Polyunsaturated Fatty Acids Including Docosahexaenoic and Arachidonic Acid Bind to the Retinoid X Receptor α Ligand-binding Domain*

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Nuclear receptors (NRs) constitute a large and highly conserved family of ligand-activated transcription factors that regulate diverse biological processes such as development, metabolism, and reproduction. As such, NRs have become important drug targets, and the identification of novel NR ligands is a subject of much interest. The retinoid X receptor (RXR) belongs to a subfamily of NRs that bind vitamin A metabolites (i.e. retinoids), including 9-cis-retinoic acid (9-cis-RA). However, although 9-cis-RA has been described as the natural ligand for RXR, its endogenous occurrence has been difficult to confirm. Recently, evidence was provided for the existence of a different natural RXR ligand in mouse brain, the highly enriched polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) (Mata de Urquiza et al. (2000) Science 290, 2140–2144). However, the results suggested that supra-physiological levels of DHA were required for efficient RXR activation.

Using a refined method for ligand addition to transfected cells, the current study shows that DHA is a more potent RXR ligand than previously observed, inducing robust receptor activation already at low micromolar concentrations. Furthermore, it is shown that other naturally occurring PUFAs can activate RXR with similar efficiency as DHA. In additional experiments, the binding of fatty acid ligands to RXRα is directly demonstrated by electrospray mass spectrometry of the noncovalent complex between the RXR ligand-binding domain (LBD) and its ligands. Data is presented that shows the noncovalent interaction between the RXR LBD and a number of PUFAs including DHA and arachidonic acid, corroborating the results in transfected cells. Taken together, these results show that RXR binds PUFAs in solution and that these compounds induce receptor activation, suggesting that RXR could function as a fatty acid receptor in vivo.


Nuclear receptors (NRs)1 are ligand-regulated transcription factors found in most higher organisms including nematodes, flies, and mammals, with important biological functions in for example embryonal development, regulation of lipid homeostasis, and in the metabolism of drugs and xenobiotics (reviewed in Refs. 1–3). NR ligands are generally small, lipophilic molecules that easily pass through the plasma membrane and into the cell, where they bind and activate their corresponding receptor. Such ligands include the steroid hormones, vitamin D₃, thyroid hormone, retinoids, cholesterol metabolites, and fatty acids. In addition, a large number of NRs are currently lacking an identified ligand and are therefore referred to as orphan receptors. Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) constitute a subfamily of NRs, each present in three isotypes (α, β, and γ) (4). While RARs become activated by both all-trans- and 9-cis-retinoic acid (RA), RXRs only respond to 9-cis-RA (5, 6). Gene targeting studies suggest that both receptor subtypes are essential during embryonal development, and that the retinoid signal is conferred by RAR/RXR heterodimers in vivo (summarized in Ref. 7). In addition, RXR also plays a unique role in the NR family as a common heterodimer partner for several other NRs (8). As such, RXR is thought to provide essential structural and signaling support to its partner during transcriptional activation.

Although 9-cis-RA has been described as the natural ligand for RXR (6), it has been difficult to detect in vivo (8–12). However, recently presented evidence indicates that RXR can also become activated by naturally occurring polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA).

1 The abbreviations used are: NR, nuclear receptor; RXR, retinoid X receptor; RA, retinoic acid; PFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; LBD, ligand-binding domain; RAR, retinoic acid receptor; ES, electro spray; DPA, docosapentaenoic acid; NiNTA, nickel-nitrilotriacetic acid; Q-TOF, quadrupole-time-of-flight; DBD, DNA-binding domain; DMSO, dimethyl sulphoxide; Mₐ, average relative molecular mass; PPAR, peroxisome proliferator-activated receptor; HNF4, hepatocyte nuclear factor 4.

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(13). Significantly, whereas 9-cis-RA is difficult to detect in vivo, DHA is abundant in the postnatal brain, making it a prime candidate as a natural ligand for RXR (reviewed in Refs. 14 and 15). Recently, the unexpected presence of fatty acid ligands in the crystal structure of several NR ligand-binding domains (LBDs) have been reported, including the presence of oleic acid in the ligand-binding pocket of a mutated version of RXRα (16–19). In addition, the crystal structure of DHA bound in the ligand-binding pocket of RXRs (20) indicates that despite its high affinity, when positioned in the ligand-binding pocket, 9-cis-RA displays a significantly lower number of ligand-protein contacts than either DHA or the synthetic ligand BMS649/SR11237 (4-[(2-(5,6,7,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl] benzoic acid). Taken together, these findings suggest that fatty acids can function as true endogenous ligands for RXR. However, while PUFAs have been shown to activate RXR and fit into the ligand-binding pocket of RXR, noncovalent interactions between these fatty acids and RXR have yet to be demonstrated in solution.

