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# Ligands of peroxisome proliferator-activated receptor-alpha promote glutamate transporter-1 endocytosis in astrocytes

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ABSTRACT

Astrocytes, a stellate-shape glial population in the central nervous system (CNS), maintain glutamate homeostasis in adult CNS by undergoing glutamate uptake at the synapse through their glutamate transporter-1 (GLT-1). Peroxisome proliferator-activated receptor-α (PPARα) can be activated by endogenous saturated fatty acids to regulate astrocytic lipid metabolism and functions. However, it is unclear if PPARα can exert the regulatory action on GLT-1 expression in astrocytes. This study showed that treatment with palmitic acid (PA) and the other two PPARα agonists (GW 7647 and WY 14,643) caused no change in the morphology of astrocytes, whereas membranous GLT-1 protein levels in astrocytes were significantly decreased by PA and PPARα agonists. Through lentivirus-mediated overexpression of GLT-1 tagged with red fluorescent protein (GLT-1-RFP), we also observed that GLT-1-RFP puncta in the processes of astrocytes were inhibited by the PPARα agonists. This reduction was prevented by the addition of the PPARα antagonist, GW6471. GLT-1-RFP was co-localized to the early endosome marker–EEA1 in astrocytes treated with the PPARα agonists. Moreover, PPARα-induced inhibition in membranous GLT-1 expression was abolished by the addition of dynamin inhibitor (dynasore). Furthermore, the co-treatment of astrocytes with PPARα agonists and dynasore, or with PPARα agonists and protein kinase C (PKC) inhibitor bis-indolylmaleimide 1 (BIS1), prevented the endocytosis of GLT-1-RFP. Based on the results, we conclude that the PPARα agonists increased GLT-1 endocytosis in astrocytes possibly through the PKC signaling pathway. In addition, our findings provide important information of PPARα involvement in the downregulation of astrocytic glutamate uptake via the promoted GLT-1 endocytosis.



## 1. Introduction

Astrocytes are the most abundant glial cell type in the central nervous system (CNS), and function as CNS neuronal supportive cells (Reid et al., 1996; Song et al., 2002). In addition to providing neurotrophic factors and growth factors to support neuronal survival and function, astrocytes are responsible for the clearance of neurotransmitters to maintain the homeostasis of neurotransmitters (Solecki et al., 2004; Voutsinos-Porche et al., 2003; Wadiche and Jahr, 2005). For instance, astrocytes maintain glutamate homeostasis in the CNS by glutamate-aspartate transporter/excitatory amino-acid transporter-1 (GLAST/EAAT1) and glutamate transporter-1/EAAT2 (GLT-1/EAAT2) that are the major two glutamate transporters in astrocytes (Anderson and Swanson, 2000; Danbolt, 2001; Schlag et al., 1998; Swanson et al., 1997). GLT-1 expression is low during CNS development, but it reaches to an abundant level in several adult brain regions, such as the caudate nucleus, cortex and hippocampus (Anderson and Swanson, 2000; Rothstein et al., 1994; Ullensvang et al., 1997). Given that GLT-1 is responsible for 90% of forebrain glutamate uptake in adult CNS (Robinson, 1998), the imbalance of glutamate homeostasis at the glutamatergic synapse could occur when astrocytic GLT-1 expression and function decline.

Peroxisome proliferator-activated receptors (PPARs) are ligand-regulated transcription factors, and belong to a nuclear hormone receptor superfamily (Desvergne and Wahli, 1999; Evans et al., 2004). There are three isoforms of PPARs, including PPARα, PPARβ/δ, and PPARγ, which are activated by fatty acids and fatty

*Abbreviations:* BIS1, bis-indolylmaleimide 1; CS, calf serum; CNS, central nervous system; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; dbcAMP, dibutyryl cAMP; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylenediaminetetra acetic acid; EAAT, excitatory amino-acid transporter-; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter-1; NF-κB, nuclear factor kappa-B; PPARα, peroxisome proliferator-activated receptor-alpha; PPRE, PPAR response element; PDL, poly-D-lysine; PKC, protein kinase C; QPCR, quantitative real-time polymerase chain reaction; RFP, red fluorescent protein; SDS, sodium dodecyl surface.

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acid-derived molecules. PPARs regulate the expression of their target genes via the hetero-dimerization with retinoid x receptor, another nuclear hormone receptor, and then bind to the PPAR response element (PPRE) located in the promoter region of the target genes. PPARs play an important regulatory role in the energy metabolism, cellular lipid/protein metabolism and development (Tyagi et al., 2011). Among PPAR isoforms, PPARα actively participates in astrocytic lipogenesis, whereas PPARγ is mainly involved in the regulation of glycogenesis (Cristiano et al., 2005). The PPARα agonists, fenofibrate and WY 14643, have shown to inhibit the production of proinflammatory mediators (i.e. nitric oxide, tumor necrosis factor-α, interleukin-1β, interleukin-6) in mouse primary astrocytes (Xu et al., 2006). PPARα activation by its agonist WY14643 has also been reported to increase the levels of a high affinity organic/carnitine transporter OCTN3 in rat astrocytes (Januszewicz et al., 2009). Moreover, OCTN3 is thought to regulate peroxisomal fatty acid metabolism and mediate lipid metabolic homeostasis in astrocytes (Januszewicz et al., 2009). These studies point to the important biological function of PPARα in the astrocytic cell metabolism and anti-inflammation. The PPARγ agonist rosiglitazone has been shown to increase the expression of GLT-1 at the transcriptional level and augment astrocytic glutamate uptake activity, thereby protecting neurons from oxygen-glucose deprivation (Romera et al., 2007). However, the involvement of PPARα in the regulation of astrocytic GLT-1 expression and glutamate uptake is not yet disclosed.

