Protective effect nitric oxide on liver circulation from ischemia reperfusion injury.

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Abbreviated title: Effects of an exogenous nitric oxide donor on liver circulation during ischemia reperfusion.

Abstract

Introduction: The reduction of endogenous nitric oxide (NO) production during hepatic ischemia-reperfusion injury (IRI), generally *via* a reduction in endothelial nitric oxide synthase activity, leads to liver injury. We hypothesized that administration of an exogenous NO donor into the portal vein may ameliorate hepatic blood flow reduction after a period of ischemia.

Material and Methods: A total of 90 min of ischemia (Portal vein and hepatic artery) was applied in 15 anesthetized pigs, using the Pringle method under sevoflurane anesthesia. All animals were administered either saline (control group, n=8) or sodium nitroprusside (SNP, n=7) as exogenous NO donor drugs into the portal vein, 30 min before and after ischemia. The portal venous blood flow and hepatic artery blood flow were measured continuously using transonic flow probes attached to each vessel. Endogenous nitric oxide ($NOx = NO_2^- + NO_3^-$) production was measured every 10 min using a microdialysis probe placed in the left lobe of the liver.

Results:

In the SNP group, portal venous flow remained unchanged and hepatic artery flow significantly increased compared to baseline. Although the production of liver tissue NOx transiently decreased to 60 % after ischemia, its level in the SNP group remained higher than the control saline group.

Conclusion: Regional administration of SNP into the portal vein increases hepatic arterial flow during ischemia reperfusion periods without altering mean systemic

arterial pressure. We speculate that administration of an exogenous NO donor may be

effective in preventing liver injury via preservation of total hepatic blood flow.

Introduction

It is well recognized that the transient reduction of constitutive NO and the excessive production of inducible NO in different phase of hepatic ischemia-reperfusion injury occurs upon restoration of hepatic blood flow after a period of ischemia. One of the earliest events associated with reperfusion of the ischemic liver is endothelial dysfunction, characterized by decreased production of endothelial cell-derived nitric oxide (NO) (1). This rapid post-ischemic decrease in NO bioavailability appears to be due to decreased synthesis of NO, enhanced inactivation of NO by the overproduction of superoxide or both (2, 3). Previous studies have demonstrated that a reduction of endogenous nitric oxide (NO) production during early stages of hepatic IRI, generally *via* a reduction in endothelial nitric oxide synthase activity, leads to liver injury that contributes to the morbidity and mortality after liver surgery (4, 5). Infusion of NO donor drugs or supplementation with NO precursors has been suggested to minimize hepatic IRI by improving microcirculation (6-8). Shimamura et al. (3) suggested that supplementation of a NO donor into the portal vein in dog models might have a protective effect on hepatic circulation and sinusoidal microcirculation during IRI, and manipulation of NO bioactivity in experimental models has revealed involvement of NO in hepatic IRI (9) (10, 11). We hypothesized that administration of an exogenous NO donor into the portal vein ameliorate the decrease in hepatic blood flow after a period of ischemia. Recently, we recently revealed that systemic administration of NO donor drug, sodium nitroprusside are novel treatments that have been used clinically to preserve liver circulation via potentiation of compensatory mechanism against reduction

of portal venous blood flow, i.e., hepatic arterial buffer responses during sevoflurane anesthesia in pigs (12). To test this hypothesis, we evaluated the effects of regional supplementation of sodium nitroprusside (SNP), an exogenous nitric oxide donor, into the portal vein on the hepatic circulation and activity of endogenous nitric oxide in hepatic tissue in the anesthetized pig model.

Materials & Methods

The study protocol was approved by the animal research committee of Nagasaki University and complied with the animal research and animal care guidelines of the Japanese Ministry of Education, Science, Sports and Culture.

Fifteen pigs with a body weight of 25-35 kg (mean, 28.6 ± 3.3 kg) were fasted for 24 h with ad libitum access to water. As described in our prior study (12, 13), anesthesia was induced by intramuscular administration of ketamine (25 mg·kg^{-1}) followed by cannulation of an ear vein and intravenous administration of fentanyl (200 µg). All surgical procedures were performed under sterile conditions. Pigs were placed in the supine position on a heated surface (38-40 °C), and tracheotomy was performed. Anesthesia was maintained with 1.5-2% sevoflurane and 50% oxygen plus air while continuously measuring the expired gas concentration of sevoflurane (Narcotica; Fukuda Denshi, Tokyo, Japan). As muscular relaxation was needed for surgical instrumentation and measurement of pressure-flow relationships without any respiratory fluctuations, neuromuscular blockade was maintained using a non-depolarizing agent (vecuronium bromide) administered whenever there was evidence of spontaneous muscle activity. No additional analgesics were given because an initial dose of fentanyl under anesthesia was enough to obtain adequate level of analgesic effect during procedure. Mechanical ventilatory support was provided using a constant volume time-cycled ventilator. Tidal volume (8-12 ml·kg^{-1}) and ventilatory rate (12-14 cycles·min^{-1}) were adjusted to maintain ETCO$_2$ between 35-45 mmHg.

The diagram of surgical procedure was shown in Fig.1. The right carotid artery

was cannulated with a fluid-filled catheter for measurement of systemic mean arterial pressure (mean $Psys_{art}$) and withdrawal of blood samples. The right subclavian vein was cannulated for fluid infusion and drug administration including antibiotics.

