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RESEARCH PAPER

Inhibition of human insulin gene transcription by peroxisome proliferator-activated receptor γ and thiazolidinedione oral antidiabetic drugs

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Background and purpose: The transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is essential for glucose homeostasis. PPARγ ligands reducing insulin levels *in vivo* are used as drugs to treat type 2 diabetes mellitus. Genes regulated by PPARγ have been found in several tissues including insulin-producing pancreatic islet β-cells. However, the role of PPARγ at the insulin gene was unknown. Therefore, the effect of PPARγ and PPARγ ligands like rosiglitazone on insulin gene transcription was investigated.
Experimental approach: Reporter gene assays were used in the β-cell line HIT and in primary mature pancreatic islets of transgenic mice. Mapping studies and internal mutations were carried out to locate PPARγ-responsive promoter regions.
Key results: Rosiglitazone caused a PPARγ-dependent inhibition of insulin gene transcription in a β-cell line. This inhibition was concentration-dependent and had an EC_{50} similar to that for the activation of a reporter gene under the control of multimerized PPAR binding sites. Also in normal primary pancreatic islets of transgenic mice, known to express high levels of PPARγ, rosiglitazone inhibited glucose-stimulated insulin gene transcription. Transactivation and mapping experiments suggest that, in contrast to the rat glucagon gene, the inhibition of the human insulin gene promoter by PPARγ/rosiglitazone does not depend on promoter-bound Pax6 and is attributable to the proximal insulin gene promoter region around the transcription start site from −56 to +18.
Conclusions and implications: The human insulin gene represents a novel PPARγ target that may contribute to the action of thiazolidinediones in type 2 diabetes mellitus.
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Keywords: β-cells; thiazolidindiones; human insulin gene transcription; PPARγ

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; RXRα, retinoid X receptor α

Introduction

The nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ), is, among other functions, an important regulator of glucose homeostasis (Desvergne and Wahli, 1999). While the endogenous ligand of PPARγ is not known, there are several synthetic compounds that bind PPARγ with high affinity and activate the receptor. These include the thiazolidinedione class of oral antidiabetic drugs, which are in use for the treatment of type 2 diabetes mellitus (Desvergne and Wahli, 1999). Thiazolidinediones like rosiglitazone and pioglitazone are effective agents for the control of glycaemia in patients with type 2 diabetes (Natali and Ferranini, 2006). In addition, in a randomized controlled clinical trial including 5238 patients, pioglitazone significantly reduced by 16% a composite end point. This included death from any cause, nonfatal myocardial infarction and stroke (Dormandy *et al.*, 2005). The overall clinical benefit from use of thiazolidinediones, however, remains to be defined (Lago *et al.*, 2007; Lincoff *et al.*, 2007; Nissen and Wolski, 2007; Singh *et al.*, 2007).

When compared with other oral antidiabetic drugs like the sulphonylureas and metformin, thiazolidinediones exhibit a unique antidiabetic effect. Through PPARγ, they reduce hepatic glucose output and increase insulin sensitivity (Natali and Ferranini, 2006). Furthermore, decreases in fasting plasma insulin levels have been reported in most trials performed with thiazolidinediones (Walter and Lübben, 2005; Wajchenberg, 2007). Reductions in fasting plasma insulin

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levels have been in the range of 10.7–31.8 pmol·L^{-1} with pioglitazone monotherapy and 6.6–27.2 pmol·L^{-1} with rosiglitazone monotherapy (Walter and Lübben, 2005). Thiazolidinediones also reverse the decline in the function of insulin-producing pancreatic islet β-cells in type 2 diabetes mellitus with improvements in islet architecture, insulin content, proinsulin to total immunoreactive insulin ratio and glucose-stimulated insulin secretion (Zeender *et al.*, 2004; Walter and Lübben, 2005; Wajchenberg, 2007). Although these effects on insulin could be explained as the beneficial sequelae of reducing hyperglycaemia and peripheral insulin resistance, direct effects of PPARγ on β-cells may contribute as well. PPARγ serves as transcription factor for a great number of genes involved in fatty acid uptake and storage, inflammation and glucose homoeostasis in adipose tissue, skeletal muscle and liver (Kostadinova *et al.*, 2005; Gervois *et al.*, 2007). Furthermore, PPARγ is expressed also in pancreatic islet β-cells (Braissant and Wahli, 1998). Nevertheless, the role of PPARγ in β-cells in the regulation of the insulin gene has received little attention so far.

