Do Cyclooxygenase-2 Knockout Mice Have Primary Hyperparathyroidism?

Manshan Xu, Shilpa Choudhary, David Goltzman, Felicia Ledgard, Douglas Adams, Gloria Gronowicz, Boguslawa Kocecz-Jaremko, Lawrence Raisz, and Carol Pilbeam

There are two enzymes for cyclooxygenase (COX) encoded by separate genes, COX-1 and COX-2 (1–3). In most tissues, COX-1 is constitutively expressed, whereas COX-2 is transiently induced. COX-2 is highly expressed in bone and is regulated by multiple local and systemic factors important in bone metabolism (4). In the absence of COX-2, little prostaglandin E2 (PGE2) is produced by cultured osteoblasts or cultured bone marrow cells (5–7). Agonists shown to induce COX-2 expression and PGE2 production in osteoblastic cells include cytokines [IL-1 (8–10), TNF-α (11), and IL-6 (12)], growth factors [TGF-α (13, 14), TGF-β (14), bone morphogenetic protein-2 (6), and basic fibroblast growth factor (15)], fetal bovine serum (16), systemic hormones [PTH (8, 17) and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (5)], extracellular calcium (7), and fluid shear stress or mechanical loading (18–20). PGs themselves induce COX-2 expression and therefore can amplify PG responses to other agonists (21).

PGs produced by COX-2 can be potent stimulators of both bone resorption and bone formation (4). In addition, the many factors that induce COX-2 expression generally have their own independent effects on bone turnover. It is therefore difficult to predict the effects of COX-2 expression on bone mass in vivo. Several studies using mice with disruption of the COX-2 gene, COX-2 knockout (KO) mice, have suggested that bone mass or density is decreased in COX-2 KO relative to WT mice (22, 23). However, it is unclear whether COX-2 KO mice can be used for studying the direct effects of COX-2 absence on bone turnover. The developers of COX-2 KO mice reported that KO mice have nephropathy severe enough to decrease the average life span to approximately 3–4 months (24, 25). A recent study reported that COX-2 KO mice have progressive renal deterioration, with 20% of mice dying between 7–23 wk of age (26). Loss of functioning renal mass in mice, as in humans, is expected to decrease 1,25(OH)2D3 production, thereby decreasing serum calcium levels, and decreased serum calcium levels are expected to increase PTH secretion. This secondary hyperparathyroidism could increase bone turnover, making it difficult to separate out the direct effects of the absence of COX-2 on bone from those secondary to nephropathy.

In the present study we studied differences in skeletal phenotype due to COX-2 absence, apart from renal dysfunction if possible. Although COX-2 KO mice did die faster than
WT mice, renal failure did not appear to be universal. Initially, we killed mice at 4 months of age and found several with renal failure associated with marked secondary hyperparathyroidism, whereas others appeared relatively healthy. On the assumption that most COX-2 KO mice with progressive renal dysfunction would die within the first 6–8 months of life and that phenotypic differences between COX-2 WT and KO mice might be most evident in older mice, we then studied 10-month-old mice. Our results suggest that COX-2 KO mice without functional renal deterioration have primary hyperparathyroidism with increased levels of 1,25(OH)2D3. The skeletal differences between COX-2 WT and KO mice are small, and we speculate that the effects of hyperparathyroidism on bone turnover compensate for the absence of COX-2 in bone.

**Materials and Methods**

**Animals**

The COX-2 KO mice used in this study were developed at University of North Carolina on a C57BL/6, 129SV background (25). Because COX-2 KO females are infertile (27) and because we wanted to obtain both WT and KO in the same litter, we bred COX-2 WT and KO mice for experiments by mating mice heterozygous for the disrupted COX-2 allele (COX-2 HET mice). To establish founders for the breeding colonies, we backcrossed the original C57BL/6, 129SV HET mice onto a pure C57BL/6 background (backcrossed more than nine generations). Colonies were maintained by mating the C57BL/6 HET founders with C57BL/6 WT mice from The Jackson Laboratory (Bar Harbor, ME), which were refreshed every 6 months to avoid genetic drift. We tried to generate COX-2 KO mice by crossing COX-2 HET mice on a C57BL/6 background, but found few or no KO mice in the litters. A similar observation was made by Dr. Scott Morham (personal communication), who developed the COX-2 KO mice. Hence, the C57BL/6-COX-2 HET mice were outbred to 129P3/J WT mice to maintain the production of COX-2 HET mice. To establish founders for the breeding colonies, we backcrossed the original C57BL/6, 129SV HET mice onto a pure C57BL/6 background (backcrossed more than nine generations). Colonies were maintained by mating the C57BL/6 HET founders with C57BL/6 WT mice from The Jackson Laboratory (Bar Harbor, ME), which were refreshed every 6 months to avoid genetic drift. We tried to generate COX-2 KO mice by crossing COX-2 HET mice on a C57BL/6 background, but found few or no KO mice in the litters. A similar observation was made by Dr. Scott Morham (personal communication), who developed the COX-2 KO mice. Hence, the C57BL/6-COX-2 HET mice were outbred to 129P3/J WT mice to maintain the production of KO mice. The final breeding protocol for COX-2 KO and WT mice used in this study is shown in Fig. 1. Mice were genotyped as described previously (5). All animal protocols were approved by the animal care and use committee of University of Connecticut Health Center (Farmington, CT).

