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Binding and activation of G protein-coupled receptor 40 by perfluorodecanoic acid

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Abstract

Perfluoroalkyl acids (PFAAs) have been shown to disrupt insulin secretion in animals, and are significantly related to serum insulin concentration and diabetes incidence in several epidemiological studies. G-protein coupled receptor 40 (GRP40) is a transmembrane receptor and is activated by medium- and long-chain fatty acid. This receptor plays an important role in insulin secretion. In this study, the direct binding interactions between perfluorodecanoic acid (PFDA) and GPR40 and the subsequent alteration of GPR40 activity were investigated. The results indicated that PFDA could bind GPR40 directly with a K_d of 2.70 μM. The $[Ca^{2+}]i$ assay indicated that the PFDA was able to induce the elevation of $[Ca^{2+}]i$, and the GW1100 could inhibit entirely the PFDA-induced $[Ca^{2+}]i$ increasing, indicating that the $[Ca^{2+}]i$ increasing induced by PFDA in this study was GRP40-mediated. This study showed the PFDA could directly bind GPR40 and activate the signal pathway.

Keywords: G-protein coupled receptor 40; perfluorodecanoic acid; Binding assay; Activation potency

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Introduction

Perfluoroalkyl acids (PFAAs) are found in the environment globally and have been detected in the liver, fat, and serum of wild animals, as well as in human serum, breast milk and semen (Houde et al., 2006; Raymer et al., 2012). Due to their environmental persistence, bioaccumulation and biomagnification through the food web, the risks of PFAAs to human health are of great concern and currently under evaluation (Conder et al., 2008).

A number of studies on acute, short term, and longer-term toxicity of PFAAs on various species suggest developmental toxicity, reproductive toxicity, immunotoxicity, and carcinogenicity in test animals (Kennedy et al., 2004; Corsini et al., 2012). Many of the toxicity are thought to be related to interactions between PFAAs and peroxisome proliferator activated receptor alpha (PPARα). Activation of the PPARα results in transcription of a range of genes including those involved in peroxisomal enzymes, peroxisome proliferation, specific lipid metabolism and biosynthesis of sterol and bile acids(Wolf et al., 2012; Takacs and Abbott, 2007). However, recent studies in PPARα knockout animals showed that the PPARα is not the only mechanism involved in transmission of some PFAAs-induced toxicity (Abbott et al., 2009; Minata et al., 2010). Some studies also revealed that PFAAs bind directly and activate estrogen receptors (ER), and induce ER-mediated transcriptions in cells (Gao et al., 2013). Besides the unclear receptors, PFAAs also can bind with some transport proteins, such as serum albumin (Bischel et al., 2010), fatty acid binding protein (Zhang et al., 2013) and thyroid hormone transport proteins (Weiss et al., 2009). Although some biological targets have been gradually revealed for exploring molecular mechanisms underlying PFAAs toxicity, there are still other proteins or pathways *in vivo* to potentially interact with PFAAs.

Lots of epidemiological studies revealed that the PFAAs were significantly related to serum insulin concentration (Halldorsson et al., 2012; Lin et al., 2009). The positive relationship between PFAAs concentration and the incidence of diabetes also was reported (Lind et al., 2014; Lundin et al., 2009). The animal studies also demonstrated that the PFAAs could induce the increasing of serum insulin (Hines et al., 2009). However, the mechanism by which the PFAAs induce diabetes and insulin disorder is unclear.

The G-protein coupled receptor 40 (GRP40), known as free fatty acid receptor 1, is a transmembrane receptor and is known for its relation to insulin secretion in the pancreas (Ma et al., 2007). This receptor has drawn considerable attention as a potential therapeutic target for type II diabetes (Alquier and Poitout, 2009). Loss of function of GPR40 has shown that the GPR40 mediates, at least in part, fatty acid-amplification of glucose-induced insulin secretion. The GPR40 is mainly expressed in pancreatic β-cells and activated by medium- and long-chain fatty acid. The activation

of GPR40 in cells induce an increase of intracellular [Ca^{2+}] ([Ca^{2+}]i) and insulin secretion (Feng et al., 2012).

