

# Palmitic Acid Reduces Circulating Bone Formation Markers in Obese Animals and Impairs Osteoblast Activity via C16-Ceramide Accumulation

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**Abstract** Obesity and impaired lipid metabolism increase circulating and local fatty acid (FA) levels. Our previous studies showed that a high high-saturated -fat diet induced greater bone loss in mice than a high high-unsaturated-fat diet due to increased osteoclast numbers and activity. The impact of elevated FA levels on osteoblasts is not yet clear. We induced obesity in 4 week old male mice using a palmitic acid (PA)- or oleic acid (OA)-enriched high fat high-fat diet (HFD) (20 % of calories from FA), and compared them to mice on a normal (R) caloric diet (10 % of calories from FA). We collected serum to determine FA and bone metabolism marker levels. Primary osteoblasts were isolated; cultured in PA, OA, or control (C) medium; and assessed for mineralization activity, gene expression, and ceramide levels. Obese animals in the PA and OA groups had significantly lower serum levels of bone formation markers PINP and OC compared to normal weight animals ( $*p < 0.001$ ), with the lowest marker levels in animals on a PA-enriched HFD ( $*p < 0.001$ ). Accordingly, elevated levels of PA significantly reduced osteoblast mineralization activity in vitro ( $*p < 0.05$ ). Elevated

PA intake significantly increased C16 ceramide accumulation. This accumulation was preventable through inhibition of SPT2 (serine palmitoyl transferase 2) using myriocin. Elevated levels of PA reduce osteoblast function in vitro and bone formation markers in vivo. Our findings suggest that saturated PA can compromise bone health by affecting osteoblasts, and identify a potential mechanism through which obesity promotes bone loss.

**Keywords** Obesity · Osteoblast · Palmitic acid · Ceramides · Mineralization

## Abbreviations

FA	Fatty acid
FFA	Free fatty acid
PA	Palmitic acid
OA	Oleic acid
HFD	High fathigh-fat diet
PINP	Procollagen type 1 N-terminal propeptide
OC	Osteocalcin
SPT2	Serine palmitoyl transferase 2

Ahmad Alsahli and Kathryn Kiefhaber have shared first co-authorship.

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## Introduction

Bone undergoes constant remodeling, in which osteoblast-mediated bone formation and osteoclast-regulated bone resorption maintain bone homeostasis and secure stability of bone mass through regulatory pathways. Systemic or local abnormalities, such as hormonal deregulation [1] or inflammation [2], compromise bone homeostasis and may enhance bone resorption by osteoclasts and decrease bone formation by osteoblasts resulting in reduced bone mass.

Systemic metabolic derangements in obesity, diabetes mellitus, and the metabolic syndrome are linked to

abnormal bone homeostasis. Although obesity itself has traditionally been associated with increased bone mass [3] mainly attributed to enhanced bone formation in response to weight-bearing mechanical stimulation [4], increased fat mass and hyperlipidemia have been shown to decrease bone mineral density and increase fracture risk in young [5, 6] and adult individuals [7–10].

One of the major characteristics of obesity is the increase in circulating fatty acid (FA) serum levels [11, 12]. How a cell or tissue respond to excess FA levels determines whether those increased levels cause lipotoxicity or whether they instigate a more neutral response [13, 14]. Most importantly, there seems to be a difference in how cells react to saturated versus unsaturated FAs. Lipotoxicity has been described as being caused by intracellular accumulation of excessive levels of saturated FAs and their metabolic intermediates [15, 16]. Our prior study focused on the impact of palmitic (PA) and oleic acid (OA) on osteoclastogenesis. Based on data found in our study, osteoclastogenesis and osteoclastic activity are enhanced by PA through pro-inflammatory activation resulting in greater reduction in bone mass and structure in obese mice on a high fathigh-fat diet enriched with PA as compared to mice on a isocaloric OA-enriched high fathigh-fat diet [17]. The effects of PA on osteoblasts, however, are not as well understood. Osteoblasts are mesenchyme-derived cells responsible for bone formation [18], and are involved in regulation of osteoclast function [2]. Osteoblast formation is inhibited by the pro-inflammatory cytokine TNF- $\alpha$  [19], and a recent study by Yeh et al. indicated that PA attenuates osteoblast differentiation through inhibition of enzymes involved in  $\beta$ -oxidation of fatty acids [20]. The aforementioned results linking PA to altered osteoclast function, and the tightly regulated relationship between osteoclasts and osteoblasts, led us to investigate the impact of elevated FA levels on osteoblasts, and attempt to identify potential mediating pathways.

