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

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Abstract

Neuron-derived clone 77 (Nur77) is an orphan nuclear receptor with currently no known natural ligands. Here, we apply 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) could enrich unsaturated fatty acids (UFAs) from tissue lipid mixtures. The interaction between 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 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 variety of physiological processes.^{1,2} Because NR transcriptional activity can be regulated by natural and synthetic small-molecule ligands, NRs have become an important target for the development of new drugs in the recent years.^{3–5} Nearly half of the 48 human NRs still have no known natural ligands, and receptors lacking ligands are referred to as orphan nuclear receptors.⁶ The identification of a natural ligand(s) for orphan receptors will help characterize the receptor, identify a new role for the ligand, and provide insights into physiological regulation of the NR.

The orphan nuclear receptor 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

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ASSOCIATED CONTENT

Supporting Information. Complete ref xx, Experimental procedures, full table of enriched metabolites, ligand enrichment experiment with GST-Nur77LBD, co-injection experiment, ANS assay, ANS displacement assay, ITC, CD spectroscopy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

mediate transcription,^{13–15} and ligands can influence the dimerization state.^{16,17} Interestingly, unlike other NRs, structural studies of Nur77 and Nurr1 suggest that these proteins do not use ligands to control transcription, because they lack a binding pocket.^{18–20}

Recent discoveries of 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 crystal structures of some orphan NRs exhibit no ligand binding site (e.g., Nur77 and TR4), NRs are 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 (Fig. 1). The gene for human Nur77LBD was first synthesized, and the protein was expressed from a vector containing a polyhistidine (His6) tag (Supporting Information) to produce 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 hydrophobic cavity of protein and shows saturation binding with folded protein samples. Titration of His6-Nur77LBD with ANS yields saturation binding, indicating that the protein is folded (Supporting Information).

Here, we used the His6-tagged LBD of Nur77 (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 hypothesize 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 and form a protein-metabolite complex. The mixture was subsequently filtered, the unbound lipids were washed away, and the protein was eluted. As a control, solid support lacking NR 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 mass ion intensity (MSII).³⁰ To cover 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 XCMS³¹ program, 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 as compared to the control (Fig. 1). From metabolite profiles of the two sample sets (i.e., His6-Nur77LBD vs. no protein), the unbiased analysis identifies the NR-enriched lipids based on their statistical significance ($p < 0.05$ and fold > 2). With a list of enriched lipids, the next step then involved characterizing the metabolites using accurate mass, retention time and co-elution (Supporting Information). Here, we found that in brain extracts, His6-Nur77LBD enriched UFAs (Fig. 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, 18:0), on the other hand, were unchanged. Next, to confirm that this result held up in other tissues with Nur77 expression, 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 enriched from 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 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 (Supporting Information). Together, these results pointed to the ability of Nur77LBD to enrich UFAs and identified UFAs as potential ligands for Nur77. Among UFAs, arachidonic acid (AA) and docosahexaenoic acid (DHA) showed the highest fold enrichment by His6-Nur77LBD in both brain and testes samples. Therefore, we chose to characterize the biochemical and biophysical interaction between Nur77 and UFAs further by using AA or DHA.

We tested AA binding to His6-Nur77LBD by displacing ANS from His6-Nur77LBD with AA (Fig. 2). In this experiment, as well as later experiments, the assay was performed alongside with 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 to UFAs, and not simply to *any* fatty acids. In addition, we tested another UFA, DHA, and an arachidonyl-containing lipid, anandamide (AEA), in this assay (Fig. 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 UFAs was necessary for the binding to Nur77, while simply having arachidonyl side chain on a lipid was not enough.

