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Improving effect of ethyl eicosapentanoate on statin-induced rhabdomyolysis in Eisai hyperbilirubinemic rats

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Abstract

The effect of ethyl eicosapentanoate (EPA-E) on statin-induced rhabdomyolysis was investigated by co-administration of EPA-E and pravastatin (PV), as a typical statin, to Eisai hyperbilirubinemic rats (EHBR). It was confirmed that the plasma PV concentration was not affected by simultaneous administration of EPA-E, and there was no cumulative increase of PV during prolonged co-administration of EPA-E and PV. Muscular degeneration was prominent (incidence 5/5; average grade 3.5 (range 2–4)) in EHBR treated with PV alone at 200 mg/kg/day for 14 days, but co-administration of EPA-E at doses of 100, 300, and 1000 mg/kg/day decreased the average grades to 1.4 (range 0.3–3.0), 0.5 (0.2–1.0), and 0.6 (0.0–1.7), respectively. Creatine phosphokinase (CPK) and myoglobin levels in plasma were well correlated with the grade of skeletal muscle degeneration. Thus, EPA-E appears to reduce the severity of statin-induced rhabdomyolysis.

Keywords: Statin; Ethyl eicosapentanoate; EPA-E; Rhabdomyolysis; Myopathy; Pravastatin; EHBR

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are used clinically to improve the lipid profile of hyperlipemic patients, thereby decreasing the incidence of primary and secondary ischemic cardiac events [1,2]. Statins decrease the concentrations of total cholesterol (T-Cho) and low-density lipoprotein (LDL) in blood, by inhibiting cholesterol synthesis in liver, and stimulating cholesterol uptake into the liver via LDL receptors [3,4]. However, they are less effective in decreasing triglyceride (TG) in blood, and some of them decrease the concentration ratio of eicosapentaenoic acid to arachidonic acid (EPA/AA) in blood, which is undesirable [5]. Moreover, one of the most important clinical adverse effects in therapy with statins is drug-induced skeletal muscle toxicity (rhabdomyolysis) [6], whose incidence is significant (e.g., 0.1–0.5% in patients treated with pravastatin (PV)). The frequency of rhabdomyolysis is further increased by co-administration of fibrates, so combined use of statins and fibrates is generally inappropriate [7].

On the other hand, EPA, one of the ω-3 unsaturated fatty acids in fish oil, has beneficial effects in patients with circulatory system diseases, such as arteriosclerosis and hyperlipemia, judging from epidemiological surveys. TG decreases with increasing EPA/AA ratio in blood, so the ethyl ester of EPA (EPA-E) is widely used as a drug to treat hyperlipemia [8–12]. Moreover, a clinical study of hyperlipemic patients with high blood concentrations of TG indicated that co-administration of EPA-E and statins was more effective in decreasing T-Cho and TG, and increasing high-density lipoprotein (HDL) than treatment with statins alone [13]. Therefore, it is important to examine the effect of EPA-E on statin-induced rhabdomyolysis.

The establishment of toxicological animal models for statin-induced rhabdomyolysis has been difficult. Pierno et al. [14,15] reported that PV treatment at 100 mg/kg/day for three months did not produce any alteration of excitation–contraction coupling of the rat skeletal muscle. Moreover, Smith et al. [16] reported that the incidence of

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induced myopathy in rats was dose-dependent, but the grade of degeneration was variable at a maximum dose of 2400 mg/kg/day (on a bid regimen) of PV for 2–4 weeks. The incidence was increased by concomitant intravenous administration of cyclosporin A at various doses for the same period, but the grade of degeneration remained variable. In a previous study [17], we examined whether the Eisai hyperbilirubinemic rat (EHBR), a mutant strain of inbred Sprague–Dawley rat lacking the expression and function of multidrug resistance-associated protein 2 (rodents, Mrp2; humans, MRP2, and ABCC2), [18–20] would be a valuable animal model for toxicity studies on organic anion compounds which are substrates of MRP2, such as PV [21–24], since systemic exposure to such drugs is expected to be increased in EHBR as compared with normal rats. Indeed, we found that drug-induced rhabdomyolysis with severe degeneration was observed in EHBR given at 200 mg/kg/day (once a day) of PV alone for 14 days. Compared with previously described models, this has several advantages: it was possible to reduce the drug administration by dosing once a day, to simplify the procedure by the dosing of PV alone, to shorten the study period, and to obtain a high rate of induction of rhabdomyolysis with a reasonably consistent degree of severity. Accordingly, the aim of the present study was to investigate the effect of EPA-E on statin-induced rhabdomyolysis, by co-administration of EPA-E and PV to EHBR.