**Analytical procedures on serum**

Mice were euthanized at 4 months (±2 wk) or 10 months (±3 wk) of age. Mice were killed in groups containing eight or more mice of both genotypes upon reaching the appropriate age. Blood was obtained by cardiac puncture at the time of death after anesthesia with gaseous carbon dioxide. Blood was not fasted. Blood was allowed to clot at room temperature, and the serum was removed after centrifugation of samples at 3000 rpm for 20 min. Serum from each animal was divided into aliquots and frozen at −80 °C. There was no repeated freezing and thawing of serum samples before measurements.

Serum creatinine, phosphorus, and calcium measurements were measured using kits (procedures 555, 350-UV, and 587, respectively) from Sigma-Aldrich Corp. (St. Louis, MO) following the manufacturer’s directions. Five microliters of serum were needed to assay calcium or phosphorus, and 30 μl serum were needed to assay creatinine. PTH was assayed on 25 μl serum using a Mouse Intact PTH ELISA Kit from Immunotopics (San Clemente, CA). This is a two-site assay, recognizing epitopes within the midregion/C-terminal portion and epitopes within the N-terminal portion of the PTH peptide. The manufacturer reports an intraassay coefficient of variation (CV) of 2.5–3.9% and an interassay CV of 7.8–8.9%. 1,25(OH)2D3 was assayed on 100 μl serum by immunoelectrotransfer followed by 125I RIA using a kit from Immunodiagnostics Systems (IDS Ltd., Tyne and Wear, UK). The manufacturer reports an intraassay CV of 5–8% and an interassay CV of 9–10%.

**Skeletal imaging**

Femurs and lumbar vertebrae were dissected free of connective tissue at the time of death and stored in 70% ethanol. Areal bone mineral density (BMD) and bone mineral content (BMC) of the femur and of the first through fifth lumbar vertebrae (L1–L5) were measured using a PIXImus2 densitometer (LUNAR, GE Medical Systems, Madison, WI). Trabecular morphology within the metaphyseal region of the distal femur and the centrum of the third lumbar vertebra (L3) was quantified using x-ray microcomputed tomography (μCT20, Scanco Medical AG, Bassersdorf, Switzerland). Volumetric analysis regions for measuring trabecular bone volume fraction (BV/TV) were selected within the endosteal borders to include the central 80% of vertebral height and the secondary spongiosa of femoral metaphyses located 760 μm (~5% of length) distal to the physis.

**Bone histomorphometry**

For static histomorphometry, femurs were fixed in 4% paraformaldehyde at 4 °C, decalcified in 15% EDTA, dehydrated in progressive concentrations of ethanol, cleared in xylene, and embedded in paraffin. Five-micrometer sections were cut, stained with tartrate-resistant acid phosphatase to visualize osteoclasts, and counterstained with hematoxylin. Measurements included cortical width, percent BV/TV, percent osteoblast surface, percent osteoclast surface, and trabecular spacing and number.

For dynamic histomorphometry, mice received calcine (Sigma-Aldrich Corp.; 10 mg/kg body weight) 7 d before death and xylenol orange (Sigma-Aldrich Corp.; 90 mg/kg body weight) 2 d before death, administered ip. Femurs were dissected free of tissue and fixed in 70% ethanol at the time of death, then dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded undecalcified in methyl methacrylate. Five-micrometer-thick longitudinal serial sections were cut on a Fölycut S microtome (Reichert-Jung, Nussloch, Germany) with a D profile knife (Delaware Diamond Knives Corp., Wilmington, DE).

Both static and dynamic measurements were made in a blinded, nonbiased manner using the OsteoMeasure computerized image analysis system (OsteoMetrics, Inc., Atlanta, GA) interfaced with an Optiphoto Nikon microscope (Nikon, Inc., Melville, NY). The terminology and units used are those recommended by the histomorphometry nomenclature committee of the American Society for Bone and Mineral Research (28). All measurements were confined to the growth plate/metaphyseal junction. Osteoblasts were identified as cuboidal cells lining the trabecular perimeter. Osteoclasts were identified as multinucleated cells on the trabecular bone surface. In static measurements, cortical width was measured in the diaphysis 4000 μm from the growth plate. Dynamic measurements included single and double-labeled surfaces, mineral apposition rate (MAR), and bone formation rate (BFR/BS). In addition, a mineral apposition rate of 0.3 μm/d was assigned to samples that had single labels, but no double labels (29).

