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Docosahexaenoic Acid Reduces Palmitic Acid-Induced Endoplasmic Reticulum Stress in Pancreatic B Cells

EMI SUZUKI[1], TOMOKAZU MATSUDA[1], TAKESHI KAWAMOTO[2], HIROAKI TAKAHASHI[2], YUSUKE MIEDA[2], YUKI MATSUURA[2], TOMOKO TAKAI[1], AYUMI KANNO[1], MAKI KOYANAGI-KIMURA[1], SHUN-ICHIRO ASAHARA[1], HIROSHI INOUE[3], WATARU OGAWA[1], and YOSHIAKI KIDO[1,2*]

[1]*Division of Diabetes and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan;*

[2]*Division of Medical Chemistry, Department of Metabolism and Diseases, Kobe University Graduate School of Health Sciences, Kobe, Japan;*

[3]*Department of Physiology and Metabolism, Brain/Liver Interface Medicine Research Center, Kanazawa University, Kanazawa, Japan; *Corresponding author*

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Abstract: Endoplasmic reticulum (ER) stress leads to peripheral insulin resistance and the progression of pancreatic beta cell failure in type 2 diabetes. Although ER stress plays an important role in the pathogenesis of diabetes, it is indispensable for cellular activity. Therefore, when assessing the pathological significance of ER stress, it is important to monitor and quantify ER stress levels. Here, we have established a novel system to monitor ER stress levels quickly and sensitively, and using this method, we have clarified the effect of differences in glucose concentration and various fatty acids on the ER of pancreatic β cells. First, we developed a cell system that secretes *Gaussia* luciferase in culture medium depending on the activation of the GRP78 promoter. This system could sensitively monitor ER stress levels that could not be detected with real-time RT-PCR and immunoblotting. This system revealed that hyperglycemia does not induce unfolded protein response (UPR) in a short period of time in MIN6 cells, a mouse pancreatic β cell line. Physiological concentrations of palmitic acid, a saturated fatty acid, induced ER stress quickly, while physiological concentrations of oleic acid, an unsaturated fatty acid, did not. Docosahexaenoic acid, an n-3 unsaturated fatty acid, inhibited palmitic acid-induced ER stress. In this study, we have established a system that can sensitively detect ER stress levels of living cells in a short period of time. This system can be used to monitor the state of the ER in living cells and lead to the investigation of the significance of physiological or pathological ER stress levels.

INTRODUCTION

The endoplasmic reticulum (ER) is an intracellular organelle located around the nucleus, and plays an important role in maintaining protein homeostasis, leading to the maintenance of cellular homeostasis. Proteins are polymers of amino acids, which are present in all living organisms, and synthesized proteins form life itself. Genetic information, which encodes proteins, exists in the nucleus as DNA. DNA is transcribed in the nucleus to become mRNA, which is translated by ribosomes located outside the nucleus to synthesize proteins; however, the primary structure of the amino acid polymer does not have a functional role. The ER has the role of quickly folding an amino acid polymer and forming an appropriate three-dimensional structure, which serves as the source of membrane proteins or secreted proteins (1). From this activity, the ER is referred to as a "protein quality control factory." In the ER, molecular chaperone proteins, such as GRP78, play a central role in the folding of the primary structure of the amino acid sequence into a three-dimensional structure. In addition, the ER acts as a store of intracellular calcium, which contributes to various important intracellular functions. Folding proteins in the ER is very important for maintaining cellular homeostasis; therefore, there is a special adaptive response for maintaining the homeostasis of the ER in the presence of unfolded proteins, which is called UPR (unfolded protein response).

Recently, it has become clear that the state of ER stress is critical in the etiology of a number of diseases, such as Alzheimer's disease, Parkinson's disease, various immune diseases, metabolic syndrome, and cancer (2–5). ER stress reportedly leads not only to peripheral insulin resistance (6) but also to the progression of pancreatic β cell failure (7–9). Control of the synthesis of insulin in pancreatic β cells, which accounts for approximately half of all

Phone: +81-78-796-4549 Fax: +81-78-796-4509 E-mail: kido@med.kobe-u.ac.jp

proteins synthesized in these cells, is very important (10). The synthesis of insulin represents an extreme burden to the ER and is considered to cause ER stress when it exceeds ER protein folding capacity of the ER, finally leading to pancreatic β cell failure (11). In addition to insulin synthesis, various stimuli, such as inflammatory cytokines (12, 13), free fatty acids (14, 15), and hyperglycemia (16, 17), reportedly cause ER stress in pancreatic β cells. It is also known that pancreatic β cell failure caused by these stimuli develops due to the complicated involvement of various stresses and signals including oxidative stress. It has also been reported that ER stress induces oxidative stress, and the production of reactive oxygen species induces ER stress (18); thus, the existence of close crosstalk between ER stress and oxidative stress has been clarified recently.

While oxidative stress can be detected from blood or urine samples on an individual level, it is difficult to detect ER stress levels in the same way, so it is not clear how much ER stress levels is directly involved in the development of pancreatic β cell failure. Furthermore, UPR plays an important role in the pathogenesis of diabetes, while it is also indispensable for cellular activity. ER stress comprises two types: pathogenic stress causing cell abnormalities, which cannot be completely treated with UPR, and physiological stress caused by biological activity, which is controllable by UPR. Therefore, when assessing the pathological significance of ER stress levels, it is important to determine physiological stress and pathological stress. Thus, we believe that further research is necessary for ER stress levels to be a therapeutic target and for the prevention of ER-associated diseases, such as diabetes.

The molecular chaperone GRP78, which is the most abundant constituent molecule of the ER, plays a central role in folding to determine the higher order structure of proteins. Therefore, we attempted to construct a system for the accurate detection of ER stress levels by monitoring the secretion of luciferase into culture medium, according to the activity of the GRP78 promoter. We clarified the effect of hyperglycemia and various fatty acids on the ER in a pancreatic β cell line (MIN6 cells) using this system.

MATERIALS AND METHODS

Cell culture

MIN6 cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 15% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator.

Reagents

Tunicamycin was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Palmitic acid, oleic acid, and docosahexaenoic acid (DHA) were purchased from Sigma-Aldrich. A stock solution of palmitic acid was dissolved in 100% ethanol at a concentration of 100 mM, and heated to 37°C. The stock solution of palmitic acid was reheated to 37°C and vortexed before use to give the final concentration shown in the figures. A stock solution of oleic acid was dissolved in 100% ethanol at a concentration of 100 mM. A stock solution of DHA was dissolved in 95% ethanol at a concentration of 1 mM, then sealed in an air-tight tube and stored at -20°C until use.

Immunoblot analysis

MIN6 cells were lysed using sonication as described previously (19, 20). The extracted proteins were loaded onto a 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to a polyvinylidene difluoride membrane, and subsequently blocked with 3% bovine serum albumin in Tris-buffered saline for 45 min. The membranes were incubated with primary antibodies for GRP78, CHOP, C/EBP-β (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich) overnight, and with the secondary antibodies (anti-rabbit IgG antibody [Promega, Madison, WI], goat anti-mouse IgG antibody [Bio-Rad Laboratories, Hercules, CA], and donkey anti-goat IgG HRP antibody [Abcam, Cambridge, UK]) for 1 h. The protein bands were visualized with an enhanced chemiluminescence detection system. Quantitative analysis of the blots was performed using Multi Gauge Version 3.0 software (Fujifilm, Tokyo, Japan), and normalized to β-actin levels.

