CHAPTER 4

ELUCIDATION OF ROLE OF NITRIC OXIDE IN 5/6<sup>th</sup> (SUBTOTAL) NEPHRECTOMIZED-INDUCED PROGRESSIVE RENAL DAMAGE
Chapter 4

Introduction

Progression to end-stage renal failure (ESRF) is the final common pathway of many forms of glomerular disease independent of the type of initial insult (Remuzzi et al., 1997). Models of renal mass reduction (RMR) have been extensively used to investigate the pathogenic mechanisms responsible for the progressive nature of chronic renal disease in humans (Hostetter et al., 1981; Klahr et al., 1988; Neuringer and Brenner, 1993; Griffin et al., 2004). The kidney’s adaptive response to surgical reduction in nephron number in the rat appears close enough to the pathophysiological characteristics of human progressive nephropathies.

The progression of renal damage resulting from reduced nephron mass has been extensively studied in the 5/6Nx model in the rat, as described originally by Shimamura and Morrison (1975). Ablation of 85-90% of the total kidney mass result in the functional and structural changes in the remnant renal tissue. Animals with a remnant kidney develop systemic hypertension and exhibit a decrease in both glomerular filtration rate (GFR) and effective renal plasma flow (ERBF) despite an adaptive increase in single Nephron GFR and plasma flow per Nephron. Rats with reduced renal mass also develop proteinuria, progressive azotemia, and structural changes in the kidney, including progressive glomerulosclerosis and tubulointerstitial disease, which eventually lead to renal insufficiency and death due to uremia (Reyes et al., 1992). Different experimental maneuvers have been employed in an effort to halt or reduce the rate of progression of renal insufficiency in this remnant model of kidney disease. The most commonly used strategies have included dietary modifications and treatment of systemic hypertension. Among the former, protein restriction (Klahr, 1989), phosphate restriction (Alfrey, 1988), and modifications of dietary lipids (Heifets et al., 1987; Schmitz et al., 1989), and among the latter, administration of angiotensin-converting enzyme inhibitors (ACEIs) (Noda et al., 1997; 1999) have consistently demonstrated a beneficial effect on the hemodynamic changes and morphological alterations that occur in this model.

Proteinuria is considered to reflect glomerular capillary wall damage and glomerular hypertension in a variety of renal diseases (Hostetter et al., 1982; Glassock, 1990). The decreased autoregulatory ability associated with the reduction in functional renal mass (Anderson et al., 1985) exposes the glomeruli to the systemic blood pressure leading to
glomerulosclerosis caused by glomerular hypertension, which is consistent with the reports that angiotensin converting enzyme inhibitors have a beneficial effect on the progression of chronic renal failure in animal models (Brunner et al., 1989; Noda et al., 1999) and in patients (Lewis et al., 1993).

Nitric oxide (NO), the main endothelium-derived relaxing factor, regulates various hemodynamic and non-hemodynamic biological phenomena. There is increasing evidence that NO is tonically synthesized within the kidney and that NO plays a crucial role in the regulation of renal hemodynamics and excretory function (Baylis et al., 1990; Thorup et al., 1996) suggesting that the modulation of renal and/or systemic NO production may affect the progression of renal diseases. In fact, long-term nitric oxide synthase inhibition has also been reported to promote proteinuria and glomerulosclerosis in normal (Baylis et al., 1992) and subtotal nephrectomized rats (Fujihara et al., 1995). The mechanism responsible for the worsening of renal disease by NO inhibition is not known, but may relate to the increased systemic and glomerular blood pressure in these rats. The modulation of systemic and/or renal NO metabolism appears to be a logical approach to change the course of chronic renal failure (CRF). Based on such an assumption, Reyes et al. (1992) found that six weeks of oral supplementation with 1% arginine solution in drinking water started immediately after 7/8th renal mass reduction resulted in a complete normalization of GFR, RBF, FEno/o protein excretion, along with lesser glomerulosclerosis and interstitial damage. Furthermore, the circulating L-arginine level was normal before and at the end of the study. In a micropuncture study, the same group (Katoh et al., 1994) suggested that the long-term administration of L-arginine in subtotally nephrectomized rats resulted in a reduction of intraglomerular capillary pressure and efferent arteriolar resistance, probably by antagonizing angiotensin-II effect.

