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Journal of Cellular Biochemistry 114:1969–1977 (2013)

Journal of Cellular Biochemistry

Regulation of Selective PPARγ Modulators in the Differentiation of Osteoclasts

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ABSTRACT

Diabetes is the most common chronic disease in the world and causes complications with many diseases, such as heart disease and osteoporosis. Osteoporosis is a systemic bone disease characterized by imbalance in bone resorption and bone formation. Osteoclast is type of bone cell that functions in bone resorption and plays a critical role in bone remodeling. Rosiglitazone and pioglitazone, which belong to Thiazolidinediones (TZDs), are commonly used antidiabetic drugs. As PPARγ full agonists, they can activate PPARγ in a ligand-dependent way. Recent studies indicate that these PPARγ full agonists have some side effects, such as weight gain and bone loss, which may increase the risk of osteoporosis. In contrast, selective PPARγ Modulators (SPPARγMs) are novel PPARγ ligands that can activate PPARγ in different ways and lead to distinct downstream genes. Mice bone marrow cells were stimulated with recombinant mouse RANKL and M-CSF to generate osteoclasts. To determine the effect on osteoclasts formation, PPARγ ligands (Rosiglitazone, Fmoc-L-Leu, and Telmisartan) were added at the beginning of the culture. Rosiglitazone significantly increased the differentiation of multinucleated osteoclasts, while osteoclasts formation triggered by SPPARγMs was much less than that displayed by rosiglitazone. We found that the enhancement of PPARγ ligands may be associated with TRAF6 and downstream ERK signal pathway. We also demonstrated osteoclasts show characteristic M2 phenotype and can be further promoted by PPARγ ligands, especially rosiglitazone. In conclusion, reduced osteoclasts differentiation characteristic of SPPARγMs highlights SPPARγMs potential as therapeutic targets in diabetes, versus traditional antidiabetic drugs. J. Cell. Biochem. 114: 1969–1977, 2013.

KEY WORDS: DIABETES; OSTEOPOROSIS; OSTEOCLAST; PPARγ; SPPARγMs

Diabetes is a group of metabolic diseases characterized by the high blood sugar (glucose) level, which results from defects in insulin secretion or action, or both. There are two major types of diabetes, Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM), and nearly 95% of diabetic patients have T2DM. Diabetes frequently results in complications with other diseases, such as heart disease, renal failure, and hepatic failure [King et al., 2012]. In addition, about 50% of diabetic patients suffer from osteoporosis. Osteoporosis is a systemic bone disease, characterized by the reduction of bone mass and an increased risk of fracture, where bone resorption exceeds bone formation. As the functional cells of bone resorption, osteoclasts are involved in the process of bone remodeling and play a critical role in maintaining bone mass homeostasis.

Osteoclasts are multinucleated cells formed by differentiated macrophages, which belong to the monocyte-macrophage lineage. High expressions of tartrate resistant acid phosphatase(TRAP) and cathepsin K (CTSK) are the hallmarks of mature osteoclasts. As first noted in 1988, the maturation of osteoclasts required the presence of bone marrow and stromal cells [Takahashi et al., 1988]. After a decade of confusion, it has been shown that these cells express two molecules which are essential and sufficient to promote osteoclastogenesis: TNF-related cytokine receptor activator of nuclear factor κB ligand (RANKL) and the polypeptide growth factor macrophage colony stimulating factor (M-CSF) [Lacey et al., 1998]. Recent studies have found the expression of PPARγ in hemopoietic stem cells [Maurin et al., 2005], suggesting that PPARγ may be involved in the differentiation of osteoclasts.

Conflicts of interest: The authors declare that they have no conflict of interest.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 91013015, 81273527, 81121062.

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Manuscript Received: 16 July 2012; Manuscript Accepted: 28 February 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 March 2013

DOI 10.1002/jcb.24534 • © 2013 Wiley Periodicals, Inc.

Peroxisome proliferator activated receptor γ(PPARγ) belongs to the nuclear receptor superfamily and is a member of the NR1C subgroup which includes PPARα and PPARδ(also termed PPARβ). PPARγ is a ligand-dependent nuclear transcription factor; the binding of ligand changes the conformation of PPARγ, recruits some of the transcriptional co-activators, and thus initiates the transcription of downstream genes. PPARγ functions to help regulate glucose and lipid metabolism, immune response, and promote cell differentiation [Tontonoz and Spiegelman, 2008]. The clinical relevance of PPARγ is the currently marketed antidiabetic drugs, rosiglitazone (Avandia) and pioglitazone (Actos). These antidiabetic drugs are PPARγ full agonist thiazolidinediones(TZDs) [Willson et al., 1996]. However, it is well established that the treatment of TZDs will cause some side effects, such as weight gain and bone loss [Chaput et al., 2000]. Rzonca et al. [2004] found that during the treatment of type 2 diabetes, the antidiabetic drug rosiglitazone might pose a significant risk of adverse skeletal effects in humans. Wan et al. [2007] further indicated that the ligand activation of PPARγ by rosiglitazone enhanced osteoclasts differentiation in a receptor-dependent manner. Taken together, these studies suggest TZD antidiabetic drugs may increase the risk of osteoporosis.

