

The Effects of Antioxidants and Nitric Oxide Modulators on Hepatic Ischemic-Reperfusion Injury in Rats

Ischemic-reperfusion injury (IRI) is thought to be caused by oxygen radicals. Nitric oxide (NO) also has been thought to play a key role in IRI. This experiment was designed to evaluate the effects of antioxidants and NO supplement on hepatic IRI. Male Sprague-Dawley rats were divided into five groups: a sham operation group, a group with IRI, and three groups with vitamin C combined with vitamin E (VC&VE), L-arginine and *N*^o-nitro-L-arginine (NNLA) injected after IRI. IRI was induced by clamping of the porta hepatis for 30 minutes and then by declamping. To prevent mesenteric blood congestion, a porto-systemic shunt had been made four weeks before the portal clamping. Biochemical assays of TNF- α level and NO₂⁻ level in the blood, malondialdehyde level, catalase activity and NO synthase activity in the liver tissue were performed. The results were as follows: IRI increased the malondialdehyde level and exhausted the catalase activity remarkably. VC&VE lowered the malondialdehyde levels and protected against catalase exhaustion, but had no significant effect on the NO production. L-arginine had a definite antioxidant effect, which was much weaker than that of VC&VE. In conclusion, antioxidants and a supplement of NO protected the liver tissue against IRI.

Key Words : Reperfusion Injury; Nitric Oxide; Antioxidants; Tumor Necrosis Factor; Ascorbic Acid

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INTRODUCTION

Ischemia-reperfusion injury (IRI) is of clinical importance in many clinical situations. Reperfused tissue after ischemia shows additional reperfusion injury, which is related to the duration of the ischemia and the tissue susceptibility. While ischemia injury stems from a deficiency of energy necessary to maintain homeostasis, reperfusion injury is thought to be primarily due to attacks by generated oxygen radicals. During reperfusion, hypoxanthine, which has been accumulated during ischemia, is metabolized to xanthine by xanthine oxidase. In this process superoxide radical ($\cdot O_2^-$) is generated and converted to hydrogen peroxide (H₂O₂) or a hydroxyl radical (OH \cdot) (1).

The endogenous antioxidant mechanism includes superoxide dismutase (SOD), selenium-dependent glutathione peroxidase, and catalase (1, 2). Exogenous antioxidants such as vitamin E, vitamin C, β -carotene, urate, albumin, and bilirubin can also scavenge potent radicals (3). Vascular endothelial injury plays an important role in the early stage of IRI (4, 5). Nitric oxide (NO) is essential to vascular homeostasis (6), and the failure of NO production from endothelial cells has been shown to be the primary mechanism of microvascular failure in IRI (7, 8). There are some data showing that NO supple-

mentation in IRI reduces the extent of IRI and protects tissues from IRI (8-11).

The effect of antioxidant therapy and NO supplement on hepatic IRI has not yet been completely elucidated. This experiment was designed to evaluate the effects of antioxidants and NO supplement on hepatic IRI in rats.

MATERIALS AND METHODS

Experimental animals & porto-systemic shunt operation

Male rats of the Sprague-Dawley strain weighing 100 to 150 g were used. All rats were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985). All surgical procedures were performed under the anesthesia of intraperitoneal ketamine injection (50 mg/kg). Four weeks before the portal clamping procedure, all rats had undergone porto-systemic shunt operations for the purpose of minimizing splanchnic vascular congestion and mesenteric venous thrombosis during portal clamping. The procedures consisted of artificial hernia formation of the cecum into the subcutaneous space between

the muscle layer and the subcutaneous fat layer. All rats survived 30 min of portal clamping due to the capillary anastomosis between the cecal mesentery and the muscle layer.

Ischemia-reperfusion injury (IRI) induction

During the entire period of IRI, the rats were placed on a thermo-regulated operation table (37°C) to maintain a constant body temperature.

The method of IRI induction was as follows: following laparotomy, the liver was made ischemic by portal triad occlusion with a small Bulldog vascular clamp for 30 min followed by reperfusion.

The rats were divided into five groups; a sham operation (SO) group, a ischemia-reperfusion (IR) group, an antioxidant (IRCE) group, a L-arginine (IRA) group, and a N^G -nitro-L-arginine (IRNA) group.

