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Specificity of Receptor – Ligand Interactions and Their Effect on Dimerisation as Observed by Electrospray Mass Spectrometry: Bile Acids Form Stable Adducts to the RXR α

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Abstract

Electrospray (ES) mass spectrometry data is presented showing that agonist binding to the nuclear receptor (NR), retinoid X receptor α (RXR α), is competitive. The competitive nature of agonist binding can be used to discriminate between the specific and non-specific binding of small lipophilic molecules to NRs. Further, data is presented showing that high affinity ligand binding to the RXR α ligand binding domain (LBD) stabilises the domain homodimer. The results indicate that homodimerisation, a functional property of the receptor associated with the binding of agonist ligands, could be used to discriminate between specific and non-specific binding events. Additionally, we report on the remarkable stability of the gas-phase complex between the RXR α LBD protein and endogenous bile acids. Gas-phase protein – bile acid interactions were found to be surprisingly strong, withstanding “in-source” fragmentation in the ES interface, and, in the case of taurocholic acid and lithocholic acid-3-sulphate, collision-induced dissociation within the collision cell of a tandem mass spectrometer. Bile acids were found to be inactive towards RXR α in transfection assays, and have not been reported to be ligands for the RXR α , although lithocholic acid has been found to be a competitor in the photoaffinity labelling of RXR β with 9-*cis*-retinoic acid (Radominska-Pandya, A. and Chen, G. 2002, *Biochemistry*, 41:4883-90). The observation of strong RXR α -bile acid non-covalent complexes in ES mass spectrometry highlight the danger of extrapolating gas-phase binding data to the solution-phase and further to a possible biological activity, particularly when surface active compounds such as bile acids are involved. The introduction of a competitive ligand binding experiment can alleviate this problem and allow the differentiation between specific and non-specific binding.

Keywords

Electrospray Ionization Mass Spectrometry; Nuclear Receptor; Non-covalent complexes; Lipids; Bile acids

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Introduction

Nuclear receptors (NRs) are ligand activated transcription factors with important biological functions in development and differentiation as well as in metabolic homeostasis in the adult.¹⁻³ Nuclear receptor ligands are generally small (i.e. molecular weight less than 500 Da) lipophilic molecules that may pass through the plasma membrane and into the cell interior. Identified endogenous NR ligands include steroids, retinoids, oxysterols, fatty acids and bile acids.^{1, 4} Further, several reports indicate that endogenous lipids including cholesterol,⁵ fatty acids⁶⁻⁹ and phospholipids^{7, 10, 11} can bind within the ligand binding pocket (LBP) of NRs during protein purification. For the peroxisome-proliferator activated receptors (PPARs) and the retinoid X receptor α (RXR α) free fatty acids have been shown to function as true receptor agonists.^{6, 12, 13}

Given the importance of NRs in physiology and disease, as well as the relative ease of administering their agonists/antagonist as pharmaceuticals, NRs make important drug targets. Several reports have shown that the binding of NR ligands to their cognate receptor can be observed directly by electrospray (ES) mass spectrometry,¹⁴⁻¹⁷ and it is now recognised that this form of mass spectrometry has the potential to be used in the identification of novel ligands to NRs.¹⁸ Further, as ES mass spectrometry is becoming ever more amenable to automation,¹⁹ it holds promise of becoming a practical tool for screening of ligand binding to receptor proteins. However, the degree of binding specificity retained in the ES process remains to be fully explored. As ES mass spectrometry takes place in the gas-phase, it follows that there may exist discrepancies between observations made by ES mass spectrometry, and the actual events in the condensed-phase. According to the current ideas of the ES process, the relative importance of intra and inter molecular ionic interactions are enhanced compared to hydrophobic interactions as solvent is removed. Thus, the mere detection of a receptor – “ligand” complex by ES mass spectrometry may not in itself be sufficient to allow extrapolation of the observation to the situation in the solution-phase. Previously, the binding of fatty acid agonists to the RXR α ligand binding domain (LBD) was investigated by ES mass spectrometry in combination with transfection assays of receptor activation.²⁰ In the current study additional mass spectrometry experiments were performed, which allow this single technique to be used to differentiate between specific and non-specific receptor – “ligand” interactions.

Moreover, experiments were performed in which the non-covalent binding of bile acids to the RXR α LBD were investigated. Endogenous bile acids are ligands for the farnesoid X receptor (FXR) and the pregnane X receptor (PXR) and are involved in the control of cholesterol elimination by regulating transcription of CYP7A1, the rate-limiting enzyme in bile acid biosynthesis.²¹⁻²⁴ Chenodeoxycholic acid (CDCA) shows the highest affinity for FXR among endogenous bile acids, while cholic acid (CA) and lithocholic acid (LCA) are less potent receptor agonists.^{22, 23} In addition to activating PXR, LCA binds to another NR, the vitamin D receptor.²⁵ To the best of our knowledge, bile acids have not been reported to function as RXR α agonist ligands. However, data has been presented using a photoaffinity labelling approach that identified LCA as a ligand for RXR β .²⁶ Further, in vitro experiments have shown that taurocholic acid (TCA) and phospholipids can inhibit estradiol and progesterone binding to myometrial estrogen and progesterone receptors, respectively.²⁷ The inhibition of ligand binding was weaker for CA than for TCA and a non-ionic detergent (Triton X-100) had no effect.²⁷ In the current study, stable bile acid - RXR α LBD complexes were observed by ES mass spectrometry. In order to ascertain if bile acids induce receptor activation in vivo, TCA, CA, CDCA and LCA were tested for RXR α activity using a reporter-gene assay.

