INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa · University of Ottawa

Effect of Early Diabetes Mellitus on the Glomerular Renin-Angiotensin System in the Rat

George J. Wehbi, B.Sc.

Thesis submitted for the degree of
Master of Science
Department of Physiology
Faculty of Medicine
University of Ottawa

July 1999

ABSTRACT

Effect of Early Diabetes Mellitus on the Glomerular Renin-Angiotensin System in the Rat

One third of all diabetic patients develop kidney disease, or diabetic nephropathy. An important site of progressive injury in diabetic nephropathy is the glomerulus. Angiotensin II (Ang II) has been implicated as a key mediator in the progression of diabetic nephropathy. Ang II influences renal hemodynamics and modulates renal transport and growth. The intrarenal renin-angiotensin system (RAS) is responsible for local production of Ang II, independent of the systemic RAS. It is not known whether the glomerular RAS is involved in altered local Ang II production. Moreover, a number of studies have suggested downregulation of Ang II receptors in early diabetes, primarily attributed to the angiotensin type 1 (AT_1) receptors. These studies determined the effect of early diabetes on the expression of components of the glomerular RAS, and on the status of a novel Ang II receptor, Ang II type 2 (AT_2) receptor. Three groups of rats were studied after two weeks: 1) control [C], 2) streptozotocin (STZ)-induced diabetes, with daily insulin to prevent ketosis but maintain hyperglycemia [D], and 3) STZ-induced diabetes, with normoglycemia maintained by insulin implants [D+I]. D rats had increased plasma glucose levels [C: 9.63±0.19 mM vs D: 37.79±1.63 mM ($p<0.001$ vs C) vs D+I: 4.88±0.43 mM ($p<0.05$ vs C); n=12] and experienced renal hypertrophy, and a decrease in body weight compared to C and D+I rats. Plasma renin activity (PRA) was decreased in D but not significantly compared to C and D+I rats. Glomerular suspensions were isolated by sequential sieving after density gradient centrifugation. By competitive RT-PCR, D had no significant effect on glomerular mRNA expression of renin [C: 2,497.50±405.03 vs D: 3,155.50±417.26 vs D+I: 2,490.00±645.80 fg mRNA/62.5 ng RNA; p=NS; n=6], angiotensinogen (n=4), or angiotensin converting enzyme (ACE: n=5). By Western analysis, glomerular AT_1 receptor protein expression was increased in D rats (341±127% of C; $p<0.05$; n=7), an effect partly reversed in D+I. By RT-PCR, AT_2 receptor mRNA was increased in the cortex of D rats [C: 58,430±6,004 vs D: 83,675±3,575 vs D+I: 56,326±3,011 arbitrary units, ($p<0.005$, D vs C and D+I); n=6-7]. To determine the effects on glomerular AT_2 receptors, a polyclonal AT_2 receptor antibody was used for immunohistochemistry and immunoblotting. In D rats, by immunohistochemistry, a consistent decrease in AT_2 receptor staining was observed within glomeruli, and in all regions of the kidney, an effect abolished by D+I. By Western analysis, glomerular AT_2 receptor protein expression was decreased in D rats (47.0±6.5% of C; $p<0.001$; n=6), with partial reversal in D+I. Moreover, in whole cortical lysates, no change was observed in AT_2 receptor protein expression by Western analysis. In summary, early diabetes has no effect on glomerular mRNA expression for renin, angiotensinogen, or ACE. AT_2 receptors are present in glomeruli, and are downregulated in early diabetes. Opposing effects of diabetes on glomerular AT_1 and AT_2 receptor expression suggest that altered Ang II-mediated glomerular signalling occurs in early diabetic nephropathy.

Dedication

To my parents, Joseph and Georgette, and to my brothers and sisters,
Roula, Nahida, Nancy, Elias, and Abraham
and also to Danielle

Acknowledgments

First and foremost, I wish to express my sincerest gratitude to my Supervisors, Dr. Kevin D. Burns and Dr. David Z. Levine for allowing me to learn from them.

I am grateful to Dr. Burns for giving me the opportunity to do research in his laboratory and for teaching me that you can never work too hard. I greatly appreciate that he always made time is his crazy schedule for me, although our early morning meetings will not be missed.

I am thankful to Dr. Levine for demonstrating a passion and dedication to science that is truly inspirational. His encouragement and support are profoundly appreciated. Thank you Dr. Levine for always making me feel like a colleague rather than a student.

I would also like to thank the members of my Advisory Committee, Dr. Richard Hébert and Dr. Bernard Jasmin for their time and insightful suggestions throughout this project.

Also, a special thanks to Joe Zimpelmann whose constant willingness to lend assistance is deeply appreciated and whose presence in the lab made the work more enjoyable. Thanks to the entire first floor "Kidney group" - Agnès, Masoud, Shawn, Rania and Gele. I would also like to express my thanks to Laurie, Michelle, and Amy for all of their help. I am also indebted to Clare and Julie for their technical help in producing the kidney sections.

I would like to thank my fellow students and friends who made the two years seem like two weeks. Thank you to Wadih, Fawzi and everyone in the Jasmin lab, and everyone on the third floor, especially Donna, Denise, and Gillian for their help.

Many thanks to the entire Animal Care staff, especially Monique, Carl, and Eileen for their invaluable help.

I would like to especially thank Danielle for always bringing me lunch whenever I forgot mine.

Finally, I would like to thank my family for their love and encouragement, especially to my brothers for their patience and their help.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
AT_1	Angiotensin type-1 receptor
AT_2	Angiotensin type-2 receptor
bp	Base pair
BUN	Blood urea nitrogen
C	Control rat
cDNA	Complimentary Deoxyribonucleic acid
D	Diabetic rat
DAB	Diaminobenzadine
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
D+I	Insulin-implanted diabetic rat
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide
ECF	Extracellular fluid
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene glycol-bis(β-Aminoethyl ether) N, N, N', N'-tetraacetic acid
eIF4E	Eukaryotic initiation factor 4E
f	Femto (10^{-15})
g	Gram
(x) g	Gravitational constant (9.8 N/kg)
h (hrs)	Hour(s)
HRP	Horseradish peroxidase
HS	High salt
IL-β1	Interleukin-β1
IMCD	Inner medullary collecting duct cells
i.p.	Intraperitoneal
kg	Kilogram
l	Litre
mg	Milligram
$MgCl_2$	Magnesium chloride
μ	Micro (10^{-6})
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
NP-40	Nonidet P40
O/N	Overnight
PBS	Phosphate buffered saline

PGS-2	Prostaglandin synthase-2
PHAS/4E-BP1	Properties of heat-and acid-stability/4E-binding protein 1
PMSF	Phenylmethylsulfonyl fluoride
RAS	Renin-angiotensin System
RNA	Ribonucleic acid
RNase	Ribonuclease
s.c.	Subcutaneous
sec	Second
sH_2O	Sterile water
STZ	Streptozotocin
TBS	Tris-buffered saline
ZS	Zero salt

1 Diabetic Nephropathy

1.1 Introduction

Diabetes mellitus is a disease of abnormal glucose metabolism and is associated with characteristic long-term complications, including nephropathy, retinopathy, neuropathy, and cardiovascular disease. Clinically, there are two types of diabetes (unless otherwise stated, diabetes refers to diabetes mellitus), Type 1 diabetes and Type 2 diabetes. Type 1 diabetes, or insulin dependent diabetes mellitus (IDDM), is an autoimmnue disease characterized by the destruction of pancreatic β-cells by the immune system. The β-cell of the pancreas is the site of insulin production in animals. The immune response which destroys these cells usually occurs early in life and is marked by a life-long dependence on daily insulin therapy to maintain normoglycemia. Type 2 diabetes, or non-insulin dependent diabetes mellitus (NIDDM) is the form of diabetes characterized by either decreased insulin secretion or decreased insulin sensitivity. This form usually develops later in life and is associated with obesity.

Long term complications develop in patients with both types of diabetes with essentially equal prevalence. Nephropathy is the diabetes-specific complication with the highest mortality rate and usually occurs in about 35 to 45 percent of patients with IDDM and in about 30 percent of individuals with NIDDM (Nathan, 1993). Diabetes initiates damage to the kidneys by inducing chronically elevated glucose levels. Persistently high glucose levels disrupt normal cellular functions both in the kidney and throughout the body. Within the kidney this injury occurs at a number of sites, most notably the glomerulus.

The glomerulus is a capillary network lined by endothelial cells, a central region of mesangial cells with their surrounding matrix material, a parietal layer of Bowman's capsule with its basement

membrane, and a visceral layer of Bowman's epithelium and its associated basement membrane (Guyton, 1991). Figure 1.1 illustrates the morphology of a human kidney, with an enlarged nephron, the functional unit of the kidney, and a glomerulus.

The endothelial cells of the glomerulus line the lumen of the capillary bed. Their function is to form fenestrae, or filtration slits that are the basis of the filtration structure. Mesangial cells, located between the basal lamina and the endothelium, are contractile and play a role in the regulation of glomerular filtration rate (GFR) (Burns, et al., 1993). They also secrete various substances, take up immune complexes, and are involved in the development of some glomerular diseases (Burns, et al., 1993).

1.2 Clinical course of human diabetic nephropathy

Diabetic nephropathy progresses through five well-defined stages (Clark and Lee, 1995), (Nathan, 1993). The first stage is characterized by glomerular hyperfiltration. Evidence supports the notion that hyperglycemia is the major stimulus for the onset of glomerular hyperfiltration. It has been shown that restoration of the normal glucose state can reverse this hyperfiltration (Nathan, 1993). Patients with persistent hyperfiltration are at a greater risk of developing clinical nephropathy (Clark and Lee, 1995).

The second stage is marked by early glomerular histologic lesions. These lesions are a result of the expansion of the glomerular mesangial matrix and thickening of the glomerular basement membrane (GBM). This is usually seen as early as 18 months after the onset of diabetes in humans and approximately 3 months after onset in the streptozotocin (STZ)-diabetic rat (Nathan, 1993), (Rerup, 1970). Stage three is termed incipient diabetic nephropathy and is characterized by the

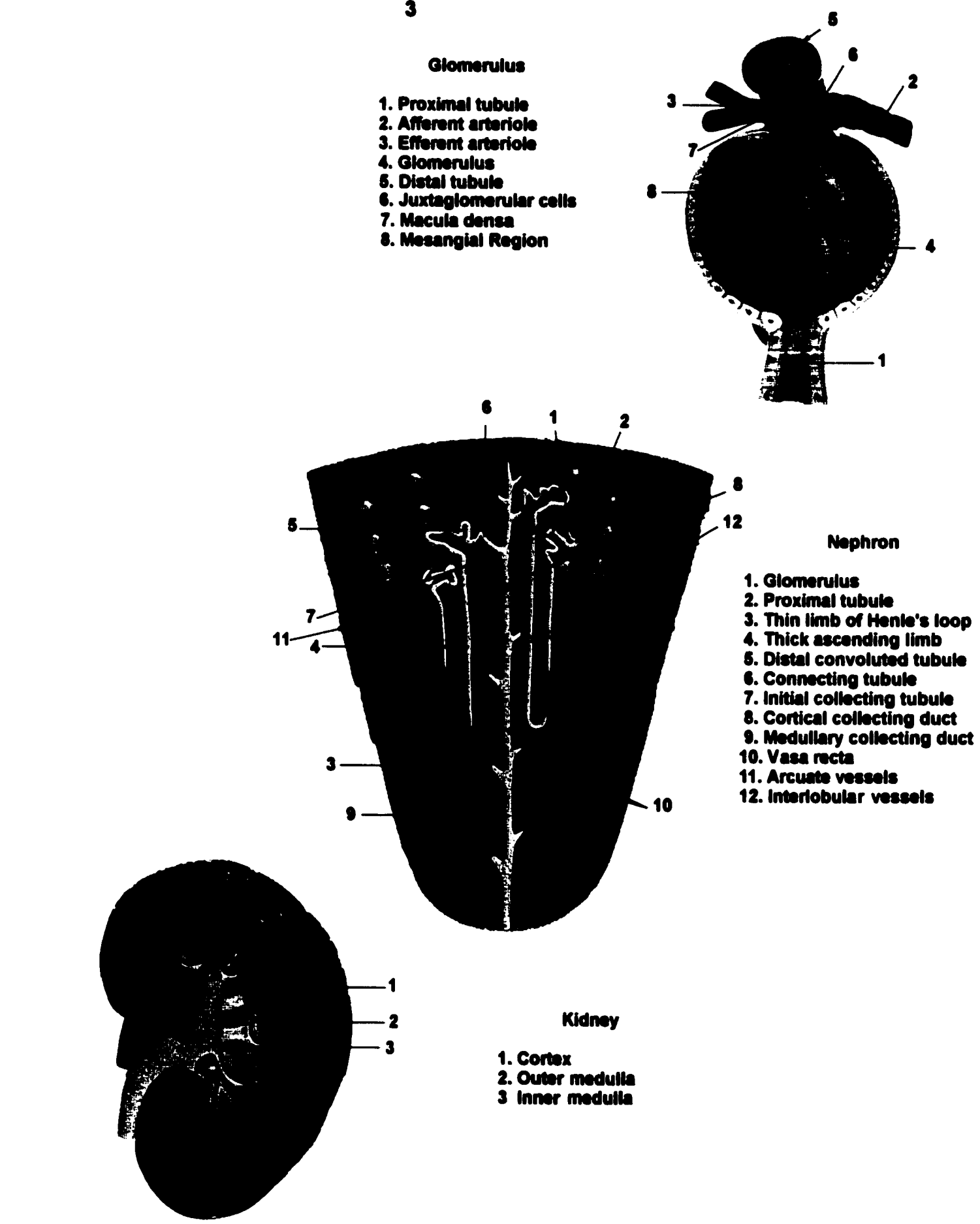

Figure 1.1 : Illustration of a human kidney with an enlarged nephron and a glomerulus (glomerulus is magnified 225x).

(From : Somso Modelle, Directional Learning, Elora,ON)

presence of protein in the urine, or microalbuminuria. In the healthy adult, albumin is not normally found in the urine. Microalbuminuria is defined as urine albumin excretion greater than 30 mg/day and less than 200 mg/day in humans, and is an indicator of glomerular injury. The amount of microalbuminuria may vary greatly and is influenced by such factors as uncontrolled hyperglycemia, hypertension, and dietary protein intake.

The fourth stage of diabetic nephropathy is characterized by persistent macroproteinuria, defined as urine protein excretion greater than 200 mg/day in humans, and a steadily declining glomerular filtration rate (GFR). In this stage hypertension is present in the majority of such individuals.

The fifth and final stage is defined as end-stage renal disease. This stage usually presents itself after approximately 20 to 30 years of diabetes, and between 30 to 40 percent of patients will have irreversible renal failure requiring dialysis or transplantation. At this stage, renal biopsies display the global glomerulosclerosis that is characteristic of long-term diabetes.

1.3 Experimental models of diabetic nephropathy

1.3.1 The streptozotocin (STZ)-induced diabetes mellitus rat model

The STZ-induced diabetes model is the most extensively studied in-vivo model of human Type 1 diabetes. STZ is a chemotherapeutic drug used in humans, however when administered to the rat at a high dose it renders these animals diabetic. It has been established that STZ induces apoptosis in pancreatic β-cells, and is responsible for the irreversible loss of insulin production in this model of IDDM (O'Brien, et al., 1996).

STZ-diabetic rats display a number of the characteristics featured in human diabetic

nephropathy. These include increased glomerular filtration rate (GFR) and renal blood flow (RBF) in the early stages, followed by a progressive reduction in GFR (Hostetter, et al., 1981). The early hyperfiltration is evident at the two week time point after induction of diabetes in the STZ-diabetes model. Increased intraglomerular pressure causes increased GRF, and this is regarded as a major pathogenic factor in the development of progressive diabetic glomerulosclerosis. As with human diabetes, the kidneys in this experimental model also undergo hypertrophy, specifically of glomerular and tubuloepithelial components (Cheng, et al., 1994), also apparent at the two week time point. Also characteristic of the early stages in both human and experimental diabetes is marked thickening of the glomerular and tubular basement membranes, associated with enhanced permeability to albumin and accumulation of extracellular matrix (ECM) components in the glomerular mesangium, and tubulointerstitial fibrosis (Ziyadeh, 1993). These characteristics of early human diabetes are all apparent at the two-week time point in the STZ-induced diabetes model. As the nephropathy evolves, there is increased mesangial expansion which leads to the destruction of the glomerular capillary lumen. This results in significant proteinuria, and a progressive reduction in glomerular filtration (Hostetter, et al., 1981). In humans, after as early as five years of diabetes, microalbuminuria is detectable. Overt proteinuria and a progressive fall in GFR occurs over the following five to ten years (Nathan, 1993). At this stage glomerulosclerosis in human renal biopsies is evident. In the STZ-diabetes model, glomerulosclerosis can be detected as early as three months after induction of diabetes (Rerup, 1970).

The two week (early) time point in the STZ-induced diabetic rat is a period of early gene activity and growth of the kidney. This stage of diabetes is characterized by the activation of cellular systems which may be involved in the later stages of diabetes.

1.3.2 The Biobreeding (BB) rat

The BB rat is an animal model of spontaneous diabetes which is not drug induced. Similar to human Type 1 diabetes, this animal model is characterized by insulin deficiency due to an autoimmune destruction of pancreatic β-cells (Velasquez, et al., 1990). Onset usually occurs seven to fifteen months after birth in 60% of animals, and rats require daily insulin treatment to prevent death. Interestingly, diabetic BB rats develop only some of the clinical manifestations of human diabetic nephropathy, including renal hypertrophy, but not increased GFR and RBF (Cohen, 1989). Also, these animals do not develop significant albuminuria despite prolonged diabetes (Cohen, et al., 1987). Moreover, diabetic BB rats are unique in that they only develop glomerular basement membrane (GBM) thickening and not glomerulosclerosis (Feld, et al., 1995). The use of this model has been restricted because of the difficulties associated with working with these rats. These animals require special facilities and care, which most institutions are not equipped to handle, making the costs associated with working with this model highly prohibitive. This model has however received a great deal of attention recently due in large part to the absence of glomerular sclerosis, a hallmark of the final stages of human diabetic nephropathy.

1.3.3 Nonobese diabetic (NOD) mouse

This animal model of human Type 1 diabetes mellitus was established two decades ago by selectively inbreeding the offspring of a spontaneously diabetic female mouse (Kikutani and Makino, 1992). Similar to the BB rat, the NOD mouse develops diabetic symptoms, including rapid weight loss, polyuria, polydipsia, and severe glucosuria, without developing obesity but does develop hypoinsulinemia secondary to autoimmune destruction of pancreatic β-cells (Velasquez, et al., 1990).

These animals manifest many of the renal characteristics observed in other models of diabetes including proteinuria, thickening of the GBM, and deposition of basement membrane-like material in glomeruli, all characteristic of the later stages of human diabetic nephropathy (Kikutani and Makino, 1992).

1.3.4 Zucker fatty (*fa*) rat

The Zucker fatty rat is a model of hyperinsulinemia, in which obesity develops at an early age due to an autosomal recessive gene. This model is different from the other models discussed in that these rats develop peripheral insulin resistance, which is similar to human NIDDM (Type 2 diabetes). These rats manifest many of the characteristics seen in human Type 2 diabetes, including mild glucose intolerance and hyperlipidemia (Velasquez, et al., 1990). However, their blood glucose level is usually normal throughout life. Also, obese Zucker rats characteristically develop albuminuria and glomerulosclerosis during the later stages of diabetes. The glomerular lesions consist of mesangial expansion, disappearance of podocytes and endothelia, and obliteration of capillary lumina (Velasquez, et al., 1990). As rats mature, albuminuria increases and GFR decreases as glomerulosclerosis becomes extensive (Velasquez, et al., 1990), also similar to the later stages of human diabetic nephropathy.

1.4 Pathogenesis of diabetic nephropathy

1.4.1 Introduction

Diabetics are under constant hemodynamic and metabolic stresses. Chronically these factors result in injury to the kidneys, specifically the glomerulus. Persistently elevated blood glucose

causes the disruption of a number of physiological signalling mechanisms. Direct glucotoxicity results in the production of cytokines and growth factors that may stimulate the expression of certain genes which may contribute to the nephropathic state. High glucose levels have been implicated in the disruption of signalling mechanisms involving angiotensin II (Ang II), transforming growth factor-β (TGF-β), protein kinase C (PKC), and endothelin (ET). The involvement of these factors in the pathogenesis of diabetic nephropathy will be outlined in greater detail in the following sections.

In addition to the direct effects of glucose on intracellular signalling, there are other pathways whereby glucose exerts adverse effects. The polyol pathway is an enzymatic pathway thought to be responsible for diabetic complications. There are also non-enzymatic reactions which have been implicated in the progression of diabetic nephropathy. The condensation reaction between glucose and reactive amino groups in target proteins yields protein-glucose complexes. These products undergo rearrangements to form what are known as advanced glycosylation end-products (AGE).

Numerous studies have demonstrated that the degree of hyperglycemia is an important predictor of renal complications, notably the Diabetes Control and Complications (DCCT) trial (DCCT, 1993). This clinical trial followed 1441 IDDM patients over a 10 year period and showed that intensive insulin therapy to maintain euglycemia effectively delayed the onset and slowed the progression of diabetic nephropathy.

1.4.2 The polyol (sorbitol) pathway in diabetic nephropathy

One of the earliest pathways proposed to mediate the complications associated with diabetes was the polyol pathway. This pathway has been implicated in the diabetes-specific complications

associated with retinopathy, neuropathy, and nephropathy. In this pathway, aldose reductase catalyzes the NADPH-dependent reduction of hexose or pentose sugars to their corresponding sugar alcohols, or polyols. The high glucose state of diabetics results in greater reduction of glucose to sorbitol, and thus a greater availability of sorbitol for the reduction of NAD^+ to NADH and fructose. The dysfunction that occurs has been linked to the hyperglycemia-induced increase in the NADH/NAD^+ ratio, and thereby the higher redox state of the cell. This state appears to be important in the de novo synthesis of diacylglycerol (DAG) and the stimulation of PKC activity (Williamson, et al., 1993). Moreover, through yet unclear mechanisms, depletion or disordered metabolism of *myo*-inositol occurs, resulting in impaired regulation of Na^+-K^+-ATPase. The use of inhibitors of aldose reductase, the rate-limiting enzyme that converts glucose to sorbitol, has been shown to prevent some of the early features of diabetic nephropathy such as glomerular hyperfiltration (Pedersen, et al., 1991), and prevents the high glucose-induced increase in collagen synthesis (Bleyer, et al., 1994). However, these inhibitors have not been successful in preventing the later stage complications associated with diabetes (Clark and Lee, 1995).

1.4.3 The presence of advanced glycosylation end-products (AGE) in diabetic nephropathy

Advanced glycosylation end-products (AGE) are generated as a result of the condensation reaction that occurs between reducing sugars and the reactive amino groups in proteins. This reaction does not occur to a significant degree under physiological conditions, but the excessively high glucose level in diabetics provides considerable levels of reducing sugars available to undergo the condensation reaction. Glucose condenses with the reactive amino groups of proteins and yields

Schiff base intermediates that undergo Amadori rearrangements to ultimately form stable protein-glucose adducts. Schiff bases are the intermediate compounds formed when a glucose and an amino group undergo a condensation reaction. Amadori products are also unstable intermediates in this condensation reaction. These compounds interchange between one another until the Amadori products undergo what are know as Amadori rearrangements to form stable AGEs. These products may undergo further condensation reactions with other proteins and produce advanced glycosylation end-products (Brownlee, 1995). This results in the cross-linking of several proteins with a glucose molecule. The most important characteristic of AGE-modified proteins is their ability to physically cross-link amino groups together, thereby prolonging their half-life as well as affecting their functional properties (Bucala and Vlassara, 1995). This cross-linking has been shown to increase basement membrane permeability to macromolecules such as albumin (Boyd-White and Williams, 1996). The net effect may be AGE modification of critical components in the kidney, such as collagen, laminin, and tubular basement membrane, thereby prolonging their half-life. The increased half-life of these renal proteins has been associated with the development of diabetic nephropathy (Bucala and Vlassara, 1995).

1.4.4 Factors implicated in the pathogenesis of diabetic nephropathy

In addition to the polyol pathway and AGEs, a number of mediators have been identified and are thought to be involved in the pathogenesis of diabetic nephropathy. A partial list of the principal factors includes Ang II, TGF-β, PKC, and ET. Altered regulation of these compounds may occur due to direct glucotoxicity, and this altered regulation may have an effect on the regulation of other factors.

1.4.4.1 Transforming growth factor (TGF-β)

TGF-β is a dimeric protein more commonly regarded as a cytokine. There are at least five distinct isoforms which have been identified (Sharma and Ziyadeh, 1994). TGF-β is the principal mediator of cell growth and extra-cellular matrix (ECM) production in the kidney (Border and Noble, 1994). There has been a great deal of interest in this factor in recent years due to emerging evidence that TGF-β is a key mediator in the pathogenesis of diabetic kidney disease. In the kidney TGF-β promotes tubular epithelial cell hypertrophy and is involved the glomerular production of collagens, fibronectin, laminin, and proteoglycans (Sharma and Ziyadeh, 1994). TGF-β also upregulates the synthesis of protease inhibitors and therefore blocks the degradation of newly synthesized ECM, and downregulates the synthesis of matrix-degrading proteases (Roberts, et al., 1992). Results from recent studies implicate Ang II as the mediator in upregulation of TGF-β. Ang II mediated constriction of the efferent arteriole results in the increased glomerular capillary hydrostatic pressure and induces the mechanical stretch of glomerular cells. This mechanical stretching of mesangial cells induces the production of TGF-β, leading to increased matrix synthesis (Riser, et al., 1996). The administration of angiotensin converting enzyme (ACE) inhibitors to diabetic rats prevented the over expression of TGF-β mRNA and the consequent tubulointerstitial injury (Gilbert, et al., 1998). ACE inhibitors are drugs used to block the enzymatic conversion of Ang I to its active form Ang II. This observed effect of ACE inhibition suggests the involvement of the rein-angiotensin system (RAS) in the prosclerotic action of TGF-β.

1.4.4.2 Protein Kinase C (PKC)

The protein kinase C (PKC) family consists of at least 11 distinct isozymes which have been

classified as serine-threonine kinases (Murphy, et al., 1998). PKC is ubiquitously expressed in all cell types and is involved in the intracellular signal transduction of numerous extracellular signals. PKC is regulated intracellularly by three principle co-factors, namely calcium, phospholipids, and DAG (Zhou, et al., 1997). Activation of PKC by high glucose and by Ang II plays a central role in the activation of target genes in the cells that are readily permeable to glucose - such as the endothelial and mesangial cells of the glomerulus.

The mechanism responsible for the activation of PKC by hyperglycemia is mediated by intracellular elevation of DAG. Intracellular levels of DAG have been shown to be elevated in a number of tissues including the renal glomeruli of diabetic animals (Ishii, et al., 1996). This elevation of DAG levels in glomeruli has been shown to be a direct consequence of elevated glucose levels (Ayo, et al., 1991). Parallel with the increase in DAG levels, activation of PKC in glomerular mesangial cells has also been reported (Ayo, et al., 1991), (Zhou, et al., 1997).

