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Discovery of a Protein−Metabolite Interaction between Unsaturated Fatty Acids and the Nuclear Receptor Nur77 Using a Metabolomics Approach

Nawaporn Vinayavekhin and Alan Saghatelian*

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, United States

Supporting Information

ABSTRACT: Neuron-derived clone 77 (Nur77) is an orphan nuclear receptor with currently no known natural ligands. Here we applied a metabolomics platform for detecting protein−metabolite interactions (PMIs) to identify lipids that bind to Nur77. Using this approach, we discovered that the Nur77 ligand-binding domain (Nur77LBD) enriches unsaturated fatty acids (UFAs) in tissue lipid mixtures. The interaction of Nur77 with arachidonic acid and docosahexaenoic acid was subsequently characterized using a number of biophysical and biochemical assays. Together these data indicate that UFAs bind to Nur77LBD to cause changes in the conformation and oligomerization of the receptor. UFAs are the only endogenous lipids reported to bind to Nur77, which highlights the use of metabolomics in the discovery of novel PMIs.

Nuclear receptors (NRs) are a class of ligand-dependent transcription factors that control a variety of physiological processes.[1,2] Because NR transcriptional activity can be regulated by natural and synthetic small-molecule ligands, NRs have in recent years become an important target for the development of new drugs.[3−5] Nearly half of the 48 human NRs still have no known natural ligands, and receptors lacking ligands are called orphan nuclear receptors.[6] The identification of a natural ligand(s) for an orphan NR helps characterize the NR, identify a new role for the ligand, and provide insights into physiological regulation of the NR.

The orphan NR Nur77 belongs to the NR subfamily 4A (NR4A). Along with the two other subfamily members (Nurr1 and NOR1), Nur77 controls critical biological functions such as apoptosis,[7,8] differentiation,[2] and gluconeogenesis.[9,10] Structurally, Nur77 shares common features with other receptors, which include (i) an N-terminal domain containing an activation function-1 (AF-1) and a DNA binding domain (DBD) and (ii) a C-terminal domain containing AF-2 and a ligand binding domain (LBD).[11,12] The LBD is responsible for the binding of the ligand as well as receptor homo- and heterodimerization.[1,12] NR monomers and dimers can recognize different DNA sequences to mediate transcription,[13−15] and ligands can influence the dimerization state.[16,17] Interestingly, unlike other NRs, structural studies of Nur77 and Nurr1 have suggested that these proteins do not use ligands to control transcription because they lack a binding pocket.[18−20]

The recent discoveries of the small-molecule Nur77 agonists 1,1-bis(3′-indolyl)-1-(*p*-methoxyphenyl)methane[21,22] and cytosporone B (csnB)[23] indicate that this assumption is inaccurate and that small molecules can interact with Nur77 and modulate its transcriptional activity after all. Even though the crystal structures of some orphan NRs exhibit no ligand binding site (e.g., Nur77 and TR4), NRs have been found to undergo conformational changes in the presence of ligands, which may unveil a binding site.[24] Prompted by the possibility that Nur77 is regulated through a ligand-dependent pathway, we set out to identify potential small-molecule ligands for Nur77 using a metabolomics strategy for elucidating protein−metabolite interactions (PMIs).[25,26]

Our metabolomics-based approach started with immobilization of the recombinant Nur77 onto a solid support (Figure 1). The gene for the LBD of human Nur77 (Nur77LBD) was first synthesized, and the protein was then expressed from a vector containing a polyhistidine (His6) tag [see the Supporting Information (SI)] to produce the His6-tagged Nur77LBD (His6-Nur77LBD). After purification by immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC),[18] we determined whether the His6-Nur77LBD was folded by using the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS).[27] ANS binds nonspecifically to the hydrophobic cavity of the protein and shows saturation binding with folded protein samples. Titration of His6-Nur77LBD with ANS yielded saturation binding, indicating that the protein was folded (see the SI). In addition, His6-Nur77LBD was able to enrich the reported Nur77 agonist csnB from brain lipid extracts with a 7-fold enrichment of csnB over the no-protein control (see the SI), indicating that the protein was folded and could interact with the known agonists.