The SO group received a sham operation which was composed of shaving, laparotomy incision, keeping the incision open for 30 min without IRI induction, and closure of the incision. The IR group was given IRI induction with no drug administration. The IRCE group received 100 mg vitamin E (α -Tocopherol, Sigma, St Louis, MO, U.S.A.) orally 24 hr before IRI induction, an intraperitoneal injection of vitamin C (L-ascorbic acid, Sigma) at a dose of 100 mg/kg, IRI induction, and then intraperitoneal injections of vitamin C at a dose of 100 mg/kg hourly following IRI induction. The IRA group received an intraperitoneal injection of L-arginine (Sigma) at a dose of 100 mg/kg 20 min before IRI induction, and then IRI induction, and intraperitoneal injections of L-arginine at a dose of 50 mg/kg hourly. The IRNA group received an intraperitoneal injection of N^G -nitro-L-arginine (Calbiochem, Darmstadt, Germany) at a dose of 30 mg/kg 20 min before IRI induction, and then IRI induction, and intraperitoneal injections of N^G -nitro-L-arginine at a dose of 30 mg/kg hourly. Five rats in each group were sacrificed at post-reperfusion 1 hr and 6 hr.

Sampling was done as follows: through a long midline incision from the chest to abdomen, the right atrium was punctured and blood was aspirated with a heparin-coated syringe. Following blood sampling, the liver was resected promptly and stored at -70°C. The blood was centrifuged, and the serum was stored at -70°C.

Biochemical assays

Blood TNF- α level was measured with the sandwich enzyme immunoassay using a solid-phase ELISA kit (Biosource, Camarillo, CA, U.S.A.). The absorbance of each well was measured by ELISA reader (CERES UV900HDI, Bio-Tek Instruments, Winooski, VT, U.S.A.) at 450 nm. The detection limit of the TNF- α measurement was 4 pg/mL.

The malondialdehyde (MDA) in liver tissue was measured with the Ohkawa's method (1979) using thiobarbituric

acid (Sigma) reaction (12, 13). Absorbance at 532 nm was measured by spectrophotometer (DU 650, Beckman). The unit of measurement was nmol/g of liver tissue.

Catalase activity in the liver tissue was measured with the Beers & Sizer's method (1952) (14). Absorbance at 240 nm was measured by spectrophotometer. The unit of measurement was units/mg of liver tissue.

Nitric oxide amount that had been produced was measured as total sum of plasma nitrite (NO_2^-) and nitrate (NO_3^-) concentrations using the Griess reaction kit (15) (R&D Systems). Nitrate was converted to nitrite by nitrate reductase. The sum of converted nitrate concentrations plus intrinsic nitrite concentrations were expressed as nitrite concentrations ($\mu\text{mol/L}$) by optical absorbances with a spectrophotometer (CERES UV900HDI) at 540 nm. Sodium nitrite solutions were used as standards.

i-NOS activity in the liver tissue was measured as the conversion rate of ^3H -L-arginine (Amersham) to ^3H -L-citrulline. Because L-arginine is a cation, L-citrulline can be separated from L-arginine by cation exchange resin. The number of β -particles from ^3H of produced ^3H -L-citrulline was measured by liquid scintillation spectrometer (TRI-CARB, Packard). This was expressed as counts per min (cpm).

Measured values were expressed as the mean \pm standard deviation. Kruskal-Wallis test and Mann-Whitney test were used to compare the means of each group using the SPSS for Windows Release 10.0 package. A p -value below 0.05 was considered statistically significant.

RESULTS

TNF- α levels in the blood

The TNF- α level of the normal rats was 12.74 ± 6.03 pg/mL (Fig. 1). The TNF- α level of the IR group after 6 hr reperfusion was significantly increased compared with that of the SO group ($p=0.001$). The TNF- α level of the IRCE group after 6 hr re-perfusion was significantly decreased compared with that of the IR group ($p<0.0001$). The TNF- α level of the IRA group after 6 hr was lower than that of the IR group ($p=0.004$). The TNF- α levels of the IRNA group after 1 and 6 hr were lower than those of the IR group ($p=0.029$ and $p=0.126$, respectively).

