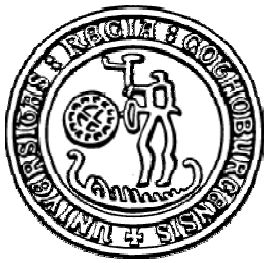
# STUDIES ON PATHOPHYSIOLOGICAL MECHANISMS IN EXPERIMENTAL MODELS OF ACUTE RENAL FAILURE

**Nicoletta Nitescu**



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# Studies on pathophysiological mechanisms in experimental models of acute renal failure

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## Abstract

Acute renal failure (ARF) affects 5-20 % of critically ill patients and is an independent risk factor for death in this patient population. Reactive oxygen species, thrombin and endothelin-1 (ET-1) are increased in ARF, and could contribute to the development of kidney failure and the poor prognosis. The aim of these studies was to investigate the effects of N-acetylcysteine (NAC; an antioxidant), thrombin inhibition and ET-1 receptor blockade on renal hemodynamics and function in experimental models of ischemic and septic ARF in rats.

N-acetylcysteine was studied in a model of renal ischemia-reperfusion (IR) injury induced by renal arterial clamping. N-acetylcysteine improved glomerular filtration rate (GFR) day 1 and 3 after IR. Furthermore, NAC decreased renal interstitial inflammation. N-acetylcysteine-treated rats had preserved renal glutathione levels and decreased plasma ascorbyl radical concentrations, indicating improved intrarenal antioxidant capacity and attenuated systemic oxidative stress. However, NAC did not improve GFR, total renal blood flow (RBF), or cortical (CLDF) and outer medullary (OMLDF) perfusion measured by laser-Doppler flowmetry, during the first 80 minutes after IR.

Thrombin inhibition with melagatran was examined in endotoxemia induced by lipopolysaccharide infusion, and in renal IR. During the first 3 h of endotoxemia, melagatran improved OMLDF, but did not attenuate the decline in GFR, RBF, CLDF and mean arterial pressure (MAP). In addition, melagatran attenuated the increase in plasma concentrations of aspartate aminotransferase, alanine aminotransferase and bilirubin, and of the cytokine tumor necrosis factor (TNF)-α. Melagatran did not diminish hepatocellular necrosis or the elevated hepatic gene expression of TNF-α, inducible nitric oxide synthase and intercellular adhesion molecule-1, evaluated by reverse transcription-polymerase chain reaction. In renal IR, melagatran did not ameliorate the decline in renal function, or attenuate renal histopathological abnormalities.

We studied the renal effects of selective endothelin type A ($ET_A$), and type B ($ET_B$), receptor antagonists during the first 2 h of normotensive endotoxemia with acute renal dysfunction. In saline-treated rats, endotoxin induced an approximate 40 % reduction in GFR, without significant changes in MAP, RBF, or in cortical perfusion and $pO_2$, measured by oxygen sensitive microelectrodes. In addition, endotoxin increased outer medullary perfusion and $pO_2$. Neither selective, nor combined, $ET_A$ and $ET_B$ receptor blockade improved GFR. However, in rats receiving selective $ET_B$ receptor antagonist, or combined $ET_A$ and $ET_B$ receptor blockade, endotoxin produced marked reductions in RBF and CLDF, without affecting MAP.

In conclusion, NAC is renoprotective in renal IR presumably by decreasing renal oxidative stress and inflammation, but not by improving kidney hemodynamics early after the ischemic insult. Thrombin seems not to be an important pathogenetic factor in the development of renal IR-injury. Thrombin inhibition with melagatran during endotoxemia preserves renal outer medullary perfusion, ameliorates liver dysfunction and attenuates the systemic inflammatory response. Endothelin-1 has beneficial effects on renal hemodynamics during early normotensive endotoxemia by activation of $ET_B$ receptors that exert a renal vasodilator influence and contribute to maintain normal RBF.

*Key words: acetylcysteine, acute renal failure, endothelin-1, endotoxin, ischemia, kidney medulla, reactive oxygen species, renal circulation, thrombin*

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# CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| ARF | acute renal failure |
| cDNA | complementary deoxyribonucleic acid |
| CLDF | cortical laser-Doppler flux |
| $^{51}$Cr-EDTA | chromium ethylenediaminetetraacetic acid |
| E Coli | Escherichia Coli |
| e/i NOS | endothelial/inducible nitric oxide synthase |
| ET-1 | endothelin-1 |
| $FE_{Na/K/H2O}$ | fractional urinary excretion rate of sodium/potassium/water |
| FF | filtration fraction |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GFR | glomerular filtration rate |
| $H_2O_2$ | hydrogen peroxide |
| HR | heart rate |
| ICAM-1 | intercellular adhesion molecule-1 |
| IM | inner medulla |
| IR | ischemia-reperfusion |
| ISOMZ | inner stripe outer medullary zone |
| KW | kidney weight |
| LPS | lipopolysaccharide |
| MAP | mean arterial pressure |
| mRNA | messenger ribonucleic acid |
| mTAL | medullary thick ascending loop of Henle |
| NAC | N-acetylcysteine |
| NO | nitric oxide |
| $NO_3^-/NO_2^-$ | nitrate/nitrite |
| $O_2^-\cdot$ | superoxide anion |
| OH· | hydroxyl radical |
| OMLDF | outer medullary laser-Doppler flux |
| OSOMZ | outer stripe outer medullary zone |
| PAR | protease activated receptor |
| $PGF_{2\alpha}$ | prostaglandin $F_{2\alpha}$ |
| PMN | polymorphonuclear neutrophil |
| $pO_2$ | partial pressure of oxygen |
| RBF | renal blood flow |
| ROS | reactive oxygen species |
| RT-PCR | reverse transcription-polymerase chain reaction |
| RVR | renal vascular resistance |
| TGF | tubuloglomerular feedback |
| TNF-α | tumor necrosis factor-alpha |
| $U_{Na/K}V$ | urinary sodium/potassium excretion |
| UV | urine flow rate |

## PUBLICATIONS AND MANUSCRIPTS

The thesis is based on the following publications and manuscripts, which will be referred to in the text by their roman numerals:

I. Nitescu N, Ricksten S-E, Marcussen N, Haraldsson B, Nilsson U, Basu S, Guron G
**N-acetylcysteine attenuates kidney injury in rats subjected to renal ischemia-reperfusion**
*Nephrol Dial Transplant 21(5):1240-1247, 2006*

II. Nitescu N, Grimberg E, Ricksten S-E, Guron G
**Effects of N-acetylcysteine on renal haemodynamics and function in early ischaemia-reperfusion injury in rats**
*Clin Exp Pharmacol Physiol 33 (1-2): 53-57, 2006*

III. Nitescu N, Grimberg E, Ricksten S-E, Marcussen N, Guron G
**Thrombin inhibition with melagatran does not attenuate renal ischemia-reperfusion injury in rats**
*Submitted*

IV. Nitescu N, Grimberg E, Ricksten S-E, Marcussen N, Nordlinder H, Guron G
**Effects of thrombin inhibition with melagatran on renal hemodynamics and function and liver integrity during early endotoxemia**
*In press Am J Physiol Regul Integr Comp Physiol*

V. Nitescu N, Grimberg E, Ricksten S-E, Herlitz H, Guron G
**Endothelin B receptors preserve renal blood flow in a normotensive model of endotoxin-induced acute kidney dysfunction**
*Submitted*

# 1. INTRODUCTION

## 1.1. Clinical aspects of acute renal failure

Acute renal failure (ARF) is the deterioration of renal function over a period of hours to days resulting in an inability of the kidney to excrete waste products of metabolism and to maintain fluid and electrolyte homeostasis [4]. Acute renal failure is an independent risk factor for death [5]. Interestingly, it has recently been demonstrated that even mild elevations in serum creatinine are associated with increased mortality [5]. Therefore, a term encompassing a continuum from subclinical renal injury, in which serum creatinine changes minimally, to complete renal failure, has been put forward – acute kidney injury (AKI) [6]. The RIFLE (Risk, Injury, Failure, Loss and End-stage renal disease [ESRD]) classification has been developed to obtain a definition and grading of the severity of AKI. Patients are classified into the RIFLE severity classes based on changes in serum creatinine or urine output from baseline [6].

Acute renal failure affects 5-7 % of hospitalized patients and 5-20 % of patients in the intensive care unit (ICU) [7]. The prevalence of ARF is particularly high, approximately 50 %, among patients with sepsis and multiple organ dysfunction syndrome [8]. Mortality in ARF patients is 7-23 % in the absence of underlying diseases [7]. However, mortality in critically ill patients with ARF is 50-80 %, and depending on the severity of co-morbidities and on the number of failing organs [7, 9]. Factors that may contribute to increased mortality in ARF patients include volume overload, acid-base derangements, insulin resistance, and enhanced oxidative stress and inflammation [7, 10-12]. Infections and cardiorespiratory failure are the main death causes in ARF patients [7]. Although the majority of patients surviving an episode of ARF recover renal function, 15-30 % progress to ESRD [9, 13].

In ICU patients, the main causes of ARF are renal ischemia in the setting of low cardiac output and hypotension (approximately 30 % of cases), and sepsis (approximately 20 % of cases) [7, 9]. Nephrotoxins contribute to about 15 % of ARF cases [7, 9]. In 50 % of cases the etiology is multifactorial [7, 9]. Conceivably, the pathogenetic mechanisms in clinical ARF are multiple and overlapping.

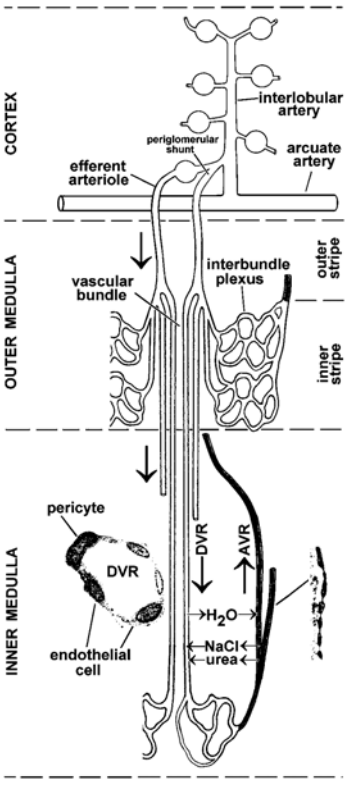
## 1.2. How do we treat acute renal failure today?

Despite the clinical seriousness of ARF, no large randomized double blind clinical study has yet shown that any pharmacological agent significantly reduces mortality or the need for renal replacement therapy in ARF. Large randomized trails have demonstrated that diuretics [14], low-dose dopamine [15] and high-dose atrial natriuretic peptide (ANP) [16] are not renoprotective. However, recent clinical trials show positive effects of N-acetylcysteine (NAC) in radiocontrast nephropathy, although results are conflicting [17, 18]. In addition, clinical studies indicate beneficial effects of low-dose ANP in ischemic ARF [19] and of fenoldopam in septic ARF [20]. Clearly, more studies are needed to identify therapeutic measures that improve renal outcome in ARF. In order to design adequate therapies the pathophysiology of AKI must be further elucidated. In addition, to enable early treatment interventions, biomarkers of AKI that can be detected earlier than increases in plasma

creatinine are needed. Early biomarkers of AKI that are currently evaluated in the clinic include urinary neutrophil gelatinase-associated lipocalin, interleukin-18 and kidney injury molecule-1 [21, 22].

### 1.3. The renal circulation – anatomy and physiology

Renal blood flow (RBF) is distributed mainly to the cortex (90 % of total RBF), where blood supply is abundant and $pO_2$ high, approximately 50 mmHg [23]. In contrast, blood flow to the renal medulla, by vasa recta capillaries formed from efferent arterioles of juxtamedullary glomeruli, or from periglomerular vascular shunt pathways [1], is low (approximately 10 % of total RBF), to preserve osmotic gradients and optimize urinary concentration [23]. Medullary blood has a reduced hematocrit compared to cortical blood, and thereby decreased oxygen transporting capacity [24]. Furthermore, countercurrent diffusion of oxygen from descending to ascending vasa recta capillaries leaves the outer medulla hypoxic [23, 25]. Oxygen consumtion in the outer medulla is high because of active reabsorption of sodium in the S3 segment of proximal tubules and in medullary thick ascending loops of Henle (mTAL) [23, 26]. Thus, a combination of limited oxygen supply and high oxygen demand renders the outer medulla hypoxic, with a $pO_2$ of 10-20 mmHg [23]. Medullary hypoxia has been demonstrated in several mammalian species including humans [23]. Since the outer medulla is hypoxic already during physiological conditions it is vulnerable to ischemia. Notably, the distribution of tubular damage in ischemic ARF appears to be determined by intrarenal oxygen gradients, with tubular injury predominantly to the S3 segment and mTAL, localized in the outer medulla [23, 27].




Cortical blood flow is efficiently autoregulated by the myogenic response and tubuloglomerular feedback (TGF) mechanism [1]. In contrast, medullary blood flow is considered poorly autoregulated [1]. However, it has been shown that during acute renal hypoperfusion (systolic arterial pressure approximately 80 mmHg) local vasodilation and down-regulation of tubular transport, mediated by nitric oxide (NO), adenosine and prostaglandins, contribute to preserve outer medullary perfusion and oxygenation [1, 28]. Vasodilating agents can act on pericytes that surround descending vasa recta and respond in a manner similar to smooth muscle cells of arterioles [1]. Furthermore, during hypoperfusion, a fall in glomerular filtration rate (GFR) diminishes filtered sodium load for reabsorption and thereby outer medullary oxygen demand [28].

**Figure 1.** Schematic illustration of the anatomy of renal microcirculation (from Ref. [1]). DVR and AVR denote descending and ascending vasa recta.

### 1.4. Pathophysiological mechanisms in ischemic acute renal failure

The clinical course of ARF has traditionally been divided into initiation, maintenance and recovery phases. Based on data from both clinical and experimental studies, an important role in the development of renal ischemic injury has recently been ascribed to microvascular injury with inflammation, coagulopathy and congestion, leading to persistent perfusion abnormalities and tissue hypoxia [29]. These pathophysiological changes occur predominantly in the renal outer medulla during a proposed extension phase that may extend the initial injury to the renal tubules causing a progressive decline in renal function even after total RBF has been restored [29, 30].

*Initiation phase*

Prerenal ARF is a reversible decline in GFR when renal perfusion is decreased [4]. If severe renal hypoperfusion is not corrected, oxygen tension becomes so diminished that mitochondrial oxidative phosphorylation and cellular adenosine 5'-triphosphate (ATP) concentrations can not be maintained. As a result, ischemic acute tubular necrosis (ATN) ensues [4]. This leads to a rapid and pronounced decline in GFR. Histopathological data from patients with ischemic ARF are scarce and of variable timing [31]. However, tubular epithelial cell injury, mainly in the S3 and mTAL segments, has been demonstrated in ARF patients [27]. Tubular injury is characterized by impaired cytoskeletal integrity and loss of normal polarity of the Na-K-ATPase [32]. Injured tubular cells are shed intraluminaly and form obstructing casts [33]. Furthermore, impaired tubular cellular tight junction integrity increases paracellular permeability and causes backleak of glomerular filtrate into circulation [34].

In the predominant experimental model of ischemic ARF, renal ischemia-reperfusion (IR), the above mentioned pathophysiological changes also occur [29]. Furthermore, it has been shown in renal IR that mislocalization of Na-K-ATPase to the apical tubular membrane decreases sodium reabsorption and increases sodium chloride delivery to the macula densa, thus activating the TGF mechanism [4].

*Extension phase*

Although analyses of outer medullary injury in human ARF are few, accumulation of leukocytes in vasa recta and interstitial oedema have been detected [35]. By the use of new research techniques, e.g. two-photon fluorescence microscopy, it has been shown in the renal IR model that endothelial cell dysfunction contributes to microvascular congestion, inflammation, hypercoagulability and increased permeability [29, 36]. These events may reduce blood flow and prolong tissue hypoxia in the outer medulla [29]. Endothelial dysfunction reduces NO generation and impairs vasodilatation [37]. Increased permeability of the injured endothelium leads to hemoconcentration, and to endothelial cell swelling and interstitial oedema that compress capillaries and tubules [36]. Coagulation is promoted by loss of endothelial anticoagulant and profibrinolytic substances [38], and inflammation is activated by up-regulation of endothelial adhesion molecules [39]. Adhesion and aggregation of leukocytes, platelets and erythrocytes obstruct the vasculature and impede blood flow [29, 40]. Moreover, enhanced leukocyte-endothelial interactions activate leukocytes to propagate

an injurious inflammatory reaction by their release of cytokines and reactive oxygen species (ROS) [39].

The extension phase has been suggested to occur hours to days after the initial renal insult [29]. During this time period interventions against inflammatory, coagulatory and hemodynamic abnormalities could be possible, and clinically relevant, since the majority of ARF patients are diagnosed during this phase [29].

*Maintenance and recovery phases*

During the maintenance and recovery phases GFR is first stable, but gradually increases. Normal kidney function can be re-established by tubular repair processes, e.g. proliferation, migration and differentiation [29]. Human ischemic ARF and experimental renal IR demonstrate several corresponding mechanisms of tubular regeneration after injury, and renal failure may be completely reversible despite an initial severe reduction in GFR [41, 42]. However, after recovery from IR, chronic renal disease may develop and predispose to ESRD [13]. The factors that determine the progression of AKI to chronic renal dysfunction are unknown. In patients, pre-existing renal disease appears to be a risk-factor for developing ESRD after ARF [13].

### 1.5. Pathophysiological mechanisms in septic acute renal failure

Although data on pathophysiological mechanisms from patients with septic ARF are sparse and inconsistent, they indicate hemodynamic [43], inflammatory [44] and coagulatory [45] abnormalities. Increased plasma concentrations of vasoconstrictors, e.g. catecholamines, angiotensin II and endothelin-1, have been demonstrated in human sepsis [8]. Presumably, their production is promoted when cytokine-induced NO synthesis reduces systemic vascular resistance and mean arterial pressure [8]. Endogenous vasoconstrictors may attenuate the decrease in systemic vascular resistance, but could cause renal vasoconstriction and reduced GFR [8]. However, human sepsis-induced ARF may also occur despite normal RBF [43].

To study pathophysiological mechanisms in sepsis during controlled conditions, animal models have been developed. Administration of endotoxin, a cell-wall component of gram-negative bacteria, is the most prevalent mode to cause many of the coagulatory, inflammatory and hemodynamic responses of gram-negative sepsis [46, 47]. Additional models such as administration of bacteria, and the cecal ligation and puncture model of polymicrobial sepsis, are also used (see “Discussion”). In endotoxemia, activation of coagulation [48] and inflammation [49] contribute to the development of ARF. Furthermore, in the majority of endotoxemia studies RBF is decreased [50], and may contribute to tubular injury [51]. In endotoxemic animals, microcirculatory abnormalities, including accentuated arteriovenous shunting and heterogeneity of RBF distribution, cause regional hypoxia [52]. This hypoxia is aggravated by increased renal oxygen consumtion [53], although studies exist indicating that endotoxemia can induce a renal cellular oxygen extraction deficit by defects in mitochondrial respiration [54]. Deteriorated renal hemodynamics during endotoxemia may be the result of renal vasoconstriction caused by activation of the sympathetic nerve system, and the renin-angiotensin and endothelin systems [8, 55]. In this setting, the vasodilating effect of renal endothelial nitric oxide synthase (eNOS) derived NO is important to maintain RBF and

GFR [56, 57]. However, renal eNOS activity may be inhibited during endotoxemia by inducible nitric oxide synthase derived NO i.e. NO autoinhibition [57].

