Title: **Electric foot-shock stress drives TNF-α production in the liver of IL-6-deficient mice**

Running head: Stress-induced IL-6 secretion is negatively correlated with liver TNF-α level

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Abstract

Objectives: Accumulating evidence has shown that interleukin-6 (IL-6) elicits pleiotropic effects on a variety of biological functions, including its anti-apoptotic potential during liver injury. Our previous work demonstrated that restraint stress-induced elevation of plasma IL-6 negatively regulates plasma tumor necrosis factor-α (TNF-α). Herein, we further clarified the mechanism of the above finding and investigated the effect of IL-6 on liver apoptosis triggered by stress.

Methods: Male C57BL/6J and IL-6-deficient C57BL/SV129 mice were exposed to 1 hr of electric foot-shock stress. Thereafter, the serum, liver, and spleen TNF-α levels were measured at several time points. Serum alanine aminotransferase (ALT), liver caspase-3, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) activities were analyzed to evaluate the severity of liver injury and apoptosis.

Results: The liver, but not the spleen, of the IL-6-deficient mice exhibited a significant increase in the TNF-α level after stress in parallel with the serum TNF-α elevation, whereas no such TNF-α responses were found in the wild animals. No significant differences in stress-induced elevation in serum ALT levels, liver caspase-3 activities, and the number of TUNEL-positive hepatocytes were found between the wild and IL-6-deficient mice.

Conclusions: Taken together, these results indicate that IL-6 may play a critical role in suppressing the TNF-α production in the liver, thereby decreasing the blood TNF-α level. In contrast, IL-6 secretion was shown to have no protective effect on stress-triggered liver injury.

Introduction

Although interleukin-6 (IL-6) may have originally evolved as a pro-inflammatory cytokine that regulates the promotion of B lymphocyte differentiation into antibody-forming cells and the stimulation of T lymphocyte proliferation and differentiation (1,2), it is becoming more evident that IL-6 elicits pleiotropic effects on a variety of biological functions, including the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis (3,4), the production of acute-phase proteins in liver cells (5), activity as an endogenous pyrogen (6), modulation of nociception (7), and neurotrophic functions (8).

Furthermore, several animal studies have demonstrated that the blood IL-6 level is increased after application of not only inflammatory stimuli, but also physiological and psychological stressors such as exposure to an open field (9), electric foot-shock, conditioned aversive stress (10), immobilization (11-13), and restraint stress (14, 15), thus suggesting that IL-6 may also play a significant role in stress response. In fact, our previous study (14) reported that the treatment of mice with anti-IL-6 antibody sensitized the TNF-α response to stress, thus indicating that the restraint stress-induced elevation of plasma IL-6 negatively modulated the plasma TNF-α level and may have thus contributed to the maintenance of homeostasis. However, the exact mechanism of this new finding remains to be clarified.

Recently, it has been established that IL-6 has a potential hepatic anti-apoptotic effect and thereby can regenerate and repair the liver after injury (16-18). Accordingly, it is reasonable to suppose that an increase in IL-6 after stress could also inhibit liver injury due to stress-triggered apoptosis.

This study aims to replicate our previous finding (14) of stress-induced blood TNF-α elevation by the use of IL-6-deficient mice, to

evaluate TNF-α response to stress in the liver and spleen, and furthermore, to investigate the possibility of a protective effect of IL-6 on stress-initiated liver injury.

Materials & Methods

Mice

Male C57BL/6J (B6) (8-10 weeks of age, Charles River Japan, Shizuoka, Japan) and IL-6-knockout C57BL/SV129 (The Jackson Laboratory, Bar Harbor, ME) mice were kept on a 12-hour light/ 12-hour dark cycle with food and water freely available. The mice were allowed to acclimatize themselves to the colony for 7 days before the experiments began. The temperature of the colony room was maintained at 22-23 °C. This experiment was reviewed by the Ethics committee on Animal Experiments of the Graduate School of Medical Sciences, Kyushu University, and was done under the control of the Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

Stress protocol

The mice were administered an electric foot-shock by placing them in a box with a floor composed of stainless grids, according to the method reported by Shi et al. (19) with some modifications. A scrambled electric shock was delivered through the floor grids by an AC stimulator, PST-001 (Star medical, Tokyo, Japan). An interval timer was connected to the stimulator to allow repeated application of shocks of an intensity of 0.5 mA for 7.5 sec periods, once every 2 min. The control animals were maintained without access to food or water during the same regimen of foot-shock. At several time points following the exposure to stress, the mice were killed by decapitation and perfused with 20 ml phosphate buffered saline (PBS) at 4 °C via the left ventricle of the heart before collection of the serum, liver, and spleen. These samples were then stored at – 80 °C before being assayed.

