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Effect of Ischemia/Reperfusion on the Rat Intrarenal Renin-Angiotensin System

by
© Jimmy Kontogiannis

Thesis submitted for the degree of
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Dedication

To my parents, Tasia and Gus, and also to my sister Helen

Declaration

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Jimmy Kontogiannis

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ABSTRACT

Effect of Ischemia/reperfusion on the Rat Intrarenal Renin-Angiotensin System

Proliferation and differentiation of tubular cells occur after renal ischemia/reperfusion. Angiotensin II has multiple growth effects on different renal cell types, and also regulates renal blood flow. In particular, proximal tubular cells locally synthesize angiotensin II, which can bind to AT_1 receptors in an autocrine/paracrine fashion. The function of the AT_2 receptor is unknown, but is linked to apoptosis in other tissues. In the current study, gene expression for intrarenal angiotensinogen, cortical AT_1 receptor and proximal tubular AT_1 receptor was determined by Northern blots, and gene expression for the AT_2 receptor was determined by RT-PCR, in rats after ischemia/reperfusion. A significant downregulation of angiotensinogen mRNA occurred from 6 to 72 hours post-ischemia/reperfusion, whereas the cortical AT_1 receptor did not change significantly. In contrast, proximal tubular AT_1 receptor mRNA significantly decreased from 0 to 24 hours post-ischemia/reperfusion, and recovered after 72 hours. New gene expression for the AT_2 receptor was observed in ischemic proximal tubular segments and in the ischemic outer medulla 120 hours after ischemia/reperfusion. By histoautoradiography, a significant decrease in the density of angiotensin II binding was noted in the ischemic cortex after 24 hours post-ischemia/reperfusion, and in the outer medulla at 3 and 24 hours. At 120 hours post-ischemia/reperfusion, intrarenal angiotensin II levels, density of angiotensin II binding and angiotensinogen gene expression recovered. In conclusion, the intrarenal renin-angiotensin system is downregulated with ischemia/reperfusion, and recovers 120 hours post-ischemia/reperfusion, with co-expression of AT_1 and AT_2 receptor mRNA at this time. These findings suggest that co-expression of AT_1 and AT_2 receptors may be involved in the remodelling of injured tissue.

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List of Abbreviations

Ab-Ang II	Immunoglobulin for angiotensin II
ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
AT1	Angiotensin type-1 receptor
AT2	Angiotensin type-2 receptor
BCIP	5-bromo-4-chloro-3-indolyl-phosphate-4-toluisin
BSA	Bovine serum albumin
Bsm I	Restriction enzyme
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary DNA
Ci, mCi, μCi	Curie, millicurie, microcurie
cpm	Counts per minute
ddH2O	Double distilled water
dNTP	Deoxy nucleoside triphosphate (N=G, A, T, C)
ddNTP	2,3-dideoxy nucleotide triphosphate (N=G, A, T, C)

DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
FLB	Formaldehyde loading buffer
g	Gravitational constant=9.8N/kg
g, mg, μg	Gram, milligram, microgram
Gi	Inhibitory G protein
Gs	Stimulatory G protein
H & E	Hematoxylin and Eosin
Hpa I	Restriction enzyme
HPLC	High performance liquid chromatography
125I-Ang II	125I-labelled [Sar1Ile8] angiotensin II
kb	Kilobase
Kpn I	Restriction enzyme
min	Minutes
Mops	3-(N-morpholino) propanesulfonic acid

mRNA	Messenger ribonucleic acid
n	Number of observations
NBT	4-nitro-blue-tetrazolium chloride
O.D.	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pCR Script SK (+)	Cloning vector (plasmid)
PRA	Plasma renin activity
Pvu II	Restriction enzyme
RAS	Renin-angiotensin system
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
sH2O	Sterile water

SEM	Standard error mean
ssDNA	Salmon sperm DNA

Overview of Renal Ischemia/Reperfusion

1.0 Preface

Angiotensin II is known to have diverse physiological effects within the kidney. These effects include regulation of ion balance, regulation of renal hemodynamics and stimulation of growth (Moe et al. 1993). Angiotensin II is formed from a cascade of enzymatic reactions beginning with cleavage of the substrate angiotensinogen, and all synthetic components have been shown to exist in the kidney (Moe et al. 1993). As such, the kidney has its own renin-angiotensin system. It is not known, however, whether the components of the kidney's renin-angiotensin system are affected by renal ischemia/reperfusion. Such information might provide insight into the regulation and physiological functions of the different components of the intrarenal renin-angiotensin system during ischemia/reperfusion. In turn, possible beneficial and/or detrimental effects of angiotensin II receptor signalling on recovery of kidney function can be deduced from this information.

1.1 Acute Renal Failure

Acute renal failure is defined as a loss of renal function over a period of hours to days, with an inability of the kidney to excrete nitrogenous wastes and to maintain electrolyte balance (Thadhani et al. 1996). An increase of plasma creatinine concentration >44 μM or a reduction of creatinine clearance by 50% are used commonly to define this condition (Solomon et al. 1994) Acute renal failure can, under different conditions, result from acute tubular necrosis (Kaufman et al. 1991). Ischemic and toxic insults to the kidney are two common intrarenal causes of acute tubular necrosis. These clinical conditions can be

manifested, for example, in a patient by extensive blood loss, after aortic clamping or with use of nephrotoxic antibiotics. Acute renal failure can also be caused by prerenal causes, in which the glomeruli and tubules are intact but clearance is reduced by impaired renal perfusion. Acute renal failure of prerenal causes is often reversed if appropriate measures are taken to correct the original cause, but can predispose the kidney to ischemic-induced acute tubular necrosis (Corwin et al. 1987). Prerenal, ischemic, and toxic insults are in fact responsible for most causes of acute renal failure and subsequent acute tubular necrosis.

1.2 Synopsis of Renal Ischemia/Reperfusion Changes

Ischemia is one of several causes of tissue hypoxia. Other forms of hypoxia include reduced binding of O_2 to hemoglobin (transport hypoxia) and impaired O_2 utilization (histotoxic hypoxia) (Granger et al. 1992). Specifically, an ischemic episode is characterized by stagnant blood flow and deficient oxygen delivery by the blood to the tissue. In contrast to the heart and brain where ischemia leads to permanent cell loss, the ischemic kidney does have the capacity to completely restore its structure and function (Thadhani et al. 1996); its recovery involves a timely co-ordinated process of epithelial cell regeneration and differentiation.

Ischemic-induced acute renal failure leads to injury and death of tubular cells. The actual events leading to the death of tubular cells are complex and not very well understood. The few studies conducted in ischemic animal models do demonstrate, however, certain common structural features and biochemical changes believed to be characteristic and important for the onset of tubular necrosis.

1.2.1 Morphological/Biochemical Changes

Proximal tubular cells undergo extensive aerobic respiration and receive a high percentage of renal blood flow to sustain energy and vectorial transport processes. These cells have a high density of mitochondria, but low glycolytic capacity and glycogen storage capacity. For this reason, proximal tubular cells are susceptible to ischemic damage. Following renal ischemia/reperfusion, morphological changes are first observed in the proximal tubular cells. Both the apical brush border and cell polarity are lost (Humes et al. 1986). The tight junctions, which serve to align the proximal tubules in the correct orientation and to control epithelial permeability, are also disrupted (Liu et al. 1993). Normally, the Na/K-ATPase is found on the basolateral membrane and generates concentration gradients for Na reabsorption across the proximal tubule cells. With ischemia/reperfusion, the Na/K-ATPase redistributes from the basolateral to the apical membrane, and apical to basolateral Na-dependent transport is lost (Molitoris et al. 1992). In other studies pertaining to renal ischemia/reperfusion, dead tubular cells have been observed to slough into the tubular lumen and to develop a cast formation with ischemia/reperfusion, a process that increases intratubular pressure and reduces the effective glomerular filtration rate (Liu et al. 1993). Also, the loss of the epithelial tight junctions can allow a backleak of glomerular filtrate, further reducing the glomerular filtration (Liu et al. 1993). Creatinine, which is normally filtered and neither reabsorbed or secreted, is elevated in plasma with ischemia-induced injury and is therefore used as a diagnostic marker for acute renal failure (Moore et al. 1984).

In addition to the proximal tubule cells, cells of the outer medulla have also been shown to undergo changes after ischemia/reperfusion (Brezis et al. 1995). During ischemia, tubular and endothelial cells swell. Rapid DNA

fragmentation is also observed along the medullary thick limbs, signifying apoptotic changes in this region during ischemia (Brezis et al. 1995). Much of the inherent susceptibility of the outer medulla to ischemic/reperfusion damage is due in part to low blood flow and low pO_2 in this region (Shanley et al. 1986).

Elevated intracellular calcium concentration has been implicated as a possible biochemical change that contributes to epithelial cell toxicity, especially in ischemic proximal tubular cells (Kribben et al. 1994). In the kidney, increased intracellular calcium concentration has vasoconstrictive effects on vasculature and is associated with cytoskeleton breakdown, as well as with interference of mitochondrial energy metabolism. In addition to its role in regulating voltage-gated channels, intracellular calcium also exerts a significant role as a cofactor in the activation of certain proteases and phospholipases. It is reasonable to postulate then, a role for intracellular calcium in ischemic-induced pathophysiology, most likely through the activation of enzymes and impairment of intracellular metabolism. Depletion of cellular ATP has been shown to increase cytosolic calcium concentrations (Snowdowne et al. 1994). During ischemia, rapid breakdown of ATP into adenosine, inosine, and hypoxanthine has been reported (Bonventre 1993). Moreover, the breakdown products of ATP during renal ischemia may exert unique physiological actions. By crossing cell membranes, these small nucleotides can bind to ligand-specific receptors of neighbouring intrarenal arteries, and imbalance renal blood flow. Whereas adenosine has a vasoconstrictor effect on the cortex of the kidney, the medulla responds with vasodilation and inhibition of tubular function (Bonventre 1993).

The cellular phospholipase A_2 enzyme is also activated with ischemia, and seems to alter the permeability of cell and mitochondrial membranes, a result that may explain ischemic-induced cell and mitochondrial swelling (Bonventre 1993). Differentially activated forms of phospholipase A_2 have

recently been reported in hypoxic proximal tubule cells, with the low molecular weight isoform dominating over the high molecular weight isoform (Choi et al. 1995). In short, these changes in phospholipase A_2 activity and/or isoforms might reflect differences in the metabolism of arachidonic acid during ischemia/reperfusion. Such an effect may lead to the preferential formation of certain eicosanoids, and possibly determine differences in eicosanoid signalling towards the modulation of the vasculature tone, the complement system and/or chemotaxis of neutrophils during ischemia/reperfusion.

1.2.2 Reperfusion and Generation of Oxygen Radicals

A significant degree of renal damage occurs with ischemia alone, but reperfusion has also been recognized to contribute to the pathophysiology of acute renal failure. Ischemia has been shown to depress the antioxidant capacity and the biological sink for oxygen radicals in the kidney (Safirstein et al. 1994), thereby allowing reperfusion to potentially increase the severity of damage in tissue, by re-introducing reactive oxygen species which damaged tissue may not be able to metabolize. Sources of reactive oxygen species include cyclooxygenases, the mitochondrial electron transport chain, the xanthine oxidase system, and infiltrating neutrophils (Thadhani et al. 1996). Tissue damage by oxygen radicals is associated with peroxidation of lipid membranes and protein dysfunction via the Amadori reaction (Linder 1991). In a study examining the role of white blood cells in renal ischemia/reperfusion, the extent of injury was decreased in animals for which white blood cells were removed from plasma; this suggested a pathogenic role for white blood cells in the maintenance of ischemic-induced acute renal failure, likely through secretion of reactive oxygen species and/or other inductive signalling factors (Klausner et al. 1989). It is of interest that reactive oxygen species may also control the gene

expression of some growth factors (Homma et al. 1995), such as heparin-binding epidermal growth factor. In this particular study, enhanced kidney gene expression of heparin-binding epidermal growth factor in the rat ischemic model was reversed by administration of antioxidant reagents (Homma et al. 1995). Precaution, however, has to be exercised with regards to the overall significance of reperfusion-mediated delivery of oxygen radicals in promoting tissue injury. In contrast to studies that have found beneficial effects with the use of anti-oxidants, some studies have also demonstrated no benefit with their use (Bonventre 1993).

1.3 Growth Factors in Regenerating Post-ischemic Kidneys

Intrarenal gene expression of growth factors has been investigated in the rat ischemic model of acute renal failure. In the ischemic kidney, damaged tubular epithelium must regenerate and differentiate into functional tubules in order for the kidney to function. Research efforts have recently focused on the use of growth factors post-ischemia, as one method to understand and specifically target the acceleration of recovery in the post-ischemic kidney. Certain growth factors are known to be expressed within the kidney, and expression of specific receptors for these growth factors has been localized to the tubules of the nephron. Additionally, studies on ischemic-induced acute renal failure in animals have demonstrated changes in the levels of gene expression for renal-derived growth factors and/or their respective receptors after ischemic injury. These observations offer valuable insight into the pathophysiology of ischemic-induced acute renal failure.

The thick ascending limb of the nephron is the major source of epidermal growth factor (EGF) in the kidney (Safirstein et al. 1993). During

ischemia/reperfusion, pre-proEGF mRNA decreases significantly and stays persistently low for a prolonged period of time. In contrast, the EGF receptor number increases (Safirstein et al. 1990). The administration of EGF post-ischemia, however, mitigates ischemic-induced acute renal failure (Humes et al. 1989). These data suggest, therefore, that correction of renal-derived EGF levels hastens recovery of the post-ischemic kidney.

Insulin-like growth factor I (IGF-I) and transforming growth factor-β (TGF-β) are renotropic factors, and both have been studied during the course of ischemia-reperfusion in the rat. TGF-β mRNA expression has been shown to be elevated for up to fourteen days post-ischemia within the regenerating tubules of the outer medulla, with levels returning to basal values twenty-eight days after ischemia/reperfusion (Basile et al. 1996). Most active TGF-β protein in the normal kidney has been immunolocalized within proximal tubular cells, but in the ischemic kidney the active TGF-β is also immunolocalized within regenerating proximal tubular cells, sloughed tubular cells and also in papillary fronds (clumps of cells that have proliferated into the lumen of the tubule) (Shimizu et al. 1993).

In contrast to TGF-β mRNA, IGF-I mRNA has been shown to decrease significantly with ischemia-reperfusion, with reappearance occurring three days onward mainly in the regenerative cells of the cortical and medullary regions (Matejka et al. 1992). Normally, IGF-I receptors are distributed throughout the nephron regions of the kidney, with the majority of receptors localized to proximal tubular cells (Matejka et al. 1992). Following ischemia/reperfusion, IGF-I receptors are increased and are distributed mainly in the injured nephron segments, as compared to the sham rats (Matejka et al. 1992). Of interest, the administration of IGF-I post-ischemia significantly lowers plasma creatinine and blood urea nitrogen levels in ischemic rats, compared to rats receiving vehicle (Miller et al. 1992). In the normal kidney, IGF-I has been shown to induce

glomerular and proximal cell hypertrophy (Hammerman et al. 1989), in addition to increasing glomerular filtration and renal plasma flow (Hirschberg et al. 1990). These results suggest beneficial effects of IGF-I in the post-ischemic regenerating kidneys, which may arise either through the growth stimulatory effects and/or hemodynamic effects of IGF-I. No information has yet been published about the clinical effects of these growth factors in the human setting.

1.4 Therapeutic Strategies

In addition to the use of growth factors for treatment of ischemic acute renal failure, other approaches for the treatment have focused on vasodilators. Previous observations have found ischemia to alter endothelial-cell function and also to decrease the response and production of local vasodilatory substances (Conger 1991). Studies have therefore concentrated on the therapeutic assessment of agents known to be vasodilatory, either in man or animal.

Dopamine, for example, dilates renal arterioles and increases the glomerular filtration rate in humans (McDonald et al. 1964). Unfortunately, clinical studies in humans have not demonstrated any benefit of administrating dopamine in human-renal ischemia (Conger 1995). Of similar interest, the use of atrial natriuretic peptide has been shown to attenuate the severity of renal failure, and actually to potentiate renal recovery when administered after an ischemic episode (Shaw et al. 1987). Calcium-channel blockers have also been assessed for therapeutic efficacy in different settings of acute renal failure, but not in ischemic-induced injury. In this regard, calcium-channel blockers have been shown to reduce the incidence of tubular necrosis in renal transplantation (Neumayer et al. 1992), thus re-inforcing a role for intracellular calcium in the

pathophysiology. Another effect of calcium-channel blockers includes renal vasodilation (Bonventre 1993).

Endothelin is a potent intrarenal vasoconstrictor that reduces renal blood flow and GFR (Badr et al. 1994). Promising advances have been made with the use of anti-endothelin antibodies and/or endothelin-receptor antagonists in protecting animals from ischemic acute renal failure (Chan et al. 1994, Gellai et al. 1994). In rats receiving an endothelin type A receptor antagonist (BQ123) after ischemia/reperfusion, the survival rate increased markedly by improving tubular Na reabsorption and increasing GFR and K excretion (Gellai et al. 1994). The K plasma levels were found to be normal at 5 days post-ischemia after treatment with the antagonist (Gellai et al. 1994), a significant finding because hyperkalemia ($K^+ > 8$ mEq/L) is the major source of fatality in ischemic rats and in humans too.

Based on these observation, post-ischemic vasodilation seems to lessen the severity of renal damage. The protection mediated by vasodilation, although not yet determined conclusively, may be a direct consequence of an enhanced ability of renal blood vessels to deliver oxygen or remove toxic substances from the vicinity of damaged tissue. Other benefits of increased renal blood flow may include reduction of medullary transport systems by disruption of the urinary concentrating mechanism.

Some therapeutic approaches have concentrated on the use of osmotic agents such as mannitol, with the rationale that reduced tissue swelling and increased intratubular flow rate might accelerate recovery from ischemic-induced renal failure. Similarily, the loop diuretic furosemide has also been used to increase intratubular flow. In addition, by inhibiting the Na/K/Cl co-transporter of the thick ascending limb and reducing the ATP utilization, furosemide protects medullary tubules from hypoxia (Brezis et al. 1984). The data, however, are

inconclusive; whereas mannitol and furosemide work well in protecting the ischemic kidneys of animals and in the preservation of transplant kidneys ex vivo, most studies in humans have failed to demonstrate effectiveness against ischemic injury (Malis et al. 1983, Conger et al. 1995).

No therapeutic gains have been published in the human setting since these findings, and this would seem to suggest that recovery from renal ischemia in humans is complex. Angiotensin II is an octapeptide hormone found in high quantities within the rat kidney (Yosipiv et al. 1996), and has also been implicated in certain renal diseases. The specific role of this peptide during ischemia/reperfusion, however, has not yet been investigated. Interestingly, angiotensin II has unique growth properties on renal cell types, and is also a potent intrarenal vasoconstrictor; thus, angiotensin II exerts control over two physiological processes that are determinants of renal recovery from ischemic acute tubular necrosis. A further understanding of the pathophysiology of ischemic acute tubular necrosis can therefore be made by studying the intrarenal renin-angiotensin system during the course of ischemia/reperfusion.

Intrarenal Renin-Angiotensin System

2.0 Renin-Angiotensin System

Debate has existed over the past few years as to whether tissue in general, or in this case renal tissue, can generate local angiotensin II independently of the circulatory endocrine route, the latter which involves the liver, the kidney and the lungs. More specifically, investigators have debated over the existence of an intrarenal renin-angiotensin system that has all necessary enzymatic pathways and substrates to sustain angiotensin II production.

The existence of angiotensin II as a circulatory endocrine hormone is well established. The liver secretes angiotensinogen into the blood where it then serves as a substrate for the renin enzyme. Angiotensinogen is a glycoprotein and currently the only identified substrate for renin (Lynch et al. 1991). The angiotensinogen gene also has many polyadenylation sites that allow for a variety of angiotensinogen mRNA sizes to be generated, but the size of these messages has not accounted for any difference in substrate affinity towards renin (Gomez et al. 1995). At the protein level, glycosylation has been noted to produce different isoforms of angiotensinogen protein. No angiotensinogen isoform, however, has been found to affect renin recognition any differently (Campbell et al. 1984).

The renin enzyme is produced from the granular cells of the afferent arteriole, and catalyzes the formation of angiotensin I (10 amino acids) from angiotensinogen (Inagami et al. 1986). Both macula densa cells and granular cells of the afferent arteriole compose the juxtaglomerular apparatus. Initially, renin is produced as pre-pro-renin and upon removal of the leader sequence in

the rough endoplasmic reticulum, the pro-renin is glycosylated and secreted from the Golgi apparatus for exocytosis (Hsueh et al. 1992).

The angiotensin-converting enzyme (ACE) of the lung endothelial cells cleaves angiotensin I (10 amino acids) to generate angiotensin II (8 amino acids), and can also cleave kinins such as bradykinin; therefore, ACE has a dual function which involves the activation of a vasoconstrictor and the inactivation of a vasodilator. Indeed, ACE has been found in most endothelial tissues examined: gut, heart, adrenal, brain and kidney (Ehlers et al. 1989). Once formed, angiotensin II can function as a vasoconstrictor and regulator of blood pressure. Also, angiotensin II can contribute to the maintenance of vascular tone, stimulate release of aldosterone from the zona glomerulosa of the adrenal gland, as well as increase Na uptake across renal proximal tubule cells (Moe et al. 1993). Figure 2.0 summarizes the renin-angiotensin cascade.

2.1 Evidence for an Intrarenal Renin-Angiotensin System

Recently, sensitive techniques have allowed more thorough studies to be conducted on tissue. Specifically, it has been shown that both proximal tubular segments and glomeruli compartments are specific regions of the nephron able to produce angiotensin II, as determined by angiotensin II purification and subsequent radioimmunoassay (Braam et al. 1993, Seikaly et al. 1992). Results have shown consistent 1000 X higher concentrations of angiotensin II originating from the proximal and glomerular compartments of the kidney, as compared to the levels found in the systemic circulation. These data have therefore served as strong evidence for the existence of an intrarenal renin-angiotensin system, that is independent of the circulatory endocrine route of angiotensin II formation .

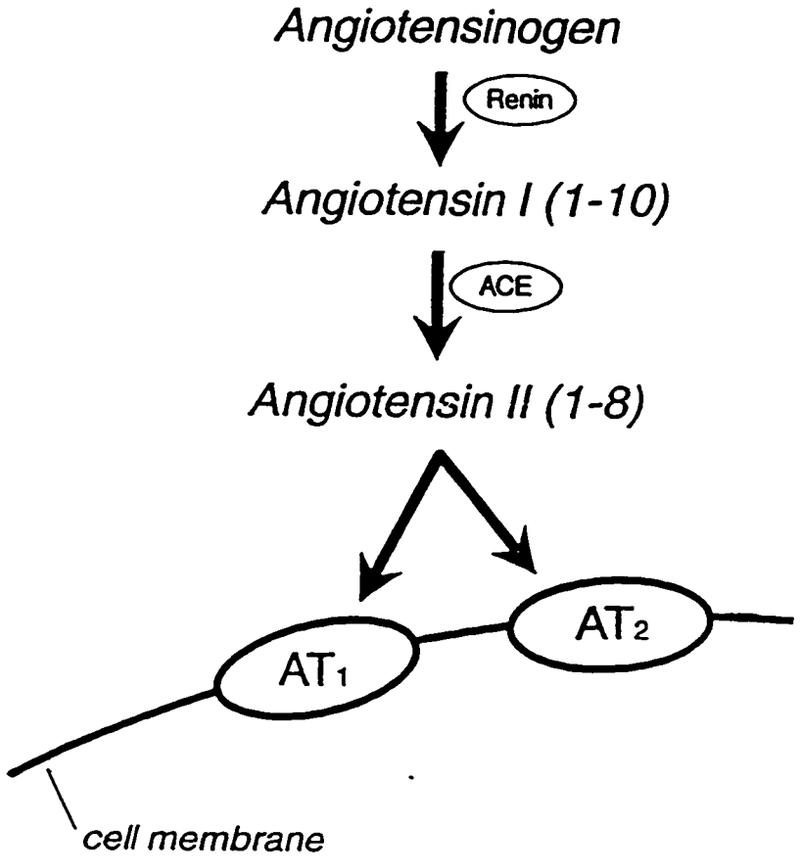


Figure 2.0: The Renin-Angiotensin System cascade. Angiotensinogen is cleaved by renin to form angiotensin I. The angiotensin converting enzyme then cleaves angiotensin I to form angiotensin II. Angiotensin II binds to either AT_1 or AT_2 receptors. (Modified from Gomez, 1995).

As expected, all the necessary renin-angiotensin components for angiotensin II production have been found within the proximal tubular cells. In situ hybridization, for instance, has revealed most renal angiotensinogen mRNA to be confined to the proximal tubular cells, and to a lesser degree in glomeruli (Ingelfinger et al. 1990). Pinocytotic vesicles of proximal tubular cells have also tested positive for renin by way of immunocytochemistry (Taugner et al. 1982). In cultured proximal tubule cells, renin mRNA has been detected by RT-PCR (Moe et al. 1994). Furthermore, immunohistochemical techniques have localized the highest concentration of ACE to the brush border and basolateral membranes of proximal tubule cells (Marchetti et al. 1987). Smaller quantities have been observed in the arterioles and glomeruli (Marchetti et al. 1987). These combined findings, together with the observation that angiotensin II receptors have been localized to the luminal and basolateral membrane of proximal tubular cells (Cheng et al. 1995), are consistent with the role of intratubular angiotensin II in paracrine/autocrine signalling of proximal tubular cells.

Some investigators have hesistated to accept the notion that renal tissue can produce such high amounts of angiotensin II, and have argued that the high concentrations of angiotensin II receptors in the kidney may protect angiotensin II from degradation and perhaps account for a longer half-life and/or higher concentration of angiotensin II (Nussenberger et al. 1992). In the rat, angiotensin II levels are higher in the kidney than in any other organ (Yosipiv et al. 1996).

In summary, the model of the rat intrarenal angiotensin-system may prove useful for the understanding of human renal diseases, as it relates to the context of intrarenal angiotensin II production and/or angiotensin II signalling. This is supported by studies that have shown human urine to contain high levels of

angiotensin II, suggesting that humans have an intrarenal renin-angiotensin system as do rats (Vos et al. 1994). The importance of the human intrarenal renin-angiotensin system in the disease state, of course, is demonstrated by the use of ACE inhibitors in diabetic patients; ACE inhibitors attenuate the onset of diabetic renal failure in the human setting (Lewis et al. 1993), suggesting a pathogenic role of intrarenal angiotensin II in diabetes. In streptozotocin-induced diabetic rats, the onset of renal failure is also delayed by use of ACE inhibitors (Anderson et al. 1989). Since human and rat intrarenal renin-angiotensin systems respond similarly in diabetes, the study of the intrarenal renin-angiotensin system in the ischemic rat may be an acceptable model for understanding the pathophysiology of ischemic acute renal failure in humans.

2.2 Function of Angiotensin II

The renin-angiotensin system has important physiological functions for bodily homeostasis, as emphasized by blockade of the angiotensin type 1 receptor (AT_1) in the developing rat kidney. In these studies, the cardiovascular system and animal development are impaired, including kidney development (Gomez et al. 1995). Fewer and thicker dilated afferent arterioles develop with AT_1 blockade, and a higher proportion of dilated tubules and immature glomeruli are observed within the kidney.

In the kidney, angiotensin II has been shown to induce hypertrophy of murine proximal tubule cells, as estimated by total protein and cell size (Wolf et al. 1990). In cultured murine proximal tubule cells, angiotensin II-induced hypertrophy has been shown to be mediated by TGF-β (Wolf et al. 1993), which suggests that angiotensin II causes the induction of growth-related factors. Further support is derived from studies showing angiotensin II to have a

stimulatory effect on the activation of growth-related proto-oncogenes in proximal and mesangial cells (Norman et al. 1991). Enhanced gene expression of the transcription factor c-jun by angiotensin II is a cited example (Naftilan et al. 1990).

Differential effects of angiotensin II are also observed, depending on the specific cell type. Whereas angiotensin II is hypertrophic for proximal and mesangial cells (Wolf et al. 1990), angiotensin II is a mitogen for cells of the thick ascending limb (Wolf et al. 1995). Moreover, angiotensin II can modulate the effects of other growth factors, such as EGF. In proximal tubular cells, the high expression of EGF receptors is thought to receive paracrine signalling from sources of EGF originating from the thick ascending limb. Recent observations have found angiotensin II to potentiate the mitogenic capacity of EGF, at least in cultured proximal tubular cells (Norman et al. 1987).

2.3 Function of the AT_1 receptor

Angiotensin II physiological responses are mediated by angiotensin II receptors localized on the cellular membrane. The use of DuP 753 and PD123177 non-peptide antagonists has permitted the identification of two classes of angiotensin II receptors, referred to as AT_1 and AT_2, respectively (Timmermans et al. 1992). The nucleotide sequence for the AT_1 receptor encodes a seven-transmembrane G-protein receptor. The signalling mechanism of the AT_1 receptor is coupled through a G_i mechanism, and some studies have also linked the AT_1 receptor of proximal tubular cells with the activation of phospholipase C and intracellular calcium at higher angiotensin II concentrations. Although contradictory in its signalling mechanism, this discrepancy may be potentially attributed to degradatory angiotensin II products. Recently, angiotensin IV (fragment 3-8) has been shown to stimulate calcium

influx in kidney opossum cells, by acting on unique receptors neither sensitive to DuP 753 or PD123177. Since the lumen of the proximal tubular lumen contains the highest concentration of peptidases in the body (Kenny et al. 1978), it has been proposed that high concentrations of angiotensin II can be degraded into different angiotensin II fragments, such as angiotensin IV, that can then signal independently of the AT_1 receptor.

In addition to being expressed on proximal, thick ascending and distal tubular segments of the nephron, the AT_1 receptor is also abundantly expressed on renal vasculature, in particular the afferent/efferent arterioles and vasa recta. In the former, the AT_1 receptor functions to control the vascular tone and the glomerular filtration rate. The efferent arteriole has been demonstrated to have an enhanced vasoconstrictive tone in the presence of angiotensin II compared to the afferent arteriole, an effect that can increase intraglomerular pressure and favour filtration (Gomez et al. 1995). In the medulla, the AT_1 receptor can influence the medullary blood flow and dictate the development of the medullary osmotic gradient, which is an important aspect for the concentration of urine. The slow medullary blood flow and the countercurrent exchange of oxygen within the vasa recta are the two most important reasons for the low pO_2 levels found in this portion of the kidney (Brezis et al. 1995). Due to this precarious situation, the medulla can be damaged at lower levels of pO_2, as seen with ischemia/reperfusion.