MDA levels in liver tissues

The MDA level of the normal rats was 143.46 ± 26.68 nmol/g (Fig. 2). The MDA levels of the IR group after 1 and 6 hr were much higher than those of the SO group ($p<0.0001$ for both comparisons). The MDA levels of the IRCE group after 1 and 6 hr were significantly decreased compared with those of the IR group ($p<0.0001$ for both comparisons). The MDA levels of the IRA group were markedly reduced

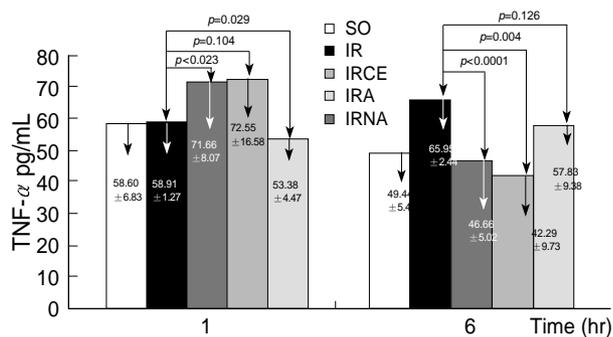


Fig. 1. The effects of vitamin C/vitamin E, L-arginine, and N^G -nitro-L-arginine on the blood TNF- α levels in ischemic-reperfusion injured rats. SO: sham operation, IR: ischemia-reperfusion, IRCE: ischemia-reperfusion with vitamin C and vitamin E, IRA: ischemia-reperfusion with L-arginine, IRNA: ischemia-reperfusion with N^G -nitro-L-arginine.

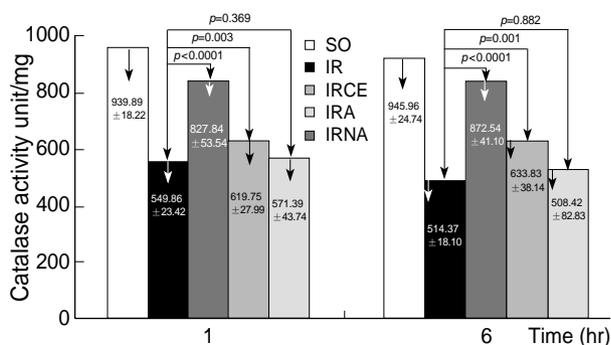


Fig. 3. The effects of vitamin C/vitamin E, L-arginine, and N^G -nitro-L-arginine on catalase activities in rat liver tissue injured by ischemic-reperfusion. SO: sham operation, IR: ischemia-reperfusion, IRCE: ischemia-reperfusion with vitamin C and vitamin E, IRA: ischemia-reperfusion with L-arginine, IRNA: ischemia-reperfusion with N^G -nitro-L-arginine.

compared with those of the IR group ($p < 0.0001$ for both comparisons), although significantly higher than those of the IRCE group ($p < 0.0001$). The MDA levels of the IRNA group were reduced when compared with those of the IR group ($p = 0.001$ and $p = 0.035$, respectively), but significantly higher than those of the IRA group ($p < 0.0001$ and $p = 0.002$, respectively).

Catalase activities in liver tissue

The catalase activity of normal rats was 1427.148 ± 104.54 unit/mg (Fig. 3). The catalase activities of the IR group were markedly decreased compared with those of the SO group for both 1 hr and 6 hr reperfusion ($p < 0.0001$ and $p = 0.0001$, respectively). The catalase activities of the IRCE group after 1 and 6 hr were much higher than those of the IR group ($p < 0.0001$ and $p = 0.0001$, respectively). The catalase activities of the IRA group after 1 and 6 hr were significantly higher than those of the IR group ($p = 0.003$ and

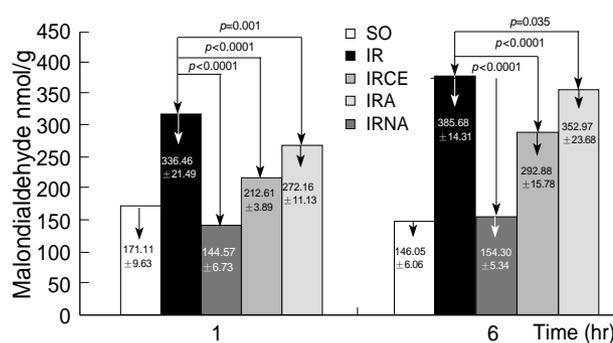


Fig. 2. The effects of vitamin C/vitamin E, L-arginine, and N^G -nitro-L-arginine on lipid peroxidation in rat liver tissues injured by ischemic-reperfusion. SO: sham operation, IR: ischemia-reperfusion, IRCE: ischemia-reperfusion with vitamin C and vitamin E, IRA: ischemia-reperfusion with L-arginine, IRNA: ischemia-reperfusion with N^G -nitro-L-arginine.

