Communication

Retinoid X Receptor Acts as a Hormone Receptor in Vivo to Induce a Key Metabolic Enzyme for 1,25-Dihydroxyvitamin D₃*

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We demonstrate here that RNA levels of 25-hydroxyvitamin D₃-24-hydroxylase (24-(OH)ase), a key catabolic enzyme for 1,25-dihydroxyvitamin D₃, are increased by a highly selective retinoid X receptor (RXR) ligand, LG100268, in mice in hours. Correspondingly, upon LG100268 treatment, kidney 24-(OH)ase enzymatic activity increases 5-10-fold. The endogenous retinoid hormone, all-trans-retinoic acid and 9-cis-retinoic acid, and the synthetic retinoic acid receptor-selective compound, TTNPB, also stimulate 24-(OH)ase activity. Additionally, we show that LG100268 stimulates transcription of a luciferase reporter plasmid driven by 24-(OH)ase promoter sequences in the presence of RXR in CV-1 cell cotransactivation assays. This first demonstration of a gene that is regulated in the intact animal through an RXR-mediated pathway confirms earlier hypotheses that RXR is a bona fide hormone receptor. Regulation of a key gene in the vitamin D signaling pathway by a retinoid transducer may provide a molecular basis for some of the documented biological effects of vitamin A on bone and vitamin D metabolism.

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Performance of Mice—BALB/c female mice (Harlan-Teklad, 8–12 weeks old, three to four per dosing group) were placed on a control diet (low calcium, 0.8% strontium; Harlan Teklad 94325) for 14 days prior to dosing of Mice—BALB/c female mice (Harlan-Teklad, 8–12 weeks old, three to four per dosing group) were placed on a control diet (low phosphate, low calcium; Harlan Teklad 94326) or a diet that renders the 1st day and 8 a.m. and noon on the 2nd day with sacrifice 3–4 h after the dose on the 8th day. The 32-h study (Fig. 2b) included drug doses that were at 8 a.m., noon, and 4 p.m. on the 1st day and 8 a.m. and noon on the 2nd day with sacrifice 3–4 h after the dose on the 8th day.

Northern Analysis—RNA was extracted (16), and Northern analyses were performed by standard techniques. Probes were as follows: 900-bp EcoR I/XbaI DNA fragment of the rat 24-(OH)ase cDNA in PUC-18 (24-OH)ase (11); 1.4-kb human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment (Clonetech); RARα probe, a 1.4-kb NcoI 5’ end fragment from YEpTRPHRARα, yeast expression vector construct used for hRARα cDNA (17); apoA1 probe, a polymerase chain reaction...

EXPERIMENTAL PROCEDURES

Compounds—9cRA, LG100268 (14), and TTNPB were synthesized and purified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc. tRA (Schweizerhall, Inc.) was repurified at Ligand Pharmaceuticals, Inc.
generated rat 142-bp PstI/HindIII fragment from pGEM-t-ApoA1 (18); mCRBP II probe, a 400-bp polymerase chain reaction product (19); and mouse β-actin probe, a 1.5-kb EcoRI/XhoI fragment from pBLU-β-actin (Stratagene). Quantification was by Phosphorimage analysis (Molecular Dynamics).

Circulating Levels of Compounds—Plasma concentrations of LG100268 and 9RRA following dosing were determined using reversed phase high pressure liquid chromatography (HPLC). Equal volumes of plasma from three mice were combined to produce each 0.5-ml sample. 9RRA determination was as described previously (20). LG100268 determination was as follows. Samples were precipitated with 5 volumes of methanol and then subjected to solid phase extraction. Solvent was evaporated, and the residue was solubilized in mobile phase (acetonitrile, 10 mM ammonium acetate, acetic acid (75:25:1)). Analyses were conducted using a Rainin Microsorb-MV C-18 column (250 × 4.6 mm) and an Alltech direct connect guard column maintained at 40 °C. Detection was at 281 nm, and the flow rate was 1.2 ml/min. The response of the instrument was linear between 0.25 and 25 μM.

