

Nitric Oxide Synthase in Heart and Thoracic Aorta After Liver Ischemia and Reperfusion Injury: An Experimental Study in Rats

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Abstract

Objectives: We tested the effects of liver reperfusion in the immunohistochemical expression of nitric oxide synthase on the thoracic aorta and the heart.

Materials and Methods: We randomized 24 male Wistar rats into 3 groups: (1) control; (2) R2 group, with 60 minutes of partial (70%) liver ischemia and 2 hours of global liver reperfusion; (3) and R6 group, with 60 minutes of partial liver ischemia and 6 hours of global liver reperfusion.

Results: In the heart, there was little, diffuse immunohistochemical endothelial staining; immunohistochemical inducible nitric oxide synthase staining was expressed in the adventitia layer of intramyocardial vessels in both cases, with a time-dependent but not statistically significant increase. In the thoracic aorta, a time-dependent decrease in endothelial nitric oxide synthase expression in the muscular layer after reperfusion, which was statistically significant in R6 versus the control. Positive immunostaining for inducible nitric oxide synthase was seen in the muscular and endothelial layers, and this varied from moderate in the control group, to light in the endothelium in groups R2 and R6.

Conclusions: We observed changes that may be implicated in heart injury and impairment of aortal tone after liver ischemia and reperfusion injury.

Key words: Lipid peroxidation, Liver transplant

Introduction

Vascular complications are a significant source of morbidity after liver reperfusion that occurs throughout hepatic transplant. Although ischemia is a local event, the mediators from ischemic tissues enter circulation during revascularization and affect other organ systems.^{1, 2} Complications involve remote organs like the lung, heart, and aorta. For example, pulmonary microvascular permeability is increased in nonischemic lungs after perfusion with effluent from reperfused ischemic liver, compared to those lungs perfused with effluent from nonischemic livers, in a double-isolated perfused rat organ model.³ Severe hepatic ischemia results in heart injury involving systemic inflammatory responses.⁴ Interestingly, ischemia and reperfusion injury impair aortic tone, and despite its vasoconstrictor capability, methylene blue provides the aorta with dose-dependent protection.⁵ Kupffer cell activation and subsequent tumor necrosis factor alpha release,⁶ and oxidative stress mediators leading to localized hepatic injury and remote organ dysfunction, are probable agonists of injury. Although remote organ injury is well documented, it is not well comprehended.

Nitric oxide (NO) is produced from L-arginine and molecular oxygen by nitric oxide synthase (NOS), an enzyme that exists in 3 isoforms encoded by distinct genes. It can be classified in 2 categories: constitutive NOS (cNOS), which includes neuronal

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NOS (nNOS) and endothelial NOS (eNOS); and inducible NOS (iNOS). Both are involved in local hepatic ischemia and reperfusion (IR) injury. Nitric oxide has been recognized as an important mediator of physiological and pathophysiological processes, but although it plays a fundamental role in the pathogenesis of local liver ischemia and reperfusion injury, its effects in remote organ injury are not completely understood.⁷⁻⁹ Remote effects by liver reperfusion have been reported, but there is a paucity of articles. Among the issues to be investigated, the precise effects of liver IR in nitric oxide synthase (NOS) expression in remote organs remains unclear. In this study, we examine the effects of liver IR injury in the immunohistochemical expression of NOS in the thoracic aorta and heart.

Materials and Methods

This study was carried out in accordance with the guidelines of the University of São Paulo Animal Experimentation and Animal Research Ethics Committee.

Twenty-four male Wistar albino rats (250 g) were randomly allocated in 3 groups: The control group (rats submitted to a sham operation); the R2 group (rats were submitted to 60 min of partial warm liver ischemia and 2 h of reperfusion); and the R6 group (in which rats were submitted to 60 min of partial warm liver ischemia and 6 h of reperfusion (8 rats in each group)).