The liver was exposed by a midline abdominal incision. A drainage catheter was inserted into the urinary bladder. The portal vein and common hepatic artery were gently dissected free from surrounding tissue. A fluid-filled catheter was inserted into the portal vein through the side branch to within 1 cm of the hilum of the liver to measure portal venous pressure. Pressure transducers were calibrated simultaneously with the same gain and zeroed to the level of the portal vein. The portal venous pressure was measured with pressure transducers (AP-611G; Nihon Kohden, Tokyo, Japan). Flow and pressure signals were continuously recorded on a Power lab (Bio Research Center, Tokyo, Japan). Appropriately sized ultrasonic flow probes were placed downstream around the common hepatic artery and portal vein. Blood flows (portal venous blood flow and hepatic arterial blood flow) were measured using ultrasonic transit time flow probes (Transonic, Ithaca, NY) attached on the vascular (Qpv and Qha $mmHg \cdot ml^{-1} \cdot min \cdot kg^{-1}$).

A catheter was placed in the anterior hepatic vein via the external jugular vein, and the location of the catheter was confirmed by direct palpation. The catheter was withdrawn 0.5-1.0 cm from the wedge position to allow measurement of hepatic venous pressure. When surgical procedures were completed, the abdominal wall was re-approximated and towels were placed on the surface to minimize heat loss.

Evaluation of Hepatic Artery Buffer Responses

The reciprocal increase of hepatic arterial blood flow against reduction of portal venous blood flow, i.e., Hepatic Artery Buffer Responses (HABR) were elicited by reducing portal venous blood flow from baseline during ischemia reperfusion. The magnitude of HABR was evaluated by comparing changes in hepatic arterial resistance ($\Delta Rha = Pmean\text{-}sys \, / \, Qha$) with changes in portal venous resistance ($\Delta Rpv = Ppv \, / \, Qpv$), using the index of change in blood flow ($\Delta Rha \, / \Delta Rpv$). The magnitude of HABR was evaluated by comparison of the percent change of calculated index ($\Delta Rha \, / \Delta Rpv$) compared to baseline values.

Biochemical assessment of liver cytolysis (Serum Enzyme analysis)

Blood samples were collected at baseline, just after ischemia and 3 h after ischemia reperfusion, to evaluate hepatic damage by measuring AST and ALT levels. Blood was centrifuged and the plasma separated. The AST and ALT determinations were performed at a professional pathology laboratory (SRL, Tokyo, Japan).

Excretory Liver Function

Bile production, as a parameter of excretory liver function, was monitored continuously during the reperfusion period. The common bile duct was cannulated (the cystic duct was occluded) and drained into an unpressurized reservoir for continuous measurement of the bile flow, which is expressed as mL/3-h reperfusion period.

Assessment of endogenous tissue Nitric oxide using microdialysis methods

The diagram of measurement system using microdialysis technique was shown in Fig.2. The endogenous liver tissue NOx (NO_2- + NO_3-) levels in the liver were measured using a microdialysis method. The principle of microdialysis is to mimic the passive function of a capillary blood vessel by the perfusion of a tubular, semipermeable membrane introduced into a tissue. The microdialysis technique that we used in this study was essentially the same as in a previous study (14, 15), but was modified for use in the pig liver. Before ischemia, a microdialysis probe (Eicom OP-175-20, Eicom Co., Kyoto, Japan) was inserted into the left median lobe of the liver. The microdialysis probe consisted of a 20-mm long dialysis fiber (220 μm OD, 200 μm ID, molecular cutoff 50,000) glued between two polyethylene tubes (0.28 mm ID), mechanical support for the fiber being provided by a platinum wire fastened at the ends of the polyethylene tubes. A perfusion fluid was pumped through the outer tube and flowed beneath the membrane, where exchange between the interstitial fluid and the perfusion fluid took place. At the tip, the fluid entered a small hole in the inner tube and flowed backwards to finally be collected into an auto-injector. The probe was perfused with Ringer's solution (Na^+ 147.0 mM, K^+ 4.0 mM, Ca^{2+} 2.3 mM, Cl^- 155.6 mM) at a constant flow rate of 2 μl/min using a micro-syringe pump. The standard solution of $NaNO_2$ 10^{-6} mol litre^{-1} and $NaNO_3$ 10^{-6} mol litre^{-1} was perfused into probe a t a flow rate of 2 μl/min to calculate basal NOx concentration. After an equilibration period of 90 min, the perfused microdialysates were collected every 10 min in the sample loop of an automated sample injector connected to an automated NO detector system. NOx (NO_2- + NO_3-) levels

were measured using an automated NO detector high-performance liquid chromatography system (ENO-10, Eicom Co., Kyoto, Japan) based on the Griess reaction (16).

Experimental protocol

The experimental protocol was explained in Fig.3. The pigs were randomly divided into three groups, sham operated group (n=3), saline control group (n=8) and pharmacological group preconditioned by SNP (n=9). In the sham-operated group, the liver was exposed for 5 h, (the animals were subjected to anesthesia and laparotomy without hepatic ischemia, the liver remaining exposed throughout the 5-h period). In the non-preconditioned group (ischemic-reperfusion: IR group), animals were subjected to 90 min of ischemia followed by a 180 min period of reperfusion. In the pharmacologically preconditioned group (sodium nitroprusside (SNP) + IR), 30 min before 90 min of ischemia and 180 min of reperfusion, the liver was subjected to a preconditioning protocol, i.e. intra-portal infusion of SNP (5 μg / kg/min). All animals were assessed 3 hr after reperfusion periods and histopathological samples were obtained at completion after 3hr of assessment. After the experiment, the animals were deeply anesthetized in order provide euthanasia due to ethical criteria with supplemental doses of intravenous injection of thiopental (20 mg/kg) under maintenance anesthesia (4% sevoflurane and 50% oxygen) and sacrificed by intravenous injection of KCL (40 meq/20 cc).

