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CLEARANCE FROM CIRCULATION AND ORGAN DISTRIBUTION OF ^{125}I-ENDOTHELIN-1 IN CARBON TETRACHLORIDE-INDUCED ACUTE LIVER INJURY AND HEPATIC FIBROSIS RATS

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Objectives: To verify whether endothelin (ET) ET_B receptors are involved in removal of circulating ET-1, and to examine whether acute liver injury (ALI) and hepatic fibrosis (HF) affect the removal rate and organ distribution of ET-1. **Methods:** ALI and HF rats were made by single and repeated injection of carbon tetrachloride, respectively. Under ketamine anesthesia, ^{125}I-ET-1 was i.v. bolus injected, then blood samples were collected as scheduled (1, 2, 5, 10, 15 and 30 min after injection). The lungs, liver, kidneys, and aorta were harvested to measure the accumulated radioactivity. Normal control (NC) rats and BQ788-pretreated (BQ) rats also underwent the same injection and sampling procedure. ^{125}I-ET-1 binding sites in the liver were studied with light-microscopic autoradiography. **Results:** BQ788 substantially decreased the removal rate of ^{125}I-ET-1 from circulation and tended to decrease the lung trapping of the tracer, while significantly increased the hepatic distribution of the tracer. Removal of ^{125}I-ET-1 from systemic circulation was impaired both in ALI and HF rats. ^{125}I-ET-1 distribution was reduced in the liver and kidneys of ALI, whereas increased in the lungs of both ALI and HF. Light-microscopic autoradiography revealed a dense distribution of grains in the periportal areas of NC and ALI rats, but such gradient was lost in HF rats. **Conclusions:** We confirmed the physiology for removal from circulation and organ trapping of ET-1. The decreased ET-1 removal from circulation may be due to reductions in hepatic and renal degradation of ET-1 in ALI rats, and may be ascribed to anatomical and/or functional diminution of lipocytes in the cirrhotic liver.

Key words: endothelin clearance, ET_B receptor, BQ788, liver diseases, carbon tetrachloride

INTRODUCTION

Endothelin (ET)-1 is an outstandingly potent vasoconstrictor peptide that was initially isolated from culture medium of porcine aortic endothelial cells (1). Subsequently, two more isoforms, ET-2 and ET-3, were identified (2,3). Two types of ET receptors (ET_A and ET_B) have been cloned, while the third receptor (ET_c) has been pharmacologically and biologically characterized, and its cDNA has been cloned from *Xenopus laevis* (4-7). ET_A has a high affinity for ET-1 and ET-2 and a low affinity for ET-3, whereas ET_B has equal affinity for all types of ETs (8). Although both types of receptors have long been known to be involved in modulation of vascular tone, some part of ET_B receptors are now regarded as a clearance receptor (9). After binding to ET_B receptors, ET molecules are internalized by receptor-mediated endocytosis and degraded by endopeptidases or excreted into urine (10-14).

In the physiological state, ET_B receptors have been detected in many organs including the lungs, kidneys, liver, spleen, heart, aorta, cerebellum, and pituitary gland. However, it is unknown what proportion of these ET_B receptors are linked to vasomotion and ET-clearance, respectively. Exogenous ET-1 was reported to be rapidly removed from circulation mainly through the lungs, kidneys and liver by binding to ET_B receptors (9), which are selectively blocked by BQ788 (9,15). Accordingly, our first aim is to

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verify that ET_B receptors are actually involved in ET-1 removal from circulation and its organ distribution (or sequestration; followed by endocytosis and degradation) by using BQ788 in normal rats.

