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Dantrolene prevents hepatic steatosis by reducing cytoplasmic Ca^{2+} level and ER stress

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ABSTRACT

Introduction: Our previous studies demonstrated that dantrolene, a ryanodine receptor stabilizer, prevents endoplasmic reticulum (ER) stress in the heart. ER stress is a strong mediator of impaired lipid metabolism in the liver, thereby contributing to fatty liver disease. In this study, we investigated the effects of dantrolene on fatty liver disease in mice and ER stress in hepatocytes.

Methods and results: Eight weeks old C57BL/6 mice were fed high-fat diet (HFD) for 8 weeks with or without the oral administration of dantrolene (100 mg/kg/day). The livers of mice without dantrolene (HFD group) showed severe fatty liver, whereas the livers of the mice treated with dantrolene (HFD + DAN group) only showed slightly fatty liver. To address the preventive effects of dantrolene, primary hepatocytes were cultured with palmitate in the presence or absence of dantrolene. Dantrolene reduced lipid load and prevents palmitate-induced increase in cytoplasmic Ca^{2+} and ER stress. Based on these findings, we propose that dantrolene is a potential new therapeutic agent against fatty liver disease.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of chronic diseases that includes fatty liver disease, steatosis, and more aggressive lesions such as steatohepatitis, lobular necro-inflammation with fibrosis, or cirrhosis [1–3]. NAFLD is often caused by obesity or lifestyle-related diseases, and it is classified into simple fatty liver and nonalcoholic steatohepatitis (NASH) [4]. NASH refers to fatty liver that progresses to cirrhosis or hepatocellular carcinoma (HCC), and it accounts for 10–20% of NAFLD [5]. It is thought that the onset of NASH progresses from NAFLD, but the associated mechanism is not clear due to various factors.

Among these factors, our attention has been on unfolded protein response and increase in cytoplasmic Ca^{2+} [6,7]. Therefore, the purpose of this study was to examine the role of cytoplasmic Ca^{2+} and endoplasmic reticulum (ER) stress in free fatty acid (FFA)-induced primary hepatocyte damage. Ryanodine receptors (RYRs) may play a role in the regulation of ER Ca^{2+} homeostasis [8].

Dantrolene is a specific agent used for the treatment of malignant hyperthermia (MH) [9]—a disease caused by a single amino acid mutation of skeletal RyR1 [10]. Dantrolene has also been shown to inhibit the leakage of Ca^{2+} from diseased RyR2 in CPVT or heart failure [11–15]. We have also demonstrated that dantrolene reduced ER stress by reducing ER Ca^{2+} leakage [in submission]. Based on these previous findings, we evaluated the effects of dantrolene on high-fat diet (HFD)-induced NAFLD in mice and FFA-induced elevation of cytoplasmic Ca^{2+} and ER stress in primary isolated hepatocytes.

2. Materials and methods

2.1. Solutions

Liver perfusion and liver digestion media were obtained from Gibco,

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MA. The attachment medium consisted of 5% fetal bovine serum (FBS) in Williams medium E. The culture medium consisted of 2 mM L-glutamine in HepatoZYME-SFM. Penicillin-streptomycin solution (Fuji Film-Wako, Osaka, Japan) was added to all the media.

2.2. Mice

Wild-type (WT) C57BL/6 mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Only male mice were used in this study. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The care of the animals and the protocols used were in accordance with the guidelines laid down by the Animal Ethics Committee of the Yamaguchi University School of Medicine.

C57BL/6 mice were fed either a high-fat diet (HFD; DIO Rodent Purified Diet w/60% Energy From Fat, TestDiet, MO, USA) or normal diet for 8 weeks, from when they were 5 weeks old (the groups were termed the HFD group and the CNT group, respectively).

Dantrolene-treated mice were fed with 100 mg/kg/day dantrolene (Fuji Film-Wako Chemicals, Tokyo, Japan). In all the groups, mice were fed using a feeding apparatus (Roden CAFÉ; Oriental Yeast Co., Ltd., Tokyo, Japan) to prevent the feed from getting wet with urine. To avoid excessive moisture from dantrolene, the feed containing dantrolene was changed at least every 3 days. Body weight was monitored during chronic administration of dantrolene; in all the mice, body weight increased similarly regardless of dantrolene administration. The weight of the food consumed by each mouse was monitored, and the amount of dantrolene powder mixed in the food was adjusted to 100 mg/kg/day, which we previously used to suppress the cardiac dysfunction and arrhythmias [16]. In most cases, the concentration of dantrolene in the food was 0.25% (w/w). No animal exhibited signs of toxicity, and no mortality was detected.

