in MC3T3-E1 cells treated with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 1 d. Nuclear Histone H1 and cytoplasmic GAPDH were used as internal controls. a, $P<0.05$ *vs.* control group; b, $P<0.05$ *vs.* ox-PAPC group; c, $P<0.05$ *vs.* Wnt-3a group. B. Immunofluorescent staining of β-catenin translocation in nucleus (red arrow) of MC3T3-E1 cells treated with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 1 d.

Immunofluorescent staining of β-catenin distribution

After 24 hours of incubation, MC3T3-E1 cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS. An antibody for β-catenin (1:100, Cell Signaling Technology, Danvers, USA) was applied overnight at 4°C. The cells were then incubated in dark with FITC-conjugated Goat anti-rabbit IgG (Merck Millipore, Darmstadt, Germany) at room temperature for 1 hour and stained with DAPI (0.5 μg/ml). The samples were visualized under a Zeiss LSM 700 confocal microscope (Zeiss Inc., Maple Grove, USA).

Data analysis

All data were expressed as the mean ± standard error of the mean (SEM). Student's T-test was taken to assess the real-time PCR results, while One-way Analysis of Variance (ANOVA) followed by LSD post hoc test was performed in the other analyses using the SPSS 19.0 Statistics Software (SPSS Inc, Chicago, IL, U.S.A.). Values of *p* lower than 0.05 were considered statistically significant.

Results

Ox-PAPC decreased mRNA levels of bone-related genes in MC3T3-E1 cells undergoing osteogenic differentiation

To study the effects of ox-PAPC on osteoblast differentiation, MC3T3-E1 cells were induced to undergo osteogenic differentiation using 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The cells were simultaneously treated with or without 15 μg/ml ox-PAPC for 3 h, 1 d, 3 d and 5 d, and mRNA levels of osterix, Runx2, ALP and OC were monitored using real-time RT-PCR. The key transcription factors in osteogenic differentiation, osterix and Runx2 were both significantly down-regulated in ox-PAPC-treated MC3T3-E1 cells when compared with the control cells (**Figure 1A**, **1B**). OC mRNA level was also decreased at all time points in cells treated with ox-PAPC when compared with the control cells. After 5 days of incubation, OC mRNA level in ox-PAPC-treated cells was only one third of that in the control cells (**Figure 1C**). ALP mRNA level in cells treated with ox-PAPC for 3 h were slightly reduced compared to that in control cells but with no statistically significant difference detected. At later time points,

