# ROLE OF DOPAMINE IN DIABETIC NEPHROPATHY

**BY**

**ANIMESH SAHAI**

A Thesis
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Doctor of Philosophy

Department of Anatomy
University of Manitoba
Winnipeg, Manitoba

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Animesh Sahai

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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**To**

**My Mother**
**for teaching me the values of life**
**And**
**My Father**
**for helping me abide by them**

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## ABSTRACT

Dopamine is a physiologically important paracrine substance produced in the kidney. In addition to modulating renal blood flow, dopamine is of consequential importance in the regulation of electrolyte homeostasis. Dopamine by virtue of its regulatory effects on water and electrolyte balance may have a crucial role in maintaining homeostasis in volume-related disorders like diabetes mellitus. Initially, experiments were designed to systematically characterize the role of dopamine in diabetes mellitus. Plasma levels were shown to be increased while endogenous content of renal dopamine was shown to be decreased in diabetic animals. Since it can be argued that this may be artefactual, turnover studies were performed in diabetic animals and compared to controls. Dopamine uptake was decreased but the turnover is increased in diabetes suggesting that there is a overall increase in dopaminergic activity in diabetes. Radioligand binding studies were performed to characterize the dopamine receptors. Scatchard analysis revealed a downregulation of the DA1 receptor in the diabetic kidney. The analysis was further extended using 10-point competition curves which revealed a best fit to a two-site model, a high-affinity low-density and a low-affinity high-density site. Both these receptor subtypes were downregulated in chronic diabetes. Since dopamine coordinates intracellular calcium concentration by modifying the transport mechanisms, attention was then directed to characterizing the status of cytosolic calcium and the regulatory influence of dopamine.

The $Ca^{2+} + Mg^{2+}$ ATPase-linked $Ca^{2+}$ extrusion pump is believed to play a crucial role in the maintenance of an optimal cytosolic concentration. Though calcium is important in cell signalling (third messenger) and other biochemical processes, the

situation of calcium overload could compromise cellular integrity and function. Studies of the calcium pump in diabetes revealed a depressed level in chronic diabetes. The specificity of this reaction was assessed by the stimulatory response of another enzyme, $Na^+ + K^+$ ATPase, to varying levels of dopamine. Phosphorylation studies further established that the dopaminergic activity is increased in diabetes as suggested by an enhanced $Na^+ + K^+$ ATPase activity in diabetes. Furthermore, there was a lack of response of the calcium pump to increasing concentrations of dopamine in the diabetic model. Furthermore, there was a lack of response of the calcium pump to increasing concentrations dopamine in the diabetic model. As a consequence of this, there exists a depressed calcium pump which favors increased intracellular calcium concentrations and preferentially activates calcium-dependent processes.

Transglutaminase is a zymogen which is activated by millimolar concentrations of calcium and catalyzes the formation of isopeptide crosslinking between endo-γ-glutaminyl and endo-ε lysyl residues. Intracellular calcium levels trigger the induction of latent transglutaminase activity leading to irreversible crosslinking. The present study also showed an increase in the kidney cortical extract of diabetic animals. Cells in the diabetic kidney have calcium levels which are capable of inducing such crosslinking of the structural proteins of the extracellular matrix. The extracellular matrix is a mirror of the metabolic history of the cell and alterations including crosslinking may play a causal role in the development of diabetic nephropathy.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| DA | Dopamine |
| ATPase | Adenosine triphosphatase |
| BLM | Basolateral membrane |
| $Ca^{2+}$ | Calcium |
| K + | Potassium |
| Mg 2+ | Magnesium |
| Na + | Sodium |
| DDH 2O | Double distilled water |
| MAO | Monoamine oxidase |
| COMT | Catecholamine-O-methyl transferase |
| L-AAAD | L-aromatic amino acid decarboxylase |
| cAMP | cyclic adenosine monophosphate |
| DA1 | Dopamine receptor type 1 |
| DA2 | Dopamine receptor type 2 |
| GFR | Glomerular filtration rate |
| RBF | Renal blood flow |
| ESRD | End Stage Renal Disease |

# 1. INTRODUCTION

Diabetic nephropathy is a condition characterized by micro- or macroalbuminuria and progressive renal dysfunction and occurs in 33-45% of patients with insulin-dependent diabetes mellitus and 15-25% of patients with non-insulin dependent diabetes mellitus (1-3). This condition, which is irreversible, may be modified, though usually results in end-stage renal disease (ESRD) (4). Twenty-seven percent of ESRD patients have a primary diagnosis of diabetes, and diabetes is the most rapidly growing cause of ESRD, with new cases increasing by almost by 11% annually (5). Hemodialysis, renal transplantation, continuous ambulatory peritoneal dialysis and treatment for anemia by erythropoietin all significantly increase direct medical costs (6). The monetary burden of ESRD is high with the 1995 US Renal Data Sytem approximating the cost at US$ 55 000 per patient per year to US$ 11 billion annually (7). Furthermore, the socio-economic impact of ESRD is far-reaching, ranging from morbidity and mortality, a rapidly growing health-care budget and unemployment, to an impaired ability of the patient and family to live a 'normal' life (8).

Diabetic nephropathy is a progressive disease with its attendant proteinuria and structural lesions leading to a decrease in glomerular filtration rate (9). The initial stages are characterized by an increased glomerular filtration rate (GFR) accompanied by renal hypertrophy (10). The appearance of proteinuria heralds the onset of clinical diabetic nephropathy accompanied by typical pathological findings (11). Studies of experimentally induced diabetes (12, 13) and of patients with diabetes (14, 15) reveal that abnormal intracellular calcium metabolism is a common defect in both insulin-dependent and non-insulin-dependent diabetes. Defective handling of cytosolic calcium is germane

to all tissues tested suggesting that the abnormal metabolism is a fundamental disorder of the diabetic state (16).

Of the many risk factors identified in the pathophysiology of diabetic nephropathy, hemodynamic alterations have been shown to be preeminent. The increase in GFR may be damaging to glomerular endothelial, epithelial and mesangial cells, thereby initiating and contributing to the progression of diabetic nephropathy (17). Numerous mediators of hyperfiltration have been proposed, and this phenomenon likely reflects a multifactorial etiology (18, 19). Dopamine has been identified as a putative factor, with its paracrine and autocrine function, in the pathogenesis of diabetic nephropathy (20). Dopamine, a precursor of norepinephrine and epinephrine, is produced at proximal tubular and possibly at neuronal sites in the kidney. The dopamine is thus released into the interstitium and available for local cell-to-cell action (21). In the kidney, dopamine exerts a tonic effect by coordinating the fluid and electrolyte balance. Although, the renal vasodilator effect of dopamine most likely contributes to the natriuretic effect, the changes in electrolyte excretion can occur in the absence of changes in renal blood flow and glomerular filtration rate (22). Thus, dopamine via its hemodynamic effects may play a modulatory role in electrolyte regulation in metabolic disorders of fluid regulation like diabetes mellitus. In addition to increasing renal blood flow, dopamine generally increases urine flow, sodium chloride, potassium, phosphate and calcium excretion (23). Altered dopamine regulation of calcium may be of consequential importance in the development of diabetic nephropathy. The ubiquitous calcium dysfunction may be the unifying theory at the biochemical level in the genesis of diabetic nephropathy. Adaptive mechanisms operating in early diabetes preserve the integrity of the calcium regulating

system ensuring the constancy of the renal cell environment. Dysregulation of the calcium environment may lead to intracellular calcium overload thus precipitating nephropathy.

The glomerular extracellular matrix, comprising the glomerular basement membrane and mesangial matrix, is a mirror of the metabolic history of the cell. Alterations in the quality and quantity of matrix proteins may modify its functional features through compromise of adhesive and self-assembly properties of its components via increases in both collagenous and noncollagenous crosslinking (24, 25). In this regard, there exists a family of enzymes that catalyze the formation of crosslinks through diamines and polyamines (26). Transglutaminase catalyzes covalent crosslinking between endo-γ-glutaminyl and endo-ε-lysyl residues of proteins resulting in the formation of γ-glutamyl-ε-lysine or ε(γ-glutamyl)lysine isopeptide side-chain bridges. It is a bifunctional protein with the possibility of an infinite degree of linear polymerization by γ-glutamyl-ε-lysine bridges, while the exclusive proteins may only serve as chain initiators or terminators. The presence of enzymes that promote irreversible crosslinking of proteins could have disastrous consequences for the cell but this is precluded as the physiologic activity of this enzyme requires $Ca^{2+}$ and is cryptic under physiologic conditions. The enzymatic activity can be detected if cytosolic $Ca^{2+}$ is elevated to millimolar range from its usual physiologic intracellular concentrations (0.1-0.2 μM). $Ca^{2+}$ ion thus acts as a physiologic activator of this enzyme (27). The abnormal cytosolic $Ca^{2+}$ in the diabetic kidney may provide optimum conditions for the activation of transglutaminase and ensuing crosslinking of extracellular matrix proteins. The formation

of insoluble complexes via the crosslinking process may alter the functional processes in the kidney and aid in the development of diabetic nephropathy.

The purpose of this study was to investigate the hypothesis that alterations in dopaminergic activity may affect the cytosolic calcium status and promote activation of latent processes to aid in the development of diabetic nephropathy. The present study was therefore designed to systematically characterize the status of dopamine in the diabetic kidney. It was further extended to identify the interplay between dopamine and calcium pump in the diabetic kidney and to show how this interaction may foster the development of diabetic nephropathy.

# 2. REVIEW OF THE LITERATURE

## 2.1. DOPAMINE

### 2.1.1 Synthesis, storage and metabolism

Dopamine is an endogenous catecholamine that serves not only as a precursor for norepinephrine and epinephrine but also as a neurotransmitter (28). The essential amino acids phenylalanine and tyrosine, derived from proteins in the diet, form the starting point for catecholamine synthesis. These amino acids are slowly oxidized to dihydroxyphenylalanine, or dopa, by the action of tyrosine hydroxylase present in the cell bodies of adrenergic neurons and the adrenal medulla. Conversion of dopa to dopamine is a fast reaction catalyzed by aromatic-L-amino-acid decarboxylase. Dopamine is thus the first product in the sequence of catecholamine synthesis reactions. Dopamine is then carried by the vesicular monoamine transporter from the cytosol into the catecholamine storage vesicle where the synthetic pathway proceeds to the formation of norepinephrine by hydroxylation of dopamine catalyzed by dopamine β-hydroxylase (29). In sympathetic axons and in 15 to 20% of chromaffin cells, norepinephrine is the final product. In 80 to 85% of chromaffin cells, a further enzymatic step occurs. Norepinephrine is further N-methylated by phenylethanolamine N-methyltransferase to epinephrine. However, in dopaminergic neurons there is no dopamine β-hydroxylase or phenylethanolamine N-methyltransferase, and as a result synthesis halts at dopamine and does not proceed further (30).

Catecholamines in noradrenergic axons and chromaffin cells are sequestered from the cytosol in membrane-limited organelles, catecholamine storage vesicles (or

chromaffin granules in chromaffin cells) (31). The process of catecholamine discharge from chromaffin cells and sympathetic axons is exocytosis, whereby all soluble components are simultaneously released and ultimately make their way into the circulation. In the bloodstream catecholamines have a very short half-life, 1 to 2 minutes, and are cleared from the circulation by reuptake and enzymatic degradation. Complete enzymatic degradation involves the sequential action of two cytosolic enzymes, monoamine oxidase, MAO, and catecholamine-O-methyltransferase, COMT, either of which may initiate the process. COMT, found mainly in the cytosol of liver and kidney cells, adds a methyl group to one of the hydroxyl oxygens on the catecholamines' dihydroxyphenyl ring yielding methoxytyramine from dopamine. Similar metanephrine compounds are obtained from norepinephrine and epinephrine. The metanephrines are then deaminated by MAO to yield homovanillic acid (in the case of dopamine) and vanillic acid. Besides enzymatic degradation, catecholamines, including dopamine, undergo clearance from the circulation by direct renal excretion and sulfoconjugation of a ring hydroxyl group (32).

### 2.1.2 Endogenous dopamine in the kidney

The amount of dopamine excreted in human urine is 5-20 fold greater than the amount of norepinephrine (33). Initial assumptions of plasma filtration as the source of dopamine in the urine were rendered invalid with the development of sensitive and specific radioisotopic techniques. Three main hypotheses have been put forward to account for the large amounts of dopamine found in human and animal urine,

a) deconjugation hypothesis (Kuchel hypothesis) (34)

Dopamine, derived from the adrenal gland, is rapidly conjugated to the sulfate and glucuronide and these compounds circulate to the kidney. Renal enzymes such as β-D-glucuronidase and arylsulphatase deconjugate these compounds releasing free dopamine into the kidney substance and then into the urine. However, this theory, though attractive, has been disputed by several studies (35-37). Inhibition of dopa decarboxylase by carbidopa reduced renal dopamine production suggesting that a normal activity of dopa decarboxylase was necessary for renal production of dopamine in man (35). Also, studies with γ-glutamyl dopamine, a renal dopaminergic prodrug, have refuted the deconjugation hypothesis (36, 37). On administration of this drug, concentrations of dopamine are not increased, implying that extrarenal cleavage of this compound is minimal. In contrast highly significant amounts of free and conjugated dopamine are found in the urine.

b) Renal dopaminergic nerves

Dopamine-containing neuronal elements have been identified in the dog kidney (38, 39). It has been suggested that dopamine released into the urine might be an overspill from the dopaminergic nerves. This theory seems unlikely in the face of rapid and effective reuptake mechanisms present in most dopaminergic neurons (40, 41). Injecting tritiated L-dopa into the proximal tubules or peritubular space of innervated and denervated kidneys showed that, though dopa was converted into dopamine, there was no difference between innervated and denervated kidneys (42). Studies on the acutely and chronically denervated kidneys have suggested that these nerves tonically inhibit renal tubular dopamine production (42, 43). Even if these nerves cannot account for the

majority of the free dopamine in the urine, they have a significant modulatory effect on overall renal dopamine contribution.

c) Renal tubular production

Dopamine is formed in the renal tubular cells by the action of L-aromatic amino acid decarboxylase (L-AAAD) on L-dopa and is then secreted into the tubular lumen (44, 45). The tubular transport of L-dopa has been characterized as an active process with great structural specificity, and might even be the rate-limiting step (46). Histochemical techniques have localized L-AAAD to the renal cortex and is extraneuronal since sympathetectomy does not alter L-AAAD activity (47). Most of the renal tubular dopamine production occurs in the S1 and S2 segments of the proximal tubule with the majority of the activity located around the apical cell membranes of the proximal tubules (48). Some activity has also been localized to the distal tubule (49). It must be pointed out that L-AAAD is not specific for dopa, since it also decarboxylates 5-hydroxytryptophan, O-tyrosine and N-tyrosine (50). The classic experiments of Ball et al. in the dog have shown renal tubular production to be a major source of urinary free dopamine (51). By studying arterio-venous differences, they demonstrated a rise in dopamine and fall in L-dopa concentration.

The above studies together with an analyses of those on plasma dopamine and dopamine prodrugs would favor renal tubular production of dopamine as the major source of free urinary dopamine and to a lesser extent from renal dopaminergic nerves (52). Urinary dopamine primarily reflects renal tubular dopamine production, whereas renal nervous dopamine output can be assessed from renal venous levels (53).

### 2.1.3 Functional regulation of endogenous dopamine

Since dopamine may serve as an intrarenal natriuretic hormone, endogenous dopamine plays a role in regulation of renal sodium excretion under markedly varying sodium intake and thus maintaining electrolyte homeostasis (54, 55). The state of hydration, the type and degree of volume expansion, age and genetics, as well as species influence the action of endogenous dopamine on renal sodium transport (56). Dietary sodium appears to be a major regulating factor in the renal tubular production of dopamine. Salt loading in humans and the animal leads to increased renal production of dopamine (57, 58). Increase from a low sodium (9 mmol $Na^+$/day) to a high sodium (249 mmol $Na^+$/day) diet resulted in a two-fold increase in L-dopa and urinary dopamine (59). Plasma L-dopa was unchanged by dietary salt manipulation, which would suggest increased delivery of L-dopa to sites of uptake by proximal tubular cells (60). Additionally, tubular transport of some aromatic amino acids is sodium-dependent and this might be one mechanism for accelerated uptake (61). Likewise, sodium may have intratubular effects on dopamine production (41). Moreover, other factors may be involved in modulating sodium excretion and renal dopamine production. α-human ANP, acting via cGMP, has been shown to inhibit dopamine production, though this mode may be under ideal conditions (62, 63). Further evidence for a physiological role of endogenous dopamine comes in conditions associated with abnormalities of the dopaminergic system. In patients with congenital dopamine β-hydroxylase deficiency, circulating L-dopa is increased and dopamine is produced in excess while epinephrine and norepinephrine are absent. These patients have high excretion rates of sodium compared to control and the pressure-natriuresis curve is shifted to the left (64).

Other crystalloids have also been evaluated in renal dopamine production. Harvey et al. (65) showed that, in man, potassium chloride is not a stimulus for increased dopamine output, though in the rat, the chlorides of sodium, potassium and ammonium augmented renal dopamine production (66). However, the discordant response to potassium may be a dose-response effect. It must also be mentioned that sodium bicarbonate inhibits dopamine production, prompting the idea that the chloride ion is of consequential importance in renal dopamine production (66).

Other regulating determinants may be intricately involved in sodium load and obligatory dopamine response (67). In comparison to crystalloids, colloids, specifically intravenous albumin, produce no such increase in urinary dopamine output (68). This implies that the renal sensing mechanism for dopamine production can distinguish between different modalities of intravascular volume expansion. Analogously, protein loading enhanced urinary dopamine production forasmuch as urinary norepinephrine and epinephrine output was unaltered. The increased dopamine production and the associated natriuresis was largely prevented by carbidopa (69). This exaggerated response occurs due largely to the tyrosine obtained from the protein load, and subsequent conversion to L-dopa. The antinatriuretic effect of dopamine blockers is not entirely due to an increase in aldosterone production, since adrenalectomy does not interfere with the antinatriuretic effect of dopamine blockade.

To summarize, the main controlling factors for renal dopamine production are sodium and/or chloride ions, dietary protein, and the intracellular electrolytic and hormonal medium.

### 2.1.4 Renal actions of dopamine

Infusion of dopamine either given systemically or directly into the renal artery produces a dose-dependent increase in renal blood flow and a less marked rise in glomerular filtration rate (70). This response is blocked by dopamine antagonists but not by other neurotransmitter antagonists, suggesting the specificity of this response. Similarly, dopamine infusion has been shown to produce an increase in para-aminohippurate clearance and inulin clearance (71). Simultaneous hemodynamic studies indicated that these changes are accompanied by prominent increases in cardiac output but no significant changes in heart rate or arterial pressure. Similar increases in renal function have not been reported after administration of other sympathomimetic amines (72). It must be pointed out that, though fewer studies have been done on humans, the results are similar to animal studies though to a lesser degree. Experiments have revealed that augmentation of renal function was not secondary to systemic hemodynamic changes, for administration of dopamine in one renal artery produced greater augmentation of glomerular filtration rate, para-aminohippurate clearance, and sodium excretion in the infused kidney than on the contralateral side (71). Furthermore, the vasorelaxing properties of dopamine have been demonstrated in the main renal, arcuate and interlobular arteries, and afferent and efferent arterioles (73-75).

