

Nitric Oxide Synthase Distribution and Expression with Ischemic Preconditioning of the Rat Liver

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Abstract

This study was undertaken to identify nitric oxide synthase (NOS) isoforms responsible for the generation of cytoprotective NO during liver ischemic preconditioning IPC. Sprague Dawley rats were subjected to 45 mins lobar ischemia followed by 2 hr reperfusion (IR). L-arginine or L-NAME was administered to stimulate or block NO synthesis. Study groups (n=6) had, (1) sham laparotomy, (2) IR, (3) IPC with 5 min ischemia and 10 min reperfusion before IR, (4) L-arginine before IR, (5) L-NAME + IPC before IR. Liver function tests, nitrite + nitrate (NO_x) and plasma amino acids were analysed. The endothelial cell and inducible isoforms of NOS (eNOS and iNOS) were identified using immunohistochemistry and Western blotting. Both IPC and L-arginine treatment increased NO_x (p<0.05) and, improved serum liver enzymes (p<0.05) when compared with IR. These effects were prevented by L-NAME. Hepatic vein NO_x was significantly higher than circulating NO_x. iNOS expression was absent within the groups. The preconditioned livers were associated with upregulation of eNOS expression and also increased L-arginine levels. The effects of L-arginine administration were similar to those evident following IPC. Thus, cytoprotective NO generation during IPC of the liver was a result of increased eNOS expression and increased L-arginine substrate availability.

Introduction

Liver injury caused by ischemia and reperfusion occurs in various surgical interventions including hepatectomy and liver transplantation (1). Ischemia reperfusion injury is associated with an acute inflammatory response and microvascular dysfunction, which finally lead to irreversible cell injury (2). Ischemic preconditioning (IPC) is an endogenous adaptive phenomenon wherein exposure of tissues to brief periods of ischemia may protect them from subsequent, prolonged ischemia. IPC was first described in the heart, and recent studies have demonstrated the existence of IPC in the liver in animal models as well as in humans (3). Peralta et al have suggested NO is a central mediator of IPC both in normal livers (4) and steatotic livers (5). Our group has recently demonstrated the association of NO with improved hepatic oxygenation (6) and microcirculation (7) after IPC. In addition NO donor had similar effect to IPC in increasing post transplant survival in a rat model of cold preservation and reperfusion injury (8). In contrast some studies have suggested that NO inhibition protects organs against ischemia reperfusion injury (9).

Overall, there is increasing evidence for the role of NO in the IPC effect and it is attractive to hypothesize this experimental evidence may eventually lead to pharmacological strategies using NO for protecting the liver from ischemic injury. It has been proposed that the hepatoprotective NO during IPC results from increased extracellular adenosine and activation of adenosine receptors (4). Another study has proposed that adenosine attenuates hepatic injury by prevention of downregulation of endothelial NOS (10). However the mechanism by which adenosine modulates NOS activity is not explained. In NO in the liver is produced from L-arginine by various isoforms of the enzyme nitric oxide synthase (NOS). It is likely that the protective NO generation with IPC occurs through the modulation of the L-arginine/ NOS pathway. However, so far isoform specific studies in relation to NO production with IPC are lacking. In a previous experimental study when NOS activity was evaluated on chromatography, IPC did not produce significant differences in endothelial or inducible isoforms of NOS when compared to ischemia reperfusion group (11). Another group has proposed that since the pretreatment with an inducible NOS inhibitor in rats undergoing ischemia reperfusion did not

aggravate liver damage, the hepatoprotective NO observed in the study was likely a result of the endothelial isoform activity (12). Clearly, defining the isoform specific pathway for NO production with IPC will allow a better understanding for the development of drug targeting to induce the preconditioning response. The present study has attempted to identify NOS isoforms responsible for the generation of cytoprotective NO during liver IPC in a rat model of lobar ischemia reperfusion injury. The distribution and expression of the endothelial and inducible isoforms of NOS and, the L-arginine substrate availability, have been measured in the present study.

Materials and Methods

Animal preparation and surgical procedure. The study was conducted under a project license granted by the home office in accordance with the Animals (Scientific Procedures) Act 1986. Male Sprague- Dawley rats, each weighing 250- 300g were used for the experiments. All animals were kept in temperature controlled environment with 12 hours light- dark cycle and allowed tap water and standard rat chow pellets ad libitum.

Animals were anaesthetised using Urethane 1mg/kg body weight intraperitoneally and prepared for aseptic surgery. Animals were allowed to breathe spontaneously via concentric mask connected to an oxygen regulator during the procedure. The animal's body temperature was maintained at 37-39 °C using a heating pad (Harvard apparatus Ltd., Kent, UK) and monitored with a rectal temperature probe. The arterial oxygen saturation (SaO₂) and heart rate were continuously monitored with pulse oxymeter (Ohmeda Biox 3740 pulse oximeter, Ohmeda, Louisville, Colorado, USA). Polyethylene catheters (PE-50, 0.38 mm inner diameter, Portex, Kent, UK) were inserted into the right femoral artery and connected to a pressure transducer for monitoring of mean arterial blood pressure (MABP) and for collecting blood samples. Normal saline was administered intraperitoneally to compensate for intraoperative blood loss.

