

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

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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. *Am J Physiol Regul Integr Comp Physiol* 292: R1117–R1124, 2007. First published October 26, 2006; doi:10.1152/ajpregu.00471.2006.—Sepsis is associated with an activation of the coagulation system and multiorgan 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 bolus dose of lipopolysaccharide (LPS; 6 mg/kg). Sham-Saline, LPS-Saline, and LPS-Melagatran study groups received isotonic saline or melagatran immediately before (0.75 $\mu\text{mol/kg}$ iv) and continuously during (0.75 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ iv) 4.5 h of endotoxemia. 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 the LPS-Saline group ($P < 0.05$, 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 ($-34 \pm 11\%$, $P < 0.05$), alanine aminotransferase ($-21 \pm 7\%$, $P < 0.05$), bilirubin ($-44 \pm 9\%$, $P < 0.05$), and TNF- α ($-32 \pm 14\%$, $P < 0.05$) 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.

acute kidney failure; acute liver failure; coagulation; multiple organ failure; sepsis

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 have 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 microthrombosis formation, and impaired blood flow to several organ systems, thereby causing multiorgan 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). PARs are a novel class of receptors that are activated by serine proteases and 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 ~ 250 g were used. All experiments were approved by the regional ethics committee in Göteborg. Chemicals were from Sigma (St. Louis, MO) unless otherwise stated. Melagatran (a generous gift from AstraZeneca, Mölndal, Sweden) was stored and prepared as described previously (13).

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Protocol 1: Renal hemodynamics and function. Rats were divided into three study groups, 1) lipopolysaccharide (LPS)-Saline ($n = 8$), 2) LPS-Melagatran ($n = 8$), and 3) Sham-Saline ($n = 9$), anesthetized with thiobutabarbital (Inactin, 120 mg/kg ip), placed on a heating table, and prepared for renal clearance experiments as described previously (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) with a data acquisition program (Biopac MP 150, Biopac Systems, Santa Barbara, CA). The left kidney was exposed by a flank incision and immobilized in a plastic cup. The left ureter was catheterized for urine collection into preweighed vials. Rectal and kidney temperatures were kept at 37°C. A perivascular ultrasonic flow probe (0.7 VB, T206, Transonic Systems, Ithaca, NY) 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 was previously 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 previously (27).

Throughout the experiment, rats received 10 ml·kg⁻¹·h⁻¹ of isotonic saline. After completion of the surgical preparation, a 40-min equilibration period was allowed before baseline clearance measurements over two consecutive 30-min periods. Subsequently, endotoxemia was induced by intravenous administration of LPS (*Escherichia coli* 0127:B8) in a bolus dose of 6 mg/kg over 30 min. 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 iv bolus) and throughout the experiment (0.75 μmol·kg⁻¹·h⁻¹ iv). On the basis of previous studies (7) and pilot experiments, this dose was expected to produce a plasma melagatran concentration of ~1 μmol/l throughout the study period, a concentration known to prolong thrombin time and activated partial thromboplastin time 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-treated 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 an intravenous bolus dose of 1.0 μmol/kg, followed by 1.0 μmol·kg⁻¹·h⁻¹ intravenously throughout.

GFR was determined by measuring renal ⁵¹Cr-EDTA clearance (⁵¹Cr-EDTA, Amersham) as described previously (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 previously (12). 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 ⁵¹Cr-EDTA × 100. Renal vascular resistance was calculated as MAP (mmHg)/RBF [ml·min⁻¹·g kidney wt⁻¹] and filtration fraction (FF) as the ratio between GFR and RBF. Presented baseline data are average values of the two clearance periods before 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 protocol 1 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 start of endotoxin administration. After 4.5 h, indexes of liver and pancreas injury were assessed by measuring plasma levels of aspartate aminotransferase (AST),