In addition to augmenting renal blood flow, exogenous dopamine generally increases urine flow, as well as sodium chloride, potassium, phosphate and calcium excretion (76-79). Dopamine induced natriuresis has been shown to be in effect in sodium surfeit states, and actually decreased sodium excretion in sodium deficit states (80). In hydropenic states, dopamine increased renal blood flow and glomerular filtration

rate without affecting sodium excretion rate. In contrast, in volume-expanded dogs dopamine did increase renal blood flow and induced a natriuresis and diuresis (81). Thus the natriuretic effect of dopamine becomes more evident under states of extracellular fluid volume expansion. Although, the renal vasodilator effect of dopamine most likely contributes to the natriuretic effect, the changes in electrolyte excretion can occur in the absence of changes in renal blood flow and glomerular filtration rate (82). Thus, dopamine via its hemodynamic effects may play a modulatory role in electrolyte regulation.

### 2.1.5 Mechanism of action

Dopamine binds to stereospecific cell receptors and evokes its physiological response in target cells. This hormone-receptor interaction leads to transduction of both cyclic adenosine monophosphate (cAMP) dependent (83, 84) and cAMP-independent (85, 86) intracellular pathways. Since cellular function is largely regulated by protein phosphorylation, these second messengers regulate phosphorylation by protein kinases and dephosphorylation by phosphatases thereby expressing the physiological actions induced by dopamine.

a) Dopamine receptors

Dopamine receptors have been localized to vascular, neuronal and tubular sites in the kidney (87, 88). Radioligand binding studies have shown two types of renal dopamine receptors, viz., DA1 and DA2 (89). DA1 And DA2 receptors are present in brushborder and basolateral membranes of proximal tubules and in cortical collecting ducts (90). DA1 receptors have been identified in the medial layer of renal microvessels, while DA2

receptors have been recognized presynaptically on sympathetic nerve terminals in the adventitia and intima of renal vessels, and in the glomerulus (89).

The transducers of the DA1/DA2 receptors involve both cAMP-dependent and cAMP-independent mechanisms. Occupation of the DA1 receptor leads to a stimulation of the adenylate cyclase activity with formation of cAMP (83). This action could be mimicked by DA1 agonists and blocked by DA1 antagonists (84). Renal DA1, but not DA2, receptors also are associated with stimulation of phospholipase C activity, which is independent of adenylate cyclase (91). Guanine nucleotides mediate the stimulation of phospholipase C or adenylate cyclase induced by dopamine and DA1 agonists (92). The mechanism by which a single receptor system can coordinate two different pathways is unknown. It is postulated that either multiple forms of the same receptor or deferential coupling of the receptor with different G proteins may be the mechanism of action. Thus DA1 receptors may couple to one type of G protein leading to stimulation of the adenylate cyclase system, whereas stimulation of another G protein may induce the phospholipase C system (93, 94).

The natriuresis associated with dopamine and DA1 agonists is due to renal hemodynamic and tubular effects (82). The tubular effects are brought about via actions at the proximal tubule and at more distal sites including the collecting ducts (90). In the proximal convoluted tubule, dopamine decreases sodium uptake at the brush-border membrane by decreasing $Na^+$ - $H^+$ antiport activity (95). This action is mediated via stimulation of membrane-bound adenylate cyclase activity resulting in, formation of cAMP which in turn leads to protein phosphorylation and inhibition of $Na^+$ - $H^+$ antiport mechanism. Furthermore, DA1 agonists decreased the $V_{max}$, but did not alter the $K_m$ of

the transporter (81). Inhibition of cAMP formation prohibits this inhibitory effect of dopamine on $Na^+$ transport. The $Na^+$ - $K^+$ ATPase at the basolateral membrane supplies the driving force for the transcellular transport of electrolytes and organic solutes and thereby plays a crucial role in the resorptive capacity of the kidney (93). At the basolateral membrane, dopamine modulates sodium transport by fine tuning the $Na^+$ - $K^+$ ATPase enzyme (96, 97). It must be pointed out that dopamine has no effect on purified $Na^+$ - $K^+$ ATPase, and therefore it is postulated that this effect may be via G proteins and signal transducers (98, 99). Dopamine and agonists stimulate phospholipase C activity and catalyze the formation of inositol phosphates and diacylglycerol. Diacylglycerol activates protein kinase C resulting in protein phosphorylation and inhibition of the $Na^+$ - $K^+$ ATPase enzyme (91). However, this compartmentalization of sodium transport may be too rigid and simplistic as inhibition of $Na^+$ - $K^+$ ATPase has been demonstrated in the rat renal proximal tubular cells (96). Akin to the brain, a third intracellular messenger was identified coupled to the DA1 chain of events. This messenger, 32 amino acid dopamine- and cAMP-regulated phosphoprotein, DARPP-32, was identified by biochemical, immunohistochemical and in situ hybridization techniques to have an anatomical distribution similar to dopaminoceptive cells possesing DA1 receptors. The mechanism postulated is that cAMP stimulates cAMP-dependent protein kinase, and phosphorylates dephospho-DARPP-32 to phospho-DARPP-32, and thus inhibits protein phosphatase-1. As a consequence of inhibition of protein phosphatase-1, the inactive phospho- $Na^+$ - $K^+$ ATPase complex is dephosphorylated to the active complex (101). The active complex is rendered to its inactive form by a protein kinase-dependent phosphorylation. Complementary studies with isolated tubule segments have also shown that dopamine

caused a change in the Michelis constant for $K^+$, suggesting a conformational change in the $Na^+ + K^+$ ATPase complex (102). Furthermore, dopamine inhibition of $Na^+ + K^+$ ATPase can be prevented by pertussis toxin, suggesting G protein involvement (93), and by sphingosine, indicating protein kinase participation (92). Dopamine may also inhibit sodium transport by several other mechanisms (99). It can antagonize the effect of mineralocorticoids in collecting ducts, or by its interactions with other hormonal systems, viz., aldosterone and the pro-opiomelanocortin biosynthesis (103, 104).

Dopamine regulates intracellular electrolyte concentration by modifying their transport mechanisms. Dopamine coordinates cytosolic calcium levels by increasing calcium excretion by the kidney though the exact mechanism of action is unknown (78). Furthermore, dopamine acts via activation of membrane phospholipases which are involved in calcium flux (78). Blockade of calcium entry by nifedipine was associated with a reduction in basal renal blood flow, suggesting that there is a link between dopamine receptors and calcium flux resulting in vasodilation and alteration of renal blood flow (105). Thus dopamine may be involved in calcium homeostasis acting via its tubular and hemodynamic effects, though its exact mechanism still remains to be elucidated.

### 2.1.6 Role of dopamine in pathology

Dopamine by its regulatory effects on water and electrolyte balance may have a role in maintaining plasma volume and electrolyte homeostasis. It can be argued that a relative failure of either its levels or end-organ hyporesponsiveness may be of pathologic significance in such disorders. Dopamine has been shown to consistently increase sodium excretion and thus improve cardiac and renal functions in patients with congestive heart

failure (106). Though adverse effects have limited this application to short term infusions, this therapeutic application offers great potential (107). Dopamine appears to be beneficial in oliguric patients with normal or low systemic resistance. In addition to the possibility of improving renal blood flow, dopamine increases peripheral resistance more than isoproterenol and less than norepinephrine. Thus dopamine conceivably has a role in different forms of shock (108). Similar improvements in renal function have been noted in patients with renal failure due to hypertension and cirrhosis (109). A role for dopamine has been shown in acute renal failure in combination with diuretic agents obviating the need for dialysis in some of these patients (110). Diabetes mellitus is another volume-related disorder, where defective mobilization of dopamine has been causative in impaired salt-loading response (111). The increase in sodium excretion on salt-loading, in diabetic patients, is markedly delayed and no significant increase in urinary dopamine output is observed (112). As a consequence of this, a cascade of events may be initiated in diabetics leading to altered volume and electrolyte handling. In addition, the changed *milieu interieur* may lend itself to cause further pathological insult in varied organs. Thus, unique renal and hemodynamic effects have been ascribed to dopamine, which may have a significant role in the pathophysiology of related disorders.

## 2.2. DIABETIC NEPHROPATHY

Diabetic nephropathy is a leading cause of chronic renal failure in the United States and is one of the leading causes of morbidity and mortality in the individual diabetic patient (113). It occurs in approximately 33-45% of patients with insulin-dependent diabetes mellitus, and 15-25 % of patients with non-insulin dependent diabetes mellitus (1). Twenty-seven percent of end-stage renal disease patients have a primary

diagnosis of diabetes, with new cases of diabetes-related end-stage renal disease growing by 11 % annually (1). The monetary cost of end-stage renal disease is high with any form of medical intervention. Hemodialysis, continuous ambulatory peritoneal dialysis and renal transplantation, directly, contribute sigificantly to the cost of medical care. Indirect costs in terms of quality of life, emotional burden to patient and families are of consequential importance.

Diabetic nephropathy may be defined as, " a multifaceted pathologic entity which spans the continuum from early renal hypertrophic changes to late stages of advanced structural distortion of glomeruli, renal vasculature, interstitium and tubules (114). It is progressive, and is characterized initially by subtle abnormalities of morphology, accompanied by increased glomerular filtration rate and renal hypertrophy. Microalbuminuria may be present in a variable percentage of such patients, while about half of them may be hypertensive even in the absence of microalbuminuria (115). This preclinical phase then progresses to a stage of incipient nephropathy, distinguished by microalbuminuria in the range of 20 - 200 μg/min, and well-established glomerular abnormalities (116). The appearance of dipstick-positive urine heralds the onset of the clinical phase of the diabetic patient (117). Diabetic nephropathy appears in its clinical form after at least 5-7 years of diabetes mellitus. The subsequent decline to renal failure after the appearance of proteinuria is rapid (117); the rate of decline in renal function may vary over a four-fold range but is linear for each patient (116). In the pre-dialysis and transplantation era, an average of four to five years elapsed between the detection of proteinuria and death from uremia (118). A consensus of reports are consistent with an average decay of GFR of 10-15 ml/min/year from the stage of proteinuria when it is 50-

90 ml/min/year (119). Thus, the evolution from the onset of proteinuria to end-stage renal disease may vary from months to decades but is on an average of 4-5 years.

Hypertension is an important prognosticator in the development and especially in the progression of renal damage in diabetics (115). There is a higher prevalence of hypertension in diabetic nephropathy compared to age-matched and sex-matched controls with diabetes mellitus but without diabetic nephropathy (1). Prospective studies in patients with diabetes mellitus have noted an increase in blood pressure with an increase in albumin excretion (120). Initially, there is a small but significant increase in blood pressure, though these patients are not necessarily hypertensive, and typically have a preservation of renal function with a normal or elevated GFR (120). Studies have also documented the correlation between the mean arterial pressure and albumin excretion rate (120, 121). Results of many cross-sectional studies have further reported the renal damage associated with hypertension in diabetes mellitus. Long-term studies with anti-hypertensives have also shown the efficacy of these drugs in reducing the incidence of overt nephropathy in normotensives and hypertensives (122, 123). Parving et al. (122) observed a limited ability in the autoregulatory property of the kidney, due to a diminution in the vasoconstrictive properties of the afferent arteriole in diabetes mellitus. Consequently, the elevated glomerular capillary pressures may contribute to glomerular hypertension; the presence of chronic hyperglycemia may further contribute to the renal damage (124). Furthermore, patients with diabetic nephropathy have been shown to have an elevated red blood cell sodium-lithium countertransport activity which may in itself be a risk factor in the development of hypertension by its ability to retain sodium (125). In addition, enhanced red blood cell sodium-lithium countertransport activity may induce

mitogenic activity in the mesangial cells, and thereby alter mesangial tone and subsequently the GFR. Alterations in the GFR may be a contributing factor in the development of end-stage renal disease secondary to diabetes mellitus (126).

### 2.2.1 Structural-functional correlations of diabetic nephropathy

An initial enlargement of the glomeruli has been demonstrated in early-onset diabetes mellitus in the face of an enlarged kidney (127). Moreover, glomerular basement membrane thickening, measured by the orthogonal intercept method, is generally uniform, both within and between glomeruli, and detectable 2 years after the onset of diabetes mellitus. The thickening tends to progress with duration of diabetes, and is maximal in the presence of nodular glomerular lesions. Immunofluorescence studies have demonstrated increased staining of α3(IV)NC and α4(IV)NC domains of type IV collagen that persist into the advanced stages of the disease (128). Similarly, mesangial expansion can be detected 3.5 to 5 years after the onset of diabetes, and most often is the dominant pathologic process in end-stage renal disease (129). The increase in mesangium derives from the increase in constituents of the matrix, with the increase in matrix material significantly more than the mesangial cells. The Kimmelsteil-Wilson nodular lesion is the culmination of pathologic mesangial expansion in diabetes mellitus (130). Though considered pathognomonic of diabetes, the Kimmelsteil-Wilson nodule is not universally present in diabetes (131). Also, capillaries can develop microaneurysms which may be the site of future of the Kimmelsteil-Wilson nodule. The nodules are oval structures that vary remarkably in size as well as number within individual glomeruli, with some glomeruli in a single specimen demonstrating several nodules while others show none. The diffuse form of intercapillary glomeruloscerosis is more common than

the nodular lesion, and represents a range of expansion of the mesangium from minimal to overt (127, 132). Histopathologically, it is similar to the nodular form being PAS positive and eosinophilic. Other common lesions in diabetes are the capsular drop and fibrin cap lesions (133). The capsular drop lesion is an exudative hyaline lesion in the subendothelial space, while the capsular drop forms under the parietal layer of the epithelium along Bowmans' capsule. Though arteriosclerosis is ubiquitous and affects all arterioles in the body, arteriolar hyalinosis of both afferent and efferent arterioles is probably the earliest renal change, and could play a significant role in glomerular scarring (127, 133). Epithelial and endothelial cell structures and the interstitium are generally well preserved in the initial stages of the disease; with continued progression of the disease diffuse epithelial and tubulo-interstitial changes occur (134). There is fusion of the foot processes of the epithelial cells, which later degenerate and become detached from the basement membrane giving rise to areas stripped of epithelial covering. The tubules atrophy with thickening of the tubular basement membrane. The interstitium thickens with infiltration of several classes of inflammatory cells. Lymphocytes, plasma cells, neutrophils and eosinophils propitiate the chronic scarring process of the diabetic kidney (135, 136). Immunofluorescence studies have demonstrated increased amounts of native antigens as well as other circulating proteins (137). Increased linear staining of IgG and IgM has been demonstrated along the basement membrane; less consistently, fibrin, C3, albumin and ceruloplasmin were also established along the glomerular basement membrane (138). Exudative lesions show the presence of large molecular weight proteins including gamma globulins, fibrinogen and lipoproteins. The glomerulus

displays positive immunofluorescence for several fundamental proteins viz., actomyosin, collagen, laminin and fibronectin (137).

Paralleling these observations, functional changes in the diabetic kidney have been documented by several studies (139-141). Micropuncture studies in diabetic rats have shown increased GFR, renal plasma flow, single-nephron GFR, and intraglomerular capillary pressure (141, 142). A causal link was established between hyperfiltration and diabetic nephropathy from single-nephron studies in animals (142). With extreme increases in single-nephron hyperfiltration, glomerular structural damage is clearly evident. Concomitantly, glomerular epithelial stripping, mesangial expansion, fusion of epithelial-cell foot protocytes, and endocytosis of plasma proteins in the form of dense droplets occured (143). These were associated with increased transcapillary glomerular hydraulic gradient, glomerular plasma flow and proteinuria (143, 144). Subsequent treatment with angiotensin-converting enzyme inhibition had a protective effect by reducing glomerular structural lesions and proteinuria. Indeed, it has been repeatedly shown that control of glomerular capillary hypertension is associated with significant slowing of structural injury (145). The increased GFR that occurs is probably caused by greater reduction in afferent than efferent arteriolar resistances and increased transcapillary hydraulic pressure gradient (146). Also, mesangial expansion is closely related to the function of the diabetic nephropathy. With increasing occupation of the glomerulus by the thickening mesangium, there is a decrease in the relative peripheral capillary wall filtration surface (147). Human studies have demonstrated that the filtration surface per glomerulus is directly related to the GFR (148). Increasing mesangial expansion (a mesangial volume >37%) is associated with overt proteinuria (149).

Hypertension is most frequently associated with increased albumin excretion and well-established glomerular lesions. Furthermore, seventy-five percent of patients with mesangial expansion have hypertension. Thus severe mesangial expansion is associated with nephropathy, altered GFR, proteinuria and hypertension (149). Similarly, arteriolar hyalinosis, glomerular sclerosis and cortical interstitial volume fraction showed a positive correlation with proteinuria and increased GFR, and subsequent development of diabetic nephropathy (150, 151).

Several factors have been identified as possible pathogenetic mechanisms in diabetic nephropathy. Glycemic control, metabolic environment, familial, ethnic and genetic predispositions, hemodynamic disturbances including systemic and renal hemodynamic alterations are possible candidates in an attempt to explain the development of diabetic nephropathy (152). The category of hemodynamic alterations may interact with metabolic and genetic factors to ultimately produce progressive diabetic nephropathy. Hyperglycemia, growth hormone, glucagon, glomerulopressin, increased kidney size, deficiency of insulin, ketosis, sorbitol accumulation are several candidates that have been implicated to explain the elevated GFR in diabetes (153). In addition to the altered levels of glucose, metabolites and associated metabolic fuel-related hormones, changes in circulating levels as well as vascular responsiveness to several classic vasoactive hormones may be altered in diabetes. Renal function in normal humans is influenced by catecholamines, renin, angiotensin II, atrial natriuretic peptide and prostaglandins, and modifications of these hormonal systems may contribute to the development of elevated GFR (153). Furthermore, use of angiotensin-converting enzyme inhibitors therapeutically adumbrate a special dependence of the diabetic kidney on the

renin-angiotensin system (154). Altered intrarenal hemodynamics may also play a significant role in the genesis of an elevated GFR. Glomerular hypertension due to altered glomerular arteriolar resistance, either a decrease in afferent or increase in efferent resistance, or increase in the ultrafiltration coefficient, $K_f$, may result in elevated GFR (155). Thus both systemic and renal hemodynamics may contribute to the development of an increased GFR. The increased GFR serves as a potential mediator of hyperfiltration and is an attempt by the body to maintain its fluid homeostasis (156). These increments in GFR average approximately 40% compared to age-matched non-diabetic controls (157). Experimental studies in rats made diabetic with streptozotocin indicate that diabetes induces a state of intrarenal vasodilation, with elevation of single-nephron glomerular capillary plasma flow rate. The mean glomerular capillary hydraulic pressure tends to rise as the reduction in afferent arteriolar resistance is proportionally greater than the efferent arteriolar resistance. Glomerular hyperperfusion and hypertension thus contribute to the single nephron hyperfiltration observed in diabetes (158, 159). However, despite extensive investigations, the precise mechanism underlying hyperfiltration remains elusive. One of the mechanisms implicates dopamine, a catecholamine, in the development of increased GFR (112). Considering the effects of endogenous dopamine in intrarenal hemodynamics, and the similarity of the altered hemodynamic state in diabetes, dopamine may well have a modulatory role in the pathogenesis of elevated GFR, and subsequent development of diabetic GFR.

### 2.2.2 Diabetic nephropathy and calcium metabolism

Accumulating data from animal models of diabetes and human patients reveal abnormal levels of calcium in most tissues (160, 161). The levels of the different

mechanisms of intracellular calcium homeostasis are also altered suggesting a potential contribution of these calcium homeostasis regulators in the pathogenesis of diabetic nephropathy. Altered intracellular calcium may be a recurrent theme in the metabolic, cardiovascular, renal, ocular and neural manifestations of diabetes mellitus (162). Renal cortical membranes are associated with an increased $Ca^{2+}$ + $Mg^{2+}$ ATPase, a regulator of intracellular calcium homeostasis (163). The increase in the enzymatic activity may reflect a compensatory response to increased intracellular calcium, and may in as yet unidentified ways account for the renal hypertrophy (164). Similarly, exposure of cells to calcium may result in selective overexpression of endoplasmic reticulum proteins that constitute the basement membrane (165). Increase in the rate of turnover of basement membrane proteins may contribute to changes in basement membrane width and composition. Additionally, altered intracellular calcium levels may alter the porosity of capillaries in diabetes by altering the contractile function of endothelial cells lining these capillaries (166). The consequence of these alterations may be in revised glomerular hemodynamics with a propensity for developing glomerular hypertension (167). The compensatory response of an elevated GFR may in itself induce further glomerular damage (168).