In recent years, electrospray (ES) mass spectrometry has been successfully applied in the study of noncovalent protein complexes (21–25). The number of applications of noncovalent interactions by affinity chromatography. Pooled eluates were dialysed against 50 mM ammonium acetate buffer, pH 8, at 4 °C. This protein stock solution (8.7 mg/ml) was stored at −70 °C. Before use, the protein stock solution was thawed and diluted 28-fold in 10 mM ammonium acetate buffer, pH 8, to a final protein concentration of 10 pmol/μl and kept on ice. If considered necessary following the acquisition of an initial ES mass spectrum, the protein solution (10

<table>
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<tr>
<th>name</th>
<th>abbreviation</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
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<tr>
<td>9-cis-retinoic acid</td>
<td>9cRA</td>
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</tr>
<tr>
<td>all-trans-retinoic acid</td>
<td>atRA</td>
<td>300.44</td>
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<tr>
<td>oleic acid / 9-cis-octadecenoic acid</td>
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<tr>
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<td>C20:4</td>
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<tr>
<td>cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid</td>
<td>C22:6 / DHA</td>
<td>328.49</td>
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<tr>
<td>8-[1-(5,6,7,8-tetrahydro-3,5,5,8,9-pentamethyl-2-naphthalenyl)cyclopentyl]-3-pyridinecarboxylic acid</td>
<td>LG268</td>
<td>363.50</td>
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**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Methanol (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkerburn, United Kingdom). Ethanol (99.5%) was obtained from Kemetyl AB (Haninge, Sweden). Stearic acid (octadecanoic acid, C18:0), oleic acid (cis-9-octadecenoic acid, C18:1), arachidonic acid (cis-9,12,15-octadecatrienoic acid, C20:3) and linoleic acid (cis-9,12-octadecadienoic acid, C18:2) and linolenic acid (cis-9,12,15-octadecatrienoic acid, C18:3) were obtained from Sigma-Aldrich (St. Louis, MO). Lolidon Fine Chemicals AB (Malmö, Sweden), LG100268 (LG268) was obtained from Tularik Inc. (South San Francisco, CA), SR11237, LG268 was obtained from GlaxoSmithKline. See Scheme 1 for structures of some of the compounds used in the ES mass spectrometry study. Deionized water was used after further purification using a Milli-Q reagent grade water system (Millipore S.A., Molsheim, France). Lipidex-1000 was obtained from Packard Instrument Company Inc. (Downers Grove, IL). Nickel-nitritriacetic acid (Ni-NTA) agarose resin was obtained from Qiagen Inc. (Valencia, CA).

**Protein Expression and Purification**—The human RXRα LBD, amino acids 203–462, was cloned into the pET-15b vector (Novagen Inc., Madison, WI) and expressed in Escherichia coli BL21 cells. The His<sub>6</sub>-tagged protein construct was purified under nonnaturating conditions by affinity chromatography. Pooled eluates were dialysed against 50 mM ammonium acetate buffer, pH 8, at 4 °C. This protein stock solution (8.7 mg/ml) was stored at −70 °C. Before use, the protein stock solution was thawed and diluted 28-fold in 10 mM ammonium acetate buffer, pH 8, to a final protein concentration of 10 pmol/μl and kept on ice. If considered necessary following the acquisition of an initial ES mass spectrum, the protein solution (10

**SCHEME 1.** Names, abbreviations, M<sub>r</sub> values, and structures of selected compounds used in ES analyses.
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pmol/µl was washed by five concentration-dilution cycles (10 mM ammonium acetate buffer, pH 8) on 10 K Nanosep spin filters (Pall Gellman Laboratory, Ann Arbor, MI). The amino acid sequence of the RXRα LBD was verified by tryptic digestion, followed by peptide mass mapping (89% sequence coverage) and tandem mass spectrometry of selected peptides. Under native conditions, i.e. in 10 mM ammonium acetate buffer, pH 8, the average relative molecular mass (M_R) was measured to be 31,370 Da, in good agreement with the theoretical M_R of 31,371 Da. In addition to the expected M_R, a second component of M_R = 31,548 Da was observed. This corresponds to the glucocylated His_{29}-tag protein (∼178.05 Da, monoisotopic mass) (39). Using non-denaturing ES mass spectrometry, receptor occupancy was shown to be better than 85% at a 5-fold excess of high-affinity ligand (LG268, data not shown).

Mass Spectrometry—Protein spectra were recorded on a quadrupole-time-of-flight (Q-TOF) instrument (Q-TOF 1; Micromass plc, Manchester, United Kingdom) fitted with the standard Z-spray ES interface. Samples were infused at a flow rate of 5 µl/min, generated by a Harvard type 22 syringe pump (Harvard Apparatus, South Natick, MA) and electrosprayed from a stainless steel capillary raised to a potential of +3 kV. The desolvation gas flow was 300 liters/h at 120 °C, and the nebulizer gas was set at 20 liters/h. Both the desolvation and nebulizer gases were nitrogen. The ES capillary tip was positioned ∼0.5 cm laterally and 1.0 cm posterior to the sampling orifice of the sampling cone. All analyses were performed in the positive-ion mode. To minimize disruption of noncovalent interactions, the cone voltage and collision voltage were kept low, i.e. at 5 and 4.2 V, respectively. The source block temperature was set to 80 °C. In order to optimize the instrument for the observation of noncovalent protein complexes, argon gas was introduced to the hexapole collection cell to bring the reading on the nearby ‘Analyser’ Penning gauge from 8.5 × 10^{-6} to 4.0–4.5 × 10^{-5} mbar. By partially closing the Edwards SpeediValve on the pumping line linking the ES interface to the rotary pump (40, 41), the ‘Analyser’ gauge reading was increased further to 5.3–5.6 × 10^{-5} mbar. The transport and collision cell multipole radio frequency offsets were set to 1.0 to increase the transmission of high m/z ions. The TOF pusher was operated in the manual mode with a pusher time of 150 µs. The m/z scale of the instrument was calibrated prior to each session by electrospraying solutions of either caesium iodide (2 mg/ml, 50% isopropanol) or horse heart myoglobin (2 pmol/µl, 1% acetic acid) from gold-coated borosilicate capillaries (Protana AS, Odense, Denmark) using the nano-ES interface.

