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# Physiological Roles of Nitric Oxide during Maturation in Conscious Lambs

by

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# *ABSTRACT*

In adult animals and humans, the endogenously produced vasoactive factor, nitric oxide, contributes to a variety of physiological processes including blood pressure regulation and maintenance of renal function. Little evidence has, however, been accumulated regarding its role as a modulator of cardiovascular and fluid and electrolyte homeostasis during the perinatal period. This thesis provides an account of the experiments conducted to investigate these physiological roles of nitric oxide. By evaluating the responses to the L-arginine analog, $N^G$-nitro-L-arginine methyl ester (L–NAME) and its inactive isomer, D-NAME, in one and six-week old lambs, we set out to determine the influence of nitric oxide on baseline cardiovascular and renal hemodynamics and function during postnatal development.

Initially, we established a dose of L-NAME that would inhibit the acetylcholine-induced increase in intra-renal nitric oxide synthesis for at least two hours in both age groups of lambs. Doses of 10, 20 and 40 mg/kg of L-NAME resulted in age and dose-dependent changes in systemic and renal hemodynamics. L-NAME at 20 and 40 mg/kg was necessary to comparably inhibit intra-renal nitric oxide synthesis in one and six-week old lambs, respectively. At these doses, the renal hemodynamic effects of L-NAME were similar between the two age groups.

Next, we observed that in one-week but not in six-week old lambs, L-NAME shifted the arterial baroreflex control of heart rate towards lower pressures. As well, maximum and minimum heart rates and the heart rate range over which the arterial baroreflex operates were reduced by L-NAME in the first week of life.

Finally, we established that L-NAME has marked age-dependent effects on glomerular and tubular function. In response to L-NAME administration, glomerular filtration rate decreased in one-week old lambs. Older lambs, however, manifested significant increases in electrolyte excretion accompanied by a reduction in urinary osmolality and an increase in free water clearance in the absence of changes in glomerular filtration rate.

Taken together, these studies provide new information that in conscious lambs, nitric oxide modulates baseline cardiovascular and renal hemodynamics and function and influences the arterial baroreflex control of heart rate in an age-dependent manner.

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# *CHAPTER ONE*

# *Introduction*

The purpose of this chapter is firstly, to provide familiarity with some of the physical characteristics of nitric oxide and of the enzyme responsible for its synthesis and secondly, to introduce selected physiological effects of this molecule on the cardiovascular and renal systems. The third focus of this chapter will be to highlight several of the cardiovascular, renal and endocrine changes that occur with postnatal maturation and furthermore discuss their possible interactions with nitric oxide during development.

## 1.1 WHAT IS NITRIC OXIDE?

In 1980, Furchgott and Zawadzki first demonstrated that endothelial cells synthesized and released a diffusible factor termed endothelium-derived relaxing factor

(EDRF), which then acted on the underlying vascular smooth muscle layer to produce relaxation (54). This pioneering work was followed by other investigations that eventually led to the identification of EDRF as nitric oxide in 1987 (136,152). Since its discovery, nitric oxide has been implicated to mediate various physiological and pathophysiological processes in the body including maintaining vascular tone, establishing long-term potentiation, modulating neuronal transmission, as well as possessing a wide range of immunological and cytotoxic effects (53,121,153).

Nitric oxide is a biologically ubiquitous molecule, which is moderately soluble in water and has a half-life of only a few seconds (53,208). It is synthesized from the amino acid L-arginine through one of three known isoforms of the nitric oxide synthase (NOS) enzyme: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). These isoforms are also commonly referred to as NOS I, NOS II and NOS III, respectively, reflecting their order of molecular characterization (23,94,224). Although these enzymes share a high degree of homology, suggesting a common lineage, their tissue distribution and differences in regulation of expression indicate that they are evolutionarily specialized. Type nNOS, iNOS and eNOS are localized on chromosomes 12, 17 and 7 respectively (220). The expression of n/eNOS is constitutive, meaning that under normal conditions, this enzyme is tonically active and that further activation is dependent on the elevation of intracellular calcium ions. Since physiological increases in intracellular calcium ion concentrations are transient, the output of n/eNOS is relatively rapid (due to the fact that it does not require new protein synthesis) but low (picomolar amounts of nitric oxide produced); both isoforms are calmodulin dependent. The third form of NOS (iNOS) is of the inducible type meaning that it is not expressed within cells under basal conditions, but

rather synthesized upon stimulation by such factors as endotoxins and cytokines. Although the inducible NOS may or may not show calcium dependency, calmodulin binding is still required. After the stimulation of iNOS, there is a lag phase of approximately eight hours before nitric oxide levels are significantly increased. Once activated, however, these synthases are capable of producing nitric oxide for prolonged periods of time, and in nanomolar quantities (7,121,217).

NOS enzymes are best characterized as cytochrome P-450-like heme proteins (217). Structurally, these enzymes consist of an oxidative domain at the amino-terminus, which is known to bind heme and a reductase domain at the carboxy-terminus. Binding sites for the necessary cofactors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine nucleotide (FAD), flavin mononucleotide (FMN), exist in the reductase domain and act to shuttle electrons to the heme center which catalyzes the oxidation of L-arginine to form nitric oxide. Tetrahydrobiopterin ($H_4B$) is also required as a necessary cofactor (7,89,195,198,217). Figure 1.1 illustrates the process of NO formation from its precursor and various cofactors.

$$\text{L-arginine} \xrightarrow[\text{(n/i/e) NOS}]{\text{O}_2,\ \text{NADPH, FMN, H}_4\text{B, FAD, heme}} \text{NO + L-citrulline}$$

**Figure 1.1** Nitric oxide synthesis. Oxygen ($O_2$), nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), tetrahydrobiopterin ($H_4B$), flavin adenine nucleotide (FAD), nitric oxide (NO), and neuronal (n), inducible (i), and endothelial (e) nitric oxide synthase (NOS).

The short half-life of nitric oxide in biological solutions is primarily due to several pathways of degeneration. Once formed, nitric oxide rapidly interacts with reactive oxygen to form nitrate and nitrite end-products, which are eliminated from the circulation via renal excretion (121). Other methods of removal include quenching of nitric oxide with superoxide radicals, to form the potent and destructive peroxynitrite anion (19), as well as the binding of nitric oxide to heme containing proteins with iron-sulfur sites such as oxyhemoglobin (88).

Much of what we know about the physiological and pathophysiological roles of nitric oxide have come from studies employing a variety of techniques including pharmacological perturbations of NOS enzymes and/or their cofactors in both organ and cellular systems (14,28), as well as several known molecular techniques. These techniques include gene knockout and recombinant DNA models to study the individual isoforms of NOS enzyme (75,76,107). Additionally, NOS enzyme induction using an array of synthase-dependent stimuli (110,132) and hybridization models, assist investigators in detecting NOS isoforms *in situ* (128). Although each of these techniques has contributed to our understanding of the roles of nitric oxide in biological systems, none provides a perfect model in every setting. In our experiments, we chose the pharmacological approach to study the role of nitric oxide in modulating cardiovascular and renal homeostasis. This technique will be discussed in further detail in the proceeding section.

## 1.2 PHARMACOLOGICAL APPROACHES TO THE STUDY OF NITRIC OXIDE

In contrast to the previously mentioned molecular approaches to the study of nitric oxide synthesis, using pharmacological means to inhibit its synthesis is much easier to perform. Since nitric oxide does not act on specific receptors to exert its effects, researchers have developed methods of accessing either specific binding sites on the NOS enzyme or on intracellular targets of nitric oxide to alter the synthesis and/or function of this molecule.

The use of agents that directly bind to specific sites on the NOS enzyme has made it possible to determine that the binding of NADPH, FMN, FAD, $H_4B$, calmodulin and heme are all critical for the mechanisms by which nitric oxide is produced. Various pharmaceutical agents have been developed which specifically target and inhibit the proper functioning of these cofactors. For example, diphenylene iodonium (DPI) can inhibit nitric oxide production by arresting all NADH or NADPH-utilizing enzymes within the cell (85). As well, since calmodulin is a necessary molecule in the production of nitric oxide, regardless of the isoform of NOS, agents such as trifluoperazine and chlorpromazine can be used as calmodulin antagonists and hence inhibit NOS activity by preventing the binding of calmodulin to the enzyme (25). Although the use of these agents does make it possible to arrest cellular nitric oxide production, these methods are neither specific to a particular isoform of the NOS enzyme nor to other cellular processes which utilize similar intracellular machinery as does the NOS enzyme. Some specificity, however, may be achieved by using agents such as ethylenediaminetetra acetic acid (EDTA) which is a calcium ion chelator to target calcium-dependent e/n NOS or 2,4-

diamino-6-hydroxy-pyrimidine or 7-nitroindazole, both of which interfere with the cellular production of $H_4B$, an important cofactor in nitric oxide production (65,117) (See Figure 1.1).

With the discovery that L-arginine analogs could effectively inhibit the production of nitric oxide, further advancements were rapidly made in the field of nitric oxide pharmacology. As their name implies, these agents act by competitively inhibiting the binding of L-arginine with the NOS enzyme (130). This inhibition is stereospecific, with only the L- and not the D-enantiomer showing any inhibitory effects on nitric oxide synthesis. In fact, following initial competitive binding, the inhibitory effects of these analogs may be reversed with the addition of excess L-arginine (14). It has been observed, nevertheless, that in instances of prolonged incubation with $N^G$-monomethyl-L-arginine, there is irreversible inhibition of neuronal and macrophage NOS, the mechanism of which still remains to be identified (85). Some examples of L-arginine analogs include $N^G$-amino-L-arginine (L-NAA), $N^G$-monomethyl-L-arginine (L-NMMA), $N^G$-nitro-L-arginine (NOARG), $N^G$-nitro-L-arginine methyl ester (L-NAME) and the naturally occurring $N^G$-dimethyl-L-arginine (ADMA). More recently, other compounds that share some structural similarity to arginine and contain a guanidino group have drawn attention for their ability to inhibit NOS. These compounds include L-canavanine, aminoguanidine, L-$N^5$-(Iminoethyl)-Ornithine (L-NIO), thiocitrulline and 7-nitroindazole (7-NI) (121).

Over the past decade, an overwhelming number of NOS inhibitors have been developed. Although some are more potent for a specific isoform of NOS than others, specificity is difficult to achieve due to the cross reactivity between the various types of

the synthase. Currently, a considerable amount of research into specific inhibitors is being aimed on inhibitors that are specific for iNOS, since these would be potential therapeutic agents for use in the treatment of septic shock. The majority of these inhibitors resemble arginine. For example, L-canavanine, aminoguanidine, L-NIO, and phenylimidazole have all been reported to be more potent as inhibitors of iNOS than nNOS or eNOS *in vitro* (85,122,219). Although the degree of specificity may vary between these compounds, effective inhibition of iNOS can be achieved with concentrations in the low micromolar range. The potential difficulty with some inhibitors such as L-canavanine is that it is a broad-spectrum inhibitor that also inhibits other arginine-utilizing enzymes such as arginase (73). In addition to aminoguanidine's ability as an effective inhibitor of iNOS, it has been shown to be 100 times less potent at producing a pressor response *in vivo* (35). Therefore, aminoguanidine may a useful tool in inhibiting iNOS induced disorders with little effect on the cardiovascular system. Recently, it was found that substituted isothioureas are potent NOS inhibitors. For example, nanomolar concentrations of S-ethylisothiourea have been reported to be very effective in inhibiting iNOS *in vitro* (127). Additionally, it was shown to be 10-40 times more potent for inhibiting iNOS than n/eNOS. Although some isothioureas do produce a weak pressor response *in vivo*, aminoethylthiourea's effect on blood pressure is negligible compared to its potent inhibition of iNOS (192).

There has been less interest in establishing specific inhibitors for the nNOS and eNOS isoforms of the synthase. Such a discovery would be extremely useful, however, in discerning whether or not the nitric oxide mediated response detected in a tissue is due to the synthesis of nitric oxide by endothelial cells or by neurons. Furfine *et al* showed

that S-ethyl-L-thiocitrulline was much more potent at inhibiting nNOS compared to both iNOS and eNOS (55). Therefore, it was suggested that this compound might be useful in controlling nitric oxide release from neuronal mechanisms *in vivo*. However, it was later found that S-ethyl-L-thiocitrulline had strong pressor effects *in vivo*, thus making its uses limited (129). Interestingly, there are agents that show different responses between their *in vivo* and *in vitro* trials. L-$N^G$-nitro-arginine-p-nitro-anilide (6) and 7-nitroindazole (122) have been shown to inhibit responses mediated by nitric oxide synthesized in sensory nerves *in vivo* without increasing blood pressure. *In vitro*, however, neither compound inhibits nNOS to a greater extent than eNOS, and although their *in vitro* actions are similar to those of L-NAME, *in vivo* they exert a fraction of the pressor responses observed with L-NAME (5). Although the reason for their selectivity for nNOS *in vivo* but not *in vitro* has not been established, it was speculated that the inhibitory effects of 7-nitroindazole may have been reduced by xanthine oxidase which is present in higher concentrations in intact endothelial cells.

As with other synthase inhibitors, advances in discovering specific inhibitors of eNOS have not resulted in definitive conclusions. For example, *in vitro* evidence suggests that ebselen, a drug that acts on the thiol group of the NOS molecule, preferentially inhibits eNOS over n/iNOS (229). Subsequent findings showed that at a certain concentration range, ebselen preferentially inhibited iNOS over eNOS (71). Although not very selective, the most commonly used inhibitors of eNOS are the nitro-arginine-derived agents. For example, *in vitro* evidence obtained from rats shows that L-NNA or L-NOARG are 100 times more potent in inhibiting e/nNOS compared to iNOS (66,213). Since L-NNA is poorly soluble at physiologic pH, its methyl ester,

L-NAME, is more often used due to its similar pharmacological effects and lesser toxicity (95,213). Another popular inhibitor is L-NAA, which is 100 times more potent for inhibiting eNOS than n/iNOS compared to the more generic inhibitor L-NMMA (66,95). L-NAA has, however, been shown to have toxic effects, at least in dogs, possibly through its active metabolites (34).

Therefore, the search for more specific inhibitors of the different isoforms of NOS is not at an end. Although some specificity may be achieved *in vitro*, it is not always confirmed *in vivo*, suggesting that as in all systems there are redundancies or checks-and-balances to ensure the proper workings of cellular and whole organ functions.

## 1.3 PHYSIOLOGICAL ROLES OF NITRIC OXIDE

As previously mentioned, much of what has been discovered regarding the physiological roles of nitric oxide has been established through the use of L-arginine analog, nitric oxide synthesis inhibitors such as L-NAME. In this next section, an overview of the interaction between nitric oxide and various physiological systems implicated in the current research will be highlighted including systemic and renal hemodynamics, the arterial baroreflex control of heart rate, and renal function.

### 1.3.1 Nitric oxide and the cardiovascular system

It is suggested that nitric oxide, generated under basal conditions in the central nervous system and in the peripheral vasculature, plays an important role in regulating blood pressure and systemic vascular resistance and hence helps to maintain cardiovascular homeostasis. For example, the infusion of the L-arginine analog L-NMMA to conscious adult rats, dogs and guinea pigs increases vascular resistance in a

variety of beds including that of the kidney, mesentery, carotids and hindquarters (1,33,56). This increase in systemic vascular resistance is accompanied by an increase in blood pressure and concomitant decreases in heart rate and in cardiac output (1,47,102,143,154). All of the aforementioned cardiovascular effects of nitric oxide synthesis inhibition can be reversed with the administration of excess L-arginine, thus confirming that the effects of the inhibitor are due to the blockade of basal nitric oxide production. Recent evidence also implicates nitric oxide in modulating blood pressure through its actions at the nucleus tractus solitarius (NTS) of the medulla oblongata (67,164). Studies in anesthetized rats and rabbits have shown that NOS inhibition increases renal sympathetic outflow (67,164), thus providing evidence that the increased blood pressure following systemic nitric oxide synthesis inhibition may be partially mediated through increased sympathetic output. This has also been confirmed by Patel *et al* in the anesthetized rat paraventricular nucleus (PVN), where intracerebroventricular L-NAME administration was shown to produce an increase in renal sympathetic nerve activity (139,230). The aforementioned studies should, however, be interpreted with caution as anesthesia is known to modify cardiovascular and humoral status (123,135,179).

Inhibition of nitric oxide production has been useful in deducing the effects of nitric oxide on renal hemodynamics. For example, the administration of nitric oxide synthesis inhibitors results in an increase in renal vascular resistance leading to a decreased renal blood flow (14,46,63,92). The observed increase in renal vascular tone following NOS inhibition may be attributed to several mechanisms including the removal of the direct vasodilator effect of nitric oxide on the renal vasculature and/or the

experimentally induced disturbance in the intrarenal balance between the vasoconstrictor effects of angiotensin II, norepinephrine and endothelin and the vasodilator effects of nitric oxide (15,104,115). Recent evidence, obtained from conscious adult rats, suggests that when systemic nitric oxide synthesis is inhibited, the renal vasoconstrictor effects of exogenous angiotensin II are exacerbated, such that greater increases in renal vascular resistance are observed than if nitric oxide synthesis was left uninhibited (15). Moreover, it appears that in the conscious adult dog where angiotensin II levels are experimentally elevated above normal, the influence of nitric oxide and other vasodilators such as prostacyclin in regulating renal hemodynamics and function become increasingly important (2). In total, therefore, these studies exemplify the critical balance between such vasoconstrictors as angiotensin II and the compensatory effects of vasodilator substances such as nitric oxide in modulating renal vascular tone and thus maintaining renal perfusion.

In one study in conscious dogs, it was shown that intravenous administration of a NOS inhibitor had a greater effect on renal hemodynamics as compared to intrarenal injection of the same inhibitor (63); the authors attributed this to possible secondary effects of systemic drug administration. It is, however, difficult to interpret the data obtained in this study, as a) different doses of the NOS inhibitor were administered intrarenally as compared to intravenously, b) inhibition of nitric oxide production within the kidney was only assessed after intrarenal NOS inhibition, c) placement of the catheter within the renal artery itself could significantly impact the degree to which NO synthesis was inhibited and d) there were no control measurements made.

### 1.3.2 Nitric oxide and the arterial baroreflex

In addition to its well-known effects in modulating cardiovascular homeostasis, nitric oxide has been postulated to act as a neuronal modulator in the central and peripheral nervous systems (67,83,148,203,227). NOS is present in almost all the sites involved in integration of the arterial baroreflex, such as the carotid sinus, the NTS, the rostral ventrolateral medulla, and the dorsal motor nucleus of the vagus (24,69,148).

The arterial baroreceptor reflex is an important cardiovascular reflex, which continuously monitors short-term changes in blood pressure and maintains it within a specific range (36,144). Signals arising from peripheral receptors including those of the arterial baroreceptors, found in the carotid sinus and aortic arch, travel via afferent fibers of the glossopharyngeal and vagal cranial nerves, respectively, to terminate in the NTS in the medulla of the hindbrain (37,193). These afferent tracts provide the NTS with moment-to-moment information about the state of the cardiovascular system. The NTS is the central integrator region responsible for receiving these afferent signals as well as others from higher brain centers including the PVN. This information is then relayed to the caudal ventrolateral medulla and subsequently to the rostral ventrolateral medulla, where an appropriate efferent output is generated via the autonomic nervous system.

Forstermann *et al* were the first to show that apart from the NOS activity present in the NTS, this area is rich in N-methyl-D-aspartate (NMDA) receptors (52). It was previously reported that the excitatory amino acid, L-glutamate, was involved in the central neurotransmission of baroreceptor afferent nerve fibers (197) and that NMDA receptors were coupled to NOS activation (57). Garthwaite *et al* then assimilated all these findings by showing that by acting on NMDA receptors in cerebellar cells,

exogenously administered glutamate induced the release of a diffusible messenger with similar properties to those of nitric oxide. The authors subsequently determined that this diffusible factor was released in a calcium-dependent manner and that cyclic GMP levels were elevated following its release, thus pointing at nitric oxide as a potential candidate as the neuronal messenger (57).

These findings were taken one step further by Sakuma *et al* who suggested from studies in anesthetized Wistar rats (164), that nitric oxide might be involved in the central regulation of sympathetic tone. They found that intravenous (I.V.) L-NMA administration resulted in an increase in blood pressure and in renal sympathetic nerve activity, accompanied by transient decreases in heart rate; these effects were reversed with excess L-arginine infusion. Following C1-C2 transection, L-NMA injection produced an attenuated increase in blood pressure, whereas the increase in renal nerve activity was abolished. These studies provide evidence to suggest that in addition to its direct effects as a vasodilator, nitric oxide may play a role in the central regulation of blood pressure by reducing sympathetic outflow, at least in the anesthetized animal. These findings were later confirmed by Harada *et al* in anesthetized rabbits microinjected with L-NMMA directly into the NTS (67). Moreover, these studies provided support for the premise that L-arginine analog inhibitors of NOS are freely able to cross the blood-brain barrier (120,164,207). Therefore, although systemically injected, an L-arginine analog inhibitor may have direct effects on the vascular walls by inhibiting the functioning of the local NOS enzyme and potential secondary effects on NOS located in the central nervous system.

In more recent studies, it was discovered that apart from inhibiting renal sympathetic outflow from the central nervous system by acting at the level of the NTS and PVN (139,230), nitric oxide also modulated the afferent discharge of carotid sinus baroreceptors. Matsuda *et al* demonstrated in anesthetized rabbits, that increases in sinus baroreceptor activity induced by increasing carotid sinus pressure were significantly attenuated in the presence of nitric oxide (111). It is therefore evident that nitric oxide may modulate the arterial baroreflex through its actions at the afferent, central integrator as well as the efferent portions of the arterial baroreflex arc.

### 1.3.3 Nitric oxide and renal function

The role of nitric oxide in influencing renal function has been ascertained using various NOS inhibitors and nitric oxide synthesis stimulators, in both *in vivo* and *in vitro* settings (2,21,91,143,145,153,162). From these studies, it is well established that nitric oxide is an important endogenous modulator of renal function through its action at key sites of the kidney including the renal arterioles, the glomerulus and the tubular epithelial cells of the nephron.

The afferent and efferent arterioles of the kidney play a major role in providing the majority of the resistance to blood flow within the renal bed (212). The diameter of these vessels, especially that of the afferent arteriole, is under myogenic, neural as well as paracrine control. Nitric oxide, as postulated by Edwards *et al* (46), is such a paracrine agent. These investigators showed that when L-NNA was administered into the bathing medium of isolated rabbit afferent and efferent arterioles, afferent arteriolar tone was reduced by 40% whereas efferent arteriolar tone was not altered. Consistent with subsequent studies (80), these authors also found that the efferent arteriole could be

stimulated to produce nitric oxide via the application of the endothelium-dependent vasodilator, acetylcholine. Overall, these results suggest that under basal conditions, nitric oxide is produced in renal afferent but not efferent arterioles and thus help maintain a consistent glomerular capillary pressure, and glomerular filtration rate (GFR).