Due to their structural similarity between PFAAs and fatty acids, we speculate the GPR40 may be a target of PFAAs toxicological actions *in vivo*. However, to date, the binding property between PFAAs and GPR40 and the subsequent alteration of GPR40 activity has not been reported yet. Therefore, in the present work, *in vitro* binding of PFAAs to GPR40 and their activity on the receptor in cells were investigated for the first time.

2. Materials and methods

2.1 Materials.

4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Dodecanoic Acid (BODIPY-C12) was purchased from Molecular Probes (Eugene, OR, USA). Oleic acid (OA), perfluorodecanoic acid (PFDA), digitonin and anti-FLAG M2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade. The plasmid of pcDNA-3-GPR40-FLAG (C-terminally tagged) was a generous gift from Prof. Graeme Miligan (University of Glasgow, Glasgow, Scotland, United Kingdom) (Stoddart et al., 2007). Magnabind Protein G beads (particle size 1~4 μm) was purchased from Pierce (Rockford). The Fura-2, AM was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade.

2.2 Cell Culture and Transfection.

HEK-293T cells were cultured in RPMI 1640 medium (Gibco, Grand Island, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin. Cells were maintained in a humidified incubator at 37 ℃ and 5% CO_2 atmosphere. The plasmid of pcDNA-3-GPR40-FLAG was transiently transfected into cells using GenJet Ver.Ⅱ (Signa Gen, Rockville, MD). After 32 h incubation, the cells were serum-starved for 4 h. The cell suspensions were washed with cold PBS, and then were lysed and the GPR40 receptor was solubilized in a buffer consisting of 10 mM PBS, 1% digitonin and 1% protease inhibitor cocktail. To approximately 2×10^7 cells, 200 μL of solution buffer was added and the mixture was placed on a rocker at 4℃. After 45 min the mixture was centrifuged at 12,000 rpm for 30 min at 4℃, and the supernatants containing GPR40 receptor were transferred to new tubes and kept frozen at -80℃ until analysis.

2.3 Flow Cytometry Analysis.

The flow cytometry analysis was performed according to the method reported by Hara et al (2009). with some modifications. Briefly, the cells lysates were incubated with 2 μg of anti-FLAG M2 antibody for 1 h on a rotator on ice. Then 15 μL of Magnabind protein G beads was added to the mixture. After 1 h of incubation, the GPR40-beads complex was collected using an external magnetic field. The complex was washed two times with PBS containing 1% digitonin and 1% protease inhibitor cocktail, and then stored in 200 μL of PBS containing 0.05% digitonin and 1% protease inhibitor cocktail.

A 10-μL aliquot of the GPR40-beads complex was added into 190 μL PBS buffer with 0.05% digitonin, 1% protease inhibitor cocktail, and different amount of BODIPY-C12. After the samples had been allowed to equilibrate for 10 min in dark, the fluorescence intensity of the beads was measured using flow cytometry.

The binding affinity of PFDA and GPR40 was determined in competitive binding assays using BODIPY-C12 as the fluorescence probe. In each assay, 10 μL of GPR40-beads complex (~10^5 counts), BODIPY-C12 and different concentrations of PFDA were mixed in a total volume of 200 μL PBS containing 0.05% digitonin and 1% protease inhibitor cocktail, and incubated for 10 min at room temperature in dark, then the fluorescence intensity of the beads was measured and recorded. The displacement of bound fluorescent probe was calculated from the decrease of fluorescence value of the beads with increasing PFDA concentrations. The competition curves were fitted with a sigmoidal model to derive an IC_{50} value. The K_d value was calculated according to Equation 1.

$$IC_{50\ ligand} / [BODIPY\text{-}C12]_{total} = K_{d\ ligand} / K_{d\ BODIPY\text{-}C12} \quad \text{(Eq.1)}$$

Where $K_{d\ BODIPY\text{-}C12}$ is the measured K_d for BODIPY-C12 obtained as described above, and $[BODIPY\text{-}C12]_{total}$ is the total concentration of BODIPY-C12 used in this experiment.

