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Effect of Metformin on Bone Marrow Progenitor Cell Differentiation: In Vivo and In Vitro Studies

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ABSTRACT

Diabetes mellitus is associated with bone loss. Patients with type 2 diabetes are frequently treated with oral antidiabetic drugs such as sulfonylureas, biguanides, and thiazolidinediones. Rosiglitazone treatment has been shown to increase adipogenesis in bone marrow and to induce bone loss. In this study we evaluated the effect of in vivo and in vitro treatment with metformin on bone marrow progenitor cells (BMPCs), as well as the involvement of AMPK pathway in its effects. The in vitro effect of coincubation with metformin and rosiglitazone on the adipogenic differentiation of BMPCs also was studied. In addition, we evaluated the effect of in vivo metformin treatment on bone regeneration in a model of parietal lesions in nondiabetic and streptozotocin-induced diabetic rats. We found that metformin administration both in vivo and in vitro caused an increase in alkaline phosphatase activity, type I collagen synthesis, osteocalcin expression, and extracellular calcium deposition of BMPCs. Moreover, metformin significantly activated AMPK in undifferentiated BMPCs. In vivo, metformin administration enhanced the expression of osteoblast-specific transcription factor Runx2/Cbfa1 and activation of AMPK in a time-dependent manner. Metformin treatment also stimulated bone lesion regeneration in control and diabetic rats. In vitro, metformin partially inhibited the adipogenic actions of rosiglitazone on BMPCs. In conclusion, our results indicate that metformin causes an osteogenic effect both in vivo and in vitro, possibly mediated by Runx2/Cbfa1 and AMPK activation, suggesting a possible action of metformin in a shift toward the osteoblastic differentiation of BMPCs. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: BONE MARROW PROGENITOR CELLS; METFORMIN; ROSIGLITAZONE; OSTEOBLASTOGENESIS; ADIPOGENESIS; DIABETES MELLITUS

Introduction

Long-standing diabetes mellitus has been associated with bone loss that can cause an increase in fracture risk.[1–3] It has been suggested that these diabetes-associated bone alterations could be, at least in part, due to a pathologic accumulation of advanced glycation end products (AGEs) on bone extracellular matrix proteins.[4,5] Different insulin sensitizers such as metformin and thiazolidinediones (used as monotherapy or in combination) have proved to be efficient for the treatment of type 2 diabetes mellitus.[6] It has been demonstrated previously that thiazolidinediones (e.g., rosiglitazone and netoglitazone) not only activate peroxisome proliferator-activated receptor gamma (PPARγ) but also suppress the expression and action of the osteoblast-specific transcription factor Cbfa1.[7] Moreover, there is evidence that thiazolidinediones promote bone marrow stromal cell adipogenesis and inhibit osteogenesis.[8–10] Mice treated in vivo with thiazolidinediones decreased their bone mineral content, bone formation, and trabecular bone volume and increased adipogenesis.[8,9,11] On the other hand, in patients with type 2 diabetes mellitus, metformin and sulfonylureas have been associated with a decreased risk of fracture.[1] However, there is little information regarding the effect of these antidiabetic drugs on bone metabolism.

Our group has recently demonstrated that metformin causes a direct osteogenic action in a model of osteoblasts in culture.[12] These actions include a dose-dependent increase in cell proliferation, type I collagen production, alkaline phosphatase (ALP) activity, and mineral deposition. These osteogenic actions of metformin appear to be mediated by an increase in the expression of nitric oxide synthases and in the activity of extracellular regulated kinases. Recently, Kanazawa and colleagues[13] have confirmed these results and suggested that metformin can induce the differentiation and mineralization of osteoblasts via activation of AMPK pathway and induction of endothelial nitric oxide synthase (eNOS) and bone morphogenetic protein-2 (BMP-2) expression.

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Bone is a highly dynamic tissue.[14] Cells of the bone marrow microenvironment appear to share common mesenchymal progenitor cells, which present the ability to differentiate into various cell types such as osteoblasts, adipocytes, and chondrocytes.[15–17] In this context, bone marrow metabolic conditions are determinants of the biologic balance between osteoblast-mediated bone formation and marrow adipogenesis.[15] Diverse factors such as endogenous hormones and drug treatment can affect this delicate balance, modifying the osteoblast-adipocyte ratio in the bone marrow.[15] In this context, a recent study has underlined the importance of PPARγ in controlling the adipocyte-osteoblast relationship within the bone marrow cavity.[18]

In the present work, we have evaluated the in vivo and in vitro actions, as well as the possible mechanism of action, of metformin on bone marrow mesenchymal progenitor cells (BMPCs) and the effect of in vitro cotreatment with metformin and rosiglitazone on BMPCs. We also have investigated the effect of in vivo metformin administration on bone tissue regeneration in control nondiabetic and streptozotocin-induced diabetic rats.

Materials and Methods

Animal treatment

Adult male Sprague-Dawley rats (190 to 210 g) were used. Animals were maintained in a temperature-controlled room at 23°C with a fixed 12 hour light:12 hour darkness cycle, and fed standard rat laboratory chow and water *ad libitum*. All experiments on animals were done in conformity with the Guidelines on Handling and Training of Laboratory Animals published by the Universities Federation for Animals Welfare (1992).[19] In half the animals, diabetes was induced by a single intraperitoneal injection of streptozotocin at a dose of 55 mg/kg freshly dissolved in citrate buffer (0.05 M, pH 4.5).[20] Four days later, blood glucose was assayed, and diabetes was verified in all streptozotocin-treated animals.

Animals then were divided into four groups of 10 animals per group. Control nondiabetic rats (C) and diabetic rats (D) received water *ad libitum*; metformin-treated nondiabetic rats (M) and metformin-treated diabetic rats (DM) received 100 mg/kg per day of metformin (Quimica Montpellier, Buenos Aires, Argentina) in drinking water for 2 weeks.

Serum glucose was measured by the glucose oxidase method; serum triglyceride and cholesterol were measured by a commercial kit (Wiener Laboratories, Rosario, Argentina).

Bone reossification model

The effect of metformin on the process of bone repair was assessed by a reossification model, as described previously.[21] Animals were divided into four groups as described earlier. Briefly, all the animals were anesthetized by intraperitoneal/intramuscular injection of 0.12 mL/100 g of 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. Animals were maintained in a thermostatized atmosphere with 12 hour light-dark cycles and fed with commercial rat chow for 15 days.

