Ligand-binding regulation of LXR/RXR and LXR/PPAR heterodimerizations: SPR technology-based kinetic analysis correlated with molecular dynamics simulation

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Abstract

Liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR) are two members of nuclear receptors involved in the nutrient metabolisms of dietary fatty acid and cholesterol. They are found to be of cross-talk function in that LXR regulates fatty acid synthesis and PPAR controls fatty acid degradation. LXRs (LXR α and LXR β) function by forming obligate heterodimers with the retinoid X receptor (RXR), and subsequently binding to specific DNA response elements within the regulatory regions of their target genes. In this work, the kinetic features concerning LXR/RXR and LXR/PPAR interactions have been fully investigated using surface plasmon resonance (SPR) technology. It is found that LXRs could bind to all the three PPAR subtypes, PPAR α , PPAR γ and PPAR δ with different binding affinities, and such receptor/receptor interactions could be regulated by ligand binding. Moreover, molecular dynamics (MD) simulations were performed on six typical complex models. The results revealed that ligands may increase the interaction energies between the receptor interfaces of the simulated receptor/receptor complexes. The MD results are in agreement with the SPR data. Further analyses on the MD results indicated that the ligand binding might increase the hydrogen bonds between the interfaces of the receptor/receptor complex.

Keywords: liver X receptor (LXR); peroxisome proliferator-activated receptor (PPAR); retinoid X receptor (RXR); surface plasmon resonance (SPR); kinetic analysis; molecular dynamics (MD) simulation

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LXRs (LXR α and LXR β) are nuclear receptors that regulate the metabolisms of several important lipids, including cholesterol and bile acids (Song et al. 1994; Willy et al. 1995). These receptors function by forming obligate het-

erodimers with the retinoid X receptor (RXR) and binding specific DNA sequences (response elements) within the promoters of the genes they regulate (Willy and Mangelsdorf 1997). It is already known that LXRs regulate the nutrient metabolism pathways through their interactions with specific, naturally occurring oxysterols, including 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S),25-epoxycholesterol (Lehmann et al. 1996; Peet et al. 1998). LXR α is expressed most highly in the liver and to a lesser extent in the kidney, small intestine, spleen, and adrenal gland (Willy et al. 1995). In contrast to the restricted expression pattern of LXR α , LXR β is ubiquitously ex-

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pressed (Kainu et al. 1996). PPAR belongs to another sort of nuclear receptor family, which plays an important role in the regulation of the storage and catabolism of dietary fats (Grossman and Lessem 1997; Rutolo et al. 1998; Willson et al. 2000). PPAR contains three subtypes: PPAR α , PPAR δ (also termed as PPAR β), and PPAR γ . PPAR α is a member of the steroid/thyroid hormone receptor and regulates the expression of a number of genes critical for lipid and lipoprotein metabolism. PPAR γ is a ligand-dependent transcription factor influencing the adipocyte differentiation and glucose homeostasis, while PPAR δ may work as a widespread regulator in fat burning and probably be used as a potential target in the treatment of obesity and related disorders (Uppenberg et al. 1998; Willson et al. 2000; Wang et al. 2003).

The involvements of PPARs in multiple and diverse cellular functions suggest these receptors may be integrated with other cellular signaling pathways apart from the well-characterized RXR (Retinoid X receptor) pathway (Miyata et al. 1996). RXR α and its cognate ligand 9cRA appear to have a critical role in hormonal signaling. RXR α can heterodimerize with many nuclear receptors including LXR and PPAR in facilitating the binding and transactivation of receptors with appropriate DNA response elements (Willson et al. 2000). Some reports have indicated that PPAR does not interact with the thyroid hormone receptor (TR), but with LXR (Juge-Aubry et al. 1995), even though the reason of such a binding selectivity is still unknown. In fact, much attention has recently been paid to PPAR/LXR interaction (Mangelsdorf et al. 1995; Ide et al. 2003; Yoshikawa et al. 2003). By gel shift and in vitro protein/protein binding assays, it has been discovered that the interactions between LXRs and PPAR α are involved in fatty acid degradation, which is a reverse of the fatty acid synthesis function of PPAR α (Mangelsdorf et al. 1995).

A nuclear receptor protein contains a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) (Zhou et al. 1998). In general, the LBD is about 200–300 amino acids in length and not conserved as well. There are at least four functions encoded in the LBD, viz. dimerization, ligand binding, binding to coactivators or corepressors, and transactivation (Bourguet et al. 1995; Renaud et al. 1995). The LBD has thus been the focus of intense study for the determination of ligand specificity (Wurtz et al. 1996). Among the 12 helices in the LBD of PPARs, the carboxyl helices are required for heterodimerization with RXR and the N-terminal helices are involved in forming ligand binding pockets (Juge-Aubry et al. 1995). Recently, by use of varied biophysical techniques such as fluorescent and circular dichroism spectral technology, the ligand binding features for some receptors have been fully characterized (Palmer and Wolf 1998; Margeat et al. 2003; Yu et al. 2004).

Since 1991, surface plasmon resonance (SPR) technology-based Biacore has been widely used to monitor the thermodynamics and kinetics for a wide variety of macromolecular interactions, including protein/protein, protein/DNA, and ligand/protein (DNA) interactions (Karlsson et al. 2000). SPR technology has been also successfully employed to study the interactions between ligands and nuclear receptors and the effects of ligand-binding on receptor dimerization (Cheskis et al. 1995; Myszkowski 1997; Robinson et al. 2000; Yu et al. 2004).

