Acta Pharmacologica Sinica

www.nature.com/aps

ARTICLE

All-*trans* retinoic acid impairs glucose-stimulated insulin secretion by activating the RXR/SREBP-1c/UCP2 pathway

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Diabetes is often associated with vitamin A disorders. All-*trans* retinoic acid (ATRA) is the main active constituent of vitamin A. We aimed to investigate whether ATRA influences diabetic progression and its mechanisms using both Goto-Kazizazi (GK) rats and INS-1 cells. Rat experiments demonstrated that ATRA treatment worsened diabetes symptoms, as evidenced by an increase in fasting blood glucose (FBG) levels and impairment of glucose homeostasis. Importantly, ATRA impaired glucose-stimulated insulin secretion (GSIS) and increased the expression of sterol regulatory element-binding protein 1c (SREBP-1c) and uncoupling protein 2 (UCP2) in the rat pancreas. Data from INS-1 cells also showed that ATRA upregulated SREBP-1c and UCP2 expression and impaired GSIS at 23 mM glucose. *Srebp-1c* or *Ucp2* silencing attenuated GSIS impairment by reversing the ATRA-induced increase in UCP2 expression and decrease in ATP content. ATRA and the retinoid X receptor (RXR) agonists 9-*cis* RA and LG100268 induced the gene expression of *Srebp-1c*, which was almost completely abolished by the RXR antagonist HX531. RXRα-LBD luciferase reporter plasmid experiments also demonstrated that ATRA concentration-dependently activated RXRα, the EC_{50} of which was 1.37 μM, which was lower than the ATRA concentration in the pancreas of GK rats treated with a high dose of ATRA (approximately 3 μM), inferring that ATRA can upregulate *Srebp-1c* expression in the pancreas by activating RXR. In conclusion, ATRA impaired GSIS partly by activating the RXR/SREBP-1c/UCP2 pathway, thus worsening diabetic symptoms. The results highlight the roles of ATRA in diabetic progression and establish new strategies for diabetes treatment.

Keywords: all-trans retinoic acid; diabetes; glucose-stimulated insulin secretion; sterol regulatory element-binding protein 1c; uncoupling protein 2; retinoid X receptor

Acta Pharmacologica Sinica (2022) 43:1441–1452; https://doi.org/10.1038/s41401-021-00740-2

INTRODUCTION

Vitamin A including retinol, retinaldehyde, and ATRA are essential endogenous substances for maintaining normal physiological processes [1]. Retinol is metabolized into retinaldehyde via alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs), and retinaldehyde is further converted to retinoic acids (RAs), the main active constituents of vitamin A, by retinaldehyde dehydrogenases (RALDHs, also known as ALDH1As). RAs exist as a variety of isomers, including all-*trans* retinoic acid (ATRA), 9-*cis* RA, 13-*cis* RA, and 9,13-*cis* RA. ATRA is the primary RA isomer in the body [2]. It is generally accepted that ATRA exerts its physiological functions by regulating retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [3]. RXRs form homodimers or heterodimers with other nuclear receptors, such as RARs, liver X receptors (LXRs), and peroxisome proliferator-activated receptors (PPARs) [4, 5].

Accumulating studies have shown that ATRA is involved in the progression of some diseases, such as obesity, diabetes, and cancer [6]. Several investigations have demonstrated that type 2 diabetes is often accompanied by an imbalance of vitamin A, as indicated by decreased levels of retinaldehyde and increased levels of RAs [7–9]. The imbalance of vitamin A caused by diabetes may result from upregulation of RALDH expression or downregulation of ADH/RDH expression [9, 10]. Diabetes has been reported to cause significant upregulation of pancreatic Raldh3 expression, leading to a 7-fold increase in the pancreatic ATRA concentration of mice [11]. It has been reported that Raldh3 overexpression impairs insulin secretion in MIN6 cells [12]. Clinical trials have demonstrated that administration of 13-*cis* RA increases serum triglyceride (TG) levels in both healthy subjects and patients [13, 14], and administration of ATRA also markedly increases the TG concentration and body weight [15]. Similarly, in rats, ATRA treatment enhances TG accumulation in serum [16, 17]. All these results infer that increased RAs concentration plays role in diabetes progression.

RAs have also been demonstrated to affect the sequential specification of pro-endocrine cells, committed preβ-cells and differentiated β-cells [18–20], in turn altering the functions of β-cells. RA signaling mediated by RARs is thought to be required for maintaining both β-cell function and mass in the adult pancreas [21]. RAs regulate insulin secretion, possibly via RAR/RXR-mediated transcriptional regulation or nongenomic mechanisms [20, 22, 23]. However, reports on the roles of RAs in glucose-stimulated insulin secretion (GSIS) are often contradictory. 9-*cis* RA, an RXR agonist, was identified to impair GSIS, promote glucose intolerance and rapidly attenuate glucose sensing and insulin secretion under 23 mM glucose stimulation in mice [24]. However, in RINm5F cells, the

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Received: 28 April 2021 Accepted: 2 July 2021
Published online: 20 August 2021

insulin secretion under 0, 0.5, 2.8, 7, and 11 mM glucose stimulation was increased by 50%–100% after exposure to 9-*cis* RA (1–1000 nM) for 48 h. ATRA treatment was found to have similar effects, but the effects of ATRA were less potent than those of 9-*cis* RA [25]. The effects of RXR activation on GSIS are dependent on glucose levels. Inhibition of insulin release by 9-*cis* RA-mediated RXR activation only occurs under high-glucose stimulation (25 mM) [26]. In islets of transgenic mice, dominant-negative RXR expression enhances insulin secretion under high glucose stimulation (27 mM). Similarly, in a pancreatic β-cell line, suppression of RXRs also enhances GSIS at a high glucose concentration (25 mM) [26]. Interestingly, the effect of ATRA on lipid metabolism is also dependent on glucose levels. In 3T3L1 cells, ATRA suppresses lipid accumulation in normal glucose medium (5.5 mM), but in high-glucose medium (25 mM), ATRA obviously promotes lipid accumulation as well as fatty acid synthase and sterol regulatory element-binding protein 1c (Srebp-1c) gene expression [27]. ATRA has been reported to induce Srebp-1c expression in INS-1 cells, rat primary hepatocytes, and HepG2 cells [28, 29]. Our preliminary data also demonstrated that ATRA impairs GSIS in INS-1 cells and induces Srebp-1c expression.

GSIS is involved in the uptake and metabolism of glucose as well as the formation of adenosine triphosphate (ATP). Uncoupling protein 2 (UCP2) plays important role in energy balance by regulating ATP synthesis [30]. Overexpression of *Ucp2* has been reported to reduce ATP content, leading to inhibition of GSIS in islets [31]. Overexpression of *Srebp-1c* induces the expression of UCP2 and reduces ATP production [32, 33]. Moreover, SREBP-1c itself regulates fatty acid and TG synthesis-related genes expression, leading to lipid accumulation in islets and thus impairing islet function [34].

The aims of the study were to investigate (1) whether ATRA worsens diabetes symptoms in Goto-Kazizazi (GK) rats (type 2 diabetic animals) by impairing GSIS and the possible underlying mechanism; (2) whether ATRA-induced impairment of GSIS involves the upregulation of UCP2 and SREBP-1c expression; and (3) whether ATRA impairs GSIS by activating RXRs. The results highlight the roles of ATRA in diabetes progression and provide new strategies for diabetes treatment.

MATERIALS AND METHODS

Reagents

ATRA and 9-cis RA were obtained from Sigma Chemical Co. (St. Louis, MO, United States). RPMI-1640 medium, fetal bovine serum (FBS), and trypsin (0.25%)-EDTA were purchased from Invitrogen (Waltham, CA, USA). β-Mercaptoethanol, butylated hydroxytoluene, and phenylmethyl sulfonyl fluoride were purchased from J&K Chemical (Shanghai, China). RIPA, loading buffer, and BCA kits were obtained from Beyotime Institute of Biotechnology (Nanjing, China). The insulin ELISA kit was purchased from Millipore Chemical (Bedford, MA, United States). TG and total cholesterol (TC) kits were obtained from Nanjing Jiancheng Bioengineering Institute. Monoclonal antibodies against SREBP-1c and UCP2 were purchased from Santa Cruz Biotechnology (CA, United States). All the other reagents were of analytical grade and were commercially available.

