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Citation	Placenta, 75, 34-41 https://doi.org/10.1016/j.placenta.2018.11.010
Issue Date	2019-01
Doc URL	https://hdl.handle.net/2115/76644
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	PLAC_2018_197_Revision 1_V0.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Highlights:

- PUFAs (AA, EPA, DHA) increased xCT/*SLC7A11* expression and function in BeWo cells
- NRF2 expression contributed to HO-1/*HMOX1* induction by PUFAs but not to xCT/*SLC7A11*
- xCT/*Slc7a11* was detected in rat placenta at the mRNA level
- xCT/*Slc7a11* expression at gestational day (GD) 12 was higher than that at GD 20

Analysis of the effects of polyunsaturated fatty acids on transporter expressions using a PCR array: induction of xCT/SLC7A11 in human placental BeWo cells

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Abbreviations: AA, arachidonic acid; DHA, docosahexanoeic acid ; EPA, eicosapentaenoic acid; GD, gestational day; HO-1, heme oxygenase-1; NRF2, Nuclear factor-erythroid 2-related factor-2; PPAR: peroxisome proliferator-activated receptor, PUFA, polyunsaturated fatty acid

Abstract

Objective

Polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), are essential for adequate fetal growth. The aim of the present study was to elucidate the effects of PUFAs on the expression and function of placental transporters, which play important roles in placental functions including the supply of nutrients to the fetus, excretion of metabolites, and protection of the fetus from xenobiotics.

Methods

Human placental choriocarcinoma BeWo cells were used as a trophoblast model. PUFA-induced alteration in the gene expression of 84 transporters was investigated by a commercially available PCR array. Protein levels and the activity of transporters were assessed by western blotting and uptake experiments, respectively. The placental expression of the transporters was analyzed using pregnant Wistar rats.

Results

PUFAs (AA, EPA, and DHA) increased cystine/glutamate transporter xCT/*SLC7A11*, which mediates the cellular uptake of cystine coupled with the efflux of glutamate in human placental choriocarcinoma BeWo cells. These PUFAs also increased [^{14}C]-cystine uptake in BeWo cells. PUFA-induced

xCT/*SLC7A11* mRNA expression was not blocked by nuclear factor-erythroid 2-related factor-2 (NRF2) knockdown. Reverse transcription (RT)-PCR analysis indicated that xCT/*Slc7a11* mRNA was detected in rat placenta and the expression level at gestational day (GD) 12 was higher than that at GD 20.

Conclusion

These results indicate that PUFAs promoted cystine uptake in placental cells by inducing xCT/*SLC7A11* expression and NRF2 did not contribute to upregulation of xCT/*SLC7A11* by PUFAs. Furthermore, xCT expression in rat placenta may change during pregnancy.

Keywords: arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), placenta, transporter, xCT/*SLC7A11*

1. Introduction

Nutrition during pregnancy is important for maternal health, pregnancy outcomes, and fetal growth [1]. It has been suggested that maternal nutritional factors also influence offspring's risk of metabolic and chronic diseases in later life [2].

Polyunsaturated fatty acids (PUFAs), including omega-6 (n-6) arachidonic acid (AA), omega-3 (n-3) eicosapentaenoic acid (EPA), and n-3 docosahexaenoic acid (DHA), are essential for fetal growth and development [3]. Recently, the associations between PUFAs and several obstetric disorders have been reported. Meher *et al*. reported that placental DHA levels were lower in mothers delivering low birth weight offspring [4]. Assumpção *et al*. showed that erythrocytes from mothers and fetuses with intrauterine growth restriction (IUGR) exhibited lower levels of AA and DHA [5]. Furthermore, the authors suggested that the reduced levels of AA and DHA upregulate fatty acid transporters in placenta. Wadhwani *et al*. demonstrated that mothers with preeclampsia at weeks 16 through 20 of gestation have lower fatty acid stores and lower placental synthesis and transport thereof, proposing the possible benefit of supplementation of n-3 PUFAs [6]. In mothers with gestational diabetes, impairment of DHA transfer to the fetus and alterations in DHA metabolism compared to controls have been reported [7]. Calabuig-Navarro *et al*. reported that supplementation of obese and overweight women with n-3 PUFAs during mid- to late pregnancy reduced placental lipid accumulation [8].

The placenta plays an important role in fetal development and an overall healthy pregnancy.

This organ has a variety of functions, such as providing nutrients to fetus, waste excretion, gas exchange, hormone secretion, etc. The critical functions involving the exchange of substances between mother and fetus are supported by several membrane transporters in placental trophoblasts [9]. Many transporters have been identified and analyzed in various organs; they are classified as ATP-binding cassettes (ABC) and solute carriers (SLC). ABC and SLC transporters are also expressed in the placenta, where they are involved in the biological processes and intrauterine environment of the fetus [10-11]. Furthermore, the expression of several transporters in placenta has been found to vary at each stage of gestation [12]. The activity and expression of placental transporters are influenced by factors such as hormones, cytokines, and nutrients [13]. A more thorough understanding of the factors affecting the expression and function of placental transporters at each stage of gestation is important in the development of strategies to improve pregnancy outcomes.

It has been reported that fatty acids affect the expression levels of several genes, including those involving transport in various cells [14-17]. Alvaro *et al*. have reported that n-3 PUFAs (EPA and DHA) downregulate Niemann-Pick C1-Like 1 (NPC1L1) and ATP-binding cassette transporter A1 (ABCA1) in the human colon cancer Caco-2 cell line [16]. Furthermore, Kuan *et al*. have reported that n-3 and n-6 PUFAs reduced P-glycoprotein (P-gp) expression in Caco-2 cells. [17].

Despite the importance of PUFAs during pregnancy, associations between placental transporter function and maternal intake of PUFAs have not been fully elucidated. Therefore, the purpose of the present study was to elucidate the effects of PUFAs on the expression and function of

placental transporters. Since several transporters exist in the placenta as mentioned above, it is important to comprehensively investigate PUFA-induced alterations in transporters. For this purpose, we employed a commercially available PCR array kit (RT^2 Profiler™ PCR Array) containing primers for a set of 84 primary drug transporter genes in a 96-well plate format.

2. Materials and methods

2.1. Chemicals

AA, EPA, and DHA (solutions in ethanol) were purchased from Cayman Chemical. (Ann Arbor, MI, USA). Fatty acid-free bovine serum albumin (BSA) was purchased from Wako (Tokyo, Japan).

2.2. Cell culture

Human placental choriocarcinoma BeWo cells were cultured as previously described [18].

2.3. Treatment of BeWo cells with PUFA

Three days after the BeWo cells were seeded on plastic plates, they were used for experiments. Once the culture medium was removed, cells were washed with 1 mL of fetal bovine serum (FBS)-free medium. After washing the cells, 100 μM of PUFA (AA, EPA, or DHA) was added to the cells. PUFAs were diluted with 1 % fatty acid-free BSA in FBS-free Ham's F-12K (Kaighn's) medium (final ethanol concentration: 0.1 %). The same concentration of fatty acid-free BSA and ethanol was used as a control. After treatment, the cells were used for further experiments as described below.

2.4. MTT assay

Cell viability was assessed by the MTT assay as described previously [19].

