

DHA upregulates FADS2 expression in primary cortical astrocytes exposed to vitamin A

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Summary

The *fads2* gene encoding $\Delta 6$ -desaturase, the rate-limiting enzyme in the LCPUFA biosynthesis is expressed in astrocytes. Dietary fatty acids, which cross the blood-brain barrier may regulate the transcription of lipogenic enzymes through activation of transcription factors such as peroxisome proliferator-activated receptors (PPARs). The PPARs form the transcription complex with retinoid X receptors (RXRs) that are activated by 9-cis retinol, a metabolite of vitamin A (VA). The study examines whether challenge of astrocytes with VA, prior 24-hour treatment with palmitic acid (PA), α -linolenic acid (ALA) or docosahexaenoic acid (DHA) has the effect on the FADS2 expression. RT-qPCR showed that in astrocytes not challenged with VA, PA increased *fads2* gene expression and DHA decreased it. However, in VA-primed astrocytes, PA doubled the FADS2 mRNA levels, while DHA increased *fads2* gene expression, oppositely to non-primed cells. Furthermore, similar changes were seen in VA-primed astrocytes with regard to $\Delta 6$ -desaturase protein levels following PA and DHA treatment. ALA did not have any effect on the FADS2 mRNA and protein levels in either VA-primed or non-primed astrocytes. These findings indicate that in the presence of vitamin A, DHA upregulates *fads2* gene expression in astrocytes.

Introduction

In mammals, the biosynthesis of long-chain polyunsaturated fatty acids (LCPUFAs) from 18-carbon chain essential fatty acids is carried out by the elongases, and $\Delta 5$ - and $\Delta 6$ -desaturases in the endoplasmic reticulum (Sprecher *et al.* 1995). These desaturases are encoded by the *fads1* and *fads2* genes, respectively, and together with the *fads3* gene on human chromosome 11 (11q12-13.1) create the cluster, which genetic variations determine the LCPUFA levels in cell membrane and in circulation (Marquardt *et al.* 2000; Mathias *et al.* 2014). The $\Delta 6$ -desaturase is the rate-limiting enzyme of the LCPUFA synthesis; it introduces the double *cis* bonds at both n-3 and n-6 LCPUFAs by O₂-dependent subtraction of two hydrogens from a methylene group of hydrocarbon chains.

Dietary fatty acids are signaling molecules that regulate biological processes via regulation of metabolic enzyme gene transcription. It has been demonstrated that LCPUFAs downregulate the transcription of key lipogenic enzymes, including $\Delta 6$ -desaturase (FADS2) (Clarke and Jump 2001), acting as feedback inhibitors of their own synthesis. In the cells, nonesterified PUFAs bind to the sensors that belong to the family of peroxisome proliferator-activated receptors (PPARs) (Bordoni *et al.* 2006). PPARs heterodimerize with retinoid X receptors (RXRs) activated by vitamin A derivatives, such as 9-*cis* retinoic acid (Zapata-Gonzalez *et al.* 2007), and bind to regulatory elements in the promoters of responsive genes (Hermanson *et al.* 2002). The PPAR isoforms (PPAR α , PPAR β / δ , and PPAR γ) and RXR β isotype have been found in cultured astrocytes by fluorescence imaging and Western blot (Chistyakov *et al.* 2015). A recent study utilizing quantitative PCR and double immunofluorescence showed high expression of PPAR α and low expression of PPAR γ in the mouse cortical astrocytes (Warden *et al.* 2016). Furthermore, interaction between DHA and RXR has been demonstrated in the brain (Lengqvist *et al.* 2004).