Activation of PKC is one of the earliest mechanisms whereby high glucose stimulates ECM production. Increased de novo synthesis of DAG greatly increases the affinity of PKC for Ca^{2+} and phosphatidyl serine at normal intracellular Ca^{2+} levels, thereby permitting sustained PKC activation (Bell, 1986).

Activation of PKC is known to induce the transcription of both *c-fos* and *c-jun* mRNA in cultured mesangial cells (Kreisberg, et al., 1994), and in the glomeruli of STZ-diabetic rats (Sharma, 1995). These transcription factors regulate gene expression through an AP-1 binding site (Franza, et al., 1988), and thus activation of PKC may lead to increased production of the Jun/Fos (AP1) transcription factor complex. The promoter regions of TGF-β (Kim, et al., 1990), fibronectin (Dean, et al., 1990), and laminin (Okano, et al., 1992) contain the AP-1 binding consensus sequences.

Recently, Ishii and colleagues demonstrated that a PKC-β selective inhibitor could reduce GFR in STZ-diabetic rats (Ishii, et al., 1996). Furthermore, the dose response in normalizing GFR parallelled the inhibitory effect on PKC activity. More importantly, they demonstrated that even at maximum doses, this inhibitor did not affect PKC activity in the glomeruli of nondiabetic rats, suggesting that PKC-β may not be significantly activated under normal circumstances. This study also implicated PKC-β as the major isoform involved in mediating the vascular changes observed in diabetes.

1.4.4.3 Endothelin (ET)

The endothelins (ET) are a family of contractile peptides made up of 21 amino acid residues. ET-1 was first identified as a potent vasoconstrictor produced by vascular endothelial cells (Yanagisawa, et al., 1988). Three distinct isoforms of ET have been identified: ET-1, ET-2, and ET-3 (Inoue, et al., 1989). Two receptor subtypes, ET receptor A (ET_A) and ET receptor B (ET_B) are widely expressed in the kidney and are responsible for the wide range of biological actions of ET. These actions include constriction of most renal vessels, contraction of mesangial cells, inhibition of sodium and water reabsorption, enhanced glomerular cell proliferation, and stimulation of extracellular matrix (ECM) accumulation (King, et al., 1989), (Badr, et al., 1989).

Data supports a pathophysiological role for ETs in diabetic nephropathy. In STZ-diabetic rats, ET_A receptor antagonists reduced GFR and urinary protein excretion, and decreased glomerular mRNA levels of collagens alpha 1(I, III, and IV), laminins B1 and B2, tumor necrosis factor (TNF)-α, platelet derived growth factor (PDGF)-B, basic fibroblast growth factor (bFGF), and TGF-β (Nakamura, et al., 1995). In STZ-diabetic rats, ACE inhibitors also reduced GFR and urinary protein

excretion, and decreased glomerular ET-1 mRNA levels, but had no effect on mRNA levels of TNF-α, PDGF-B, bFGF, or TGF-β (Fukui, et al., 1994). In cultured mesangial cells, elevated glucose levels have been reported to enhance the secretion of ET-1 (Yamauchi, et al., 1990), and downregulate ET_A receptors (Inishi, 1991).

In Type 2 diabetic humans, treatment with the ACE inhibitor captopril reduced plasma ET-1 levels (Ferri, et al., 1995). These data provide a link between ETs and the RAS. The mechanisms of ET-1 action in diabetes remain to be clarified, but are undoubtedly due to a number of factors, one of which may be Ang II.

A major factor implicated in the pathogenesis of diabetic nephropathy and the focus of this thesis is the octapeptide Ang II. The following chapter outlines the role of the RAS in diabetic nephropathy.

2 The Renin-Angiotensin System (RAS) in Diabetic Nephropathy

2.1 Overview of the RAS

The classical RAS cascade is comprised of a series of intermediate compounds generated from enzymatic reactions ultimately producing the active peptide Ang II. This model of Ang II production involves a complex system in which the substrate and enzymes are produced in different tissues. Briefly, angiotensinogen, which is produced in the liver, is hydrolysed by an enzyme known as renin, which itself is produced by the juxtaglomerular cells of the afferent arteriole in the kidney (Burns, et al., 1993). Renin functions solely as a proteolytic enzyme. Angiotensinogen is acted on by renin to produce a 10 amino-acid peptide known as angiotensin I (Ang I). Ang I, which has no

known physiologic function, is further cleaved to an 8 amino-acid peptide known as Ang II by a converting enzyme or ACE found on the surface of vascular endothelial cells (both renal and pulmonary endothelial cells are important sites for the conversion of Ang I to Ang II in this circulating model) (Burns, et al., 1993). Moreover, this cascade does not represent the only manner through which this pathway proceeds. Enzymes such as tonin, cathepsin G, chymase, aminopeptidases (AMP) and neutral endopeptidases (NEP) can act on the intermediates and the final product to produce an array of angiotensins (Gomez and Norwood, 1995). Figure 2.1 illustrates this putative scheme.

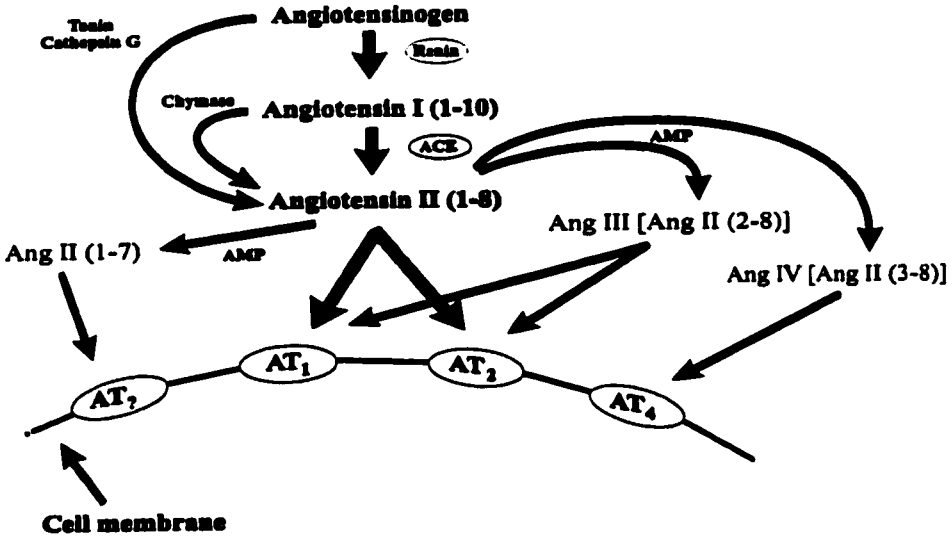

Figure 2.1: The RAS cascade, with the traditional cascade indicated by thick arrows.

This figure also illustrates that once formed, either within tissue or by the circulating RAS, Ang II exerts its actions by binding to specific receptors, termed type-1 (AT_1) or type-2 (AT_2) receptors. Recently, we and others have identified a high affinity receptor, in the rat kidney for Ang

II (3-8) (Ang IV) which has been termed the type-4 (AT_4) receptor (Ardaillou, 1999).

2.2 Functions of Ang II

The potent vasoconstrictor actions of Ang II have long been recognized. Ang II also has important non-renal actions. In the central nervous system, Ang II stimulates thirst and modulates the release of pituitary hormones (Gomez and Norwood, 1995). Ang II also facilitates adrenergic neurotransmission, causes catecholamine secretion from the adrenal medulla, stimulates adrenal glucocorticoid and aldosterone synthesis (Tufro-McReddie and Gomez, 1993). In the liver, Ang II stimulates glycogenolysis and gluconeogenesis.

The intrarenal actions of Ang II include modulation of RBF, GFR, tubular epithelial transport, renin release and cellular growth. Ang II ensures adequate RBF by maintaining vascular tone, and therefore arterial blood pressure through the AT_1 receptor. In addition, Ang II maintains GFR by differentially contracting the efferent arteriole more than the afferent arteriole (Burns, et al., 1993). This leads to an increase in glomerular filtration pressure and a rise in GFR, even in the face of reduced RBF.

In the proximal tubule, Ang II, via AT_1 receptors stimulates the apical Na^+/H^+ antiporter at low concentrations ($< 10^{-9}$ M) via two identified signal transduction pathways. The first to be described was that involving decreased cAMP concentrations (Douglas, et al., 1990). The other mechanism involves the activation of PKC, whereby PKC ζ is the main isoform implicated (Karim, et al., 1995). Via this pathway, Ang II is an extremely potent stimulator of proximal tubule bicarbonate reabsorption via the Na^+/H^+ antiporter regulating as much as 60% of reabsorbed bicarbonate (Cogan, 1990). High concentrations of Ang II ($>10^{-9}$ M), inhibit Na^+/H^+ antiporter

activity, mediated by the P450 cytochrome-dependent mono-oxygenase pathway (Houillier, et al., 1996).

Ang II also modulates bicarbonate transport. Following systemic infusion of Ang II, bicarbonate reabsorption in the loop of Henle was increased (Capasso, et al., 1994). Similarly systemic Ang II infusion stimulated distal tubule bicarbonate reabsorption (Levine, et al., 1994). The authors speculated that this may be due to either enhanced Na^+/H^+ antiporter activity or Ang II mediated mineralocorticoid stimulation of H^+/ATPase.

Ang II modulates cellular growth and may act as a growth factor for several renal cells. In mesangial cells and tubular epithelial cells, Ang II promotes growth changes and synthesis of extracellular matrix proteins (Ruiz-Ortega and Egido, 1997). The characteristic glomerular hypertrophy seen in the early stages of diabetes in rats has also been shown to be attenuated significantly by ACE inhibitors (Anderson, et al., 1989).

2.3 Ang II receptors

Ang II elicits responses by binding to specific receptors located on the surface of cellular membranes of target cells, leading to the activation of multiple intracellular signal transduction pathways. The use of non-peptide receptor antagonists to Ang II receptors has allowed the characterization and subsequent cloning of at least two distinct Ang II receptor subtypes, termed AT_1 and AT_2 (Timmermans, et al., 1992).

2.3.1 Ang II type 1 (AT_1) receptors

Physiological and pharmacological studies have demonstrated that most of the known actions

of Ang II are mediated by the AT_1 receptor (Timmermans, et al., 1992). The AT_1 receptor is a 359-amino acid protein with a predicted molecular weight of 40.9 kD and is a member of the seven transmembrane domain G protein-coupled receptor family (Sasaki, et al., 1991). AT_1 stimulation by Ang II activates phospholipase C_β (PLC_β), which results in increased intracellular calcium and inositol 1,4,5-trisphosphate (IP_3) concentrations (Sasaki, et al., 1991). Also, Ang II activates mitogen- activated protein (MAP) kinases, such as extracellular-regulated kinases (ERK ½) via Src and Ras, as well as the JAK/STAT pathways (Schmitz and Berk, 1997).

In rodents, there are two highly homologous subtypes of the AT_1 receptor, termed AT_{1A} and AT_{1B} (Iwai and Inagami, 1992). These isoforms share 94% homology in amino acid sequence, yet are only 60% identical in the 5' and 3' untranslated regions of the gene. AT_{1A} receptors are expressed predominantly in vascular smooth muscle, liver, lung, and kidney, whereas the AT_{1B} receptors are found mainly in the adrenal and anterior pituitary (Sasaki, et al., 1991). In humans however, there is only one AT_1 receptor gene.

The distribution of renal AT_1 receptors has been most extensively studied in the rat, but this distribution is highly conserved across species. AT_1 have been localized to various regions of the kidney, including the glomerulus, and all tubular segments (with the greatest expression occurring in the proximal tubule), medullary interstitial cells and the renal vasculature (Harrison-Bernard, et al., 1997). Using a monoclonal antibody specific to the AT_1 receptor, Harrison-Bernard and colleagues demonstrated prominent immunostaining in the proximal tubule brush-border and basolateral membrane (Harrison-Bernard, et al., 1997). Also, distal tubules, cortical and medullary collecting ducts exhibited specific immunoreactivity. Glomerular staining for AT_1 was observed in mesangial cells and podocytes. Macula densa cells also exhibited positive staining. This study

demonstrated the widespread distribution of this receptor within the kidney, greater than previously thought.

Ang II stimulation of AT_1 receptors is known to mediate a myriad of effects in the kidney. Through vasoconstriction of the glomerular afferent arteriole, Ang II decreases cortical RBF and glomerular plasma flow. Constriction of the efferent arteriole by Ang II also causes increased glomerular capillary pressure. The efferent arteriole has been shown to have a higher vasoconstrictory sensitivity to Ang II in comparison to the afferent arteriole (Gomez and Norwood, 1995), which will result in a net force favouring increased filtration.

2.3.2 Ang II type 2 (AT_2) receptors

The AT_2 receptor is considerably different from the AT_1 receptor and only shares 32% nucleotide sequence homology (Mukoyama, et al., 1993). This receptor exhibits a significant interspecies conservation in sequence homology, and has been cloned in the mouse, rat, and human (Mukoyama, et al., 1993). The AT_2 receptor is also of the seven transmembrane domain family of receptors, and is composed of 363 amino acids with a predicted molecular weight of 41 kD. Whether this receptor is G protein-coupled is still a matter of debate. The AT_2 receptor appears to activate one or a number of cellular tyrosine phosphatases. Activation of MAP kinase phosphatase 1 results in MAP kinase inhibition and apoptosis (Yamada, et al., 1996).

The predominant and widespread distribution of AT_2 receptors in fetal tissue has suggested an important role in embryonic development and growth. However, mice with a disrupted AT_2 receptor gene appear to grow normally and present no abnormal morphology in the brain, blood vessels, heart, or kidney (Ichiki, et al., 1995). The expression of AT_2 receptors declines throughout

the course of gestation with a concomitant increase in AT_1 receptor expression.

The expression of the AT_2 receptor in adult non-renal tissue has been identified in the adrenal medulla, heart, aorta, and various brain regions (Inagami, 1999). In the majority of adult mammals investigated, AT_2 receptor density in the kidney cortex and medulla is very low or undetectable (Allen, et al., 1999). In humans, however, considerable AT_2 receptors have been identified in the adventitia of large preglomerular arcuate and interlobular arteries (Zhuo, et al., 1996). A recent immunohistochemical study has demonstrated that AT_2 receptors are expressed in the kidneys of adult rats (Ozono, et al., 1997). Although intrarenal AT_2 receptor expression is diminished in adult life, it is stimulated in the adult rat in response to dietary sodium depletion.

The major function of AT_2 receptors in the adult has thus far been linked to antagonism of the vasoconstrictor actions of AT_1 receptors by modulating vascular sensitivity to Ang II (Ichiki, et al., 1995), inhibition of the proliferative and growth-promoting effects of AT_1 receptors (Stoll, et al., 1995), and mediation of programmed cell death (Yamada, et al., 1996). Additionally, AT_2 receptors have been linked to sodium retention by the kidney. Lo and colleagues demonstrated that AT_2 receptor antagonists effectively and rapidly increased diuresis and natriuresis in the rat kidney *in vivo* (Lo, et al., 1995). Moreover, the administration of AT_2 agonists was able to suppress diuresis and natriuresis. The functions of the AT_2 receptor are thus far proving to be quite intriguing in that this receptor serves to promote sodium retention but at the same time mediates vasorelaxation.

Finally, activation of the RAS during sodium depletion increases intrarenal nitric oxide (NO) production through stimulation of the AT_2 receptor (Siragy and Carey, 1997). Enhanced production of NO mediated increased renal interstitial fluid cGMP production. It would be fair to speculate that increased NO production from the actions of Ang II on the AT_2 receptor is one mechanism mediating

the vasorelaxation associated with this receptor.

2.4 Diabetes, dietary salt, and the RAS

It is well established that sodium restriction upregulates renin production (Tank, et al., 1997). This may influence systemic and local Ang II production and in this manner affect renal hemodynamics. Peripheral renin activity is significantly increased largely due to increased renal production by the juxtaglomerular cells of the afferent arteriole (Tank, et al., 1997). Conversely, a high salt diet suppresses renin production.

Aside from the abnormalities in renal hemodynamics, the early stages of diabetic nephropathy are characterized by alterations in renal sodium handling (Bank, et al., 1988). Despite net increases in urinary sodium excretion, clinical and experimental diabetes are both associated with enhanced tubular sodium reabsorption (Pollock, et al., 1991). Modifications in tubular sodium handling, such as increased sodium-glucose co-transport, will alter distal sodium delivery and may alter activity of the tubuloglomerular feedback (TGF) mechanism with subsequent changes in glomerular hemodynamics. TGF is a flow-dependent mechanism of autoregulating GFR and RBF. This mechanism involves a feedback loop whereby the rate of tubular fluid flow is sensed by the macula densa cells of the juxtaglomerular apparatus and converts this rate of fluid flow into a signal that affects afferent arteriole resistance and thus GFR. Therefore, one possible mediator in the abnormal renal hemodynamics seen in early diabetes may be the abnormal sodium handling by renal cells.

In a key human study by Miller, it was found that dietary sodium restriction in diabetics increased GFR, thereby exacerbating the underlying hemodynamic abnormalities, rather than

reducing GFR and RBF to levels found in control subjects (Miller, 1997). This study demonstrated that the renal response to sodium restriction was qualitatively different in diabetic subjects, who seem to respond with an increase in GFR and RBF, whereas control subjects exhibit little change. The mechanism for this response is unknown, but it has been speculated that salt restriction reduces salt delivery to the macula densa which in turn results in afferent arteriole vasorelaxation and increased GFR. Ordinarily salt restriction would result in a decreased GFR.

2.5 The intrarenal RAS

2.5.1 Proximal tubule production of Ang II

There is now considerable evidence that within the kidney all of the components of the RAS are synthesized independently of the systemic RAS. The epithelial cells of the proximal tubule were one of the earliest cell types to demonstrate the capacity to form Ang II. The epithelial cells of the proximal tubule contain all of the components necessary for synthesis of Ang II, including the mRNAs for the enzymes renin and ACE, and for the renin substrate angiotensinogen (Burns, et al., 1993). The proximal tubule lumen contains high levels of Ang II (10^{-8} M) which are approximately 1000 times higher than those found in circulation (Braam, et al., 1993). These high levels are likely due to local synthesis and secretion from the apical surface of proximal tubule cells. This locally produced Ang II can then bind to specific receptors for Ang II present on the apical membrane of proximal tubule cells. Ang II can also be modified by aminopeptidases, which are abundant on the surface of proximal tubule cells, forming Ang II degradation products which may have distinct receptors.

2.5.2 Glomerular production of Ang II

Recently, Atiyeh and colleagues reported that isolated rat glomeruli are also capable of *de novo* Ang II synthesis (Atiyeh, et al., 1995). This group demonstrated that ACE inhibition caused a decreased production of Ang II in suspensions of rat glomeruli, in a concentration-dependent manner. The cells within the glomerulus which may be responsible for local Ang II production have not yet been identified. However, the glomerulus contains mRNA for all components of the RAS. For example, mesangial cells in culture are capable of synthesizing renin (Chansel, et al., 1987), and renin mRNA has been localized to the glomerular mesangium (Atiyeh, et al., 1995). Also, the glomerular tuft is known to contain mRNA for angiotensinogen, and ACE has been localized to glomerular capillaries (Ingelfinger, et al., 1990). Therefore, the glomerulus possesses all of the components necessary for Ang II production.

In freshly microdissected glomeruli from rats fed a low salt diet, Tank and colleagues demonstrated increased renin mRNA by quantitative competitive reverse transcriptase polymerase chain reaction (RT-PCR) (Tank, et al., 1997). Conversely, renin mRNA in the glomeruli of rats fed a high salt diet was decreased. This demonstrated that glomerular renin transcript levels were regulated by chronic changes in dietary sodium intake, independent of juxtaglomerular cell renin production. This critical study convincingly demonstrated that intrarenal RASs could contribute to renal adaptations in response to stimuli.

3 Diabetes and the intrarenal RAS

3.1 ACE inhibition slows the progression of diabetic nephropathy

One of the earliest indications that Ang II may be mediating some of the injury observed in

diabetic nephropathy was determined by interrupting the formation of Ang II with ACE inhibitors in STZ-diabetic rats (Zatz, et al., 1986). In this pivotal study, ACE inhibition led to normalization of glomerular capillary hypertension and a consequent protection against the development of albuminuria and glomerular injury. Subsequently, numerous experimental and clinical studies have demonstrated the renoprotective effects of ACE inhibition in slowing the progressive renal injury in diabetes.

Initially it was thought that the renal injury was prevented simply from the lowering of systemic blood pressure. Studies employing antihypertensive agents other than ACE inhibitors did not prevent nephropathy, demonstrating that it was in fact interruption of the RAS which provided these beneficial effects (Anderson, et al., 1989). This has demonstrated a clear involvement of the RAS in the progression of diabetic nephropathy.

3.2 AT_1 Antagonists

Further evidence that the protective effects of ACE inhibitors are primarily due to decreased Ang II generation, rather than other ACE-mediated effects (such as enhanced bradykinin generation) comes from observations that Ang II AT_1 receptor antagonists reproduce the same beneficial hemodynamic and structural effects observed with ACE inhibition (Remuzzi, et al., 1993).

Experimental studies have demonstrated that AT_1 receptor blockade effectively reduces systemic blood pressure in rats throughout the course of diabetes (Remuzzi, et al., 1993). This reduction of blood pressure decreased the load being transduced to the kidney and decreased the injury inflicted on the glomerulus. In diabetic animals that were treated with an AT_1 receptor antagonist, glomerular permeability to macromolecules was effectively prevented as reflected by a

significant reduction in proteinuria at various time intervals in comparison to untreated diabetic rats (Remuzzi, et al., 1993). In addition to the positive effect of reduced proteinuria, diabetic rats treated with the AT_1 receptor antagonist losartan had less glomerular and tubulointerstitial lesions than did both untreated diabetics and normal controls (Remuzzi, et al., 1993). Untreated diabetic rats displayed major abnormalities in kidney tissue consisting of focal and segmental glomerulosclerosis with tubular atrophy and interstitial inflammation. Therefore, AT_1 receptor blockade reproduces both the beneficial hemodynamic, and structural effects first observed with ACE inhibition in diabetic models.

The use of ACE inhibitors in the management of established as well as early diabetic renal disease is however considered the first line of therapy. ACE inhibitors decrease the production of Ang II, whereas AT_1 receptor blockers increase Ang II levels (Zou, et al., 1996). The use of AT_1 receptor blockers would increase the availability of Ang II to act on accessible AT_2 receptors. Therefore, in the clinical setting, ACE inhibitors remain the preferred choice in RAS interruption, until further information is available on the effects of diabetes on AT_2 receptors.

3.3 Altered regulation of the intrarenal RAS due to diabetes

The beneficial effects of ACE inhibition suggest that the intrarenal RAS is activated in diabetic nephropathy. However, studies attempting to assess the local changes occurring in RAS components due to hyperglycemia have yielded seemingly contradictory data.

A number of groups have attempted to characterize intrarenal, cell-specific changes in RAS components at various time points in diabetic nephropathy. At an early time point (2-weeks after induction of diabetes) one study found no significant difference in either angiotensinogen or renin

mRNA in the whole kidneys of STZ-diabetic rats (Kalinyak, et al., 1993). In contrast, a long-term (8-weeks after induction of diabetes) study has demonstrated that while plasma renin and ACE activities were not significantly different from control levels, total intrarenal renin concentration and whole kidney angiotensinogen and renin mRNA levels were elevated in diabetes (Anderson, et al., 1993). This study also demonstrated reduced proximal tubule ACE staining, although ACE protein was increased within glomeruli. Additionally, Brown and colleagues demonstrated a three-fold decrease in plasma angiotensinogen 4 weeks after STZ-induced diabetes, which remained decreased at 12 weeks (Brown, et al., 1997). Furthermore, at the 12 week time point an approximate four-fold increase in plasma renin concentration was observed, with no change in plasma renin activity. In contrast, Cassis documented a significant decrease in plasma renin activity at 4 weeks after STZ-induced diabetes (Cassis, 1992). This study also demonstrated a significant decrease in whole kidney renin mRNA. Finally, Kigoshi and colleagues demonstrated reduced plasma renin activity (PRA), renal renin activity, and plasma aldosterone levels in STZ-diabetic rats (Kigoshi, et al., 1986). In summary, some data characterizing the intrarenal RAS appear to be contradictory.

These conflicting data with regards to the state of the intrarenal RAS in diabetes may merely reflect different regulatory phases of the intrarenal RAS during the various stages of diabetic nephropathy. It may also reflect differential regulation of these local RASs within such a heterogeneous organ such as the kidney. For example, ACE inhibition upregulates renin in the juxtaglomerular apparatus but not in proximal tubules (Chen, et al., 1994). It is also possible that local changes in tissue RAS may mediate altered regulation of Ang II receptors.

Ang II is known to regulate the expression of its own receptor. In glomeruli, Ang II downregulates the AT_1 receptor, but in proximal tubules Ang II upregulates this receptor (Cheng,

et al., 1994). Also, in response to sodium depletion in adult rats, the AT_2 receptor is increased in the whole kidney (Ozono, et al., 1997). This implies that differential levels of endogenous Ang II exist within various renal compartments.

A number of studies have demonstrated the apparent downregulation of renal Ang II receptors in diabetes. In a study by Brown and colleagues, a decrease in whole kidney AT_1 receptors was observed at 4 weeks, and suppressed even further at 12 weeks after STZ-induced diabetes (Brown, et al., 1997). Similarly, decreased Ang II binding in the glomeruli of STZ-diabetic rats has been demonstrated by a number of groups (Ballermann, et al., 1984), (Wilkes, 1987). More recently, it has been demonstrated that AT_1 mRNA and ^{125}I Ang II binding is reduced in the proximal tubule of two-week STZ-diabetic rats (Cheng, et al., 1994). These studies suggest an apparent downregulation of glomerular and tubular Ang II AT_1 receptors in STZ-diabetic rats. These seemingly contradictory data may simply be a result of cell specific changes in the RAS. Despite numerous studies providing convincing evidence of altered regulation, there has not been a comprehensive assessment of the state of the intraglomerular RAS in early diabetes.

4 Purpose

4.1 Summary of introduction

There are numerous factors which contribute to the progression of diabetic nephropathy. One of the key mediators of this injury appears to be Ang II. The mediators of diabetic nephropathy, precipitated by the initiating stimulus of hyperglycemia, are illustrated in figure 4.1.

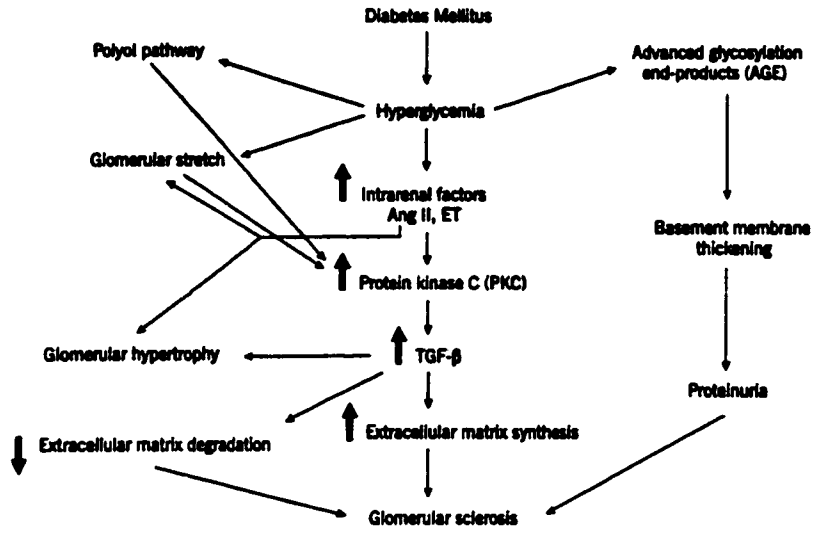

Figure 4.1: Summary of events taking place in the kidney in response to hyperglycemia.