Therefore, the direct effect of PPARγ and thiazolidinediones on insulin gene transcription in pancreatic islet β-cells was examined in the present study. Using transfection studies in the β-cell line HIT and in primary islets of mature transgenic mice, this study demonstrates that PPARγ ligands inhibit insulin gene transcription. This study suggest that, in contrast to the glucagon gene, the inhibition of the insulin gene does not depend on promoter-bound Pax6 and is conferred by the proximal insulin gene promoter region around the transcription start site.

Methods

Plasmid constructs

The plasmids pT81Luc, –350GluLuc (Schwaninger *et al.*, 1993), –410rInsLuc (Siemann *et al.*, 1999), –336hInsLuc, –258hInsLuc, –222hInsLuc, –193hInsLuc, –140hInsLuc, –93hInsLuc, –56hInsLuc, –336/+18hInsLuc (Oetjen *et al.*, 2007), PPRE-Luc, pPPARγ (Schinner *et al.*, 2002) and pcDNA3-retinoid X receptor (RXR)α, pcDNA3-PPARγ1-475N, pcDNA3-PPARγ175-475N (Krätzner *et al.*, 2008) have been described previously. The expression vector pBAT14.mPax6 was kindly provided by Dr M. German (University of California, San Francisco, CA) (Sander *et al.*, 1997). The plasmid pCMV-GFPtpz was purchased from Canberra-Packard (Dreieich, Germany). To generate –336/–31P-Luc, the fragment from –336 to –31 of the human insulin gene promoter was amplified by PCR using –336hInsLuc as template and cloned in front of the heterologous minimal promoter –85rInsLuc (P) (Siemann *et al.*, 1999). All constructs were verified by sequencing using the enzymic method.

Cell culture and transfection of DNA

The insulin-producing pancreatic islet β-cell line HIT-T15 (Santerre *et al.*, 1981) was grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 5% horse serum, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). JEG-3 human choriocarcinoma cells (Oetjen *et al.*, 2007) were grown in DMEM supplemented with 10% foetal calf serum, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). HIT cells were trypsinized and transfected in suspension by the DEAE-dextran method (Schwaninger *et al.*, 1993) with 2 μg of reporter gene plasmids and, when indicated, 1 μg of expression vector per 6 cm dish. Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin and antibiotics as described above. Thiazolidinediones were added 24 h before harvest. JEG cells were transfected by the calcium phosphate precipitation method (Oetjen *et al.*, 2007) with 3 μg of reporter gene plasmid and, when indicated, 207 ng of expression vector per 6 cm dish. In all experiments (HIT and JEG), cotransfections were carried out with a constant amount of DNA, which was maintained by adding Bluescript (Stratagene, La Jolla, CA). In all experiments 1 μg (HIT) or 50 ng (JEG) of cytomegalovirus–GFP (plasmid pCMV-GFPtpz) per 6 cm dish was cotransfected to check for transfection efficiency. Cell extracts (Schwaninger *et al.*, 1993) were prepared 48 h after transfection. The luciferase assay was performed as described previously (Schwaninger *et al.*, 1993). For the transfection of small interference RNA (siRNA) against Pax6, cells were transfected by metafectene (Biontex, Munich, Germany) with 50 or 100 pmole per 2 cm dish of siRNA as indicated according to the manufacturer's recommendations. The following sequences were employed: siRNA 1 – GGGACCACUUCAACAGGACUCAUUU, siRNA 2 – GGAGUGAACCUGACAUGUCUCAGUA, siRNA 3 – ACCACACCUGUCUCCUCCUUCAC AU and their respective complementary strands. The efficiency of Pax6 knock down was tested by immunoblot using an antibody against Pax6 (Santa Cruz, Heidelberg, Germany). Green fluorescent protein was measured in the cell extracts using the FluoroCount^{TM} microplate fluorometer (Packard) with a 485 nm (excitation)/530 nm (emission) filter pair.