Real-time RT-PCR analysis

Real-time RT-PCR analysis was performed as described previously (21, 22). Total cellular RNA was extracted from MIN6 cells using an RNeasy Kit (QIAGEN, Valencia, CA). Real-time RT-PCR was performed with the SYBR Green reagent (Promega) and quantified with an ABI 7900 sequencer. Each experiment was repeated three times. The relative abundance of mRNAs was calculated with cyclophilin mRNA as the invariant control. The sequences for the primer pairs (Invitrogen, Carlsbad, CA) were as follows (forward and reverse, respectively): 5′-ACCGGGTTTCGGGACTTGA-3′ and 5′-GTTGCGTAGTCCCGTGTCCA-3′ for mouse C/EBPβ; 5′-TGGAGTTCCCCAGATTGAAG-3′ and 5′-CCTGACCCACCTTTTTCTCA-3′ for mouse GRP78; and 5′-AGCTGGAAGCCTGGTATGAGGA-3′ and 5′-AGCTAGGGACGCAGGGTCAA-3′ for mouse CHOP.

Plasmid construction and transfection

On the basis of an examination of the published sequence of human GRP78, we amplified by PCR a 311-bp fragment of the GRP78 promoter (nucleotides -304 to +7 relative to the transcription start site) from genomic DNA of HeLa cells and cloned it into the *Kpn*I-*Hind*III sites of the pBlueScript II vector (Agilent Technologies, Santa Clara, CA). Next, we generated a DNA fragment with floxed yellow fluorescent protein-*Gaussia* luciferase (YFP-Gluc) by using PrimeSTAR® Max DNA Polymerase (Takara Bio Co., Shiga, Japan), and inserted this fragment into the *Eco*RI-*Hind*III site of the GRP78-luciferase vector to generate the pBS-GRP78 promoter floxed YFP-Gluc vector (pBS-Gluc vector). The GRP78-luciferase reporter vector, as well as vectors for C/A ATF6α and pcDNA-Cre, were transfected into the cells by using Lipofectamine 3000 (Invitrogen). The cells were exposed to various reagents and assayed for luciferase activity at 24 h after transfection.

Luciferase assay

MIN6 cells and HEK293 cells were transfected with the GRP78-luciferase reporter vector and Cre vector by lipofection. In transfectants that were treated with reagents, luciferase activity in the culture media was measured using Biolux® *Gaussia* Luciferase Assay Kit reagents (New England Biolabs, Ipswich, MA).

Statistical analysis

The results are expressed as the mean ± standard error. Statistical significance was assessed with analysis of variance and, when appropriate, Student's *t* test. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Establishment of a system that can detect ER stress levels sensitively and timely

To establish a cell line in which Gluc was secreted by ER stress, we generated a DNA fragment that included the sequence of the GRP78 promoter and floxed YFP-Gluc. To introduce genes into organisms such as mice and detect ER stress levels in each organ in future experiments, we inserted the loxP sequence into the DNA fragment.

First, in order to confirm the efficiency of Gluc secretion after lipofection, the above sequence was inserted into a pcDNA vector as an expression vector (Fig. 1A). This vector and pcDNA-Cre were co-transfected into MIN6 cells by lipofection (1 day after passage). At 24 h after transfection, the cells were divided into groups incubated in culture media with different glucose concentrations of 100, 300, 450, 600, and 800 mg/dL (2 days after passage; Fig. 1B). Gluc activity in the supernatant of the culture medium was measured after 24 h (3 days after passage), the medium was replaced, and after 24 h (4 days after passage), Gluc activity was measured again, and the number of cells was measured at the same time (Fig. 1C, D). For the effect of glucose concentration, Gluc activity was significantly increased after 24 h when the glucose concentration was 300 mg/dL compared with 100 mg/dL glucose; however, there was no significant difference over 300 mg/dL glucose (Fig. 1C, D). Gluc activity tended to decrease in all glucose concentration groups after 48 h compared with after 24 h (Fig. 1D). While this system can evaluate the activity of the GRP78 promoter of transfected cells without being influenced by changes in the number of cells after lipofection, long-term evaluation over 24 h has decreased sensitivity. Furthermore, when glucose concentration in the culture medium was low, it was suggested that there was a mechanism inhibiting the secretion of Gluc itself (Fig. 1D).

Next, we inserted a DNA fragment containing GRP78 promoter-floxed YFP-Gluc into the pBlueScript II vector (pBS-Gluc; Fig. 1E). We aimed to establish a system that could evaluate the activity of the GRP78 promoter in cell culture supernatant by co-transfecting the cells with pBS-Gluc and pcDNA-Cre (Fig. 1E). When pBS-Gluc and pcDNA-Cre were introduced independently into MIN6 cells, Gluc activity in the supernatant did not increase; however, when the pBS-Gluc/pcDNA-cre ratio was approximately 5, 10, and 20, a remarkable increase in Gluc activity was observed to the same degree, respectively (Fig. 1F). Next, ATF6α, which is a transcriptional activator of GRP78, was introduced into HEK293 cells together with pBS-Gluc and pcDNA-Cre, Gluc activity increased by 4- to 8-fold depending on the expression of ATF6α (Fig. 1G). The increased activity of Gluc induced by ATF6α continued until 24 h. In addition, we performed the same experiment using MIN6 cells. By co-expressing ATF6α, Gluc activity increased by 2- to 5-fold in 30 min depending on the expression of ATF6α. The increased activity of Gluc induced by ATF6α also continued until 24 h in MIN6 cells (Fig. 1H). Furthermore, when HEK293 cells were treated with tunicamycin, an inducer of ER stress, an extremely low concentration (0.01 μg/mL) of tunicamycin increased Gluc activity approximately twice as fast than in the absence of tunicamycin at 30 min (Fig. 1I). From the above results, we concluded that this system can detect a small amount of ER stress levels sensitively and timely.

