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Troglitazone Treatment Increases Bone Marrow Adipose Tissue Volume but Does not Affect Trabecular Bone Volume in Mice

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Abstract. Aging is associated with decreased trabecular bone mass and increased adipocyte formation in bone marrow. As osteoblasts and adipocytes share common precursor cells present in the bone marrow stroma, it has been proposed that an inverse relationship exists between adipocyte and osteoblast differentiation. In order to test this hypothesis, we studied mice treated with troglitazone (n = 9) given as a 0.2% of food admixture (2.0 g troglitazone per kg food) for 10 months and control mice (n = 9). Troglitazone is a potent stimulator of adipogenesis acting at the nuclear receptor: peroxisome proliferator activated receptor-gamma (PPARγ). Histomorphometric analysis of proximal tibia was performed in order to quantitate the amount of trabecular bone volume per total volume(BV/TV %), adipose tissue volume per total volume (AV/TV %), and hematopoietic marrow volume per total volume (HV/TV %) using the point-counting technique. Bone size did not differ between the two groups. In troglitazone-treated mice, AV/TV was significantly higher than in control mice (4.7 ± 2.1% vs. 0.2 ± 0.3%, respectively, mean ± SD, $P < 0.001$). BV/TV was similar in the two groups (16.9 ± 5.6% for troglitazone-treated group vs. 14.9 ± 4.7% for control group) as well as ash weight of the vertebrae. HV/TV was reduced in troglitazone-treated mice compared with control mice (78.4 ± 6.8% vs. 84.9 ± 4.7%, respectively, $P < 0.05$) and the presence of vascular sinusoids was reduced (7.3 ± 1.7% vs. 16.1 ± 5.6%, respectively, $P < 0.05$). Our data demonstrate that adipogenesis and osteogenesis can be regulated independently. Troglitazone-induced adipogenesis in the bone marrow may be caused by changes in the bone marrow vascularity.

Key words: Troglitazone — Adipocyte — Osteoblast — Differentiation — Mesenchymal stem cell.

Aging of the human skeleton is characterized by a progressive decrease in bone mass and increased risk for fragility fractures [1]. The mechanisms of age-related bone loss are not known in detail. Decreased bone formation seems to play an important role, as evidenced by several histomorphometric studies in humans [2–4] and may be caused by a decrease in the number of recruited osteoblasts needed for bone formation [5].

Osteoblasts are derived from mesenchymal stem cells present in the bone marrow stroma [termed "mesenchymal stem cells" or "marrow stromal cells" (MSC)]. These precursor cells are capable of differentiating into several cell lineages including osteoblasts, adipocytes, chondrocytes, smooth muscle cells, and fibroblasts [6–9] . In human MSC cultures, differentiation into osteoblastic or adipocytic lineage is possible through manipulation of culture conditions [8, 10].

In several human and animal studies, age-related decrease in bone mass has been associated with increased adipocyte cell population in the bone marrow [4, 11, 12]. Also, in SAMP6 mice, which are a model for accelerated senescence, decreased bone mass was associated with increased bone marrow fat volume [13]. Thus, it has been postulated that an inverse relationship exists between adipocyte and osteoblast differentiation and that increased adipocyte differentiation with age may lead to an impaired osteoblast differentiation and a decreased bone formation [14–16] .

In order to test this hypothesis, we studied the effects of inducing adipogenesis on osteoblastic differentiation by quantitating changes in bone marrow tissue composition in a group of mice treated with troglitazone. Troglitazone is a member of thiazolidinediones which are known ligands for peroxisome proliferator-activated receptor-gamma (PPARγ) and are able to induce adipocyte differentiation *in vitro* and *in vivo* [17–19].

Material and Methods

Animals

This study was part of a large experiment investigating the effect of troglitazone on atherosclerosis in the apoE-deficient ($apoE^{-/-}$) mice (delivered from Bomholtgaard Breeding and Research Center, Denmark). Male $apoE^{-/-}$ mice were back-crossed nine generations into the C57BL/6 background and thus are phenotypically and genetically identical to the C57BL/6 mice except for the apo-E gene [20]. The mice were fed regular pelleted mouse chow (13%

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calories as fat) until 2 months of age. Thereafter they were given non-pelleted chow with or without a 0.2% troglitazone (2 g/kg food) added for the next 10 months. This dose of troglitazone has been previously demonstrated to exert significant biological effects in rats [21]. At the end of the study, the animals were sacrificed and the tibiae were removed, cleaned from soft tissues, and preserved in 70% ethanol.

