CHAPTER 5
CHRONIC NITRIC OXIDE SYNTHASE
INHIBITION-INDUCED HYPERTENSION AND
RENAL ALTERATIONS
Introduction
The relative risk of serious renal damage in patients with uncomplicated essential hypertension is low as compared with other cardiovascular complications. Nevertheless, given the huge prevalence of hypertension in the general population, it still remains the second leading cause of end-stage renal disease (ESRD), with the risk being substantially higher in blacks. Historically, hypertension-induced renal damage in patients with uncomplicated essential hypertension has been separated into the 2 distinct clinical and histological patterns of "benign" and "malignant" nephrosclerosis (Olson, 1998). Benign nephrosclerosis is the pattern observed in the majority of patients with uncomplicated primary hypertension. The somewhat nonspecific vascular lesions of hyaline arteriosclerosis develop slowly without overt proteinuria. Although focal ischemic glomerular occlusion and nephron loss occur over time, renal function is not seriously compromised except in susceptible individuals such as blacks in whom the process tends to follow a more severe and accelerated course. By contrast, "malignant" nephrosclerosis is observed with very severe hypertension (malignant phase of essential hypertension) and has a characteristic renal phenotype of acute disruptive vascular and glomerular injury with prominent fibrinoid necrosis and thrombosis. Ischemic glomeruli are frequent because of vascular injury. Renal failure can develop rapidly in the absence of adequate therapy. Although episodes of malignant nephrosclerosis undoubtedly contribute to the development of ESRD in untreated or noncompliant patients, the full-blown clinical phenotype has fortunately become uncommon with the wide availability of effective antihypertensives.

A major advance over the past two decades has been the recognition that the spectrum of hypertension-induced renal damage extends beyond benign and malignant nephrosclerosis. There is abundant evidence that coexistent hypertension plays a predominant role in the progression of most chronic kidney diseases (CKD), including diabetic nephropathy, presently the leading cause of ESRD (Neuringer and Brenner, 1993; Olson, 1998; Bidani and Griffin, 2002). These deleterious effects are observed with even mild-to-moderate blood pressure (BP) elevations in CKD patients, indicating an enhanced vulnerability to hypertensive renal damage with a lower BP threshold for damage and a steeper slope of the relationship between BP increase and renal damage.
However, it has been difficult to quantitate the contribution of hypertension to progressive renal disease because of the lack of a specific histological phenotype. Vascular pathology, considered the hallmark of hypertensive injury, often is not prominent in this setting of CKD. Instead, an accelerated segmental or global glomerulosclerosis (GS) seems to be superimposed on the intrinsic phenotype of the underlying renal disease (Neuringer and Brenner, 1993; Olson, 1998; Bidani and Griffin, 2002). Nevertheless, recent investigations in experimental animal models have increased our understanding of the mechanisms that underlie the observed differences in histological phenotypes and susceptibility to hypertensive renal damage.

**Pathophysiology of Hypertensive Renal Damage**

The direct adverse consequences of hypertension on any vascular bed are expected to be a function of the degree to which it is exposed to the increased pressures. The pathogenetic determinants of hypertensive renal damage can thus be broadly separated into 3 categories: (1) the systemic BP "load"; (2) the degree to which such load is transmitted to the renal vascular bed; and (3) local tissue susceptibility to any given degree of barotrauma. It seems self-evident that because the ambient BP profile in conscious animals is characterized by spontaneous, rapid, and often large fluctuations in BP, conventional isolated BP measurements are inherently inadequate to define quantitative relationships between BP and renal damage. The availability of BP radiotelemetry by allowing chronic BP monitoring in conscious unrestrained animals has provided a major advance in hypertensive target organ damage research (Bidani *et al.*, 1993; Dominiczak *et al.*, 1998; Bidani and Griffin, 2002; Kurtz, 2003).

**BP Load and Its Transmission to the Renal Microvasculature**

Normally, increases in systemic BP, episodic or sustained, do not have marked effect on the renal microvasculature by proportionate autoregulatory vasoconstriction of the preglomerular vasculature such that renal blood flow and glomerular hydrostatic pressures ($P_{GC}$) are maintained relatively constant. These autoregulatory responses therefore provide the primary protection against hypertensive renal damage (Navar, 1978; Bidani *et al.*, 1987; Bidani *et al.*, 1993; Dominiczak *et al.*, 1998; Bidani and Griffin, 2002; Kurtz, 2003). As long as BP remains below a certain limit (within the autoregulatory range), only benign nephrosclerosis is observed; however, if this threshold is exceeded, acute
disruptive injury (malignant nephrosclerosis) is expected to result despite intact autoregulation (Bidani and Griffin, 2002). However, once vascular injury develops, autoregulatory responses can be secondarily compromised and result in the amplification of renal damage (vide-infra) (Karlsen et al., 1997; Long and Price, 2004). A clear illustration of such a threshold relationship between BP and malignant nephrosclerosis has recently been demonstrated using BP radiotelemetry in the stroke-prone spontaneously hypertensive rat model (Griffin et al., 2003). Moreover, as would be predicted, even modest BP reductions to below this threshold were shown to prevent such damage (Griffin et al., 2003). In general, chronic hypertension tends to shift both the upper and lower limits of autoregulation to the right and represents a protective adaptation (Bidani and Griffin, 2002). Therefore, an acute severe elevation in BP is more likely to exceed the autoregulatory threshold and cause injury than equally severe hypertension that develops more gradually (Bidani and Griffin, 2002; Griffin et al., 2003).

