Modest Decreases in Endogenous All-trans-Retinoic Acid Produced by a Mouse Rdh10 Heterozygote Provoke Major Abnormalities in Adipogenesis and Lipid Metabolism

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Pharmacological dosing of all-trans-retinoic acid (atRA) controls adiposity in rodents by inhibiting adipogenesis and inducing fatty acid oxidation. Retinol dehydrogenases (Rdh) catalyze the first reaction that activates retinol into atRA. This study examined postnatal contributions of Rdh10 to atRA biosynthesis and physiological functions of endogenous atRA. Embryonic fibroblasts from Rdh10 heterozygote hypomorphs or with a total Rdh10 knockout exhibit decreased atRA biosynthesis and escalated adipogenesis. atRA or a retinoic acid receptor (RAR) pan-agonist reversed the phenotype. Eliminating one Rdh10 copy in vivo (Rdh10+/−) yielded a modest decrease (≤25%) in the atRA concentration of liver and adipose but increased adiposity in male and female mice fed a high-fat diet (HFD); increased liver steatosis, glucose intolerance, and insulin resistance in males fed an HFD; and activated bone marrow adipocyte formation in females, regardless of dietary fat. Chronic dosing with low-dose atRA corrected the metabolic defects. These data resolve physiological actions of endogenous atRA, reveal sex-specific effects of atRA in vivo, and establish the importance of Rdh10 to metabolic control by atRA. The consequences of a modest decrease in tissue atRA suggest that impaired retinol activation may contribute to diabesity, and low-dose atRA therapy may ameliorate adiposity and its sequelae of glucose intolerance and insulin resistance.

All-trans-retinoic acid (atRA) controls proliferation, differentiation, and cell function by regulating multiple genes through the following nuclear receptors: retinoic acid receptor (RAR)α, -β, and -γ and PPARδ (1). Two reactions generate atRA from vitamin A (retinol). Retinol dehydrogenases (Rdh) of the short-chain dehydrogenase/reductase gene family (Rdh1, Rdh10, and Dhrs9) convert retinol into retinal, whereas retinal dehydrogenases (Raldh) of the aldehyde dehydrogenase gene family (Raldh1, -2, and -3) convert retinal into atRA (2,3). Retinal reductases (e.g., Dhrs3) also contribute to maintaining steady-state retinal concentrations, as does carotenoid cleavage (4). atRA concentrations are maintained by the steady-state concentration of retinal set by Rdh and reductases and activities of Raldh and atRA catabolic enzymes (Cyp26A1, -B1, and -C1) (5,6). The distinct but overlapping spatial and temporal expression patterns of Rdh suggest that specific isoforms satisfy discrete functions (7). Differences in the phenotypes of ablated retinoid metabolon proteins provide further support for this hypothesis (8,9).

Rdh10 was cloned from the retinal pigment epithelium and retinal Muller cells as an all-trans- and 11-cis-Rdh in the visual cycle (10,11). Rdh10, however, has widespread expression during embryogenesis, localizing with many sites of atRA signaling (12). Most homozygous Rdh10-null mouse pups die by embryo day 13 (E13) from severe forelimb, internal organ, and forebrain abnormalities (13). Long-term retinal dosing to dams allows the birth of Rdh10-null mice in Mendelian frequency but does not rescue all aspects of atRA deficiency in neonates. An Rdh10 hypomorph, produced by a missense mutation (A196V), produces pups that die mostly by E17.5 with edema, a midline facial cleft, forebrain defects, impaired cerebrovasculature, and absence...
of olfactory bulbs (14,15). Thus, atRA production during mid-to-late gestation requires Rdh10, but additional Rdh also are important during embryogenesis.

Postnatal contributions of Rdh10 to atRA biosynthesis have not been studied extensively. A Sertoli and/or germ cell–specific Rdh10 knockout in juvenile mice prevents spermatogenesis equivalent to that during vitamin A deficiency, but upon reaching adulthood, these mice exhibit normal spermatogenesis, indicating age-specific Rdh10 functions (16). Overexpression of Rdh10 in primary human neonatal keratinocytes produced an aberrant phenotype, consistent with the inhibition of differentiation and upregulation of atRA-induced genes (17). The same phenomena were not duplicated by overexpression of the human ortholog (RDH16) of mouse Rdh1, suggesting that Rdh10 serves as the major Rdh in neonate keratinocytes. In contrast, sequential knockdown of endogenous Rdh in primary rat hippocampus astrocytes showed that Rdh10, Dhrs9, and Rdh2 (rat ortholog of mRdh1) contribute to atRA biosynthesis (18).

The mRdh1 knockout illustrates the contribution of endogenous atRA to energy balance (19). When fed a low-fat diet (LFD), mRdh1-null mice have a 59% (12.7 g) increase in fat at 36 weeks old relative to wild type (WT). Rdh1-null mice, however, do not have other symptoms of atRA deficiency. Pharmacological atRA dosing to mice fed high-fat diets (HFDs) further suggests that retinoids regulate energy expenditure, consistent with the inhibition of differentiation and upregulation of atRA-induced genes (17). The same phenomena were not duplicated by overexpression of the human ortholog (RDH16) of mouse Rdh1, suggesting that Rdh10 serves as the major Rdh in neonate keratinocytes. In contrast, sequential knockdown of endogenous Rdh in primary rat hippocampus astrocytes showed that Rdh10, Dhrs9, and Rdh2 (rat ortholog of mRdh1) contribute to atRA biosynthesis (18).