Crystal structures of human Nur77LBD revealed that the binding pocket of Nur77 was completely occupied with bulky hydrophobic aromatic residues.¹⁹ With this data, if a ligand were to reside at the binding site, then the protein must adjust its conformation such that a cavity is created. To examine whether AA caused 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 (Fig. 3 and Supporting Information). Treatment of His6-Nur77LBD with 10 molar equivalents of AA resulted in a substantial change in the CD spectra between 205 nm and 230 nm, as compared to the non-treated control. A similar change was also observed in the experiment with sodium arachidonate and DHA; however, no significant difference was measured 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 indicates that the LBD is undergoing 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 (Supporting Information), we investigated the interaction of AA with Nur77LBD monomer and oligomers (i.e., mixture of monomer, dimer, trimer, and tetramer) separately, since previous work with other nuclear receptors (e.g., estrogen receptor) indicated that 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. The result suggested either that the interaction between Nur77 monomer and AA did not exist, or that Nur77 monomer bound to AA only with low affinity, which exceeded the measurement capability of the instrument. By contrast, ITC experiment with solution of His6-Nur77LBD oligomers showed significant differences in the heat of formation, which indicated that AA is binding to a higher order complex. The oligomers bound to AA with a binding constant (K_a) of 3×10^5 M and the binding enthalpy (ΔH) of -15.9 kCal/mol. As a control, His6-Nur77LBD oligomers were titrated with PA, but no change in heat was detected. Taken together, the biophysical data suggests that AA binds to an oligomeric form of His6-Nur77LBD. An ANS displacement assay with an oligomeric solution of Nur77LBD showed a complete displacement of ANS from Nur77LBD at lower concentrations of AA (i.e., $5 \mu\text{M}$ for oligomers vs. $100 \mu\text{M}$ for monomer) to support the notion that AA preferentially binds to oligomers (Supporting Information).

As mentioned, NR ligands are able to influence the dimerization states of the LBD, and our hypothesis is that AA can stabilize His6-Nur77LBD oligomers. To test this hypothesis, we determined oligomeric states of His6-Nur77LBD with or without the addition of AA (or PA as a control) using SEC (Fig. 4). The samples were run independently on an analytical Superdex 200 column, which allowed separation of different oligomers of the protein. The resulting FPLC chromatograms were then used to calculate 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 of known protein markers.

At all time points (0, 4, and 8 h), His6-Nur77LBD samples without addition of any lipids and the PA-control samples yielded similar FPLC chromatograms (Fig. 4). The major peak in both His6-Nur77LBD samples eluted as a species with experimental MW of 26 kDa, corresponding to a monomer (predicted 28 kDa). Similarly, a trace amount of the protein in the samples exhibited MW of 50 kDa, leading to 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 equivalents of AA, the FPLC chromatograms became visually distinct from those in samples without AA (Fig. 4). Specifically, new peaks were detected at earlier V_e . These peaks yielded experimental MW of 79 and 108 kDa, closely matched with that 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 h to 4 h 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 toward that direction. Importantly, these conditions did not lead to uncontrolled aggregation, as the proteins never

came out of solution. Taken together, the SEC data provides 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 at 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. Based on 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 UFAs binding on Nur77 transcriptional activity using cell-based transcriptional assays or RT-PCR assays with known Nur77 target genes.^{11,21–23,33} Additionally, we imagine that 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A 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. Negative- and positive-mode MS ions are represented as green circles and blue diamonds, respectively, with the corresponding unsaturated fatty acids (UFAs) ions highlighted in red circles. (c) UFAs were enriched by His6-Nur77LBD when compared to 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.

Figure 2.

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

Figure 3.
(a, b) CD spectra showed changes in conformation of His6-Nur77LBD when it was treated with 10 molar equivalents 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 size exclusion chromatography. (a) Standard curve was plotted as a function of the logarithm of the molecular weight (MW) and elution volume (V_e). The MW of the proteins used as MW markers were indicated along the plot in blue. The data were fitted to a linear regression model (black line) and subsequently employed to calculate experimental (Exp.) MW of His6-Nur77LBD in various samples (in red and in the table). (b, c, d) UV traces at 280 nm of His6-Nur77LBD samples showed AA-induced oligomerization of His6-Nur77LBD from a monomer (1) to a trimer (3) and tetramer (4), whereas no change in oligomeric states of the protein was detected in the no-lipid control (b) or in PA-treated (d) samples.