Electrophoretic mobility shift assay

Synthetic complementary oligonucleotides (hIns PPRE wt: 5′-GGCCCAGCAGCCCTCAGCCCTCCAGGACAGGCT-3′, hIns PPRE mut: 5′-GGCCCAGCAGCAAGCATCTTGCCAGG ACAGGCT-3′) were annealed and labelled by a fill-in reaction using [α-^{32}P]dCTP and Klenow enzyme. Fifteen microlitres of islet extracts was pre-incubated with 2 μg poly dI/dC in binding buffer (20 mmol·L^{-1} HEPES, 1 mmol·L^{-1} EDTA) and when indicated with a 200-fold molar excess of the competitors (PPRE wt: 5′-GGTAAAGGTCAAAGGTCAAT-3′, PPRE mut: 5′-GGTAAAGAACAAAGAACAAT-3′) for 10 min at room temperature, followed by a 15 min incubation with the labelled oligonucleotides at room temperature. The binding reaction was subjected to electrophoresis on a 5% non-denaturing poly acrylamide gel.

Generation and analysis of transgenic mice

All animal studies were conducted according to the National Institutes of Health's guidelines for care and use of experimental animals and were approved by the Committee on Animal Care and Use of the local institution and state. The generation and analysis of transgenic mice carrying a transgene with the luciferase reporter gene under the control of the

human insulin gene promoter from –336 to +112 have been described before (Oetjen *et al.*, 2003a).

Isolation and culture of islets

Pancreatic islets from mice or transgenic mice were isolated and incubated as described previously (Oetjen *et al.*, 2003a). In short, isolated islets were pre-incubated in a humified atmosphere of 95% air/5% CO_2 for 12 h in RPMI 1640 medium containing 5 mmol·L^{-1} glucose and supplemented with 10% foetal calf serum, penicillin (100 U·mL^{-1}) and streptomycin (100 µg·mL^{-1}). Rosiglitazone was added 7 h, glucose (final concentration 20 mmol·L^{-1}) 6 h before harvest. Islet collection and extraction, as well as the measurement of luciferase activity and protein content were performed as has been described (Oetjen *et al.*, 2003a). Following this protocol, the human insulin promoter has been shown to confer a normal, physiological glucose response to reporter gene expression in isolated islets (Oetjen *et al.*, 2003a). For the electrophoretic mobility shift assay, approximately 800 islets were lysed in 150 µL lysis buffer (50 mmol·L^{-1} HEPES, 150 mmol·L^{-1} NaCl, 1.5 mmol·L^{-1} $MgCl_2$, 1 mmol·L^{-1} EGTA, 10% glycerol, 0.5% NP-40, 1 mmol·L^{-1} $NaVO_4$, 50 mmol·L^{-1} NaF, 20 mmol·L^{-1} β-glycerophosphate), passed five times through a 20 G needle, incubated on ice for 30 min and centrifuged at 4°C, 20 800× *g* for 5 min. The supernatant was used for the binding reaction.

Statistical analysis

All results are expressed as means ± SEM. Statistical significance was calculated with ANOVA, followed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Materials

Rosiglitazone was kindly provided by GlaxoSmith-Kline (Welwyn Garden City, Hertfordshire, UK). Darglitazone and englitazone (CP-72,467-02, sodium salt) were provided by Pfizer Inc (Groton, CT). RNAi was obtained from Invitrogen (Karlsruhe, Germany). Luciferin was purchased from Promega (Mannheim, Germany).

Results

Effect of PPARγ and thiazolidinediones on human insulin gene transcription in HIT β-cells

The expression of PPARγ is very high in normal pancreatic islets, approximately two-thirds of the expression level in white adipose tissue (Braissant and Wahli, 1998; Rosen *et al.*, 2003; Lupi *et al.*, 2004). In contrast, HIT cells express low levels of PPARγ as indicated by the observation that activation of a PPAR-dependent promoter (PPRE-Luc) by the thiazolidinedione rosiglitazone required transfection of a PPARγ expression vector (Figure 1). Consequently, this cell line allowed a direct assessment of the role of PPARγ in insulin gene transcription. Similarly, low-level expression of PPARγ in cell lines derived from tissues with high-level expression has been reported previously (Ricote *et al.*, 1998) including the