This study was undertaken to determine postnatal contributions of Rdh10 to atRA biosynthesis and function. We used heterozygous knockouts (Rdh10<sup>+/–</sup>) and embryonic fibroblasts from Rdh10 hypomorphs to study the impact of Rdh10 on atRA generation and function. Heterozygotes were used to circumvent homozygote embryonic lethality. Knockout one Rdh10 copy yielded only a modest decrease in tissue atRA but produced an array of metabolic and tissue abnormalities, some of which were sex specific. These results reveal Rdh10 contributions to atRA metabolic functions, resolve physiological actions of endogenous atRA in vivo and its sex-specific effects, and predict health consequences of a modest decrease in tissue atRA.

**RESEARCH DESIGN AND METHODS**

**Mice**

Rdh10-targeted C57BL/6N embryonic stem cells were injected into BALB/C blastocysts by the University of California (UC) Davis Mouse Biology Program with loxP sites that flank Rdh10 exon 2 (Supplementary Fig. 1). Rdh10 floxed mutants were back-crossed into C57BL/6J mice >12 generations and bred with mice expressing CMV-Cre [B6.C-Tg(CMV-cre)1Cgu/J; The Jackson Laboratory] to generate Rdh10<sup>+/–</sup>- mice. Controls were littermates (Rdh10<sup>+/+</sup> and CMV-Cre<sup>+</sup>).

Mice were fed an AIN93G diet with 4 IU/g vitamin A (180614; Dyets, Inc) and 9% fat (LFD) or 50% fat as lard (HFD). Unless noted otherwise, mice were 4 months old and fed either diet from weaning. Body and food weights were measured weekly. Body composition was determined with an EchoMRI 100V whole-body magnetic resonance analyzer. Energy expenditure and activity were assessed with a laboratory animal monitoring system. VO<sub>2</sub> and VCO<sub>2</sub> were normalized to the lean body mass of each mouse. Femur marrow was harvested by centrifugation (22). Animal experiments were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Protocols were approved by the UC Animal Care and Use Committee.

**atRA Dosing**

Five milligrams of 90-day slow-release atRA pellets or placebo (Innovative Research of America) were implanted under the skin on the lateral side of the neck in 6-week-old mice.

**Glucose Tolerance Test/Insulin Tolerance Test**

Glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were performed on 4-month-old mice fed an HFD. Mice were fasted 16 h before GTT and 4 h before ITT and placed in individual cages without food, but with free access to water. Either glucose (1.5 g/kg body weight) or insulin (0.5 IU/kg body weight) was injected. Blood glucose from tail tips was measured with a glucometer.

**Serum Measurements**

Serum was sent to the UC Davis Mouse Metabolic Phenotyping Center for quantification of insulin, leptin, adiponectin, and β-hydroxybutyrate. Serum nonesterified fatty acid (NEFA) was quantified with an EnzyChrom Free Fatty Acid Assay Kit (EFFA-100).

**TAG Assay**

Triacylglycerol (TAG) was measured in homogenized livers using a Triglyceride Colorimetric Assay Kit (10010303; Cayman Chemical). Values were normalized to liver wet weight.

**Quantitative PCR**

mRNA expression was normalized to the geometric means of Gusb and Tbp. Primer and quantitative PCR details are included in the Supplementary Data.

**Histology**

Formalin-fixed, paraffin-embedded tissues were cut into 8-μm sections. Deparaffinized sections were stained with hematoxylin-eosin (H-E). Fibrosis was visualized with Lillie’s modified Masson’s Trichrome Stain for Connective Tissue. Tissue sections were imaged using a Zeiss Axio Imager M1 microscope equipped with QImaging SPMPix MicroPublisher.

**F4/F80 Immunohistochemistry**

Liver was embedded in Tissue-Tek optimal cutting temperature compound (OCT), snap frozen in liquid nitrogen, and sectioned into 12-μm slices. Slides were fixed by submerison in ice-cold acetone. Endogenous peroxidase was blocked with 0.3% H<sub>2</sub>O<sub>2</sub>. Slides were incubated with F4/80 antibody (Immunocruz ABC Staining System) and counterstained in H-E.

**Bone Histology**

Femurs were fixed for 24 h at 4°C in Bouin solution (50:1) and decalcified in 10% EDTA at 4°C. Femurs were bisected
at the diaphysis center into proximal and distal halves. Distal femurs were dehydrated, embedded in paraffin, and sectioned longitudinally starting 10 μm from the frontal (coronal) plane. Sections 50–150 were collected onto Premier Charged Microscope slides in a water bath at 40–42°C and H-E stained.

Liver Oil Red O Staining
Liver was embedded in OCT in cryostat molds, snap frozen in liquid nitrogen, sectioned at 12 μm, fixed with 10% neutral-buffered formalin, submerged in Oil Red O, and counterstained in Harris Modified Hematoxylin Solution. Sections were treated with aqueous mounting medium (Shurmount). Slides were imaged within 24 h to avoid interference from Oil Red O precipitation.