GFR is determined both by the net filtration pressure across the glomerular capillaries and by the ultrafiltration coefficient ($K_f$) (212). Studies suggest that apart from influencing glomerular capillary pressure by acting on pre-glomerular vessels, nitric oxide may be involved in modulating baseline $K_f$ (14,167). In micropuncture studies in rats and rabbits (40,228), a diminished $K_f$ and a decreased GFR have been found consistently after nitric oxide synthesis inhibition, suggesting that nitric oxide may modulate baseline GFR by regulating mesangial cell tone. Studies in conscious rats and dogs have demonstrated that GFR either decreases or does not change following the administration of NOS inhibitors (2,14,63). The general consensus is, however, that there is a reduction in GFR following the injection of L-arginine analogs, which in conjunction with the greater decrease in renal plasma flow, results in an increase in filtration fraction (9,14). There is also agreement that the autoregulatory capacity of the kidney in the face of nitric oxide synthesis inhibition is unaffected (12,162). Recent studies in rats have revealed the presence of neuronal NOS enzymes in *macula densa* cells (79,204,218). In microperfused preparations of the afferent arteriole and *macula densa*, selective inhibition of *macula densa* NOS augments the afferent arteriolar vasoconstriction brought about by a high sodium chloride concentration at the renal tubular lumen side of *macula densa* cells (78). This suggests that nitric oxide generated by the *macula densa* may in turn modulate tubuloglomerular feedback.

Inhibition of nitric oxide synthesis has variable effects on urine flow rate and electrolyte clearance, depending on the dose of inhibitor used, and level of activation of the renin-angiotensin system (2,82,162,165). For example, the injection of sub-pressor doses of NOS inhibitors to anesthetized dogs (125) and rats (93) results in a decrease in urine flow rate as well as in sodium and potassium excretion rates. These findings are, however, difficult to interpret considering the multitude of physiological variables that anesthetics are known to alter (112,135). In contrast, I.V. injections of higher doses of nitric oxide synthesis inhibitors produce an increase in blood pressure accompanied by increases in both urine flow and in sodium excretion rates in conscious rats (14), but not in conscious dogs (47). The discrepancy between the findings of these studies most likely resides in the dosage of inhibitor used; 10 mg/kg of L-NAME was administered to the rats, whereas only 5 mg/kg L-NNA was injected into the dogs. In a further attempt to examine the phenomenon of NOS inhibitor induced natriuresis, Majid and Navar (108) described that in anesthetized animals, an elevation in renal perfusion pressure, as manifested by increases in blood pressure following NOS inhibition, stimulates the intrarenal production of nitric oxide. This newly formed nitric oxide then acts on renal tubular sites to induce natriuresis and diuresis (this phenomenon is described below). In the above mentioned studies, however, there were no steps taken to ensure that intrarenal nitric oxide synthesis was attenuated or altered following NOS inhibition. Therefore, taken together, these studies do not provide a clear consensus on the effects of nitric oxide synthesis inhibition on renal tubular fluid reabsorption, justifying the need for further experiments to elucidate this matter.

Several lines of evidence point to direct tubular effects of nitric oxide in modulating urinary electrolyte excretion (21,194). The concentrations of NOS are greatest in the renal medulla, a region densely populated both by the *vasa recta* and collecting tubules, than in any other region in the kidney (7,21,77,91,165). Biondi *et al* demonstrated in *ex vivo* kidney of dogs, that the endothelium dependent increase in cyclic GMP activity was greatest in the renal medulla (21). Moreover, Stoos *et al* found that in cultured collecting duct cells, an elevation of intracellular cyclic GMP activity decreased sodium reabsorption by inhibiting amiloride-sensitive $Na^+/H^+$ antiporters located on the apical cell membrane (194). More recently, Wang *et al* showed the presence of nNOS in the principal cells of microdissected cortical collecting duct cells of rats (216). Additionally, recent evidence obtained from isolated rat nephron segments has demonstrated that nitric oxide may decrease sodium and chloride flux in the cortical thick ascending limb of the loop of Henle (146). Furthermore, Liang *et al* have reported that in cultured opossum proximal tubular cells, nitric oxide inhibits $Na^+/K^+$ ATPase activity via cyclic GMP mechanisms (98). This evidence suggests that nitric oxide produced by tubular epithelial and by peritubular capillary endothelial cells may inhibit sodium and chloride reabsorption at various nephron sites; whether this occurs *in vivo* remains to be seen.

## 1.4 CARDIOVASCULAR, RENAL AND ENDOCRINE FUNCTION DURING MATURATION

Numerous changes take place throughout maturation in all species studied to date. This next section will describe some of these changes and discuss how they may be influenced by nitric oxide during development.

As maturation proceeds there are marked systemic and renal hemodynamic alterations that occur such as a steady increase in blood pressure, due to an increasing systemic vascular resistance, accompanied by a decrease in heart rate (84,99,159). Lister *et al* showed in conscious lambs that there is also a considerable decrease in cardiac output (99) which reaches a nadir around five weeks of age. These findings were supported by Klopfenstein *et al* who showed in conscious lambs that the age-dependent fall in cardiac output was a result of decreases in both stroke volume and heart rate (84).

The changes mentioned above occur simultaneously with decreases in vascular resistance in a variety of beds including that of the kidney (84,172). The distribution of cardiac output to the kidney increases as maturation proceeds from the fetal stage, when it is about 2-3%, to approximately 6% in the first week of life and to almost 18% by 30 days after birth (159). At birth, renal vascular resistance is high and declines with maturation (29,159,172). Blood flow within the kidney itself is also age-dependent with renal blood flow becoming distributed progressively in a centrifugal pattern from the medulla to the cortex, reaching adult perfusion levels between three to six weeks in the dog (29). In sheep, however, this redistribution of blood to cortical regions of the kidney occurs much sooner, reaching adult levels between three and nine days after birth (4).

The changes in renal blood flow patterns that occur with development are closely accompanied by postnatal modifications in renal function. The measure of renal filtering capacity, GFR, increases up to and then remains constant over the last third of gestation, when corrected for kidney weight (159). After birth, GFR increases threefold during the first 24h after birth (183) and continues to increase until approximately four weeks in dogs (172) and sheep (3,184). Using ferrocyanide as an indicator and inspecting the precipitate in dissected renal tubules of lambs, Aperia *et al* showed that filtering capacity of cortical nephrons increased from 22% in the fetus to 77% after birth. This value continued to increase to 98% by the first week of life (4), whereas 100% of the juxtamedullary nephrons were filtering even at birth (3,161). Furthermore, compared to the adult whose kidney excretes less than 1% of filtered sodium, fetal sheep excrete nearly 11% at 130 days gestation, which nears 5% at term (160). After birth, both urinary sodium excretion and fractional excretion of sodium follow a biphasic pattern. There is an initial increase in both parameters at one hour after birth, which then decline to adult levels by 24 h (126). Additionally, there is convincing evidence that there is a developmental transfer of tubular sodium reabsorption from more distal to proximal regions of the nephron shortly after birth (103).

The postnatal period is also characterized by many changes in circulating levels of various hormones and vasoactive substances. For example, in a variety of species including the piglet, dog, human and sheep, the renin-angiotensin system is highly activated in the perinatal period and declines with postnatal maturation (8,64,178,211). Although it is not known why this system is so highly active in the newborn period, elevated levels of angiotensin II may be partially responsible for the characteristically

high renal vascular resistance of the period, although this remains to be determined. Interestingly, studies in our laboratory have shown that circulating angiotensin II may be necessary for promoting normal renal perfusion pressure and glomerular function in the conscious newborn lamb (42). We have also measured circulating levels of endothelin and arginine vasopressin, both of which are not altered with age in the conscious lamb (182,185). It should, however, be noted that although circulating levels of these compounds may not be altered with maturation, there may be age-dependent changes in their receptor distribution and/or sensitivity which may then influence their effects on postnatal changes in cardiovascular and renal function. For example, circulating levels of catecholamines are elevated in the newborn period and decline to adult levels in the first few days of life (226). Nevertheless, in the dog, this newborn period is characterized by an overly sensitive renal bed to adrenergic agonists (50).

It is often difficult to extrapolate between species, especially in terms of organ system development and function. The purpose of the experiments described in this thesis is not to extrapolate our findings in newborn lambs to the human neonate. We are, however, fortunate that the changes which occur in both cardiovascular and renal morphological development and function, as well as endocrine function, as described above, are in many instances comparable between the two species. The findings described herein will directly impact the clinical management of perturbations in cardiovascular, endocrine and kidney function in the human infant and child, since these experiments will provide -- for the first time -- new and important information regarding nitric oxide and its role in normal mammalian physiological development.

It is therefore evident from this section that the changes which take place during maturation in cardiovascular and renal function result from an interplay between many of the above mentioned factors. One factor that has been demonstrated to interact with all of the above physiological parameters and substances is nitric oxide. In the adult, nitric oxide synthesis inhibition has been shown to increase circulating levels of endothelin, arginine vasopressin, and prostaglandins (43,62,158), whereas plasma catecholamines and renin activity have been reported to decrease (125,147). It is also known that renal NOS mRNA activity and levels are increased in the developing animal and decline with postnatal maturation in piglets, rats and guinea pigs (51,188,189,200). The only studies that have investigated the role of nitric oxide in modulating cardiovascular and renal hemodynamics and function have been conducted on anesthetized piglets (190,191) and rabbits (9). Interpretation of these results is, however, difficult due to the confounding influences of anesthesia on cardiovascular, endocrine and renal function, as well as the known differences in the vascular effects of nitric oxide under anesthesia (123,135,231).

Therefore, the primary objective of the studies described herein was to investigate some of the physiological effects of nitric oxide in the conscious lamb during postnatal maturation. The next four chapters will provide a detailed description of each experimental series conducted. Each chapter contains the introduction, experimental methods, results, and discussion including a thorough interpretation of the data obtained in achieving this objective.

# *CHAPTER TWO*

## *Materials and Surgical Procedures*

The following section provides a description of catheter preparation, as well as a detailed account of the necessary surgical procedures performed prior to each of the experimental protocols that will be introduced in subsequent chapters.

### 2.1 PREPARATION OF CATHETERS

This section describes the preparation of all catheters used in each of the surgical procedures outlined in Section 2.2.

The femoral vessel catheters were constructed from polyethylene tubing (PE 160, Intramedic, Sparks, Maryland). Catheters were cut to a predetermined length of 110 cm. One end of the catheter was chosen as the tip, and marks were made using a permanent

ink marker at five-centimeter intervals from the tip, for 20 cm. These marks were used during surgery to assist the surgeon in determining the distance that the tip of the catheter was inserted into the femoral vessel. The distal end of the catheter was then fitted with a five-centimeter long sleeve adapted from silastic tubing (Dow Corning, Midland, Michigan), which served to protect the 18-gauge blunt needle (Monoject, St. Louis, Missouri) that was inserted into the distal portion of the catheter. At the 20 cm mark from the tip of the vessel catheter, a one-centimeter cuff of silastic tubing was glued in place using crazy glue.

Individual sterilization pouches (Propper Manufacturing Co., Long Island City, New York) were packed with four of the above mentioned catheters, five three-way stopcocks with Luer-lock fittings, one 18-gauge needle (Becton Dickinson, Franklin Lakes, New Jersey), four slip-tip plastic five-milliliter syringes (Becton Dickinson, Franklin Lakes, New Jersey), and one slip-tip plastic 10 cc syringe.

The bladder catheter was adapted from an 8F Feeding tube (Meditron, Don Mills, Ontario). The catheter was cut to a predetermined length of 40 cm and fitted with a 14-gauge tubing adapter (Becton Dickinson, Rutherford, New Jersey). Using crazy glue, two one-centimeter long cuffs of silastic tubing were glued 0.5 cm apart at a distance of five-centimeters from the tip of the catheter. Using fine-tipped scissors, several additional holes were cut out of the catheter body at regular intervals between its tip and the cuff to allow for the free-flow of urine during experiments. Individual sterilization pouches were packed with one bladder catheter, as described above, and two three-way stopcocks.

The ultrasonic renal blood flow transducer sized 3S-4S (Transonics Systems, Inc., Ithaca, New York) were individually placed in sterilization pouches containing a small Philips screw driver, extra screws and a five-milliliter plastic syringe containing acoustic coupling jelly (K-Gel, Toro Pharmaceuticals, Downsview, Ontario).

The sterilization pouches containing the catheters and flow transducers were placed in a plastic bag and were sterilized under a fume-hood using ethylene oxide gas (Amprolene, Haw River, North Carolina) for 12 h. Upon successful sterilization, the pouches were allowed to aerate under the fume-hood for at least 12 h before being utilized in surgery.

## 2.2 SURGICAL PROCEDURES

In this section, both the general and specific surgical procedures conducted for each of the listed experiments will be described. The first section describes surgical protocol that was common to all of the described studies.

All lambs were obtained from a local source (Sheep Advisory Service, Alberta) and housed with their mothers in individual pens in the vivarium of the Health Sciences Center except during surgery and experiments. All surgical and experimental procedures were carried out in accordance with the "Guide to the Care and Use of Experimental Animals" provided by the Canadian Council on Animal Care, and with the approval of the Animal Care Committee of the University of Calgary.

### 2.2.1 General surgical procedures

The day prior to surgery, the lamb's wool was shaved in accordance to the appropriate surgical protocol. A minimum of three days before experiments, surgery was

performed on lambs using aseptic techniques, as previously described (174,180). The lamb was brought into the surgery suite and anesthesia was induced with a mask and halothane (3-4%) in oxygen. Once the lamb became sufficiently anesthetized, its vocal cords were sprayed with lidocaine topical anesthetic (Xylocaine, Astra Pharma, Inc., Mississauga, Ontario) and its trachea was intubated (Size 4.0-6.0, Willy Rusch, Kernen, Germany). Anesthesia was maintained by ventilating the lungs with halothane (0.5-1%) in a mixture of nitrous oxide and oxygen (3:1) on a dual phase control respirator pump (Harvard apparatus, South Natick, Massachusetts) set at a volume of 15-20 mL/kg, and at a rate of respiration of 12 breaths per minute.

Through an inguinal incision made using an adjustable cautery (Model 771-2C, Birtcher, El Monte, California), the left femoral vessels were isolated from surrounding connective tissues and were catheterized. Catheters were advanced to the level of the inferior *vena cava*, for later I.V. infusions of 5% dextrose in 0.9% NaCl during experiments, and to the abdominal aorta for aortic blood pressure recordings. Catheters were tunneled subcutaneously to exit the lamb on its left flank. Catheters were contained in pouches on a specially designed lamb body jacket (Lomir, Inc., Montreal, Quebec). The lamb was allowed to recover form the effects of surgery and anesthesia in a critical care unit for small animals (Shor-Line, Schroer Manufacturing Co., Kansas City, Missouri), with adjustable oxygen supply.

Antibiotics (0.5 mg/kg enrofloxacin, Baytril, Etibicoke, Ontario) were administered intramuscularly at surgery and at 12 h intervals thereafter, for 48 h. Analgesics were not administered to any of the animals. All lambs were able to stand within 60 min of completion of surgery upon which they were returned to the vivarium

where they were housed with their mothers until the time of the experiments, at least three days later. During this time, animals were trained to rest quietly in a supportive sling in the laboratory environment. This three day recovery period has previously been shown by Sidi *et al* (177) to be more than adequate for the return of blood pressure, heart rate, cardiac output to pre-surgical values in sheep; reports from our laboratory also support these findings (180,182).

### 2.2.2 Cardiovascular effects of nitric oxide synthesis inhibition

In addition to the surgical protocol mentioned in Section 2.2.1, the following procedures were followed to prepare the animal for this experimental protocol.

Through an inguinal incision, additional catheters were placed in the right femoral artery and advanced to lie superior to the junction of the renal artery to allow for the close-arterial injection of drugs to the kidney during experiments. The right femoral vein was also cannulated and catheter advanced to the inferior *vena cava* for central venous pressure measurements during the experiment. By means of a right flank incision, the right renal artery was exposed and a pre-calibrated ultrasonic flow transducer (3S-4S) was placed around it as previously described (181) for later measurement of renal blood flow. This flank incision was closed and the transducer cable was contained in a pouch on a lamb jacket between experiments.

### 2.2.3 Nitric oxide and the arterial baroreflex control of heart rate

In addition to the surgical procedures described in Section 2.2.2, a separate catheter was placed into the right femoral vein and advanced to the level of the inferior *vena cava* for I.V. administration of drugs during the experiments. The catheter was tunneled to exit the lamb on its right flank, and stored in a pouch on the lamb body jacket

for safe keeping between experiments.

### 2.2.4 Renal effects of nitric oxide synthesis inhibition

In addition to the surgical procedures described in Sections 2.2.2, by means of an abdominal midline incision, the urinary bladder was exposed and a catheter inserted directly across the bladder wall. The catheter was secured in place using a figure-eight knot. This incision was closed and the catheter stored in a special pouch on the lamb body jacket.

# *CHAPTER THREE*

## *Systemic and Renal Hemodynamic Effects of Nitric Oxide Synthesis Inhibition during Maturation in Conscious Lambs*

### 3.1 INTRODUCTION

The vascular endothelium participates in the regulation of regional vascular tone through the production and release of various vasoconstrictor and vasodilator factors (53,104). Endothelium derived nitric oxide, as first described by Furchgott and Zawadzki (54), is one such vasodilator substance.

As previously described in Chapter One, nitric oxide is a biologically ubiquitous molecule that is synthesized from the amino acid L-arginine by one of three isoforms of the NOS enzyme (121,153). The endothelial form of this synthase (eNOS), found primarily in vascular endothelial cells, generates nitric oxide under basal conditions and is most responsible for the cardiovascular effects of nitric oxide.

In the adult, nitric oxide produced in the central nervous system as well as in the systemic vasculature plays an important role in regulating blood pressure and vascular resistance (2,14,56,74,164). In conscious dogs for example, the administration of L-NAME is associated with an increase in systemic vascular resistance and mean arterial pressure, and consequently a decrease in heart rate and in cardiac output (47). Moreover, Gardiner *et al* demonstrated that in conscious rats, nitric oxide is an important modulator of organ perfusion in several vascular beds including that of the kidney (56). Nitric oxide's role in modulating renal hemodynamics is further accentuated by chronic NOS inhibition studies which demonstrate that intrarenally produced nitric oxide is necessary for promoting normal tissue perfusion (16,153,167).

Intrarenally generated nitric oxide may also function as a buffer against angiotensin II induced vasoconstriction (2,115,179). For example, when nitric oxide synthesis is inhibited in conscious adult animals, the renal vasoconstrictor effects of exogenous angiotensin II are exacerbated (2,15). Therefore, there appears to be a critical balance between the renal vasoconstrictor effects of angiotensin II and the renal vasodilator effects of nitric oxide, especially in face of elevated levels of angiotensin II. In the newborn period, circulating levels of renin and angiotensin II are elevated in all species studied (8,64,178,211). Taken together, the above mentioned studies led us to speculate that in the immediate newborn period, when circulating levels of angiotensin II are elevated, the role of nitric oxide in modulating renal hemodynamics may be exacerbated, compared to that seen later in life.

The aim of the present study was, therefore, to determine the role of nitric oxide in modulating systemic and renal hemodynamics during maturation in conscious lambs.

We hypothesized that this role would be more pronounced in newborn lambs than in older animals. To achieve this aim, we measured various parameters of systemic and renal hemodynamics before and after the administration of L-NAME, or its inactive isomer D-NAME, in conscious, chronically instrumented newborn and older lambs.

## 3.2 METHODS

Experiments were carried out at least three days after surgery in conscious, chronically instrumented newborn lambs (n=10) and older lambs (n=14). The surgical procedures are described in Chapter Two.

On the day of an experiment, the lamb was brought to the laboratory and placed in a supportive sling for at least 60 min to allow for it to become accustomed to its surroundings. An I.V. infusion of 5% dextrose in 0.9% sodium chloride (4.17 mL/kg/h) was started, and continued for the duration of the study, to assist in maintaining fluid balance. The left femoral arterial and venous catheters were connected to pressure transducers for measurement of arterial and venous pressures, respectively; the flow transducer was connected to a flowmeter for measurement of renal blood flow. Arterial and venous blood pressures and renal blood flows were recorded onto a polygraph and simultaneously to a computer at 200 Hz using the data acquisition and analysis software package, CVSOFT. Experiments were carried out as follows:

### 3.2.1 Experimental protocol

Parameters of systemic and renal hemodynamics were measured in each lamb before (Control) and for 4 h after the I.V. injection of L-NAME or D-NAME (Precision Biochemicals, Vancouver, British Columbia) administered at the following doses: 10

mg/kg (experiment one), 20 mg/kg (experiment two), 40 mg/kg (experiment three). Each experiment was carried out on separate days at intervals of 24 to 48h, and in random order. Drugs were diluted from a 1000 mg/mL stock solution. Care was taken to insure that same volume of L-NAME or D-NAME was injected to the animal at every dose.

We also measured the renal blood flow response to I.A. injection of 1 μg/kg of acetylcholine chloride at various time points throughout the experiments as follows: 5, 10, 20, 40, 60, 90, 120, 150, 180, 210 and 240 min. This dose was determined by us in previous experiments as the $ED_{75}$ renal blood flow response to acetycholine chloride over the range of doses 0 to 10 ug/kg (173). This allowed us to assess the degree to which renal arterial nitric oxide synthesis activity was influenced by the administration of L-NAME or D-NAME.

At the end of the three experiments, lambs were administered a lethal dose of sodium pentobarbitone. Upon *post mortem* inspection, the placement of the catheters and the zero offset of the renal blood flow transducer were determined. The right and left kidneys were removed and weighed.

### 3.2.2 Computations and Statistical Analyses

Systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressures, mean venous pressure (MVP), renal blood flow (RBF) and renal vascular resistance (RVR=MAP-MVP/RBF) were calculated using CVSOFT, and spreadsheets (Excel, Microsoft Office, Version 7.0). Heart rate (HR) was determined from the systolic peak of the arterial pressure waveform.

The effects of age, drug and dose on the measured variables were determined using MANOVA procedures for repeated measures. For all MANOVA tests, where the F

values were significant, Newman-Keuls multiple comparison tests were applied to determine where the significant differences occurred. Significance was accepted at the 95 % confidence interval. All data are presented as mean±one standard deviation.

## 3.3 RESULTS

Baseline physiologic variables and measurements are provided in Table 3.1. As maturation proceeded, renal blood flow and heart rate decreased, whereas renal vascular resistance increased.

