Effect of BQ-123 and Nitric Oxide Inhibition on Liver in Rats after Renal Ischemia-Reperfusion Injury

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Abstract. Ischemia-reperfusion (I/R) injury induces an inflammatory response and production of oxygen-derived reactive species which affect many organs including heart, brain, kidney and gastrointestinal tract. The aim of this study was to assess the hepatic changes after renal I/R injury.

Male Sprague Dawley rats were subjected to either sham operation or treatment with L-NAME, L-arginine and BQ-123 during 30 min renal ischemia and 2 h reperfusion injury. Hepatic superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) activities, and thiobarbituric acid-reactive substances (TBARS) and nitric oxide (NO) levels were evaluated to show hepatic response to renal I/R injury.

Catalase and SOD activities showed significant differences between the control and the other groups after I/R. On the other hand, GSH-Px activity did not show any significant changes between the control and the other experimental groups mentioned under above conditions. Meanwhile, levels of TBARS were not different between the control and the other experimental groups, whereas NO level showed changes between the control and experimental groups except the one to which endothelin receptor antagonist agent (BQ-123) subjected.

Experimental period may not be enough to determine the changes in GSH-Px activity and level of TBARS. However, catalase and SOD activities decreased in experimental groups treated by chemical agents. NO level decreased in chemicalagent-applied experimental groups but not in the group to which endothelin receptor antagonist BQ-123 wasapplied alone.

Key words: Renal ischemia-reperfusion — Liver — Antioxidant enzymes — Oxidation

Introduction

The liver plays a central role in a large number of metabolic and immune processes. It has become clear in recent years that normal and pathologic liver functions

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depend on the complex interactions among the heterogeneous sub-populations of liver cells. The liver is composed of true hepatic parenchymal cells (hepatocytes), vascular endothelial cells, resident hepatic macrophages (Kupffer cells), bile duct cells and fat-storing cells (Stellate or Ito cells). All of these cell types control an array of physiological responses and influence neighbouring cells in a number of ways (Taylor et al. 1998).

As the largest organ in the body, the liver commands a substantial fraction of the cardiac output. More than two thirds of this flow enters through the portal vein. However, the very low vascular resistance of the liver and minimal portal vein pressure creates an insufficient top guarantee uniform perfusion of such a large organ. It is therefore likely the mechanisms responsible for the blood distribution in the liver cannot ensure a homogeneous perfusion throughout the liver. Uneven perfusion would not only limit the ability of the liver to efficiently remove toxins from the blood, but could lead to hepatocellular dysfunction in underperfused areas (Baron et al. 2000).

It is now widely accepted that the nitric oxide (NO) pathway is involved in the functional regulation of any organ, tissue and cell in the body (Muriel and Sandoval 2000). NO is an important molecule involved in several different physiological processes such as vasodilatation, neurotransmission, immunological defence and coagulation pathway. Furthermore, NO inhibits proliferation and migration of endothelial and smooth muscle cells (Böhm 2002). In normal rat livers, inhibition of NO synthesis causes a marked increase in perfusion pressure, suggesting that NO maintains hepatic perfusion under physiological conditions (Baron et al. 2000). Having a short half-life, NO is a free radical that may interact with reactive oxygen intermediates to form more toxic species. The reaction of NO with superoxide anion can produce the peroxynitrite anion, which can decompose to generate a strong oxidant such as the hydroxyl radical. Peroxynitrite can induce sulphydryl oxidation and lipid peroxidation (Muriel and Sandoval 2000).

Endothelin (ET) has been the most potent vasoconstrictive peptide so far and characterized by long-lasting action. Initially, the sustained increase in blood pressure elicited by the peptide appeared to suggest involvement of the peptide in mechanism of hypertension or maintenance of blood pressure. Recent reports demonstrated that endogenous ET conferred basal constrictor tone of the peripheral vascular bed and played a fundamental physiological role in the maintenance of blood pressure in humans (Masaki 1998).

It has been reported that ischemia-reperfusion (I/R) induces an inflammatory response which elicits tissue damage in number of organs such as heart, brain and kidney, although the pathophysiological mechanisms leading to acute ischemia renal failure are not well understood. The acute inflammatory response is characterized by induction of proinflammatory cytokinesis, expression of different adhesion molecules and neutrophil infiltration. Recruitment of neutrophil to the sites of inflammation has been shown to be associated with production of cytokinesis such as tumor necrosis factor-α or interleukin-1. Moreover, reactive oxygen species (ROS), which are produced at the sites of inflammation by neutrophils augment the tis-

sue injury by operating with proteases. The other important effect of ROS is the regulation of cytokine gene expression (Serteser et al. 2002).