Two receptor subtypes have been identified for the AT_1 receptor in mouse and rat, named AT_{1a} and AT_{1b} (Iwai et al. 1992). In the kidney, expression of the AT_{1a} receptor predominates over the AT_{1b} receptor, both at the protein and mRNA level. Elsewhere, the mRNA expression of these two receptor subtypes has been shown to be influenced independently of one another, especially in salt diets (Du et al.1995); here, transcriptional regulation is

believed to account for the differential gene expression of the two AT_1 subtypes with different salt diets. The AT_{1b} receptor is more abundant than the AT_{1a} receptor in the pituitary gland, the testis and the adrenal glands (Gomez et al. 1995). From an evolutionary point of view, the AT_{1b} receptor gene is considered to have resulted from gene duplication of the AT_{1a} receptor. The two AT1 receptor isoforms are 95% homologous at the amino acid level, and this is cited as evidence for gene duplication. Interestingly, the 5' and 3' flanking nucleotide regions of the two isoforms are low in homology (Iwai et al. 1992), an observation that may explain some differences in the transcriptional control between the two isoforms under different physiological conditions.

2.4 Function of the AT_2 Angiotensin II Receptor

The AT_2 angiotensin II receptor is another angiotensin II receptor recently cloned and found to encode a 363 amino acid protein having seven transmembrane domains, and exhibiting a 32% amino acid homology with the AT_1 receptor (Kambayashi et al. 1993). The AT_2 receptor gene is located on the X chromosome and unlike the prolific expression of the AT_1 receptor which can be found in the vasculature and most tissues, Northern blot analysis has revealed mRNA of the rat AT_2 receptor to be confined to the adrenal gland, the ovarian system and certain brain nuclei of the adult rat (Ichiki et al. 1995). Some investigators have debated over the existence of AT_2 mRNA in the adult kidney, but no data have yet substantiated its existence directly.

The AT_2 receptor cDNA has been transfected into COS-7 (monkey kidney cells) and PC12w cells (rat pheochromocytoma cell line which expresses AT_2 receptor abundantly), and found in both cases to have an inhibitory effect on protein phosphotyrosine phosphatase (PTP) activity, as determined by the release of ^{32}Pi from ^{32}P-phosphorylated substrates (Kambayashi et al. 1993).

In short, angiotensin II-mediated inhibition of PTP activity could only be blocked by the AT_2 receptor antagonist PD123319, and not by AT_1 receptor antagonist DuP 753. Also, pretreatment with pertussin toxin could eliminate angiotensin II-induced inhibition of PTP activity, indicating that AT_2 regulates PTP activity through a G_o or G_i mechanism (Kambayashi et al. 1993).

No biological functions for the AT_2 receptor have been rigorously established. It is conceivable that the AT_2 receptor may exert a differentiation and/or anti-proliferation role. Much of the evidence is derived from studies conducted on developing tissues. In developing fetal tissue, for example, AT_2 mRNA is prominent in the skin, tongue, kidney and other organs as well (Kambayashi et al. 1993). In the fetal kidney from day E12 (embryonic) to day 15 postpartum (D15), in situ hybridization shows prominent AT_2 mRNA expression in the kidney cortex and outer medulla, confined to undifferentiated nephrogenic mesenchymal tissue, but not in the immature or mature glomeruli and tubules of the kidney (Shanmugam et al. 1995). By day 22 postpartum, no AT_2 mRNA is evident in the kidney, and the AT_1 receptor mRNA is expressed in abundance, mediating the well-described physiological functions of angiotensin II, as discussed earlier (Shanmugam et al. 1995). Similiar patterns have been noted in the protein expression of the AT_2 receptor (Aguilera et al. 1994).

More recently, the AT_2 receptor has been shown to induce apoptosis in PC12W cells and R3T3 cells (mouse fibroblast cell line) transfected with the AT_2 cDNA (Yamada et al. 1996). In both cell-lines, the AT_2 receptor was involved in the dephosphorylation of mitogen-activated protein kinase (MAP kinase), and the apoptotic conditions could be reversed using vanadate (protein-tyrosine-phosphatase inhibitor) or antisense oligonucleotide to MAP kinase phosphatase 1. Thus, the data suggested that MAP kinase phosphatase 1,

activated by the AT_2 receptor, caused MAP kinase inactivation (dephosphorylation) and thereby induced apoptosis.

In knockout studies conducted on the mouse AT_2 receptor, effects on blood pressure and exploratory behaviour were noted. Most notably, resting systolic and diastolic pressures were elevated in the knockout mice for the AT_2 receptor (Ichiki et al. 1995). Also, administration of different doses of angiotensin II caused significant rises in systolic and diastolic pressures in these knockout mice, as compared to normal mice. Furthermore, the development of the kidney and other organs showed no morphological changes in the knockout mice (Ichiki et al. 1995), which is suprising considering that the AT_2 receptor is developmentally regulated. The precise role of the AT_2 receptor still remains obscure. As an example, current literature indicates that AT_2 receptor activation decreases diuresis and natriuresis in the rat (Lo et al. 1995), thus contradicting the functional significance of the knockout studies carried out on mice. In another study further exploring the role of AT_2 receptor, AT_2 receptor activation was found to increase urinary cGMP (guanosine 3',5'-cyclic monophosphate) levels in rats (Siragy et al. 1996).

In other more pertinent studies, expression of the AT_2 receptor is known to occur after myocardial infarction and in skin wound healing (Nio et al. 1995). Moreover, granulosa cells of atretic follicles, which are known to express high levels of the AT_2 receptor, also undergo marked apoptosis (Pucell et al. 1991). It also appears that the AT_2 receptor can modulate the activity of the AT_1 receptor, because AT_2 receptor activation mediates anti-growth effects on rat coronary endothelial cells, and opposes AT_1 receptor-mediated mitogenesis (Stoll et al. 1995). These combined observations certainly suggest an apoptotic/anti-proliferative role for the AT_2 receptor in the physiological setting, and the enhanced gene expression of this receptor within injured tissue may

influence AT_1-mediated growth responses. As of yet, no information exists addressing these effects of renal ischemia/reperfusion on the intrarenal renin-angiotensin components and angiotensin II-mediated signalling.

Purpose

3.0 Rationale

At present, little information is available on the effects of ischemia/reperfusion on the intrarenal renin-angiotensin system. Enalapril, a specific ACE inhibitor, has been shown to delay the onset of renal insufficiency in dogs with ischemic kidneys (Graham et al. 1993), perhaps suggesting that the AT_1 receptor has a detrimental effect in renal ischemia/reperfusion. Given the important role of the intrarenal renin-angiotensin system in regulation of hemodynamics and kidney growth modulation, and the importance of these two parameters on the status of renal recovery, a logical extension would be to characterize the intrarenal renin-angiotensin system during the course of ischemia/reperfusion.

3.1 Purpose

The purpose of this project was divided into two principal categories:

First, our objective was to investigate the effects of renal ischemia/reperfusion on the relative state of mRNA expression for the intrarenal renin-angiotensin system components. Specifically, the following component mRNAs were studied: angiotensinogen, cortical and proximal tubular AT_1 receptors, proximal tubular AT_2 receptors and outer medullary AT_2 receptors.

Secondly, it was the objective of the project to investigate the effects of renal ischemia/reperfusion on angiotensin II binding and total renal angiotensin II peptide levels.

3.2 Hypothesis

All intrarenal renin-angiotensin system components were hypothesized to be upregulated following ischemia/reperfusion. The rationale for this is based on studies of other growth factors, such as TGF-β and heparin-binding EGF (Basile et al. 1996, Homma et al. 1995).

3.3 Approach

Angiotensinogen gene expression was assayed by Northern blot analysis. The relatively high abundance of the AT_1 receptor in both renal cortical and proximal tubular segments made the use of Northern blot analysis an ideal choice for the assay of this mRNA following ischemia/reperfusion. The assay of the AT_2 receptor mRNA, however, required a different approach. Given the debate over the existence of this mRNA in the kidney and its presumable low expression, the assay of this mRNA involved the use of RT-PCR. The identity of the RT-PCR product had to be verified through DNA sequencing.

The effect of ischemia/reperfusion on angiotensin II binding was assayed by histoautoradiography on renal slices. In addition, total angiotensin II peptide was quantitated at 120 hours after ischemia/reperfusion. This method involved the purification of angiotensin II from peptides and other angiotensin II-related degradatory products using a reverse phase HPLC column. Total angiotensin II was subsequently quantitated by radioimmunoassay.

Methods

4.0 Experimental Model and Animals

Male Sprague-Dawley rats (250-350 g) and differing in age by at most one week were used in these studies. Data were obtained from kidneys of sham and post-ischemic rats at 0, 1, 3, 6, 24, 72 or 120 hours after reperfusion. All rats were housed in the University of Ottawa Animal Care Unit, and the protocol used in this study was approved by the University of Ottawa Animal Care Committee.

4.1 Surgery

Male Sprague-Dawley rats were anesthetized with somnotol. The anesthestic was administered by injection into the peritoneal cavity at a dose of 65 mg/kg body weight, and complete anesthesia was determined by complete loss of jaw tone, corneal reflex and paw reflex. Extracellular fluid volume was maintained by a 5 ml bolus injection of saline subcutaneously prior to surgical manipulation.

A smooth continuous skin incision was made along the mid-line using a Barb Parker knife. Toothed forceps were used to grasp and lift the underlining rectus abdominis muscle. Metzembaum scissors were then used to cut along the mid-line of the muscle. The left and right renal arteries and veins were exposed with the use of a curved mosquito clamp, and then clamped with vascular clips for 60 minutes to induce ischemia. Sham rats were operated on in the same fashion, but the renal arteries/veins were not clamped. All rats were kept on a heated pad at 37^{o}C for the duration of the surgery . After 60 minutes, the clamps were removed and the kidneys were allowed to reperfuse. The rectus abdominis muscle was closed by a horizontal interrupted suture and the skin was closed by a subcutaneous suture. Vicryl 3/0 was used as suture

material. All rats had access to water and food ad libitum, and all surgeries were conducted in a paired fashion, such that each ischemic rat was paired with a respective sham rat.

4.2 Blood Collection

Prior to animal sacrifice, blood samples were obtained at the completion of the timed reperfusion by an intracardiac puncture. A 20 1/2" gauge needle connected to a 10 ml syringe was inserted at an angle of 45^{o} to the xiphisternum into the heart ventricle. Blood volumes in the range of 4-6 mls were drawn and injected into an EDTA-coated vial. The samples were centrifuged at 4000 X g for 20 minutes in order to obtain the plasma, which was then aliquoted into sterile tubes and preserved at -20 ^{o}C. Assays of plasma [creatinine], [blood urea nitrogen] and plasma renin activity were kindly performed by an independent lab (University of Ottawa, Department of Biochemistry, Ottawa General Hospital).

4.3 Isolation of Proximal Tubular and Outer Medullary Tissue

Proximal tubular segments were isolated by Percoll gradient centrifugation, as previously described (Vinay et al. 1981). The renal cortices from kidneys of sham and post-ischemic rats were dissected free from the outer and inner medullary regions of the kidney, and then gently minced and suspended in a solution containing (in mM) 115 NaCl, 24 $NaCO_3$, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 2.0 NaH_2PO_4, 5 glucose, 1 alanine, 10 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.4), 0.03% collagenase (type IV; Sigma, St. Louis, MO) and 0.01% soybean trypsin inhibitor (Sigma) (buffer A). The suspension was gassed with 95% O_2 and 5% CO_2 and maintained at 37^{o}C for 30 minutes. After digestion, the cortical suspension was

strained through a 250 μm brass sieve and centrifuged for 1 minute at 100 X g. The pellet was resuspended in buffer A without collagenase or trypsin inhibitor, and centrifuged for 1 minute. This was repeated three times. Next, the pellet was resuspended in a 40% Percoll solution of identical ionic compostion as buffer A, which had been previously chilled to 4 ^{o}C. The Percoll solution was centrifuged at 26000 X g for 30 minutes at 4 ^{o}C, and the digested tissue separated into four distinct bands (F1-F4) after centrifugation.

As described previously, the different densities of the various tubular segments allowed for isolation of enriched proximal tubular segments by removing the lowermost band after centrifugation (Vinay et al. 1981). In order to verify the presence of proximal tubular segments in the lowermost band of the Percoll gradient, total RNA was isolated and assayed for the presence of angiotensinogen mRNA by RT-PCR, as shown in figure 4.0 (see section 4.6 for details on primers and RT-PCR). Since kidney angiotensinogen mRNA has been shown to exist predominantly in proximal tubular cells (Ingelfinger et al. 1990), its presence served as a positive control. Cell death was also excluded from the preparation of sham and ischemic proximal tubular segments. As shown in figure 4.1, more than 95% of proximal tubular segments did not incorporate the cell death marker trypan blue, indicating a high rate of viability; however, in the presence of 0.1% SDS, proximal tubular segments did incorporate the trypan blue dye (figure 4.1C, positive control).

The outer medullary tissue was dissected from the cortex, using fine scissors. Total RNA was isolated according to the methodology described in section 4.4.

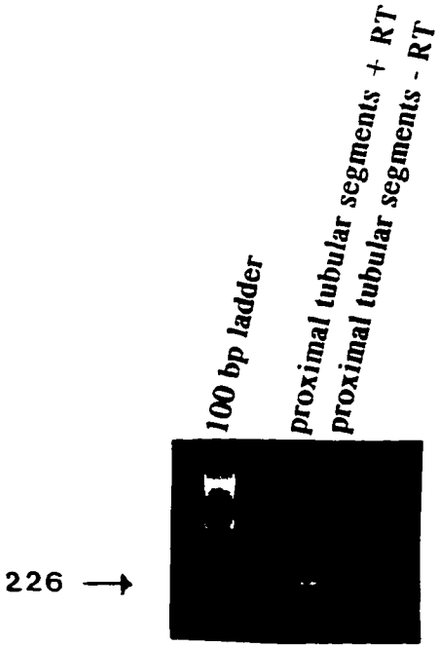


Figure 4.0: Proximal tubular segments express angiotensinogen mRNA. Total RNA was extracted from proximal tubular segments collected from the Percoll gradient. A 226 bp fragment of the angiotensinogen mRNA was amplified by RT-PCR and used as a positive control for the presence of proximal tubular segments. + RT=presence of reverse transcriptase, - RT=absence of reverse transcriptase.

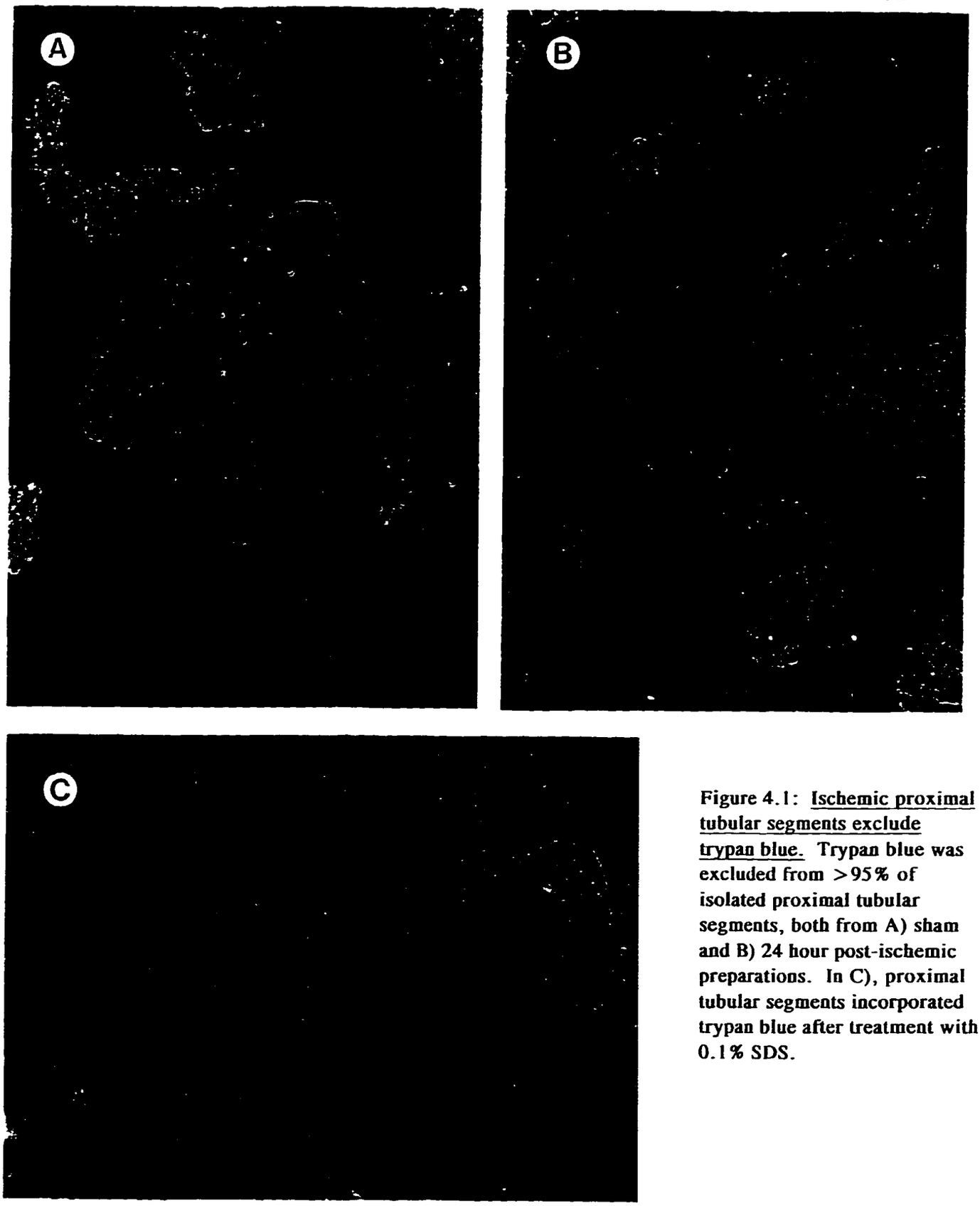


Figure 4.1: Ischemic proximal tubular segments exclude trypan blue. Trypan blue was excluded from >95% of isolated proximal tubular segments, both from A) sham and B) 24 hour post-ischemic preparations. In C), proximal tubular segments incorporated trypan blue after treatment with 0.1% SDS.

4.4 RNA Isolation

The method of CsCl RNA extraction was used temporarily at the start of these studies, but the RNA isolation protocol was later switched to using commercially available spin columns from Qiagen (Chatsworth, CA), which was less time consuming and more cost effective than the method of CsCl RNA extraction. This protocol was also advantageous in that it permitted isolation of RNA only above 200 bp size, excluding smaller RNA fragments. This condition was ideal in maximizing the AT_2 receptor RT-PCR signal (see section 4.6). The quality of the RNA was visualized with ethidium bromide staining on a 1% agarose formaldehyde gel. Equivalent amounts of RNA were loaded from RNA samples of sham and post-ischemic rat kidneys, as verified by equal ethidium bromide staining of both the 28S and 18S ribosomal RNA bands.

4.4.1 CsCl Method

The method of CsCl RNA extraction was used to extract cortical RNA from the kidney following collection of blood samples (Maniatis et al. 1989). Renal cortical tissue was dissected and placed into a Petri dish containing a strong denaturing solution to inhibit RNase activity [4 M guanidinium thiocyanate (Sigma, St. Louis, MO), 25 mM sodium citrate (Sigma), 0.5% lauryl sarcosine (Sigma) and 0.1 M 2-mercaptoethanol (Sigma)]. Cortical tissue was finely minced with a razor blade and then homogenized (Dounce homogenizer). Final homogenization of the tissue was carried out with a tissue grinder (5 X 5 sec bursts). To remove insoluble debris, homogenates were centrifuged 10000 X g for 20 minutes at room temperature. The supernatant was recovered and subsequently passed through a 20 1/2" needle in order to shear chromosomal DNA.

The solution was aliquoted onto CsCl solution [5.7 M CsCl, 5.2 mM EDTA, pH=5.7] in a 2 mm x 18 mm diethylpyrocarbonate (DEPC)-pretreated flexible ultracentrifuge tube. Effort was made to maintain the interphase between the CsCl solution and the denaturing solution at the time of transfer. This gradient was centrifuged in a Beckmann ultracentrifuge at 26000 X g at 14^{o}C for 24 hours.

RNA was isolated by aspirating the supernatant from the RNA-containing pellet, and allowing the pellet to air dry for 5 minutes. The RNA pellet was subsequently washed with 100% ethanol and reconstituted into DEPC-treated water. A 1:1 volume of phenol and 49:1 chloroform:isoamyl alcohol solution was added to the RNA sample, and the tubes were vortexed and centrifuged 12000 X g at 4^{o}C for 5 minutes; this step removed contaminating protein and/or DNA from the RNA sample (Maniatis et al. 1989). The RNA in the upper aqueous phase was then transferred to a new tube.

To initiate precipitation of RNA, 0.1 X volume of sodium acetate [3 M, pH=5.2] and 2.5 X volume of 100% ice-cold ethanol were added to the aqueous phase. Tubes were vortexed and then stored at -80 ^{o}C for 20 minutes. Thereafter, the contents were centrifuged at 14000 X g at 4 ^{o}C for 15 minutes, leading to the precipitation of RNA against the side of the tube. This RNA pellet was washed in 500 µl of ice-cold 70% ethanol and then dissolved in DEPC-treated water.

The concentration (µg/µl) of RNA was determined by a Beckmann UV spectrophotometer, using the relationship 1 O.D.=40 µg/1000 µl of RNA (O.D.=260 nm). RNA purity was determined by O.D. ratio of 260/280. Careful attention was made to obtain RNase-free samples of RNA. It was important, therefore, to treat equipment for RNase contamination and to wear gloves throughout the duration of the protocol.

4.4.2 Qiagen RNA Columns

A second method of RNA isolation involved the use of Qiagen RNA columns (Qiagen, Chatsworth, CA). A small amount of tissue (<0.7g) was placed into 700 μl of lysis buffer supplied by the kit. Tissue was finely minced by a razor blade and then homogenized in a tissue grinder for 2 minutes. Homogenized tissue was further processed by passing it through a Qiagen shredder column and centrifuging at 10500 X g for 2 minutes. Equal volume of 70% ethanol was added to the eluant, and this solution was passed through a Qiagen RNA column by centrifuging at 10500 X g for 20 seconds. According to instructions within the kit, the eluant was discarded and the same procedure was repeated twice more using patent buffers. The Qiagen RNA column was placed inside an eppendorf tube and 30 μl of DEPC-H_2O was added onto the column. The RNA was eluted into the eppendorf tube by centrifuging at 10500 X g for 1 minute. All procedural steps were carried out at room temperature, and the final RNA sample was stored at -80 ^{o}C .

4.5 Northern Blot Analysis

4.5.1 RNA Electrophoresis

RNA samples were electrophoresed on gels as follows. Samples of RNA were added to gel sample buffer that consisted of 20 mM MOPS [3-(N-morpholino) propanesulfonic acid], 50 mM Na acetate, 10 mM EDTA (pH=7.0), 17% formaldehyde, 50% formamide, 1 mM EDTA (pH=8.0), 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol. To ensure the presence of high quality RNA and equal RNA loading between sham and respective ischemic RNA samples, ethidium bromide was added to the sample buffers at 1-2 μg/ml.

Prior to gel loading, samples of RNA were heat-denatured at 65 ^{o}C for 15 min in a heating block, and then placed on ice; this step prevented any non-specific secondary coiling of RNA. All gels consisted of 1% agarose/2.2 M formaldehyde in 20 mM MOPS. A constant voltage of 58 mV for 2-3 hours was sufficient for complete electrophoresis of the RNA samples. Following electrophoresis, polaroid photographs were taken of ethidium bromide-stained gels under UV illumination.

4.5.2 Northern Blot

Gels were washed extensively in double distilled water for 1 hour after complete electrophoresis, to remove residual amounts of formaldehyde from the gel. The gel was transferred thereafter into 10 X SSC [1.5 M NaCl, 0.15 M sodium citrate, pH=7.0], and washed twice for 30 minutes each.

RNA was transferred from the gel onto a nylon membrane through capillary action, as described elsewhere (Reed 1985). Briefly, the RNA gel was positioned onto a 2 X thick Whatman 3MM filter paper presoaked in 10 X SSC, with ends overhanging and making contact with a 10 X SSC buffer solution. A positively-charged nylon membrane (Mandel, Guelph, ON) of identical dimensions was fitted over the RNA gel, followed by 2 X 3 MM Whatman filter paper and then paper towels. A small weight was applied over the paper towels, and transfer was carried out overnight at room temperature.

Following transfer, the nylon membrane was removed carefully and washed for 2 minutes in 6 X SSC at room temperature. A small corner was also cut from the membrane for orientation purposes. RNA was permanently fixed onto the nylon membrane using a UV crosslinker (BioRad, Montreal, Quebec). This reaction involved the formation of cross-links between bases of RNA and the positively charged amine groups on the surface of the membrane (Church

1984). The absence of RNA staining by ethidium bromide on the gel, was used to determine the efficiency of RNA transfer onto the nylon membrane.

4.5.3 cDNA Labelling

The Amersham multiprime labelling system was used for the labelling of a 1 kb rat angiotensinogen cDNA (gift from Dr. K. Lynch, University of Virginia) and a 1.2 kb rat AT_{1a} receptor cDNA (gift from Dr. T. Inagami, Vanderbilt University). Approximately 50 ng of cDNA was required per labelling reaction, or an equivalent of 1.5 µl from the stock cDNA solution.

An 1.5 µl aliquot from the cDNA stock solution of either the angiotensinogen or AT_1 receptor was added to a tube containing 26.5 µl of sterile water. This solution was boiled for 5 minutes, followed by rapid cooling on ice; this procedure preserved cDNA in a single-stranded form, as required by the Klenow enzyme to generate complementary radioactive probes. To make a radioactive probe, denatured cDNA was incubated with buffer, primer, [^{32}P]-dCTP (0.22 µCi/µl) and Klenow DNA polymerase for 1 hour at 37 ^{o}C. All radioactive precautions were adhered to in this experiment. For the removal of non-incorporated radioactive nucleotides, the probe was purified by centrifugation through a Sephadex G-50 column (Boehringer Mannheim, Laval, Quebec). A Sephadex G-50 column was a convenient form of gel chromatography in which mononucleotides were readily trapped in the pores of the column, with exclusion of larger-sized polymers like cDNA. A 1 µl aliquot of the probe was counted by scintillation spectrometry, and a probe with specific activity greater than 2 X 10^{8} cpm/µg cDNA was used for Northern blot analysis.

4.5.4 Northern Hybridization

Northern hybridization is a method adapted and slightly modified from its counterpart called Southern hybridization (Southern 1975). The nylon membranes, now containing fixed samples of RNA, were prehybridized for 1-2 hours at 42 ^{o}C in a solution consisting of 30% formamide, 0.1% sodium dodecyl sulfate, 50 mM NaH_2PO_4 (pH=7.0), 5 X Denhardt's solution [0.1% BSA, 0.1% Ficoll and 0.1% polyvinylpyrrolidine), 100 μg/ml salmon sperm DNA and 5 X SSC. To eliminate non-specific hybridization of the cDNA probe to the nylon membrane, salmon sperm DNA was also included with the cDNA probe. The prehybridization solution was removed from the membrane after the elapsed 1-2 hours, and replaced with a hybridization solution. The hybridization solution was of identical composition to the prehybridization solution, but in addition contained the ^{32}P-labelled cDNA probe and 10% dextran sulfate; dextran sulfate was used to increase the efficiency of hybridization between the cDNA probe and desired sequence of mRNA. All labelled probes and salmon sperm DNA were denatured for 5 minutes by boiling, and then immediately cooled on ice prior to being added into the hybridization solution. This preserved all DNA in the form of single strands, a requirement for hybridization between the cDNA probe and the single-stranded mRNA. All hybridization reactions were performed overnight in a 42 ^{o}C water bath.

Hybridized membranes were initially washed at low stringency (2 X SSC, 0.1% SDS for 40 minutes at room temperature), followed by a high stringency wash (0.2 X SSC, 0.1% SDS for 25 minutes at 65 ^{o}C). Membranes were exposed overnight at -70 ^{o}C to Kodak X-OMAT film with two intensifiers. Autoradiography of Northern blots was linear for the AT_1 receptor over a range of different concentrations of kidney cortical RNA (1-20 μg), as shown in figure

4.2. The linearity of the curve in figure 4.2 confirmed ideal conditions for Northern blot analysis of the cortical AT_1 receptor.