In the following, we report the in vitro study of the kinetic binding characterizations of LXR-LBDs/RXR α -LBD and LXR-LBDs/PPAR-LBDs using SPR technology-based Biacore 3000. The experimental results are quite consistent with the molecular dynamics (MD) simulations on these protein/protein interactions, which are also reported here. The results demonstrate that the protein/protein interactions exist between LXRs and PPARs, and these interactions could be regulated by the bindings of the corresponding ligands.

Results

SPR assay

To obtain the kinetic and thermodynamic features for the receptor/receptor interactions, the association (k_a) and dissociation (k_d) rate constants and the dissociation equilibrium constants (K_D s) for the bindings of LXR-LBDs to RXR α -LBD and PPAR-LBDs were determined by using the Biacore 3000 biosensor (Biacore AB). The binding responses in resonance units (RUs) were continuously recorded and presented graphically as a function of time. The heterogeneous ligand-parallel reaction (HLPR) fit model (Cheskis and Freedman 1996) was used to calculate the k_a , k_d , and K_D parameters. In this fit model, one analyte interacts with two independent binding sites, i.e., $A + B1 + B2 = AB1 + AB2$.

LXR-LBDs/RXR α -LBD interactions

In the investigation of LXR-LBDs/RXR α -LBD interactions, two experimental strategies have been applied. During the first determination, RXR α -LBD was immobilized on the sensor chip by the standard amine coupling protocol, and LXR-LBDs (LXR α -LBD and LXR β -LBD) were treated as analytes. A surface with approximately 800 RU of immobilized RXR α -LBD was obtained. Injections of *apo*- or ligand-bound LXR α (β)-LBD at concentrations from 1.28 nM to 4 μ M were run over the chip surface. As two typical examples, Figure 1A shows the sensorgrams of serial injections of *apo*-LXR α -LBD over the immobilized RXR α -LBD surface, and Figure 1B presents the sensorgrams of serial injections of LXR α -LBD preincubated with

Figure 1. Unliganded and liganded LXRα-LBD/RXRα-LBD interactions evaluated by Biacore 3000. Sensorgrams obtained from the injections of LXRα-LBD over the immobilized RXRα-LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μM (A); injections of LXRα-LBD incubated with 20 μM 22RHC over the immobilized RXRα-LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μM (B); injections of RXRα-LBD over the immobilized LXRα-LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μM (C); and injections of RXRα-LBD incubated with 20 μM 9cRA over the immobilized LXRα-LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μM (D).

22RHC over the immobilized RXRα-LBD surface. The sensorgrams were then processed for computing the related kinetic and thermodynamic parameters of protein/protein interactions by the HLPR fit model, and the results are listed in Table 1.

In general, the HLPR fit model may produce two sets of binding constants (Cheski and Freedman 1996). The *apo*-

LXRα-LBD binding to the immobilized RXRα-LBD produced association and dissociation rate constants by $k_{a1} = 57.7 \text{ M}^{-1}\text{sec}^{-1}$ and $k_{d1} = 1.67 \times 10^{-5} \text{ sec}^{-1}$, and $k_{a2} = 1.7 \times 10^3 \text{ M}^{-1}\text{sec}^{-1}$ and $k_{d2} = 1.76 \times 10^{-3} \text{ sec}^{-1}$; their corresponding equilibrium dissociation constants K_{D1} (k_{d1}/k_{a1}) and K_{D2} (k_{d2}/k_{a2}) could thus be calculated as 2.9×10^{-7} and $3.75 \times 10^{-7} \text{ M}$, respectively. For the binding

Table 1. Association (k_a) and dissociation (k_d) rate constants and apparent equilibrium dissociation (K_D') constants of unliganded and liganded LXR-LBDs/RXRα-LBD interactions with RXRα-LBD immobilized (A) and LXRs-LBD immobilized (B)

A

	Immobilized receptor RXRα		
Analyte	k_a (M ⁻¹ S ⁻¹)	k_d (S ⁻¹)	K_D' (M)
LXRα	57.70 ± 4.39	1.67 ± 0.13 × 10 ⁻⁵	2.90 ± 0.22 × 10 ⁻⁷
LXRα + 22RHC	1.85 ± 0.16 × 10 ⁴	4.03 ± 0.40 × 10 ⁻⁵	2.17 ± 0.22 × 10 ⁻⁹
LXRα + T0901317	1.70 ± 0.12 × 10 ⁴	3.69 ± 0.23 × 10 ⁻⁷	2.17 ± 0.13 × 10 ⁻¹¹
LXRβ	4.35 ± 0.24 × 10 ³	4.93 ± 0.34 × 10 ⁻⁴	1.14 ± 0.08 × 10 ⁻⁷
LXRβ + 22RHC	2.47 ± 0.15 × 10 ⁵	5.74 ± 0.52 × 10 ⁻⁴	2.32 ± 0.21 × 10 ⁻⁹
LXRβ + T0901317	2.47 ± 0.13 × 10 ⁵	6.95 ± 0.59 × 10 ⁻⁴	2.82 ± 0.24 × 10 ⁻⁹