In the physiological liver, ET_B mRNA has been detected in lipocytes, sinusoidal endothelial cells, and Kupffer cells. Light- and electron-microscopic autoradiographic studies have shown the presence of ET-1 binding sites in normal rat liver tissue and its association with lipocytes (9,16-20). The hepatic ET_B receptors, at least in part, are assumed to contribute to ET clearance. In fact, plasma ET-1 concentration was elevated in patients with chronic liver diseases (21). Although the mechanism of such ET-1 elevation is unproved, ET-1 over-production is unlikely a mechanism because there are only few studies that have found ET-1 over-expressed in acute liver injury and cirrhosis. On the other hand, ET-1 clearance has been shown to be decreased *ex vivo* in isolated livers from cirrhotic rats (22-24), suggesting a possible involvement of ET_B receptors. Since it is yet unknown whether or not the ET-1 clearance and its organ- and intrahepatic distribution *in vivo* are affected by liver diseases, the second aim of our present study is to examine whether and how the plasma clearance, organ distribution, and intrahepatic distribution of exogenous ^{125}I-ET-1 are altered by acute liver injury (ALI) and hepatic fibrosis (HF) in the rat.

MATERIALS AND METHODS

Chemicals

BQ788, whose chemical name is N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyl-D-norleucine, binds to almost total ET_B receptors at a concentration of 1-10 nmol/l in some cell lines (15). The dose and route of administration (0.1 mg/kg/min, i.v.) of BQ788 used in the present study were the same as those by Fukuroda, et al (9).

Making disease model rats

Male Wistar rats weighing 210-280 g were used. ALI rats (n=3) were made by bolus intraperitoneal injection of carbon tetrachloride (CCl_4 2 mg/kg) 24 h before the experiment day. HF rats (n=3) were made by subcutaneous injection of 50% CCl_4-olive oil solution (2 mg/kg) twice a week for 10 weeks, and subjected to the experiment for radioactive ET-1 distribution on the next day of the end of 10-week period. Normal control rats (NC, n=3) and BQ788-pretreated rats (BQ, n=3) received no CCl_4 at all.

Experiment protocol

Rats were anesthetized with ketamine hydrochloride (12 mg/kg, i.m.). Cannulae were inserted into a unilateral femoral artery for blood sampling, and into the contralateral femoral vein for injection of ^{125}I-ET-1 and infusion of BQ788 (for BQ group) or physiological saline (for NC, ALI, and HF groups). A bolus dose of ^{125}I-ET-1 (22,000 nmol, 1.85 MBq) in 0.3 ml of 10 mM phosphate-buffered saline (PBS) containing 0.05% bovine serum albumin (BSA), pH 7.4 was injected 15 min after beginning the infusion of physiological saline for NC, ALI, and HF groups, while 15 min after beginning the infusion of BQ788 for BQ group. Blood samples (0.1 ml each) were collected at 1, 2, 5, 10, 15, and 30 min after ^{125}I-ET-1 injection, and their radioactivity was measured with a gamma counter (MINAX AUTO-GAMMA 5000 SERIES, Packard, USA) for 1 min. Following exhaustive bleeding and washing out of blood with PBS, the lungs, liver, kidneys, and abdominal aorta were resected, weighed, and measured of their radioactivity in the gamma counter for 1 min.

All experiments were performed according to the Animal Experiment Guidelines of Shimane Medical University.

Light-microscopic autoradiography

Resected organs were immediately fixed in Bouin solution, and then dehydrated, penetrated, and embedded in paraffin. For light-microscopic autoradiography, NTB (Kodak, Tokyo, Japan) was dissolved in a water bath at 45℃ for 30 min in a dark room. Sections cut from the embedded organ specimens were placed on glass slides and dipped in NTB emulsion for 3 sec. After the exposure process in a shaded box at 4℃ for 3 weeks, the slides were developed for 3 min at 25℃ using Konidol X (Konica, Tokyo, Japan) and post-staining was performed with hematoxylin-eosin.

^{125}I-ET-1 binding

Light-microscopic autoradiographs of the liver sections magnified 100 times were prepared for a study of the distribution of ^{125}I-ET-1 binding sites (represented as radiophotochemical grains) in the hepatic lobules. The number of grains in a 100 μm square area in 10 randomly selected sites of each of the periportal, middle, and pericentral areas were counted. In the HF rats, grain counting was performed in the interstitial and parenchymal areas because of the difficulty in differentiating the periportal area from the pericentral area.

Statistical analyses

Statistically significant differences between NC group and BQ group were analyzed by paired t-test, and those among respective groups (NC, ALI, and HF groups) were analyzed by ANOVA and modified t-test for multiple comparison. Data were expressed as mean ± standard deviation (S.D.), and a difference of $p<0.05$ was regarded as significant.