Here we used His6-Nur77LBD to select for small molecules that bind specifically to the ligand-binding pocket of Nur77. Because the reported Nur77 agonist csnB contains functional groups reminiscent of mammalian lipids (alkyl chains and carbonyl groups), we hypothesized that Nur77 natural ligands are lipophilic molecules. Therefore, the bound His6-Nur77LBD was incubated with a lipophilic metabolite extract from mouse brain or testes where Nur77 is expressed.[28] During the incubation step, Nur77 ligands could bind to the NR to form a protein−metabolite complex. The mixture was subsequently filtered, after which the unbound lipids were washed away and the protein was eluted. As a control, a solid support lacking NR

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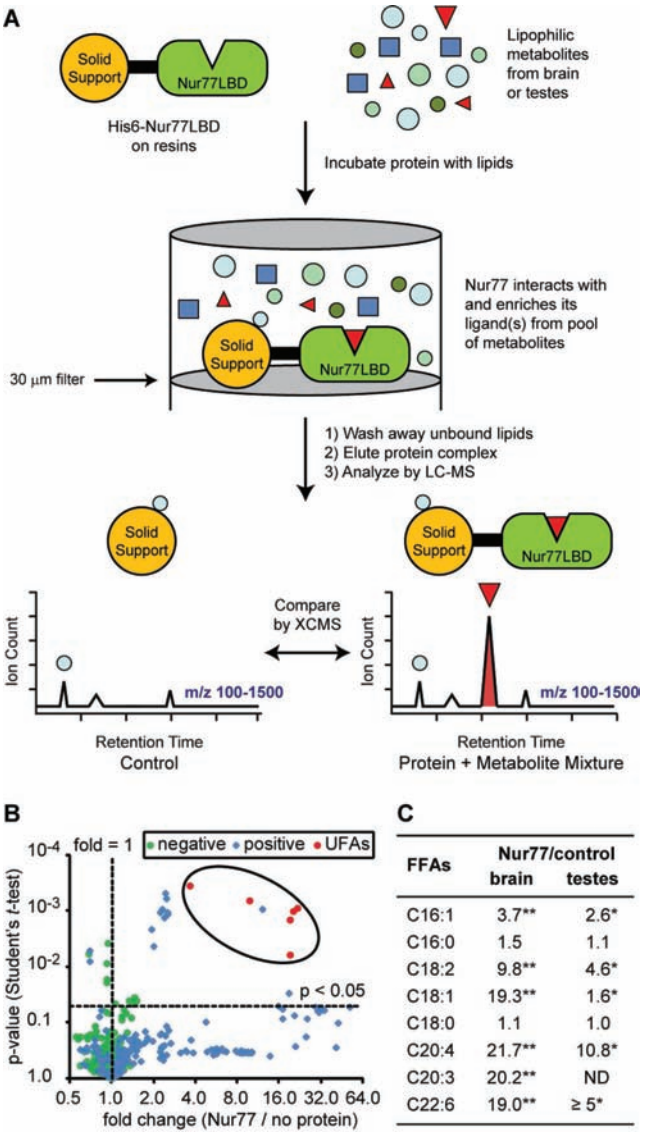

FFAs	Nur77/control brain	Nur77/control testes
C16:1	3.7**	2.6*
C16:0	1.5	1.1
C18:2	9.8**	4.6*
C18:1	19.3**	1.6*
C18:0	1.1	1.0
C20:4	21.7**	10.8*
C20:3	20.2**	ND
C22:6	19.0**	≥ 5*

Figure 1. Metabolomics strategy for identification of potential Nur77 ligands. (A) Metabolomics workflow for the discovery of PMIs with Nur77LBD. (B) Plotting each metabolite ion based on its statistical significance and fold enrichment value identifies significantly enriched ions. Ions in negative- and positive-mode MS are represented as green circles and blue diamonds, respectively, and the corresponding unsaturated fatty acid (UFA) ions as red circles. (C) UFAs were enriched by His6-Nur77LBD relative to the no-protein control in samples incubated with lipid extracts from brain and testes. The experiment identified UFAs as potential Nur77 ligands. Fold changes (Nur77/control) represent statistically significant differences between His6-Nur77LBD and no-protein control samples (Student's *t* test; *, *p*-value <0.05; **, *p*-value <0.01; $N = 3$). FFA, free fatty acid; ND, not detectable.

was used to account for any background from lipids binding to resin.

