Insulin Regulates Retinol Dehydrogenase Expression and All-trans-retinoic Acid Biosynthesis through FoxO1*

Kristin M. Obrochta, Charles R. Krois, Benito Campos, and Joseph L. Napoli

From the Department of Nutritional Sciences and Toxicology, Graduate Program in Metabolic Biology, University of California, Berkeley, California 94720

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**Background:** Retinoic acid regulates energy balance and induces phosphoenolpyruvate carboxykinase gene expression.

**Results:** Refeeding, glucose, and insulin decrease retinoic acid in vivo. Insulin suppresses retinol dehydrogenase gene expression through suppressing FoxO1.

**Conclusion:** Insulin inhibits retinoic acid biosynthesis through inhibition of FoxO1-induced Rdh10 gene expression.

**Significance:** Insulin and retinoic acid exert counter balancing effects in regulating energy status.

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All-trans-retinoic acid (atRA), an autacoid derived from retinol (vitamin A), regulates energy balance and reduces adiposity. We show that energy status regulates atRA biosynthesis at the rate-limiting step, catalyzed by retinol dehydrogenases (RDH). Six h after re-feeding, Rdh1 expression decreased 80–90% in liver and brown adipose tissue and Rdh10 expression was decreased 45–63% in liver, pancreas, and kidney, all relative to mice fasted 16 h. atRA in the liver was decreased 44% 3 h after reduced Rdh expression. Oral gavage with glucose or injection with insulin decreased Rdh1 and Rdh10 mRNA 50% or greater in mouse liver. Removing serum from the medium of the human hepatoma cell line HepG2 increased Rdh10 and Rdh16 (human Rdh1 ortholog) mRNA expression 2–3-fold by 4 h, by increasing transcription and stabilizing mRNA. Insulin decreased Rdh10 and Rdh16 mRNA in HepG2 cells incubated in serum-free medium by inhibiting transcription and destabilizing mRNA. Insulin action required PI3K and Akt, which suppress FoxO1. Serum removal increased atRA biosynthesis 4-fold from retinol in HepG2 cells, whereas dominant-negative FoxO1 prevented the increase. Thus, energy status via insulin and FoxO1 regulate Rdh expression and atRA biosynthesis. These results reveal mechanisms for regulating atRA biosynthesis and the opposing effects of atRA and insulin on gluconeogenesis, and also suggest an interaction between atRA and insulin signaling related diseases, such as type II diabetes and cancer.

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A wide scope of biological processes, including embryonic development, cell differentiation and proliferation, immune function, neurogenesis, and energy metabolism, depend on the vitamin A (retinol) metabolite all-trans-retinoic acid (atRA). (1–5). atRA controls energy balance by inhibiting differentiation of pre-adipocytes into mature white adipose, regulating the function of white adipose cells, and by regulating whole body lipid and carbohydrate metabolism (6–9). Dosing atRA to mice fed a chow diet, which contains ample vitamin A, affords resistance to diet-induced obesity (10, 11). Impairing atRA homeostasis causes abnormalities in intermediary metabolism. Mice with ablative cellular retinol-binding protein 1 (encoded by Rbp1), which regulates retinol homeostasis, experience glucose intolerance from enhanced gluconeogenesis, resulting from hyperglucagonemia, and also undergo increased adipocyte differentiation (12, 13). atRA functions through nuclear hormone receptors RARα, RARβ, and -γ and peroxisome proliferator-activated receptor δ, which affect transcription and translation (14, 15). Genes regulated by atRA through nuclear receptors include: Pck1, which expresses phosphoenolpyruvate carboxykinase, the enzyme that catalyzes the committed step in gluconeogenesis (16); Ucp1, which expresses uncoupling protein 1, a mediator of adaptive thermogenesis (17); and inhibitors of adipogenesis, including Pref1, Klf2, and Sox9 (18). Despite the impact of atRA on energy balance, little is known about whether energy balance might regulate atRA homeostasis.