Docosahexaenoic acid (DHA) esterified to *sn*-2 position of glycerophospholipids is the most abundant LCPUFA in the brain (Stillwell and Wassall 2003). The high level of DHA-containing phospholipids plays an important role in maintaining neuronal membrane fluidity, which creates a molecular environment suitable for the operation of transmembrane proteins with membrane-associated signaling proteins (Akbar *et al.* 2005). As astrocytes, unlike neurons, express FADS2 and possess activity in synthesis

of 22-carbon PUFAs (Moore *et al.* 1991), it has been proposed that astrocytes supply lipids to neurons (Taberero *et al.* 2001). The regulation of *fads2* gene expression by dietary fatty acids has been extensively investigated in the liver (Nara *et al.* 2002), however, the mechanism of fatty acid regulation of this gene in astrocytes remains unknown. To better understand this process, the present study examines the effect of palmitic acid (PA; C16:0), α -linolenic acid (ALA; 18:3 n-3), and DHA (22:6 n-3) on FADS2 mRNA and protein levels in rat astrocytes. Since vitamin A derivatives activate RXRs, the obligatory heterodimer of the PPAR transcription factor, the study investigates whether pretreatment of astrocytes with vitamin A may influence *fads2* gene transcription, which is regulated by fatty acids.

Materials and methods

Primary astrocytes were prepared from newborn 1-2 days old Wistar rats as previously described (Zawadzka and Kaminska 2003). The study protocol as approved by the Government Ethical Committee for Animal Care. Cells were cultured in high-glucose DMEM with 10% FBS and antibiotics in 75 cm² flasks at 37°C and 5% CO₂ atmosphere then seeded into 6-well plates (5x10⁵ cells/well), or into 80 mm Petri dishes (1.5x10⁶ cells/dish). After the growth phase, astrocytes were pretreated with 10 μ M vitamin A (VA, Medana Pharma SA. Poland) or cultured in DMEM for 24 hours, followed by incubation with PA, ALA, and DHA at concentration of 50 μ M for next 24 hours. Control cells were cultured in DMEM.

The *fads2* gene expression was examined by reverse transcription quantitative polymerase chain reaction PCR (RT-qPCR). Total RNA was isolated from astrocytes using TRIZOL RNA reagent (Invitrogen Life Technologies USA). The quantity and purity of the extracted RNA was checked with a PicoDrop Microliter UV/Vis Spectrophotometer. A ratio A 260/280 of 1.9 – 2.1 was taken as pure RNA. The RNA quality was determined using a 2100 Bioanalyzer (Agilent Technologies). The RNA samples used in the reverse transcription reaction were calculated and diluted to the same concentration and quality (RIN value was > 7). Reverse transcription of 1 μ g of total RNA was performed in a total 20 μ l using Superscript III reverse/ ImProm-IITM reverse transcriptase (Promega USA), dNTPs and random hexamer primers (Invitrogen Life Technologies USA) for 1 h at 50°C. The obtained cDNA was used for qPCR reaction. Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The following primer pairs: 5'GTTCTTCTTTCTCCTCCTGTCCC-3' (forward) and 5'-

CATTGCCGAAGTACGAGAGGAT-3' (reverse); 5'-CATGGCCTTCCGTGTTTCCTA-3' (forward) and 5'-CCTGCTTACCACCTTCTTGA-3' (reverse) were used to detect the mRNA of FADS2 and GAPDH, respectively. Gene expression was quantified using an ABI Prism 7000 Sequence Detection System (Applied Biosystems), using a Brilliant III SYBR QPCR kit (Agilent Technologies) according to the manufacturer's protocol. Each sample was assayed in triplicate in independent reactions. PCR efficiency was validated with a standard curve. The results were analysed according to the $2^{-\Delta\Delta C_t}$ method. Relative gene expression was normalized to GAPDH expression and presented as relative quantification (RQ) i.e. fold change compared to the untreated control with RQ value of 1.

The FADS2 protein level was determined by Western blot using Thermo Scientific–Pierce reagents and antibodies supplied by Santa Cruz Biotechnology. Astrocytes were lysed with RIPA buffer (25mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 and protease inhibitors cocktail) and total protein levels were determined by BCA Protein Assay Kit. The protein samples (20 μ g) were separated by SDS-PAGE, transferred onto the nitrocellulose membrane and incubated overnight with rabbit polyclonal anti-FADS2 antibodies (sc-98480) followed by two-hour incubation with mouse anti rabbit IgG horseradish peroxidase–conjugated secondary antibodies (sc-2357). The luminescence signal of bands was obtained using Pierce ECL Western Blotting Substrate and visualized on Amersham Hyperfilm ECL. Densitometric analysis was performed with Image-J software. Beta-actin was used as a loading control. To detect β -actin protein, the same blot was re probed with mouse anti- β actin monoclonal antibody (sc-47778), after stripping in buffer containing DTT; the detection was performed using secondary antibody-HRP conjugate, as described above.