alanine aminotransferase (ALT), bilirubin, and pancreas-specific α-amylase. Concurrently, arterial blood gases were taken (ABL 510 blood gas analyzer, 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 killed, and lungs, kidneys, and 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). TNF-α was analyzed with an enzyme-linked immunosorbent assay (ELISA) kit (Rat TNF ELISA Kit II, BD Biosciences), and NO₃⁻/NO₂⁻ was measured spectrophotometrically (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical), following the manufacturer's instructions. Plasma melagatran concentrations were measured with 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 and eosin and Masson trichrome, and processed for analysis by light microscopy. Necrotic/apoptotic hepatocytes and polymorphonuclear neutrophils (PMNs) were counted in 20 consecutive high-power fields (×400). No distinction was made between cell necrosis and apoptosis. Analyses were made by an investigator blinded to 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 with 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) with SYBR Green I as described previously (14). Primer sequences for TNF-α (6), inducible nitric oxide synthase (iNOS) (35), intercellular adhesion molecule (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 housekeeping 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 and eosin and Masson trichrome and processed for semiquantitative 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 the inner medulla: tubular atrophy and dilatation, PMN infiltration, interstitial edema, interstitial inflammation and fibrosis, vascular fibrin deposition and microthrombosis, and vascular congestion. Analyses were made by an investigator blinded to 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 previously (12).

Statistics. Values are means ± SE except for semiquantitative data, which are presented as medians 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 prespecified between-group analyses were performed: Sham-Saline vs. LPS-Saline and LPS-Saline vs. LPS-Melagatran. Histological data were analyzed by nonparametric Kruskal-Wallis and Mann-Whitney *U*-tests. A value of $P < 0.05$ was considered statistically significant. The statistical software program SPSS 11.5.1 was used (SPSS, Chicago, IL).

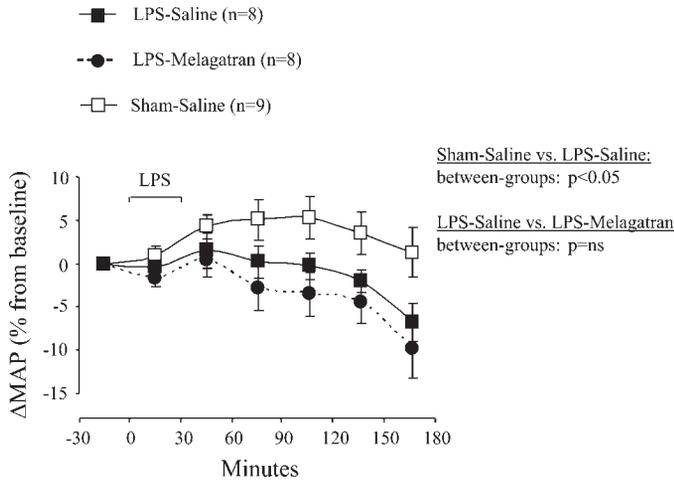


Fig. 1. Effect of melagatran or isotonic saline on mean arterial pressure (MAP) in thiobutabarbital-anesthetized rats infused with lipopolysaccharide (LPS). LPS at a dose of 6 mg/kg was infused intravenously over 30 min from time 0 to 30 min (see MATERIALS AND METHODS). Values are means \pm SE. Statistical analyses were performed by ANOVA for repeated measurements. ns, Not statistically significant.

RESULTS

Systemic hemodynamics. Endotoxin administration caused a significant 10% decrease in MAP ($P < 0.05$; Fig. 1) and a concomitant approximate 15% increase in HR ($P < 0.001$; data not shown). Melagatran had no significant effects on MAP (Fig. 1) or HR in endotoxemic rats throughout the study period. A transient increase in MAP (peak increase of $\sim 5\%$) was observed in the Sham-Saline group (Fig. 1). There were no significant differences in lung wet-to-dry weight ratio between