Laparotomy was carried out through a midline incision. The ligamentous attachments from the liver to the diaphragm were severed and the liver was exposed. Ischemia of the median and left lateral

lobes of the liver was produced by clamping the corresponding vascular pedicle containing the portal vein and hepatic artery branches using an atraumatic microvascular clamp. The other hepatic lobes were not handled during the procedure. This method produces ischemia to the left and median lobes of the liver (about 70% of the liver) while leaving the blood supply to the right and caudate lobes uninterrupted (13). At the end of the ischemia period the vascular clamp was removed and reperfusion was allowed. The animal's abdomen was covered with a plastic wrap to prevent fluid evaporation. At the end of the experiment hepatic venous blood, and arterial blood, and hepatic tissue samples were collected, and the animals killed by exsanguination.

Data from the pulse oximeter and pressure transducer were collected continuously on a laptop computer. The data were calculated as one minute averages at baseline, at the end of ischemia and at the end of reperfusion (post-ischemia).

At termination, samples of ischemic lobes of liver were collected on dry ice and immediately stored at -80 C. for immunohistochemistry and western blotting.

Experimental protocols. The rats were randomly allocated to 5 study groups:

Group 1. Control (sham) group (n=6): The liver was exposed for 3 hrs. There was no liver ischemia.

Group 2. Ischemia-reperfusion (IR) (n=6): Ischemia was induced in the median and left lateral hepatic lobes for 45 minutes, followed by a 2 hour period of reperfusion at room temperature.

Group 3. Ischemic preconditioning (IPC) + IR (n=6): The median and left lateral lobes were preconditioned with 5 minutes ischemia followed by 10 minutes of reperfusion. This was followed by IR (group 2 procedure).

Group 4. L-arginine + IR (n=6): Animals were treated with L-arginine (300 mg/kg body weight), intravenously 10 minutes prior to ischemia and intraperitoneal bolus at reperfusion.

Group 5. L-NAME + IPC + IR: Animals were treated with N ω -Nitro-L-arginine methyl ester (L-NAME) (30 mg/kg body weight, intravenously) 10 minutes prior to group 3 procedure.

Measurement of hepatocellular injury. Plasma alanine aminotransferase (ALT) was measured on a Hitachi 747 auto-analyzer using commercially available enzymatic kit tests. The tests were determined using reagents supplied by Boehringer Mannheim Ltd UK.

Measurement of nitric oxide production. Plasma nitrite + nitrate (NO_x) from arterial blood and hepatic venous blood samples were measured using a 280 Nitric Oxide Analyser (Sievers Instruments) by chemiluminescence method (14).

NOS immunohistochemistry. Commercially available polyclonal antibodies against the specific NOS isoforms (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to identify the isoforms on tissues sections.

Cryostat-cut sections thaw-mounted onto polylysine-coated slides were used. Sections were allowed to equilibrate at room temperature for 30 min and fixed in acetone for 20 min at -20°C and rinsed in 0.01M PBS. Endogenous enzyme activity was inhibited by pre-incubating sections in 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were incubated in 5% normal goat serum in PBS at room temperature for 20 min to block background staining. Tissue was then incubated in the appropriate primary antibody (rabbit anti-e NOS, anti-I NOS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature. After incubation sections were rinsed with PBS and incubated with biotinylated secondary goat antibody for 30 minutes. Following further rinses, sections were incubated with streptavidin/biotinylated horseradish peroxidase solution for 30 min (StreptABC Complex/HRP duet, Mouse/Rabbit kit, DAKO). DAB was used as the chromogenic substrate solution and sections were counterstained with Mayer's haematoxylin and prepared for microscopic examination. For negative controls, sections were processed without primary antibody.