Materials and methods

Chemicals and animals. PV (sodium salt) was purchased from APIN Chemicals (Oxon, UK). Eicosapentaenoic acid ethyl ester(ethyl all-*cis*-5,8,11,14,17-icosapentaenoate, EPA-E; purity 96.9%) was obtained from Mochida Pharmaceutical. The other reagents and solvents used in this study were commercially available products of analytical grade or chromatographic grade. The animal study was performed according to the Guidelines for the Care and Use of Laboratory Animals in the Pharmaceutical Research Center, Mochida Pharmaceutical and approved by the Committee of Ethics of Animal Experimentation of the Pharmaceutical Research Center, Mochida Pharmaceutical. Male EHBR, 4 weeks of age, were obtained from Japan SLC (Hamamatsu, Japan). The animals were individually housed at constant temperature (23 ± 3 °C) and humidity (50 ± 20%), with 12 h of light per day. They were allowed access to water and were given commercially available diet, CE-2 (Clea Japan, Tokyo, Japan).

Toxicokinetic study of PV. The toxicokinetic study for PV was designed as follows; Group A: PV was administered to 7-week-old rats at a single dose of 200 mg/kg ($n = 3$). Group B: EPA-E (1000 mg/kg) and PV (200 mg/kg) were administered, each as a single dose, to 7-week-old rats ($n = 3$). Group C: EPA-E was administered to 5-week-old rats at a dose of 1000 mg/kg for 14 days, then EPA-E at the same dose and PV (200 mg/kg) were administered for 14 days (as a satellite group of group E mentioned below, $n = 1$). EPA-E was emulsified in 5% aqueous gum arabic solution by using a dispersing agent (T-25, IKA Japan K.K.) and an ultrasonic homogenizer (VC-130, Sonics and Materials). PV was dissolved in 0.5% hydroxypropyl methylcellulose. After single or final dosing of PV to rats, blood samples were withdrawn via the jugular vein with heparinized syringes at designated times and centrifuged (1700*g*) for 15 min at 4 °C to obtain plasma samples. Probenecid (internal standard) and 50 mM phosphate buffer (pH 4.5) were added, and PV was extracted from rat plasma using ethyl acetate. The organic layer was collected and evaporated, then the dried residue was dissolved in aqueous methanol. Aliquots were analyzed in an HPLC system (HP-1050; Agilent Technologies, Palo Alto, CA, USA) equipped with a CAPCELL PAK C8 DD column (150 × 2.0 mm i.d., Shiseido, Tokyo, Japan) using isocratic elution at 0.2 mL/min with 37% of 5 mM ammonium acetate aq./63% methanol. PV was detected using a triple quadrupole mass spectrometer (TSQ700; Thermo Electron, San Jose, CA, USA) fitted with an electrospray ionization source. Negative ion selected reaction monitoring was done using the transitions 423–321 for PV and 284–240 for the internal standard. The values of the area under the plasma concentration time-curve from time 0 to infinity ($AUC_{0-\infty}$) of groups A and B were estimated by the trapezoidal method with extrapolation to infinite time using the nonlinear estimation program WinNonlin (Pharsight, Mountainview, CA, USA). AUC from time 0 to 24 h ($AUC_{0-24\ h}$) of group C was also calculated by use of the trapezoidal method. The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were determined directly from the observed data. The total body clearance corrected for absorption (CL_{tot}/F) was calculated by dividing the dose by the AUC value. Each value in groups A and B represents the mean ± SD of three animals.

