The Effects of Ulinastatin on the Reticuloendothelial System (RES) of Rabbits in Endotoxin Shock

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Carbon clearance, concentration of fibronectin (Fn) in the blood and arterial ketone body ratio (AKBR) have been measured in the rabbit in order to evaluate the RES phagocytic activity and the effect of ulinastatin on it in endotoxin (ET) shock. The following results were obtained: 1) carbon deposition by ET injection was found in organs (lung and kidney) which did not show it in the control group. The reasons for this might be considered that the RES was blocked because of phagocytizing ET and tissue debris, so that the carbon which could not be processed by the RES spilled over and became clogged; 2) the RES phagocytic activity were found to decline from an early stage after injection of a lethal dose of ET due to a decline in Fn productivity and an increase in Fn consumption; and, 3) ulinastatin suppressed the decrease of Fn and the carbon deposition in the kidney in ET shock. These results are suggestive of the usefulness of ulinastatin as an anti-shock agent, preserving the RES phagocytic activity through the inhibition of Fn consumption. (Key words: fibronectin, arterial ketone body ratio, carbon clearance, endotoxin shock, ulinastatin)

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The decline of the RES function is commonly observed in the patients with shock, suggesting a weakness of their host defense capacity. However, the RES function is not easy to ascertain in clinical patients.

Lancer et al.[1,2] have pointed out the importance of plasma Fn as an indicator of the RES phagocytic activity. However, a discrepancy between the RES phagocytic activity and Fn concentration in the serum was observed[3,4]. It has also become clear that the activity of Kupffer cells plays an important role in the RES[3,5].

Clearance of carbon particles from the blood and their deposition into the organs, the venous blood level of fibronectin (Fn), and the arterial ketone body ratio (AKBR) have been determined as being representative of the RES phagocytic activity in the present study[6,7].

Ulinastatin, a polyvalent protease inbitor, has been reported to exert an anti-shock action through stabilizing lysosomes. The present research was undertaken to clarify the effect of ulinastatin on the phagocytic activity of RES in rabbits in the state of ET shock.

Materials and Methods (table 1)

1. Experimental animals

Fifty five buck rabbits (Japanese white), weighing between 2.5 to 3.0 kg, were used for this study. Anesthesia was induced by 20 mg/kg of Nembutal injected via an ear vein. A tracheostomy was performed to facilitate stable spontaneous respiration. A polyethylene catheter was

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Table 1. Methods

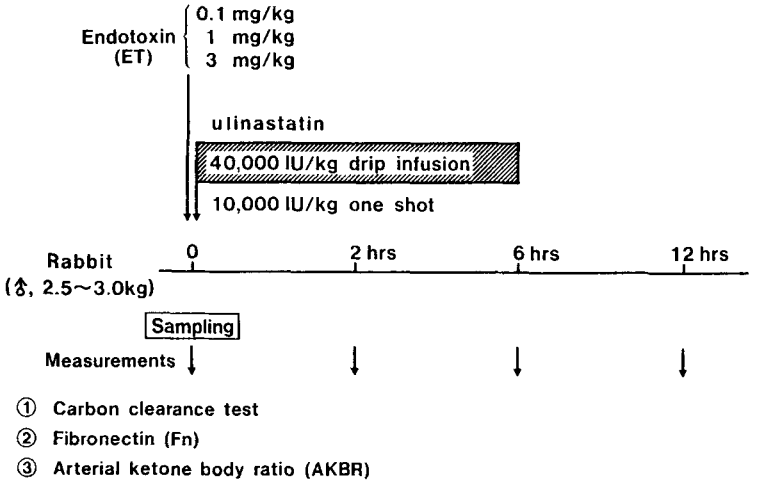
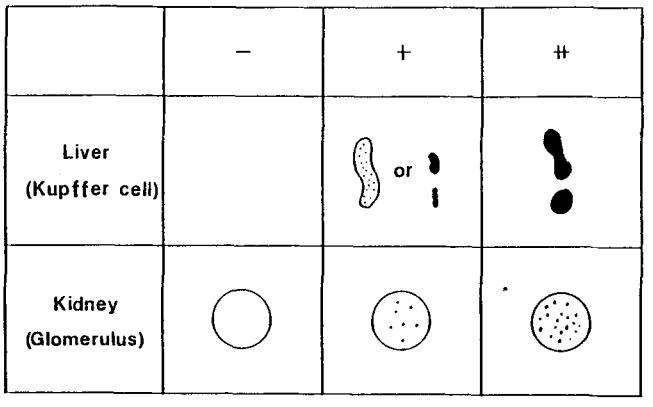


