



## Fish oil constituent eicosapentaenoic acid inhibits endothelin-induced cardiomyocyte hypertrophy via PPAR- $\alpha$



Nobutake Shimojo<sup>a,c,\*</sup>, Subrina Jesmin<sup>a,c</sup>, Satoshi Sakai<sup>a</sup>, Seiji Maeda<sup>a,b</sup>, Takashi Miyauchi<sup>a</sup>, Taro Mizutani<sup>c</sup>, Kazutaka Aonuma<sup>a</sup>, Satoru Kawano<sup>a,c</sup>

<sup>a</sup> Department of Cardiovascular Medicine, Division of Clinical Medicine, Faculty of Medicine, University of Tsukuba

<sup>b</sup> Division of Sports Medicine, Faculty of Health and Sport Sciences, University of Tsukuba

<sup>c</sup> Department of Emergency and Critical Care Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

### ARTICLE INFO

#### Article history:

Received 13 November 2013

Accepted 21 April 2014

Available online 2 May 2014

#### Keywords:

Cardiomyocyte

hypertrophy

eicosapentaenoic acid

endothelin

peroxisome proliferator-activated receptor -  $\alpha$

### ABSTRACT

**Aims:** A growing body of evidence shows the cardiovascular benefits of fish oil ingredients, including eicosapentaenoic acid (EPA), in humans and experimental animals. However, the effects of EPA on endothelin (ET)-1-induced cardiomyocyte hypertrophy and the involved signaling cascade are largely unknown. A previous study has demonstrated that peroxisomal proliferator-activated receptor (PPAR)- $\alpha$  ligand (fenofibrate) prevents ET-1-induced cardiomyocyte hypertrophy. Although EPA is a ligand of PPAR- $\alpha$ , to date, no study has examined a relationship between EPA and PPAR- $\alpha$  in cardiomyocyte hypertrophy. Here, we investigated whether EPA can block ET-1-induced cardiomyocyte hypertrophy and the possible underlying mechanisms.

**Main methods:** At day 4 of culture, neonatal rat cardiomyocytes were divided into four groups: control, control cells treated with EPA (10  $\mu$ M), ET-1 (0.1 nM) administered only and EPA-pre-treated ET-1 administered groups. Also, the cardiomyocytes were treated with PPAR- $\alpha$  siRNA in order to elucidate the mechanisms that may underlie suppression of hypertrophy via the EPA-PPAR system.

**Key findings:** Following ET-1 treatment, 2.12- and 1.92-fold increases in surface area and total protein synthesis rate in cardiomyocytes, respectively, were observed and these changes were greatly blocked by EPA pre-treatment. Further, the expression of PPAR- $\alpha$  increased in EPA-treated groups. PPAR-PPRE binding activity was suppressed in ET-1 administered cardiomyocyte and this suppression was improved by EPA treatment. Lastly, pre-treatment of cardiomyocytes with PPAR- $\alpha$  siRNA prior to EPA treatment attenuated the suppressing effects of EPA on cardiomyocyte hypertrophy.

**Significance:** In conclusion, the present study shows that EPA attenuates ET-1 induced cardiomyocyte hypertrophy by up regulating levels of PPAR- $\alpha$  pathway.

© 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

### Introduction

Cardiac hypertrophy is a major predictor of progressive heart disease, an indicator of a poor prognosis (Rohini et al., 2010), as well as constitutes an independent risk factor for heart failure, hypertension and ischemic heart disease (Sadoshima et al., 1992; Rohini et al., 2010; Ho et al., 1998). In general, pathological hypertrophy occurs in response to aberrant stress signals, e.g. neuro-hormonal activation, aortic

stenosis, inflammation or cardiac injury (Rohini et al., 2010). Specifically, one of the most important vascular hormones that contribute to the development of hypertrophy is endothelin (ET)-1. The ET system is comprised of 3 active ETs, i.e., ET-1-3 (Yanagisawa et al., 1988; Giannessi et al., 2001). ET-1, the main isoform, is synthesized in multiple cell types, including endothelial, muscular coat of arterial wall, as well as in the heart, kidney and central nervous system (Yanagisawa et al., 1988). The actions of ET are mediated through its interaction with two classes of cell surface receptors, namely ET type A (ET<sub>A</sub>) and ET type B (ET<sub>B</sub>). ET<sub>A</sub> receptor mediates vasoconstriction and cell growth, whereas ET<sub>B</sub> receptor is linked to endothelial-cell mediated vasodilatation and release of other neuro-hormones, such as aldosterone (Giannessi et al., 2001). In vitro studies have shown that ET-1-induced cardiac hypertrophy involves various signaling cascades mediated by ET<sub>A</sub> receptors, such as Raf-1 and mitogen-activated protein kinases in the neonatal rat (Shimojo et al., 2006; Yamazaki et al., 1996). Other signaling pathways mediating ET-1-induced cardiac hypertrophy include

\* Corresponding author at: Dept. of Emergency and Critical Care Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, 305-8575 Japan, Tel.: +81 29 853 3210; fax: +81 29 853 5984.

