Age, gender and diet effects on gene expression regulated by 1,25-dihydroxyvitamin D₃

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Age, gender and diet effects on gene expression regulated by 
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Age, gender and diet effects on gene expression regulated by 1,25-dihydroxyvitamin D₃

by

Julie Ann Johnson

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For the Graduate College

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**PAPER I.** AGE AND GENDER EFFECTS ON 1,25-DIHYDROXYVITAMIN D₃-REGULATED GENE EXPRESSION

**ABSTRACT**

**INTRODUCTION**
GENERAL INTRODUCTION

Primary osteoporosis is an age-related disorder characterized by a decrease in bone mass, causing susceptibility to bone fractures (1). It affects an estimated 15-20 million people in the United States (1), both men and women, although women have a much greater risk (1, 2). Of women over 65, one-third will suffer vertebral fractures (2). Some risk factors that can lead to development of osteoporosis include genetic background (3, 4, 5), aging (2), female gender (1, 2), estrogen or testosterone deficiency (1, 6, 7), poor nutrition (1, 2), low peak bone density (1, 8), lack of exercise (2), and disease (2).

The active hormonal form of vitamin D3, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] plays a major role in maintenance of bone mineral homeostasis (9). Intracellular 1,25(OH)2D3 receptors (VDR) mediate the effects of 1,25(OH)2D3 in the major target tissues of kidney, bone, and intestine (9). Changes in 1,25(OH)2D3 metabolism or VDR expression may affect target tissue responsiveness. The 1,25(OH)2D3 up-regulates its receptor in vitro (10, 11). Vitamin D-dependent calcium (Ca) binding proteins (CaBP-28K and CaBP-9K), which seem to be involved in regulation of Ca absorption, also are regulated by 1,25(OH)2D3 through its receptor (12, 13). The 25-hydroxyvitamin D3 [25(OH)D3] is converted to 1,25(OH)2D3 in the kidney by the 25-hydroxyvitamin D-1α-hydroxylase enzyme (1α-hydroxylase) (14, 15). Inactive metabolites [24,25(OH)2D3, 1,24,25(OH)3D3] are produced by the 1α,25-dihydroxyvitamin D-24-hydroxylase (24-hydroxylase) in target tissues such as kidney and intestine (14, 15). In the kidney, parathyroid hormone (PTH) induces the activity of 1α-hydroxylase and suppresses 24-hydroxylase activity (16, 17). The 1,25(OH)2D3 induces 24-hydroxylase activity in both kidney and intestine (18, 19, 20, 21). Parathyroid hormone does not down-regulate 24-hydroxylase activity in intestine (22). Additionally, sex steroids may play a role in regulation of Ca balance, because of their direct effects on bone cells in vitro (23, 24).

With aging, plasma PTH concentrations tend to be chronically elevated (25, 26), production of 1,25(OH)2D3 decreases (27, 28), and intestinal Ca absorption becomes less efficient (29, 30). Concentrations of sex steroids, particularly estrogen after the menopause in females, also decrease with aging (6, 7).

The purpose of the experiments described in this dissertation was to examine more in depth the regulation of factors involved in maintenance of bone mineral homeostasis at the cellular and molecular level. Specifically, gene products whose expression are regulated by
1,25(OH)$_2$D$_3$ were examined, including VDR, CaBP-9K, CaBP-28K, and 24-hydroxylase in both kidney and duodenum. Differences in regulation of these proteins was examined in male and female rats, both young and old, in response to dietary Ca deficiency and supplementation, and dosing with 1,25(OH)$_2$D$_3$.

It is hoped that further knowledge gained by these studies will provide a better understanding of the processes involved in development of osteoporosis, and so lead to improved treatment and prevention.

**Explanation of Dissertation Format**

This dissertation consists of two separate manuscripts. Each paper is complete in itself, with an abstract, introduction, materials and methods, results, discussion, and references. A literature review for the dissertation is included before the first manuscript, and a general discussion of the combined results is included after the third manuscript. An appendix containing additional information pertinent to the manuscripts is included at the end of the dissertation. References cited in the general introduction, literature review, general discussion, and appendix are listed following the general discussion.
LITERATURE REVIEW

The purpose of this review is to summarize current knowledge regarding the effects of aging and gender differences on factors involved in the regulation of bone mineral homeostasis, and how these factors are involved in the development of involutional osteoporosis. Better understanding of the mechanisms by which these factors regulate bone mineral homeostasis can lead to more effective measures for prevention and treatment of osteoporosis.

Osteoporosis

Osteoporosis is a bone disorder characterized by an absolute decrease in the amount of bone, leading to heightened susceptibility to fractures (1, 31, 32). Idiopathic osteoporosis can occur in juveniles and young adults (31). Osteoporosis also can be caused by medical conditions, certain drugs, or surgery, such as ovariectomy, hypogonadism, hyperthyroidism, and treatment with glucocorticoids (31). This is termed secondary osteoporosis. Osteoporosis generally, however, is considered a disease of aging (i.e., involutional osteoporosis). Hip fractures have been recognized as a result of osteoporosis in the elderly for over a century (33), and vertebral fractures have been associated with postmenopausal osteoporosis for more than 50 yr (34). In the United States alone, an estimated 1.5 million fractures occur every year as a result of osteoporosis, leading to an estimated cost to society of $7-$10 billion annually. This cost is mostly related to hip fracture, which is fatal in 12-20% of cases, with 25% of survivors confined to long-term nursing home care (31, 35).

Bone mass in humans increases during development and growth until around age 30, followed by a short period of stability, after which age-related bone loss begins (31, 36, 37). Over the life span, women can expect to lose about 35% of their cortical bone and 50% of trabecular bone, and men can expect to lose about 23% and 33%, respectively (31, 38). Bone is a dynamic tissue, constantly turning over. This remodeling takes place within bone remodeling units (BRU). In a normal cycle of bone remodeling, osteoclasts resorb bone, creating lacunae in the bone (37, 39). Osteoclasts attach tightly to the bone surface, forming a sealed compartment into which lysosomal enzymes and protons are secreted (39). Osteoclast attachment to bone is mediated by integrins, which are cell surface adhesion
molecule receptors (39). This resorption process occurs over a period of about 2 wk. Osteoblasts then replace the osteoclasts, filling in the resorptive cavities, over a 3-4 mo period, to create a new structural unit of bone (37). During bone loss, resorption and formation are uncoupled, with a relative increase in resorption over formation (3, 31). Trabecular bone is found primarily in the vertebrae, pelvis, flat bones, and the ends of long bones. Cortical bone predominates in the shafts of long bones. With its greater surface area, trabecular bone is much more metabolically active than cortical bone, and so is more responsive to changes in mineral homeostasis (3).

Bone loss occurs biphasically. First, there is a slow rate of bone loss over the lifetime, after achievement of peak bone density (3). This slow phase occurs in both men and women, and is age-dependent. The slow phase starts around age 40 in cortical bone, and possibly as early as age 30-35 in trabecular bone. Bone loss in the slow phase results primarily from impaired osteoblast activity (3). That is, the osteoblasts fail to completely fill the resorptive cavities made by osteoclasts in the cycle of bone remodeling.

The second type of bone loss is an accelerated phase of bone loss that occurs in women after the menopause (31). Bone loss in the accelerated phase is due mostly to high turnover rate, when a greater number of osteoclasts are active and create deeper lacunae (3, 31). Bone formation also may increase, but the increase in rate of resorption is even greater. The accelerated phase occurs immediately following the menopause in both cortical and trabecular bone. The rapid rate of bone loss decreases to slow phase rates after 8-10 yr in cortical bone. In trabecular bone, however, there may be a greater initial rate of accelerated bone loss compared with cortical bone, but the duration of accelerated bone loss is shorter. A major portion of trabecular bone loss occurs even before the onset of menopause, and cortical bone loss occurs mostly post-menopausally (7, 31, 40).

Osteoblasts in osteoporotic patients seem to respond normally to proliferative stimuli (31, 37, 41). It therefore seems that changes that occur during aging are in the stimulating factors. For example, growth hormone and insulin-like growth factor-I have been shown to decrease with age (42, 43). Many other factors involved in maintenance of bone mineral content undergo changes with aging. These will be discussed in later in this review.

Several steps can be taken to prevent bone loss that occurs with aging and/or menopause. Estrogen replacement therapy, along with dietary Ca supplementation, is the most effective treatment currently available for slowing bone loss following menopause (31, 32, 44). Menopause, however, is only one of several causal factors; vertebral bone loss
begins well before menopause (7, 31, 40). Estrogen and Ca act mainly to decrease the activity of new BRU (45, 46). Calcium also may act to decrease PTH secretion (44, 47). Because bone cells contain estrogen receptors (23, 48), it is likely that estrogen also acts directly on bone cells to counteract the stimulation of resorptive activity by PTH. An alternative treatment to estrogen replacement is calcitonin (CT), which may act directly on osteoclasts to decrease resorption (31, 49). Several different bisphosphonates also are being tested (31, 50, 51). These compounds are taken up by the skeleton, actually increasing bone mass, and possibly inhibiting resorption through toxicity to osteoclasts, or by making bone more resistant to digestion by osteoclasts. More recently, echistatin, an integrin antagonist, has been shown to prevent osteoclast attachment to bone surface and to completely inhibit osteoclast-mediated bone resorption \textit{in vivo} (52). Regular weight-bearing exercise stimulates osteoblast function, and increases bone density (53, 54). Muscle mass and bone mass, in fact, are directly related (55).

Despite the great benefits of estrogen replacement therapy in treatment of postmenopausal osteoporosis, estrogen deficiency does not entirely explain the accelerated bone loss associated with menopause. Serum concentrations of sex steroids are similar in postmenopausal women both with and without osteoporosis (31, 32, 56). Some postmenopausal osteoporotic patients do not respond to currently available therapies (57). It is hypothesized that these patients may have intrinsic abnormalities in osteoblast function, preventing stimulation of bone formation much above basal levels (31, 57). These observations, along with the fact that slow phase bone loss begins even before menopause (7, 40), suggest that other factors involved in regulating bone mineral metabolism also must play a part in determining individual susceptibility to involutional osteoporosis.

**Factors Involved in Regulation of Bone Mineral Homeostasis**

The PTH-vitamin D endocrine system is the major regulator of Ca balance (58). Concentrations of Ca in extracellular fluid (ECF) and plasma are maintained within a relatively narrow range, and deviations from this range can have serious consequences (58, 59). When deviations do occur, this system acts to normalize plasma Ca concentrations through multiple mechanisms.

In a state of normal Ca balance, net intestinal Ca absorption and renal Ca excretion are approximately equal (59). An average adult human ingests around 15 mg/kg body
weight of Ca daily. Of this, about 11 mg/kg is excreted in the feces, and 4 mg/kg enters the
ECF and plasma Ca pool. This extracellular Ca pool equilibrates with bone Ca pools, and,
to a lesser extent, with intracellular Ca pools. Upon filtration through the kidney, about 4
mg/kg of Ca is excreted in urine (59).

In response to decreased plasma Ca concentrations, greater amounts of PTH are
secreted (44, 47). This PTH acts to decrease renal Ca excretion, increase mobilization of Ca
from bone, stimulate renal production of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (47),
and suppress 24-hydroxylase catabolism of 1,25(OH)₂D₃ (9, 22, 60, 61). Resulting
1,25(OH)₂D₃ increases CaBP-9K and CaBP-28K expression (58). This may increase
intestinal Ca absorption and decrease kidney Ca excretion (58, 62, 63). The 1,25(OH)₂D₃
also acts directly on bone cells to stimulate Ca mobilization (64, 65). The 1,25(OH)₂D₃
receptors (VDR) have been found in osteoblasts but are not present in osteoclasts (66).

In the case of excessive plasma Ca concentrations, PTH release is suppressed, and
release of CT is stimulated (44, 47, 59). The CT acts to increase renal excretion of Ca,
decrease mobilization of bone Ca, and stimulate renal 1,25(OH)₂D₃ synthesis (59, 66, 67).
The subsequent drop in plasma 1,25(OH)₂D₃ concentration leads to suppression of PTH
secretion (68), a decrease in mobilization from bone and decreased absorption of Ca in the
intestine (64, 66).

**Vitamin D**

Vitamin D has been found to have many roles in addition to its classical role in
regulation of mineral homeostasis. In addition to the classical target tissues of bone,
intestine, and kidney (64), more recent research has shown 1,25(OH)₂D₃ to have activity in
many other tissues, including, but not limited to, skeletal and smooth muscles (69, 70, 71),
brain, and pituitary gland (72), pancreas (73, 74), liver (69, 75), and hematopoietic and
immune system cells (76, 77, 78, 79). The 1,25(OH)₂D₃ enhances cardiac and vascular
muscle contractile function, modulates blood pressure, and inhibits excessive expression of
myocardial collagen (70). In skeletal muscle, 1,25(OH)₂D₃ affects cell division and
differentiation, alters phospholipid metabolism, and induces specific proteins (71, 80). The
1,25(OH)₂D₃ affects hormone production and secretion in the pituitary, playing a
permissive role in enhancing agonist-induced production of prolactin releasing hormone and
thyrotropin-stimulating hormone (72). In the pancreas, 1,25(OH)₂D₃ stimulates insulin
secretion by a direct effect on the β-cells (73, 74). The 1,25(OH)₂D₃ restores the ability of
hepatocytes to induce DNA polymerase-α activity and initiate DNA synthesis after partial hepatectomy (75). The 1,25(OH)₂D₃ regulates growth and differentiation of several cell types in the hematopoietic and immune systems (76). It enhances immune responses in some cases (77, 78) and suppresses them in others (79). This brief description is not intended to cover all that is currently known about the numerous actions of vitamin D. The focus of this review will be on the role of vitamin D in regulation of bone mineral homeostasis.

**Vitamin D metabolism**

Vitamin D is not truly a vitamin but currently is classified as a steroid hormone (9, 60, 64, 81). Vitamin D is a seco-steroid, which is ingested in the diet or can be synthesized in the skin from 7-dehydrocholesterol upon exposure to UV irradiation (9, 60, 64). Recognition that sunlight exposure could produce adequate amounts of vitamin D eliminated rickets as a major health problem (1). Vitamin D metabolites are carried in the bloodstream by the vitamin D binding protein (DBP) (9, 64, 82). In the liver, vitamin D is converted to the inactive metabolite 25(OH)D₃ by 25-hydroxylase, a cytochrome P450-containing enzyme (9, 60, 64).

