CARDIOVASCULAR

Nitric oxide and liver microcirculation during autoregulation and haemorrhagic shock in rabbit model

F. Lhuillier1 2*, M.-O. Robert1, P. Crova2, J. Goudable2, F. Arnal2, R. Cespuglio3, G. Annat2 and J.-P. Viale1 2

1Département d’Anesthésie-Réanimation, Hôpital de la Croix Rousse, 103 Grande Rue de la Croix-Rousse, 69317 Lyon Cedex 04, France. 2UPRES EA 1896 and 3Neurobiology INSERM U480, 8 Avenue Rockefeller, 69008 Lyon, France

*Corresponding author. E-mail: franck.lhuillier@chu-lyon.fr

Background. Direct evidence of nitric oxide (NO) involvement in the regulation of hepatic microcirculation is not yet available under physiological conditions nor in haemorrhagic shock.

Methods. A laser Doppler flowmetry was used to measure liver perfusion index and a specific NO-sensitive electrode was inserted into liver parenchyma of anaesthetized rabbits. Hepatic autoregulation during moderate hypovolaemia (mean arterial pressure at 50 mm Hg without liver perfusion alteration; blood withdrawal 17.7 (4.2) ml [mean (SD)] or haemorrhagic shock [mean arterial pressure at 20 mm Hg associated with liver perfusion impairment and lactic acidosis; blood withdrawal 56.0 (6.8) ml] were investigated over 60 min and were followed by a rapid infusion of the shed blood. Involvement of NO synthases was evaluated using a non-specific inhibitor, NAPNA (Nv-nitro-L-arginine P-nitro-anilide).

Results. In the autoregulation group, a decrease [30.0 (4.0) mm Hg] of mean arterial pressure did not alter liver perfusion index, whereas the liver NO concentration increased and reached a plateau [125 (10)%; compared with baseline; P<0.05]. This NO concentration was reduced to zero by the administration of NO synthase inhibitor. Haemorrhagic shock led to a rapid decrease in liver perfusion index [60 (7)%; compared with baseline; P<0.05] before an immediate and continuous increase in NO concentration [250 (50)%; compared with baseline; P<0.05]. Infusion of NO inhibitor before haemorrhagic shock reduced the NO concentration to zero and hepatic perfusion by 60 (8)% (P<0.05) of the baseline. Mean arterial pressure increased simultaneously. In these animals, during haemorrhage, a continuous increase in NO concentration still occurred and liver perfusion slightly increased. In all groups but NAPNA+haemorrhagic shock, blood replacement induced recovery of baseline values.

Conclusions. NO plays a physiological role in the liver microcirculation during autoregulation. Its production is enzyme-dependent. Conversely, haemorrhagic shock induces a rapid increase in hepatic NO that is at least partially enzyme-independent.

Keywords: complications, haemorrhage; liver, blood flow; microcirculation; pharmacology, nitric oxide

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Under physiological conditions, the total liver blood flow is a stable value despite variations in blood pressure, implicating an autoregulatory mechanism.1

The contribution of nitric oxide (NO) to the regulation of basal vascular tone is diverse and tissue-specific.2 In the liver, NO has already been shown to modify hepatic vascular resistance at the arterial level and in the microcirculation during arterial pressure variations.3-6 However, in these studies, the role of NO was estimated by methodologies using cumulative measurements of derived products of NO metabolism or pharmacological inhibition of NO synthases (NOS). This inhibition itself
modifies liver blood flow, precluding any evaluation of NO in autoregulation. The availability of a specific NO electrode directly inserted into the hepatic parenchyma allows continuous measurement of the intrahepatic concentration of NO.

This work was designed to verify the involvement of hepatic NO in the microcirculation. We hypothesized that there was basal NO production and that the latter was further increased during autoregulation and haemorrhagic shock.

Materials and methods

Surgical procedure

This study was performed on 42 New Zealand White rabbits (weight 2.5 kg) in an authorized animal care laboratory approved by the French Health Authority and was approved by the University research committee.