Selective PPARγ modulators(SPPARγMs) differ from PPARγ full agonists. They are unlikely to bind PPARγ in the traditional way; distinct conformational change recruits different co-activators and expresses disparate downstream genes. Unlike PPARγ full agonists, the activation of PPARγ elicited by SPPARγMs is much less than that displayed by PPARγ full agonists, implying SPPARγMs may decrease the side effects caused by traditional PPARγ full agonists. Ma et al. [2010] demonstrated that SPPARγMs telmisartan could alleviate rosiglitazone-induced bone loss in ovariectomized spontaneous hypertensive rats. However, the mechanisms are largely unknown. Here we assessed the role of PPARγ full agonists (Rosiglitazone) and SPPARγMs (Fmoc-L-Leu, Telmisartan) in the differentiation of osteoclasts and the associated signal transduction mechanisms, which may provide new strategies for the development of antidiabetic drugs.

MATERIALS AND METHODS

REAGENTS

Alpha-minimum essential medium (α-MEM) and fetal bovine serum (FBS) were supplied by Gibco (Grand Island, NY). Rosiglitazone, Fmoc-L-Leu, Telmisartan, and tartrate-resistant acid phosphatase (TRAP) kit were purchased from Sigma (St.Louis, MO). Recombinant mouse M-CSF and RANKL were purchased from Peprotech (London, UK) and resuspended in PBS before use. Ssofast evagreen supermix was provided by BIO-RAD (CA). Final cytokine concentrations used are described in detail in results specific for each experiment.

IN VITRO DIFFERENTIATION

Osteoclasts were differentiated from bone marrow mononuclear cells using 8-week-old BALB/C mice as described [Kawano et al., 2003]. Briefly, we differentiated bone marrow mononuclear cells with 100 ng/ml of M-CSF and 100 ng/ml of RANKL in α-MEM containing 10%FBS for 4D in a humidified atmosphere of 5% CO_2 at 37°C, which allowed the bone marrow mononuclear cells to develop into pre-osteoclasts. We then removed the supernatant and further extended the culture for 5D under the same conditions. For TRAP staining, we cultured osteoclasts on coverslips.

HISTOCHEMICAL CHARACTERIZATION OF OSTEOCLAST FORMATION

At day 9, cells were stained for the expression of the osteoclasts hallmark, tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase, leukocyte (TRAP) kit (Sigma). Coverslips were washed in warm ddH_2O, fixed in citrate/acetone solution for 1 min, and stained for acidphosphatase in the presence or absence of 1.0 mol/l tartrate, using naphthol AS-BI phosphate as a substrate. Then reacted with Fast-Garnet GBC salt and the cells were counterstained with hematoxylin stain. We identified mature osteoclasts as multinucleated (>3 nuclei); TRAP-positive cells and the number of osteoclasts on each coverslip were counted as previously described [Neale et al., 2000].

REAL-TIME QUANTITATIVE PCR

Total RNA was extracted from osteoclasts by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA 1 μg was reverse-transcribed by M-MLV reverse transcriptase (Trans, China) and submitted to real-time quantitative PCR. RNA samples were normalized by the house keeping gene β-actin. This normalization provided the control for the efficiency of RNA extraction as well as the integrity and the amount of RNA. PCR primers were synthesized by Invitrogen Corporation (Carlsbad, CA) and presented in Table I.

CYTOKINE ASSAYS BY SPECIFIC ELISA

Tumor necrosis factor-alpha (TNF-α), interleukin-1β(IL-1β), interleukin-12 (IL-12), interleukin-4(IL-4), interleukin-6(IL-6), interleukin-10(IL-10), interleukin-12(IL-12), and interleukin-13(IL-13) in supernatants were quantified using standard sandwich enzyme-linked immunosorbent sorbent assays (ELISA) according to instruction manuals. Cytokines concentrations are expressed in nanogram per milliliter, as calculated from calibration curves from serial dilutions of murine recombinant standards (eBioscience, San Diego) in each assay. The sensitivity of ELISA was 20 pg/ml.

WESTERN BLOT

Cell cultures were harvested and then suspended in whole cell lysis buffer purchased from Beytotime (Haimen, Jiangsu, China). Protein concentration was determined by BCA reagent from Pierce (Rockford, IL). The proteins (50 μg per sample) were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels, and then electro-transferred onto polyvinylidene fluoride membrane (Amersham, UK). Antibodies were used against the following proteins: PPARγ(Santa Cruz, CA), ERK1/2, p-ERK1/2, and GAPDH (Kangcheng, Shanghai, China).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). All values were expressed as the mean ± standard error (SEM). Data for mRNA expression were tested by Student *t*-test. A value of $P < 0.05$ or $P < 0.01$ was

TABLE I. Primer Sequences Used for RT-PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
TRAP	CACTCCCACCCTGAGATTTGT	CATCGTCTGCACGGTTCTG
CTSK	AATACCTCCCTCTCGATCCTACA	TGGTTCTTGACTGGAGTAACGTA
IGF-1	GACCGAGGGGCTTTTACTTCA	GGACGGGGACTTCTGAGTCTT
YM1	AGAAGGGAGTTTCAAACCTGGT	GTCTTGCTCATGTGTGTAAGTGA
CD206	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
TRAF6	ATGCAGAGGAATCACTTGGCA	ACGGACGCAA4GCAAGGTT
PPARγ	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA
CD36	ATGGGCTGTGATCGGACTG	GTCTTCCCAATAAGCATGTCTCC
c-Fos	GAATCCGAAGGGAACGGAATAA.G	CAATCTCAGTCTGCAACGCA
PGC-lb	TGACGTGGACGAGCTTTCAC	GGGTCTTCTTATCCTGGGTGC

considered statistically significant and was indicated by * or **, respectively.