## Materials and Methods

### Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Columbia University. Mice were maintained under appropriate barrier conditions in a 12-hr light-dark cycle and received food and water ad libitum.

### *Fatty-Acid (FA)-Enriched High Fathigh-Fat Diet to Induce Weight Gain*

Four weeks old male C57BL/6 mice were randomly divided into groups ( $n = 5$ /group) and put on either PA- or

OA-enriched diet (Research Diets, Inc., New Brunswick, NJ) or normal chow diet for 3 months. The high caloric FA-enriched diets contained an increased amount of FA (20 % of calories from fatty acids whereas chow contains a total of 10 %) to induce weight gain (Supplemental Table 1).

### ELISA

Serum protein levels were determined using ELISA according to the manufacturers' recommendation ( $n = 5$ /group; R&D Systems, Minneapolis, MN, Abcam, Cambridge, MA, myBioSource, San Diego, CA).

### DEXA Analysis

Total body fat was measured using dual-energy X-ray absorptiometry (DEXA) on a Lunar PIXImus X-ray densitometer for small animals using Lunar PIXIMUS2 version 2.10 software. Obese mice were scanned at 10 mm/s with a resolution of 0.5 mm  $\times$  0.5 mm. Total body fat was determined in a window that excluded the head area.

### Osteoblast Cell Cultures

Cells were isolated from 2 to 3 day-old C57BL/6 mice (Charles River, Kingston, NY) as previously described [21]. Briefly, calvarias were subjected to two sequential digestion cycles, 5 and 55 min long using an enzyme medium consisting of 0.1 % Collagenase (Worthington, Lakewood, NJ) and 0.05 % trypsin EDTA. The supernatant was filtered with 100  $\mu$ l cell strainers and then mixed in a 1:1 ratio with  $\alpha$ -Minimal Essential Medium ( $\alpha$ -MEM), which contained 10 % Fetal Bovine Serum, FBS, (Life Technologies, Grand Island, NY), 1 % Antibiotic-Antimycin (Life Technologies), 1 % MEM Non-Essential Amino Acids (Corning Cellgro, Manassas, VA), and 1 % L-Glutamine (Life Technologies). The mixture was centrifuged at 2000 rpm for 5 min and the supernatant was discarded. Cells were re-suspended in 10.0 ml  $\alpha$ -MEM.  $5.0 \times 10^5$  cells were added to wells in a 48-well-plates and incubated at 37  $^{\circ}$ C degrees and 5 % CO<sub>2</sub>. The medium was replaced after 2 days with culture medium that contained  $\alpha$ -MEM, 5 mM  $\beta$ -glycerol-phosphate (Sigma-Aldrich St. Louis, MO), and 100  $\mu$ g/ml ascorbic acids (Sigma-Aldrich). The culture medium was changed every other day.

### Fatty Acid Cultures

Bovine Serum Albumin (BSA) (Sigma-Aldrich) was added to PBS to yield a 20 % BSA solution. Palmitic acid (PA) or sodium oleate (OA) powder (both Sigma-Aldrich) were

added to sodium hydroxide and DEPC water to yield a 20 mM FA solution. The FA solutions were then complexed with the 20 % BSA solution in a 2:1 molar ratio and added to  $\alpha$ -MEM culture medium to result in a final concentration of 0.2 mM PA and 0.2 mM OA. Control conditions received only the solutions without the FA complexes. The medium was changed every 2 days and cells were cultured for a total of 7 days. Ceramide accumulation was inhibited in cultures by adding 2.0  $\mu$ M myriocin (Sigma-Aldrich) to the culture medium at the time of PA stimulation. In detail, cell cultures were changed to culture medium to induce differentiation. After 2 days, medium was renewed and PA and myriocin was added to the cultures. Cells were harvested 48 h later.

### RNA Purification and Gene Expression Analysis

Total RNA purification, cDNA generation, and quantitative real-time PCR were performed as previously described [22]. Incorporation of SYBR Green dye into the PCR products was monitored with an iCycler BioRad sequence detection system. Samples were normalized against 18 S. The sequences of the primers can be provided upon request.

### Alizarin Red Staining

Alizarin Red staining, (Osteogenesis Assay Kit, EMD Millipore, Billerica, MA), was used to quantify differences in osteoblast mineralization activity between the conditions according to the manufacturer's instructions.

### Lipid Analysis

FA levels in serum and bone were analyzed as previously described by LC/MS/MS [23]. Bone was homogenized in 0.5 mL of methanol containing internal standard by using a tissue tearor prior FA analyses. Ceramide content was determined by using the DAG kinase method for total ceramide content and an LC/MS/MS-based analysis for ceramide subspecies analysis as previously described [24, 25]. Values were normalized over protein content.