2.5. PCR array

Alterations in mRNA expression by PUFAs in BeWo cells were assessed using the RT^2 Profiler PCR Array Kit (Qiagen, Hilden, Germany). This commercial array includes 84 preselected genes involved in drug transport (PAHS-070Z). Cells were treated with AA or EPA (100 μM) for 24 hours as described in 2.3. Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen). The A260/A280 and A260/A230 ratios for RNA isolated from BeWo cells were 2.06 ± 0.01 and 2.21 ± 0.04 (mean ± S.D.), respectively. Single-stranded (ss) cDNA was synthesized from 0.5 μg of total RNA using an RT^2 First Strand Kit (Qiagen) following the manufacturer's protocols. Analysis was carried out using an Mx3000^{TM} Real-Time PCR System (Stratagene, San Diego, CA, USA) following the manufacture's protocols. Fold changes were calculated using the ΔΔCt method with the software provided by the manufacturer (Qiagen). Ct values > 35 were considered as no expression. The five housekeeping genes (*β2M*, *GAPDH*, *HPRT1*, *RPL13A*, and *ACTB*) included in the array were used as reference genes. All five housekeeping genes and their average Ct values did not greatly change by the treatment with PUFAs (data not shown). In accordance with the manufacturer's instructions, the average Ct value of all housekeeping genes was used for normalization.

2.6. Quantitative reverse transcription PCR

Total RNA was extracted using an RNeasy Mini Kit and cDNA was prepared from 1 μg of total RNA by reverse transcription using ReverTra Ace® (Toyobo Co., Ltd., Osaka, Japan). Quantitative PCR was performed using an Mx3000™ Real-Time PCR system with a KAPA SYBR® Fast qPCR kit (Kapa Biosystems, Wilmington, MA). The cycling conditions were as follows: 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 15 seconds (except for HO-1); 40 cycles of 95 °C for 3 seconds and 60 °C for 20 seconds for HO-1. The relative mRNA levels of the target genes were normalized to β-actin.

2.7. Western blotting

BeWo cells were lysed in a lysis buffer supplemented with cOmplete™ Mini protease inhibitor cocktail tablets (MilliporeSigma, Burlington, MA, USA) and 1 mM PMSF. The lysate was kept for five minutes on ice and sonicated briefly at 4 °C. Then it was centrifuged at 14 000 × g for ten minutes at 4 °C, and the resulting clear supernatant was collected. The protein concentration was determined using a Pierce® bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The cell lysates were denatured at 100 °C for three minutes using blue loading buffer (Cell Signaling Technology, Beverly, MA, USA). Proteins (20 μg protein/well) were subjected to SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, USA). The membranes were blocked with TBS containing 0.05 %

Tween 20 (TBS/T) and 5 % non-fat dry milk for one hour at room temperature. After being washed with TBS/T, the membranes were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: rabbit anti-xCT monoclonal antibody (1:500, Cell Signaling Technology, #12691) and mouse anti-actin monoclonal antibody (1:500, Clone C4/MAB1501, Chemicon, Temecula, CA, USA). The bands were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (anti rabbit/mouse immunoglobulin G (1:4000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized with ECL™ Western Blotting Detection Reagents (GE Healthcare Life Science, Pittsburgh, PA, USA). Band intensities were analyzed using ImageJ analysis software (NIH, Bethesda, MD, USA).

2.8. Uptake experiment

For the uptake experiment, BeWo cells were seeded onto 24-well collagen-coated plastic plates. Once the culture medium was removed, cells were washed with Na^+-free transport buffer and pre-incubated at 37 °C with 0.5 mL of the transport buffer. The buffer consisted of 1.26 mM calcium chloride, 0.49 mM magnesium chloride, 0.41 mM magnesium sulfate, 5.3 mM potassium chloride, 0.44 mM potassium phosphate monobasic, 137 mM N-methyl-D-glucamine chloride, 0.34 mM dipotassium hydrogen phosphate, 5.6 mM D-glucose, and 10 mM HEPES, adjusted to a pH of 7.4. The cells were incubated for ten minutes at 37 °C with the transport buffer containing 0.1 μCi/mL [^{14}C]-cystine. After incubation, the buffer was aspirated, and the cells were immediately rinsed twice with

ice-cold transport buffer. To measure the radioactivity of the [^{14}C]-cystine taken up by the cells, the cells were solubilized in 1 % SDS/0.2 N NaOH. The samples were mixed with 3 mL of a scintillation cocktail to measure the radioactivity using a liquid scintillation counter. The amount taken up by the cells was normalized to the cell protein. The protein concentration was determined by the BCA assay.

2.9. Nuclear factor-erythroid 2-related factor-2 (NRF2) knockdown by small interfering RNA (siRNA)

NRF2 siRNA (Oligo ID: HSS107130) and negative control (NC) (Stealth™ RNAi Negative Control medium GC Duplex) were purchased from Thermo Fisher Scientific. The siRNA-mediated knockdown of NRF2 expression was performed using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA). BeWo cells were transfected with the siRNA (reverse-transfection) at a final concentration of 10 nM according to the manufacturer's instructions. The cells were incubated for 72 hours at 37 °C under 5% CO_2. The culture medium was aspirated after siRNA treatment and the cells were further treated with PUFAs for 24 hours as described in 2.3.

2.10. RT-PCR analysis in placental rat samples

Timed-pregnant female Wistar rats (12–13 week old) were obtained from CLEA Japan (Tokyo, Japan). The rats were housed at 23 ± 2 °C and 60 ± 10 % relative humidity, with a 12-hour light/dark cycle. Rats were allowed free access to food and water. The experimental protocols were

reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals." Total RNA was prepared from rat whole placenta using ISOGEN II (Nippon Gene, Tokyo, Japan) and an RNase-Free DNase Set (Qiagen). cDNA was prepared from 1 μg of total RNA by reverse transcription using ReverTra Ace® (Toyobo). PCR was performed using HotStar Taq DNA polymerase (Qiagen) and specific primers under the following cycling conditions: 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 10 seconds. The primer sequences are shown in Table 1. The PCR products were subjected to electrophoresis on a 2 % agarose gel and then visualized by ethidium bromide staining.

2.11. Statistical analysis

Experiments were repeated at least three times. Data are presented as mean ± standard deviation (SD) of independent experiments. A Student's t-test was used to determine the significance of the differences between the means of two groups. Statistical significance among means of more than two groups was evaluated using a one-way analysis of variance (ANOVA) followed by Holm's test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of PUFAs on cell viability and the expression of adipose differentiation-related protein in BeWo cells

Cell viability after treatment with PUFAs was assessed with the MTT assay. Treatment with 100 μM of AA, EPA, or DHA for 24 hours did not affect the viability of BeWo cells (Figure 1A). It has been reported that adipose differentiation-related protein (ADRP/*PLIN2*) expression is induced by PUFAs [20, 21]. We confirmed the effects of PUFAs on ADRP mRNA expression under our experimental conditions. In accordance with the previous reports, the expression of ADRP mRNA was induced by AA, EPA, and DHA to 191 %, 169 %, and 174 % of the control value, respectively (Figure 1B).

3.2. PCR array analysis of the effects of PUFAs on the expression of transporters

In the present study, the expression levels of 84 transporters in BeWo cells were comprehensively assessed by a PCR array kit. The distribution of Ct values of BeWo cells without treatment with PUFAs (control) is shown in Figure 2A. Approximately 70 % of the transporter genes in the PCR array kit were detected in BeWo cells. Twenty percent of genes were highly expressed (Ct value < 25), 28 % were intermediate (Ct value = 25-30), and 23 % of genes exhibited low expression levels (Ct value = 30-35). BeWo cells to 180 %, 209 %, and 250 %. Twenty-nine percent of genes were deemed absent calls (Ct value > 35) in BeWo cells. Detailed data are shown in supplemental

table 1. Figure 2B and 2C show scatter plots of the expression level of each gene in the control sample versus the AA-treated (B) or EPA-treated sample (C). A standard three-fold change in expression was used as a cut-off. *SLC7A11* expression was significantly increased by both AA (fold change: 5.03, p = 0.043) and EPA (fold change: 6.03, p = 0.002). Although *ABCC4* expression was increased by EPA treatment, the alteration was not statistically significant.