### 2.2.3 Diabetic nephropathy and extracellular matrix

Alterations in the quality and quantity of matrix proteins may modify its characteristics, both in terms of generation as well as function, in the face of a changing metabolic environment. The glomerular basement membrane and mesangial matrix are together referred to as the glomerular extracellular matrices. Mesangial expansion plays a pivotal role in diabetic glomerulosclerosis because the initial enlargement of the

mesangium is a function of the enlarged glomerulus (169), while it is only later that expansion of the mesangium occurs at the expense of the glomerular capillary lumen and filtration surface, thus leading to deterioration of the glomerular function (170). A growing body of evidence suggests a sequential progression of biochemical and metabolic events central to intracellular disposal of excess glucose via enzymatic and nonenzymatic pathways is involved in diabetic insult (171-173). These alterations may affect cellular integrity either directly or by interfering with the synthesis, release and action of paracrine and autocrine factors that influence remodelling. Increased levels of sugars produce enhanced non-enzymatic glycosylation that alter both the structure and function of macromolecules reacting with these sugars, and lead to the formation of advanced glycosylation end products (174, 175). Increased synthesis of extracellular matrix components have been identified subsequent to binding of these glycosylation end products to specific receptors (167, 177). Besides there are three other pathways for the further metabolism of glucose, viz., polyol pathway, glycolysis, and pentose phosphate shunt (179-182). As a result of these pathways, there is enhanced de novo synthesis of diacylglycerol from glucose. Moreover, the increased cytosolic NADH/$NAD^+$ ratio associated with sorbitol oxidation to fructose could divert glyceraldehyde-3-phosphate away from the glycolytic route and towards the formation of dihydroxyacetone phosphate and diacylglycerol (181). Diacylglycerol induces protein kinase C which has two fundamental effects. First, it impairs agonist-stimulated cytosolic calcium response, possibly via down-modulation of phospholipase C. Second, it augments extracellular matrix overproduction as shown in rat mesangial cells (183). Finally, oxidative stress due to generation of free radicals by disposal of excess glucose, may also play a role in

mesangial proliferation (184). Thus, thickening of the glomerular basement membrane and mesangial matrix occur within 2 to 5 years of onset of diabetes and also occur in diabetics receiving kidney transplants (185). Excess glycation can result in a resistance to collagenase digestion, an interference with normal crosslink formation, a change in physico-chemical properties such as thermal stability and tensile strength and the formation of abnormal crosslinks between glycated moieties (173, 174, 186, 187). Furthermore, the reactive groups formed as a consequence of collagen glycation can trap, condense or form covalent bonds with nonglycated soluble proteins such as serum albumin, IgG, or lipoproteins, which may help to explain the increased concentration of albumin in basement membranes of patients with diabetic nephropathy (187). It has also been suggested that such trapped or bound proteins retain the ability to form immune complexes with appropriate antibodies or antigens, suggesting a mechanism whereby non-enzymatic glycation could initiate or perpetrate immune injury (187). Fibronectin is an extracellular matrix glycoprotein with biologic activities that include influencing cell-substratum adhesion/attachment and binding to collagen and glycosaminoglycans such as heparin. Non-enzymatic glycosylation of fibronectin inhibits its binding to heparin, decreases its ability to bind to denatured collagen, and comprises its binding with heparin and gelatin (188). Thus, it is believed that excess non-enzymatic glycation of fibronectin and probably of other noncollagenous matrix components in diabetes influences organizational features of the basement membrane (186). This could be accomplished through compromise of adhesive, attachment, and self-assembly properties of its macromolecules. Furthermore, collagen proteins, which are major constituents of the glomerular extracellular matrix, are rich in lysine and hydroxylysine rsidues, and have a

long half-life. Since the number of free amino groups and the biological half-life of a protein determine the extent of glycation in vivo, it is expected that collagen would undergo increased glycation in vivo when subjected to a hyperglycemic milieu (189). Glomerular basement collagens also readily undergo non-enzymatic glycation in vitro in a reaction that is dependent on time and glucose concentration, although the extent and sites may differ in different collagens. The formation of insoluble complexes by the crosslinking process may alter the functional process in the kidney and aid in the development of diabetic nephropathy (189-191). In this regard, a calcium dependent enzyme, transglutaminase, has been identified that crosslinks proteins into large molecular weight polymers (26, 27). This enzyme is widespread in different cells of the body, and has also been localised to the kidney by immunocytochemical studies. Transglutaminase crosslinks proteins by the formation of ε-(γ-glutamyl)lysine isopeptide bonds (26). Furthermore, nonenzymatic glycation, with the formation of glycation products and crosslinking of both glomerular basement membrane and mesangial matrix proteins by transglutaminase may play a causal role in the thickening of the extracellular matrix in diabetes.

## 2.3. RENAL HANDLING OF CALCIUM

Calcium has only been recently recognised as an universal cell messenger. It was the classical work by Ringer in 1882 (192) on the role of $Ca^{2+}$ in muscle contraction which showed that $Ca^{2+}$ was fundamental to cell signalling mechanisms. Subsequently, the developments in the field of $Ca^{2+}$ ionophores, the discovery of calmodulin and other intracellular $Ca^{2+}$ binding proteins, the progress in studies on membrane transport of $Ca^{2+}$ and the development of precise techniques for measuring intracellular $Ca^{2+}$ concentration

have contributed to the continued expansion of interest in the role of $Ca^{2+}$. Paralleling these developments, there has been an autocatalytic growth in the understanding of mechanisms that are involved in the regulation of cytosolic $Ca^{2+}$ concentration.

Considering the importance of $Ca^{2}$ as an intracellular messenger, it is conceivable that mechanisms exist to maintain an optimal cytosolic $Ca^{2+}$ concentration. $Ca^{2+}$ homeostasis is maintained by the integrated actions of the intestine, bone and kidneys. Interchange of $Ca^{2+}$ between skeleton and blood, its absorption from the intestine, and renal reabsorption of filtered $Ca^{2+}$ are all involved, but the relative contribution of each is still controversial. Although the gut and skeleton do play significant roles, the kidney has been shown to be the chief regulator of $Ca^{2+}$ homeostasis (193, 194).

In the blood, 35 to 45% of total $Ca^{2+}$ is bound to plasma proteins, and only the remaining fraction passes freely across the glomerular basement membrane. In the healthy man, this free fraction of $Ca^{2+}$ is approximately 11,000 mg. It is obvious from this that there are effective mechanism(s) for tubular reabsorption of $Ca^{2+}$. Data from micropuncture and microperfusion studies (195-197) also indicate active $Ca^{2+}$ reabsorption along the tubule. Both micropuncture studies and the evaluation of urine samples collected following transient ureteral occlusion reveal tubular fluid/ultrafiltrable plasma ratios of $Ca^{2+}$ well below 1.0 (193,198-200), despite an electric potential difference across the tubule that should lead to the appearance of tubular fluid/ultrafiltrable plasma ratios above 1.0. These findings suggest that a very effective active transport mechanism exists for $Ca^{2+}$ in the renal tubule.

Under basic conditions, nearly all the $Ca^{2+}$ in the glomerular filtrate is reabsorbed along the nephron. Though the maximal load of the filtered $Ca^{2+}$ is absorbed along the

proximal portion of the tubule, reabsorption is considerable along the distal segments of the nephron (201). Both active and passive modes of transport have been postulated to exist for tubular reabsorption of $Ca^{2+}$, even along in the same segment, though the cellular events responsible for active $Ca^{2+}$ transport by the renal tubule remain undefined.

However, it is important to understand a few essential concepts in the area of cellular $Ca^{2+}$ homeostasis. Most of the $Ca^{2+}$ is reabsorbed from the tubular lumen into the cell across the brush-border membrane along an electrochemical gradient by a facilitated diffusion transport mechanism which is powered by an electrogenic sodium transport system (202, 203). In other words, $Ca^{2+}$ reabsorption into the cell is a passive mechanism. $Ca^{2+}$ is maintained at low concentrations in the cell with the background concentration of free $Ca^{2+}$ in most cytosol oscillating between 0.1 and 0.2 μM (204-206). Though $Ca^{2+}$ is important in cell signalling and other biochemical processes, the situation of $Ca^{2+}$ overload could jeopardise cellular function and integrity (207). Indeed, inundation of the cytosol with $Ca^{2+}$ is a frequent and early event in cell pathology (207). It might be even challenging to say that eucaryotic cells have elected to live in a permanent state of controlled risk, a dynamically convenient but nevertheless dangerous choice where the margin separating cells from $Ca^{2+}$ catastrophe may, on occasion, be very narrow. This obviously necessitates the presence of systems that eject $Ca^{2+}$ from the cells thereby offsetting its downhill penetration. Moreover, the electrochemical gradient at the basolateral side is reversed, requiring energy-dependent mechanisms for the extrusion of $Ca^{2+}$ (208, 209). And it is by concerted actions of the importing system ($Ca^{2+}$ channel) and the exporting systems ($Ca^{2+}$ pump and $Na^{+}$-$Ca^{2+}$ exchanger) that an optimal cytosolic $Ca^{2+}$ concentration is maintained. The $Na^{+}$-$Ca^{2+}$ exchanger is a large-capacity, low-affinity electrogenic antiporter (210).

However, kinetic studies have shown that it is in equilibrium at cytosolic free $Ca^{2+}$ levels and does not contribute greatly to $Ca^{2+}$ extrusion (211). The $Ca^{2+}$ pump on the other hand is a low capacity system with a high affinity for $Ca^{2+}$ (209). Its high affinity enables it to interact with $Ca^{2+}$ even at the very low background intracellular concentration of resting cells. It therefore functions continuously, satisfying the demands for the fine tuning of intracellular $Ca^{2+}$.

## 2.4. RENAL $Ca^{2+}$-ATPASE

Cytoplasmic free $Ca^{2+}$ concentration (0.1 to 0.2 μM) is several orders of magnitude lower than in the extracellular milieu. Though the diffusion of $Ca^{2+}$ into the cell is passive, the electrochemical gradient at the basolateral side is reversed, requiring energy-dependent mechanisms for $Ca^{2+}$ exit (208). In transporting epithelia, pump and exchanger systems participate in the vectorial translocation of $Ca^{2+}$ for maintaining intracellular $Ca^{2+}$ homeostasis (208, 211). Thus, membrane-bound $Ca^{2+}$ transporting enzymes have been identified in cortex homogenates (212), suspensions of cortical tubules (213) and microsomes (214-217) from rat, rabbit and hog kidneys. Strong corroborative evidence for a function of $Ca^{2+}$-ATPase in renal tubular $Ca^{2+}$ transport has been obtained from two studies on plasma tubules. First, separation of vesicles from the luminal and basolateral aspect of the cell has shown that these enzymes are located exclusively in the basolateral membrane (214, 216, 217), where they extrude $Ca^{2+}$ into the peritubular space against steep gradients. Second, $Ca^{2+}$ uptake by (inside-out) basolateral membrane vesicles (corresponding in vivo to $Ca^{2+}$ extrusion from the cell), depends on, and is supported by,

ATP found in the cytosol and in concentrations optimal for $Ca^{2+}$-dependent ATPase activity (202).

### 2.4.1 General properties

The presence of a $Ca^{2+}$-stimulated ATPase in the renal basolateral membrane has been identified and shown to participate in the vectorial transport of $Ca^{2+}$ from the tubular side to the basolateral membrane of the tubular cell (214). Studies showed that the enzyme was present along the entire nephron and was activated by millimolar concentrations of either $Ca^{2+}$ or $Mg^{2+}$, neither of which was necessary for activation by the other cation (218). The physiologic role of this low-affinity enzyme in transmembrane calcium transport is not clear, but is activated by $Ca^{2+}$ concentrations in the millimolar range, whereas the cytosolic free $Ca^{2+}$ concentration in kidney cells is of a lower magnitude (206). It is conceivable that the high capacity of the enzyme compensates for its low affinity (214), or that $Ca^{2+}$ may be "compartmentalized" at the inner surface of the membrane to a higher concentration than in the rest of the cytosol (213). The exact status of this low-affinity $Ca^{2+}$-ATPase in $Ca^{2+}$ transport still remains to be determined.

A more acceptable hypothesis is the presence of a high-affinity $Ca^{2+}$-ATPase for extruding $Ca^{2+}$ out from the renal tubular cells, activated by normal intracellular $Ca^{2+}$ concentrations. In rat renal cortical basolateral membranes and rabbit renal tubules, a $Mg^{2+}$-dependent, high-affinity, low capacity calmodulin-sensitive $Ca^{2+}$-ATPase has been identified (210, 216, 219-222). Moreover, the relationship between the $K_m$ for $Ca^{2+}$ of this ($Ca^{2+}$+$Mg^{2+}$) ATPase (0.68 mM) and that of the ATP-dependent $Ca^{2+}$ uptake (0.5 mM) in vesicle preparations lends further credence to the existence and involvement of this enzyme in transmembrane $Ca^{2+}$ transport (202, 216).

### 2.4.2 $Ca^{2+}$ + $Mg^{2+}$ ATPase and $Ca^{2+}$ transport

The general mechanism of the $Ca^{2+}$ pump follows the pattern of that of all other P-type ion pumps (223, 224). The cycle begins with the $Ca^{2+}$ dependent transfer of the terminal phosphate of ATP to an aspartic acid residue in the pump (225, 226). The phosphorylated intermediate has a high $Ca^{2+}$ affinity. The pump bearing the phosphorylated intermediate ($E_1$-P in the conventional formalism) undergoes a conformation transition promoted by $Mg^{2+}$: the new conformer is called $E_2$-P (227). The matter of the affinity for ATP is more complex. The hydrolysis to $E_2$ and $P_i$ is assumed to be accelerated by the binding of ATP to a site on the pump that has less affinity (228, 229) than the site that binds ATP to initiate phosphorylation (228, 229). In the last step of the reaction cycle, the dephosphorylated $E_2$ conformer reverts back to the initial $E_1$ conformation. Analogous to other P-type ion pumps, vanadate (a pentacoordinate, trigonal bipyramid stereo analogue of phosphate), also inhibits the $Ca^{2+}$-ATPase at very low concentrations (230). It is generally assumed that vanadate inhibits by binding to the aspartic acid residue. Not only has it been shown to act as a non-competitive inhibitor of the binding of ATP to the high affinity site (234), but also as a mixed, partially competitive inhibitor at the low affinity site (231). Vanadate interacts with the $E_2$ conformer of the ATPase, blocking the last step of the reaction (i.e. $E_2$--$E_1$ transformation) thereby stabilizing the enzyme in the $E_2$ conformation.

One aspect of the reaction cycle which is still open to question is the step during which translocation of $Ca^{2+}$ across the hydrophobic barrier of the membrane takes place. Two steps in the reaction cycle have been tentatively identified. The first refers to a conformational change of the pump protein, i.e. $E_1$-P to $E_2$-P, and the second, from conformer $E_2$ to $E_1$. In either case, the $Ca^{2+}$ binding site, located on the interior of the

membrane before the translocation step ($E_1$ conformation), will be located on the external side of the plasma membrane at the end of the translocation step, i.e. the $Ca^{2+}$ binding site on the $E_2$ conformer of the pump faces the exterior of the cell (232). Apart from the dispute regarding the site of $Ca^{2+}$ transfer, considerable debate also persists in the number of $Ca^{2+}$ atoms translocated during the cycle. The observation of a Hill coefficient greater than one for the activation of ATPase, saturation of the pump and the fact that the rate of $Ca^{2+}$ transfer in erythrocytes is proportional to the square of cytosolic $Ca^{2+}$, indicates there are two high-affinity $Ca^{2+}$ binding sites (233, 234). It is possible that the sigmoidal kinetics of this pump could be attributed to other factors, e.g. the $Ca^{2+}$-dependent binding of the activator calmodulin (235). Even if two $Ca^{2+}$ binding sites existed, whether the translocation step involves one to two $Ca^{2+}$ atoms is still an open issue. Kinetic studies under optimal conditions (236) or in purified preparations (237) favor a 1:1 stoichiometry. Others have found that $Ca^{2+}$: ATP stoichiometry depends on the $Ca^{2+}$ concentration and approach a value of two at [$Ca^{2+}$] above 500 mM (238, 239).

### 2.4.3 $Ca^{2+}$ transport in the diabetic kidney

The action of insulin, like other hormone systems, involves the initial binding of the hormone to a specific receptor on the cell membrane, which would initiate a series of biochemical events. The potential role of $Ca^{2+}$ in mediating insulin action still remains to be elucidated (240). A potential role for $Ca^{2+}$ in insulin-mediated cellular responses is suggested by observations of direct modulation by insulin of high affinity $Ca^{2+}$+$Mg^{2+}$ATPase activity in adipocyte plasma membranes (241). If there is some regulatory role for this enzyme in mediating insulin action, it is reasonable to assume that in situations of insulin resistance, insulin's effect on $Ca^{2+}$+$Mg^{2+}$ATPase may be different from

that found under normal conditions. Levy et al. (240) examined the direct effect of insulin on the high affinity $Ca^{2+}$+$Mg^{2+}$ATPase of kidney basolateral membranes in streptozotocin-induced diabetic rats. The enzyme activity in basolateral membranes from diabetic rats was higher at all $Ca^{2+}$ concentrations tested due to a higher maximum velocity of the enzyme from diabetic rats. The enzyme was inhibited by trifluperazine and no difference was noted in the calmodulin content in membranes from diabetic and control rats. Furthermore, insulin significantly increased the activity of the $Ca^{2+}$+$Mg^{2+}$ATPase in the kidney basolateral membranes in control rats, though there was a lack of such response from the membranes of diabetic rats. These findings not only demonstrate a defect in the ability of insulin to regulate the $Ca^{2+}$+$Mg^{2+}$ATPase activity in diabetic rats, but also inculpate an inherent defect in enzyme activity as responsible for the impaired insulin action in diabetic rats. It may also be pointed out that in the presence of an increased $Ca^{2+}$ pump in diabetes, rats are resistant to the development of congestive heart failure unless superimposed with hypertension (242). However, a causal relationship has not yet been established. Thus it is clear from the foregoing discussion that an impaired intracellular $Ca^{2+}$ homeostasis represents a crucial factor in the pathogenesis of diabetic nephropathy.

## 2.5. TRANSGLUTAMINASE

Transglutaminases belong to a family of enzymes that catalyze the formation of large protein aggregrates (26, 27). The term, "transglutaminase" has been used to describe a $Ca^{2+}$-dependent transamidating activity present in the liver and some other tissues of the guinea pig which could be measured by the covalent incorporation of amines into proteins, and also by a reaction between αN and αC-blocked glutamine peptides and hydroxylamine (243). Since then several different transglutasminases have

been localized to distinct physiologic compartments. Transglutaminase activity has been demonstrated in the blood (erythrocytes and platelets), placenta, epidermis, prostate, lung, fibroblasts, and the kidney (244). These different enzymes share significant homology in the active site but with significant differences in the remainder of their protein configuration (26). Functionally, all these various forms react with specific glutamine residues in substrate proteins and catalyze the transamination of the glutamine residues with the ε-amino group of a lysine from a second substrate protein to form protein-polyamine conjugates with its resultant effects on cell function.