Two successive dehydrogenations produce atRA from retinol (19). During limited or normal vitamin A nutriture, retinol dehydrogenases (RDH) of the short-chain dehydrogenase/reductase gene family catalyze the first and rate-limiting reaction to produce all-trans-retinal. Retinal dehydrogenases, of the aldehyde dehydrogenase gene family, catalyze the second reaction to produce atRA. Multiple isoforms of retinoid-metabolizing enzymes occur in both families, often in the same cell types, but these enzymes differ in subcellular expression loci, and can show differential cell expression patterns. RDH1 and RDH10 are the most characterized RDH in the path of atRA biosynthesis: both are expressed early in embryogenesis and throughout life in multiple tissues, and both contribute to atRA biosynthesis from retinol in intact cells in the presence of retinal dehydrogenases (20–22). Dehydrogenase reductase 3 (DHRS3) functions as a retinal reductase, which interacts with RDH10 to control retinoid homeostasis (23). Rdh1-null mice are born in Mendelian frequency, but experience increased weight and adiposity, which is prevented by feeding copious vitamin A (24). In
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contrast, most Rdh10-null mice die by embryonic day 12.5, but can be rescued by maternal administration of retinoids (25). Notably, like Rdh1-null mice, Rdh10-null mice do not exhibit total atRA deficiency, suggesting complementary actions of the two and/or occurrence of additional RDH.

Liver exerts a central function in maintaining whole body metabolic homeostasis (26). During feeding, liver catalyzes increased energy storage by generating glycogen and triacylglycerol. During fasting, liver generates glucose and ketone bodies from gluconeogenesis, glycogenolysis, and fatty acid oxidation. Glucose, insulin, and glucagon control the balance between energy storage during feeding and production of fuels during fasting. A combination of portal vein glucose, insulin, and neuronal signal(s) causes liver to transition from catabolic to anabolic metabolism (27). Insulin binding to its cell surface receptors activates two canonical pathways in hepatocytes: the mitogen-activated protein kinase and phosphoinositide 3-kinase 3-kinase (PI3K). The mitogen-activated protein kinase pathway includes extracellular-signal regulated kinase (ERK) signaling and regulates transcription involved in differentiation, growth, and survival (28). The PI3K pathway activates Akt (PKB) to mediate metabolic transitions, including promotion of glycogen and lipid synthesis, and suppression of gluconeogenesis. Effectors downstream of PI3K/Akt include: glycogen synthase kinase-3 (GSK-3), which regulates glycogen synthesis; mammalian target of rapamycin complex 1 (mTORC1), which regulates protein synthesis; BCL2-associated agonist of cell death (BAD), which regulates cell survival; and forkhead box other (FoxO), which regulates transcription of genes required for gluconeogenesis, including glucose-6-phosphatase (Glc-6-P) (29, 30).

This report compares Rdh1 and Rdh10 expression in tissues of fasted versus re-fed mice, and focuses on atRA in liver. Liver served as the focus of this study because of its central contributions to both energy and retinoid homeostasis. We found that insulin, via inactivating FoxO1, represses Rdh1 and Rdh10 expression, with an associated decrease in atRA biosynthesis. These observations are consistent with the recent analysis that FoxO1 may link gluconeogenesis to retinoid homeostasis (31). Our results provide direct evidence showing that energy status regulates atRA biosynthesis, and provides new insight into the relationship between atRA biosynthesis and its regulation of energy balance.

EXPERIMENTAL PROCEDURES

Animals—C57Bl/6j male mice, age 2–3 months, bred in-house were housed up to 5 per cage with littermates, and fed an AIN93G semi-purified diet with 4 IU of vitamin A/g (Dyets, catalog number 110700), unless noted otherwise. The exceptions were: mice in the 12-h re-fed experiment were both male and female (no significant difference in atRA values was detected by sex); mice used for fasting and insulin or exendin-4 treatments were purchased from Jackson Laboratories (catalog number 110010237), 200 nm rapamycin (Cayman number 13346), 20 μM PD98059 (CalBiochem number 513000), Adenovirus constructs dnFoxO1 (FoxO1Δ256) and lacZ were infected into 70% confluent HepG2 cells and collected for analysis after 48 h. Virus doses were tested empirically and selected based on the highest amount tolerated in HepG2 cells, according to published protocols (32).

