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Journal of Cellular Biochemistry 116:998–1006 (2015)

Journal of Cellular Biochemistry

# Androgen Receptor Silences Thioredoxin-interacting Protein and Competitively Inhibits Glucocorticoid Receptor-Mediated Apoptosis in Pancreatic β-Cells

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## ABSTRACT

Androgen receptor (AR) is known to bind to the same *cis*-element that glucocorticoid receptor (GR) binds to. However, the effects of androgen signaling on glucocorticoid signaling have not yet been elucidated. Here, we investigated the effects of testosterone on dexamethasone (DEX, a synthetic glucocorticoid)-induced apoptosis of pancreatic β-cells, which might be involved in the pathogenesis of type 2 diabetes mellitus in males. We used INS-1 #6 cells, which were isolated from the INS-1 pancreatic β-cell line and which express high levels of AR. Testosterone and dihydrotestosterone inhibited apoptosis induced by DEX in INS-1 #6 cells. AR knockdown and the AR antagonist hydroxyflutamide each diminished the anti-apoptotic effects of testosterone. AR was localized in the nucleus of both INS-1 #6 cells and pancreatic β-cells of male rats. Induction of thioredoxin-interacting protein (TXNIP) is known to cause pro-apoptotic effects in β-cells. Testosterone suppressed the DEX-induced increase of TXNIP at the transcriptional level. A Chromatin immunoprecipitation assays showed that both AR and GR competitively bound to the *TXNIP* promoter in ligand-dependent manners. Recombinant DNA-binding domain of AR bound to the same *cis*-element of the *TXNIP* promoter that GR binds to. Our results show that AR and GR competitively bind to the same *cis*-element of *TXNIP* promoter as a silencer and enhancer, respectively. These results indicate that androgen signaling functionally competes with glucocorticoid signaling in pancreatic β-cell apoptosis. J. Cell. Biochem. 116: 998–1006, 2015. 

**KEY WORDS:** ANDROGEN RECEPTOR; SILENCER; GLUCOCORTICOID RECEPTOR; THIOREDOXIN-INTERACTING PROTEIN; PANCREATIC β-CELLS; TYPE 2 DIABETES MELLITUS

Androgens such as testosterone and dihydrotestosterone (DHT) have roles in the development and maintenance of male reproductive tissues [Zhou, 2010] as well as roles in non-reproductive tissues [De Gendt and Verhoeven, 2012; Chang et al.,

Abbreviations: AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; ChREBP, carbohydrate response element-binding protein; DBD, DNA-binding domain; DEX, dexamethasone; DHT, dihydrotestosterone; EMSA, electrophoresis mobility sift assay; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HF, hydroxyflutamide; T2DM, type 2 diabetes mellitus; TPI, triosephosphate isomerase; TXNIP, thioredoxin-interacting protein.

Conflict of interest: The authors declare that there is no conflict of interest.

Grant sponsor: Japan Society for the Promotion Science; Grant numbers: 23780141, 25450176.

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Manuscript Received: 4 September 2014; Manuscript Accepted: 18 December 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 5 January 2015

DOI 10.1002/jcb.25054 • © 2015 Wiley Periodicals, Inc.

2013]. The physiological functions of androgens are predominantly mediated by the activation of androgen receptor (AR), which is a member of the steroid hormone receptor superfamily of ligand-activated transcription factors [Lamb et al., 2001]. Androgen-activated AR binds to a specific androgen response element (ARE) of target gene promoters, which induces transcription of their genes [Heemers and Tindall, 2007]. In some cases, AR acts as ligand-dependent repressor of target genes through recruitment of co-repressor proteins such as NCoR [Cheng et al., 2002; Prescott et al., 2007]. The consensus sequence of ARE (5′-GGTACAnnnTGTTCT-3′) is commonly recognized by glucocorticoid receptor (GR) [Claessens et al., 2001]. The genes that are specifically regulated by these steroidal hormone receptors are determined by the tissue in which the receptors are expressed or by the sequence variability surrounding the ARE [Claessens et al., 2001].

Pancreatic β-cells secrete insulin, which is the principal hormone for regulating glucose homeostasis [Saltiel and Kahn, 2001]. Pancreatic β-cell mass is steadily decreased with the development of type 2 diabetes mellitus (T2DM) [Butler et al., 2003]. Therefore, to maintain β-cell mass, reducing the death of β-cells is crucial for the prevention and delay of T2DM [Ackermann and Gannon, 2007]. Notably, low blood testosterone levels are a risk factor of T2DM in men [Ding et al., 2006], and androgen deprivation therapy for prostate cancer patients is associated with the risk of T2DM [Keating et al., 2006]. In male rats, androgens increase insulin mRNA levels and protect β-cells from oxidative stress [Morimoto et al., 2001, 2005]. However, it remains unclear how androgen affects the function of β-cells.

Thioredoxin-interacting protein (TXNIP; also known as thioredoxin-binding protein-2 or vitamin$D_3$-upregulated protein 1) exerts pro-apoptotic effects in β-cells [Shalev, 2008]. Binding of TXNIP to thioredoxin causes the dissociation of the apoptosis signal regulating kinase 1 from thioredoxin, and the released kinase triggers apoptosis. Glucocorticoid induces the expression of TXNIP and stimulates apoptosis in pancreatic β-cells [Reich et al., 2012]. Notably, the induction of apopotosis by glucocorticoid is diminished without induction of TXNIP [Reich et al., 2012].

AR binds to the same *cis*-element that GR binds to. However, the competitive regulation of a gene that is commonly regulated by AR and GR has not yet been reported. In the present paper, we report that testosterone suppressed DEX-induced apoptosis in pancreatic β-cells. Furthermore, AR and GR competitively bind to the same *cis*-element of the *TXNIP* promoter as a silencer and enhancer, respectively, and differentially regulate apoptosis.