### 2.5.1 Transglutaminase and crosslink formation

Transglutaminase catalyzes a nucleophilic displacement reaction by crosslinking endo-γ-glutaminyl and endo-ε-lysyl residues of proteins resulting in the formation of γ-glutamyl-ε-lysine or ε(γ-glutamyl)lysine isopeptide side-chain bridges (245). The Enzyme Commission recommends the term "R-glutaminyl-peptide:amine-γ-glutamyl-transferase" with designation (EC 2.3.2.13). In the process of dimerization of two protein molecules by transglutaminase, if the protein molecules are of the same species, a homologous ensemble is obtained; otherwise, a heterologous ensemble is obtained. Transglutaminase-specific donor and acceptor crosslinking functionalities may exist on a single protein, when it is referred to as bifunctional, or may exist exclusively as donor or acceptor towards crosslink formation (246-248). The significance of this is that with bifunctional proteins, the possibility of an infinite degree of linear polymerization by γ-glutamyl-ε-lysine bridges may occur, while the exclusive proteins may only serve as chain initiators or terminators. This property of labeling of proteins by labeled amines,

e.g. dansylcadaverine, has been utilized in probing the crosslink domains of proteins (246).

The presence of enzymes that promote irreversible crosslinking of proteins could have disastrous consequences for the cell. This potential danger is precluded as the physiologic activity of this enzyme requires $Ca^{2+}$ and is cryptic under physiologic conditions. The enzymatic activity can be detected if cytosolic $Ca^{2+}$ is elevated to millimolar range from its usual physiologic intracellular concentrations. $Ca^{2+}$ ion thus acts as a physiologic activator of this enzyme (249). Metal-ion induced conformational changes in the enzyme proteins occur and are essential for binding of glutamine substrates and for other steps in catalysis (250). The intracellular transglutaminase shows an apparent requirement for half-maximal activation in the 0.1 mM to 0.5 mM $Ca^{2+}$ range (26, 249). These levels are not manifested under normal conditions in living cells. In the diabetic state, the depression of the $Ca^{2+}$ + $Mg^{2+}$ ATPase (calcium pump) favors the intracellular accumulation of calcium up into the micromolar range due to its inability to transport calcium ions out of the cell (250-253). Calcium has been shown to be a *universal activator* for all latent transglutaminases studied so far (26, 27). Thus the cytosolic milieu favors the induction of these latent enzymes or zymogens. It must be pointed out that the exact mechanism favoring transformation from the sequestered zymogen to the active enzyme is not known. Under any circumstance, the conscription of transglutaminase activity is favored with the resultant γ-glutamyl-ε-lysine crosslink formation. Codistribution of fibronectin and collagen has been observed in cultured fibroblasts derived from rat kidney thus providing optimal conditions for enzyme action

(254). The resultant covalent crosslinking in vivo could thus have important bearings on the cell conformation and function individually and the organ performance as a whole.

## 3. AIMS AND OBJECTIVES

Cytoplasmic free $Ca^{2+}$ concentration (0.1 to 0.2 μM) is several orders of magnitude lower than in the extracellular milieu (16). The low intracellular free $Ca^{2+}$ concentration and an electronegative cell interior infer there is passive diffusion of $Ca^{2+}$ across the brushborder membrane into the cell from the tubular lumen. The electrochemical gradient of $Ca^{2+}$ at the basolateral side is reversed suggesting the presence of energy-dependent processes for $Ca^{2+}$ exit (238). In transporting epithelia, pump and exchanger systems participate in the vectorial translocation of $Ca^{2+}$ for maintaining intracellular $Ca^{2+}$ homeostasis. An ATP-dependent $Ca^{2+}$ transporting enzyme has been identified in the rat kidney cortical basolateral membrane (212). This energy-requiring ion pump may provide an optimal cytoplasmic concentration lower than that in the extracellular fluid. The present study was designed to characterise the $Ca^{2+}$ pump in the diabetic kidney.

Dopamine has been recognized as an important modulator of renal function inasmuch as it modulates GFR and renal plasma flow and electrolyte homeostasis (70). In addition to increasing renal blood flow, exogenous dopamine generally increases urine flow, sodium chloride, potassium, phosphate, and calcium excretion (78). Although the renal vasodilator effect of dopamine most likely contributes to the natriuretic effect, the changes in electrolyte excretion can occur in the absence of changes in renal blood flow and GFR (77). Dopamine regulates intracellular electrolyte concentration by modifying their transport mechanisms. Dopamine coordinates cytosolic calcium levels by increasing calcium excretion by the kidney though the exact mechanism of action is unknown. Furthermore, dopamine acts via activation of membrane phospholipases which

are involved in calcium flux (83). Dopamine, by its regulatory effects on water and electrolyte balance, may have a role in maintaining plasma volume and electrolyte homeostasis (89). Diabetes mellitus is another volume-related disorder, where defective mobilization of dopamine or lack of its modulatory effect on the electrolyte environment may be causative in the development of diabetic nephropathy. To this effect, experiments were initially designed to systemically characterize dopamine in the kidney.

Nephropathy is a serious complication of diabetes mellitus and is an important cause of morbidity and mortality in diabetic patients (1). It is progressive, characterized by subtle abnormalities including an increase in GFR (hyperfiltration) and renal hypertrophy (120). Micropuncture studies have documented an increased single-nephron GFR, renal plasma flow and increased intraglomerular capillary pressure (121). Increased GFR appears to be involved in the initiation and progression of renal disease (122). Although no single mechanism has yet been identified to explain the hemodynamic alteration, candidate defects include dopamine (111). As a consequence of dopamine's participation in the fluid and electrolyte balance, the present study was proposed to investigate the hypothesis that alterations in dopaminergic activity may affect the cytosolic calcium status and promote activation of latent processes to aid in the development of diabetic nephropathy. The present study was therefore designed to systematically characterize the status of dopamine in the kidney in early and chronic diabetes. It was extended to investigate the precise modulation of the electrolyte status in diabetes mellitus by dopamine. The interplay between dopamine and calcium pump was examined and how this interaction may foster the development of nephropathy in the setting of chronic diabetes.

The specific objectives of the research are

A. To examine and characterize dopamine receptors in the kidney in diabetic nephropathy

   a. Perform receptor assays with specific radioligands in kidney cortex membranes in control and diabetic rats.

   b. Determine specificity of dopamine receptors involved in regulating the effects of dopamine.

   c. Endogenous dopamine content of the kidney will be measured to provide a correlation with the receptor status.

B. Characterize the role of dopamine in diabetic nephropathy.

This correlation between dopamine and diabetic nephropathy would be based on an analysis of interactions of:

a) Dopamine and sodium transport:

   i) We will examine $Na^+ + K^+$ ATPase in kidney cortex membranes in control and diabetic animals.

   ii) The effect of dopamine on $Na^+ + K^+$ ATPase will be examined in control and diabetic state to determine the specificity of the action.

   iii) Perform phosphorylation studies to establish the post-receptor action of dopamine.

b) Dopamine and calcium transport.

i) Examine the status of the $Ca^{2+} + Mg^{2+}$ ATPase in the acute and chronic stage of diabetes.

ii) Determine the response of $Ca^{2+}$ + $Mg^{2+}$ ATPase to dopamine in the early and chronic state of diabetes.

C. Determine the levels of transglutaminase activity in the control and diabetic animals.

# 4. MATERIALS AND METHODS

## 4.1. Experimental animals

Male Sprague-Dawley rats weighing 200 ± 10 g were used. The total number of animals used in the study were 229. They were grouped as control (C) (n = 89), and diabetic (D) (n=140). Diabetes was induced by streptozotocin injection (65 mg/kg body wt., via the femoral vein) whereas the control animals received the buffered vehicle (0.1 M citrate, pH 4.5) (256). Nine animals that were injected with streptozotocin did not become diabetic, and were initially treated as a separate group. Since values for the different parameters between control and streptozotocin-injected non-diabetic animals were overlapping, the data in these two groups were combined. The animals were sacrificed 2 and 6 weeks later. A time frame of 2 and 6 weeks was chosen after pilot studies in our laboratory showed these time periods as representative of early and chronic stages of diabetic nephropathy.

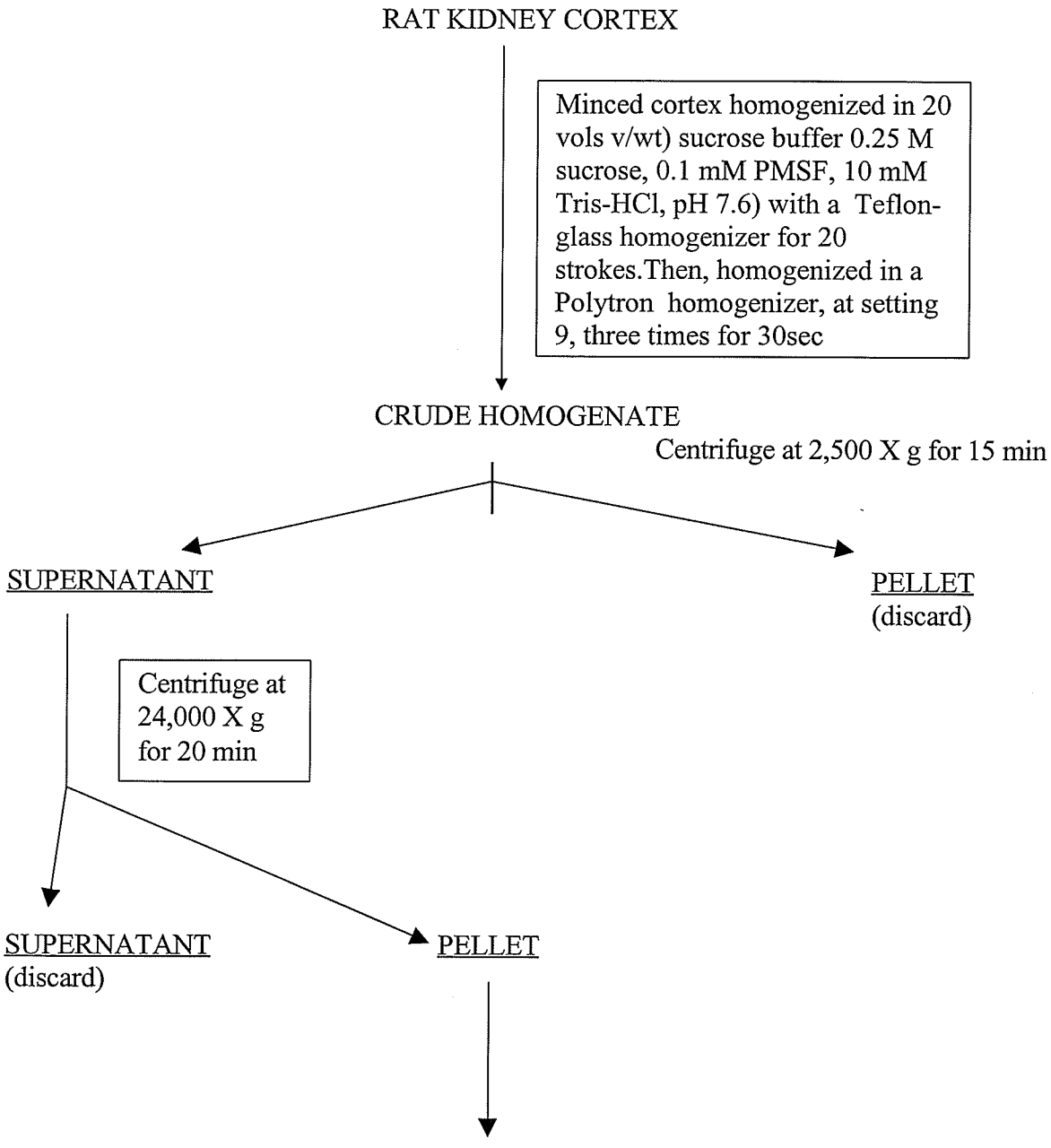
## 4.2. Chemicals

All the chemicals used were of reagent grade and obtained from Sigma Chemical Co., St. Louis, MO.

## 4.3. Membrane preparation

Basolateral membrane was isolated from kidney cortex according to Sacktor et al. (257). The kidney cortex was homogenized in 20 volumes of buffer (0.25 M sucrose, 0.1 mM PMSF (phenylmethyl sulfonyl fluoride), and 10 mM Tris-HCl, pH 7.6), at a polytron setting of 9, three times for 30 seconds each and then centrifuged (Beckman centrifuge, Model J2-21, California) at 2500 g (Beckman JA 20 rotor) for 15 min at 0-5 °C. The supernatant was recentrifuged at 24,000 g for another 20 min. The pellet was resuspended in

sucrose buffer, homogenized and centrifuged at 30,000 X g through a density gradient of Percoll for 35 min. Fraction II was diluted with 4 volumes of another buffer containing 100 mM mannitol, 100 mM KCl and 5 mM Hepes-Tris, pH 7.2, and centrifuged at 34,000 X g for 30 min. The loose fluffy pellet was isolated, resuspended in 4 volumes of Hepes-Tris buffer and centrifuged at 34,000 X g for 30 min again. The final pellet was diluted in a small volume (1 ml) of Hepes-Tris buffer. The membranes were stored at -80 $^{0}$C until used (Chart I).

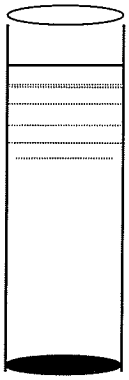


Resuspend the fluffy upper layer of pellet in 32.2 ml sucrose buffer. Homogenize in Teflon-glass homogenizer for 20 strokes

CRUDE PLASMA MEMBRANES

Add 2.8 ml 100% Percoll and mix vigorously.
Centrifuge at 30,000 X g 35 min.

PERCOLL GRADIENT



FRACTION I

FRACTION II
(BASOLATERAL MEMBRANES)

FRACTION III

Fractions were collected from the top by pumping a 50% sucrose solution into the bottom of the centrifuge tube with a peristaltic pump. Fractions were diluted with 4 vol of medium containing 100 mM KCl, 100 mM Mannitol, 5 mM Hepes-Tris, pH 7.2.

Centrifuge at 34,000 X g for 30 min.

After centrifugation, the Percoll had formed a very dense glassy pellet. The membranes formed a loose fluffy pellet above the Percoll. The membranes were removed, resuspended in the same medium and, then, recentrifuged at 34,000 X g for 30 min.

BASOLATERAL MEMBRANES
Membranes were suspended in a small medium.

**Chart I. Protocol for preparing basolateral membranes from rat renal cortex**

### 4.4. Plasma Glucose

Plasma glucose was estimated by a reagent kit (Sigma). Briefly, the glucose in the serum was determined by a coupled enzymatic method using the enzymes hexokinase and glucose-6-phosphate dehydrogenase (258). In principle, glucose is phosphorylated to glucose-6-phosphate by hexokinase using ATP. The glucose-6-phosphate is then reduced to 6-phosphogluconate by glucose-6-phosphate dehydrogenase in the presence of nicotinamide dinucleotide (NAD). The consequent increase in absorbance due to an equimolar reduction of NAD to NADH at 340 nm is directly proportional to glucose concentration. The concentration can thus be determined based on millimolar absorptivity of NADH (6.22 at 340 nm).

### 4.5. Dopamine Turnover (259)

Each rat was injected with 12 μCi/100 g of $^{3}$H-dopamine intraperitoneally. The rats were sacrificed at 4 and 8 hours following injection, and the kidney cortex processed for dopamine content determination by homogenizing in 0.4 N perchloric acid. Dopamine was then extracted by the addition of alumina and washed twice to remove the buffer. 500 μl of the supernatant was taken and quantified by addition of 10 cc of scintillation fluid and counting in a scintillation spectrometer. Dopamine activity was determined by counting the difference between the tritiated specimens and those without.

### 4.6. Endogenous dopamine content (259)

Kidney cortex was homogenized (1:5 wt/vol in 0.4 N perchloric acid in the presence of 1% sodium metabisulphite) with a Polytron. The homogenate was centrifuged at 30,000 X g for 20 min. The supernatant was collected and measured. 500 μl of the supernatant was taken and pH adjusted to 8.6 with 2M Tris-HCl.

Catecholamines were then subjected to alumina extraction and measured by high-pressure liquid chromatography.

### 4.7. $Na^+ + K^+$ ATPase estimation

The $Na^+ + K^+$ ATPase was measured as shown previously (260). 100 µg of membrane protein was incubated in an assay mixture containing 100 mM NaCl, 10 mM KCl and 50 mM Tris-HCl, pH 7.6. The reaction was started by the addition of 4mM ATP and stopped after 20 min by the addition of 12% trichloroacetic acid. Phosphate determination was then carried out by a colorimetric method using 1 mM potassium dihydrogen phosphate as a standard. ATPase activity was expressed as nanomoles of $P_i$ per mg protein per min and was calculated by subtracting basal $Mg^{2+}$ATPase from that achieved in the presence of 1 mM sodium and potassium. The ATPase activity was also measured when necessary in the presence and absence of various substrates, stimulators and inhibitors.

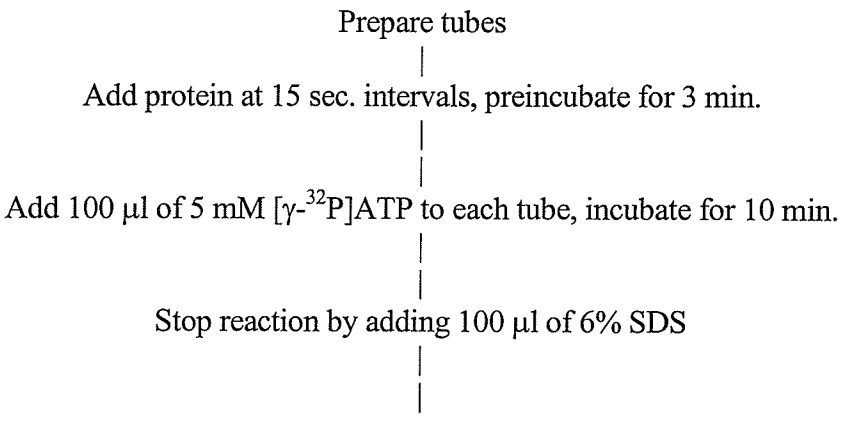
### 4.8. Radioligand receptor studies (261)

Membrane preparations were preincubated at 37 °C for 10 minutes. Each assay tube contained 200 µl of membrane protein (approximately 0.1 mg of protein) and 100 µl of various concentrations of [$^3$H]-spiperone (0.09-16 nM) with or without an excess (100 µl) of unlabelled 10 µM spiperone, and buffer (50 mM Tris-HCl buffer containing 120 mM NaCl, 5 mM KCl, 1 mM Mg $Cl_2$, 1 mM $CaCl_2$ and 250 KIE/ml aprotinin, pH 7.6) in a total volume of 500 µl. The tubes were incubated at 37 °C for 20 minutes, and the reaction terminated by rapid filtration through Whatman GF/C glass fiber filters. The filters were washed twice with 5 ml of cold Tris-HCl buffer, pH 7.6, and placed in scintillation vials containing 10 cc of scintillation fluid containing methylcellulose. The radioactivity on the filters was then measured by a liquid scintillation spectrometer. Specific binding is the

difference in radioactivity bound to the receptor in the presence of excess spiperone and that in its absence. Scatchard analysis of the data was done to arrive at the binding affinity ($K_d$) and receptor number ($B_{max}$).