Cytokine measurement

Sections of the liver and spleen removed from one end of the organs were weighed (80 to 120 mg) and homogenized in 400 μl of RIPA buffer (150 mM NaCl, 1.0 % TritonX-100, 0.1 % SDS, 10 mM Tris-HCl, and1 mM EDTA pH 7.2, 4 ℃) with a protease inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan). The homogenate was centrifuged at 15,000 rpm for 15 min to collect supernatants. The IL-6 and TNF-α amounts in the samples were quantified using the sandwich ELISA technique according to a method described previously (20). Briefly, 96-well ELISA plates were coated with anti-cytokine Ab at predetermined optimal concentrations. After washing and blocking, the wells were incubated with samples and standards, and then biotinylated anti-cytokine Abs was added. Then, streptavidin-peroxidase and a peroxidase substrate were added for the colorimetrical reactions. The cytokine concentrations of the test samples were determined with reference to a standard curve constructed using serial dilutions of the standard cytokines. For the assay of each cytokine, the following coating Abs, biotinylated Abs, and standards, respectively, were used: IL-6, MP5-20F3 (PharMingen, San Diego, CA), MP5-32C11(PharMingen), and recombinant IL-6 (Boehringer Mannheim, Mannheim, Germany); TNF-α, TN3-19.12 (PharMingen), rabit anti-mouse TNF-α(PharMingen), and recombinant TNF-α(Boehringer Mannheim). The liver and spleen IL-6 levels were normalized to the amount of protein in the sample measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Alternatively, since the serum TNF-α levels did not reach measurable levels by the sandwich ELISA assay, this concentration was determined in a cytolytic cell assay using the mouse fibrosarcoma cell line WEHI 164, clone 13 (purchased from ATCC, Manassas, VA), as previously described (21). In brief, the WEHI cells (2×10^4/100μl) were cultured with serial dilutions of

serum samples in a 96-well microtiter plate. After 18 hr of incubation at 37 °C with 5 % CO_2, 50 □l of MTT tetrazolium (1mg/ml; Research Organics, Cleveland, OH) was added to determine the cytolysis. After being allowed to incubate for an additional 4 h, the supernatants were removed, and 100 □l SDS with 0.01 N HCl was added to dissolve the crystals. The level of cytolysis was measured using a microplate ELISA reader (NJ-2300, System Instrument, Tokyo, Japan) at 570 nm. The TNF-α activity in the serum samples was calculated based on a recombinant TNF-α □ Boehringer Mannheim□ standard run in the same assay.

Measurement of serum alanine aminotransferase (ALT) activity

The ALT activity in serum was determined with a Hitachi 7170 automatic analyzer (Hitachi, Tokyo, Japan).

Measurement of liver caspase-3 activity

Liver caspase-3 activity was analyzed by a commercially available kit (CaspACE assay system, Promega, Madison, WI, USA). Briefly, one hundred milligrams of the liver was homogenized in 200 μl of a cell lysis buffer (CaspACE assay system, Promega) with a protease inhibitor cocktail (Sigma). The homogenates were centrifuged at 15,000 × rpm at 4 °C for 15 min. The supernatant was incubated with an enzyme substrate, Ac-DEVD-pNA at 37 °C for 4 hrs. The released pNA was determined by the changes of absorbance at 405 nm using a microplate ELISA reader (NJ-2300, System Instrument). To verify the specificity of the reaction, the enzyme activity in each sample was measured in the presence or absence of the caspase-3 inhibitor, Ac-DEVD-CHO. The results were expressed as pmol of the substrate cleaved per mg of liver protein.

Assessment of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

Formaline-fixed sections of liver tissue (5-μm) were collected from either wild or IL-6-deficient mice immediately after 1 hr of foot-shock stress, then deparaffinized in xylene and hydrated in graded ethanol. The apoptotic cells were identified using a Cell Death Detection kit (Roche Molecular Biochemicals, Rotekreuz, Switzerland). Five random ×20 fields from three slides/group were examined and the TUNEL positive brown nuclei (apoptotic) within hepatocytes were counted.

