RAGE supports parathyroid hormone-induced gains in femoral trabecular bone

Binu K. Philip, Paul J. Childress, Alexander G. Robling, Aaron Heller, Peter P. Nawroth, Angelika Bierhaus, and Joseph P. Bidwell

Department of Anatomy and Cell Biology, Indiana University School of Medicine; Department of Biomedical Engineering, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana; and Department of Internal Medicine and Clinical Chemistry, University of Heidelberg, Heidelberg, Germany

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Philip BK, Childress PJ, Robling AG, Heller A, Nawroth PP, Bierhaus A, Bidwell JP. RAGE supports parathyroid hormone-induced gains in femoral trabecular bone. Am J Physiol Endocrinol Metab 298: E714–E725, 2010. First published December 22, 2009; doi:10.1152/ajpendo.00564.2009.— Parathyroid hormone (PTH) renews bone mass to the osteopenic skeleton, but significant questions remain as to the underlying mechanisms. The receptor for advanced glycation end products (RAGE) is a multiligand receptor of the immunoglobulin superfamily; however, recent studies indicate a role in bone physiology. We investigated the significance of RAGE to hormone-induced increases in bone by treating 10-wk-old female Rage-knockout (KO) and wild-type (WT) mice with human PTH (1–34) at 30 μg·kg⁻¹·day⁻¹ or vehicle control, 7 days/wk, for 7 wk. PTH produced equivalent relative gains in bone mineral density (BMD) and bone mineral content (BMC) throughout the skeleton in both genotypes. PTH-mediated relative increases in cortical area of the midshaft femur were not compromised in the null mice. However, the hormone-induced increase in femoral cancellous bone was significantly attenuated in Rage-KO mice. The loss of RAGE impaired PTH-mediated increases in femoral cancellous bone volume, connectivity density, and trabecular number but did not impact increases in trabecular thickness or decreases in trabecular spacing. Disabling RAGE reduced femoral expression of bone formation genes, but their relative PTH-responsiveness was not impaired. Neutralizing RAGE inhibited bone formation and decreased mineralization in vivo. However, RAGE is not essential for bone modeling and remodeling, leading to increases in cortical bone thickness and improvements in trabecular architecture (1, 29).

The observation that the receptor activator of NF-κB ligand (RANKL) and its receptor RANK play roles in bone modeling and remodeling and dendritic cell activation gave rise to the field of osteoimmunology and potential new therapeutic targets for various bone diseases (reviewed in Refs. 20 and 25). The receptor for advanced glycation end products (RAGE) is a part of a signaling axis that is active in a variety of immune responses and regulates bone cell activity under some circumstances (Ref. 4 and Refs. 3, 36, and 37 therein). RAGE is a transmembrane receptor, a member of the immunoglobulin superfamily, and is expressed by numerous cell types, including monocytes, macrophages, T lymphocytes, neurons, endothelial cells, osteoclasts, and osteoblasts (3, 4, 27, 37). RAGE binds to several ligands, including advanced glycation end products (AGEs), high-mobility group box 1 protein (HMGB1), S100 proteins, and β-amyloids. As an adhesion receptor, RAGE ligands include types I and IV collagens as well as the β₂-integrin Mac-1 (reviewed in Ref. 31). RAGE signal transduction pathways typically activate NF-κB-mediated responses involved in inflammation, and this receptor plays significant roles in the alarmin response of the innate immune system and contributes to diabetic complications and neurodegenerative disorders (3, 4, 27).

The significance of RAGE signaling in the skeleton is unclear, but recent studies suggest roles in both normal bone homeostasis and inflammation-associated bone loss. RANKL induces the release of HMGB1 from immature osteoclasts, and its activation of RAGE or the Toll-like receptors TLR2 and TLR4 is important for osteoclast actin ring development (37). Additionally, HMGB1 enhances osteoclast progenitor sensitivity to RANKL (37). Diabetic mice infected with the human periodontal pathogen Porphyromonas gingivalis exhibited a significant decrease in alveolar bone loss when treated with soluble RAGE, the extracellular domain of the receptor that blocks its activation (22). The apoptotic osteoblast releases HMGB1, and once released by the dying osteoblast, HMGB1 stimulates the secretion of the proresorption cytokines RANKL, TNFα, and IL-6 from bone marrow stromal osteoblasts (36). PTH regulation of osteoblast apoptosis is a significant part of mechanisms underlying the anabolic response, and thus the HMGB1–RAGE signaling axis may support some aspects of hormone-mediated bone remodeling.
In this study, we inquired whether RAGE activity is necessary for any aspect of PTH-induced gains in bone. Treatment of female Rage-null and wild-type (WT) mice with anabolic doses of hormone for 7 wk revealed the surprising observation that abrogation of receptor activity attenuated the skeleton’s response to anabolic PTH. Specifically, RAGE was required for hormone-mediated improvement of femoral trabecular architecture but not intrinsically necessary for increasing cortical thickness. Additionally, RAGE appeared to contribute to the mechanisms that regulate fat and bone accrual in the growing mouse. These data reveal a heretofore unrecognized role for RAGE in normal skeletal homeostasis and specifically in the mechanisms underlying its response to anabolic PTH.

MATERIALS AND METHODS

Mice. Our local Institutional Animal Care and Use Committee approved all experiments and procedures involving the use of the experimental mice described in this study. Rage-knockout (KO) mice were prepared as described previously and backcrossed onto a C57BL6 background at least eight generations (8). Briefly, two loxP sites in the same orientation flanked exons 2–7 of the Rage gene so that upon exposure to Cre the intervening genomic sequences were deleted. These mice were mated to Cre deleter mice to remove Rage from all tissues (8). The absence of RAGE expression was confirmed upon exposure to Cre the intervening genomic sequences were deleted. These mice were mated to Cre deleter mice to remove Rage from all tissues (8). The absence of RAGE expression was confirmed using PCR analysis of mouse-tail DNA and quantitative real-time PCR (qRT-PCR) analysis of mRNA isolated from bone marrow cell cultures (data not shown). WT C57BL/6J mice from The Jackson Laboratories (Bar Harbor, ME) were used as controls, accordant with previously published studies on RAGE and bone phenotype (11, 37).

