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Eicosapentaenoic acid attenuates statin-induced ER stress and toxicity in myoblast

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ABSTRACT

We previously reported that eicosapentaenoic acid (EPA) improved statin-induced rhabdomyolysis in rats (Naba et al. [6]). In this study, we report for the first time direct improvement by EPA of statin-induced toxicity in cultured myoblasts and the mechanistic involvement of endoplasmic reticulum (ER) stress. Differentiated rhabdomyosarcoma cells (RD cells) were treated with statins and EPA for 1–4 days. Statins induced various toxic changes in RD cells, and EPA attenuated all of these changes. Interestingly, statins increased mRNA expression of ER stress markers (XBP-1 and CHOP) and EPA attenuated both. Further, in a statin-induced rat model of rhabdomyolysis, these markers in skeletal muscle were significantly correlated with plasma CPK activity. In RD cells, statins also increased p-c-Jun protein content and caspase-3/7 activity, while 4-PBA, an ER stress attenuator, PPAR-δ agonist, and EPA attenuated them. These findings suggest that EPA attenuates statin-induced ER stress, JNK activation and toxicity in cultured myoblast cells, and that PPAR-δ may mechanically involved in the effects of EPA.

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1. Introduction

Statins are reversible and competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and widely used for the clinical treatment of hypercholesterolemia. Adverse effects of them, although uncommon, include myalgia and elevated creatine phosphokinase (CPK) and myoglobin (Mgb) levels, and in rare cases life-threatening rhabdomyolysis may develop. Many theories exist concerning the mechanism of toxicity of statins, though the mechanism itself remains unknown [1,2]. Eicosapentaenoic acid (EPA), an n-3 polyunsaturated fatty acid (PUFA), has also been used for the treatment of hyperlipidemia, and there have been several reports that coadministration of n-3 PUFA and statins synergistically yielded beneficial effects in treating hyperlipidemia and arteriosclerosis in an animal model [3] and in human studies [4,5]. Nevertheless, few studies have examined the effects of n-3 PUFA on statin-induced myotoxicity. We previously reported that EPA can improve statin-induced rhabdomyolysis in rats [6]. However, whether EPA has direct effects on statin-induced toxicity in myoblasts is unclear. In this study, we show the first time that EPA directly inhibits statin-induced toxicity in cultured myoblast cells, and that endoplasmic reticulum (ER) stress, JNK, and PPAR-δ may play some roles in the mechanism responsible for this phenomenon.

2. Materials and methods

2.1. Materials

RD cells (human rhabdomyosarcoma cell line, passage Nos. 4–7) were purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM, low glucose: D-6046) was from Sigma–Aldrich (St Louis, MO). Non-essential amino acids (neAA, 11140), horse serum (HS, 16050-122), and fetal bovine serum (FBS, 10437-028) were purchased from GIBCO-BRL (Carlsbad, CA). Bovine serum albumin (BSA, fraction V, fatty acid-free, 531-78711), CPK-II-test-Wako, and Hoechst 33258 for DNA determination were purchased from Wako (Osaka, Japan). M-PER mammalian protein extraction reagent and the BCA protein assay kit were purchased from Pierce (Rockford, IL); the myoglobin EIA kit from Life Diagnostics (West Chester, PA); the SAPK/JNK assay kit from Cell Signaling (Danvers, MA); and the CASE-kit for S73-phosphorylated c-Jun (p-c-Jun) from SuperArray Bioscience (Frederick, MD). Caspase-Glo-3/7, -8 and -9, Calpain-Glo, and the DeadEnd colorimetric TUNEL assay system was purchased from Promega (Madison, WI). Simvastatin was extracted from Lipovas purchased from Banyu (Tokyo, Japan); cerivastatin sodium salt was purchased from Sequoia Research (Pangbourne, United Kingdom); pitavastatin calcium salt and pravastatin were from Apin (Abingdon, United Kingdom); and rosuvastatin calcium salt and atorvastatin calcium salt were from Wako. Eicosapentaenoic acid sodium salt (EPA) was purchased from NU-CHEK-PREP (Elysian, MN); eicosapentaenoic acid ethyl ester (EPA-E) was obtained from Nippon Suisan (Tokyo, Japan); ryanodine, squalene, L(+)-ascorbic acid (VtC) and AACOCF3 were from Wako;

