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Retinaldehyde represses adipogenesis and diet-induced obesity

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Abstract

The metabolism of vitamin A and the diverse effects of its metabolites are tightly controlled by distinct retinoid-generating enzymes, retinoid-binding proteins and retinoid-activated nuclear receptors. Retinoic acid regulates differentiation and metabolism by activating the retinoic acid receptor and retinoid X receptor (RXR), indirectly influencing RXR heterodimeric partners. Retinoic acid is formed solely from retinaldehyde (Rald), which in turn is derived from vitamin A. Rald currently has no defined biologic role outside the eye. Here we show that Rald is present in rodent fat, binds retinol-binding proteins (CRBP1, RBP4), inhibits adipogenesis and suppresses peroxisome proliferator-activated receptor- α and RXR responses. *In vivo*, mice lacking the Rald-catabolizing enzyme retinaldehyde dehydrogenase 1 (Raldh1) resisted diet-induced obesity and insulin resistance and showed increased energy dissipation. In *ob/ob* mice, administering Rald or a Raldh inhibitor reduced fat and increased insulin sensitivity. These results identify Rald as a distinct transcriptional regulator of the metabolic responses to a high-fat diet.

Although vitamin A and its metabolite retinoic acid have therapeutic applications, frequent side effects limit their use^{1–3}. In clinical trials involving β -carotene supplementation, worrisome increases in cardiovascular events and mortality have been noted, despite evidence suggesting possible beneficial vascular effects of this treatment³. These variable responses to retinoids probably derive from the fact that β -carotene and vitamin A (retinol) and their major metabolites—retinaldehyde (Rald) and retinoic acid—regulate diverse cellular responses, including development, immune function and vision^{4,5}. The tight control of retinoid biology is evident in the elaborate system that governs the absorption, formation, transportation and action of these structurally and functionally distinct retinoid metabolites. Despite this, retinoids

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and their effects remain poorly understood^{4,5}. Of note, retinoid metabolism can vary among tissues, for instance in terms of the expression and activity of specific families of enzymes that govern the transition from retinol to Rald to retinoic acid (**Supplementary Fig. 1** online and ref. ⁵). Alcohol dehydrogenases (Adh) oxidize retinol to Rald, and retinaldehyde dehydrogenases (Raldh) participate in the determination of cellular concentrations of free Rald by oxidizing Rald to retinoic acid⁵. Differences in the concentrations of specific retinoid metabolites may underlie their effects in various settings.

Retinoic acid, the most studied metabolite in the vitamin A pathway, exerts its broad range of biologic effects in large part by controlling gene expression. Retinoic acid binds to and activates members of the nuclear receptor family, including retinoic acid receptor (RAR) and retinoid X receptor (RXR) - transcription factors that link vitamin A metabolism to the transcriptional regulation of specific gene cassettes^{6–8}. RXR also controls key metabolic pathways by serving as the obligate heterodimeric partner for several members of the steroid hormone nuclear receptor family, including peroxisome proliferator-activated receptors (PPARs)⁸. Adipogenesis is a differentiation process regulated by the complex interaction of some 11 RXR heterodimeric partners (**Supplementary Fig. 1** and ref. ⁹). In 3T3-L1 adipocyte differentiation assays, retinoic acid effects vary as a function of the stage of adipogenesis and relative RAR, PPAR- γ and RXR expression^{9,10}. Early in adipogenesis, retinoic acid blocks differentiation, whereas after 48 h of differentiation, it promotes fat cell formation¹⁰. Inhibition of endogenous retinoic acid production by the Raldh inhibitor citral reduces weight *in vivo* in animal models^{11,12}. Moreover, although 9-*cis*-retinoic acid has been generally accepted as a natural ligand for RXR (refs. 7,8), its role in adipogenesis and its existence *in vivo* have been challenged by some biochemical and genetic studies⁵.

Rald is primarily considered to be a precursor for retinoic acid formation^{5,13}. Although Rald (11-*cis*-Rald) is essential for molecular signaling in vision, a role for Rald outside the eye remains essentially unknown⁴. We hypothesized that Rald itself might be present *in fat in vivo*, where it could function as a specific but previously unrecognized regulator of adipogenesis, independent of its conversion to retinoic acid.

RESULTS

Rald is present in rodent fat

Rald is generated by the action of alcohol dehydrogenase-1 (Adh1) on retinol, and its concentration is determined in large part through its subsequent catabolism by retinaldehyde dehydrogenase-1 (Raldh1, the gene for which is officially designated *Aldh1a1*) to retinoic acid. We found that both *Adh1* and *Raldh1* were differentially expressed during 3T3-L1 preadipocyte differentiation (Fig. 1a). Whereas preadipocytes expressed mainly *Adh1*, differentiated 3T3-L1 cells expressed predominantly *Raldh1*, suggesting specific and temporally regulated Rald production and catabolism in fat. These enzymes were also expressed in white fat from both lean (C56/BL6) and obese (*ob/ob*) mice (Fig. 1a). *Adh1* expression was significantly higher in lean mice than in *ob/ob* mice, whereas *Raldh1* expression was not significantly different between the groups (Fig. 1a, $P < 0.05$). Given the differential regulation between *Adh1* and *Raldh1* in lean versus genetically obese mice, we tested further for the presence and functional effects of Rald in fat tissue.