Preparation of protein extracts. Samples of liver tissue (100- 200 mg) were homogenised in ice-cold lysis buffer (20 mM HEPES [pH 7.2], 1 mM EDTA, 0.2 M Sucrose, 20 µg/ml Soybean trypsin inhibitor, 20 µg/ml leupeptin, 5 µg/ml peptastin, 5 mg/ml DTT, 5 µg/ml E-64, 5 µg/ml bestatin, 5 µg/ml aprotinin, 5 µg/ml antipain, 0.1 mM PMSF). After centrifugation at 10,000g at 4°C for 30 min, the supernatants were stored at -80°C. Protein determination of samples was performed using the Bicinchoninic acid protein assay (15)

SDS gel electrophoresis and Western blotting. Solubilized proteins were subjected to Nu-polyacrylamide gel electrophoresis (NuPAGE) system (Invitrogen life technologies Ltd, Paisley, UK) on precast NuPAGE 4- 12% gradient gels using NuPAGE MOPS running buffer and

electrophoretically transferred onto nitrocellulose membranes using NuPAGE western blot buffer. After blotting the membranes the transfer of proteins was checked using Ponceau S stain. The membranes were then blocked using PBS containing 0.5% bovine serum albumin, 1% polyvinylpyrrolidone 10, 1% polyethylene glycol, 0.2% Tween 20 and 10 mM NaF. Next, the membranes were incubated with appropriate primary antibody (polyclonal rabbit anti e-NOS, anti i-NOS, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Next the membranes were washed for 3 x 5 min and 1 x 15 min with PBS Tween 0.05%. After this the membranes were incubated for 1 hr at room temperature with anti rabbit IgG horseradish peroxidase secondary antibody (1:200 dilution). Next the washing steps were repeated. To detect any proteins which have bound the antibody, the membranes were incubated with West Dura reagents (Perbio, Cheshire, UK) for 5 min according to manufacturers instructions. After this the membrane was exposed to a digital camera as part of an electronic imaging system to visualise the proteins bound to the antibody.

Measurement of plasma amino acids. Plasma amino acids were analyzed by an automated amino acid analyzer (Beckman Instruments, Brea, CA)

Statistical analysis. The experimental results are expressed as mean \pm SD of 6 animals in each group. Data was analyzed using analysis of variance (ANOVA) for multiple comparisons. Analysis between two groups was performed using unpaired Student's t test (two-tailed) where ANOVA indicated significance for the multiple comparison. Statistical significance was accepted when $p < 0.05$. Blots were quantified by densitometric analysis of each protein band compared to a background reading from each membrane using an image analysis system and the data is expressed in relative optical density (OD) units.

Results

The baseline MABP was 85.0 ± 3.5 mmHg in the animals. It increased by 25.2 mmHg after L-NAME administration. The heart rate however did not change significantly after L-NAME injection. In the L-arginine treated group, a transient fall in blood pressure was observed immediately after L-arginine

administration, but this was statistically not significant. In the other groups blood pressure did not change significantly throughout the experiment. In all animals in the experimental groups the heart rate and arterial oxygen saturation did not change significantly throughout the experiment ($p > 0.05$).

The changes in ALT and NO_x are shown in fig. 1. Both IPC and L-arginine treatment produced similar decrease in plasma ALT and increase in NO_x. L-NAME treatment with IPC aggravated the increase in ALT and decreased NO_x. To determine whether the NO generation was intrahepatic, NO_x levels were measured in the hepatic venous blood and in the femoral arterial blood at the same time points in the animals. Hepatic vein NO_x was significantly higher than arterial (circulating) NO_x in IPC and L-arginine treated groups (hepatic vein NO_x 40.3 ± 2.7 (group 3), 31.1 ± 2.3 (group 4) vs. circulating NO_x 28.6 ± 2.1 (group 3), 21.4 ± 2.2 (group 4), both $p < 0.05$).

With eNOS and iNOS immunohistochemistry, the negative controls had no positive staining. Both immunohistochemistry (fig.2.) and western blotting (fig.3.) showed increased eNOS expression with IPC and L-arginine treatment. The immunostaining was localized to hepatocytes and vascular endothelium. There was no eNOS expression in IR and L-NAME + IPC groups by either immunohistochemistry or western blotting (fig.2 and 3). There was no iNOS expression within the experimental groups (fig.3.).

With IR there was a significant decrease in arginine and a significant increase in ornithine levels. When compared with IR group IPC resulted in increased plasma arginine levels. L-arginine administration increased its plasma levels but also resulted in increased ornithine levels. Plasma arginine levels with IPC and L-arginine treatment were not different from sham group. L-NAME decreased arginine and increased ornithine levels (fig.4.).

Discussion

This study has addressed the relationship of endothelial isoform (eNOS) and inducible isoform (iNOS) of nitric oxide synthase with the ischemic preconditioning effect on early (< 2 hrs) ischemia reperfusion injury of the liver. The major finding of the study is that cytoprotective nitric oxide is produced by eNOS during ischemic preconditioning of the liver. The study has shown that eNOS

expression was upregulated and iNOS induction was absent in the ischemically preconditioned livers during early ischemia reperfusion injury. The results also demonstrate that IPC was associated with an increased availability of the L-arginine substrate.