For NRs, agonist ligand binding results in conformational changes of the NR LBD. Importantly, stabilisation of the C-terminal helix H12 (i.e. the “activation function-2”, AF-2 helix) in its active conformation is critical in formation of the co-activator protein interaction surface and thus for transcriptional activation.³ In the absence of ligand, the RXR LBD protein has been shown to be dominantly tetrameric in solution, while ligand binding induces homodimer formation.²⁸⁻³⁰ It has been proposed that changes of the oligomeric state could serve regulatory functions in vivo.³¹⁻³³

To screen multiple test compounds for receptor binding, the LBD of the protein is necessarily purified in the absence of ligand. However, this purification procedure results in a mixed solution of monomer and, for the main part, the highly stable *apo*-tetramer.²⁸ For the RXR LBD purified in the absence of agonist, a mixture of monomer and tetramer species was observed in the mass spectrum.²⁰ Though ligand binding to the monomer could be observed, upon ligand addition there were no clear changes in the oligomeric state of the receptor (i.e. no high degree of dimerisation).²⁰ Recently, a method for the purification of homodimeric *apo*-RXR was described based on the use of a zwitterionic detergent (3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS).³⁴ Using this alternative method for protein purification, we have evaluated the propensity for dimerisation of the RXR α in the presence of the high affinity ligand LG268 or the endogenous bile acid, TCA. As agonist ligands induce RXR homodimerisation, dimer abundance could serve as a marker for ligand efficiency that would be readily detectable by non-covalent ES mass spectrometry.

Experimental

Reagents and solutions

Dithiothreitol (DTT), CHAPS, lithocholic acid-3-sulphate (LCA-3-sulphate), sodium lauryl sulphate (sodium dodecylsulphate, SDS), *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), *cis*-7,10,13,16-docosatetraenoic acid (DTA) and 9-*cis*-retinoic acid (9-*cis*-RA) were from Sigma (Sigma-Aldrich Sweden AB, Stockholm, Sweden). RXR-antagonist LG1001208, retinoic acid receptor antagonist RO41-5253, 17 β -estradiol (E₂) and the bile acids CDCA, CA, LCA, TCA as their sodium salts were from previous studies. LG100268 (LG268) was from Tularik Inc. CA, USA. Stock solutions of 10 mM were prepared of all compounds in 99.5% ethanol, except for TCA which was dissolved in 50% aqueous methanol. See Scheme 1 for structures of selected compounds used.

Cell culture and transfections

HEK293 and JEG-3 cells were maintained in Dulbecco's modified Eagle's medium and minimal essential medium, respectively (both from Gibco BRL, Life Technologies, Täby, Sweden), supplemented with 10% stripped fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine. Transfections were performed in triplicate in 24-well plates using Lipofectamine Reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). Briefly, each well was transfected with 100 ng of effector plasmid encoding the LBD of RXR α (CMX-RXR α) and 200 ng of a luciferase reporter plasmid containing three RXR-binding sites from the apolipoprotein 1 gene (ApoA1-tk-luc) or the RAR β gene (β RE-tk-luc), followed by a minimal thymidine kinase promoter. As a reference, 200 ng CMX- β gal plasmid containing the β -galactosidase gene was used.³⁵ 4-5 hours after transfection, potential ligands were added to a final volume of 1 mL in each well. Fatty acid and bile acid stock solutions were diluted in dimethyl sulphoxide and added directly into each well. High affinity agonists LG268 and 9-*cis*-RA, as well as RXR-antagonist LG1001208 and RAR-antagonist RO41-5253 were diluted in medium prior to addition. The cells were harvested 24 hours later, and the extracts were assayed for

luciferase and reference β -galactosidase activity in a microplate luminometer/photometer reader (Lucy-1, Anthos, Salzburg, Austria).

Protein expression and purification

The human RXR α LBD, amino acids 203-462, (hereafter “RXR α ”) was expressed as a His₆-tagged fusion protein in *E. coli* and purified by affinity chromatography. Protein-containing fractions were pooled and buffer exchanged to 50 mM ammonium acetate, pH 8.1, by dialysis. Aliquots of this protein stock solution (~8.7 mg/mL) were stored at -70 °C until analysis.

Mass spectrometry

All mass spectra were acquired on a Micromass QTOF 1 mass spectrometer (Micromass plc, Manchester, UK) operated in the positive-ion mode. When using conventional-ES, the capillary voltage was kept constant at approximately 3.0 kV. The potential offset on the collision cell was 4.2 V unless otherwise indicated. The desolvation gas-flow was 300 L/hr at a temperature of 120 °C. The collision cell was pressurised using argon to give a reading of 3.5×10^{-5} mbar on the nearby ‘analyser’ Penning gauge. Furthermore, the pumping to the ES interface was reduced to increase the interface pressure such that the reading on the ‘analyser’ gauge was increased to $\sim 5.5 \times 10^{-5}$ mbar to preserve protein – ligand non-covalent interactions.³⁶ The m/z scale of the instrument was calibrated using caesium iodide clusters by spraying a 5 mg/mL caesium iodide solution (50 % aqueous isopropanol) from a metal coated borosilicate capillary (Protana AS, now Proxeon, Odense, Denmark) using the nano-ES interface.

The abundance ratio of *holo*- / *apo*-receptor ($R_{\text{holo-/apo}}$) was calculated using the peak-top abundance value for each peak measured relative to the peak-top intensity of the background noise at the onset of the peak in the mass spectrum. As the high mass tailing to the RXR monomer peaks was observed to vary considerably between different analysis sessions, two control samples (RXR 10 μ M, 10 mM ammonium acetate, pH 8.1, with and without the addition of either DHA or LG268) were included in each session. Variations in the degree of adducting (i.e. desolvation efficiency) which explain these tailing effects may be the result of slight variations of the interface conditions between sessions. It should be noted that on the QTOF1 instrument, the ‘analyser’ Penning gauge (used in this work to monitor the ES interface pressure) is located close to the collision cell. Thus, the distance between the ES atmosphere-vacuum interface region and the gauge itself is considerable and small variations in the gauge reading may correspond to significant variations in the actual source pressure.

Sample analysis

For each analysis session the protein stock solution was thawed on ice and diluted in 10 mM ammonium acetate, pH 8.1, to give a final protein concentration of 10 μ M. For each sample, lipid stock solution (10 mM) was added to 300 μ L of 10 μ M RXR α LBD solution to give a final lipid concentration of 10 – 50 μ M, keeping the alcohol concentration of the sample at or below 0.5%. Samples were then equilibrated for 25 min at room temperature before starting sample infusion. Sample solutions were delivered to the ES interface at a flow rate of 5 - 10 μ L/min using a Harvard type 22 syringe pump (Harvard Apparatus, South Natick, MA, USA).