### 1.6. Reactive oxygen species

Reactive oxygen species is a term that includes both oxygen radicals, i.e. oxygen species containing one or more unpaired electrons e.g. the superoxide anion ($O_2^{-}\cdot$) and the hydroxyl radical (OH·), and nonradicals that are oxidizing agents and/or are easily converted into radicals e.g. hydrogen peroxide ($H_2O_2$) [58]. Reactive oxygen species are generated by the stepwise addition of electrons to oxygen [58]. The superoxide anion is generated as oxygen accepts a single electron, and the dismutation of superoxide yields hydrogen peroxide. Superoxide may react with nitric oxide to form the reactive nitrogen species peroxynitrite ($ONOO^-$). The main sources of ROS include electron transport chains in mitochondria and endoplasmatic reticulum, and the cytosolic xanthine oxidase and plasma membrane NAD(P)H oxidase systems [59, 60]. Reactive oxygen species are produced under normal conditions and are degraded by the endogenous antioxidant systems e.g. superoxide dismutase, glutathione, glutathione peroxidase and catalase. Oxidative stress is an imbalance resulting from increased production of ROS and/or reduced antioxidant capacity, leading to accumulation of ROS and potential damage to cell constituents lipids, proteins and DNA [59].

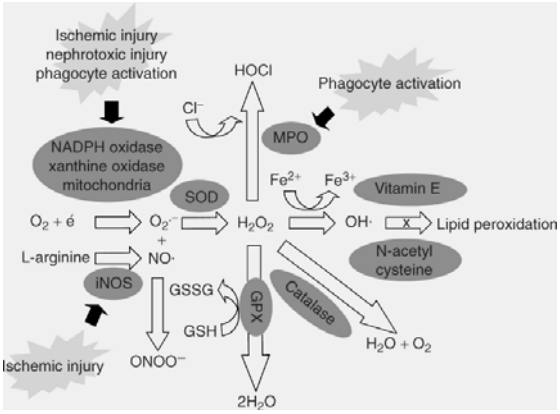



**Figure 2.** Reactions that generate and degrade ROS (from [3]). $O_2^{-}\cdot$ denotes superoxide, SOD superoxide dismutase, MPO myeloperoxidase, NO nitric oxide, $ONOO^-$ peroxynitrite, OH· hydroxyl radical, $H_2O_2$ hydrogen peroxide, GSH glutathione, GPX glutathione peroxidase, HOCl hypochlorous acid, and iNOS inducible nitric oxide synthase.

### 1.7. Reactive oxygen species in renal ischemia-reperfusion

In patients with ARF, oxidative stress is enhanced and may contribute to kidney injury [10, 12]. In renal IR, the intrarenal production of ROS is increased, and intrarenal antioxidant levels diminished, and this can cause cellular damage [61, 62]. Activated leukocytes and endothelial and tubular cells may produce $O_2^{-}\cdot$ by NAD(P)H-oxidase [59]. Also, during ischemia the enzyme xanthine dehydrogenase is converted to xanthine oxidase. The latter metabolizes hypoxanthine, generated from the breakdown of ATP, to xanthine, producing $O_2^{-}\cdot$ in the reaction. In addition, myeloperoxidase released by activated leukocytes generates the ROS hypochlorous acid (HOCl).

In renal IR, injury to cellular membrane lipids and proteins by ROS increase membrane permeability and reduce ion transport e.g. by Na-K-ATPase [63]. Oxidative injury impairs the actin cytoskeleton, the tight junction function, and the integrin dependent attachment of cells to the basement membrane [64]. Renal hemodynamics can be deteriorated by ROS-induced production of vasoconstrictors, e.g. isoprostanes that are formed when ROS

non-enzymatically oxidize arachidonic acid, and by increased vascular tone and reactivity to vasoconstrictors due to ROS effects on intracellular calcium handling in smooth muscle cells [65]. Reactive oxygen species may also impair vasodilatation by reducing NO availability [66]. Hydrogen peroxide can damage DNA and inhibit ATP synthesis, thereby predisposing to cell death [67]. In addition, ROS can act as second messengers and activate redox-sensitive transcription factors, e.g. NF-kappa B, thereby inducing inflammatory gene expression [68].

### 1.8. N-acetylcysteine

N-acetylcysteine is a precursor for the biosynthesis of the antioxidant glutathione and also yields sulfhydryl (-SH) groups that directly scavenge ROS [69]. N-acetylcysteine has been shown to attenuate renal oxidative stress after IR [70]. Furthermore, NAC inhibits the IR-induced immediate early gene response, and thereby apoptosis [69, 71]. *In vitro,* NAC has antiinflammatory effects [69, 72]. N-acetylcysteine can increase the expression of eNOS [73] and may increase the bioavailability of NO [66]. Accordingly, NAC attenuates renal vasoconstriction in experimental ischemic ARF caused by inferior vena cava occlusion [66]. Renal protective effects of NAC have previously been demonstrated in experimental models of toxic [74], cholestasis-induced [75] and ischemic [66] ARF, although the results are not conclusive [73]. Clinically, NAC has been extensively used and shown to have few side-effects, and recent trials suggest that NAC may prevent radiocontrast-induced ARF [17].

### 1.9. The coagulation factor thrombin

Coagulation is activated when circulating factor VII binds to tissue factor (TF) expressed on monocytes or activated endothelium, or on extravascular tissue exposed to blood by injured endothelium [76]. Activation of coagulation results in increased thrombin production. Thrombin promotes clot formation by stimulating platelet activation and aggregation, by catalyzing the conversion of fibrinogen to fibrin, and by activating factor XIII [76]. In addition, thrombin reduces fibrinolysis [76]. Thrombin can also activate protein C [76]. Furthermore, thrombin can cause activation and chemotaxis of leukocytes [77]. Cellular effects of thrombin have been shown to be mediated by protease activated receptors (PARs) 1, 3 and 4 [77].

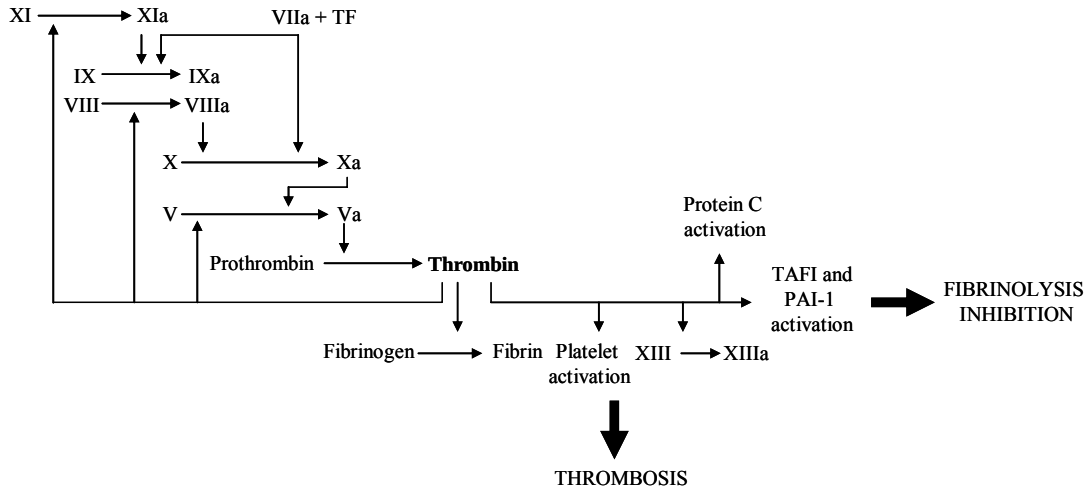



**Figure 3.** Schematic and simplified representation of the generation and effects of thrombin. TF denotes tissue factor, TAFI thrombin activatable fibrinolysis inibitor and PAI-1 plasminogen activator inhibitor 1.

### 1.10. Thrombin in endotoxemia

Endotoxemia is associated with disseminated intravascular coagulation, and reduced anticoagulant activity and fibrinolysis [47, 78]. Increased thrombin generation may cause intravascular fibrin deposition, leukocyte activation and adhesion, and platelet aggregation [78]. This may lead to microvascular injury, endothelial dysfunction, generalized microthrombi formation, and impaired blood flow to several organ systems thereby causing hypoxic injury and multiorgan failure [78]. In addition, thrombin has been shown to exert direct vasoconstrictor effects, e.g. in the kidney *in vitro* [79]. Thrombin inhibitors improve microvascular perfusion in striated muscle and the mesentery during endotoxemia in some [80, 81], but not in all [82], studies. In addition, previous studies suggest beneficial effects of thrombin inhibition on kidney and liver function, and survival, in endotoxemic animals [83, 84], although the results are not conclusive [85].

### 1.11. Thrombin in ischemia-reperfusion

Renal IR is associated with activation of the coagulation system [86]. Injured renal endothelium has a reduced ability to inhibit coagulation and promote fibrinolysis [29]. Furthermore, activated leukocytes release factors that inhibit fibrinolysis [29]. Increased thrombin generation has been demonstrated in the kidney microvasculature and tubuli in both experimental and clinical ischemic ARF [45, 87]. Fibrin deposition and platelet aggregation in glomerular and peritubular capillaries may cause microthrombosis leading to decreased GFR and impaired renal perfusion and oxygenation [45, 87]. Fibrin-containing tubular casts could lead to tubular obstruction and decreased GFR [87, 88]. Moreover, thrombin has been shown to elicit proinflammatory responses after renal IR by activation of PAR-1 [89]. Interestingly, inhibiting activation of the coagulation system by tissue factor antisense oligonucleotides ameliorates renal IR-injury [86].

### 1.12. The direct thrombin inhibitor melagatran

Melagatran is a selective, reversible and powerful low-molecular weight (429 Da) active site inhibitor of thrombin activity [90]. As a consequence, melagatran inhibits platelet activation and the conversion of fibrinogen to fibrin [90, 91]. Melagatran can also inhibit thrombin's activation of PAR-1 and PAR-4 [92], and of thrombin activatable fibrinolysis inhibitor [93]. Melagatran can be used to more specifically determine the role of thrombin in pathophysiological processes since thrombin inhibitors heparin and antithrombin inhibit additional coagulation factors, e.g. Xa [94, 95]. Factor Xa has been shown to signal via PAR-1 [96]. Melagatran ameliorates experimental ischemic heart injury in mice [97] and has been suggested to improve kidney function in endotoxemic pigs as indicated by reduced plasma creatinine levels [98]. Hypothetically, melagatran could improve renal hemodynamics and function in endotoxemia and renal IR by inhibiting microthrombosis formation and PAR-1 mediated renal vasoconstriction and inflammation. Although melagatran, the first direct thrombin inhibitor extensively investigated for prevention of thromboembolic events, was withdrawn from further clinical development in February 2006 due to concerns over liver safety, the results obtained with melagatran could be applicable for other thrombin inhibitors with similar molecular properties.

### 1.13. The endothelin system

Endothelins 1, 2 and 3 constitute a family of peptides, of which endothelin-1 (ET-1), which is produced in a number of cell types including endothelial and vascular smooth-muscle cells [99], has been shown to be important in the regulation of renal hemodynamics and function [100]. Endothelin-1 is able to constrict or dilate the kidney vasculature depending on the relative contribution of endothelin type A ($ET_A$) and type B ($ET_B$) receptors [100]. Endothelin-1 is rapidly synthesized and secreted in response to various stimuli e.g. endotoxin and ischemia [99]. In the vasculature, $ET_A$ receptors are localized on smooth muscle cells whereas $ET_B$ receptors are distributed on both endothelial and smooth muscle cells [99]. Exogenous ET-1 elicits renal cortical vasoconstriction and reduces total RBF primarily through $ET_A$ activation [100]. Endothelin type B receptor activation is able to produce both constrictor and dilator actions in the kidney, and vasodilation is mediated primarily by receptors on endothelial cells involving the release of NO [100]. Endothelin type B receptors in the collecting duct exert natriuretic effects [101]. Also, ET-1 has been shown to have proinflammatory effects, and to increase ROS production and cell proliferation [99].

### 1.14. Endothelin-1 in sepsis

Enhanced ET-1 synthesis has been demonstrated in both experimental and clinical sepsis, and results from previous studies suggest a role for ET-1 in sepsis-induced ARF [102, 103]. Endothelin type A receptor inhibition, and non-selective ET-1 receptor blockade, have been shown to improve RBF [102], cortical and outer medullary perfusion [104], and GFR [102] in endotoxemia associated with hypotension. However, hypothetically, ET-1 might also exert protective effects in the kidney during endotoxemia through $ET_B$ mediated release of renal vasodilator substances NO and prostaglandin $I_2$ [100], and by decreasing sodium reabsorption [101] and consequently oxygen consumtion. The precise roles of $ET_A$ and $ET_B$ receptor subtypes in the renal response to endotoxemia have not yet been defined.

## 2. AIMS

The overall aim of the thesis was to elucidate the role of reactive oxygen species, thrombin and endothelin-1 in the regulation of renal hemodynamics and function in experimental models of ischemic and septic acute renal failure, and to identify possible clinical therapeutic implications.

The specific aims were:

1. to determine if N-acetylcysteine is renoprotective in an experimental model of severe ischemic acute renal failure, i.e. ischemia-reperfusion, and if so by what mechanism

2. to evaluate whether systemic oxidative stress is increased in rats with ischemic acute renal failure, and if so, whether this could be attenuated by N-acetylcysteine treatment

3. to study the effects of thrombin inhibition with melagatran on renal ischemia-reperfusion injury

4. to study the effects of thrombin inhibition with melagatran on renal hemodynamics and function, and liver integrity, during early endotoxemia

5. to examine the role of endothelin type A and type B receptors in regulating renal hemodynamics and function, and intrarenal oxygenation, in early normotensive endotoxemia with acute renal dysfunction

# 3. MATERIALS AND METHODS

## 3.1. Animals

Experiments were performed on male Sprague-Dawley rats weighing 240-330 g obtained from Scanbur BK, Sollentuna, Sweden (I-II) or Harlan, Horst, The Netherlands (III-V). Animals were acclimatized for one week after arrival. Rats had free access to normal rat chow ($Na^+$, 120 mmol/kg; $K^+$, 153 mmol/kg) and tap water and were kept in rooms with a controlled temperature of 24-26° C and a 12:12 h dark-light cycle. All experiments were approved by the regional ethics committee in Göteborg.

## 3.2. Experimental models of acute renal failure

*Ischemia-reperfusion (I-III)*

Renal ischemia-reperfusion (IR) was carried out in animals anesthetized with ketamine (75 mg/kg, intraperitoneally (i.p.), Ketalar®, Pfizer, NY, USA) and xylazine (10 mg/kg, i.p., Rompun®, Apoteket AB, Stockholm, Sweden). In studies I and II, through flank incisions, the left renal artery was clamped for 40 minutes by a non-traumatic microvascular clip, and a right-sided nephrectomy was performed. During sham surgery the right kidney was excised and the left renal artery was dissected and manipulated but no clip applied.

In study III, a model of less severe renal failure was employed, and renal IR-injury was induced by bilateral clamping of the renal arteries for 35 minutes. Rectal temperature was kept at 37-38° C throughout. After surgery, fluid losses were replaced by administration of 5 ml of warm (37° C) isotonic saline i.p.

*Endotoxemia (IV, V)*

In study IV, endotoxemia was induced in thiobutabarbital (100 mg/kg i.p., Inactin®, Sigma, St. Louis, MO, USA) anesthetized rats by an intravenous (i.v.) bolus dose of lipopolysaccharide (LPS; E Coli 0127:B8, Sigma; 6 mg/kg) given during 30 minutes. This dose of LPS causes acute kidney dysfunction and liver injury in a well characterized model of endotoxemia [105].

A normotensive endotoxemia model was obtained in study V by continuous infusion of a lower dose of LPS (E Coli 0111:B4, Sigma; 1 mg/kg/h i.v.). Control animals received equivalent volumes of isotonic saline (IV, V). In this model, LPS caused a marked decline in glomerular filtration rate (GFR) in the absence of reduced renal blood flow (RBF).

## 3.3. Renal clearance experiments in anesthetized rats

*General procedures*

Rats were anesthetized with thiobutabarbital (100-120 mg/kg i.p.), placed on a heating table, and tracheotomized to facilitate spontaneous breathing. A polyethylene (PE) catheter (PE50) was inserted into the femoral artery and connected to a pressure transducer (Smiths Medical, Kirchseeon, Germany) for monitoring of MAP and heart rate (HR) using a data acquisition program (Biopac MP 150, Biopac Systems, Santa Barbara, CA, USA). The urinary bladder (PE160), and the left ureter (PE25; II, IV, V), were catheterized. After completion of the surgical preparations, a 40-45 minute equilibration period was allowed before renal clearance

measurements began. Kidney (II, IV, V) and rectal temperatures were monitored and kept at 37-38° C. Fluid substitution was provided by continuous infusions of 10 ml/kg/h isotonic saline (I, III-V) or 15 ml/kg/h 2% bovine serum albumin (BSA) in isotonic saline (II). The higher infusion rate in study II was used to promote urine production and enable clearance measurements in rats with severely diminished GFR immediately after renal arterial declamping.

*Clearance measurements*

Glomerular filtration rate was determined by measuring renal $^{51}$Cr-EDTA clearance ($^{51}$Cr-ethylenediaminetetraacetic acid, Amersham Laboratories, Buckinghamshire, UK). $^{51}$Cr-EDTA was injected intravenously at the start of the equilibration period in a bolus dose of 10 μCi/kg followed by an infusion of 15 μCi/kg/h throughout. Urine was collected during each clearance period into pre-weighed vials. Blood (~150 μl) was sampled in heparin coated tubes at the start and completion of each clearance period. Plasma was obtained by centrifugation at 2000 rpm for 5 minutes. Drawn blood samples were replaced by equivalent volumes of 4 % (I-IV) or 2 % (V) BSA in isotonic saline. Urine and plasma samples were analyzed for sodium and potassium concentrations and for radioactivity *(vide infra)*. The mean of plasma radioactivity measured at the start and at the completion of each clearance period was used to calculate GFR.

*Renal blood flow measurements (II, IV, V)*

Renal blood flow was measured on the left kidney exposed by a subcostal flank incision, immobilized in a plastic cup, and embedded in cotton wool soaked in warm (37° C) saline. The surface of the kidney was covered with warm (37° C) paraffin oil. A perivascular ultrasound transit time flow probe (0.7 VB, T206, Transonic Systems Inc., Ithaca, NY, USA) was placed around the renal artery for measurement of RBF. The probe was calibrated by the manufacturer using a gravity-fed constant flow set-up, and a zero calibration control was performed before each experiment by placing the probe in unstirred water. The ultrasound transit time method has been shown to provide accurate measurements of RBF, and to be relatively insensitive to changes in blood hematocrit levels [106]. The coefficient of variation for RBF measurements during baseline conditions in the present studies was 13 %.