The status of the glomerular RAS in diabetes is unknown. What is known however, is that differential regulation of the RAS occurs in the kidney during diabetic nephropathy. Moreover, the glomerulus is the site where most of the injury occurs. There are currently no data on the state of the glomerular RAS and the status of AT_2 receptors in the kidneys of early diabetes.

4.2 Purpose

4.2.1 Rationale

The glomerulus is the major site of progressive injury in diabetes. Ang II has been implicated as a mediator of this injury. Presently, little is known about the level of activation of the glomerular RAS in early diabetes. Moreover, the status of regulation of Ang II receptors in glomeruli is unknown or conflicting. Given the important role of the intrarenal RAS in regulating

hemodynamics, and growth within the kidney, and the impact which diabetes has on the kidney, especially the glomerulus, it would be important to characterize the intrarenal RAS in the glomerulus during early diabetes.

4.2.2 Objectives

My thesis had three major aims.

1 - To characterize the mRNA of the components of the RAS (renin, ACE , and angiotensinogen) in freshly isolated glomeruli from STZ-induced diabetic rats.

2 - To determine if early diabetes alters the expression of rat whole kidney AT_1 and AT_2 receptors.

3 - To determine the effect of varying salt diet on the glomerular RAS in two-week STZ-induced diabetic rats.

4.2.3 Hypotheses

I hypothesize that,

1 - The mRNA for components of the glomerular RAS (renin, ACE , and angiotensinogen) in two-week STZ-diabetic rats will be upregulated.

2 - The expression of the AT_2 receptor will be down-regulated in the glomerulus of STZ-induced diabetic rats, similar to AT_1 receptors.

3 - Five days of high salt diet will suppress renin mRNA expression, and conversely a zero salt diet will stimulate renin mRNA expression in the glomeruli and cortex of normal rats. Similarly, in the glomeruli of two-week STZ-diabetic rats, a zero salt diet will result in an increase in the mRNA for renin and high salt diet will result in the decrease of renin mRNA.

5 Methods

5.1 Animal model

All studies were approved by the Animal Care Committee of the University of Ottawa. Age-matched male Sprague-Dawley rats weighing 200 to 225 g were used for all animal studies and were housed in the University of Ottawa Animal Care Facility. Prior to any animal studies, rats were permitted to acclimatize for 5 days. Animals were allowed free access to distilled water and standard rat chow, except when placed on restricted salt diets, in which case the standard rat chow was replaced by the restricted salt diet. Data were obtained from control, diabetic, and insulin-implanted diabetic rats at 2 weeks after induction of diabetes. For the diabetic group, only rats with plasma glucose levels greater than 20 mM at the time of sacrifice were used in further analyses. In all groups, after sacrifice, both kidneys were removed and used for analyses.

5.2 Induction of diabetes

Rats were rendered diabetic with *N*-[methylnitrosocarbamoyl]-D-glucosamine (streptozotocin; STZ) (65 mg/kg ip; Sigma, St. Louis, MO) dissolved in 0.1 M sodium citrate buffer (pH 4.0) (Ihm, et al., 1992). All rats used in these studies were rendered diabetic with STZ. Therefore, STZ-diabetic rats will simply be referred to as diabetic rats. The following day, urine was assessed for glucose and ketones with a Keto-Diastix (Bayer Inc., Etobicoke, ON) reagent strip, and only those animals with sustained glucosuria as described (Everett, et al., 1992) were classified as diabetics and were included in further experiments. Diabetic rats were randomly separated into two groups: 1) rats implanted with a sustained release insulin implant, Linplant (Linshin Canada,

Scarborough, ON) to maintain euglycemia and 2) diabetic rats treated daily with 1-2 units of insulin (Humulin L, Eli Lilly and Co., Indianapolis, IN) subcutaneous (s.c.) to maintain hyperglycemia, but prevent ketosis. Urine was monitored daily for ketones and glucose, in order to adjust the daily insulin injections. Each group of rats was paired with a vehicle-injected control animal (injected with an equivalent volume of 0.1 M sodium citrate pH 4.0). Therefore, one group of rats consisted of a vehicle-injected control rat, a diabetic rat, and a insulin-implanted diabetic rat. Rats were maintained for 2 weeks with free access to distilled water and standard rat chow. Moreover, diabetic rats which had lost significant body weight overnight (> 5%) were given 1-2 mls of 0.9 % saline solution intraperitoneal (i.p.).

5.3 High and zero salt diets

In this portion of the study 36 rats were used. Two groups of animals were placed on special diets containing different amounts of salt. One group was placed on a zero-salt (ZS) (no NaCl) diet and the other on a high-salt (HS) (2.9% NaCl) diet. Each group consisted of a control, a diabetic, and an insulin-implanted diabetic animal. After 9 days of diabetes, and 5 days prior to sacrifice, rats were fed either a zero-salt or high-salt synthetic diet. One kg of ZS diet contained 1.74 g K_2CO_3, 1.81 g K_2SO_4, 1.59 g $4MgCO_3{\cdot}Mg(OH)_2{\cdot}5H_2O$, and 989.72 g Teklad electrolyte-free diet (Harlan Teklad, Madison, WI). The high salt diet (2.9%) was identical in composition to that of the ZS diet except that it also contained 1.15 g of Na_2CO_3 and 28.0 g of NaCl per kilogram. Rats were monitored closely for weight gain during the 5 day dietary period and were allowed free access to water. Blood glucose in these rats was monitored by the Chemstrip bG (Boehringer Mannheim Canada, Laval, QC) blood glucose test strip. All diabetic rats had blood glucose levels greater than

27 mM after induction of diabetes. One day prior to sacrifice, rats were placed in metabolic cages for overnight measurements of urine volume, food intake, and water intake.

5.4 Blood collection

Two weeks after induction of diabetes, rats were killed by CO_2 narcosis, followed by decapitation. Blood volumes ranging from 8-12 mls were collected in glass culture tubes and immediately centrifuged at 4000 x g for 20 min in order to collect the plasma. The plasma was divided into two tubes, a sterile green top sodium heparin vacutainer for glucose, BUN, and creatinine analysis and a sterile purple top ethylenediamine tetra-acetic acid (EDTA) vacutainer for renin activity analysis. The plasma was immediately placed on ice and sent to the Ottawa Hospital Biochemistry Laboratory (General Campus) for analysis.

5.5 Isolation of glomeruli and cortical tissue

Glomeruli were isolated by Percoll gradient centrifugation, as previously described (Vinay, et al., 1981) with minor modifications. Renal cortices from both rat kidneys were dissected free from the outer and inner medullary regions of the kidney, and then were gently minced in a glass petri dish on ice. Approximately 0.3 g of cortical tissue was kept and used for RNA isolation. The remaining tissue was then suspended in a solution containing (in mM) 115 NaCl, 24 $NaCO_3$, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 2.0 NaH_2PO_4, 5.0 glucose, 1.0 alanine, 10.0 *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES, pH 7.4), 0.03% collagenase (type IV; Sigma) and 0.01% soybean trypsin inhibitor (Sigma) (Buffer A). The suspension was gassed with 95% O_2 and 5% CO_2 and placed in a 37°C water bath for 45 min. After digestion, the cortical suspension was strained

through a 250 μm brass sieve (Mesh No. 60, Newark Wire Cloth Co., ESBE Scientific, Markham, ON) and centrifuged for 1 min at 100 x g. The pellet was resuspended in Buffer A without collagenase or trypsin inhibitor, and centrifuged for 1 min. This was repeated three times. Next, the pellet was resuspended in a 40 % Percoll (Sigma) solution of identical ionic composition as Buffer A, which had been chilled to 4°C. The Percoll solution was centrifuged at 26,000 x g for 30 min at 4°C, and the digested tissue separated into four distinct bands (F1-F4) after centrifugation, as described (Vinay, et al., 1981). The different densities of the various nephron segments allowed for the isolation of a highly enriched band of glomeruli present in the uppermost band of the Percoll gradient (the F1 layer). This band has been shown to be highly enriched with glomeruli (> 95%) (Badr, et al., 1989). Purification of this band by multiple sieving was performed as described (Atiyeh, et al., 1995) with modifications. Our laboratory has developed a method of washing and sieving which results in an essentially pure preparation of glomeruli. Briefly, the F1 band of the Percoll gradient was removed and washed with PBS (8.5 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, and 145 mM NaCl, pH 7.4) buffer. This tissue was then strained through a 106 μm brass sieve (Mesh No. 150) with PBS buffer so that the glomeruli passed through this sieve and were collected on a 75 μm brass sieve (Mesh No. 200) immediately below it. Tubules and other cellular debris remained on the upper 106 μm sieve (Figure 5.1). The tissue collected consisted of a pure (100 %) collection of glomeruli, as determined by light microscopy. Glomerular cells did not undergo significant cell death in this preparation as determined by their ability (> 95%) to exclude the vital dye Trypan blue (10 mg/dL), demonstrating a high rate of viability (Figure 5.2). However, as a positive control, in the presence of 0.1% sodium dodecyl sulphate (SDS) glomeruli did incorporate the Trypan blue dye.

pho
is
con
glo
by

did
95%
(Pan
(SD

5.6 Isolation of RNA

Total RNA was isolated from kidney cortex and glomeruli using a commercially available kit, RNeasy (Qiagen, Chatsworth, CA). Briefly, a small amount of tissue (~ 0.3 g) was gently minced with a blade and placed in 550 μl of lysis buffer supplied with the kit. The tissue was then homogenized for approximately 1 min at 15 sec pulses with a tissue shredder (Biospec Products, Bartlesville, OK) . This lysate was then passed through a Qiagen shredder column by centrifuging at 16,500 x g for 75 sec. An equal volume of 70% ethanol was then added to the eluant. This mixture was then passed through a Qiagen RNA column by centrifuging at 11,000 x g for 30 sec. The eluant was then discarded and the column was washed 3 times with buffers supplied with the kit, each time discarding the eluant, according to the instructions provided with the kit. In order to elute the RNA from the column, the RNA column was placed in an eppendorf tube and 20 μl of RNase free water, also supplied with the kit, was pipetted directly onto the column. The RNA was eluted into the eppendorf tube by centrifuging the column at 16,500 x g for 75 sec. RNA quality was assessed by running 1 μg of the samples on ethidium bromide-stained 2% agarose-formaldehyde gels. RNA concentration and purity was determined by optical density analysis at 260 and 280 nm. RNA yield varied from 5-25 μg per experiment and all samples were of high quality as assessed by these standards. The RNA isolation was carried out at room temperature, and the RNA was subsequently stored at -80 °C until used.

5.7 Competitive reverse transcriptase-polymerase chain reaction (RT-PCR)

Cortex and glomerular mRNA were assayed by competitive RT-PCR from total RNA, using deletion mutant cRNA from renin, angiotensinogen, and ACE. Prior to RT, RNA samples were treated in order to digest any residual genomic DNA. RNA samples were digested using amplification grade Deoxyribonuclease I (Life Technologies, Burlington, ON) for 15 min at room temperature. To 1 μg of RNA sample, 1 μl of 10X DNase I reaction buffer, 1 μl DNase I (Amp Grade, 1 U/μl) and ribonuclease-free water was added to a total volume of 10 μl. The DNase was inactivated by the addition of 2.5 mM EDTA (final concentration) to the reaction mixture and then heating for 10 min at 65°C. Samples of total RNA were reverse transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ), and the PCR was performed in a Perkin-Elmer Gene Amp 2400 PCR thermocycler. The RT mix contained 5 mM $MgCl_2$ solution, 1X PCR Buffer II, 1 mM dNTPs, 1 U/μl RNase inhibitor, 2.5 U/μl murine leukemia virus reverse transcriptase (MuLV Reverse Transcriptase), 2.5 μM random hexamers and RNase free water to adjust to a total volume of 20 μl. The samples were left at room temperature for 10 min to allow sufficient time for annealing. The samples were then incubated for 15 min at 42°C, after which reverse transcriptase (RT) was inactivated by heating to 99°C for 5 min. Following the RT step, the cDNA mixture was amplified by PCR. Briefly, 2.5 U/100 μl Ampli*Taq* DNA polymerase, 2 mM $MgCl_2$ solution, 1X PCR Buffer II, 65.5 μl of water and 1 μM each of the upstream and downstream oligonucleotide primers for the mRNA of interest were added to each sample, resulting in a total volume of 100 μl. PCR was performed using 62.5 ng of total RNA by denaturing at 94 °C for 30 s, annealing at 63 °C for 30 s, and extending at 72 °C for 45 s for 35 cycles using a hot start method. The cycle number

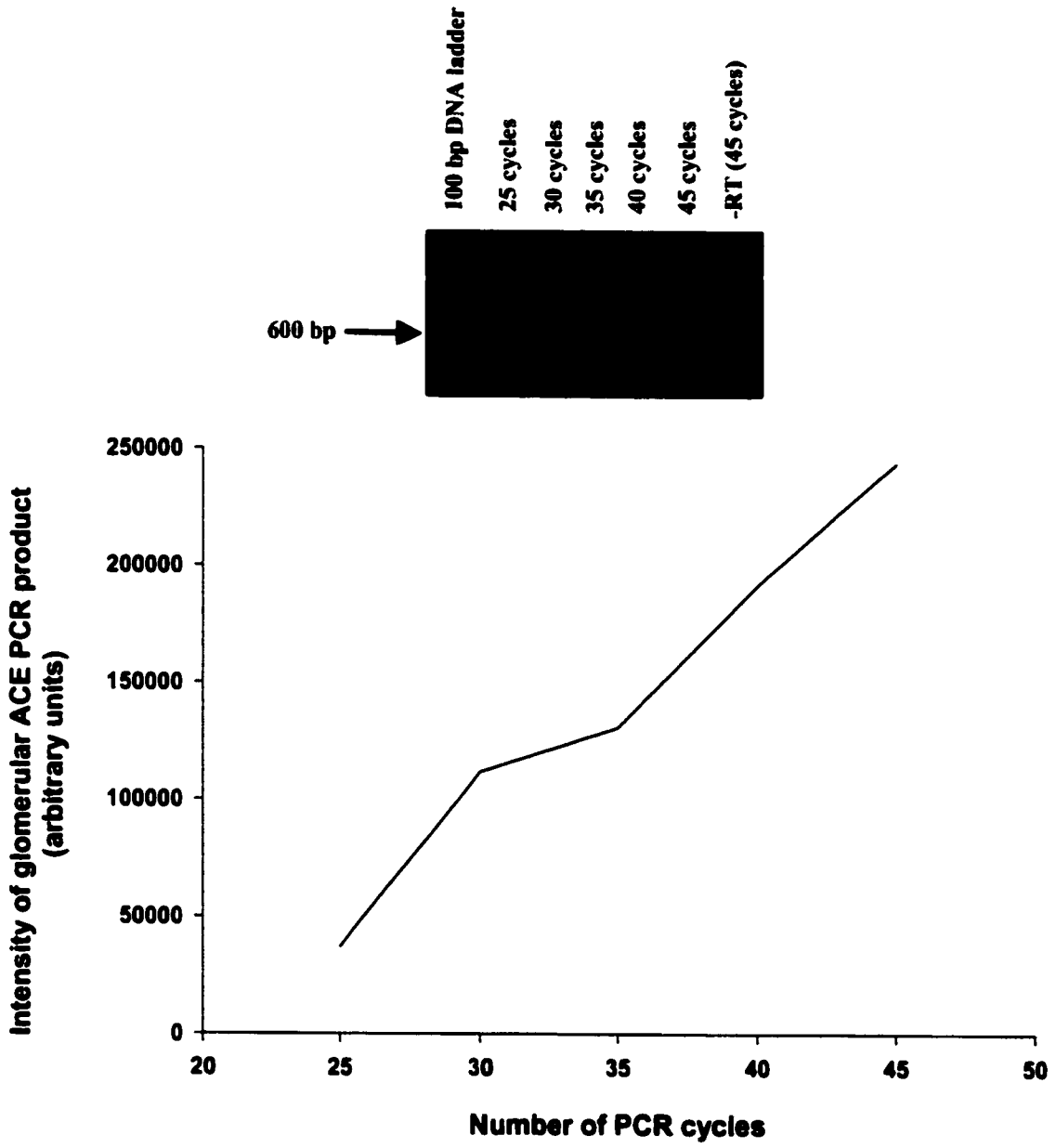

Figure 5.3: Effect of increased PCR cycle number on amplification glomerular ACE mRNA. Efficiency of RT-PCR for ACE mRNA as a function of different PCR cycle numbers. A 442 bp partial cDNA was amplified from total glomerular RNA (62.5 ng) using increasing PCR cycle numbers. Band intensities were quantitated using 3% agarose vistra green gels and a phosphorimager. A representative example is shown in the inset. All samples included reverse transcriptase (RT) unless otherwise indicated with a -RT designation.

and amount of RNA for each target varied according to preliminary PCR experiments (Figure 5.3).

5.7.1 Competitive RT-PCR for renin, angiotensinogen, and ACE mRNA

A competitive reverse-transcriptase polymerase chain reaction method was used to measure absolute levels of renin, angiotensinogen, and ACE mRNA from total glomerular RNA. Each sample of RNA (62.5 ng) was simultaneously reverse transcribed with serial dilutions of deletion mutant cRNA (Figure 5.4). The use of a deletion mutant not only permitted the absolute quantification of mRNA levels but also served as an internal control. These deletion mutants and their cRNA products were previously generated from partial cDNA sequences in our laboratory using inverse PCR, essentially as described (Dostal, et al., 1994). Briefly, a partial cDNA sequence for each mRNA was inserted into the pCR-Script Amp SK(+) (Stratagene, La Jolla, CA) cloning vector. The inserted renin cDNA sequence corresponded to bases 1033 to 1296 of the rat renin mRNA and was amplified using the following primers, upstream (sense) primer 5'-[CTG CCA CCT TGT TGT GTG AG]-3' (bases1033-1052) and downstream (anti-sense) primer 5'-[CCA GTA TGC ACA GGT CAT CG]-3' (bases 1296-1277), resulting in a 264-base product (Dostal, et al., 1994). The inserted angiotensinogen cDNA sequence corresponded to bases 737 to 962 of the rat angiotensinogen mRNA and was amplified using the following primers, upstream (sense) primer 5'-[CCT CGC TCT CTG GAC TTA TC]-3' (bases 737-756) and downstream (anti-sense) primer 5'-[CAG ACA CTG AGG TGC TGT TG]-3' (bases 962-941), resulting in a 226-base product (Dostal, et al., 1994). Finally, the inserted ACE cDNA sequence corresponded to bases 3013 to 3454 of the rat ACE mRNA and was amplified using the following primers, upstream (sense) primer 5'-[GCC ACA TCC AGT ATT TCA TGC AGT]-3' (bases 3013-3036) and downstream (anti-sense) primer 5'-[AAC TGG AAC TGG ATG ATG AAG CTG A]-3' (bases 3454-3430), resulting in a 442-base product

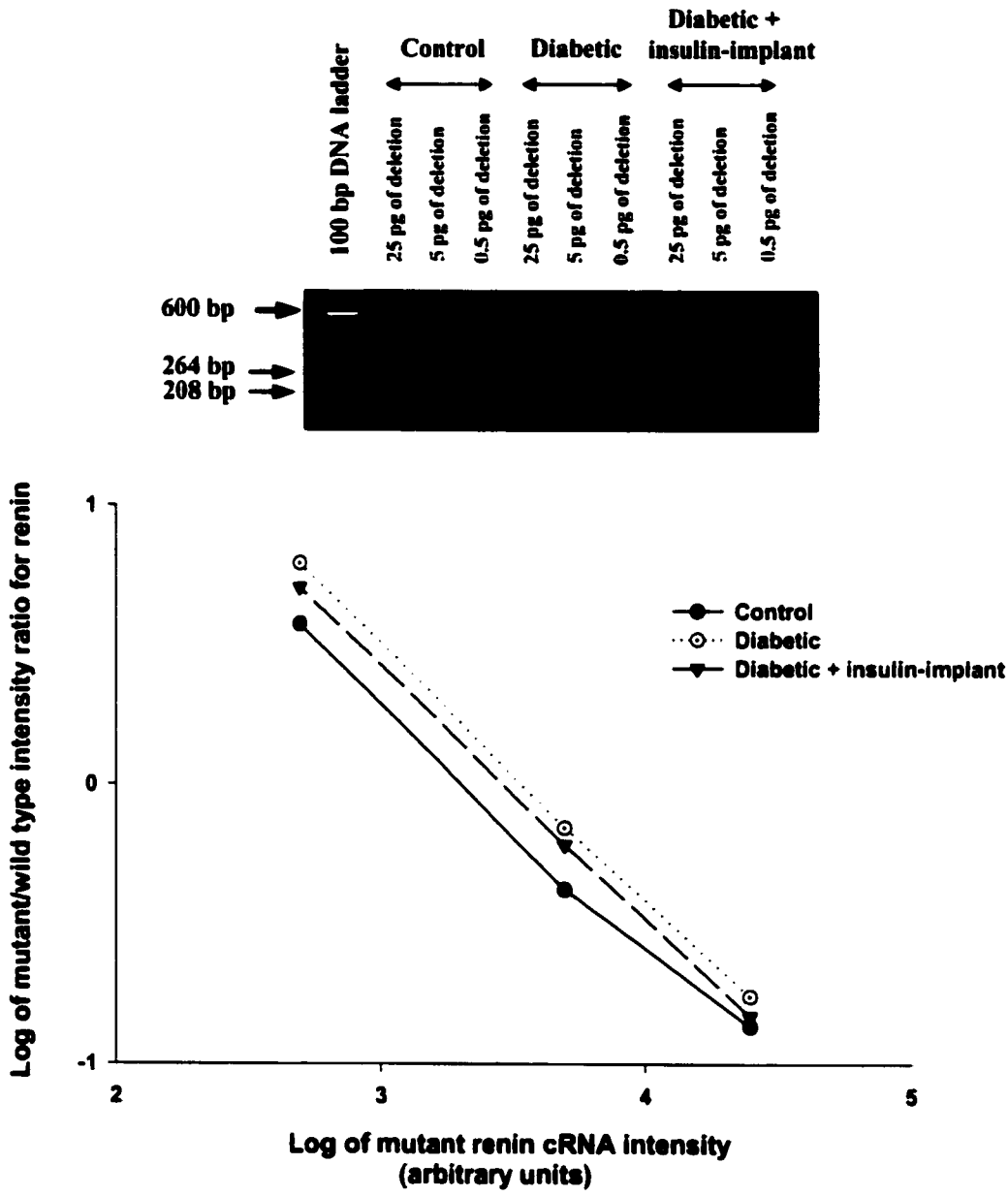

Figure 5.4: Quantitation of glomerular renin mRNA by competitive RT-PCR. Competitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) from a representative experiment in glomeruli after 2 weeks of diabetes. Total glomerular RNA (62.5 ng) was coamplified with serial dilutions of a known concentration of mutant renin template. Amplified cDNA was visualized on a 3% agarose vistra green gel and quantitated with a phosphorimager. The unknown amount of mRNA was determined by interpolating to where the log of the ratio is zero.

(Koike, et al., 1994).

Inverse PCR was performed on the plasmids containing the renin, angiotensinogen, and ACE partial cDNA sequences. For inverse PCR, the sense and antisense primers were inverted in a tail-to-tail direction in order to amplify the cloning vector together with the cDNA of interest, thereby generating gaps between the 5' ends of the PCR products (Figure 5.5).

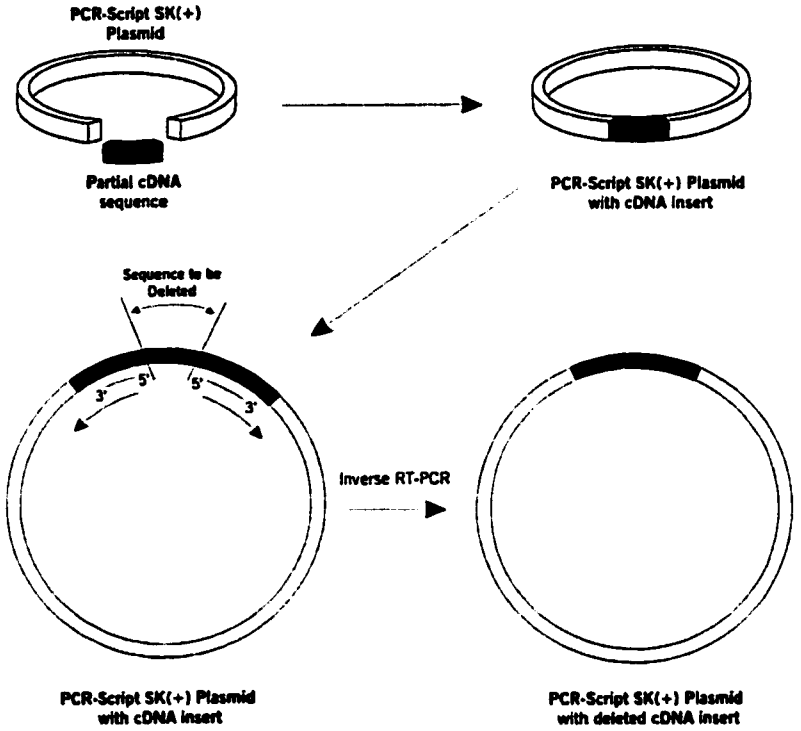

Figure 5.5: Simplified overview of method used to generate the deletion mutants for renin, ACE, and angiotensinogen used in the competitive RT-PCR.

The oligonucleotide primers used in the inverse PCR reaction which generated gaps in the sequence were as follows. The renin sense primer was 5'-[CGA CTG AGC GTT GTG AAC TGT AGC CA]-3', corresponding to bases 1166-1183, and the anti-sense primer was 5'-[CGA CTG AGA

TAT AGG ATG TGC CAG TG]-3', corresponding to bases 1093-1076, resulting in a 208 base mutant PCR product, representing a 56 base deletion (Dostal, et al., 1994). The angiotensinogen sense primer was 5'-[AGC GTC GTT CCA AGG GAA GAT GAG AGG C]-3', corresponding to bases 847-864, and the anti-sense primer was 5'-[CGA CTG AGT GTC ACA GCC TGC ACA AAC C]-3', corresponding to bases 811-791, resulting in a 169 base mutant PCR product, representing a 57 base deletion (Dostal, et al., 1994). The ACE sense primer was 5'-[GTC TCT GCC CTC CAG TGC CTA G]-3', corresponding to bases 3337-3358, and the anti-sense primer was 5'-[GAA AGT TGA TGT CAT GCT C]-3', corresponding to bases 3195-3177, resulting in a 301 base mutant PCR product, representing a 141 base deletion (Koike, et al., 1994). In order to create a large quantity of these mutant plasmids, these vectors were ligated and transformed into Escherchia coli cells (Stratagene). The resulting plasmids generated mutant mRNA templates. The inverse PCR was performed for 35 cycles, consisting of denaturation at 96°C for 1 min, annealing at 63°C for 1 min, and elongation at 72°C for 90 sec. Each deletion mutant was sequenced in order to verify its sequence.