RESULTS

PPARγ LIGANDS REGULATED OSTEOCLASTOGENESIS

Our objective was to investigate the specific role of PPARγ on the differentiation of osteoclasts. Bone marrow cells were obtained from 8-week-old BALB/C mice and cultured in the presence of recombinant mouse M-CSF (100 ng/ml) and RANKL (100 ng/ml) with and without PPARγ agonists on coverslips. At day 9, cells were stained with Acid Phosphatase, Leukocyte (TRAP) kit (Sigma) and the number of TRAP-positive osteoclasts (>3 nuclei) were counted. As expected, microscopy and cell count showed that the number of mature multinucleated osteoclasts were significantly increased by PPARγ full agonist rosiglitazone (ROS), while SPPARγMs Fmoc-L-Leu(Fmoc) and telmisartan (TM) treatment reduced the pro-differentiation effect compared with ROS (Fig. 1).

Meanwhile, total mRNA was extracted from osteoclasts and the RNA expression of TRAP and CTSK, two typical functional indicators of osteoclasts [Kiviranta et al., 2001; Matsuo et al., 2004] were detected. Consistent with the results mentioned above, we found that the expression of TRAP was only enhanced a little by Fmoc and TM, while ROS further raised the expression. A similar trend appeared in CTSK expression (Fig. 2A,B).

In order to assess whether PPARγ ligands influenced the formation of osteoblasts, we detected the osteoblast-related factor insulin-like growth factors-1(IGF-1) expressed by mature osteoclasts [Kasukawa et al., 2002].Interestingly, the treatment of ROS decreased the expression of IGF-1 in osteoclasts, while Fmoc and TM treatment showed no significant difference (Fig. 2C). These results suggested that PPARγ ligands might play an important role in decreasing the formation of osteoblasts, thereby promoting the process of osteoporosis.

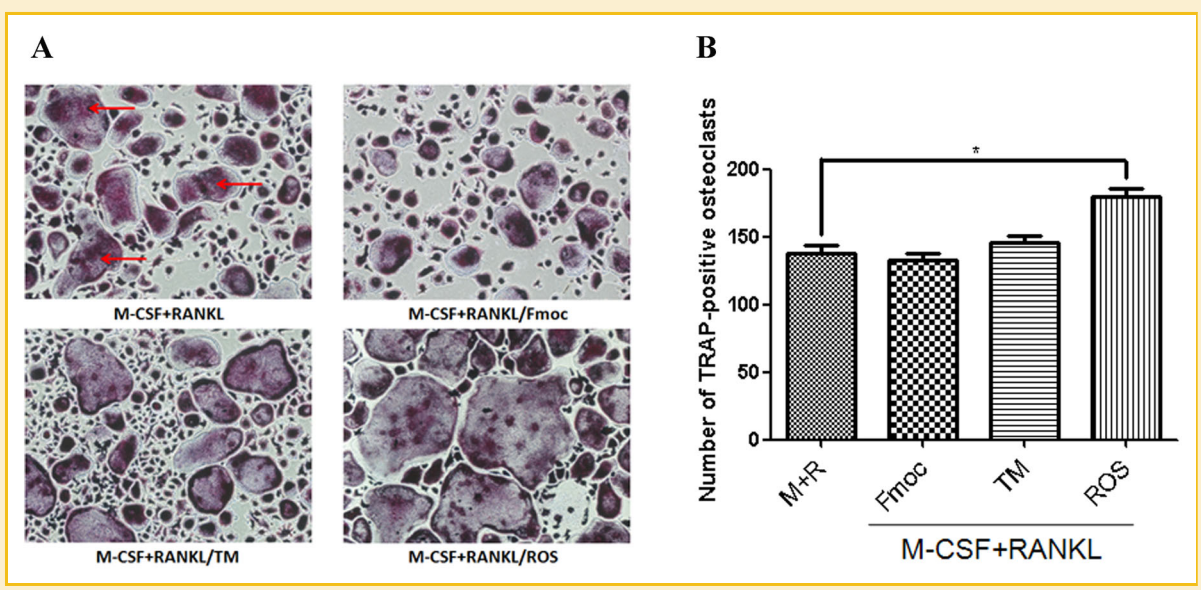

Fig. 1. TRAP staining of mature osteoclasts. We differentiated mouse bone marrow cells with 100 ng/ml of M-CSF and 100 ng/ml of RANKL in α-MEM containing 10% FBS for 4D, then removed the supernatant and further extended the culture for 5D under the same conditions; 10 μM Fmoc, 10 μM TM and 5 μM ROS were added respectively during the course and TRAP staining was carried out at day 9 (A). We identified mature osteoclasts as multinucleated (>3 nuclei), TRAP+ cells. The number of osteoclasts on the coverslips was counted (B), $^{*}P < 0.05$.