Statistical analysis

All data are expressed as the means ± SE. The data were analyzed by a factorial analysis of variance (ANOVA), followed by the unpaired t-test. For the data on the serum IL-6 and TNF-α levels, we used the Mann-Whitney *U* test. A P value of < 0.05 was considered to be significantly different from the corresponding value.

Results

Foot-shock stress elevated the liver TNF-α level of IL-6-deficient mice in parallel with the serum TNF-α level

First, we confirmed the consistency of the results of our previous animal studies (13-15). The present stress protocol also induced a significant increase in the serum IL-6 level that was maximal at 1 hr; the IL-6 level had decreased by 2 hr after the beginning of foot-shock stress (Fig. 1).

Moreover, the serum TNF-α activity in the foot-shock stress-exposed IL-6-deficient mice was statistically increased at both 2 and 4 hr after the introduction of 1 hr of stress, but not in the wild animals (Table 1). Similarly, foot-shock stress led to an elevation of the liver TNF-α level that was first seen at 2 hr and was significantly enhanced at 4 hr following the start of stress, while no such TNF-α response was apparent in the wild group (Fig. 2). In contrast, no increase in the spleen TNF-α was found in comparison of the levels before (11.7 ± 1.8 pg/mg protein) and at 4 hr (10.9 ± 0.8 pg/mg protein) after the beginning of stress in IL-6-deficient mice. Thus, these findings indicate that IL-6 may be critically involved in suppressing the elevation of serum and liver TNF-α levels during foot-shock stress.

Stress induces liver injury in IL-6-deficient mice similarly to wild mice

Since some recent studies have demonstrated that IL-6 has an anti-apoptotic effect in the liver (16-18), we examined whether or not the stress-induced increase in IL-6 had the capability of preventing hepatic apoptosis triggered by stress.

As shown in Fig. 3, the serum ALT activity in the IL-6-deficient mice did not significantly increase after stress, compared with that of the wild

animals. Likewise, the stress-induced elevation of liver caspase-3 activity, an obvious marker of apoptotic response, in the IL-6-deficient mice was almost identical to that in the wild animals (Fig. 4). In addition, a histological analysis by TUNEL also showed no significant difference of the number of apoptotic hepatocytes between these two groups of animals after stress (Wild mice 2.6 ± 0.5; IL-6-deficient mice 3.2 ± 0.7 per high per field, × 200), thus indicating that an increase in IL-6 in response to stress has no protective effect on stress-triggered liver injury.

Discussion

In this study, 1 hr of electric foot-shock stress was shown to result in significantly elevated serum IL-6 levels in wild mice. In addition, the stress-exposed IL-6-deficient mice, but not the wild mice, exhibited a TNF-α increase in the liver as well as in the serum, whereas no such a TNF-α response was found in the spleen. Thus, these findings indicate that IL-6 may play a critical role in blocking the TNF-α secretion in the liver after stress, which subsequently decreases the TNF-α level in the blood.

Several previous studies of animals have provided evidence for the involvement of the hepatic sympathetic nerve system in IL-6 up-regulation in the serum during various kinds of stress. Indeed, we and our colleagues have reported that the stress-induced elevation of plasma IL-6 is suppressed by the administration of 6-hydroxydopamine (6-OHDA), which is able to ablate the sympathetic nerve system, a partial (70 %) hepatectomy, or a hepatic sympathectomy (12,13,15). In addition, Kitamura et al. (22) proved by that immobilization stress induces IL-6 mRNA expression in the liver in parallel with an elevation in the plasma IL-6 level and that IL-6 positive signal is mainly present in the hepatocytes, but not in non-parenchymal cells. Together with an *in vitro* experiment (23) showing that norepinephrine (NE) increases IL-6 mRNA expression in hepatocytes, but not in non-parenchymal liver cells, these findings thus indicate that the endogenous NE, which is released from the end terminal of the hepatic sympathetic nerve, is responsible for stress-induced IL-6 production in the liver, thereby increasing the IL-6 level in the blood.