**Renal histology**

Mouse kidneys were halved; fixed in 10% neutral-buffered formalin; processed through 70%, 95%, and absolute ethanol; embedded in a paraffin block; and sectioned at 5-μm intervals. The paraffin sections were deparaffinized in toluene and rehydrated through graded alcohols to distilled water, then stained with hematoxylin and eosin.
Mortality of COX-2 KO mice

Previous studies had suggested that renal abnormalities in COX-2 KO mice were present shortly after birth and resulted in death by 3–4 months of age (24–26). We wanted to study the COX-2 KO phenotype in mice without compromised renal function so that the effects of COX-2 disruption on skeletal phenotype could be studied without being complicated by the secondary hyperparathyroidism associated with renal failure. We hypothesized that COX-2 KO mice surviving to 4 months of age might be free of renal abnormalities.

When we found some 4-month-old KO mice with renal failure, we expanded the study to include mice killed at 9–10 months of age. Cohorts of COX-2 WT and KO mice were identified at 2 months of age and set aside to be killed at 4 or 10 months of age.

The estimated mortality in these cohorts from 2 months of age is shown in Table 1. Deaths were generally reported by the animal care facility, and animals were removed without additional investigation. The total numbers of mice do not reflect the distribution of COX-2 genotypes born because some mice died or were removed before 2 months of age. From these data, it is estimated that 0.5–1.5% of WT or HET mice died per month and 4–6% of KO mice died per month from 2–10 months of age. Hence, the death rate among KO mice was about 4 times greater than the rate among WT or HET mice. Contrary to our initial hypothesis, there was not a decrease in the death rate among KO mice after 4 months of age.

Results

Statistics

Analysis of data was performed using SigmaStat for Windows, version 2.03 (San Rafael, CA). When the data were distributed normally, means of groups were compared by ANOVA, with post-hoc testing of pairwise multiple comparisons by Bonferroni’s method. When the data failed to meet the normality test, Kruskal-Wallis ANOVA on ranks was performed, followed by Dunn’s test for all pairwise multiple comparisons.

TABLE 1. Deaths in mice with both COX-2 alleles intact (WT), one COX-2 allele disrupted (HET), or both COX-2 alleles disrupted (KO)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>A) 2–4 Months</th>
<th>B) 4–10 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2 WT</td>
<td>3/114 (3)</td>
<td>4/58 (7)</td>
</tr>
<tr>
<td>COX-2 HET</td>
<td>1/73 (1)</td>
<td>4/79 (5)</td>
</tr>
<tr>
<td>COX-2 KO</td>
<td>6/78 (8)</td>
<td>14/41 (34)</td>
</tr>
</tbody>
</table>

Deaths were recorded in mice followed from about 2 months of age until mice were killed at 4 months of age and in mice from about 4 months of age until mice were killed at 10 months of age. Data are ratios of the deaths during the indicated time period to the total number of animals of that genotype being followed. The percentage is in parentheses.

Serum creatinine, calcium, phosphorus, and PTH levels in COX-2 WT and KO mice

Body weights and serum data for the 4-month-old mice are shown in Table 2. Two of the KO mice killed at 4 months of age had serum creatinine levels greater than 1.5 mg/dl. Data for these mice were excluded from the summary in Table 2 and are shown in Table 3. These mice looked sick at time of death. They had low body weight, low serum calcium, high serum phosphorus, and extremely high PTH levels. Thus, these mice appeared to have renal failure with secondary hyperparathyroidism.

After exclusion of these two mice, the mean serum creatinine level was still 30% higher in COX-2 KO males compared with WT males (Table 2). Fewer females were examined, and although not statistically significant, a similar trend toward higher serum creatinine was seen in female KO mice. PTH levels in KO mice also tended to be higher than those in WT mice, and this difference was statistically significant in female mice. It was difficult to explain the trend toward higher PTH levels in KO male mice as being due to renal failure in view of the low phosphorus level in KO males, but the role of renal dysfunction in the elevated PTH in KO females was unclear.

We examined gross renal histology from some healthy 4-month-old WT and KO mice. Although some KO kidneys had lymphocytic infiltrates not seen in WT kidneys, we found no differences in glomerular or tubular structures between WT and KO kidneys (data not shown). It seemed, therefore, that renal disease affecting COX-2 KO mice was not universal. Hence, we decided to examine older mice, with the hope that most or all KO mice surviving to 10 months of age might have intact renal function, and other metabolic abnormalities might become more evident.

Body weights and serum parameters in 10-month-old COX-2 WT and KO mice are shown in Table 4. Weights tended to be lower in KO mice compared with WT mice, but this difference was statistically significant only for females (8%). There was a nonstatistically significant trend for creatinine levels to be higher (10%) in KO mice compared with WT mice. Weights and creatinine levels were normally distributed around the medians, as indicated by the box plots shown in Fig. 2. Box plots for serum calcium, phosphorus, and PTH data are shown in Fig. 3. There was a small, but significant, increase (8%) in mean serum calcium in COX-2 KO male mice compared with WT male mice, but variability was also greater in KO mice (Fig. 3A). WT females had higher calcium and phosphorus levels than WT males, but there was no difference between WT and KO females. The mean PTH level was 50% higher in 10-month-old COX-2 KO males compared with WT males (Table 4). There was also a trend for female KO mice to have higher PTH levels than WT mice. Unlike the other serum parameters, PTH values for males, females, or both combined, as shown in Fig. 3C, were not normally distributed.

