Identification of 9-cis-retinoic acid as a pancreas-specific autacoid that attenuates glucose-stimulated insulin secretion

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The all-trans-retinoic acid (atRA) isomer, 9-cis-retinoic acid (9cRA), activates retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in vitro. RARs control multiple genes, whereas RXRs serve as partners for RARs and other nuclear receptors that regulate metabolism. Physiological function has not been determined for 9cRA, because it has not been detected in serum or multiple tissues with analytically validated assays. Here, we identify 9cRA in mouse pancreas by liquid chromatography/tandem mass spectrometry (LC/MS/MS), and show that 9cRA decreases with feeding and after glucose dosing and varies inversely with serum insulin. 9cRA reduces glucose-stimulated insulin secretion (GSIS) in mouse islets and in the rat β-cell line 832/13 within 15 min by reducing glucose transporter type 2 (Glut2) and glucokinase (GK) activities. 9cRA also reduces Pdx-1 and HNF4α mRNA expression, ~8- and 80-fold, respectively: defects in Pdx-1 or HNF4α cause maturity onset diabetes of the young (MODY4 and 1, respectively), as does a defective GK gene (MODY2). Pancreas β-cells generate 9cRA, and mouse models of reduced β-cell number, heterozygous Akita mice, and streptozotocin-treated mice have reduced 9cRA. 9cRA is abnormally high in glucose-intolerant mice, which have β-cell hyper trophy, including mice with diet-induced obesity (DIO) and db/db and ob/ob mice. These data establish 9cRA as a pancreas-specific autacoid with multiple mechanisms of action and provide unique insight into GSIS.

Impaired glucose-stimulated insulin secretion (GSIS) develops through multiple mechanisms, including actions of metabolic hormones and inflammatory cytokines, products of metabolic overload, and endoplasmic reticulum stress; however, mechanisms of GSIS and impaired glucose tolerance remain incompletely understood (1–4). Also uncertain is the contribution of impaired glucose tolerance to diminished pancreatic β-cell function and mass associated with type 2 diabetes (5). GSIS relies on the pancreas, and pancreas development, islet formation, and function require normal vitamin A nutriture (6–8). Vitamin A restriction during development impairs islet development and promotes glucose intolerance in adult rodents. On the other hand, restricting vitamin A in mature diabetes-prone rats reduces diabetes and insulin sensitivity through enhancing glucose sensing and metabolism. All-trans-retinoic acid (atRA), an activated metabolite of vitamin A, regulates pancreas development, and atRA does not enhance the incidence of diabetes in diabetes-prone rats fed a vitamin A-deficient diet (7, 9, 10). Although the contribution of vitamin A to pancreas development through atRA seems clear, mechanisms whereby vitamin A affects mature pancreas function have not been determined in depth, nor have the specific vitamin A metabolites been identified that contribute to GSIS control. atRA induces differentiation and regulates cell processes by activating the nuclear receptors RAR α, -β, and -γ, which regulate transcription and translation (11). atRA does not activate the nuclear receptors RXR α, -β, and -γ, which serve as obligatory partners for RAR and numerous other nuclear receptors that regulate metabolism and energy balance (12). 9-Cis-retinoic acid (9cRA), an atRA isomer, binds both retinoic acid receptors (RARs) and retinoid X receptors (RXRs) with high affinity in vitro and has diverse pharmacological actions distinct from atRA (13). For example, treating embryo day-11 pancreas organ cultures with 9cRA inhibits stellate cell activation more potently and quickly than atRA and inhibits acini differentiation, but prompts ductal differentiation and endocrine maturation (9, 10). atRA, in contrast, induces acini rather than ductal differentiation. As a panagonist of six nuclear receptors, 9cRA has undergone extensive pharmacological assessment. As the drug alitretinoin, it is effective against chronic hand dermatitis and T-cell lymphoma (14). Used systemically, it alters energy metabolism (15). 9cRA also shows promise in reducing ischemic brain injury in a rat model and in immunosuppressing human dendritic cells (16, 17). Regardless of the pharmacological utility of 9cRA, sensitive assays capable of quantifying individual RA isomers in biological matrices have not detected 9cRA in serum and in a variety of tissues (18, 19). This leaves uncertain whether 9cRA functions in vivo as an activated vitamin A metabolite with discrete physiological functions.