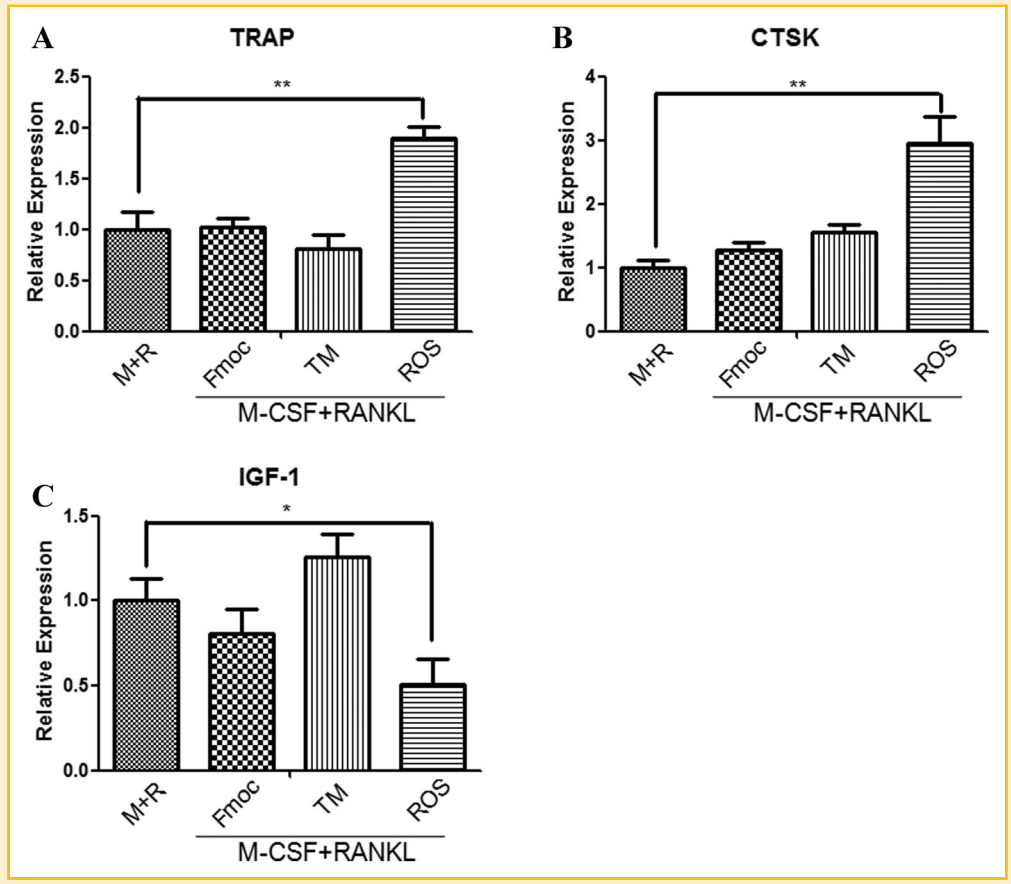

Fig. 2. mRNA expression of osteoclast functional and osteoblast-related genes. We differentiated mature osteoclasts as described in Figure 1, 10 μM Fmoc, 10 μM TM, and 5 μM ROS were added respectively during the course. At day 9, total RNA was extracted from osteoclasts. The mRNA expression of osteoclast functional genes (TRAP and CTSK) were detected by qRT-PCR (A and B).The mRNA expression of osteoblastrelated gene IGF-1 was also detected by qRT-PCR (C), $^{*}P < 0.05$ and $^{**}P < 0.01$.

THE PHENOTYPE OF OSTEOCLASTS IS SILIMAR TO M2

It is well established that macrophages have many phenotypes and are generally divided into three groups: classically activated macrophage (M1 phenotype), alternatively activated macrophage (M2 phenotype), and deactivated macrophage. As one of the resident tissue macrophages, the phenotype of osteoclasts is still unclaimed. Therefore, the cytokines released by osteoclasts were analyzed by ELISA kits. As expected, our results showed that the release of IL-10 and IL-13, considered to be M2 related anti-inflammation cytokines, were detected in osteoclasts (Fig. 3D,E). Meanwhile, we detected low levels of many pro-inflammation cytokines, including TNF-α, IL-1β, and IL-6 (Fig. 3A–C). Taken together, these results indicated the phenotype of osteoclasts was similar to M2 phenotype. Accordingly, the treatment of PPARγ ligands, especially ROS, promoted the release of IL-10 and downregulated the production of pro-inflammation cytokines (Fig. 3A–D), implying an anti-inflammation capacity of PPARγ ligands.

To further assess the phenotype of osteoclasts, we measured the expression of typical M2 macrophage surface markers mannose receptor (CD206) and YM 1 by qRT-PCR. Osteoclasts consistently expressed CD206, which could be further promoted by PPARγ ligands, especially ROS (Fig. 4A). YM1 could also be detected, however, not as significantly as CD206 (Fig. 4B).

PPARγ LIGANDS REGULATED THE ACTIVITY OF PPARγ DURING THE DIFFERENTIATION OF OSTEOCLASTS

To determine the activation of PPARγ, the expression of CD36, a downstream effector marker regulated by the activated PPARγ, was investigated [Nicholson et al., 2004]. As seen in Figure 5A, ROS significantly activated PPARγ and increased CD36 expression. Fmoc and TM could also promote the activity of PPARγ and expression of CD36. We also examined the expression of PPARγ, notably; the RNA expression of PPARγ was extremely increased by ROS (Fig. 5C). However, the western blot result indicated that PPARγ ligands did not influence the protein expression of PPARγ (Fig. 5D,E).