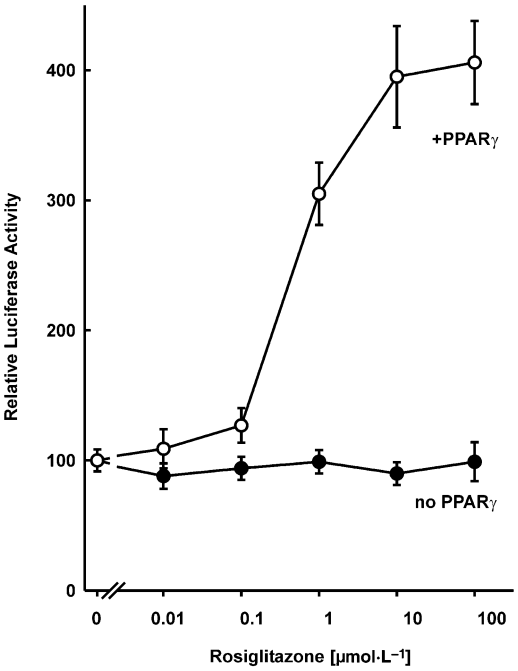


Figure 1 Activation of a peroxisome proliferator-activated receptor (PPAR)-dependent promoter by rosiglitazone and PPARγ in HIT β-cells. A luciferase reporter gene under the control of three copies of a PPARγ response element (plasmid PPRE-Luc) was transfected into HIT cells together with and without an expression vector encoding PPARγ. Increasing concentrations of rosiglitazone were added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± SEM of three independent experiments, each in duplicate.

islet α-cell line InR1-G9 (Schinner *et al.*, 2002) and β-cell line MIN6 (Nakamichi *et al.*, 2003). To study the effect of PPARγ and thiazolidinediones on insulin gene transcription, a fragment from –336 to +112 of the human insulin gene was fused to the luciferase reporter gene (construct –336hInsLuc) (Oetjen *et al.*, 2007; 2003a). This insulin promoter fragment is sufficient to confer tissue-specific gene expression and regulation of gene transcription by cAMP-, calcium-, glucose-, calcineurin- and mitogen-activated protein kinase-induced signalling pathways (Melloul *et al.*, 2002; Hay and Docherty, 2006; Oetjen *et al.*, 2003a,b; 2007). In the absence of cotransfected PPARγ expression plasmid, treatment of HIT cells with rosiglitazone at concentrations of up to 100 µmol·L^{-1} had no effect on insulin gene transcription (data not shown). This is consistent with the reported lack of effect of rosiglitazone on insulin gene transcription in the MIN6 β-cell line (Richardson *et al.*, 2006). However, when a PPARγ expression plasmid was cotransfected, rosiglitazone inhibited insulin gene transcription (Figure 2). Consistently, rosiglitazone activated a PPRE-driven reporter gene only when PPARγ was cotransfected (Figure 1). Thus, rosiglitazone inhibits insulin gene transcription by a PPARγ-dependent mechanism. Inhibition of insulin gene transcription by rosiglitazone was concentration-dependent with an IC_{50} value of about 1 µmol·L^{-1} (Figure 2). Cotransfection of an expression vector encoding the RXRα together with PPARγ did not alter the concentration–response curve for inhibition of insulin gene transcription by rosiglitazone (data not shown). The concentrations of rosiglitazone

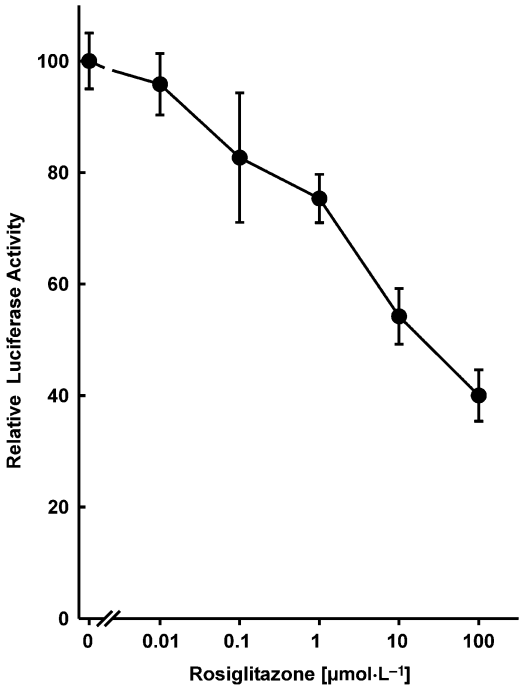


Figure 2 Inhibition of human insulin gene promoter activity by rosiglitazone and peroxisome proliferator-activated receptor γ (PPARγ). Plasmid –336hInsLuc was transfected into HIT β-cells together with pPPARγ. Rosiglitazone was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± SEM of three independent experiments, each in duplicate.