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mevalonate (lactone form), geranylgeranylpyrophosphate ammonium salt (GGPP, used after evaporation of organic solvent), docosahexaenoic acid sodium salt (DHA), arachidonic acid sodium salt (AA), 2-Br-palmitic acid, palmitic acid sodium salt, oleic acid, Sandoz 58-035, *N*-methyl-L-arginine acetate salt (L-NMMA), α-tocopherol (VtE), 4-phenylbutyric acid (4-PBA), and wortmanin were purchased from Sigma–Aldrich; U-73122, BAPTA/AM, Z-VAD-FMK, bongkrekic acid (BA), ALLN, SP600125, SB203580, L165041, tauroursodeoxycholic acid sodium salt (TUDCA) and PD98059 were purchased from Calbiochem; tunicamycin was from Santa Cruz Biotechnology (Santa Cruz, CA), coenzyme Q10 (CoQ10) was from MP Biomedicals (Solon, OH); dolichol phosphate (dolichol-P, used after evaporation of organic solvent) was purchased from Doosan SRL (Toronto, ON, Canada), and *N*-6-isopentenyl-adenine (IPA) was from Alexis Biomedicals (San Diego, CA). Eisai hyperbilirubinemic (EHBR) rats were from Japan SLC (Hamamatsu, Japan).

2.2. Methods for RD cells

RD cells were suspended in growth medium (DMEM, low glucose, supplemented with 50 U/mL penicillin, 50 ng/mL streptomycin, 1% neAA, and 10% FBS), and inoculated in 24-well or 96-well plates. After the cells had grown to complete confluence in each well, the medium was changed to differentiation medium (growth medium containing 2% HS substituted for 10% FBS), and the cells were cultured another 9–45 days. Then, drug-supplemented medium (differentiation medium supplemented with 10 mM Hepes, 0.5% BSA, and test-drugs or solvent; ~0.3% ethanol, DMSO, water, or saline) was added for 7 h to 4 days. Medium volume per well was 100 μL for 96-well plates and 500 μL for 24-well plates. Medium was changed every 2 or 3 days in the case of growth or differentiation medium, and every 1 or 2 days in the case of drug supplementation medium. The medium was then sampled for measurement of CPK activity and Mgb. The cells were washed twice with PBS and harvested with MPER reagent (100 μL for 96-well plates and 300 μL for 24-well plates). The cells were homogenized on ice by sonication, and then protein, CPK activity, DNA, and Mgb were measured with the BCA protein assay kit, CPK-II test Wako, Hoechst 33258, and myoglobin EIA kit, respectively.

For measurement of other parameters, the cells were centrifuged (1000 rpm, 1 min), washed once with PBS, and recentrifuged. The PBS was discarded, and each respective procedure was performed. For TUNEL staining, measurement of JNK activity and p-c-Jun protein content, the DeadEnd colorimetric TUNEL assay system, the SAPK/JNK assay kit, and the CASE-kit were used, respectively. For measurement of caspase-3/7, -8 and -9 activities and calpain activity, the cells were homogenized with MPER reagent or caspase-lysis buffer [7,8], then commercial kits (Caspase-Glo-3/7, -8 and -9, Calpain-Glo) were used, and values were corrected for protein content. For determination of mRNA of some genes, total cellular RNA was isolated and purified by RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was generated using an SuperScript first-strand synthesis system (Invitrogen). Quantitative real-time PCR was performed using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). Levels of gene expression were measured using TaqMan gene expression assays (Applied Biosystems). The assay IDs of TaqMan gene expression assays used in this study are X box-binding protein 1 (XBP1, Hs00964359_m1) and C/EBP-homologous protein (CHOP, Hs99999172_m1). GAPDH mRNA was measured by using Pre-Developed Taqman Assay Reagents Human GAPDH (Applied Biosystems) as an internal control.

2.3. Effects of statin and EPA on ER stress marker in rat skeletal muscle

Treatments were performed in our previous reports [6]. Briefly, EHBR rats were administered pravastatin (200 mg/kg/day for 14 days), and pre- and co- administered EPA-E (100–1000 mg/kg/day for 14 and 14 days, respectively). At 24 h after the last dosing, rats were anesthetized for blood sampling, and skeletal muscles were taken from hind limbs after euthanasia. No-treatment rats were used as control rats. Frozen samples of skeletal muscle were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA from the homogenates was purified with RNeasy mini kit (Qiagen). Generation of cDNA and quantitative real-time PCR were performed as described above for RD cells. The assay IDs of TaqMan gene expression assays used in this study are XBP1 (Rn01443523_m1), CHOP (Rn00492098_g1), and immunoglobulin heavy chain-binding protein (Bip, Grp78, Rn00565250_m1). rRNA was measured by using TaqMan ribosomal RNA control reagents (Applied Biosystems) as an internal control. All experiments were carried out in accordance with the guideline for the care and use of laboratory animals of Mochida Pharmaceutical.