However, even in the absence of severe hypertension, renal damage can still develop if there is an enhanced transmission of elevated systemic pressures to the renal microvasculature. Any significant preglomerular vasodilation, as observed after uninephrectomy or in early type 1 diabetes (before significant nephropathy), is expected to result in a greater fractional transmission of the ambient systemic pressures (Bidani et al., 2003). However, if such vasodilation is not accompanied by impaired renal autoregulation or severe hypertension, only a modest increase in the vulnerability to hypertensive injury is expected (Bidani et al., 2003). This may account for the largely benign renal course in most uninephrectomized individuals and possibly the long delay in the development of overt diabetic nephropathy (Parving et al., 1996; Bidani et al., 2003).

However, if renal autoregulation is additionally impaired, as seen after more severe (~75%) renal mass reduction in animals or in humans with diabetic or nondiabetic CKD (Bidani et al., 1987; Griffin et al., 1994; Christensen and Hansen, 1997; Christensen and Hommel, 1999), the susceptibility to hypertensive injury is markedly enhanced with the greatly reduced BP threshold for damage and the steeper relationship between BP and renal damage. Additionally, such enhanced glomerular pressure transmission in the
absence of hypertension severe enough to cause vascular injury primarily leads to accelerated glomerulosclerosis (GS) (Olsen, 1998; Bidani and Griffin, 2002). The clearest demonstration of this phenomenon has been provided in the most extensively investigated model of CKD, the rat 5/6 renal ablation model (Neuringer and Brenner, 1993; Bidani et al., 1993; Bidani and Griffin, 2002). Through the use of BP radiotelemetry, it has been shown that the progressive GS of the initially normal remnant glomeruli in these rats follows the quantitative relationships with BP (Bidani et al., 1993; Griffin et al., 1994). The importance of autoregulatory capacity as a determinant of the susceptibility to hypertensive injury is further illustrated by the effects of the dihydropyridine calcium channel blockers (CCBs) in this model. Given the critical dependence of autoregulatory response on voltage-gated calcium channels, these agents, not unexpectedly, further impair the already impaired renal autoregulation in the 5/6 ablation model (Griffin et al., 1995; Bidani and Griffin, 2002). Predictably, CCBs also further reduce the BP threshold and increase the slope of the relationship between GS and BP such that greater GS is observed at any given BP elevation as compared with untreated rats, and protection is not achieved without achieving normotension (Griffin et al., 1995; Bidani and Griffin, 2002). Conversely, if preglomerular vasodilation and autoregulatory impairment are prevented in this model through the substitution of a low-protein diet, GS is also ameliorated despite continued hypertension (Bidani et al., 1987; Griffin et al., 2003). However, if CCBs are given to the low-protein diet-fed rats, renal autoregulation is impaired and the protection against GS is also abolished (Griffin et al., 2003). Similar adverse effects of dihydropyridine CCBs, and protective effects of a low-protein diet on GS, have also been noted in the streptozotocin-induced diabetes model (Anderson et al., 1992; Neuringer and Brenner, 1993).

Of note, differences in autoregulatory efficiency have also been postulated to account for some of the strain (genetic) differences in susceptibility to hypertensive injury (Karlsen et al., 1997; Bidani and Griffin, 2002; Van Rodijen et al., 2002). However, it needs to be emphasized that these adverse effects of impaired renal autoregulation on susceptibility to hypertensive renal damage are only observed in a vasodilated vascular bed. In a vasoconstricted bed, the consequences of impaired autoregulation primarily result in a diminished capacity to maintain renal blood flow and glomerular filtration rate (GFR).
when systemic pressures are reduced, with an enhanced potential for ischemic tubulointerstitial injury. A similar ischemic pathogenesis may underlie the tubulointerstitial injury observed in angiotensin infusion models (Long et al., 2004).

**Local BP-Independent Determinants of Tissue Susceptibility**

Although still poorly defined, genetic or acquired differences in intrinsic structure or function may result in differences in the severity of damage expressed at any given degree of increased pressure exposure (barotrauma) (Karlsen et al., 1997; Bidani and Griffin, 2002; Kurtz, 2003). For instance, there is evidence that glomerular hypertrophy may be an independent risk factor for GS (Fogo, 2000; Bidani and Griffin, 2002; Griffin et al., 2003). In addition to the expected increase in wall tension (Laplace Law: tension = pressure x radius), hypertrophy of glomerular capillaries may also compromise their ability to withstand mechanical stress (Kriz et al., 1995; Pavenstadt et al., 2003). It has been proposed that the glomerular capillary epithelial cell (podocyte) through its interdigitating foot processes provides structural support against pressures that are substantially higher than in systemic capillaries (≈45 versus ≈20 mm Hg). The limited replication potential of this terminally differentiated cell during glomerular hypertrophy may limit its ability to maintain physical integrity and mechanical support during hypertensive stress.

