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FEBS Letters 588 (2014) 1071–1079

FEBS Letters
journal homepage: www.FEBSLetters.org

Identification of insulin as a novel retinoic acid receptor-related orphan receptor α target gene

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A R T I C L E I N F O

Article history:
Received 22 December 2013
Revised 27 January 2014
Accepted 14 February 2014
Available online 25 February 2014

Edited by Laszlo Nagy

Keywords:
Retinoic acid receptor-related orphan receptor α
Insulin
Promoter
Gene transcription

A B S T R A C T

Insulin plays an important role in regulation of lipid and glucose metabolism. Retinoic acid receptor-related orphan receptor α (RORα) modulates physiopathological processes such as dyslipidemia and diabetes. In this study, we found overexpression of RORα in INS1 cells resulted in increased expression and secretion of insulin. Suppression of endogenous RORα caused a decrease of insulin expression. Luciferase and electrophoretic mobility shift assay (EMSA) assays demonstrated that RORα activated insulin transcription via direct binding to its promoter. RORα was also observed to regulate BETA2 expression, which is one of the insulin active transfactors. In vivo analyses showed that the insulin transcription is increased by the synthetic RORα agonist SR1078. These findings identify RORα as a transcriptional activator of insulin and suggest novel therapeutic opportunities for management of the disease.

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1. Introduction

Insulin is a peptide hormone, produced by beta cells of the pancreas and plays an important role in regulation of lipid metabolism and glucose metabolism [1,2]. The variation of insulin level can induce many metabolism diseases, such as obesity and dyslipidemia, especially diabetes [3]. Therefore, identification and characterization of the factors regulating insulin transcription and secretion will provide insights into the pathogenesis of these diseases. The expression of the insulin gene in pancreatic beta cell is regulated by combination of transcription factors [4], such as PDX1 [5], MAFA [6,7], BETA2 [8].

Nuclear hormone receptors (NHRs) function as ligand-dependent DNA binding factors that translate hormonal, nutritional and pathophysiological signals into gene regulation [9–11]. In the context of metabolism, NHRs control lipid and glucose homeostasis in an organ- or tissue-specific manner [12]. Dysfunctional NHR signalling has been associated with dyslipidaemia, insulin resistance, diabetes and obesity [13–15]. Hence NHR has an important role in regulating insulin biosynthesis and is increasingly recognized as an important target for combating type 2 diabetes [15].

Retinoic acid receptor-related orphan receptor (ROR) alpha is a member of the NHR superfamily [16]. RORα has the typical structure of a nuclear receptor including a ligand-binding domain linked to a DNA-binding domain via a hinge region and an N-terminal region that modulates its transcriptional activity. RORα regulate gene expression by binding as a monomer to ROR-responsive elements (ROREs) found in target gene promoters. A typical RORE is composed of a 5-bp AT-rich sequence preceding the half-core (A/G)GGTCA motif [16]. RORα has been identified to control the transcription of the genes important in regulation of lipid and glucose metabolism, such as genes encoding apolipoprotein A1, A5 and C3, and glucose 6-phosphatase [17–20]. A spontaneous mutation consisting of a deletion within the RORα gene that prevents translation of the RORα ligand-binding domain has been identified in the staggerer mouse [21]. In addition to severe neurological disorders, the homozygous staggerer ($ROR\alpha^{sg/sg}$) mice display metabolic abnormalities. These mice have severe hypoalphalipoproteinemia and atherosclerosis on an atherogenic diet [22,23]. Phenotypes observed in $ROR\alpha^{sg/sg}$ mice also revealed a role for RORα in modulating diet-induced obesity, insulin sensitivity and glucose uptake [24,25]. Moreover, RORα was identified as a diabetes susceptibility locus in Mexican Americans [26]. We recently found that the plasma insulin levels were regulated by locus containing RORα on mouse chromosome 9 [27]. However, the functional mechanism of RORα in the regulation of insulin synthesis is remains unclear.

In the present study, we demonstrated that RORα positively and directly regulates insulin gene expression. In addition, we have

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http://dx.doi.org/10.1016/j.febslet.2014.02.029
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provided evidence that RORα regulate insulin expression indirectly by stimulating the expression of BETA2, which is one of the insulin gene active transcription factors. Our data identify insulin as a novel target gene for RORα and suggest a new role for RORα in diabetes.

2. Materials and methods

2.1. Plasmid and virus

The rat Ins2 gene promoter (−754 to +188) was amplified from genomic DNA of INS-1 cells and cloned into pGL3-Basic reporter vector (Promega, USA). The cDNA encoding full-length mouse RORα was amplified by RT-PCR and then subcloned into the pAdTrack-CMV vector through SalI and HindIII restriction sites. Mouse RORα has 98% identity with rat RORα at the amino acid level. The resultant plasmid was then transformed into BJ5183 cells containing the Adeasy-c1 adenoviral backbone plasmid to produce the recombinant adenovirus plasmid, which was then transfected into Hek293 cells by Lipofectamine™2000 (Invitrogen, USA) to allow packaging and amplification of the recombinant adenovirus (Ad-RORα). The adenovirus was purified using Adeno-x™ Virus Mini Purification Kit (Clontech) and the infective titer was determined by a limiting dilution plaque assay. The efficiency of adenoviral infection was examined by using the adenovirus Ad-RORα tagged with green fluorescence protein.