The role of the endogenous L-arginine/NOS pathway through the use of NO donors and NOS inhibitors has been studied in animal models of both warm Ischemia reperfusion injury as well as cold preservation injury of the liver. Inhibition of NO production by administration of a nonselective NOS inhibitor increased hepatocellular injury (12), whereas augmentation of NO synthesis with NO donor and L-arginine supplementation decreased hepatic ischemia reperfusion injury and increased post transplant survival in rats (8). Inhibition of the L-arginine pathway increased graft damage, whereas augmentation of this pathway decreased the necrotic and apoptotic cell death during preservation injury in a rat liver transplant model (16). In relation to preconditioning, NO has been implicated in the protection induced by IPC in models of warm ischemia reperfusion injury, preservation injury and reperfusion injury in steatotic livers. However, isoform specific studies identifying which isoform of NOS is associated with NO generation during hepatic IPC are lacking. The identification of these mechanisms is important for developing strategies involving targeted pharmacological intervention or genetic manipulation to protect the liver from ischemic injury. In the heart NO generation from the iNOS enzyme has been implicated in the late phase (>24 hrs) of IPC (17). In the liver there are only two previous studies, which give indirect evidence that hepatic IPC is associated with eNOS activity. In the present study, isoforms of NOS were measured to determine which one is associated with cytoprotective hepatic NO generation.

In the present study, 45 minutes of ischemia followed by 2 hours reperfusion (group 2) resulted in decreased NO production. This was associated with decreased eNOS expression and decline in L-arginine levels. iNOS expression was absent. The mechanisms for decreased NO production after ischemia reperfusion could therefore be inhibition of eNOS and/or decreased substrate availability. The NO synthases catalyze the conversion of L-arginine and oxygen to NO and citrulline. Activated endothelium and neutrophil activation after ischemia reperfusion results in production of reactive oxygen species, which can inhibit NO synthases (18). The delivery of L-arginine to eNOS is also impaired after ischemia reperfusion (19). It has been proposed that L-arginine could be a rate limiting

factor for eNOS activity during ischemia and reperfusion (20). The decreased eNOS expression results in decreased NO production during ischemia and reperfusion (21). Further, the local depletion of the L-arginine substrate around eNOS may decrease or derange eNOS expression or activity leading to overproduction of reactive oxygen species (22). This may contribute to the hepatocellular injury indicated by the significant increase in ALT in ischemia reperfusion group. Another possibility for the L-arginine depletion is its catabolism by hepatic arginase I enzyme during reperfusion. Arginase is responsible for the hydrolysis of arginine to urea and ornithine and is present in highest concentration in the liver. Although arginase was not measured in this study, hepatic ischemia and reperfusion has been shown to be associated with an increased arginase I activity (16,23). In a model of liver transplantation in rats, increased level of circulating arginase I protein in the serum 1 hour after reperfusion was associated with a 10 fold decrease in serum arginine levels (16). In the present study the increased ornithine levels in group 2 would suggest an increased arginase activity.

This study has suggested that the preconditioned livers (group 3) had significantly decreased hepatocellular injury and were associated with upregulation of eNOS expression. Significant increases in both NO production and L-arginine levels were observed in this group. Given that ischemia reperfusion (group 2) had resulted in decreased eNOS expression, the increased eNOS expression in the preconditioned livers would suggest that IPC prevents the decline of eNOS or restores eNOS expression during ischemia reperfusion. Since iNOS expression was not detected, eNOS is the likely potential enzymatic sources for the increased NO production in the preconditioned livers. Further, the results show that the NO generation with IPC was specific to the liver since the hepatic vein NO levels were higher than circulating NO levels. An increased NO production immediately after the preconditioning treatment is well documented in previous studies (4,6). The transient ischemia of IPC induces protection for subsequent prolonged ischemia. The short time course for triggering IPC suggests that IPC events are triggered at posttranslational level since transcriptional activity takes several hours. A precedent exists for this hypothesis; in the myocardium, transcription of new proteins and subsequent iNOS generation has been linked to the late effect (>24 hrs) of preconditioning (17). eNOS is constitutively expressed and directly regulated by calcium (24) and therefore NO production from eNOS is quicker whereas iNOS upregulation is protein expression dependent and requires

several hours for induction (24). In addition, the increased L-arginine levels with IPC would indicate an increased substrate availability for eNOS. The increased L-arginine could be a result of either increased synthesis or decreased degradation. Circulating citrulline maintains endogenous synthesis of arginine in rats and humans (25). Since the citrulline levels were not significantly different between the groups, this would suggest that the arginine synthesis was not significantly modified. Arginine is degraded by arginase to ornithine and sufficient quantities of arginase can limit the availability of arginine for NO synthesis (26). The low ornithine levels in the preconditioned livers could suggest the possibility that the effect of IPC may involve modulation of arginase to increase substrate availability for NO biosynthesis. Arginase has two distinct isoenzymes and the interplay between arginase and NOS isoenzymes is more complex than the fact that they use a common substrate and, the mechanisms by which IPC may modulate this interplay is beyond the scope of this study. Although eNOS upregulation at the end of preconditioning treatment has not been demonstrated in the present study, our data supports the notion that IPC prevents the decline of eNOS during early ischemia reperfusion injury and this together with increased L-arginine substrate availability leads to increased cytoprotective NO production. Further, the aggravated hepatocellular injury with L-NAME administration additionally substantiates a role for eNOS derived NO in the preconditioning effect.