Rald is an unstable molecule, making its detection in tissues challenging¹⁴. To counter this, Rald reduction to Rald oximes has been used to generate a stable biochemical Rald signature for purposes of quantification^{14–16}. We used this approach to measure the presence of Rald in fat from C57/BL6 mice on either a standard (lean) or a high-fat diet. High-fat fed mice doubled their body weight as compared with those on regular chow (Fig. 1b). We dissected equal amounts of fat tissue from lean and obese mice, immediately reduced the isolated tissues

with hydroxylamine, and analyzed the concentrations of retinol and Rald oxime using chromatographic and spectral analysis (for comparison, we used retinol and Rald oxime standards; **Supplementary Fig. 2** online). Our analysis revealed that not only did fat contain Rald, but that retinol and Rald were reduced approximately 60% and 82%, respectively, in obese versus lean mice (Fig. 1b). Rald was also present in rabbit fat (data not shown), obviating any species-specific artifact. Mass spectrometric analysis confirmed Rald oxime structures in pooled HPLC-purified fractions from wild-type rodent fat extracts as evidenced by comparison with a Rald oxime standard (Fig. 1c). The presence of Rald oxime in fat was supported by the similar molecular weight of a protonated molecule at 300 mass-to-charge ratio (m/z), the loss of the oxime group at 242 m/z and the overall fragmentation pattern (94–208 m/z). Finally, our analysis showed that Rald concentration in white fat ranged from 100 nM to ~1 μ M, based on an analysis of 13 mice on regular chow (data not shown).

The cognate interaction between retinoids and retinol-binding proteins can influence the amounts and effects of retinoids both within and outside the cell⁴. As retinol is the known binding partner for human cellular retinol-binding protein-1 (CRBP1) and retinol-binding protein 4 (RBP4)^{17,18}, we used standard fluorescence quenching assays to compare Rald versus retinol binding to CRBP1 and RBP4. All-*trans*-Rald had a similar binding profile as all-*trans*-retinol to both CRBP1 and RBP4 (Fig. 1d). The existence of mechanisms for Rald binding and transport as well as its presence in nanomolar concentrations in fat supported further investigation into a functional role for Rald in adipogenesis at similar concentrations to those demonstrated *in vivo*.

Rald inhibits adipogenesis *in vitro*

To test Rald effects on adipogenesis *in vitro*, we performed 3T3-L1 mouse preadipocyte differentiation assays using Rald concentrations similar to those found in fat *in vivo*. Rald concentrations as low as 100 nM suppressed mRNA expression of the adipogenic target genes *Cd36*, *Adipoq* (encoding adiponectin) and *Fabp4* (also known as *aP2*); this repression occurred in a concentration-dependent manner in both the presence (Fig. 2a) and the absence (**Supplementary Fig. 3a** online) of the PPAR- γ agonist BRL49653 (BRL), a potent adipogenic stimulus.

As the effects of retinoic acid on adipogenesis vary as a function of the differentiation stage¹⁰, we compared responses to 9-*cis*-retinoic acid, all-*trans*-retinoic acid (the most abundant form of retinoic acid) and all-*trans*-Rald in both the early and late phases of adipocyte differentiation. The expression and release of the adipocytokine adiponectin (encoded by *Adipoq*) is an indicator of the extent of adipogenesis^{19,20}. Whereas 9-*cis*-retinoic acid, all-*trans*-Rald and to a lesser extent all-*trans*-retinoic acid (~15% inhibition, $P < 0.05$) all inhibited *Adipoq* expression when added early to differentiating 3T3-L1 preadipocytes (day 0, Fig. 2b), both isomers of retinoic acid had no inhibitory effect when added later (day 2, Fig. 2b) as previously reported¹⁰. In contrast to retinoic acid, Rald also inhibited *Adipoq* expression when added during later stages of differentiation, even at nanomolar concentrations (Fig. 2a,b). In these same experiments, Rald stimulation either early or late in adipocyte differentiation also decreased adiponectin secretion, in a concentration-dependent manner (Fig. 2c). Indeed, nanomolar concentrations of Rald mitigated the sixfold increase in adiponectin induced by BRL stimulation (Fig. 2c). Rald also decreased lipid accumulation during 3T3-L1 preadipocyte differentiation in both the absence and the presence of BRL (Fig. 2d).

Rald regulates distinct nuclear receptor responses

Given the suppression of PPAR- γ agonist-stimulated adipogenesis and adiponectin release by Rald, we tested whether Rald regulates RAR and RXR activity, and, if so, whether it does so in a manner distinct from that of other retinoids. We performed ligand-binding domain (LBD)-

GAL4 transfection assays in 3T3-NIH fibroblasts, in the presence and absence of known specific nuclear receptor agonists and Rald. As previously reported²¹, all-*trans*-Rald weakly activated the RAR- α LBD, but did not alter activation of RAR- α LBD by its known ligand, 9-*cis* retinoic acid (Fig. 3a). Rald alone had no effect on RXR- α LBD activation (Fig. 3a), but, in contrast to its effect on RAR- α LBD responses, it significantly inhibited RXR- α LBD activation by 9-*cis*-retinoic acid (Fig. 3a).

