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Osteopenia and impaired fracture healing in aged EP4 receptor knockout mice

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Abstract

The EP4 receptor, one of the subtypes of the prostaglandin E_2 (PGE_2) receptor, plays a critical role in the anabolic effects of PGE_2 on bone. However, its role in the maintenance of bone mass in aged animals and its role in fracture healing is not well known. Our studies addressed these issues by characterizing the skeletal phenotype of aged, EP4 receptor knockout (KO) mice, and by comparing fracture healing in aged KO mice versus wild type (WT) mice. There was no significant difference in body weight and femoral length between KO and WT mice at 15 to 16 months of age. Lower bone mass was seen radiographically in both axial and long bones of KO mice relative to WT mice. Micro-CT images of the distal femurs showed thinner cortices, fewer trabeculae, and a deteriorated trabecular network in KO mice. Total bone content, trabecular content, and cortical content, as assessed by pQCT in the distal femur, were lower in KO mice than WT controls. Histomorphometric measurements showed that trabecular bone volume and bone formation rate were significantly decreased whereas osteoclast number on trabecular surface and eroded surface on endocortical surface were significantly increased in KO mice. These data indicated that deleting the EP4 receptor resulted in an imbalance in bone resorption over formation, leading to a negative bone balance. The lower bone formation rate in EP4 KO mice was primarily due to decreased mineralizing surface, suggesting that the defect in overall bone formation was mainly due to the defect in osteoblastogenesis. Fracture healing was examined in KO and WT mice subjected to a transverse femoral fracture. Callus formation was significantly delayed as evidenced both radiographically and histologically in the fractured femurs of KO mice compared with those of WT mice. KO mice had significant decreases in total callus area, cartilaginous callus area, and bony callus area 2 weeks after fracture. By 4 weeks, complete bony bridging was seen in WT mice but not in KO mice. These data demonstrate that the absence of the EP4 receptor decreases bone mass and impairs fracture healing in aged male mice. Our findings indicate that the EP4 receptor is a positive regulator in the maintenance of bone mass and fracture healing.

Keywords: EP4 receptor; EP4 KO mice; Bone formation; Bone resorption; Fracture healing

Introduction

It is well known that prostaglandin E_2 (PGE_2) induces significant increases in bone formation, bone mass, and bone strength when administered systemically or locally to the skeleton [1–3]. It has also been reported that endogenous PGE_2 increases locally after fracture [4], and inhibition of PGE_2 production impairs bone healing [5–7]. In addition, studies have shown that local administration of PGE_2 stimulates bone formation and callus development in animal models [6,8]. However, the associated side effects, such as diarrhea, lethargy, and flushing, limit the therapeutic usage of PGE_2 in humans.

It is now known that the pharmacological activities of PGE_2 are mediated through at least four receptor subtypes, EP1–EP4 [9]. In recent years, a number of studies have

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demonstrated that the EP4 receptor subtype plays a critical role in PGE_2's bone anabolic effects. Weinreb et al. [10] showed that the EP4 receptor is expressed not only in embryonic and neonatal bone tissue of mice but also is expressed in bone tissue of young adult rats and osteoblastic cell lines. Its expression in bone tissues is upregulated by PGE_2 [11]. In bone marrow cell cultures derived from EP4 receptor knockout (EP4 KO) mice, mineralized nodule formation was absent and could not be increased by treatment with PGE_2 [12]. The results of several in vivo studies confirmed and extended these in vitro findings. When PGE_2 was infused onto the periosteal surfaces of femurs, it caused local bone formation in WT but not in EP4 KO mice [12]. In addition, an EP4-specific antagonist suppressed the increase in bone mass induced by PGE_2 in young rats [13]. Furthermore, local infusion of a selective EP4 agonist markedly increased local bone formation [12]. Lastly, systemic administration of selective EP4 agonists increased bone formation and augmented bone mass in ovariectomized (OVX) and immobilized rats [12,14].