Animal experiments

Male GK rats (weighing 280 ± 10 g) and age-matched male Wistar rats (weighing 300 g) were obtained from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, China) and housed at a controlled temperature (22 ± 2 °C) and relative humidity (50% ± 10%) on a 12-h light/dark cycle with free access to food and water. Following 3 days of acclimation, the GK rats were randomly divided into 3 groups (5 of each group): the diabetic control (GK-CON), low-dose ATRA (GK-LRA, 2.5 mg/kg), and high-dose ATRA (GK-HRA, 10 mg/kg) groups. The dose of ATRA was selected according to previous reports [35, 36]. ATRA was dissolved in olive oil before use and then intraperitoneally (i.p.) administered to GK rats once daily for 5 weeks. Wistar rats (normal control rats) and GK-CON rats received vehicle. Fasting blood glucose (FBG) levels were measured every week. The food and water consumption, as well as body weight, were recorded every day. On day 35, the experimental rats, which had been fasted overnight, were sacrificed under light diethyl ether anesthesia 1 h following ATRA administration. The blood, liver, and pancreas were immediately collected to measure biochemical parameters and the expression of target proteins/mRNAs as well as for histopathological examination of the pancreas. Animal experiments were carried out according to the Institutional Guidelines for the Care and Use of Laboratory Animals and were authorized by the Animal Ethics Committee of China Pharmaceutical University.

Intraperitoneal insulin tolerance test (ITT), intraperitoneal glucose tolerance test (IPGTT), and intraperitoneal pyruvate tolerance test (IPTT), and analysis of GSIS

The ITT, IPGTT, and IPTT were respectively performed on days 29, 31, and 33 of ATRA treatment. For the ITT, the rats were fasted for 6 h and then administered insulin (1 IU/kg) i.p. For the IPGTT and IPTT, the rats were fasted for 12 h and then administered glucose (1.5 g/kg) or pyruvate sodium (1 g/kg) i.p., respectively. A GSIS test was performed simultaneously with the IPGTT.

Measurement of biochemical parameters

The levels of TG, TC, low-density lipoprotein, high-density lipoprotein, glutamic oxalacetic transaminase, and glutamic pyruvic transaminase were measured with test kits according to the instructions. Insulin levels in serum and pancreas were measured using an insulin ELISA kit. The concentrations of free fatty acids (FFAs) in the serum and pancreas were determined using HPLC [37]. The concentrations of ATRA in the pancreas were measured using HPLC as previously described [38].

qRT-PCR analysis

The mRNA levels of target genes were measured by quantitative real-time polymerase chain reaction (qRT-PCR). Total mRNA was extracted from the rat pancreas and INS-1 cells using TRIzol reagent (Takara, Japan) and reverse transcribed into cDNA using Hiscript III-RT SuperMix for qRT-PCR (Vazyme, Nanjing). qRT-PCR was performed using SYBR Green Master Mix (Yeasen, Shanghai) and corresponding primers (Table 1) on a LightCycler 96 real-time PCR system from Roche Applied Science (Indianapolis, IN, USA) according to the instructions. The mRNA levels of the corresponding target genes were normalized to the mRNA level of β-*actin*.

Western blot analysis

Proteins were extracted with RIPA lysis buffer. Equal amounts of proteins were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes. After blocking in 5% nonfat dry milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at 25 °C, the membranes were incubated with primary antibodies against SREBP-1c (1:500 dilution), UCP2 (1:200), and β-actin (1:6000 dilution) overnight at 4 °C; washed three times with TBST; and incubated with an HRP-conjugated secondary anti-mouse antibody (1:3000 dilution) for 2 h. The protein bands were visualized using a high-sensitivity ECL Western blotting substrate (Vazyme, Nanjing) with a gel imaging system (Tanon Science & Technology Co., Ltd., Shanghai).

Cell culture and drug treatment

INS-1 cells from LiXing Institute of Biotechnology (Nanjing, China) were placed in 6-well, 12-well, or 24-well plates at a density of 6×10^4 cells/mL and cultured with RPMI-1640 medium containing 10% FBS, 50 μM β-mercaptoethanol, 2 g/L $NaHCO_3$, 2 mM glutamine, 100 mg/L streptomycins, and 62.5 mg/L penicillin in a humidified incubator with a 5% CO_2 and 95% air atmosphere at 37 °C. When the

Table 1. Primer sequences for qRT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
β-actin	GCTATGTTGCCCTAGACTTCG	GCCACAGGATTCCATACCCAG
Srebp-1c	GGAGCCATGGATTGCACATT	AGGCCAGGGAAGTCACTGTCT
Fas	CTGGACTCGCTCATGGGTG	CATTTCCTGAAGCTTCCGCAG
Pdx-1	CGCCGCATGAAGTGGAAAAA	AGCCACAAACAACGCCAATC
Insulin-1	CCCGGCAGAAGCGTGGCATT	CATTGCAGAGGGGTGGGCGG
Insulin-2	GTGGTTCTCACTTGGTGGAAGCTC	CTCCAGTGCCAAGGTCTGAAGGT
Pc3	CTTCTTTTCTCTCAGCCCTTCCTAC	CATTCATTGACAAACTGCCTCTTC
Ucp2	GCTGGTGACCTATGACCTCATCAA	GTACTGGCCCAAGGCAGAGTTC
RARα	AACCCCTTCCTAGTGGTGGA	TCGATGGAGTGGTTTGAGCC
RARβ	TGGATTTCTACACCGCGAGC	GGATTGAGCAGTATGCCGGT
RXRα	CGCTCCTCAGGCAAACACTA	TTGTTGTCACGGCAGGTGTA
RXRβ	AGCTCCTCATTGCGTCCTTC	TGTCAGCACCCGATCAAAGA

cells reached 60% confluence, they were incubated with a culture medium containing ATRA (0.3–10 μM) for another 72 h, and the medium was replaced every day. After ATRA treatment, the cells were used to assess glucose uptake, glucose consumption, insulin secretion, ATP, TG, and FFA concentrations, and the expression of corresponding target proteins/genes. The incubation time was selected according to a preliminary experiment. 9-*Cis* RA (10 μM) served as a positive control. The roles of RXR, RAR, and PPARγ in the induction of SREBP-1c expression by ATRA were also investigated using the receptor antagonists Ro 41-5253 (RAR), HX531 (RXR), and GW9662 (PPARγ). Well-known agonists of the corresponding receptors TTNPB (RAR), 9-*cis* RA (RXR), LG100268 (RXR), and troglitazone (PPARγ) served as positive controls.

Measurement of insulin secretion levels, glucose uptake/glucose consumption, TG/FFA content, and ATP content in INS-1 cells treated with ATRA

Glucose-stimulated insulin secretion. GSIS was elicited in INS-1 cells according to a previously described method [26]. In brief, the INS-1 cells cultured with ATRA were washed twice with phosphate-buffered saline (PBS, pH = 7.4). Following starvation for 1 h in Krebs–Ringer bicarbonate buffer (KRBB) containing 3 mM glucose and ATRA, the buffer was replaced with KRBB containing ATRA and 23 mM glucose, and the cells were incubated for another 15 min. Insulin levels in the buffer solution, which was an index of insulin release, were measured using insulin ELISA kits. Insulin release was also measured in the presence of 5.5 mM glucose or 30 mM KCl. Insulin release and intracellular insulin levels were also measured at 15, 30, and 60 min stimulated with 23 mM glucose.

Glucose uptake. Cells cultured with ATRA were starved in low-glucose (3 mM) KRBB solution for 1 h. Then, the KRBB solution was replaced with high-glucose (23 mM) KRBB solution, and the cells were incubated for 15 min. Glucose levels in the KRBB solution were measured using glucose test kits, and the decrease in glucose levels was measured as an index of glucose uptake by cells.

Glucose consumption. Cells cultured with ATRA were starved for 1 h in low-glucose (3 mM) KRBB solution. Then, the cells were cultured in RPMI-1640 medium without FBS for 6 h. The residual glucose content in the medium was determined as an index of glucose consumption by the cells.