The effects of L-arginine administration were similar to those evident following IPC. L-arginine supplementation increased eNOS expression. L-arginine has been shown to increase eNOS expression and NO production both in cells and in vivo in both humans and mice (27). L-arginine supplementation modulates the NO dependent vascular functions and clinical studies have reported that L-arginine administration reduces the symptoms of coronary heart disease in patients (28). The similar effect to IPC following the stimulation of NO formation by supplementation with L-arginine suggests that NO might inhibit the downregulation of eNOS during reperfusion. This is consistent with the observation that the prevention of eNOS downregulation by exogenous adenosine attenuated hepatic ischemia reperfusion injury(10). However the mechanism by which adenosine prevents the downregulation of eNOS is not explained. Further, in this study the administration of exogenous L-arginine significantly increased its plasma level but also led to increased ornithine levels. The higher production of ornithine would indicate arginase activity. This raises the possibility that some of the NO generation after L-

arginine administration may be independent of the L-arginine/ NOS pathway. A nonenzymatic pathway for NO generation by the reaction of hydrogen peroxide and arginine has been proposed (29). In a previous study, Peralta et al (30) have shown that continuous L-arginine infusion not only reverted effects of NO inhibition but decreased hepatocyte injury induced by ischemia reperfusion. The NOS inhibitor L-NAME can to a variable levels inhibit arginine binding and metabolism as well as its transport across the hepatocyte plasma membrane (31). Cellular arginine may be shunted into different metabolic pathways including the ornithine cycle and this may explain the reduction in plasma arginine and increase in ornithine in the L-NAME group in this study.

The ALT and NO_x measurements indicate that the IPC induced hepatoprotection was effective for a time course of 2 hrs reperfusion following 45 mins ischemia. The sustained protection shown by the results raises the possibility of other molecules in addition to eNOS contributing to the preconditioning signal. The possibility of compensatory up-regulation of iNOS isoform is excluded by the results. The other possibility is enzymatic products of eNOS activity may contribute towards the multi factorial trigger/ mediator of IPC. Exogenous nitric oxide (L-arginine treated group) produced similar rise in NO_x and up-regulation of eNOS as with IPC. NO is not the sole product of e-NOS and free radicals may be generated (32). Recent evidence suggests that free radicals were essential for preconditioning (33). The possibility of free radicals synthesized as a by product of eNOS activity contributing to the preconditioning effect cannot be excluded in this study.

The NOS inhibition used in this study was not isoform specific. It was assumed that the role of iNOS would be negligible since the enzyme takes several hours to be induced and the results indicate that NOS inhibition was complete and effective for eNOS as well as iNOS. The observation from this study that eNOS may contribute to early preconditioning may have potential importance in the clinical application of preconditioning in man. Patients who have developed acute myocardial infarction have increased eNOS polymorphisms (34). Such polymorphism may adversely affect the nitric oxide generation resulting from a transient ischemic insult and may therefore attenuate protection potentially afforded by IPC. There is no evidence that such polymorphism exists in liver ischemia reperfusion injury so far and this area warrants further investigation. Nonetheless, eNOS may prove to be a useful target in hepatoprotection from ischemia reperfusion injury.

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Captions to figures

Fig. 1. Plasma Alanine aminotransferase (ALT) and hepatic vein nitrite + nitrate, NO_x (μM). Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, #p<0.05 vs. IR, Students t test.

Fig. 2. Representative photomicrographs eNOS immunostaining staining in liver sections taken at the end of 2 hrs reperfusion phase. The brown stain in hepatocytes and vascular endothelium indicates eNOS expression. The groups were A) Sham, B) Ischemia reperfusion, C) Ischemic preconditioning, D) L-arginine + IR, and E) L-NAME + IPC + IR.

Fig. 3. Western blotting for eNOS and iNOS at the end of 2 hrs reperfusion phase. The groups were A) Sham, B) Ischemia reperfusion, C) Ischemic preconditioning, D) L-arginine + IR, and E) L-NAME + IPC + IR. The blot is representative of 3 independent experiments. *p< 0.05 vs. sham, #p< 0.05 vs. IR..

Fig. 4. Plasma amino acids (μM). Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, #p<0.05 vs. IR, Students t test.

Table 1. Heart rate (HR) (beats/ min), arterial oxygen saturation (SaO2) (%), mean arterial blood pressure (MABP) (mmHg). Values are mean \pm SD of 6 animals in each group.