Mass spectra of the acidic lipids were recorded in the negative ion mode on a Quattro Micro triple-quadrupole instrument (Micromass plc). The instrument was fitted with a nano-ES interface, and samples were electrosprayed from gold-coated borosilicate capillaries. Tandem mass spectra of the fatty acid [M-H+2Li]^- ions were recorded on the Q-TOF instrument in the positive ion mode. The collision gas was Ar and the collision energy 25–35 eV.

Sample Analysis—RXRα LBD stock solution (8.7 µg/ml) was diluted with buffer (10 mM ammonium acetate, pH 8) to give a 10 pmol/µl solution and kept on ice. Ligand stock solutions (10 mM) were made up in 99.5% ethanol and stored at −25 °C. Compounds were tested with a 5:1 ligand-protein molar ratio in a sample volume of 300 µl. Each sample was vortexed briefly and left to equilibrate for 25 min at room temperature before starting sample infusion to the mass spectrometer and data acquisition. To prevent contamination by memory effects from the syringe or sample inlet capillary, three wash steps were included before each sample infusion: 1) 150 µl of 70% methanol, 5% formic acid, and 2) 150 µl of water, followed by 3) 150 µl of 10 mM ammonium acetate buffer, pH 8. Prior to all analysis sessions, one sample of RXRα without ligand (10 pmol/µl) was infused. Samples containing 9-cis-RA, all-trans-RA, or LG268 were analyzed under low-light conditions due to their photosensitive nature.

Data Analysis—Mass spectral data was analyzed using the manufacturer’s MassLynx v3.5 software, which includes the maximum entropy deconvolution algorithm (42).

Cell Culture and in Vitro Activity Assays—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium and minimal essential medium, (both from Life Technologies, Inc., Taby, Sweden), supplemented with 10% stripped fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine. Transfections were performed in triplicates in 24-well plates using LipofectAMINE reagent according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). Briefly, each well was transfected with 100 ng of effector plasmid encoding the full-length human RXRα (CMX-RXRα) or the LBD of human RXRα fused to the DNA-binding domain (DBD) of the yeast transcription factor GAL4 (CMX-GAL4-RXRα) and 200 ng of a luciferase reporter plasmid containing three RXR-binding sites from the apolipoprotein 1 gene (ApoA1-tk-luc), the RARβ gene (iPcE-tk-luc), or four copies of the GAL4-binding sites from the yeast upstream activating sequence (UAS-tk-luc) followed by a minimal thymidine kinase promoter. As a reference, 200 ng of CMX-jpgal plasmid containing the β-galactosidase gene was used (43). Four to 5 h after transfection, ligands were added to a final volume of 1 ml in each well. Fatty acids and high-affinity ligands were dissolved in dimethyl sulfoxide (DMSO), and 1 µl was added directly into each well. The cells were harvested 24 h later, and the extracts were assayed for luciferase and reference β-galactosidase activity in a microplate luminometer/photometer reader (Lucy-1; Anthos, Salzburg, Austria). Brain-conditioned medium was prepared as described (13).

Affinity Capture of Ligands from Brain-conditioned Medium by RXRα LBD—RXRα LBD protein stock solution (4.95 mg/ml) was dialyzed against 50 mM ammonium acetate, pH 8.1. Aliquots of RXRα LBD stock solution (1.05 ml) were incubated for 40 min on ice and for 20 min at room temperature in the presence or absence of a 1.5-fold molar excess of LG268 to protein (10 mM in ethanol, 24.9 µl added). Subsequent steps were carried out at room temperature unless otherwise indicated, and buffer solutions were heated to 37 °C prior to use. A 1.5-fold excess of LG268 was maintained in subsequent steps while correcting for volumetric changes. RXRα solutions (1.05 ml), with or without added LG268, were mixed with 2.56 ml of brain-conditioned medium (13) to give an RXRα concentration of 46 µM in a sample volume of 3.61 ml. Samples were incubated for 30 min at 37 °C, before adding 0.75 ml of Ni-NTA agarose (50% slurry, v/v) washed with lysis buffer (300 mM NaCl, 50 mM NaHPO₄, 10 mM imidazole, pH 8.0) and incubating for 45 min at 37 °C. The samples were decanted to fritted disposable plastic columns (Bio-Rad, Hercules, CA), and the resin beads were washed twice with 1 ml of lysis buffer. Protein was eluted with 5 × 0.5 ml of elution buffer (300 mM NaCl, 50 mM NaHPO₄, 250 mM imidazole, pH 8.0). Eluted fractions were pooled and desalted on a PD-10 gel filtration column (Amersham Biosciences, Uppsala, Sweden) using 10 mM ammonium acetate buffer, pH 8.1. Protein concentration was determined (Bradford assay) before storing the samples at −25 °C. Recovery was ~50% of the RXRα LBD protein.