## MATERIALS AND METHODS

### ANIMAL TISSUES

Wistar male rats (Kwl: Wistar, 8 weeks old, n = 3, Kiwa Laboratory Animals, Wakayama, Japan) were sacrificed by exsanguination under anesthesia. Pancreatic islets were isolated by intraductal collagenase digestion and Ficoll–Conray density gradients. Isolated islets and dissected the ventral prostate or the pancreas were frozen by liquid $N_2$ and kept at −80°C until use. Animal experiments were approved by the Animal Care and Use Committee of Osaka Prefecture University in which veterinarian participated and were performed in compliance with its guidelines.

### CELL CULTURE

INS-1 rat pancreatic β-cells [Asfari et al., 1992] and newly established INS-1-derived cell line, INS-1#6 cells, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 11.1 mM D-glucose, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml sodium penicillin G, and 100 μg/ml streptomycin sulfate. INS-1 cells are known to exhibit phenotypic heterogeneity [Hohmeier et al., 2000; Merglen et al., 2004]. Several clones of the cells were grown from single cells and their AR expressions were measured by Western blotting. One of the colonies, INS-1 #6 cells, strongly expressed AR and expressed β-cell markers (*e.g.*, *PDX-1*, *MafA*, and *insulin-1*, and *insulin-2*) and was thus selected for further studies. Steroid-free medium was prepared using dextran-coated charcoal-treated fetal bovine serum (10%) and phenol red-free RPMI1640 medium. Cells were maintained at 37°C in a 5% $CO_2$/95% air atmosphere at 98% humidity.

### QUANTITATIVE RT-PCR

Total RNA was isolated and cDNA was synthesized. Quantitative RT-PCR was performed using SYBR Premix Ex Taq II (TAKARA Bio, Shiga, Japan) with the set of primers for *TXNIP* (sense: 5′-CGAGTCAAAGCCGTCAGGAT-3′ and antisense: 5′-TTCATAGCGCAAGTAGTCCAAGGT-3′) and β-*actin* (sense: 5′-TGTCACCAACTGGGACGATA-3′ and antisense: 5′-GGGGTGTTGAAGGTCTCAAA-3′) at an annealing temperatures of 60°C. The Ct values were transformed into relative quantification data using the $2^{-\Delta\Delta Ct}$ method.

### WESTERN BLOTTING

Isolated pancreatic islets or cultured cells were sonicated in IP buffer [Harada et al., 2007]. The centrifuged supernatant was analyzed by Western blotting. Anti-AR (N-20), anti-GR (P-20), and anti-TXNIP/VDUP1 (C-18) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-actin (137CT26.1.1) antibody was from ABGENT (San Diego, CA); anti-triosephosphate isomerase (TPI) antibody was described previously [Yamaji et al., 2003]; and anti-caspase-3 (#9662), anti-cleaved caspase-3 (#9661), and anti-PARP (46D11) antibodies were from Cell Signaling Technology (Beverly, MA). Immunoreactive bands were developed as described previously [Harada et al., 2012].

### IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE MICROSCOPY

Fresh rat pancreas (n = 3) were fixed and embedded in paraffin. Sliced serial microtome sections (4 μm thick) were dewaxed and endogenous peroxidase was inactivated by incubation with 5% $H_2O_2$. Tissue sections were heated by microwave exposure at 600 W for 10 min in 10 mM citrate buffer (pH 6.0) and blocked. After reaction with anti-AR (N-20), anti-AR (C19, Santa Cruz Biotechnology), or anti-insulin (D6C4, Hytest, Turku, Finland) antibodies, immunostaining was performed using Histofine (Nichirei, Tokyo, Japan) and 3,3′-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA), followed by counterstain with hematoxylin. INS-1 #6 cells were incubated in the presence of 100 nM testosterone for 12 h. Immunofluorescence staining was performed as described previously [Harada et al., 2011].

### CELL VIABILITY ASSAY

INS-1 and INS-1 #6 cells were seeded in 48-well plates with steroid-free medium [Harada et al., 2007]. After attachment to the plates, cells were incubated in the presence or absence of AR ligands for 72 h. Dexamethasone (DEX, a synthetic glucocorticoid) was co-incubated with testosterone for the last 48 h. Testosterone and DEX were used at final concentrations of 100 nM and 10 nM, respectively, throughout this study. The cells were transfected with double-strand siRNAs for AR (AR#1 sense: 5′-GACUCAGCUGCCCCAUCCA(dTdT)-3′ and AR#2 sense 5′- ACCAUGCAGAAUACAAAU(dTdT)-3′) or control double-strand siRNA (Sigma, SIC001) as described previously [Harada et al., 2012; Mitani et al., 2012]. Briefly, the cells were transfected using 10 nM siRNA and 0.5 μl of Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD), incubated for 24 h, and then incubated in the presence or absence of AR ligand for 72 h. Cell viability was determined using AlamarBlue Dye (TREK Diagnostics Systems, Cleveland, OH) and survival rate was determined as described previously [Horiuchi et al., 2014].