2.4. Miscellaneous procedures

All experiments were carried out at least in triplicate, and all values in figures are presented as the mean ± SE. Statistical analysis were performed by Dunnett's or Tukey–Kramer's multiple comparison tests. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ represent statistical differences between the control group and statin-alone-treated group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent those between the statin-alone-treated group and statin with various compound-treated groups.

3. Results and discussion

The toxicity of each statin in this model was evaluated by measurement of protein content in residual cell lysate after removal of dead cells by washing. The potency of the 7 types of statins evaluated in this study varied, but all had toxic effects on RD cells (Fig. 1A). In particular, cerivastatin, which was taken off the market because of severe rhabdomyolysis in patients [1], exhibited marked toxicity in our model. The severity of statin toxicity was similar to that reported previously [9]. Interestingly, when 50 μM EPA was added with each statin, the degree of toxicity, regardless of statin, was decreased (Fig. 1B). Further testing was performed using 30 μM simvastatin. Simvastatin has generally been used in statin toxicity studies with cultured myoblasts [8,10,11]. This concentration (30 μM) was similar to that in previous studies demonstrating toxicity of simvastatin in myoblasts [8,10], and also similar to that in other studies demonstrating simvastatin's pleiotropic effects on vascular endothelial or smooth muscle cells [12–14]. Fig. 1C shows parameters other than cellular protein on day 4. Like cellular protein, cellular DNA, CPK, and Mgb levels were also decreased by simvastatin treatment. Levels of CPK and Mgb in culture media were increased by simvastatin treatment. EPA attenuated the changes in these parameters. These results show for the first time that EPA directly inhibits statin-induced toxicity in cultured myoblast cells. Many previous studies showed that statins induced apoptosis in cultured myoblasts [7–10]. In our model, after 2 days of simvastatin treatment, TUNEL stain-positive cells, indicating intracellular DNA fragmentation and apoptosis, were increased, and EPA prevented these morphological changes (data not shown). This suggested that simvastatin induced apoptosis in RD cells. In addition, the activity of cellular caspase-3/7, the central effector enzymes of apoptosis, was increased by simvastatin, and this increase was also prevented by EPA (Fig. 1C).

Next, we tested the effect of many low-molecular-weight compounds with reported efficacy in preventing statin toxicity in cultured myoblasts [8,10,11,15]. All test compounds were confirmed not to have toxic or proliferative effects solely in differentiated