[Ca^{2+}]i Assay.

The changes of [Ca^{2+}]i were measured simultaneously by the fura-2 ratiometric method. Briefly, HEK-293T cells were seeded in a 96-cell plate at a density of 1×10^4 cells/well. When the cells reached 80~90% confluency, they were transfected with pcDNA-3.0-GPR40 plasmid. After 20 h incubation, the cells were serum-starved for 2 h. The cells were loaded with 5 μM Fura-2, AM, for 30 min in culture medium, washed with HBSS buffer. Then the cells were immediately exposed in PFDA with various concentrations for 10 min. Fluorescence at 510 nm was measured by using alternating excitation of 340 and 380 nm. The [Ca^{2+}]i was expressed as the ratio of fluorescence intensity at excitation wavelengths 340 and 380 nm.

3. Results

3.1 BODIPY-C12 binding property

The mild nonionic detergent of digitonin was used to lyse the cells and solubilize the GPR40 receptor. The FLAG-tagged GPR40 receptor was expressed in the HEK-293T cells and captured on the Magnabind Protein G beads *via* an anti-FLAG antibody (Fig. 1). The fluorescence intensity of the GPR40-beads complex was measured by flow cytometry analysis. As shown in Fig. 2, the fluorescence intensity of the GPR40-beads complex increased significantly as the BODIPY-12 added, while fluorescence intensity for the Naked-beads (which incubated with the lysates of untransfected HEK-293T cells) had no changes. This result indicating that the fluorescence probe can specially bind the

GPR40 receptor on the GPR40-beads complex. The K_d value obtained from the binding curve was 0.92 μM.

In order to test whether the flow cytometry method can be used in competitive ligand binding assays, we used OA as ligand to displace the fluorescent probe. As can be seen from Fig. 3, the addition of OA resulted in decrease of fluorescence intensity of the GPR40-beads complex, indicating that BODIPY-C12 was displaced from the receptor. The IC_{50} value was determined as 2.07 μM. The calculated K_d of OA was 1.90 μM, which is close to the value of 1.3 μM reported by Hara et al (2009).

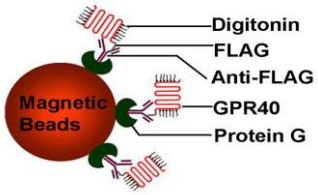

Fig. 1 Schematic diagram of the GPR40-beads complex

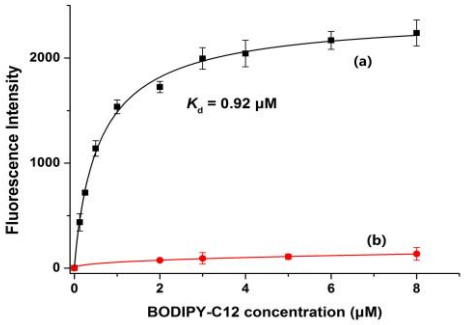

Fig.2 Fluorescence intensity of (a) GPR40-beads complex and (b) Naked-beads with increasing BODIPY-C12

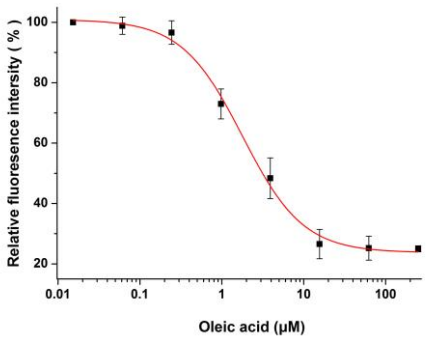

Fig.3 Competition curves of BODIPY-C12 with OA

3.2 Assessment of PFDA binding affinity with GPR40

After the flow cytometry method was established and validated, the binding interaction of PFDA with GPR40 was investigated. The fluorescence displacement curve for PFDA was shown in Fig. 4. The PFDA alone has no effect on the probe fluorescence intensity, so the decrease of fluorescence intensity of the beads is attributed to the competitive binding of PFDA and BODIPY-C12 to the receptor. The IC_{50} and K_d values of PFDA were obtained as 29.4 and 27.0 μM, respectively.