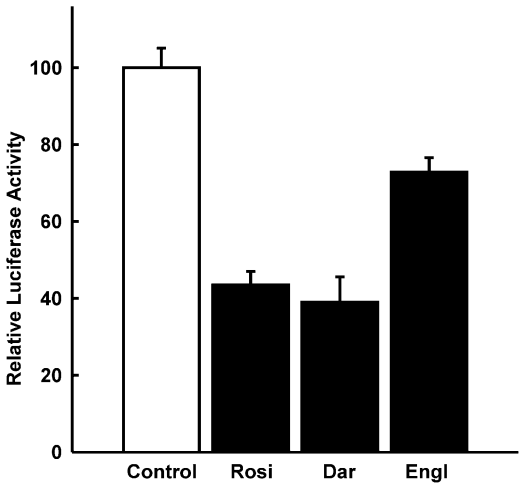


Figure 3 Inhibition of insulin gene transcription by the thiazolidinediones rosiglitazone, darglitazone and englitazone. HIT β-cells were transfected with –336hInsLuc and pPPARγ. They were treated with rosiglitazone (Rosi, 10 μmol·L^{-1}), darglitazone (Dar, 30 μmol·L^{-1}) or englitazone (Engl, 100 μmol·L^{-1}) 24 h before harvest as indicated. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± SEM of three independent experiments, each in duplicate.

that inhibit insulin gene transcription (Figure 2) are similar to those that activate the PPARγ-dependent promoter (Figure 1). The maximum inhibition of insulin gene transcription by rosiglitazone was about 60% (Figure 2). In addition to rosiglitazone, two other thiazolidinediones, darglitazone and englitazone, also inhibited insulin gene transcription (Figure 3). The specificity of the effect of PPARγ/rosiglitazone on insulin gene transcription is further supported by the lack of effect of PPARγ/rosiglitazone on CMV-promoter activity (not shown). These data indicate that PPARγ inhibits insulin gene transcription in response to the binding of thiazolidinediones.

Effect of rosiglitazone on glucose-stimulated insulin gene transcription in primary pancreatic islets

HIT cells are a well-established β-cell line and very useful in studies of insulin gene transcription (Santerre *et al.*, 1981; Melloul *et al.*, 2002; Hay and Docherty, 2006). As a tumour cell line, they may differ in critical aspects from normal β-cells. To investigate the effect of rosiglitazone in normal primary β-cells and under stimulation by glucose as the major physiological stimulus of insulin gene transcription (Melloul *et al.*, 2002; Hay and Docherty, 2006), islets of adult mice carrying a luciferase reporter transgene under the control of the human insulin gene promoter (from –336 to +112) were used. The expression of the human insulin gene within the islets of these transgenic mice has previously been shown to be regulated by glucose within the physiological concentration range (Oetjen *et al.*, 2003a). In the present study, we show that the treatment of the isolated islets with rosiglitazone abolished glucose-induced human insulin gene transcription (Figure 4).

Mapping of the PPARγ-responsive segment in the human insulin gene promoter

The transcription factor Pax6 has been shown to interact with PPARγ through its transactivation domain and to mediate the inhibition by PPARγ and thiazolidinediones of rat glucagon gene transcription (Schinner *et al.*, 2002; Krätzner *et al.*, 2008). Pax6 also binds to the rat insulin I gene promoter (Knepel *et al.*, 1991; Sander *et al.*, 1997). To examine whether Pax6 may bind to and activate also the human insulin gene promoter, hInsLuc was transfected into the heterologous cell line JEG, with and without cotransfection of an expression vector encoding Pax6. The luciferase reporter gene under the control of the rat insulin I (rInsLuc) or rat glucagon gene promoter (GluLuc) was transfected as a control. As shown in Figure 5A, Pax6 activated the rat glucagon gene promoter 117-fold and, less so, the rat insulin I gene promoter. In contrast, the activation of the human insulin gene promoter by Pax6 was only fivefold (Figure 5A). This activation appears to be non-specific, as similar slight increases were produced by Pax6 using the luciferase reporter gene under the control of promoters that lack a Pax6 binding site such as the truncated viral thymidine kinase promoter (pT81Luc) (not shown). Reduction of the cellular Pax6 content of HIT cells by siRNA did not decrease the transcriptional activity of the human insulin gene promoter (100 ± 5.6%, control: 92.3 ± 1.8% in the presence of siRNA, $n = 6$; $P < 0.05$) nor did it interfere with the inhibitory effect of rosiglitazone on human insulin gene transcription (Figure 5B). In addition, rosiglitazone inhibited human insulin gene transcription only when the overexpressed PPARγ contained its DNA binding domain, indicating