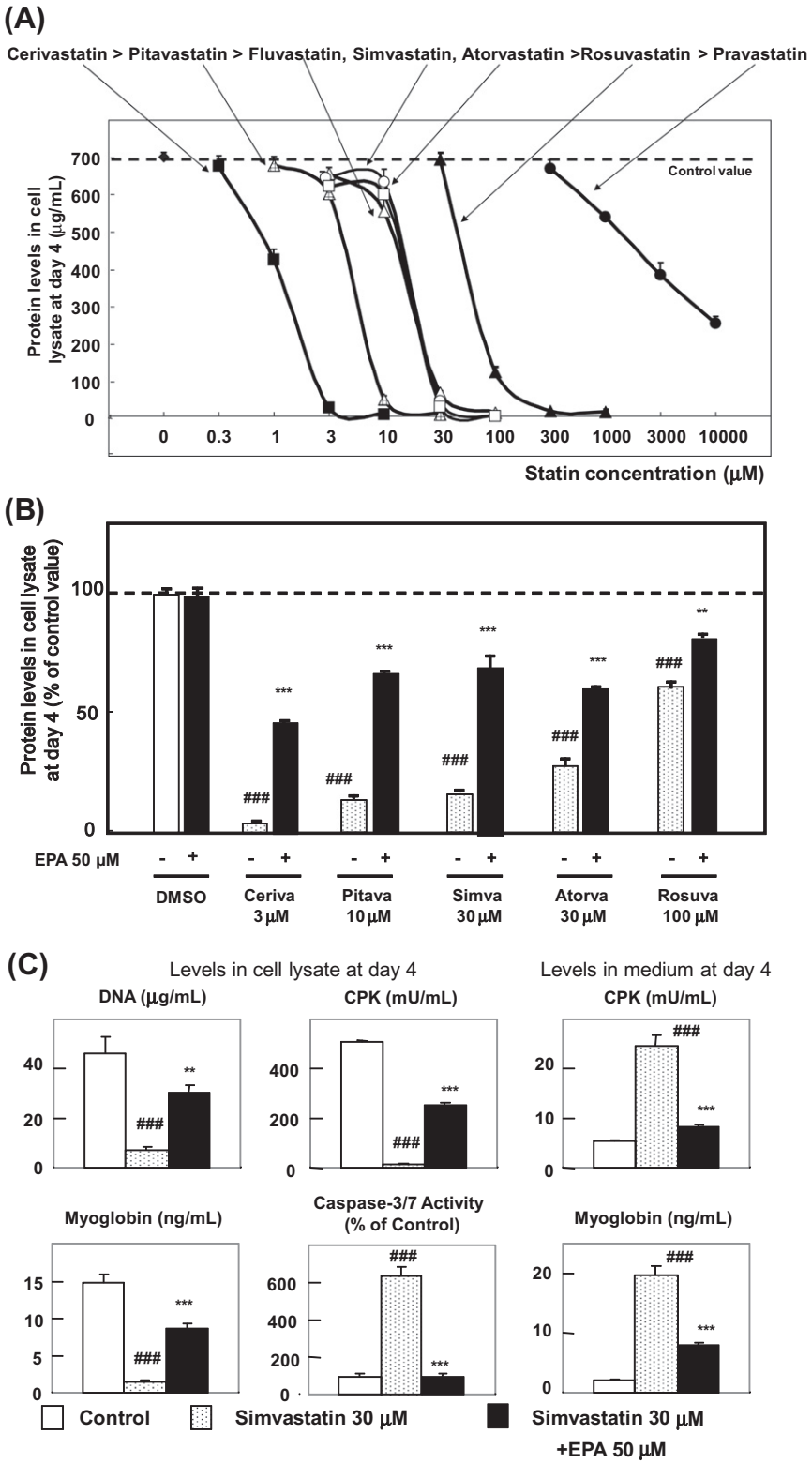

Fig. 1. (A) Various statins induce toxicity in RD cells. Differentiated RD cells were treated with various statins for 4 days, and cells were then washed with PBS and lysed with MPER reagent. Protein content in cell lysate was evaluated to determine the toxicity of various statins. (n = 3–12, mean ± SE). (B) EPA inhibits various types of statin-induced toxicity in RD cells. Differentiated RD cells were treated with various statins and 50 μM EPA for 4 days, and cells were then treated the same as in A. Values are shown as the% of control value. (2 experiments, n = 3–7, mean ± SE). (C) EPA prevents all statin-induced changes other than that in cellular protein content. Differentiated RD cells were treated with 30 μM simvastatin and 50 μM EPA for 4 days. Then DNA, Mgb content, CPK, and Caspase-3/7 activity in cell lysate and Mgb content and CPK activity in medium were measured (2 experiments, n = 4, mean ± SE).

RD cells (data not shown). Among them, mevalonate and GGPP completely prevented toxicity, while squalene did not prevent toxicity at all (Fig. 2, Nos. 2–4). Thus, within the mevalonate cascade, not a decrease in cholesterol but a decrease in GGPP, which

no	name	category	μM
1	EPA	n-3, PUFA	50
2	Mev	product of HMG-coR	100
3	GGPP	metabolite of HMG-coR	100
4	Squalene	metabolite of HMG-coR	100
5	U-73122	PLC inhibitor	5
6	Ryanodine	inhibitor of Ca release from SR	50
7	BAPTA-AM	Ca kilator	1
8	ALLN	Calpain inhibitor	10
9	B.A	inhibitor of CytC release from Mitochond.	4
10	Z-VAD-FMK	pan-caspase inhibitor	100
11	CoQ10	anti oxidant, metabolite of HMG-coR	100
12	VtC	antioxidant	100
13	VtE	antioxidant	100
14	4-PBA	ER stress attenuator	500
15	TUDCA	ER stress attenuator	100
16	Dolicol-P	metabolite of HMG-coR	60 [1)]
17	Sandoz 58-035	ACAT inhibitor	30 [1)]
18	IPA	metabolite of HMG-coR	100
19	L-NMMA	NOS inhibitor	100
20	DHA	n-3, PUFA	50
21	Oleic acid	MUFA	50
22	Palmitic acid	SA	50
23	AA	n-6、PUFA	50
24	AACOCF3	cPLA2 inhibitor	2
25	2-Br-Palmitic acid	non-metabolizing FFA, PPAR-δ agonist	50
26	L-165041	PPAR-δ agonist	100
27	SP-600125	JNK inhibitor	10
28	SB203580	P38 MAPK inhibitor	20
29	PD98059	MEK inhibitor	25

