Effect of nitric oxide synthase (NOS) inhibition on macro- and microcirculation in a model of rat endotoxic shock

Soni Savi Pulamsetti1, Daniel Maring1, Hossein Ardeschir Ghofrani1, Konstantin Mayer1, Norbert Weissmann1, Bernhard Rosengarten2, Martin Lehner3, Christian Schudt2, Rainer Boer3, Friedrich Grimminger1, Werner Seeger1, Ralph Theo Schermuly1

1Department of Internal Medicine, and 2Department of Neurology, Justus-Liebig-University Giessen, Giessen, Germany; 3Altana Pharma, Konstanz, Germany

Summary
Treatment of hemodynamic instability in septic shock often demands the administration of vasopressor agents, although these may have deleterious effects on microcirculatory homeostasis. Inhibition of nitric oxide synthase (NOS) has been suggested as an alternative therapeutic approach, as NO formation may be excessively increased in sepsis. To compare the effects of epinephrine titration, non-selective NOS inhibition by L-NMMA and selective inhibition of inducible NOS (iNOS) by 1400W on hemodynamics and on the regulation of microcirculation in a rat model of endotoxic shock, we intravenously injected endotoxin (LPS) or saline to male Wistar rats and after 2 hours randomized LPS treated rats into four different groups that received either saline, norepinephrine, L-NMMA or 1400W (n=6 per group). Three hours after LPS administration, rats presented with severe systemic arterial hypotension (64 ± 3 vs. 115 ± 4 mmHg, p<0.001), unresponsiveness to volume treatment, lactate acidosis and a marked increase in plasmatic nitrite and nitrate levels (15 ± 8 vs. 263 ± 47 µM, p<0.001). Measurement of the tissue oxygenation in the ileum mucosal layer by the Erlangen micro-lightguide spectrophotometer (EMPHO) technique demonstrated marked heterogeneity of hemoglobin saturation, with appearance of low oxygenated areas. Norepinephrine, usually stabilizing blood pressure (99 ± 7 vs. 67 ± 4 mmHg 60 min after infusion, p<0.01), increased lactate formation (7.9 ± 0.2 vs. 3.7 ± 0.5 mM, p<0.001) and drastically increased low oxygenated regions in the ileum mucosal layer. L-NMMA similarly increased blood pressure (92 ± 6 vs. 67 ± 4 mmHg 60 min after infusion, p<0.05), but did not enhance lactate acidosis. However, some further deterioration of mucosal oxygenation was again noted. 1400W forwarded stabilization of blood pressure (88 ± 5 vs. 67 ± 4 mmHg 60 min after injection, p<0.05), reduced plasmatic nitrite and nitrate levels similar to L-NMMA, without an aggravation of lactate acidosis. In addition, mucosal oxygenation did not deteriorate in response to this agent. Thereby, we conclude that in a rat model of endotoxic shock selective iNOS inhibitors are superior to non-specific NOS inhibitors and in particular to norepinephrine for the treatment of macro- and microcirculatory abnormalities in experimental septic shock.

Keywords
Sepsis, iNOS, EMPHO, 1400W, L-NMMA, norepinephrine

Introduction
Nitric oxide (NO) is a potent vasodilator derived from the enzymatic oxidation of L-arginine by a family of enzymes, the NO synthases (NOS) (1). The constitutive isofom (cNOS) of these is intimately involved in the physiological regulation of vascular tone and blood flow distribution, whereas an inducible isofom (iNOS) is strongly upregulated in response to inflammatory stimuli such as cytokines and bacterial lipopolysaccharides. Enhanced generation of NO, due to widespread induction of iNOS, has been implicated in the cardiovascular failure in septic shock (2, 3). This view is supported by experimental studies using "selective" iNOS-inhibitors, which prevented the decrease in blood pressure and the accumulation of the metabolic end products of NO, nitrite and nitrate, in septic animals (2–5).