Subsequently, 25(OH)D₃ is hydroxylated again in the kidney by the 1α-hydroxylase enzyme complex, which is made up of a flavoprotein, ferredoxin, and a cytochrome P450 (60) and is located mainly in mitochondria of the proximal renal tubules (60, 83, 84). This reaction produces 1,25(OH)₂D₃, the metabolically active hormonal form of vitamin D. The 1α-hydroxylation is the rate-limiting reaction in 1,25(OH)₂D₃ synthesis (1, 83). The activity of 1α-hydroxylase is regulated by several factors. Low plasma Ca concentrations stimulate PTH secretion (60, 83), possibly through a mechanism mediated by a Ca-activated, phospholipid-dependent protein kinase (PKC) (85). Parathyroid hormone stimulates the 1α-hydroxylase enzyme by increasing the intracellular messenger cyclic adenosine monophosphate (cAMP) (86, 87). Activation of 1α-hydroxylase by PTH also is associated with dephosphorylation of the renal ferredoxin (83, 88, 89). Phosphorous (P) deficiency increases 1α-hydroxylase activity (90, 91, 92). Through a feedback mechanism, high plasma concentrations of 1,25(OH)₂D₃ inhibit 1α-hydroxylase activity (60, 87, 92), and 1,25(OH)₂D₃ deficiency stimulates 1α-hydroxylase activity (93).

Both 25(OH)D₃ and 1,25(OH)₂D₃ can be hydroxylated by kidney 24-hydroxylase to form 24,25(OH)₂D₃ and 1,24,25(OH)₃D₃, respectively (1, 9, 60). It is debated whether or not 24,25(OH)₂D₃ has any biological activity, but synthesis of 1,24,25(OH)₃D₃ is
considered the first step in a catabolic inactivation pathway (9, 64). The kidney 24-hydroxylase is also cytochrome P450-dependent and is regulated reciprocally with 1α-hydroxylase (9, 60, 64, 87, 94). That is, adequate plasma Ca or elevated plasma PTH concentrations suppress kidney 24-hydroxylase activity (9, 22, 60, 61, 64), and high concentrations of plasma 1,25(OH)₂D₃ stimulate activity by increasing activity of the enzyme (60, 61, 87). Vitamin D deficiency results in suppression of renal 24-hydroxylase activity (93). The 24-hydroxylase is also found in several other tissues, including the major target tissue of the intestine, where it is regulated differently from the kidney enzyme (22). Parathyroid hormone does not down-regulate intestinal 24-hydroxylase activity (22), but 1,25(OH)₂D₃ does induce the enzyme in intestine as in kidney (22, 90). Dietary Ca restriction leads to up-regulation of intestinal 24-hydroxylase through increased plasma 1,25(OH)₂D₃ concentrations. However, despite equivalently increased plasma 1,25(OH)₂D₃ concentrations, dietary P restriction did not result in up-regulation of intestinal 24-hydroxylase (90). Dietary P restriction also does not affect renal 24-hydroxylase activity (91, 92).

Genomic actions of vitamin D

In the major target tissues of bone, intestine, and kidney, 1,25(OH)₂D₃ exerts its biological effects primarily through intracellular VDR (9, 64, 81). The VDR are part of the steroid-thyroid receptor gene superfamily of nuclear transcription factors (9, 95, 96, 97, 98). This classification is based on structural similarities with the other receptor proteins, including a highly conserved DNA-binding domain (9, 95, 96, 97, 98). The VDR exist predominantly in the nucleus (9, 97). The receptor protein contains a hormone-binding domain near the carboxy-terminus and a DNA-binding domain near the amino-terminus (9, 95, 97). The DNA-binding domain contains two "Zinc (Zn) finger" structures, in which a Zn atom is tetrahedrally coordinated through the sulfhydryl moieties of four conserved cysteine residues (9, 95, 97). This DNA-binding domain is highly evolutionarily conserved and resembles that seen in other DNA-binding proteins, such as transcription factor IIIA (9, 95, 97). Interaction of 1,25(OH)₂D₃ with VDR results in ligand-dependent phosphorylation of certain serine residues (85, 99). Ligand-dependent phosphorylation on serine residues also is seen with the progesterone receptor (100).

The interaction of 1,25(OH)₂D₃ with its receptor mediates changes in gene transcription of specific proteins. The steroid-receptor complex interacts with a specific region of the promoter of a gene, designated as a vitamin D response element (VDRE), and
can result in both positive and negative regulation of gene transcription (9, 95, 97). This VDRE consists of two direct hexameric repeats, suggesting that VDR may bind as a homo- or heterodimer (101, 102, 103). Some evidence implies that VDR does not interact with the VDRE as a homodimer (101). Indeed, a 59-64 kDa nuclear activating protein (NAP) is essential for VDR binding to VDRE (104, 105). Formation of the NAP-VDR complex is independent of 1,25(OH)\(_2\)D\(_3\) and DNA, and subsequent interaction with the VDRE does not require binding of 1,25(OH)\(_2\)D\(_3\), although binding of 1,25(OH)\(_2\)D\(_3\) greatly enhances both complex formation and DNA binding (104, 105). The retinoid X receptors \(\alpha\) and \(\beta\) (RXR-\(\alpha\) and RXR-\(\beta\)) have been identified as possible candidates for this NAP, because they form heterodimers with VDR and increase subsequent VDRE binding and transcriptional activation (106, 107). Another study, however, gives evidence that the NAP essential for VDR interaction with VDRE is not likely to be an RXR (108). Recently, two functionally distinct classes of response elements (RE) for vitamin D have been demonstrated (107). In the class of REs found in the osteocalcin gene (termed VDRE), transcriptional activation is induced by 1,25(OH)\(_2\)D\(_3\) alone, probably through binding of VDR homodimers to the VDRE. In the class of REs present in the osteopontin gene (termed VD/RXRE), 1,25(OH)\(_2\)D\(_3\) and 9-cis-retinoic acid (the ligand for RXR) act together to induce a synergistic response in transcription, through VDR/RXR heterodimers. These RXR-dependent and RXR-independent mechanisms represent two separate nuclear signalling pathways for 1,25(OH)\(_2\)D\(_3\), offering more complex control of 1,25(OH)\(_2\)D\(_3\)-regulated genes (107). The disparity in results from the earlier studies that examined RXR-VDR interactions may be the result of looking at REs for 1,25(OH)\(_2\)D\(_3\) in only one gene, which would yield different results depending on the type of RE present in the particular gene studied.

The 1,25(OH)\(_2\)D\(_3\) has been shown to regulate transcription of over 60 genes, including CaBP-9K (13, 58), CaBP-28K (13, 58), 24-hydroxylase (22, 60), and osteocalcin (9, 109, 110). The 1,25(OH)\(_2\)D\(_3\) also up-regulates its own receptor (61, 111). Treatment of cells \textit{in vitro} with 1,25(OH)\(_2\)D\(_3\) decreases the binding affinity of VDR for the hormone (112). In addition to catabolic degradation pathways, this offers a mechanism for controlling the effects of 1,25(OH)\(_2\)D\(_3\) on the cell. Ketoconazole, an inhibitor of the degradative 24-hydroxylase enzyme, increases homologous up-regulation of VDR, resulting in an increased proportion of occupied VDR and prolonged duration of occupation by 1,25(OH)\(_2\)D\(_3\) (10).
Up-regulation of intestinal VDR and CaBP-9K leads to a corresponding increase in absorption of dietary Ca (51, 58, 63, 64). When plasma concentrations of 1,25(OH)_{2}D_{3} are increased endogenously by dietary Ca deficiency to concentrations similar to those attained with exogenous treatment with 1,25(OH)_{2}D_{3}, different results are obtained (111). Kidney VDR and VDR mRNA are actually down-regulated, and intestinal VDR and VDR mRNA are not affected. This most likely is a result of the increase in plasma PTH, which occurs during states of Ca deprivation (44, 47). Parathyroid hormone negatively regulates transcription of VDR both in vitro and in vivo in kidney cells (61). No PTH receptors have been found in intestine, however. PTH possibly regulates VDR, and 24-hydroxylase as well, through a PKC-mediated cAMP production, resulting in suppression of gene transcription (22, 87, 113, 114, 115).

**Non-genomic actions of vitamin D**

More recent studies have implicated another pathway by which 1,25(OH)_{2}D_{3} might exert its effects on target tissues. This mode of action involves a signal transduction pathway, which is independent of the regulation of gene transcription (81, 92). The hormone interacts with the cell membrane (possibly through a cell surface receptor), resulting in the opening of Ca channels in a response that is too rapid to be a result of gene transcription. The main observed biological responses thought to be a result of this rapid non-genomic action are transcalcitachia (defined as "very rapid stimulation of intestinal Ca transport") and the opening of voltage-gated Ca channels in rat osteoblast-like osteosarcoma cells (81). This biological response appears within seconds to minutes after treatment with 1,25(OH)_{2}D_{3} compared with one or more hours required for genomic responses (81, 92).

**Parathyroid Hormone**

Parathyroid hormone is an 84 amino acid (a.a.) straight chain polypeptide hormone synthesized by the parathyroid glands (47). Its secretion is stimulated by a fall in plasma Ca concentration and suppressed by above normal Ca concentrations (44, 47). The mechanism for control of PTH secretion by extracellular Ca concentrations is not clear but seems to involve regulation of Ca channels in the plasma membrane (44). The signal generated by cell membrane sensors, which sense changes in extracellular Ca concentrations, regulates the intracellular Ca concentration, and it is the intracellular Ca pool that interacts to mediate PTH secretion (44). This occurs at an intracellular Ca concentration of about 200 nM, regardless of the extracellular Ca concentrations (44). The VDR have been found in the parathyroid glands and have been shown to bind upstream of the PTH gene (44, 116). The
1,25(OH)₂D₃ also suppresses pre-proPTH mRNA expression (44). Both in vitro and in vivo studies show that 1,25(OH)₂D₃ inhibits PTH secretion (44, 68). This suppression seems to be the result of increased sensitivity of the parathyroid gland to extracellular Ca (68). In rat osteoblast-like osteosarcoma cells, glucocorticoid increases and 1,25(OH)₂D₃ decreases the activation of adenylate cyclase and cAMP-dependent protein kinase (PKA) by PTH (117). Parathyroid hormone has reciprocal regulatory effects on VDR, down-regulating both VDR and VDR mRNA in vitro, and blocking the homologous up-regulation of VDR by 1,25(OH)₂D₃ in vivo (61). It thus seems that PTH and 1,25(OH)₂D₃ have opposing effects on certain genes. In a study examining the effects of a 3-wk period of dietary Ca and vitamin D deficiency on PTH, Ca deficiency resulted in a 5-fold increase in expression of PTH and its mRNA (118). Vitamin D deficiency increased PTH mRNA abundance 2-fold (118). Combined Ca and vitamin D deficiency, however, caused a 10-fold rise in PTH mRNA (118). In control rats, parathyroid gland cells made up 25% of the total number of cells from parathyroid-thyroid tissue, whereas parathyroid gland cells made up 42% of parathyroid-thyroid tissue in rats deficient in both Ca and vitamin D (118). Studies in the bovine parathyroid gland suggest that estrogen and progesterone also stimulate PTH secretion (44).

Parathyroid hormone interacts with high affinity receptors in cells in the renal distal tubule to increase Ca reabsorption, urinary excretion of cAMP, and 1α-hydroxylation of 25(OH)D₃ and to inhibit reabsorption of phosphate and bicarbonate (47). PTH receptors also are found on osteoblasts in bone where PTH acts to increase bone resorption (47, 59).

The ability of PTH to stimulate renal cAMP formation upon interaction with its receptor resides in the 1-27 a.a. region of the hormone's amino-terminus, which contains domains for receptor binding and activation (47). Normal adult plasma contains both intact PTH-(1-84) and various fragments of PTH that lack the amino-terminal 1-27 a.a. region (47). These fragments are present at a concentration 4-20-fold greater than that of intact PTH. Although the amino-terminal 1-27 a.a. region is considered the bioactive portion of PTH, recent studies have reported in vitro biological activity of fragments lacking this region, including the 53-84 a.a. region and the 30-34 a.a. region (47). In vivo, these fragments are released from the parathyroid glands even when secretion of the intact PTH-(1-84) is suppressed by high plasma Ca concentrations (47). It is not known, however, whether or not these fragments have any true biological activity in vivo. For the purposes of this review, PTH is defined as hormone fragments containing the putative biologically active
1-27 a.a. region (including the intact 1-84 a.a. hormone), and PTH receptors refer to the receptors for these fragments.

The PTH receptor is a plasma membrane glycoprotein with asparagine-linked oligosaccharides (119). It is linked to a guanine nucleotide triphosphate-binding protein (G-protein) (26, 120, 121), and evidence shows that transduction of the PTH signal from the cell surface receptor may involve both PKA and PKC signal transduction pathways (122).

**Vitamin D-Dependent Ca-Binding Proteins**

The CaBP-9K and CaBP-28K belong to the troponin-C superfamily of proteins (58). They are highly conserved across species and occur in highest concentration in several Ca-transporting tissues, such as kidney distal tubules, placenta, and intestine (58). They also are found in brain, peripheral nervous system, pancreas, parathyroid gland, and bone, although at much lower concentrations (58, 123, 124). Whereas induction of CaBP-9K and CaBP-28K is dependent on 1,25(OH)2D3 in the tissues where they are most abundant (13, 58, 123, 125), CaBP-28K is present in the brain in the absence of 1,25(OH)2D3 (58, 123, 125), suggesting alternate functions in this tissue. This review will focus on the role of CaBP-9K and CaBP-28K in regulation of Ca homeostasis, however.

Both CaBP-9K and CaBP-28K are found in avian intestine and mouse kidney. In other mammals, such as rat and pig, however, CaBP-9K is present in intestine but not kidney, and CaBP-28K is found in kidney but not intestine (58). The CaBP-9K also has been found in rat osteoblast, osteoclast, and bone matrix, where its presence is dependent on vitamin D (126). The 1,25(OH)2D3 has been shown to modulate rat intestinal CaBP-9K gene expression in two ways. The first is by rapid stimulation of transcription, seen within 15 min of treatment and peaking at one h (13). The second is a post-transcriptional stabilizing effect, which prevents degradation of the CaBP-9K transcript (13). This stabilization accounts for the observed accumulation of CaBP-9K mRNA several hours after 1,25(OH)2D3 treatment.

The CaBP-9K and CaBP-28K bind Ca with high affinity in "EF hand" structures and undergo conformational change when Ca binds (58). Although not well defined, their function seems to be to enhance vitamin D-dependent transcellular Ca transport (58, 62, 63, 65), and they are frequently present in tissues also containing a plasma membrane ATP-dependent Ca pump (127, 128, 129, 130). The CaBP-28K in avian intestine and the CaBP-9K in rat intestine are distributed similarly to the plasma membrane Ca pump (58, 129,
Correlations have been demonstrated between 1,25(OH)2D3 induction of CaBP-9K and CaBP-28K and the rate and time course of Ca transport (58, 132). The amount of CaBP-9K in rat and pig intestine, as well as the efficiency of Ca absorption, increases in response to a Ca-deficient diet (133, 134). The basolateral membrane of chick intestinal enterocytes contains an ATP-dependent Ca pump, which has been shown to be initially phosphorylated, and then quantitatively increased after dosing chicks with 1,25(OH)2D3 (63, 132). The basolateral membrane of rat renal distal tubules also contains an ATP-dependent Ca pump (58, 127). Interestingly, the CaBP-28K is confined exclusively to the distal tubule and co-localizes with this pump (127). In the proximal tubule, Ca and sodium (Na) transport is a coupled process. In the distal tubule, however, Ca and Na transport can be dissociated, and Ca transport proceeds against much larger concentration and electrical gradients (127). Whereas 50-60% of filtered Ca is reabsorbed by the proximal tubule, the 10% reabsorbed in the distal tubule is the fraction regulated by PTH (127), implying a greater physiological importance than mere quantity may suggest.