For analysis, the eluted sample was analyzed by liquid chromatography−mass spectrometry (LC−MS) using an untargeted metabolomics platform.[29] In contrast to a targeted approach where only known metabolites of interest are selected for monitoring and quantitation, an untargeted platform allows quantitation of all ionizing metabolites simultaneously, measuring both known and structurally novel metabolites based on their

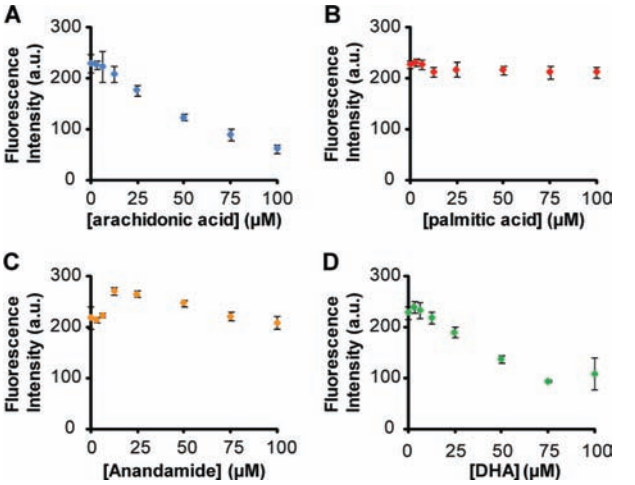

Figure 2. ANS displacement assays showing the displacement of 800 μM ANS from 0.5 μM His6-Nur77LBD by increasing concentrations of (a) AA or (d) DHA but not by (b) PA or (c) AEA.

MS ion intensity (MSII).[30] To cover the broadest range of metabolites, LC−MS was also performed in both the negative- and positive-ion modes. The metabolite profiles of the protein samples were then compared to those of the control samples using the XCMS program[31] to identify metabolites bound to and enriched by Nur77 in an unbiased manner.

Using His6-Nur77LBD in the metabolomics-based ligand-enrichment experiment, we found elevated levels of unsaturated fatty acids (UFAs) in the eluate of protein samples relative to the control (Figure 1). From the metabolite profiles of the two sample sets (i.e., His6-Nur77LBD vs no protein), the unbiased analysis identified the NR-enriched lipids on the basis of their statistical significance ($p < 0.05$ and fold > 2). With a list of enriched lipids, the next step involved characterizing the metabolites using accurate mass, retention time, and coelution data (see the SI). Here we found that in brain extracts, His6-Nur77LBD enriched UFAs (Figure 1), which included palmitoleic acid (C16:1), linolenic acid (C18:2), oleic acid (C18:1), arachidonic acid (AA, C20:4), eicosatrienoic acid (C20:3), and docosahexaenoic acid (DHA, C22:6). Saturated fatty acids (i.e., C16:0, C18:0), on the other hand, were unchanged. Next, to check whether this result would hold up in other tissues expressing Nur77, we performed the same experiments with lipid extracts from testes. His6-Nur77LBD also enriched UFAs in this case. In fact, UFAs were the only class of lipids consistently enriched in both brain and testes extracts in these experiments.

To eliminate the possibility that the result depended in any way on the protein tag or beads, we repeated the experiments using a glutathione *S*-transferase (GST)-fused Nur77LBD (GST-Nur77LBD). As expected if the lipid were binding to the Nur77LBD, GST-Nur77LBD enriched UFAs in brain lipids in this case as well (see the SI). Together, these results pointed to the ability of Nur77LBD to enrich UFAs and identified UFAs as potential ligands for Nur77. Among UFAs, AA and DHA showed the greatest enrichment by His6-Nur77LBD in both brain and testes samples. Therefore, we chose to characterize the biochemical and biophysical interactions between Nur77 and UFAs further by using AA and DHA.

We tested AA binding to His6-Nur77LBD by displacing ANS from His6-Nur77LBD with AA (Figure 2). In this experiment, as

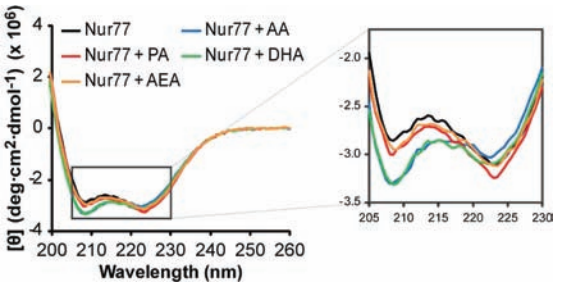

Figure 3. CD spectra showing changes in the conformation of His6-Nur77LBD upon treatment with 10 molar equiv of AA or DHA but not of PA or AEA.