However, of the local mechanisms, the BP-independent tissue damage promoting effects of angiotensin II (ANG-II) and, more recently, aldosterone have received the greatest emphasis (Neuringer and Brenner, 1993; Ketteler et al., 1995; Fogo, 2000; Epstein, 2001; Long et al., 2004). The triggering of several downstream deleterious cellular and molecular pathways is postulated to lead to oxidative stress and the activation of growth factors and fibrogenic mediators such as transforming growth factor-β and plasminogen activator inhibitor-1. Despite the considerable *in vitro* data demonstrating these pathways, the primary evidence to support their *in vivo* importance is derived from the very large number of studies in animal models that have claimed to show glomeruloprotection by renin-angiotensin system (RAS) blockade and/or aldosterone antagonists over and beyond that achieved by "equivalent" BP reductions with other antihypertensive regimens (Neuringer and Brenner, 1993; Ketteler et al., 1995; Fogo, 2000; Taal et al., 2000; Epstein, 2001). However, when BP has been measured more continuously by
radiotelemetry instead of intermittently by tail-cuff, the renoprotection can be entirely accounted for by the achieved BP reductions with little evidence of additional BP-independent protection (Griffin et al., 1994; Bidani et al., 2000; Bidani and Griffin, 2002). This is true of both the malignant nephrosclerosis and the accelerated GS models (5/6 ablation). No evidence of a shift to a higher BP threshold for damage or a decrease in the slope of the relationship between BP and GS is seen with RAS blockade, as would be expected with significant BP-independent protection. In this context, it is relevant to note that isolated $P_{\text{gc}}$ measurements like isolated BP measurements may not accurately reflect chronic pressure exposure. Such limitations probably account for the lack of consistent correlations between $P_{\text{gc}}$ and GS (Fogo, 2000), even in models demonstrating excellent correlation with radiotelemetrically measured systemic BP (Bidani et al., 1993; Bidani and Griffin, 2002).

Collectively, these data suggest that the activation of downstream molecular mediators of tissue injury may not be exclusive to angiotensin II and/or aldosterone but may represent a response to tissue stress and/or injury per se. There is evidence that pressure alone can activate many of these downstream pathways (Xu et al., 1996; Sjogren et al., 2000; Griffin et al., 2000; Bidani et al., 2000), and the histological phenotype of hypertensive renal damage exhibits little difference in models with or without overt RAS activation (Olson, 1998). Conversely, little evidence of the activation of these deleterious pathways or renal damage is observed in the absence of elevated pressures despite substantial angiotensin and aldosterone increases during low salt intake, congestive heart failure, or cirrhosis, or in the clipped kidney of the 2-kidney–1-clip model of Goldblatt hypertension (Hall et al., 2004). In fact, the administration of even very large amounts of exogenous aldosterone results in little target organ damage in animals maintained normotensive on a low-salt diet. Moreover, investigations into the pathogenic role of aldosterone have usually not adequately controlled for changes in potassium balance, which can independently impact renal damage (Tobin et al., 1985). Thus, although it remains possible that angiotensin II and aldosterone may amplify hypertensive renal damage through BP-independent mechanisms in certain situations and/or models, definitive evidence remains to be obtained.
Unresolved Issues

Despite the progress that has been achieved, certain fundamental issues of hypertensive target organ damage remain unresolved. The term "BP load" is used generically because the relative pathogenic importance of individual BP parameters (mean, systolic, diastolic, pulse pressure, and BP variability) remains undefined (Bidani and Griffin, 2002). Although recent clinical data have indicated that systolic and possibly pulse pressures are more closely correlated with target organ damage than mean arterial or diastolic pressures, the pathophysiological basis of such empirical observations remains unknown. It is also possible that the relative pathogenic potential of these individual BP parameters may differ for different target organs. Moreover, the transmission of fluctuating systemic pressures to target organs in real time must also be a dynamic process with the transmission of individual BP fluctuations depending on their rate (frequency) and the kinetics of the autoregulatory responses. The fluctuations in microvascular pressures (pressure transients and/or peak pressures) may have a greater pathogenic potential than sustained steady elevations. The unusually rapid activation kinetics of the afferent arteriolar myogenic response noted recently seems to be consistent with this protective function. Moreover, the fact that systolic, rather than mean, BP seems to be the trigger signal for this response (Loutzenhiser et al., 2002) may be indicative of a greater pathogenetic potential of systolic (peak) pressures. Biophysical approaches are being developed to separate the BP energy into its component parts and to assess the potential renal microvascular transmission and pathogenic importance of these individual components of BP power (energy/unit time) (Bidani and Griffin, 2002; Loutzenhiser et al., 2002; Bidani et al., 2003).