RESULTS

^{125}I-ET-1 clearance from circulation

Fig. 1 shows the time course of radioactivity decay in the plasma following ^{125}I-ET-1 injection in NC, BQ, ALI, and HF groups. Injected ^{125}I-ET-1 was rapidly cleared from the systemic circulation in NC rats. In the BQ group, clearance of ^{125}I-ET-1 was highly inhibited ($p<0.05$, 1 min after injection; $p<0.001$, 2 min after injection). The clearance rates in ALI and HF groups were decreased: radioactivity in both ALI and HF groups 10 min after ^{125}I-ET-1 injection were higher than that of NC rats ($p<0.05$ for both groups vs. NC group).

Organ distribution of ^{125}I-ET-1

Figures 2a and 2b show radioactivity accumulations in the liver, lungs, kidneys, and aorta 30 min after ^{125}I-ET-1 injection. In the BQ rats, radioactivity tended to be decreased in the lungs, whereas it was markedly increased in the liver (368.1%, $p<0.01$ vs. NC) and tended to be increased in the kidneys (Fig. 2a). It should be noted that the lungs are as yet the principal organ to clear the circulating ET even in the presence of BQ788, as can be seen from the relative magnitude of the scale in Y-axis (Fig. 2a).

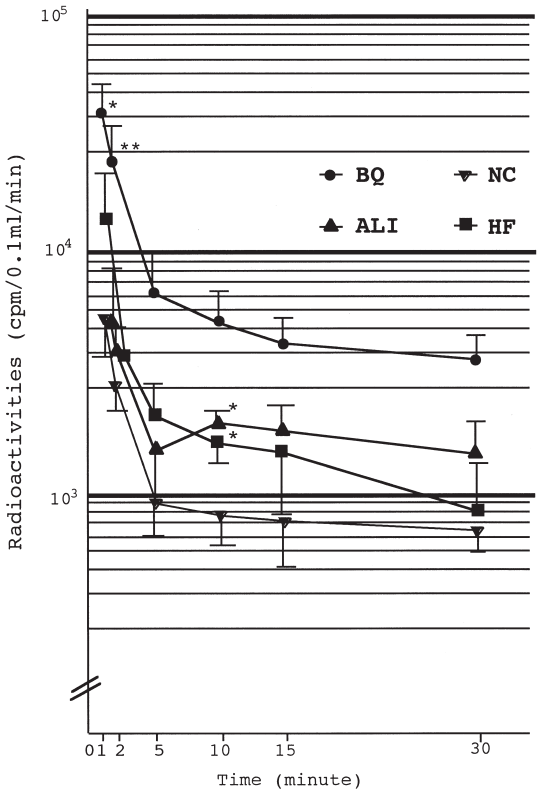

Fig. 1. Decay in blood radioactivity after ^{125}I-ET-1 injection in normal control group (NC), BQ788-pretreated group (BQ), acute liver injury group (ALI), and hepatic fibrosis group (HF). Each group consisted of 3 rats. The data are expressed as mean ± S.D. * $p<0.05$ and ** $p<0.001$ vs. NC.

In the ALI rats, radioactivity in the liver, kidneys and aorta tended to be decreased as compared to those in NC (-49.9% in the liver, -59.7% in the kidneys, and -28.7% in the aorta; Fig. 2b), whereas radioactivity in the lungs was significantly increased in ALI (+153.3%, $p<0.05$ vs. NC). In the HF rats, accumulation in the liver was not decreased. Moreover, the accumulation of radioactivity in the lungs and kidneys were significantly increased (+140.9% in the lungs, +120.4% in the kidneys; $p<0.05$ for both organs vs. NC group; Fig. 2b).