3.3. Alterations in expression and function of xCT/*SLC7A11* by PUFA

Subsequently, changes in *SLC7A11* mRNA by PUFAs in BeWo cells were validated by RT-qPCR. Expression levels of *SLC7A11* were induced by AA and EPA to 414 % and 636 % of the control value, respectively (Figure 3A). Furthermore, treatment with DHA also increased *SLC7A11* to 294 % of the control value in BeWo cells. *SLC7A11* encodes cystine /glutamate transporter xCT protein [22]. Western blot analysis showed that PUFAs (AA, EPA, and DHA) increased xCT protein levels to 209 %, 222 %, and 219 % of the control, respectively (Figure 3B). xCT/*SLC7A11* Na^+-independently mediates cystine uptake into cells coupled with the efflux of glutamate [22]. Consequently, the function of xCT/*SLC7A11* was assessed by [^{14}C]-cystine transport activity under Na^+-free conditions. In accordance with the induction of xCT protein levels, AA, EPA, and DHA significantly increased [^{14}C]-cystine uptake into BeWo cells to 180 %, 209 %, and 250 % of the control, respectively (Figure 3C).

3.4. NRF2/*NFE2L2* knockdown did not affect the alterations of xCT/*SLC7A11* by PUFA

It has been reported that the transcriptional factor NRF2/*NFE2L2* is involved in xCT/*SLC7A11* expression in several cell lines [23]. Therefore, we investigated the involvement of NRF2 in alterations of xCT/*SLC7A11* by PUFA in BeWo cells using siRNA. Treatment of siRNA targeted to NRF2/*NFE2L2* for 72 hours markedly decreased NRF2 mRNA expression to approximately 20% of the negative control level (Figure 4A). Heme oxygenase-1 (HO-1/*HMOX1*), another target gene of NRF2, was induced by PUFAs (especially AA and DHA) under negative control siRNA conditions (Figure 4B). NRF2 siRNA treatment attenuated HO-1 induction by these PUFAs. On the contrary, NRF2 siRNA treatment did not attenuate xCT/*SLC7A11* induction by PUFAs (AA and EPA) (Figure 4C).

3.5. Expression of xCT/*Slc7a11* in rat placenta

It has been reported that xCT is over-expressed in various cancer cells and tissues [22]. Therefore, we investigated xCT/*Slc7a11* expression in normal rat placenta by RT-PCR. xCT/*Slc7a11* was detected in rat placenta both at GD 12 and at GD 20 (Figure 5). Furthermore, the data suggested the expression of xCT at GD 20 was lower than that at GD 12.

4. Discussion

PUFAs are essential for fetal growth and development [3]. Placental transporters play important roles in placental function such as supplying nutrients to the fetus, excreting metabolites, and protecting the fetus from xenobiotics [9-11]. However, the effects of PUFAs on placental transporters during pregnancy have not been well studied. Therefore, the current study aimed to investigate the effects of PUFAs, including AA, EPA, and DHA, on the expression of transporters in BeWo cells as an *in vitro* placental model.

Prior to evaluating transporter expression, we confirmed the effects of PUFAs on ADRP expression. In line with previous reports [20, 21], the expression of ADRP mRNA was induced by 100 μM of AA, EPA, and DHA (Figure 1B). This concentration of PUFAs did not affect the viability of BeWo cells (Figure 1A). The concentration is relevant to physiological levels of PUFAs. It has been reported that concentration ranges in plasma phosphatidylcholine at 34 weeks of gestation were 79.7–132.3 μg/mL (262–434 μM) for AA, 3.2–7.7 μg/mL (10.6–25.5 μM) for EPA, and 38.8–70.5 μg/mL (108–215 μM) for DHA [24].

In the present study, a PCR array kit was applied to thoroughly assess the profiles of transporter expression. There are many reports that investigate gene expression in a specific pathway by PCR array in various type of cells [25-28]. Berveiller *et al*. revealed the changes in some drug transporters during differentiation of cytotrophoblast to syncytiotrophoblast [27]. Park *et al*. demonstrated that tetrabromobisphenol A increased some genes involved in inflammatory in first

trimester extravillous trophoblast HTR-8/SVneo cells using a PCR array kit focused on an immune response pathway [28]. Using the drug transporter PCR array kit, we revealed that *SLC7A11* was upregulated by treatment with AA and EPA. On the contrary, other transporters did not show marked changes (i.e., > three-fold change). EPA tended to alter the *ABCC4* gene that encodes multidrug resistance-associated protein 4 (MRP4); however, it was not statistically significant. Using RT-qPCR (standard curve method), we validated the *ABCC4* alteration data (Supplemental Figure 1). Accordingly, PUFAs (AA, EPA, and DHA) did not affect *ABCC4* mRNA expression. The alterations of some genes exhibiting low expression levels originally detected by a PCR array were not validated by RT-qPCR [28]. Since *ABCC4* expression in BeWo cells was relatively low (high Ct value > 30), the disagreement between the results of the PCR array and RT-qPCR might be due to expression level. We used a standard three-fold change in expression as a cut-off to avoid a false-positive results and to identify genes with significantly different expression, a method similar to that detailed in many previous reports [26]. Another commonly described method is to use two-fold change as a cut-off [28]. A list of genes that were changed more than two-fold is shown in supplemental table 2.

SLC7A11 encodes xCT that Na^+-independently takes up cystine into cells with the efflux of glutamate and functions by disulfide-linkage to 4F2 heavy chain (4F2hc) as a heterodimer [23]. Our results indicated that the upregulation of *SLC7A11* increased protein levels and function of xCT (Figure 3). PCR array analysis indicated that the chaperone protein 4F2hc/*SLC3A2* was not significantly changed by AA and EPA (fold change: -1.28 for AA; fold change: 1.39 for EPA).

xCT/*SLC7A11* is thought to be important for the regulation of cellular redox balance [23]. The cystine transported into cells is quickly converted to cysteine that acts as a precursor for glutathione. As a possible mechanism of the induction of xCT/*SLC7A11* by PUFAs, we focused on NRF2, which is a major transcription factor of xCT/*SLC7A11* expression that mediates antioxidant response [22]. In addition, it has been reported that HO-1, which is another target gene of NRF2, was NRF2-dependently induced by AA in rat astrocytes [29] and by DHA in rat neurons [30]. Contrary to our hypothesis, NRF2 siRNA did not attenuate xCT/*SLC7A11* induction by PUFAs, at least AA and EPA (Figure 4C). On the other hand, NRF2 siRNA attenuated induction of HO-1 by PUFAs (Figure 4B). Furthermore, NRF2 siRNA did not decrease baseline expression of xCT/*SLC7A11* (Supplemental Figure 2). On the other hand, NRF2 knockdown decreased baseline expression of HO-1. Although additional investigations are required, these results imply that NRF2 is not the main contributor of the regulation of the expression of xCT/*SLC7A11* by PUFAs in BeWo cells. In addition to NRF2, several regulatory mechanisms of xCT/*SLC7A11* expression have been reported at the transcriptional, post-transcriptional, and post-translational levels [22]. Activating transcription factor 4 (ATF4) upregulates xCT/*SLC7A11*, whereas the tumor suppressor p53 represses it. MicroRNAs such as miR-27a, miR-26b, and miR-375, and nonsense-mediated mRNA decay have been reported to regulate xCT/*SLC7A11*. Future studies are needed to address the molecular mechanisms responsible for the induction of xCT/*SLC7A11* expression in placental cells.

xCT/*SLC7A11* was upregulated by both AA and EPA. On the other hand, PCR array results

implied that the patterns of gene variation by AA or EPA showed differences to some extent. It has been widely known that there are differences between n-6 AA and n-3 EPA in terms of biochemical and physiological properties. For example, n−6 PUFAs derived eicosanoids have proinflammatory effects, whereas n−3 PUFAs derived eicosanoids have anti-inflammatory activities [31]. Furthermore, it has been reported that EPA but not AA increases nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) in adipose tissue [32], although these PUFAs have similar affinities to PPARs [33]. Although the reason is not clear at present, the differences between AA and EPA in terms of biochemical and physiological properties might affect the results. Similar to regulation mechanisms of transporters by PUFAs in other cell line, it has been reported that LXR and RXR might contribute to the downregulation of NPC1L1 in Caco-2 [16]. It has also been reported that increase in nuclear receptors CAR and PXR might be involved in the upregulation of P-gp/*MDR1* in Caco-2 cells [17]. Future studies are required to clarify whether there are differences between AA and EPA with respect to effects on transporters and mechanisms.