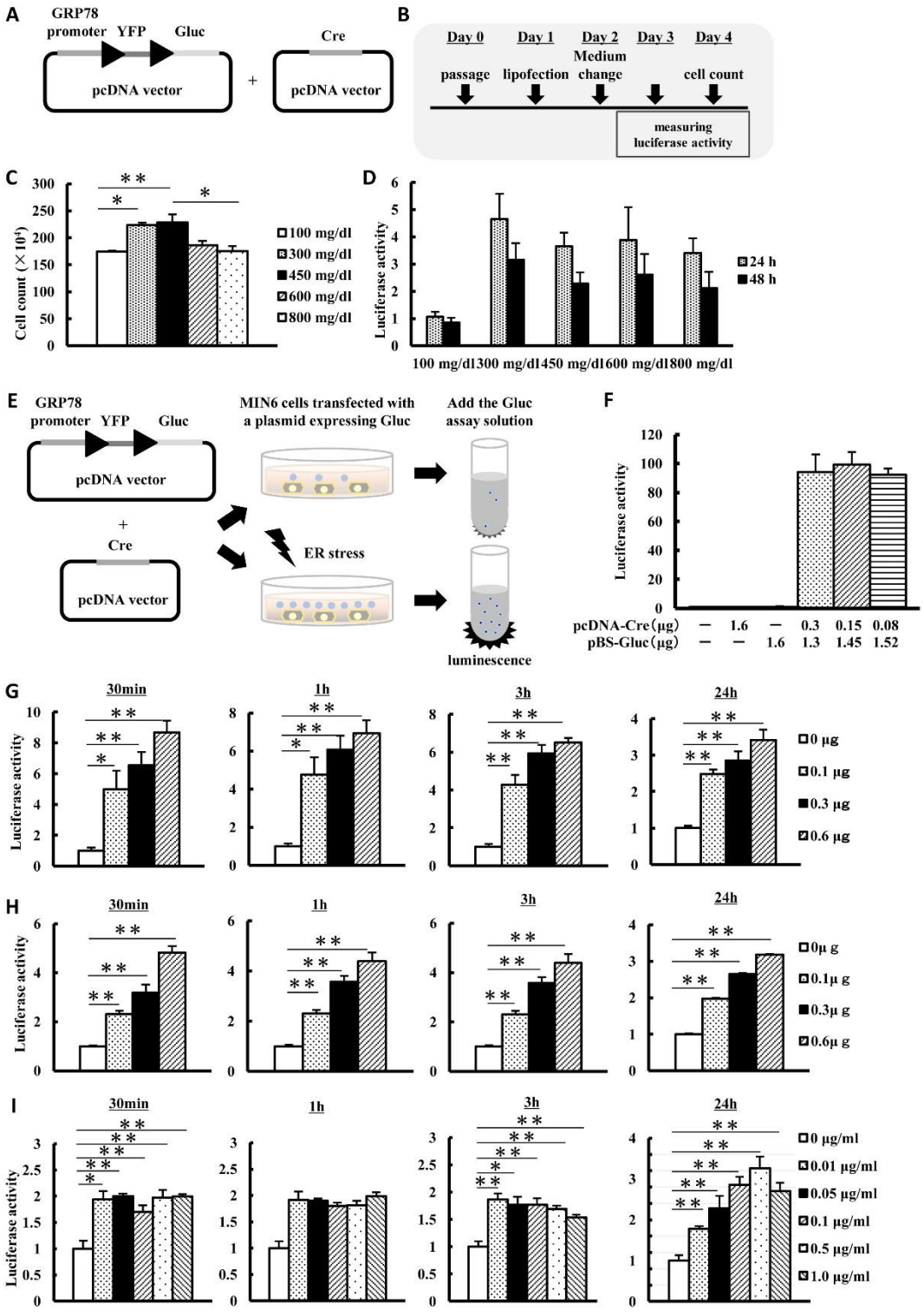

Figure 1. Development of a system that can detect ER stress levels sensitively and timely. (A) Cells were transfected with the pcDNA-GRP78 promoter-floxed YFP-Gluc vector (pcDNA-Gluc) and pcDNA-Cre vector by lipofection. (B) MIN6 cells were passaged, and 24 h later, the pcDNA-Gluc vector and pcDNA-Cre vector were co-transfected by lipofection. At 24 h after transfection, the glucose concentration in the medium of MIN6 cells was changed to 100, 300, 450, 600, 800 mg/dL. (C) The number of cells was counted after 48 h treatment. (D) Luciferase activity of the cell culture supernatant was measured after 24 h and 48 h treatment. (E) The cells were co-transfected with the pBS-GRP78 promoter-floxed YFP-Gluc vector (pBS-Gluc vector) and pcDNA-Cre vector by lipofection. The cells were treated with various compounds at 24 h after transfection. Luciferase activity of the cell culture supernatant was measured, and the presence or absence of ER stress was evaluated. (F) The cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection, and the medium was exchanged after 24 h. Luciferase activity of the medium supernatant was measured at 24 h after medium exchange. (G) HEK293 cells were co-transfected with

pBS-Gluc, pcDNA-Cre, and 0, 0.1, 0.3, or 0.6 μg pcDNA-CA-ATF6α by lipofection. After 24 h, the medium was exchanged. Luciferase activity of the cell culture supernatant was measured at 30 min, 1 h, 3 h, and 24 h. (H) MIN6 cells were co-transfected with pBS-Gluc, pcDNA-Cre, and 0, 0.1, 0.3, or 0.6 μg pcDNA-CA-ATF6α by lipofection. After 24 h, the medium was exchanged. Luciferase activity of the cell culture supernatant was measured at 30 min, 1 h, 3 h, and 24 h. (I) HEK293 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. The cells were treated with tunicamycin at 24 h post-transfection. Luciferase activity of the cell culture supernatant was measured at 30 min, 1 h, 3 h, and 24 h after treatment. *P < 0.05, **P < 0.01

Sensitive detection of ER stress levels in MIN6 cells using this system

In order to detect ER stress levels in pancreatic β cells, MIN6 cells were treated with low concentrations of tunicamycin. First, ER stress-related proteins were evaluated. For 0.01 and 0.5 μg/mL tunicamycin, the expression of C/EBPβ and CHOP did not change at 30 min after treatment. C/EBPβ and CHOP expression was increased only in the 0.5 μg/mL tunicamycin group after 24 h. However, there was no change in GRP78 expression at any point (Fig. 2A). Next, changes in the mRNA expression of ER stress-related molecules were examined by real-time RT-PCR. Similar to the evaluation at the protein level, in the 0.01 and 0.5 μg/mL tunicamycin groups, the expression of ER stress-related CHOP, C/EBPβ, and GRP78 mRNA was not significantly changed at 30 min after treatment (Fig. 2B). The expression of C/EBPβ, CHOP, and GRP78 mRNA was increased only in the 0.5 μg/mL tunicamycin group after 24 h. However, similar to the protein expression experiments, in the 0.01 μg/mL tunicamycin group, no change of mRNA could be detected even at 24 h after treatment (Fig. 2B).

