Social disruption stress exacerbates α-galactosylceramide-induced hepatitis in mice

Running head: Aggravation of liver injury by psychosocial stress

Junko Sonoda[1)], Yoichi Chida[1)], Nobuyuki Sudo[1)], and Chiharu Kubo[1)]

Department of Psychosomatic Medicine[1)], Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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Corresponding author: Chiharu Kubo, Department of Psychosomatic Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

TEL: 81-92-642-532, FAX: 81-92-642-5336

E-mail: chidayo@cephal.med.kyushu-u.ac.jp

Abstract

Objective: Psychosocial stress has been suggested as a possible aggravating factor in liver diseases, however the underlying mechanism has yet to be clarified. Recently, our research revealed that electric foot-shock stress aggravated NKT cell-dependent α-galactosylceramide (α-GalCer)-induced hepatitis in mice via a mechanism mediated by endogenous glucocorticoids. In this study, we examined whether or not such aggravation could be applied to *psychosocially* stressful situation, social disruption stress. **Methods:** Male wild C57BL/6 (B6) or B6 hepatitis virus type B surface antigen-transgenic (HBs-tg) mice, a hepatitis B virus carrier mouse model, were exposed three times in one week to social disruption stress in which an 8-month-old aggressive male intruder was placed into their home cage (5 mice per group) for 2 hr. At 12 hr after the final exposure to the stress, the wild and HBs-tg mice were intravenously injected with α-GalCer. **Results:** The stress-exposed wild mice exhibited significantly reduced thymus weight loss compared with the control animals. Moreover, this stress regimen led to a significant increase in the serum alanine aminotransferase levels of both the wild and HBs-tg mice, although the increase in the HBs-tg mice was higher than that in the wild mice. **Conclusion:** These findings demonstrated that, just as with electric foot-shock stress, social disruption stress exacerbated α-GalCer-induced hepatitis.

Introduction

Some early clinical reports suggested that psychosocial stress exacerbates the course and outcome of liver disorders. Indeed, a few cross-sectional studies have shown a significant association between the psychological state and the severity of certain liver diseases, such as alcoholic liver injury, chronic hepatitis type B, and chronic hepatitis type C [1-3]. In addition to these researches on humans, we recently conducted animal studies to clarify the physiological mechanisms behind the detrimental effect of stress on the liver [4-6]. Notably, electric foot-shock stress has been reported to aggravate α-galactosylceramide (α-GalCer)-induced hepatitis in mice [4]. α-GalCer, a glycolipid, has been reported to specifically stimulate liver NK1.1 Ag^+ T (NKT) cells and to subsequently induce hepatocyte apoptosis via the Fas-Fas ligand signaling pathway [7-9]. This Fas signaling-dependent liver injury is an experimental model of hepatitis that has been shown to be involved in the intrahepatic immunity to several infections and certain hepatic disorders [10-15]. Our recent work indicated that electric foot-shock stress elevated endogenous glucocorticoids, and consequently exacerbated α-GalCer-induced liver injury via both the expansion of liver NKT cells and the up-regulation of hepatocyte Fas antigen [4]. However, the application of these results to psychosocial stress in humans is limited because electric foot-shock stress is generally considered to include many physical factors.

Social disruption (SDR) stress, conflict during the social interaction of animals, is a model of psychosocial stress that may be comparable to the situation of people who feel a lack of control in human relationships. In rodents, several kinds of social stress have been shown to induce changes in the immune system, such as altered leukocyte subset populations [16-18], decreased mitogen-induced proliferation [19], decreased cytokine production [19], and decreased antibody production [16, 20].

In this study, we employed SDR stress as a mode of psychosocial stress and investigated whether or not SDR stress affects the pathologic state of α-GalCer-induced hepatitis in both wild mice and hepatitis virus type B surface antigen transgenic (HBs-tg) mice, a hepatitis B virus (HBV) carrier animal model.

