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Metformin prevents anti-osteogenic in vivo and ex vivo effects of rosiglitazone in rats

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ABSTRACT

Long-term treatment with the insulin-sensitizer rosiglitazone reduces bone mass and increases fracture risk. We have recently shown that orally administered metformin stimulates bone reossification and increases the osteogenic potential of bone marrow progenitor cells (BMPC). In the present study we investigated the effect of a 2-week metformin and/or rosiglitazone treatment on bone repair, trabecular bone microarchitecture and BMPC osteogenic potential, in young male Sprague–Dawley rats. Compared to untreated controls, rosiglitazone monotherapy decreased bone regeneration, femoral metaphysis trabecular area, osteoblastic and osteocytic density, and TRAP activity associated with epiphyseal growth plates. It also decreased the ex vivo osteogenic commitment of BMPC, inducing an increase in PPARγ expression, and a decrease in Runx2/Cbfa1 expression, in AMP-kinase phosphorylation, and in osteoblastic differentiation and mineralization. After monotherapy with metformin, with the exception of PPARγ expression which was blunted, all of the above parameters were significantly increased (compared to untreated controls). Metformin/rosiglitazone co-treatment prevented all the in vivo and ex vivo anti-osteogenic effects of rosiglitazone monotherapy, with a reversion back to control levels of PPARγ, Runx2/Cbfa1 and AMP-kinase phosphorylation of BMPC. In vitro co-incubation of BMPC with metformin and compound C—an inhibitor of AMPK phosphorylation—abrogated the metformin-induced increase in type-1 collagen production, a marker of osteoblastic differentiation. In conclusion, in rodent models metformin not only induces direct osteogenic in vivo and ex vivo actions, but when it is administered orally in combination with rosiglitazone it can prevent several of the adverse effects that this thiazolidenedione shows on bone tissue.

1. Introduction

Diabetes mellitus has been shown to be associated with a reduction in bone strength, an increase in bone fracture rate and impairment in bone healing, both in human (de Liefde et al., 2005; Melton et al., 2008; Vestergaard et al., 2005) and in experimental animal models (Kawashima et al., 2009; Santana et al., 2003).

Patients with type 2 diabetes mellitus are frequently treated with oral insulin-sensitizing drugs such as biguanides and thiazolidenediones, in monotherapy or combined therapy. We have recently demonstrated that the biguanide metformin induces a direct osteogenic action in a model of osteoblasts in culture (Cortizo et al., 2006). These actions include a dose-dependent increase in cell proliferation and type-I collagen production, as well as an increase both in alkaline phosphatase activity (ALP), and in extracellular mineral deposition. These osteogenic actions of metformin have been shown to be associated with an activation of ERK and AMPK pathways, an induction of endothelial nitric oxide synthase (eNOS) and an increase in bone morphogenetic protein-2 (BMP-2) expression (Cortizo et al., 2006; Kanazawa et al., 2008).

In other previous studies we have also found that the in vivo administration of metformin to Sprague–Dawley rats induces an increased rate of differentiation towards the osteoblast phenotype of bone marrow progenitor cells (BMPC) obtained from the animal's tibiae vs. control rats. In vivo metformin administration enhances the expression by BMPC of the osteoblast-specific transcription factor Runx2/Cbfa1, as well as activation of AMPK (Molinuevo et al., 2010). In addition, using a model of minimal bone defect we demonstrated that metformin treatment stimulates bone reossification in both control and diabetic rats (Molinuevo et al., 2010).

Osteoblasts and adipocytes derive from a common bone marrow progenitor cell (Pittenger et al., 1999). In vitro, the thiazolidinedione rosiglitazone has been demonstrated to induce murine bone marrow stromal cell adipogenesis while inhibiting osteogenesis, by binding to PPARγ2 receptors (Lecka Czernik et al., 2002). In vivo, treatment of mice with rosiglitazone has been found to decrease bone mineral content, bone formation, and trabecular bone volume, while increasing adipogenesis, by decreasing Cbfa1 and enhancing aP2 expression (Ali et al., 2005; Rzonca et al., 2004). Interestingly, we have shown that metformin partially inhibits the adipogenic actions of rosiglitazone on BMPC in vitro (Molinuevo et al., 2010).

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Several clinical studies have recently examined the effect of thiazolidinediones on bone mass and on the risk of bone fractures. In an observational study, bone mass was shown to be decreased in thiazolidinedione users (Schwartz et al., 2006). Additionally, both pioglitazone and rosiglitazone were associated with an increased bone fracture risk in other large prospective clinical trials (Dormuth et al., 2009; Kahn et al., 2008; Meier et al., 2008).

In the present work we have investigated the in vivo actions of metformin and rosiglitazone monotherapy—as well as their co-treatment or combined therapy—on rat bone reossification and on the micro-architecture and histomorphometry of femoral metaphysis. We have also evaluated the ex vivo effects of these drug combinations on BMPC obtained from the femoral diaphysis: in particular, their action on the lineage commitment of BMPC by measuring PPARγ and Runx2/Cbfa1 expression, ALP activity, type-I collagen production and extracellular mineral deposition. In order to study possible mechanisms of action, we have measured the activation of the AMPK signal transduction pathway.

2. Materials and methods

2.1. Animal treatment

Adult male Sprague–Dawley rats (190–210 g) were used. Animals were maintained in a temperature-controlled room at 23 °C, with a fixed 12 h light:12 h darkness cycle, and fed standard rat laboratory chow and water *ad libitum*. All experiments on animals were performed in conformity with the Guidelines on Handling and Training of Laboratory Animals published by the Universities Federation for Animals Welfare (Guidelines of the UFAW, 1992).

Animals were divided into four groups of 10 animals per group: control rats (C) received water ad libitum; metformin-treated rats (M) received 100 mg/kg/day of metformin (Quimica Montpellier, Buenos Aires, Argentina) in drinking water for 2 weeks (Molinuevo et al., 2010); rosiglitazone-treated rats (R) received 4 mg/kg/day of rosiglitazone (Quimica Montpellier, Buenos Aires, Argentina) in drinking water for 2 weeks (Wang et al., 2008); and metformin and rosiglitazone-treated rats (M-R) received a combination of both treatments.

Serum glucose (glucose oxidase method) (Lott and Turner, 1975), triglyceride and cholesterol were measured by commercial kits (Wiener Laboratories, Argentina). No significant differences between experimental groups were found for these serum metabolites.

2.2. Evaluation of bone micro-architecture and reossification

We evaluated the effect of metformin and/or rosiglitazone treatment on (a) femoral metaphysis micro-architecture, and (b) the process of bone repair using a reossification model of minimal bone lesion as described previously (Santana et al., 2003). Briefly, all animals were anesthetized by intraperitoneal/intramuscular injection of 0.12 ml/100 g body weight with 62.5 mg/ml ketamine hydrochloride and 6.25 mg/ml xylazine (Laboratorios Richmond, Buenos Aires, Argentina). Circular craniotomy defects of 1.0 mm diameter were made in parietal bones of animals with a cylindrical low-speed carbide bur (Santana et al., 2003). Animals were then divided into four groups as described in Animal treatment section, maintained in a thermostatized atmosphere with 12 h light–dark cycles and fed with commercial chow. After 15 days of treatment all rats were sacrificed under anesthesia by cervical dislocation, prior to dissection of femoral and parietal bones for evaluation of metaphyseal micro-architecture and bone re-ossification, respectively, as described in the following section.