Except PPARγ ligands, the activation of PPARγ also needed the help of other co-activators, such as PGC-1β [Wei et al., 2010]. PCR analysis demonstrated that the RNA expression of PGC-1β was increased by ROS. SPPARγMs had no significant role in the promotion (Fig. 5B), suggesting that PGC-1β may have taken part in the differentiation of osteoclasts.

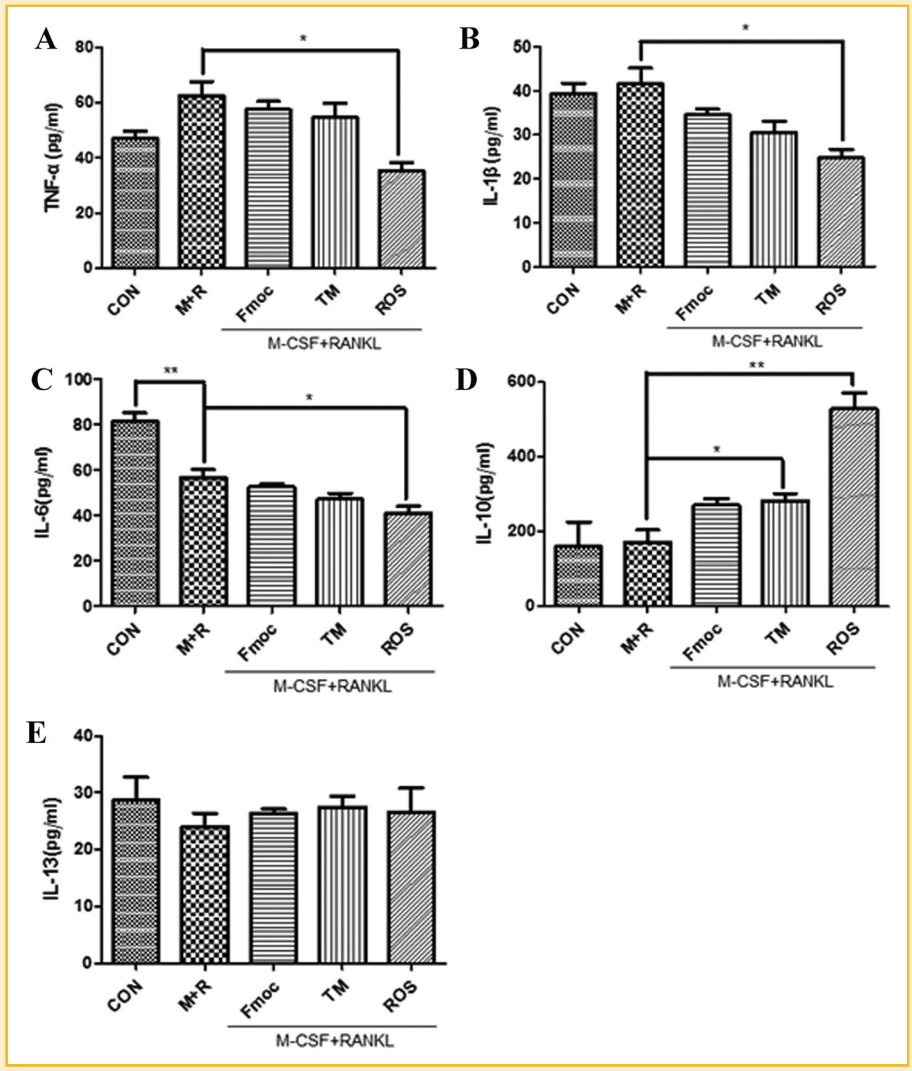

Fig. 3. Determination of cytokine released by osteoclasts. We differentiated mature osteoclasts as described in Figure 1, 10 μM Fmoc, 10 μM TM, and 5 μM ROS were added respectively during the course. At day 9, the culture supernatants were harvested, M1-related cytokines (A–C) and M2-related cytokines (D and E) were determined by ELISA, $^{*}P < 0.05$ and $^{**}P < 0.01$.

PPARγ LIGANDS REGULATED THE SIGNAL PATHWAYS IN OSTEOCLASTS DIFFERENTIATION

Previous studies have suggested roles for TRAF6 and MAPK signal pathways in the differentiation of osteoclasts [Kobayashi et al., 2001]. To assess the mechanisms underlying the regulation of PPARγ ligands, we next evaluated the expression of TRAF6 and MAPK signal pathways related genes. Our data demonstrated that the expression of TRAF6 was significantly increased by ROS; this verified the pro-differentiation of osteoclasts by PPARγ full agonists. SPPARgMs had a little effect on the expression of TRAF6 (Fig. 6A). Furthermore, c-Fos, the downstream of JNK signal pathway, also involved in the differentiation of osteoclasts [Grigoriadis et al., 1994]. PPARγ ligands promoted the expression of c-Fos, consistently with the results of TRAF6, but not so significant (Fig. 6B). Taken together, these results suggested that PPARγ ligands might regulate the differentiation of osteoclasts by JNK signal pathway.