Table 2. Degree of carbon accumulation in liver and kidney



− : Absolutely no carbon particles were recognized
+ : Some carbon particles were recognized
++ : Many carbon particles were recognized

placed into femoral artery to withdraw blood specimens. The endotoxin shock models were produced by an intravenous administration of varying amounts of endotoxin (Escherichia Coli lipopolysaccharide 0127:B8, Difco Lab. USA). These models were classified into three ET groups, based on the administered doses, 0.1, 1 and 3 mg/kg, respectively.

Ulinastatin was injected immediately after the ET injection into the 3 mg/kg ET group a one-shot dose of 10,000 unit/kg. Then, a dose of 40,000 unit/kg of ulinastatin was drip-infused for a periods of 2 (carbon clearance test) or 6 (AKBR, Fn) hours.

2. *Determination of the RES phagocytic activity (carbon clearance test, Fn, AKBR)*

(a) Carbon clearance and deposition of carbon in the RES

Carbon particles of Perikan 17 black ink were diluted to a concentration of 16 mg/ml in saline according to Halpern[8] and 8 mg/100g body weight was administered intravenously into the animals. Blood was withdrawn at 6 different times to determine the concentration of carbon particles. This

blood then was hemolyzed in 80-fold diluted 1% Na_2CO_3 and colorimetry analysis was made, using a spectrum analyzer (UV-2014, Shimazu Co., Ltd.). The concentrations of carbon particle were analyzed to obtain phagocytic indices by the exponential regression method[8,9].

At 60 min after injection of carbon particles, the animals were sacrificed to remove organs involved in RES such as the liver, the lungs, and the kidneys. The organs were fixed in 10% neutral formalin and cut sections were stained with Hemotoxylin-eosin (H&E) to examine the deposition of carbon particles microscopically. The accumulation of carbon particles in the Kupffer cells in the liver and the gromeruli in the kidney was quantitatively evaluated using the standards shown in table 2. The deposition of carbon particles on the lung capillary beds was evaluated for the presence or absense of particles.

(b) Fibronectin (Fn) concentration in the blood

Quantitative analysis of the blood Fn in 15 rabbits was performed by immune turbidimetry, using human plasma Fn. With regard to cross immunity, we already have confirmed in our previous report[10] that the blood Fn of the rabbit can react to the human Fn antibody by the Ouchterlony method.