### PLASMIDS

Luciferase reporter vector pGL4.19-*TXNIP*(−1081/+20) containing −1081/+20 of mouse *TXNIP* promoter (GenBank AF282825.1) was constructed using pGL3B-1081 [Wang et al., 2006] and pGL4.19 (Promega, Madison, WI). ARE/glucocorticoid response element (GRE) site (−851/−837)-mutated reporter vector, pGL4.19-*TXNIP* (−1081/+20)-ARE/GREm, was constructed by site-directed mutagenesis using sense primer (5′-GGAACATATACAAAAGCTTCCC-CAACTTCACAG-3′) and antisense primer (5′-CTGTGAAGTTGGGGAAGCTTTTGTATATGTTCC-3′) and pGL4.19-*TXNIP* (−1081/+20) as a template. pGL4.19-2xARE/GRE-SV40 was constructed by inserting annealed oligonucleotides (sense: 5′-GATCTGAACATATACAAAATGTTCCCCAACGAACATATA-CAAAATGTTCCCCAACA-3′ and antisense: 5′-GATCTGTTGGG-GAACATTTTGTATATGTTCGTTGGGGAACATTTTGTATATGTTCA-3′) and SV40 promoter from pGL4.13 into pGL4.19 vector. In the DNA-binding deficient AR(C619Y), Cys at amino acid 619 is substituted for Tyr in the Zinc finger of the DNA-binding domain (DBD) [Nazareth et al., 1999]. The expression vector of the AR(C619Y) mutant was constructed by site directed mutagenesis using sense primer 5′-GTCGTCTTCGGAAATATTATGAAGCAGG-GAT-3′, antisense primer 5′-ATCCCTGCTTCATAATATTTCCGAA-GACGAC-3′, and pcDNA3.1-AR [Harada et al., 2007] as a template.

### REPORTER ASSAY

INS-1 #6 cells that had been incubated in steroid free medium(a 48-well plate) were transiently transfected with the *TXNIP* promoter-driven luciferase reporter vector, pcDNA3.1-AR [Harada et al., 2007] or pcDNA3.1-AR(C619Y), pcDNA3.1-GR [Harada et al., 2007], and pGL4.75[*hRluc*/CMV] using GenePORTER (Gene Therapy Systems, San Diego, CA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were stimulated by ligand for 3 h. Luciferase reporter assay was performed as described previously [Harada et al., 2007].

### DNA LADDER

INS-1 #6 cells that had been cultured in 12-well plates with testosterone and/or 10 μM hydroxyflutamide (HF) for 24 h were incubated in the presence of DEX for 24 h. Cells were harvested and lysed with 10 mM Tris–HCl, pH 7.5 containing 10 mM EDTA and 0.5% Triton X-100, followed by incubation with 1% SDS, 0.2 mg/ml RNaseA, and 0.2 mg/ml Proteinase K. After ethanol precipitation, DNA ladder was evaluated with agarose gel electrophoresis and ethidium bromide staining.

### CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP assay was performed as described previously [Harada et al., 2011] with minor modifications. Briefly, INS-1 #6 cells were preincubated with testosterone for 24 h and then stimulated with DEX for 15 min, followed by fixation with paraformaldehyde. Sonicated lysates (400 μl) were reacted with 1 μg of anti-AR (N-20) or anti-GR (P-20) antibodies by overnight incubation. Lysates were further incubated with pre-blocked (0.5 mg/ml bovine serum albumin and 0.25 mg/ml salmon sperm DNA) Dynabeads protein G (Life Technologies). After washing, the precipitated DNA was released by ChIP elution buffer. The eluted fraction or the sonicated lysate (50 μl, referred as an input fraction) was incubated with RNaseA and proteinase K. After ethanol precipitation, all fractions were dissolved in 50 μl of DNase-free water. The *TXNIP* promoter containing ARE/GRE were amplified by quantitative PCR with a SYBR Premix ExTaq II on Thermal Cycler Dice, TP-800 (Takara) using sense primer (5′-CAGTGTAAAGGTACACACCTCACTAAA-3′) antisense primer (5′-CCATTACGTTCTATTCTGTTCTGTTCT-3′), and 1 μl of template according to the following program: 1 cycle, 94°C for 1 min; 40 cycle, 94°C for 15 s, 60°C for 30 s, 72°C for 30 s. The Ct values of the ChIP sample were divided by the Ct value of an input sample and were transformed into quantification data using the $2^{-\Delta Ct}$ method.

### ELECTROPHORESIS MOBILITY SIFT ASSAY (EMSA)

Amplified cDNA encoding DBD (amino acid 557–622) of AR [Harada et al., 2007] was subcloned into pGEX-5X-1 vector. Recombinant GST or GST-AR-DBD was expressed in *Escherichia coli* BL21 by inducing 0.1 mM isopropyl β-*D*-thio-galactopyranoside for 5 h at 30°C in the presence of 10 μM $ZnCl_2$. Recombinant proteins were bound to Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ) and washed five times with PBS containing 10 μM $ZnCl_2$. After elution with 50 mM Tris–HCl, pH 8.8 containing 50 mM reduced glutathione and 10 μM $ZnCl_2$, recombinant proteins were dialyzed with PBS containing 10 μM $ZnCl_2$ and stored in 15% glycerol solution at −80°C until use. At the 3′-terminal of annealed oligonucleotides (wild-type, sense: 5′-CATATA-CAAAATGTTCCCCAAC-3′ and antisense: 5′-GTTGGGGAACATTTTG-TATATG-3′ or mutant, sense: 5′-CATATACAAAAGCTTCCCCAAC-3′ and antisense: 5′-GTTGGGGAAGCTTTTGTATATG-3′) were labeled with DIG according to manufacturer's protocol (Roche, Mannheim, Germany). Each 0.5 μg of GST or GST-AR-DBD was reacted with DIG-labeled probe (100 fmol) in the presence or absence of non-labeled probe (10 pmol) for 30 min at room temperature in 20 μl mixture containing 10 mM Hepes-NaOH, pH 7.9, 2% glycerol, 80 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, and 10 μM $ZnCl_2$. The mixtures were separated on a non-reducing acrylamide gel (5%) and transferred to a Hybond $N^+$ membrane (GE Healthcare). DIG-labeled probe was detected using alkaline phosphatase-labeled anti-DIG antibody (Roche) and Tropix CDP star reagent (Applied Biosystems, Foster City, CA).