It is also reported that the ERK pathway is involved in the negative regulation of osteoclastogenesis [Boyle et al., 2003], therefore, the protein expression of ERK1/2 and P-ERK1/2 were examined. We discovered that ROS reduced the phosphorylation of ERK1/2 without impacting the total expression of ERK1/2 (Fig. 6C,D); this indicated the activity of ERK1/2 was suppressed, which in turn promoted the osteoclastogenesis. SPPARγMs also decreased the phosphorylation of ERK1/2, however, less than that displayed by ROS (Fig. 6C,D).

DISCUSSION

Thiazolidinediones (TZDs) have been used to treat hyperglycemia in type II diabetes since 1997. Despite their powerful insulin-sensitizing effects, these drugs presented a number of deleterious side effects in clinical practice, including significant weight gain,

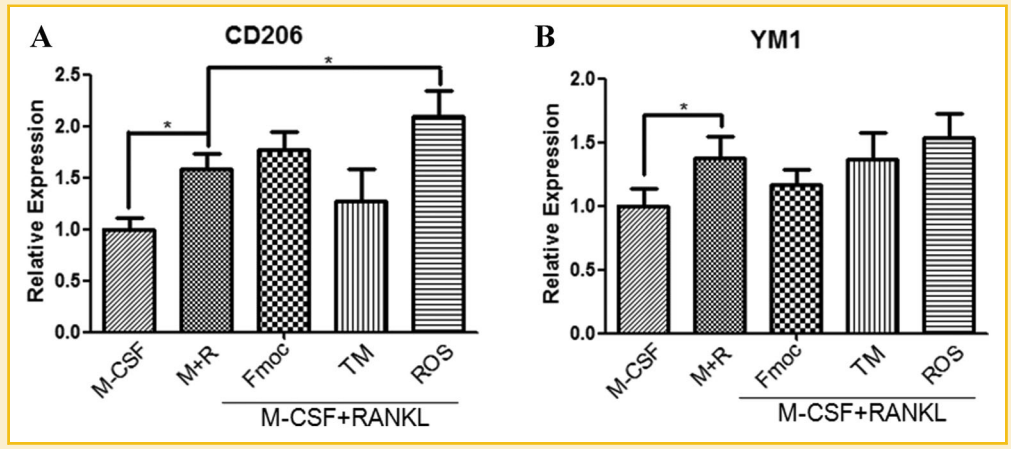

Fig. 4. M2 phenotype genes expressed by osteoclasts. We differentiated mature osteoclasts as described in Figure 1, 10 μM Fmoc, 10 μM TM and 5 μM ROS were added respectively during the course. At day 9, total RNA was extracted from osteoclasts. The mRNA expression of macrophage M2 phenotype genes (CD206 and YM1) were detected by real-time quantitative PCR and $^{**}P < 0.01$.

peripheral edema and bone loss [Nesto et al., 2003; Rzonca et al., 2004; Wan et al., 2007]. Fmoc-L-Leu was found to be the ligand of PPARγ in 2001, but the co-activators recruited were different from traditional agonists which can only partly activate PPARγ, therefore people named it the selective PPARγ modulator. Until now, there have been about 20 kinds of SPPARγMs, including Fmoc-L-Leu and Telmisartan. Fmoc-L-Leu is a chemically distinct PPARγ ligand. Two molecules of Fmoc-L-Leu bind to the ligand-binding domain of a single PPARγ molecule, making its mode of receptor interaction distinct from that of other nuclear receptor ligands. Fmoc-L-Leu induces a particular allosteric configuration of PPARγ, resulting in differential cofactor recruitment and translating in distinct pharmacological properties. Rocchi et al. [2001] have shown that Fmoc-L-leu can improve insulin sensitivity via activation of PPARγ, with a lower adipogenic activity compared with rosiglitazone. Angiotensin AT1 receptor antagonists (sartans) are widely used in the treatment of hypertension and related diseases; they account for 15% of all antihypertensive prescriptions in US. Telmisartan is one of the angiotensin AT1 receptor antagonists noted for its unique favorable effects on carbohydrate and lipid metabolism. Of note, Telmisartan was shown to be able to abolish the excess weight gain induced by pioglitazone without interfering with its insulin-sensitizing properties, thus identify it to be another SPPARγM [Benson et al., 2004; Derosa et al., 2004]. In the current study, we used in vitro differentiation model and our data indicated that rosiglitazone could significantly promote the differentiation of osteoclasts, resulting in bone loss. More importantly, we first demonstrated two SPPARγMs, Fmoc-L-Leu and Telmisartan, had partly abolished the side effect compared with rosiglitazone in vitro.

As we all know, macrophages can be divided into three phenotypes: classically activated macrophage (also named M1 phenotype), alternatively activated macrophage (also named M2 phenotype), and deactivated macrophage. Although osteoclasts belong to the long-lived resident tissue macrophages, the phenotype has not been identified. To evaluate this, we detected the cytokines released by osteoclasts and the expression of some M2 phenotype markers, such as CD206 and YM 1. As expected, we found that osteoclasts revealing characteristic M2 phenotype showed high expression of CD206, YM1, and IL-10; this might be triggered by M-CSF during the cell culture. Furthermore, PPARγ ligands could promote the process, especially rosiglitazone. These results indicated that PPARγ ligands might have the potential to promote the differentiation of macrophages to the M2 phenotype and display anti-inflammation effect.

