Original article

Real-time monitoring of liver damage during experimental ischaemia–reperfusion using a nitric oxide sensor

A. Nakagawa[1], Y. Yokoyama[1], H. Suzuki[5], K. Shoji[3], Y. Watanabe[4], A. Imamura[2], T. Kokuryo[1] and M. Nagino[1]

[1]Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, and [2]Inter Medical Company Limited, Nagoya, [3]Faculty of Human Arts and Science, University of Human Arts and Science, [4]Department of Pharmacology, High Technology Research Centre, Showa Pharmaceutical University, Tokyo, Japan, and [5]Department of Life and Reproduction Science, Section of Biochemistry, University of Verona, Verona, Italy

Correspondence to: Dr Y. Yokoyama, Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan (e-mail: yyoko@med.nagoya-u.ac.jp)

Background: Hepatic ischaemia–reperfusion (IR) injury may lead to liver damage during liver surgery, and intrahepatic nitric oxide (NO) levels may play a role in this context. The aim of this study was to demonstrate real-time changes in intrahepatic NO concentration during IR and to correlate potential hepatic NO production with liver damage using a selective NO sensor.

Methods: Wistar rats were exposed to 15 min of hepatic ischaemia followed by reperfusion, after which changes in intrahepatic NO levels were measured using an NO sensor. Additionally, rats were exposed to five successive periods of IR, each consisting of 15 min ischaemia followed by 5 or 15 min reperfusion, and hepatic damage was evaluated by blood tests and histological examination. Hepatic expression of Akt, phosphorylated Akt, endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS was examined at different time points during and after IR by western blot and immunohistochemical analysis.

Results: During ischaemia, intrahepatic NO levels increased and reached a plateau at approximately 10 min. Repeated 15 min ischaemia–5 min reperfusion cycles reduced the maximum amount of NO produced during ischaemia gradually, and almost no NO production was observed during the fifth period of ischaemia. NO production following repeated ischaemia was proportional to the degree of hepatic viability. Phosphorylated eNOS was upregulated and correlated with the level of NO production during hepatic ischaemia.

Conclusion: Intrahepatic NO levels decrease during repeated IR in rats. Real-time monitoring of intrahepatic NO levels is useful for the prediction of IR-related liver injury during experimental liver surgery.

Surgical relevance

Hepatic ischaemia–reperfusion (IR) injury may induce variable degrees of postoperative liver damage because tolerance to IR varies between patients. Therefore, a device that can predict liver damage during hepatic IR would be desirable in order to perform safe liver surgery.

This study explored the real-time dynamics of intrahepatic nitric oxide (NO) levels during repeated hepatic IR in rats using a selective NO sensor. After repeated episodes of 15 min ischaemia and subsequent reperfusion, the maximum amount of NO produced during ischaemia gradually decreased. The levels of NO produced following repeated ischaemia were proportional to the degree of hepatic viability.

Real-time monitoring of intrahepatic NO levels using selective NO sensors is useful for the prediction of IR-related liver injury and may be employed in the future as a tool to prevent liver injury during liver surgery.

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Introduction

Nitric oxide (NO) is a key protective molecule that plays a crucial role in maintaining the hepatic microcirculation. Nitric oxide synthase (NOS) metabolizes L-arginine to citrulline to produce NO. Two isozymes of NOS, endothelial (eNOS) and inducible (iNOS) NOS, play an important pathophysiological role in the liver. The relatively small (picomolar to nanomolar) quantities of NO produced

by the calmodulin/calcium-dependent activation of eNOS, constitutively expressed in sinusoidal endothelial cells, are important in maintaining the hepatic microcirculation under steady-state conditions[1] (*Fig. 1*). In contrast, during acute ischaemia the liver upregulates iNOS and produces large (micromolar) quantities of NO[2]. Upregulation of iNOS is probably necessary to cope with the sudden hepatic ischaemic stress, but time (hours) is required to upregulate gene expression and protein synthesis[3,4]. Previous reports have clarified the mechanism of calmodulin/calcium-independent regulation of NO production by phospho-Akt-dependent eNOS phosphorylation under ischaemic conditions[5–8] (*Fig. 1*). Via this mechanism, large quantities of NO may be produced in seconds, because this process is regulated by phosphorylation of associated proteins and does not require upregulation of gene expression or protein synthesis.

Intrahepatic NO levels may change following hepatic IR[9], but the real-time dynamics are still unclear. In particular, the dynamics of intrahepatic NO immediately after initiation of ischaemia have never been evaluated. Recently, selective NO sensors were developed to measure NO levels both *in vivo*[10–12] and *in vitro*[11,13], and have been used to monitor real-time NO production in brain[10], heart[11] and kidney[12]. However, *in vivo* studies demonstrating the real-time dynamics of intrahepatic NO levels during repeated IR have not been performed.

