Effects of thrombin inhibition with melagatran on renal hemodynamics and function and liver integrity during early endotoxemia

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Abstract

Sepsis is associated with an activation of the coagulation system and multi-organ failure. The aim of the study was to examine the effects of selective thrombin inhibition with melagatran on renal hemodynamics and function, and liver integrity, during early endotoxemia. Endotoxemia was induced in thiobutabarbital anesthetized rats by an intravenous (i.v.) bolus dose of lipopolysaccharide (LPS; 6 mg/kg). Study groups (1) Sham-Saline, (2) LPS-Saline and (3) LPS-Melagatran, received isotonic saline, or melagatran, immediately before (0.75 µmol/kg i.v.) and continuously during 4.5 h of endotoxemia (0.75 µmol/kg/h i.v.). Kidney function, renal blood flow (RBF), and intrarenal cortical and outer medullary perfusion (OMLDF) measured by laser-Doppler flowmetry, were analyzed throughout. Markers of liver injury and tumor necrosis factor (TNF)-α were measured in plasma after 4.5 h of endotoxemia. In addition, liver histology and gene expression were examined. Melagatran treatment prevented the decline in OMLDF observed in group LPS-Saline (p<0.05, LPS-Melagatran vs. LPS-Saline). However, melagatran did not ameliorate reductions in mean arterial pressure, RBF, renal cortical perfusion and glomerular filtration rate, or attenuate tubular dysfunctions, during endotoxemia. Melagatran reduced the elevated plasma concentrations of aspartate aminotransferase (-34±11 %, p<0.05), alanine aminotransferase (-21±7 %, p<0.05), bilirubin (-44±9 %, p<0.05) and TNF-α (-32±14 %, p<0.05) in endotoxemia. Melagatran did not diminish histological abnormalities in the liver, or the elevated hepatic gene expression of TNF-α, intercellular adhesion molecule-1, and inducible nitric oxide synthase, in endotoxemic rats. In summary, thrombin inhibition with melagatran preserved renal OMLDF, attenuated liver dysfunction and reduced plasma TNF-α levels, during early endotoxemia.

Key words: Acute kidney failure; Acute liver failure; Coagulation; Multiple organ failure; Sepsis
Introduction

Acute renal failure occurs in 20-50% of critically ill patients with sepsis and multiple organ dysfunction syndrome and is an independent risk factor contributing to the high mortality of 50-70% in this patient group (33).

Activated coagulation factors are likely to be important mediators of sepsis-associated organ injury (30, 33, 40). In support of this notion, activation of the coagulation system and increased thrombin generation in sepsis has been associated with intravascular fibrin formation, leukocyte activation and adhesion, and platelet aggregation (30, 40). These events may lead to microvascular injury, endothelial dysfunction, generalized microthrombi formation, and impaired blood flow to several organ systems thereby causing multi-organ failure (37, 40). Accordingly, thrombin inhibitors have been shown to improve microvascular perfusion in striated muscle and the mesentery during endotoxemia (19, 21), although the results are not conclusive (20, 39). Furthermore, thrombin inhibition has demonstrated beneficial effects on liver function and survival in endotoxemic animals in some (3, 31), but not in all (10, 32), studies. In addition to its roles in the coagulation pathway, and in platelet and leukocyte activation, thrombin has also been shown to cause renal vasoconstriction (11). This effect seems to be mediated by activation of protease-activated receptor (PAR)-1 (5, 11, 36). Protease-activated receptors are a novel class of receptors that are activated by serine proteases and that mediate numerous cellular actions of thrombin (4).

Melagatran is a selective and powerful inhibitor of thrombin activity and the conversion of fibrinogen to fibrin (13). Moreover, melagatran inhibits thrombin’s activation of PAR-1 and PAR-4 (28). Interestingly, thrombin inhibition with melagatran has been suggested to improve kidney function in endotoxemic pigs as indicated by reduced plasma creatinine levels (9). However, in that study no detailed analyses of renal hemodynamics and function were carried out. Hence, we hypothesized that melagatran, by inhibiting
microthrombosis formation and PAR-1 mediated renal vasoconstriction, might improve renal blood flow (RBF) and glomerular filtration rate (GFR) in endotoxemia.