The CaBP-9K and CaBP-28K have been hypothesized to act as buffers for intracellular Ca, but their response to dietary Ca changes is inconsistent with the activity of a buffer (58). Another hypothesis is that CaBP-9K and CaBP-28K shuttle Ca from the intestinal brush border to the basolateral membrane (135). This assumes Ca uptake occurs through endocytic vesicles. The Ca then would be transported in lysosomes through the cell along microtubules to the basolateral membrane, where it would undergo exocytosis. Evidence for this mechanism comes from the regulatory effect of 1,25(OH) on α-tubulin mRNA expression (135). In a time course corresponding to that of enhanced vesicular Ca transport, four tubulin isotypes (including α-tubulin) were increased in microtubules isolated from intestinal epithelium of chicks treated with 1,25(OH)2D3. Glucocorticoids, which suppress 1,25(OH)2D3-stimulated Ca transport, decreased the quantity of total tubulin rather than altering specific isotypes (135). Additionally, CaBP-9K and CaBP-28K may be involved in intracellular signalling pathways. The presence of CaBP-28K increases the release of Ca from mitochondria in greater amounts than could be bound by the CaBP-28K (136).
Age Effects on Factors Involved in Regulation of Bone Mineral Homeostasis

**Vitamin D**

Concentrations of $1,25(\text{OH})_2\text{D}_3$ in plasma have been shown to decline with aging in humans and several animal species (27, 137, 138, 139). The defect seems to be in decreased activity of the kidney $1\alpha$-hydroxylase enzyme (28). When treated with PTH to stimulate $1,25(\text{OH})_2\text{D}_3$ synthesis, no change was seen in elderly osteoporotic patients, whereas young healthy adults had almost doubled $1,25(\text{OH})_2\text{D}_3$ concentrations (28). Seasonal variations have been shown in plasma concentrations of $1,25(\text{OH})_2\text{D}_3$ and $25(\text{OH})\text{D}_3$, with decreases in winter months when daylight hours are fewer (25, 137). The decreased concentrations of $25(\text{OH})\text{D}_3$ were even lower in older adults (mean age 78 yr) than in young adults (137).

The VDR mRNA first appears in rat intestine at about 3 wk of age, the same age at which intestinal Ca transport increases (140, 141). In kidney, VDR mRNA is induced sometime between birth and one wk of age (141). Both the VDR and its mRNA decline in rat intestine with aging after about one mo of age (138, 142, 143). The VDR also declines with aging in rat bone (142). The apparent affinity of intestinal and bone VDR for $1,25(\text{OH})_2\text{D}_3$ remained unchanged (138, 142). In contrast, the VDR content of kidney does not change with aging, but the affinity of kidney VDR for DNA-cellulose has been shown to decline in aging (144). This decline in VDR affinity for DNA is not seen in duodenum, however (138, 144). Two different VDR bands (50K and 52K) were detected by immunoblotting in kidney, but only a single 52K band was detected in duodenum (144). The apparent size difference between the bands may be the result of phosphorylation (85, 99). The 50K form elutes from DNA-cellulose at higher salt concentrations than the 52K form and is decreased in old rats (144).

**Parathyroid Hormone**

Plasma PTH concentration is chronically elevated with aging (25, 137, 139, 145, 146, 147), and renal PTH binding sites are decreased in old animals (145, 148, 149). Stimulation of renal Na/Ca exchange by PTH, stimulation of adenylate cyclase activity by PTH, and activity of $G_5$ and $G_1$ proteins, as detected by ADP-ribosylation, also are decreased in aging (145, 149). Parathyroid hormone increased synthesis of $1,25(\text{OH})_2\text{D}_3$ in renal slices from young (3-mo-old) rats but had no effect in renal slices from 12- to 24-mo-old rats (150, 151). Parathyroidectomy can partially negate these age effects,
suggesting that the elevated plasma PTH concentrations down-regulate renal PTH receptors, leading to the blunting of kidney response to PTH in aging (148, 149, 151).

Clearance of PTH is not changed by aging, so increased PTH concentrations must be caused by increased secretion (152). Evidence shows that perhaps the "set point" of Ca concentration for PTH release increases with aging (146, 152). That is, PTH can be secreted at higher plasma Ca concentrations than those that usually stimulate its secretion. Age-related changes in regulation of secretion by Ca also have been shown for CT (146, 153). Thyroid and parathyroid glands from old (24- to 27-mo-old) rats secreted more PTH and CT than did glands from young (2- to 3-mo-old) or adult (12- to 13-mo-old) rats (146, 153). When exposed to 2.5 mM Ca, suppression of PTH secretion and stimulation of CT secretion occurred to a lesser degree in thyroid and parathyroid glands from old rats than from young rats (146). Dietary Ca depletion results in increased plasma PTH concentrations in young rats, but not in adult or old rats, who have greater plasma PTH concentrations compared with young rats regardless of dietary Ca content (147). Interestingly, food restriction suppresses increased PTH (153, 154) and CT (153) secretion as well as delaying decreases in plasma 25(OH)D3 and 1,25(OH)2D3 and bone loss that occur with aging (153).

Vitamin D-Dependent Ca-Binding Proteins

Intestinal Ca absorption and the ability to adapt to a Ca-deficient diet decrease with age in rats and humans (12, 30, 155, 156, 157, 158), probably at least in part as a result of decreased production of 1,25(OH)2D3 (151, 159), which stimulates uptake of dietary Ca by the intestine (64, 65). The decrease in absorption is specific for Ca and is not because of decreased size of intestinal epithelial cells or decreased total cell number (29, 160). Aging also results in accelerated Ca turnover rate, with increased Ca excretion in feces and urine (161).

Both kidney CaBP-28K and intestinal CaBP-9K and their mRNAs decrease with aging (157, 160, 162). In cultured rat renal tubules, treatment with 1,25(OH)2D3 induced CaBP-28K to a greater extent in tubules from adult (10- to 12-mo-old) and old (20- to 24-mo-old) rats than in tubules from young (1-mo-old) rats (163). These results imply that the in vivo decrease in kidney CaBP-28K seen with aging is probably a result of decreased plasma 1,25(OH)2D3 concentrations rather than a lack of responsiveness to 1,25(OH)2D3.
In intestine, 1,25(OH)₂D₃ treatment increased Ca transport to a lesser extent in old rats compared with young rats (156, 164).

In isolated rat intestinal membrane vesicles, CaBP-9K decreased from 3 to 24 mo of age (160). Although ATP-dependent Ca uptake did not decrease until between 12 and 24 mo of age, it was much greater in 3-mo-old rats compared with adults. In the absence of ATP, no age difference was observed. The apparent affinity of vesicles for Ca did not change with age, but the apparent maximal Ca uptake capacity decreased (155, 160). In another experiment, young (6-wk-old) and adult (12-mo-old) rats were made vitamin D deficient by feeding a 0.8% strontium diet (12). They then were given oral doses of different amounts of 1,25(OH)₂D₃, and intestinal Ca active transport and CaBP-9K content were measured. Young rats responded with greater stimulation of active Ca transport compared with adult rats for every dose of 1,25(OH)₂D₃ examined (12). Induction of CaBP-9K was similar for both young and adult rats.

Gender and Sex Hormone Effects on Factors Involved in Regulation of Bone Mineral Homeostasis

Few studies have been done comparing males and females in regard to regulation of Ca homeostasis, although the specific roles of estrogens and androgens are now being studied more closely.

Calcium and PTH concentrations in plasma follow circadian rhythms, which are different in men and women (165). Calcium concentrations are lower in women than in men in the early morning. Calcium excretion decreases at night in both men and women, but more so in men, and the percentage reduction is greater. Secretion of PTH increases at night in both sexes, but increases earlier and to a greater extent in men. Because of these differences, women may be more dependent on dietary Ca intake than are men to maintain Ca homeostasis. Although differences in Ca excretion between men and women were small (165), daily differences of about 20 mg Ca/d over decades may contribute to gender differences in rate of bone loss.

Plasma Ca concentrations increase from 4 to 12 mo of age in vitamin D-deficient female but not in vitamin D-deficient male rats, and vitamin D-deficient females live longer than do vitamin D-deficient males (166). It was hypothesized that this age response may be a result of females having greater circulating concentrations of prolactin, a known stimulator of intestinal Ca absorption (166). Contrary to expected results, however, prolactin
treatment stimulated passive Ca uptake in isolated intestinal cells only from vitamin D-
deficient male and not from vitamin D-deficient female rats (166).

Estrogen increases nuclear VDR in rat liver, decreases nuclear VDR in rat kidney, and has no effect on nuclear VDR in rat intestine (167). Ovariectomized women, however, show a blunted response of intestinal Ca absorption to 1,25(OH)2D3, which can be prevented by estrogen replacement (168). Because estrogen does not affect intestinal VDR content (169), these data support the hypothesis for a vitamin D-independent action of estrogen on intestinal Ca absorption (168). Estrogen receptors are present in parathyroid tissue and thyroid C cells, and treatment of ovariectomized rats with 17β-estradiol stimulates expression of the mRNAs for PTH and CT (170). The combined anabolic effects of PTH and CT on bone provide an indirect pathway for estrogen action in prevention of bone loss (170).

Estrogen has direct effects on bone cells in vitro (23, 46), and estrogen receptors have been demonstrated in bone cells both in vivo and in vitro (23, 48, 171). Nuclear binding of estrogen is steroid-specific, saturable, and cell-type specific (23, 48). In bone cells in vitro, estrogen may exert anabolic effects by decreasing PTH receptor responsiveness and may exert anabolic effects by stimulating matrix synthesis and cell proliferation (46, 171). In osteoblasts in vitro, 17β-estradiol inhibits the expression of interleukin-6 (IL-6) and IL-6 mRNA induced by the bone resorbing cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) (172). The IL-6 stimulates osteoclast development, so, bone loss caused by estrogen depletion may be because of a loss of inhibition of these bone resorbing factors (172). Estrogen enhances 1,25(OH)2D3 action in rat osteoblast-like osteosarcoma cells by increasing VDR number (169), and also induces progesterone receptors and nuclear binding of progesterone in normal cultured human osteoblast-like cells (48). Estrogen receptors are present in osteoclasts as well as in osteoblasts (173). In vitro, estrogen decreases resorption of bone particles, and decreases lysozyme protein concentrations and the expression of lysosomal protein mRNA in osteoclasts (173). These findings suggest yet another mechanism by which estrogen acts to prevent bone loss in vivo (173).

Androgens also have direct effects on bone cells (24, 45, 174). Specific androgen binding and androgen receptor mRNA have been demonstrated in bone cells in vitro (24). In human osteoblast-like osteosarcoma cells, androgens decrease cell proliferation and increase transcripts for α1(I)-procollagen and transforming growth factor-β (24).
Testosterone deficiency in humans (175, 176) and castration in rats (177) causes marked bone loss, likely through increased resorption (178). Additionally, pharmacologic doses of a 19-nortestosterone and a 17α-hydroxyprogesterone independently influence bone formation and resorption and enhance 17β-estradiol stimulation of endosteal bone formation in the mouse (45). Testosterone and progesterone also inhibit expression of IL-6 by osteoblasts, as does 17β-estradiol (172), although twice the effective concentration of 17β-estradiol was required for this effect (172). These pharmacological effects of testosterone and progesterone may be the result of non-specific binding with estrogen receptors.
PAPER I. AGE AND GENDER EFFECTS ON 1,25-DIHYDROXYVITAMIN D₃-REGULATED GENE EXPRESSION
The aging process changes many factors involved in regulation of bone mineral metabolism, including concentrations of 1,25-dihydroxyvitamin D3 [1,25(OH)₂D₃] and parathyroid hormone (PTH) in plasma, and of 1,25(OH)₂D₃ receptors (VDR), vitamin D₃-dependent calcium binding proteins (CaBP-28K and CaBP-9K), and the 1α,25-dihydroxyvitamin D-24-hydroxylase (24-hydroxylase) in target tissues. These changes may affect the process which results in age-associated disease, such as osteoporosis.

Osteoporosis occurs much more frequently in females than in males, in part because of changes that occur at menopause. Females attain lower peak bone density than do males. The extent of adverse effects of age-related bone loss is less if peak bone density is greater.

In the present study, various physiological factors in both female and male Fischer 344 rats of different ages (1, 2.5, 6, and 18 mo) were compared. Plasma 1,25(OH)₂D₃ concentrations decreased with age in rats of both genders. Abundance of the CaBP-28K and its mRNA in kidney, and CaBP-9K and CaBP-9K mRNA in intestine also decreased (P <0.05) with age in both male and female rats. These decreases would be expected with low plasma 1,25(OH)₂D₃ concentrations. Kidney 24-hydroxylase activity and 24-hydroxylase mRNA content were elevated significantly in 18-mo-old males and females, compared with younger ages. These data suggest increased renal catabolism of 1,25(OH)₂D₃ may be responsible for low plasma 1,25(OH)₂D₃ concentrations observed in the older animal.

Most differences between males and female rats were found at 2.5 mo of age, which represents sexual maturity for rats. Plasma PTH concentrations, plasma 1,25(OH)₂D₃ concentrations, 24-hydroxylase enzyme activity and 24-hydroxylase mRNA content in kidney, abundance of 24-hydroxylase mRNA in intestine, and amount of CaBP-9K and CaBP-9K mRNA in intestine were greater (P <0.05) in males than in females at 2.5 mo of age. The lower plasma 1,25(OH)₂D₃ concentrations in females help to explain observed gender differences in the expression of genes stimulated by 1,25(OH)₂D₃. The combined effects of these gender differences at ages when peak bone density is being developed may contribute to the greater incidence of osteoporosis in females compared with males.
INTRODUCTION

Involutional osteoporosis is an age-related disorder characterized by an absolute decrease in bone mass, causing susceptibility to bone fractures (1). The disease affects an estimated 15-20 million men and women in the United States (1), although women have a much greater risk (1, 2). Of women over 65, one-third will suffer vertebral fractures during their lifetime (2).

Females are at a greater risk for osteoporosis than are males, in part because of accelerated bone loss that occurs after menopause. Females also attain lower peak bone mass than do males, and lose a greater percentage of bone over the lifetime. Estrogen depletion is related to development of osteoporosis in females. Estrogens and androgens have direct effects on bone cells in vitro (7, 8), and estrogens modulate 1,25(OH)₂D₃ activity (9, 10), but little is known about the specific mechanisms that lead to the development of osteoporosis. Not everyone with estrogen deficiency, however, develops osteoporosis (2). This fact suggests that other factors may be responsible for changes in calcium (Ca) absorption and bone mineral maintenance.