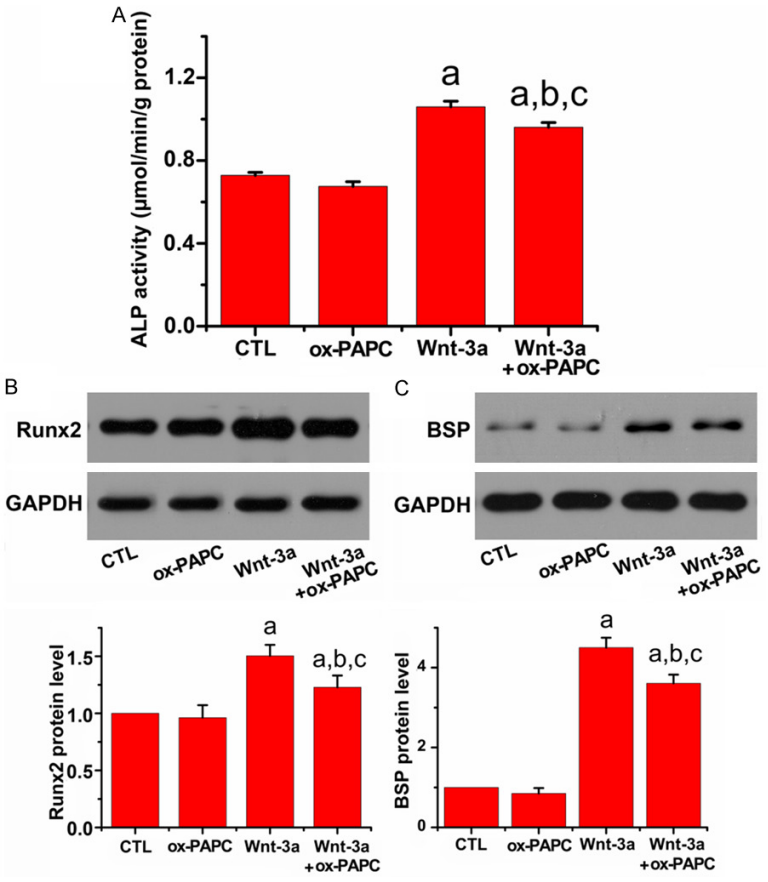

Figure 3. Ox-PAPC inhibited Wnt-3a-induced osteogenic differentiation. (A) ALP activity in MC3T3-E1 cells after treated with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 2 days. (B, C) Changes in protein levels of Runx2 (B) and BSP (C) in MC3T3-E1 cells after treated with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 2 days and 3 days, respectively. a, $P<0.05$ *vs.* control group; b, $P<0.05$ *vs.* ox-PAPC group; c, $P<0.05$ *vs.* Wnt-3a group.

ALP mRNA levels showed statistically significant decreases in cells treated with ox-PAPC when compared with those in the control cells (**Figure 1D**). These results clearly indicated that ox-PAPC inhibited osteogenic differentiation of MC3T3-E1 pre-osteoblast cells towards mature bone cells.

Ox-PAPC inhibited canonical Wnt signaling pathway and Wnt-3a-induced osteogenic differentiation in MC3T3-E1 cells

To investigate the effects of ox-PAPC on the canonical Wnt signaling pathway, MC3T3-E1 cells were treated with the regular culture medium supplemented with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 1 day. Cells treated only with the regular culture medium served as the control, while the other three groups were named as ox-PAPC group, Wnt-3a group and Wnt-3a+ox-PAPC group. Western blot was performed to quantitatively analyze the distribution of β-catenin in nucleus and cytoplasm. We found that Wnt-3a group showed a prominent increase in the nuclear translocation of β-catenin when compared with the control group. And Wnt-3a+ox-PAPC group showed significant attenuation of Wnt-3a-induced nuclear translocation of β-catenin (**Figure 2A**). These results were further confirmed by immunofluorescent staining of β-catenin. The increased number of cell nuclei with positive β-catenin fluorescence signals in the Wnt-3a group was decreased by addition of ox-PAPC (**Figure 2B**). Taken together, our results indicated that ox-PAPC inhibits Wnt-3a-induced activation of the canonical Wnt signaling pathway.

To investigate the effects of ox-PAPC on canonical Wnt signaling-induced osteogenic differentiation, MC3T3-E1 cells were treated with the regular culture medium supplemented with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml). ALP activity and protein levels of Runx2 and BSP were then evaluated. Two days after the treatment, we found that the ALP activity in the Wnt-3a group was enhanced when compared with the control group. However, the addition of ox-PAPC in the Wnt-3a+ox-PAPC group significantly down-regulated the Wnt-3a-activated ALP activity (**Figure 3A**). No statistically significant difference was detected between the ox-PAPC group and the control group during the study period. Changes in the protein levels of Runx2 and BSP demonstrated a similar pattern (**Figure 3B**, **3C**). These results indicated that ox-PAPC inhibited osteogenic differentiation induced by canonical Wnt signaling.