Thus, the aim of the present study was to examine the effects of thrombin inhibition with melagatran on renal hemodynamics and function, and liver integrity, in a well characterized rat model (25) of endotoxemia with multiple organ dysfunctions.
Materials and Methods

General procedures
Male Sprague-Dawley rats (Harlan, Horst, The Netherlands) weighing approximately 250 g were used. All experiments were approved by the regional ethics committee in Göteborg. Chemicals were from Sigma (St. Louis, MO, USA) if not stated otherwise. Melagatran (a generous gift from AstraZeneca, Mölndal, Sweden) was stored and prepared as described (13).

Protocol 1: Renal hemodynamics and function
Rats were divided into three study groups: (1) LPS-Saline, n=8; (2) LPS-Melagatran, n=8; and (3) Sham-Saline, n=9, anesthetized with thiobutabarbital (Inactin, 120 mg/kg intraperitoneally), placed on a heating table, and prepared for renal clearance experiments as described (27). An arterial line was connected to a pressure transducer (Smiths Medical, Kirchseeon, Germany) for monitoring of mean arterial pressure (MAP) and heart rate (HR) using a data acquisition program (Biopac MP 150, Biopac Systems, Santa Barbara, CA, USA). The left kidney was exposed by a flank incision and immobilized in a plastic cup. The left ureter was catheterized for urine collection into pre-weighed vials. Rectal and kidney temperatures were kept at 37° C. A perivascular ultrasonic flow probe (0.7 VB, T206, Transonic Systems Inc., Ithaca, NY, USA) was placed around the left renal artery for measurement of RBF. Renal cortical (CLDF) and outer medullary (OMLDF) perfusion were estimated by laser-Doppler flowmetry (PF5000, Perimed, Stockholm, Sweden). The laser-Doppler signal has previously been demonstrated to provide a reliable estimate of regional tissue blood flow in the kidney (34). Renal outer medullary laser-Doppler flux was measured by a needle probe (411, Perimed) inserted 3.5 mm into the kidney, as described (27).
Throughout the experiment, rats received 10 ml/kg/h of isotonic saline. After completion of the surgical preparation, a 40 minute equilibration period was allowed before baseline clearance measurements during two consecutive 30 minute periods. Subsequently, endotoxemia was induced by intravenous (i.v.) administration of lipopolysaccharide (LPS; E Coli 0127:B8) in a bolus dose of 6 mg/kg during 30 minutes. This dose of LPS causes acute kidney dysfunction and liver injury in a well characterized model of endotoxemia in rats (25). The LPS-Melagatran group received melagatran immediately before LPS administration (0.75 µmol/kg i.v. bolus), and throughout the experiment (0.75 µmol/kg/h i.v.). Based on previous studies (7), and pilot experiments, this dose was expected to produce a plasma melagatran concentration of approximately 1 µmol/L throughout the study period, a concentration known to prolong thrombin time (TT) and activated partial thromboplastin time (APTT), and to exert pronounced antithrombotic effects in vivo (8). Control rats received equivalent volumes of isotonic saline. Renal hemodynamics and function were measured continuously for 3 h during endotoxemia.

In a supplementary group of sham animals (Sham-Melagatran, n=8) we investigated the effects of melagatran on renal hemodynamics and function according to the protocol described above. Adjusting for normal GFR, these animals received melagatran in a bolus dose of 1.0 µmol/kg i.v. followed by 1.0 µmol/kg/h i.v. throughout.

Glomerular filtration rate was determined by measuring renal $^{51}$Cr-EDTA clearance ($^{51}$Cr-ethylenediaminetetraacetic acid, Amersham, Buckinghamshire, UK), as described (12). Arterial blood samples (0.3 ml) were replaced by equivalent volumes of 4 % bovine serum albumin in isotonic saline. Urine and plasma samples were analyzed for sodium, potassium, and radioactivity, as described (12). Fractional urinary excretion rates of sodium (FE$_{Na}$, %), potassium (FE$_{K}$, %), and water (FE$_{H_{2}O}$, %), were estimated as the ratio of their respective clearances to that of $^{51}$Cr-EDTA, x 100. Renal vascular resistance (RVR) was
calculated as MAP (mmHg)/RBF (ml/min/g kidney weight [KW]), and filtration fraction (FF) as the ratio between GFR and RBF. Presented baseline data are average values of the two clearance periods prior to LPS administration.

