EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 29, No 1, 19-23, March 1997

Modulation by aspartate of ischemia/reperfusion-induced oxidative stress in rat liver

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Accepted 6 January 1997

Abbreviations: XO, xanthine oxidase; XDH, xanthine dehydrogenase; TBARS, thiobarbituric acid-reactive substance; TBA, thiobarbituric acid; AST, aspartate aminotransferase; MDH, malate dehydrogenase

Abstract

Ischemia-reperfusion injury is related with oxygen free radicals; a reason which has been suggested for this is the conversion of xanthine dehydrogenase (XDH) into xanthine oxidase (XO). In the present study, metabolic control of the enzymic conversion by modulating the cellular redox potential was attempted. An amino acid, aspartate, was tested as a possible candidate on the assumption that as a participant in the malate/aspartate shuttle, it might modify the cellular NADH/NAD+ balance. Its effect was studied by measuring the level of lipid peroxidation as a thiobarbituric acid-reactive substance (TBARS) and the conversion ratio of XDH to XO in the perfused-rat livers. The experimental animals, male Sprague Dawley rats were divided into three groups: control, ischemia and ischemia/reoxygenation. To each group, aspartate was infused at 2 mM level. ischemia alone did not affect the level of TBARS or the conversion ratio of the enzyme, regardless of aspartate infusion. In contrast, reoxygenation of previously ischemia liver significantly elevated the level of TBARS and decreased the ratio of XDH to XO; both this level and this ratio were ameliorated by aspartate. The protective role of aspartate against oxidative stress induced by ischemia/ reoxygenation can be explained by the fact that aspartate may correct the increased NADH/NAD ratio by facilitating NAD regeneration from NADH through the coupled aspartate aminotransferase/malate dehydrogenase reaction and the malate-aspartate shuttle. Aspartate application may thus contribute to the development of a preventive strategy against ischemia/reperfusion-induced oxidative damages.

Key words: ischemia/reperfusion injury, rat liver, oxidative stress, aspartate, xanthine oxidase

Introduction

Ischemia/reperfusion injury is associated with the production of oxygen-derived free radicals inducing lipid peroxidation, which cause alterations in biomembrane-associated functions of the cell or subcellular organelles. Ischemia/reperfusion-induced radical generation has been partially explained by the conversion of xanthine dehydrogenase (XDH) into xanthine oxidase (XO) (Parks *et al*., 1982; Roy and McCord, 1983). XDH can be converted to XO irreversibly by proteolysis or reversibly by sulfhydryl oxidation during the ischemia/reperfusion process. And the ischemia causes the cellular increase of NADH, which has been shown to inhibit the activity of NAD^+-dependent XDH (Ballard, 1971; Kato *et al*., 1990). In consequence, the oxygen-dependent XO pathway is activated to oxidize hypoxanthine and xanthine with a burst of superoxide. Moreover, the increased breakdown of ATP during ischemia aggravates accumulation of xanthines, requiring their metabolic conversion to uric acid by xanthine oxidase (Kamiike *et al*., 1982; Engerson *et al*., 1987)

It was recently reported that treatment of previously hypoxic hepatocytes with ethanol increased cellular toxicity (Khan and O'Brien, 1995). Ethanol further aggra-vated the ischemia-induced aberrant ratio of NADH to NAD^+, since the bulk of the reducing power (NADH) is produced during ethanol oxidation. It is therefore reasonable to speculate that the involvement of both ischemia and ethanol in the alteration of the cellular redox state is one of the important factors in free radical-mediated liver injury. The control of cytosolic NADH in relation to the conversion of XDH to XO can thus contribute to the development of new therapeutic strategies against ischemia/reperfusion injury.

We have recently suggested that aspartate can protect against oxidative stress-induced tissue damage in ethanol-perfused rat livers (Park and Park, 1995; Park *et al*., 1996). Aspartate reduces the ethanol-induced conversion of XDH into XO by modulating the cytosolic NADH/NAD ratio, since aspartate is a direct substrate for the malate-aspartate shuttle, which is the main pathway for transferring reducing equivalents originating from the cytosol to the mitochondria.