2.2. Overexpression and knockdown

The rat insulinoma cell line (INS-1) was cultured in RPMI-1640 (Gibco) containing 11.2 mmol/l glucose and 2 mmol/l L-glutamine at 37 °C with 5%CO_2. The medium was supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 unit/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 50 μmol/l 2-mercaptoethaol (Gibco). INS-1 cells were plated in 6-well plate one day before infection. The cells were infected with adenovrius at the moi of 40 and cultured in 2% FBS medium for 4 h, and then switched to regular growth media. 48 h later, the media were collected for ELISA, and the cells were harvested for quantitative PCR or Western blot. The water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Mannheim, Germany) was performed to assess the effects of adenovirus and adenovirus-mediated RORα overexpression on the INS-1 cell viability. For knockdown assay, INS-1 cells were transfected with 4 μg/well control shRNA or RORα shRNA plasmid (GenePharma, Shanghai, China) using Lipofectamine™2000. After 48 h, the cells were collected to perform quantitative PCR assay or Western blot. The media were subjected to ELISA.

2.3. RNA and protein analysis

Total RNA was extracted from INS-1 cells using Trizol reagent (Invitrogen). We performed real-time quantitative PCR analysis as previously described (primers in Table 1) [28].

For Western blot assay, INS-1 cells were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholic sodium salt, 50 mM Tris HCl, pH 7.4, 2 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, one tablet of complete inhibitor cocktail (Roche Diagnostics) in 30 ml). Protein concentration was measured using a BCA protein assay kit (Qiagen), 20 μg proteins from each sample was separated by 10% SDS–PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore) and immunoblotted with the respective primary antibodies. Antibodies were anti-RORα antibody (Santa Cruz), anti-BETA2 antibody (Cell Signal technology) and anti-β-actin (Milipore). Blots were then washed and subsequently incubated with the secondary horseradish peroxidase-conjugated antibodies (Sigma–Aldrich). Signals were revealed by using an enhanced chemiluminescence kit according to manufacturer's instructions (Millipore).

Table 1
Sequence of primers.

Gene	Primers sequence (5′-3′)	
	Forward	Reverse
Ins1	AGCAAGCAGGTCATTGTTCC	ACGACGGGACTTGGGTGT
Ins2	GGAGCGTGGATTCTTCTACAC	TGCCAAGGTCTGAAGGTCAC
Pdx1	CCAGAGTTCAGTGCTAATCC	CCCAGTCTCGGTTTCATTCG
Beta2	TCCAGGGTTATGAGATCGTCAC	TTCTTGTCTGCCTCGTGTTCC
Mafa	CTGCTCCTCGGTGCTCTCTT	GGTTCAGGTGGTGCTGATACC
Gapdh	CTCTCTGCTCCTCCCTGTTC	GACTGTGCCGTTGAACTTGC

2.4. Transient transfection and dual luciferase activity assay

INS-1 cells plated in 96-well plate were transiently transfected with 20 ng/well of control or Ins2 promoter reporter plasmid using Lipofectamine™2000 (Invitrogen). 12 h post-treatment, the cells were infected with vehicle or RORα adenovirus for an additional 24 h. For RNAi assay, RORα shRNA or control shRNA was individually, with Ins2 promoter reporter plasmid, cotransfected into INS-1 cells. The luciferase activity was measured by using the Dual-Glo™ luciferase assay system according to the manufacturer's protocols (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity (firefly luciferase/Renilla luciferase) and presented as relative luciferase activity.

2.5. Enzyme linked immunosorbent assay (ELISA)

To examine insulin secretion, the cells were washed twice with Krebs-Ringer–bicarbonate–HEPES (KRBH) buffer (117 mM NaCl, 4.75 mM KCl, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.18 mM KH_2PO_4, 20 mmol/l HEPES, 2.54 mM $CaCl_2$, 5.6 mM glucose, pH 7.4). The cells were then starved in KRBH buffer containing 5.6 mM glucose for 30 min and subsequently incubated for 1.5 h in KRBH buffer with 16.7 mM glucose. Insulin in the media was measured using rat/mouse insulin ELISA kit (Millipore). To determine the cellular insulin content, the cells were washed with phosphate-buffered saline and lysed in ice-cold lysis buffer. After sonication and centrifugation, the insulin levels in the supernatant were determined by using ELISA assay.

2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins from INS-1 cells with or without RORα adenovirus were extracted by NE-PER® nuclear and cytoplasmic extraction reagents kit (Thermo). The 5′ end Biotin-labeled wild-type probe 5′-AAACAGCAAAGTCCAGG<u>GGTCA</u>GGG-3′ or mutant probe 5′-AAACAGCAAAGTCCAGG<u>AAGGC</u>GGG-3′ (potential RORE indicated in underlined) (Qiagen) was incubated with nuclear protein in binding buffer for 20 min using a LightShift chemiluminescent EMSA kit (Thermo). For oligonucleotide competition experiments, unlabeled oligonucleotides were added to the reaction at 200-fold molar excess to the labeled probes. For RORα supershift analysis, RORα antibody (Santa Cruz, USA) was added. The DNA–protein complexes were separated on a 5% non-denaturing poly-acrylamide gel. Then the DNA–protein complexes were transferred onto nylon membranes, and visualized by a Chemiluminescent nucleic acid detection module (Thermo).

2.7. Animal experiments

All experiments were in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals and the International Association for the Study of Pain Research Guidelines. Animal care and experimental procedures were approved by the Ethics Committee of Animal Experimentation of Sichuan University. Eight-week-old male C57BL/6J mice were housed in a temperature- and humidity-controlled room with a 12 h light/12 h dark cycle, and were given standard chow containing 6% fat and water ad libitum.