Table 1. Renal hemodynamics and function at baseline

	Sham-Saline (n = 9)	LPS-Saline (n = 8)	LPS-Melagatran (n = 8)
BW, g	369 \pm 11	322 \pm 16*	320 \pm 17*
MAP, mmHg	116 \pm 3	119 \pm 3	124 \pm 2
HR, beats/min	358 \pm 5	366 \pm 8	373 \pm 8
GFR, ml·min ⁻¹ ·g KW ⁻¹	1.01 \pm 0.06	1.01 \pm 0.05	1.10 \pm 0.04
RBF, ml·min ⁻¹ ·g KW ⁻¹	8.4 \pm 0.6	7.2 \pm 0.2	8.6 \pm 0.4
RVR, mmHg/ (ml·min ⁻¹ ·g KW ⁻¹)	14.5 \pm 1.2	16.6 \pm 0.7	14.5 \pm 0.7
FF, %	12.4 \pm 0.8	14.0 \pm 0.7	12.9 \pm 0.8
CLDF, PU	673 \pm 17	631 \pm 28	678 \pm 32
OMLDF, PU	155 \pm 7	136 \pm 7	153 \pm 10
UV, μ l·min ⁻¹ ·g KW ⁻¹	3.58 \pm 0.54	3.92 \pm 0.64	4.40 \pm 0.44
FE _{Na} , %	0.23 \pm 0.04	0.32 \pm 0.10	0.40 \pm 0.09
FE _K , %	18.6 \pm 2.3	18.9 \pm 2.8	26.5 \pm 2.7
FE _{H₂O} , %	0.37 \pm 0.07	0.38 \pm 0.06	0.40 \pm 0.04
U _{Na} V, μ mol·min ⁻¹ ·g KW ⁻¹	0.22 \pm 0.05	0.26 \pm 0.10	0.41 \pm 0.08
U _K V, μ mol·min ⁻¹ ·g KW ⁻¹	0.47 \pm 0.06	0.44 \pm 0.08	0.74 \pm 0.08*†

Values are means \pm SE of baseline renal clearance data in thiobutabarbital-anesthetized rats presented as the average values of two 30-min clearance periods before lipopolysaccharide (LPS) administration. BW, 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_{H₂O}, fractional urine flow rate; U_{Na}V, urinary sodium excretion; U_KV, urinary potassium excretion. * $P < 0.05$ vs. sham treated; † $P < 0.05$ vs. LPS-Saline.

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, before 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 the LPS-Melagatran group ($P < 0.05$; Table 1).

Endotoxin produced significant reductions in RBF, CLDF, and GFR compared with sham-treated animals ($P < 0.05$, Figs. 2 and 3). In addition, LPS decreased FF by $28 \pm 5\%$ in the LPS-Saline group ($P < 0.05$ vs. sham treated). Endotoxin caused a progressive decline in OMLDF over time compared with sham-treated animals (group \times time interaction $P < 0.001$, between groups $P = 0.07$; Fig. 2). In the LPS-Saline group, both absolute [data not shown for U_{Na}V and U_KV] and FF_{H₂O}, FF_{Na}, and FF_K showed a significantly different pattern