5.7.2 RT-PCR for Angiotensin II type 2 (AT_2) receptor

The absolute levels of AT_2 mRNA were not quantified since a deletion mutant template was not available for the AT_2 receptor mRNA. Relative mRNA levels between groups were compared using conventional RT-PCR with amplification of serial dilutions of RNA. The upstream AT_2 sense primer was 5'-TGA GTC CGC ATT TAA CTG C-3', and the downstream antisense primer was 5'-ACC ACT GAG CAT ATT TCT CAG G3', resulting in a 536-base pair product, representing nucleotides 226-761 of the rat AT_2 cDNA (Kambayashi, et al., 1993). PCR was performed using 1 μg of total RNA by denaturing at 94 °C for 30 s, annealing at 65 °C for 30 s, and extending at 72

°C for 45 s for 40 cycles using a hot start method. Preliminary RT-PCR experiments were done in order to determine if the number of cycles used for the PCR were within the linear range, and a dilution curve was also done in order to verify if the amount of RNA used was sufficient and remained in the linear portion of the dilution curve (Figure 5.6). RT-PCR products were visualized on a UV transilluminator after being run on a 2% agarose gel and stained with ethidium bromide.

In order to quantitate all PCR products, samples were run on a 2% agarose gel stained with Vistra Green nucleic acid gel stain (Amersham) and were visualized using the Molecular Dynamics (Sunnyvale, CA) Storm phosphorimager. The data were analysed using ImageQuant image analysis software. All RT-PCR reactions included negative controls whereby the inclusion of reverse transcriptase in the reaction tube was omitted in order to exclude the possibility of genomic DNA amplification. Moreover, all samples were run with a 100 base pair ladder in order to ascertain the size of the amplification product.

5.8 Immunohistochemistry

Both kidneys were removed, cut longitudinally with a razor blade, and immediately placed in Zamboni's fixative (2% paraformaldehyde, 15% picric acid in PBS, pH 7.4) for 2 hrs. The solution was replaced with fresh fixative and placed at 4 °C overnight. The following day the tissue was washed with 10% sucrose in 0.1 M phosphate buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, pH 7.4). The sucrose phosphate buffer was replaced daily during the following 7 days. One kidney was then paraffin-embedded, and 10 μm sections were cut and stained with a rabbit polyclonal anti-rat AT_2 receptor antibody, kindly obtained from Dr. Robert M. Carey (University of Virgina). Sections were first deparaffinized and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 100%

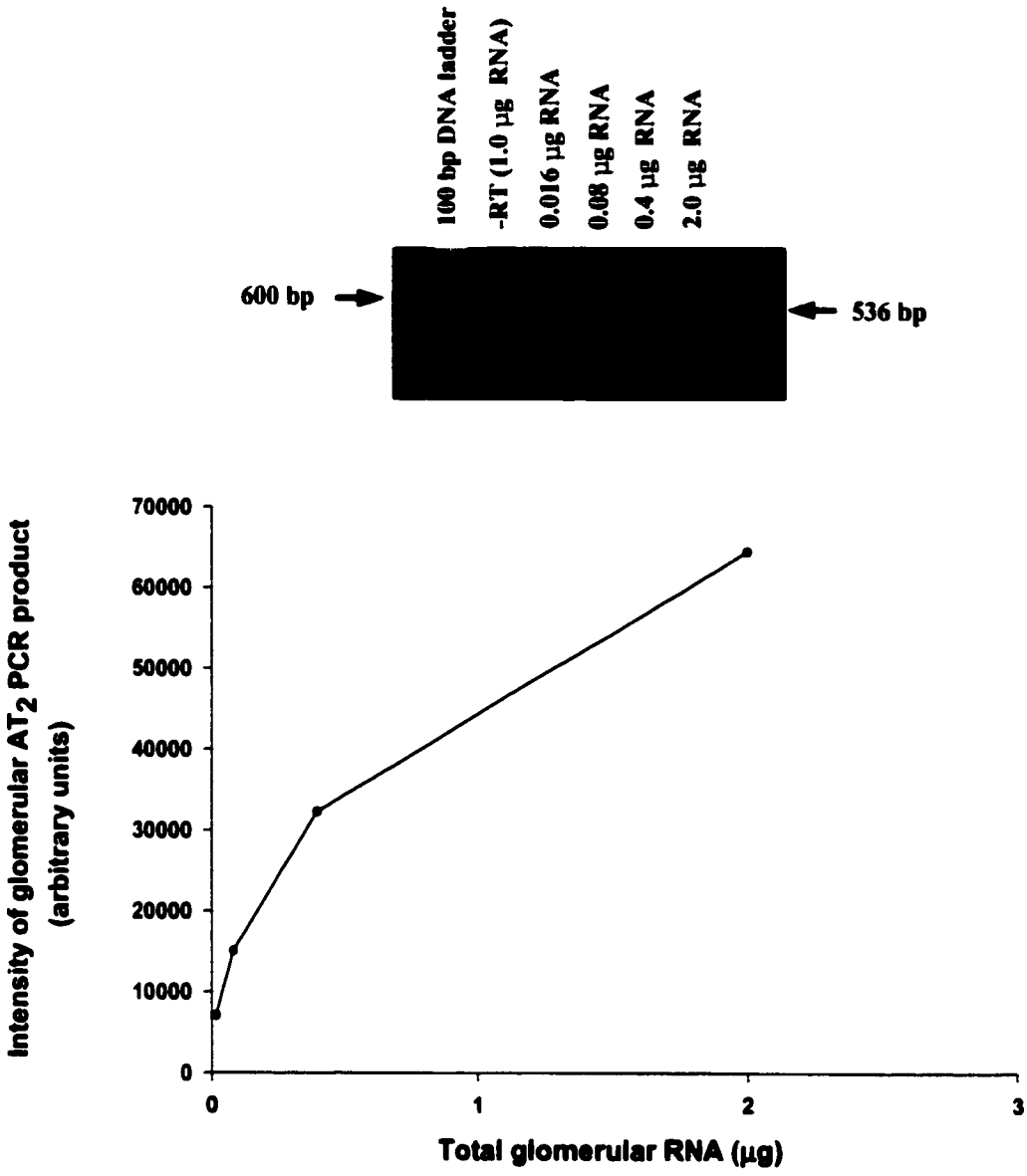

Figure 5.6: Cortex RNA standard curve for AT_2 receptor mRNA amplification by RT-PCR. Standard curve for mRNA analysis of renal cortical AT_2 receptor mRNA. RT-PCR was performed on a five-fold serial dilution of total glomerular RNA. A 536 bp partial AT_2 cDNA was amplified from total cortical RNA at various concentrations of total RNA. Band intensities were quantitated using a 3% agarose vistra green gels and a phosphorimager. A representative example is shown in the inset. All samples included RT unless indicated otherwise with a -RT designation.

MeOH. Sections were incubated in 3% goat serum + 1% milk in PBS to block nonspecific binding sites of secondary goat antibody. The sections were then incubated with primary antibody (1:100) in 1.5% goat serum + 0.5% milk in PBS for 48 hrs at 4°C in a humidified chamber. Next the slides were incubated with the secondary antibody, anti-rabbit IgG, biotinylated (1:50) in PBS for 30 min at room temperature in a humidified chamber. The slides were subsequently placed in 3% H_2O_2 for 10 min prior to incubation with streptavidin-horseradish peroxidase (HRP) diluted 1:50 in PBS for 30 min at room temperature in a humidified chamber. Finally the slides were incubated with 50 μl of diaminobenzadine (DAB) (BioGenex, San Ramon, CA) as substrate. The slides were counterstained with hematoxylin (Sigma), dehydrated and fixed with Permount (Fisher Scientific, Ottawa, ON) histological mounting medium, and viewed with a Zeiss Axiophot microscope.

In order to exclude non-specific binding, the primary antibody was incubated with a 20-fold excess of immunizing peptide for 1 hr at 37°C and then placed on the slide. Slides were qualitatively examined utilizing a 0, 1+, 2+, 3+ grading system. A score of 0 signified the absence of staining, 1+ light staining, 2+ medium staining, and 3+ heavy staining. Each slide was observed in 3 distinct areas of the kidney, which included the cortex, outer medulla, and inner medulla, and viewed in 4 different fields for each area. Observations were made with the viewer blinded to the origin of the slide.

5.9 Western blot analysis of the AT_1 and AT_2 receptor

Proteins from the cortex and isolated glomeruli of control, diabetic, and insulin- implanted diabetic rats were isolated as described (Ozono, et al., 1997). Briefly, cortex and isolated glomeruli were homogenized in a buffer containing 10% glycerol, 20 mM Tris-HCl, 100 mM NaCl, 2 mM

phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 2 mM ethylene glycol-bis(β-Aminoethyl ether) *N, N, N', N'*-tetraacetic acid (EGTA), 10 mM sodium orthovanadate,10 μg/L leupeptin, and 10 μg/L aprotinin. The homogenate was then centrifuged at 30,000 x g for 30 min at 4°C. The resulting pellet was resuspended in the identical buffer described above, but which also contained 1% Nonidet P40 (NP-40) and was then stirred for 2 hrs at 4°C. This lysate was then centrifuged at 30,000 x g for 30 min at 4°C. The supernatant was removed and kept at -80 °C until it was used for further analysis.

Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad, Montreal, QC) using bovine serum albumin (Sigma) as the standard. Solubilized tissue samples (40 μg) along with prestained standards as molecular weight markers (Bio-Rad) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (5% acrylamide stacking gel and 10% running gel). The resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were then blocked overnight at 4 °C with 3% skim milk in Tris-buffered saline (TBS), pH 7.6. For AT_2 receptor immunodetection, membranes were incubated with a rabbit polyclonal anti-rat AT_2 receptor antibody diluted 1:2000 in TBS with 2% skim milk and 0.01% sodium azide for 2 hrs at room temperature. Membranes were then washed with TBS containing 0.01% Tween 20 (TBS-T) and incubated with anti-rabbit secondary antibody conjugated to HRP (Amersham, Oakville, ON) diluted 1:2000. For AT_1 receptor immunodetection, membranes were incubated with a rabbit polyclonal anti-rat AT_1 receptor anti-body (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in TBS-T with 2% skim milk for 2 hrs at room temperature. Subsequent to washing with TBS-T, membranes were incubated with anti-rabbit secondary antibody conjugated to HRP (Amersham) diluted 1:2000. Proteins were detected by enhanced chemiluminescence (ECL,

Amersham) on Hyperfilm (Amersham) according to the instructions provided with the kit.

In order to ensure equal protein loading, all membranes were stripped and probed with a monoclonal anti-β-actin antibody (mouse ascites fluid; Sigma) which recognizes the β-actin protein at approximately 45 kD. Signals on Western Blots were quantified by densitometry and corrected for the β-actin signal using the Kodak Digital Science Image Station 440CF and the 1D Image Analysis program.

5.10 Statistical analysis

Results are expressed as mean ± SE. Data were analysed by either an unpaired Student's *t*-test, by one-way ANOVA followed by Bonferonni correction for all pairwise comparisons or as in the case of the Western blots by the Mann-Whitney rank sum test for non-parametric data. A value of $p < 0.05$ was deemed significant.

5.11 Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Materials for blood collection, immunohistochemistry, and PCR were purchased from VWR Canlab (Mississauga, ON), and all centrifuge tubes were purchased from Sarstedt (St-Leonard, QC). All primers for PCR were obtained from Oligos Etc. (Wilsonville, OR). RNA was quantified with a Spectronics Genesys 5 spectrophotometer from ESBE Scientific (Markham, ON). Gels were resolved on Bio-Rad's (Montreal, QC) Wide Mini-Sub Cell GT electrophoresis apparatus and visualized with a variable intensity transilluminator from Fisher Scientific (Ottawa, ON).

6 Results

6.1 Whole animal data

Table 6.1 illustrates whole animal data for the three groups of rats: control (C), 2-week diabetic (D), and 2-week diabetic + insulin-implant (D+I). Control animals displayed normoglycemia, whereas diabetic rats were severely hyperglycemic and demonstrated glucosuria [blood glucose, C: 9.6±0.2 mM vs D: 37.8±1.6 mM; $p<0.001$ vs C; n= 12], similar to data reported by Ballermann and colleagues (Ballermann, et al., 1984). In the insulin-implanted diabetic rats, blood glucose values returned to normal, and were in fact significantly less than control values [D+I: 4.9±0.4 mM; $p<0.05$ vs C; n=12]. There were significant decreases in body weight and increases in kidney weight in the diabetic group. Moreover, the kidney weight to body weight ratio was significantly increased in the diabetic group. Insulin treatment completely reversed the decreases in body weight, as well the increases in kidney weight, and returned the kidney weight to body weight ratio to control values. Blood urea nitrogen (BUN) was significantly increased in the diabetic group. Serum creatinine levels, however did not change in any of the groups. In the diabetic group, plasma renin activity (PRA) was slightly suppressed, although this was not significantly different from values in control rats (figure 6.1).

Table 6.1: Summary of physiological data from 2-week control, diabetic, and insulin-implanted diabetic rats.

	Control (C)	Diabetic (D)	Diabetic + insulin-implant (D+I)
Blood glucose (mM)	9.6±0.2	37.8±1.6[a]	4.9±0.4[c]
Body weight (g)	359.3±4.7	267.9±5.6[a]	350.6±7.6
Kidney weight (g)	1.31±0.03	1.49±0.05[b]	1.25±0.02
Kidney weight as % of total body weight	0.37±0.01	0.56±0.02[a]	0.36±0.01
BUN (mM)	5.7±0.4	8.8±0.6[b]	6.4±0.3
Serum creatinine (μM)	52.5±1.1	53.1±1.4	51.7±1.5

Values represent means ± SE from 12 experiments where one experiment represents one group of C, D, and D+I rats. [a]$p<0.001$ vs C and D+I; [b]$p<0.005$ vs C; and D+I; [c]$p<0.05$ vs C

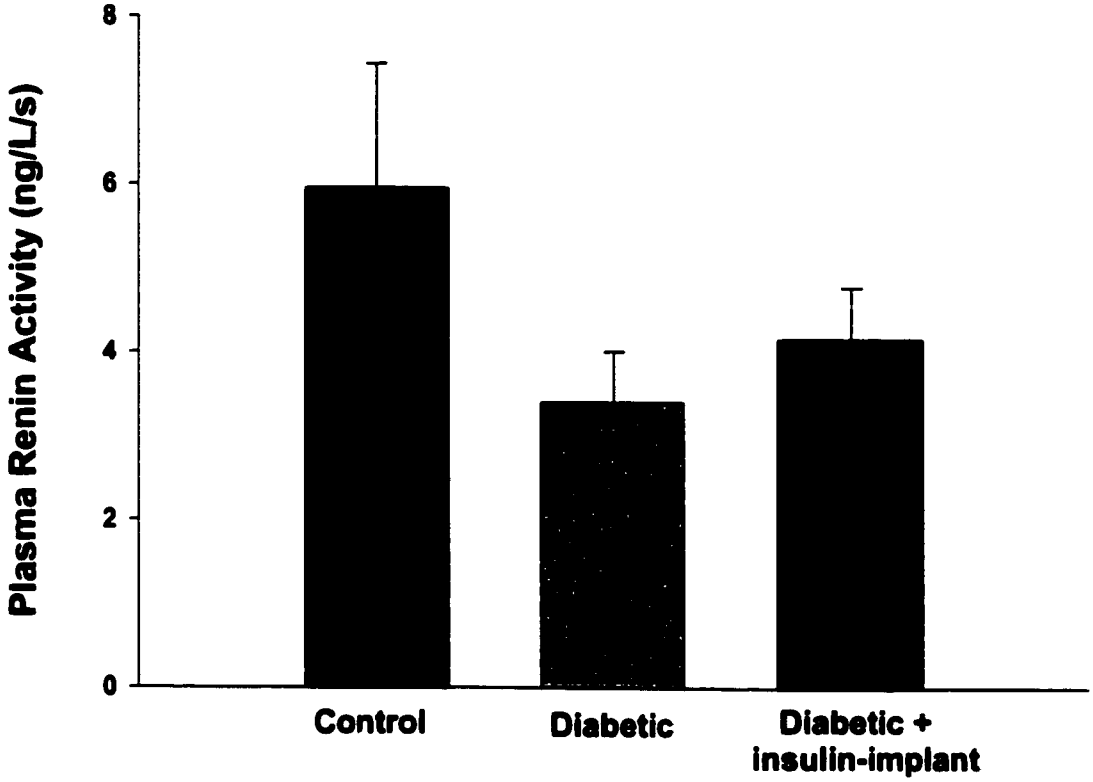

Figure 6.1: Effect of 2-week diabetes on peripheral renin activity (PRA). Plasma renin activity from control, diabetic, and insulin-implanted diabetic rats. Plasma was obtained from rats in each group after 2 weeks of study. Data represent means ± SE from 12 rats for each group.

6.2 Competitive RT-PCR assay

In order to establish that the competitive RT-PCR assay could detect predicted changes in mRNA expression, normal rats were placed on diets consisting of either 2.9% NaCl (high salt or HS) or 0% NaCl (zero salt or ZS), since sodium depletion is known to stimulate glomerular renin mRNA expression (Tank, et al., 1997). Competitive RT-PCR for renin mRNA was performed on total RNA isolated from cortex and glomeruli. In both cortex and glomeruli, a zero salt diet stimulated an approximate 3-fold increase in renin mRNA [cortex: 925.2±54.7 fg mRNA/62.5 ng total RNA for HS vs 2773.7±348.8 fg mRNA/62.5 ng total RNA for ZS; $n=6$; $p<0.001$ and glomeruli: 2241.0±582.2 fg mRNA/62.5 ng total RNA for HS vs 6223.8±1320.5 fg mRNA/62.5 ng total RNA for ZS; $n=5$; $p<0.025$] (figure 6.2). Moreover, in subsequent experiments whereby competitive RT-PCR for renin mRNA was repeated several times on the same RNA sample, it was determined that this assay was sensitive enough to detect differences greater than 15% in mRNA levels between samples (data not shown).

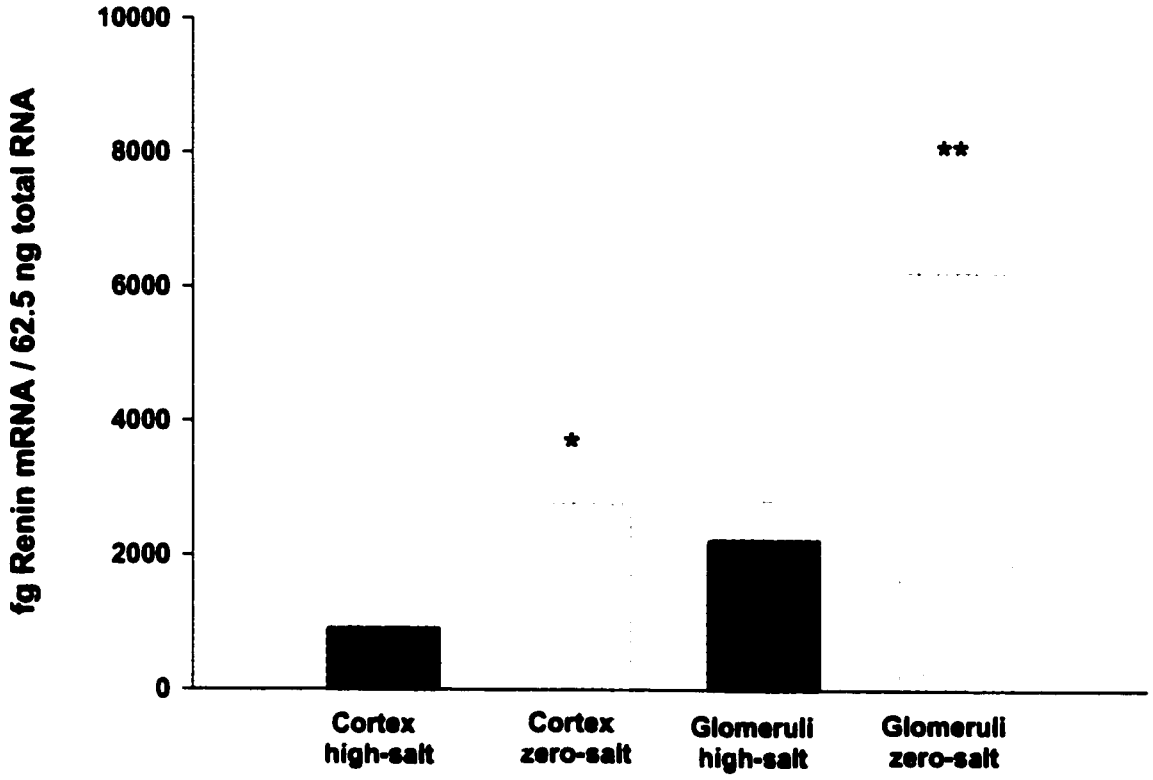

Figure 6.2: Determination of validity of competitive quantitative RT-PCR assay. Competitive RT-PCR for renin mRNA from cortex and glomeruli of rats on a 5-day diet of either high or zero salt rat chow. Values are means ± SE from 6 experiments for cortex and 5 experiments for glomeruli. *$p<0.001$ vs cortex high salt, and **$p<0.025$ vs glomeruli high salt. Statistical significance was assessed by one-way ANOVA followed by Bonferroni t-test.

6.3 Effect of early diabetes on components of the RAS in the glomerulus

Preliminary experiments attempting to identify the proteins for all RAS components (renin, ACE, and angiotensinogen) in the kidney were performed by immunohistochemistry. Within the literature, studies attempting to identify the proteins for RAS components in glomeruli have been unsuccessful (Anderson, et al., 1993). This may have been primarily due to the absence of satisfactory antibodies for these proteins. Renin immunostaining has been localized to the juxtaglomerular cells of the afferent arteriole but not within glomeruli (Barajas, 1997). Similarly, we observed renin immunostaining in the afferent arterioles, but in only one experiment out of six, and not within glomeruli. Moreover, ACE protein has been localized to endothelial cells and glomeruli (Anderson, et al., 1993). However, in our experiments ACE immunostaining, and Western blotting for ACE using a commercially available antibody was not successful. Finally, in experiments performed in our laboratory, angiotensinogen immunostaining was localized to proximal tubule cells, but not within glomeruli.

Therefore, we attempted to quantitate the mRNA for components of the glomerular RAS in response to early diabetes by competitive RT-PCR. Preliminary experiments were performed to determine the optimal conditions for competitive RT-PCR (the standard curve for renin PCR cycle numbers is shown in figure 5.3). These experiments included standard curves for each component of the RAS for quantity of RNA used, cycle number for PCR, and range of concentrations of deletion mutants used so that all parameters were within the linear portion of the standard curves. Moreover, the annealing temperature in the PCR step was optimized so that a single band of the expected size was produced. In all cases 62.5 ng of total RNA was used and the RT-PCR conditions were the same for all components of the RAS (as outlined in the methods).

As shown in figure 6.3, our data suggested that there were increased levels of renin mRNA in the glomeruli of diabetic rats compared to control, however this did not reach statistical significance by one-way ANOVA [control: 2497.5±405.0 fg mRNA/62.5 ng RNA vs diabetic: 3155.5±417.3 fg mRNA/62.5 ng RNA (p=NS vs control) vs insulin-implanted diabetic: 2490.0±645.8 fg mRNA/62.5 ng RNA; n=6]. Moreover, there was no significant difference in glomerular renin mRNA between control and insulin-implanted diabetic rats.

With regard to glomerular angiotensinogen mRNA levels, there was no significant difference between control, diabetic, and insulin-implanted diabetic rats (figure 6.4). Finally, there was no significant difference in glomerular ACE mRNA levels between control, diabetic, and insulin-implanted diabetic rats (figure 6.5). Therefore, these data suggest that the mRNA for glomerular components of the RAS are not altered in early diabetes.

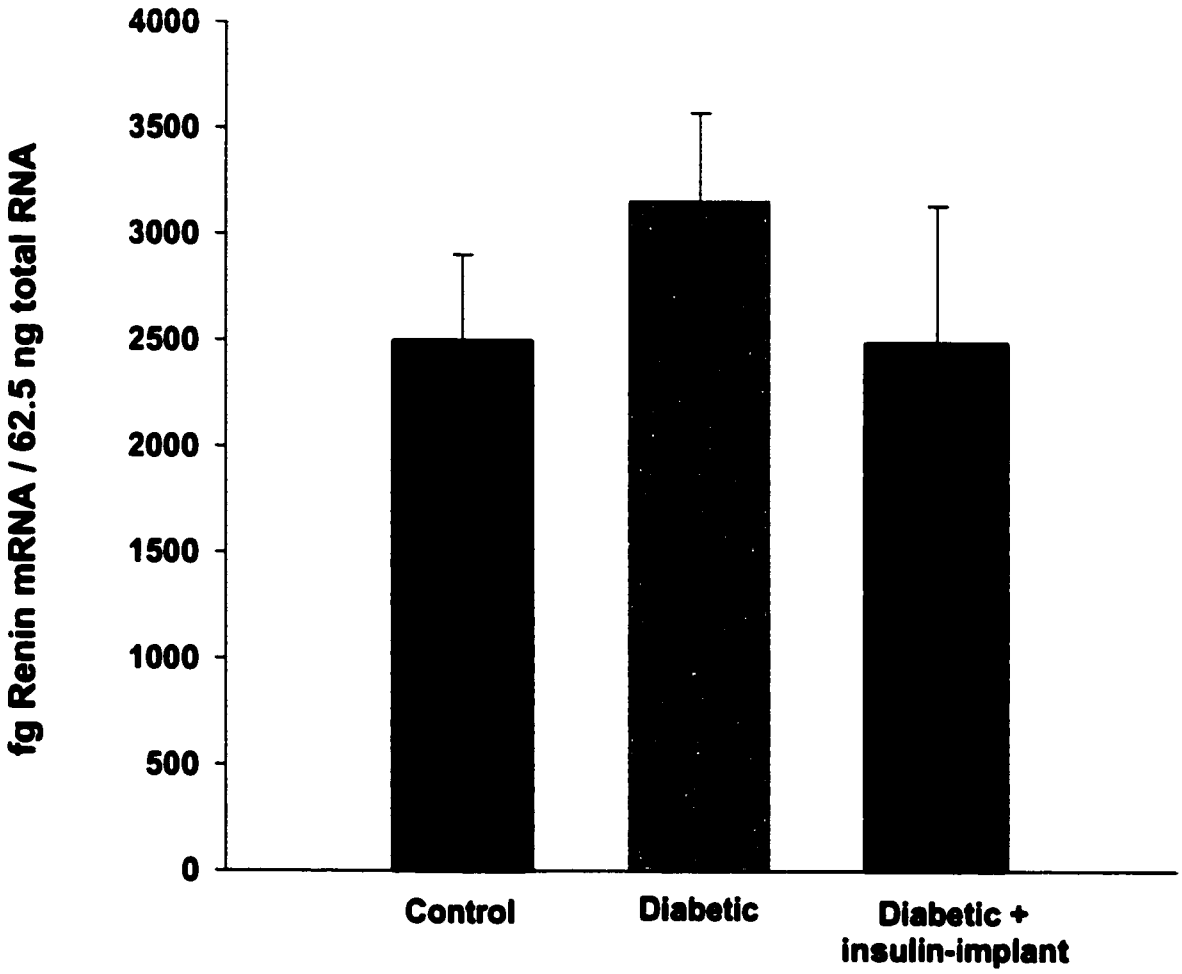

Figure 6.3: Effect of 2-week diabetes on glomerular renin mRNA. Competitive RT-PCR for renin mRNA from the glomeruli of 2-week control, diabetic, and insulin-implanted diabetic rats. Values are means ± SE from 6 experiments.