*Intrarenal perfusion measurements with laser-Doppler technique (II, IV, V)*

Renal cortical (CLDF) and outer medullary (OMLDF) perfusion were estimated by laser-Doppler (LD) flowmetry (model PF5000, Perimed, Stockholm, Sweden). A fiber optic LD probe (diameter 1.0 mm, model 407, Perimed) was applied on the kidney surface for measurement of CLDF, and a needle-probe (diameter 0.45 mm, model 411, Perimed) was inserted 3.5 mm into the kidney for assessment of OMLDF, using micromanipulators. Both probes were stabilized, but not fixed, allowing them to follow changes in kidney volume. Correct placement of the outer medullary probe was verified by dissecting the kidney at the end of each experiment. Calibration of the LD probes was performed as recommended by the manufacturer at 0 perfusion units (PU) on a plastic disc for optical zero and at 250 PU by

immersion in a motility standard latex solution. The coefficient of variation for CLDF and OMLDF measured at baseline in the present studies were 10 % and 15 %, respectively.

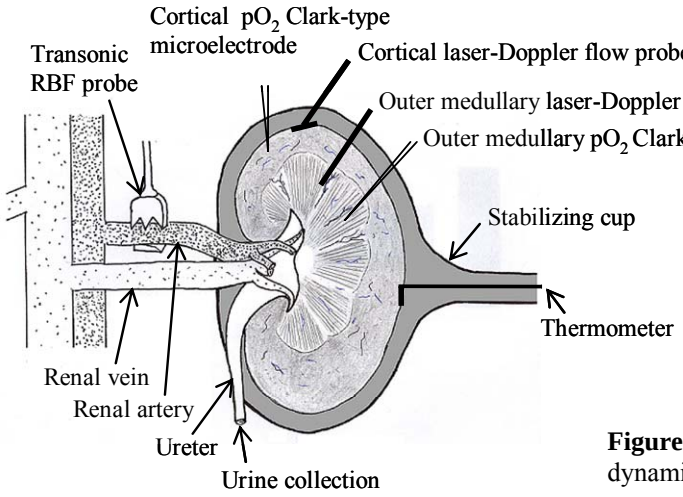



**Figure 4.** Experimental set-up for intrarenal hemodynamics and oxygen tension registrations.

The LD instrument scatters near-infrared monochromatic light into a hemisphere of tissue in the direct proximity of the probe, and registers and analyzes the reflected light to determine perfusion. Moving red blood cells (RBC) in the tissue vasculature reflect the LD light with a shift in frequency (the Doppler shift) that depends on their speed and direction. The different frequencies of reflected light are summed up and added to the unaltered frequency from static tissue matrix. The mean change in frequency is linearly related to the mean velocity of moving RBC in the tissue. The intensity of the reflected light at the mean Doppler frequency is linearly related to the volume fraction of moving RBC in the tissue (i.e. hematocrit). Thus, the LD signal is the product of the average speed of moving cells and their concentration in the measured tissue volume.

The measured volume of the LD instrument is influenced by the wavelength of the transmitted light, the probe configuration (fiber diameter and the distance between transmitting and receiving fibers), and the biophysical properties of the investigated tissue e.g. light absorption and scattering. In the present studies, the measured volume was estimated to be a hemisphere of 0.3-0.5 $mm^3$ [40, 107].

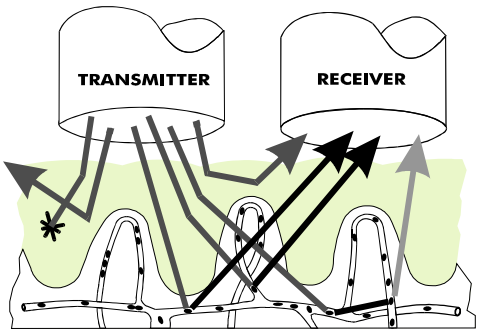



**Figure 5.** The principle for laser-Doppler (LD) measurements. Light emitted by the LD instrument is backscattered by the tissue, and the optical mixing of the reflected light of different frequencies at the photodetector surface produces an electrical signal that is proportional to tissue perfusion, defined as the number of erythrocytes x $area^{-1}$ x $time^{-1}$. Figure from Perimed.

*Renal oxygen tension measurements (V)*

Renal oxygen tension was measured using Clark-type oxygen sensitive microsensors with an outer tip diameter of 10 μm (OX10, Unisense, Aarhus, Denmark) attached to

micromanipulators and inserted at depths of approximately 1.0 mm in the cortex and at approximately 3.5 mm in the outer medulla, as described [2]. The design of the electrode is shown in figure 6. The oxygen microsensor has a silicone membrane at the tip through which oxygen can diffuse and subsequently be reduced at a golden plated platinum cathode polarized at −0.8 V against an internal silver/silver-chloride anode. The reduction of oxygen results in a small current (<0.50 picoampere) that is linearly related to the $pO_2$ around the electrode tip [108]. The current is measured by a picoamperemeter. The electrodes were calibrated in water at 37° C, saturated with $N_2$ gas or air, before and after each experiment. The coefficient of variation for oxygen tension measurements in the cortex ($CpO_2$) and outer medulla ($OMpO_2$) at baseline in our studies were 12 % and 14 %, respectively.

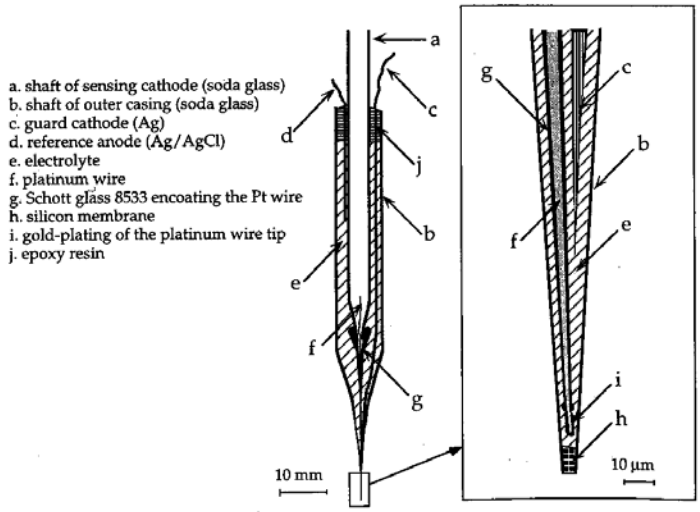



**Figure 6.** Oxygen sensitive Clark-type microelectrode (from Ref. [2]).

### 3.4. Kidney histology (I, III, IV)

Kidneys were excised, decapsulated, weighed, and immersion-fixed in 4 % formaldehyde in phosphate buffer (pH 7). The paraffin-embedded kidneys were sliced in 3 µm thick coronal sections and stained with hematoxylin-eosin and masson-trichrome (IV) for examination by light microscopy. For each kidney, the four sections most proximal to the centre were examined (x 4 objective). Analyses were made by an investigator blinded for treatment group. Renal histopathological changes were evaluated semi-quantitatively using an arbitrary scale where 0 = no changes, 1 = mild focal changes, 2 = modest diffuse changes, and 3 = severe diffuse changes, as described [109].

Histological variables including tubular atrophy and dilatation, interstitial inflammation and fibrosis, interstitial oedema, polymorphonuclear (PMN) neutrophil infiltration, vascular fibrin deposition and microthrombosis, and vascular congestion, were quantitated in the renal cortex, outer and inner stripe of the outer medulla, and inner medulla. The zonal definition determined by Kriz et al was used [110]. In study I, a semi-quantitative estimation of the type of leukocyte (i.e. lymphocyte, plasma cell and granulocyte) in the inflammatory infiltrate of the cortex and outer medullary zone was performed. For this purpose an arbitrary scale was utilized where 0 = no or only a few scattered cells, 1 = cell type present but in minority, 2 = cell type dominating and in majority, 3 = cell type heavily dominating.

### 3.5. Liver histology (IV)

Liver tissue sampled from each lobe was immersion-fixed in 4 % formaldehyde in phosphate buffer (pH 7), stained with hematoxylin-eosin and masson-trichrome and processed for analyzes by light microscopy. Necrotic/apoptotic hepatocytes and PMN neutrophils were counted in 20 consecutive high-power fields (x 400). No distinction was made between cell necrosis (i.e. increased eosinophilia, cell swelling, lysis, karyohexis, or karyolysis), and apoptosis (i.e. cell shrinkage, chromatin condensation and/or margination, or formation of apoptotic bodies). Analyzes were made by an investigator blinded for treatment group.

### 3.6. Reverse transcription-polymerase chain reaction (RT-PCR) of liver tissue (IV)

Liver tissue was snap frozen in liquid nitrogen and stored at -80° C until analyzed. RNA was extracted using Trizol®Reagent (Invitrogen, Paisley, Scotland) according to the manufacturer's protocol. The concentration of RNA was determined spectrophotometrically at 260 nm (SPECTRAmax Plus$^{384}$ microplate reader, Molecular Devices Corp, Sunnyvale, CA) and its purity verified by the 260/280 nm absorbance ratio. With this extraction protocol RNA purity was >1.7.

cDNA synthesis was performed by reverse transcription using ThermoScript™ RT-PCR system (Invitrogen). For gene amplification, the cDNA was added to FastStart Master SYBR®green I reaction mixture (Roche Diagnostics GmbH, Mannheim, Germany) and relative quantification of mRNA was performed on a LightCycler (Roche), as described [111]. Oligonucleotide primer sequences for tumor necrosis factor (TNF)-α [112], inducible nitric oxide synthase [113], intercellular adhesion molecule-1 [112] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [111] were obtained from the literature and synthesized by Invitrogen. Amplification conditions for cDNA were as described in the reference literature [111-113]. GAPDH was chosen as endogenous control to correct for potential variation in RNA loading and efficiency of the amplification reaction.

Standard curves for each gene was obtained by plotting log dilution (*x*-axis) against crossing point (Cp) values (*y*-axis). The initial amount (IA) of gene product was then calculated by the formula IA=Cp-b/m where b is the *y*-intercept and m is the slope of the individual standard curve. An inclusion criterion for analysis was an intersample difference of Cp <0.5 cycles. The relative gene expression level of the target gene was the ratio between target and house-keeping gene (GAPDH) cDNA. Specificity of the PCR product was validated by melting curve analysis. Finally, the PCR product was verified as a single band on an agarose gel.

### 3.7. Markers of oxidative stress (I)

The urinary excretion of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was measured since it has been shown to be a reliable *in vivo* biomarker of systemic, whole body, lipid peroxidation [58]. In addition, 8-iso-$PGF_{2\alpha}$ is a bioactive molecule that can exert renal vasoconstriction and reduce RBF and GFR [58]. Rats were kept in metabolic cages to enable collection of urine over 24 h periods. Urine was collected in vials kept on ice containing 0.01 % butylated hydroxytoluene to prevent *ex vivo* lipid autooxidation, and was stored 4-6 months at -80° C

until analyzed for 8-iso-$PGF_{2\alpha}$ using a highly specific and sensitive radioimmunoassay, as described [114]. Urinary 8-iso-$PGF_{2\alpha}$ levels were adjusted for creatinine concentrations.

In anesthetized rats (pentobarbital sodium, 60 mg/kg, i.p.; Pentobarbital natrium®, Apoteket AB), blood was sampled from the aorta, immediately centrifuged (2000 rpm for 5 min at 4° C), and plasma snap-frozen in liquid nitrogen. Plasma samples were stored at -80° C and analyzed within 6 months from collection for ascorbyl radical concentrations by electron spin-resonance spectroscopy (ECS 106 ESR spectrometer, Bruker, Billerica, MA, USA), as described [115]. A free radical standard solution of 4-hydroxy-2,2,6,6-tetrametylpiperidin-1-oxyl (4-Hydroxy-TEMPO) was used to calculate the absolute concentration of the ascorbyl radical. The ascorbyl radical is an oxidation product of ascorbic acid that has been used as a marker of oxidative stress in both experimental and clinical settings [58]. As it is quickly metabolized to ESR-silent species (e.g. ascorbate and dehydroascorbate) [58], plasma ascorbyl radical levels appear to be independent of renal function.

Whole kidney glutathione concentrations were measured on normal right kidneys excised immediately prior to the induction of left-sided IR, and on injured left kidneys from the same animal 24 h after IR. Total glutathione concentrations, an index of renal antioxidative capacity, were measured with an assay based on a reaction using Ellman's reagent, according to the manufacturer's instructions (Glutathione assay kit, Cayman Chemicals, Ann Arbor, Michigan, USA).

### 3.8. Analytical procedures

Radioactivity was analyzed by a Packard 3-channel scintillation counter (model 5019, Packard Co., Amana, IA, USA). Sodium and potassium concentrations were measured by flame spectrophotometry (Flame Spectrophotometer, model FLM, Radiometer, Copenhagen, Denmark). Arterial blood gases were analyzed using the ABL 510 blood-gas analyzer (Radiometer). Creatinine, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) concentrations were determined enzymatically and bilirubin spectrophotometrically (Modular PP, Roche). Plasma melagatran concentrations were measured using liquid chromatography-mass spectrometry as described [116]. Commercially available analytic kits were used to measure plasma TNF-α (Rat TNF ELISA Kit II, BD Biosciences, Franklin Lakes, NJ, USA) and endothelin-1 (ET-1; Human Endothelin-1 Immunoassay, R&D Systems, Minneapolis, USA) by enzyme-linked immunosorbent assay, and plasma $NO_3^-/NO_2^-$ spectrophotometrically (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemicals).

### 3.9. Drugs

N-acetylcysteine (NAC; Acetylcysteine NM Pharma®, Merck NM, Stockholm, Sweden) was diluted in isotonic saline (I, II) or in 2 % BSA in isotonic saline (II). Melagatran (AstraZeneca, Mölndal, Sweden), the endothelin A ($ET_A$) receptor antagonist BQ-123 (Peptides International, Louisville, KY, USA) and the endothelin B ($ET_B$) receptor antagonist BQ-788 (Peptides International) were dissolved in isotonic saline.

In studies I and II, NAC was administered in doses previously shown to reduce renal oxidative stress after IR [70, 117]. Treatment was started before IR to establish adequate

intracellular levels of the antioxidant glutathione in kidneys prior to the ischemic insult [70]. This mode of administration mimics protocols in clinical studies demonstrating beneficial effects of NAC in the prevention of radiocontrast nephropathy.

In study III, plasma melagatran concentrations of approximately 0.5 μmol/L were targeted as this concentration has been shown to markedly prolong thrombin time (TT) and activated partial thromboplastin time (APTT), to reduce platelet activation, and to exert potent antithrombotic effects in models of arterial and venous thrombosis *in vivo* [90, 91, 118-123]. As melagatran is eliminated mainly (approximately 80 %) by the kidneys [124], lower doses were administered to rats subjected to renal IR than to sham-operated animals.

Melagatran dosing in study IV was according to a protocol expected to produce a plasma melagatran concentration of approximately 1 μmol/L throughout the study. Melagatran in this plasma concentration has been shown to attenuate increases in plasma creatinine levels in endotoxemic pigs [98].

In study V, animals were pre-treated with endothelin antagonists in doses that, based on previous studies [125-128], were expected to result in steady-state concentrations that completely block responses to both endogenous and exogenous (0.3 nmol/kg i.v. bolus) ET-1 mediated by the $ET_A$ and $ET_B$ receptors.

### 3.10. Study protocols

*Study I*

Rats were divided into four treatment groups: (1) IR-Saline, (2) IR-NAC, (3) Sham-Saline and (4) Sham-NAC. For measurement of plasma creatinine concentrations, venous blood samples (~200 μl) were collected from tail veins under brief isofluran (Isofluran®, Baxter Inc., Deerfield, IL, USA) anesthesia on day 1 and 3 after renal IR, or blood samples taken from the aorta of anesthetized animals on day 7 after IR. In separate groups of rats, renal clearance experiments were performed during three consecutive 20 minute clearance periods 24 h after IR.

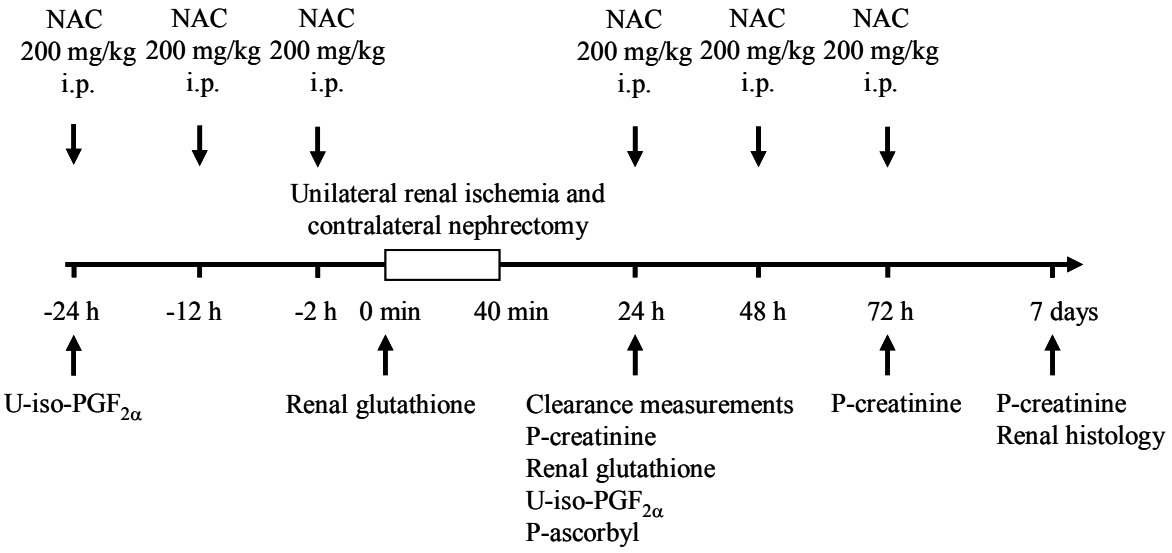



**Figure 7.** Experimental protocol, study I.

*Study II*

Rats were divided into two treatment groups: IR-Saline and IR-NAC. Animals were anesthetized, unilaterally nephrectomized through a flank incision, and prepared for renal clearance measurements and analyses of RBF, CLDF and OMLDF.

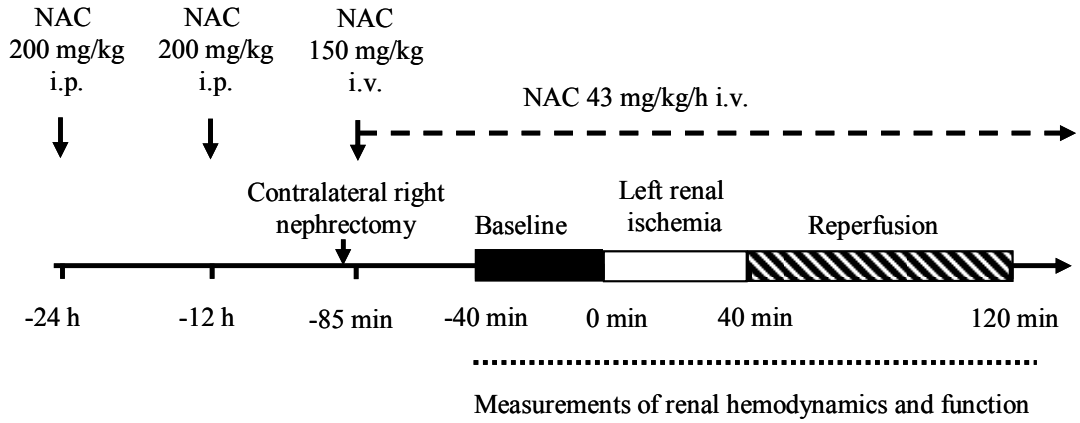



**Figure 8.** Experimental protocol, study II.

*Study III*

Rats were divided into four study groups: (1) IR-Saline, (2) IR-Melagatran, (3) Sham-Saline and (4) Sham-Melagatran. Prior to renal IR, or sham surgery, melagatran was administered in a subcutaneous (s.c.) bolus dose of 0.5 μmol/kg in group IR-Melagatran, and 0.7 μmol/kg in group Sham-Melagatran, respectively. After the bolus dose, a subcutaneous infusion of melagatran (0.08 μmol/kg/h in group IR-Melagatran and 0.4 μmol/kg/h in group Sham-Melagatran) was initiated and maintained throughout (Alzet pump 1003D, B&K Universal, Sollentuna, Sweden). Measurements of renal function were performed during three consecutive 20 minute periods 48 h after renal IR.