After an overnight fast, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Additional amounts were injected when necessary. After a midline laparotomy, the portal triad was exposed, and a vascular clamp was applied cephalad to the branches of the right and caudate lobes; thus, occluding the portal vein, hepatic artery, and bile duct (about 70% of the liver mass). This method allows for portal decompression through the right and caudate lobes and prevents mesenteric venous congestion. The abdomen was humidified with saline solution, and the muscular layer was approximated with single stitches.

After 60 minutes of ischemia, the clamp was removed and the abdomen was closed in a single layer. Control animals were treated in an identical fashion, but with the omission of vascular occlusion. After 2 hours for the R2 group and 6 hours for the R6 group, 5 mL of blood was drawn from suprahepatic

veins for analysis, and the animals were killed by exsanguination. The liver, heart, and thoracic aorta were recovered immediately after exsanguination. Small portions of the liver were kept at -70°C until the moment of assay for lipid peroxide, whereas other portions were separated and immersed in 10% buffered formalin solution for histologic examination. Tissue samples from the heart and thoracic aorta were fixed in a 10% buffered formalin solution and embedded in paraffin until the moment of immunohistochemical or histologic analysis. A blood sample was immediately centrifuged, and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with a commercial kit (Labormed Co, Ltd., Guarulhos, SP, Brazil) and are reported as SI units.

Assay for lipid peroxide in liver

Tissue samples of ischemic and nonischemic liver were homogenized with ice-cold Ringer's solution to determine the malondialdehyde (MDA) levels. The MDA levels were assayed for products of lipid peroxidation using a commercially available kit (Calbiochem, San Diego, CA, USA). The results are expressed as nmol/g tissue.

Immunohistochemical assay

Tissue samples were immediately fixed in 10% buffered-formalin for 24 hours and embedded in paraffin. Paraffin embedded tissue blocks were sectioned at 3 µm. Sections were processed for eNOS, iNOS, and nNOS staining using commercially available kit (DAKO LSAB2 Kit, peroxidase for use on RAT specimens, DAKO Corporation, Carpinteria, CA, USA).

In brief, sections were fixed to slides pretreated with [3-aminopropyl] triethoxysilane (Sigma, St. Louis, MO, USA). Subsequently, sections were deparaffinized and rehydrated through a descending alcohol series followed by distilled water. Endogenous peroxidase activity was inactivated with hydrogen peroxide, and the sections were incubated with citrate buffer in a humidified heat chamber (Optisteam Plus, Krups North America Inc., New Jersey, USA) for antigen retrieval. The unspecific bindings were blocked with swine normal serum. Sections were incubated with polyclonal eNOS antibody (NOS3 (H-159): sc-8311, Santa Cruz Biotechnology, CA, USA) at a dilution of 1:25 or monoclonal iNOS antibody (NOS2 (C-11): sc-

7271, Santa Cruz Biotechnology) at a dilution of 1:5 or monoclonal nNOS antibody (NOS1 (A-11): sc-5302, (Santa Cruz Biotechnology) at a dilution of 1:5. In sequence, sections were incubated with secondary antibody of LSAB2 kit and then with the streptavidin peroxidase of the same kit. Finally, the reactions were revealed by 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA), and the sections were counterstained with Harris' hematoxylin. As negative controls, sections were processed with the above procedures, omitting the primary or secondary antibodies.

All the slides were observed and photographed using a high-definition camera (AxioCam HRc, Zeiss, German) coupled to a microscope (Axioskop 2 plus, Zeiss, German). Immunostaining was graduated semiquantitatively using an arbitrary 5-tiered scale (0, +, ++, +++, and +++) by an observer who was not aware of the animal group.

Histologic method

Selections were stained with hematoxylin and eosin and Masson's trichrome staining, and examined histologically by light microscopy.

