VITAMIN D RECEPTORS,

TISSUE GROWTH

AND

INSULIN DEPENDENT DIABETES

by

LISA A. STONE

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UNIVERSITY OF OTTAWA

ABSTRACT

The studies described in this thesis evaluate the effect of insulin dependent diabetes on vitamin D receptors (VDR) and tissue growth in two models of insulin dependent diabetes: the streptozotocin-induced diabetic rat and the spontaneously diabetic BB rat. In both models, untreated diabetes was associated with decreased circulating 1,25-dihydroxycholecalciferol (1,25 DHCC), disturbed mineral homeostasis and up regulation of intestinal VDRs. The increase in unoccupied VDRs was associated with compensatory tissue growth in the intestine and hyperplasia. Low circulating 1,25 DHCC prevented amplification of the action of 1,25 DHCC despite up-regulation of intestinal VDRs, as evidenced by a reduction in calbindin D-9K in the diabetic intestine. The above parameters were normalized in STZ diabetic rats treated with exogenous insulin. There were no alterations in VDR numbers in kidneys or thymus of untreated diabetic animals, indicating that up-regulation of VDRs did not occur in all vitamin D target tissues. Unlike the intestine, compensatory growth in the kidney was not associated with hyperplasia, suggesting tissue specific changes in 1,25 DHCC metabolism during untreated diabetes which may be related to hyperplasia.

DEDICATION

This thesis is dedicated to

David & Simon

and

Mom & Dad

for all your love & support

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LITERATURE REVIEW

I. VITAMIN D METABOLISM

1,25 Dihydroxycholecalciferol (1,25 DHCC), the active metabolite of vitamin D_3 (cholecalciferol), functions as a steroid hormone with a wide range of biological activities. Its classical role in mineral homeostasis has been well documented; evidence is now emerging to indicate that 1,25 DHCC has other functions, such as in immunoregulation and in the modulation of cell differentiation and proliferation (Minghetti and Norman, 1988).

a) **Synthesis and Catabolism of Vitamin D_3:** Although vitamin D_3 may be obtained through diet (which resulted in its early classification as a vitamin), it is also synthesized in the skin by the action of ultraviolet light on 7-dehydrocholesterol. It is then hydroxylated (at carbon 25 in the liver, and at carbon 1 in the kidney) to form 1,25 DHCC, the most potent naturally produced metabolite of vitamin D_3 (Reichel et al, 1989). The production of 1,25 DHCC from 25 hydroxycholecalciferol (25 HCC) in the kidney is catalyzed by 25 HCC-1-hydroxylase, a mixed function oxidase similar to the adrenal steroid hydroxylases. The activity of this enzyme is the control point of 1,25 DHCC production, and is directly related to the calcium status of the individual. Parathyroid hormone (PTH), which is released in response to low blood calcium, stimulates 1-hydroxylase activity; the resulting increase in 1,25 DHCC stimulates mineral absorption at the level of the intestine and mineral

conservation in the kidney. The subsequent increase in the blood calcium decreases 1,25 DHCC production by inhibiting further PTH release; 1,25 DHCC itself inhibits PTH synthesis at the transcriptional level (Reichel et al, 1989). 1,25 DHCC also regulates its own production by directly inhibiting the renal 1-hydroxylase and inducing a 24-hydroxylase in kidney and extrarenal tissues. This 24- hydroxylase can act on both 25 HCC and 1,25 DHCC to produce intermediates in the catabolism of vitamin D (Reichel et al, 1989). The biosynthesis of 1,25 DHCC is affected by other hormones as well, such as insulin, estrogen, prolactin and growth hormone (Pike, 1991).

1,25 DHCC and the other metabolites of vitamin D_3 are carried in the blood by two proteins, the vitamin D binding protein (DBP) and albumin. DBP binds 85 - 88% of the circulating 25 HCC and 1,25 DHCC; albumin binds 12 - 15%. Although only a small fraction (0.4%) of 1,25 DHCC circulates in the free form it has been suggested (Bikle and Gee, 1988) that cells respond to the free, not the protein-bound fraction of circulating 1,25 DHCC. However, more recent studies suggest a role for DBP in entry or intracellular transport of 1,25 DHCC and 25 HCC (McLeod and Cooke, 1989).

b) Biological Actions of 1,25 DHCC: The presence of the vitamin D receptor (VDR) in target tissues unrelated to calcium homeostasis has suggested additional functions for 1,25 DHCC. To date, these functions include stimulation of insulin secretion from the pancreas and modulation of prolactin synthesis in the pituitary gland as well as effects on thyroids, gonads and

muscle (Reichel et al, 1989). 1,25 DHCC is also involved in immunoregulation; it suppresses production of interleukin-2, granulocyte-macrophage colony-stimulating factor and interferon-gamma by activated human lymphocytes, and inhibits the synthesis of immunoglobulins (Reichel et al, 1989). This literature review will focus on two of the major functions of 1,25 DHCC; namely, maintenance of mineral homeostasis and modulation of cell differentiation.

(i) **Mineral homeostasis:** 1,25 DHCC maintains serum calcium and phosphate levels and provides minerals for bone formation primarily by stimulating their absorption from the intestine and reabsorption in the kidney. Calbindin D-9K, a 1,25 DHCC-induced calcium binding protein present in mammalian intestine, is thought to be involved in the increased calcium absorption that occurs in response to 1,25 DHCC treatment. Levels of calbindin D-9K mRNA are higher in the absorptive enterocytes (the highly differentiated cells at the tips of duodenal villi) than in the undifferentiated proliferative crypt cells. A 28kD form of calbindin, which is also sensitive to 1,25 DHCC, has been implicated in active calcium transport in the distal tubules of the kidney (Thomasset et al, 1987); however, its precise function remains unknown.

As discussed in section (a) the feedback of 1,25 DHCC on its own synthesis and catabolism, as well as its effect on serum PTH levels, allow stringent control of the production of 1,25 DHCC and thus of blood calcium.

Maintenance of extracellular mineral homeostasis explains, in part, the effects of 1,25 DHCC on bone since bone mineralization is impaired during hypocalcemia or hypophosphatemia. In addition, 1,25 DHCC directly affects bone formation by stimulating differentiation of osteoblasts (bone-forming cells) and production of alkaline phosphatase, osteocalcin and other bone matrix proteins. 1,25 DHCC also induces formation of osteoclast (bone-resorbing cells) from bone marrow stem cells (Reichel et al, 1989). The importance of 1,25 DHCC in bone metabolism is graphically illustrated in cases of vitamin D deficiency, which result in osteomalacia and rickets.

(ii) Cell differentiation: Miyaura et al (1981) first suggested that 1,25 DHCC could induce the differentiation of myeloid leukemia cells into macrophages; since then, the anti-proliferative, differentiation-promoting effect of 1,25 DHCC has been demonstrated in a variety of cancer and normal cells (lung, colon, melanoma, sarcoma) (Reichel et al, 1989). 1,25 DHCC promotes the differentiation of bone marrow stem cells into multinucleated osteoclasts (involving the formation of monocytes and macrophages as precursor cells); these results suggest a link between the skeletal and immune systems with 1,25 DHCC as a common factor (Minghetti and Norman, 1988). This link is supported by reports that states of vitamin D deficiency or resistance are accompanied by decreased resistance to infection.

1,25 DHCC exerts anti-proliferative effects in other cell types as well. Studies with cultured bovine aortic endothelial cells have revealed a vitamin

D microendocrine system with both VDR expression and 1-hydroxylase activity (Merke et al, 1989). These cells exhibit upregulation of VDR and increased 1,25 DHCC production during rapid proliferation, followed by a decrease in VDR and proliferation rate in response to 1,25 DHCC.

The mechanisms for 1,25 DHCC's effects on cell differentiation are still unclear; however, 1,25 DHCC inhibits the transcription of histone H4, c-myc and c-mcb (replication-linked genes) while up-regulating the expression of c-fos and c-fms (differentiation-linked genes) (Minghetti and Norman, 1988). 1,25 DHCC has been shown to induce protein kinase C (PKC) activity in HL-60 and MDBK cells (Obeid et al, 1990; Simboli-Campbell et al, 1992). Since PKC activation appears to be necessary for the differentiation/anti-proliferative effect of 1,25 DHCC on HL-60 cells, 1,25 DHCC may exert these effects through PKC (Martell et al, 1988).

c) **The 1,25 DHCC Receptor (VDR):** The VDR was discovered in 1969 by Haussler and Norman, whose research provided the link between 1,25 DHCC and its actions in the nucleus. Their studies revealed a protein present in chromatin extracts of intestinal mucosa that exhibited high affinity binding of 1,25 DHCC; this protein was later shown to bind other vitamin D metabolites with affinities reflecting their biological potency in vivo (Brumbaugh and Haussler, 1974).

(i) Biochemical properties and subcellular distribution: The VDR is a single polypeptide ranging in size from 52 to 55 kDa in mammals, which binds 1,25 DHCC with high affinity (Kd = 1 to $50x10^{-11}$) (Reichel et al, 1989; Mangelsdorf et al, 1987). The VDR is classified as a member of the nuclear trans-acting receptor family based on its protein structure, functional activity and chromosomal gene organization (Pike et al, 1988). The receptors of this family (which include the steroid hormone receptors as well as the receptors for thyroid hormone and retinoic acid) exert their effects at the genomic level by interacting with DNA and modulating gene expression (Pike, 1991). The VDR possesses a cysteine-rich DNA binding domain in its N-terminal region which forms a "zinc- finger" motif; this domain is the most highly conserved within the receptor family (Pike and Haussler, 1979; Pike et al, 1988). The C-terminal 30kD of the VDR protein contains a hydrophobic pocket which binds 1,25 DHCC; this hormone binding region is less highly conserved among the steroid receptor family (Pike et al, 1988). The region between the hormone binding domain and the DNA binding domain is antigenic and extremely sensitive to proteolysis (Haussler et al, 1988).

Until recently, evidence indicated that the unoccupied VDR was located in the cytoplasm while the steroid-bound receptor was found in the nucleus; a "nuclear translocation mechanism" was proposed as the necessary and perhaps rate-limiting step of steroid hormone action (Brumbaugh and Haussler, 1974; Brumbaugh and Haussler, 1975). Recent immunocytochemical evidence has

indicated that the occupied receptor is found predominantly in the nucleus (Clemens et al, 1988). Upon binding of 1,25 DHCC, the affinity of the DNA-binding site for DNA increases and the steroid-receptor complex binds to target cell DNA; it may then act either to up-regulate or to down-regulate the transcription of genes containing the sequence specific vitamin D response element (Minghetti and Norman, 1988).

(ii) Cellular distribution: The VDR is found in all tissues known to play a role in mineral homeostasis; these include intestine, bone, kidney, parathyroid glands, avian shell gland, chorioallantoic membrane and the mammalian placenta (Haussler, 1986). However, recent studies with a monoclonal antibody specific to the VDR have confirmed that it is also present in most other tissues; these include brain, liver, lung and pancreas (Clemens et al, 1988; Nguyen et al, 1990; Clark et al, 1980). These data are not surprising considering the diverse biological effects now attributed to 1,25 DHCC.

Both in vivo and in vitro studies (Haussler, 1986) have shown that the distribution of the VDR within tissues is limited to specific cell types. For example, in the pancreas, only the beta cells are the target of 1,25 DHCC action (Clark et al, 1980); in bone, osteoblasts but not osteoclasts have detectable levels of VDR (Clemens et al, 1988). In the intestinal epithelium, the VDR is more abundant in the undifferentiated crypt cells than in the highly differentiated enterocytes near the tip of the villus (Clemens et al,

1988). These findings suggest an association between the state of enterocytic cell differentiation and VDR expression.

(iii) VDR regulation: The response of a cell to 1,25 DHCC is determined primarily by the level of the VDR; thus, regulation of VDR expression represents a means of controlling tissue responsiveness. This concept is demonstrated most strikingly in the developing rat intestine. Newborn rat pups show no active intestinal calcium transport and do not express calbindin; neither active calcium transport nor calbindin can be induced by 1,25 DHCC administration. The lack of sensitivity to 1,25 DHCC reflects the lack of VDRs in the intestinal epithelium at this age. As the intestine matures, the VDR becomes expressed in the intestinal epithelium simultaneously with the onset of active calcium transport, calbindin expression and responsiveness to 1,25 DHCC (DeLuca et al, 1982; Burmester et al, 1988). These developmental changes correspond to the time of weaning, when calcium intake is dramatically decreased and active transport of calcium is essential to ensure adequate absorption. Although the underlying basis for these changes is unclear, administration of epidermal growth factor to suckling rats causes precocious intestinal maturation accompanied by induction of the VDR and calbindin expression (Bruns et al, 1989).

1,25 DHCC regulates its own receptor in several ways. Binding of 1,25 DHCC "activates" the VDR and increases its affinity for DNA (Pike et al, 1983). It has been shown that exposure of mouse 3T6 cells to 1,25 DHCC

increases the amount of VDR mRNA and protein (Mangelsdorf et al, 1987; Haussler et al, 1988). Homologous up regulation of the VDR has been confirmed in vivo (Costa and Feldman, 1986; Strom et al, 1989; Sandgren and DeLuca, 1990). Posttranslational receptor phosphorylation occurs in response to 1,25 DHCC (Mangelsdorf et al, 1987; Haussler et al, 1988) but does not seem to affect hormone or DNA binding. Instead, it has been suggested (Hsieh et al, 1991; Jones et al, 1991) that VDR phosphorylation may play a role in transcriptional control or receptor degradation. These studies have shown that the VDR is phosphorylated by PKC and casein kinase II, and that these phosphorylations may be essential for transcriptional activation.