Only one mouse was excluded from the summary of mice killed at 10 months of age. This was a female mouse with a serum PTH level of 624 ng/ml, which was an outlier (>2 sd from the mean). Although this mouse had low body weight (19.6 g), the highest serum creatinine in the female KO group (0.81 mg/dl), and high phosphorus (12.4 mg/dl), consistent with renal dysfunction, its serum calcium (12.0 mg/dl) was high. Inclusion of this mouse in the summary data would have made the PTH difference between WT and KO females statistically significant. Regression of serum calcium (dependent variable) for 10-month-old male and female KO mice against PTH (independent variable) showed a small, significant, positive correlation (r = 0.35; P < 0.05; n = 43; data not shown). No other statistical significant correlations between
pairs of parameters or between weights and parameters were found.

**Serum 1,25(OH)₂D₃ levels in COX-2 WT and KO mice**

Overall, the serum data from the 10-month-old mice suggested primary, rather than secondary, hyperparathyroidism in COX-2 KO mice. In an effort to clarify this, we tested whether the KO mice had impaired 1,25(OH)₂D₃ synthesis, which is expected in chronic renal dysfunction, or increased 1,25(OH)₂D₃ levels, which is expected in primary hyperparathyroidism. We measured 1,25(OH)₂D₃ levels for all mice for 10-month-old males and females, KO data, and variability was high. In 4-month-old males and females, there was no difference between WT and KO mice, but there were few KO data, and variability was high. In 4-month-old males and 10-month-old males and females, KO mice had 1,25(OH)₂D₃ levels 1.8- to 2.3-fold greater than the WT mice.

Because we had no serum for measurement of 1,25(OH)₂D₃ levels from the few mice identified with definite renal dysfunction, we followed a small group of female mice as they continued to age, and when a KO mouse appeared to become sick at 12 months of age, we killed it. At the same time, we killed seven female WT mice ranging in age from 13–16 months. Serum from these mice was processed simultaneously, and results are shown in Table 6. The sick KO mouse had a serum creatinine level of 1.04, with lower body weight, lower serum calcium, and higher serum phosphorus than the WT mice. Although the sick KO mouse had an elevated PTH level, its calcium, and higher serum phosphorus than the WT mice.

The kidneys from this sick KO mouse were smaller than those from WT mice (Fig. 4). As we had noticed frequently in kidneys from 4- and 10-month-old KO mice that had no structural abnormalities (data not shown), the KO mouse had lymphocytic infiltrates especially evident around blood vessels (Fig. 4). There was also evidence for enlarged renal tubules and dilated calices in the sick KO mouse.

**Skeletal analysis of COX-2 WT and KO mice: imaging**

BMD was measured on dissected left femurs and lumbar vertebrae (L1–L5) from 4- and 10-month-old mice (Table 7). BMD tended to be lower in COX-2 KO mice compared with WT mice in all groups. The difference was statistically significant for 4-month-old female lumbar vertebral BMD (12%) and 10-month-old female femur BMD (9%). In some groups, vertebral (4-month-old female KO mice) and femoral (10-month-old WT females) BMDs were higher (6%) in females than in males. Lumbar BMD, but not femur BMD, decreased about 9% with age in WT mice, suggesting that trabecular, but not cortical, bone mass decreased with age.

The 10-month-old female COX-2 KO mouse, excluded from the summary analysis because of possible renal dysfunction, as discussed above, had low femur BMD (58.4 mg/cm²) compared with the remaining mice in this group (65.3 mg/cm²). Femur BMD was also measured in one of the 4-month-old KO mice excluded for renal failure, and this value (59.9 mg/cm²) was low compared with that in the remaining group (67.6 mg/cm²). These observations suggest that renal abnormalities associated with effects of COX-2 absence can decrease BMD.

**Skeletal analysis of COX-2 WT and KO mice: histomorphometry**

Static histomorphometric analyses on the distal femur showed decreased cortical width in 4-month-old male KO mice compared with WT (9%), but not in 10-month-old males (Table 9). There was also a significant decrease in cortical width in 10-month-old KO females compared with WT females (9%). No effect of genotype on percent BV/TV was seen. Female mice tended to have lower BV/TV than males in all groups, with a significant difference (35%) between WT

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**TABLE 2. Comparison of body weights and serum parameters in COX-2 WT and KO mice killed at 4 months of age**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>27.9 ± 0.6 (41)</td>
<td>26.8 ± 0.6 (31)</td>
<td>25.0 ± 0.7 (17)*</td>
<td>23.5 ± 0.8 (10)*</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.37 ± 0.03 (41)</td>
<td>0.49 ± 0.04 (31)*</td>
<td>0.32 ± 0.06 (17)</td>
<td>0.43 ± 0.06 (10)</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>10.5 ± 0.2 (41)</td>
<td>10.7 ± 0.2 (31)</td>
<td>10.8 ± 0.2 (17)</td>
<td>11.3 ± 0.3 (10)</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>10.1 ± 0.3 (41)</td>
<td>9.3 ± 0.3 (31)*</td>
<td>10.0 ± 0.5 (17)</td>
<td>10.7 ± 1.1 (10)*</td>
</tr>
<tr>
<td>PTH (ng/dl)</td>
<td>44.2 ± 5.4 (41)</td>
<td>51.6 ± 9.4 (31)</td>
<td>39.6 ± 3.8 (17)</td>
<td>118 ± 35.3 (10)*</td>
</tr>
</tbody>
</table>

Results for two 4-month-old KO mice with serum creatinine levels greater than 1.5 were excluded from this summary and are shown in Table 3. Data are the mean ± SEM for the number of mice in parentheses.