2.3. Histological analysis of the liver

Liver specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h, stored overnight in 10% formalin, and embedded in paraffin. Cross-sections (3 μm) of the tissue were stained with hematoxylin and eosin (H&E). Images were taken using a BZ-9000 microscope (Keyence, Tokyo, Japan).

2.4. Isolation and culture of primary hepatocytes

Hepatocytes were isolated from the livers of the mice, as described in the manufacturer's instructions. Briefly, after the mice were anesthetized, the inferior vena cava (IVC) and portal vein were exposed. The IVC was cannulated with a 24G soft needle and perfused with Liver Perfusion Medium (Gibco) at 4 mL/min for 5 min. Next, the perfusion medium was replaced with liver digestion medium (Gibco) at 3.5 mL/min for 10 min. The liver was collected into a dish with DMEM, and the liver sac was cut to release hepatocytes. The liver cell-suspension was filtered through a 70-μm mesh filter and spun at 100×*g* for 5 min. The pellet was re-suspended in attachment medium and added to collagen I-coated dishes (Matsunami, Osaka, Japan). After cell attachment (approximately 3 h after plating), primary hepatocytes were cultured at a density of 1.0×10^5 cells/well in HepatoZYME-SFM (Thermo-Fisher) containing 1 × penicillin-streptomycin, 2 mM L-glutamine and 1.25 μg/cm^2 type I collagen, at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. Palmitate-induced steatosis in primary hepatocytes

To assess the effects of dantrolene against hepatocyte damage, primary hepatocytes were incubated in 0.2 mM palmitate for 24 h with or without dantrolene (3 μM) in culture medium. Palmitate was first dissolved with bovine serum albumin (BSA) at a concentration of 1 mM with 0.17 mM BSA at 70 °C. Thereafter, the medium was added at a final concentration of 0.2 mM. Control dishes did not contain palmitate, BSA, and dantrolene.

2.6. Oil red O staining

The cultured hepatocytes were fixed with 10% paraformaldehyde in PBS for 10 min and washed three times with PBS. The cells were washed with deionized water and incubated in 60% isopropanol for 1 min, and subsequently stained with Oil Red O solution (60% Oil Red O in DW) for 10 min at room temperature. The cells were rinsed twice with PBS and counterstained with Gill's hematoxylin for 1 min and imaged using a BZ-9000 microscope (Keyence, Tokyo, Japan).

2.7. Immunocyto-fluorescence analysis

Antibodies against GRP78 were used for immunohistochemistry: PA5-19503 1/600, Thermo Fisher Scientific, Waltham, USA. Cultured hepatocytes were fixed with 4% paraformaldehyde in PBS for 5 min, washed three times with PBS, and permeabilized in 0.1% Triton X-100. Then, the cells were incubated in 1% BSA and 3% Protein Block (DAKO, CA, USA) for 1 h. The cells were next incubated with the GRP78 antibody (1/1000) in 0.1% BSA and 0.3% Protein Block for overnight at 4 °C, followed by labeling with an Alexa 488-conjugated secondary antibody (1/300; Molecular Probes, OR, USA). The cells were washed three times with PBS.

2.8. Analysis of cytoplasmic Ca^{2+} in isolated hepatocytes

The primary isolated hepatocytes were loaded with fluo-4 AM (20 μM; Molecular Probes, OR, USA) for 20 min at 37 °C. After washing the cells twice with Tyrode solution, the cytoplasmic Ca^{2+} images were obtained using a BZ-9000 microscope (Keyence, Tokyo, Japan).

2.9. Quantification of images

Quantification of fatty droplets, Oil Red O stained area, and fluorescence intensity of the cells were carried out using Image-J software. For fatty droplets and Oil Red O stained area, adequate threshold was determined by the blinded researcher, and area over the threshold was calculated. For fluorescent intensity, ROIs for each cell was manually set and mean fluorescent intensity of each cell was calculated.

2.10. Statistical analyses

We employed Kruskal-Wallis with a post-hoc Tukey's test for the comparison of 3 groups with 3 mice each (Fig. 1). One-way ANOVA with a post-hoc Tukey's test was used for statistical comparison of 3 groups with 60 cells each (Figs. 2–4). All data are expressed as the mean ± SEM. A probability value less than 0.05 indicated statistical significance. GraphPad Prism ver. 5 (Graph Rad Software, CA, USA) was used for the statistical analysis.