Nitric Oxide (NO) is an endogenous autacoid that exerts a basal tonic vasodilatory effect on the vascular wall by increasing cyclic GMP levels in smooth muscle cells (Moncada, 1997). Thus acute and chronic inhibition of NO synthesis produced by oral administration of NO synthase inhibitors such as Nω-nitro-L-arginine methyl ester (L-NAME) increase vasoconstrictor tone and consequently the blood pressure (Lahera et al., 1991; Beierwalters and Carretero, 1992; Navarro-cid et al., 1996). NO also controls a variety of biological processes, including neurotransmission, cell growth, apoptosis, inflammation and renal function (Moncada, 1997; Zou and Cawley, 1999; Tassorelli et
The progression of renal failure is related to both systemic and glomerular haemodynamic changes and the activation of vasoactive hormones, growth factors and cytokines (Johnston et al., 1998).

**Fig.1. Mechanism for progressive renal damage**

The significance of NO as a mediator of renal function has been extensively studied. Numerous studies have shown that NO is an important mediator of natriuresis, which is not only involved in the control of sodium excretion under physiological conditions, but also exerts an important role in the chronic adaptation to a high sodium intake (Lahera et al., 1991; Zou and Cawley, 1999). In addition, it has also been shown that NO can modulate the renal excretory response to changes in arterial pressure, the pressure-diuresis-natriuresis mechanisms (Garcia-Estan et al., 1996; Guarasci and Kline, 1996;
Fortepiani et al., 1999). NO exerts these effects by controlling not only tubular iron transport, but also the renal vascular tone. Indeed this endothelial factor is an important modulator of renal vasculature because it controls the diameter of several vessels, including afferent and efferent arterioles and vasa recta (Imig and Roman, 1992; Edwards and Trizna, 1993; Harrison-Bernard et al., 1999). Thus NO can play an important role in the regulation of renal hemodynamics.

Acute blockade of NO produces a dose-dependent and prolonged increase in arterial blood pressure and renal vasoconstriction with a subsequent reduction in renal plasma flow and a smaller decline in glomerular filtration rate (Lahera et al., 1991). Systemic administration of NO blockers produces widespread inhibition of NO synthesis and an increase in blood pressure, both of which have indirect effects on the kidney (Baylis et al., 1992; Ribeiro et al., 1992). Local intrarenal inhibition of NO generation leads to much smaller increases in renal vascular resistance than those seen during systemic NO blockade. Chronic treatment with NOS inhibitors leads to severe hypertension, renal damage and decreased survival in experimental animals (Baylis et al., 1992; Ribeiro et al., 1992; Salazar et al., 1992). Chronic blockade of NO produces dose-dependent chronic hypertension. Partial NO blockade over a two-month period produced a moderate, stable systemic hypertension with renal vasoconstriction, proteinuria and mild glomerular sclerotic injury (Baylis et al., 1992). More complete NO blockade for four to six weeks produced severe hypertension with renal vasoconstriction and substantial microvascular and glomerular damage, characteristic of hypertensive microangiopathy (Ribeiro et al., 1992). After only two weeks of severe NO blockade, blood pressure and glomerular pressure were higher than after two months of a more moderate dose of NO blocker. With severe chronic NO blockade, there is a progressive increase in blood pressure and renal vascular resistance, a decline in the glomerular filtration rate and development of proteinuria, all occurring within three weeks.

Thus, the present study was carried out to evaluate the effect of nitric oxide donors or the agents which can upregulate the NOS enzyme, on the hypertension and renal damage induced by chronic inhibition of nitric oxide synthase.
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) 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 L-NAME administration, the animals were housed in metabolic cages. A 24 hour urine collection was obtained from each rat for laboratory investigations.The animals were randomly assigned to all groups (n = 7, each group). The first five groups received L-NAME (Sigma-Aldrich, USA) at the doses of 1, 5, 10, 20 and 40 mg/kg/day respectively for 6 weeks in drinking water. Another six groups were administered L-arg (125 mg/kg), Mol (10 mg/kg), Neb (1 mg/kg), Pro (10 mg/kg), Rvt (5 mg/kg) and Pra (20 mg/kg) to see their per se effect.

Evaluation of renal damage due to hypertension:
To evaluate the effect of development of hypertension due to chronic nitric oxide synthase inhibition on kidney, the animals were divided in the following groups:

Group 1 (C) animals received only drinking water (vehicle for L-NAME).

Group 2 (L-NAME) animals received L-NAME (40 mg/kg/day) in drinking water for six weeks.

Group 3 (L-arg+L-NAME) animals were treated with L-arg (125 mg/kg, i.p.) along with L-NAME for six weeks.