	Group 1 (Sham)	Group 2 (IR)	Group 3 (IPC)	Group 4 (L-arginine + IR)	Group 5 (L-NAME + IPC)
HR	235.0 \pm 11.9	229.6 \pm 7.7 ^{NS1}	230.0 \pm 6.2 ^{NS2}	233.9 \pm 7.7 ^{NS2}	230.0 \pm 9.7 ^{NS2}
SaO2	98.4 \pm 1.1	97.7 \pm 0.6 ^{NS1}	97.2 \pm 0.9 ^{NS2}	96.9 \pm 1.4 ^{NS2}	97.4 \pm 1.6 ^{NS2}
MABP	85.0 \pm 3.5	85.6 \pm 1.4 ^{NS1}	85.3 \pm 1.6 ^{NS2}	81.0 \pm 2.1 ^{NS2}	110.2 \pm 3.4* [†]

*p<0.01 vs. Group 1; [†]p<0.05 vs. Group 2; ^{NS1}Not significant (p>0.05 vs. group 1);

^{NS2}Not significant (p>0.05 vs. group 2) using unpaired t test.

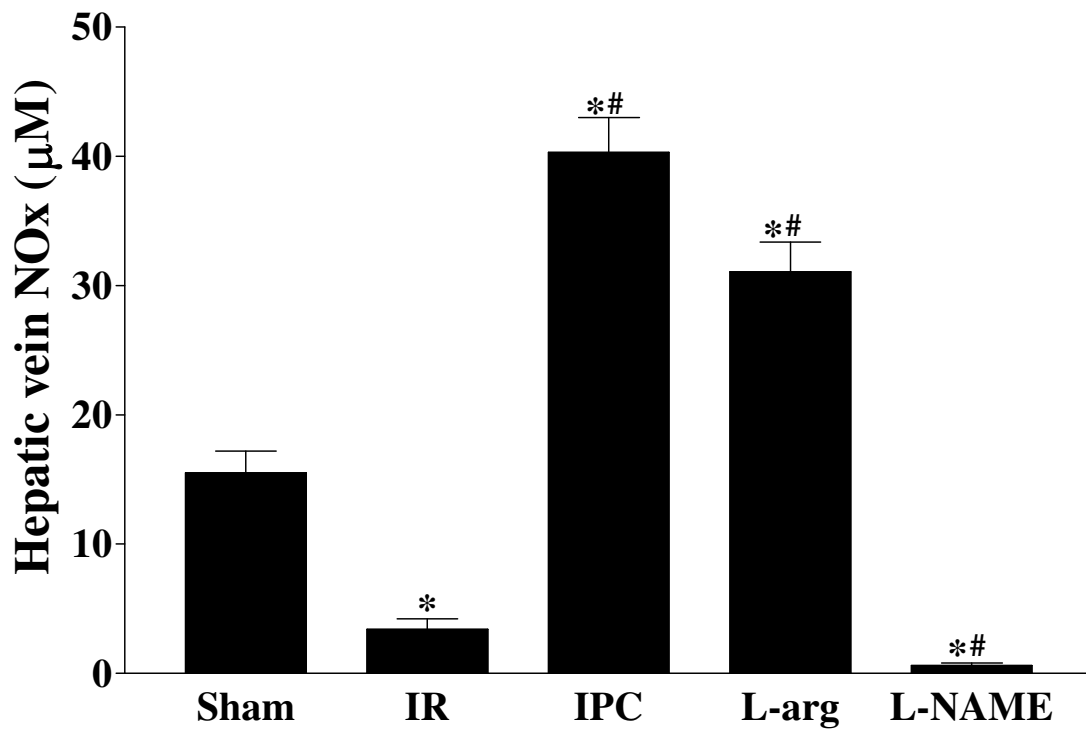
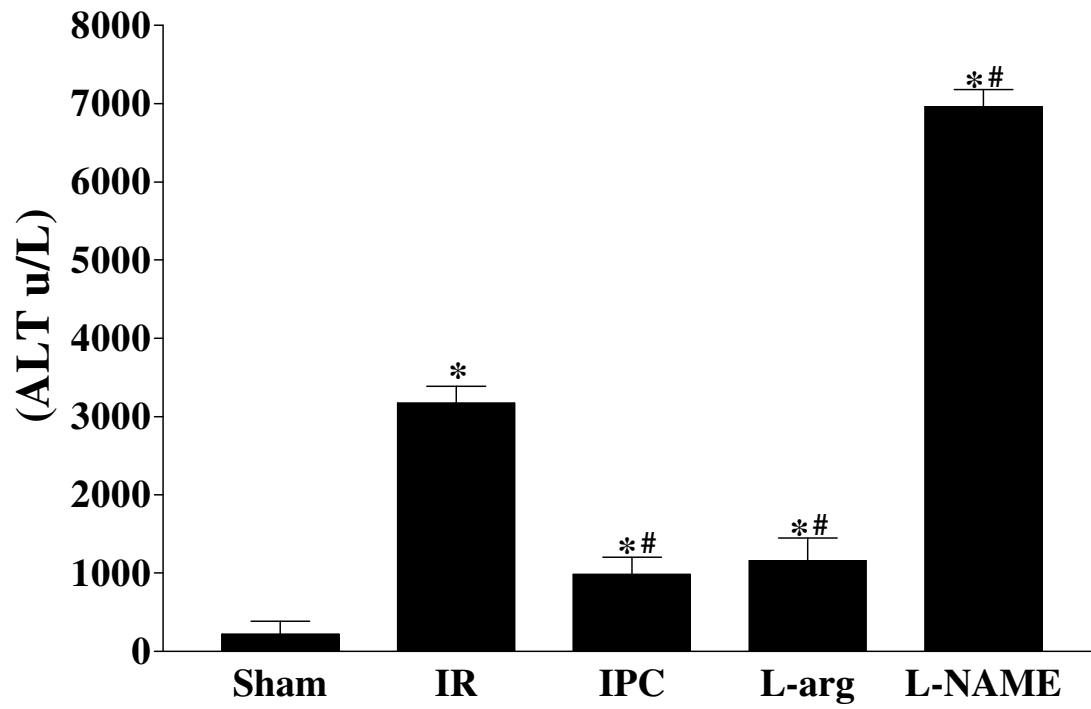