4.6 Reverse Trancriptase (RT)-PCR assay

The AT_2 receptor mRNA was assayed using RT-PCR. Total RNA was isolated using the commercially available kit as described above, followed by DNase digestion to remove any contaminating DNA. PCR oligonucleotide primers (0.5 μM) were designed from the cDNA sequence of rat AT_2 receptor and chosen such that the GC% content ranged between 45-50%; the upstream sense strand was (5'-TGAGTCCGCATTTAACTGC-3') and the downstream antisense strand was (5'-ACCACTGAGCATATTTCTCAGG-3'), generating a 536 bp product [226-761 bp of rat AT_2 receptor cDNA] (Kambayashi et al. 1993). In addition, RT-PCR was performed on AT_{1a}, AT_{1b} and angiotensinogen mRNA using rat specific primers, as described elsewhere (Matsubara et al. 1994, Dostal et al. 1994): [rat AT_{1a}: upstream sense (5'-GCACACTGGCAATGTAATGC-3') and downstream antisense (5'-GTTGAACAGAACAAGTGACC-3'), generating a 385 bp product], [rat AT_{1b}: upstream sense (5'-GCCTGCAAGTGAAGTGATTT-3') and downstream antisense (5'-TTTAACAGTGGCTTTGCTCC-3'), generating a 204 bp product] and [rat angiotensinogen: upstream sense (5'-CCTCGCTCTCTGGACTTATC-3') and downstream antisense (5'-CAGACACTGAGGTGCTGTTG-3'), generating a 226 bp product, from bp 737-962 of rat angiotensinogen cDNA]. All RT-PCR products were resolved on 1.5% agarose ethidium bromide gels. The identity of the AT_2 RT-PCR reaction was confirmed by partially sequencing the PCR product (154 bp), after it was subcloned into the plasmid pCR-Script SK(+) (Stratagene, California); a 100% homology was achieved with the known cDNA

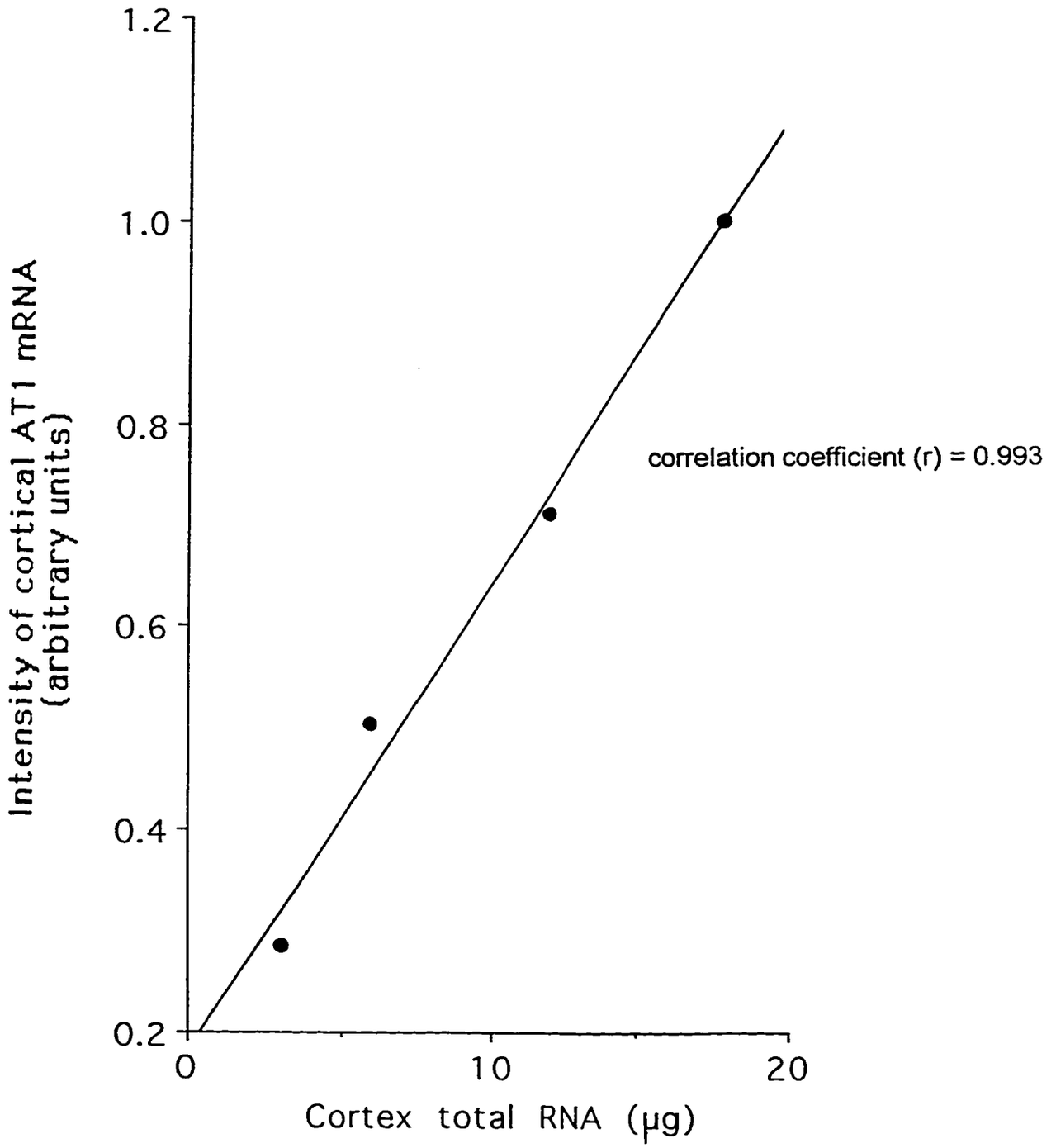


Figure 4.2: Standard curve for Northern blot analysis of renal cortical AT_1 receptor mRNA. Different amounts of cortical total RNA (µg) were prepared for Northern blot analysis of the AT_1 receptor mRNA, as described in the methodology. Signal intensities were determined by densitometry, normalized against the most intense signal and plotted against total RNA amount (µg). Linearity of curve confirmed ideal Northern blot conditions for analysis of the cortical AT1 receptor mRNA.

sequence for the AT_2 receptor. DNA contamination from the RNA sample was excluded as a source of error, because PCR without RT yielded no product after 40 cycles.

The Perkin Elmer GeneAmp Kit (Perkin Elmer, Norwalk, Connecticut) was used for RT-PCR, and contained all the necessary reagents required for reverse transcription and polymerase chain reaction. A RT mastermix was prepared with the following components per reaction tube: 5 mM $MgCl_2$, 1 X PCR buffer, 1 mM dGTP, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 1 U RNase inhibitor and 2.5 μM of random hexamers.

The RT mastermix was divided into two, with one half receiving RT and the other half receiving DEPC-H_2O (negative control). A 17 μl aliquot of either solution was placed into a PCR reaction eppendorf tube, followed by the addition of 1 μg (3 μl) of DNase-digested RNA sample. Samples were allowed to equilibrate to room temperature for 10 minutes, sufficient time for random hexamers to anneal to regions of all mRNAs. All mRNA species were reverse transcribed at 42 ^{o}C for 15 minutes, followed by denaturation at 99 ^{o}C for 5 minutes. Samples were immediately placed on ice to preseve the cDNA in a single stranded form. Samples then received a 80 μl aliquot of a PCR mastermix consisting of the following (final concentration): 2 mM $MgCl_2$, 1 X PCR buffer, 2.5 U TAQ DNA polymerase, 0.5 μM upstream primer and 0.5 μM downstream primer.

All PCR reaction tubes were vortexed, centrifuged and placed into a 94 ^{o}C preheated thermal cycler that was programmed to proceed for 40 cycles at 94 ^{o}C denaturing for 30 sec, 60 ^{o}C annealing for 30 sec and 72 ^{o}C extension for 45 sec. Total RNA samples from the sham and ischemic rat kidneys stained equally in intensity with ethidium bromide on formaldehyde agarose gels, verifying equal RNA loading into the RT-PCR reaction. Amplification of a partial

β-Actin cDNA [upstream sense (5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3') and downstream antisense (5'-AGCAGCCGTGGCCATCTCTTGCTGGAAGTC-3'), generating a 352 bp product] was also used as an internal control for the RT-PCR reactions. Efficiency of RT-PCR for the AT_2 receptor was shown to be within the linear range of amplification, as determined by amplification of different dilutions of starting total RNA (figure 4.3) and different PCR cycle numbers (figure 4.4). This method of determining efficiency has been described elsewhere (Sveistrup et al. 1995).

4.7 Subcloning of AT_2 RT-PCR Product

The 536 bp RT-PCR product for the AT_2 receptor was resolved on a 1.5% agarose ethidium bromide gel, and purified using a Geneclean Kit (Bio/Can Scientific, Mississauga, ON). Briefly, the excised band was placed in 3 X volume of 5 M NaI at 55 ^{o}C until the agarose had fully dissolved. An aliquot of 5 μl of glassmilk was added to physically bind to the cDNA molecules. The DNA/glassmilk pellet was recovered after brief centrifuging, and redissolved in sterile water at 37 ^{o}C for 20 minutes to detach cDNA molecules from the glassmilk. The glassmilk was removed by brief centrifuging, and the purified partial AT_2 cDNA within the supernatant was removed and stored at -20 ^{o}C.

The RT-PCR product for the AT_2 receptor was inserted into the plasmid pCR-Script SK(+) (Stratagene, La Jolla, CA), using a unique Srf I polylinker restriction site flanking either side of the T_7 and T_3 promoters as the site of insertion. The reaction mix consisted of: 1 ng of pCR-Script SK(+) cloning vector, 1 mM pCR-Script reaction buffer, 0.5 mM rATP, 0.5 ng of purified RT-PCR product, 0.5 U of Srf I restriction enzyme and 0.5 U of T4 DNA ligase (to

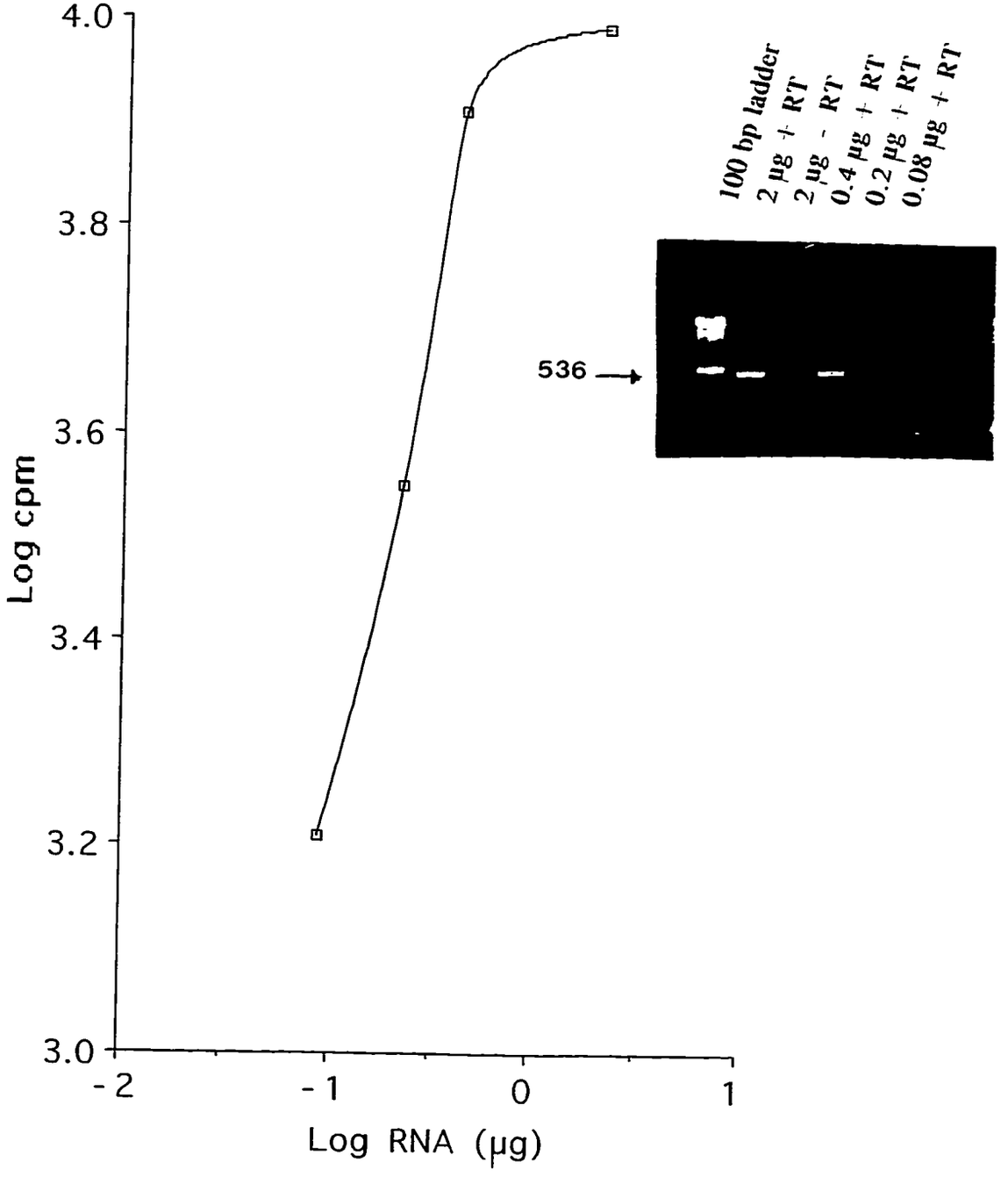


Figure 4.3: Efficiency of RT-PCR for the AT2 mRNA as a function of starting total RNA amount (µg). A 536 bp partial AT2 cDNA was amplified from proximal tubular total RNA for different dilutions of starting total RNA (2 µg). Band intensities were quantitated using trace amounts of [^{32}P]dCTP (5 µCi), excised from the agarose gel and measured in a scintillation counter. A representative example is shown in the inset. + RT=presence of reverse transcriptase, - RT=absence of reverse transcriptase.

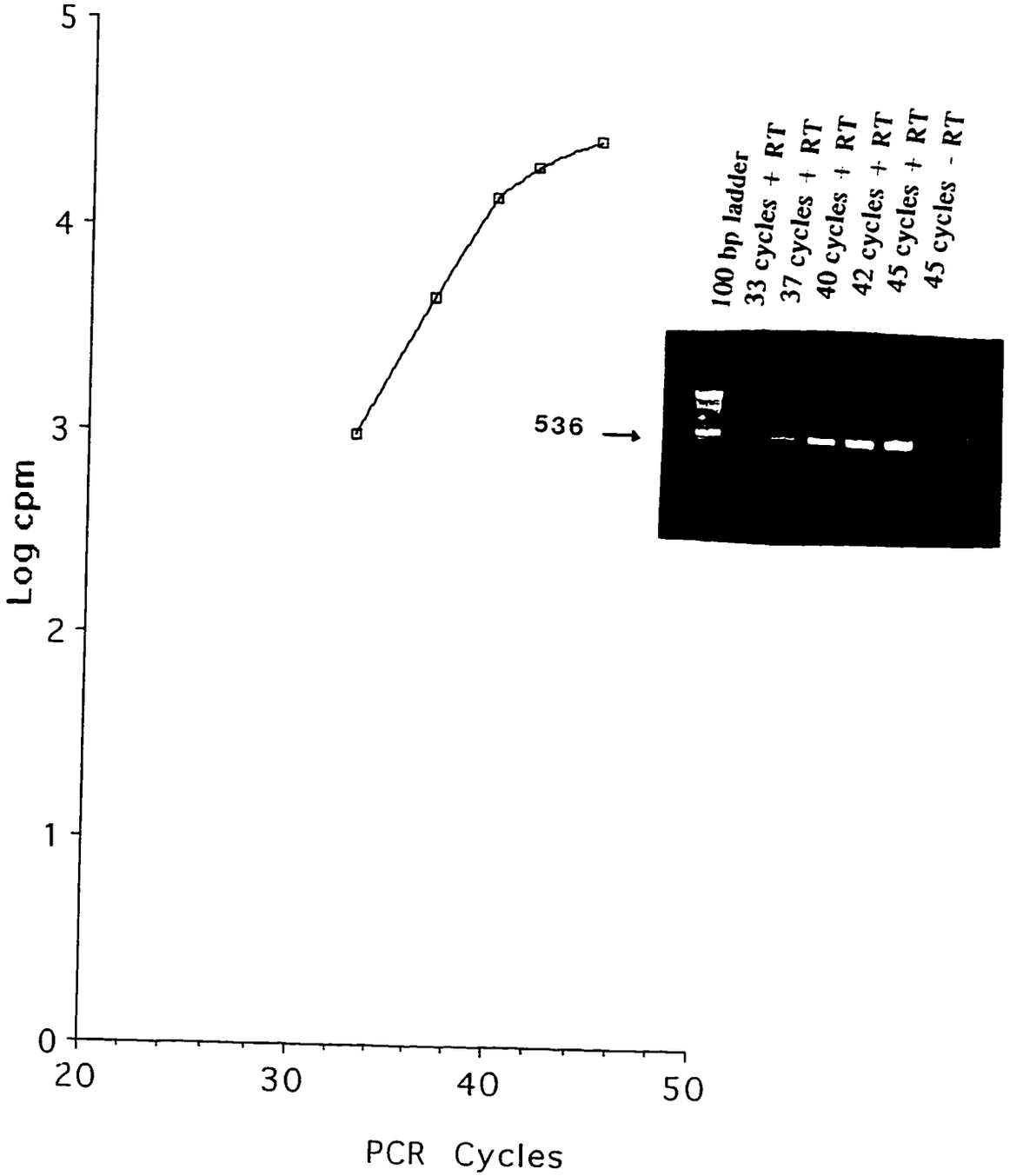


Figure 4.4: Efficiency of RT-PCR for the AT2 mRNA as a function of diffferent PCR cycle numbers. A 536 bp partial AT2 cDNA was amplified from proximal tubular total RNA (0.5 µg) for different PCR cycle numbers. Band intensities were quantitated using trace amounts of [^{32}P]dCTP (5 µCi), excised from the agarose gel and measured in a scintillation counter. A representative example is shown in the inset. + RT=presence of reverse transcriptase, - RT=absence of reverse transcriptase.

ligate onto both blunt ends of linearized plasmid). The contents were gently mixed and and incubated at room temperature for one hour. The reaction was terminated by heating to 65 ^{o}C for 10 minutes. The structure of the plasmid and the unique restriction sites for the plasmid and AT_2 insert are shown in figure 4.5A. Figure 4.5B graphically illustrates the steps involved in inserting the partial AT_2 cDNA into the plasmid pCR-Script SK (+).

In order to obtain large quantities of the recombinant plasmid, Escherichia coli supercompetent cells (Stratagene, La Jolla, CA) had to transformed for the recombinant plasmid and then cultured. In the transformation process, Escherichia coli cells were removed from -80 ^{o}C and allowed to thaw on ice. A 40 µl aliquot of cells was placed into a prechilled polypropylene tube and gently mixed with 0.7 µl of β-mercaptoethanol for 10 minutes on ice. The presence β-mercaptoethanol increased the transformation efficiency. A 50 ng amount of recombinant pCR-Script SK(+) was added to the competent Escherichia coli cells and incubated on ice for 30 minutes. Both the recombinant plasmid and Escherichia coli were then heat-shocked to 42 ^{o}C for 42 seconds and immediately placed on ice for 2 minutes. The length of heat-shock was critical, because heat-shocking over 42 seconds or under 42 seconds significantly reduced the transformation efficiency, according to the manufacturer. The newly transformed Escherichia coli were cultured in 0.45 ml of SOC medium [2% tryptone, 0.5% yeast extract, 0.05% NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose] for 1 hour at 37 ^{o}C with shaking at 225-250 rpm. Aliquots of 50 µl, 75 µl and 100 µl of the transformation mixture were plated onto individual ampicillin-resistant agar plates using a sterile spreader, and allowed to incubate overnight at 37 ^{o}C.

The ampicillin-resistant agar plates were inspected the following day for any visible appearance of bacterial colonies. Since the plasmid carried an

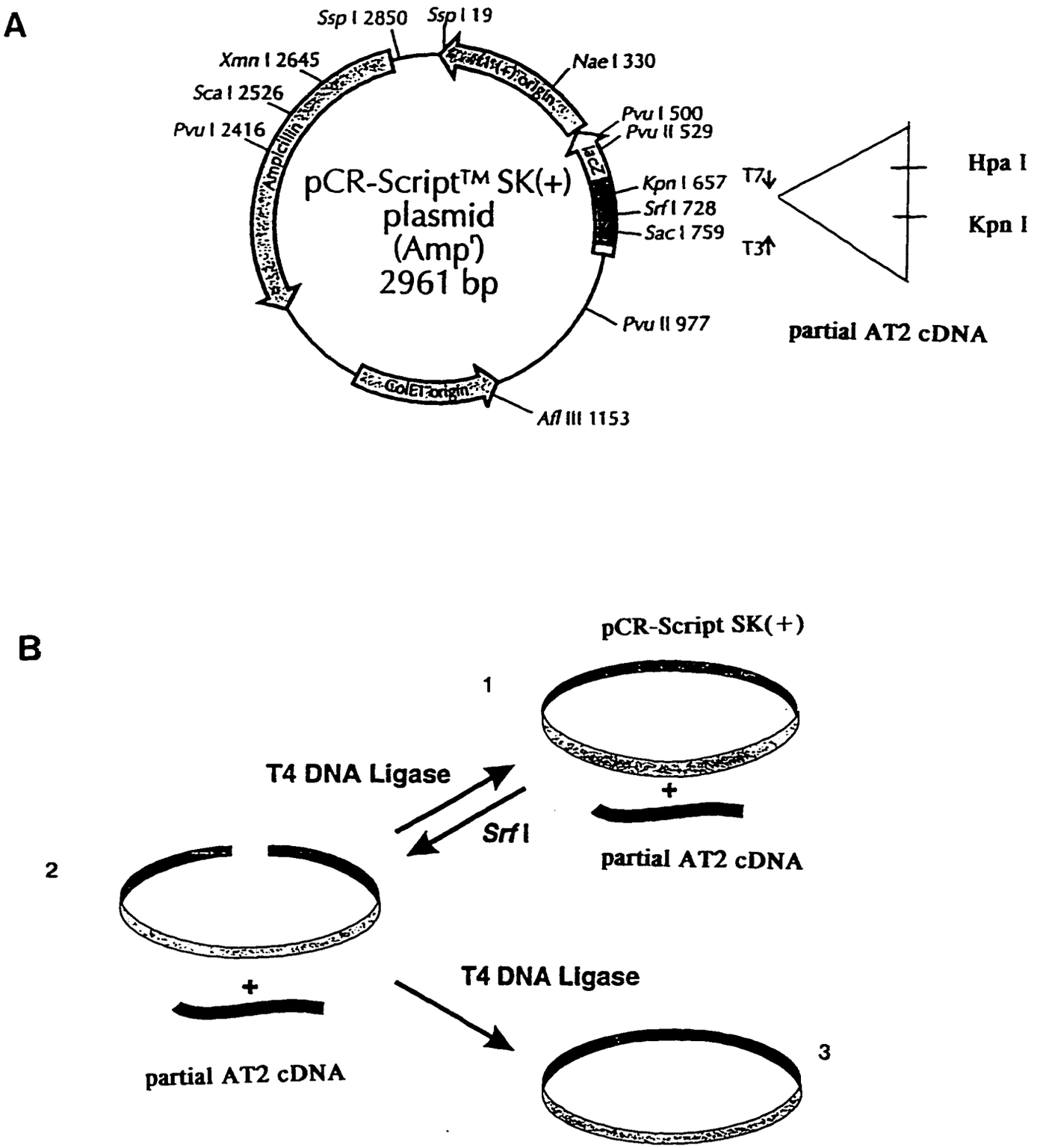


Figure 4.5: Insertion of the partial AT2 cDNA into the pCR-Script SK(+) plasmid. The pCR-Script SK(+) plasmid was used to subclone the 536 bp RT-PCR product of the AT2 receptor mRNA. A) The pCR-Script SK(+) plasmid carries an ampicillin resistant gene and also has two Pvu II restriction sites and one Kpn I restriction site. The partial AT2 cDNA carries one Hpa I and one Kpn I restriction site, and this fragment is inserted within the Srf I restriction site. B) illustrates graphically the steps required to insert the partial AT2 cDNA. The partial AT2 cDNA is co-incubated with the pCR-Script SK(+) plasmid, as shown in 1), and the pCR-Script SK(+) plasmid is then linearized by Srf I digestion, as shown in 2). In 3), both ends of the partial AT2 cDNA are annealed to both ends of the pCR-Script SK(+) plasmid by T4 DNA ligase enzyme.

ampicillin-resistant gene, the presence of a colony implied successful transformation of Escherichia coli cells. A single visible colony was removed and allowed to culture by transferring it to 250 ml of SOC medium (containing 50 µl/ml of ampicillin) for 16 hours. The use of ampicillin ensured the continual selection of the transformed Escherichia coli cells.

The bacterial pellet was collected by brief centrifugation. The plasmid DNA from the Escherichia coli was isolated using the alkali hydrolysis principal described elsewhere (Maniatis et al. 1989). The Qiagen plasmid midi kit used to isolate the plasmid was based on this principle (Qiagen, California). Briefly, lysozyme enzyme and SDS were used in small concentrations to gently disrupt the bacterial cell membrane and expose the cytoplasm. The bacterial chromosomal DNA became denatured, whereas the plasmid DNA did not, making the plasmid DNA physically possible to separate by centrifugation. After separation, plasmid DNA was further purified by phenol-chloroform extraction as described earlier, and finally precipitated with 0.7 X volumes of isopropanol by centrifuging at 20000 X g at 4 ^{o}C for 15 minutes. The supernatant was discarded and the plasmid DNA pellet was resuspended in sH_2O (sterile water) and stored at -20 ^{o}C.

Successful insertion of the partial AT_2 cDNA into the plasmid pCR-Script SK(+) was determined by restriction digestion, as shown in figure 4.6A. The restriction enzyme Pvu II recognized two restriction sites spanning the Srf I insertion site, and did not recognize the AT_2 cDNA insert (see figure 4.5A). Restriction digestion of the recombinant plasmid with Pvu II generated a 986 bp fragment, and not a 449 bp fragment; this confirmed an insertion. The restriction enzyme Kpn I also recognized two restriction sites, with one restriction site localized to the partial AT_2 receptor cDNA insert and the other localized to the plasmid. Restriction digestion of the recombinant plasmid with Kpn I generated

an expected 449 bp fragment. The recombinant plasmid was also linearized by Hpa I, a sequence unique only to the partial AT_2 receptor cDNA.

4.7.1 Deletion Mutant of Partial AT_2 Receptor cDNA

A 132 bp deletion mutant for the recombinant pCR-Script SK(+) plasmid was also created. Prior to the studies, it was assumed that analysis of the AT_2 receptor gene expression would also require co-amplification of a mutant cRNA transcript of the partial AT_2 receptor cDNA as an internal control, in what is referred to as competitive RT-PCR. This procedure was ultimately not essential because of the absence of AT_2 receptor mRNA in sham proximal tubular segments. In brief, a 132 bp deletion mutant for the partial AT_2 receptor cDNA was created by restriction digestion of the recombinant pCR-Script SK(+) plasmid with two unique restriction enzymes only confined to within the partial AT_2 receptor cDNA, but not to the native plasmid. The restriction enzymes Hpa I and Bsm I were used to delete 404 bp from within the partial AT_2 receptor cDNA. To create a large quantity of the 132 bp deletion mutant of the partial AT_2 cDNA, the mutant plasmid was transformed into Escherichia coli cells as described earlier. Plasmid DNA was isolated using the same procedure described earlier.

Figure 4.6B provides direct evidence of a 132 bp mutant of the original recombinant plasmid. Restriction digestion with Pvu II generated a 583 bp fragment for the deletion mutant, and the expected 986 bp fragment for the recombinant plasmid, thus confirming a 404 bp deletion from within the partial AT2 receptor cDNA.

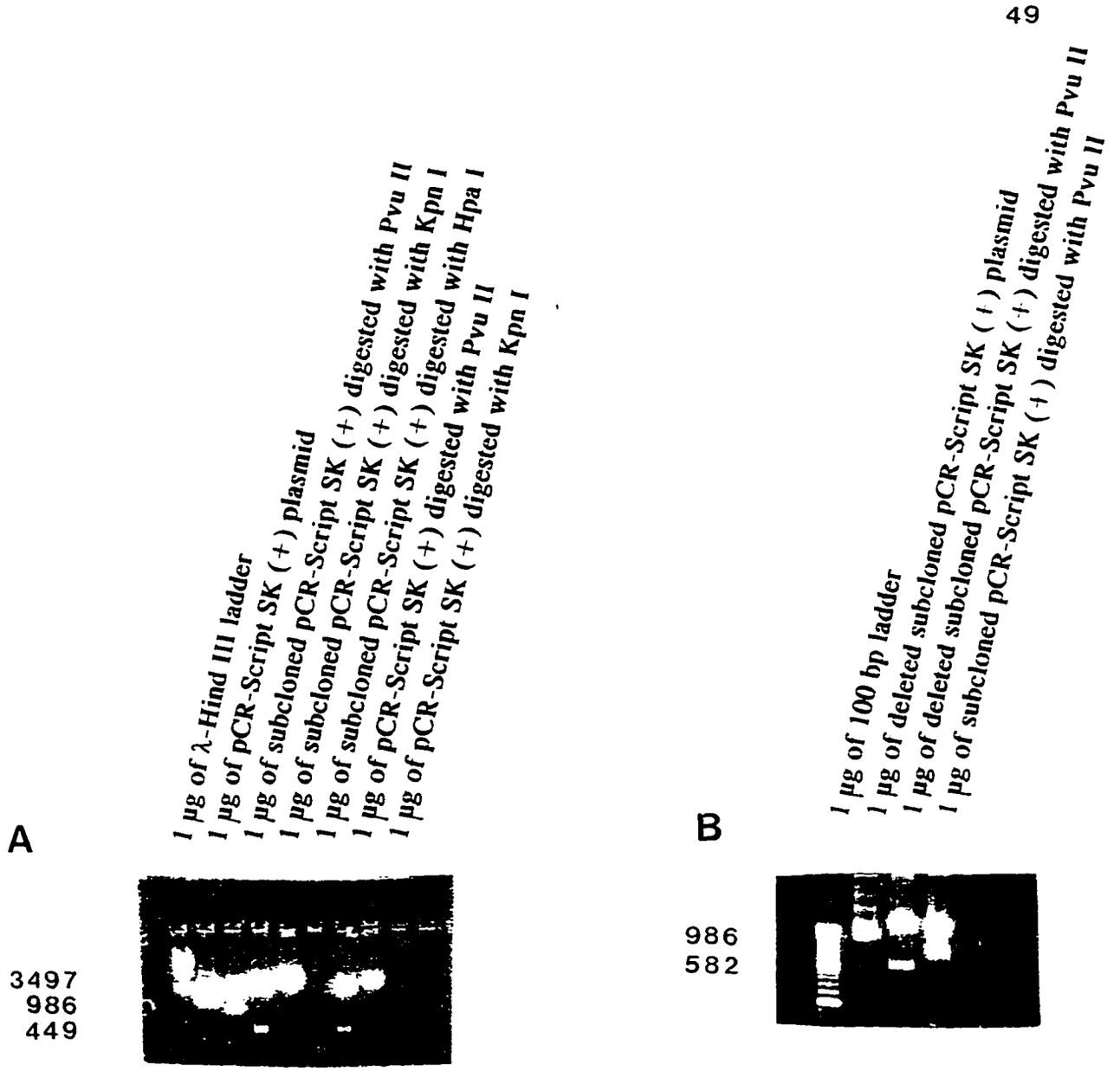


Figure 4.6: Verification of partial AT_2 cDNA insertion into the pCR-Script SK(+) plasmid by restriction digestion. In A), the expected 986 bp and 449 bp fragments were generated from the 3497 bp recombinant pCR-Script SK(+) plasmid by Pvu II and Kpn I restriction digestion, respectively. These fragments did not appear in the native plasmid. The recombinant pCR-Script SK(+) plasmid was also linearized by Hpa I, a sequence unique only to the partial AT_2 cDNA. A 132 bp mutant for the recombinant pCR-Script SK(+) was also created, by a 404 bp deletion spanning Hpa I and Bsm I restriction sites. In B), Pvu II restriction digestion generated a 583 bp fragment for the deletion mutant, and a 986 bp fragment for the recombinant plasmid, thus confirming a loss of DNA sequences within the partial AT_2 cDNA insertion.