Analogs of L-arginine such as monomethyl-L-arginine (L-NMMA) or L-arginine-methylester (L-NAME) have been shown to increase blood pressure and partially restore adrenergic vascular reactivity in experimental and human septic shock (2, 3, 6–9). In a multicenter clinical study, L-NMMA has been shown to enable a 60 to 80% reduction in norepinephrine dosage in patients with sepsis (10). However, a phase-III study including 797
patients was recently discontinued because the treatment group with L-NMMA showed higher mortality (11). Therefore, interest is now focused on the identification of compounds that will allow selective inhibition of the iNOS-dependent NO production while preserving eNOS activity. 1400W, for which such an efficacy profile was shown in vitro and in vivo (12), was recently reported to reduce organ damage and mortality during rodent endotoxic shock. Of interest, there is evidence for resident macrophages within the intestinal wall to become strongly activated in endotoxemia (13), thereby largely contributing to motility changes and in particular microcirculatory disturbances within the mucosal layer. Such abnormalities have been implicated in severe loss of gut barrier function, further perpetuating septic sequelae (14).

In this study, we employed 1400W and compared data to L-NMMA, a non-selective NOS inhibitor, and to norepinephrine for nonspecific vasoconstriction. We investigated the impact of these agents on hemodynamics in a rat endotoxic shock model, paying particular attention to the microcirculatory abnormalities in the intestinal mucosa in response to the bacterial agent. Injection of lipopolysaccharide is commonly used to induce an inflammatory response and systemic hypotension in rats and other species (15, 16). However, these experimental models cannot mimic the clinical condition of severe human sepsis, which was shown for several "successful" therapeutic approaches that failed to be effective in the clinical setting.

Material and methods

Surgical preparation and measurements

Adult male Wistar rats weighing 350 to 400 g were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). The rats were trachotomized and ventilated with a frequency of 60 s⁻¹ and a tidal volume of 3 ml with a FIO₂ of 0.5 (SAP3830P, IITC Lifesciences, CA, USA). Body temperature was maintained at 37°C by a heating pad. The left carotid artery was cannulated and connected to a pressure transducer for the online measurement of arterial blood pressure (SAP). A polyethylene catheter (OD 0.8 mm, ID 0.5 mm) was inserted into the right ventricle through the right external jugular vein for online measurement of right ventricular systolic pressure (RVPsys). However, for the best transparency SAP and RVPsys values were provided only at a specific time interval (every 15 min). Blood samples were obtained to measure the plasma concentrations of lactate and nitrate. Plasma nitrate was determined by a chemiluminescence technique as described (2). Each blood sample was replaced by the same volume of isotonic saline. The total volume of sampled blood amounted to 2 ml.

Reflectance spectrophotometry for mucosal hemoglobin oxygenation

As described previously, an Erlangen Microlightguide Spectrophotometer (EMPHO II, Bodenseewerk Geräteotechnik, Überlingen, Germany), was employed for measurement of ileal mucosal hemoglobin oxygenation (HbO₂;i) and relative hemoglobin concentration (rel Hb_conc;i) (17–21). Briefly, a small abdominal incision was performed and the flexible micro-lightguide was placed in the ileum under sterile techniques. Light from a xenon arc lamp was transmitted to the tissue and backscattered light was collected resulting in a detecting area of approximately 0.09 mm². This small catchment volume along with a high recording frequency allows the assessment of hemoglobin oxygenation (HbO₂;i) in intact tissues. Furthermore, these ongoing measurements were performed in 4 distinct areas of the upper intestinal tract by changing the position of the micro-lightguide every 30 min to detect the heterogeneity of the intracapillary hemoglobin oxygenation. rel Hb_conc reflects changes in capillary blood volume, and was calculated as previously described (22).

Western blotting for detection of iNOS

Rat aorta and diaphragm tissue lysates obtained from three animals from each experimental group (control, 3h and 5h septic shock) were prepared by fine mincing and homogenization of snap-frozen tissues in 5 volumes (wt/vol) of lysis buffer containing 10 mM Tris HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% deoxycholine, 1% Triton X-100, and a mixture of protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN, USA). The lysates were clarified at 13,000 x g for 20 min at 4°C. The protein concentration of the supernatants was determined with the use of the Dye Reagent Concentrate (Bio-Rad). Extracts containing equal amounts of protein were denatured by boiling for 10 min in Laemmli’s buffer containing β-mercaptoethanol, separated on 7.5% SDS-polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membrane. Membranes were blocked for 1 h at room temperature with antibody buffer (PBS containing 0.1% Tween 20 and 5% nonfat dried milk) and then incubated with primary antibodies (rabbit IgG (Sigma) for 1 h at room temperature and developed by enhanced chemiluminescence. Quantification of the immunoblot was performed with a computerized densitometer (Biometra, Germany). Density values are expressed relative to the GAPDH control level of each sample. All densities reported are means and SEM of three separate experiments.