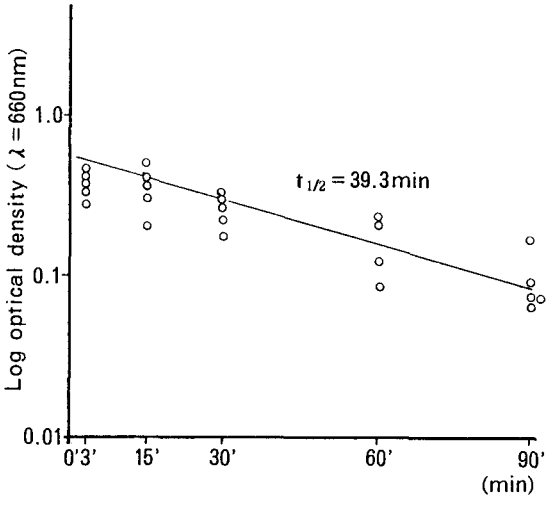


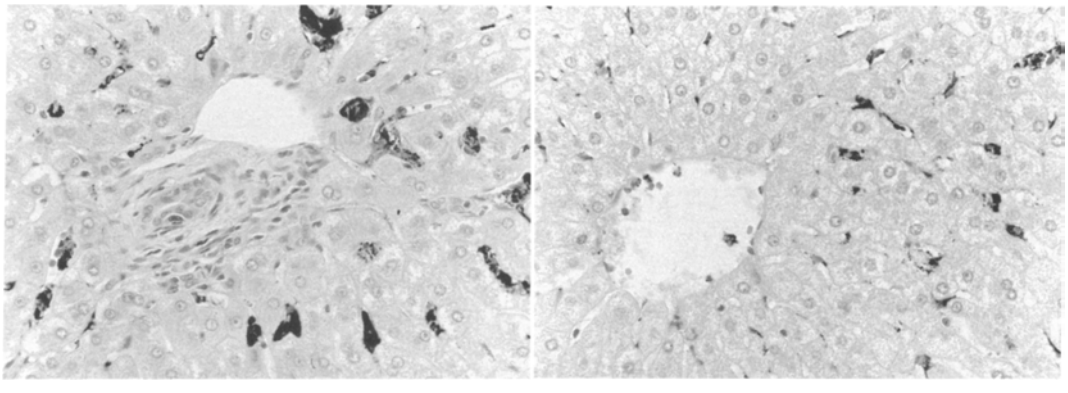
Fig. 1. Carbon clearance of control rabbit
The quenching curves of carbon particles in the blood were highly exponential with a regression coefficient of 0.982. The decay curve was expressed as followed formula:

$$C = C_o e^{-kt},$$

in which the phagocytic index (k) was 0.0181 and half-life of carbon particle in the blood (t) was 39.5 min in the intact control group (n = 5).

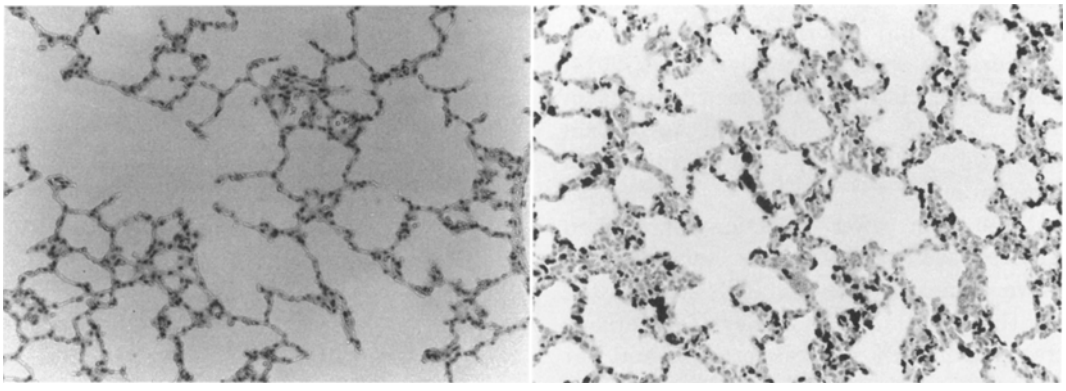
(c) Arterial ketone body ratio (AKBR)

AKBR stands for the ratio of acetoacetate (mg/ml) to β-hydroxybutyrate



Control (++) 2 hrs after ET 3 mg/kg injection (+)

Fig. 2. Carbon deposition in the liver of control and ET injected rabbits.
The uptake of carbon particles by the Kupffer cells in the ET injected groups was less than that of the control group. This is suggestive of a fall in the RES phagocytic activity in the ET injected groups. (×400)