PTH treatment regimen. To initiate an experiment, 8-wk-old female WT and Rage-KO mice were given 100 μl of sterile saline by subcutaneous (sc) injection once daily to acclimatize them to handling. At 10 wk of age, mice were sorted into four groups based on equivalent mean group body weight (7–9 mice/group). The four treatment groups were 1) vehicle-treated WT, 2) PTH-treated WT, 3) vehicle-treated Rage-KO, and 4) PTH-treated Rage-KO. Mice were injected with human PTH-(1–34) [hPTH-(1–34); Bachem Biosciences] at 30 μg·kg⁻¹·day⁻¹ sc daily or vehicle control (0.2% BSA/0.1% 1.0 mM HCl in saline; Abbott Laboratories, North Chicago, IL) for 14 or 48 days and euthanized on day 15 or day 49, respectively, 24 h after the last injection.

Dual energy X-ray absorptiometry. Bone mineral content (BMC; g) and areal bone mineral density (BMD; g/cm²) were measured in the postcranium skeleton, once weekly from 8 to 17 wk of age, by dual-energy X-ray absorptiometry (DEXA) using a PIXImus mouse densitometer (PIXImus II; GE-Lunar, Madison, WI). Mice were anesthetized with isoflurane (2% at 1.5 l/min) and placed in a horizontal position, with limbs outstretched on the PIXImus platform. From these scans, whole body (WB), femur, tibia, and spine (L3–L5) BMD and BMC, as well as whole body lean and fat mass, were calculated. Body weight was recorded at each scan using a standard laboratory scale.

**Fig. 1.** Female receptor for advanced glycation end products (Rage)-knockout (KO) and wild-type (WT) mice are equally responsive to parathyroid hormone (PTH), as assessed by whole body (WB) bone mineral density (BMD) and bone mineral content (BMC). Mice were treated with vehicle (VEH) or human (h)PTH-(1–34) 30 μg·kg⁻¹·day⁻¹ for 48 days from 10 to 17 wk of age. The 4 treatment groups (n = 7–9/group) were 1) VEH-treated WT (WT VEH), 2) PTH-treated WT (WT PTH), 3) VEH-treated Rage-KO (KO VEH), and 4) PTH-treated Rage-KO (KO PTH). Dual-energy X-ray absorptiometry (DEXA) was used to obtain weekly values of WB BMD (A) and WB BMC (C). From these data, the relative %change in WB BMC (B) and WB BMC (D) from 8 to 17 wk of age was determined for the 4 treatment groups. Analysis of both the raw BMD and BMC data and the %change data reveal a strong treatment effect but no genotype × treatment interaction. A slower rate of bone accrual is indicated for WB BMC by a significant genotype effect for the %change data (D). A repeated-measures multivariate analysis of variance (RM MANOVA) was used to determine the listed P values for the raw data (A and C), and a 2-factor ANOVA (2-W-ANOVA) was used to determine the listed P values for the %change data (B and D).
Microcomputed tomography. Femurs and vertebra were dissected from the mice immediately after euthanization, the muscle and connective tissue removed, and the bones transferred to 10% neutral buffered formalin and stored at 4°C. After 48 h the bones were transferred to 70% ethanol and stored at 4°C until analysis. We have previously described our methodology for assessing the trabecular microarchitecture at the distal femoral metaphysis and for evaluating cortical architecture at the midshaft femur, including total area, cortical area, medullary area, and second moments of inertia ($I$) (30). To assess cancellous microarchitecture at the fifth lumbar vertebra, a 270-$\mu$m-thick region in the caudal portion of each vertebral body was scanned on a desktop microcomputed tomographer ($\mu$CT20; Scanco Medical, Bassersdorf, Switzerland) at 13-$\mu$m resolution. A microfocus X-ray tube with a focal spot of 10-$\mu$m was used as a source. For each slice, 600 projections were taken over 216° (180° plus half of the fan angle on either side). Each slice was obtained at an increment of 13-$\mu$m. After manually defining the cancellous compartment, the trabecular stacks (21 slices) were reconstructed to the third dimension using.

Fig. 2. Female RAGE-KO and WT mice are equally responsive to PTH, as assessed by femur BMD (A) and femur BMC (C). From these data, the relative %change in femur BMD (B) and femur BMC (D) from 8 to 17 wk of age was determined for the 4 treatment groups. Analysis of both the raw BMD and BMC data and the %change data reveal a strong treatment effect but no genotype $\times$ treatment interaction. A slower rate of bone accrual is indicated for femur BMC by a significant genotype $\times$ time interaction from the raw data (C) and a significant genotype effect for the %change data (D). A RM MANOVA was used to determine the listed $P$ values for the raw data (A and C), and a 2-W-ANOVA was used to determine the listed $P$ values for the %change data (B and D).

Fig. 3. Female RAGE-KO and WT mice are equally responsive to PTH, as assessed by tibia BMD and BMC. Mice were treated as described in Fig. 1 from the same 4 treatment groups. DEXA was used to obtain weekly values of tibia BMD (A) and tibia BMC (C). From these data, the relative %change in tibia BMD (B) and tibia BMC (D) from 8 to 17 wk of age was determined for the 4 treatment groups. Analysis of both the raw BMD and BMC data and the %change data reveal a strong treatment effect but no genotype $\times$ treatment interaction. A slower rate of bone accrual is indicated for tibia BMC by a significant genotype $\times$ time interaction from the raw data (A and C) and a significant genotype effect for the %change data (B and D). A RM MANOVA was used to determine the listed $P$ values for the raw data (A and C), and a 2-W-ANOVA was used to determine the listed $P$ values for the %change data (B and D).
a standard-convolution back projection procedure with a Shepp-Logan filter, using a threshold value of 270. From the 3D constructs of the femur and spine, trabecular bone total volume (%BV/TV), connectivity density (Conn D; mm⁻³), structure model index (SMI), trabecular number (Nb N; mm⁻¹), trabecular thickness (Nb Th, mm), and trabecular spacing (Nb Sp, mm) were calculated using the Scanco software.