The present results demonstrated, for the first time, that liver TNF-α level increase after stress is negatively correlated with secretion of IL-6, which thus indicates that IL-6 may negatively regulate TNF-α□ □□□□□□□□□□□□.

In this study, the precise mechanism of this novel finding was not able to be established; however, one possible mechanism is that, in an indirect fashion, IL-6 inhibits liver TNF-α production through an activation of HPA axis. Actually, IL-6 was reported to elicit a stimulation of the HPA axis (3,4), thus suggesting that IL-6 may suppress the liver TNF-α production by enhancing the blood corticosterone secretion, which is known to have anti-inflammatory and immunosuppressive properties. Nevertheless, this possibility seems unlikely in our experimental system because radioimmunoassay did not show a significant difference in the serum corticosterone levels of the wild and IL-6-deficient animals after stress (data not shown), which is consistent to several previous studies demonstrating that IL-6 did not participate in stress-triggered HPA axis stimulation (14, 24).

An alternative possible mechanism is that IL-6 directly reduces the production of TNF-α, because previous studies have reported that, in *in vitro* human mononuclear cells (25) or monocytes (26) culture, IL-6 dose-dependently suppressed the TNF-α production induced by lipopolysaccharide (LPS). Moreover, Schindler et al. (25) also proved that IL-6 were capable of decreasing a transcriptional activation of TNF-α after treatment with LPS. In *in vivo* animal experiment, Fattori et al. (27) showed that LPS-treated IL-6-deficient mice produced threefold more TNF-α than did wild controls. Recently, an overexpression of IL-6 in IL-6 transgenic mice was shown to impair the TNF-α elevation induced by myocardial injury (28). Clearly, further studies are needed to verify the direct suppressive effect of IL-6 on TNF-α production in the liver and thereby to reveal the exact mechanism regulating the hepatic IL-6-TNF-α interaction.

Recent reports have demonstrated that IL-6 exerts the following anti-apoptotic effects in the liver: pretreatment with IL-6 protected mice from fulminant hepatitis by various kinds of lethal challenges such as warm ischemia /reperfusion (29), *Staphylococcus aureus* endotoxin B following D-Galactosamin sensitization (30), and a high dose of lipopolysaccharide (31). However, the present observation that stress-induced elevation of serum ALT and liver caspase-3 activities failed to show any differences between wild and IL-6-deficient mice indicates that elevated IL-6 after stress may have no protective effect on stress-triggered liver apoptosis.

In conclusion, this is the first report to indicate that increased IL-6 secretion after stress may negatively regulate liver TNF-α level, but that IL-6 has no capability to protect the liver from injury induced by stress. The present results may shed light on the role of IL-6 in the maintenance of homeostasis during stress.

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Legends

Fig. 1. Kinetics of serum IL-6 levels after stress

The wild mice were exposed to 1 hr of electric foot-shock stress. The serum IL-6 level was measured at 0, 1, 2, and 4 hr following the beginning of stress. All values were expressed as the mean ± S.E. (n=6-7/time points). A ** *P* value of < 0.01 was considered to be significantly different from the corresponding value.

N.D. indicates not detectable

Fig. 2. Stress increases the hepatic level of TNF-α in IL-6-deficient mice

The open- and closed-circles indicate the wild and IL-6-deficient mice, respectively. The liver TNF-α level was measured at 0, 1, 2, and 4 hr after the introduction of stress. All values were expressed as the mean ± S.E. (n=5-7/time points). A * *P* value of < 0.05 was considered to be significantly different from the basal value in the IL-6-deficient mice. # *P* < 0.05 and ##*P* < 0.01 were considered to be significantly different from the corresponding values in the wild mice.

Fig. 3. Stress increases the serum ALT activity in IL-6-deficient mice to an extent similar to that apparent in wild mice

The open- and closed-circles indicate the wild and IL-6-deficient mice, respectively. The serum ALT activity was measured at 0, 1, 2, and 4 hr after the start of stress. All values were expressed as the mean ± S.E. (n=5-7/time points). A * *P* value of < 0.05 was considered to be significantly different from the basal value.

Fig. 4. Kinetics of the liver caspase-3 activity after stress

The open- and closed-columns indicate the wild and IL-6-deficient mice, respectively. The liver caspase-3 activity was measured at 1, 2, 4 hr after the beginning of stress. All values were expressed as the mean ± S.E. (n = 5-7).