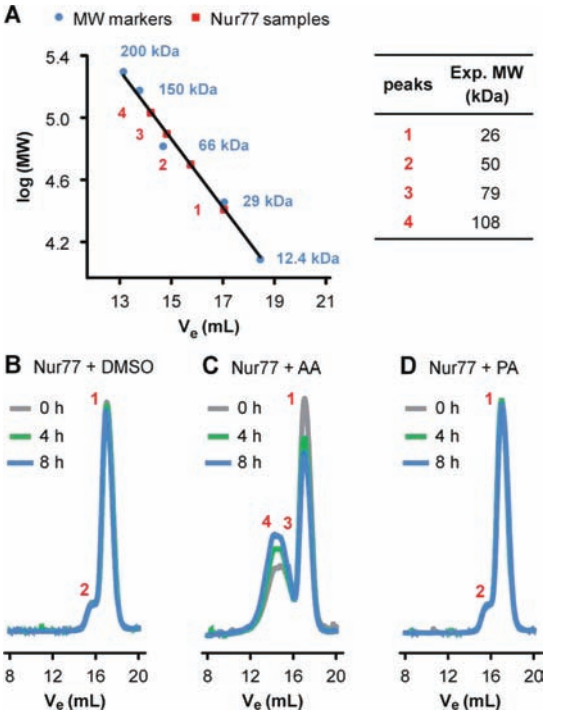

Figure 4. Determination of oligomeric states of His6-Nur77LBD in the presence or absence of lipids by SEC. (A) Standard curve plotted as a function of the logarithm of the molecular weight (MW) and the elution volume (V_e). The MWs of the proteins used as MW markers are indicated along the plot in blue. The data were fitted to a linear-regression model (black line) and subsequently employed to calculate the experimental (Exp.) MW of His6-Nur77LBD in various samples (in red and in the table). (B–D) UV traces at 280 nm of His6-Nur77LBD samples showed (C) AA-induced oligomerization of His6-Nur77LBD from a monomer (1) to a trimer (3) and tetramer (4), whereas no change in the oligomeric state of the protein was detected in (B) the no-lipid control or (D) a PA-treated sample.

well as later experiments, the assay also included a saturated fatty acid, palmitic acid (PA, C16:0). At the same concentrations, AA displaced ANS from His6-Nur77LBD while PA did not, suggesting that Nur77LBD interacts specifically with UFAs and not simply with *any* fatty acid. In addition, we tested DHA and an arachidonyl-containing lipid, anandamide (AEA), in this assay (Figure 2). DHA displaced ANS from His6-Nur77LBD in a similar fashion to AA, whereas AEA did not, suggesting further that the carboxyl group on the UFA is necessary for binding to Nur77, while simply having an arachidonyl side chain on a lipid is not enough.

Crystal structures of human Nur77LBD revealed that the binding pocket of Nur77 is completely occupied with bulky hydrophobic aromatic residues.[19] Thus, if a ligand were to reside at the binding site, then the protein must adjust its conformation to create a cavity. To examine whether AA causes a conformation change in His6-Nur77LBD, we collected circular dichroism (CD) spectra in the presence and absence of AA as well as PA and other appropriate controls (Figure 3; also see the SI). Treatment of His6-Nur77LBD with 10 molar equiv of AA resulted in a change in the CD spectrum between 205 and 230 nm in comparison with the nontreated control. Similar changes were also observed in the experiments with sodium arachidonate and DHA; however, no significant differences were observed in the case of PA, AEA, or *cis*-9-retinoic acid (RA), a ligand for retinoic acid receptor (RAR) and retinoid X receptor (RXR). Overall, the data supported our hypothesis that Nur77 binds UFAs and indicated that the LBD undergoes conformational changes to mediate small-molecule binding.

