Regulation of thyroid hormone activation via the liver X-receptor/retinoid X-receptor pathway

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Abstract

Thyroid hormone receptor (TR) and liver X-receptor (LXR) are the master regulators of lipid metabolism. Remarkably, a mouse with a targeted deletion of both LXR α and LXR β is resistant to western diet-induced obesity, and exhibits ectopic liver expression of the thyroid hormone activating type 2 deiodinase (D2). We hypothesized that LXR/retinoid X-receptor (RXR) signaling inhibits hepatic D2 expression, and studied this using a luciferase reporter containing the human *DIO2* (*hDIO2*) promoter in HepG2 cells. Given that, in contrast to mammals, the chicken liver normally expresses D2, the chicken *DIO2* (*cDIO2*) promoter was also studied. 22(R)-OH-cholesterol negatively regulated *hDIO2* in a dose-dependent manner (100 μ M, approximately twofold), while it failed to affect the *cDIO2* promoter. Truncations in the *hDIO2* promoter identified the region –901 to –584 bp as critical for negative regulation. We also

investigated if 9-*cis* retinoic acid (9-*cis* RA), the ligand for the heterodimeric partner of TR and LXR, RXR, could regulate the *hDIO2* promoter. Notably, 9-*cis* RA repressed the *hDIO2* luciferase reporter (1 μ M, approximately fourfold) in a dose-dependent manner, while coexpression of an inactive mutant RXR abolished this effect. However, it is unlikely that RXR homodimers mediate the repression of *hDIO2* since mutagenesis of a DR-1 at –506 bp did not interfere with 9-*cis* RA-mediated repression. Our data indicate that *hDIO2* transcription is negatively regulated by both 22(R)-OH-cholesterol and 9-*cis* RA, which is consistent with LXR/RXR involvement. *In vivo*, the inhibition of D2-mediated tri-iodothyronine (T₃) production by cholesterol/9-*cis* RA could function as a feedback loop, given that T₃ decreases hepatic cholesterol levels.

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Introduction

Thyroid hormone receptor (TR) and liver X-receptors (LXRs) are the key regulators of lipid metabolism. Both these receptors prefer to bind to a direct repeat of the consensus DNA-binding site separated by a 4 bp spacing (DR-4), and thus can coordinately regulate gene expression through this motif (Wu & Koenig 2000, Kalaany & Mangelsdorf 2006). LXR can be activated by cholesterol, while tri-iodothyronine (T₃) binding to TR has profound effects on the expression profile of thyroid hormone-dependent genes. Both the receptors can heterodimerize with retinoid X-receptor (RXR), and thus their signaling is also regulated by 9-*cis* retinoic acid (9-*cis* RA; Glass 1994, Willy *et al.* 1995, Castillo *et al.* 2004).

The *DIO2* gene encodes type 2 deiodinase (D2), a tightly regulated oxidoreductase selenoenzyme that catalyzes thyroid hormone activation by converting thyroxine (T₄) to T₃, thus generating ligand for TR (Gereben *et al.* 2008). Remarkably, a mouse with a targeted disruption of LXR α and LXR β (double LXR knockout (KO) mice) is resistant to western diet-induced obesity, and exhibits ectopic liver expression of D2 accompanied by increased expression of T₃ target genes involved in fatty acid utilization (Kalaany *et al.* 2005). In this model, hepatic induction of D2 expression in LXR KO mice was dependent on dietary cholesterol, but not on SREBP-1c-mediated lipogenic pathways. Furthermore, it has been demonstrated that bile acids, the principal products of cholesterol catabolism, prevent obesity in wild-type mice, but not in D2 KO mice, by indirectly increasing D2

expression via the G-protein-coupled receptor TGR5 (*GPBAR1*; Watanabe *et al.* 2006).

