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Erythropoietin production in acute and chronic obstructive nephropathy

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For my parents,

Mohammad, his family,

Hassan and Hasan

Content

1 Abbreviations

ACE	angiotensin converting enzyme
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
ANG II	angiotensin II
AIDS	acquired immune deficiency syndrome
AT1	angiotensin II type 1 receptor
AT2	angiotensin II type 2 receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
AVD	arterio-venous difference
BMP-7	bone morphogenetic protein-7
BS\OE	blood samples\organs excision
Caspases	cysteine aspartate-specific proteases
CFU-E	colony-forming units-erythroid
CO	carbon Monoxide
COHb	carboxy hemoglobin
GFR	glomerular filtration rate
ECM	extracellular matrix
EGF	epidermal growth factor
EPO	Erythropoietin
h	hour
Hb	hemoglobin
HIF	hypoxia inducible factor
HRE	hypoxia response elements
IGF-1	insulin-like growth factor-1
min	minute
MMP	matrix metalloproteinase
MP	methylation potential
mRNA	messenger ribonucleic acid
n	number
NFκB	nuclear factor κB

NO	nitric oxide
NOS	nitric oxide synthase
NX	nephrectomy
O_2	oxygen
ODD	oxygen-dependent degradation
P_{GC}	hydrostatic pressure in glomerular capillary
P_{BS}	hydrostatic pressure in Bowman's space
pO_2	O_2 partial pressure
pVHL	von Hippel Lindau protein
RBF	renal blood flow
rHuEPO	recombinant human erythropoietin
TGF- ß1	transforming growth factor-ß1
SNGFR	single nephron glomerular filtration rate
TAD	terminal transactivation domain
TNF	tumor necrosis factor
UUL	unilateral ureteral Ligation
π_{GC}	glomerular capillary oncotic pressure
π_{BS}	Bowman's space oncotic pressure

2 Introduction

2.1 Obstructive nephropathy

Urinary tract obstruction is of great importance to clinicians because it is a common entity that occurs at all ages. Urinary tract obstruction is a common cause of acute and chronic renal failure and the primary cause of end-stage renal disease in children (Manucha 2007).

Obstructive nephropathy is a renal disease caused by the presence of structural or functional changes in the urinary tract that impede the normal flow of urine. It is due to functional or anatomic lesions that can be located anywhere in the urinary tract between the renal tubules to the urethral meatus. Because the degree and duration of obstruction are the main determinants of renal dysfunction, early diagnosis and treatment are the keys to prevent progression of renal impairment. Acquired obstructive nephropathy in humans is usually partial and prolonged in its time course. Only few studies addressed the question of erythropoietin (EPO) secretion by hydronephrotic kidneys. Therefore, the present study was designed to explore the hypoxia-induced EPO secretion response following unilateral ureteral ligation (UUL) in rats.

2.1.1 Epidemiology

In an autopsy series on 59.064 patients aged 0-80 years, the frequency of hydronephrosis was 3.1% (2,9% in females and 3,3% in males) (Klahr 2000). There was no significant sex difference until the age of 20 years. Between the age of 20 and 60, the frequency of urinary obstruction was higher in women than in men, mainly due to the effects of pregnancy and urinary tract compression by tumors. After age of 60, prostatic disease raised the frequency of the urinary tract obstruction among men above that among women. The incidence of hydronephrosis at autopsy is somewhat lower in children than adults, being 2% in one series of 16.100 autopsies (Klahr 2000). Recurrent infections of the urinary tract are commonly associated in pediatric patients with urinary tract malformation. Any newborn with a urinary tract infection, regardless

of sex, should be presumed to have urinary obstruction or reflux until proven otherwise (el-Dahr and Lewy 1992).

2.1.2 Classification and causes of obstructive nephropathy

Urinary obstruction is classified according to cause, duration, degree, and the localization (Docherty *et al.* 2006). Renal calculi are the most common cause of acute obstruction. Chronic obstruction may develop slowly and is long lasting. Causes of obstructive nephropathy can be functional or mechanical. Ureter or bladder dysfunction can be induced by myelodysplasia, injury or congenital defect of the spinal cord, tabes dorsalis, diabetes mellitus, multiple sclerosis, and autonomic neuropathy including drug-induced neuropathy (such as disopyramide). Mechanical causes can be divided to intrinsic and extrinsic causes. Intrinsic mechanical causes can be induced by intrarenal tubular obstruction such as precipitation of uric acid, sulfuonamide, paraprotien crystals, drugs (such as indinavir) or by extrarenal pelvic or ureteral obstruction which may be caused by calculus, thrombus, papillary necrosis and tumor or structural lesions of the ureter or bladder include stricture, tumor, urethral valves, ureteroceles and foreign body. Extrinsic mechanical causes are compression of the urinary tract or surgical misadventures include accidental ureteral ligation. Compression of the urinary tract can be caused by prostatic hypertophy or carcinoma, uterine prolapse or tumor, ovarian abscess, cyst, or tumor, endometriosis, pregnancy, enlarged or aneurysmal pelvic vessels, retroperitoneal tumor, infection, lymphadenopathy, and fibrosis.

2.1.3 Renal function in obstructive nephropathy

2.1.3.1 Changes in renal hemodynamics

Obstruction can alter one or more of the determinants of glomerular filtration rate (GFR). As known, GFR is expressed by the following equation:

$$GFR = K_f * P_{UF}$$

where: $P_{UF} = [(P_{GC} - P_{BS}) - (\pi_{GC} - \pi_{BS})]$

K_f is the hydrolic conductivity.

P_{UF} is the effective filtration pressure.

P_{GC} is glomerular capillary hydrostatic pressure.

P_{BS} is Bowman's space hydrostatic pressure.

π_{GC} is glomerular capillary oncotic pressure.

π_{BS} is Bowman's space oncotic pressure.

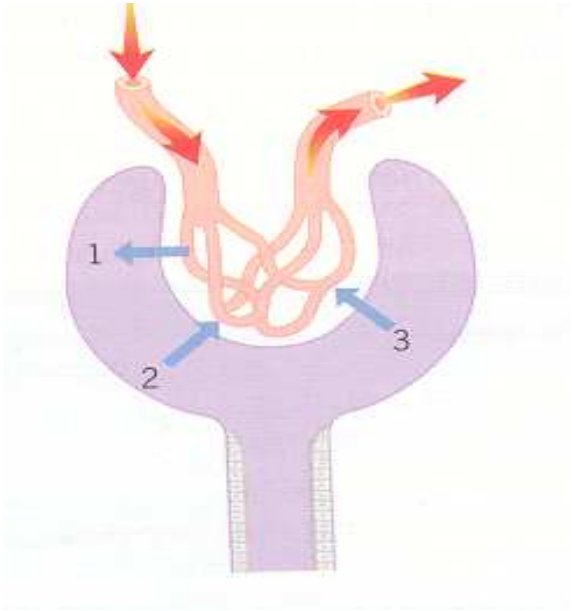


Figure 1. Pressures controlling glomerular filtration. Hydrostatic pressures in the glomerular capillary, P_{GC}, (**1**), in the Bowman space, P_{BS}, (**2**) and colloid oncotic pressure in the glomerular capillary, π_{GC}(**3**). Arrows indicate the direction of a pressure gradient.

Acute ureteral obstruction is characterized by a transient increase in renal blood flow (RBF) and in ureteral hydrostatic pressure of the affected kidney (Vaughan, Jr. *et al.* 2004; Vaughan, Jr. *et al.* 1970). During the acute obstruction, the afferent arterioles respond with vasodilation lasting approximately 1 to 1,5 h after the onset of UUL (Felsen *et al.* 2003; Dal *et al.* 1977; Ichikawa 1982). The acute consequences of partial urinary tract obstruction on glomerular filtration dynamics depend upon the extent of P_{GC} increase and the reactive reduction in preglomerular resistance (vasodilation of the afferent arterioles). An increase of P_{BS} from 12-20 mmHg may lead to increase of P_{GC} from 45 to 53 mmHg which results in an unchanged single nephron glomerular filtration rate (SNGFR). However, if P_{BS} rises to 35 mmHg as seen in acute obstruction SNGFR is largely reduced. It has been shown that nitric oxide (NO) plays a central role in afferent vasodilation response to acute urinary obstruction (Hegarty *et al.* 2001). Administration of indomethacin blocks the hyperemic response. Thus, vasodilating prostglandins are also involved in afferent vasodilation (Allen *et al.* 1978; Gaudio *et al.* 1980).

RBF starts to decline progressively after 3 h of unilateral obstruction through 12 to 24 h (Moody *et al.* 1975; Yarger and Griffith 1974) eventually resulting in a 50% reduction of RBF. As a consequence GFR is greatly reduced and intratubular pressure may normalize to preobstruction values (Gaudio *et al.* 1980; Dal *et al.* 1977). After 24 h of obstruction, P_{GC} falls from 45 to 30 mmHg by preglomerular vasoconstriction, SNGFR tends to be zero. This decrease of GFR is due to both a decrease in SNGFR and a decrease in the number of filtering nephrons (Klahr 2000).

Changes in RBF in acute and chronic urinary tract obstruction are shown schematically in figure 2.

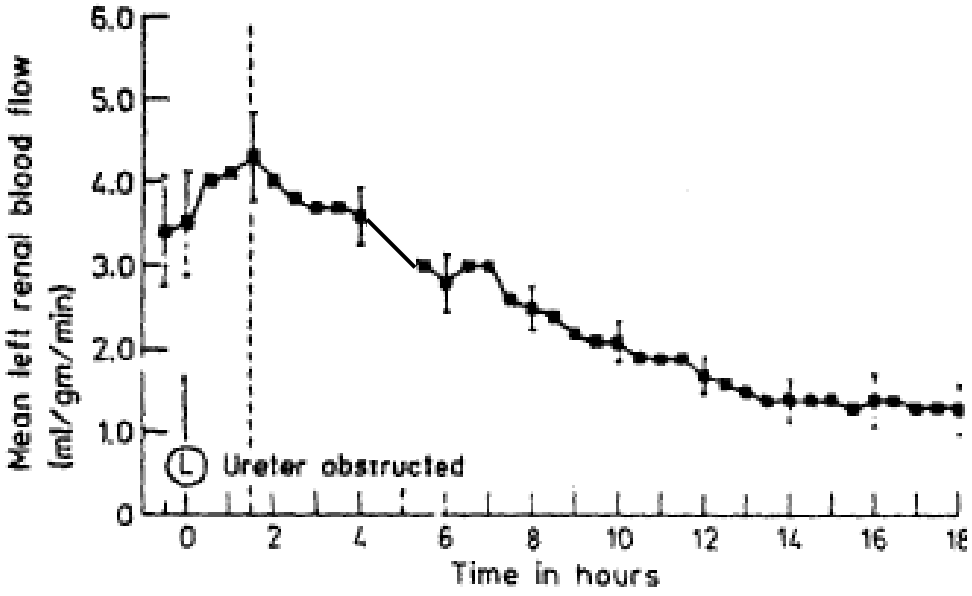


Figure 2. Time course of renal RBF in dogs following unilateral obstruction of the left ureter. Changes are characterized by transient increase of RBF and a subsequent progressive decrease modified from (Vaughan, Jr. *et al.* 2004).

The progressive vasoconstriction in the late phase of UUL is mediated by several vasoactive features such as angiotensin II (ANGII), Thromboxan A_2 and antidiuretic hormone and mediators of tubuloglomerular feedback (Yarger *et al.* 1980; Reyes *et al.* 1991; Arendshorst *et al.* 1974). It has been shown an increased eicosanoid synthesis, induction of thromboxane A_2 in the isolated perfused kidney and changes in microsomal metabolism of arachidonic acid in obstructed kidneys of rabbits and humans (Morrison *et al.* 1977) but not in dogs (Loo *et al.* 1986; Felsen *et al.* 1990). Infusion of N-monomethyl-L-arginine, a nitric oxide synthase (NOS) inhibitor, during ureteral obstruction results in a decrease in RBF and an increase in the ratio of renovascular resistance to total peripheral resistance (Chevalier *et al.* 1992). The reduction in glomerular hydrostatic pressure after chronic ureteral obstruction leads also to reduction in the glomerular size as shown in figure 3.