Ox-PAPC activated ERK and p38 mitogen-activated protein kinases (MAPK) pathways in MC3T3-E1 cells

To investigate whether ERK and p38 MAPK signaling pathways were activated by ox-PAPC,

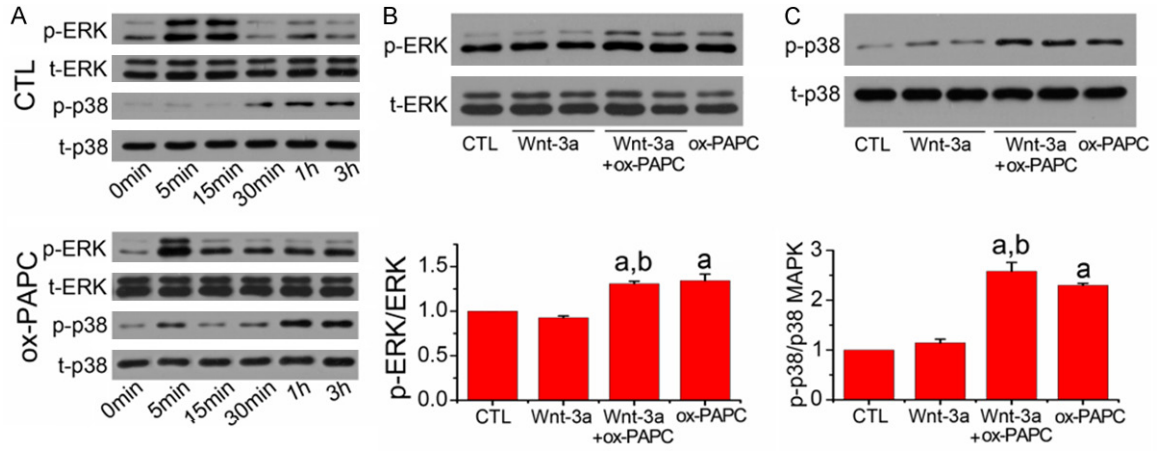

Figure 4. Ox-PAPC activated both ERK and p38 MAPK pathway. (A) Changes in the phosphorylation levels of ERK and p38 MAPK in MC3T3-E1 cells after treated with ox-PAPC (15 μg/ml) for 5 min, 15 min, 30 min, 1 h and 3 h. (B, C) Phosphorylation levels of ERK (B) and p38 MAPK (C) in MC3T3-E1 cells treated with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 1 h. Total-ERK and total-p38 MAPK were used as internal controls. a, $P<0.05$ *vs.* control group; b, $P<0.05$ *vs.* Wnt-3a group.

MC3T3-E1 cells were incubated with the regular culture medium supplemented with ox-PAPC (15 μg/ml). The phosphorylation levels of ERK and p38 MAPK were then determined using western blot analysis at different time points. We found that ox-PAPC treatment immediately increased the phosphorylation levels of both ERK and p38 MAPK, and these enhancing effects continued until 3 hours after the ox-PAPC treatment (**Figure 4A**). We then treated MC3T3-E1 cells with the regular culture medium supplemented with Wnt-3a (20 ng/ml) and/or ox-PAPC (15 μg/ml) for 1 hour, and determined the phosphorylation levels of ERK and p38 MAPK. The quantitative analysis confirmed that the phosphorylation levels of ERK and p38 MAPK were both up-regulated by ox-PAPC but not by Wnt-3a (**Figure 4B**, **4C**).

Blockage of ERK pathway reversed the inhibitory effects of ox-PAPC on canonical Wnt signaling pathway and wnt-3a-induced osteogenic differentiation

To investigate the roles of activated ERK and p38 MAPK pathways in ox-PAPC-treated cells, MC3T3-E1 cells were pretreated with regular culture medium supplemented with ERK inhibitor PD98059 (10 μM) or p38 MAPK inhibitor SB203580 (10 μM) for 1 hour, and then treated with Wnt-3a (20 ng/ml), ox-PAPC (15 μg/ml) and/or MAPK inhibitors. ALP activity assay and western blot analysis were used to assess the osteogenic differentiation. Our results showed that ERK inhibitor PD98059 successfully reversed the inhibitory effects of ox-PAPC on Wnt-3a-enhanced expressions of ALP, Runx2 and BSP. In contrast, the addition of p38 inhibitor SB203580 slightly enhanced the negative effects of ox-PAPC on ALP activity and expression of Runx2 (**Figure 5A-C**). Nuclear and cytoplasmic distribution of β-catenin was also monitored using western blot and immunofluorescence staining. We found that ERK inhibitor PD98059 completely restored the inhibitory effect of ox-PAPC on Wnt-3a-triggered nuclear translocation of β-catenin. In contrast, SB203580 showed no effects on nuclear translocation of β-catenin (**Figure 5D**, **5E**).