Lipid Extraction and ES Analysis of Ligands Bound to RXRα LBD—Desalted protein fractions from the affinity capture experiments above were thawed, and bound lipids were extracted using Lipidex-1000 gel (44). Before use, Lipidex-1000 was washed with: 1) 50% methanol, 2) 100% methanol, and 3) water, and then stored as a 50% (v/v) slurry in water. For each extraction, 300 µl of Lipidex-1000 slurry was transferred to a 1.5-ml Eppendorf tube, and excess water was removed after centrifugation. One milliliter of the thawed desalted protein fraction from the affinity capture experiment above and 1432.2 ng of [14C]DHA in 2 µl were added to the gel bed of [14C]DHA in ethanol,
specific activity 55 mCi/mmol, from American Radiolabelled Chemicals Inc., St Louis, MO). The concentration of [14C]DHA was determined by counting 1 μl of the ethanol solution and calculated to be 716.1 ng/μl using the specific activity value. The slurry was acidified to pH 2–3 using acetic acid and incubated for 30 min at 37 °C. The supernatant was removed, and the Lipidex bed was washed with 1.2 ml of water, which was discarded. The lipids bound to the Lipidex bed were extracted using 3 ml of water, which was discarded. The lipid solution was evaporated under a stream of nitrogen, and the residue was reconstituted in 0.1 ml of methanol. The resulting sample was analyzed by negative ion nano-ES mass spectrometry. The amount of unlabeled extracted DHA was calculated from the intensity ratio of deprotonated [M-H] ions at m/z 281, 303, and 327, respectively). The amounts of oleic and arachidonic acids extracted were determined from the relative intensity of their respective dilithiated adducts ([M-2Li]+) (45).

reference compound (theoretically calculated for DHA to be 0.0264).

The amounts of oleic and arachidonic acids extracted were determined from the relative intensity of their respective deprotonated molecules (m/z 281 and 303, respectively) as compared with deprotonated DHA. To determine the fatty acid composition of the original brain-conditioned medium, 1 ml of brain-conditioned medium was extracted using the Lipidex-1000 procedure described above. The ions at m/z 281, 303, and 327 were identified by low-energy collision-induced dissociation of their respective didithiated adducts ([M-H-2Li]+) (45).

**RESULTS**

**Fatty Acid Activation of RXRα in Transfected Cells**—We have previously shown that RXR can be activated by certain fatty acids, including DHA, in cells transfected with an RXRα expression vector and a luciferase reporter gene preceded by an RXR-response element from the apolipoprotein A1 gene (13). According to these observations, the fatty acid concentration required to reach half maximal activation (EC50) of mouse RXRα was in the 50–100 μM range, raising the question whether such levels of free fatty acids are present in cells in vivo. In these original experiments, fatty acid solutions were
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Fig. 2. Unsaturated fatty acids activate RXRα through RXR homodimers and act synergistically on RXR-RAR heterodimers. A, various concentrations (1, 2, 4, 8, 16, and 32 μM) of DHA were titrated either alone (□) or in the presence of 1 μM RXR antagonist (LG1208; ▲) or 1 μM RAR antagonist (RO-415253; ▼) on 293T cells transfected as in Fig. 1. B, 293T cells transfected as in Fig. 1 were treated with RXR-specific ligand LG268 (1, 10, 100, and 1000 nM; ■), DHA (1, 2, 4, 8, 10, 16, and 32 μM; ◆), PPARα-specific ligand GW7647 (▼), PPARβ/δ-specific ligand GW7407 (▲), or PPARγ-specific ligand GW7845 (●) (0.1, 1, and 10 μM for PPAR ligands). All values are shown as fold induction after normalization to β-galactosidase (β-gal) similar to Fig. 1. Note that the symbols for PPAR ligands overlap on the y-axis. C, 293T cells transfected with a luciferase reporter carrying three RAR/RXR-binding sites from the RARβ gene (βRE-tk-luc) were treated with atRA (10 nM) or with increasing concentrations of DHA (1, 5, 10, and 20 μM) alone or in combination with atRA (10 nM). Error bars indicate SEM values for each triplicate experiment.