2.3. Histological and histochemical evaluation of bone

Femoral and parietal bones obtained from rats of all four groups were processed for histological and quantitative histomorphometric analysis. Bones were fixed in 10% formalin and decalcified in 10% EDTA. The bones were embedded in paraffin and 5 μm sections were obtained with an SM 2000R Leica microtome. The sections were stained with either hematoxylin–eosin (H–E) for routine evaluations, or tartrate resistant acid phosphatase (TRAP) (Sigma, St. Louis, MO) histochemistry to specifically identify osteoclasts (Schell et al., 2006). Pictures were taken with a Nikon Coolpix 4500 digital camera on an Eclipse E400 Nikon microscope. Images were analyzed using the Image J program (www.macbiophotonics.ca/imagej) with a Microscope scale plugin.

In femur metaphysis, trabecular bone percentage/density was determined as the quotient between the area of trabecular bone and the total area (trabecular bone plus bone marrow cavities). In parietal bones at the site of the previously induced lesion, bone reossification was calculated as the ratio between the newly reossified area and the average bone thickness (H–E).

Additionally, osteoblastic density was evaluated by counting the number of osteoblasts per unit of bone surface (H–E); osteocyte density was assessed by counting the number of osteocytes per area of bone (H–E); and osteoclastic density was calculated as the positive TRAP area per square millimeter of bone tissue (Oc/mm^2) (Molinuevo et al., 2010).

2.4. Bone Marrow Progenitor Cell (BMPC) isolation and osteogenic induction

The ex vivo effect of metformin and/or rosiglitazone on BMPC was evaluated (that is, the effect of insulin-sensitizers administered in vivo, on the phenotypic expression of BMPC isolated from animals of all experimental groups and cultured in vitro). BMPC were obtained from rats of all four groups, as previously described (Molinuevo et al., 2010). Briefly, animals were sacrificed under anesthesia by rapid neck dislocation. Bone marrow cells were collected by flushing femur and tibiae of the animals with Dulbecco's modified essential medium (DMEM) (Invitrogen, Buenos Aires, Argentina) under sterile conditions. The obtained suspension was seeded in a 25 cm^2 tissue culture flask and incubated in DMEM supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina) at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Non-adherent cells were removed by changing the medium after 24 h. The culture medium was changed twice a week until cells reached confluence (10–15 days). In some experiments, the cell monolayer was detached using 0.12% trypsin–1 mM EDTA, replated in 6- or 24-well plates containing 10% FBS-DMEM media and incubated at 37 °C. After cells reached confluence, they were induced to differentiate into osteoblasts using an osteogenic medium (DMEM–10% FBS containing 25 μg/ml ascorbic acid and 5 mM sodium β-glycerol–phosphate) for a further 15 or 21 days. Medium was changed twice a week.

2.5. Evaluation of osteoblastic differentiation of BMPC

After 15 days of osteogenic induction, cell monolayers grown in 24-well plates were washed with phosphate buffered saline (PBS) and lysed with 200 μl of 0.1% Triton-X100. An aliquot of the lysate was used to evaluate alkaline phosphatase activity (ALP) by the hydrolysis of p-nitrophenylphosphate (p-NPP) into p-nitrophenol (p-NP) at 37 °C in an appropriate alkaline buffer. The absorbance of p-NP was recorded at 405 nm (Cortizo and Etcheverry, 1995). Aliquots of the same extract were used for protein determination by Bradford's technique (Bradford, 1976). Type I collagen production was evaluated as previously reported (Tullberg-Reinert and Jundt, 1999). Briefly, after 21 days of osteogenic differentiation cell monolayers were fixed with Bouin's solution and stained with Sirius red dye for 1 h. The stained material was dissolved in 1 ml of 0.1 N sodium hydroxide and the absorbance of the solution was recorded at 550 nm. Extracellular calcium deposits (mineralization nodules) were also measured after 21 days of osteogenic differentiation using Alizarin S red staining (Ueno et al., 2001). Stained calcium deposits were extracted with 1 ml of 0.1 N sodium hydroxide, recording the optical density at 548 nm.

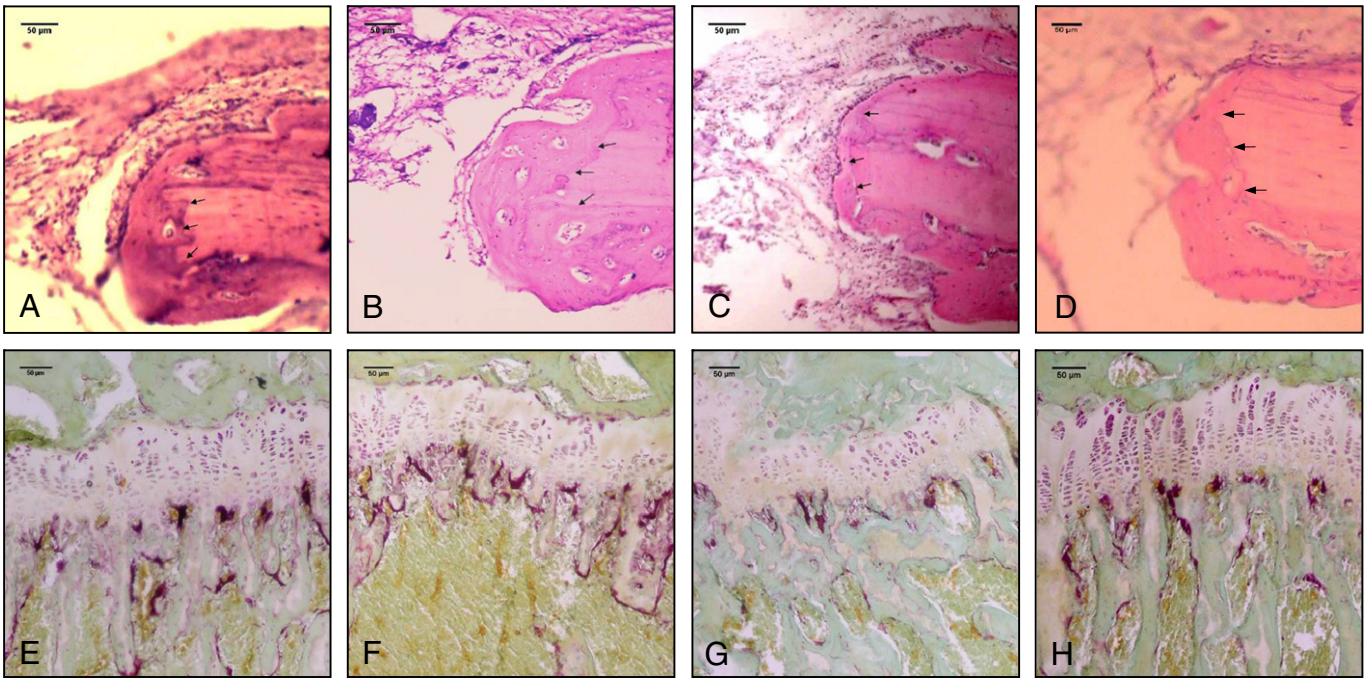

Fig. 1. Effects of metformin and/or rosiglitazone on bone healing and on TRAP activity of femoral metaphyses. As described in Materials and methods, animals were anaesthetized and a 1 mm circular lesion practiced in one of their parietal bones, after which they were submitted for 2 weeks to no treatment (A, E), 100 mg/kg/day of metformin in drinking water (B, F), 4 mg/kg/day of rosiglitazone in drinking water (C, G), or combined treatment with both insulin-sensitizing agents (D, H). After all treatments, animals were sacrificed and their parietal bones and their femurs were dissected and demineralized for staining with hematoxylin–eosin (A–D) or for histochemical staining of the femoral metaphyses to determine TRAP activity (E–H). A–D: Arrows indicate new bone formation; the bone lesion is at the left hand side of each photograph, with a vertical (top-to-bottom) direction.

