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PPAR γ Regulates Pharmacological but not Physiological nor Pathological Osteoclast Formation

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The fracture predisposition of women treated with the insulin sensitizing drugs, thiazolidinediones (TZDs), which activate the nuclear receptor, PPAR γ , is a major skeletal complication of the metabolic syndrome [1]. Thus, the means by which TZDs and PPAR γ impact bone cells have come under particular scrutiny. For example, it is claimed that PPAR γ is essential for physiological osteoclastogenesis and that pharmacological induction of these bone resorptive cells is a (the) principal means by which TZDs promote fracture [2, 3]. The conclusion that PPAR γ mediates physiological osteoclast formation is, however, derivative of a study in which the gene was deleted in myeloid lineage cells, from which the osteoclast is derived, using Tie2 Cre [2]. Because this recombinase also targets endothelium [4] we asked if the same skeletal phenotype would obtain with more myeloid-specific Cre recombinases. To this end, we crossed PPAR $\gamma^{\text{fl/fl}}$ mice to those expressing 2 copies of LysM Cre^{+/+} (LysM KO) which exclusively targets myeloid cells, including those of osteoclast lineage [5]. Naïve LysM Cre^{+/+} (Cont) mice served as control. As demonstrated by FACS, PPAR γ deletion by LysM Cre does not affect abundance of marrow-residing, committed osteoclast granulocyte/macrophage progenitors (GMP) (not shown). Bone marrow macrophages (BMMs) were then induced to undergo osteoclastogenesis by M-CSF and RANK ligand (RANKL). PPAR γ diminishes with osteoclast differentiation in control mice but is virtually undetectable, regardless of duration of exposure to osteoclastogenic cytokines, in LysM KO mice (Fig 1A). Predictably, osteoclastogenic differentiation marker proteins increase with exposure to the cytokines. These proteins are, however, similarly expressed in the presence and absence of PPAR γ . Importantly, induction of c-Fos, postulated to mediate the putative physiological, osteoclast-forming properties of PPAR γ

[2], as well as the key osteoclastogenic transcription factor, NFATc1, are unaltered in face of LysM Cre-mediated deletion of the nuclear receptor. Moreover, osteoclast-inductive signaling molecules, whether stimulated by RANKL or M-CSF, are indistinguishable in both genotypes (Fig S1A,B).

Indicating PPAR γ does not regulate basal osteoclastogenesis, in vitro, formation of the bone resorptive cell mirrors that of their control counterparts (Fig 1B). To determine if the same holds in vivo, we histomorphometrically quantitated the tibiae of control and conditionally deleted mice. We find that trabecular bone volume and the abundance of osteoclasts, whether expressed as number/mm of trabecular bone surface or the percentage of trabecular surface bearing TRAP-expressing cells, are the same in both genotypes (Fig 1C). We next subjected LysM KO and Cont mice to skeletal uCT analysis. Confirming histomorphometry, trabecular bone volume and parameters of cancellous architecture are similar in both groups (Fig 1D).

These data establish that LysM-Cre-mediated deletion of PPAR γ alters neither physiological osteoclastogenesis nor skeletal architecture. To examine this issue in a pathological context we asked if the same obtains in estrogen deficiency. Thus, female mice were ovariectomized or subjected to sham surgery. Four weeks later, skeletal parameters were quantified by uCT. As expected, ovariectomy reduced trabecular bone volume by approximately 1/3 in control mice (Fig S1C). The same magnitude of osteoporosis occurred, however, in their LysM KO counterparts. Next, we induced K/BxN serum transfer inflammatory arthritis in both groups of animals [4]. As seen in Fig S1D, osteoclast abundance appears identical in arthritic mice regardless of the presence of PPAR γ in myeloid lineage cells.