Animals were premedicated with xylazine (5 mg kg⁻¹ administered i.m.) and anaesthetized with ketamine hydrochloride (50 mg kg⁻¹ administered i.m.). Anaesthesia was maintained by a continuous administration of a mixture of ketamine (3 mg kg⁻¹ h⁻¹) and xylazine (1.5 mg kg⁻¹ h⁻¹). After tracheotomy, animals lungs were mechanically ventilated (Servo ventilator 900B; Siemens-Elema, Sweden) with 50% oxygen and the ventilatory frequency was adjusted to keep the end-tidal carbon dioxide within the physiological range. Body temperature was maintained between 38.0 and 39.5 °C by means of a heater located above the animal. Systemic blood pressure was monitored using a Gould pressure transducer connected to a 1 mm fluid-filled catheter inserted in the right carotid artery. Left carotid was cannulated for blood withdrawal with a heparinized 60 ml syringe. After a right sub-costal laparotomy, a NO electrode was inserted into the right upper hepatic lobe (5 mm depth). Tissue perfusion was then measured using direct laser-Doppler velocimetry probe (Perimed, Jarfalla, Sweden). At the end of the reperfusion period, rabbits were killed by an i.v. injection of KCl.

NO measurement in the rabbit liver

NO generated in the liver parenchyma was measured using a pulse voltammetric method that is differential normal pulse voltammetry as previously described. This method is based on the measurement of the oxidation current produced by the application of a potential ramp to oxidizable compounds. The NO sensor was prepared on the basis of the carbon fibre sensor described elsewhere for catecholamines and 5-hydroxyindoles. Measurements were obtained every 2 min. Voltammetric measurements (peak height) were performed by using a Biopulse (Radiometer-Tacussel Society, Villeurbanne, France). After 45 min of steady NO signal, the mean of the five measurements over the last 10 min was used as the baseline reference.

Measurement of hepatic perfusion

Liver perfusion was measured using a direct laser-Doppler flowmetry probe (Perimed, Jarfalla, Sweden). The probe was placed on the surface of the right upper hepatic lobe. Stability of the probe was provided by a small plastic disc allowing a non-traumatic non-swinging contact on the surface of the liver. Doppler signals were continuously recorded throughout the study.

Blood samples

In all groups, arterial blood samples (5 ml) were obtained from the left carotid at T0 (45 min before intervention), just before blood withdrawal (T1), before resuscitation (T2) and finally, 60 min after resuscitation (T3). Transaminases [alanine amino transferase (ALT)] were determined in blood samples according to the International Federation of Chemical Chemistry technique using a pyridoxal phosphate activation (Roche Diagnostics, Meylan, France) on analyzer Modular P (Roche Diagnostics, Meylan, France). Lactic acid was determined by an enzymatic reaction with conversion of lactate to pyruvate and measurements at 660 nm (Roche Diagnostics) on a Hitachi 912 analyzer (Roche Diagnostics). Partial arterial pressure of carbon dioxide (Paco₂), oxygen (Pao₂) and pH were immediately measured with an ABL 5 (Radiometer SA, France).

Drugs

A non-specific long-lasting NOS inhibitor, Nω-nitro-L-arginine p-nitro-anilide (NAPNA; Sigma Aldrich, Paris, France) dissolved in 400 μl dimethylsulfoxide (Sigma Aldrich) was diluted in 10 ml saline.

Animal protocol

Forty-two rabbits were randomly assigned to six groups. In the control group (control; n=7), NO electrode was inserted into the liver parenchyma without any other intervention. In the NAPNA control group (n=7), NAPNA (40 mg kg⁻¹) was slowly infused i.v. over 45 min through the marginal ear vein without withdrawal of blood. In the autoregulation groups, blood withdrawal was performed to bring mean arterial pressure (MAP) down to 50 mm Hg, while keeping the hepatic Doppler signal constant. This pressure target was chosen according to a pilot experiment performed before this study. During the study, the following procedures were planned: if MAP increased above 50 mm Hg, additional blood withdrawals were performed, if hepatic perfusion decreased, blood volume necessary to restore hepatic perfusion to baseline was reinfused. This procedure was
maintained for 60 min, followed by a rapid infusion (30 s) of the shed blood in the autoregulation group \((n=7)\), or by administration of NAPNA \((40 \text{ mg kg}^{-1})\) over 45 min in the autoregulation+NAPNA group \((n=7)\). Data were further recorded for 60 min after blood re-infusion or NOS inhibitor administration.