The stability of human skeleton is maintained by the balance between osteoclasts and osteoblasts. The excessive differentiation of osteoclasts or inhibition of osteoblasts differentiation will lead to osteoporosis. On the contrary, it will cause osteopetrosis. In our study, we demonstrated that PPARγ ligands truly increased the number of osteoclasts and the related gene expression. But what was the effect on osteoblasts? As the formation and differentiation of osteoblasts are related with osteoblast-inducing factors, such as insulin-like growth factors-1(IGF-1) and bone morphogenetic protein-2(BMP-2) [Kasukawa et al., 2002],we examined the RNA expression of IGF-1 in osteoclasts. It is of interest that the treatment of PPARγ ligands (especially rosiglitazone) decreased the expression of IGF-1, which may promote osteoporosis.

Both PPARγ agonists and SPPARγMs are ligands of PPARγ, however co-activators are recruited differently. It is well established that PGC-1β is a co-activator of PPARγ and PGC-1β can be recruited to promote the activation of PPARγ. Wei et al. [2010] have reported that PGC-1β mediated the osteoclastogenesis through PPARγ. We next evaluated if the expression of PGC-1β was regulated by PPARγ ligands. Our results indicated that the expression of PGC-1β was reduced after the treatment of SPPARγMs instead of rosiglitazone. Meanwhile, as one of the downstream hallmarks of PPARγ activation, SPPARγMs triggered lower expression of CD36 than that displayed by rosglitazone, suggesting PPARγ was partly activated, which may be related with the reduction of co-activator PGC-1β mentioned above.

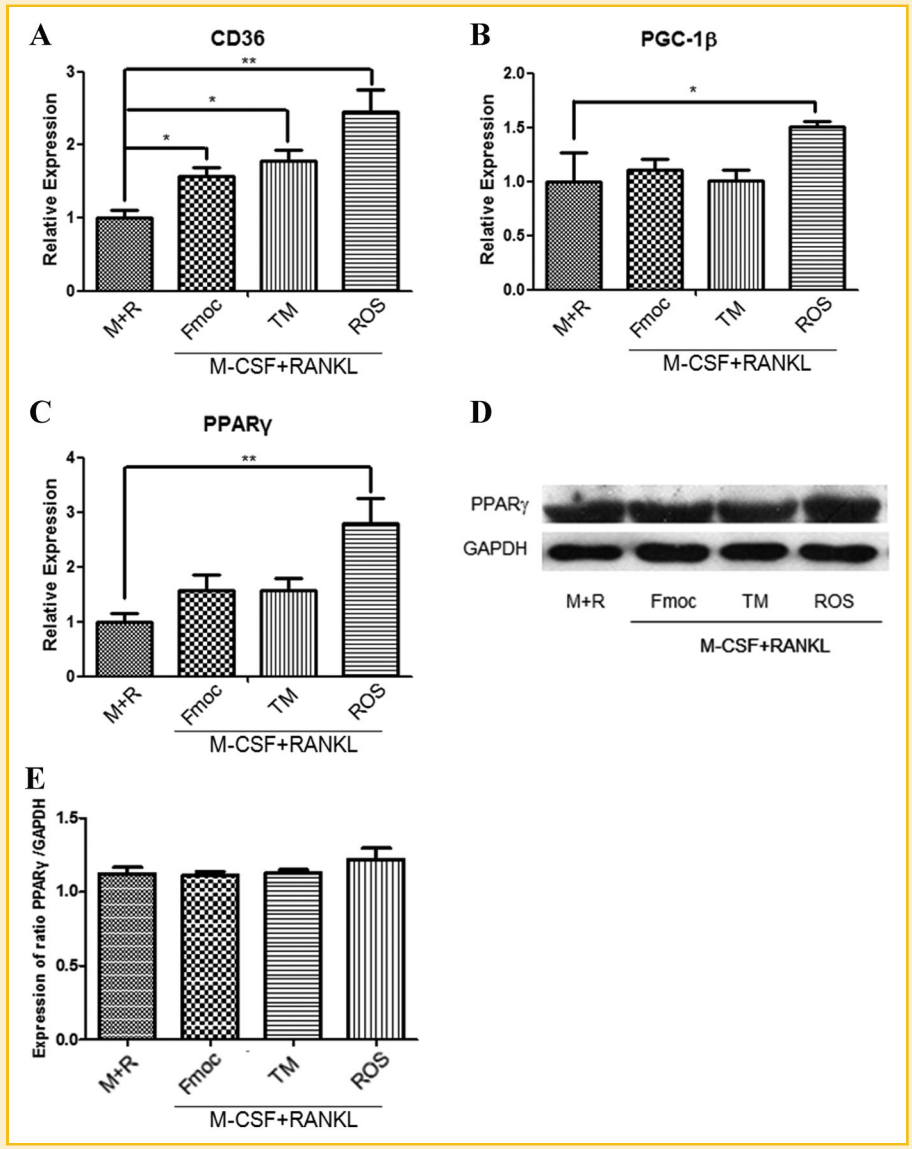

Fig. 5. The activity of PPARγ during the differentiation of osteoclasts. Mature osteoclasts were obtained as described in Figure 1 and PPARγ ligands were added. At day 9, total RNA was extracted from osteoclasts. The mRNA expression of PPARγ downstream gene CD36 (A) and PPARγ co-activator PGC-1β (B) were detected by qRT-PCR. The mRNA and protein expression of PPARγ itself were also detected by qRT-PCR (C) and Western blot (D). Data were quantified by ImageJ densitometry analysis (E), $^{*}P < 0.05$ and $^{**}P < 0.01$.