Protocol 2: Liver integrity, blood gases and plasma analyses

Separate groups of rats (LPS-Saline, n=12; LPS-Melagatran, n=12; and Sham-Saline, n=11) were anesthetized and subjected to endotoxemia, and saline or melagatran treatment, following a protocol identical to the one described above. However, animals were not prepared for analyses of renal hemodynamics or clearance measurements, although MAP and HR were measured throughout. In addition, the protocol was extended to 4.5 h after starting endotoxin administration. After 4.5 h, indices of liver and pancreas injury were assessed by measuring plasma levels of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), bilirubin and pancreas-specific α-amylase. Concurrently, arterial blood gases were taken (ABL 510 blood-gas analyser, Radiometer, Copenhagen, Denmark) and blood was collected for analyses of plasma concentrations of melagatran, tumor necrosis factor (TNF)-α, and nitrate (NO₃⁻) and nitrite (NO₂⁻). Animals were sacrificed and lungs, kidneys and the liver were excised. Lung wet-to-dry weight ratio was determined after 12 h at 100° C.

Aminotransaminases were analyzed by an enzymatic method, and bilirubin and α-amylase were measured spectrophotometrically (Modular, Roche Diagnostics, Mannheim, Germany). Tumor necrosis factor-α was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Rat TNF ELISA Kit II, BD Biosciences, NJ, USA), and NO₃⁻/NO₂⁻ was measured spectrophotometrically (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Michigan, USA), following the manufacturer’s instructions. Plasma melagatran concentrations were measured using liquid chromatography-mass spectrometry (24).
Liver histology
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 analyses by light microscopy. Necrotic/apoptotic hepatocytes and polymorphonuclear (PMN) neutrophils were counted in 20 consecutive high-power fields (x 400). No distinction was made between cell necrosis and apoptosis. Analyses were made by an investigator blinded for treatment group.

Reverse transcription - polymerase chain reaction of liver tissue
Liver tissue was snap frozen in liquid nitrogen and stored at -80° C until analyzed. RNA was extracted using Trizol Reagent (Invitrogen, Paisley, UK), and subsequently cDNA was synthesized with the Thermoscript reverse transcriptase-polymerase chain reaction (RT-PCR) system (Invitrogen), following the manufacturer’s protocol. Relative quantification of mRNA was performed on a LightCycler (Roche) using SYBR-Green I, as described (14). Primer sequences for TNF-α (6), inducible nitric oxide synthase (iNOS) (35), intercellular adhesion molecule-1 (ICAM-1) (6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14) were obtained from the literature and synthesized by Invitrogen. Amplification conditions for TNF-α, iNOS, ICAM-1 and GAPDH cDNA were as described in the reference literature (6, 14, 35). Melting-curve analysis was performed to ensure single product amplification, and verified by a single band on an agarose gel. Relative expression of the target gene is shown as the ratio between target and house-keeping gene (GAPDH) cDNA.

Kidney histology
Kidneys were decapsulated, weighed, and immersion-fixed in 4 % formaldehyde in phosphate buffer (pH 7). Kidneys were stained with hematoxylin-eosin and masson-trichrome, and
processed for semi-quantitative assessments by light microscopy as previously described (12). The following variables were quantified separately in the renal cortex, outer and inner (ISOMZ) stripe of the outer medullary zone and in the inner medulla: tubular atrophy and dilatation, PMN neutrophil infiltration, interstitial edema, interstitial inflammation and fibrosis, vascular fibrin deposition and microthrombosis, and vascular congestion. Analyses were made by an investigator blinded for treatment group using an arbitrary scale where 0 = no changes, 1 = mild focal changes, 2 = modest diffuse changes, and 3 = severe diffuse changes, as described (12).