Histopathology

The formalin-fixed liver specimens were embedded in paraffin, 3-μm-thick sections were made and stained with hematoxylin and eosin. Histopathological analysis was carried out by pathologists. Histopathological damage was evaluated on the basis of the lesion of edema surrounding sinusoidal congestion in the hepatic lobule. Under the microscope, we evaluated a percentage of the area of the lesion of edema surrounding sinusoidal congestion to the total area. The area of the lesion was estimated using the area circumscribed with the connecting line of central veins of the affected hepatic lobules.

Statistical analysis

All statistical analyses were performed using Prism 5 (GraphPad Software, Inc.) to test the two-tailed hypothesis. We used one-way ANOVA to examine the effects of SNP and saline on hemodynamic parameters, portal venous flow, hepatic arterial flow, index of HABR, i.e., $\Delta Rha /\Delta Rpv$ and tissue NOx levels at each examined points compared to baseline value. When significant differences were detected, a post-hoc protected Dunnett test was used to isolate the differences. We also used two-way ANOVA to examine the difference between saline control group and SNP preconditioning group. Statistical significance was assumed to be $P < 0.05$. The data are presented as mean ± standard deviation (SD), unless otherwise noted.

Result

There was no significant difference of mean systemic arterial pressure (mean $Psys_{art}$) and heart rate (HR) of control group and SNP preconditioning group during baseline and ischemia reperfusion. Fig.4-A, 4-B, 4-C shows the change of regional hemodynamics of the hepatic artery and portal vein, i.e., hepatic arterial blood flow (Qha), portal venous blood flow (Qpv) and total liver blood flow (Qtotal) of control group and SNP preconditioning group during baseline and ischemia reperfusion. There was significant difference of Qha at administration of SNP in SNP preconditioning group and at post ischemia phase in control group (Fig.4-A). There was significant difference of Qpv at post ischemia, 1hr reperfusion and 2 hr reperfusion phase in control group. But there was no significant difference of Qpv in SNP preconditioning group (Fig4-B). There was no significant difference of Qtotal at any phase in both groups. Fig.5 shows magnitude of HABR throughout baseline and reperfusion phase. There was significant difference of HABR at 1hr reperfusion and 2hr reperfusion phase compared to baseline value. Fig.6 shows the change of tissue NOx level throughout ischemia reperfusion phase. There was significant difference of NOx during ischemia compared to basal level and there was significant difference between two groups. Fig.7 represents the influence of exogenous NO on the hepatic tissue. Histologic analysis showed different pathological findings in two groups. Hematoxylin and eosin stains reveals an ischemic change, such as edematous changes at the hepatic lobule with sinusoidal congestion in NS group (A and B). It is mild in SNP group (C and D). Although the edema area with sinusoidal congestion was seen in a wide area in liver tissue at the NS group ($70.8 \pm 17\%$), at the SNP group it was seen in a small area in liver tissue ($42.9 \pm 21\%$). Significant difference was seen in between the two groups ($p<0.01$) (Fig. 8).

Discussion

The major finding of this study is that pharmacological preconditioning using a nitric oxide donor such as SNP may preserve both portal venous blood flow and hepatic arterial flow during the early stages of ischemia reperfusion after 90 min of total hepatic ischemia by the Pringle method. This study also indicates that the beneficial effect of SNP infusion is due to reduction of hepatic arterial and portal venous resistance associated with preservation of hepatic tissue NOx levels, and that a possible site of action of the NO donor might exist in the pre-sinusoidal region of the liver. Furthermore, we speculate that hepatic arterial buffer responses may play an important role in the maintenance of total hepatic blood flow in case of a reduction in portal venous blood flow in the early stages of reperfusion in non-pharmacologically preconditioned animals.

In this study, SNP, the NO donor, well preserved portal venous blood flow and hence, total liver blood flow, without any involvement of HABR. Our findings suggest that pharmacological preconditioning using a NO donor (SNP) can protect hepatic circulation during the early stages of ischemia reperfusion by preserving total liver blood flow.

Endogenous tissue nitric oxide levels

This is the first study to evaluate tissue NOx levels in sevoflurane anesthetized pig liver. We found NOx level might be approximately 0.1 ± 0.03 μmol litre^{-1} of the liver in sevoflurane anesthetized pig liver. Previously, Isobe et al. (15) reported changes in

tissue NOx levels, expressed as a percentage of the pre-ischemic level, in a control group of pigs anesthetized with a mixture of oxygen, nitrous oxide and isoflurane without indicating the basal NOx levels in the pig liver. In this study, we used standard sample injections of known NOx in the control phase. Hence, we could estimate tissue NOx levels in the sevoflurane anesthetized pig liver. Previously the basal NOx concentration has been reported in the rat hippocampus of 0.15 μmol litre^{-1} similar value found in the liver tissue of pig in this study. However, we are unsure about the applicability to the human liver of this value of tissue NOx levels in the resting condition during sevoflurane anesthesia.