### 4.9. $Ca^{2+}$ + $Mg^{2+}$ ATPase estimation

The $Ca^{2+}$+$Mg^{2+}$ATPase was measured by a modification of the method of Pershadsingh and McDonald (241, 263). The assay medium contained 5 μg of basolateral membrane protein, 50 mM Tris-HCl (pH 7.2), 1 mM ATP, about 0.2 μCi [$\gamma$-$^{32}$P]ATP (New England Nuclear Corp.), 2 mM $MgCl_2$ and $10^{-10}$ M free $Ca^{2+}$. The incubation was done at 37 °C for 10 minutes and the reaction stopped by 6% SDS. The released $^{32}$P was converted to a phosphomolybdate complex using phosphate reagent (2 volumes of 10 N sulfuric acid, 2 volumes of 10% ammonium molybdate and 1 volume of 0.1 M silicotungstic acid). The phosphomolybdate complex was extracted into xylene: isobutanol (65:35) (264) and further quantified by liquid scintillometry. ATPase activity was expressed as nanomoles of $P_i$ per mg protein per min and was calculated by subtracting basal $Mg^{2+}$ATPase from that achieved in the presence of 1 μM free $Ca^{2+}$. The ATPase activity was also measured as and when necessary in the presence and absence of various substrates, stimulators and inhibitors. (Chart II).



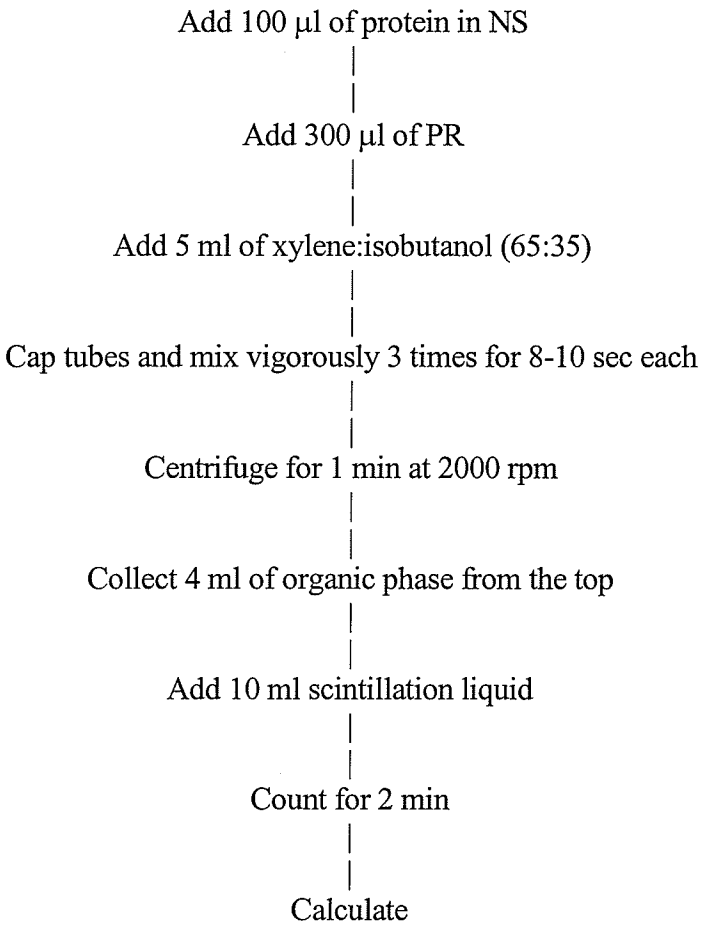



**Chart II. Isotopic assay for $Ca^{2+} + Mg^{2+}$ ATPase**

### 4.10. Transglutaminase assay (26)

Membranes were incubated with 0.5 mM $^{14}$C-putrescine dihydrochloride in 10 mg/ml DMC, 150 mM NaCl, 40 mM DTT, and 40 mM Tris-HCl, pH 7.4. The reaction was terminated by the addition of 7.5% TA, and the tubes were allowed to sit for an hour on ice. The precipitate was then collected on Whatman GF/A filters, washed four times with 5% TCA and quantified by liquid scintillometry. Specific activity was expressed as nmol putrescine incorporated into membranes.

## 5. RESULTS

Table I shows the general characteristics of experimental animals. Presence of diabetes in streptozotocin-injected animals was confirmed by the presence of increased levels of plasma glucose compared with the control group at 6 weeks. The data at 1 week is not shown as it was not significantly different from control. It must be pointed out that not all animals injected with streptozotocin became diabetic. These streptozotocin-injected non-diabetic animals were included in the control group as their parameters were not different from the control group. The diabetic animals showed a significant decrease in body weight while their kidney weight and kidney to body weight ratio was increased. In chronic diabetes, the animals showed a significant increase in their plasma creatinine.

Diabetic animals showed a decrease in the endogenous kidney dopamine content (1.76 ± 0.79 ng/g) compared to control (2.87± 0.81 ng/g) (Fig I). Figure I also shows the kidney weight in control and diabetic animals in the early and chronic diabetes. This indicates that the endogenous dopamine content expressed as a proportion of the kidney weight is decreased. Plasma dopamine levels were increased in the chronic diabetic rats (0.63 ± 0.03 ng/ml, vs 0.44 ± 0.02 ng/ml) (Fig II). Evaluation of the rate constant revealed an increase in the diabetic state (0.085 ± 0.002 K/hr, vs 0.041 ± 0.004 K/hr). The turnover rate and half-life were not significantly different in the two groups (Table II). Dopamine uptake and disappearance levels were lower in diabetic animals suggesting that the decrease in endogenous dopamine content in diabetic animals was real (Fig. III).

$Na^+ + K^+$ ATPase assay in diabetic animals in the early and chronic stages exhibited an increase when compared to controls (Fig IV). The lower panel shows the time-dependency of the enzyme in control and diabetic animals which suggests that there

was no change in inherent characteristics of the enzyme. Increased levels of dopamine displayed an inhibitory effect on the $Na^+ + K^+$ ATPase in diabetic animals (Figure V).

Figure VI shows the Scatchard analysis of the DA1 receptor binding in the renal cortical membranes of control and diabetic animals in chronic diabetes. There was a downregulation of the DA1 receptor in the diabetic animals without any change in affinity. The lower panel clearly shows the dose-dependent nature of this increase in the receptor density. Further examination of the DA1 subtype with 10-point competition curves indicated that the binding was best fit to a two-site model. The two components can be identified as a high-affinity, low-density site (Fig VII) and a low-affinity, high-density binding site (Fig VIII). Radioligand binding studies showed that there as a downregulation of both the high-affinity (Bmax 138 ± 11, Kd 7.19 ± 0.48) and low-affinity (Bmax 220 ± 19, Kd 39.81 ± 1.13) in diabetics compared to the high-affinity (Bmax 208 ± 17, Kd 7.08 ± 0.37) and low-affinity (Bmax 448 ± 28, Kd 38.51 ± 38.46) in control animals. It must be pointed out that the downregulation was without any changes in affinity of the ligand for its receptor site. The lower panel shows the concentration-dependency of specific binding to DA1 receptors. Evaluation of the DA2 subtype unveiled no differences in the control (Bmax 460 ± 38, Kd 15.8 ± 1.28) and diabetic animals (Bmax 421 ± 33, Kd 16.2 ± 1.91) (Fig. IX). The lower panel in Fig. IX reflects the concentration-dependency of specific binding and the increased $Na^+ + K^+$ ATPase activity. This would favor an increased retention of sodium in diabetes, which is consistent with the increased sodium body stores seen in this state in animal and human subjects.

The $Ca^{2+}$ + $Mg^{2+}$ ATPase was analyzed in early and chronic diabetes and contrasted to control animals. The calcium pump activity was depressed in the chronic diabetic mode (124 ± 12 nmol Pi/mg/min) as compared to control (220 ± 16 nmol Pi/mg/min) (Fig. X). The response of the calcium pump to dopamine unveiled a lack of response of this enzyme to dopamine at 6 weeks (Fig. XI).

Transglutaminase level was measured using radiolabelled $^{14}C$-putrescine and dimethylcasein as the substrate. The result showed a significantly greater activity in the pellet fraction in diabetics (323 ± 40 dpm/hr/μg protein) in comparison to diabetic animals at 6 weeks (121 ± 12 dpm/hr/μg protein) (Fig XII). This would suggest that the transglutaminase activity was largely associated with the insoluble fraction of the homogenate, perhaps co-sedimenting with extracellular matrix proteins.

**Table I. General characteristics of experimental animals**

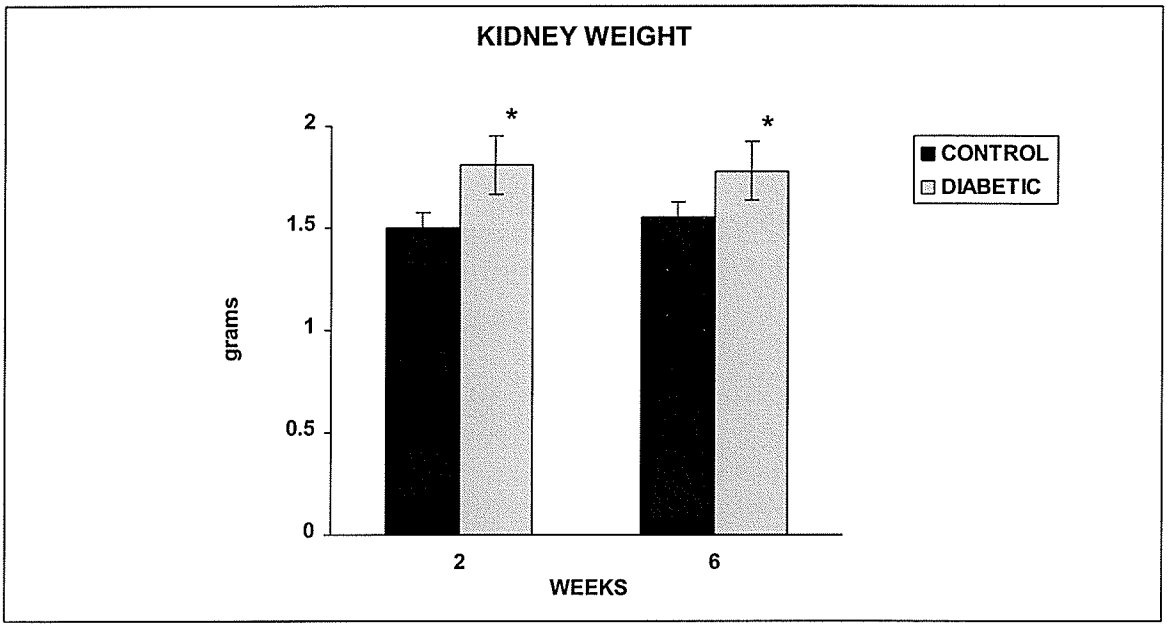
| | **CONTROL (n=89)** | **DIABETIC (n=140)** |
|---|---|---|
| Body wt, g | 447 ± 15 | 235 ± 14* |
| Kidney wt, g | 1.5 ± 0.04 | 1.7 ± 0.02* |
| Kidney wt/body wt ratio, mg/g | 3.36 ± 0.12 | 7.23 ± 0.11* |
| Plasma glucose, mg/dl | 138 ± 8 | 462 ± 12* |
| Plasma creatinine, μmol/L | 40.5 ± 1.3 | 61.6 ± 0.9* |
| Urine creatinine, mmol/L | 0.4 ± 0.008 | 0.95 ± 0.01* |

**Values are means ± SE of 15 experiments. Only one kidney was used for measuring weight.**
*** Significantly different ($p<0.05$) from control.**

**Table II. Dopamine turnover in control and diabetic animals**

| | CONTROL | DIABETIC |
|---|---|---|
| Endogenous dopamine,ng/g | $2.87 \pm 0.81$ | $1.76 \pm 0.79$* |
| Rate constant, (K), $h^{-1}$ | $0.041 \pm 0.004$ | $0.085 \pm 0.002$* |
| Turnover rate, ng/g/hr | 0.114 | 0.172 |
| Half-life ($t_{1/2}$) | 26.42 | 12.08 |

**Values are means ± SE of 5 experiments (n = 5 animals per experiment at 6 weeks of diabetes).**
**Endogenous dopamine content was determined by homogenizing kidney cortex (1:5 wt/vol) in 0.4 N perchloric acid using 1% sodium metabisulphite as an antioxidant with a Polytron. The homogenate was centrifuged at 30,000X g for 20 minutes. The supernatant was collected and the pH adjusted to 8.6 with 2 M Tris-HCl. Dopamine was extracted by the addition of alumina followed by washing it twice with the buffer. The supernatant was then collected and subjected to high-pressure liquid chromatography for dopamine determination.**
**For turnover studies, each rat was injected with 12 μCi/100g of ($^3$H) dopamine intraperitoneally and sacrificed at 4 and 8 hours following injection. The kidneys were then processed for dopamine content by homogenizing in 0.4 N perchloric acid. Dopamine was extracted by the addition of alumina followed by washing it twice with the buffer. After centrifugation the supernatant was taken and quantified by scintillation spectrophotometry. The results were then expressed as ng/g/hr of kidney tissue. Dopamine activity was determined by counting the difference between the tritiated specimens and those without. Half-life was calculated as 0.69/K.**
*** Significantly different ($p<0.05$) from control.**



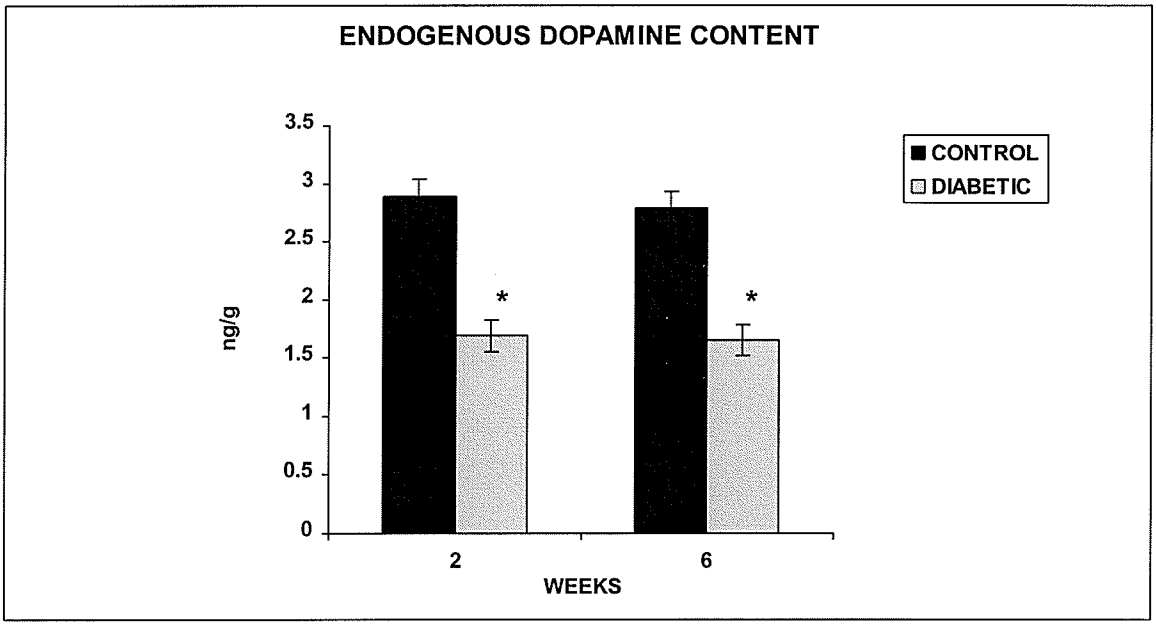




**Figure I.** **Relationship between kidney weight and endogenous dopamine level at 2 and 6 weeks of diabetes. Upper panel, kidney weight. Lower panel, endogenous dopamine content standardized to the kidney weight. Results are means ± SE of 5 experiments (n = 5 animals per experiment).**
*** Significantly different ($p<0.05$) from control.**

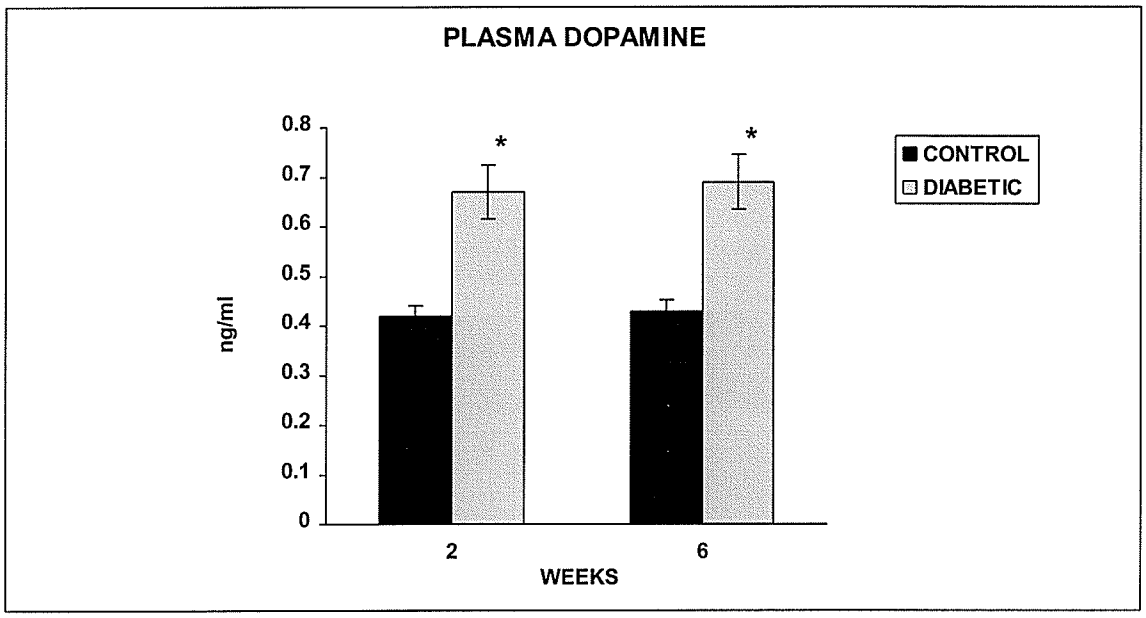



**Figure II.** **Plasma dopamine level of experimental animals at 2 and 6 weeks of diabetes. Results are means ± SE of 5 experiments.**
*** Significantly different ($p<0.05$) from control.**