Intrahepatic distribution of ^{125}I-ET-1

In the normal liver, a majority of grains were detected in the periportal area, whereas they were significantly fewer in the pericentral area (Fig. 3). Similar distribution was also observed in the liver of ALI rats (Fig. 4). In HF rats, grains diffusely distributed in all areas (Fig. 5). When the numbers of

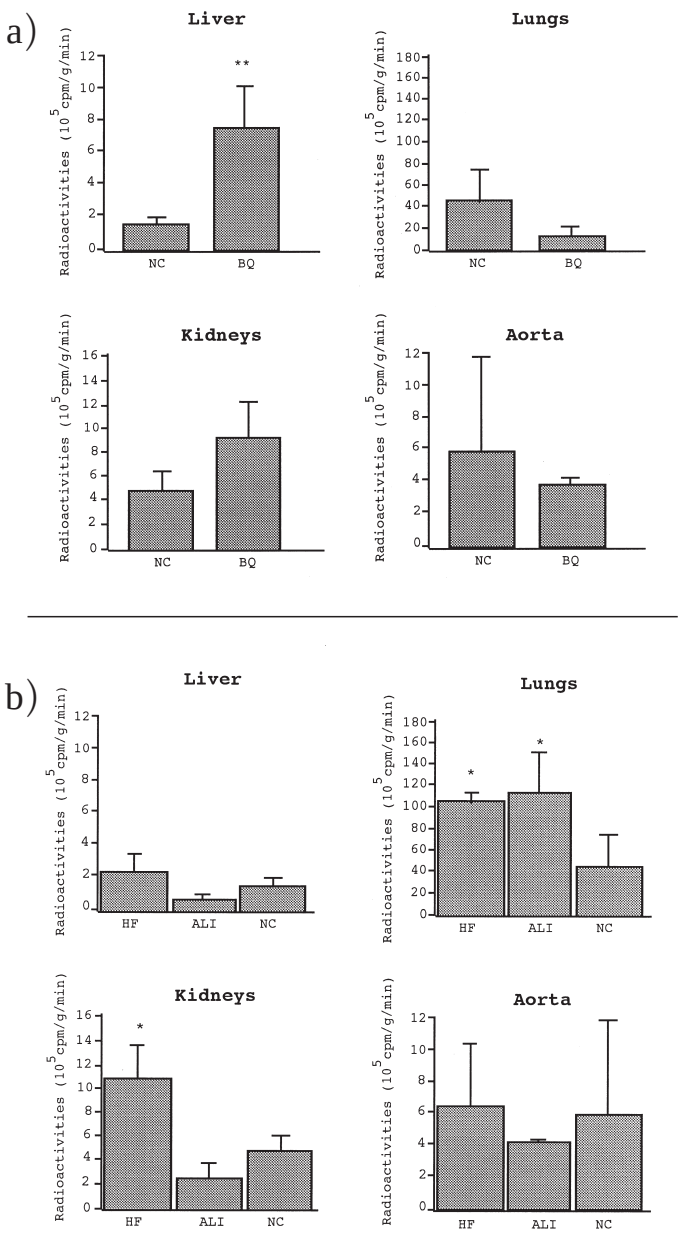

Fig. 2. Distribution of radioactivity in the liver, lungs, kidneys, and aorta 30 min after ^{125}I-ET-1 injection. The panel 2a) shows the effects of BQ788 on tissue distribution of radioactive endothelin-1. The panel 2b) compares the distribution of the tracer in the organs isolated from hepatic fibrosis rats (HF) and acute liver injury rats (ALI) with that of normal control rats (NC). The data are expressed as mean ± S.D. * $p<0.05$ and ** $p<0.01$ vs. NC group.

grains found in each area were compared among the groups, there was a gradient from the periportal toward the pericentral area in NC and ALI groups, whereas no such a gradient was seen in the HF group (Fig. 6).