Toxicity study. The toxicity study was designed as follows; Group D: 0.5% HPMC solution was administered to 7-week-old rats once a day for 14 days ($n = 3$). Group E: PV (200 mg/kg) was administered to 7-week-old rats for 14 days ($n = 5$). Groups F-H: EPA-E was administered to 5-week-old rats at a dose of 100 (group F, $n = 5$), 300 (group G, $n = 5$) or 1000 mg/kg (group H, $n = 3$) for 14 days, then EPA-E at the same dose and PV (200 mg/kg) were administered for 14 days, Group I: EPA-E was administered to 5-week-old rats at a dose of 1000 mg/kg for 28 days ($n = 3$). Drug solutions were prepared in the same manner as mentioned above. The body weight, food consumption, and general symptoms of each animal were monitored daily during the study period. At 24 h after the end of the last dosing, the animals were anesthetized with 2.5% pentobarbital sodium(Somnopentyl; Schering-Plough, Kenilworth, NJ, USA) and heparinized blood samples were collected from the abdominal aorta for biochemical analysis. Creatine phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), T-Cho, and TG were determined with autoanalyzers (COBAS MIRA PLUS, Roche Diagnostics, Basel, Switzerland, or Abaxis EA; Abaxis, Union City, CA, USA). In addition, plasma myoglobin was measured with an ELISA kit (Cat. No. 2110-2, Life Diagnostics). Body weight gain, food consumption and biochemical parameters are shown as the means ± SD of three or five animals. Subsequently, each animal was killed and skeletal muscles were taken from each of three sites, musculus rectus femoris, musculus biceps femoris, and musculus gastrocnemius, of the right and left hind limbs (total of six specimens). All muscles were preserved in 10% phosphate-buffered formaldehyde solution, routinely processed, embedded in paraffin, and sectioned at 3-μm thickness. These sections were stained with hematoxylin and eosin. In histopathological evaluation, the degree of muscular degeneration was evaluated according to the following scoring system [16,17]: grade 0, within normal limits; grade 1, small amounts of muscle fibers showing a few vacuoles but no necrosis; grade 2, scattered necrosis of muscle fibers; grade 3, necrotic area reaching one-third of the observed area; and grade 4, necrotic area exceeding one-third of the observed area. The mean of the scores of the six specimens was taken as representative of the individual. Moreover, the average of these individual values was regarded as representative of the group.

Statistical analysis. Statistical analysis of kinetic parameters (Table 1, Group A vs B) was performed by means of Student's or Welch's *t* test after the *F* test for equality of variance. For body weight gain, food consumption and biochemical parameters, Student's or Welch's *t* test was used after the *F* test to confirm homoscedasticity (Table 2, Group D vs E and Group D vs I), or Dunnett's or Steel's test after Bartlett's test for homoscedasticity (Table 2, Group E between F, G, or H). In histopathological evaluation of muscular degeneration, Mann–Whitney's *U* test (Table 3, Group D vs E and Group D vs I) or Steel's test (Table 2, Group E between F, G, or H) was used. All analyses were two-tailed, and a difference between means was considered to be significant when the *P* value was less than 0.05.

Table 1
Pharmacokinetic parameters of PV after oral administration to male EHBR

Group		Dose (mg/kg)		C_{max} (μg/mL)	T_{max} (h)	AUC (μg·h/mL)	CL_{tot}/F (L/h/kg)
		PV	EPA-E				
A	Single	200	0	2.22 ± 0.41	0.8 ± 0.3	8.30 ± 0.36	24.12 ± 1.06
B		200	1000	1.80 ± 0.57	1.2 ± 0.8	9.07 ± 2.77	23.44 ± 6.91
C	Day 13	200	1000	2.61	0.5	10.15	19.70

Each value of group A and B represents the mean ± SD of three animals.

Table 2
Body weight gain, food consumption, and biochemical parameters in plasma of EHBR treated with PV and EPA-E

Group	No. of animals	Dose (mg/kg/day)		Body weight gain (g)	Food consumption (g/day)	CPK (U/L)	Myoglobin (ng/mL)	AST (U/L)	ALT (U/L)	T-Cho (mg/dL)	TG (mg/dL)
		PV	EPA-E								
D	3	0	0	93.2 ± 15.2	25.7 ± 1.8	582 ± 153	4.43 ± 1.63	112 ± 17	35 ± 6	102 ± 3	51 ± 3
E	5	200	0	29.5 ± 32.8*	19.2 ± 3.4*	9660 ± 7665	124.67 ± 68.78*	281 ± 121*	235 ± 38*	99 ± 13	39 ± 19
F	5	200	100	61.0 ± 10.7	25.1 ± 1.4#	1932 ± 1939	13.23 ± 16.1#	456 ± 435	135 ± 65#	111 ± 9	21 ± 8
G	5	200	300	68.6 ± 5.2	25.2 ± 1.1#	647 ± 135#	3.04 ± 1.31#	166 ± 28	80 ± 17#	115 ± 7#	36 ± 21
H	3	200	1000	68.3 ± 19.9	26.2 ± 2.1#	942 ± 454	4.54 ± 2.19	172 ± 60	67 ± 13#	106 ± 11	28 ± 9
I	3	0	1000	94.7 ± 2.5	26.7 ± 1.0	598 ± 150	5.33 ± 2.09	91 ± 6	43 ± 2	85 ± 3*	54 ± 14

Each value represents the mean ± SD of three or five animals.
* $p < 0.05$, significantly different from the group D by Student's t test or Welch's test.
# $p < 0.05$, significantly different from the group E by Dunnett's or Steel's test.