In the present study, BeWo cells, a continuous cell line originating from human placental choriocarcinoma, were used as a trophoblast model. This cell line is widely used to study the mechanisms of placental transport of compounds and the role of transporters in these processes [34]. The cells have some advantages, such as the ability to maintain the morphological characteristics of trophoblasts and to produce placental hormones, as well as ease of manipulation. However, differences between BeWo cells and normal trophoblasts in gene expression and responses to various stimuli have

been reported [34]. Although xCT/*SLC7A11* plays physiological roles in normal tissues such as brain, immune cells, and skin, the transporter is over-expressed in various cancerous cells, rendering it a potential therapeutic target for cancer [35]. Given the lack of research on the expression and function of xCT in normal placenta, we investigated its expression in the placenta of pregnant rats. Our results indicated that xCT/*Slc7a11* was expressed in rat placentas at the mRNA level (Figure 5). Furthermore, our data suggested the mRNA expression of xCT/*Slc7a11* at GD 20 was lower than that at GD 12. Recently, Simner *et al*. reported the gene expression of xCT/*SLC7A11* in human placenta, revealing that the expression decreased as gestation progressed [36]. However, reasons for the variation of xCT expression in placenta throughout gestation and an understanding of its involvement in physiological and pathological processes are not clear at present. It has been observed that oxidative stress increases during gestation [37]. Further studies are needed to investigate the expression, function, and role of xCT in placenta. In addition, future investigations should address whether the transporter expression in the placenta is affected by the composition of PUFAs in the diet (supplementation or deficiency) fed to pregnant rats.

In conclusion, we used a PCR array analysis to conduct a comprehensive evaluation of changes in transporter expression by PUFA in BeWo cells. The present study showed that treatment with PUFA (AA, EPA, and DHA) increased cystine transporter xCT/*SLC7A11* in BeWo cells. Furthermore, the upregulation of xCT/*SLC7A11* in BeWo cells was independent of NRF2 expression. In addition, xCT/*Slc7a11* mRNA was detected in rat placenta, and the expression level at GD 12 was

higher than that at GD 20. As previously mentioned, PUFAs are important for adequate fetal growth in a healthy pregnancy. In addition, their importance has been suggested in some obstetric disorders, such as diabetes, preeclampsia, and intrauterine growth restriction, thought to be associated with oxidative stress [38, 39]. The involvement of the induction of xCT/*SLC7A11* in physiological and pathological processes could be a subject of future research. However, because of certain disadvantages in the use of cell lines, the extrapolation of the present findings observed in BeWo cells to normal trophoblasts must be made with caution. Further studies are necessary using normal trophoblasts and *in vivo* models.

Acknowledgments

Funding

This work was supported by the Mishima Kaiun Memorial Foundation Research Grant (provided to A.F.).

Conflicts of Interest

The authors declare no conflicts of interest.

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Figure captions

Figure 1. Cell viability (A) and ADRP mRNA expression (B) after treatment with PUFAs. BeWo cells were treated with 100 μM of AA, EPA, or DHA for 24 h. Each column represents the mean with SD of three independent experiments. **: significantly different from the control at $p < 0.01$.

Figure 2. PCR array analysis of effects of AA and EPA on transporter expression. (A) Distribution of Ct values of BeWo cells (control sample). (B and C) BeWo cells were treated with 100 μM of AA or EPA for 24 h. The scatter plot graphs the expression level ($2^{-\Delta Ct}$) of each gene in the control versus the test sample AA (B) or EPA (C). Each dot represents the mean value of three independent experiments. The bold lines indicate the three fold-change in gene expression.

Figure 3. Effects of PUFAs on expression and function of xCT/*SLC7A11*. (A) BeWo cells were treated with 100 μM of AA, EPA, or DHA for 24 h. After the treatment, expression of xCT/*SLC7A11* was investigated by RT-qPCR. Each column represents the mean with SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared to the control. †$p < 0.01$, ††$p < 0.01$ between PUFAs. (B) Western blotting of BeWo cells treated with PUFAs. Each column represents the mean with SD of five independent experiments. Representative image data are shown. **$p < 0.01$ compared to the control. (C) After treatment with the PUFAs, the uptake activity of [^{14}C]-cystine was investigated. BeWo cells were incubated for 10 min with a Na^+-free transport buffer containing [^{14}C]-cystine (0.1

μCi/m). Each column represents the mean with SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared to the control. †$p < 0.05$ between PUFAs.

Figure 4. Effects of NRF2 siRNA knockdown on alteration in xCT/*SLC7A11* by PUFA. BeWo cells were transfected with negative control siRNA or NRF2/*NFE2L2* siRNA (HSS107130) for 72 h and then treated with PUFAs (AA, EPA, or DHA) for 24 h. (A) NRF2/*NFE2L2*, (B) HO-1/*HMOX1*, and (C) xCT/*SLC7A11* mRNA expression of BeWo cells were evaluated by RT-qPCR. Each column represents the mean with SD of three to four independent experiments. *$p < 0.05$, **$p < 0.01$ compared to the control. †$p < 0.05$, ††$p < 0.01$ between PUFAs.

Figure 5. RT-PCR analysis of rat placenta at GD 12 and GD 20. PCR was performed using specific primers. β-actin was used as a loading control.

Supplemental Figure 1. Effects of PUFAs on expression of MRP4/*ABCC4*. BeWo cells were treated with 100 μM of AA, EPA, or DHA for 24 h. The expression of MRP4/*ABCC4* was investigated by RT-qPCR. Each column represents the mean with SD of three independent experiments.

Supplemental Figure 2. Effects of NRF2 siRNA knockdown on the baseline expression of HO-1/*HMOX1* and xCT/*SLC7A11*. BeWo cells were transfected with negative control siRNA or

NRF2/*NFE2L2* siRNA for 72 h. (A) HO-1/*HMOX1* and (B) xCT/*SLC7A11* mRNA expression of BeWo cells were evaluated by RT-qPCR. Each column represents the mean with SD of three to four independent experiments. $^{**}p < 0.01$ compared to the negative control.

Table 1. Primer sequences

Name		Primer sequence	Product size (bp)	References
Human				
β-actin (*ACTB*)	Forward	5'-TGGCACCCAGCACAATGAA-3'	186	[40]
	Reverse	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'		
ADRP (*PLIN2*)	Forward	5'-TGTGGAGAAGACCAAGTCTGTG-3'	197	[41]
	Reverse	5'-GCTTCTGAACCAGATCAAATCC-3'		
xCT (*SLC7A11*)	Forward	5'-CCTGGCATTTGGACGCTACAT-3'	182	[42]
	Reverse	5'-TCAGAATTGCTGTGAGCTTGCA-3'		
MRP4 (*ABCC4*)	Forward	5'-GGAATATCAGAAACGCCCACCACCAG-3'	168	[43]
	Reverse	5'-GAGGTCTTTACGGATGTCAACG-3'		
HO-1 (*HMOX1*)	Forward	5'-CAGGATTTGTCAGAGGCCCTGAAGG -3'	139	[44]
	Reverse	5'-TGTGGTACAGGGAGGCCATCACC -3'		
NRF2 (*NFE2L2*)	Forward	5'-AAACCAGTGGATCTGCCAAC-3'	135	[45]
	Reverse	5'-ACGTAGCCGAAGAAACCTCA-3'		
Rat				
β-actin (*Actb*)	Forward	5'-CTATCGGCAATGAGCGGTTC-3'	134	[46]
	Reverse	5'-GAGGTCTTTACGGATGTCAACG-3'		
xCT (*Slc7a11*)	Forward	5'-GACAGTGTGTGCATCCCCTT-3'	110	[47]
	Reverse	5'-GCATGCATTTCTTGCACAGTTC-3'		