The role of Hepatic Arterial Buffer Response

It is well known that if portal blood flow decreases, the hepatic artery dilates, while the hepatic artery constricts if portal flow increases (17, 18). In normal pig liver, activation of HABR may compensate for reduction of portal venous blood flow, resulting in maintenance of total liver blood flow (12, 13). In this study, we found significant increases in magnitude of HABR evaluated by index (ΔRha /ΔRpv) at 1hr reperfusion and 2hr reperfusion phase in control group. This result indicate that the hepatic arterial blood flow in the face of reduction in portal venous blood flow during the early phase of reperfusion increased by activation of HABR function in control group. However, in the SNP preconditioning group, HABR activation was not found at any phase of reperfusion, indicating that increase of hepatic arterial blood flow was not required because of well preserved portal venous blood flow. Although we do not know why the

exogenous NO donor SNP modulate HABR function, several possible mechanisms, including the influence of NO on adenosine release (19-22) or possible interactions between NO and hydrogen sulfide (H_2S) (23-27) may explain the contribution of NO on regulation of HABR. We predict that if the function of HABR is depressed by cirrhosis of the liver or other diseases, total liver blood flow may be significantly depressed during the early stages of ischemia reperfusion.

The influence of exogenous NO on circulation of liver

Mechanisms by which NO provided hepatoprotective effects against ischemia and reperfusion injury were not fully determined in this study, however several possible explanation could be indicated. First, it could be due to improvement of microcirculation by the vasodilatory effect of NO. NO has been proved to regulate hepatic sinusoidal circulation through morpho-functional influences on Ito cells, rather than through a direct effect on portal venous and hepatic arterial vascular smooth muscle cells. Histopathological examination demonstrated the value of SNP preconditioning on the microcirculation of the liver during the early phase of reperfusion. We found significant edema surrounding sinusoidal congestion with partial trabecular derangement and centrilobular hepatocyte necrosis at 3 h after reperfusion in the control group. In contrast, there was less edema formation of tissue with the trabecular arrangement remained normal and severe hepatic damage was not found in the SNP preconditioning group at 3 h after reperfusion. These results suggested that pharmacological preconditioning using an exogenous nitric oxide donor (SNP)

ameliorated edema formation and hepatic tissue damage via protection and preservation of total hepatic blood flow during early phase of ischemia reperfusion. This protective role of exogenous nitric oxide might be consistent with the pathophysiology in ischemia reperfusion in rat kidney (2). These findings were consistent with previous studies that revealed protective role of NO on the liver microcirculation.

Second, ameliorating effects of NO on liver ischemia and reperfusion relates to function controlling scavenging properties of superoxide. Superoxide radicals are implicated as an initial and potential mediator of ischemia and reperfusion injury. Although it is also known that NO can combine with superoxide radicals to form the more highly toxic free radicals species, peroxynitrite, peroxinitrite is unlikely to be involved in this experimental model, because a very high level of peroxinitrite (i.e., at least 500 maicro mol/L) is required to produce cytotoxic effects.

Thirdly, another explanation for improvement of liver circulation might be involvement of energy metabolism during ischemia and reperfusion. NO modulates a key enzyme of the Krebs cycle (mitochondrial aconitase) and several mitochondrial electron transport cascades. NO might act as an energy-sparing agent during ischemia and reperfusion phase. However, in order to deliver more oxygen and nutrients to the liver and to improve the restoration of energy metabolism, an increase in hepatic arterial blood flow elicited by HABR function may play an important role.

Possible site of action of exogenous NO

Interestingly, we found that supplementation of a NO donor provided significant influences on both portal venous and hepatic arterial blood flow. Furthermore, our data indicates that liver tissue NOx remain unchanged due to supplementation of SNP into the portal vein. The fact that systemic infusion of SNP equally altered both portal venous blood flow and hepatic arterial blood flow indicates that the possible site of action of SNP is pre-sinusoidal (12). Therefore, we speculate that the possible site of action of exogenous SNP infused into the portal vein may be in the pre-sinusoidal region of the liver rather than in the post-sinusoidal region.

Morphologically, HA and PV branches run parallel to each other within the liver, and supply blood to the hepatic sinusoids via terminal hepatic arterioles and terminal portal venules. Direct hepatic arteriolo-portal venous connections and hepatic arteriolo-sinusoidal connections have been suggested to exist in the pig liver (28-32). The site of the hepatic arterial blood flow-mediating mechanism was thus concluded to exist upstream from the microvasculature of each acinus, but downstream from branch points supplying the different acini. The existence of such anatomically regulated vascular pathways may account for our experimental observations.