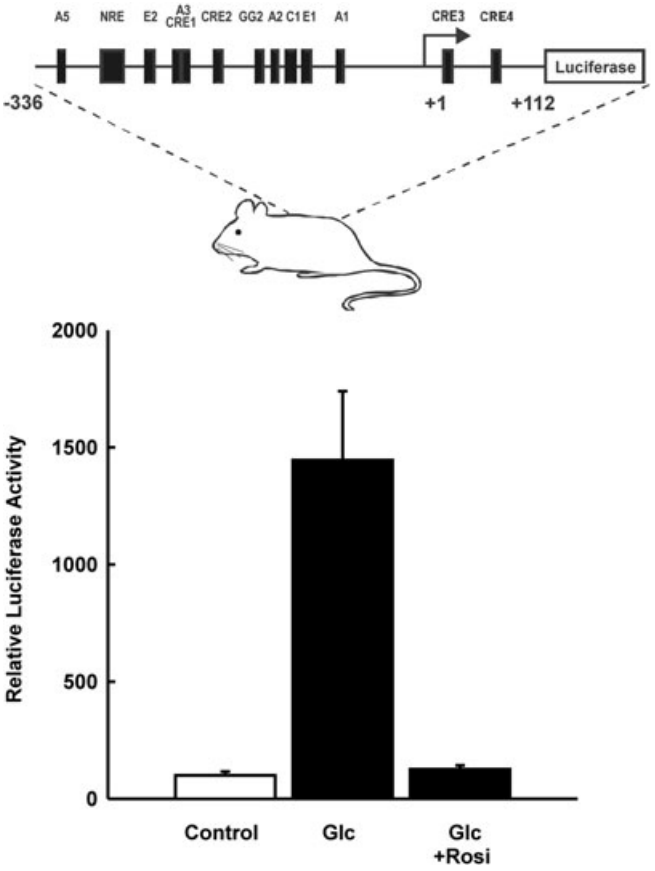
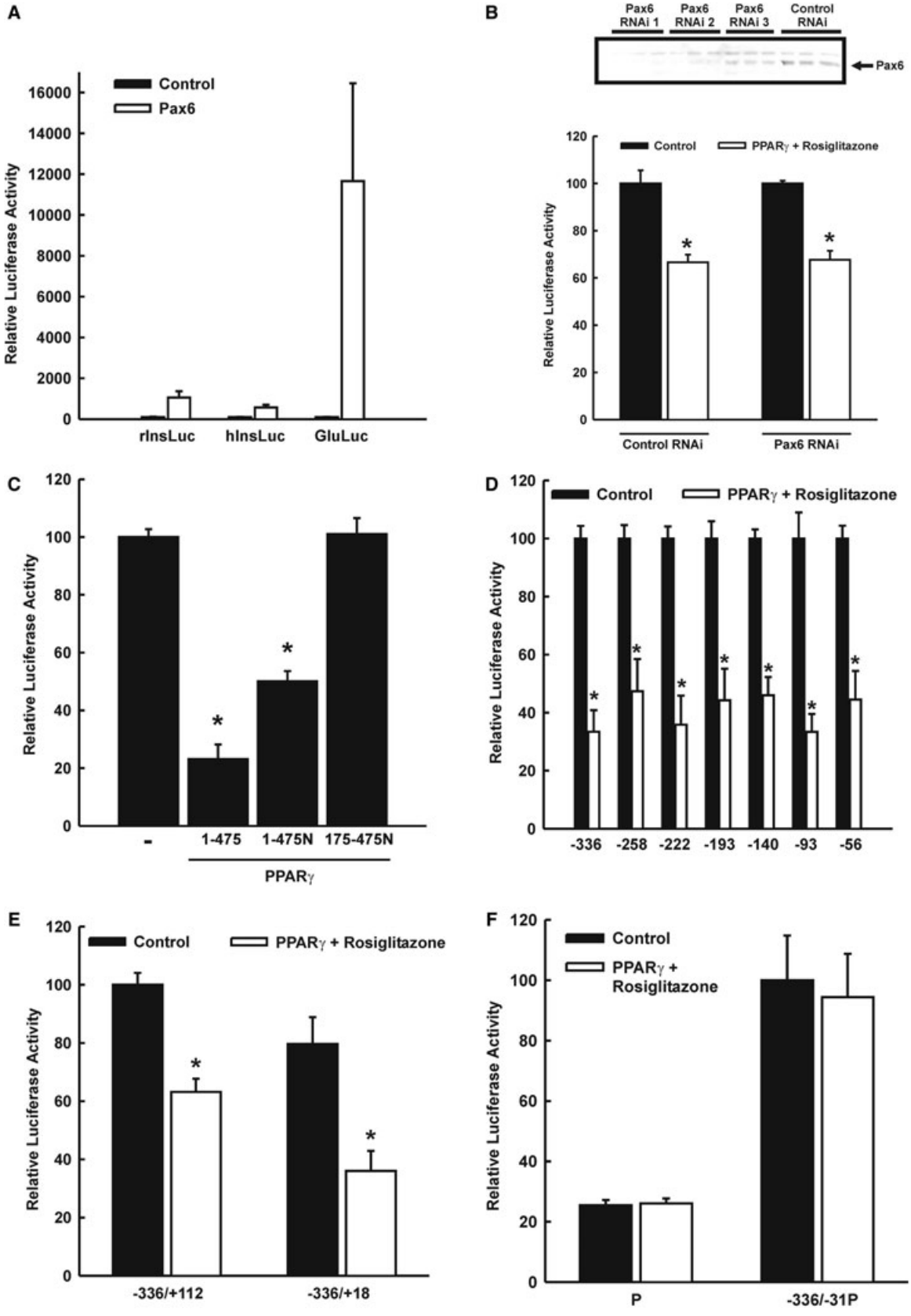