Modified procedure for the analysis of homodimers

When experiments were performed to investigate the stability of the RXR α LBD homodimer upon addition of agonist, a slightly modified sample purification and analysis protocol was used.

Protein purification—Protein purification was essentially as above with modifications according to published procedures.³⁴ Briefly, the His-tagged protein was eluted from the affinity column into a tube holding 0.5 M EDTA and 0.1 M CHAPS to give final concentrations of 5 and 0.5 mM, respectively. The eluate was then subjected to one round of gel filtration (PD-10 column, Bio-Rad, Hercules, CA, USA) to obtain the LBD protein (~9.8 mg/mL, Bradford assay) in 500 mM KCl, 50 mM potassium phosphate (pH 7.4) with 10 mM DTT and 0.5 mM CHAPS and was stored at -70 °C until use.

Sample preparation, RXR homodimerisation—Protein stock solution was thawed and 50 μ L was buffer exchanged to 10 mM ammonium acetate (pH 8.0) using micro-biospin p6 spin columns (Bio-Rad, Hercules, CA, USA) and kept on ice. Just before analysis, this solution was diluted to ~20 μ M (assuming 100% recovery in the gelfiltration) RXR LBD in the same buffer. This dilution was determined empirically to give adequate signal intensity for RXR. Ligand stock solution was then added to give a final ligand concentration of 40 μ M. The sample solution (200 μ L) was incubated for 25 min at room temp before being loaded into a borosilicate glass nanospray capillary (Protana AS, now Proxeon, Odense, Denmark) for analysis by nano-ES.

Mass spectrometry, RXR homodimerisation—Nano-ES analysis was essentially as described above, but with the following modifications. Argon was introduced into the collision cell such that the 'analyser' Penning gauge reading increased from 7.8×10^{-6} to 1.3×10^{-5} mbar. To increase the source pressure, the backing rotary pump gas line was throttled bringing the 'analyser' gauge reading to 5.0×10^{-5} mbar. The cone voltage was set to either 20 or 80 V as is indicated in the figure legends. A gentle flow of nitrogen gas was applied to the back of the nanospray capillary to start the electrospray.

Results

Bile acids do not induce RXR α activation

The bile acids CA, TCA, CDCA and LCA did not induce RXR α receptor activity when tested on transfected cells. The RXR α agonists LG268 and DHA were used as positive controls. Shown in Fig. 1 are titrations over indicated concentration ranges for LG268 (10^{-10} – 10^{-6} M), DHA (C22:6) and DTA (C22:4) (10^{-6} – 10^{-4} M), and the bile acids TCA, CDCA and CA (10^{-5} – 10^{-4} M) and LCA (10^{-6} – 10^{-4} M). As shown, LG268 induces RXR α activation with an effective concentration at half maximum response (EC_{50}) value in the nanomolar range (Fig. 1). This activity could be abolished by addition of an RXR α antagonist (LG1001208) but was not affected by an RAR antagonist (RO41-5253, data not shown). The low affinity agonists DHA and DTA show receptor activation at micromolar concentrations (Fig. 1). The bile acids CA, TCA, CDCA (Fig. 1) and LCA (data not shown) were all inactive up to the highest concentrations tested.

Mass spectra of RXR α LBD

When an aqueous 10 mM ammonium acetate solution, pH 8.1, of 10 μ M RXR α LBD protein was analysed at a low cone-voltage, i.e. 50 V, the resulting spectrum is dominated by an envelope of multiply charged ion peaks in the m/z range 2400 - 2900 (Fig. 2A). The peaks observed at m/z values 2414, 2615 and 2853 correspond to the 13+, 12+ and 11+ charge-states of monomeric RXR α LBD. The observation of just a few charge states at high

m/z is characteristic of a protein in a folded conformation.³⁷ Deconvolution of this peak series onto a true mass scale gives an observed molecular mass of 31370 Da, which is in good agreement with the expected value (31371 Da). When a 10 μ M RXR α LBD solution was analysed under more energetic ES conditions, i.e. at a cone voltage of 195 V and a collision voltage of 50 V, the 13+ to 11+ charge state envelope remained as the main peak envelope (data not shown). However, the minor ion series corresponding to RXR α dimer (m/z 3250 – 4000, Fig. 2A) and tetramer (m/z 4350 – 5300, data not shown) observed under the milder conditions, were not stable under the more energetic conditions (data not shown). This shows that the latter instrument conditions are sufficiently harsh to dissociate non-covalent protein – protein complexes. Each RXR α monomer peak shows a satellite peak at higher m/z which corresponds to the addition of 178 Da to the monomeric molecules, and has been suggested to correspond to gluconoylation (+C₆H₁₀O₆, M_r 178.14) of the His₆-affinity tag.³⁸ This modification is indicated by a filled circle in Figs. 2-8. Additional satellite peaks are present which correspond to the addition of 60 Da to the RXR α monomer e.g. at m/z 2620 for the 12+ charge state. Presumably these correspond to acetic acid adducts to the protein.³⁹ The +60 Da adducts were unstable at increased cone voltages, indicating their non-covalent nature.

Binding specificity is preserved in ES mass spectrometry of fatty acid ligand - RXR complexes

The polyunsaturated fatty acid DHA (C22:6) has been identified as a natural low affinity agonist for RXR α ,^{7, 12, 13} while the synthetic aromatic acid LG268 is a high affinity agonist. Ligand binding to RXR α was investigated at a 2.5-fold excess concentration of ligand to protein (25 μ M and 10 μ M respectively), under experimental conditions optimised to preserve RXR α – agonist non-covalent complexes (i.e. increased ES source pressure, a cone voltage of 50 V and a collision cell potential of 4.2 V). Receptor – ligand binding was clearly observed for the high affinity ligand LG268 (Fig. 2C, m/z 2645.9) and for DHA (Fig. 2D m/z 2643.0). The m/z increments correspond in the respective cases to the added mass of LG268 and DHA (for relative molecular mass, M_r , values of tested compounds see Table 1). Under these conditions, the observed receptor – agonist ligand complexes show predominantly a 1:1 stoichiometry (Fig. 2C and D). The *holo* / *apo*-receptor ion abundance ratio ($R_{\text{holo/apo}}$) values were calculated to 1.15 (LG268) and 0.58 (DHA). A number of other polyunsaturated fatty acids including docosapentaenoic acid, arachidonic acid, linoleic acid and linolenic acid as well as high affinity RXR α agonists 9-*cis*-RA and SR11237 (BMS 649) were similarly shown to bind to RXR α .²⁰