Histomorphometry

The tibiae were embedded in methylmetacrylate (technovit 9100, Heraeus Kulzer, Wehrheim/Ts., Germany) and cut into 10 μm thick vertical sections by a Jung Model K microtome. Twelve consecutive sections were cut and stained with toluidine blue. From these 12 section, the 4–6 best sections were selected for analysis, depending on the size of the section of the proximal tibia (4 sections were selected if the section of the proximal tibia was large and 6 sections if the section of the proximal tibia was small). Measuring adipose tissue volume per total volume (AV/TV, %), trabecular bone volume per total volume (BV/TV, %) and hematopoietic marrow volume per total volume (HV/TV, %) were performed using a point-counting method described previously [22]. Point counting was conducted on a computer screen where the region of interest (ROI) of 25 pixels (5 × 5) was drawn and an equal magnification was employed for all sections (×200). The ROI was the proximal tibia from underneath the growth zone. Cortical bone was not included. The number of points hitting fat cells, bone tissue, and hematopoietic cells were counted and were divided by the total number of ROI.

In order to assess the size and number of adipocytes and vascular siusoids in the marrow tissue, all sections were evaluated at a higher magnification (×400). The ROI was the secondary spongiosa, including the metaphyseal area from 0.2 to 2.2 mm under the growth zone cartilage. Twelve fields of interest were investigated from each animal. Photographs were taken from each field (Olympus® digital camera, DP11) and were captured on a personal computer. A custom-made software program was created for the point-counting procedure (10 × 8 points in each field). From the point counting, the following measurements were determined: BV/TV, AV/TV, and the tissue volume of vascular sinusoids per total volume (VS/TV). In addition, the number of adipocytes was counted on screen printouts of each field of interest and the mean adipocyte size was calculated from the absolute adipocyte area and the number of adipocytes.

Assessment of Bone Mass

In order to study the effects of troglitazone treatment on bone mass, bone ash weight was determined. Six lumbar vertebrae were removed from each animal, cleaned from the attached soft tissues, and ashed (2 hours at 105°C and 580°C successively).

Plasma Analysis

Plasma glucose was determined by the glucose oxidase method (YSI 2300 STAT Plus Glucose analyzer). Plasma insulin was measured using a sensitive rat insulin RIA kit (Linco Research Inc., St. Louis MO, USA). Plasma total cholestrol (TC), HDL-C, and triglyceride (TG) were measured on a Cobas Fara Analyzer (Roche) using kits from Roche Diagnostica (Copenhagen, Denmark).

Statistical analysis

Differences between groups were examined using Student's *t*-test or Mann-Whitney's test. The results are presented as mean ± standard deviation (SD). Relationship between variables was examined using simple regression analysis.

Results

Troglitazone-treated (n = 15) and control mice (n = 17) did not differ in their body weight at baseline. Plasma levels of troglitazone in troglitazone-treated animals ranged from 4.6 to 11.0 μg/ml (mean 7 μg/ml) and there was no detectable troglitazone in plasma of control animals (detection limit <0.1 μg/ml). Troglitazone treatment did not change body weight, plasma levels of glucose, or HDL-C. On the other hand, it increased levels of TC (21 ± 7 mmol/L for troglitazone-treated mice versus 13 ± 3 mmol/L for control mice, $P < 0.001$), TG (4.8 ± 2.2 mmol/L for troglitazone-treated mice versus 1.3 ± 0.4 mmol/L for controls, $P < 0.01$) and insulin (1.3 ± 0.8 ng/ml for troglitazone-treated mice vs. 0.6 ± 0.2 ng/ml for controls, $P < 0.01$).