The aim of this study was to investigate activities of liver antioxidant enzyme and the levels of NO and thiobarbituric acid-reactive substances (TBARS), as an indicator of lipid peroxidation, when NO synthase (NOS) inhibition and ET-A$_1$ receptor blockade were produced during renal I/R injury.

Materials and Methods

Animals

Male Sprague Dawley rats (155–220 g) were used in this study. The rats were bred in Animal laboratory at Inonu University. The rats were quarantined for 8 days at 22–24 ℃ with 12 h light/12 h dark cycle before operation (7 a.m. to 7 p.m.). Standard laboratory chow and water were available *ad libitum*. The experimental protocol was conducted after obtaining permission from local ethical committees (17.12.2002–2002/11).

Surgical procedure

The rats were anaesthetized with urethane (Sigma Chemical Co.) at an intraperitoneal dose of 1.2 g/kg. After the rats were placed on a heating pad, their abdominal regions were shaved with a safety razor and sterilized with povidone iodine solution. Body temperature was maintained at 38 ± 1 ℃. The left carotid artery was cannulated and connected to a pressure transducer (Harvard EM751) to monitor mean arterial blood pressure (MABP). Heart rate and MABP were continuously recorded by a Harvard model 50–8952 transducer and displayed on a Harvard universal pen-recorder. A midline incision was made and Bulldog clamp was applied between abdominal aorta and renal hilus.

- Group 1 control group ($n = 7$): the rats were not undergone any process.
- Group 2 shame operated ($n = 7$): the rats were undergone 30 min ischemia and 2 h reperfusion.
- Group 3 ($n = 7$): the rats were undergone 30 min ischemia and 2 h reperfusion and treated with 20 mg/kg L-NAME (Saito and Miyagawa 2000).
- Group 4 ($n = 7$): the rats were undergone 30 min ischemia and 2 h reperfusion and treated with 1.7 mg/kg BQ-123 during the experiment (Mino et al. 1992).
- Group 5 ($n = 7$): the rats were undergone 30 min ischemia and 2 h reperfusion and treated with both 20 mg/kg L-NAME and 1.7 mg/kg BQ-123 (Mino et al. 1992; Saito and Miyagawa 2000).
- Group 6 ($n = 7$): the rats were undergone 30 min ischemia and 2 h reperfusion and treated with 20 mg/kg L-NAME and 1.7 mg/kg BQ-123 during the experiment and 400 mg/kg L-arginine (L-Arg) (Mino et al. 1992; Myers et al. 1995; Saito and Miyagawa 2000).

At the end of the surgical procedure, the livers were excised and then weighed up. After weighing the livers, homogenate, supernatant and extracted samples were

prepared as described elsewhere (Irmak et al. 2001), and the following determinations were made on the samples using commercial chemicals supplied by Sigma.

Determination of catalase activity

Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi's method (Aebi 1974). The principle of the assay is based on the determination of the rate constant $k\,(\mathrm{s}^{-1})$, or the H_2O_2 decomposition rate at 240 nm. Results were expressed as rate constant *per* gram protein.

Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px, EC 1.6.4.2) activity was measured by the method of Paglia and Valentine (1967). The enzymatic reaction in the tube, which is containing following items: NADPH, reduced glutathione, sodium azide, and glutathione reductase, was initiated by addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units *per* gram protein.

Determination of TBARS level

The tissue TBARS level was determined by a method (Esterbauer and Cheeseman 1990) based on the reaction with thiobarbituric acid (TBA) at 90–100 °C. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances react with TBA producing a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with 2 volumes of cold 10 % (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67 % (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nanomole *per* gram wet tissue according to a standard graph which was prepared from the measurements done with a standard solution (1,1,3,3-tetramethoxypropane).

Determination of superoxide dismutase activity

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. (1988). The principle of the method is based on the inhibition of nitro blue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lyzate after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50 % inhibition in the NBT reduction rate. SOD activity was also expressed as units *per* milligram protein.

