Attenuation of Ischemic Liver Injury by Monoclonal Anti-Endothelin Antibody, AwETN40

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Abstract

Background—Enhanced production of endothelin-1 (ET-1), vasoconstrictive 21 amino acids produced by endothelial cells during ischemia and after reperfusion of the liver, is known to cause sinusoidal constriction and microcirculatory disturbances, which lead to severe tissue damage. Using a 2-hour hepatic vascular exclusion model in dogs, we tested our hypothesis that neutralization of ET-1 by monoclonal anti-ET-1 and anti-ET-2 antibody (AwETN40) abates vascular dysfunction and ameliorates ischemia/reperfusion injury of the liver.

Study Design—After skeletonization, the liver was made totally ischemic by cross-clamping the portal vein, the hepatic artery, and the vena cava (above and below the liver). Veno-venous bypass was used to decompress splanchnic and inferior systemic congestion. AwETN40, 5 mg/kg, was administered intravenously 10 minutes before ischemia (treatment group, n = 5). Nontreated animals were used as controls (control group, n = 10). Animal survival, hepatic tissue blood flow, liver function tests, total bile acid, high-energy phosphate, ET-1 levels, and liver histopathology were studied.

Results—Treatment with AwETN40 improved 2-week animal survival from 30% to 100%. Hepatic tissue blood flow after reperfusion was significantly higher in the treatment group. The treatment significantly attenuated liver enzyme release, total bile acid, and changes in adenine nucleotides. Immunoreactive ET-1 levels in the hepatic venous blood of the control group showed a significant increase and remained high for up to 24 hours after reperfusion. Histopathologic alterations were significantly lessened in the treatment group.

Conclusions—These results indicate that ET-1 is involved in ischemia/reperfusion injury of the liver, which can be ameliorated by the monoclonal anti-ET-1 and anti-ET-2 antibody AwETN40.

Endothelins (ETs), a group of vasoconstrictive 21 amino acids identified by Yanagisawa and colleagues (1), are involved in the pathogenesis of various disorders of the liver. They play regulatory roles in the hepatic microcirculation and exert glycogenolytic actions in the liver (2). In particular, ET-1, synthesized by sinusoidal cells and catabolized by hepatocytes (3), induces Ca^{2+} release and stimulation of glycogen phosphorylase in hepatocytes (4), constriction of Ito cells (5), and stimulation of platelet-activating factor in Kupffer cells (6). More important, production of ET-1 is enhanced during ischemia and after reperfusion in the liver and causes sinusoidal constriction, leading to severe microcirculatory disturbances and tissue damage (7).

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In this study, we examined the role of ET-1 in ischemia-induced liver damage in dogs by blocking its action with a novel anti-ET-1 and anti-ET-2 monoclonal antibody, AwETN40.

Methods

Animals

Adult female beagle dogs weighing 8–12 kg were used. This study was conducted with the approval of the Institutional Animal Care and Use Committee, and all animals were cared for according to National Institutes of Health guidelines. After overnight fasting, the animals were anesthetized with thiopental sodium (25 mg/kg) for induction, and anesthesia was maintained with isoflurane, nitrous oxide, and oxygen by positive pressure mechanical ventilation. The right carotid artery and right jugular vein were cannulated for monitoring arterial pressure and central venous pressure and for obtaining blood samples. Electrocardiograph results and esophageal temperatures were monitored during the operation. Electrolyte solution (Plasmalyte, Travenol Laboratories, Deer-field, IL) was infused (20 mL·kg^{-1}·h^{-1}) through the operation to maintain adequate hydration and hemodynamic indices. Blood gases and electrolytes were measured frequently and corrected if necessary.

Operative procedures

After entering the abdomen through a midline incision, we skeletonized the liver by dividing all of the suspensory hepatic ligaments. A venous catheter, Intracath 16GA (Becton Dickinson Vascular Access, Sandy, UT), was inserted into the suprahepatic inferior vena cava and positioned in the left hepatic vein for collecting hepatic venous blood samples. The catheter was tunneled beneath the skin and exteriorized between the scapulae to allow serial blood sampling after the operation. Total hepatic vascular exclusion was initiated by cross-clamping the portal vein, hepatic artery, and infra- and supra-hepatic inferior vena cava. Pump-driven venovenous bypass (Biomedics, Minetonka, MN) was used to decompress the splanchnic venous bed and infrahepatic inferior vena cava (8). The bypass circuit connected the left femoral vein and the splenic vein to the left jugular vein. The animals were given heparin (50 U/kg) 5 minutes before the initiation of ischemia. After 2 hours of ischemia, the liver was reperfused, the bypass system was removed, and a splenectomy was performed. Cefamandole nafate (1 g) was administered intraoperatively and continued for 3 postoperative days. The animals were allowed to eat and drink the morning after the operation and were followed for 2 weeks.