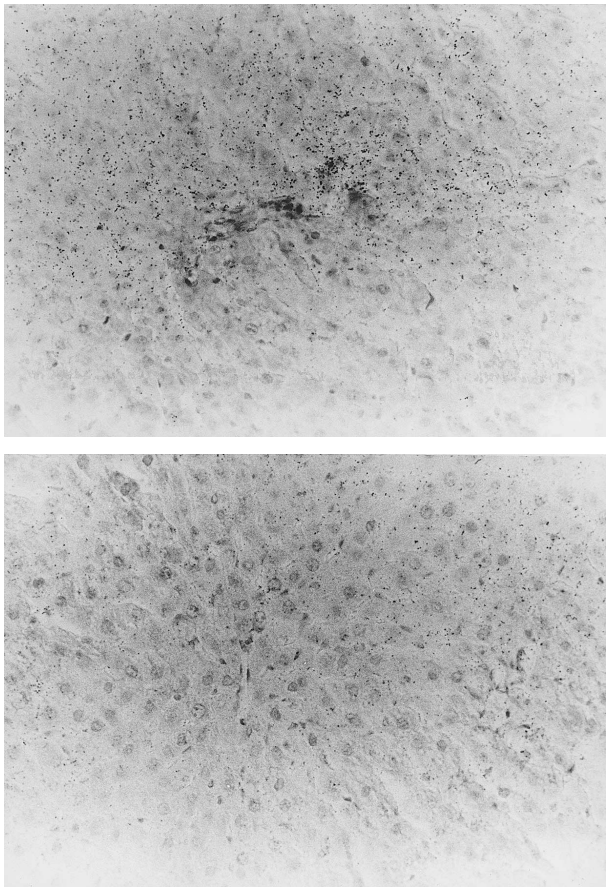

Fig. 3. Representative light-microscopic autoradiograph of the liver taken from a rat of the normal control group, showing grains in the periportal area (upper panel) and in the pericentral area (lower panel). Post-stained by Hematoxylin-Eosin. Original magnification = ×100.

DISCUSSION

The first part of the present study demonstrated that the physiological clearance (in the NC group) of exogenous ^{125}I-ET-1 from systemic circulation was substantially inhibited by BQ788 pretreatment (Fig. 1), presumably by suppressing the ET_B receptor-mediated ET-1 trapping by the lungs (Fig. 2a). This result is consistent with that of Fukuroda et al (9), and verifies that ET_B receptors, particularly of the lungs, play a pivotal role in removing ET from the systemic circulation. In contrast, BQ788 potently increased the hepatic trapping of ET-1: this result on the liver is also consistent with those investigators. These results suggest three possible explanations for hepatic ET-1 trapping: 1) mediation by the ET receptors other than ET_B (i.e., ET_A and/or ET_c); 2) down-regulation of ET_B receptors; or 3) the participation of other clearance systems. Our data that

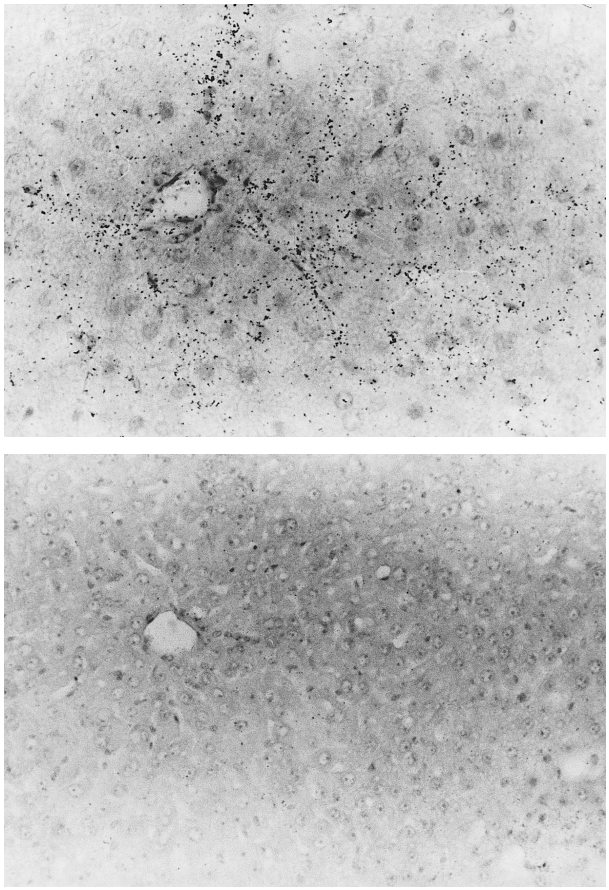

Fig. 4. Representative light-microscopic autoradiograph of the liver from a rat of acute liver injury group, showing grains in periportal area (upper panel) and in pericentral area (lower panel). Original magnification = ×100.