Materials & Methods

Mice

Male C57BL/6 (B6) and C57BL/6J-TgN (Alb1HBV) 44Bri-transgenic (HBs-tg) mice were obtained from Charles River Japan (Shizuoka, Japan) and Jackson Laboratory (Bar Harbor, ME), respectively. All animals were maintained on a 12-hour light/ 12-hour dark cycle with food and water freely available. The temperature of the colony room was maintained at 22-23 °C. Mice were allowed to acclimatize to the colony for 7 days before the experiments began, and were used at 8 weeks of age. This experiment was reviewed by the Ethics in Animal Experiments Committee of the Graduate School of Medical Sciences, Kyushu University and was carried out under 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.

Experimental protocol

Mice were exposed to SDR stress according to the method of Stark et al. [21] with some modifications. Briefly, cages of 5 mice were randomly assigned to either the control or SDR group. An 8-week-old or an 8-month-old mouse was introduced into a cage with the control or stressed mice for 2 hr (5:00-7:00 PM) three times (every second day) during a period of one week. The behavior of the stressed mice was observed to ensure that the

intruder attacked the residents and that the residents showed submissive postures. If the intruder did not attack or was attacked by any of the residents, he was replaced with a new intruder. The health status of both animal groups was checked at the end of each stress procedure. Approximately 5-10 % of the SDR mice received visible tail injuries. In some experiments, the body and thymus weight of mice from both groups were measured 12 hrs after the last stress exposure. In a separate experiment, blood for corticosterone (CORT) measure was taken by cardiac puncture immediately after the last 2-h SDR. The blood samples were collected in EDTA-treated tubes and the separated plasma was stored at –80 °C until analysis.

α–GalCer, (2S,3S,4R)-1-O-(α-D-galactopyronosyl)-N-hexacosyl-2-amino-1,3,4-octadecanetriol, synthesized by the Kirin Brewery (Gunma, Japan), was dissolved in 0.5 ml of phosphate buffered saline (PBS). Referring to previous protocols [12, 22], a preliminary experiment was done to determine the optimal dose for inducing the most severe and reproducible liver injury. As a result, 50 ng and 25 ng of α–GalCer were intravenously injected to the wild and HBs-tg mice, respectively, 12 hrs. after the last stress procedure. Thereafter, blood samples were collected from the tail at the indicated time points and the sera were stocked.

Measurement of the serum alanine aminotransferase level

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

Measurement of the corticosterone levels

The plasma concentrations of CORT were measured with commercially available corticosterone enzyme-immuno assay kit (Cayman Chemical, MI).

Preparation of liver mononuclear cells

Hepatic mononuclear cells (MNCs) were prepared as previously described (4). In brief, the liver was collected immediately after the final SDR and was passed through a stainless steel mesh, and the resulting dissociated cells were suspended in phosphate buffered saline (PBS), washed once, resusupended in an isotonic 30% Percoll solution (Amersham, Wikströms, Sweden) containing heparin (100 U/ml, Shimizu Pharmaceutical, Shizuoka, Japan), and then centrifuged at 500 × g for 15 min at room temperature. The cell pellet was resuspended in 0.83 % NH_4Cl solution for 10 min and then was washed once in PBS supplemented with 2% FCS and 0.1% sodium azide (staining buffer). The number of MNCs was counted in a Bürker haemocytometer.

Flow cytometric analysis

The surface phenotypes of liver MNCs were characterized by a three-color flow cytometric analysis. Before staining with Abs, the MNCs were preincubated for 30 min at 4 °C with an anti-mouse FcgR II/III receptor antibody (2.4 G2) to prevent nonspecific binding. The cells were then washed twice in staining buffer, and incubated at 1×10^5 cells/sample for 30 min at 4 °C with a fluoresceninisothiocyanate (FITC)-conjugated mouse mAb to CD3 (145-2C11, PharMingen), a phycoerythrin (PE)-conjugated mouse mAb to NK1.1 (LMM6604, Caltag, Buringame, CA), and a biotin-conjugated mouse mAb to Fas ligand (MFL1, PharMingen), followed by being labeled with a peridinin chlorophyll protein (PerCP)-streptavidin (PharMingen). After washing, 10^4 cells were analyzed by flow cytometry using a FACScalibur (Becton-Dickinson, San Jose, CA).