RNA extraction, cDNA synthesis, and qRT-PCR analysis. RNA from the femurs of 12-wk-old female WT and Rage-null mice that had been treated with PTH or vehicle was harvested and prepared for qRT-PCR (30). Real-time PCR primers and probes were obtained from Assays-on-Demand (Applied Biosystems, Foster City, CA) for the following genes: alkaline phosphatase (Mm01187117_m1), the pro-α1(I) chain (Col1a1) (Mm00801666_g1), fatty acid-binding protein 4 (Fabp4; Mm00445880_m1), Lef1 (Mm00550265_m1), Opg (Mm00435452_m1), osterix (Mm00441908_m1), and Runx2 (Mm00501578_m1). Custom design primer/probe was prepared for nuclear matrix protein-4/cas interacting zinc finger protein (Nmp4/CIZ): forward 5'-CACAGTCTCA-GGGCAGATTGAA-3', reverse 5'-CTCTGGCAACAGCTGATCCTT-3', probe 5'-ACACTATGTTCATCAACAAG-3', reverse 5'-CTCTGGGCAACAGCTGATCCTT-3'. The ΔΔCₜ method was employed for comparing the relative expression of genes of interest between WT and Rage-KO bone using Rplp2 (Mm03059047_gH) as the normalizer (30).

Statistical analyses. The JMP Version 7.0.1 (SAS Institute, Cary, NC) was used to process statistical analyses. We used a repeated-measures multivariate analysis of variance (MANOVA) to assess whether genotype or treatment had a significant impact on phenotypic measures multivariate analysis of variance (MANOVA) to assess whether genotype or treatment had a significant impact on phenotypic measures. We used a repeated-measures multivariate analysis of variance (MANOVA) to assess whether genotype or treatment had a significant impact on phenotypic measures.

RESULTS

Ablation of RAGE did not impact the PTH-induced relative increase in BMD and BMC but did alter the rate of bone and fat accrual. Intermittent PTH treatment induced equivalent relative increases in BMC and BMD in the WT and Rage-KO mice. Mice were treated with vehicle or hPTH-(1–34) 30 µg·kg⁻¹·day⁻¹ for 48 days from 10 –17 wk of age. The four treatment groups (n = 7–9/group) were 1) vehicle-treated WT, 2) PTH-treated WT, 3) vehicle-treated Rage-KO, and 4) PTH-treated Rage-KO. Figures 1–4 show the longitudinal raw BMD and BMC data, obtained weekly using DEXA, of the four treatment groups accompanied by the respective relative percent change histograms representing these parameters. Analysis of the longitudinal raw data and the percent change data indicated a strong treatment effect for WB (Fig. 1), femur (Fig. 2), and tibia (Fig. 3) BMD and BMC but no significant genotype × treatment interaction; i.e., the WT and null mice responded similarly to PTH for these parameters. Similarly, the spine (L3–L5) BMD and BMC longitudinal data showed a treatment effect but no genotype × treatment interaction (Fig. 4). The percent change data approached a significant treatment effect for spine BMC (P = 0.0863; Fig. 4).

![](Fig. 4. Female Rage-KO and WT mice are equally sensitive to PTH, as assessed by spine (L3–L5) BMD and BMC. Mice were treated as described in Fig. 1 from the same 4 treatment groups. DEXA was used to obtain weekly values of spine BMC (A) and spine BMC (C). From these data, the relative %change in spine BMC (B) and spine BMC (D) from 8 to 17 wk of age was determined for the 4 treatment groups. Analysis of both the raw BMD and BMC data reveal a treatment effect but no genotype × treatment interaction; the %change data approach a significant treatment effect for spine BMC. There was an enhanced rate of accrual of spine BMC in the Rage-null mice, as evidenced by a significant genotype effect in the %change analysis (B) and a near-significant genotype × time interaction effect in the raw longitudinal analysis (A). A RM MANOVA was used to determine the listed P values for the raw data (A and C), and a 2-W-ANOVA was used to determine the listed P values for the %change data (B and D).
difference in statistical outcome between the spine longitudinal and percent change analyses is likely due to the relatively large standard deviations in these measurements combined with the difference in the number of experimental observations used in the longitudinal and percent change analyses.

The Rage-KO mice exhibited a slower rate of bone accrual than the WT mice in many of the skeletal sites. The accrual rate, represented by the genotype × time interaction term for the longitudinal raw data and by the genotype term for percent change analysis, was significant for WB BMC (Fig. 1), femur BMC (Fig. 2), and tibia BMD and BMC (Fig. 3). Conversely, there was an enhanced rate of accrual of spine BMD in the Rage-null mice, as evidenced by a significant genotype effect in the percent change analysis and a near-significant genotype × time interaction effect in the raw longitudinal analysis (Fig. 4).