E-mail addresses: [nokeshimojo@yahoo.co.jp](mailto:nokeshimojo@yahoo.co.jp) (N. Shimojo), [jsubrina@gmail.com](mailto:jsubrina@gmail.com) (S. Jesmin), [ssakai@md.tsukuba.ac.jp](mailto:ssakai@md.tsukuba.ac.jp) (S. Sakai), [maeda@taiiku.tsukuba.ac.jp](mailto:maeda@taiiku.tsukuba.ac.jp) (S. Maeda), [t-miyauc@md.tsukuba.ac.jp](mailto:t-miyauc@md.tsukuba.ac.jp) (T. Miyauchi), [mizutani@md.tsukuba.ac.jp](mailto:mizutani@md.tsukuba.ac.jp) (T. Mizutani), [kaonuma@md.tsukuba.ac.jp](mailto:kaonuma@md.tsukuba.ac.jp) (K. Aonuma), [kawano\\_s@md.tsukuba.ac.jp](mailto:kawano_s@md.tsukuba.ac.jp) (S. Kawano).

phospholipase C, PKC, ERK1 and ERK2, c-Fos and c-Jun, c-Jun NH2-terminal kinase (JNK) and p38 (Irukayama-Tomobe et al., 2004; Shimojo et al., 2006).

Recent studies show strong and compelling evidence that consumption of fish-derived fatty acids, such as eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3), provide cardioprotective benefits. Specifically, these fatty acids (EPA and DHA) alter membrane fluidity, interact with transcription factors, such as peroxisomal proliferator-activated receptor (PPAR) and sterol regulatory element binding protein (Krey et al., 1997), and are substrates for enzymes, including cyclooxygenase, lipoxygenase and cytochrome P450 (Cottin et al., 2011). In the late 1990s, EPA, as well as other  $\omega$ -3 polyunsaturated fatty acid (PUFA), was first described as a ligand of PPAR (Krey et al., 1997), which (PPAR) has three isoforms, namely  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$ , and belongs to a superfamily of nuclear hormone receptors of the heart. PPAR is also activated by a diverse group of ligands, including unsaturated fatty acids and isoforms of specific drugs, such as fibrates (PPAR- $\alpha$ ) and anti-diabetic drugs of thiazolidinediones class (PPAR- $\gamma$ ) (Planavila et al., 2005). PPAR- $\alpha$ , the predominant PPAR isoform in the heart, has been implicated in hypertrophic signaling. PPAR- $\alpha$  activator, fenofibrate, interferes with the signaling pathway of ET-1-induced cardiac hypertrophy through negative regulation of activator protein-1 (AP-1) binding activity, partly via inhibition of the JNK pathway in cultured cardiomyocytes (Irukayama-Tomobe et al., 2004). However, it is unclear whether EPA-activated PPAR- $\alpha$  can ameliorate cardiomyocyte hypertrophy.

The present study investigated whether activation of the PPAR- $\alpha$  pathway mediates the suppressive effects of EPA in ET-1-induced hypertrophy of cardiomyocytes.

## Materials and methods

### Myocyte isolation and culture

Ventricular cardiac myocytes were isolated from 2- to 3-day-old Sprague-Dawley rats, as described previously (Shimojo et al., 2006), and were incubated on fibronectin-coated dishes in DMEM-Ham's F-12 medium supplemented with 0.1% fatty acid-free BSA (Sigma, St. Louis, MO). Cells were then cultured for 3 days after differential adhesion and later used for further experiments. Initially, myocytes were only exposed to vehicle prior to treatments (control, in 95% air-5% CO<sub>2</sub>), and at day 4, the cardiomyocytes were treated either with 10  $\mu$ M EPA, 0.1 nM ET-1 or pre-treated with 10  $\mu$ M EPA, followed by 0.1 nM ET-1 on day 5 of culture for 24 h and harvested for analysis. In order to determine the optimal doses of ET-1 and EPA, dose response studies were performed, as described here: 1) *ET doses*: a) 10<sup>-8</sup>, b) 10<sup>-9</sup>, c) 10<sup>-10</sup> and d) 10<sup>-11</sup> M and 2) *EPA doses*: a) 1, b) 3, c) 10, d) 30 and e) 100  $\mu$ M (Shimojo et al., 2006). All animal experiments were carried out in a humane manner and were pre-approved by the local Institutional Animal Experiment Committee of the University of Tsukuba.

### Cardiomyocyte surface area

In order to determine changes in cell size, images of cells were captured using a charge-coupled device camera (Olympus, Tokyo, Japan) and the peripheries of the cells were traced and analyzed on the captured images using the NIH Image software (National Institutes of Health, Bethesda, MD). These values were then doubled to account for the portion of the cell surface in contact with the dish. All cells from randomly selected fields in two or three dishes were examined for each experimental group. A total of 100 cells in each experimental group were examined, as described in our previous study (Shimojo et al., 2006, 2007).

### Protein synthesis

The effects of various treatments on the rate of protein synthesis in cardiomyocytes were investigated, as described previously (Shimojo et al., 2006). Briefly, protein synthesis in cultured neonatal rat ventricular myocytes was evaluated by measuring incorporation of [<sup>14</sup>C] leucine into acid-insoluble cellular material. The cells were plated in 24-well dishes at a density of 10<sup>5</sup> cells/well. After treatment with ET-1 or vehicle for 24 h, 0.1  $\mu$ Ci/ml [<sup>14</sup>C] leucine was added and cells were incubated for 24 h. The cells were washed twice with cold PBS, and 5% TCA was added for 10 min. The cells were then incubated with 0.25% trypsin at 37 °C for 30 min, and cell residues were solubilized in 0.5 N NaOH for 10 min. Aliquots were then counted with a scintillation counter (Beckman LS-6500 scintillation counter; Beckman Coulter, Fullerton, CA).