Statistical analysis

All data are expressed as the means ± SE. The data on the time course of the serum ALT levels were analyzed by the repeated measure analysis of variance (ANOVA) followed by the Scheffé test. For other data, the unpaired t-test was used. A value of $P < 0.05$ was considered to be significantly different from the corresponding value.

Results

Effect of social disruption stress on body weight, thymus weight, and CORT in the wild type mice

Psychosocially stressful stimuli have been reported to decrease thymus weight [23-25]. Therefore, the thymus weight and the ratio of thymus weight to body weight were first evaluated to confirm if our SDR stress paradigm would actually place a stressful burden on the mice. As shown in Table 1, both the thymus weight and the ratio of thymus weight to body weight on the wild type mice were significantly reduced in the stress mice than the control animals, whereas no significant difference in body weight was found between the two animal groups. In line with these findings, the CORT, a well-known critical stress hormone, levels in the SDR stress mice were significantly higher than the control animal (CORT, Control group, 74.0 ± 33.5 ng/ml; SDR group, 375.0 ± 155.3ng/ml; $P < 0.01$).

Effect of social disruption stress on α-GalCer-induced liver injury in the wild and HBs-tg mice

In the wild mice, SDR stress accelerated an α-GalCer-induced increase in the serum ALT level, with the level significantly increased at 24hr after α-GalCer injection (Fig 1).

As for the HBs-tg mice, the increase in the serum ALT level by α-GalCer was more pronounced than that of the wild animals, even though the dose of this material in the HBs-tg mice (25ng/mice) was half the amount given to the wild animals (50ng/mice). More interestingly, exposure to SDR stress remarkably enhanced this exacerbation, as shown by the significantly elevated serum ALT levels at more time points (24, 48, and 72hr) in the HBs-tg mice than in the wild animals (Fig 2).

Discussion

Accumulating evidence has linked psychosocial factors to the onset, course and outcome of various physical diseases. In the digestive organs, peptic ulcer and inflammatory bowel disease have been investigated in numerous previous studies [26, 27]. However, little is known about the role of psychosocial stress in liver disorders. In this study, we demonstrated that SDR stress aggravated α-GalCer-induced hepatitis in mice, indicating that psychological stress may have a deleterious effect on the progress of acute liver injury and the acute deterioration seen in chronic hepatitis.

Possible mechanisms underlying the injurious effect of SDR stress on α-GalCer-induced hepatitis remain unclear; however, our recent study showed that electric foot-shock stress elevated endogenous glucocorticoids, which consequently exacerbated α-GalCer -induced liver injury via both the expansion of liver NKT cells and the up-regulation of hepatocyte Fas antigen [4]. At present, we confirmed that SDR stress significantly elevated the serum CORT levels in the wild mice. In addition, together with the current result that SDR stress failed to increase any expansion of liver NKT cells in the wild mice (NKT cells, Control group, $0.62 \times 10^4 \pm 0.11$; SDR group, $0.53 \times 10^4 \pm 0.21$), the previous finding suggests that, in an SDR stress paradigm, elevated endogenous glucocorticoids might mediate a stress-induced exacerbation of α-GalCer-triggered liver injury

via an up-regulation of hepatocyte Fas antigen, but not an expansion of liver NKT cells. This possible explanation needs to be supported by some further experiments using a glucocorticoid antagonist or adrenalectomy, and assessing the hepatocyte Fas expression.