Given the inconsistency of the effects of LysM Cre deletion of PPAR γ on osteoclastogenesis with that reported to accompany Tie2 Cre deletion [2] we bred male Tie2 Cre PPAR $\gamma^{fl/wt}$ to female PPAR $\gamma^{fl/fl}$ mice (Tie2 KO). As reported, Tie2 KO mice have a significant increase in spleen weight relative to body mass (Fig S2A). Similar to LysM KO mice, the abundance of marrow-residing committed GMP osteoclast precursors was virtually unaltered by Tie2 Cre-mediated deletion (not shown). Moreover, the in vitro and in vivo osteoclastogenic capacity and bone mass of Tie2 Cre+ PPAR $\gamma^{fl/fl}$ mice were essentially identical to their Cont counterparts (Figs 1 E,F,G,H). We extended these studies to the Tie2 Cre PPAR $\gamma^{fl/fl}$ mice used by Wan et al in the original study of the role of the nuclear receptor on murine osteoclastogenesis (Tie2^{original}) (kindly provided by Dr. Yihong Wan) [2]. Again we observed normal osteoclast formation in vitro and unaltered bone mass (FigS2 B,C,D) as well as absence of a pale bone phenotype (Fig S2E). Finally, to confirm the lack of effect of PPAR γ deletion on physiological osteoclast formation we crossed PPAR $\gamma^{fl/fl}$ mice to those expressing RANK Cre (kindly provided by Dr. N. Takahashi) or Vav1 Cre. The former targets all osteoclast lineage cells [6] and the latter, hematopoietic stem cells and thus all their derivatives [7]. In both circumstances we observed no effect on the bone resorptive cell (Fig S2 F,G,H,I,J,K,L). All Cre recombinases reduced PPAR γ mRNA more than 99%, confirming the absence of mosaicism (not shown).

TZDs bind PPAR γ and it has been long assumed that these drugs promote insulin sensitivity via activation of this adipogenic nuclear receptor. Recent evidence, however, challenges this

posture [8]. Thus, a TZD with low PPAR γ affinity promotes glucose sensitivity as effectively as its standard counterparts [9]. Unlike rosiglitazone, however, the same PPAR γ -sparing TZD fails to induce osteoporosis in mice [3]. Confirming that unlike physiological or pathological osteoclastogenesis, PPAR γ mediates pharmacological induction of bone resorptive cells, rosiglitazone-stimulated osteoclast differentiation as determined by cathepsin K expression, is arrested in LysM KO BMMs (Fig 11).

These data call to question the concept that PPAR γ mediates osteoclast generation in physiological or pathological conditions. Similarly, the normal differentiation of PPAR γ -deficient precursors is inconsistent with the posture that osteoclast progenitors reside almost exclusively in PPAR γ -expressing cells [10]. They also challenge our conclusion that the polycomb protein, ASXL2, stimulates osteoclast formation in a cell autonomous manner by serving as a PPAR γ co-activator, raising the possibility that non-osteoclast autonomous mechanisms exert a more profound influence than previously appreciated [11]. Regardless of PPAR γ 's lack of effect on basal and pathological osteoclastogenesis it is central to pharmacologically-induced bone resorption, thus contributing to the fracture-producing properties of TZDs.

Online Methods

Mice

PPAR $\gamma^{fl/fl}$ mice, Lys M-Cre mice, Tie2-Cre and Vav1-Cre mice were obtained from Jackson laboratory. RANK Cre mice were generously provided by Dr. N. Takahashi (Matsumoto Dental University, Japan) [12]. Tie2^{original} KO mice were generously provided by Dr. Y. Wan (University of Texas Southwestern Medical School). All mice were housed in the animal care unit of Washington University School of Medicine, where they were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of Washington University School of Medicine.