4.8 Partial Sequencing of RT-PCR Product

The 536 bp RT-PCR product for the AT_2 receptor was subcloned into pCR-Script SK(+) and sequenced according to the dideoxy termination method (Lehninger et al. 1993). To perform the sequencing, the plasmid had to be alkali-denatured to obtain a single-stranded form of DNA. This form of DNA would allow a rat-specific AT_2 primer to anneal to the partial AT_2 cDNA inserted within the plasmid. To denature the plasmid, 3 μg of plasmid DNA was treated with 0.2 M NaOH, 0.2 mM EDTA for 30 minutes at 37 ^{o}C, and then neutralized by adding 0.1 X volumes of 3 M sodium acetate (pH=5.0). Subsequently, the plasmid DNA was precipitated with 3 X volumes of 70% ethanol and collected by centrifuging at 15000 X g at 4 ^{o}C. The pellet was washed with 70% ethanol and resuspended in sH_2O. Sequenase reaction buffer (Amersham) and a rat specific AT_2 primer were added to the plasmid DNA and then heated to 65 ^{o}C for 2 minutes. The heated mixture was then allowed to equilibrate to room temperature for 30 minutes, a process that enabled the rat specific AT_2 primer to effectively anneal to its homologous plasmid DNA sequence. The following were then added to the primed plasmid DNA, according to instructions provided by the kit Sequenase 2.0 kit (Amersham): 0.1 M DTT, diluted labelling mix, [α-^{35}S]ATP and sequenase enzyme.

The mixture was incubated at room temperature for 2 minutes, and then aliquoted and incubated in four distinct tubes containing either 2,3-dideoxyribonucleoside-5-triphosphates ddGTP, ddATP, ddTTP or ddCTP at 37^{o}C for 3 minutes. The reaction was terminated by using an aliquot of stop solution, as described by the Sequenase kit. In this overall reaction, the synthesis of complementary DNA strands was terminated by the incorporation of

ddNTPs (2,3-dideoxynucleoside 5 triphosphate), which lack 3-OH necessary for DNA synthesis (McMurray et al. 1986). Using different ddNTPs, the DNA strands were terminated into a fraction of the population of chains at sites where incorporation of a ddNTP was possible. Since radioactive dATP was used in the reaction, each termination chain was radioactive; therefore, autoradiography could then be used as a means to visualize each termination site in increasing size by electrophoretic separation.

For electrophoretic separation, each ddNTP reaction was loaded onto a 8% urea polyacrylamide gel and separated by 3000 V. The gel was dried onto Whatmann 3MM filter paper using a BioRad Gel Drier and exposed to standard X-ray film 24 hours. Figure 4.7 shows a representative partial sequence reaction for the recombinant pCR-Script SK(+) plasmid. This partial sequence was 100% homologous to the rat AT_2 cDNA (Kambayashi et al. 1993).

4.9 Histoautoradiography

Sham and ischemic kidneys were perfused with phosphate buffered saline [pH=7.4, 0.9% NaCl] through a heart puncture and then immediately preserved on dry ice. Tissue was sectioned into 20 μm thickness at -14 ^{o}C, thaw-mounted onto Fisher brand histological microscope slides and then dried overnight in a dessicator under reduced pressure at 4 ^{o}C. Tissue was then preincubated with an isotonic solution consisting of (in mM) 150 NaCl, 5 Na_2EDTA, 0.3 bacitracin and 0.2% BSA (bovine serum albumin) for 15 minutes, followed by one hour of incubation with (2 μCi or 100 pM) of ^{125}I-labelled [Sar^1Ile^8]Ang II (2000 Ci/mmol, Amersham) dissolved in the same isotonic solution used in the preincubation step. The preincubation step served to remove debris from the sectioned tissue prior to binding, and bacitracin was

5'-GTTAACATTGTTGTGGTCTCACTGTTTTGTTGTCAAAAGGGCCCTAAAAGG-3'

Figure 4.7: Partial DNA sequencing of recombinant pCR-Script SK(+) plasmid showing 100% homology to known sequence of AT_2 cDNA. The dideoxy termination method was used to sequence the recombinant plasmid by using a rat specific 5' primer from the rat AT_2 cDNA sequence. Terminated strands for different dideoxyribonucleotides (G=dideoxyguanosine triphosphate, A=dideoxyadenosine triphosphate, T=dideoxythymidine triphosphate, C=dideoxycytodine triphosphate) were resolved in individual lanes on a 8% urea polyacrylamide gel. Nucleotide sequence shown below gel refers to the sequence read between the two arrows, and which is 100% homologous to the AT_2 cDNA.

used prevent degradation of the labelled angiotensin II from occurring in the incubation step. The use of ^{125}I-labelled [Sar^{1}Ile^{8}]Ang II was preferred over ^{125}I-labelled Ang II because of the slightly higher affinity of binding of the modified form (Mendelsohn et al. 1993). No significant binding was observed on sections of tissue co-incubated in the presence of 10 μM of non-radioactive (cold) Ang II, thus demonstrating the specificity of binding in these studies.

After incubation, tissue slices were washed for one minute each in four consecutive ice-cold 50 mM Tris buffer solutions [pH=7.4], dried under warm air and then exposed to ^{3}H-Hyperfilm for 1-2 days. The cold rapid washes helped reduce any non-specific binding of ^{125}I-labelled [Sar^{1}Ile^{8}]Ang II. ^{3}H-Hyperfilm was developed with D19 (Kodak) and fixed by Kodak rapid fixer for 3.5 minutes each, respectively. Figure 4.8 shows a typical histoautoradiograph of angiotensin II binding observed in a sham rat. Binding predominates in glomeruli, proximal convoluted tubules and medullary vasa recta.

4.10 Histology

Freshly frozen tissue was sectioned into 20 μm thickness and stored in -80 ^{o}C until tissue processing. To view tissue histology, freshly frozen sections were brought to room temperature, dried and then fixed with 10% formalin for 10 minutes. Fixed tissue was removed, washed in distilled water and stained with hematoxylin for 4 minutes. Tissue was then washed with luke warm water and treated with the following solutions in order: 25% EtOH 30 sec, eosin 30 sec, 50% EtOH 30 sec, 75% EtOH 30 sec, 90% EtOH 30 sec, 2X 95% EtOH 1 min and finally 2X toluene for 5 min each. The stained tissue was then mounted with a coverslip and viewed under light microscopy for examination.

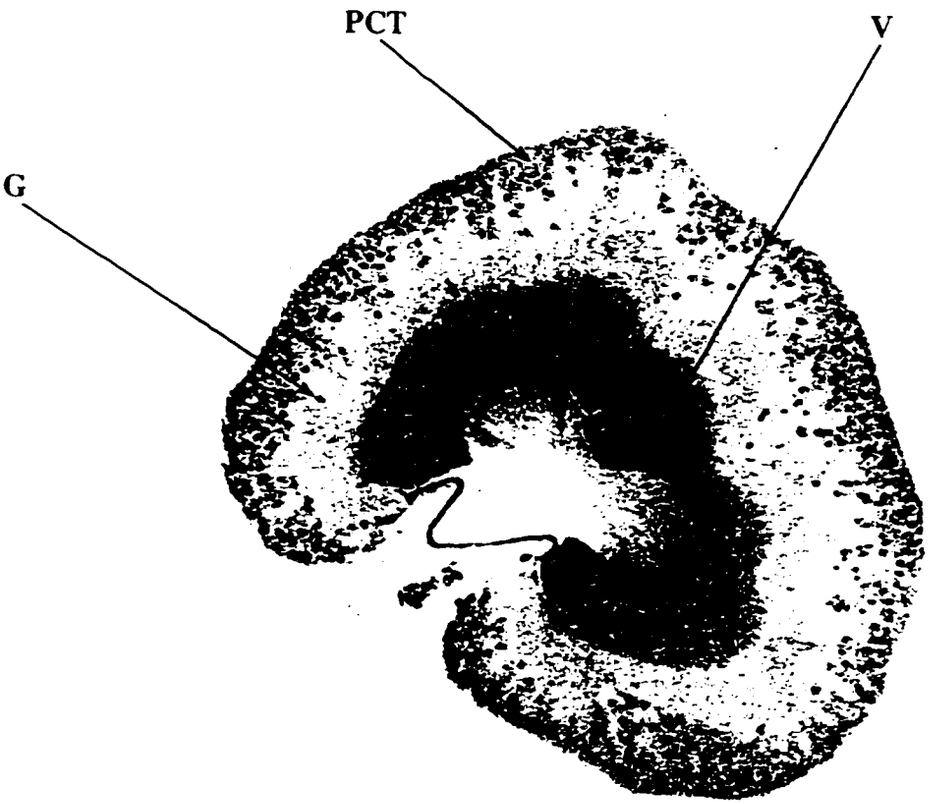


Figure 4.8: Representative histoautoradiograph of a sham renal section showing detailed binding of ^{125}I-angiotensin II. Histoautoradiographs were prepared from renal sham sections (20 μm) incubated in an isotonic solution containing ^{125}I-angiotensin II for one hour. After extensive washing and exposure to ^{3}H-Hyperfilm, detailed binding for angiotensin II was observed. G=glomeruli, V=vasa recta bundles, PCT=region of proximal convoluted tubules

Toluidine blue is a vital dye known to stain cells undergoing apoptosis (Kim et al. 1996). To stain for toluidine blue, freshly frozen sections (20 μm) were fixed with 10% formalin for 10 minutes and subsequently washed in distilled water. Fixed sections were stained in toluidine blue for 5 minutes, and then rinsed in absolute EtOH and xylene for 3 minutes each. Sections were mounted and examined under light microscopy.

4.11 Angiotensin II Peptide Quantification

Angiotensin II was quantitated using a radioimmunoassay method (Amersham). Ischemic or sham rat kidneys were flushed free of blood via an infusion of phosphate buffered saline [pH=7.4] into the heart, which lasted no more than 50 seconds. The kidneys were removed and snap frozen in liquid nitrogen until further processing.

Each snap frozen kidney was weighed prior to experimental manipulation. To isolate angiotensin II, the kidneys were removed from liquid nitrogen and placed in boiling 1 M acetic acid. The tissue was minced with sterile blades and allowed to boil for 20 minutes. This crucial initial step, as cited elsewhere (Ruzicka et al. 1996), ensured that all degradatory enzymes (ie. aminopeptidases) were inactivated and did not interfere with angiotensin II isolation and degradation (Ruzicka et al. 1996). Tissue was then placed on ice, homogenized for 1 minute and centrifuged at 10000 X g at 4 ^{o}C for 10 minutes. The aqueous phase was removed and prepared for cartridge extraction.

C_{18} Sep-Pak cartridges (Waters, Milford, MA) were used to remove peptide angiotensin II from cellular debris on the basis of affinity chromatography. Ideal conditions were prepared by pre-conditioning the cartridge with 10 ml of distilled water, 10 ml pure methanol (BDH, Toronto, ON)

and another 10 ml of distilled water, at a rate of 5 ml/min. Efficiency of cartridge extraction was determined by % recovery of ^{125}I-Ang II after extraction, and found to be 80-90%. The sample supernatant was applied to the cartridge at a rate of 1 ml/min. The sample-loaded cartridge was rinsed with 5 ml of 0.1% acetic acid in distilled water, and then eluted in 6 ml of 4% acetic acid in ethanol at a rate of 1 ml/min. Eluants were dried in a Speed Vac concentrator (Savant Inc., SVC100D) and prepared for further purification by high performance liquid chromatography (HPLC), because separation of angiotensin II-related peptides by cartridge extraction was not possible based on the similiar chemical properties for these peptides. Separation of angiotensin II from other related peptide fragments by HPLC eliminated the possibility of cross-reactivity of the angiotensin II antiserum during radioimmunoassay. All work related to the HPLC extraction of angiotensin II was kindly performed by Roselyn White of Dr. Leenen's laboratory at the Ottawa Civic Hospital Heart Institute, using a protocol previously described (Ruzicka et al. 1996).

Angiotensin II was assayed by radioimmunoassay after HPLC extraction. Antiserum and ^{125}I-angiotensin II were obtained from Amersham (Mississauga, ON). Each assay tube consisted of 100 μl of sample, 100 μl diluted ^{125}I-angiotensin II (1:25), and 50 μl of antiserum (1:625) dissolved in assay buffer [50 mM Tris, 0.3% BSA pH=7.5]. All assay tubes were incubated at 4 ^{o}C for 24 hours. After incubation, non-bound sample and labelled angiotensin II were removed by activated charcoal solution [2.5 g of activated charcoal (Sigma) dissolved in 250 ml of assay buffer], and precipitated by centrifugation at 12000 X g for 10 min at 4 ^{o}C. The supernatant was retrieved and counted in a gamma scintillation counter. Calculation of % bound was based on the equation 100 X [counts(sample)-counts(blank)/counts (maximum binding)]. Samples assayed for angiotensin II were diluted to within the 50 fg range, well above the 0.5-1 fg

limit of sensitivity for the antibody. Figure 4.9 shows a representative standard curve for the angiotensin II radioimmunoassay. By the principle of mass action, the higher amount of unlabelled angiotensin II competes more readily for the binding of antiserum than does the lower amount of sample angiotensin II, leaving behind less bound labelled angiotensin II after charcoal precipitation. As a result, counts decrease with increasing amounts of angiotensin II and the slope becomes negative. This same principle can then be applied for determining the amount of angiotensin II from sham or ischemic kidneys.

4.12 Densitometry Analysis

Images of Northern blots and histoautoradiographs were captured and analyzed using the Image 1.47 software package. Total intensities for sham and ischemic Northern blot signals were quantitated by determing mean pixel density and area, and expressed in relative terms as band intensity [ischemia/sham].

For the analysis of histoautoradiographs, the renal cortical and medullary areas were selected by outlining the contour of each region. The mean density of angiotensin II binding, as estimated by mean pixel density, was determined for each region of the kidney, and values were then expressed in relative terms as mean density of angiotensin II binding [ischemia/sham]. Minimal variability in mean pixel density was observed for different regions within cortex or outer medulla of single sections of kidney.

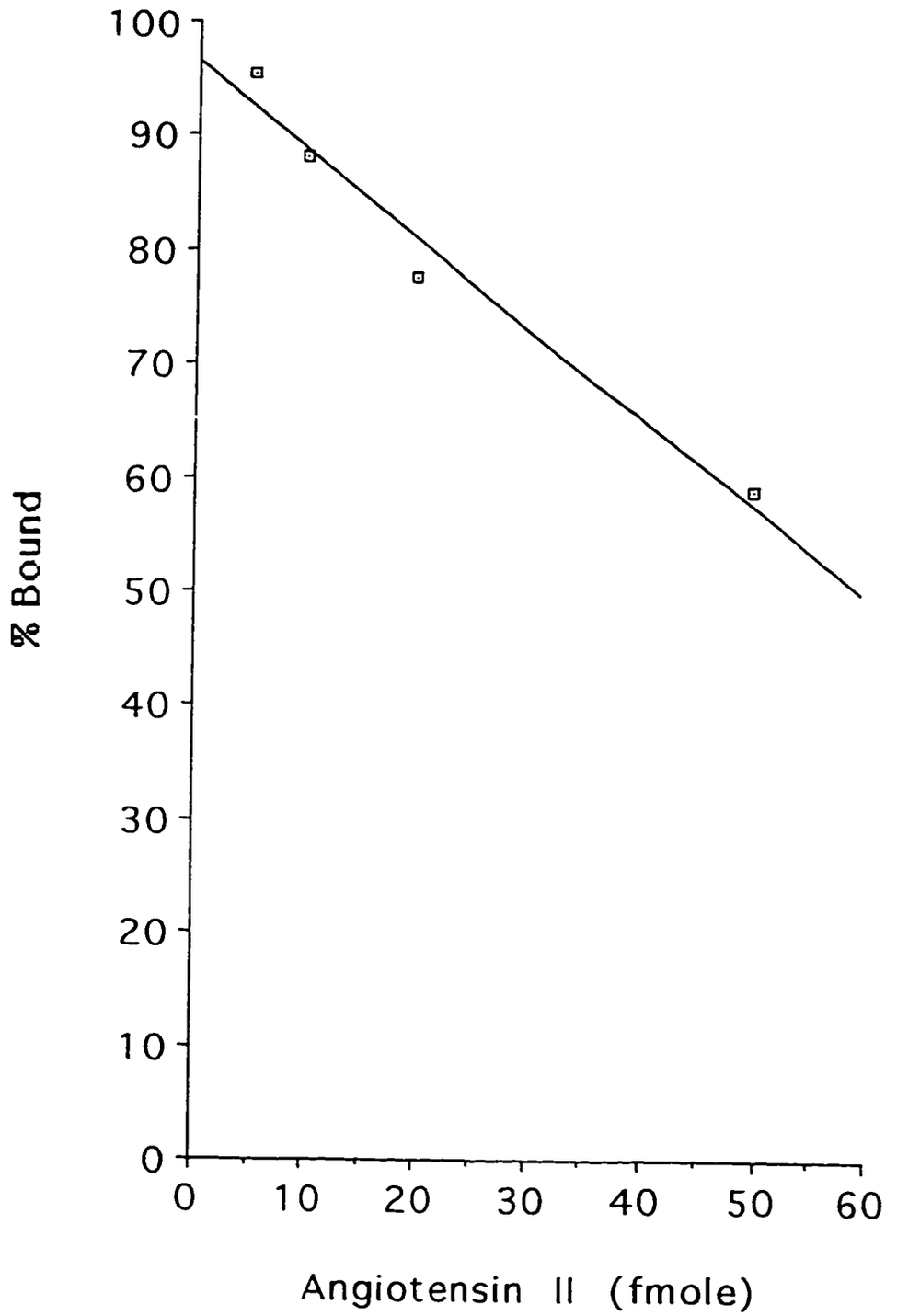


Figure 4.9: Representative standard curve for the assay of angiotensin II by radioimmunoassay. Different amounts of unlabelled angiotensin II (fmole) were incubated in a fixed amount of ^{125}I-angiotensin II and angiotensin II antiserum for 4 hours. Maximal binding was determined by incubation with no unlabelled angiotensin II. After removal of non-bound labelled and sample angiotensin II by activated charcoal, the bound labelled angiotensin II was measured in a gamma scintillation counter. % Bound was defined as (cpm standard/cpm maximal binding) X 100%.

4.13 Statistics

Values are expressed as mean $\pm$ SEM. The analysis of Northern blots and histoautoradiographs was performed by z-score statistics, which uses a standardized normal distribution. Serum [creatinine], [blood urea nitrogen], plasma renin activity and angiotensin II levels were compared by a students t-test. A value of $P<0.05$ was accepted as significant.

Results

5.0: Measurement of Creatinine and BUN

To characterize the intrarenal renin-angiotensin system in post-ischemic renal injury, kidneys were obtained from sham-operated rats and ischemic rats 0 hours, 3 hours, 6 hours, 24 hours, 72 hours and 120 hours after ischemia/reperfusion. The extent of functional renal injury in the rats was evaluated by measurement of serum [creatinine] (figure 5.0). Significant increases in serum [creatinine] levels were observed for rats at 3, 6, 24 and 72 hours after reperfusion, compared to the respective sham rats [3 hours after ischemia/reperfusion: 116.7 $\pm$ 5.8 μM vs. sham: 48.3 $\pm$ 2.2 μM; $P<0.0005$ (n=6), 6 hours after ischemia/reperfusion: 123 $\pm$ 15.4 μM vs. sham: 47.3 $\pm$ 3.0 μM; $P<0.005$ (n=5), 24 hours after ischemia/reperfusion: 298.0 $\pm$ 29.0 μM vs. sham: 46.8 $\pm$ 1.2 μM; $P<0.0005$ (n=6) and 72 hours after ischemia/reperfusion: 401.7 $\pm$ 104.7 μM vs. sham: 44.8 $\pm$ 1.6 μM; $P<0.0005$ (n=10)]. By 120 hours post-ischemia, the mean serum [creatinine] levels returned to baseline values, and did not differ significantly from levels in the sham rats.

Similarly, the extent of functional renal injury was evaluated by measurement of [blood urea nitrogen] (figure 5.1). Significant increases in [blood urea nitrogen] levels were observed for rats at 3, 6, 24 and 72 hours after reperfusion, compared to the respective sham rats [3 hours after ischemia/reperfusion: 8.3 $\pm$ 0.4 mM vs. sham: 4.3 $\pm$ 0.3 mM; $P<0.005$ (n=5), 6 hour after ischemia/reperfusion: 14.2 $\pm$ 1.9 vs. sham: 4.7 $\pm$ 0.5 mM; $P<0.01$ (n=4) , 24 hours after ischemia/reperfusion: 42.9 $\pm$ 6.1 mM vs. sham: 5.1 $\pm$ 0.4 mM; $P<0.005$ (n=5) and 72 hours after ischemia/reperfusion: 67.50 $\pm$ 16.5 mM vs. sham: 4.7 $\pm$ 0.2 mM; $P<0.005$ (n=10)]. By 120 hours post-ischemia, the mean [blood urea nitrogen] mM levels returned to baseline values, and did not differ significantly from levels in sham rats.

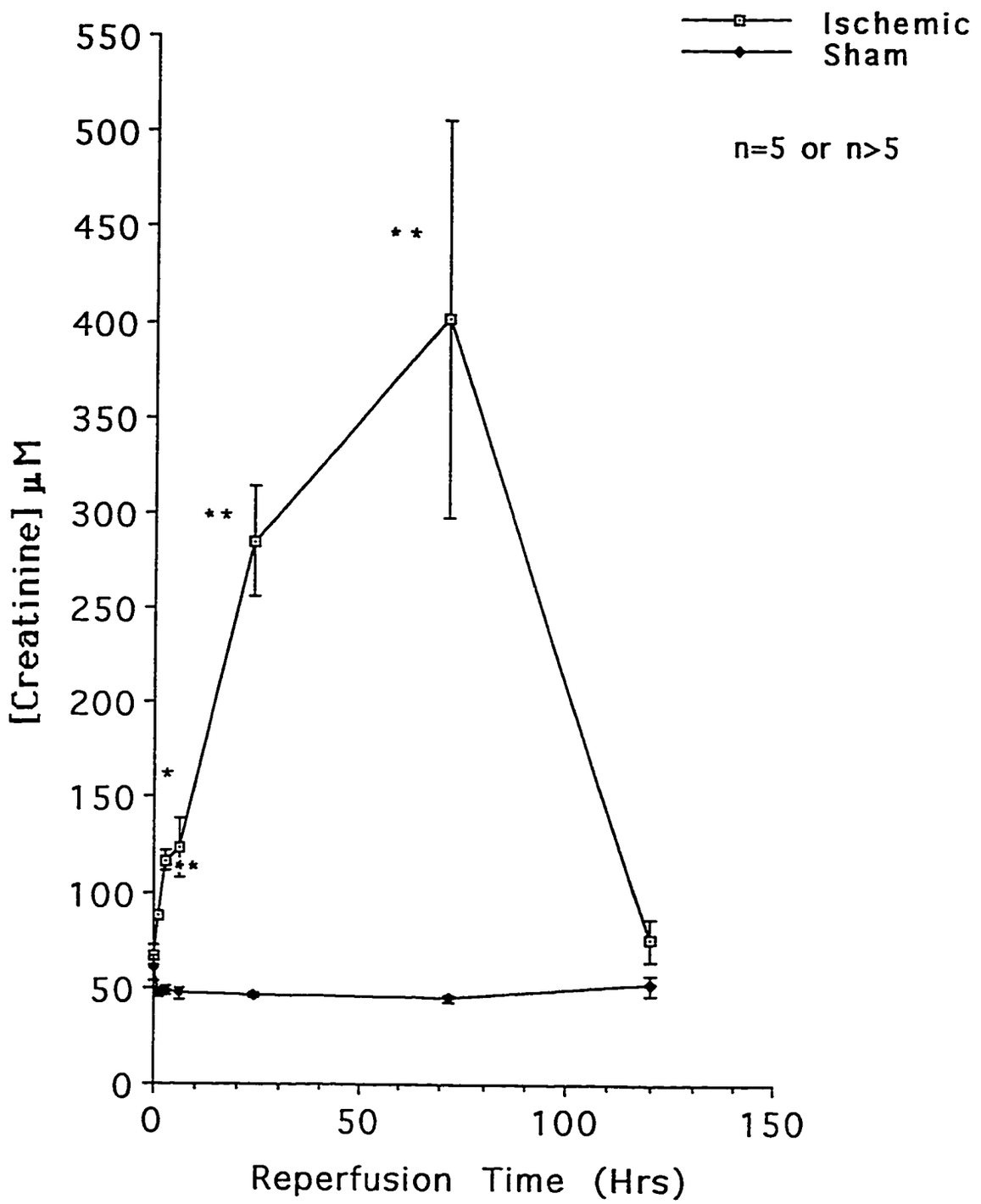


Figure 5.0: The effect of ischemia/reperfusion on plasma creatinine levels. Plasma creatinine levels were significantly higher in the 3 hour, 6 hour, 24 hour and 72 hour post-ischemic rats, compared to the sham rats. Data are expressed as mean ± SEM, and n refers to number of experiments. *P<0.005, **P<0.0005 vs. sham levels.

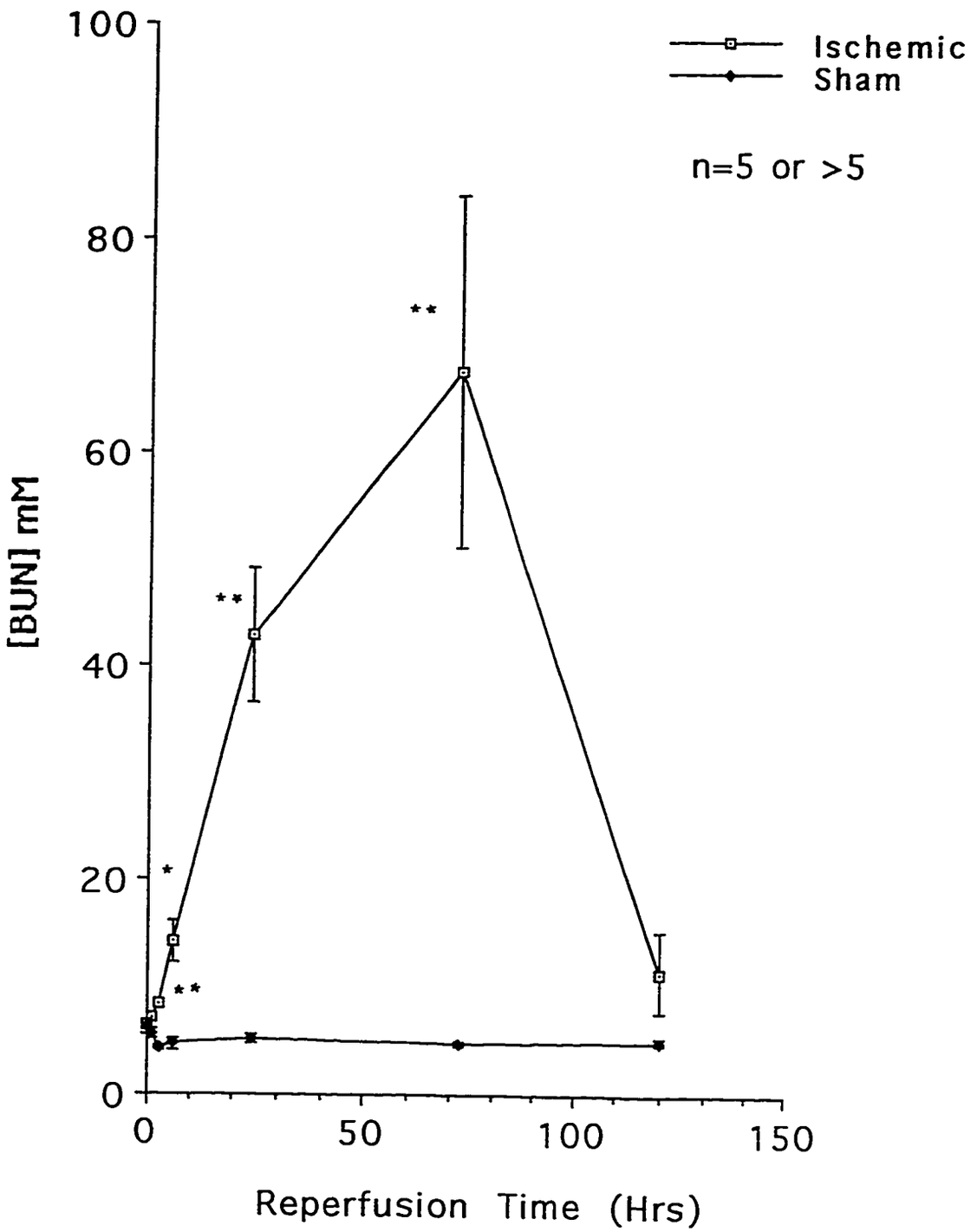


Figure 5.1: The effect of ischemia/reperfusion on blood urea nitrogen levels. Blood urea nitrogen levels were significantly higher in the 3 hour, 6 hour, 24 hour and 72 hour post-ischemic rats, compared to the sham rats. Data are expressed as mean ± SEM, and n refers to number of experiments. *P<0.01, **P<0.005 vs. sham levels.

5.1 Histological changes and plasma renin activity

To define further the extent of histological injury, sections of kidneys (20 μm) from sham and ischemic rats were stained with hematoxylin and eosin, or toluidine blue, and evaluated histologically. No differences in histology were noted among kidney sections obtained from sham rats at different times after surgery (figure 5.2). At 24 hours post-ischemia/reperfusion, an abundance of necrotic cells were observed within the cortical tubules (figure 5.3). Most glomeruli had remained intact and showed no signs of necrosis at this time point (figure 5.3). By 120 hours post-ischemia/reperfusion, tubular epithelial cells had regenerated, with the appearance of normal tubular structures (figure 5.4). At this particular time point, many apoptotic-like bodies were observed within the tubular lumens (figure 5.4), and the development of tubular cell papillary fronds was also noted (figure 5.5, figure 5.6), the latter representing overgrowth of tubular cells (Shimizu et al. 1993).