*P < 0.05, effect of gender.

**TABLE 3. COX-2 KO male mice, killed at 4 months of age, with apparent renal failure**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>18.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.64</td>
<td>5.14</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.79</td>
<td>8.41</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>18.5</td>
<td>25.0</td>
</tr>
<tr>
<td>PTH (ng/dl)</td>
<td>2393</td>
<td>4079</td>
</tr>
</tbody>
</table>
females and males at 10 months of age. There was a trend toward higher osteoblast and osteoclast surfaces in KO mice compared with WT mice. The difference (40–60%) was significant for percent osteoclast surface in females.

Dynamic histomorphometry of the distal femur is shown in Table 10. There was a significant increase of 55% in MAR and 70% in BFR/BS in 10-month-old KO male mice compared with WT mice, but not in female mice. Interestingly, both MAR and BFR/BS were 1.5- to 2.6-fold higher in females than in males at 10 months of age. This difference appeared to be largely due to the lack of age-related decreases in MAR and BFR/BS in females. There were no differences between genotypes in the other parameters measured (data not shown).

**Discussion**

We found no increase in mortality among COX-2 HET mice, but there was high mortality among COX-2 KO mice, as reported in other studies (24–26, 30). One study reported

**TABLE 4. Comparison of body weights and serum parameters measured in COX-2 WT and KO mice killed at 10 months of age**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>31.6 ± 0.5 (49)</td>
<td>30.8 ± 0.5 (24)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.41 ± 0.02 (49)</td>
<td>0.46 ± 0.03 (24)</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.7 ± 0.1 (49)</td>
<td>10.5 ± 0.3 (24)*</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>9.0 ± 0.2 (44)</td>
<td>9.6 ± 0.6 (20)</td>
</tr>
<tr>
<td>PTH (ng/dl)</td>
<td>49.6 ± 4.8 (49)</td>
<td>75.3 ± 10.6 (24)*</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM for the number of animals in parentheses.

* P < 0.01, effect of gender.

* P < 0.05, effect of gender.

Data are the mean ± SEM for the number of animals in parentheses.

* P < 0.01, effect of gender.

* P < 0.05, effect of gender.

* P < 0.05, effect of genotype.

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that 35% of COX-2 KO mice died with a patent ductus arteriosus within 48 h of birth (30). Other studies have not found a high incidence of neonatal death among COX-2 KO mice, but have found shortened life span secondary to renal dysplasia (25, 26). Renal development in COX-2 KO mice appeared normal until postnatal d 10, after which there was progressive renal architectural disruption and functional deterioration, and about 20% of KO mice died between 7 and 23 wk of age from renal failure (26). We found that approximately 40% of COX-2 KO mice died between 2 and 10 months of age. All of these studies used mice in a mixed C57BL/6 and 129 genetic background, and it is unclear what impact various degrees of inbreeding may have had on the results. For example, C57BL/6 mice lack type IIA secreted phospholipase A2; as a result, PG production associated with this enzyme is decreased (31). In COX-2 KO mice backcrossed into an outbred CD-1 background, our preliminary observations suggest that there is no increased mortality (data not shown). Hence, the severity of problems associated with the absence of COX-2 could be increased in a pure C57BL/6 genetic background, and this might account for our observation that backcrossing \textit{cox-2}\textsuperscript{-/-} onto a C57BL/6 genetic background resulted in decreased numbers of COX-2 KO mice in litters after weaning.

As expected, some COX-2 KO mice had evidence of renal tubular impairment at the time of death. These mice had decreased serum calcium, increased serum phosphorus, and markedly elevated PTH, similar to the changes seen in secondary hyperparathyroidism in humans (32–35), dogs (36), rats (37–41), and mice (42). The mechanisms proposed to account for secondary hyperparathyroidism in renal failure include 1) phosphorus retention, which inhibits the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) that converts 25(OH)\textsubscript{2}D\textsubscript{3} to 1,25(OH)\textsubscript{2}D\textsubscript{3} by 1α-hydroxylase that converts 25(OH)\textsubscript{2}D\textsubscript{3} to 1,25(OH)\textsubscript{2}D\textsubscript{3} as a result of phosphorus retention and decreased renal mass, 3) increased parathyroid hyperplasia due to phosphorus retention and reduced 1,25(OH)\textsubscript{2}D\textsubscript{3}, 4) resistance to the actions of 1,25(OH)\textsubscript{2}D\textsubscript{3} and PTH as a result of reduced numbers of vitamin D receptors and calcium sensors, and 5) subsequent decrease in serum calcium resulting from all of the above (32–35). Thus, decreased 1,25(OH)\textsubscript{2}D\textsubscript{3} should be an important factor in the development of secondary hyperparathyroidism. Although we could find no studies of 1,25(OH)\textsubscript{2}D\textsubscript{3} in renal failure in mice, there are several studies in rats showing the same relationships as those in humans (37, 38, 40, 41). We were able to measure serum 1,25(OH)\textsubscript{2}D\textsubscript{3} in one of the COX-2 KO mice with evident renal failure and found it to be very low compared with WT mice and KO mice without renal failure.