### Western blot analysis

Cardiomyocytes were plated at a field density of 2  $\times$  10<sup>6</sup> cells/cm<sup>2</sup> on 60-mm culture dishes with 2 ml of culture medium. Cardiomyocytes from the different treatment groups were lysed on ice with buffer (10 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate and 0.1% SDS). The protein concentration of the supernatant was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Samples were run on SDS-PAGE, using 7.5–10% polyacrylamide gel, and electrotransferred to polyvinylidenedifluoride (PVDF) filter membrane. To reduce non-specific binding, the PVDF membrane was blocked for 2 h at room temperature with 5% non-fat milk in PBS containing 0.1% Tween 20 (TPBS). Thereafter, the PVDF membrane was incubated overnight at 4 °C with primary antibodies in TPBS. After washing the membrane three times in TPBS, we incubated it in horseradish peroxidase-conjugated anti-rabbit (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK), anti-mouse (Amersham Biosciences), or anti-goat antibodies (Santa Cruz Biotechnology, Dallas, TX), diluted at 1:2,000–10,000 in TPBS at room temperature for 60 min. The blots were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences), exposed to X-ray film, and analyzed by free NIH Image software produced by Wayne Rasband (National Institutes of Health). Only negligible loading or transfer variations were observed between samples.

Commercial rabbit polyclonal PPAR- $\alpha$  antibody (Santa Cruz Biotechnology) and mouse monoclonal  $\beta$ -actin antibody (Abcam, Cambridge, UK) were used.

### RNA interference

Small interference RNA (siRNA) molecules were designed using Invitrogen's website and transfected into cardiomyocytes using Oligofectamine. A fluorescein isothiocyanate-labeled non-sense siRNA molecule was used as control. Transfected cells were recovered for 24 h and treated with/without EPA as described before. Knockdown in gene expression was identified after isolating the RNA and performing protein blots 48 h after expression of the siRNAs (Fig. 2).  $\beta$ -actin was used as an internal control.

### PPAR- $\alpha$ to DNA binding activity assay

The degree of DNA binding of PPAR- $\alpha$  was determined by an immunosorbent assay (ELISA) utilizing PPAR- $\alpha$  transcription factor assay kits (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. Briefly, a specific double stranded DNA sequence containing the peroxisome proliferator responsive element (PPRE) was immobilized onto the bottom of the wells of the 96-well plates. Aliquots of harvested cells diluted to obtain 50  $\mu$ g protein in each assay were added to each well, so that PPAR contained in the extract bind specifically to the PPRE. PPAR- $\alpha$  was detected by the addition of specific

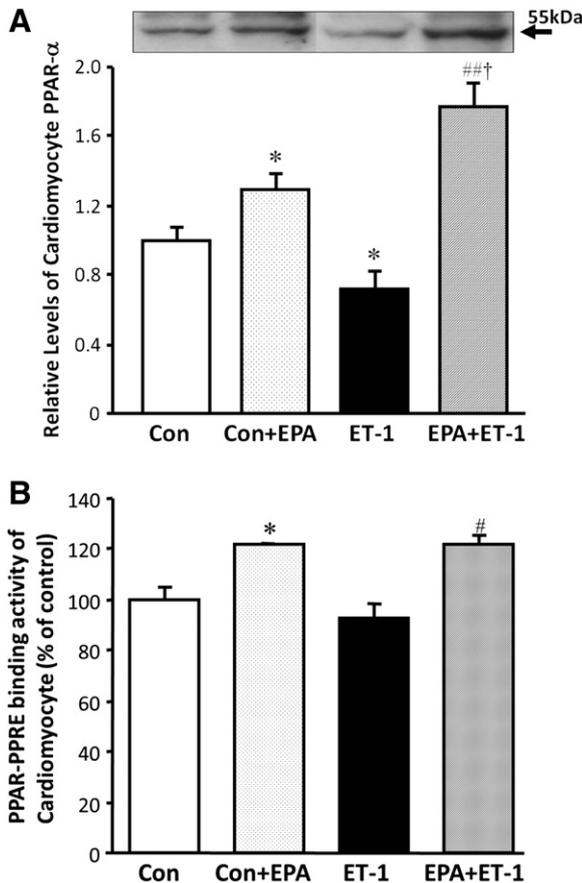
primary antibodies directed against PPAR- $\alpha$ . A secondary antibody conjugated to HRP was added to provide a sensitive colorimetric readout at 450 nm.