Table 1. Effect of stress on serum TNF-α activity

Mice		Serum TNF-α □ □□□□□y (□□□□□□		
		Time (hr)		
		0	2	4
B6	Wild	< 1.0	< 1.0	< 1.0
	IL-6-deficient	< 1.0	13.7 ± 7.1 *	24.6 ± 17.8 *

After both the wild and IL-6-deficient mice were exposed to 1 hr of electric foot-shock stress, measurement of serum TNF-α activity was done at 0, 2, and 4 hr following the introduction of stress. *$P < 0.05$ was considered to be significantly different from the basal value.

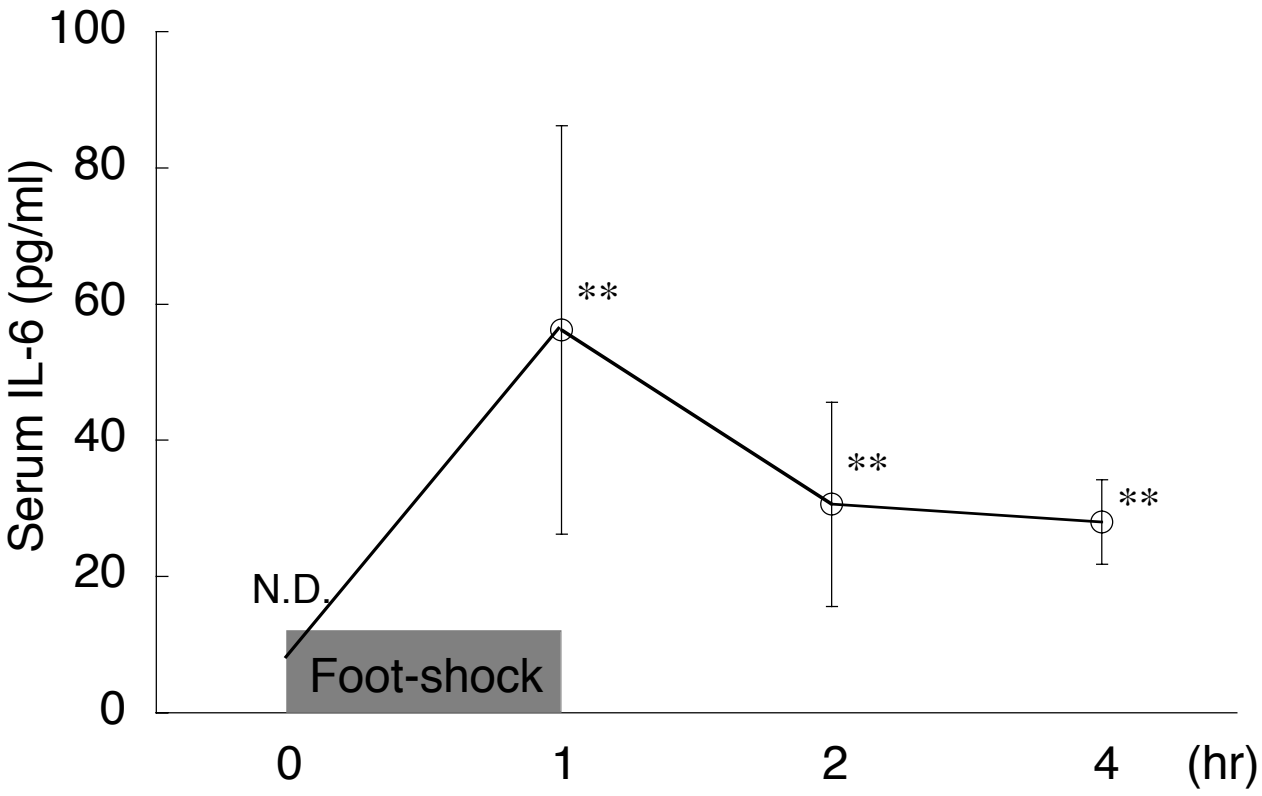