2.6. Western blot analysis for Cbfa-1 and PPARγ expression

BMPC from all experimental groups were grown to confluence in 6-well plates in DMEM–10% FBS. In some cases, cells were further differentiated for 21 days into osteoblasts as described above. After these culture periods, cells were lysed in Laemmli's buffer (Laemmli, 1970). These lysates were heated to 100 °C for 3 min, and 30 μg of protein subjected to 12% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. After washing and blocking, the membranes were incubated overnight at 4 °C with an antibody directed against Cbfa-1/Runx-2 for evaluation of osteoblastogenesis, or with an antibody specific for PPARγ to evaluate adipocytic commitment (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In order to normalize results, all blots were stripped and re-probed with an anti β-actin antibody (Sigma, St. Louis, MO, USA).

2.7. Mechanisms of action for metformin

BMPC from all experimental groups were cultured in 6-well plates in DMEM–10% FBS until confluent, then further submitted to a 3-week osteogenic differentiation. Cells were then lysed in Laemmli's buffer (Laemmli, 1970), electrophoresed and blotted to PVDF membranes. Blots were probed with either an anti-total AMPK or anti-P-AMPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Kanazawa et al., 2008). In all cases, immunoblots were developed by an enhanced chemiluminescence method. The intensity of the specific bands was quantified by densitometry after scanning the photographic film. Images were analyzed using the Scion-beta 2 program.

In other in vitro experiments, confluent BMPC from control rats were submitted to an osteogenic induction for 15 days as described above, in the presence or absence of 100 μM metformin and/or 0.5 μM compound C (an inhibitor of AMPK activation/phosphorylation) added to the culture medium (Shah et al., 2010). After these incubations, cells were evaluated as described above for type 1 collagen secretion and AMPK/P-AMPK levels.

2.8. Statistical analysis

Results are expressed as the mean ± S.E.M. Differences between the groups were assessed by one-way ANOVA using the Tukey *post hoc* test. For non-normal distributed data, the non-parametrical Kruskal–Wallis test with Dunn *post hoc* test was performed, using GraphPad In Stat, version 3.00 (Graph Pad Software, San Diego, CA, USA). $P<0.05$ was considered significant for all statistical analyses.

3. Results

3.1. Metformin prevents rosiglitazone-induced decrease in bone healing

As described in Materials and methods, minimal bone lesions were induced in the parietal bones of rats. The animals were then submitted to an oral treatment with rosiglitazone and/or metformin, or no treatment. After 2 weeks, histological (H–E) analysis of the lesion site showed that, while metformin monotherapy greatly increased bone healing vs.

Table 1
Effect of oral metformin and/or rosiglitazone on parietal bone healing.

Oral treatment	[Reossified area/parietal thickness] (% of control)
None (control)	100 ± 8
Metformin	207 ± 15 [a]
Rosiglitazone	22 ± 3 [a]
Metformin + rosiglitazone	93 ± 9 [b]

Animals were anesthetized and a 1 mm circular lesion practiced in one of their parietal bones, after which they were treated for 2 weeks as described in Materials and methods, with or without metformin and/or rosiglitazone. After all treatments, animals were sacrificed and their parietal bones were dissected and demineralized for staining with hematoxylin–eosin and quantitation of reossification. Values are expressed as the mean ± S.E.M. of sections.

[a] $P<0.01$ versus control.

[b] $P<0.02$ versus rosiglitazone.

control, rosiglitazone monotherapy impaired it. Moreover, in animals submitted to the co-treatment, metformin completely prevented the deleterious effects of rosiglitazone on bone reossification (Fig. 1A–D, Table 1). Fig. 1A–D shows typical images of para-sagittal sections through a portion of the 1 mm lesion, for each experimental group.

We also evaluated possible treatment-related changes in the TRAP activity of parietal bone lesions. In accordance with our previously published results (Molinuevo et al., 2010) we found an increase in this parameter in metformin-treated rats versus control. However, rosiglitazone had no effect on the TRAP activity at this site of reossification: rosiglitazone monotherapy was not different from control, while rosiglitazone–metformin combined therapy was not different from metformin alone (data not shown).

3.2. Femoral metaphysis micro-architecture is impaired by rosiglitazone, and restored by metformin

In all experimental groups, histological (H–E) and histochemical (TRAP) analysis were performed on femoral metaphysis in order to evaluate their micro-architecture.

Femoral relative trabecular area was found to be influenced by a 2-week treatment with metformin and/or rosiglitazone (Fig. 2A). Treatment with metformin alone significantly increased relative trabecular area by 26% versus control, while rosiglitazone monotherapy decreased it by 31% versus control. Metformin–rosiglitazone combined therapy prevented the effect of rosiglitazone alone. Similar tendencies were found for density of osteocytes, specialized in bone maintenance: namely, an increase for metformin alone versus control, a decrease for rosiglitazone alone, and a complete reversion back to control levels for rosiglitazone–metformin combined therapy (Fig. 2C). When we evaluated the density of bone-forming osteoblasts, we found them to be reduced versus control by rosiglitazone treatment, while metformin was able to increase osteoblast number versus control even in the presence of rosiglitazone (Fig. 2B).

We also evaluated osteoclast activity by TRAP histochemical staining and found treatment-related differences principally in areas proximal to the femoral cartilaginous growth plate. As can be seen in Figs. 1E–H and 2D, metformin treatment increased TRAP activity versus control, while rosiglitazone monotherapy induced a decrease in this marker of osteoclast number. Interestingly, TRAP activity after rosiglitazone–metformin combined therapy showed a complete reversion back to control levels.