Discussion

According to previous findings, a variety of oxidized lipids in bone tissue impact the decision of MSC differentiation towards osteoblast lineage *versus* other cell lineages. Instead of undergoing osteogenic differentiation, BM-MSCs isolated from C57BL/6 mice fed with a high fat, atherogenic diet are directed to undergo adipogenic differentiation *in vitro*. The effects are ascribed to the oxidation of LDL, based on the fact that MM-LDL rather than native LDL inhibits osteogenic differentiation and promotes adipogenic differentiation of murine marrow stromal cells [12, 22]. Similarly, ox-PAPC enhances expressions of adipogenic transcription factor peroxisome proliferator-activated receptor γ2 (PPARγ2) and adipogenic

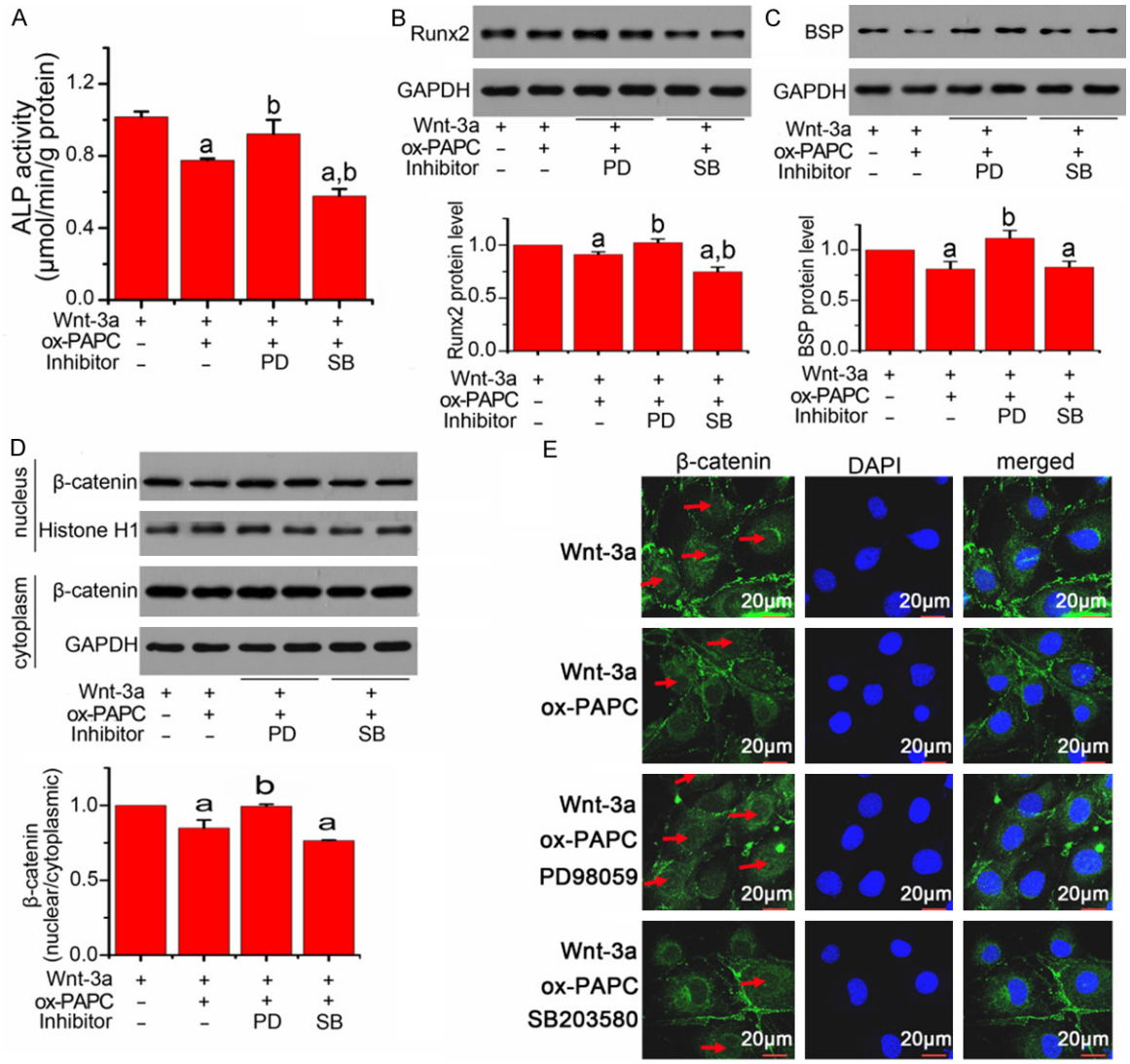

Figure 5. The inhibitory effects of ox-PAPC on canonical Wnt signaling pathway and Wnt-3a-induced osteogenic differentiation were mediated by the activation of the ERK pathway. MC3T3-E1 cells were pretreated with ERK inhibitor PD98059 (PD, 10 μM), p38 MAPK inhibitor SB203580 (SB, 10 μM) for 1 h, and then treated with Wnt-3a (20 ng/ml), ox-PAPC (15 μg/ml) and/or different MAPK inhibitors for 1 to 3 days. A. ALP activity was determined after 2 days. B. Protein level of Runx2 was determined after 2 days. C. Protein level of BSP was determined after 3 days. D. Western blot analysis of β-catenin distribution in MC3T3-E1 cells after 1 day of treatment. Histone H1 and GAPDH were used as internal controls. A-D. a, $P<0.05$ *vs.* Wnt-3a group; b, $P<0.05$ *vs.* Wnt-3a+ox-PAPC group. E. Immunofluorescent staining of β-catenin nuclear translocation (red arrow) in MC3T3-E1 cells after 1 day of treatment.