Experimental groups

AwETN40, a monoclonal anti-ET-1 and anti-ET-2 antibody, was supplied by Takeda Chemical Industries Ltd. (Osaka, Japan). AwETN40 dissolved in saline was administered intravenously, at a dose of 5 mg/kg, 10 minutes before the initiation of ischemia (treatment group, n = 5). Animals receiving saline alone were used as controls (control group, n = 10).

Determinations

Two-week animal survival, liver function tests, total bile acid (TBA), hepatic tissue blood flow (HTBF), tissue biochemistry, histopathology, and ET levels were used to evaluate the efficacy of the treatment in this study. Peripheral venous blood samples were collected serially for determination of liver enzymes, including aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase, by a Technicon RA500 autoanalyzer (Bayer, Tarrytown, NY). Serum TBA was measured by the enzymatic fluorometric method using the Sterongnost-alpha Flu Kit (Nyegaad, Oslo, Norway) (9). Serial measurements of HTBF were performed intraoperatively using a laser Doppler flowmeter (Advance Laser Flowmeter, ALF21; Advance Company Ltd., Tokyo, Japan). The probe was placed on three lobes of the

liver until a stable flow value was obtained. To avoid a misreading in HTBF measurements related to respiratory motion, the ventilation was always stopped briefly. The mean of these three values was used as the representative value and was expressed as a percentage of the value of the preischemia level.

Wedge liver biopsies collected before ischemia, at the end of ischemia, and 15 minutes and 60 minutes after reperfusion were diced into small pieces and immediately transferred into liquid nitrogen for the determination of adenine nucleotides. Concentrations of adenine nucleotides were measured using a Waters high-performance liquid chromatography system (Waters Chromatography Division/Millipore Corp., Milford, MA; Model 484 absorbance module and Model 717 WISP system) at 254 nm (Waters 484, Tunable Absorbance Detector) (10). Energy charge was calculated using the equation $[ATP + \frac{1}{2} ADP]/[ATP + ADP + AMP]$, in which ATP = adenosine triphosphate, ADP = adenosine diphosphate, and AMP = adenosine monophosphate. The other tissues were fixed in buffered formalin and stained with hematoxylin and eosin. Histopathology was examined by a single pathologist without knowledge of the groups or the timing of tissue sampling. Histologic assessment was performed semiquantitatively based on the degree of sinusoidal congestion, sinusoidal derangement, ischemic hepatocyte injury, and hepatocyte necrosis as follows: none = 0, mild = 1, moderate = 2, and severe = 3. The number of neutrophils infiltrated in the tissue was counted after Leder's staining (11) and expressed as that per 1,000 hepatocyte nuclei.

Plasma concentration of immunoreactive ET-1 (irET-1) in the hepatic venous blood was measured by radioimmunoassay (12). The 4-mL blood samples, collected in a Vacutainar 6452 tube (Becton Dickinson Vacutainer Systems, Rutherford, NJ) with 7.5 mg of ethylenediamine tetraacetic acid, were centrifuged at $2,800 \times g$ and 4°C for 10 minutes and were stored at − 80° C until the assay. Samples and standards (ET-1; Peninsula Laboratories, Belmont, CA) were reconstituted in assay buffer and incubated for 24 hours with rabbit anti-ET-1 serum (RIK-6901; Peninsula Laboratories) at 4°C. The addition of iodine 125 ET-1 (Peninsula Laboratories) was followed by a second 24-hour incubation, and bound and free radioligand were separated using the second antibody method. Bound radioactivity data were evaluated after logit/log transformation, and the irET-1 data were presented after correction for recovery. The rabbit antiserum exhibited a cross-reactivity of 17% with human big ET-1 and of 7% with ET-3, but no cross-reactivity with unrelated peptides (ie, atrial natriuretic peptide, brain natriuretic peptide, vasopressin, and angiotensins I, II, and III). The standard curve was very stable, with a midpoint (50% inhibitory concentration) of 14 pg/tube.

Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM). Animal survival was compared using χ^2 test. Intra-group analysis was performed using Wilcoxon signed-rank test; intergroup analysis was performed using Mann-Whitney U test. A p value < 0.05 was considered statistically significant.

Results

Survival

All of the animals treated with AwETN40 survived for 14 days after the operation, but 7 of 10 control dogs died of liver failure. The survival rate in the treatment group was significantly higher than that in the control group (p < 0.05) (Fig. 1).