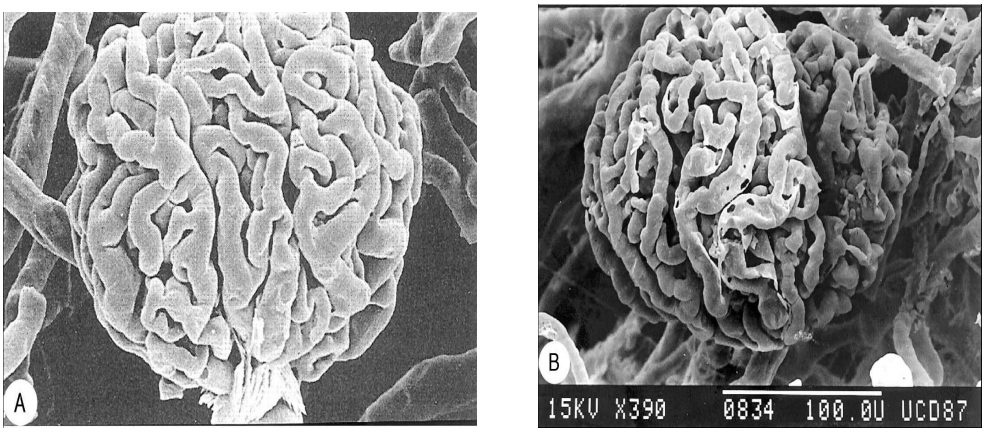


Figure 3. A, scanning electron microscopic appearance of normal glomerular cast. B, appearance of glomerular microvascular cast after obstruction shows capillary collapse and irregularity. Reproduced form (Vaughan, Jr. *et al.* 2004)

2.1.3.2 Changes in tubular function

Hydronephrosis can cause several abnormalities in tubular function. Obstruction impairs the reabsorption of solutes and water and reduces the ability to concentrate or dilute the urine (McDougal 1982). Obstruction reduces the net reabsorption of salt in several nephron segments (Sonnenberg and Wilson 1976; Hanley and Davidson 1982; Hwang *et al.* 1993), by down-regulation of the expression and activities of specific transport protiens (Hwang *et al.* 1993; Li *et al.* 2003). Several signal chains mediate this down regulation, including cessation of urine flow with the reduction of the rate of sodium entry across the apical membrane (Zeidel 1993), increased levels of natriuretic substances such as prostaglandin E2 (PGE_2) (Lear *et al.* 1990; Strange 1989), and infiltration of the kidney by mononuclear cells (Harris *et al.* 1989). The fractional excretion of potassium is decreased in patients with obstructive nephropathy. Thus, patients with this disorder may develop hyperkalemic/hyperchloremic acidosis, particularly those with chronic partial obstruction (Batlle *et al.* 1981). Obstruction dramatically reduces urinary acidification. In animal with ureteral obstruction, there is a decrease of H^+ ATPase pumps in the apical surface of intercalated

cells, this may account for the acidifying defect seen after release of ureteral obstruction (Purcell *et al.* 1991). The inability to concentrate the urine results from inability of the thick ascending limb of Henle's loop to generate a high osmolality in the medullary interstitum.

2.1.4 Inflammatory cell infiltration, apoptosis and renal fibrosis in obstructive nephropathy

Progression of obstructive nephropathy involves tubulointersitial injury, characterized by inflammatory cell infiltration, tubular atrophy and interstitial fibrosis.

2.1.4.1 Inflammatory mediators in obstructive renal injury

The interstitial inflammation constitutes an early response to obstructive nephropathy (Misseri *et al.* 2004). This response is largely mediated by renal infiltration with macrophages (Kluth *et al.* 2004). These macrophages constitute a source for a number of growth factors and inflammatory agents such as, interleukin 1 and interleukin 6, transforming growth factor ß (TGF-ß), tumor necrosis factor a (TNF-a), platelet-derived growth factor (Klahr 1998). While many actions of activated macrophages are detrimental to the kidney, some macrophage populations can attenuate inflammation (Kluth *et al.* 2004). Macrophages that infiltrate renal tissue after obstructive nephropathy are attracted by a variety of cytokins and chemokines. Adhesion molecules are responsible for the localization of monocytes to an area of inflammation as studies using knockout selectin mice subjected to UUL have shown (Ogawa *et al.* 2004; Lange-Sperandio *et al.* 2002). Smad3 (Mothers against decapentaplegic homolog 3) deficiency attenuates inflammation after UUL through decreasing renal interstitial infiltration by monocytes and CD4/CD8 T cells (Inazaki *et al.* 2004). Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction (Wada *et al.* 2004). The intrarenal renin–angiotensin system is highly activated following ureteral obstruction (Chevalier and Cachat 2001), and monocyte recruitment is mediated, at least in part, through angiotensin II type 2

receptor (AT_2) receptor activation of the transcription factor nuclear factor-kappa B (NFκB) (Esteban *et al.* 2004).

2.1.4.2 The role of apoptosis in the pathogenesis of obstructive nephropathy

Apoptosis, or programmed cell death, is the major mechanism by which renal tubular cell death and a reduction in renal mass occur following ureteral obstruction (Gobe and Axelsen 1987; Truong *et al.* 1998).

Renal tubular cell apoptosis begins after 4 days and is peaking after 15 days, of ureteral obstruction, while interstitial cell apoptosis increases progressively over the duration of obstruction over 45 days (Choi *et al.* 2000). Glomerular cells, on the other hand, are very resistant to obstruction-induced apoptosis, with no evidence of glomerular cell apoptosis occurring after 90 days of ureteral (Truong *et al.* 1998). Caspases (cysteine aspartate-specific proteases), especially caspase 3 are involved in mediation of renal cell apoptosis in obstructive nephropathy (Truong *et al.* 2001). In animal models of UUL, use of angiotensin converting enzyme (ACE) inhibitors and genetic ANG II type1 receptor (AT_1) deletion cause a reduction in early apoptosis and late fibrosis (Jones *et al.* 2000; Satoh *et al.* 2001). It has been also found that chronic ureteral obstruction inhibits Bcl-2 expression and thereby promotes obstruction-induced renal tubular cell apoptosis (Chevalier *et al.* 2000). Epidermal growth factor (EGF) levels was reduced during experimental hydronephrosis (Walton *et al.* 1992). Furthermore, administration of EGF during ureteral reduces the expression of TGF-ß1 and suppresses apoptosis in distal tubular and collecting duct cells (Kennedy *et al.* 1997; Chevalier *et al.* 1998).

2.1.4.3 Renal interstitial fibrosis resulting from UUL

Tubulointerstitial fibrosis is one of the major pathologic components of renal injury following partial ureteral obstruction, and its presence contributes to obstruction-induced renal dysfunction (Misseri *et al.* 2004). This process is controlled, in part, by matrix metalloproteinases (MMPs), whose function is to degrade the collagenous and noncollagenous components of extracellular matrix. MMPs are maximally inhibited during ureteral obstruction due to the

increased expression of tissue inhibitors of metalloproteinases (Morrissey *et al.* 1996; Engelmyer *et al.* 1995). Increased collagen deposition in the interstitial space also occurs during ureteral obstruction and contributes to the development of tubulointerstitial fibrosis (Alvarez *et al.* 1992; Miyajima *et al.* 2000). Infiltration by macrophages in turn, produce a variety of inflammatory cytokines and growth factors, including TGF, TNF, interleukin-1, interleukin-6 and PDGF (platelet-derived growth factor), that further contribute to tubulointerstitial inflammation and fibrosis (Diamond *et al.* 1998). Figure 4 illustrates the various factors that involved in interstitial fibrosis in obstructive nephropathy.

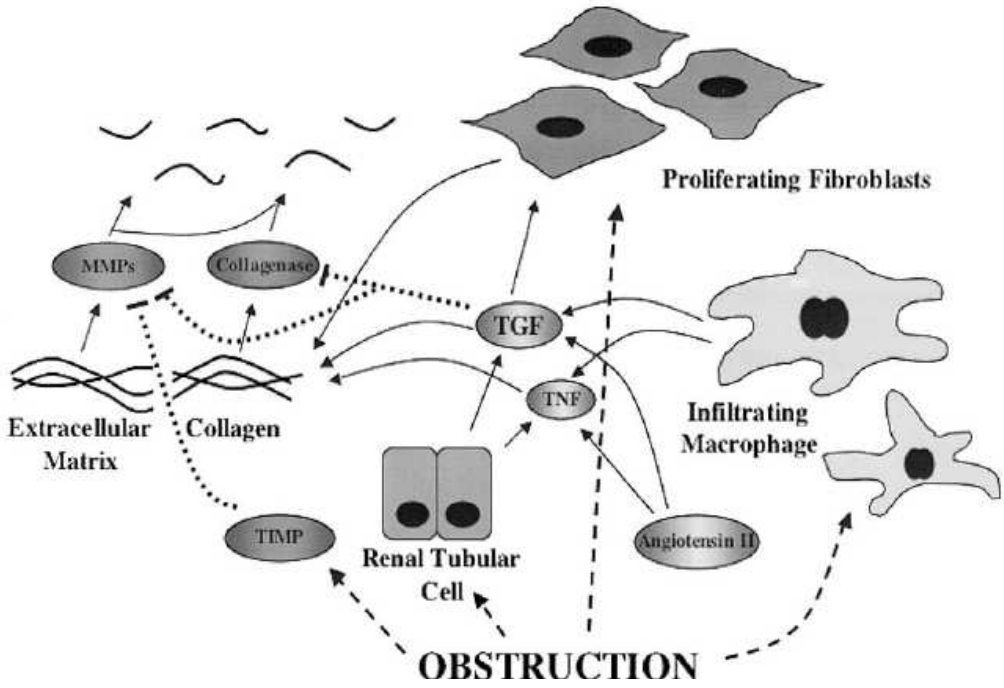


Figure 4. Schematic drawing of factors that are involved in interstitial fibrosis obstructive nephropathy (see text for further explanation).

TGF-ß1 : Increased TGF-ß1 expression has been observed in response to ureteral in both fetal and adult animal models (Diamond *et al.* 1994; Roberts *et al.* 1992; Chung and Chevalier 1996). TGF-ß1 stimulates the proliferation of

fibroblasts (Kuncio *et al.* 1991; Postlethwaite *et al.* 1987), and triggers a complex signal cascade, which involves processes leading to stimulate fibrosis (Alvarez *et al.* 1992; Miyajima *et al.* 2000; Chandrasekhar and Harvey 1988; Roberts *et al.* 1992; Border and Noble 1994; Malyankar *et al.* 1997). TGF-ß1's interacts with other known growth factors and inflammatory mediators, such as EGF and ANGII which have been shown to be contributed to the obstruction-induced tubulointerstitial fibrosis (Kaneto *et al.* 1993; Chevalier and Cachat 2001; Pimentel, Jr. *et al.* 1993; Ishidoya *et al.* 1995; Ricardo *et al.* 1996).

ANGII : ANGII production is rapidly stimulated following the onset of ureteral obstruction and has been linked to many of the pathophysiologic processes involved in ureteral , including alterations in renal hemodynamics, fibrosis, and apoptosis (Klahr 1991; Klahr *et al.* 1988). The most of the damaging effects of ANG II are mediated by the AT1 receptor (Klahr and Morrissey 2002). Studies performed in AT_1a receptor knockout mice or after of ANGII inhibition have reported that collagen expression and deposition decreases supporting the important role of ANGII in the renal fibrosis induced by urinary tract obstruction (Klahr and Morrissey 1997; Morrissey and Klahr 1998b).

TNF-a: TNF-a is a potent pro-inflammatory cytokine implicated in the pathophysiology of a wide variety of renal and glomerular diseases, including ischemia–reperfusion injury, diabetic nephropathy, lupus nephritis and in obstructive nephropathy (Donnahoo *et al.* 1999; Donnahoo *et al.* 2000; Ortiz *et al.* 1995). ANG II in turn stimulates TNF-a-related pathophysiological events leading to renal fibrosis, and together they can account for 70–80% of the pathophysiological changes in obstructive nephropathy (Guo *et al.* 2001).

NF-κB : An increase in obstruction-induced NF-κB activation has been correlated with an increase in tubular and interstitial proliferation as well as interstitial fibrosis (Miyajima *et al.* 2003; Morrissey and Klahr 1998a). NF-κB activation up-regulates the expression of a number of chemokines and cytokines implicated in the fibroproliferative changes of ureteral. NF-κB appears to be an important upstream regulator of ANG II expression in obstructive renal injury, and the role of ANG II in renal fibrosis is well established.

2.2 Erythropoietin (EPO)

EPO is an essential growth and survival factor for erythroid progenitor cells. The kidneys are the main sites of EPO production in adult humans. The liver contributes to EPO production by approximately 5%. EPO is not stored in vesicles but is produced on demand following hypoxic stimulus, resulting in extensive EPO mRNA expression. Hypoxia inducible factor-1 (HIF-1) is the most important transcription factor for hypoxic stimulation of EPO production, leading to increased EPO gene transcription.

2.2.1 Structure of EPO

EPO is an essential growth factor for the regulation and differentiation of erythrocytic progenitor cells in bone marrow with molecular mass of 30.4 kDa (Jelkmann 2004). The carbohydrate portion amount to 40% of its mass (Bauer *et al.* 1989). It circulates in plasma of the human with 165 amino acids with three N-linked and one O-linked acidic oligosaccharide side chains (Fisher 1997). The O-linked oligosaccharide is present in human EPO but not in rodent EPO (Wasley *et al.* 1991; Higuchi *et al.* 1992) and therefore it appears to lack functional importance (Jelkmann 2004) while the 3 N-linked oligosccharides are essential in EPO stabilizing in the circulation (Takeuchi *et al.* 1989; Misaizu *et al.* 1995). Two disulfide bridges are formed between cysteine 7 and 161 and between cysteine 29 and 33. Reduction of the disulfide bridges results in complete loss of bioactivity (Sasaki *et al.* 2000; Jelkmann and Hellwig-Burgel 2001; Bauer *et al.* 1989). EPO in plasma is relatively heat and pH stable (Jelkmann 1992). EPO human gene is located on chromosome 7 and is composed of four introns and five exons (Jelkmann and Hellwig-Burgel 2001; Powell *et al.* 1986; Lin *et al.* 1985).