We applied a liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay developed to distinguish and quantify RA isomers in biological matrices to identify retinoids in the pancreas and detected not only atRA, but also 9cRA. Pancreases 9cRA, but not atRA, reacts within minutes to blood glucose fluctuations and attenuates the impact of glucose on GSIS through multiple mechanisms, including rapid action. These data validate 9cRA as a naturally occurring metabolite of vitamin A with a physiological function unique among retinoids, broadening insight into mechanisms of GSIS, and provide unique perspective into vitamin A and islet function.

Results

9cRA as an Endogenous Pancreas Retinoid. We applied a sensitive LC/MS/MS assay to compare pancreas RA isomers to those in serum and liver, because the endocrine pancreas expresses nuclear receptors that recognize RA isomers and responds to retinoid-induced signaling, and liver serves as the principal storage site of retinoids and contributes to retinoid homeostasis (20, 21). Consistent with previous work, prominent physiological RA isomers in serum and liver included atRA and 9,13-di-cis-RA (9,13dcRA), an RA isomer without known biological activity, but
9cRA was not detected (18, 19). In contrast, we identified 9cRA in pancreas, along with atRA and 9,13dcRA (Fig. 1A). We confirmed that analysis did not generate 9cRA by adding retinoids to pancreas before homogenization, extraction, and assay (Fig. 1B). Only 9cRA increased the 9cRA signal, excluding oxidation of the RA precursor retinal and/or isomerization of atRA during analyses as sources of 9cRA. Concentrations of 9cRA in pancreas occur within the range of concentrations of other RA isomers in tissues and serum (Fig. 1C). These data provide an analytically rigorous identification of 9cRA as a naturally occurring retinoid. If 9cRA occurs in the tissues assayed other than pancreas, amounts would be <0.05 pmol/g, on the basis of the LC/MS/MS assay’s limit of detection in biomatrices.

9cRA Varies with Fasting, Feeding, and Glucose Challenge. The fasted-to-fed transition resulted in a 36% decrease in 9cRA, which accompanied the increase in blood glucose and serum insulin, but caused no changes in pancreas atRA, 9,13dcRA, or retinol (Fig. 2A and Fig. S1). Consistent with this observation, challenging fasted mice with a bolus of glucose decreased 9cRA >80% within 15 min, coinciding with the rapid rise in blood glucose (Fig. 2B). 9cRA recovered markedly by 30 min and continued to rise thereafter. In contrast, glucose challenge had no impact on pancreas atRA or 9,13dcRA. During glucose challenge, 9cRA correlated inversely with serum insulin, further suggesting a contribution to pancreas function consistent with decreasing GSIS (Fig. 2C). In addition, exogenous 9cRA reduced serum insulin during glucose challenge (Fig. 2D).

9cRA Promotes Glucose Intolerance. The inverse relationship between serum insulin and pancreas 9cRA during the glucose tolerance test (GTT), and ability of 9cRA to reduce serum insulin, prompted testing whether 9cRA decreases glucose disposal. Mice were injected with 9-cis retinol, a potential precursor of 9cRA, or 9cRA before a GTT. Mice injected with 9-cis retinol responded with a 2- to 2.7-fold increase in pancreas 9cRA, sustained at least 120 min (Fig. 3A). Mice injected with 9cRA responded with an ~30-fold increase in pancreas 9cRA, which declined by 120 min to ~fourfold above control. Note that the decrease in endogenous 9cRA in the control (dosed only with glucose) at 30 min reflected the same degree of decrease (~40%) observed at 30 min in the GTT experiment of Fig. 2B. Increases in pancreas 9cRA caused by both 9-cis retinoids resulted in glucose intolerance, such that 120 min after the glucose challenge, blood glucose was at least twofold higher than in vehicle-dosed mice (Fig. 3B). The lowest concentrations of 9cRA (40 nM) achieved after dosing either 9cRA or 9-cis retinol arrested glucose disposal to the same extent as the higher concentrations achieved, indicating a dose–response relationship with a maximum effect near or below 40 nM 9cRA. These data suggest that the physiological decrease in pancreas 9cRA as glucose increases
DMSO). 9-

GTT in mice dosed with 9-
inoids as described in

Respectively. Glucose (2 g/kg) was injected at 0 min:

Pancreas 9cRA after dosing with 9-

Rapidly Attenuates Glucose Sensing and Insulin Secretion.