To determine the effects of renal ischemia on the systemic renin-angiotensin system, plasma renin activity (ng/L/s) was measured in ischemic and sham rats by radioimmunoassay at 0 hours, 3 hours, 6 hours and 24 hours post ischemia/reperfusion (table 5.0). No significant changes in renin activity were noted between ischemic and respective sham rats at 0, 1, 3, 6 or 24 hours after ischemia/reperfusion.

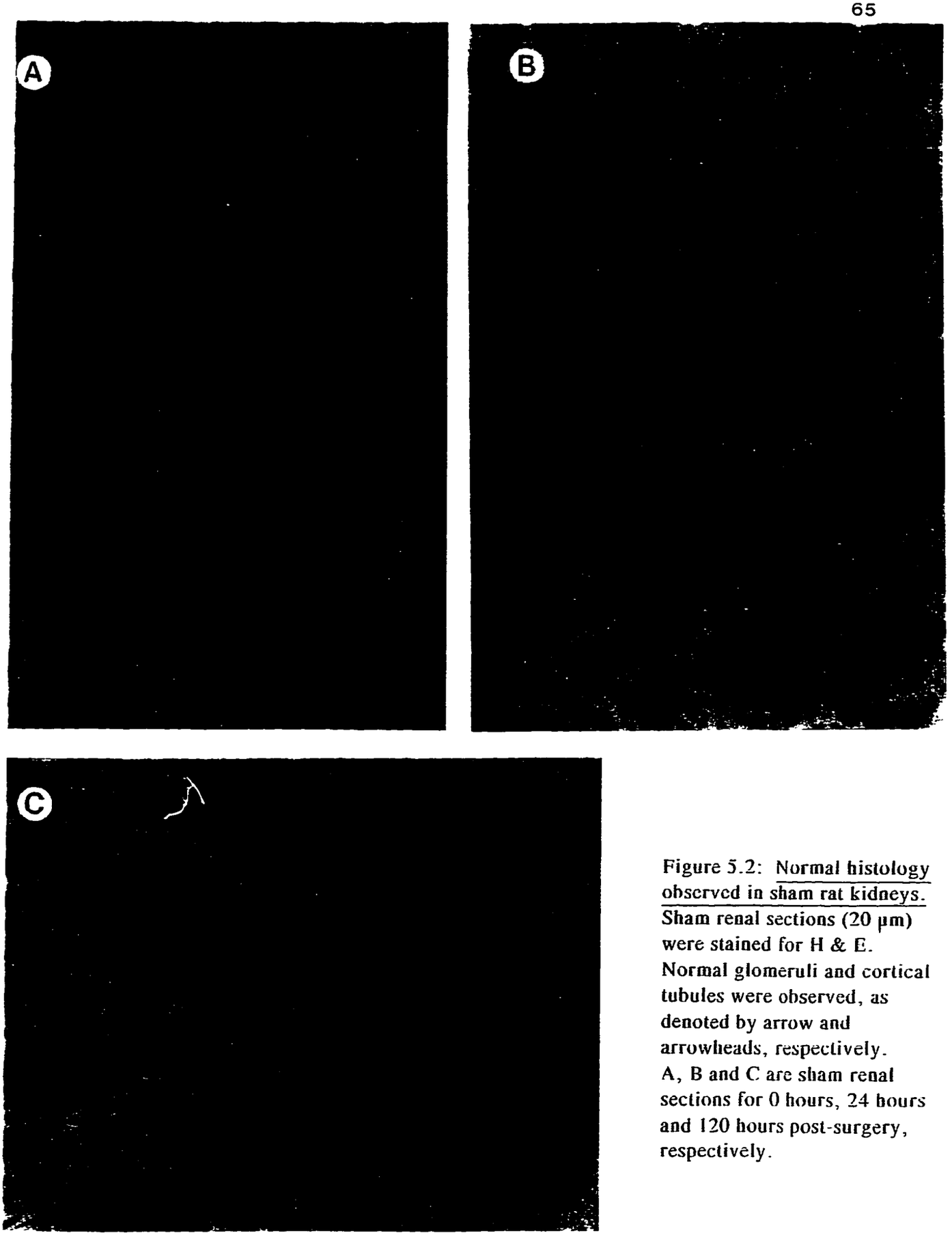


Figure 5.2: Normal histology observed in sham rat kidneys. Sham renal sections (20 μm) were stained for H & E. Normal glomeruli and cortical tubules were observed, as denoted by arrow and arrowheads, respectively. A, B and C are sham renal sections for 0 hours, 24 hours and 120 hours post-surgery, respectively.

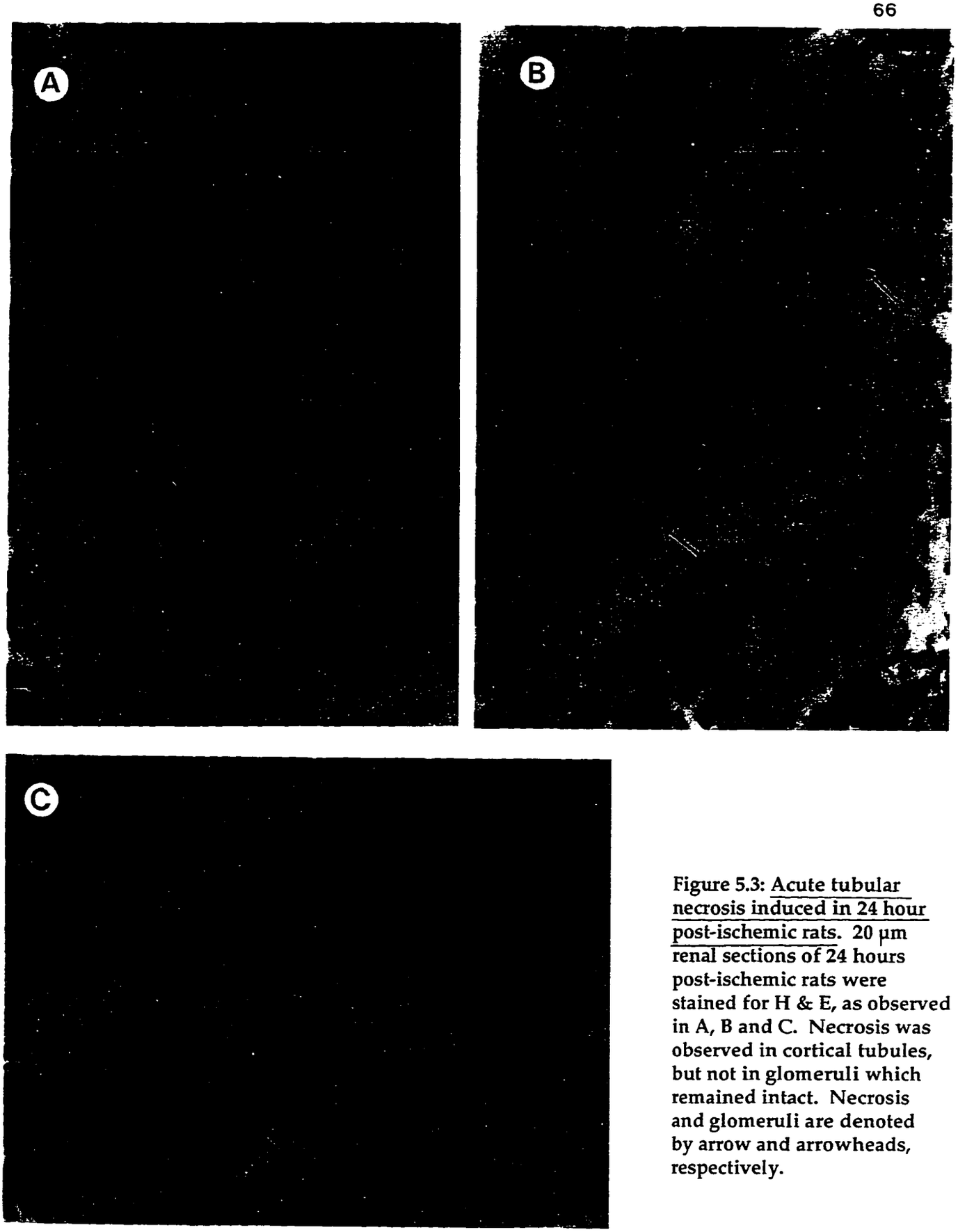


Figure 5.3: Acute tubular necrosis induced in 24 hour post-ischemic rats. 20 μm renal sections of 24 hours post-ischemic rats were stained for H & E, as observed in A, B and C. Necrosis was observed in cortical tubules, but not in glomeruli which remained intact. Necrosis and glomeruli are denoted by arrow and arrowheads, respectively.

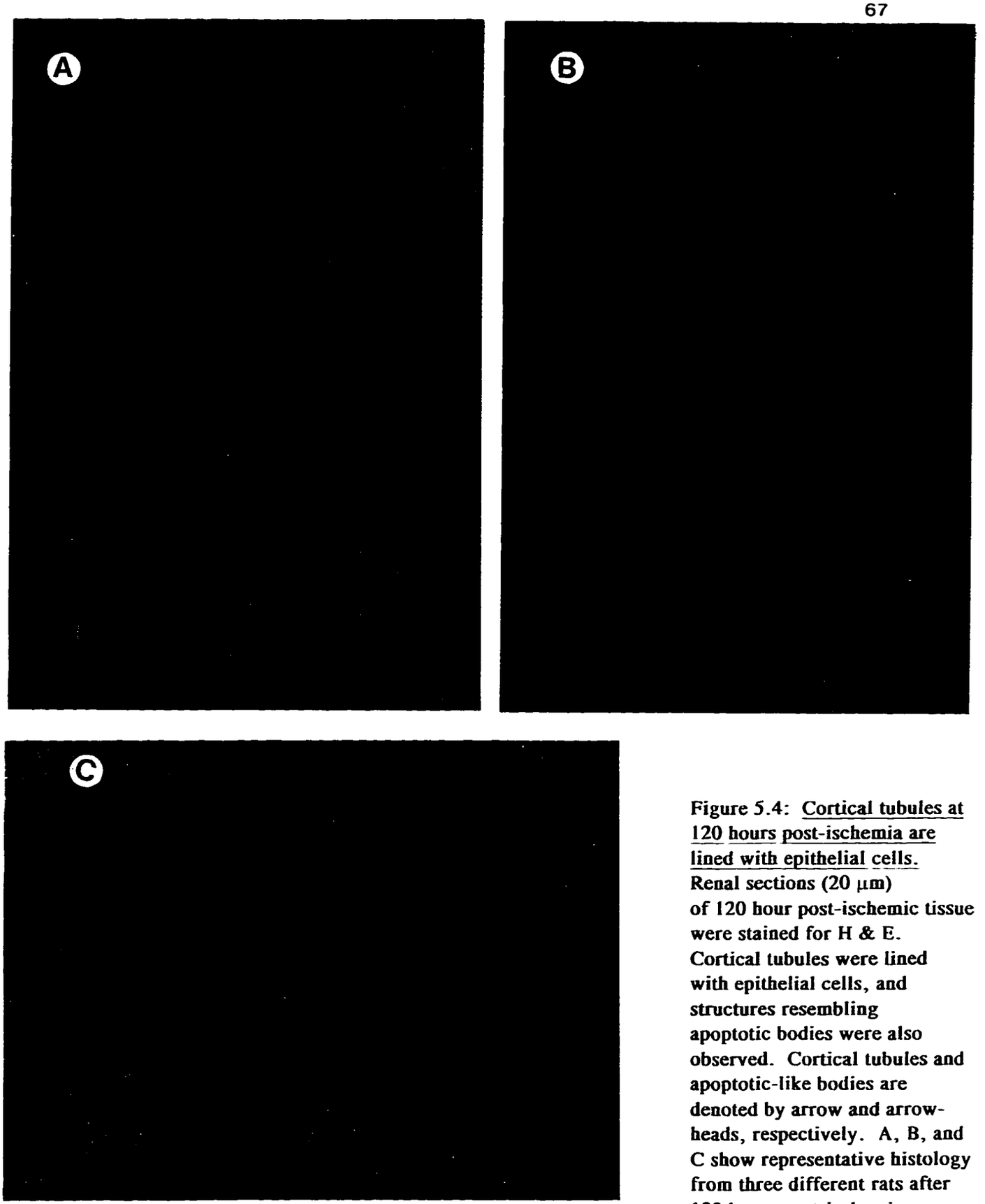


Figure 5.4: Cortical tubules at 120 hours post-ischemia are lined with epithelial cells. Renal sections (20 μm) of 120 hour post-ischemic tissue were stained for H & E. Cortical tubules were lined with epithelial cells, and structures resembling apoptotic bodies were also observed. Cortical tubules and apoptotic-like bodies are denoted by arrow and arrow-heads, respectively. A, B, and C show representative histology from three different rats after 120 hours post-ischemia.

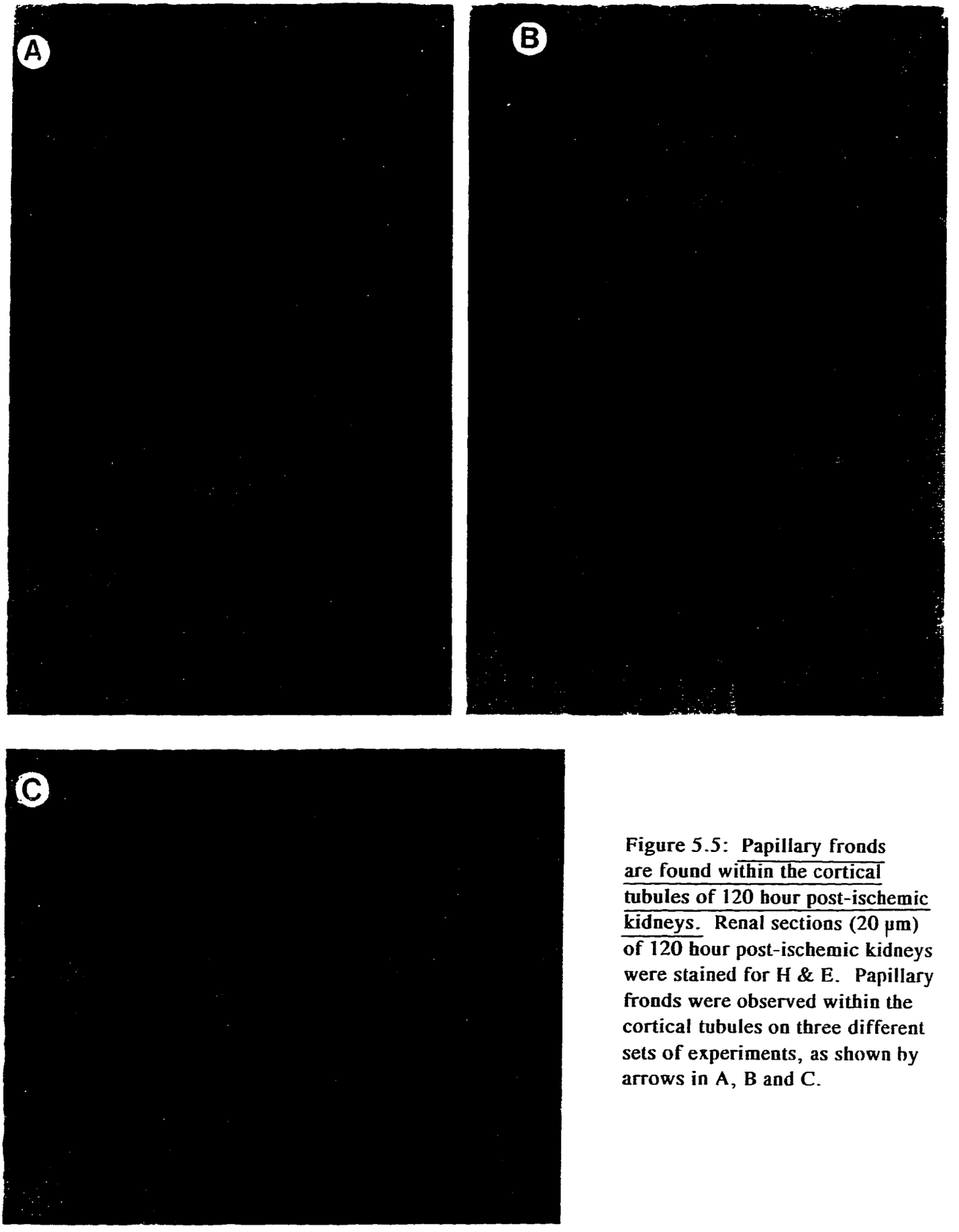


Figure 5.5: Papillary fronds are found within the cortical tubules of 120 hour post-ischemic kidneys. Renal sections (20 μm) of 120 hour post-ischemic kidneys were stained for H & E. Papillary fronds were observed within the cortical tubules on three different sets of experiments, as shown by arrows in A, B and C.

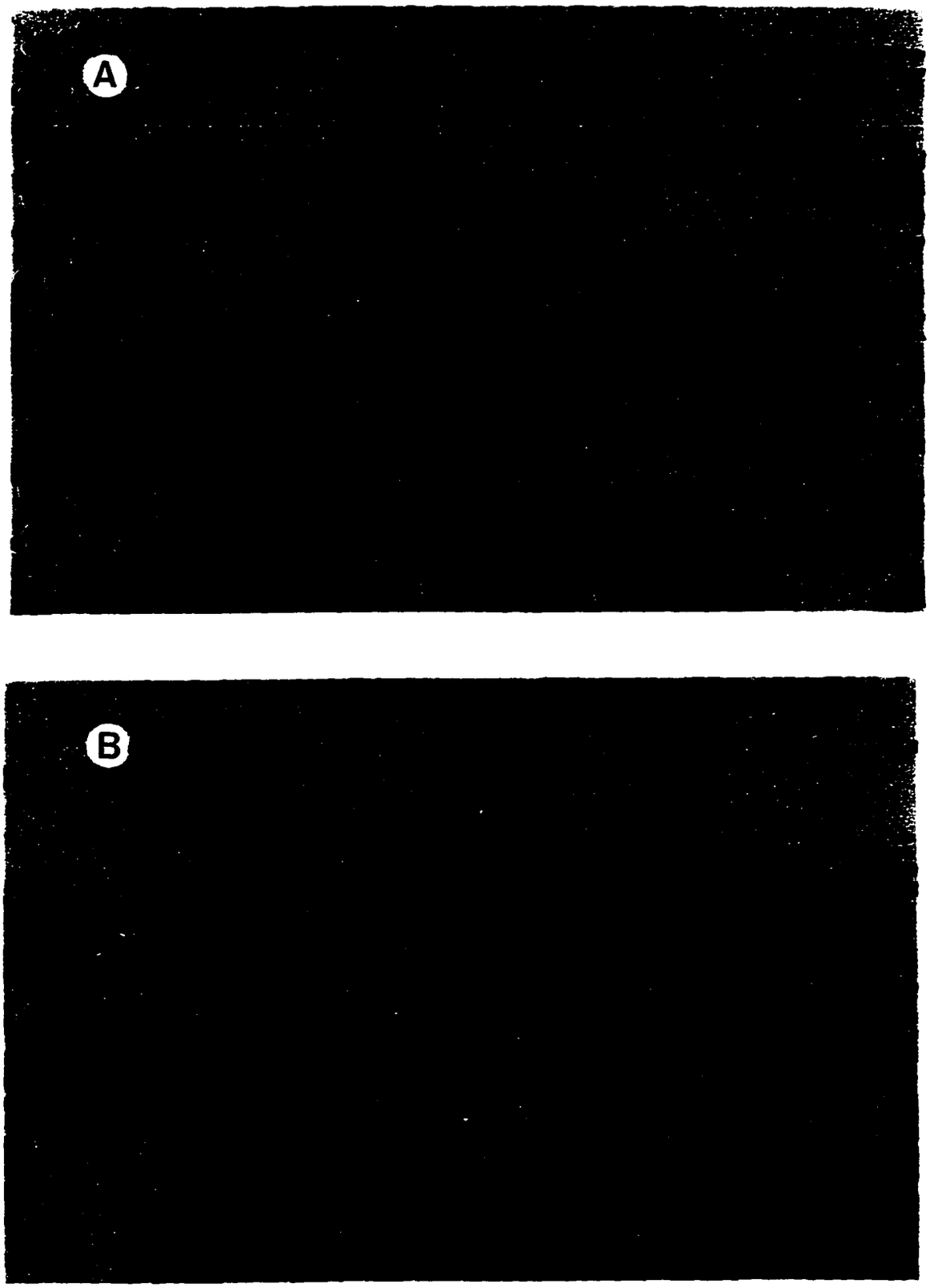


Figure 5.6: Papillary fronds and apoptotic-like structures are found within the lumens of 120 hour post-ischemic rats. Renal sections (20 μm) of kidneys after 120 hours post-ischemia were stained for toluidine blue. Papillary fronds and structures resembling apoptotic bodies were observed, as shown in A and B. Papillary fronds and structures resembling apoptotic bodies are denoted by arrow and arrowheads, respectively.

Time (Hrs)	Sham	Ischemia/Reperfusion
0	0.72 ± 0.13	0.52 ± 0.14
1	0.96 ± 0.10	0.74 ± 0.10
3	0.94 ± 0.12	0.69 ± 0.31
6	0.74 ± 0.05	0.68 ± 0.10
24	0.70 ± 0.05	0.75 ± 0.08

Table 5.0: The effect of ischemia/reperfusion on plasma renin activity. Plasma renin activity was quantitated by radioimmunoassay. The values of plasma renin activity did not differ significantly between the ischemic and sham rats at any reperfusion time up 24 hours post-ischemia/reperfusion (n=5-8).

5.2 Gene Expression of Intrarenal Renin-Angiotensin System

5.2.1: Intrarenal Angiotensinogen mRNA

Expression of the angiotensinogen gene after ischemia/reperfusion was determined by Northern blot analysis on sham and ischemic kidney cortical RNA (figure 5.7). Decreased gene expression for angiotensinogen was observed in ischemic kidneys with acute renal failure, relative to sham kidneys 6, 24 and 72 hours after reperfusion [angiotensinogen mRNA band intensity [ischemia/sham]: 6 hours: 0.42 ± 0.14; $P<0.02$ (n=4), 24 hours: 0.013 ± 0.008; $P<0.0001$ (n=5), 72 hours: 0.020 ± 0.01; $P<0.0001$ (n=4)]. Recovery of angiotensinogen gene expression in ischemic kidneys, relative to the respective sham rats, was observed at 120 hours post-ischemia/reperfusion [angiotensinogen mRNA band intensity [ischemia/sham]: 120 hours: 0.82 ± 0.15; P=NS (n=5)]. Representative Northern blots for angiotensinogen mRNA originating from ischemic and respective sham kidney cortices are shown in figure 5.8. Ethidium bromide-stained formaldehyde agarose gels of RNA are also shown in figure 5.8, demonstrating equal RNA loading between ischemic and sham groups.

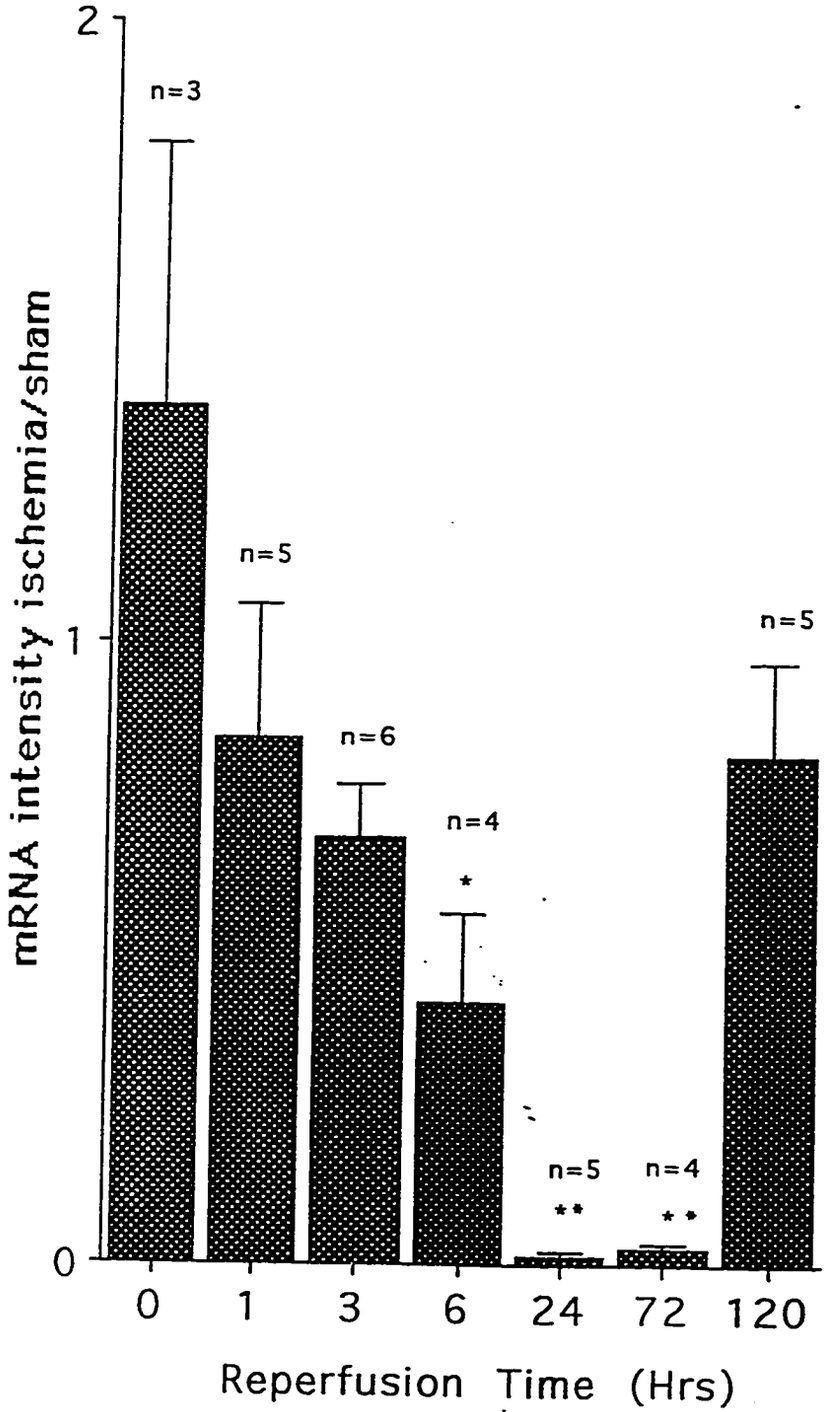


Figure 5.7: Effect of ischemia/reperfusion on angiotensinogen gene expression in renal cortex. Angiotensinogen gene expression was quantified by Northern blot analysis. Levels of angiotensinogen mRNA originating from ischemic kidneys were compared to levels originating from sham kidneys for each reperfusion time (relative signal). Data are expressed as mean $\pm$ SEM, and n refers to number of experiments. *P<0.05, **P<0.0001 vs. sham.

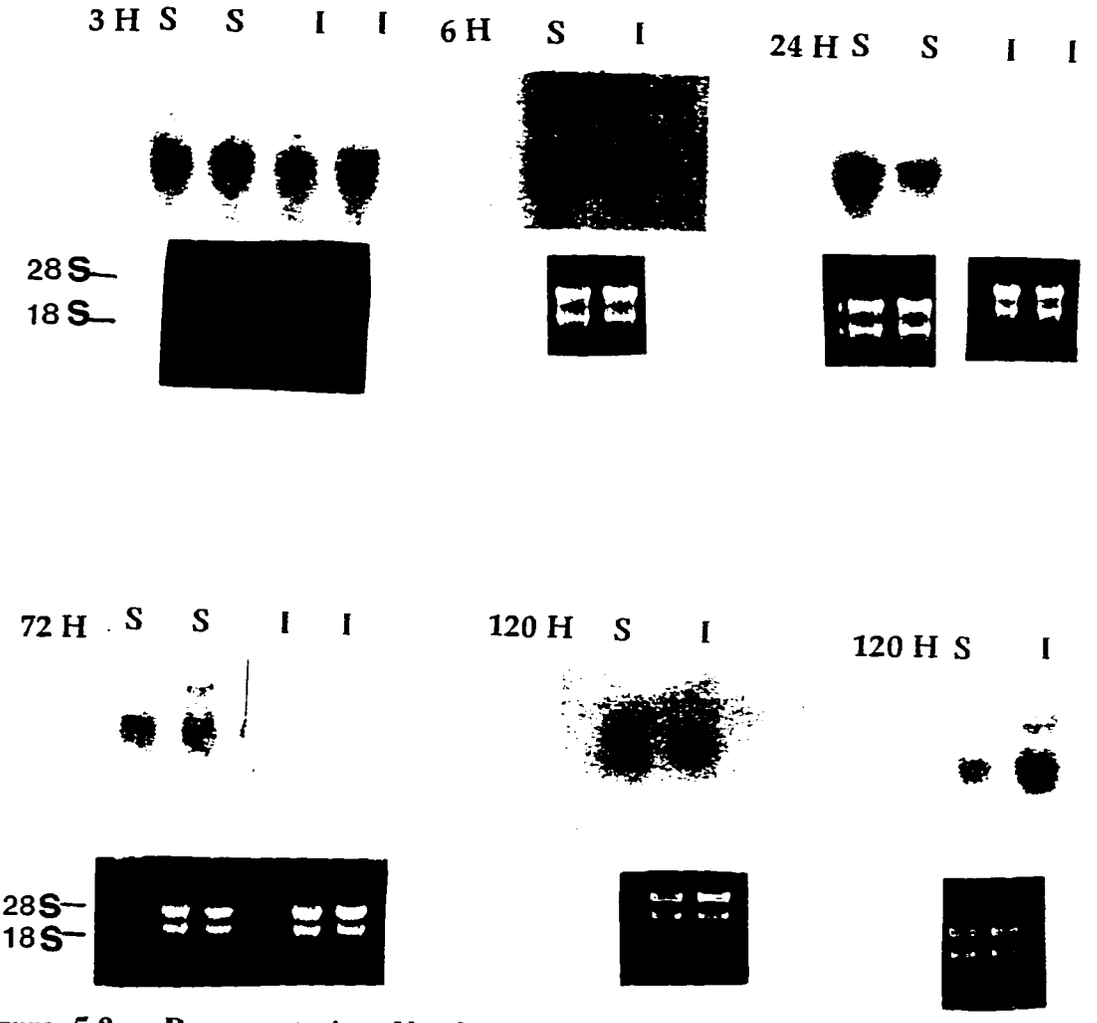


Figure 5.8: Representative Northern blot signals for angiotensinogen mRNA. Northern blot signals of ischemic and respective sham renal cortices for angiotensinogen mRNA, are shown for 6, 24, 72 and 120 hours post-ischemia/reperfusion. Ethidium bromide-stained RNA for the 28S and 18S ribosomal RNA bands are also shown for ischemic and sham samples, demonstrating equal band intensities; S=sham, I=ischemia/reperfusion.