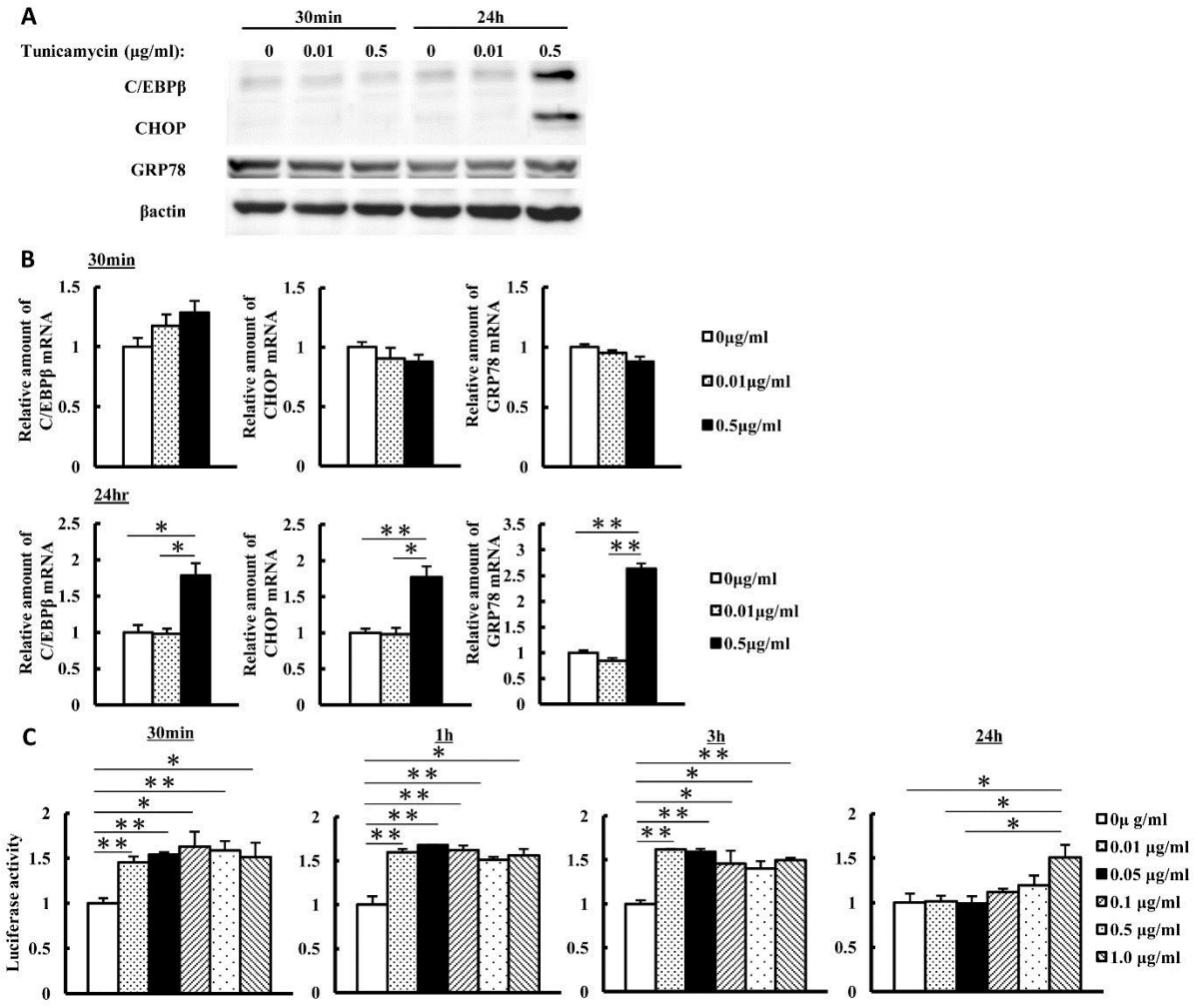

Figure 2. Effect of tunicamycin on MIN6 cells. (A) MIN6 cells were treated with tunicamycin for 30 min and 24 h. The expression of C/EBPβ, GRP78, and CHOP was analyzed by western blotting. (B) MIN6 cells were treated with tunicamycin for 30 min and 24 h. RNA was extracted from MIN6 cells, and the expression of CHOP, C/EBPβ, and GRP78 mRNA was evaluated by real-time RT-PCR. (C) MIN6 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. The cells were treated with tunicamycin at 24 h post-transfection. Luciferase activity of the cell culture supernatant was measured at 30 min, 1 h, 3 h, and 24 h after treatment. *P < 0.05, **P < 0.01

On the other hand, Gluc activity increased by 1.5-fold in the 0.01 μg/mL tunicamycin group as compared with control at 30 min (Fig. 2C), as in the case of HEK293 cells (Fig. 1I). Compared with HEK293 cells, the low transfection efficiency of MIN6 cells was considered to be the cause of their lower rate of increased Gluc activity. This effect persisted until 3 h after treatment (Fig. 2C), but it was attenuated after 24 h (Fig. 2C). This may have been caused by a decrease in the number of transfected cells due to ER stress over a long period of time after treatment. Therefore, these findings clarified that this system can detect a small amount of ER stress levels in MIN6 cells sensitively, which cannot be detected by previous methods.

Differences in short-term glucose concentrations do not induce ER stress in MIN6 cells

Next, we investigated the effect of differences in glucose concentration on the ER. ER stress-related proteins were evaluated in MIN6 cells cultured with glucose concentrations of 100, 300, 450, 600, and 800 mg/dL. There were no significant changes in the expression of C/EBPβ or CHOP at glucose concentrations of 300, 450, 600 and 800 mg/dL (Fig. 3A). The expression of CHOP, C/EBPβ, and GRP78 mRNA was increased only at 100 mg/dL glucose (Fig. 3B). However, at other concentrations of glucose, there was no significant change in the mRNA levels of ER-related molecules (Fig. 3B). When Gluc activity was evaluated under the same conditions, there was no significant difference in Gluc activity according to glucose concentration at 30 min and 1 h after glucose load. In contrast, at 3 h and 24 h, increased Gluc activity was observed at a glucose concentration of 300 mg/dL or more compared with 100 mg/dL glucose (Fig. 3C). We consider that the decrease in Gluc activity at 100 mg/dL glucose in the culture medium represented a decrease in Gluc secretion rather than a change due to ER stress (Fig. 1B, C). These results suggest that glucose concentrations of 300 mg/dL or more in culture medium do not cause UPR in MIN6 cells during a short term (i.e., within 24 h). In contrast, our system could not evaluate ER stress levels with a glucose concentration of 100 mg/dL and long-term loading over 24 h.