Experimental protocols

Endotoxemia was induced by i.v. injection of 1.5 mg/kg endotoxin (Salmonella abortus equi LPS, Sigma, 5mg/ml in isotonic saline) into the tail vein (n=36). Control animals received sham application of saline instead of LPS (n=6). The animals were set in their cage with food and water ad libitum. Two hours after LPS injection, animals were divided into four groups and prepared as described in the "Surgical preparation and measurements" section.

Control

Hemoglobin oxygenation and relative hemoglobin concentration were assessed with the EMPHO technique. Data from individual animals were recorded with 2 Hz and the frequency of HbO₂;i values was calculated in intervals between 0 and >70 % hemoglobin oxygenation. The final data shown represent the average of variables determined from all individual distributions (n=6).
Group 1
The rats received an infusion of 15 ml/kg x h saline for stabilization of blood pressure (n=6).

Group 2
The rats received an infusion of norepinephrine (NE; 0.01 to 10 µg/kg x min). This infusion was continuously given over 2 h and titrated to achieve stable blood pressure in the range of 90 mmHg and avoids both progressive hypotension and overcorrection of mean BP (n=6).

Group 3
The rats were treated with a continuous infusion of L-NMMA (150 µg/kg x min) to achieve stable BP in the range of 90 mmHg (n=6).

Group 4
The rats were treated initially with three different doses of 1400W (1, 3 or 9 mg/kg, applied as bolus) in preliminary experiments to select the drug dose that can optimally restore systemic hypotension in response to endotoxin (n=6 each). Final experiments were performed with 3mg/kg 1400W (n=6). In addition, this dose has recently been shown to reduce plasma nitrite/nitrate levels in LPS treated rats significantly (23).

Form measurement of hemoglobin oxygenation and relative hemoglobin concentration the micro-lightguide probe was placed at the mucosal layer of the ileum and this position was changed every 30 min. Arterial blood sampling at baseline, 60 and 120 min after beginning of saline/drug infusion for the determination of blood gases and lactate were taken from the rats of all above mentioned experimental groups. In addition EMPHO measurement was performed in all these rats throughout the experimental observation period.

Statistical analysis
The data are expressed as means ± SEM. Data were tested for normality with the Kolmogorov-Smirnov test and analyzed by using one-way analysis of variance (ANOVA) and the Turkey-HSD multiple comparisons test for post hoc analysis. A value of p < 0.05 was considered to be significant.

Results
Controls and baseline conditions
In sham-injected animals, all hemodynamic parameters and arterial blood gases were in the physiologic range (Fig. 1A, Fig. 2, Table 1). Systemic blood pressure and right ventricular pressure remained stable over the whole observation period of 120 min. Arterial pO₂ did not change significantly and ranged from 200 to

![Figure 1: Influence of norepinephrine (A) or NO-synthase (B) inhibitors on mean systemic arterial pressure measured in the carotid artery. Systemic arterial pressure (SAP) is given (mean ± SEM of 6 independent experiments each, SEM bars are missing when falling into symbol). Lipopolysaccharide injection or application of 1400W is indicated by arrows. Horizontal bars indicate infusions of saline, norepinephrine or L-NMMA. ***, p<0.001 versus LPS/saline treated animals.](#)

![Figure 2: Influence of norepinephrine or NO-synthase inhibitors on systolic right ventricular pressure. Systolic right ventricular pressure (RVP₂₅) is given (mean ± SEM of 6 independent experiments each, SEM bars are missing when falling into symbol). Lipopolysaccharide injection or application of 1400W is indicated by the arrows. Horizontal bars indicate infusions of saline, norepinephrine or L-NMMA.](#)
240 mmHg. Arterial CO₂ values in the low normal range were maintained by artificial ventilation; this resulted in an arterial pH of ~7.34. A close distribution of hemoglobin oxygenation between 40-60% was measured in the ileal mucosa (Fig. 3A). Relative hemoglobin concentration did not change significantly during the experiments. This was also true for lactate. Plasma nitrate levels were 14.5 ± 7.5 µM at the end of the experiments (Fig. 4).