**Statistical analysis**

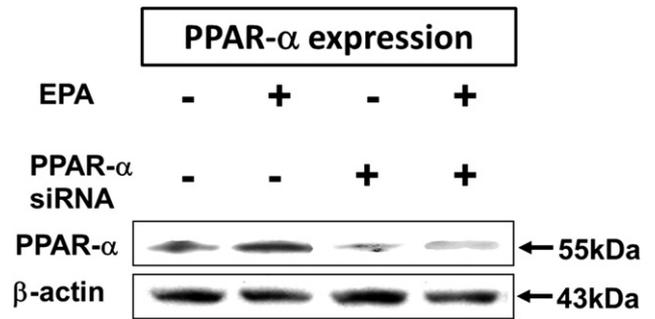
Values for data generated in the present study were expressed as means  $\pm$  SE. Intergroup differences were compared using one-way ANOVA, adjusted by Bonferroni's using the IBM-SPSS version 19.0 software (IBM-SPSS Inc, Chicago, IL). The same total number of sample size for each experimental group was determined as reported in our previous study (Shimojo et al., 2006, 2007). Differences were considered significant at  $p < 0.05$ .

**Results**

In our current experimental design, we determined PPAR- $\alpha$  expression in four experimental groups. Here, we show a differential expression of PPAR- $\alpha$ , based on four different treatments (Fig. 1). Specifically, we show that PPAR- $\alpha$  expression was significantly up regulated in EPA pre-treated groups, as determined by immunoblotting, irrespective of ET-1 administration. Notably, a marked increase of PPAR- $\alpha$  expression was observed in the EPA pre-treated group that was later administered with ET-1 than the group (EPA) without ET-1. Interestingly, the expression of PPAR- $\alpha$  was significantly down-regulated in ET-1-induced hypertrophied cardiomyocytes compared to control (Fig. 1A). Similar types of results were obtained from DNA-binding assay when evaluating



**Fig. 1.** Typical Western blot and intensity of the bands of PPAR- $\alpha$  of control (Con), EPA-treated (Con + EPA) control cells, ET-1-treated (ET-1) only, and EPA-pretreated ET-1-administered (EPA + ET-1) cardiomyocytes (A). In each experiment, Con value is normalized as 1.0. Values are means  $\pm$  SE of 5–7 separate experiments. DNA binding activity of PPAR- $\alpha$  determined by an immunosorbent assay (B) in Con, Con + EPA, ET-1, and EPA + ET-1 cardiomyocytes. Values are means  $\pm$  SE. \* $P < 0.01$  vs. Con; <sup>##</sup> $P < 0.01$  vs. ET-1; <sup>†</sup> $p < 0.05$  vs. Con + EPA.



**Fig. 2.** Representative image of immunoblotting of PPAR- $\alpha$  in cardiomyocyte extracts in presence or absence of EPA or PPAR- $\alpha$  siRNA.  $\beta$ -actin was used as internal control.

PPAR- $\alpha$ 's DNA-binding activity to the PPAR response elements (PPRE), in both EPA pre-treated groups. PPAR- $\alpha$ 's DNA-binding activity to PPRE was enhanced with or without ET-1 administration (Fig. 1B).

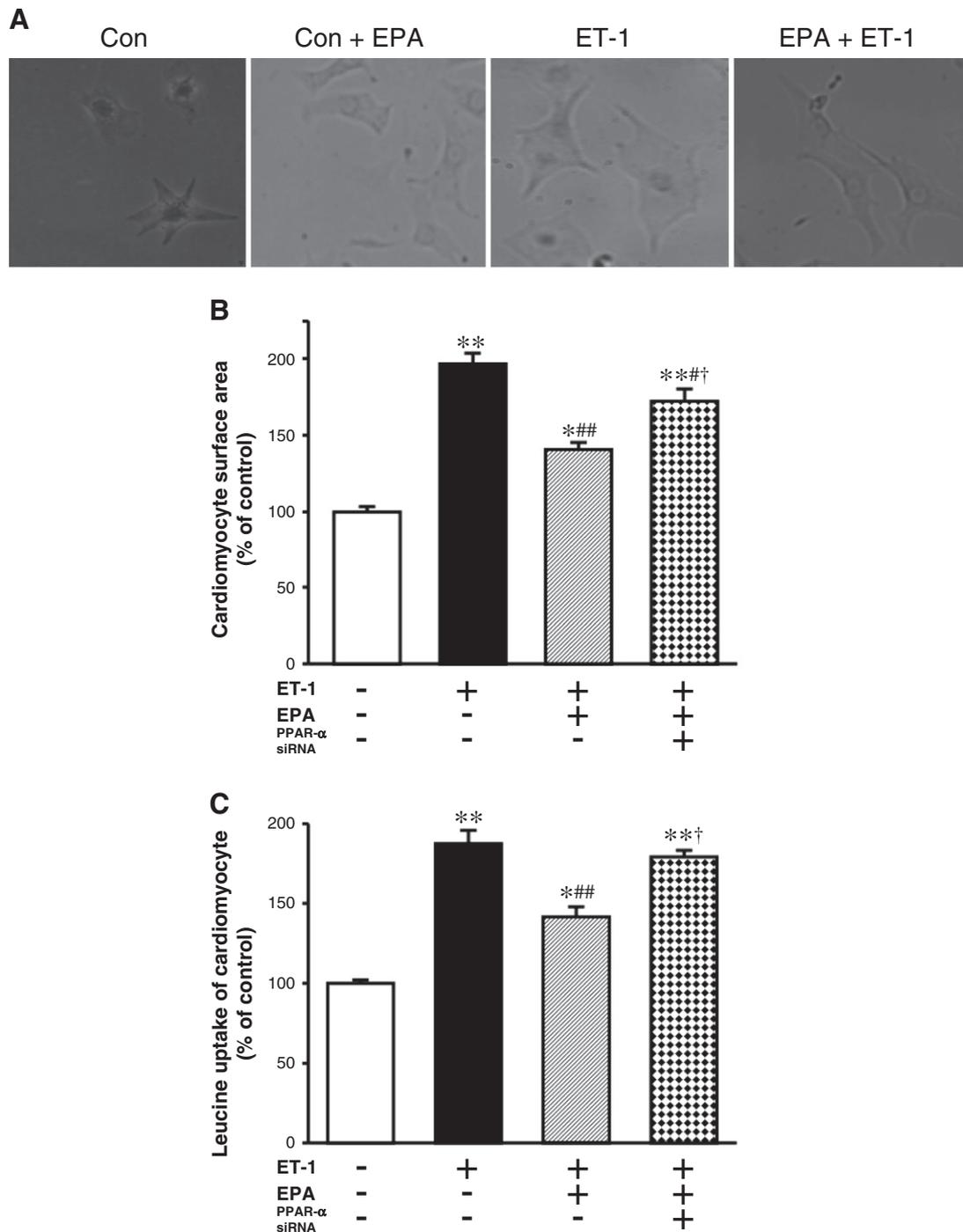
Under the current experimental setting, the expression of PPAR- $\alpha$  was down-regulated by siRNA, with or without EPA administration in neonatal cardiomyocytes. PPAR- $\alpha$  expression in siRNA-transfected cardiomyocyte was almost reduced by 80% compared to the EPA-only treated group (Fig. 2). Thus, the same PPAR- $\alpha$  siRNA was used in subsequent experiments.