3.3. In vivo administration of metformin and rosiglitazone induce opposing effects on the in vitro osteoblastic differentiation of BMPC

We evaluated the effects of in vivo treatment with rosiglitazone and/or metformin, on the osteoblastic differentiation of BMPC in vitro (i.e., the ex vivo effect of these drugs on BMPC), after 15 or 21 days of

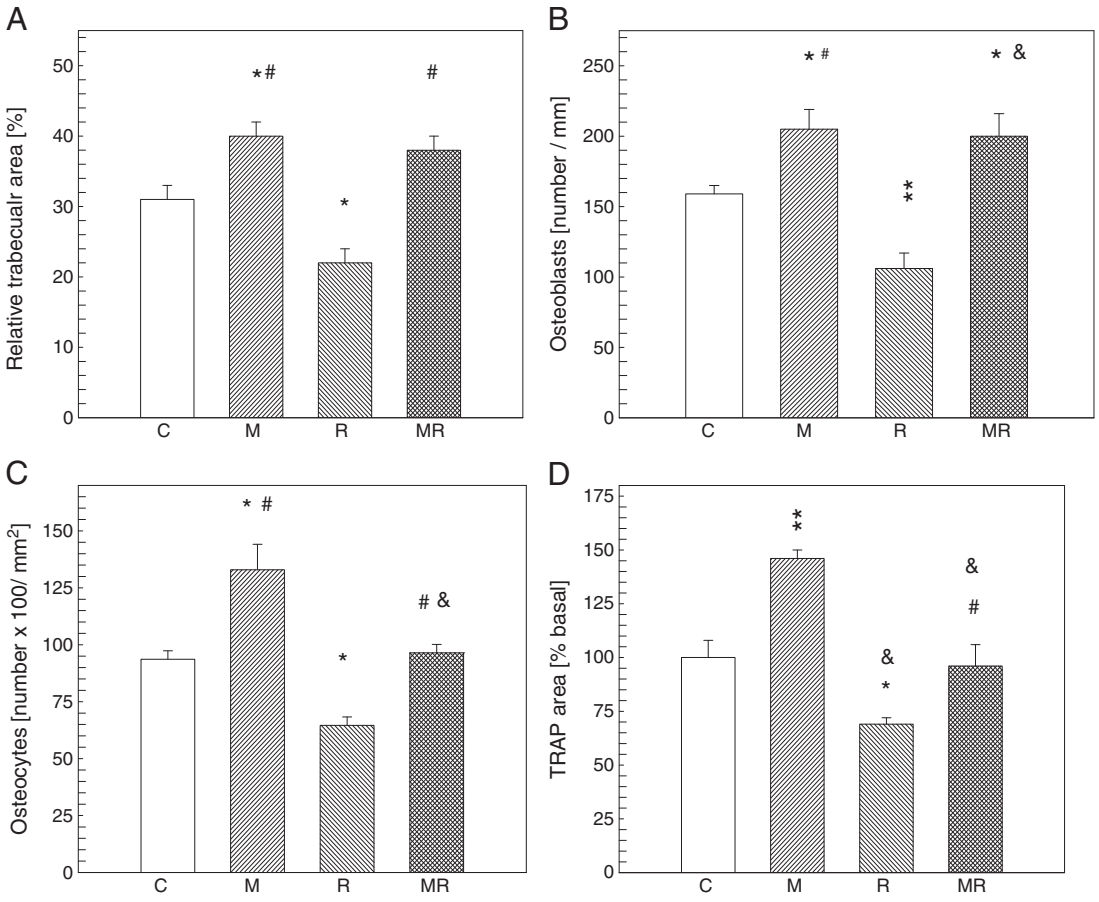

Fig. 2. Metformin and rosiglitazone show opposing effects on osteoblast number, osteocyte density and trabecular volume of femoral metaphysis. Animals were submitted for 2 weeks to no treatment (C), 100 mg/kg/day of metformin in drinking water (M), 4 mg/kg/day of rosiglitazone in drinking water (R), or combined treatment with both insulin-sensitizing agents (MR). After all treatments, animals were sacrificed and their femurs dissected and demineralized for staining of the metaphyses with hematoxylin–eosin (A-C) or analyzed for TRAP activity (D). As described in Materials and methods, relative trabecular area, [%] (A), osteoblast number per mm of bone surface (B), osteocyte number per mm^2 of bone area (C) and positive TRAP area per mm^2 of bone area [% basal] were determined for each experimental condition. Results are expressed as the mean ± S.E.M. *P<0.05; **P<0.001 vs. control; #P<0.01 vs. rosiglitazone; &P<0.001 vs. metformin.

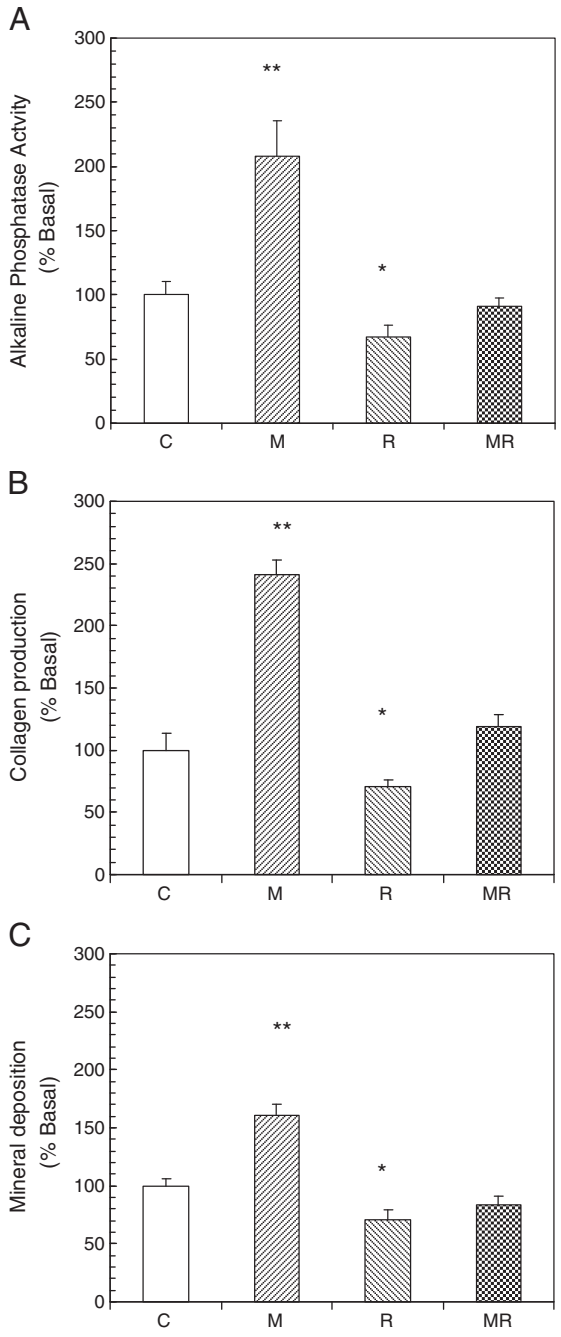

Fig. 3. Ex vivo effects of metformin and/or rosiglitazone on the differentiation and mineralization of BMPC. Animals were submitted for 2 weeks to no treatment (C), 100 mg/kg/day of metformin in drinking water (M), 4 mg/kg/day of rosiglitazone in drinking water (R), or combined treatment with both insulin-sensitizing agents (MR), after which they were sacrificed and processed to obtain BMPC. The resulting cells were then incubated in an osteogenic medium for 2 or 3 weeks, after which they were either lysed or fixed as described in Materials and methods, and evaluated for specific alkaline phosphatase activity (A), type 1 collagen secretion (B) or extracellular mineral accumulation (C). Results are expressed as % of basal (shown as the mean ± S.E.M.). $^{*}P<0.05$ vs. control; $^{**}P<0.01$ vs. control.

incubation of cells in an osteogenic medium as described in Materials and methods. Thus, we measured their ALP activity, type 1 collagen production and accumulation of extracellular mineral nodules. For all three parameters we found a significant increase vs. control for metformin monotherapy and a decrease vs. control for rosiglitazone monotherapy (Fig. 3). In the co-treatment group, a complete reversion to control values was observed for collagen production, while ALP activity and mineralization was only partially reverted (in the latter case only a non-significant tendency was observed).