Mean arterial pressure and hepatic tissue blood flow

The mean arterial pressure of both groups fell moderately soon after the initiation of ischemia and fell considerably immediately after reperfusion. Our operational procedure produced

complete liver ischemia, but there was false-positive tissue blood flow by Doppler flow measurement during ischemia, ranging from 8–11% of the pre-ischemia levels (Fig. 2). The restoration of tissue blood flow after reperfusion was significantly better in the treated animals than in the controls.

Liver enzyme levels

After reperfusion, levels of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were significantly lower in the AwETN40-treated animals than in control animals (Fig. 3; Table 1).

Total bile acid

Table 1 details the changes in the TEA levels in the control and treatment groups. TEA levels in the treatment group reached a peak value at 6 hours after reperfusion and declined thereafter; levels in the control group continued to increase until 24 hours after reperfusion.

Endothelin levels

Changes in irET-1 levels in the hepatic venous blood in the control group are shown in Figure 4. Reperfusion of the liver after 2 hours of ischemia induced a significant rise in irET levels, by 4 times from 5 minutes to 60 minutes, 7 times at 3 hours, and > 18 times thereafter. Measurement of irET-1 in the treatment group failed because of the competitive nature of AwETN40 against the radioimmunoassay method used in this study.

Adenine nucleotides

Treatment with AwETN40 slowed the degradation of adenine nucleotides during ischemia and enhanced energy resynthesis after reperfusion (Table 2). Adenosine triphosphate levels and total adenine nucleotide levels in the treatment group at 15 minutes and 60 minutes after reperfusion were significantly higher than those in the control group. Energy charge showed no significant difference between the groups.

Histology

Although no significant histologic differences were found at the end of 2-hour ischemia and at 15 minutes after reperfusion, the treatment group showed significantly less structural damage and neutrophil infiltration in the liver than the control group. In particular, hepatocyte necrosis was not seen in any of the treatment-group animals (Table 3).

Discussion

In this study, we demonstrated that the neutralization of ET-1 by AwETN40 lessened microcirculatory disturbance, attenuated ischemia/reperfusion injury, and improved animal survival. In addition, pretreatment with AwETN40 slowed enzyme leakage, inhibited adenine nucleotide degradation in ischemic tissues, enhanced ATP resynthesis, and improved histopathologic findings.

Endothelins are a group of related 21-amino acid peptides (ET-1, ET-2, and ET-3), which bind to three different types of ET receptors (ET_A, ET_B, and ET_C) (13). In addition, two subtypes of ET_B receptors have been identified, ET_{B1} and ET_{B2} (14). ET_A has a high affinity for ET-1 and ET-2~ but a low affinity for ET-3. ET_B has an equally potent affinity for ET-1, -2, and -3 (15). The ET_A receptor generally has been accepted as the predominant receptor subtype associated with vasoconstriction. The ET_{B1} receptor is located in the endothelial cell and mediates vasodilation through the release of nitric oxide and prostacyclin. The ET_{B2} receptor is located on the vascular smooth muscle cells and mediates vasoconstriction directly (16). In

the liver, the number of these ET receptors per cell is 30–60-fold higher on Ito cells than on sinusoidal endothelial cells or hepatocytes (17). The abundance of ET receptors on Ito cells suggests that these cells are a major target for ET in the liver. Ito cells extend their numerous long cytoplasmic processes to surround the sinusoidal wall and are involved in reducing the hepatic sinusoidal microcirculation by contraction (18). Among various ETs, it has been shown that ET-1 is a major ET produced by ischemia and reperfusion, and that both ET_A and ET_B receptors are located on the cell membrane of Ito cells (15).

Endothelin can be divided into two structural portions: (1) the N-terminal 15 amino acids, having a rigid structure with two disulfide bonds; and (2) the C-terminal heptapeptide, consisting of relatively hydrophobic amino acids. AwETN40 is a monoclonal antibody for ET-1 and ET-2 that recognizes only the N terminal and not the C terminal. Although AwETN40 was first developed for the sandwich enzyme immunoassay for ET-1 (19), it has also proved effective in preventing myocardial infarction (20), acute renal failure (21), liver ischemia (22,23), and endotoxin-induced liver injury (24) by its neutralizing activity against ET-1 and ET-2.