Control (−) 2 hrs after ET 3 mg/kg injection (+)

Fig. 3. Carbon deposition in the lung of control and ET injected rabbits
In the ET injected groups, the carbon deposition was observed on the capillary beds in the lungs. (×100)

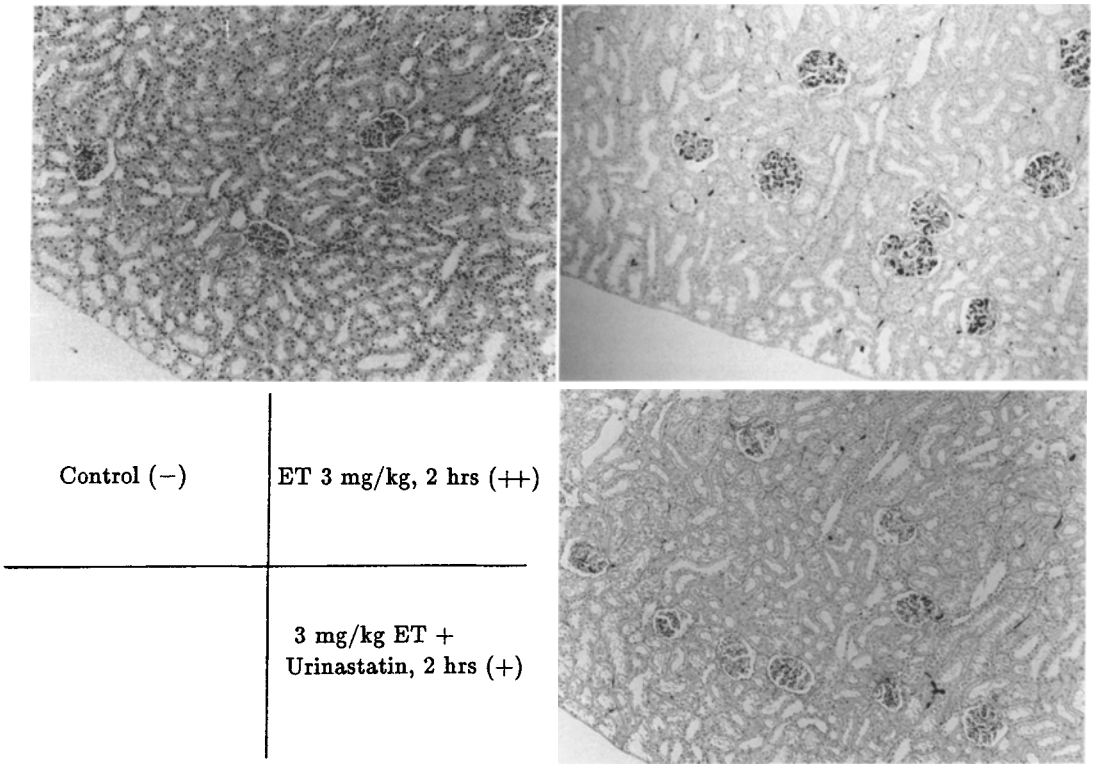


Fig. 4. Carbon deposition in the kidneys of control, ET injected and ulinastatin treated rabbits at 2 hrs after ET injection.
The carbon deposition was observed in the glomeruli in the ET only injected group. In the ulinastatin treated groups, the carbon deposition in the glomeruli was less than that in the ET only injected group. (×100)

(mg/ml) in the arterial blood. Acetoacetate and β-hydroxybutyrate were measured enzymatically[11] in fifteen rabbits. The amount of ketone bodies in the blood of the

fed rat in contrast to that of the starved rat differs considerably[12] and individual differences in the AKBR can be expected to be large. Therefore, the observation of the AKBR in the same test rabbits was considered to be the most appropriate procedure.

3. Data analysis

The values of the Fn and the AKBR were expressed as percentages of the initial value, though the differences between the controls and each treated group were determined by the actual measured value. Results were given as the mean ± S.D. The comparison of the means was performed by the Student's T-test.