3.4. Ex-vivo effect of metformin and/or rosiglitazone on the expression of osteoblast- and adipocyte-promoting transcription factors

We evaluated the expression of the osteoblast-specific transcription factor Runx2/Cbfa1, and of the adipocyte-promoting transcription factor PPARγ, by Western blotting of cell lysates obtained from confluent BMPC of all experimental groups (i.e., rats with or without metformin and/or rosiglitazone; BMPC prior to—or after 3 weeks of—osteogenic differentiation). As expected, after 3 weeks of osteogenic induction, BMPC from control rats showed an increase in the expression of Runx2/Cbfa1 and a decrease of PPARγ. Additionally, we found that at both time points (before and after osteogenic differentiation) Runx2/Cbfa1 was significantly decreased vs. control by rat treatment with rosiglitazone as monotherapy, while metformin (a) increased its expression vs. control when administered as monotherapy, and (b) in the co-treatment group prevented the rosiglitazone-induced decrease in expression to control levels (Fig. 4 A and C). When we evaluated expression of PPARγ, we found that prior to osteogenic differentiation its levels were elevated vs. control in BMPC obtained from rosiglitazone-treated rats, while addition of metformin curbed this effect of rosiglitazone (Fig. 4B). On the other hand, after osteogenic differentiation we found no differences between groups for PPARγ expression (Fig. 4D).

3.5. Effect of rosiglitazone and metformin on the AMPK signaling pathway in BMPC

We obtained BMPC from control rats and from rosiglitazone and/or metformin-treated rats, and submitted them to an osteogenic differentiation medium for 21 days, as described in Materials and methods. After this period of time, we evaluated both AMPK and P-AMPK in cell extracts by Western blotting. As expected from our previous results (Molinuevo et al., 2010), we found that ex vivo metformin monotherapy induced a significant increase vs. control in the [P-AMPK/total AMPK] ratio of BMPC (Fig. 5). On the other hand, this ratio was decreased vs. control in BMPC from the rats submitted to rosiglitazone monotherapy. However in BMPC obtained from the metformin–rosiglitazone co-treatment group, the [P-AMPK/total AMPK] ratio reverted back to control levels.

In order to confirm that AMPK activation/phosphorylation mediates metformin osteogenic action at least in part, in other experiments we submitted BMPC from control animals to a 15-day osteoblastic induction, in the presence or absence of metformin and/or compound C (an inhibitor of AMPK activation). As can be seen in Fig. 6, metformin significantly elevated the [P-AMPK/total AMPK] ratio of BMPC and their type 1 collagen production (a marker of osteoblastic differentiation), while co-incubation of metformin and compound C abrogated all of these increases.

4. Discussion

Bone is a specialized connective tissue that includes an organic extracellular matrix mineralized by hydroxyapatite crystals, and a delicately balanced cellular compartment that comprises osteoblasts, osteocytes and osteoclasts. Bone-forming osteoblasts can be recruited from bone marrow progenitor cells (BMPC), which under proper stimuli have the capabilities to differentiate into different cell types. This process can be influenced by different agents such as cytokines, growth factors, hormones, or drugs.

Bone tissue is constantly being renewed by a precise coupling of resorption, formation and maintenance, in a process that has been termed remodeling. If osteoblastic and osteoclastic actions are exactly balanced, bone volume and mineral density remain constant. Bone quality, however, also depends on the activity of osteocytes that have

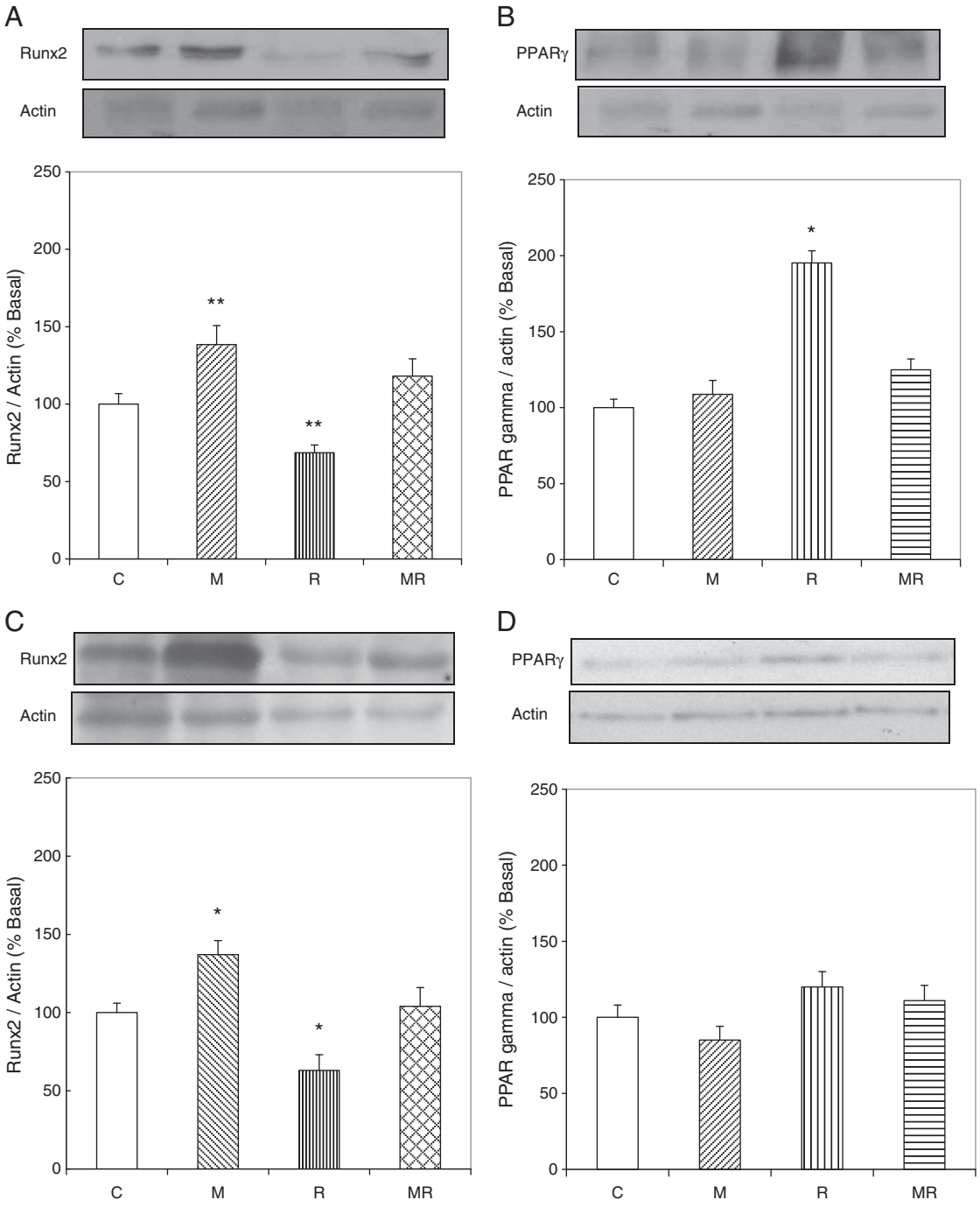

Fig. 4. Ex vivo effects of metformin and/or rosiglitazone on the expression of Cbfa1/Runx2 and PPARγ by BMPC. Animals were submitted for 2 weeks to no treatment (C), 100 mg/kg/day of metformin in drinking water (M), 4 mg/kg/day of rosiglitazone in drinking water (R), or combined treatment with both insulin-sensitizing agents (MR), after which they were sacrificed and processed to obtain BMPC. The resulting cells were cultured until confluence in DMEM-FBS (A, B). In certain experiments, cells were further incubated in an osteogenic medium for an additional 3 weeks (C, D). In all cases, monolayers were then solubilized in Laemmli's buffer and submitted to SDS-PAGE and Western blotting, in order to evaluate the expression either of the osteoblast-specific transcription factor Cbfa1/Runx2 (A, C), or the adipogenic factor PPARγ (B, D). All blots were then stripped and re-probed for house-keeping actin expression, in order to normalize results. Typical Western blots are shown, together with quantitation of all blots for each experimental condition. $^{*}P<0.05$ vs. control; $^{**}P<0.01$ vs. control.