The dosage of AwETN40 used in this experiment was based on previous reports. Watanabe and associates (20) showed that the effect of exogenous ET-1 administration (0.75 mg/kg) on blood pressure was neutralized by pretreating animals with 1.5–4.5 mg/kg of AwETN40. They also found that the effect of 22.5 mg/kg AwETN40 lasted for > 24 hours. Using a 1-hour partial (70%) liver ischemia model in dogs, Kawamura and colleagues (23) reported that pretreatment with 2 mg/kg of AwETN40 was effective in attenuating ischemia/reperfusion injury. Thus, we considered that the dosage of AwETN40 in this study, 5 mg/kg, would be sufficient for neutralization of ET-1 in ischemia/reperfusion injury in the 2-hour total hepatic vascular exclusion model in dogs.

In this study, the concentration of irET-1 in hepatic venous blood increased immediately after reperfusion and remained markedly elevated for up to 24 hours above 15.9 ± 7.1 pg/mL. Nakamura and associates (22) reported that irET-1 increased to 12 pg/mL at 3 hours after reperfusion using a 60-minute total liver ischemia model in rats. Kawamura and colleagues (23) reported that irET-1 increased to 6 pg/mL at 3 hours after reperfusion using the 1-hour partial liver ischemia model in dogs. The different concentrations of irET-1 between these studies may depend on the difference in species, the duration of ischemic time, the timing of the measurement, and the amount of ischemic liver damage. Levels of ET-1 in the circulation after ischemia and reperfusion are influenced by the balance between production and its clearance by the injured liver. It has been demonstrated that hypoxia induces ET-1 gene expression and secretion in endothelial cells (25), which is regulated by the degree of ischemia and the duration of reperfusion. In addition, interleukin-1, tumor necrosis factor, and transforming growth factor-β, all of which are up-regulated by ischemia and reperfusion, stimulate ET-1 production and release from sinusoidal endothelial cells (26). Clearance of ET is markedly reduced in injured livers as compared with normal livers (23).

Exogenous ET-1 has been shown to cause a pronounced elevation of intrahepatic vascular resistance, resulting in a profound decrease in hepatic blood flow and a refractory increase in portal pressure (2). In this study, although there was no difference in mean arterial pressure between the groups, preischemic administration of AwETN40 markedly improved HTBF after reperfusion. However, HTBF did not reach the preischemic level during the 1-hour observation period, suggesting that the effect of this agent may not be sufficient or that there are other factors involved. First, AwETN40 barely moves from the bloodstream into the interstitial space because of its large molecular weight. It may bind easily to ET in the circulation, but it is more difficult to trap ET in the interstitial space, even if a sufficient dose of the agent is given intravenously. Second, ET-1 exerts its action by an autocrine or paracrine mechanism. Ito cells

have a very low level of ET-1 mRNA in the normal liver, but it increases significantly once the liver receives ischemic insults (27). In addition, when ET-1 is produced by endothelial cells, 80% of the ET-1 is released into the interstitial space rather than into the circulation (28). Finally, insufficient restoration of HTBF in this study may relate to other impaired endothelial cell functions, such as reduced synthesis of nitric oxide and prostaglandins (29).

The determination of serum TBA is a sensitive and specific index for hepatobiliary disorders that depends on the synthesis and excretory functions of hepatocytes and the hepatic blood flow (30). As shown in the present study, TBA increased immediately after reperfusion and remained high in the control group for up to 24 hours; TBA levels in the treatment group began to decrease after 6 hours, indicating better hepatocyte function and greater hepatic blood flow.

Adenosine triphosphate is the most basic factor in maintaining functional and structural integrity of hepatocytes (31). Reduction of ATP levels leads directly to the disturbance of cellular integrity and exacerbation of cellular dysfunction. Endothelin-1 lowers ATP levels by increased consumption (2) or inhibits ATP synthesis by suppressing the oxygen supply through vasoconstriction (32). Pretreatment with AwETN40 abated these adverse effects of ET-1 in this study.

The number of polymorphonuclear neutrophils (PMNs) infiltrated in postischemic liver tissue was significantly reduced by AwETN40 treatment. Polymorphonuclear neutrophils play a crucial role in the progression of ischemia/reperfusion injury (33). During interactions of PMNs and ET-1, ET-1 has been shown to stimulate migration and aggregation of PMNs (34) and to enhance superoxide anion production by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (35). On the other hand, PMNs increase the induction of ET-1 mRNA expression in endothelial cells (36) and convert big ET to ET-1 (37). Furthermore, ET-1 stimulates the expression of adhesive molecules, CD11/CD18 integrin, on the neutrophil surface (38). The expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on cerebromicrovascular endothelial cell lines is also up-regulated by ET-1 (39). These observations suggest that ET-1 exerts influence on PMNs by facilitating their adhesion to endothelial cells and infiltration into the tissue. Consequently, the neutralization of ET-1 by AwETN40 appears, in part, to suppress PMNs' adhesion to endothelial cells, lessen PMNs' activation, and attenuate liver injury.