Given these effects of Rald on RXR-LBD activation, we questioned whether Rald could inhibit the activation of a transfected canonical PPAR response element (PPRE) luciferase construct after transfection of RXR or PPAR- γ and agonist stimulation (9-*cis* retinoic acid or BRL, respectively). Rald significantly inhibited PPRE activation by each agonist, with the most potent effects seen after PPAR- γ and RXR co-transfection and PPAR- γ agonist stimulation (60% inhibition, Fig. 3b). Given these results, we next investigated the direct interaction between PPAR- γ and all three major Rald isomers (9-*cis*-, 13-*cis*- and all-*trans*-Rald), using cell-free radioligand displacement assays²². All three isomers displaced high affinity PPAR- γ agonists ($K_d = 5.9 \pm 0.7$, 9.7 ± 1.2 , and 11.9 ± 1.9 μ M, respectively; mean $\pm$ s.d.), consistent with direct but weak binding of these molecules to the PPAR- γ -LBD (Fig. 3b). We observed similar effects in cell-based PPAR- γ -LBD assays in the presence of BRL (data not shown). Given Rald's distinct effects on adipogenesis, and known discrete roles for RXR and PPAR- γ in this process, we evaluated Rald effects in an RXR loss-of-function model, repeating standard 3T3-L1 adipogenesis assays in the presence of Rald, but after decreasing RXR- α and RXR- β using short interfering RNA (siRNA) to the RNA encoding each RXR isotype. These RXR isoforms are expressed early in adipogenesis (48 h), helping to initiate subsequent adipocyte differentiation, as evident *in vitro*²³ and *in vivo*²⁴. We measured triglyceride accumulation and adiponectin secretion as specific, distinct indicators of adipogenesis^{20,25}. After siRNA exposure, total RXR was undetectable by western blotting (Fig. 3c). As expected, 3T3-L1 adipocytes treated with RXR- α siRNA and RXR- β siRNA showed decreased triglyceride accumulation, which Rald decreased further, independent of RXR expression. In contrast, whereas RXR- α siRNA and RXR- β siRNA significantly decreased adiponectin secretion (~92% less), Rald had no further effect (Fig. 3d), consistent with an RXR-dependent Rald effect on adiponectin release.

Rald metabolism controls adipocyte biology

Endogenous concentrations of Rald are dictated by enzymes controlling its production (Adh1) and catabolism (Raldh1)^{5,14}. *Raldh1*-deficient mice (*Raldh1*^{-/-}) have been well characterized as a model for Rald overproduction¹⁴. These mice have impaired Rald oxidation, as evident in their markedly decreased retinoic acid and increased Rald concentrations while on a vitamin A-containing diet¹⁴. *Raldh1*^{-/-} mice fed a standard chow diet with 4 IU vitamin A per g had twice the plasma Rald of those in age- and sex-matched wild-type controls (8.6 ± 2.6 and 3.8 ± 2.6 nM, respectively). To evaluate whether *Raldh1* deficiency also affects fat cell differentiation, we performed adipogenesis assays in primary embryonic fibroblasts isolated from *Raldh1*^{-/-} and wild-type mice. Adipogenesis was markedly decreased in *Raldh1*^{-/-} as compared with wild-type cells, as evidenced by oil-redO staining for lipid accumulation (Fig. 4a). Adiponectin secretion was 52% less in *Raldh1*^{-/-} versus wild-type cells, a difference that was even more pronounced after treatment with BRL at all concentrations tested (63% less in *Raldh1*^{-/-} versus wild-type cells with 300 nM BRL, Fig. 4b). With higher BRL concentrations (10 μ M), these effects of Rald on adiponectin suppression and lipid accumulation approached wild-type levels (Supplementary Fig. 3b).

Mass spectroscopy analyses demonstrated Rald in white fat from wild-type and *Raldh1*^{-/-} mice (242.9 *m/z*; *n* = 12 per genotype; Fig. 4c). This spectral pattern was identical to that seen with a Rald oxime standard (Supplementary Fig. 4a online). To study Rald effects on adipogenesis

in vivo, we placed wild-type and *Raldh1*^{-/-} mice on a high-fat diet (45% fat, standard vitamin A 4 IU/g), measuring their weight weekly until tissue analysis at after 6 months of experimental diets (age 8 months). White fat of *Raldh1*^{-/-} mice had significantly higher retinol (152%) and Rald (206%) as compared with that from wild-type mice (Fig. 4d). Adipocytes from *Raldh1*^{-/-} fat were half the size of those from wild-type fat (Fig. 4e,f). In these same fat samples, adipocyte size correlated inversely with Rald concentrations (Fig. 4g).