Retinoid Quantification—Liver atRA was quantified in 6 to 9 mice per group. Tissues were collected under yellow light and frozen immediately in liquid nitrogen. On the day of analysis, tissues were weighed, thawed on ice, and hand homogenized in 0.9% saline. HepG2 cells were collected under yellow light in 1× reporter lysis buffer (Promega E3971), and stored at −80 °C. Cell protein was quantified with the Pierce BCA Protein Assay Kit (Thermo Scientific number 23227). For atRA biosynthesis in HepG2 cells, 50 nM (100 pmol) all-trans-retinol (Sigma R7632) was delivered in dimethyl sulfoxide 4 h before cell harvest. Cells were homogenized by pipetting and vortexing. Retinoids were recovered by a two-step acid and base extraction (33). All materials in contact with samples were glass or stainless steel. Internal standards were used to calculate extraction efficiency of retinoids. atRA was extracted and quantified by LC/MS/MS, with 4,4-dimethyl retinoic acid as an internal standard (34). Retinol was extracted and quantified by HPLC/UV, with 3,4-didehydroretinol as internal standard (35).

RNA isolation and qPCR—Isolation of RNA from liver and cells was by the TRIzol reagent (Invitrogen) method. Liver was homogenized using a Qiagen Tissue Lyser II set at 30 Hz for 1 min. cDNA was prepared using iScript reagent kit (Bio-Rad 170-8891). Real-time qPCR was prepared with 2× TaqMan master mix (Life Technologies 4369016), and TaqMan Gene Expression Assays: mouse ACTB, Mm00607939_s1; Rdh10, Mm00467150_m1; G6pc, Mm00839363_m1; Rhdl, Mm00650636_m1; Dhrs3, Mm00488080_m1; human ACTB Hs01060665_g1; RDH10, Hs00416907_m1; G6-c-6-P, Hs00609179_m1; Rdh16, Hs00559712_m1; Dhrs3, Hs00191073_m1 (Life Technologies), and run on an ABI 7900 thermocycler. Gene expression was ana-
lyzed by the ΔΔCt method, normalized to β-actin, and expressed as fold-change relative to expression in fasted liver or the reference condition described in cells.

**Protein Expression**—Western blots were done with the Mini PROTEAN system (Bio-Rad), 10% TGX gels (Bio-Rad number 456-1033), and semi-dry transfer (Bio-Rad Trans-blot SD) to nitrocellulose membranes (Whatman). Western blots were visualized with the Licor Odyssey system, and quantified by densitometry in reference to actin expression. Primary antibodies were actin (ProSci number 3779) and HA (12CA5) (Roche number 11583816001). Secondary antibodies were LI-COR IR Dye (680LT number 926-68020, 800CW number 926-32211).

**Immunofluorescence**—HepG2 cells were fixed in ice-cold acetone for 10 min, then stained with FoxO1 primary antibody (Santa Cruz number 11350) for 2 h at 37 °C, and incubated with Alexa Fluor 488 secondary antibody (Life Technologies number A11034) for 1 h at 37 °C. Coverslips were mounted onto glass slides with Vectashield mounting medium containing DAPI (Vector Labs number H-1200) and imaged by fluorescence microscopy (Zeiss Axiosmager).

**Statistics**—Data are presented as mean ± S.E. and analyzed using two-tailed, unpaired Student’s t tests or linear regression analysis.

**RESULTS**

**Re-feeding Decreases Rdh mRNA in Multiple Mouse Tissues and atRA in Liver Versus Fasted Levels**—To determine whether changes in energy status regulate atRA biosynthesis, we measured expression of *Rdh1* and *Rdh10* in tissues of mice fasted 16 h or re-fed *ad libitum* 6 h after a 16-h fast. In liver, *Rdh1* and *Rdh10* mRNA were decreased in re-fed animals 86 and 57%, respectfully, relative to expression in fasted animals (Fig. 1, A and B). In brown adipose tissue of re-fed mice, *Rdh1* was decreased 77%, whereas *Rdh10* expression was unchanged. In pancreata of re-fed mice, *Rdh10* mRNA was decreased 43%, and *Rdh1* expression was below the limit of detection. In epididymal white adipose tissue of re-fed mice, *Rdh10* expression was unchanged and *Rdh1* expression was below the limit of detection. In kidney of re-fed mice, *Rdh10* expression was decreased 62%. Focusing on the liver, the atRA concentration was reduced 77%, whereas *Rdh1* mRNA was decreased 43%, and *G6pc* expression similar to re-feeding. We tested the standard protocol of fasting and re-feeding, blood glucose from mice fasted 16 h and refeed for 6 h increased significantly versus mice fasted 16 h (Fig. 2D).