2.2.2 Sites of EPO production, metabolism and clearance

EPO is predominantly produced by the kidney in the adult and to a smaller extent by the liver whereas the liver is the main site of EPO production in the fetus (Sasaki *et al.* 2000; Jelkmann 1992). Minor amounts of EPO mRNA are expressed in spleen, lung, testis and brain (Tan *et al.* 1992; Fandrey and Bunn

1993). EPO is produced by the peritubular fibroblasts in the renal cortex and outer medulla (Maxwell *et al.* 1997; Bachmann *et al.* 1993; Eckardt and Kurtz 2005). Morphometric quantitation of EPO-producing cells in the renal cortex revealed an approximately exponential increase in the number of EPO-producing cells as the hypoxic stimulus increased. The greater the hypoxic stimulus the larger number of cortical-medullary junction containing EPO-producing cells (Koury *et al.* 1989).

Serum EPO level in health human range from 5 to 20 U/l (Jelkmann 1992; Rege *et al.* 1982; Garcia *et al.* 1979). This level can increase under anaemia and hypoxia conditions up to thousand-fold (Jelkmann 1992). EPO has a physiological circadian rhythm, with maximum in the midnight and minimum at noon (Cotes and Brozovic 1982; Marti *et al.* 1996). The serum EPO concentration is similar in healthy women and men (Jelkmann and Wiedemann 1989; Cotes 1982; Mason-Garcia *et al.* 1990). The aging process does not influence the physiological diurnal fluctuations (Hellebostad *et al.* 1988) (Pasqualetti and Casale 1997). The half time of intravenously applicated homologous EPO was calculated to be 1,5 hour (h) in rats (Emmanouel *et al.* 1984; Naets and Wittek 1969). In humans, the half time of EPO after exposure to hypoxia is calculated with an average of 5.2 h (Eckardt *et al.* 1989). But this may be overestimated, because the production of EPO does not cease immediately at the end of a hypoxia stimulation but continues for nearly 2 h (Kubatzky *et al.* 2001; Cahan *et al.* 1990).

Like other glycoprotiens, EPO is cleared relatively slowly from the blood (Jelkmann 1992). EPO is predominantly metabolized by the liver (Dinkelaar *et al.* 1981). Less than 10% of EPO is excreted by the kidney (Rosse and Waldmann 1964; Jelkmann 1992).

2.2.3 Stimulation of EPO production

2.2.3.1 Regulation of EPO production by HIF

EPO expression is known to be controlled by several transcription factors and thus possesses several regulatory DNA elements. Of primary importance are hypoxic response elements (HRE) in the 3`-EPO enhancer to which specific heterodimeric hypoxia-inducible transcription factors (HIF-alpha/beta) can bind to the non-coding DNA (Grenz *et al.* 2007). HIF are heterodimers composed of alpha and beta (ARNT-subunit) subunits that belong to the basic helix-loop-helix-PAS super family (Hara *et al.* 2001). There are at least three subtypes of the HIF-alpha subunit (-1 alpha, -2 alpha, -3 alpha) (Jelkmann 2004). HIF 1 alpha/beta is generally considered the primary mediator of hypoxia-induced EPO gene expression (Wang and Semenza 1993; Wang *et al.* 1995). mRNA levels of HIF-1alpha and HIF-1beta remain essentially unaltered by the induction of hypoxia (Wood *et al.* 1996; Gradin *et al.* 1996). HIF-1beta remains abundant irrespective of oxygen (O_2) concentration and present constantly in the nucleus (Huang *et al.* 1996). HIF-1alpha is not detectable in normoxic cells (Jewell *et al.* 2001). Hypoxia induces a striking increase in the abundance of HIF-1a protein. Thus, accumulation of HIF-1alpha is a prerequisite to the activation of HIF-1. The activation of HIF-1 depends primarily on hypoxia-induced stabilization of HIF-1alpha, which is otherwise rapidly degraded in normoxic cells (Huang *et al.* 1998; Huang *et al.* 1996). HIF-alpha possesses two oxygen-dependent proteolytic degradation domains (ODD) and two terminal transactivation domains (TADs). In the presence of O_2, the alpha-subunit is rapidly prolyl- and asparaginyl-hydroxylated, targeted by the von-Hippel-Lindau protein/E3 ubiquitation ligase complex, and degraded by the ubiquitin-proteasome system (Ivan *et al.* 2001). In hypoxia, however the iron containing prolyl-hydroxylases are inactivated, thus HIF-1alpha abundance increases and can subsequently enter the nucleus and to form the active heterodimer with HIF-1beta.

In cells under normoxia, the ODD domain confers an extremely short half-life (>5 minutes) of HIF-1alpha while under hypoxia the half-life of HIF-1alpha reaches 30 minutes. Under hypoxic conditions HIF-1alpha is not subject to ubiquitination and proteasomal degradation (Semenza 2001). Thus, both the half-life and specific activity of HIF-1alpha are determined by its oxygen-dependent hydroxylation, which provides a direct mechanism for the transduction of changes in O_2 partial pressure (pO_2) to the nucleus, leading to changes in gene transcription (Semenza 2007). See figure 5 for further explanation.

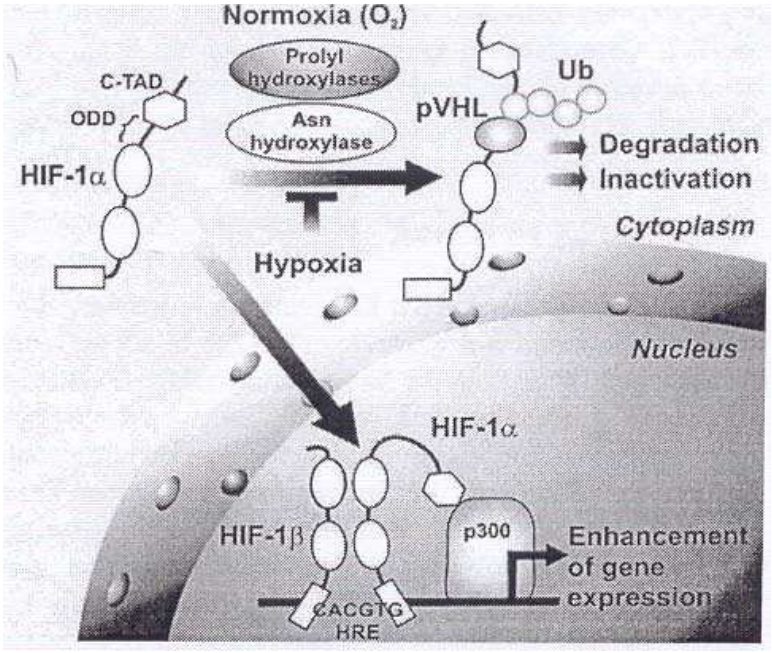


Figure 5. Regulation of HIF by prolyl and asparaginyl hydroxylation. In normxia, prolyl-hydroxylation in the oxygen-dependent degradation domain (ODD) results in binding of von-Hippel-Lindau protein (pVHL) and the ubiquitin ligase complex (UB) with subsequent proteasomal degradation and asparaginyl hydroxylation in the C-terminal transactivation domain (C-TAD) prevents binding of the p300/CBP transcriptional coactivator. In hypoxia, HIF1 alpha enters the nucleus to form the active transcription complex with p300/CREP and HIF1 beta (Jelkmann 2004).

2.2.3.2 Hypoxia stimulation of EPO production

Tissue hypoxia is the primary stimulus of EPO production. The concentration of red blood cells is not a directly controlled variable in the feedback regulation of EPO production. Instead, the blood O_2 availability is the trigger. In turn, this depends upon the O_2 capacity of the blood, the O_2 tension, and the O_2 affinity of the blood. In addition, the rates of the local blood flow and of the cellular O_2 consumption also contribute to local pO_2 (Jelkmann 1992).

Plasma EPO activity increases exponentially with lowered O_2 carrying capacity of erythrocyte-Hb. Multiple studies in chronically anaemic humans have shown that there is an inverse log/linear relationship between serum EPO activity and hemoglobin (Hb) concentration (Baer *et al.* 1987; Cotes 1982) or hematocrit (Erslev *et al.* 1987; Erslev *et al.* 1980). Acute haemorrhage leads to increase EPO production in humans (Mayeux *et al.* 1990; Adamson 1968) and animals (Beru *et al.* 1986; Bondurant and Koury 1986).

On lowering the inspiratory pO_2 tension, plasma EPO increases exponentially with the degree of hypoxemia in humans (Abbrecht and Littell 1972) and animals (Schooley and Mahlmann 1972). Both sojourners and natives living at high altitude exhibit elevated red blood cell counts (Lenfant and Sullivan 1971). A fall in serum EPO has been observed in anaemic patients inspiring almost pure O_2 (Embury *et al.* 1984).

EPO plasma levels were shown to rise in rats following exchange transfusion of normal blood with high-O_2 affinity (Lechermann and Jelkmann 1985). In the other hand, a lowering of the affinity of the blood is associated with decreased EPO levels, such as in patients with sickle cell anaemia (Macdougall *et al.* 1989). Patients with sickle cell anemia who have red cells with low O_2 affinity produce less EPO at a given Hb concentration than patients with non-hemoglobinopathy anemias (Sherwood *et al.* 1986).

The exposure to carbon monoxide (CO) constitutes a clear example for the increase of EPO production associated with an increased O_2 affinity to Hb. This effect is the result from the high binding affinity of CO to Hb and the reduced O_2 dissociation from Hb causing a reduction of pO_2 in the tissue (Gorman *et al.* 2003). As result, a long functional anemic hypoxia will arise. Thus, CO exposure constitutes a well established experimental method for stimulation of EPO production in human and animals (Caro *et al.* 1981; Fried *et al.* 1984).

CO does not only decrease the dissociation of O_2 from Hb but is also released from Hb at low rate (250-fold) (Dolan 1985). Thus, intoxication with CO is long lasting. In addition, CO binds to intracellular myoglobin in the myocardium and impairs the oxidative phosphorylation in mitochondria. The affinity of CO for myoglobin is even greater than for Hb (Blumenthal 2001). It interferes with cellular metabolism not only by binding to Hb and myoglobin but also by the inhibition of cytochrome a_3 and cytochrome P-450 (Greingor *et al.* 2001; Hawkins *et al.* 2000). In the presence of carboxy hemoglobin (COHb), the dissociation curve of the remaining HbO_2 shifts to the left further decreasing the amount of the O_2 released (Prockop and Chichkova 2007).

The clinical symptoms of CO poisoning are often non-specific and can mimic a variety of common disorders. The most important effect of CO is tissue hypoxia. This effect is most significant in areas of high blood flow and high O_2 demand. For this reason, heart and brain are the tissues at greatest risk in CO intoxication (Ilano and Raffin 1990). After inhalation of CO, the body reacts by increasing blood levels of catecholamins and cortisol (Sylvester *et al.* 1979) and by increasing of heart rate and cardiac ejection fraction (Adams *et al.* 1973; Ayres *et al.* 1973). The diagnosis is normally made by measuring the percentage of COHb in blood samples. A low baseline level of 1%–3% COHb in venous blood is detectable under normal conditions (Ernst and Zibrak 1998). A small fraction from the baseline COHb levels is produced endogenously. The endogenous CO is produced as result of heme oxidation which is catalyzed by

the heme oxygenase system (Maines 1997). Endogenous CO can relax vascular smooth muscle through the pathway from CO to Cyclic guanosine monophosphate (cGMP) production (Guo *et al.* 2006). Tobacco smoke and exposure to CO in the environment elevate the baseline level (Varon *et al.* 1999). The normal levels of COHb can reach 5% in smokers, during pregnancy and in people with haemolytic anaemia. Heavy smoking can cause levels as high as 13% (Harper and Croft-Baker 2004). COHb levels do not correlate linearly with clinical symptoms. There is only limited correlation between the low COHb level with severity of clinical features, and cases have been reported of severe CO toxicity at only “low levels of poisoning” (Norkool and Kirkpatrick 1985). Thus, COHb levels are valuable for confirming CO exposure but cannot be used to stratify severity of poisoning, predict prognosis, or indicate a specific treatment plan (Hardy and Thom 1994) because the effect of CO in reducing O_2 delivery to the tissue is rather exponential than linear. EPO plasma concentration after CO exposure to produce 50% COHb was increased much more than EPO plasma concentration following a 50% reduction in blood O_2 carrying capacity was produced by isovolemic hemodilution.