Histologic examination of bone reossification

After 15 days of metformin treatment, rats were sacrificed under anesthesia by neck dislocation, and the parietal bones were processed for histologic and quantitative histomorphometric analysis. Parietal 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 (Leica Microsystems, Wetzlar, Germany) microtome. The sections were stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP) (Sigma, St. Louis, MO, USA) histochemistry to specifically identify osteoclasts.[22] Photographs were taken with a Nikon Coolpix 4500 digital camera on an Eclipse E400 Nikon microscope (Nikon, Tokyo, Japan). Images were analyzed using the Image J program (*www.macbiophotonics.ca/imagej*) with a microscope scale plugin. Reossification was calculated as the ratio between either the newly reossified area or thickness and the average bone thickness (H&E). In addition, osteoblastic density was evaluated by counting the number of osteoblasts per unit of reossification surface, osteocyte density was assessed by counting the number of osteocytes per area of newly reossificated bone, and osteoclastic density was calculated as the positive TRAP area per square millimeter of newly reossificated bone tissue (Oc/mm^2).[22]

Bone marrow progenitor cell (BMPC) isolation and incubation

BMPCs were obtained from control nondiabetic and metformin-treated rats as described previously.[16] Briefly, animals were sacrificed under anesthesia by rapid neck dislocation. Bone marrow cells were collected by flushing the femurs and tibiae of the animals with Dulbecco's modified essential medium (DMEM) (Invitrogen, Buenos Aires, Argentina) under sterile conditions. The resulting 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. Nonadherent cells were removed by changing the medium after 24 hours. The culture medium was changed twice a week. When cells reached confluence (after 10 to 15 days), the cell monolayer was detached using 0.12% trypsin and 1 mM EDTA and subcultured in tissue culture plates. After 5 days, the basal level of alkaline phosphatase activity (i.e., prior to osteogenic differentiation) was evaluated as described below.

In order to investigate the role of AMPK as a signal-transduction pathway for metformin in BMPCs, cells were plated onto six-well plates and cultured until 70% confluence. They were then serum-starved for 2 hours and incubated with the doses of metformin indicated in the figure legends for 60 mintes. At the end of this incubation period, the cells were processed by immunofluorescence and Western blotting to evaluate expression and activation of AMPK, as described below.

Osteogenic differentiation of rat BMPCs

BMPCs were plated at a density of 5×10^4 cells/well in 24-well plates containing 10% FBS–DMEM medium 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.[12] The medium was changed twice a week. Osteoblastic differentiation was evaluated by measuring alkaline phosphatase activity, type I collagen production, osteocalcin expression, and extracellular calcium deposition, as described below.

After 15 days of osteogenic differentiation, cell monolayers were washed with phosphate-buffered saline (PBS) and lysed with 200 μL 0.1% Triton-X100. An aliquot of 100 μL of the lysate was used to evaluate alkaline phosphatase activity by the hydrolysis of *p*-nitrophenylphosphate (*p*-NPP) into *p*-nitrophenol (*p*-NP) at 37°C for 1 hour. The absorbance of *p*-NP was recorded at 405 nm.[23] Aliquots of the same extract were used for protein determination by Bradford's technique.[24] Type I collagen production was evaluated as reported previously.[25] Briefly, after 21 days of osteogenic differentiation, cell monolayers were fixed with Bouin's solution and stained with Sirius red dye for 1 hour. The stained material was dissolved in 1 mL 0.1 N sodium hydroxide, and the absorbance of the solution was recorded at 550 nm. Osteocalcin expression was evaluated after 21 days of culture by Western blotting. Extracellular calcium deposits (mineralization nodules) also were evaluated after 21 days of culture using alizarin S red staining.[26] Stained calcium deposits were extracted with 1 mL 0.1 N sodium hydroxide, recording the optical density at 548 nm. Alternatively, stained cultures were observed using a Nikon microscope and photographed.

Adipogenic differentiation of rat BMPCs

To initiate adipocyte differentiation, BMPCs were grown to confluence in 24-well plates in DMEM–10% FBS. Differentiation to adipocytes was induced by further culturing the cells for 10 days with DMEM–10% FBS supplemented with 0.5 μM rosiglitazone (Quimica, Montpellier, Argentina), 1 μM dexamethasone (Decadron, Sidus, Argentina), and 200 nM insulin (Lilly, Buenos Aires, Argentina). In certain experiments, rosiglitazone was substituted by 3-isobutyl-1-methylxanthine (IBMX) 0.5 mM as inducer of adipogenesis. To evaluate the effect of metformin on rosiglitazone-induced adipogenesis, in some cases cells were simultaneously incubated with metformin (1 or 10 μM) and 0.5 μM rosiglitazone for 10 days. After these culture periods, intracellular triacylglyceride deposits were analyzed with an enzymatic commercial kit (Wiener, Rosario, Argentina). Briefly, 100 μL 0.1% Triton-X100 cell monolayer lysates were incubated for 1 hour with reaction buffer according to the manufacturer's instructions, and absorbance was measured at 505 nm. Aliquots of the same extract were used for protein determination by Bradford's technique.[24] In some cases, after 10 days of differentiation, the medium was aspirated, and nonlysed cells were stained with oil red O to detect accumulation of intracellular lipid deposits. The cell monolayers were observed using an Eclipse E400 Nikon microscope and photographed with a Nikon Coolpix 4500 digital camera.

Western blot analysis

BMPCs were grown to confluence in 6-well plates in DMEM–10% FBS and then differentiated into either osteoblasts or adipocytes as described earlier. At the end of different culture periods, cells were lysed in Laemmli's buffer.[27] These lysates were heated to 100°C for 3 minutes, and 30 μg of protein was subjected to 12% SDS-PAGE. The separated proteins then were transferred to PVDF membranes. After washing and blocking, the membranes were incubated overnight at 4°C with an antibody directed against Cbfa1/Runx2 or osteocalcin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for evaluation of osteoblastogenesis or against PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for evaluation of adipogenesis. In order to normalize results, all blots were stripped and reprobed with an anti-β-actin antibody (Sigma, St. Louis, MO, USA). In experiments designed to investigate AMPK, blots were probed with either an anti-total AMPK or anti-P-AMPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to assess the in vitro mechanism of action of metformin.[13] Blots were developed by an enhanced chemiluminescence method. The intensity of the specific bands was quantified by densitometry after scanning of the photographic film. Images were analyzed using the Scion beta 2 program (Scion Corporation, Frederick, MD, USA).