The Rage-KO mice showed an enhanced rate of accrual of body fat, as measured by DEXA during the experimental period, compared with the WT mice, as indicated by a strong genotype effect in the percent change analysis and a genotype × time interaction in the longitudinal raw analysis (Fig. 5, A and B). Hormone treatment significantly attenuated the increase in percent fat in the Rage-null mice but had no impact on this parameter in the WT animals; i.e., there was a strong treatment effect and a significant genotype × treatment interaction in the

![Graph](image)

**Fig. 5.** Intermittent PTH regulates %fat in female Rage-KO mice but not in WT mice. The rate of body weight gain is lower in Rage-null mice than in WT mice. Mice were treated as described in Fig. 1 from the same 4 treatment groups (n = 8–10/group). A: DEXA was used to obtain weekly values of %fat. C: body weight was recorded at the time of the DEXA measurement. From these data, the relative %change in %fat (B) and body weight (D) from 8 to 17 wk of age was determined for the 4 treatment groups. Analysis of both the raw %fat data and the %change data reveal a strong treatment effect and a strong genotype × treatment interaction. A higher rate of fat accrual is indicated by a significant genotype × time interaction from the raw data (A) and a significant genotype effect for the %change data (B). Hormone did not impact body weight in either genotype (C and D). However, the rate of weight gain was slower in the null mice, as evidenced by a significant genotype effect in the %change analysis (D) and significant genotype × time interaction effect in the raw longitudinal analysis (C). A RM MANOVA was used to determine the listed P values for the raw data (A and C), and a 2-W-ANOVA was used to determine the listed P values. A Tukey’s honestly significantly different (HSD) post hoc test to determine significant differences between the experimental groups was performed on the %change data in B.
analyses of the longitudinal raw and percent change data (Fig. 5, A and B). Disabling RAGE significantly decreased the rate of weight gain, but PTH had no significant impact on body weight or its rate of increase in either genotype (Fig. 5, C and D).

The absolute differences in raw BMD and BMC between 
Rage-KO and WT mice appeared to depend on the bone site and age of the animals, the result of site-specific differences in the rate of accrual of bone mass. Similarly, although the rate of weight gain was lower in the female null mice, they were

Table 1. Values for cortical architecture at the midshaft femur obtained with μCT from 17-wk-old female mice treated with hPTH-(1–34) 30 μg·kg⁻¹·day⁻¹ or VEH control for 7 wk (10–17 wk of age)

<table>
<thead>
<tr>
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<th>Treatment</th>
<th>2-Way ANOVA P Values</th>
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Data are means ± SD, μCT, microcomputed tomography; hPTH-(1–34), human parathyroid hormone-(1–34); WT, wild type; KO, knockout; VEH, vehicle; PTH, parathyroid hormone; T HSD, Tukey’s honestly significantly different post hoc test; MA, marrow area; CA, cortical area; TA, total area; IMAX, IMIN, and J, maximum, minimum, and polar moments of inertia, respectively. The 4 treatment groups (n = 7–8 mice/group) were 1) VEH-treated WT (WT VEH), 2) PTH-treated WT (WT PTH), 3) VEHTreated receptor for advanced glycation end products (Rage)-KO (KO VEH), and 4) PTH-treated Rage-KO (KO PTH). The parameters were CA (mm²), MA (mm²), and TA (mm²) and the IMAX, IMIN, and J (mm⁴). A 2-factor ANOVA was used to evaluate the impact of genotype and treatment on the individual parameter. The 2-factor ANOVA indicated a significant genotype × treatment interaction for MA; therefore, we used a T HSD to determine significant differences between the experimental groups. Groups not connected by the same letter are significantly different.

Fig. 7. Female Rage-KO mice gain less femoral cancellous bone than WT animals in response to anabolic PTH. Microcomputed tomography (μCT)-acquired femoral trabecular architecture, including bone volume per total volume (BV/TV; A), connectivity density (Conn D, mm⁻³; B), structure model index (SMI; C), trabecular number (Tb N, mm⁻¹; D), trabecular thickness (Tb Th, mm; E), and trabecular spacing (Tb Sp, mm; F), was compared between WT and Rage-KO 17-wk-old animals that had been treated with VEH or hPTH-(1–34) 30 μg·kg⁻¹·day⁻¹ for 48 days. The 4 treatment groups (n = 7–8/group) were 1) VEH-treated WT, 2) PTH-treated WT, 3) VEH-treated Rage-KO, and 4) PTH-treated Rage-KO. PTH enhanced BV/TV and Conn D in the WT mice but not in the null mice. Hormone treatment increased Tb N in WT mice to a greater extent than in Rage-KO animals. Tb Th, Tb Sp, and SMI were equally responsive to hormones in both genotypes. There were no differences in any of these parameters between the VEH-treated WT and Rage-KO 17-wk-old mice. To evaluate the early hormone response, we compared bones from 12-wk-old WT and Rage-KO mice that had been treated with VEH or PTH for 14 days; the same experimental design was used (4 treatment groups, 7–8 mice/group). Tb Th was equally enhanced in both genotypes after 14 days of PTH, but no hormone response was observed in the other parameters. The 12-wk-old WT mice exhibited an enhanced trabecular architecture (BV/TV, Conn D, Tb N, Tb Th, and Tb Sp) compared with Rage-KO animals. The listed P values were determined with a 2-W-ANOVA. A Tukey’s HSD post hoc test was used to determine differences between the experimental groups if a significant genotype × treatment interaction was indicated.
actually heavier than the WT controls during the experimental period. We compare absolute differences in skeletal parameters between WT and null mice with caution since the animals were not littermates. However, our experimental design accounted for any differences in genetic and environmental factors; i.e., the genotype × treatment (difference in WT and null response to PTH) and genotype × time (difference in WT and null rate of bone or fat accrual) terms were independent of such influences. Nevertheless, the lower rate of bone accrual is consistent with the modest but significantly decreased femur length (Fig. 6) and attenuated midshaft cortical geometry (Table 1; also see below).

Rage-KO mice were refractive to PTH-induced expansion of the marrow area at the midshaft femur. Next we examined the impact of anabolic PTH on midshaft femur cortical bone geometry; bones were obtained from the 17-wk-old WT and Rage-KO mice of the four treatment groups and analyzed by µCT. Hormone treatment enhanced marrow area only in the WT mice; i.e., there was a significant genotype × treatment interaction (Table 1). Although PTH treatment enhanced the cortical area and total area of the midshaft femur in both genotypes, the lack of hormone-mediated medullary area expansion in the null mice resulted in a larger increase in cortical area in these animals that approached significance as reflected by the genotype × treatment interaction value of $P = 0.0773$ (Table 1). The maximum, minimum, and polar moments of inertia ($I_{\text{MAX}}$, $I_{\text{MIN}}$, and $J$) were responsive to hormones in both genotypes (Table 1). Finally, the Rage-null mice exhibited significantly attenuated values for nearly all of the midshaft femur parameters (genotype effect, $P < 0.05$), which is consistent with the lower rate of bone accrual in the femur.