In both newborn and older lambs, L-NAME administration at all doses produced an increase in mean arterial pressure above control values by five minutes (Figure 3.1, $p<0.001$). Although the magnitude of this response was not dose-dependent in the newborn animals, the peak response was dose-dependent in older lambs ($p<0.05$). The duration of this increase in blood pressure to L-NAME was dose dependent ($p<0.001$) but not age-dependent. In newborn lambs, blood pressure returned to control levels by 40 min and 150 min following the administration of 10 mg/kg and 20 mg/kg L-NAME, respectively. Blood pressure did not return to baseline values following 40 mg/kg L-NAME after four hours. Older lambs demonstrated similar responses to 10 mg/kg, 20 mg/kg and 40 mg/kg doses of L-NAME, where blood pressure remained elevated for 20 min, 120 min and 240 min, respectively. The observed changes in mean arterial pressure at all doses and ages was due to changes in both systolic and diastolic blood pressures, although the magnitude of the change in diastolic blood pressure was greater than that of the systolic. D-NAME administration had no effect on mean arterial pressure in newborn

**Table 3.1 Baseline measurements in conscious lambs.**

| | NEWBORN LAMBS | OLDER LAMBS |
|---|---|---|
| Age (days) | 10 ± 1 | 44 ± 5 * |
| Body weight (kg) | 8.1 ± 1.8 | 12.3 ± 2.1* |
| MAP (mmHg) | 84 ± 5 | 86 ± 7 |
| SAP (mmHg) | 113 ± 10 | 105 ± 13 |
| DAP (mmHg) | 65 ± 7 | 69 ± 5 |
| HR (bpm) | 185 ± 12 | 118 ± 24* |
| RBF (mL/min/gkw) | 2.6 ± 0.2 | 3.5 ± 0.6* |
| RVR (mmHg/mL/min/gkw) | 31.2 ± 3.1 | 25 ± 4.7* |

Mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), heart rate (HR), renal blood flow (RBF), renal vascular resistance (RVR), and gram kidney weight (gkw). Values are means ± SD. *$p<0.05$ compared to newborn lambs.

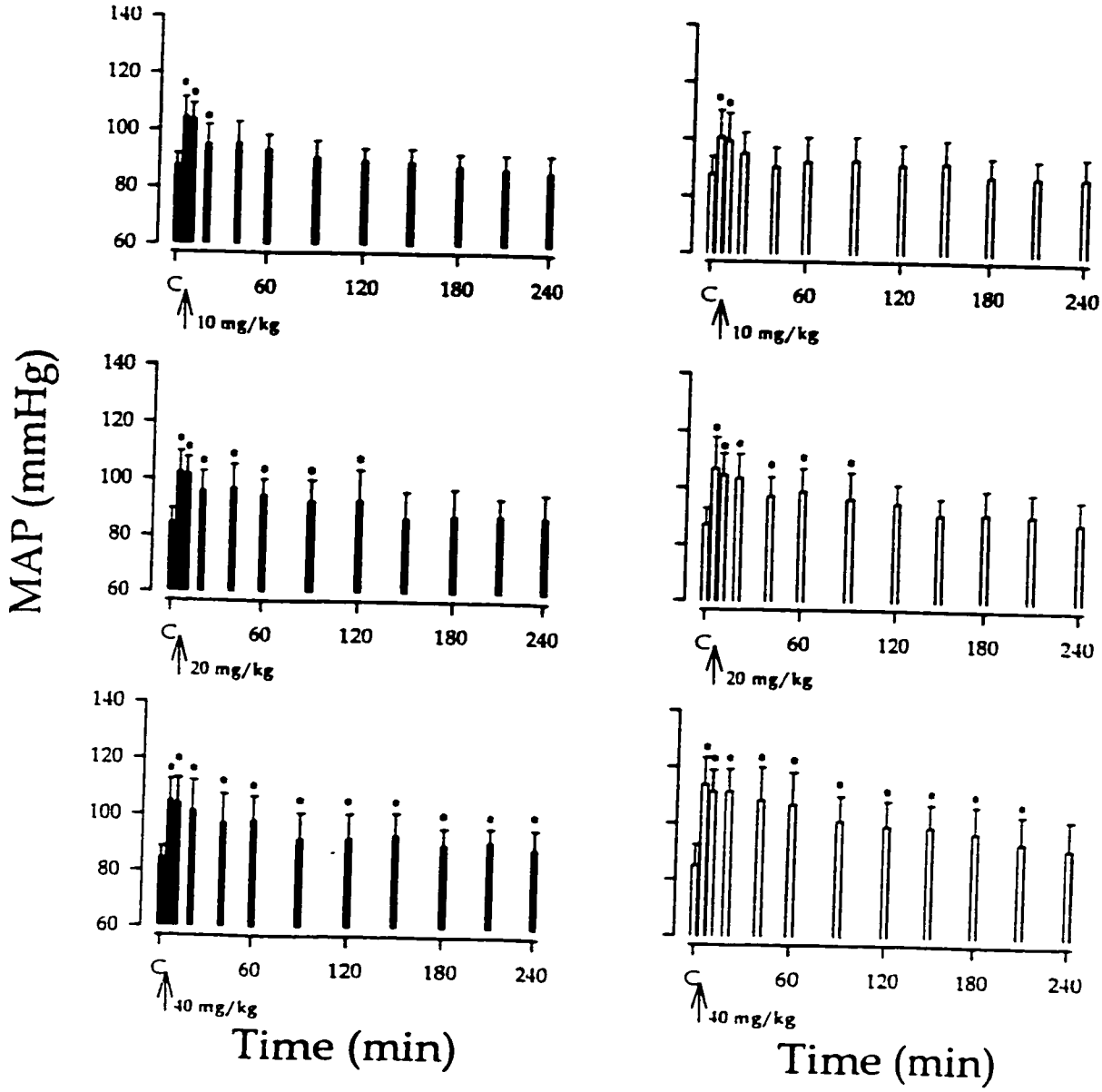


**Figure 3.1** Mean arterial pressure (MAP) measured before (C) and after I.V. injection of L-NAME at various doses in newborn (left, solid bars) and older lambs (right, open bars). Values are mean ± SD. *p<0.05 compared to C.

lambs, whereas it resulted in a slight increase in pressure at 180 min in older animals ($p<0.001$), which persisted until the end of the experiment.

Neither of the three doses of L-NAME had significant effects on mean venous pressure in any age group studied.

Heart rate decreased within five minutes of L-NAME injection at all three doses in newborn lambs and remained decreased for four hours. In contrast, heart rate remained constant after 10 mg/kg and declined only after 20 mg/kg and 40 mg/kg L-NAME in the older animals (Figure 3.2, $p<0.001$). Although this response was not dose-dependent in both groups of lambs ($p=0.63$), there was a significant temporal relationship with respect to the bradycardia between the two ages ($p<0.001$). In older lambs, however, the bradycardia persisted for 90 min following 20 mg/kg and for at least 240 min following 40 mg/kg. Only in older lambs, 40 mg/kg D-NAME administration produced a slight decrease in heart rate at 210 min, which persisted until the end of the study.

As illustrated in Figure 3.3, L-NAME administration resulted in a decrease in renal blood flow, the duration of which was both age ($p=0.055$) and dose-dependent ($p<0.05$). In newborn lambs, renal blood flow decreased by five minutes after L-NAME administration for all doses studied. The duration of this response was 150 min and 180 min after 10 mg/kg and 20 mg/kg L-NAME injection, respectively, but persisted for at least four hours following 40 mg/kg L-NAME. Renal blood flow was unaltered following 10 mg/kg L-NAME in the older lambs, but decreased below control levels for 40 min and 150 min after 20 mg/kg and 40 mg/kg L-NAME, respectively. D-NAME did not alter renal blood flow in any of the age groups studied.

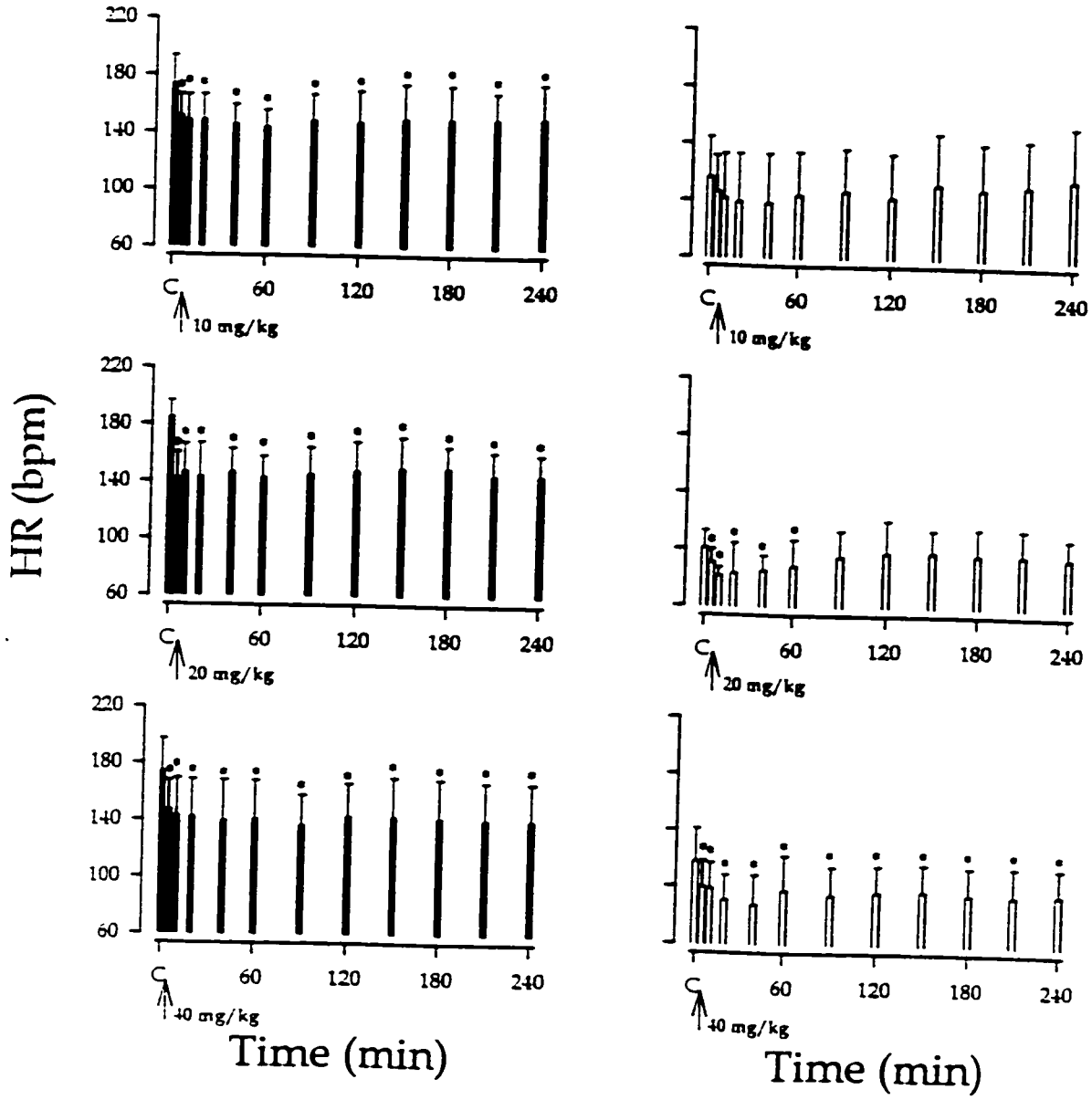


**Figure 3.2** Heart rate (HR) measured before (C) and after I.V. injection of L-NAME at various doses in newborn (left, solid bars) and older lambs (right, open bars). Values are mean ± SD. *$p<0.05$ compared to C.

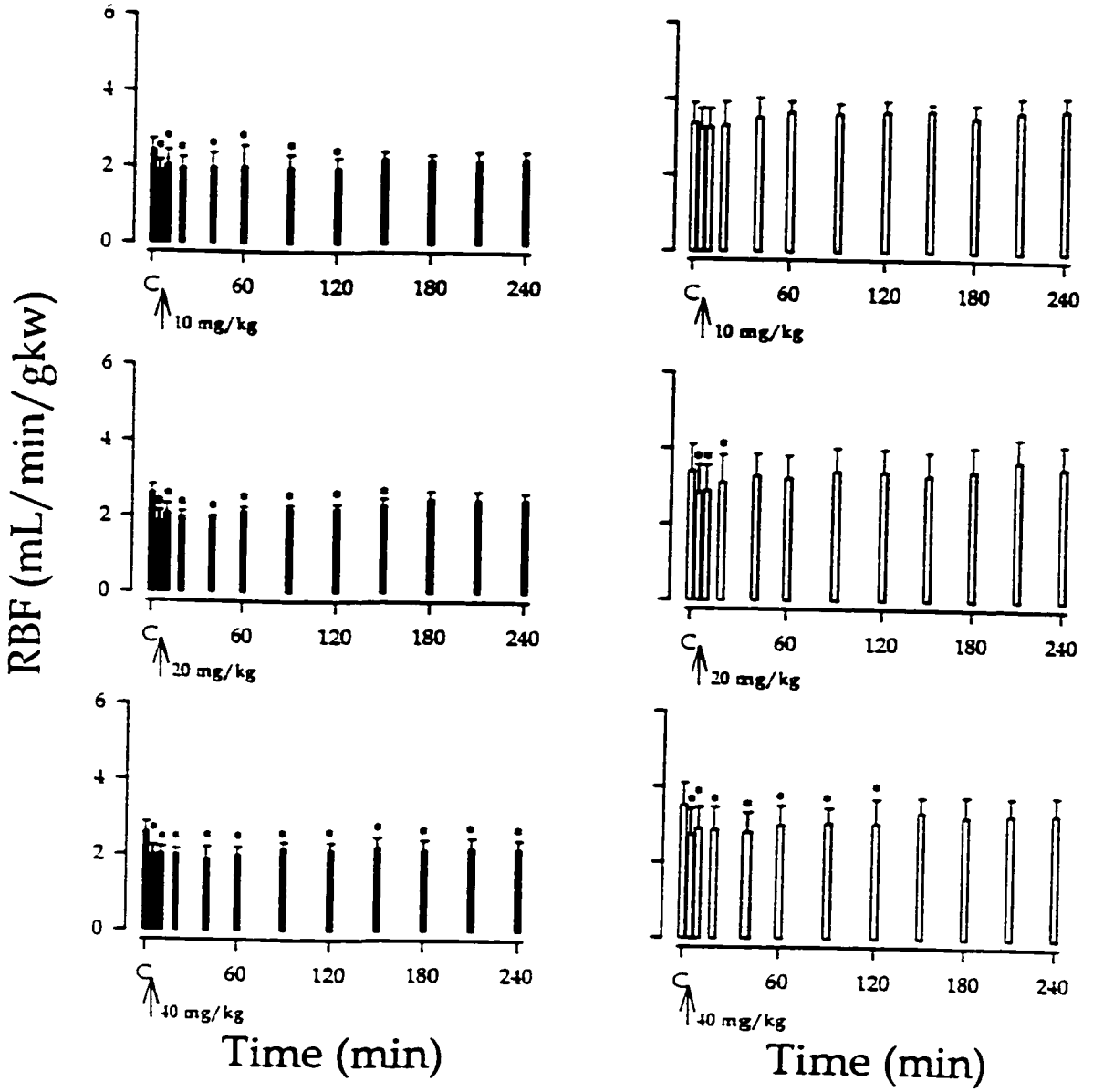


**Figure 3.3** Renal blood flow (RBF) measured before (C) and after I.V. injection of L-NAME at various doses in newborn (left, solid bars) and older lambs (right, open bars). Values are mean ± SD. *p<0.05 compared to C.

Renal vascular resistance increased by five minutes after L-NAME administration in both ages of lambs (Figure 3.4, $p<0.001$). The duration of this response was age ($p<0.001$) and dose-dependent ($p<0.001$), and the profile of the renal vascular resistance response to L-NAME mirrored that of the renal blood flow response in both newborn and older lambs. D-NAME administration did not alter renal vascular resistance at any of the doses tested.

### 3.3.1 Summary of systemic and renal hemodynamic effects of L-NAME

The above section provides a detailed account of the age and dose- dependent nature of the systemic and renal hemodynamic effects of L-NAME in conscious lambs during maturation. The proceeding portion of the result's section will attempt to consolidate this information:

Figure 3.5 illustrates the renal blood flow response to intra-arterial administration of acetylcholine chloride. This demonstrates the effect of L-NAME but not D-NAME in inhibiting the renal vasodilation induced by administration of acetylcholine chloride. Figure 3.6 shows that the I.A. acetylcholine induced changes in renal blood flow following L-NAME administration were attenuated in an age-, time- and dose-dependent manner ($p<0.001$). After administration of 10 mg/kg of L-NAME, the renal vasodilatation response to acetylcholine was attenuated for only 20 min in both age groups. After 20 mg/kg, this response was attenuated for 150 min in newborns and for 90 min in older lambs. After 40 mg/kg, the renal blood flow response to acetylcholine was attenuated for at least four hours in newborns, whereas the control response was reached by approximately 150 min in older lambs. Therefore, in order to effectively compare the

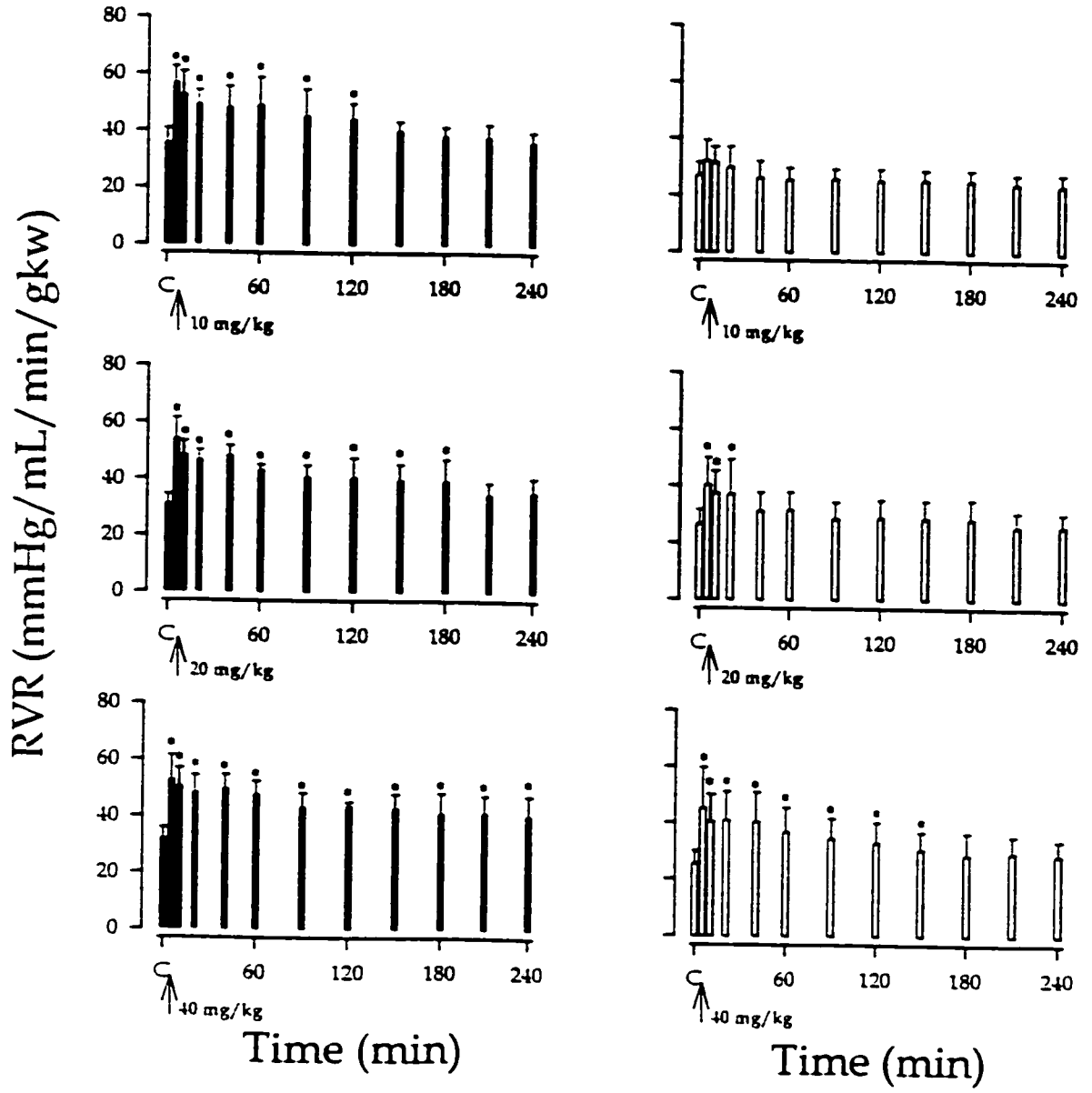


**Figure 3.4** Renal vascular resistance (RVR) measured before (C) and after I.V. injection of L-NAME at various doses in newborn (left, solid bars) and older lambs (right, open bars). Values are mean ± SD. *$p<0.05$ compared to C.