Immunofluorescence microscopy

Subconfluent osteoblasts grown on glass coverslips were washed in PBS, fixed with 4% paraformaldehyde in PBS (10 minutes at room temperature), and permeabilized with methanol for 4 minutes at −20°C.[28] Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 2 hour. Cells then were incubated with anti-AMPK or anti-P-AMPK antibodies (1:100 in blocking buffer) overnight at 4°C. After washing, cells were exposed to a fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:200) for 1 hour at room temperature. Cells were mounted in VECTA-SHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) and observed with a Nikon fluorescence microscope. Fluorescence intensity of each cell was semiquantified with an MBF-Image J program (*www.macbiophotonics.ca*) using green global calibration.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) and were obtained from three separate experiments performed in sextuplicate. Differences between the groups were assessed by one-way analysis of variance (ANOVA) using the Tukey post hoc test. For nonnormally distributed data, the non parametric Kruskal-Wallis test with the Dunn post hoc test was performed using GraphPad In Stat version 3.00 (Graph Pad Software, San Diego, CA, USA). $p < .05$ was considered significant for all statistical analyses.

Results

In vitro direct effect of metformin on BMPC osteoblastic differentiation

In a first series of experiments, we evaluated the direct effect of metformin on BMPC differentiation to osteoblastic phenotype.

BMPCs from control rats were incubated in osteogenic medium containing increasing doses of metformin (0.5 to 10 μM). These doses are in the order of the plasma concentrations of metformin found in patients (10 μM).[29] Osteogenic differentiation was evaluated by assessing the expression of extracellular type I collagen, ALP activity, and mineral deposits after 7, 15, and 21 days of culture. No differences were observed between control and metformin-treated cells after the first week of osteogenic differentiation (data not shown). After 15 days of differentiation, metformin significantly increased ALP activity, in a dose-dependent manner (Fig. 1*A*). As explained in "Materials and Methods," production of type I collagen was first qualitatively assessed by Sirius red staining of cell monolayers (see Fig. 1*B*) and then quantified by extraction of the dye. As shown in Fig. 1*C*, metformin dose-dependently increases collagen production between 0.5 and 10 μM. Additionally, collagen production of metformin-treated cells is significantly greater than in untreated cells at all metformin doses tested. This effect correlates with a greater mineralizing activity of metformin-treated cells, as evaluated by alizarin red staining of the cell monolayer (see Fig. 1*D*) and its quantification after extraction (see Fig. 1*E*). After 21 days of osteogenic differentiation, extracellular calcium deposits (nodules of mineralization) were increased at all metformin doses tested versus untreated cells, with the highest calcium deposits obtained at 10 μM of metformin (160 ± 18% basal; see Fig. 1*E*).

Metformin partially abolishes rosiglitazone-induced adipogenesis

To determine the effect of metformin on rosiglitazone-induced adipogenesis, BMPCs were committed to differentiate into adipocytes by treatment for 10 days with either rosiglitazone alone (0.5 μM) or rosiglitazone plus metformin (1 or 10 μM). Adipogenesis was evaluated through the enzymatic determination of intracellular lipid deposits in cell extracts and the staining of lipid droplets with oil red O in cell monolayers. BMPCs cultured in adipogenic medium without the addition of rosiglitazone presented intracellular lipid content below the detection limit of our enzymatic assay (data not shown). We found that metformin caused a significant dose-dependent decrease in the rosiglitazone-induced accumulation of intracellular triacylglycerides (Fig. 2*A*) and of lipid droplets (see Fig. 2*B*) when compared with cells treated with rosiglitazone alone. In parallel experiments in which we substituted rosiglitazone with IBMX as inducer of adipogenesis, metformin also was found to induce a dose-dependent decrease in the accumulation of intracellular lipids (data not shown).

In vivo administration of metformin increases the in vitro osteoblastic differentiation of BMPCs

We next evaluated the effect of in vivo treatment with metformin on the osteogenic potential of BMPCs in vitro (i.e., the ex vivo effect of metformin on BMPCs). This drug was administered to rats in drinking water, as described in "Materials and Methods." After 15 days, BMPCs were obtained from control and metformin-treated rats and cultured until they reached confluence. After the first passage, the basal-specific activity

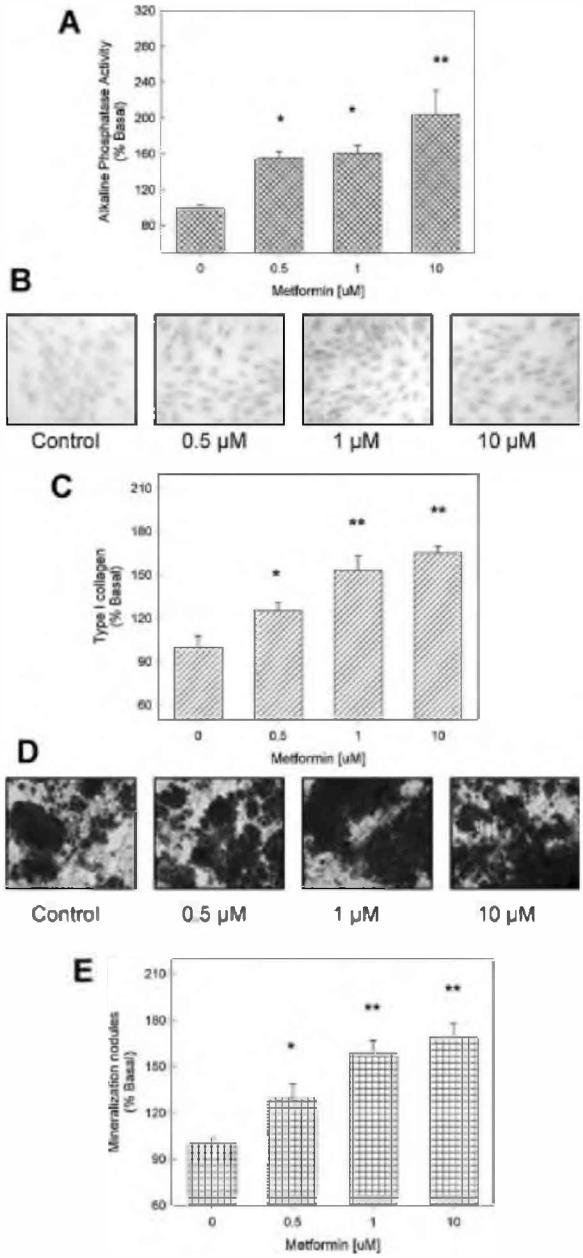

Fig. 1. Effect of metformin on osteoblastogenesis. BMPCs isolated from control rats were cultured in an osteogenic medium for 15 or 21 days with or without different doses of metformin. After 15 days, ALP (*A*) and type I collagen production (*B*, *C*) were measured to evaluate osteoblastic differentiation. After 21 days of culture, mineralization was assessed by the alizarin red method (*D*, *E*). Basal ALP activity (0 μM metformin) = 4.2 ± 0.5 nmol *p*-nitrophenol/mg protein per minute. Basal type I collagen production (0 μM metformin) = 4.2 ± 0.2 μg collagen/mg protein. Values are expressed as mean ± SEM. $^{*}p < .05$, $^{**}p < .01$.

of ALP was assessed for all cultures (i.e., prior to osteogenic differentiation). Cells obtained from metformin-treated rats showed a significantly greater expression of ALP (8.9 ± 0.8 nmol *p*-nitrophenol/mg protein per minute) than cells obtained from control (untreated) rats (4.7 ± 0.2 nmol *p*-nitrophenol/mg protein per minute; $p < .05$).