Mouse Embryonic Fibroblasts
Mouse embryonic fibroblasts (MEFs) were isolated from E13.5–14 embryos (23) and cultured in ultraviolet-irradiated growth medium of DMEM (Gibco BRL), 10% bovine calf serum (30-2030; American Type Culture Collection), and 100 units/mL penicillin/streptomycin (Gibco BRL) in six-well plates at 37°C. Differentiation was induced 2 days after confluence (differentiation day 0 [dd0]), with growth medium containing 0.5 nmol/L methylisobutylxanthine (IBMX), 1 μmol/L dexamethasone, 0.85 mmol/L insulin, and 100 nmol/L rosiglitazone. After 3 days (dd3), the medium was changed to growth medium with 0.85 mmol/L insulin and 100 nmol/L rosiglitazone, renewed every other day. Each experiment was performed with MEFs from two to three dams and repeated two to three times with MEFs from different dams. MEFs from littersmates were compared.

Effects of nuclear receptor ligands were tested with vehicle (DMSO), 500 nmol/L atRA (refreshed daily), 100 nmol/L TTNPB (RAR pan-agonist; T3757; Sigma-Aldrich), or 100 nmol/L GW0742 (PPARδ agonist; G3295; Sigma-Aldrich) (refreshed every 2 days).

atRA biosynthesis was quantified in MEFs treated for 2 h with 250 nmol/L retinol, a physiological concentration (24). Cells were collected with 1× reporter lysis buffer (E397A; Promega) and flash frozen. atRA was quantified by liquid chromatography with tandem mass spectrometry (25).

Oil Red O staining was performed on dd7. MEFs were fixed with 10% formalin/PBS and stained with 60% Oil Red O/isopropanol. Excess stain was removed by washing with water. Stained oil droplets were dissolved in isopropanol. Absorbance was read at 510 nm.

MEF Rdh10 Ablation
A guide RNA targeting exon 3 in a lentiCRISPR v2 plasmid (plasmid 52961; Addgene) was introduced into SV40-immortalized MEFs (26). Cells that survived puromycin selection were plated individually. DNA was sequenced for Cas9-induced breaks repaired by nonhomologous end joining. Both alleles of Rdh10 were affected, resulting in deletion of exons 3 (active site) to 6, i.e., amino acid residues beyond 184. Colonies with random mutations were used as controls. The guide RNA sequence was as follows:

forward 5′-CACCACGATGTCAGGATTAATCA-3′; reverse 5′-AAACTGTTATCTTCAGCATCGTC-3′. The probe to verify CRISPR knockout was Mm.PT58/9541215. Four control and four knockdown clones were isolated (Supplementary Fig. 2). One clone of each was chosen for detailed study (WT6 and KO27).

Statistics
Data are expressed as means ± SD, unless noted otherwise. Significance was determined by two-tailed, unpaired Student t tests or two-way ANOVA.

RESULTS
Rdh10 Generates atRA in Preadipocytes
Rdh10 expression is >22-fold more intense than Dhrs9 in primary undifferentiated MEFs (dd0) (Fig. 1A). Rdh10 mRNA decreases by 50% after MEF differentiation, but Dhrs9 mRNA does not change. Rdh1 and RdhE2 mRNA were not detected. The net atRA concentration in WT MEFs after incubation with retinol also decreases ~50% from dd0 during adipogenesis (Fig. 1B), suggesting that Rdh10 generates atRA to inhibit adipogenesis.

To test whether downregulation of Rdh10 allows adipogenesis, we used a mouse model harboring a mutated Rdh10 (Rdh10<sup>5306ΔAsp</sup>), which encodes a hypomorph with lower activity than WT because of a single nucleotide mutation in the substrate binding pocket (V196A) (14). This model has the benefit of reducing catalytic activity without altering Rdh10 expression, which avoids off-target effects that might result from gene ablation. Homozygous hypomorphs die before E13.5–14. Therefore, we isolated MEFs from heterozygous hypomorphs (HYPO). HYPO MEFs had a 40% reduction in the steady-state atRA concentration relative to WT after incubation with retinol (Fig. 1C). Only Dhrs9 mRNA among retinoid metabolon genes compensated for the Rdh10 ablation, with a twofold increase (P < 0.03) (Supplementary Fig. 3). Basal expression of Pparg in HYPO MEFs did not differ from WT on dd0 (Fig. 1D). After differentiation (dd7), HYPO MEFs had ~40% more adipocytes than WT (Fig. 1E and F); expression of adipogenic markers (Pparg and ap2) was ~2.5- to 3.8-fold higher in HYPO versus WT MEFs (Fig. 1G). Total knockout of Rdh10 with CRISPR CAS9 in immortalized MEFs (CKO) revealed an ~18-fold increase in Oil Red O-positive cells compared with immortalized WT MEFs (Fig. 1H–J), with Pparg and Fabp4 expression ~3-fold higher (Fig. 1K). These data are consistent with Rdh10 generating atRA in mesenchymal stem cells to suppress adipogenesis.