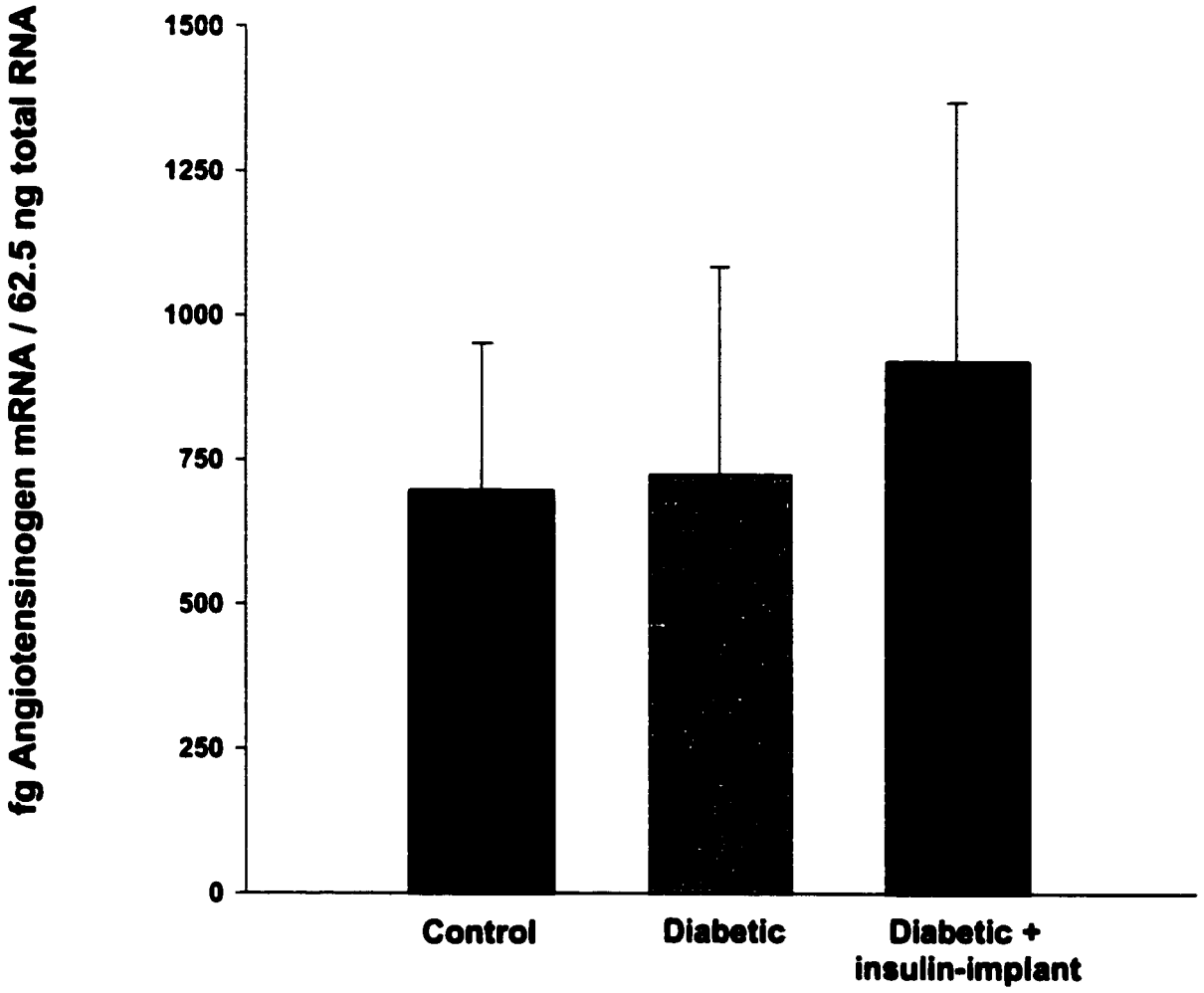

Figure 6.4: Effect of 2-week diabetes on glomerular angiotensinogen mRNA. Competitive RT-PCR for angiotensinogen mRNA from the glomeruli of 2-week control, diabetic, and insulin-implanted diabetic rats. Values are means ± SE from 6 experiments.

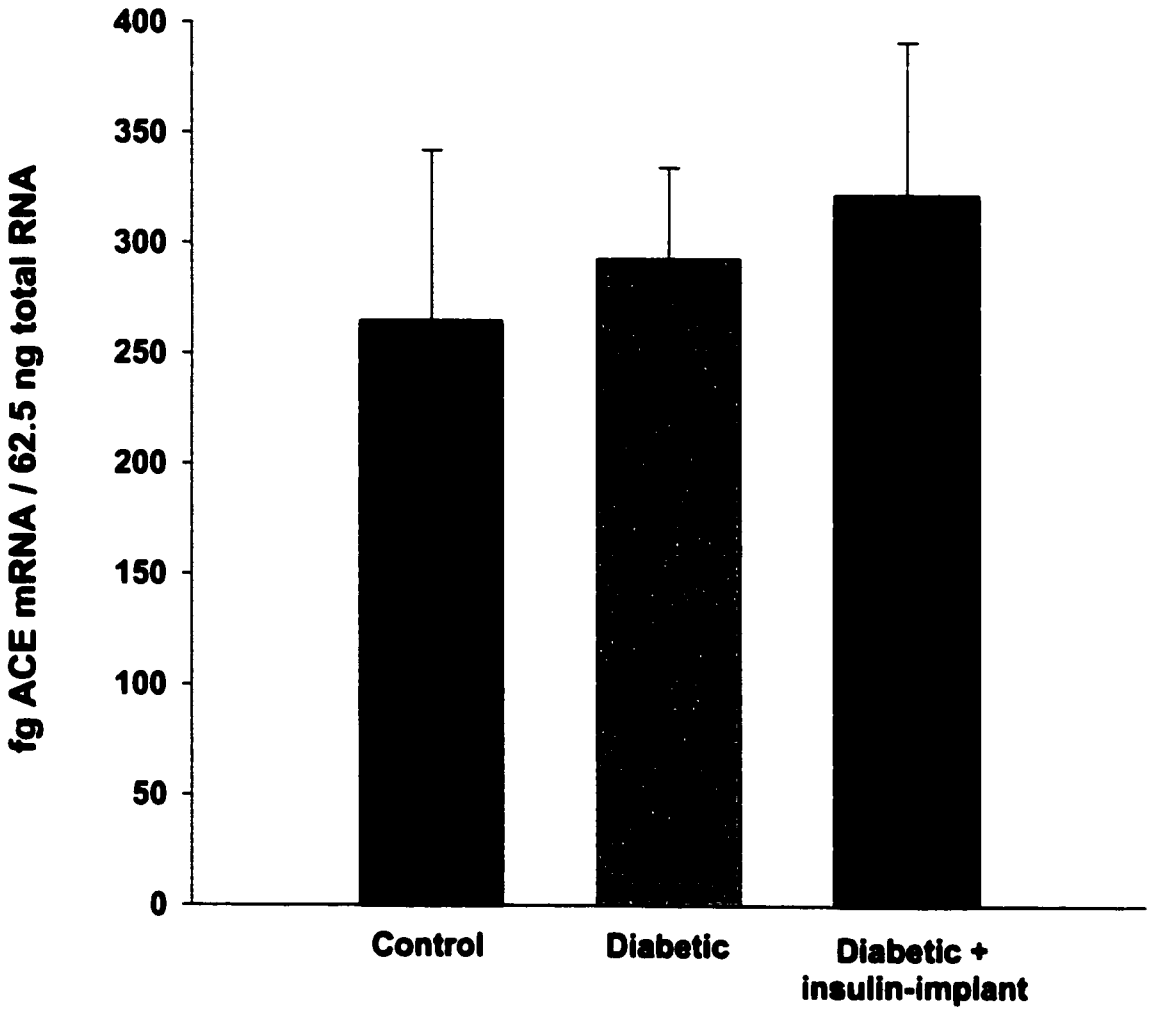

Figure 6.5: Effect of 2-week diabetes on glomerular ACE mRNA. Competitive RT-PCR for ACE mRNA from the glomeruli of 2-week control, diabetic, and insulin-implanted diabetic rats. Values are means ± SE from 6 experiments.

6.4 Effect of varying salt diet on the components of the RAS

The effect of either a high salt (HS) or zero salt (ZS) diet on components of the RAS in 2-week control, diabetic, and insulin-implanted diabetic rats was investigated. In these studies, a group of control, diabetic, and insulin-implanted diabetic rats was placed on a zero salt diet and another group of control, diabetic and insulin-implanted diabetic rats was placed on a high salt diet 5 days before sacrifice (9 days after the onset of diabetes). The day before sacrifice, rats were placed in metabolic cages and overnight physiological parameters were measured as shown in table 6.2. All rats on the zero salt diet did not show any traces of Na in their urine, whereas all rats on the high salt diet demonstrated significant urinary Na excretion. Moreover, diabetic rats on the high salt diet showed a significantly higher Na excretion rate and urinary volume than the control and insulin-implanted diabetic rats on the same high salt diet [C: 4.71±.088 mmol Na^+ vs D: 12.17±1.13 mmol Na^+ ($p<0.001$ vs C and D+I) vs D+I: 6.85±0.87 mmol Na^+ (p=NS vs C); n=6]. Diabetic rats on both the high and zero salt diets demonstrated significant body weight decreases in comparison to control and insulin-implanted diabetic rats on either diet [C: 344.5±8.8 g (ZS) and 357.3±12.4 g (HS) vs D: 265.0±8.3 g (ZS) and 268.7±13.1 g (HS); $p<0.001$vs C rats on ZS and HS diet; n=5;] (Table 3.2).

Plasma renin activity (PRA) was increased with the institution of a zero salt diet in control, diabetic, and insulin-implanted diabetic rats, but this only reached statistical significance in the diabetic and insulin-implanted diabetic rats [C: (HS) 5.4±0.9 ng/L/s vs (ZS) 10.9±0.8 ng/L/s; p=NS vs HS and D: (HS) 2.1±0.1 ng/L/s vs (ZS) 13.7±2.3 ng/L/s; $p<0.001$ vs HS] (Figure 6.6).

Competitive RT-PCR data for renin mRNA in this study were not obtained. The absence of consistent positive control data required that these data be discarded. Speculation about the reasons for inconsistent data will be made in the discussion.

Table 6.2: Summary of physiological data from control (C), diabetic (D), and diabetic + insulin-implant (D+I) rats placed on either a zero salt (0% NaCl) or high salt (2.9% NaCl) diet 5 days before sacrifice.

	Control (C)		Diabetic (D)		Diabetic + insulin-implant (D+I)	
	Zero salt	High salt	Zero salt	High salt	Zero salt	High salt
Body weight (BW) (g)	344.5±8.8	357.3±12.4	265.0±8.3[a]	268.7±13.1[a]	328.0±7.3	341.5±9.1
Kidney weight (KW) (g)	1.25±0.08	1.33±0.08	1.54±0.03[a]	1.68±0.13[a]	1.27±0.08	1.35±0.03
KW as % BW	0.36±0.01	0.39±0.02	0.60±0.03[a]	0.64±0.02[a]	0.40±0.02	0.40±0.02
O/N urine Na excretion (mmol)	0 (undetectable)	4.71±0.88	0 (undetectable)	12.17±1.13[b]	0 (undetectable)	6.85±0.87
O/N urine output (ml)	12.2±2.0	11.2±2.4	170.4±26.1[a]	266.2±48.7[a]	11.3±2.2	32.8±9.1
O/N water intake (ml)	32.7±2.6	33.5±3.5	190.6±28.5[a]	288.8±49.6[a]	28.0±5.6	59.0±7.2
O/N food intake (g)	18.2±0.5	30.0±6.3	28.2±3.4	29.0±3.8	14.8±1.3	21.7±1.9

Values represent means ± SE from 6 experiments. [a]P<0.001 vs C and D+I (both high and zero salt groups), [b]P<0.001 vs C and D+I high salt group. Abbreviation: O/N, overnight.

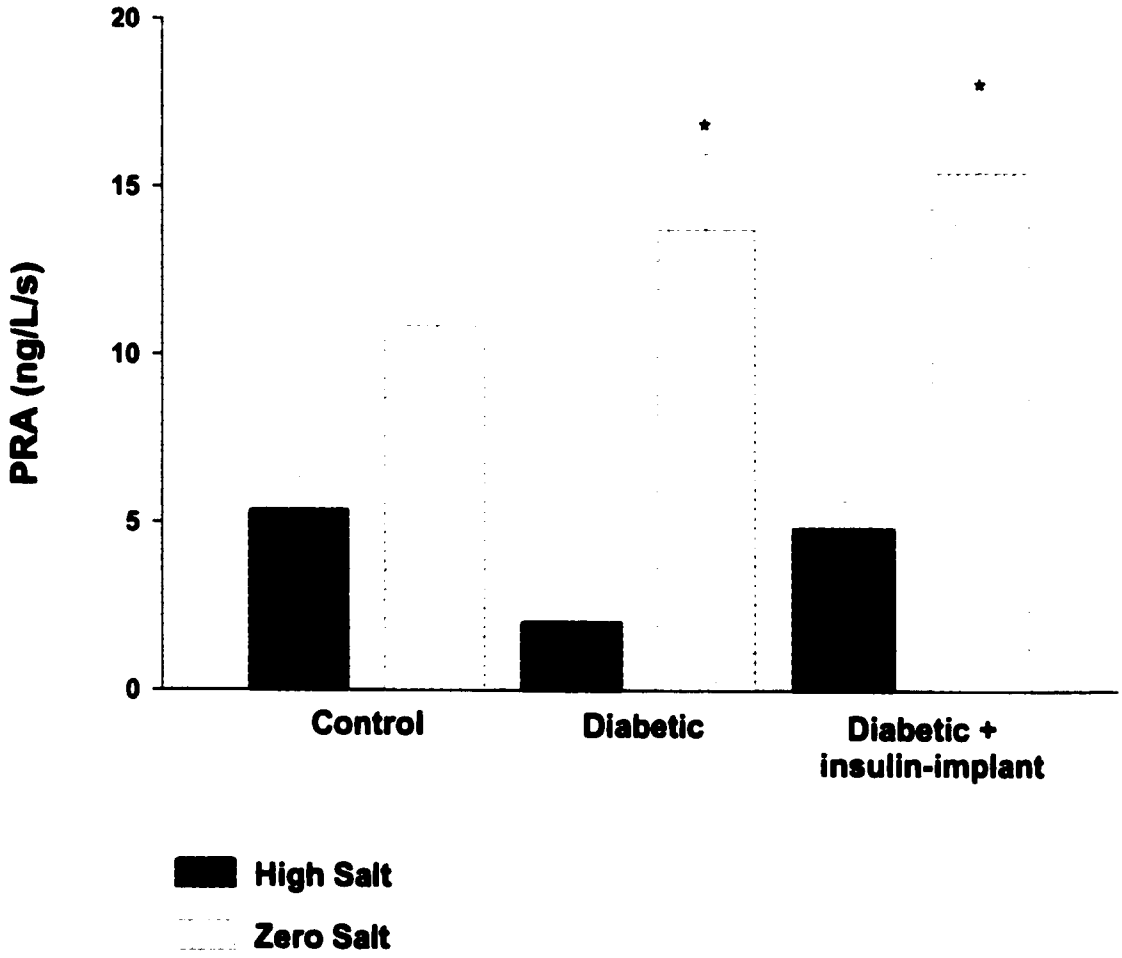

Figure 6.6: Effect of either high or zero salt diet on 2-week control, diabetic, and insulin-implanted diabetic rat plasma renin activity (PRA). Plasma renin activity (PRA) from 2-week control, diabetic, and insulin-implanted diabetic rats on either a high or zero salt diet 5 days prior to sacrifice. Values are means ± SE from 6 experiments. *$p<0.001$ vs high salt diet within the group. Statistical significance was assessed by one-way repeated measure (RM) ANOVA followed by Bonferroni t-test for all pairwise multiple comparisons.

6.5 Western blot analysis of the glomerular AT_1 receptor

A number of studies have documented a decrease in glomerular Ang II receptors, as early as 1 day and persisting up to two months of diabetes (Kalinyak, et al., 1993), (Wilkes, 1987), and (Ballermann, et al., 1984). These groups did not identify which receptor subtype was decreased, but merely described a decrease in Ang II receptors in response to diabetes at various stages of this disease. In order to determine if this decrease was due to the AT_1 receptor, Western blot analysis was performed on glomerular cell lysates from control, diabetic, and insulin-implanted diabetic rats for the AT_1 receptor. A single band of the predicted molecular mass (41 kD) (Harrison-Bernard, et al., 1997) was observed on all Western blots (figure 6.7 inset). In the 2-week diabetic rat group, AT_1 receptor protein expression was significantly increased in glomeruli [D: 354.4±127.4% of C ($p<0.005$ vs C); n=7], with only a partial reversal to control values in the insulin-implanted diabetic rat group [D+I: 212.3±41.1% of C ($p<0.05$ vs C); n=7] (figure 6.7). These data demonstrate that the protein for the AT_1 receptor is not decreased in glomeruli after two weeks of diabetes and perhaps the AT_1 receptor may not be the subtype responsible for the documented decrease in glomerular Ang II receptors in diabetes.

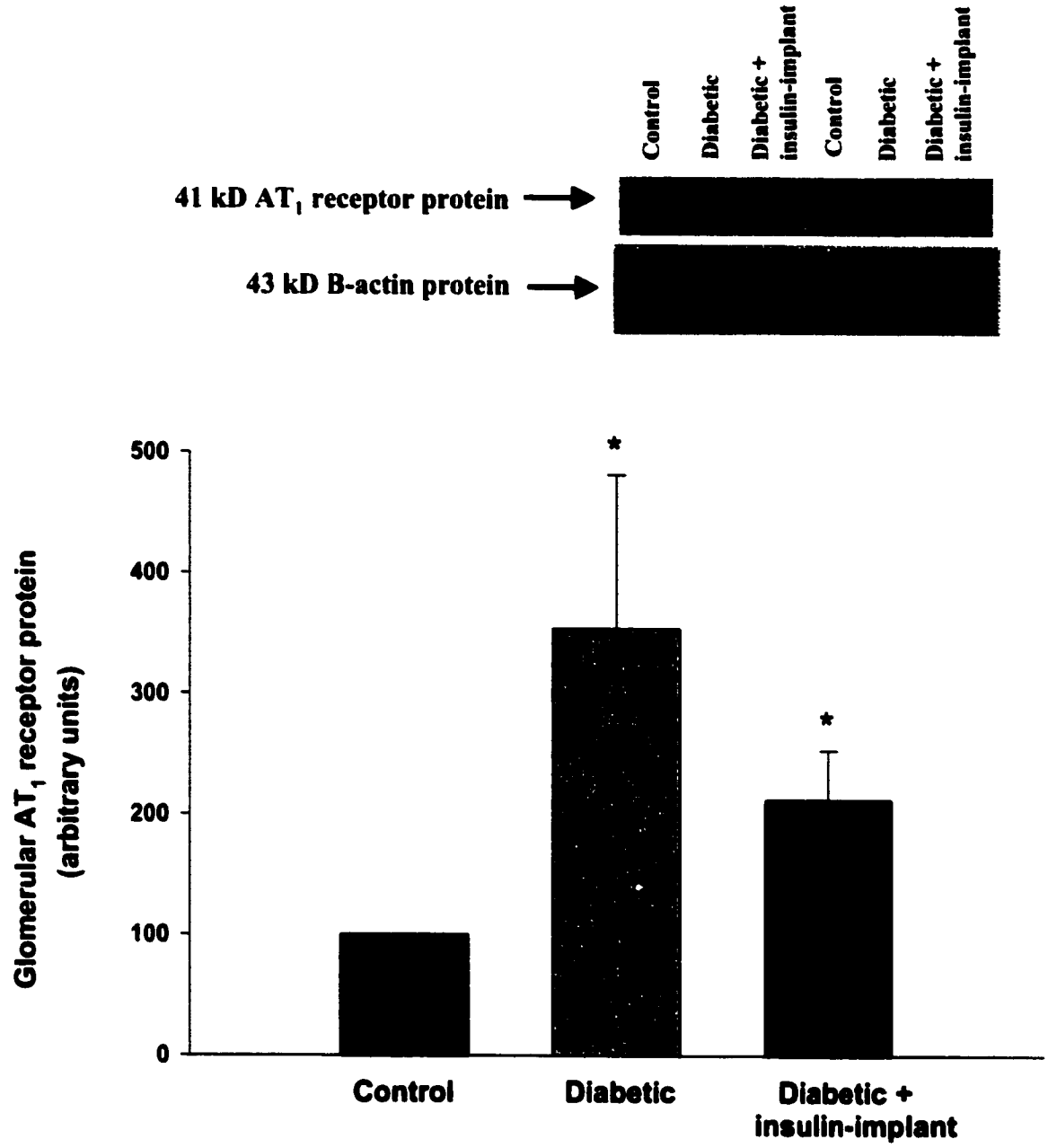

Figure 6.7: Effect of 2-week diabetes on glomerular AT_1 receptor protein expression by Western blot. Western blot analysis of glomerular protein (30 μg) for AT_1 receptor expression in 2-week control, diabetic, and insulin-implanted diabetic rats. AT_1 receptor protein was detected as a single band of ~41 kD. All bands were normalized to control and were corrected for β-actin. A representative Western blot of 2 sample experiments is shown in the inset. Data represent the means ± SE from 7 experiments. *$p<0.05$ vs control. Statistical significance was assessed by the Mann-Whitney rank sum test for nonparametric data.

6.6 Effect of diabetes on AT_2 receptor mRNA and protein

6.6.1 Effect of diabetes on mRNA for the AT_2 receptor in the renal cortex

There is presently no information on the state of the intrarenal AT_2 receptor in diabetes. This is the first study to examine the status of the AT_2 receptor in the kidneys of adult rats in early diabetes. To determine the effect of diabetes on renal AT_2 receptor gene expression, RT-PCR was performed for AT_2 receptor mRNA in the cortex and glomeruli of 2-week diabetic rats. Preliminary experiments to determine the conditions for RT-PCR for AT_2 receptor gene expression in the cortex and glomeruli were performed, including a standard curve to determine the quantity of total RNA to be used, the number of cycles for PCR, and the temperature conditions for the PCR. All values chosen were within the linear portion of the standard curves and are as outlined in the methods section.

In the cortex, a 536 bp nucleotide cDNA was detected corresponding to the expected size of the amplification product of the partial AT_2 receptor cDNA sequence. After 2 weeks of diabetes, cortical AT_2 receptor mRNA was significantly increased, with a complete reversal in the insulin-implanted diabetic group [C: 58,430±6,004 vs D: 83,675±3,575 ($p<0.005$ vs C and D+I) vs D+I: 56,326±3,011 (p=NS vs C) arbitrary units; n=6-7] (figure 6.8). Analysis of β-actin by RT-PCR revealed no significant difference in RNA loading between all groups.

In glomeruli, a 536 bp nucleotide product was also detected, but in only 3-4 out of 7 RNA samples for each control, diabetic, and insulin-implanted diabetic group. This did not permit quantitative analysis of glomerular AT_2 receptor mRNA.

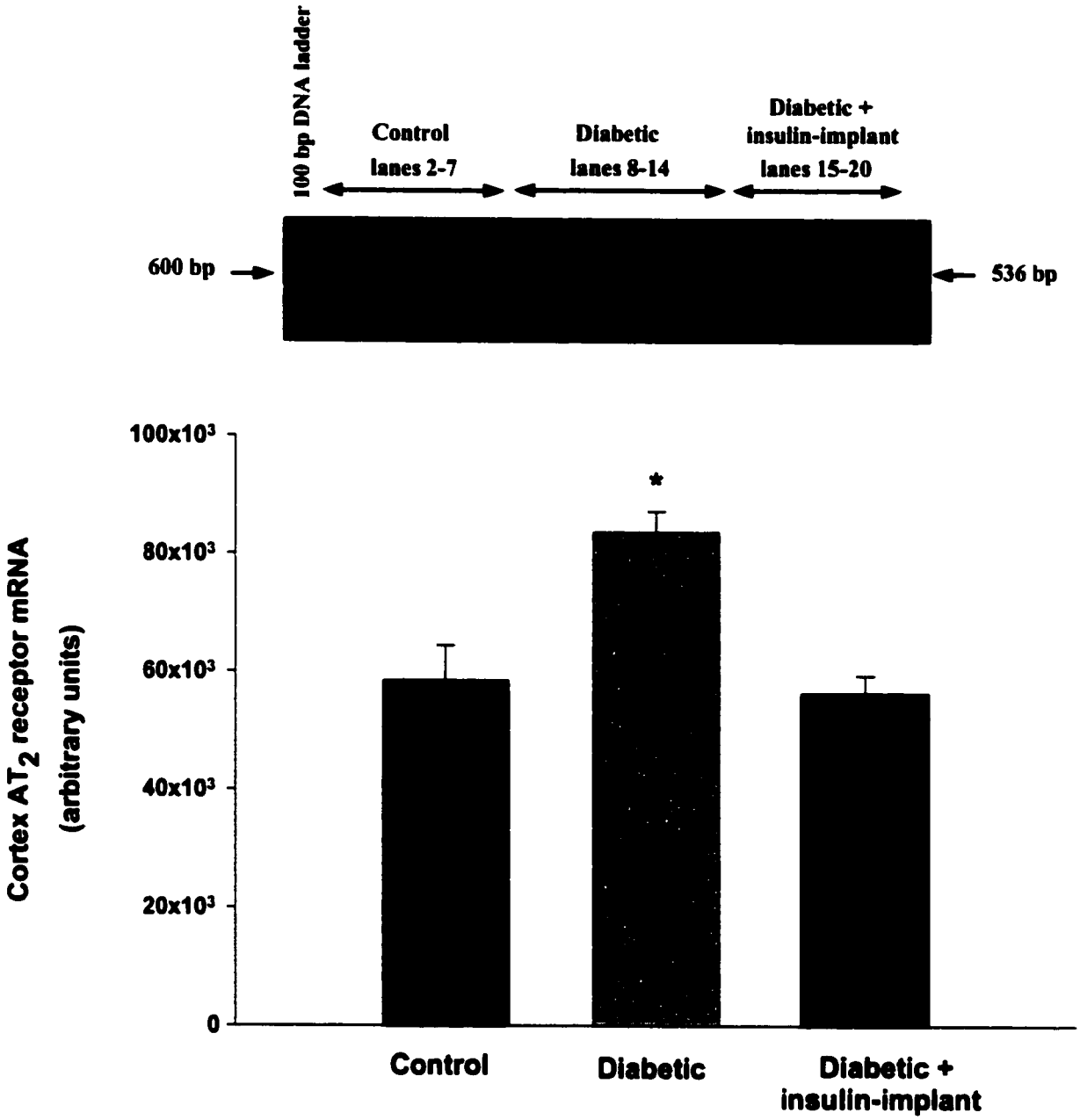

Figure 6.8: Effect of 2-week diabetes on cortex AT_2 receptor mRNA. Total cortex RNA from 2-week control, diabetic, and insulin-implanted diabetic rats assayed for AT_2 receptor mRNA. A 536 bp cDNA was quantitated on a 3% agarose vistra green gel. RT-PCR for β-actin was performed on each sample to ensure equal sample loading. The inset illustrates these experiments with each band representing AT_2 receptor PCR product from a different experimental group. *$p<0.005$ vs control and insulin-implanted diabetic rats. Statistical significance was assessed by one-way ANOVA.