Rage-KO mice exhibited a significantly diminished PTH-induced acquisition of femoral but not vertebral trabecular bone. We further examined the bones from the 17-wk-old WT and Rage-KO mice of the four treatment groups to compare the PTH response of the femoral trabecular compartment. The parameters of %BV/TV (Fig. 7A), Conn D (mm$^{-3}$; Fig. 7B), SMI (Fig. 7C), Tb N (mm$^{-1}$; Fig. 7D), Tb Th (mm; Fig. 7E), and Tb Sp (mm; Fig. 7F) were evaluated using µCT. Hormone treatment for 48 days enhanced BV/TV and Conn D in the WT mice but did not significantly increase these parameters in the null mice, resulting in strong genotype × treatment interactions for these parameters (Figs. 7, A and B). Similarly, anabolic PTH increased Tb N in WT mice to a greater extent than in Rage-KO animals (Fig. 7D). Interestingly, the parameters of SMI, Tb Th, and Tb Sp were equally responsive to hormone in both genotypes (Fig. 7, C, E, and F, respectively).

To characterize the early hormone response of the femoral trabecular compartment, we compared bones from 12-wk-old WT and Rage-KO mice that had been treated with vehicle or PTH for 14 days; the same experimental design was used (4 treatment groups, 7–8 mice/group; Fig. 7). The Tb Th was equally enhanced in both genotypes after 14 days of PTH, but no hormone response was observed in the other parameters this early in the treatment (Fig. 7). The Rage-KO mice had less femoral trabecular bone than the WT animals (genotype effect for BV/TV, Conn D, Tb N, Tb Th, and Tb Sp; Fig. 7) at 12 wk of age, but there were no differences by 17 wk of age, indicating that in the absence of anabolic hormone treatment the younger female Rage-KO mice exhibited deficits in the femoral trabecular compartment that were normalized as they aged. The typical µCT scans of bones from 17-wk-old mice of the four treatment groups are shown in Fig. 8.

Disabling RAGE did not impair PTH responsiveness of vertebral cancellous bone. We used µCT analysis of the L5 vertebra to compare %BV/TV (Fig. 9A), Conn D (mm$^{-3}$; Fig. 9B), SMI (Fig. 9C), Tb N (mm$^{-1}$; Fig. 9D), Tb Th (mm; Fig. 9E), and Tb Sp (mm; Fig. 9F) between WT and Rage-KO 17-wk-old animals from our four treatment groups. Tb Sp was the only parameter significantly responsive to 48 days of hormone treatment, but there was no genotype × treatment interaction; i.e., both WT and null mice responded comparably to PTH (Fig. 9F). Despite this equivalent Tb Sp response to hormone, all vertebral parameters appeared more sensitive to PTH in the null mice but not to such a degree as to produce a significant treatment effect or genotype × treatment interaction (Fig. 9). To evaluate the early hormone response, we compared bones from 12-wk-old WT and Rage-KO mice that had been treated with vehicle or PTH for 14 days, as described above. As was observed in the 17-wk-old mice treated with hormone for 48 days, Tb Sp was equally responsive to hormone in 12-wk-old WT and null mice after only 14 days of treatment (Fig. 9F). Interestingly, Conn D was significantly increased in response to PTH after 14 days of treatment in both WT and Rage-KO...
mice (treatment, \( P = 0.0162 \)), although the null mice were modestly but not significantly more sensitive to hormone (genotype \( \times \) treatment, \( P = 0.0819 \); Fig. 9B). Finally, the Rage-KO mice (12 and 17 wk old) exhibited more vertebral trabecular bone than the WT mice (genotype effect for Bv/TV, Conn D, and Tb Sp; Fig. 9, A, B, and F, respectively). The typical \( \mu \)CT scans of the L5 vertebrae from the 17-wk-old mice of the four treatment groups are shown in Fig. 10.

Disabling RAGE attenuates the expression of genes supporting the osteoblast phenotype but not their PTH responsiveness. To evaluate the impact of RAGE on the initial PTH response of genes that support the osteoblast phenotype, we analyzed femoral RNA from the bones of 12-wk-old female Rage-KO and WT mice that had been treated with vehicle or hPTH-(1–34) 30 \( \mu \)g/kg \( \times \) day \(-1\) for 14 days; RNA was harvested 24 h after the last PTH/vehicle injection. The four treatment groups (\( n = 4–6/\text{group} \)) were 1) vehicle-treated WT, 2) PTH-treated WT, 3) vehicle-treated Rage-KO, and 4) PTH-treated Rage-KO. Compared with the WT mice, the Rage-null animals showed a significant attenuated expression (strong genotype effect) of the genes that support osteoblast differentiation and bone formation, including alkaline phosphatase (Fig. 11A), the pro-\( \alpha \)-I (I) chain (Col1a1; Fig. 11B), Runx2 (Fig. 11C), and osteonectin (Fig. 11D). However, these genes exhibited a strong treatment effect but no genotype \( \times \) treatment interaction, indicating that PTH responsiveness of these genes was not compromised in the null mice. The Rankl/Opg ratio, osteoblast-derived cytokines involved in regulating osteoclastogenesis, showed no genotype or treatment effects (Fig. 11E). The transcription factors Lef1 and Nmp4/CIZ, associated with bone formation and inhibition of bone formation (30), respectively, both exhibited a significant decrease in expression but no response to hormone, although the treatment effect for Nmp4/CIZ was nearly significant (\( P = 0.0771 \); Fig. 11F and G).