The present work was undertaken to study the protective effect of nitric oxide donors L-arginine, molsidomine, nebivolol, resveratrol, pravastatin and the nitric oxide synthase inhibitors L-NAME (non specific NOS inhibitor), aminoguanidine (specific iNOS inhibitor) and L-NIO (specific eNOS inhibitor) on the progressive renal dysfunction of the remnant kidney model and to explore the involvement of specific nitric oxide synthase isoform in this model.
MATERIALS AND METHODS

Male Wistar rats (Central animal house, Panjab University, Chandigarh, India) with initial body weight of 200 to 250 g. were used in these studies. Animal care and treatment were conducted in conformity with Institutional Animal Ethical Committee (IAEC), Panjab University, Chandigarh. All animals were housed in standard light/dark cycle with free access to rat chow and tap water ad libitum.

Drugs: L-arginine (Himedia, Mumbai), Pravastatin (Ranbaxy Research Laboratories, Gurgaon, India), L-NAME (Sigma, USA), aminoguanidine (Himedia, Mumbai), L-NIO (Caymen Chemicals, USA) were dissolved in distilled water. Nebivolol (Jenessan Pharmaceutica, Belgium), resveratrol (Sigma, St. Louis, MI, USA), molsidomine (Caymen Chemicals, USA) were suspended in 0.25% carboxy methyl cellulose (CMC). All the drugs were freshly prepared at the beginning of each experimental protocol.

EXPERIMENTAL PROTOCOLS: Before surgery, the animals were housed in metabolic cages. A 24 hour urine was collected from each rat for laboratory investigations. Afterwards, animals underwent a two-stage 5/6 nephrectomy (5/6 NX) (interval of one week) under ketamine anesthesia (50 mg/kg, i.p.).

5/6th Nephrectomy (Subtotal nephrectomy):

Animal Preparation:
✓ Animals were fasted night before surgery.
✓ Animals were prepared for aseptic surgery using sterile instruments

Surgical Protocol:
✓ Laparotomy to expose left kidney was performed
✓ Top 1/3 and bottom 1/3 of left kidney was removed (Fig. A)
✓ Two pieces of gel foam were applied to cover the cut surface of the kidney
✓ Upper and lower 1/3 portion of kidney was cut off in one stroke and were immediately covered with gel foam, exerting mild pressure.
✓ After cutting off second pole of kidney, mild pressure on gel foam pads was maintained for approximately 1 minute.
Laparotomy was closed.

1 Week Later:

- Right side nephrectomy was performed by retroperitoneal approach.
- Kidney area was shaved.
- Palpate right kidney.
- Longitudinal incision 2 cm long parallel to spine and 1 cm lateral was made.
- Back muscles were dissected to expose kidney.
- All vessels of right kidney were ligated and extirpated.
- Skin was closed.

Experimental Groups:

Group 1: (Sham) (n=6) comprised of Sham-operated rats.
Group 2: (STNx) (n=8) 5/6th NX rats, no drug therapy.
Group 3: (STNx + L-arg) (n=8) 5/6 NX rats were treated with L-arginine (125 mg/L, in drinking water) and L-NAME (10 mg/kg, i.p.)
Group 4: (STNx + Mols) (n=8) 5/6th NX rats, but the animals were treated with molsidomine (10 mg/kg, p.o.)
Group 5: (STNx + Neb) (n=8) 5/6th NX rats were treated with nebivolol (1 m
Group 6: (STNx + RVT) (n=8) 5/6th NX rats were treated with resveratrol (5
Group 7: (STNx + Pra) (n=8) 5/6th NX rats were treated with pravastatin (20
Group 8: (STNx + L-NAME) (n=8) 5/6th NX rats were treated with L-NAM
i.p.)
Group 9: (STNx + L-arg + L-NAME) (n=8) 5/6th NX rats were treated wit
Group 10: (STNx + Mol + L-NAME) (n=8) 5/6th NX rats were treated with molsidomine (10 mg/kg, p.o.) and L-NAME (10 mg/kg, i.p.)

Group 11: (STNx + Neb+ L-NAME) (n=8) 5/6th NX rats were treated with nebivolol (1 mg/kg, p.o.) and L-NAME (10 mg/kg, i.p.)

Group 12: (STNx + RVT + L-NAME) (n=8) 5/6th NX rats were treated with resveratrol (5 mg/kg, p.o.) and L-NAME (10 mg/kg, i.p.)

Group 13: (STNx + Pra + L-NAME) (n=8) 5/6th NX rats were treated with pravastatin (20 mg/kg, i.p.) and L-NAME (10 mg/kg, i.p.)