Raldh1 regulates lipid and glucose metabolism

We questioned if *Raldh1* deficiency would have systemic metabolic consequences. Indeed, after high-fat feeding (6 months), *Raldh1*^{-/-} mice gained significantly less weight (93%) than the wild-type mice (weight gain in wild-type = 26.6 ± 1.9 g; in *Raldh1*^{-/-} = 13.7 ± 3.6 g; Fig. 5a). Weight differences were evident beginning at 1 month of high fat feeding (data not shown). Of note, *Raldh1*^{-/-} females weighed significantly less than males (relative to wild-type mice of the corresponding sex, 57% versus 41% less, $P < 0.001$). Given sex differences in metabolic parameters related to fat, we performed additional metabolic analyses in wild-type ($n = 5$) and *Raldh1*^{-/-} females ($n = 4$). DEXA scanning revealed decreased whole-body fat accumulation in *Raldh1*^{-/-} mice (Supplementary Fig. 4b). The decreased white fat accumulation in *Raldh1*^{-/-} versus wild-type mice was evident in both subcutaneous and visceral fat pads (76% and 74% less, respectively; Fig. 5b). *Raldh1*^{-/-} mice also had significantly lower plasma free fatty acids than wild-type mice (0.21 ± 0.1 mmol/l versus 0.53 ± 0.3 mmol/l, respectively; $P < 0.04$, Wilcoxon rank test). Livers from *Raldh1*^{-/-} mice also had decreased lipid accumulation compared with wild-type mice, as evident from H&E staining (Fig. 5c) and total liver weight (Supplementary Fig. 4c).

The differences in fat accumulation in *Raldh1*^{-/-} versus wild-type mice occurred despite similar food and water intake in both groups (Fig. 5d), indicating a shift in total energy balance in *Raldh1* deficiency. Indeed, *Raldh1*^{-/-} mice had a significantly higher metabolic rate (Fig. 5e), respiratory quotient (Fig. 5f), and body temperature (Fig. 5g) compared with wild-type controls. Consistent with the role of thermogenesis in determining body weight²⁶, amounts of uncoupling protein-1 (UCP-1) in brown fat were significantly higher in *Raldh1*^{-/-} mice than wild-type mice (Fig. 5g).

Fat tissue regulates whole body insulin sensitivity through various mechanisms, including adipokine release²⁷⁻³⁰. As such, we measured changes in adiponectin, leptin and RBP4 in *Raldh1*^{-/-} mice. Total adiponectin, leptin and RBP4 were significantly decreased in the plasma of *Raldh1*^{-/-} mice as compared with wild-type mice (Fig. 6a). On high fat diet, *Raldh1*^{-/-} mice were protected from the increased insulin resistance evident in wild-type mice as seen in glucose and insulin tolerance testing (Fig. 6b). Insulin concentrations did not differ between genotypes (Fig. 6c). To further consider if Rald effects on fat accumulation and insulin resistance were due to Rald itself and not to subsequent generation of retinoic acid or other vitamin A metabolites, we used *ob/ob* mice that experience progressive weight gain on regular chow^{28,31} to compare responses to Rald, the Rald parent compound vitamin A, all-*trans*-retinoic acid and citral, a known inhibitor of Raldh enzymes¹¹. After 3 weeks, we quantified subcutaneous fat mass by magnetic resonance imaging (MRI). The extent of visceral fat accumulation in these mice precluded its accurate quantitative measurement. Mice receiving Rald or citral had significantly less subcutaneous fat relative to total body fat ($15.5 \pm 0.6\%$ and $14.8 \pm 0.6\%$, respectively), than those receiving vehicle, vitamin A or all-*trans*-retinoic acid ($18.8 \pm 1.4\%$, $17.3 \pm 1.3\%$ and $19.1 \pm 1.6\%$) (all $P < 0.05$; Fig. 6d,e). Rald administration also repressed adipogenesis in preadipocytes isolated from human visceral fat depots (data not shown). RBP4 amounts also varied in *ob/ob* mice exposed to these different retinoids. Whereas RBP4 amounts did not vary in response to retinoic acid or vehicle, they doubled in response to vitamin A (Fig. 6f). In contrast, both Rald and citral significantly suppressed circulating

RBP4 (versus vehicle), recapitulating the RBP4 pattern seen in *Raldh1*^{-/-} mice (Fig. 6a). Consistent with the changes seen in adiposity and RBP4, both Rald and citral administration improved glucose tolerance in *ob/ob* mice (Fig. 6g).

DISCUSSION

We demonstrate here that Rald plays a distinct metabolic role in adipocyte differentiation *in vitro*, and in diet-induced insulin resistance and obesity *in vivo*. Rald is present in fat at nanomolar concentrations (~1 nmol/g) and can interact with CRBP1 and RBP4, binding proteins involved in intracellular and circulating retinoid transport. Rald suppresses adipogenic gene expression, adipocyte lipid accumulation, RXR- α and PPAR- γ responses, all at concentrations (<1 μ M) similar to those in rodent fat *in vivo*. Despite the widely held assumption that Rald outside of the eye serves mainly as a precursor for retinoic acid⁴, the differences in the effects of Rald versus those of retinoic acid *in vitro* and *in vivo* argue for Rald itself as a distinct mediator in fat. When Rald concentrations were increased *in vivo*, either in the absence of *Raldh1* or after direct Rald administration, fat formation was decreased. Inhibition of Rald catabolism (citral treatment) *in vivo* had similar effects although again distinct from retinoic acid or vitamin A administration. Together this data identifies Rald as a biologically active metabolite present in fat that may regulate adipogenesis through its action on RXR- α and PPAR- γ responses and in a manner opposite of retinoic acid effects.