Table 3
Incidence and grade of skeletal muscle degeneration in male EHBR treated with PV and EPA-E

Group				Grade of skeletal muscle degeneration[a]							
	Dose (mg/kg/day)		Animal No.	Right side			Left side			Average	
	PV	EPA-E		R[b]	B[b]	G[b]	R[b]	B[b]	G[b]	Individual	Group
D	0	0	1	0	0	0	0	0	0	0.0	0.0
			2	0	0	0	0	0	0	0.0	
			3	0	0	0	0	0	0	0.0	
E	200	0	1	4	3	2	4	4	2	3.2	3.5#
			2	3	4	3	3	4	2	3.2	
			3	4	4	3	4	4	3	3.7	
			4	4	4	2	4	4	3	3.5	
			5	4	4	3	4	4	4	3.8	
F	200	100	1	1	0	0	1	0	0	0.3	1.4#
			2	2	1	0	0	0	0	0.5	
			3	3	3	3	3	3	3	3.0	
			4	2	3	1	2	3	1	2.0	
			5	2	2	0	1	2	0	1.2	
G	200	300	1	0	0	1	0	0	0	0.2	0.5#
			2	0	0	0	1	0	0	0.2	
			3	0	2	0	0	2	0	0.7	
			4	1	2	0	0	2	1	1.0	
			5	0	0	0	1	1	0	0.3	
H	200	1000	1	0	0	0	0	0	0	0.0	0.6
			2	0	0	0	0	0	0	0.0	
			3	2	3	0	2	3	0	1.7	
I	0	1000	1	0	0	0	0	0	0	0.0	0.0
			2	0	0	0	0	0	0	0.0	
			3	0	0	0	0	0	0	0.0	

[a] The grade of skeletal muscle degeneration is described in the Materials and methods section: grade 0, within normal limits; grade 1, small amounts of muscle fibers showed a few vacuoles but no necrosis; grade 2, scattered necrosis of muscle fibers; grade 3, necrotic area up to one-third of observed area; and grade 4, necrotic area exceeded one-third of observed area.
[b] R, musculus rectus femoris; B, musculus biceps femoris; and G, musculus gastrocnemius.

Results

Toxicokinetics of PV

The plasma concentration–time data of PV are shown in Fig. 1 for single administration of PV (groups A and B) and in Fig. 2 for the final dosing of multiple administrations (group C). The derived kinetic parameters are summarized in Table 1. In the case of a single oral administration of PV alone at a dose of 200 mg/kg, the C_{max} was 2.22 ± 0.41 μg/mL at 0.8 ± 0.3 h after dosing, while $AUC_{0-\infty}$ and CL_{tot}/F were 8.30 ± 0.36 μg·h/mL and 24.12 ± 1.06 L/h/kg, respectively. After a single co-administration of PV (200 mg/kg) and EPA-E (1000 mg/kg), the plasma concentration profile of PV was similar to that after administration of PV alone; there was no significant difference of kinetic parameters between them. In the case of multiple co-administrations of PV (200 mg/kg/day) and EPA-E (1000 mg/kg/day), the C_{max} of PV was 2.61 μg/mL at 0.5 h after last dosing, while $AUC_{0-24\,h}$ and CL_{tot}/F were 10.15 μg·h/mL and 19.70 L/h/kg, respectively. There was no remarkable difference of kinetic parameters between single and multiple dosing. Moreover, the multiple-dosing profile of plasma PV concentration simulated from the kinetic parameters after single administration was consistent with the observed data.

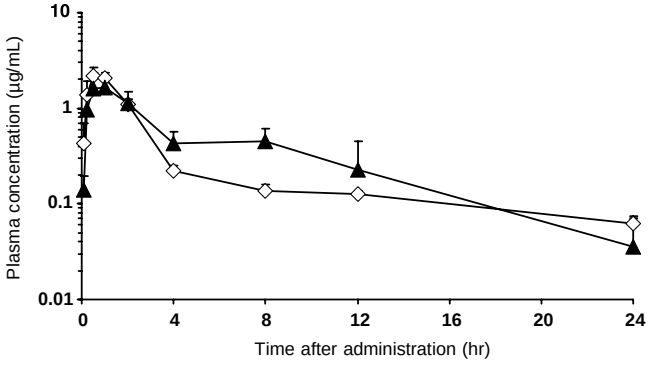

Fig. 1. Plasma concentrations of unchanged drug after a single oral administration of PV at a dose of 200 mg/kg to male EHBR. Open squares and closed triangles represent the case of administration of PV alone and that of co-administration of EPA-E 1000 mg/kg, respectively. Each point represents the mean ± SD of three animals.