Group 14: (STNx + Amg) (n=8) 5/6th NX rats were treated with aminoguanidine (100 mg/kg, i.p.)

Group 15: (STNx + L-NIO) (n=6) 5/6th NX rats were treated with L-NIO (5 mg/kg, i.p.)

The drug therapy lasted for 12 weeks, during which 24-hour urine was collected from all seven groups at weeks 0, 4, 8 and 12. Systolic Blood Pressure (SBP) was recorded by tail cuff method (UGO Basile, Italy) at week 0, 4, 8 & 12. Urinary protein excretion was measured by twenty four-hour urine collection using metabolic cages & proteinuria was determined by the biuret method at week 0, 4, 8 & 12.

Renal Function Measurement:
Blood urea nitrogen (BUN) and creatinine were determined in all blood samples (from tail vein) by using standard diagnostic kits (Span Diagnostics, Gujarat, India) along with urea and creatinine clearances.

Estimation of urine nitrite and nitrate levels:
As per chapter 1.

Evaluation of renal histology:
At the end of experiment (at week 12), the rats were sacrificed and their kidneys were removed and washed with ice-cold saline. It was then fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. 5 μm thick sections were cut, deparaffinized, hydrated and stained with periodic acid shiff stain (PAS). At least 50 consecutive cortical glomeruli were counted. Each glomerulus was assigned to one to five categories based on the degree of detectable morphological damage: 0, no visible pathologic change; 1, mesangial deposits with some mesangial hypercellularity; 2, appearance of focal areas of sclerosis with or without generalized
mesangial thickening; 3, segmental sclerosis with loop collapse, and blebs in epithelial cells; & 4 global sclerosis, hyalinosis and fibrosis.

STATISTICAL ANALYSIS
The data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test for comparing means from different treatment groups. The data were expressed as mean ± SEM and a value of $p<0.05$ was considered statistically significant.

RESULTS:
Food intake & Body weight:
The food intake was similar in all the experimental groups for the entire study period (12 weeks). Rats with reduced renal mass treated with NO donors gained weight in a similar manner as untreated rats, however animals treated with L-NAME and L-NIO exhibited a slight fall in body weight after 12 weeks (not significant).

Animal Survival:
The survival in the untreated 5/6th nephrectomized group, L-NAME and L-NIO treated group was 75% and 60% respectively, as compared to sham-operated rats at week eight, which was increased significantly in animals treated with L-arginine, molsidomine, nebulol, resveratrol, pravastatin and aminoguanidine till the end of study. However animals treated with L-NAME and L-NIO had a significant lower survival.

Systolic Blood Pressure:
Serial values of SBP are shown in Fig.1. The STNx animals exhibited a significant increase in systolic blood pressure (SBP) starting from week 4 onwards. The administration of L-arginine (125 mg/L), molsidomine (5 mg/kg), nebulol (1 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) in STNx rats resulted in a significant lower systolic blood pressure after 4,8 and 12 weeks of therapy compared with the untreated STNx rats. The combination of L-arginine + L-NAME, resveratrol + L-NAME, pravastatin and L-NAME resulted in a significant increase in SBP as compared to L-arginine, resveratrol and pravastatin in 5/6th nephrectomized rats, however animals
treated with molsidomine + L-NAME and nebivolol and L-NAME had similar SBP as that of molsidomine and nebivolol alone in 5/6th nephrectomized rats. The administration of non specific NOS inhibitor (L-NAME) and specific eNOS inhibitor (L-NIO) in the 5/6th nephrectomized rats further increased the systolic blood pressure (SBP) which lasted throughout the study period compared with the STNx rats, however animals treated with aminoguanidine exhibited similar SBP as that of STNx group.

**Urinary protein excretion:**
Changes in urinary protein excretion during the study period are shown in Fig.2. Proteinuria values were similar in all animals at the beginning of the experiment. After 12 weeks of therapy, the group treated with L-arginine (125 mg/L), molsidomine (5 mg/kg), nebivolol (1 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) in STNx rats resulted in a significant lower urinary protein excretion after 4,8 and 12 weeks of therapy compared with the untreated STNx rats. The addition of L-NAME in L-arginine, resveratrol and pravastatin treated groups resulted in a significant increase in urinary protein excretion as compared to L-arginine, resveratrol, pravastatin in 5/6th nephrectomized rats. However animals treated with molsidomine + L-NAME and nebivolol and L-NAME showed similar levels of proteinuria as that of molsidomine and nebivolol alone in 5/6th nephrectomized rats. The administration of L-NAME, aminoguanidine and specific eNOS inhibitor (L-NIO) did not influence the course of protein excretion in 5/6th nephrectomized rats.