In haemorrhagic shock groups, 14 rabbits were subjected to haemorrhagic shock according to Wiggers’s model. Haemorrhagic shock was obtained by withdrawing blood in order to bring MAP down to 20 mm Hg for 60 min. Animals were then resuscitated by infusion of the shed blood over around 4 min (haemorrhagic shock group, \(n=7)\). In the second haemorrhagic shock group, NAPNA was first infused i.v. over 45 min before haemorrhage (NAPNA+haemorrhagic shock group, \(n=7)\).

**Statistical analysis**

Results are expressed as mean \((\text{SD})\). Comparisons between means were performed by a two-way ANOVA followed by a Newman–Keuls test when the ANOVA showed significance. Statistical significance was assumed at a \(P\)-value <0.05. Statistical analysis was performed using Statistica version 5 software (StatSoft, Maisons-Alfort, France).

**Results**

**Impact of hepatic autoregulation or haemorrhagic shock on biological data**

In the autoregulation and control groups, \(P_{\text{aO2}}, P_{\text{aCO2}}, \text{ALT}, \text{lactic acid}\) and \(\text{pH}\) remained unchanged throughout the

![Graph showing time course of ALT, lactic acid, and pH for six groups of animals](https://example.com/graph.png)

**Fig 1** Time course of ALT, lactic acid and pH \([\text{mean (SD)}]\) in arterial blood for the six groups of animals. For all groups, \(T_0\) values were initial values. \(T_1\) values were recorded 45 min later in control groups (control and NAPNA control) or just before intervention in autoregulation (autoregulation and autoregulation+NAPNA) and haemorrhagic shock groups (haemorrhagic shock and NAPNA+haemorrhagic shock). \(T_2\) were recorded 60 min later whatever the group; thus at the end of the blood withdrawal in autoregulation and haemorrhagic groups. \(T_3\) were recorded 60 min later whatever the group. Two groups were pretreated with NAPNA \([\text{NAPNA control group (dotted bars) and NAPNA+haemorrhagic shock group (horizontal bars)}]\).

\[\text{†} P<0.05\text{ for comparison of values of haemorrhagic shock group vs control group.}\]

\[\text{‡} P<0.05\text{ for comparison of values of haemorrhagic shock group vs autoregulation group.}\]

\[\text{§} P<0.05\text{ for comparison of values of haemorrhagic shock group vs NAPNA+haemorrhagic shock group.}\]
procedure. $P_aCO_2$ decreased during blood shedding. This haemorrhagic shock led to lactic acidosis and transaminase increases after 60 min of shock which persisted at 60 min of reperfusion (Fig. 1). Pre-treatment of the shocked animals with NAPNA reduced plasma transaminases but not lactic acidosis. $P_aCO_2$ and $P_aO_2$ values are listed in Table 1.

Table 1 Time course of $P_aCO_2$ and $P_aO_2$ in arterial blood for the six groups of animals. For all groups, T0 values were initial values. T1 were values recorded 45 min later in control groups (control and NAPNA control) or just before intervention in autoregulation (autoregulation and autoregulation+NAPNA) and haemorrhagic shock groups (haemorrhagic shock and NAPNA+haemorrhagic shock). T2 were recorded 60 min later whatever the group; thus at the end of the blood withdrawal in autoregulation and haemorrhagic groups. T3 were recorded 60 min later whatever the groups. Two groups were pretreated by NAPNA (NAPNA control group and NAPNA+haemorrhagic shock group). *$P<0.05$ vs control; †$P<0.05$ vs baseline. Results are expressed in mean (SD)