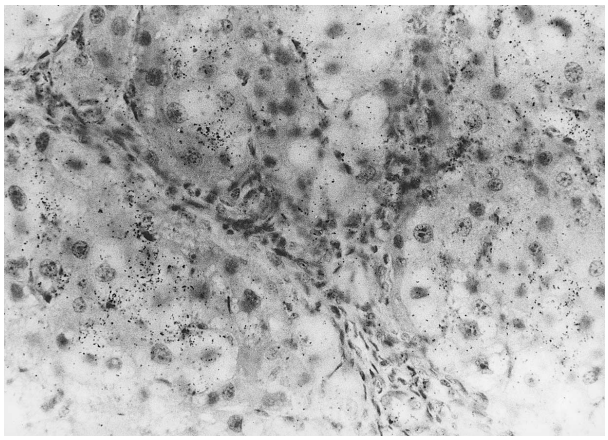

Fig. 5. Representative light-microscopic autoradiograph of the liver isolated from a rat of hepatic fibrosis group. Original magnification = ×100.

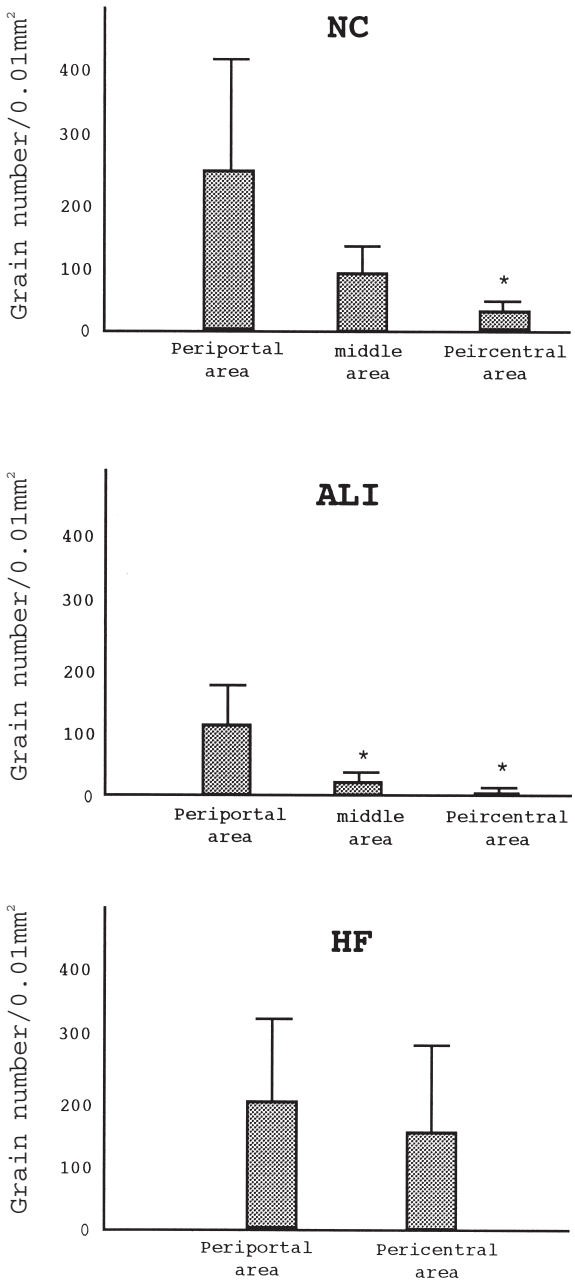

Fig. 6. Intrahepatic distribution of grains (radioactive labeling index) in the normal control group (NC), acute liver injury group (ALI), and hepatic fibrosis group (HF). Means ± SD (n = 3 for each group) are shown. * $p<0.05$ vs. the periportal area of respective groups.

accumulation of radioactivity in the kidneys was increased by BQ788 is inconsistent with the result by those investigators. The reason for this discrepancy is unknown at the moment. In any way, it should be noted that the lungs are the primary organ to clear the circulating ET, as judged from the magnitude of Y-scale (Fig. 2a).