TG and FFA content. Cells cultured with ATRA were washed twice with cold PBS. Intracellular TG and FFA contents were measured using the Folch's method and HPLC, respectively, as described above.

ATP levels. The levels of intracellular ATP were simultaneously determined with GSIS using commercial ATP kits according to the manufacturer's instructions (Cayman Chemical, USA).

siRNA-mediated silencing of SREBP-1c and UCP2 in INS-1 cells

To confirm the contributions of SREBP-1c and UCP2 to the impairment of GSIS by ATRA, INS-1 cells were transfected with 100 nM SREBP-1c or UCP2 siRNA with Lipofectamine 3000. The siRNAs targeting SREBP-1c (5′–3′ sense sequence: AAUCUUGUCAUUG AUAGAG) and UCP2 (5′–3′ sense sequence: CGUAGUAAUGUUUGU CACC) were provided by GenePharma Co., Ltd., (Shanghai, China). After 6 h of transfection, the culture medium was replaced with fresh RPMI-1640. After 24 h, the mRNA levels of SREBP-1c and UCP2 were measured to confirm the silencing efficiency. After 24 h of transfection, the cells were subjected to the corresponding experiments as described above.

Analysis of RXR activation by ATRA by a luciferase reporter assay

HEK293T cells were cultured with DMEM containing 10% FBS. HEK293T cells were seeded in 6-well plates and incubated for 16 h. A total of 2 μg (1:1) Gal4-hRXRα LBD expression plasmid and pGL4.35[luc2P/9XGAL4UAS/Hygro] luciferase reporter plasmid were transfected into HEK293T cells using Lipofectamine 3000 and P3000 according to the manufacturer's instructions. After 24 h of transfection, the cells were digested and incubated in 96-well plates for 12 h. The cells were again incubated with ATRA (0.04–10 μM) for 24 h. The cultured cells were lysed, and luciferase activity was assayed according to the instructions of a dual luciferase assay kit (Yeasen, Shanghai, China).

Data analysis

All data are presented as the mean ± standard deviation (SD). Statistical significance among groups was evaluated by one-way ANOVA followed by Fisher's LSD multiple comparison test. $P < 0.05$ indicated a significant difference.

RESULTS

ATRA accelerates disease progression in diabetic rats

The food and water consumption, body weight, and FBG levels of experimental rats were measured during ATRA treatment. The results showed that compared with Wistar rats, GK-CON rats showed higher food and water consumption and FBG levels (Fig. 1). ATRA treatment decreased the water consumption of diabetic rats (Fig. 1b) but significantly accelerated the exacerbation of FBG levels in a dose-dependent manner (Fig. 1d). Biochemical parameters were further measured on day 35

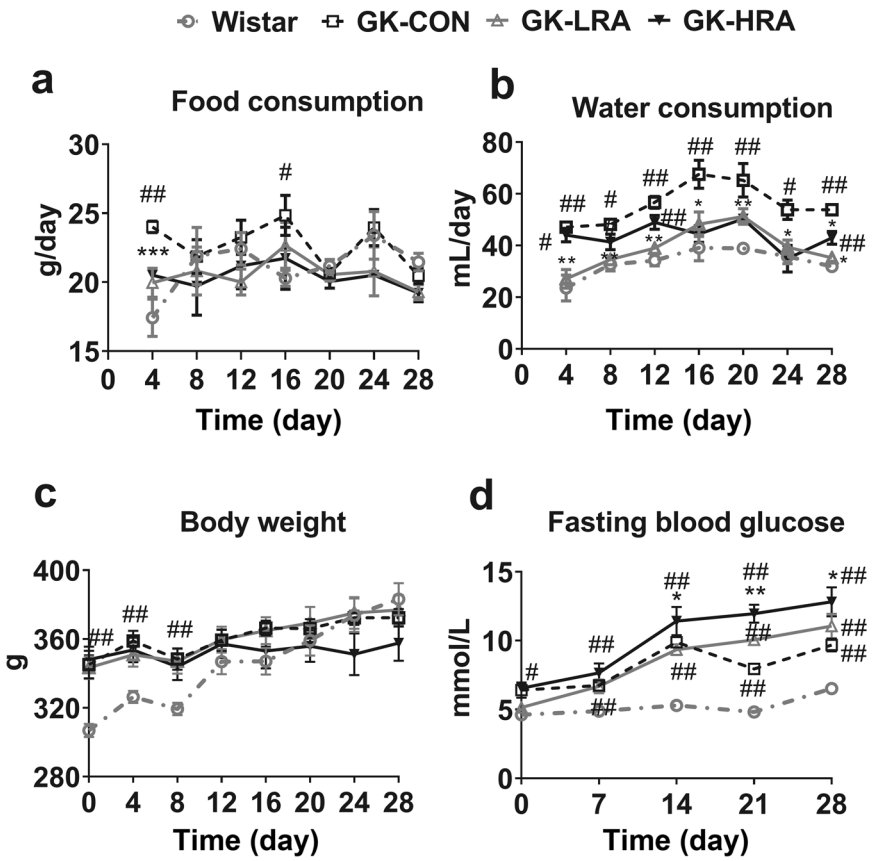

Fig. 1 ATRA worsens diabetes symptoms in GK rats. Changes in food consumption (**a**), water consumption (**b**), body weight (**c**), and fasting blood glucose (FBG) levels (**d**) in Wistar, GK-CON, GK-LRA, and GK-HRA rats during the experiment. Data are presented as means ± SD ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GK-CON rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Wistar rats.

following ATRA treatment (Table 2). ATRA dose-dependently increased the levels of FBG, and the contents of TG and FFAs in both the serum and pancreas of GK rats. Moreover, ATRA treatment also significantly enhanced HOMA-IR. All these results demonstrated that ATRA accelerates diabetes progression.

ATRA treatment impairs glucose homeostasis and GSIS in GK rats The ITT, IPTT, and IPGTT/GSIS tests were carried out on day 29, day 31, and day 33 during ATRA treatment, respectively. The results showed that compared with Wistar rats, GK-CON rats showed higher levels of blood glucose, serum insulin, and insulin resistance, which are characteristics of type 2 diabetes. The ITT results showed that ATRA enhanced insulin resistance in GK rats, leading to slower glucose elimination and higher levels of blood glucose (Fig. 2a). The IPTT results also demonstrated that ATRA enhanced gluconeogenesis (Fig. 2b), as evidenced by the significantly higher concentrations of blood glucose in ATRA-treated GK rats compared with GK-CON rats at 15 min following administration of pyruvate sodium. ATRA treatment tended to further impair glucose metabolism following administration of a loading dose of glucose (Fig. 2c), although no significant difference was observed. Serum insulin levels were also measured during the IPGTT. The results showed that in Wistar rats, a loading dose of glucose (i.p.) stimulated insulin release, with serum insulin secretion peaking at 15 min following glucose loading, but this insulin secretion peak was not observed in GK-CON rats or in GK rats treated with ATRA, indicating that GSIS was impaired. More importantly, ATRA treatment decreased serum insulin levels at 15 min and 30 min following glucose loading (Fig. 2d), inferring that ATRA treatment further enhanced the impairment of GSIS by diabetes. On day 35, insulin concentrations in the rat pancreas were measured, and the results showed that diabetes elevated pancreatic insulin concentrations, which were also further enhanced by ATRA treatment (Fig. 2e).

Factors related to insulin synthesis and release in the rat pancreas To further explore the mechanism by which ATRA impairs GSIS, the concentrations of ATRA in the pancreas were measured on day 35 following ATRA treatment. Compared with Wistar rats, GK-CON rats showed higher levels of pancreatic ATRA, and ATRA treatment further increased ATRA levels in the pancreas of GK rats (Fig. 3a). Histopathological examination demonstrated that the islets of Wistar rats were regular, showing a round shape with clear boundaries, while many vacuoles were found in the islets of GK-CON rats, and the islets of GK rats treated with ATRA were atrophic with blurred boundaries (Fig. 3b). Moreover, ATRA treatment also showed a trend toward a lower pancreas weight (Table 2). These results inferred that ATRA treatment enhanced the impairment of islets by diabetes, although a significant increase in insulin content was found in the pancreas of GK rats treated with ATRA (Fig. 2e).