The molecular links between cholesterol and D2-catalyzed thyroid hormone activation are presently unclear. We hypothesized that the LXR/RXR signaling pathways inhibit hepatic D2 expression in mammals. In order to better understand the regulation of fat metabolism, we investigated the effect of the LXR/RXR pathway on D2 expression by characterizing the response of human *DIO2* (*hDIO2*) promoter to both 22(R)-OH-cholesterol/LXR and 9-*cis* RA/RXR pathways in human hepatic HepG2 cells. Notably, while D2 is not normally expressed in the mammalian liver (Croteau *et al.* 1996), it is expressed in the chicken (Gereben *et al.* 1999). Given this contrast, we also cloned the chicken *DIO2* (*cDIO2*) promoter, and studied its responsiveness to the LXR/RXR signaling pathways. Our results indicate that the *hDIO2* promoter is potently down-regulated at the transcriptional level by both LXR and RXR ligands, while in contrast, the *cDIO2* promoter is unaffected by 22(R)-OH-cholesterol treatment.

Materials and Methods

Cell culture and transfection

HepG2 cells of human hepatoma origin were maintained under standard conditions in DMEM supplemented with 10% FBS. To remove lipids for experiments with 22(R)-OH-cholesterol (Sigma), serum was double stripped as described (Larsen *et al.* 1986). The concentration of 22(R)-OH-cholesterol and 9-*cis* RA (Sigma) used in the treatments is described in the legends, and treatments were performed for at least 20 h. The vehicles for 22(R)-OH-cholesterol and 9-*cis* RA were ethanol and DMSO respectively. Transient transfection was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

Constructs and mutagenesis

The human RXR α and LXR β expression vectors were described earlier (Seol *et al.* 1995). The human RXR α Δ 19C mutant lacks the last 19 amino acids (aa 444–462) and the AF-2 region, but retains heterodimerization (Zavacki *et al.* 1997). The 6.9 kb 5' flanking region (FR) *hDIO2* luciferase promoter constructs and their truncated forms were described previously (Zeold *et al.* 2006). PCR-based site-directed mutagenesis was used to mutate the DR-1 site in the *hDIO2* 5'FR. In short, overlapping PCR was used to generate the mutant cassette that was inserted between the PacI/NheI sites of the 6.9 kb 5' FR *hDIO2* luciferase constructs.

Cloning of the *cDIO2* promoter

The GenBank no. AF125575 chicken D2 RNA sequence (Gereben *et al.* 1999) was aligned to the chicken genomic sequence to locate the *DIO2* promoter in the chicken

genome with *DIO2* being identified on chromosome #5. A ~5.7 kb *cDIO2* 5' FR fragment was isolated using the Expand Long Template PCR System (Roche) with chicken genomic DNA as the template, and it was cloned into pGemT vector. A ~3.6 kb *cDIO2* 5'FR was amplified on this template using the same kit and the following oligonucleotides: sense, TGCACGTGGATAATCCATC-CAGGTACCACTCT; antisense, GTTTTAGCTTGC-TTCCTTGAAGCCTTTTATACATTC. The resulting fragment was cloned between the KpnI and Klenow blunted NheI sites of the pGL3-basic vector, and was confirmed by sequencing (Promega).

Promoter studies

Promoter studies using a luciferase reporter were performed as described previously (Fekete *et al.* 2004, Zeold *et al.* 2006). As internal controls, hGH and *Renilla* expression vectors (pRL-h β actin-213+932) were used (Gereben *et al.* 2001, Zeold *et al.* 2006, Doleschall *et al.* 2007). The CRE was induced with the coexpression of the α -catalytic subunit of protein kinase A (PKA; Maurer 1989) as described (Gereben *et al.* 2001). Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Where indicated, an empty CDM8 expression vector was used in the control transfections. The results are expressed as *Luciferase*/hGH or *Luciferase*/*Renilla* ratios.

Statistical analysis

Results are presented as means $\pm$ S.D. Analysis was statistically preformed using an unpaired *t*-test, or one-way ANOVA followed by Newman-Keuls *post hoc* testing when multiple comparisons were made. $P < 0.05$ was considered statistically significant.