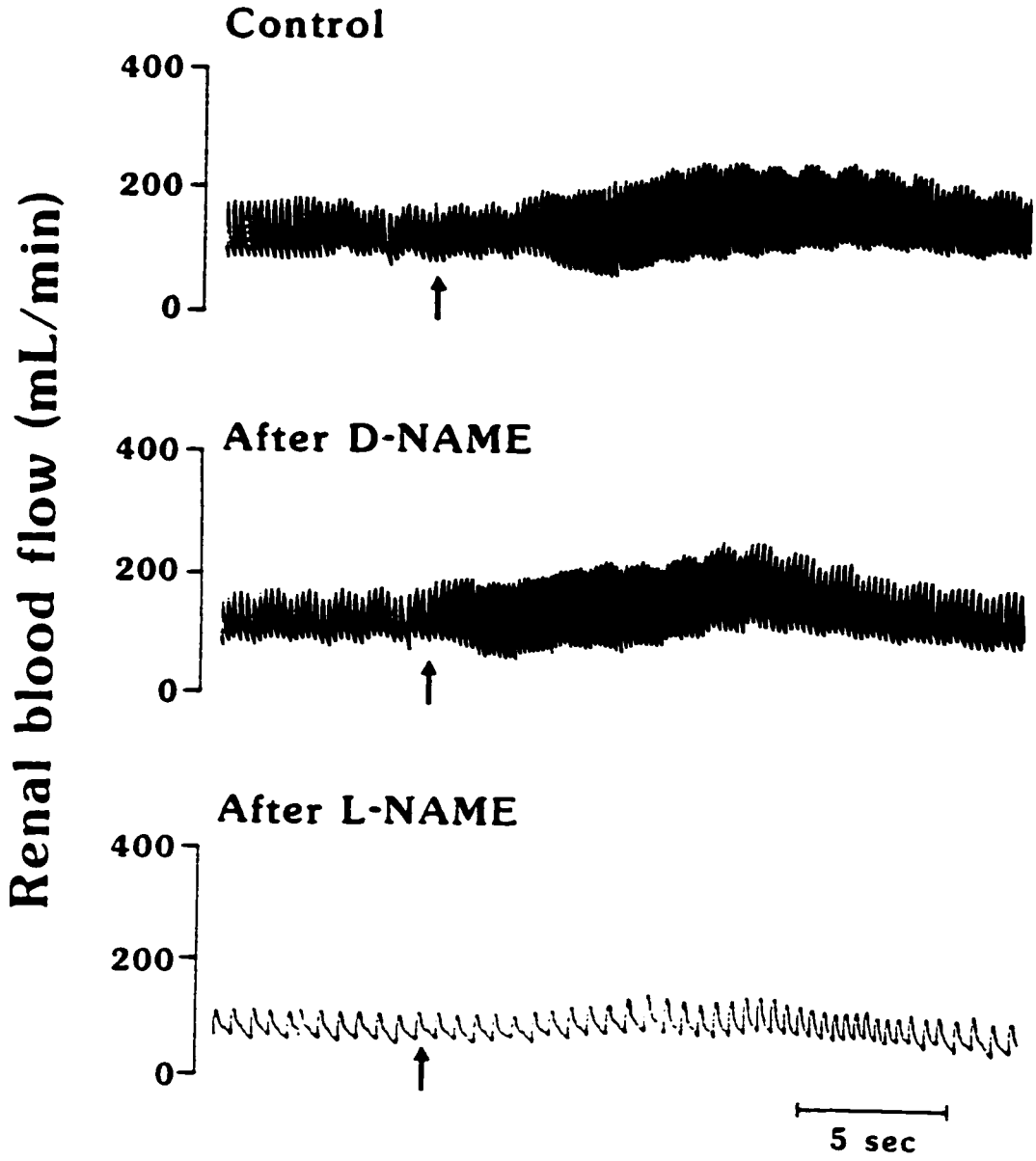


**Figure 3.5** Experimental tracings illustrating the renal blood flow responses to close renal arterial injections of 1 μg/kg acetylcholine chloride (arrow) during Control measurements (top) and at 60 min after the I.V. administration of 40 mg/kg D-NAME (middle) and L-NAME (bottom) in older lambs (Adapted from Sener, A. and Smith, F.G., 1999).

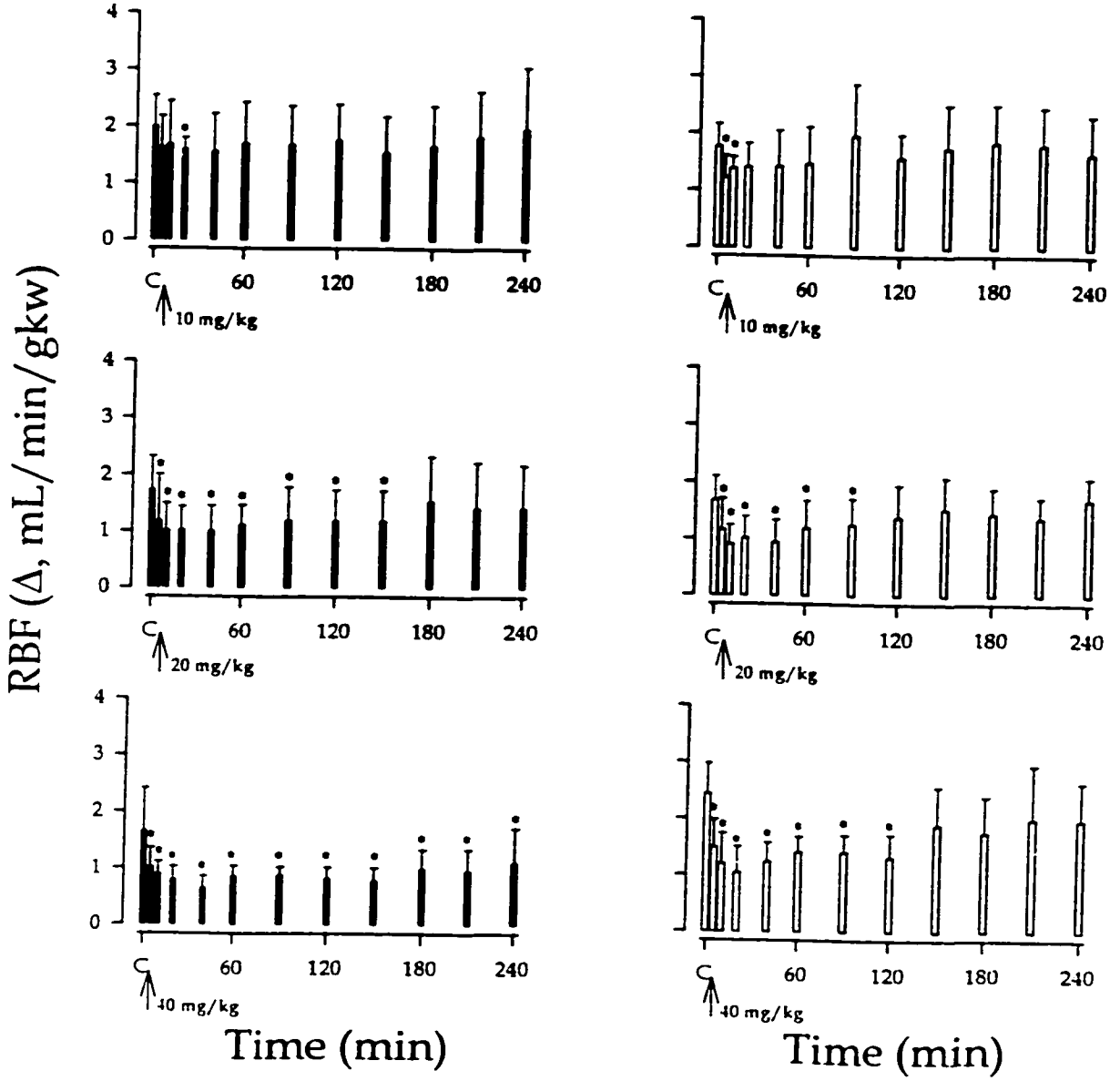


**Figure 3.6** Changes in renal blood flow (RBF) after I.A. injection of 1 μg/kg acetylcholine chloride measured before (C) and after I.V. injection of L-NAME at various doses in newborn (left, solid bars) and older lambs (right, open bars). Values are mean ± SD. *$p<0.05$ compared to C.

renal hemodynamic responses to L-NAME in the two age groups, it is necessary to compare these responses at the same degree of NOS inhibition.

As assessed by the attenuation of the acetylcholine-induced renal vasodilatation (Figure 3.6), intra-renal arterial NOS activity was attenuated for 120 min in both newborn and older lambs after I.V. administration of 20 mg/kg and 40 mg/kg L-NAME, respectively. It is at these doses of L-NAME then, that we chose to compare the influences of nitric oxide in modulating systemic and renal hemodynamics in the newborn and older lambs.

As demonstrated in Figure 3.7, when the changes in renal blood flow and in renal vascular resistance for both age groups were calculated following comparable intra-renal NOS inhibition by L-NAME, there were no significant differences found in the magnitude of the responses.

## 3.4 DISCUSSION

The purpose of the present study was to determine the role of nitric oxide in modulating systemic and renal hemodynamics during maturation in chronically instrumented conscious lambs. Our aim was to test the hypothesis that nitric oxide's influence in regulating renal hemodynamics would be exacerbated in the immediate newborn period when plasma levels of angiotensin II are markedly elevated, compared to older lambs. We found that administration of L-NAME resulted in dose-dependent increases in blood pressure and decreases in heart rate in both newborn and older animals. Blood pressure effects were, however, greatest in older lambs, whereas heart rate effects were greatest in newborn lambs. These findings demonstrate that nitric oxide

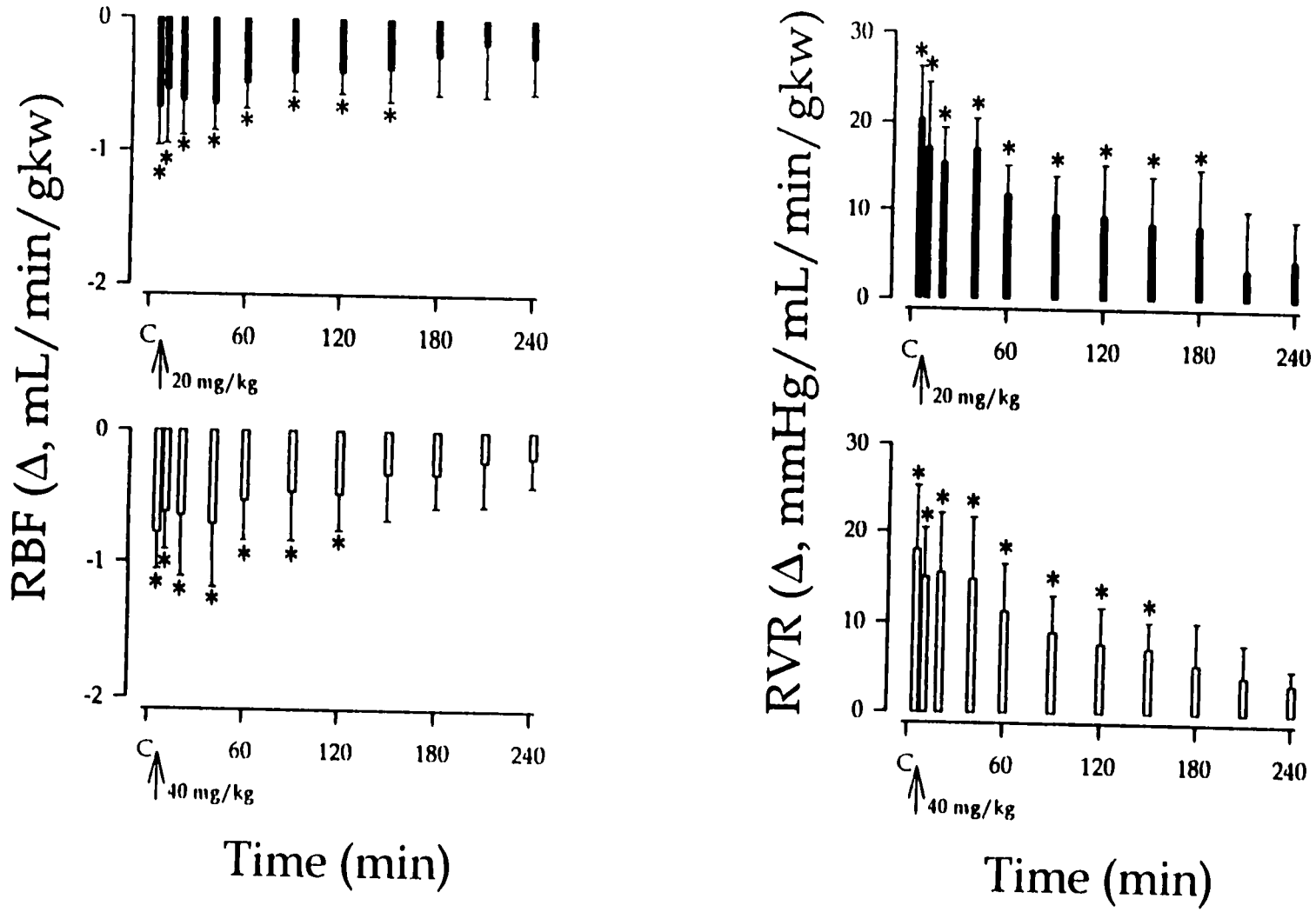


**Figure 3.7** Changes in renal blood flow (RBF), on the left, and in renal vascular resistance (RVR), on the right, measured before (C) and after I.V. injection of L-NAME in newborn (solid bars) and older lambs (open bars). Values are mean ± SD. *p<0.05 compared to C.

modulates systemic hemodynamics in an age-dependent manner. We also observed that under conditions of comparable intrarenal NOS inhibition, L-NAME injection to newborn and older lambs, respectively, was associated with an increase in renal vascular tone, which was comparable in both age groups. Therefore, we reject our hypothesis and conclude that nitric oxide does not modulate renal hemodynamics in an age-dependent fashion. Our results are the first to demonstrate the systemic and renal hemodynamic effects of nitric oxide during early postnatal life and during maturation in conscious lambs.

Previous studies in adult animals and humans (14,31,63,90) have shown that inhibition of nitric oxide production leads to an increase in blood pressure over the short term, and chronically leads to hypertension (16). Therefore, it is apparent that nitric oxide plays a vital role in blood pressure regulation, resulting primarily from the removal of endothelial derived nitric oxide. It has also recently been suggested that nitric oxide may play a role in blood pressure regulation by altering sympathetic output from the central nervous system (203), or by acting as a neurotransmitter both centrally and peripherally (106,202). It is suggested that, in rats and rabbits (45,67,164), nitric oxide acts through NMDA receptors coupled to NOS activation (58) at the level of the NTS to influence blood pressure by modulating sympathetic output to the periphery. Recent reports by Patel *et al* in rats (139,230) also suggest that nitric oxide may act at the PVN, via the gamma amino butyric acid pathway, to inhibit sympathetic outflow to the kidney, at least in adult rats. Moreover, Matsuda *et al* (111) have shown that nitric oxide directly modulates baroreceptor activity in the isolated carotid sinus of anesthetized rabbits. Taken together, these studies lead us to postulate that nitric oxide may modulate the

afferent, central, and/or efferent limbs of the autonomic nervous system; whether this role is altered with maturation, however, is not known.

The potential role of nitric oxide as a modulator of the autonomic nervous system is intriguing, as our data show that L-NAME administration appears to have altered the arterial blood pressure:heart rate relationship, at least in newborn lambs (Figures 3.1 and 3.2). Minami *et al* reported that in conscious Wistar rats, I.V. injection of L-NAME resulted in a shift of the arterial baroreflex control of heart rate towards higher pressures (119). In contrast, Liu *et al* showed that in conscious rabbits, L-NNA administration resulted in a decrease in heart rate whereas blood pressure did not change, suggesting a shift in the arterial baroreflex control of heart rate towards lower pressures (100). Although the aforementioned studies provide contradicting findings, our data provide evidence to suggest that nitric oxide modulates the arterial baroreflex control of heart rate, and that this role of nitric oxide may be age-dependent. We continued to investigate this phenomenon further by assessing the arterial baroreflex control of heart rate before and after L-NAME during postnatal maturation. The results of this study will be discussed in detail in Chapter Four of this thesis.

Previous studies in conscious adult dogs and rats have shown that inhibition of nitric oxide synthesis is associated with a dramatic increase in renal vascular resistance (15,47). Our data support these findings and provide the first information that nitric oxide also influences renal vascular tone under normal physiological conditions soon after birth, in conscious lambs. The influence of L-NAME was, however, more prolonged in the newborn lambs. One can not rule out the possibility that the prolonged effects of L-NAME in newborn lambs may be a reflection of a maturational change in

L-NAME metabolism; to date, however, there have been no studies to investigate this possibility. If this were the case, however, one might expect that blood pressure and heart rate effects would follow the same pattern as the renal vascular effects; this was not seen.

We hypothesized that the role of nitric oxide in modulating renal vascular tone would be greatest soon after birth due to the presence of high circulating levels of angiotensin II. This premise was based on the studies by Baylis *et al* who showed that nitric oxide plays an increased role in maintaining renal perfusion in conscious adult rats when angiotensin II levels are acutely elevated under experimental conditions (15). To the contrary, we found that the effects of L-NAME were similar in both newborn and older lambs when evaluated at comparable NOS inhibition. Therefore, we conclude that nitric oxide modulates renal vascular tone to a similar extent throughout postnatal maturation in conscious lambs.

Solhaug *et al* have previously investigated the role of nitric oxide in modulating renal hemodynamics in anesthetized piglets (190,191). The authors reported that nitric oxide's influence over renal hemodynamics was increased in the anesthetized newborn piglet compared to older pigs. Since anesthesia is known to alter many physiological parameters, including the animal's humoral status, the results obtained by Solhaug *et al* should, therefore, be interpreted with caution (112,231). It should also be noted that in the study by Solhaug *et al*, the degree of intrarenal NOS inhibition was not evaluated following L-NAME injection, thus potentially adding another confounding factor to their results. A second explanation for the discrepancy between our findings and those mentioned above may reside in the different stimulatory effects of acute versus chronic

elevations in circulating angiotensin II on NOS activity. Although acute infusions of angiotensin II may upregulate NOS and hence the production of nitric oxide (17), following chronic elevations in circulating angiotensin II, such as that which occurs in the newborn period under normal physiological conditions, NOS activity may become dissociated from the stimulatory effects of angiotensin II. For example, Deng *et al* showed that in anesthetized rats chronically administered angiotensin II via a subcutaneous osmotic minipump for five days, urinary nitrate/nitrite (metabolites of nitric oxide degradation) excretion were not significantly different from those measured in control rats who received vehicle. When rats were acutely administered angiotensin II, however, urinary nitrate/nitrite levels were markedly elevated compared to values obtained from control rats (41). It is therefore likely that under experimental conditions when angiotensin II levels are acutely elevated due to injection or such as that which occurs as a side effect of anesthesia, the capacity of nitric oxide to modulate renal hemodynamics may be exacerbated. Our findings, therefore, provide a significant contribution to the current knowledge on this topic.

Interestingly, renal neuronal NOS mRNA activity is increased at birth and declines with postnatal maturation in piglets, rats and guinea pigs (51,189,200). As well, we have previously demonstrated that the ability of the renal vasculature to generate acetylcholine-induced vasodilatation is greatest in the newborn lamb compared to its older counterpart (173). Similar findings were also reported by Torok and Gerova in aortic ring preparations obtained from newborn and adult dogs (206). These findings are not surprising since evidence provided by Deng *et al* suggests that although chronic elevations in circulating angiotensin II may no longer stimulate compensatory nitric

oxide production, the renal vasculature then becomes increasingly sensitive to the stimulatory effects of such endothelium-dependent vasodilators as acetylcholine (41). It is unclear as to why intra-renal NOS levels are elevated in the newborn period. It is possible that the presence of high NOS activity early in life may signify a greater role for nitric oxide in modulating glomerular and/or tubular function during this stage of development, compared to later in life. To elucidate this matter, we measured the effects of L-NAME on various parameters of renal function during development. These experiments are reported in Chapter Five of this thesis.

It has previously been shown that the renal vasodilatory effects of acetylcholine are either partly attenuated or completely abolished by the administration of L-arginine analog inhibitors of NOS. For example, Radermacher *et al* (151), and Reid and Rand (157) showed that the acetylcholine induced renal vasodilatation of the isolated perfused rat kidney was attenuated by administration of L-arginine analogues. Additionally, Lahera *et al* reported that in meclofenamate treated anesthetized dogs (92), the renal vasodilatation induced by intra-renal infusion of acetylcholine was abolished by pre-treatment with L-NMMA. Although our results are in accordance with the previous findings listed above, our's are the first to show the time-course of the attenuated acetylcholine-induced renal vasodilatation following nitric oxide synthesis inhibition. In the current study, increasing doses of L-NAME did not alter the magnitude of the attenuated response to acetylcholine chloride injection, but did prolong the duration of attenuation. The mechanism of this is not known.

In conclusion, these experiments provide the first investigation of the systemic and renal hemodynamic responses to nitric oxide synthesis inhibition in conscious,

chronically instrumented lambs during maturation. Our observations provide new evidence that nitric oxide is an important modulator of baseline cardiovascular homeostasis during postnatal maturation. Although nitric oxide appears to modulate systemic hemodynamics in an age-dependent manner, its role in influencing renal hemodynamics is not altered with postnatal maturation.

# *CHAPTER FOUR*

# *Effects of Nitric Oxide Synthesis Inhibition on the Arterial Baroreflex Control of Heart Rate during Maturation in Conscious Lambs*

## 4.1 INTRODUCTION

The arterial baroreflex is an important cardiovascular reflex that compensates for second-to-second changes in blood pressure by eliciting alterations in both heart rate and systemic vascular resistance, thus ensuring cardiovascular homeostasis (37). Arterial baroreceptors, located in the carotid sinus and regions of the aortic arch, are stretch receptors which monitor changes in arterial blood pressure by sensing changes in transmural pressures across the vascular wall and hence alter their afferent output to the NTS of the hind brain accordingly. The NTS is responsible for receiving, integrating and

finally generating an appropriate efferent output based upon these and other afferent signals from higher brain centers, including the PVN of the hypothalamus.

In addition to its role as a vasoactive molecule, nitric oxide has been implicated as a potential modulator of the arterial baroreflex (104) through its actions at the afferent, central and efferent portions of the arterial baroreflex arc (67,111,164,203,215). For example, Matsuda *et al* demonstrated in anesthetized rabbits, that increases in sinus baroreceptor activity induced by increasing carotid sinus pressure were significantly attenuated in the presence of nitric oxide (111). Furthermore, histochemical mapping techniques have identified the presence of nitric oxide synthase enzymes in numerous sites responsible for cardiovascular function, including the PVN, NTS and the rostral ventrolateral medulla (214), thereby implicating nitric oxide as a potential central modulator of the arterial baroreflex. Additionally, Harada *et al* (67) reported that in anesthetized rabbits, microinjection of L-NMMA directly onto the NTS produced an increase in renal sympathetic nerve activity, which could be reversed by microinjections of the nitric oxide precursor, L-arginine. Likewise, recent studies by Patel *et al* in anesthetized rats showed that nitric oxide appears to act at the PVN to inhibit sympathetic outflow (139,230).

As described in Chapter Three, our investigation into the cardiovascular effects of L-NAME during postnatal maturation in conscious lambs revealed an age-dependent effect of nitric oxide synthesis inhibition on blood pressure and heart rate (176). Firstly, the increase in blood pressure after L-NAME administration was greatest in older lambs, whereas the resultant decrease in heart rate was greatest in newborn animals. Secondly, in newborns but not in older lambs, this decrease in heart rate persisted even though

blood pressure returned to control values. Our data therefore suggested to us that nitric oxide normally modulates the arterial baroreflex control of heart rate early in life and that this effect is altered with postnatal maturation. Although there is compelling evidence that implicates nitric oxide as a potential modulator of all segments of the baroreflex arc, whether this role is altered with maturation in the conscious animal remains to be investigated.

The purpose of the current study was, therefore, to test the hypothesis that the role of nitric oxide in modulating the arterial baroreflex control of heart rate is age-dependent. To test this hypothesis, we assessed the arterial baroreflex before and after I.V. administration of L-NAME and its inactive isomer, D-NAME, in chronically instrumented conscious newborn and older lambs.

## 4.2 METHODS

Experiments were carried out in conscious, chronically instrumented newborn (9 ± 1 days; 6.8 ± 0.6 kg body weight; n=5) and older (42 ± 2 days; 12.4 ± 1.7 kg body weight; n=5) lambs. Two experiments were carried out on each lamb at least 24 hours apart and in random order, at least three days after surgery, details of which can be found in Chapter Two.