Finally, the expression of Fabp4, a marker for mature adipocyte function (18), was not decreased in the null mice (no genotype effect; Fig. 11H). Although Fabp4 expression appeared to be more sensitive to hormone (B and F). The 12-wk-old Rage-KO mice exhibited an enhanced vertebral trabecular architecture compared with the WT mice (genotype effect for BV/TV, Conn D, and Tb Sp). To evaluate the early hormone response, we compared bones from 12-wk-old WT and Rage-KO mice that had been treated with vehicle or PTH for 14 days using the same experimental design (4 treatment groups, 7–8 mice/group). Both Conn D and Tb Sp were responsive to hormone (B and F). The 12-wk-old Rage-KO mice exhibited an enhanced vertebral trabecular architecture compared with the WT mice (genotype effect for BV/TV, Conn D, and Tb Sp; A, B, and F, respectively). The listed \( P \) values were determined with a 2-W-ANOVA.
nulls. However, the male null mice exhibited a significantly attenuated femoral trabecular architecture compared with WT mice at both 12 and 28 wk of age (Table 2). Thus, unlike the female null mice, the development of femoral cancellous bone in the male Rage-KO animals did not ultimately match the WT architecture as the animals aged. Additionally, in contrast to the female null mice, the male Rage-KO animals showed a spare vertebral trabecular architecture compared with the WT male mice, although the comparative differences between the measured parameters were rarely statistically significantly different (Table 2).

DISCUSSION

Intermittent PTH treatment increases cortical bone thickness and improves trabecular architecture. This occurs via hormone-induced remodeling, a process by which osteoclast-mediated resorption precedes osteoblast-mediated formation at the same skeletal site or bone multicellular unit. Modeling also contributes to the anabolic response and is a mechanism whereby resorption and formation take place independently at different bone sites (1, 29). The enhanced cortical thickness is primarily the result of renewed modeling that restores periosteal and/or endocortical apposition (29). Improvements in cancellous architecture include increased trabecular connectivity, thickening of the trabeculae, and converting the trabeculae from a more rod-like to a more normal plate-like appearance (1, 29). These hormone-stimulated improvements in cancellous architecture are the result of both renewed apposition of bone on preexisting architecture (modeling) and an increase in the frequency of bone remodeling associated with a positive balance; i.e., the amount of bone laid down at each bone multicellular unit is enhanced (1, 29). This PTH-induced remodeling of cancellous bone may include osteoclast-mediated longitudinal tunneling of thickened individual trabeculae, which converts them into multiple trabeculae, increasing Conn D and Tb N and in some cases normalizing Tb Th; this remodeling phenomenon has been reported in humans, monkeys, dogs, and C57BL/6 mice (6, 9, 14, 16). The initiation of remodeling is dependent on osteoclast activation, but both remodeling and modeling involve the recruitment of osteoprogenitor cells, mobilization of osteoblasts to bone surfaces, and stimulation of osteoblast differentiation and apposition of matrix along either existing bone surfaces or recently excavated bone surfaces (14, 17, 21, 23).

The present data demonstrate that RAGE is necessary for certain key aspects of the skeleton’s response to anabolic PTH. Specifically, RAGE is required for hormone-mediated improvement of femoral trabecular architecture but not intrinsically necessary for increasing cortical bone thickness. Bone histomorphometric analysis of specimens taken throughout the treatment regimen is necessary to clarify the cellular basis of RAGE action, and this is underway.

Four intriguing results from our data include the following observations. 1) The rate of bone accrual was significantly lower in the null mice compared with the WT animals, but the rate of fat accrual was comparatively enhanced in the nulls; 2) the PTH-induced relative increases in skeletal BMD and BMC (measurements primarily for cortical bone) were unaffected by the absence of RAGE; 3) cortical bone geometry of the midshaft femurs was attenuated in the null mice, as was femur length, but the PTH-induced increase in cortical area was proportionate in WT and null mice; and 4) in vivo femoral gene profiles showed a decreased expression of genes that support the osteoblast phenotype in the Rage-KO mice, but expression of femoral Fabp4, a mediator of fatty acid metabolism and marker for mature adipocytes (18), was unimpaired. Leading our ongoing histomorphometric analyses is the hypothesis that these observations are part of the same phenomenon, i.e., RAGE ablation undermines some aspects of PTH-induced osteoblast mobilization and differentiation. Moreover, the decreased rate of bone accrual coupled to the increased rate of fat accrual in the null mice raises the question as to whether disabling RAGE shifts the balance between bone and fat, i.e., between osteoblasts and adipocytes. A reciprocal interdependence between osteoblastogenesis and adipogenesis is well established, although the complex interrelationships between bone density, marrow adiposity, and peripheral fat stores remain to be elucidated (Ref. 24 and references therein), and we are currently investigating these relationships in the Rage-KO mice. Adipogenesis may be the default lineage fate in the absence of RAGE. The nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) drives adipogenesis and suppresses osteoblastogenesis partly through inhibiting the function of Runx2, resulting in a reduced number of osteoblasts in the bone marrow (19); indeed, we observed that Runx2 expression is reduced in the null mice. Interestingly, PPARγ also attenuates RAGE expression (34), but whether this is critical to its regulation of osteoblast number remains to be determined. Finally, further work is needed to explain our intriguing observation that anabolic PTH suppresses the accrual of fat in the null but not the WT mice.
The most striking effect of disabling RAGE on the anabolic response was the impairment of PTH-induced improvement of femoral trabecular architecture. Hormone treatment failed to significantly enhance trabecular BV/TV or Conn D and produced only a small increase in Tb N in the Rage-KO mice; however, hormone-mediated increases in Tb Th and Tb Sp were intact in the null mice. This raises the question as to whether PTH-induced osteoclast-mediated intratrabecular tunneling, which enhances Conn D and Tb N, was defective in the absence of RAGE. Histomorphometric assessment may clarify whether PTH-stimulated recruitment and activation of Rage-KO osteoblasts and osteoclasts to the femoral trabecular compartment were insufficient to enhance BV/TV but adequate for adding bone to existing trabeculae, thus not impacting changes in hormone-induced increases in Tb Th and Tb Sp.