Tissue NO levels determination

NO has a half-life of only a few seconds, because it is readily oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) which serve as an index parameter of NO production. The method for plasma nitrite and nitrate levels was based on the
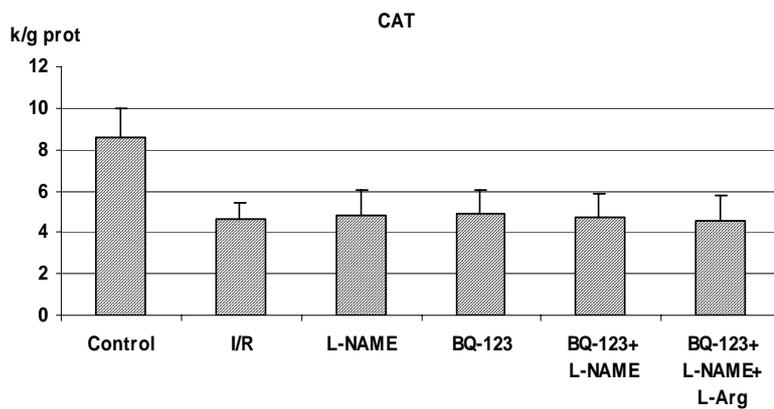
Figure 1. Activities of catalase (CAT) in liver tissue of the groups.

Griess reaction (Cortas and Wakid 1990). Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured by spectrophotometer at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. A standard curve was established with a set of serial dilutions (10^{-8}–10^{-3} mol/l) of sodium nitrite. Linear regression was done by using the peak area from nitrite standards. The resulting equation was used to calculate the unknown sample concentrations. Results were expressed as micromole *per* gram wet tissue.

Determination of protein content

Protein measurements were made at all stages according to the Lowry's method (Lowry et al. 1951).

Statistical analysis

Data were analyzed by using a commercially available statistics software package (SPSS® for Windows version 9.0, Chicago, USA). Distribution of the groups was analyzed with one sample Kolmogrov–Smirnov test. All groups showed normal distribution, so that parametric statistical methods were used to analyze the data. One-way ANOVA test was performed and *post-hoc* multiple comparisons were done with least significant difference. Results were presented as means $\pm$ SEM. p values < 0.05 were regarded as statistically significant.

Results

The results are expressed in Table 1. The activities of catalase and SOD were significantly higher in control group than the other experimental groups ($p < 0.05$). On the other hand, GSH-Px activity was not statistically significant between control and the other experimental groups. Figure 3 shows changes in hepatic GSH-Px

Table 1. Activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and the thiobarbituric acid reactive substance (TBARS) and nitric oxide (NO) levels in I/R (renal ischemia-reperfusion), L-NAME (a nitric oxide inhibitor), BQ-123 (an ET-A receptor antagonist), L-NAME+BQ-123 and L-NAME+BQ-123+L-Arg groups

Groups	CAT (k/g prot.)	SOD (U/mg prot.)	GSH-Px (U/g prot.)	TBARS (nmol/g wet tissue)	NO (nmol/g wet tissue)
Group 1 (Control)	8.622 ± 1.375	0.279 ± 0.027	3.903 ± 2.241	15.514 ± 4.733	0.170 ± 0.026
Group 2 (I/R)	4.635 ± 0.773	0.171 ± 0.021	3.750 ± 1.461	17.662 ± 3.420	0.176 ± 0.025
Group 3 (L-NAME)	4.856 ± 1.149	0.181 ± 0.028	3.590 ± 0.985	17.227 ± 2.309	0.108 ± 0.017
Group 4 (BQ-123)	4.888 ± 1.152	0.180 ± 0.034	3.841 ± 1.514	17.608 ± 2.679	0.180 ± 0.014
Group 5 (L-NAME+ BQ-123)	4.704 ± 1.207	0.177 ± 0.025	3.835 ± 1.168	17.550 ± 1.853	0.113 ± 0.010
Group 6 (L-NAME+ BQ-123+L-Arg)	4.597 ± 1.190	0.182 ± 0.036	3.695 ± 0.928	17.682 ± 1.916	0.113 ± 0.011
			p		
1–2	0.036	0.030	n.s.	n.s.	n.s.
1–3	0.047	0.047	n.s.	n.s.	0.030
1–4	0.049	0.046	n.s.	n.s.	n.s.
1–5	0.039	0.040	n.s.	n.s.	0.044
1–6	0.034	0.050	n.s.	n.s.	0.044
2–3	n.s.	n.s.	n.s.	n.s.	0.015
2–4	n.s.	n.s.	n.s.	n.s.	n.s.
2–5	n.s.	n.s.	n.s.	n.s.	0.023
2–6	n.s.	n.s.	n.s.	n.s.	0.023
3–4	n.s.	n.s.	n.s.	n.s.	0.003
3–5	n.s.	n.s.	n.s.	n.s.	n.s.
3–6	n.s.	n.s.	n.s.	n.s.	n.s.
4–5	n.s.	n.s.	n.s.	n.s.	0.005
4–6	n.s.	n.s.	n.s.	n.s.	0.005
5–6	n.s.	n.s.	n.s.	n.s.	n.s.