We postulated the following scenario on the basis of our findings. Two hours of ischemia causes extensive damage to sinusoidal endothelial cells in the liver. The damage triggers the production and release of endogenous ET-1 that induces the constriction of Ito cells, leading to sinusoidal constriction and microcirculatory disturbances. The reduction in blood flow augments postischemic tissue injury. In addition, ET-1 enhances the various adverse actions of PMNs. The neutralization of ET-1 by the monoclonal antibody AwETN40 is considered to contribute to the attenuation of liver damage by inhibiting sinusoidal constriction and PMN action, which are involved in the pathogenesis of ischemia/reperfusion injury of the liver.

Acknowledgements

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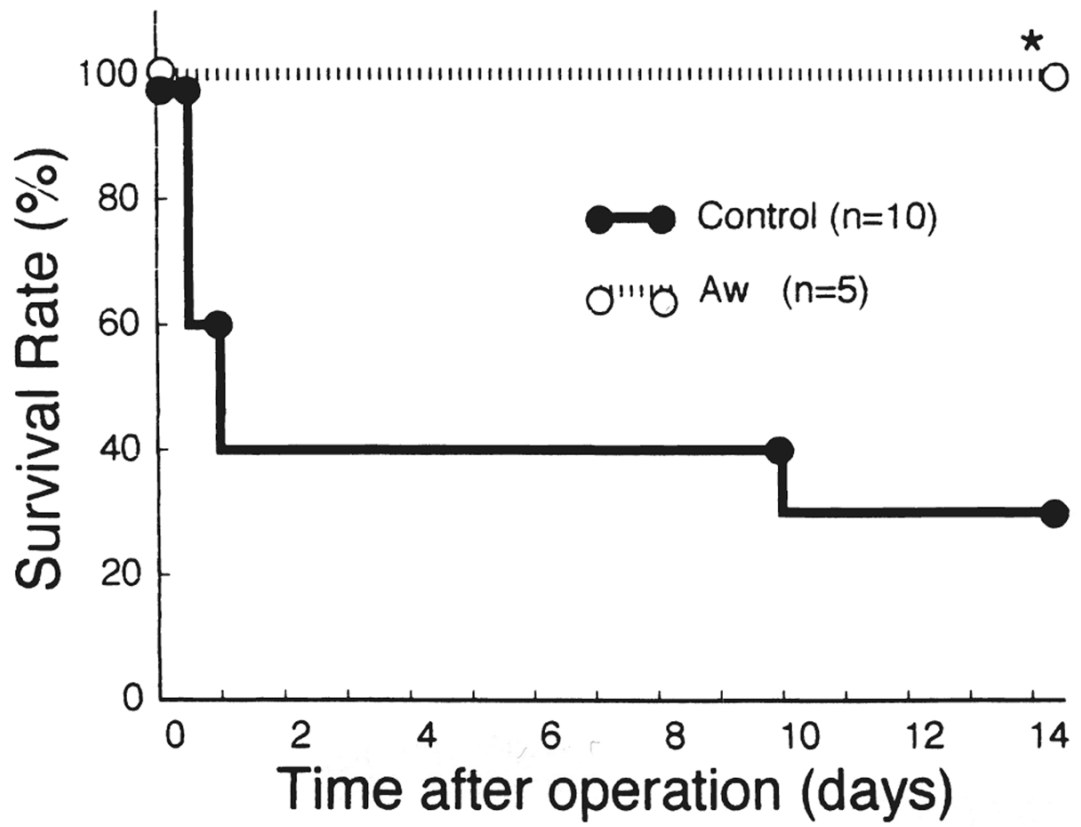
Fig 1.
Animal survival after the operation. *p < 0.05 versus control. Aw, AwETN40 (treatment group).
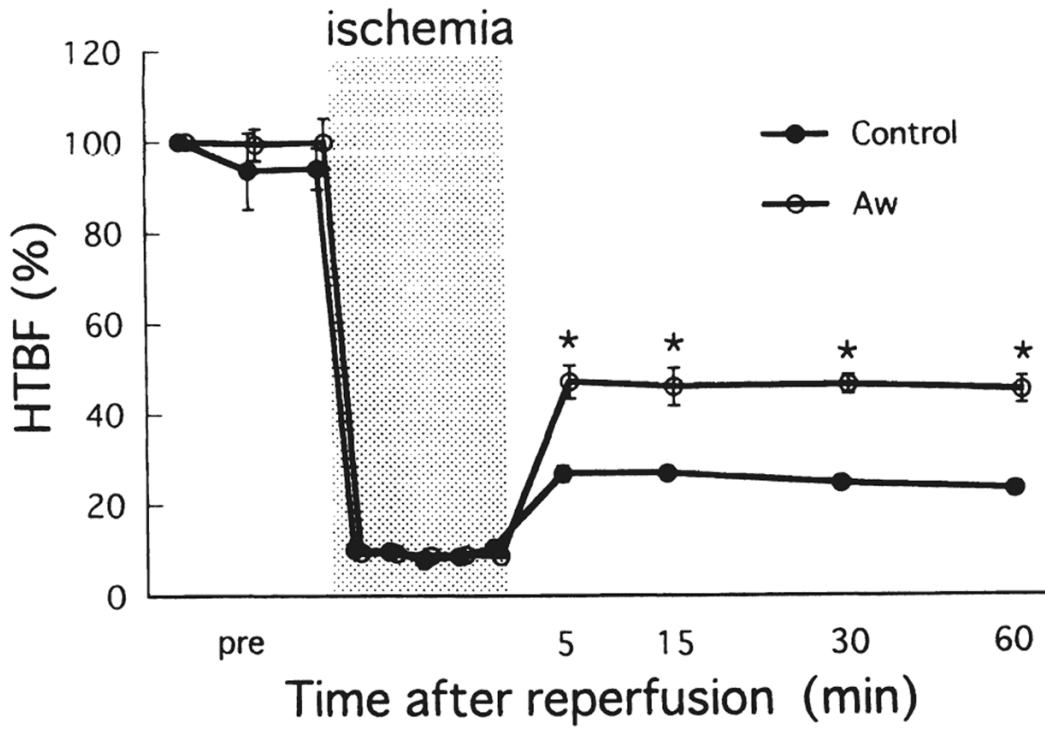
Fig 2.
Changes in hepatic tissue blood flow (HTBF) during ischemia and after reperfusion. Values are expressed as a percentage