Rald's unique effects on adipokine expression, adipogenesis and body weight focus attention on the parameters regulating the relative cellular concentrations of Rald and retinoic acid. The balance between Rald and retinoic acid is determined by factors such as the concentration of vitamin A in the body, the expression and activity of enzymes that metabolize Rald and retinoic acid, other retinol-modifying enzymes (such as esterases and hydrolases), as well as retinol-binding proteins and the redox status in cells^{4,5,32}. Each of these factors may have functional consequences, as seen with the recently reported association between RBP4 and diabetes in mice and humans^{29,33}. In terms of the enzymes metabolizing Rald, we have demonstrated the genetic absence of *Raldh1* results in a metabolic phenotype involving marked alterations in fat accumulation, glucose homeostasis and adipokine production after high-fat feeding. These metabolic changes are probably due at least in part to the increased concentrations of Rald in fat, as demonstrated here, especially as direct administration of Rald reproduced the metabolic profile evident in *Raldh1*^{-/-} mice. Notably, direct administration of retinoic acid and Rald had different effects *in vitro* and *in vivo*, further supporting a role for Rald that is distinct from its function as a retinoic acid precursor.

Rald seems to exert its effects through both RXR-dependent and RXR-independent mechanisms. Rald repressed adiponectin production, but not after RXR expression had been reduced. In contrast, Rald-mediated repression of triglyceride accumulation persisted regardless of RXR expression. Rald inhibited LBD activation and cellular responses to both RXR and PPAR- γ agonists, with effects on early and late adipogenesis that are consistent with the reported temporal expression of PPAR- γ and RXR^{9,10}. Although Rald binding to the RXR- and PPAR- γ -LBDs was weak, it potently suppressed adipogenesis *in vitro* and *in vivo*. It has been reported that selective synthetic PPAR and RXR modulators also show a similar divergence between the potency of receptor binding and adipogenic effects^{34,35}. The ability of higher BRL concentrations to overcome the effects of Rald suggests that Rald responses are mediated at least part through PPAR- γ . Various mechanisms may underlie how certain molecules influence nuclear receptor responses independent of receptor-binding potency, including conformational changes in the receptor, salt bridge formation, accessory molecule recruitment and release, and post-translation protein modification³⁶. Rald's metabolic effects make further studies on Rald derivatives, the factors determining Rald concentrations and Rald transport all of considerable interest. More broadly, these data suggest a regulatory pathway

in which a given molecule (vitamin A) can yield both a nuclear receptor agonist (retinoic acid) and a molecule (Rald) capable of inhibiting specific nuclear receptor responses (PPAR- γ , RXR).

These studies provide a link between vitamin A metabolism and responses to a high-fat diet, including pathologic complications such as obesity and insulin resistance. The metabolic changes evident with increased Rald have several potential clinical implications. The protection against diet-mediated obesity and insulin resistance identified in *Raldh1*^{-/-} mice suggests this inducible enzyme of vitamin A metabolism as a potential candidate for therapeutic targeting and/or a source of body weight variation. Similarly, Rald generation might influence therapeutic responses to vitamin A, retinoic acid or other retinoid-based treatments as well as the effects of PPAR- γ agonists. The presence of Rald in fat and its association with RBP4 suggest that Rald might contribute to the relationship between RBP4 and insulin sensitivity^{29,33}.

In the models studied here, Rald concentrations in fat *in vivo* correlated tightly with changes in fat accumulation and metabolic responses, providing one probable explanation for the metabolic phenotype of *Raldh1*-deficient mice. Rald has effects on both visceral and subcutaneous fat, as seen in *Raldh1*^{-/-} mice. In the absence of Raldh1, energy balance appears shifted toward increased energy dissipation, as suggested by the increased body temperature, metabolic rate⁴² and UCP-1 expression manifest in these mice. Interestingly, in some animal models, increased UCP-1 has not been associated with an increased respiratory quotient³⁷. Regardless, the changes in energy balance seen with Rald could result from its actions in various Rald-and retinoic acid-sensitive tissues, including brown fat, immune cells and the central nervous system (CNS). Recent work suggests a possible role for Raldh2 in dendritic cells⁴³, although Raldh1 appears the predominant determinant of Rald metabolism in response to vitamin A intake¹⁴. The CNS is a particularly important regulator of body weight in general and adipocyte biology specifically; for example, in determining rates of lipolysis³⁸. Our data do not specifically exclude the possibility of Rald exerting some effects via the CNS. Here we focused on demonstrating the presence of Rald and its effects on adipocyte responses. The linear relationship between Rald concentrations and adipocyte size, the lower concentrations of Rald in obese as compared with lean mice, and the impaired adipogenesis seen in *Raldh1*^{-/-} preadipocytes all suggest that Rald effects in adipose tissue probably contribute to the protection against diet-induced obesity and insulin resistance evident in Rald1-deficient mice. Certainly Rald's actions in adipose tissue, including the regulation shown here of specific adipokines, can also provide feedback to other systems, including the CNS and the immune system, with subsequent effects on adipose biology.

Taken together, these findings allow Rald to join retinoic acid as a distinct biologically active mediator of energy balance and insulin sensitivity. The integration of these critical metabolic pathways by a natural molecule such as Rald may provide new opportunities for understanding the complex interaction between vitamin A, its metabolites and the transcriptional regulation of metabolism.