Fig. 11. Disabling RAGE attenuates the expression of genes supporting the osteoblast phenotype but not their PTH responsiveness. The PTH-stimulated responses of the genes alkaline phosphatase (Alk phos; A), the pro-alpha(I) chain (Col1a1; B), Runx2 (C), osterix (D), receptor activator of NF-kB (Rankl)/Opg (E), Lef1 (F), nuclear matrix protein-4/cas interacting zinc finger protein (Nmp4/CIZ; G), and fatty acid-binding protein 4 (Fabp4; H) were evaluated using qRT-PCR analysis of femoral RNA isolated from 12-wk-old female Rage-KO and WT mice that had been treated with vehicle or hPTH(1–34) 30 μg·kg⁻¹·day⁻¹ for 14 days; RNA was harvested 24 h after the last PTH/vehicle injection. The four treatment groups (n = 4–6/group) were 1) VEH-treated WT, 2) PTH-treated WT, 3) VEH-treated Rage-KO, and 4) PTH-treated Rage-KO. Rage-KO mice exhibited a significantly attenuated expression of Alk phos, Col1a1, Runx2, osterix, Lef1, and Nmp4/CIZ compared with WT mice (genotype effect), but those genes showing a treatment effect showed no genotype × treatment interaction, suggesting that PTH-responsiveness of Rage-KO osteoblasts is intact. A 2-W-ANOVA was used to determine the listed P values.

The observed weak response of the rodent spine to PTH, compared with that of the femur, is consistent with previous observations on 10- to 17-wk-old C57BL/6 mice (15). Interestingly, this hormone-driven decrease in femoral SMI was attenuated in the Rage-KO mice, and the genotype × treatment interaction term approached significance. Bone histomorphometry may be able to determine whether this apparent nominally enhanced hormone sensitivity in the null spine results from PTH-induced growth on the greater number...
of preexisting trabeculae present at the beginning of the treatment. This would be consistent with the observed PTH-induced appositional growth of the highly trabecularized human vertebra (28). It is not clear why the trabecular compartments of the femur and spine respond differently to PTH in the absence of RAGE, but there is precedent for this phenomenon. Mice with conditional osteoblast deletion of connexin 43 showed a site-specific impact on PTH responsiveness (7). The molecular basis of RAGE’s contribution to the PTH anabolic response remains to be elucidated but may involve this receptor’s interaction with HMGB1. This cytokine is required for endochondral bone formation and is a chemotactic agent for both osteoblasts and osteoclasts (33), consistent with our hypothesis that RAGE is critical for PTH-induced mobilization of these cells. The HMGB1-RAGE signaling axis supports osteoclastogenesis (37) and thus may contribute to the proposed deficits in PTH-mediated remodeling in the null mice. PTH governs the osteogenic release of HMGB1 via its regulation of apoptosis, a critical component to the anabolic response, and upon its release from these cells, HMGB1 in turn stimulates the secretion of the proresorbing cytokines RANKL, TNFα, and IL-6 from bone marrow stromal osteoblasts (36).

Our comprehensive longitudinal analysis of the Rage-KO mouse during anabolic PTH treatment reveals a more complex skeletal phenotype than reported previously (11, 38) and suggests deficits in both the osteogenic and osteoclastic arms of skeletal homeostasis, which is consistent with recent studies showing a role for RAGE in the development and activities of both osteoclasts and osteoblasts (11, 12, 26, 37, 38). Bone histomorphometry analysis is required to elucidate the cellular basis of RAGE’s role in the skeleton’s response to anabolic hormone. Clearly, RAGE’s impact on bone is site, age, and sex specific. This is not surprising in light of fact that RAGE is a multifunctional multiligand receptor expressed on numerous cell types. Our work suggests that RAGE functionality is not limited to inflammatory and other pathological pathways but has an important physiological role in normal skeletal biology.