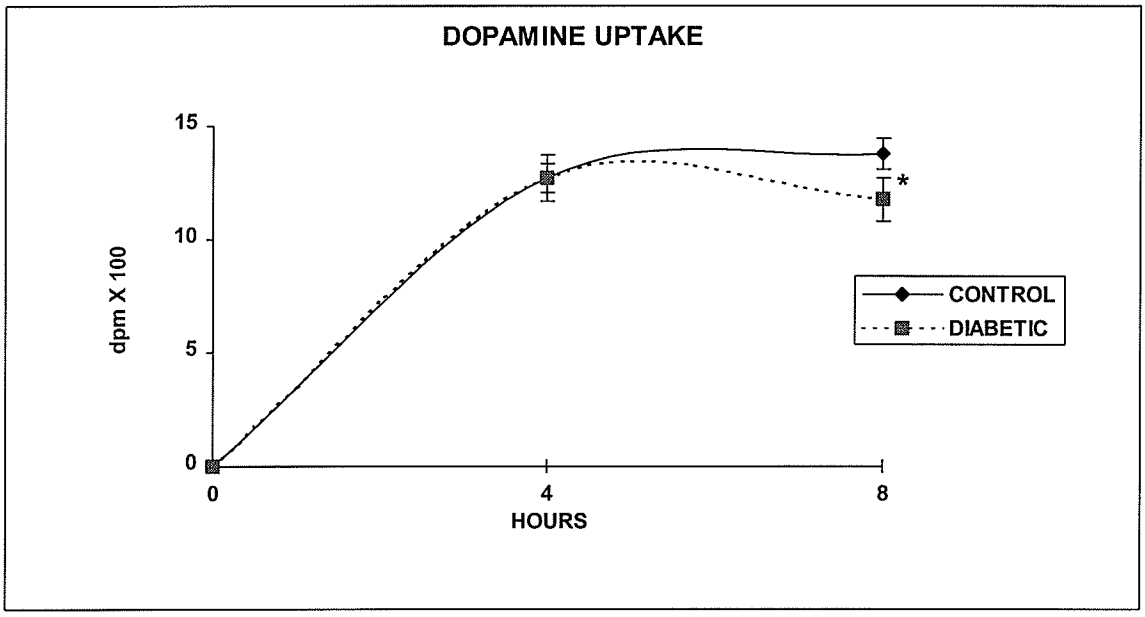
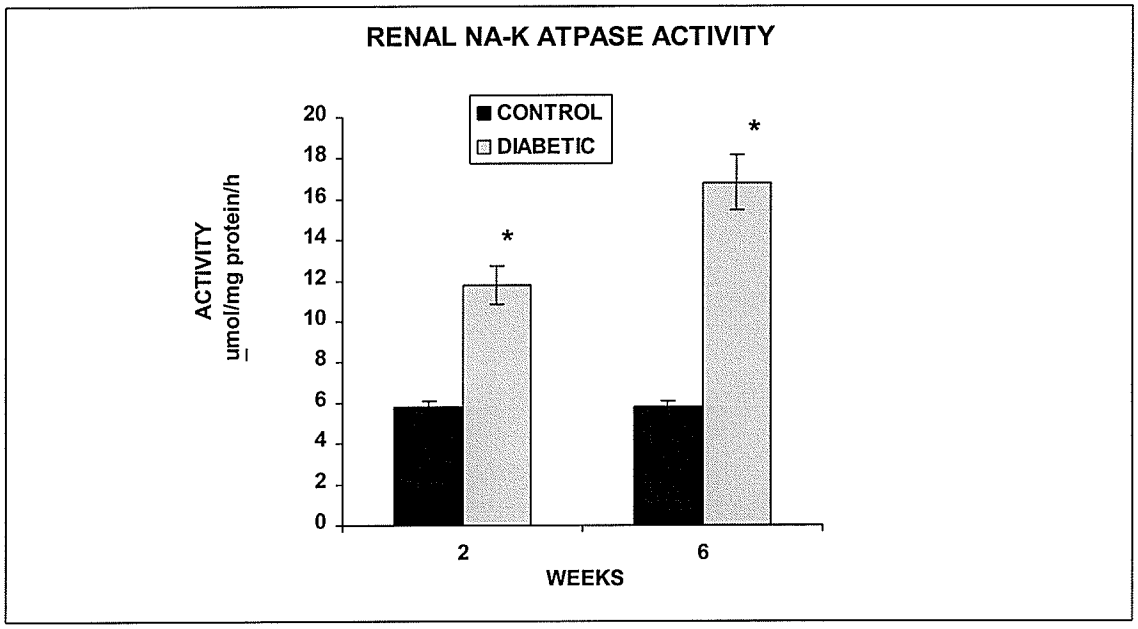



**Figure III.** **Uptake of ($^3$H) dopamine in control and diabetic kidney at 6 weeks. Slices of renal cortex of known weight were incubated in a buffer medium containing 2 ng/ml of ($^3$H)dopamine and 0.75 mg/ml of ascorbic acid in 15 ml Krebs bicarbonate buffer (1.2 m $MgSO_4$ , 120 mM NaCl, 25 mM $NaHCO_3$ , 1.2 mM KCl, 1.2 mM $KH_2PO_4$ , 1.25 $CaCl_2$ and 11.1 mM dextrose ) prewarmed at 37°C and preequilibrated with 95% $O_2$ – 5% $CO_2$ , pH 7.4. The slices were removed at timed intervals and catecholamines extracted by alumina extraction and quantified by liquid scintillometry. Results are means ± SE of 5 experiments (n = 5 animals per experiment).**
*** Significantly different ($p<0.05$) from control.**



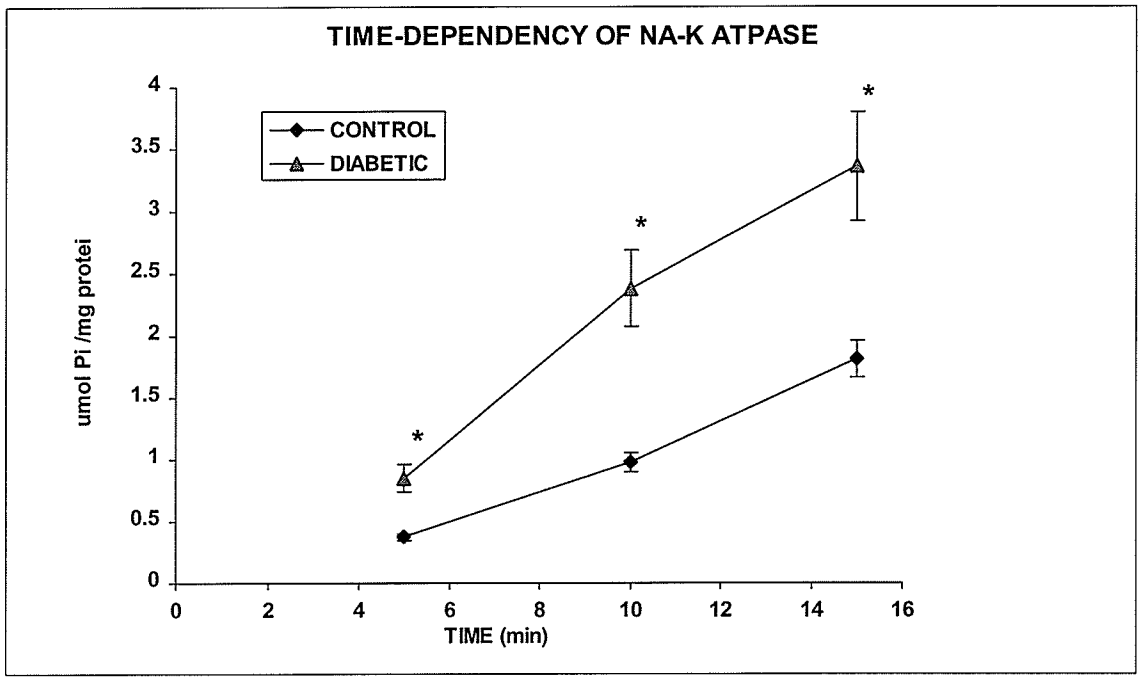




**Figure IV.** **$Na^+ + K^+$ ATPase activity at 2 and 6 weeks of diabetes (upper panel). Membrane protein was incubated in a mixture containing 4mM ATP and measured in the presence of 1 mM ouabain. Phosphate determination was carried out using a colorimetric method. Time-dependency of $Na^+ + K^+$ ATPase at 6 weeks of diabetes (lower panel). Results are means ± SE of 5 experiments (n = 5 animals per experiment)**

*** Significantly different from control ($p<0.05$)**

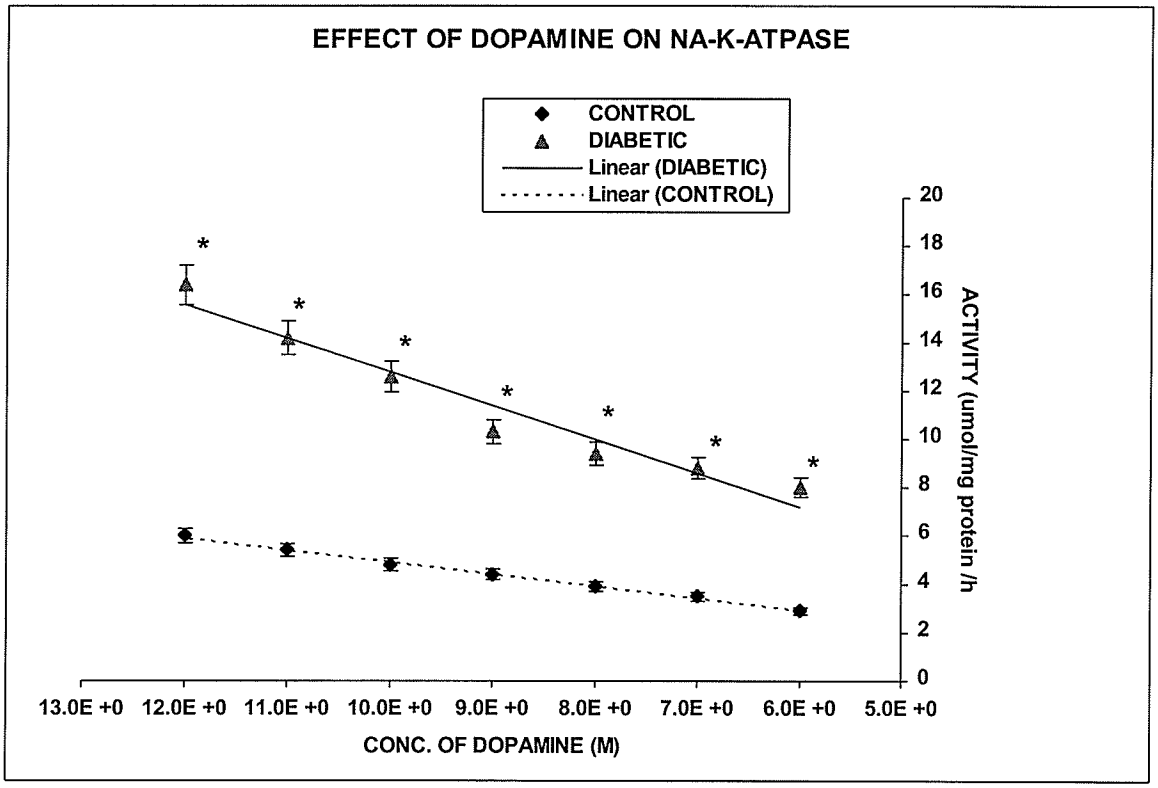



**Figure V.** **Effect of dopamine on $Na^+ + K^+$ ATPase in control and chronic diabetes. Membrane protein was incubated in a mixture containing 4 mM ATP and measured in the presence of 1 mM ouabain. Phosphate determination was carried out using a colorimetric method. The effect of dopamine was determined in the presence and absence of various concentrations of dopamine ($10^{-12} - 10^{-6}$). Results are means ± SE of 5 experiments (n= 5 animals per experiment)**
*** Significantly different from control ($p<0.05$)**

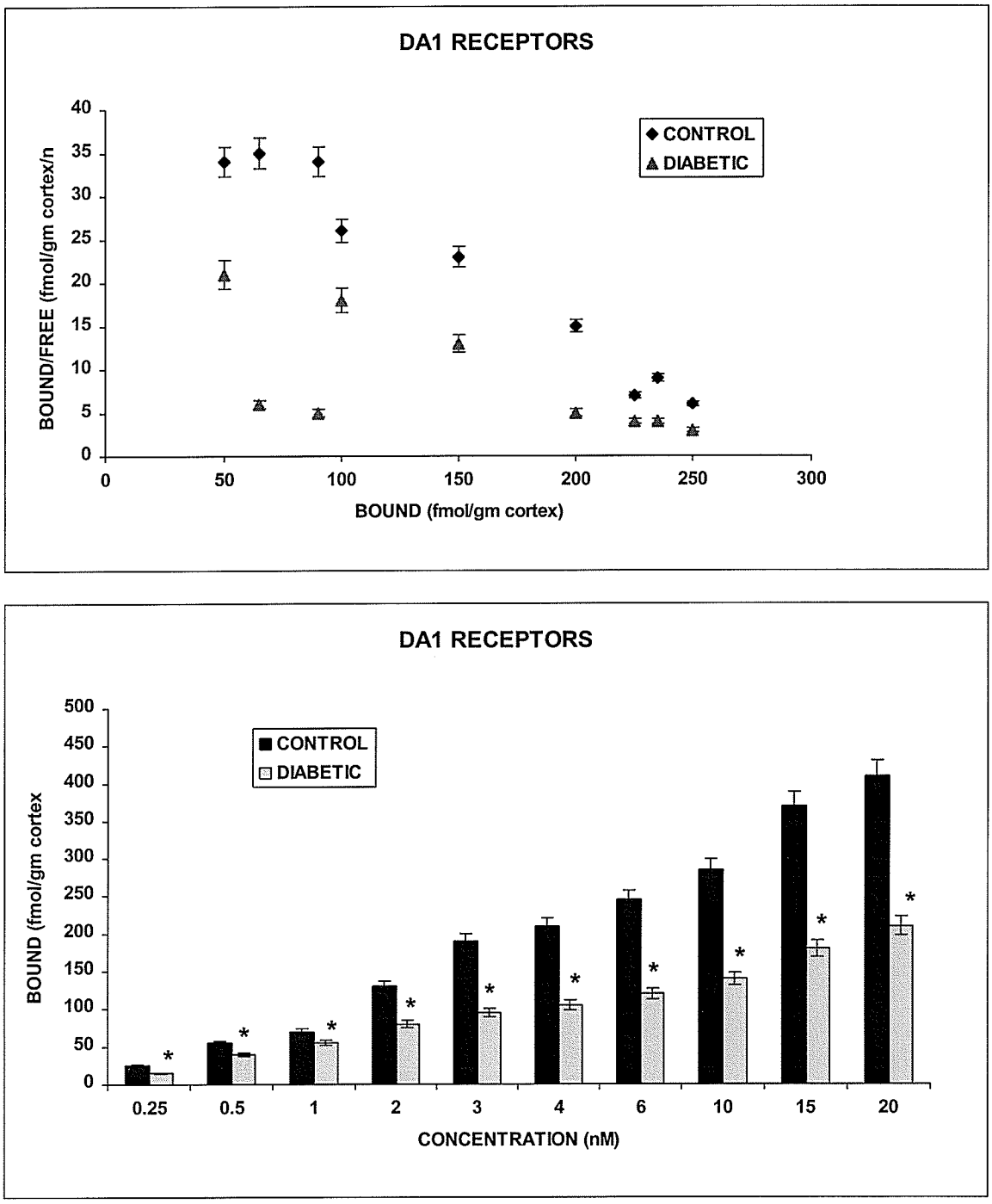
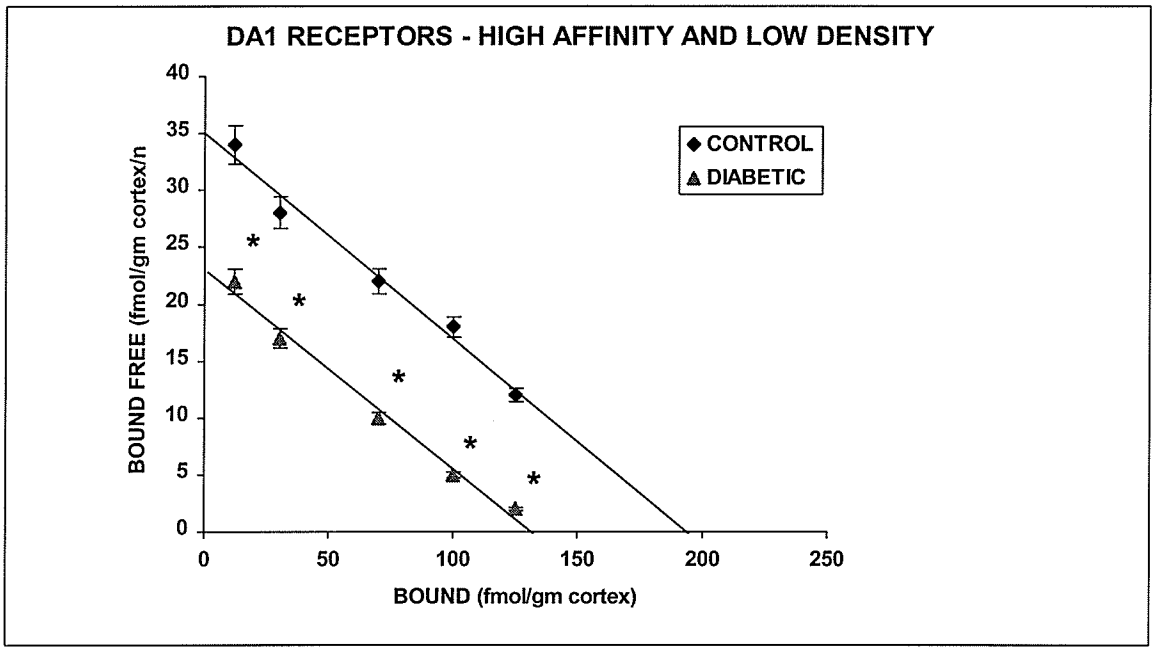



**Figure VI.** **Scatchard analysis of dopamine receptor binding at 6 weeks of diabetes. Membranes were incubated with different concentrations of (3H) 23390. Specific binding was the difference between the radioactivity bound to receptors in the presence and absence of SCH 23390. The lower panel shows the concentration dependency of specific binding. Results are means ± SE of 5 experiments (n = 5 animals per experiment)**