Recent studies suggested antioxidative, anti-inflammatory, antiproliferative, anti-apoptotic, and vasodilating characteristics of low CO levels (Hoetzel and Schmidt 2006). In vivo, CO confers tissue protection in animal models of lung disease, including those with oxidative and inflammatory lung injury and ischemia/reperfusion injury. Furthermore, low-dose CO ameliorates vascular injury and reduces the rejection rate of lung and vascular grafts (Ryter and Choi 2006). Those effects are based on the ability of CO to depress the fibrinolytic axis (Fujita *et al.* 2001).

The kidney is the main site of EPO synthesis. Thus, local blood flow and the cellular O_2 consumption also contribute to intrarenal pO_2. The O_2 consumption of the kidney depends mainly on GFR and subsquent tubular sodium reabsorption. Increased plasma EPO levels were increased in animals with diminished RBF induced either surgically by renal artery constriction (Fisher and

Samuels 1967; Gross *et al.* 1976) or pharmacologically by application of vasoconstrictive agents such as ANGII (Fisher *et al.* 1971; Gould *et al.* 1973). It has been shown that decreasing O_2 supply to the kidney through reduction of RBF is less effective in increasing EPO production than reducing of Hb concentration (Pagel *et al.* 1988).

2.2.3.3 Other factors regulating EPO production

EPO expression is known to be controlled by several transcription factors and thus possesses several regulatory DNA elements. Of primary importance are HRE in the EPO prmotor to which HIF-alpha/beta can bind. Besides HIF, the EPO promotor has binding sites for GATA factors (Stockmann and Fandrey 2006). GATA-2 and GATA-3 are thought to suppress EPO secretion whereas GATA-4 induces EPO secretion by binding at the promotor (Blanchard *et al.* 1992). Moreover, the EPO promoter and the 5`-flanking region contain binding sites for NF-κB inhibiting EPO gene expression in inflammatory diseases (La *et al.* 2002). Pro-inflammatory cytokines, such as TNF-α and Interleukin-1, have also been shown to inhibit EPO expression (Stockmann and Fandrey 2006).
The increases of EPO secretion during limited O_2 availability are not affected by extracellular adenosine generation or signalling as recently shown by (Grenz *et al.* 2007). Renal nerves also contribute to the half-maximal EPO secretory response to CO exposure, possibly via Neuropeptid Y receptors (Gebhard *et al.* 2006). Thyroid hormones have been shown to enhance hypoxia induced EPO produdtion in isolated perfused rat kidney and HepG2 cells (Fandrey *et al.* 1994). EPO secretion was higher following administration of fenoterol, a ß2 receptor agonist, under normoxic conditions and following haemorrhage (Gleiter *et al.* 1997; Gleiter *et al.* 1998). Studies in human volunteers demonstrated that elevation of plasma renin activity and elevated ANGII level increase EPO concentration in dose-dependent manner. These effects were greatly reduced by administration of the ANGII receptor antagonist losartan (Freudenthaler *et al.* 1999; Gossmann *et al.* 2001).

2.2.4 EPO production and hydronephrosis

Previous studies have yielded conflicting data about EPO production by the hydronephrotic kidney. Reissmann et al. provided evidence suggesting that erythropoietin production is preserved after ureter ligation (Reissmann *et al.* 1960). Jelkmann et al. demonstrated normal response to hypoxia in the rat kidney with the ligated ureter after 4 days, as well as lack of the response after 3 months (Jelkmann *et al.* 1988). Maxwell et al. reported a diminished EPO secretory respoce to hypoxia in rats 5–10 days after ureter ligation and a reduced number of interstitial cells expressing the EPO gene (Maxwell *et al.* 1997). Nečas has shown an early but transient suppression of EPO gene responsiveness to hypoxia after ureter ligation (Necas and Ponka 1998).

2.2.5 Multiple physiological functions of EPO

2.2.5.1 EPO and erythropoiesis

EPO is the essential growth factor for the erythrocytic pogenitors, controls their proliferation, differentiation and survival as well. The most primitive EPO-responsive progenitor cells are the burst-forming unit-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E). CFU-Es are the most EPO-sensitive cells with the highest density of EPO receptors on their surfaces (Jelkmann 2004). EPO supports the survival of erythrocytic pogenitors cells and permits their final differentiation by the prevention of programmed cell death (Koury and Bondurant 1992; De *et al.* 1999).

The EPO receptor is a member of the cytokine class I receptor superfamily, exists as a dimer with 66-78 KD (Livnah *et al.* 1999). EPO binding to the receptor triggers a complex signal cascade which contains many transcription and signal transduction proteins (Bonanou-Tzedaki *et al.* 1987; Bouscary *et al.* 2003; Miura *et al.* 1994). Significant reticulocytosis becomes apparent about 3-4 days after an acute increase in plasma EPO (Jelkmann 2004).

2.2.5.2 Tissues protective functions of EPO

EPO has recently been shown to modulate cellular signal transduction pathways to perform multiple functions other than erythropoiesis. EPO has been

identified as a neurotrophic and neuroprotective agent in a wide variety of experimental paradigms, from neuronal cell culture to in vivo models of brain injury (Juul 2002). These neuroprotective effects have been demonstrated in different animal models (Bernaudin *et al.* 1999; Brines *et al.* 2000) and in patients with cerebral ischemia (Ehrenreich *et al.* 2002).

In addition, EPO has been shown to protect vascular integrity and promote angiogenesis, this role extends beyond the direct preservation of endothelial cells integrity and the formation of new blood vessels (Chong *et al.* 2002). The antiapoptotic effects of recombinant human erythropoietin (rHuEPO) on cardiomyocytes have been reported in tissue culture and in vivo animal models of ischemia-reperfusion injury (Bogoyevitch 2004; Smith *et al.* 2003).

2.2.6 Pathophysiology of EPO production

2.2.6.1 EPO production deficiency

End stage renal failure is the most common cause of EPO production deficiency (Kendall 2001). EPO concentrations are decreased in patients with malignant, infectious, autoimmune, or renal diseases (Means, Jr. and Krantz 1992; Jelkmann 1992). Therapy with rHuEPO has become a standard therapy for correction of renal and non-renal anemias. RHuEPO can correct the anemia in all patients with renal failure. Potential non-renal indications for rHuEPO include the anemias associated with cancer, autoimmune disease, AIDS, bone marrow transplantation and myeldoplastic syndromes (Jelkmann 2000). Darbepoetin alfa is an analogue of rHuEPO produced by recombinant DNA technology. It has a prolonged half-life and an enhanced in vivo biological activity compared with rHuEPO and permits a reduction in the administration frequency (Siddiqui and Keating 2006).

2.2.6.2 EPO overproduction

Excessive EPO production leads to secondary erythrocytosis. Because peripheral O_2 delivery is the main regulatory parameter, EPO production increases at lowered arterial O_2 tension at high altitude. Certain tumors can produce EPO and may lead to erythrocytosis, e.g. in renal carcinomas (Da Silva et al. 1990; Sufrin et al. 1977) and hepatocellular carcinomas (Kew and Fisher 1986; McFadzean et al. 1967). Enhanced EPO production can be also found in 8-18 % of the recipients of kidney transplants associated with post transplantation polycythemia (Gleiter 1996; Aeberhard et al. 1990). In contrast, polycythemia vera is associated with normal or lowered EPO levels (Garcia et al. 1982; de et al. 1981). Thus, polycythemia vera is caused by EPO-independent growth of erythrocytic progenitors from abnormal stem cell clones (Reid et al. 1988; Cashman et al. 1983).

2.3 Aim of thesis

There is no systematic analysis in the literature on EPO secretion in response to systemic hypoxia in rats subjected to ureteral ligation for various time intervals. Therefore, we wanted to establish a dose-response-relationship of EPO secretion in UUL rats following CO exposure and low O_2 (8%) breathing. We also wanted to assess the influence of different length of UUL from 6 to 72 hours and the recovery from UUL by stenting the ligated ureter to re-establish the urine flow.

3 Methods

3.1 Surgical procedures

Experiments were performed in Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 250 to 350 g. Rats had free access to standard rat chow (Altromin 1320, Altromin, Lage, Germany) and tap water. Experimental protocols were approved in accordance with the German Animal Protection Law.

3.1.1 Unilateral ureteral ligation (UUL)

All animals were anesthetised with intraperitoneal injection Ketamin (DeltaSelect, Pfullingen, Germany) 70 mg/kg body weight and Xylazin (Rompun 2%, Bayer Vital, Leverkusen, Germany) 15 mg/kg body weight. The animals were placed in a dorsal position on a servo-controlled heated table (Effenberger, München, Germany) to keep the rectal temperature constant at 37.2℃. When corneal reflex and painful stimuli wer e no longer observed, lower midline abdominal incision was made to expose the bladder and the ureter. The right urerter was carefully isolated from connective tissues and ligated in the upper third. Thereafter, the incision was closed with sutures of 3-0 vicryl thread (Johnson & Johnson Jult, Brusseles, Belgium). Duration of this procedure took 10-12 minutes. Rats were allowed to recover from surgical procedure in a warmed cage until they began restoring the normal behavioural activities. In the sham-operated rats the ureter was carefully dissected from surrounding tissues but not ligated.

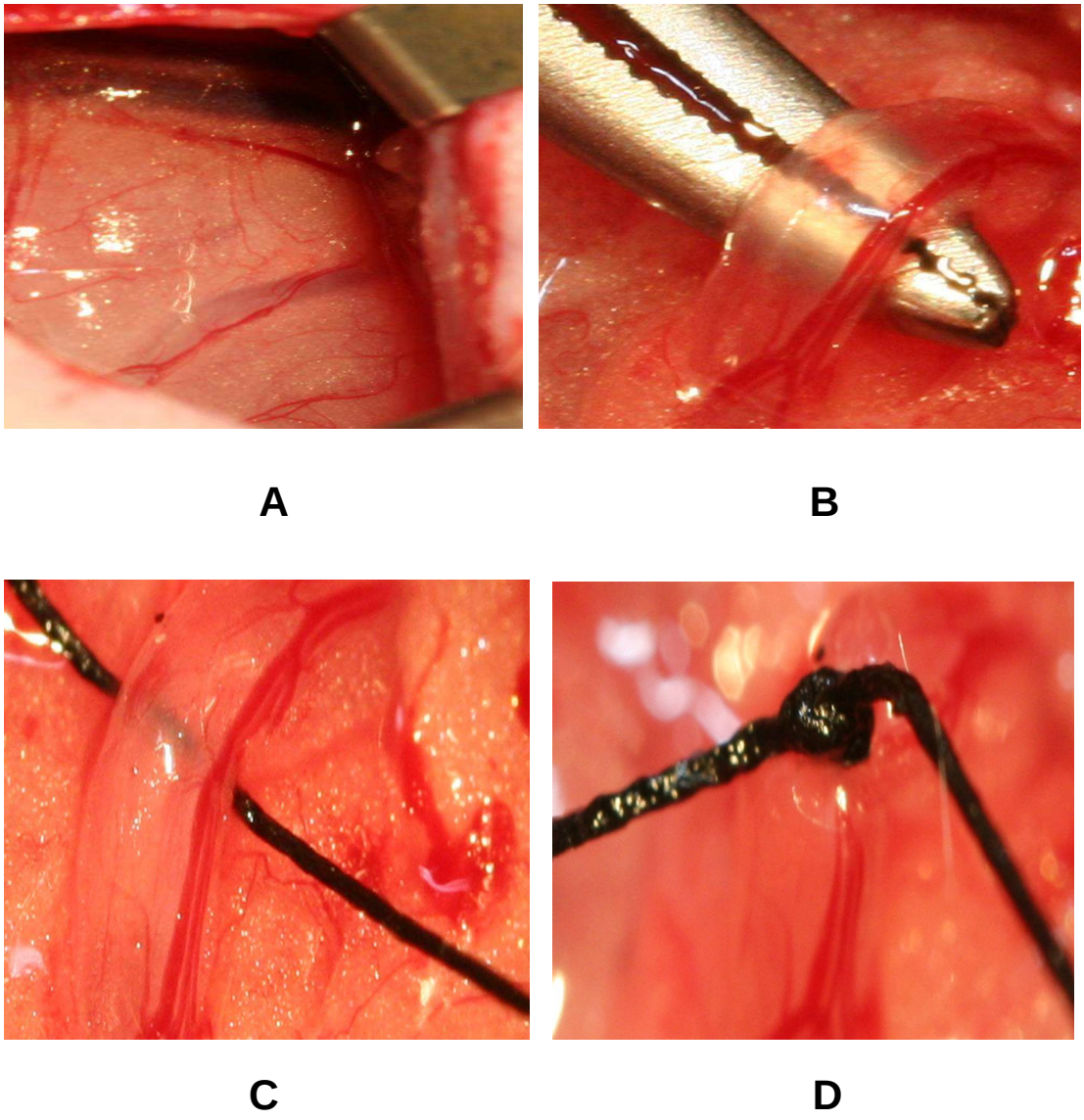


Figure 6. Steps of UUL A: exposure of the right ureter; B: isolation of the ureter; C and D: ligation of the ureter at the upper third.