To determine whether the route of glucose dosing affected *Rdh10* expression, we compared the impact of oral gavage and intraperitoneal injection. Glucose injected intraperitoneally had no effect on *Rdh10* expression (Fig. 2D). Insulin, however, caused a dose-dependent decrease in liver *Rdh10* expression that reached statistical significance at 0.5 IU/kg (Fig. 2F), a dose recommended for the insulin tolerance test (36). Because nutrients trigger incretin secretion, we tested the impact of glucagon-like peptide 1. Glucagon-like peptide 1 is short lived in circulation from inactivation by dipeptidyl peptidase-4 (37). Therefore, we used the stable glucagon-like peptide 1 receptor agonist, exendin-4 (38). Treatment with exendin-4 had no impact on *Rdh10* expression (Fig. 2G). These data demonstrate that re-feeding, oral gavage with glucose, and intraperitoneal insulin each are sufficient to reduce liver *Rdh10* expression from the higher fasted levels.

**RDH10 Transcription Increases with Serum Removal and Is Attenuated by Insulin in HepG2 Cells**—We used the human hepatoma cell line, HepG2, to study mechanisms of *RDH10* regulation. The presence or absence of serum in the medium can model the impact of growth factors, including insulin (39, 40). Therefore, serum was removed from the medium, which prompted a 2–3-fold increase in *RDH10* mRNA 4 h after the medium change (Fig. 3A). *RDH10* mRNA remained elevated at least 16 h. The increase in *RDH10* mRNA correlated with that of glucose-6-phosphatase (*Glc-6-P*), which is induced tran-
scriptionally in liver during fasting (41). DHRS3 expression increased 1.5-fold after 4 h of serum-free medium, and insulin prevented the increase (Fig. 3B). Although insulin prevented the increase in RDH10 mRNA prompted by serum-free medium, neither high nor low glucose affected RDH10 expression (Fig. 3C).

Glucagon had no effect on RDH10 expression in either growth (10% serum) or serum-free medium. These data validate HepG2 cells as a model for insulin regulation of RDH10 expression in liver.

The transcription inhibitor actinomycin D (ActD) prevented the increase in RDH10 mRNA by removing serum (Fig. 3D). ActD effects were neither additive nor synergistic with those of insulin, indicating that both serum and insulin repress RDH10 transcription. The translation initiation inhibitor cyclohexi-
mide (CHX) prevented the increase in RDH10 expression upon serum exclusion, consistent with a requirement for protein synthesis for an increase in, or stabilization of, the mRNA. The actions of CHX and insulin were additive, suggesting insulin regulates RDH10 independently of translation.

Insulin Inhibits RDH10 Transcription via PI3K, Akt, and Suppression of FoxO1—PI3K or Akt inhibitors had no impact on RDH10 expression in serum-free medium, but prevented repression by insulin (Fig. 4A). Inhibition of mTORC1 or MEK/ERK did not prevent the increase in RDH10 expression induced by serum-free medium and did not ameliorate insulin inhibition. These data demonstrate that PI3K and Akt are required for suppression of RDH10 transcription by insulin and suggest that FoxO1, which insulin suppresses, induces RDH10 transcription. To test the need for FoxO1, we infected HepG2 cells with an adenovirus expressing a dominant-negative FoxO1 construct (dnFoxO1), which produces a truncated mutant that binds DNA, but lacks the transcription activation domain (32). Cells infected with dnFoxO1 did not increase RDH10 expression when exposed to serum-free medium. This is consistent with induction of FoxO1 activity by serum-free medium and prevention of FoxO1 activity by dnFoxO1 (32, 42). Insulin had no further effect on RDH10 expression in these cells. Expression of dnFoxO1 did not affect RDH10 expression in cells when exposed to growth medium. In contrast, adenovirus expressing lacZ responded to serum-free medium with an increase in RDH10 mRNA that was inhibited by insulin. G6pc expression followed the same pattern as RDH10 in the adenovirus experimental conditions (Fig. 4B). Western blot analysis confirmed the expression of HA-tagged dnFoxO1 (Fig. 4C). Insulin signal-
ing induces phosphorylation and nuclear export of FoxO1 (43). Cells in serum-free medium had FoxO1 located in puncta within the nucleus. Cells treated with insulin had FoxO1 expressed in a diffuse pattern within the cytoplasm, consistent with nuclear export (Fig. 4D).