To fully delete PPAR γ using LysM-Cre, PPAR $\gamma^{fl/fl,LysMCre+/-}$ mice were crossed again to obtain animals bearing two copies of the LysM-Cre allele (PPAR $\gamma^{fl/fl,LysMCre+/+}$). LysMCre $+/+$ mice served as control. All Tie2, Tie2^{original}, RANK and Vav1 Cre experiments involved PPAR $\gamma^{fl/fl,Tie2Cre+/-}$ (Tie2 KO), PPAR $\gamma^{fl/fl,Tie2Cre+/-original}$ (Tie2^{original} KO), PPAR $\gamma^{fl/fl,RANK-Cre+/-}$ (RANK KO) and PPAR $\gamma^{fl/fl,Vav-Cre+/-}$ (Vav KO), respectively. PPAR $\gamma^{fl/fl}$ littermate without Cre, generated by breeding male Cre $+/+$ and female PPAR $\gamma^{fl/fl}$ mice, served as control. All in vivo bone phenotype experiments were analyzed in 2 month old mice, except Tie2 Cre^{original} which were analyzed at 3 months of age. Power analysis revealed 4 mice/group was required for 80% assurance and 5 mice/group for 90% assurance. No animals were excluded from the study. All animals were assigned to groups on a genetic basis and were not randomized.

Reagents

Recombinant murine M-CSF was obtained from R&D Systems (Minneapolis, MN). Glutathione S-transferase (GST)-RANKL was expressed in our laboratory as described [13].

The source of antibodies and reagents is as follows: c-Fos, Phospho-AKT (Thr 308), phospho-ERK (44/42), integrin $\beta 3$, AKT, phospho-P38, phospho-I κ B α , I κ B α , PPAR γ (Cell Signaling); c-Fos, NFATc1 (Santa Cruz); beta-actin (Sigma), Rosiglitazone from Cayman chemicals (Ann Arbor, MI). All other chemicals were obtained from Sigma.

Macrophage isolation and osteoclast culture

Primary BMMs were prepared as described [13] with slight modification. Marrow was extracted from femora and tibiae of 6- to 8-wk-old mice with α -MEM and cultured in α -MEM containing 10% inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (α -10 medium) with 1:10 CMG condition media on petri-plastic dishes. Cells were incubated at 37⁰C in 6% CO₂ for 3 days and then washed with PBS and lifted with 1x trypsin/EDTA in PBS. A total of 1.2x10⁴ BMMs were cultured in 500 μ l α -MEM containing 10% heat inactivated FBS with GST-RANKL and 30 ng/ml of mouse recombinant M-CSF in 48-well tissue culture plates, some containing sterile bone slices. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity after 5 days in culture, using a commercial kit (Sigma 387-A, St. Louis, MO).

Immunoblot

Cultured cells were washed twice with ice-cold PBS and lysed in radioimmune precipitation assay (RIPA) buffer containing 20 mm Tris-HCl, pH 7.5, 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 1% Triton X-100, 2.5 mm sodium pyrophosphate, 1 mm β -glycerophosphate, 1 mm Na₃VO₄, 1 mm NaF, and 1 $\times$ protease inhibitor mixture (Roche Applied Science). After incubation on ice for 10 min, cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. Forty micrograms of total lysates were subjected to 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Filters were blocked in 0.1% casein in PBS for 1 h and incubated with primary antibodies at 4 $^{\circ}$ C overnight followed by probing with fluorescence-labeled secondary antibodies (Jackson ImmunoResearch Laboratories). Proteins were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Microcomputed tomography (μ CT)

The trabecular volume of the distal femoral metaphysis or proximal tibia was measured using a Scanco μ CT40 scanner (Scanco Medical AG, Bassersdorf, Switzerland). A threshold of 250 was used for evaluation of all male scans and a threshold of 212 was used for evaluation of all female scans. 50 slices were analyzed, starting with the first slice in which condyles and primary spongiosa were no longer visible. Researchers were blinded in these experiments.

RNA Extraction and quantitative qPCR

RNA from cultured cells was isolated and purified using the RNeasy RNA purification kit (Qiagen); RLT lysis buffer was supplemented with β -mercaptoethanol (1%). Purified RNA was treated with DNase I (Invitrogen) before reverse transcription. cDNA was synthesized from RNA (1 μ g) using iScriptTM cDNA Reverse Transcription kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green

Master Mix kit and gene specific primers. Quantitative PCR was performed on ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). All reactions were performed in triplicates and relative mRNA levels were calculated by the comparative threshold cycle method using cyclophilin as an internal control. The primer sequences are as follows: *cathepsin K*: 5'-aggcagctaaatgcagagggtaca-3' and 5'-agcttgcacatgatggacacagaga-3'; *cyclophilin A*: 5'-agcatcacaggtcctggcatc-3' and 5'-ttcaccttcccaaagaccac-3'.