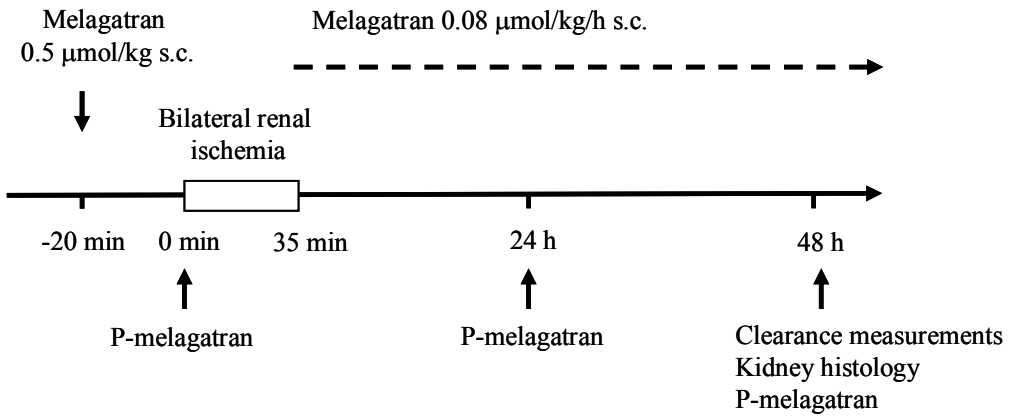



**Figure 9.** Experimental protocol, study III.

*Study IV*

Rats were divided into four study groups: (1) LPS-Saline, (2) LPS-Melagatran, (3) Sham-Saline and (4) Sham-Melagatran, anesthetized and prepared for measurements of renal clearance, RBF, CLDF, and OMLDF. In separate groups of anesthetized rats, plasma concentrations of melagatran, TNF-α and $NO_3^-/NO_2^-$ were analyzed. In addition, liver integrity and gene expression, and kidney histology, were assessed.

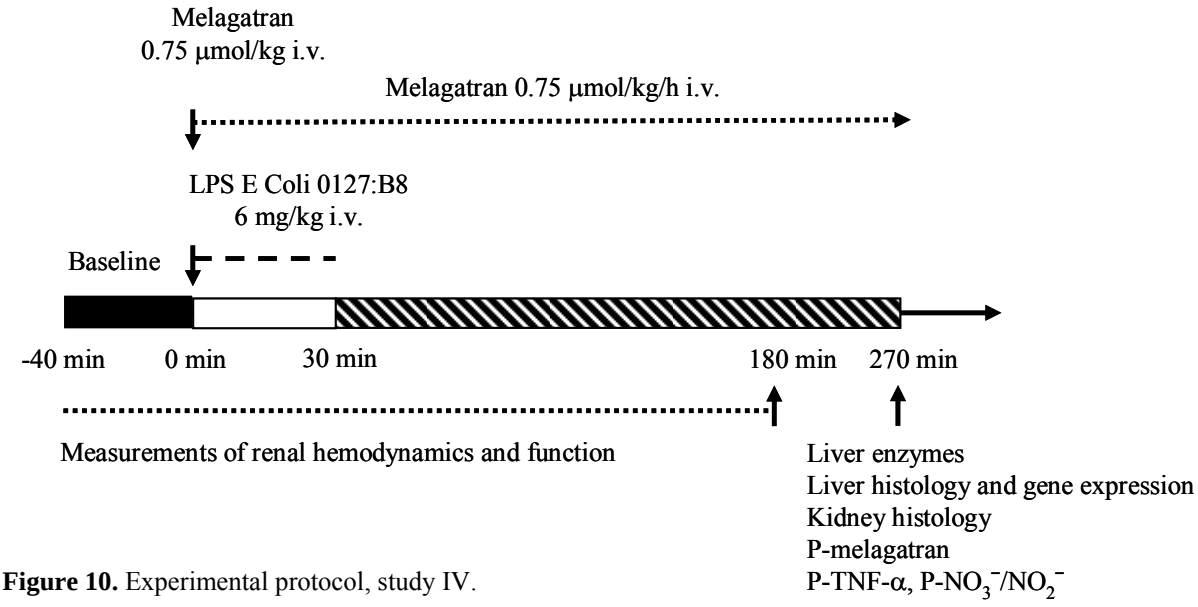



**Figure 10.** Experimental protocol, study IV.

*Study V*

Animals were divided into five study groups: (1) Sham-Saline, (2) LPS-Saline, (3) LPS-BQ123, (4) LPS-BQ788 and (5) LPS-BQ123+BQ788. Rats were anesthetized and prepared for measurements of renal clearance, RBF, CLDF, OMLDF, $CpO_2$ and $OMpO_2$.

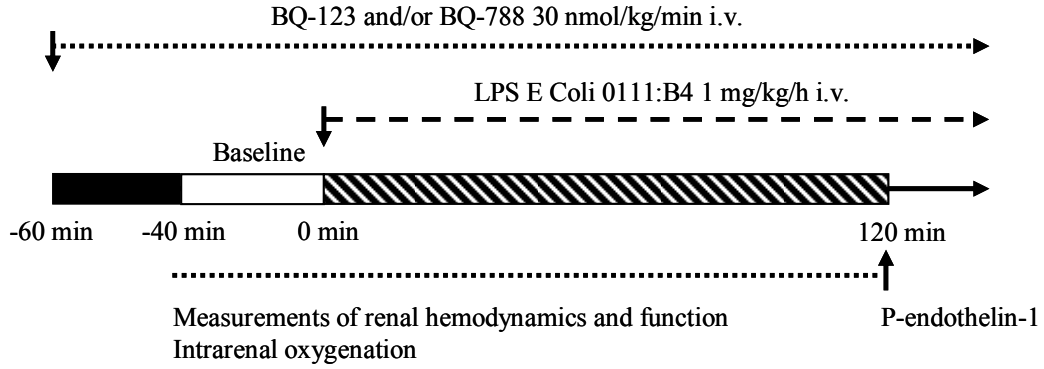



**Figure 11.** Experimental protocol, study V.

## 3. 11. Calculations

Glomerular filtration rate was calculated from renal $^{51}$Cr-EDTA clearance using the equation $C_{EDTA} = (U_{EDTA} \times UV)/P_{EDTA}$. Renal vascular resistance was calculated as MAP (mmHg)/RBF

(ml/min/g kidney weight), and filtration fraction (FF) as the ratio between GFR and RBF. Renal plasma flow could not be used to calculate FF since blood hematocrit was not measured during each clearance period. Fractional urinary excretion rates of sodium ($FE_{Na}$, %), potassium ($FE_K$, %), and water ($FE_{H2O}$, %), were estimated as the ratio of their respective clearances to that of $^{51}$Cr-EDTA, taken as GFR, x 100. Continuous data (HR, arterial pressure, RBF, and laser-Doppler and $pO_2$ data) were sampled 15 times per minute during clearance experiments. Mean values were calculated for each clearance period. Baseline data are presented as the average of two baseline clearance periods.

### 3.12. Statistics

Values are presented as mean ± SEM except for semi-quantitative data which are expressed as the median with 25th and 75th percentiles.

Differences between groups were evaluated by one-way and two-way analyses of variance (ANOVA) or ANOVA for repeated measurements. Adjustments for multiple comparisons were made by Bonferroni's correction (I, II, III, V) or Fisher's post-hoc test (IV).

The following pre-specified between-group analyses were performed, in study IV: (1) LPS-Saline vs. Sham-Saline and (2) LPS-Melagatran vs. LPS-Saline.

In study V, analyses were between groups: (1) LPS-Saline vs. Sham-Saline, (2) groups LPS-BQ123, LPS-BQ788, and LPS-BQ123+BQ788 vs. LPS-Saline, and (3) groups LPS-BQ123 and LPS-BQ788 vs. LPS-BQ123+BQ788.

The non-parametric Kruskal-Wallis' and Mann-Whitney's tests were used on renal histopathological parameters. Cumulative survival was examined by Kaplan-Meier analysis.

The intra-assay coefficient of variation for baseline RBF, LD and $pO_2$ measurements was calculated as the standard deviation divided by the mean, x 100.

A p-value of less than 0.05 was considered statistically significant in all experiments. Analyses were performed using the statistical software program SPSS 11.5.1 (SPSS Inc., Chicago, IL, USA).

# 4. REVIEW OF RESULTS

## 4.1. Effects of N-acetylcysteine on renal ischemia-reperfusion injury (I)

*Kidney function*

N-acetylcysteine (NAC) ameliorated the decline in renal function day 1 and 3 after renal ischemia-reperfusion (IR; Figure 12). Furthermore, NAC reduced hyperkalemia day 1 after renal IR (plasma potassium concentrations were 6.4±0.6 mmol/L and 8.0±0.4 mmol/L in groups IR-NAC and IR-Saline, respectively; $p<0.05$), but did not influence the increases in $FE_{Na}$, $FE_K$, and $FE_{H2O}$.

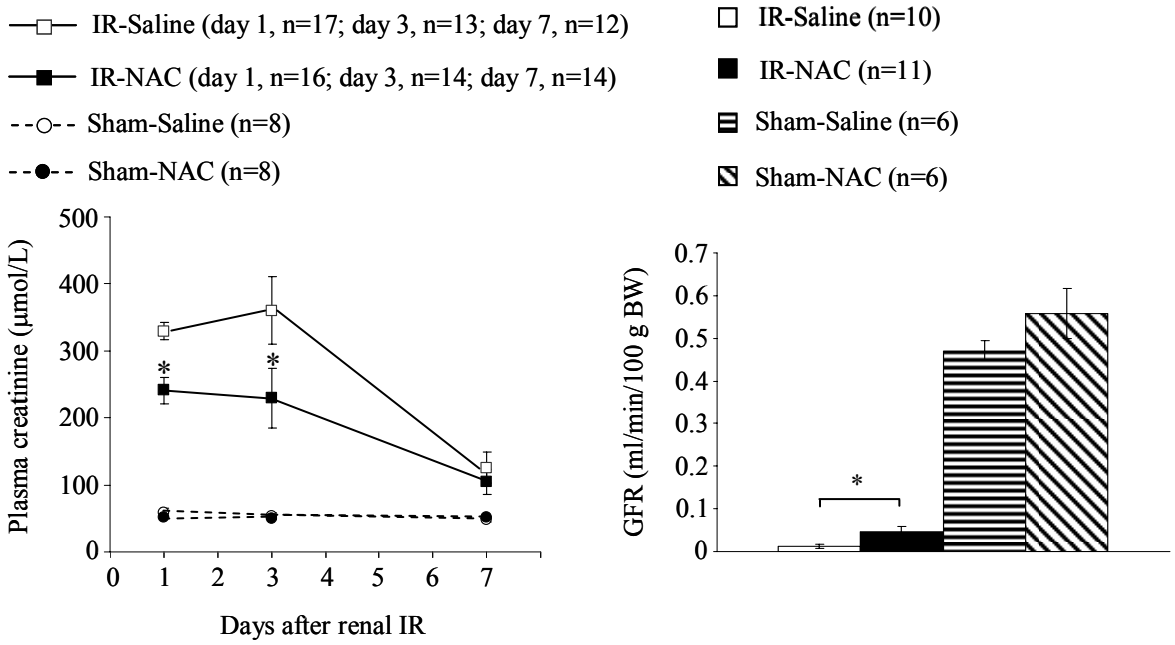



**Figure 12.** Effects of renal ischemia-reperfusion (IR), or sham surgery, on plasma creatinine concentrations day 1, 3 and 7 after IR (left panel), and on glomerular filtration rate (GFR) day 1 after IR (right panel), in rats treated with N-acetylcysteine (NAC) or isotonic saline. * $p<0.05$ between IR groups.

*Kidney histology*

Kidney histology day 7 after renal IR was characterized by tubular atrophy and dilatation, interstitial inflammation mainly consisting of lymphocytes, and fibrosis ($p<0.05$ vs. sham, Figure 13). N-acetylcysteine significantly reduced interstitial inflammation in the cortex and outer medulla of IR-injured kidneys ($p<0.05$).

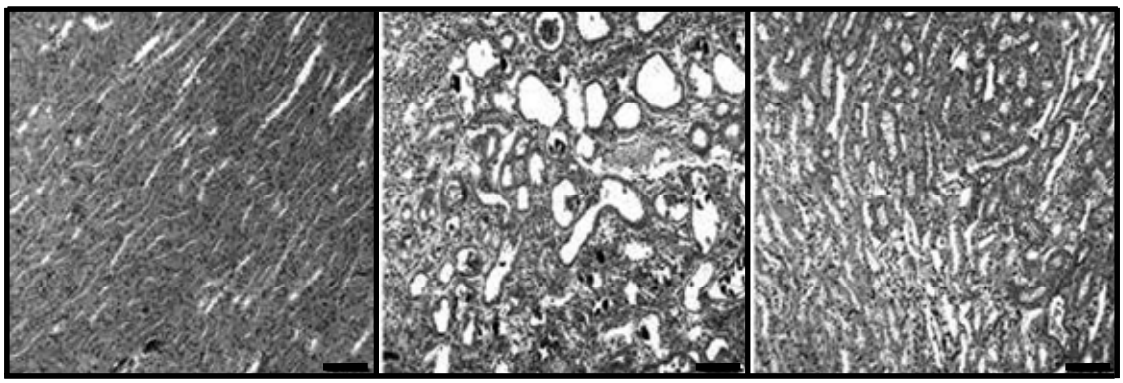



**Figure 13.** Kidney histology in the outer stripe of the outer medulla 7 days after renal ischemia-reperfusion (IR; magnification x 90, scale bar denotes 100 μm). In the saline-treated rat subjected to IR (middle panel) severe morphological injury is seen. In the corresponding region of an N-acetylcysteine (NAC)-treated rat (right panel), morphological changes tended to be less pronounced. No signs of injury are detected in the sham-operated kidney (left panel).

*Mortality*

The number of deaths during the 7 day study period was 8/20 (40 %) in group IR-Saline and 3/17 (18 %) in group IR-NAC. Cumulative survival tended to be higher in group IR-NAC compared to group IR-Saline, although not reaching statistical significance (p=0.13).

*Markers of oxidative stress*

Plasma levels of the ascorbyl radical were significantly increased 24 h after renal IR, although concentrations were significantly reduced in group IR-NAC compared to IR-Saline (Figure 14). Urinary excretion of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was markedly increased, compared to baseline, during the first 24 h after renal IR (Figure 14). However, there were no significant effects of NAC on the rate of urinary 8-iso-$PGF_{2\alpha}$ excretion (Figure 14). Kidney glutathione concentrations decreased significantly in group IR-Saline in response to IR (Figure 14). However, NAC completely prevented the drop in kidney glutathione levels, 24 h after IR (Figure 14).

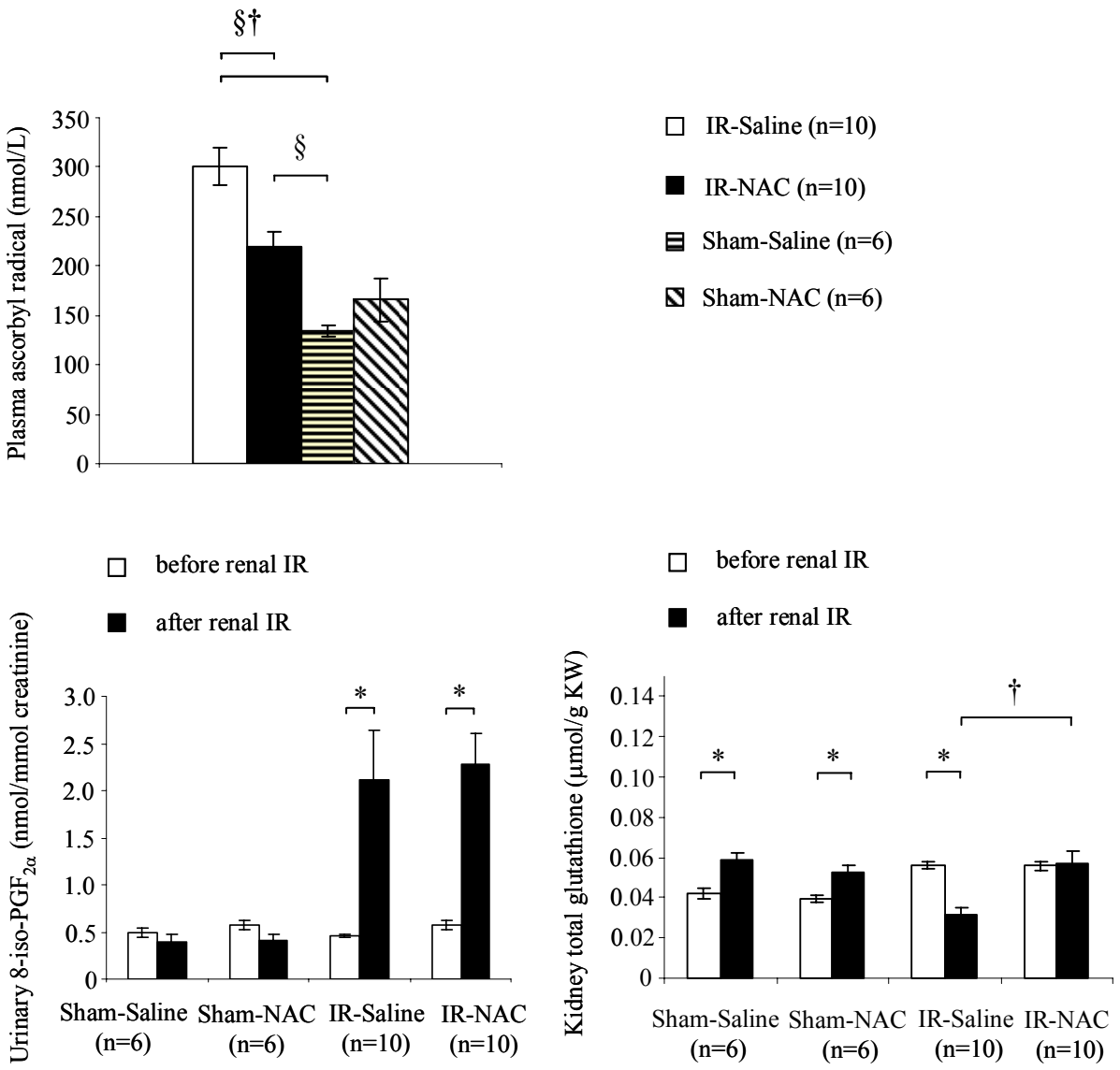



**Figure 14.** Plasma ascorbyl radical levels, urinary 8-iso-$PGF_{2\alpha}$ excretion and kidney total glutathione concentrations, in rats subjected to renal ischemia-reperfusion (IR), or sham surgery, and treated with N-acetylcysteine (NAC) or isotonic saline. Plasma ascorbyl radical levels were measured at 24 h after IR, urinary 8-iso-$PGF_{2\alpha}$ excretion during 24 h before and after IR, and renal glutathione immediately before and at 24 h after IR. KW denotes kidney weight. * $p<0.05$ vs. before renal IR or sham surgery, § $p<0.05$ vs. sham, and † $p<0.05$ between IR-groups.

