Hepatic Ischemia Is Associated with an Increase in Liver Parenchyma Nitric Oxide That Is in Part Enzyme-Independent

Franck Lhuillier, M.D., *Pierre Parmantier, Pharm.Dr.,* *Joelle Goudable, Ph.D.,†* *Philippe Crova, M.D.,*
Bertrand Delafosse, M.D., Ph.D.,‡ *Guy Annat, M.D., Ph.D.,§* *Raymond Cespuglio, Ph.D.,‖* *Jean Paul Viale, M.D., Ph.D.#*

Background: Nitric oxide (NO) might be involved in liver response to local ischemia–reperfusion injury.

Methods: A specific NO-sensitive electrode was inserted into liver parenchyma of anesthetized rabbits. After a 45-min period of stable NO signal, the vascular pedicle of the caudal lobe of the liver was clamped for 45 min, then the clamp was removed. Perfusion of the right upper lobe was left unchanged. The same procedure was applied in other animals after administration of a long-acting nonspecific NO synthase inhibitor NAPNA.

Results: Occlusion of the caudal pedicle was associated with a mean threefold increase in NO signal measured in the caudal lobe. After unclamping, this signal returned within 8 min to baseline value and remained stable for the next 6 h. In the right upper lobe, NO signal was unaffected by caudal lobe ischemia. By the end of the 6-h reperfusion period, administration of the NO inhibitor L-NAME led to a suppression of the NO signal, thus demonstrating the specificity of the measurement. Plasma nitrate and nitrite concentrations remained almost unchanged during the study period in all groups. In animals whose NO synthases had been previously inhibited by NAPNA, clamping the caudal pedicle for 45 min was still associated with a significant increase in caudal lobe NO signal.

Conclusion: Nitric oxide is present in liver parenchyma, and its generation is dramatically affected by an ischemia injury. The increased NO generation during local ischemia is, at least in part, independent of NO synthases.

LIVER ischemia–reperfusion injury is a clinical syndrome occurring most commonly as a result of trauma, hypovolemic shock, liver resection, or liver transplantation. Several studies have suggested that nitric oxide (NO) is involved in liver response to local ischemia–reperfusion injury, but controversial results have been published. In certain circumstances, NO has been reported to have beneficial effects on ischemia–reperfusion injury,[1,2] while other groups have described a cytotoxic role of NO.[3–5]

From a methodologic point of view, estimation of NO production has been usually performed by tissue measurement of the NO synthase (NOS) activity or of derived products such as nitrites and nitrates in blood or

biologic fluids. These methods suffer at least from two main limitations: no results could be obtained during the ischemic period itself, and, moreover, the information they give is delayed with respect to the time of ischemia.

In the current study we describe the kinetics and magnitude of NO production occurring in the liver parenchyma as well as the NOS contribution to NO signal by using a NO-specific electrode in an experimental model of ischemic–reperfusion injury.

Materials and Methods

Acute Liver Ischemia Reperfusion Model

The study was performed on 32 New Zealand White rabbits of either sex (weight, 3.0 kg) in an authorized animal care laboratory according to the French health authority and was approved by the University research committee.

The animals were premedicated with xylazine (5 mg/kg administered intramuscularly) and anesthetized with ketamine HCl (50 mg/kg administered intramuscularly). The marginal ear vein was cannulated (20-gauge cannula) for the continuous administration of medium-molecular-weight hydroethyl starch (15 ml/h Elohes 6%; Fresenius France Pharma, Sèvres, France) and to maintain anesthesia by infusion of a mixture of ketamine ($3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and xylazine ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Adequate depth of anesthesia was ensured before any surgical procedures by the absence of pedal and palpebral reflexes. After tracheostomy, animals were mechanically ventilated (Servo ventilator 900B; Siemens-Elema, Sweden) with 50% oxygen. The tidal volume was set at 15 ml/kg and the respiratory rate at 35 strokes/min. Then the respiratory rate was adjusted to keep the end-tidal carbon dioxide within the physiologic range. End-tidal gas concentrations were measured continuously using a gas analyzer (Capnomac Ultima; Datex, Helsinki, Finland). Systemic blood pressure was monitored using a Gould pressure transducer connected to a 1-mm fluid-filled catheter inserted in the right carotid artery. This catheter was used to take blood samples during the experiment. Body temperature, recorded through a thermistor inserted into the esophagus, was maintained between 38.0 and 39.5°C by means of a heater located above the animal. Limb lead II of the electrocardiogram was continually monitored by means of subcutaneous needle electrodes. Local Xylocaine anesthetic (Astra-Zeneca, Rueil-Malmaison, France) was injected before a