<table>
<thead>
<tr>
<th>$P_aCO_2$ (kPa)</th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.7 (3.1)</td>
<td>31.2 (3.0)</td>
<td>31.1 (3.1)</td>
<td>31.4 (3.4)</td>
</tr>
<tr>
<td>NAPNA control</td>
<td>32.2 (3.5)</td>
<td>31.4 (3.1)</td>
<td>31.4 (3.1)</td>
<td>31.7 (3.2)</td>
</tr>
<tr>
<td>Autoregulation</td>
<td>31.7 (3.1)</td>
<td>31.4 (3.0)</td>
<td>31.4 (3.4)</td>
<td>31.2 (3.5)</td>
</tr>
<tr>
<td>Autoregulation+NAPNA</td>
<td>32.6 (3.7)</td>
<td>32.0 (3.4)</td>
<td>31.7 (3.5)</td>
<td>31.7 (3.7)</td>
</tr>
<tr>
<td>Haemorrhagic shock</td>
<td>32.0 (3.0)</td>
<td>31.7 (3.1)</td>
<td>28.0 (3.7)</td>
<td>28.6 (4.1)</td>
</tr>
<tr>
<td>NAPNA+haemorrhagic shock</td>
<td>32.6 (3.7)</td>
<td>32.0 (3.2)</td>
<td>30.0 (2.8)</td>
<td>29.3 (3.4)</td>
</tr>
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</table>

Liver perfusion and NO concentration during autoregulation or haemorrhagic shock

Liver perfusion and liver NO concentrations were stable during the whole experiment for control group. In the NAPNA control group, NOS inhibitor administration induced a disappearance of the NO signal lasting 100 min. In the two autoregulation groups (autoregulation with and without NAPNA), decreased MAP did not alter liver perfusion index, whereas liver NO concentration increased as early as the 4th minute and reached a plateau [125 (10)% of baseline; Fig. 3]. Re-infusion of shed blood induced recovery to baseline values. Haemorrhagic shock led to a rapid decrease in liver perfusion index prior to an immediate and continuous increase in NO concentration [250 (50)% of baseline]. Within 30 min of infusion of shed blood, NO concentration decreased to baseline values (Fig. 4).

Effect of NAPNA administration on hepatic blood flow and NO concentrations in liver parenchyma

In the NAPNA haemorrhagic shock group, pre-treatment with a 45 min continuous infusion of NAPNA (40 mg kg$^{-1}$) induced a decrease in NO concentration to zero in the liver parenchyma, a decrease in hepatic perfusion to 60 (7)% of the baseline value, and an increase in the mean systemic arterial pressure (Fig. 3). When the administration was performed at the 60th minute of autoregulation, the same phenomenon was observed (Fig. 2).

In haemorrhagic shock animals pretreated with NAPNA, a continuous increase in NO concentration occurred from the 8th minute of shock until resuscitation (Fig. 4). In this condition, liver perfusion increased slightly during haemorrhagic shock, reaching the level of the non-inhibited haemorrhagic group. Within 30 min of infusion of shed blood, NO concentration decreased to zero and liver perfusion increased to about 60% of the baseline values.

Discussion

The main findings of this study simultaneously using a laser-Doppler and an NO-sensitive electrode were:

First, NO played a physiological role in the liver microcirculation, as enzymatic inhibition reduced hepatic microcirculation despite an increase in systemic arterial pressure.

Second, enzymatic production of NO was involved in hepatic autoregulation in vivo occurring during moderate blood withdrawal.

Third, haemorrhagic shock induced a rapid increase of NO in the liver during the course of the shock that was at least partially enzyme-independent.