Group 4 (Mol+L-NAME) animals were treated with molsidomine (10 mg/kg, p.o.) along with L-NAME for six weeks.
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**Group 5** (Neb+L-NAME) animals were treated with nebivolol (1 mg/kg, p.o.) along with L-NAME for six weeks.

**Group 6** (Pro+L-NAME) animals were treated with propranolol (10 mg/kg, i.p.) along with L-NAME for six weeks.

**Group 7** (RVT+L-NAME) animals were treated with resveratrol (5 mg/kg, p.o.) along with L-NAME for six weeks.

**Group 8** (Pra+L-NAME) animals were treated with pravastatin (20 mg/kg, i.p.) along with L-NAME for six weeks.

Blood pressure was determined by the tail cuff method using blood pressure recorder (Ugo Basile, Italy). The measurements were made the day before starting the treatment and then repeated every week. After the blood pressure measurement, the animals were housed in the metabolic cages for 24 hr to collect urine. Proteinuria was measured every week by biuret method.

At the end of this study period, the animals were anaesthetised with ketamine (50 mg/kg, i.p.) and blood was collected through carotid artery. Just after sacrificing the animals, both kidneys were isolated. Left kidney was used to estimate the renal antioxidative enzymes (reduced glutathione, SOD and catalase) along with thiobarbituric acid reactive substances (TBARS). The right kidney was used for histopathological studies. The serum was separated and stored at -20°C to measure creatinine and blood urea nitrogen.

**Assessment of renal function**

Serum samples were assayed for blood urea nitrogen (BUN) and serum creatinine by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

**Estimation of tissue and urine nitrite and nitrate levels:**

As described in chapter 1.

**Post mitochondrial supernatant preparation (PMS)**

As described in chapter 1.

**Estimation of lipid peroxidation**

As described in chapter 1.

**Estimation of antioxidant enzymes (AOE)**

As described in chapter 1.

**Histological studies**
The right kidney was isolated immediately after sacrificing the animal 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-micrometer (µm) thick sections were cut, deparaffinized, hydrated and stained with periodic acid-schiff (PAS) stain. The renal sections were examined in blind fashion for typical lesions of malignant hypertension such as thickening of walls of glomeruli, fibrosis, dilatation and atrophy of renal tubules and proliferation of epithelial cells in all treatments.

STATISTICAL ANALYSIS
Data are presented as means ± S.E.M. One way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was applied to calculate the statistical significance between various groups. A value of \( p<0.05 \) was considered to be statistically significant.

RESULTS
The L-NAME-treated rats exhibited a significant mortality as compared with other groups with administration of 40 mg/kg dose in drinking water. About 50% of the L-NAME rats were alive at the end of the experiment. Treatment with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin showed a protective effect on survival of the rats receiving L-NAME.

Treatment with L-NAME induced a progressive and dose dependent elevation in systolic blood pressure (SBP), as shown in Fig.2a. Lower doses of L-NAME did not result in elevation of SBP even after 6 weeks, but the SBP increased by 23.8 ± 3.6 mmHg with the dose of 20 mg/kg/day and by 39.9 ± 4.2 mmHg with the dose of 40 mg/kg/day after six weeks of L-NAME administration. The increase in SBP became evident after 3 weeks of L-NAME administration and remained elevated till the end of study. There was a significant increase in proteinuria in L-NAME (20 and 40mg/kg/day) administered rats from third week onwards (Fig.3a.). Prior treatment of animals with L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) decreased the SBP (Fig. 2b.) as well as the proteinuria (Fig.3b.) significantly till the end of study. In per se groups, when L-arg, Mol, Neb, Pro, Rvt and
Pra were administered alone for six weeks, only nebivolol and propranolol significantly reduced the SBP whereas other agents did not alter SBP (Fig.2c.).

**Effect of L-arginine, molsidomine, nebivolol, resveratrol and pravastatin on L-NAME induced urine and tissue total NO levels**

The animals treated with L-NAME exhibited a significant reduction in urine total NO levels from third week onwards. Tissue total NO levels were also decreased significantly (measured at the end of the study). Treatment of animals with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin significantly improved the urine (Fig. 4a., 4b.) as well as tissue total NO levels (Fig. 4c.).

**Effect of L-arginine, molsidomine, nebivolol, resveratrol and pravastatin on L-NAME induced renal dysfunction**

Administration of 40 mg/kg/day of L-NAME led to a significant increase in serum creatinine and blood urea nitrogen (BUN) levels. Further, creatinine and urea clearance was significantly reduced after administration of L-NAME for six weeks. Treatment with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin significantly attenuated this decrease in renal function and also improved the urea and creatinine clearance (Fig.5a-d.).

**Effect of L-arginine, molsidomine, nebivolol, resveratrol and pravastatin on L-NAME induced changes in TBARS and in the antioxidant pool**

Administration of 40 mg/kg of L-NAME produced a significant increase in TBARS levels and reduced the activity of renal antioxidants enzymes (GSH, SOD and Catalase). Treatment of animals with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin along with L-NAME significantly reduced the elevated TBARS levels and restored the activity of renal endogenous antioxidants (Table 1).