prot., protein; k, rate constant; n.s., non significant; $p < 0.05$ was considered as statistically significant
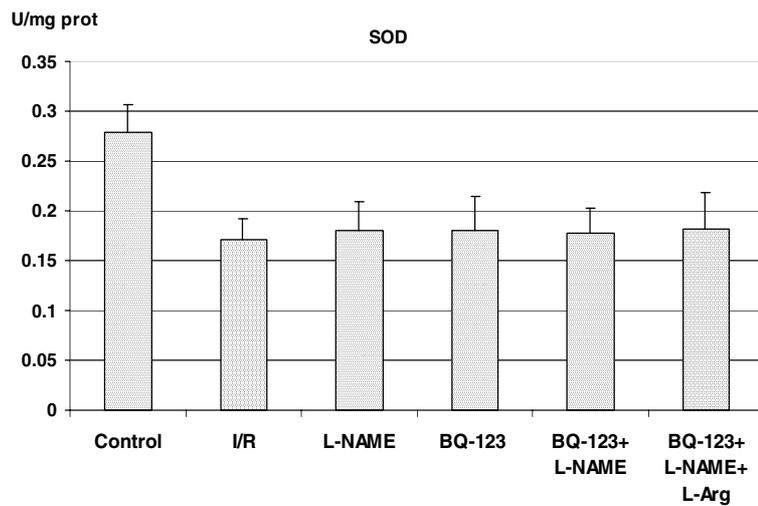
Figure 2. Activities of superoxide dismutase (SOD) in liver tissue of the groups.
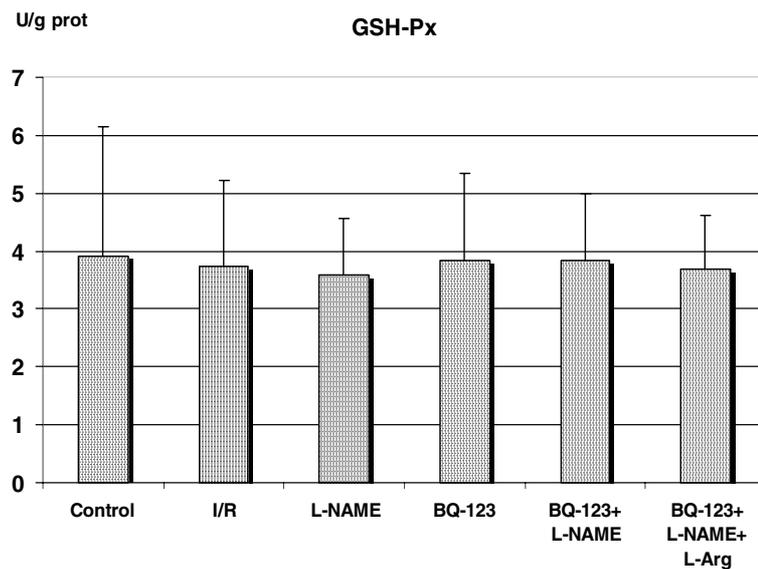

Figure 3. Activities of glutathione peroxidase (GSH-Px) in liver tissue of the groups.