Prepared in plastic tubes by diluting a fatty acid/DMSO stock in cell culture medium prior to addition to the transfected 293T cells (13). However, in the present study, the concentrated ligand stock was added directly to transfected 293T cells in culture without prior dilution in cell culture medium. A comparison between the direct addition of fatty acid ligand and the previous method of addition via dilution in plastic tubes shows that DHA is almost 10-fold more effective in activating RXRα when added directly (Fig. 1A). Accordingly, by the latter method of ligand addition, the EC50 for RXRα activation by DHA is about 5–10 μM fatty acid (Fig. 1, A and C). Interestingly, activation of RXRα by the high-affinity ligands 9-cis-RA and LG268 was not affected by the method of ligand addition (Fig. 1B). Using this alternative direct addition protocol for ligand administration, we tested additional fatty acids for their ability to activate RXR in transfected 293T cells. Unsaturated fatty acids such as DHA, docosapentaenoic acid (C22:5), and arachidonic acid (C20:4) lead to a robust activation of RXRα, whereas saturated fatty acids like arachidic acid (C20:0) and stearic acid (C18:0) did not (Fig. 1C). The EC50 values for the polyunsaturated fatty acids were between 5 and 10 μM. The RXRα response to high-affinity agonists (SR11237) or DHA was independent of whether full-length RXRα (CMX-RXRα) in combination with the ApoA1-tk-luc reporter or a RXRα LBD-GAL4 DBD fusion construct (CMX-GAL4-RXRα) with the UAS-tk-luc reporter was used to transfect the 293T cells (Fig. 1D).

Reporter Activation Is a Direct Effect of Fatty Acids on RXRs—RXRα is a common heterodimer partner for several NRs, including RAR and peroxisome proliferator-activated receptors (PPARs) whose natural ligands include retinoids and fatty acids, respectively (46–48). The possibility therefore existed that the fatty acid ligands were inducing the reporter by activating a heterodimer partner of RXR expressed in the transfected 293T cells. However, while the RXR-specific antagonist LG1208 completely blocked reporter induction by DHA, the RAR-specific antagonist RO41-5253 had no effect, suggesting that RAR is not involved in fatty acid-mediated reporter induction (Fig. 2A). Similarly, synthetic PPAR-specific ligands did not stimulate induction of the reporter, suggesting that PPARs are not involved in reporter induction (Fig. 2B). The same negative results were obtained when treating the cells with ligands for liver X receptors, farnesoid X receptor, thyroid receptor, and vitamin D3 receptor (data not shown). All-trans-RA activation of RXR-RAR heterodimers has been reported to be potentiated by RXR agonist 9-cis-RA (49). In the present study, a synergistic effect between DHA and atRA was observed in 293T cells transfected with a luciferase reporter carrying three RAR/RXR-binding sites from the RARβ gene (βRE-tk-luc) were treated with atRA (10 nM) or with increasing concentrations of DHA (1, 5, 10, and 20 μM) alone or in combination with atRA (10 nM). Error bars indicate SEM values for each triplicate experiment.

ES Mass Spectrometric Analysis of Unliganded RXRα LBD—During recent years, ES mass spectrometry has become a powerful method for studying noncovalent interactions between NR proteins and their ligands (26, 28, 30). A mixture of native protein and ligand is injected into the mass spectrometer and analyzed with respect to mass, allowing noncovalent complexes to be detected directly from solution. The results from the activity assays presented in Figs. 1 and 2 suggested that RXRα binds several fatty acids. We therefore proceeded to verify the interaction between the RXRα LBD and different ligands using ES mass spectrometry. Initially,
optimum conditions were established for the acquisition of mass spectra of a solution of nondenatured RXR LBD (10 pmol/µl, 10 mM ammonium acetate, pH 8) in the absence of added ligand (Fig. 3A). Three major peaks are observed in the m/z range 2000–3000 Th, at 2414, 2615, and 2853 Th, corresponding to the 13+, 12+, and 11+ charge states of the monomeric protein, respectively (see Fig. 3B for an expanded view of the 12+ peak area). The presence of only a few peaks in a charge state envelope at high m/z indicates that the protein is present in a folded conformation, presumably the native conformation. A minor charge state envelope is also observed in the m/z range 1200–2300 Th corresponding to the multiply protonated forms (25+ through 14+) of the denatured monomeric protein (Fig. 3A). In the higher m/z ranges 3000–4000 and 4500–5500 Th, two further charge state envelopes are observed, which are assigned to RXR LBD homodimers (3000–4000) and homotetramers (4500–5500), respectively, showing a high degree of adducting (Fig. 3A).

Analysis of RXR LBD Ligand Noncovalent Complexes—Once it was established that the RXR LBD could be electro-sprayed and analyzed in its native conformation, conditions were further optimized for ligand-binding analysis. Initial experiments were performed with the high-affinity RXR ligand 9-cis-RA, M_r 300.44 Da (Fig. 3A). Using the optimized analysis procedure, a protein solution (10 pmol/µl) containing ligand (50 pmol/µl 9-cis-RA) was analyzed. In the presence of 9-cis-RA, a new series of peaks was resolved at m/z 2437, 2640, and 2880 Th, corresponding to the 13+, 12+, and 11+ charge states of the monomeric RXR LBD-9-cis-RA noncovalent complex, respectively. This is shown for the 12+ charge state in Fig. 3C. As expected, addition of the weak RXR agonist DHA (C22:6, M_r 328.49 Da) to the RXR LBD
solution gave a peak corresponding to a noncovalent complex between the protein and the fatty acid ligand (Fig. 3D). In contrast, all-trans-RA, which does not bind RXR, did not give a significant peak corresponding to the noncovalent complex (Fig. 3E), verifying the validity of the experimental design.