B

	Immobilized receptor					
	LXRα			LXRβ		
Analyte	k_a (M ⁻¹ S ⁻¹)	k_d (S ⁻¹)	K_D' (M)	k_a (M ⁻¹ S ⁻¹)	k_d (S ⁻¹)	K_D' (M)
RXRα	63.90 ± 3.90	3.94 ± 0.27 × 10 ⁻⁵	6.17 ± 0.42 × 10 ⁻⁷	64.50 ± 5.71	4.98 ± 0.30 × 10 ⁻⁵	7.75 ± 0.47 × 10 ⁻⁷
RXRα + 9cRA	7.18 ± 0.51 × 10 ³	3.78 ± 0.34 × 10 ⁻⁷	5.26 ± 0.47 × 10 ⁻¹¹	7.25 ± 0.58 × 10 ³	2.95 ± 0.24 × 10 ⁻⁶	4.07 ± 0.33 × 10 ⁻¹⁰

of 22RHC-bound LXR α -LBD to RXR α -LBD, $k_{a1} = 2.19 \times 10^3 \text{ M}^{-1}\text{sec}^{-1}$, $k_{d1} = 1.43 \times 10^{-3} \text{ sec}^{-1}$, $k_{a2} = 1.85 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$, and $k_{d2} = 4.03 \times 10^{-5} \text{ sec}^{-1}$; the corresponding K_{D1} and K_{D2} are 6.54×10^{-7} and $2.17 \times 10^{-9} \text{ M}$, respectively. Usually, the binding parameter corresponding to the smaller K_D value representing the majority of the binding is considered as the apparent equilibrium dissociation constant (denoted by K_D' herein after) (Cheskis and Freedman 1996). The kinetic data and K_D' values of other *apo*- and ligand-bound LXR-LBDs/RXR α -LBD (immobilized) interaction are listed in Table 1A.

To determine the effect of ligand 9cRA binding to RXR α -LBD on the interactions between RXR α -LBD and LXR-LBDs, LXR-LBDs were immobilized on the sensor chips with resonance unit around 800 RU by the standard amine coupling method mentioned above, and RXR α -LBD was regarded as analyte. *apo*- or 9cRA-Bound RXR α -LBD was injected over the immobilized LXR-LBD surface at different concentrations from 1.28 nM to 4 μM . Figure 1C shows the sensorgrams of serial injections of RXR α -LBD to the immobilized LXR α -LBD surface, and Figure 1D presents the sensorgrams of serial injections of RXR α -LBD preincubated with 9cRA to the immobilized LXR α -LBD surface. The kinetic and thermodynamic parameters were also obtained by fitting the sensorgrams with the HLPF fit model, and the binding parameters of *apo*- and 9cRA-bound RXR α -LBD to the immobilized LXR β -LBD were obtained. The determined data are listed in Table 1B.

SPR results indicate that both the LXR α -LBD and LXR β -LBD have high binding affinities with RXR-LBD by K_D' values around 0.1 μM (Table 1). Ligands of both LXR and RXR are able to increase the binding affinities of LXR-LBDs to RXR α -LBD by two to four orders of magnitude. However, it is noticed that different ligands exert different promotion activity against one given protein/protein interaction. The binding of T0901317 increases the LXR α -LBD/RXR α -LBD interaction by four orders of magnitude, while the binding of 22RHC only enhances this interaction by two orders of magnitude (Table 1A). Both T0901317 and 22RHC promote the LXR β -LBD/RXR α -LBD interaction by two orders of magnitude (Table 1A). Moreover, the kinetic data indicate that ligands of either LXR or RXR largely increase the association rate constant (k_a) and decrease the dissociation rate constant (k_d) of the LXR-LBDs/RXR α -LBD binding processes, thereby increasing the binding affinities between the receptors (Table 1).

LXR-LBDs/PPAR-LBDs interactions

Similar to the above assays, the effects of LXR ligand-binding to the LXR-LBDs/PPAR-LBDs interactions could

be analyzed by immobilizing PPAR-LBDs (PPAR α -LBD, PPAR β -LBD, and PPAR δ -LBD) on the sensor chips with *apo*- and ligand-bound LXR-LBDs as analytes. By the standard amine coupling protocol, surfaces with ~1000 RU of immobilized PPAR-LBDs were prepared. The *apo*- or ligand-bound LXR-LBDs at concentrations ranging from 1.28 nM to 4 μM were run over the chip surface. As two representative examples, Figure 2A shows the sensorgrams of serial injections of *apo*-LXR α -LBD over the immobilized PPAR α -LBD surface, and Figure 2B displays the sensorgrams of serial injections of LXR α -LBD preincubated with the LXR ligand, 22RHC, over the immobilized PPAR α -LBD surface. The sensorgrams were fitted by the HLPF fit model, and the obtained kinetic and thermodynamic parameters are listed in Table 2.

SPR determinations indicate that LXR-LBDs may tightly bind to PPAR-LBDs, the K_D' values range from ~1–0.01 μM . Similarly, these receptor/receptor interactions may also be regulated by the ligands: (1) The binding of the LXR natural ligand, 22RHC, promotes the LXR α -LBD/PPAR α -LBD and LXR α -LBD/PPAR γ -LBD interactions by three orders of magnitude and the LXR α -LBD/PPAR δ -LBD interaction by one order of magnitude; (2) the binding of the synthetic ligand, T0901317, exhibits little effect on the LXR α -LBD/PPAR-LBDs interactions; (3) the binding of 22RHC to LXR β -LBD may promote the LXR β -LBD/PPAR α -LBD interaction by one order of magnitude, but

Figure 2. Interaction of *apo*- or ligand-bound LXR α -LBD with immobilized PPAR α -LBD evaluated by Biacore 3000. Sensorgrams obtained from the injections of LXR α -LBD over the PPAR α -LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μM (A); injections of LXR α -LBD incubated with 20 μM 22RHC over the PPAR α -LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μM (B).