**Urinary levels of NO metabolites:**
At the end of the study, the untreated 5/6th nephrectomized rats had a much lower urinary NO$_2$ + NO$_3$ excretion (1.95±0.72 μmol/L) compared with untreated sham-operated animals (26.2 ± 0.8 μmol/L) (p<0.001). L-arginine, molsidomine, nebivolol, resveratrol, and pravastatin therapy raised the urinary excretion of NO$_2$ + NO$_3$ to normal values (same as untreated sham-operated rats) at the end of the study. The addition of L-NAME significantly diminished this effect of L-arginine, resveratrol, pravastatin but this was not observed in case of animals treated with molsidomine and nebivolol. Treatment of 5/6th nephrectomized rats with L-NAME and L-NIO alone had significant lower NO$_2$ + NO$_3$ levels, however same was not in the case of aminoguanidine treated rats (Fig.3.).
Renal Function Test:
Renal function (comprising of serum creatinine levels, blood urea nitrogen (BUN) levels, creatinine and urea clearance) was impaired significantly at the end of the study in the 5/6th nephrectomized rats (Fig.4a,b,c,d.). Treatment with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin significantly limited the renal function deterioration, while the animals treated with L-NAME in combination with L-arginine, resveratrol and pravastatin had impaired renal dysfunction, which was not observed in case of animals treated with L-NAME along with molsidomine and nebivolol. The administration of L-NAME and L-NIO to the STNx rats further worsened the renal function; however same was not the case in the animals treated with aminoguanidin.

Renal Histology:
The kidneys of untreated rats exhibited a significant increase in the number of glomeruli with sclerotic lesions and interstitial fibrosis as compared to sham-operated rats. Treatment of animals with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin inhibited the progression of glomerulosclerosis and tended to prevent the interstitial fibrosis, however rats treated with L-NAME along with L-arginine, resveratrol and pravastatin exhibited a significant increase in the glomerulosclerosis, which was not in case of animals treated with L-NAME along with molsidomine and nebivolol. Treatment of STNx rats with L-NAME, aminoguanidin and L-NIO alone exhibited a similar degree of morphological damage as that of untreated STNx rats (Fig.5.).
Fig. 1. Effect of L-arginine (125 mg/L, in drinking water), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), aminoguanidine (100 mg/kg, i.p.) and L-NIO (5 mg/kg, i.p.) on systolic blood pressure in 5/6 nephrectomized rats. The values are expressed as mean ± S.E.M. *p<0.05 as compared to week 0, a p<0.05 as compared to STNx group (one-way ANOVA followed by Newman Keuls test).
**Fig. 2.** Effect of L-arginine (125 mg/L, in drinking water), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), aminoguanidine (100 mg/kg, i.p.) and L-NIO (5 mg/kg, i.p.) on proteinuria in 5/6th nephrectomized rats. The values are expressed as mean ± S.E.M. *p<0.05 as compared to week 0, a p<0.05 as compared to STNx group (one-way ANOVA followed by Newman Keuls test).
Fig. 3. Effect of L-arginine (125 mg/L, in drinking water), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), aminoguanidine (100 mg/kg, i.p.) and L-NIO (5 mg/kg, i.p.) on urinary \( \text{NO}_2 + \text{NO}_3 \) in 5/6\(^{th}\) nephrectomized rats. The values are expressed as mean ± S.E.M. *\( p < 0.05 \) as compared to week 0, a
Effect of L-arginine (125 mg/L, in drinking water), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), aminoguanidine (100 mg/kg, i.p.) and L-NIO (5 mg/kg, i.p.) on plasma creatinine (Fig. 4A.), blood urea nitrogen (BUN) (Fig. 4B.), creatinine (Fig. 4C.) and urea clearance (Fig. 4D.) in 5/6 nephrectomized rats. The values are expressed as mean ± S.E.M. *p<0.05 as compared to Sham, a p<0.05 as compared to STNx group (one-way ANOVA followed by Newman Keuls test).
Periodic Acid-Schiff (PAS) stained longitudinal sections of kidneys of sham rat (A), 5/6 subtotal Nephrectomized (STNx) rat (B), L-arg+STNx treated rat (C), Mol+STNx treated rat (D), Neb+STNx treated rat (E) and RVT+STNx treated rat (F) (312.5X)
Periodic Acid-Schiff (PAS) stained longitudinal sections of kidneys of Pra+St treated rat (G), L-NAME+STNx treated rat (H), L-NAME+L-arg+STNx treated (I), L-NAME+Mol+STNx treated rat (J), L-NAME+Neb+STNx treated rat (K), L-NAME+RVT+STNx treated rat (L) (312.5X)
Periodic Acid-Shiff (PAS) stained longitudinal sections of kidneys NAME+Pra+STNx treated rat (M), Amg+STNx treated rat (N) and L-? treated rat (O) (312.5X)