2.7.1. Immunofluorescence staining of mouse pancreas

Mouse pancreas was dissected and flash frozen in liquid nitrogen, and then embedded in OCT compound (Tissue-Tek). Serial cryosections were mounted on chrome-alun-coated slides for Immunofluorescence staining. Slides were fixed with 3.7% formaldehyde, and then blocked in phosphate-buffered saline (PBS, pH 7.4) with 6% normal donkey serum and 0.3% Triton X-100 (Sigma–Aldrich). Thereafter, sections were incubated with a goat anti-mouse insulin antibody (Santa Cruz, 1:50 dilution) or with a rabbit anti-mouse RORα antibody (Santa Cruz, 1:100 dilution). After washing in PBS-Tween 0.02%, sections were immunostained with Alexa Fluor conjugated secondary antibodies (1:500, Life technologies). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Life technologies). Sections were mounted with Entellan (Merck Millipore). Image analysis was performed with a fluorescence upright microscope (ZEISS Axio Imager, Germany).

2.7.2. Determination of the effect of SR1078 on gene expression

SR1078 is a synthetic ligand that is able to function as an ROR-specific agonist [29]. Mice were fasted for 4 h and then injected intraperitoneally (i.p.) with SR1078 (10 mg kg^{-1}, Sigma–Aldrich) or dimethyl sulfoxide (DMSO) (5 mice/group). 2 h after SR1078 treatment, mice were injected with glucose (2 g kg^{-1} i.p.). The pancreas was harvested at the 30 min time point after the glucose injection. The tissues were mechanically disrupted and simultaneously homogenized in the presence of Trizol reagent (Invitrogen) for RNA preparation or RIPA buffer for protein extraction. RNA concentrations were measured with the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), while RNA quality was assessed by agarose gel electorphoresis (Fig. S1). Protein concentration was measured using a BCA protein assay kit (Qiagen). The expressions of RORα, Ins1, Ins2 and BETA2 were assessed by quantitative PCR assay or Western blot.

2.7.3. Intraperitoneal glucose tests

For glucose tolerance test, mice were fasted for 4 h and then treated with the RORα agonist SR1078 (10 mg kg^{-1} i.p.) or DMSO (5 mice/group). 2 h after treating with the compound SR1078, mice were injected with glucose (2 g kg^{-1} i.p.). Blood was collected for glucose determination from the tip of the tail at 0, 15, 30, 60, and 120 min after the glucose injection.

2.8. Statistical analysis

All data were shown as mean ± S.D with at least three independent experiments. The trapezoidal rule was used to determine the area under the curve (AUC). Statistical analysis was performed using GraphPad Prism and Student's *t*, $P < 0.05$ was considered statistically significant.

3. Results

3.1. RORα over-expression increases insulin expression and secretion

To identify the potential role of RORα in insulin regulation, we over-expressed adenoviral RORα in INS-1 cells and measured the expressions of Ins1 and Ins2 genes by real-time quantitative PCR. As shown in Fig. 1A, the RORα overexpression significantly increased the expression of Ins2 gene but not Ins1 gene compared with control adenovirus. Furthermore, we examined whether RORα overexpression would result in an alteration of cellular insulin content and insulin secretion. INS-1 cells were infected with RORα adenovirus or control virus, and after 48 h the amount of insulin in both cell and medium were measured using an ELISA. Overexpression of RORα led to a 1.4-fold increase in cellular insulin (Fig. 1B) and a 2.2-fold increase in secreted insulin (Fig. 1C) compared with the control virus. To rule out possible effects of RORα overexpression against apoptosis of INS-1 cells, the effect of adenovirus and adenoviral RORα on the viability of INS-1 cells was assessed using the WST-1 assay. As shown in Fig. 1D, there was no change in cell viability in cells transfected with adenoviral RORα compared with the empty vector group in almost all statistical evaluations. Collectively, these data indicate that RORα up-regulates insulin gene expression.

3.2. shRNA-mediated inhibition of endogenous RORα expression decreases insulin expression and secretion

To gain more insight into the involvement of RORα in the regulation of the insulin gene, we used shRNA transfection to knockdown endogenous RORα expression and examined its effect on endogenous insulin mRNA expression. As depicted in Fig. 2A, Western blot analysis confirmed that the RORα shRNA significantly decreased RORα protein expression. Furthermore, Fig. 2B demonstrated that the silence of RORα led to significant decrease in both Ins1 and Ins2 genes expression compared to the control shRNA as measured by quantitative PCR. In addition, we measured the amount of insulin in both cell and medium using an ELISA. A loss of RORα expression led to a 15.8% decrease in cellular insulin (Fig. 2C) and a 26.7% decrease in secreted insulin (Fig. 2D) as compared with the control shRNA. Taken together, these data confirm that RORα plays a key role in the physiological control of insulin gene expression in pancreatic beta cells.

3.3. RORα enhances the activity of Ins2 gene promoter by binding to RORE consensus

Data from overexpression and knockdown of RORα in INS-1 cells indicate that RORα regulates insulin expression. Next, we determined whether RORα can modulate Ins2 gene promoter transcriptional activity. Rat INS-1 cells were transiently co-transfected with a luciferase reporter vector driven by the rat Ins2 promoter fragment (−754 bp to +188 bp) and the adenoviral RORα. The results in Fig. 3A shows that RORα can induce a markedly higher Ins2 promoter activity. Inclusion of RORα resulted in an approximate 3-fold increase in Ins2 promoter-driven transcription. Then, we showed that the transcriptional activity of the Ins2 reporter construct was strongly increased in a dose-dependent manner by the cotransfection of RORα expression vector in rat INS-1 cells (Fig. 3B). This indicates that RORα regulates Ins2 positively. To confirm the role of RORα in the regulation of Ins2 promoter activity, we used shRNA transfection to knock down endogenous RORα

