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Interaction kinetics of liposome-incorporated unsaturated fatty acids with fatty acid-binding protein 3 by surface plasmon resonance

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ABSTRACT

The role of heart-type fatty acid-binding protein (FABP3) in human physiology as an intracellular carrier of fatty acids (FAs) has been well-documented. In this study, we aimed to develop an analytical method to study real-time interaction kinetics between FABP3 immobilized on the sensor surface and unsaturated C18 FAs using surface plasmon resonance (SPR). To establish the conditions for SPR experiments, we used an FABP3-selective inhibitor 4-(2-(1-(4-bromophenyl)-5-phenyl-1*H*-pyrazol-3-yl)-phenoxy)-butyric acid. The affinity index thus obtained was comparable to that reported previously, further supporting the usefulness of the SPR-based approach for evaluating interactions between FABPs and hydrophobic ligands. A pseudo-first-order affinity of FABP3 to K^+ petroselinate (C18:1 Δ6 *cis*), K^+ elaidate (C18:1 Δ9 *trans*), and K^+ oleate (C18:1 Δ9 *cis*) was characterized by the dissociation constant (K_d) near micromolar ranges, whereas K^+ linoleate (C18:2 Δ9,12 *cis*/*cis*) and K^+ α-linolenate (C18:3 Δ9,12,15 *cis*/*cis*/*cis*) showed a higher affinity to FABP3 with K_d around 1×10^{-6} M. Interactions between FAPB3 and C18 FAs incorporated in large unilamellar vesicles consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and FAs (5:1 molar ratio) were also analysed. Control DMPC liposomes without FA showed only marginal binding to FABP3 immobilized on a sensor chip while liposome-incorporated FA revealed significant responses in sensorgrams, demonstrating that the affinity of FAs to FABP3 could be evaluated by using the liposome-incorporated analytes. Significant affinity to FABP3 was observed for monounsaturated fatty acids (K_d in the range of 1×10^{-7} M). These experiments demonstrated that highly hydrophobic compounds in a liposome-incorporated form could be subjected to SPR experiments for kinetic analysis.

1. Introduction

The leading cause of human mortality is cardiovascular disease, which affects populations in both developed and developing countries, with an estimated predicted death rate of nearly 23.6 million by the year 2030.[1] Research in animal models has shown that fatty acid binding protein 3 (FABP3) maintains the energy homeostasis in the heart,[2] regulates lipid metabolism and adipose tissue development,[3] increases insulin sensitivity,[4] and controls dopamine D2 receptor function in the brain.[5] FABP3 levels in the serum have been shown to strongly correlate with body mass index but weakly with hypertension.[6] FABP3 presence in the blood has been identified as an early biochemical marker for acute myocardial infarction and Creutzfeldt–Jakob disease; FABP3 role as a possible tumor suppressor in breast adenocarcinoma has also been suggested.[6] In addition, it has been shown that FABP3 may interfere with the treatment of cardiovascular disease because certain specific drugs can act as FAPB3 inhibitors by interacting with the fatty acid binding site, which renders the drug inactive and causes

Abbreviations: CMC, critical micelle concentration; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; FABP3, fatty acid binding protein 3; FAs, fatty acids; k_a, association rate constant; k_d, dissociation constant; K_d, equilibrium dissociation constant; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; MUFAs, mono-unsaturated FAs; NHS, N-hydroxysuccinimide; PI, 4-(2-(1-(4-bromophenyl)-5-phenyl-1*H*-pyrazol-3-yl)-phenoxy)-butyric acid; PUFAs, poly-unsaturated fatty acids; SPR, surface plasmon resonance.

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dyslipidaemia. Very recently, Kumagai et al. has revealed the potential clinical use of Una-G belonging to the FABP family as a ligand-activated fluorescent probe for detecting a biomarker.[7]

Fatty acids (FAs) in the cell cytosol originate from dietary lipids and de novo lipogenesis from carbohydrates. FA metabolism by β-oxidation in peroxisomes and mitochondria and ω-oxidation in microsomes require carrier proteins. Cytosolic carriers are also necessary for FAs to act as effectors of nuclear transcription and to be stored in adipocytes as triacylglycerides. FABPs represent a family of abundantly produced intracellular proteins involved in the transport of FAs. FABPs are relatively small 15-kDa polypeptides containing an N-terminal helix-turn-helix motif (αI–αII) that caps one end of the β-barrel formed by 10 anti-parallel strands.[8] Among 12 FABP isoforms, FABP3 constitutes 4–8% of the total cytosolic proteins in the mammalian heart and is ubiquitously expressed in both cardiac and skeletal muscle and marginally in the stomach, brain, lungs, and mammary glands.[9,10] The helical N-terminus was shown to participate in the regulation of FA transfer to intracellular membranes through collision transfer interactions.[11,12] This 'portal hypothesis' states that an FA molecule enters solvent-accessible area of FABP through a dynamic region comprising the α-helix II and βC–βD and βE–βF turns before binding to the pocket.[13] Non-specific interactions with FABP3 hydrophilic surface as well as conformational changes facilitate FA binding and subsequent entering into the hydrophobic cavity of FABP3[14,15] while the carboxylate group as shown by X-ray crystallography is buried in the core of the protein.[16]

Surface plasmon resonance (SPR) is considered one of the most powerful techniques for evaluating the affinity kinetics of molecular interactions in biological systems. Kinetic analysis enables label-free, real-time investigation of biomolecular hydrophobic interactions; SPR-based biosensors allow accurate estimation of distinct association/dissociation rate constants and equilibrium status parameters in different reaction models. In order to mimic biochemical/biomedical conditions, SPR analysis has to be carried out in aqueous media and is therefore largely applied to binding studies of water-soluble ligands immobilized on a sensor chip. Recently, considerable progress has been made toward structure elucidation of membrane-associated receptors and lipid bilayers by casting membranes on the sensing surface.[17] On the other hand, highly hydrophobic biomolecules such as lipids and sterols, which often play an essential role in signal transductions and other physiological events, are still troublesome analytes because of their poor water solubility. In the present study, we have established the experimental conditions for evaluating interactions between FABP3 and a hydrophobic inhibitor by SPR, and attempted to estimate the binding affinity between FABP3 and long-chained FAs incorporated into large unilamellar vesicles (LUVs).