Supplemental Table 1. Ct values of control sample

Gene	Ct value (Mean, n=3)	Gene	Ct value (Mean, n=3)
ABCA1	32.58	*SLC19A2*	26.25
ABCA12	33.53	*SLC19A3*	27.74
ABCA13	> 35	*SLC22A1*	33.66
ABCA2	28.82	*SLC22A2*	> 35
ABCA3	31.55	*SLC22A3*	> 35
ABCA4	28.41	*SLC22A6*	> 35
ABCA9	33.39	*SLC22A7*	> 35
ABCB1	> 35	*SLC22A8*	> 35
ABCB11	> 35	*SLC22A9*	> 35
ABCB4	> 35	*SLC28A1*	24.85
ABCB5	34.79	*SLC28A2*	> 35
ABCB6	> 35	*SLC28A3*	32.20
ABCC1	25.47	*SLC29A1*	29.18
ABCC10	26.50	*SLC29A2*	23.03
ABCC11	28.05	*SLC2A1*	26.25
ABCC12	> 35	*SLC2A2*	21.08
ABCC2	> 35	*SLC2A3*	33.12
ABCC3	31.71	*SLC31A1*	20.10
ABCC4	34.52	*SLC38A2*	24.45
ABCC5	28.47	*SLC38A5*	22.22
ABCC6	26.10	*SLC3A1*	33.52
ABCD1	27.08	*SLC3A2*	31.91
ABCD3	24.83	*SLC5A1*	22.55
ABCD4	26.28	*SLC5A4*	> 35
ABCF1	23.96	*SLC25A13*	> 35
ABCG2	22.70	*SLC7A11*	28.85
ABCG8	> 35	*SLC7A5*	22.27
AQP1	31.68	*SLC7A6*	26.41
AQP7	31.64	*SLC7A7*	22.29
AQP9	> 35	*SLC7A8*	25.66
ATP6V0C	24.36	*SLC7A9*	26.74
ATP7A	28.47	*SLCO1A2*	30.02
ATP7B	24.54	*SLCO1B1*	> 35
MVP	29.66	*SLCO1B3*	> 35
SLC10A1	33.58	*SLCO2A1*	> 35
SLC10A2	> 35	*SLCO2B1*	32.73
SLC15A1	> 35	*SLCO3A1*	> 35
SLC15A2	30.92	*SLCO4A1*	26.04
SLC16A1	21.30	*TAP1*	25.11
SLC16A2	> 35	*TAP2*	25.22
SLC16A3	28.19	*VDAC1*	21.78
SLC19A1	28.33	*VDAC2*	21.90

Supplemental Table 2. Genes that were changed more than two-fold

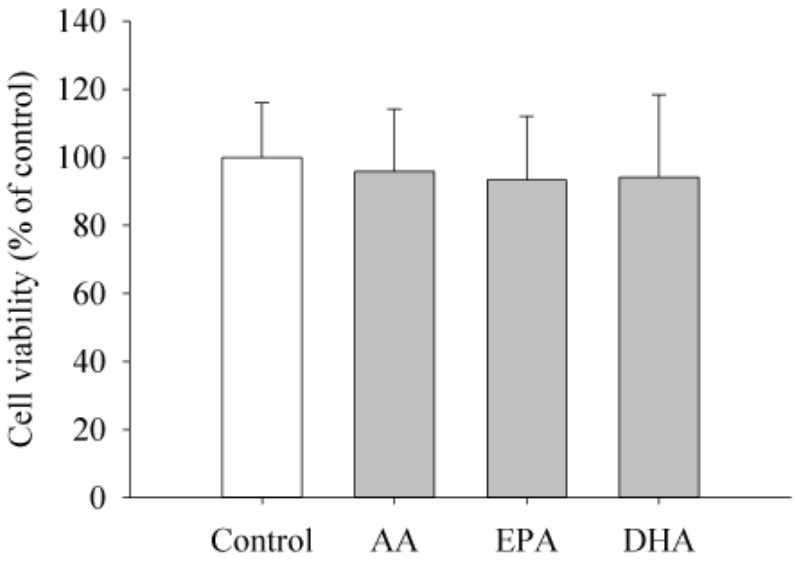
		Fold change	*p*-value
AA			
	ABCA2	-2.35	0.786
	SLC7A11	5.03	0.043
	SLCO2B1	2.57	0.185
EPA			
	ABCA3	2.89	0.076
	ABCC4	4.76	0.159
	SLC7A11	6.03	0.002

1

2 Data shown are mean of fold change relative to control from three independent experiments.

Figure 1

(A)


Cell viability (% of control)
140
120
100
80
60
40
20
0
Control
AA
EPA
DHA

(B)

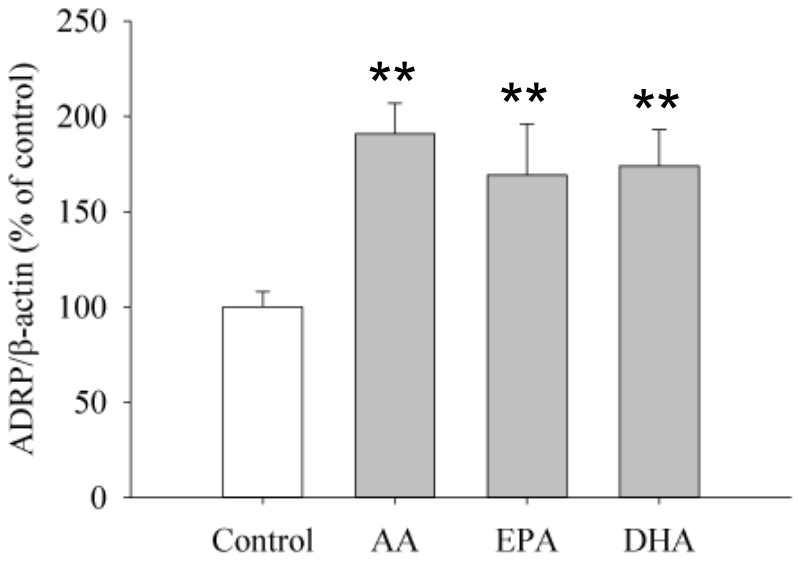
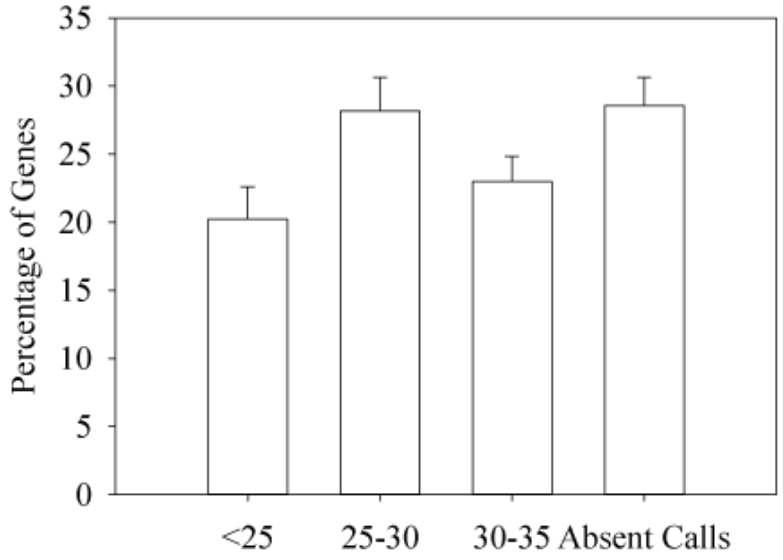
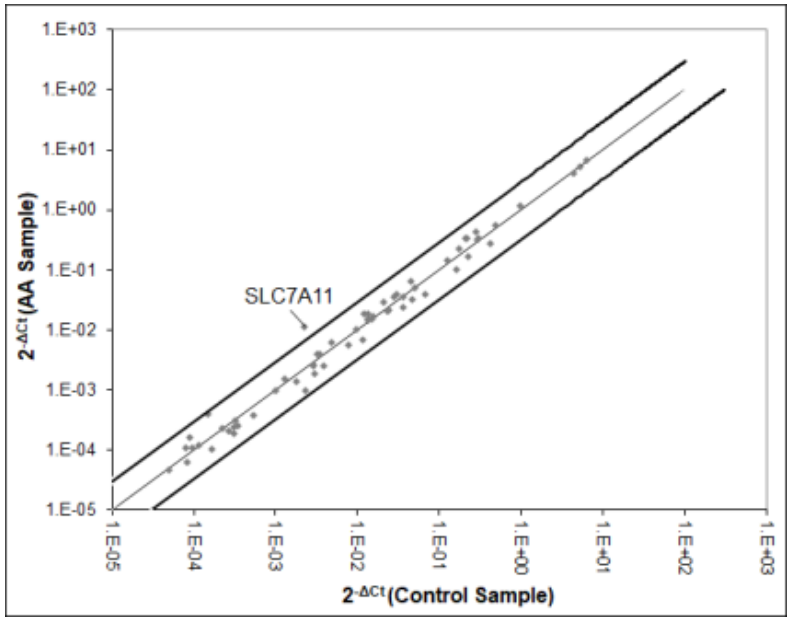