Bone samples of 9 mice from the troglitazone-treated group and 9 mice from the control group were chosen randomly for histological analysis. Representative sections of tibiae from troglitazone-treated and control animals are shown in Figure 1. The ROI employed to compare the tissue composition of an area covering both primary and secondary spongiosa was similar in the two groups. In troglitazone-treated animals, AV/TV was significantly increased to 4.7 ± 2.1% compared with 0.2 ± 0.3% in the control group ($P < 0.001$). The increase in AV in troglitazone-treated mice was due to changes in adipocyte number and not in their size. The number of adipocytes was increased from 22 in a total of 108 ROI in control mice to 1029 in a total of 108 ROI in troglitazone-treated mice. We found no difference in the size of adipocytes between control and the troglitazone-treated mice: diameter 30.7 μm for control and 31.3 μm for troglitazone-treated adipocytes. Also, we found no significant correlation between AV/TV and p-insulin, TG or p-TC in the troglitazone-treated animals.

There was no statistically significant differences in the BV/TV between the troglitazone-treated and control mice (16.9 ± 5.9% and 14.9 ± 4.7% respectively, n.s.). Also, the bone ash weight, which is a real estimate of bone mass, was similar in the two groups: 4.82 ± 0.77 mg/vertebra for the control group and 4.78 ± 0.53 mg/vertebra for the troglitazone-treated group.

HV/TV was significantly decreased in troglitazone-treated animals compared with control animals (78.4 ± 6.8% vs. 84.9 ± 4.7%, respectively, $P < 0.05$). Examining the hematopoietic tissue in the control group revealed the presence of a large number of sinusoids lined by endothelial cells (Fig. 1C) that were markedly decreased in bones of the troglitazone-treated animals (Fig. 1F). Quantitation of the VS/TV showed a decrease from 16.1 ± 5.6% in control mice to 7.3 ± 1.7% in troglitazone-treated mice ($P < 0.05$) (Fig. 2).