[1)]: μg/mL

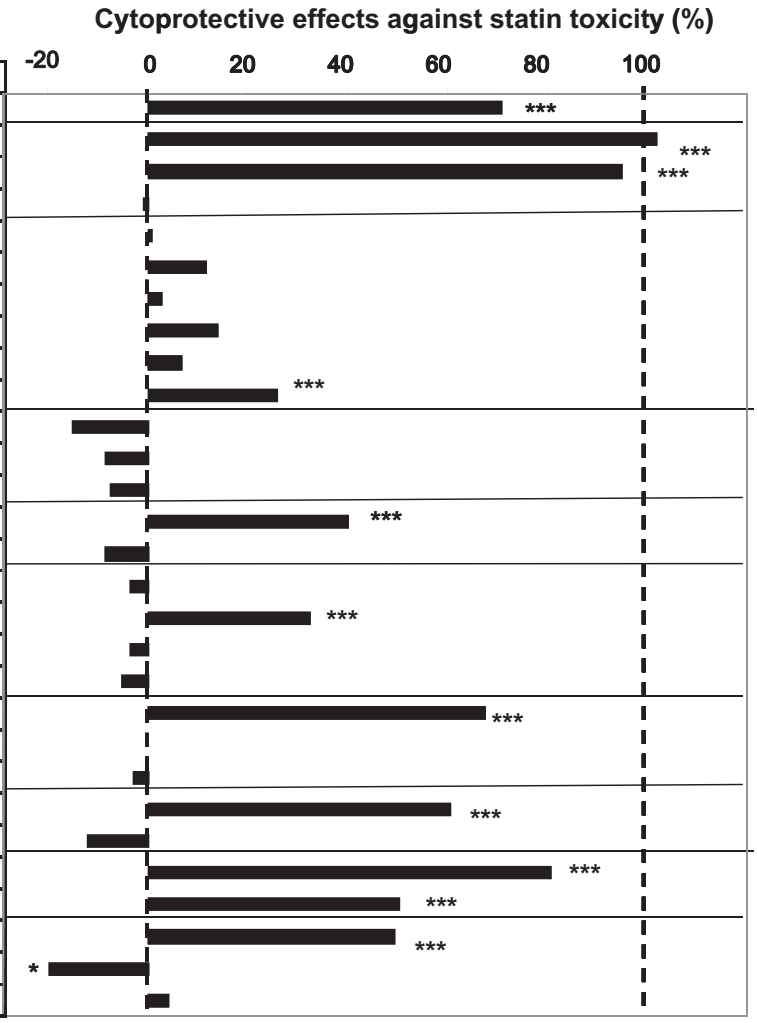

Fig. 2. Cytoprotective effects of various compounds in our model. Differentiated RD cells were treated with 30 μM simvastatin and various compounds for 4 days, and cells were then treated the same as in Fig. 1A (8 experiments, $n = 3–50$). Values presented are cytoprotective effects against statin toxicity (with level of control (non-treated) group as 100% and level of statin-alone-treated group as 0%).