## 4.2. Effects of N-acetylcysteine on renal hemodynamics and function during early ischemia-reperfusion (II)

*Kidney function*

Renal IR caused a pronounced decline in GFR and a marked increase in $FE_{Na}$ during the first 80 minutes of reperfusion (Figure 15). N-acetylcysteine had no significant effects on GFR or $FE_{Na}$ during this time period (Figure 15). There were no significant differences between groups in any of the investigated variables at baseline.

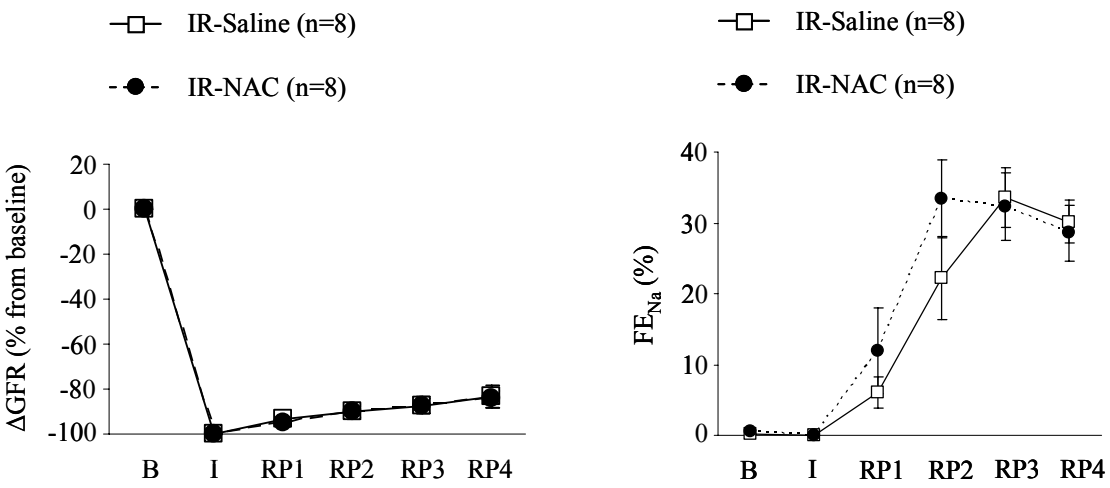



**Figure 15.** Effects of renal ischemia-reperfusion (IR) on glomerular filtration rate (GFR) and fractional urinary excretion rate of sodium ($FE_{Na}$) in anesthetized rats treated with N-acetylcysteine (NAC) or isotonic saline. B indicates baseline; I, 40 minutes of renal artery clamping; and RP1-RP4, 4 consecutive 20 minute clearance periods during reperfusion.

*Renal blood flow*

After release of the renal arterial clamp, RBF, CLDF and OMLDF rapidly returned to values not significantly different from baseline (Figure 16). N-acetylcysteine had no significant effects on RBF, CLDF or OMLDF during the first 80 minutes after renal ischemia (Figure 16).

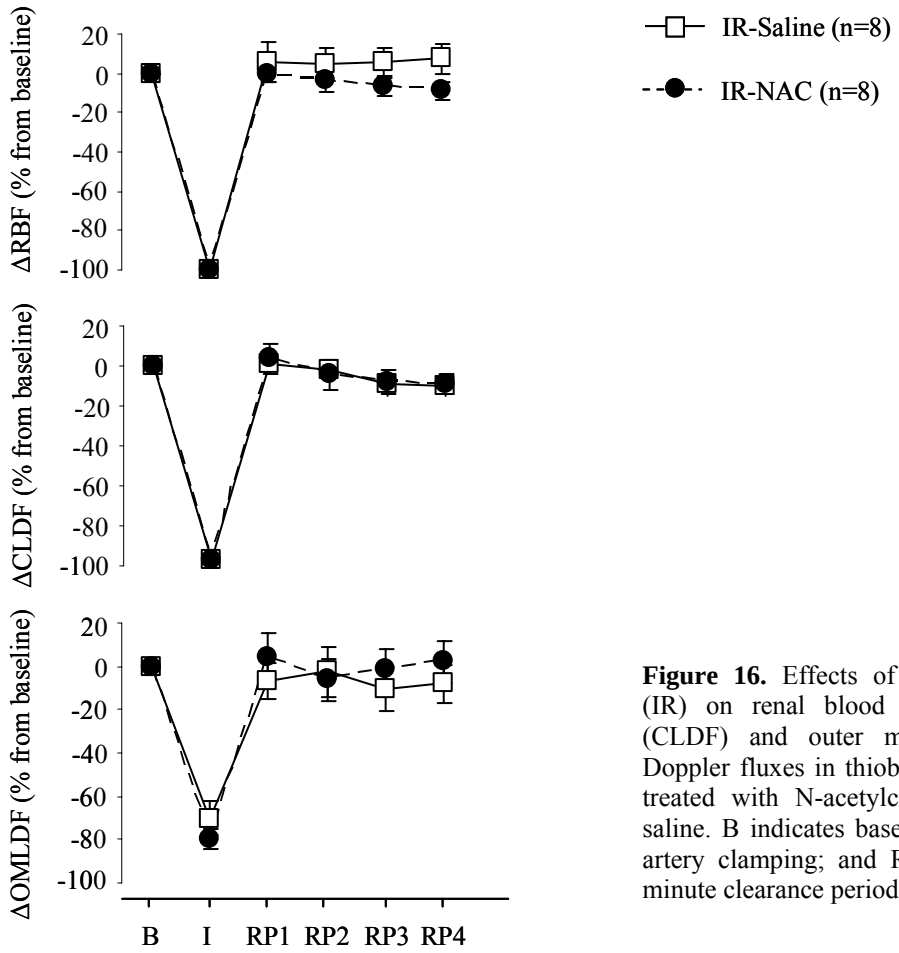



**Figure 16.** Effects of renal ischemia-reperfusion (IR) on renal blood flow (RBF), and cortical (CLDF) and outer medullary (OMLDF) laser-Doppler fluxes in thiobutabarbital anesthetized rats treated with N-acetylcysteine (NAC) or isotonic saline. B indicates baseline; I, 40 minutes of renal artery clamping; and RP1-RP4, 4 consecutive 20 minute clearance periods during reperfusion.

### 4.3. Thrombin inhibition in renal ischemia-reperfusion injury (III)

*Kidney function, systemic hemodynamics and blood gases*

Forty-eight hours after renal IR, rats demonstrated a marked decline in GFR, and increases in $FE_{Na}$, $FE_K$ and $FE_{H2O}$ ($p<0.05$ vs. sham, Figure 17). In addition, urinary potassium excretion in absolute values ($U_KV$) was reduced in rats subjected to renal IR ($p<0.05$ vs. sham). Thrombin inhibition with melagatran decreased $U_KV$ ($p<0.05$), but had no statistically significant effects on GFR, $FE_{Na}$, $FE_K$, $FE_{H2O}$ and plasma potassium concentrations, compared to saline treatment (Figure 17).

There were no significant differences between groups in mean arterial pressure (MAP; 116±4 mmHg in group IR-Melagatran and 117±4 mmHg in group IR-Saline), heart rate (HR), arterial $pO_2$ or oxygen saturation.

Melagatran treatment significantly decreased arterial hemoglobin concentrations (Hb), and Hb was 129±4 g/L in group IR-Melagatran and 143±2 g/L in group IR-Saline, respectively ($p<0.05$). No deaths occurred throughout the study.

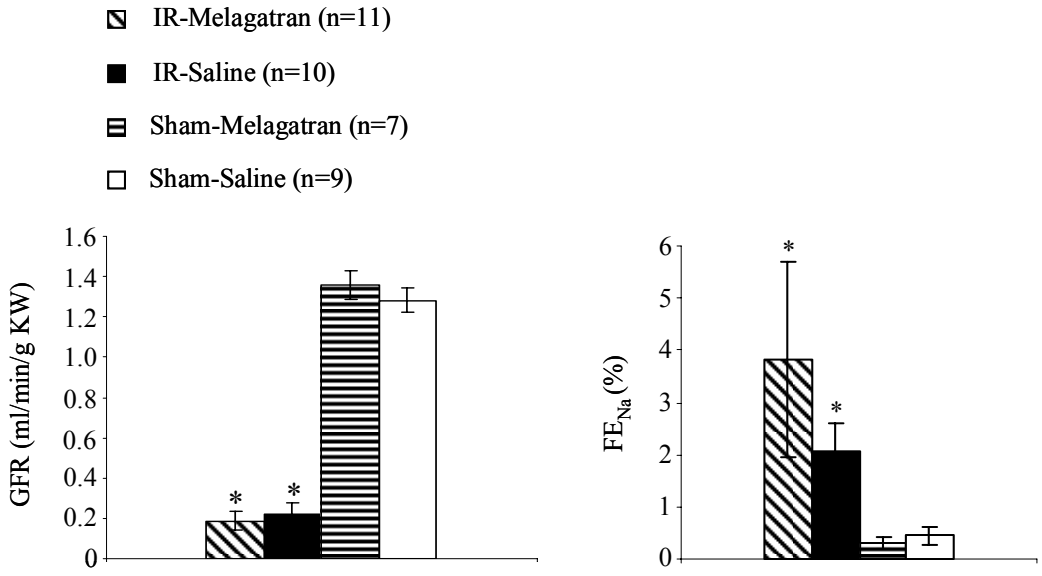



**Figure 17.** Glomerular filtration rate (GFR) and fractional urinary excretion rate of sodium ($FE_{Na}$) 48 h after renal ischemia-reperfusion (IR), or sham surgery, in thiobutabarbital anesthetized rats treated with melagatran or isotonic saline. KW denotes kidney weight. * $p<0.05$ vs. sham.

*Kidney histology*

Both groups subjected to renal IR showed significant tubular necrosis, tubular atrophy and dilatation, tubular cast formation, and interstitial mononuclear leukocyte inflammation, in the cortex and outer medulla ($p<0.05$ vs. sham, Figure 18). In addition, vascular congestion with erythrocytes and interstitial polymorphonuclear (PMN) neutrophil infiltration was evident in the inner stripe of the outer medullary zone of kidneys with IR-injury ($p<0.05$ vs. sham,

Figure 18). Renal fibrin depositions or microthrombi were not detected in any of the study groups. There were no statistically significant differences between groups IR-Saline and IR-Melagatran in any of the investigated histological variables (Figure 18).

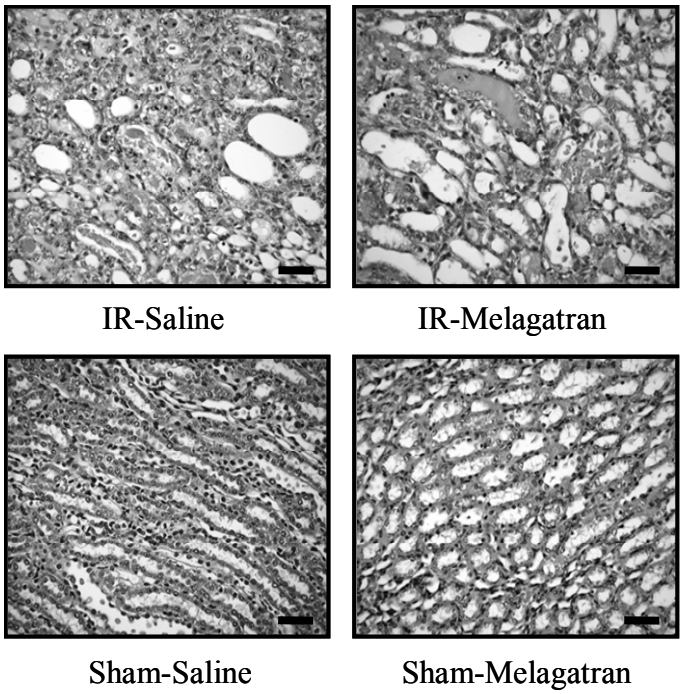



**Figure 18.** Kidney histology in the outer medulla 48 h after renal ischemia-reperfusion (IR; magnification x 20, scale bar denotes 250 μm). A similar degree of renal histopathological changes was observed in groups IR-Saline and IR-Melagatran.

*Plasma melagatran concentrations*

Plasma melagatran concentrations in group IR-Melagatran were: 0.72±0.11 μmol/L, 0.57±0.06 μmol/L and 0.18±0.03 μmol/L, at 0 h, 24 h and 48 h of reperfusion, respectively. The lower plasma concentration at 48 h could be explained by the fact that rats with renal IR-injury recover kidney function during the second day after the ischemic insult [129] as melagatran is eliminated from plasma principally by glomerular filtration [124]. The Sham-Melagatran group had plasma melagatran concentrations of 0.51±0.08 μmol/L at 48 h of reperfusion.

### 4.4. Effects of thrombin inhibition on renal hemodynamics and function and liver integrity during early endotoxemia (IV)

*Systemic hemodynamics, kidney function and intrarenal blood flow*

Lipopolysaccharide (LPS) administration caused a decrease in MAP (Figure 19), and an approximate 15 % increase in HR ($p<0.001$), in both melagatran- and saline-treated rats.

Endotoxin produced significant reductions in GFR, RBF and CLDF compared to sham (Figures 19 and 20). In addition, LPS caused a progressive decline in OMLDF over time in group LPS-Saline compared to sham (Figure 20). Melagatran significantly increased OMLDF in LPS-injected animals, but had no significant effects on RBF, CLDF, or GFR (Figures 19 and 20). Melagatran had no significant effects on renal hemodynamics and function in saline-treated control rats.

Plasma melagatran concentrations in group LPS-Melagatran were 1.04±0.05 μmol/L at 4.5 h after the start of LPS administration.

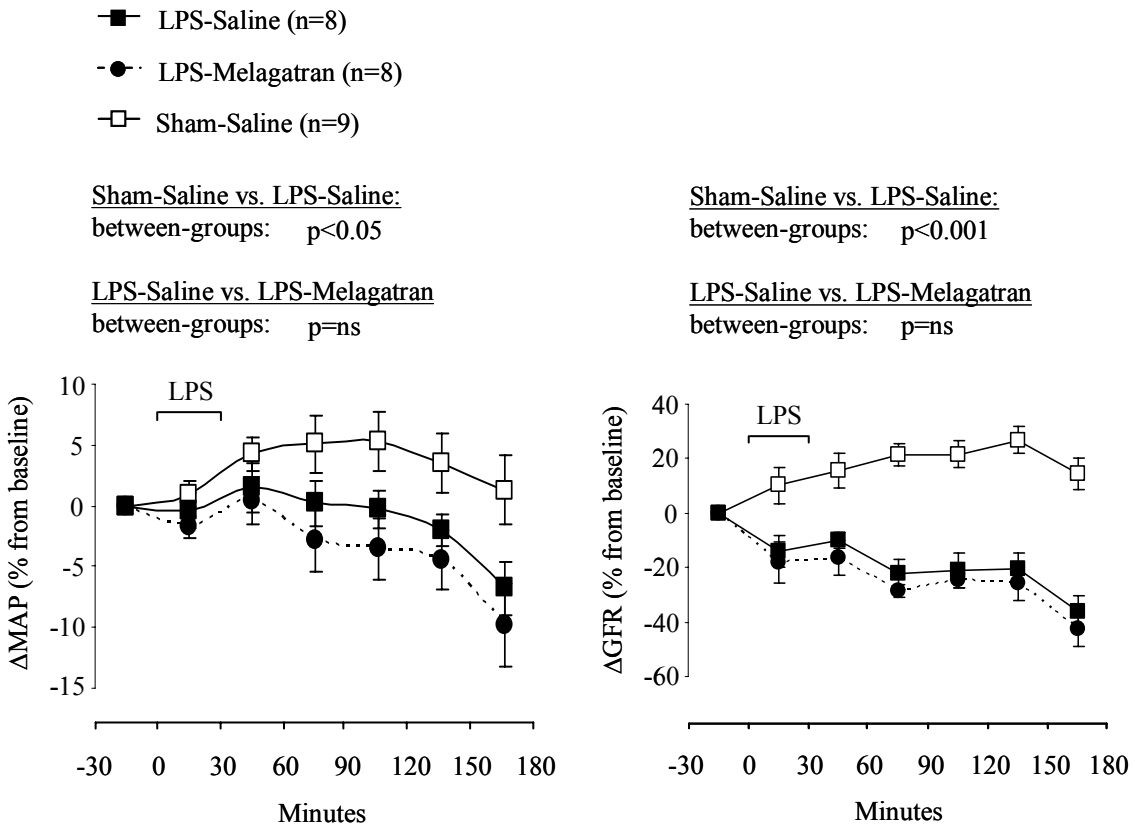



**Figure 19.** Effects of lipopolysaccharide (LPS) infusion on mean arterial pressure (MAP) and glomerular filtration rate (GFR) in thiobutabarbital anesthetized rats treated with melagatran or isotonic saline. Statistical analyses were performed by ANOVA for repeated measurements. ns denotes not statistically significant.