On the day of an experiment, the lamb was brought into the laboratory and placed in a supportive sling for at least 60 min to allow it to become accustomed to its surroundings. An I.V. infusion of 5% dextrose in 0.9% sodium chloride (4.17 mL/kg/h) was started, and continued for the duration of the study, to assist in maintaining fluid balance. The left femoral arterial catheter was connected to a pressure transducer and

systolic arterial pressure was recorded continuously onto a polygraph and simultaneously to a computer at 200 Hz using the data acquisition and analysis software package, CVSOFT.

Systolic arterial pressure and heart rate were measured before (Control) and for 2 hours after I.V. injection of 20 mg/kg of L-NAME (experiment 1) or its inactive isomer D-NAME (experiment 2) constituted from a stock solution of 1000 mg/mL. This dose was chosen from our previous experiments described in Chapter Three (174,175). Administration of 20 mg/kg of L-NAME results in comparable increases in systolic blood pressure in terms of both magnitude and duration in both groups of lambs. Since systolic blood pressure is the independent variable in this series of experiments, the administration of this dose of L-NAME ensured that the cardiovascular system would be perturbed in a similar manner in both age groups of lambs.

During the control period and at 30, 60, 90 and 120 min after the administration of either L-NAME or D-NAME, the arterial baroreflex control of heart rate was assessed as follows: We measured the heart rate responses to changes in systolic arterial pressure induced by I.V. injections of 10 μg/kg of phenylephrine hydrochloride (PHE, Sabex, Inc., Boucherville, QC) and 10 μg/kg of sodium nitroprusside (SNP, Nipride, Hoffmann-La Roche, Mississauga, ON), to increase and decrease systolic arterial pressure, respectively.

At the end of the two experiments, lambs were injected with a lethal dose of sodium pentobarbitone and, upon *post mortem*, the placement of the catheters was confirmed.

### 4.2.1 Computations and Statistical Analyses

Data were analyzed over consecutive cardiac cycles using CVSOFT; heart rate (HR) was calculated from the peak of the systolic pressure waveform, and data were entered into spreadsheets (Excel, Microsoft Office, Version 7.0). Beat to beat changes in heart rate induced by changes in systolic arterial pressure (SAP), using PHE and SNP, were plotted against the preceding SAP for all animals using a graphics package (Sigma Plot, Jandel Scientific, Version 4.0). The following four-parameter sigmoidal logistic function was applied to the raw data to generate the best-fit curve based upon the equation of Kent *et al* (81): $HR = [(a - d) / 1 + (SAP/c)^b] + d$. The parameters "a" and "d" represent the asymptotic maximum and minimum heart rate values, respectively. The parameter "c" is the systolic arterial pressure value at the mid-point of the heart rate range and "b" is the slope or gain of the curve. To determine whether these four parameters were altered with respect to control, statistics were carried out using a paired Student's t-test with Bonferroni correction. A non-paired Student's t-test was applied to determine whether these four parameters were different between one and six-week old lambs. Significance was accepted at the 95% confidence interval.

## 4.3 RESULTS

Administration of L-NAME resulted in a significant increase in systolic arterial pressure (Table 4.1) from control values in both newborn and older lambs, which lasted for 120 min and 90 min, respectively ($p<0.05$). Furthermore, this L-NAME induced increase in systolic arterial pressure was accompanied by a reduction in heart rate in both age groups which persisted for the duration of the study in newborns and for 90 min in

**Table 4.1 Effects of L-NAME on baseline cardiovascular measurements.**

| | Age | Control | 30 min | 60 min | 90 min | 120 min |
|---|---|---|---|---|---|---|
| SAP (mmHg) | NEWBORN | 109 ± 10 | 120 ± 13* | 116 ± 7* | 124 ± 13* | 118 ± 10* |
| | OLDER | 111 ± 9 | 124 ± 5* | 124 ± 12* | 123 ± 10* | 117 ± 12 |
| HR (bpm) | NEWBORN | 204 ± 25 | 156 ± 17* | 144 ± 19* | 148 ± 13* | 150 ± 20* |
| | OLDER | 115 ± 14† | 97 ± 6*† | 99 ± 12*† | 97 ± 13*† | 109 ± 13† |

Systolic arterial pressure (SAP) and heart rate (HR). Values are mean ± SD. *$p<0.05$ compared to Control. †$p<0.05$ compared to newborn lambs.

older lambs (Table 4.1).

Baseline values for the various parameters describing the arterial baroreflex control of heart rate are shown in Table 4.2. The maximum and minimum heart rates, as well as the heart rate range over which the arterial baroreflex operates, were significantly less in older than in newborn lambs. Systolic arterial pressure at the mid-point of the heart rate range was greater in older lambs compared to newborn lambs ($p<0.05$). The gain was, however, similar between the two age groups studied.

At 30 min after L-NAME administration, there was a decrease in the maximum and minimum heart rates as well as a reduction in the heart rate range over which the arterial baroreflex operates in newborns; these effects were sustained for two hours (Figure 4.1). L-NAME did not alter the gain of the systolic arterial pressure:heart rate relationship, or the systolic arterial pressure at the mid-point of the heart rate range in these newborn animals (Figure 4.1, Table 4.2).

Interestingly, none of the parameters of the arterial baroreflex control of heart rate measured was influenced by L-NAME administration in older animals (Figure 4.2, Table 4.2) at 30, 60, 90 or 120 min.

D-NAME administration did not alter any of the parameters governing the arterial baroreflex control of heart rate in both newborn and older lambs (Appendix A).

## 4.4 DISCUSSION

The purpose of the current study in conscious lambs was to assess the arterial baroreflex control of heart rate during postnatal maturation and to determine whether the role of nitric oxide in modulating the arterial baroreflex control of heart rate is age-

**Table 4.2 Effects of L-NAME on parameters of the arterial baroreflex control of heart rate.**

| | Age | Control | 30 min | 60 min | 90 min | 120 min |
|---|---|---|---|---|---|---|
| HR range (bpm) | NEWBORN | 175 ± 34 | 115 ± 38* | 129 ± 39* | 131 ± 41* | 107 ± 27* |
| | OLDER | 91 ± 19† | 105 ± 29 | 95 ± 22 | 101 ± 25 | 103 ± 31 |
| HR maximum (bpm) | NEWBORN | 288 ± 25 | 187 ± 30* | 238 ± 31* | 223 ± 38* | 217 ± 21* |
| | OLDER | 162 ± 15† | 174 ± 20 | 173 ± 15† | 169 ± 17† | 173 ± 21† |
| HR minimum (bpm) | NEWBORN | 123 ± 14 | 72 ± 13* | 99 ± 10* | 82 ± 21* | 110 ± 7* |
| | OLDER | 71 ± 10† | 69 ± 11 | 78 ± 7† | 68 ± 8† | 70 ± 10† |
| Gain (bpm/mmHg) | NEWBORN | -17 ± 5 | -19 ± 11 | -22 ± 8 | -21 ± 10 | -18 ± 7 |
| | OLDER | -14 ± 5 | -16 ± 6 | -17 ± 6 | -20 ± 9 | -20 ± 10 |
| $SAP_{50}$ (mmHg) | NEWBORN | 109 ± 5 | 134 ± 13* | 107 ± 10 | 114 ± 12 | 115 ± 7 |
| | OLDER | 116 ± 4† | 110 ± 6† | 111 ± 7 | 116 ± 7 | 108 ± 13 |

Heart rate (HR), systolic arterial pressure (SAP), and systolic arterial pressure at the mid-point heart rate range ($SAP_{50}$). Values are mean ± SD. *$p<0.05$ compared to Control. †$p<0.05$ compared to newborn lambs.

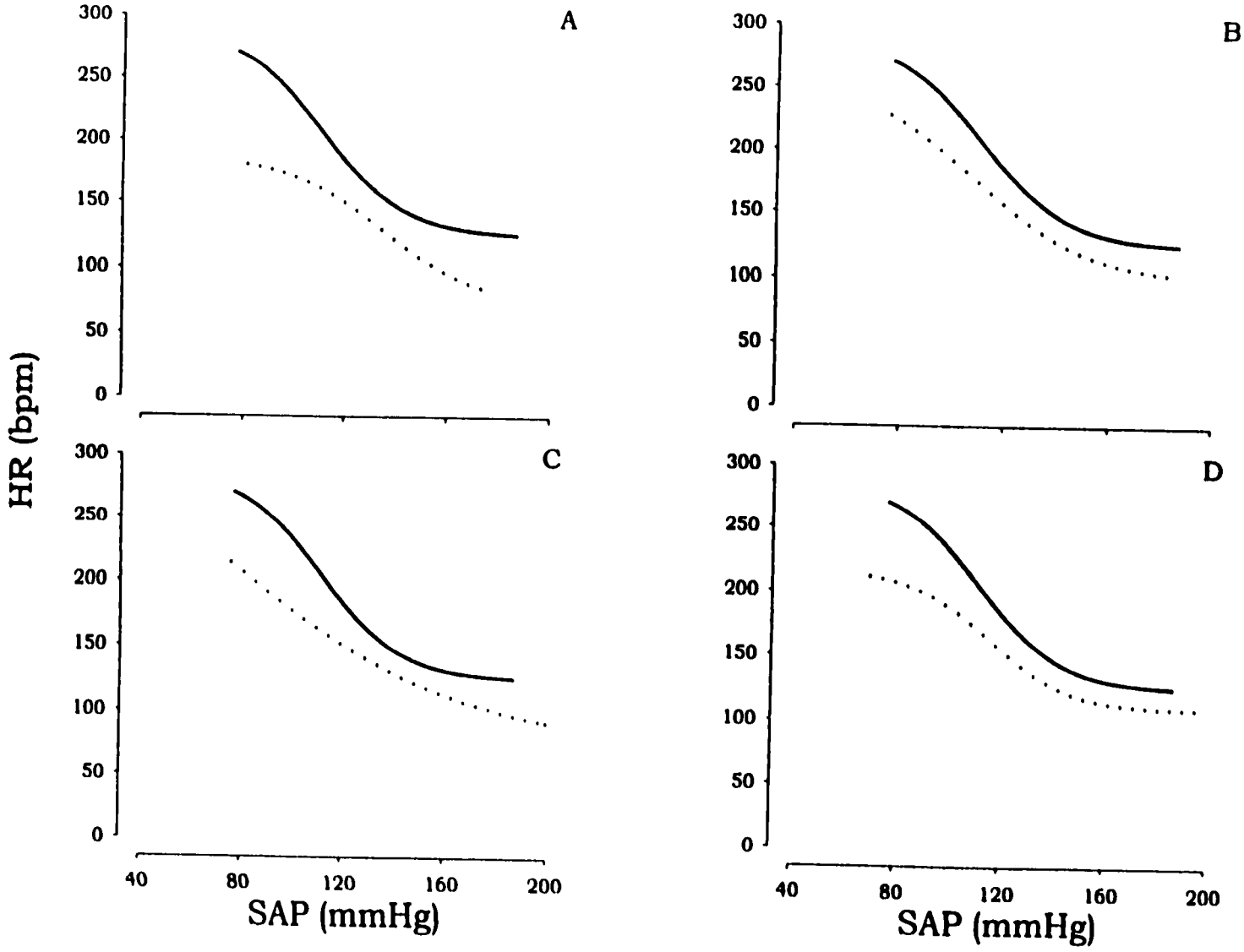


**Figure 4.1** Arterial baroreflex control of heart rate measured before (control, solid line) and at 30 min (A), 60 min (B), 90 min (C), and 120 min (D) after (dotted line) L-NAME administration in newborn lambs.

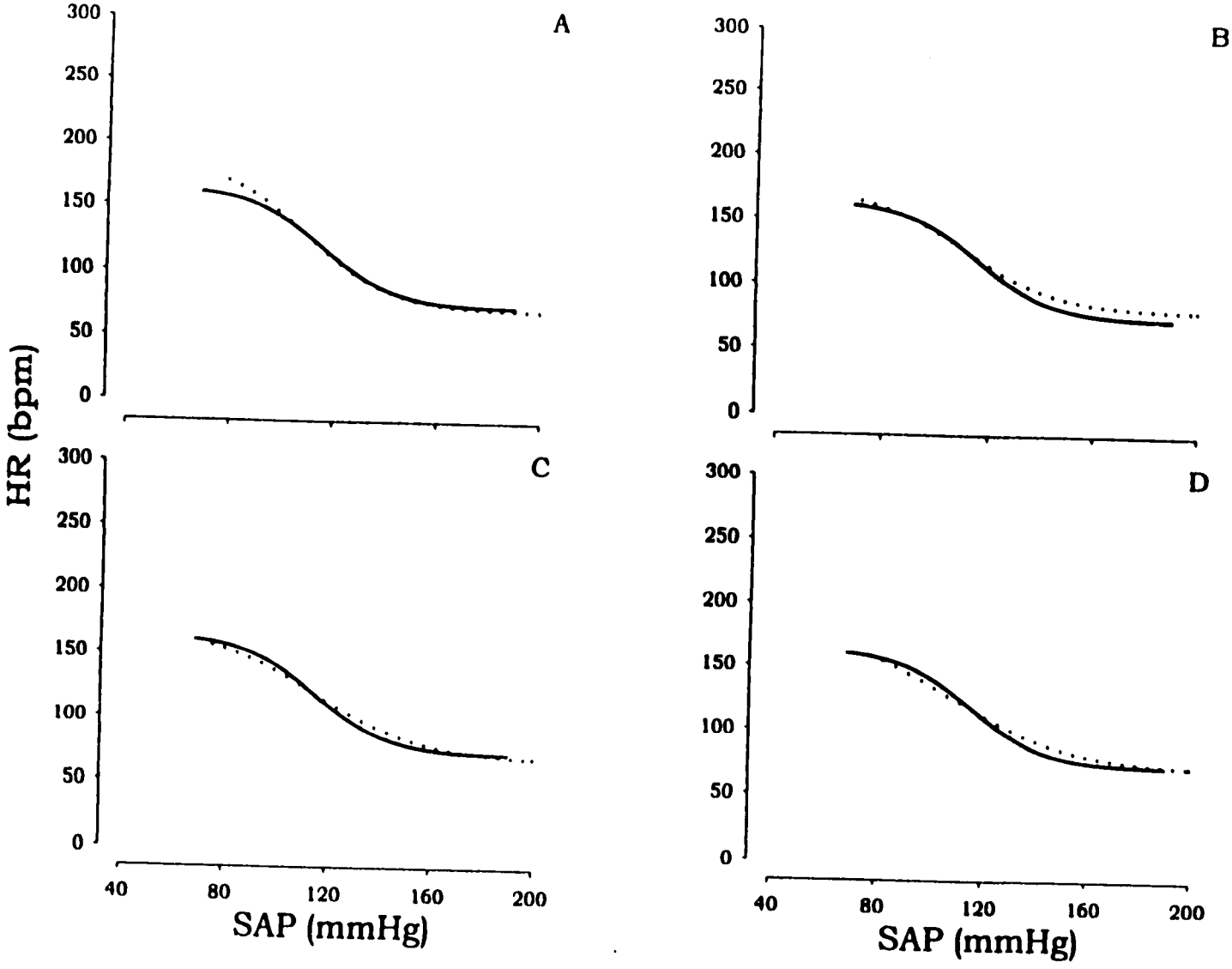


**Figure 4.2** Arterial baroreflex control of heart rate measured before (control, solid line) and at 30 min (A), 60 min (B), 90 min (C), and 120 min (D) after (dotted line) L-NAME administration in older lambs.

dependent. The novel findings of this study are that nitric oxide synthesis inhibition alters the parameters governing the arterial baroreflex control of heart rate in an age-dependent fashion as follows: In newborn, but not in older lambs, L-NAME administration was associated with a shift of the systolic arterial pressure:heart rate relationship towards lower pressures, as well as a reduction in the maximum and minimum heart rates and the range over which the arterial baroreflex operates. In addition, we provide new information that in newborns, the arterial baroreflex control of heart rate operates over a wider heart rate range compared to older lambs, whereas the gain of the relationship between systolic arterial pressure and heart rate is not altered with age. These data provide new information on the arterial baroreflex control of heart rate in conscious lambs throughout postnatal maturation. Our findings also provide important novel evidence that nitric oxide modulates the arterial baroreflex control of heart rate early in life and that this effect appears to be less predominant as maturation proceeds.

Our data are the first to show that the arterial baroreflex control of heart rate is modulated by nitric oxide in the newborn period. Interestingly, we discovered that in newborn but not in older lambs, the range over which the arterial baroreflex controls heart rate was diminished following L-NAME administration and that this effect was due to decreases in both the upper and lower heart rate plateaus of the systolic arterial pressure:heart rate relationship. Although the mechanisms behind these findings were not investigated in the current study, they may stem from the combination of the effects of nitric oxide synthesis inhibition on the afferent, central and/or efferent limbs of the baroreflex arc.

Previously, Matsuda *et al* showed that in anesthetized rabbits, the administration

of nitric oxide and of the nitric oxide donor, S-nitrosocysteine, directly onto the carotid sinus produced a reduction in carotid sinus afferent nerve activity, even in the face of increased carotid sinus pressures (111), suggesting that nitric oxide reduces the activity of the carotid sinus nerve. When carotid sinus pressures were, however, elevated in the presence of L-NAME, carotid sinus afferent nerve activity was not accentuated. Therefore, although large increases in local nitric oxide levels may attenuate carotid sinus afferent nerve activity, endogenously produced nitric oxide does not appear to reduce the firing rate of these afferent fibers, at least in rabbits. This would suggest that in our experiments, the age-dependent effects of L-NAME on the arterial baroreflex control of heart rate likely do not result from differences in carotid sinus afferent nerve activity.

The presence of NOS enzymes in medullary brain sites associated with integration (NTS), sympathetic and parasympathetic nerve output (rostral ventrolateral medulla and nucleus ambiguus, respectively) as well as in higher brain centers such as the PVN of the hypothalamus is well established (96,214). Importantly, studies by Traystman *et al* have confirmed in anesthetized dogs, cats and pigs, that the peripheral administration of L-NAME at a dose of 20 mg/kg can cross the blood brain barrier and reduce cortical NOS activity to ~30% of controls within 30 min, as measured by a reduction in the formation of $^{14}$C-L-citrulline; this effect lasts for up to six hours (207). If this holds true for the conscious state, and for the sheep, we can infer from these data that I.V. administration of L-NAME to both age-groups of lambs would have accessed the central nervous system within the time frame of our study to modulate the influence of endogenous nitric oxide on the brain regions listed above. Although the blood brain barrier of piglets has been reported to be more permeable to unbound bilirubin at two-

days of age compared to two-week old animals (97), there is no information regarding the permeability of the regions of the blood-brain barrier in the age-groups of our lambs. It is not known whether the observed age-dependent effects of L-NAME on the arterial baroreflex control of heart rate result from maturational changes in the permeability of these regions. None the less, the transport of L-NAME across the blood brain barrier is not limited by the size of fenestrations. It has been demonstrated that in rat chroroid plexus isolates, the transport of L-NA and its methyl ester L-NAME across the blood brain barrier are dependent either on a quaternary ammonium cation transporter or a basic amino acid transporter which were shown to be non-saturable (166,196). Therefore, although an age-dependent change in transporter number and function is possible, it is unlikely that an age-dependent change in the permeability of the blood brain barrier to L-NAME is responsible for our age-dependent observations.

Studies in both conscious (100) and anesthetized (67,164,203,230) animals have demonstrated that the administration of nitric oxide synthesis inhibitors, either peripherally or centrally to specific medullary and hypothalamic sites mentioned above, is associated with a marked increase in renal sympathetic nerve activity. Sympathetic outflow to different organ systems is, however, not always uniform. Ma *et al* showed in anesthetized rats, that I.V. L-NAME administration decreased the discharge rate of 12 of 14 NTS neurons, whereas it increased neuronal activity in two (105); these effects were reversed with L-arginine administration. The authors observed similar results when they performed the experiment in superfused rat brain slices. Therefore, it is important to note that depending on the nerve population at the NTS, the role of nitric oxide in influencing efferent output may be altered. In the context of our findings, whether or not L-NAME

administration reduced the modulatory influence of the NTS on sympathetic outflow and hence the parameters governing the arterial baroreflex control of heart rate in conscious lambs remains to be investigated.

Research into the influence of nitric oxide synthesis inhibition on the arterial baroreflex control of heart rate in conscious animals is limited to studies carried out in rabbits (44,45,100) and rats (119,168). In both conscious rabbits and rats, the administration of L-NNA or L-NAME significantly reduces both the upper and lower heart rate plateaus as well as the heart rate range over which the arterial baroreflex operates, as we observed in the present study in one-week old lambs. The influence of nitric oxide synthesis inhibition on the gain of this reflex in adult animals is not fully understood (45,100,168). For example, a recent study by Scrogin *et al* showed that in conscious rats, L-NAME administration decreased the gain of the arterial baroreflex control of heart rate (168). In contrast, Du *et al* showed in conscious rabbits, that L-NNA did not alter the gain of this reflex (45), whereas Liu *et al* found that L-NNA increased it (100). The discrepancy between these findings appears to depend upon differences in experimental design, namely the method of assessing the arterial baroreflex control of heart rate. For example, Scrogin *et al* assessed the upper and lower ranges of blood pressure on separate days, whereas Liu *et al* used perivascular occluder cuffs to manipulate blood pressure. The methods of Du *et al* were similar to those employed in our study, and interestingly, appear to have produced comparable results to what we observed in newborn lambs.

In light of these studies in adult rats and rabbits, it was surprising to see that L-NAME administration had no significant effects on any of the parameters of the

arterial baroreflex control of heart rate in older lambs. As experimental discrepancies have already been discussed, other factors such as maturational changes in the presence and/or activity of central NOS enzymes also need to be addressed. Studies in both rat (114,142) and sheep (133) brain have demonstrated that NOS activity is altered with maturation. Matsumoto *et al* reported that in rat cerebellar and cerebral homogenates, NOS activity as assessed by the formation of $^{3}$H-L-citrulline, was significantly higher in the cerebrum during the first week of life and declined with maturation, whereas NOS activity in the cerebellum was low in newborns and increased dramatically in adult rats (114). Similar findings were reported by Pearce *et al* in whole brain homogenates obtained from spontaneously hypertensive rats (142). On the contrary, recent evidence provided by Northington *et al* in sheep, indicates that NOS activity in the thalamus and medulla, as measured by the formation of $^{14}$C-L-citrulline, reaches values greater than that observed in the adult by 70 days of gestation; activity then declines with postnatal maturation (133). Therefore, these studies demonstrate that species differences are present in the development and functioning of the NOS enzyme in the central nervous system. Furthermore, it can be inferred from these studies that the similarity between the L-NNA and L-NAME induced alterations in the arterial baroreflex control of heart rate in adult rats and newborn lambs may rest upon the activity of central NOS.