We also evaluated the ex vivo effect of metformin on BMPC osteogenic potential after 15 or 21 days of incubation of cells

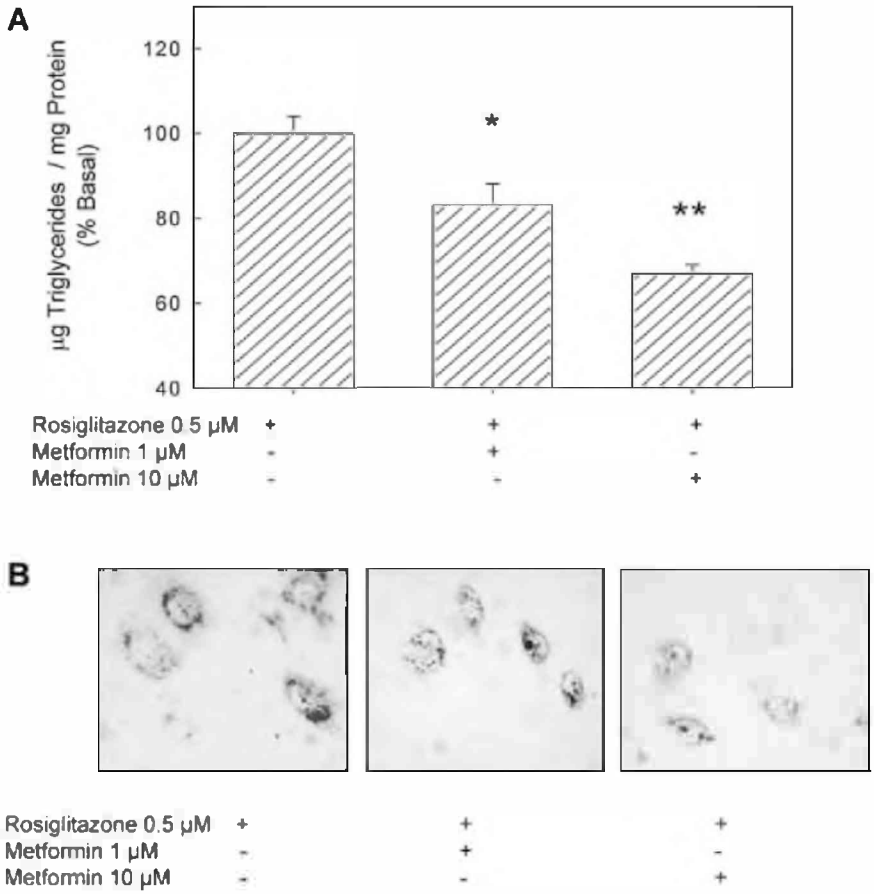

Fig. 2. Effect of metformin on rosiglitazone-induced adipogenesis. BMPCs isolated from control rats were induced to differentiate into adipocytes with a medium containing rosiglitazone, insulin, and dexamethasone in the presence or absence of different doses of metformin. After 10 days of culture, cell extracts were obtained, and intracellular triacylglyceride accumulation was quantified as described in "Materials and Methods" (*A*). Alternatively, lipid droplets were stained with oil red O in whole cells and observed with a light microscope (*B*). Basal triacylglyceride content (0 µM metformin) = 178 ± 6 µg/mg protein. Values are expressed as mean ± SEM. $^{*}p < .05$, $^{**}p < .01$.

obtained from either control or metformin-treated rats in an osteogenic medium. After 15 days of differentiation, we found that BMPCs from metformin-treated rats expressed higher levels of ALP than BMPCs from control rats (Fig. 3*A*) and a significant increase in type I collagen production (see Fig. 3*B*). Similarly, after 21 days of differentiation, we found a significant increase in osteocalcin expression (see Fig. 3*C*) and in the formation of mineralization nodules (see Fig. 3*D*) in BMPCs from metformin-treated rats in comparison with BMPCs from control rats.

Metformin increases bone healing in diabetic and nondiabetic rats

We used a model of bone reossification to evaluate the effect of the in vivo administration of metformin on bone tissue regeneration. To this effect, we performed uniform craniotomy defects in parietal bones as described in "Materials and Methods" and allowed them to heal for 15 days. After this period of time, this bone—in which mechanical loading can be expected to be low—went through different stages in the repair of intramembranous bone[30]: namely, an inflammatory infiltrate followed by formation of granulatory and fibrous tissue and osteoclastic resorption of bone fragments and, finally, bridging of the lesion by a hard callus of intramembranous primary bone. In the time span of our experiments, we were unable to find any significant remodeling of the primary bone callus to form mature cancellous bone.

In our experiments, both control (nondiabetic) and streptozotocin-induced diabetic rats were used. The metabolic status of the different animal groups was assessed by the biochemical parameters showed in Table 1. At the time of sacrifice, diabetic animals (treated or untreated with metformin) showed a 15% reduction in body weight. Plasma glucose levels of the D and DM groups were significantly elevated. In addition, diabetic animals had significantly elevated cholesterol and triglyceride levels compared with the nondiabetic control and metformin-treated groups.