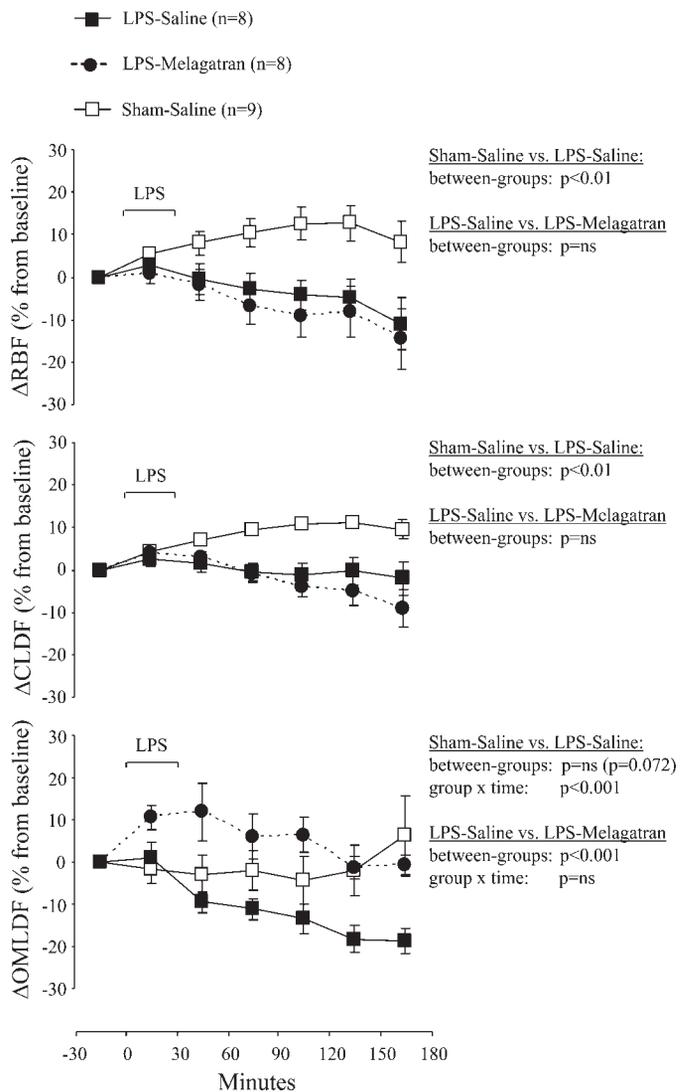


Fig. 2. Effect of melagatran or isotonic saline on renal blood flow (RBF; top) and renal cortical (CLDF; middle) and outer medullary (OMLDF; bottom) laser-Doppler fluxes in thiobutabarbital-anesthetized rats infused with LPS. LPS at a dose of 6 mg/kg was infused intravenously over 30 min from time 0 to 30 min (see MATERIALS AND METHODS). Values are means \pm SE. Statistical analyses were performed by ANOVA for repeated measurements. Group \times time, interaction between time and treatment group effects.

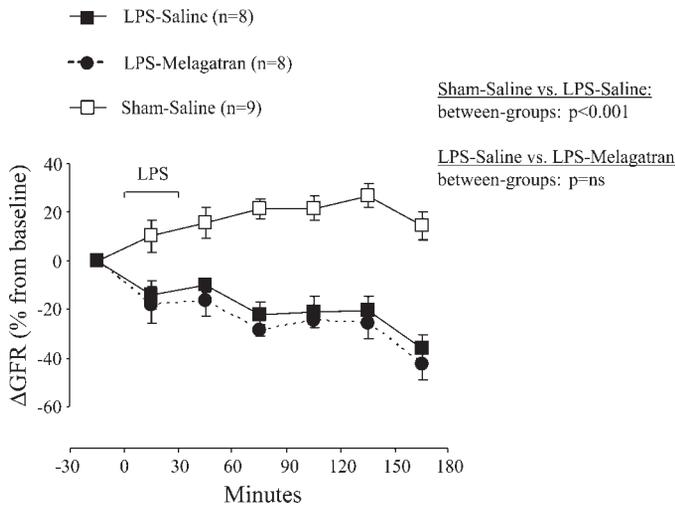


Fig. 3. Effect of melagatran or isotonic saline on glomerular filtration rate (GFR) in thiobutabarbital-anesthetized rats infused with LPS. LPS at a dose of 6 mg/kg was infused intravenously over 30 min from time 0 to 30 min (see MATERIALS AND METHODS). Values are means \pm SE. Statistical analyses were performed by ANOVA for repeated measurements.

over time compared with the Sham-Saline group (group \times time interaction $P < 0.05$; Fig. 4). Excretory responses in LPS-treated animals were characterized by initial reductions followed by pronounced increases starting at ~ 90 min after initiation of LPS infusion (Fig. 4).

Melagatran treatment significantly increased OMLDF in LPS-injected animals ($P < 0.05$, LPS-Melagatran vs. LPS-Saline; Fig. 2). In addition, melagatran decreased $U_{K/V}$ and FE_K ($P < 0.05$; Fig. 4). However, there were no significant differences between LPS groups in absolute levels of $U_{K/V}$ and FE_K 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 (Figs. 2–4). In the Sham-Saline group, RBF, CLDF, GFR, urine volume (UV), FE_{Na} , and FE_K increased significantly over time compared with baseline values ($P < 0.05$; Figs. 2–4).