In both male and female COX-2 KO mice with relatively intact renal function, there was still a trend for mean PTH levels to be higher than levels in WT mice at all ages. This difference was statistically significant for 4-month-old KO female mice and 10-month-old male KO mice. In addition, in no group was the serum calcium level in KO mice lower than that in WT mice. Hence, the higher PTH levels in KO mice were not driven by decreased serum calcium, suggesting that the KO mice had primary hyperparathyroidism. Although serum calcium was only significantly elevated in 10-month-old male KO mice compared with WT mice, serum calcium could be said to be inappropriately high, perhaps corresponding to normocalcemic primary hyperparathyroidism in humans (43).

As another indication of whether the increased PTH levels were due to renal insufficiency or to primary hyperparathyroidism, we measured 1,25(OH)\textsubscript{2}D\textsubscript{3} levels. PTH stimulates the conversion of 25(OH)\textsubscript{2}D\textsubscript{3} to 1,25(OH)\textsubscript{2}D\textsubscript{3} by 1α-hydroxylase, and in primary hyperparathyroidism, elevated PTH levels are associated with increased serum calcium, decreased or normal serum phosphorus, and increased 1,25(OH)\textsubscript{2}D\textsubscript{3} levels (44, 45).

TABLE 5. Comparison of serum 1,25(OH)\textsubscript{2}D\textsubscript{3} (picograms per deciliter) values measured in COX-2 WT and KO mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Males</th>
<th>KO</th>
<th>Females</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>75.4 ± 5.9 (18)</td>
<td>177.6 ± 22.3 (19)*</td>
<td>58.5 ± 9.5 (11)</td>
<td>52.5 ± 23.0 (4)</td>
</tr>
<tr>
<td>10</td>
<td>85.8 ± 9.9 (21)</td>
<td>152.7 ± 16.7 (12)*</td>
<td>88.9 ± 11.8 (18)</td>
<td>166.1 ± 14.8 (14)*</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM for the number of animals in parentheses.

*P < 0.01 vs. WT.

As expected, some COX-2 KO mice had evidence of renal tubular impairment at the time of death. These mice had decreased serum calcium, increased serum phosphorus, and markedly elevated PTH, similar to the changes seen in secondary hyperparathyroidism in humans (32–35), dogs (36), rats (37–41), and mice (42). The mechanisms proposed to account for secondary hyperparathyroidism in renal failure include 1) phosphorus retention, which inhibits the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) that converts 25(OH)\textsubscript{2}D\textsubscript{3} to 1,25(OH)\textsubscript{2}D\textsubscript{3} as a result of phosphorus retention and decreased renal mass, 3) increased parathyroid hyperplasia due to phosphorus retention and reduced 1,25(OH)\textsubscript{2}D\textsubscript{3}, 4) resistance to the actions of 1,25(OH)\textsubscript{2}D\textsubscript{3} and PTH as a result of reduced numbers of vitamin D receptors and calcium sensors, and 5) subsequent decrease in serum calcium resulting from all of the above (32–35). Thus, decreased 1,25(OH)\textsubscript{2}D\textsubscript{3} should be an important factor in the development of secondary hyperparathyroidism. Although we could find no studies of 1,25(OH)\textsubscript{2}D\textsubscript{3} in renal failure in mice, there are several studies in rats showing the same relationships as those in humans (37, 38, 40, 41). We were able to measure serum 1,25(OH)\textsubscript{2}D\textsubscript{3} in one of the COX-2 KO mice with evident renal failure and found it to be very low compared with WT mice and KO mice without renal failure.

In both male and female COX-2 KO mice with relatively intact renal function, there was still a trend for mean PTH levels to be higher than levels in WT mice at all ages. This difference was statistically significant for 4-month-old KO female mice and 10-month-old male KO mice. In addition, in no group was the serum calcium level in KO mice lower than that in WT mice. Hence, the higher PTH levels in KO mice were not driven by decreased serum calcium, suggesting that the KO mice had primary hyperparathyroidism. Although serum calcium was only significantly elevated in 10-month-old male KO mice compared with WT mice, serum calcium could be said to be inappropriately high, perhaps corresponding to normocalcemic primary hyperparathyroidism in humans (43).