Neuronal signaling and cAMP-dependent pathway participate in the regulation of GLT-1 expression in astrocytes (Schlag et al., 1998; Zhou and Sutherland, 2004). The cAMP-induced increase in GLT-1 expression is through the $PI_3K$-dependent pathway that activates nuclear factor kappa-B (NF-κB) to promote GLT-1 transcription (Sitcheran et al., 2005; Zelenaia et al., 2000). Thus, a membrane-permeable cAMP analogue, dibutyryl cyclic-AMP (dbcAMP), is widely used to mimic neuronal effect on an increase in GLT-1 expression and trafficking (Chan et al., 2012; Kalandadze et al., 2002; Li et al., 2006; Zhou and Sutherland, 2004). Based on the findings as stated above, in this study we used dbcAMP-primed astrocytes as an in vitro culture model to examine the expression of GLT-1 after exposure to the saturated fatty acid palmitic acid (PA) or to the two PPARα agonist (GW7647 and WY14643). Our findings demonstrate that the PPARα ligands caused no effect on GLT-1 translation in astrocytes, but increased the endocytosis of GLT-1 molecules on the cell processes of astrocytes along with the reduction in astrocytic glutamate uptake ability. Moreover, the increase in GLT-1 internalization is possibly through the protein kinase C (PKC)-mediated pathway. The findings provide important evidence that the activation of PPARα in astrocytes might attenuate the clearance of extracellular glutamate molecules at the glutamatergic synapse.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium, trypsin- ethylenediaminetetraacetic acid (EDTA), and antibiotics (penicillin and streptomycin) were purchased from Gibco (Invitrogen Corporation, Carlsbad, USA). Calf serum (CS) and fetal bovine serum (FBS) were from HyClone Laboratories (Logan, UT, USA). Bovine serum albumin (BSA), N6,2′-*O*-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP), dynasore, palmitic acid (PA), poly-D-lysine (PDL), and protease inhibitor cocktail were purchased from Sigma-Aldrich. Bisindolylmaleimide I (BISI) was from Calbiochem (Cambridge, MA, USA). TRIzol™ was from Invitrogen (Carlsbad, CA, USA). PPARα antagonist (GW6471) and PPARα agonists (GW7647 and WY14643) were from Tocris Bioscience (Ellisville, MS, USA). [$^3$H]-L-Glutamate was purchased from Amersham Biosciences (Piscataway, NJ, USA). Mem-PER™ plus a membrane protein extraction kit and Pierce cell surface protein isolated kit were obtained from Thermo Scientific Biotechnology (Rockford, IL, USA).

### 2.2. Cell culture

The preparation of mixed glia cultures followed a previously described protocol (Chan et al., 2012). Cerebral cortices were collected from one- to two-day-old Sprague-Dawley rat pups. The preparation was performed in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, National Cheng Kung University. The tissues were homogenized and filtered through a 70-μm nylon filter mesh. After centrifugation, the cell pellets were re-suspended in DMEM/F-12 medium containing 10% FBS and seeded onto PDL-coated T-75 cell culture flasks. The cultures were maintained in a 5% $CO_2$ incubator at 37 °C, and the medium was renewed every two to three days. Seven to eight days later, microglia were removed using the shake-off method. The cultures were maintained in DMEM/F-12 medium containing 10% CS. The medium was changed every three days until it was used. The remaining cells in the cultures consisted of 92% primary rat astrocytes with glial fibrillary acidic protein (GFAP)-positive immunostaining, and about 5–7% of Iba1-positive microglia. After subculture, primary astrocytes were primed by 300 μM of dbcAMP for 24 h to induce their stellation and GLT-1 expression prior to treatment.

### 2.3. Preparation of palmitic acid solution

Palmitic acid (PA) stock solution (100 mM) was prepared in 0.1 M NaOH at 70 °C for 10 min, and then mixed at 55 °C for 10 min with 50 g/L BSA at the ratio of 1:19. The final stock concentration of PA was 5 mM.

### 2.4. Cell viability assay

The cells were replated onto a 24-well plate with DMEM/F-12 medium containing 10% CS. After treatment, the cultures were subjected to 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) cell viability assay. The assay was performed by the incubation of the cells in MTT solution (0.5 mg/mL per well) for 4 h. The product (formazan) was lysed in dimethyl sulfoxide (DMSO) for 10 min at 37 °C. MTT absorbance was measured using an ELISA reader at 595 nm.

### 2.5. Quantitative real-time polymerase chain reaction (QPCR)

The cells were seeded at the density of $1 \times 10^6$ cells onto a 60 mm petri-dish. After treatment, cell lysis was conducted using TRIzol™ solution following a previously described procedure (Chan et al., 2012). One μg of RNA was then converted into cDNA by MMLV-reverse transcriptase (Invitrogen). PCR amplification was performed using a LightCycler® FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) for 10 min at 95 °C, followed by 40 cycles set for 10 s at 95 °C, annealing for 10 s at 65 °C, and extending for 2 s at 72 °C. The data (Cycle at threshold, Ct) were normalized to those of the housekeeping gene GAPDH as ΔCt ($Ct_{GLT\text{-}1}$-$Ct_{GAPDH}$), and were expressed as the relative mRNA levels by comparison of $\Delta Ct_{\ treatment}$ to $\Delta Ct_{\ control}$ ($2^{\Delta Ctreatment\ -\ \Delta Ctcontrol}$). The primers used in this study were designed using the Primer-BLAST software provided by National Center for Biotechnology