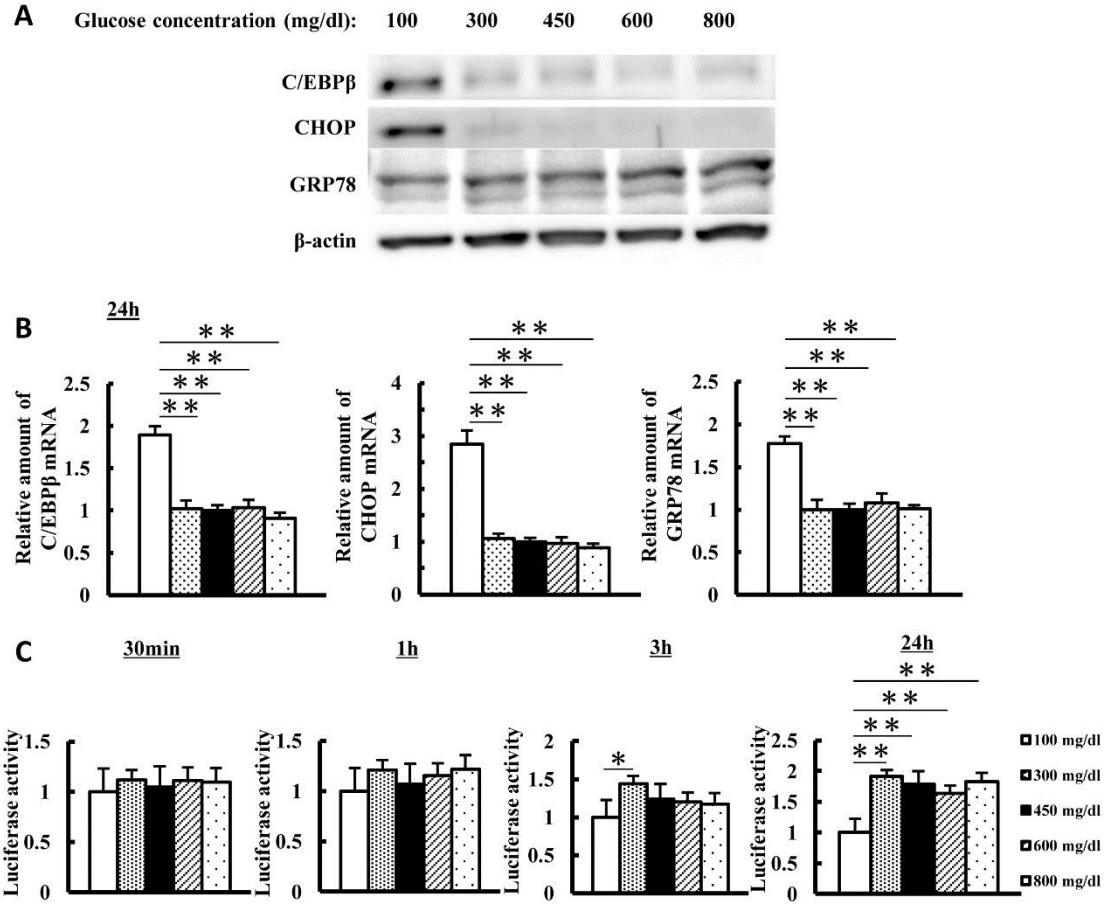

Figure 3. Effect of glucose concentration on MIN6 cells. (A) MIN6 cells were cultured with glucose concentrations of 100, 300, 450, 600, and 800 mg/dL. The expression of C/EBPβ, GRP78, and CHOP was evaluated at 24 h after treatment by western blotting. (B) MIN6 cells were cultured with glucose concentrations of 100, 300, 450, 600, and 800 mg/dL. RNA was extracted from MIN6 cells after 24 h, and the expression of CHOP, C/EBPβ, and GRP78 mRNA was evaluated by real-time RT-PCR. (C) MIN6 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. At 24 h after transfection, the cells were cultured with glucose concentrations of 100, 300, 450, 600, and 800 mg/dL. Luciferase activity of the cell culture supernatant was measured at 30 min, 1 h, 3 h, and 24 h after treatment. $*P < 0.05$, $**P < 0.01$

Palmitic acid induces ER stress even at a physiological concentration in MIN6 cells

In pancreatic β cells, lipotoxicity along with glucotoxicity induce ER stress. Therefore, we investigated the effect of a saturated fatty acid, palmitic acid, on the ER in pancreatic β cells. When MIN6 cells were treated with a physiological concentration of 100 μM palmitic acid and a mildly elevated concentration of 500 μM palmitic acid, the expression of C/EBPβ, CHOP, and GRP78 did not change at 30 min after treatment (Fig. 4A). After 24 h, CHOP and C/EBPβ expression increased in a palmitic acid concentration-dependent manner, but there was no change of GRP78 expression (Fig. 4A). Next, mRNA expression levels were evaluated by real-time RT-PCR. At 30 min, no change was observed in the mRNA levels of CHOP, C/EBPβ, and GRP78 at both 100 and 500 μM palmitic acid, but after 24 h, CHOP and C/EBPβ mRNA was increased at both 100 and 500 μM in a concentration-dependent manner. However, no change was observed in the mRNA levels of GRP78 at both 100 and 500 μM palmitic acid, even after 24 h (Fig. 4B). On the other hand, 100 μM palmitic acid increased Gluc activity at 30 min by approximately 1.7-fold; 500 μM palmitic acid increased Gluc activity by approximately 2.5-fold (Fig. 4C). These results suggest that palmitic acid induces ER stress in pancreatic β cells even at a physiological concentration.

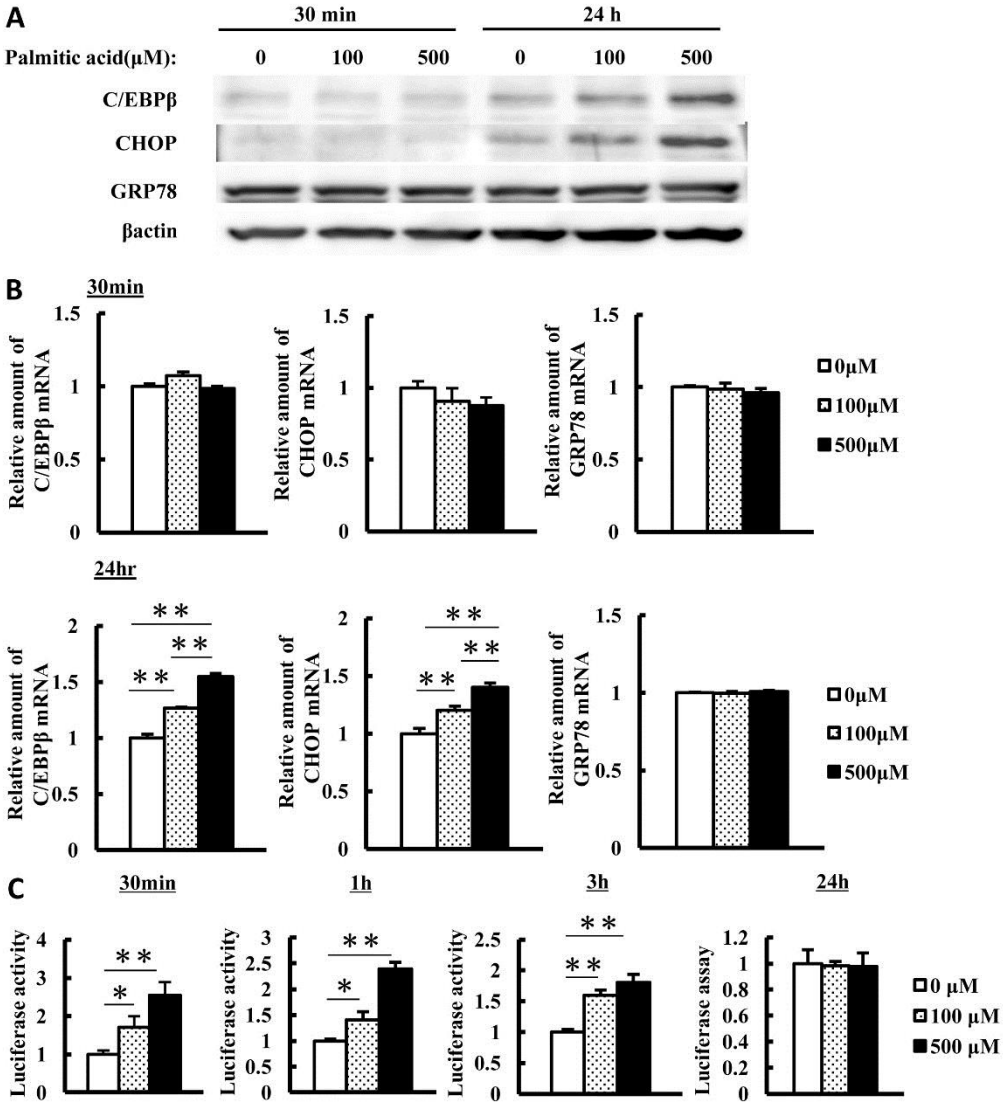

Figure 4. Effect of palmitic acid on MIN6 cells. (A) MIN6 cells were treated with palmitic acid for 30 min and 24 h, and the expression of C/EBPβ, GRP78, and CHOP was analyzed by western blotting. (B) MIN6 cells were treated with palmitic acid for 30 min and 24 h. RNA was extracted from the cells, and the expression of CHOP, C/EBPβ, and GRP78 mRNA was evaluated by real-time RT-PCR. (C) MIN6 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. At 24 h after transfection, the cells were treated with palmitic acid. Luciferase activity of the cell culture supernatants was measured at 30 min, 1 h, 3 h, and 24 h after treatment. **P* < 0.05, ***P* < 0.01