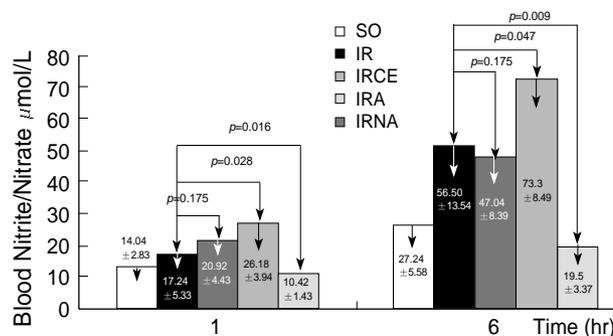


Fig. 4. The effects of vitamin C/vitamin E, L-arginine, and N^G -nitro-L-arginine on the blood nitrite and nitrate levels in ischemic-reperfusion injured rat. SO: sham operation, IR: ischemia-reperfusion, IRCE: ischemia-reperfusion with vitamin C and vitamin E, IRA: ischemia-reperfusion with L-arginine, IRNA: ischemia-reperfusion with N^G -nitro-L-arginine.

$p = 0.001$, respectively), but much lower than those of the IRCE group ($p < 0.0001$ and $p = 0.0001$, respectively). The catalase activities of the IRNA group were not different from those of the IR group ($p = 0.369$ and $p = 0.882$, respectively), and lower than those of the IRA group ($p = 0.077$ and $p = 0.024$, respectively).

Nitrite concentrations in serum

The nitrite concentration of normal rats was 10.84 ± 2.08 unit/mg (Fig. 4). The nitrite concentration of the IR group were markedly increased compared with those of the SO group at 6 hr of reperfusion ($p = 0.009$). The concentration of the IRCE group was higher at 1 hr of reperfusion and lower at 6 hr of reperfusion than those of the IR group, but without statistical significance ($p = 0.175$ and $p = 0.175$, respectively). The nitrite concentrations of the IRA group after 1 and 6 hr were significantly higher than those of the

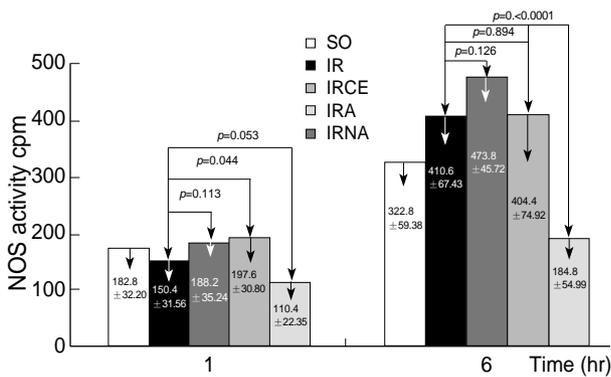


Fig. 5. The effect of vitamin C/vitamin E, L-arginine, and N^G -nitro-L-arginine on NOS activity in rat liver tissue injured by ischemic-reperfusion. SO: sham operation, IR: ischemia-reperfusion, IRCE: ischemia-reperfusion with vitamin C and vitamin E, IRA: ischemia-reperfusion with L-arginine, IRNA: ischemia-reperfusion with N^G -nitro-L-arginine.