Fig. 3. Exogenous 9cRA induces glucose intolerance. (A) Increases in total

pancreas 9cRA after dosing with 9-cis retinol or 9cRA (0.5 mg/kg in 100 μL

dMSO). 9-cis retinol and 9cRA were injected 60 and 15 min before glucose,

respectively. Glucose (2 g/kg) was injected at 0 min: five to eight mice per

group; *P = 0.01 and **P < 0.003 vs. vehicle control; ***P < 0.005 vs. 0 min. (B)

GTT in mice dosed with 9-cis retinol or 9cRA: five to seven mice per group;

*P < 0.04, **P < 0.002, ***P < 0.005 vs. control. Mice were dosed with ret-
inoids as described in A. All data are ±SEM.

permits optimum insulin secretion, and reveal that 9-cis retinol can serve as a precursor to 9cRA.

9cRA Rapidly Attenuates Glucose Sensing and Insulin Secretion. Glucose uptake by the pancreas rapidly affects both Glut2 and

GK activities through posttranslational mechanisms (22, 23). Although GK activity limits the rate of glucose uptake by the

β-cell, Glut2 contributes more than passive glucose transport by

signaling glucose concentrations: phosphorylation decreases both

Although GK activity limits the rate of glucose uptake by the

Pancreas 832/13 cells and islets were preincubated

2 h with 3 mM glucose. At 0 min, the medium was

exchanged for medium containing 23 mM and agents

indicated for the duration of experiments. (A) 9cRA reduces Glut2 activity in 832/13 cells after 15 min in-

duction: 4–8 replicates per group; *P ≤ 0.0003 vs. no addition. (B) 9cRA reduces GK activity in 832/13

cells: 3–7 replicates per group. *P < 0.02, **P < 0.002 vs. control. (C) 9cRA reduces ATP content in 832/13 cells: 2–4 replicates per group. *P < 0.008 vs. control. (D) 9cRA decreases Ca

 influx into 832/13 cells: 2 replicates per group; *P < 0.02. (E) 9cRA decreases GSIS by 832/13 cells: 3–11 replicates per group; *P < 0.01 vs. control. (F) 9cRA decreases GSIS by pancreatic islets. The graph shows baseline insulin secretion during

3 mM glucose and the effect of 9cRA on stimulation of insulin secretion by 23 mM glucose: 8–9 replicates per group; *P < 0.02, **P < 0.002 vs. control. (G) 9cRA does not affect KCl-stimulated insulin secretion from islets; *P < 0.02 vs. 0 time. (H) 9cRA reduces Pdx-1 and HNF4α mRNA after 2 h in 832/13 cells: 3 replicates per group. One hundred nanomolar 9cRA was used in all experi-

ments, unless noted otherwise. All data are ±SEM.
cells (Fig. S3) (26, 27). In contrast to 9cRA, neither decreased the fold increase in insulin secretion after 1-h incubation with 23 mM of glucose vs. 5 mM, but both decreased basal insulin secretion stimulated by 3 mM.

After 2 h, 9cRA decreased expression of Pdx-1 and HNF4α mRNA, 7- and 77-fold, respectively (Fig. 4H). Pdx-1 induces glucokinase, Glut2, and insulin gene expression in the mature pancreas (28, 29). HNF4α regulates insulin release through controlling mitochondrial metabolism of glucose and HNF1α, Glut2, and insulin gene expression (30, 31).

Pancreas β-Cells Produce 9cRA. We analyzed pancreata from mice with decreased numbers of β-cells to identify sources of 9cRA. A point mutation in the insulin 2 (Ins2) gene of Ins2Akita mice induces β-cell apoptosis, which reduces the number of β-cells, indicated by a 46% reduction in pancreas insulin (32). Pancreata from fed heterozygous Akita mice had 40% lower 9cRA than wild type (WT), consistent with the decrease in β-cells (Fig. 5A and Fig. S4). In contrast, pancreas atRA increased and retinol did not differ from WT, demonstrating a unique relationship between 9cRA and β-cells. To confirm this insight, we injected mice with streptozotocin (Stz), which causes β-cell necrosis (33). 9cRA in pancreas of Stz-treated mice decreased with time in direct proportion to the decrease in β-cells, assessed by insulin content (Fig. 5B and C). Seventy-two hours after Stz dosing, β-cell numbers decreased 67%, accompanied by a 58% decrease in 9cRA, impaired glucose tolerance, and elevated nonfasting blood glucose (Fig. 5C and Fig. S5). By 96 h, β-cells decreased 95% and 9cRA decreased 70%, consistent with β-cells serving as a major source of pancreas 9cRA. On the basis of the 9cRA remaining after β-cell destruction, other pancreas cells may contribute ~20–25% to the 9cRA pool.