II. ABERRATIONS IN VITAMIN D, BONE AND MINERAL METABOLISM IN DIABETES MELLITUS

Diabetes mellitus type I (insulin dependent diabetes) results in a variety of systemic complications; in recent years it has become evident that bone and mineral metabolism is yet another system affected by this disease. Although adverse skeletal effects of diabetes have been well documented throughout the life cycle, the biochemical basis for these changes is not yet fully understood. This literature review will focus on the abnormalities in vitamin D and mineral metabolism which have been reported during diabetes and their possible relevance to skeletal complications.

a) Human Studies:

(i) Skeletal effects: Fetuses of diabetic mothers have a higher than normal incidence of skeletal malformations such as acrania, cleft palate, polydactylism and caudal regression syndrome (Silberberg, 1986). Children who have been diabetic for about 5 years show a growth deficit of up to 5-6 cm, and are correspondingly underweight (9-12kg). This puts these children about 5 years behind in growth compared to non-diabetic children of the same age (Silberberg, 1986). In a study by Levin et al (1976), 50% of diabetic children had a bone deficit of greater than 10%; Santiago et al (1977) showed that 25% of diabetic children had metacarpal cortical thickness values below the 5% limit for normal children. Bone loss seems to be most rapid at the onset of diabetes, and slows after about 5 years; thereafter bone mineral content remains fairly constant (Rosenbloom, 1977) but is reduced compared to age-matched controls. Similar findings have been reported in adult-onset type I diabetes; again, bone loss is most severe at the onset of diabetes, and stabilizes after 3-5 years. There seems to be greater bone mineral loss with poorly regulated diabetes (McNair, 1988).

The clinical significance of the decrease in bone mass associated with diabetes has not been clearly elucidated. Although some studies have shown an increased rate of skeletal fractures among diabetic patients, much of the data is conflicting. (Hough, 1987). Contradictory data from clinical studies may arise from the heterogeneity of the diabetic populations studied; not only

are there variations in severity and duration of disease, many other factors, such as insulin resistance, alcoholism, intestinal malabsorption, pregnancy and renal disease, can also affect bone metabolism.

(ii) Calcium regulating hormones and plasma minerals: Despite insulin therapy, many diabetics display wide fluctuations in plasma and urinary glucose. Poorly controlled diabetic patients have high urinary calcium, phosphate and magnesium excretion, which is partly accounted for by the osmotic effect of glycosuria. They also exhibit correspondingly reduced plasma levels of calcium and magnesium; however, plasma phosphate levels are normal or increased (McNair, 1988). PTH, which is normally released in response to hypocalcemia, has been found to be normal or low (Frazer et al, 1981; McNair, 1988), although high PTH levels have also been observed in some studies (Nyomba et al, 1986). It has been consistently shown that 1,25 DHCC levels are lower in diabetic patients (Frazer et al, 1981; Nyomba et al, 1986; Aksnes et al, 1988) and that 24,25 dihydroxycholecalciferol (24,25 DHCC), an intermediate in the catabolism of vitamin D_3 is higher (Frazer et al, 1981; Aksnes et al, 1988); moreover, 24,25 DHCC is highest in the first five years after diagnosis of diabetes, and is positively correlated to the severity of bone loss (Frazer et al, 1981). Since 25 HCC is the precursor for both 1,25 DHCC and 24,25 DHCC, this suggests a defect in the regulation of vitamin D hydroxylation in the early stages of diabetes, which may be related to an inappropriate response of PTH to hypocalcemia.

b) Experimental Diabetes:

(i) Animal models: Most studies of experimental diabetes have used beta cell toxins to chemically-induce insulin deficiency. Although alloxan has been utilized in the past, streptozotocin (STZ) is now more commonly used to induce diabetes in rats. This antibiotic has a rapid, irreversible cytotoxic action which is highly specific for the pancreatic beta cells; necrosis of these cells can be seen within seven hours of STZ injection. The degree of diabetes can be controlled by varying the dose of STZ injected; 25mg/kg intravenously produces a mild form of diabetes, while 100mg/kg results in severe diabetes (Junod et al, 1967). Induction of diabetes is achieved with very little nephrotoxicity, as evidenced by the ability of exogenous insulin treatment to normalize blood glucose and weight gain in STZ induced diabetic rats.

The discovery in 1974 of a spontaneously diabetic strain of Wistar rat has provided an excellent animal model for human diabetes mellitus type I. The "BB" rat (discovered at the BioBreeding Laboratories in Ottawa) develops insulitis with selective pancreatic beta cell destruction, resulting in insulin dependent diabetes at 40-140 days of age. As is thought to be the case in human diabetes, the onset of diabetes in BB rats seems to involve both genetic and immunological factors. Inheritance may be autosomal recessive and is linked to the major histocompatibility locus. The involvement of the altered immune system is suggested by the presence of insulitis, lymphopenia, passive

transfer of insulitis, presence of islet cell surface antibodies, thyroiditis, and increased susceptibility to infection. The morphological changes in the pancreas are strikingly similar to those which occur in human type I diabetic patients soon after diagnosis (Marliss et al, 1982). However, there are problems associated with this diabetic model: BB diabetic rats are often severely diabetic, exhibiting weight loss and ketosis; the age of onset of diabetes is variable and uncontrollable; BB rats must be housed in semibarrier conditions due to their compromised immune system. Although for these reasons the BB diabetic rat has been used less frequently than the STZ-induced diabetic rat, its resemblance in etiology to human diabetes makes it an invaluable model.

(ii) Skeletal effects: Chronically untreated diabetic rats exhibit impaired bone turnover as indicated by decreased osteoblast number and function and decreased osteoclast number (Hough et al, 1981; Bouillon et al, 1988). Biochemical parameters of bone turnover (skeletal alkaline phosphatase, urinary hydroxyproline excretion) are also decreased (Hough et al, 1981). These are accompanied by reductions in bone mass although the composition of the bone does not seem to be altered (Verhaeghe et al, 1989).

(iii) Calcium regulating hormones and plasma minerals: Plasma calcium has been shown to be normal (Schneider et al, 1976; Hough et al, 1983; Wood et al, 1984; Nyomba et al, 1985) or decreased (Schedl et al, 1978;

Wilson et al, 1982; Ishidia et al, 1983) in STZ diabetic rats; similar variations are observed in plasma phosphorus levels. Urinary calcium excretion is increased as much as three-fold compared to controls (Wood et al, 1984). 1,25 DHCC and 1-hydroxylase are consistently decreased while 25 HCC is normal (Schneider et al, 1977b; Wilson et al, 1982; Seino et al, 1983; Schedl et al, 1984; Nyomba et al, 1985). Similar results for calcium and vitamin D metabolites have been obtained using BB rats (Nyomba et al, 1989). 24,25 DHCC levels have not been assessed in most studies; Wilson et al (1982) reported decreased 24,25 DHCC in untreated diabetic rats compared to control animals, however the activity of the 24-hydroxylase was increased in diabetes (Hough et al, 1983; Wongsurawat et al, 1983). Data on PTH levels is scarce and conflicting; Schedl et al (1978) found PTH was higher in diabetic rats, while Hough et al (1981) observed decreased PTH levels with diabetes. Exogenous insulin therapy to normalize blood glucose corrects the abnormalities in plasma minerals and vitamin D metabolites.

Chronically untreated diabetes is associated with decreased protein synthesis; therefore both STZ-induced diabetic rats and BB rats have decreased levels of vitamin D binding protein (DBP). Calculation of free 1,25 DHCC suggests it may be in normal (Nyomba et al, 1985) or increased (Nyomba et al, 1989) in chronically diabetic rats.

(iv) Vitamin D receptors and target organ responsiveness: The intestinal mucosa is the classic target tissue for 1,25 DHCC. 1,25 DHCC acts

to increase absorption of calcium from the intestine, possibly by increasing the amount of intestinal calbindin. Although diabetic rats have depressed body growth, they exhibit enhanced intestinal growth, with increased absorption of nutrients such as hexoses (Schedl et al, 1971) and amino acids (Lal et al, 1974). However, calcium absorption is decreased in short-term diabetes (Schneider et al, 1972; Schneider et al, 1977a; Schneider et al, 1976; Wood et al, 1984). Chronic diabetes in the rat has been associated with both increased (Hough et al, 1982) and decreased (Nyomba et al, 1989) calcium absorption. Correspondingly, duodenal calbindin levels are drastically decreased in both short-term (Schneider et al, 1973; Schneider et al, 1974) and chronic (Nyomba et al, 1989) diabetes. Treatment with insulin or with 1,25 DHCC, but not with 25 HCC, restores calcium transport to normal (Schneider et al, 1977a; Schneider et al, 1976). Thus the response of the intestine to 1,25 DHCC does not seem to be impaired in the diabetic state.

There is limited data available on the characteristics of the VDR in the diabetic state. Seino et al (1983) found an increase in intestinal VDR in untreated STZ diabetic rats. Conversely, chronically untreated BB diabetic rats exhibited decreased levels of VDR in the intestine (Nyomba et al, 1989). The conflicting data obtained with these two diabetic models indicates that further studies are required to elucidate whether insulin deficiency per se affects the expression or function of the VDR. Although in vitro data suggests a link between the rate of cell proliferation and VDR expression, previous

studies did not correlate changes in VDR number with indices of intestinal growth during diabetes. To date, no studies have thoroughly examined the effect of diabetes on VDRs in target tissues other than the intestine.

The aims of the studies described in this thesis were to determine:

(a) the effect of short term diabetes on circulating levels of vitamin D metabolites and minerals;

(b) the effect of diabetes on VDR in intestine, kidney and thymus in relation to tissue growth indices;

(c) the relevance of altered VDR during diabetes to intestinal function using indices such as sucrase, alkaline phosphatase and calbindin;

(d) the ability of insulin to correct abnormalities in vitamin D metabolism during STZ diabetes.

In order to determine the generality of the effects of insulin deficiency, two models of insulin dependent diabetes were chosen for these studies: the STZ-induced diabetic rat and the spontaneously diabetic BB rat.

MATERIALS AND METHODS

I. ANIMALS, DIETS AND HOUSING

a) Streptozotocin-Induced Diabetic Rats: Male weanling Sprague Dawley rats (Charles River, Montreal, Quebec) were individually housed in stainless steel hanging cages and fed AIN semi-purified (pelleted) normal rat diet and tap water ad libitum. The diet, containing 1 I.U. cholecalciferol per gram diet, 0.6% calcium and 0.4% phosphorus, was obtained from US Biochemicals (Clevelend, Ohio). After consuming this diet for approximately one week, rats weighing 120-130 grams were injected intraperitoneally with 80 mg/kg body wt streptozotocin (STZ) in citrate buffer (pH 4.5) to induce diabetes; control rats were injected with an equal volume of citrate buffer. Animals developing diabetes within three days of STZ injection (as determined by urinary glucose levels > 55 mmol/l using Clinistix) were used for the studies. With this dose of STZ, untreated diabetic animals grew at a reduced rate, but did not lose weight or develop ketosis during the experimental period.

In some experiments, diabetic animals were treated every morning with NPH insulin (Eli Lilly, Indianapolis, IN); the amount given was adjusted daily to produce early morning trace glucosuria (less than 2.8 mmol/l).

Control, untreated and treated diabetic animals were sacrificed within 20 days of induction of diabetes.

b) Spontaneously Diabetic BB Rats: Spontaneously diabetic male BB

Wistar rats were obtained from Dr. P. Thibert, of the Health Protection Branch, Health and Welfare Canada, Ottawa and housed in semi-barrier conditions. Groups of five to six diabetic rats (mean age varying from 45 to 60 days) were received one day after detection of diabetes by glucosuria and were not treated with insulin. Male age-matched Wistar rats bred from the same Wistar colony from which the diabetic rats were originally bred in 1967 were used as controls.

Animals were housed in individual stainless steel hanging cages with free access to Purina lab chow and tap water. They were weighed daily, and diabetic animals were tested with Clinistix each day for glucosuria and ketonuria. Controls and diabetics were sacrificed within seven days of receipt; those exhibiting ketonuria were not used in the studies.

II. EXPERIMENTAL DETAILS

After an overnight fast, animals were sacrificed by decapitation, and blood from the neck was collected into heparinized tubes for assays of vitamin D metabolites, minerals, glucose and insulin. Intestine, thymus and kidneys were removed and placed in ice-cold phosphate buffered saline (PBS).

III. BLOOD ANALYSIS

a) **Plasma minerals:** Plasma calcium and magnesium were analysed by atomic absorption spectrophotometry (Instrumentation Laboratories model

#551) in the presence of 1% LaCl, using Fisher certified atomic absorption standards for calcium and magnesium. Ionized calcium in fresh plasma was determined using a CIBA Corning Ca^{++} analyser (Model 634). Phosphorus was measured by the colorimetric method of Fiske and Subbarow (1925).

b) Vitamin D Metabolites: Using the method of Rheinhardt and Hollis (1986) vitamin D metabolites were first extracted from plasma with acetonitrile and then isolated using C18 and silica Sep-Paks (Waters). 1,25 DHCC was measured by radioreceptor assay (Rheinhardt and Hollis, 1986) with calf thymus cytosol as source of VDR. 25 HCC levels were determined in separate samples by competitive binding assay with rachitic rat plasma as a source of vitamin D binding protein (Shephard and DeLuca, 1980).

c) Insulin and Glucose: Serum insulin was measured, using rat insulin as a standard, with a commercially available radioimmunoassay kit obtained from Immunonuclear Corp. (Stillwater, MINN). Plasma glucose was analysed using a reagent kit (Sigma) based upon the glucose oxidase method.

IV. VITAMIN D RECEPTOR BINDING ASSAY

a) Tissue Preparation:

(i) Intestine: The first 10cm of the small intestine distal to the stomach was removed, flushed with PBS, split longitudinally, blotted dry and gently scraped with a glass slide. The mucosa obtained was weighed, washed

with PBS and homogenized in 20 volumes of a high salt buffer (TKEDMS, pH 7.4) containing 10mM Tris-HCl, 300mM KCl, 1mM EDTA, 10mM sodium molybdate and 0.02% soybean trypsin inhibitor. The homogenate was centrifuged at 200,000g for 45 min (Beckman L2-65B) and the supernatant (intestinal cytosol) was used for 1,25 DHCC binding studies.