The purpose of this study was to characterize real-time changes in intrahepatic NO levels during repeated hepatic IR using a sensitive and selective NO sensor in rat liver. As NO is protective during ischaemic stress, it was hypothesized that hepatic NO production is associated with liver viability and that monitoring of intrahepatic NO levels could be used to predict liver damage during repeated IR stress.

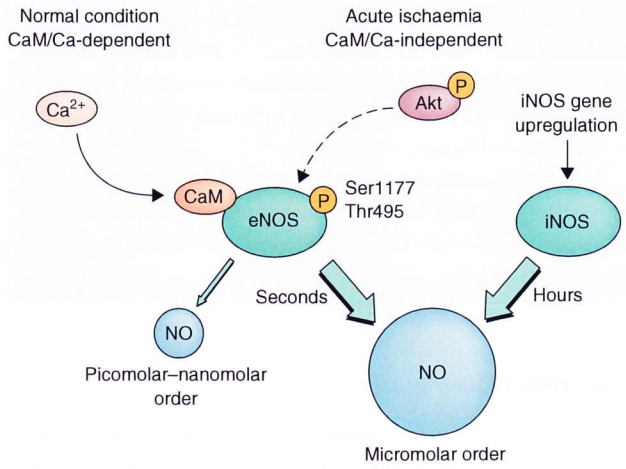

Fig. 1 Nitric oxide (NO) production in the normal condition and in acute ischaemia. CaM, calmodulin; Ca, calcium; P, phosphate; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase

Methods

Animals

A total of 162 male Wistar rats (250–300 g, 8 weeks old; SLC JAPAN, Nagoya, Japan) were housed in a temperature- and humidity-controlled environment on a 12-h light–dark cycle and allowed access to food and sterilized water *ad libitum*. All experiments were approved by the Nagoya University Committee on Animal Research, and all experimental animals received humane care in accordance with the National Institutes of Health publication, *Guide for the Care and Use of Laboratory Animals*.

Specific nitric oxide sensor

The two-electrode potentiostatic IMN-111 NO sensor (probe diameter 200 μm) was purchased from Inter Medical (Nagoya, Japan). This NO sensor (*Fig. S1*, supporting information) can detect NO concentrations as low as 1 nmol/l. Before and after all experiments, the NO sensor was polarized and calibrated to confirm its accuracy.

Surgical procedures and experimental protocol

Rats were anaesthetized with pentobarbital sodium (15 mg/kg intraperitoneal injection) on a heating pad. After upper abdominal transverse laparotomy, a working electrode was inserted 5 mm deep into the left lobe of the liver and a reference electrode was attached to the surface of the liver. Thereafter, the working electrode was allowed to stabilize for 30 min until the recording line was balanced. This level was set as zero and the relative change in intrahepatic NO levels was measured in subsequent experiments. To stop hepatic inflow, the hepatoduodenal ligament was clamped with a vascular clip (BEAR Medic Corporation, Chiba, Japan) and recirculation was established by releasing the vascular clip. The response of the electrode to NO was recorded as electrical current. Blood and liver samples were harvested at different time points during IR. In a separate experiment, repeated cycles of 15 min of ischaemia followed by 5 or 15 min of reperfusion were applied in some rats ($n = 12$).

Sample preparation

Tissue samples for western blot analysis and eNOS activity assays were obtained after 0, 1, 3, 5, 7, 10, 15 and 30 min of

total ischaemia. Samples were also obtained after 15 min of ischaemia followed by 5 min of reperfusion. Each sample was collected from one rat, which was then killed. Tissue samples were cut immediately into 5-mm cubic sections and kept at −80 °C until use. Six to ten rats were used for each group and time point.

Administration of nitric oxide synthase inhibitor

To inhibit NOS activity, the non-specific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Cell Signaling Technology, Danvers, Massachusetts, USA), which inhibits both iNOS and eNOS, 15 mg/kg intraperitoneally 60 min before hepatic ischaemia[14], or the selective inhibitor of iNOS N^6-(1-iminoethyl)-L-lysine (L-NIL; Cell Signaling Technology), 3 mg/kg intravenously 15 min before hepatic ischaemia followed by an infusion of 1 mg per kg per h[15], was injected in separate animals.