Statistical Analyses

Transaminase and MDA values in the text, Figures, and Tables are expressed as means $\pm$ SD of 8 independent experiments. Each biochemical parameter was compared using a 1-way ANOVA. Bonferroni significant difference tests were used for comparisons within the groups. Immunohistochemical data are expressed as median $\pm$ range of distribution (min-max) of positive staining cells of 5 independent experiments. Statistical analyses were performed using the Kruskal-Wallis test of a 1-way ANOVA for multiple comparisons, followed by Dunn's test for comparison between groups. A *P* value less than .05 was considered significant. Data were analyzed with Prism 4.0 (GraphPad, San Diego, CA, USA).

Results

Liver injury

Changes in serum transaminases

At 2 hours and 6 hours after liver reperfusion, the serum ALT and AST levels were significantly higher in group R2 (1.867 ± 935.9 U/L and 1.489 ± 169.4 U/L; *P* < .05) and in group R6 (3.688 ± 2.420 U/L and

2.773 ± 659.6 U/L; *P* < .01) versus the control group (33.63 ± 2.06 U/L and 110 ± 5.5 U/L).

Changes in malondialdehyde levels

Malondialdehyde levels were found to be significantly higher in the R2 and R6 groups versus the control group for ischemic liver (5.15 ± 0.37 nmol/g; *P* < .001 and 8.00 ± 0.44 nmol/g; *P* < .01 vs 3.5 ± 0.06 nmol/g) and nonischemic liver (6.89 ± 1.46 nmol/g; *P* < .05 and 7.99 ± 2.96 nmol/g; *P* < .01, vs 3.60 ± 1.43 nmol/g).

Changes in heart immunohistochemical staining of eNOS, iNOS, and nNOS

Immunohistochemical staining showed little, diffuse eNOS expression with a time-dependent and not statistically significant increase after reperfusion. Immunohistochemical iNOS staining showed a time-dependent and not statistically significant increase, expressed mainly in the adventitia layer of intramyocardial vessels. There was no nNOS immunohistochemical staining (Figure 1, Table 1).

Changes in thoracic aorta immunohistochemical staining

Immunohistochemical staining showed diffuse eNOS formation in the adventitia, endothelium, and muscular layer of the thoracic aorta. A time-dependent decrease in the muscular layer after reperfusion was observed, which was statistically significant after 6 hours of reperfusion versus the control group (*P* < .05). Endothelial eNOS staining was not changed by reperfusion. Positive immunostaining for iNOS was observed in the muscular and endothelial layers, and this varied from moderate in the sham group to light in the endothelium in groups R2 and R6. There was no iNOS expression in the muscular layer after 2 hours

Table 1. Values represent median (min-max) of marks in an arbitrary 5-tiered scale (0, +, ++, +++, and +++) from 5 experiments in each group. Observations correspond to immunohistochemical staining for eNOS, iNOS, and nNOS in thoracic aorta and heart. **P* < .05.

		Control	Reperfusion 2 h	Reperfusion 6 h
Heart	eNOS	0 (0-2)	1 (0-1)	1 (0-2)
	iNOS	1 (0-2)	2 (1-3)	2 (1-2)
	nNOS	0 (0-0)	0 (0-0)	0 (0-0)
Endothelium	eNOS	2 (0-3)*	0 (0-1)*	1 (0-3)
Thoracic	iNOS	2 (0-3)	0 (0-2)	0 (0-3)
Aorta	nNOS	0 (0-2)	0 (0-1)	0 (0-0)
Muscular	eNOS	3 (2-4)	1 (0-2)	0 (0-3)
Thoracic	iNOS	2 (0-3)	0 (0-0)	1 (0-3)
Aorta	nNOS	1 (0-2)	2 (1-3)	2 (1-2)

of reperfusion, $P < .05$ versus the control group, and a light and not statistically significant decrease in immunohistochemical staining after 6 hours of reperfusion. Minimal nNOS expression was observed, which was not altered by reperfusion (Figure 2, Table 1).