Clinical implications of SNP infusion into the portal vein

Several studies regarding the role of NO in hepatic ischemia reperfusion injury, focusing on supplementation of constitutive NOS (cNOS) and inhibition of inducible NOS (iNOS), have been published. Pannen et al. (33) reported that a relatively selective

cNOS inhibitor aggravated IR injury in isolated perfused rat liver. Ferraresso et al. (5) suggested that a physiological NO precursor attenuated IR injury in the pig liver. These results suggest that endogenous NO produced by eNOS has hepatoprotective effects on hepatic IRI. Supplementation of a NO donor into the portal vein has drawn much attention as an alternative treatment for preventing IRI, because of reduction in NO production in early stages of ischemia reperfusion. The hepatoprotective effects of NO donors in the early stages of ischemia-reperfusion makes them of potential use in pathophysiological conditions such as IRI (12). Most recently, Walsh et al. (34) and Phillips et al. (35) suggested that nitric oxide may protect liver function against IRI. There are a number of studies that revealed the protective effects of NO donors on liver function during ischemia reperfusion injury (1, 3, 36-40). Shimamura et al. (3) demonstrated the marked hepato-protective effects of the NO donor L-arginine against severe ischemia and reperfusion injury in canine livers. They strongly indicated that supplementation with L-arginine appears to be a possible strategy in clinical settings because of the lack of adverse hypotensive effects on systemic circulation with its use. Consistent with previous findings, we found that preservation of total liver blood flow by intra portal infusion of SNP may lead to the maintenance of splanchnic circulation during sevoflurane anesthesia, leading to stable hemodynamic management. We speculate that maintenance of liver blood flow may cause less induction of stress-induced variables, such as iNOS and endothelin in the sinusoidal region.

Possible Limitations of the Current Study (validation of this study)

There are several limitations to this study. First, we used an intact liver model without any vascular shunt to prevent systemic hypotension and intestinal congestion. Most previous studies suggest that hepatic IRI includes portal congestion during ischemia (7, 41). Intestinal congestion accelerates transmigration of endotoxins from the intestinal tract into the portal blood (42), which in turn induces inducible nitric oxide synthase (iNOS) expression in various types of cells. Hence, our experimental model may be partly affected by endotoxin translocation. However, we believe that the influence of this intestinal congestion would have occurred equally in both the saline control group (ischemia only) and SNP preconditioned group.

Second, we observed only the early stage (first 3 h) of ischemia reperfusion. We cannot deny that liver circulation may be altered in the late stages of ischemia reperfusion, in association with increased levels of iNOS induced by ischemic stress. It has been suggested that iNOS is activated after 3 h of ischemia or other pathophysiological stress. Isobe et al. (15) suggested that at 3 h after reperfusion, (1) NO is produced by iNOS after reperfusion of Kupffer cells or neutrophils; (2) NO reacts with superoxide and forms peroxinitrite in the centrilobular region; (3) peroxinitrite causes severe hepatic damage. However, since we did not evaluate tissue levels of iNOS, we cannot estimate the influence of iNOS on liver circulation during the late phase of reperfusion injury.

In conclusion, we have indicated that regional administration of SNP into the portal vein increases hepatic arterial flow during ischemia reperfusion periods without altering

mean systemic arterial pressure. We speculate that administration of an exogenous NO donor may be effective in preventing liver injury via preservation of total hepatic blood flow.

Conflict of interest

The authors report no conflicts of interest.

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Figure legend

Fig.1

The diagram of experimental procedure.

Fig.2

The diagram of measurement system using microdialysis technique of tissue nitric oxide.

Fig.3

The protocol of experiment.

Fig.4-A, 4-B, 4-C

The change of regional hemodynamics.   Portal venous blood flow (Qpv) and hepatic arterial blood flow (Qha) and total liver blood flow (Qtotal).

Fig.5

The change of HABR assessed by the index of (ΔRha /ΔRpv).   Note that the index of ΔRha /ΔRpv was calculated by (ΔRha = Pmean-sys / Qha) devided by (ΔRpv = Ppv / Qpv)with changes in portal venous resistance (ΔRpv = Ppv / Qpv), using the index of change in blood flow (ΔRha /ΔRpv).   The magnitude of HABR was evaluated by comparison of the percent change of calculated index (ΔRha /ΔRpv) compared to baseline values.

Fig.6

The change of tissue NOx level troughout experiment.

Fig.7

Histological examination of liver tissue in both groups.

Comparisons of histopathology for pig liver between NS (A and B) and SNP (C and D) groups. A and C indicate the periphery of the hepatic lobule (Original magnification ×), B and D indicate the periphery of Glisson's capsule (Original magnification ×).