**Statistics**

Values are means ± SEM except for semi-quantitative data which are presented as the median with 25th and 75th percentiles. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Fisher’s post-hoc test, or ANOVA for repeated measurements, when appropriate. The following pre-specified between-group analyses were performed: Sham-Saline vs. LPS-Saline, and LPS-Saline vs. LPS-Melagatran. Histological data were analyzed by non-parametric Kruskal-Wallis’ and Mann-Whitney’s tests. A value of p<0.05 was considered statistically significant. The statistical software program SPSS 11.5.1 was used (SPSS Inc., Chicago, IL, USA).
Results

Systemic hemodynamics

Endotoxin administration caused a significant 10% decrease in MAP (p<0.05, Figure 1) and a concomitant approximate 15% increase in HR (p<0.001, data not shown). Melagatran had no significant effects on MAP (Figure 1) or HR in endotoxemic rats throughout the study period. A transient increase in MAP (peak increase of approximately 5%) was observed in group Sham-Saline (Figure 1). There were no significant differences in lung wet-to-dry weight ratio between groups (data not shown). No significant hemorrhages, and no deaths, occurred during experiments in any of the study groups.

Renal hemodynamics and function

At baseline, prior to LPS administration, there were no statistically significant differences between groups in renal hemodynamics or function except for an elevated rate of urinary potassium excretion (\(U_KV\)) in group LPS-Melagatran (p<0.05, Table 1).

Endotoxin produced significant reductions in RBF, CLDF and GFR compared to sham (p<0.05, Figures 2-3). In addition, LPS decreased FF by 28±5% in group LPS-Saline (p<0.05 vs. sham). Endotoxin caused a progressive decline in OMLDF over time compared to sham (group x time interaction p<0.001, between-groups p=0.07, Figure 2). In group LPS-Saline, both absolute (data not shown for \(U_NaV\) and \(U_KV\)) and fractional urinary excretion rates of water, sodium, and potassium showed a significantly different pattern over time compared to in group Sham-Saline (group x time interaction p<0.05, Figure 4). Excretory responses in LPS-treated animals were characterized by initial reductions followed by pronounced increases starting at approximately 90 minutes after initiation of LPS-infusion (Figure 4).
Melagatran treatment significantly increased OMLDF in LPS-injected animals (p<0.05, LPS-Melagatran vs. LPS-Saline, Figure 2). In addition, melagatran decreased UKV and FEK (p<0.05, Figure 4). However, there were no significant differences between LPS-groups in absolute levels of UKV and FEK throughout the endotoxemic period (data not shown). Melagatran had no statistically significant effects on RBF, CLDF, GFR, FF, or absolute or fractional urinary excretion rates of sodium or water, in endotoxemic rats (Figures 2-4). In group Sham-Saline, RBF, CLDF, GFR, UV, FE_Na and FE_K increased significantly over time compared to baseline values (p<0.05, Figures 2-4).

**Markers of liver injury**

Administration of LPS caused significant increases in ASAT, ALAT and bilirubin compared to sham (p<0.05, Figure 5). Treatment with melagatran significantly reduced the elevated plasma concentrations of ASAT (-34±11 %, p<0.05), ALAT (-21±7 %, p<0.05) and bilirubin (-44±9 %, p<0.05), in endotoxemic rats (Figure 5). Lipopolysaccharide had no statistically significant effect on plasma concentrations of pancreas-specific α-amylase (data not shown).

**Effects of melagatran in sham animals**

Melagatran had no statistically significant effects on RBF (+2±3 % vs. baseline), GFR (+4±2 % vs. baseline), CLDF (+1±2 % vs. baseline), and OMLDF (+3±6 % vs. baseline) in group Sham-Melagatran. Melagatran produced a modest reduction in MAP evident after 3 h (-6±1 % vs. baseline, p<0.05). Similar to in group Sham-Saline, UV, FE_Na and FE_K increased significantly over time compared to baseline values (p<0.05, data not shown). Plasma levels of ASAT, ALAT and bilirubin were not significantly different from in group Sham-Saline (data not shown). Plasma concentrations of melagatran were 1.20±0.06 μmol/L and not significantly different from in group LPS-Melagatran.
Plasma TNF-α, nitrate/nitrite, and melagatran concentrations

Rats with endotoxemia demonstrated a marked, approximately 7-fold increase in plasma TNF-α concentrations (p<0.05, Figure 6). Melagatran treatment significantly decreased the elevated plasma concentrations of TNF-α by 32±14 % (p<0.05, Figure 6). Endotoxin administration produced an approximately 15-fold increase in plasma NO₃⁻/NO₂⁻ compared to sham (p<0.05), with no significant difference between groups LPS-Saline and LPS-Melagatran (Figure 6). Plasma melagatran concentrations were 1.04±0.05 µmol/L in group LPS-Melagatran, when measured 4.5 h after the start of LPS administration.