### STATISTICS

Data were analyzed by Student's *t*-test or one-way or two-way analysis of variance followed by Tukey's post-hoc test using JMP statistical software version 8.0.1 (SAS Institute, Cary, NC). Data are shown as means ± SD, and statistically significant differences are considered when $P$ values were less than 0.05.

## RESULTS

### EFFECTS OF TESTOSTERONE ON GLUCOCORTICOID-INDUCED APOPTOSIS

Pretreatment of testosterone (100 nM) significantly increased the survival of INS-1 cells after stimulation of apoptosis by 10 nM DEX for 48 h (Fig. 1A). As shown in Fig. 1B, the expression level of AR in INS-1 cells was comparable to that in rat pancreatic islet. We isolated INS-1 #6 cells that expressed AR more strongly than did the parent INS-1 cells. Testosterone (10 nM and 100 nM) and DHT (10 nM) also protected against 10 nM DEX-induced apoptosis in INS-1 #6 cells (Fig. 1C), and the protective effect of 100 nM testosterone in INS-1 #6 cells (approximately 18% increase in survival) was stronger than that in INS-1 cells (approximately 9% increase in survival). The protective effects of testosterone were significantly reduced by AR knockdown (Fig. 1D) and by the AR antagonist HF (10 μM)(Fig. 1E). DNA ladder formation was induced by exposure to DEX for 24 h and suppressed by testosterone (Fig. 1F). Testosterone also reduced DEX-induced cleavages of caspase-3 and PARP-1 (Fig. 1G). HF blocked the suppression of these apoptotic features by testosterone.

### NUCLEAR LOCALIZATION OF AR IN PANCREATIC β-CELLS

AR was accumulated in the nucleus in the presence of 100 nM testosterone in INS-1 #6 cells (Fig. 2A). Immunohistochemical analysis

