Protective Effect of Ulinastatin against Liver Injury Caused by Ischemia-Reperfusion in Rats

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ABSTRACT—The effects of ulinastatin (ULN), a human urinary protease inhibitor, on liver injury caused by ischemia-reperfusion were studied in rats. In the liver ischemia-reperfusion model, ULN suppressed the elevation of serum transaminase levels and tissue lipid peroxide levels in the liver. ULN did not exhibit a radical-trapping action on the superoxide and hydroxyl radicals as measured by electron spin resonance (ESR). ULN suppressed formylmethionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA)-induced superoxide production from polymorphonuclear leukocytes (PMNs) as measured by the cytochrome c assay. ULN did not inhibit either xanthine oxidase (XO) activity or the conversion of xanthine dehydrogenase (XDH) to XO during the ischemic period. ULN also strongly protected against the hypotonic hemolysis of rat erythrocytes. These results suggest that ULN's membrane stabilizing action and suppressive effect against PMNs superoxide production might be attributed to its suppressive effect on the liver's lipid peroxidation caused by ischemia-reperfusion.

Keywords: Ulinastatin, Ischemia-reperfusion, Lipid peroxidation, Polymorphonuclear leukocyte, Membrane stabilization

Oxygen-derived free radicals induced by ischemia-reperfusion have recently attracted increased attention as factors which exacerbate cellular injury in the liver (1, 2). Ischemia itself can cause tissue damage and eventual death; however, reintroducing oxygen to the tissue can result in further injuries. During the ischemic period, adenosine triphosphate (ATP) is catabolized to hypoxanthine, which accumulates in the tissues, and xanthine dehydrogenase (XDH), which is highly concentrated in the venular endothelium, is converted to xanthine oxidase (XO) via a protease. In the presence of oxygen, xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine and simultaneously generates superoxide (3). Another important source of free radical production is believed to be the generation of active oxygen species from polymorphonuclear leukocytes (PMNs) (4).

Recently, it has been reported that some protease inhibitors had a radical-trapping action or suppressed superoxide production from PMNs (5–8). In addition, protease inhibitors suppressed the conversion of XDH to XO via a protease (9). Ulinastatin (ULN), an acidic glycoprotein with a molecular weight of 67,000, is a protease inhibitor purified from fresh human urine (10, 11). ULN suppresses proteases such as trypsin, chymotrypsin and elastase (12). ULN also stabilizes lysosomal membranes and suppresses the release of lysosomal enzymes (13). Moreover, ULN is beneficial in the treatment of experimental acute pancreatitis and shock, and it has been used clinically as an antishock drug (12, 13). Therefore, it is of great interest whether ULN suppresses the tissue injury induced by oxygen-derived free radicals. In the present study, we examined the effects of ULN on superoxide production from the hypoxanthine-xanthine oxidase (HPX-XO) system and PMNs, and also investigated the effects of ULN against lipid peroxidation in the liver caused by ischemia-reperfusion.

MATERIALS AND METHODS

Animals

Male Wistar rats (200–250 g; Seiwa Laboratory Animals, Inc., Japan) were housed in an environmentally-controlled room (20–23°C, 50–60% humidity, illuminated from 7:00 to 19:00 hr) with food and water available ad libitum. ULN (100,000 U/kg) or physiological saline solution (0.5 ml/200 g body weight) was
gradually injected via the tail vein 5 min before the induction of ischemia.

**Preparation of ischemic rats**

To prepare the ischemic rats, the abdomen was opened with a middle incision under light ether anesthesia, and the left portal vein and hepatic artery were occluded with a microvessel clip. The abdomen was then closed and the animals were allowed to awaken. After 15 min of liver ischemia, the vascular clip was released, and the right lateral and caudate lobes were removed, leaving only the ischemic left lateral and median lobes intact (14). After 60 min of liver reperfusion, the rats were sacrificed by decapitation. Sham-operated rats, which experienced resected right lateral and caudate lobes but not the ischemic procedure, were used as controls.