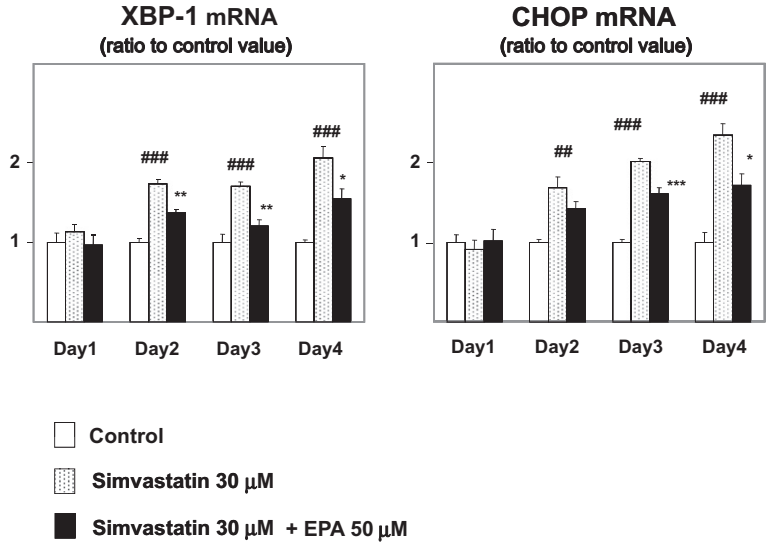

Fig. 3. Statin increased ER stress markers in RD cells and EPA prevented them. Differentiated RD cells were treated with 30 μM simvastatin and 50 μM EPA for 1–4 days, and cellular XBP-1 and CHOP mRNA content were measured ($n = 3–4$). GAPDH mRNA content was also measured as an internal control.

is essential for protein prenylation, played an important role in simvastatin-induced toxicity in our model, similar to another study [15]. Pan-caspase inhibitor weakly inhibited statin-induced toxicity (Fig. 2, No. 10), though other compounds reported to prevent statin toxicity in cultured myoblasts [8,11] had no effect on simvastatin-induced toxicity (Fig. 2, Nos. 5–9). Evaluation of changes in activity of various proteases involved in caspase-3/7 activity [8,10], i.e., caspase-8, caspase-9, and calpain, revealed no effects due to statins or EPA in our model (data not shown).

Some reports have suggested a correlation between statin-induced toxicity and reactive oxygen species [1,2]. However, antioxidants tended to worsen toxicity rather than prevent it (Fig. 2, Nos. 11–13). In some models, antioxidants or reducing agents have been shown to be initiators or activators of ER stress [16], an intracellular abnormal condition mainly induced by abnormal protein accumulation or intra-endoplasmic Ca^{2+} ion imbalance [16,17]. ER stress often activates caspase-3/7 and apoptosis [16,17]. We therefore tested two types of ER stress attenuator (4-PBA and TUDCA), and found that 4-PBA attenuated statin-induced toxicity (Fig. 2, Nos. 14–15). Cellular mRNA levels of the ER stress markers XBP-1 and CHOP [16,17] were increased by statins and EPA attenuated both (Fig. 3). In our model, the widely used ER stress inducer tunicamycin [16] (10 μg/mL, 24 h) increased XBP-1 (2.8-fold) and CHOP (13-fold) (data not shown). This is the first report that statins induce ER stress, and also the first report that EPA attenuates ER stress. We then measured mRNA levels of ER stress markers in skeletal muscle of EHBR rats administered statin and EPA (Fig. 4A). Plasma CPK activities in statin-administered rat (statin-alone and statin + EPA-E treated rats) were significantly correlated with mRNA levels of XBP-1, CHOP, and Bip (Fig. 4B). These results suggest that statins may induce ER stress and EPA attenuate it not only in cultured myoblast cells but also in vivo rat skeletal muscles.

What is the mechanism of statin-induced ER stress in cultured myoblasts? Increase in NOS activity [18] and scavenger receptors [19], deficiency of translation and *N*-glycosylation of some proteins have been considered causes of ER stress [16]. In some cases, statins increase NOS [20] and scavenger receptors [20,21]. Statins may induce abnormality of protein glycosylation and tRNA production via decrease in dolichol [22] and IPA [23], both of which are metabolites of mevalonate. In our model, dolichol phosphate, IPA and