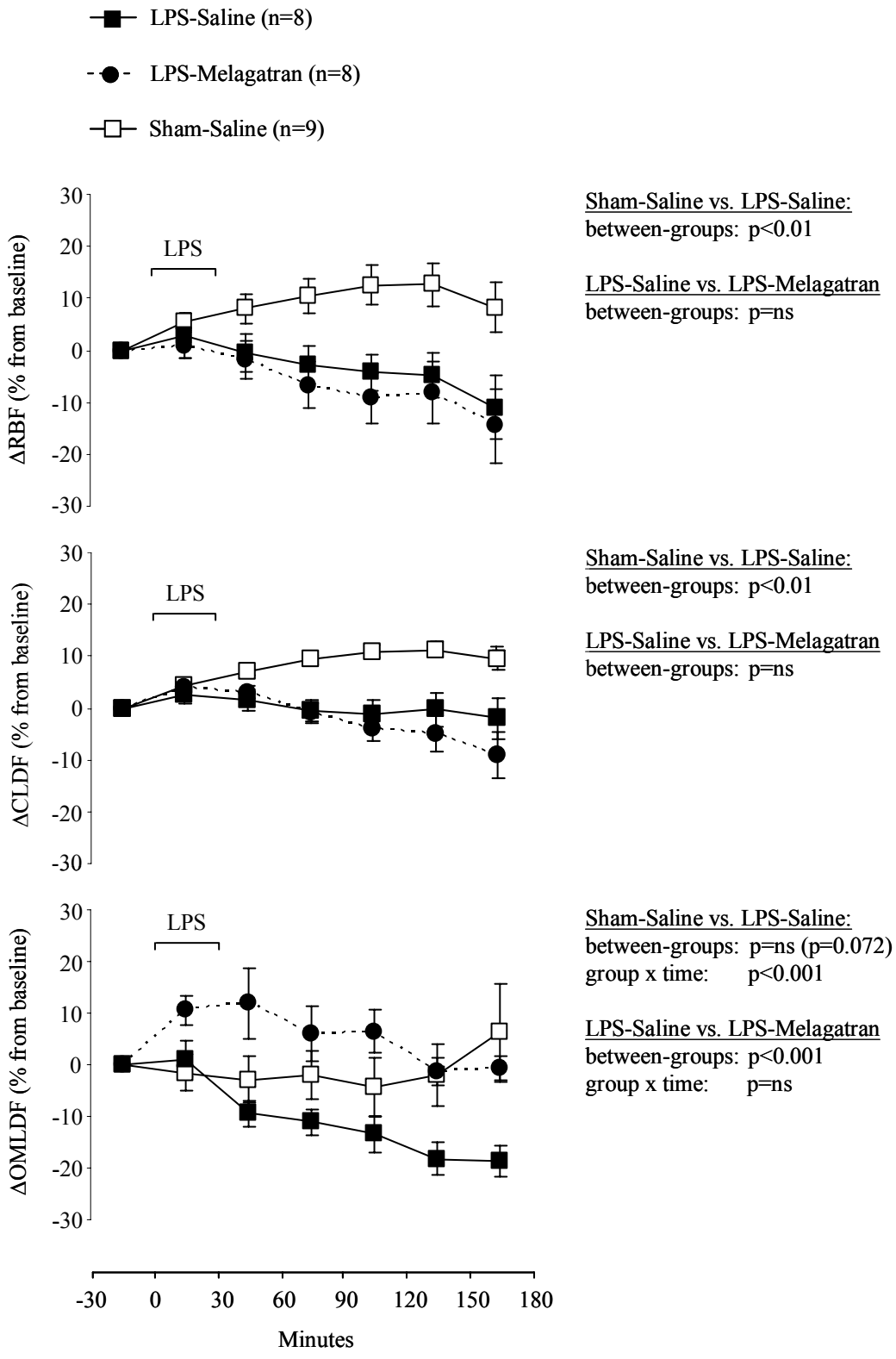



**Figure 20.** Effects of lipopolysaccharide (LPS) infusion on renal blood flow (RBF), and cortical (CLDF) and outer medullary (OMLDF) laser-Doppler fluxes, in thiobutabarbital anesthetized rats treated with melagatran or isotonic saline. Statistical analyses were performed by ANOVA for repeated measurements. "Group x time" denotes interaction between time, and treatment group, effects. ns denotes not statistically significant.

*Liver function and histology*

Endotoxin administration caused significant increases in ASAT, ALAT and bilirubin compared to sham (Figure 21). Melagatran reduced the elevated plasma concentrations of ASAT (-34±11 %), ALAT (-21±7 %) and bilirubin (-44±9 %), in endotoxemic rats (Figure 21). Melagatran had no significant effects on liver function tests in saline-treated control rats.

Endotoxemia caused a 5-fold increase in hepatocyte necrosis/apoptosis and a 25-fold increase in PMN neutrophil accumulation, compared to sham ($p<0.05$). There were no significant increases in fibrin deposition or microthrombi formation in livers of endotoxemic rats. There were no statistically significant differences in hepatocyte necrosis/apoptosis or hepatic PMN neutrophil sequestration between groups LPS-Saline and LPS-Melagatran.

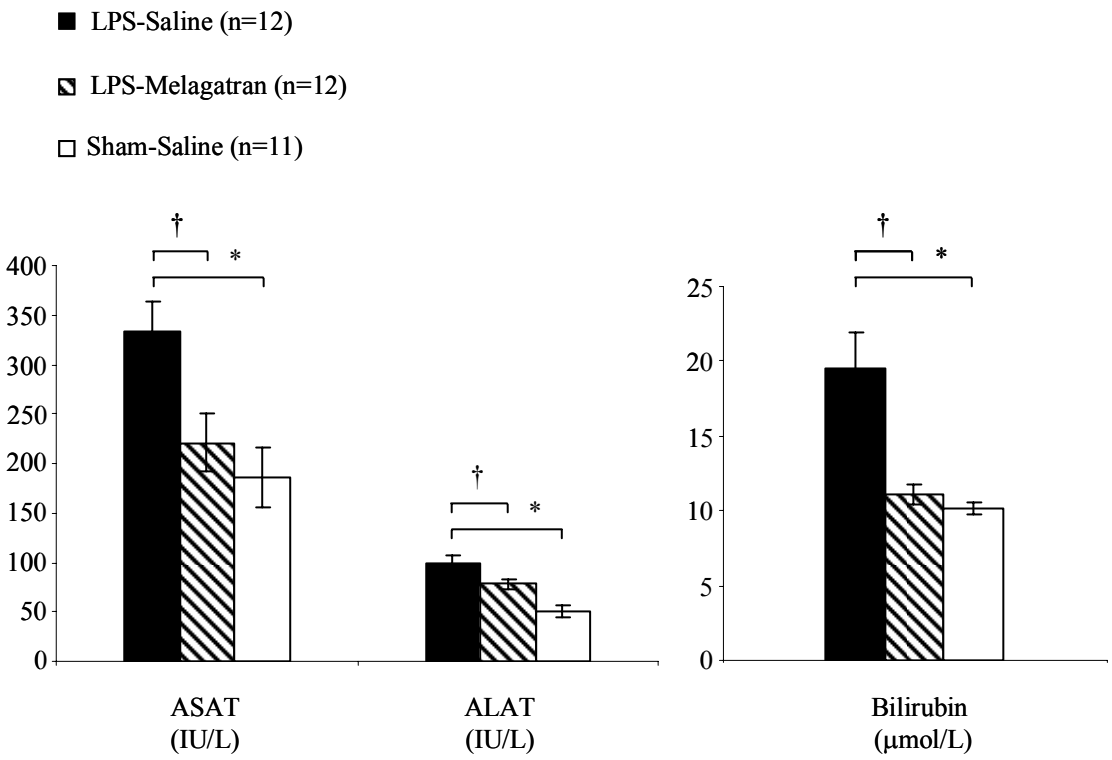



**Figure 21.** Effects of lipopolysaccharide (LPS) infusion on plasma concentrations of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and bilirubin in thiobutabarbital anesthetized rats treated with melagatran or isotonic saline. Blood was collected 4.5 h after starting LPS administration. * $p<0.05$ vs. sham, † $p<0.05$ between LPS-groups.

*Plasma tumor necrosis factor-α and nitrate/nitrite*

Rats with endotoxemia demonstrated a marked, approximately 7-fold increase in plasma tumor necrosis factor (TNF)-α concentrations (Figure 22). Melagatran significantly decreased the elevated plasma concentrations of TNF-α by 32±14 % (Figure 22). Endotoxin administration produced an approximately 15-fold increase in plasma nitrate/nitrite ($NO_3^-/NO_2^-$) compared to sham ($p<0.05$), with no significant difference between groups LPS-Saline and LPS-Melagatran.

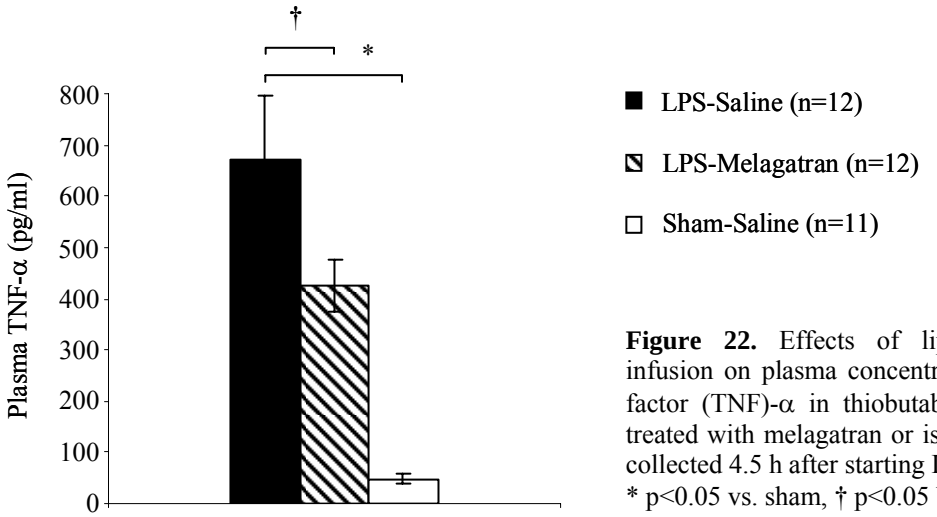



**Figure 22.** Effects of lipopolysaccharide (LPS) infusion on plasma concentrations of tumor necrosis factor (TNF)-α in thiobutabarbital anesthetized rats treated with melagatran or isotonic saline. Blood was collected 4.5 h after starting LPS administration.
* $p<0.05$ vs. sham, † $p<0.05$ between LPS-groups.

*Hepatic gene expression of TNF-α, iNOS and ICAM-1*

Endotoxin infusion increased hepatic mRNA levels of TNF-α, inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1; Figure 23). Melagatran had no statistically significant effects on TNF-α, iNOS or ICAM-1 gene expression (Figure 23).

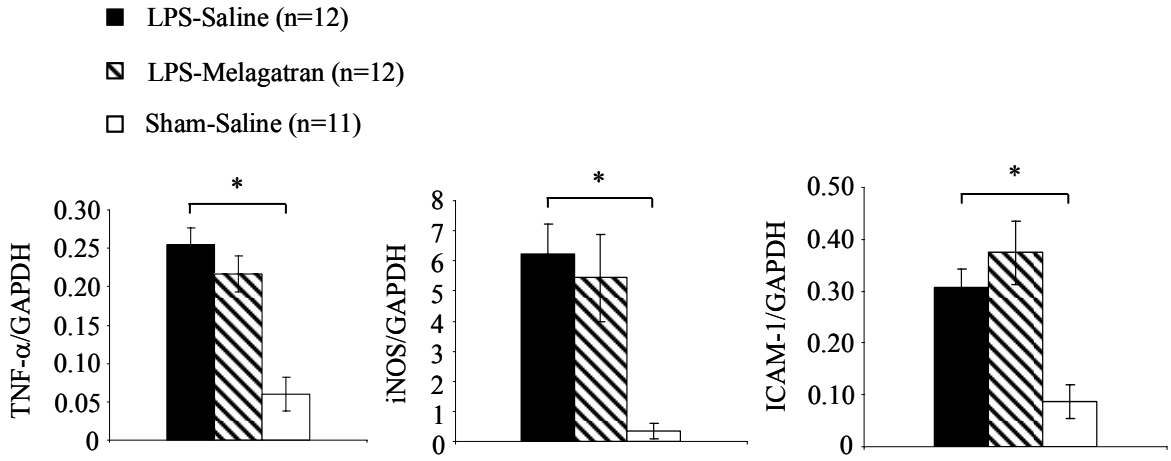



**Figure 23.** Effect of lipopolysaccharide (LPS) infusion on hepatic gene expression of tumor necrosis alpha (TNF)-α, inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1), relative to the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in thiobutabarbital anesthetized rats treated with melagatran or isotonic saline. Liver tissue was sampled 4.5 h after starting LPS administration. * $p<0.05$ vs. sham.

### 4.5. Role of endothelin receptor subtypes $ET_A$ and $ET_B$ in normotensive endotoxemia with acute renal dysfunction (V)

*Mean arterial pressure, heart rate and glomerular filtration rate*

Lipopolysaccharide infusion did not significantly affect MAP in groups LPS-Saline, LPS-BQ788 or LPS-BQ123+BQ788 (Figure 24). Endotoxin reduced MAP in group LPS-BQ123 by approximately 20 % compared to groups LPS-Saline and LPS-BQ123+BQ788 (Figure 24). Endotoxin caused a significant, approximately 15 %, increase in HR in all groups ($p<0.05$ vs. Sham-Saline).

Endotoxin reduced GFR and FF by approximately 40 % in all LPS-treated groups ($p<0.05$ vs. Sham-Saline, Figure 24).

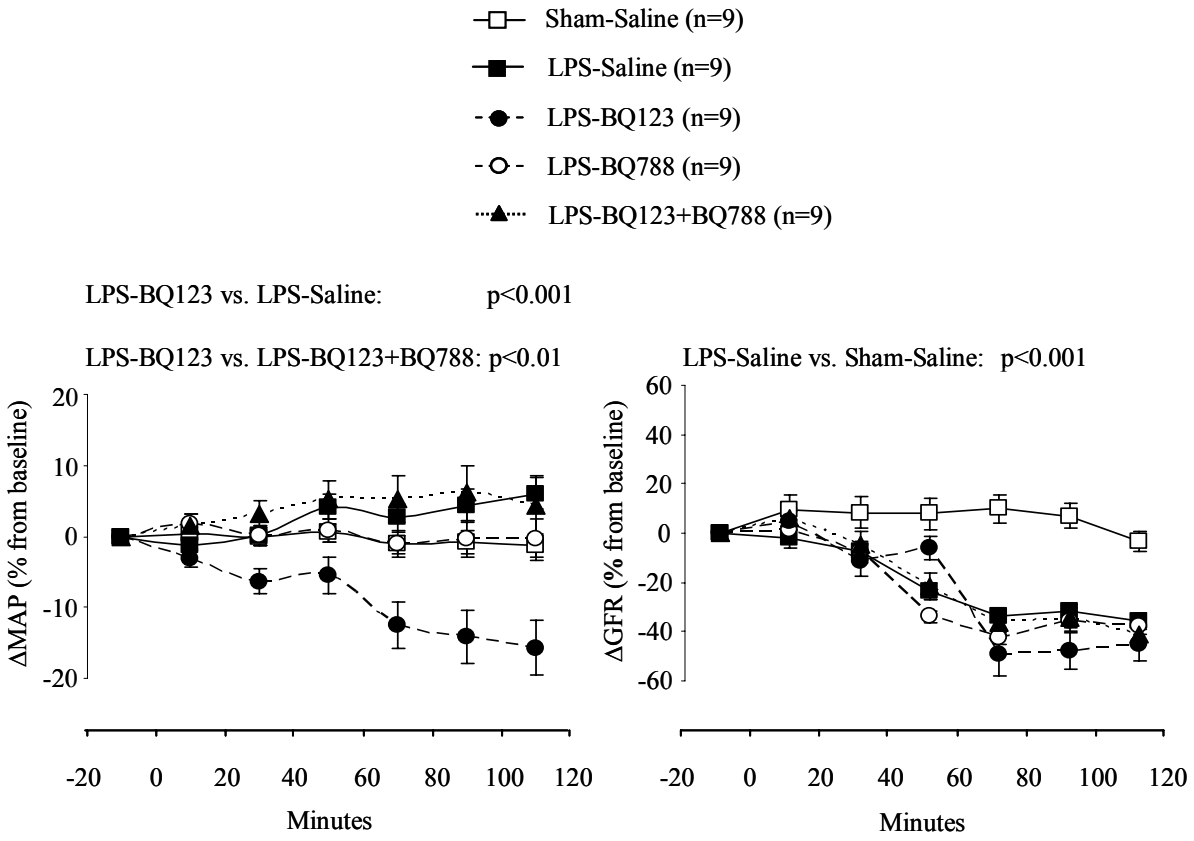



**Figure 24.** Mean arterial pressure (MAP) and glomerular filtration rate (GFR) in response to intravenous lipopolysaccharide (LPS) infusion (1 mg/kg/h starting at time 0 minutes) in thiobutabarbital anesthetized rats treated with BQ-123, BQ-788, BQ-123+BQ-788, or isotonic saline. P-values denote between-group differences for repeated measurements ANOVA.

*Intrarenal blood flow and renal vascular resistance*

There was no statistically significant difference in RBF and RVR between groups LPS-Saline and Sham-Saline (Figures 25 and 26). Endotoxin decreased RBF in groups LPS-BQ788 (-18±4 %) and LPS-BQ123+BQ788 (-14±3 %), and tended to increase RVR in these groups ($p=0.11$ for group LPS-BQ788 and $p=0.14$ for LPS-BQ123+BQ788), compared to LPS-Saline

(Figures 25 and 26). Endotoxin infusion in BQ-123-treated rats did not significantly influence RBF (Figure 25). However, group LPS-BQ123 had significantly decreased RVR compared to LPS-Saline and LPS-BQ123+BQ788 (Figure 26).

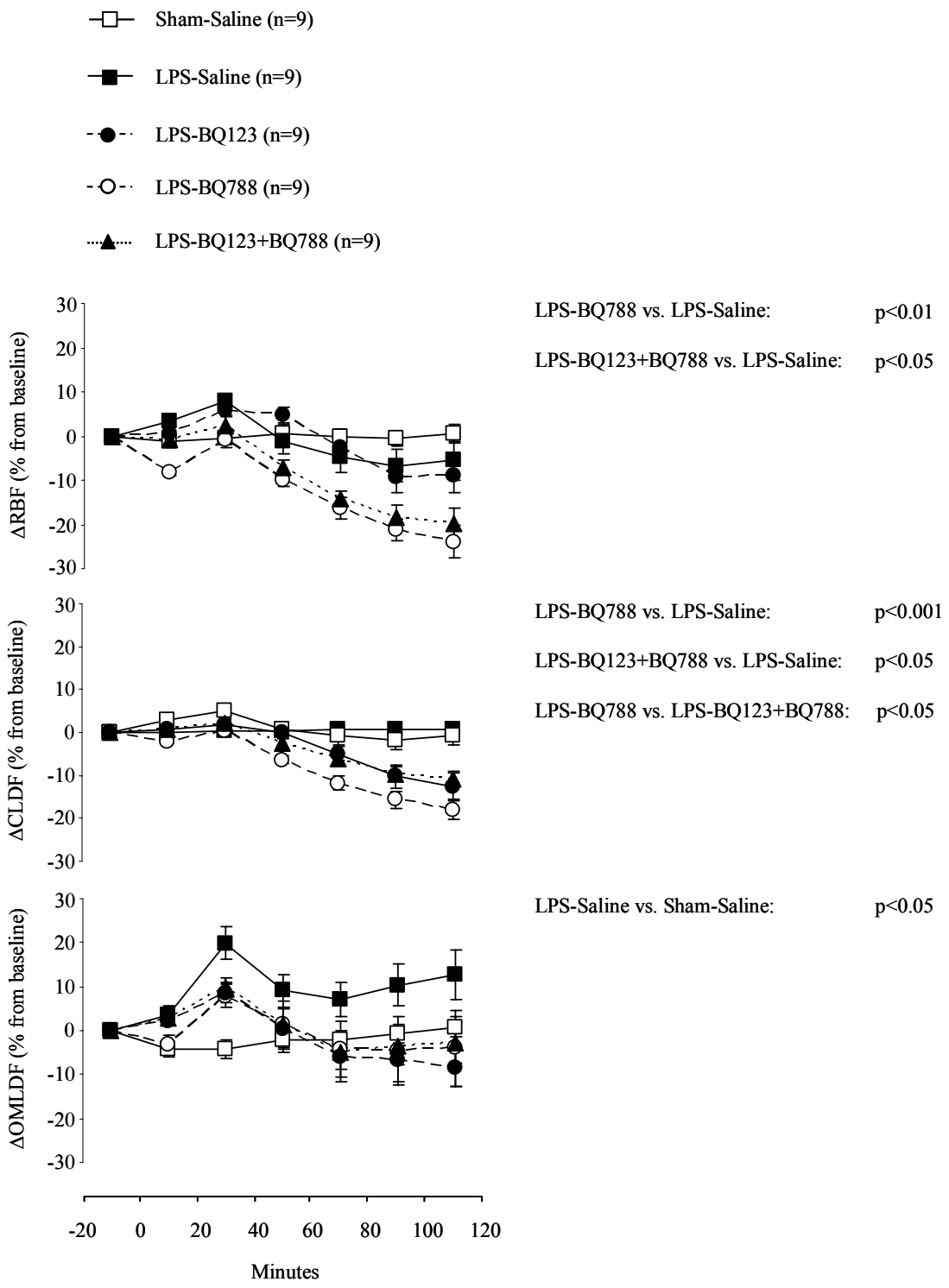



**Figure 25.** Renal blood flow (RBF), cortical laser-Doppler flux (CLDF) and outer medullary laser-Doppler flux (OMLDF) in response to intravenous lipopolysaccharide (LPS) infusion (1 mg/kg/h starting at time 0 minutes) in thiobutabarbital anesthetized rats treated with BQ-123, BQ-788, BQ-123+BQ-788, or isotonic saline. P-values denote between-group differences for repeated measurements ANOVA.