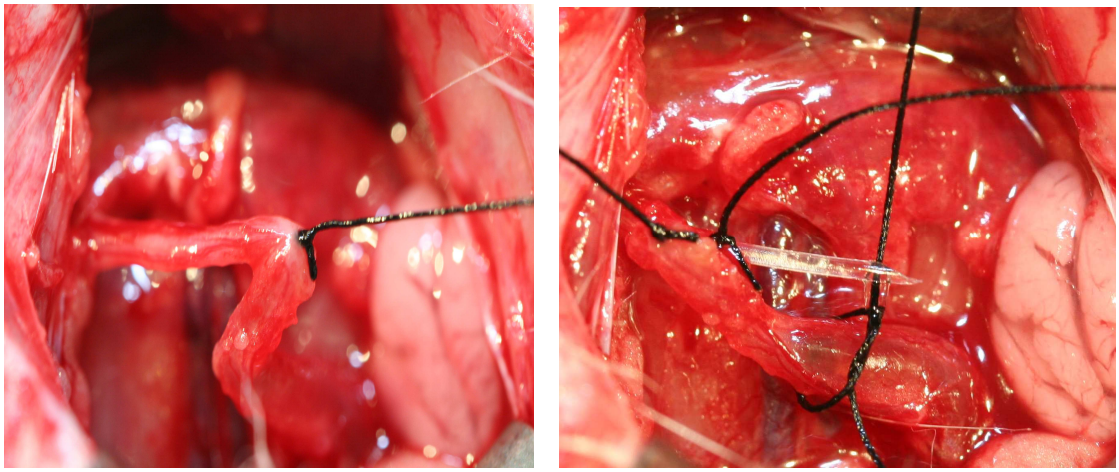
3.1.2 Contralateral nephrectomy

In order to assess the EPO secretory response of the ligated right ureteral kidney, the left kidney was excised 6 hours before the animals were challenged with CO or 8% O_2. Anesthesia was induced as described above (3.1.1). Left flank incision was made and the kidney was carefully isolated from connective tissue, the adrenal gland was left intact. Renal vessels and ureter were isolated

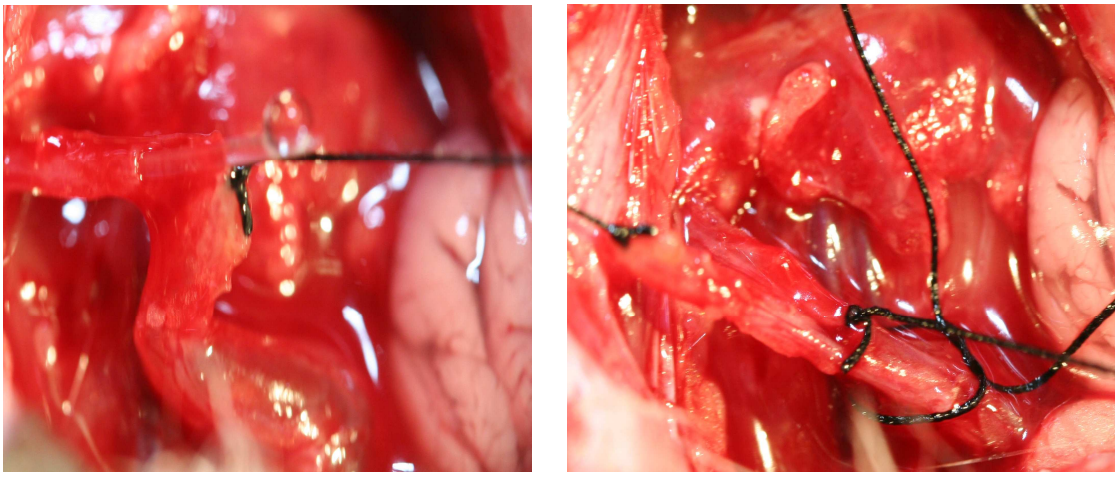
and ligated and the left kidney was removed. The incision was closed with 3-0 vicryl sutures. The animals were allowed to recover from surgical procedure in a warmed cage until they began restoring the normal behaviour activities.

3.1.3 Re-establishment of urine flow after UUL

Aim of this procedure was to asses CO-stimulated EPO secretion after relief of the ureteral obstruction. Anesthesia was induced a described above (3.1.1). The animals were placed in a dorsal position on a servo-controlled heated table to keep the rectal temperature constant at 37.2℃. The midline incision was reopened and the ligated segment of the ureter was carefully exposed and excised. Thereafter a small polyethylene tubing (5-7 mm in length, 0,4 mm Ø) was placed into the open ends of the distal and proximal part of the ureter. Figure 8 illustrates this recanulating procedure. The re-establishment of urine flow by stenting the ligated segment of the ureter was completely achieved and lasted for at least 5 days.



A **B**



C D

Figure 7. Steps of re-establishment of urine flow by canulation of the ligated ureter. A: exposure of the ligated ureter segment; B and C: incision of the ureter proximal of its ligation site and insertion of a stent; D: insertion of the stent into the distal segment of the ureter which resulted in unrestricted urine flow.

3.1.4 Measurement of plasma EPO and EPO mRNA

At the end of the exposure to hypoxic stimuli the rats were anesthetised as described above (3.1.1). Blood obtained by intracardiac puncture was collected with pre-heparinized syringes (Braun, Germany). The blood was transferred to pre-cooled Eppendorf cups (2 ml, Eppendorf) and centrifuged (Eppendorf centrifuge 5415R) at 4000 rpm, 4℃ for 10 minutes. The obtained plasma was stored at -80℃ until EPO measurement. The kidney was quickly removed and shock-frozen with a clamp pre-cooled to the temperature of liquid nitrogen. The clamped kidneys were then stored at -80℃ till further analysis.

3.1.5 Exposure of rats to Hypoxia

3.1.5.1 Hypoxic Cage

The conscious animals were placed in a specially designed ventilated chamber. This chamber was manufactured by Mr. Stieler in the Institute of Pharmacology

and Toxicology of Tübingen University. The chamber consists of transparent Plexiglas and allowed to expose eight rats simultaneously. A perforated wall has been placed in the chamber dividing it to two compartments allowing separation of different animals if required. The cage was used to apply two types of hypoxia. The first type is based upon tissue hypoxia by exposing the animals to different concentrations of CO (0, 400, 600, 800, 1000 ppm) for 4 h. The second type was hypobaric hypoxia by exposing the animal to O_2 8% for 2 h. The actual CO concentration in the cage was monitored and recorded by a CO sensor (Testo GmbH & Co, Reutlingen). CO flow was regulated with two valves placed on the CO line and air line. Before the animals were placed into the chamber the CO concentration was adjusted at the required concentration. Two apertures at the top of the cage allow to place the animals into the cage and taking them out with only minor fluctuation of CO/O_2 concentration as shown in figure 8.

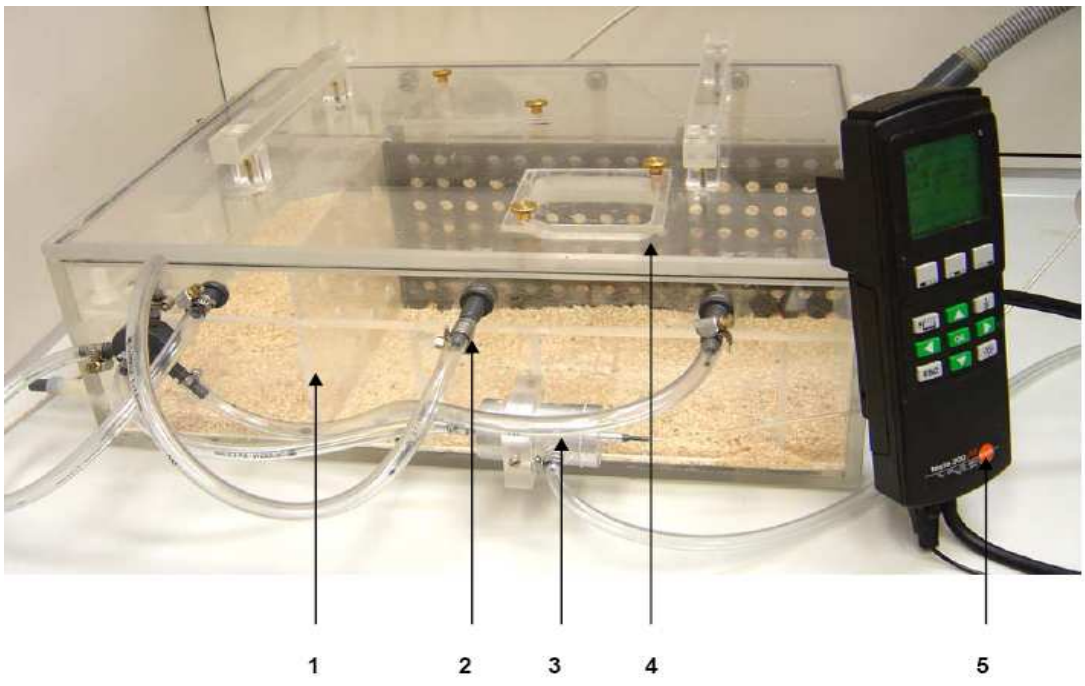


Figure 8. The hypoxia cage: 1. dividing perforated wall. 2. gas inlet. 3. mixer jet. 4. inlet for rats. 5. CO and oxygen sensor device.

3.2 Experimental protocols

3.2.1 Time dependency

The time course of the experimental design is depicted in figure 9.

EPO production was studied depending on the duration of ureteral obstruction under CO exposition. For UUL we choose a period of 6, 12, 24 and 72 h before CO exposure. 6 h before CO exposure, the contralateral kidney was removed under anaesthesia as described in (3.1.1). This procedure was applied in order to prevent the response of the intact kidney to CO since this response would mask EPO secretion by the UUL kidney. The animals took 2-3 h until they recovered from the anaesthesia. Thereafter hypoxia was induced by exposing animals to CO at concentration of 600 ppm for 4 h. Immediately after 4 h CO exposure the rats were anaesthetized, blood was taken and kidney were excised. Sham-operated rats were subjected to left nephrectomy without UUL under the same anaesthesia.

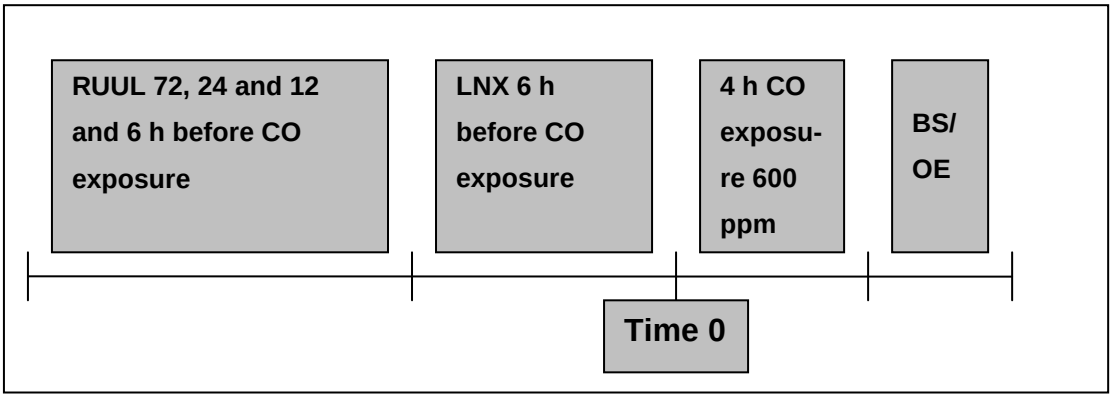


Figure 9. Time intervals of the different experimental types: RUUL (right unilateral ureteral ligation) 6, 12, 24 and 72 h before CO exposure, LNX (left nephrectomy) 6 h before CO exposure and then 4 h CO exposure, BS/OE blood taking and kidney excision.

3.2.2 Dose dependency

The dose dependent effect of hypoxia (CO exposure) on the plasma level of EPO was studied in the hydronephrotic rats and their respective controls. The hypoxia degree was changed by altering the CO dose. UUL was performed 24 h before CO exposure. 6 h before CO exposure the contralateral kidney was removed. EPO production was stimulated by exposing the animals for 4 h to different CO concentrations (0, 400, 600, 800, 1000 ppm). After CO exposure the rats were anaesthetized, blood was collected and kidney was excised. The time course of the experimental design is depicted in figure 10.