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right subcostal laparotomy to provide adequate analgesia. The caudal lobe was identified, and elastic was passed around the caudal pedicle of the liver (around 30% of the total weight of the liver). The elastic ends were threaded through a vinyl tube to make a snare to perform further occlusion and reperfusion.

At the end of the reperfusion period, a nonspecific NOS inhibitor was injected to check the specificity for NO of the voltametric signal recorded. The caudal pedicle was then reoccluded, and diluted Uniperse blue (Ciba-Geigy, Hawthorne, NY) was injected in the ear vein to verify the vascular exclusion of the caudal lobe during ischemia. With this technique, the previously perfused lobes during all the experiments appear blue, whereas the caudal lobe remains unstained. Anesthetized rabbits were then killed by an intravenous injection of KCl.

Measurement of Blood Flow

Blood flow was measured using a direct laser Doppler velocimetry probe (Perimed, Jarfalla, Sweden). After calibration with a Perimed motility standard kit, a laser Doppler probe, including a small plastic suction disc allowing a nontraumatic contact, was placed on the surface of the hepatic caudal lobe. This probe was connected to a 632-nm helium-neon laser tube and measured flow velocity to a depth of 1 mm before, during, and after the ischemic period.[6]

Nitric Oxide Measurement of the Rabbit Liver

Nitric oxide generated in the liver parenchyma was measured using a pulse voltametric method, *i.e.*, differential normal pulse voltametry. This method is based on the measurement of the oxidation current yielded by the application of a potential ramp to oxidizable compounds.[7]

Measurements were performed using a three-electrode potentiostatic system displaying the NO sensor as working electrode, a platinum wire (diameter = 100 μm) as auxiliary electrode, and an Ag/AgCl (diameter = 200 μm) as reference electrode.

The NO sensor was prepared on the basis of the carbon fiber sensor described elsewhere for catecholamines and 5-hydroxyindoles.[7,8] Its active part, consisting of a carbon fiber (diameter = 30 μm, length = 500 μm; Textron systems, Wilmington, MA), was first pretreated with a triangular current in 10 mM phosphate-buffered saline and then coated successively with porphyrine-nickel (Interchim, Montluçon, France) and Nafion® (Sigma-Aldrich chimie, Lyon, France), as described previously.[9] The specific NO oxidation current occurred at a potential of +650 mV and was proportional to the NO concentration of the medium.

Differential normal pulse voltametry measurements were performed in the caudal part or the upper right lobe of the liver. The working electrode was inserted into the parenchyma (5-mm depth). Reference and auxiliary electrodes were placed in contact with the subcutaneous cellular tissue.

Measurements were taken every 2 min. The carbon fiber surface treatments and the voltametric measurements (peak height) were performed using a Biopulse® (Radiometer-Tacussel Society, Villeurbanne, France). After a 45-min steady state of the NO signal, the mean of the last five measurements (10 min) was used as the baseline reference.

Blood Samples

Transaminases (aspartate amino transferase and alanine amino transferase) were determined in blood samples with a biochemical multianalyzer. The concentration of nitrites (NO_2^-) and nitrates (NO_3^-) in plasma was determined by the Griess reaction, as follows. After deproteination by a solution of zinc sulfate, samples were incubated with cadmium granules to reduce nitrate to nitrite; the total nitrite was measured at 540 nm absorbance by diazotization with Griess reagent.[10] The results are expressed as the sum of the *N*-oxides of NO (NOx). Serum total proteins were measured to detect a hemodilution by volume loading during the whole procedure. This allowed us to correct the biologic value according to the initial protein concentration and to rule out any dilution effect.