HBs-tg mice, an HBV carrier transgenic mouse model, have been shown to display a characteristic liver pathology because of their overproduction of abnormally large amounts of the large envelope protein of HBV in the endoplasmic reticulum of their hepatocytes and to develop ground glass cells and spontaneous liver disease [28-30]. In addition, some recent studies reported that the same dose of α-GalCer gave rise to more severe liver injury in the HBs-tg mice than in wild mice [12, 22]. Likewise, the present study showed that an α-GalCer-induced increase in serum ALT level was more pronounced in the HBs-tg mice. Furthermore, this exacerbation was considerably enhanced by exposure to SDR stress, which arouses our interest in the influence of psychosocial status on the course and outcome of chronic hepatitis virus diseases in humans. Indeed, Kunkel et al. showed that alanine aminotransferase levels were positively correlated with the degree of depression, measured by the short form of the Beck Depression Inventory, in patients with chronic hepatitis B [2]. In addition, Nagano et al. recently indicated that chronic psychosocial stress relevant to the type I personality, as proposed by Grossarth-Maticek, was strongly related to the incidence of cancer and mortality and that it affected the course of chronic hepatitis C [3]. Together with the present

results in HBs-tg mice, these findings indicate that psychosocial stress may more easily and severely reduce liver function and induce liver injury in hepatitis virus carriers and in patients with chronic virus hepatitis than in healthy persons.

In conclusion, SDR stress was shown to exacerbate α-GalCer-induced hepatitis in the HBs-tg mice as well as the wild mice. Further research is needed to clarify possible mechanisms that might explain such an SDR-induced effect. The present experimental design using an animal model proved useful for investigating the association between psychosocial stress and liver injury.

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Legends

Fig. 1. α-GalCer-induced response of the serum ALT activity of the stress-pre-exposed wild mice

The animals were injected intravenously with α-GalCer (50 ng/mouse) 12 hr after the final exposure to either an 8-week-old (Control group) or an 8-month-old mouse (SDR group). All values were expressed as the mean ± S.E. (n=8-10/group). * $P < 0.05$ was considered to be significantly different from the corresponding value.

Fig. 2. α-GalCer-induced response of the serum ALT activity of the stress-pre-exposed HBs-tg mice

The animals were injected intravenously with α-GalCer (25 ng/mouse) 12 hr after the final exposure to either an 8-week-old (Control group) or an 8-month-old mouse (SDR group). All values were expressed as the mean ± S.E. (n=8-10/group). * $P < 0.05$ was considered to be significantly different from the corresponding value.

Table 1. Effect of social disruption stress on body weight and thymus weight **of the wild type mice**

Mice		Body weight (g)	Thymus weight (mg)	Thymus/Body (mg/g)
Wild mice	Control (n=7)	21.2 ± 0.2	59.0 ± 3.0	2.78 ± 0.15
	SDR (n=7)	20.8 ± 0.5	49.2 ± 2.1 *	2.36 ± 0.06 *

Measurement of body weight and thymus weight was done at 12 hr following the final exposure to social disruption (SDR) stress. *$P < 0.05$ was considered to be significantly different from the value of the control mice.