To confirm that the observed gas-phase RXR α - ligand complexes were a result of specific ligand binding to the RXR α LBD, an RXR α non-agonist, 17 β -estradiol (E_2) was analysed under the same experimental conditions (i.e. 10 μ M RXR α and 25 μ M E_2) in a control experiment (Fig. 2E, E_2 is incapable of activating the RXR α in transfection experiments, but is a high affinity agonist for the estrogen receptor).⁴⁰ By comparing Fig. 2E (25 μ M E_2) to Fig. 2B (no ligand added) it is evident that a major peak corresponding to an RXR α - E_2 complex is not observed. A minor peak is observed at m/z 2638.4 for the 12 + charge state, corresponding to an added mass of 272 Da, i.e. the mass of E_2 , and a $R_{\text{holo/apo}}$ value of 0.17 was determined. However, it is probable that this value of $R_{\text{holo/apo}}$ is anomalously high, as the base line in the region of spectrum around m/z 2638 is elevated even in the absence of ligand (Fig. 2B). This base-line elevation is probably due to a covalent modification i.e. phosphogluconoylation (addition of 258 Da), to the His₆-affinity tag.³⁸ Nevertheless it appears that some non-specific binding of E_2 to the receptor is being observed. When the spectra in Figs. 2C and D are examined closely in the region above m/z 2660, low abundance peaks, can be discerned corresponding to the binding of a second molecule of LG268 ($R_{\text{holo/apo}}$ = 0.23) or DHA ($R_{\text{holo/apo}}$ = 0.07) to the protein. Together, with the

observed RXR α - E₂ interactions, it appears that a minor degree of non-specific binding occurs under the experimental conditions used.

Competitive binding between RXR α high and low affinity agonists

To further investigate the binding specificity of RXR α agonist ligands in ES mass spectrometry, experiments were performed in which the competitive binding between the high affinity ligand LG268 and the low affinity ligand DHA was investigated, cf ref 41. When DHA was added at an equimolar concentration to protein (DHA concentration 10 μ M), the RXR α - DHA complex was observed at m/z 2642.9 for the 12+ charge state (Fig. 3B, $R_{\text{holo-/apo}} = 1.08$). (It should be noted that $R_{\text{holo-/apo}}$ values vary from session to session, this may be on account of slightly different instrumental conditions used at different times, and/or a change in the amount of correctly folded protein with time. Only correctly folded protein can accommodate ligand in its LBP). When LG268 was added together with DHA at an equimolar concentration (i.e. 10 μ M each) to RXR α , only a single RXR α - ligand complex was observed i.e. at m/z 2645.8 (Fig. 3C, $R_{\text{holo-/apo}} = 2.47$), corresponding to the addition of the high affinity ligand LG268 to RXR α . As the binding affinity of LG268 is in the nanomolar range⁴² compared to the micromolar affinity of DHA,¹³ these results indicate that LG268 and DHA are both competing to occupy the LBP of RXR α , and specific ligand - receptor interactions are being observed. The ability of a high affinity ligand to displace a low affinity ligand from the NR LBP, can be used as a test for the specificity of "ligand" - NR interactions observed by mass spectrometry.

Bile acid binding to RXR α

As the bile acids CA, TCA, CDCA and LCA were not found to activate RXR α in transfected cells, it was not expected that they would be shown to bind to the RXR α LBD with any great affinity in ES mass spectrometry experiments. However, these three bile acids were observed to strongly bind to the receptor in ES experiments. When the bile acid TCA was added in combination with the high affinity ligand LG268 to the RXR α LBD, but at a three fold higher concentration (Fig. 4C), it was not able to displace LG268 from the LBP, and no evidence was obtained for competitive binding. Remarkably, however, when the same mixture was analysed at a high cone voltage (195 V cf. 50 V), TCA - protein adduct peaks were observed at m/z 2658.3 and m/z 2701.2 (indicated by a filled diamond in Fig. 4D) which had been previously masked by the high mass tailing of the receptor - agonist peaks and other undissociated adducts (Fig. 4C), but no evidence was obtained for remaining LG268 - RXR α LBD non-covalent complex. This shows that TCA forms a very stable complex with RXR α in the gas-phase, but when analysed under mild conditions its inability to displace LG268 from the LBP suggests that the binding is not in this pocket. It should be noted that the RXR α LBD is being analysed as a His₆-tagged fusion protein, and bile acids, particularly taurine conjugated bile acids, may interact with the His₆-tag.

A subsequent competition experiment was then performed between TCA and the weak RXR α agonist DHA, both at 10 μ M concentrations. Comparison of the spectra in Fig. 5B and 5C shows that the $R_{\text{holo-/apo}}$ value of 0.63 for DHA binding does not decrease when TCA is additionally present ($R_{\text{holo-/apo}} = 0.71$). This indicates that competitive ligand binding is not occurring. Similar results were obtained when a 3-fold excess of TCA to DHA (30 μ M TCA) was tested (data not shown). It can be noted in Fig. 5C that the peak corresponding to the RXR α protein with a single TCA adduct is essentially unresolved from the peak corresponding to one DHA molecule bound to gluconoylated protein. However, when the cone voltage is increased to 195 V, under which conditions the protein - DHA complex is dissociated, the binding of one molecule of TCA can be readily observed at m/z 2658.6 (Fig 5D). From the above data, despite binding strongly to the RXR α LBD, it can be concluded that TCA does not bind in the LBP.