Peak bone density is achieved by about age 30-35 yr (3, 4, 5). Age-related bone loss, which begins soon after (3, 4, 5), is more problematic if less bone is present. A loss of 30% of peak bone density will lead to fracture susceptibility if peak bone density was low, but may not be a problem if peak bone density was great. Although many cases of osteoporosis in women are related to changes that occur at menopause (3), the slow phase of bone loss begins well before menopause (6). For these reasons, it is important to study factors involved in the regulation of bone mineral homeostasis throughout the life span, and not just in senescence, when bone loss has already occurred. Prevention of the disease would be preferable to treatment after the fact.

The active hormonal form of vitamin D₃, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], plays a major role in regulation of bone mineral metabolism (11). Both formation and catabolism rates determine the circulating concentrations of 1,25(OH)₂D₃. In the kidney, parathyroid hormone (PTH) induces the activity of 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), and suppresses 1α,25-dihydroxyvitamin D-24-hydroxylase (24-hydroxylase) activity (12, 13). PTH does not down-regulate 24-hydroxylase in the intestine (14). The 1,25(OH)₂D₃ induces 24-hydroxylase activity in both kidney and intestine (15, 16, 17, 18). Intracellular 1,25(OH)₂D₃ receptors (VDR) mediate the effects
of 1,25(OH)₂D₃ in the major target tissues of kidney, bone, and intestine (11). Changes in VDR expression may affect target tissue responsiveness. The 1,25(OH)₂D₃ up-regulates its receptor in vitro (19, 20). Vitamin D-dependent Ca binding proteins (CaBP-28K and CaBP-9K), which seem to be involved in regulation of Ca absorption, also are regulated by 1,25(OH)₂D₃ through its receptor (21, 22).

During the aging process, changes occur in many of the factors involved in regulation of Ca homeostasis. Plasma PTH concentration is elevated chronically (23, 24), whereas renal PTH responsiveness decreases (24). Intestinal Ca absorption decreases (25, 26), and production of 1,25(OH)₂D₃ in the kidney decreases (27, 28).

Male and female rats were compared to determine whether any gender differences exist in the factors studied. The purpose of this study was to more carefully examine factors involved in the regulation of bone mineral homeostasis at ages representative of various stages of the life span. Specifically, we examined gene products regulated by 1,25(OH)₂D₃, including the VDR, CaBP-28K and CaBP-9K, and the 24-hydroxylase enzyme.
MATERIALS AND METHODS

Materials

Vitamin D metabolites [25(OH)D₃, 1,25(OH)₂D₃, 24,25(OH)₂D₃, 1,24,25(OH)₃D₃, and 1,25,28(OH)₃D₂] were gifts from Milan Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Complementary DNA (cDNA) for rat VDR was provided by Dr. Wesley Pike (Ligand Pharmaceuticals, San Diego, CA). Mouse CaBP-28K cDNA was a gift from Sylvia Christakos (University of Medicine and Dentistry of New Jersey, Newark, NJ). M. Elizabeth Bruns (University of Virginia Medical School, Charlottesville, VA) provided the cDNA for mouse CaBP-9K. Kyuichiro Okuda (Hiroshima University School of Dentistry, Hiroshima, Japan) provided the cDNA for rat 24-hydroxylase. The α-[³²P]-dCTP (3,000 Ci/mmol, 10 mCi/ml) and γ-[³²P]-ATP (4,500 Ci/mmol, 10 mCi/ml) was purchased from Amersham Corp. (Arlington Heights, IL). Oligo-d(T)₁₈ was from Pharmacia (Piscataway, NJ). All other chemicals were of reagent grade or better.

Animals

Animal studies were conducted in accord with NIH Guidelines for the Care and Use of Laboratory Animals, and specific animal protocols were approved by the National Animal Disease Center Animal Care and Use Committee. Six male and six female Fischer 344 rats of each age were used. Eighteen-mo-old rats were purchased from the NIH (Bethesda, MD) aging colony. Rats of all other ages were purchased from Harlan-Sprague-Dawley (Madison, WI). Rats were maintained on Teklad (Madison, WI) stock diet for one wk after arrival. Final ages were 4-wk-, 2.5-mo-, 6-mo-, and 18-mo-old. Rats were anesthetized with CO₂:O₂ (1:1) and decapitated. Blood was collected for analysis of plasma Ca, PTH, and 1,25(OH)₂D₃. Mucosa was scraped from the proximal 20 cm of the small intestine. One-half was used to assay VDR content, and one-half was frozen immediately in liquid N₂ for later RNA extraction. Kidneys also were collected: one for assay of VDR concentration, and one frozen in liquid N₂ for RNA extraction.

We also compared 2.5- and 18-mo-old male and female Fischer 344 rats for response to treatment with 1,25(OH)₂D₃. Rats were fed a purified diet (Teklad) containing 1% Ca for one wk, then were injected intraperitoneally (i.p.) with 72 ng/kg body weight of 1,25(OH)₂D₃ (in 5% ethanol, 95% propylene glycol) 6 hours before death. Mucosa from
the proximal 20 cm of the small intestine was either assayed for 24-hydroxylase activity or frozen in liquid N$_2$ for later RNA extraction. Kidneys also were collected for later RNA extraction, or cortices were removed and assayed for 24-hydroxylase activity.

**VDR Assay**

Kidneys or intestinal mucosa were minced and washed in ice cold Tris/NaCl buffer (10 mM Tris, pH 7.4, 50 mM NaCl) with 50 KIU/ml proteinase inhibitor (Trasylol, Mobay Chemical Corp., New York, NY). Tissue then was homogenized (20% w/v) in 500 mM KTD (500 mM KCl, 10 mM Tris, pH 7.4, 5 mM dithiothreitol, 200 µg/ml soybean trypsin inhibitor) (29) and centrifuged at 230,000 X g. Aliquots (50 µl) of the resulting supernatant (cytosol) were incubated with 8 nM [3H]-1,25(OH)$_2$D$_3$ ± 100-fold excess of 1,25(OH)$_2$D$_3$ in triplicate at 4 C for 16 h to estimate VDR binding sites. Bound and free hormone were separated using hydroxyapatite. All VDR assays were corrected for non-specific binding. Cytosol protein concentration was determined by the Bradford method, with a bovine serum albumin (BSA) standard (30).

**CaBP Assays**

Kidney and intestinal cytosols were prepared as described for the VDR assay. Kidney cytosol then was analyzed for CaBP-28K content by radioimmunoassay as previously described (31). Intestinal cytosol from 1-, 2.5-, 6-, and 18-mo-old male and female rats first was equilibrated into 10 mM Tris buffer, pH 7.4 by using PD-10 sepharose columns (Pharmacia) and then lyophilized in a SpeedVac Concentrator (Savant Instruments, Inc., Farmingdale, NY). Lyophilized samples were redissolved in 1 ml of 10 mM Tris, pH 7.4 and assayed for protein concentrations (30). Intestinal CaBP-9K content was analyzed (32) by using specific polyclonal antisera to CaBP-9K (33).

**Intestinal 24-Hydroxylase Assay**

Intestinal 24-hydroxylase activity was determined by measuring the amount of [3H]-1,24,25(OH)$_3$D$_3$ produced during incubation of intestinal mucosa homogenates with [3H]-1,25(OH)$_2$D$_3$ (34). Intestinal mucosa was homogenized (5% w/v) in ice cold 0.25 M sucrose, 40 mM HEPES pH 7.4, 2 mM dithiothreitol (DTT). Aliquots of homogenate were
incubated for 5-15 min at 37 C with 6 µCi [3H]-1,25(25-OH)2D3, 7.7 mM glucose-6-phosphate, 7 mM ATP, 56 mM nicotinamide, 147 µM NAD+, 130 µM NADP+, 2.6 mM succinate, 35 mM KHPO4, 2.8 mM MgCl2, 35 mM KCl, 0.07 U/ml glucose-6-phosphate dehydrogenase, 0.30 U/ml alcohol dehydrogenase (18, 34). An additional 144 nM 1,25(25-OH)2D3 was added to reactions with homogenates from animals treated with 1,25(25-OH)2D3. The reaction was stopped by addition of 3.8 volumes of MeOH:MeCl2 (2:1). An aliquot of 1,25,28(28-OH)3D2 was added to each tube as a marker for the efficiency of extraction with MeCl2. Extracts were dried in a SpeedVac Concentrator (Savant Instruments, Inc., Farmingdale, NY) and stored under N2 at -20 C. For HPLC analysis, extracts were resuspended in the column running solvent [hexane:isopropanol:MeOH (88:10:2)], and run over a 4.6 mm x 250 mm Spherisorb NH2 column (Isco, Inc., Lincoln, NE) at a rate of 2 ml/min. One-min eluate fractions were collected, dried, and 3H peaks were quantitated by using liquid scintillation counting. Recovery of 1,25,28(28-OH)3D2 was measured by UV absorbance at 254 nm. Protein concentrations were measured in intestinal homogenates by the Bradford method, with a bovine serum albumin (BSA) standard (30). Amount of [3H]-1,24,25(25-OH)3D3 produced per min per mg protein was calculated and corrected for recovery of 1,25,28(28-OH)3D2.

**Kidney 24-Hydroxylase Assay**

Kidney 24-hydroxylase activity was determined by measuring the amount of 24,25(25-OH)2D3 produced during incubation of kidney cortex homogenates with 25(OH)D3 (35). Kidney cortex was homogenized (10% w/v) in ice cold 0.25 M sucrose, 50 mM HEPES, pH 7.4, and centrifuged at 12,900 X g. The resulting pellet was resuspended by homogenizing (10% w/v) in ice cold solution of 0.15 M sucrose, 40 mM HEPES, pH 7.4, 2 mM MgCl2, and 10 mM malate. Aliquots of homogenate were incubated for 15 min at 37 C with 15.6 mM 25(OH)D3. The reaction was stopped by addition of 1 volume of MeOH:MeCl2 (2:1). An aliquot of [3H]-24,25(25-OH)2D3 was added to each tube as a marker for the efficiency of extraction with MeCl2. Extracts were dried in a SpeedVac Concentrator (Savant Instruments, Inc.) and stored under N2 at -20 C. For HPLC analysis, extracts were resuspended in the column running solvent [hexane:MeCl2:EtOH:acetonitrile (90:6:3:1)], and run over a 4.6 mm x 250 mm Spherisorb NH2 column (Isco, Inc.) at a rate of 2 ml/min. One-min eluate fractions were collected, and an aliquot was analyzed for recovery of [3H]-
24,25(OH)_2D_3 by using liquid scintillation counting. Elution time for 24,25(OH)_2D_3 was determined by UV absorbance at 254 nm of a 24,25(OH)_2D_3 standard. Fractions containing the 24,25(OH)_2D_3 peak were pooled and concentrated. A protein binding assay (36) was used to quantitate 24,25(OH)_2D_3 in the peak. Protein concentrations were measured in kidney cortex homogenates by the Bradford method, with a BSA standard (30). Amount of 24,25(OH)_2D_3 produced per min per mg protein was calculated and corrected for recovery of [3H]-24,25(OH)_2D_3.

**Northern and Slot Blot Analyses**

An acid guanidinium thiocyanate-phenol-chloroform extraction was used to isolate RNA from kidney and intestinal mucosa (37). Poly(A)^+ RNA then was prepared by oligo(dT)-cellulose chromatography. Poly(A)^+ RNA (10-20 µg) was separated on a 1.2% formaldehyde-agarose gel and transferred to an Optibind reinforced nitrocellulose membrane (Schleicher and Schuell, Keene, NH) or to a Magna NT nylon membrane (Micron Separations Inc., Westboro, MA) for Northern blots. For slot blot analysis, poly(A)^+ RNA (1.25-5 µg) was blotted onto Optibind membranes by using a Minifold II slot blot apparatus (Schleicher and Schuell). The RNA was cross-linked to the membranes by irradiation with a Stratagene UV Stratalinker (Stratagene Cloning Systems, La Jolla, CA) or by a 5-min exposure to UV light. Restriction enzyme digestion was used to obtain a 1.7 kb rat VDR cDNA insert from the EcoRI site of pIBI76 (38), a 1.2 kb mouse CaBP-28K cDNA insert from the EcoRI site of pIBI76 (39), a 0.2 kb mouse CaBP-9K cDNA insert from the EcoRI site of Bluescribe (40), and a 3.4 Kb rat 24-hydroxylase cDNA insert from the EcoRI site of pUC19 (41). A random primer oligolabeling kit (Pharmacia) was used to label the cDNA to a specific activity of 10^8-10^9 dpm/µg of DNA. Membranes were prehybridized for 20-60 min (Optibind), or for 1-2 h (Magna NT), at 42 C in 5X SSPE (SSPE = 0.18 M NaCl, 10 mM Na_2PO_4, 1 mM EDTA, pH 7.4), 50% formamide, 5X Denhardt's reagent ([X Denhardt's = 0.02% Ficoll (type 400), 0.02% polyvinylpyrrolidone, 0.02% BSA (fraction V)], 0.5% sodium dodecyl sulfate (SDS) and 100 µg/ml denatured herring sperm DNA. Hybridization was carried out overnight at 42 C in fresh prehybridization buffer plus 250 µg of transfer RNA as a carrier and 1-5 X 10^6 cpm/ml of ^32P-labeled cDNA probe. Membranes were washed twice for 10 min at room temperature in 2X SSPE/0.1% SDS and then twice for 10 min at room temperature in 0.1X SSPE/0.1% SDS. For CaBP-9K, and
CaBP-28K probes, membranes sometimes were washed twice for 10 min at room temperature in 2X SSPE/0.1% SDS only. Imaging and quantitation of bands was done with an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). Autoradiography was carried out at -70°C for 12-168 h with XOMAT X-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen. To normalize for the amount of poly(A)^+ RNA loaded, membranes were probed with a pd(T)_{18} probe (42). The pd(T)_{18} was labeled with either γ-[^{32}P]-ATP, by using a DNA 5'-terminus labeling system (BRL Laboratories Life Technologies, Inc., Gaithersburg, MD), or with α-[^{32}P]-dCTP, by using a DNA 3'-end-labeling system from Promega (Madison, WI). Membranes were prehybridized for 10 min at room temperature in 5X SSPE and then hybridized for 1-2 h at room temperature in 5X SSPE and 5X Denhardt's reagent plus 200 pmol of [^{32}P]-labeled pd(T)_{18} per 11 x 14 cm membrane. Washing was four times for 5 min at room temperature in 2X SSPE for the 5'-end labeled probe or twice for 10 min at room temperature in 2X SSPE/0.1% SDS for the 3'-end labeled probe. Imaging, quantitation, and autoradiography were performed as described for cDNA probes.