In vitro endothelial nitric oxide synthase activity assay

All *in vitro* experiments were performed in a box filled with nitrogen gas at 32°C because NO is unstable in the presence of oxygen. Each liver sample was soaked in a column with 1 ml phosphate buffered saline (PBS) containing 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 1 mol/l EDTA-free complete protease inhibitor, 1 mmol/l sodium orthovanadate (all from Calbiochem Merck, San Diego, California, USA) and 10 mmol/l sodium fluoride (Cell Signaling Technology). Tissue samples were homogenized using Bead Smash 12® (Wakenyaku, Kyoto, Japan), centrifuged at 10 000 *g* at 4°C for 10 min, and the supernatant was collected. While the supernatant was being stirred, the NO sensor was dipped into the supernatant. Subsequently, 1 mmol/l flavin–adenine dinucleotide (Cell Signaling Technology), 10 mmol/l tetrahydrobiopterin (Calbiochem Merck) and 10 mmol/l reduced nicotinamide–adenine dinucleotide phosphate (Calbiochem Merck) were added. This solution was made with, or without 10 μmol/l calmodulin (Cell Signaling Technology) and 1 mmol/l calcium (Sigma, St Louis, Missouri, USA). Finally, 1 mmol/l L-arginine (substrate of NOS; Cell Signaling Technology) was added and changes in NO levels were measured.

In situ visualization of nitric oxide

To visualize NO, 4,5-diaminofluorescein diacetate (DAF2-DA; Sigma) staining was performed on 5-μm thick frozen hepatic tissue sections (before and after 15 min ischaemia). The sections were incubated in 10^{-5} mol/l DAF2-DA diluted in PBS buffer for 2 h. Samples were then washed out three times using PBS and mounted. DAF2-DA staining was examined under fluorescence on an Olympus fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with an excitation (447–517 nm)

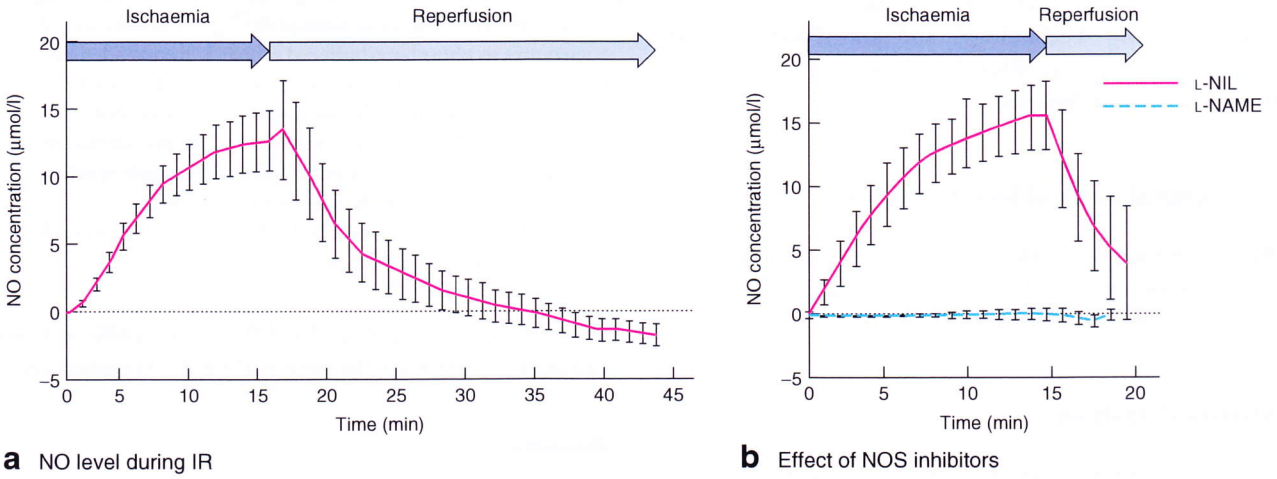

Fig. 2 **a** Intrahepatic nitric oxide (NO) level during ischaemia–reperfusion (IR) in the rat liver using a selective NO sensor ($n = 8$). **b** Effects of the non-specific nitric oxide synthase (NOS) inhibitor (N^G-nitro-L-arginine methyl ester, L-NAME) or specific inducible NOS inhibitor (N^6-(1-iminoethyl)-L-lysine, L-NIL) on intrahepatic NO levels during IR ($n = 3$ per group). Values are mean(s.e.m.). Negative values for NO observed during reperfusion indicate that NO levels were lower than at the start of ischaemia

and emission (496–576 nm) green filter for DAF-2 fluorescence[16].