It is therefore assumed that aspartate supplementation would also contribute to restoring the cellular balance of

NADH/NAD, disturbed during ischemia/reperfusion, through which oxidative tissue damage might be reduced. In the present investigation, aspartate was infused to rat liver during the ischemia/reoxygenation period, and the level of lipid peroxidation and activities of XDH and XO were monitored in order to evaluate the modulation of ischemia/reperfusion-induced liver damage.

Materials and Methods

Experimental protocol

Rats were divided into three main groups: control, ischemia, and reoxygenation, as shown in Figure 1. Each group was subdivided according to aspartate supplementation in perfusion into a (without) and b (with aspartate). In the control group, after 15 min of washing, the livers were perfused with oxygenated buffer for 60 min. Hypoxia of the liver was produced by replacing oxygen-saturated buffer with nitrogen-saturated buffer. During the first 30 min of perfusion the ischemia state was maintained. Reoxygenation of the ischemia liver was achieved by replacing the perfusate with the oxygen-saturated buffer. Each group was also infused with 2 mM aspartate from the starting point or after the ischemia state.

Liver Perfusion

Male Sprague-Dawley rats weighing 240 ± 10 g were used for all experiments. An *in situ* perfusion technique employed was a modification of that previously described (Reinke *et al*., 1982; Deaciuc *et al*., 1992). The portal vein and the inferior vena cava were cannulated; the basic perfusate consisted of a standard Krebs-Ringer bicarbonate buffer containing 0.1% glucose at pH 7.4. For one hour prior to and throughout the perfusion, the perfusate was gassed with a mixture of 95% O_2 and 5% CO_2, and stirred at a hydrostatic pressure of 20 cm of water to ensure complete saturation with oxygen; its flow rate was 5 ml/min.

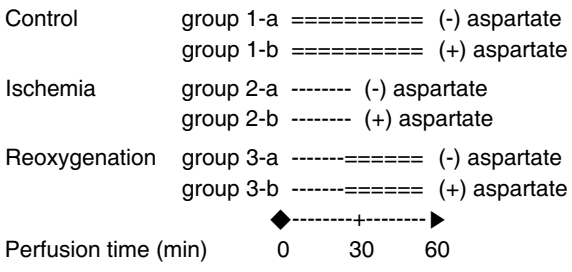

Figure 1. Diagram of experimental procedures. Single dotted lines indicate the ischemic state, while double dotted lines, reoxygenation state.

Sample Preparation

After perfusion, livers were excised and kept frozen at -70˚C. In order to analyze for lipid peroxidation, 10% (w/v) liver homogenates were prepared in 0.15 M NaCl. Meanwhile, 0.1 M Tris-HCl buffer (pH 8.1) containing 0.1 mM EDTA, 0.2 mM phenylmethanosulfonyl fluoride, 1 mM benzamidine hydrochloride, 1 mM aprotinin and 1 mM dithiothreitol were used for XDH and XO assay.

Analytical procedure and enzyme assay

Lipid peroxidation was assessed by the measurement of thiobarbituric acid-reactive substance (TBARS) (Esterbaur *et al*., 1990). The homogenates were added to an equal volume of thiobarbituric acid (TBA) reagent (0.375% TBA, 15% trichloroacetic acid, 0.25 M HCl). After mixing, samples were heated for 15 min in a boiling water bath and centrifuged at 800 *g* for 10 min after cooling. The supernatant was monitored for its absorbance at 535 nm, and its TBARS (malondialdehyde, MDA) content was determined by using an extinction coefficient of 156,000 $M^{-1}cm^{-1}$.

XDH and XO activities were measured using a slight modification of the methods described previously (Kato *et al*., 1990; Waud *et al*., 1976). The samples (100 μl) was added to the reaction mixture containing 0.2 mM xanthine, 0.1 M Tris-HCl buffer (pH 8.1) with 0.1 mM EDTA in a final volume of 1 ml. Activity was monitored by reading optical densities at 295 and 340 nm in the absence or presence of 0.5 mM NAD^+ at 30˚C. One unit (U) of the activity of either enzyme is defined as the amount of enzyme required to produce 1 μmol of NADH or 1 μmol of uric acid per min, respectively. Specific activity was calculated as milliunit (mU) per mg protein. Protein was determined by the method of Lowry *et al*. (1951).