We stained the tissue sections with H&E to evaluate the following parameters: relative quantity of newly reossified bone (Fig. 4*A–D*), the number of osteocytes immersed in reossified bone tissue (see Fig. 4*E*), and the number of osteoblasts on the surface of reossified bone (see Fig. 4*E*). In addition, TRAP staining was performed to evaluate the proportion of reossified surface covered by osteoclasts (see Fig. 4*F–J*). Histomorphometric analysis of the tissue sections is shown in Table 2. Diabetic rats showed a decrease in the thickness, area, and osteoblast and

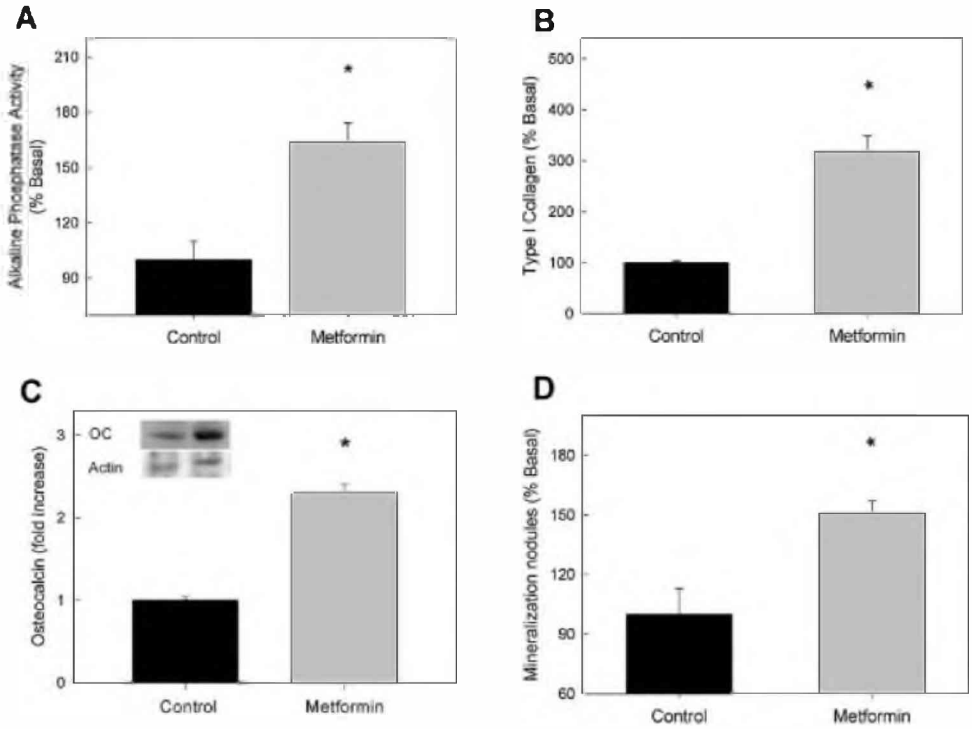

Fig. 3. Ex vivo effect of metformin on the osteoblastic potential of BMPCs. BMPCs isolated from control or metformin-treated (100 mg/kg per day) rats were incubated in an osteogenic medium for 15 days, after which ALP activity (*A*) and type I collagen production (*B*) were measured, or BMPCs were cultured for 21 days, after which osteocalcin (*C*) and mineralization nodules (*D*) were evaluated. Values are expressed as mean ± SEM. $^{*}p < .05$.

osteocyte density, of newly reossified bone when compared with nondiabetic rats. Metformin treatment induced a significant increase in the thickness and area of reossification in both control and diabetic rats without affecting osteoblast and osteocyte density. Interestingly, a significant 3.8-fold increase was observed in the TRAP activity of nondiabetic rats after 15 days of treatment with metformin.

Effect of ex vivo metformin treatment on osteoblastic and adipogenic transcription factors

We next evaluated possible changes in the expression of the two major transcription factors for osteoblastogenesis and adipogenesis, Cbfa1/Runx2 and PPARγ (Fig. 5). BMPCs from either control or metformin-treated rats were cultured in an osteogenic or adipogenic medium for the times indicated in the figures. Western immunoblot analysis showed that when compared with BMPCs from basal conditions (undifferentiated cells, time 0), BMPCs from metformin-treated rats expressed significantly more of the osteoblastic transcription factor Runx2 (see Fig. 5*A*) but a similar amount of the adipogenic transcription factor PPARγ (see Fig. 5*B*). In addition, there was a time-dependent increase in Runx2 expression after 15 and 21 days of osteoblastic differentiation. In contrast, no changes in PPARγ expression were seen after 10 days of adipogenic differentiation in BMPCs from metformin-treated or untreated rats.

Effect of metformin on the AMPK signaling pathway in BMPCs

In order to investigate possible signal-transduction mechanisms, we assessed the in vitro and ex vivo effect of metformin on AMPK signaling. We first studied the direct effect of metformin on the activation of AMPK in BMPCs derived from control rats by both immunofluorescence and Western blot techniques.

When analyzed by immunofluorescence, total AMPK was found to localize in the nucleus and the cytoplasm of both untreated and metformin-treated cells, showing no observable

Table 1. Weight and Plasma Profile after 15 days of Treatment with or without Metformin 100 mg/kg in Control Nondiabetic and Diabetic Animals

Group	Weight (g)	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Control	190 ± 9	110 ± 10	43 ± 4	45 ± 4
Control + metformin	187 ± 6	103 ± 5	36 ± 1	37 ± 3
Diabetic	160 ± 7*	257 ± 30*	59 ± 2***	62 ± 5***
Diabetic + metformin	155 ± 8***	213 ± 23***	35 ± 3	48 ± 4

Values are mean ± SEM, $n = 10$ per group.
$^{*}p < .05$ versus C group,
$^{**}p < .05$ versus C + M group.