**DHA Titrations Using ES Mass Spectrometry**—The spectrum presented in Fig. 3D was of a solution containing RXR LBD (10 μM) and DHA (50 pmol/μl). Ligand binding could be observed at other ligand concentrations. Accordingly, when the protein solution was incubated with different amounts of DHA (12.5–100 pmol/μl), the relative intensity of the peaks corresponding to the receptor-ligand complex were observed to increase with increasing ligand concentration (Fig. 4, A–D, and data not shown). Interestingly, at a DHA concentration of 12.5 pmol/μl, the complex was still clearly observed (Fig. 4B).

**RXRα LBD Forms Noncovalent Complexes with Other PUFAs**—Next, the ES mass spectrometric analysis was extended to include other potential fatty acid ligands. As shown previously (13) and in Fig. 1C, not only DHA but also other PUFAs activate RXRα, while monounsaturated and saturated fatty acids have little or no effect. Therefore, increasing concentrations of various fatty acids were incubated with the RXRα LBD and ligand binding investigated by ES mass spectrometry. The PUFA, arachidonic acid (C20:4, Mr 304.47 Da), was found to bind to the RXRα LBD (Fig. 4, F–H), whereas the saturated fatty acid, stearic acid (C18:0, Mr 284.48 Da) did not (Fig. 4I). This is in accordance with the activity measurements presented above (Fig. 1C). Similarly, other PUFAs including docosapentaenoic (C22:5, M, 330.51 Da), linolenic (C18:2, 312.53 Da), and arachidic acid (C20:0; K), or 50 μM docosanoic acid (C22:0; L). The glucuronoylated variant of RXRα LBD is labeled with a solid circle and the RXRα LBD-fatty acid complex by an arrow.

**Fig. 4.** RXRα LBD binds unsaturated fatty acid ligands in solution. ES mass spectra of the RXRα LBD (10 μM) in the absence (A and E) and presence of 12.5, 25, and 50 μM DHA (C22:6; B–D), arachidonic acid (C20:4; F–H), or 50 μM stearic acid (C18:0; I), 50 μM oleic acid (C18:1; J), 50 μM arachidic acid (C20:0; K), or 50 μM docosanoic acid (C22:0; L). The glucuronoylated variant of RXRα LBD is labeled with a solid circle and the RXRα LBD-fatty acid complex by an arrow.
ligand-binding pocket of the RXR of all-
-trans-RA to 9-
-cis-RA (50).
Two NRs phylogenetically closely related to RXR have recently been shown to co-purify with a receptor-ligand complex for the high-affinity ligand 9-
-cis-RA, the main peak observed corresponds to the RXR LBD (Fig. 5A). This suggests that we observe specificity of binding and stearic acid (expected m/z 2638.96 for the 12+ charge state), while a peak corresponding to the RXR LBD-DHA complex is observed (m/z 2643.25; Fig. 5B). Similarly, when an RXR LBD solution is incubated with 25 μM DHA and 25 μM 9-cis-RA, the main peak observed corresponds to the receptor-ligand complex for the high-affinity ligand 9-cis-RA (Fig. 5C). This suggests that we observe specificity of binding to the RXR LBD for DHA and 9-cis-RA, respectively. As expected, the results also indicate that 9-cis-RA has a higher affinity for the RXR ligand-binding site because 9-
-cis-RA is indicated by a solid circle.

Identification of Specific RXR Ligands in Mixtures of Potential Ligands—The above results show that receptor-ligand binding can be observed in a simple mass spectrometric experiment. The methodology can theoretically be extended to the analysis of mixtures of potential ligands. This is illustrated for the RXR LBD and a mixture of ligands consisting of DHA (C22:6), stearic acid (C18:0), and erucic acid (C22:1), all at 12.5 pmol/μl concentration (Fig. 5B). As expected, there is no evidence for a complex formed between the RXR LBD and stearic acid (expected m/z 2638.96 for the 12+ charge state), while a peak corresponding to the RXR LBD-DHA complex is observed (m/z 2643.25; Fig. 5B). This suggests that the PUFAs are released from brain lipids during incubation in the medium and that their binding to the RXR LBD is sufficiently strong to withstand the purification procedure, most notably a gel-filtration step (see “Experimental Procedures”). To show binding specificity of the fatty acids, a 1.5-fold excess of high-affinity ligand LG268 was added to the medium prior to the addition of receptor protein. Formation of the RXR LBD-fatty acid complex could thus be inhibited by about 60%, as would be expected if the fatty acids were acting as true ligands competing with LG268 for binding to the RXR LBD (Fig. 5C). As shown in Fig. 6C, the total concentration of the three bound fatty acids in the recovered protein fraction was roughly equimolar to the protein concentration indicating a 1:1 binding stoichiometry (Fig. 6C). This shows that RXR is specifically binding these fatty acids in the brain-conditioned medium, and that binding is probably occurring through the RXR ligand-binding pocket. Direct lipid extraction of brain-conditioned medium in the absence of RXR LBD suggests that oleic acid, arachidonic acid, and DHA are the major fatty acid constituents in brain (data not shown).