Analyte	Immobilized receptor				K_D' (M)	K_D' (M)	PPAR δ	
	PPAR α		PPAR γ				k_d (S $^{-1}$)	K_D' (M)
	k_a (M $^{-1}$ S $^{-1}$)	k_d (S $^{-1}$)	K_D' (M)	k_a (M $^{-1}$ S $^{-1}$)	k_d (S $^{-1}$)	K_D' (M)	K_D' (M)	
LXR α	$6.28 \pm 0.52 \times 10^4$	$1.84 \pm 0.24 \times 10^{-3}$	$2.92 \pm 0.038 \times 10^{-8}$	$1.45 \pm 0.14 \times 10^4$	$2.53 \pm 0.32 \times 10^{-4}$	$1.74 \pm 0.22 \times 10^{-8}$	$9.55 \pm 1.29 \times 10^{-5}$	
LXR α + 22RHC	$3.56 \pm 0.65 \times 10^4$	$9.44 \pm 1.24 \times 10^{-7}$	$2.65 \pm 0.35 \times 10^{-11}$	$2.1 \pm 0.18 \times 10^4$	$2.93 \pm 0.21 \times 10^{-7}$	$1.39 \pm 0.10 \times 10^{-11}$	$4.80 \pm 0.42 \times 10^{-4}$	
LXR α + T0901317	$3.33 \pm 0.43 \times 10^4$	$3.42 \pm 0.21 \times 10^{-4}$	$1.03 \pm 0.063 \times 10^{-8}$	$2.04 \pm 0.25 \times 10^4$	$6.7 \pm 0.52 \times 10^{-5}$	$3.28 \pm 0.21 \times 10^{-9}$	$3.76 \pm 0.29 \times 10^{-4}$	
LXR β	187.00 ± 27.00	$6.09 \pm 0.17 \times 10^{-5}$	$3.26 \pm 0.091 \times 10^{-7}$	66.80 ± 6.45	$8.28 \pm 0.67 \times 10^{-5}$	$1.24 \pm 0.10 \times 10^{-6}$	$1.28 \pm 0.12 \times 10^{-4}$	
LXR β + 22RHC	67.90 ± 4.30	$1.94 \pm 0.15 \times 10^{-6}$	$2.86 \pm 0.22 \times 10^{-8}$	67.30 ± 3.25	$2.76 \pm 0.14 \times 10^{-4}$	$4.10 \pm 0.21 \times 10^{-6}$	$2.58 \pm 0.27 \times 10^{-4}$	
LXR β + T0901317	96.60 ± 8.70	$5.04 \pm 0.62 \times 10^{-7}$	$5.21 \pm 0.64 \times 10^{-9}$	202.00 ± 18.00	$2.47 \pm 0.23 \times 10^{-4}$	$1.22 \pm 0.11 \times 10^{-6}$	$7.09 \pm 0.69 \times 10^{-5}$

Figure 3. Interaction of *apo*- or ligand-bound PPAR γ -LBD interaction with immobilized LXR α -LBD evaluated by Biacore 3000. Sensorgrams obtained from the injections of PPAR γ -LBD over the LXR α -LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μ M (A); injections of PPAR γ -LBD incubated with 20 μ M 15dPGJ2 over the LXR α -LBD surface at concentrations of 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μ M (B).

has little effect on the PPAR γ -LBD/PPAR δ -LBD interactions; (4) the binding of T0901317 to LXR β -LBD increases the LXR β -LBD/PPAR δ -LBD and LXR β -LBD/PPAR α -LBD interactions by one and two orders of magnitude, respectively, while it has little effect on the LXR β -LBD/PPAR γ -LBD interaction. In addition, the large increase of the LXR α -LBD/PPAR α (γ)-LBD interactions by 22RHC binding to LXR α -LBD may be mainly ascribed to the high decrease of the dissociation constants (Table 2).

To investigate the effect of the PPAR ligand binding on the PPAR-LBDs/LXR-LBDs interactions, LXR α (β)-LBD was immobilized on the sensor chip with the resonance unit $\sim$ 1000 RU, and PPAR α (γ / δ)-LBD was treated as analytes. As typical examples, the sensorgrams of the serial injections of the *apo*- PPAR γ -LBD over the immobilized LXR α -LBD surface are shown in Figure 3A, and the sensorgrams of the serial injections of PPAR γ -LBD preincubated with 15dPGJ2 over the immobilized LXR α -LBD surface are shown in Figure 3B. All the sensorgrams were also fitted by the HPLR fit model as described above. The obtained kinetic and thermodynamic parameters are listed in Table 3.