Animal Protocol

Thirty-two rabbits were divided into five groups. In the caudal ischemia–reperfusion group (n = 7), NO electrode was inserted into the caudal parenchyma before a 45-min caudal pedicle occlusion and 6 h of reperfusion. In the upper right lobe group (n = 7), NO electrode was inserted into the hepatic upper right lobe parenchyma before a 45-min caudal pedicle occlusion and 6 h of reperfusion. In the control group (n = 7), the NO electrode was inserted into the caudal parenchyma without any ischemia. For these first three groups, the specificity for NO of the electrode was tested by injecting intravenously a nonspecific NOS inhibitor, L-NAME (200 mg/kg N^{ω}-nitro-L-arginine methyl ester; Sigma-Aldrich, Saint Quentin Fallavier, France) at the end of the reperfusion period.

To determine whether NOSs are involved in NO production during ischemia–reperfusion, NAPNA (N^{ω}-nitro-L-arginine P-nitro-anilide; Sigma-Aldrich), a long life nonspecific NOS inhibitor, was used. In the NAPNA ischemia–reperfusion group (n = 6), the NO electrode was inserted into the caudal parenchyma before infusion of 40 mg/kg intravenous NAPNA during 45 min. This group underwent a 45-min caudal pedicle occlusion and 6 h of reperfusion. In the NAPNA control group (n = 5), the NO electrode was inserted into the caudal parenchyma before infusion of 40 mg/kg intravenous NAPNA during 45 min. No ischemia was performed in this group. Blood samples (3 ml) were taken on the right

carotid before ischemia, before reperfusion, 2 min after reperfusion, and then each hour after reperfusion.

Drug Treatments

NAPNA, dissolved in 400 μl dimethylsulfoxide (Sigma-Aldrich), was diluted in 50 ml saline and slowly administered (45 min) intravenously.

Statistical Analysis

Results are expressed as mean ± SEM. For biologic data, effect of time was analyzed using a two-way analysis of variance for repeated measurements. Interaction analysis revealed whether effects of ischemia were different between groups. For the NO concentrations, a two-way analysis of variance for repeated measurements was also performed to analyze the effects of ischemia: one for the three groups of animals experiencing ischemia or control, and another one for the two groups experiencing NAPNA inhibition. When the F value was significant, comparisons between means were conducted by a Newman-Keuls test. Statistical significance was assumed at a P value < 0.05. Statistical analysis was performed using Statistica version 5 software (StatSoft, Maisons-Alfort, France).

Results

Biologic Data

The time course of protein concentration in plasma showed a continuous decrease ($P < 0.05$ after 2 h of reperfusion *vs.* time before ischemia for each group) relating to hemodilution during the anesthesia of the rabbits (approximately 9 h). The decrease of plasma protein concentration was not statistically different between the groups.

Aspartate amino transferase and alanine amino transferase plasma concentrations were, respectively, three and five times higher ($P < 0.05$) after 4 h of reperfusion than in the control group (fig. 1). NOx concentrations were significantly increased at 6 h, but interaction analysis of the analysis of variance did not show any group effect (fig. 2).

Hepatic Blood Flow and Nitric Oxide
Concentrations during Ischemia–Reperfusion

Clamping the caudal pedicle of the liver led to a 60% decrease in caudal blood flow. From the 10th minute after the unclamping, the blood flow returned to preclamping level (fig. 3). The evolution of hepatic blood flow during ischemia–reperfusion was the same when rabbits were treated with NAPNA (data not shown).