The expression of target genes and proteins related to insulin synthesis/release was also measured. Compared with Wistar rats, GK-CON rats showed significantly lower mRNA expression of Pancreatic duodenal homeobox-1 (*Pdx-1*) (Fig. 3c), *Insulin-1* (Fig. 3d), *Insulin-2* (Fig. 3e) and Proinsulin convertase enzyme 3 (*Pc-3*) (Fig. 3f). ATRA treatment increased the expression of three genes related to insulin synthesis (Fig. 3d–f), which partly explained the increased pancreatic insulin concentration. Insulin release is considered to be linked to glucose metabolism and uptake, so the mRNA levels of *Glut2* and *Gck* were also measured. The results were not consistent with our expectation that ATRA

Table 2. Alterations in physiological and biochemical parameters in experimental rats.

	Wistar	GK-CON	GK-LRA	GK-HRA
Serum				
FBG (mmol/L)	5.84 ± 0.33	8.02 ± 1.30$^{\#\#}$	9.48 ± 1.43$^{\#\#}$	11.14 ± 1.90$^{**\#\#}$
Insulin (ng/mL)	1.35 ± 0.14	1.63 ± 0.42	2.34 ± 0.51$^{*\#\#}$	3.69 ± 0.73$^{**\#\#}$
HOMA-IR	7.45 ± 0.83	12.49 ± 4.42$^{\#}$	20.68 ± 4.22$^{*\#\#}$	39.40 ± 13.41$^{**\#\#}$
TG (mmol/L)	0.73 ± 0.14	0.78 ± 0.34	1.20 ± 0.35$^{*\#\#}$	2.54 ± 1.53$^{*\#\#}$
TC (mmol/L)	1.04 ± 0.07	1.55 ± 0.42$^{\#\#}$	1.42 ± 0.23$^{\#\#}$	1.31 ± 0.30
HDL (mmol/L)	1.83 ± 0.10	2.16 ± 0.54	2.17 ± 0.18$^{\#\#}$	1.72 ± 0.35
LDL (mmol/L)	0.73 ± 0.12	1.10 ± 0.26$^{\#\#}$	0.85 ± 0.25	0.94 ± 0.32
GPT (IU/L)	6.40 ± 0.78	6.64 ± 2.60	7.69 ± 2.71	12.56 ± 8.15
GOT (IU/L)	10.06 ± 6.14	10.84 ± 3.09	13.27 ± 4.14	16.03 ± 5.92
Palmitic acid	0.29 ± 0.048	0.17 ± 0.032$^{\#\#}$	0.27 ± 0.055**	0.39 ± 0.11**$^{\#}$
Leinoleic acid	0.38 ± 0.049	0.21 ± 0.042$^{\#\#}$	0.37 ± 0.11**	0.47 ± 0.13**
Stearic acid	0.032 ± 0.0086	0.027 ± 0.0064	0.038 ± 0.017	0.046 ± 0.013**$^{\#}$
Oleic acid (mmol/L)	0.27 ± 0.042	0.17 ± 0.048$^{\#\#}$	0.28 ± 0.071**	0.36 ± 0.12**
Pancreas				
P/B (%)	0.28 ± 0.019	0.22 ± 0.040$^{\#}$	0.18 ± 0.025$^{\#\#}$	0.19 ± 0.030$^{\#\#}$
TG (mmol/kg)	67.16 ± 5.1	27.18 ± 8.25$^{\#\#}$	38.69 ± 17.35$^{\#}$	50.04 ± 18.35*
TC (mmol/kg)	0.98 ± 0.030	2.36 ± 0.49$^{\#\#}$	3.31 ± 0.95$^{\#\#}$	5.73 ± 4.0$^{\#}$
Palmitic acid	0.46 ± 0.076	0.23 ± 0.11$^{\#\#}$	0.39 ± 0.090*	0.46 ± 0.21*
Leinoleic acid	0.43 ± 0.050	0.19 ± 0.095$^{\#\#}$	0.28 ± 0.074$^{\#\#}$	0.30 ± 0.097$^{\#}$
Stearic acid	0.074 ± 0.010	0.050 ± 0.020$^{\#}$	0.077 ± 0.019*	0.084 ± 0.029
Oleic acid (mmol/L)	0.80 ± 0.10	0.26 ± 0.096$^{\#\#}$	0.48 ± 0.097**$^{\#\#}$	0.46 ± 0.19$^{\#\#}$

P/B represents the ratio of pancreas weight to body weight. Data are presented as means ± SD ($n = 5$).
$^{*}P < 0.05$.
$^{**}P < 0.01$ vs. GK-CON.
$^{\#}P < 0.05$.
$^{\#\#}P < 0.01$ vs. Wistar.

treatment has little effect on the mRNA expression of *Glut2* and *Gck* in the pancreas of GK rats (Fig. 3g, h).

ATRA did not affect the expression of *Fas* mRNA (Fig. 3i) but significantly induced the expression of SREBP-1c mRNA (Fig. 3j) and protein (Fig. 3k) in the pancreas of rats. Significantly increased levels of TG and FFAs were also found in the pancreas of GK rats treated with ATRA (Table 2).

Effects of ATRA on GSIS in INS-1 cells

GSIS was measured in INS-1 cells treated with ATRA, and 9-*cis*-RA served as a positive control. First, time-dependent insulin release was measured between 0 and 15 min, between 15 and 30 min, and between 30 and 60 min after 23 mM glucose stimulation (Fig. 4a). The results showed that insulin secretion peaked at 15 min. At this point in time, ATRA concentration-dependently impaired insulin secretion stimulated by 23 mM glucose (Fig. 4b). Thus, the time point for measuring insulin release and the concentration of ATRA was set as 15 min and 10 μM, respectively. Importantly, we found that the effects of ATRA and 9-cis RA (10 μM) on GSIS were dependent on glucose levels. Both ATRA and 9-cis RA enhanced GSIS at low glucose levels (5.5 mM) without affecting insulin release by 30 mM KCl (Fig. 4c and d) but obviously impaired GSIS at high glucose levels (23 mM) (Fig. 4e). Furthermore, ATRA treatment did not affect the total insulin content in INS-1 cells (Fig. 4f), which was not consistent with the increased mRNA levels of *Insulin-1* and *Pc3* (Fig. 4g). Moreover, ATRA had little effect on the expression of GLUT2 and GCK mRNA (Fig. 4g) and protein (Fig. 4h), glucose uptake (Fig. 4i) and glucose consumption (Fig. 4j). All of the above results inferred that ATRA did not affect insulin secretion by influencing insulin synthesis or glucose metabolism and uptake.

In line with the in vivo data, ATRA also concentration-dependently increased the levels of TG (Fig. 4k) and FFAs (Fig. 4l) in INS-1 cells, and this effect was accompanied by obvious induction of SREBP-1c mRNA and protein expression (Fig. 4m and n). Several reports have demonstrated that elevated levels of FFAs, especially palmitic acid, can suppress GSIS [39, 40]. Therefore, the effect of palmitic acid on 23 mM GSIS was assessed. We found that a 48-h incubation with 0.35 mM palmitic acid, which is similar to the concentrations of palmitic acid in the pancreas and serum of experimental rats (Table 2), did not influence GSIS in INS-1 cells (Fig. 4o). The results were not consistent with a previous report [40]. The effects of higher concentrations of palmitic acid were not investigated due to the cytotoxicity of this FFA (Fig. 4p).