**Assay of lipid peroxides**

The liver was perfused with 0.9% NaCl via the left ventricle to the inferior vena cava before excision. The lipid peroxides formed in the liver were determined according to the method of Ohkawa et al. (15). A reaction mixture containing 0.1 ml of 10% tissue homogenate was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetate buffer (adjusted to pH 3.5 with NaOH) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. After heating at 95°C for 60 min in a water bath, 5 ml of a solution of n-butanol-pyridine (15:1) was added, and the resulting mixture was mixed vigorously. After centrifugation at 3000 rpm for 10 min, the optical density of the organic layer was measured at 532 nm with a spectrophotometer. Tetramethoxypropane was used as a standard for the assay, and the lipid peroxide content was expressed as nmol of malondialdehyde (MDA) formation per mg protein.

**Assay of serum GOT, GPT and LDH levels**

The blood samples were collected after 60 min of reperfusion, and serum markers such as glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) activities were measured with a Shimadzu CL20 Auto Analyzer.

**Radical-trapping ability**

The radical-trapping ability of ULN was estimated by the electron spin resonance (ESR) spectrometer (JEOL JES FE-1X) using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) as a radical-trapping reagent (16, 17). For determining the superoxide anion-trapping ability, 15 μl of 9.2 M DMPO was mixed with 50 μl of 2 mM hypoxanthine in phosphate buffer solution (PBS), pH 7.4, 35 μl of 5.5 mM diethylenetriaminepentaaetic acid (DETPAPAC) in PBS, and various concentrations of ULN (50 μl). Then, 50 μl of 0.4 U/ml xanthine oxidase was added and the solution was mixed. The reaction mixture was transferred into a quartz cell and ESR spectra of DMPO-OOH, the spin-trapped adduct of the superoxide anion, were observed with the spectrophotometer. For determination of the hydroxyl radical-trapping ability, 20 μl of 0.92 M DMPO was mixed with 75 μl of 1 mM FeSO4 plus 1 mM DETAPAC in PBS, 75 μl of 1 mM hydrogen peroxide and various concentrations of ULN. The ESR spectra of DMPO-OH, the spin-trapped adduct of the hydroxyl radical, were observed with the spectrophotometer.

**Measurement of superoxide generation from PMNs**

After 15 min of ischemia followed by 60 min of reperfusion of the liver, the blood was collected from the inferior vena cava with a heparinized syringe. The concentration of neutrophils in Hank’s solution was then adjusted to 3 × 10⁷ cells/ml. Superoxide production was measured by the cytochrome c method (18). Four hundred microliters of Krebs Ringer phosphate buffer with 5.4 mM glucose, 20 μl of 1.3 mM ferricytochrome c, 100 μl of cell suspension (3 × 10⁶ cells) and various concentrations of ULN were preincubated at 37°C for 3 min. The cells were then stimulated by formylmethionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA) at a final concentration of 1.9 × 10⁻⁷ M and 190 ng/ml, respectively. The reduction of ferricytochrome c was measured by the increase in optical density at 550 nm using a dual beam spectrophotometer (Hitachi, model 557) for 3 and 5 min after addition of FMLP and PMA, respectively. The amount of superoxide production was calculated using a molar extinction coefficient of 21.0 × 10³ M⁻¹ cm⁻¹.