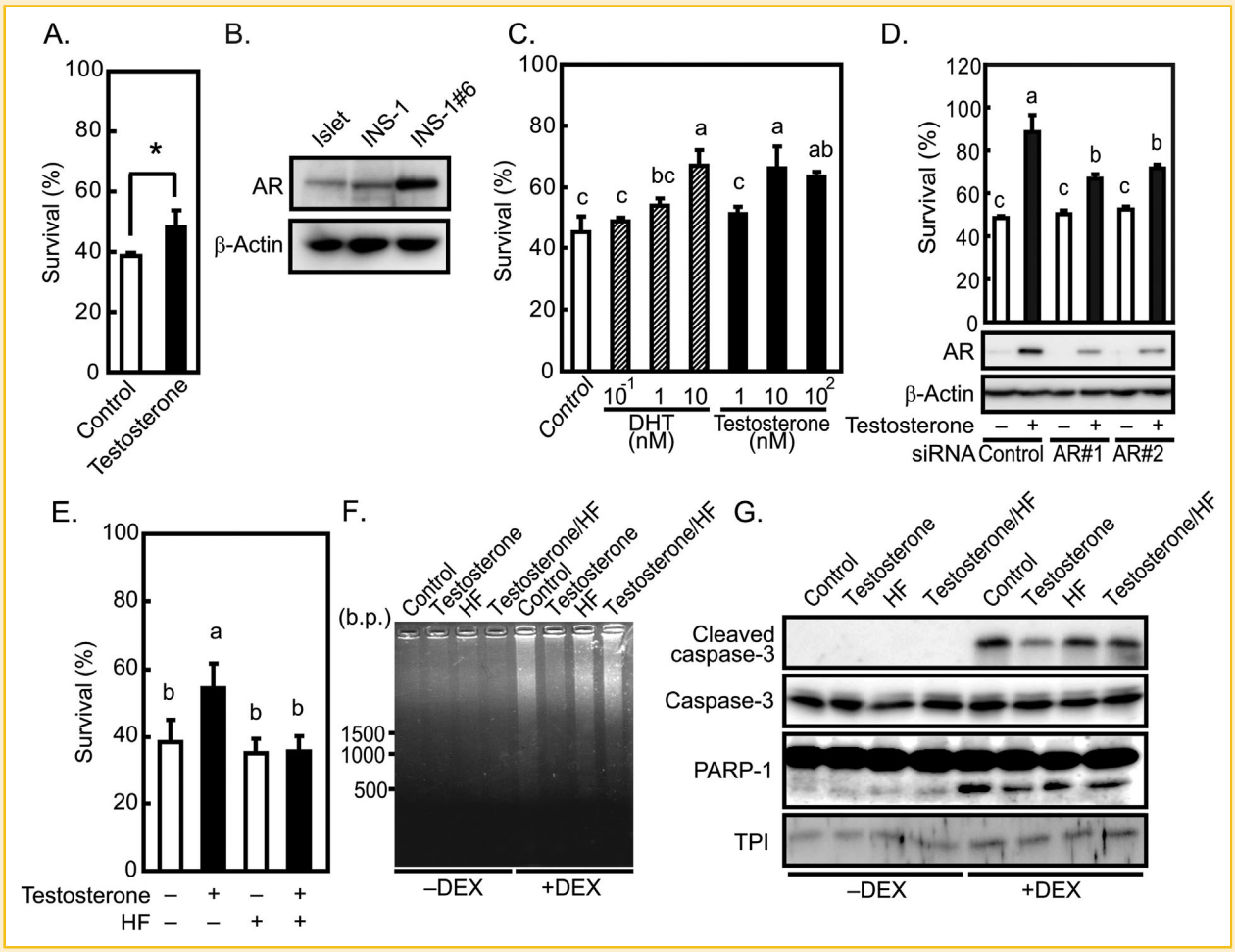


**Fig. 1.** Effects of androgens on DEX-induced cell death. A: Effects of 100 nM testosterone on INS-1 cell survival. B: AR expression in pancreatic islet and β-cell lines. Figure is a representative of three independent experiments. C: Effects of various concentrations of androgens on INS-1 #6 cell survival. D: Effects of siRNA-mediated knockdown of AR in INS-1 #6 cells survival. Cell lysate was analyzed by Western blotting. E: Effects of HF (10 μM) on 100 nM testosterone-increased INS-1 #6 survival. F and G: Effects of 100 nM testosterone and 10 μM HF on 10 nM DEX-induced DNA laddering or the cleavage of caspase-3 and PARP-1. INS-1 #6 cells were treated with 100 nM testosterone and/or 10 μM HF for 24 h, and further incubated in the presence of 10 nM DEX for 24 h, and cell lysates were analyzed. Each of the graphs in the figure is a representative of three independent experiments with four replicates. Asterisk or different letters above bars indicate statistically significant differences whereas bars with the same letter are not statistically different ($P < 0.05$).

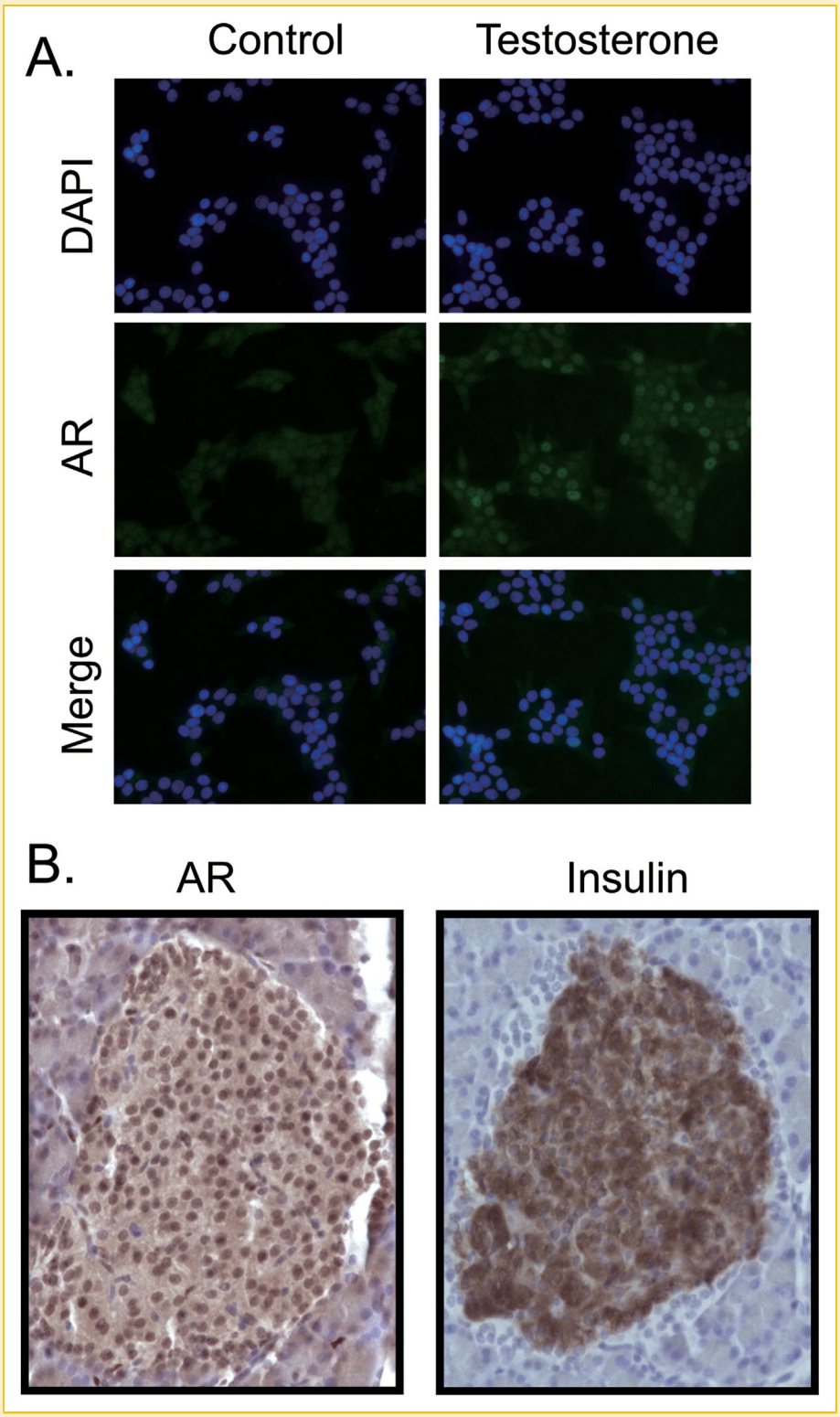


**Fig. 2.** Localization of AR in pancreatic β-cells. A: Immunofluorescence staining of AR in INS-1 #6 cells. The DAPI and AR panels shown are representative of three independent experiments. B: Immunohistochemical analysis of AR and insulin in pancreatic β-cells of male rats. The images are representative of three male rats.

using serial microtome sections indicated that AR was expressed in the nucleus of insulin (β-cell marker)-positive cells (Fig. 2B). Similar results were observed with another anti-AR antibody (C-19) (data not shown). These results suggest that AR acts in the nucleus of pancreatic β-cells.

### EFFECTS OF TESTOSTERONE ON DEX-INDUCED TXNIP EXPRESSION

Testosterone (100 nM) did not affect the expression of GR (Fig. 3A), indicating that the reduction of DEX-induced apoptosis is not a result of down-regulation of GR. *TXNIP* gene expression was greatly increased by 10 nM DEX, and the increase was significantly blocked by 100 nM testosterone in INS-1 #6 cells (Fig. 3B, upper panel).