Western blot analysis

Tissue samples were mixed with Laemmli sample buffer containing 1 mmol/l PMSF, 1 mmol/l EDTA-free complete protease inhibitor, 1 mmol/l sodium orthovanadate and 10 mmol/l sodium fluoride, and then homogenized and centrifuged as described above. The supernatant fluid was collected as a lysate and electrophoresed on sodium dodecyl sulphate–polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Immobilon; Millipore, Billerica, Massachusetts, USA) and then probed with antibodies; signals were detected with an enhanced chemiluminescence system (Nakalai, Kyoto, Japan).

Histological evaluation

Tissue samples were fixed immediately in neutral buffered formalin, embedded in paraffin, and then either stained with haematoxylin and eosin or subjected to immunohistochemistry for eNOS, phospho-eNOS, and iNOS using the automated slide preparation system Discovery XT (Ventana Medical Systems, Tucson, Arizona, USA). Before staining, paraffin sections were heated at 65°C for 15 min in a paraffin oven and blocked with non-fat milk. The staining procedure was carried out according to the manufacturer's protocol (Ventana Medical Systems). The anti-eNOS antibody (BD Bioscience, San Diego, California, USA), anti-phospho-eNOS antibody (Cell Signaling Technology) and anti-iNOS antibody (BD Bioscience) were diluted in Discovery® Ab Diluent (Ventana Medical Systems).

Biochemical assay of blood samples

Blood samples were taken for measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by standard laboratory methods.

Statistical analysis

Data are expressed as mean(s.e.m.). Statistical differences between two groups were compared with Student's t test. The level of significance was set at $P < 0{\cdot}050$. The integrated value of the NO curve (the area under the curve; AUC) was calculated to estimate total NO production. The slope at each time point (the differential coefficient) was calculated to estimate the potential for NO production.

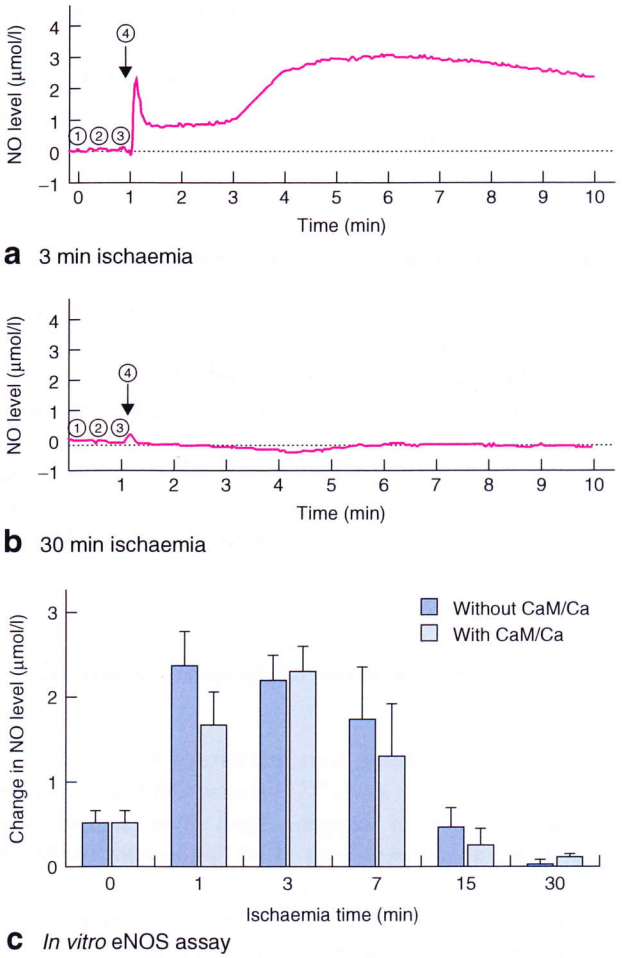

Fig. 3 Representative *in vitro* data showing changes in nitric oxide (NO) levels in liver homogenates of samples following **a** 3 min and **b** 30 min of ischaemia during administration of coenzymes in phosphate-buffered saline: 1, flavin–adenine dinucleotide 1 mmol/l; 2, tetrahydrobiopterin 10 mmol/l; 3, reduced nicotinamide–adenine dinucleotide phosphate 10 mmol/l; 4, the substrate L-arginine. **c** NO production in homogenates in the *in vitro* endothelial nitric oxide synthase (eNOS) activity assay with or without calmodulin (10 μmol/l)/calcium (1 mmol/l) (CaM/Ca) ($n = 6$–7 in each group); values are mean(s.e.m.)