Discussion

In the present study we tested the hypothesis that adipocyte

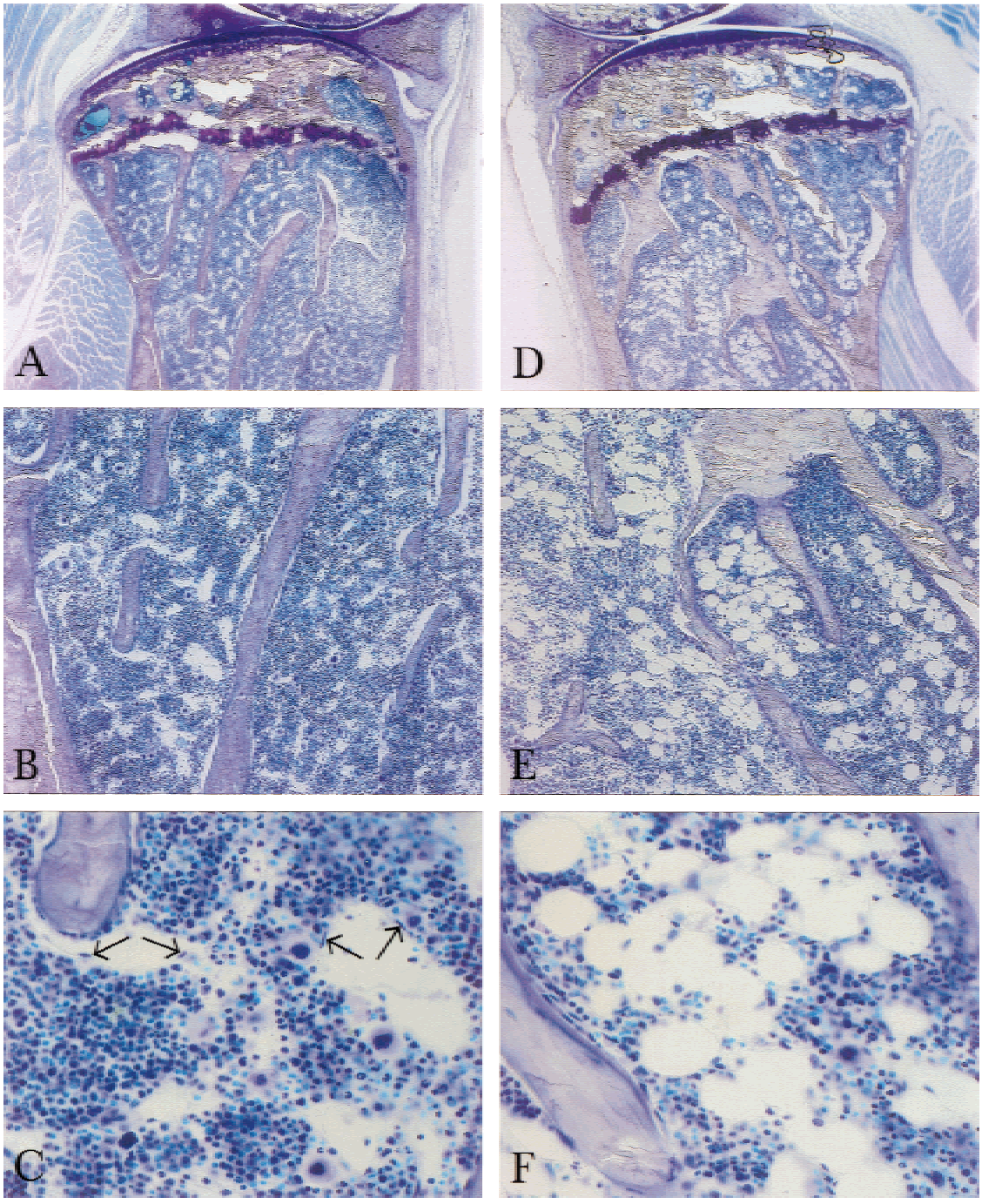

Fig. 1. An example of a control animal (**A,B,C**) and a troglitazone-treated animal (**D,E,F**). (**A**) Proximal part of tibia from a control animal including: joint cartilage, epiphysis, growth zone, and metaphysis. There is a thin layer of cancellous bone underneath the growth zone and a few vertically oriented trabeculae in the metaphysis (magnification ×40). (**B**) Metaphyseal region at a higher magnification (×100). The bone marrow is dominated by hematopoietic cells and large sinusoids and contains very few adipocytes. (**C**) Large sinusoids lined by endothelial cells indicated by arrows (magnification ×400). (**D**) Proximal tibia from a troglitazone-treated animal, the bone marrow contains a large number of adipocytes (magnification ×40). (**E**) Higher magnification of D. The bone marrow is dominated by adipocytes. There are only few visible sinusoids (×100). (**F**) The abundant adipocytes in the bone marrow. Only few visible sinusoids can be detected. There is an active bone-forming surface in the left side of the image with osteoblasts covering a thin layer of osteoid (magnification ×400).

and osteoblast differentiation exhibits a reciprocal relationship *in vivo*, as demonstrated previously in several *in vitro* studies [14–16] . According to this hypothesis, exposure of stromal cells in the bone marrow to an adipogenic stimulus should increase the number of adipocytes and decrease the number of osteoblasts and hence bone formation. The opposite should be true—that an increase in bone formation would decrease the number of bone marrow adipocytes. Our

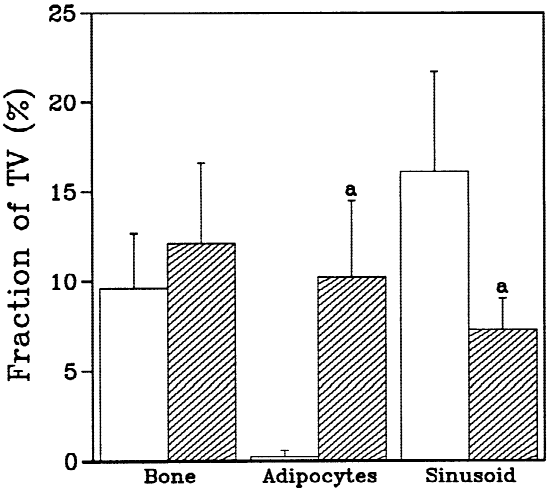

Fig. 2. Bone volume, adipocyte volume, and sinusoid volume as fractions of total volume (TV) in percentage. Variables were measured in a region of interest (ROI) covering the secondary spongiosa alone at a magnification ×400. a = $P < 0.05$.

study showed that troglitazone induced adipocyte differentiation and increased AV/TV in the bone marrow without compromising the osteoblastic cell differentiation as suggested by the absence of changes in trabecular bone volume and bone ash weight.