level. There was also no significant difference in TBARS level, index of lipid per-oxidation between groups, either (Figure 4). However, level of NO was increased in I/R+BQ-123 group in comparison with other groups except control and IR
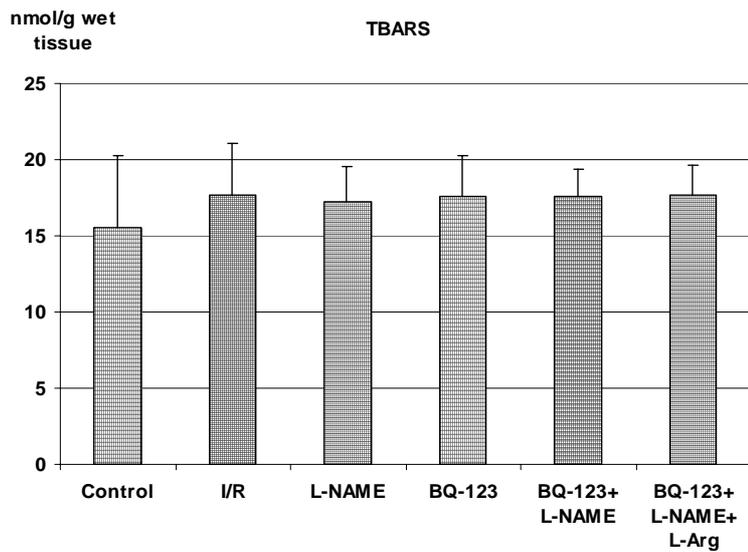
Figure 4. Level of thiobarbituric acid reactive substance (TBARS) in liver tissue of the groups.
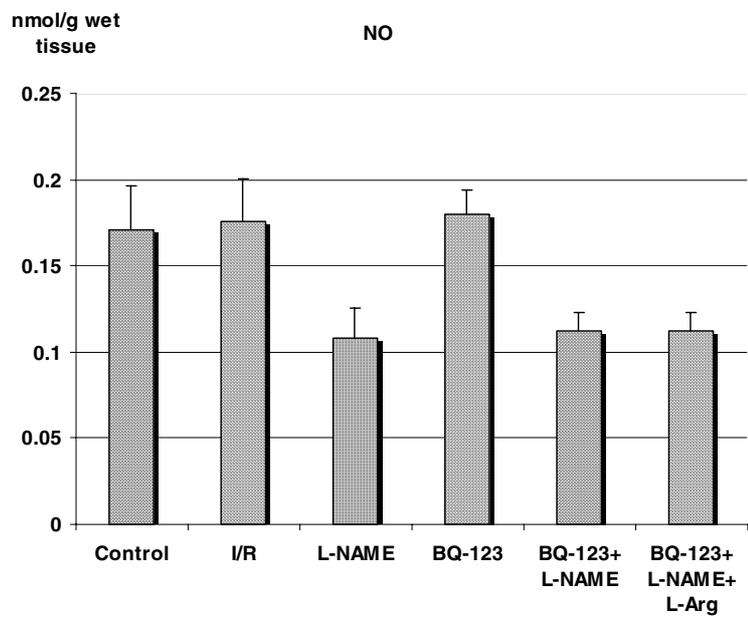
Figure 5. Level of nitric oxide (NO) in liver tissue of the groups.

groups ($p < 0.05$). There was significant difference in NO level between control and I/R+L-NAME, I/R+L-NAME+BQ-123, I/R+L-NAME+BQ-123 +L-Arg groups. However, NO level of control group was not significantly different from Groups 2 and 4. But, NO levels of Groups 5 and 6 were lower than the other groups except Group 3 (Figure 5) ($p < 0.05$).

Discussion

It has been known that during ischemia, cells and tissues undergo rapid changes which lead to perturbations in signalling pathways and surface molecule expression. Depending on the time and severity of ischemia, toxic products accumulate intracellular, leading to apoptosis and necrosis, resulting in loss of organ function. During the time of reperfusion, accumulated toxic metabolites are flushed into the system, which may affect other organs and may negatively influence the process of regeneration in the ischemic organ (Riedemann and Ward 2003). On injury due to ischemia, two major components involved in events leading to injury are well know, namely, complement activation and neutrophil stimulation with accompanying oxygen radical-mediated injury. Under ischemic condition, reduced oxygen supply leads to enhanced neutrophil adherence to endothelial cells due to increased surface expression of adhesion molecule on endothelial cells. This ultimately results in diapedesis of neutrophil and their oxidative burst, which result in oxygen radical production. These events are thought to contribute to the tissue damage during I/R in various organs (Riedemann and Ward 2003).

NO is produced from the amino acid L-Arg in a reaction catalyzed by enzymes NOS (Muriel and Sandoval 2000). Three NOS have been identified: two constitutive (cNOS: type 1 or neuronal and type 3 or endothelial) and one inducible (iNOS: type 2). The activity of cNOS is normally detectable in Kupffer cell, whereas no cNOS is ever encoded in hepatocytes. However, hepatocytes, Kupffer and stellate cells are prompted to express an intense iNOS activity once exposed to effective stimuli such as bacterial lipopolysaccharide (LPS) and cytokines. There is a close interaction between ET-1 and NO: ET-1 stimulates NO release by activation of endothelial NOS (eNOS) *via* the ET-B receptor, while NO inhibits ET-1 release by blocking ET-1 gene expression (Hirata and Ishimaru 2002).