An immediate and continuous increase in NO concentration in the caudal lobe was induced by the clamping of the caudal hepatic pedicle (fig. 3). By 10 min after clamping, a plateau was reached. It remained stable until
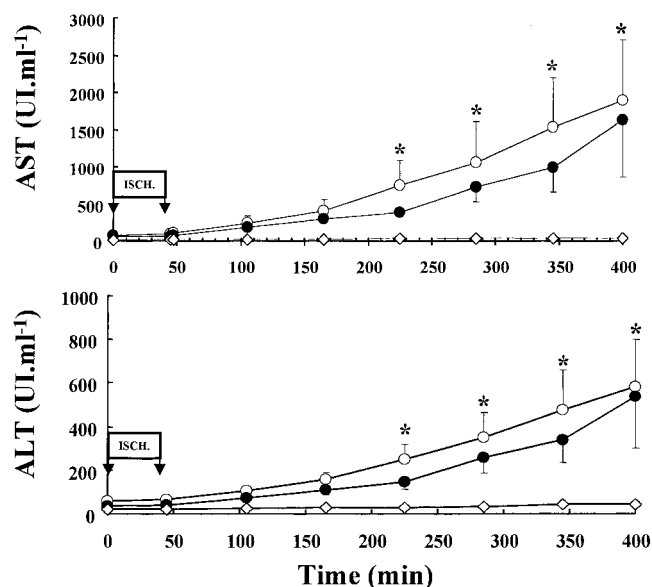

Fig. 1. Time course of aspartate amino transferase and alanine amino transferase (mean ± SEM) following a 45-min period of ischemia (Isch) in three groups of animals: animals with a caudal ischemia while the nitric oxide (NO) concentration of the caudal lobe was recorded (open circles), same ischemia while the upper lobe NO concentration was recorded (closed circles), and no ischemia (open diamonds). Plasma activity is expressed in international units per milliliter of plasma at constant protein concentration to include hemodilution effect. $P < 0.05$ after 4 h of reperfusion compared with the control group.

reperfusion was initiated. However, as indicated by a large SEM, the maximum NO increase varied from one animal to another, ranging from 175 to 480% of the preclamping value. Within 8 min after unclamping, the NO concentration decreased to baseline value and remained stable during the 6 h of reperfusion (fig. 3). Conversely, the NO concentration remained stable in the upper right lobe during the caudal lobe ischemia and reperfusion and in the control group (fig. 3). At the end of the reperfusion period, administration of L-NAME led to a complete suppression of the NO signal.
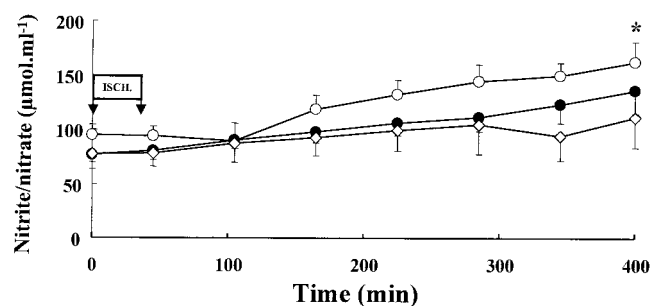

Fig. 2. Time course of nitrites–nitrates concentration (mean ± SEM) following a 45-min period of ischemia (Isch). Same symbols as in figure 1 for the three groups of animals. Concentration was statistically higher than baseline after 6 h of reperfusion. No difference was found between the groups.
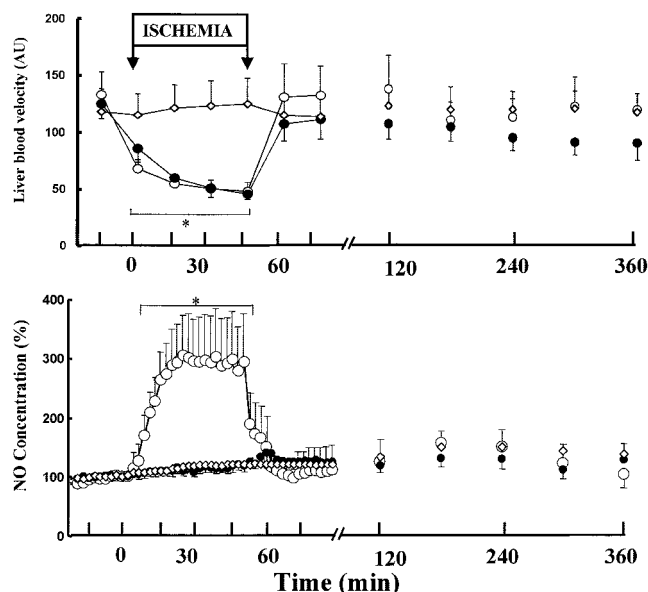
Fig. 3. Liver blood velocity and nitric oxide (NO) concentration before, during, and after ischemia (Isch) in three groups of animals. In the caudal ischemia–reperfusion group (open circles), the NO recordings were performed in the caudal lobe; in the upper right lobe group (closed circles), the NO concentration recordings were made in the upper lobe; in the control group (open diamonds), no ischemia was performed. Values of blood velocity are expressed in arbitrary units (AU). For clarity, SEM of the control group are not displayed. *P < 0.05 for comparison of blood velocities and NO concentration in the caudal lobe before *versus* during ischemia for caudal and upper groups.