Markers of liver injury. Administration of LPS caused significant increases in AST, ALT, and bilirubin compared with sham-treated animals ($P < 0.05$; Fig. 5). Treatment with melagatran significantly reduced the elevated plasma concentrations of AST ($-34 \pm 11\%$, $P < 0.05$), ALT ($-21 \pm 7\%$, $P < 0.05$), and bilirubin ($-44 \pm 9\%$, $P < 0.05$) in endotoxemic rats (Fig. 5). LPS had no statistically significant effect on plasma concentrations of pancreas-specific α -amylase (data not shown).

Effects of melagatran in sham-treated animals. Melagatran had no statistically significant effects on RBF ($+2 \pm 3\%$ vs. baseline), GFR ($+4 \pm 2\%$ vs. baseline), CLDF ($+1 \pm 2\%$ vs. baseline), and OMLDF ($+3 \pm 6\%$ vs. baseline) in the Sham-Melagatran group. Melagatran produced a modest reduction in MAP evident after 3 h ($-6 \pm 1\%$ vs. baseline, $P < 0.05$). Similar to the Sham-Saline group, UV, FE_{Na} , and FE_K increased significantly over time compared with baseline values ($P < 0.05$; data not shown). Plasma levels of AST, ALT, and bilirubin were not significantly different from those in the Sham-Saline group (data not shown). Plasma concentrations of

melagatran were $1.20 \pm 0.06 \mu\text{mol/l}$ and not significantly different from those in the LPS-Melagatran group.

Plasma TNF- α , NO_3^-/NO_2^- , and melagatran concentrations. Rats with endotoxemia demonstrated a marked, approximately sevenfold increase in plasma TNF- α concentrations ($P < 0.05$; Fig. 6). Melagatran treatment significantly decreased the elevated plasma concentrations of TNF- α by $32 \pm 14\%$ ($P < 0.05$; Fig. 6). Endotoxin administration produced an ~ 15 -fold increase in plasma NO_3^-/NO_2^- compared with sham treatment ($P < 0.05$), with no significant difference between LPS-Saline and LPS-Melagatran groups (Fig. 6). Plasma melagatran concentrations were $1.04 \pm 0.05 \mu\text{mol/l}$ in the LPS-Melagatran group 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 P_{CO_2} accompanied by respiratory alkalosis ($P < 0.05$; Table 2). There were no significant differences in P_{O_2} (Table 2), base excess, or plasma