Finally, our study explored the mechanisms underlying the regulation of PPARγ ligands. It has been widely reported that the differentiation of osteoclasts is associated with RANK/TRAF6 axis, which mediates many signaling pathways, including MAPK pathway, NF-κB pathway, and JNK pathway [Boyle et al., 2003]. We demonstrated that rosiglitazone significantly increased the expression of TRAF6, while the enhancement by SPPARγMs was relatively lower. At the same time, c-FOS, as the downstream of JNK signal pathway, also displayed the same trend. On another aspect, the ratio of P-ERK1/2 and ERK1/2 was decreased by ROS, suggesting the ERK signal pathway was suppressed. Thus promoted the differentiation of osteoclasts according to the previous report [Hotokezaka et al., 2002]. All these results suggested that PPARγ ligands might promote the differentiation of osteoclasts through RANK/TRAF6 axis, MAPK, and JNK pathways.

However, there are still many unresolved problems. Is it possible that some other co-activators are recruited by SPPARγMs to activate PPARγ? Are there different downstream genes regulated by SPPARγMs, which influence the differentiation of osteoclasts? What is the pathway relationship between TRAF6 and PPARγ? Are post-translational modifications of PPARγ involved? All these questions need to be addressed.

In summary, we provided evidence that SPPARγMs had less pro-differentiation activity in osteoclasts compared with PPARγ full agonist rosiglitazone. In addition, we demonstrated the phenotype of osteoclasts was similar to M2 phenotype, which can be further

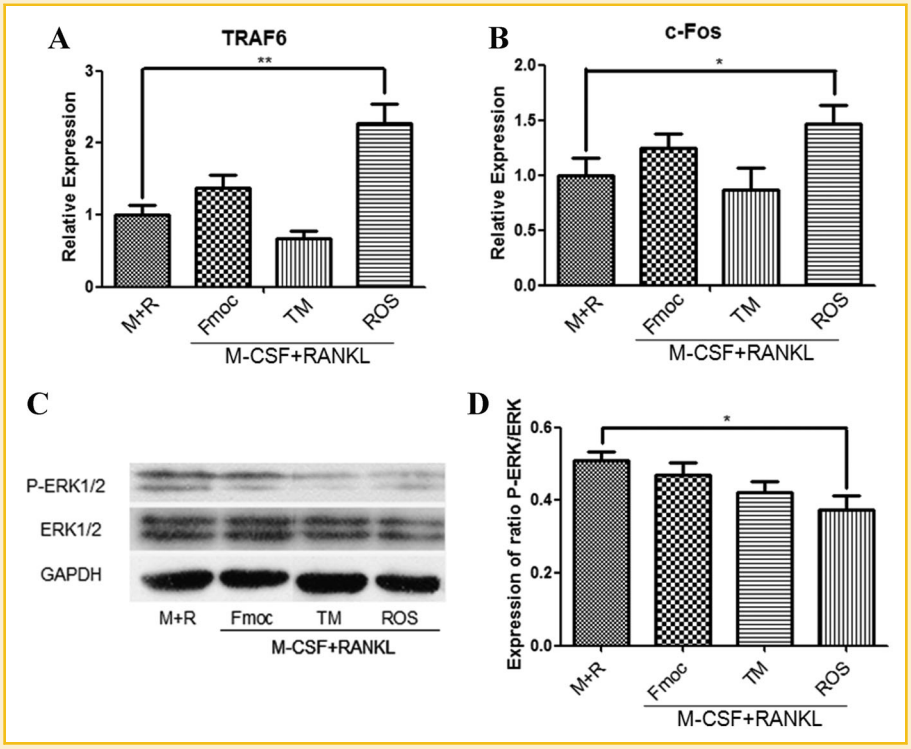

Fig. 6. PPARγ ligands regulated the signal pathways in osteoclasts differentiation. We obtained mature osteoclasts as described in Figure 1, 10 μM Fmoc, 10 μM TM, and 5 μM ROS were added respectively during the course. At day 9, total RNA and protein were extracted from osteoclasts. The mRNA expression of genes in osteoclasts differentiation signal pathways (TRAF6 and c-FOS) were detected by qRT-PCR (A and B). The protein expression of ERK1/2 and P-ERK1/2 were examined by Western blot and quantified by ImageJ densitometry analysis (C and D), $^{*}P < 0.05$ and $^{**}P < 0.01$.

promoted by PPARγ ligands. The effects might be regulated through TRAF6 and MAPK signal pathways by the activation of PPARγ. These findings have potential clinical implications, as they imply that long-term rosiglitazone usage as antidiabetic drug may cause osteoporosis, owing to a combination of increased bone resorption and reduced bone formation. Our data also indicated that SPPARγMs might provide a new strategy for the development of antidiabetic drugs that have little side effects. On the other hand, given the potential importance of this finding to increased expression of osteoblasts formation related genes, the pro-differentiation activity of PPARγ ligands might be beneficial in certain diseases, like osteosclerosis.

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