As another indication of whether the increased PTH levels were due to renal insufficiency or to primary hyperparathyroidism, we measured 1,25(OH)\textsubscript{2}D\textsubscript{3} levels. PTH stimulates the conversion of 25(OH)\textsubscript{2}D\textsubscript{3} to 1,25(OH)\textsubscript{2}D\textsubscript{3} by 1α-hydroxylase, and in primary hyperparathyroidism, elevated PTH levels are associated with increased serum calcium, decreased or normal serum phosphorus, and increased 1,25(OH)\textsubscript{2}D\textsubscript{3} levels (44, 45).

TABLE 6. Comparison of a group (n = 7) of COX-2 WT female mice, killed at 13–16 months of age, with a sick COX-2 KO female mouse, killed at 12 months of age

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Creatinine (mg/dl)</th>
<th>Calcium (mg/dl)</th>
<th>Phosphorus (mg/dl)</th>
<th>PTH (ng/ml)</th>
<th>1,25(OH)\textsubscript{2}D\textsubscript{3} (pg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>27.7 ± 1.7</td>
<td>0.41 ± 0.04</td>
<td>9.0 ± 0.2</td>
<td>8.9 ± 0.4</td>
<td>55.1 ± 6.4</td>
</tr>
<tr>
<td>KO</td>
<td>23.5</td>
<td>1.04</td>
<td>8.7</td>
<td>10.2</td>
<td>455.7</td>
</tr>
</tbody>
</table>

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</thead>
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<td>8.7</td>
<td>10.2</td>
<td>455.7</td>
</tr>
</tbody>
</table>
Many studies in transgenic mice have now shown that the PTH-vitamin D-calcium regulation is similar in mice and humans (42, 50–54). The simplest explanation consistent with the data is that COX-2 KO mice have primary hyperparathyroidism. Early renal insufficiency in the KO mice might have increased variability in the measurements. A study in humans of the influence of renal function on the features of primary hyperparathyroidism found that PTH and serum calcium were higher and 1,25(OH)2D3 levels were lower in patients with renal insufficiency and primary hyperparathyroidism compared with those in patients with primary hyperparathyroidism alone (55).

PGs have been shown to affect parathyroid function. Early studies of cells and fragments from bovine PTG showed that PTG cells secreted PGs (6-keto-PGF1α, PGF2α, PGE2) and that PGF2α could inhibit, and PGE2 could stimulate, PTH secretion (56, 57). This modulation of PTH secretion by endogenous PGs might depend on COX-2 expression. Extracellular Ca2+ is a potent inducer of COX-2 in kidney and bone cells (7, 58). In the kidney, PGE2 and extracellular Ca2+ can inhibit the same pool of adenylyl cyclase via different mechanisms, leading to the suggestion that PGE2 increases the sensitivity of the cells to Ca2+ so that effects can be seen at physiological concentrations of extracellular Ca2+ (59). Hence, absence of COX-2-produced PGE2 might decrease the Ca2+ sensitivity of PTG cells, leading to increased PTH secretion.

The absence of COX-2 expression might be expected to affect both bone resorption and formation. PGE2 is abundantly produced by osteoblasts in response to multiple agonists, largely as a result of the induction of COX-2. PGE2 is a potent stimulator of bone resorption and can mediate in part the resorptive activities of other agonists (4). When the specific role of COX-2 was examined in marrow cultures from COX-2 KO mice, the ability of 1,25(OH)2D3 or PTH to stimulate osteoclast formation was reduced 60–70% in KO cultures compared with WT cultures (5). PTH injected above the calvariae caused hypercalcemia in COX-2 WT mice, but not in COX-2 KO mice (5). In contrast, PGE2 can also stimulate bone formation. In vivo studies in rats have demonstrated a potent anabolic effect of exogenous PGE2 (60, 61). Exogenous PGE2 stimulates osteoblastic differentiation in

**TABLE 7.** Comparison of BMD (milligrams per square centimeter), measured by Dexa, in COX-2 WT and KO mice

<table>
<thead>
<tr>
<th></th>
<th>4-month-old mice</th>
<th>10-month-old mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Femur</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68.2 ± 1.3 (25)</td>
<td>67.8 ± 1.6 (18)</td>
</tr>
<tr>
<td>Female</td>
<td>68.7 ± 1.5 (17)</td>
<td>65.4 ± 1.9 (8)</td>
</tr>
<tr>
<td>Lumbar vertebrae (L1–L5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>56.4 ± 1.3 (18)</td>
<td>53.0 ± 1.7 (14)</td>
</tr>
<tr>
<td>Female</td>
<td>64.0 ± 1.7 (17)</td>
<td>56.3 ± 1.7 (10)</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM for the number of mice in parentheses.

a P < 0.05, effect of gender.
b P < 0.01, effect of genotype.
c P = 0.05, effect of age.
d P < 0.01, effect of gender.
e P < 0.01, effect of age.

**FIG. 4.** Sections of kidneys, stained with hematoxylin and eosin, from a 13-month-old female COX-2 WT mouse (A) and a 12-month-old female COX-2 KO mouse (B) with serum parameters consistent with renal failure and secondary hyperparathyroidism. Higher magnification shows enlarged tubules and lymphocytic infiltration in the KO kidney (D) compared with the WT kidney (C).
Comparison of static histomorphometric parameters measured in the distal femur in COX-2 WT and KO mice

TABLE 9.