Representative photographs of the morphological evaluation of four experimental groups were also obtained (Fig. 3). ET-1 caused significant increase in the cell surface area of cardiomyocyte (2.12 fold;  $p < 0.0001$  vs. control) and this morphological change was greatly ameliorated by pre-treating cells with EPA (45% decrease;  $p < 0.0001$  vs. ET-1) (Fig. 3B). It is important to note that no significant morphological change was observed between the control and EPA-only administered cardiomyocyte groups ( $p = 0.93$ ) (Fig. 3A), based on cell surface area. Next, we examined the effects of PPAR- $\alpha$  siRNA on EPA pre-treated cardiomyocytes that were later administered with ET-1, which induced hypertrophy. Pre-treatment of the EPA + ET-1-administered group with the siRNA of PPAR- $\alpha$  significantly inhibited the EPA-mediated anti-hypertrophic action in ET-1-induced hypertrophied cardiomyocyte (Fig. 3B). Consistent with changes observed in the cell surface area, <sup>14</sup>C-leucine incorporation was markedly increased in ET-1-administered groups, and these increases were resolved by EPA pre-treatment (Fig. 3C). Pre-treatment of EPA + ET-1-administered hypertrophied cardiomyocytes with PPAR- $\alpha$  siRNA has significantly attenuated the regression of cardiomyocyte hypertrophy exerted by EPA treatment, as observed in <sup>14</sup>C-leucine incorporation (Fig. 3C).

**Discussion**

The novelty of the present study is the demonstration that ET-1-induced hypertrophic remodeling in rat neonatal cardiomyocyte is effectively prevented by the pre-treatment of cells by EPA and that the anti-hypertrophic effects of EPA are likely mediated by PPAR- $\alpha$  activation. EPA treatment induced both the expression and DNA binding activity of PPAR- $\alpha$  in ET-1 administered hypertrophied cardiomyocytes. Furthermore, treatment with PPAR- $\alpha$  siRNA significantly attenuated the amelioration exerted by EPA on ET-1-induced cardiomyocyte hypertrophy.

In the present study, we found that ET-1 induced significant hypertrophy in neonatal rat cardiomyocytes. There was significant increase in cell size, cell surface area, as well as increased protein synthesis rate in ET-1 administered cardiomyocytes compared to control cardiomyocyte. The most potential finding in the present study is that pre-treatment of cells with EPA regresses ET-1-induced cardiomyocyte hypertrophy through the activation of PPAR- $\alpha$ . These current findings are consistent with our previous report that showed that ET-1 can induce significant hypertrophic changes in cardiomyocytes by up-regulating levels of key hypertrophic markers (ANP and BNP)



**Fig. 3.** Representative charge-coupled device camera images (A) of control (Con), EPA-treated (Con + EPA) control cells, ET-1-treated (ET-1) only, and EPA-pretreated ET-1-administered (EPA + ET-1) cardiomyocytes. Magnification  $\times 400$ . Effect of pre-treatment of PPAR- $\alpha$  si-RNA on EPA treated ET-1 administered cell surface area (B) and [ $^3$ H]leucine uptake (C) in rat ventricular cardiomyocytes. si-RNA transfected cells were recovered for 24 h and treated with/without EPA and ET-1. Data are obtained from control (untreated) cardiomyocytes are normalized as 100%. Data are means  $\pm$  SE; n = 100 (B) and n = 10 (C). \*p < 0.05 vs. Con; \*\*p < 0.001 vs. Con; #p < 0.05 vs. ET-1; ###p < 0.001 vs. ET-1; †p < 0.05 vs. ET-1 + EPA.