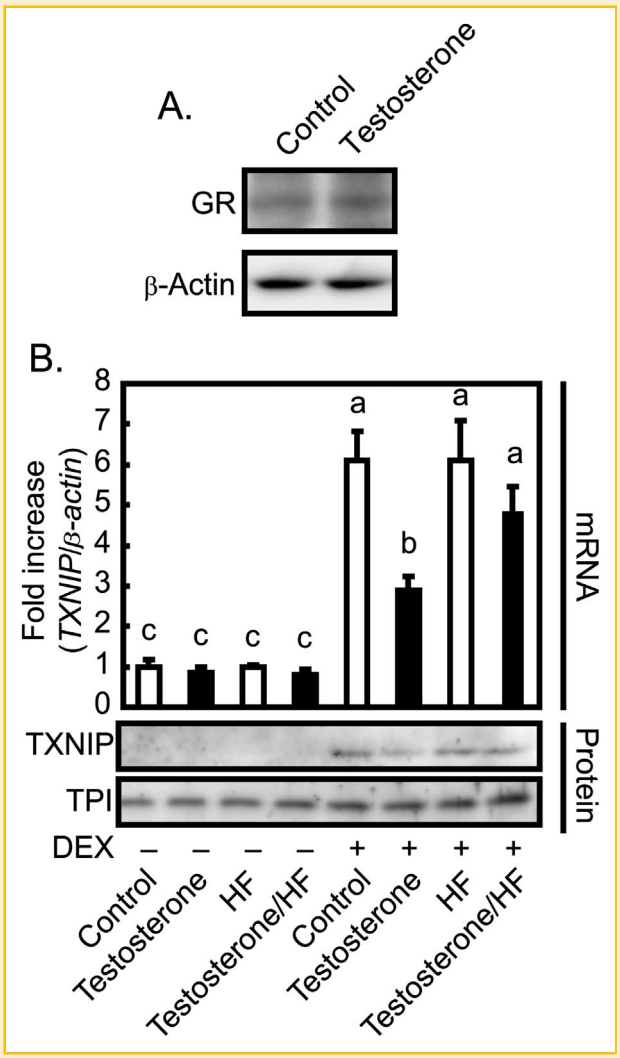


Fig. 3. Effects of testosterone on DEX-induced TXNIP expression. A: Effects of testosterone (100 nM) on GR expression in INS-1 #6 cells. INS-1 #6 cells were incubated with testosterone (100 nM) for 24 h and the cell lysates were analyzed by Western blotting. B: Effects of DEX (10 nM) and testosterone on *TXNIP* mRNA and protein expression levels. INS-1 #6 was incubated with testosterone (100 nM) for 24 h and then stimulated 10 nM DEX for 1 (for RNA analysis) or 6 h (for protein analysis). The graphs are a representative of three independent experiments with three replicates. Different letters above bars represent statistical differences whereas bars with the same letter are not statistically different ($P < 0.05$).

Testosterone also suppressed DEX-induced *TXNIP* expression in parent INS-1 cells (data not shown). TXNIP expression was also suppressed at the protein level (Fig. 3B, lower panel). In addition, HF (10 μM) diminished the suppression of *TXNIP* by testosterone both at mRNA and protein levels.

### EFFECT OF TESTOSTERONE ON *TXNIP* PROMOTER ACTIVITY

As shown by the luciferase reporter assay, testosterone (100 nM) attenuated 10 nM DEX-induced *TXNIP* (−1081/+20) promoter activity (Fig. 4, left panel). Then, we constructed a luciferase reporter, pGL4.19-*TXNIP* (−1081/+ 20)-ARE/GREm, which mutated the GR-binding site (−851/−837 in mouse *TXNIP* promoter) [Wang et al., 2006]. DEX did not activate *TXNIP* (−1081/+20)-ARE/GREm promoter in β-cells (Fig. 4, middle panel). DEX-induced activation was attenuated by testosterone when reporter assay was performed using two-tandem repeat of the ARE/GRE fused with SV40 minimum promoter (Fig. 4, right panel). These results indicate that AR and GR acts on the ARE/GRE site of *TXNIP* promoter.

### BINDING OF AR TO THE PROMOTER REGION OF *TXNIP*

In a ChIP assay of INS-1 #6 cells, anti-AR IgG, but not control IgG, precipitated the *TXNIP* promoter containing the ARE/GRE site when the cells were treated with 100 nM testosterone (Fig. 5A), and anti-GR IgG, but not control IgG, precipitated the *TXNIP* promoter when cells were treated with 10 nM DEX (Fig. 5B). Notably, testosterone-treatment significantly suppressed DEX-induced binding of GR to the *TXNIP* promoter. Mutant AR (C619Y), which is defective in DNA-binding [Nazareth et al., 1999], did not suppress DEX-induced *TXNIP*