Fig.8

Box plots graph represent a percentage of the area of the lesion of edema surrounding sinusoidal congestion to the total area. ($p<0.01$).
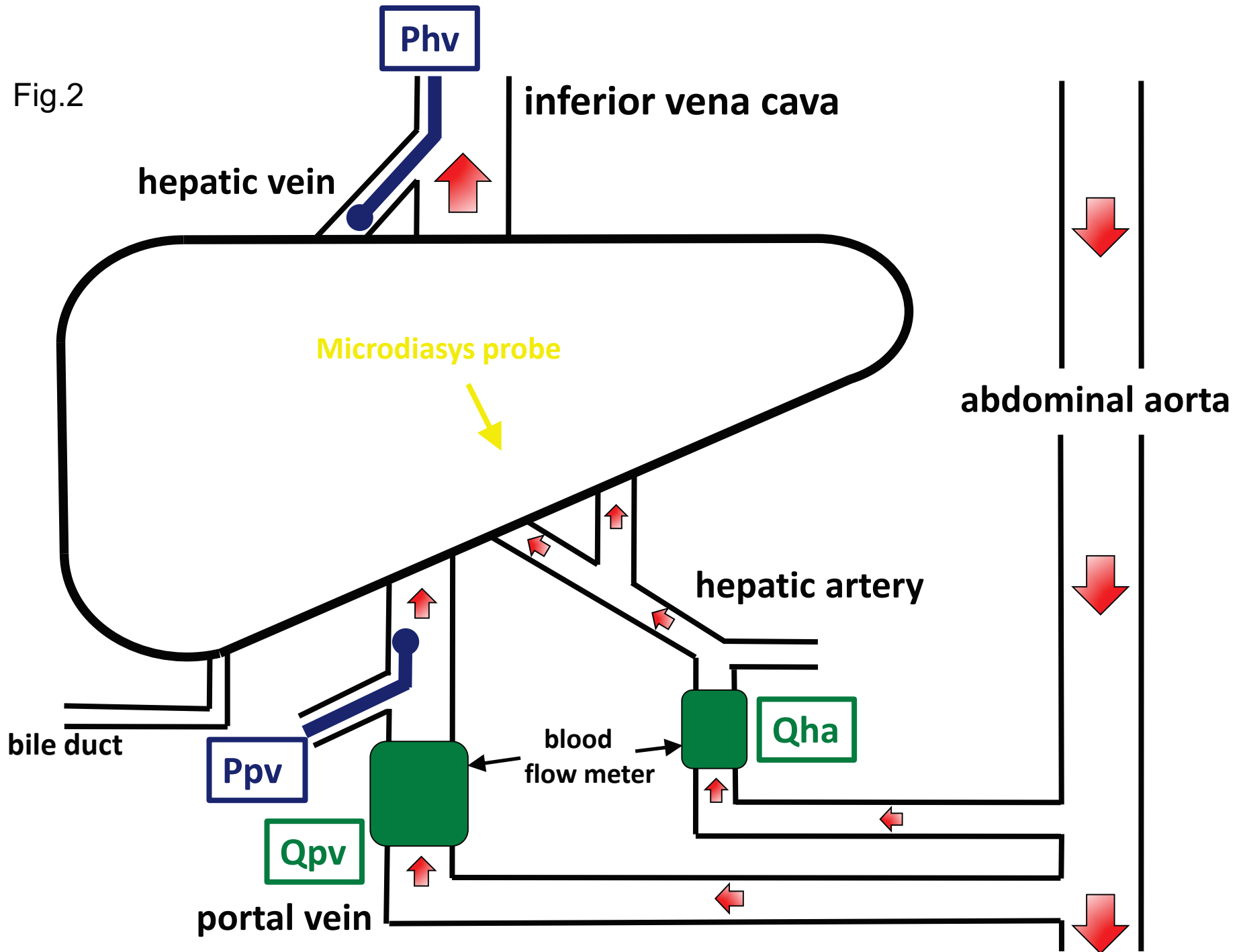
Fig.1