(Shimojo et al., 2006). Further, we show here that such remodeling could be effectively prevented by pre-treating cells with EPA. EPA exerted its effects in that study by suppressing phosphorylated JNK, and c-Jun (Shimojo et al., 2006). Phosphorylated JNK activates the transcription factor, AP-1, which could increase ET-1 gene expression in cardiomyocyte (Kudoh et al., 1997; Irukayama-Tomobe et al., 2004). Such a positive feedback system of phosphorylated JNK, AP-1 and ET-1 may play crucial roles in the development of cardiomyocyte

hypertrophy (Fujisaki et al., 1995). On the other hand, PPARs have been suggested to interfere negatively with levels of AP-1, Stat and NF- $\kappa$ B signaling pathways by competing for essential co-factors (Delerive et al., 1999). Therefore, activated PPAR- $\alpha$  is thought to bind to AP-1 or its cofactor and that AP-1 is prevented from binding to the cis-elements of the promoter region, which results in impairment of ET-1 gene induction. Thus, one possible mechanism of the inhibitory action of PPAR- $\alpha$  activation on ET-1-induced cardiac hypertrophy may

be through interference of AP-1 binding to the ET-1 gene promoter (Irukayama-Tomobe et al., 2004; Takemoto et al., 1999; Ogata et al., 2002). Collectively, these facts led us to speculate that EPA may interfere in the ET-1 positive feedback system as stated above via EPA-PPAR- $\alpha$ -AP-1 pathway.

Recent reports may shed light on the mechanism underlying the role of activated PPAR- $\alpha$  in the suppression of ET-1-induced cardiomyocyte hypertrophy. Activation of PPAR- $\alpha$  prevents ET-1 induced-cardiac hypertrophy in neonatal rat cardiomyocytes through regulating PI3K/Akt/glycogen synthase kinase (GSK)3 $\beta$  and nuclear factor of activated T cells (NFATc4) signaling pathway (Li et al., 2007). Le et al. also suggest that activated PPAR- $\alpha$  can compete with GATA-4 binding to NFATc4, thereby decreasing trans-activation of NFATc4, and interfering with ET-1-induced cardiomyocyte hypertrophy (Le et al., 2012). On the other hand, in piglet cerebral microvascular endothelial culture, it was demonstrated that PPAR- $\alpha$  activation attenuated ET-1 production by agents that mediate brain injury. The likely mechanism for such a process could probably be mediated by the PPAR- $\alpha$ -induced increase in eNOS expression/NO production and the complex PKC signaling pathways (Yakubu et al., 2007), thus suggesting some potential beneficial effects in using PPAR- $\alpha$  agonists in addressing cardiovascular remodeling.

ET-1 also induces NF- $\kappa$ B via two independent cascades (via ET<sub>A</sub> receptor, or via diacylglycerol) in human renal tubular epithelial cells (Gerstung et al., 2007), and NF- $\kappa$ B represents a key factor that mediates both neuro-hormonal and pro-inflammatory signals, leading finally to hypertrophic and/or fibrotic processes in the cardiovascular system (Takemoto et al., 1999; Pechanova and Simko, 2010). Thus, ET-1 also induced cardiomyocyte hypertrophy via NF- $\kappa$ B-mediated pathways. Further, myocardial fibrosis and diastolic dysfunction in deoxycorticosterone acetate-salt hypertensive rats is ameliorated by PPAR- $\alpha$  activator fenofibrate, partly by suppressing inflammatory responses associated with the nuclear NF- $\kappa$ B pathway (Ogata et al., 2004). Thus, EPA may also prevent ET-1-induced hypertrophy via ET-1-NF- $\kappa$ B pathway in rat neonatal cardiomyocytes.

We also found here that EPA treatment, in both control and ET-1-administered cardiomyocytes, enhanced the expression of PPAR- $\alpha$  compared to either control group or ET-1 administered group only. But the extent of PPAR- $\alpha$  up regulation is greater in ET-1-administered cells than the control group after EPA treatment. In addition, PPAR- $\alpha$  expression is significantly diminished in ET-1 administered cardiomyocyte hypertrophy group compared to the control cardiomyocytes. Based on these results, we speculate that pre-treatment of cells in the control group by EPA induced a more pronounced increase of PPAR- $\alpha$  than that of ET-1-administered hypertrophied cardiomyocytes. This unexpected effect of EPA pre-treatment regarding the expression of PPAR- $\alpha$  cannot be explained in the present study. Cardiac energy metabolic shifts occur as a normal response to diverse physiologic and dietary conditions and as a component of the pathophysiological processes that accompany cardiac hypertrophy, heart failure and myocardial ischemia. The capacity to produce energy via the utilization of fats by the mammalian postnatal heart is controlled in part at the level of nuclear gene encoding enzymes that are involved in mitochondrial fatty acid  $\beta$ -oxidation, including PPAR- $\alpha$  (Barger and Kelly, 2000). In the present study, the decrease of PPAR- $\alpha$  expression in ET-1-administered cardiomyocyte group implies that ET-1 treatment caused the myocytes to turn from aerobic to anaerobic metabolism because of mitochondrial dysfunction. ET-1 is one of signal-transducer-effector involved in the subsequent induction of oxidative stress originating within its mitochondria that dictates the opening of the mitochondrial permeability transition pore (Shahbaz et al., 2010). The exaggerated expression of PPAR- $\alpha$  in EPA plus ET-1 group may indicate the presence of a physiological compensatory response under the current experimental setting. Indeed, ET-1 activates not only vaso-constrictive, pro-inflammatory and proliferative factors in the cardiomyocytes, but also cardioprotective factors, including ANP and BNP (Fujisaki et al., 1995). Liang et al. suggest that PPAR- $\alpha$