Endotoxin did not significantly affect CLDF in saline-treated rats (Figure 25). However, endotoxin decreased CLDF in groups LPS-BQ788 (-18±2 %) and LPS-BQ123+BQ788 (-10±2 %, Figure 25). In addition, CLDF was significantly reduced in group LPS-BQ788 vs. LPS-BQ123+BQ788 (Figure 25).

Endotoxin increased OMLDF (peak increase 24±4 % vs. Sham-Saline) in saline-treated rats (Figure 25). For the duration of LPS infusion, there were no statistically significant differences in OMLDF between rats treated with endothelin receptor antagonists and saline (Figure 25). However, in groups LPS-BQ123 and LPS-BQ123+BQ788, OMLDF was progressively reduced over time compared to in group LPS-Saline (group x time interaction $p<0.05$, Figure 25).

LPS-BQ123 vs. LPS-Saline: $p<0.001$

LPS-BQ123 vs. LPS-BQ123+BQ788: $p<0.001$

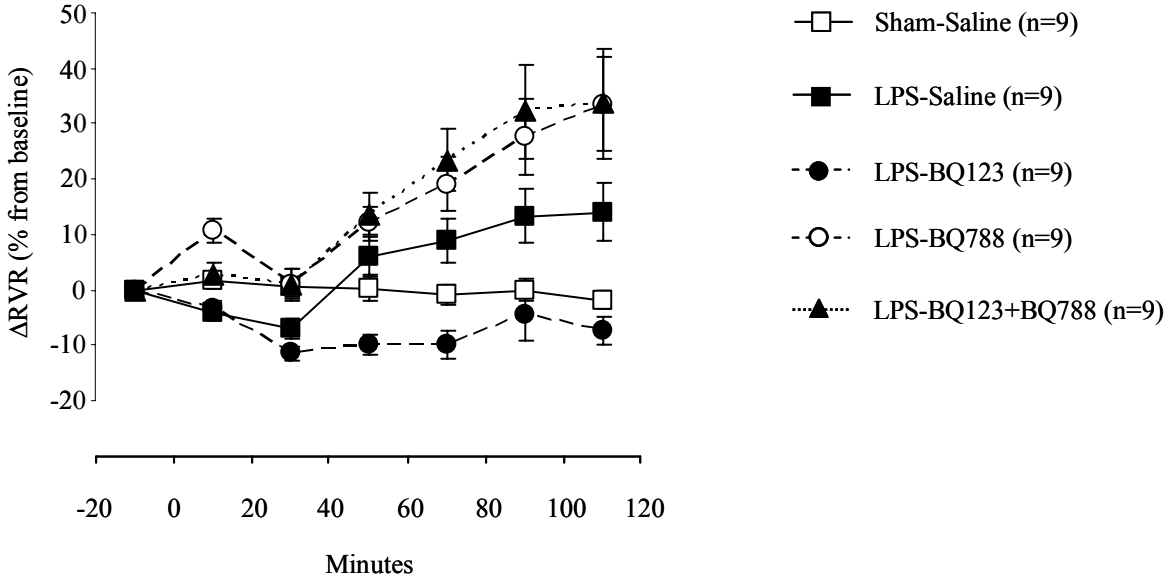



**Figure 26.** Renal vascular resistance (RVR) in response to intravenous lipopolysaccharide (LPS) infusion (1 mg/kg/h starting at time 0 minutes) in thiobutabarbital anesthetized rats treated with BQ-123, BQ-788, BQ-123+BQ-788, or isotonic saline. P-values denote between-group differences for repeated measurements ANOVA.

*Intrarenal oxygen tension*

Endotoxin did not have a statistically significant effect on cortical $pO_2$ in saline-treated rats (Figure 27). In addition, there were no significant differences in cortical $pO_2$ between groups treated with LPS (Figure 27).

Endotoxin dramatically increased outer medullary $pO_2$ (45±11 % vs. Sham-Saline at 120 minutes) in group LPS-Saline (Figure 27). Outer medullary $pO_2$ was normalized in group LPS-BQ123+BQ788 and significantly reduced compared to groups LPS-Saline and LPS-BQ788 (Figure 27).

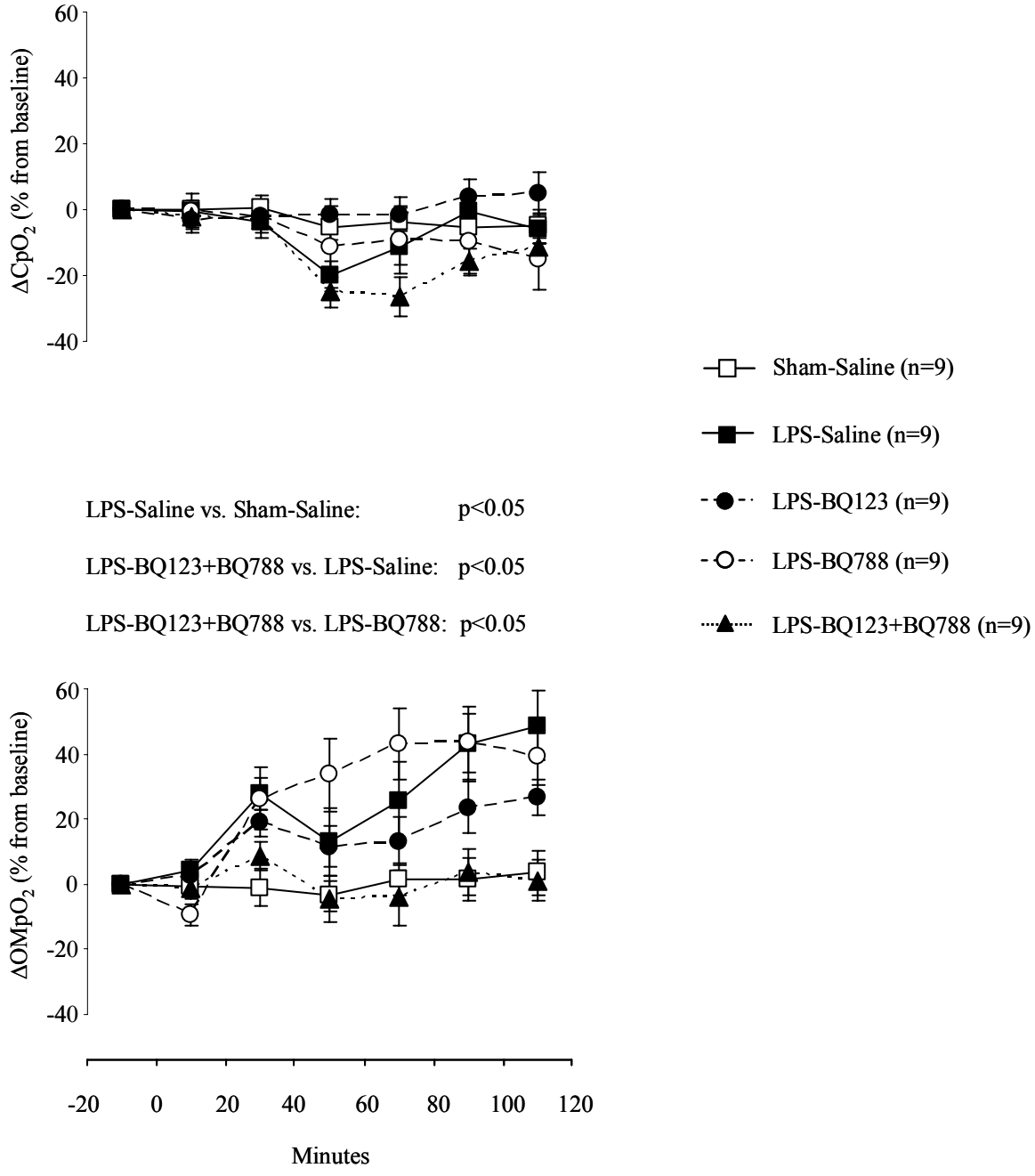



**Figure 27.** Renal cortical ($CpO_2$) and outer medullary ($OMpO_2$) oxygen tension in response to intravenous lipopolysaccharide (LPS) infusion (1 mg/kg/h starting at time 0 minutes) in thiobutabarbital anesthetized rats treated with BQ-123, BQ-788, BQ-123+BQ-788, or isotonic saline (see "Methods"). P-values denote between-group differences for repeated measurements ANOVA.

*Plasma endothelin-1 concentrations*

Lipopolysaccharide administration increased plasma endothelin-1 (ET-1) concentrations (7.5±0.2 pg/ml in group LPS-Saline and 6.1±0.4 pg/ml in group Sham-Saline, Figure 28). In addition, groups treated with selective, or combined, endothelin receptor antagonism had significantly elevated plasma ET-1 levels compared to LPS-Saline (Figure 28).

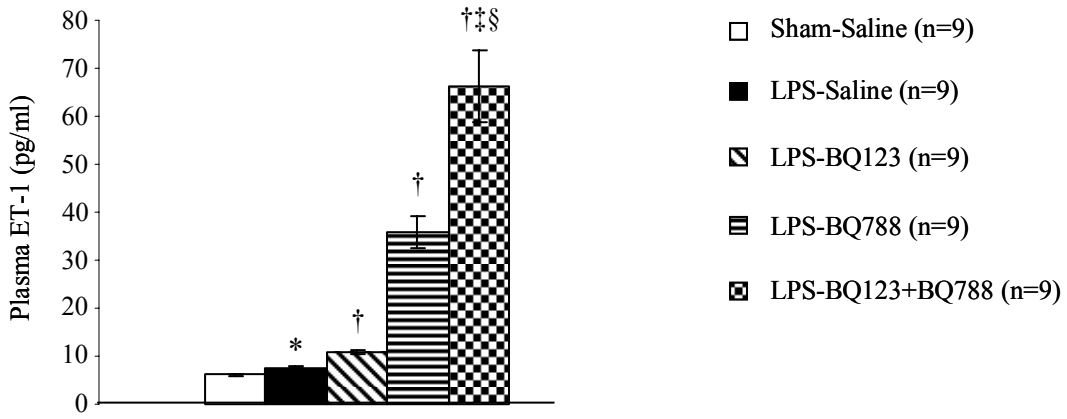



**Figure 28.** Plasma endothelin-1 (ET-1) concentrations 2 h after the start of intravenous lipopolysaccharide (LPS) infusion (1 mg/kg/h), in thiobutabarbital anesthetized rats treated with BQ-123, BQ-788, BQ-123-BQ-788, or isotonic saline. * $p<0.05$ vs. Sham-Saline; † $p<0.05$ vs. LPS-Saline; ‡ $p<0.05$ vs. LPS-BQ123, and § $p<0.05$ vs. LPS-BQ788.

# 5. DISCUSSION

## 5.1. Methodological considerations

*Experimental models of ischemic ARF*

At present there are no techniques to non-invasively and continuously establish the status of renal parenchymal microcirculation and oxygenation in humans. Although new techniques are evolving e.g. blood oxygenation level dependent magnetic resonance imaging and positron emission tomography, animal models seem indispensable to gain knowledge of the pathophysiology of ischemic ARF and to identify possible therapeutic targets.

However, although a large number of drugs have been shown to be renoprotective in experimental models of ischemic ARF, no convincingly effective therapy has yet emerged for patients. This could partly be explained by the fact that in clinical ARF the diagnosis is often delayed and therapy initiated after renal injury has been established. Also, the design of the experimental studies and the clinical relevance of the animal models could be questioned. For instance, experimental models may not address the multi-factorial etiology and predisposing co-morbidities of clinical ARF [31].

Renal ischemia-reperfusion (IR) is the predominant, and most well characterized, experimental model of ischemic ARF. However, except for in renal transplantation and aortic surgery, complete cessation of RBF followed by abrupt reperfusion is a rare cause of human ARF. The main histological differences between renal IR-injury and human ischemic ARF is that in patients, tubular necrosis is often limited, and although both proximal and distal nephron segments are affected, most severe injury appears in the distal nephron [31, 130]. In contrast, in renal IR there is extensive cellular necrosis with predilection for the proximal tubule in the outer medulla and distal nephron injury is often sparse [31, 42]. However, if the duration of renal arterial clamping is prolonged, with arterial occlusion times similar to in studies I-III of this thesis, distal nephron injury is evident.

Of the existing animal models, renal IR seems to most accurately resemble human ischemic ARF [131]. Acute kidney injury with selective outer medullary ischemia induced by co-administration of radiocontrast and inhibitors of NO and prostaglandin synthesis [132] has no clinical counterpart. Similarly, ARF after inferior vena cava occlusion is seldom encountered clinically [66]. Unfortunately, marked and prolonged renal hypoperfusion does not induce severe renal injury, at least not in healthy animals [133].

Taken together, although imperfect, the renal IR model has many similarities to human ischemic ARF, during all phases of renal injury (see “Introduction”, paragraph 1.3.). Previous studies applying the IR model have provided valuable knowledge of the pathophysiology of human ischemic ARF [131].

*Experimental models of sepsis-induced ARF*

Gram-negative bacteremia is a common cause of human sepsis [134]. In patients, increased plasma concentrations of bacterial endotoxin predict the development of multiorgan failure and mortality [135]. Hence, administration of endotoxin to animals, or employing low doses

to human volunteers, is the most prevalent mode to mimic gram-negative sepsis. In addition, it has been shown that the responses evoked by endotoxin in humans and in research animals are comparable [136]. The use of whole, viable and proliferating bacteria has the disadvantage that the endotoxin dose is difficult to control [42].

In patients, sepsis is often caused by a combination of gram-negative and gram-positive bacteremia. However, activation of coagulation and inflammation are universal responses to infection, and similar between different microorganisms [137]. Models that generate polymicrobial sepsis, e.g. cecal ligation and puncture (CLP), evoke a cytokine response somewhat different in kinetics and magnitude from that in endotoxemia [138]. Indeed, CLP is regarded as a model of subacute sepsis. However, in the CLP model the septic reaction is a combined result of bacterial contamination and bowel necrosis [139]. In addition, the response to CLP peritonitis is variable since the dose of bacteria is difficult to control and quantify. Consequently, only a minority of animals develop ARF after CLP and the severity of ARF is highly variable [140].

In the present studies well characterized models of endotoxemia (lipopolysaccharide from gram-negative E Coli) with acute renal failure were used. To resemble the clinical situation, animals were fluid resuscitated. Measurements were performed during early endotoxemia to investigate early pathophysiological mechanisms. Furthermore, we studied the effects of thrombin inhibition during the first 3-4 hours of endotoxemia since it has been shown that thrombogenesis surpasses fibrinolysis, and a proinflammatory state occurs, during this time period [47, 105, 122].

*Effects of anesthesia on renal hemodynamics and function*

To enable direct, continuous and simultaneous measurements of renal hemodynamics and function, and intrarenal oxygenation, we performed studies in anesthetized animals. These registrations are currently not technically feasible in conscious animals. It may be argued that anesthesia can influence the systemic and renal response to ischemia and endotoxin, and to the drugs investigated [141]. To take into account possible effects of anesthesia, time-control animals were included. The anesthetic thiobutabarbital was used in all studies. Thiobutabarbital has previously been shown not to significantly influence RBF and GFR [142]. We observed increases in UV, $FE_{Na}$ and $FE_K$ in saline-treated sham animals in our studies. This could presumably, at least to some extent, be an effect of inhibited tubular reabsorption by thiobutabarbital [142, 143]. However, the changes in urinary excretory function could also be a consequence of plasma volume expansion.

*Renal perfusion measurements with the laser-Doppler technique*

The laser-Doppler (LD) technique has been used to estimate relative changes in regional renal blood flow since the early 1980s. The major advantage of the LD technique is that it enables continuous measurements in a manner that is impossible with other methods. The main limitation of the method is that the LD signal does not provide absolute blood flow data in ml/min. To give absolute flows, the LD signal requires calibration against another method and knowledge of the local hematocrit [144]. Hence, in our studies LD data were presented in arbitrary perfusion units.

The LD device measures perfusion within a hemisphere of 0.3-0.5 $mm^3$, and this could be a limitation when microvascular perfusion is heterogeneous. In addition, LD flowmetry can not determine if changes in LD flux are due to capillary recruitment or rarefaction [144]. However, the LD method has previously been shown to be highly correlated to absolute renal cortical blood flow values measured by electromagnetic flowmeter (r=0.91) and radioactive microspheres (r=0.91) [145], and to medullary blood flow measured from the accumulation of $^{51}$Cr-labeled erythrocytes (r=0.92) and videomicroscopy [146, 147]. Thus, the LD signal provides a reliable estimate of regional tissue blood flow in the kidney.

### 5.2. N-acetylcysteine has renoprotective effects in ischemic acute renal failure

*Long-term effects of N-acetylcysteine*

In study I, we showed that N-acetylcysteine (NAC) attenuates the decline in renal function in a model of severe ischemic ARF. DiMari et al [71] have previously demonstrated that NAC given in high doses i.v. ameliorates the decline in GFR in a model of less severe renal IR-injury. Recently, NAC in a dose similar to the one used in our study was shown to attenuate tubular dysfunction (increases in $FE_{Na}$ and $FE_{H2O}$) in a model of mild renal IR-injury [73]. In that study, NAC prevented the down-regulation of renal medullary Na-K-2Cl co-transporter and aquaporin-2 expression after IR [73]. However, protective effects of NAC on tubular function were not evident in our study of severe renal IR.

In a clinical study, NAC demonstrated renoprotective effects in patients with ARF after cardiac surgery [148]. However, other studies have shown a lack of effect of NAC on the decline in renal function after cardiac surgery [149] and aortic aneurysm repair [150]. Notably, since indices of oxidative stress were not measured in these studies, it is difficult to determine if the doses of NAC were appropriate. Thus, further clinical trials are needed to determine if prophylactic administration of appropriate doses of NAC could protect renal integrity in patients at risk of developing ischemic ARF.