To compare the effects of three different fatty acids, one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test was used. P-values < 0.05 were considered significant.

Results

The effects of fatty acids differing in the numbers of double bonds on fads2 gene expression in astrocytes non-primed and primed with vitamin A are shown in figure 1A. The level of the FADS2 transcript in astrocytes incubated with DHA was reduced by half ($p < 0.01$), but was increased in PA-treated

cells ($p < 0.01$) compared to controls. ALA did not change the FADS2 mRNA level compared to control cells.

However, instead of reducing the level of FADS2 mRNA in astrocytes preincubated with vitamin A, DHA upregulated the levels fivefold compared to control cells ($p < 0.0001$). Likewise, PA doubled the FADS2 mRNA in VA-primed astrocytes compared to cells untreated with vitamin A. The administration of ALA in combination with retinol did not affect the FADS2 mRNA levels.

These effects on *fads2* gene expression in VA-primed astrocytes were reflected in the levels of FADS2 protein. As figure 1B shows, densitometric analysis of the bands corresponding to FADS 2 revealed an increase in FADS2 protein levels after PA ($p < 0.01$) and DHA ($p < 0.001$) treatment in VA-primed astrocytes compared to the control cells. However, in astrocytes not primed with VA, the FADS2 protein levels after incubation with fatty acids did not differ from those observed in the control cells. Similarly, incubation with ALA did not influence the FADS2 protein levels in VA-primed astrocytes.

Discussion

In the brain, a high level of DHA should be maintained after the intense DHA accretion in late fetal and infant period (Innis 2008). However, during aging and under adverse conditions, such as the production of inflammatory mediators by activated microglia and astrocytes, which enhances phospholipid deacylation via activation of phospholipase A2, the DHA level in neuronal membranes decreases; this results in an imbalance in the cellular homeostasis, which is known to be associated with various types of neuropathology. Since astrocytes express a functional FADS2 (Moore 2001) they may be a potential source of DHA for neurons, similarly to their role in cholesterol supply to neurons (Pfrieger and Ungerer 2011). Therefore, it is important to recognize the regulation of FADS2 expression by exogenous fatty acids in astrocytes.

The molecular mechanism of gene transcription regulation by fatty acids, well recognized in hepatocytes, involves the activation of PPARs and sterol regulatory elements binding protein 1 (SREBP-1) (Georgiadi and Kersten 2012). It has been demonstrated that in rat hepatocytes, various PUFAs differently activate PPARs. Oleic acid and DHA had little or modest effects on the PPAR-regulated target genes, while eicosapentaenoic acid significantly enhanced the PPAR-dependent target gene transcription (Pawar and

Jump 2003). Regarding the activation of genes involved in *de novo* lipogenesis by SREBP-1c, it has been found that PUFAs generally suppress the transcription of a wide range of hepatic lipogenic genes, either by inhibition of SREBP gene transcription (Xu *et al.* 1999), or by suppression of SREBP-1 proteolytic processing (Takeuchi *et al.* 2010). Moreover, SREBP-1 expression has been documented in different regions of rodent brains (Camargo *et al.* 2012), as well the contribution of SREBPs in cholesterol and fatty acid synthesis in astrocytes (Okamoto *et al.* 2006).

The present results demonstrate that in the absence of vitamin A, fatty acids regulate FADS2 gene expression in astrocytes in different ways: saturated PA slightly increased FADS2 transcript levels while DHA diminished them. The inhibitory effect of DHA on FADS2 gene expression may have a similar mechanism to that observed in hepatocytes, where DHA acts as a potent suppressor of SREBP-1 abundance in the nucleus by accelerating its proteasomal degradation (Botolin *et al.* 2006). However, following DHA treatment, FADS2 mRNA and protein levels in VA-primed astrocytes were higher than in those not primed with VA; similarly, following PA treatment, FADS2 expression was enhanced more in VA-primed cells than in non-primed with VA astrocytes.