### Statistical Analysis

Comparisons between groups were performed using GraphPad/InStat3. Comparisons between two groups were performed using unpaired two-tailed Student's *t* tests and from more than two groups one-way ANOVA. Pearson correlation coefficient and linear regression analysis was used to check for dependences. All values are presented as

means  $\pm$  STDEV. Differences between groups were considered statistically significant at  $p < 0.05$ .

## Results

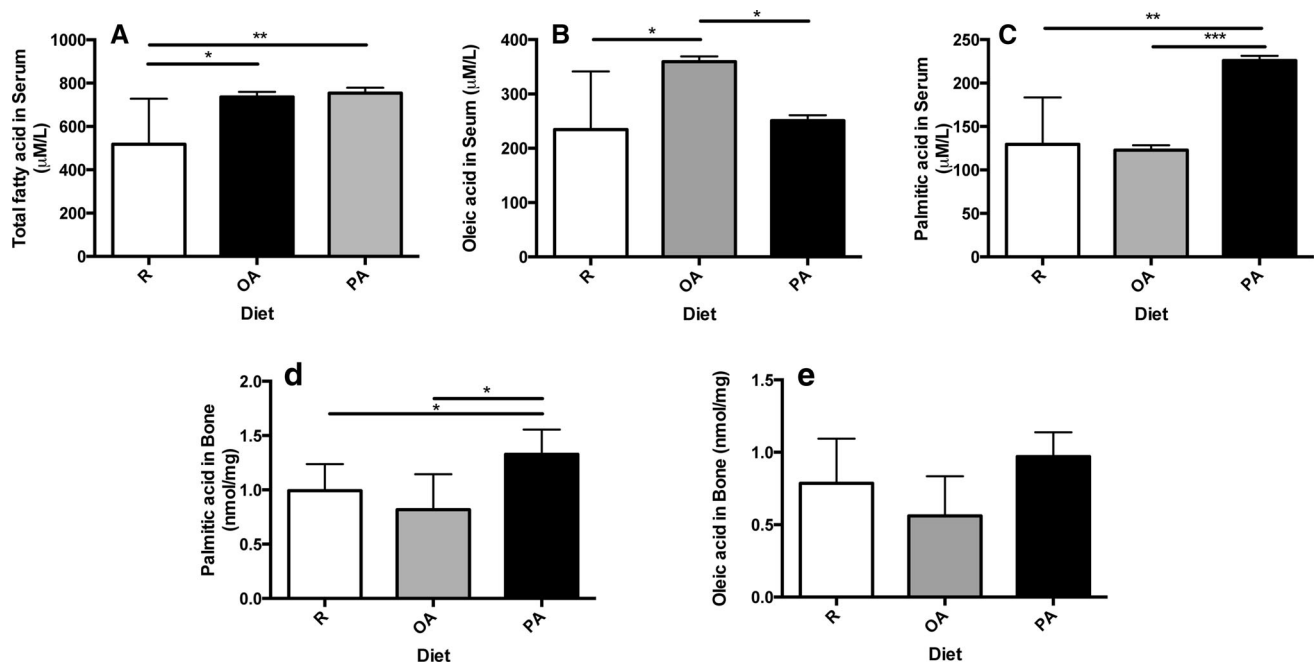
### Increased Systemic Circulating PA is Associated with Increased PA in Bone in Obese Mice

One characteristic of obesity is an increase in circulating FA levels due to excessive dietary food intake [12]. To investigate this further, C57BL/6 mice were fed isocaloric, high-fat diets (HFD) enriched in either OA or PA (20 % of calories from FA) vs a normal (C) caloric diet (10 % of calories from FAs) for 4 months. After 12 weeks, all animals on HFD, irrespective of fat source, weighed more compared to animals on a normal caloric standard chow diet (C/OA/PA in g:  $31.7 \pm 1.5$  vs.  $41 \pm 1.2^*$ ,  $39.3 \pm 1.0^*$ ,  $*p < 0.05$  vs control). Total body fat accumulation was comparable between obese animals on a PA versus an OA-enriched diet, with mean percent body fat of  $47.1 \pm 3.1$  % in OA animals and  $46.4 \pm 5.1$  % in PA animals ( $p = 0.51$ ).