Results

Monitoring of intrahepatic nitric oxide levels during ischaemia–reperfusion

During ischaemia, intrahepatic NO levels gradually increased and reached a plateau at approximately

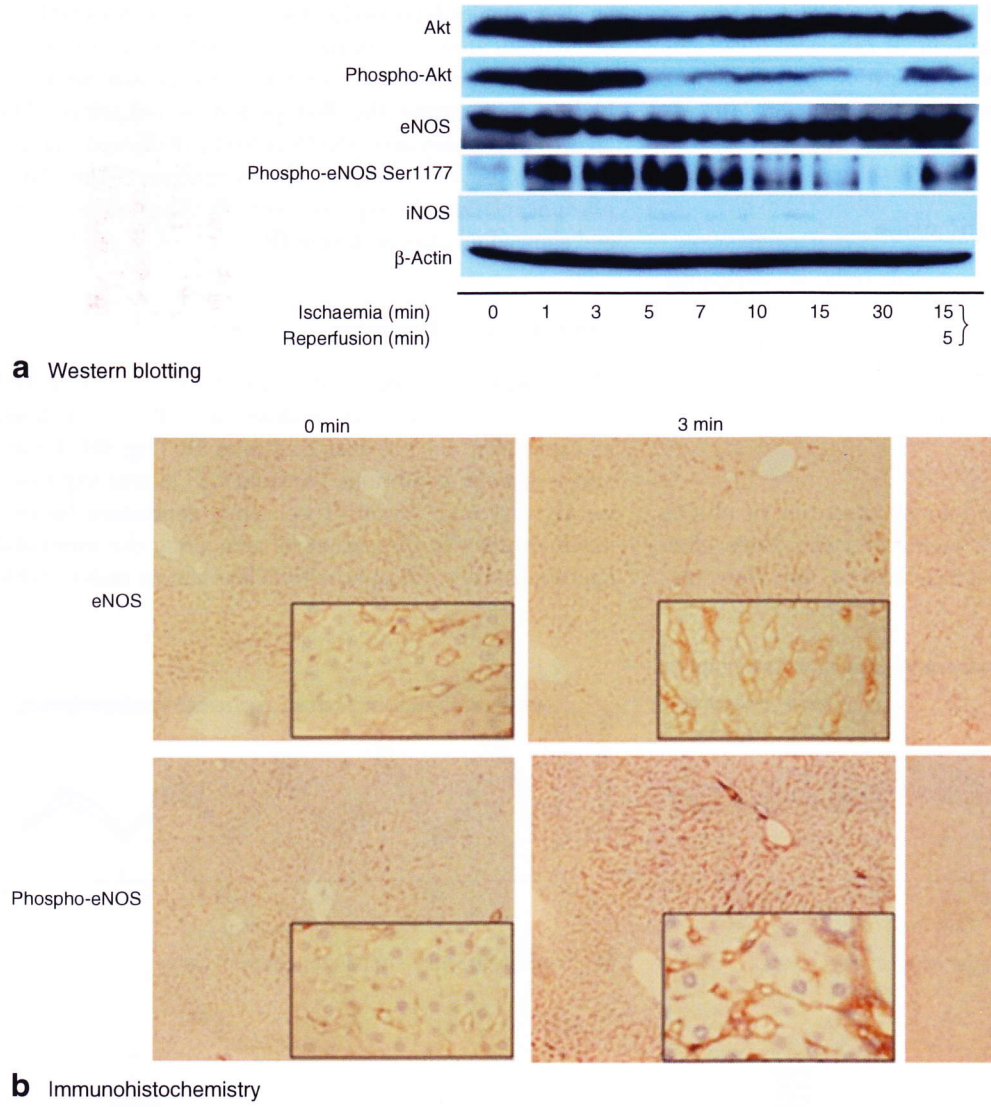

Fig. 4 **a** Expression of Akt, phosphorylated Akt (phospho-Akt), endothelial nitric oxide synthase (eNOS), phosphorylated eNOS (phospho-eNOS) and inducible nitric oxide synthase (iNOS) at different time points of hepatic ischaemia–reperfusion (western blot). β-Actin was used as a control. **b** Immunohistochemistry for eNOS and phospho-eNOS protein in the 0-, 3- and 30-min hepatic ischaemia groups (original magnification ×100, inset magnification ×400)

10 min (*Fig. 2a*). There was no further increase in NO concentration even after 30 min of ischaemia (data not shown). On reperfusion, after a small increase, NO levels quickly returned to baseline. Negative values for NO observed during reperfusion indicate that NO levels were lower than at the start of ischaemia. Following administration of the non-specific NOS inhibitor L-NAME, NO production was almost completely inhibited (*Fig. 2b*), indicating that NO biosynthesis during IR was dependent on NOS. L-NIL (specific inhibitor of iNOS) did not inhibit NO production (*Fig. 2b*), suggesting that the high NO production during ischaemia was not dependent on iNOS activity.