Blood gases

Endotoxin produced an increase in plasma lactate levels (p<0.05), and a decrease in pCO₂ accompanied by respiratory alkalosis (p<0.05, Table 2). There were no significant differences in pO₂ (Table 2), base excess or plasma bicarbonate levels (data not shown) between study groups. Both LPS-injected groups had significantly lower hemoglobin concentrations compared to sham (p<0.05, Table 2).

Liver histology

Endotoxemia caused an approximate 5-fold increase in hepatocyte necrosis/apoptosis and a 25-fold increase in PMN neutrophil accumulation, predominantly in periportal areas, compared to sham (p<0.05, Figure 7). There were no apparent abnormalities in Kupffer cell morphology, and no increases in fibrin deposition, microthrombi formation, or sinusoidal congestion, in endotoxemic rats (data not shown). Group LPS-Melagatran had a similar degree of histopathological injury compared to LPS-Saline, and there were no statistically significant differences in hepatocyte necrosis/apoptosis or hepatic PMN neutrophil sequestration between LPS-treated groups (Figure 7).
**Hepatic gene expression of TNF-α, iNOS and ICAM-1**

Endotoxin administration significantly increased hepatic mRNA levels of TNF-α, iNOS and ICAM-1, compared to sham (p<0.05, Figure 8). Melagatran had no statistically significant effects on TNF-α, iNOS or ICAM-1 gene expression (Figure 8). There were no significant differences between groups in the expression of the house-keeping gene GAPDH (data not shown).

**Kidney histology**

There was no significant difference in kidney weight between groups (data not shown). Group LPS-Saline showed no statistically significant abnormalities in any of the investigated renal histological variables compared to sham (data not shown). There was a tendency towards an increased vascular congestion of erythrocytes in the ISOMZ in group LPS-Saline compared to sham (p=0.24, data not shown). Melagatran significantly decreased vascular congestion of erythrocytes in the ISOMZ of LPS-injected animals (0 [0-0.25] vs. 1 [0-2], in groups LPS-Melagatran and LPS-Saline, respectively, p<0.05).
Discussion

The main findings of the present study were that selective thrombin inhibition with melagatran preserved renal outer medullary perfusion, and reduced markers of liver injury and plasma TNF-α levels, during early endotoxemia in rats.

In the present study, endotoxin caused an approximate 50 % decline in GFR that was paralleled by significant reductions in RBF, CLDF and OMLDF. As the renal outer medulla is hypoxic already during physiological conditions and therefore vulnerable to reductions in blood flow (2), it is feasible to hypothesize that the observed 20 % decrease in OMLDF in endotoxemic rats could have threatened outer medullary integrity. Although melagatran did not improve GFR or attenuate tubular dysfunctions during the first 3 h of endotoxemia, we speculate that melagatran, by completely preventing the decrease in OMLDF, could prevent ischemic tissue injury and improve kidney function long-term. However, this hypothesis needs to be addressed in future studies by measuring outer medullary oxygen tension in this model, and by examining kidney function at later stages of endotoxemia.

The mechanisms by which melagatran improved OMLDF specifically during endotoxemia were not elucidated in the present study. However, as melagatran did not have any significant effects on MAP or total RBF in endotoxemic rats, our results suggest that melagatran exclusively affected the renal medullary microcirculation. Blood flow to the renal medulla is regulated by a number of vasoactive substances that are synthesized within the medulla and act through paracrine and autocrine mechanisms (29). Based on anatomical considerations, descending vasa recta seem to be an important site of regulation of renal medullary blood flow. Descending vasa recta are surrounded by contractile pericytes and respond to an array of vasoactive agents in a manner similar to arterioles (29). Interestingly, thrombin has been shown to cause renal vasoconstriction through activation of PAR-1
receptors (5, 11, 36). In a recent study (28) it was demonstrated that melagatran inhibited thrombin-induced PAR-1 cleavage in platelets in a dose-dependent manner. Thus, it is reasonable to speculate that melagatran could inhibit thrombin-induced renal vasoconstriction through inhibition of PAR-1 activation. However, to our knowledge the effects of thrombin, and PAR activation, on renal medullary microcirculation have not been examined. Clearly, the mechanisms by which melagatran improved OMLDF in the present study need to be investigated further.