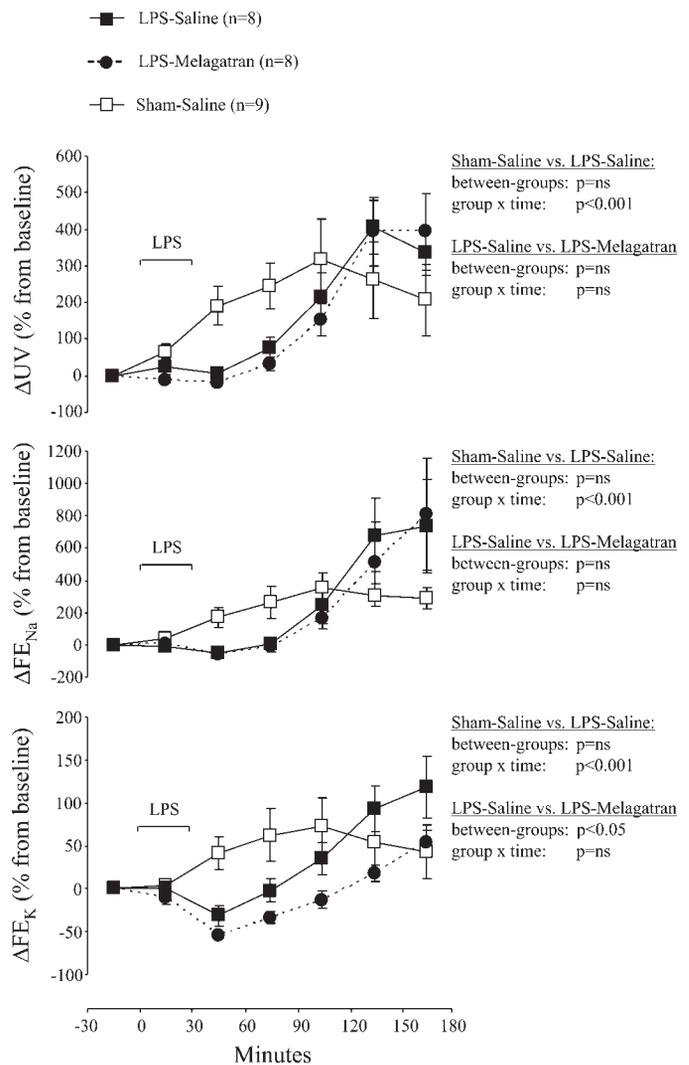


Fig. 4. Effect of melagatran or isotonic saline on urine volume (UV; top) and fractional urinary sodium (FE_{Na} ; middle) and potassium (FE_K ; bottom) excretion in thiobutabarbital-anesthetized rats infused with LPS. LPS at a dose of 6 mg/kg was infused intravenously over 30 min from time 0 to 30 min (see MATERIALS AND METHODS). Values are means \pm SE. Statistical analyses were performed by ANOVA for repeated measurements.

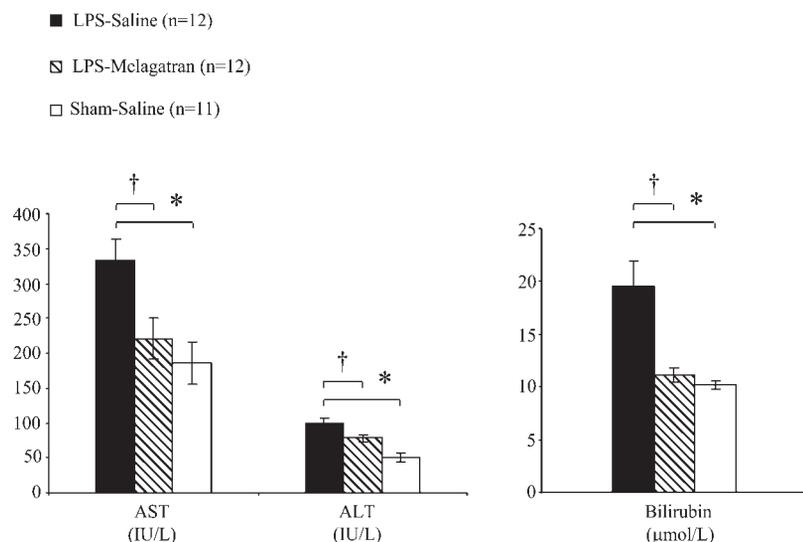


Fig. 5. Effect of melagatran or isotonic saline on plasma concentrations of aspartate aminotransferase (AST; *left*), alanine aminotransferase (ALT; *center*), and bilirubin (*right*) in thiobutabarbital-anesthetized rats infused with LPS. Blood was collected 4.5 h after start of LPS administration. Values are means \pm SE. * $P < 0.05$ vs. sham treated; † $P < 0.05$ vs. LPS-Saline.

bicarbonate levels (data not shown) between study groups. Both LPS-injected groups had significantly lower hemoglobin concentrations compared with sham-treated animals ($P < 0.05$; Table 2).