We found that the arterial baroreflex control of heart rate was shifted towards lower pressures following L-NAME administration in newborns, but not in older lambs. This age-dependent effect of L-NAME might result from differences in circulating levels of angiotensin II and arginine vasopressin. Previous experiments in conscious rabbits have shown that both centrally and peripherally administered angiotensin II can act on

$AT_1$ receptors to reset the blood pressure:heart rate relationship towards higher pressures (156,221). These receptors are known to be responsible for most of the physiological actions of angiotensin II and are located in the area postrema of the hindbrain (113). Furthermore, the role of angiotensin II in resetting the arterial baroreflex control of heart rate towards higher pressures under basal conditions is well known, as the administration of the angiotensin converting enzyme inhibitor, captopril, to rabbits resets the blood pressure:heart rate relationship towards lower pressures (155). Of relevance to our age-dependent observations is that receptor binding and $AT_1$ mRNA levels in rats and sheep in the medulla is higher in newborns compared to the adult (169,209). Moreover, Segar *et al* have demonstrated that in newborn lambs, the capacity of angiotensin II to influence the arterial baroreflex control of heart rate is similar to that observed in adult sheep (171); these results should be interpreted with caution, however, since they were conducted in animals that were sedated, paralyzed and artificially ventilated. Nevertheless, this evidence suggests that endogenous angiotensin II may play a similar if not a greater role in modulating the baroreflex control of heart rate in the newborn period compared to the adult, although this remains to be investigated under normal physiological conditions.

In addition to angiotensin II, arginine vasopressin has been shown to be an important modulator of the arterial baroreflex control of heart rate in conscious animals. For example, Undesser *et al* showed that in conscious rabbits, I.V. infusion of arginine vasopressin produces an increase in blood pressure and a decrease in heart rate (210). Interestingly, the authors reported that the arginine vasopressin induced decrease in heart rate was significantly greater than that observed in response to a comparable increase in blood pressure by phenylephrine administration (210). These data therefore suggest that

increased circulating levels of arginine vasopressin may reset the arterial baroreflex control of heart rate towards lower pressures. Additionally, these authors found that lesioning the area postrema resulted in equal cardiovascular responses to both arginine vasopressin and phenylephrine, thus providing further evidence that arginine vasopressin acts at the area postrema, possibly on $V_1$ receptors (186), to influence the arterial baroreflex control of heart rate. In contrast, however, Nuyt *et al* showed that the administration of arginine vasopressin to newborn sheep resulted in a decrease in heart rate, for a given increase in blood pressure, which was similar to that observed with a bolus injection of phenylephrine (134); the nature of these experiments does, however, limit the interpretability of the findings since again, the animals were sedated, paralyzed and artificially ventilated. In total, current evidence suggests that although arginine vasopressin does play an important role in modulating arterial baroreflex control of heart rate in the older animal, it does not appear to share the same role in the newborn lamb.

We have previously found that an I.V. injection of 20 mg/kg L-NAME decreases plasma renin activity by 20 min, which then remains below control levels for one hour in both newborn and older conscious lambs (Sener, A. and Smith, F.G., unpublished observations). This translates to an L-NAME induced reduction in circulating levels of angiotensin II in both groups of lambs. Additionally, although we did not measure plasma levels of arginine vasopressin following nitric oxide synthesis inhibition, our data from Chapter Five provide significant evidence to suggest that in older but not in newborn lambs, L-NAME markedly decreases plasma arginine vasopressin levels for at least 60 min, as we observed significant decreases in urinary osmolality and marked increases in free water clearance following L-NAME injection (See Figure 5.6). Based

on these findings the shift of the arterial baroreflex control of heart rate in newborn lambs towards lower pressures following L-NAME administration may be attributed to decreased levels of circulating angiotensin II acting on the area postrema. Likewise, the lack of effects of L-NAME in resetting of the blood pressure:heart rate relationship in older lambs may be due to the net effect of the opposing actions of a decrease in circulating levels of angiotensin II (shift towards lower pressures) and a decline in plasma arginine vasopressin levels (shift towards higher pressures) following L-NAME administration. Further investigation is required to fully understand the mechanisms behind this age-dependent effect of L-NAME on the arterial baroreflex control of heart rate.

Investigation into the functionality of the arterial baroreflex control of heart rate throughout pre- and post-natal maturation has revealed that it is functional during fetal life, at least in the sheep (26). Although it is agreed upon that the arterial baroreflex is functional at all stages of postnatal maturation, it is still unclear as to the changes that occur in the parameters which determine the blood pressure:heart rate relationship during maturation. Unfortunately, many of the studies have been conducted on anesthetized (22,140,205) or sedated (118,170) animals, making interpretation of these results difficult as both anesthetics and tranquilizers have been shown to alter the arterial baroreflex (112,123,222). Although select studies in conscious swine (137) and sheep (39) have attempted to determine the maturational changes in the arterial baroreflex control of heart rate, these results are also not fully devoid of such confounding factors as allowing some of the animals to sleep during the experiments (shown to alter the reflex control of heart rate and blood pressure (49,187) or drawing conclusions about the entire range of the

blood pressure:heart rate relationship after only assessing the upper portion of the blood pressure range using phenylephrine (39). Our study is, therefore, the first to describe the maturational changes in the arterial baroreflex control of heart rate during postnatal development in conscious animals, free of the confounding effects brought upon by the various techniques described above. Under normal physiological conditions, we observed that the maximum and minimum heart rate as well as the range over which the arterial baroreflex control of heart rate operates were significantly greater in newborn lambs compared to that seen in older animals. Although there is not much agreement between any of the aforementioned studies with respect to these three parameters, our findings are in concert with those performed in sedated newborn sheep (118,170) and in conscious piglets (137). A larger heart rate range in the newborn may signify a more pronounced role for the arterial baroreflex control of heart rate in maintaining cardiovascular homeostasis in face of perturbations in blood pressure, compared to later in life. Such a wide operating range in heart rate is perhaps not required in the older animal, as its mechanisms for restoring blood pressure may not only be dependent on compensatory changes in heart rate, but also on an increased ability to alter sympathetic nerve activity to various vascular beds to modulate peripheral vascular resistance (27).

The gain of the arterial baroreflex control of heart rate was similar between newborn and older lambs. Previous results by other investigators provide inconclusive data for comparison, as they have shown the gain to decrease (137), not change (170) or increase (39) depending on the study in question. Since the systolic pressure at the mid-point heart rate range increased slightly between the newborn and older lambs, whereas the midpoint of the heart rate range decreased significantly with age, it appears that with

maturation, the set-point of the resting heart rate decreases. This phenomenon may be a physiological adaptation to the changes that occur with postnatal development, including a reduced requirement of the older lamb for the thermogenic benefits of an elevated metabolism induced basal tachycardia compared to the newborn sheep (99). Moreover, this shifting of the arterial baroreflex control of heart rate towards lower pressures with maturation may stem from a maturational decrease in circulating levels of angiotensin II. The evidence for this postulate comes from our own data which shows that following L-NAME administration, the heart rate range of the blood pressure:heart rate relationship became similar between younger and older lambs. Since L-NAME has been shown to reduce circulating levels of renin and angiotensin II in numerous studies (38,147) and in our own unpublished observations, the alteration in the arterial baroreflex control of heart rate during postnatal maturation may be a reflection of decreasing circulating angiotensin II levels. Further studies are required, however, to fully understand this phenomenon.

In conclusion, the current study in chronically instrumented conscious lambs provides the first evidence that nitric oxide modulates the arterial baroreflex early in life in conscious animals. In addition, we showed that the effects of L-NAME on parameters governing the arterial baroreflex control of heart rate were age-dependent. Our findings provide additional information that various parameters governing the arterial baroreflex control of heart rate are altered with postnatal maturation in conscious sheep.

# *CHAPTER FIVE*

# *Renal Effects of Nitric Oxide Synthesis Inhibition during Maturation in Conscious Lambs*

## 5.1 INTRODUCTION

Numerous studies in conscious dogs (63) and rats (15) as well as our work in conscious lambs (174) have demonstrated that in addition to its effects on systemic hemodynamics, nitric oxide is an important modulator of renal vascular tone. Nitric oxide is not only necessary for promoting normal renal perfusion (16), but it's role in maintaining renal hemodynamics becomes exceedingly important as circulating levels of angiotensin II are experimentally elevated (2,15).

In addition to its effects on renal hemodynamics, nitric oxide plays an important role in modulating renal function (153,163). In adult rats, nitric oxide influences glomerular filtration rate (14) and tubuloglomerular feedback (78) through its vasodilator

actions on renal afferent arterioles (46) and mesangial cells (14,167). Nitric oxide also modulates fluid and electrolyte balance through its direct inhibitory actions on $Na^+/H^+$ antiporters located on proximal tubular epithelial cells (223), on apical and basolateral membranes of epithelial cells located on the thick ascending limb of the loop of Henle (60) as well as on apical membranes of principal cells of the medullary collecting duct (21,165,194,216). Moreover, it interacts with other vasoactive substances such as angiotensin II, endothelin and arginine vasopressin, which themselves influence renal function. For example, in adult rats and dogs, nitric oxide synthesis inhibition is associated with an increase in circulating levels of endothelin and arginine vasopressin (62,158), whereas plasma renin activity has been reported to decrease (125,147).

Recently, we showed that the role of nitric oxide in modulating renal hemodynamics was not altered with age (175). This finding is intriguing as renal NOS mRNA activity is elevated in newborn piglets, rats and guinea pigs and declines with postnatal maturation (51,189,200). Evidence that nitric oxide production and release may be altered during development, combined with our findings that the renal hemodynamic influence of nitric oxide is similar between newborn and older lambs, led us to speculate that high renal NOS activity in the newborn may result in age-dependent effects of nitric oxide on glomerular and tubular function. To date, the influence of nitric oxide on renal function in the immediate newborn period and during postnatal maturation has not been investigated under normal physiological conditions in conscious animals.

The purpose of the present in chronically instrumented conscious lambs was, therefore, to test the hypothesis that the effects of nitric oxide on glomerular and tubular function are developmentally regulated. To this end, we measured the effects of

L-NAME or its inactive isomer, D-NAME, on various parameters of renal function in newborn and older lambs.

## 5.2 METHODS

Experiments were carried out in conscious, chronically instrumented lambs at one week (8 ± 1 days; 6.3 ± 0.4 kg body weight; n=8) and six weeks (40 ± 2 days; 11.6 ± 1.5 kg body weight; n=8) of age. Two experiments were carried out on each lamb at least 24 h apart and in random order, at least three days after surgery. Details of the surgical procedures are described in Chapter Two.

On the day of an experiment, the lamb was brought into the laboratory and placed in a supportive sling for at least 60 minutes to allow it to become accustomed to its surroundings. The urinary bladder was drained and an I.V. infusion of 5% dextrose in 0.9% sodium chloride (4.17 mL/kg/h) was started, and continued for the duration of the study, to assist in maintaining fluid balance. During this time, a priming dose of $^{14}$C-inulin (0.5 μCi/kg, Amersham, Oakville, Ontario) in dextrose was injected I.V. followed by a constant I.V. infusion at 2.5 μCi/kg/h, for later measurement of glomerular filtration rate. A bolus dose of lithium chloride (2M, 100 μL/kg) was also injected I.V. for later calculation of proximal tubular sodium reabsorption (103,201). Left femoral arterial and venous catheters were connected to pressure transducers for measurement of arterial and venous pressures; the flow transducer was connected to a flow meter for measurement of renal blood flow. Arterial and venous pressures and renal blood flows were recorded continuously onto a polygraph and simultaneously to a computer at 200 Hz using the data acquisition and analysis software package, CVSOFT.

The above listed parameters of systemic and renal hemodynamics were measured for one hour before (Control) and for one hour after I.V. injection of L-NAME (experiment one) or its inactive isomer D-NAME (experiment two) at a dose of 20 mg/kg, obtained from a 1000 mg/mL stock solution. The dose of 20 mg/kg was selected from our previous dose-response curves, the results of which were detailed in Chapter Three. We found that this dose inhibited the acetylcholine-induced stimulation of intra-renal arterial nitric oxide synthesis for 60 min in both age groups of lambs (Figure 3.6) (176). Care was taken to ensure that the L-NAME and D-NAME were administered in the same volume for both experiments.

Following the 60-min equilibration period, the experiment was started. Consecutive 20 min urinary collections were made during the control period, and at 20 min intervals following L-NAME or D-NAME injection; urine volume was recorded and samples stored at –70°C for later determination of urinary electrolytes ($Na^+$, $K^+$, $Cl^-$, $Li^+$) and urinary osmolality. At the end of each urinary collection, 2.5 mL of arterial blood were removed of which ~0.1 mL was used for the measurement of hematocrit (Hct) using capillary tubes and a micro-hematocrit centrifuge (Clay Adams, Parsippany, New Jersey). The remaining blood was centrifuged; supernatant removed and stored at -70°C for later determination of plasma electrolytes ($Na^+$, $K^+$, $Cl^-$, $Li^+$) and plasma osmolality. All blood was replaced with previously obtained maternal blood to avoid any hemodynamic effects of sampling.

At the end of both experiments, lambs were injected with a lethal dose of sodium pentobarbitone and the flow transducer was monitored to measure any zero offset. Upon

*post mortem*, the placement of the catheters and of the flow transducer was confirmed, and the right and left kidneys were removed and weighed.

### 5.2.1 Analytical Procedures

Urinary and plasma $^{14}C$-inulin levels were determined immediately after each experiment using a liquid scintillation counter (Wallace 1410, Turku, Finland). Urine and plasma samples were later thawed to room temperature and urinary and plasma electrolytes ($Na^+$, $K^+$, $Cl^-$, $Li^+$) and osmolalities were measured using a flame photometer (IL-943, Lexington, Massachusetts), chloridometer (Labconco, Kansas City, Missouri) and micro-osmometer (Advanced Instruments Model 3MO, Needham Heights, Massachusetts), respectively.

### 5.2.2 Computations and Statistical Analyses

Glomerular filtration rate (GFR) was calculated as the clearance of [$^{14}C$]-inulin, renal plasma flow (RPF) as RPF = Renal blood flow (RBF) x (1- Hct), and filtration fraction (FF) as FF = GFR/RPF.

Total ($FR_{Na}$), proximal ($PFR_{Na}$), and distal ($DFR_{Na}$) fractional sodium reabsorption were calculated as follows: $FR_{Na}$ (%) = [1 - ($C_{Na}$ / GFR)] x 100; $PFR_{Na}$ (%) = [1 - ($C_{Li}$ / GFR)] x 100; $DFR_{Na}$ (%) = $FR_{Na}$ - $PFR_{Na}$. The urinary sodium ($U_{Na}$) to potassium ($U_K$) excretion ratio was determined from $U_{Na}:U_K = U_{Na} / U_K$. The urinary osmolality ($U_{Osm}$) to plasma osmolality ($P_{Osm}$) ratio was calculated as $U_{Osm}:P_{Osm} = U_{Osm} / P_{Osm}$. The transtubular potassium gradient (TTKG) was calculated as $TTKG = U_{K+} / (U_{Osm} / P_{Osm}) / P_{K+}$. Free water clearance ($C_{H_2O}$) was calculated as follows: $C_{H_2O}$ (mL/min) = $V - C_{Osm}$. Renal vascular resistance (RVR) was calculated as follows: RVR

(mmHg/mL/min/gkw) = (MAP - MVP) / RBF. Renal variables were corrected for gram kidney weight (gkw) to allow for comparison between age groups.

Statistical analysis was carried out using SPSS-PC Version 8.0 and MANOVA procedures for repeated measures, factors being treatment (L-NAME or D-NAME) and age (one and six-weeks). Where the F value was significant, Newman-Keuls multiple comparison tests were applied to determine where the significant difference(s) occurred. Significance was accepted at the 95% confidence interval. All data are presented as mean±one standard deviation.

## 5.3 RESULTS

Glomerular filtration rate significantly increased from one to six-weeks of age ($p<0.001$, Figure 5.1). There were age-dependent effects on glomerular filtration rate ($p<0.001$), such that in one-week old lambs, but not in older animals, L-NAME administration resulted in a decrease in glomerular filtration rate which remained below control values for the duration of the study ($F=2.98$, $p<0.05$). Renal plasma flow was greater in older compared to newborn lambs ($p<0.05$, Figure 5.1). Following L-NAME administration, renal plasma flow decreased below control levels for at least 60 min in the newborn lambs, whereas it declined for only 20 min in the older group of animals ($F=6.60$, $p<0.001$). In response to L-NAME administration, filtration fraction also declined in one-week old lambs by 20 min and did not return to controls by the end of the experiment ($F=2.92$, $p<0.05$); L-NAME did not influence filtration fraction in six-week old lambs (Figure 5.1).

Urine flow rate increased with age in conscious lambs ($p<0.001$, Figure 5.2). In

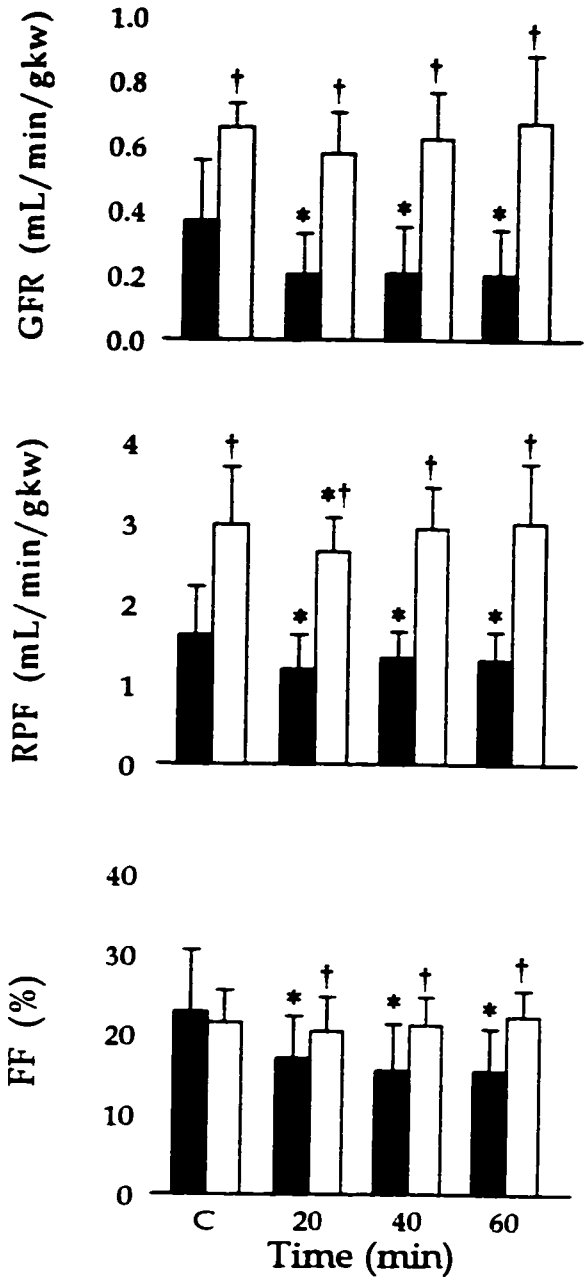


**Figure 5.1** Glomerular filtration rate (GFR), renal plasma flow (RPF) and filtration fraction (FF) measured before (C) and after I.V. administration of L-NAME in one-week (solid bars) and six-week (open bars) old lambs. Values are mean ± SD. *p<0.05 compared to C. †p<0.05 compared to one-week old lambs.

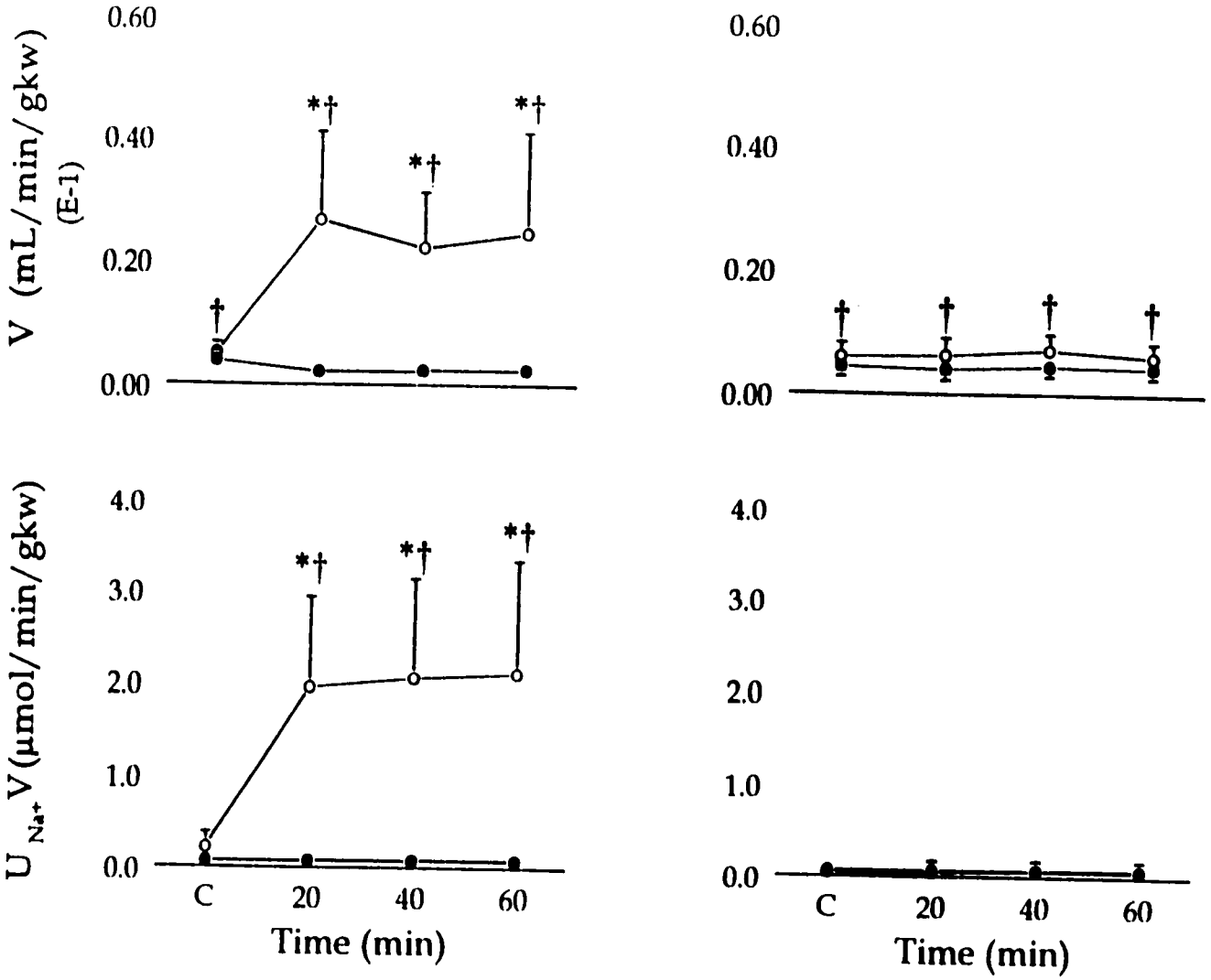


**Figure 5.2** Urine flow rate (V) and urinary excretion rate of sodium ($U_{Na+}V$) measured before (C) and after I.V. administration of L-NAME (left) and D-NAME (right) in one-week (solid circles) and six-week (open circles) old lambs. Values are mean ± SD. *$p<0.05$ compared to C. †$p<0.05$ compared to one-week old lambs.

response to L-NAME injection, there was a marked increase in urine flow rate by 20 min which persisted for 60 min in six-week old lambs ($F=45.67$, $p<0.001$, Figure 5.2). This diuresis was accompanied by a natriuresis in six-week old animals, which also lasted for 60 min ($F=15.40$, $p<0.001$, Figure 5.2). L-NAME did not influence urinary flow rate or sodium excretion in one-week old lambs (Figure 5.2).