of the preischemic HTBF level. Bars express SEM. *p < 0.01 versus control. Aw, AwETN40.
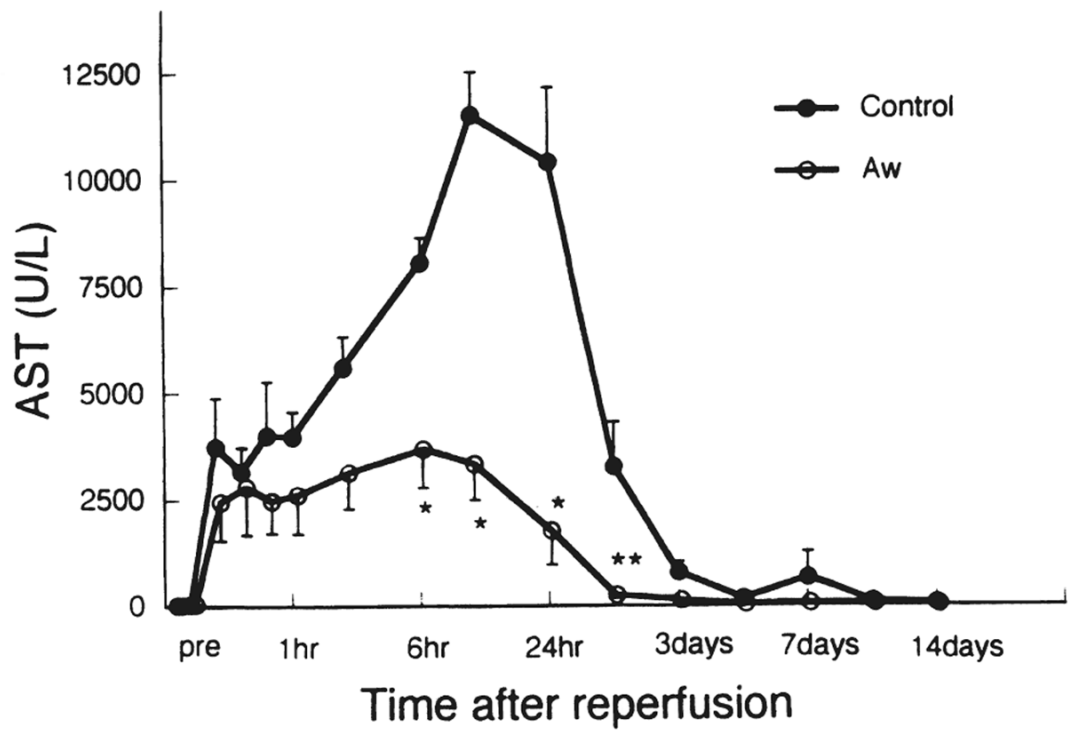
Fig 3.
Changes in aspartate aminotransferase (AST) after reperfusion. Bars express SEM; only one-sided SEM is presented. *p < 0.05 versus control: **p < 0.01 versus control. Aw, AwETN40.
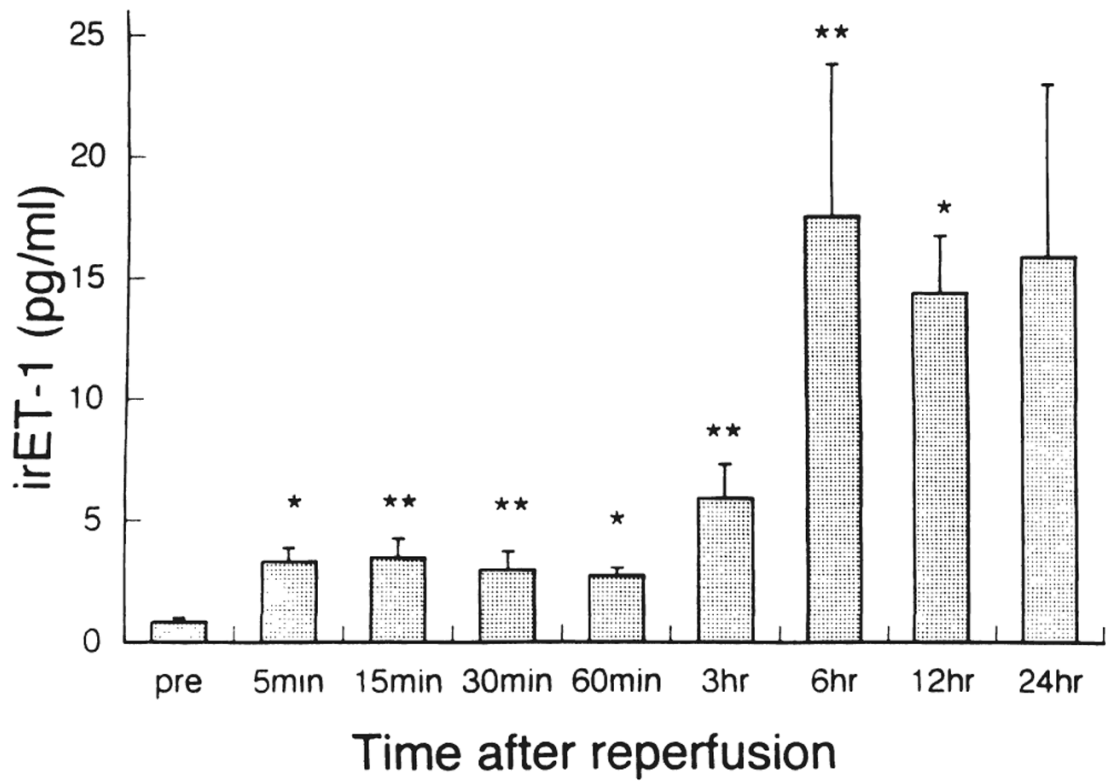
Fig 4.
Concentration of immunoreactive endothelin-1 (irET-l) in the hepatic venous blood alter reperfusion. Bars express SEM. *p < 0.05 versus preischemic value; **p < 0.01 versus preischemic value.