As shown in Table 1, activities of CAT and SOD were significantly different from control group and other groups. Hepatic CAT and SOD activities were observed to decrease in all experimental groups compared to control group (Figures 1 and 2). Similar results have been reported by Sun et al (2004). It can be said that using the same I/R period for all groups, the activities of SOD and CAT are affected. This was also verified by Serteser et al. (2002) showing that using different I/R period resulted in different level of SOD and CAT activities. As a result, period of I/R is a crucial parameter for determination of antioxidant enzymes level. On the other hand, GSH-Px activity was similar among I/R groups and the others. However, this result is contradictory to the findings of authors Serteser et al. (2002) and Sun et al. (2004). Due to the fact that I/R period was same in all groups,

level of superoxide anion produced by neutrophils was same leading to insignificant changes in the GSH-Px level which acts on H_2O_2 to convert it into water. Using some chemicals such as BQ-123 and L-NAME may affect the production of NO, which reacts with superoxide anion. The reaction between NO and superoxide anion can produce the peroxynitrite anion, which can decompose to generate a strong oxidant such as the hydroxyl radical. Therefore, using of such chemicals can also affect GSH-Px activity. Additionally, it is well known that membrane lipid peroxidation is induced by oxidative stress (I/R, in turn, it will cause the changes in structure of membrane and changes in the activities of essential antioxidant enzymes. Therefore, antioxidant enzymes activities change depend on more than one condition.

As can be seen from the Table 1, renal I/R resulted in a constant level of hepatic tissue TBARS, indicating changes in lipid oxidation among I/R and other groups. This result was also different from those in the literature. According to Serteser et al. (2002), the rate of lipid oxidation changes depends on I/R period.

NO is an unstable free radical produced in the liver at low levels by constitutive eNOS and at high levels by iNOS unregulated by LPS and cytokines in both parenchymal and non-parenchymal cells. NO exerts protective or toxic effects. iNOS-dependent production of NO contributes to liver cell necrosis in I/R and shock (Kaplowitz 2000). NO production is known as is an important parameter for tissue injury. We found that hepatic NO level was decreased by administering NO inhibitor to rats subjected to renal I/R. These results confirmed those of Tome et al. (1999) and Yang et al. (2003). NO level of I/R group was higher than other groups except those treated with BQ-123, an endothelial receptor antagonist. Neutrophils are accumulated in various organs after I/R (Kelly et al. 1996; Miyazawa et al. 2002). Several mechanisms may contribute to neutrophils-mediated reperfusion injury. Activated neutrophils can release cytokines, ROS, proteases, elastases, myeloperoxidase, and other enzymes. These substances upregulate adhesion receptors, are chemotactic, increase vascular permeability for other inflammatory cells, damage tissue directly and impair endothelial function. Neutrophils increase injury to endothelial cells subjected to anoxia and reoxygenation (Kelly et al. 1996).

Increased NADPH oxidase activity of activated phagocytic cells (Kupffer cells, macrophages, neutrophiles) release reactive oxygen metabolites that can attack neighboring cells (Kaplowitz 2000) and hepatic endothelial cells, Ito cells and macrophages can also generate NO through iNOS expression (Taylor et al. 1998; Alcaraz and Guillen 2002).

On the other hand, L-Arg treatment could not recover the inhibition of NO synthesis due to L-NAME treatment in this study. We thought that this dose of L-NAME resulted in inhibition of NO production despite 400 mg/kg dosage of L-Arg treatment. This might indicate that NO production is likely reduced because of reduced availability of the NO substrate L-Arg. Tome and co-workers demonstrated that L-NAME treatment caused decreased NO production in proximal tubules of rats that underwent to renal I/R injury (Tome et al. 1999).

In conclusion, it is very clear that the renal I/R period has a significant effect

on the liver injury. That is to say, renal I/R period can increase or decrease accumulation of neutrophils, which produces superoxide anion that can create ROS which could cause multiorgan failure. According to our results, chemical substances such as L-NAME alone and L-NAME plus BQ-123 inhibit NO production which can protect organs against any damage due to the existence of peroxylnitrit.

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