Histopathologic examination of the heart

The heart was histologically normal, with no change found in the control group. Subendocardial and

subepicardial injury and focal necrosis of myocardial cells were observed after 2 hours of liver reperfusion. Subendocardial and subepicardial injury, with scattered foci of intramural reperfusion and necrosis, and no inflammatory infiltrate were found after 6 hours of liver reperfusion (Figure 3).

Histopathologic examination of the ischemic liver

No changes were observed in the control group. Moderate congestion, vacuolization of hepatocytes,

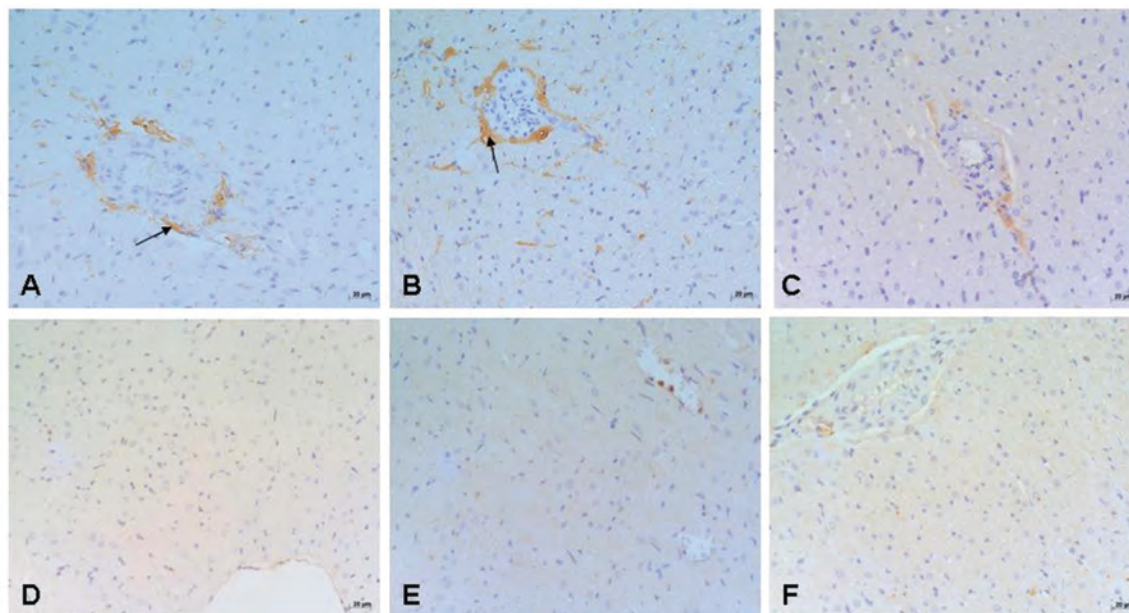


Figure 1. Photomicrographs of immunohistochemical evaluation for iNOS in the heart observed in the tunica adventitia of intramyocardial vessels (→) in animals of groups R2 (A), R6 (B), and control (C) $\times 400$, and the evaluation of eNOS, showing diffuse expression in the groups R2 (D), R6 (E), and controls (F) $\times 400$.