(ii) Kidney and thymus: To eliminate contamination of cytosol from these tissues with plasma vitamin D binding protein, the method of Ishida et al (1988) was used. Tissues were homogenized in 40 volumes of low salt buffer, TEDMS (TKEDMS without KCl), and centrifuged for 10 min at 5,000g (Sorvall RC-5B). The resulting pellet was weighed, homogenized in 10 volumes of TKEDMS and centrifuged for 10 min at 5,000g. The supernatant was then spun at 200,000g for 45 min and the supernatant (a clear chromatin extract) was used for 1,25 DHCC binding studies.

b) 1,25 DHCC Binding Assay: 160µl of intestinal cytosol or renal and thymic chromatin extracts were incubated with 20µl of ^{3}H-1,25 DHCC, to achieve final concentrations ranging from 0.06- 1.2nM 1,25 DHCC for 3 hours at 4°C. Non-specific binding was assessed in parallel tubes which also contained a 200-fold excess of unlabelled 1,25 DHCC. Bound and free hormone were separated by addition of 200µl dextran coated charcoal, incubation for 20 min and centrifugation at 3,500g for 10 min (Beckman J6-B). The supernatants containing the bound ^{3}H-1,25 DHCC were poured into

scintillation vials and counted in a Beckman liquid scintillation counter. Saturation plots of 1,25 DHCC binding to tissues from control and diabetic animals indicated that saturation of the VDR was achieved under these conditions (see appendix figure (i)). Data was analysed by the method of Scatchard (using computerized linear regression of data points to obtain best fit lines) to determine 1,25 DHCC specific binding sites and the dissociation constant (Kd) of binding. In some experiments, only specific binding sites were quantitated by incubation with a saturating concentration (1.2nM) of ^{3}H-1,25 DHCC.

c) FPLC Determination of Molecular Weight of the 1,25 DHCC Binding Component: Intestinal cytosol (2mg protein/ml) was incubated with 6nM ^{3}H-1,25 DHCC for 3 hours at 4°C. Unbound hormone was removed with charcoal treatment (as described above); bound hormone was applied to a Superose 12 column on a Pharmacia FPLC (P3500 pump) and eluted with TKEDMS containing aprotinin (24 trypsin inhibitor units/ml, 1ml/100ml buffer) and leupeptin (0.02%) at a flow rate of 0.3ml/min. One ml fractions were collected and counted for radioactivity. ^{14}C-albumin (MW=66kD) was used as an internal standard.

V. CALBINDIN D-9K AND CALBINDIN D-28K

The proximal 10cm of the intestine (duodenum) was removed, rinsed with PBS containing aprotinin (24 trypsin inhibitor units/ml, 1ml/100ml buffer)

and phenylmethylsulfanyl fluoride (0.05 mg/ml), and frozen in liquid nitrogen. The samples were sent on dry ice to Dr. M.E. Bruns, University of Virginia, and calbindin D-9K was analysed by radioimmunodiffusion assay using anti-rat intestinal calbindin D-9K polyclonal antiserum (Bruns et al, 1989). Kidneys were frozen in liquid nitrogen and analysed by radioimmunoassay in collaboration with Dr. S. Christakos at the University of Medicine and Dentistry in New Jersey, using anti-rat renal calbindin D-28K polyclonal antiserum (Sonenberg et al, 1984).

VI. INTESTINAL ENZYME ASSAYS

a) **Sucrase:** Intestinal mucosa was homogenized in five volumes of PBS, and sucrase was assayed by the method of Dahlqvist (1968). Briefly, a 0.056M sucrose solution in maleate buffer (1.161g maleic acid + 17.4 ml 1N NaOH/100ml, pH6.4) was added to samples and blank, and allowed to incubate at room temperature for 15 minutes. The samples were then diluted with water and boiled to inactivate the enzyme. The glucose produced by the action of sucrase was then assayed using a glucose oxidase reagent kit obtained from Sigma.

b) **Alkaline Phosphatase:** The mucosal homogenate prepared for sucrase analysis was diluted 50 times, and 20µl was added to 1ml of 0.5M AMP buffer containing (per 100ml) 0.2105g p-nitrophenylphosphate, 0.05g bovine serum albumin (BSA) and 0.0407g $MgCl_2$. Tissue blanks, to which 100µl 5N NaOH

were added before addition of homogenate, were run in parallel. The production of p-nitrophenol was monitored as absorbance at 410nm using a kinetic program (Quant II Linear) and a Beckman model #DU-65 spectrophotometer.

VII. OTHER ANALYSES

a) **DNA Analysis:** DNA was extracted from homogenates of intestinal mucosa, kidney and thymus, and quantitated by fluorescence spectroscopy, using the method of Downs and Wilfinger (1983). Homogenates were sonicated briefly, and a volume containing approximately 5mg of protein was added to 1ml of AT extraction solution (1N NH_4OH, 0.2% Triton X-100). After incubation for 10min at 37°C, a 200µl aliquot of the cell lysate was added to 1ml of assay buffer (100mM NaCl, 10mM EDTA, 10mM Tris), centrifuged (2500g, 30 min, 4°C), and placed on ice. DNA standard (200µl, 0.5mg/ml) was added to 1ml AT solution, incubated as above, and a 500µl aliquot was added to 500µl of assay buffer. Sample supernatants (10-100µl) and DNA standard (10-50µl) were added to 3ml of Hoechst 33258 dye solution (2mM EDTA, 50mM Na_2HPO_4, 2M NaCl, 1µg/ml Hoechst 33258 from stock solution of 1mg/ml). Fluorescence was then measured in a Turner model #430 spectrofluorometer (Palo Alto, CA) with an excitation wavelength of 350nm and an emission wavelength of 455nm.

b) **Protein Analysis:** Total protein was assayed in tissue homogenates,

cytosol and chromatin extracts by the method of Bradford (1976) using BSA as a standard.

VIII. STATISTICS

Statistics were analysed using the True Epistat computer program. For comparison between two treatment groups, Student's unpaired t test was used. Comparison of three groups was by one-way ANOVA followed by Newman-Keuls analysis to determine differences between means when a significant F value was found using ANOVA. Differences were considered significant if a p value of less than 0.05 was determined. Data are expressed as mean +/- standard error.

CHAPTER I

EFFECT OF UNCONTROLLED STZ DIABETES ON 1,25 DHCC METABOLISM

EXPERIMENTAL DESIGN

In this preliminary study, rats were studied 10 and 20 days after induction of diabetes by STZ administration. Each group (control and diabetic, days 10 and 20) contained four rats and all were fed AIN semi-purified diet. Indices of glucose homeostasis, plasma minerals and vitamin D metabolites were assayed at each time point. Specific binding sites for 1,25 DHCC were determined by Scatchard analysis of equilibrium binding data.

The objectives of this study were to establish conditions for STZ diabetes studies, and to confirm reports of alterations in 1,25 DHCC, plasma minerals and VDRs in the diabetic state. Two time points were chosen to determine when these changes occurred; this was necessary to establish conditions for subsequent experiments.

RESULTS

a) **Body Weight and Blood Parameters** (Table I-1, I-2): At both time points, STZ diabetic rats exhibited decreased body weights and plasma insulin levels and increased plasma glucose compared to control animals. Ten days after STZ, diabetic rats had significantly reduced circulating 1,25 DHCC and

increased 25 DHCC; however, they were normocalcemic and normophosphatemic. After 20 days, 1,25 DHCC remained low and 25 HCC remained high in diabetic rats; at this point diabetic rats were also hypocalcemic and hypophosphatemic relative to control animals.

b) **VDR Analysis:** Intestinal VDR content was elevated approximately two-fold in diabetic rats compared to control (control=120±19; diabetic=242±43 fmoles/mg protein), but no correlation to the duration of diabetes was evident. Despite the lower body weight in diabetic animals, the absolute intestinal mucosal weights were significantly elevated in diabetic animals compared to control.

Table I-1: Body weights and blood parameters in fasted control and STZ diabetic rats 10 days after administration of streptozotocin[1].

	CONTROL	DIABETIC
BODY WEIGHT (g)	217 ± 7	156 ± 10*
PLASMA		
Glucose (mg%)	95.0 ± 2.4	371.3 ± 16.6*
Insulin (ng/ml)	4.04 ± 1.15	0.64 ± 0.13*
Calcium (mg%)	12.25 ± 0.56	11.83 ± 0.46
Phosphorus (mg%)	9.85 ± 0.17	9.28 ± 0.51
25 HCC (ng/ml)	8.0 ± 1.0	26.3 ± 12.2
1,25 DHCC (pg/ml)	111.5 ± 15.7	27.9 ± 9.8*

[1]Data are mean ± standard error; n = 4 animals per treatment group.
*Significantly ($p < 0.05$) different control vs diabetic, Student's unpaired t test.

Table I-2: Body weights and blood parameters in fasted control and STZ diabetic rats 20 days after administration of streptozotocin[1].

	CONTROL	DIABETIC
BODY WEIGHT (g)	318 ± 13	143 ± 19*
PLASMA		
Glucose (mg%)	105.5 ± 1.7	483.0 ± 89.1*
Insulin (ng/ml)	5.05 ± 1.24	0.52 ± 0.05*
Calcium (mg%)	12.43 ± 0.33	10.56 ± 0.43*
Phosphorus (mg%)	9.80 ± 0.11	7.78 ± 0.62*
25 HCC (ng/ml)	9.4 ± 1.3	24.5 ± 7.3
1,25 DHCC (pg/ml)	171.0 ± 22.3	24.7 ± 4.8*

[1]Data are mean ± standard error; n = 4 animals per treatment group
*Significantly ($p < 0.05$) different control vs diabetic, Student's unpaired t test

DISCUSSION

In this preliminary study we confirmed that uncontrolled STZ diabetes results in decreased circulating 1,25 DHCC and that this decrease preceded the onset of hypocalcemia and hypophosphatemia. This data suggested a relationship between low circulating 1,25 DHCC and impaired extracellular calcium and phosphorus homeostasis during untreated diabetes. A previous study by Seino et al (1983) also found reduced 1,25 DHCC in untreated diabetic rats; however, both short term and long term diabetes were associated with hypercalcemia and hyperphosphatemia. The varying results obtained for circulating calcium and phosphorus levels may be due to variations in the duration and severity of diabetes and the degree of impaired 1,25 DHCC synthesis.

During insulin deficiency, decreased circulating 1,25 DHCC may be responsible for some of the long term complications in bone metabolism found in experimental diabetes, such as impaired bone turnover (Hough et al, 1981; Bouillon et al, 1988) and reduced bone mass (Verhaeghe et al, 1989). As discussed in the literature review, human diabetes mellitus is also associated with decreased bone mass and is accompanied by reduced circulating 1,25 DHCC.

In our study, the two-fold increase in intestinal VDR levels was apparently unable to compensate for low circulating 1,25 DHCC and did not prevent the eventual aberrations in mineral homeostasis. Our data for

intestinal VDR levels confirm those of Seino et al (1983) who reported an increase in intestinal VDR in untreated diabetic rats. Although these studies did not address the mechanism of up-regulation of intestinal VDR we observed that an increase in intestinal mucosal weight accompanied the increase in VDR levels. Thus VDR up-regulation could be related to the compensentory tissue growth occurring in the diabetic intestine, or could be secondary to metabolic alterations. To distinguish between these possibilities, the effect of diabetes on the VDR and growth indices in several 1,25 DHCC target tissues was investigated in the next series of studies. In addition, the relevance of alterations in VDR levels to intestinal function was examined.

CHAPTER II

VITAMIN D RECEPTORS AND COMPENSATORY TISSUE GROWTH IN THE STZ DIABETIC RAT

EXPERIMENTAL DESIGN

The effect of diabetes (uncontrolled and insulin-treated) on 1,25 DHCC metabolism was studied in STZ diabetic rats 10 days after induction of diabetes. This time point was chosen since the preliminary study indicated that alterations in intestinal VDR were evident within 10 days, yet plasma calcium and phosphorus remained normal. Rats were fed an AIN semi-purified diet; the number of animals per group varied from four to fifteen, depending on the parameter being analysed. VDR number and characteristics as well as growth indices were analysed in intestine and kidney (two tissues which exhibit compensentory growth during untreated diabetes) and compared to thymus (which atrophies in untreated diabetes). The effect of the alterations in VDR number on intestinal function was determined by analysis of vitamin D dependent calbindin D-9K, sucrase and alkaline phosphatase.

RESULTS

a) **Body Weight and Blood Parameters** (Table II-1): Untreated diabetes was associated with a lower final body weight (due to decreased growth rate) and higher blood glucose compared to controls. Insulin therapy generally

restored body weight and blood glucose to control levels; however, some rats exhibited weight loss, elevated blood glucose and ketosis despite insulin therapy. Data from these rats was analysed separately and when presented was labelled "diabetic + insulin, poor control". The data on insulin treated rats shown in the tables and figures refer to animals who exhibited trace glucosuria for seven consecutive days of insulin therapy.

Plasma 1,25 DHCC and magnesium were decreased in the untreated diabetic animals, but no change in 25 HCC, calcium or phosphorus was observed. Although insulin treatment significantly increased 1,25 DHCC and magnesium, levels were still lower in diabetic animals than in control animals.

b) **Tissue Weights, Protein and DNA** (Table II-2): Intestinal mucosal weight, both absolute and as a percentage of body weight (somatic index), was higher in diabetic untreated rats compared to controls. The increase in tissue weight was associated with an increase in total mucosal protein and a concomitant increase in total DNA; thus, the DNA:protein ratio of the intestinal mucosa was not changed in the diabetic state. At sacrifice, insulin treated diabetic rats did not exhibit evidence of altered intestinal growth; mucosal weight, protein and DNA levels were comparable to control values.