Both HYPO and WT MEFs responded to increasing retinol concentrations, but HYPO MEFs differentiated to a greater extent than WT (Fig. 2A). The IC<sub>50</sub> values of retinol were 290 and 333 nmol/L for WT and HYPO, respectively, for Oil Red O quantification (Fig. 2B). The marked difference in total lipids, but the small difference between the IC<sub>50</sub> values, may result from Oil Red O reflecting total lipid accumulation, caused by both adipogenesis and de novo lipogenesis. The absolute values of Oil Red O concentrations...
would differ because of differences in adipogenesis, whereas the IC_{50} values would reflect the distinct process of lipid accumulation in differentiated cells. The IC_{50} values of adipocyte-expressing genes Pparg (454 and 915 nmol/L, respectively, for WT and HYPO) and Ap2 (65 and 703) indicate enhanced adipogenesis associated with impaired HYPO atRA biosynthesis (Fig. 2C and D). Higher retinol concentrations produced differences in expression of atRA-inducible genes Rarb, Cyp26a1, and Cyp26b1, consistent with impaired HYPO atRA biosynthesis from retinol.

Rdh10^{+/−} Mice Have More Severe Diet-Induced Obesity and Insulin Resistance
To determine Rdh10 functions in vivo, we generated mice with a whole-body deletion. Heterozygotes (Rdh10^{+/−}) were
studied because homozygotes are not viable. Decreases in Rdh10 mRNA in liver (52%), inguinal white adipose tissue (iWAT; 87%), and epididymal WAT (eWAT; 64%) verified knockdown (Fig. 3A). Rdh10⁻/⁻ mice fed an LFD did not differ from WT in weight, fat body mass, or GTT (Supplementary Fig. 5). Rdh10⁻/⁻ mice fed an HFD displayed substantial weight differences relative to WT (Fig. 3B). Males weighed >5 g more than WT littersmater, whereas females weighed ~4.7 g more. Increases in body weight were caused primarily by increases in fat, with no significant differences (<1 g) in lean weight (Fig. 3D). No significant differences occurred in food intake between Rdh10⁻/⁻ and WT (Fig. 3E and F). Serum insulin increased nearly twofold in Rdh10⁻/⁻ males (Fig. 3G). A 3.5-fold increase in serum leptin and a 40% increase in NEFA reduced 50%, a decrease that reflects an increase in Hif1α (Fig. 4B) (27). The inflammatory indicators Saa3 and Tnfa increased two- to threefold. Rdh10⁻/⁻ mice had larger adipocytes in eWAT and iWAT (Fig. 4C, D, and F), reflected in the average adipocyte sizes (Fig. 4E and G). Histologically, Rdh10⁻/⁻ males also had increased inflammation (Fig. 4H) and fibrosis (Fig. 4I). These results suggest that adipose expansion exceeds angiogenesis, which induces hypoxia, inflammation, oxidative stress, and fibrosis (28). Fibrosis from persistent inflammation damages adipocytes, which increases NEFA release, resulting in nonadipose tissues accumulating TAG. The inflammation and fibrosis of adipose, with the increase in serum NEFA (Fig. 3I), would contribute to liver steatosis.

Males Fed an HFD Develop Liver Steatosis

Histological analysis showed that 14 of 19 (~74%) Rdh10⁻/⁻ males fed an HFD had increased fat accumulation in liver, relative to 7 of 18 (39%) WT, without enhancement of the inflammation marker F4/80 (Fig. 5A). Females fed the HFD

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**Figure 2**—Rdh10 HYPO MEFs are less sensitive to retinol than WT. A: Oil Red O (ORO) images of WT and HYPO MEFs treated with retinol every 24 h during differentiation imaged on dd7. Scale bars, 100 μm. B-G: Open diamonds, HYPO; filled circles, WT. B: Quantification of Oil Red O from A. MEFs from three embryos per group per dose. Two-way ANOVA, P < 0.0001, genotype and dose. OD, optical density. C-G: WT and HYPO MEFs treated with retinol as illustrated in A and B. Cells were harvested on dd7. Representative data of two experiments, each performed with duplicate cultures of MEFs from individual pups. C and D: Two-way ANOVA, P < 0.0001, genotype and dose. E-G: ***P < 0.001.
showed a much more modest reaction (Fig. 5B). Rdh10\(^{+/2}\) males fed the HFD had a greater than twofold increase (5.2 mg) in liver TAG compared with WT (Fig. 5C). Three of the Rdh10\(^{+/2}\) males reacted more intensely than the others. Excluding these three from the analysis still showed a significant difference from WT, with a 44% increase in TAG (\(P, 0.02\)). In contrast, livers of female Rdh10\(^{+/2}\) fed an HFD showed no significant difference in TAG content from WT (Fig. 5D). Rdh10\(^{+/2}\) male livers were larger than WT (Fig. 5E), weighing 20% more (0.2 g) (Fig. 5E and G). Rdh10\(^{+/2}\) female liver weights did not differ from WT (\(P > 0.1\)) (Fig. 5F). Decreases in gene expression occurred in Acadm (52% decrease), which encodes the medium-chain acyl-CoA dehydrogenase; Ppara (32% decrease), which stimulates \(\beta\)-oxidation; and Saa1 (63% decrease), which designates acute inflammation (Fig. 5H). Serum \(\beta\)-hydroxybutyrate in fasted males and females did not differ with genotype (Supplementary Fig. 8).