In vitro assay

High NO production was observed in liver homogenates of samples taken following 3 min of hepatic ischaemia (*Fig. 3a*). NO production decreased gradually as the length of ischaemia increased, and almost no increase in NO

concentration was observed after 15 or 30 min of hepatic ischaemia (*Fig. 3b*). The addition of calmodulin/calcium did not alter NO production, indicating that NO production during ischaemia was not dependent on calmodulin/calcium (*Fig. 3c*).

In situ visualization of nitric oxide

NO was visualized in endothelial cells in periportal areas; this was more prominent after 15 min ischaemia than at baseline (before ischaemia) (*Fig. S2*, supporting information).

Western blot analysis

Phospho-Akt levels, assumed to be upstream of eNOS phosphorylation, were much higher at 1 and 3 min after initiation of ischaemia than at baseline (*Fig. 4a*). However, levels gradually decreased and were almost undetectable 15 or 30 min after the initiation of ischaemia. Phospho-Akt levels were restored after reperfusion but were not higher than during the first period of ischaemia. The level of phosphorylated eNOS at Ser1177 showed changes similar to those of phospho-Akt. In contrast to the eNOS phosphorylation, the expression of iNOS protein was weak and showed no change during IR.

Immunohistochemistry

eNOS was expressed predominantly in the periportal area along the sinusoidal endothelial cells in the liver; its expression did not change during IR (*Fig. 4b*). Under non-ischaemic conditions phospho-eNOS was expressed weakly, but after 3 min of ischaemia expression became much stronger in the periportal area along the sinusoidal endothelial cells. Phospho-eNOS was almost undetectable