As illustrated in Figure 5.3, there were age-dependent effects of L-NAME on total, proximal and distal fractional reabsorption of sodium ($p<0.001$). Following L-NAME administration, total ($F=13.08$, $p<0.001$) and proximal ($F=7.48$, $p<0.001$) fractional reabsorption of sodium decreased, whereas distal ($F=5.91$, $p=0.001$) fractional reabsorption of sodium increased by 20 min and remained elevated for 60 min. L-NAME had no significant effects on any of these variables in one-week old lambs.

Under basal conditions, urinary excretion of potassium was greater in six-week old compared to one-week old lambs ($p<0.001$, Figure, 5.4). There was an age-dependent effect of L-NAME on urinary potassium excretion ($F=67.81$, $p<0.001$) and on the fractional reabsorption ($F=6.35$, $p=0.019$) of potassium. At 20 min after L-NAME administration, six-week old lambs showed a marked kaliuresis which lasted until the end of the study ($F=7.22$, $p<0.001$). In the older animals, fractional reabsorption of potassium decreased in response to L-NAME and persisted for 60 min ($F=8.22$, $p<0.001$, Figure 5.4). L-NAME did not alter either variable in one-week old lambs.

L-NAME produced an age-dependent response in urinary chloride excretion ($F=83.79$, $p<0.001$) and in the fractional reabsorption of chloride ($F=14.82$, $p=0.001$, Figure 5.5). At 20 min following nitric oxide synthesis inhibition, there was a marked chloriuresis in the six-week old lambs, which lasted for the entire study ($F=19.08$,

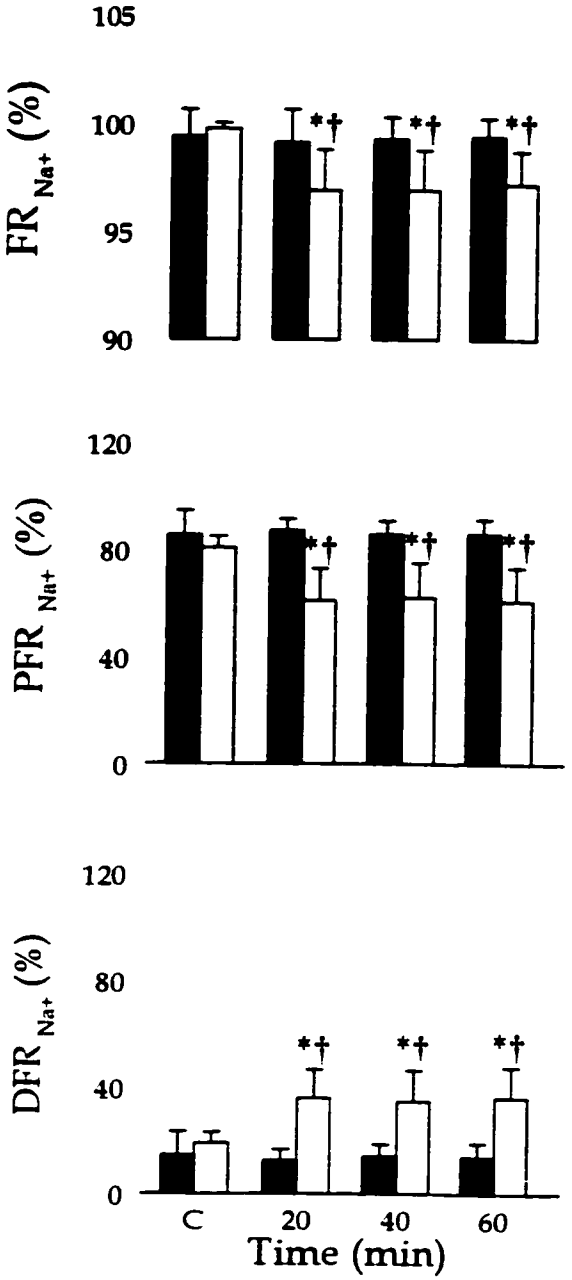


**Figure 5.3** Total ($FR_{Na+}$), proximal ($PFR_{Na+}$) and distal ($DFR_{Na+}$) fractional reabsorption of sodium measured before (C) and after I.V. administration of L-NAME in one-week (solid bars) and six-week (open bars) old lambs. Values are mean ± SD. *$p<0.05$ compared to C. †$p<0.05$ compared to one-week old lambs.

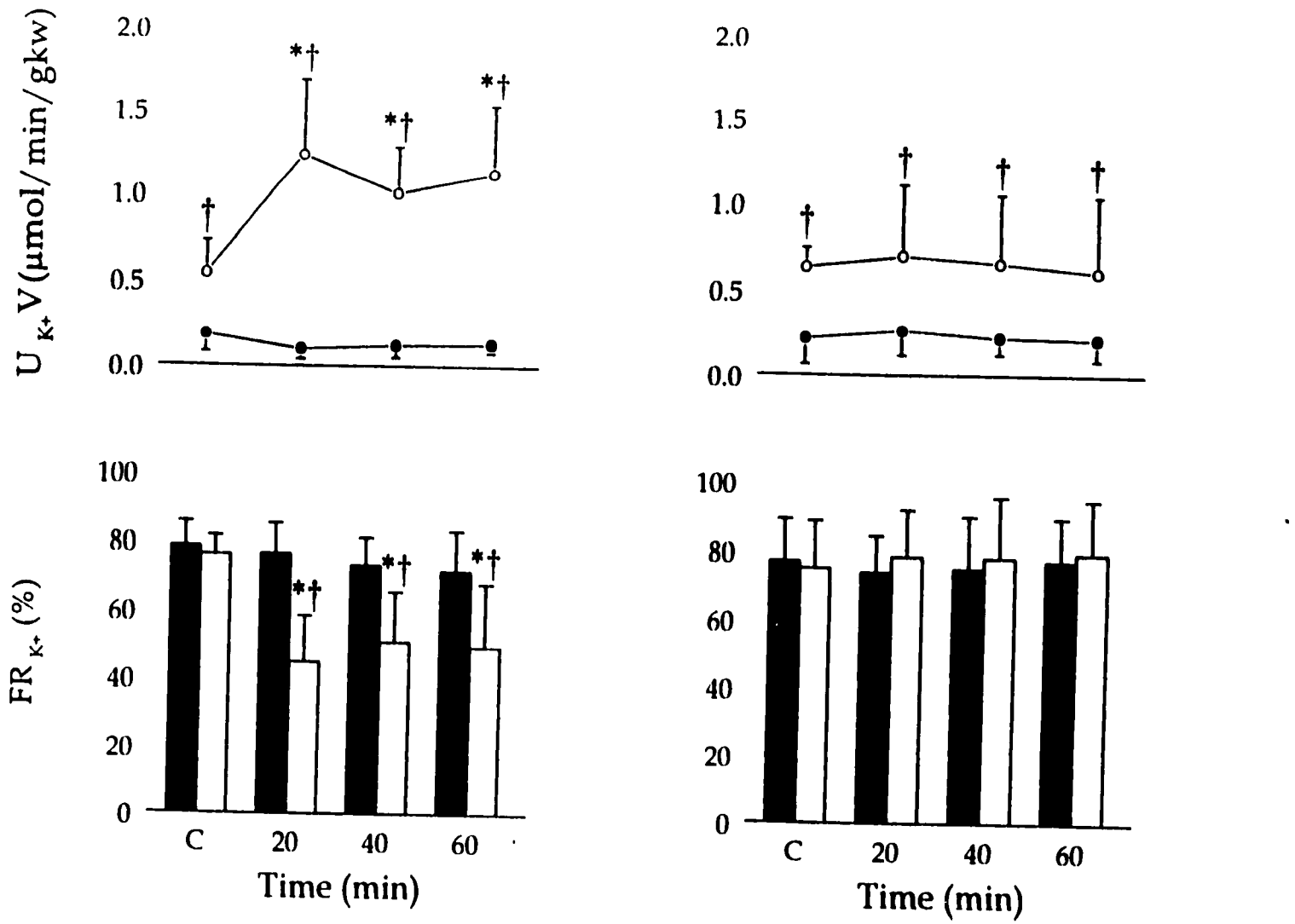


**Figure 5.4** Urinary potassium excretion rate ($U_{K+}V$) and fractional reabsorption of potassium ($FR_{K+}$) measured before (C) and after I.V. administration of L-NAME (left) and D-NAME (right) in one-week (solid circles) and six-week (open circles) old lambs. Values are mean ± SD. *$p<0.05$ compared to C. †$p<0.05$ compared to one-week old lambs.

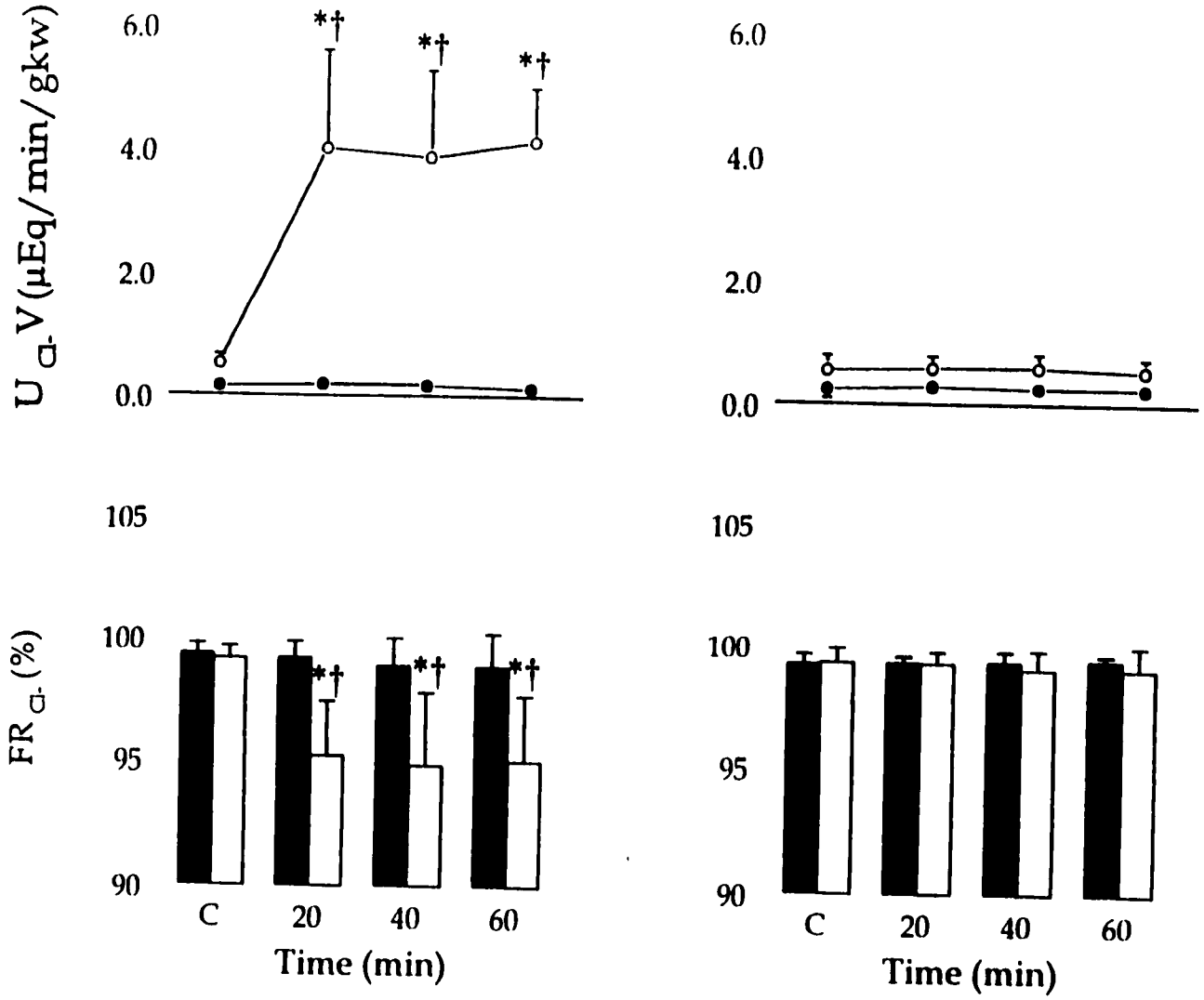


**Figure 5.5** Urinary chloride excretion rate ($U_{Cl^-}V$) and fractional reabsorption of chloride ($FR_{Cl^-}$) measured before (C) and after I.V. administration of L-NAME (left) and D-NAME (right) in one-week (solid circles) and six-week (open circles) old lambs. Values are mean ± SD. *p<0.05 compared to C. †p<0.05 compared to one-week old lambs.

$p<0.001$). In the older animals, fractional reabsorption of chloride also decreased in response to L-NAME for the duration of the experiment ($F=9.75$, $p<0.001$). L-NAME had no significant effects on chloride excretion or on its fractional reabsorption in the newborn animals.

There were also age-dependent effects of L-NAME on the clearances of sodium, potassium and chloride ($p<0.001$, Appendix B). In six week old, but not in one-week old animals, clearances of all electrolytes increased markedly after L-NAME and lasted for the duration of the study. L-NAME administration was also associated with a significant increase in the urinary sodium to potassium excretion ratio ($0.4 \pm 0.4$ at control to $2.3 \pm 1.3$ at 20 min, $p<0.001$) as well as the transtubular potassium gradient ($4.8 \pm 1.7$ at control to $16.6 \pm 6.2$ at 20 min, $p<0.001$) in six-week old lambs; this response persisted for the remainder of the study (Appendix B). There was no change in the urinary sodium to potassium excretion ratio or in the transtubular potassium gradient following L-NAME administration to one-week old lambs.

In response to L-NAME administration, urinary osmolality significantly decreased ($F=2.78$, $p=0.047$) and free water clearance increased ($F=3.04$, $p=0.034$) by 20 and 40 min, respectively in six-week old lambs (Figure 5.6); these responses lasted for the duration of the study. Figure 5.7 demonstrates that in response to L-NAME administration, the relationship between osmolar clearance and free water clearance was shifted to more positive free water clearances in six-week old lambs. The ratio between osmolar and free water clearance was unaffected by L-NAME administration in one-week old lambs. Furthermore, in six-week old but not in one-week old lambs, L-NAME injection was associated with a decline in the ratio between urinary and plasma

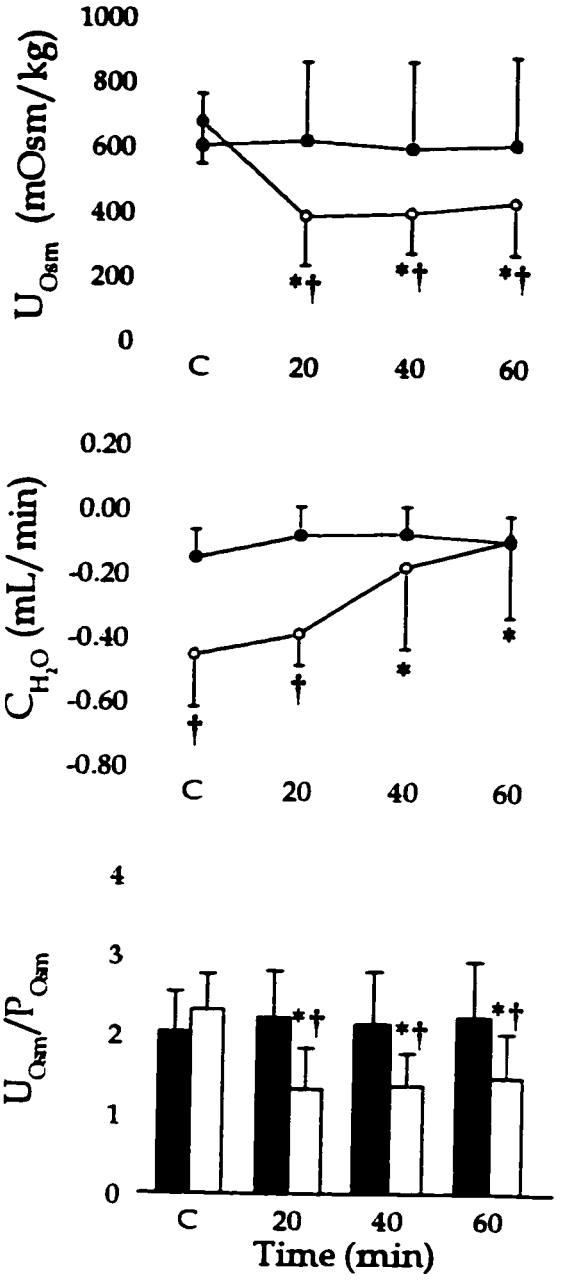


**Figure 5.6** Urinary osmolality ($U_{Osm}$), free water clearance ($C_{H_2O}$), and urinary/plasma osmolality ratio ($U_{Osm}/P_{Osm}$) measured before (C) and after I.V. administration of L-NAME in one-week (solid bars) and six-week (open bars) old lambs. Values are mean ± SD. *$p<0.05$ compared to C. †$p<0.05$ compared to one-week old lambs.

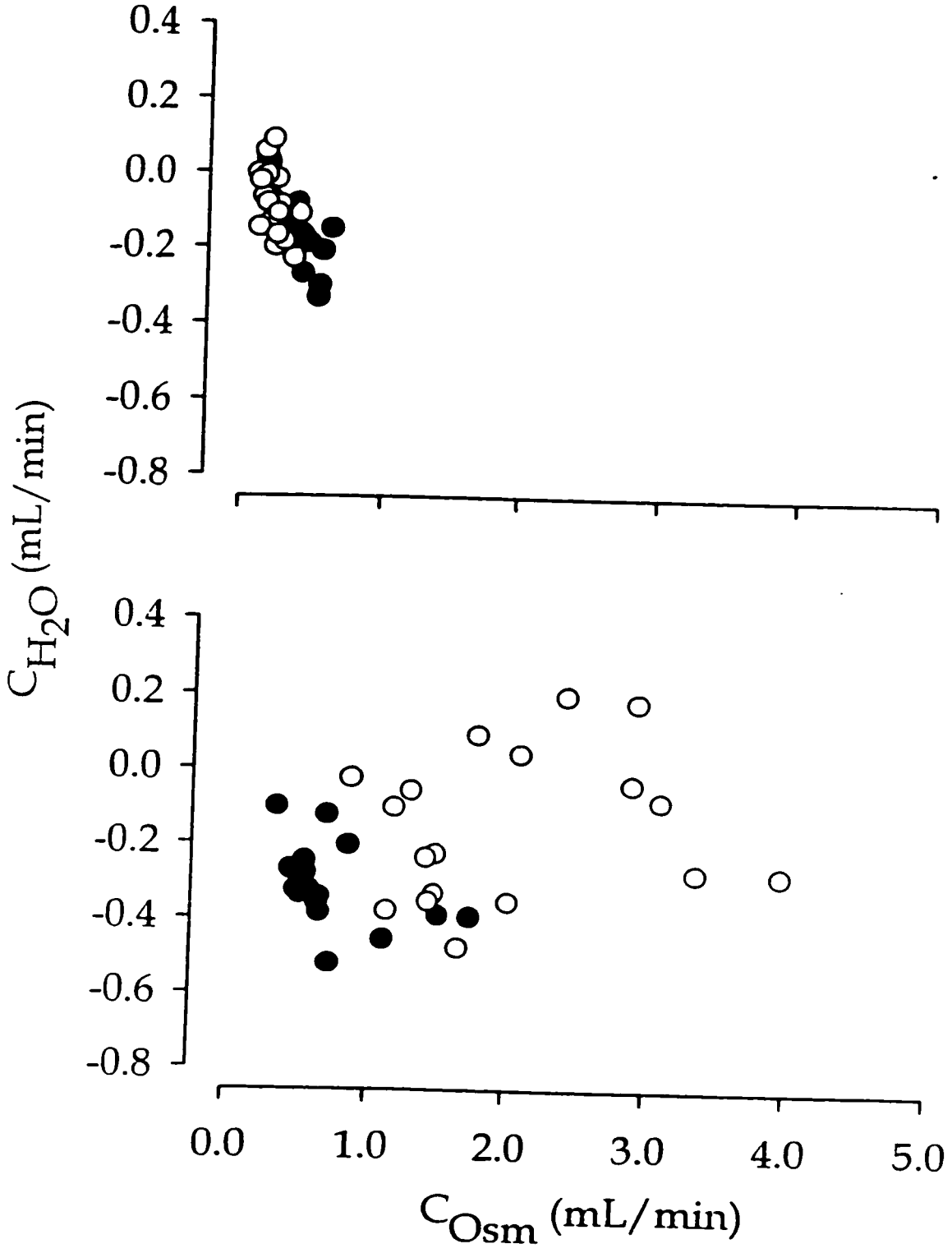


**Figure 5.7** Relationship between osmolar clearance ($C_{Osm}$) and free water clearance ($C_{H_2O}$) during control measurements (closed circles) and after (open circles) I.V. administration of L-NAME in one-week (top) and six-week (bottom) old lambs.

osmolality in six-week old lambs which persisted for 60 min ($F=4.78$, $p=0.013$, Figure 5.6).

The administration of L-NAME had no significant effects on plasma sodium and chloride concentrations, nor did it influence plasma osmolality and hematocrit in either of the age groups studied (Appendix C). Interestingly, however, L-NAME administration produced a decrease in plasma potassium levels by 40 min in six-week old lambs which persisted until the end of the study ($F=14.74$, $p<0.001$); nitric oxide synthesis inhibition did not alter plasma potassium levels in one-week old lambs.

D-NAME had no significant effects on any of the measured parameters of renal function in both one and six-week old lambs.