**Other Analyses**

Plasma PTH concentration was measured with an N-terminal specific radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA). Atomic absorption spectrophotometry was used to determine plasma Ca content (43). Plasma extracts were assayed for 1,25(OH)_{2}D_{3} as described previously (44). Statistical differences were determined by using Analysis of Variance and Bonferroni Multiple Comparisons tests. Values were considered to be significantly different when P <0.05.
RESULTS

Expression of 1,25(OH)₂D₃-regulated genes was examined in kidneys of male and female rats from weanlings to senescence. As seen in Figure 1, kidney VDR concentration did not change over the age span studied. The abundance of VDR mRNA in kidney was similar to the VDR protein (Fig. 1). No gender differences were found in VDR mRNA or VDR protein in kidney, except at 2.5 mo of age, when females had more (P <0.05) VDR, but not VDR mRNA, in kidney than did males (Fig. 1).

Kidney CaBP-28K concentration decreased (P <0.05) with age in both males and females (Fig. 2). The message for CaBP-28K also declined with age in both males and females (Fig. 2). The magnitude of decline in renal CaBP-28K mRNA was less in females than in males, however, because of the lower amounts present in kidneys of females compared with males at one mo of age (Fig. 2).

Expression of the mRNA for the 1,25(OH)₂D₃-catabolizing enzyme, 24-hydroxylase, increased dramatically (P <0.05) with aging in kidneys of both male and female rats (Fig. 3). This age increase was greater (P <0.05) in males than in females (Fig. 3). Additionally, abundance of 24-hydroxylase mRNA in kidney was greater (P <0.05) in males than in females at 2.5 and 6 mo of age (Fig. 3). Renal 24-hydroxylase enzyme activity also increased (P <0.05) with aging in both males and females (Table 1). In contrast to 24-hydroxylase message abundance, however, the age increase in 24-hydroxylase activity was not greater in males than in females (Table 1). At sexual maturity (2.5-mo-old), males had both greater (P <0.05) renal 24-hydroxylase activity (Table 1) and 24-hydroxylase mRNA content (Fig. 3) than did females.

Expression of 1,25(OH)₂D₃-regulated genes also were examined in intestinal mucosa of weanling through senescent male and female rats. Intestinal VDR concentration decreased (P <0.05) with age for rats of both genders (Fig. 4). The abundance of VDR mRNA in intestine followed a similar pattern to that of VDR protein concentration over age in both males and females (Fig. 4). Intestinal VDR, but not VDR mRNA, content decreased with age to a lesser extent in females compared with males (Fig. 4).

As seen in Figure 5, intestinal CaBP-9K concentration decreased greatly (P <0.05) with age in both males and females. Correspondingly, the abundance of intestinal CaBP-9K mRNA also decreased (P <0.05) with age in rats of both genders (Fig. 5). These decreases in CaBP-9K and CaBP-9K mRNA content of intestine were not as dramatic in
FIGURE 1. Effect of age and gender on kidney VDR and VDR mRNA expression. Values for VDR are the mean ± SEM for six rats. VDR mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old males (control), after correction for background and normalization to pd(T)18. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between male and female rats of the same age.
FIGURE 2. Effect of age and gender on kidney CaBP-28K and CaBP-28K mRNA expression. Values for CaBP-28K are the mean ± SEM for six rats. CaBP-28K mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old males (control), after correction for background and normalization to pd(T)18. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P < 0.05) different between male and female rats of the same age.
Kidney CaBP-28K mRNA
Fraction of Control

Kidney CaBP-28K ng/μg Protein

Males
Females

1 Month 2.5 Months 6 Months 18 Months

1 Month 2.5 Months 6 Months 18 Months

2.1 Kb 2.8 Kb 2.0 Kb

*
FIGURE 3. Effect of age and gender on kidney 24-hydroxylase mRNA. 24-Hydroxylase mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old males (control) after correction for background and normalization to pd(T)_{18}. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between male and female rats of the same age. ° Indicates means significantly (P <0.05) different between different aged rats of the same gender.
Kidney 24- Hydroxylase mRNA Fraction of Control
FIGURE 4. Effect of age and gender on intestinal VDR and VDR mRNA expression. Values for VDR are the mean ± SEM for six rats. VDR mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old males (control), after correction for background and normalization to pd(T)$_{18}$. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between male and female rats of the same age.
FIGURE 5. Effect of age and gender on intestinal CaBP-9K and CaBP-9K mRNA expression. Values for CaBP-9K are the mean ± SEM for six rats. CaBP-9K mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old males (control), after correction for background and normalization to pd(T)18. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between male and female rats of the same age.
TABLE 1. Kidney 24-hydroxylase activity in 2.5- and 18-mo-old male and female Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>2.5 mo</th>
<th>18 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>48.8 ± 10.8</td>
<td>392.7 ± 98.1a</td>
</tr>
<tr>
<td>Male</td>
<td>144.2 ± 29.2b</td>
<td>193.8 ± 37.8</td>
</tr>
</tbody>
</table>

Values are pg 24,25(OH)₂D₃/min • mg of protein (mean ± SEM for six rats).

aMeans significantly different (P <0.05) between 2.5- and 18-mo-old rats of the same gender.

bMeans significantly different (P <0.05) between male and female rats of the same age.

females, however, because females had lesser (P <0.05) amounts of CaBP-9K and CaBP-9K mRNA at the younger ages than did males (Fig. 5).

Abundance of the 24-hydroxylase mRNA in intestinal mucosa decreased (P <0.05) between one and 18 mo of age in male rats (Fig. 6). Intestinal 24-hydroxylase mRNA content was similar between males and females, except at 2.5 mo of age, when females had less (P <0.05) 24-hydroxylase mRNA in intestine than did males (Fig. 6). Activity of the intestinal 24-hydroxylase enzyme, which is constitutively low unless stimulated, did not change with age in rats of either gender (Table 2). Intestinal 24-hydroxylase activity was not different between males and females at the detectability limits of the assay used (Table 2). Male and female rats aged 2.5- and 18-mo-old all responded to an i.p. injection of 1,25(OH)₂D₃ with an increase (P <0.05) in intestinal 24-hydroxylase activity (Table 2). Eighteen-mo-old females with a greater (P <0.05) increase in intestinal 24-hydroxylase activity than did 2.5-mo-old females to 1,25(OH)₂D₃ treatment (Table 2). This age difference in response to 1,25(OH)₂D₃ injection was not seen in males (Table 2). The 2.5-mo-old males treated with 1,25(OH)₂D₃ had greater (P < 0.05) intestinal 24-hydroxylase
FIGURE 6. Effect of age and gender on intestinal 24-hydroxylase mRNA. 24-
Hydroxylase mRNA values are from slot blots and are presented as a
fraction of the value for 1-mo-old males (control) after correction for
background and normalization to pd(T)18. Quantitation was done by using
an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA).
* Indicates means significantly (P <0.05) different between male and female
rats of the same age. ° Indicates means significantly (P <0.05) different
between different aged rats of the same gender.
Intestine 24-Hydroxylase mRNA Fraction of Control

**Females**
- 16 Months
- 6 Months
- 3 Months
- 1 Month

**Males**
- 18 Months
- 3 Months
- 2.5 Months
- 1 Month

Intestine 24-Hydroxylase mRNA Fraction of Control
TABLE 2. Intestinal 24-hydroxylase activity in 2.5- and 18-mo-old male and female Fischer 344 rats injected intraperitoneally with 72 ng/kg of body weight of 1,25(OH)$_2$D$_3$ (in 5% ethanol, 95% propylene glycol) 6 h before death

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ 1,25(OH)$_2$D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 mo</td>
<td>18 mo</td>
</tr>
<tr>
<td>Female</td>
<td>10.4 ± 2.0</td>
<td>8.1 ± 0.8</td>
</tr>
<tr>
<td>Male</td>
<td>10.9 ± 0.9</td>
<td>14.5 ± 2.8</td>
</tr>
</tbody>
</table>

Values are pg 1,24,25(OH)$_3$D$_3$/min • mg of protein (mean ± SEM for six rats).

$^a$Means significantly different (P < 0.05) between treated and untreated rats of the same age and gender.

$^b$Means significantly different (P < 0.05) between 2.5- and 18-mo-old rats of the same gender and treatment.

$^c$Means significantly different (P < 0.05) between male and female rats of the same age and treatment.

activity than did 2.5-mo-old females treated with 1,25(OH)$_2$D$_3$ (Table 2). At 18 mo of age, however, males treated with 1,25(OH)$_2$D$_3$ had lower (P < 0.05) intestinal 24-hydroxylase activity compared with 1,25(OH)$_2$D$_3$-treated females of the same age (Table 2).

As expected, plasma PTH concentrations increased (P < 0.05) with age in both males and females (Table 3). Females, however, had lower (P < 0.05) plasma PTH concentrations at 2.5 mo of age than did males (Table 3).

In both male and female rats, plasma 1,25(OH)$_2$D$_3$ concentrations decreased (P < 0.05) with age (Table 4). Females had lower (P < 0.05) concentrations of plasma 1,25(OH)$_2$D$_3$ than did males at both 2.5 and 18 mo of age (Table 4). Concentrations of 1,25(OH)$_2$D$_3$ in plasma increased (P < 0.05) similarly in all age groups treated in response to 1,25(OH)$_2$D$_3$ injection (Table 4).

No gender or age differences were found for plasma Ca concentrations.
TABLE 3. Plasma PTH concentrations in 2.5- and 18-mo-old male and female Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>2.5 mo</th>
<th>18 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>125.8 ± 16.1</td>
<td>264.2 ± 25.7</td>
</tr>
<tr>
<td>Male</td>
<td>220.7 ± 20.0b</td>
<td>285.8 ± 56.7</td>
</tr>
</tbody>
</table>

Values are pg PTH/ml of plasma (mean ± SEM for six rats).

a Means significantly different (P < 0.05) between 2.5- and 18-mo-old rats of the same gender.

b Means significantly different (P < 0.05) between male and female rats of the same age.

All Northern and slot blots used for quantitation of mRNA abundance were normalized by using a pd(T)18 probe. This probe was chosen because β-actin mRNA was found to change with age in the tissues examined here (intestinal mucosa and kidney) (Figs. A1, A2, A3). For this reason, β-actin cDNA was determined to be an inappropriate probe for normalization of quantity of poly(A)+ RNA applied to membranes in aging experiments. Poly(A)+ RNA, as measured by probing blots with pd(T)18, did not change with age in either intestinal mucosa or kidney (Fig. A3). See the Appendix for a complete explanation and data on choice of probes for normalization in aging studies.
**TABLE 4.** Plasma 1,25(OH)$_2$D$_3$ concentrations in 2.5- and 18-mo-old male and female Fischer 344 rats injected intraperitoneally with 72 ng/kg of body weight of 1,25(OH)$_2$D$_3$ (in 5% ethanol, 95% propylene glycol) 6 h before death

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ 1,25(OH)$_2$D$_3$</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>2.5 mo</td>
<td>18 mo</td>
<td>2.5 mo</td>
<td>18 mo</td>
</tr>
<tr>
<td>Female</td>
<td>48.7 ± 2.3</td>
<td>34.5 ± 4.8$^a$</td>
<td>249.2 ± 12.7$^b$</td>
<td>259.3 ± 3.9$^b$</td>
</tr>
<tr>
<td>Male</td>
<td>80.0 ± 7.4$^c$</td>
<td>60.4 ± 1.2$^a, c$</td>
<td>303.1 ± 15.1$^b, c$</td>
<td>307.2 ± 9.1$^b, c$</td>
</tr>
</tbody>
</table>

Values are pg 1,25(OH)$_2$D$_3$/ml of plasma (mean ± SEM for six rats).

$^a$Means significantly different (P <0.05) between 2.5- and 18-mo-old rats of the same gender and treatment.

$^b$Means significantly different (P <0.05) between treated and untreated rats of the same age and gender.

$^c$Means significantly different (P <0.05) between male and female rats of the same age and treatment.
The extent of adverse effects of age-related bone loss in an individual is dependent in part on the absolute peak bone density achieved before net bone loss begins. It is important, therefore, to study factors that regulate bone mineral homeostasis at ages when bones are developing, as well as at later ages, when bone diseases, such as osteoporosis, cause adverse bone loss. Females are more susceptible to osteoporosis, and also achieve lower peak bone density compared with males. For these reasons, this study examined factors involved in the regulation of bone mineral homeostasis in rats of ages representative of various stages of the life span. Both male and female rats were compared to determine whether any gender differences exist in the factors studied. Specifically, we examined factors involved in regulation of Ca homeostasis whose gene expression is regulated by 1,25(OH)\textsubscript{2}D\textsubscript{3}.

Kidney CaBP-28K and its mRNA decreased (P <0.05) with age in both male and female rats (Fig. 2). Amount of intestinal CaBP-9K and its mRNA also decreased (P <0.05) with age (Fig. 5). Similar results were found in male rats by Armbrecht et al (45) for changes in the expression of kidney CaBP-28K and intestinal CaBP-9K in aging rats. These changes may be a response to the decreased (P <0.05) concentration of plasma 1,25(OH)\textsubscript{2}D\textsubscript{3} in older rats (Table 4), because 1,25(OH)\textsubscript{2}D\textsubscript{3} regulates the expression of CaBP-28K and CaBP-9K through its receptor (21, 22). Expression of intestinal CaBP-9K is absolutely dependent on the presence of 1,25(OH)\textsubscript{2}D\textsubscript{3} (21,22). Additionally, the decrease (P <0.05) in intestinal VDR concentration with age (Fig. 4) would contribute to decreased expression of CaBP-9K in the intestine, because 1,25(OH)\textsubscript{2}D\textsubscript{3} exerts its effects on gene transcription through the VDR (11). These findings also help to explain the decline in Ca absorption in the intestine that occurs with aging (25,26), because CaBP-9K is involved in this process (21,22), although the specific mechanism by which the CaBP-9K facilitates Ca absorption is unknown.

Concentration of VDR and abundance of VDR mRNA in intestine also decreased (P <0.05) with age in both females and males, but neither changed in kidney (Figs. 1 and 4). Horst et al. (46) and Koszewski et al. (47) have published similar results for intestinal and kidney VDR, respectively, in one- and 18-mo-old male rats. Although kidney VDR (Fig. 1) did not decrease with age as did intestinal VDR (Fig. 4), CaBP-28K and its message (Fig. 2) did decline with age in kidney. This difference may be explained by a
decrease in VDR binding to DNA-cellulose that occurs in kidneys, but not intestine, of aged rats (47). The decreased DNA binding capacity of VDR in kidneys of old rats, in addition to the age-related decrease in plasma 1,25(OH)_{2}D_{3} concentrations, would likely result in decreased stimulation of expression of kidney CaBP-28K.