ADRP/β-actin (% of control)
250
200
150
100
50
0
**
**
**
Control
AA
EPA
DHA

Figure 2

(A)


Percentage of Genes
35
30
25
20
15
10
5
0
<25
25-30
30-35 Absent Calls

(B)


SLC7A11
2-ΔCt (AA Sample)
2-ΔCt (Control Sample)
1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
1.E-05

(C)

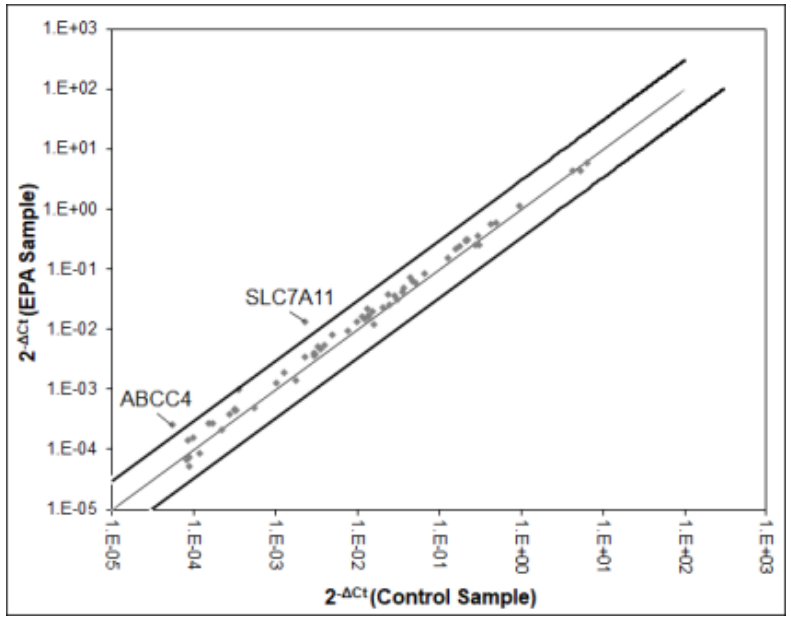
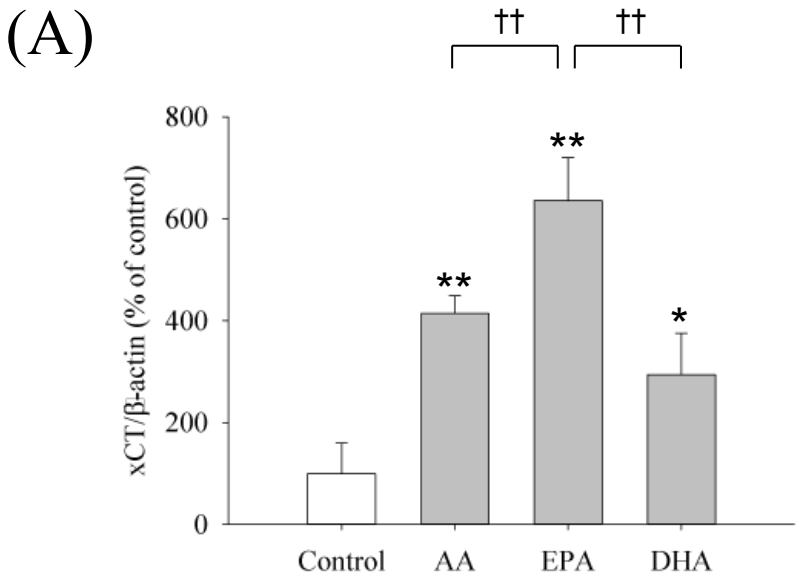
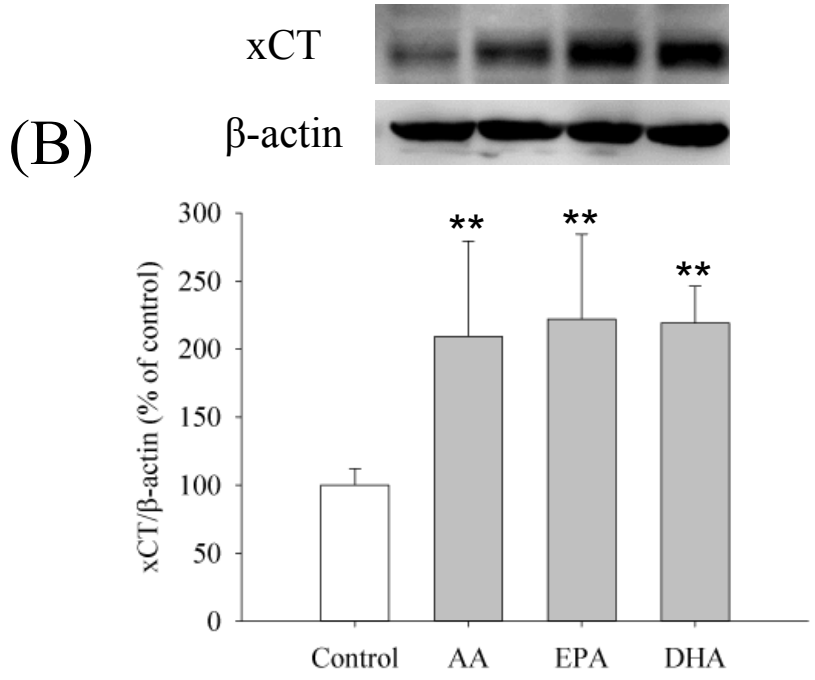

SLC7A11
ABCC4
2-ΔCt (EPA Sample)
2-ΔCt (Control Sample)
1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
1.E-05

Figure 3


(A)
††
††
800
600
400
200
0
xCT/β-actin (% of control)
**
**
*
Control
AA
EPA
DHA


(B)
xCT
β-actin
300
250
200
150
100
50
0
xCT/β-actin (% of control)
**
**
**
Control
AA
EPA
DHA