Measurement of liver perfusion and microcirculation

Blood flow in the hepatic microcirculation was measured by laser-Doppler flowmetry which enabled continuous
measurement without interfering with the circulation. Because of the technique used, this flowmetry was dependent on the red blood cell velocity and their concentration in the microvasculature.\textsuperscript{12} This concentration itself is in turn dependent on the density and haematocrit of the microvessels in the tissue. As a consequence, besides haematocrit, the laser-Doppler signal is dependent on red blood cell velocity and density of the microvasculature that are

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**Fig 2** Time course of MAP, liver perfusion index and hepatic NO concentration in control groups. Results are expressed in mean (SD). NAPNA infusion increased MAP, decreased liver perfusion and reduced to zero NO concentration for at least 100 min. *P*<0.05 for control vs NAPNA control.
both likely to be modified during haemorrhagic shock. Laser-Doppler flowmetry could not discriminate which mechanism was predominant. On the other hand, any stable laser-Doppler signal implies a constant blood cell velocity and microvascular density or an absolute compensation of the one by the other, indicating in both situations a constant red blood cell supply. Considering the reliability of surface laser-Doppler flowmetry, experimental studies showed that

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**Fig 3** Time course of MAP, liver perfusion index and hepatic NO concentration in autoregulation groups. Results are expressed in mean (SD). Despite a lower MAP, liver perfusion index was unchanged in the autoregulation group during blood withdrawal. Liver NO concentration increased. MAP, NO and liver perfusion parameters reached baseline values at the restitution period. NAPNA infusion increased MAP, decreased liver perfusion and reduced to zero NO concentration for at least 100 min. *P<0.05 for autoregulation vs autoregulation+NAPNA.

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Lhuillier et al.
Fig 4 Time course of MAP, liver perfusion index and hepatic NO concentration before, during and after haemorrhagic shock for two groups of animals undergoing haemorrhagic shock with restitution 60 min later. Results are expressed as mean (SD). In the first group (haemorrhagic shock, closed circle), animals underwent only blood withdrawal whereas the second group received NOS inhibition 45 min before blood withdrawal (NAPNA+haemorrhagic shock, open square). In the haemorrhagic group blood withdrawal led to a 50% decrease in liver perfusion and an immediate continuous increase in NO concentration. Within 30 min of infusion of the shed blood, NO concentration decreased to baseline value, whereas liver perfusion was restored. Soon after NAPNA infusion, liver blood flow decreased by 60% whereas the MAP increased by 40%. During haemorrhagic shock, NO concentration rapidly increased, and liver blood flow increased slightly reaching the same level as in the non-inhibited haemorrhagic shock group. Infusing the shed blood reduced NO signal to zero whereas the MAP reached baseline values and the liver perfusion was incompletely restored. *P<0.05 haemorrhagic shock vs NAPNA+haemorrhagic shock.
this signal was linearly related to the total liver blood flow measured using independent means in isolated rat livers or in in situ pig livers. However, the variability of the regression coefficients precluded any conversion of the laser-Doppler flow signal into an absolute value of flow.

**Evaluation of liver NO production**

In this study, we used a pulse voltammetric method yielding a continuous recording of NO concentration. NO electrodes, used together with this voltammetric method have been confirmed as having an excellent sensitivity and specificity towards biologically released NO in vivo. This method has already been used in an in vivo hepatic ischaemia–reperfusion rabbit model. The main source of enzymatic production is likely to be the endothelial cell, but there are also other cells, for example monocytes, Kupffer cells and hepatocytes, which have the ability to synthesize NO under physiological conditions or after stimulation. However, our measurements could not determine the type of hepatic cells involved in this NO production. Indeed, the space of diffusion of NO is estimated to be in the range 100–200 μm, well beyond the diameter of the NO-sensitive electrode (30 μm). This space diffusion is smaller than the liver lobular dimension, and therefore any effect of lobular heterogeneity of NO production could have increased the variability of the NO signal. This NO concentration was measured superficially, around 5 mm in depth, close to the depth of the laser-Doppler flow measurement, and did not necessarily represent the NO concentration of the whole liver. However, an absence of a gradient of flow between the periphery and the core of the liver has been demonstrated. Moreover, it has been suggested that NO could act not only as a paracrine agent with local effects, but also following release into the blood stream. Therefore, NO recorded at the liver surface could have been produced upstream of the recording site.