The data listed in Table 3 indicate that, compared with the interactions of *apo*- LXR-LBDs with the immobilized PPAR-LBDs, the binding affinities of PPAR-LBDs to the immobilized LXR-LBDs are found to decrease by about one order of magnitude. This may ascribe to the fact that the immobilization of LXR-LBDs to the sensor chip has some unfavorable effect on the formation of LXR-LBDs/PPAR-LBDs complexes (Cheski and Freedman 1996). As indicated above, ligand regulation also exists for these receptor/receptor interactions. For example, the two PPAR α ligands WY14643 and Bezafibrate promotes PPAR α -LBD/LXR α (β)-LBD interactions by about one order of magnitude; the binding of 15dPGJ2 to PPAR γ -LBD increases the PPAR γ -LBD/LXR α -LBD and PPAR γ -LBD/LXR β -LBD interactions by four and three orders of magnitude, respectively. It is interesting that the binding of Troglitazone to PPAR γ -LBD increases the PPAR γ -LBD/LXR α -LBD binding affinity by two orders of magnitude but decreases the PPAR γ -LBD/LXR β -LBD interaction by about one order of magnitude. However, the binding of GI262570 to PPAR γ -LBD does not affect the PPAR γ -LBD/LXR α (β)-LBD interaction, while the binding of Bezafibrate to PPAR δ -LBD may strengthen the PPAR δ -LBD/LXR α (β)-LBD interaction by one or two orders of magnitude.

Table 3. Association (k_a) and dissociation (k_d) rate and apparent equilibrium dissociation (K_D') constants of unliganded and liganded PPARs-LBD/LXRs-LBD (immobilized) interactions

Analyte	Immobilized receptor					
	LXR α			LXR β		
	k_a ($M^{-1}S^{-1}$)	k_d (S^{-1})	K_D' (M)	k_a ($M^{-1}S^{-1}$)	k_d (S^{-1})	K_D' (M)
PPAR α	69.50 $\pm$ 7.40	1.92 $\pm$ 0.12 $\times 10^{-5}$	2.77 $\pm$ 0.17 $\times 10^{-7}$	103.00 $\pm$ 7.30	1.97 $\pm$ 0.45 $\times 10^{-5}$	1.92 $\pm$ 0.44 $\times 10^{-7}$
PPAR α + WY14643	1.18 $\pm$ 0.27 $\times 10^4$	1.97 $\pm$ 0.21 $\times 10^{-4}$	1.66 $\pm$ 0.18 $\times 10^{-8}$	9.88 $\pm$ 1.34 $\times 10^4$	1.68 $\pm$ 0.23 $\times 10^{-3}$	1.70 $\pm$ 0.23 $\times 10^{-8}$
PPAR α + Bezafibrate	37.40 $\pm$ 5.76	3.15 $\pm$ 0.35 $\times 10^{-6}$	8.40 $\pm$ 0.94 $\times 10^{-8}$	1.09 $\pm$ 0.97 $\times 10^5$	2.97 $\pm$ 0.28 $\times 10^{-3}$	2.72 $\pm$ 0.26 $\times 10^{-8}$
PPAR γ	51.10 $\pm$ 3.27	4.68 $\pm$ 0.47 $\times 10^{-5}$	9.17 $\pm$ 0.92 $\times 10^{-7}$	57.50 $\pm$ 5.85	1.40 $\pm$ 0.13 $\times 10^{-5}$	2.43 $\pm$ 0.23 $\times 10^{-7}$
PPAR γ + 15dPGJ2	2.15 $\pm$ 0.19 $\times 10^4$	6.43 $\pm$ 0.78 $\times 10^{-7}$	2.99 $\pm$ 0.36 $\times 10^{-11}$	2.45 $\pm$ 1.86 $\times 10^4$	1.18 $\pm$ 0.17 $\times 10^{-5}$	4.81 $\pm$ 0.69 $\times 10^{-10}$
PPAR γ + Troglitazone	4.64 $\pm$ 0.38 $\times 10^3$	1.31 $\pm$ 0.12 $\times 10^{-5}$	2.82 $\pm$ 0.26 $\times 10^{-9}$	88.10 $\pm$ 9.65	4.96 $\pm$ 0.45 $\times 10^{-4}$	5.62 $\pm$ 0.51 $\times 10^{-6}$
PPAR γ + GI262570	40.90 $\pm$ 5.89	2.05 $\pm$ 0.25 $\times 10^{-5}$	5.03 $\pm$ 0.61 $\times 10^{-7}$	43.40 $\pm$ 3.42	2.86 $\pm$ 0.35 $\times 10^{-5}$	6.58 $\pm$ 0.81 $\times 10^{-7}$
PPAR δ	2.06 $\pm$ 0.45 $\times 10^3$	2.98 $\pm$ 0.36 $\times 10^{-3}$	1.45 $\pm$ 0.17 $\times 10^{-6}$	1.57 $\pm$ 0.14 $\times 10^3$	6.01 $\pm$ 0.54 $\times 10^{-4}$	3.83 $\pm$ 0.34 $\times 10^{-7}$
PPAR δ + Bezafibrate	1.36 $\pm$ 0.085 $\times 10^3$	1.33 $\pm$ 0.13 $\times 10^{-4}$	9.80 $\pm$ 0.96 $\times 10^{-8}$	569.00 $\pm$ 34.00	3.46 $\pm$ 0.31 $\times 10^{-5}$	6.10 $\pm$ 0.54 $\times 10^{-8}$

Figure 4. Profiles for the receptor/receptor interactions of the six typical complexes derived from the MD simulations. (A) Unliganded and liganded RXR α -LBD/LXR α -LBD interactions. (B) Unliganded and liganded PPAR γ -LBD/LXR α -LBD interactions.