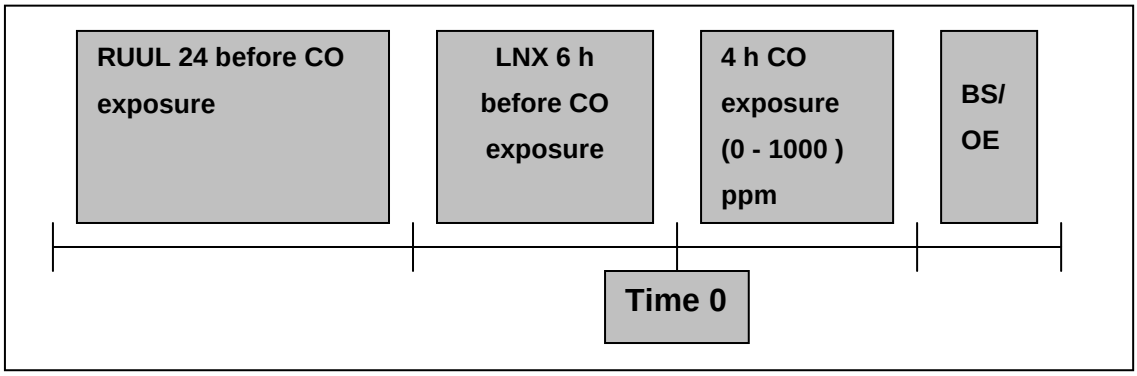


Figure 10. Dose dependency experimental design: RUUL (right unilateral ureteral ligation) 24 h before CO exposure, LNX (left nephrectomy) 6 h before CO exposure and then 4 h CO exposure; BS/OE blood taking and kidney excision.

3.2.3 Effect of systemic hypoxia by low pO_2

The time course of the experimental design of this study is depicted in figure 11. UUL was performed 24 h before hypobaric hypoxia. 6 h before hypobaric hypoxia the contralateral kidney was removed. Hypobaric hypoxia was induced by exposing animals to O_2 8% for 2 h. Thereafter rats were allowed to breath room air for 2 h and then blood was sampled and kidney was excised.

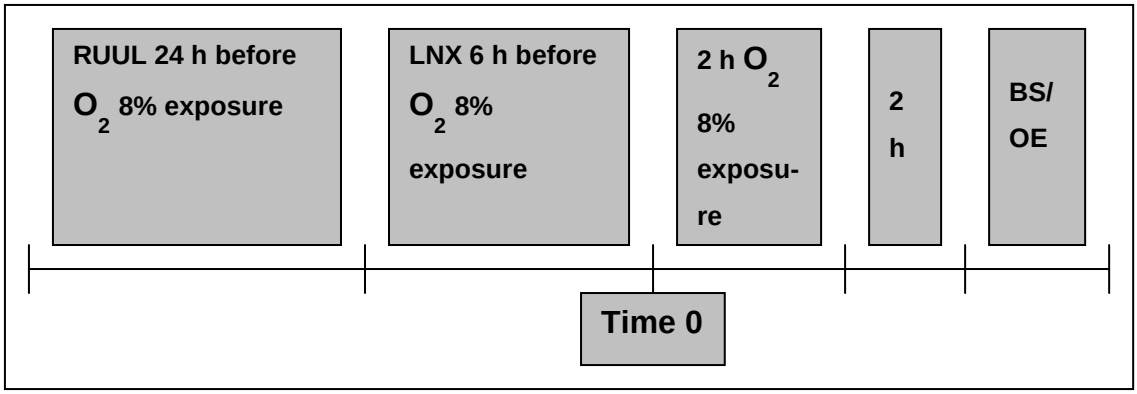


Figure 11. Experimental design for low O_2 exposure: RUUL (right unilateral ureteral ligation) 24 h before 8% O_2 exposure, LNX (left nephrectomy) 6 h before 8% O_2 exposure and then 2 h O_2 8% exposure, 2 h room air breathing, BS/OE blood taking and kidney excision.

3.2.4 Re-establishment of urine flow after UUL

3.2.4.1 24h UUL plus 24h or 72h recovery phase

The time course of the experimental design is depicted in figure 12. Right ureter was obstructed as described in 3.1.1. 24 h later the ureter was released and urine flow was re-establishment as described in (3.1.3). The animals were allowed to recover from the obstruction for 24 h and 72 h in two separated groups. Thereafter, NX was done and the animals were allowed to recover from the surgery for 6 h. Hypoxia was induced by placing the animals in CO chamber and exposing them to CO 600 ppm for 4 h. Thereafter, blood was sampled and kidneys were excised.

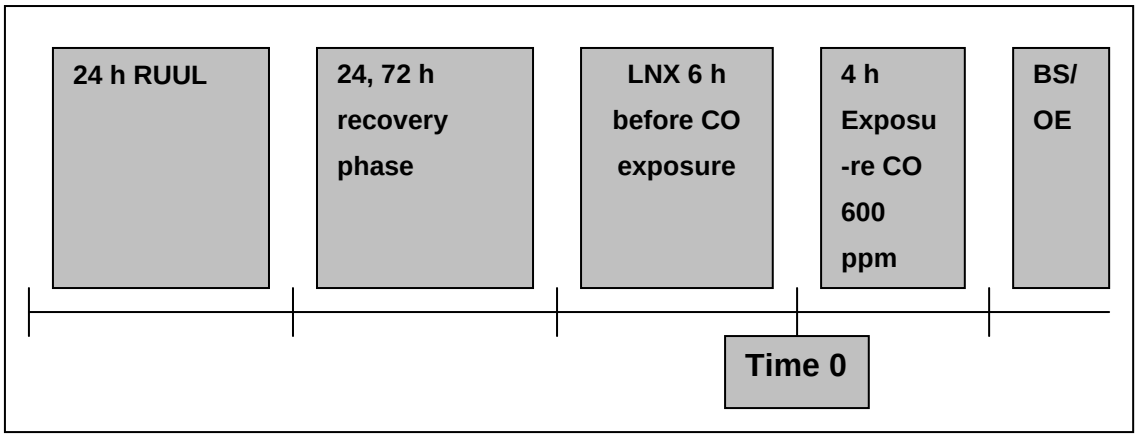


Figure 12. Re-establishment of urine flow experimental design: RUUL (right unilateral ureteral ligation) for 24 h, surgical insertion of a ureteral stent followed by 24 or72 h of recovery phase, LNX (left nephrectomy) 6 h before CO exposure, 4 h CO exposure, BS/OE blood sampling and kidney excision.

3.3 Analytical procedures

3.3.1 EPO in Plasma

Plasma EPO concentrations were determined via quantitative enzyme linked immunoassay (R&D Systems, GmbH, Wiesbaden-Nordenstadt Germany) according to the manufacturer's instructions.

3.3.1.1 Principle of the Assay

The assay is based on the double-antibody sandwich method. A monoclonal antibody specific for rat EPO has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and the EPO being present will be bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for rat EPO is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The amount of color generated is directly

proportional to the amount of conjugate bound to the EPO antibody complex, which in turn is directly proportional to the amount of EPO in the specimen or standard. The sample values are then read off the standard curve. The absorbance of this complex is measured with the program Magellan 3 by photometer (Tecan, A-5028 Austria) set to 450nm wavelength. A standard curve is generated by plotting absorbance versus the concentration of EPO standards. The EPO concentration of the unknown specimen is determined by comparing the optical density of the specimen to the standard curve. EPO plasma levels were expressed in pq/ml.

3.3.2 mRNA in renal tissue

3.3.2.1 Isolation total RNA and mRNA

The hypoxic tissue was given in mortar mixed with liquid nitrogen and crushed. Powdered tissue (100 mg) was taken to isolate total RNA, the rest was stored at -80℃ till further analysis. Total mRNA was extract ed from the clamped hypoxic kidney tissue with RNA pure (PeqLab, Erlangen), tissue probes were weighted and the total mRNA was isolated with this way, 100 mg powdered tissue was mixed with 1 ml peqGold RNA-Pure and in Potter homogenated. The homogenate was incubated at room temperature for 5 minutes afterwards 200 µl Chloroform was added to 1 ml homogenate. The mixture was centrifuged with 12 000 × g (4℃) for 5 minutes. The supernatant was transferred to Eppendorf cup.

To precipitate RNA, 0.5 ml isopropanol was added pro ml supernatant and incubated 10 minutes at room temperature. Then centrifugation for 10 minutes was performed with 12 000 × g (4℃). The supernatan t discharged and the obtained pellet was mixed with per 1 ml 75 % ethanol then centrifuged (10 min; 12 000 × g; 4℃) and washed twice. The extracted RN A was absorbed in 60 µl DEPC-Water and warmed in water bath at 60℃ for 10 minutes. The RNA solution was stored at -80℃.

3.3.2.2 RNA Quantification

RNA-solution (1 µl) was added to199 µl $NaHPO_4$ 2mM and the optical solution density was measured photometretically with 260 nm wavelength. The concentration was calculated depending on the following equation:

Concentration RNA [mg/ml] = $Extinction_{260nm}$ × 0.04 mg/ml × dilution factor

3.3.2.3 c DNA synthesis

Reverse transcription was performed with 300 ng of total RNA as template and 0.25 µg random hexamers (promega) as primer using 12.5 U AMV reverse transcriptase (PeqLab, Erlangen) in a total volume of 20 µl (20℃ for 10 min, 42℃ for 1 h and 85℃ for 5min).

3.3.2.4 Real-time–PCR analysis

For LightCycler reaction, a master mix of the following reaction components was prepared to the indicated end concentration: 12.6 µl H_2O, 2.4 µl $MgCl_2$ (4 mM). 0.5 µl forward primer (0.5 µM), 0.5 µl reverse primer (0.5 µM), and 2.0 µl fast start DNA Master SYBR Green I (Roche Diagnostics). Two microliter c DNA was added to 18 µl LightCycler master mix as PCR template. The primer sequences for EPO gene is given as follows:

Gene	primer Sequences (F, Forward; R, Reverse)	Product size (bp)
EPO	F: CAC GAA GCC ATG AAG ACA GA R: GGC TGT TGC CAG TGG TAT TT	100

Following LightCycler experimental run protocol was used: denaturation program (95℃ for 10 min), amplification, and quant ification program repeated 45 times (95℃ for 10 s “touch down PCR” 68℃ -58℃ with a step size of 0.5℃ /cycle for 10 s, 72℃ for 16 s with a single fluore scence measurement) and

finally, a cooling step at 37℃. “Fit point method” was performed in the LightCycler software 3.5.3 (roche). Gene expression was calculated according to a mathematical model for relative quantification depending on the following method (Pfaffl 2001):

$$\text{Ratio} = \frac{(E_{tar})^{\triangle CP_{tar}(\text{control-sample})}}{(E_{ref})^{\triangle CP_{ref}(\text{control-sample})}}$$

The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene. E_{tar} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{tar} is the CP deviation of control – sample of the target gene transcript; ΔCP_{ref} is the CP deviation of control – sample of reference gene transcript. The reference gene could be a stable and secure unregulated transcript, e.g. a house- keeping gene transcript.

3.4 Statistical methods

All values are presented as means ± SEM. Statistical significance was determined by one way analysis of variance (ANOVA) using the program JMP 7.

4 Results

4.1 Effect of UUL duration on EPO secretion

The time course of CO-stimulated EPO production by the hydronephrotic kidney is shown in Figure 13. The major finding was that EPO secretion is reduced by 70% after 24h of UUL Compared with the sham group, with further reduction (to values <10%) after 72 h of UUL. However, within (6-12h) EPO plasma levels were even higher than sham group but they do not reach statistical significance. The changes in EPO plasma level were parallel to changes in EPO mRNA content of the renal hypoxic tissue.

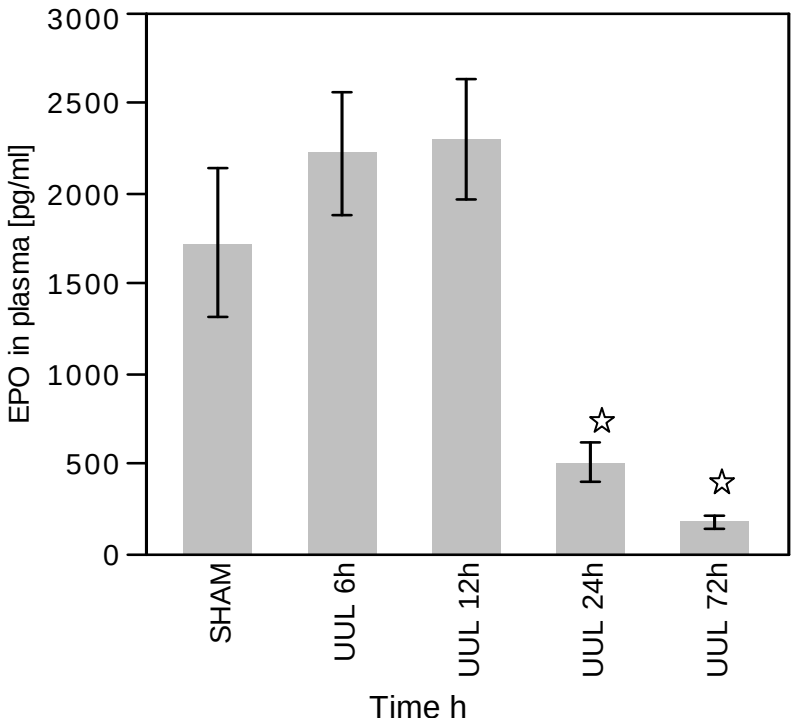


Figure 13. Time course of EPO plasma levels following exposure to CO (600 ppm) for 4 hours in sham or hydronephrotic kidney (UUL) (*p<0.01 vs. SHAM). Note that EPO plasma level after 72 h UUL is nearly absent.