Liver histology. Endotoxemia caused an ~ 5 -fold increase in hepatocyte necrosis/apoptosis and a 25-fold increase in PMN accumulation, predominantly in periportal areas, compared with sham-treated animals ($P < 0.05$; Fig. 7). There were no apparent abnormalities in Kupffer cell morphology and no increases in fibrin deposition, microthrombosis formation, or sinusoidal congestion in endotoxemic rats (data not shown). The LPS-Melagatran group had a similar degree of histopathological injury compared with the LPS-Saline group, and there were no statistically significant differences in hepatocyte necrosis/apoptosis or hepatic PMN sequestration between LPS-treated groups (Fig. 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 with sham-treated animals ($P < 0.05$; Fig. 8). Melagatran had no statistically significant effects on TNF- α , iNOS, or ICAM-1 gene expression (Fig. 8). There were no significant differences

between groups in the expression of the housekeeping gene GAPDH (data not shown).

Kidney histology. There was no significant difference in kidney weight between groups (data not shown). The LPS-Saline group showed no statistically significant abnormalities in any of the investigated renal histological variables compared with sham-treated animals (data not shown). There was a tendency toward an increased vascular congestion of erythrocytes in the ISOMZ in the LPS-Saline group compared with sham-treated animals ($P = 0.24$; 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 LPS-Melagatran and LPS-Saline groups, 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.

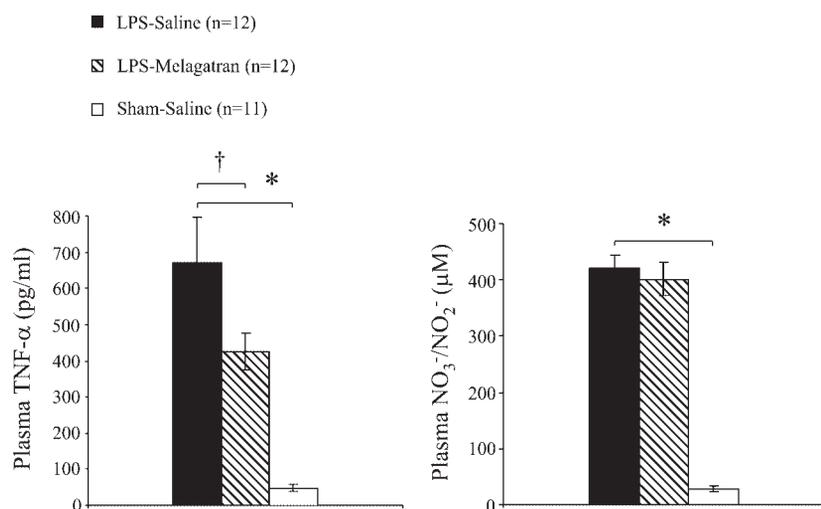


Fig. 6. Effect of melagatran or isotonic saline on plasma concentrations of tumor necrosis factor (TNF- α (*left*)) and nitrate (NO₃⁻)/nitrite (NO₂⁻) (*right*) in thiobutabarbital-anesthetized rats infused with LPS. Blood was collected 4.5 h after start of LPS administration. Values are means \pm SE. * $P < 0.05$ vs. sham treated; † $P < 0.05$ vs. LPS-Saline.

Table 2. Arterial blood gases

	Sham-Saline (n = 11)	LPS-Saline (n = 12)	LPS-Melagatran (n = 12)
Hb, g/l	134±2	117±2*	112±6*
Lactate, mmol/l	0.73±0.04	1.77±0.18*	1.67±0.11*
pH	7.42±0.01	7.51±0.01*	7.50±0.01*
PCO ₂ , kPa	4.75±0.14	3.65±0.18*	3.66±0.13*
PO ₂ , kPa	10.51±0.32	11.56±0.35	10.90±0.28

Values are means ± SE for arterial blood gases in thiobutobarbital-anesthetized rats 4.5 h after start of LPS infusion. Hb, hemoglobin; PCO₂, partial pressure of carbon dioxide; PO₂, partial pressure of oxygen. **P* < 0.05 vs. sham treated.