METHODS

Reagents

We obtained reagents and media from Sigma-Aldrich and BioWhittaker unless otherwise indicated. All media contained amphotericin B, penicillin and streptomycin. BRL49653 (rosiglitazone) was a gift from GlaxoSmithKline. Unless otherwise indicated, retinoids used were all-*trans* isomers. All diets were from Research Diet, Inc.

Animal studies

C57/BL6 mice (weight: 19.6 ± 2 g) versus *ob/ob* mice (41.7 ± 2 g), all 12-week-old females on regular chow, were used for gene expression studies and retinoid analysis ($n = 3$ per genotype). For retinoid MS analysis, we studied pooled subcutaneous and visceral fat from male mice (129S3/SvImJ, 6 months of age, $n = 4$) and a New Zealand White rabbit. For metabolic studies, we compared age- (8 weeks old) and sex-matched *Raldh1*^{-/-14} and wild-type mice (five per sex and genotype). The D12451 high-fat diet contained 45% of calories derived from fat and standard vitamin A (4 IU/g). Water was *ad libitum*.

For MRI fat distribution studies, we administered retinoids (all-*trans*-Rald, vitamin A or all-*trans*-retinoic acid, all 500 nM) or citral (10 μ M, equal to 240 nmol/g), all in ethanol:PBS (2:200 μ l), by daily intraperitoneal injections for 3 weeks. The Standing Committee on Animals at Harvard Medical School approved all protocols.

Cell culture

We cultured and differentiated mouse 3T3-L1 preadipocytes and primary fibroblasts isolated from 16-d-old embryos using standard adipogenesis and isolation protocols²⁵. Cells were differentiated (7 d) with or without BRL (1 μ M). Rald was added at the indicated concentrations in either early (5 h) or late (48 h) stages of adipogenesis, as timed to initiation of differentiation.

Transient transfections

For transient transfection of NIH 3T3 cells (2.3×10^4 cells, 24-well plates), we used pCMX- β -galactosidase and LBD-yeast Gal 4-luciferase constructs and Fugene (Roche) as before³⁹. For siRNA transfections, we administered scrambled control (sequences C, D) or specific RXR- α and RXR- β siRNA sequences (Santa Cruz) to 3T3-L1 cells (90% confluence, antibiotic-free DMEM, 10% calf serum, 24-well plates) using Lipofectamine 2000 (8 h) and OptiMEM medium (Invitrogen). We supplemented the medium with 10% FBS 5 h after transfection. We evaluated RXR amounts by Western blotting 48 h after transfection.

Human recombinant RBP and fluorescence binding assays

Human RBP4 and mouse CRBP1 subcloned in pET expression vectors were expressed and purified as described previously⁴⁰, but without retinol. We dialyzed refolded protein against binding assay buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0) overnight, and quantified proteins at 280 nm (RBP4 40,400 M⁻¹cm⁻¹, CRBP1 26,720 M⁻¹cm⁻¹)^{17, 41}. We measured tryptophan fluorescence (Aminco, Spectronic Unicam) by excitation (285 nm and emission (335 nm), both in 0.05 M sodium phosphate buffer¹⁸ which indicated retinoid binding to RBP4 or CRBP1 (1 μ M).

Scintillation proximity assay

We studied Rald displacement of a ³H₂-labeled known synthetic PPAR- γ agonists (nTZD3, $K_d = 2.5$ nM) from human full-length PPAR- γ_2 as before²².

RNA analysis

We determined mouse *Adh1*, *Raldh1* and *Actb* (β -actin) mRNA (RNeasy, Qiagen) in white fat using semi-quantitative RT-PCR and the following primers:

Adh1: 5'-ATGAGCACTGCGGGAAAAGT-3', 5'-ACTTTATTGGCCGTGT CTCTAA-3';
Raldh1: 5'-TGGGTAACTGCTATATCATGTTG-3', 5'-GGGTG
 CCTTTATTAAGCTTTGCG-3'. Results were obtained within the linear range for each gene (31 and 29 cycles, respectively) and normalized to *Actb* (β -actin) expression. We performed northern blotting with HyBond (Amersham) as before³⁹.

Protein and intracellular lipid analysis

We determined triglyceride content in lysed cells (RIPA buffer, complete protease inhibitor cocktail, Roche) using enzymatic colorimetric assay (Wako) and measured adiponectin using ELISA (R&D Systems). For western blotting, we performed a reducing gel separation (10% acrylamide) on cell lysates, plasma and tissue lysates, before hybridization with antibodies to RXR (Santa Cruz), to mouse RBP4 (Alpco) or to UCP-1 (Chemicon).