Very surprisingly for an interaction that does not involve the LBP, the gas-phase RXR α – TCA complex proved to be more stable than the either the RXR α – LG268 or RXR α – DHA complexes which do involve LBP interactions (Fig. 4D and 5D). This is further illustrated in Fig. 6, where DHA and TCA were added in combination to give a solution of 25 μ M of each acid and 10 μ M of RXR α and analysed at a cone voltage of 50 V and 195 V. In the 50 V spectrum peaks were observed at m/z 2642.5 and 2658.0, corresponding to the RXR α – DHA complex (indicated by an arrow) and the RXR α – TCA complex (indicated by a filled diamond), respectively (Fig. 6A). When the cone voltage was increased to 195 V the RXR α – DHA complex dissociated, while the RXR α – TCA complex remained stable (Fig. 6B). Under these more energetic desolvation conditions, additional TCA molecules binding to the protein (up to 3 per monomer) became visible. It is interesting to note how the improved desolvation conditions (i.e. an increase of the cone voltage by 145 V from 50 V to 195 V) allows the observation of protein adducts otherwise obscured by the extensive high mass tailing to the protein peaks usually present when spectra are recorded under the “softer” non-covalent conditions (Fig. 6A).

Stoichiometry of RXR α LBD - bile acid complexes

Although bile acids are ligands for the FXR, evidence from our transfection studies indicate that they are not agonists for RXR α , and the conclusion from the competitive studies described above is that they do not bind to the RXR α LBP. However, the fact that bile acids bind to other proteins, e.g. serum albumin, and also have surfactant properties, does raise the possibility that bile acid - RXR α interactions are of significance in solution, and that the bile acid - RXR α complexes observed by ES are a reflection of solution interactions. To further investigate the behaviour of bile acids as protein adducts under non-denaturing ES conditions, we analysed the natural bile acids TCA, CA, CDCA and LCA in five-fold excess to protein. When TCA, CDCA, CA or LCA was added in five-fold excess to a 10 μ M RXR α solution and analysed at a cone-voltage of 50 V (collision voltage 4.2 V), series of peaks were observed for each charge state at higher m/z than those of the free protein (Fig. 7C-F). The m/z increment in each case corresponds to the added mass of the respective bile acid, i.e. TCA, CA, CDCA and LCA. In each spectrum, binding of multiple bile acid molecules to RXR α LBD monomers is evident. In Fig. 7 bile acid adducts are labelled by filled diamonds, while the presence of the gluconoylated protein (+178 Da modification) is marked by a filled circle. Note that bile acids also bind to the gluconoylated protein. Similar spectra were obtained for lithocholic acid-3-sulphate (LCA-3-sulphate, data not shown). As the number of bound bile acid molecules increases there is an apparent exponential decline in adduct peak intensity (Fig. 7C-F). This binding stoichiometry is markedly different from that of the known fatty acid RXR α agonists, e.g. DHA, which essentially forms a 1:1 complex (Fig. 7B)

Gas phase stability of protein-adduct complexes

As the RXR α – TCA complexes proved to be surprisingly stable in the gas phase (Fig. 4-6), further experiments were performed in an attempt to explain this phenomenon. At a cone-voltage of 50 V, fatty acid agonist – receptor, synthetic agonist – receptor and bile acid – receptor complexes are stable (Fig. 2-7). When the cone voltage was elevated to 85 V, the complex between the fatty acid agonist and RXR α were fully dissociated (data not shown). In contrast, at this cone-voltage the gas phase complexes formed between RXR α and the bile acids were still stable (data not shown). In fact, CA, CDCA and LCA formed RXR α - bile acid complexes that were stable to cone-voltages of up to 130 V, and cone-voltages of 140-185 V were required to fully dissociate these complexes. With respect to cone-voltage, the relative order of stability of bile acid - RXR α interactions is LCA<CDCA<CA, and on this scale, fatty acid agonist – RXR α non-covalent interactions are weaker than bile acid – RXR α interactions.

LCA - RXR α complexes were essentially dissociated at a cone voltage of 140 V while the CA - RXR α complexes were still stable at a cone-voltage of 180 V. This difference in the stability of bile acid - RXR α complexes suggests that the α -side hydroxyl groups of the bile acids are important interacting components in stabilising the RXR α - bile acid complexes in the gas-phase. In addition to α -hydroxyl groups, the bile acids CA, CDCA and LCA have a carboxylic acid group at C-24, while TCA has an aminoethanesulphonic group attached via an amide bond at C-24. When the TCA - RXR α complex was investigated, it showed greater gas-phase stability than CA-, CDCA- or LCA - RXR α complexes. At a cone voltage of 195 V (which is the maximum value allowed on the Q-TOF instrument), the binding of 1-4 molecules of TCA per protein monomer was still evident (Fig. 4D, 5D and 6B). In addition, when the collision cell voltage was increased from 4.2 V to 50 V in an attempt to disrupt the RXR α - TCA complex, the RXR α - TCA complex was found to be stable (Fig. 8A). The increased stability of the TCA - RXR α complexes (Fig. 8A) compared to the CA - RXR α complexes provide an example of the importance of ionic interactions in stabilising non-covalent complexes in the gas-phase. To further study the effect of a strongly acidic group, at a position other than on the bile acid side-chain, on the gas-phase stability of complexes with RXR α , the stability of the RXR α - LCA and RXR α - LCA-3-sulphate complexes were compared (Fig. 8B and C). Under the highly energetic conditions of a cone voltage of 195 V and a collision voltage of 50 V, the LCA sample showed only peaks corresponding to the *apo*-protein (Fig. 8B), while LCA-3-sulphate gave adduct peaks corresponding to 1-5 molecules of LCA-3-sulphate per monomer subunit (labelled with filled diamonds in Fig. 8C). This shows that addition of a strongly acidic group to the bile acid structure drastically increases the stability of a protein - bile acid complex in the gas-phase.

The results of the competitive ligand binding studies indicate that the strong bile acid - protein interactions are unlikely to be the result of bile acid binding within the LBP. It is more plausible that the stability of the bile acid - RXR α complexes in the gas-phase is a consequence of the surfactant nature of the bile acid, i.e. possessing hydrophobic and hydrophilic regions. To test this hypothesis, the surfactant sodium lauryl sulphate (SDS) was incubated with the RXR α . As in the experiments with the bile acids, a solution of 50 μ M of SDS and 10 μ M RXR α was analysed. In the resulting ES mass spectrum, protein - SDS interactions were observed (Fig. 8D, RXR α - SDS complexes are indicated by filled diamonds), and the spectra strongly resembled those of the protein with TCA and LCA-3-sulphate (Fig. 8A and C). At a cone voltage of 195 V and a collision voltage of 50 V, 1-3 bound SDS molecules per RXR monomer subunit were observed (Fig. 8D).