**Increased Adipocyte Formation in Female Femur Marrow**

We examined femur bone marrow, because marrow stem cells serve as progenitors to adipocytes. Rdh10\(^{+/2}\) males fed an HFD had a slight increase in adipocytes near the growth plate, which was not statistically significant (Fig. 6A and B), and showed no significant differences in genes typically expressed in adipocytes (Fig. 6C). Females fed an HFD had a threefold increase in adipocytes clustered near the growth plate (Fig. 6D and E) and enhanced expression of Fabp4 (>50%), Pparg (100%), Ffab5 (100%), and AdipoQ (>80%) (Fig. 6F). Females fed an LFD also had threefold increased adipocytes in femur marrow (Fig. 6H and I). Increased expression of AdipoQ did not increase circulating adiponectin (Supplementary Fig. 9). Femur lengths of males did not differ with genotype, but femurs from the Rdh10\(^{+/2}\) females fed an HFD were ~8.5% longer than WT (Fig. 6J).
atRA Rescues the Phenotype

atRA caused a greater than fivefold decrease in the number of Oil Red O–positive cells in HYPO MEFs and a greater than threefold decrease in WT, and suppressed differentiation to a similar base value in both (Fig. 7A and B). Pparg and Fabp4 expression was 4- and 14-fold higher in HYPO MEFs than WT, which was reduced by atRA to 4- and 12-fold less than vehicle-treated HYPO MEFs (Fig. 7C and D). Because atRA activates both RAR and PPARα, we tested receptor-specific ligands in CKO MEFs, which differentiated to a much greater extent than WT (Fig. 7C and D). The RAR pan-agonist TTNPB reduced differentiation by fivefold, whereas the PPARα agonist was ineffective. TTNPB also reduced Pparg and Fabp4 expression by 5- and 40-fold, respectively (Fig. 7G and H). Thus, HYPO and CKO MEFs respond robustly to atRA with robust decreases in adipogenesis.

We next determined the impact of atRA on Rdh10+/− mice treated with low-dose atRA weighed 9 g less than placebo-treated mice and had body weights equivalent to mice fed an LFD (Fig. 7I). atRA dosing also improved glucose tolerance and insulin sensitivity (Fig. 7J and K). A 50% decrease in fat relative to vehicle-treated mice reflected the major weight difference, with no significant change in lean mass (Fig. 7L). atRA rescued the liver steatosis phenotype and reduced the sizes of white adipocytes in eWAT (Fig. 7M). These data suggest the potential of chronic low-dose atRA treatment for metabolic disorders.

DISCUSSION

We undertook this research to reveal postnatal Rdh10 contributions to atRA function and to assess endogenous atRA actions. Previous studies focusing on specific organs or using vitamin A deficiency models revealed that atRA has widespread impact on metabolism, consistent with organ
autonomous effects (15–17,20,29–32). Total Rdh10 ablation impairs the biogenesis of multiple organs, including the pancreas, and causes embryonic lethality between E10.5 and E13, but Rdh10+/− mice reportedly were “indistinguishable” from WT (33). This conclusion, referring to the appearance of mice fed a chow diet, coincides with our observations of the appearance of Rdh10+/− mice fed an LFD. Despite evaluating heterozygotes, the metabolic phenotype reported here involved multiple tissues and was surprisingly severe. More surprising were the modest decreases in tissue atRA that led to the metabolic defects. This reveals that minor decreases in tissue atRA have a major impact on metabolic health and can predispose to obesity, glucose intolerance, insulin resistance, and ectopic adipose formation and fat deposition. This finding, as well as rescue with low-dose atRA, indicates the importance of establishing the connection between retinol activation and atRA concentrations and metabolic diseases, and suggests the potential of low-dose atRA for metabolic disease therapy.

Our observations support the proposed functions of atRA in arresting adipogenesis and regulating lipid metabolism (19,21). Previous studies to define atRA function in energy homeostasis, however, were performed either with established cell lines, which may not faithfully model their origins, or through dosing pharmacological and/or toxic amounts of atRA to animals fed a diet copious in vitamin A (lab chow). Diets with copious vitamin A obscure knock-out effects and could contribute to vitamin A toxicity when dosing pharmacological atRA (19,34). Vitamin A and atRA represent prototypical examples of hormesis, an inverted U-shaped dose-response curve in which increasing concentrations increase beneficial effects to a point, but beneficial effects ultimately subside with high concentrations and toxicity ensues. Thus, dosing high amounts of atRA confounds toxicology with physiology, especially in animals fed chow diets, which contain at least fourfold more vitamin A than used here, which we based on recommendations by the National Research Council.

The dimorphic impact of atRA exposed another unanticipated result. Distinctions between males and females
in diet-dependent liver steatosis and bone marrow adipocyte formation provide new insight into interaction(s) between retinoids and steroid sex hormones. Complex interactions between the two estrogen receptors ER\textsubscript{a} and ER\textsubscript{b} and the atRA receptors RAR\textsubscript{a} and RAR\textsubscript{b} occur in breast cancer cell lines (35,36). Moreover, estrogen induces atRA biosynthesis in primary human endometrial cells (37), whereas atRA induces estrogen biosynthesis in placental cells (38). Little is known about these interactions in adipose. atRA plus estrogen, however, additively induce Rarg, estrogen receptor \textsubscript{b} (Esr2), and aromatase (Cyp19a1) in NIH3T3-L1 cells (39). It is reasonable to propose that atRA impairs estrogen function in female bone marrow, in addition to having direct effects. Interactions between androgens and atRA also occur. atRA downregulates androgen receptor mRNA in breast cancer cell lines. Vitamin A restriction increases androgen receptor expression in rat testis (40). Castration increases Rara and Rarg mRNAs in rat prostate, which testosterone dosing represses (41). Testosterone reduces loss of bone density in mice dosed chronically with pharmacological atRA (10 mg/kg/day) (42). These interactions might contribute to differences in the bone phenotype between male and female Rdh10\textsuperscript{−/−} mice.