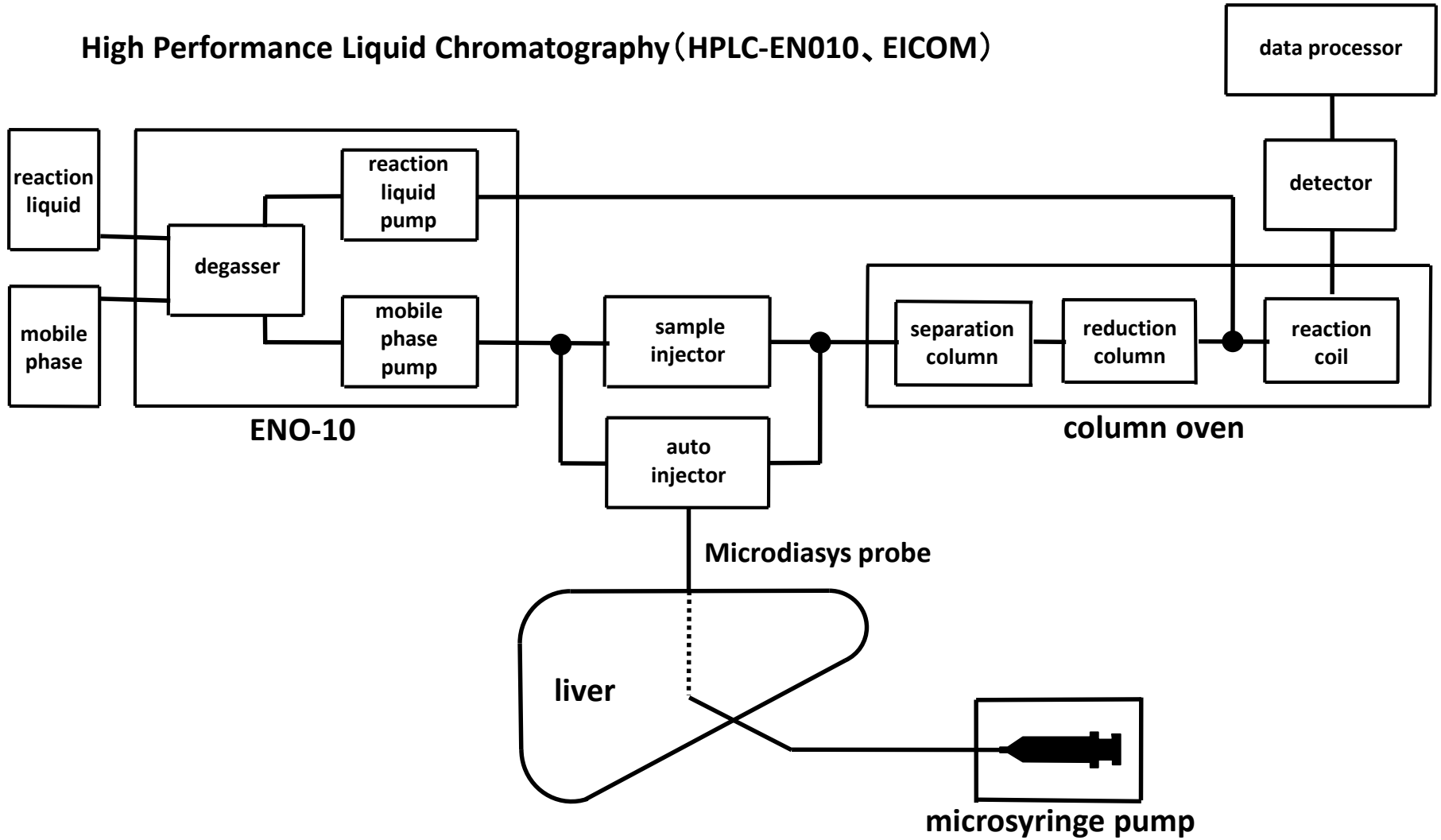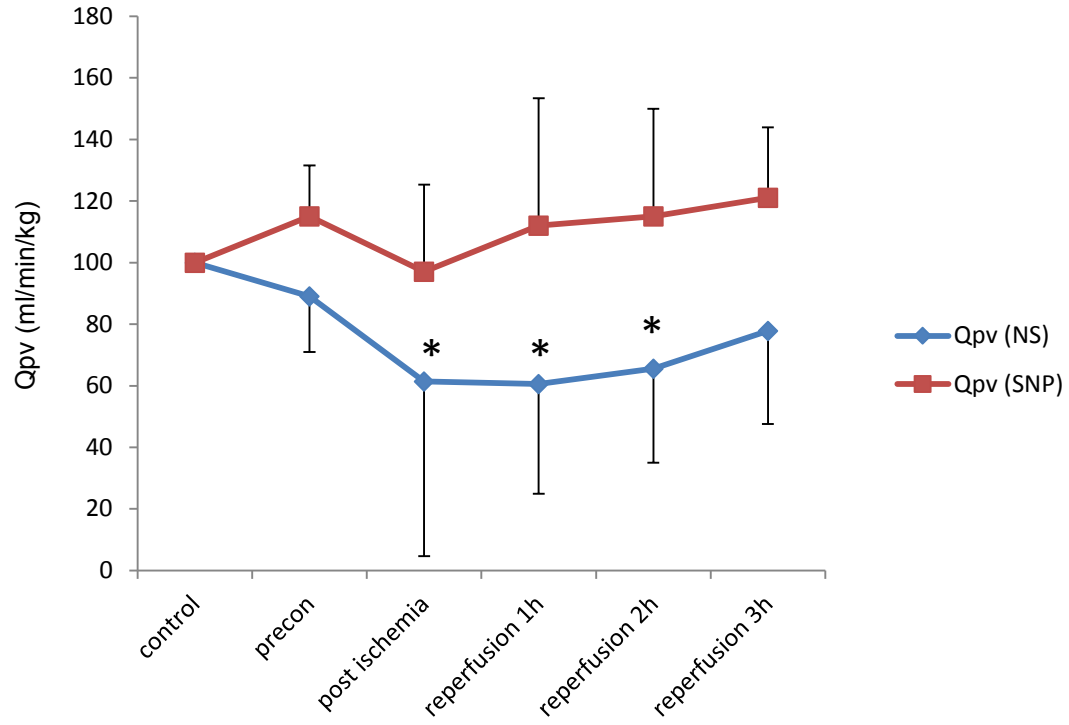experimental protocol
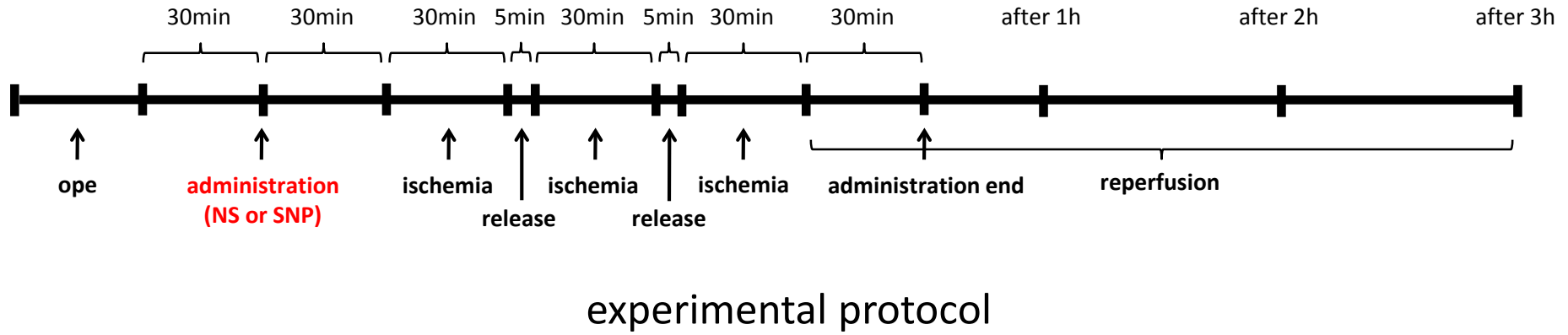
Fig.2