been postulated to eliminate small imperfections from their surrounding mineralized ECM. As these imperfections accumulate, eventually the whole osteon will be remodeled. All osteons undergo remodeling cycles periodically, and it has been estimated that the entire human skeleton is renewed every 10 years (Teitelbaum, 2000). Thus, it is evident that bone tissue can show an increased tendency for micro-fractures or clinical fractures, as a consequence of a decrease in its volume and mineral density (when resorption systematically exceeds bone formation); and/or a decrease in bone quality (when the activity and number of osteocytes is diminished, or when the remodeling cycle of osteons becomes less frequent).

There has recently been an accumulation of basic and clinical evidence linking thiazolidinedione therapy to a decrease in bone mineral density and quality, and to an increase in fracture risk (Ali et al., 2005; Dormuth et al., 2009; Kahn et al., 2008; Lecka Czernik et al., 2002; Meier et al., 2008; Rzonca et al., 2004; Schwartz et al., 2006). Additionally, in July 2010 an expert joint advisory committee to the FDA agreed that treatment with rosiglitazone was associated with a significant increase

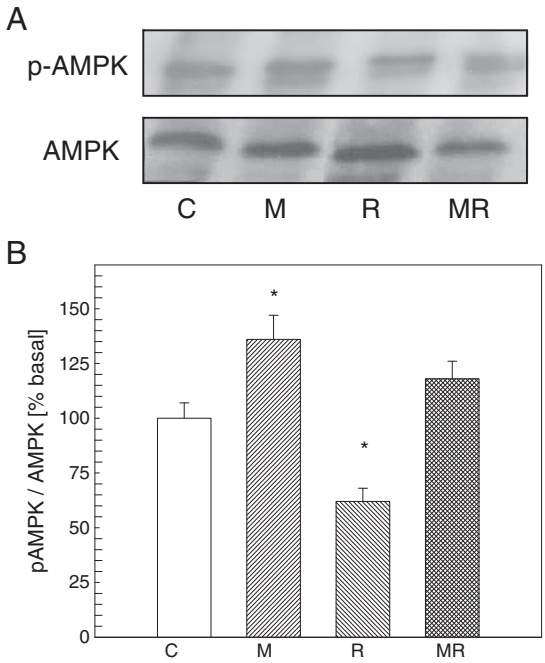

Fig. 5. Ex vivo effects of metformin and/or rosiglitazone on the activation / phosphorylation of AMPK in BMPC. Animals were submitted for 2 weeks to no treatment (C), 100 mg/kg/day of metformin in drinking water (M), 4 mg/kg/day of rosiglitazone in drinking water (R), or combined treatment with both insulin-sensitizing agents (MR), after which they were sacrificed and processed to obtain BMPC. The resulting cells were then incubated in an osteogenic medium for 3 weeks, after which they were solubilized in Laemmli's buffer and submitted to SDS-PAGE and Western blotting, in order to evaluate the expression of the signal-transduction protein AMPK (and of its activated form pAMPK). The corresponding blots for total- and phosphorylated-AMPK were quantitated in order to calculate their ratio for each experimental condition. Typical Western blots are shown in (A), and the P-AMPK/AMPK ratio of all blots for each experimental condition is shown in (B). *$P<0.05$ vs. control.

in cardiovascular risk (Rosen, 2010), after which the FDA decided to restrict the use of this drug. On the other hand, we and others have recently shown that metformin exerts direct osteogenic effects on osteoblasts in culture, via activation of AMPK and ERK (Cortizo et al., 2006; Kanazawa et al., 2008). We have additionally found that when metformin is administered to rats in drinking water, it increases the reossification of small bone lesions, as well as promoting the osteoblastic differentiation of BMPC via AMPK activation (Molinuevo et al., 2010). As metformin is frequently combined with a thiazolidinedione for the treatment of patients with type 2 Diabetes mellitus, in the present study we sought to determine what consequences the simultaneous administration of these two drugs with apparently opposing effects on bone tissue, might have on rat bone micro-architecture and regeneration, and on the osteogenic commitment of BMPC.

Our present in vivo and ex vivo results confirm the findings of previous studies (i.e., the deleterious effects on bone of the thiazolidinedione rosiglitazone; and the osteogenic actions of metformin). In particular, in this study we have found that rosiglitazone administered as monotherapy for 2 weeks significantly decreases vs. control: (a) parietal bone healing; (b) femoral metaphysis trabecular area, osteoblast number and osteocyte density; (c) osteoclastic TRAP activity associated with the metaphyseal growth plate; and (d) the osteogenic commitment of BMPC. On the contrary, after 2 weeks of monotherapy with metformin all of the above parameters are significantly increased (when compared to untreated controls). However, the most remarkable result of the present study is our finding that metformin, when administered orally to rats in combination with rosiglitazone, partially or totally prevents all of the in vivo and ex vivo deleterious effects that this thiazolidinedione shows (as monotherapy) on bone tissue. These findings are in line with our previously published results (Molinuevo et al., 2010), in which we found that when incubated with BMPC in vitro, metformin dose-dependently inhibited the rosiglitazone-induced adipogenesis (triacylglyceride accumulation) of these cells.

In further experiments we set out to investigate possible mechanisms involved in these effects of metformin and/or rosiglitazone on bone. To begin with, we evaluated the ex vivo effect of these insulin-sensitizing drugs on the expression of osteoblastic- and adipocytic-promoting factors by BMPC, both in basal culture conditions and after their in vitro osteogenic induction. As expected from our previous results (Molinuevo et al., 2010) we found that: (a) after 3 weeks of osteogenic induction, BMPC from control rats showed an increase in the expression of Runx2/Cbfa1 and a decrease of PPARγ; and that (b) metformin monotherapy induced an increase at both time points in the expression of Runx2/Cbfa1 but had no effect on the expression of PPARγ, indicating that this drug stimulates osteoblastogenesis in the bone marrow micro-environment. On the other hand, rosiglitazone monotherapy simultaneously increased PPARγ while decreasing Runx2/Cbfa1 expression, suggesting that this thiazolidinedione stimulates adipogenesis and reduces the osteoblastic commitment of BMPC. Interestingly, BMPC obtained after metformin/rosiglitazone combined therapy showed a reversion of both Runx2/Cbfa1 and PPARγ expression back to control (non-treated) levels.