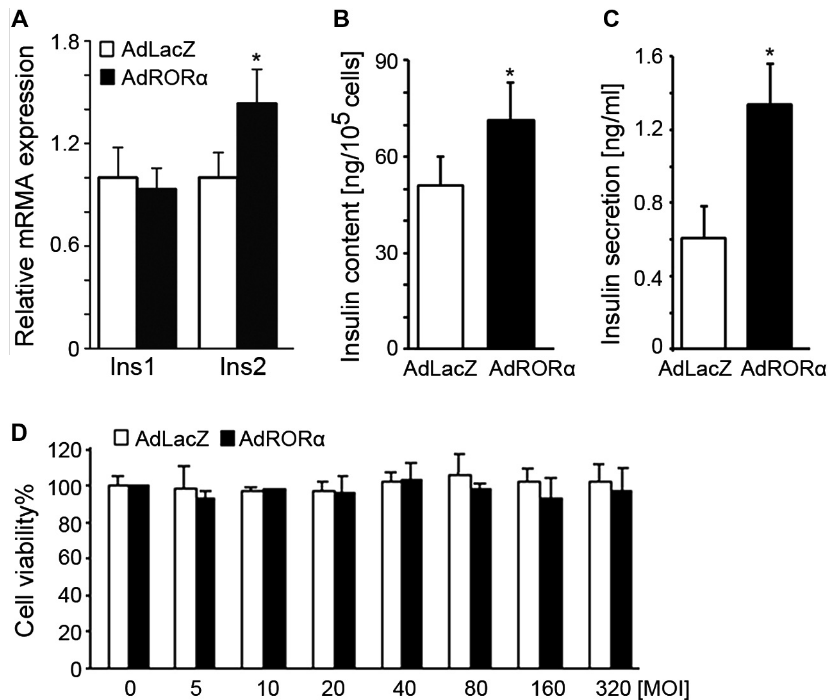

Fig. 1. Adenovirus-mediated RORα overexpression increases insulin expression and secretion. INS-1 cells were treated with control adenovirus or RORα adenovirus at multiplicity of infection of 40 MOI/cell for 48 h. (A) RORα overexpression in INS-1 cells increases Ins2 gene expression but no influence on Ins1 at the mRNA level as measured by quantitative PCR. The expression in controls (AdLacZ) was normalized to a value of 1.0. (B) Cellular contented insulin and (C) secreted insulin measured by ELISA. (D) Effect of adenoviral RORα on the viability of INS-1 cells was assessed by WST-1 assay. Values were presented as the means ± S.D. and representative of three separate experiments. *, indicated $P < 0.05$ relative to control adenovirus.

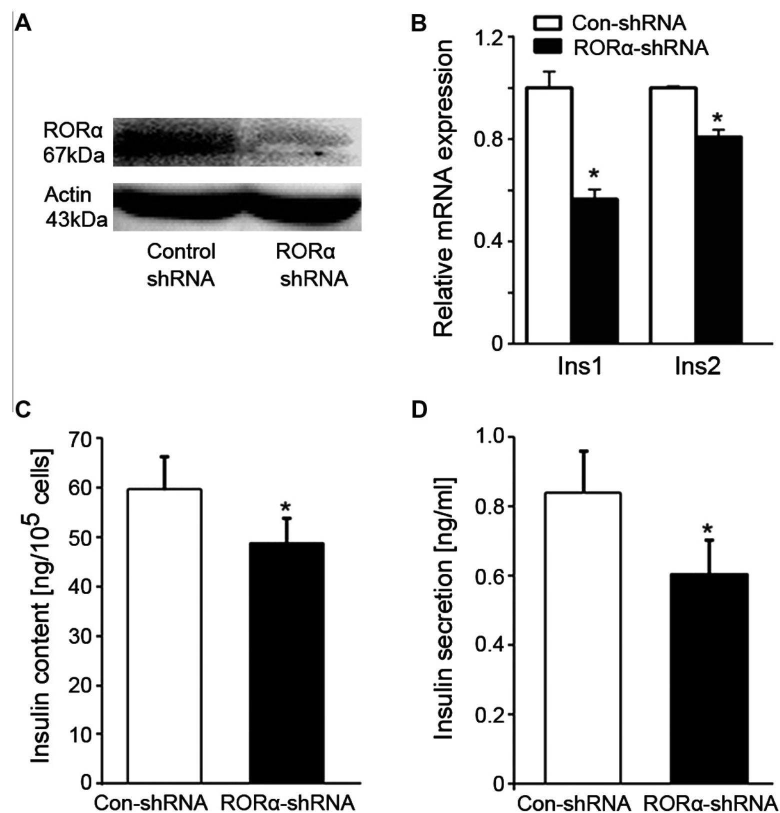

Fig. 2. Silence of RORA by shRNA results in decreased insulin expression and secretion. INS-1 cells were treated with control shRNA or RORα shRNA for 48 h. (A) Knockdown of RORα expression was confirmed by Western blot. β-actin was used as a loading control. (B) Decreased RORα expression in INS-1 cells leads to decreased expression of both Ins1 and Ins2 genes as measured by quantitative PCR. The control shRNA was normalized to a value of 1.0. Decreased RORα expression leads to both the cellular contented insulin (C) and secreted insulin (D) decreased as measured by ELISA. Values were presented as the means ± S.D. and representative of three separate experiments. *, indicated $P < 0.05$ relative to control shRNA.