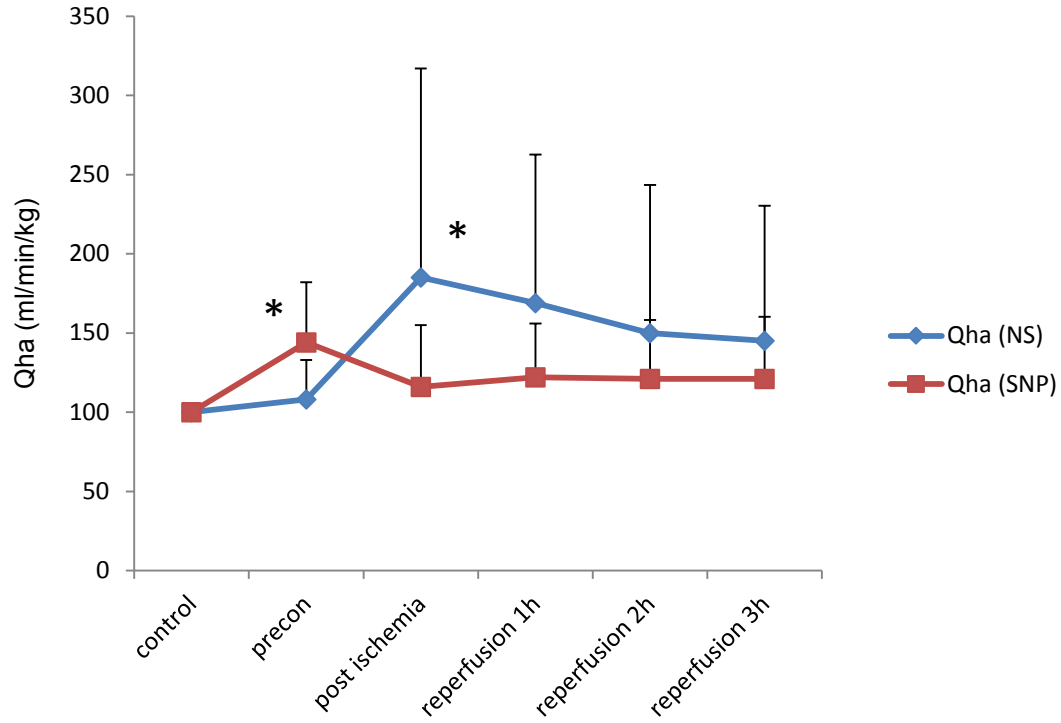Fig.3

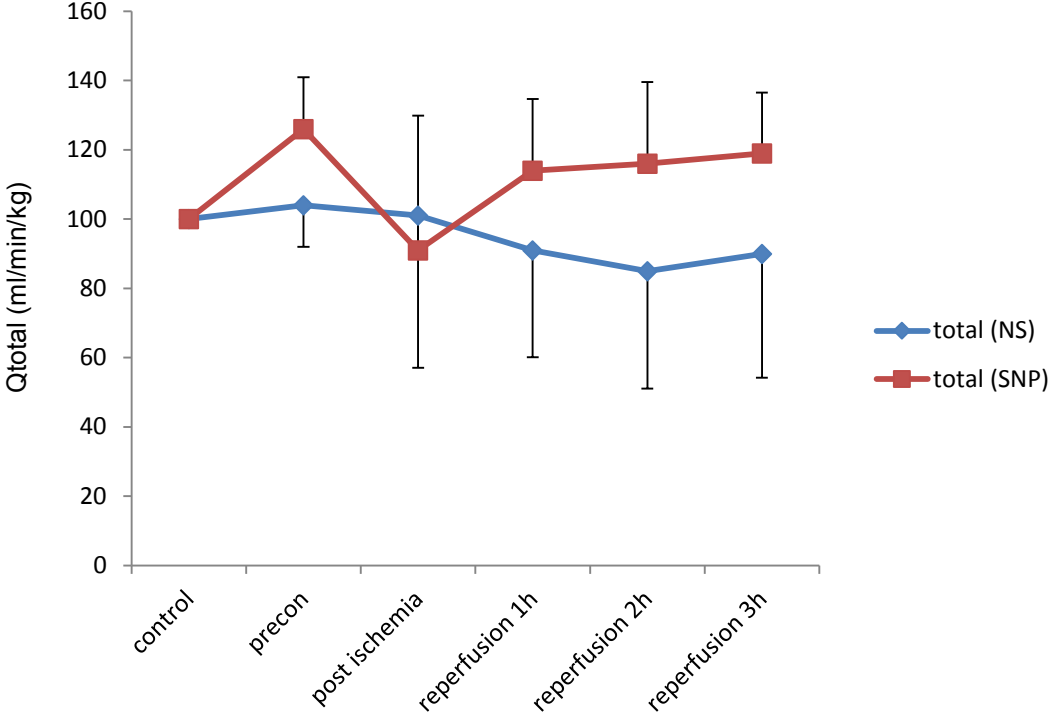**High Performance Liquid Chromatography（HPLC-EN010、EICOM）**

Fig.4-A

Fig.4-B

Fig.4-C

Fig.5

Fig.6

Fig.7

Fig.8