![Figure 3: Distribution of HbO₂ values in the ileal mucosa of A) control animals, B)-D) septic animals and those undergoing the different treatment regimes. For each group, data are pooled. The histograms represent the distribution of HbO₂ values (14,000 values per experiment) of 6 independent experiments each (mean ± SEM). SEM bars are missing when falling into symbol). Three hours after intravenous injection of LPS, animals were subjected to infusion of saline (B), norepinephrine (C), L-NMMA (D) or bolus application of 1400W (E). *, p<0.05, **, p<0.01, †††, p<0.001 versus saline-treated animals: †, p<0.05, ††, p<0.01, †††, p<0.001 versus saline-treated animals: ‡, p<0.05, ‡‡, p<0.01 versus LPS/norepinephrine-treated animals:

### Table 1: Arterial blood gases and lactate in rats with LPS-induced septic shock in response to different treatment strategies. PₐO₂, arterial PO₂; pHₐ, arterial pH; PCO₂, arterial PCO₂; rel Hb_conc, relative hemoglobin concentration. The columns give summarized data for the groups pre- (0') and post (60', 120') infusion of saline, norepinephrine, L-NMMA and 1400W. Asterix indicates significant differences between LPS/norepinephrine and LPS/NaCl (n=6 each group, ††† = p<0.001); Crosses indicate significant differences among LPS/norepinephrine, LPS/L-NMMA and LPS/1400W (n=6 each group, ††† = p<0.001).

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<th>Control</th>
<th>LPS/NaCl</th>
<th>LPS/norepinephrine</th>
<th>LPS/L-NMMA</th>
<th>LPS/1400W</th>
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<td>PₐO₂ (mmHg)</td>
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<td>231 (±12)</td>
<td>242 (±12)</td>
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<td>Lactate (mM)</td>
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<td>3.8 (±0.2)</td>
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<tr>
<td>rel Hb_conc</td>
<td>1.00</td>
<td>1.02 (±0.15)</td>
<td>1.07 (±0.23)</td>
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LPS induced circulatory failure (Group 1)

Intravenous LPS resulted in severe systemic hypotension (64 ± 3 vs. 115 ± 4 mmHg, p<0.001) (Fig. 1A) with right ventricular systolic pressures being largely unchanged (Fig. 2). Systemic hypotension was not substantially influenced by sole fluid resuscitation. As compared to control animals, significant arterial acidosis occurred, accompanied by an increase in plasma lactate levels (Table 1). Plasma nitrate increased >10-fold (Fig. 4) and a marked broadening of the distribution of hemoglobin oxygenation in the ileal mucosa was observed (Fig. 3B). This is reflected by an increase in frequency of mucosal hemoglobin oxygenation intervals of 30–40% (p<0.01), 50–60% (p<0.001) and >70% (p<0.05) in these animals. Furthermore, there was a slight increase in relative hemoglobin concentration in the further course of the experiments. Western blot analysis confirmed the presence of iNOS in both aorta and diaphragm samples from septic shock (after both 3h and 5 h of LPS exposure), although no signal for iNOS was found in control animals (Fig. 5).

LPS/norepinephrine (Group 2)

As in the preceding group, LPS-treated animals showed significant systemic hypotension. Continuous infusion of norepinephrine was titrated (0.01 to 10 µg/kg x min) to achieve restoration of systemic blood pressure (99 ± 7 vs. 67 ± 4 mmHg 60 min after infusion, p<0.001) (Fig. 1A). No significant changes in right ventricular pressure, blood gases, or plasma nitrate levels were noted in response to the catecholamine therapy (Fig. 2, Fig. 4, Table 1). As compared to group 1, plasma lactate levels were even further increased (7.9 ± 0.2 vs. 3.7 ± 0.5 mM, p<0.001). Moreover, the spectrum of the overall hemoglobin oxygenation within the ileal mucosa further deteriorated. The frequency of areas with hemo...
globin oxygenations between 0–10% (p<0.01), 10–20% (p<0.001), 40–50% (0.001) and >70% (p<0.05) increased significantly as compared to saline-treated septic rats (Fig. 3C). In consequence, there was a significant decrease in the frequency of areas with hemoglobin oxygenations between 30–70%. The relative hemoglobin concentration increased to 1.43 at the end of the experiments.