Enzyme Activity Studies—Mice were treated twice daily (4 h between doses) with 3 mg/kg LG100268 or vehicle for 8 h or 3.5 days (eight mice per group). Sacrifice was 3 h after the last dose. Kidneys were perfused with cold 0.25 M sucrose in phosphate-buffered saline, pH 7.4, pooled from four mice, homogenized, and precipitates were prepared as described previously (21); incubations (0.5 ml; 12 mg/ml protein) with 50 μM 25-(OH)D3 were for 1 h at 37 °C (21). Purification and quantitation of generated 24,25-(OH)2D3 were by solid phase extraction and HPLC (21).

Co-transactivation Assays—Co-transfection/transactivation assays were performed in CV-1 cells as described previously (2, 22).

RESULTS AND DISCUSSION

Mice were treated with the RXR-selective compound, LG100268 (Fig. 1), a documented highly specific, high affinity binder and activator of RXRs (14), and kidney 24-(OH)ase RNA levels were monitored by Northern analysis (Figs. 2 and 3). Vitamin D-deficient mice were treated with 1,25-(OH)2D3 or vehicle and with and without LG100268. After an 8-day regimen with once daily doses, 1,25-(OH)2D3-treated mice exhibited increased levels of kidney 24-(OH)ase RNA (~6-fold), as expected (11) (Fig. 2a, lanes 7–9) versus vehicle-treated mice (Fig. 2a, lanes 4–6). LG100268 in combination treatment with 1,25-(OH)2D3 resulted in an almost complete inhibition (Fig. 2a, lanes 10–12) of the increase in RNA levels that was observed with 1,25-(OH)2D3 alone (Fig. 2a, lanes 7–9). Surprisingly, LG100268 treatment alone resulted in a ~2-fold increase in 24-(OH)ase RNA levels (Fig. 2a, lanes 13–16) in comparison with the vitamin D-deficient mice that received vehicle (Fig. 2a, lanes 4–6). Interestingly, the effect of LG100268 to repress 1,25-(OH)2D3 induction of 24-(OH)ase RNA observed after 8 days of treatment was not observed after dosing periodically for 32 h (Fig. 2b, lanes 10–12) in comparison with 1,25-(OH)2D3-treated mice (Fig. 2b, lanes 7–9). However, the induction of 24-(OH)ase RNA by LG100268 alone was observed at 32 h (Fig. 2b, lanes 13–15), and the fold induction was greater (~3.3-fold) than the 2-fold induction seen at 8 days (Fig. 2a, lanes 13–16). Since the repression effect of LG100268 on 1,25-(OH)2D3 induction of 24-(OH)ase occurred only after longer time periods, a direct negative effect of the RXR ligand on the ability of the VDR-RXR heterodimer to stimulate 24-(OH)ase seemed unlikely. An alternate possibility might be that liganded RXR does not heterodimerize with VDR; complete depletion of the pool of unliganded RXR available to VDR may take longer than
Gavage in sesame oil; sacrifice occurred four per dosing group) were treated with two doses 4 h apart by oral 24-(OH)ase.

These genes are regulated in our system, Northern analyses were performed using RNA from liver (apoA1) and intestine (CRBPII) of the mice treated with two or five doses of the various retinoids for 8 or 32 h, respectively (Fig. 3c). ApoA1 (Fig. 3c, lanes 1–16) and CRBPII (Fig. 3c, lanes 17–24) RNA levels did not change with retinoid treatment on the 8- or 32-h schedule in comparison with untreated animals. Therefore, in BALB/c mice under the conditions of these experiments no significant up-regulation of apoA1 and CRBPII was observed with retinoid treatment. Additionally, apoA1 RNA levels from rat liver also were not observed to change after dosing of rats with three different concentrations of RXR-selective compound for durations ranging from 4 h to 5 days. Therefore, to date, 24-(OH)ase is the only gene that has been shown to be regulated by RXR-selective ligands in the intact animal.