Meta-analysis of epidemiological studies indicate a U-shaped relationship between serum retinol and hip fracture risk (hormesis), i.e., either high or low serum retinol increases osteoporosis risk (43). Skeletal growth relies on RAR. Excess atRA accelerates bone maturation and decreases bone density, especially in orchiectomized rodents (29,42). The mechanism seems related to atRA effects on multipotent stem cells in bone marrow differentiating into osteoblasts or adipocytes. Increased marrow adiposity associates with adverse effects on skeletal function (44); atRA may provide a link in this connection. Multiple studies with cell lines demonstrate that atRA inhibits mesenchymal cell differentiation into adipocytes and promotes differentiation into osteoblasts, and indicate that adipocyte formation impairs osteoblast production (30,45). Increased adipocyte differentiation during osteoporosis may contribute to bone deterioration by occupying the marrow niche and by reducing osteoblastogenesis. A recent study, using primary bone marrow stem cells, extended the observation that atRA inhibits adipocyte differentiation but concluded that atRA inhibits osteoblast differentiation (46). Our study did not address adipogenesis versus osteoblastogenesis but showed that a decrease in endogenous atRA stimulates adipocyte formation in bone marrow independently of dietary fat that affects females. Thus, the Rdh10\textsuperscript{−/−} mouse introduces a model for studying adipocyte formation in bone marrow and atRA effects on osteoblastogenesis and osteoporosis.
Figure 7—atRA through RAR ameliorates excess adipogenesis. A: Oil Red O imaging of MEFs differentiated in the presence of vehicle or atRA. MEFs from four embryos per genotype. Representative result of two experiments. Scale bars, 100 μm. B: Quantification of Oil Red O (ORO) in A. **P < 0.006 from vehicle (V); #P < 0.01, WT vs. HYPO. C and D: Expression of adipogenic genes (Pparg and Fabp4) in MEFs differentiated in the presence of vehicle or atRA; n = 2–3 embryos per group. Representative result of two experiments. **P < 0.006 from vehicle; #P < 0.008, WT vs. HYPO. E: Oil Red O images of differentiated CKO on dd7 treated with vehicle, TTNPB, or GW0742 throughout differentiation. Representative result of two experiments with duplicates for each. Scale bars, 200 μm. F: Quantification of images in E. #, *P < 0.007 from WT and vehicle-treated CKO, respectively. G and H: Expression of Pparg and Fabp4 in cells in E. Representative result of two experiments. n = 3 per group per experiment. #, *P < 0.007 from WT and vehicle-treated CKO, respectively. I and J: Rdh10+/− mice fed an HFD and treated with time-released placebo or atRA. n = 6–7 mice per genotype. I: Body weights. Two-way ANOVA, dose and time, P < 0.0001. J: GTT, two-way ANOVA, dose and time, P < 0.0001. K: ITT, two-way ANOVA, dose and time, P < 0.0001. L: EchoMRI (eMRI). Fat: *P < 0.0001, placebo vs. atRA. M: Representative H-E images of tissues. Scale bars, 130 μm. OD, optical density.
The relationship of retinol and atRA with liver steatosis has been unclear. Some reports concluded that RAR induces liver steatosis, whereas others reported beneficial actions; this may reflect retinoid hormesis causing dose-dependent outcomes (47). The intervention of specific atRA receptors offers some clarity. Transgenic male mice that express a dominant-negative RARα develop liver steatosis by 4 months old without obesity and develop hepatocarcinoma by 12 months (48). The former was associated with decreased fatty acid oxidation. Overexpression of the atRA-inducible RARβ in mouse liver enhances fatty acid oxidation and energy expenditure (31), whereas the RARβ2-specific agonist AC261066 reduces liver steatosis in HFD-fed mice (49). Increasing endogenous atRA concentrations by inhibiting the atRA-catabolic Cyp26 isoforms enhances expression of mitochondrial biogenesis markers Nrf1 and Pgc1β 5- to 10-fold (32). These data are consistent with atRA enhancing fatty acid oxidation and mitochondrial biogenesis in vivo and suggest processes contributing to the liver steatosis phenotype.

In contrast to increased adiposity of Rdhl and Rdh10 knockouts, Rdhl-knockout mice resist adiposity. Rdhl1 ablation decreases atRA in liver and in some female fat pads of 6- to 7-week-old mice fed an HFD, which should enhance adiposity but does not affect retinal in these mice. These and other data indicate that the multifunctional Rdhl1, which also has a diverse array of substrates, acts independently of retinal and RA in adipose to permit adiposity (50).