Fig.1.

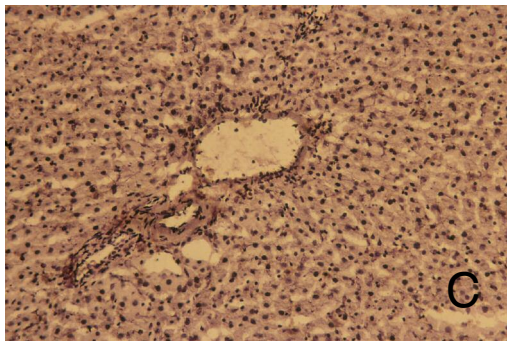
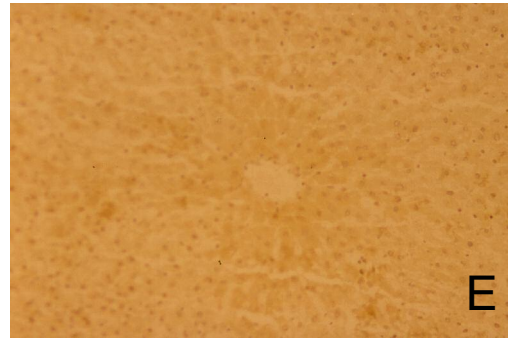
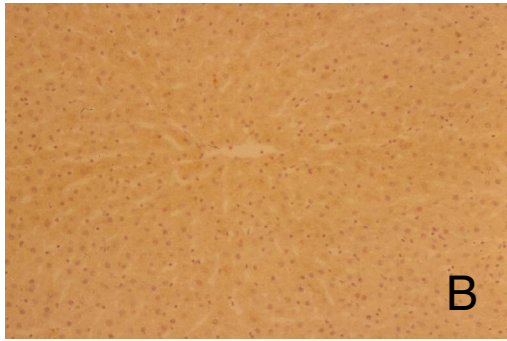
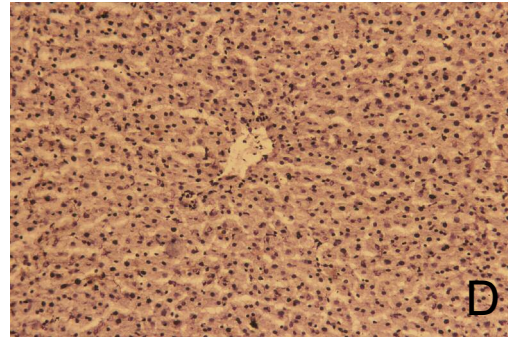
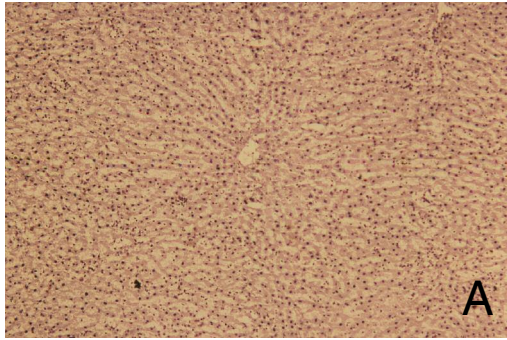