5.2.2 Cortical and Proximal Tubular AT_1 Receptor mRNA

Northern blot analysis was used to determine the effect of renal ischemia/reperfusion on the gene expression of the cortical AT_1 receptor. Analysis showed that the intensity of cortical AT_1 receptor mRNA of ischemic animals, relative to sham, did not differ significantly at any reperfusion time (figure 5.9). Representative Northern blots for kidney cortical AT_1 receptor mRNA originating from ischemic and respective sham kidney cortices are shown in figure 5.10. Ethidium bromide-stained formaldehyde agarose gels of RNA are also shown in figure 5.10, demonstrating equal RNA loading between ischemic and sham groups. To determine the specific levels of AT_{1a} receptor gene expression in ischemic and respective sham kidney cortex at 120 hours post-ischemia/reperfusion, total RNA was assayed for the AT_{1a} receptor mRNA by RT-PCR. As shown in figure 5.11, band intensities for AT_{1a} did not differ between the ischemic and respective sham kidney cortex at this time point.

Northern blot analysis was used to determine the effect of renal ischemia/reperfusion on the proximal tubular AT_1 receptor gene expression, as shown in (figure 5.12). Decreased gene expression for the AT_1 receptor was observed in proximal tubular segments of kidneys with ischemic acute renal failure, relative to sham kidneys, after 0, 1, 3 and 24 hours of ischemia/reperfusion [proximal tubular AT_1 receptor mRNA band intensity [ischemia/sham]: 0 hour 0.45 ± 0.09; $P<0.0002$ (n=3), 1 hour: 0.60 ± 0.06; $P<0.0002$ (n=3), 3 hours: 0.40 ± 0.07; $P<0.0002$ (n=3), 24 hours: 0.40 ± 0.17, $P<0.02$ (n=4)]. Gene expression for the AT_1 receptor did not differ significantly in ischemic proximal tubular segments at 72 hours and 120 hours post-ischemia/reperfusion, relative to respective sham rats. Representative Northern blots of ischemic and respective sham proximal tubular segments for the AT_1 receptor are shown in figure 5.13. Ethidium bromide-stained formaldehyde

agarose gels of RNA are also shown in figure 5.12, demonstrating equal RNA loading between ischemic and sham groups.

Northern blot analysis for the proximal tubular AT_1 receptor could not discriminate between both receptor subtypes. To determine the levels of AT_{1a} and AT_{1b} receptor gene expression in ischemic and respective sham proximal tubular segments at 120 hours post-ischemia/reperfusion, total RNA was assayed for each receptor subtype by RT-PCR. As shown in figure 5.14, band intensities for AT1a (figure 5.14A) and AT1b (figure 5.14B) receptor mRNA did not differ between the ischemic and sham proximal tubular segments at this time point. This indicated that the gene expression of both AT_1 receptor subtypes had recovered at 120 hours post-ischemia/reperfusion.

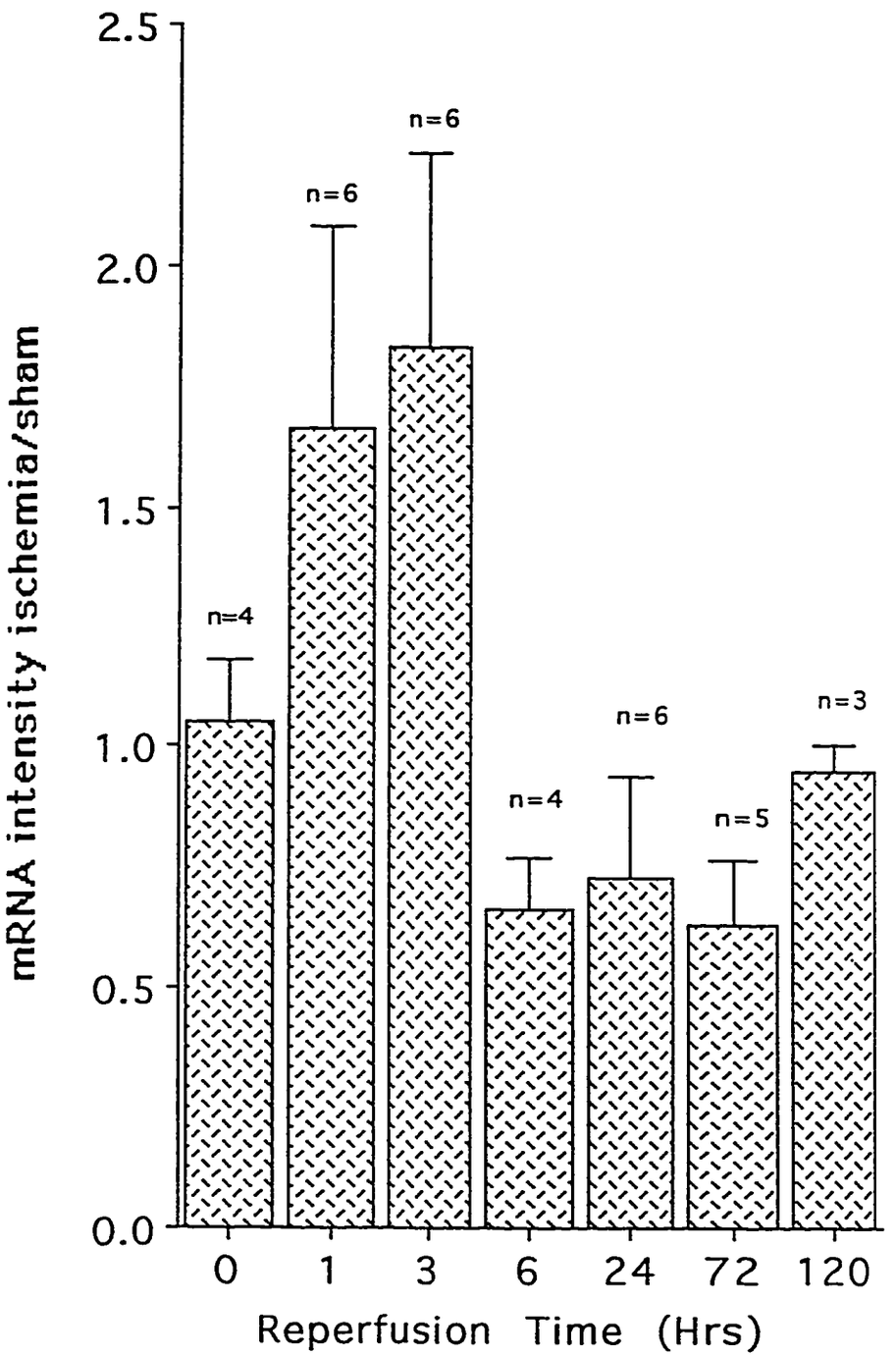


Figure 5.9: Effect of ischemia/reperfusion on AT_1 receptor gene expression in the renal cortex. Cortical AT_1 receptor gene expression was quantified by Northern blot analysis. Levels of cortical AT_1 receptor mRNA originating from ischemic kidneys were compared to levels originating from sham kidneys for each reperfusion time (relative signal). Data are expressed as mean $\pm$ SEM, and n refers to number of experiments.

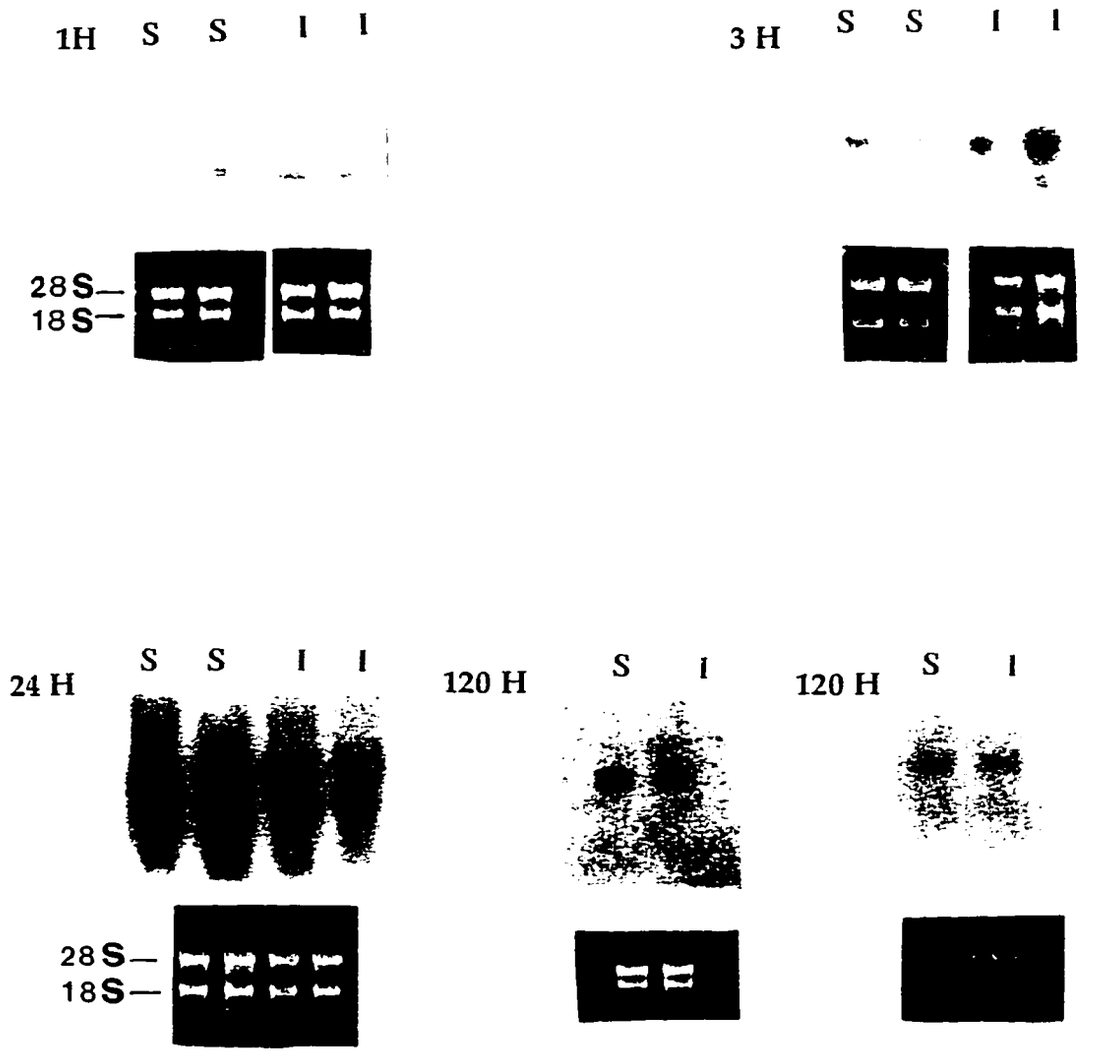


Figure 5.10: Representative Northern blot signals for cortical AT_1 receptor mRNA. Northern blot signals of ischemic and respective sham renal cortices for AT_1 receptor mRNA, are shown for 3, 24 and 120 hours post-ischemia/reperfusion. Ethidium bromide-stained RNA for the 28S and 18S ribosomal RNA bands are also shown for ischemic and sham samples, demonstrating equal band intensities; S=sham, I=ischemia/reperfusion.

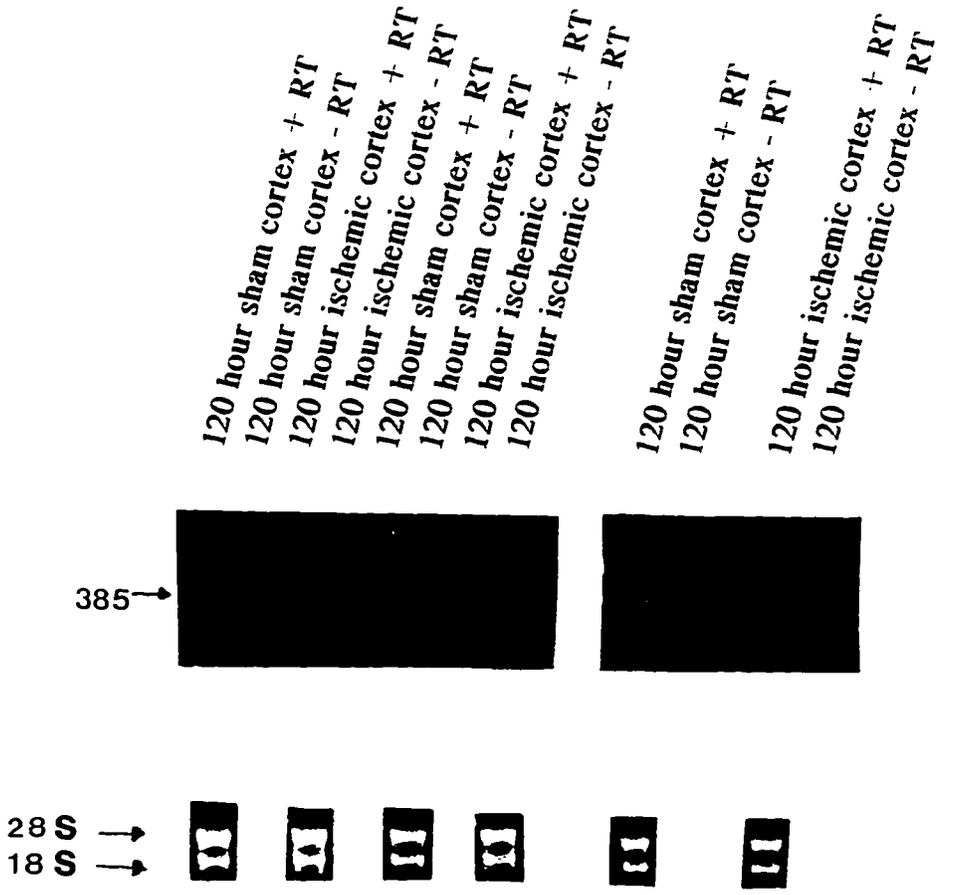


Figure 5.11: Effect of ischemia/reperfusion on the cortical AT_{1a} receptor mRNA at 120 hours post-ischemia/reperfusion. Cortical AT_{1a} receptor mRNA was determined by RT-PCR. Band intensities of the 385 bp RT-PCR product for the cortical AT_{1a} receptor mRNA did not differ between the ischemic and sham groups. Ethidium bromide-stained RNA samples for the 28S and 18S ribosomal RNA bands is also provided, demonstrating equal band intensities; + RT=presence of reverse transcriptase, - RT=absence of reverse transcriptase.

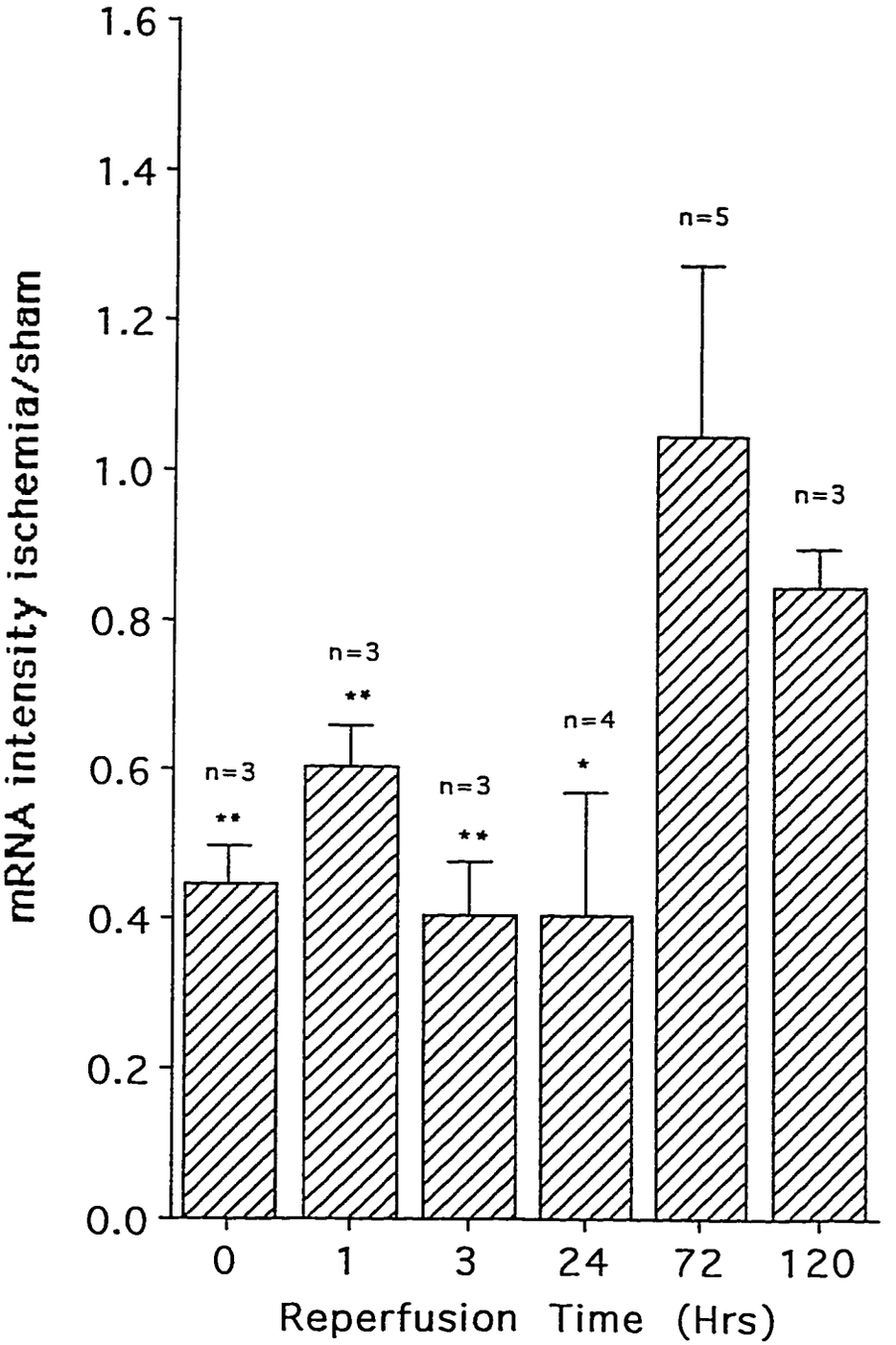


Figure 5.12: Effect of ischemia/reperfusion on proximal tubular AT_1 receptor gene expression. Proximal tubular AT_1 receptor gene expression was quantified by Northern blot analysis. Levels of proximal tubular AT_1 receptor mRNA originating from ischemic kidneys were compared to levels originating from sham kidneys for each reperfusion time (relative signal). Data are expressed as mean $\pm$ SEM, and n refers to number of experiments. *P<0.05, **P<0.0002 vs. sham.

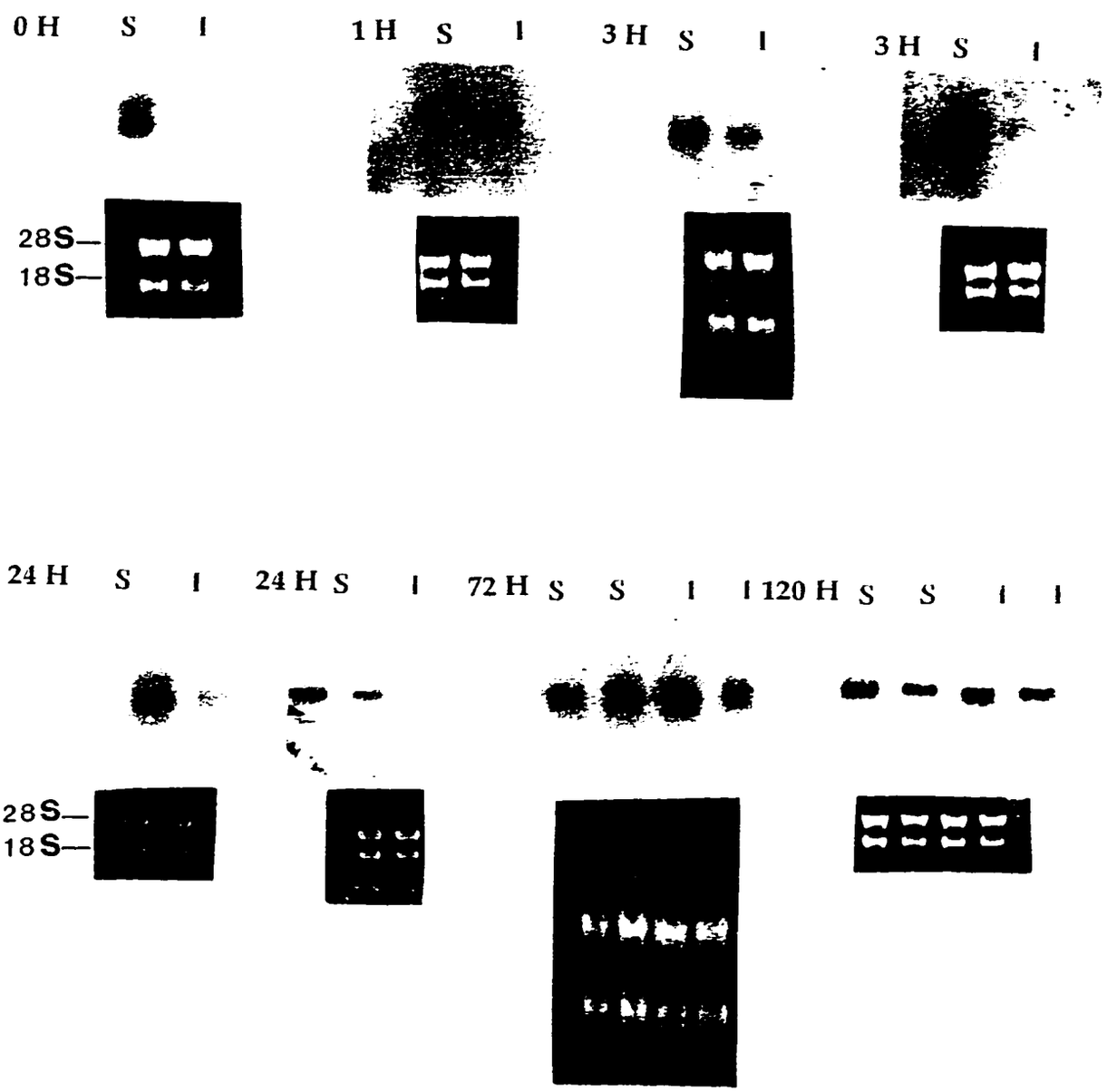


Figure 5.13: Representative Northern blot signals for proximal tubular AT_1 receptor mRNA. Northern blot signals of ischemic and respective sham proximal tubular AT_1 receptor mRNA, are shown for 0, 1, 3, 24, 72 and 120 hours post-ischemia/reperfusion. Also shown are ethidium bromide-stained RNA samples of ischemic and sham proximal tubular segments for the 28S and 18S ribosomal RNA bands, demonstrating equal band intensities; S=sham, I=ischemia/reperfusion.

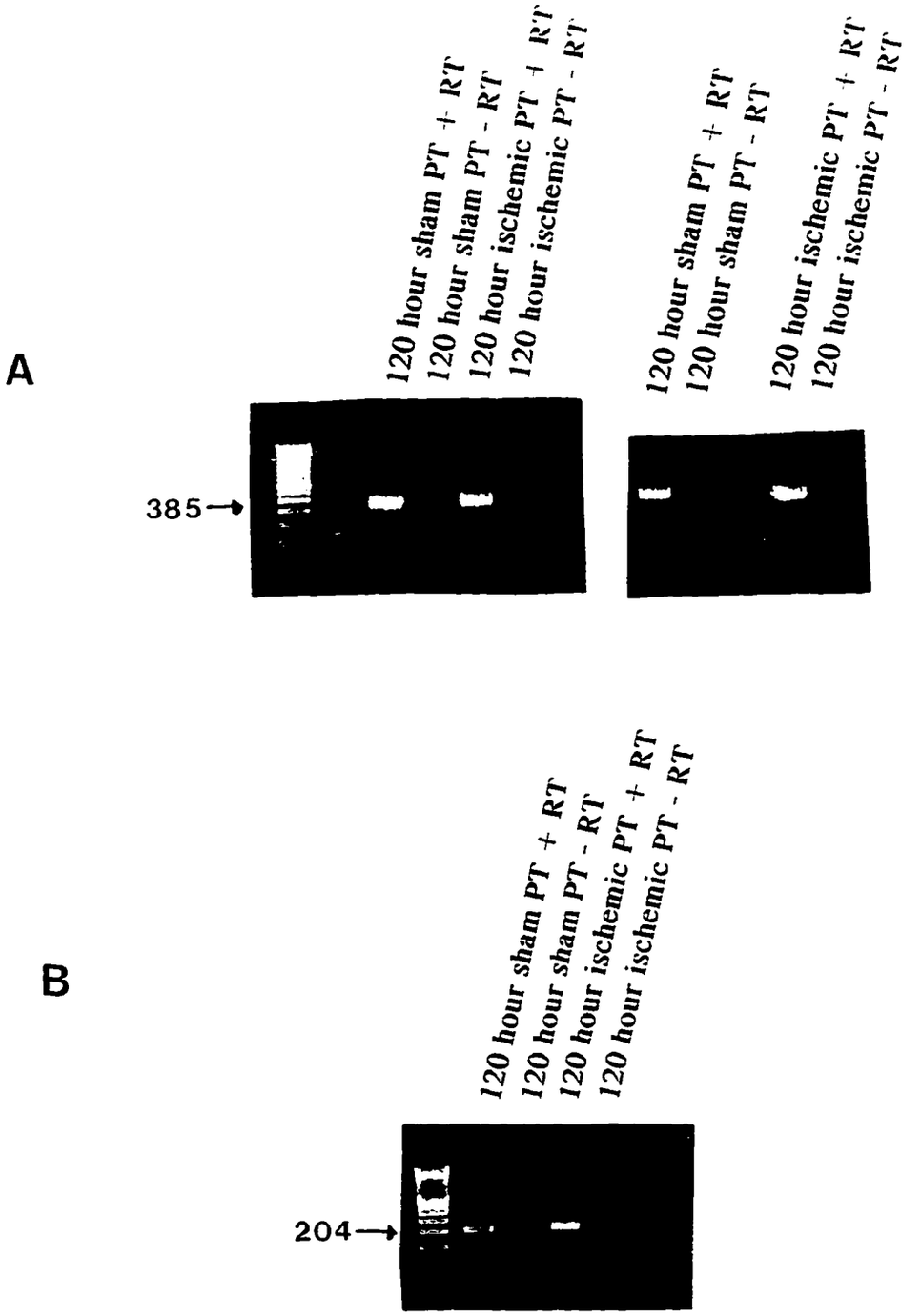


Figure 5.14: Effect of ischemia/reperfusion on proximal tubular AT_{1a} and AT_{1b} receptor mRNA 120 hours after ischemia/reperfusion. Levels of proximal tubular AT_{1a} and AT_{1b} receptor mRNA were determined by RT-PCR. In A), band intensities for the 385 bp RT-PCR product of the AT_{1a} receptor mRNA did not differ between the ischemic and sham groups. In B), band intensities for the 204 bp RT-PCR product of the AT_{1b} receptor mRNA did not differ between the ischemic and sham groups; + RT=presence of reverse transcriptase, - RT=absence of reverse transcriptase.

5.2.3 AT_2 Receptor mRNA

AT_2 receptors have not been demonstrated in adult kidney tubules (Aguilera et al. 1994). To determine if ischemia/reperfusion could induce AT_2 receptor gene expression, RT-PCR was performed for the AT_2 receptor mRNA in ischemic and sham proximal tubular segments at 0, 72 and 120 hours post-ischemia/reperfusion. In proximal tubular segments of ischemic and sham rats, no RT-PCR signal for the AT_2 receptor mRNA was observed at 0 or 72 hours after ischemia/reperfusion (figure 5.15A). Significant band intensities for the AT_2 receptor mRNA were consistently observed in the ischemic proximal tubular segments at 120 hours post-ischemia/reperfusion, but not in the respective sham rats (n=5) (figure 5.15B). This indicated that 120 hours of ischemia/reperfusion had a stimulatory effect on the AT_2 receptor gene expression.

To further characterize the AT_2 receptor mRNA expression, RT-PCR for the AT_2 mRNA was also performed on total RNA isolated from the kidney outer medulla at 0, 24, 72 and 120 hours of ischemia/reperfusion. Signal intensities for the AT_2 receptor mRNA in the ischemic outer medulla did not change relative to the sham outer medulla, either at 0, 24 or 72 hours after ischemia/reperfusion. Consistent enhanced AT_2 receptor gene expression was observed in the ischemic outer medulla taken at 120 hours post-ischemia/reperfusion, relative to the respective sham rats (figure 5.16).