The β-cell line 832/13 generated 9cRA and atRA from their respective 9-cis- or all-trans-retinol and retinyl precursors at similar rates (Fig. 5D). Pancreas microsomes contain both 9-cis- and all-trans-retinol (33 ± 1.4 and 76 ± 2 pmol/g protein, respectively; three replicate analyses of a five-pancreata pool) (Fig. 5E). Thus, β-cells have the capacity and a substrate to biosynthesize 9cRA.

Elevated 9cRA in Models of Glucose Intolerance. To determine whether mouse models of glucose intolerance are accompanied by 9cRA increases, we assayed pancreata from ob/ob and db/db mice and mice with diet-induced obesity (DIO) (34). ob/ob mice lack leptin, are obese, and have high blood glucose and serum insulin (Fig. S6 A and B). ob/ob mice had 2.2-fold higher 9cRA than WT controls (Fig. 6A). Mice with DIO were glucose intolerant, weighed ~50% more than controls, and had ~twofold higher 9cRA than controls (Fig. 6B and Fig. S6 C and D). db/db mice, which lack the leptin receptor and have elevated blood glucose and serum insulin (Fig. S6E), had 34% higher 9cRA than lean controls (Fig. 6C). Although atRA also increased in pancreas of ob/ob mice, the increase was modest, and atRA did not increase in pancreas of db/db mice. In DIO pancreata, the atRA increase exceeded the 9cRA increase. Neither the atRA nor the 9cRA changes in pancreas correlated with changes in retinol. Thus, 9cRA was the only endogenous retinoid assayed (9cRA, atRA, and retinol) that changed consistently in pancreas with changes in glucose tolerance, regardless of the underlying cause of impaired glucose tolerance.

Discussion
With an analytically rigorous assay, we determined that 9cRA is a naturally occurring retinoid in pancreas. This result addresses the long-standing questions of whether or not 9cRA contributes to the biological activity of vitamin A in vivo, and if so, in what capacity. We found that 9cRA localizes to the pancreas, where it attenuates sensitivity to glucose through a rapid reduction of GLUT2 and GK activities, without a concomitant rapid change in their mRNA. Reduction of glucose uptake and phosphorylation led to reduced ATP and insulin release. In the longer term, 9cRA reduces transcription of two genes required for insulin synthesis and secretion—Pdx-1 and HNF4α. Notably, defects in either of these two genes and the gene that expresses GK cause the monogenic diseases known as maturity onset diabetes of the young (MODY): defects in HNF4α, GK, and Pdx-1 cause MODY1, 2, and 4, respectively (35, 36). Therefore, at least three of six well-characterized MODY are caused by defects in genes regulated by 9cRA or in a gene that encodes a protein regulated by 9cRA. These data clarify insight into the effects of vitamin A on pancreas function, i.e., atRA programs pancreas development and 9cRA attenuates GSIS in the developed pancreas. This is consistent with the need for vitamin A during pancreas development and with alleviation of glucose intolerance by restricting vitamin A in the mature pancreas. The observation of 9cRA in the pancreas justifies future work to determine when the pancreas begins producing 9cRA, to clarify its function during pancreas development.

β-cells, which are integral to GSIS, serve as a major source of 9cRA, and 9cRA effects on β-cell function were observed by
Autocoid action in pancreas appears specific to 9cRA. Pancreas atRA did not change with an increase in blood glucose after the fasted-to-the-fed transition, or after glucose injection, indicating that atRA does not regulate short-term modulation of GSIS. The increase in atRA in the pancreases during DIO and in ob/ob mice seems to be a compensating reaction to obesity, because disrupting atRA biogenesis by ablating Rdh1 enhances adiposity, whereas chronic dosing of atRA to mice with DIO reduces weight by ~15% (15, 50).

In summary, this work demonstrates that 9cRA occurs as an endogenous pancreas retinoid, establishes a function for 9cRA in GSIS, and provides unique insight into retinoid function and glucose homeostasis. Of interest are the likely nongenomic effects of 9cRA. These studies provide insight into an essential element of pancreas-mediated glucose homeostasis, which should prove useful for understanding mechanisms of GSIS and diabetes.