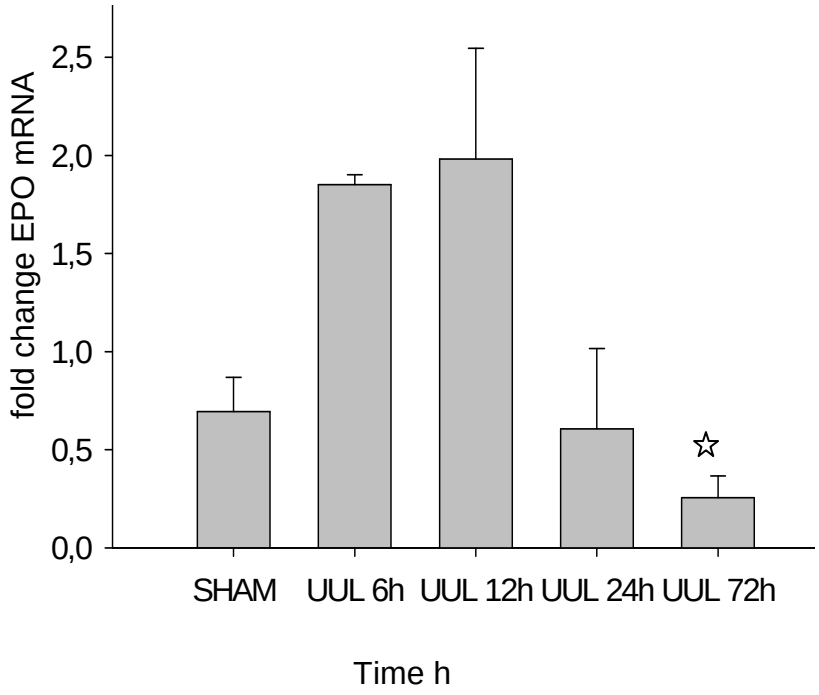


Figure 14. Time dependent changes of EPO mRNA expression in renal tissue UUL rats (*p<0.01 vs. SHAM). It is obvious that the time dependent changes in EPO plasma concentration is paralleled by the changes in EPO mRNA.

4.2 Dose dependent EPO response following CO exposure

The capacity of the hydronephrotic kidney to secrete EPO was studied following increased CO concentration. As shown in figure 14, EPO plasma level is significantly decreased by 70% after 24 h UUL compared to sham operated rats. Based on these time dependency experiments, all dose dependency experiments were performed using the 24 h UUL group.

The capacity of EPO production in response to different CO concentrations is significantly impaired as shown in figure 15.

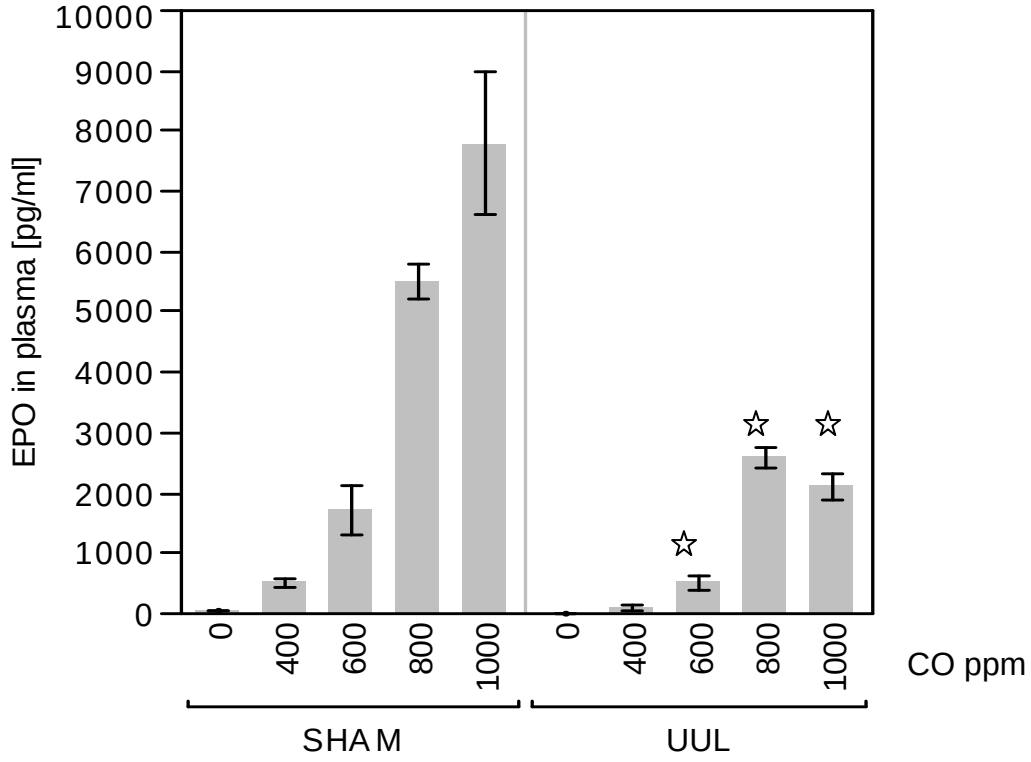


Figure 15. EPO plasma levels following 4 h exposure to increasing concentration of CO (*p<0.01 vs. SHAM). Note that UUL reduces EPO secretory response to large extent.

4.3 Comparison of EPO production of the hydronephrotic kidneys following CO and low pO_2 exposure

EPO secretion is induced by CO exposure and hypobaric hypoxia. These studies provide feasibility to investigate EPO secretion in two models of limited O_2 availability. After having demonstrated a similar EPO secretory response after 24 h of UUL under exposure to different doses of CO, we next exposed these rats to hypobaric hypoxia. After 24 h UUL EPO secretion is reduced by 70% after stimulation by 8% O_2 for 2h as shown in figure 16. EPO production decreases to the same extent by 70 % after 24 UUL in the both two models of limited O_2 availability (hypobaric hypoxia and functional anemic hypoxia).

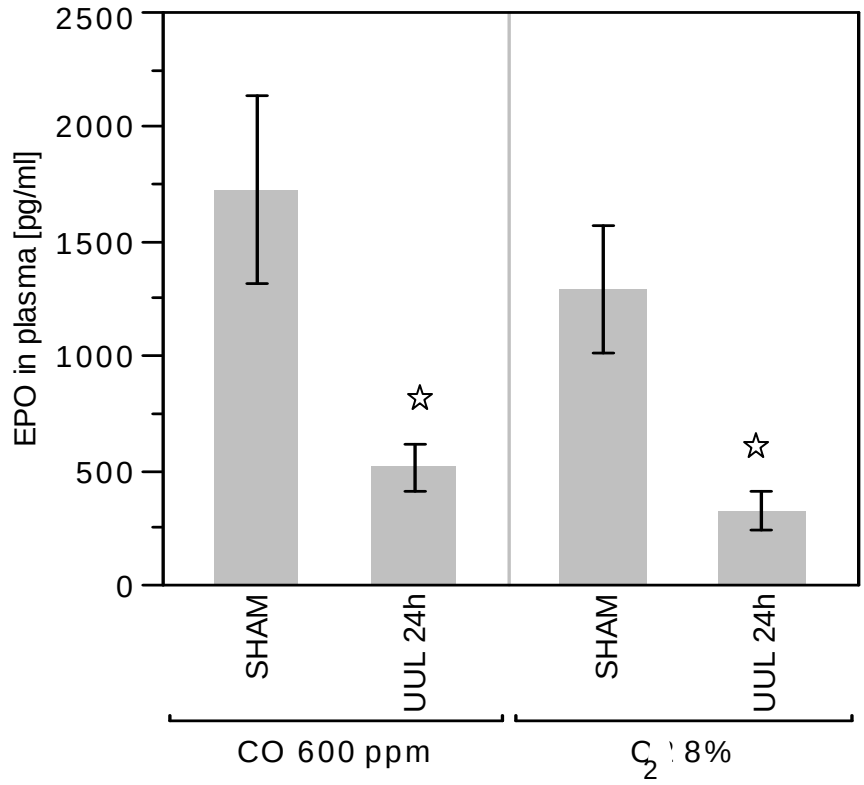


Figure 16. Comparison of EPO in plasma after 24h UUL and control groups after 4 h stimulation by CO 600 ppm and 2 h hypobaric hypoxia stimulation (*p<0.01 vs. SHAM).

4.4 EPO plasma levels after re-establishment of urine flow

The recovery of EPO secretion after relief of UUL was assessed. The ureter was ligated for 24 h. Afterwards the obstruction was released by stenting the ligated ureteral segment and the animals were subjected to recovery phase for 24 and 72 hours. The major finding was that 72 h after re-establishment of urine flow, the EPO secretory response to CO recovers up to 50%. Whereas after 24 h of re-establishment of urine flow no recovery of EPO secretory response to CO was noted as shown in figure 17.

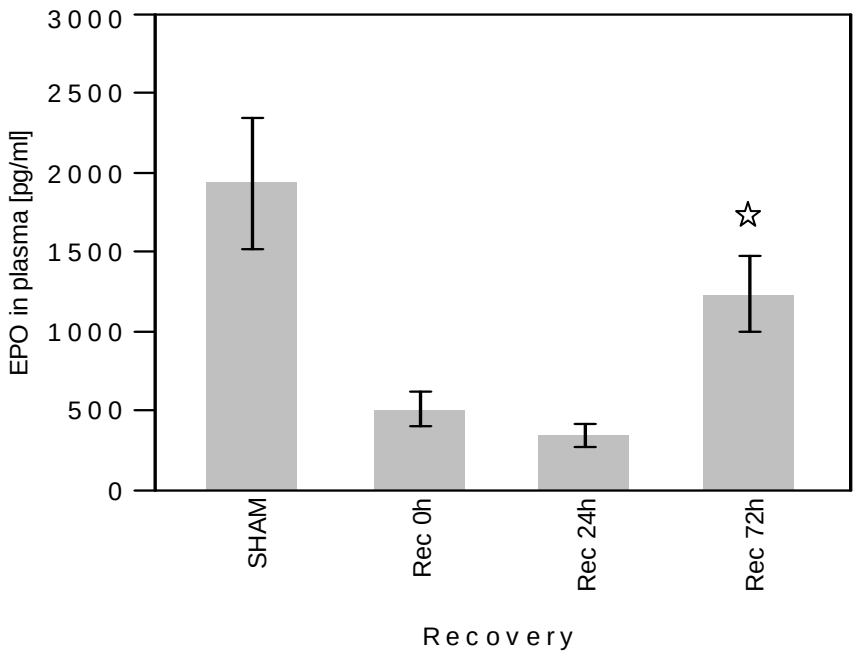


Figure 17. Hypoxia (CO 600 ppm)-stimulated EPO plasma levels after re-establishment of urine flow after 24 UUL (*p >0. 01 vs. Rec 0h).

5 Discussion

5.1 Mechanisms of reduced EPO secretion

Despite the numerous studies about the pathophysiology of the urinary tract obstruction and the kidney function after obstructive nephropathy, little is known about the endocrine function of the kidney after urinary tract obstruction. In addition, contradictory results have been reported in previous studies with regard to the capacity of hydronephrotic kidney to secrete EPO. Therefore, the present study was designed to explore systematically the hypoxia induced-EPO secretion response following UUL in rats.

In this study, we examined the effect of hypoxia on EPO plasma and EPO mRNA levels in kidneys with a ligated ureter compared with normal kidneys. Our data show clearly a dose-dependent reduced capacity of the hydronephrotic kidneys to secrete EPO and EPO mRNA expression after CO exposure. EPO secretion is markedly reduced by 70% after 24h of UUL compared with the sham group, with further reduction (to values <10%) after 72h of UUL. Changes in EPO mRNA of the renal tissue were also parallel to changes in EPO plasma level. Similar reduction was found after stimulation of EPO by hypobaric hypoxia (pO_2 8%). The reduction in hypoxia-stimulated EPO production after UUL was partially reversible. 72 h after re-establishment of urine flow EPO secretory response to CO recovers up to 50% in the 24 h hydronephrotic kidney.