DISCUSSION:
The remnant kidney model is widely considered to be a classical model of renal disease. In this model, the Nephron number is reduced by surgical infarction of kidney tissue. Then 5/6Nx model in the rat has been widely used the effect of treatment strategies on the progression scarring of the kidney associated with extensive renal ablation. Because the initial effects of the Nephron mass involves hypertension and hyperfiltration of remnant nephron majority of research efforts have been directed to determine if the corre altered hemodynamics improves the renal damage.
Given the vasodilator, antiproliferative and antiplatelet actions of NO, basal, continuous NO production is believed to be central to circulatory regulation and to the maintenance of renal structural integrity (Zatz and Baylis, 1998). Accordingly, NO deficiency has been consistently shown in association with human (Schmidt and Baylis, 2000) and experimental (Ashab et al., 1995; Aiello et al., 1997) renal insufficiency. Administration of a NO donor attenuated renal injury in the Nephrectomized (Nx) model (Benigni et al., 1999). Conversely, it is well established that chronic inhibition of NO synthesis promotes systemic hypertension and severe renal injury in intact rats (Ribeiro et al., 1992; Baylis et al., 1992) and aggravates renal damage in Nx rats (Fujihara et al., 1995).

The mechanisms by which the overall synthesis of NO is diminished in chronic nephropathies and in the Nx model have not been completely elucidated. Low NO production could reflect the renal mass reduction as evident in this model, although the contribution of the kidneys to the total NO synthesis may be small even in the normal condition when compared with the output of other organs (Schmidt and Baylis, 2000). It should be stressed that enhanced iNOS expression has been reported in association with a number of clinical and experimental nephropathies (Sugimoto et al., 1999; Li et al., 1996; Goto et al., 1995) as well as in inflammatory disorders occurring in other tissues (Connor et al., 1995; Vane et al., 1994).

Previous studies have shown that in the remnant kidney model, the progressive development of renal insufficiency was associated with lesser NO formed in the kidney, paralleled by a reduction in the amount of inducible NOS expressed by the kidney (Aiello et al., 1997). A previous study has reported that blockade of NO production in this model is associated with increased systemic and glomerular pressure and a more rapid course of renal scarring (Fujihara et al., 1995). At 12 weeks after 5/6th renal mass reduction, the untreated rats were characterized by marked systemic hypertension and almost three fold lower daily urinary NO$_2$ + NO$_3$ excretion, compared to the sham-operated rats. This reduction had started becoming evident from fourth week onwards. These findings favor the hypothesis that the experimental CRF caused by severe renal mass reduction is characterized by a low renal NO production. The development of hypertension in CRF has been attributed to the accumulation of an endogenous NO synthase inhibitor (Hostetter et al., 1981). Previous in vitro studies have shown that the potency of naturally
occurring inhibitor(s) of NOS in renal insufficiency was overcome by increasing the arginine concentration (MacAllister et al., 1994). In favor of this hypothesis was the high urinary excretion of NO metabolites in the 5/6th nephrectomized rats treated as well as in the sham-operated animals treated with L-arginine. This effect was significantly diminished with the addition of exogenous NOS inhibitor (Ashab et al., 1995).