2. Materials and methods

2.1. Material

4-(2-(1-(4-Bromophenyl)-5-phenyl-1*H*-pyrazol-3-yl)-phenoxy)-butyric acid, a protein inhibitor (PI) for FABP3, was synthesized according to the previously reported method.[18] Free petroselinic, elaidic, oleic, linoleic, and α-linolenic acid were purchased from Sigma–Aldrich (St. Louis, MO), and treated with 1 M potassium hydroxide in methanol to be converted into potassium (K^+) salts. N-hydroxysuccinimide (NHS), ethanolamine, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 10 mM sodium acetate buffer (pH 4.5), 50 mM sodium hydroxide (NaOH), 0.5% (w/v) sodium dodecyl sulfate (SDS), and 10× PBS running buffer (detergent free, pH 7.4) were obtained from GE Healthcare (Uppsala, Sweden). Dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich. All other chemicals were of analytical grade.

2.2. Purification and delipidation of FABP3

The human *FABP3* gene (hFABP3) was synthesized with an N-terminal *Nde*1 site and C-terminal *Bam*H1 site. The 399-bp fragment was ligated into the *Nde*1/*Bam*H1-digested pET21a vector (Novagen, Madison, WI) and the expression plasmid pET21a hFABP3 was used to transform *Escherichia coli* BL21 (DE3). The expression of hFABP3 was induced by adding isopropyl-β-D-thiogalactopyranoside to the culture medium. Bacteria were grown as previously described,[19] harvested by centrifugation, sonicated, and centrifuged at 100,000*g* for 30 min at 4 °C. The supernatant was fractionated with ammonium sulphate and the appropriate fraction was dialyzed against 50 mM Tris–HCl (pH 8.0). The dialysate was passed through an anion exchange Hitrap DEAE FF column equilibrated with 50 mM Tris–HCl (pH 8.0), concentrated by ultrafiltration, and purified by size-exclusion column chromatography; refolding after delipidation was then performed.

2.3. Liposome preparation

LUVs were prepared by thin film hydration, freeze-thaw and subsequent extrusion. Lipid thin films were formed by dissolving 30 mg of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) without or with FA K^+ salts (5:1 molar ratio) in 6 mL $CHCl_3$ in a round bottom flask. The resultant solution was mixed thoroughly, evaporated, and the obtained film was further dried under vacuum for 12 h. The lipid film was then hydrated and suspended in 1 mL of running buffer (PBS, pH 7.4, with or without 5% DMSO). The suspension was sonicated and subjected to 5 cycles of freezing (−80 °C), thawing (60 °C), and vortexing (5 s) to form multilamellar vesicles (MLVs). The MLV suspension was passed through a double 0.1-μm-polycarbonate membrane filter 19 times with LiposoFast-Basic extruder (Avestin Inc., Ottawa, Canada) to form LUVs. The FA and DMPC concentrations of the solution were determined by the Free Fatty Acid Quantification Colorimetric/Fluorometric Kit (BioVision, Inc., Milpitas, CA) and Phospholipid *C*-Test (Wako Pure Chemical Industries Ltd, Japan) respectively, and the liposomes were diluted with PBS (pH 7.4) to give final FA concentrations of 20, 40, 60, and 80 μM.

2.4. Interaction measurements by SPR

The SPR biosensing investigations were performed at a controlled temperature of 25 °C using an FABP3-modified carboxylated dextran matrix (CM5) chip and analysed using the BIAcore T200 system (GE Healthcare). The unmodified CM5 sensor chip was washed 3 times with 50 mM NaOH at a flow rate of 20 μL/min for 2 min. Pre-concentration tests using 10 mM sodium acetate buffer at pH 4.0, 4.5, 5.0, and 5.5 showed the optimum binding at pH 4.5. FABP3 was covalently immobilized on the CM5 sensor chip using a modified amine coupling technique on a flow cell 2 or 4; cells 1 and 3 were unaltered and used as a reference. Surface activation was induced by injecting 70 μL of 0.1 M NHS mixed with 0.39 M EDC (1:1, v/v), at a flow rate of 5 μL/min for 7 min. FABP3 (100 μg/mL) was passed at 2 μL/min for 30 min over the sensor chip surface. NHS ester groups were deactivated with 1 M ethanolamine hydrochloride (pH 8.5) and the resulting surface was washed 3 times with running buffer (PBS pH 7.4 with or without 5% DMSO) to remove unbound species. The acceptable immobilization levels (referred to as bound and final FABP3 responses) were between 9000 and 13,000 response units (RU). Mass transfer limitation analysis did not show any significant variations in the rate constants depending on flow rates (5, 15, or 75 μL/min); therefore, all experiments were performed at a flow rate of 10 μL/min. Dissociation time (complete elution of the sample) was either 50 or 120 s. In each kinetic analysis, a blank run with only running buffer