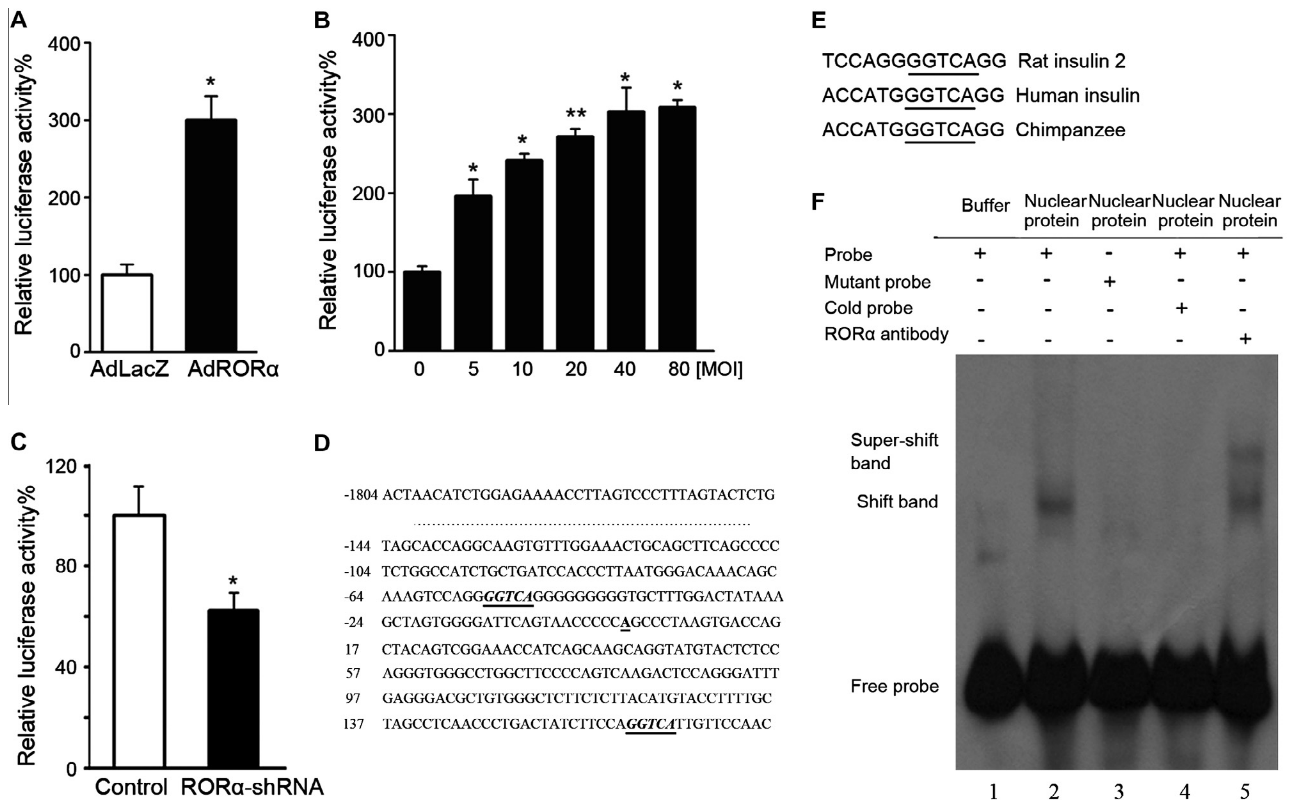

Fig. 3. RORα activates Ins2 gene promoter activity by binding to the RORE consensus. (A) INS-1 cells were transfected with control or RORα adenovirus along with the Ins2 reporter construct. The overexpression of RORα dramatically increases Ins2 promoter activity. (B) RORα increases Ins2 gene promoter activity in a dose-dependent manner. Luciferase values were normalized using Renilla luciferase. (C) INS-1 cells were transfected with control shRNA or RORα shRNA along with the Ins2 reporter construct. Knockdown of RORα expression reduces Ins2 gene promoter activity. (D) Schematic of the Ins2 promoter. Sequence analysis revealed the presence of two putative RORE located at nucleotides −54 bp and +162 bp from the transcription start site, respectively. (E) The RORE at nucleotide −54 bp is evolutionary conserved among rat, human and chimpanzee. (F) Using an EMSA assay, it is determined that RORα binding to the Ins2 RORE is specific and competitive. The labeled wild-type probe was incubated with control buffer without nuclear proteins (lane1). The labeled wild-type probe (lane 2) or labeled mutant-type probe (lane 3) was incubated with nuclear proteins in the absence of competitor. For competition, unlabelled oligonucleotide containing the consensus RORE (cold probe) was added at 200-fold excess (lane 4). Supershift assays with biotin labeled probe and nuclear extracts in the presence of anti-RORa antibody (lane 5). Values (a–c) are mean ± S.D. and representative of three separate experiments; $^{*}P < 0.05$ compared with corresponding controls.

expression in INS-1 cells. As shown in Fig. 3C, a 40% reduction of luciferase activity in the presence of RORα shRNA compared to the control shRNA was observed. These data further suggest that RORα may up-regulate Ins2 promoter activity by binding its promoter.

It has been identified that RORα regulates genes expression by binding a promoter special site which display characteristics of a classical RORE consensus containing an (A/G)GGTCA sequence. We searched for putative ROREs within the 2 kb of the Ins2 promoter and found two putative ROREs at nucleotides −54 bp and +162 bp from the transcription start site, respectively (Fig. 3D), the −54 bp site is conserved in human, rat, and chimpanzee (Fig. 3E). To confirm the physical interaction of RORα with the putative RORE sequences in Ins2 promoter, we performed the electrophoretic mobility shift assay (EMSA). Biotinylated double-strand oligonucleotide containing the Ins2 RORE (−71 to −47) was used as probe. A binding complex was formed between RORα and the labeled wild-type Ins2 RORE (Fig. 3F, lane 2). However, the binding was not observed with mutated probe (Fig. 3F, lane 3). Similarly, RORα binding was completely abolished by the competitive unlabeled oligonucleotides corresponding to Ins2 RORE (cold probe) (Fig. 3F, lane 4). Furthermore, RORα was also identified in the RORE/RORα complex by supershift analysis using anti-RORα antibody (Fig. 3F, lane 5). Collectively, these data indicate that RORα specifically binds to the RORE site presents between −71 and −47 bp in Ins2 gene promoter.