marker genes while reducing expressions of Runx2 and other bone-related genes in differentiating human mesenchymal stem cells [23]. In pre-osteoblasts, ox-PAPC is also found to negatively affect the osteogenic differentiation [13]. Besides, ox-PAPC is reported to attenuate osteogenic signaling induced by bone morphogenetic protein-2 (BMP-2) and PTH in MC3T3-E1 cells [24]. Consistent with these previous findings, we found that ox-PAPC treatment significantly down-regulated expressions of osteogenic transcription factors and bone matrix proteins in MC3T3-E1 cells undergoing osteogenic differentiation. And canonical Wnt signaling-induced osteogenic differentiation was also depressed by ox-PAPC.

β-catenin-dependent canonical Wnt signaling is a critical signaling pathway regulating bone development and regeneration. Defects on the components of canonical Wnt pathway induce osteoporosis-pseudoglioma syndrome, while

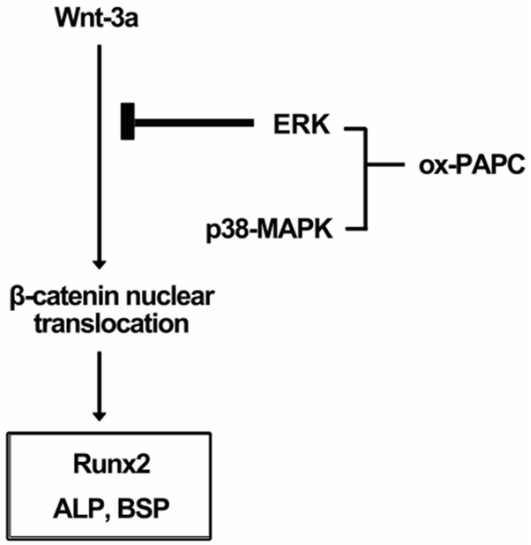

Figure 6. Molecular mechanisms underlying the inhibitory effects of ox-PAPC on canonical Wnt signaling pathway and Wnt-3a-induced osteogenic differentiation. Ox-PAPC depressed canonical Wnt signaling by inhibiting nuclear translocation of β-catenin *via* activation of ERK pathway, which consequently inhibited expressions of bone-related genes.