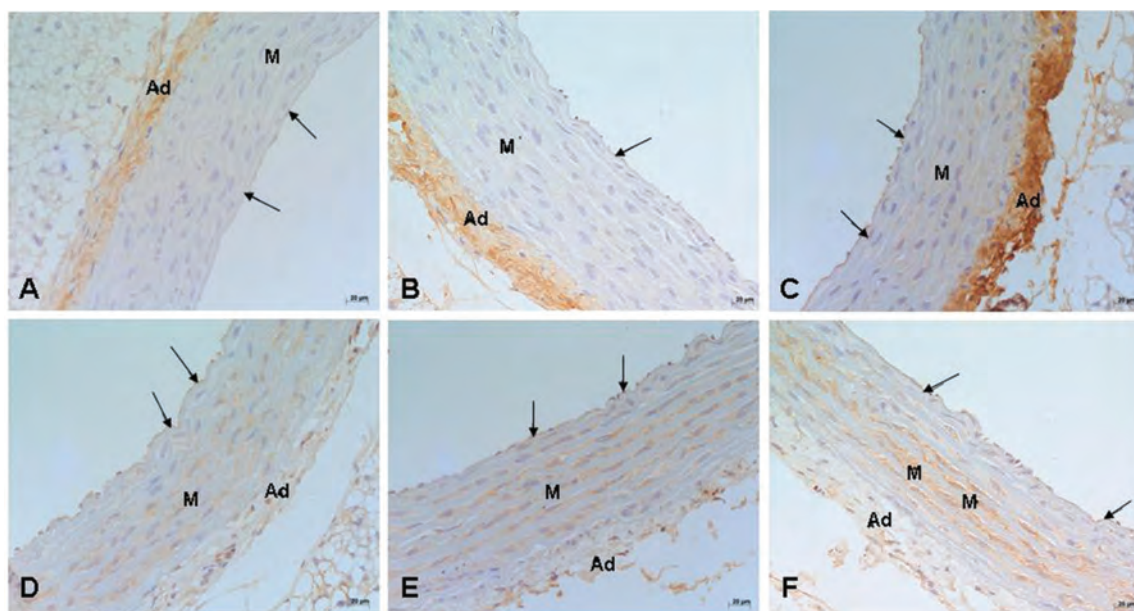


Figure 2. Photomicrographs of immunohistochemical evaluation for iNOS in the thoracic aorta in animals of groups R2 (A), R6 (B), and controls (C), with little marking endothelial (→) and muscle (M), adventitia (Ad) $\times 400$, and evaluation of eNOS. Diffuse expression in the endothelial layer (→), muscle (M), and adventitia (Ad) in the control group (F) with time-dependent decrease in groups R2 (D) and R6 (E) $\times 400$.

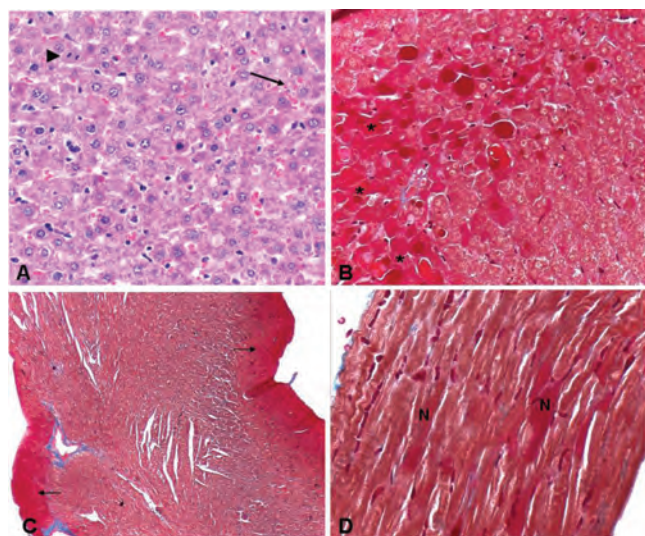


Figure 3. Effects of liver ischemia reperfusion injury by indicated hematoxylin and eosin (A) and Masson's trichrome staining (B). Histopathologic changes in liver: R2 group (A) improves the appearance of hydropic hepatocytes, presence of moderate congestion ($\rightarrow$) and occasional mitoses ($\blacktriangle$), and the R6 group (B) intracytoplasmic inclusions in injured or necrotic hepatocytes (*). Effects of heart ischemia injury indicated by Masson's trichrome staining (C and D). Accentuation of lesions in the subendocardial and subepicardial myocardial cells ($\rightarrow$) in the R2 group (C) and isolated foci of myocyte cell necrosis (N) with no inflammatory response in the R6 group (D). Increases are $\times 100$ (C) and $\times 400$ (A, B, and D).

apparent increase of interstitial cells, sporadic mitoses, and apoptotic cells were seen after 2 hours of liver reperfusion. After 6 hours of reperfusion, moderate congestion, severe diffuse hepatocellular necrosis with inflammatory infiltrate causing disruption of the microarchitecture, increase in interstitial cells, neutrophilic infiltration in the portal zone, and cytoplasmic inclusions in injured or necrotic hepatocytes were observed (Figure 3).