In our study, L-arginine, molsidomine, nebivolol, resveratrol and pravastatin administration in the 5/6th nephrectomized rats resulted in an increase in urinary $NO_2 + NO_3$ excretion, improved the renal function, decreased proteinuria & systolic blood pressure and reduced the glomerulosclerosis as compared with untreated animals. These protective effects of L-arginine, resveratrol and that of pravastatin were partially blocked by co-administration of L-NAME while the protective effect of molsidomine and nebulol was not blocked by co-administration of L-NAME. The reason for this may be that L-arginine, resveratrol and pravastatin increase NO levels by upregulating the NOS activity, while molsidomine and nebulol are direct NO donors. The 5/6th nephrectomized animals treated with L-NAME and L-NIO alone, further worsened these effects as compared to that of untreated animals, while the 5/6th nephrectomized animals treated with aminoguanidine exhibited almost similar degree of damage as that of untreated STNx rats. Since L-NIO (a specific eNOS inhibitor) worsened the renal damage observed in the STNx animals, this indicates that it is the eNOS inhibition which is primarily responsible for the damaging effect in this progressive renal damage model of chronic renal failure.

The kidneys and, particularly the proximal tubules are a major source of L-arginine synthesis (Boudy et al., 1993). A decrease in NO production or an impaired response to NO may contribute to the initiation or maintenance of the increased intraglomerular high pressure. The present study showed that oral supplementation of L-arginine for 12 weeks in 5/6th nephrectomized rats resulted in a complete normalization of the levels of creatinine, BUN, urea & creatinine clearance, significantly reduced proteinuria and increased the urinary excretion of total nitric oxide contents. These results were accompanied by a relatively good control of the SBP. The protective effect of L-arginine was evident at four weeks after the initiation of therapy and persisted until the end of the study. The progression of glomerulosclerosis was completely abolished by L-arginine
treatment compared with untreated STNx rats, as evident from the morphological studies. When a NOS inhibitor was given in combination with L-arginine, the recovery of renal function was partially abolished.

Molsidomine (MOL) is a prodrug, and a potent vasodilator, has been used widely as an antianginal agent. In the liver, it decarboxylates enzymatically to form SIN-1 (Kukovetz and Holzmann, 1986). In the present study, we found that chronic administration of the NO donor, molsidomine, to rats with remnant kidney, hypertension and overt proteinuria decreased blood pressure, significantly increased the urinary excretion of NO₂⁺NO₃, and had a marginally favorable effect on the progressive deterioration of renal function. However, and of great interest, molsidomine administration significantly prolonged animal survival and almost completely prevented the progression of glomerulosclerosis, as evident from the renal morphological studies. The administration of L-NAME did not abolish the protection afforded by the molsidomine clearly indicating that NOS is not involved in the protection afforded by this compound.

Nebivolol, a new selective β₁-adrenergic blocking agent, is endowed with peripheral vasodilating properties mediated by the modulation of the endogenous production of nitric oxide (NO), as well as additional antioxidative effects (Mangrella et al., 1998). In particular, it has been demonstrated that nebivolol vasodilates human forearm vasculature via the L-arginine/NO pathway (Mangrella et al., 1998). Similar to molsidomine, nebivolol decreased blood pressure, reduced proteinuria, increased urinary NO₂⁺NO₃ excretion, prevented the renal damage and animal survival to a greater degree and almost restored the normal morphology of animals. The addition of L-NAME did not abolish this protective effect of nebivolol, clearly indicating that NOS is not involved in its protective effect. To further confirm that the protective effect observed with nebivolol is due to its nitric oxide donating property and not due to lowering of blood pressure, an extra group was employed consisting of animals treated with propranolol (10 mg/kg, i.p.), where propranolol decreased the blood pressure to a significant extent, but it failed to show any protection in any of the other parameters, thus confirming that effect produced by nebivolol is not only due to its vasodilating property but involves the contribution of non-hemodynamic effects of NO.
Chapter 4

Resveratrol administration in the 5/6th nephrectomized rats resulted in an increase in urinary NO₂+NO₃ excretion, improved the renal function, decreased proteinuria & systolic blood pressure and reduced the glomerulosclerosis as compared with untreated animals. This effect of resveratrol was already present at four weeks of therapy and remained unchanged for the entire duration of the therapy. The concomitant administration of NOS inhibitor, L-NAME, with resveratrol partially prevented the beneficial effects of resveratrol. Also, the long-term administration of resveratrol administration resulted in a high NO₂+NO₃ urinary excretion in the 5/6th nephrectomized rats, which was inhibited in the resveratrol + L-NAME treated rats indicating that resveratrol exerts its effect by increasing the NO levels, may be via upregulating the NOS activity.