ligands could prove to be useful in the management of disorders associated with hypertrophy and remodeling of the myocardium via regulating BNP transcription activity (Liang et al., 2003). It is possible that such a compensatory system, working together with PPAR- $\alpha$ , may take place in the cardiomyocytes of ET-1-induced hypertrophy that are pre-treated with EPA. However, future studies are needed to adequately address this issue.

Here, we also found that the reversing effects of PPAR- $\alpha$  siRNA on EPA-induced reduction of cardiomyocyte size (surface area) are only partial (approximately 50%) (Fig. 3B). Such findings are comparable to EPA's action on ET-1-induced increase in cell size. These outcomes indicate that such a regulation may involve other ET-1-dependent mechanisms, consistent with our earlier comments expressing our surprise on data showing that EPA solely stimulates PPAR- $\alpha$ , an effect blocked by ET1 (Fig. 1), whereas both potentiate expression of PPAR- $\alpha$ . Previously, we have also shown that that EPA inhibits the important hypertrophic pathway involving TGF- $\beta$ 1, JNK and c-Jun in ET-1-induced hypertrophied cardiomyocytes (Shimojo et al., 2006). Kitamura et al showed that treatment with EPA attenuated atrial fibrosis, ERK phosphorylation, and TGF- $\beta$ 1 expression of atrium in a rabbit model of atrial fibrillation (Kitamura et al., 2011). Furthermore, rats fed the 5% n-3 PUFA, including EPA diet had lower enterocytic phosphorylated JNK protein and secreted less cholesterol into the mesenteric lymph compared with the control (Lu et al., 2011). In addition, the work by Fujisaki (Fujisaki et al., 1995), who demonstrated an ET1-ETA mediated autocrine loop (positive feedback) maintaining cell growth in cardiac fibroblasts, may also explain for the partial attenuation of EPA exerted antihypertrophic effect by PPAR- $\alpha$  siRNA on ET-1 induced hypertrophied cardiomyocytes. Collectively, these data imply the existence of other mechanisms of anti-hypertrophic effects of EPA on ET-1-induced hypertrophied cardiomyocyte.

There are some obvious limitations in this study besides the limitations already stated above. First, the present study only utilized the cardiomyocyte culture. Future studies should also use non-cardiomyocytes (fibroblast, endothelial cell, smooth muscle cell etc.), either alone (pure) or co-cultured. It is likely that different cells, alone or co-cultured may generate findings that are different from the current study. In addition, there is also a need to replicate the current experiment in vivo.

## Conclusion

In conclusion, the present study shows that ET-1 induces significant cardiomyocyte hypertrophy, and that pretreatment with EPA attenuated these remodeling events by up regulating levels of PPAR- $\alpha$  pathway.

## Conflict of interest

None.

## Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research B and C from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20462210, 22390334, 23592025, 23406037, 23406016, 23406029, 24406026, 25305034) and Japan Society for the Promotion of Science.

## References

- Barger PM, Kelly DP. PPAR signaling in the control of cardiac energy metabolism. *Trends Cardiovasc Med* 2000;10:238–45.
- Cottin SC, Sanders TA, Hall WL. The differential effects of EPA and DHA on cardiovascular risk factors. *Proc Nutr Soc* 2011;70:215–31.
- Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, et al. Peroxisome proliferator-activated receptor  $\alpha$  negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF- $\kappa$ B and AP-1. *J Biol Chem* 1999;274:32048–54.