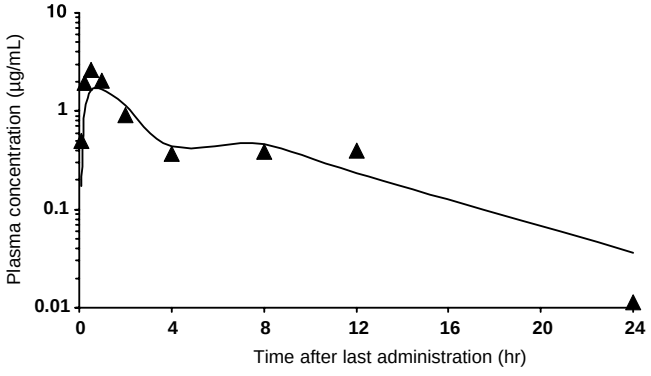

Fig. 2. Plasma concentrations of unchanged drug after the last dosing of multiple administrations (on day 13) of PV at a dose of 200 mg/kg/day with EPA-E (1000 mg/kg/day) to male EHBR. Closed triangles represent the observed data, while the solid line represents the simulation using the kinetic parameters obtained after single administration of PV with EPA-E at the same doses.

Toxicity of PV

Body weight gain, food consumption (between 7 and 9 weeks old), and biological parameters at 24 h after final dosing in groups D–I are shown in Table 2. The incidence and grade of skeletal muscle degeneration in EHBR treated orally with PV for 14 days are shown in Table 3. The correlations among degeneration scores, plasma CPK activity, and myoglobin concentration are shown in Fig. 3. Body weight gain in the control group administered the vehicle (group D) was 93.2 ± 15.2 g. In group E treated with PV alone at 200 mg/kg/day for 14 days, body weight gain was suppressed (29.5 ± 32.8 g). Suppression in group F, G, and H treated with PV and EPA-E (body weight gain 61.0–68.6 g) was modest. Body weight gain in group I, administered EPA-E alone, was not significantly different from that of group D. Food consumption, which was 25.7 ± 1.8 g/day in the control group, was reduced in the PV-alone group. However, it recovered to the control level in the groups co-administered PV and EPA-E. There was no significant difference of food consumption between the EPA-E alone group and the control group. No general symptoms were apparent in any group. The plasma CPK level, which was 582 ± 153 U/L in the control group, was greatly increased in group E (9660 ± 7665 U/L) and was decreased in groups F–H, with apparent EPA-E dose dependency at the lower doses, i.e., 1932 ± 1939, 647 ± 137, and 942 ± 454 U/L for groups F, G, and H, respectively. The CPK level in group I, 598 ± 150 U/L, was not significantly different from that of group D. The plasma myoglobin concentration, which was greatly increased in group E (124.67 ± 68.78 ng/mL) compared with the control group, was decreased in group F–H with apparent EPA-E dose dependency at the lower doses, i.e., 13.23 ± 16.10, 3.04 ± 1.31, and 5.90 ± 5.37 ng/mL for groups F, G, and H, respectively. The myoglobin level in group I was not significantly different from that of group D. AST and ALT activities, which were 112 ± 17 and 35 ± 6 U/L, respectively, in the control group, were increased in the PV-alone group. These values were decreased EPA-E dose-dependently in the PV and EPA-E co-administered groups. No significant difference between the control and EPA-E-alone administered group was observed. T-Cho concentration, which was 102 ± 3 mg/dL in the control group, showed almost the same value in all groups except, the EPA-E-alone administered group (85 ± 3 mg/dL). No significant difference of TG concentration between the control and PV-alone or EPA-E-alone group, or among PV-administered groups, was observed. Histopathologically, the incidence and extent of muscular degeneration were prominent in EHBR treated with PV