High affinity agonists promote RXR homodimers

When non-liganded RXR LBD was purified in the presence of 0.5 mM CHAPS and analysed by nano-ES, the abundance of monomer (M) and dimer (D) ions was approximately equal (Fig. 9A). The protein peaks are broad due to non-dissociated adducts incompletely removed in the gel-filtration process. Nevertheless, individual charge states can be unambiguously identified as indicated in Fig. 9A. It is noteworthy that peaks corresponding to homotetramers are not apparent in the spectrum. Further, the addition of CHAPS in the purification protocol leads to an about five-fold increase in the relative abundance of the dimer as compared to purification performed in the absence of detergent (data not shown).

When a high affinity agonist (LG268) was added at two-fold excess to protein, there was a distinct decrease in the relative abundance of RXR monomer (Fig. 9B). Alternatively, when the bile acid TCA was added this effect was not observed (Fig. 9C). This indicates that the high affinity RXR agonist LG268 is stabilising the protein homodimer.

Ligand binding to the homodimer is observed upon addition of the high affinity agonist LG268. Considering the most abundant homodimer species, i.e. the 16+ charge state, upon the addition of LG268 there is a distinct shift in peak position (cf. Fig 9D & 9E). This m/z shift corresponds to the addition of two molecules of LG268 per homodimer. Using more energetic ES interface conditions (i.e. a cone voltage of 80 V), the component responsible for this mass shift is dissociated, and only the *apo*-homodimer peak is observed (Fig. 9F). Interestingly, in a similar experiment, the addition of TCA did not cause any increase in mass of the homodimer (cf. Fig. 9D & Fig. 9G). This suggests that, either bile acid binding is blocked by dimerisation, or the bile acid binding sites are already occupied by CHAPS which itself is a cholic acid conjugate. In summary, the results of these experiments on RXR homodimers show that binding of the high affinity agonist increases the relative stability of RXR α homodimers.

Discussion

In recent years, non-denaturing or “non-covalent” ES mass spectrometry has been used to study several types of biologically relevant molecular assemblies, such as protein – DNA, 43-45 and protein – ligand^{15, 46, 47} complexes. Non-denaturing ES mass spectrometry has now become amenable to high throughput techniques, and is becoming increasingly reproducible.¹⁹ As such, its use as a technique to screen for ligand binding is expected to escalate. Within this context, the issue of discriminating between true- and false-ligand binding (i.e. specific and non-specific binding) becomes ever more important.

In this work, the ES response for both true receptor agonist compounds and non-specific binders has been investigated for the NR RXR α . As RXR α ligands bind within a largely hydrophobic LBP, we expected that the folding of the protein around its cognate ligand would result in a higher gas-phase stability of the receptor – ligand complex than for a non-specifically bound adduct. We envisaged that the relative dissociation energies of gas phase non-covalent complexes could be used to discriminate between specific and non-specific binding. In this work bile acids were selected as compounds with non-specific binding properties, while the high affinity agonist LG268 and the low affinity agonist DHA were selected as true ligands. Surprisingly stable bile acid - protein non-covalent complexes were observed in the gas-phase. For the more strongly acidic bile acids TCA and LCA-3-sulphate, the non-covalent interactions between bile acid and RXR α were exceptionally strong, the complexes being sufficiently stable to withstand a cone-voltage of 195 V in combination with a collision voltage of 50 V (600 eV for the 12+ charge state). Fatty acid agonist ligands bound to RXR α required significantly lower energy for complex dissociation. From the bile acid experiments it was concluded that the presence of strongly acidic (sulphonic acid or sulphate ester) groups incorporated into the bile acid structure, drastically increases the gas-phase stability of the protein - bile acid complexes. The relative strengths of the protein – bile acid interactions determined in the gas-phase were in the reverse order to the published data for bile acid – albumin binding in solution. Protein – bile acid binding in solution depends to a large extent on hydrophobic interactions, i.e. on protein hydrophobicity.⁴⁸ Bile acid binding to albumin has been extensively studied and at least two bile acid binding sites have been described.⁴⁹ Interestingly, LCA, but not CDCA or deoxycholic acid, has been shown to compete with 9-*cis*-RA for binding to RXR β using a photoaffinity labelling approach.²⁶ However, the question of receptor activation was not addressed. In the present study, LCA did not activate RXR α and in ES mass spectrometry the binding stoichiometry was the same for all bile acids studied. As discussed below, the binding stoichiometry was different for true ligands and the bile acids. Thus our results indicate that LCA is not a true ligand for RXR α .

In solution, bile acid binding affinity for albumin and plasma lipoproteins decreases with an increasing number of hydroxyl groups in the steroid nucleus while the pK_a of the acidic group is of little importance.^{50, 51} As shown in the present study, the monohydroxylated bile acid LCA formed significantly less stable complexes in the gas-phase than the trihydroxylated bile acid CA. Gas-phase binding strength increased further with the presence of a strongly acidic group. Thus, the order of binding affinity in the gas-phase is opposite to that found in solution. This illustrates that the relative importance of hydrogen bonding and ionic interactions is enhanced upon the removal of solvent in going from the solution- to the gas-phase. Our bile acid data clearly indicates that measurements of relative gas-phase dissociation energies for receptor – “ligand” complexes in this case do not give an indication of binding affinity in solution. Similar examples of discrepancy between solution phase and gas-phase interaction as studied by electrospray mass spectrometry have also been reported for phospholipid interactions with phospholipid transfer protein.⁵²

It should be noted that the RXR α LBD used in the present study is a His₆-tagged fusion protein, and the possibility of ionic interactions between the His-tag and the deprotonated bile acids exists.