Kidney 24-hydroxylase activity (Table 1) and 24-hydroxylase mRNA (Fig. 3) increased (P <0.05) with aging in both males and females. An increase in 24-hydroxylase activity in the kidney has been shown before in male rats; renal [3H]-24,25(OH)_{2}D_{3} production was increased in 13- and 25-mo-old rats compared with that in 2.5-mo-old rats (48, 49). PTH is a negative regulator of 24-hydroxylase activity (12, 50); so, down-regulation of PTH receptors seen in old rats (24, 51, 52) likely would result in an increase in 24-hydroxylase activity by way of decreasing suppression of the enzyme. Plasma 1,25(OH)_{2}D_{3}, a positive regulator of 24-hydroxylase activity and 24-hydroxylase mRNA expression (53, 54), was lower (P <0.05) in old rats (Table 4) than in young rats. It seems then that the PTH effects must override the effects of 1,25(OH)_{2}D_{3} in this case. The fact that the mRNA follows the same pattern as the enzyme activity would suggest that PTH regulates the enzyme at the pre-translational level either by regulating transcription or by stabilizing the untranslated mRNA. The degree of stimulation of production of cAMP by PTH was decreased by 25% in 12-mo-old male rats compared with 1-mo-old rats (49). These results support a decrease in PTH responsiveness in kidney as a reason for increased 24-hydroxylase expression in older rats. Contrasting results were found by Armbrecht and Boltz (55) for changes in kidney 24-hydroxylase mRNA expression as a result of aging. They found that the expression of 24-hydroxylase mRNA in kidney was lower in old rats throughout a 48-h period after a dose of 1,25(OH)_{2}D_{3}. The differences in their results may be because the rats in this experiment (55) were first made vitamin D deficient by feeding a low Ca, 0.8% strontium diet for 6 d. It is unknown what other physiological effects this treatment may have. The physiological state of rats treated in this manner is far from normal. Data from this study (55), therefore, are not representative of what is occurring in a normal physiological state; so, the observed decrease in 1,25(OH)_{2}D_{3}-induced expression of kidney 24-hydroxylase mRNA in old rats is irrelevant when used to explain what is happening in aging of normal animals. Age-related increases in renal 24-hydroxylase 24-hydroxylase mRNA suggest that renal catabolism of 1,25(OH)_{2}D_{3} increases in aging animals. Therefore, the low plasma 1,25(OH)_{2}D_{3} concentrations observed in the aged animal could be the result of increased turnover of 1,25(OH)_{2}D_{3}.
Intestinal 24-hydroxylase mRNA content declined (P <0.05) with age in both males and females (Fig. 6), but no change was seen in activity of the intestinal enzyme at the detectability limits of the enzyme assay. Activity of intestinal 24-hydroxylase stimulated by an i.p. injection of 1,25(OH)2D3 also did not change with age in male rats. (Table 2). Plasma 1,25(OH)2D3 decreased (P <0.05) with age (Table 4, 48, 49) in both sexes. Intestine has not been shown to contain PTH receptors; so, it is likely that 1,25(OH)2D3 is the primary regulator of 24-hydroxylase in this tissue. In fact, PTH inhibits 24-hydroxylase mRNA expression stimulated by 1,25(OH)2D3 in kidney but not in intestine (14). The positive stimulation of 24-hydroxylase expression would decrease with the reduction in plasma 1,25(OH)2D3 concentration that occurs in aging. The VDR, through which 1,25(OH)2D3 exerts its transcriptional effects, also decreases with age in the intestine (46). A confusing result is that, in females, 1,25(OH)2D3-stimulated intestinal 24-hydroxylase activity was greater (P <0.05) in 18-mo-old compared with 2.5-mo-old female rats (Table 2). This age increase in females, but not in males, cannot be explained by regulation of the enzyme by 1,25(OH)2D3, because plasma 1,25(OH)2D3 concentrations decrease (P <0.05) with age (Table 4), and are lower (P <0.05) in females than in males at both 2.5 and 18 mo of age (Table 4). It would be expected, therefore, that 18-mo-old females would have lower 24-hydroxylase activity in intestine compared with all other groups studied. Some other regulator must be at work in this instance.

Plasma PTH concentration increased with age in both male and female rats (Table 3). This may be a response to, or cause of, the down-regulation of PTH receptors reported in old rats (51, 52). Age-related increases in plasma PTH concentrations must be caused by increased secretion, because clearance of PTH is not changed by aging (54). The decline in intestinal Ca absorption with age also could result in increased PTH secretion, because low plasma Ca concentration stimulates PTH secretion (62). In this study, however, plasma Ca concentrations did not change with age. An alternative explanation for increased plasma PTH concentrations in older animals is that the "set point" of plasma Ca concentration for PTH release increases with aging (56, 57). That is, PTH can be secreted at higher plasma Ca concentrations, which usually suppress PTH secretion (56, 57, 58).

Plasma 1,25(OH)2D3 concentration declined (P <0.05) with age for both male and female rats (Table 4). The activity of 1α-hydroxylase, which converts 25(OH)D3 to the biologically active 1,25(OH)2D3 in the kidney (12, 13), decreases with aging (59, 60). In male rats from 6- to 18-mo-old, Wada et al (61) found that both metabolic clearance rate and
production rate of 1,25(OH)₂D₃ increased with age, resulting in no change in plasma 1,25(OH)₂D₃ concentrations after 6 mo of age. An increase in metabolic clearance rate would support our findings of increased activity of renal 24-hydroxylase with aging. Metabolic clearance rate is determined by catabolism rates in all tissues. Although we found decreased 24-hydroxylase activity in intestine of older rats, the activity rates in kidney are much greater, making renal 1,25(OH)₂D₃ catabolism more important in determining metabolic clearance rate. Production rate reflects synthesis rates. The age-related decrease in concentration of plasma 1,25(OH)₂D₃ that we found is a reflection of both synthesis and degradation rates, and, in this case, catabolism rate must be greater than synthesis rate. This decrease took place over a wider age range than that studied by Wada et al (61). We also saw no significant decrease in plasma 1,25(OH)₂D₃ concentrations of rats between 6 and 18 mo of age. Although PTH stimulates 1α-hydroxylase activity in kidney (12, 13), the down-regulation of PTH receptors seen in aging could explain the lower plasma 1,25(OH)₂D₃ concentrations in older animals, despite the greater plasma PTH concentrations. In combination with an age-related decrease in 1,25(OH)₂D₃ synthesis, the increased activity of renal 24-hydroxylase in old rats (Table 1) would contribute to the observed decline in plasma 1,25(OH)₂D₃ concentrations with aging. Other factors influencing age-related declines in plasma 1,25(OH)₂D₃ concentration include non-absorption of dietary precursors or a decline in synthesis of precursors in skin.

At 2.5 mo of age, females had lower (P <0.05) abundance of CaBP-9K and CaBP-9K mRNA in intestinal mucosa than did the males (Fig. 5). Because the expression of CaBP-9K is absolutely dependent on 1,25(OH)₂D₃ (21, 22), this gender difference is likely to be the result of lower (P <0.05) plasma 1,25(OH)₂D₃ concentrations in females compared with males at this age (Table 4). Additionally, amounts of kidney CaBP-28K and CaBP-28K mRNA, whose expression also is stimulated by 1,25(OH)₂D₃, were lower (P <0.05) in females than in males at 2.5 mo of age (Fig. 2). Gender differences in expression of genes regulated by 1,25(OH)₂D₃ during ages at which bones are developing may be important in determining peak bone density and development of osteoporosis later in life.

Kidney 24-hydroxylase activity (Table 1) and 24-hydroxylase mRNA abundance (Fig. 3) was lower (P <0.05) in females than in males at 2.5 mo of age, but only kidney 24-hydroxylase mRNA abundance (Fig. 6) was lower (P< 0.05) in females than in males at 18 mo of age. At 2.5 mo of age, PTH suppression of kidney 24-hydroxylase expression seems to override stimulation by 1,25(OH)₂D₃, resulting in males having greater 24-
hydroxylase activity than do females. At 18 mo of age, PTH effects on kidney 24-
hydroxylase are blunted because of down-regulation of PTH receptors (51, 52). At this
age, plasma 1,25(OH)2D3 concentrations may be more influential in regulation of 24-
hydroxylase expression.

Intestinal 24-hydroxylase mRNA and 1,25(OH)2D3-stimulated 24-hydroxylase
activity was greater (P <0.05) in males than in females at 2.5 mo of age (Table 2 and Fig.
6). Parathyroid hormone does not regulate 24-hydroxylase mRNA expression in intestine,
as it does in kidney (14). Differences between males and females in intestinal 24-
hydroxylase activity (Table 2) at 2.5 mo of age, therefore, can be explained by differences
in plasma 1,25(OH)2D3 concentrations between genders (Table 4).

Plasma 1,25(OH)2D3 concentrations were similar or lower (P <0.05) in females
than in males at all ages (Table 4). At younger ages, this may in part be a result of greater
plasma PTH concentrations in males than in females, because PTH stimulates 1α-
hydroxylase synthesis of 1,25(OH)2D3 in kidney (12, 13). The down-regulation of PTH
receptors, which occurs with aging (51, 52), could eliminate PTH differences as an
explanation for differences in plasma 1,25(OH)2D3 concentrations between older males and
females. The 24-hydroxylase enzyme regulates 1,25(OH)2D3 concentrations by
catabolizing the hormone (60). In 2.5-mo-old rats, however, 24-hydroxylase activity in
kidney (Table 1) and 1,25(OH)2D3-stimulated 24-hydroxylase activity in intestine (Table 2)
both were lower (P <0.05) in females than in males. If this catabolic activity were the major
determinant of plasma 1,25(OH)2D3 concentrations at this age, females should have more
plasma 1,25(OH)2D3 compared with males. In fact, the opposite is true. Synthesis rates,
therefore, must be more important than catabolism rates in determining plasma
1,25(OH)2D3 concentrations at 2.5 mo of age. In contrast to results in 2.5-mo-old rats, at
18 mo of age, both renal 24-hydroxylase activity (Table 1) and 1,25(OH)2D3-stimulated
intestinal 24-hydroxylase activity were greater (P <0.05) in females than in males. At this
age, when PTH effects on 1α-hydroxylase activity are blunted by down-regulation of renal
PTH receptors (51, 52), it would make sense that 24-hydroxylase catabolism would be the
major determinant of plasma 1,25(OH)2D3 concentrations.

Plasma PTH concentration was less (P <0.05) in females than in males at 2.5 mo of
age (Table 3). This gender difference may contribute to the lower plasma concentrations of
1,25(OH)2D3 in females compared with males at this age (Table 4), because PTH stimulates
the 1α-hydroxylase enzyme to synthesize 1,25(OH)₂D₃, and suppresses 24-hydroxylase catabolism of 1,25(OH)₂D₃ in the kidney (12, 13).

The original observation that kidney 24-hydroxylase activity (Table 1) and 24-hydroxylase mRNA abundance (Fig. 3) are greatly increased in old rats suggests that increased renal metabolism of 1,25(OH)₂D₃ may contribute to the age-related decline in plasma 1,25(OH)₂D₃ concentrations observed in older animals. This in turn may contribute to age-related bone loss.

Male and female rats were similar over the age span studied for many of the factors examined. Most gender differences were found at sexual maturity (2.5-mo-old). The differences between males and females at this age may be a result of lower plasma 1,25(OH)₂D₃ concentrations in females compared with males. The combined effects of these gender differences at ages when peak bone density is being developed may contribute to the greater incidence of osteoporosis in females compared with males.
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PAPER II. EFFECTS OF AGE AND DIETARY CALCIUM DEPLETION ON GENES REGULATED BY 1,25-DIHYDROXYVITAMIN D₃
Age-related changes in the factors that regulate Ca homeostasis may affect how older animals respond to stresses such as dietary Ca deficiency. Young (3-wk-old) and old (18-mo-old) male Fischer 344 rats were fed diets containing either 1% or 0.02% Ca for 4 wk. In young rats, renal 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] receptors (VDR) and VDR mRNA content significantly declined as dietary calcium (Ca) declined, as has been previously shown by Goff et al. (1). In contrast, renal VDR in old rats was not affected by dietary Ca deficiency. Expression of the renal vitamin D$_3$-dependent calcium binding protein (CaBP-28K) mRNA also decreased in response to dietary Ca deficiency in young, but not in old, rats. Dietary Ca deficiency caused a decrease in kidney 1α,25-
dihydroxyvitamin D-24-hydroxylase (24-hydroxylase) mRNA abundance in 1-mo-old rats but resulted in a slight increase or no change in kidney 24-hydroxylase mRNA from the already elevated amounts in 18-mo-old rats. Intestinal CaBP-9K mRNA abundance increased in response to Ca deficiency only in young rats. Dietary Ca deficiency caused an increase in intestinal 24-hydroxylase mRNA content in 1-mo-old males, but either a slight decrease or no change was seen in 18-mo-old males. Plasma 1,25(OH)$_2$D$_3$ concentrations increased in response to dietary Ca deficiency in young rats. In old rats, however, plasma 1,25(OH)$_2$D$_3$ concentrations were lower and increased much less in response to dietary Ca changes. Increased renal catabolism of 1,25(OH)$_2$D$_3$ may be responsible for low plasma 1,25(OH)$_2$D$_3$ concentrations observed in the older animal. These results indicate that the ability of old rats to adapt to dietary Ca deficiency is severely impaired. The reason for this inability to adapt seems to be because they are unable to respond to Ca deficiency by increasing renal production of 1,25(OH)$_2$D$_3$. Without this response, the old rats are unable to appropriately regulate genes whose products are responsible for maintenance of Ca homeostasis.
INTRODUCTION

Plasma and extracellular fluid calcium (Ca) concentrations are maintained within a relatively narrow range (2, 3). When deviations from this range occur, such as when inadequate amounts of Ca are available through absorption from the diet, the parathyroid hormone (PTH)-vitamin D endocrine system acts to maintain plasma Ca concentrations through multiple mechanisms (2, 3). These mechanisms include increasing the efficiency of Ca absorption in the intestine (2, 4, 5) and increasing Ca mobilization from bone (6, 7, 8).

Aging animals often exhibit changes in factors that are part of the PTH-vitamin D endocrine system. Efficiency of absorption of Ca from the diet is poor (9, 10), plasma 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] concentrations have been found to both decrease (11, 12) and remain unchanged (13, 14), and plasma PTH concentrations tend to be elevated chronically (15, 16). These age-related changes in factors that maintain plasma Ca homeostasis may result in inappropriate responses to perturbations of Ca balance. If Ca homeostasis is not adequately maintained, diseases such as osteoporosis can develop.