Discussion

Liver IR injury is associated with remote organ reperfusion injury attributable to the systemic inflammatory or oxidative mediators released into the circulation during reperfusion.⁹⁻¹¹ Even though NO is an important mediator of local reperfusion injury and local NOS expression is clearly modified by liver IR, the effects of liver IR in NOS expression in remote organs are poorly recognized.¹²⁻¹⁵

We studied the effects of liver IR damage in the immunohistochemical expression of NOS in rat thoracic aortas and hearts. Rats experienced severe liver IR injury, which was confirmed by measuring the release of ALT and AST, and the elevated hepatic tissue lipid peroxide in ischemic and nonischemic liver. We found a decrease in the immuno-

histochemical expression of eNOS and iNOS after liver reperfusion in the muscular layer of the thoracic aorta. It has been previously demonstrated that hepatic IR impairs aortic tone.^{16, 17} Prolonged aortic spasm and abnormal vascular response to phenylephrine were observed in aortic rings of rats exposed to postischemic liver perfusate in an ex vivo rat liver-aortic ring model. In addition, liver IR interferes with aortic tone and its response to vasoactive drugs. This effect can be reversed by methylene blue in a dose-dependent fashion.⁴ Noticeably, a loss in NOS expression could have a significant effect on regulation of vasomotion and could explain, at least in part, changes in the aortic tonus observed by others.

Our study also has shown a mild increase in the heart eNOS and iNOS expression that is not statistically significant by the semiquantitative method, and we demonstrated damage by histopathologic examination of the heart. Myocardial injury after liver IR has been reported in medical literature. Liu and associates observed that prolonged IR of the liver induced enlarged mitochondria in myocardial cells, thickened vascular walls, and scattered necrotic lesions in myocardial tissue.¹⁸ Liver IR also induced changes in blood cardiac troponin I level, an index of cardiovascular injury. Inducible nitric oxide synthase expression is involved in heart and lung tissue injury after liver IR injury.⁴ We showed a time-dependent but not statistically significant increase, expressed mainly in the adventitia layer of intramyocardial vessels. An increasing level of nitric oxide arising from per vascular iNOS activity may further react with superoxide to form peroxynitrite and hydroxyl radicals, resulting in damage to the heart tissue. On the other hand, NO from per vascular iNOS can help prevent further tissue injury because of its effects as an antioxidant and anti-inflammatory agent, along with its beneficial effects on cell signaling and inhibition of nuclear proteins.¹⁴

The experimental design used here has some limitations. Immunohistochemical is a finest method for localization of an antigen in tissue. We did not test the function of NOS nor its biological availability during liver reperfusion, so the experimental design did not permit us to draw conclusions about the function or physiological effects of NOS in remote organs after liver reperfusion injury. Changes in NOS expression were

estimated by a semiquantitative method. Whether the small expression of NOS observed in the thoracic aorta is followed by a decrease in NO synthesis or availability, and whether this effect could deprive the tissues of the beneficial effects of NO, and NO from per vascular myocardial cells could induce heart damage, are subjects for future experiments. In particular, experiments should be designed to quantify NOS in remote organs after liver reperfusion and test their function. These efforts are justifiable because hepatic NOS is notorious for having a significant contribution to local liver IR injury in an animal model.¹⁴ Hence, it is plausible that liver IR reperfusion results in a change in the NOS turnover in remote organs, an effect that should contribute to the pathophysiological phenomena observed in the clinical setting during liver IR injury.

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