Figure 4 Effect of rosiglitazone on glucose-stimulated insulin gene transcription in normal primary pancreatic islets of transgenic mice. The upper panel depicts a scheme of the reporter gene used to generate the transgenic mice. Islets of transgenic mice were isolated and treated with glucose (Glc, 20 mmol·L^{-1}) 6 h prior to harvest with and without rosiglitazone (Rosi, 30 μmol·L^{-1}; 7 h prior to harvest). The control received 5 mmol·L^{-1} glucose only. Luciferase activity is expressed as percentage of the activity measured in the untreated controls. Values are means ± SEM of four experiments.

that binding of PPARγ to the insulin gene promoter is necessary for the inhibitory effect of the thiazoldindione (Figure 5C). These data do not support the view that PPARγ and thiazolidinediones may inhibit the human insulin gene promoter through inhibition of the transcriptional activity of promoter-bound Pax6, as is the case with the rat glucagon gene promoter (Schinner *et al*., 2002; Krätzner *et al*., 2008).

To define the cis-acting DNA sequences within the human insulin gene promoter that mediate transcriptional repression by PPARγ, a deletion analysis was performed. As shown in Figure 5D, expression of 5′-deleted mutant plasmids in HIT cells revealed that the repression by PPARγ and rosiglitazone was unimpaired when the 5′ end was shortened from –336 to –56. Basal activity was eventually reduced by 5′-deletion; for –56hInsLuc it was low but still detectable (2.4 ± 0.2% of –336hInsLuc). These results indicate that DNA sequences that allow repression by PPARγ may reside 3′ to –56. When the 3′ end of the human insulin gene promoter was shortened from +112 to +18 (construct –336/+18hInsLuc), the inhibition by PPARγ and rosiglitazone was fully preserved (Figure 5E). Bearing in mind the results of the 5′-deletion analysis, these data suggest that DNA sequences that are critical for repression by PPARγ to the human insulin gene may be located between –56 and +18. This conclusion was further supported by the lack of inhibition by PPARγ and rosiglitazone when the DNA sequences around the transcription start site of the human insulin gene promoter (sequences 3′ to –31) were replaced by a heterologous non-responsive minimal promoter (Figure 5F). The electrophoretic mobility shift assay showed that protein(s) of primary islet extracts bind in a mutation-sensitive way to the proximal sequence of the human insulin gene promoter (Figure 6). The protein complex was competed for by the consensus PPRE sequence (Desvergne and Wahli, 1999) but less so by a mutated PPRE sequence (Figure 6B), indicating that PPARγ is among the binding proteins.