Table 1

Alanine Aminotransferase, Lactate Dehydrogenase, and Total Bile Acid After Reperfusion

Measurement	Group	Preischemia	30 min	60 min	3h	6h	12 h	24 h
ALT (U/L)	Control	32.2 ± 2.2	$4,846 \pm 1,678$	$4,961 \pm 798$	$6,575 \pm 1,007$	$9,332 \pm 1,128$	$13,563 \pm 1,379$	$12,680 \pm 1,451$
	Aw	32.0 ± 4.5	$2,222 \pm 699$	$2,373 \pm 897$	$2,842 \pm 807^{*}$	$3,344 \pm 914^{\dagger}$	$3,505 \pm 925^{\dagger}$	$3,439 \pm 1,612^{\dagger}$
LDH (U/L)	Control	80.6 ± 15.7	$3,209 \pm 1,076$	$3,385 \pm 474$	$3,128 \pm 479$	$2,906 \pm 559$	$3,045 \pm 634$	$1,440 \pm 216$
	Aw	53.0 ± 15.7	$2,378 \pm 624$	$2,004 \pm 732$	$1,533 \pm 423$	$1,148 \pm 161^{*}$	$561 \pm 125^{*}$	$261 \pm 25.5^{\dagger}$
TBA (μmol/L)	Control	0.3 ± 0.3	36.3 ± 7.9	48.5 ± 11.7	100 ± 11.0	133.8 ± 11.8	124.3 ± 13.1	175.4 ± 39.0
	Aw	2.4 ± 1.0	$67.2 \pm 5.0^{*}$	59.4 ± 14.9	97.4 ± 31.1	102.4 ± 24.6	$44.6 \pm 14.9^{\dagger}$	$30.4 \pm 12.3^{*}$

*
p < 0.05 versus control value.

†
p < 0.01 versus control value.

ALT, alanine aminotransferase; Aw, AwETN40; LDH, lactate dehydrogenase; TBA, total bile acid.

Table 2

Tissue Concentrations of Adenine Nucleotides During Ischemia and After Reperfusion

Time	Group	ATP (nmol/g tissue)	ADP (nmol/g tissue)	AMP (nmol/g tissue)	TAN (nmol/g tissue)	EC (nmol/g tissue)
Preischemia	Control	$2,666.2 \pm 283.5$	894.0 ± 111.2	213.6 ± 26.4	$3,773.7 \pm 286.7$	0.8229 ± 0.017
2 h after ischemia	Control	236.9 ± 40.4	335.0 ± 29.1	$1,147.1 \pm 95.9$	$1,718.9 \pm 132.2$	0.2337 ± 0.024
	Aw	357.0 ± 61.5	422.6 ± 94.2	$1,537.7 \pm 98.2$[*]	$2,317.3 \pm 165.1$[*]	0.2401 ± 0.031
15 min after reperfusion	Control	846.8 ± 152.2	409.9 ± 65.6	160.0 ± 15.9	$1,416.8 \pm 209.3$	0.7229 ± 0.036
	Aw	$1,905.3 \pm 233.9$[†]	623.7 ± 82.3[*]	252.7 ± 21.1[*]	$2,781.6 \pm 275.2$[†]	0.7943 ± 0.015
60 min after reperfusion	Control	$1,227.2 \pm 123.7$	329.4 ± 33.4	112.2 ± 13.1	$1,668.8 \pm 143.2$	0.8324 ± 0.014
	Aw	$1,994.2 \pm 171.8$[*]	517.9 ± 73.0[*]	268.4 ± 48.5	$2,780.6 \pm 137.1$[*]	0.8093 ± 0.030

[*]
p < 0.05 versus control value.

[†]
p < 0.01 versus control value.

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; TAN, total adenine nucleotides; EC, energy charge; Aw, AwETN40.

Table 3

Histologic Findings

Finding	Group	Preischemia	120 min of ischemia	15 min after reperfusion	60 min after reperfusion
Histologic abnormality					
Sinusoidal congestion	Control	0	1.0 ± 0.15	1.4 ± 0.31	2.0 ± 0.21
	Aw	0.2 ± 0.2	1.0 ± 0	1.6 ± 0.24	1.6 ± 0.24
Sinusoidal derangement	Control	0	1.5 ± 0.17	1.3 ± 0.15	2.1 ± 0.1
	Aw	0	1.2 ± 0.2	1.6 ± 0.24	1.8 ± 0.2
Ischemic hepatocyte injury	Control	0	0	1.1 ± 0.18	1.9 ± 0.18
	Aw	0	0	0.6 ± 0.24	$1.0 \pm 0.32^{*}$
Hepatocyte necrosis	Control	0	0	0.1 ± 0.1	1.3 ± 0.15
	Aw	0	0	0	$0^{\dagger}$
Total score	Control	0	2.5 ± 0.22	3.8 ± 0.42	7.3 ± 0.33
	Aw	0.2 ± 0.2	2.2 ± 0.2	$3.8 \, 0.73$	$4.4 \pm 0.6^{\dagger}$
Number of PMNs	Control	16.7 ± 2.2	15.7 ± 2.2	39.4 ± 5.5	57.2 ± 6.4
	Aw	17.3 ± 3.4	18.2 ± 4.2	22.5 ± 5.2	$22.3 \pm 3.2^{*}$

[*] $p < 0.05$ versus control value.

[†] $p < 0.01$ versus control value.

Aw, AwETN40; PMN, polymorphonuclear neutrophil.