Serum-free Medium Increases and Insulin Decreases RDH10 mRNA Stability—We determined the elimination half-life ($t_{1/2}$) of RDH10 mRNA in cells cultured 16 h in serum-free medium, or maintained in growth medium, and then exposed to ActD for 24 h (Fig. 5A). RDH10 mRNA had a $t_{1/2}$ of 35 h in the absence of serum and insulin. In the absence of serum, but in the presence of insulin, the $t_{1/2}$ decreased to 19 h. In the presence of serum and absence of insulin (growth medium), RDH10 mRNA had a bipsic $t_{1/2}$. The mRNA was decreased 50% by 8 h; after 8 h the mRNA was stabilized with a $t_{1/2}$ of 118 h. Adding serum for 8 h to cells exposed to serum-free medium for 16 h also decreased the amount of RDH10 mRNA by 50%. Because RDH10 expression is elevated 2–3-fold in serum-free medium, the amounts of message in cells with serum-free medium, with or without insulin, continued to exceed those of cells maintained in growth medium. Destabilization of RDH10 mRNA by insulin was attenuated by inhibition of PI3K or expression of dnFoxO1, and completely prevented by inhibition of Akt (Fig. 5B). In the absence of ActD, RDH10 expression in cells exposed to serum-free medium 16 h, then treated 4 h with insulin, was not changed significantly (Fig. 5C). In contrast, treatment with serum or serum and insulin reduced RDH10 mRNA to the amount in growth medium. These data show that mRNA induced by long-term exposure to serum-free medium is more sensitive to serum than insulin. In cells exposed to serum-free medium for 16 h, then treated 8 h with CHX and ActD versus ActD alone, maximum RDH10 mRNA stability required trans-

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RDH116 is Regulated Similarly to RDH10—Because mouse Rdh1 expression was also reduced in liver of re-fed versus fasted
mice, we examined its human ortholog RDH16 in HepG2 cells. Removing serum from the medium increased RDH16 expression within 2 h, rising to 4-fold by 16 h (Fig. 6A). Insulin prevented the increase in RDH16 mRNA that occurred in serum-free medium, but neither glucagon nor glucose had an effect (Fig. 6B). The increase in RDH16 mRNA that occurs in serum-free medium is dependent on transcription, as shown by ActD treatment (Fig. 6C). Interestingly, ActD treatment reduced RDH16 expression more than that of growth medium or insulin. Insulin had no effect in the presence of dnFoxO1, which reduced RDH16 mRNA to a greater extent than insulin treatment in serum-free medium (Fig. 6D).

FoxO1 Is Required for Elevated atRA Biosynthesis in Serum-Free Medium—To test the impact of reduced RDH mRNA expression on atRA biosynthesis from retinol, HepG2 cells infected with dnFoxO1 or lacZ control were maintained 16 h in growth or serum-free medium, and then incubated 4 h with all-trans-retinol. atRA was increased ~4-fold in cells incubated in serum-free relative to growth medium (Fig. 7). The increase was prevented by dnFoxO1. Quantification of retinol recovered in cells revealed greater uptake in cells maintained in serum-free relative to growth medium. Thus, despite the increased intracellular retinol concentration, atRA biosynthesis was...
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**FIGURE 8. Regulation of Rdh expression by insulin signaling.** Both atRA and FoxO1 induce Pck1 transcription to increase gluconeogenesis. FoxO1 induces atRA biosynthesis by inducing Rdh mRNA. Insulin suppresses FoxO1 activity, thereby suppressing gluconeogenesis and atRA biosynthesis through decreasing Rdh transcription and mRNA stability. Activation of PI3K/Akt represents a molecular indicator of cancer, implicating decreased atRA.

decreased by dnFoxO1, relative to the rate observed in serum-free medium.

**DISCUSSION**

A vitamin A-deficient diet results in glycogen deficiency because of impaired gluconeogenesis, caused by low Pck1 expression (44–46). Stimulation of gluconeogenesis is mediated directly by the vitamin A metabolite atRA through RA receptor response elements in the key gluconeogenic gene Pck1 (44, 45). Here we demonstrate reciprocal regulation by energy status of Rdh mRNA and atRA concentrations via insulin effects on FoxO1. Fasting-induced coordinate induction of both Rdh and Pck1 by FoxO1 stimulates gluconeogenesis (Fig. 8). In the fed state, insulin repression of FoxO1 suppresses gluconeogenesis via repressing both Rdh and Pck1. Although serum factor(s) other than insulin control Rdh expression and mRNA stability, we focused on insulin because of its critical role in regulating gluconeogenesis (9, 47).