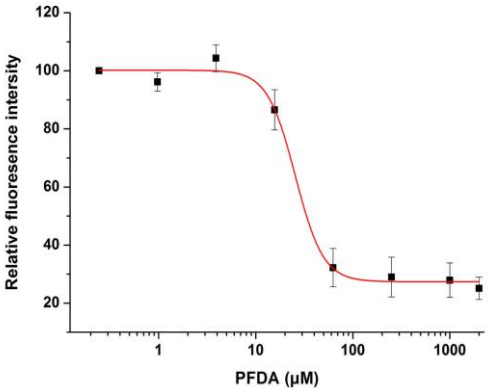

Fig. 4 Competition curves of BODIPY-C12 with PFDA

3.3 Effect of PFDA on $[Ca^{2+}]i$

We further examined the effect of PFDA on the $[Ca^{2+}]i$ in HEK-293T cells either transfected or untransfected GPR40, and the results was shown in Fig.5. For the untransfected cells, PFDA exposure cannot induce any changes of $[Ca^{2+}]i$, but For the GPR40-transfected cells, the PFDA exposure could obviously change the $[Ca^{2+}]i$.

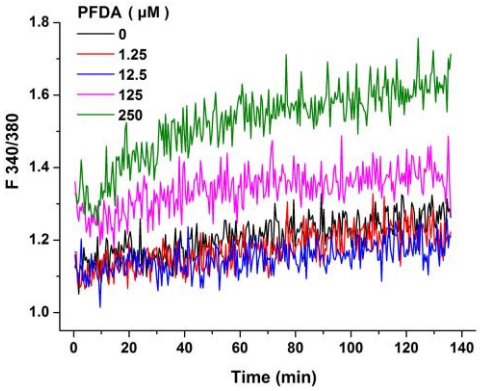

Fig.5 PFDA induced $[Ca^{2+}]i$ changes in HEK-293T cells.

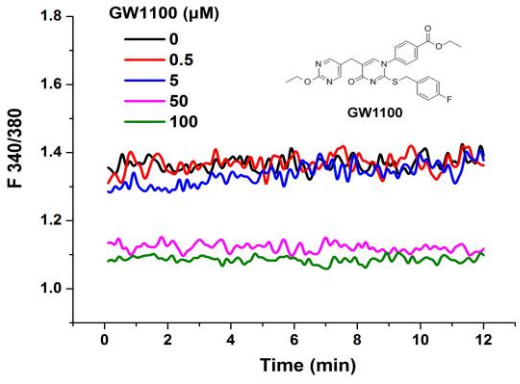

Fig.6 The effect of GW1100 on the PFDA induced $[Ca^{2+}]I$ changes.

In order to further examine whether the PFDA induced $[Ca^{2+}]i$ increasing is GPR40-mediated, the GW1100, a selective antagonist of GPR40-mediated Ca^{2+} elevations, was used in the experiment. As shown in Fig.6, the GW1100 could inhibit the PFDA induced $[Ca^{2+}]i$ increasing at the concentration of 50 or 100 μM. These results indicated that the PFDA induced $[Ca^{2+}]i$ elevation was GPR40-dependent.

Conclusion

In the present study, we determined the binding potency of PFDA with GPR40 by flow cytometry method based competitive binding assay. The results indicated the PFDA could directly bind GPR40 with a K_d of 2.70 μM. The GPR40-binding can further induce $[Ca^{2+}]i$ increase. We first prove in this paper that, besides the nuclear receptor, transporter, and enzyme, the G-protein coupled receptor also is a target of PFAAs and can mediate the PFAAs-induced toxicity, this means that the PFAAs can cause toxicity even without entering the cells. We hope that our study would help to improve our understanding on the mechanisms of PFAAs interference on G-protein coupled receptors and may on slight on the mechanisms of PFAAs induced diabetes and insulin disruption.

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