Kidney weight was also elevated in untreated diabetic rats (expressed as either wet weight or somatic index). The increased weight was associated with an increase in total renal protein, however there was no increase in total renal DNA. Therefore, the DNA:protein ratio in the diabetic untreated rat

kidney was reduced. Insulin treatment normalized these changes in renal growth.

Although thymic weight, protein and DNA were lower in untreated diabetic than in control rats, the somatic index and DNA:protein ratio were comparable to control values. Insulin therapy restored thymic weights of diabetic rats to control levels.

c) VDR Analysis:

(i) Intestine: Table II-3 summarizes data from ^{3}H-1,25 DHCC equilibrium binding assays for intestinal cytosol from control and diabetic animals; representative Scatchard plots are shown in Figure II-1. As demonstrated in the preliminary study, untreated diabetic rats exhibited significant increases in intestinal 1,25 DHCC specific binding sites per mg protein. Since total intestinal mucosal protein was increased in untreated diabetic as compared to control rats, total 1,25 DHCC specific binding sites in the diabetic intestine were increased to an even greater degree than specific binding sites expressed per mg protein (control=654±122, diabetic=2761±802 fmol; $p < 0.05$). In this study, Scatchard analysis indicated that the Kd of the intestinal VDR in untreated diabetic animals was increased compared to controls.

The effect of insulin therapy on 1,25 DHCC binding to intestinal cytosol is also shown in Figure II-1. When good control of diabetes was achieved

(defined as seven days trace glucosuria and normalization of weight gain), binding of 1,25 DHCC was similar to that of control rats. When diabetes could not be controlled with insulin treatment (glucosuria 55mmol after 14 days of treatment) the Scatchard plot of 1,25 DHCC binding was very similar to that for a diabetic untreated rat. Table II-3 summarizes the data for insulin treated diabetic rats (good control only) and indicates that good control was accompanied by normalization of the number of intestinal 1,25 DHCC binding sites; however, the Kd was not completely normalized by insulin therapy.

When the data for control, insulin treated diabetic and untreated diabetic animals was combined, a significant correlation ($r=0.69$, $p<0.05$) was found between mucosal weight and 1,25 DHCC specific binding sites using Pearson's correlation coefficient (Figure II-2).

To determine whether increased 1,25 DHCC binding to diabetic intestinal cytosol was related to changes in the number or size of the ^{3}H-1,25 DHCC binding component, intestinal cytosol from control and untreated diabetic rats was incubated with ^{3}H-1,25 DHCC and analysed by FPLC (Figure II-3). In both control and diabetic samples, specifically bound ^{3}H-1,25 DHCC eluted in one major peak of approximately 66 kD, which compares well with published reports for the mammalian VDR. In Figure II-3, the increased size of the ^{3}H-1,25 DHCC binding peak from diabetic intestine is also evident.

(ii) Kidney: Binding data for renal chromatin extracts from control and STZ diabetic untreated rats indicate that neither 1,25 DHCC specific

binding sites per mg protein (Table II-3) nor total binding sites (control=555±60, diabetic=752±122 fmol) were affected by uncontrolled STZ diabetes. The Kd for renal VDR was also unchanged. Representative Scatchard plots for kidney VDRs are illustrated in Figure II-4.

(iii) Thymus: Data for 1,25 DHCC specific binding to control and diabetic untreated thymic extracts is summarized in Table II-3 and representative Scatchard plots are shown in Figure II-5. No significant effect of diabetes on thymic 1,25 DHCC specific binding sites or Kd was observed.

d) Effect of Diabetes on Intestinal Mucosal Function (Table II-4): Since alterations in VDR during diabetes seemed to be unique to the intestine, the physiological relevance of these alterations was further investigated. The amount of calbindin D-9K per mg protein was significantly lower in STZ diabetic untreated rat intestine as compared to control and insulin treated rats. Alkaline phosphatase specific activity (an intestinal brush border enzyme) tended to be lower in uncontrolled diabetic rat intestine and higher in insulin treated diabetic rat intestine compared to controls although the differences were not statistically significant. The specific activity of sucrase, another brush border enzyme, was significantly increased in uncontrolled diabetic mucosal homogenates as compared to controls and was normalized by insulin therapy.

Table II-1: Body weights and blood parameters in fasted control, STZ diabetic untreated and STZ diabetic insulin treated rats[1].

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
BODY WEIGHT (g)	209.7 ± 6.3[a] (9)	153.6 ± 5.4[b] (9)	217.0 ± 4.7[a] (11)
PLASMA			
Glucose (mg%)	121.2 ± 3.8[a] (9)	294.2 ± 42.2[b] (8)	113.7 ± 14.3[a] (11)
Calcium (mg%) Total Ionized	 11.2 ± 0.4 (8) 5.28 ± 0.08 (8)	 10.1 ± 0.4 (7) 4.96 ± 0.12 (5)	 10.2 ± 0.3 (11) 5.24 ± 0.08 (11)
Magnesium (mg%)	1.82 ± 0.06[a] (8)	1.48 ± 0.06[b] (7)	1.67 ± 0.05[c] (11)
Phosphorus (mg%)	7.56 ± 0.44 (8)	6.69 ± 0.32 (7)	6.89 ± 0.19 (11)
25-HCC (ng/ml)	15.3 ± 1.4 (10)	18.7 ± 1.9 (8)	12.4 ± 2.2 (11)
1,25-DHCC (pg/ml)	120.8 ± 12.3[a] (9)	54.2 ± 12.5[b] (7)	97.2 ± 12.9[c] (11)

[1]Data are mean ± standard error, numbers in parentheses indicated actual number of samples analyzed.
[a,b,c]Means with different letters in their superscripts are significantly ($p < 0.05$) different by one-way ANOVA followed by Neuman-Keuls analysis.

Table II-2: Indices of tissue growth in intestinal mucosa, kidney and thymus from control, STZ diabetic untreated and STZ diabetic insulin treated rats[1].

INTESTINAL MUCOSA (n = 4-6)

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
Weight (g)	0.395 ± 0.034[a]	0.566 ± 0.031[b]	0.364 ± 0.019[a]
Somatic Index[2]	1.85 ± 0.17[a]	3.64 ± 0.20[b]	1.75 ± 0.10[a]
Total Protein (mg)	29.4 ± 2.5[a]	46.2 ± 2.3[b]	30.4 ± 1.8[a]
µg DNA/mg protein	12.7 ± 1.0	13.1 ± 0.5	10.6 ± 0.7

KIDNEY (n = 6)

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
Weight (g)	1.07 ± 0.05[a]	1.25 ± 0.05[b]	1.01 ± 0.06[a]
Somatic Index[2]	10.1 ± 0.3[a]	16.1 ± 0.6[b]	9.6 ± 0.3[a]
Total Protein (mg)	96.0 ± 3.4[a]	118.7 ± 5.1[b]	92.9 ± 4.5[a]
µg DNA/mg protein	9.5 ± 0.5[a]	7.4 ± 0.3[b]	10.5 ± 0.8[a]

THYMUS (n = 6)

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
Weight (mg)	750.8 ± 28.5[a]	482.8 ± 37.5[b]	705.7 ± 33.2[a]
Somatic Index[2]	3.6 ± 0.2	3.1 ± 0.2	3.4 ± 0.2
Total Protein (mg)	43.3 ± 1.7[a]	22.4 ± 1.5[b]	31.9 ± 2.3[a]
µg DNA/mg protein	5.9 ± 1.1	4.4 ± 0.8	5.7 ± 1.0

[1]Data are means ± standard error, n = number of samples analyzed per group
[2]mg tissue/g body weight
[a,b]Means with different letters in their superscripts are significantly ($p < 0.05$) different by one way ANOVA followed by Neuman-Keuls analysis

Table II-3: Specific binding sites (SBS) for 1,25 DHCC and Kd of the vitamin D receptor in intestinal mucosa, kidney and thymus from control, STZ diabetic untreated and STZ diabetic insulin treated rats[1].

INTESTINAL MUCOSA (n = 5-9)

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
SBS (fmol/mg protein)	178.4 ± 29.5[a]	408.7 ± 82.0[b]	167.8 ± 32.8[a]
Kd (nMolar)	0.11 ± 0.02[a]	0.29 ± 0.03[b]	0.19 ± 0.03[a,b]

KIDNEY (n = 10-15)

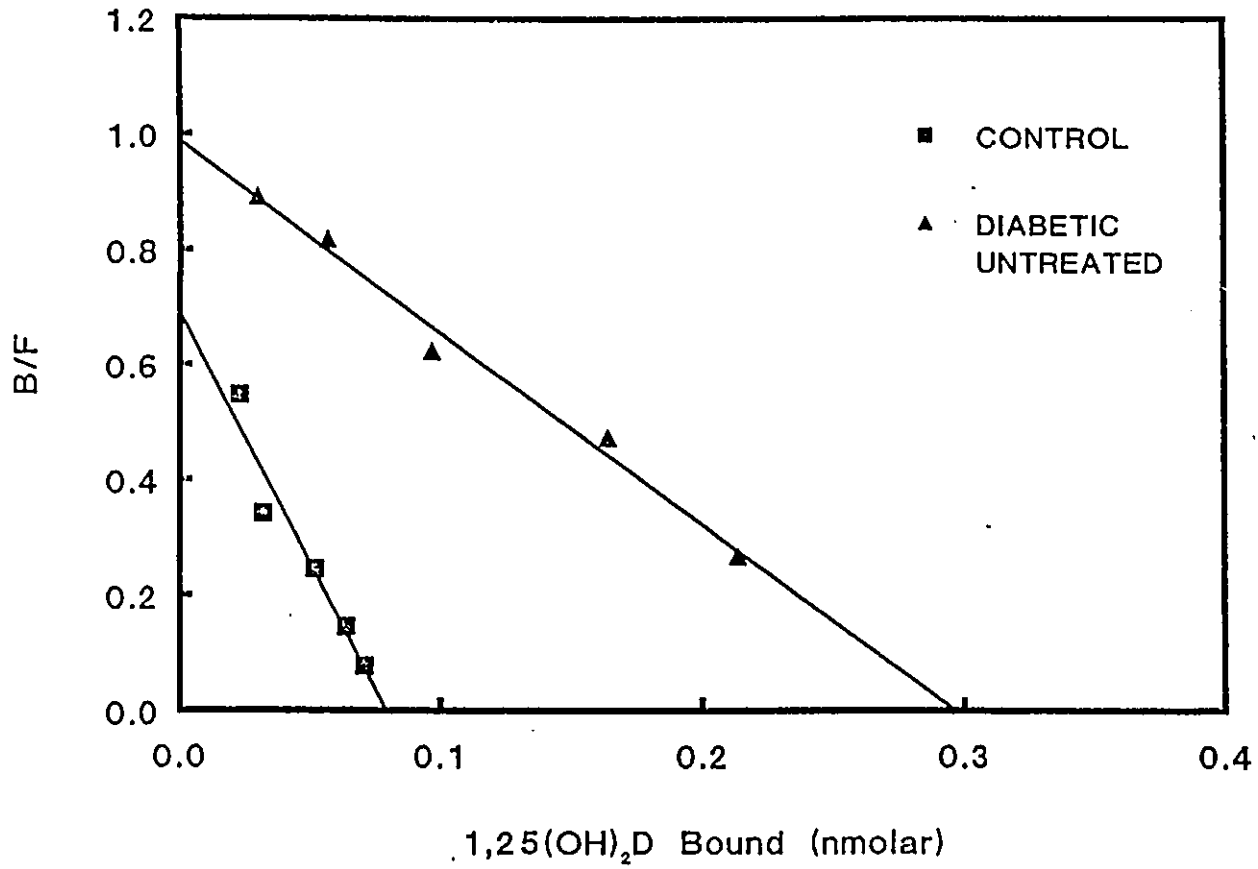
	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
SBS (fmol/mg protein)	54.2 ± 6.1	60.8 ± 6.2	48.9 ± 7.1
Kd (nMolar)	0.063 ± 0.008	0.052 ± 0.004	0.047 ± 0.009

THYMUS (n = 7)

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
SBS (fmol/mg protein)	12.5 ± 0.8	9.3 ± 1.5	nd
Kd (nMolar)	0.033 ± 0.009	0.039 ± 0.014	nd

[1]Data are mean ± standard error, n = number of samples analyzed per group
[a,b]Means with different letters in their superscripts are significantly ($p < 0.05$) different by one way ANOVA followed by Neuman-Keuls analysis
nd = not determined