ATRA induces the expression of *Srebp-1c* by activating RXR

Srebp-1c overexpression itself can impair insulin release [41, 42]. RXRs have been reported to mediate the expression of Srebp-1c [29]. It is generally accepted that RAs exert their biological effects via RARs and RXRs. Whether ATRA induces *Srebp-1c* expression by activating the above nuclear receptors was further investigated in INS-1 cells. The results showed that 9-*cis* RA, ATRA, and the RXR agonist LG100268 markedly induced the expression of *Srebp-1c* mRNA, which was almost completely abolished by an RXR antagonist HX531 (Fig. 5a, b). Neither the RAR antagonist Ro 41-5253 nor the PPARγ antagonist GW9662 affected the induction of *Srebp-1c* mRNA by 9-*cis* RA and ATRA (Fig. 5a, c). Moreover, the RAR agonist TTNPB and PPARγ agonist troglitazone had little

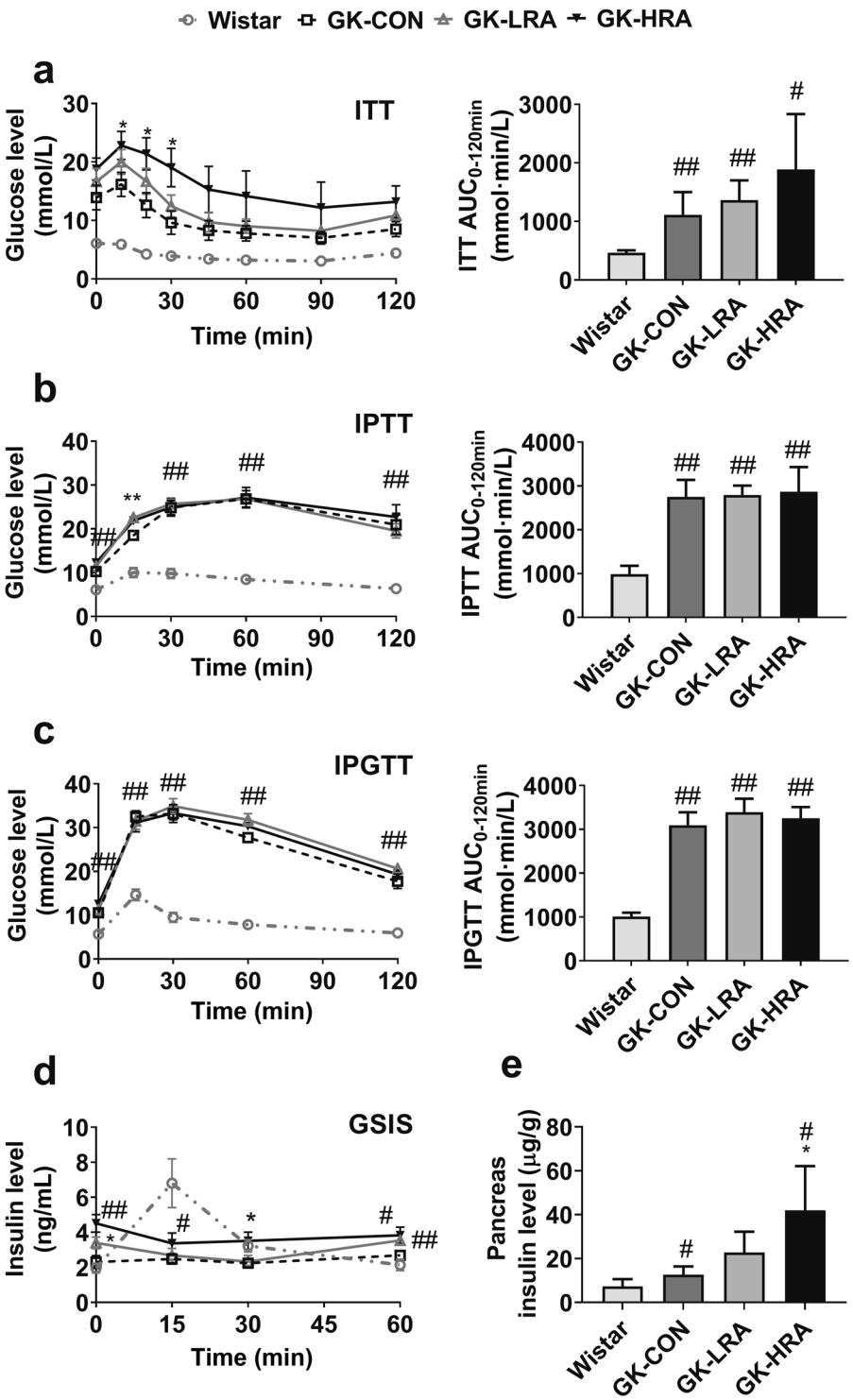

Fig. 2 Changes in glucose homeostasis and insulin levels in Wistar, GK-CON, GK-LRA, and GK-HRA rats. Blood glucose levels after an intraperitoneal insulin load (1.0 IU/kg) (**a**), pyruvate sodium load (1.0 g/kg) (**b**) and glucose load (1.5 g/kg) (**c**). Serum insulin concentrations during the intraperitoneal glucose tolerance test (**d**). Insulin levels in the pancreas of rats on day 35 after ATRA treatment (**e**). Data are presented as means ± SD ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GK-CON rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Wistar rats.

effect on the expression of *Srebp-1c* mRNA (Fig. 5b, c). Besides, higher expression of RXRs was detected in INS-1 cells (Fig. 5d). All these results indicated that ATRA induced *Srebp-1c* expression by activating RXR.

Activation of RXR by ATRA was further evaluated using HEK293T cells transfected with an RXRα-LBD luciferase reporter plasmid. The results demonstrated that ATRA concentration-dependently activated luciferase expression and that the estimated EC_{50} was 1.37 μM (Fig. 5e), which was lower than the observed ATRA concentrations (approximately 3 μM) in the pancreas of GK rats treated with a high dose of ATRA (Fig. 3a), inferring that ATRA can act as an agonist of RXRs in the rat pancreas.