**Measurement of XO and XDH activities**

XO and XDH activities were measured according to the method described by Della Corte and Stirpe (19). The livers were homogenized with 5 ml of ice-cold 0.1 M Tris buffer (pH 8.1) per g of liver. The homogenate was centrifuged at 600 × g for 20 min at 4°C. The supernatant solution was recentrifuged at 105,000 × g for 60 min at 4°C, and the remaining supernatant solution was dialyzed against three changes of 100 volumes of 0.1 M Tris buffer (pH 8.1) for 3 hr at 4°C. The dialyzed solution was used for determining enzyme activities. Dia
yzed solution (0.2 ml) was added to 0.6 ml of 0.3 mM xanthine and 2.2 ml of 0.136 M Tris buffer, pH 8.1. The XO and XDH activities were determined spectrophotometrically at 292 nm by examining urate formation without NAD⁺ (XO activity) and with NAD⁺ (XDH activity).
+ XO activity). The enzyme activities were expressed in milliunits (mU), with one unit corresponding to the formation of 1 μmol of uric acid/min.

**Stabilizing effect on erythrocyte membrane**

Protective effects of ULN on hypotonic hemolysis were performed according to a modification of Parpart's method (20). Phosphate buffer containing the appropriate amount of NaCl adjusted to induce 70% hemolysis was prepared. Various concentrations of ULN were dissolved in this phosphate buffer. A 5-ml aliquot of the test drug or control buffer solution containing 50 μl rat erythrocytes was incubated at 37°C for 1 hr and then centrifuged at 2,000 rpm for 5 min. The absorbance of the supernatant at 540 nm was measured by a spectrophotometer.

**Protein determination**

The protein concentration was determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

**Chemicals**

ULN was a gift from Mochida Co., Ltd. (Tokyo Japan). 2-Thiobarbituric acid, sodium dodecyl sulfate, tetramethoxypropane and Tris hydroxymethyl amonemethane were obtained from Wako Pure Chemical Industries, Ltd. DMPO, FMLP, PMA, ferricytochrome c, xanthine, xanthine oxidase and human erythrocytes superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NAD⁺ was obtained from Boehringer Mannheim GmbH.

**Statistical analysis**

Data were expressed as the mean ± S.E. The mean values of the SEM index were analyzed for significant differences by Student’s t-test.

**RESULTS**

**Lipid peroxide level in liver tissue**

As previously described (22, 23), the MDA levels, which are derived from lipid peroxidation in liver tissue, increased after 60 min of reperfusion following 15 min of ischemia. However, when 100,000 U/kg of ULN was administered before ischemia, the MDA level increase was significantly suppressed (Fig. 1).

**Serum GOT, GPT and LDH levels**

The serum GOT, GPT and LDH levels in the ischemia-reperfusion group were significantly increased as compared with those of the sham-operated group. When ULN was administered before ischemia, the elevation of serum GPT was significantly suppressed. In addition, the elevation of serum GOT and LDH levels also showed a tendency to be suppressed (Table 1).

**Radical trapping ability**

To investigate whether these suppressive effects of ULN were due to a radical scavenging action, the possibility of the radical-trapping ability of ULN against superoxide and hydroxyl radical was estimated by the ESR method. ULN at a concentration of 100,000 U/ml exhibited neither superoxide nor hydroxyl radical-trapping action (Fig. 2).

**Superoxide production by rat PMNs**

Superoxide production by rat PMNs stimulated by FMLP and PMA were 0.24 ± 0.01 and 2.13 ± 0.12 nmol/3 X 10⁶ cells/min, respectively. The electron donors were believed to be superoxide anions produced by PMNs, since this reduction was completely inhibited by the addition of 30 U/ml of SOD (data not shown). As shown in Fig. 3, superoxide production by rat PMNs stimulated by FMLP and PMA was inhibited by ULN dose-dependently.
Table 1. Effects of ULN on serum GOT, GPT and LDH levels after ischemia-reperfusion of the liver

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Sham-ope</td>
<td>362 ± 54</td>
<td>108 ± 26</td>
<td>2690 ± 461</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>1820 ± 240</td>
<td>1367 ± 139</td>
<td>24350 ± 1894</td>
</tr>
<tr>
<td>Ischemia-reperfusion (ULN treated)</td>
<td>1245 ± 123</td>
<td>865 ± 83</td>
<td>18841 ± 2781</td>
</tr>
</tbody>
</table>

K.U.: Karmen units. W.U.: Wroblewski units. Ischemia-reperfusion: rats treated with 15-min ischemia followed by 60-min reperfusion. Sham-ope: sham-operated rats treated in the same manner except for clamping. ULN treated: ULN (100,000 U/kg) was administered 5 min before inducing ischemia in the liver. Each value represents the mean ± S.E. of 5 rats per group. *P < 0.05, **P < 0.01.