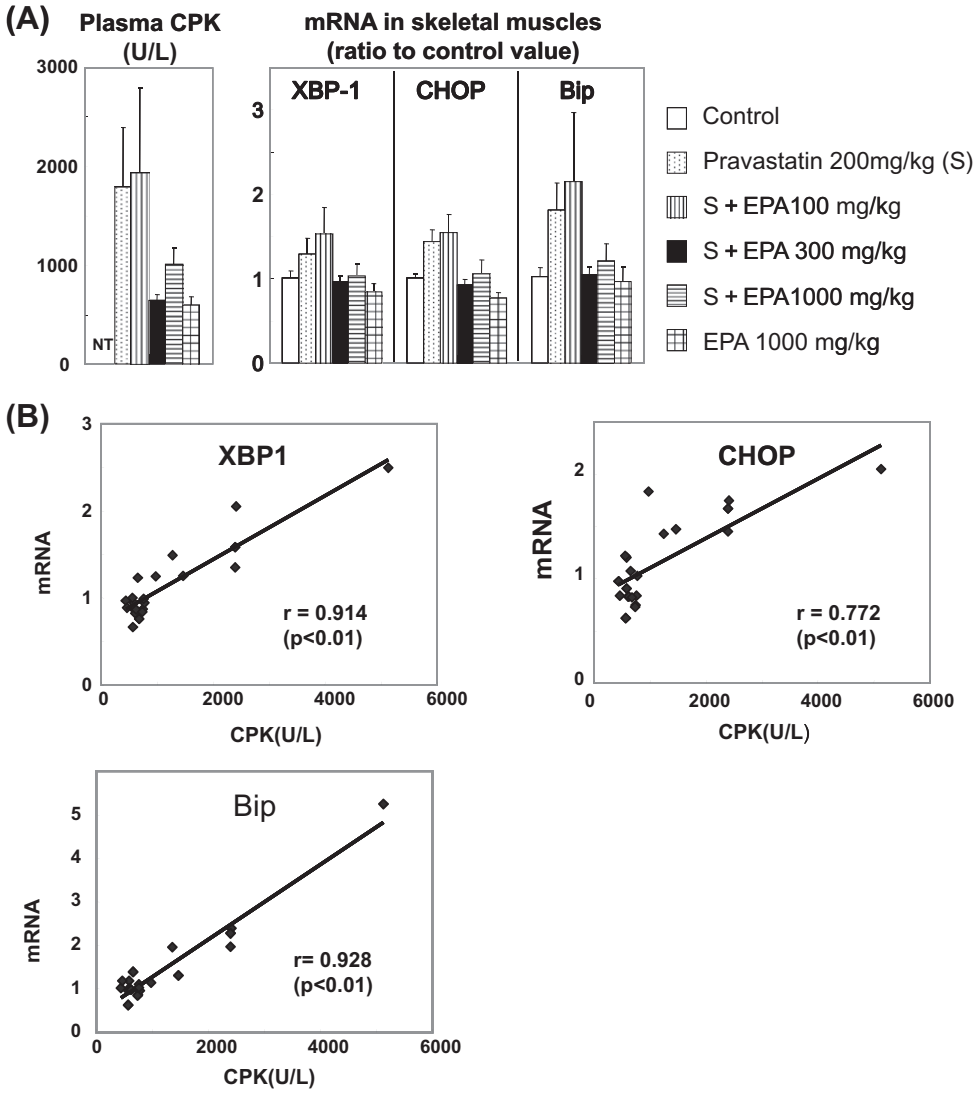

Fig. 4. (A) The effects of EPA on plasma CPK and skeletal muscle ER stress markers in rats with statin-induced rhabdomyolysis. Eisai hyperbilirubinemic rats (EHBR) were administered 200 mg/kg/day pravastatin alone or co-administered 100–1000 mg/kg/day EPA-E for 14 days. Plasma CPK activity and skeletal muscle mRNA of XBP-1, CHOP, and Bip were measured. Skeletal muscle rRNA content was also measured as an internal control for mRNAs (n = 3–5). NT: Plasma CPK activity of control rat in this experiment was not measured, while that in another experiment was 582 ± 88 U/L (n = 3). (B) Plasma CPK activities were significantly correlated with skeletal muscle mRNA levels of XBP-1, CHOP, and Bip. The values in statin-treated rats used in (A) are plotted (n = 18).

Table 1
Effects of various compounds on statin-induced increases of XBP-1 mRNA, p-c-Jun protein content and caspase-3/7 activity. Differentiated RD cells were treated for 3 days (p-c-Jun) or 4 days (caspase-3/7, XBP-1) with 30 μM simvastatin and various compounds. Then mRNA of XBP-1 (using quantitative PCR methods, corrected by rRNA, $n = 6–8$), p-c-Jun protein content (using the CASE-kit, corrected by cellular protein, 2 experiments, $n = 6–11$) and caspase-3/7 activity (by Caspase-3/7 GRO, corrected by cellular protein, $n = 3–4$) were measured.