We and others have recently shown the activation (phosphorylation) of AMPK to be a relevant mechanism of action for metformin on bone cells, both in vivo and ex vivo (Jang et al., 2011; Kanazawa et al., 2008; Molinuevo et al., 2010; Shah et al., 2010). In particular, Jang and co-workers found in a series of elegant experiments that metformin can stimulate the differentiation of MC3T3E1 osteoblasts through the trans-activation of Runx-2 via an AMPK/USF-1/SHP regulatory cascade. Thus, in the present study we evaluated this signal-transduction pathway in our ex vivo model, measuring the [P-AMPK/total AMPK] ratio in BMPC obtained from treated or untreated rats. As expected from our previous studies (Molinuevo et al., 2010), we found that this ratio was increased by metformin monotherapy. On the other hand, it was decreased by rosiglitazone monotherapy, and reverted back to control (untreated) levels by metformin/rosiglitazone combined therapy. We further supported the involvement of AMPK in the metformin-mediated osteogenic induction of osteoblasts in vitro, by showing that co-incubation with the AMPK inhibitor compound C can prevent the increase the AMPK phosphorylation and type 1 collagen secretion of BMPC cultured for 15 days in the presence of metformin.

Interestingly, other authors have previously shown that PPARγ is sensitive to AMPK activity. Phosphorylation by AMPK of p300 (a coactivator protein for PPARγ) represses both the ligand-dependent and ligand-independent transactivating functions of PPARγ (Yang et al., 2001). Through this mechanism, activation of AMPK by metformin could induce a decrease in the PPARγ-mediated response of BMPC to rosiglitazone, thus diminishing the capacity for this thiazolidinedione to induce adipogenic differentiation of BMPC. On the other hand, in our ex vivo system rosiglitazone monotherapy simultaneously induces a decrease in active (phosphorylated) AMPK and an increase in PPARγ expression. Since a lower AMPK activity would be expected to decrease the repression of PPARγ transactivation, this could be interpreted as a mechanism whereby rosiglitazone monotherapy can potentiate its adipogenic effect on BMPC.

To our knowledge, this is the only study that has evaluated the basic pathophysiological consequences on bone metabolism (and the possible mechanisms of action), of therapy with metformin and/or rosiglitazone. On the other hand, the effects of this treatment strategy on bone have been addressed in several recent clinical studies. In the 5-year follow-up multicentric ADOPT study (Kahn et al., 2008), long-term treatment (>12 months) with rosiglitazone alone was associated with an approximate doubling in the risk of upper and lower limb bone fractures in women with type 2 diabetes (but not in men), compared with those taking metformin as monotherapy. In a retrospective study (Tzoulaki et al., 2009), combined rosiglitazone therapy (versus metformin

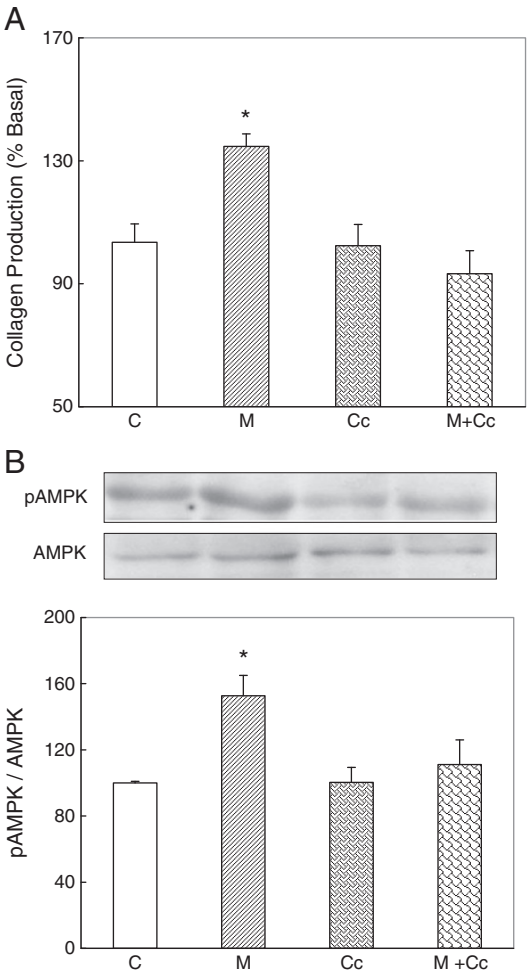

Fig. 6. In vitro inhibition of AMPK phosphorylation prevents metformin-induced osteogenic effects on BMPC. BMPC were obtained from control (untreated) animals as described in Materials and methods. The cells were then submitted to an osteogenic induction for 15 days, in the presence or absence of 100 μM metformin and/or 0.5 μM compound C (an inhibitor of AMPK activation). Thus, cells were fixed as described in Materials and methods, and evaluated for type 1 collagen secretion (A). Alternatively, cells were solubilized in Laemmli's buffer and submitted to SDS-PAGE and Western blotting for total AMPK and phosphorylated AMPK, after which the corresponding blots were quantitated in order to calculate their ratio for each experimental condition (B). C: control; M: metformin; Cc: compound C. *$P<0.05$ vs. control.

monotherapy) was associated with a 50% increase in upper and lower limb bone fractures, although fractures were not discriminated by patient gender. In the RECORD study (Home et al., 2009), combined rosiglitazone therapy appeared to increase the frequency of limb fractures in women (versus patients treated with metformin plus sulphonylureas), although this was only established by self-referral and in a subset of the entire cohort. In a small cross-sectional study (Mancini et al., 2009), rosiglitazone plus metformin appeared to increase the prevalence of osteoporotic vertebral fractures in elderly males, versus treatment with metformin alone. Taken together, these clinical studies seem to indicate that the incidence of fractures may be greatest in patients under long-term treatment with rosiglitazone alone, lower in patients on metformin monotherapy, and intermediate when rosiglitazone is administered in combination with metformin or a sulphonylurea.

5. Conclusions

In the present study, we have found that treatment with rosiglitazone alone decreases bone regeneration, femoral metaphysis trabecular area, osteoblastic and osteocytic density, osteogenic commitment of BMPC, and TRAP activity associated with growth plates (presumably associated with resorption of calcified cartilage and/or of newly formed primary bone tissue). All of these decreases over time would be expected to impair bone growth while decreasing bone quality, thus rendering it more prone to fractures. In addition, we have found that metformin as monotherapy increases all of these parameters versus control, whereas our results for metformin plus rosiglitazone tend to be intermediate. If these results may be extrapolated to a clinical setting, they could provide a possible explanation for the clinical findings of other authors.

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References

Ali, A.A., Weinstein, R.S., Stewart, S.A., Parfitt, A.M., Manolagas, S.C., Jilka, R.L., 2005. Rosiglitazone causes bone loss in mice by suppressing osteoblast differentiation and bone formation. Endocrinology 146, 1226–1235.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cortizo, A.M., Etcheverry, S.B., 1995. Vanadium derivatives act as growth factor-mimetic compounds upon differentiation and proliferation of osteoblast-like UMR106 cells. Mol. Cell. Biochem. 145, 97–102.

Cortizo, A.M., Sedlinsky, C., McCarthy, A.D., Blanco, A., Schurman, L., 2006. Osteogenic actions of the anti-diabetic drug metformin on osteoblasts in culture. Eur. J. Pharmacol. 536, 38–46.

de Liefde, I.I., van der Klift, M., de Laet, C.E., van Daele, P.L., Hofman, A., Pols, H.A., 2005. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporosis Int. 16, 1713–1720.