Figure II-1. Scatchard plots of ^{3}H-1,25 DHCC binding to intestinal mucosal cytosol from control and STZ diabetic rats. Cytosol was incubated with 0.06-1.2 nMolar ^{3}H-1,25 DHCC for 3 hr and treated as described in Methods. In II-1a (top) is shown ^{3}H-1,25 DHCC binding to cytosol from control and untreated diabetic animals. In these representative plots, the specific binding sites (fmol/mg protein) and Kd (nMolar) were 584 and 0.29 for diabetic and 182 and 0.14 for control cytosol, respectively. In II-1b (bottom), ^{3}H-1,25 DHCC binding to cytosol from control and insulin treated STZ diabetic rats is shown. Diabetic+insulin, good control refers to a rat which exhibited trace glucosuria for seven consecutive days of insulin therapy. Diabetic+insulin, poor control refers to a diabetic rat which never achieved trace glucosuria despite insulin therapy for 14 days. For these representative plots, the specific binding sites (fmol/mg protein) and Kd (nMolar) were 313 and 0.31 for diabetic+insulin (poor control); 127 and 0.09 for diabetic+insulin (good control) and 182 and 0.14 for control cytosol, respectively. Summary and statistical analysis of data from control, diabetic untreated and diabetic insulin treated (good control) is presented in Table II-3.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0.6
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B/F
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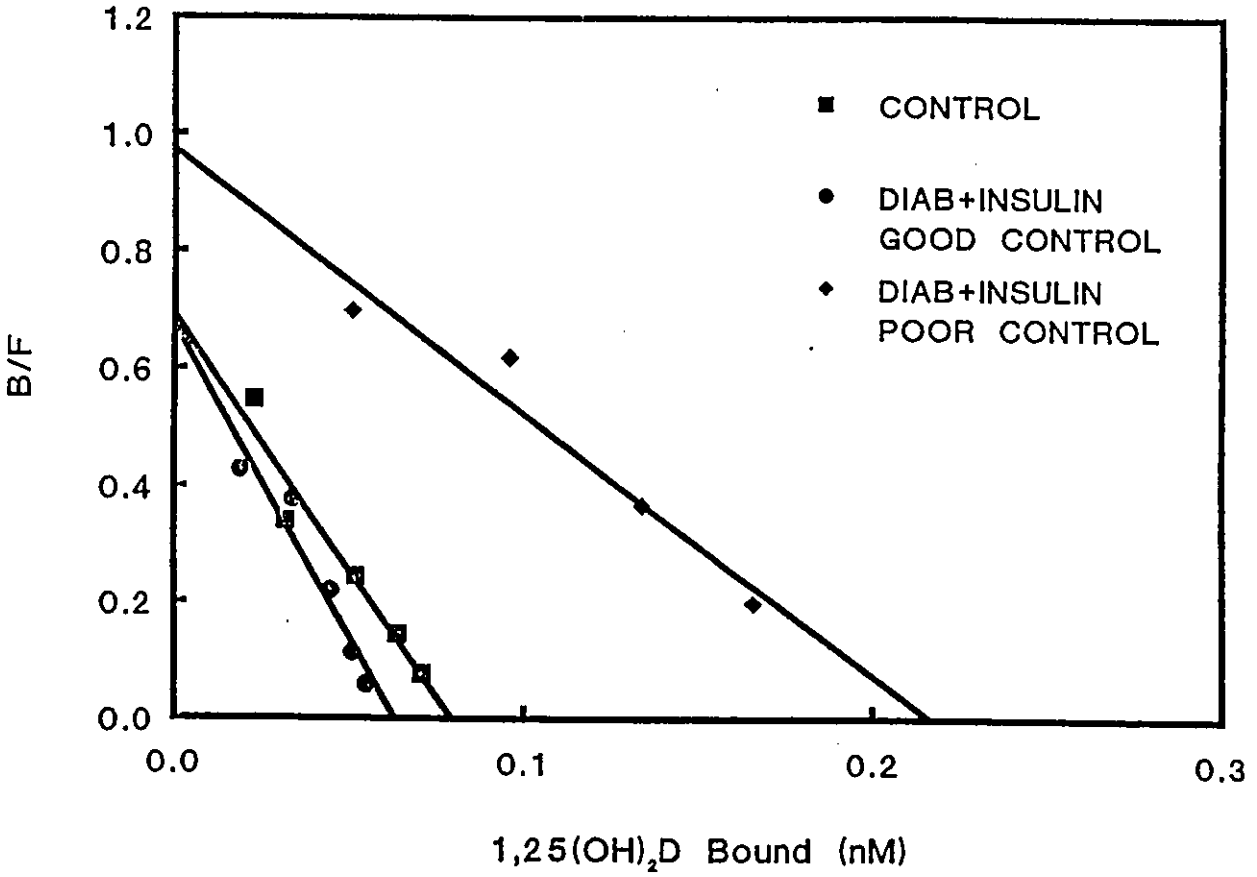
CONTROL
DIABETIC
UNTREATED
1,25(OH)2D Bound (nmolar)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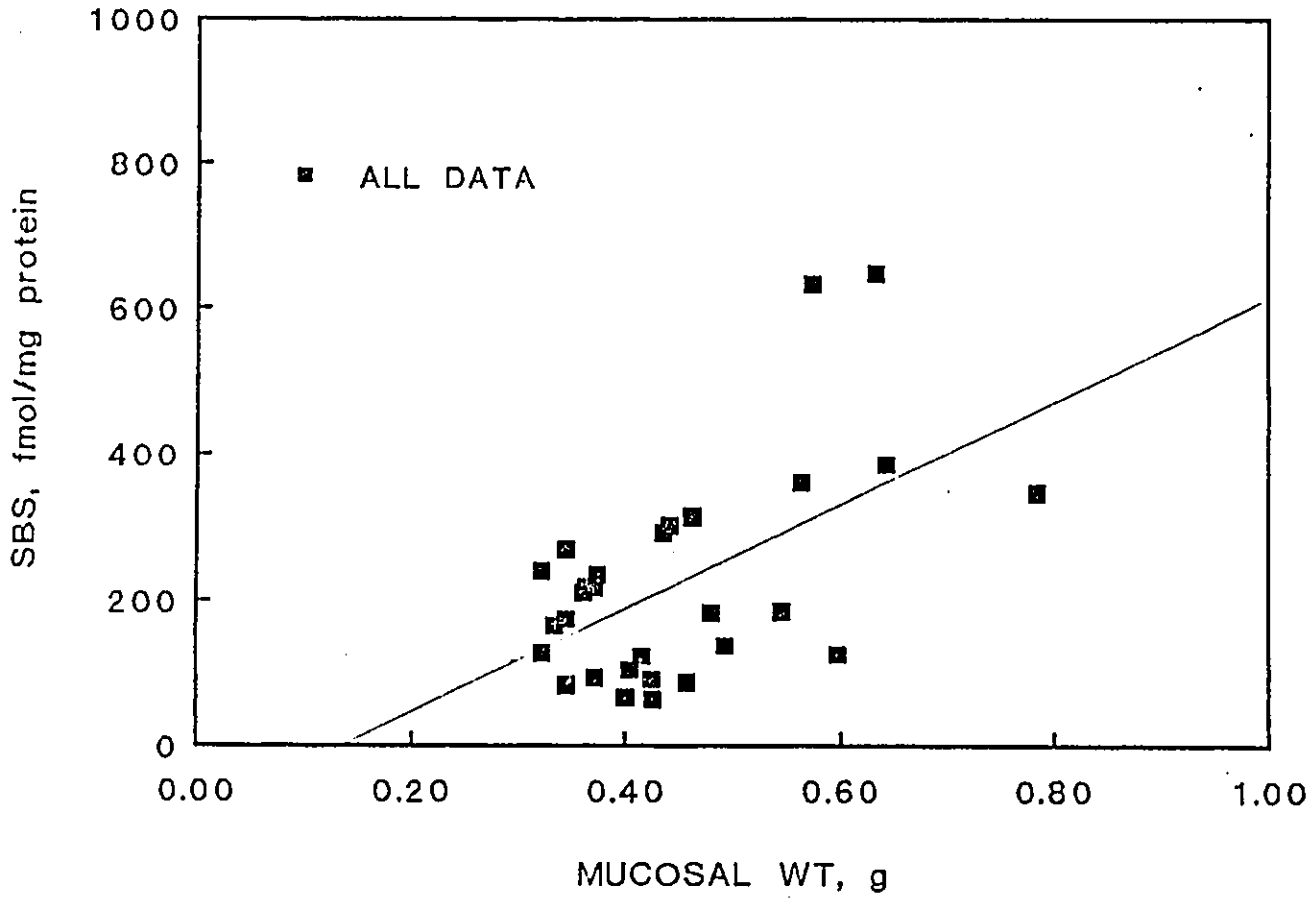
CONTROL
DIAB+INSULIN
GOOD CONTROL
DIAB+INSULIN
POOR CONTROL
1,25(OH)2D Bound (nM)

Figure II-2. Correlation between VDR number and intestinal mucosal weight. Specific binding sites for 1,25 DHCC (fmol/mg protein) (see Methods for details) was plotted against mucosal weight (g) for control, insulin treated STZ diabetic and untreated STZ diabetic rats. A significant correlation ($r=0.69$, $p<0.05$) was found between mucosal weight and specific binding sites using Pearson's correlation coefficient.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600
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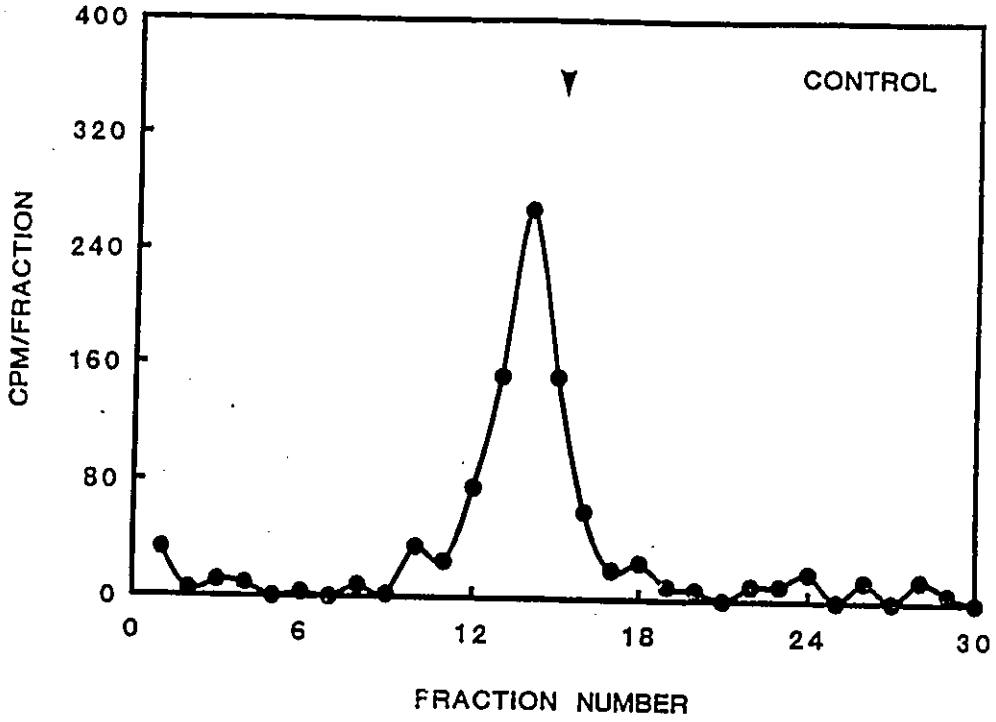
0.80
1.00
MUCOSAL WT, g

Figure II-3. FPLC profile of ^{3}H-1,25 DHCC binding to intestinal cytosol from control and STZ diabetic untreated rats. Cytosol was incubated with 6nM ^{3}H-1,25 DHCC, applied to a Superose 12 FPLC column and eluted with TKEDMS buffer (see Methods). Arrow indicates elution position of ^{14}C-albumin (MW=66Kd).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18
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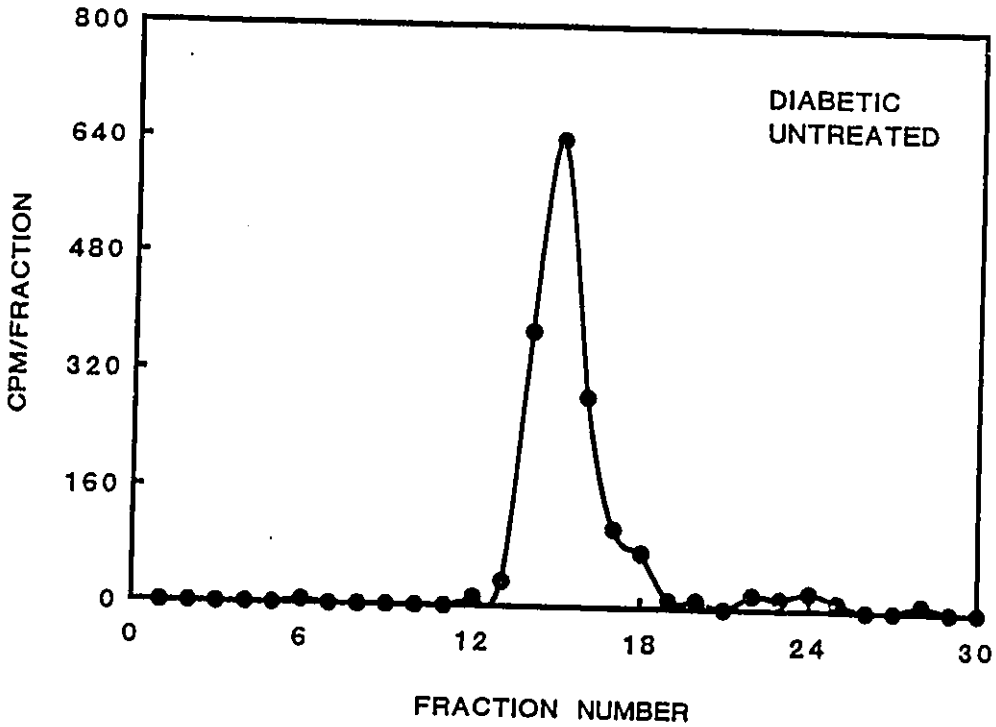
30
FRACTION NUMBER