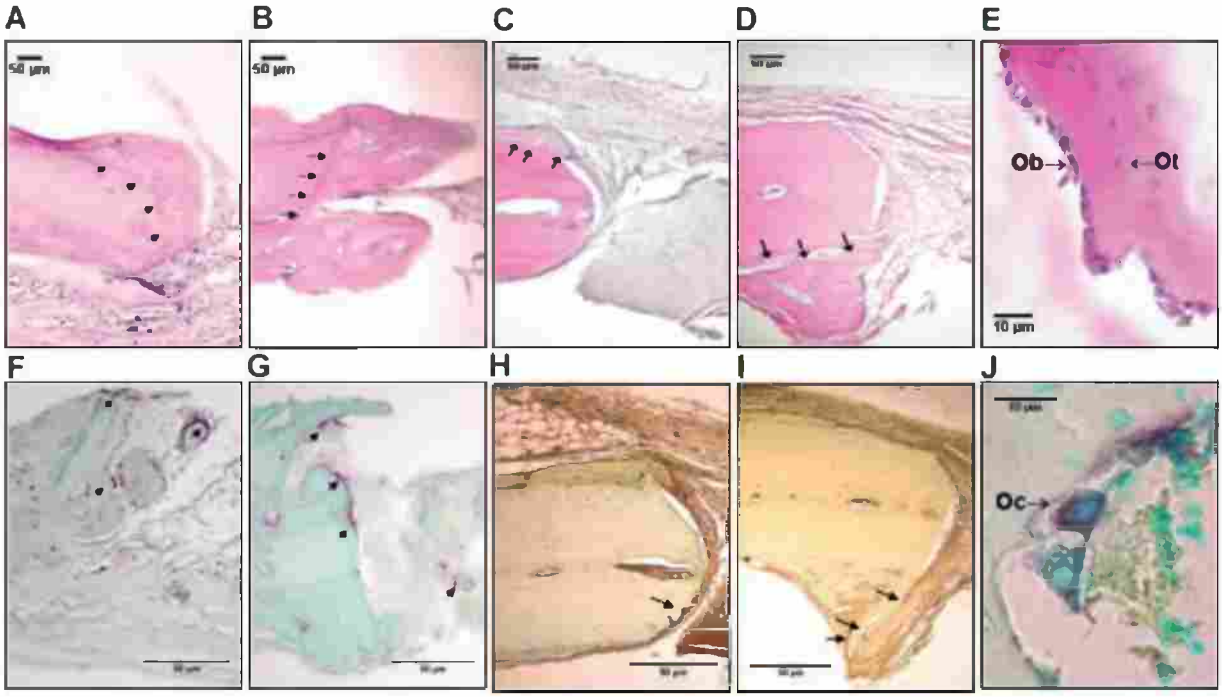

Fig. 4. Bone repair in a bone reossification model. A 1 mm circular bone defect was performed under anesthesia in the parietal bone of control nondiabetic rats (*A*, *F*), metformin-treated control rats (100 mg/kg per day) (*B*, *G*), streptozotocin-induced diabetic rats (*C*, *H*), or metformin-treated diabetic rats (*D*, *I*). After 15 days, all animals were sacrificed, and the parietal bones were examined by the histologic procedures detailed in "Materials and Methods." Bone sections were stained with hematoxylin & eosin (*A–E*) or analyzed for TRAP activity (*F–J*) to assess new bone formation (*arrows*), as well as the density of osteocytes (*E*), osteoblasts (*E*), and osteoclasts (*J*). Panels (*E*) and (*J*) (control rats) are representative images of osteoblasts, osteocytes, and osteoclasts.

difference in fluorescence intensity in either case (data not shown). However, when we probed with an antibody that recognizes the phosphorylated Thr172 in the α-subunit of AMPK, we found different results (Fig. 6*A*). Metformin increased P-AMPK-associated fluorescence in both the cytoplasm and the nucleus. A semiquantitation of P-AMPK- associated fluorescence in metformin-treated BMPCs showed that P-AMPK expression was increased 1.5-fold versus untreated control cells in both the nucleus and the cytoplasm.

The results obtained by immunofluorescence were further confirmed by Western immunoblotting (see Fig. 6*B*). After 1 hour of incubation, 100 μM metformin significantly increased by 1.6-fold the P-AMPK:AMPK ratio in BMPCs.

In other experiments we obtained BMPCs from both control and metformin-treated rats and submitted them to an osteogenic differentiation medium for 0, 15, or 21 days. After these periods of time, we evaluated both AMPK and P-AMPK in cell extracts by Western blotting. We found that ex vivo metformin induced a significant 2.8-fold increase in the P-AMPK:AMPK ratio after 21 days, with no observable difference for 0 or 15 days (see Fig. 6*C*).

Discussion

Bone is a complex tissue containing several cell types that is continuously undergoing a process of self-renewal and repair termed *bone remodeling*.[1–4] In aged people and in patients suffering from diabetes mellitus, bone remodeling is altered, and consequently, there is an increased skeletal fragility and fracture risk.[1–3]

Table 2. Quantitative Histomorphometric Analysis of Tissue Sections of the Lesion Area From Parietal Bones

	Control	Control + Metformin	Diabetic	Diabetic + Metformin
Reossification thickness/Bone thickness [%]	30 ± 1	$46 \pm 3^{*}$	$19 \pm 1^{*,\#}$	$43 \pm 4^{*,\#,\bullet}$
Reossification area/Bone thickness [mm]	69 ± 9	$143 \pm 11^{*}$	$16 \pm 4^{*,\#}$	$160 \pm 10^{*,\bullet}$
Osteocyte number/Reossification area [mm^{-2}]	2440 ± 194	2943 ± 226	$993 \pm 132^{*,\#}$	$854 \pm 103^{*,\#}$
Osteoblast number/Reossification length [mm^{-1}]	145 ± 11	156 ± 16	$60 \pm 6^{*,\#}$	$49 \pm 9^{*,\#}$
TRAP area/Reossification area [%]	2.3 ± 0.4	$8.8 \pm 1.7^{*}$	$2.9 \pm 0.1^{\#}$	$2.4 \pm 0.9^{\#}$

Sections of decalcified bone were stained with haematoxylin–eosin or tartrate resistance acid phosphatase (TRAP) (for osteoclasts) and quantitated using Image J program. Values are expressed as mean ± SEM of sections.

$^{*}p < .05$ vs. C group;

$^{\#}p < .05$ vs. C + M group;

$^{\bullet}p < .05$ vs D.