Molecular dynamics simulations for the receptor/receptor interactions

To gain insights into the detailed influence of the ligands on the receptor/receptor interactions at the molecular and atomic levels, 3-nsec molecular dynamics (MD) simulations were performed on six typical receptor/receptor binary complexes and ligand-receptor/receptor (or receptor/receptor-ligand) ternary complexes, viz. *apo*-RXR α -LBD/*apo*-LXR α -LBD, *apo*-RXR α -LBD/LXR α -LBD-22RHC, RXR α -LBD-9cRA/*apo*-LXR α -LBD, *apo*-PPAR γ -LBD/*apo*-LXR α -LBD, *apo*-PPAR γ -LBD/LXR α -LBD-22RHC, and PPAR γ -LBD-15dPGJ2/*apo*-LXR α -LBD.

The energetic properties of the systems with respect to time give an indication of the overall stability of the MD trajectory. The profiles of total energies of the above six models versus simulation time are shown in Figure S1 in

the Supplemental Material, which indicate that all the systems are stable during the simulations. The profiles of the receptor/receptor interaction energies of the complex systems versus simulation time are shown in Figure 4. For each complex, the receptor/receptor interaction energy fluctuates dramatically along the MD trajectory. Along the interaction energy profile of a complex, we can address the lowest minimum point that corresponds to the configuration with the maximum interactions. Such lowest minimum points of *apo*-RXR α -LBD/*apo*-LXR α -LBD, *apo*-RXR α -LBD/LXR α -LBD-22RHC, RXR α -LBD-9cRA/*apo*-LXR α -LBD, *apo*-PPAR γ -LBD/*apo*-LXR α -LBD, *apo*-PPAR γ -LBD/LXR α -LBD-22RHC, and PPAR γ -LBD-15dPGJ2/*apo*-LXR α -LBD are, respectively, located at around 1080, 2710, 1970, 1380, 1110, and 740 psec in their own interaction energy profiles (Fig. 4). The interaction energies corresponding to the lowest minimum points are listed in Table 4, which indicate that these ligands (22RHC, 9cRA, and 15dPGJ2) may increase the receptor/receptor interaction strengths. This is in good agreement with the SPR determinations (Tables 2, 3). To address the reason of the ligand binding-caused increase of the receptor/receptor interactions, we monitored the hydrogen bond number and hydrophobic interaction pairs on the snapshots corresponding to the lowest interaction energies using the Ligplot program (Wallace et al. 1995). The results are listed in Table 4 and in Figure S2 in the Supplemental Material, which suggest that the ligand binding only slightly affects the hydrophobic interaction, but dramatically enhances the hydrogen-bond interaction.

Discussion

RXR is an obligate heterodimeric partner of LXRs, and it has been reported that the LXR/RXR complex is a permissive heterodimer that can be activated by the binding of RXR ligand 9cRA (Gampe et al. 2000), and the binding of the synthetic LXR ligand T0901317 and natural ligand 22RHC to LXR is able to reinforce the LXR/RXR heterodimerization at the cellular level (Bethany et al. 1999), even though no more detailed quantitative data have been obtained. Other facts regarding the activation of LXR α /RXR α interaction by LXR and RXR ligands were proved by using the GST-pulldown assay or other protein/protein interaction detecting methods (Song et al. 1995; Lehmann et al. 1996). In the present work, we have comprehensively investigated the interactions of LXRs with RXR α and the effects of the ligand binding on the receptor/receptor interactions by using the SPR technique and MD simulation. Our SPR and MD indicate that both LXR and RXR ligands can greatly regulate the LXR-LBDs/RXR α -LBD interactions. From the determined binding data, we can tentatively propose that LXR-LBD and RXR α -LBD may form a complex, and the liganded receptors may form a tighter one.

Table 4. Interaction energies, hydrogen bond number, and hydrophobic interaction pairs for the receptor/receptor interaction derived from the molecular dynamics simulations

Plot ^a	Complex	Interaction energy (kJ/mol)	Hydrogen bond number	Hydrophobic interaction pairs
A	apo-RXR α -LBD/apo-LXR α -LBD	-1935.63	3	28
B	apo-RXR α -LBD/LXR α -LBD-22RHC	-2310.15	10	29
C	RXR α -LBD-9cRA/apo-LXR α -LBD	-2130.29	6	23
D	apo-PPAR γ -LBD/apo-LXR α -LBD	-1954.81	6	26
E	apo-PPAR γ -LBD/LXR α -LBD-22RHC	-2037.07	14	26
F	PPAR γ -LBD-15dPGJ2/apo-LXR α -LBD	-2008.41	11	22

^a The Ligplot pictures for the interactions are shown in Figure S2 in the Supplemental Material. A–F correspond to Figures S2A–F.

The above conclusions may also be true for the interactions between LXRs and PPARs. Since it has been known that LXRs/PPARs complexes have no specific DNA binding elements, they cannot directly modulate the transcription activity of the related genes (Miyata et al. 1996). Protein/protein interaction with ligand modulation may therefore be pivotal for their role in vivo. The SPR determination indicates that Bezafibrate can enhance both the PPAR α -LBD/LXR α -LBD and PPAR δ -LBD/LXR α -LBD interactions. This is in agreement with the fact that Bezafibrate is a dual agonist of PPAR α and PPAR δ (Willson et al. 2000). It is reported that 15dPGJ2 can strengthen the association of PPAR γ with its coactivator, but Troglitazone cannot, indicating that the mode of coactivator interacting with PPAR γ is ligand type-specific (Kodera et al. 2000). We found that 15dPGJ2 can enhance the PPAR γ /LXR α (β) interactions; Troglitazone reinforces the PPAR γ /LXR α heterodimerization, but slightly inhibits the formation of the PPAR γ /LXR β complex (Table 3). Surprisingly, GI262570, the PPAR γ agonist with high binding affinity, does not affect on the PPAR γ /LXR α and PPAR γ /LXR β interactions (Table 3). These data demonstrate that the ligand regulated binding mode of LXRs to PPAR γ is also ligand type-specific.