As expected, obese animals on a high fathigh-fat OA- and PA-enriched diet had significantly higher levels of circulating FAs compared to animals on a regular fat diet (Fig. 1a). Additionally, the FA profile of each specific high fathigh-fat diet was reflected in the FA serum profile we detected in the obese animals. Animals on a PA-enriched high fathigh-fat diet had significantly higher levels of PA (C16) in serum versus animals on an OA-enriched high fathigh-fat diet, which had significantly higher serum levels of OA (C18:1) (Fig. 1b, c). We also found that increased serum levels of PA translated into increased PA in bone (Fig. 1d), while increased serum levels of OA did not (Fig. 1e). OA levels in bone were similar in all diet groups, suggesting differential FA metabolism for PA and OA in bone.

### Hyperlipidemic PA Levels Decrease Serum Bone Formation Markers and Increase Inflammatory TNF- $\alpha$ in Obese Animals

Our previous study showed that obese mice on a high fathigh-fat saturated PA-enriched diet have a greater reduction in bone mass and structure than mice on a high fathigh-fat OA-enriched diet, potentially due to an increase in bone-resorbing osteoclastic activity [17]. To investigate whether bone-forming osteoblasts are also impacted by the increased FA levels in serum, we collected serum from all animals and determined systemic levels of bone formation markers PINP and osteocalcin. Despite gaining the same



**Fig. 1** Animals on a high fathigh-fat PA diet have increased PA levels in serum and bone. FA levels in serum of obese animals on a high fathigh-fat OA/PA-enriched diet vs normal weight animals on a normal caloric diet (R) were determined using LC/MS/MS. Obesity

increases circulating FA levels (a), OA-diet increases serum OA levels (b) PA diet increases PA serum levels (c) and PA levels in bone (d), whereas OA-diet does not increase OA levels in bone (e). \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ,  $n = 5$ /group

amount of weight, obese mice on a high fathigh-fat PA-enriched diet had a greater reduction in osteocalcin and PINP than mice on a high fathigh-fat OA-enriched diet, suggesting changes in osteoblast activity (Fig. 2a, b). These changes in bone remodeling markers were accompanied by a significant increase in circulating TNF- $\alpha$  levels in animals on a PA-enriched diet (Fig. 2c) that correlated negatively with PINP and osteocalcin (PINP:  $r = -0.76$ ,  $p = 0.0009$ ; osteocalcin:  $r = -0.67$ ,  $p = 0.0063$ ). Our findings indicate that weight gain and obesity compromise levels of bone formation markers and potentially osteoblasts, and that the specific dietary FA profile influences the extent of reduction in bone formation markers.

### PA-Induced Reduction of Serum Bone Formation Markers Translates to Reduced Osteoblast Mineralization Activity In Vitro

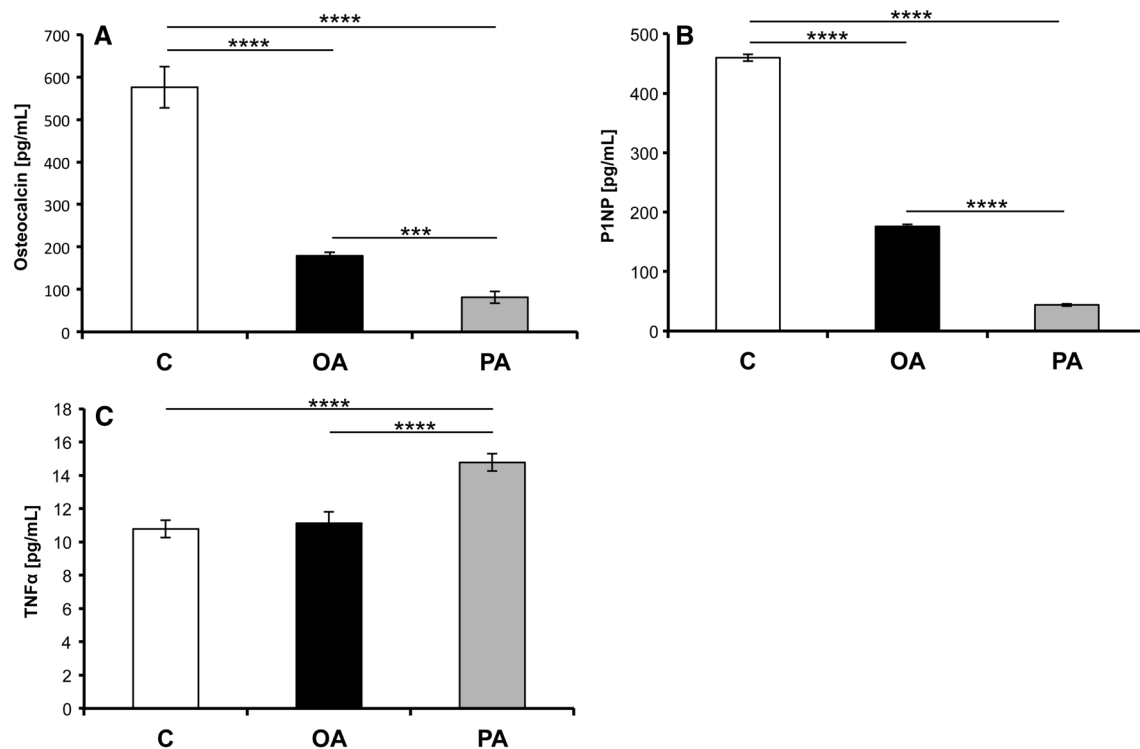
To more closely examine osteoblast function in vitro, osteoblast-precursor cells isolated from murine calvariae were cultured under hyperlipidemic FA conditions that had been described in our previous study [17]. Osteoblast precursor cells were cultured for 7 days in the presence of 0.2 mM PA and 0.2 mM OA and compared to control cells. Afterwards, mineralization activity of harvested cells was determined using an Alizarin Red colorimetric assay. As shown in Fig. 3a, PA significantly reduces osteoblast mineralization activity, whereas OA does not.