In the present study, endotoxin caused an ~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 dysfunction 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). On the basis of anatomic 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 (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 min 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 HR. 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 present study there was a transient, modest rise in MAP and progressive increases in RBF, GFR, UV, FE_{Na}, and FE_K, over time in the Sham-Saline group. 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 was previously 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). Corroborat-

■ LPS-Saline (n=12)
 ▨ LPS-Melagatran (n=12)
 □ Sham-Saline (n=11)

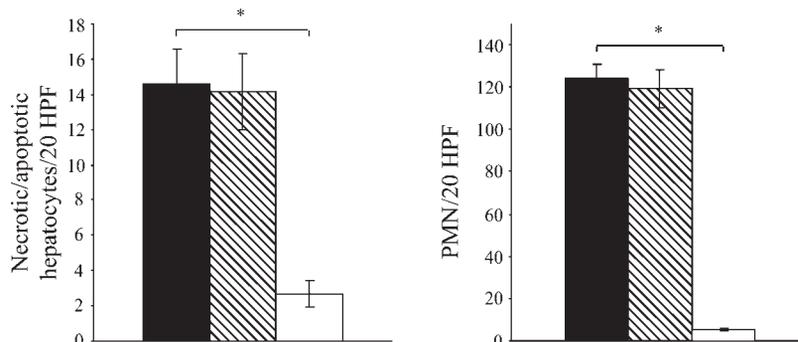


Fig. 7. Effect of melagatran or isotonic saline on hepatocyte necrosis/apoptosis (left) and hepatic polymorphonuclear neutrophil (PMN) infiltration (right) in thiobutobarbital-anesthetized rats infused with LPS. Liver tissue was sampled 4.5 h after start of LPS administration. Necrotic/apoptotic hepatocytes and PMN were counted in 20 consecutive high-power fields (HPF) at ×400 magnification. Values are means ± SE. **P* < 0.05 vs. sham treated.

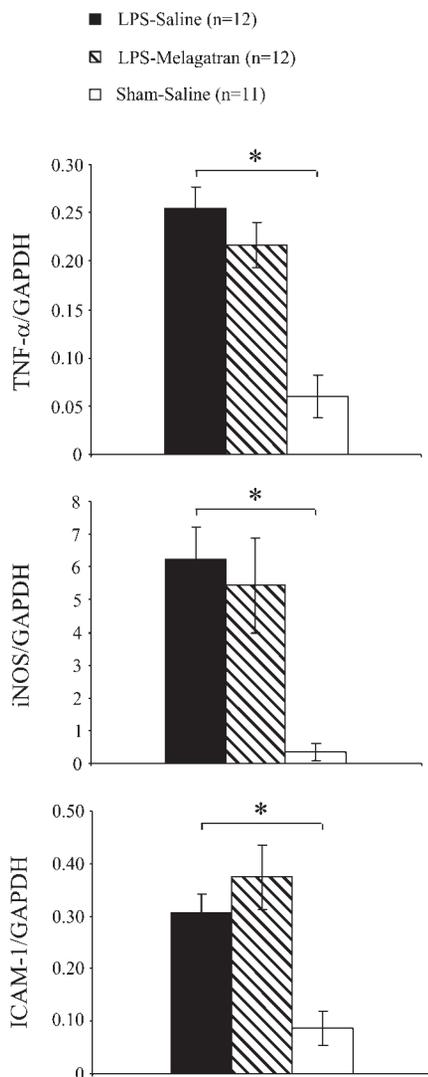


Fig. 8. Effect of melagatran or isotonic saline on hepatic gene expression of TNF- α (top), inducible nitric oxide synthase (iNOS; middle), and intercellular adhesion molecule (ICAM)-1 (bottom), relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in thiobutabarbital-anesthetized rats infused with LPS. Liver tissue was sampled 4.5 h after start of LPS administration. Analyses were performed by reverse transcription-polymerase chain reaction. Values are means \pm SE. * P < 0.05 vs. sham treated.

ing these findings, thrombin inhibition with melagatran decreased plasma AST, ALT, 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 with 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 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 accumulation or by reducing microthrombosis formation. We speculate that melagatran could have attenuated liver dysfunction in the present 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, the thrombin inhibitors heparin and anti-thrombin 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 that 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 \sim 30% in endotoxemic rats. TNF- 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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