**Physiological role of NO on microvascular hepatic perfusion**

Results obtained under baseline conditions showed a substantial basal NO-mediated vasodilatory tone in the microvascular hepatic bed. Suppression of this tonic production modifies the balance between vasoconstrictors and vasodilators at the expense of the latter. This tonic production could be further enhanced by a slight decrease in MAP induced by a moderate hypovolaemia. As the hepatic microcirculation remained unchanged despite decreased MAP, a reduction of the resistance could be inferred, associated with increased NO concentration. This observation was direct evidence of the involvement of NO in the autoregulatory mechanism of hepatic microcirculation. Demonstration of full involvement should have included the effect of NO suppression on autoregulation. Unfortunately, any NO suppression by NOS modifies at the same time the liver microcirculation.

This NO production is dependent on NOS, as NOS inhibitors suppressed the NO signal. Owing to the delay of appearance, constitutive NOS is likely to be responsible for this production. The presence of these enzymes has been demonstrated in isolated hepatic sinusoidal endothelial cells; its activity being triggered by modification of flow. Other factors could induce NO release during autoregulation such as hypoxia or local variation in pH or blood CO₂. These results are consistent with previous studies reporting a functional role for NO in the regulation of hepatic vascular tone. However, the liver cells involved in the regulatory mechanism could not be deduced from our results. In contrast to most other organ beds, several lines of evidence suggest that microvasculature is the main site of resistance changes in the liver. NO may regulate sinusoidal blood flow via two target cells: the hepatic stellate cells which have a perisinusoidal location or the smooth muscle cells which are situated pre- or post-sinusoidally. It should be noted that our experiments were conducted in haemorrhagic shock models involving microvascular vasoconstriction and any conclusions could not be extended to hypotension induced by vasodilation.

**Effects of severe haemorrhagic shock on liver NO concentration**

The induced shock in animals was severe and likely to lead to hepatic ischaemia, as indicated by the marked increase in lactic acid. In this study, NO concentrations increased very soon after the onset of shock. The short delay existing between the onset of hypoperfusion and the increase in NO signal is consistent with the involvement of constitutive NOS. However, non-selective NOS inhibitors administered before ischaemia failed to inhibit the increase in NO, suggesting the presence of enzyme-independent sources of NO. Such an enzyme independence has already been observed in previous studies. NO stores are available in tissues and could be mobilized during shock. The potential stores included the S-nitroso-compounds, nitrosyl-haemoglobin and nitrate ions which may serve as sources of bioactive NO.

The potential role of this non-enzymatic production is speculative. Owing to its diffusibility and short lifetime, NO has two main physiological functions: acting as a messenger to inhibit mitochondrial respiration and as an effector modulating oxygen delivery to tissue. Considering the latter, it could be thought that NO contributes to maintain relaxation of vascular smooth muscles and hence vasodilatation in underperfused areas. This role in the protection of microvascular hepatic flow is suggested by our own data. Indeed, during haemorrhagic shock, the hepatic flow measured soon after the onset of shock was lower in the inhibited group than that in the non-inhibited group. This difference could be ascribed to the presence of NO in the non-inhibited group. Moreover, in the inhibited group, as NO concentration increased, Doppler hepatic flow...
reached the value of the non-inhibited group. Turning to NO as a messenger, its production during shock could be viewed as part of a general mechanism brought into play to adapt to hypoxic conditions. As a whole, this enzyme-independent production of NO could be a part of the protective role of NO brought into play during ischemic conditions. In this respect, the observation that the inhibition of NO production led to a less severe hepatic injury appeared paradoxical. The protective effect of inhibition of NO production could be secondary to the already described cytotoxic effects of enzymatic NO or to the preconditioning effect of the decreased microcirculation induced by inhibitor administration. Such a reduction in reperfusion injury has been reported after liver ischemic preconditioning. However, the protective effect of NO inhibition pre-treatment should be confirmed by other liver injury markers. Indeed, the lower perfusion of pretreated livers compared with non-treated livers may have caused a reduction of cytolytic enzymes and acid release.

In summary, using a NO-sensitive electrode in an in vivo model, our study brings direct evidence of the involvement of NO in hepatic autoregulation. Haemorrhagic shock triggers a time-dependent production of NO which is at least partly independent of NOS.

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