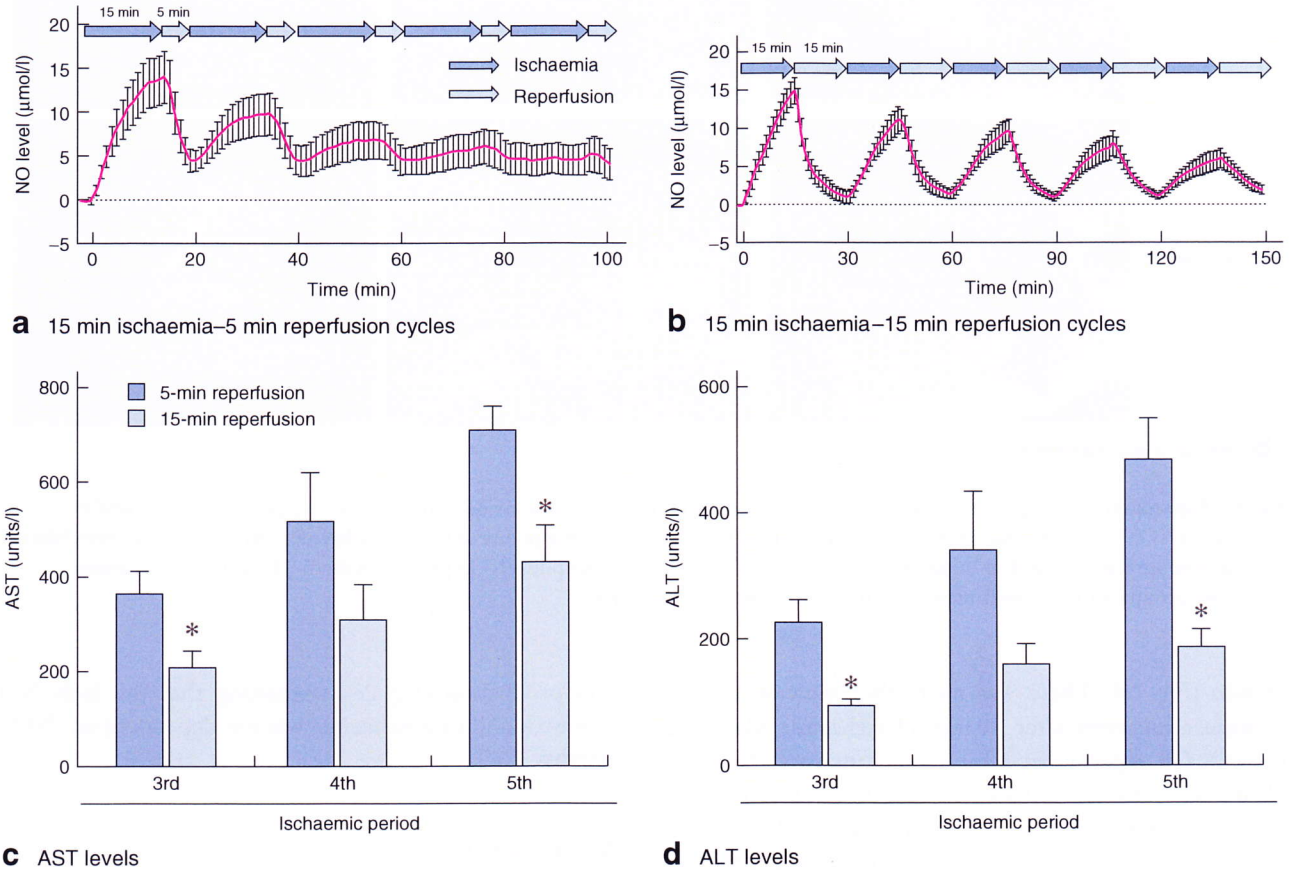

Fig. 5 **a** Changes in intrahepatic nitric oxide (NO) levels during ischaemia–reperfusion in the rat liver by repeating 15 min of ischaemia and a subsequent 5 min of reperfusion or **b** 15 min of ischaemia and a subsequent 15 min of reperfusion. **c**,**d** Serum levels of **c** aspartate aminotransferase (AST) and **d** alanine aminotransferase (ALT) after the third, fourth and fifth periods of ischaemia. Values are mean(s.e.m.). *$P < 0{\cdot}050$ *versus* 5 min reperfusion (Student's *t* test)

after 30 min of ischaemia (*Fig. 4b*). Expression of iNOS by immunohistochemistry was weak during IR (data not shown).

Monitoring of intrahepatic nitric oxide levels during repeated ischaemia–reperfusion

The peak of NO produced during ischaemia decreased in a stepwise manner by repeating 15 min ischaemia −5 min reperfusion cycles (*Fig. 5a*). Almost no increase in the level of NO was detectable during the fifth period of ischaemia. Levels did not return to baseline and remained at around 5 μmol/l even after reperfusion. However, NO production was significantly greater with a longer (15 min) than a shorter (5 min) reperfusion period (*Fig. 5b*). During each reperfusion period, NO levels returned almost to baseline.

Biochemical assay of blood samples and tissue histology

Serum levels of AST and ALT were significantly greater after 5 min reperfusion than after 15 min reperfusion (*Fig. 5c,d*). These differences were even more prominent 6 h after the fifth period of IR, and histology confirmed more sinusoidal congestion and hepatic parenchymal damage in the short reperfusion group, indicating the presence of severe circulatory failure (*Fig. S3*, supporting information).

Conceptual diagram of the nitric oxide curve analysis

The AUC and the peak differential coefficient in each ischaemic period gradually decreased with repeated IR. This decrease was more prominent following 5 min reperfusion than after 15 min reperfusion (*Fig. S4*, supporting information).

Discussion

NO is one of the most potent vasodilators and is highly upregulated in response to hepatic IR stress in the liver[17]. Hepatic generation of NO may attenuate sinusoidal perfusion failure and improve liver tissue oxygenation[18,19]. The upregulation of NO production during ischaemia is presumably a necessary step to cope with the sudden termination of organ blood flow.

The instability of NO in aqueous solutions and its high reactivity with other molecules makes its measurement difficult. Consequently, the kinetics and mechanisms that determine NO levels during hepatic IR are still poorly understood. This study explored the real-time dynamics of intrahepatic NO levels during repeated hepatic IR using a selective NO sensor. Intrahepatic NO levels increased gradually during ischaemia and reached steady state approximately 10 min after hepatoduodenal ligament occlusion. On reperfusion, after a small increase, NO levels quickly decreased to around baseline level. The small increase of NO at the beginning of reperfusion may be due to a sudden supply of oxygen from the gastrointestinal circulation, as production of NO requires the substrate L-arginine and oxygen. A notable observation using the NO sensor was the high amount of NO, in the order of micromolar concentrations, produced in response to ischaemic stress. This indicates that the liver has an enormous potential to produce large quantities of NO quickly in response to ischaemic stress.

eNOS is the NOS isoform responsible for maintaining systemic blood pressure, vascular remodelling and angiogenesis[20]. Under normal physiological conditions, relatively small quantities of NO are produced through the activation of eNOS in sinusoidal endothelial cells in the liver and diffuse a short distance to hepatic stellate cells, causing their relaxation and subsequent sinusoidal dilatation[21]. During ischaemic stress, iNOS is upregulated in the liver and produces large quantities of NO via a calmodulin/calcium-independent mechanism[3]. As iNOS upregulation requires several hours, the immediate change in intrahepatic NO concentration observed after the initiation of ischaemia is unlikely to be explained by iNOS function. In fact, in the *in vivo* study, a specific iNOS inhibitor (L-NIL) did not inhibit the increase in intrahepatic NO levels during ischaemia, and western blotting and immunohistochemistry for iNOS protein expression did not show any increase during ischaemia.