**LPS/L-NMMA (Group 3)**
Continuous infusion of L-NMMA resulted in an increase of systemic arterial pressure to the level of norepinephrine-treated rats (92 ± 6 vs. 67 ± 4 mmHg 60 min after infusion, p<0.05) (Fig. 1B). No significant changes in right ventricular systolic pressure occurred under this regime (Fig. 2). Compared to norepinephrine treated rats, a significant decrease in the frequency of areas with hemoglobin oxygenations between 0 to 10% was measured (Fig. 3D). Plasma nitrate as well as lactate levels were significantly reduced as compared to the norepinephrine group (150 ± 33 vs. 240 ± 21 µM, p<0.05, 2.8 ± 0.6 vs. 7.9 ± 0.2 mM, respectively, p<0.001) (Fig. 4, Table 1).

**LPS/1400W (Group 4)**
The rats were treated initially with three different doses of 1400W (1, 3 or 9 mg/kg, applied as bolus) to choose the drug dose that can optimally restore systemic hypotension in response to endotoxin (Fig. 6). For further studies, the selective iNOS inhibitor 1400W 3 mg/kg was applied as single bolus injection to the LPS treated rats, which resulted in a sustained increase in blood pressure (88 ± 5 vs. 67 ± 4 mmHg 60 min after injection, p<0.05) (Fig. 1B). No significant changes in right ventricular pressures were noted (Fig. 2), while significantly lower levels of plasma nitrate (Fig. 4) and lactate were measured as compared to norepinephrine-treated animals (160 ± 16 vs. 240 ± 21 µM, p<0.05, 3.1 ± 0.2 vs. 7.9 ± 0.2 mM, respectively, p<0.001) (Table 1). The frequency of areas with hemoglobin oxygenations between 0–10% (p<0.01) and 10–20% (p<0.01) was significantly reduced in comparison to catecholamine treatment, while the frequency of higher oxygenated areas was significantly increased (50–60%, p<0.05 and >70%, p<0.05) (Fig. 3E).

**Discussion**
As anticipated, all endotoxin-treated animals presented with severe circulatory shock when being instrumented three hours after administration of the bacterial agent: blood pressure was severely depressed, virtually not reacting to volume load, marked metabolic acidosis was noted, and the blood lactate levels were consistently increased. Moreover, the employment of the spectrophotometric technique to assess perfusion heterogeneity in the intestinal mucosal layer demonstrated severe microcirculatory abnormalities already at this early period of septic sequelae. In contrast to the very homogeneous tissue oxygenation pattern of the control animals, severely deoxygenated mucosal areas were detected, along with regions demonstrating increased tissue oxygenation, and this broad scattering of oxygenation patterns strongly suggests maldistribution of perfusion in this most critical mucosal surface layer of the body. This finding corroborates previous studies employing different technology, which also signaled that the gut mucosal compartment is very sensitive to microcirculatory abnormalities appearing in sepsis or systemic inflammatory response syndrome (24–26). Notably, these abnormalities persisted in spite of substantial volume treatment undertaken in the endotoxic animals, suggesting local imbalance of vasoactive mediator generation.

Non-physiological regional NO generation may well contribute to these microcirculatory events and is mostly attributed to strong, early upregulation of iNOS as demonstrated in the jejunum, ileum and colon of endotoxic rats (27). In agreement with previous reports, increased expression of iNOS was also observed in our studies. Upregulation of iNOS in response to LPS was noted in both aorta and diaphragm within 3 hours after LPS administration with a further increase after 6 hours, thereby supporting the notion that excessive production of NO via iNOS is the key mechanism in the pronounced systemic vasodilation of sepsis. Furthermore, LPS responsive macrophages in the intestinal wall may also play a major role in regional NO excess (13, 28), and together these mechanisms explain well the drastic increase of plasmatic nitrite and nitrate levels noted in the rats undergoing endotoxin challenge. Such an overproduction of nitric oxide is in accordance with previous observations in experimental and clinical sepsis (4, 6, 29).

The administration of adrenergic agents in patients with septic shock not adequately responding to volume therapy, is a mainstay of cardiovascular support, with norepinephrine being preferred to antagonize the loss in peripheral vascular resistance. In fact, near normalization of systemic blood pressure was achieved by titration of this agent in the endotoxemic animals. However, this stabilization of macrohemodynamics was paid for with a dramatic aggravation of microcirculatory abnormalities. This is already reflected by rising lactate levels and further aggravation of metabolic acidosis, and it is most impressively directly demonstrated by the EMPHO technique: the appearance of extremely low oxygenated mucosal areas further increased, with approximately 75% of the ileum mucosal layer presenting...
with hemoglobin saturation values below 30%, which are virtually absent in control animals. Considering the high energy demand of the mucosa, this dramatic local oxygen debt must, indeed, be assumed to result in a loss of barrier function at this critical body surface, which may then enable translocation of bacteria or bacterial products from the intestinal lumen into the systemic circulation.