3.4. RORα stimulates transcription factor BETA2 expression

PDX1, MAFA and BETA2 are major transcription factors in beta cell, they directly regulate insulin gene expression [6,7,30]. Therefore, we examined whether RORα regulated insulin expression by modulating the expression of these insulin transactivators. Quantitative PCR showed that the mRNA expression of Beta2 was significantly up-regulated while Pdx1 and Mafa have no variation in RORα overexpressing cells as compared to Lac-expressing control cells (Fig. 4A). Western blot analysis confirmed that RORα overexpression significantly enhanced BETA2 protein expression (Fig. 4B). When we knocked down the endogenous RORα expression with shRNA in rat INS-1 cells, BETA2 in both message and protein levels were significantly decreased whereas PDX1 and MAFA were not affected (Fig. 4C and D). These data suggest that RORα also has an indirect role in the regulation of the insulin promoter through its effect on the trans-activator BETA2.

3.5. RORα induces insulin expression and secretion in vivo

We first investigated the subtissular distribution of RORα in mouse pancreas. Immunofluorescence detection of RORα in this tissue demonstrated a localization in β-cells as shown by insulin costaining (Fig. 5A). To better understand the in vivo functions of RORα in the regulation of insulin expression, mice were treated with a synthetic RORα agonist SR1078 (10 mg kg^{-1} i.p.), and 2 h

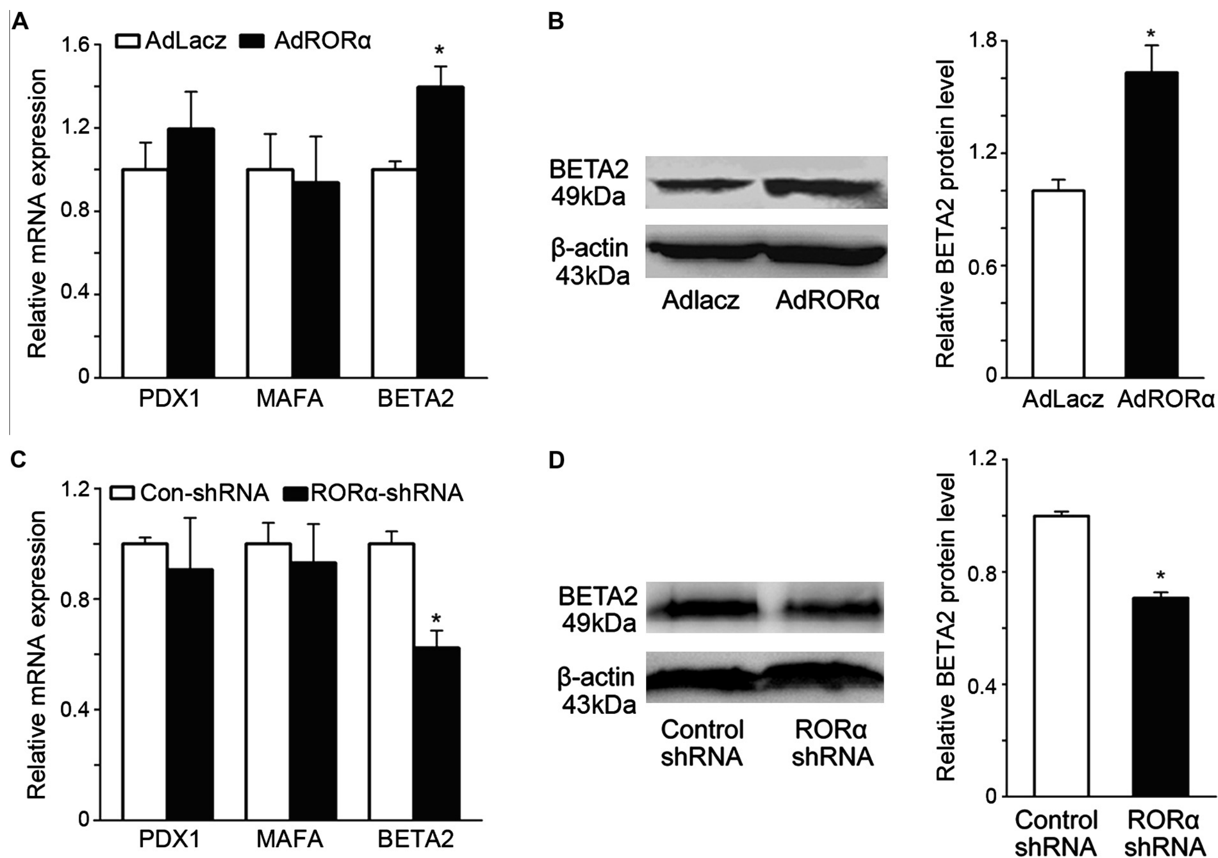

Fig. 4. RORα regulates insulin transcription factors expression. INS-1 cells were treated with adenovirus or shRNA for 48 h. (A) and (C) Pdx1, Mafa and Beta2 mRNA levels were measured by quantitative PCR relative to the control. (B) and (D) The protein level of BETA2 regulated by RORα was measured by Western blot. β-Actin was used as a loading control. The level for control was normalized to a value of 1.0. Data in the bar graph were the means ± S.D. of three separate measurements and $^{*}P < 0.05$ relative to controls.