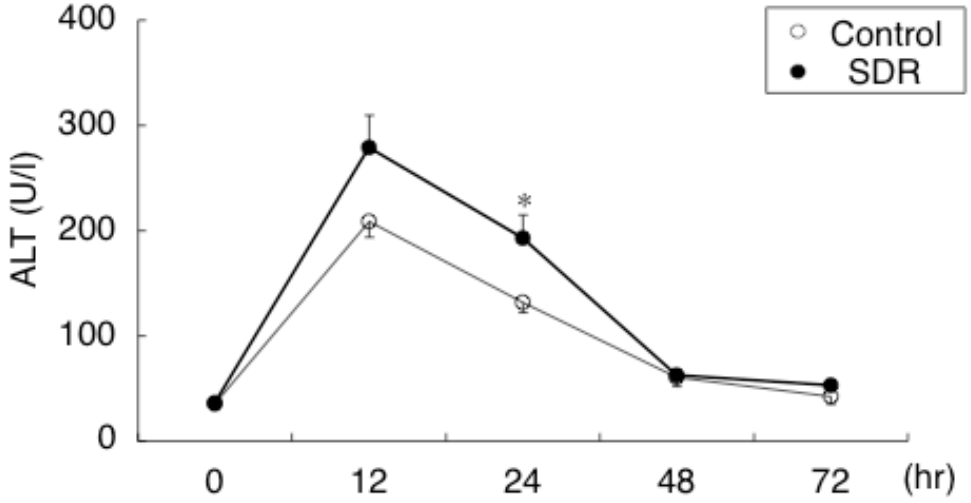

Fig 1

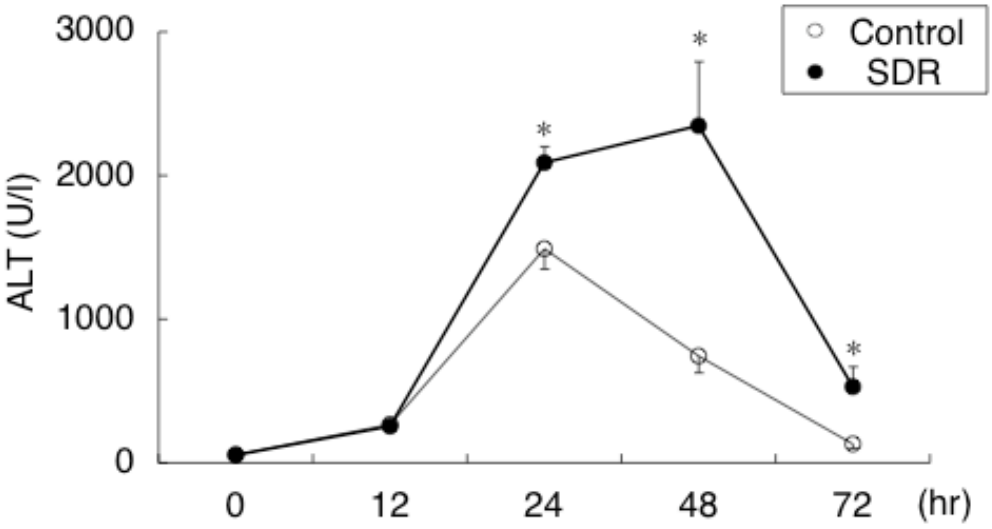

Fig 2