Methods

Animals. Male C57BL/6 mice were used, unless noted otherwise, in accordance with institutional guidelines. Mice were fed either a stock diet with 30 IU/g vitamin A (Global 18% protein rodent diet 20158; Harlan Laboratories Teklad) or an AIN-93G purified rodent diet with 30 IU/g vitamin A (119139; Dyets). The fatty acyl composition of the stock diet was (% of diet): 16:0, 0.7%; 18:0, 0.2%; 18:1 1.2%; 18:2, 2.3%; and 18:3, 0.3%. The fatty acid content of the AIN-93G diet has been published (51). These diets are referred to as vitamin A-ampile diets and produced similar results. Seven- to 12-wk-old WT, ob/ob mice, and mice heterozygous for the Akita spontaneous mutation (Insc2Akita) were purchased from The Jackson Laboratory. To establish DIO, 1-mo-old mice were fed a high-fat diet (HFD) for 5 mo. The HFD was an AIN-93G purified rodent diet (diet no. 180014; Dyets) with 50% fat-derived calories. Lard served as the fat source. The fatty acyl composition of the lard was: 14:0, 1.5%; 16:0, 25.6%; 16:1, 3.4%; 18:0, 13.1%; 18:1, 14.1%; 18:2, 10.8%; and 18:3, 1.5%. To deplete β-cells, Stz (170 mg/kg) was dosed i.p. once in 10 mM sodium citrate, pH 4.5. For GTTs, glucose (2 g/kg) was doped i.p. in saline. The db/db mice were 5 mo old and fed ad libitum. Insulin levels in this db/db colony at this age average threefold higher than controls, P < 0.01 (52). Fasting was done for 12–16 h. Mice were killed during the early light cycle.

Retinoids and Retinoid Quantification. Retinoids were purchased from Sigma, except for 9-cis retinol, which was synthesized and characterized as described (18). Retinoids were used under yellow light as described (19, 53). Samples were harvested under yellow lights, frozen immediately in liquid nitrogen, and kept at –80 °C until extraction and retinoid quantification within 1 d by LC/MS/MS using selected reaction monitoring (RA isomers) or HPLC/UV (retinol isomers) as described in detail (19, 53).

β-Cell Line Function Assays. The pancreatic β-cell line 832/13 was cultured in growth medium (RPMI 1640 with 11 mM glucose and 10% FCS, 10 mM Heps, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 mM β-mercaptoethanol, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO2 in 100-mm Petri dishes as described (25)). The medium was refreshed every 2–3 d. Cells were subcultured when they approached ≥70% confluence. Endogenous 9cRA in 832/13 cells at the time of experiments was <0.04 ± 0.0012 pmol/106 cells (n = 9 plates).

To assay Glut2, GK, and ATP, cells were transferred to 12-well plates until reaching 85% of confluence. Medium with 5 mM glucose was substituted for growth medium for 16 h. Before assays, this medium was substituted with 2 mL HBSS containing 0.2% fatty-acid-free BSA, pH 7.2 (sensing buffer), and 3 mM glucose for 2 h (except for the glucose uptake assay). Experiments were then initiated by changing the sensing buffer to 0.8 μL per well of either fresh buffer with 3 mM glucose or 23 mM glucose and/or 100 mM 9cRA, delivered in 5–10 μL DMSO, or vehicle alone. Incubations were done at 37 °C under yellow light.

9cRA Biosynthesis. 832/13 cells were cultured in 12- or 6-well plates. At confluence, the growth medium was replaced with serum-free medium (11 mM glucose) and retinoids (1 μM) in DMSO (0.1% vol/vol) or vehicle alone were added and incubated for 1 h. Cells were lysed using Reporter lysis buffer (Promega) and combined with their medium for retinoid analyses.

Insulin Secretion Studies. Islets were isolated by the University of California, San Francisco Diabetes and Endocrinology Research Center. Fifteen islets of...
similar size were incubated at 37 °C in 12-well plates with 5 mM glucose in nonadherent cells and frozen at 80 °C. Endogenous 9cRA in islets at the time of the experiment (after isolation and handling of islets) did not exceed 0.12 ± 0.008 pmol per plate (n = 5).


Data Analysis. Mean values ± SEM. Statistical significance was assessed by two-tailed, unpaired Student’s t tests for comparison of two groups, or by two-way ANOVA for comparison of two curves.

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