In the present study, beneficial effects of NAC were likely to have been mediated by restoration of renal levels of the antioxidant glutathione. In support of this notion, Paller et al have shown that glutathione infusion attenuates renal IR-injury [62]. Furthermore, we found decreased renal interstitial infiltration of lymphocytes in the cortex and outer medulla of NAC-treated animals 7 days after IR. This finding is in accord with previously reported antiinflammatory effects of NAC, e.g. attenuated NF-kappa B-induced expression of intercellular adhesion molecule-1, monocyte chemoattractant protein-1 and E-selectin [69], and with recent results of de Araujo et al [73] showing that NAC decreases lymphocyte and monocyte/macrophage infiltration into rat kidneys 48 h after IR. It is therefore plausible that reduced interstitial inflammation contribute to renoprotective effects of NAC [151].

In study I, NAC diminished systemic oxidative stress in rats with renal IR-injury, as was indicated by reduced plasma ascorbyl radical levels. However, and as previously suggested in studies of radiocontrast nephropathy [152] and cholestasis-induced

ARF [75], our results indicate that NAC may not exert protective effects by reducing lipid peroxidation as urinary 8-iso-$PGF_{2\alpha}$ levels were unaffected.

Little is known about systemic oxidative stress in animals with established ischemic ARF. Our finding of increased systemic oxidative stress (i.e. elevated urinary 8-iso-$PGF_{2\alpha}$ excretion and plasma ascorbyl radical levels) in rats with renal IR-injury is in accord with observations that systemic oxidative stress is enhanced in patients with ARF [10]. Interestingly, it has been suggested that elevated oxidative stress is associated with higher risk for death in patients with ARF [12]. One might hypothesize that NAC could attenuate systemic oxidative stress, and possibly improve survival, in patients with established ARF. Interestingly, clinical studies indicate that NAC may reduce mortality in patients with radiocontrast nephropathy [17], and attenuate the severity of illness in septic shock patients [153]. However, data are conflicting [154] and further studies are required to determine the clinical significance of NAC treatment.

*Acute effects of N-acetylcysteine on renal hemodynamics*

In study II, NAC did not attenuate the marked reductions in GFR and tubular function, during the first 80 minutes of reperfusion. Thus, NAC seems to inhibit the progression of tissue damage, or enhance repair mechanisms, during later stages of renal IR-injury. N-acetylcysteine did not affect renal blood flow, which rapidly returned to baseline levels in both NAC- and saline-treated groups after IR. N-acetylcysteine in a dose similar to the one used in the present study has been shown to attenuate the decline in RBF in models of ischemic ARF induced by inferior vena cava occlusion [66] or by co-administration of radiocontrast and inhibitors of prostaglandin and NO production [132]. However, these models are characterized by pronounced renal vasoconstriction and this might be a prerequisite for acute renal vasodilatory effects of NAC.

We found that the reduction in GFR early after IR appears to be independent of renal hypoperfusion. Previous studies have indicated the existence of a "no-reflow" phenomenon, i.e. capillary perfusion failure, after renal IR [40, 155]. Hellberg et al used laser-Doppler flowmetry to demonstrate persistent reductions in outer medullary perfusion 1 h after renal arterial declamping in rats [40]. The discrepancy between results could be explained by the fact that in our study rats were subjected to approximately two-fold higher infusion rates of saline, and this could have contributed to the rapid normalization of total and regional RBF during reperfusion. Plasma volume expansion might have caused inhibition of renal vasoconstrictor systems, and induction of endogenous renal vasodilators. Finally, the 15 % increase in MAP in our study, a finding not reported in studies showing renal hypoperfusion after IR, might also have contributed to the rapid normalization of OMLDF since medullary blood flow is poorly autoregulated [156].

### 5.3. Thrombin inhibition does not ameliorate renal ischemia-reperfusion injury

The beneficial effects of preventing activation of the coagulation system by inhibiting tissue factor (TF) have previously been demonstrated in renal IR [86]. However, it has not been clarified if improved renal function was due to decreased thrombin activity. In study III, it

was shown that selective thrombin inhibition with melagatran does not attenuate the decline in GFR, or outer medullary vascular congestion and tubular obstruction, 48 h after renal IR. In previous studies, heparin [157] and antithrombin [38] similarly did not prevent outer medullary vascular stasis after IR. However, heparin reduced tubular obstruction 1 h after IR [88], although thrombin inhibition with antithrombin did not diminish tubular dilatation 24 h after IR [38]. Thus, thrombin may contribute to tubular obstruction early after IR, but this effect seems not to be sustained until later time points.

Recently, it has been shown that selective thrombin inhibition with hirudin reduces renal inflammation and plasma creatinine levels after IR by preventing protease activated receptor (PAR)-1 activation, but not by decreasing intrarenal fibrin deposition [89]. In our study, melagatran did not reduce renal interstitial inflammation or polymorphonuclear (PMN) neutrophil infiltration after IR. This finding suggests that melagatran, at least not in the concentrations of the present study, did not effectively inhibit PAR activity and downstream inflammatory pathways. Furthermore, one might speculate that antiinflammatory effects of thrombin inhibitors are a prerequisite for renoprotection in renal IR injury.

In the present study, plasma melagatran concentrations at the time of renal arterial declamping were 0.72 μmol/L, and at 24 h after reperfusion 0.57 μmol/L, in group IR-Melagatran. Melagatran in these concentrations has previously been shown to cause almost complete thrombin inhibition and to markedly inhibit platelet activation and thrombosis formation *in vivo* [90, 91, 118-123]. For instance, plasma melagatran concentrations of approximately 0.6 μmol/L completely prevented the development of thrombosis formation in a model of vena cava thrombosis in the rat [119]. Furthermore, plasma concentrations in the range of 0.6-0.8 μmol/L have been shown to markedly reduce tissue fibrin depositions in endotoxemic rats with disseminated intravascular coagulation [122]. In the current study plasma melagatran concentrations were below 1.0 μmol/L, thus ruling out the possibility of melagatran-induced inhibition of endogenous fibrinolysis [90]. At 48 h after IR, plasma melagatran concentrations had declined to approximately 0.2 μmol/L, a level at which melagatran still effectively inhibits thrombin activity and thrombosis formation [118, 120, 121]. In a rat model of arterial thrombosis it was found that plasma concentrations of 0.15 μmol/L produced a 50 % antithrombotic effect [120]. A relatively low maintenance dose of melagatran was chosen as pilot studies revealed that doses any higher caused drug accumulation and fatal bleeding complications in rats with renal IR-injury. Taken together, plasma melagatran concentrations in the present study were clearly within the range of those well known to cause pronounced thrombin inhibition, in particular during the first 24 h after the ischemic insult when kidney injury is initiated [29] and the coagulation system activated [158].

It may be argued that bleedings and decreased hemoglobin concentrations in melagatran-treated rats could have counteracted renoprotective effects by reducing renal oxygen delivery. However, although RBF was not measured, there were no significant reductions in MAP, arterial $pO_2$ or oxygen saturation in animals receiving melagatran. In addition, it has been shown that renal oxygenation is maintained constant when hemoglobin concentrations decline to the level observed in group IR-Melagatran (129 g/L, vs. 143 g/L in group IR-Saline), by increased oxygen extraction [159]. It is therefore unlikely that the

modest reduction in hemoglobin levels caused a significant decrease in renal oxygenation that could have contributed to hypoxic renal injury.

### 5.4. Thrombin inhibition has beneficial effects during early endotoxemia

*Renal effects*

In study IV, thrombin inhibition with melagatran completely prevented the decline in OMLDF early during endotoxemia, although MAP, RBF and CLDF were not improved. These results indicate that melagatran specifically affects the renal medullary microcirculation, presumably by attenuating thrombin-induced constriction of descending vasa recta. Thrombin has been shown to cause renal vasoconstriction in the isolated perfused kidney through activation of PAR-1 receptors [79], and melagatran inhibits thrombin-induced PAR-1 cleavage *in vitro* [92]. Protease activated receptor-1 is expressed in the renal medulla [79], although its specific localization has not yet been determined. Thus, melagatran could attenuate thrombin-induced renal vasoconstriction by inhibiting PAR-1 activation. However, PAR-1 activation has been shown to have both vasoconstrictor and vasodilator effects in the isolated porcine renal interlobar artery *in vitro* [160]. PAR-4 activation appears not to influence renal vascular tone [79]. It can not be excluded that melagatran increased OMLDF by reducing microvascular thrombosis, although we could not provide evidence for this with the histological method used in our study. In addition, melagatran could cause vasodilation by preventing thrombin's reduction of tissue plasminogen activator (tPA) activity [93, 161]. Clearly, the mechanisms by which melagatran improved OMLDF need to be investigated further. Interestingly, since thrombin, by PAR-1 activation, constricts the human renal artery *in vitro* [162], it is possible that thrombin inhibition could have beneficial effects on renal hemodynamics in human sepsis.

Melagatran did not attenuate the decline in GFR, or impairment in tubular function, during the first 3 h of endotoxemia. However, since the renal outer medulla is hypoxic already during physiological conditions [23] it is plausible that the 20 % decline in OMLDF in endotoxemic rats could threaten outer medullary integrity. Consequently, we speculate that melagatran by normalizing OMLDF could prevent hypoxic tissue injury and improve kidney function long-term.

*Hepatic effects*

Thrombin has been implicated in the pathogenesis of liver injury in endotoxemia [84]. Corroborating these findings, melagatran decreased plasma ASAT, ALAT and bilirubin levels by 20-45 % during endotoxemia, although we could not detect beneficial effects on liver morphology. However, it is well established that impaired hepatocellular integrity, and not necessarily cell death, can cause release of aminotransferases into the circulation. Thus, we hypothesize that the positive effects of melagatran on liver function tests reflected less severe hepatocellular injury.

Melagatran did not attenuate liver dysfunction in endotoxemia by decreasing PMN neutrophil accumulation, microthrombosis formation, or the hepatic gene expression of

TNF-α, ICAM-1 or iNOS. However, it is possible that melagatran attenuated liver dysfunction by inhibiting PAR activation and/or by preserving liver blood flow. In support of this notion, Copple et al [84] have shown that thrombin's activation of PAR-1 on sinusoidal endothelium and Kupffer cells causes hepatic injury after LPS-infusion. Decreased liver blood flow has been demonstrated in a similar model of endotoxemia in rats [163]. Furthermore, thrombin inhibitors heparin and antithrombin have been shown to improve microvascular perfusion in the splanchnic circulation during endotoxemia [80].

It is reasonable to assume that melagatran might have exerted beneficial effects in the present study by reducing plasma TNF-α levels. The cytokine TNF-α is up-regulated early in both experimental [105] and clinical [44, 164] sepsis, and propagates inflammation and organ damage e.g. acute kidney injury [44, 49]. Tumor necrosis factor-α neutralization attenuates the decline in GFR and RBF in endotoxemic animals [49]. Furthermore, pre-treatment of rats with TNF-α antiserum affords protection against liver injury early after LPS exposure [165].

Although melagatran reduced plasma TNF-α levels, hepatic gene expression of TNF-α was unaffected. However, apart from the liver, monocytes are a major source of plasma TNF-α in endotoxemia [166]. It has been demonstrated that thrombin increases the secretion of TNF-α from monocytes exposed to endotoxin [167], and that thrombin inhibition decreases TNF-α production in these cells [168].

Recent studies suggest that activation of the coagulation system enhances systemic inflammation in endotoxemia. Although the anticoagulants tissue factor pathway inhibitor, antithrombin and activated protein C have been shown to have antiinflammatory properties in endotoxemia [78], the coagulation factors participating in the proinflammatory response have not been defined. Our results clearly indicate a role for thrombin-dependent signaling in this process.

In summary, our results indicate beneficial renal, hepatic and antiinflammatory effects of thrombin inhibition in endotoxemic rats. Previous studies have shown improved pulmonary function [169] and survival [48] in septic animals receiving thrombin inhibitors. Furthermore, thrombin inhibition attenuated endotoxin-induced disseminated intravascular coagulation in humans [47]. Results from a pilot study indicate that antithrombin supplementation reduces the frequency of renal replacement therapy and attenuates the increase in plasma bilirubin concentrations in septic patients [170]. In addition, in a subgroup analysis of a large, randomized, placebo-controlled, double blind phase III trial, the thrombin inhibitor antithrombin improved survival in patients with severe sepsis, despite increased incidence of bleeding [171].

### 5.5. Endothelin B receptor activation preserves renal blood flow in normotensive endotoxemia with acute kidney dysfunction

*Effects of endothelin B receptor antagonism*

In study V, endotoxemia in rats receiving selective $ET_B$ receptor blockade with BQ-788 caused an approximate 20 % reduction in RBF and CLDF in the absence of significant

changes in MAP. These results imply that $ET_B$ receptors preserve RBF during early endotoxemia by causing renal cortical vasodilation. One might argue that renal vasoconstriction during $ET_B$ receptor blockade could have been mediated by increased plasma ET-1 levels acting on $ET_A$ receptors [172], since $ET_B$ receptors are important in clearing ET-1 from the circulation [173]. Still, combined $ET_A$ and $ET_B$ receptor blockade did not prevent the reduction in RBF caused by LPS in rats treated with BQ-788 only. Thus, renal vasoconstriction in endotoxemic, BQ-788-treated rats, was primarily caused by a lack of $ET_B$ stimulation. Presumably, endothelial $ET_B$ receptors cause renal vasodilation by triggering the release of NO and prostaglandin $I_2$ [172]. Our results are in accord with previous studies in non-septic animals showing that $ET_B$ receptor activation can increase RBF and CLDF in response to endogenous [127] and exogenous [100, 174] ET-1.

Notably, our data show that LPS-induced decreases in RBF and CLDF in BQ-788 treated rats were not paralleled by significant reductions in GFR and FF. These results suggest that $ET_B$ receptor blockade constricted afferent and efferent arterioles to the same extent without a net effect on glomerular capillary pressure. However, our results could also be explained by $ET_B$ mediated afferent vasodilation accompanied by a reduction of the glomerular capillary ultrafiltration coefficient, as previously demonstrated in non-septic rats [128]. Thus, the precise effects of $ET_B$ receptor activation on renal afferent and efferent arteriolar tone, and on the glomerular ultrafiltration coefficient, during endotoxemia remain to be determined. Although $ET_B$ receptor antagonism did not seem to affect GFR, at least short-term in our study, it is feasible to speculate that $ET_B$-mediated renal cortical vasodilation could have renoprotective effects in endotoxemia by preserving renal oxygen delivery [51, 175].

The effects of selective $ET_B$ receptor blockade on renal hemodynamics during endotoxemia have not previously been examined. However, in a model of normotensive endotoxemia in conscious rats, combined $ET_A$ and $ET_B$ receptor blockade was shown not to significantly influence RBF [176]. Mitaka et al [102] showed that the non-selective endothelin receptor antagonist TAK-044 attenuated the decrease in RBF during endotoxemic shock. However, this finding does not necessarily contradict our results since TAK-044 inhibits the $ET_A$ receptor with a 100-fold greater potency than the $ET_B$ receptor [177].

*Effects of endothelin A receptor antagonism*

In animals treated with the $ET_A$ receptor antagonist BQ-123, LPS produced an approximately 20 % decrease in MAP compared to in saline-treated animals. This finding is in accord with a previous observation in normotensive endotoxemia [178]. Still, in the LPS-BQ123 group, RBF was preserved and RVR reduced. Notably, combined $ET_A$ and $ET_B$ receptor antagonism completely prevented the drop in MAP in response to LPS and caused a marked increase in RVR. These data clearly indicate that the hypotensive effect, and renal vasodilator response, in endotoxemic rats treated with BQ-123, was dependent on intact $ET_B$ receptor signaling.

*Effects of endothelin-1 on outer medullary perfusion*

In accord with previous studies in models of normotensive endotoxemia [104, 179], LPS caused pronounced reductions in GFR and FF in our study without significant alterations in

RBF. This could be explained by LPS causing a simultaneous constriction of afferent arterioles and dilatation of efferent arterioles leading to reduced glomerular capillary pressure. In addition, LPS-induced tubular dysfunction could increase sodium delivery to the distal tubule and activate the tubuloglomerular feedback mechanism resulting in afferent arteriolar constriction and decreased GFR [180]. Compatible with LPS-induced vasodilation of renal efferent arterioles of juxtamedullary nephrons, and possibly also direct effects on the descending vasa recta, LPS increased OMLDF in the present study. Our data suggest a role for ET-1 in mediating the increase in OMLDF, since OMLDF returned to baseline levels after 1 h of endotoxemia in rats receiving combined treatment with BQ-123 and BQ-788. Combined endothelin receptor blockade has previously been shown to have only transient and minor effects on OMLDF in normotensive endotoxemia [104]. The discrepancy between results could be explained by the degree of activation of the endothelin system, since it has been shown that ET-1 increases OMLDF dose-dependently [172]. Silldorff et al [181] have shown that ET-1, mainly by $ET_A$ receptor activation, constricts *in vitro* perfused outer medullary descending vasa recta. However, in that model, *in vivo* modulating vasodilator systems were absent. Notably, outer medullary hyperperfusion during early endotoxemia has previously been demonstrated also by others [104, 179] and a role for NO in this process has been indicated [179].

*Effects of endothelin-1 on outer medullary oxygenation*

We found that LPS increased outer medullary $pO_2$. Presumably this was a consequence of both increased OMLDF, and reduced GFR, as decreased filtered load reduces tubular solute reabsorption, and hence oxygen consumtion, in the outer medulla [26, 28]. Furthermore, increased outer medullary NO production could have contributed to the increase in $pO_2$ by reducing mitochondrial oxygen consumtion [182]. Interestingly, combined $ET_A$ and $ET_B$ receptor antagonism prevented the increase in outer medullary $pO_2$ caused by LPS. This observation is compatible with the results for OMLDF and suggests that ET-1 contributes to the increase in outer medullary perfusion and oxygenation during early normotensive endotoxemia. As previously suggested [172], activation of the endothelin system in the renal outer medulla could be a homeostatic mechanism to preserve perfusion and oxygenation.

# 6. CONCLUSIONS

1. N-acetylcysteine improves kidney function and reduces renal interstitial inflammation in rats subjected to renal IR-injury. These effects are associated with repletion of renal glutathione levels. N-acetylcysteine has no positive effects on GFR or renal blood flow during the first 80 minutes after renal IR. Presumably, NAC exerts renoprotective effects by antioxidative and antiinflammatory pathways, and not by improving renal hemodynamics during early reperfusion.

2. Systemic oxidative stress is increased in rats with ischemic ARF and is attenuated by NAC treatment.

3. The selective thrombin inhibitor melagatran does not ameliorate abnormalities in kidney morphology or function 48 h after renal IR. Thrombin is apparently not an important factor in the development of renal IR-injury in rats.

4. Thrombin inhibition with melagatran preserves renal outer medullary perfusion, ameliorates liver dysfunction and reduces plasma TNF-α levels during early endotoxemia.

5. During early normotensive endotoxemia $ET_B$ receptors exert a renal vasodilator influence and contribute to maintain normal RBF and cortical perfusion. Endogenous ET-1, via $ET_B$ receptors, may have renoprotective effects during this stage of endotoxemia.

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