Results

Treatment with 22(R)-OH-cholesterol and 9-*cis* RA negatively regulated the *hDIO2* promoter in HepG2 cells transfected with LXR β and RXR α in a dose-dependent manner with approximately twofold suppression of transcriptional activity at a dose of 100 and 1 μ M respectively (Fig. 1A). Treatment with 22(R)-OH-cholesterol alone in LXR β -transfected HepG2 cells caused a similar effect (Fig. 1B). In contrast, a 3 $\times$ DR-4 binding site containing luciferase promoter control construct was induced under similar conditions in the presence of only LXR β by approximately threefold, while coexpression of LXR β and RXR α induced the control by approximately fourfold (Fig. 1C). In the control experiments, we also tested the responsiveness of a pGL3-basic construct containing 880 bp of the mouse type 1 deiodinase (D1) promoter to 22(R)-OH-cholesterol and no change was observed, further

Figure 1 Cholesterol negatively regulates *hDIO2* in a dose-responsive manner. (A) HepG2 cells were transiently transfected with 6.9 kb 5'FR-*hDIO2*-Luc and 10 ng RXR α and treated with 1 μM 9-*cis* retinoic acid in the presence or absence of 10 ng LXR β . (B) HepG2 cells were transiently transfected with 6.9 kb 5'FR-*hDIO2*-Luc, 10 ng LXR β , and 10 ng PKA. (C) Induction of a 3 $\times$ DR-4-Luc-positive control construct in the presence of 22(R)-OH-cholesterol. Luc/hGH ratios are shown as arbitrary units (a.u.). Experiments were repeated at least three times. Mean $\pm$ s.d.; * $P < 0.001$, ** $P < 0.01$, and *** $P < 0.05$ versus vehicle by ANOVA followed by the Newman-Keuls *post hoc* test; # $P < 0.001$ versus vehicle by *t*-test.

confirming the specificity of this repression of the *hDIO2* promoter (data not shown).

Based on the markedly different hepatic expression of the human and chicken D2, we also cloned the *cDIO2* promoter and tested its response to 22(R)-OH-cholesterol in the presence of LXR β . The sequence of the cloned *cDIO2* 5'FR fragment has a low homology to *hDIO2*, and only the proximal ~ 160 bp can be aligned. However, this portion has both a putative CRE and a TATA box in the same position as the human promoter (Fig. 2A). Strikingly, coexpression of the *cDIO2* promoter with PKA resulted in ~ 10 -fold increase in promoter activity, but 22(R)-OH-cholesterol treatment in the presence of LXR β did not suppress the promoter either with or without PKA coexpression (Fig. 2B).

A series of truncations of the *hDIO2* promoter were used to map the region necessary for negative regulation by LXR β and cholesterol (Fig. 3). Deletion of the region at -117 bp to -3.9 kb resulted in a sharp decrease in the overall promoter activity and a loss of 22(R)-OH-cholesterol responsiveness, despite the presence of the putative DR-4 at -4616 bp. A combined deletion of the regions between -6.9 to -2.1 kb and -912 to -117 bp also resulted in a similar effect. The activity of a construct containing -901 bp of the *DIO2* 5' FR was decreased by 22(R)-OH-cholesterol treatment, while a fragment containing -584 bp was unresponsive, indicating that the region between -584 and -901 bp plays an important role in the negative response to cholesterol.

We also studied whether 9-*cis* RA, the other ligand of LXR/RXR heterodimer, could also negatively regulate the *hDIO2* promoter in a dose-dependent manner. One-micromolar 9-*cis* RA caused an approximately fourfold suppression of *hDIO2* transcription both in the absence and in the presence of PKA-mediated CRE stimulation (Fig. 4A and B). However, coexpression of an inactive RXR α mutant completely abolished the suppressive effect of 9-*cis* RA on the *hDIO2* promoter (Fig. 4C). In the control experiments,

we also tested the response of the 880 bp mouse D1 promoter pGL3-basic construct to 9-*cis* RA, and no change was observed (data not shown).