### Hyperlipidemic Levels of PA Increase Saturated Ceramide Accumulation in Osteoblasts

Especially in excessive amounts, lipids and their metabolites can impact molecular pathways that potentially result in cellular lipotoxicity [15, 16] and apoptosis [26–28], possibly via the de novo formation of ceramides. Ceramides are primarily formed from palmitate, with de novo synthesis mediated by serine palmitoyl transferase (SPT) and dihydro-ceramide synthase [28]. To determine ceramide levels in osteoblast cultures exposed to hyperlipidemic levels of PA and OA, osteoblast precursor cells were cultured in the presence of either 0.2 mM PA or 0.2 mM OA. PA-cultured osteoblasts had significantly higher levels of ceramides C16, C20, and C22 compared to both control and OA-cultured osteoblasts (Fig. 3b), as well as significantly higher levels of total saturated ceramide levels (Fig. 3c). OA cultures showed a trend of having higher levels of unsaturated ceramides compared to controls (Fig. 3d), therefore, PA is associated with increased saturated ceramide production, while OA is not.

### Inhibition of C16 Ceramide Accumulation Restores Osteoblast Mineralization Activity in PA-Treated Cells

Serine palmitoyl transferase 2 (SPT2) is responsible for the formation of C16 ceramide [28]. Because no accumulation



**Fig. 2** PA-enriched diet reduces serum bone formation markers and increases inflammation. Serum drawn from obese animals on PA, OA-enriched HFD, or normal weight animals on normal diet.

Osteocalcin (a), P1NP (b), TNFα (c) levels determined by ELISA. \*\*\*\* $p < 0.0001$ ;  $n = 5/\text{group}$

of this ceramide was observed in OA-treated cells, we examined SPT2 expression and inhibition only in PA-treated cells compared to controls, and found a non-significant elevation of SPT2 expression levels in PA-treated cells compared to control cells (Fig. 4a). To further investigate this, we used myriocin, a specific SPT2 inhibitor, to assess the effect of SPT2 on osteoblast metabolism in the presence of elevated PA [29]. Accumulation of C16 ceramide, which is directly formed from PA [30], was significantly reduced in PA-cultured osteoblasts that had been supplemented with myriocin compared to PA-cultured osteoblasts without myriocin (Fig. 4b). Notably, osteoblast mineralization activity was restored when myriocin was added to PA-treated osteoblast cultures (Fig. 4c).