activation of the pathway leads to a high bone mass and density phenotype [25, 26]. In this study, the canonical Wnt signaling was significantly blocked and the Wnt-3a-induced osteogenic differentiation was inhibited by ox-PAPC treatment, indicating that ox-PAPC has negative effects on osteogenic differentiation by restraining canonical Wnt signaling pathway in MC3T3-E1 pre-osteoblasts. Meanwhile, previous study shows that lipid oxidation products promote calcifying vascular cell osteogenic differentiation [13]. And some recent studies report that activation of Wnt/β-catenin signaling by ox-LDL promotes transformation of aortic valve myofibroblasts towards the osteoblast-like phenotype [27, 28]. Together with these previous findings, our results suggested that different responses of Wnt signaling pathway to oxidized lipids in vascular cells and osteoblasts contribute to the opposite tendency of osteogenic differentiation in these two different cell lines [8].

Further investigations revealed that ox-PAPC treatment activated both ERK and p38 MAPK pathways in MC3T3-E1 cells. Blockage of the ERK pathway successfully reversed the inhibitory effects of ox-PAPC on the nuclear translocation of β-catenin and the expressions of bone-related genes, indicating that the negative influence of ox-PAPC on canonical Wnt signaling pathway and osteogenic differentiation was mediated by ERK (**Figure 6**). ERK pathway has been showed to participate in the inhibitory effects of ox-LDL on osteogenic differentiation [14]. In addition, the activation of ERK by ox-PAPC depresses the anabolic BMP-2 signaling in pre-osteoblasts [24]. Our findings further confirmed the role of ERK in the inhibitory effects of oxidized phospholipids on osteogenic differentiation, with canonical Wnt signaling pathway serving as one of its targets. On the other hand, the p38 inhibitor SB203580 slightly aggravated the negative effects of ox-PAPC on osteogenic differentiation while showing no effects on canonical Wnt signaling. The activation of p38 pathway by ox-PAPC may enhance osteogenic differentiation *via* activation of osteogenic pathways other than the canonical Wnt signaling. However, the activation of p38 pathway by ox-PAPC was weaker and later than that of ERK pathway, resulting in an overall inhibitory effect on Wnt signaling and osteogenic differentiation.

As mentioned above, the inhibitory effects of ox-PAPC on osteogenic differentiation is also mediated by inhibition of SMAD 1/5/8 and the subsequent blocking of BMP-2 signaling *via* activation of the ERK pathway [24]. Furthermore, MM-LDL attenuated Hedgehog signaling and osteogenic differentiation of BM-MSCs by inducing oxidative stress [15]. Another lipid oxidation product, 4-hydroxynonenal (4-HNE) increased oxidative stress, which in turn diverted β-catenin from TCF to Forkhead box O (FoxO) transcription factors, thereby attenuating Wnt signaling and Wnt-stimulated osteoblast differentiation in C2C12 osteoblast progenitor cells [29]. Taken together, our findings and others indicate that diverse signaling pathways and their interactions together function in mediating the inhibitory effects of oxidized lipids on osteogenic differentiation. Further research is still necessary to fully elucidate the underlying molecular mechanisms.

In summary, ox-PAPC inhibited canonical Wnt signaling pathway and Wnt-3a-induced osteogenic differentiation through activation of ERK pathway in MC3T3-E1 pre-osteoblasts. Our findings significantly expand current knowledge of the effects of oxidized phospholipids on

osteogenic differentiation and the molecular mechanism.

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Disclosure of conflict of interest

None.

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