In ALI and HF rats, the accumulation of radioactivity in the lungs was increased most among all the organs studied, and was much higher than in NC

(Fig. 2b). The lungs play a vitally important role in ET-1 clearance from systemic circulation (25-30). In rat lung alveoli, ET_A and ET_B sites have been detected in similar proportion (31), but alterations in pulmonary clearance by liver diseases has not been reported so far. Hepato-pulmonary syndrome accompanying liver cirrhosis is thought to be caused by pulmonary vascular dilatation following an over-production of nitric oxide (31-34). Over-production of ET-1 and up-regulation of ET receptors may arise from the competition with pulmonary vascular dilatation. In addition, ET-1 has been reported to contribute to liver injury induced by galactosamine and endotoxin in a study using isolated perfused rat livers (35). Thus, as a result of an up-regulation of ET receptors, the trapping of ^{125}I-ET-1 in the lungs might be increased in ALI and HF rats.

Radioactivity in the kidneys as well as liver tended to be decreased in ALI, but significantly increased in HF than in NC.

Increased urinary excretion of ET-1 in liver cirrhosis patients has been reported and is considered primarily dependent on elevated plasma ET-1 levels (21): the increased ET load onto the kidney may result in the increased urinary output. On the other hand, Lange et al. reported that patients with hepatorenal syndrome (HRS) had markedly elevated plasma ET-1 and -3 concentrations as compared with normal subjects (32). These facts suggest that urinary excretion of ET-1 may be increased due to hyperendothelinemia in the liver cirrhosis patients who are not complicated with HRS, but if complicated with HRS, the ET-1 clearance from systemic circulation would be decreased.

Hepatic accumulation of radioactivity was less in ALI than NC, but tended to be increased in HF rats. Thus, it is suggested that the hepatic ET-1 removal from circulation is impaired in ALI. Trends of increment in hepatic ET accumulation in HF rats may be explained as follows. As discussed earlier, ET receptors other than ET_B type may play a dominant role in hepatic ^{125}I-ET-1 trapping and such type of ET receptors could be up-regulated by hepatic diseases. Uchida (22) evaluated the mechanisms of elevated plasma ET-1 levels in CCl_4-treated rats and found that ET-1 levels in the sera from both portal vein and inferior vena cava (IVC) were increased in ALI rats, while the levels were increased only in the IVC in HF rats. In addition, serum ET-1 levels from the IVC after transportal ET-1 infusion with isolated perfused liver models were higher in HF than those in NC and ALI (22). These results by Uchida et al indicate that intrahepatic clearance of ET-1 may be decreased in liver fibrosis. However, endogenous ET-1 levels produced in the liver have not been measured in their study nor in other studies on liver diseases. Thus, the exact mechanisms for plasma ET elevation in liver cirrhosis await further investigations.

As for the intrahepatic distribution of ^{125}I-ET-1, most grains were detected in the periportal area of the liver, whereas they were significantly decreased toward the pericentral area of the liver in NC and ALI. The distribution of grains was consistent with that of lipocytes, which are located in Disse's spaces in the periportal area and are reported to have an influence on sinusoidal blood flow (36-38). In addition, lipocytes play a profibrotic role in liver cirrhosis (39). In the present study, gradation of grains which was regarded as physiological was lost in HF rats. Pinzani et al. (23) demonstrated that progressive activation and phenotype modulation of human lipocytes in culture were associated with a progressive shift from a relative predominance of E T_A to that of ET_B. In liver fibrosis, transformation of lipocytes into myoblasts and fibroblasts along with the redistribution of lipocytes associated with lobular restructuring might influence the distribution of grains.

In conclusion, we confirmed by using a selective ET_B antagonist (BQ788) the physiology for removal from circulation and organ trapping of ET-1 in NC rats. We also demonstrated that a decrease in hepatic and renal degradation of ET-1 may lead to a decrease in ^{125}I-ET-1 removal from circulation in ALI rats, whereas the distribution of grains may reflect anatomical and/or functional diminution of lipocytes in the liver of HF.

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