6.6.2 Effect of diabetes on AT_2 receptor protein expression

A highly specific AT_2 receptor antibody (kindly provided by Dr. Robert M. Carey, University of Virginia) was used to evaluate AT_2 receptor protein expression in the kidneys of 2-week control, diabetic, and insulin-implanted diabetic rats. By Western blot analysis, a single band of the predicted molecular mass for the AT_2 receptor (44 kD) (Ozono, et al., 1997) was detected in cell lysates from the cortex and glomeruli.

AT_2 receptor protein expression in the cortex of diabetic rats did not demonstrate any significant change from control values (figure 6.9). A single band of the predicted molecular mass was detected for all samples (n=5), and all samples were corrected for protein loading by measurement of signals for β-actin (figure 6.9 inset).

In the glomeruli of diabetic rats, a significant decrease in AT_2 receptor protein expression was observed by Western blot (figure 6.10) [D: 47.0±6.6% of C ($p<0.001$ vs C); n=6]. This decrease in AT_2 receptor expression was only partially reversed in the insulin-implanted diabetic group [D+I: 66.8±8.4% of C ($p<0.005$ vs C); n=6]. All bands were corrected for equal protein loading by measurement of signals for β-actin.

By immunohistochemistry, staining was observed in all kidney sections, indicating that the AT_2 receptor is expressed in the adult kidney. Slides were observed using a 4 level grading system for staining analysis, and were randomly observed in a blinded manner. Each slide was examined in three distinct regions (cortex, outer medulla, and inner medulla) of the kidney, and each region was examined in 4 different fields for staining. In all slides, within the cortex, glomeruli were specifically evaluated for staining. The absence of staining in the field was arbitrarily assigned a value of 0, whereas light staining was assigned a value of 1+, medium staining was assigned a value

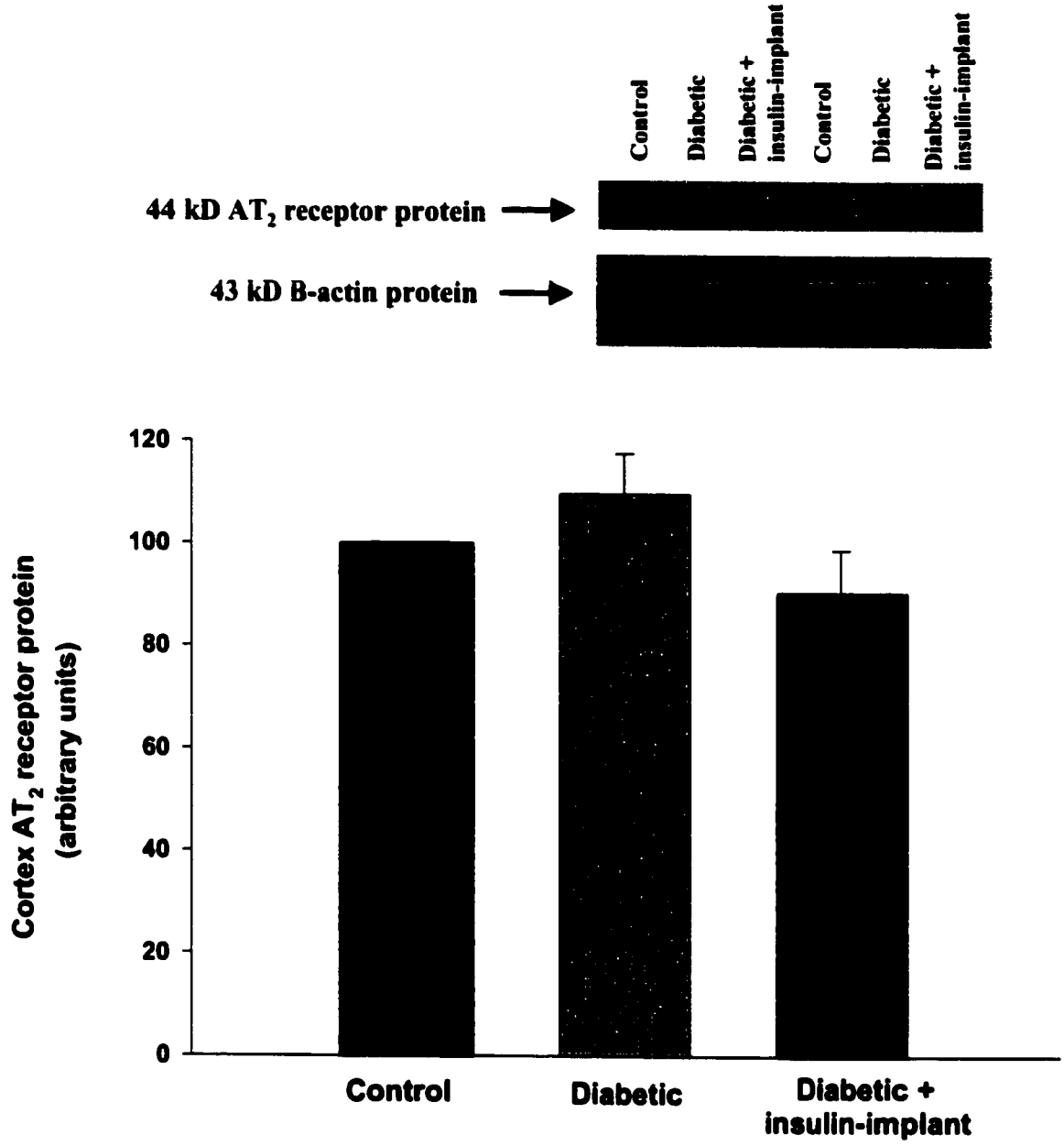

Figure 6.9: Effect of 2-week diabetes on cortex AT_2 receptor protein expression by Western blot. Western blot analysis of cortex protein (30 μg) for AT_2 receptor expression in 2-week control, diabetic, and insulin-implanted diabetic rats. AT_2 receptor protein was detected as a single band of ~44 kD. All bands were normalized to control and were corrected for β-actin. A representative Western blot of 2 sample experiments is shown in the inset. Data represent the means ± SE from 5 experiments.

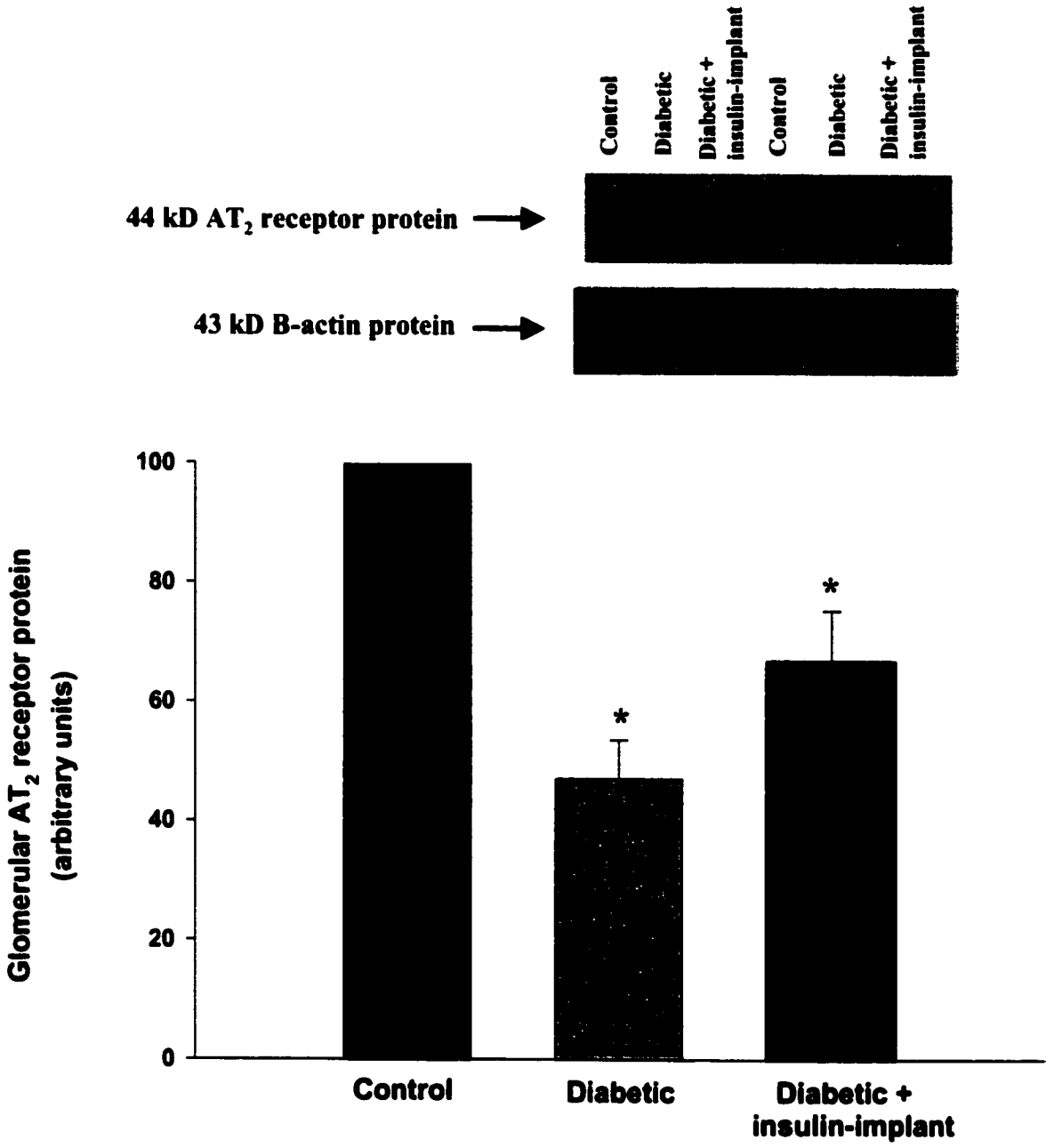

Figure 6.10: Effect of 2-week diabetes on glomerular AT_2 receptor protein expression by Western blot. Western blot analysis of glomerular protein (30 μg) for AT_2 receptor expression in 2-week control, diabetic, and insulin-implanted diabetic rats. AT_2 receptor protein was detected as a single band of ~44 kD. All bands were normalized to control and were corrected for β-actin. A representative Western blot of 2 sample experiments is shown in the inset. Data represent the means ± SE from 6 experiments. *$p<0.05$ vs control. Statistical significance was assessed by the Mann-Whitney rank sum test for nonparametric data.

of 2+, and heavy staining was assigned was assigned a value of 3+. These data are summarized in table 6.3. As indicated, AT_2 receptor staining was decreased in all regions of the diabetic kidney. For example, 21 out of the 32 fields examined (4 fields in each of the 8 different sections) in the cortex of control rats were given a 2+ score, whereas in diabetic sections, only 7 out of 32 fields observed was given a 2+ score. In all regions of the kidney from diabetic sections, greater than 25 out of 32 fields were given a 1+ or 0 score. Although these data were not analysed statistically, the trend suggested a decrease in AT_2 receptor protein expression in early diabetes.

In figure 6.11 panel A, prominent AT_2 receptor staining is visible within the glomerulus of a representative section from a control rat. Although some diffuse staining of tubular segments was observed, staining was predominantly localized to glomeruli in the cortex of all sections (n=8). In diabetic rats, glomerular staining for the AT_2 receptor was markedly decreased (figure 6.11 panel B). This was consistently observed in all sections (n=8). Similar to the control sections, insulin-implanted diabetic rat sections displayed significant AT_2 receptor staining in glomeruli in all sections (n=8) (figure 6.11 panel C).

Figure 6.12 illustrates representative photomicrographs of the outer medullary region of control, diabetic, and insulin-implanted diabetic rat kidney sections stained for the AT_2 receptor. Focal staining was observed in the interstitial cells of this region, both in control and insulin-implanted diabetic rats (figure 6.12, panels A and C respectively). In both control and insulin-implanted diabetic groups, prominent staining was visible in all sections (n=8). In diabetic rats, AT_2 receptor staining was decreased in the outer medullary region of the kidney at 2-weeks after induction of diabetes (figure 6.12 panel B) (n=8).

Figure 6.13 illustrates representative photomicrographs of the inner medullary region of

control, diabetic, and insulin-implanted diabetic rat kidney sections stained for the AT_2 receptor. Staining in this region of the kidney for AT_2 receptor protein was also decreased in diabetic kidney sections. Both control and insulin-implanted diabetic rat kidney sections stained for the AT_2 receptor exhibited distinct tubular staining (figure 6.13, panels A and C for control and insulin-implanted diabetic respectively). The staining pattern suggested that AT_2 receptors were localized to the apical region and cytoplasm of inner medullary collecting duct cells (figure 6.13, panel A). The insulin-implanted diabetic rat kidney sections demonstrated similar staining to the control sections. However, the staining pattern was not as definite in these sections, perhaps due to the manner in which the kidney sections were cut. In the sections from diabetic rats (figure 6.13, panel B), staining was noticeably decreased in all sections in this region of the kidney (n=8).

As a negative control, preadsorption of the primary antibody with the immunizing peptide completely eliminated glomerular staining for the AT_2 receptor (figure 6.14) in serial sections from a control rat kidney. As expected, there is an almost complete absence of staining in the glomerulus of a representative section using the primary antibody which was preadsorbed with a 20-fold excess of the immunizing peptide prior to incubation (figure 6.14 panel B). Figure 6.14, panel C represents a control rat kidney section in which the primary antibody was omitted, as a negative control. A complete absence of staining is seen in this figure. This experiment demonstrated the specificity of the antibody for the AT_2 receptor protein.

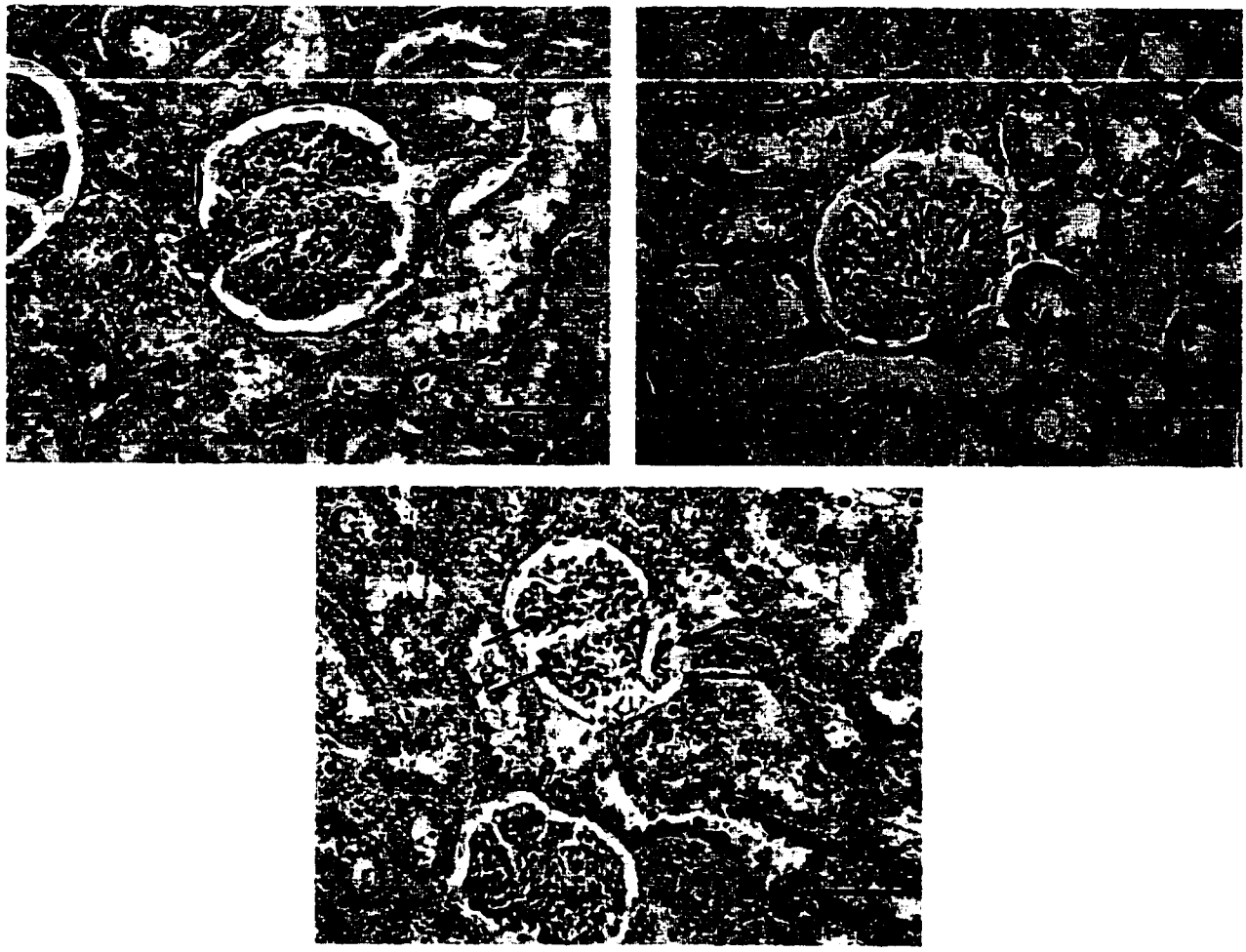

Figure 6.11: Effect of 2-week diabetes on cortex and glomerular AT_2 receptor protein expression by immunohistochemistry. Photomicrographs of cortical region from 10 μm sections of Zamboni fixed and paraffin embedded rat kidneys stained for the AT_2 receptor. A, control rats demonstrated marked glomerular staining (indicated by arrows). B, 2-week diabetic rats exhibited decreased staining for the AT_2 receptor in glomeruli. C, insulin-implanted diabetic rats demonstrated AT_2 receptor staining similar to levels seen in control. In all sections cortical staining was predominantly localized to the glomerulus, with some diffuse tubular staining. Images are representative from n=8 rats for each group. Magnification x 400.

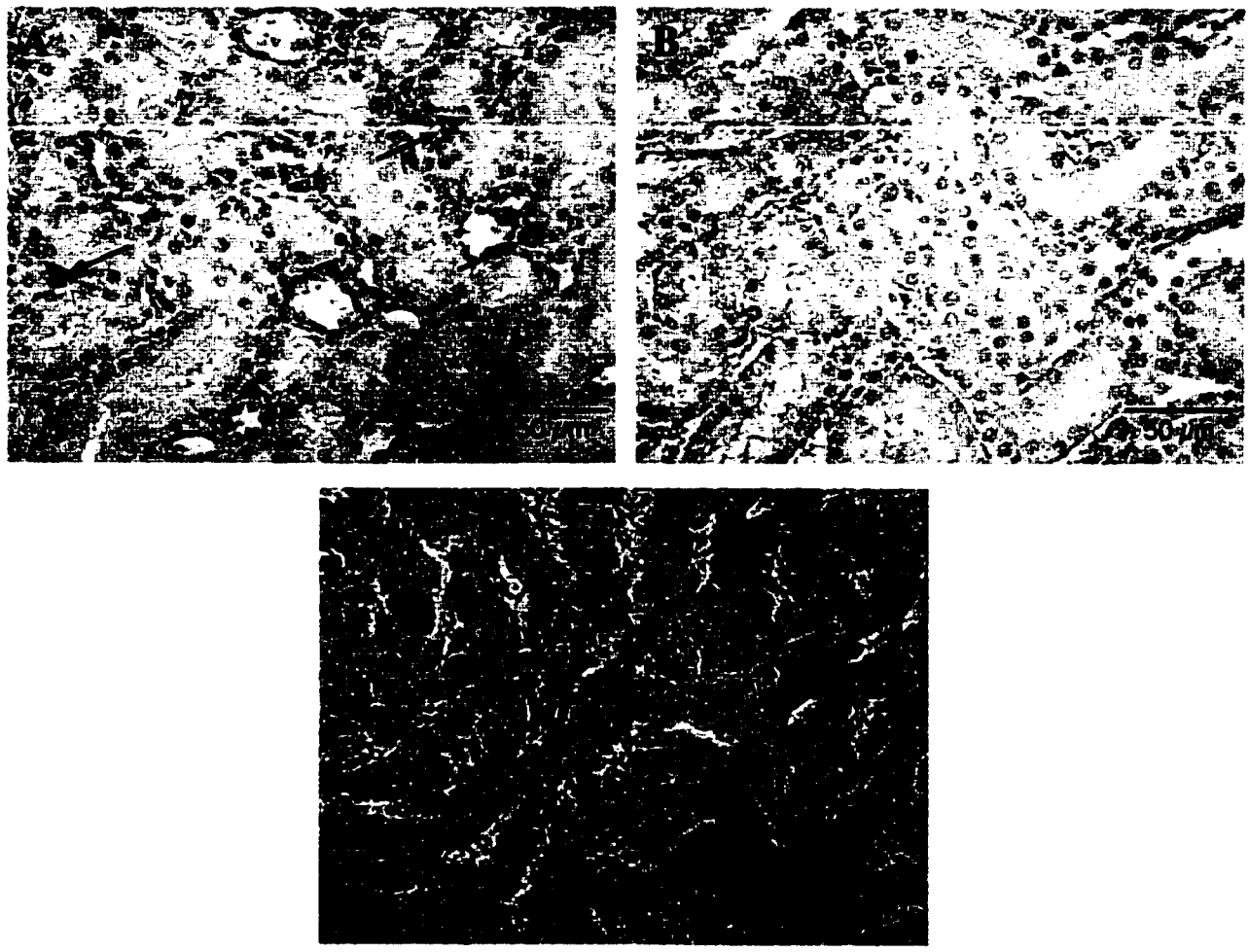

Figure 6.12: Effect of 2-week diabetes on outer medullary AT_2 receptor protein expression by immunohistochemistry. Photomicrographs of outer medullary region from 10 μm sections of Zamboni fixed and paraffin embedded rat kidneys stained for the AT_2 receptor. A, control; B, 2-week diabetes; C, insulin-implanted diabetes. All sections demonstrated diffuse AT_2 receptor staining with some focal staining (indicated by arrows) appearing to be localized to the interstitial cells. Images are representative from n=8 rats for each group. Magnification x 400.

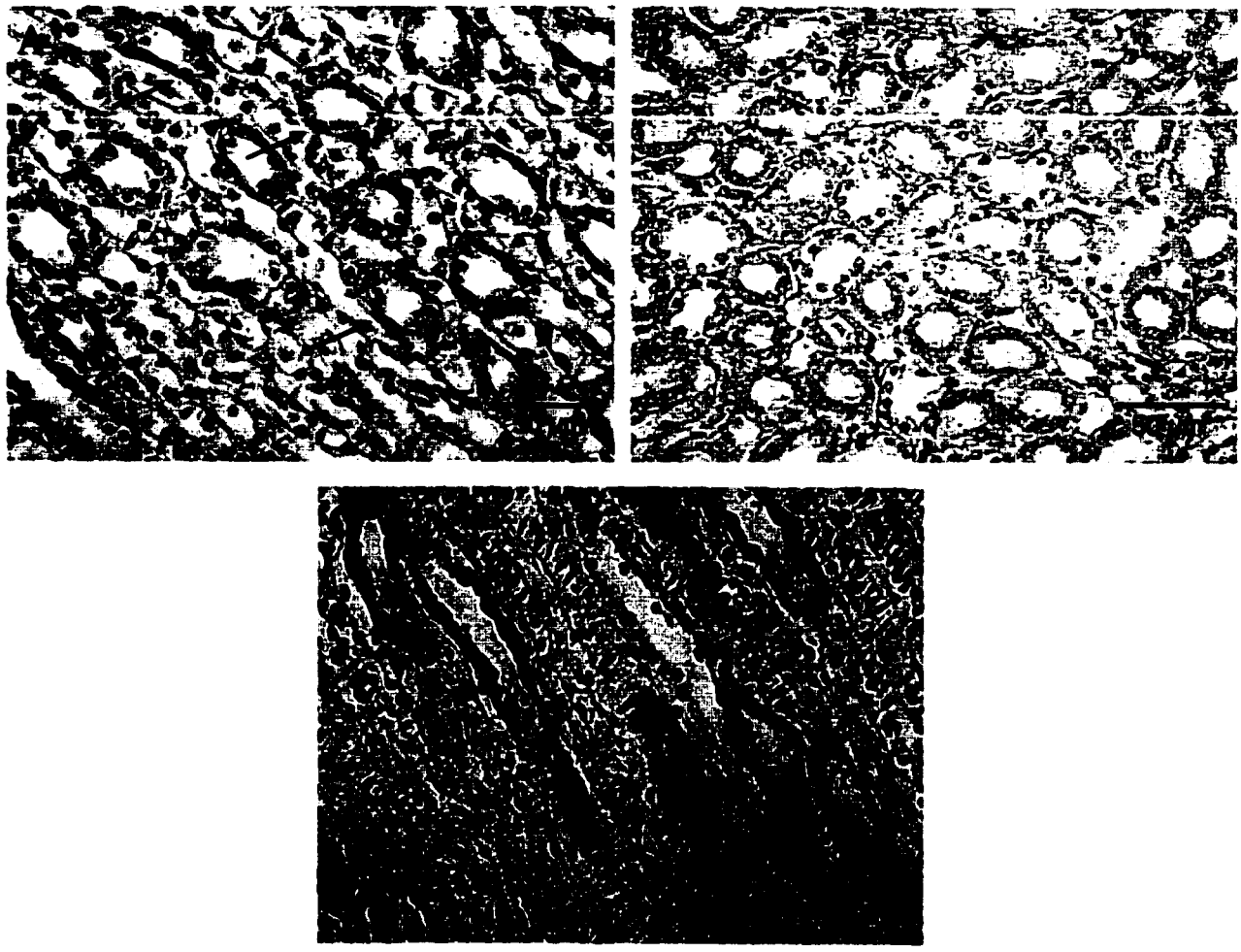

Figure 6.13: Effect of 2-week diabetes on inner medullary AT_2 receptor protein expression by immunohistochemistry. Photomicrographs of inner medullary region from 10 μm sections of Zamboni fixed and paraffin embedded rat kidneys stained for the AT_2 receptor. A, control rats demonstrated heavy staining (indicated by arrows). This appeared to be localized to the cytoplasm and apical membrane of inner medullary collecting ducts (IMCD). B, 2-week diabetic rats demonstrated little or no staining for the AT_2 receptor. C, insulin-implanted diabetic rats demonstrated heavy staining throughout the inner medullary region, an apparent return to levels observed in control. Images are representative from n=8 rats for each group. Magnification x 400.