	Parameters (% of control value)		
Group	XBP-1 mRNA	p-c-Jun protein	Caspase-3/7 activity
Control	100 ± 6	100 ± 5	100 ± 6
Simvastatin 30 μM (S30)	248 ± 12$^{\#\#\#}$	209 ± 15$^{\#\#\#}$	691 ± 53 $^{\#\#\#}$
S30 + mevalonate 500 μM	107 ± 8***	105 ± 8***	88 ± 12***
S30 + EPA 50 μM	132 ± 7**	153 ± 9***	63 ± 5***
S30 + 4-PBA 1000 μM	171 ± 14***	147 ± 6***	348 ± 43***
S30 + L-165,041 100 μM	178 ± 11***	160 ± 10**	307 ± 21***
S30 + SP-600,125 10 μM		110 ± 8***	
S30 + Z-VAD-FMK 100 μM			11 ± 1***

L-NMMA (inhibitor of NOS) did not exhibit cytoprotective effects against statin toxicity, though ACAT inhibitor did (Fig. 2, Nos. 16–19), similar to some oxidized LDL-induced apoptosis models [24]. Statins may increase scavenger receptors and result in cellular accumulation of cholesterol ester, though more detailed experiments are needed to test this.

What is the mechanism of the cytoprotective effects of EPA? We first found no changes in concentration of the active form of simvastatin in RD cells (data not shown). DHA, a n-3 PUFA in the same category as EPA, protected against statin toxicity, while saturated fatty acids (SA), palmitic acid, and mono-unsaturated fatty acids (MUFA), oleic acid, did not protect against it (Fig. 2, Nos. 20–22). In our model, AA prevented toxicity, and PLA2 inhibitor did not (Fig. 2, Nos. 23–24) and PI3 K inhibitor did not abolish the cytoprotective effects of EPA (data not shown). Contrary to some reports [25–27] on cytoprotective effects of EPA or DHA in certain types of cells, decrease in intracellular AA content or PLA2 activity and activation of PI3K did not appear to play roles in the mechanism of the cytoprotective effects of EPA in our model.

PPARs agonists have been shown to inhibit some apoptotic stimuli [28–30]. Furthermore, EPA has been reported to exhibit agonistic activity at PPAR-α and -δ [31]. The predominantly expressed subtype of PPAR in skeletal muscle was reported to be PPAR-δ [32], and PPAR-δ agonist prevents cytotoxicity of thapsigargin [30], which is known to induce ER stress in certain types of cells [16]. Based on these reports, we hypothesized that EPA exhibited cytoprotective effect via PPAR-δ in our model. 2-Br-palmitic acid [31] and L165041, both of which are PPAR-δ agonists, prevented statin toxicity (Fig. 2, Nos. 25–26). L165041 also decreased statin-induced XBP-1 mRNA as similar to EPA, 4-PBA, and mevalonate (Table 1). In some reports, ER stress activated JNK and induced apoptosis [17], EPA decreased JNK activity [33] and statins activated JNK [34]. In our model, the JNK inhibitor SP600125 prevented statin toxicity, while inhibitors of P38 MAPK or MEK did not (Fig. 2, Nos. 27–29). Indeed, statins increased cellular JNK activity, and EPA and SP600125 attenuated it (data not shown). Cellular content of p-c-Jun, one of the products of JNK, was also increased by statin, and mevalonate, EPA, 4-PBA, L165041, and SP600125 each attenuated it (Table 1). Furthermore, mevalonate, EPA, 4-PBA, L165041, and Z-VAD-FMK each attenuated the activation of caspase-3/7 induced by statin (Table 1). These findings suggested that statins induce ER stress via decrease in GGPP content, and result in activation of JNK and caspase-3 and induction of apoptosis. In addition, it was suggested that EPA attenuates statin-induced toxicity, at least in part via attenuation of ER stress, perhaps through PPAR-δ activation.

No report has described the relationship between EPA and ER stress. However it is of great interest that a similar correspondence has been reported between the diseases induced by ER stress [16,17] and the diseases amenable to treatment with fish oils or n-3 PUFAs (e.g., Huntington disease and bipolar disorder [35], diabetes and insulin resistance [36], defects in insulin release [37], atherosclerosis [4,5], steatosis and hepatopathy [38], and renal disease [39]).

In conclusion, these findings suggest that EPA directly attenuates statin-induced ER stress, JNK activation and toxicity in cultured myoblast cells, and that PPAR-δ may mechanically involved in the effects of EPA.

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