3. Results

3.1. Dantrolene administration prevents fatty liver in mice

As shown in Fig. 1A, although the intake amount of the meals of the HFD and HFD + DAN groups were almost the same except for the first week, the body weight of the mice in the HFD + DAN group was much lower than that in the HFD group. Figure 1B shows a representative image of the liver histology of each group. In the HFD group, the mice on the dantrolene diet had a significant reduction in hepatic steatosis compared with the HFD group. Hepatic lipid partitioning was calculated from the H&E stained image and shown in Fig. 1C.

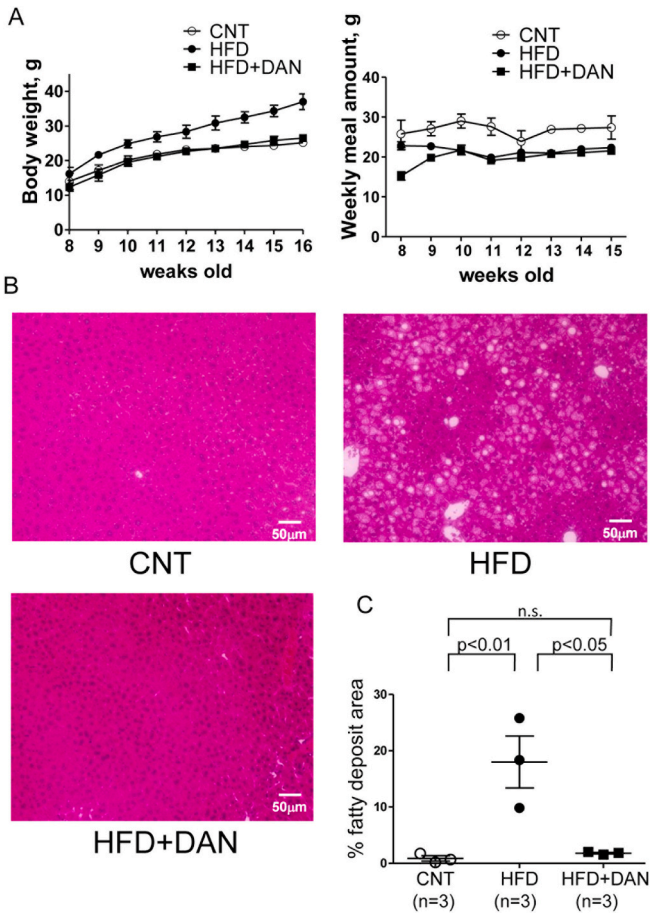

Fig. 1. A high-fat diet induce hepatic steatosis. Wild-type C57BL/6 mice were fed either normal control diet (CNT group) or high-fat diet (HFD group) for 8 weeks. A: Body weight and meal intake amount of the mice. B:Liver histology (magnification ×100); C: Summarized data of hepatic lipid partitioning are calculated from the Hematoxylin and eosin images. There were significant differences between HFD group and HFD + DAN group. Values are the mean ± SEM of three different mice.

3.2. Dantrolene reduces FFA intake in hepatocytes

We modeled lipotoxicity *in vitro* by culturing hepatocytes in the presence of 0.2 mM palmitate with or without dantrolene. Accumulation of lipid droplets was evident in the palmitate-treated primary hepatocytes (FFA group) at 24 h, whereas the lipid droplets reduced in the palmitate and dantrolene-treated cells (FFA + DAN group), as examined by Oil red O staining. Figure 2A shows a representative image of Oil Red O staining of hepatocytes. In the FFA + DAN group, the hepatocytes showed a significant reduction in lipid droplets compared with the FFA group. Hepatocytic lipid partitioning was calculated from Oil red O staining image and shown in Fig. 2B.

3.3. Dantrolene prevents FFA-induced increase in cytoplasmic Ca^{2+} in hepatocytes

Figure 3A shows a representative image of Fluo-4 in hepatocytes. In the FFA + DAN group, the hepatocytes showed a significant reduction in Fluo-4 intensity compared with the FFA group. The Fluo-4 fluorescence intensities are summarized in Fig. 3B.