Another technique commonly used to assess the thermodynamics of protein–ligand binding is isothermal titration calorimetry (ITC).[32] In these ITC experiments (see the SI), we investigated the interaction of AA with Nur77LBD under conditions favoring the formation of monomer and oligomers (e.g., a mixture of monomer and dimer) separately, since previous work with other NRs (e.g., estrogen receptor) indicated that the ligand can influence the oligomerization state of the receptor.[16,17] Titration of a monomeric solution of His6-Nur77LBD with AA gave no detectable change in enthalpy. This result suggested either that the interaction between Nur77 monomer and AA does not exist or that Nur77 monomer is bound to AA only with low affinity that exceeded the measurement capability of the instrument. By contrast, an ITC experiment with solution of His6-Nur77LBD oligomers showed significant differences in the heat of formation, indicating that AA is bound to a higher-order complex. The oligomers bound to AA with a binding constant (K_a) of 3×10^5 M and a binding enthalpy (ΔH) of −15.9 kcal/mol. As a control, His6-Nur77LBD oligomers were titrated with PA, but no change in enthalpy was detected. Taken together, the biophysical data suggest that AA binds to an oligomeric form of His6-Nur77LBD. An ANS displacement assay with an oligomeric solution of Nur77LBD showed complete displacement of ANS from Nur77LBD at lower concentrations of AA (i.e., 5 μM for oligomers vs 100 μM for monomer), in support of the notion that AA preferentially binds to oligomers (see the SI).

As mentioned above, NR ligands are able to influence the dimerization state of the LBD, and we hypothesized that AA can stabilize His6-Nur77LBD oligomers. To test this hypothesis, we determined the oligomeric states of His6-Nur77LBD with or without the addition of AA (or PA as a control) using SEC (Figure 4). The samples were run independently on an analytical Superdex 200 column, which allowed separation of different oligomers of the protein. The resulting fast protein LC (FPLC) chromatograms were then used to calculate the molecular weight (MW) of each oligomeric species by locating the elution volume (V_e) of each peak on the standard curve constructed using V_e values for known protein markers.

At all time points (0, 4, and 8 h), His6-Nur77LBD samples without addition of lipid and the PA control samples yielded similar FPLC chromatograms (Figure 4). The major peak in both His6-Nur77LBD samples eluted as a species with an experimental

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MW of 26 kDa, corresponding to a monomer (predicted 28 kDa). Similarly, a trace amount of the protein in the samples exhibited a MW of 50 kDa, leading to the assignment of the species as a dimer (predicted 57 kDa). However, when the experiment was conducted with His6-Nur77LBD in the presence of 10 molar equiv of AA, the FPLC chromatograms became visually distinct from those in samples without AA (Figure 4). Specifically, new peaks were detected at earlier V_e. These peaks yielded experimental MW of 79 and 108 kDa, closely matching those of a trimer (predicted 85 kDa) and a tetramer (predicted 114 kDa), respectively. Furthermore, as His6-Nur77LBD was incubated longer with AA (i.e., from 0 to 4 to 8 h), the ratio of the oligomeric peaks to monomeric peak increased, suggesting that AA might help stabilize higher oligomers of Nur77, thus driving the equilibrium in that direction. Importantly, these conditions did not lead to uncontrolled aggregation, as the proteins never came out of solution. Taken together, the SEC data provide evidence that UFAs impact the oligomerization state of His6-Nur77LBD.

UFAs represent the first endogenous lipids reported to partake in a protein−metabolite interaction with Nur77. This finding demonstrates the value of the metabolomics platform in providing novel biological insight. Subsequent biophysical studies demonstrated that the UFAs bind to His6-Nur77LBD, leading to changes in the conformation and oligomerization state of the receptor. On the basis of this activity, we hypothesize that these lipids may influence the homo- or heterodimerization state of the Nur77 to modulate transcriptional activity. Future work will involve investigating the impact of UFA binding on Nur77 transcriptional activity using cell-based transcriptional assays or RT-PCR assays with known Nur77 target genes.[11,21−23,33] Additionally, structural studies with Nur77 and AA will be important in defining the UFA−Nur77 binding site, which may differ from the traditional NR binding site. More generally, the ability to discover novel PMIs will spur the continued use of metabolomics for the discovery of protein-binding lipids for other orphan receptors.

■ ASSOCIATED CONTENT

Supporting Information. Complete citations of all references, experimental procedures, full table of enriched metabolites, ligand enrichment experiment with GST-Nur77LBD, coinjection experiment, ANS assay, ANS displacement assay, ITC data, and CD spectroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
saghatelian@chemistry.harvard.edu

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