Site-directed mutagenesis of a putative DR-1 site at -506 bp in the 6.9 kb *hDIO2* promoter construct did not interfere with the 9-*cis* RA-mediated suppression of *hDIO2*, suggesting that binding of a RXR homodimer at this site is not involved in this process (Fig. 5); however, we cannot rule out that signaling of RXR through another heterodimer complex is not involved. Deletion constructs containing -2.1 kb, -901 bp, and -584 bp of the *hDIO2* promoter remained responsive to 9-*cis* RA-mediated suppression, although only the higher dose was effective. In addition, while the proximal -117 bp of the *hDIO2* promoter had a low basal activity, this was suppressed by 9-*cis* RA treatment. Interestingly, 9-*cis* RA could also suppress the ~ 3.5 kb *cDIO2* promoter, although basal activity of this reporter construct was very low.

Discussion

The LXR/FXR/TGR5 system plays a key role in both the maintenance of cholesterol and lipid homeostasis (Houten *et al.* 2006, Kalaany & Mangelsdorf 2006). LXR and FXR coordinately regulate cholesterol and bile acid metabolism, with LXR being activated by oxysterols, while FXR is activated by the clearance products of cholesterol, bile acids. In addition, LXR also transcriptionally controls genes associated with hepatic lipogenesis including SREBP-1c, a key regulator of fatty acid and triglyceride synthesis. On the other hand, bile acids, acting through both FXR and TGR5, decrease triglyceride levels through a variety of mechanisms including the repression of SREBP-1c expression and modulation of fatty acid uptake and β -oxidation (Houten *et al.* 2006, Kalaany & Mangelsdorf 2006).

Figure 2 Cholesterol does not affect the chicken *DIO2* promoter. (A) Sequence alignment of the proximal region of the *cDIO2* and *hDIO2* 5' flanking regions with the CRE and the TATA box is shown in bold. (B) HepG2 cells were transiently transfected with 3.6 kb 5'-*cDIO2*-Luc and 10 ng LXRβ. Ten nanograms of PKA were used to induce basal promoter activity as indicated, while PKA-uninduced cultures received CDM8. Luc/hGH ratios are shown as arbitrary units. Experiments were repeated three times, mean ± s.d.

Thyroid hormones also play a crucial role in the regulation of body composition and lipid homeostasis (Baxter & Webb 2009). Recently, it has emerged that a key point in thyroid hormone action is the local generation of thyroid hormone by T_4 to T_3 conversion by D2 (Gereben *et al.* 2008). Thus, it is not surprising that local T_3 generation by D2 can also be modulated by regulators of fat metabolism. Bile acids, via TGR5, have been shown to up-regulate D2 expression in brown adipose tissue, leading to resistance to diet-induced obesity (Watanabe *et al.* 2006). In addition, the double LXR KO mouse is resistant to western diet-induced obesity, and exhibits ectopic liver expression of D2 (Kalaany *et al.* 2005). Taken together as a whole, this suggests that bile acid-mediated pathways act to promote pathways associated with lipolysis, which would include the T_3 -generating D2. In contrast, sterols via LXR would promote lipogenesis and the down-regulation of factors such as D2.

To test this hypothesis, we investigated whether D2 was negatively regulated by the LXR/RXR pathway in HepG2 cells. The human LXRα and LXRβ share almost 80% amino acid identity in their DNA-binding domain and ligand-binding domain (Zhao & Dahlman-Wright 2010). In these studies, we used LXRβ, which is the more ubiquitously expressed LXR isoform (Annicotte *et al.* 2004).

When cells were cotransfected with LXRβ, treatment with 22(R)-OH-cholesterol negatively regulated the *hDIO2* promoter in HepG2 cells in a dose-dependent manner with a approximately twofold suppression of transcriptional activity at 100 μM (Fig. 1A and B). While sequence analysis identified a putative DR-4 at -4616 bp of *hDIO2* promoter that showed significant similarity to the LXR response element of ATP-binding cassette subfamily G member 1 (Varga & Su 2007), deletion of this element demonstrated that this DR-4 is not involved in the suppression of *hDIO2* (Fig. 3). Notably, it has been recently shown that LXR response elements could be more diverse than previously thought and beyond the classical DR-4, and that DR-1 and inverted repeat 1 motifs may also bind to LXR (Varga & Su 2007). Deletion mapping further localized this negative regulation by 22(R)-OH-cholesterol to a region between -901 and -584 bp of the *hDIO2* promoter, although no obvious LXR-consensus binding sequences could be identified in this region (Fig. 3). Although this region contains a functional binding site for the homeodomain-containing transcription factor TTF1/Nkx2.1 (-619 to -627; Gereben *et al.* 2001), it is presently not clear how Nkx2.1 could be involved in the demonstrated mechanism.