In addition, the MD simulations on six typical complex models indicate that ligand binding to the receptors does not affect the hydrophobic interactions but increases the number of hydrogen bonds (Table 4; Fig. S2 [Supplemental Material]), which may be the major reason that the ligand-binding increases most of the receptor/receptor interactions in this study (Tables 2, 3).

Materials and methods

Chemicals and enzymes

The restriction and modifying enzymes were purchased from TaKaRa. The bacterial strains BL21pLysS and DH5 α , as well as the vector pET15b, were from Qiagen. The chelating affinity column and lower molecular weight (LMW) marker were purchased from Amersham Pharmacia Biotech, Inc. Isopropyl β -D-thio-

lactoside (IPTG) was purchased from Promega. All other chemicals were from Sigma in their analytical grade. The ligands of LXR α and LXR β , T0901317 (*N*-methyl-*N*-[3-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl)-phenyl]-benzenesulfonamide) and 22RHC (22(R)-hydroxycholesterol), as well as the PPARs ligands WY14643, 15dPGJ2 (15-deoxy- $\Delta_{12,14}$ -prostaglandin J₂), Troglitazone, GI262570, and Bezafibrate were from CAYMAN Pharmaceutical Co. Ltd.

Plasmids

All the recombinant DNA manipulation methods were performed according to the standard method (Maniatis 1989). The plasmids of pCMX-hLXR α and pCMX-hLXR β were kindly provided by Dr. Mangelsdorf (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center), pAdTrack-PPAR δ by Dr. Vogelstein (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center), pET15b-hPPAR γ -LBD (aa 204–477) by Dr. Uppenberg (Department of Structural Chemistry, Pharmacia and Upjohn, Sweden), pSG-hPPAR α by Dr. Lu (Shenzhen Chipscreen Biosciences Ltd.), and pGEX-KG-RXR α by Dr. Christopher (University of California, San Diego). The cDNAs of LXR α -LBD (aa 200–447), LXR β -LBD (aa 213–461), PPAR α -LBD (aa 199–468) and PPAR δ -LBD (aa 287–477) were amplified by PCR, and subcloned into vector pET15b followed by sequencing.

Protein expression and purification

LXR α (β)-LBD protein was expressed and purified according to the method in the literature (Causevic et al. 1999) with some modifications. *Escherichia coli* BL21pLysS cells transformed with plasmids of pET15b-LXR α (β)-LBD were grown in 100 mL of LB medium containing 100 mg/L of ampicillin at 37°C overnight, and then incubated into 1 L of LB supplemented with ampicillin. The expression of LXR α (β)-LBD was induced by the addition of 0.5 mM of isopropyl β -D-thiogalactoside (IPTG). After induction for 5 h at 25°C, the cells were harvested by centrifugation at 4000g, 4°C for 30 min. The pellet was washed, frozen, and then disrupted by sonication against Buffer A (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole [pH 7.5]). The lysed cells were centrifuged at 14,000g at 4°C for 30 min. The supernatant was kept and the pellet discarded. One milliliter (1 mL) of HiTrap Ni²⁺ chelating column was equilibrated with 10 mL of sterile deionized water, 50 mM NiSO₄, and finally 10 mL of Buffer A. The supernatant was passed over the column at a flow rate of 5 mL/min, followed by washing it with 20 mL of Buffer A and 20 mL of Buffer B (60 mM imidazole in Buffer A), respectively. The protein of interest was thus eluted with 10 mL of Buffer C (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole [pH 7.5]).

The expression and purification of the PPAR-LBDs (PPAR α -LBD, PPAR γ -LBD, and PPAR δ -LBD) and RXR α -LBD proteins were carried out using similar procedures to those of LXR α (β)-LBD proteins. The protein concentration was measured by use of the standard Bradford method.

Surface plasmon resonance (SPR) determinations

The binding affinities of LXR-LBDs with PPAR-LBDs and RXR α -LBD were evaluated by using a Biacore 3000 instrument (Biacore AB). Immobilization of the corresponding proteins to the hydrophilic carboxymethylated dextran matrix of the sensor chip CM5 (Biacore) was performed by the standard primary amine coupling reaction according to the supplied manual by Biacore Co. The proteins to be covalently bound to the matrix were diluted in 10 mM sodium acetate buffer (pH 5.0) to a final concentration of 0.30 mg/mL. Equilibration of the baseline was completed by a continuous flow of HBS running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% [v/v] surfactant P20 [pH 7.4]) through the chip for 1–2 h. Samples were automatically injected into the flow cells with concentration increased gradually. All the Biacore data were collected at 25°C with HBS as a running buffer at a constant flow of 20 μ L/min. The sensorgrams were processed using automatic correction for nonspecific bulk refractive index effects.

In general, the equilibrium dissociation constant (K_D) evaluating the protein/protein binding affinity could be determined by the 1:1 Langmuir binding fit model or the HLPR fitting analysis model (<http://www.biacore.com>). To assess the two models for analyzing the sensor data in this work, several parameters such as the standard error (SE) value, the residual plot and χ^2 were analyzed by the Biacore evaluation software package. The residual plot describes the differences between the measured and calculated data. The χ^2 value denotes the fitting degree between the estimative and the experimental curves. The smaller the χ^2 , the smaller their variation.