**Effect of L-arginine, molsidomine, nebivolol, resveratrol and pravastatin on L-NAME induced changes in renal morphology**

The animals treated with L-NAME revealed marked histological damage observed in the form of severe interstitial fibrosis, hemorrhages and thrombosis in the glomeruli, thickening of the walls of glomeruli. L-arginine, molsidomine, nebivolol, resveratrol and pravastatin treatment significantly prevented these morphological alterations (Fig.6.).
Effect of L-NAME on systolic blood pressure (SBP) in rats treated with different doses of L-NAME (Fig. 2a). Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on SBP in rats treated with L-NAME (40 mg/kg) (Fig. 2b). The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, **p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).
Effect of L-NAME and *per se* effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on systolic blood pressure (SBP). The values are expressed as mean ± S.E.M. *p*<0.05 as compared to Week 0 group, (one-way ANOVA followed by Newman Keuls test).
Effect of L-NAME on proteinuria in rats treated with different doses of L-NAME (Fig.3a). Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on proteinuria in rats treated with L-NAME (40 mg/kg) (Fig.3b). The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, **p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).

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Fig. 4a.

**Fig. 4b.**

Effect of L-NAME on urinary total NO levels in rats treated with different doses of L-NAME (Fig. 4a). Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on urinary total NO levels in rats treated with L-NAME (40 mg/kg) (Fig. 4b). The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, **p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on tissue total NO contents (μmol/mg protein) in rats treated with L-NAME (40 mg/kg). The values are expressed as S.E.M. *p<0.05 as compared to control group, **p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on serum creatinine (Fig. 5a) and blood urea nitrogen (BUN) (Fig. 5b) in rats treated with L-N/ (40 mg/kg). The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, **p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).
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Fig. 5d.

Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 mg/kg), propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on creatinine (Fig. 5c) and urea clearance (Fig. 5d) in rats treated with L-NAME (40 mg/kg). The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, **p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).
Table 1

Effect of L-arginine (125 mg/kg), molsidomine (10 mg/kg), nebivolol (1 n propranolol (10 mg/kg), resveratrol (5 mg/kg) and pravastatin (20 mg/kg) on antic pool in rats treated with L-NAME (40 mg/kg). The values are expressed as n S.E.M. *<p<0.05 as compared to control group, **<p<0.05 as compared to L-NAME treated group (one-way ANOVA followed by Newman Keuls test).

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS (nmol/mg protein)</th>
<th>GSH (Moles/mg protein X 10^-3)</th>
<th>SOD (units/mg protein)</th>
<th>Catalase (k/min)</th>
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<tr>
<td>Control</td>
<td>37.5±4.5</td>
<td>18±0.35</td>
<td>1.5±0.12</td>
<td>0.38±0.01</td>
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<tr>
<td>L-NAME 40</td>
<td>83.45±7.2*</td>
<td>11.28±0.42*</td>
<td>0.58±0.1*</td>
<td>0.2±0.02*</td>
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<tr>
<td>L-arg+L-NAME</td>
<td>42.8±3.1**</td>
<td>17.1±3.1**</td>
<td>1.38±0.09**</td>
<td>0.37±0.01**</td>
</tr>
<tr>
<td>Mol+L-NAME</td>
<td>53.6±2.9**</td>
<td>15.9±0.28**</td>
<td>1.27±0.095**</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>Neb+L-NAME</td>
<td>45.9±3.8**</td>
<td>16.85±0.35**</td>
<td>1.32±0.12**</td>
<td>0.36±0.01</td>
</tr>
<tr>
<td>Prop+L-NAME</td>
<td>85.6±8.5*</td>
<td>11.5±0.41*</td>
<td>0.65±0.09*</td>
<td>0.2±0.01</td>
</tr>
<tr>
<td>RVT+L-NAME</td>
<td>35.24±4.2**</td>
<td>18.56±0.42**</td>
<td>1.53±0.1**</td>
<td>0.4±0.01*</td>
</tr>
<tr>
<td>Pra+L-NAME</td>
<td>39.41±3.3**</td>
<td>17.8±0.28**</td>
<td>1.42±0.12**</td>
<td>0.37±0.01</td>
</tr>
</tbody>
</table>

Periodic Acid-Shiff (PAS) stained longitudinal sections of kidneys of normal rat (A) and L-NAME treated rat (B) (312.5X)
Periodic Acid-Schiff (PAS) stained longitudinal sections of kidneys of L-a NAME treated rat (C), Mol+L-NAME treated rat (D), Neb+L-NAME treated rat (E), RVT+L-NAME treated rat (F) and Pra+L-NAME treated rat (G) (3
DISCUSSION

Previous evidence obtained after acute inhibition of NO biosynthesis has suggested that NO exerts a basal relaxing effect on renal and systemic microvessels, thus modulating the effects of local and systemic vasoconstrictors (Rees et al., 1989; Baylis et al., 1990; Zatz and Nucci, 1991). Chronic inhibition of NO has been reported to induce an increase in blood pressure, confirming the tonic vasodilatory effect exerted by NO in the control of vascular tone (Moncada, 1997). It has been shown that renal vasculature is more sensitive than other vascular beds because the acute inhibition of NO synthesis reduced renal plasma flow even before it produced changes in systemic blood pressure (Labera et al., 1991). This concept is supported by the present finding that chronic L-NAME administration increased systolic blood pressure in a dose-dependent form.