Table 6.3: Summary of AT_2 receptor staining in kidney sections of control (C), diabetic (D), and diabetic + insulin-implant (D+I), n=8 for each. Observations were made in a blinded manner, using a 0 (no staining), 1+ (light staining), 2+ (medium staining), and 3+ (heavy staining) grading system. Each kidney section was observed in three distinct regions of the kidney: 1) cortex, specifically glomeruli 2) outer medulla, and 3) inner medulla. Four fields were observed for each of the three regions of the kidney for each section. Each number below represents the number of times the observation was made in all sections in that particular field.

	Degree of staining	Cortex (glomeruli)	Outer medulla	Inner medulla
Control (C)	0	0	0	0
	1+	9	16	4
	2+	21	16	17
	3+	2	0	11
Diabetic (D)	0	10	11	13
	1+	15	15	15
	2+	7	6	4
	3+	0	0	0
Diabetic + insulin-implant (D+I)	0	6	4	0
	1+	5	11	8
	2+	19	16	14
	3+	2	1	10

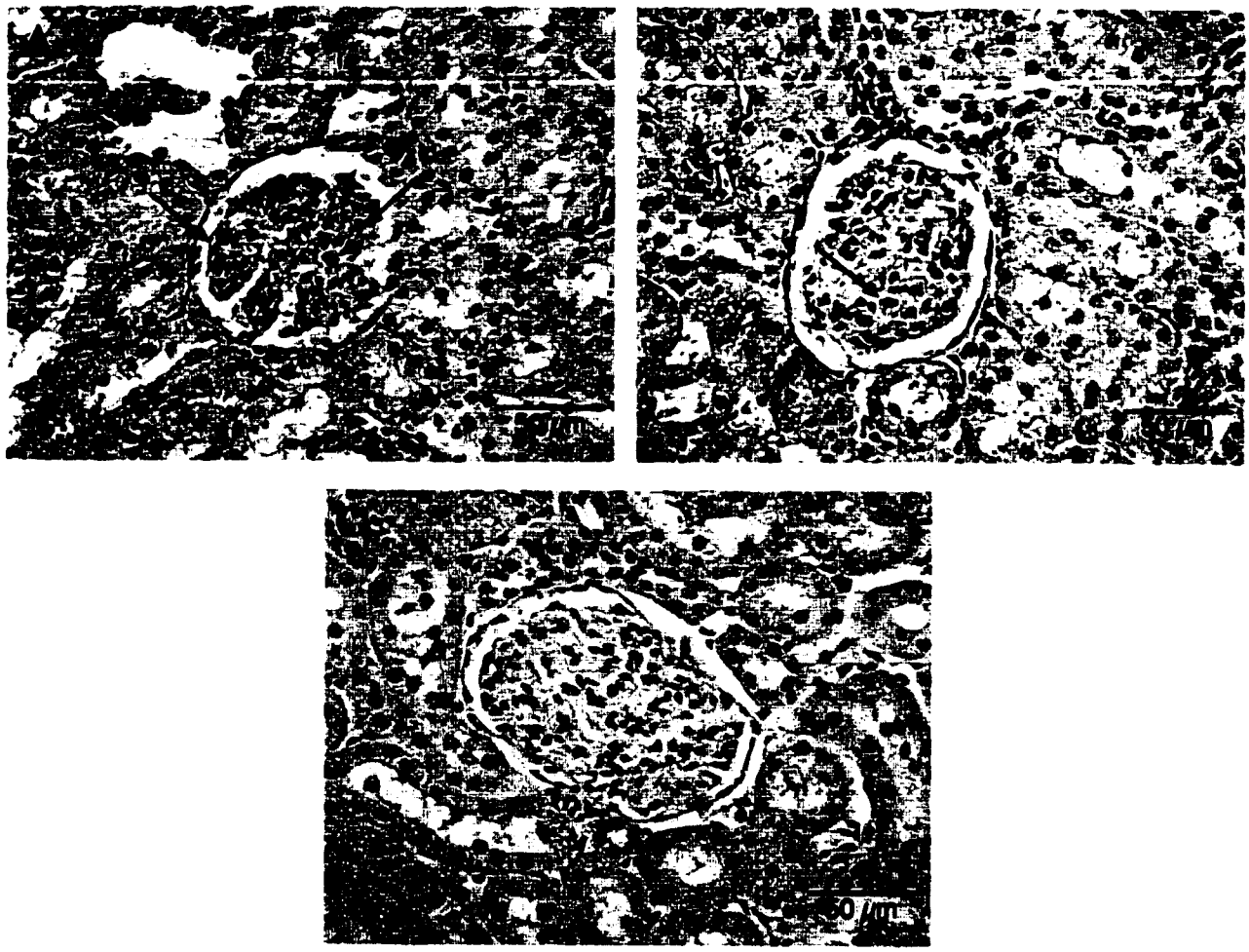

Figure 6.14: Negative controls for AT_2 immunostaining. Serial 10 μm sections from a control kidney stained for the AT_2 receptor. Positive control A, 1:100 dilution of primary antibody demonstrating staining in the glomerulus; Negative control B, effect of preincubation of 1:100 primary antibody with a 20-fold excess of immunizing peptide, revealing no glomerular staining; Negative control C, absence of primary antibody, demonstrating no staining in glomeruli. Representative photomicrographs of n=2 experiments. Magnification x 400.

7 Discussion

7.1 Summary

In the present studies, diabetic rats exhibited the expected changes in blood glucose levels, body weights, and kidney weight. Diabetic rats had significantly elevated blood glucose levels, decreased body weight, and increased kidney weight. Insulin treatment in diabetic rats returned these values to control levels. PRA appeared to be decreased, although not significantly in diabetic rats, suggesting that these rats were not ECF volume contracted.

Diabetes did not alter the mRNA in glomeruli for components of the RAS. Although renin mRNA appeared to increase in glomeruli, this increase was not significant. Both angiotensinogen and ACE mRNA levels did not change.

PRA was increased in control rats on a zero salt diet (C-ZS) in comparison to control rats on a high salt diet (C-HS), although not significantly. PRA was however, significantly increased in both diabetic rats on a zero salt diet (D-ZS) and insulin-implanted diabetic rats on a zero salt diet (D+I-ZS) in comparison to their high salt (HS) counterparts. This finding suggested a salt sensitivity in diabetic rats in terms of renin activity, an effect not completely reversed by insulin therapy.

Western blot analysis of AT_1 receptor protein in the glomeruli of diabetic rats revealed an approximate 3.5-fold increase in protein levels. Insulin therapy partly reversed this increase, but the increase remained significant. A number of studies have suggested decreased Ang II receptors in glomeruli of early diabetes, implying that perhaps this receptor subtype is not the primary Ang II receptor in glomeruli.

Analysis of AT_2 receptor mRNA by RT-PCR and AT_2 receptor protein by Western blot in the renal cortex illustrated increased mRNA levels with no increase in protein levels in diabetic rats.

Subsequent analysis of glomerular cell lysates for AT_2 receptor protein levels demonstrated significant decreases in diabetic rats with a partial reversal to control levels with insulin therapy.

Immunohistochemical analysis of whole kidney sections for AT_2 receptors revealed a consistent decrease in staining throughout the kidney in diabetic rats. In the cortex, glomerular staining was decreased in diabetic rats, and returned to levels observed in control with insulin treatment. In the outer medulla, focal staining of interstitial cells was not observed in diabetic rats but was apparent in control and insulin-implanted diabetic rats. Finally, apical membrane and cytoplasmic staining of IMCD cells was observed in control and insulin-implanted diabetic rat sections but not in diabetic rat sections. These data indicate a downregulation of AT_2 receptor protein throughout the kidney in early diabetes.

7.2 Whole animal data

Induction of diabetes resulted in expected changes in physiological parameters (outlined in table 6.1). Control animals exhibited normal blood glucose and did not demonstrate glucosuria during the 2-week period of investigation. Although control rat blood glucose levels are high relative to the normal values for humans, this value is not different from values for normal rats cited by others (Ballermann, et al., 1984), (Cassis, 1992) and (Kalinyak, et al., 1993). Furthermore, diabetic rats demonstrated severe hyperglycemia and chronic glucosuria. These data are also not different from the observations of others (Ballermann, et al., 1984), (Cassis, 1992) and (Kalinyak, et al., 1993). Diabetic rats given a sustained release insulin implant showed a reversal to control in almost all parameters measured in table 6.1. Interestingly, blood glucose values in these rats were significantly less than control. This was most likely due to overestimation of the size of the insulin-

implant. A conservative estimate as to the size of the implant was made so that blood glucose levels would not approach those of the diabetic rats. It should be noted that no insulin-implanted rats died and rarely did these rats become hypoglycemic. Insulin-implanted diabetic rats were used to determine if insulin could reverse any changes observed in diabetic rats.

Diabetic rats exhibited the characteristic decrease in body weight. This is possibly due to abnormal glucose metabolism and not likely due to rats being volume contracted, since plasma renin activity (PRA) was not elevated. In fact PRA was decreased, although not significantly in diabetic rats (table 6.1). Since diabetes induces osmotic diuresis, there was a concern that these animals were losing extracellular fluid (ECF) volume and this might be the primary factor responsible for weight loss. If ECF volume loss due to osmotic diuresis was the main contributor to weight loss in diabetic rats, then PRA in these rats is expected to be elevated, which was not the case. At the time of sacrifice examination of the abdominal cavity of diabetic rats revealed the absence of abdominal fat and these rats clearly appeared to be growth retarded. Weight loss due to osmotic diuresis was prevented by treating rats which lost greater than 5% body weight overnight with 0.9% saline (1-2 ml i.p. bolus injections as required).

Serum creatinine was used to assess kidney function. In clinical practice, serum creatinine concentration is used to estimate glomerular filtration rate (GFR). Serum creatinine and GFR follow an inverse relationship, so that an increase in serum creatinine indicates a fall in GFR (Rehberg, 1926). Serum creatinine levels did not change after two weeks of diabetes in any of the rats studied. This indicated that GFR was not significantly altered. With regards to blood urea nitrogen (BUN), another parameter used to assess kidney function, diabetic rats exhibited elevated levels, significantly higher than control rats. This increase in BUN was reversed in insulin-implanted diabetic rats.

Increased BUN is likely due to increased protein metabolism in diabetic rats since glucose metabolism has been disrupted in these animals. The catabolic state of diabetic rats may result in abnormal rates of amino acid uptake and release from tissues. This in turn can alter rates of hepatic urea production from those amino acids (Dossetor, 1966). In the absence of increased protein metabolism, an elevated BUN would suggest volume depletion (Dossetor, 1966), however in this case it is unlikely due to the absence of an elevated PRA.

7.3 Early diabetes did not alter glomerular mRNA levels for RAS components

Preliminary experiments to determine if our competitive RT-PCR assay could detect predicted changes in glomerular mRNA levels were performed. It is known that salt restriction stimulates glomerular renin mRNA levels. Tank and colleagues demonstrated an approximate 4-fold increase in glomerular renin mRNA in response to sodium depletion in normal rats (Tank, et al., 1997). In these experiments glomeruli were microdissected and not isolated by graded sieving and washing. Furthermore, this group measured the number of copies of renin mRNA per glomerulus. We did not determine the number of renin mRNA copies per glomerulus since we did not estimate the number of glomeruli in each preparation. Our observation of an approximate 3-fold increase in response to sodium depletion however, demonstrated that our method of glomerular isolation was valid and that using this assay we were capable of detecting predicted changes in glomerular mRNA levels.

The magnitude of change in renin mRNA in both the cortex and glomeruli in response to sodium depletion was the same. In both regions of the kidney we were able to demonstrate a 3-fold increase in renin mRNA. It is interesting to note however, that glomeruli contained more renin

mRNA per total RNA than the cortex. This was not expected since the cortex is comprised of glomeruli and a number of tubular segments which are capable of producing renin. In addition, the cortex includes the afferent arteriole which is the site within the kidney responsible for most renin production (Tank, et al., 1997). However, the higher levels of renin mRNA in the glomerulus in comparison to the cortex may be explained by a number of reasons. The most likely explanation is that glomeruli simply possess more renin mRNA per total RNA than the cortical region. The cortex of the kidney is a heterogenous region comprised of a number of distinct tubular segments which may not be capable of producing renin. Since the cells making up these specialized tubules would contribute their RNA pool but not renin mRNA, this would decrease the amount of renin mRNA per total RNA observed in this region. Consequently, since we were using total RNA in the competitive RT-PCR experiments, more renin mRNA per total RNA in glomeruli would account for this observed difference. Another possibility is that glomerular renin mRNA is more stable than cortical renin mRNA and is not degraded as quickly during and after isolation.

These experiments allowed us to quantitate glomerular changes in RAS components in early diabetes. We did not observe any changes in renin, angiotensinogen, or ACE mRNA in glomeruli in early diabetes (figures 6.3, 6.4, and 6.5 respectively). It did appear however, that renin mRNA was slightly increased in glomeruli but this did not reach statistical significance. Nonetheless, this system may be activated but we are not able to detect these changes, due largely to the sensitivity of this assay. As speculated previously, glomerular renin mRNA may be more stable than that in cortex, and so increased renin mRNA, even though not very dramatic, may influence intraglomerular production of Ang II considerably.

A major concern in the quantitation of renin mRNA within glomeruli by RT-PCR was the

possible contamination of the glomerular preparation by the juxtaglomerular (JG) cells of the afferent arteriole. Since these sites are in close proximity to the glomerulus and they are a major site of renin synthesis, any contamination of these elements would significantly alter the mRNA measured. It was highly unlikely that arteriolar contamination occurred in these studies because of the high purity of our preparation. First, the repeated washing and sieving glomerular isolation we developed generated highly pure glomerular preparations when viewed by light microscopy. We visually identified preparations by light microscopy that were always essentially 100% pure glomeruli with no attached afferent arterioles. Furthermore, we attempted to stain these preparations for renin protein in order to identify possible afferent arteriolar segments that remained attached. Although we were unable to consistently obtain a positive control (renin staining occurred only 2 out of 7 times in the positive control), we did not see any renin staining in any glomerular preparation. Second, the amount of renin mRNA in the JG cells of the afferent arteriole is much greater than in glomeruli (Tank, et al., 1997). Therefore, tissue contamination, which is a random event, would introduce large variations in the amount of renin mRNA measured in glomerular preparations. This did not occur since the intersample variation of glomerular renin was no different from that in the cortex. Therefore, JG cell contamination of our glomerular preparation was highly unlikely.

In all of these studies, we assayed whole glomeruli for RAS mRNA. Cells responsible for synthesis of Ang II within the glomerulus have not been identified. Glomeruli are composed of a number of cell types including endothelial cells, mesangial cells, and epithelial cells. At present, no study has identified a specific cell type within glomeruli which may be responsible for any alterations occurring as a result of diabetes. However, a few studies have localized certain RAS components

to specific cell types within glomeruli. For example, ACE protein has been identified in the vascular endothelial cells of glomeruli (Anderson, et al., 1993) and angiotensinogen mRNA has been identified in cultured human mesangial cells (Lai, et al., 1995). Until other studies are able to identify specific glomerular cell types responsible for synthesis of individual RAS components, it is only possible to speculate which cell types are responsible for the observed changes in the diseased state.

The dilemma of using alterations in mRNA levels to predict changes in protein levels rests in the fact that increases in mRNA do not always correspond to increases in protein, and vice versa. This phenomenon was demonstrated in this thesis with the renal cortex AT_2 receptor. A highly significant increase in renal cortex AT_2 receptor mRNA in diabetic rats did not predict a likewise increase in protein levels by Western blot. The translation of mRNA to protein is dependent on many factors and is not based solely on the amount of mRNA available for translation. However, in the case of the glomerular RAS where there is an absence of adequate techniques to assess changes in glomerular protein levels for the components of the RAS, assessing mRNA levels can provide some insight on the status of the RAS in early diabetes.

The ultimate purpose of assessing glomerular changes in mRNA for components of the RAS is for possible therapeutic approaches in diabetes, but also to predict the status of intraglomerular Ang II levels. If dramatic changes in mRNA were observed in early diabetes then it would be reasonable to suggest that the RAS is altered and this may influence intraglomerular Ang II levels. The direct measure of intraglomerular Ang II levels in early diabetes has not yet been documented in the literature. However, Seikaly and colleagues have measured intraglomerular Ang II concentrations directly by *in vivo* micropuncture in normal Munich-Wistar rats (Seikaly, et al.,

1990). The measure of intraglomerular Ang II concentrations using this technique requires the use of Munich-Wistar rats which have characteristically large glomeruli located near the surface of the kidney.

7.4 Effect of salt diet on RAS components in early diabetes

These studies were undertaken to determine whether the ingestion of a sodium-restricted diet could modify glomerular production of renin mRNA in early diabetes. In a recent study by Miller, it was demonstrated that in non-diabetic human subjects, sodium depletion did not affect GFR, whereas in diabetic subjects, sodium depletion resulted in an increased GFR (Miller, 1997). Increased GFR in diabetes in response to sodium depletion was also observed in a study by Vallon and colleagues, involving STZ-induced diabetic rats (Vallon, et al., 1995). In another study by Vallon and colleagues, it was shown that a high salt diet significantly decreased GFR in diabetic rats, yet increased GFR in control rats (Vallon, et al., 1997). Increased GFR in early diabetes is known to mediate some of the injury observed in long-term diabetes. The observation that dietary sodium could influence GFR in diabetics in a manner opposite to that of non-diabetics in the three studies cited previously, led us to assess glomerular renin mRNA levels in control, diabetic, and insulin-implanted diabetic rats placed on either a high salt diet or a zero salt diet. We speculated that the increased GFR in sodium depleted diabetic rats was a result of increased local Ang II production, mediated in part by increased local renin activity. Increased local Ang II production may result in the constriction of the efferent arteriole and thus an increase in GFR (Burns, et al., 1993). Our studies were designed to determine if diabetic rats responded to zero dietary salt by increasing glomerular renin mRNA and in this respect possibly influencing local production of Ang II.

With regards to the physiological data (summarized in table 6.2), the two salt diets (high and zero) did not have an effect on body weight, kidney weight, urine output, water intake, or food intake when comparing rats within the same group (eg. high salt diet control vs zero salt diet control). However, as expected, rats on the zero salt diet did not excrete any sodium whereas the rats on the high salt diet excreted a substantial amount of sodium in the urine.

The difference in PRA was not significantly different between control rats on a high salt diet and control rats on a zero salt diet. PRA was significantly different however, between diabetic rats on a high salt diet and diabetic rats on a zero salt diet. Although PRA was significantly elevated in insulin-implanted diabetic rats on the zero salt diet in comparison to their high salt counterparts, this difference was not as significant statistically as that in the diabetic group. Therefore, diabetic rats demonstrated an enhanced ability to increase PRA in response to dietary changes of sodium, suggesting an increased salt sensitivity. This finding corresponds well with the observations of Vallon and colleagues and Miller (Vallon, et al., 1995), (Miller, 1997). The absence of PRA stimulation in control rats on a zero salt diet in these studies is in agreement with the finding by both groups that GFR is not altered, according to the hypothesis proposed above. Briefly, we hypothesized that an increase in local renin activity would correspond to an increase in local Ang II production, and a consequent increase in GFR mediated by efferent arteriolar constriction by Ang II. Moreover, the significant stimulation of PRA by sodium depletion in our studies and the increase in GFR in diabetes in the studies by Vallon and colleagues and Miller also follow this hypothesis (Vallon, et al., 1995), (Miller, 1997). However, these data represent circulating levels of renin activity and do not reflect what is happening within the glomerulus. Therefore, we went on to assess glomerular changes of renin mRNA in diabetic rats on either a high salt or a zero salt diet.

These studies did not provide any data with respect to glomerular and cortical renin mRNA levels in response to high and zero salt diets in diabetic rats. The absence of a consistent positive control (i.e. stimulation of renin mRNA in either the cortex or glomeruli with zero salt) did not permit the inclusion of these data. Speculation about the failure of these experiments suggested a number of possibilities. One possibility was that the RNA was not used soon enough after isolation and degradation of the RNA resulted in the high variability that we observed. The isolation of RNA from these 36 rats took place over a period of approximately 6-8 weeks. Another possibility considered was that too many samples were used at once in the RT-PCR step. In order to reduce RT-PCR variability and error, all RNA samples from the same condition were reverse transcribed and then subject to PCR at the same time. This was done in order to ensure uniformity of conditions for RT-PCR for all experiments. For example, the RNA from all six high salt diet control rats was reverse transcribed simultaneously. Each of these six RNA samples required three different concentrations of deletion mutant cRNA and a negative control (the absence of the RT enzyme) for each sample. This required the RT and then PCR of 24 samples. The RT-PCR of 24 samples resulted in inconsistent incubation times between the first tube and the last tube and this may have contributed to the observed error.

7.5 Early diabetes increases glomerular AT_1 receptors

Several studies have suggested that a downregulation of glomerular Ang II receptors occurs in diabetes. At present no study has identified which Ang II receptor subtype is decreased within the glomeruli of diabetic rats, although it has been suggested that it is AT_1 receptor due to its abundance in the adult kidney. At two weeks after STZ-induced diabetes, Kalinyak and colleagues found

significantly reduced glomerular Ang II receptors by radioligand binding in frozen kidney sections (Kalinyak, et al., 1993).

Also by radioligand binding, Wilkes demonstrated decreased Ang II receptor density in the glomeruli of diabetic rats (Wilkes, 1987). In the study by Wilkes, a time course of glomerular Ang II receptor expression was performed after STZ-induced diabetes, which demonstrated reduced glomerular Ang II receptor density as early as twenty-four hours after the induction of diabetes. The reduction of glomerular Ang II receptors persisted for 2 months at which time receptor density was normalized.

Furthermore, at 4 weeks after STZ-induced diabetes, Ballermann and colleagues demonstrated that Ang II receptors are also decreased within the glomeruli of diabetic rats (Ballermann, et al., 1984). In this study, glomeruli were harvested by differential centrifugation and graded sieving techniques and receptor density was determined by competitive binding assays. Using a technique similar to the one we employed for glomerular isolation, this group also demonstrated a significant reduction in glomerular Ang II receptors but did not identify which Ang II receptor subtype was decreased. All of these studies suggest that Ang II receptors are downregulated within glomeruli in early diabetes.

To identify the Ang II receptor subtype which was decreased in glomeruli we performed Western blots on glomerular cell lysates for the AT_1 receptor. Surprisingly, we identified an increase in glomerular AT_1 receptor expression after two-weeks of STZ-induced diabetes. In the glomeruli of diabetic rats we observed an approximate 3.5-fold increase in AT_1 receptor protein in 7 experiments. This increase was partially reversed in insulin-implanted diabetic rats, although it did remain significantly above control. This finding, in addition to the binding data from the literature,

suggests that AT_1 receptors may not be translocated to the cell membrane. Since binding studies assess receptors present in the cell membrane, the decreased binding observed in previous studies and the increased protein levels for the AT_1 receptor which we observed suggest that the presence of this receptor in the cell membrane is decreased. Another possibility is that diabetes increases the expression of the AT_1 receptor but at the same time decreases the expression of another Ang II receptor subtype to a greater extent. It is possible that the abundance of the AT_2 receptor within glomeruli is greater than that of the AT_1 receptor, and that the decrease of the AT_2 receptor within glomeruli has a greater impact on the observed decrease in Ang II receptors than the increase in AT_1 receptors. This speculation led to the investigation of the status of the AT_2 receptor within glomeruli of diabetic rats.

7.6 Effect of early diabetes on AT_2 receptors in the kidney

7.6.1 AT_2 receptor mRNA but not protein is increased in the cortex

Total RNA from the renal cortex was isolated from 2-week control, diabetic, and insulin-implanted diabetic rats (1 group represents one control, one diabetic, and one insulin-implanted diabetic rat; n=7 groups). By RT-PCR, we observed increased AT_2 receptor mRNA in the cortex of diabetic rats. In glomeruli however, we were not able to consistently amplify AT_2 receptor mRNA in all samples. Despite numerous attempts, only 3-4 out of the 7 RNA samples for each condition (C, D, or D+I) resulted in an amplified PCR product of the desired size when visualized on an ethidium bromide gel. Since all samples were reverse transcribed (RT) and then subjected to PCR at the same time, and since repeated attempts to amplify RNA samples were not successful, error due to the RT-PCR procedure was not a likely possibility. RNA degradation however, may account for

the observed amplification in some RNA samples and not in others. This seems to be the most likely possibility since the amplification of AT_2 receptor mRNA resulted in PCR product signals from RNA samples in a random manner with varying signal intensities.

Increased AT_2 receptor mRNA levels in the renal cortex of diabetic rats by RT-PCR led to the investigation of AT_2 receptor protein levels in renal cortex cell lysates. Western blot analysis revealed that there was no change in AT_2 receptor protein levels in the cortex. An observed increase in mRNA levels but no change in protein levels was unexpected and suggested that the translation of this protein may be downregulated. Although not unusual, an increase in the mRNA but not in the protein has been documented within the kidney. For example, it has been shown that in mesangial cells, in the presence of the glucocorticoid dexamethasone, interleukin-β1(IL-β1) stimulates prostaglandin synthase-2 (PGS-2) mRNA levels but has no effect on PGS-2 protein levels (Rzymkiewicz, et al., 1994). The increased renal cortical mRNA for AT_2 receptors in diabetes may be a consequence of transcriptional activation and/or enhanced stability of this message, perhaps through the induction of cytoplasmic or nuclear factors which may prolong the half-life of the message.

This finding suggested a decrease in the translation of the AT_2 receptor protein may be occurring in diabetes through the involvement of factors which may inhibit the translation of the protein. This decline in translation may be mediated through the eukaryotic initiation factor 4E (eIF4E) protein whose activity is regulated by insulin (Lawrence and Abraham, 1997). This protein is responsible for recognition of the mRNA cap, a structure that is found at the end of almost all eukaryotic messages (Pain, 1996). Recognition of the mRNA cap is important for initiating translation of most eukaryotic mRNAs. Insulin is known to increase the availability of eIF4E by

promoting the dissociation of eIF4E from its carrier protein PHAS/4E-BP1 (properties of heat-and acid-stability/4E-binding protein 1) (Lawrence and Abraham, 1997). Therefore in the diabetic state, decreased insulin levels may result in decreased translation of this protein partly via this mechanism.

7.6.2 AT_2 receptor expression is decreased in glomeruli

A highly specific polyclonal antibody was used to investigate AT_2 receptor protein expression in the kidneys of diabetic rats. Both Western blot and immunohistochemical analysis demonstrated decreased AT_2 receptor protein expression in the glomeruli of diabetic rats, with a partial reversal to control levels in the insulin-implanted diabetic group.