For both the 1:1 Langmuir binding fit model and HLPR fit model, the association rate constant (k_a) and dissociation rate constant (k_d) were fitted simultaneously by rate equation 1, and the equilibrium dissociation constant K_D was calculated according to equation 2; the difference between these two models exists in that for HLPR fit model it has two association and two dissociation reactions as shown in equation 3

$$\frac{dR}{dt} = k_a \times C \times (R_{\max} - R) - k_d \times R \quad (1)$$

where C is the concentration of the ligand, R represents the response unit (RU), for heterogeneous ligand parallel reaction fit model, R (observed) = $R_1 + R_2$.

$$K_D = k_d/k_a \quad (2)$$

$$A + B_1 + B_2 = AB_1 + AB_2 \quad (3)$$

The SPR data collection and process regarding the ligand binding to the receptors were also according to the same strategies as the above-mentioned. The ligands were preincubated with the proteins for 30 min before flowing over the sensor chip surfaces. When LXR α (β)-LBD protein was immobilized on the CM5 sensor chip (Biacore), the PPAR α (γ / δ)-LBD protein alone or incubated with the corresponding PPAR ligand was let to flow through the chip surface. When PPAR α (γ / δ)-LBD protein was immobilized on the CM5 sensor chip, the LXR α (β)-LBD protein alone or

incubated with the corresponding LXR ligand was let to flow through the chip surface.

The assay for the interactions between LXR α (β)-LBD and RXR α -LBD is similar to the above procedures.

Molecular dynamics simulations

The monomer structures of PPAR γ -LDB, RXR α -LDB, and LXR α -LDB were isolated from the crystal structures of PPAR γ -LDB/RXR α -LDB (PDB entry 1FM6) and RXR β -LDB/LXR α -LDB (PDB entry 1UHL) complexes. Missing residues and atoms in the crystal structures were repaired by using the Biopolymer module encoded in Sybyl 6.8 (Tripos Inc.). The models of RXR α -LDB/LXR α -LDB and PPAR γ -LDB/LXR α -LDB were constructed as follows: By replacing the coordinates of RXR β -LDB in the crystal structure of the RXR β -LDB/LXR α -LDB complex with those of the RXR α -LDB crystal structure, we obtained a 3D model of the RXR α -LDB/LXR α -LDB complex. Similarly, substituting the coordinates of RXR α -LDB in the crystal structure of the PPAR γ -LDB/RXR α -LDB complex with those of LXR α -LDB crystal structure resulted in a 3D model of PPAR γ -LDB/LXR α -LDB complex. To construct the 3D models of ligand/receptor/receptor complexes, 22RHC and 15dPGJ2 were separately docked into the binding pockets of LXR α -LDB and PPAR γ -LDB of the above receptor/receptor complexes using the FlexX program (Rarey et al. 1996). The X-ray crystal structure of the 9cRA/RXR α -LDB complex was directly used in constructing the 3D models of ligand/receptor/receptor complexes. Thus we obtained the 3D models of six complexes, viz. apo-RXR α -LDB/apo-LXR α -LDB, apo-RXR α -LDB/LXR α -LDB-22RHC, RXR α -LDB-9cRA/apo-LXR α -LDB, apo-PPAR γ -LDB/apo-LXR α -LDB, apo-PPAR γ -LDB/LXR α -LDB-22RHC, and PPAR γ -LDB-15dPGJ2/apo-LXR α -LDB, for further molecular dynamics (MD) simulations.

All MD simulations were performed using the parallelized MD program GROMACS 3.1.4 (Berendsen et al. 1995; Erik et al. 2001). Before MD simulations, explicit polar and aromatic hydrogen atoms were added for all models. The GROMOS87 force field was used for the proteins (van Gunsteren and Berendsen 1987). The geometries of the ligands (22RHC, 9cRA, and 15dPGJ2) were optimized at the B3LYP/6-31G** level and then their partial atomic charges were determined using the CHelpG algorithm encoded in the Gaussian 98W package. Topology file and other force field parameters except the charges of the ligands were generated using the PRODRG program (van Aalten et al. 1996; <http://davapc1.bioch.dundee.ac.uk/programs/prodrp>).

Each complex was solvated into a rectangular periodic box filled with SPC water molecules (Berendsen et al. 1981). The minimum distance between the protein atoms and the box walls was set to be >10 Å. Counterions were added to neutralize the net charges of the systems. Then each system was subjected to an energy minimization using the steepest descent method until energy convergence to 100 kJ/(mol·nm). Afterward, each system was equilibrated for 100 psec. Finally, a 3-nsec MD simulation was performed on each system. During the MD simulations, the Lincs and Settle methods (Miyamoto and Kollman 1992; Hess et al. 1997) were applied to constrain covalent bond lengths, allowing an integration step of 2 fsec. The Particle-mesh Ewald (PME) method was used to calculate the electrostatic interactions (Darden et al. 1993). The simulated systems were coupled into an external temperature bath at 300 K with a coupling constant of $\tau_T = 0.1$ psec and isotropic pressure coupling with time constant of $\tau_P = 1$ psec was applied to keep the pressure at 1.0 bar (Berendsen et al. 1984). The structures for analysis were saved every 500 steps (1 psec). MD simulations were run on a 128-CPU Silicon Graphics Ori-

gin3800 server. Analyses were performed using facilities within the GROMACS package and the Ligplot program (Wallace et al. 1995).

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