*** Significantly different from control ($p<0.05$)**



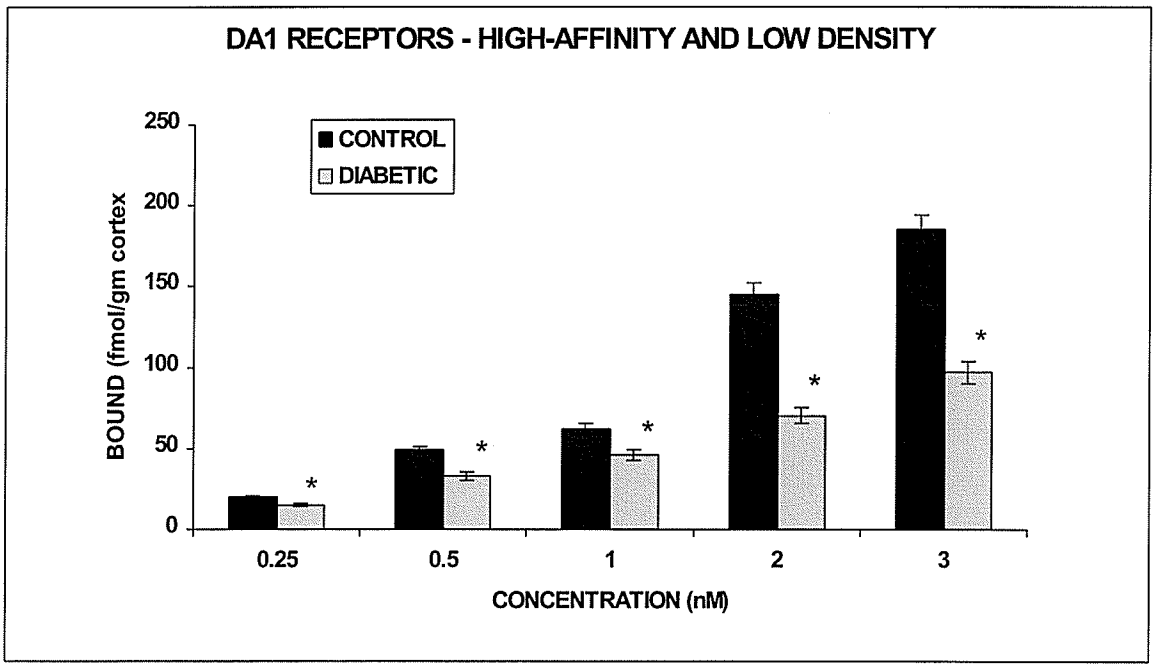
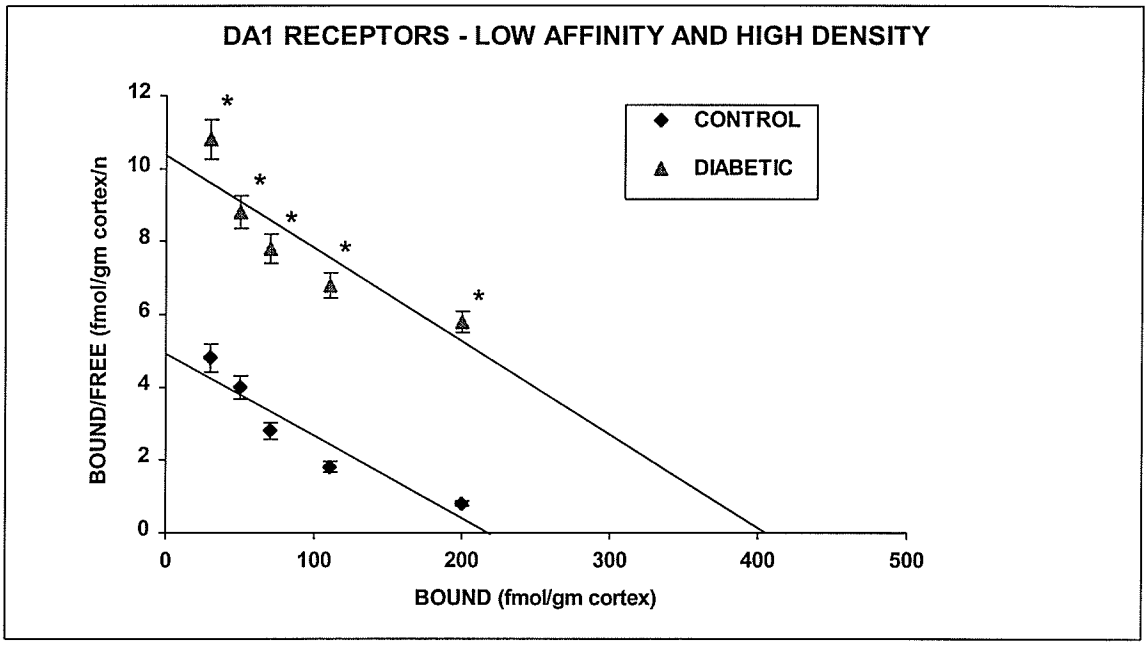




**Figure VII.** **Scatchard analysis of high affinity low density DA1 receptor binding at 6 weeks of diabetes. Membranes were incubated with different concentrations of (3H) 23390. Specific binding was the difference between the radioactivity bound to receptors in the presence and absence of SCH 23390. The lower panel shows the concentration dependency of specific binding. Results are means ± SE of 5 experiments (n = 5 animals per experiment)**
*** Significantly different from control ($p<0.05$)**



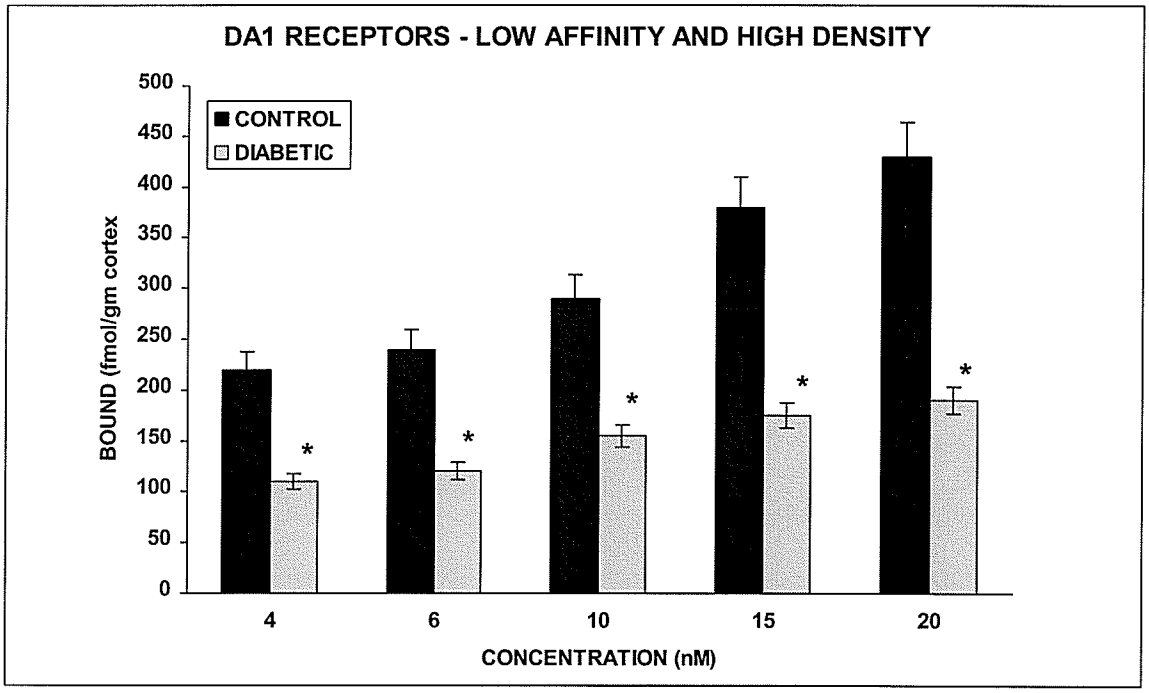
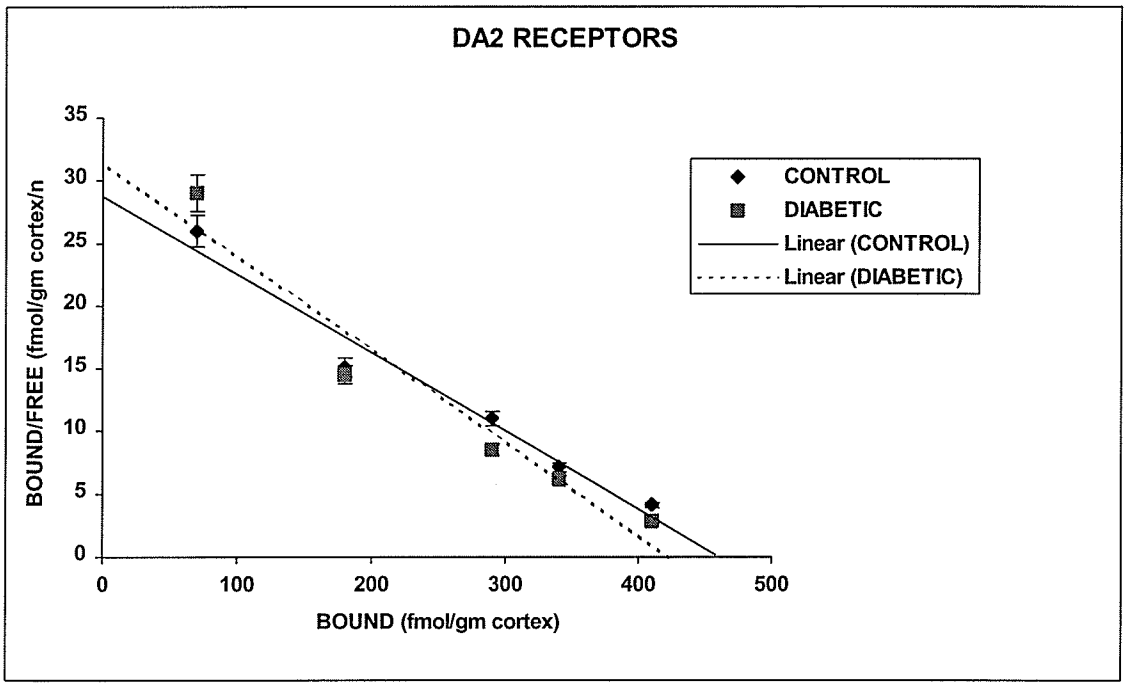




**Figure VIII.** **Scatchard analysis of low affinity high density DA1 receptor binding at 6 weeks of diabetes. Membranes were incubated with different concentrations of ($^3$H) 23390. Specific binding was the difference between the radioactivity bound to receptors in the presence and absence of SCH 23390. The lower panel shows the concentration dependency of specific binding. Results are means ± SE of 5 experiments (n = 5 animals per experiment)**
*** Significantly different ($p<0.05$) from control.**



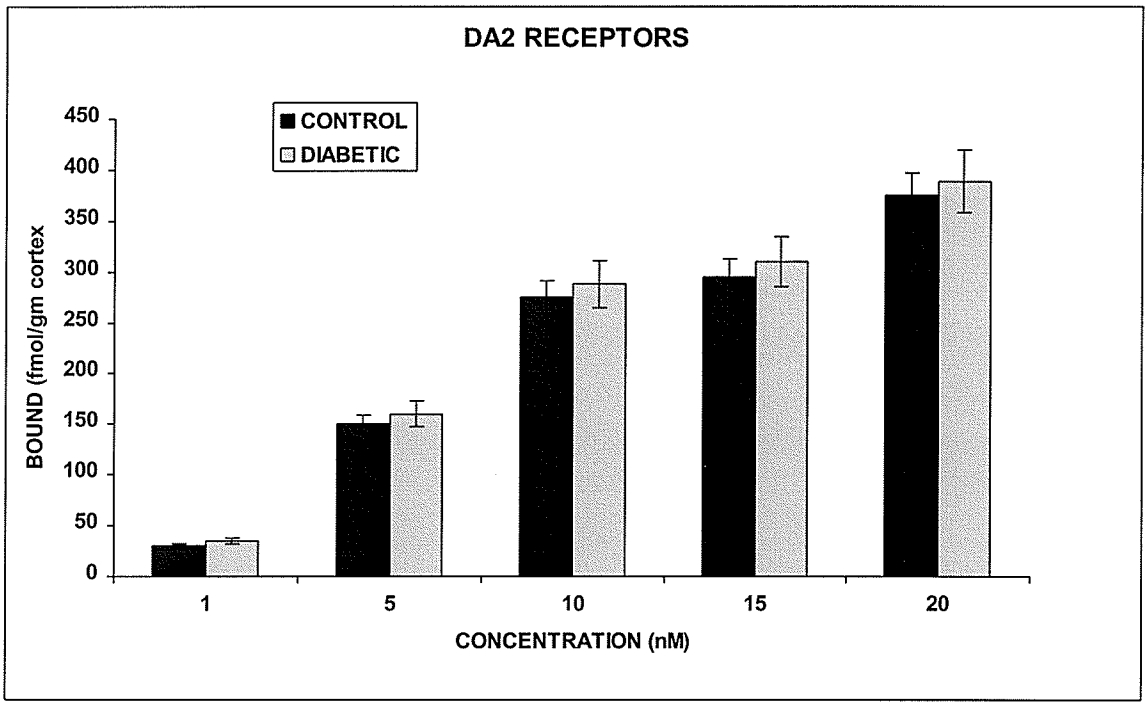




**Figure IX.** **Scatchard analysis of DA2 receptor binding at 6 weeks of diabetes. Membranes were incubated with different concentrations of ($^3$H) sulpiride. Specific binding was the difference between the radioacivity bound to receptors in the presence and absence of l-sulpiride. Results are means ± SE of 5 experiments (n = 5 animals per experiments).**

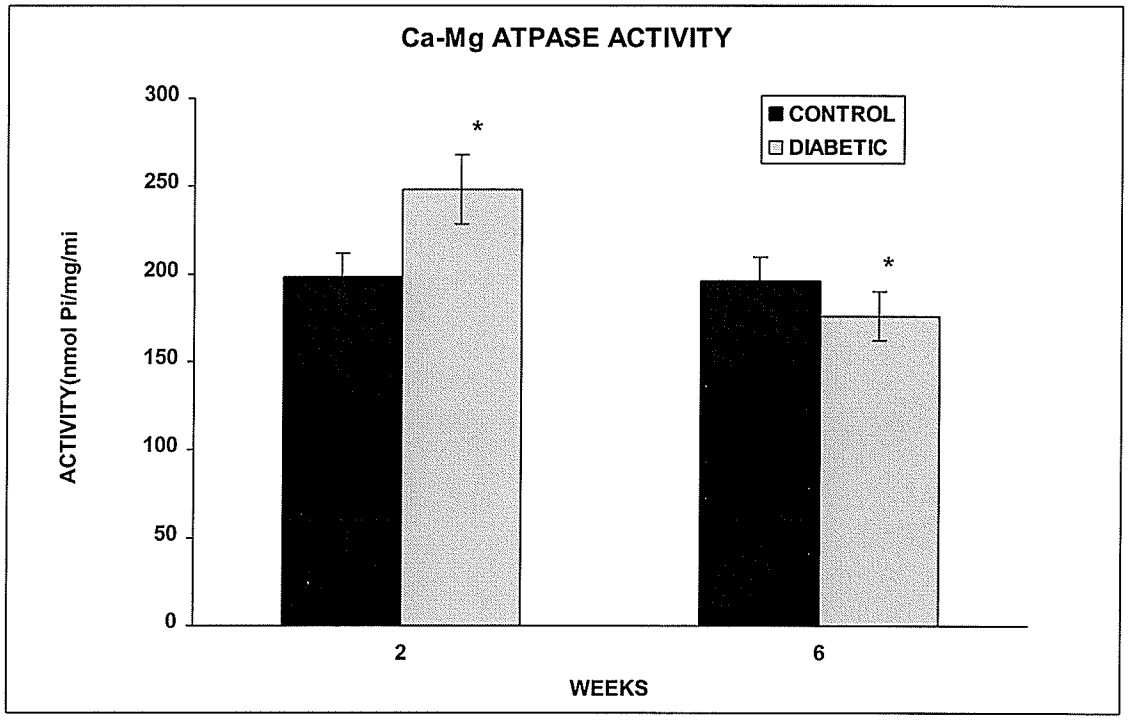
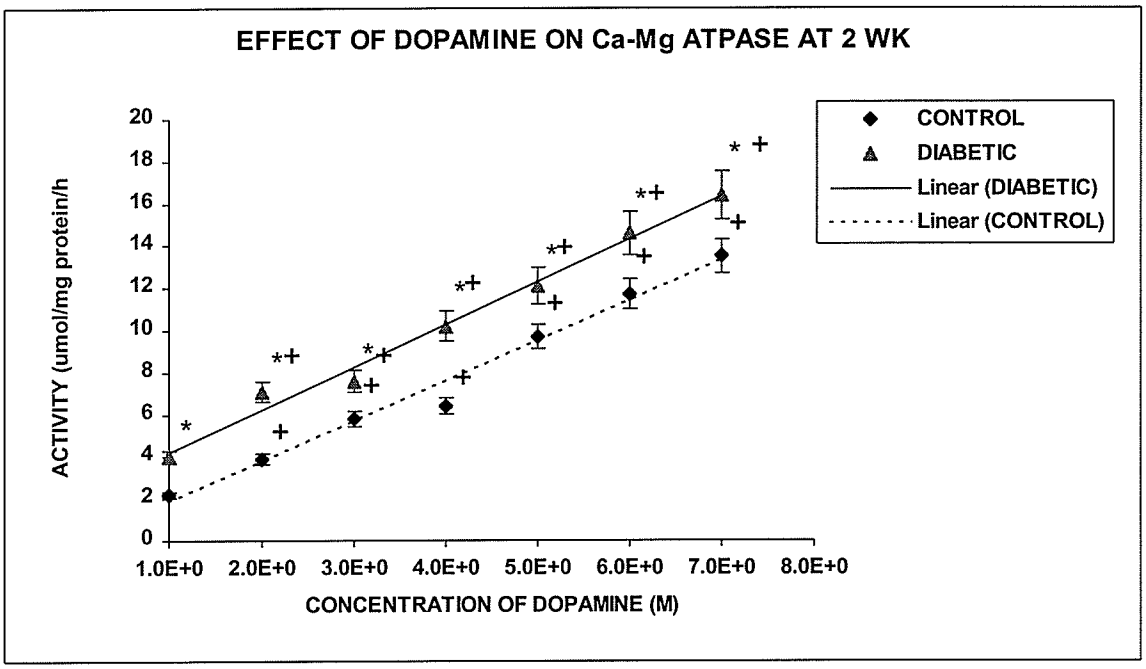



**Figure X.** **$Ca^{2+}$ + $Mg^{2+}$ ATPase activity of experimental groups at 2 and 6 weeks of diabetes. The $Ca^{2+}$ + $Mg^{2+}$ ATPase was determined by subtracting the basal $Mg^{2+}$ ATPase from the total $Ca^{2+}$ + $Mg^{2+}$ ATPase activity. Results are means ± SE of 5 experiments (n = 5 animals per experiments).**
*** Significantly different ($p<0.05$) from control.**



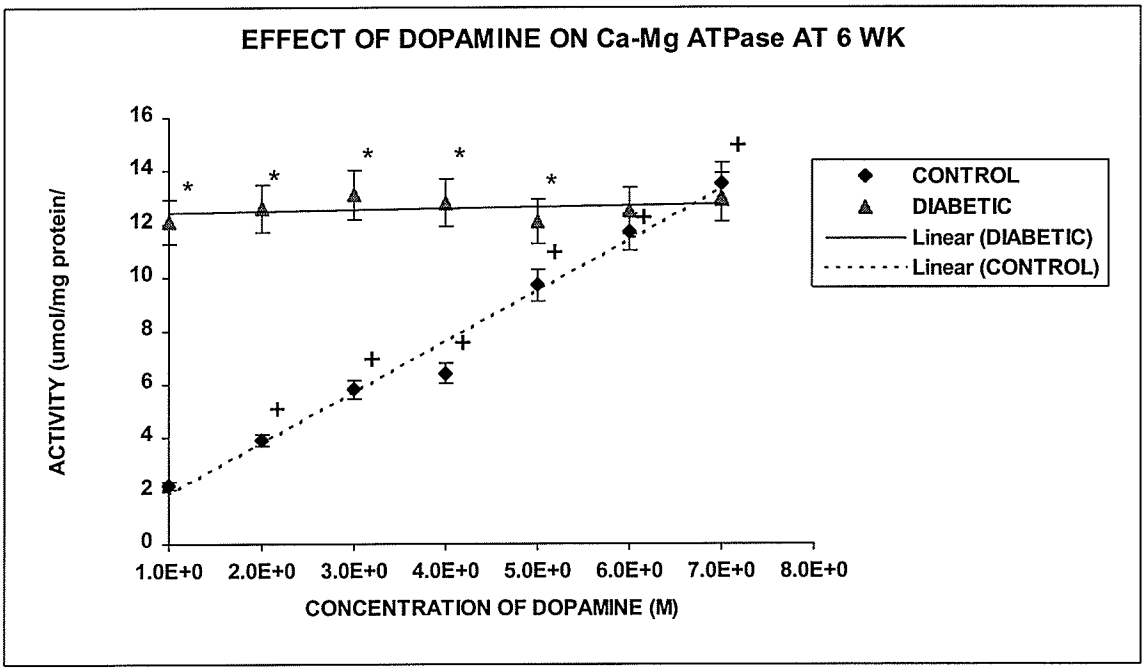




**Figure XI.** **Effect of different concentrations of dopamine at 2 weeks (upper panel) and 6 weeks (lower panel) of diabetes. The $Ca^{2+}$ + $Mg^{2+}$ ATPase was determined by subtracting the basal $Mg^{2+}$ ATPase from the total $Ca^{2+}$ + $Mg^{2+}$ ATPase activity. Results are means ± SE of 5 experiments (n = 5 animals per experiment).**
*** Significantly different ($p<0.05$) from control.**
**+ Significantly different ($p<0.05$) from previous lower concentration of dopamine.**