Fig. 2. Effects of ULN on the formation of DMPO spin-trapped adduct, DMPO-OOH (left) and DMPO-OH (right). ESR spectrum recorded before (A) and after (B) addition of 100,000 U/ml of ULN. MnO: Mn²⁺ signal.

Fig. 3. Effects of ULN on superoxide production by rat PMNs. Cell suspension and various concentrations of ULN were preincubated at 37°C and stimulated by FMLP and PMA. Superoxide productions in the absence of ULN (control values) were 0.24 ± 0.01 and 2.13 ± 0.12 nmol/3 × 10⁶ cells/min, respectively. Each value represents the mean ± S.E. of 5 separate experiments. ○: FMLP, ●: PMA.
Table 2. Effects of ULN on XDH and XO activities after 15-min liver ischemia

| Groups         | XDH + XO (mU/100 mg protein) | XO (mU/100 mg protein) | %XO  
|----------------|-------------------------------|------------------------|------
| No ischemia    | 180.0 ± 18.6                  | 46.8 ± 7.5             | 26.6 ± 3.4 |
| 15 min ischemia| 182.4 ± 10.2                  | 56.4 ± 5.4             | 31.3 ± 3.3 |
| (ULN treated)  | 189.3 ± 7.5                   | 52.5 ± 3.9             | 28.1 ± 2.5 |

Control livers and those treated with 15-min ischemia were removed and homogenized for the assay. ULN treated: ULN (100,000 U/kg) was administered 5 min before inducing ischemia in the liver. Each value represents the mean ± S.E. of 5 rats per group.

Table 3. Stabilizing effects of ULN and dl-propranolol on rat erythrocyte membrane

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Final conc.</th>
<th>inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dl-Propranolol</td>
<td>10 µM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>39.7</td>
</tr>
<tr>
<td></td>
<td>500 µM</td>
<td>78.5</td>
</tr>
<tr>
<td>ULN</td>
<td>200 U/ml (0.3 µM)</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>500 U/ml (0.8 µM)</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>1,000 U/ml (1.5 µM)</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>5,000 U/ml (7.5 µM)</td>
<td>93.6</td>
</tr>
<tr>
<td></td>
<td>10,000 U/ml (15.0 µM)</td>
<td>96.3</td>
</tr>
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</table>

The inhibitory effects of these drugs against 70% hypotonic hemolysis are shown.

Conversion of XDH to XO in the liver

To investigate whether or not liver ischemia and ULN in rats affected the conversion of XDH to XO, the XDH and XO activities of liver tissue were measured. As shown in Table 2, the quantity of the total XDH + XO and XO activities remained unchanged even after 15 min ischemia. Moreover, administration of 100,000 U/kg of ULN did not have any effect on this conversion.

Stabilizing effect on erythrocyte membrane

To study the membrane-stabilizing properties of ULN, rat erythrocytes were used as a model. dl-Propranolol is recognized as a potent membrane stabilizer in hypotonic hemolysis (24). ULN showed a greater range of protection than dl-propranolol against 70% hemolysis in rat erythrocytes in a dose-dependent manner (Table 3).