Results

1. Carbon clearance test

(a) Control group (fig. 1)

The quenching curves of carbon particles in the blood were found to be highly exponential with a regression coefficient of 0.982, as illustrated in figure 1. The decay curve was expressed as followed formula:

$$C = Coe^{-kt}$$

Where, the phagocytic index (K) was 0.0181, and the half-life of carbon particles in the blood (t) was 39.5 min in the control group (n = 5).

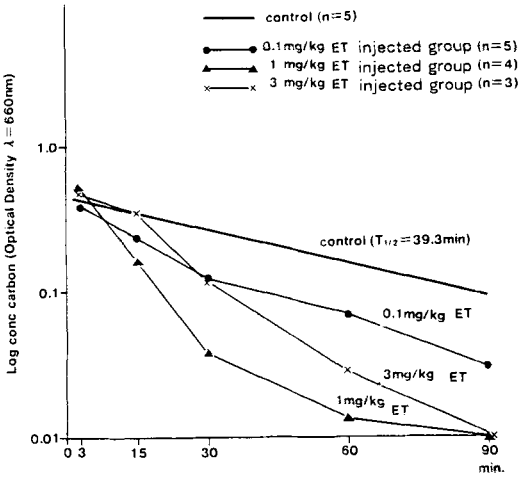


Fig. 5. Blood disappearance of carbon particle in control and endotoxin injected rabbits

The carbon quenching from the blood in all ET groups (0.1, 1 and 3 mg/kg) became rapid but the O.D. values were irregular and could not be expressed as a function of time. Therefore in all ET groups, both the phagocytic index and the half time could not be calculated.

The deposition of carbon particle was observed only in the hepatic cells and could not be seen in either the lungs or the kidneys

Table 3. Carbon accumulation in the liver, lungs and kidneys

In all ET injected group, the carbon deposition was observed not only in the liver but also in the lungs and kidneys. However, the amount of ET and the time after ET injection did not affect the deposition of carbon particle in the liver, lungs and kidneys.

classification of rabbits		Liver	Lung	Kidney
Control (n=5)		++	−	−
0.1mg/kg ET	2hrs (n=5)	+	+	++
	6hrs (n=5)	+	+	++
1 mg/kg ET	2hrs (n=4)	+	+	++
	6hrs (n=4)	+	+	++
3 mg/kg ET	2hrs (n=3)	+	+	++
3 mg/kg ET + ulinastatin	2hrs (n=3)	+	+	+