The present data showed that renal NO inhibition, as suggested by blunted total urinary NO excretion, was associated with a reduction in GFR, as demonstrated by a significant decrease in creatinine and urea clearance, indicating that NO exerts a major role in regulation of renal hemodynamics. The present data also shows that the chronic administration of L-NAME causes a massive proteinuria, supporting the concept of a renoprotective role of NO. This renal damage seems to be a consequence of functional rather than structural disruption of glomerular wall, because these alterations can be reversed by restoration of NO levels.

Hypertension can damage the kidney in multiple ways (Johnston et al., 1998). It produces hemodynamic and mechanical stress, which may lead to glomerular endothelial dysfunction. The high blood pressure may activate vasoactive substances that directly stimulate the production of cytokines and growth factors, leading to the production of extracellular matrix proteins. A number of studies have suggested that higher levels of blood pressure are associated with a faster decline in renal function.

The mechanisms underlying the NO-deficient hypertension are not completely established but there is evidence suggesting that the rennin-angiotensin system is mainly responsible for the renal and systemic alterations induced by chronically reduced NO availability (Pollock et al., 1993; Morton et al., 1993; Qiu et al., 1994; Bank et al., 1994; Zanchi et al., 1995; Ortiz et al., 1998). Since NO is a potent inhibitor of rennin release, its inhibition should elevate rennin production. Both Jover et al. (1993) and Uhlenius et
al. (1999) have demonstrated that chronic AT1 receptor blockade with losartan largely prevents the hypertension and renal injury after several weeks of NO blockade in the rat. In another study, Pollock et al. (1993) show that chronic AT1 blockade not only completely prevents the hypertension but also reverses an established hypertension in this model, suggesting that chronic NO blockade-induced hypertension is ANG-II dependent. ANG-II exerts a range of hemodynamic and non-hemodynamic effects, as vasoconstriction, stimulation of synthesis and release of aldosterone, smooth muscle cell hypertrophy (Geisterfer et al., 1988), extracellular matrix synthesis (Kato et al., 1991), increased platelet aggregation (Feener et al., 1995), monocyte adhesion (Hernandez-Presa et al., 1998) and activation (Hahn et al., 1994), and release of inflammatory cytokines (Kranzhofer et al., 1999). All of these events are crucial steps in control of vascular homeostasis. More recent data suggests that many effects of angiotensin II are mediated by ROS. For example, rats rendered hypertensive with angiotensin II infusion show a marked elevation in vascular ROS production (Rajagopalan et al., 1996). NAD(P)H oxidases are a particularly important ROS source in the vasculature, and it is known today that angiotensin II is a potent stimulus for the activation of NAD(P)H oxidases (Griendling et al., 1994, 2000).

An important consequence of enhanced ROS formation is the disruption of delicate balance between the steady state levels of NO and O$_2^{-}$. When excessive O$_2^{-}$ is formed, NO can rapidly react at or near the diffusion-limited rate to form potent peroxynitrite radical (Murad, 1994). The affinity of NO for O$_2^{-}$ is far greater than of superoxide dismutase for O$_2^{-}$ (Wink et al., 1993). A significant correlation was seen between NO bioavailability, peroxynitrite formed and O$_2^{-}$ production (Guzik et al., 2002). Both NO and PGI$_2$ are inactivated by O$_2^{-}$. Thus, O$_2^{-}$ by decreasing the half-life of NO and PGI$_2$ and lowering their circulating concentrations could produce vasoconstriction and initiate the development of hypertension.

In the present study, chronic inhibition of NO produced a dose dependent increase in blood pressure associated with renal damage, increased proteinuria, decreased urinary total nitric oxide levels. The increase in blood pressure was associated with marked renal histological changes in our model. Some of these, such as glomerular wall thickening, are characteristics of prolonged arterial hypertension (Ribeiro et al., 1992). The most
frequent structural abnormality encountered in these rats was a global collapse of glomerular tuft, consistent with severe glomerular ischemia. Pretreatment of animals with L-arginine significantly counteracted these parameters including the structural abnormalities, thus indicating that basal NO biosynthesis was associated with marked lowering of blood pressure, which ultimately leads to the development of renal injury. Further, pretreatment of animals with molsidomine and nebivolol were also able to prevent the increase in blood pressure to almost same extent as that of L-arginine. When L-arg, Mol, Neb, Pro, Rvt and Pra were administered alone for six weeks, only nebivolol and propranolol significantly reduced the SBP whereas other agents did not alter SBP. These interventions when administered alone did not alter renal function or renal oxidative stress (data not shown). Molsidomine and nebivolol treatment significantly counteracted the effects of L-NAME. Molsidomine has been shown to be a tolerance-devoid exogenous NO donor (Hinz and Schroder, 1998; Megson and Webb, 2000) and thus in this NO-deficient hypertension model, it probably corrects the deficiency of endogenous NO and thus tilts the balance in favor of vasodilators, thus counteracting the deleterious effects of ANG-II and ROS.