Figure 3 Mapping of cholesterol-mediated suppression of the *hDIO2* 5'FR. HepG2 cells were transiently transfected with 6.9 kb 5'FR-*hDIO2*-Luc and its truncated forms as indicated along with 10 ng LXR. Luc/hGH ratios are shown as arbitrary units. Experiments were repeated three times. Mean $\pm$ s.d.; * $P < 0.05$ versus vehicle by *t*-test. The DR-1 at -506 bp (AGGACC t AGGTCA; indicated as $\circ$ and DR-4 at -4616 bp (TGACTT tttt TGACCA; indicated as Δ) are putative, and were determined using NHR SCAN (Sandelin & Wasserman 2005) supplemented with manual analysis.

While numerous LXR target genes have been identified, our knowledge of LXR-suppressed genes is still rather limited (Baranowski 2008). It is known that LXR activation down-regulates hepatic enzymes essential for gluconeogenesis including phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase (Stulnig *et al.* 2002). In the murine intestine, LXR agonists decreased the expression of Niemann-Pick C1-like 1 protein, a critical player of intestinal cholesterol absorption (Duval *et al.* 2006). To our knowledge, the negative regulation of the *hDIO2* gene represents the first link between T_3 generation and LXR/RXR signaling.

In the liver, D1 is expressed abundantly, but due to its high K_m for T_4 , its contribution to circulating T_3 in euthyroid humans is considered to be minimal (Maia *et al.* 2005).

It has also been suggested that hepatic D1 might contribute to local T_3 generation in the liver (St Germain *et al.* 2009), and thus the local regulation of the *DIO1* gene via the LXR/RXR pathway could be important. However, we did not find any evidence in HepG2 cells that the *DIO1* gene could be regulated via this pathway.

While the *hDIO2* promoter was down-regulated by the LXR/22(R)-OH-cholesterol pathway (Fig. 1A and B), this suppression was not observed with the *cDIO2* promoter (Fig. 2B). This finding is in accordance with observations that D2 is not normally expressed in the mammalian liver (Croteau *et al.* 1996), while the chicken liver has D2 expression and activity (Gereben *et al.* 1999). Although the *cDIO2* 5' promoter fragment that was tested was shorter than the *hDIO2* promoter (3.7 vs 6.9 kb of 5' FR),

Figure 4 9-*cis* Retinoic acid negatively regulates *hDIO2* in a dose-responsive manner. HepG2 cells were transiently transfected with 6.9 kb 5'FR-*hDIO2*-Luc, empty CDM8 vector as a control, and (A) wild-type RXRα, (B) wild-type RXRα plus PKA, or (C) mutant RXRα plus PKA. Luc/hGH ratios are shown as arbitrary units. Experiments were repeated three times. Mean $\pm$ s.d.; * $P < 0.01$, ** $P < 0.001$ versus vehicle by ANOVA followed by the Newman-Keuls *post hoc* test.

Figure 5 Effect of 9-*cis* retinoic acid on the suppression of the *DIO2* 5'FR. HepG2 cells were transiently transfected with the 5'FR-h*DIO2*-Luc deletion constructs and *cdio2*-Luc along with 200 ng RXR α as indicated. A putative DR-1 (indicated as ○) was mutated for Att ACC TA tt TCA, while the putative DR-4 is shown as Δ. Luc/*Renilla* ratios are shown as arbitrary units. Experiments were repeated four times. Mean $\pm$ s.d.; * P <0.05 versus vehicle by ANOVA followed by the Newman-Keuls *post hoc* test; ** P <0.01 versus vehicle; and *** P <0.001 versus vehicle.

the 22(R)-OH-cholesterol-responsive region in the h*DIO2* promoter was mapped between -901 and -584 bp, which would also have been included in the 3.7 kb *cDIO2* reporter construct. In both chickens and humans, the liver is the major site of cholesterol synthesis (Hermier 1997), and thus further studies will be required to elucidate the potential physiological consequences of the differential sensitivity of the two *DIO2* promoters to 22(R)-OH-cholesterol-mediated repression between these species.