after the injection the pancreas were harvested and mRNA and protein purified for assessment of RORα, Ins1, Ins2 and Beta2 gene expression. As shown in Fig. 5B–D, pancreas mRNA and protein levels of RORα were dramatically elevated over 2-fold in mice treated with SR1078, compared with control mice. Commensurate with the induction of RORα, the levels of Ins2 mRNA and BETA2 protein were significantly increased versus vehicle control, which are consistent with previous observations in INS1 cells. SR1078 also significantly improved glucose tolerance in mice, because the plasma glucose concentration during the glucose tolerance test was lower at each time point in SR1078- than in vehicle-treated animals (Fig. 5E). Accordingly, the area under the curve (AUC) was also significantly smaller in SR1078-treated than in vehicle-treated mice (Fig. 5F). Together with in vitro studies, these results confirm that RORα plays a key role in the physiological control of insulin gene expression.

4. Discussion

In this study, we have established Ins2 as a novel RORα target gene. Endogenous Ins2 mRNA expression was up-regulated by overexpression of RORα in rat INS-1 cells. Knock-down of RORα by shRNA produced a decrease in Ins2 mRNA. Furthermore, the altered expression of RORα also led to the alteration of secreted insulin and cellular content insulin accordingly. Moreover, the Ins2 promoter-driven luciferase activity was upregulated by RORα expression. As expected, a potential RORα response element (RORE) was found to be located in Ins2 promoter. EMSA proved that RORα has DNA-binding ability to Ins2 promoter. In addition, our in vivo experiment also showed that RORα activation increases insulin production. Taken together, our study identifies a new signaling pathway regulating insulin expression.

Manipulation of RORα levels through stable over-expression or shRNA knock-down also affected the level of transcription factor BETA2 in beta cells. BETA2 is a crucial factor for the assembly and function of the insulin gene transcription complex. This leads us to conclude that RORα modulates insulin gene expression, both directly through binding to the insulin promoter and indirectly by modulating the activity of other beta-cell-enriched transcription factors. The mRNA expression of Beta2 was up-regulated in RORα over-expressed cells, and down-regulated in RORα-knockdown cells, suggesting that Beta2 could be a RORα target gene. We further analyzed Beta2 promoter for the presence of putative ROREs and found five putative ROREs (Fig. S2), however, only the site at −2661 bp is conserved among human, mouse, and chimpanzee. Whether RORα binds specifically to the RORE in the 5′ regulatory region of Beta2 gene will require further investigation.

Rat islets of Langerhans contain two insulins (Ins1 and Ins2) that are products of non-allelic genes [31]. Our study showed that over-expression of RORα led to a markedly increase of Ins2 expression but had no influence on Ins1 in INS-1 cells. However, both Ins1 and Ins2 were down-regulated when endogenous RORα gene was knocked down by shRNA, suggesting that it is involved directly or indirectly in the regulation of both genes. Our data demonstrated that RORα is able to directly bind the RORE sequence of Ins2 promoter, so it likely has a direct effect. The lack of response of Ins1 to RORα over-expression may indicate that its expression levels