Oleic acid induces ER stress in MIN6 cells in a short period of time only at a high concentration

Then, we investigated the effect of a monounsaturated fatty acid, oleic acid, which is known to reduce the lipotoxicity induced by saturated fatty acids, in MIN6 cells. When MIN6 cells were treated with a physiological concentration of 100 μM oleic acid and a mildly elevated concentration of 500 μM, the expression levels of CHOP and C/EBPβ did not change at 100 μM after 24 h, but their expression was significantly increased at 500 μM (Fig. 5A). Next, mRNA expression levels were evaluated by real-time RT-PCR. At 30 min, no change was observed in the transcription levels of CHOP, C/EBPβ, and GRP78 at both 100 and 500 μM. Even after 24 h, the expression levels of ER-related molecules did not change at 100 μM, but those of CHOP, C/EBPβ, and GRP78 were markedly increased at 500 μM (Fig. 5B). On the other hand, Gluc activity at 30 min did not change at 100 μM oleic acid; however, at 500 μM, Gluc activity increased by approximately 1.7-fold at 30 min (Fig. 5C). These results indicate that a physiological concentration of oleic acid did not induce UPR even with this system, whereas at a high concentration, mild ER stress levels was detected in a short period of time.

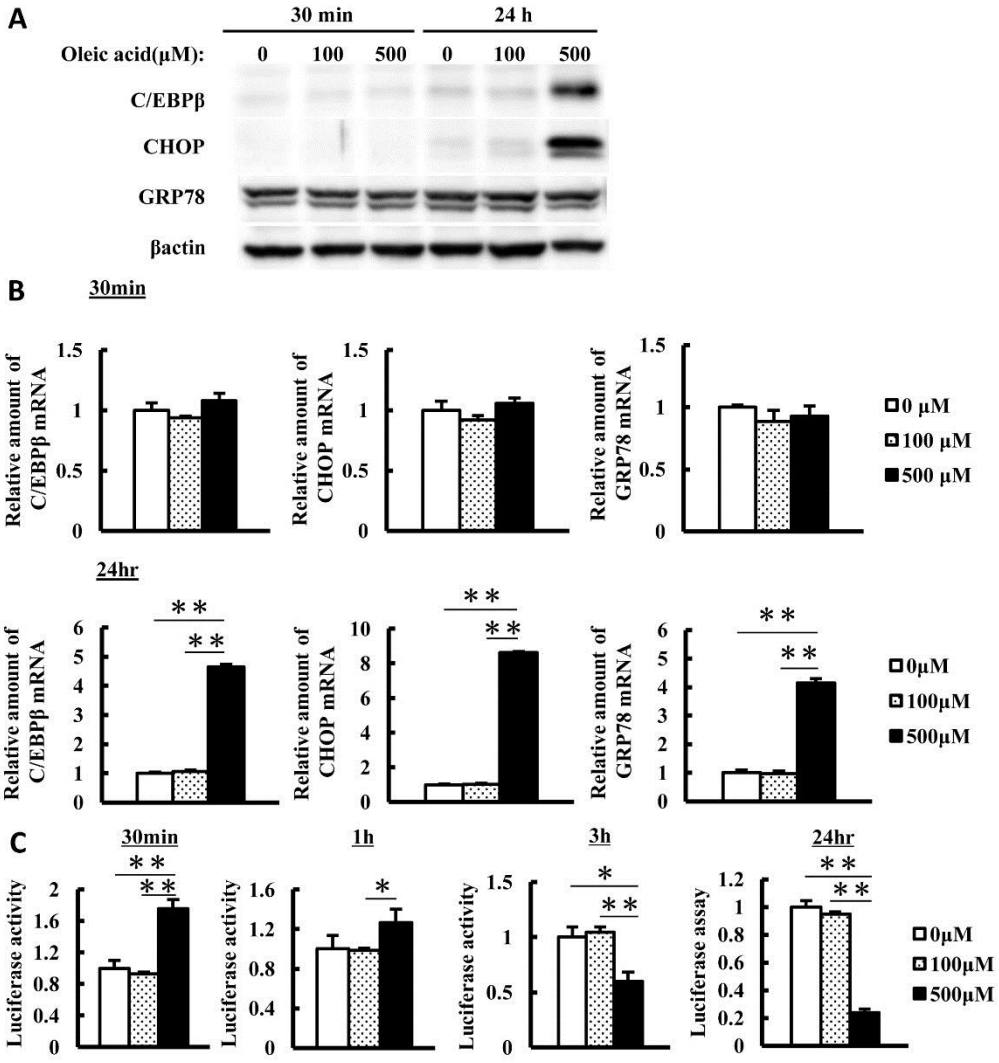

Figure 5. Effect of oleic acid on MIN6 cells. (A) MIN6 cells were treated with oleic acid for 30 min and 24 h, and the expression of C/EBPβ, GRP78, and CHOP was analyzed at 30 min and 24 h by western blotting. (B) MIN6 cells were treated with oleic acid for 30 min and 24 h. RNA was extracted from MIN6 cells, and the expression of CHOP, C/EBPβ, and GRP78 mRNA was evaluated by real-time RT-PCR. (C) MIN6 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. At 24 h after transfection, the cells were treated with oleic acid. Luciferase activity of the cell culture supernatants was measured at 30 min, 1 h, 3 h, and 24h after treatment. $*P < 0.05$, $**P < 0.01$

A high concentration of DHA induces ER stress in MIN6 cells

We examined the effect of DHA, a polyunsaturated fatty acid, on MIN6 cells. When MIN6 cells were treated with a low concentration of 500 nM DHA and a high concentration of 2 μM, the expression levels of CHOP and

C/EBPβ did not change at 500 nM after 24 h, but at 2 μM, their expression levels were significantly increased (Fig. 6A). Next, mRNA expression was evaluated by real-time RT-PCR. At 30 min, there was no change in the levels of CHOP, C/EBPβ, and GRP78 mRNA at both 500 nM and 2 μM DHA. Even after 24 h, there was no change at 500 nM DHA, but CHOP, C/EBPβ, and GRP78 mRNA expression levels were significantly increased at 2 μM (Fig. 6B). Conversely, Gluc activity at 30 min did not change at 500 nM and 2 μM DHA (Fig. 6C). These results indicate that a low concentration of DHA did not induce UPR, while a high concentration of DHA induced ER stress.