LXR/RXR forms a 'permissive heterodimer' that can be independently activated by ligands of either heterodimerization partner (Willy *et al.* 1995). We also demonstrated that the RXR/9-*cis* RA pathway strongly represses the h*DIO2* promoter in HepG2 cells (Fig. 4). This is in agreement with previous findings that the RXR-specific retinoid LG268 suppressed D2 expression in the mouse pituitary and the T α T1 cell line (Sharma *et al.* 2006). However, we were unable to clearly define the region responsible for the negative regulation of the h*DIO2* promoter using deletion mapping, since despite a drastic loss of basal activity, a construct containing -117 bp of the h*DIO2* promoter was still repressed by 9-*cis* RA treatment unlike the pGL3-basic backbone vector. Furthermore, mutagenesis of a putative DR-1-binding site at -506 site in h*DIO2* promoter did not interfere with 9-*cis* RA-mediated suppression, indicating that either this site is not functional, or RXR homodimers, which prefer the DR-1 motif, are not involved in the observed suppression (Fig. 5). Notably, while the *cDIO2* promoter could not be suppressed with 22(R)-OH-cholesterol,

its expression was decreased with 9-*cis* RA (Figs 3 and 5). Taken together, our data with regard to both deletion mapping of the 22(R)-OH-cholesterol and 9-*cis* RA and the responsiveness of the h*DIO2* and *cDIO2* promoters, and the fact that 22(R)-OH-cholesterol will only repress the h*DIO2* promoter but not the *cDIO2* promoter while both are negatively regulated by 9-*cis* RA, might suggest that these two modes of repression are independent of one another.

Both the mammalian (Gereben & Salvatore 2005) and the *cDIO2* 5'FRs are cAMP sensitive (Fig. 2). A cross-talk between the LXR- and cAMP-mediated gene expression has been suggested (Kalaany & Mangelsdorf 2006). However, we did not find any evidence that the effects of the LXR/RXR pathway on *DIO2* expression were modulated by PKA (Figs 1B, 2B and 4B).

LXR β expression is ubiquitous, leading to the conclusion that the suppression of D2-mediated T₃ generation via LXR/RXR might not be confined to the liver. Further studies are required to understand the potential importance of this pathway in different tissues. For example, brain should be considered as an important target for future studies of this pathway based on the fundamental importance played by D2 in the brain T₃ content (Gereben *et al.* 2008), and on the findings demonstrating cholesterol and RA synthesis in brain tissue (Wagner *et al.* 2002, Dietschy & Turley 2004). Furthermore, 22(R)-OH-cholesterol and 9-*cis* RA have also been shown to modulate specific LXR/RXR targets in the brain such as the ATP-binding cassette transporter A1 (Koldamova *et al.* 2003).

In conclusion, the hDIO2 promoter appears to be an LXR/RXR target gene, since it can be negatively regulated by either 22(R)-OH-cholesterol or 9-*cis* RA. Moreover, the repression of this promoter appeared to map to a region between -584 and -901 bp for 22(R)-OH-cholesterol and before -117 bp for 9-*cis* RA, although no classical binding sites with LXR/RXR heterodimers or RXR/RXR homodimers could be defined. Furthermore, these effects are independent of a known strong activator of DIO2 gene transcription, cAMP. Previous studies demonstrated that the LXR double KO mice on a western diet exhibit resistance to diet-induced obesity along with ectopic hepatic D2 expression; T₃ decreases hepatic cholesterol levels, and CYP7a1, the rate-limiting enzyme in hepatic bile acid synthesis, is positively regulated by both LXR and TR (Lehmann *et al.* 1997, Gullberg *et al.* 2000). Taking these findings together, it can be speculated that *in vivo* the cholesterol/9-*cis* RA-mediated inhibition of D2-catalyzed T₃ production could function as a negative feedback loop.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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