Results

In the present study, rats were divided on the basis of perfusion time and pattern into three groups as follows: control, ischemia and reoxygenation. Each group was also divided into two subgroups, depending on the addition of aspartate (Figure 1). The level of lipid peroxidation in each group is shown in Table 1. In control groups, the amounts of TBARS were 0.174 ± 0.009 and 0.166 ± 0.013 nmol/mg protein, respectively, showing no significant difference in the absence or presence of aspartate. Their amounts of TBARS were slightly less than those in the ischemia groups but not significant ($P > 0.05$). In the ischemia groups, the addition of aspartate did not cause any significant changes in the level of lipid peroxidation ($P > 0.05$). However, the amount of TBARS in the reoxygenation group in the absence of aspartate was 0.214 ± 0.003 nmol/mg protein, resulting in 25% increase compared to the control and ischemia

groups. This increase was not observed in aspartate-treated reoxygenation group. The amount of TBARS in this group was 0.180 ± 0.001 nmol/mg protein. These results indicate that aspartate may play a role in preventing ischemia/reperfusion injury by reducing the level of TBARS.

To identify their role in the change of TBARS levels, specific activities of the enzymes, XDH and XO, were measured and the degree of conversion of XDH to XO was expressed as a ratio, as shown in Table 2. In control groups, the ratio of XDH to XO was about 7.17 to 7.7 without any significant difference. A similar tendency was observed in the ischemia groups, where the ratio was 6.7 to 6.9 in the absence or presence of aspartate. Decrease in the ratio of XDH to XO was observed in the reoxygenation group (30 min of oxygenated reperfusion following 30 min ischemia) compared to the control and ischemia groups. In addition, the ratios in the reoxygenation group were significantly different in response to aspartate infusion, being 4.36 for non-aspartate treatment and 5.22 for aspartate treatment, respectively ($P < 0.05$). This indicates that aspartate infusion can partially correct the ischemia/reperfusion-induced decrease of the XDH/XO ratio.

Discussion

The effect of aspartate infusion on ischemia/reperfusion induced lipid peroxidation in the liver is shown in Table 1. The levels of TBARS, indicative of lipid peroxidation, was not much different between ischemia groups and control groups. The aspartate treatment to both groups did not cause any changes in the level of TBARS. However, it was found that TBARS level in the reoxygenation group was about 25% higher than those in the control or ischemia groups. The major mechanism for ischemia/reperfusion injury was suggested to be the activation of XO. It is thus reasonable to consider modulation of conversion of XDH to XO to reduce the elevated level of TBARS.

In the present study, measured activity of XDH was consistent with that previously reported, showing approx. 90% activity of total XDH plus XO (Marubayashi *et al*., 1991). At the final time point, after 1 h ischemia/reoxygenation, approximately 20% of total enzyme is present in XO form (Table 2). These results thus indicate that the increased activity of XO was induced by the subsequent reoxygenation of the previously ischemic state. The increased activity of XO may be due to conversion of XDH into XO, as suggested by no significant changes of the total activity of XDH plus XO in those groups.

It has been generally accepted that energy-rich adenine nucleotides are rapidly catabolized to hypoxanthine as oxygen deprivation occurs during ischemia (Malis & Bonventre, 1986). Subsequent metabolism of purines by XO causes oxidative tissue damages. On the basis of this fact, the inhibition of XO and the salvage of substrates for nucleotides have been suggested as a mechanism to protect against ischemia/reperfusion injury (Manning *et al*., 1984; Stein *et al*., 1990). On the other hand, an increase in ischemia-induced NADH inhibits NAD^+-dependent XDH activity. As mentioned above, increased NADH is associated with the increased breakdown of nucleotide and a shift from the XDH to the XO pathway in ischemia-reperfusion injury (Ballard, 1971; Engerson *et al*., 1987; Kato *et al*., 1990). NADH after ethanol treatment has been shown to inhibit XDH activity *in vitro* as well as *in vivo*; a similar observation in an ethanol study has shown enhanced degradation of purine, providing substrates for XO. It has recently been

Table 1. Levels of lipid peroxides in homogenate prepared from perfused rat liver. All the values are means ± SD for three to four rats per group.