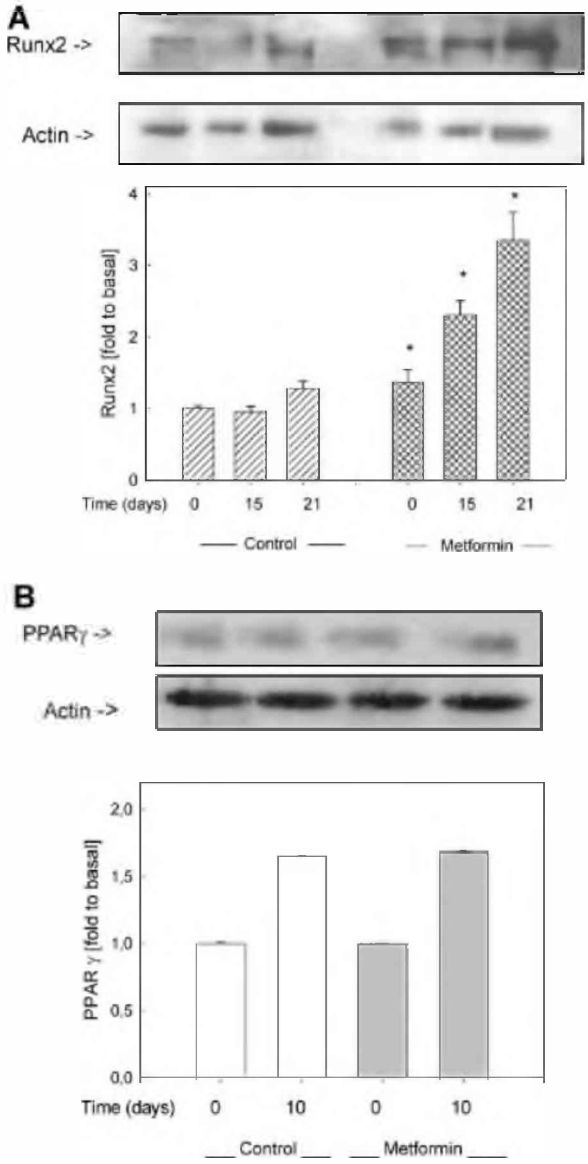

Fig. 5. Expression of Runx2 and PPARγ in BMPCs from control or metformin-treated rats. BMPCs isolated from either control or metformin-treated (100 mg/kg per day) rats were cultured in osteogenic or adipogenic medium for the periods of time indicated in the figures. The expression of osteogenic transcription factor Runx2 and the housekeeping protein actin (*A*) or adipogenic transcription factor PPARγ and actin (*B*) was evaluated by Western immunoblot. The blots were quantified with the Scion beta 2 image program, data were normalized against actin, and the corresponding values are shown as bar graphs. Values are expressed as mean ± SEM. $^{*}p < .05$.

Recently, considerable interest has focused on bone marrow mesenchymal cells for different applications in the field of bone tissue engineering. Prior studies have demonstrated that in the bone marrow microenvironment, reciprocal regulation of PPARγ and Runx2 underlies the age-related increase in both marrow adiposity and bone loss.[18] Since an increase in bone marrow adipocytes is associated with different conditions that lead to bone loss,[15] and under the perspective of recent studies that indicate that thiazolidinediones not only inhibit osteogenesis but also stimulate adipogenesis,[7] evaluation of the actions of metformin—a widely used antidiabetic drug—on bone marrow adipogenic and osteogenic differentiation is of clinical relevance.

Under proper stimuli, BMPCs have the capability to differentiate into different cell types such as osteoblasts, chondrocytes, and adipocytes. The commitment to differentiate into a specific cell type can be influenced by different agents such as cytokines, growth factors, hormones, or drugs. We have previously demonstrated that metformin enhances the proliferation, differentiation, and mineralization of osteoblast cell lines in culture.[12] In the present study we have found that metformin causes a direct increase in BMPC osteoblastic maturation: It stimulates both ALP activity and type I collagen synthesis after 15 days of culture, as well as the deposition of extracellular mineral nodules after 21 days of culture (see Fig. 1). We also have found that the osteogenic stages of development for BMPCs from control rats are similar to those previously described by other authors for mesenchymal stem cells and osteoblastic cell lines.[31–33] Those authors have reported that initially the proliferation of osteoblasts coincides with production of type I collagen and fibronectin and is followed sequentially by an enhanced expression of ALP and extracellular calcium deposition.[31,33] The increased expression of ALP mediates the formation of insoluble phosphate salts, thereby initiating the calcification process.[31]

In this study we have found that metformin also increases the ex vivo osteogenic potential of BMPCs; that is, the in vivo administration of metformin enhances BMPC osteoblastic ALP, type I collagen, osteocalcin, and mineral deposition in vitro (see Fig. 3) probably by increasing the expression of the Runx2/Cbfa1 transcription factor (see Fig. 5). Additionally, we created a parietal bone defect in nondiabetic and streptozotocin-induced diabetic rats, either untreated or treated with metformin. In untreated animals, we found that the diabetic state per se induces a significant decrease in bone healing, as evaluated by histomorphometric analysis (see Fig. 4 and Table 2). These results are in agreement with the previous report of Santana and colleagues.[21] We also observed that metformin causes an increase in bone reossification at the lesion site in both diabetic and nondiabetic animals (see Fig. 4 and Table 2). In addition, metformin increases total osteoclastic activity in nondiabetic animals. These results with metformin probably reflect a concerted increase in bone formation and remodeling at the lesion site in a precisely regulated process that involves the localized coupling of osteoclasts with osteoblasts.[22,34] Although we found that metformin induces an increase in bone reossification, it does not alter the glycemic profiles of either diabetic or nondiabetic rats (see Table 1). These results, as well as the results of our previous studies with osteoblastic cells in culture,[12] provide strong evidence that metformin has a direct osteogenic effect on bone cells. Our present in vivo and ex vivo findings are in agreement with previous clinical studies by other authors that indicate that metformin treatment could be associated with a decrease in the fracture risk of diabetic patients.[1]

We also performed studies to elucidate the mechanism of action of metformin on bone cells. Our present findings show that metformin directly enhances the phosphorylation/activation of AMPK, as assessed by immunofluorescence and Western