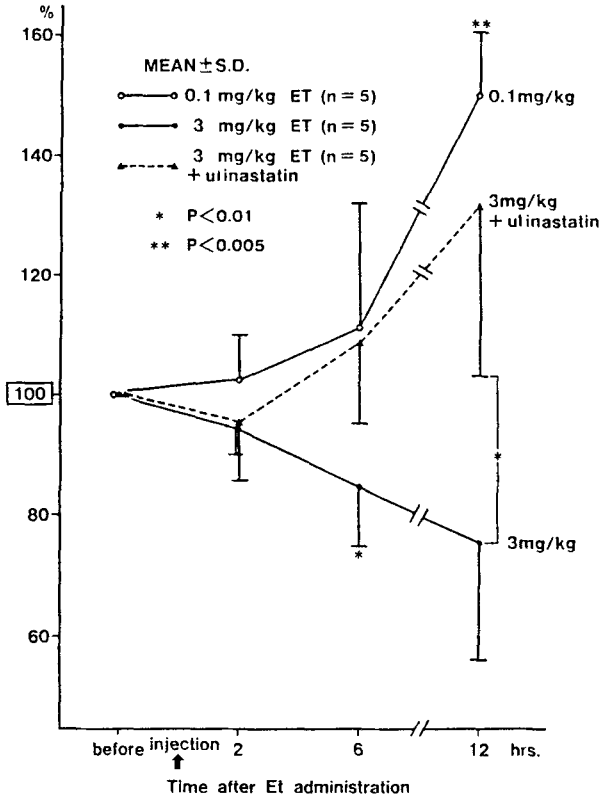


Fig. 6. The percentage change of fibronectin of rabbit

The Fn concentration in the blood of the 0.1 mg/kg ET group was significantly increased at 12 hours after the injection ($P<0.005$). On the other hand, that of the 3 mg/kg ET group was significantly decreased at 6 hours after the injection ($P<0.01$).

Ulinastatin significantly suppressed the lowering of Fn in the blood at 12 hours after the 3 mg/kg ET injection ($P<0.05$).

under an optical microscope (figs. 2–4).

(b) ET group

Carbon quenching from the blood in all three ET groups (0.1, 1, and 3 mg/kg) became rapid but the O.D. values were irregular and could not be expressed as a function of time (fig. 5). Therefore, in all three ET groups, both the phagocytic index and the half time could not be calculated.

As for the distribution of carbon in organs, carbon deposition was observed not only in the liver but also in the lungs and kidneys under the optical microscope (figs. 2–4). The microscopic observations clarified that the uptake of carbon particles by the Kupffer cells in all the ET injected groups was less than that of the control group (fig. 2).

The amount of ET and the time after ET injection, however, did not affect the deposition of carbon particle in the liver, the lungs and the kidneys (table 3).

(c) ET + ulinastatin group

In the liver and the lungs, there was no difference in the degree of carbon deposition between the ET-only group and the group that also had been given ulinastatin.

As for the kidneys, the carbon deposition in the group that also had been given ulinastatin was less than that found in the ET-only group (fig. 4).

2. Fibronectin concentration in the blood (fig. 6)

The Fn concentration in the blood of the 0.1 mg/kg ET group was significantly increased at 12 hours after the injection ($P<0.005$). On the other hand, the Fn concentration in the 3 mg/kg ET group was markedly decreased at 6 hours after the injection ($P<0.01$).

Ulinastatin notably suppressed the lowering of Fn in the blood at 12 hours after the 3 mg/kg ET injection ($P<0.05$).

3. AKBR (fig. 7)

In the 0.1 mg/kg ET group, the AKBR rose significantly after 2 hours ($P<0.05$), but

Table 4. Summary of results

In the kidney, the carbon deposition of the group added ulinastatin was less than that of ET-only group. Ulinastatin significantly suppressed the lowering of Fn in the blood at 12 hours after the 3 mg/kg ET injection.

1. Carbon clearance

	Control	ET Group	3 mg/kg ET + ulinastatin
Liver (RES)	++	+	+
Kidney	−	++	+
Lung	−	+	+

2. Fn, AKBR

	hrs.	0.1 mg/kg ET	3 mg/kg ET	3 mg/kg ET + ulinastatin
Fn	2	↑	↓	↓
	6	↑	⇓	↑
	12	⇑	↓	↑
AKBR	2	⇑	⇓	↑
	6	⇓	⇓	⇓
	12	⇓	⇓	↓

↑ Weak increase ⇑ Significant increase
↓ Weak decrease ⇓ Significant decrease

decreased at 6 and at 12 hours ($P<0.01$ in both cases). In the 3 mg/kg ET group, the AKBR began to decline significantly from 2 hours ($P<0.01$) and continued to fall with time ($P<0.01$ at 6 hours, and $P<0.05$ at 12 hours).

Ulinastatin did not affect the fall of the AKBR in the 3 mg/kg ET group.

The results of the present study are summarized in table 4.

Discussion

1. AKBR and Fn (figs. 6, 7)

In a previous study[6], it was concluded that the AKBR reflected the hepatic mitochondorial redox potential and the energy charge in the liver. It also has been reported that there is a correlation between the AKBR and the RES function[13].