Isolation of Specific RXR Ligands from Brain-conditioned Medium—Two NRs phylogenetically closely related to RXR, i.e. hepatocyte nuclear factor 4 α and γ (HNF4 α and γ, respectively), have recently been shown to co-purify with a mixture of C14–18 fatty acids (17, 18). In the current study, we have incubated RXR LBD with brain-conditioned medium (13), which acts as a source of fatty acids, and then purified the resulting protein-ligand complexes. After extraction, the ligands were analyzed by negative ion ES (Fig. 6A). Three major [M–H]− ions were observed at 281, 303, and 327 Th, corresponding to deprotonated molecules of oleic acid, arachidonic acid, and DHA, respectively. The ions at m/z 255 and 283 Th were mainly due to contaminants, as they were also present in spectra of blank samples (data not shown). In contrast, when the RXR was purified after incubation with nonconditioned medium, the ions at m/z 303 and 327 were not observed and that at m/z 281 was only detected in low abundance (data not shown). This suggests that the PUFAs are released from brain lipids during incubation in the medium and that their binding to the RXR LBD is sufficiently strong to withstand the purification procedure, most notably a gel-filtration step (see “Experimental Procedures”). To show binding specificity of the fatty acids, a 1.5-fold excess of high-affinity ligand LG268 was added to the medium prior to the addition of receptor protein. Formation of the RXR LBD-fatty acid complex could thus be inhibited by about 60%, as would be expected if the fatty acids were acting as true ligands competing with LG268 for binding to the RXR LBD (Fig. 5B and C). As shown in Fig. 6C, the total concentration of the three bound fatty acids in the recovered protein fraction was roughly equimolar to the protein concentration indicating a 1:1 binding stoichiometry (Fig. 6C). This shows that RXR is specifically binding these fatty acids in the brain-conditioned medium, and that binding is probably occurring through the RXR ligand-binding pocket. Direct lipid extraction of brain-conditioned medium in the absence of RXR LBD suggests that oleic acid, arachidonic acid, and DHA are the major fatty acid constituents in brain (data not shown).

DISCUSSION

The signaling capacity of RXR in vivo remains unclear, as its proposed natural ligand, 9-cis-RA, has been difficult to detect in mammalian tissue (8–12). However, a number of studies have indicated that active signaling by RXR itself is of biological importance (51–55). One of the main fatty acids in brain, DHA, was previously identified as an endogenous ligand for

Fig. 5. Electrospray mass spectrometry can be used to detect RXR ligands in simple ligand mixtures. ES mass spectra of the RXR LBD (10 pmol/μl) alone (A) or with the addition of mixtures containing either 12.5 pmol/μl each of stearic acid (C18:0), erucic acid (C22:1), and DHA (C22:6) (B), or 9-cis-RA (9cRA) and DHA, both at 25 μM (C). The arrow indicates the position on the m/z scale of the receptor-ligand complex. Gluconoylated RXR is indicated by a solid circle.
Polyunsaturated Fatty Acids Bind to the RXR Ligand-binding Domain

**Fig. 6.** Affinity capture of RXRα ligands from brain-conditioned medium. **A**, negative ion electrospray spectrum of lipid mixture extracted from RXRα LBD protein after incubation with brain-conditioned medium. The peaks at m/z 281, 303, and 327 correspond to the [M-H] ions of oleic acid, arachidonic acid, and DHA, respectively. The peak at m/z 329 represents the [M-H] ions of the added internal standard, [14C]DHA. Peaks at 255 and 283 represent fatty acid contaminants, presumably palmitic and stearic acids. **B**, spectrum of lipid extract as in A but in the presence of a 1.5-fold excess of LG268 to protein. Diagram **C** shows concentrations (µM) of DHA (C22:6), arachidonic acid (C20:4), and oleic acid (C18:1), as well as their summed concentration and the protein concentration in the respective purified RXRα LBD fractions (A and B), i.e. without (light gray bars) and with 1.5-fold excess of LG268 (black bars). Note that the spectrum in B is shown at 1.7-fold magnification to normalize the intensities of the peaks for the internal standard (m/z 329, i.e. [14C]DHA) in the two spectra (A and B).

RXR (13). The current study using *in vitro* activity assays and ES mass spectrometry has provided additional evidence for the existence of a fatty acid-stimulated RXR-dependent signaling pathway *in vivo*. Importantly, it is shown that additional unsaturated fatty acids, including docosapentaenoic, arachidonic, and oleic acids, also bind to and activate RXR, suggesting that this ability is not exclusive for DHA.

Furthermore, DHA was shown to share the functional property of 9-cis-RA to synergistically activate RXR-RAR heterodimers in combination with all-trans-RA (Fig. 2C). This finding further supports the function of unsaturated fatty acids as true RXR agonist ligands.