Tissue-specific changes in Rdh10 and Rdh1 expression during fasting and re-feeding are consistent with a complex contribution of atRA to regulating intermediary metabolism. Epididymal white adipose tissue and pancreata do not express Rdh1 (data not shown), but express Rdh10. The non-responsiveness of Rdh10 in epididymal white adipose tissue to changes in energy balance allows several interpretations, including the presence of another Rdh that responds to changes in energy status, or feeding versus fasting does not modify atRA epididymal white adipose tissue concentrations in adult mice. These possibilities are the subjects of ongoing studies. The decrease in Rdh10 expression in pancreas during re-feeding is consistent with the requirement for retinoids in pancreatic development (48, 49) and function (50, 51), and also a subject of ongoing studies. Suppression of Rdh1 in liver by re-feeding, and Rdh10 in liver and kidney, relate insulin action with atRA signaling in two gluconeogenic tissues. Decreased Dhrs3 expression in liver of re-fed versus fasted mice is consistent with a mutually activating interaction of Rdh10 and Dhrs3 (23), and the reduction in atRA biosynthesis. Evidently, energy metabolism affects Rdh expression in multiple tissues to regulate retinoid signaling effects as feedback to regulation of energy disposition by atRA.

Oral dosing has a singular effect on glucose uptake and insulin action in liver (27). Glucose delivery by the portal vein combined with insulin, and an as-yet unidentified neuronal signal, increases net hepatic glucose uptake compared with peripheral delivery, with a concomitant impact on insulin-regulated processes (52, 53). A comparison of oral glucose dosing to infusion via the portal vein revealed similar enhancement in hepatic glucose uptake relative to peripheral presentation, which excludes a contribution from a gut-secreted factor, such as Glp-1 (54). Yet, secretion of Glp-1 upon feeding contributes to reduced glucose production in liver by augmenting glucose-stimulated insulin secretion from pancreas (55). Our data of oral but not peripheral glucose dosing reducing Rdh mRNA are consistent with these observations and support the physiological significance of the observation.

Reduced Rdh expression preceded the reduction in liver atRA, and was accompanied by a lower rate of atRA biosynthesis in HepG2 cells, consistent with a cause and effect relationship. atRA homeostasis autoregulates via induction of both cata- bolic cytochromes P-450 and the retinyl ester-forming lecithin:retinol acyltransferase (56–58). The former decreases atRA itself, whereas the latter decreases the amount of retinol available to support atRA biosynthesis. Changes in lecithin:retinol acyltransferase and cytochrome P-450 may have buffered atRA differences in the re-fed relative to the fasted liver. The 2-fold decrease in liver atRA resulting from insulin action seems remarkable in the context of this impetus to sustain atRA homeostasis.

FoxO (forkhead box “Other”) proteins constitute a subgroup of a family of evolutionarily conserved transcription factors that mediate insulin signaling in mammals, but also mediate metabolism and longevity in primitive organisms such as Caenorhabditis elegans and Drosophila (59, 60). FoxO proteins contribute to metabolic regulation through effects in liver, muscle, adipose tissue, and pancreas. Of these, FoxO1 stimulates a committed step in hepatic gluconeogenesis, catalyzed by phosphoenolpyruvate carboxykinase (PCK1), and also the last step catalyzed by glucose-6-phosphatase (Glc-6-P) (30, 32, 61, 63). Haploinsufficiency of FoxO1 restores insulin sensitivity in insulin-resistant mice (64). A constitutively active gain-of-function FoxO1 mutant targeted to liver and pancreas manifests a diabetic phenotype (64). Liver-specific inactivation results in decreased serum glucose at birth, and upon fasting in adult mice from impaired glycogenolysis and gluconeogenesis (63). A liver-specific FoxO1-null mouse crossed with an insulin receptor-null mouse has reduced glucose production and lacks the neonatal diabetes and hepatosteatosis that occur in insulin receptor-null mice (63). Liver-specific FoxO1-null mice treated with the β-cell toxin streptozotocin, have elevated VLDL secretion, cholesterol, and plasma-free fatty acids (65).