Liquid chromatography (LC), mass spectrometry (MS)

We generated a Rald oxime standard using reduction of retinaldehyde (100 μ M, 22 $^{\circ}$ C, 2 h under argon protected from light) with hydroxylamine (1 M) and EDTA (25 μ M) in PBS as before^{14,15}. White fat (~200 μ g) was dissected from relevant animals, immediately reduced with hydroxylamine, purified by solid-phase extraction (Bakerbond amino column) and analyzed for retinol and Rald oxime content using high performance LC (HPLC) analysis (see also legend to **Supplementary Fig. 2**). Pooled Rald oxime fractions then underwent structural analysis using LC and tandem MS (LC-MS-MS) using atmospheric pressure chemical ionization in positive mode (Bruker Daltonics, Esquire LC) to detect Rald oxime ion molecular ion peak $[M+H]^+$ at 300 m/z . The ionization parameters included capillary voltage, 3,000 V; APCI temperature, 350 $^{\circ}$ C; source temperature, 300 $^{\circ}$ C; and scanning ions in the 82 to 306 m/z range.

Histology

We embedded fat and liver tissue in paraffin before hematoxylin and eosin (H&E) staining followed by quantification of adipocyte size or liver lipid accumulation (ImageJ software).

Magnetic resonance imaging (MRI)

We obtained MRI scans (1-mm slices) using a Bruker Avance 500 wide-bore spectrometer (11.7 T; 500 MHz for proton) fitted with a gradient amplifier and a 30 mm ‘birdcage’ transmitter/ receiver coil before processing the data (Paravision). The spin-echo parameters for T1-weighted images were as follows: TE = 15 ms, TR = 300 ms, matrix = 256, FOV = 30 mm; for RARE images, TE = 51 ms, TR = 2,500 ms, matrix = 256, FOV = 30 mm. Abdominal fat measurements used axial slices ($n = 8$) at the level of the left renal pelvis.

Dual-energy X-ray absorptiometry (DEXA)

We used the GE Lunar Corporation PIXImus2 DEXA Scanner, normalizing the data to a quality control plot (Charles River Laboratories).

Metabolic parameters

After mouse acclimation to a powdered high-fat diet (4 d), we measured food and water intake, oxygen consumption and carbon dioxide production in metabolic cages (Ancare, Charles River Laboratories). The calculated metabolic rate (Weir equation) is expressed per g body weight⁴². We performed insulin (ITT) and glucose tolerance tests (GTT) after fasting (16 h), using intraperitoneal insulin injections (ITT, 0.1 U/ml, 0.005 ml/g body weight) or a single 25% dextrose injection (GTT, 0.004 ml/g body weight), and a glucometer for measurements (Accu-Chek Advantage, Roche).

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Figure 1.

Rald and Rald-metabolizing enzymes in rodent fat. **(a)** Northern blot analysis (left) of *Adh1* and *Raldh1* expression in 3T3-L1 preadipocytes before and after differentiation; fatty acid binding protein 4 gene (*Fabp4*), differentiation control. Representative (middle) and quantified (right) RT-PCR analysis of *Adh1* and *Raldh1* expression in white fat from female lean *C57/BL6* (weight: 19.6 ± 2 g) versus *ob/ob* mice (41.7 ± 2 g) normalized to *Actb* (β -actin) expression. * $P < 0.05$ (Wilcoxon rank test). **(b)** Weight (left), retinol (middle) and Rald concentrations (HPLC) in white fat from lean (standard chow) and high fat (HF)-fed *C57/BL6* female mice. * $P < 0.001$, ** $P < 0.003$ (Mann-Whitney *U*-test). **(c)** LC-MS-MS analysis of Rald oxime in a standard (left, STD, 500 nM) and in pooled rodent fat (right, $n = 12$; representative example **Supplementary Fig. 2**). BG, background. **(d)** Fluorescence binding assays between the retinol-binding proteins CRBP1 (left) and RBP4 (right) and all-*trans*-Rald, as compared with all-*trans*-retinol. Error bars in **a** and **b**, s.d.

Figure 2. Rald and retinoic acid differentially regulate PPAR- γ -induced adipogenic genes. **(a)** Northern blotting for *Cd36*, *Adipoq*, *Fabp4* and *Gapdh* performed in 3T3-L1 cells differentiated with BRL in the presence of Rald. **(b)** *Adipoq* mRNA expression after Rald, 9-*cis*-retinoic acid or all-*trans*-retinoic acid (all 500 nM) stimulation during either early or late stage of adipogenesis ($n = 3$ per condition). **(c)** As in **b** but in the absence (left) or presence (right) of BRL and varying Rald concentrations before measurement of adiponectin in media (ELISA). * $P < 0.05$ (for both early and late stages; adiponectin concentrations after Rald stimulation versus initial levels; Mann-Whitney U -test). **(d)** Oil red O staining of lipid accumulation in cells used in **c**. Scale bar, 300 μ m.

Figure 3.

Rald regulates nuclear receptor responses *in vitro*. (a) RAR-α(left) and RXR-α(right) LBD-GAL4 assays performed in NIH-3T3 cells in the absence (Veh) or presence of 9-*cis* retinoic acid (9-*cis*-RA) (300 nM) and increasing Rald concentrations. $n = 3$ measurements. $*P < 0.05$ (RXR-αLBD activation by 9-*cis*-RA in the absence versus presence of Rald). (b) Left, activation of a transfected PPAR response element (PPRE) luciferase construct transfected with constructs for RXR-α, PPAR-γ or both, followed by BRL or 9-*cis*-RA (1 μM) stimulation in the presence of Rald (left). Data normalized to receptor activation induced by the respective ligand (BRL or 9-*cis* retinoic acid alone, set at 100%). $*P < 0.05$ (Wilcoxon rank test). Right, binding of Rald isomers to full-length PPAR-γ protein in radioligand displacement assay.