In our experiments, the AKBR of the group given 0.1 mg/kg ET rose at 2 hours after the injection and then began to fall at 6 hours. The reason for this is thought to be that at 2 hours after the ET injection, the metabolic load that had been increased by the ET was compensated temporarily by an accelerated ATP productivity of the mitochondria[14]; however, at 6 hours after ET injection, the acceleration of ATP productivity could no longer meet the ATP consumption and the energy charge in the liver fell.

Following an ET injection of a lethal dose of 3 mg/kg ET, the fall of the AKBR began at 2 hours after the injection. This suggests that when the administered ET amounted to a lethal dose, a state of irreversible shock already had begun by 2 hours after the injection. It can be assumed therefore that tissue perfusion had fallen and that the ATP productivity of the hepatic mitochondria was disturbed by a lack of oxygen.

The fall on Fn concentration in the blood after an injection of a lethal dose of 3 mg/kg ET in the present experiments is consistent with a previous theory[2,15], that sepsis tends to cause Fn deficiency. The reason for the Fn fall could be that phagocytizing tissue debris (fibrin aggregates, effete platelates, FDP and collagen), generated by the ET injection in the blood, accelerated the consumption of Fn.

It is said that Fn is produced mainly by liver cells and the vascular endothelial cells[16]. Therefore another reason for this Fn fall could be that as the fall in the AKBR

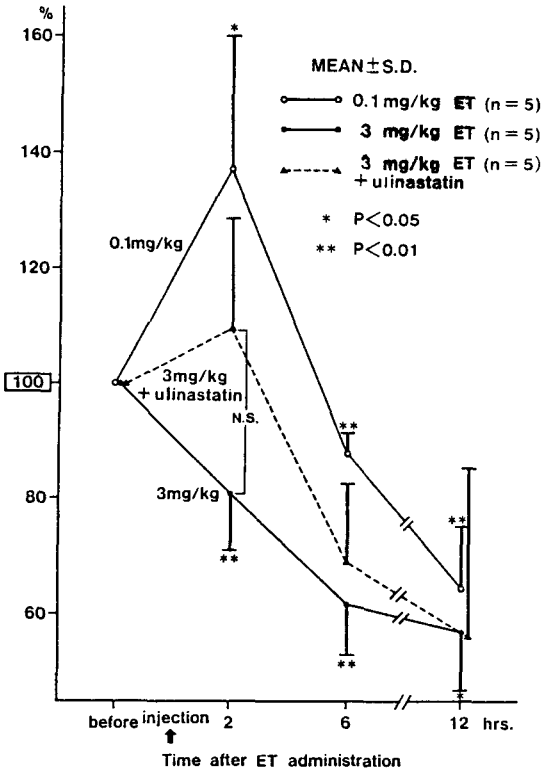


Fig. 7. The percentage change of AKBR of rabbit (arterial ketone body ratio)

In the 0.1 mg/kg ET group, AKBR rose significantly after 2 hours, ($P<0.05$) but was significantly decreased at 6 and 12 hours ($P<0.01$ in both cases). In the 3 mg/kg ET group, AKBR began to decline significantly from 2 hours ($P<0.01$) and continued to fall significantly with time ($P<0.01$ at 6 hours, and $P<0.05$ at 12 hours).

Ulinastatin did not affect the fall of AKBR of 3 mg/kg ET.

occurred, due to metabolic ataxia of the liver induced by ET shock, the protein synthesis ability and the Fn productivity in the liver fell.

Thus, when a lethal amount of ET was injected, the Fn concentration in the blood fell. Yet, when a very small amount of ET (0.1 mg/kg) was inejcted, the Fn concentration in the blood rose significantly at 12 hours after the injection.

Judging from the rise in the AKBR at 2 hours after injection of a very small amount of ET in our experiments, the liver entered a hypermetabolic state in compensation. Therefore, in a similar manner, the rise in Fn also could be considered to have resulted from a compensatory increase in the productivity of Fn in the liver.