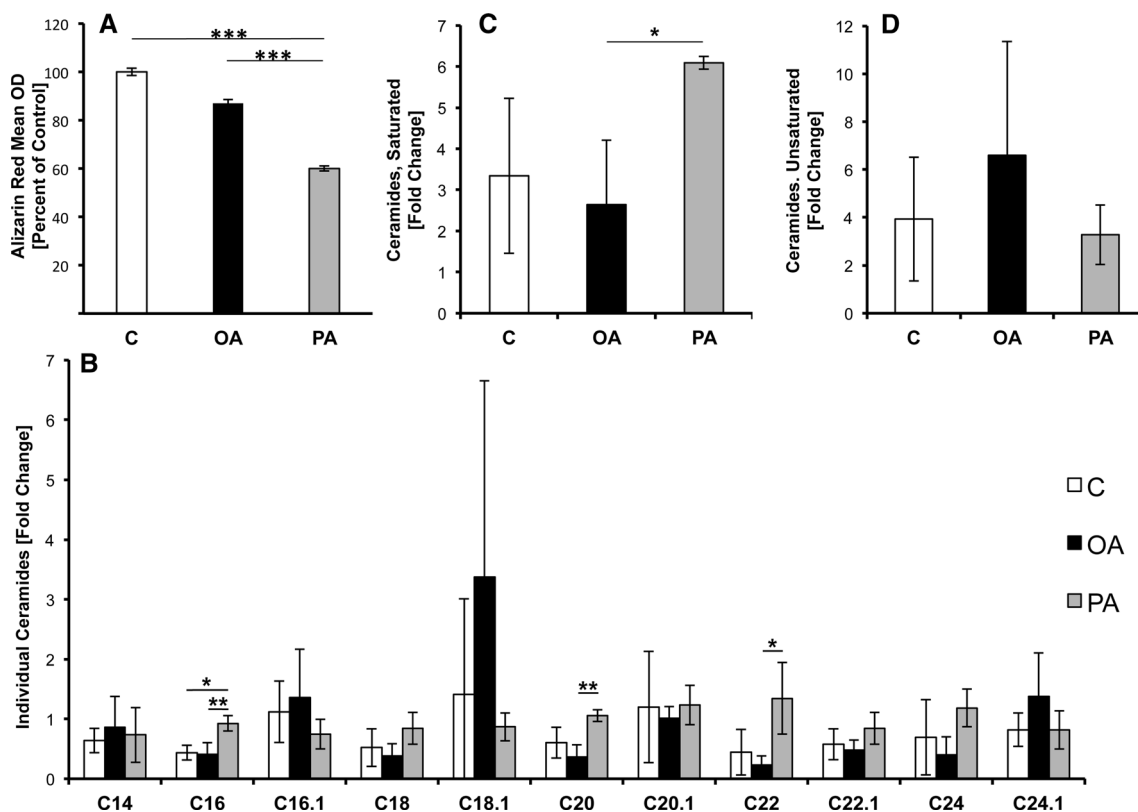
## Discussion

Palmitic acid and oleic acid are the most abundant saturated and unsaturated fatty acids, respectively, in the western diet and in adipose tissue [31] and, therefore, they are important topics of study in regards to diet impact on aspects of general health. Bone health can be compromised by perturbing the balance between osteoblast and

osteoclast activity, and PA has been previously demonstrated to enhance osteoclast formation and activity, leading to greater bone resorption [17]. Therefore, this study focused on the potential effects of hyperlipidemic levels of PA and OA on osteoblast development and activity. We propose that elevated levels of PA lead to decreased osteoblast function through accumulation of saturated ceramides, specifically C16 ceramides, and that inhibition of C16 ceramides rescues osteoblast activity.

Obesity is associated with elevated serum free FA (FFA) levels. This was recently demonstrated by Lu et al., who showed that rats on a HFD had significantly elevated serum FFA (particularly PA), and that this translated to an increase in fatty acid deposition in blood vessel walls [32]. Our results mirror this, showing that high PA feeding results in high PA in serum and in bone, while high OA feeding results in elevated OA levels only in serum (Fig. 1). Because of this increase in PA in bone, we hypothesized that PA could be directly affecting bone cell function.

In mice fed a high fat-high-fat diet, enrichment with either OA or PA caused significantly reduced levels of P1NP and osteocalcin (Fig. 2), commonly used markers of bone formation [33]. P1NP is indicative of collagen formation, while osteocalcin is secreted by osteoblasts to be



**Fig. 3** Osteoblasts cultured in the presence of fatty acids have reduced mineralization activity and show trend of increased ceramide production. **a** Alizarin red staining for mineralization activity. Data presented as percent of control absorbance at 405 nm.  $n = 6/\text{group}$ ;

**b–d** ceramides measured within osteoblasts. **b** Separate quantification of specific ceramides. **c** Total levels of saturated ceramides. **d** Total levels of unsaturated ceramides.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.0001$

incorporated into the growing bone matrix. The significant reduction in the detectable levels of both of these markers under HFD conditions suggests that the fatty acids are impairing osteoblast function. These results concur with previous research, which has demonstrated a negative correlation between serum levels of bone formation markers and obesity status, fat percentage, or visceral fat mass in both animal and human models [34–36]. Interestingly, although both saturated and unsaturated conditions caused a significant decrease in P1NP and OC compared to control, PA treatment led to significant reduction below that seen with OA-enriched HFD treatment. This suggests that, while any hyperlipidemic environment could be harmful to osteoblasts, a high-saturated-fat environment may be significantly worse.