	TBARS (nmol/mg protein)
Control groups	
Group 1	0.174 ± 0.009
Group 2	0.166 ± 0.013
Ischemia groups	
Group 3	0.148 ± 0.010[a]
Group 4	0.159 ± 0.006[a]
Reoxygenation groups	
Group 5	0.214 ± 0.003[b]
Group 6	0.180 ± 0.001[b,c]

[a] $P > 0.05$ versus control groups.
[b] $P < 0.05$ versus control groups.
[c] $P < 0.05$ versus group 5.

Table 2. Comparison of activities of XDH and XO in perfused rat liver tissues. Units of enzymatic activities are mU/mg protein. All the values are means ± SD for three to four rats per group.

	XDH	XO	XDH/XO
Control groups			
Group 1	12.45 ± 0.15	1.74 ± 0.11	7.17 ± 0.36
Group 2	16.25 ± 2.15	2.18 ± 0.55	7.69 ± 0.96
Ischemia groups			
Group 3	14.25 ± 0.05	2.08 ± 0.04	6.86 ± 0.04[a]
Group 4	15.20 ± 0.40	2.30 ± 0.36	6.74 ± 1.24[a]
Reoxygenation groups			
Group 5	12.30 ± 0.40	2.82 ± 0.16	4.36 ± 0.37[b]
Group 6	12.05 ± 0.95	2.37 ± 0.33	5.22 ± 0.20[b,c]

[a] $P > 0.05$ versus control groups.
[b] $P < 0.05$ versus control groups.
[c] $P < 0.05$ versus group 5.

reported that the treatment of previously ischemia hepatocytes with ethanol increased cellular toxicity (Khan *et al*., 1995); this was explained by the fact that ethanol further increased the cellular aberrant ratio of NADH to NAD^+ induced by ischemia. In the present study, modulation in the altered redox state was assumed to be one of the mechanisms protecting against ischemia/reperfusion injury.

As shown in our previous papers (Park & Park, 1995; Park *et al*., 1996), aspartate was effective in modulating the increased ratio of NADH to NAD^+ in ethanol-perfused rat liver by activating the malate-aspartate shuttle. That aspartate modulated the altered redox state in ethanol-perfused liver was evidenced by the change in the lactate/pyruvate ratio in vascular effluents, a change which accurately reflects the cellular-free NAD^+/NADH ratio. As a result of the corrected ratio of NADH/NAD^+ resulting from aspartate treatment, the increased XO activity resulting from ethanol infusion was significantly reduced with lower level of oxidative stress than control group. It is conceivable that aspartate may correct the increased NADH/NAD^+ ratio by facilitating NAD^+ regeneration from NADH through AST- and MDH-coupled reactions and by augmenting the malate-aspartate shuttle between the cytosol and mitochondria (Park, 1993). By the same reason, it would be possible to extend the idea that NAD^+/NADH imbalance caused by any other reason might be corrected by aspartate supplementation. One of the typical disorders associated with the nucleotide coenzyme imbalance is the ischemia/ reperfusion injury, where the resulting oxidative tissue damage is well known.

Thus, in the present study, the ischemia/reperfusion injury in the liver tissue was studied to elucidate whether aspartate infusion would ameliorate the oxidative injury. As expected, the addition of aspartate to the liver perfusion system during the whole period of ischemia/ reoxygenation resulted in a reduced level of TBARS, and simultaneously decreasd the ratio of XDH to XO compared to that without aspartate ($P < 0.05$). The significant prevention by aspartate of lipid peroxidation in this ischemia/reperfusion liver tissue could be directly related with the inhibition of enzymic conversion of XDH to XO due to aspartate-induced cellular redox changes. Furthermore, the general possibility of modulating cellular ratio of NADH to NAD^+ by simple application of an amino acid suggests the wider application to varying ischemia/ reperfusion injuries.

In conclusion, our results indicate that aspartate would be effective in reducing the level of oxidative stress induced by ischemia/reperfusion. The effective role of aspartate in oxidative stress was shown by the reduced level of TBARS and the normalization of XDH to XO ratio. This result may thus contribute to the development of a possible therapy in the pathology generated by ischemia and reperfusion such as in the cases of organ transplantation, myocardiac infarction, muscle degeneration and various thrombo-embolic disorders, etc.

Acknowledgement

This work was partially supported by the grants from the Ministry of Education for Basic Medical Research Promotion Fund and from the Korea Research Foundation for Health Science.

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