2. *Carbon clearance test (fig. 1, fig. 5, table 3)*

Unlike the control group, in all the ET injected groups, because the carbon quenching from the blood could not be expressed as a function of time, the phagocytic indices could not be calculated. These findings appear to reflect the difference in distribution of carbon in the organs between the two groups.

Carbon deposition in the lungs and kidneys induced by ET injection may be due to a fall in the RES phagocytic activity such as the direct lesional action of ET on the liver mitochondria[17], and the phagocytizing effect of ET[18] and the tissue debris generated by the injection. It also has been shown that the uptake of carbon in the liver of the ET-injected groups was less than that of the control group, as shown in figure 2. It appears that the carbon particles, which could not be processed by the RES, overflowed and clogged the capillary blood vessels. The possibility also exists that the fall in the AKBR and the Fn after ET injection, as shown in figures 6, 7 may be referable to that in the RES phagocytic activity.

Since the AKBR and Fn did not fall within 2 hours after a very small injection of ET, shown in table 3, it is difficult to assume, however, a fall of the RES phagocytic activity during this period, but even though carbon deposition was observed in the lungs and kidneys. The reason for this is thought to be that the ET activated blood coagulation cascades containing both intrinsic[19] and exogenous[20] factors and microthrombi were so formed.

In the group that received a lethal dose of ET (3 mg/kg), AKBR began to fall at 2 hours after injection, and the rabbits appeared to enter a state of irreversible shock. Under such a shock, the possibility exists that the RDS (reticuloendothelial depressant substance)[21], which specifically

suppresses the RES functions, may have been generated from the ischemic viscera, and that the fall in the RES functions might have become remarkable.

The carbon deposition in the lungs and kidneys in the ET-injected group suggests that under a state of the lowered RES phagocytic activity, or due to hypercoagulability induced by the ET injection, the ET and tissue debris that cannot be processed tends to clog the capillary blood vessels of the lungs and other organs. In the lungs, this clogging raises the pulmonary vascular resistance, which can cause ARDS (adult respiratory distress syndrome). Further, it can also be a cause of MOF (multiple ogan failure).

3. Effects of ulinastatin (table 4)

In sever case of shock, various proteolytic enzymes that are released into the blood, due to destroyed lysosome membranes, can led to an irreversible treatment. For treatment, inhibitors of proteolysis are said to be effective. It has been reported that ulinastatin, a kind of glycoprotein with a molecular weight of about 67.000, separated from fresh human male urine[22], has an inhibiting effect against various proteolytic enzymes[23] and, therefore, may be use in anti-shock therapy.

Ulinastatin was found to suppress the decrease of Fn at 12 hours after ET injection. The reason for this could be that the Fn had been decomposed by a protease such as plasmin[24], and that this decomposition might have been inhibited by ulinastatin, a protease inhibitor. There is a report which says that ulinastatin has an anti-DIC action which remarkably suppresses the activity of thromboplastin increased by ET. Therefore, it is also possible to suppose that ulinastatin might decrease the fibrin generation in blood vessels and the combined Fn consumption.

Ulinastatin also has suppressed the carbon deposition in the glomeruli of the kidneys in the ET-injected group. The reason for this might be due to two considerations that follow:

① In view of the effect of ulinastatin on Fn, as shown in table 4, ulinastatin may have preserved the RES phagocytic activity through the inhibition of the Fn consumption, thus reducing the amount of carbon particles that spilled over; and,

② ulinastain might have suppressed the formation of microthrombi in the capillary blood vessels due to its anti-DIC action.

Therefore ulinastatin can be expected to suppress the decrease of fibronectin by ET and to recover the RES phagocytic activity.

Consequently, ulinastatin may be considered as a therapeutic weapon to combat for ARDS and MOF in cases of ET shock.

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