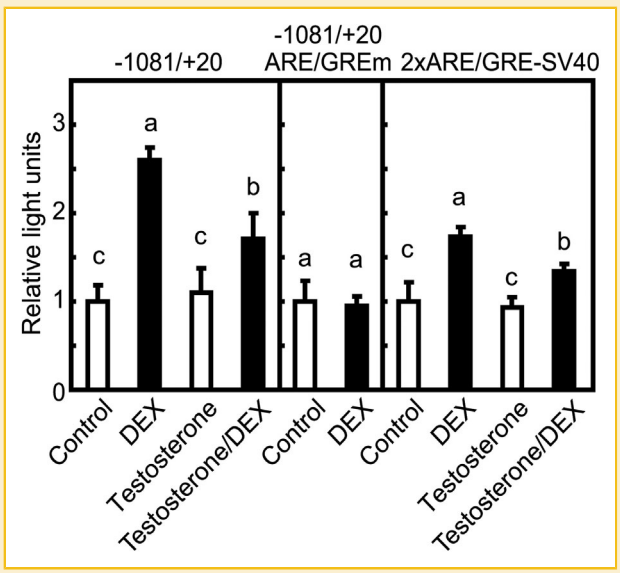


Fig. 4. Effects of testosterone on *TXNIP* promoter activity. *TXNIP* promoter (−1081/+20), ARE/GRE site-mutated *TXNIP* promoter (−1081/+20), and 2xARE/GRE-SV40 promoter activities were determined after incubation with 100 nM testosterone and 10 nM DEX using luciferase reporter assay. The graph is a representative of three independent experiments with four replicates. Different letters above bars represent statistical differences whereas bars with the same letter are not statistically different ($P < 0.05$).

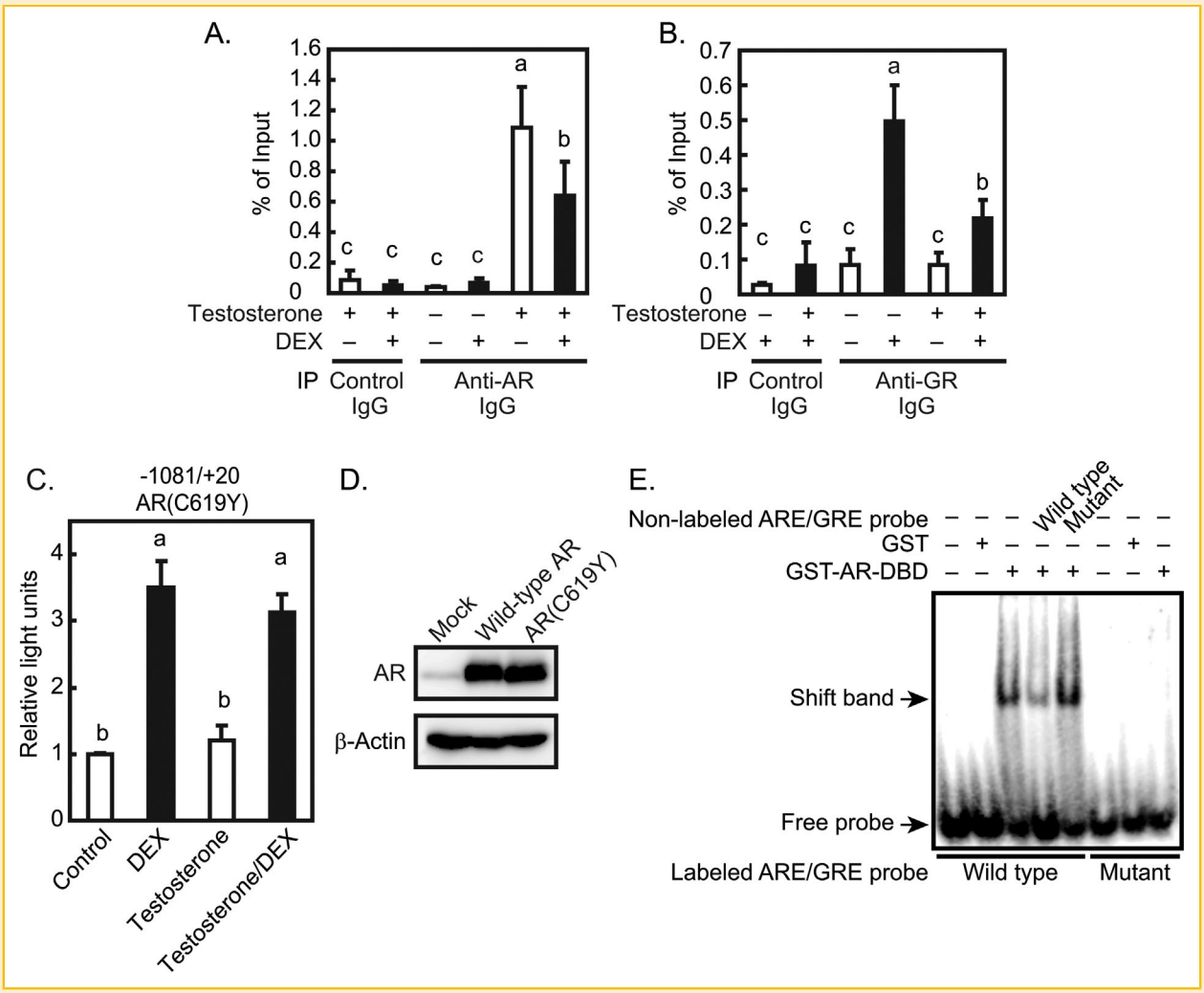


**Fig. 5.** Binding of AR and GR to the *TXNIP* promoter. A and B: Physical interaction between AR (A) or GR (B) and the *TXNIP* promoter determined by ChIP assay in INS-1 #6 cells. Immunoprecipitation (IP) was performed using control, anti-AR, or anti-GR IgG. Data is presented as percent input of each co-precipitated sample relative to input chromatin sample. The graph is a representative of three independent experiments with three replicates. C: Effect of DNA-binding-deficient AR(C619Y) on the DEX-induced *TXNIP* promoter (−1081/+20) activity. The graph is a representative of three independent experiments with four replicates. D: Expression levels of AR(C619Y) after stimulation with testosterone for 3 h was assessed by Western blotting. E: EMSA assay with GST-AR-DBD and ARE/GRE oligonucleotides. The graph is a representative of three independent experiments. Different letters above bars represent statistical differences whereas bars with the same letter are not statistically different ($P < 0.05$).

promoter (−1081/+20) activity (Fig. 5C), even though expression level of AR (C619Y) was comparable to that of the wild-type (Fig. 5D). In EMSA assay, the band sift of the wild-type ARE/GRE probe shifted in the presence of GST-AR-DBD, but not in the presence of GST (Fig. 5E). The band sift was decreased by a non-labeled wild-type ARE/GRE probe, but not by a non-labeled ARE/GRE site mutant probe. In addition, GST-AR-DBD did not cause a band sift of the mutant ARE/GRE probe. These results indicate that AR directly binds to the ARE/GRE of *TXNIP* promoter.