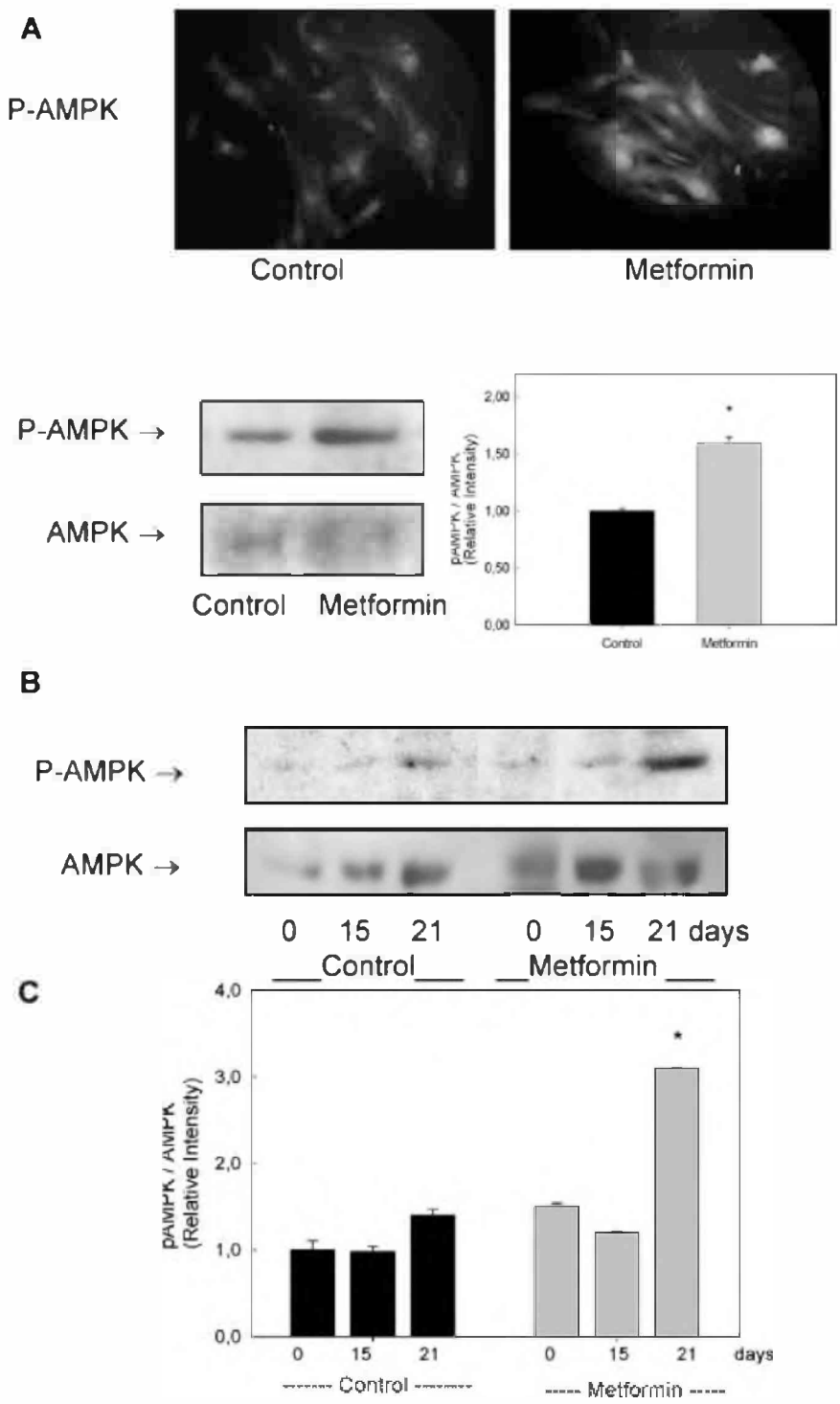

Fig. 6. Effect of metformin on AMPK activation in BMPCs derived from contol or metformin-treated rats. BMPCs were isolated from untreated control rats (*A*, *B*). After reaching confluence, cells were serum starved for 2 hours and further incubated for 1 hour in the absence (control) or the presence of 100 μM metformin. After this incubation period, cells were processed by immunofluorescence (*A*) or Western immunoblot (*B*) to detect AMPK and/or P-AMPK. In other experiments, BMPCs were isolated from untreated control or metformin-treated rats (*C*). After they reached confluence, cells were induced to differentiate in an osteogenic medium for 0, 15, or 21 days. At the end of this culture period, cells were extracted with Laemmli buffer and processed for P-AMPK and AMPK detection by Western immunoblot. The blots were quantified with the Scion beta 2 image program, and the P-AMPK:AMPK ratio was determined for each experimental condition. Data are representative of three different experiments. $^{*}p < .05$ versus control.

blotting in BMPCs. These results agree with the previous observations of Kanazawa and colleagues[13] in MC3T3E1 osteoblastic cells. These authors showed that metformin-induced osteoblastic differentiation was associated with AMPK activation and with enhanced expression of eNOS and BMP-2 mRNA. Using this same osteoblastic cell line, we also have demonstrated previously[12] that metformin exerts a direct osteogenic effect associated with MAPK activation and an

increase in e/iNOS protein expression. In our present results, we have found that BMPCs obtained from metformin-treated rats and submitted to an osteogenic medium for 15 to 21 days express significantly higher levels of osteoblastic markers in comparison with BMPCs from untreated rats (see Fig. 3). These findings can be associated with a parallel increase in Runx2/Cbfa1 expression (see Fig. 5*A*) and in the phosphorylation/activation of AMPK (see Fig. 6*C*) after 15 to 21 days of osteogenic differentiation. Altogether, our previous and present observations suggest that metformin can induce commitment of osteoprogenitor cells to the osteoblastic phenotype and bone formation by exerting direct effects on bone cells. The mechanism of action of metformin appears to be mediated by MAPK, e/iNOS, and AMPK signaling.

In addition, our present results indicate that metformin at clinically relevant doses[29] can partially block the proadipogenic effect of rosiglitazone on BMPCs (see Fig. 2); however, treatment with metformin does not affect expression of the transcription factor PPARγ versus control (see Fig. 5*B*). Rosiglitazone is an efficient insulin-sensitizing drug that unfortunately presents adipogenic side effects in the bone marrow microenvironment and that is administered frequently as monotherapy or in combination with other oral antidiabetic drugs such as metformin for the treatment of patients with type 2 diabetes mellitus.[6] Large prospective clinical trials have shown recently that rosiglitazone intake is associated with an increased fracture rate.[35,36] In the A Diabetes Outcome Progression Trial (ADOPT), this was demonstrated in subjects with recently diagnosed type 2 diabetes, previously naive to oral antidiabetic drugs, who were randomized to taking either rosiglitazone, metformin, or glyburide as monotherapy for an average 4 years.[35] Fractures were seen predominantly in the lower and upper limbs (in the hip and wrist). However, it should be noticed that in the present studies we have used an in vivo model of parietal bone defects that does not support mechanical loading, as is the case for long bones. In addition, the healing of the defect used in our study occurs in a relatively short period of time (15 days), and the reossification process is intramembranous, meaning that its healing does not include soft callus formation, as in endochondral bone repair, but involves direct bridging of the lesion by a hard callus of primary bone, which is then remodeled into mature bone by the combined action of osteoclasts and osteoblasts.[30] In our model of bone healing, metformin was found to simultaneously increase reossification and osteoclastic activity in the lesion site, suggesting a concerted increase in bone formation and remodeling.

Additionally, our results show in an in vitro model a reversion of rosiglitazone proadipogenic effects on BMPC differentiation by cotreatment with metformin. However, we cannot extrapolate these results to humans. Further clinical investigations are needed to prove that this drug association has similar effects in human patients.

In conclusion, our results show that metformin increases the osteogenic induction of BMPCs both in vitro and in vivo and enhances the process of bone repair in diabetic and nondiabetic rats. The osteogenic actions of metformin on BMPCs are associated both with an increase in the expression of osteoblast-specific transcription factor Runx2/Cbfa1 and with an increase in the phosphorylation of AMPK. Moreover, metformin is able to partially inhibit the adipogenic action of rosiglitazone on BMPCs. Further investigations are needed to elucidate the in vivo significance of the inhibition by metformin of rosiglitazone-induced bone marrow adipogenesis.

Disclosures

The authors state that they have no conflicts of interest.

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