Results expressed as relative displacement of $^3\text{H}_2$ -labeled PPAR- γ agonist nTZD3 by Rald isomers. **(c)** Total RXR protein abundance 48 h after RXR- α siRNA and RXR- β siRNA (siRXR- α , siRXR- β) or control siRNA transfection in 3T3-L1 cells (western blotting). **(d)** Trigly-ceride content (left) and secreted adiponectin protein abundance (right) in cells as in **c**. RXR siRNA treatment decreased adiponectin concentrations in both the absence (data not shown) and presence of BRL but Rald had no further effect in these cells. NS, nonsignificant. For all studies, error bars represent s.d., $n = 3$ per condition, Wilcoxon rank test. * $P < 0.001$, ** $P < 0.01$.

Figure 4.

Raldh1 deficiency is associated with suppressed adipogenesis *in vitro* and increased Rald and reduced adipocyte size *in vivo*. **(a)** Lipid accumulation in differentiated primary fibroblasts from *Raldh1*^{-/-} as compared with wild-type (WT) embryos. Scale bar, 200 μ m. **(b)** Left, adiponectin concentrations in nondifferentiated (Non-D) and differentiated (D) cells as in **a**. Right, BRL effects on adiponectin secretion in similar *Raldh1*^{-/-} versus WT cells. $n = 3$ per condition and genotype. $*P < 0.001$ (Mann-Whitney *U*-test; error bars here and below, s.d.). **(c)** LC-MS-MS of pooled fat from wild-type mice (left) contained less Rald oxime (242 m/z fragment, arrow) than fat from *Raldh1*^{-/-} mice (right). $n = 4$ mice per genotype. **(d)** Retinol (left) and Rald oxime (right) concentrations by HPLC in high fat-fed female *Raldh1*^{-/-} versus wild-type mice ($n = 4$ mice per genotype) $*P < 0.03$, $**P < 0.005$ (Mann-Whitney *U*-test). **(e)** Adipocyte size in white fat of mice studied in **d**. $n = 3$ mice per genotype. $*P < 0.001$ (Mann-Whitney *U*-test). **(f)** Representative H&E sections of fat from mice studied in **d**. Scale bar, 200 μ m. **(g)** Correlation between Rald concentrations and adipocyte size in mice studied in **d** ($P < 0.03$, Spearman correlation test).

Figure 5.

Raldh1^{-/-} mice resist high-fat diet-induced obesity. **(a)** Weight of wild-type and *Raldh1*^{-/-} mice after high-fat diet (180 d). *P < 0.001 (Wilcoxon rank test). Representative X-ray images of each genotype. Scale bar, 2 cm. **(b)** Weight of subcutaneous and visceral fat pads (left) from wild-type and *Raldh1*^{-/-} female mice (*n* = 5, 4 respectively; *P < 0.001, Mann-Whitney *U*-test). Representative isolated visceral (epididymal) and subcutaneous (proximal and scapular) fat pads (right). **(c)** Liver lipid area density from the same mice as in **b** *P < 0.03, *n* = 3 females per genotype; a.u., arbitrary units. Representative H&E-stained liver sections (right). Scale bar, 200 μm. **(d–g)** Food and water consumption, metabolic rates, respiratory quotients, rectal temperatures and UCP-1 protein amounts in mice as in **b**. *P < 0.05, **P < 0.04 (Wilcoxon rank test). Error bars, s.d. except in **f** (s.e.m.); a.u., arbitrary units.

Figure 6.

Raldh1 deficiency and direct Rald administration protect against insulin resistance. Mice analyzed in **a–c** are the same as in Figure 5. **(a)** Plasma protein concentrations of adiponectin and leptin and amounts RBP4. * $P < 0.048$, ** $P < 0.002$, *** $P < 0.04$ (Wilcoxon rank test). Top, representative RBP4 western blots in females and males. **(b)** Glucose (left) and insulin (right) tolerance tests. **(c)** Insulin concentrations. * $P < 0.05$ or NS, Mann-Whitney U -test. **(d,e)** MRIs of *ob/ob* mice receiving vehicle, vitamin A (Vit A), all-*trans*-retinoic acid (RA), all-*trans*-Rald (Rald) or citral injections daily for 4 weeks. Arrows indicate the largest subcutaneous fat pad, quantified in **e** (four scans per mouse). Similar cross-sectional location indicated by kidney (K) and vertebra (V) sizes. * $P < 0.001$, ** $P < 0.002$, *** $P < 0.003$ (Wilcoxon rank test). **(f)** Western analysis of plasma RBP4 in mice as in **d** (one representative blot shown). * $P < 0.05$ (treatment with vehicle versus Rald or citral), # $P < 0.05$ (treatment with vitamin A versus Rald or citral) (Mann-Whitney U -test). **(g)** Glucose tolerance in mice as in **d**. * $P < 0.05$ (treatment with vitamin A versus citral), # $P < 0.05$ (treatment with vehicle versus citral) (Wilcoxon rank test). Error bars, s.d. except in **b** (s.e.m.).