Dormuth, C.R., Carney, G., Carleton, B., Bassett, K., Wright, J.M., 2009. Thiazolidinediones and fractures in men and women. Arch. Intern. Med. 169, 1395–1402.

Guidelines on the handling and training of laboratory animals. The Biological Council of Animal Research and Welfare Panel. Universities Federation for Animal Welfare (UFAW, Potters Bar, England.

Home, P.D., Pocock, S.J., Beck-Nielsen, H., Curtis, P.S., Gomis, R., Hanefeld, M., Jones, N.P., Komajda, M., McMurray, J.J.V., 2009. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 Diabetes (RECORD): a multicentre, randomised, open-label trial. Lancet 373, 2125–2135.

Jang, W.G., Kim, E.J., Bae, I.H., Lee, K.N., Kim, Y.D., Kim, D.K., Kim, S.H., Lee, C.H., Franceschi, R.T., Choi, H.S., Koh, J.T., 2011. Metformin induces osteoblast differentiation via orphan nuclear receptor SHP-mediated transactivation of Runx2. Bone 48, 885–893.

Kahn, S.E., Zinman, B., Lachin, J.M., Haffner, S.M., Herman, W.H., Holman, R.R., Kravitz, B.G., Yu, D., Heise, M.A., Aftring, R.P., Viberti, G., 2008. Diabetes Outcome Progression Trial (ADOPT) Study Group. Rosiglitazone-associated fractures in type 2 diabetes: an Analysis from A Diabetes Outcome Progression Trial (ADOPT). Diabetes Care 31, 845–851.

Kanazawa, I., Yamaguchi, T., Yano, S., Yamauchi, M., Sugimot, T., 2008. Metformin enhances the differentiation and mineralization of osteoblastic MC3T3-E1 cells via AMP kinase activation as well as eNOS and BMP-2 expression. Biochem. Biophys. Res. Commun. 375, 414–419.

Kawashima, Y., Fritton, J.C., Yakar, S., Epstein, S., Schaffler, M.B., Jepsen, K.J., LeRoith, D., 2009. Type 2 diabetic mice demonstrate slender long bones with increased fragility secondary to increased osteoclastogenesis. Bone 44, 648–655.

Laemmli, U.K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lecka Czernik, B., Moerman, E.J., Grant, D.F., Lehman, J.M., Manolagas, S.C., Jilka, R.L., 2002. Divergent effects of selective peroxisome proliferator-activated receptor-γ2 ligands on adipocyte *versus* osteoblast differentiation. Endocrinology 143, 2376–2384.

Lott, J.A., Turner, K., 1975. Evaluation of Trinder's glucose oxidase method for measuring glucose in serum and urine. Clin. Chem. 21, 1754–1760.

Mancini, T., Mazziotti, G., Doga, M., Carpinteri, R., Simetovic, M., Vescovi, P.P., Giustina, A., 2009. Vertebral fractures in males with type 2 Diabetes treated with rosiglitazone. Bone 45, 784–788.

Meier, C., Kraenzlin, M.E., Bodmer, M., Jick, S.S., Jick, H., Meier, C.R., 2008. Use of thiazolidinediones and fracture risk. Arch. Intern. Med. 168, 820–825.

Melton III, L.J., Leibson, C.L., Achenbach, S.J., Therneau, T.M., Khosla, S., 2008. Fracture risk in type 2 diabetes: update of a population-based study. J. Bone Miner. Res. 23, 1334–1342.

Molinuevo, M.S., Schurman, L., McCarthy, A.D., Cortizo, A.M., Tolosa, M.J., Gangoiti, M.V., Arnol, V., Sedlinsky, C., 2010. Effect of metformin on bone marrow progenitor cell differentiation: in vitro and in vivo studies. J. Bone Miner. Res. 25, 211–221.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., Marshak, D.R., 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284, 143–147.

Rosen, C.J., 2010. Revisiting the rosiglitazone story—lessons learned. N. Engl. J. Med. 363, 803–806.
Rzonca, S.O., Suva, L.J., Gaddy, D., Montague, D.C., Lecka-Czernik, B., 2004. Bone is a target for the antidiabetic compound rosiglitazone. Endocrinology 145, 401–406.
Santana, R.B., Xu, L., Chase, H.B., Amar, S., Graves, D.T., Trackman, P.C., 2003. A role for advanced glycation end products in diminished bone healing in type 1 diabetes. Diabetes 52, 1502–1510.
Schell, H., Lienau, J., Epari, D.R., Seebeck, P., Exner, C., Muchow, S., Bragulla, H., Haas, N.P., Duda, G.N., 2006. Osteoclastic activity begins early and increases over the course of bone healing. Bone 38, 547–554.
Schwartz, A.V., Sellmeyer, D.E., Vittinghoff, E., Palermo, L., Lecka-Czernik, B., Feingold, K.R., Strotmeyer, E.S., Resnick, H.E., Carbone, L., Beamer, B.A., Park, S.W., Lane, N.E., Harris, T.B., Cummings, S.R., 2006. Thiazolidinedione use and bone loss in older diabetic adults. J. Clin. Endocrinol. Metab. 91, 3349–3354.
Shah, M., Kola, B., Bataveljic, A., Arnett, T.R., Viollet, B., Saxon, L., Korbonits, M., Chenu, C., 2010. AMP-activated protein kinase (AMPK) activation regulates in vitro bone formation and bone mass. Bone 47, 309–319.
Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science 289, 1504–1508.
Tullberg-Reinert, H., Jundt, G., 1999. In situ measurement of collagen synthesis by human bone cells with a Sirius red-based colorimetric microassay: effects of transforming growth factor b2 and ascorbic acid 2-phosphate. Histochem. Cell. Biol. 112, 271–276.
Tzoulaki, I., Molokhia, M., Curcin, V., Little, M.P., Millett, C.J., Ng, A., Hughes, R.I., Khunti, K., Wilkins, M.R., Majeed, A., Elliott, P., 2009. Risk of cardiovascular disease and all cause mortality among patients with type 2 diabetes prescribed oral antidiabetes drugs: retrospective cohort study using UK general practice research database. BMJ 339, b4731.
Ueno, A., Kitase, Y., Moriyama, K., Inoue, H., 2001. MC3T3-E1-conditioned medium-induced mineralization by clonal rat dental pulp cells. Matrix Biol. 20, 347–355.
Vestergaard, P., Rejnmark, L., Mosekilde, L., 2005. Relative fracture risk in patients with diabetes mellitus, and the impact of insulin and oral antidiabetic medication on relative fracture risk. Diabetologia 48, 1292–1299.
Wang, H.N., Wang, Y.R., Liu, G.Q., Liu, Z., Wu, X.P., Wei, X.L., Hong, T.P., 2008. Inhibition of hepatic interleukin 18 production by rosiglitazone in a rat model of nonalcoholic fatty liver disease. World J. Gastroenterol. 14, 7240–7246.
Yang, W., Hong, Y.H., Shen, X.-Q., Frankowski, C., Camp, H.S., Leff, T., 2001. Regulation of transcription by AMP-activated protein kinase. J. Biol. Chem. 276, 38341–38344.