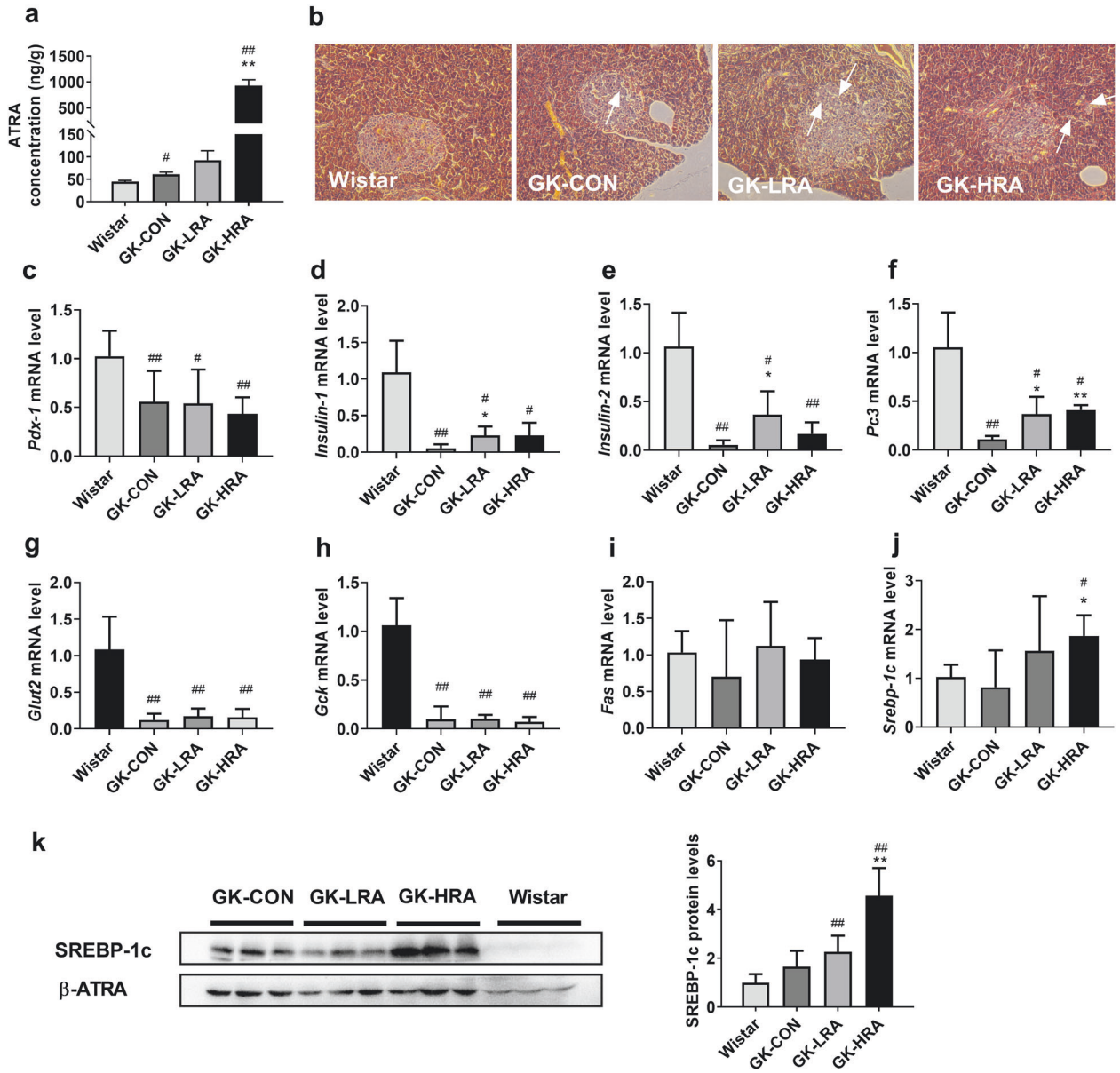

Fig. 3 Effects of ATRA on pancreatic insulin synthesis and release. The concentrations of ATRA in the pancreas of Wistar, GK-CON, GK-LRA, and GK-HRA rats (**a**). Histopathological examination of the pancreas of above rats (**b**), arrows indicate typical damage. mRNA levels of *Pdx-1* (**c**), *Insulin-1* (**d**), *Insulin-2* (**e**), *Pc3* (**f**), *Glut2* (**g**), *Gck* (**h**), and *Fas* (**i**), and expression of *Srebp*-1c mRNA (**j**) and protein (**k**) in the pancreas of above rats. Data are presented as means ± SD ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GK-CON rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Wistar rats.

ATRA impairs GSIS by inducing the expression of SREBP-1c and UCP-2

GSIS is considered to be dependent on ATP generation, which is highly controlled by UCP2 [43]. Overexpression of Srebp-1c has been reported to significantly enhance the expression of UCP2 mRNA, leading to a decrease in ATP content [41]. These results indicated that ATRA might impair GSIS by activating the SREBP-1c/UCP2 pathway. To confirm this speculation, UCP2 expression and ATP levels were measured in INS-1 cells treated with ATRA. Consistent with our expectation, ATRA significantly induced the expression of UCP2 mRNA and protein (Fig. 6a, b) and dose-dependently decreased the ATP content (Fig. 6c). The roles of SREBP-1c in ATRA-mediated impairment of GSIS were confirmed by silencing of *Srebp-1c* or by using the SREBP-1c inhibitor fatostatin (FA). The mRNA and protein levels of SREBP-1c in INS-1 cells transfected with SREBP-1c siRNA were decreased to 30% and 52% of those in control cells, respectively (Fig. 6d, e). *Srebp-1c* silencing downregulated UCP2 expression, increased ATP levels, and induced GSIS. More importantly, *Srebp-1c* silencing significantly attenuated the ATRA-induced increase in UCP2 expression, decrease in ATP content, and impairment of GSIS (Fig. 6e, g, h). Consistent with the effects of *Srebp-1c* silencing, 10 μM SREBP-1c inhibitor FA also significantly reversed the ATRA-induced changes described above (Fig. 6f, i, j), further demonstrating that ATRA impairs GSIS by inducing SREBP-1c. The role of UCP2 in ATRA-mediated impairment of GSIS was also demonstrated in *Ucp2*-silenced INS-1 cells. The UCP2 mRNA (Fig. 6n) and protein (Fig. 6k) levels in cells treated with UCP2 siRNA were decreased to 30% and 50% of those in control cells, respectively. *Ucp2* silencing increased the ATP content and GSIS. Furthermore, *Ucp2* silencing almost completely abolished the ATRA-mediated induction of UCP2 expression, decrease in ATP content, and impairment of GSIS (Fig. 6k–m). In vivo data also showed that ATRA treatment increased *Ucp2* mRNA (Fig. 6o) and SREBP-1c mRNA and protein

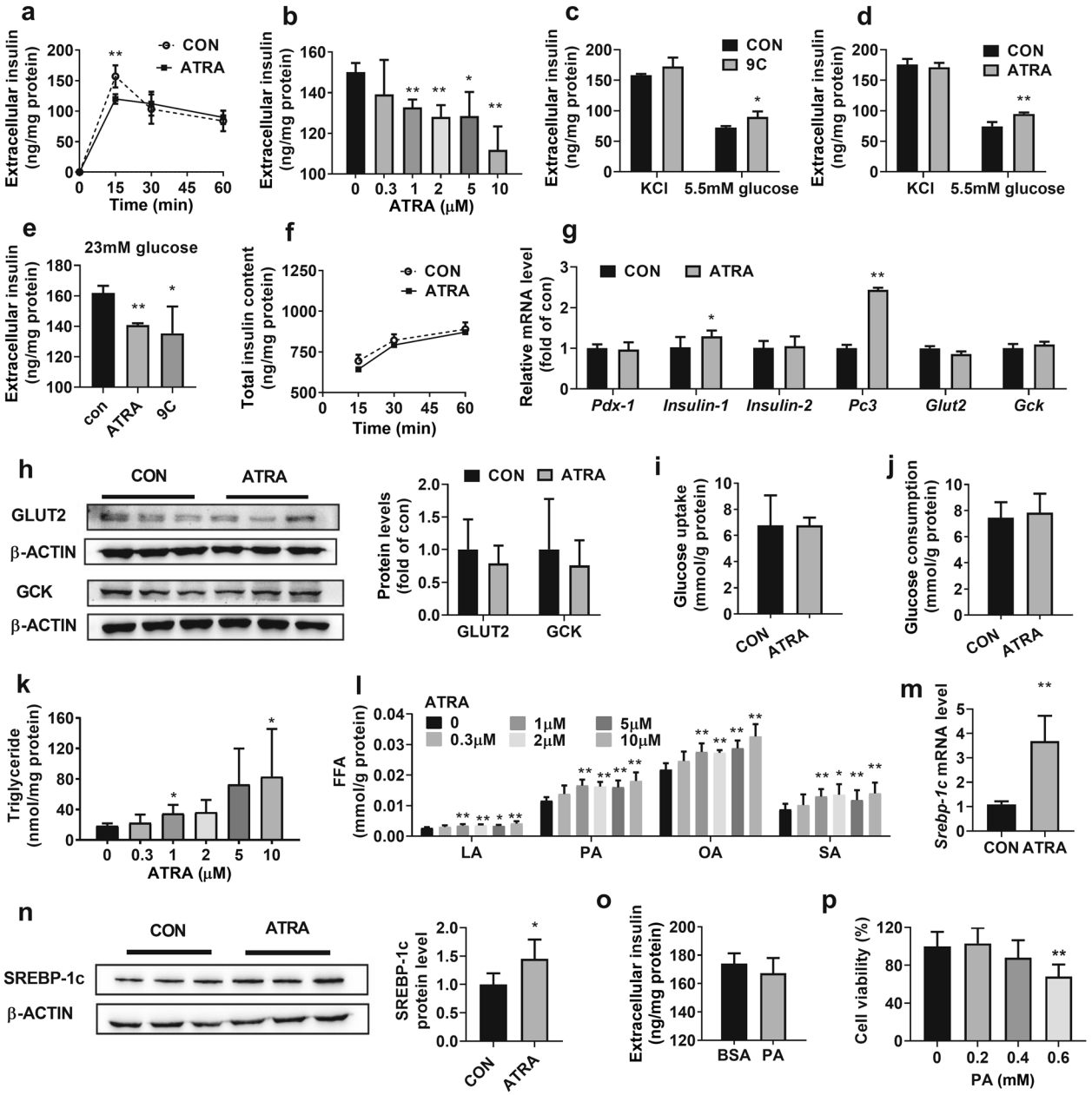

Fig. 4 Effects of ATRA on GSIS and glucose and lipids metabolism in INS-1 cells. Time- and concentration-dependent effects of ATRA on 23 mM glucose-stimulated insulin secretion (GSIS) (**a**, **b**, $n = 4$). Effects of 9-*cis* RA and ATRA on insulin secretion at 5.5 mM glucose, 30 mM KCl (**c**, **d**), or 23 mM glucose stimulation (**e**) ($n = 4$). Effects of ATRA on total insulin content in INS-1 cells at 23 mM glucose stimulation (**f**, $n = 4$). Effects of ATRA on the expression of insulin synthesis- and secretion-related genes (**g**), levels of GLUT2 and GCK protein (**h**), glucose uptake (**i**), and glucose consumption (**j**) in INS-1 cells ($n = 6$). Effects of ATRA on the levels of TG (**k**, $n = 4$), FFAs (**l**, $n = 6$), and expression of SREBP-1c mRNA (**m**, $n = 4$) and protein (**n**, $n = 6$) in INS-1 cells. Effects of palmitic acid (PA) on GSIS (**o**) and cell viability (**p**) ($n = 4$) in INS-1 cells. Data are presented as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. CON or 0.