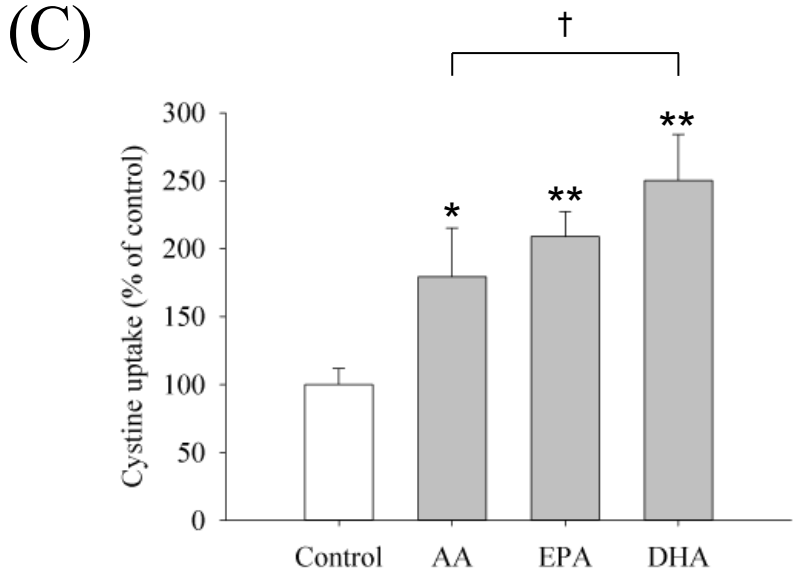

(C)
†
300
250
200
150
100
50
0
Cystine uptake (% of control)
*
**
**
Control
AA
EPA
DHA