Interestingly, urinary sodium excretion increased markedly 90 minutes after LPS administration in endotoxemic rats although MAP, RBF and GFR were reduced, and the sympathetic nervous system activated as indicated by pronounced elevations in heart rate. These endotoxin-induced abnormalities in tubular function were not affected by melagatran treatment although melagatran improved OMLDF, indicating that tubular dysfunctions were not caused by outer medullary ischemia. In the current study there was a transient, modest, rise in MAP, and progressive increases in RBF, GFR, UV, FE_{Na}, and FE_{K}, over time in group Sham-Saline. These findings could probably be explained by extracellular fluid volume expansion as animals were resuscitated with intravenous saline to resemble the clinical situation in septic patients. Indeed, when comparing infused volume to urine output for each clearance period, all study groups were in clear positive fluid balance throughout.

It may be argued that the modest effect of melagatran on kidney function in the present study could be explained by low rates of thrombin generation during early endotoxemia. However, it has previously been shown that the coagulation system is markedly activated, and thrombin generation increased, already during the first hours after endotoxin administration (3, 31). Furthermore, since thrombin has a high affinity for PAR-1 (4, 28), increased thrombin generation would most likely also result in PAR-1 activation. In addition, the dose of melagatran in the present study was appropriate since plasma melagatran
concentrations of 1 µmol/L have been shown to exert almost complete thrombin inhibition and pronounced antithrombotic effects in vivo (8), and to inhibit thrombin’s activation of PAR-1 and -4 in vitro (28).

Activated coagulation factors have been implicated in the pathogenesis of liver injury in endotoxemia (26). Corroborating these findings, thrombin inhibition with melagatran decreased plasma ASAT, ALAT and bilirubin levels by 20-45 % in endotoxemic animals, although we could not detect beneficial effects on liver morphology. Presumably, we would have been able to detect less severe hepatocellular injury in melagatran-treated rats using more sensitive histological methods, e.g. electron microscopy. It is well established that impaired hepatocellular integrity, and not necessarily cell death, can cause release of aminotransferases into the circulation. Thus, it is feasible to hypothesize that the positive effects of melagatran on liver function tests reflected less severe hepatocellular injury. Furthermore, beneficial effects of melagatran on liver integrity might have been detected earlier with liver function tests than by histological analyses. This is supported by observations in other models of hepatic injury in rats (23).

In the present study melagatran did not decrease PMN neutrophil accumulation in the liver, or the hepatic gene expression of TNF-α, ICAM-1 or iNOS, in endotoxemic rats. In addition, LPS-injected animals showed no apparent hepatic microthrombosis. Therefore, and as suggested by others (3, 26), our data imply that thrombin inhibition does not attenuate liver dysfunction in endotoxemia by decreasing PMN neutrophil accumulation or by reducing microthrombosis formation. We speculate that melagatran could have attenuated liver dysfunction in the current study by inhibiting PAR-1 activation and/or by preserving liver blood flow. In this regard, Copple et al (3) have shown that PAR-1 activation by thrombin causes hepatic injury after LPS-infusion. In addition, decreased liver blood flow has been demonstrated in a similar model of endotoxemia in rats (38). Furthermore, thrombin inhibitors
heparin and antithrombin have been shown to improve microvascular perfusion in the splanchnic circulation during endotoxemia (21). Thus, melagatran might have had positive effects on liver blood flow, similar to in the renal outer medulla, in the present study. Additional studies are required to determine how melagatran reduced liver dysfunction during early endotoxemia.

Finally, melagatran treatment reduced plasma TNF-α concentrations by approximately 30% in endotoxemic rats. Tumor necrosis factor-α is a cytokine known to propagate inflammation and organ damage in sepsis (15). Furthermore, plasma TNF-α levels correlate with mortality in septic patients (15). Knotek et al (22) found that TNF-α neutralization ameliorated early renal dysfunction in endotoxemic mice. Similarly, pretreatment of rats with TNF-α antiserum afforded protection against liver injury early after LPS exposure (16). Thus, it is reasonable to speculate that melagatran may have beneficial effects in endotoxemia through its effect on plasma TNF-α levels. Interestingly, although melagatran reduced plasma TNF-α levels, hepatic gene expression of TNF-α was unaffected. This finding could be explained by the fact that monocytes are a major source of plasma TNF-α in endotoxemia (1). In support of this hypothesis, it has been demonstrated that thrombin increases the secretion of TNF-α from monocytes exposed to endotoxin (18), and that thrombin inhibition decreases TNF-α production in these cells (17). Recent studies suggest that activation of the coagulation system enhances systemic inflammation in endotoxemia, although the coagulation factors participating in the proinflammatory response have not been defined (30, 31). Our results clearly indicate a role for thrombin-dependent signaling in this process.