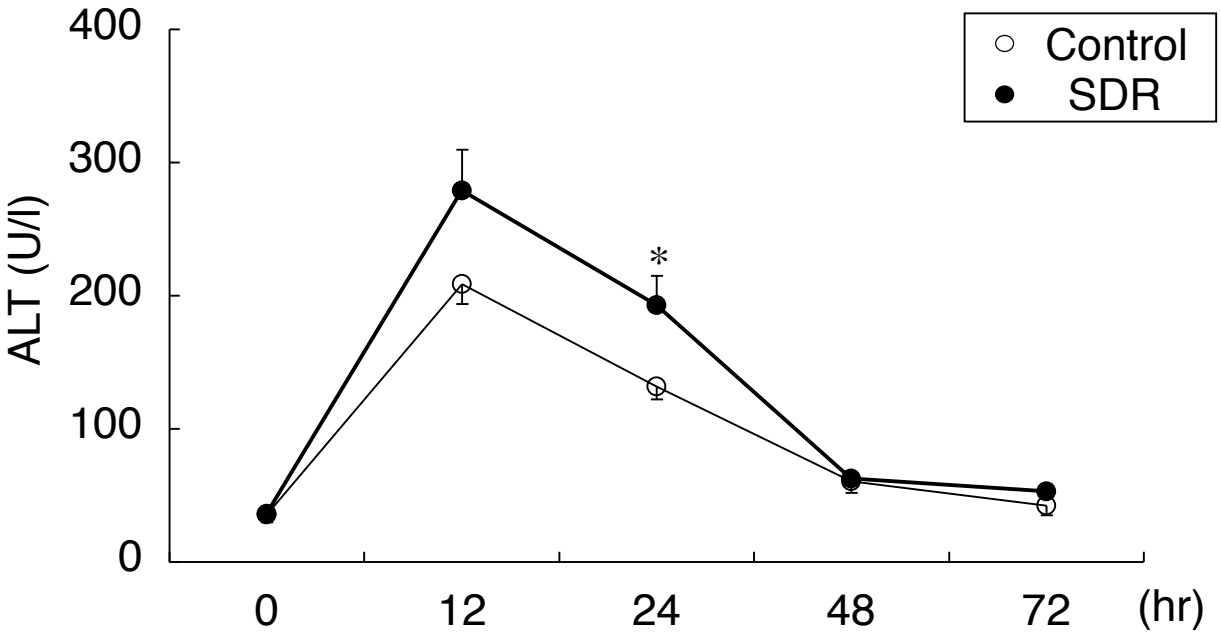

Fig 1

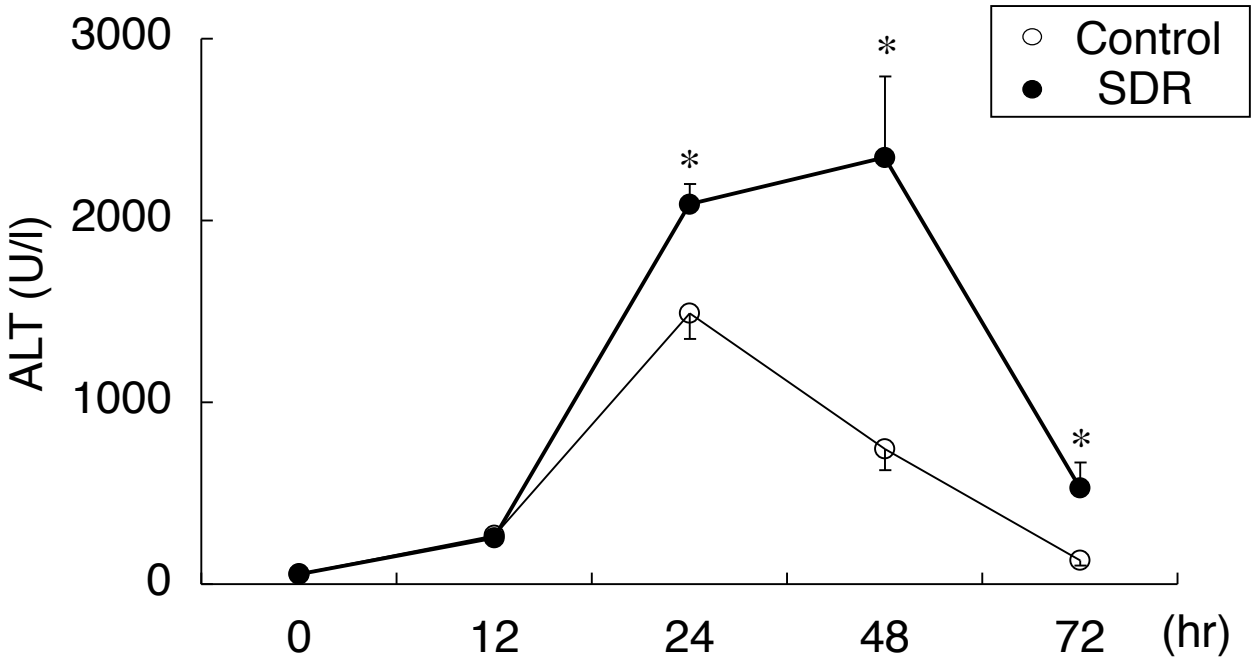

Fig 2