3.4. Dantrolene decreases FFA-induced ER stress in hepatocytes

Cellular ER stress was measured using immunofluorescence staining of hepatocytes. Figure 4A shows a representative image of the

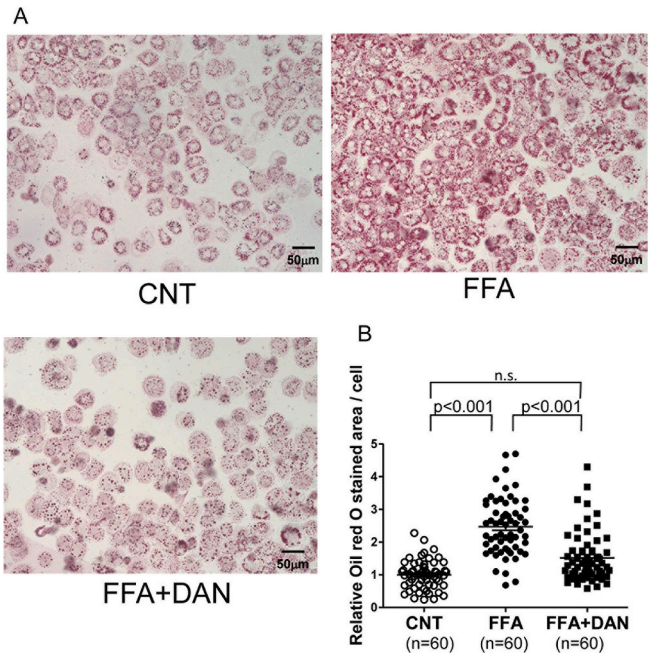

Fig. 2. Lipid accumulation in hepatocytes. Primary murine hepatocytes were isolated from mice and incubated for 24 h in either media alone (CNT group), media containing 0.2 mM palmitate (FFA group), or media containing 0.2 mM palmitate and 3 μM dantrolene (FFA + DAN group) and stained with Oil Red O. A: Representative image of Oil Red O stained hepatocytes. B: Oil Red O stained area per cell were calculated. There were significant differences between FFA group and FFA + DAN group. Values are the mean ± SEM of 60 cells from three different experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

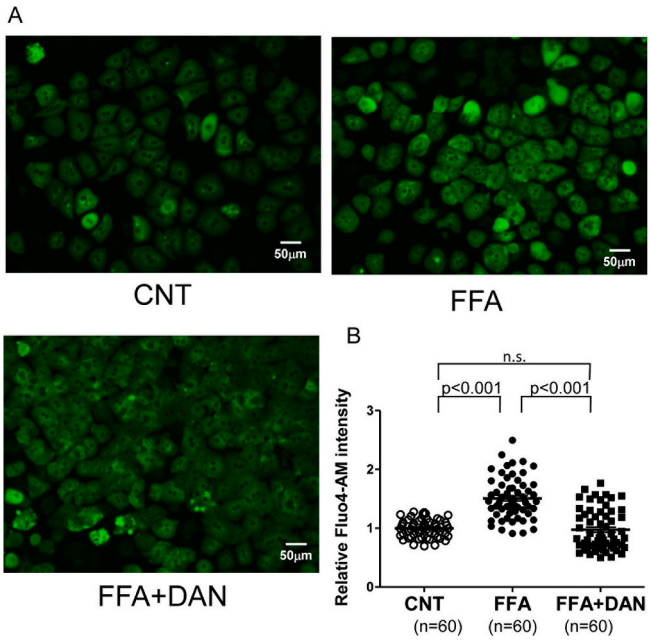

Fig. 3. Cytoplasmic Ca^{2+} level in hepatocytes. Fluo-4 AM was loaded in primary murine hepatocytes after incubation for 24 h in either media alone (CNT group), media containing 0.2 mM palmitate (FFA group), or media containing 0.2 mM palmitate and 3 μM dantrolene (FFA + DAN group). A: Representative images of Fluo-4 loaded hepatocytes. B: Fluo-4 fluorescent intensity were calculated. There were significant differences between FFA group and FFA + DAN group. Values are the mean ± SEM of 60 cells from three different experiments.