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key enzyme that regulates the synthesis of cholesterol from mevalonic acid by suppressing the conversion of HMG-CoA (Goldstein and Brown, 1990). Because of this activity, the clinical use of statins has produced a significant reduction in cardiovascular-related morbidity and mortality in patients with established cardiovascular disease and hypercholesterolemia (Byington et al., 1995; Shepherd et al., 1995). However, mevalonate is the precursor not only of cholesterol but also of many nonsteroidal compounds; inhibition of HMG-CoA reductase by statins may therefore result in pleiotropic effects (Rosenson and Tangney, 1998; Faggiotto and Paoletti, 1999). Statins may thus exert anti-inflammatory and antiarteriosclerotic actions beyond lipid reduction (Ni et al., 2001). The beneficial effects of statins are likely to result from stabilization of unstable atheroma prone to rupture, because cholesterol-lowering therapy with statins increases markers of plaque stability (Shiomi et al., 1995; Libby et al., 1998). Although such effects of statins are predominantly attributed to their lipid-lowering effects, subgroup analysis of the data from clinical trials suggests that there may be beneficial effects independent of their cholesterol-lowering effects (Shepherd et al., 1995; Sacks et al., 1996). Endothelium-dependent nitric oxide (NO)-mediated vasodilatation has been demonstrated to improve in the early phase of statin treatment in humans (Egashira et al., 1994; Treasure et al., 1995; Anderson et al., 1995). It is reported that addition of statins to cultured human endothelial cells increases endothelial-type NO
synthase expression and activity (Laufs et al., 1998). Thus, one may hypothesize that some of the beneficial effects of statins may be due to their effects on endothelial cells which are independent of cholesterol-lowering effects. However, it is unclear whether statins attenuate cardiovascular inflammation and arteriosclerosis in vivo through their cholesterol-lowering independent effects. The renoprotective effects of statins have also been reported in streptozotocin-induced diabetic nephropathy (Kim et al., 2000), puromycin-induced nephrosis (Harris et al., 1990), and unilateral ureteral obstruction (Moriyama et al., 2001). In the present study, the treatment with pravastatin for 12 weeks in 5/6\textsuperscript{th} nephrectomized rats resulted in a normalization of the levels of creatinine, BUN, urea & creatinine clearance, significantly reduced proteinuria and significantly increased the urinary excretion of total nitric oxide contents. These results were accompanied by a relatively good control of the SBP. The effect of pravastatin became evident at four weeks after the initiation of therapy and remained unchanged until the end of the study. The progression of glomerulosclerosis was completely abolished by pravastatin treatment compared with untreated STNx rats, as evident from the morphological studies. When a NOS inhibitor was given in combination with pravastatin, the recovery of renal function as well as prevention of glomerulosclerosis was partially abolished.

Extensive studies by Floege et al. (1992) and Kliem et al. (1996) have shown that in the remnant kidney model, proliferation of intrinsic glomerular and tubulointerstitial cells is an early event in the development of renal damage, being mediated by lymphocytes and macrophage-derived cytokines and growth factors as platelet-derived growth factors and TGF-β. Endogenous NO produced by glomerular and mesangial cells has an inhibitory effect on TGF-β production (Craven, 1997). Thus, NO modulators which enhance renal levels of NO should have a beneficial effect on glomerulosclerosis by limiting the synthesis and favoring the depletion of extracellular matrix by renal metalloproteinases (Roberts and Noble, 1994; Schlondroff, 1995). In addition to the role of NO, increased oxidant stress has been demonstrated in the 5/6 Nx-model (Nath et al., 1990) and this serves as a stimulus for apoptosis, which is an early event in the remnant kidney model (Sugiyama et al., 1996). Although we did not measure oxidative stress in this model, we have established that NO donors combat the oxidative stress in the kidneys and this effect might have played some role in their renoprotective effect in this experimental model.
Increased renal and systemic NO availability after NO donor administration would help limit renal vasoconstriction and reduce proliferation of smooth muscle cells and matrix accumulation that lead to vessel wall thickening and insufficiency (Fabris et al., 1995; Amann et al., 1997). With disease progression and development of overt nephropathy, other mechanisms might prevail, rendering NO no longer capable of counteracting the effect of so many cellular and humoral mediators of organ damage.

In conclusion, chronic renal failure after 5/6th nephrectomy is a low nitric oxide production state. Chronic administration of NO donors and NOS upregulators protects the renal function, possibly by adding the necessary substrate to the endothelial cells damaged by the resultant microcirculatory changes.