As mentioned earlier, mice lacking the EP4 receptor have been utilized to study the effects of exogenous PGE_2 on bones. We have previously examined the skeletal phenotype of EP4 KO mice at 4 to 5 months of age and found no pronounced changes in bone length, bone mass, and bone turnover [15]. However, the skeletal phenotype of EP4 KO mice at an older age is unknown. In addition, the role of the EP4 receptor in fracture healing, a biological cascade involving PGE_2 production, is not well documented. Therefore, the current studies were carried out to address these issues by characterizing the skeletal phenotype of aged, EP4 KO mice and by comparing fracture healing in KO mice with wild type (WT) mice.

Materials and methods

Animals

EP4 receptor knockout mice (KO) and wild type controls (WT) were generated as previously described [16]. Homozygous null animals were generated in a selected mixed background of 129/SvEv, C57BL/6, and DBA2 strains by sib × sib or sib × offspring crosses that exhibited the best neonatal survival. Eventually, this recombinant inbred line, designated EP4A, was bred KO × KO to generate the animals used for this study. A line of WT control mice derived from the same mixed strain background was designated EP4B. KO and WT mice were intercrossed every 3 generations to minimize genetic drift between the two lines. The animals were housed at 24°C with a 12-h light/12-h dark cycle and allowed free access to water and a commercial diet (Purina laboratory Rodent Chow 5001, Purina-Mills, St. Louis, MO) containing 0.95% calcium, 0.67% phosphorus, and 4.5 IU/g vitamin D_3. The experiments were conducted according to Pfizer animal care-approved protocols, and animals were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals.

Experiment 1

Male mice at 15–16 months of age from each of strain and age-matched WT ($N = 1$) and KO ($N = 11$) groups were used for the characterization of the skeletal phenotype of EP4 KO. All mice were subcutaneously injected with calcein at a dose of 5 mg/kg (Sigma Chemical Co., St. Louis, MO) on −11 and −1 days prior to euthanasia. This regimen resulted in deposition of a single or double fluorochrome label at bone surfaces that were actively mineralizing at the time of the injections. All mice were sacrificed by CO_2 asphyxiation and both right and left femurs, right tibia, and lumbar spine were harvested. Radiographs of the right femurs and lumbar spines were taken at necropsy using a Specimen Radiography System (MX-20; Faxitron X-ray Corporation, Wheeling, IL, USA). The right femurs were scanned by a micro-CT machine (Micro-CT40, Scanco Medical, Auenring 6–8, Bassersdorf, Switzerland) with software version 3.1. A cross-section of distal femur metaphysis (a total of 50 slices in thickness of 16 μm each, total thickness = 0.8 mm) was taken at 2.3 to 3.1 mm proximal to the distal end (~1.3 to 2.1 mm from the growth plate) for the determination of trabecular bone volume and trabecular connectivity density. The right femurs were then scanned by peripheral quantitative computed tomography (pQCT, Stratec XCT Research M; Norland Medical Systems, Fort Atkison, WI, USA) with software version 5.40 as previously described [17]. A 1-mm-thick cross-section of each distal femoral metaphysis was taken at 2.5 mm proximal to the distal end (~1.5 mm proximal to the growth plate, a cancellous bone enriched site), and 1-mm-thick cross-section of each femoral diaphysis was taken at 8 mm proximal from the distal end (a cortical bone enriched site) with a voxel size of 0.10 mm. Volumetric bone content, density, and area were determined for total, trabecular, and cortical bone. In addition, cortical thickness, periosteal and endocortical circumferences were determined at the femoral diaphysis.

The left femurs were processed for histomorphometric assessment on cancellous bone as previously described [18–20]. Briefly, the left femurs were dehydrated in graded concentrations of ethanol and embedded undecalcified in methyl methacrylate. Longitudinal frontal sections of the distal femur were cut at 4- and 10-μm thickness using a Reichert-Jung Polycut S microtome (Leica Corp., Heidelberg, Germany). The 4-μm sections were stained with a modified Masson's Trichrome stain and the 10-μm sections remained unstained. All histomorphometric measurements were performed in cancellous bone tissue of the distal