IR group ($p=0.028$ and $p=0.047$, respectively), and higher than those of the IRCE group ($p=0.117$ and $p=0.009$, respectively). The concentrations of the IRNA group were markedly decreased compared with the IR group ($p=0.016$ and $p=0.009$, respectively).

i-NOS activities in liver tissue

The activities of the IR group were lower at 1 hr of reperfusion and higher at 6 hr of reperfusion than those of the SO group, but the difference were not statistically significant ($p=0.147$ and $p=0.061$, respectively) (Fig. 5). The activities of the IRCE group were higher than those of the IR group, but the differences were not significant ($p=0.113$ and $p=0.126$, respectively). The activity of the IRA group after 1 hr was higher than that of the IR group ($p=0.044$). The activities of the IRNA group were lower than those of the IR group at 1 hr and 6 hr of reperfusion ($p=0.053$ and $p<0.0001$, respectively).

DISCUSSION

The hepatic IRI model used in this study was portal triad occlusion for 30 min with a subsequent reperfusion period of 60 and 360 min. Without porto-systemic shunt operation, the mortality of rats was over 50% and severe mesenteric vascular congestion and edema was observed in all surviving rats. With this shunt operation, mesenteric vascular congestion was markedly reduced and no rat died.

Vitamin C and vitamin E were used as antioxidants. Vitamin C is known to participate in antioxidant action more rapidly than bilirubin, urate, the sulfhydryl group and α -tocopherol, and lipid peroxidation occurs after vitamin C has been depleted (3). Vitamin C repairs vitamin E, thereby permitting vitamin E to recycle (16). A high dose of vitamin C (14 mg/kg·hr) is

necessary to detoxify the large amount of oxygen radicals in burn (17). The usual dosage is ineffective (18). This experiment also used a high-dose of vitamin C. It was demonstrated that a high-dose vitamin C with pre-treatment of vitamin E was very effective in preventing catalase exhaustion and protecting against IRI in the liver. A few studies reported that administration of antioxidants afforded complete protection against IRI in various organs (5, 19-21).

TNF- α is thought to play some role in IRI because it is highly expressed in the transplanted liver regardless of rejection (22). TNF- α induces i-NOS, although it suppresses constitutive NOS (23).

The TNF- α level in the blood rather than TNF- α gene expression was measured in this study. A greater increase of the TNF- α level after 6 hr of reperfusion rather than 1 hr may indicate that TNF- α does not play an important role in the very early stage of hepatic IRI.

As an index of lipid peroxidation and oxidative tissue injury, MDA is reliable (24). It has been demonstrated that the administration of L-arginine had some antioxidant effects in hepatic IRI. NO acts as a signal to activate glutathione peroxidase and increase reduced glutathione. Glutathione peroxidase and catalase convert H_2O_2 to H_2O and O_2 , and this may explain the mechanism of reducing catalase exhaustion by administration of L-arginine. If this is true, administration of N^G -nitro-L-arginine should show a negative antioxidant effect. However, the same antioxidant effect as that of L-arginine was observed, although the antioxidant effect of N^G -nitro-L-arginine was much weaker than that of L-arginine. Actually NO combines with $O_2^{\cdot -}$ to make $ONOO^{\cdot -}$, and this $ONOO^{\cdot -}$ is known to be more toxic than $O_2^{\cdot -}$. This may suggest another bi-directional action of NO.

Although many reports have demonstrated the protective effect of NO supplement in IRI (8, 10, 11), a few reports proposed that the blocking of NO synthesis gave protection against IRI (5, 25). The result that N^G -nitro-L-arginine had a similar antioxidant effect as that of L-arginine was self-contradictory and should be elucidated in further studies.

The NOS activity was depressed after 1 hr of reperfusion but increased after 6 hr of reperfusion. This induction of i-NOS was thought to be due to oxygen radicals and IL-1 β , and to play a self-protective role against IRI (26). Reducing the oxidative stimulus by administration of an antioxidant would decrease i-NOS induction, but the i-NOS activity of the IRCE group in this study was increased. The reason for this result may be as follows. First, this study was an in vivo experiment, and antioxidant therapy had a more profound protective effect on the i-NOS system. Second, vitamin C may be an inducing factor of i-NOS by itself.

The results of this study suggest the following points. First, both antioxidants and a nitric oxide donor have definite protective effects on ischemia-reperfusion injury of the liver. Second, the administration of antioxidants is a much more

effective method of reducing ischemia-reperfusion injury than the supplement with a NO donor. Further studies are necessary to elucidate the mild antioxidant effect of blocking of NO synthesis in hepatic ischemia-reperfusion injury.

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