In addition to the conventional NO formation through calmodulin/calcium-dependent eNOS activation, phosphorylation of eNOS produces large amounts of NO following cellular stimulation[22]. Therefore, it was hypothesized that NO production immediately after ischaemic stress was mediated by eNOS phosphorylation, because this process does not require gene expression and protein synthesis. In the present study, phospho-eNOS expression was much higher 1 and 3 min after starting ischaemia than at baseline. However, levels gradually decreased and were almost undetectable after 15 or 30 min of ischaemia. These results are consistent with the results of the *in vitro* eNOS activity assay and immunohistochemistry. This hypothesis was supported by the results of *in vivo* studies using NOS inhibitors. The increase in intrahepatic NO levels during hepatic ischaemia was probably due to the accumulation of NO produced by phosphorylated eNOS. The plateau that occurred approximately 10–15 min after initiation

of ischaemia may be explained by the disappearance of hepatic ability to activate the phosphorylated eNOS pathway. These results also indicate that the cellular capability to produce NO is limited when ischaemic time extends beyond 10 min.

Hepatectomy is a challenging surgical procedure because of the high risk of blood loss. To reduce blood loss during hepatectomy, the hepatoduodenal ligament is frequently clamped (Pringle manoeuvre)[23], either continuously or intermittently. In general, an ischaemia time of 15 min and a reperfusion time of 5 min are employed. However, the tolerance of the liver to ischaemic stress is still unclear and may vary in each patient. Therefore, the optimal time for ischaemia and reperfusion should be determined for individual patients, and a device that predicts liver damage during hepatic IR may help perform safe liver surgery.

The peak of NO production during ischaemia gradually decreased in successive 15 min ischaemia–5 min reperfusion cycles, and almost no NO production was detectable by the fifth period of ischaemia. Nevertheless, NO levels were maintained around 5 μmol/l. NO production was significantly greater with 15 min reperfusion than with 5 min reperfusion. NO is quickly scavenged by haem molecules in the blood. Therefore, the NO dynamics during reperfusion may represent the degree to which accumulated NO is washed out by the recovery of the hepatic macro/microcirculation. It can be speculated that 15 min of reperfusion may be sufficient to wash out accumulated NO almost completely and to restore NO levels to baseline, even after the fifth ischaemic period.

The repeated ischaemic stress with short intervals induces a disturbance of the hepatic microcirculation. This reduces oxygen supply in the liver and may lead to reduced adenosine 5′-triphosphate generation[24] and subsequent phosphorylated eNOS formation, resulting in NO peaks during ischaemia. Incomplete washout of excessively accumulated NO in the liver during reperfusion may result in raised intrahepatic NO levels even during the reperfusion interval. This may finally damage the liver through the process of lipid peroxidation[25]. Therefore, an adequate reperfusion interval may be necessary to allow better recovery of the macro/microcirculation and tissue oxygen levels. The results of this study imply that intrahepatic NO measurements can be used to monitor liver damage during hepatic surgery using a selective NO sensor. By further characterizing the dynamic curves of intrahepatic NO levels using this device, it may become possible to determine an optimal ischaemic time and reperfusion interval that minimize liver damage induced by repeated IR. It should be noted, however, that the present data simply provide a conceptual idea and that this probe has not yet been applied during real-time hepatectomy in large animals or humans.

Disclosure

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Schematic diagram of the selective nitric oxide sensor (Word document)

Fig. S2 *In situ* visualization of nitric oxide by 4,5-diaminofluorescein diacetate staining (Word document)

Fig. S3 A Serum level of aspartate aminotransferase and alanine aminotransferase 6 h after the fifth period of ischaemia. **B** Histology samples harvested after the fifth period of ischaemia (Word document)

Fig. S4 A Conceptual diagram of the nitric oxide (NO) curve analysis. **B** Area under the curve of NO level during each ischaemic period. **C** Peak differential coefficient at each time point (Word document)

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