The precise mechanism of this effect is not recognized yet, however, we speculate that it may be similar to the mechanisms of the regulation of nuclear receptor by fatty acids (Jump 2013). The PPAR response element in the promoter of the FADS2 gene was identified as direct repeat sequence 1 (DR1) (Tang *et al.* 2003; Rakhshandehroo *et al.* 2010). To carry out most of their functions, the PPARs must heterodimerize with RXR; as this is a heterodimerization partner of about twenty other nuclear receptors, some heterodimers also share the same DR1 element. Both PPAR and RXR mRNAs have been found in cortical astrocytes (Cullingford *et al.* 1998). Dietary retinol is converted to 9-*cis* isomer and all-*trans* retinoic acid (Conaway *et al.* 2013), the ligands of RXRs and RAR, respectively, which could form a variety of heterodimers competing to bind to the DR1 and for cross-talk with the PPAR:RXR heterodimer (Chan and Wells 2009). As a result, the coactivators can have better access to the PPAR:RXR complex to enhance FADS2 promoter activity.

In conclusion, vitamin A may affect LCPUFA production in astrocytes, which is especially important for upregulation of *fads2* gene expression by DHA. These findings can contribute to the development of a molecular strategy to increase LCPUFA synthesis by astrocytes.

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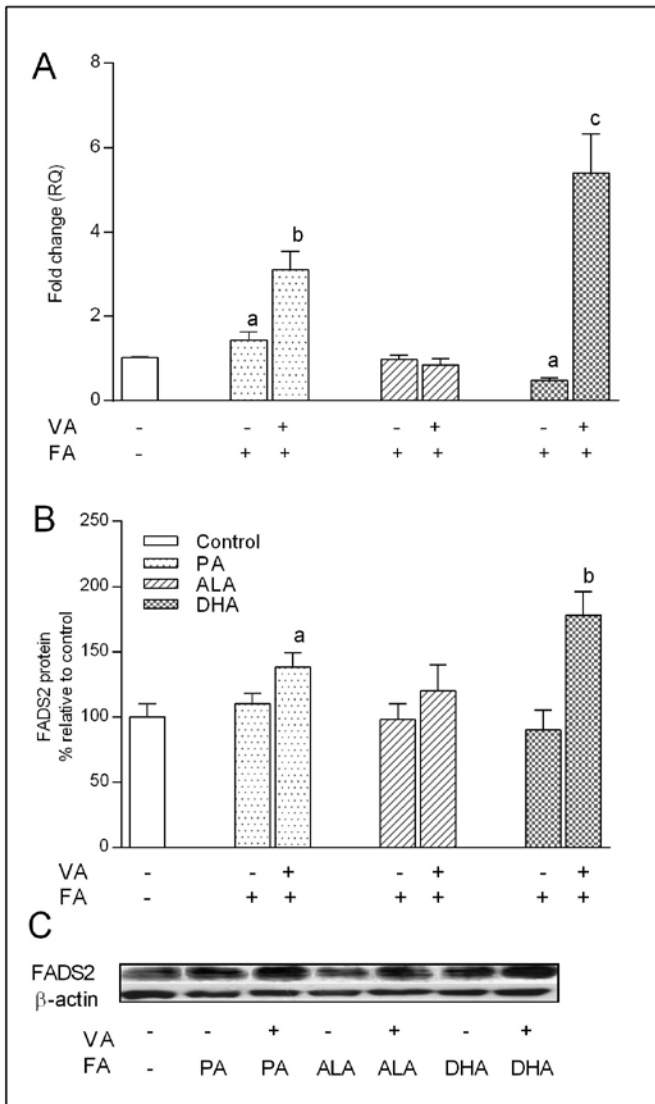


Figure description

Fig. 1. *FADS2* mRNA (**A**) and *FADS2* protein (**B**) levels after palmitic acid (PA), α -linolenic acid (ALA) and docosahexaenoic acid (DHA) treatment in astrocytes primed or non-primed with vitamin A (VA).

Bottom (**C**), the representative immunoblot for *FADS2* and β -actin proteins. Data are means \pm SEM (n = 6 for *FADS2* mRNA and n=2 for *FADS2* protein). ^a p< 0.01, ^b p< 0.001, ^c p<0.0001 compared to astrocytes cultured in DMEM alone.