Initially, a high concentration (50–100 µM) of free fatty acid was shown to be required for efficient receptor activation in transfected cells (13). However, after refining the method of ligand addition, the present study has shown that several fatty acids activate RXR with EC₅₀ values of about 5–10 µM (Fig. 1C). Such levels of free fatty acids are likely to exist *in vivo* as they correlate with the concentrations required for proper function of fatty acid metabolizing enzymes such as cyclooxygenase 1 and 2 (reviewed in Ref. 56). Previous studies have shown that phospholipases A₂ and C can mediate an autocrine release of free fatty acid from membrane-bound phospholipids, for example in response to neurotransmitters (57) and after neuronal damage (Ref. 57 reviewed in Ref. 58).

Recently, a DHA-specific phospholipase A₂ has been reported, which could be involved in supplying RXR with sufficient free DHA (59, 60). Alternatively, it has been suggested that neighboring cells could supply neurons with free DHA in a paracrine fashion (61). Taken together, these results suggest that sufficiently high levels of free fatty acid could accumulate in cells to allow efficient activation of RXR.

Also recently, a number of articles have reported the unexpected presence of fatty acids in the crystal structure of several NR LBDs. For example, the two receptor isotypes HNF4α and γ were shown to bind a mixture of saturated and monounsaturated C₁₄–₁₈ fatty acids (17, 18). Similarly, the RA-related receptor β was crystallized containing a stearic acid molecule in the ligand-binding pocket (19). A mutated version of mouse RXRα was crystallized containing a fortuitous fatty acid ligand (oleic acid) in the ligand-binding pocket (16). Finally, the insect ortholog of RXR, Ultraspiracle, was recently crystallized carrying a phospholipid ligand in its ligand-binding pocket (62, 63). In the present study, the copurification of the RXRα LBD along with fatty acid receptor agonists (DHA, arachidonic acid, and oleic acid) derived from brain is demonstrated. The PPAR and liver X receptor subgroups of NRs have previously been shown to bind and be regulated by PUFAs having apparent dissociation constants in the low micromolar range (48, 64). These results indicate that the ability to use fatty acids as a stabilizing structural element might be a widespread phenomenon within the NR family. In this context, it is interesting to note that RXRs are phylogenetically most related to HNFs, suggesting that perhaps these receptors evolved from a common receptor ancestor with such a capacity for fatty acid binding (65). According to the present results, the RXRs would then have gained
the ability to use the fatty acids as true ligands to regulate their transcriptional activity.

An important concern regarding the mass spectrometric studies presented here is whether or not the noncovalent interactions observed reflect direct binding of the fatty acid ligand in the ligand-binding pocket of RXR. Due to the lipophilic nature of fatty acid molecules, the possibility exists that the observed interactions are merely reflecting nonspecific binding to the receptor surface. With this in mind, the present investigation also included control molecules that did not activate the receptor in transfection assays, including the saturated fatty acids stearic acid (C18:0) and arachidic acid (C20:0), docosanoic acid (C22:0), as well as the monounsaturated fatty acid erucic acid (C22:1) (Fig. 1C, and data not shown). These fatty acids were not found to bind to the RXRα LBD (Fig. 4, I, K, and L, and data not shown). However, the spectrum shown in Fig. SB illustrates the limitation of ES mass spectrometry when analyzing mixtures of ligands. The non-binder erucic acid (C22:1) has a mass of 338 Da, just 10 Da more than DHA, and on the m/z scale a 10-Da mass difference for a 12+ ion translates to only a 0.8 Th difference. This degree of resolution and mass accuracy can be difficult to achieve in the study of native proteins, suggesting that investigations of ligand binding should be restricted to mixtures of potential ligands that differ sufficiently in mass to be resolved (at least partially) in the resulting mass spectra. However, Rai and colleagues have recently demonstrated that masses of proteins within a mixture can be accurately determined (10 ppm) by the use of an internal calibrant, even when individual protein masses cannot be resolved (66). In the current study, the free RXRα LBD could be used as such an internal calibrant, in which case the mass of a bound ligand could be accurately determined to ±0.3 Da.

Taken together the mass spectrometric results increase our confidence in the validity of the described mass spectrometric method for observing specific receptor ligand interactions. The results indicate that protein affinity purification coupled with ES mass spectrometry could be used in future studies to detect interactions between other NRs and their ligands. Specific ligands could thus be identified and isolated from complex mixtures such as tissue homogenates or conditioned media.

The present results show that several unsaturated fatty acids, including DHA, arachidonic acid, and oleic acid, have the capacity to specifically bind and activate the RXRα LBD and thereby act as in vivo ligands for this receptor. These findings indicate that RXRs could play important roles as fatty acid sensors in vivo. Thus, the data challenges the current view of 9-cis-RA as the main RXR ligand in vivo and suggests that fatty acid ligands have the potential to exert important effects on RXR-mediated gene transcription. Indeed, RXRs are expressed in tissues known to be involved in lipid metabolism, and RXR-specific ligands have been shown to have potent effects on lipid homeostasis (67–70). Thus, our results provide additional evidence that RXR plays an active role as a signaling receptor with the capacity to become activated by free fatty acids in vivo.

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