Circulating levels of LG100268 and 9cRA were determined by performing pharmacokinetic studies on mice treated with LG100268 or 9cRA. Two doses of 30 mg/kg LG100268, 3 mg/kg 9cRA, or 10 mg/kg 9cRA were given 4 h apart, mice were sacrificed, and blood was obtained at various time points. Table I shows that doses of 30 mg/kg LG100268 resulted in plasma concentrations of ~1–13 μM LG100268 throughout the duration of the study. Ten-fold lower doses of LG100268 (3 mg/kg) effected the same -fold increase in 24-(OH)ase RNA levels as did higher LG100268 doses of 12 mg/kg (Fig. 3a) and 30 mg/kg (data not shown) and resulted in circulating LG100268 levels below the limits of quantitation of the assay (<250 nm). Doses of 10 mg/kg of 9cRA resulted in plasma concentrations of ~100–600 nm 9cRA at the indicated time points. Ten-fold lower doses of 9cRA (1 mg/kg) also effected induction of 24-(OH)ase RNA (Fig. 3a); circulating levels were not measured at that dose. Therefore, the determined circulating levels of LG100268 and 9cRA after administration of effective doses for exertion of a biological effect (i.e. increase of 24-(OH)ase RNA) are in the low nanomolar range.

To ascertain if the observed increases in 24-(OH)ase RNA corresponded with an increase in kidney 24-hydroxylase enzyme activity, metabolic conversion assays were performed. Table II shows that enzymatic conversion of 25-(OH)D₃ to 24,25-(OH)₂D₃ in kidney extracts from mice treated with LG100268 (3 mg/kg twice daily for 8 h or 3.5 days) was 5-10-fold greater than the conversion observed in kidney extracts from normal untreated mice. The retinoids utilized in this study were characterized as RXR-selective (LG100268) or RAR-selective (9cRA) based on our in vitro transcription assays (Ref. 14 and see Fig. 4, lanes 1–4) to ensure that there were no significant differences in biological activity between LG100268 and 9cRA.

Certain genes including apoA1 and CRBPII have been postulated to be activated through RXR-driven pathways by virtue of the fact that reporter plasmids driven by sequences from within their promoters have been shown to be stimulated by 9cRA or RXR-selective compounds in the presence of cotransfected RXR through a defined element in cell-based cotransactivation assays (2–7). However, since this initial work 4 years ago, there have not been any published reports showing that these genes are regulated in vivo. To test whether these genes are regulated in vivo in our system, Northern analyses were performed using RNA from liver (apoA1) and intestine (CRBPII) of the mice treated with two or five doses of the various retinoids for 8 or 32 h, respectively (Fig. 3c). ApoA1 (Fig. 3c, lanes 1–16) and CRBPII (Fig. 3c, lanes 17–24) RNA levels did not change with retinoid treatment on the 8- or 32-h schedule in comparison with untreated animals. Therefore, in BALB/c mice under the conditions of these experiments no significant up-regulation of apoA1 and CRBPII was observed with retinoid treatment. Additionally, apoA1 RNA levels from rat liver also were not observed to change after dosing of rats with three different concentrations of RXR-selective compound for durations ranging from 4 h to 5 days. Therefore, to date, 24-(OH)ase is the only gene that has been shown to be regulated by RXR-selective ligands in the intact animal.

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Fig. 4. Retinoids stimulate transcription from human 24-(OH)ase promoter sequences in the presence of retinoid receptors. RXRα (lanes 1, 2, 5, 6, 10, and 12), RARγ (lanes 3, 4, and 7), VDR (lanes 9 and 11), or RARγ and RXRα (lane 8) expression plasmids (13, 22) were cotransfected along with CRBPII (2, 22) (lanes 1 and 2), TRE-pal (22) (lanes 3 and 4), 24-(OH)-6000 (13) (lanes 5–8), 24-(OH)-400 (13) (lanes 9 and 10), or 24-(OH)-100 (13) (lanes 11 and 12) containing reporter plasmids. Fold induction was determined by dividing the normalized response (2, 22) at saturating concentrations of 1,25-(OH)2D3, leading to reduced activity of VDR (Fig. 4, lane 12) as well as it did through the wild type promoter sequence (Fig. 4, lane 10). Therefore, retinoid ligands acting through their respective receptors are able to stimulate transcription from the 24-(OH)ase promoter in a heterologous system utilizing cis element(s) that appear to be distinct from or overlapping with that utilized by VDR. Hence, effectors of vitamin A signal transduction, including the RXR-selective ligand, LG100268, acting through the nuclear retinoid receptors, induce a key gene involved in vitamin D signaling, thereby adding a new level of complexity to the cross-talk between the two systems.

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