(Fig. 3j, k) expression. All these results demonstrated that ATRA impairs GSIS by upregulating SREBP-1c expression and promoting UCP-2 expression, leading to a decrease in ATP content.

DISCUSSION

Extensive clinical data have suggested vitamin A disorders in diabetic patients [44, 45], indicating that vitamin A may be involved in diabetes progression. However, the roles of RAs, especially ATRA, in diabetes are complex. The main findings of the present study were that ATRA treatment worsened diabetes symptoms and impaired GSIS, glucose metabolism, and insulin resistance. Several reports have shown that ATRA treatment decreases blood glucose levels, prevents the progression of diabetes, and improves β-cell functions in streptozotocin-induced diabetic mice and rats [46–48]. The discrepancies in the findings might result from differences in the type of diabetes studied and the diabetic models used. It is generally accepted that glucose and lipid metabolism disorders are the main side effects of clinical use of RAs, occurring in greater than 30% of individuals treated with RAs [49, 50], which partly supports our findings that ATRA worsens diabetes symptoms.

The present study showed that normal rats exhibited an obvious peak in insulin levels 15 min following administration of a

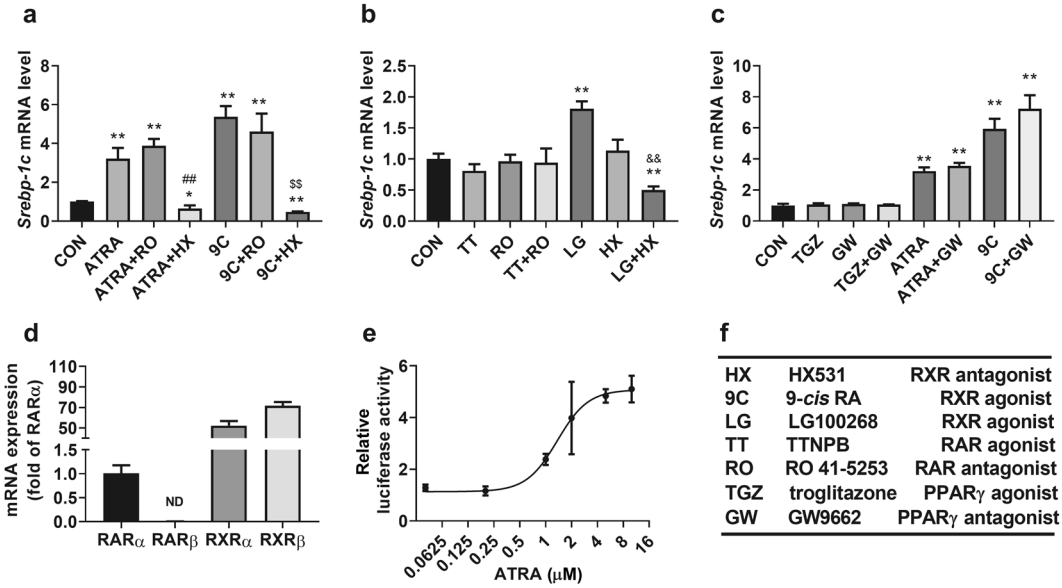

Fig. 5 ATRA activates RXR to induce the expression of *Srebp-1c*. Effects of ATRA, 9-*cis* RA, HX531, and RO 41-5253 (**a**), TTNPB, LG100268, HX531, and RO 41-5253 (**b**), ATRA, 9-*cis* RA, troglitazone, and GW9662 (**c**) on *Srebp-1c* mRNA expression in INS-1 cells. The mRNA expression of RARα, RARβ, RXRα, and RXRβ in INS-1 cells (**d**). Concentration-dependent effects of ATRA on the RXRα relative luciferase activity in HEK293T cells transfected with RXRα-LBD luciferase reporter plasmid (**e**). The abbreviations of agonists and antagonists we used (**f**), the concentrations of ATRA and 9-cis RA were 10 μM, concentrations of all other agonists and antagonists were 2 μM. Data are presented as means ± SD ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. CON. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ATRA. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. 9 C; $^{\&\&}P < 0.01$ vs. LG.

loading dose of glucose, but insulin levels did not peak in GK-CON rats or GK rats treated with ATRA. More importantly, at 15 min following administration of a loading dose of glucose, the insulin levels of GK rats treated with a high dose of ATRA were significantly lower than basal insulin levels, which demonstrated that diabetes impaired GSIS and that the impairment of GSIS was enhanced by ATRA. The expression of RALDH3 (ALDH1A3) has been reported to be obviously upregulated in the islets of patients with type 2 diabetes, and elevated pancreatic RALDH3 expression is thought to be related to insulin secretion impairment [51]. Similar results have also been found in diabetic *db/db* mice, which is in line with the significantly increased ATRA concentration in the pancreas of *db/db* mice [11]. The present study demonstrated that GK rats also showed higher pancreatic ATRA levels than normal control rats (Wistar rats). These results indicated that impairment of GSIS partly resulted from increased pancreatic ATRA levels.

We also found that ATRA treatment aggravated insulin resistance, which may be one of the causes of diabetes symptoms aggravation and needs to be further investigated. The relationships between insulin resistance and ATRA have been demonstrated, but the results are inconsistent. One report demonstrated that ATRA treatment relieves insulin resistance in high-fat diet-fed animal models [52], but other studies have shown that ATRA can induce insulin resistance and enhance TG accumulation in the rat serum [16, 17]. And clinical data have proven that elevated ATRA concentrations tend to increase lipid accumulation [15], thus inducing insulin resistance. These discrepancies might come from differences in diabetes-related conditions.

The present study also showed that ATRA increased insulin concentrations in the pancreas of GK rats, which was partly attributed to the induction of insulin synthesis-related gene expression. However, this phenomenon did not occur in INS-1 cells. ATRA impaired GSIS without affecting the cellular insulin concentration. These inconsistencies may result from differences in in vivo and in vitro conditions. In vivo, the pancreatic insulin content is influenced by multiple factors, such as the number of pancreatic β-cells and insulin synthesis and secretion. ATRA has been reported to influence β-cell function and mass [18–20], and the increase in insulin concentration in the pancreas induced by ATRA was comprehensive. Importantly, ATRA treatment obviously inhibited insulin secretion 15 min after glucose stimulation. Thus, we focused on the impairment of GSIS by ATRA. GSIS is thought to be linked to glucose metabolism. However, ATRA treatment had little effect on the expression of *Glut2* and *Gck* in both GK rats and INS-1 cells, which cannot explain why ATRA treatment impaired GSIS.

The mechanisms by which ATRA impairs GSIS were further investigated using INS-1 cells. Interestingly, we found that the effects of ATRA or 9-*cis* RA on GSIS were dependent on glucose levels, as insulin release was impaired under a high concentration of glucose (23 mM) but stimulated by a low concentration of glucose (5.5 mM), which is consistent with previous reports [21, 26, 53]. It is worth noting that the effect of ATRA on lipid metabolism and SREBP-1c expression has also been reported to be dependent on glucose levels [27], inferring a connection between GSIS and SREBP-1c expression mediated by ATRA. The present study also demonstrated that incubation with ATRA concentration-dependently increased the levels of FFAs and TG and induced SREBP-1c expression. However, it is inconsistent with a previous report [40] that palmitic acid at a concentration of 0.35 mM, which is similar to the level found in the rat pancreas, does not impair GSIS. KCl depolarization is widely used to mimic depolarization during GSIS [54]. Our results showed that ATRA did not affect insulin release induced by 30 mM KCl. All these results indicated that ATRA impairs GSIS via a novel mechanism.