<table>
<thead>
<tr>
<th></th>
<th>4-month-old mice</th>
<th>10-month-old mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Distal femur</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21.4 ± 1.8 (28)</td>
<td>21.9 ± 2.5 (15)</td>
</tr>
<tr>
<td>Female</td>
<td>16.4 ± 1.2 (11)</td>
<td>15.9 ± 2.0 (5)</td>
</tr>
<tr>
<td>L3 vertebra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are the mean ± SEM for the number of mice in parentheses.

a P < 0.01, effect of age.
b P < 0.05, effect of genotype.
c P < 0.01, effect of gender.

dominantly cortical bone, suggested that COX-2 KO mice had small decreases (<10%) in cortical bone density compared with WT mice. Cortical bone width measured by histomorphometry also tended to be decreased in COX-2 KO mice. Trabecular bone volume in KO mice was either no different from that in WT mice or increased relative to that in WT mice. Measurements of osteoblastic and osteoclastic surfaces suggested increased turnover in KO mice. Both trabecular bone formation rate and mineral apposition rate were significantly elevated in 10-month-old male KO mice compared with WT mice, but not in female mice. However, formation and mineral apposition rates did not decrease with age in WT females as they did in males and were higher in females than in males at 10 months of age. Perhaps the effects of mild hyperparathyroidism are more apparent in 10-month-old male KO mice because rates of formation had

TABLE 8. Comparison of BV/TV (percentage), measured by μCT, in COX-2 WT and KO mice

<table>
<thead>
<tr>
<th></th>
<th>4-month-old mice</th>
<th>10-month-old mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Cortical width (μm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>226.4 ± 4.8 (21)</td>
<td>209.6 ± 5.4 (17)c</td>
</tr>
<tr>
<td>Female</td>
<td>212.1 ± 18.5 (4)</td>
<td>202.6 ± 13.3 (5)</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10.8 ± 1.0 (21)</td>
<td>11.0 ± 1.5 (17)</td>
</tr>
<tr>
<td>Female</td>
<td>8.5 ± 1.6 (4)</td>
<td>7.9 ± 0.9 (5)</td>
</tr>
<tr>
<td>Osteoblast surface (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14.2 ± 1.6 (14)</td>
<td>18.5 ± 2.0 (12)</td>
</tr>
<tr>
<td>Female</td>
<td>15.4 ± 1.9 (4)</td>
<td>19.1 ± 1.6 (5)</td>
</tr>
<tr>
<td>Osteoclast surface (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10.7 ± 1.0 (14)</td>
<td>12.0 ± 1.2 (12)</td>
</tr>
<tr>
<td>Female</td>
<td>8.3 ± 1.3 (4)</td>
<td>12.9 ± 0.8 (5)c</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM for the number of mice in parentheses.

a P < 0.05, effect of genotype.
b P < 0.05, effect of age.
c P < 0.01, effect of genotype.
d P < 0.05, effect of gender.
e P = 0.05, effect of genotype.
argued that the effects seen of fresh serum to cultures or addition of PTH, and that under agonist secretion, a transiently inducible gene such as COX-2 would not be expected to be highly expressed. How-however, mechanical loading of bone occurs regularly, and mechanical loading can induce COX-2 and COX-2-derived PGs, which are thought to be important mediators of the anabolic response to mechanical loading (67). In vitro studies suggest that the absence of COX-2 should reduce both resorption and formation. If the net balance of resorption and formation remained unchanged, there might be only a delay in returning to steady state conditions after perturbation or challenge. Such an effect would fit with the delayed healing seen after fracture in COX-2 KO mice or in mice in which COX-2 activity has been inhibited by NSAIDs (68, 69). However, bone turnover is not decreased in the COX-2 KO mice. Hence, another explanation for the difference between in vitro and in vivo results is that the effects of absent COX-2 on bone turnover are compensated by increased PTH and/or 1,25(OH)2D3, both of which can stimulate bone resorption.

It is also possible that COX-1 plays a compensatory role in the absence of COX-2 (70). Although COX-1 is constitutively expressed, we have seen little PGE2 production in cells cultured from COX-2 KO mice (5, 6, 14), and we have not seen differential mRNA expression of COX-1 in tissues from COX-2 KO mice (data not shown). However, we cannot rule out some compensation in vivo. Because double COX-2 KO, COX-1 KO mice are not expected to survive, secondary to failure of the patent ductus arteriosus to close, we are generating COX-2 KO mice that are heterozygous for COX-1 for future study.

In conclusion, our study suggests that although COX-2 KO mice can have severe renal dysfunction, associated with marked secondary hyperparathyroidism, about 60% of COX-2 KO mice that reach 2 months of age survive to 10 months of age with relatively normal renal function. These surviving mice appear to have primary hyperparathyroidism, with elevated PTH and 1,25(OH)2D3 levels and increased rates of trabecular bone formation and mineral apposition, which may compensate for the absence of COX-2 in preserving bone mass.

### Acknowledgments

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