Table 2. Comparison of male Rage-KO and WT phenotype

<table>
<thead>
<tr>
<th></th>
<th>12 Wk WT</th>
<th>12 Wk KO</th>
<th>23.5 Wk WT</th>
<th>23.5 Wk KO</th>
<th>28 Wk WT</th>
<th>28 Wk KO</th>
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<tr>
<td>DEXA BMD/BMC</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>WB BMD</td>
<td>0.0492 ± 0.0018</td>
<td>0.0471 ± 0.0014*</td>
<td>0.0533 ± 0.0030</td>
<td>0.0496 ± 0.0023*</td>
<td>0.0545 ± 0.0031</td>
<td>0.0502 ± 0.0024*</td>
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<tr>
<td>WB BMc</td>
<td>0.4180 ± 0.0354</td>
<td>0.3411 ± 0.0128*</td>
<td>0.4437 ± 0.0377</td>
<td>0.3593 ± 0.0242*</td>
<td>0.4346 ± 0.0362</td>
<td>0.3717 ± 0.0251*</td>
</tr>
<tr>
<td>Femur BMD</td>
<td>0.0756 ± 0.0036</td>
<td>0.0659 ± 0.0029*</td>
<td>0.0736 ± 0.0080</td>
<td>0.0690 ± 0.0052*</td>
<td>0.0754 ± 0.0062</td>
<td>0.0699 ± 0.0039*</td>
</tr>
<tr>
<td>Femur BMC</td>
<td>0.0330 ± 0.0027</td>
<td>0.0266 ± 0.0018*</td>
<td>0.0316 ± 0.0040</td>
<td>0.0289 ± 0.0033*</td>
<td>0.0349 ± 0.0043</td>
<td>0.0290 ± 0.0030*</td>
</tr>
<tr>
<td>Tibia BMD</td>
<td>0.0531 ± 0.0031</td>
<td>0.0466 ± 0.0014*</td>
<td>0.0561 ± 0.0040</td>
<td>0.0495 ± 0.0029*</td>
<td>0.0539 ± 0.0043</td>
<td>0.0507 ± 0.0046</td>
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<tr>
<td>Tibia BMC</td>
<td>0.0306 ± 0.0026</td>
<td>0.0253 ± 0.0017*</td>
<td>0.0308 ± 0.0034</td>
<td>0.0286 ± 0.0033*</td>
<td>0.0328 ± 0.0031</td>
<td>0.0285 ± 0.0028*</td>
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<td>Spine BMD</td>
<td>0.0566 ± 0.0041</td>
<td>0.0572 ± 0.0030</td>
<td>0.0598 ± 0.0055</td>
<td>0.0567 ± 0.0048</td>
<td>0.0619 ± 0.0040</td>
<td>0.0543 ± 0.0026*</td>
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<tr>
<td>Spine BMC</td>
<td>0.0244 ± 0.0026</td>
<td>0.0213 ± 0.0013*</td>
<td>0.0251 ± 0.0038</td>
<td>0.0211 ± 0.0026*</td>
<td>0.0253 ± 0.0022</td>
<td>0.0212 ± 0.0019*</td>
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<tr>
<td>%Fat</td>
<td>13.34 ± 1.21</td>
<td>18.28 ± 2.27*</td>
<td>16.08 ± 3.87</td>
<td>25.10 ± 4.43*</td>
<td>17.23 ± 2.94</td>
<td>23.48 ± 4.19*</td>
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<tr>
<td>Weight</td>
<td>28.16 ± 1.71</td>
<td>24.84 ± 1.43*</td>
<td>31.51 ± 1.86</td>
<td>29.62 ± 2.76*</td>
<td>29.51 ± 1.87</td>
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<tr>
<td>Femur length</td>
<td>15.70 ± 0.32</td>
<td>14.95 ± 0.39*</td>
<td></td>
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<td>16.34 ± 0.37</td>
<td>15.53 ± 0.37*</td>
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<tr>
<td>µCT femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BV/TV</td>
<td>0.0662 ± 0.0185</td>
<td>0.0232 ± 0.0074*</td>
<td></td>
<td></td>
<td>0.0514 ± 0.0214</td>
<td>0.0222 ± 0.0079*</td>
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<tr>
<td>Conn D</td>
<td>8.71 ± 7.13</td>
<td>2.28 ± 2.55*</td>
<td></td>
<td></td>
<td>14.6675 ± 10.1360</td>
<td>2.2750 ± 2.4659*</td>
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<tr>
<td>SMl</td>
<td>2.96 ± 0.23</td>
<td>3.60 ± 0.26*</td>
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<td></td>
<td>0.286 ± 0.3679</td>
<td>0.331 ± 0.2499*</td>
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<tr>
<td>Tb N</td>
<td>2.57 ± 0.29</td>
<td>1.97 ± 0.26*</td>
<td></td>
<td></td>
<td>0.253 ± 0.3572</td>
<td>1.91 ± 0.3586*</td>
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<tr>
<td>Tb Th</td>
<td>0.0553 ± 0.0065</td>
<td>0.0428 ± 0.0028*</td>
<td></td>
<td></td>
<td>0.0487 ± 0.0049</td>
<td>0.0460 ± 0.0047</td>
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<tr>
<td>Tb Sp</td>
<td>0.3980 ± 0.0460</td>
<td>0.5178 ± 0.0695*</td>
<td></td>
<td></td>
<td>0.4036 ± 0.0614</td>
<td>0.5418 ± 0.1019*</td>
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<tr>
<td>µCT L5</td>
<td></td>
<td></td>
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<tr>
<td>BV/TV</td>
<td>0.2041 ± 0.0538</td>
<td>0.1640 ± 0.0246</td>
<td></td>
<td></td>
<td>0.1814 ± 0.0544</td>
<td>0.1541 ± 0.0362</td>
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<tr>
<td>Conn D</td>
<td>154.97 ± 35.61</td>
<td>128.21 ± 40.92</td>
<td></td>
<td></td>
<td>123.53 ± 24.96</td>
<td>87.73 ± 50.81</td>
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<tr>
<td>SMl</td>
<td>2.32 ± 0.44</td>
<td>2.79 ± 0.27*</td>
<td></td>
<td></td>
<td>0.209 ± 0.38</td>
<td>0.357 ± 0.37*</td>
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<tr>
<td>Tb N</td>
<td>9.00 ± 0.23</td>
<td>10.18 ± 0.24</td>
<td></td>
<td></td>
<td>10.08 ± 0.36</td>
<td>10.22 ± 0.41</td>
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<tr>
<td>Tb Th</td>
<td>0.0482 ± 0.0051</td>
<td>0.0429 ± 0.0021*</td>
<td></td>
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<td>0.0478 ± 0.0050</td>
<td>0.0436 ± 0.0054</td>
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<tr>
<td>Tb Sp</td>
<td>0.1147 ± 0.0056</td>
<td>0.1107 ± 0.0015</td>
<td></td>
<td></td>
<td>0.1159 ± 0.0033</td>
<td>0.1188 ± 0.0048</td>
</tr>
</tbody>
</table>

Data are means ± SD. DEXA, dual-energy X-ray absorptiometry; BMD, bone mineral density; BMC, bone mineral content; BV/TV, bone volume to total volume; Conn D, connectivity density; SMl, structure model index; Tb N, trabecular number; Tb Th, trabecular thickness; Tb Sp, trabecular spacing. DEXA (BMC and BMD) results at 12 (n = 15 KO/10 WT), 23.5 (n = 34 KO/18 WT), and 28 wk of age (n = 16 KO/10 WT). Femur length at 12 (n = 13 KO/10 WT) and 28 wk of age (n = 19 KO/10 WT). µCT of femoral and vertebral (L5) parameters in 12-wk-old femur (n = 19 KO/10 WT), 23.5-wk-old femur (n = 20 KO/10 WT), 12-wk-old L5 (n = 7 KO/6 WT), and 28-wk-old L5 (n = 20 KO/10 WT). A t-test was used to determine significance; *t < 0.05.
DISCLOSURES
No conflicts of interest are declared by the author(s).

REFERENCES