These studies provide the first insight into the physiological effects of endogenous atRA in energy balance and the function of Rdh10 in postnatal intermediary metabolism. The data reveal that Rdh10 contributes to normal glucose and insulin sensitivity, control of adiposity and adipogenesis, and preservation of bone health. The surprisingly modest decrease in atRA that caused these effects suggests that low-dose atRA therapy may help ameliorate adiposity and its sequelae of glucose intolerance and insulin resistance.

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References

1. Isakova M, Karbyshev M, Piskunov A, Rochette-Egly C. Nuclear and extra-nuclear effects of vitamin A. Annu Rev Pharmacol Toxicol 2015;55:13–53
2. Napoli JL. Physiological insights into all-trans-retinoic acid biosynthesis. Biochim Biophys Acta 2012;1821:152–167
3. Kadishvili NY. Retinoic acid synthesis and degradation. Subcell Biochem 2016;81:127–161
4. Harrison EH. Mechanisms involved in the intestinal absorption of dietary vitamin A and provitamin A carotenoids. Biochim Biophys Acta 2012;1821:70–77
5. Ross AC, Zolfaghari R. Cytochrome P450s in the regulation of cellular retinoic acid metabolism. Annu Rev Nutr 2011;31:65–87
6. Nelson CH, Buttrick BR, Ishihara N. Therapeutic potential of the inhibition of the retinoic acid hydroxylases CYP26A1 and CYP26B1 by xenobiotics. Curr Top Med Chem 2013;13:1402–1428
7. Zhai Y, Higgins D, Napoli JL. Coexpression of the mRNAs encoding retinol dehydrogenase isoforms and cellular retinol-binding protein. J Cell Physiol 1997;173:36–43
8. Niedereiter K, Vermot J, Fraulob V, Chambon P, Dolle P. Retinaldehyde dehydrogenase 2 (RALDH2)–independent patterns of retinoic acid synthesis in the mouse embryo. Proc Natl Acad Sci U S A 2002;99:16111–16116
9. Dupoux V, Matt N, Gamlier JM, Chambon P, Mark M, Ghyselinck NB. A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment. Proc Natl Acad Sci U S A 2003;100:14036–14041
10. Wu BX, Chen Y, Chen Y, et al. Cloning and characterization of a novel all-trans retinol short-chain dehydrogenase/reductase from the RPE. Invest Ophthalmol Vis Sci 2002;43:3365–3372
11. Wu BX, Moiseyev G, Chen Y, Rohrer B, Crouch RK, Ma JK. Identification of RDH10, an all-trans retinol dehydrogenase, in retinal Muller cells. Invest Ophthalmol Vis Sci 2004;45:3857–3862
12. Romand R, Kondo T, Cammas L, Hashino E, Dolle P. Dynamic expression of the retinoic acid-synthesizing enzyme retinol dehydrogenase 10 (rdh10) in the developing mouse brain and sensory organs. J Comp Neurosci 2008;508:879–892
13. Rhinn M, Schulzaur B, Niedereiter K, Dolle P. Involvement of retinol dehydrogenase 10 in embryonic patterning and rescue of its loss of function by maternal retinaldehyde treatment. Proc Natl Acad Sci U S A 2011;108:16687–16692
14. Ashique AM, May SR, Kane MA, et al. Morphological defects in a novel Rdh10 mutant that has reduced retinoic acid biosynthesis and signaling. Genesis 2012;50:415–423
15. Bonney S, Harrison-Uy S, Mishra S, et al. Diverse functions of retinoic acid in brain vascular development. J Neurosci 2016;36:7786–7801
16. Tong MH, Yang Q, Davis JC, Greweld MD. Retinol dehydrogenase 10 is indispensable for spermatogenesis in juvenile males. Proc Natl Acad Sci U S A 2013;110:543–548
17. Lee SA, Belyaeva OV, Wu L, Kadishvili NY. Retinol dehydrogenase 10 but not retinol/sterol dehydrogenase(s) regulates the expression of retinoic acid-responsive genes in human transgenic skin raft culture. J Biol Chem 2011;286:13550–13560
18. Wang C, Kane MA, Napoli JL. Multiple retinol and retinal dehydrogenases catalyze all-trans-retinoic acid biosynthesis in astrocytes. J Biol Chem 2011;286:6542–6553
19. Zhang M, Hu P, Krois CR, Kane MA, Napoli JL. Altered vitamin A homeostasis and increased size and adiposity in the rdh1-null mouse. FASEB J 2007;21:2886–2896
20. Amengual J, Ribot J, Bonet ML, Palou A. Retinoic acid treatment enhances lipid oxidation and inhibits lipid biosynthesis capacities in the liver of mice. Cell Physiol Biochem 2010;25:657–666
21. Berry DC, DeSantis D, Soltanian H, Croniger CM, Noy N. Retinoic acid upregulates preadipocyte genes to block adipogenesis and suppress diet-induced obesity. Diabetes 2012;61:1112–1121.

22. Kelly NH, Schimenti JC, Patrick Ross F, van der Meulen MC. A method for isolating high quality RNA from mouse cortical and cancellous bone. Bone 2014;68:1–5.

23. Xu J. Preparation, culture, and immortalization of mouse embryonic fibroblasts. Curr Protoc Mol Biol 2005;Chapter 28:Unit 28.1.