An alternative approach to allow the differentiation between specific and non-specific receptor binders would be to use the ratio of the ion abundance for the *holo*- to the *apo*-receptor species. Assuming that specific binding gives the greater $R_{holo/apo}$ value, a cut-off value can be set above which binding can be classified as specific. In the experiments presented in Fig. 2, the $R_{holo/apo}$ value was 1.15 for a high affinity RXR α agonist, 0.58 for a low-affinity agonist, and 0.17 for a non-agonist (17 β -estradiol). Since the base-line of the spectrum may fluctuate due to alterations in the ES interface conditions used, setting an arbitrary cut-off value for what should be considered specific binding may be problematic. Such a general cut-off value would need to be empirically determined based on the analysis of a large number of samples under standardised and reproducible conditions. However, as is evident by comparing the spectra presented in Fig. 2D, 3B, 5B and 7B, small variations in instrumental conditions or protein age can drastically affect the $R_{holo/apo}$ value. Only spectra recorded under identical condition in one analytical session with protein from an identical stock could be effectively compared.

Non-specific binding of small molecules to proteins generally gives concentration dependent binding-stoichiometry i.e. the higher the concentration of the small molecule, the greater the number that can be attached to the protein. In the bile acid binding experiments, the stoichiometry is that of multiple bile acids binding per protein monomer (Fig. 7). In contrast, it is clear from published structural data that cognate ligands bind to the RXR α LBD with a 1:1 stoichiometry.⁷ Even if the correct binding stoichiometry is unknown, the observation of a near exponential decline of ion intensity for increasing numbers of bound bile acid molecules would strongly suggest non-specific binding. The variation of binding stoichiometry of potential ligands to a receptor in a concentration-dependent manner at the very least indicates that binding is non-specific.

In the current study, we show that the best approach to identify specific ligands to a given receptor is to perform competition experiments against a known receptor ligand, in agreement with the proposals of Tjernberg et al.⁴¹ The results indicate that LG268 and DHA bind in a competitive manner to a single site on the protein, presumably the LBP of the RXR α LBD. The relative binding affinities for LG268 and DHA correspond well to reported dissociation constants determined using solution-based methods. In contrast, the bile acid TCA, although binding more strongly to the receptor in the gas-phase, was unable to compete away the receptor agonist DHA from the LBP, even though the bile acid was present at a concentration three-fold excess to DHA and protein. The bile acid did strongly

bind to the protein, but this was not at the expense of DHA, and was thus not in the LBP. These results indicate that when a competitive-binding control experiment is performed, the ES mass spectrometry approach to the measurement of receptor – ligand binding is valid.

True ligand binding to the RXR α LBD affects the multimeric assembly of the protein and leads to formation of homodimers in the absence of heterodimer partner proteins such as the retinoic acid receptor (RAR) LBD.^{29, 30} Ligand-induced dimerisation allows DNA binding and subsequent transcriptional activation by RXR as either a homo- or a heterodimer complex.³² Here we show that receptor dimerisation is stabilised by the high affinity agonist LG268 (Fig. 9) and that the non-specific binder TCA did not stabilise dimerisation. These observations provide further evidence that data from non-covalent ES experiments can be used to assess ligand binding in solution. At least for RXR, analysis of the degree of dimerisation can aid in distinguishing between specific and non-specific NR “ligands”.

Protein folding and protein interactions in solution are greatly influenced by the buffer system used and by additives such as reducing agents and detergents. In this respect, mass spectrometric analyses are at a disadvantage due to their inherent incompatibility with non-volatile salts and detergents. It is certainly possible that overzealous desalting prior to ES analysis can negatively influence the outcome of non-covalent ES experiments, or at least the interpretation of the result. In the sample preparation protocol for the homodimerisation experiments subsequently performed by nano-ES, a one-step gel filtration procedure in the microliter format (50 μ L sample volume) was included to allow desalting. The protein sample was rapidly brought from high ionic strength conditions (0.5 M potassium chloride) to a weakly buffered ammonium acetate solution (10 mM), diluted (to $\sim$ 20 μ M protein concentration) and analysed. This desalting method was sufficient for obtaining mass spectra of acceptable signal intensity under non-denaturing conditions. As the gel-filtration step is readily automated and amenable to scaling down, this represents a simple alternative to more elaborate on-line methods for buffer exchange.⁵³

Finally, it is worth noting that bile acid - RXR α LBD binding may occur in solution, but not in the LBP. Several NR LBD proteins have only been crystallised in the presence of co-activator peptides.⁵⁴ The hydrophobic groove of the co-activator interaction surface⁵⁵ is then covered by the amphipathic α -helix of the co-activator peptide, effectively increasing protein solubility. Significantly, Klaholz and co-workers found a highly ordered detergent molecule (dodecyl- α -D-maltoside) occupying the co-activator binding groove in the crystal structure of the human RAR γ LBD.⁵⁶ This indicates that the globular RXR α LBD protein may exhibit hydrophobic properties due to its co-activator interaction surface. Given the tendency of bile acids to form multimers, in solution⁵⁷⁻⁵⁹ as well as in the gas-phase,⁶⁰ the possibility exists that a multimer of bile acid molecules is interacting with few or a single site rather than several molecules interacting at multiple sites. A single such site may arise in the RXR α LBD from the hydrophobic co-activator binding groove.

In conclusion, our findings show the need for caution in assigning biological functionality to protein – “ligand” interactions as observed by ES mass spectrometry, particularly when the “ligands” are low molecular weight surfactants. For such compounds, the results indicate a poor correlation between gas-phase dissociation energy and solution phase binding affinity. We present a method that will allow the differentiation of specific ligand binding to the LBP from non-specific binding. Taken together, the results of the current study increase our confidence in the value of ES mass spectrometry as a direct ligand binding assay.

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Scheme 1.

Structures of compounds analysed for RXR α binding by ES mass spectrometry, (A) cholic acid, (B) taurocholic acid, (C) chenodeoxycholic acid, (D) lithocholic acid, (E) lithocholic acid-3-sulphate, (F) LG268, (G) docosahexaenoic acid and (H) dodecylsulphate.

Figure 1.

Titration of RXR agonists and bile acids on transfected cells. High affinity LG268 (solid square) induces RXR activation in the nM range. The fatty acids DHA (C22:6, solid wedge) and DTA (C22:4 solid triangle) show EC₅₀-values in the μM range, whereas the bile acids TCA (solid diamond), CDCA (solid circle) and CA (open square) are all inactive. Note that the bile acid data points overlap on to the x-axis.

Figure 2.