Serum transfer arthritis

Arthrogenic serum was obtained from K/B $\times$ N mice (6 to 12 weeks old). Serum (100 μ l per mouse) was injected intraperitoneally on days 0 and 5. The mice were sacrificed 2 weeks after first injection. Five or more mice were used for each group.

Histology and histomorphometry

The tibiae and femur of 2-month-old mice were fixed with 10% neutral buffered formalin, followed by decalcification in 14% EDTA for 10 days, paraffin embedding, and TRAP staining. Static and dynamic histomorphometric parameters were measured using BioQuant OsteoII (BioQuant Image Analysis Corporation, Nashville, TN) in a blinded fashion. Researchers were blinded in these experiments.

Statistics

Statistical significance was determined using Student's t test and expressed as $\pm$ S.D. Precision was expressed as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Normality tests were used to test the assumption of a normal distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PPAR γ deficiency does not affect osteoclast differentiation in Vitro and in Vivo

A) Immunoblot analysis of osteoclast differentiation markers temporally expressed by Lys M Cre (Cont) and LysM Cre PPAR $\gamma^{fl/fl}$ (LysM KO) BMMs (M ϕ) cultured with RANKL and M-CSF. Actin serves as loading control. Experiment was performed 4 times. B) Representative histological appearance of tartrate resistant acid phosphatase (TRAP)-stained osteoclasts (red reaction product) derived from Cont and LysM KO BMMs cultured for 5 days with M-CSF and increasing amounts of RANKL. Experiment was performed 5 times. Scale bar: 1 mm. C) Histomorphometric analysis of trabecular bone volume (BV/TV) and osteoclast number expressed as % of trabecular bone surface juxtaposed to osteoclasts (OC.S/BS) and osteoclast number/mm trabecular bone (OC. No./mm) of Cont (n=5) and LysM KO (n=5) mice. D) μ CT analysis of femoral BV/TV and BMD of Cont (n=5) and LysM KO (n=5) mice. E) Representative histological appearance of TRAP-stained osteoclasts derived from PPAR $\gamma^{fl/fl}$ (Cont) and Tie2 Cre PPAR $\gamma^{fl/fl}$ (Tie2 KO) BMMs cultured for 5 days with M-CSF and increasing amounts of RANKL. Scale bar: 1 mm. Experiment was performed 3 times. F) Immunoblot analysis of osteoclast differentiation markers temporally expressed by Cont and Tie2 KO BMMs cultured with RANKL and M-CSF. Actin serves as loading control. Experiment was performed 3 times. G) Histomorphometric analysis of trabecular bone volume (BV/TV) and osteoclast number expressed as % of trabecular bone surface juxtaposed to osteoclasts (OC.S/BS) and osteoclast number/mm trabecular bone (OC. No./mm) of Cont (n=5) and Tie2 KO (n=5) mice. H) μ CT analysis of femoral BV/TV and BMD of Cont (n=5) and Tie2 KO (n=5) mice. I) Cathepsin K expression (ROS) in Cont and LysM KO mice. Cont: blue bar, LysM KO: red bar. ** indicates statistical significance.

Histomorphometric analysis of trabecular bone volume and osteoclast number of Cont (n=5 or 6) and Tie2 KO (n=9) mice. H) μ CT analysis of femoral BV/TV and BMD of Cont (n=6) and Tie2 KO (n=6) mice. I) qPCR analysis of osteoclast cathepsin K mRNA of Cont and Lys M KO BMMs cultured 4 days with RANKL and M-CSF +/- rosiglitazone (R0SI). (Mean $\pm$ SD., ** $p < 0.01$). Experiment was performed 2 times.