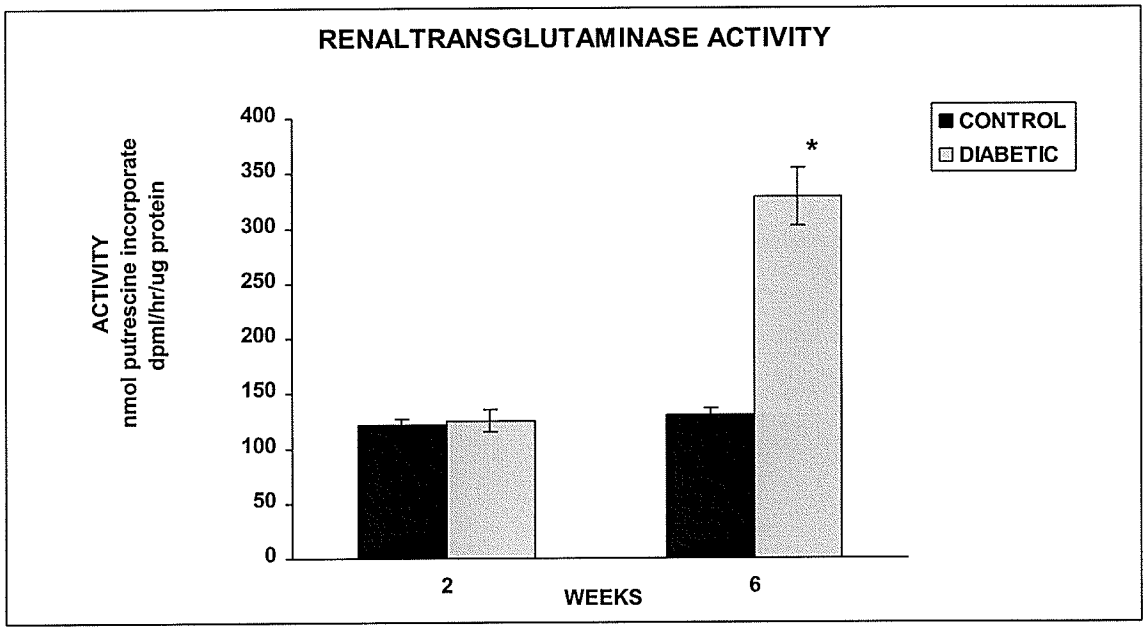



**Figure XII.** **Renal transglutaminase activity at 2 and 6 weeks of diabetes. The membrane protein was incubated in a mixture containing 0.5 mM $^{14}$C-putrescine dihydrochloride and the specific activity was expressed as the nmol of putrescine incorporated. Results are means ± SE of 5 experiments (n = 5 animals per experiment).**
*** Significantly different ($p < 0.05$) from control.**

## 6. DISCUSSION

Considering the importance of calcium as an intracellular messenger, it is conceivable that mechanisms exist to maintain an optimal cytosolic calcium concentration (223). Calcium homeostasis is orchestrated by the integrated actions of the intestine, bone and kidney. Although the gut and skeleton do play significant roles, studies have shown that the kidney is the chief regulator of calcium (193, 194). Under basic conditions, nearly all of the calcium in the glomerular filtrate is reabsorbed via both active and passive mechanisms of transport along the proximal and distal segments of the nephron (161). Most of the calcium is reabsorbed from the tubular lumen into the cell across the brush-border membrane along the electrochemical gradient by a facilitated diffusion transport mechanism, which is powered by an electrogenic sodium transport system (164). The calcium is maintained at low concentrations in the cell, with the background concentration of free calcium in most cytosol oscillating between 0.1 and 0.2 μM. However, with calcium as important as it is in cell signalling and other biochemical processes, the situation of calcium overload could jeopardise cellular function and integrity (192). This obviously necessitates the presence of systems that eject calcium from the cells thereby offsetting its downhill penetration. Furthermore, the electrochemical gradient is reversed at the basolateral membrane, requiring energy-dependent processes. Therefore the implication that the kidney contains a calcium pump for extruding calcium is of physiologic importance (208). This pump is involved in the vectorial translocation of calcium out of the cell across the basolateral membrane of the cell. It thus provides a fine tuning mechanism for maintaining optimal cytosolic calcium concentrations and provides an environment for cell function (216, 242).

Dopamine has been recently recognized as important regulator of renal function due to its modulation of the GFR, renal plasma flow and electrolyte homeostasis (78). This ascribes a potential role for dopamine in fluid and electrolyte homeostasis. Thus, in volume-related disorders, like diabetes mellitus, dopamine may have significant influence on cellular homeostasis and integrity. Dopamine, by tempering $Ca^{2+}$ transport, may influence cellular environment in diabetes mellitus (111). It is also postulated that target hyporesponsiveness or insensitivity to dopamine may also be accountable for disordered homeostasis favoring downhill progression of the cell.

The results of the present study showed that dopamine levels are altered in diabetes mellitus and is representative of the compensatory response of the sympathetic nervous system to diabetes mellitus. A consensus including studies in our laboratory, reveal that there is increased sympathetic nervous system activity in diabetes mellitus (263, 265, 266). Altered catecholamine concentrations may represent a mechanism to move towards an euvolemic status with increased filtration of the osmotic load of increased glucose. However, it can be argued that measured levels of this catecholamine may merely reflect a decreased degradation of dopamine. Our studies showed an increase in dopamine turnover rate in diabetic kidney cells with decreased endogenous dopamine concentrations consistent with an unquestionable increase in dopamine levels. The increased rate constant would also favor an increased production of dopamine as part of the redemptory response in diabetes mellitus. The increased dopamine can elicit preferential post-glomerular vasodilation to balance the pre- and post-glomerular resistances, characteristic in early diabetic nephropathy (111), and thus reestablish normal GFR. Studies have shown that increased intrarenal production of dopamine could elicit

preferential post-glomerular vasodilation and thus correct for the GFR. An effect of dopamine on the filtration fraction has also been envisaged (112).

Hyperfiltration with its attendant increased glomerular capillary pressure has received considerable mention as a pathogenetic factor in the development of diabetic nephropathy (124). The consequence of the early suppression of this hyperfiltration is that the progression to diabetic nephropathy is precluded (123). Our studies have also documented that in early stages of the diabetic state, histology is essentially normal with no evidence of diabetic nephropathy. Hostetter et al. (126) documented elevations of single-nephron GFR in diabetic rats with subsequent development of proteinuria and progressive glomerular sclerosis. Remnant kidney model studies have also shown an elevation of intraglomerular capillary pressure prior to the development of glomerular sclerosis (141).

Hyperfiltration reflects a multifactorial etiology involving alterations in glucose and metabolites, associated metabolic fuel-related hormones, vasoactive substances, and vascular responsiveness. Altered glomerular hemodynamic forces acting through physical and mechanical means modify the growth and activity of glomerular cells causing their destruction. Given that shear stress is proportional to flow velocity, the hyperfiltration would be expected to impart increased force on the endothelial cell wall and thereby alter its structure (267-269). Expression of peptide vasoregulatory growth factor genes is differentially regulated by level of fluid shear stress. Endothelin-derived relaxing factor, tissue plasminogen activator, plasma derived growth factor-B, endothelin-1 have been incriminated to induce structural, physiological and biochemical adaptations in endothelial cell function that may prove detrimental over time (270). Hyperfiltration may

also influence mesangial and epithelial cell function by possible increases in protein, total collagen collagen and key components of mesangial extracellular matrix including collagen IV, collagen I, laminin and fibronectin (271). Possible mediators of such an influence on basement membrane thickening include alteration of ion-flow at the cell membrane level through mechanosensitive cation channel and/or integrin-induced cytoskeletal changes (272).

In spite of these impressive results, evidence exists against the role of elevated glomerular capillary pressure as the sole factor in the development of diabetic nephropathy. In rats who are severely diabetic and are not treated with insulin, typical histological changes occur but glomerular pressures are normal. Furthermore, most of the studies have used either proteinuria development or the development of glomerulosclerosis as the end-points in their studies. However, urinary albumin excretion and focal segmental glomerulosclerosis are not entirely specific to diabetes (270). Proteinuria is a non-specific indicator of renal disease, and does not necessarily indicate diabetic etiology. Similarly, focal segmental glomerulosclerosis is not an essential indicator of diabetic nephropathy; at best, its presence may additionally reinforce the diagnosis of diabetic nephropathy (270). Fogo et al. (273) have also shown that captopril can prevent glomerular sclerosis in adriamycin-treated rats without affecting glomerular pressure. Consistent with this, numerous studies have documented the role of converting enzyme inhibitors in the prevention of diabetic nephropathy with no effect on glomerular hemodynamics (124). Besides, other therapeutic interventions such as protein restriction have been shown to have a beneficial effect on preserving renal function without

affecting glomerular pressures (126). These findings suggest that other non-hemodynamic factors may also contribute in the pathogenesis of diabetic nephropathy.

Glucose reacts nonenzymatically with amino groups of structural and circulating proteins to initiate a posttranslation modification process known as nonenzymatic glycation or glycation reaction. This condensation reaction proceeds through the formation of a Schiff base resulting in an aldimine linkage, which is stabilized by an Amadori rearrangement to form a stable, covalent-bound ketoamine (274). Glycated products may bind greater amounts of circulating proteins such as albumin and low-density lipid proteins and thus alter filtration characterisitics (274). The degradation of both trapped circulating proteins and the structural proteins native to the glomerulus may be diminished due to reduced susceptibility to proteolytic degradation (271, 274). Additionally, dietary imbalance, presence of ketone bodies and the intracellular accummulation of sorbitol may alter the metabolic milieu and filtration characteristics. The pathogenesis of afferent arteriolar dilation is undoubtedly multifactorial and numerous mediators have been proposed.

Dopamine is a physiologically important paracrine substance and its compartmentalization within the kidney may present an important role in the specific paracrine actions of dopamine (52). Although the source of urinary dopamine has not been defined, considerable evidence exists to show that renal dopamine is produced largely within renal tubular cells (23). Plasma concentration of dopamine is insufficient to provide the quantity of urinary dopamine even if all the dopamine in the circulation could be extracted from the plasma flowing through the kidney. Therefore, urinary dopamine output has been assumed to be an index of renal dopamine secretion (20). The

present study also showed a decreased endogenous dopamine content in the diabetic rats. Furthermore, the rate constant of dopamine turnover is increased in the diabetic model further establishing that the decrease in endogenous dopamine is indisputable.

Though dopamine is synthesized, stored and released within the kidney, and dopamine receptors are localized in the renal vasculature, glomerulus and tubular system, its actions at dopamine receptors (DA1 and DA2) and adrenergic receptors limit its effectiveness as a heuristic tool and pharmacologic agent (21). In order to further delineate the role of dopamine, an analysis of dopamine receptors was done in the diabetic models and comparisons made with controls. DA1 receptor subtype is the predominant subtype in the kidney, and exerts a significant influence in the diabetic milieu (111). Our results showed that the DA1 receptors are downregulated in the chronic diabetic state. This would be consistent with the fact the endogenous dopamine content is decreased, while the systemic sympathetic activity is increased in the chronic diabetes in both animals and humans (20, 164). Murabayashi et al. (20) have also shown a decrease in the endogenous renal dopamine content in diabetic patients with nephropathy compared to diabetics without nephropathy. The consequence of such a decrease in available dopamine might promote sodium retention, which may in turn lead to high blood pressure. It must be pointed out that renal sympathetic nerve activity, renal-angiotensin-aldosterone system and other factors might be involved in renal sodium handling in the diabetic patients with nephropathy.

A positive correlation has been shown between the increased tubular sodium excretion and an increase of the nephrogenic component of AMP. Such a correlation would not be compatible with a β-adrenergic receptor site of action, since β-adrenergic

stimulation in the kidney, even if resulting in natriuresis by an inhibition of proximal sodium reabsorption, is at the same time accompanied by a decreased renal excretion of cAMP, probably by an increased tubular reabsorption of cAMP (91). Decreased urinary dopamine associated with decreased nephrogenic cAMP and excessive sodium retention appears to be just the opposite of changes observed in the kidney following dopamine infusion. A naturally corollary to this would entail a sodium-retaining effect in endogenous dopamine deficiency as seen in diabetics (275). Again, it has been shown that tubular DA1 receptors can be activated at lower doses than vascular DA1 receptors due to higher density of tubular DA1 receptors (89, 275). Such an observation is consistent with the postulation that renal dopamine probably plays a greater role in the control of renal sodium excretion rather than renal hemodynamics. In the present study, subset examination of the DA1 receptors revealed that there are two types of the DA1 receptors and both are downregulated in the diabetic state. This would favor a decreased sodium excretion in diabetics which has amply been borne out by both animal and human studies (80, 97, 99, 103). Contributions from the renal-angiotensin-aldosterone and renal sympathetic nerve activity probably contribute to the overall sodium renal handling in diabetics.

Dopamine regulates cytosolic calcium levels by increasing calcium excretion through the kidney though the exact mechanism still remains to be elucidated (78). Since the $Ca^{2+}$ + $Mg^{2+}$ ATPase is the chief regulator of cytosolic calcium by acting as the calcium pump in the vectorial translocation of calcium (208), the effects of dopamine on this calcium pump was the logical next step in this study. Dopamine normally stimulates the calcium pump favoring extrusion of calcium ions out of the cell. As a consequence,

the cellular integrity is maintained. In the early diabetic stage, this again is true, which is concordant with the fact that renal function is maintained and diabetic nephropathy is precluded (12). In the chronic state, there is a lack of response of the dopamine on this enzyme, in spite of increased availability of dopamine, and the changes of diabetic nephropathy ensue. As a consequence of lack of response of this calcium pump to dopamine, a situation of calcium overload occurs. An increased intracellular calcium may decrease oxidative phosphorylation in mitochondria (12, 192). As a result, the capacity of the renal cell to generate ATP becomes attenuated which in turn compromises renal cellular function or precipitates nephropathy.

It can be argued that this lack of response of the calcium pump to dopamine may represent part of a generalized response to diabetes. However, another ubiquitous enzyme, $Na^+ + K^+$ ATPase is stimulated by dopamine both in the acute and chronic stage of diabetes. Both in vitro and in vivo studies further verified that the $Na^+ + K^+$ ATPase is stimulated in diabetes which is congruous with increased sodium transport in diabetes mellitus (276, 277). Numerous studies have shown that there is an increase in whole body sodium in diabetes mellitus (20, 125). This is of noteworthy importance. First, it establishes that the lack of effect of dopamine on calcium transport in diabetes is a specific effect. Second, it reiterates the idea of increased sodium stores in diabetes (278, 279). According to the theory of DeWardener and McGregor (280), sodium increase of any origin will be answered by the release of natriuretic factor. This will be followed, according to the hypothesis of Blaustein, by an increase in intracellular calcium (281). As mentioned previously, calcium loading in the millimolar range compromises cellular integrity and leads to loss of tissue function (17). These effects are seen clinically in

human subjects who in chronic diabetes show hypertension and progressive loss of renal function.

The question now arises as to what is the mechanism by which increased calcium jeopardizes cellular integrity? Changes in oxidative phosphorylation with consequent alterations in ATP reserves have been perpetrated as a possible mechanism (12, 32). Besides, calcium, by virtue of its ability to alter other enzymatic machinery may modify cellular metabolism and function. The metabolism of the cell is mirrored by its extracellular matrix and alterations in the quality and quantity of matrix proteins may modify its characteristics, both in terms of generation as well as function. The glomerular basement membrane and mesangial matrix are together referred to as the glomerular extracellular matrices. Thickening of the glomerular basement membrane and increase in mesangial matrix are cardinal events in diabetes mellitus (130, 132, 133). Discernible basement membrane changes follow the onset of the metabolic disturbances and progress with the duration of the disease. Basement membrane is composed of glycoprotein material of a collagenous nature with varying amino acid content (190). This subunit polydiversity of the basement membrane represents one of its most striking structural features and may have important bearing on the chemical changes that this membrane undergoes in disease processes. Although the physiologic basis is not yet understood, it seems unlikely that each of the isolated polypeptides comprising the basement membrane is a separate biosynthetic component. Similarly, postribosomal modification of the basement membrane proteins may not be responsible for the observed heterogeneity observed in the diabetic state. It is reasonable to assume then that a limited physiologic

proteolysis would favor an increased deposition of structural proteins promoting basement membrane thickening.

Transglutaminases are latent calcium-dependent enzymes that promote crosslinking of matrix proteins by isopeptide bond formation (27). In the present study, diabetic animals showed increased levels of transglutaminase levels when compared with control animals. Collagen proteins of the basement membrane are rich in lysine and hydroxylysine residues and have a long biological half-life favoring increased glycation (282). Such denatured extracellular matrix proteins could be particularly vulnerable to transglutaminase crosslinking further insolubilizing the crosslinked products. Increased crosslinking of proteins by transglutaminase in the mesangial matrix and glomerular basement membrane would favor a thickening of the glomerular matrices. Indeed, denatured fibronectin is a better substrate for transglutaminase than native fibronectin (283).

Expansion of the glomerular mesangium leads to a decrease in GFR by impinging on the glomerular capillary vasculature, thereby decreasing the glomerular filtration surface (284). The increase in mesangial matrices is due primarily to the accummulation of normal matrix proteins. Increased pressures, physical stress and shear forces have all been speculated to lead to disruption in the selectivity of the glomerular barrier allowing proteins to cross the barrier as well as appear in the mesangium (26, 27). Translocation of the transglutaminase to the mesangium increases the potential for transglutaminase-catalyzed crosslink formation with the proteins of the mesangial matrix (285). Thus crosslinking influences organizational features of the basement membrane through compromise of adhesive, attachment and self-assembly properties of its macromolecules.

Therefore, conditions exist for activation of transglutaminases due to increased cytosolic calcium concentrations secondary to depression of the calcium pump in chronic diabetes. Dopamine, which can stimulate the calcium pump and thus prevent the calcium overload in the acute state, is unable to sanction such an effect in chronic diabetes. As a consequence, intracellular calcium rises leading to activation of transglutaminase with the crosslinking of structural proteins in the extracellular matrix. Crosslink formation results in the deposition of insoluble large molecular weight polymeric protein structures in the extracellular matrix of the kidney. These polymers are resistant to proteolysis and result in the expansion of the extracellular matrix with its attendant alteration of glomerular function thus contributing to the development of diabetic nephropathy.

## 7. CONCLUSION

Alterations in dopaminergic activity in chronic diabetes inhibit $Ca^{2+}$ + $Mg^{2+}$ ATPase. $Ca^{2+}$ + $Mg^{2+}$ ATPase (calcium pump) is a membrane-bound enzyme involved in the vectorial translocation of calcium from the intracellular site to the blood. This energy utilizing mechanism maintains an optimum intracellular calcium concentration. In chronic diabetes, dopaminergic activity is increased in the kidney. There is availability of increased plasma dopamine in the situation of decreased uptake and increased turnover. This favors an inhibition of the calcium pump which in turn decreases calcium extrusion and results in a steep rise of intracellular calcium levels. This increase in cytosolic calcium triggers the induction of latent transglutaminase activity. Transglutaminase activation favors the catalysis of isopeptide crosslinks between matrix proteins. These crosslinks result in the deposition of insoluble, protease-resistant, large molecular weight polymeric protein structures in the extracellular matrix of kidney. These polymers are resistant to proteolysis and result in thickening of the extracellular matrix and alteration in glomerular filtration thus contributing to the development of diabetic nephropathy.

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