Analysis of RXR α under non-denaturing conditions in the absence and presence of agonist ligand. (A) ES mass spectrum of a 10 μ M solution of RXR α LBD in 10 mM ammonium acetate, pH 8.1, covering the m/z range 1000 – 4500. Shown in B – E is the most intense charge state of monomeric RXR α ($[M+12H]^{12+}$) in the absence of ligand (B); or in the presence of 25 μ M LG268 (C); DHA (D); or 17 β -estradiol (E). The +178 Da modification (His₆-tag gluconoylation) is indicated by a filled circle. The spectra were recorded with a sample flow rate of 5 - 10 μ L/min, a cone-voltage of 50 V and a collision voltage of 4.2 V, with a source block temperature 80 - 120 $^{\circ}$ C.

Figure 3.

Agonist binding to RXR α is competitive. ES mass spectra showing the 12+ monomer charge state for RXR α in the absence of ligand (A); in the presence of 10 μ M DHA (B); and in the presence of 10 μ M DHA and LG268 (C). The receptor concentration was 10 μ M. The +178 Da modification (His₆-tag gluconoylation) is indicated by a filled circle. The sample flow-rate was 5 μ L/min, and data was acquired at a cone voltage of 50 V, a collision voltage of 4.2 V with a source block temperature of 80 $^{\circ}$ C.

Figure 4.

Bile acid and LG268 binding to RXR α is non-competitive. ES mass spectra showing the RXR α monomer 12+ charge state (receptor concentration 10 μ M) in the absence of ligand (A); in the presence of 10 μ M LG268 (B); or in the presence of 10 μ M LG268 and 30 μ M TCA (C and D). The +178 Da modification (His₆-tag gluconoylation) is indicated by a filled circle and bound bile acid molecules by a filled diamond. Sample flow rate was 5 μ L/min, in (A), (B) and (C) the cone-voltage was 50 V, while in (D) it was 195 V. In all spectra the collision voltage was 4.2 V with a source block temperature of 80 $^{\circ}$ C.

Figure 5.

Bile acid and fatty acid binding to RXR α is non-competitive. Spectra of the RXR α monomer 12+ peak in the absence of ligand (A); in the presence of 10 μ M DHA (B); and in the presence of 10 μ M DHA and 10 μ M TCA (C and D). The sample flow rate was 5 μ L/min, in (A), (B) and (C) the cone-voltage was 50 V, while in (D) it was 195 V. In all spectra the collision voltage was 4.2 V with a source block temperature of 80 $^{\circ}$ C.

Figure 6.

Analysis of a RXR α with a mixture of DHA and TCA. ES mass spectra of a 10 μM RXR α solution, 10 mM ammonium acetate, pH 8.1, in the presence of an equimolar mixture of DHA and TCA (25 μM / compound) using a cone-voltage of 50 V (A); and 195 V (B), and at a collision cell voltage of 4.2 V. The sample was cleaned-up by on-line dialysis⁵³ by pumping the sample solution (5 $\mu\text{L}/\text{min}$) through a dialysis fibre of molecular weight cut-off 5000, just prior to on-line ES analysis. The RXR - DHA complex is labelled with an arrow, RXR α - TCA complexes by a filled diamond, and the +178 Da modification with a filled circle. The multiple binding of TCA molecules to the protein becomes visible at the higher cone-voltage.

Figure 7.

Protein binding of DHA and bile acids under non-denaturing ES conditions. ES mass spectra of a 10 μ M solution of RXR α in 10 mM ammonium acetate, pH 8.1, in the absence of ligand (A); in the presence of 50 μ M DHA (B); TCA (C); CDCA (D); CA (E), and LCA (F). The cone-voltage was 50 V, and the collision cell voltage 4.2 V. The sample was cleaned-up by on-line dialysis by pumping the sample solution (5 μ L/min) through a dialysis fibre of molecular weight cut-off 5000, just prior to on-line ES analysis. The +178 Da modification is labelled with a filled circle, the RXR α - DHA complex by an arrow, and the RXR α - bile acid complexes with filled diamonds. Each panel shows the 13+ and 12+ charge states of monomeric RXR α LBD.

Figure 8.

Bile acid – protein complexes are stable under energetic ES conditions, (cone voltage 195 V and collision cell voltage 50 V). ES mass spectra of a 10 μ M solution of RXR α in 10 mM ammonium acetate, pH 8.1, in the presence of 50 μ M TCA (A); 50 μ M LCA (B); 50 μ M LCA-3-sulphate (C); and 50 μ M SDS (D). The sample was cleaned-up by on-line dialysis by pumping the sample solution (5 μ L/min) through a dialysis fibre of molecular weight cut-off 5000, just prior to on-line ES analysis. The +178 Da modification is labelled with a filled circle, and the RXR – bile acid or SDS complexes with a filled diamond.

Figure 9.

The high affinity agonist ligand LG268 stabilises homodimerisation of the RXR α LBD. Nano-ES mass spectra were recorded on protein samples desalted by gel filtration and diluted to $\sim 20 \mu\text{M}$ with 10 mM ammonium acetate, pH 8.0. Shown in (A) is the high m/z range spectrum (2000 – 6500) of $apo\text{-RXR}\alpha$. Shown in (B) is the effect of the addition of two-fold excess LG268 (40 μM final concentration) to protein. Panel (C) shows the effect of adding TCA to 40 μM . Monomer (M) and dimer (D) charge states are indicated. Shown in (D-G) is the homodimer $16+$ peak. The spectrum in (D) was recorded in the absence of ligand, while those in (E and F) were recorded in the presence of 40 μM LG268. The mass shift upon LG268 addition, corresponding to the mass of two molecules of LG268, is

indicated by the black bar. Shown in (G) is the result of the addition of TCA to 40 μM . The spectra shown in (A-E and G) were recorded at a cone voltage of 20 V and that in (F) was at a cone voltage of 80V.

Table 1Relative molecular mass (M_r) values for test compounds.

compound	relative molecular mass (M_r)
LG268	363.50
DHA	328.49
17- β -estradiol (E_2)	272.39
TCA	515.71
CA	408.57
CDCA	392.57
LCA	376.57
LCA-3-sulphate	456.64
SDS	266.16