Discussion

It is well-known that lipid peroxidation has been implicated in the pathogenesis of liver injury caused by ischemia-reperfusion. Oxygen-derived free radicals produced after reperfusion following ischemia promote the chain reaction of lipid peroxidation in biomembranes, which results in the functional and structural damage to liver cells (25–28). If ULN has some suppressive effects on superoxide production in the HPX-XO system or PMNs, then it is expected that ULN also suppresses lipid peroxide formation in liver tissue and subsequent liver injury. In the present study, administration of ULN suppressed the elevation of lipid peroxide levels and serum parameters in the liver ischemia-reperfusion model. With respect to radical production by PMNs, ULN dose-dependently suppressed the FMLP and PMA-induced superoxide production in the cytochrome c study in vitro. It has been reported that a protease might be involved in the activation of NADPH oxidase and that some protease inhibitors suppressed superoxide production by PMNs (5-8, 29). Kawamoto et al. (4) reported that superoxide production by PMNs was activated by ischemia-reperfusion and played an important role in liver injury. Therefore, the suppressive effect of ULN on superoxide production by PMNs may be one of the factors that inhibit lipid peroxidation caused by ischemia-reperfusion of the liver.

Another important examination is based on the fact that protease inhibitors inhibit the conversion of XDH to XO during ischemia. Roy and McCord (30) have shown that significant conversion of XDH to XO occurs in ischemic rat liver within 30 min, and XO newly generated during ischemia plays an important role in oxygen-free radical production. If XO is newly generated during 15 min of ischemia, ULN might inhibit the conversion of XDH to XO and suppress free radical production. However, XO activity remained unchanged after 15 min of hepatic ischemia in the present study, and ULN did not have any effect on XO activity in vivo. Recently, Marubayashi et al. (31) showed that no significant changes were observed in XO activity even after 90 min of ischemia, and when hepatic ischemia was prolonged for 6 hr, XO activity rose to 37% of the
total activity. They therefore concluded that the period of ischemia required for maximal conversion was significantly longer than that reported by Roy and McCord. In addition, since ULN did not suppress superoxide production by the HPX-XO system and did not have a trapping action of superoxide anions in this study, apparently ULN never suppresses XO itself.

Membrane stabilizing and radical scavenging effects have been reported as mechanisms of action for liver protection by agents in drug-induced models of hepatic injury (32, 33). In this study, the effects of ULN on free radical and membrane stabilizing actions were examined to clarify the mechanisms of liver protection. It has been reported that some drugs which do not have a radical-trapping action, but which do have a membrane stabilizing effect, suppress lipid peroxidation in liver tissue (34–36). Ohnishi et al. (13) reported that ULN significantly reversed the increase of serum $\beta$-glucuronidase activity and protected against the aggravation and mortality induced by experimental shock. Yao et al. (37) also reported that ULN attenuated the elevation of serum creatine phosphokinase (CPK) levels during hemorrhagic shock. This effect may be attributed to ULN’s plasma membrane stabilizing action. In our hypotonic crythrocyte hemolysis test, ULN remarkably inhibited hemolysis at concentrations over 500 U/ml (corresponding to 0.8 \mu M). In addition, the effects of ULN on radical-trapping ability were studied by ESR using a spin trapper, DMPO, but neither superoxide nor hydroxyl radical-trapping action were observed. This finding indicates that ULN does not scavenge these radicals.

These results suggest that ULN’s membrane stabilizing action, as well as its suppressive effect against PMNs superoxide production, may play an important role in protecting the liver against lipid peroxidation caused by oxygen-derived free radicals in the liver ischemia-reperfusion model. Although further studies are needed to support this hypothesis, the present data indicate that the direct action of ULN is distinct from its scavenging actions.

REFERENCES

4 Kawamoto, S., Inoue, M., Tashiro, S., Morino, Y. and
19 Della Corte, E. and Stirpe, F.: The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into
oxidase (type O) and purification of the enzyme. Biochem. J. 126, 739–745 (1972)


24 Hajikano, M., Tonooka, M., Horie, K. and Yasuhara, H.: Relationship between hepatotoxicity and membrane effects on erythrocytes by various drugs. Med. J. Showa Univ. 40, 309–319 (1980) (Abs. in English)