## DISCUSSION

In the present study, we showed that DEX-induced apoptosis was inhibited by testosterone or DHT by an AR-dependent mechanism in INS-1 #6 cells. On the other hand, GR stimulates the expression of TXNIP, a critical protein for the DEX-induced apoptosis, in a DEX-dependent manner [Reich et al., 2012]. Our results showed that AR directly bound to the ARE/GRE site of *TXNIP* promoter which GR binds to [Wang et al., 2006], and suppressed DEX-stimulated induction of TXNIP. Taken together, these results indicate that GR and AR exerted pro-apoptotic and anti-apoptotic effects, respectively, and that AR represses GR-induced apoptosis, at least in part, through repressing the expression of TXNIP in male pancreatic β-cells.

A novel finding of this study is that AR and GR bind competitively to the same *cis*-element of the target gene promoter and act as silencer and enhancer, respectively. Because androgens and glucocorticoids are generally known as anabolic and catabolic hormones, respectively, functional competition between AR and GR

is probably involved in the regulation of many cellular processes. Association of AR with a ligand is important for the dominant negative activity of AR against GR activity [Yen et al., 1997], and the heterodimerization of AR with GR is considered to result in the decrease of GR activity by DHT [Chen et al., 1997; Zhao et al., 2004]. Although it is well known that the consensus sequences of ARE and GRE are identical [Claessens et al., 2001], competitive action of AR and GR on the ARE/GRE site has not yet been reported. In muscle, testosterone suppresses DEX-induced FOXO1 expression [Qin et al., 2010]; however, it is unclear whether AR and GR compete with each other on the same ARE/GRE of the *FOXO1* gene promoter. In contrast, GR compensates for the loss of AR activity in the prostate, and confers resistance to antiandrogens by bypassing the AR blockade [Arora et al., 2013]. Whether AR and GR have competitive or compensative functions may depend on the cell type or target gene. Overall, the results of this study provide evidence of a functional competition between androgen and glucocorticoid signaling through direct competition between AR and GR on the target gene promoter.

The ARE/GRE site of the *TXNIP* promoter [Wang et al., 2006] is conserved among rat, mouse, and human according to MatInspector (Genomatix Software, Munich, Germany, http://www.genomatix.de), highlighting the importance of this site. In T-cell lymphocyte, DEX also induces apoptosis by inducing TXNIP [Reich et al., 2012], and AR is expressed and regulates cell develoment [Olsen and Kovacs, 2001], suggesting that AR regulates the development of T-cell by controlling the expression of TXNIP. Pancreatic β-cell mass decreases with the development of T2DM [Butler et al., 2003], and the decrease is accelerated by glucotoxicity. Testosterone suppresses β-cell apoptosis induced under high glucose (i.e., glucotoxic) conditions [Hanchang et al., 2013] and TXNIP is also essential for the induction of glucotoxicity [Shalev, 2008]. In a separately study (unpublished data by Harada N.), testosterone was found to down-regulate the expression of TXNIP induced under high glucose conditions. This result suggest that the repression of TXNIP by AR also helps to decrease glucotoxicity. TXNIP is induced by carbohydrate response element-binding protein (ChREBP) under hyperglycemia. Therefore, these results suggest that AR acts not only as a silencer for GR but also as a repressor against ChREBP and protects pancreatic β-cells from apoptosis. Microarray analysis show that *TXNIP* is negatively regulated by androgen in prostate cancer cells [Velasco et al., 2004] and in dermal papilla cells [Park et al., 2007], suggesting that AR suppresses *TXNIP* expression induced by multiple stimuli. The expression of TXNIP is down-regulated by PI3K/Akt/FOXO1 signaling and PKA signaling [Shaked et al., 2009; Kibbe et al., 2013], both of which are important for β-cell survival. In addition, *TXNIP* knockout in β-cells results in an increase of β-cell mass [Chen et al., 2008]. Taken together, our results suggest that AR regulates β-cell mass by silencing *TXNIP* gene expression.

Testosterone is important for controlling blood glucose levels because its levels in T2DM are lower than those in healthy men [Ding et al., 2006] and androgen deprivation causes T2DM in men [Keating et al., 2006]. Castration decreases insulin mRNA [Morimoto et al., 2001], insulin secretion [Xia et al., 2013], and increases oxidative stress-induced β-cell death [Morimoto et al., 2005] in male rats. We immunohistochemically showed that AR is expressed and located in the nucleus of pancreatic β-cells of male rats. These results suggest that β-cells are a direct target of testosterone.

The present results show that testosterone reduces the expression of TXNIP in pancreatic β-cells by an AR-dependent mechanism, and plays a key role in the survival of the cells. These results strengthen the notion that androgens contribute to the prevention and treatment of T2DM in men.

## ACKNOWLEDGMENTS

pGL3B-1081 (Addgene plasmid #18759) was a kind gift of Dr. Clark Distelhorst. This work was supported by Grants-in-Aid (23780141 and 25450176) for scientific research (to N.H.) from the Japan Society for the Promotion Science.

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