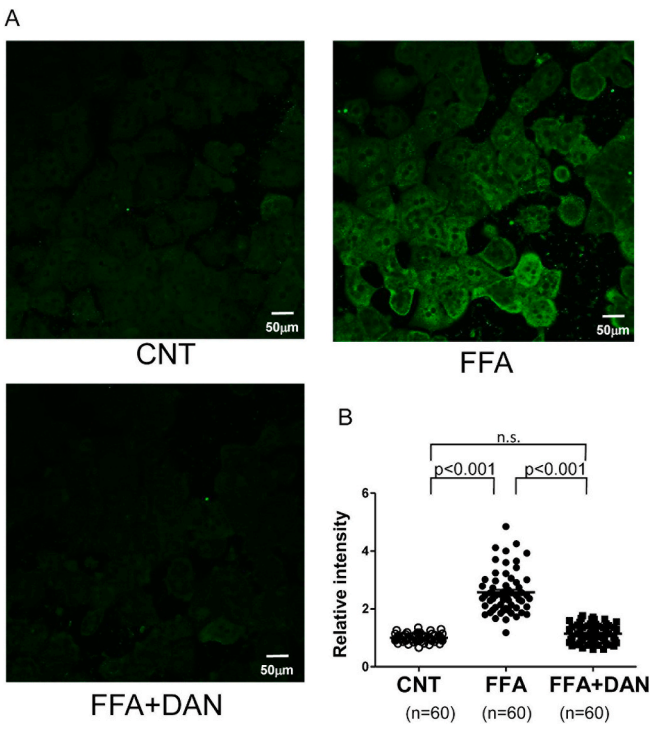

Fig. 4. GRP78 in hepatocytes. Immunocyte-fluorescent study of GRP78 was done for primary murine hepatocytes after incubation for 24 h in either media alone (CNT group), media containing 0.2 mM palmitate (FFA group), or media containing 0.2 mM palmitate and 3 μM dantrolene (FFA + DAN group). A: Representative images of immunocyte-fluorescent study for GRP78 hepatocytes. B: GRP78 fluorescent intensity were calculated. There were significant differences between FFA group and FFA + DAN group. Values are the mean ± SEM of 60 cells from three different experiments.

immunocyto-fluorescent staining of GRP78. In the FFA + DAN group, the hepatocytes showed a significant reduction in GRP78 intensity compared with FFA group. GRP78 expression was calculated from the image and summarized in Fig. 4B.

4. Discussion

The principal finding of this study is that dantrolene prevents NAFLD in a HFD-fed murine model. The mechanism of the dantrolene effect is discussed here.

The ER is an organelle important for protein synthesis and folding, lipid synthesis, and Ca^{2+} homoeostasis. It is known that palmitate induces ER stress via calcium depletion in the ER [17,18]. Elevated ER stress in the liver could contribute considerably to the alteration of lipid metabolism, thereby leading to hepatic steatosis [19] and apoptosis [20]. This appears to be a malignant cycle initiated by palmitate, ER stress, decreased lipid metabolism, and increased lipid precipitation. Therefore, the regulation of hepatic ER stress is viewed as a promising therapeutic strategy for the treatment of NAFLD.

Several studies have analyzed ryanodine receptor expression in the liver by [^{3}H]-ryanodine binding [21], ryanodine induced Ca^{2+} release [22], or mRNA expression [23]. In summary, only RyR1 is thought to be expressed in the liver, and it plays an important role in the regulation of ER Ca^{2+} levels [8].

The present study evaluated the effects of oral administration of dantrolene in an experimental NASH model. This study revealed that dantrolene suppressed lipid accumulation in the liver. Our conclusion is that dantrolene maintains ER Ca^{2+} levels to reduce ER stress, thereby closing the malignant loop of fatty precipitation in the hepatocytes. As far as we know, only one study has reported the effects of dantrolene on fatty liver disease [18]. This study used a Gaussia luciferase-based reporter protein system which was responsive to ER calcium depletion. They injected 15 mg/kg dantrolene (i.p.) daily for 7 days to the rats and found that ER calcium depletion was prevented by dantrolene. Although the method of administration was different, our data are consistent with their findings.

5. Conclusions

Oral administration of dantrolene prevents fatty liver disease by stabilizing ryanodine receptor on the ER and consequently preventing ER stress.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Masaki Tamitani: Data curation, Methodology. **Takeshi Yamamoto:** Writing - original draft, Supervision. **Naoki Yamamoto:** Supervision. **Koichi Fujisawa:** Supervision. **Shinji Tanaka:** Data curation. **Yoshihide Nakamura:** Data curation. **Hitoshi Uchinoumi:** Visualization. **Tetsuro Oda:** Data curation. **Shinichi Okuda:** Visualization. **Taro Takami:** Supervision. **Shigeki Kobayashi:** Supervision. **Isao Sakaida:** Supervision. **Masafumi Yano:** Supervision, Writing - review & editing.

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