DIABETIC
UNTREATED
CPM/FRACTION
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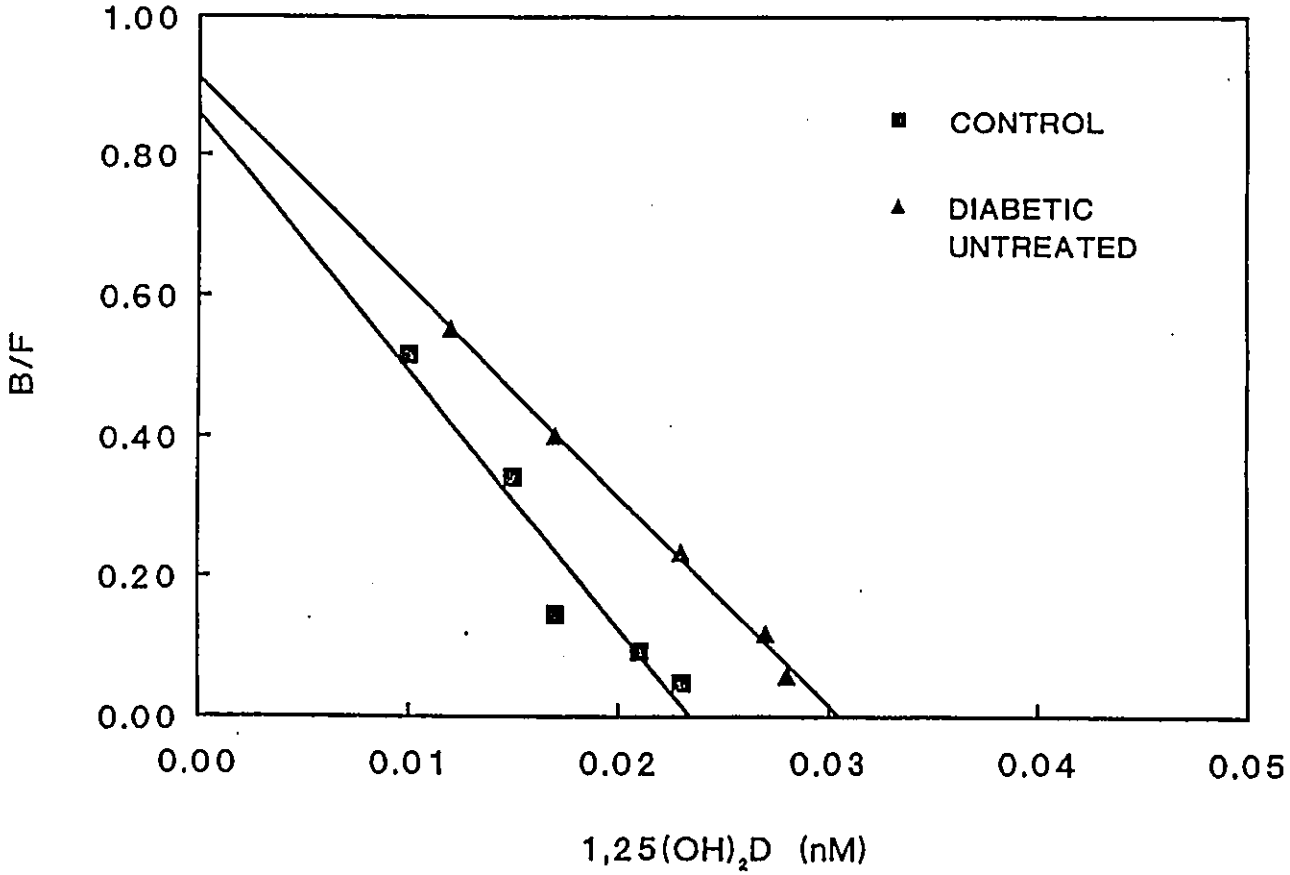
640
480
320
160
0
0
6
12
18
24
30
FRACTION NUMBER

Figure II-4. Scatchard plots of ^{3}H-1,25 DHCC binding in renal chromatin extracts from control and untreated diabetic rats. See legend for Figure II-1 for assay details.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0.03
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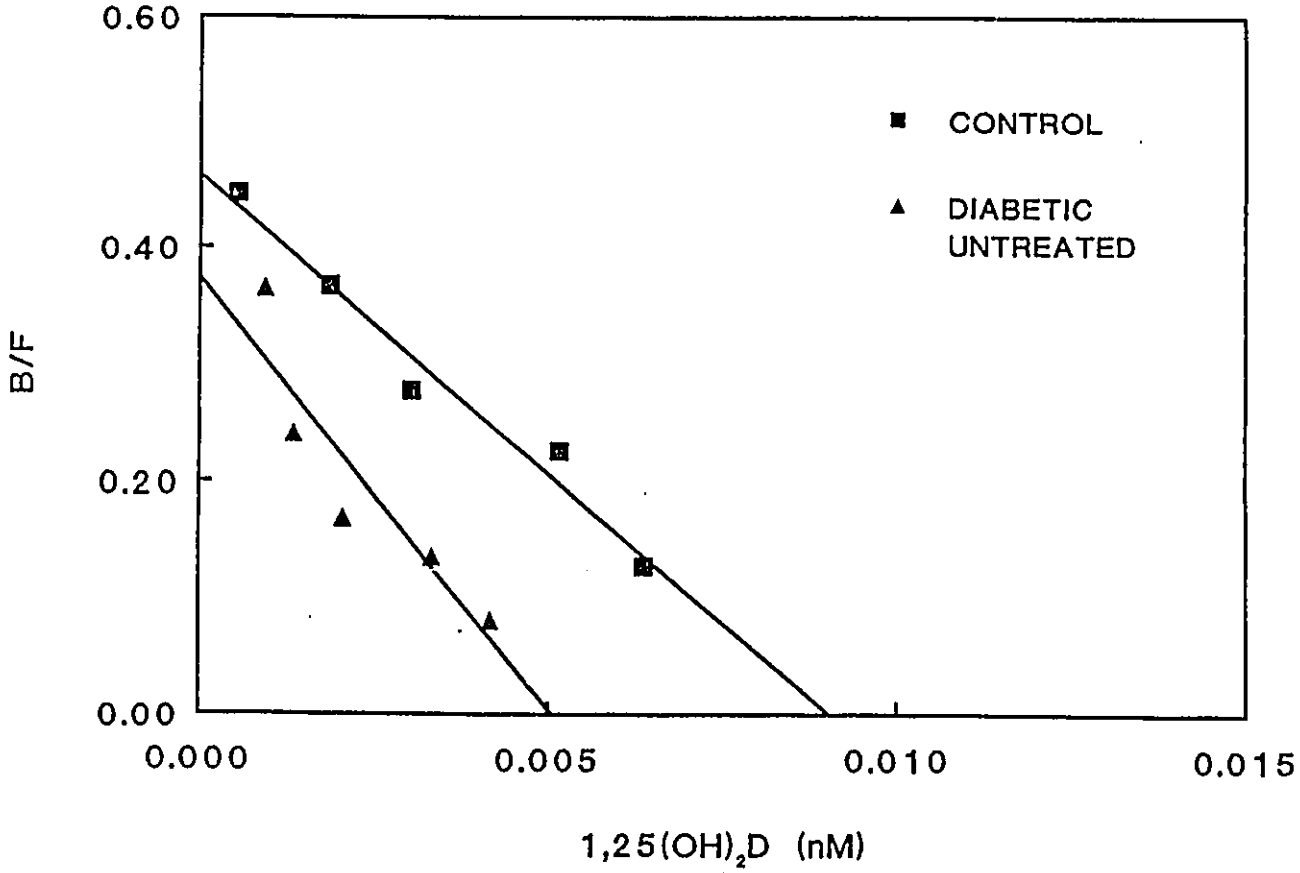
0.05
1,25(OH)2D (nM)
CONTROL
DIABETIC
UNTREATED

Figure II-5. Scatchard plots of ^{3}H-1,25 DHCC binding in thymic chromatin extracts from control and untreated diabetic rats. See legend for Figure II-1 for assay details.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0.20
0.00
B/F
0.000
0.005
0.010
0.015
1,25(OH)2D (nM)
CONTROL
DIABETIC
UNTREATED

Table II-4: Intestinal calbindin D-9K, alkaline phosphatase and sucrase in control and STZ diabetic rats[1].

	CONTROL	DIABETIC UNTREATED	DIABETIC TREATED
Calbindin D-9K (μg/mg protein)	23.0 ± 0.9[a]	12.1 ± 1.5[b]	19.5 ± 1.0[a]
Alkaline Phosphatase (μmol/mg/min)	5.6 ± 0.9	3.3 ± 0.4	4.2 ± 1.4
Sucrase (μg/mg/min)	4.5 ± 0.4[a]	7.0 ± 0.4[b]	4.8 ± 0.5[a]

[1]Data are mean ± standard error; n = 5-6 animals per treatment group
[a,b]Means with different letters in their superscripts are significantly different ($p < 0.05$) by one way ANOVA followed by Neuman-Keuls analysis

DISCUSSION

Up-regulation of intestinal VDR in untreated STZ diabetic rats has been previously reported (Seino et al, 1983); in this study, we examined other tissues to determine if VDR up-regulation was a general effect of diabetes. Our data clearly indicate that VDRs were increased in intestine but were not increased in kidney and thymus, two other vitamin D target tissues (Table II-3). These findings suggest that the alterations in VDR levels occurring in untreated diabetes may be unique to the intestine, and therefore that local factors in the intestine are likely involved in VDR up-regulation.

To determine the mechanisms involved in VDR up-regulation, we assessed whether the increase in intestinal VDR was related to the changes in intestinal growth which occurred in untreated diabetes. Both kidney and intestine exhibited compensatory growth (Table II-2); however, only intestinal VDR were increased (Table II-3). This data suggested that the nature of the compensatory growth might be different in the two tissues; thus, we compared the relative contributions of hypertrophy (increase in cell size) and hyperplasia (increase in cell number) to tissue growth (Table II-2). In the diabetic intestine, both protein and DNA were increased and the DNA:protein ratio was unchanged, suggesting that the increase in tissue weight resulted primarily from hyperplasia. This suggestion is in agreement with previous studies (Miller et al, 1977) which showed increased intestinal DNA synthesis (as measured by in vivo ^{3}H-thymidine incorporation) in STZ diabetic rats compared to control. The mechanism underlying the development of intestinal hyperplasia is unclear. Although it has been suggested that hyperplasia is

related to hyperphagia, recent studies in our laboratory (Gagnon and Welsh, unpublished) indicate that hyperplasia in STZ diabetic rats was temporally related to the onset of hyperglycemia rather than to hyperphagia. Hyperglycemia may in turn, modulate synthesis or action of growth factors such as epidermal growth factor, which enhances enterocytic proliferation and up regulates the VDR (Bruns et al, 1989).

In contrast to the intestine, we observed that kidney growth during STZ diabetes was associated with an increase in total protein without a concomitant increase in total DNA (Table II-2). This resulted in a reduced DNA:protein ratio, suggesting that compensatory growth in the kidney was due to hypertrophy rather than hyperplasia. This suggestion is consistent with the work of Seyer-Hansen (1983) who reported increased kidney cell size accompanied by a decreased DNA:protein ratio in the diabetic state. These findings indicate that neither hyperplasia nor up-regulation of VDRs occurred in the kidney. In contrast to intestine and kidney, the thymus was atrophied in diabetic animals, however there were no alterations in thymic somatic index or DNA:protein ratio. This suggests that decreased thymic weight in STZ diabetic animals was related to their lower body weight. Diabetes did not affect VDR levels in thymus (Table II-3).

From the above results we can postulate that hyperplasia is associated with up-regulation of the VDR, although tissue hypertrophy or atrophy do not seem to affect VDR numbers. This association is supported by recent in vitro studies which have suggested a link between cell proliferation and VDR up-regulation in bone cells (Chen et al, 1981), thymus derived lymphocytes

(Provvedini et al, 1984) and epithelial cells (Merke et al, 1989). Our data suggest that this relationship may exist in vivo, although further studies are required to elucidate the mechanism(s) involved.

In the gut, proliferation occurs in the stem cell compartment of the crypt. These rapidly proliferating cells, which contain high levels of VDRs, migrate up the villus and undergo terminal differentiation into absorptive enterocytes. During the differentiation process, the VDR is down regulated. If diabetes alters the cell population along the crypt villus axis, the increased VDR we observed might actually reflect an increase in the number of cells expressing the VDR, rather than an increase in the number of VDRs per cell. Further studies to map VDR expression along the crypt villus axis, using techniques such as in situ hybridization or immunocytochemistry, will be necessary to test this possibility.

If VDR number is related to hyperplasia, discrepancies for VDR number among different diabetic models and tissues may be due to factors such as severity of diabetes, age and diet, all of which may affect compensatory tissue growth (and thus the degree of hyperplasia/hypertrophy) during diabetes (Seyer-Hansen, 1983; Back et al, 1990; Jobin et al, 1986). Further research is required to clarify this issue, since previous studies using STZ (Seino et al, 1983) and BB (Nyomba et al, 1989) diabetic rats did not include data on tissue weights, protein or DNA.

The possibility that increased 1,25 DHCC binding to intestinal cytosol from diabetic rats represented additional proteins which could specifically bind ^{3}H-1,25 DHCC was investigated by FPLC. ^{3}H-1,25 DHCC binding to intestinal

cytosol of diabetic and control rats (Figure II-3) indicated that in both cases a single peak at approximately 66 Kd was observed. Thus, it is unlikely that the increased ^{3}H-1,25(OH)$_2$D$_3$ binding observed in intestinal cytosol from untreated diabetic rats represents binding to proteins other than the VDR. On FPLC, the ^{3}H-1,25 DHCC binding peak was larger in the diabetic intestinal cytosol compared to controls, consistent with our data from equilibrium binding assays.