Interestingly, the non-specific NO synthase inhibitor L-NMMA effectively restored systemic blood pressure in the absence of any adrenergic substance, thereby providing further evidence that excess NO formation largely contributes to the loss of vascular tone occurring under conditions of endotoxia, as a mimicry of septic events. In contrast to epinephrine, this effect was not coupled to a further increase in lactate formation or to an aggravation of metabolic acidosis. However, concerning the regulation of intestinal mucosal perfusion, an enhanced appearance of low oxygenated areas was again detected when comparing the HbO$_2$ profile to the animals with only saline infusion, although this shift to low oxygenation patterns was not as prominent as with norepinephrine. As may be anticipated, a significant decrease in plasma nitrite and nitrate levels was observed in the L-NMMA-treated animals, though normal values were not achieved, signaling that – at the given dosage restoring macrohemodynamics – excess NO formation was not yet fully blocked by this agent.

As a major finding of this study, the most advantageous therapeutic effects were achieved by the selective iNOS inhibitor 1400W, which restored the systemic blood pressure similar to L-NMMA, supporting the view that the endotoxin-elicited excess NO formation is largely attributable to iNOS upregulation. This interpretation is also in line with the finding that the decrease in plasma nitrite and nitrate levels forwarded by 1400W corresponded to that observed in the presence of L-NMMA. The stabilization of systemic blood pressure by the iNOS inhibitor occurred in the absence of any further increase in lactate formation or aggravation of metabolic acidosis. Moreover, the analysis of the intestinal mucosal oxygen saturation pattern did not reveal enhanced appearance of low oxygenated areas, but the overall distribution of HbO$_2$ values was very similar to the pattern observed in the animals with saline infusion only. This beneficial effect of the iNOS inhibitor is well in line with the protective effects of 1400W on vascular leakage in lung, ileum, colon and liver in a septic rat model (12, 30). However, normalization of ileum mucosal tissue oxygenation was not achieved by the iNOS inhibitors, as is apparent from the comparison of the HbO$_2$ values with those of the control animals.

Several studies addressed the role of nitric oxide synthase inhibitors in septic models for treatment of systemic hypotension and reduction of plasma nitrite and nitrate levels (4, 6, 31). In a similar experimental setup to that used in the present study, infusion of L-NMMA after endotoxin challenge was reported to reduce the vascular injury in jejunum, ileum and colon (32). In the current study, however, taking a closer view of the oxygenation of the intestinal mucosal layer, the stabilization of macrohemodynamics by L-NMMA was paralleled by a worsening of perfusion distribution with enhanced appearance of low oxygenation areas. This was not as extreme as that observed in response to norepinephrine, but still must be assumed to be disadvantageous for the maintenance of the mucosal barrier properties. The capability of an iNOS inhibitor to provide stabilization of blood pressure without deterioration of intestinal mucosal oxygenation characterizes these agents as most interesting tools for treatment of hemodynamic instability in septic shock. iNOS inhibition may thus turn out to be the better alternative to non-specific NO synthase inhibition for hemodynamic treatment of septic patients, in whom the L-NMMA trial recently failed (11), although preclinical data promised high efficacy. Similarly, numerous mediators were pharmacologically targeted in animal models of infection or endotoxiaemia and successfully prevented lethality of this experimental challenge, while clinical trials failed. Exemplarily, targeting of tumor necrosis factor α (TNF-α) by neutralizing antibodies prevented mortality in a baboon model of exogenous E. coli infection (33) but failed to be effective in a set of different clinical trials (34, 35). Another key mediator is interleukin 1, which was successfully targeted experimentally by a receptor antagonist (36, 37). However, clinical trials demonstrated only modest and non-significant effects (38, 39). In summary, beneficial effects of therapeutic agents in animal models can hardly be translated into success in human clinical trials, and future studies have to prove efficacy of selective iNOS inhibitors in septic shock and organ dysfunction.

References