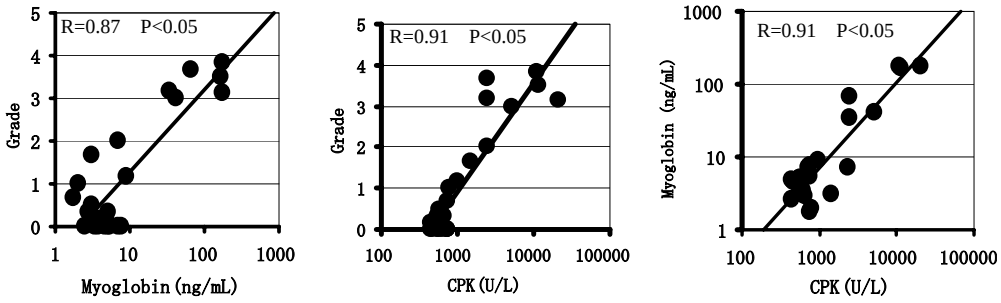

Fig. 3. Relationships among grade of skeletal muscle degeneration, plasma CPK activity, and myoglobin concentration in EHBR at 24 h after completion of 14-day multiple oral administrations of PV.

alone at 200 mg/kg/day for 14 days (incidence 5/5, average grade 3.5). The degree of muscular degeneration in these animals ranged from grade 2 to grade 4, and the individual averages were within grades 3.2–3.8. The extent of muscular degeneration was decreased in groups F–H with apparent EPA-E dose dependency at the lower doses. For groups F, G, and H, the average grades were 1.4 (individual range 0.3–3.0), 0.5 (0.2–1.0), and 0.6 (0.0–1.7), respectively. No sign of muscular degeneration was observed in group F. Significant positive correlations (correlation coefficients greater than 0.87) were seen among CPK activity, myoglobin concentration, and grade of muscular degeneration.

Discussion

After a single co-administration of PV (200 mg/kg) and EPA-E (1000 mg/kg), the plasma concentration profile of PV was similar to that after administration of PV alone, with no significant difference of kinetic parameters. Moreover, there was no significant difference of kinetic parameters for PV plasma concentration, such as AUC and CL/F, between single and multiple dosing of PV and EPA-E. Indeed, the multiple-dosing profile simulated with the kinetic parameters obtained after single administration agreed well with the observed data. It was considered that plasma PV concentration was not affected by simultaneous use of EPA-E, and there was no cumulative effect of multiple dosing in case of co-administration of EPA-E and PV.

In EHBR treated with PV alone at 200 mg/kg/day for 14 days, the plasma CPK level and myoglobin concentration (indices of muscular damage) were greatly increased. Histopathological examination revealed prominent muscular degeneration (incidence 5/5, average grade 3.5). The degree of muscular degeneration in these animals ranged from grade 2 to grade 4, and the individual averages were within grades 3.2–3.8. The incidence and extent of muscular degeneration were improved dose-dependently by EPA-E administration in the range from 0 to 300 mg/kg/day. CPK and myoglobin levels in plasma were correlated with the grade of skeletal muscle degeneration. In other words, parameters improved dose-dependently in group E–G (dose of EPA-E: 0-300 mg/kg/day), while the improvement reached a plateau in groups G–H (dose of EPA-E: 300–1000 mg/kg/day).

The AST and ALT activity in plasma also decreased depending upon the dose of EPA-E. These enzymes, which are considered to be markers of hepatic damage, are also present in muscle cells [25]. It is considered that their activity in plasma might reflect the degeneration of muscle, rather than liver injury, in the present model. In group E, treated with PV alone, suppression of body weight gain and food consumption with wide scattering were observed, and the suppression was reversed by EPA-E co-administration, though without apparent dose-dependency. The suppression in groups F–H treated with both PV and EPA-E was modest.

In this paper, we investigated the improving effect of EPA-E on statin-induced rhabdomyolysis, by co-administration of EPA-E and PV to EHBR. The incidence and extent of muscular degeneration were decreased dose-dependently by EPA-E. Thus, we conclude that EPA-E reduces statin-induced rhabdomyolysis. The mechanism underlying statin-induced rhabdomyolysis remains unclear [26], though possibilities include: (1) depletion of secondary metabolic intermediates such as coenzyme Q10 and ubiquinone [27,28]; (2) induction of apoptotic cell death [29,30]; (3) participation of chloride ion channels [31,32]; (4) genetic polymorphism of transporters whose substrate is pravastatin [33,34]. Therefore, it is difficult at present to speculate as to the mechanism of the improving effect of EPA-E on statin-induced rhabdomyolysis. In this investigation, we examined the effect of EPA-E pre-treatment before co-administration of PV and EPA-E. The possible healing effect of EPA-E on existing myopathy will be the subject of further study. Since all statins currently in clinical use have myopathy as a potential side effect, it would also be worthwhile to investigate the effect of EPA-E on rhabdomyolysis induced any other statins.

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