One explanation of reduced EPO secretion after hydronephrosis may be the change in pO_2 of the renal tissue after UUL since during hydronephrosis GFR ceases and tubular electrolyte transport may fall to a minimum. Thus renal O_2 consumption may be very low and pO_2 may be not low enough for stimulation of EPO production. There is evidence that considerable inhomogeneities of O_2 supply within the kidney cortex exist, despite the fact that the kidney is supplied

with large amounts of O_2 (Bauer and Kurtz 1989). EPO is produced by the peritubular fibroblasts in the renal cortex. Physiologically, this location would allow a continuous monitoring of pO_2 which is the result of both O_2 supply to O_2 consumption (Bauer and Kurtz 1989). It has been reported that renal artery constriction in rats associated with diminished EPO secretion following hypoxia (Zivny *et al.* 1972). This might be related to the fact that the kidney GFR falls during reduced renal perfused pressure which would in turn reduce tubular transport work and thus O_2 consumption. Other studies have reported increased plasma EPO levels in animals with diminished RBF induced either surgically by renal artery constriction (Fisher and Samuels 1967; Gross *et al.* 1976) or pharmacologically by application of vasoconstrictive agents such as ANGII (Fisher *et al.* 1971; Gould *et al.* 1973).

Pagel et al. have shown that decreasing O_2 supply to the kidney through reduction in RBF (ischemic hypoxia), using Goldblatt clips with graded inner diameters, is less effective in increasing EPO production than reducing the Hb concentration (anemic hypoxia) (Pagel *et al.* 1988). According to these investigators maximum EPO values were obtained, when RBF was reduced till 10% of normal. Moderate hypoxia (hematocrit approximately 20% in anemic hypoxia) does not change RBF and GFR rate significantly (Grupp *et al.* 1972). Thus, it is likely that the exposure to moderate systemic hypoxia has no effect on GFR and RBF and but stimulates EPO production. Ureteral obstruction reduces the net reabsorption of salt in several nephron segments (Sonnenberg and Wilson 1976; Hanley and Davidson 1982; Hwang *et al.* 1993). In addition, previous studies have reported that RBF is reduced by roughly 50 % after 24 h of urinary tract obstruction; SNGFR tends to be zero which associates with decrease of transport work nearly to zero. Thus, the global O_2 consumption of the kidney would be expected to be significantly lowered after UUL compared to controls. Since RBF is reduced by 50% after 24 h UUL, O_2 delivery to the UUL kidney is also reduced by 50%. If O_2 consumption in the UUL kidney would be

reduced by 50 % then arterio-venous difference of oxygen (AVD-O_2) may be similar as in controls. In fact, recent studies from our laboratory have shown that AVD-O_2 of the 24 UUL kidneys is unchanged compared to controls (Laszlo *et al.* 2007). Thus, O_2 extraction of the kidney is unaltered after UUL. This finding supports the assumption of normal pO_2.

In addition, it was reported a significant preglomerular shunting of O_2 within the cortical vasculature between interlobular vessels which are arranged in a countercurrent fashion and represent quantitatively the largest contact area between arteries and veins within the renal cortex (Schurek *et al.* 1990). A recent study has reported no significant changes in renal tissue pO_2 by reducing of RBF to 40% in rats and rabbits (Leong *et al.* 2007). Since AVD-O_2 of the 24 UUL kidneys was unchanged compared to controls and O_2 extraction of the kidney was also unaltered after UUL. Thus, it is unlikely that an elevated pO_2 in the hydronephrotic kidney could explain the observed reduction of EPO production.

Another explanation for the mechanism of reduced EPO secretion in hydronephrosis could be based upon an impairment of mRNA production and reduced half life of EPO mRNA. Effective translation of mRNA to the protein requires unimpaired mRNA export from the nucleus into the cytosol which depends upon the capping process (Hermes *et al.* 2004). The methylation of the 5’ end of mRNA (capping) is vital process to create mature and stable messenger RNA which is then able to intiate translation. Capping is a highly regulated process which ensures the messenger RNA's stability while it serves translation in the process of protein synthesis. The transmethylation reaction of mRNA capping involves a S-adenosylmethionine SAM-dependent methyl-transferase. The product of this transmethylation is S-adenosylhomocysteine (SAH) which acts as a potent product inhibitor of SAM-dependent transmethylation. This effect is due to binding of SAH to the active site of these

methyltransferases with a higher affinity than SAM (Hoffman *et al.* 1979). The ratio of SAM/SAH is termed “Methylation Potential” (MP) which is considered to be a reliable indicator of the flow of methyl groups transferred from SAM to methyl receptors within the cells (Kloor *et al.* 2002). This ratio is also used as a general indicator of cellular methylation capacity, whereby a decrease in this ratio predicts reduced cellular methylation activity (Hermes *et al.* 2005). The product of these methylation reactions, SAH, is hydrolyzed solely by S-adenosylhomocysteine hydrolase (SAHH) in eukaryotic cells. SAHH is present in the nucleus of transcriptionally activated cells (Radomski *et al.* 1999) suggesting that the hydrolysis of SAH removes a potent block of SAM-dependent transmethylation activity. A deletion of SAHH gene is associated with embryonic lethality in mice which shows the biological importance of this enzyme (Miller *et al.* 1994). Hydrolysis of SAH results in adenosine (Ado) and homocysteine formation and SAHH catalyzes the reversible reaction of Ado and homocysteine to SAH (Kloor *et al.* 2002), see also Fig. 19. Ado has been suggested to play an important role in renal (Osswald *et al.* 1991), cardiovascular (Kroll *et al.* 1993), and neuronal functions (Fredholm and Hedqvist 1980), and homocysteine has been implicated in causing vascular disease (Hankey and Eikelboom 2000). Thus, SAHH is an essential enzyme in regulating processes like transmethylation (Shatkin 1976), transsulfuration (Finkelstein and Martin 1984) and purine metabolism (Kroll *et al.* 1993). Figure 19 shows the metabolic pathway of SAH formation and degradation.

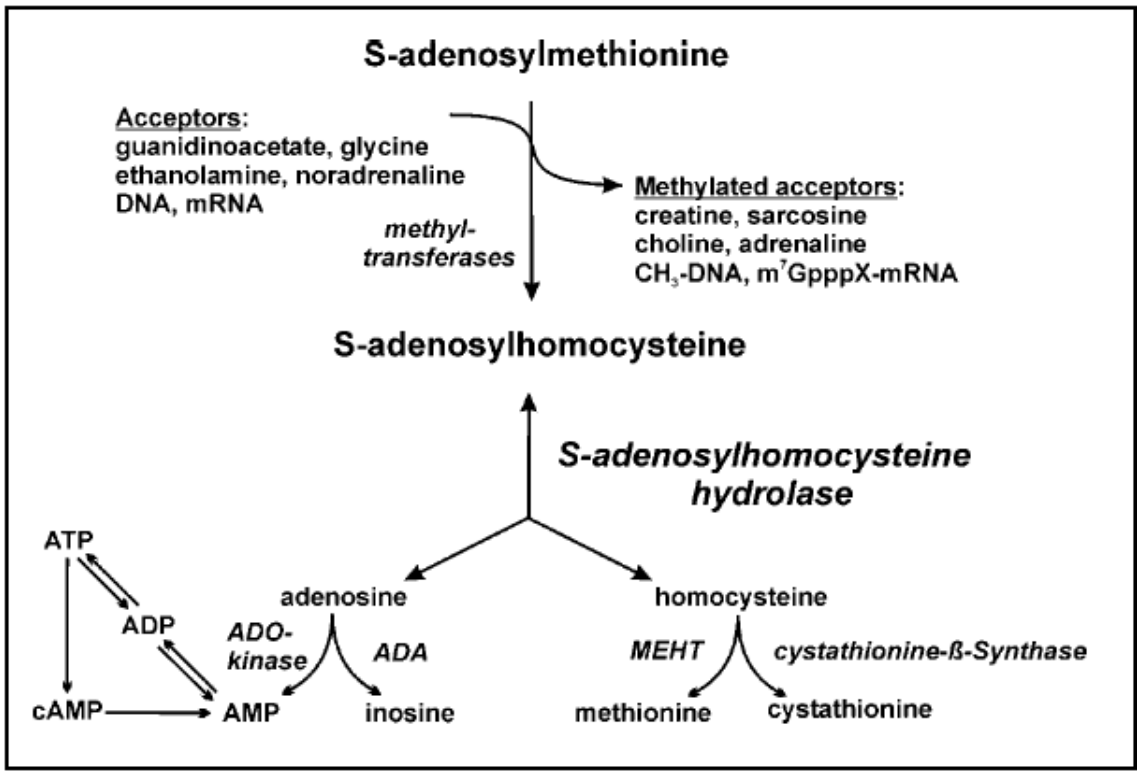


Figure 18. Metabolic pathway of SAH formation and degradation. Since SAH is a potent inhibitor of methyltransferases and since SAH controls the intracellular SAH concentration, inhibition of this enzyme leads to marked changes in the methylation potential and thus in transmethylation reactions.

Laszlo and co workers have observed an 18-fold increase in SAH tissue levels from 0.97 to17.7 nmol/wet weight leading to a dramatic fall of MP after UUL (Laszlo *et al.* 2007). The concept of SAH-induced inhibition of mRNA methylation was supported by the finding in HepG2 cells that inhibition of SAHH, which reduces MP to a similar degree as seen in our in vivo experiments with UUL, leads to decrease EPO expression by about 40% under both normoxic and hypoxic conditions (Hermes *et al.* 2004). It was also found in cells that SAH is involved in the process of apoptosis by altering gene expression. By irreversible inhibition of SAHH apoptosis was induced (Hermes *et al.* 2007). Extrapolating these findings to our observations that UUL leads to an 18-fold increase in SAH tissue levels, it is suggested that the apoptosis in UUL kidneys may, at least in part, also be the result of greatly impaired capping of mRNA

due to inhibition of SAM-dependent transmethylation capacity of the cell. Thus, our data provide a new mechanism of progressive apoptosis of the renal paranchyme seen in obstructive nephropathy.

5.2 Recovery of renal EPO secretion

As shown in Figure17 CO-stimulated EPO plasma levels recovered to roughly 50 % of normal values when urine flow was re-established after stenting the ligated upper part of the ureter. This partial EPO secretory recovery was associated with a substantial decrease in SAH tissue levels from 18 to 2 nmol/wet weight (Laszlo *et al.* 2007). This observation supports the notion that tissue SAH and thus the methylation potential (MP) is critically involved in the acute response of EPO secretion to reduced O_2 delivery to the kidney (see also Fig. 18).

Besides the influence of SAH/SAM on renal EPO secretory response of hydronephrotic kidneys described in this thesis, other factors have been identified to modulate EPO secretion. Among these factors that interfere with tubular and vascular components of the kidney, one has to mention nitric oxide (NO), angiotensin II, prostaglandins, thromboxan A_2, endothelin, TGF-ß, TNF-a, interleukin-1. Clearly more studies are required to elucidate the patho-mechanisms responsible for the renal response to ureteral obstruction.

6 Summary

EPO is an essential growth factor for the regulation and differentiation of erythrocytic progenitor cells in bone marrow. It is predominantly synthesized in the kidneys and to a smaller extent (~5%) also in the liver. EPO is not stored in vesicles but is produced on demand following hypoxic stimulus mainly through transcriptional activation of EPO gene. In turn, EPO gene expression is controlled by a HIF-1alpha by means of O_2-dependent regulation process.

Previous studies have yielded conflicting results with regard to the capacity of EPO secretion by the hydonephrotic kidneys. The main purpose of this work was a systematic assessment of EPO secretion in response to systemic hypoxia in rats subjected to ureteral ligation. The influence of different duration of UUL from 6 to 72 hours and the recovery from UUL by stenting the ligated ureter to re-establish the urine flow were analysed. In addition, a dose-response-relationship in UUL rats following CO exposure was performed. EPO production by the hydronephrotic kidney was also studied with the model of limited O_2 availability (CO exposure and hypobaric hypoxia).

The influence of different duration of UUL from 6 to 72 h on EPO secretion shows clearly a reduced EPO production by 70% after 24 h of UUL with further reduction after 72 h UUL. Early after UUL (6-12h) EPO secretion was slightly increased. The same reduction of EPO secretion in 24 UUL rats is observed after exposure to hypobaric hypoxia. The capacity of EPO production in response to different CO concentrations is strong impaired in hydronephrotic kidneys. CO-stimulated EPO production was reversible after re-establishment of urine flow in the obstructed kidney. The EPO secretory response to CO recovers up to 50% after 72 h of re-establishment of urine flow. In conclusion, our findings obtained with UUL rats show that EPO secretory response to hypoxic stimuli is greatly reduced after UUL in rats.

With respect to the possible mechanisms of impaired EPO secretion in UUL it could be excluded that a diminished fall in tissue pO_2 following systemic hypoxia contribute to the reduced EPO response in UUL.

Another factor for efficient EPO secretion is EPO mRNA production or stability. Efficient export of mRNA out of the nucleus into the cytosol requires the cap formation which depends upon a normal transmethylation capacity. Recent studies from our laboratory show a great increase in SAH tissue levels up to 18 fold with a great decrease of MP after UUL. Since the 5' end of mRNA requires SAM-dependent methylation (capping) for efficient translation, inhibition of this transmethylation reaction would lead to an impaired EPO mRNA translation which in turn impairs EPO production in UUL.

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9 Curriculum vitae

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Education

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