Figure 4

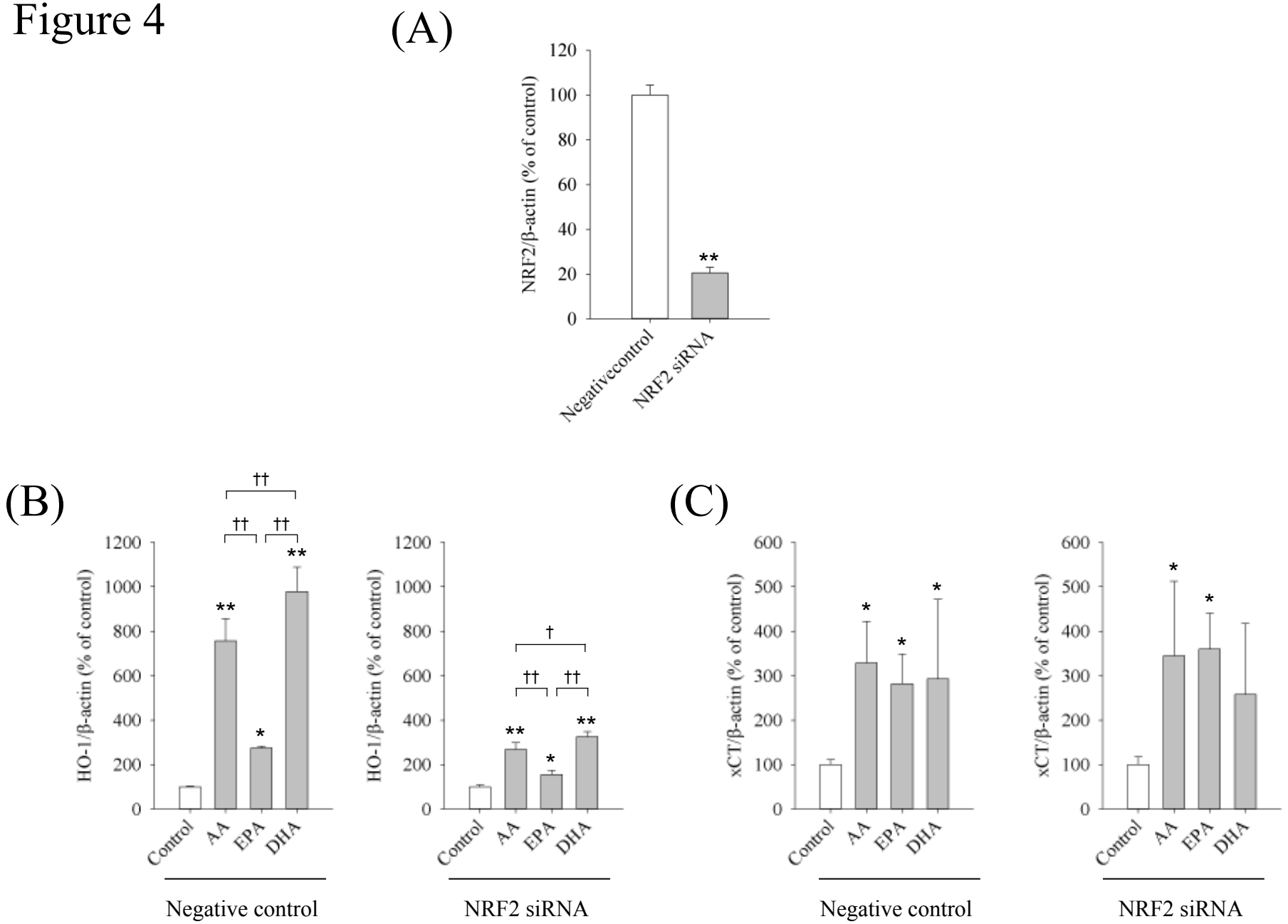
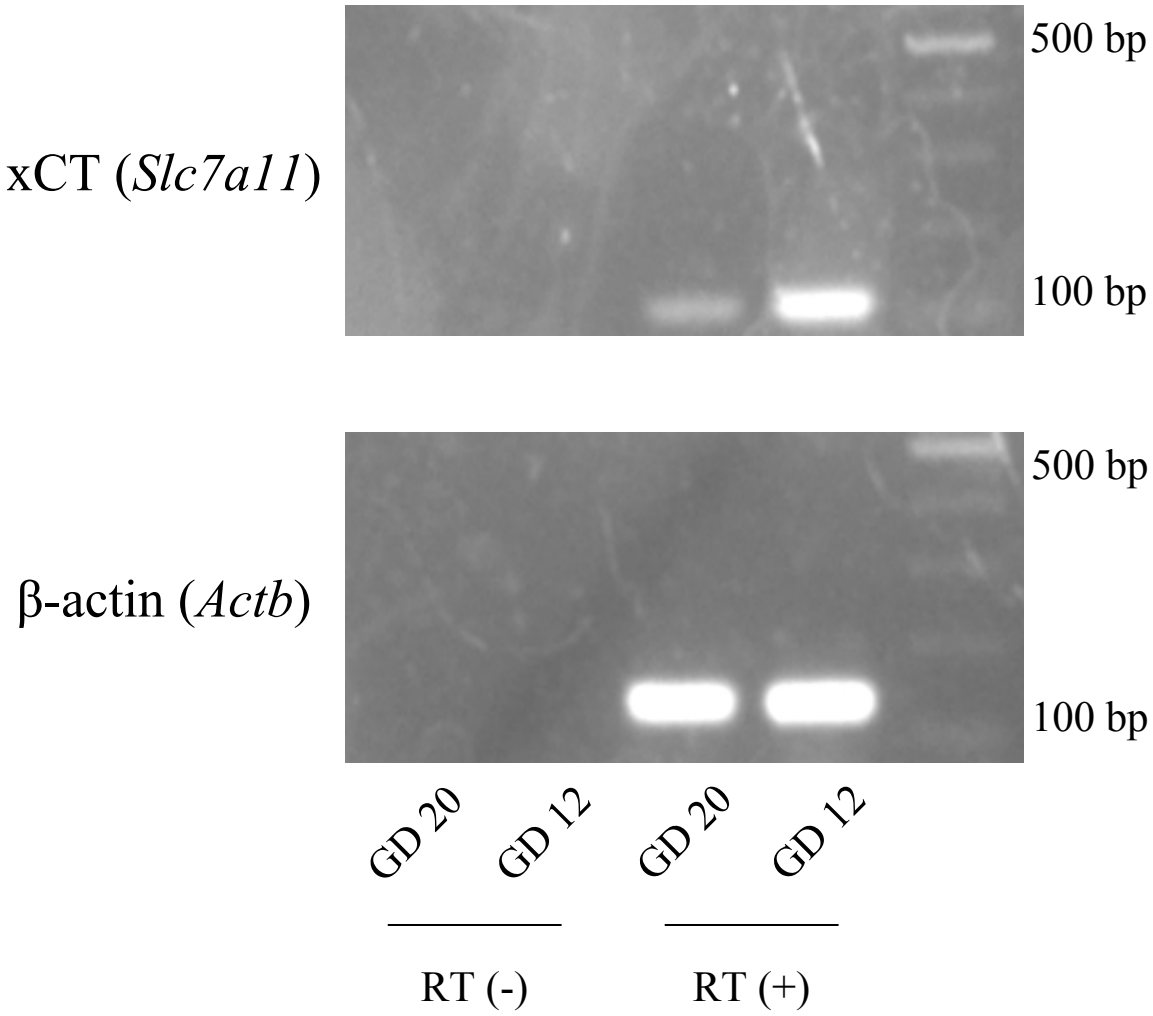
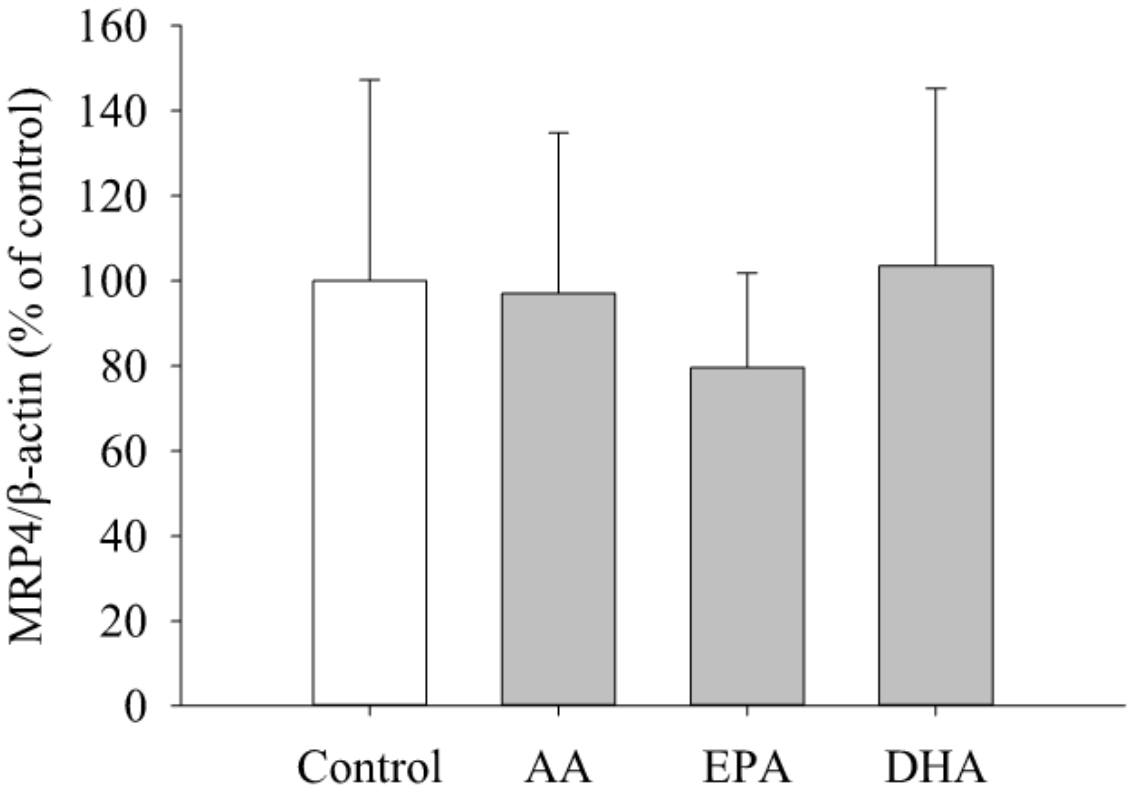
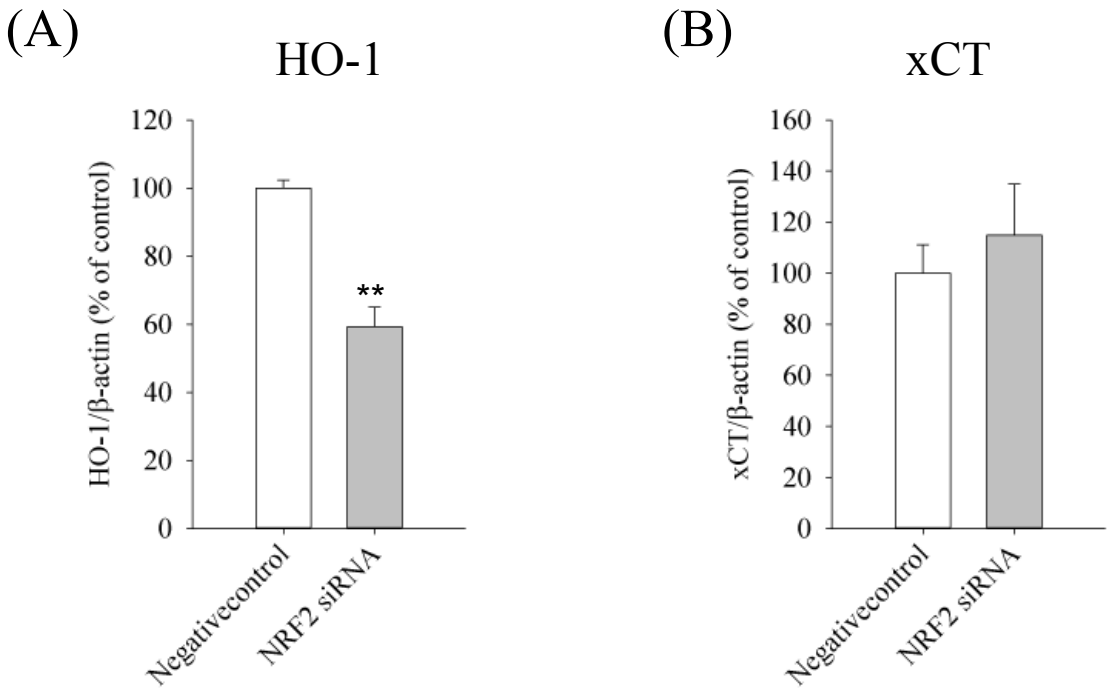

(A)
NRF2/β-actin (% of control)
120
100
80
60
40
20
0
**
Negativecontrol
NRF2 siRNA
(B)
HO-1/β-actin (% of control)
1200
1000
800
600
400
200
0
††
††
††
**
**
*
Control
AA
EPA
DHA
Negative control
HO-1/β-actin (% of control)
†
††
††
**
**
*
Control
AA
EPA
DHA
NRF2 siRNA
(C)
xCT/β-actin (% of control)
600
500
400
300
200
100
0
*
*
*
Control
AA
EPA
DHA
Negative control
xCT/β-actin (% of control)
*
*
Control
AA
EPA
DHA
NRF2 siRNA

Figure 5


xCT (*Slc7a11*)
500 bp
100 bp
β-actin (*Actb*)
500 bp
100 bp
GD 20
GD 12
GD 20
GD 12
RT (-)
RT (+)

Supplemental Figure 1



Supplemental Figure 2