7.6.2.1 Western blot analysis

Western blot analysis revealed that AT_2 receptor protein expression was decreased significantly in glomerular cell lysates from diabetic rats. This decrease in AT_2 receptors amounted to 47.0±6.5% of the value from control rats, with a partial reversal to control levels in the insulin-implanted diabetic group. Similar to what was observed with the glomerular AT_1 receptor, insulin therapy did not completely reverse the decrease of glomerular AT_2 receptors to control levels.

The failure of insulin therapy to reverse the effects of diabetes in these studies suggests that insulin had direct effects, independent of glucose levels. Apart from its most commonly associated function of increasing cellular uptake of glucose in most cells, insulin exerts a number of effects, including regulating translation, as was discussed previously. In the control group of rats, as with most animals, the levels of insulin in circulation depend on the levels of glucose in the blood. For example, high levels of glucose in the blood, such as after a meal, would result in increased insulin in circulation. In this manner, insulin levels fluctuate in response to glucose levels in non-diabetic

subjects. In the insulin-implanted diabetic rats however, the release of insulin into circulation was not dependent on glucose levels but merely on the rate of dissolution of the insulin implant. The implant ensured a constant delivery of insulin into circulation to maintain glucose levels. In this regard, the physiologically unregulated delivery of insulin and its direct effect in the insulin-implanted diabetic rats may have been responsible for the lack of complete reversal to control levels.

7.6.2.2 Immunohistochemical analysis

Throughout the cortex, AT_2 receptor staining was decreased in diabetic sections. In particular, staining was consistently decreased within glomeruli. Moreover, the mild tubular staining observed in control animals was also consistently decreased in diabetics. The decreased staining observed in diabetic rat sections was reversed with insulin therapy in the insulin-implanted diabetic rat group.

Similarly, outer medullary staining for the AT_2 receptor was decreased in diabetic kidney sections. The focal staining observed throughout this region in control rat sections was markedly decreased in diabetic rat sections. The staining pattern in the insulin-implanted diabetic group was similar to that observed in control rats.

Finally, inner medullary staining for the AT_2 receptor was observed in control rats and appeared to be localized to the apical membrane and cytoplasm of inner medullary collecting duct (IMCD) cells. Similar to observations in the other regions of the kidney, AT_2 receptor staining was also decreased in this region. Inner medullary staining was significantly decreased in diabetic rats and the pattern of staining returned to those seen in control with insulin therapy.

Negative control experiments confirmed the specificity of the antibody for the AT_2 receptor. The preadsorption of the antibody with a 20-fold excess of the immunizing peptide prior to

incubation of the antibody with the kidney section completely prevented the staining of any region from a control rat kidney. Moreover, no staining was observed in sections where the primary antibody was omitted. These experiments along with the observation of a single band at the predicted molecular mass for the receptor demonstrated the specificity of the antibody for the AT_2 receptor.

The apparent global downregulation of AT_2 receptors in the kidney, observed by immunohistochemistry and the glomerular downregulation of AT_2 receptors observed by Western blot in diabetes suggest that this receptor is primarily responsible for the documented decrease in Ang II receptors. This finding suggests that this receptor may have functional significance in the adult kidney and its expression is altered in early diabetes.

The AT_2 receptor has been shown to inhibit cellular proliferation and growth (Stoll, et al., 1995), an action opposite to that of AT_1 receptors. AT_2 receptors have also been shown to antagonize the vasoconstrictor actions of AT_1 receptors (Ichiki, et al., 1995), and promote sodium retention by the kidney (Lo, et al., 1995). Also, Ozono and colleagues demonstrated by immunohistochemistry and by Western blot (using the same antibody used in these studies) that the AT_2 receptor is upregulated in response to sodium depletion, mainly in glomeruli and interstitial cells (Ozono, et al., 1997). The altered expression of the AT_2 receptor in the kidney of diabetic rats, as demonstrated in the present studies, may explain some of the documented changes which occur in early diabetes, in terms of the known physiological functions of the AT_2 receptor as outlined previously.

Decreased AT_2 receptors throughout the kidney and within glomeruli may be responsible for the rapid renal hypertrophy observed in early diabetes. A decrease in AT_2 receptors throughout the

kidney in early diabetes may result in less inhibition of cellular growth and proliferation. Furthermore, in a study by Remuzzi and colleagues, the use of a highly specific AT_1 receptor antagonist did not prevent the renal hypertrophy observed in early diabetes (Remuzzi, et al., 1993). This suggests that renal hypertrophy is not likely due to the upregulation of AT_1 receptors in glomeruli, as shown in the present study, in spite of the fact that AT_1 receptors have been shown to stimulate cellular hypertrophy and hyperplasia (Ardaillou, 1999).

Moreover, downregulation of AT_2 receptors in diabetes may influence the vasodilatory functions associated with this receptor. Within the glomerulus, reduced AT_2 receptors and increased AT_1 receptors may contribute to the increased intraglomerular pressure commonly observed in early diabetes. Indeed, AT_1 receptor antagonism has been shown to reverse the increased GFR observed in early diabetes (Remuzzi, et al., 1993). Increased intraglomerular pressure is known to mediate the progressive glomerular injury seen in the later stages of diabetes.

Finally, decreased AT_2 receptors throughout the kidney in early diabetes may influence sodium retention by the kidneys and therefore, may be responsible for the suggested salt sensitivity of diabetic rats. Lo et al. demonstrated that AT_2 receptor antagonism resulted in the increased sodium excretion by the kidney (Lo, et al., 1995). Similarly, a decrease in AT_2 receptors in early diabetes may result in increased sodium excretion, consistent with the observations of these studies. Diabetic rats on a high salt diet excreted significantly more sodium than control rats on the same diet (table 6.2). In this regard, increased sodium excretion in diabetic rats on the high salt diet may result in a decreased ECF volume and thus may account for their ability to significantly increase PRA in comparison to control rats (figure 6.6). This finding implies an apparent salt sensitivity in diabetic rats in regard to PRA. Downregulation of AT_2 receptors in early diabetes in the inner medullary

region may be partly responsible for increased sodium excretion. The collecting duct is the site where final adjustments in sodium excretion are made in order to maintain a balance between the amount of sodium ingested and the amount excreted (Koeppen and Stanton, 1997). Therefore, decreased AT_2 receptor staining of IMCD cells (figure 6.13), indicating a decrease in receptor expression, may be responsible for the increased excretion of sodium in diabetic rats.

7.7 Conclusions

The STZ-induced diabetes rat model demonstrated that early diabetes did not significantly alter the glomerular expression of mRNA for components of the RAS. Moreover, by RT-PCR, AT_2 receptor mRNA was increased but not the protein in the renal cortex of diabetic rats. This finding suggested that there was perhaps a block in the translation of the AT_2 receptor occurring in diabetes. With regards to Ang II receptors in the glomerulus, Western blot analysis revealed that AT_1 receptors were increased whereas AT_2 receptors were decreased in diabetes. Decreased AT_2 receptor expression was also observed by immunohistochemistry, and the decrease was not limited to the glomerulus but was observed throughout the kidney. These data suggest an apparent differential regulation of Ang II receptors in the kidney of diabetic rats.

The findings of the present study may lead to therapeutic approaches to prevent some of the physiological changes occurring in early diabetes which result in the progression of diabetic nephropathy. The development of specific AT_2 receptor agonists, in order to selectively activate this receptor, may prevent the renal hypertrophy characteristic of early diabetes, mediated by its antiproliferative actions. These data also suggest that the use of AT_1 receptor blockade in diabetes as a means of preventing injury may be beneficial. Blocking the AT_1 receptor would prevent the

growth and constrictor actions of this receptor and make available more Ang II to act on the AT_2 receptor, since AT_1 receptor blockade results in increased circulating Ang II levels (Zou, et al., 1996). Thus, more Ang II is able to act on the remaining AT_2 receptors and enhance their ability to prevent growth and mediate vasodilation. The activation of this receptor in the face of downregulation by diabetes may be another approach to prevent the injury associated with diabetic nephropathy.

8 References

Allen AM, Zhuo J, and Mendelsohn FA. Localization of angiotensin AT1 and AT2 receptors. *J Am Soc Nephrol* 10 Suppl 11: S23-9, 1999.

Anderson S, Jung FF, and Ingelfinger JR. Renal renin-angiotensin system in diabetes: functional, immunohistochemical, and molecular biological correlations. *Am J Physiol* 265: F477-86, 1993.

Anderson S, Rennke HG, Garcia DL, and Brenner BM. Short and long term effects of antihypertensive therapy in the diabetic rat. *Kidney Int* 36: 526-36, 1989.

Ardaillou R. Angiotensin II receptors. *J Am Soc Nephrol* 10 Suppl 11: S30-9, 1999.

Atiyeh BA, Arant BS, Jr., Henrich WL, and Seikaly MG. In vitro production of angiotensin II by isolated glomeruli. *Am J Physiol* 268: F266-72, 1995.

Ayo SH, Radnik R, Garoni JA, Troyer DA, and Kreisberg JI. High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am J Physiol* 261: F571-7, 1991.

Badr KF, DeBoer DK, Takahashi K, Harris RC, Fogo A, and Jacobson HR. Glomerular responses to platelet-activating factor in the rat: role of thromboxane A2. *Am J Physiol* 256: F35-43, 1989.

Badr KF, Murray JJ, Breyer MD, Takahashi K, Inagami T, and Harris RC. Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney. Elucidation of signal transduction pathways. *J Clin Invest* 83: 336-42, 1989.

Ballermann BJ, Skorecki KL, and Brenner BM. Reduced glomerular angiotensin II receptor density in early untreated diabetes mellitus in the rat. *Am J Physiol* 247: F110-6, 1984.

Bank N, Lahorra G, Aynedjian HS, and Wilkes BM. Sodium restriction corrects hyperfiltration of diabetes. *Am J Physiol* 254: F668-76, 1988.

Barajas L. Cell-specific protein and gene expression in the juxtaglomerular apparatus. *Clin Exp Pharmacol Physiol* 24: 520-6, 1997.

Bell RB. Protein Kinase C activation by diacylglycerol second messengers. *Cell* 45: 631-632, 1986.

Bleyer AJ, Fumo P, Snipes ER, Goldfarb S, Simmons DA, and Ziyadeh FN. Polyol pathway mediates high glucose-induced collagen synthesis in proximal tubule. *Kidney Int* 45: 659-66, 1994.

Border WA, and Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331: 1286-92, 1994.

Boyd-White J, and Williams JC, Jr. Effect of cross-linking on matrix permeability. A model for AGE- modified basement membranes. *Diabetes* 45: 348-53, 1996.

Braam B, Mitchell KD, Fox J, and Navar LG. Proximal tubular secretion of angiotensin II in rats. *Am J Physiol* 264: F891-8, 1993.

Brown L, Wall D, Marchant C, and Sernia C. Tissue-specific changes in angiotensin II receptors in streptozotocin- diabetic rats. *J Endocrinol* 154: 355-62, 1997.

Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* 46: 223-34, 1995.

Bucala R, and Vlassara H. Advanced glycosylation end products in diabetic renal and vascular disease. *Am J Kidney Dis* 26: 875-88, 1995.

Burns KD, Homma T, and Harris RC. The intrarenal renin-angiotensin system. *Semin Nephrol* 13: 13-30, 1993.

Capasso G, Unwin R, Ciani F, De Santo NG, De Tommaso G, Russo F, and Giebisch G. Bicarbonate transport along the loop of Henle. II. Effects of acid- base, dietary, and neurohumoral determinants. *J Clin Invest* 94: 830-8, 1994.

Cassis LA. Downregulation of the renin-angiotensin system in streptozotocin- diabetic rats. *Am J Physiol* 262: E105-9, 1992.

Chansel D, Dussaule JC, Ardaillou N, and Ardaillou R. Identification and regulation of renin in human cultured mesangial cells. *Am J Physiol* 252: F32-8, 1987.

Chen M, Harris MP, Rose D, Smart A, He XR, Kretzler M, Briggs JP, and Schnermann J. Renin and renin mRNA in proximal tubules of the rat kidney. *J Clin Invest* 94: 237-43, 1994.

Cheng HF, Burns KD, and Harris RC. Reduced proximal tubule angiotensin II receptor expression in streptozotocin-induced diabetes mellitus. *Kidney Int* 46: 1603-10, 1994.

Clark CM, Jr., and Lee DA. Prevention and treatment of the complications of diabetes mellitus [published erratum appears in N Engl J Med 1995 Sep 21;333(12):810]. *N Engl J Med* 332: 1210-7, 1995.

Cogan MG. Regulation and control of bicarbonate reabsorption in the proximal tubule. *Semin Nephrol* 10: 115-21, 1990.

Cohen AJ, Rossetti, R.G., Stoff, J.S. Glomerular hyperfiltration occurs in streptozotocin-diabetic (STZ-D) but not in spontaneously diabetic rats. (abstract) *Kidney Int* 35: 425, 1989.

Cohen AJ, McGill PD, Rossetti RG, Guberski DL, and Like AA. Glomerulopathy in spontaneously diabetic rat. Impact of glycemic control. *Diabetes* 36: 944-51, 1987.

DCCT: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med* 329: 977-86, 1993.

Dean DC, McQuillan JJ, and Weintraub S. Serum stimulation of fibronectin gene expression appears to result from rapid serum-induced binding of nuclear proteins to a cAMP response element. *J Biol Chem* 265: 3522-7, 1990.

Dossetor JB. Creatininemia versus uremia. The relative significance of blood urea nitrogen and serum creatinine concentrations in azotemia. *Ann Intern Med* 65: 1287-99, 1966.

Dostal DE, Rothblum KN, and Baker KM. An improved method for absolute quantification of mRNA using multiplex polymerase chain reaction: determination of renin and angiotensinogen mRNA levels in various tissues. *Anal Biochem* 223: 239-50, 1994.

Douglas JG, Romero M, and Hopfer U. Signaling mechanisms coupled to the angiotensin receptor of proximal tubular epithelium. *Kidney Int Suppl* 30: S43-7,1990.

Everett AD, Scott J, Wilfong N, Marino B, Rosenkranz RP, Inagami T, and Gomez RA. Renin and angiotensinogen expression during the evolution of diabetes. *Hypertension* 19: 70-8, 1992.

Feld LG, Cachero S, Ellis EN, Dedeoglu O, Ueda Y, Leonard C, Letizia R, Mallon V, and Van Liew JB. Resistance to glomerular injury in the diabetic biobreeding rat. *Exp Physiol* 80: 991-1000, 1995.

Ferri C, Laurenti O, Bellini C, Faldetta MR, Properzi G, Santucci A, and De Mattia G. Circulating endothelin-1 levels in lean non-insulin-dependent diabetic patients. Influence of ACE inhibition. *Am J Hypertens* 8: 40-7, 1995.

Franza BR, Jr., Rauscher FJd, Josephs SF, and Curran T. The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. *Science* 239: 1150-3, 1988.

Fukui M, Nakamura T, Ebihara I, Makita Y, Osada S, Tomino Y, and Koide H. Effects of enalapril on endothelin-1 and growth factor gene expression in diabetic rat glomeruli. *J Lab Clin Med* 123: 763-8, 1994.

Gilbert RE, Cox A, Wu LL, Allen TJ, Hulthen UL, Jerums G, and Cooper ME. Expression of transforming growth factor-beta1 and type IV collagen in the renal tubulointerstitium in experimental diabetes: effects of ACE inhibition. *Diabetes* 47: 414-22, 1998.

Gomez RA, and Norwood VF. Developmental consequences of the renin-angiotensin system. *Am J Kidney Dis* 26: 409-31, 1995.

Guyton AC. Textbook of medical physiology, Eighth edition. W.B. Saunders Company, Philadelphia, Pennsylvania, p.286-288, 1991.

Harrison-Bernard LM, Navar LG, Ho MM, Vinson GP, and el-Dahr SS. Immunohistochemical localization of ANG II AT1 receptor in adult rat kidney using a monoclonal antibody. *Am J Physiol* 273: F170-7, 1997.

Hostetter TH, Troy JL, and Brenner BM. Glomerular hemodynamics in experimental diabetes mellitus. *Kidney Int* 19: 410-5, 1981.

Houillier P, Chambrey R, Achard JM, Froissart M, Poggioli J, and Paillard M. Signaling pathways in the biphasic effect of angiotensin II on apical Na/H antiport activity in proximal tubule. *Kidney Int* 50: 1496-505, 1996.

Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa I, Hogan BL, and Inagami T. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* 377: 748-50, 1995.

Ihm CG, Lee GS, Nast CC, Artishevsky A, Guillermo R, Levin PS, Glassock RJ, and Adler SG. Early increased renal procollagen alpha 1(IV) mRNA levels in streptozotocin induced diabetes. *Kidney Int* 41: 768-77, 1992.

Inagami T. Molecular biology and signaling of angiotensin receptors: an overview. *J Am Soc Nephrol* 10 Suppl 11: S2-7, 1999.

Ingelfinger JR, Zuo WM, Fon EA, Ellison KE, and Dzau VJ. In situ hybridization evidence for angiotensinogen messenger RNA in the rat proximal tubule. An hypothesis for the intrarenal renin angiotensin system. *J Clin Invest* 85: 417-23, 1990.

Inishi Y, Uchida, S., Kaname, S., Ogata, E., Kurokawa, K. High ambient glucose down-regulates endothelin-1 receptors in cultured rat mesangial cells. (abstract) *J Am Soc Nephrol* 2: 291, 1991.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, and Masaki T. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A* 86: 2863-7, 1989.

Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, and King GL. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* 272: 728-31, 1996.

Iwai N, and Inagami T. Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett* 298: 257-60, 1992.

Kalinyak JE, Sechi LA, Griffin CA, Don BR, Tavangar K, Kraemer FB, Hoffman AR, and Schambelan M. The renin-angiotensin system in streptozotocin-induced diabetes mellitus in the rat. *J Am Soc Nephrol* 4: 1337-45, 1993.

Kambayashi Y, Bardhan S, Takahashi K, Tsuzuki S, Inui H, Hamakubo T, and Inagami T. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem* 268: 24543-6, 1993.

Karim Z, Defontaine N, Paillard M, and Poggioli J. Protein kinase C isoforms in rat kidney proximal tubule: acute effect of angiotensin II. *Am J Physiol* 269: C134-40, 1995.

Kigoshi T, Imaizumi N, Azukizawa S, Yamamoto I, Uchida K, Konishi F, and Morimoto S. Effects of angiotensin II, adrenocorticotropin, and potassium on aldosterone production in adrenal zona glomerulosa cells from streptozotocin-induced diabetic rats. *Endocrinology* 118: 183-8, 1986.

Kikutani H, and Makino S. The murine autoimmune diabetes model: NOD and related strains. *Adv Immunol* 51: 285-322, 1992.

Kim SJ, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, and Roberts AB. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. *Mol Cell Biol* 10: 1492-7, 1990.

King AJ, Brenner BM, and Anderson S. Endothelin: a potent renal and systemic vasoconstrictor peptide. *Am J Physiol* 256: F1051-8, 1989.

Koeppen BM, and Stanton BA. Renal Physiology, Second edition. Mosby-Year Book Inc., St. Louis, Missouri, p. 35 and p. 95-106, 1997.

Koike G, Krieger JE, Jacob HJ, Mukoyama M, Pratt RE, and Dzau VJ. Angiotensin converting enzyme and genetic hypertension: cloning of rat cDNAs and characterization of the enzyme. *Biochem Biophys Res Commun* 198: 380-6, 1994.

Kreisberg JI, Radnik RA, Ayo SH, Garoni J, and Saikumar P. High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cell cultures. *Kidney Int* 46: 105-12, 1994.

Lai KN, Lai KB, and Leung JCK. Gene expression of the renin-angiotensin system (RAS) in human glomerular cells. (abstract) *XIIIth International Congress of Nephrology*: 102, 1995.

Lawrence JC, Jr., and Abraham RT. PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem Sci* 22: 345-9, 1997.

Levine DZ, Iacovitti M, Buckman S, and Harrison V. In vivo modulation of rat distal tubule net HCO3 flux by VIP, isoproterenol, angiotensin II, and ADH. *Am J Physiol* 266: F878-83, 1994.

Lo M, Liu KL, Lantelme P, and Sassard J. Subtype 2 of angiotensin II receptors controls pressure-natriuresis in rats. *J Clin Invest* 95: 1394-7, 1995.

Miller JA. Renal responses to sodium restriction in patients with early diabetes mellitus. *J Am Soc Nephrol* 8: 749-55, 1997.

Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE, and Dzau VJ. Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. *J Biol Chem* 268: 24539-42, 1993.

Murphy M, McGinty A, and Godson C. Protein kinases C: potential targets for intervention in diabetic nephropathy. *Curr Opin Nephrol Hypertens* 7: 563-70, 1998.

Nakamura T, Ebihara I, Fukui M, Tomino Y, and Koide H. Effect of a specific endothelin receptor A antagonist on mRNA levels for extracellular matrix components and growth factors in diabetic glomeruli. *Diabetes* 44: 895-9, 1995.

Nathan DM. Long-term complications of diabetes mellitus. *N Engl J Med* 328: 1676-85, 1993.

O'Brien BA, Harmon BV, Cameron DP, and Allan DJ. Beta-cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. *J Pathol* 178: 176-81, 1996.

Okano R, Mita T, and Matsui T. Characterization of a novel promoter structure and its transcriptional regulation of the murine laminin B1 gene. *Biochim Biophys Acta* 1132: 49-57, 1992.

Ozono R, Wang ZQ, Moore AF, Inagami T, Siragy HM, and Carey RM. Expression of the subtype 2 angiotensin (AT2) receptor protein in rat kidney. *Hypertension* 30: 1238-46, 1997.

Pain VM. Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem* 236: 747-71, 1996.

Pedersen MM, Christiansen JS, and Mogensen CE. Reduction of glomerular hyperfiltration in normoalbuminuric IDDM patients by 6 mo of aldose reductase inhibition. *Diabetes* 40: 527-31, 1991.

Pollock CA, Lawrence JR, and Field MJ. Tubular sodium handling and tubuloglomerular feedback in experimental diabetes mellitus. *Am J Physiol* 260: F946-52, 1991.

Rehberg PB. Studies on kidney function. The rate of filtration and reabsorption in the human kidney. *Biochem J.*, 20: 447, 1926.

Remuzzi A, Perico N, Amuchastegui CS, Malanchini B, Mazerska M, Battaglia C, Bertani T, and Remuzzi G. Short- and long-term effect of angiotensin II receptor blockade in rats with experimental diabetes. *J Am Soc Nephrol* 4: 40-9, 1993.

Rerup CC. Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol Rev* 22: 485-518, 1970.

Riser BL, Cortes P, Heilig C, Grondin J, Ladson-Wofford S, Patterson D, and Narins RG. Cyclic stretching force selectively up-regulates transforming growth factor-beta isoforms in cultured rat mesangial cells. *Am J Pathol* 148: 1915-23, 1996.

Roberts AB, McCune BK, and Sporn MB. TGF-beta: regulation of extracellular matrix. *Kidney Int* 41: 557-9, 1992.

Ruiz-Ortega M, and Egido J. Angiotensin II modulates cell growth-related events and synthesis of matrix proteins in renal interstitial fibroblasts. *Kidney Int* 52: 1497-510, 1997.

Rzymkiewicz D, Leingang K, Baird N, and Morrison AR. Regulation of prostaglandin endoperoxide synthase gene expression in rat mesangial cells by interleukin-1 beta. *Am J Physiol* 266: F39-45, 1994.

Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, and Inagami T. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature* 351: 230-3, 1991.

Schmitz U, and Berk BC. Angiotensin II signal trasduction: Stimulation of multiple mitogen-activated protein kinase pathways. *Trends Endocrinol Metab* 8: 261-266, 1997.

Seikaly MG, Arant BS, Jr., and Seney FD, Jr. Endogenous angiotensin concentrations in specific intrarenal fluid compartments of the rat. *J Clin Invest* 86: 1352-7, 1990.

Sharma K, Danoff, T.M., DePiero, A., et. al. Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: possible mediation via protein kinase C. *Biochem Biophys Res Commun* 207: 80-88, 1995.

Sharma K, and Ziyadeh FN. The emerging role of transforming growth factor-beta in kidney diseases. *Am J Physiol* 266: F829-42, 1994.

Siragy HM, and Carey RM. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 100: 264-9, 1997.

Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R, and Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest* 95: 651-7, 1995.

Tank JE, Henrich WL, and Moe OW. Regulation of glomerular and proximal tubule renin mRNA by chronic changes in dietary NaCl. *Am J Physiol* 273: F892-8, 1997.

Timmermans PB, Chiu AT, Herblin WF, Wong PC, and Smith RD. Angiotensin II receptor subtypes. *Am J Hypertens* 5: 406-10, 1992.

Tufro-McReddie A, and Gomez RA. Ontogeny of the renin-angiotensin system. *Semin Nephrol* 13: 519-30, 1993.

Vallon V, Kirschenmann D, Wead LM, Lortie MJ, Satriano J, Blantz RC, and Thomson SC. Effect of chronic salt loading on kidney function in early and established diabetes mellitus in rats. *J Lab Clin Med* 130: 76-82, 1997.

Vallon V, Wead LM, and Blantz RC. Renal hemodynamics and plasma and kidney angiotensin II in established diabetes mellitus in rats: effect of sodium and salt restriction. *J Am Soc Nephrol* 5: 1761-7, 1995.

Velasquez MT, Kimmel PL, and Michaelis OEt. Animal models of spontaneous diabetic kidney disease. *Faseb J* 4: 2850-9, 1990.

Vinay P, Gougoux A, and Lemieux G. Isolation of a pure suspension of rat proximal tubules. *Am J Physiol* 241: F403-11, 1981.

Wilkes BM. Reduced glomerular angiotensin II receptor density in diabetes mellitus in the rat: time course and mechanism. *Endocrinology* 120: 1291-8, 1987.

Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, van den Enden M, Kilo C, and Tilton RG. Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42: 801-13, 1993.

Yamada T, Horiuchi M, and Dzau VJ. Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci U S A* 93: 156-60, 1996.

Yamauchi T, Ohnaka K, Takayanagi R, Umeda F, and Nawata H. Enhanced secretion of endothelin-1 by elevated glucose levels from cultured bovine aortic endothelial cells. *FEBS Lett* 267: 16-8, 1990.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411-5, 1988.

Zatz R, Dunn BR, Meyer TW, Anderson S, Rennke HG, and Brenner BM. Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J Clin Invest* 77: 1925-30, 1986.

Zhou X, Li C, Dlugosz J, Kapor-Drezgic J, Munk S, and Whiteside C. Mesangial cell actin disassembly in high glucose mediated by protein kinase C and the polyol pathway. *Kidney Int* 51: 1797-808, 1997.

Zhuo J, Dean R, MacGregor D, Alcorn D, and Mendelsohn FA. Presence of angiotensin II AT2 receptor binding sites in the adventitia of human kidney vasculature. *Clin Exp Pharmacol Physiol Suppl* 3: S147-54, 1996.

Ziyadeh FN. Renal tubular basement membrane and collagen type IV in diabetes mellitus. *Kidney Int* 43: 114-20, 1993.

Zou LX, Imig JD, von Thun AM, Hymel A, Ono H, and Navar LG. Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension* 28: 669-77, 1996.