Effect of NAPNA on Nitric Oxide Concentrations in Liver Parenchyma

Treatment with a 45-min continuous infusion of NAPNA (40 mg/kg) reduced the NO concentration in the liver parenchyma to zero after 45 min, and this effect persisted for at least 150 min (fig. 4). When caudal pedicle was clamped, a continuous increase in NO concentration occurred from the eighth minute of ischemia
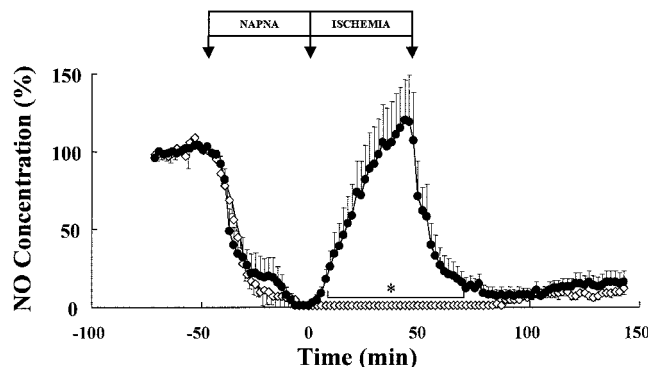

Fig. 4. Time course evolution of nitric oxide (NO) concentrations after infusion of a nonselective NO synthase inhibitor (NAPNA) over a 45-min period in two groups of animals. This administration was followed by an ischemia for 45 min (Ischemia) in a group (closed circles), while the other group experienced no ischemia (open diamonds). Values are expressed as percentage of the reference values. *P < 0.05 for comparison of values measured before *versus* during ischemia.

until reperfusion. Within 40 min after unclamping, the NO concentration decreased to zero and remained stable for 90 min. Of the animals treated with NAPNA, four developed intermittent ventricular tachycardia at the end of the NAPNA inhibition period, and four in each group died beyond the 150th minute of reperfusion (data not reported).

Discussion

The main findings of this study using an NO-sensitive electrode are three. First, NO is present in the normally perfused liver. Second, the concentration of NO increases rapidly following ischemia in the liver, remains elevated during the full time course of ischemia, and is reduced upon reperfusion. Third, the NO production triggered by ischemia is at least partially NOS–independent.

Evaluation of Nitric Oxide Production by the Liver

In this study we used a pulse voltametric method yielding a continuous recording of NO concentration. The NO electrode, used together with this voltametric method, was first developed to define the NO concentration in the brain tissue. It has been checked for having an excellent sensitivity and specificity toward the NO biologically released *in vivo*. A linear relation between the voltametric height of the NO signal and the NO concentration has been shown over a range of pH from 4 to 9.[7] The specificity was assessed by its suppression following the injection of nonspecific inhibitors of NOSs at the end of the procedure (L-NAME) or before ischemia (NAPNA). However, our measurements could not presume the type of hepatic cell involved in this NO production because of the space of diffusion of this molecule. Indeed, the diameter of the NO-sensitive electrode was 30 μm, and the space diffusion of NO is usually estimated to be in the range of 100 to 200 μm.[11]

Considering the model of ischemia–reperfusion injury, we used hydroethyl starch infusion for volume support. Indeed, this compound has been shown to decrease organ injury after hepatoenteric ischemia–reperfusion.[12]