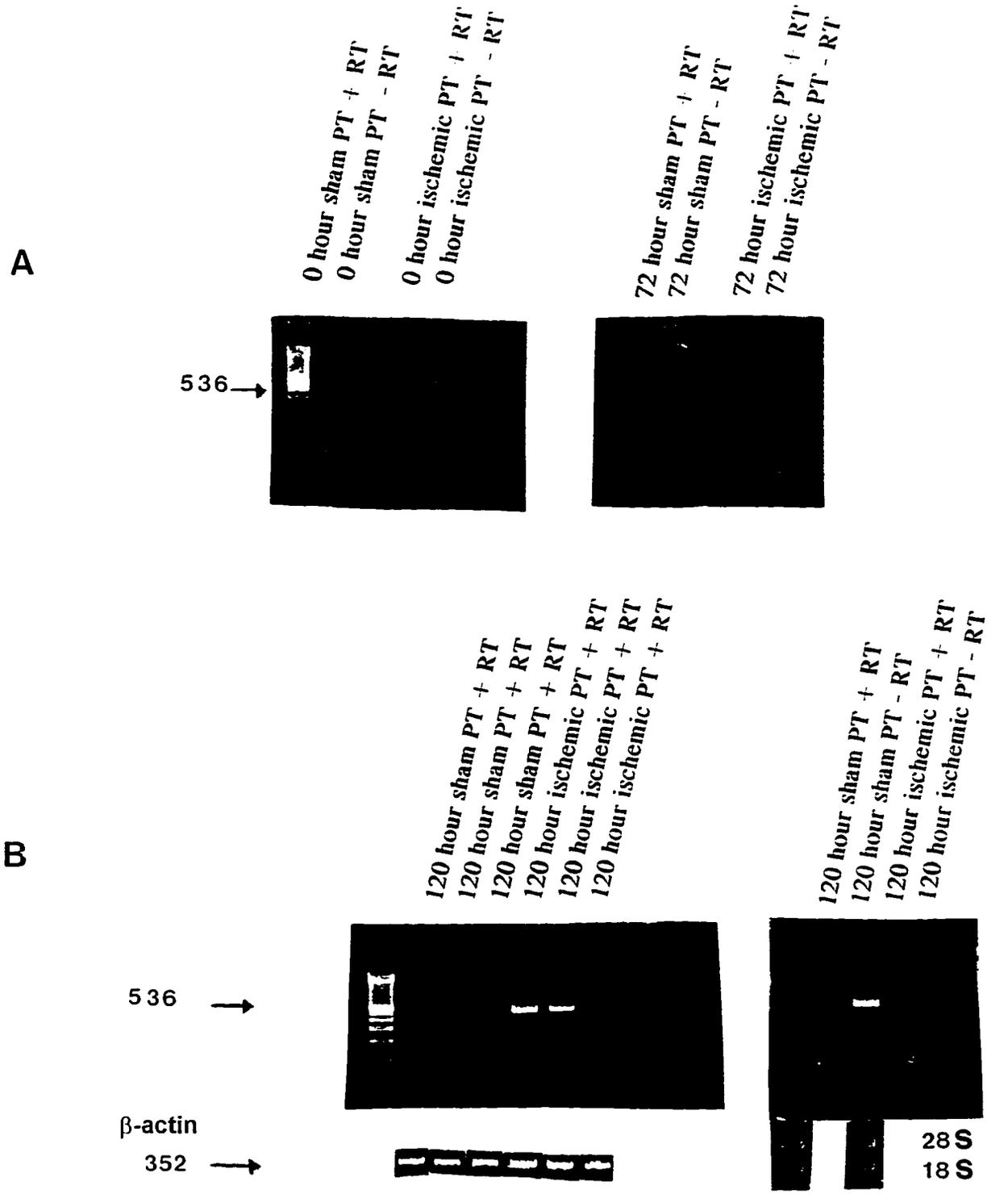


Figure 5.15: AT_2 receptor gene expression is stimulated in ischemic proximal tubular segments at 120 hours post-ischemia/reperfusion. Total RNA from sham and ischemic samples were assayed for AT_2 receptor mRNA. The 536 bp RT-PCR product for the AT_2 receptor mRNA did not appear in sham or ischemic samples at 0 or 72 hours post-ischemia/reperfusion. The 536 bp RT-PCR product for the AT_2 receptor mRNA appeared in ischemic proximal tubular segments 120 hours after ischemia/reperfusion, but not in the sham samples. Equal band intensities of the 352 bp RT-PCR product for β-actin are shown below for the 120 hour post-ischemic and sham samples, demonstrating equal RNA loading. Also shown are ethidium bromide-stained RNA samples of 120 hour post-ischemic and sham samples for 28S and 18S ribosomal RNA bands, showing equal RNA band intensities; + RT= presence of reverse transcriptase, - RT= absence of reverse transcriptase, PT=proximal tubular segments

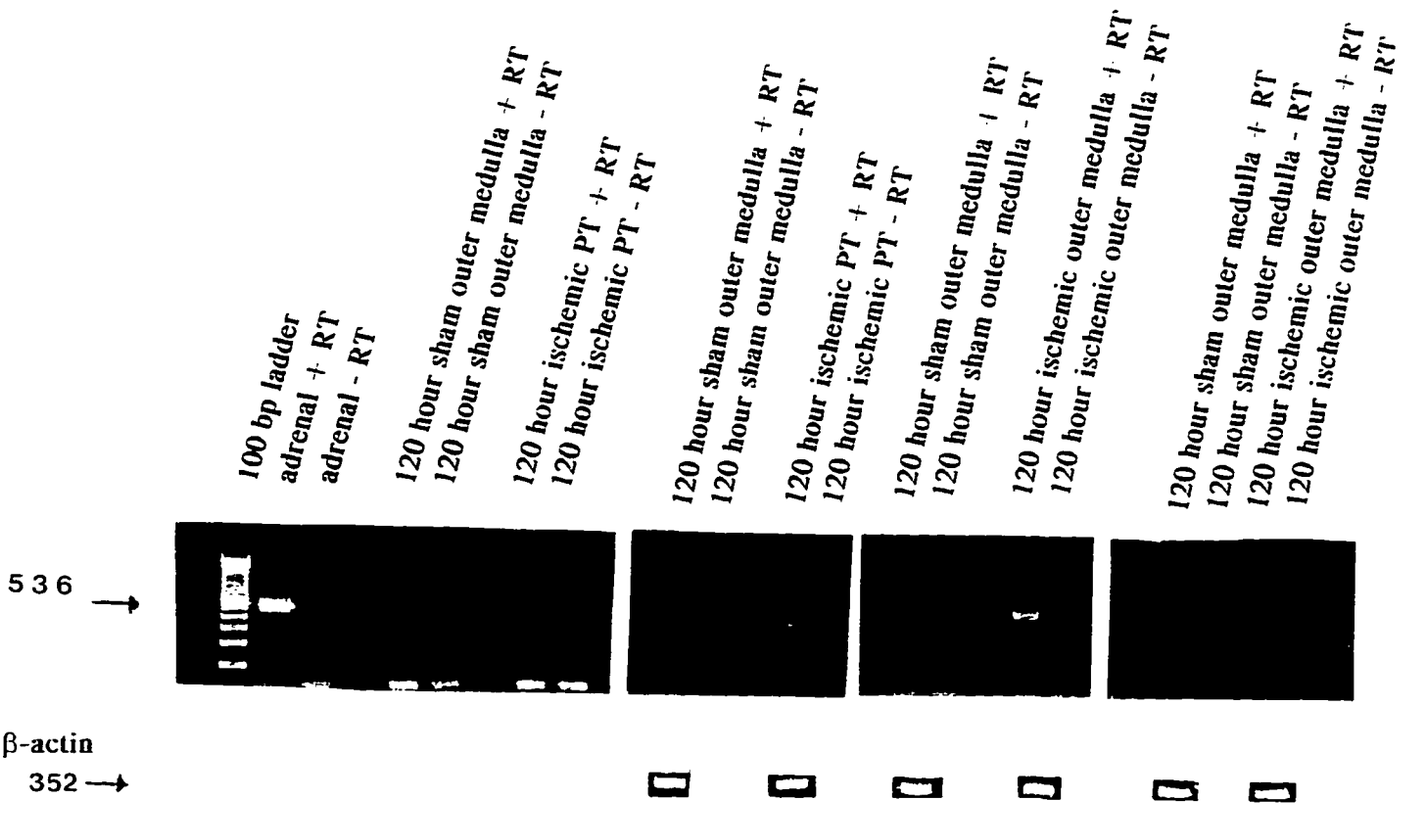


Figure 5.16: AT_2 receptor gene expression is stimulated in ischemic outer medulla 120 hours after ischemia/reperfusion. Band intensities for the 536 bp RT-PCR product for the AT_2 receptor mRNA were enhanced in the ischemic outer medulla after 120 hours post-ischemia/reperfusion, relative to the sham samples. Equal band intensities of the 352 bp RT-PCR product for β-actin are shown below for the 120 hour post-ischemic and sham samples, demonstrating equal RNA loading; + RT=presence of reverse transcriptase, - RT=absence of reverse transcriptase.

5.3 Histoautoradiography of Ang II Binding Sites

The effect of ischemia/reperfusion on the mean density of angiotensin II binding was determined by histoautoradiography. As shown in figure 5.17, the mean density of angiotensin II binding in the ischemic cortex decreased significantly, relative to sham rats, at 24 hours after ischemia/reperfusion [cortical density of angiotensin II binding [ischemia/sham]: 24 hours: 0.48 ± 0.06; $P<0.0001$ ($n=4$)]. The mean density of angiotensin II binding in the cortex did not differ significantly between ischemic and sham kidneys at 0 or 120 hours post-ischemia/reperfusion.

The mean density of angiotensin II binding was also determined in the outer medulla by histoautoradiography, as shown in figure 5.18. Significant decreases in the outer medullary density of angiotensin II binding were observed in ischemic rats at 3 hours and 24 hours post-ischemia/reperfusion, relative to sham rats [medullary density of angiotensin II binding [ischemia/sham]: 3 hours: 0.49 ± 0.10; $P<0.01$ ($n=5$), 24 hours: 0.35 ± 0.07; $P<0.0001$ ($n=4$)]. The mean density of angiotensin II binding in the outer medulla did not differ significantly between ischemic and sham kidneys at 0 or 120 hours post-ischemia/reperfusion.

Representative histoautoradiographs of angiotensin II binding for ischemic and sham kidneys are shown in figures 5.19-5.22, demonstrating the reduced density of angiotensin II binding in the cortex and medulla after ischemia/reperfusion.

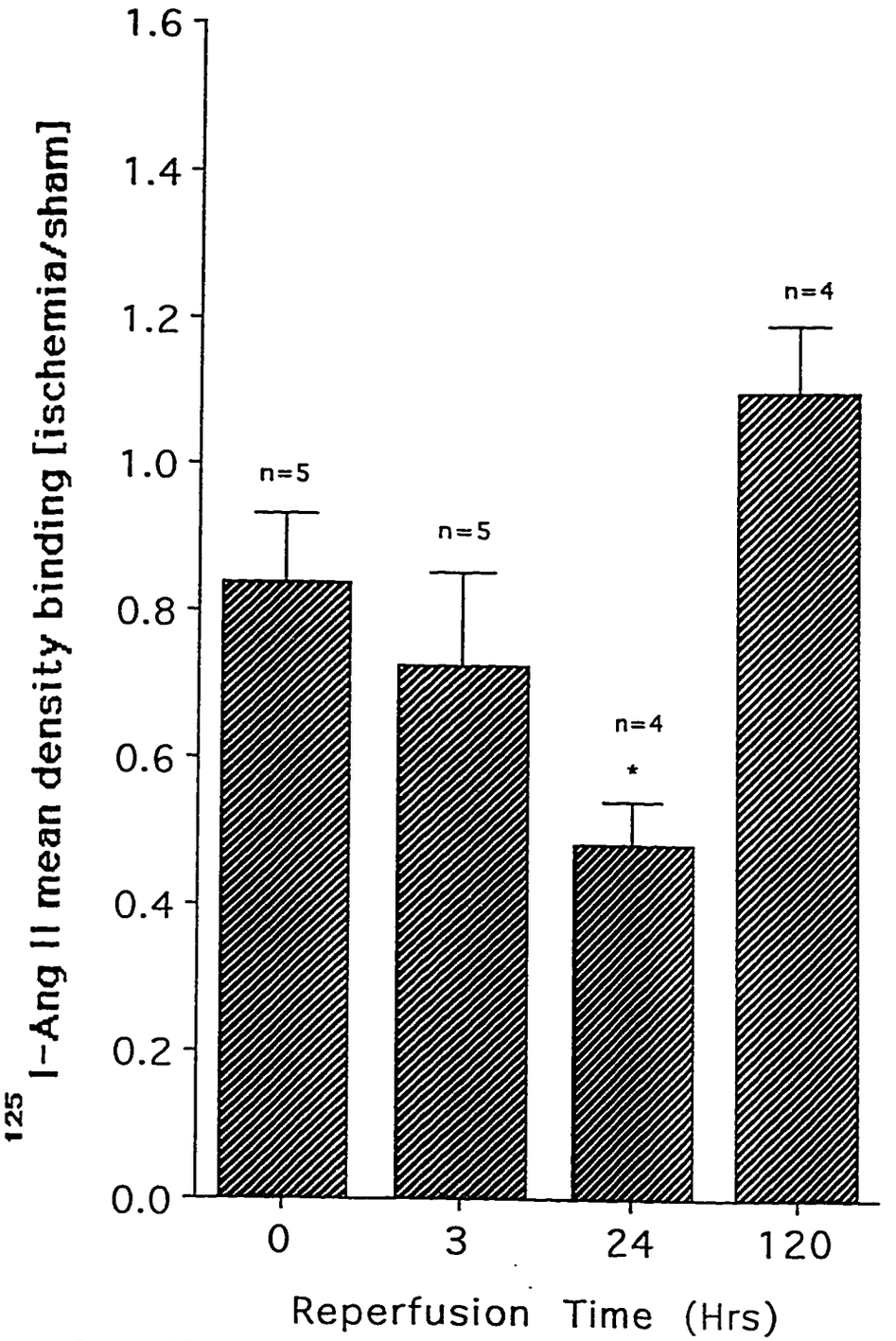


Figure 5.17: Effect of ischemia/reperfusion on the mean density of angiotensin II binding in the cortex. The mean density of angiotensin II binding in kidney cortex was determined by histoautoradiography. Mean density of angiotensin II cortical binding in ischemic kidneys was compared to the mean density of cortical angiotensin II binding originating from sham kidneys for each reperfusion time (relative signal). Data are expressed as mean ± SEM, and n refers to number of experiments. *P<0.0001 vs. sham.

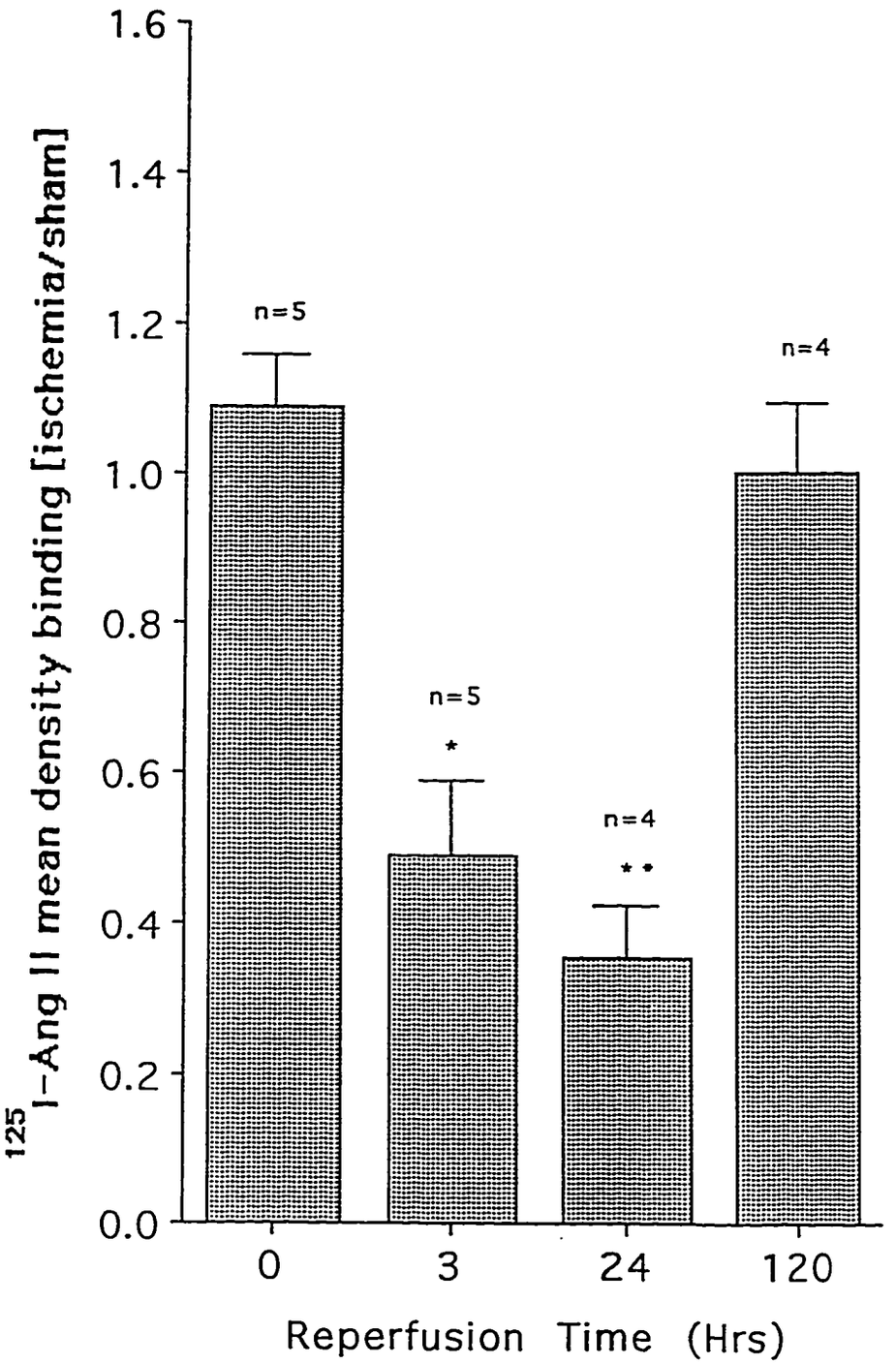
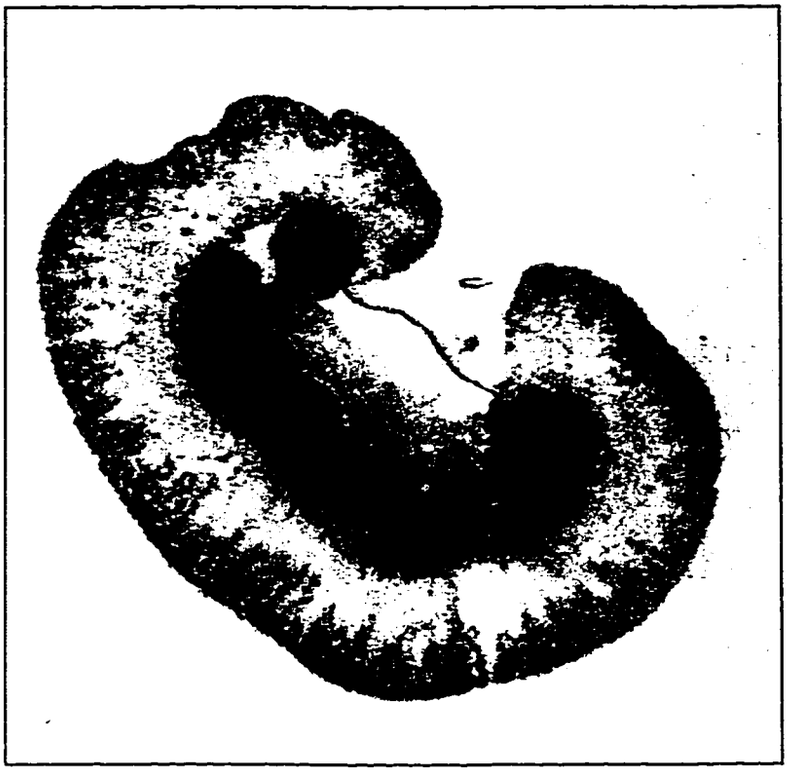
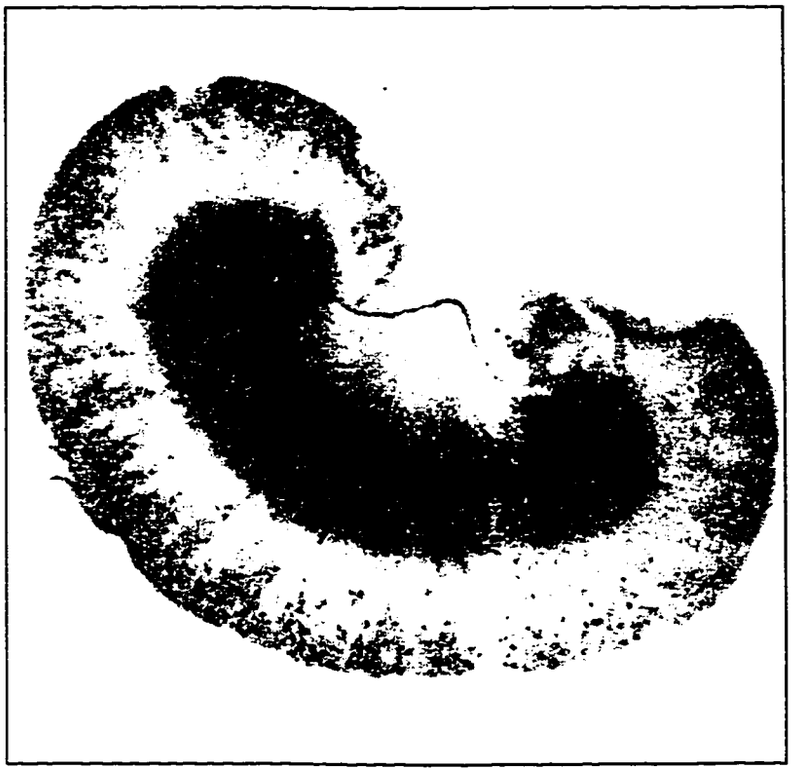


Figure 5.18: Effect of ischemia/reperfusion on the mean density of angiotensin II binding in the medulla. The mean density of angiotensin II binding in the medulla was determined by histoautoradiography. Mean density of angiotensin II medullary binding in ischemic kidneys was compared to the mean density of angiotensin II medullary binding originating from sham kidneys for each reperfusion time (relative signal). Data are expressed as mean ± SEM, and n refers to number of experiments. *P<0.01, **P<0.0001 vs. sham.

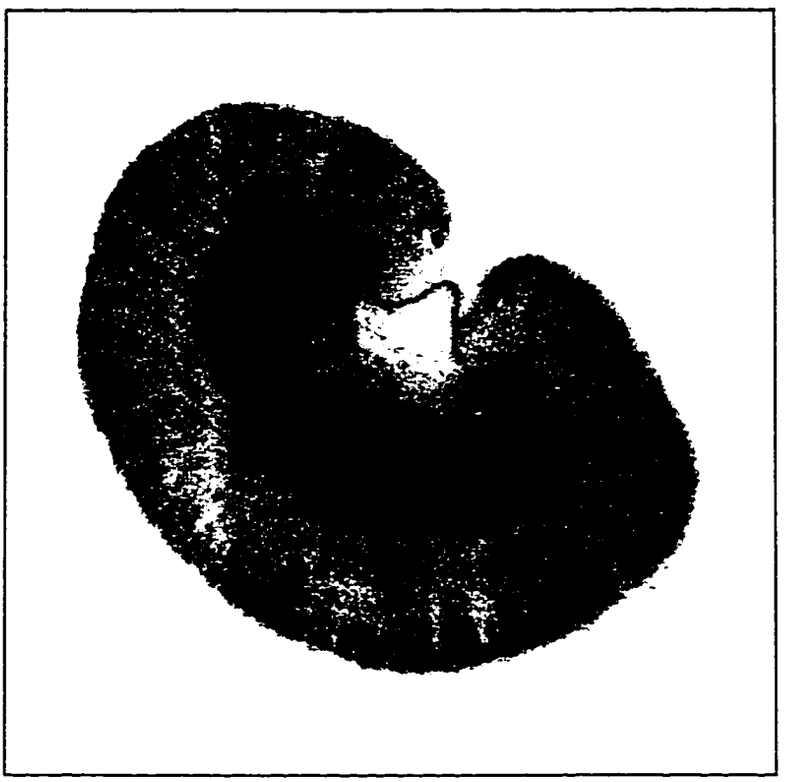


Sham



0 Hour Ischemia / Reperfusion

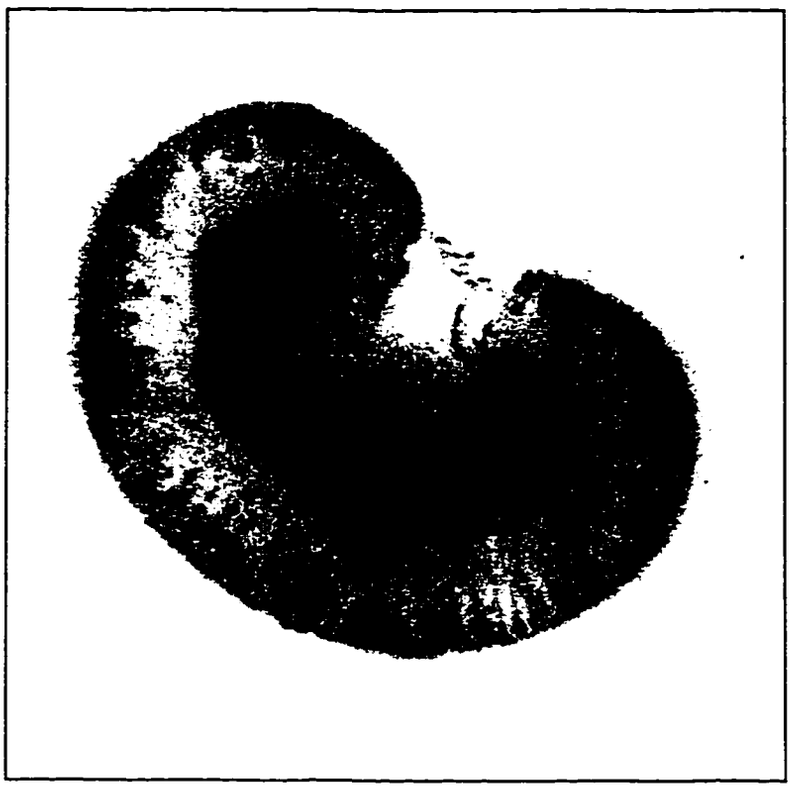
Figure 5.19: Representative histoautoradiograph for kidney sections at 0 hour post-ischemia/reperfusion. Sectioned renal tissue (20 μm) for sham and 0 hour post-ischemic kidneys was labelled with ^{125}I-[Sar^{1}Ile^{8}]angiotensin II, washed and exposed to ^{3}H-Hyperfilm. The mean density of angiotensin II binding did not differ between the 0 hour post-ischemic and sham kidneys, either in the cortex or medulla.



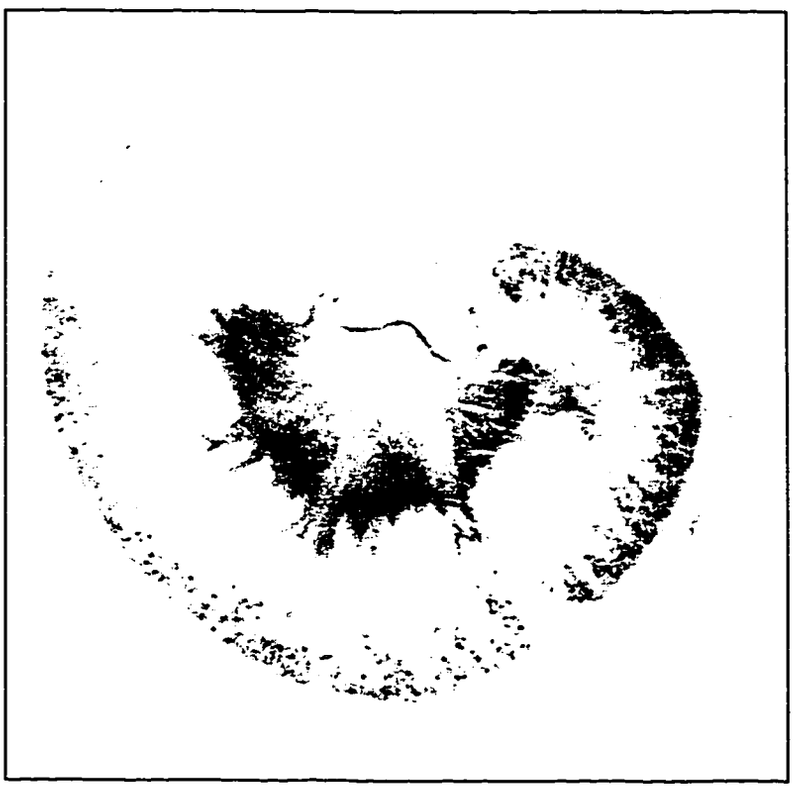
Sham

3 Hours Ischemia / Reperfusion

Figure 5.20: Representative histoautoradiograph for kidney sections at 3 hours post-ischemia/reperfusion. Sectioned renal tissue (20 μm) for sham and 3 hour post-ischemic kidneys was labelled with ^{125}I-[Sar^{1}Ile^{8}]angiotensin II, washed and exposed to ^{3}H-Hyperfilm. A significant decrease in the mean density of medullary angiotensin II binding was observed in the 3 hour post-ischemic kidney relative to the sham, but not in the cortex.