The relevance of intestinal VDR up-regulation to intestinal function in STZ diabetes is still unclear. The vitamin D-dependent calbindin D-9K was reduced in the diabetic intestine and was restored to normal levels by insulin treatment (Table II-4), confirming data of Schneider et al (1973). This finding is consistent with studies associating untreated diabetes with impaired calcium transport, which is corrected by insulin treatment (Schneider et al, 1977b). The decrease in calbindin D-9K does not seem to be due to a generalized impairment of intestinal function in diabetes, since protein synthesis and sucrase activity were not reduced. The reduction in calbindin D-9K levels despite VDR up-regulation suggests that 1,25 DHCC action was reduced in the diabetic intestine, possibly secondary to low circulating 1,25 DHCC. Reduced affinity of the VDR for 1,25 DHCC (as indicated by the increase in Kd suggested by Scatchard analysis) could contribute to this impairment although further studies are required to investigate this possibility. Alternatively, if, as discussed above, diabetes is associated with altered cell populations along the crypt villus axis, the decreased calbindin D-9K might reflect this change, since calbindin D-9K is expressed at high levels in enterocytes and at low levels in

crypt cells.

Untreated diabetic rats did not have reduced serum calcium (total or ionized), despite the decrease in 1,25 DHCC and intestinal calbindin D-9K. The implications of this finding will be discussed further in Chapter III.

To summarize, the major finding of this series of studies was that STZ diabetic rats exhibited increased intestinal VDRs in association with intestinal hyperplasia. VDRs were not increased in kidney or thymus, tissues which did not exhibit hyperplasia secondary to insulin deficiency. In order to determine whether the observed changes in vitamin D metabolism and intestinal growth were related to insulin deficiency per se, another animal model of diabetes was investigated. In agreement with our findings in STZ diabetic rats, previous studies have reported decreased circulating 1,25 DHCC in spontaneously diabetic BB rats; however, intestinal VDRs were reportedly decreased in chronically diabetic BB rats (Nyomba et al, 1989) although intestinal growth indices were not reported in that study. Since we hypothesize that altered VDR number is related to disturbed tissue growth, we reasoned that discrepancies in VDR number in these two studies could be due to differences in compensatory growth rather than to the diabetic model used. Thus the next series of studies was designed to complement our data derived from STZ diabetic rats by characterizing both the VDR and alterations in tissue growth in the BB diabetic rat.

CHAPTER III

VITAMIN D RECEPTORS AND COMPENSATORY TISSUE GROWTH IN THE SPONTANEOUSLY DIABETIC BB RAT

EXPERIMENTAL DESIGN

1,25 DHCC, plasma minerals and indices of glucose homeostasis were assessed in acutely diabetic, untreated BB rats (mean age 45 to 60 days) seven days after onset of diabetes, and compared to Wistar controls. Groups contained five to six diabetic or control rats which were fed Purina lab chow. VDRs were assessed by equilibrium binding and Scatchard analysis in intestine and kidney and compared to tissue growth indices. The effect of spontaneous diabetes on calbindins and intestinal enzymes was assessed for comparison to the data derived from the STZ model.

RESULTS

a) **Body Weight and Blood Parameters** (Table III-1): Although control and BB diabetic rats had comparable initial body weights, the average body weight of BB diabetic rats decreased slightly during the test period while control rats gained weight. This resulted in significantly lower final body weights in BB diabetic rats compared to controls. Fasting blood glucose was significantly higher in BB diabetic animals than in controls. In addition, the degree of diabetes, as indicated by the fasting blood glucose, was higher in the BB diabetic rat model than in the STZ diabetic rats.

Plasma 1,25 DHCC, calcium and magnesium were significantly lower in

BB diabetic rats than in controls although plasma phosphorus was unchanged.

b) Tissue Weights, Protein and DNA (Table III-2): Both absolute intestinal mucosal weight and somatic index were increased in BB diabetic rats, as was total mucosal protein. Since DNA was comparably increased in diabetic intestine, there was no significant difference in the intestinal DNA:protein ratio between diabetic and control animals.

Kidney weight (absolute and somatic index) and total protein were increased in diabetic rats although there was no comparable increase in DNA. Thus the DNA:protein ratio was significantly lower in the kidneys of diabetic animals as compared to controls.

c) VDR Analysis (Table III-3): BB diabetes was associated with a two-fold increase in intestinal VDRs (per mg protein); total binding sites were similarly increased (control=929±125, BB diabetic=1869±255 fmol; $p < 0.05$). Representative Scatchard plots of the ^{3}H-1,25 DHCC equilibrium binding assays for intestinal cytosol of control and BB diabetic rats are shown in Figure III-1. In the kidney, there was no significant difference in VDR number between control and BB diabetic animals (Figure III-2), whether expressed per mg protein or as total binding sites (control=1088±71, BB diabetic=1185±178 fmol).

d) Effect of Diabetes on Calbindins and Intestinal Mucosal Function (Table III-4): Calbindin D9-K (the intestinal vitamin D-dependent calcium binding protein) was significantly lower in the duodenum of BB diabetic rats as compared to controls. In contrast, the renal vitamin-D dependent calbindin

D-28K was not affected by diabetes (control=5.4±1.6, BB diabetic=5.6±1.7 μg calbindin/mg protein; n=3).

Alkaline phosphatase activity was significantly lower in the intestinal mucosa of BB diabetic rats, while sucrase specific activity was slightly (but not significantly) higher in diabetic rats than in controls.

Table III-1: Body weights and blood parameters in fasted control Wistar and BB Wistar diabetic rats[1].

	CONTROL	DIABETIC
BODY WEIGHT (g)	318 ± 8 (16)	242 ± 14* (15)
PLASMA		
Glucose (mg%)	108.8 ± 2.7 (11)	471.0 ± 9.7* (11)
Calcium (mg%)	12.71 ± 0.25 (7)	10.40 ± 0.57* (9)
Magnesium (mg%)	1.79 ± 0.24 (7)	1.63 ± 0.04* (8)
Phosphorus (mg%)	4.45 ± 0.11 (10)	4.42 ± 0.07* (9)
1,25 DHCC (pg/ml)	95.7 ± 10.5 (8)	36.0 ± 5.1* (8)

[1]Data are mean ± standard error; number in parentheses indicates number of samples analyzed per group

*Significantly ($p < 0.05$) different control vs BB diabetic, Student's unpaired t test

Table III-2: Indices of tissue growth in intestinal mucosa and kidney of control Wistar and BB Wistar diabetic rats[1].

INTESTINAL MUCOSA

	CONTROL	DIABETIC
Weight (g)	0.321 ± 0.008 (18)	0.419 ± 0.022*(15)
Somatic Index[2]	1.2 ± 0.1 (18)	1.9 ± 0.2* (15)
Total Protein (mg)	23.2 ± 2.4 (6)	47.5 ± 2.4* (6)
µg DNA/mg Protein	4.9 ± 0.5 (6)	5.5 ± 0.5 (6)

KIDNEY

	CONTROL	DIABETIC
Weight (g)	1.30 ± 0.03 (19)	1.36 ± 0.06 (19)
Somatic Index[2]	8.5 ± 0.2 (20)	10.1 ± 0.3* (18)
Total Protein (mg)	97.8 ± 3.0 (6)	114.0 ± 7.7* (7)
µg DNA/mg Protein	18.6 ± 0.7 (6)	15.7 ± 0.7* (7)

[1]Data are mean ± standard error, figures in parentheses indicate actual number of samples analyzed
[2]mg tissue/g body weight
*Significantly ($p < 0.05$) different, control vs BB diabetic rats, Student's unpaired t test

Table III-3: Specific Binding Sites (SBS) for 1,25 DHCC in intestinal mucosa and kidney from control Wistar and BB diabetic rats[1].

	CONTROL	DIABETIC
Intestinal Mucosal SBS (fmol/mg protein)	109.3 ± 7.1 (11)	253.8 ± 33.7* (10)
Kidney SBS (fmol/mg protein)	43.7 ± 4.5 (14)	58.2 ± 7.1 (15)

[1]Data are means ± standard error; number in parentheses indicates actual number of samples analyzed

*Significantly ($p < 0.05$) different control vs BB diabetic, Student's unpaired t test

Figure III-1. Scatchard plots of ^{3}H-1,25 DHCC binding to intestinal mucosal cytosol from control and BB diabetic rats. Cytosol was incubated with 0.06-1.2 nMolar ^{3}H-1,25 DHCC for 3 hr and treated as described in Methods. Summary and statistical analysis of data from control and BB diabetic rats is presented in Table III-3.

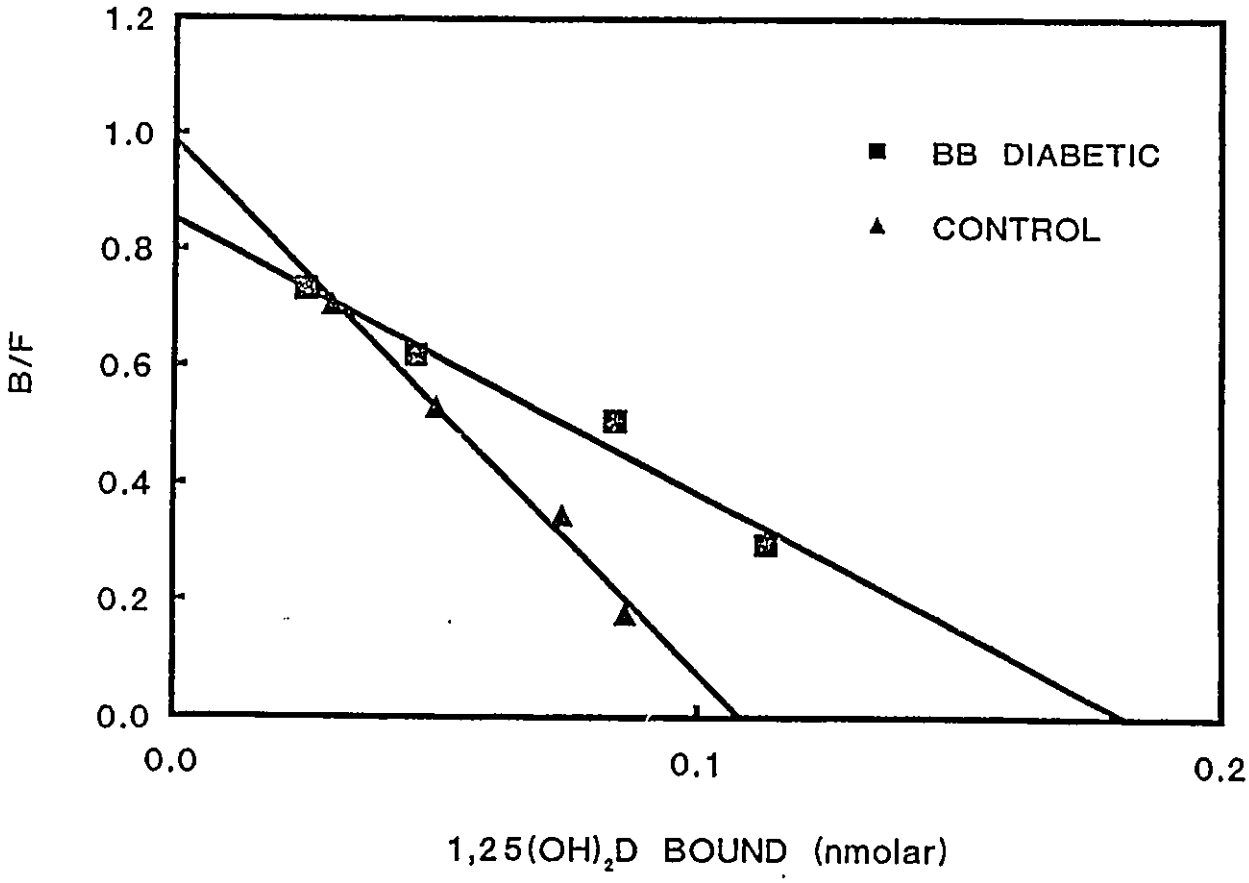

1.2
1.0
0.8
0.6
0.4
0.2
0.0
B/F
0.0
0.1
0.2
BB DIABETIC
CONTROL
1,25(OH)2D BOUND (nmolar)

Figure III-2. Scatchard plots of ^{3}H-1,25 DHCC binding to renal chromatin extracts from control and BB diabetic rats. See legend for Figure III-1 for assay details.