UCP2 has been reported to negatively regulate GSIS by affecting ATP generation [43]. Importantly, *Srebp-1c* overexpression induces the expression of *Ucp2* mRNA [41]. In contrast, *Srebp-1c* knockout significantly downregulates *Ucp2* expression [40]. The present study demonstrated that ATRA markedly induced the expression of SREBP-1c, increased UCP2 expression, decreased the ATP content, and impaired GSIS and that these effects were almost completely abolished by both treatments with an SREBP-1c inhibitor and *Srebp-1c*/*Ucp2* silencing. Rat experiments also

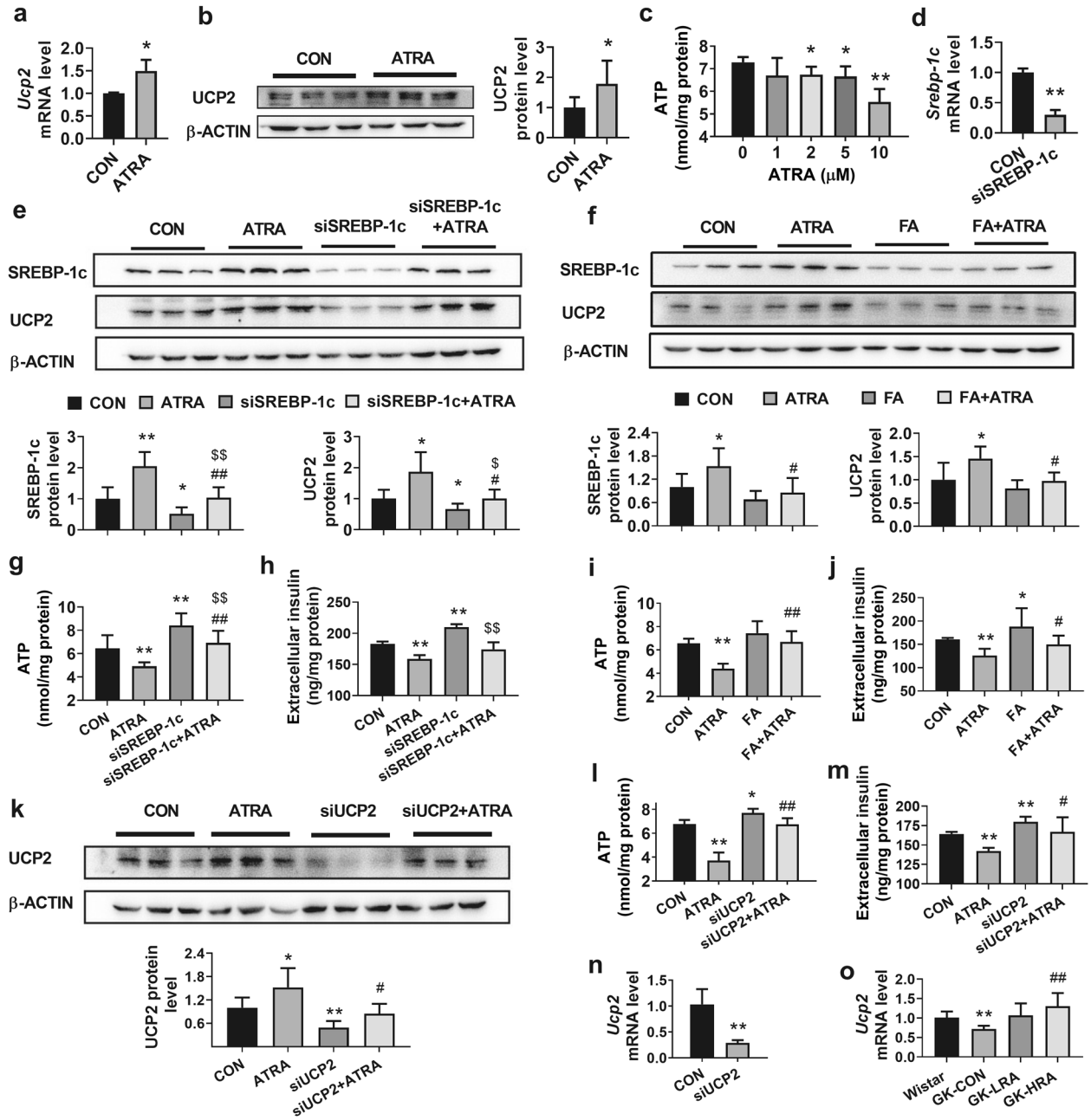

Fig. 6 ATRA impairs GSIS by activating the SREBP-1c/UCP2 pathway. Effects of ATRA on the expression of UCP2 mRNA (**a**, $n = 3$) and protein (**b**, $n = 6$) and ATP content (**c**, $n = 6$) in INS-1 cells. The mRNA level of *Srebp-1c* in INS-1 cells transfected with SREBP-1c siRNA (siSREBP-1c) (**d**, $n = 3$). The levels of SREBP-1c and UCP2 protein (**e**, $n = 6$), ATP (**g**, $n = 6$) and GSIS (**h**, $n = 4$) in siSREBP-1c and ATRA treated INS-1 cell, or in Fatostatin (FA) and ATRA treated INS-1 cell (**f**, **i**, **j**). Effects of ATRA and siUCP2 on levels of UCP2 protein (**k**, $n = 6$), ATP (**l**, $n = 6$), and GSIS (**m**, $n = 4$). Expression of *Ucp2* mRNA in INS-1 cells under siUCP2 treatment (**n**, $n = 3$), or in the pancreas of experimental rats (**o**, $n = 5$). Data are presented as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control cells (CON) or Wistar rats. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ATRA treatment cells (ATRA) or GK-CON rats; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. siSREBP-1c or siUCP2 groups.

showed that ATRA dose-dependently increased pancreatic *Ucp2* mRNA and SREBP-1c mRNA and protein expression, which is consistent with GSIS impairment. All these results demonstrated that ATRA impaired GSIS by inducing the expression of SREBP-1c and then promoting UCP-2 expression, ultimately leading to a decrease in ATP content.

Overexpression of RXR has been reported to enhance the expression of *Srebp-1c* mRNA by 9-*cis* RA [55]. The present data also showed that similar to 9-*cis* RA and LG100268, ATRA markedly induced the mRNA expression of Srebp-1c, which was almost completely abolished by HX531 but not Ro 41–5253, demonstrating that ATRA may induce *Srebp-1c* mRNA expression by activating RXR, which is in line with a previous report [29]. ATRA was thought to only activate RAR, but a recent study showed that ATRA can also recruit coactivators to activate RXR [56]. High expression of RXRα and RXRβ has been detected in INS-1 cells, and ATRA concentration-dependently induced RXRα-LBD luciferase activity, showing an EC_{50} of 1.37 μM. These results indicated that pancreatic ATRA levels, especially those in diabetic rats treated with a high dose of ATRA (approximately 3 μM), were high enough to activate RXR. Although 9-*cis* RA is considered a strong endogenous ligand of RXRs, the in vivo level of pancreatic 9-*cis*

RA is much lower than that of ATRA [11]. The roles of 9-*cis* RA in pancreatic GSIS still need to be further investigated.

In conclusion, our research revealed and proved that ATRA upregulates the expression of SREBP-1c by stimulating RXR and then inhibiting GSIS through the SREBP-1c/UCP2/ATP pathway in both GK rats and INS-1 cells, which can partly explain the aggravation of diabetes symptoms induced by ATRA. The results highlight the roles of ATRA in diabetes progression and establish new strategies for diabetes treatment. Moreover, ATRA has been widely used in the treatment of some cancers and skin diseases, and patients receiving ATRA treatment are at high risk of glucose and lipid metabolism abnormalities [15, 57], which may also be partly attributed to ATRA-induced impairment of GSIS.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (Nos. 81673505, 81872930, and 82073922), "333" Project of Jiangsu Province (BRA2020287).

AUTHOR CONTRIBUTIONS

HYY, ML, LL, and XDL designed the research. ML, HYY, YS, LZ, MMJ, TXJ, LY, and PHL completed the experiments. ML analyzed the data. HYY, ML, LL, and XDL wrote the paper. All authors reviewed the manuscript and approved the final version.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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