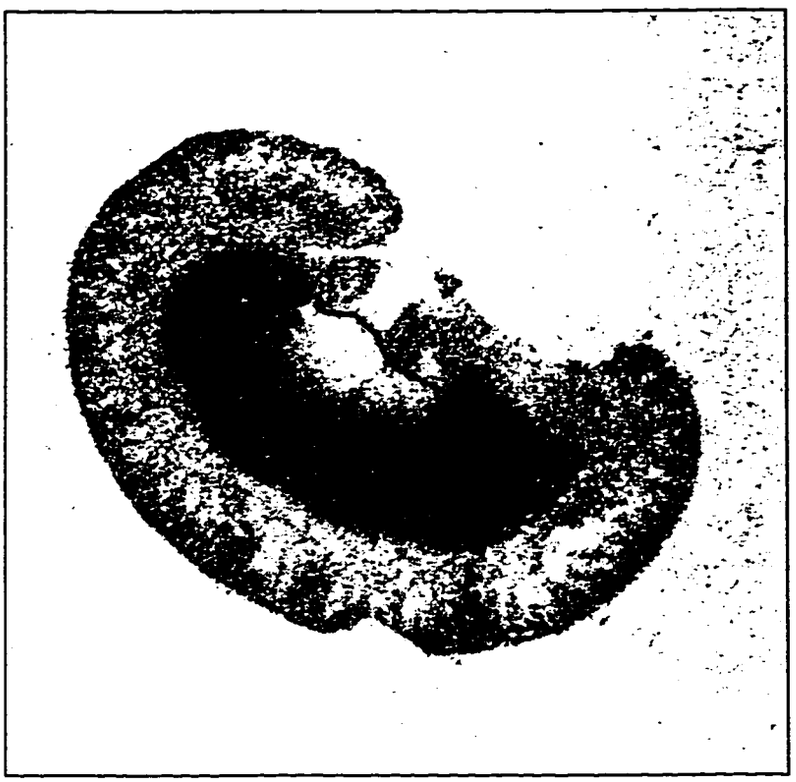


Sham

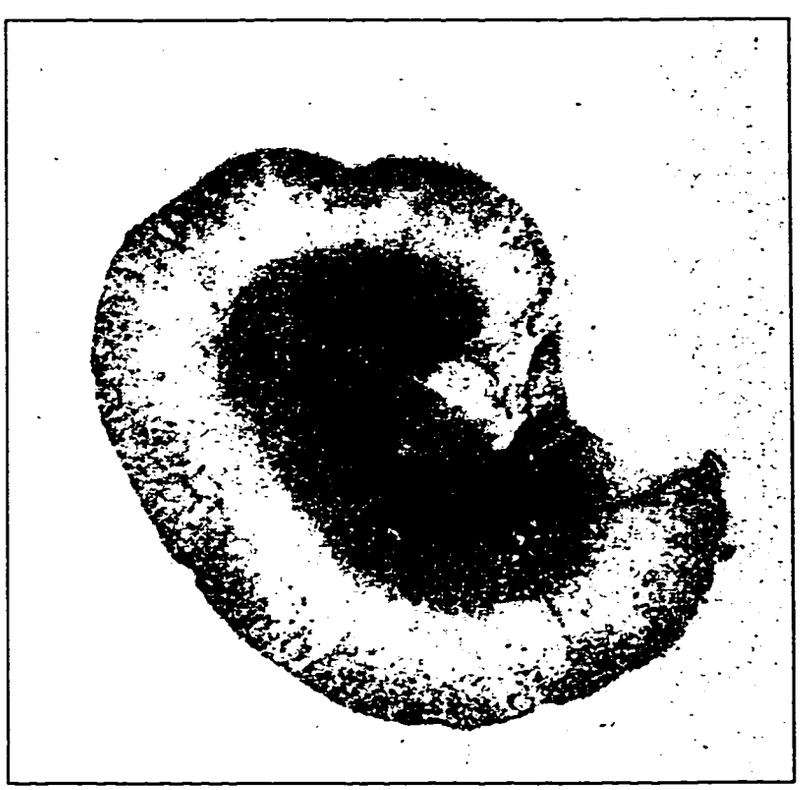


24 Hours Ischemia / Reperfusion

Figure 5.21: Representative histoautoradiograph for kidney sections at 24 hours post-ischemia/reperfusion. Sectioned renal tissue (20 μm) for sham and 24 hour post-ischemic kidneys was labelled with ^{125}I-[Sar^{1}Ile^{8}]angiotensin II, washed and exposed to ^{3}H-Hyperfilm. A significant decrease in the mean density of cortical and medullary angiotensin II binding was observed in the 24 hour post-ischemic kidneys, relative to the sham.



Sham



120 Hours Ischemia / Reperfusion

Figure 5.22: Representative histoautoradiograph for kidney sections at 120 hours post-ischemia/reperfusion. Sectioned renal tissue (20 μm) for sham and 120 hour post-ischemic kidneys was labelled with ^{125}I-[Sar^{1}Ile^{8}]angiotensin II, washed and exposed to ^{3}H-Hyperfilm. The mean density of angiotensin II binding did not differ significantly between the 120 hour post-ischemic and sham kidneys, in the cortex or medulla.

5.4 Measurement of Intrarenal Angiotensin II

To determine the effects of ischemia/reperfusion on the levels of intrarenal angiotensin II, total renal tissues from ischemic and sham kidneys at 120 hours post-ischemia/reperfusion were processed and quantified for angiotensin II by HPLC and radioimmunoassay. Purification involved cartridge extraction and reverse HPLC. As shown in figure 5.23, levels of angiotensin II were not significantly different between the ischemic and sham kidneys at 120 hours post-ischemia/reperfusion. These levels were similar to those reported in other studies (Yosipiv et al. 1996). Sham and ischemic kidney weights did not differ significantly (not shown).

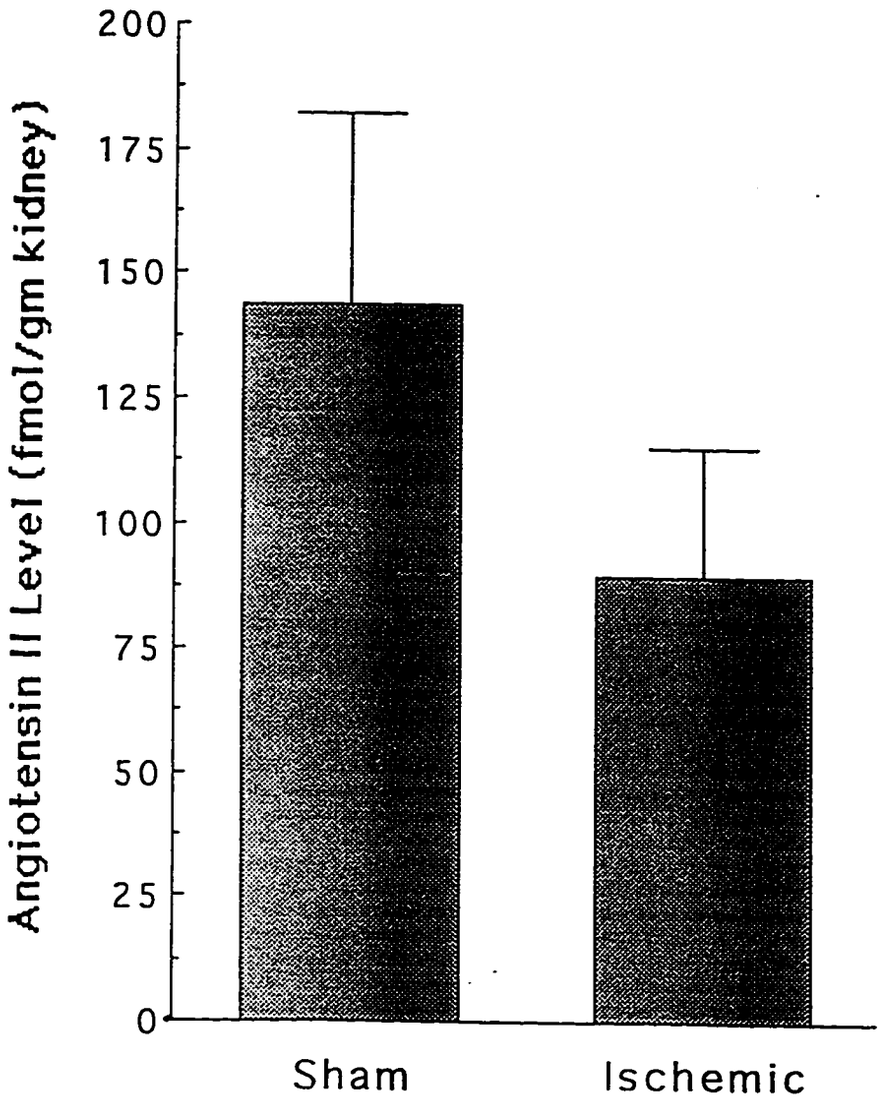


Figure 5.23: The effect of ischemia/reperfusion on intrarenal angiotensin II peptide levels 120 hours post-ischemia/reperfusion. Angiotensin II peptide levels were quantitated from sham and ischemic kidneys 120 hours post-ischemia/reperfusion. The levels of angiotensin II did not differ significantly between the two groups (n=6).

Discussion

6.0 Summary

In the present study, downregulatory changes in the expression of certain intrarenal renin-angiotensin components were observed after renal ischemia/reperfusion. Intrarenal angiotensinogen mRNA was decreased at 6, 24 and 72 hours after ischemia/reperfusion, but gene expression recovered at 120 hours post-ischemia/reperfusion. The proximal tubular AT_1 receptor mRNA was also shown to be downregulated after ischemia/reperfusion, but recovery of this gene expression occurred by 72 hours post-ischemia/reperfusion, earlier than the recovery of the angiotensinogen mRNA. Unlike proximal tubular AT_1 receptor mRNA, the ischemic kidney cortical AT_1 receptor mRNA did not change significantly relative to the sham kidneys. This suggested a different mechanism of regulation for the AT_1 receptor mRNA between proximal tubular segments and other cell types in the cortex after ischemia/reperfusion.

The mean density of angiotensin II binding was determined by histoautoradiography, and was shown to decrease with ischemia/reperfusion both in the cortex and medulla. In the cortex, mean density of angiotensin II binding remained unchanged at 0 hours and 120 hours post-ischemia/reperfusion, but decreased significantly in the cortex 24 hours post-ischemic/reperfusion, relative to sham kidneys. Similarly, the mean density of angiotensin II binding in the medulla decreased significantly at 3 and 24 hours post-ischemia/reperfusion, relative to the sham kidneys, but remained unchanged between the two groups at 0 hours and 120 hours post-ischemia/reperfusion.

Analysis of the AT_2 receptor mRNA was also performed, and new gene expression for this receptor was demonstrated in the ischemic proximal tubular segments and the outer medullary region of the kidney at 120 hours post-ischemia/reperfusion, relative to sham kidneys. Intrarenal angiotensin II was

also assayed at 120 hours post-ischemia/reperfusion, and no significant difference was observed in angiotensin II levels between ischemic and sham rat kidneys.

These data demonstrate downregulation of expression for certain intrarenal renin-angiotensin components and possible impairment in the formation of intrarenal angiotensin II during the early phases of ischemia/reperfusion. These changes may have induced adjustments in renal hemodynamics during the early phase of ischemia/reperfusion, as suggested by the reduced mean density of angiotensin II binding in the ischemic cortex and medulla, particularily in the medullary vasa recta structures. At 120 hours post-ischemia/reperfusion, expression of intrarenal renin-angiotensin system components and levels of angiotensin II levels were found to not differ from those in sham kidneys, including gene expression for the AT_{1a} and AT_{1b} receptors in ischemic proximal tubular segments. New AT_2 receptor gene expression was co-expressed with the AT_1 receptor mRNA in 120 hour post-ischemic proximal tubular segments. These data suggest possible unique physiological effects of angiotensin II on the 120 hour post-ischemic kidney, by interaction with these two different receptors, which do not normally co-exist in the adult kidney.

6.1 Experimental model of ischemia/reperfusion

Acute tubular necrosis was induced in the present model of rat renal ischemia/reperfusion, as demonstrated by the precipitous rise in plasma creatinine levels of rats with ischemia/reperfusion, compared to sham levels. The concentration of plasma creatinine reached maximum levels at 72 hours post-ischemia/reperfusion, and returned to basal levels at 120 hours post-

ischemia/reperfusion. As noted in other studies, this change in the profile of plasma creatinine concentration was indicative of sudden acute renal failure brought about by ischemic acute tubular necrosis (Humes et al. 1989). By 120 hours post-ischemia/reperfusion, however, no significant difference was observed between the plasma creatinine values of the two groups, demonstrating complete restoration of GFR. Blood urea nitrogen (BUN) was another parameter used in the diagnosis of acute renal failure. As expected, BUN concentration significantly increased with renal ischemia/reperfusion compared to the shams, following a time course similar to creatinine. Levels of BUN concentration did not differ significantly between the two groups at 120 hours post-ischemic/reperfusion. This suggested possible restoration of tubular function at this particular time, with regards to handling of urea.

Conclusive evidence for tubular necrosis in the present model of renal ischemic rats was obtained from histological sections of sham and ischemic kidneys at different reperfusion times. Sham kidneys displayed intact glomeruli and intact tubules at different times after surgery. In contrast, kidneys from ischemic rats showed prominent tubular necrosis at 24 hours post-ischemia/reperfusion. Glomeruli of ischemic kidneys, however, remained intact in this model at 24 hours and 120 hours post-ischemia/reperfusion. Based on the histological data, complete restoration of tubules was observed 120 hours post ischemia/reperfusion, with a substantial proportion of the tubular epithelial cells and lumens containing apoptotic cells. Some tubular epithelial cells also exhibited inward growth into the tubular lumen (papillary fronds), as described previously (Shimizu et al. 1993). These data therefore confirmed necrosis in this model of renal ischemia at 24 hours post-ischemia/reperfusion, and furthermore showed complete restoration of tubular epithelial lining at 120 hours post-ischemia/reperfusion. It is also evident from the histological data that most

proliferation must have occurred prior to 120 hours post-ischemia/reperfusion. As suggested by these data, the process of differentiating cells into functional tubular epithelial cells may be occurring by 120 hours post-ischemia/reperfusion.

6.2 Ischemia/Reperfusion did not change plasma renin activity

The majority of plasma renin activity is maintained by secretion from the juxtaglomerular cells of the kidney. In the present study, plasma renin activity did not change significantly between the ischemic and sham kidneys groups at any particular reperfusion time up to 24 hours post-ischemia/reperfusion. This provided indirect evidence suggesting that ischemia/reperfusion did not alter renin secretion from the juxtaglomerular cells. Moreover, histological evaluation in the current study showed juxtaglomerular cells to be intact following ischemia/reperfusion.

6.3 Ischemia/Reperfusion decreases angiotensinogen mRNA

To determine the effects of renal ischemia/reperfusion on the components of the intrarenal renin-angiotensin system, angiotensinogen mRNA was assayed by Northern blot analysis. Ischemia/reperfusion significantly decreased the levels of angiotensinogen mRNA in ischemic kidneys relative to the sham kidneys, beginning at 6 hours and continuing for 24 hours and 72 hours post-ischemia/reperfusion. Recovery of angiotensinogen mRNA gene expression in ischemic kidneys was observed at 120 hours post-ischemia/reperfusion.

Previous studies have demonstrated most angiotensinogen mRNA to be localized to proximal tubular cells, and to a lesser degree in glomeruli and vasculature (Ingelfinger et al. 1990). The current results, therefore, demonstrated that the proximal tubular renin-angiotensin system, in particular

angiotensinogen mRNA, was extremely sensitive to ischemia/reperfusion. Cell death could not account for these observations, because the proximal tubular segments retrieved from the Percoll gradient almost uniformly excluded trypan blue. In this study, the mechanism of decrease for the angiotensinogen mRNA was not investigated, but likely involved negative transcriptional regulation and/or effects on mRNA stability. Of interest, oxygen radicals have been shown to regulate gene activity (Toledano et al. 1994); therefore, the use of a transcription run-off assay could be important to support or exclude this mechanism of regulation of the angiotensinogen gene.

The mechanism of angiotensinogen mRNA downregulation remains to be determined, but given the mechanism of regulation of other growth factors, transcriptional inactivation appears a likely mechanism. Transcriptional inactivation has been noted in the ischemic kidney, especially for growth factors that are known to accelerate renal recovery, such as EGF (Price et al. 1995). It is of particular interest to note the recent isolation of a 12 KDa polysomal protein that stablizes the angiotensinogen mRNA by binding to the 3' end of the mRNA transcript (Klett et al. 1995). Originally isolated from liver, this stabilizing protein has been shown to increase angiotensinogen gene expression 3 to 4 fold in cell-free media containing acid phosphatase (Klett et al. 1995). It is conceivable, therefore, that ischemia could disrupt potentially important classes of mRNA stabilizing proteins, leading to altered angiotensinogen gene expression.

The downregulation of the angiotensinogen gene was due to a specific tissue response to ischemia/reperfusion, and not to necrosis. Some pertinent studies have established different modes of regulation for certain genes during ischemia/reperfusion. Instances of change during ischemia/reperfusion include the rapid upregulation of the hepatocyte growth factor gene, and the proto-oncogenes c-myc and egr-1 (Safirstein et al. 1994). Downregulatation of gene

expression have also been demonstrated during ischemia/reperfusion, such as for superoxide dismutase and EGF (Safirstein et al. 1994). These studies provide evidence that necrosis does not mediate downregulation of gene expression by reduction in cell number, but suggest rather that unique physiological pathways are stimulated in the ischemic kidney to affect mRNA expression.

6.4 Effects of ischemic/reperfusion on cortical AT_1 receptor mRNA

The cortical AT_1 receptor mRNA from ischemic kidneys did not change significantly from levels in shams, at any reperfusion time. This observation was quite interesting, in view of the marked decreases observed for the AT_1 receptor mRNA in the ischemic proximal tubular segments. These observations suggest the presence of other cell types in ischemic cortex, besides proximal tubular segments, that express a high degree of the AT_1 receptor mRNA.

In the cortex, glomeruli and proximal tubular segments express the AT_1 receptor in relative abundance (Mujais et al. 1986). Based on the current results, glomeruli may be responsible for preventing cortical AT_1 receptor mRNA from decreasing, but further studies are needed to conclude this. Mesangial cells are localized within the extracellular matrix of glomerular capillaries, and are candidate cells for expression of the AT_1 receptor after ischemia/reperfusion. In normal kidneys, these cell types are thought to contract and reduce the filtration surface area of glomerular capillaries (Ichikawa et al. 1991), and moreover have been hypothesized to secrete excess extracellular matrix causing glomerular scarring in certain renal diseases (Yamamoto et al. 1993). It is possible, therefore, that upregulation of mesangial AT_1 receptor mRNA might affect

glomerular filtration and/or tubular work load in the injured nephron segments after ischemia/reperfusion.

6.5 Ischemia/reperfusion decreases proximal tubular AT_1 mRNA

Proximal tubular segments from ischemic and sham kidneys were isolated using the Percoll gradient method, which has been previously shown to yield enriched preparations of proximal tubular segments (Vinay et al. 1981, Roczniak et al. 1996). Trypan blue exclusion was used to exclude cell death in the preparation, and to show that necrotic cells did not contaminate the layer of proximal tubular segments in the gradient. Significant decreases in ischemic proximal tubular AT_1 receptor mRNA were observed at 0, 1, 3 and 24 hours post-ischemia/reperfusion.

Impairment of proximal tubular angiotensin II formation was suggested by the downregulation of proximal tubular AT_1 receptor gene expression during ischemia/reperfusion. Angiotensin II regulates proximal tubular AT_1 receptor by positive feedback, with higher concentrations of angiotensin II stimulating proximal tubular AT_1 expression (Cheng et al. 1995). Impaired tubular flow and necrosis of the tubular epithelium are two mechanisms by which intratubular concentrations of angiotensin II may decrease in the early phase of ischemia/reperfusion, possibly accounting for the observed reduction in proximal tubular AT_1 gene expression. Under conditions of reduced tubular flow, excess exposure of angiotensin II to the high density of peptidases of the proximal tubular brush border might cause the rapid degradation of this peptide (Kenny et al. 1978).

The physiological consequences of the downregulation in proximal tubular AT_1 receptor mRNA are not understood. The proximal tubular renin-angiotensin system exerts demanding effects on proximal tubular segments, primarily by regulating the apical energy-dependent Na/H antiporter and the basolateral Na/HCO_3 co-transporter (Douglas et al. 1990). Decreased activity of these transporters might be expected to reduce cellular energy consumption, prevent cellular swelling and lower intracellular Na concentrations. Inhibition of the Na/H exchanger can also theoretically enhance the availability of ATP, because the basolateral Na/K ATPase relies on ATP utilization and the presence of intracellular Na. By the same token, reduced activity of the apical Na/H antiporter might also minimize calcium entry by inhibition of the Na/Ca exchanger. This could impede the deleterious effect of high intracellular calcium concentration on ischemic proximal tubular cells.

The recovery of proximal tubular AT_1 receptor mRNA had occurred at 72 hours post-ischemia/reperfuison, whereas angiotensinogen mRNA had not yet recovered. This was of particular interest, because the recovery of the AT_1 receptor mRNA within proximal tubular cells was synchronous with the highest incorporation of thymidine in this model of ischemia (Humes et al. 1989), reflecting a possible role for the AT_1 receptor in mediating proliferation and/or hypertrophy. Flattened tubular epithelial cells were noted in the present study and in other studies at 120 hours post-ischemia/reperfusion (Basile et al. 1996), revealing a distinct appearance compared to normal tubular morphology. To further demonstrate the full recovery of proximal tubular AT_1 receptor mRNA at 120 hours post-ischemia/reperfusion, RT-PCR was performed for the AT_{1a} and AT_{1b} receptor subtypes, and signal intensities were found in both cases to be the same. The gene expression of proximal tubular AT_1 receptor at 72 and 120 hours post-ischemia/reperfusion may therefore commit cells to hypertrophy in

order to restore proximal tubular function. These important questions were not, however, addressed in the current study.

6.6 Decreased ^{125}I-Ang II binding with ischemia/reperfusion

^{125}I-Ang II labelling was performed on renal slices obtained from sham and ischemic kidneys. At 0 hours and 120 hours post-ischemia/reperfusion, no significant differences in the mean density for angiotensin II binding were observed between sham and ischemic kidneys, either in the cortex or medulla. Significant decreases in the mean density of outer medullary binding for angiotensin II were observed in ischemic kidneys at 3 and 24 hours after reperfusion, relative to shams. In the ischemic cortex, the mean density of angiotensin II binding decreased significantly at 24 hours post-ischemia/reperfusion, relative to shams.

The reduced mean density of binding for angiotensin II could have been attributed to either reduced affinity or reduced number of angiotensin II receptors. A definite conclusion was not reached in the present study since angiotensin II was not used in saturating amounts during binding assays. Instead, ^{125}I-angiotensin II was used in amounts (0.1 nM) corresponding to the receptor Kd value, and this reduced the presence of non-specific binding (Mendelsohn et al. 1987). Angiotensin II receptor number has been documented to change under different physiological conditions. The downregulation and upregulation of glomerular angiotensin II receptors by sodium restriction and sodium loading, respectively, are two examples of changes in receptor number (Skorecki et al. 1983). Since mRNA for the AT_1 receptor was downregulated in proximal tubular segments and significant tubular necrosis was also observed histologically in the 24 hour post-ischemic kidney, reduced numbers of

angiotensin II receptor are likely to account for the reduced mean binding of angiotensin II observed in the ischemic cortex and medulla. Changes in affinity cannot be ruled out at present. In addition, the angiotensin II binding observed in the 120 hour post-ischemic kidneys did not discriminate between AT_1 and AT_2 receptors. It is possible, therefore, that the new AT_2 receptor mRNA is converted into AT_2 receptor protein. This requires further study.

The method of histoautoradiography provided useful information with respect to the structures affected by ischemia/reperfusion. In particular, the resolution of binding permitted a quantatitive analysis of the different regions of the kidney, especially the cortex and outer medulla, which showed reduced mean density of binding for angiotensin II after ischemia/reperfusion. Based on previous observations, angiotensin II has been shown to have a detrimental effect on the medullary oxygen balance after ischemia/reperfusion (Brezis et al. 1990). The reduced mean density of binding in the medulla may promote a relative vasodilatory response, and may represent a mechanism for injured medullary tissue to enhance oxygen delivery and/or to reduce transport work. As cited previously, medullary vasodilation has been observed as a protective mechanism for the medulla after ischemia/reperfusion (Brezis et al. 1995).

Glomerular angiotensin II binding still persisted on histoautoradiographs, even at 24 hours post-ischemia/reperfusion. According to the histology of 24 hour post-ischemic tissue, glomeruli remained protected from ischemic damage, and this might explain persistent angiotensin II binding in these structures. In the immediate vicinity of glomerular binding, reduced mean density of angiotensin II binding was also apparent, possibly representing proximal tubular convoluted segments; however, density of binding in these regions could not be quantified because of equipment resolution limitations. Angiotensin II is known to maintain filtration by predominant vasoconstriction of the efferent arteriole.

The reduced mean density of angiotensin II binding in the cortex may limit GFR and the workload of tubular segments, particularly for proximal tubular segments and the thick ascending limb.

6.7 Kidney AT_2 gene expression is stimulated in ischemic rats

The direct location of AT_2 receptor mRNA within the adult kidney has not yet been determined, nor has its existence been confirmed. Therefore, RT-PCR of adrenal mRNA for the AT_2 receptor was necessary to establish reliable amplification of a postivie control (Kambayashi et al. 1993). In the kidney cortex, RT-PCR for the AT_2 mRNA yielded a faint signal, with the same expected bp product as did the adrenal. Subsequent subcloning and sequencing of this product showed it to indeed be the the AT_2 receptor mRNA. Having established a reliable RT-PCR protocol for the AT_2 receptor mRNA, this method was then applied to the current model of renal ischemia/reperfusion.

To limit variability, total RNA from proximal tubular segments was isolated. This had the benefit of reducing the cell-type variability, that would otherwise occur by using cortex tissue. In addition, total RNA from the outer medulla was also isolated. No signals for the AT_2 mRNA were generated for proximal tubular segments from sham and ischemic kidneys, at any time up to and including 72 hours post-ischemia/reperfusion.

Analysis of the AT_2 mRNA was also carried out at 120 hours after ischemia/reperfusion. In this instance, new AT_2 gene expression was observed in the ischemic proximal tubular segments, but no signal was ever observed for the respective shams. A negative RT control was always carried out simultaneously during the analysis of the AT_2 receptor mRNA, thus eliminating any genomic DNA contamination. AT_2 receptor gene expression was also

stimulated in the outer medulla of the 120 hour post-ischemic kidney, compared to sham. This suggested the presence of the AT_2 receptor mRNA in the S_3 segments of the proximal tubules, or perhaps in other cells types originating from this region of the kidney, such as the thick ascending limb. In short, the AT_2 receptor mRNA stimulation in the 120 hour post-ischemic kidney is the first demonstration of this receptor mRNA after acute tubular necrosis.

Histological sections of 120 hour post-ischemic kidneys verified the presence of structures resembling apoptotic bodies and papillary fronds. In the rat post-ischemic kidney, original studies characterizing the kidney observed a marked nine-fold increase in the apoptotic activity within the kidney at 144-168 hours post-ischemia/reperfusion, with a gradual decline occurring until 6 months post-ischemia/reperfusion (Shimizu et al. 1993). These results placed a significant emphasis on apoptosis as an important mechanism for the remodelling phase of the ischemic kidney. Unlike the kidney, the heart and brain undergo rapid apoptosis in the early phase of ischemia/reperfusion. The new AT_2 receptor gene expression in ischemic proximal tubular segments and in the outer medulla of 120 hour post-ischemic kidneys coincides with the apoptotic activity. In other studies, apoptosis in mouse fibroblast cells and PC12W cells has been shown to be mediated through the AT_2 receptor (Yamada et al. 1996). Furthermore, the majority of papillary fronds are found within proximal tubular segments after ischemia/reperfusion, and these robust ingrowths have been noted to regress by apoptosis (Basile et al. 1996). It remains to be answered, therefore, whether the AT_2 receptor mediates apoptosis in papillary fronds and in the tubular epithelium, and whether this activity promotes remodelling of the proximal tubular epithelium.

The mechanism for induction of the AT_2 receptor gene expression in proximal tubular segments and in the outer medulla was not investigated, but

may have involved reduced levels of angiotensin II peptide during the early phase of reperfusion. Recently, a hypothesis has been put forward suggesting that the AT_1 receptor can signal and activate cis-acting gene products causing the suppression of the AT_2 receptor (Ichiki et al. 1995). Considering the prolonged downregulation of the proximal tubular AT_1 receptor mRNA during the early phase of ischemia/reperfusion, this hypothesis still has validity.

Many growth factors have been shown to induce the AT_2 receptor gene expression in R3T3 cells (fibroblast cells). These factors include insulin, interleukin 1-β and phorbol esters (Ichiki et al. 1995). Furthermore, quiescent R3T3 cells in the growth arrest phase are known to express AT_2 mRNA in abundance, but not in the proliferative phase. Interferon exists in two forms, yet only enhanced expression of interferon-1 in the quiescent phase was found to induce expression of the AT_2 receptor mRNA, while interferon-2 suppressed the AT_2 receptor mRNA during the proliferative phase (Horiuchi et al. 1995).

In vascular smooth muscle cells, insulin-like growth factor-I (IGF-I) receptors have been shown to induce expression of the AT_2 receptor. This has relevance to the current observations, because intrarenal expression of IGF-I recovers after 72 hours post-ischemia/reperfusion (Matejka et al. 1992). IGF-I growth factor induces differentiation of proximal tubular segments (Hammerman et al. 1992), and it is interesting to speculate a role for this growth factor in the induction of the AT_2 receptor after ischemia/reperfusion.

The presence of the AT_2 receptor mRNA within proximal tubular segments raises the question about the role of this receptor and the function of the intrarenal renin-angiotensin system at 120 hours post-ischemia/reperfusion. In many aspects, nephrogenic repair resembles the late stages in conversion of the embryonic mesenchyme to a tubular epithelium (Hammerman et al. 1992). The AT_2 receptor is abundantly expressed in fetal kidney and heart tissue, but

not in the adult stage. The induction of the AT_2 receptor after renal ischemia/reperfusion may therefore represent a reversion to the fetal state. A similar induction of AT_2 receptor gene expression has been noted in the rat heart after infarction (Nio et al. 1995). Knockout mice for the AT_2 receptor have recently been developed (Ichiki et al. 1995), and the recovery of renal function in these mice following ischemia/reperfusion, as compared to normal mice, could provide useful information to interpret the functional role of the AT_2 receptor.

6.8 Angiotensin II levels in ischemic rat kidneys

Renal tissue angiotensin II levels were assayed from sham and 120 hour post-ischemic kidneys, to determine whether or not intrarenal angiotensin II levels were changed during the period of co-expression of the AT_1 and AT_2 receptor mRNA. Angiotensin II levels did not significantly differ between sham and ischemic kidneys after 120 hours, and this observation demonstrated that the 120 hour post-ischemic kidney could make normal amounts of angiotensin II (Yosipiv et al. 1996), in the presence of AT_1 and AT_2 receptor gene expression. In fact, this result supports the important potential role that the intrarenal renin-angiotensin system may have in remodelling the ischemic kidney through AT_1 and AT_2 receptors. An array of new functions may apply to the intrarenal renin-angiotensin system in this pathophysiological setting, given that complete functional and histological recovery is not fully reached until 6 months post-ischemia.

6.9 Conclusion

In the current study, downregulation was demonstrated for the gene expression of renal angiotensinogen and proximal tubular AT_1 receptor mRNA after renal ischemia/reperfusion. Similarly, downregulation was observed for the mean density of angiotensin II binding in the outer medulla and cortex after ischemia/reperfusion. All these components of the intrarenal renin-angiotensin system recovered at 120 hours post-ischemia. New gene expression for the AT_2 receptor was detected in ischemic proximal tubular segments and in the outer medulla. In addition, levels of AT_{1a} and AT_{1b} receptor gene expression in proximal tubular segments had recovered at 120 hours post-ischemia/reperfusion. These results suggest downregulation of the intrarenal renin-angiotensin system in the early phase of ischemia/reperfusion, and restoration of the system at 120 hours post-ischemia/reperfusion, with activation of the AT_2 receptor mRNA. The intrarenal renin-angiotenisn system may be involved in remodelling mechanisms through the co-expression of AT_1 and AT_2 receptor mRNA.

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