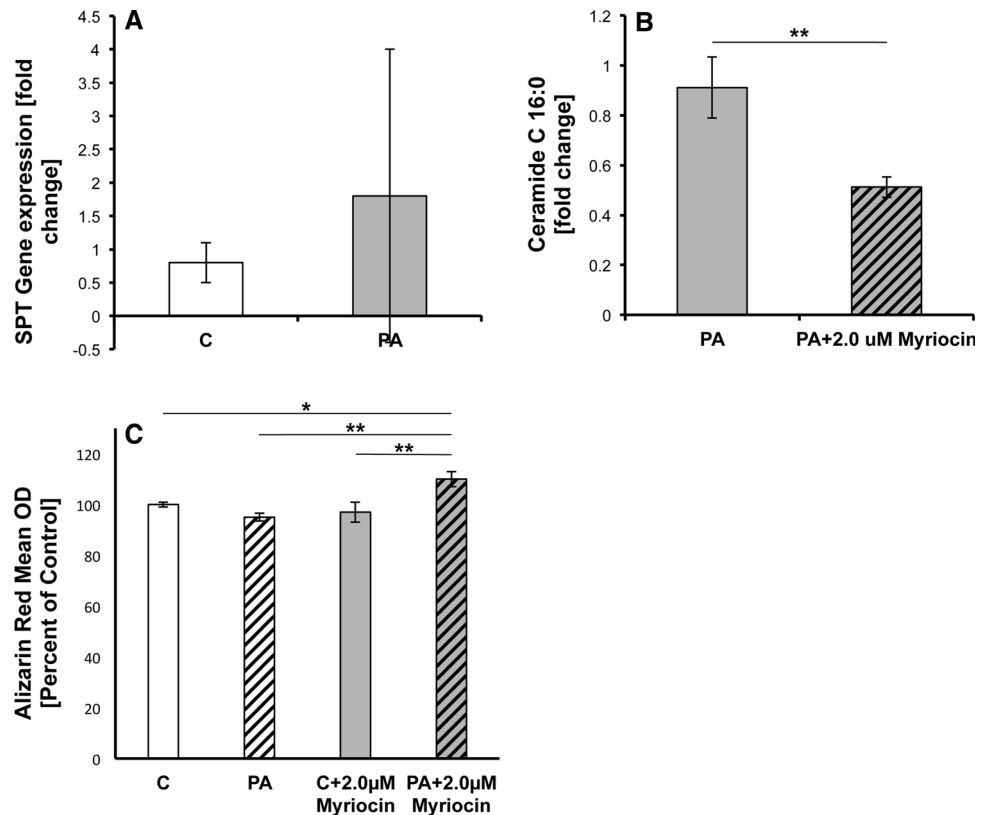
As was seen in our previous work with osteoclasts [17], in vivo treatment with PA led to increased production of the inflammatory cytokine TNF- $\alpha$  compared with both control and OA conditions. In addition, TNF- $\alpha$  levels negatively correlate with P1NP and osteocalcin in the serum. TNF $\alpha$ , a marker of inflammation is the main pro-inflammatory cytokine produced by adipose tissue, particularly visceral adipose tissue, and has been previously tied

to obesity [37]. Our finding further supports a possible link between the body's inflammatory response and impairment of bone homeostasis via effects on both osteoclasts and osteoblasts, and led us to more closely examine osteoblast function in vitro. Assessment of mineralization activity revealed that, as expected, PA led to significantly decreased mineralization activity compared to control and OA. However, despite OA treatment resulting in significantly decreased bone formation markers in vivo, mineralization activity of these cells was not significantly lower than control cells in vitro. This suggests that it is the saturation of the accumulated FA that is impacting osteoblast function. This conclusion is supported by previous studies which have shown that PA can cause cell dysfunction and premature apoptosis of hamster ovary cells [38], bovine retinal pericytes [39], and cardiomyocytes [26], in addition to osteoblasts [40].

PA is known to be the primary substrate for de novo ceramide synthesis [28], and ceramides have been linked to apoptosis [27, 39], leading us to assess whether the presence of ceramide molecules could be responsible for the PA-induced osteoblast dysfunction. We therefore evaluated formation of ceramides in osteoblast cultures exposed to



**Fig. 4** Addition of myriocin inhibits ceramide accumulation in the presence of PA. **a** Fold change gene in expression of serine palmitoyl transferase. **b** Analysis of C16 ceramide levels within osteoblasts. **c** Mineralization activity of osteoblasts cultured in the presence of myriocin and control or PA. Data presented as percent of control absorbance at 405 nm.  $n = 6/\text{group}$ . \* $p < 0.05$ , \*\* $p < 0.01$



hyperlipidemic levels of PA and OA, based on chain length and degree of saturation [41] (Fig. 3b). Our results showed that PA led to a significant increase in saturated ceramides C16, C20, and C22 compared to both control and OA. Given that these three ceramides are within the same synthesis pathway, it is logical that a significant increase in C16 would also increase downstream synthesis products [30]. Our results suggest that it is not the overall increased synthesis of ceramides that leads to compromised cell activity, but possibly the increased synthesis of specific ceramides whose production is increased by exposure to PA.