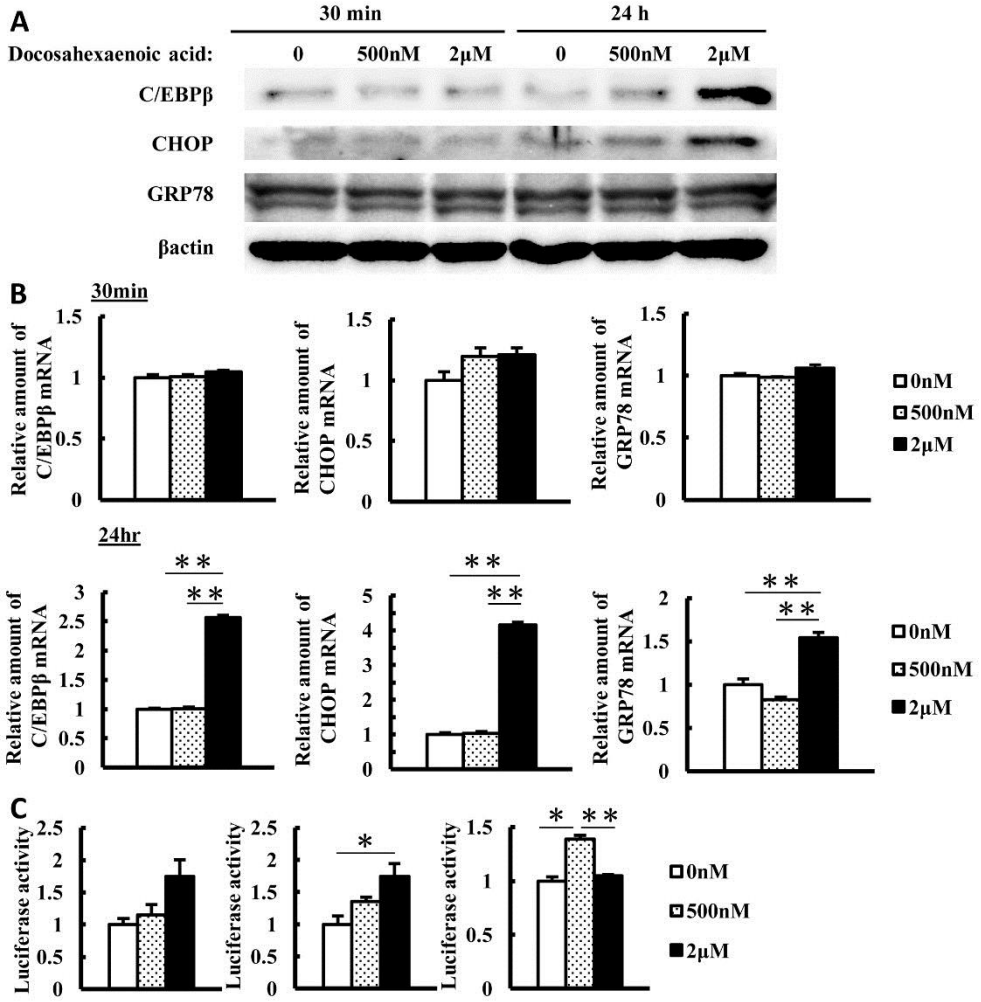

Figure 6. Effect of DHA on MIN6 cells. (A) MIN6 cells were treated with DHA for 30 min and 24 h, and the expression of C/EBPβ, GRP78, and CHOP was analyzed by western blotting. (B) MIN6 cells were treated with DHA for 30 min and 24 h. RNA was extracted from MIN6 cells, and the expression of CHOP, C/EBPβ, and GRP78 mRNA was evaluated by real-time RT-PCR. (C) MIN6 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. At 24 h after transfection, the cells were treated with DHA. Luciferase activity of the cell culture supernatants was measured at 30 min, 1 h, and 3 h after treatment. $*P < 0.05$, $**P < 0.01$

Palmitic acid-induced ER stress is reduced by DHA in MIN6 cells

We investigated the interaction between palmitic acid and DHA. The expression levels of CHOP and C/EBPβ were increased when MIN6 cells were treated with 500 μM palmitic acid for 24 h, but this upregulation decreased with the combined treatment of 500 μM palmitic acid and 500 nM DHA (Fig. 7A). Next, mRNA expression was evaluated by real-time RT-PCR. At 30 min, CHOP, C/EBPβ, and GRP78 mRNA expression levels were not changed. After 24 h, the upregulation of CHOP and C/EBPβ mRNA was decreased by the combination of palmitic acid and DHA (Fig. 7B). On the other hand, the Gluc activity observed with 500 μM palmitic acid treatment was attenuated by the combination of palmitic acid and DHA at 30 min (Fig. 7C).