## 5.4 DISCUSSION

The purpose of the current investigation was to determine the role of nitric oxide in modulating renal function during maturation in conscious, chronically instrumented lambs. We set out to test the hypothesis that, since renal NOS levels and NOS mRNA activity are both elevated at birth and decline with postnatal age, the role of nitric oxide in influencing renal function would be greater in the immediate postnatal period. The novel findings of this study are that L-NAME had significant age-dependent effects on renal function as follows: a) In one-week old, but not in six-week old lambs, glomerular filtration rate and filtration fraction decreased; b) In six week-old, but not in one-week old lambs, there was a marked diuresis, natriuresis, kaliuresis and chloriuresis; c) Urinary osmolality decreased and free water clearance increased in six-week old, but not in one-week old animals. These findings are the first to describe the role of nitric oxide in

modulating renal function in conscious lambs. These data provide new and important information that nitric oxide appears to only influence glomerular function in the newborn animal, whereas its effects appear to be directed at tubular function later in life.

Previous studies in conscious adult rats and dogs have reported that glomerular filtration rate either decreases or does not change (2,13,63) following the administration of nitric oxide synthesis inhibitors. The fact that glomerular filtration rate remained constant in older animals in the present study was therefore not too surprising. What was quite surprising, however, was the dramatic decrease in glomerular filtration rate and filtration fraction observed in newborn lambs. An age-dependent effect of nitric oxide on glomerular ultrafiltration is supported by evidence obtained from guinea pig renal cortices where constitutive NOS activity, as measured by the formation of [$^{14}$C]-L-citrulline, was significantly elevated at eight to ten days of age and declined with postnatal development (200). Since the glomeruli and hence the mesangial cells of all nephrons are located in the renal cortex, the high cortical NOS activity in the newborn period may signify nitric oxide's role as an important modulator of glomerular function at this age. Our findings provide evidence to suggest that nitric oxide may play a protective role in the newborn glomerulus, whereby it helps to maintain glomerular filtration in the face of a high renal vascular resistance (4,226) and a low glomerular filtration rate (3,172).

Since glomerular filtration rate is directly governed by two parameters – (a) the coefficient of ultrafiltration ($K_f$), determined by the permeability and the available surface area for filtration of the glomerular capillaries, and (b) the net filtration pressure across the glomerulus, which is dependent upon the difference between the net hydrostatic and

net oncotic pressures acting across the glomerular membrane (212), the decrease in glomerular filtration rate seen in one-week old lambs could have resulted from either a decrease in $K_f$, a decrease in net filtration pressure, or a decrease in both. *In vivo* glomerular micropuncture studies in rats have shown that systemic nitric oxide inhibition causes marked increases in both pre- and post-glomerular arteriolar resistances and decreases in $K_f$, probably mediated by mesangial cell contraction (40). Furthermore, *in vitro* evidence from rat glomerular mesangial cells co-cultured with endothelial cells demonstrated that nitric oxide administration increased mesangial cell cross-sectional area, whereas L-NMMA reduced it, suggesting that under basal conditions, nitric oxide relaxes glomerular mesangial cells (167). L-NAME induced alterations in other local vasoactive peptides such as an increase in thromboxane (20,232) or a decrease in dopamine (72) levels may all have contributed to decreasing $K_f$, as all these substances are known to alter the contractility of the renal mesangium (10,141). Endothelin has also been shown to act on the renal mesangium to decrease $K_f$ (149), although circulating levels of endothelin are not altered in response to L-NAME administration in both one and six week old lambs (Sener A. and Smith F.G., unpublished observations).

Hemodynamic data from newborn lambs suggest that renal perfusion pressure may have also increased in response to L-NAME administration. Therefore, if there were no changes in afferent and/or efferent arteriolar resistance, net filtration pressure would either remain unaltered, or increase; both of these would serve to increase glomerular filtration rate, not to decrease it. From our experiments in conscious, chronically instrumented lambs, afferent and/or efferent arteriolar resistances cannot be measured so that it is not possible to determine whether net filtration pressure was altered following

L-NAME administration to newborn lambs. Moreover, we cannot exclude the possibility that age-dependent changes in renal sympathetic nerve activity could account for the decrease in glomerular filtration rate observed in one-week old lambs, through the stimulatory effects of adrenergic receptors on afferent arteriolar resistance.

It should be considered that extrarenal effects of I.V. L-NAME administration might have potentiated our age-dependent observations in glomerular function. The only report comparing the I.V. versus intrarenal injections of L-NAME was performed by Granger *et al* in conscious dogs (63). The authors describe that an I.V. infusion of L-NAME has a more pronounced effect on glomerular function compared intrarenal infusions of L-NAME, which they attributed solely to extrarenal effects of NOS inhibition. Although important, the conclusions drawn from these findings should be interpreted with caution, as the authors make several assumptions that confound their findings, including: a) there is no rationale or dose response study provided for the large I.V. dose of L-NAME used; b) there was no assessment of intrarenal NOS activity following the two treatment types. This point is crucial, as it is only when the two treatment types affect the renal bed similarly that their influence may be compared; c) although the authors collected data over three hours, only one time point is reported as percent change from control; and d) the authors used a one-kidney animal model (intrarenal L-NAME) and compared these results to data obtained from a two-kidney model (I.V. L-NAME). This alone may have produced the observed results based on the phenomenon of reno-renal reflux. It is important to acknowledge, however, that the systemic administration of L-NAME may potentially have secondary effects on glomerular function, but this has yet to be shown under controlled experimental

conditions. As discussed in the proceeding pages, our data provide strong evidence that the age-dependent effects of L-NAME on renal function are not due to secondary systemic effects, but rather support a developmental alteration in renal NOS activity.

The above discussion has provided possible explanations for the age-dependent observations of L-NAME on glomerular filtration rate. The proceeding paragraphs focus on the age-dependent effects of nitric oxide on tubular function.

The natriuretic response to L-NAME administration in older lambs was associated with a decrease in proximal tubular fractional sodium reabsorption and an increase in distal tubular fractional sodium reabsorption; the net effect, however, was a marked reduction in the total fractional reabsorption of sodium. It is possible that the observed natriuretic response to L-NAME seen in older lambs was due to the phenomenon of pressure natriuresis previously observed in anesthetized dogs and in the isolated perfused kidney (48,108,116). To date, however, no data to support this premise have been obtained under normal physiological conditions.

A decline in circulating angiotensin II levels following administration of L-NAME could also explain why we observed a decrease in proximal sodium reabsorption in six-week old lambs. In fact, Goyer *et al* have shown a decrease in plasma angiotensin II levels in conscious rabbits following an I.V. infusion of L-NAME (62). Angiotensin II increases proximal tubular sodium reabsorption through its stimulatory actions on the $Na^+/H^+$ antiporter located on the apical membrane of these cells (11,150). This hypothesis is strengthened by findings of Baylis *et al* who showed that under basal conditions, angiotensin II enhances renal sodium reabsorption in the conscious rat (13). This explanation for the age-dependent effects of L-NAME on

tubular sodium reabsorption also seems unlikely; our preliminary data show that L-NAME administration at a dose of 20 mg/kg significantly reduces plasma renin activity at 20 min, renin levels then remain decreased for at least one hour in both one and six-week old lambs (Sener and Smith, unpublished observations). Since renin is the rate-limiting enzyme in the synthesis of angiotensin II, one may therefore infer that angiotensin II levels are decreased to a similar extent in both age-groups of lambs following L-NAME administration. Moreover, Garvin *et al* recently demonstrated that in isolated proximal tubules of young and adult rats, angiotensin II administration had a greater effect on fluid flux in younger compared to older rats (59). In our experiments, however, NOS inhibition was only associated with a change in proximal tubular sodium reabsorption in the older lambs. An age-dependent difference in renal sympathetic outflow after L-NAME administration could also account for the age-dependent effects on tubular sodium reabsorption, although this also seems unlikely in light of the similar renin and renal vascular responses observed in the two age-groups, and the dissimilar responses seen in glomerular filtration rate.

Another much more likely explanation for the age-dependent effects of L-NAME on tubular function resides in an age-dependent renal distribution of the NOS enzyme (51,188,189,199). In the newborn rat (51) and piglet (189), renal tubular distribution of neuronal NOS mRNA is elevated in the thick ascending limb of Henle as well as in *macula densa* cells. As maturation proceeds, in addition to maintaining its presence at the *macula densa* cells, neuronal NOS appears to migrate to the more distal cortical and medullary collecting duct cells (199). One other possible explanation for age-dependent effects of L-NAME on tubular sodium reabsorption is that there are age-

dependent changes in $Na^+/H^+$ antiporters through which nitric oxide normally modulates sodium transport. In support of this premise is that proximal tubular $Na^+/H^+$ antiporters in the neonatal rabbit possess lower activity compared to proximal tubules of the adult (11,18). The $Na^+/H^+$ antiporter is located on the apical membrane of epithelial cells of the thick ascending limb of Henle as well as in principal cells of the cortical and medullary collecting ducts (21,60,194). Recently, Garvin *et al* showed that the addition of the nitric oxide donor, spermine NONOnate, to isolated perfused thick ascending limbs of rat kidneys reduced intracellular pH significantly (60), suggesting that under baseline conditions, nitric oxide acts at this site to inhibit the $Na^+/H^+$ exchanger. Furthermore, locally produced nitric oxide is a known stimulator of intracellular cyclic GMP production in principal cells; a rise in cyclic GMP levels has been shown to be a potent inhibitor of the $Na^+/H^+$ antiporter (21,194). Taken together, these studies provide evidence to suggest that the increase in post-proximal tubular sodium reabsorption in six-week old lambs may be attributed to the removal of the tonic inhibitory influence of nitric oxide on these antiporters following L-NAME administration. Since renal chloride reabsorption and secretion is paralleled to that of sodium (87), it is promising that the increase in chloride clearance observed following L-NAME administration is in direct correlation to this anion's isoelectric transport with sodium across the tubular epithelium. Therefore, it appears that the influence of L-NAME on chloride transport is secondary to its action on tubular sodium reabsorption. In recent studies on isolated thick ascending limbs of the loop of Henle obtained from rats, however, Plato *et al* showed that administration of the nitric oxide donor, spermine NONOate, produced decreased chloride flux at this tubular site (146). Whether this occurs in the conscious animal, still

remains to be investigated.

We also observed age-dependent effects of nitric oxide inhibition on renal potassium handling, which was associated with a significant decrease in plasma potassium levels. Although to date there has not been any investigation into a direct influence of nitric oxide on renal potassium handling, the possibility that nitric oxide synthesis inhibition may also have altered adrenal production of mineralocorticoids can not be ruled out when explaining the observed kaliuresis in the six-week old animals. In support of this, studies by Natarajan *et al* in isolated rat and cultured human adrenal glomerulosa cells (the region of the adrenal gland responsible for aldosterone synthesis and secretion), showed significant immuno-staining for NOS (131). As well, the authors reported that the administration of nitric oxide donors inhibited the angiotensin II induced aldosterone synthesis from this region of the gland. Therefore, it is also possible that L-NAME administration directly stimulated aldosterone release, hence producing the kaliuresis observed in our six-week old lambs. This is a significant finding, as urinary potassium loss continued even in the face of hypokalemia in six-week old lambs. Under normal circumstances, hypokalemia is an important negative feedback mechanism for inhibiting aldosterone production and release. Therefore, our study shows that L-NAME administration appears to have over-ridden that safeguard mechanism, at least in the six-week old lambs, suggesting a new and important role for nitric oxide as a modulator of negative-feedback mechanisms. Further investigation is necessary to fully understand this phenomenon.

Another potential explanation for the kaliuresis may reside in an elevation in plasma aldosterone levels. There are three main stimuli for the production and release of

aldosterone from the adrenal cortex: increased levels of angiotensin II, hyperkalemia and increased levels of plasma adrenocorticotropic hormone (ACTH) (86). Since L-NAME produces a decrease in plasma renin activity (147), and hence a decrease in circulating angiotensin II, and considering that plasma potassium also declined in response to L-NAME administration in six-week old lambs, ACTH is left as the only known potential stimulator for aldosterone release. In fact, Giordano *et al* reported that circulating levels of ACTH are significantly increased following an intra-peritoneal injection of L-NAME to normal mice (61). Moreover, the increase in ACTH occurred concomitantly with a four-fold increase in adrenal steroidogenesis, as measured by elevations in plasma corticosterone. Our calculations of the transtubular potassium gradient further strengthen the proposed increase in aldosterone and its action on the collecting tubules. Our data show that in the six-week old lambs, L-NAME administration significantly increased the potassium concentration in the lumen of the collecting tubule threefold, when compared to control measurements. Since only aldosterone activity could produce such a gradient, these calculations provide additional evidence to suggest that aldosterone played a significant role in mediating the L-NAME induced kaliuresis seen in the older animals. It can not be overlooked, however, that there may yet be unidentified factors which may be rapidly produced in response to nitric oxide synthesis inhibition and which have similar actions as those manifested by aldosterone.

The lack of an influence of L-NAME on renal potassium handling in the newborn may rest on age-dependent effects of ACTH on aldosterone release. Moritz *et al* showed that in chronically catheterized fetal sheep at mid-gestation, I.V. administration of ACTH produced very small increases in plasma aldosterone levels, whereas it produced a

marked increase in cortisol production and release (124). These data suggest that the adrenal cortical cells responsible for aldosterone production and release are not very responsive to ACTH in the prenatal period. This statement is further supported by the findings of Papanek *et al* who demonstrated that in rabbit adrenal cells at different stages of maturation, cyclic AMP-stimulated aldosterone production was significantly lower in cells obtained from fetal and newborn tissue compared to those from older tissue (138). Further studies are necessary, however, to determine if these findings also hold true for the conscious lamb during postnatal maturation.

We also observed age-dependent effects of L-NAME on tubular water reabsorption. Although L-NAME administration resulted in an early reduction in urinary osmolality and urinary flow rate, free water clearance did not increase until 40 minutes after L-NAME injection in the six-week old lambs. This slow onset for the increase in free water clearance suggests a time delay in the inhibition and/or clearance of circulating levels of arginine vasopressin and hence the removal of its effects on water reabsorption from the renal collecting tubules. Under normal conditions, the release of arginine vasopressin from the posterior pituitary gland can be stimulated by a number of mechanisms including an increase in plasma osmolality, a decrease in effective circulating blood volume and an increase in circulating levels of angiotensin II (68). In our study, it is unlikely that the above mentioned stimuli were in force, as plasma renin activity declined, and plasma osmolality and blood volume (assessed by blood hematocrit) were not altered following L-NAME administration. Therefore, our data provide direct evidence to suggest that nitric oxide synthesis inhibition altered arginine vasopressin production and release from hypothalamic and/or posterior pituitary sites in

six-week old lambs.

Previous studies in conscious rabbits (62) reported that following a two-hour I.V. infusion of L-NAME, there was a marked increase in circulating levels of arginine vasopressin. On the contrary, findings from conscious humans and dogs have shown that bolus L-NAME administration had no effect on plasma levels of arginine vasopressin (32;109), whereas in rats it induced a significant decline in plasma vasopressin concentrations (30). Although we did not measure plasma levels of arginine vasopressin in the current study, we have estimated its relative activity by calculating the ratio between urinary and plasma osmolality. Since this ratio declined after nitric oxide synthesis inhibition, our findings suggest that L-NAME administration resulted in a significant decrease in water reabsorption from the collecting duct as compared to D-NAME treated controls, thus providing additional evidence that plasma levels of arginine vasopressin had declined. Determining the relationship between osmolar and free water clearance further reinforced this speculation, as L-NAME resulted in a shift of this relationship towards a more positive free water clearance for a given osmolar clearance in older lambs but not in newborn lambs. This implies that there was an age-dependent effect of L-NAME on the activity and/or action of arginine vasopressin on collecting duct cells.

An age-dependent modulatory role for nitric oxide in directly influencing the release of arginine vasopressin through effects on the magnocellular neurons in the hypothalamus is not known. The presence of neuronal NOS mRNA in the magnocellular neurons of the hypothalamus, mainly in the paraventricular and supraoptic nuclei, has previously been shown in adult rats (70,225). However, the role of nitric oxide in

directly influencing neural output from these sites is not certain, as L-NAME decreased neural activity 71% and increased activity in 29% of supraoptic neurons obtained from slices of rat hypothalami (101).

In conclusion, the present study is the first to show that the role of nitric oxide in modulating renal function is age-dependent in the conscious lamb. Our findings provide evidence to suggest that nitric oxide plays a predominant role in modulating glomerular function under basal conditions in the newborn lamb with little influence on tubular handling of water and electrolytes. As postnatal maturation proceeds, nitric oxide becomes less important in maintaining glomerular function and emerges as a profound modulator of tubular function. In older animals, we showed that nitric oxide modulates sodium, potassium and chloride reabsorption along the nephron, and appears to alter either the release and/or the effects of arginine vasopressin on tubular water reabsorption, in the absence of any changes in glomerular filtration rate. The mechanisms underlying these fascinating age-dependent effects of nitric oxide on renal function remain to be determined.

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Appendix A. Effects of D-NAME on parameters of the arterial baroreflex control of heart rate.

| | Age | Control | 30 min | 60 min | 90 min | 120 min |
|---|---|---|---|---|---|---|
| HR range (bpm) | NEWBORN | 139 ± 19 | 138 ± 13 | 126 ± 8 | 143 ± 33 | 143 ± 29 |
| | OLDER | 110 ± 13 | 93 ± 9 | 126 ± 30 | 113 ± 22 | 109 ± 19 |
| HR maximum (bpm) | NEWBORN | 258 ± 16 | 259 ± 11 | 250 ± 7 | 269 ± 21 | 261 ± 17 |
| | OLDER | 185 ± 7 | 178 ± 7 | 202 ± 19 | 193 ± 16 | 188 ± 14 |
| HR minimum (bpm) | NEWBORN | 119 ± 12 | 121 ± 8 | 124 ± 6 | 126 ± 8 | 118 ± 4 |
| | OLDER | 75 ± 6 | 85 ± 5* | 76 ± 7 | 80 ± 10 | 79 ± 9 |
| Gain (bpm/mmHg) | NEWBORN | -13 ± 4 | -12 ± 2 | -8 ± 2* | -14 ± 4 | -15 ± 5 |
| | OLDER | -16 ± 4 | -12 ± 4 | -20 ± 6 | -20 ± 8 | -17 ± 5 |
| $SAP_{50}$ (mmHg) | NEWBORN | 118 ± 3 | 120 ± 2 | 115 ± 2 | 112 ± 5 | 115 ± 4 |
| | OLDER | 115 ± 3 | 116 ± 2 | 104 ± 9* | 107 ± 9 | 113 ± 4 |

Heart rate (HR), systolic arterial pressure (SAP), and systolic arterial pressure at the mid-point heart rate range ($SAP_{50}$). Values are mean ± SD. *$p<0.05$ compared to Control.

Appendix B. Effects of L-NAME on some baseline parameters of renal function.

| | Age (weeks) | Control | 20 min | 40 min | 60 min |
|---|---|---|---|---|---|
| $C_{Na+}$ ($x10^{-3}$ mL/min/gkw) | One | 0.65 ± 0.61 | 0.55 ± 0.51 | 0.58 ± 0.39 | 0.65 ± 0.56 |
| | Six | 1.25 ± 1.13 | 15.88 ± 9.23*† | 16.87 ± 9.38*† | 16.97 ± 8.29*† |
| $C_{K+}$ (mL/min/gkw) | One | 0.045 ± 0.026 | 0.036 ± 0.01 | 0.041 ± 0.02 | 0.044 ± 0.01 |
| | Six | 0.14 ± 0.060† | 0.36 ± 0.11*† | 0.30 ± 0.09*† | 0.36 ± 0.14*† |
| $C_{Cl-}$ ($x10^{-3}$ mL/min/gkw) | One | 2.2 ± 1.1 | 1.9 ± 1.1 | 1.9 ± 0.7 | 2.1 ± 1.2 |
| | Six | 3.9 ± 2.1 | 33.1 ± 11.7*† | 30.3 ± 12.5*† | 34.1 ± 11.5*† |
| $U_{Na+}$ : $U_{K+}$ | One | 1.1 ± 1.2 | 1.3 ± 1.1 | 1.3 ± 1.1 | 1.1 ± 1.0 |
| | Six | 0.4 ± 0.4† | 2.3 ± 1.3*† | 2.7 ± 1.3*† | 2.6 ± 1.3*† |
| TTKG | One | 1.7 ± 0.3 | 1.7 ± 0.2 | 1.6 ± 0.3 | 1.7 ± 0.3 |
| | Six | 4.8 ± 1.7† | 16.6 ± 6.2*† | 14.3 ± 3.5*† | 14.2 ± 6.3*† |

Sodium, potassium and chloride clearances ($C_{Na+}$, $C_{K+}$, $C_{Cl-}$), urinary sodium to potassium excretion ratio ($U_{Na+}$:$U_{K+}$) and transtubular potassium gradient (TTKG) measured before (Control) and after the administration of L-NAME in conscious lambs. Values are mean ± SD. *$p<0.05$ compared to Control; †$p<0.05$ compared to one-week old lambs.

**Appendix C. Effects of L-NAME on baseline plasma electrolyte, osmolality and blood hematocrit values.**

| | Age (weeks) | Control | 20 min | 40 min | 60 min |
|---|---|---|---|---|---|
| $P_{Na+}$ (mmol/L) | One | 144 ± 2 | 145 ± 4 | 145 ± 3 | 144 ± 3 |
| | Six | 143 ± 4 | 144 ± 2 | 144 ± 7 | 143 ± 4 |
| $P_{K+}$ (mmol/L) | One | 3.8 ± 0.5 | 3.8 ± 0.4 | 3.7 ± 0.3 | 3.7 ± 0.5 |
| | Six | 3.8 ± 0.4 | 3.6 ± 0.4 | 3.5 ± 0.3* | 3.4 ± 0.3*† |
| $P_{Cl-}$ (mmol/L) | One | 141.8 ± 14.4 | 142.8 ± 15.9 | 142.8 ± 11.2 | 142.1 ± 16.0 |
| | Six | 135.7 ± 27.3 | 138.6 ± 28.9 | 143.1 ± 29.6 | 140.0 ± 31.9 |
| $P_{Osm}$ (mOsm/kg $H_2O$) | One | 294 ± 6 | 298 ± 5 | 296 ± 5 | 296 ± 6 |
| | Six | 293 ± 3 | 293 ± 6 | 296 ± 8 | 294 ± 5 |
| Hct (%) | One | 30.1 ± 1.5 | 29.6 ± 0.9 | 30.1 ± 1.0 | 29.9 ± 1.7 |
| | Six | 28.9 ± 1.8 | 28.5 ± 1.7 | 28.4 ± 1.4 | 28.3 ± 1.1 |

Plasma sodium ($P_{Na+}$), plasma potassium ($P_{K+}$), plasma chloride ($P_{Cl-}$), plasma osmolality ($P_{Osm}$), and blood hematocrit (Hct) measured before (Control) and after I.V. administration of L-NAME in conscious lambs. Values are mean ± SD. *$p<0.05$ compared to Control. †$p<0.05$ compared to one-week old lambs.