Fig. 1

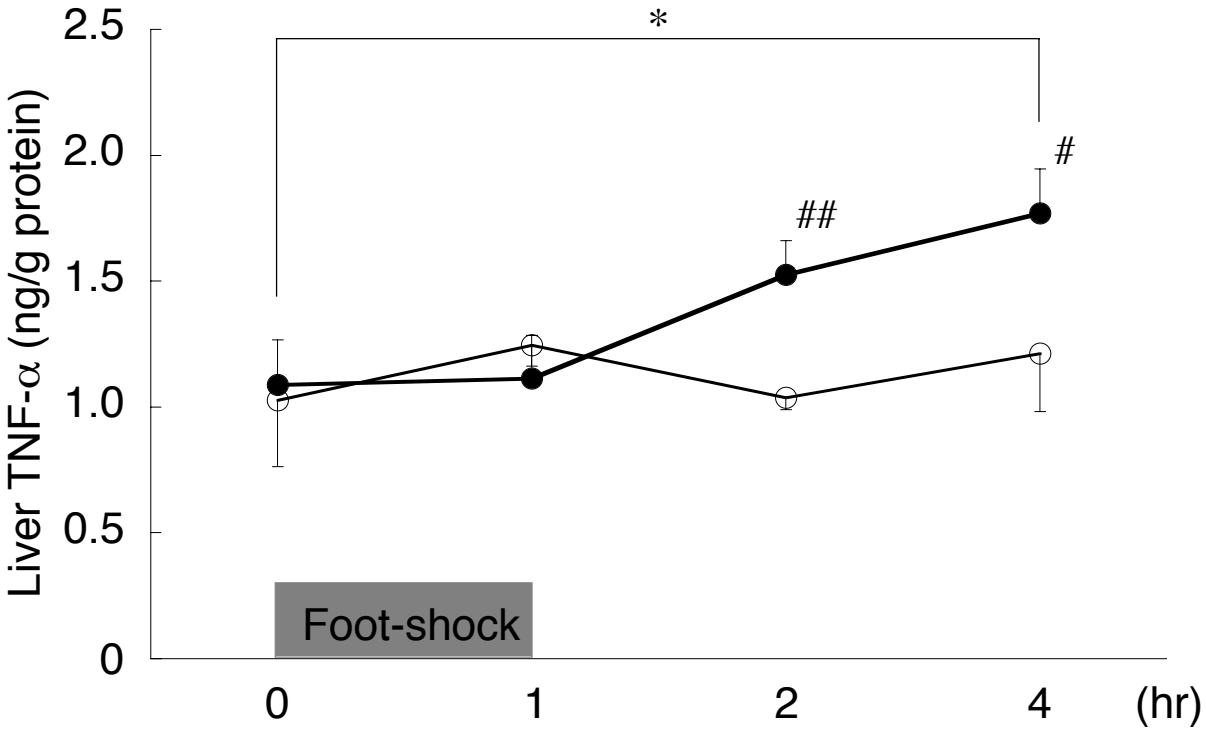

Fig. 2

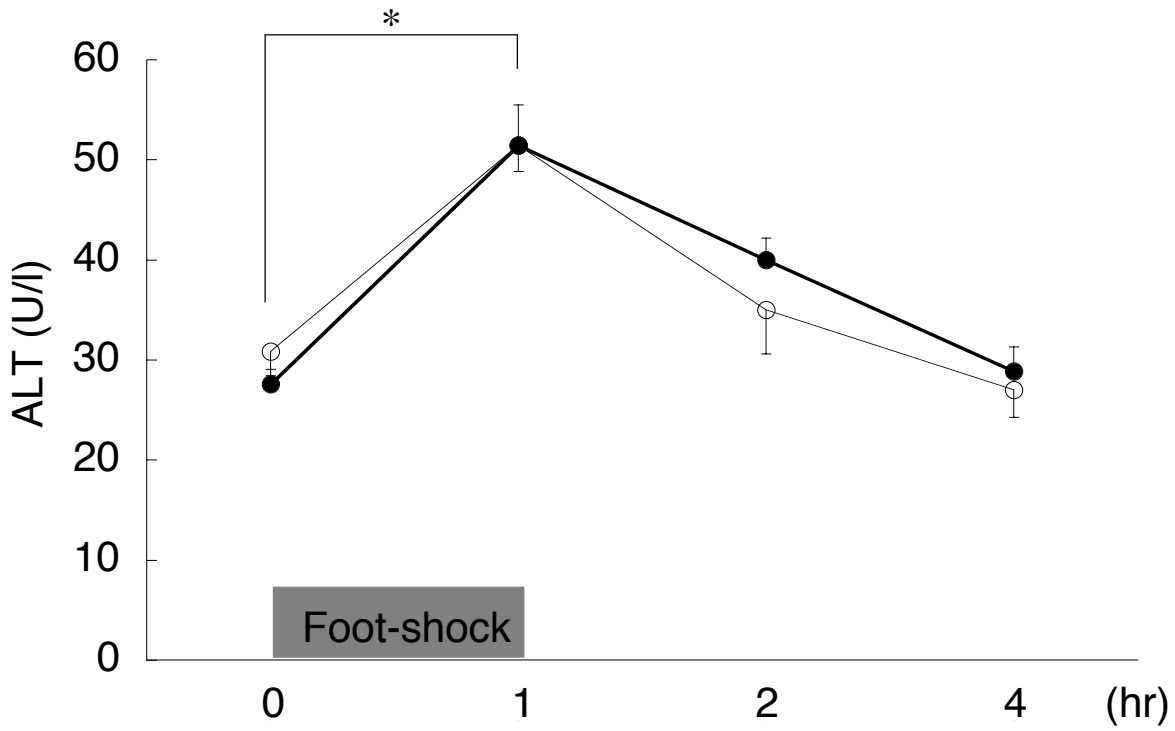

Fig. 3

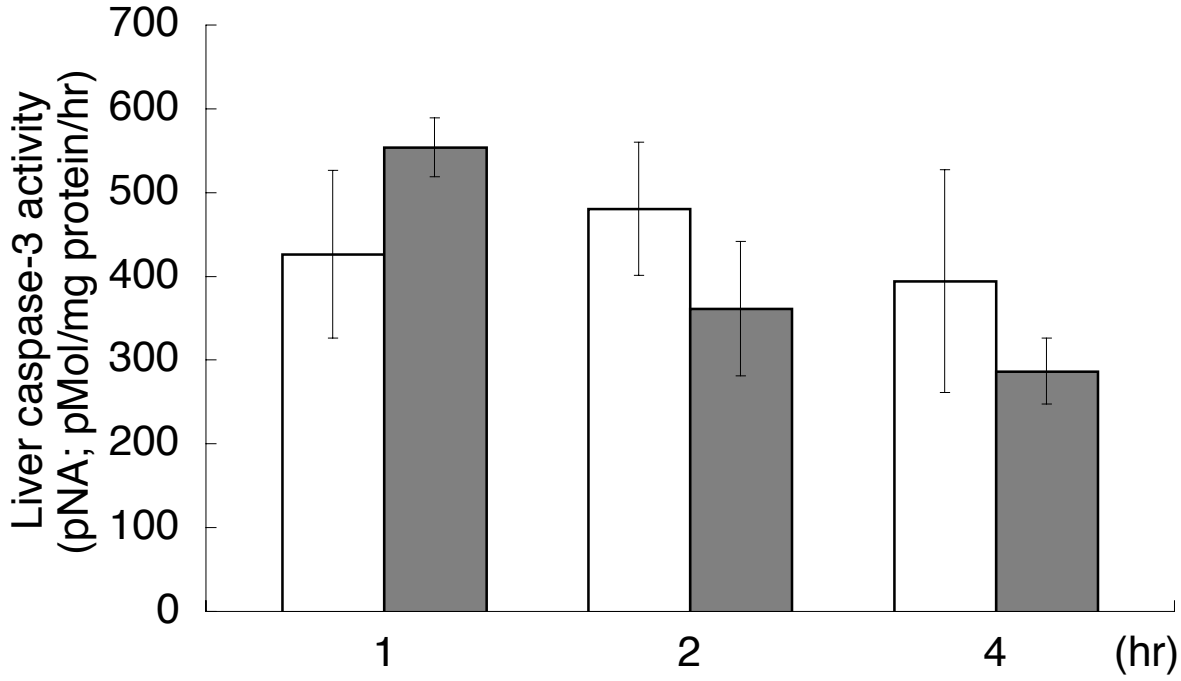
Liver caspase-3 activity
(pNA; pMol/mg protein/hr)
700
600
500
400
300
200
100
0
1
2
4
(hr)

Fig. 4