Both formation and catabolism rates determine the circulating concentrations of 1,25(OH)₂D₃. In a feedback mechanism, 1,25(OH)₂D₃ induces its own catabolism by stimulating the 1α,25-dihydroxyvitamin D-24-hydroxylase enzyme (24-hydroxylase) in target tissues of kidney and intestine (17, 18). High plasma 1,25(OH)₂D₃ concentrations also inhibit the activity of the 25-hydroxyvitamin D-1α-hydroxylase enzyme (1α-hydroxylase), which synthesizes 1,25(OH)₂D₃ from 25(OH)D₃ (19, 20, 21). In the kidney, PTH induces the activity of 1α-hydroxylase and suppresses 24-hydroxylase (22, 23, 24). PTH does not regulate 24-hydroxylase in the intestine (24). Age-related decreases in plasma 1,25(OH)₂D₃ concentrations are partly a result of decreased renal 1α-hydroxylase activity (25, 26, 27). How aging affects renal 24-hydroxylase activity has remained controversial. Armbrecht et al (13) showed that renal production of 24,25(OH)₂D₃ is increased in 13- and 25-mo-old compared with 3-mo-old male rats. Wada et al (14) have shown that both the production rate and metabolic clearance rate of 1,25(OH)₂D₃ increase between 6 and 18 mo of age, resulting in no net change in plasma 1,25(OH)₂D₃ concentrations. These data suggest that 24-hydroxylase activity is increased with aging. More recently, however, Armbrecht and Boltz (28) showed that the expression of 24-hydroxylase mRNA in kidney was lower in vitamin D deficient 12-mo-old compared with 2-mo-old rats throughout a 48-h period after a dose of 1,25(OH)₂D₃.
Evidence suggests that PTH regulates renal VDR and 24-hydroxylase, likely at the level of gene expression (1, 24, 29). Goff et al (1) showed that when plasma 1,25(OH)₂D₃ concentrations are increased endogenously, by dietary Ca deficiency, renal VDR content decreased, despite equal or greater plasma 1,25(OH)₂D₃ concentrations in the Ca deficient rats (1). Dietary Ca deficiency stimulates PTH secretion (6, 30), resulting in the increased plasma 1,25(OH)₂D₃ concentrations (31). Reinhardt and Horst (29) showed that PTH down-regulates VDR and VDR mRNA in vitro, and also blocks up-regulation of VDR by 1,25(OH)₂D₃ in vivo (29). Shinki et al (24) found that PTH inhibits 24-hydroxylase mRNA expression stimulated by 1,25(OH)₂D₃ in rat kidney. Increased plasma PTH concentrations as a result of Ca depletion seem to act in opposition to 1,25(OH)₂D₃ in regulation of gene expression (1, 24, 29). Kidneys of old rats are less responsive to PTH, because PTH receptors are down-regulated with aging (32, 33). This down-regulation probably is a result of chronically elevated plasma PTH concentrations in old rats (32).

In light of the many age-related changes in factors that are part of the PTH-vitamin D endocrine system, the purpose of this study was to determine whether these factors respond differently to the stress of dietary Ca deficiency in young (weanling) and old (senescent) rats. Specifically, we examined gene products regulated by 1,25(OH)₂D₃, including the VDR, CaBP-28K, CaBP-9K, and the 24-hydroxylase enzyme.
MATERIALS AND METHODS

Materials

Crystalline 1,25(OH)\textsubscript{2}D\textsubscript{3} was a gift from Milan Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Complementary DNA (cDNA) for rat VDR was provided by Wesley Pike (Ligand Pharmaceuticals, San Diego, CA). Mouse CaBP-28K cDNA was a gift from Sylvia Christakos (University of Medicine and Dentistry of New Jersey, Newark, NJ). M. Elizabeth Bruns (University of Virginia Medical School, Charlottesville, VA) provided the cDNA for mouse CaBP-9K. Kyuichiro Okuda (Hiroshima University School of Dentistry, Hiroshima, Japan) provided the cDNA for rat 24-hydroxylase. The α-[\textsuperscript{32}P]-dCTP (3,000 Ci/nmol, 10 mCi/ml) was purchased from Amersham Corp. (Arlington Heights, IL). Oligo-d(T)\textsubscript{18} was from Pharmacia (Piscataway, NJ). All other chemicals were of analytical grade or higher.

Animals

Six male Fischer 344 rats were used for each experimental group. Weanling (3-wk-old) rats were purchased from Harlan-Sprague-Dawley (Madison, WI), and 18-mo-old rats were purchased from the NIH (Bethesda, MD) aging colony. All rats were fed purified diets (Teklad, Madison, WI) containing either 1% or 0.02% Ca for 4 wk. Rats then were anesthetized with CO\textsubscript{2}:O\textsubscript{2} (1:1) and decapitated. Blood was collected for analysis of plasma 1,25(OH)\textsubscript{2}D\textsubscript{3} concentrations. Mucosa was scraped from the proximal 20 cm of small intestine and divided in half. One-half was frozen in liquid N\textsubscript{2} for later RNA extraction, and one-half was used to assay for VDR content. One kidney was removed and frozen in liquid N\textsubscript{2} for RNA extraction, and the other kidney was assayed for VDR concentration.

VDR Assay

Kidneys or intestinal mucosa were minced and washed in ice cold Tris/NaCl buffer (10 mM Tris, pH 7.4, 50 mM NaCl) with 50 KIU/ml proteinase inhibitor (Trasylol, Mobay Chemical Corp., New York, NY). Tissue then was homogenized (20% w/v) in 500 mM KTD (500 mM KCl, 10 mM Tris, pH 7.4, 5 mM dithiothreitol, 200 μg/ml soybean trypsin inhibitor) (34) and centrifuged at 230,000 X g. Aliquots (50 μl) of the resulting supernatant
(cytosol) were incubated with 8 mM $[^{3}H]_{-}1,25(OH)_{2}D_{3}$ ± 100-fold excess of $1,25(OH)_{2}D_{3}$ in triplicate at 4°C for 16 h to estimate VDR binding sites. Bound and free hormone were separated by using hydroxyapatite. All VDR assays were corrected for non-specific binding. Cytosol protein concentration was determined by the Bradford method, with a bovine serum albumin (BSA) standard (35).

**Northern and Slot Blot Analyses**

RNA was isolated from kidney and intestinal mucosa by using an acid guanidinium thiocyanate-phenol-chloroform extraction (36). Oligo-d(T)-cellulose chromatography then was used to prepare poly(A)$^{+}$ RNA. Poly(A)$^{+}$ RNA (10-20 µg) was separated on a 1.2% formaldehyde-agarose gel and transferred to a Magna NT nylon membrane (Micron Separations Inc., Westboro, MA). For slot blot analysis, poly(A)$^{+}$ RNA (1.25-5 µg) was blotted onto Magna NT membranes by using a Minifold II slot blot apparatus (Schleicher and Schuell, Keene, NH). Poly (A)$^{+}$ RNA was cross-linked to membranes by irradiation (UV Stratalinker, Stratagene Cloning Systems, La Jolla, CA). Restriction enzyme digestion was used to obtain a 1.7 kb rat VDR cDNA insert from the EcoRI site of pIBI76 (37), a 3.2 Kb rat 24-hydroxylase cDNA insert from the EcoRI site of pUC19 (38), a 1.2 kb mouse CaBP-28K cDNA insert from the EcoRI site of pIBI76 (39), and a 0.2 kb mouse CaBP-9K cDNA insert from the EcoRI site of Bluescribe (40). A random primer oligolabeling kit (Pharmacia) was used to label the cDNA to a specific activity of 10$^{8-10^{9}}$ dpm/µg of DNA. Membranes were prehybridized for 1-2 h at 42°C in 5X SSPE (SSPE = 0.18 M NaCl, 10 mM NaPO$_{4}$, 1 mM EDTA pH 7.4), 50% formamide, 5X Denhardt's reagent [1X Denhardt's = 0.02% Ficoll (type 400), 0.02% polyvinylpyrrolidone, 0.02% BSA (fraction V)], 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml denatured herring sperm DNA. Hybridization was carried out overnight at 42°C in fresh prehybridization buffer plus 250 µg of transfer RNA (as a carrier) and 1.5 X 10$^{6}$ cpm/ml of $^{32}P$-labeled cDNA probe. Membranes were washed twice for 10 min at room temperature in 2X SSPE/0.1% SDS and then twice for 10 min at room temperature in 0.1X SSPE/0.1% SDS. Imaging and quantitation of bands was done using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). Autoradiography was carried out at -70°C for 12-168 h with XOMAT X-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen. To normalize for the amount of poly(A)$^{+}$ RNA loaded onto gels, membranes were hybridized
with a pd(T)_{18} probe (41). The pd(T)_{18} was labeled with $\alpha-[^{32}\text{P}]-\text{dCTP}$ by using a DNA 3'-end-labeling system from Promega (Madison, WI). Membranes were prehybridized for 10 min at room temperature in 5X SSPE and then hybridized for 1-2 h at room temperature in 5X SSPE, 5X Denhardt's reagent plus 200 pmol of $[^{32}\text{P}]-$labeled pd(T)_{18} per 11 x 14 cm membrane. Washing was twice at room temperature in 2X SSPE/0.1% SDS. Imaging, quantitation, and autoradiography were performed as described for cDNA probes.

Other Analyses

A protein binding assay was used to measure 1,25(OH)$_2$D$_3$ concentrations in plasma extracts and then corrected for nonspecific binding (42). Statistical differences were determined by using an Analysis of Variance test. Values were considered to be significantly different when $P < 0.05$. 
RESULTS

We studied the expression of genes regulated by 1,25(OH)2D3 in kidneys of one- and 18-mo-old male rats fed either a normal Ca control diet or a Ca deficient diet. Kidney VDR concentration was not changed by age in rats fed a normal Ca diet. When rats were fed a Ca deficient diet, however, kidney VDR concentration decreased (P <0.05) in young (1 mo), but not old (18 mo), rats (Fig. 1). The mRNA for VDR in kidney responded in the same way to Ca deficiency as did the VDR protein (Fig. 2).

Similarly to VDR and VDR mRNA in kidney, the amount of CaBP-28K mRNA in kidney decreased (P <0.05) in young, but not old, rats fed a Ca deficient diet (Fig. 3). Differently from VDR and VDR mRNA, however, renal CaBP-28K mRNA content was decreased (P <0.05) in old rats compared with young rats (Fig. 3).

As seen in Figure 4, 24-hydroxylase mRNA abundance in kidney was increased markedly (P <0.05) in old rats compared with young rats. In response to dietary Ca depletion, 24-hydroxylase mRNA content in kidneys of young rats declined (P <0.05) (Fig. 4). Kidney 24-hydroxylase mRNA content remained unchanged by age or by dietary Ca deficiency from the already elevated (P <0.05) amounts in old rats (Fig. 4).

The effect of Ca deficiency on the expression of 1,25(OH)2D3-regulated genes also was examined in intestine of one- and 18-mo-old male rats. As seen in Figure 5, intestinal VDR concentration was unchanged by age or by dietary Ca deficiency. Intestinal VDR mRNA also did not change with age or dietary Ca depletion (Fig. 6).

In contrast, intestinal CaBP-9K mRNA content increased significantly (P <0.05) in response to dietary Ca deficiency in young rats (Fig. 7). In old rats, however, dietary Ca deficiency did not affect the abundance of intestinal CaBP-9K mRNA (Fig. 7). Intestinal CaBP-9K mRNA content decreased slightly, but not significantly, with age (Fig. 7).

Similarly to intestinal CaBP-9K mRNA, the amount of message for 24-hydroxylase in intestine decreased (P <0.05) with age (Fig. 8). Also, in response to dietary Ca depletion, intestinal 24-hydroxylase mRNA content increased significantly (P <0.05) in young rats (Fig. 8). This increase in intestinal 24-hydroxylase mRNA abundance in response to depletion of dietary Ca was not seen in old rats (Fig. 8).

Dietary Ca deficiency resulted in a significant increase (P <0.05) in plasma 1,25(OH)2D3 concentrations in young rats (Fig. 9). Plasma 1,25(OH)2D3 concentrations
FIGURE 1. Effect of age and Ca deficiency on kidney VDR. Values are the mean ± SEM for six rats. * Indicates means significantly (P <0.05) different between rats of the same age fed different diets. ° Indicates means significantly (P <0.05) different between 1- and 18-mo-old rats fed the same diet.
FIGURE 2. Effect of dietary Ca deficiency on kidney VDR mRNA expression. VDR mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old rats fed the 1% Ca diet after correction for background and normalization to pd(T)18. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P < 0.05) different between rats of the same age fed different diets. ° Indicates means significantly (P < 0.05) different between 1- and 18-mo-old rats fed the same diet.
FIGURE 3. Effect of dietary Ca deficiency on kidney CaBP-28K mRNA expression.
CaBP-28K mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old rats fed the 1% Ca diet after correction for background and normalization to pd(T)₁₈. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA).
* Indicates means significantly (P <0.05) different between rats of the same age fed different diets. ° Indicates means significantly (P <0.05) different between 1- and 18-mo-old rats fed the same diet.
Kidney CaBP-28K mRNA Fraction of 1-Month / 1% Ca

1 Month / 1% Ca
1 Month / 0.02% Ca
18 Months / 1% Ca
18 Months / 0.02% Ca
FIGURE 4. Effect of age and Ca deficiency on kidney 24-hydroxylase mRNA. 24-Hydroxylase mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old rats fed the 1% Ca diet after correction for background and normalization to pd(T)$_{18}$. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). 0 Indicates means significantly (P <0.05) different between 1- and 18-mo-old rats fed the same diet. # Indicates means significantly (P <0.05) different between rats of the same age fed different diets.
FIGURE 5. Effect of age and Ca deficiency on intestinal VDR. Values are the mean ± SEM for six rats.
FIGURE 6. Effect of dietary Ca deficiency on intestinal VDR mRNA expression. VDR mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old rats fed the 1% Ca diet after correction for background and normalization to pd(T)_{18}. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between rats of the same age fed different diets. ° Indicates means significantly (P <0.05) different between 1- and 18-mo-old rats fed the same diet.
Intestine VDR mRNA Fraction of 1-Month / 1% Ca

18 Months / 0.02% Ca
18 Months / 1% Ca
1 Month / 0.02% Ca
1 Month / 1% Ca
FIGURE 7. Effect of dietary Ca deficiency on intestinal CaBP-9K mRNA expression. CaBP-9K mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old rats fed the 1% Ca diet after correction for background and normalization to pd(T)18. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between rats of the same age fed different diets. ° Indicates means significantly (P <0.05) different between 1- and 18-mo-old rats fed the same diet.
Intestine CaBP-9K mRNA Fraction of 1-Month / 1% Ca

18 Months / 0.02% Ca
18 Months / 1% Ca
1 Month / 0.02% Ca
1 Month / 1% Ca
FIGURE 8. Effect of age and Ca deficiency on intestinal 24-hydroxylase mRNA. 24-
Hydroxylase mRNA values are from slot blots and are presented as a fraction of the value for 1-mo-old rats fed the 1% Ca diet after correction for background and normalization to pd(T)_{18}. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA).