In conclusion, thrombin inhibition with melagatran preserved renal outer medullary perfusion, ameliorated liver dysfunction and reduced plasma TNF-α levels in
endotoxemic rats. These findings may have important implications as they suggest protective effects of thrombin inhibition in sepsis, a clinical condition associated with a bad prognosis.

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References


human plasma and urine by liquid chromatography-mass spectrometry. J Chromatogr B


Legends to figures

Figure 1. Effect of melagatran, or isotonic saline, on mean arterial pressure (MAP) in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Lipopolysaccharide in a dose of 6 mg/kg was infused intravenously during 30 minutes from time 0 to 30 minutes (see “Methods”). Values are means ± SEM. Statistical analyses were performed by ANOVA for repeated measurements. ns denotes not statistically significant.

Figure 2. Effect of melagatran, or isotonic saline, on renal blood flow (RBF), and renal cortical (CLDF) and outer medullary (OMLDF) laser-Doppler fluxes in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Lipopolysaccharide in a dose of 6 mg/kg was infused intravenously during 30 minutes from time 0 to 30 minutes (see “Methods”). Values are means ± SEM. 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.

Figure 3. Effect of melagatran, or isotonic saline, on glomerular filtration rate (GFR) in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Lipopolysaccharide in a dose of 6 mg/kg was infused intravenously during 30 minutes from time 0 to 30 minutes (see “Methods”). Values are means ± SEM. Statistical analyses were performed by ANOVA for repeated measurements. ns denotes not statistically significant.

Figure 4. Effect of melagatran, or isotonic saline, on urine volume (UV), and fractional urinary sodium (FE_{Na}) and potassium (FE_{K}) excretion, in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Lipopolysaccharide in a dose of 6 mg/kg was infused
intravenously during 30 minutes from time 0 to 30 minutes (see “Methods”). Values are means ± SEM. 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.

**Figure 5.** Effect of melagatran, or isotonic saline, on plasma concentrations of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and bilirubin in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Blood was collected 4.5 h after starting LPS administration. Values are means ± SEM. * denotes p<0.05 vs. sham. † denotes p<0.05 vs. LPS-Saline.

**Figure 6.** Effect of melagatran, or isotonic saline, on plasma concentrations of tumor necrosis factor (TNF)-α and nitrate (NO$_3^-$)/nitrite (NO$_2^-$) in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Blood was collected 4.5 h after starting LPS administration. Values are means ± SEM. * denotes p<0.05 vs. sham. † denotes p<0.05 vs. LPS-Saline.

**Figure 7.** Effect of melagatran, or isotonic saline, on hepatocyte necrosis/apoptosis and hepatic polymorphonuclear (PMN) neutrophil infiltration in thiobutabarbital anesthetized rats infused with lipopolysaccharide (LPS). Liver tissue was sampled 4.5 h after starting LPS administration. Necrotic/apoptotic hepatocytes and PMN neutrophils were counted in 20 consecutive high-power fields (HPF) at x 400 magnification. Values are means ± SEM. * denotes p<0.05 vs. sham.

**Figure 8.** Effect of melagatran, or isotonic saline, 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 thiobutabarbitral anesthetized rats infused with lipopolysaccharide (LPS). Liver tissue was sampled 4.5 h after starting LPS administration. Analyses were performed by reverse transcription-polymerase chain reaction. Values are means ± SEM. * denotes p<0.05 vs. sham.
Tables

Table 1. Renal hemodynamics and function at baseline.