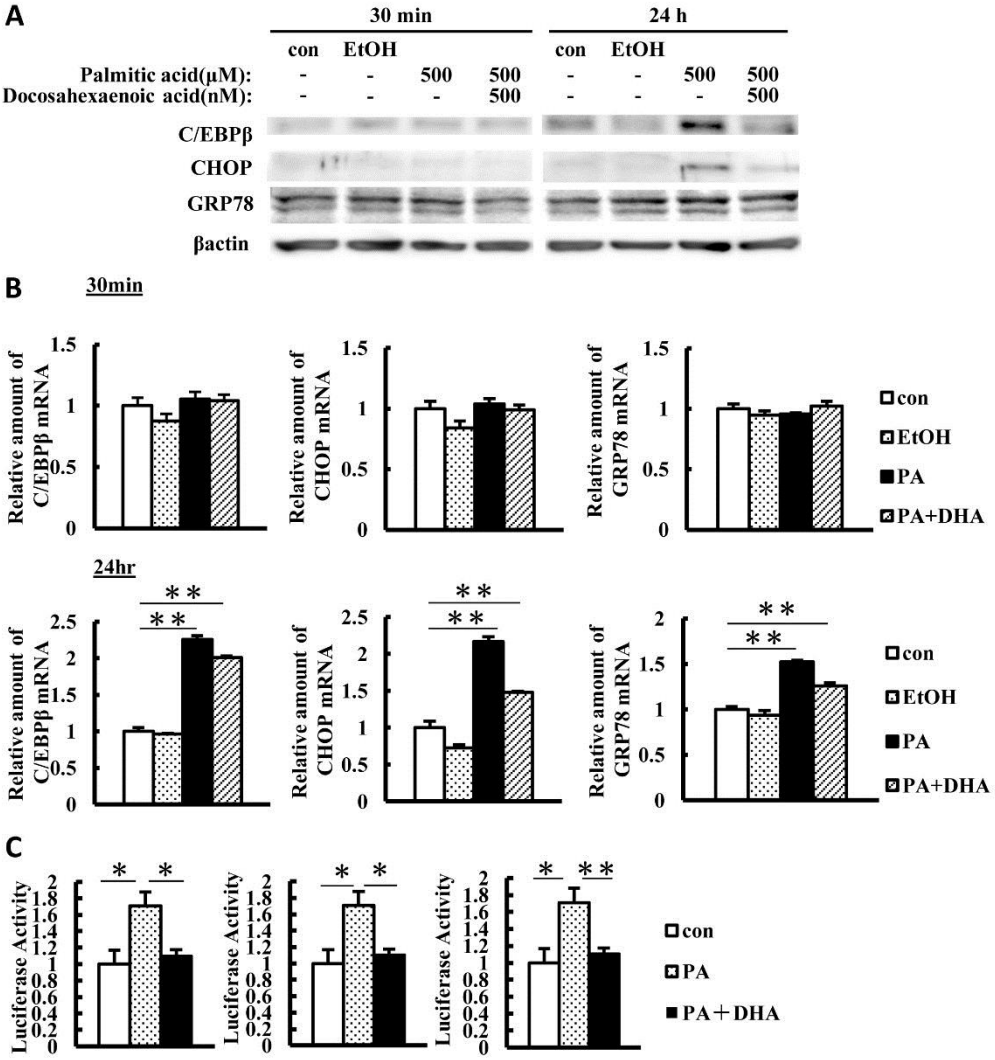

Figure 7. Effect of palmitic acid and DHA on MIN6 cells. (A) MIN6 cells were treated with ethanol, 500 μM palmitic acid, and 500 μM palmitic acid and 500 nM DHA for 30 min and 24 h, and the expression of C/EBPβ, GRP78, and CHOP was analyzed by western blotting. (B) MIN6 cells were treated with ethanol, 500 μM palmitic acid, and 500 μM palmitic acid and 500 nM DHA for 30 min and 24 h. RNA was extracted from MIN6 cells, and the expression of CHOP, C/EBPβ, and GRP78 mRNA was evaluated by real-time RT-PCR. (C) MIN6 cells were transfected with pBS-Gluc and pcDNA-Cre by lipofection. At 24 h after transfection, the cells were treated with ethanol, 500 μM palmitic acid, and 500 μM palmitic acid and 500 nM DHA. Luciferase activity of the cell culture supernatants was measured at 30 min, 1 h, and 3 h after treatment. $*P < 0.05$, $**P < 0.01$

DISCUSSION

In this study, we developed a system that can evaluate ER stress levels sensitively by detecting secretory luciferase depending on the activity of the GRP78 promoter. This system revealed that the activity of the GRP78 promoter was increased in MIN6 cells by extremely mild ER stress levels, which could not be detected by immunoblotting or real-time RT-PCR. In addition, a high concentration of glucose in the culture medium of MIN6 cells did not induce UPR in a short period of time. Furthermore, palmitic acid, a saturated fatty acid, induced ER stress in a short period of time even at a physiological concentration. While oleic acid and DHA, unsaturated fatty acids, did not cause ER stress at physiological concentrations, they induced ER stress at high concentrations. Furthermore, our results suggested that DHA, a polyunsaturated fatty acid, when used in combination with palmitic acid at physiological concentrations, reduced ER stress levels induced by palmitic acid.

Recently, it has become clear that UPR plays an important role in the pathogenesis of various diseases such as neurodegenerative diseases, immune diseases, metabolic diseases, cancer, and so on. Many studies indicating its importance at the cellular level have been published, and its relevance is unquestioned. However, when ER stress levels is evaluated in an individual, it is necessary to extract RNA and protein from the cells of each tissue. Iwawaki et al. established transgenic mice in which ER stress can be detected by analyzing the expression of the Venus

gene and green fluorescence based on XBP-1 mRNA splicing (23). Studies using this mouse revealed that ER stress plays an important role in various organs and diseases, but it cannot be evaluated in living organisms or quantify the variation and amount of ER stress levels.

While oxidative stress can be evaluated at the individual level by analyzing samples of blood or urine, there are few methods for evaluating ER stress levels in an individual. In addition, since UPR itself is an adaptive cell response and functions to maintain homeostasis, it is important to distinguish whether the stress is physiological or not. Therefore, in order to target ER stress for the treatment and prevention of diseases, it is necessary to clarify the distinction between physiological and pathological stress from the viewpoint of cellular or environmental factors.

In this study, we revealed that UPR was not induced in MIN6 cells in short-term treatment with a range of glucose concentrations and a physiological concentration of oleic acid, which is an unsaturated fatty acid. Chronic hyperglycemia and oleic acid treatment are known to induce ER stress. In fact, in this study, CHOP and C/EBPβ were significantly increased by treatment with 500 μM oleic acid for 24 h. In other words, we believe that ER stress is increased secondarily through oxidative stress and so on. It is known that not only oxidative stress but also various forms of intracellular stress, such as autophagy, hypoxia, and mitochondrial dysfunction, are closely linked to ER stress (24, 25). We think that our system is useful for evaluating these different forms of stress and ER stress separately.

DHA, which is a polyunsaturated fatty acid, reportedly has an anti-inflammatory effect and is associated with calcium homeostasis within the ER (26–28). However, the mechanism underlying the effect of DHA on the ER has not yet been clarified. In our system, a low concentration of DHA (500 nM) did not induce ER stress, but under a high concentration of DHA (2 μM), ER stress was induced. Conversely, DHA was found to reduce the ER stress induced by palmitic acid when used in combination with palmitic acid. In this system, the activity of the GRP78 promoter was reduced after 30 min combined treatment with palmitic acid and DHA compared with palmitic acid alone. Considering that DHA alone did not affect the activity of the GRP78 promoter in a short period of time, it is presumed that DHA acted directly on palmitic acid and reduced its influence on the ER, but it is necessary to perform further research to clarify the underlying mechanism.

It is well known that DHA has various effects. DHA and its derivatives protectins and resolvins provide neuroprotection and resolve inflammation (26), and omega-3 fatty acids (eicosapentaenoic acid and DHA) decrease oxidative stress and β cell apoptosis induced by palmitate (29). In addition, DHA inhibits palmitic acid- or thapsigargin-induced IL-1β secretion in THP-1 cells (30). DHA can alleviate the fructose-induced ER stress response, as evidenced by the down-regulation of the ER stress marker GRP78 and total IRE or p-IRE (31). The protective effect of DHA against the ER, which was clarified in this study, may be one of the mechanisms underlying the anti-inflammation and anti-stress effects of DHA.

Since this system utilizes a transfected gene introduced by lipofection, if the number of transfected cells is decreased, this system does not work well. In addition, Gluc activity was markedly decreased even when the glucose concentration of the culture medium was 100 mg/dL; thus, a decrease in the secretion of Gluc was suggested, considering the discrepancy from the results of real-time RT-PCR and the number of cells (Fig. 1C, D). In other words, this system is not useful for assessing long-term stress and low glucose concentration loading. Furthermore, we need to keep in mind the possibility that in addition to low glucose, with administration of extracellular stimuli that can affect systems that regulate intracellular transport such that secretion of luciferase protein is impaired, the susceptibility to ER stress can be reduced.

However, from the present study, our established system can detect physiological ER stress levels in a short period of time and does not induce apoptosis even after a long period of time, such as a low concentration of tunicamycin and a physiological concentration of palmitic acid. We believe that this system would be a very useful tool for detecting ER stress levels at the early phase.

In this research, we have established a system that can detect ER stress levels of living cells sensitively in a short period of time. It was suggested that physiological ER stress levels could be detected using this system. In addition, it is considered that it is possible to evaluate whether ER stress is primary or secondary, since it can detect a change in ER stress over a short period of time. Furthermore, we have included Cre-loxP within our system, and in the future, we will make transgenic mice with this system that can be mated with various tissue-specific Cre transgenic mice to enable the evaluation of ER stress levels in individual organs by blood sampling. We believe that the role of physiological or pathological ER stress levels in each organ can be elucidated by this method.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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