To further characterize induction of ceramide production by PA, we analyzed the gene expression level of SPT2, the rate-limiting enzyme of de novo ceramide synthesis from palmitoyl moieties [42]. Inflammation and stress up-regulate SPT2 activity in cells [43], increasing production of lipid metabolites that are associated with lipotoxicity [44]. Our results showed a non-significant increase in SPT gene expression under PA condition (Fig. 4a), suggesting that high FFA may in general up-regulate this enzyme. We then used myriocin to inhibit SPT2 activity in cells cultured in high PA conditions, and saw a significant decrease in C16 ceramide production compared to cells cultured with PA only (Fig. 4b). These results parallel our in vivo findings correlating dietary and serum FA profiles (Fig. 1).

To assess whether the reduction in C16 ceramide synthesis achieved with myriocin treatment also affected the actual functioning of osteoblasts, we repeated alizarin red staining to examine mineralization activity. Our results show that, not only does addition of myriocin to PA cultures rescue mineralization activity of osteoblasts, it appears to increase mineralization activity to levels significantly greater than control conditions (Fig. 4c). This restoration of mineralization through inhibition of de novo ceramide synthesis strongly supports a link between C16 ceramide accumulation and osteoblast dysfunction.

Our results support previous findings that exposure to hyperlipidemic levels of palmitic acid leads to cellular dysfunction. Although mice fed isocaloric HFDs enriched with either saturated or unsaturated fat gain the same amount of weight, they have disparate bone mineral density changes. Our results indicate a possible cause of this discrepancy, showing that in culture, it is only PA that leads to an accumulation of saturated ceramide C16. Further, by inhibiting the de novo synthesis of C16 ceramides, mineralization activity of osteoblasts is not only restored, it significantly surpasses that of control cells. These results strongly support a link between ceramide accumulation in osteoblasts and their impaired functioning. Combined, our in vitro and in vivo results demonstrate a pathway by which hyperlipidemic levels of dietary PA lead to accumulation

of PA in bone, and subsequently to the increased de novo synthesis of saturated ceramides. This synthesis then impairs osteoblast mineralization activity, leading to decreased bone mineral density only in animals on a PA-HFD. Although our experimental diets were not pure in their source of fat, with OA present in the PA-HFD mixture, we selected these diets in order to more closely mimic true eating patterns rather than purely experimental ones, and to maintain equal levels of other fatty acids such as linoleic acid. In this way, we feel our *in vivo* results are more generalizable to future dietary interventions. Indeed, future studies should examine the effects of varying ratios of OA:PA to investigate OA's potential attenuation of the effects of PA (see Supplemental Fig. 1) in addition to other saturated fatty acids.

Our *in vivo* results suggest possible future therapeutic applications for dietary intervention to promote healthy bone cell function, potentially by displacing common saturated dietary FAs with unsaturated. This approach is supported by the results of a recent study in which elderly men on a Mediterranean diet enriched with olive oil (OA) exhibited increased levels of OC and P1NP [45]. Further research should follow up in animal models investigating the effects of specific ceramide accumulation versus ceramides as a family of molecules and associated cellular pathways, to further characterize their impact on global bone homeostasis.

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**Authors Contribution** Ulrike Schulze-Späte designed the study. Ahmad Alsahli, Kathryn Kiefhaber, Tziporah Gold, Munira Muluke, Hongfeng Jiang, and Serge Cremers contributed to experimental work and data analysis. Kathryn Kiefhaber and Ulrike Schulze-Späte prepared the first draft of the paper. Ahmad Alsahli, Kathryn Kiefhaber, Tziporah Gold, Munira Muluke, Hongfeng Jiang analyzed data. Ahmad Alsahli, Kathryn Kiefhaber, Tziporah Gold, Munira Muluke, Hongfeng Jiang, Serge Cremers, and Ulrike Schulze-Späte interpreted results, and Ulrike Schulze-Späte is guarantor. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

#### Compliance with Ethical Standards

**Conflict of Interest** The authors have no conflict of interest to declare.

**Human and Animal Rights and Informed Consent** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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