Among the possible mechanisms by which nebivolol can exert its hypertensive effect is that since it is a NO producing beta-adrenergic blocker, so it’s quiet possible that NO producing property of nebivolol is involved in the lowering of blood pressure and prevention of renal damage rather than the beta-blockade by this compound. This fact was further confirmed by the findings that group of animals chronically treated with propranolol, a non specific beta blocker, exhibited a significant reduction in blood pressure as well as heart rate as observed with nebivolol (there was 15-20% decrease in heart rate with both drugs, data not shown), but renal protection observed with nebivolol was not seen in case of animals treated with propranolol, suggesting that NO releasing property rather than that of beta blocking activity is involved in the protective effect of this compound. Regarding the renal function data, both molsidomine and nebivolol significantly reduced the excretion of urinary proteins, and prevented the development of renal function. The antioxidative enzyme activities as well as the increase in TBARS and the structural alterations were significantly abolished by treatment of animals with molsidomine as well as that of nebivolol.
Resveratrol, a polyphenolic phytoalexin has been shown to be a potent scavenger for peroxyl radicals (Das et al., 1999; Ray et al., 1999). It has been also reported to exert its protective effect in the kidney cells through upregulation of NO (Giovannini et al., 2001). In the present study, treatment of rats with resveratrol (5 mg/kg, p.o.) along with L-NAME, significantly decreased the elevated blood pressure, rendered rats less susceptible to kidney damage induced by treatment with L-NAME. This protection was evidenced in the serum as the elevated levels of both BUN and creatinine were markedly lowered below those elicited by L-NAME treatment. In addition, the urinary protein excretion was reduced to a significant extent and the urea and creatinine clearance were markedly improved. The resveratrol treatment greatly ameliorated the structural alterations produced with L-NAME treatment in kidney tissues. In addition, the tissue and urinary excretion of nitric oxide levels were significantly enhanced by treatment with resveratrol. These findings may indicate a possible protective effect of resveratrol, however, since it was also able to ameliorate the reduced levels in GSH, SOD and catalase activities and prevented the rise in lipid peroxides, so antioxidative action of resveratrol cannot be ruled out. Since reactive O$_2^-$ species themselves are vasoconstrictor, they lead to development of renal dysfunction, so protection observed with resveratrol may be due to both its eNOS upregulating property as well as due to its antioxidative property.

Statins are the most potent and widely prescribed lipid-lowering agents used in the management of dyslipidemia and hypercholesterolemia (Rosenson and Tangney, 1998). Experimental work has demonstrated that these agents exert beneficial effects on endothelial cell, macrophage, platelet, and smooth muscle cell function (Tannous et al., 1999; Laufs et al., 1999; Pruefer et al., 1999). Moreover, in vivo models have confirmed putative improvements in flow-mediated dilatation with increased bioavailability of NO (O’Driscoll et al., 1997; Endres et al., 1998). In the present study, the pretreatment of animals with pravastatin, an HMG CoA reductase inhibitor, significantly reduced the elevated blood pressure observed with L-NAME administration. Similarly, the proteinuria was reduced to a significant extent with pravastatin treatment. In our lab, we have shown that pravastatin uregulate the eNOS enzyme, so the protection observed with pravastatin might be due to the increase in NO through upregulation of eNOS enzyme, which prevented the increase in SBP. This fact was further confirmed by an increase in
urinary and tissue total NO levels. Pravastatin treatment significantly improved the renal dysfunction, restored the enzyme activities of GSH, SOD, catalase, reduced the elevated TBAR levels and prevented the structural alterations observed with L-NAME treatment, to a significant extent. Statins strongly prevent ROS generation, either by directly decreasing cholesterol levels or due to their well-established pleiotropic effects. In particular, statins are able to prevent lipid oxidation (Giroux et al., 1993; Aviram et al., 1998; Suzumura et al., 1999) and they decrease AT-1 receptor dependent ROS generation (Wassmann et al., 2001). Moreover, they also directly increase eNOS activity. As a consequence, statins have been shown to improve endothelial function in humans (Anderson et al., 1995; O’Driscoll et al., 1997; Tsunekawa et al., 2001; John et al., 2001). Given the important prognostic significance of endothelial dysfunction, part of the beneficial pravastatin effects may be explained by their antioxidant and NO elevating properties.

In summary, the present data show that the kidney is an important target of chronic inhibition of NO because L-NAME-treated rats present important alterations in renal function which culminate in renal damage.