Troglitazone is a member of the thiazolidinediones which are ligands to the nuclear transcriptional factors PPARγ [17–19] . PPARγ are expressed in adipose tissues and upon activation they increase adipocyte cell differentiation [23]. This is further supported by the presence of obesity in activated mutations of PPARγ gene [24] or the absence of adipose tissue in PPARγ knock-out mice [19]. Induction of adipocyte differentiation in the bone marrow, as demonstrated in our study, suggests that medullary adipocyte precursor cells are responsive for troglitazone action. It also corroborates previous *in vitro* findings showing that thiazolidinediones can induce adipogenesis in cultured marrow stromal cells [25].

The increase in AV/TV during troglitazone treatment can be due to either an increase in adipocyte cell size, cell number, or both. We found that the adipocyte cell size did not change in troglitazone-treated mice and that the increase in AV/TV can be explained by a significant increase in adipocyte cell number. Similar to our findings in marrow adipocytes, Okuno et al. [26] have demonstrated that troglitazone treatment in rats can increase the number of small adipocytes in the white adipose tissue. Also, the increase in AV was not correlated to changes in plasma levels of insulin or TG, suggesting that troglitazone effects are direct through induction of adipocyte cell differentiation from precursor cells in the bone marrow.

Troglitazone treatment induced adipogenesis but it did not affect bone formation in the bone marrow. While previous studies performed on human iliac bone biopsy specimens have demonstrated an inverse relationship between fat tissue volume and trabecular bone volume [4, 12, 27], these association studies do not prove causality. According to the stromal cell hypothesis [28], osteoblastic and adipocytic differentiation occur at the level of mesenchymal stem cells in the bone marrow. Since the cellular targets for troglitazone in the bone marrow are not known, troglitazone may induce proliferation and differentiation of committed adipocyte precursors without affecting the mesenchymal stem cells and osteobast differentiation. Gene expression of PPAR-γ was not detectable in cultured human MSC and it was induced by culturing the cells in adipogenesis-inducing medium [34]. Alternatively, adipogenesis and osteoblastogenesis may be independent processes. The increase in bone marrow adipogenesis in conditions of rapid bone loss [29] and aging [4, 12, 27] could be a passive process where adipocytes occupy the space left by the absent trabecular bone elements [16]. In support for this view, some experimental studies have demonstrated that marrow adipocytes do not participate in the overall fat metabolism. Marrow adipocytes are not affected by long periods of starvation [30] or by insulin which is quite different from the changes observed in extramedullary adipocytes [31, 32].

In addition to its effects on adipogenesis, troglitazone treatment decreased the HV/TV and VS/TV in the bone marrow. The inhibitory effects of troglitazone on bone marrow vasculature may be mediated by direct effects on vascular smooth muscle cells as they do express PPAR-γ and their growth and migration can be inhibited by troglitazone [33]. Decreased marrow vascularity may be a factor contributing to enhanced adipogenesis during troglitazone treatment because of the effects of hypoxia on the adipocyte precursor cells, as suggested by Burkhardt et al. [12].

Since thiazolidinediones are increasingly employed in the treatment of type II diabetes mellitus, studying their effects on bone is clinically relevant. Two previous studies have demonstrated that troglitazone and other members of the thiazolidinediones can inhibit osteoclast formation and bone resorption *in vitro* [34, 35]. Also, biochemical markers of bone turnover were found to decrease during troglitazone treatment in diabetic patients [36]. Our findings of the maintenance of bone mass in mice after long-term treatment with troglitazone suggest that the increased bone marrow adipogenesis is not detrimental to osteoblast differentiation and function. However, similar long-term studies need to be performed in humans.

In conclusion, our study does not support the hypothesis of the presence of a reciprocal relationship between osteoblastic and adipocytic cell differentiation in the bone marrow, suggesting that the two processes can be regulated independently.

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