- Fujisaki H, Ito H, Hirata Y, Tanaka M, Hata M, Lin M, et al. Natriuretic peptides inhibit angiotensin II-induced proliferation of rat cardiac fibroblasts by blocking endothelin-1 gene expression. *J Clin Invest* 1995;96:1059–65.
- Gerstung M, Roth T, Dienes HP, Licht C, Fries JW. Endothelin-1 induces NF-kappaB via two independent pathways in human renal tubular epithelial cells. *Am J Nephrol* 2007;27:294–300.
- Giannessi D, Del Ry S, Vitale RL. The role of endothelins and their receptors in heart failure. *Pharmacol Res* 2001;43:111–26.
- Ho YL, Wu CC, Lin LC, Huang CH, Chen WJ. Assessment of the coronary artery disease and systolic dysfunction in hypertensive patients with the dobutamine-atropine stress echocardiography effect of the left ventricular hypertrophy. *Cardiology* 1998;95:1592–600.
- Irukayama-Tomobe Y, Miyauchi T, Sakai S, Kasuya Y, Ogata T, Takanashi M, et al. Endothelin-1-induced cardiac hypertrophy is inhibited by activation of peroxisome proliferator-activated receptor-alpha partly via blockade of c-Jun NH2-terminal kinase pathway. *Circulation* 2004;109:904–10.
- Kitamura K, Shibata R, Tsuji Y, Shimano M, Inden Y, Murohara T. Eicosapentaenoic acid prevents atrial fibrillation associated with heart failure in a rabbit model. *Am J Physiol Heart Circ Physiol* 2011;300:H1814–21.
- Krey G, Braissant O, L'Horsset F, Kalkhoven E, Perroud M, Parker MG, et al. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Mol Endocrinol* 1997;11:779–91.
- Kudoh S, Komuro I, Mizuno T, Yamazaki T, Zou Y, Shiojima I, et al. Angiotensin II stimulates c-Jun NH2-terminal kinase in cultured cardiac myocytes of neonatal rats. *Circ Res* 1997;80:139–46.
- Le K, Li R, Xu S, Wu X, Huang H, Bao Y, et al. PPAR $\alpha$  activation inhibits endothelin-1-induced cardiomyocyte hypertrophy by prevention of NFATc4 binding to GATA-4. *Arch Biochem Biophys* 2012;518:71–8.
- Li R, Zheng W, Pi R, Gao J, Zhang H, Wang P, et al. Activation of peroxisome proliferator-activated receptor-alpha prevents glycogen synthase 3beta phosphorylation and inhibits cardiac hypertrophy. *FEBS Lett* 2007;581:3311–6.
- Liang F, Wang F, Zhang S, Gardner DG. Peroxisome proliferator activated receptor (PPAR) alpha agonists inhibit hypertrophy of neonatal rat cardiac myocytes. *Endocrinology* 2003;144:4187–94.
- Lu J, Borthwick F, Hassanali Z, Wang Y, Mangat R, Ruth M, et al. Chronic dietary n-3 PUFA intervention improves dyslipidaemia and subsequent cardiovascular complications in the JCR:LA-cp rat model of the metabolic syndrome. *Br J Nutr* 2011;105:1572–82.
- Ogata T, Miyauchi T, Sakai S, Irukayama-Tomobe Y, Goto K, Yamaguchi I. Stimulation of peroxisome-proliferator-activated receptor alpha (PPAR alpha) attenuates cardiac fibrosis and endothelin-1 production in pressure-overloaded rat hearts. *Clin Sci (Lond)* 2002;103(Suppl. 48):284S–8S.
- Ogata T, Miyauchi T, Sakai S, Takanashi M, Irukayama-Tomobe Y, Yamaguchi I. Myocardial fibrosis and diastolic dysfunction in deoxycorticosterone acetate-salt hypertensive rats is ameliorated by the peroxisome proliferator-activated receptor-alpha activator fenofibrate, partly by suppressing inflammatory responses associated with the nuclear factor-kappa-B pathway. *J Am Coll Cardiol* 2004;43:1481–8.
- Pechanova O, Simko F. The role of nuclear factor kappa B and nitric oxide interaction in heart remodelling. *J Hypertens* 2010;28(Suppl. 1):S39–44.
- Planavila A, Laguna JC, Vázquez-Carrera M. Atorvastatin improves peroxisome proliferator-activated receptor signaling in cardiac hypertrophy by preventing nuclear factor- $\kappa$ B activation. *Biochim Biophys Acta* 2005;1687:76–83.
- Rohini A, Agrawal N, Koyani CN, Singh R. Molecular targets and regulators of cardiac hypertrophy. *Pharmacol Res* 2010;61:269–80.
- Sadoshima J, Takahashi T, Jahn L, Izumo S. Roles of mechanosensitive ion channels, cytoskeleton and contractile activity in stretch-induced immediate early gene expression and hypertrophy of cardiac myocytes. *Proc Natl Acad Sci U S A* 1992;9:9905–9.
- Shahbaz AU, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC, McGee JE, et al. Fibrosis in hypertensive heart disease: molecular pathways and cardioprotective strategies. *J Hypertens* 2010;28(Suppl. 1):S25–32.
- Shimojo N, Jesmin S, Zaedi S, Maeda S, Soma M, Aonuma K, et al. Eicosapentaenoic acid prevents endothelin-1-induced cardiomyocyte hypertrophy in vitro through the suppression of TGF-beta 1 and phosphorylated JNK. *Am J Physiol Heart Circ Physiol* 2006;291:H835–45.
- Shimojo N, Jesmin S, Zaedi S, Otsuki T, Maeda S, Yamaguchi N, et al. Contributory role of VEGF overexpression in endothelin-1-induced cardiomyocyte hypertrophy. *Am J Physiol Heart Circ Physiol* 2007;293:H474–81.
- Takemoto Y, Yoshiyama M, Takeuchi K, Omura T, Komatsu R, Izumi Y, et al. AP-1 and NF-kappa B DNA binding activities in isoproterenol-induced cardiac remodeling. *J Mol Cell Cardiol* 1999;31:2017–30.
- Yakubu MA, Nsaif RH, Oyekan AO. Peroxisome proliferator-activated receptor alpha activation-mediated regulation of endothelin-1 production via nitric oxide and protein kinase C signaling pathways in piglet cerebral microvascular endothelial cell culture. *J Pharmacol Exp Ther* 2007;320:774–81.
- Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Hiroi Y, et al. Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J Biol Chem* 1996;271:3221–8.
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411–5.