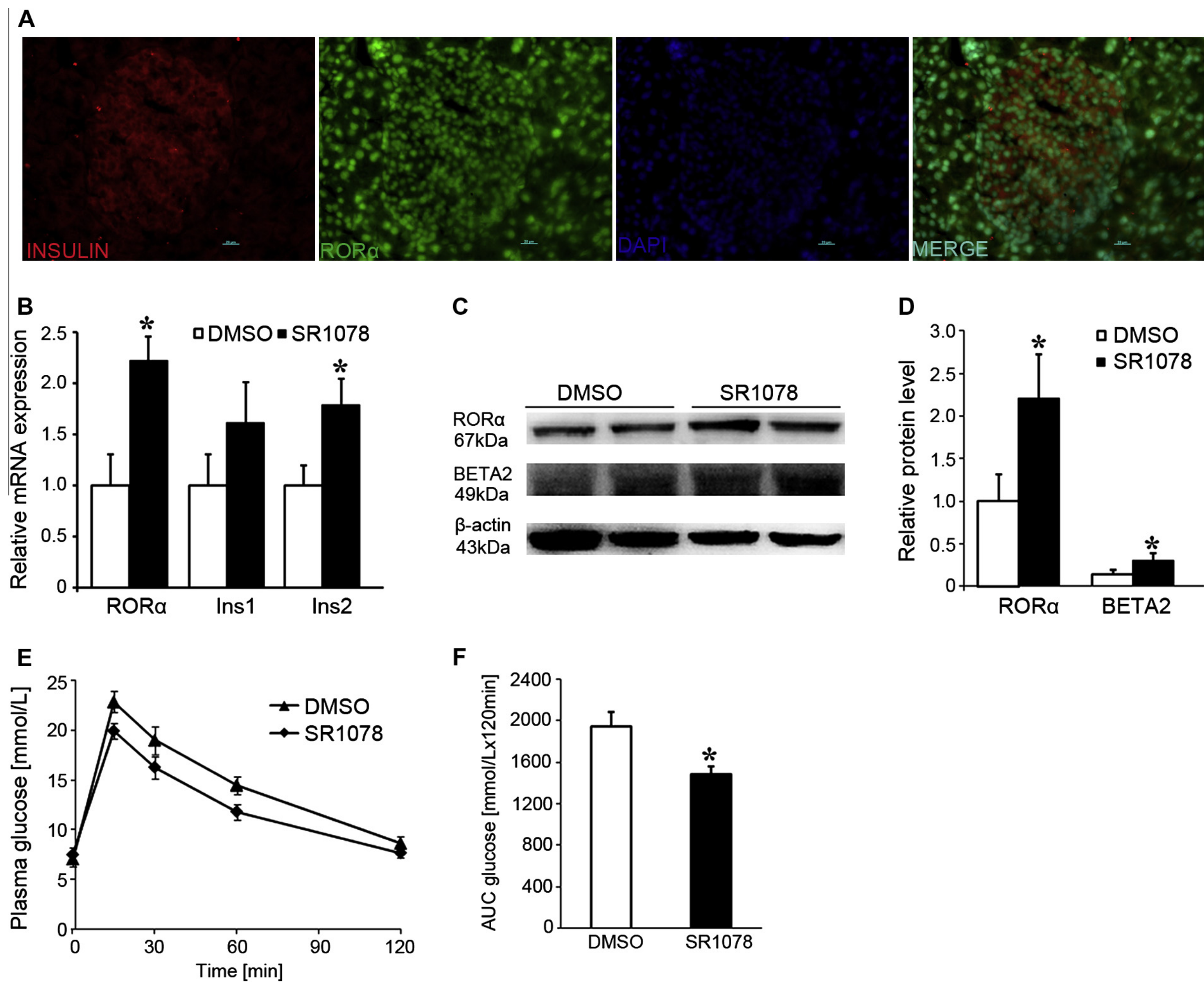

Fig. 5. RORα agonist SR1078 elevates the expressions of Ins2 and BETA2 in vivo. Mice were treated with a synthetic RORα agonist SR1078 (10 mg kg^{-1} i.p.) or DMSO for 2 h, and then were injected with glucose (2 g kg^{-1} i.p.). Thereafter, the mice were divided 2 groups. In one group, at the 30 min time point after glucose injection, the pancreas was harvested and mRNA and protein purified for assessment of gene expression. In another group, blood was collected for glucose determination from the tip of the tail at 0, 15, 30, 60, and 120 min after the glucose injection. (A) RORα is expressed in mouse pancreatic β-cells. Insulin (red) and RORα (green) were detected by indirect immunofluorescence. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars represent 20 μm. (B) RORα, Ins1 and Ins2 mRNA levels were measured by quantitative PCR relative to the control. (C) and (D) The protein levels of RORα and BETA2 were measured by Western blot. β-actin was used as a loading control. The level for control was normalized to a value of 1.0. (E) Intraperitoneal glucose tolerance test in SR1078-treated and vehicle-treated mice. (F) Area under the curve for glucose (AUC glucose) was calculated using the trapezoidal rule. The data were presented as the means ± S.D. of five mice. $^{*}P < 0.05$ relative to controls.

are near or at maximum in INS-1 cells and an increase in RORα cannot further enhance Ins1 expression. We cannot identify a RORE sequence in Ins1 promoter region. However, RORα can increase Beta2 message and protein levels and as it is known that BETA2 is capable of activating Ins1 promoter, we speculate that RORα has effects on other β-cell transcription factors which may in turn influence Ins1 expression.

RORα was initially described as an orphan nuclear receptor and has long been considered a constitutive activator of transcription in its exogenous ligands [16]. Later, natural ligands such as melatonin, cholesterol and cholesterol sulfate were shown to bind and trans-activate RORα, although there were controversies [32]. Recently, the amide SR1078 has been identified as a synthetic RORα agonist, which is expected to bind to the RORα protein and thus alters conformation and transactivating properties. However, our studies showed that the amount of RORα mRNA increased after mice was treated with SR1078 (Fig. 5B). A possible explanation is that SR1078 increases RORα expression, which subsequently regulates insulin expression. Actually, many RORα natural agonists, such as melatonin and cholesterol sulfate, are known to modulate the activity of RORα by up-regulation of RORα transcription [33,34].

RORα plays an essential role in regulation of the mammalian clock that in turn coordinates various physiological processes [35–37]. Studies have suggested that the expressions of both RORα and insulin are regulated in a circadian manner [38–41], leading to the possibility that RORα may be contributing to its circadian pattern of expression. We observed that RORα was highly expressed in mouse pancreas β-cells (Fig. 5A) and rat insulinoma INS-1 cells (Fig. 2A). This differs from those of Mühlbauer et al. who showed RORα was preferentially located in rat α-cells [41]. The nature of this difference is still not known. The circadian rhythm is tightly coupled to metabolic regulation, and among several stimuli that can entrain the clock is food [42]. This food-en-trainable oscillator controls food-anticipatory behavior as well as other physiological alterations in anticipation of a meal. Insulin levels are induced by feeding, it is possible that there may also be a circadian regulatory component to insulin secretion mediated by RORα. An aberrant circadian rhythm can also lead to development of metabolic syndrome. Clock mutant mice display hepatic steatosis, hyperlipidemia, high glucose levels, and low insulin levels [43,44]. In humans, epidemiological data indicates that shift work is associated with increased risk for development of diabetes, obesity,

and cardiovascular disease [45–47]. Recent studies in humans demonstrated that controlled misalignment of the circadian rhythm led to metabolic alterations, with some of the healthy subjects exhibiting postprandial glucose responses expected of individuals in a prediabetic state [48–50].

In conclusion, we have demonstrated for the first time that RORα regulates insulin gene transcription by directly binding to the insulin promoter at RORE site, which is highly conserved between rat and human. Furthermore, our data indicate that RORα expression also may modulate insulin gene transcription by effects on the levels of insulin transcription factor BETA2.

Acknowledgements

This study was supported by the National Natural Science Foundation of China [Grant 31071108] and the Program for New Century Excellent Talents in University [Grant NCET-10-0600].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.029.

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