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<td>7.2±0.2</td>
<td>8.6±0.4</td>
</tr>
<tr>
<td>RVR (mmHg/[ml/min/g KW])</td>
<td>14.5±1.2</td>
<td>16.6±0.7</td>
<td>14.5±0.7</td>
</tr>
<tr>
<td>FF (%)</td>
<td>12.4±0.8</td>
<td>14.0±0.7</td>
<td>12.9±0.8</td>
</tr>
<tr>
<td>CLDF (PU)</td>
<td>673±17</td>
<td>631±28</td>
<td>678±32</td>
</tr>
<tr>
<td>OMLDF (PU)</td>
<td>155±7</td>
<td>136±7</td>
<td>153±10</td>
</tr>
<tr>
<td>UV (µl/min/g KW)</td>
<td>3.58±0.54</td>
<td>3.92±0.64</td>
<td>4.40±0.44</td>
</tr>
<tr>
<td>FE\textsubscript{Na} (%)</td>
<td>0.23±0.04</td>
<td>0.32±0.10</td>
<td>0.40±0.09</td>
</tr>
<tr>
<td>FE\textsubscript{K} (%)</td>
<td>18.6±2.3</td>
<td>18.9±2.8</td>
<td>26.5±2.7</td>
</tr>
<tr>
<td>FE\textsubscript{H2O} (%)</td>
<td>0.37±0.07</td>
<td>0.38±0.06</td>
<td>0.40±0.04</td>
</tr>
<tr>
<td>U\textsubscript{Na}V (µmol/min/g KW)</td>
<td>0.22±0.05</td>
<td>0.26±0.10</td>
<td>0.41±0.08</td>
</tr>
<tr>
<td>U\textsubscript{K}V (µmol/min/g KW)</td>
<td>0.47±0.06</td>
<td>0.44±0.08</td>
<td>0.74±0.08*†</td>
</tr>
</tbody>
</table>

Baseline renal clearance data in thiobutabarbitual anesthetized rats presented as the average values of two 30 minute clearance periods prior to lipopolysaccharide (LPS) administration. BW indicates body weight; MAP, mean arterial pressure; HR, heart rate; GFR, glomerular filtration rate; KW, kidney weight; RBF, renal blood flow; RVR, renal vascular resistance;
FF, filtration fraction; CLDF, cortical laser-Doppler flux; PU, perfusion units; OMLDF, outer medullary laser-Doppler flux; UV, urine flow rate; FE_{Na}, fractional urinary sodium excretion; FE_{K}, fractional urinary potassium excretion; FE_{H_{2}O}, fractional urine flow rate; U_{Na}V, urinary sodium excretion, and U_{K}V, urinary potassium excretion. Values are means ± SEM. * denotes p<0.05 vs. sham. † denotes p<0.05 vs. LPS-Saline.
### Table 2. Arterial blood gases.

<table>
<thead>
<tr>
<th></th>
<th>Sham-Saline (n=11)</th>
<th>LPS-Saline (n=12)</th>
<th>LPS-Melagatran (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/L)</td>
<td>134±2</td>
<td>117±2*</td>
<td>112±6*</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>0.73±0.04</td>
<td>1.77±0.18*</td>
<td>1.67±0.11*</td>
</tr>
<tr>
<td>pH</td>
<td>7.42±0.01</td>
<td>7.51±0.01*</td>
<td>7.50±0.01*</td>
</tr>
<tr>
<td>pCO₂ (kPa)</td>
<td>4.75±0.14</td>
<td>3.65±0.18*</td>
<td>3.66±0.13*</td>
</tr>
<tr>
<td>pO₂ (kPa)</td>
<td>10.51±0.32</td>
<td>11.56±0.35</td>
<td>10.90±0.28</td>
</tr>
</tbody>
</table>

Arterial blood gases in thiobutabarbital anesthetized rats 4.5 h after starting lipopolysaccharide (LPS) infusion. Hb denotes hemoglobin; pCO₂, partial pressure of carbon dioxide, and pO₂, partial pressure of oxygen. Values are means ± SEM. * denotes p<0.05 vs. sham.
Figure 1.
Figure 2.
Figure 5.

- LPS-Saline (n=12)
- LPS-Melagatin (n=12)
- Sham-Saline (n=11)

![Bar graph showing enzyme activities](image-url)
Figure 6.

[Bar chart showing different groups and their comparisons with error bars.]
Figure 7.
Figure S.

- LPS-Saline (n=12)
- LPS-Melagufen (n=12)
- Sham-Saline (n=11)

Legend for Y-axis:
- PKR
- PERK
- ERS ASR

* indicates statistical significance.