Insulin-stimulated phosphorylation of FoxO1 by Akt causes nuclear export, resulting in ubiquitination-mediated proteasomal degradation (66), and down-regulation of target gene transcription (43). Indeed, we confirmed the ability of insulin to cause nuclear export of FoxO1 in HepG2 cells (Fig. 4D). FoxO1 activity is also stimulated by deacetylation, catalyzed by sirtuin1.
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(67). Inhibition of sirtuin1 in HepG2 cells with Ex-527 incubated in serum-free medium did not affect RDH10 expression (data not shown), indicating that acetylation is not involved in regulation of RDH10, at least under the conditions tested.

Recent insight into the functions of insulin receptor substrates IRS1 and IRS2 indicate that both coordinate responses to insulin via FoxO1 (68, 69). During the re-fed state, insulin activates IRS1 to suppress FoxO1 and allow an increase in glucokinase and sterol regulatory element-binding transcription factor 1 (SREBF1, a.k.a. SREBP1) expression that promotes glycolysis and lipid biosynthesis, respectively. In contrast, insulin inhibits IRS2. Because IRS2 stimulates gluconeogenesis by inducing PCK1 and G6PC in liver through FoxO1, the relatively low levels of insulin in the fasted state allow IRS2 to induce gluconeogenesis. Based on these insights, the higher insulin levels in the re-fed state would function through IRS1 to suppress RDH expression, whereas the lower insulin levels in the fasted state would allow IRS2 to induce RDH expression.

The actions of insulin and serum both prevented transcription and destabilized RDH mRNA. The effects differed in degree, and resulted in different levels of mRNA, but did not totally eliminate RDH mRNA. In both serum-free medium and insulin-containing serum-free medium, the amounts of RDH mRNA after 24 h remained 2–3-fold higher compared with serum-containing medium, because the initial concentrations were 2–3-fold higher. Destabilization of RDH mRNA by insulin dependent on active PI3K, Akt, and inhibition of FoxO1. The reduction but not elimination of RDH mRNA by insulin and serum is compatible with the continued presence and biosynthesis of atRA, and a need for RDH to modulate basal gluconeogenesis and/or its other multiple functions, whether related or unrelated to energy balance, such as regulating proliferation (70).

FoxO1 binding sites have been identified in promoters of genes associated with retinoid metabolism, including dehydrogenase/reductase SDR family member 9 (Dhrs9), cellular retinol-binding protein type 1 (encoded by Rbp1), and Rdh8, but not in Rdh1 or Rdh10 (31). To address the impact of FoxO1-regulated expression of retinoid genes, atRA target genes Pck1 and Pdk4 were measured in cells with FoxO1 knocked down. Induction of both genes in response to atRA or to its precursor, retinol, were blunted. The dose (20 μM) of atRA used, however, exceeded those found in tissues by 400–4000-fold, and those used in vitro by 20-fold. Nevertheless, these results are also consistent with FoxO1 linking retinoid metabolism and hepatic gluconeogenesis. Multiple putative FoxO/A binding sites were found in the 5′-untranslated region of Rdh10 (Encyclopedia of DNA Elements Consortium, ENCODE; University of California Southern California genome browser). Future work will determine whether these or other sites are true response elements.

Regulation by insulin signaling prompts the question whether abnormal RDH expression associates with human disease. For example, impaired insulin secretion and insulin resistance during diabetes predicts elevated RDH expression and atRA synthesis (71). Conversely, enhancement of insulin signaling through PI3K/Akt from loss of phosphatase and tensin homolog deleted on chromosome 10 in cancer implicate decreased RDH (72). Reduced RDH expression would decrease atRA biosynthesis, which could affect tumor differentiation and/or aggressiveness. Various cancers show alterations in the atRA signaling pathway, such as loss of atRA receptors (73, 74) and down-regulation of atRA chaperons (75, 76). atRA biosynthesis is impaired in breast cancer cell lines relative to normal cells (77). Reduced atRA synthesis in the Rbp1-null mouse is consistent with increased mammary tumors, and suggests consequences of the epigenetic silencing of Rbp1 in 25% of human breast cancers (62). In contrast, overexpression of RDH10 in HepG2 cells reduces proliferation (70).

In summary, despite the importance of atRA in vertebrate physiology, little is known about regulation of the rate-limiting enzymes (Rdh) that catalyze its biosynthesis. This report reveals a reciprocal interaction in which energy status regulates atRA biosynthesis through insulin that would attenuate energy balance regulation by atRA. These data predict altered atRA concentrations in diseases characterized by dysfunctional insulin signaling, including diabetes and cancer.

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