Effects of Ischemia-Reperfusion on Liver Nitric Oxide Concentration

The effect of tissue ischemia on NO production has been already observed in at least two other studies. Using an electron paramagnetic resonance spectroscopy method, Zweier *et al.*[13] described a 10-fold increase in NO in the ischemic heart. More recently, an increase in NO in gastric tissue was shown after clamping the celiac artery.[13,14] Conversely, Chan *et al.*[15] failed to observe any increase in NO in a model of intestinal ischemia tissues. In the current study, NO concentrations increased after the onset of ischemia to reach a plateau by

10 min. The short delay existing between the onset of ischemia and the increase in NO signal is consistent with the involvement of endothelial nitric oxide synthase (eNOS). However, nonselective NOS inhibitors administered before ischemia failed to inhibit the increase in NO, whereas NO production remained inhibited in the control group, thereby suggesting the presence of enzyme-independent sources of NO. NO stores are available in tissues and could be mobilized during ischemia. Indeed, the metabolic pathways of NO include the formation of *S*-nitrosothiols, *S*-nitroproteins, as well as nitrosylhemoglobin, which are potential enzyme-independent sources of NO.[16] Another enzyme-independent source for NO might be the endogenous reduction of nitrite and nitrate resulting in the appearance of NO. This mechanism has been demonstrated in ischemic heart and could be the source of NO in the ischemic liver, as the pH is likely to be in the same range as the one observed in the ischemic heart.[17] However, this NO production was dependent on the amount of nitrite–nitrate available in the tissue before ischemia. This could explain the lack of NO increase during ischemia in the intestinal tissue, owing to the low value of the basal NO production observed in this tissue.[15] In addition, NO might be generated from the interaction of hydroxyurea with hemoproteins[18] or by heme-bound NO.[19]

The relevance of this finding could be at least twofold. The first is to cast some doubt on the value of nitrite–nitrate concentrations as an index of NO formation during ischemia–reperfusion injury, as the process of ischemia itself could reduce the amount of nitrite–nitrate. In fact, throughout our study the concentrations of nitrite and nitrate remained fairly stable. The second clinical relevance is a potential explanation of the conflicting results observed in studies estimating the role of NOS inhibition in septic shock. Numerous lines of evidence led to the suggestion that NO plays a major role in the circulatory alterations occurring in septic shock. As a consequence, many experimental studies[20–22] as well as some clinical trials[23–25] have aimed at blocking the effects of NO by using selective or nonselective NOS inhibitors. According to our results, the administration of NOS inhibitors does not rule out any NO production in some areas or in some particular experimental model of septic shock with ischemia and acidosis. This could be a contributing factor to the discrepancies observed among these studies.

The exact role of this NOS-independent production of NO remains unanswered and speculative. The first refers to the physiologic effects of NO.[26] It could be thought that this NO contributes to maintain relaxation of vascular smooth muscles and hence vasodilatation in underperfused areas. This could lead at the same time to inhibition of platelet aggregation and leukocyte adhesion and activation. Turning to other roles of NO, this production during ischemia could be viewed as part of a general mechanism brought into play to adapt to hypoxic conditions. It has been shown that NO reversibly inhibits mitochondrial respiration by competing with oxygen at cytochrome oxidase.[27] It seems likely that this inhibition mediates a general metabolic suppression, including oxygen consumption, glucose and protein synthesis, and ion transport.[28] All of these cellular processes are thought to be regulated in conditions where NO is produced by ischemia.

The decrease of NO concentration associated with the reperfusion phase, already observed in previous studies,[14] could be explained by several mechanisms. Besides the washout of NO brought about by blood flow following reperfusion, the waning of the acidotic period could account for the decrease in NO. Another contributing factor could be the reaction of NO with superoxide radicals generated during the reperfusion period.[29,30]

Our study demonstrates that the use of an NO-sensitive electrode *in vivo* could be a new specific tool to investigate more accurately changes in NO production, especially when rapid alterations of NO concentration are expected. This work demonstrates that the generation of NO is a local, time-dependent response to liver ischemia and is at least partly independent of NOS.

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