Fig.2

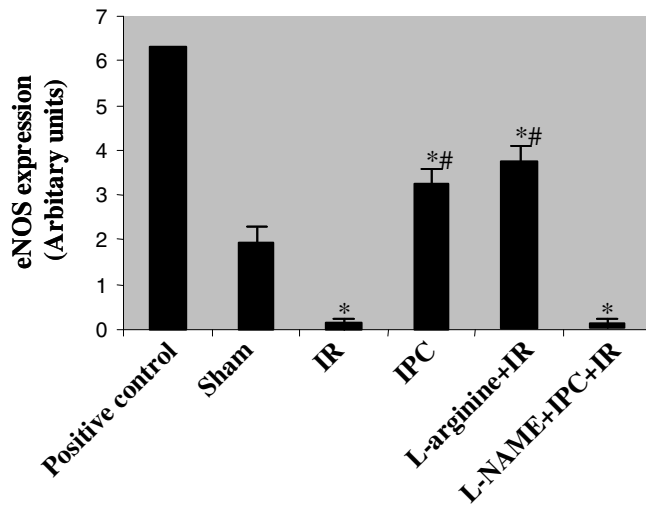
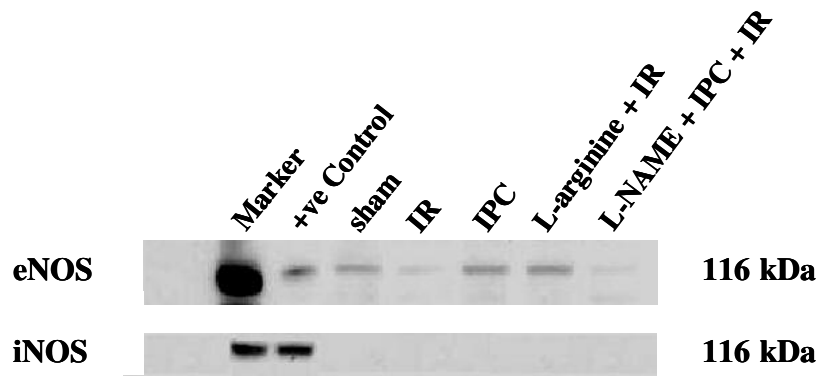


Fig. 3

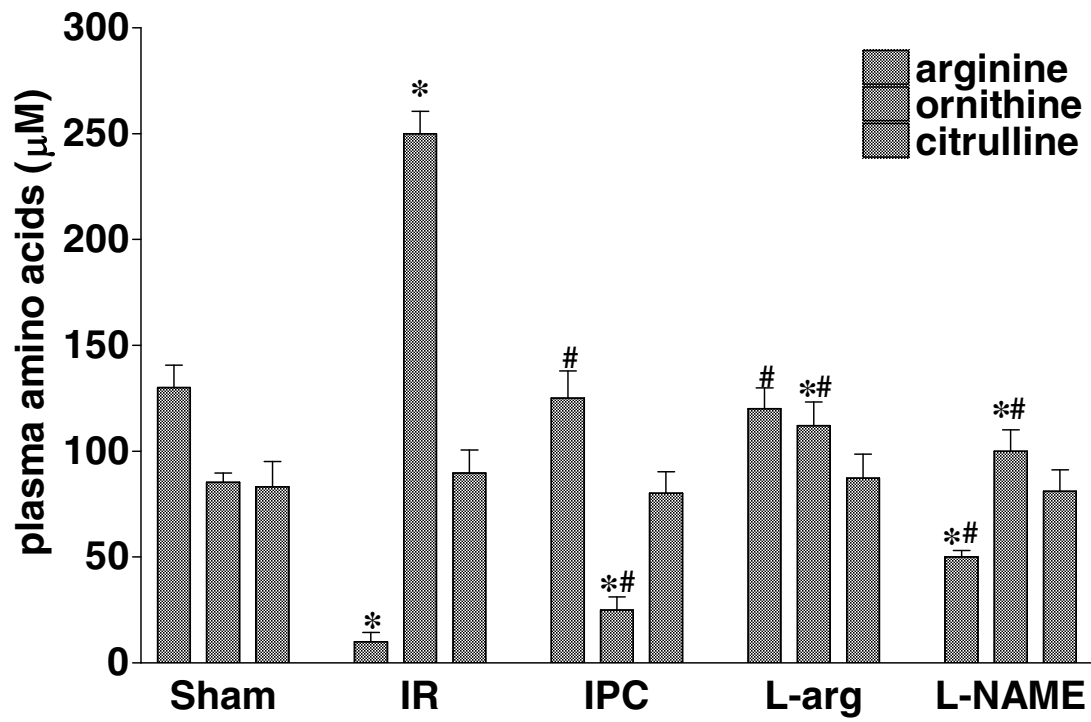


Fig. 4.