24. Kane MA, Folias AE, Napoli J. HPLC quantitation of retinal, retinol, and retinyl esters in serum and tissues. Anal Biochem 2008;378:71–79.

25. Kane MA, Folias AE, Wang C, Napoli J. Quantitative profiling of endogenous retinoic acid in vivo and in vitro by tandem mass spectrometry. Anal Chem 2008;80:1702–1708.

26. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 2013;8:2281–2308.

27. Halberg N, Khan T, Trujillo ME, et al. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. Mol Cell Biol 2009;29:4467–4483.

28. Kusminski CM, Bickel PE, Scherer PE. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat Rev Drug Discov 2016;15:639–660.

29. Nilsson O, Isoherranen N, Guo MH, et al. Accelerated skeletal maturation in disorders of retinoic acid metabolism: a case report and focused review of the literature. Horm Metab Res 2016;48:737–744.

30. Skillington J, Choy L, Derynick R. Bone morphogenetic protein and retinoid acid signaling cooperate to induce osteoblast differentiation of preadipocytes. J Cell Biol 2002;159:135–146.

31. Li Y, Wong K, Walsh K, Gao B, Zang M. Retinoic acid receptor β stimuliates hepatic induction of fibroblast growth factor 21 to promote fatty acid oxidation and control whole-body energy homeostasis in mice. J Biol Chem 2013;288:10490–10504.

32. Tripathy S, Chapman JD, Han CY, et al. All-trans-retinoic acid enhances mitochondrial function in models of human liver. Mol Pharmacol 2016;89:560–574.

33. Arregi I, Clermont M, Ilev D, et al. Retinol dehydrogenase-10 regulates pancreas organogenesis and endocrine cell differentiation via paracrine retinoic acid signaling. Endocrinology 2016;157:4615–4631.

34. E X, Zhang L, Lu J, et al. Increased neonatal mortality in mice lacking cellular retinol-binding protein II. J Biol Chem 2002;277:36617–36623.

35. Rousseau C, Nichol JN, Pettersson F, Couture MC, Miller WH Jr. ERβelta sensitizes breast cancer cells to retinoic acid: evidence of transcriptional crosstalk. Mol Cancer Res 2004;2:523–531.

36. White KA, Yore MM, Deng D, Spinella MJ. Limiting effects of RlP140 in estrogen signaling: potential mediation of anti-estrogenic effects of retinoic acid. J Clin Endocrinol Metab 2005;208:7829–7835.

37. Deng L, Shipley GL, Loose-Mitchell DS, et al. Coordinate regulation of the production and signaling of retinoic acid by estrogen in the human endometrium. J Clin Endocrinol Metab 2003;88:2157–2163.

38. Zhu SJ, Li Y, Li H, et al. Retinoic acids promote the action of aromatase and 17beta-hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17beta-estradiol in placental cells. J Endocrinol 2002;172:31–43.

39. Shearer KD, Morrice N, Henderson C, et al. Fenretinide prevents obesity in aged female mice in association with increased retinoid and estrogen signaling. Obesity (Silver Spring) 2015;23:1655–1662.

40. Zhuang YH, Bläuer M, Ylikomi T, Tuohimaa P. Spermatoogenesis in the vitamin A-deficient rat: possible interplay between retinoic acid receptors, androgen receptor and inhibit alpha-subunit. J Steroid Biochem Mol Biol 1997;60:67–76.

41. Huang HF, Li MT, Von Hagen S, Zhang YF, Irwin RJ. Androgen modulation of the messenger ribonucleic acid of retinoic acid receptors in the prostate, seminal vesicles, and kidney in the rat. Endocrinology 1997;138:553–559.

42. Broulik PD, Raška I, Brouliková K. Prolonged overdose of all-trans retinoic acid enhances bone sensitivity in castrated mice. Nutrition 2013;29:1166–1169.

43. Wu AM, Huang CJ, Lin ZK, et al. The relationship between vitamin A and risk of fracture: meta-analysis of prospective studies. J Bone Miner Res 2014;29:2032–2039.

44. Veltman-Vlug AG, Rosen CJ. Mechanisms of marrow adiposity and its implications for skeletal health. Metabolism 2017;67:106–114.

45. Zhang W, Dong ZL, Chen L, et al. Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. PLoS One 2010;5:e11917.

46. Green AC, Kocovič P, Jovic T, et al. Retinoic acid receptor signaling directly regulates osteoblast and adipocyte differentiation from mesenchymal progenitor cells. Exp Cell Res 2017;350:284–297.

47. Mellor CL, Steinmetz FP, Cronin MT. The identification of nuclear receptors associated with hepatic steatosis to develop and extend adverse outcome pathways. Crit Rev Toxicol 2016;46:138–152.

48. Yanagita A, Yamada S, Yasui S, et al. Retinoic acid receptor alpha dominant negative form causes steatohepatitis and liver tumors in transgenic mice. Hepatology 2004;40:366–375.

49. Tressino SE, Tang XH, Jessurun J, Gudas LJ. Retinoic acid receptor β2 agonists restore glycemic control in diabetes and reduce steatosis. Diabetes Obes Metab 2016;18:142–151.

50. Yang D, Kros CR, Huang P, et al. Raldh1 promotes adiposity during adolescence independently of retinal signaling. PLoS One 2012;7:e0187669.