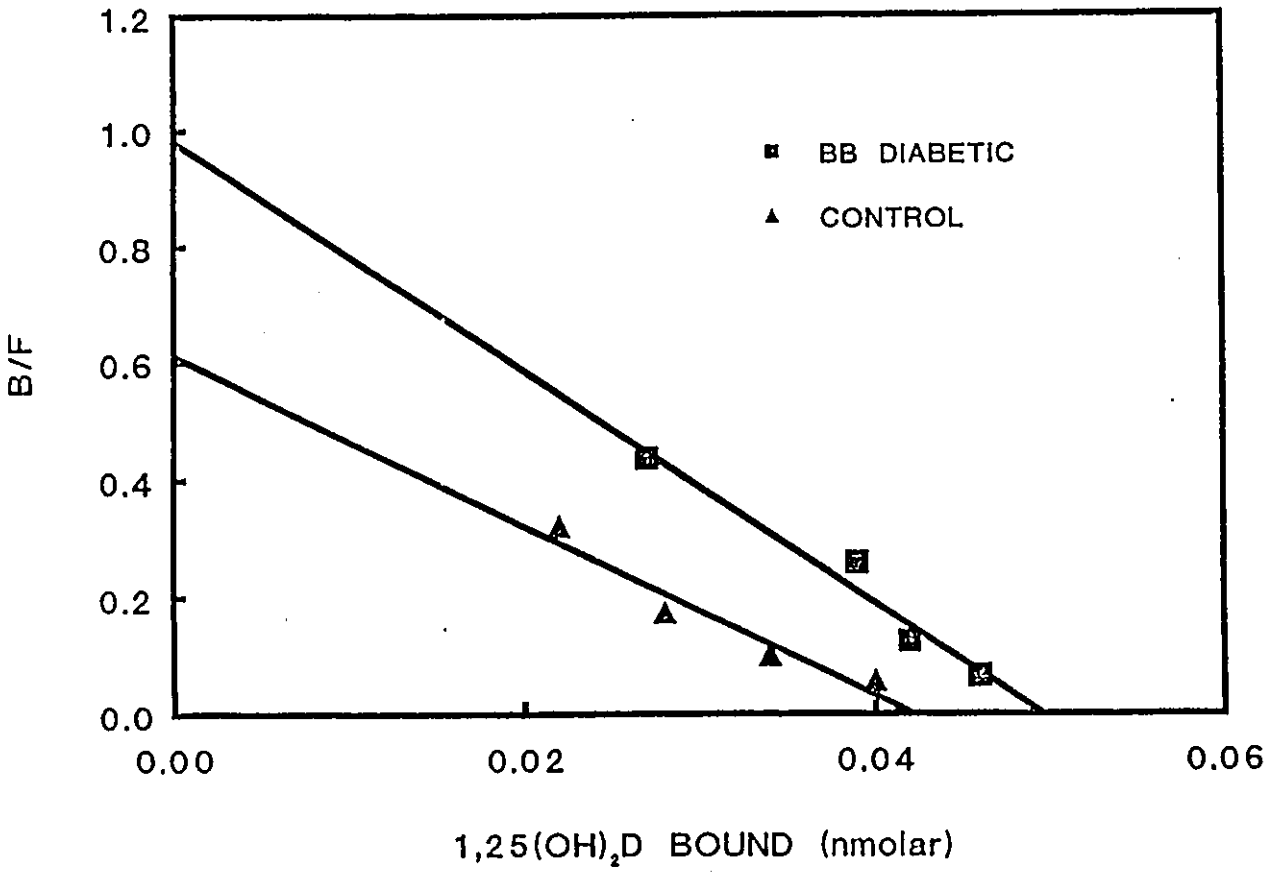

1.2
1.0
0.8
0.6
0.4
0.2
0.0
B/F
BB DIABETIC
CONTROL
0.00
0.02
0.04
0.06
1,25(OH)2D BOUND (nmolar)

Table III-4: Intestinal calbindin D-9K, alkaline phosphatase and sucrase in control Wistar and BB diabetic Wistar rats[1].

	CONTROL	DIABETIC
Calbindin D-9K (µg/mg protein)	5.58 ± 0.53 (5)	4.16 ± 0.27* (4)
Alkaline phosphatase (µmol/mg/min)	4.82 ± 0.37 (6)	3.46 ± 0.49* (6)
Sucrase (µg/mg/min)	15.2 ± 0.8 (6)	21.7 ± 4.8 (6)

[1]Data are means ± standard error; number in parentheses indicates actual number of samples analyzed

*Significantly ($p < 0.05$) different control vs BB diabetic, Student's unpaired t test

DISCUSSION

In this study, we have shown that like STZ diabetic rats, untreated BB diabetic rats have increased intestinal VDRs seven days after the development of diabetes (Table III-3). These studies indicate that up-regulation of VDRs in diabetic intestine is related to insulin deficiency per se, and is not unique to the STZ diabetic model. The data from BB diabetic rats presented here is consistent with the results of Chapters I and II and previous studies of intestinal VDRs (Seino et al, 1983) obtained with STZ diabetic rats. In contrast, Nyomba et al (1989) reported decreased VDRs in the intestine of untreated BB rats who were diabetic for 3-4 weeks. The discrepancy between Nyomba's data and that presented here may be due to varying degrees of disease severity and duration; Nyomba's study used rats that were chronically diabetic and exhibited weight loss and ketonuria. As mentioned in Chapter II, these factors may influence compensatory tissue growth which was not reported in previous BB studies (Nyomba et al, 1989). If, as we have postulated, altered VDR numbers are related to hyperplasia, the differences in disease states may account for the varying intestinal VDR numbers obtained in the BB studies in different labs.

In BB diabetes, as in STZ diabetes, intestinal but not renal VDR were increased by insulin deficiency. Since both kidney and intestine exhibit compensatory growth, we again compared the relative contributions of hyperplasia and hypertrophy to growth in these tissues (Table III-3). In the BB diabetic intestine, both protein and DNA increased similarly; thus the DNA:protein ratio was unchanged. This suggests that in BB diabetes, like in

STZ diabetes, the increase in intestinal mass was due to hyperplasia rather than hypertrophy. In the kidney, the DNA:protein ratio decreased (due to an increase in total protein without an increase in total DNA), indicating hypertrophy and not hyperplasia was occurring. These results are also in agreement with our studies in STZ rats and previous reports as discussed in Chapter II, and support the hypothesis that alterations in VDR numbers are associated with hyperplasia.

Assessment of intestinal function showed reduced vitamin D-dependent calbindin D-9K associated with BB diabetes despite the up-regulation of VDR (Table III-4). Alkaline phosphatase (which also displays vitamin D dependency) was also reduced in the BB diabetic intestine. However, protein synthesis in general was not impaired since total mucosal protein was increased and sucrase, a brush border enzyme, was not decreased in the BB intestine. Taken together these results suggest that intestinal function in general was not impaired, although the action of 1,25 DHCC in the intestine appeared to be reduced by BB diabetes. The low circulating 1,25 DHCC in the BB diabetic rats could account for this impairment. The observed decrease in calbindin D-9K levels in the BB diabetic intestine is in accordance with our studies using STZ diabetic rats and confirms previous reports (Nyomba et al, 1989; Schneider et al, 1973). Renal calbindin levels, like renal VDRs, were not affected by BB diabetes, suggesting tissue specific regulation of calbindins by 1,25 DHCC as has been previously reported (Christakos et al, 1989).

Plasma calcium was significantly reduced in BB rats (Table III-1). This is in contrast to data from STZ diabetic rats (Chapter II) in which plasma

calcium was not affected by acute diabetes. Nyomba et al (1989) reported normocalcemia in BB diabetic rats; they suggested calcemia is maintained in diabetes since hyperphagia results in increased passive calcium absorption and calcium requirements are reduced due to decreased growth rate. This would suggest that decreased calbindin D-9K and reduced circulating 1,25 DHCC during diabetes are an adaptation to increased total calcium absorption. Indeed, reduced plasma PTH has been observed in BB diabetic rats (unpublished results, our laboratory). In contrast STZ diabetic rats exhibit normal or elevated PTH (unpublished results, our laboratory; Schedl et al, 1978). It may be that the temporal changes in calcium homeostasis differ in STZ and BB diabetic rats, perhaps due to differences in disease etiology or severity. In our studies, BB diabetic rats were more severely diabetic, as indicated by a greater increase in blood glucose and a greater decrease in circulating 1,25 DHCC as compared to the STZ diabetic rats. In our preliminary study, plasma calcium was normal after 10 days of STZ diabetes, but significantly reduced by day 20. The reduction in plasma calcium was preceded by a reduction in circulating 1,25 DHCC which was evident by day 10. Other studies have shown net calcium absorption (Schneider and Schedl, 1972; Wood et al, 1984), retention (Wood et al, 1984) and plasma calcium (Schneider et al, 1977b) to be lower in untreated diabetic rats than in controls. Further research is required to determine the temporal relationship between alterations in circulating 1,25 DHCC, calbindin D-9K and calcium transport in the two diabetic models. However, our data clearly demonstrate that intestinal calbindin is reduced, despite up regulation of VDRs, in both models.

CONCLUSIONS

The studies described in this thesis suggest the following conclusions:

(a) untreated diabetes is associated with decreased circulating 1,25 DHCC, disturbed mineral homeostasis and up regulation of intestinal VDRs;

(b) VDR up regulation in intestine is associated with tissue hyperplasia;

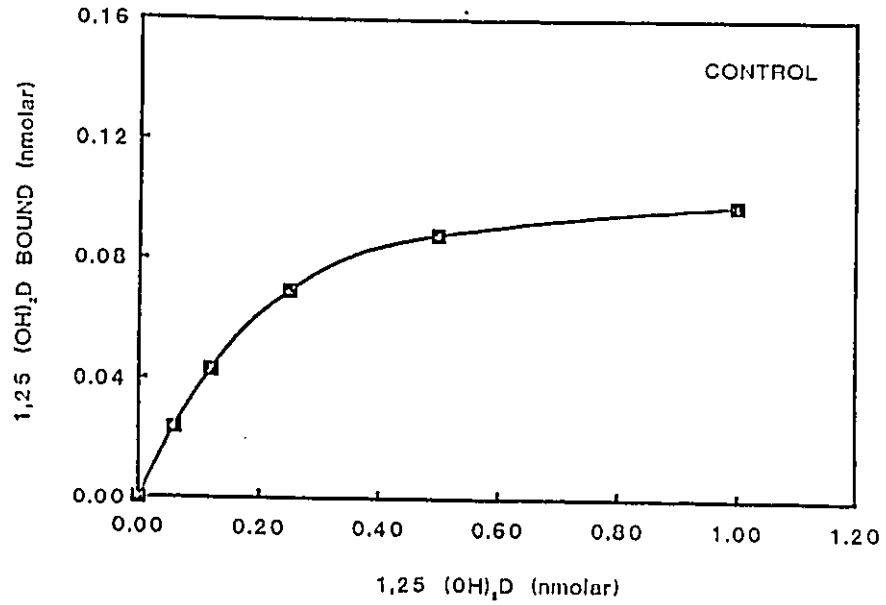
(c) although the action of 1,25 DHCC in the intestine seems to be impaired, intestinal function in general is not;

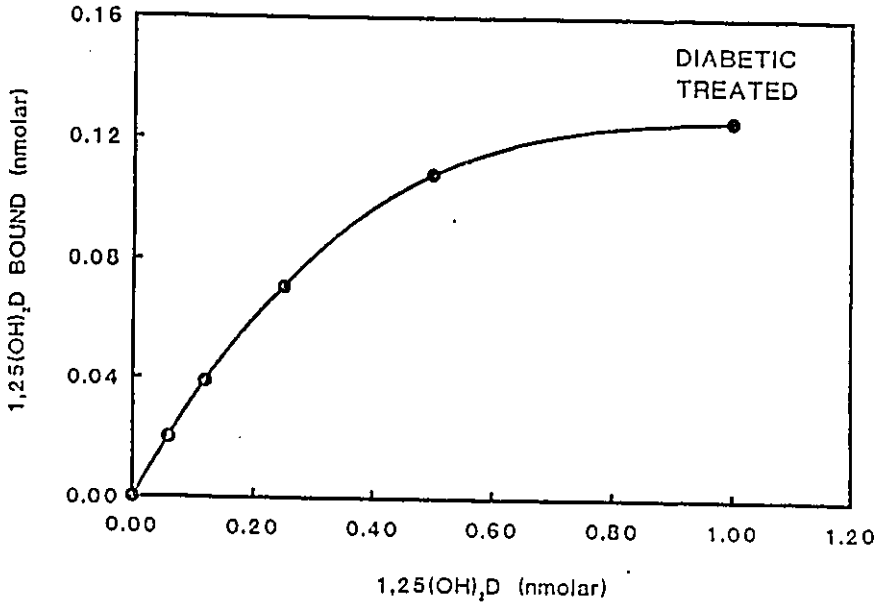
(d) a temporal relationship exists between the decrease in circulating 1,25 DHCC, decreased calbindin D-9K and aberrations in mineral homeostasis;

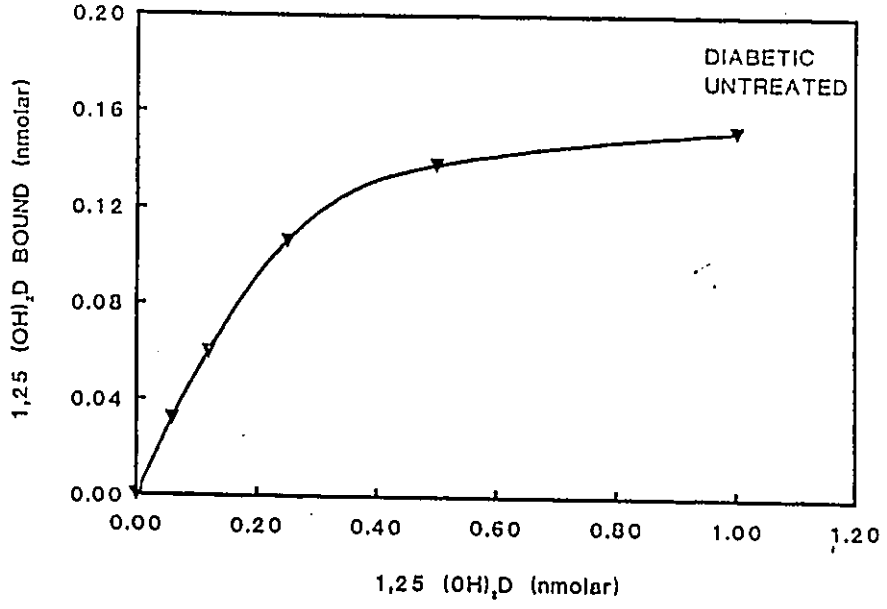
(e) the above changes are related to insulin deficiency since most are observed in two distinct models of insulin deficiency, and are corrected by exogenous insulin treatment.

APPENDIX

Figure (i). Saturation plot of ^{3}H-1,25 DHCC equilibrium binding to intestinal mucosal cytosol obtained from control (top), untreated STZ diabetic (middle) and insulin treated STZ diabetic rats. See legend Figure II-1 for assay details.


CONTROL
1,25 (OH)2D BOUND (nmolar)
0.16
0.12
0.08
0.04
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1,25 (OH)2D (nmolar)


DIABETIC
TREATED
1,25(OH)2D BOUND (nmolar)
0.16
0.12
0.08
0.04
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1,25(OH)2D (nmolar)


DIABETIC
UNTREATED
1,25 (OH)2D BOUND (nmolar)
0.20
0.16
0.12
0.08
0.04
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1,25 (OH)2D (nmolar)

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