\(^{o}\) Indicates means significantly (P <0.05) different between 1- and 18-mo-old rats fed the same diet. \(^{\#}\) Indicates means significantly (P <0.05) different between rats of the same age fed different diets.
Intestine 24-Hydroxylase mRNA
Fraction of 1-Month / 1% Ca

1 Month / 1% Ca
1 Month / 0.02% Ca
18 Months / 1% Ca
18 Months / 0.02% Ca
FIGURE 9. Effect of dietary Ca deficiency on plasma 1,25(OH)₂D₃ concentration. Values are means ± SEM for six rats. * Indicates means significantly (P < 0.05) different between rats of the same age fed different diets. 0 Indicates means significantly (P < 0.05) different between 1- and 18-mo-old rats fed the same diet.
were not changed by Ca deficiency, however, in old rats (Fig. 9). In this experiment, plasma \(1,25(\text{OH})_2\text{D}_3\) concentrations were not affected by aging (Fig. 9).
DISCUSSION

Changes often occur with aging in factors that are part of the PTH-vitamin D endocrine system (9, 10, 11, 12, 15). Because this system maintains homeostasis of plasma and extracellular fluid Ca (2, 3), changes in these factors may result in inappropriate responses to perturbations of Ca balance. Inadequate maintenance of Ca homeostasis can lead to increased incidence of diseases such as osteoporosis. Because of these age-related changes in regulation of Ca balance, this study compared adaptive responses of young (1-mo-old) and old (18-mo-old) male rats to dietary Ca deprivation. Specifically, we examined the expression of 1,25(OH)₂D₃-regulated genes whose products are involved in regulation of Ca homeostasis.

Dietary Ca deficiency caused a tremendous increase in plasma 1,25(OH)₂D₃ concentrations in young, but not in old, rats (Fig. 9). This response influenced genes stimulated by 1,25(OH)₂D₃ in the intestine. Expression of both CaBP-9K mRNA (Fig. 7) and 24-hydroxylase mRNA (Fig. 8) was increased in intestine of young, but not old rats in response to Ca depletion. Intestinal VDR and VDR mRNA contents, however, were not changed by Ca deficiency in rats of either age (Figs. 5 and 6). Goff et al (1) found similar results for intestinal VDR concentrations in young rats fed a Ca deficient diet. Increased 24-hydroxylase activity, as a result of increased expression 24-hydroxylase mRNA, could cause a decrease in the tissue concentrations of 1,25(OH)₂D₃ in intestine (18, 43), effectively preventing up-regulation of VDR by 1,25(OH)₂D₃ in intestine.

The 1,25(OH)₂D₃ hormone acts to stimulate rat intestinal CaBP-9K gene expression by both rapid stimulation of transcription and post-transcriptional stabilization of the CaBP-9K transcript (44). These different actions of 1,25(OH)₂D₃ may explain the differences in expression of VDR and CaBP-9K in intestine in response to Ca depletion. Huang et al (45) found that, in vitamin D deficient adult rats, increased expression of intestinal CaBP-9K mRNA in response to exogenous 1,25(OH)₂D₃ treatment was not accompanied by a corresponding increase in VDR synthesis.

Increased intestinal 24-hydroxylase mRNA content during Ca deficiency likely leads to greater 24-hydroxylase activity as part of a mechanism by which 1,25(OH)₂D₃ regulates its own plasma concentrations (19, 46). Additionally, in a state of Ca depletion, when plasma PTH concentrations are elevated, 24-hydroxylase activity in kidney is suppressed.
Therefore, the intestine would become more important as a catabolic site for excess 1,25(OH)_{2}D_{3} during Ca deficiency.

In contrast to what happened in the intestine, the expression of genes regulated by 1,25(OH)_{2}D_{3} in kidney was decreased in young rats in response to Ca deficiency (Figs. 1-4). These decreases did not occur in kidneys of old rats (Figs. 1-4). Others have found that Ca deficiency results in decreased activity of renal 24-hydroxylase in young rats (13, 43, 48, 49) but not in old rats (13). These findings correlate with our results for the 24-hydroxylase message. The difference between intestine and kidney in response to Ca depletion can be explained in part by the role of PTH. In the kidney, PTH suppresses 1,25(OH)_{2}D_{3}-stimulated expression of 24-hydroxylase, but PTH has no effect on 1,25(OH)_{2}D_{3}-stimulated expression of 24-hydroxylase in intestine (24). The PTH also negatively regulates VDR expression (29). Along with the fact that PTH receptors have been found in kidney, but not in intestine, these results suggest that PTH has no direct effects on intestine.

It is not known whether PTH directly affects CaBP-28K gene expression in kidney. Expression of renal CaBP-28K is dependent on 1,25(OH)_{2}D_{3} (2), however, and VDR mediate 1,25(OH)_{2}D_{3}-stimulated gene expression (7, 50). Therefore, PTH could indirectly down-regulate renal CaBP-28K expression through suppression of the VDR gene, despite high plasma 1,25(OH)_{2}D_{3} concentrations. In contrast to our results, Huang and Christakos (51) showed that abundance of rat renal CaBP-28K and CaBP-28K mRNA was unchanged by low dietary Ca, although plasma 1,25(OH)_{2}D_{3} concentrations were markedly elevated. Additionally, Bogden et al (52) showed increased renal CaBP-28K concentrations in weanling rats fed a 0.1% Ca diet compared with those fed a 2.5% Ca diet for one yr.

Renal PTH receptors are down-regulated in old rats (32), causing a blunting of response to changes in PTH concentrations (33). Armbrecht et al (53) showed that the degree of stimulation of cAMP production by PTH was decreased by 25% in 12-mo-old male rats compared with 1-mo-old rats. This down-regulation of PTH receptors in old rats can explain the lack of change in kidney content of VDR, VDR mRNA, 24-hydroxylase mRNA, and CaBP-28K mRNA, as well as the lack of increase in plasma 1,25(OH)_{2}D_{3} concentrations in old rats fed a Ca deficient diet.

Abundance of CaBP-28K mRNA was lower in old rats than in young rats (Fig. 3), despite unchanged plasma 1,25(OH)_{2}D_{3} concentrations (Fig. 9), kidney VDR content (Fig. 1), and kidney VDR mRNA abundance (Fig. 2). Koszewski et al (54) also found that renal
VDR concentration did not change with aging in male rats. Additionally, however, Koszewski et al (54) found that VDR from kidneys of old rats had decreased affinity for binding to DNA-cellulose compared with VDR from kidneys of young rats. This decrease in expression of renal CaBP-28K mRNA may be a result of decreased binding of VDR to the DNA (54).

Abundance of 24-hydroxylase mRNA in kidneys (Fig. 4) increased with aging. An increase in renal 24-hydroxylase activity also occurs with aging (see Paper I). These results correspond with observed increases in renal 24,25(OH)₂D₃ production (13, 53) and metabolic clearance rate of 1,25(OH)₂D₃ (14) with aging. In contrast, Armbrecht and Boltz (28) found that the expression of 24-hydroxylase mRNA in kidney was lower in old rats throughout a 48-h period after a dose of 1,25(OH)₂D₃. The rats in this experiment (28), however, were first made vitamin D deficient by feeding a low Ca, 0.8% strontium diet for 6 d. Any other physiological effects of this treatment are unknown; so, this data cannot be used to explain what is happening during aging of normal animals. Down-regulation of PTH receptors seen in old rats (16, 32, 33) would result in an increase in 24-hydroxylase activity by way of decreasing suppression of the enzyme, and plasma 1,25(OH)₂D₃ concentrations were unchanged by aging in this experiment (Fig. 9). It seems then that the PTH effects must override the effects of 1,25(OH)₂D₃.

These results suggest that old rats are unable to adapt to dietary Ca deficiency. The reason for this inability to adapt seems to be because they are unable to respond to Ca deficiency by increasing renal production of 1,25(OH)₂D₃. Without an adaptive response to Ca deficiency, old rats are unable to appropriately regulate genes whose products are responsible for maintenance of Ca homeostasis. This may contribute to age-related bone loss.
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The most notable finding from the first paper was that females had less intestinal CaBP-9K and CaBP-9K mRNA than did males at sexual maturity (2.5-mo-old), when a large part of bone development takes place. Females also had lower plasma 1,25(OH)_2D_3 concentrations compared with males at this age. Females may have decreased 1α-hydroxylase activity compared with males. Concentrations of plasma PTH, which stimulates 1α-hydroxylase activity, also were greater in males than in females at 2.5 mo of age. Both kidney and intestinal 24-hydroxylase activities were lower in females than in males at 2.5 mo of age. Lower plasma 1,25(OH)_2D_3 levels in females, therefore, are not a result of increased catabolism of 1,25(OH)_2D_3. Because females have less CaBP-9K and CaBP-9K mRNA intestine than do males in at developing ages, they are likely to have poorer absorption of dietary Ca and may not adapt as well as do males to Ca deprivation. This could contribute to failure of females to attain as great of peak bone mass as do males. This study of males and females suggests that it is important to look at factors which may contribute to involutional osteoporosis during bone development, before age-related bone loss begins, and not just after the disease has taken over.

An important observation from these studies is that expression of the "constitutively" expressed β-actin message changes with aging. This also is true for another "constitutive" protein, cyclophilin. This is a very important fact to know when choosing controls for aging studies, because if the control also is changing, results will be altered. These observations offer a good example of why not to just assume that the way "everyone else" does it is necessarily the right way.

These studies confirm at the message level that old rats adapt less well than do young rats to dietary Ca restriction and seem to have a blunted responsiveness to PTH. This lack of adaptation to dietary Ca depletion is at least in part a result of decreased expression of intestinal CaBP-9K, which important for absorption of dietary Ca, in older animals.

The increased catabolism of 1,25(OH)_2D_3 in kidney via 24-hydroxylase found in old rats is a unique new observation. This increased catabolism would add to the already decreased 1,25(OH)_2D_3 synthesis rates found in older animals to further decrease plasma 1,25(OH)_2D_3 concentrations in older animals. Because 24-hydroxylase activity declined with age in intestine, the mechanisms of enzyme control must be tissue-specific. An
interesting future project would be to determine the specific cause of increased renal 24-hydroxylase activity in old animals. It could be a result of simply lack of suppression by PTH, or possibly increased sensitivity to 1,25(OH)₂D₃. Perhaps other stimulatory or inhibitory factors are involved as well.
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APPENDIX. AGE EFFECTS ON PROBES USED FOR NORMALIZATION OF NORTHERN BLOTS
AGE EFFECTS ON PROBES USED FOR NORMALIZATION OF NORTHERN BLOTS

When these studies were begun, a β-actin cDNA probe was used to check for accuracy of spectrophotometric quantitation and loading of poly(A)+ RNA onto gels for Northern blots. In these beginning studies, RNA from intestine of young male rats was used. In order to determine what quantity of poly(A)+ RNA was needed to detect VDR mRNA, a Northern blot was run with 5, 10, 20, and 40 μg of poly(A)+ RNA from the intestine of a 1-mo-old male rat (Fig. A1). Considering results from probing the blot with β-actin and VDR cDNAs, quantitation and gel-loading seem to be accurate, and β-actin mRNA seems to be an appropriate control.

When comparing VDR mRNA in intestine between 1-mo-old (young) and 18-mo-old (old) male rats, we observed that VDR mRNA content declined with age in intestine, as does the concentration of VDR protein. The β-actin mRNA content also seemed to decline with age in intestine, however. To clarify what was happening, poly(A)+ RNA was very carefully re-quantitated, and a Northern blot was run with 20 μg of poly(A)+ RNA from young, and 40 μg of poly(A)+ RNA from old rat intestine (Fig. A2). Abundance of VDR and β-actin mRNAs appears to be approximately equal in the two lanes (Fig. A2). This result would imply that abundance of both messages decreases with age. Additionally, a cyclophilin cDNA probe was tried as an alternative control. The cyclophilin mRNA content also seemed to decrease with age in intestine (Fig. A2).

The β-actin mRNA is commonly used for normalization of Northern blots, because β-actin mRNA is considered to be constitutively expressed, and so is not altered by most experimental treatments. Cyclophilin mRNA also is considered to be constitutively expressed. The observation that abundance of β-actin and cyclophilin mRNAs in rat intestine seem to decrease with age, however, would incorrectly alter quantitative results from Northern blots of RNA from animals of different ages that were corrected for mRNA loading with either β-actin or cyclophilin mRNA. For example, if the intestinal VDR mRNA, which declines with age, were normalized for β-actin mRNA content, it would seem quantitatively that no change occurred in intestinal VDR mRNA with aging. This is the result we observed.

We also considered the possibility that the degree of enrichment of poly(A)+ RNA prepared by oligo-d(T)-cellulose chromatography was different for RNA from young and
FIGURE A1. Northern blot of 5, 10, 20, and 40 μg of intestinal poly(A)+ RNA from a 1-month-old male rat. The blot was probed with both VDR cDNA and β-actin cDNA to test the accuracy of loading poly(A)+ RNA on gels.
Poly (A) + RNA
5 μg  10 μg  20 μg  40 μg

Intestine from 1-mo-old Rat
FIGURE A2. Northern blot of 20 μg of intestinal poly(A)+ RNA from a 1-mo-old male rat, and 40 μg of intestinal poly(A)+ RNA from an 18-mo-old male rat. The blot was probed with VDR cDNA, β-actin cDNA, and cyclophilin cDNA.
Poly (A) + RNA

Young  Old
20 µg  40 µg

4.5 Kb  VDR

2.1 Kb  β-Actin

1.0 Kb  Cyclophilin
old rats, or that old rats had less poly(A)+ RNA in than did young rats, because every mRNA that had been tested so far seemed to decline with age in intestine. For these reasons, we decided to try using a pd(T)$_{18}$ probe to normalize for quantity of poly(A)$^+$ RNA applied to membranes. Because the pd(T)$_{18}$ binds to poly(A)$^+$ tails on mRNA, the pd(T)$_{18}$ probe measures the total amount of poly(A)$^+$ RNA on the blot instead of only the message from one gene. Use of the pd(T)$_{18}$ probe also allows for correction for any differences in the degree of recovery of poly(A)$^+$ RNA from each total RNA sample in the poly(A)$^+$ RNA selection process. Unlike β-actin mRNA, poly(A)$^+$ RNA, as detected by using a pd(T)$_{18}$ probe, did not change with age in intestine or kidney of rats (Fig. A3). Therefore, we chose to use pd(T)$_{18}$ as a control probe for aging studies.
FIGURE A3. Effect of age on kidney and intestinal β-actin mRNA and poly(A)+ RNA expression. Values for intestinal β-actin mRNA are the mean ± SEM for 6 rats. Values for kidney β-actin mRNA are the mean ± SEM for 12 rats. Values for intestinal poly(A)+ RNA are the mean ± SEM for 24 rats. Values for kidney poly(A)+ RNA are the mean ± SEM for 27-28 rats. Values are from Northern and slot blots and are presented as a fraction of the lane on the blot with the greatest number of counts, after correction for background. Quantitation was done by using an AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA). * Indicates means significantly (P <0.05) different between rats of different ages.