Figure 5 Mapping of the segment of the human insulin gene promoter that mediates the responsiveness to peroxisome proliferator-activated receptor γ (PPARγ)/rosiglitazone. (A) The transcription factor Pax6 is known to activate the rat glucagon gene and to confer responsiveness to PPARγ. This figure shows the effect of Pax6 on promoter activity of the rat insulin I, rat glucagon and human insulin gene. Luciferase reporter genes under the control of the rat insulin I (rInsLuc), rat glucagon (GluLuc) or human insulin gene promoter (hInsLuc) were transfected into heterologous JEG cells with and without cotransfection of an expression vector encoding Pax6. Luciferase activity is expressed as percentage of the activity measured in the controls (no Pax6). Values are means ± SEM of three experiments, each done in duplicate. (B) Reduction of cellular Pax6 does not interfere with rosiglitazone-induced inhibition of insulin gene transcription. Upper panel, HIT cells were transiently transfected with three different small interference RNA (shown as RNAi 1–3, see *Methods*; 50 and 100 pmole per dish) against Pax6. Cells were harvested after 48 h and an immunoblot using a Pax6 antibody was performed. RNAi 1 and 2 decrease the content of Pax6 in HIT cells. Lower panel, HIT cells were transiently transfected with RNAi 1 (50 pmole per dish), the luciferase reporter gene under control of the humane insulin gene promoter and the expression vector for PPARγ. Cells were treated with rosiglitazone (30 μmol·L^{-1}) for 24 h or left untreated. Luciferase activity is expressed as percentage of the mean value measured in the control (without PPARγ cotransfection, without rosiglitazone). Values are means ± SEM of two independent experiments, each in triplicate; *$P < 0.05$. (C) Inhibition of insulin gene transcription by rosiglitazone depends on the DNA binding domain of PPARγ. A luciferase reporter gene under control of the human insulin gene promoter from –336 to +112 bp was transiently cotransfected into HIT cells with expression vectors for PPARγ wild type (1–475 PPARγ), PPARγ with extended carboxyl terminus by a nuclear localization signal (1–475N PPARγ), PPARγ lacking the AF-1 and the DNA binding domain and carrying at the carboxyl terminal a nuclear localization signal (175–475N PPARγ). Rosiglitazone (30 μmol·L^{-1}) was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value measured in the control (without PPARγ cotransfection, without rosiglitazone). Values are means ± SEM of two independent experiments, each in triplicate. *$P < 0.01$ versus PPARγ wild type. (D) 5′-Deletion analysis. Human insulin gene promoter-luciferase reporter genes with 5′ ends as indicated were transfected into HIT β-cells with and without cotransfection of pPPARγ and rosiglitazone treatment (30 μmol·L^{-1}). Luciferase activity is expressed as percentage of the activity measured in the respective untreated controls. Values are means ± SEM of three independent experiments, each in duplicate, *$P < 0.05$. (E) Effect of a 3′-deletion of the human insulin gene promoter from +112 to +18 on PPARγ/rosiglitazone responsiveness. The plasmids –336hInsLuc (–336/+112) or –336/+18hInsLuc were transfected into HIT β-cells with or without cotransfection of pPPARγ and rosiglitazone treatment (30 μmol·L^{-1}). Luciferase activity is expressed as percentage of the activity measured in the untreated –336hInsLuc controls. Values are means ± SEM of four independent experiments, each in duplicate, *$P < 0.05$. (F) The fragment of the human insulin gene promoter from –336 to –31 was placed in front of a heterologous nonresponsive promoter (P). The plasmids were transfected into HIT β-cells with and without cotransfection of pPPARγ and rosiglitazone treatment (30 μmol·L^{-1}). Values are means ± SEM of three independent experiments, each in duplicate.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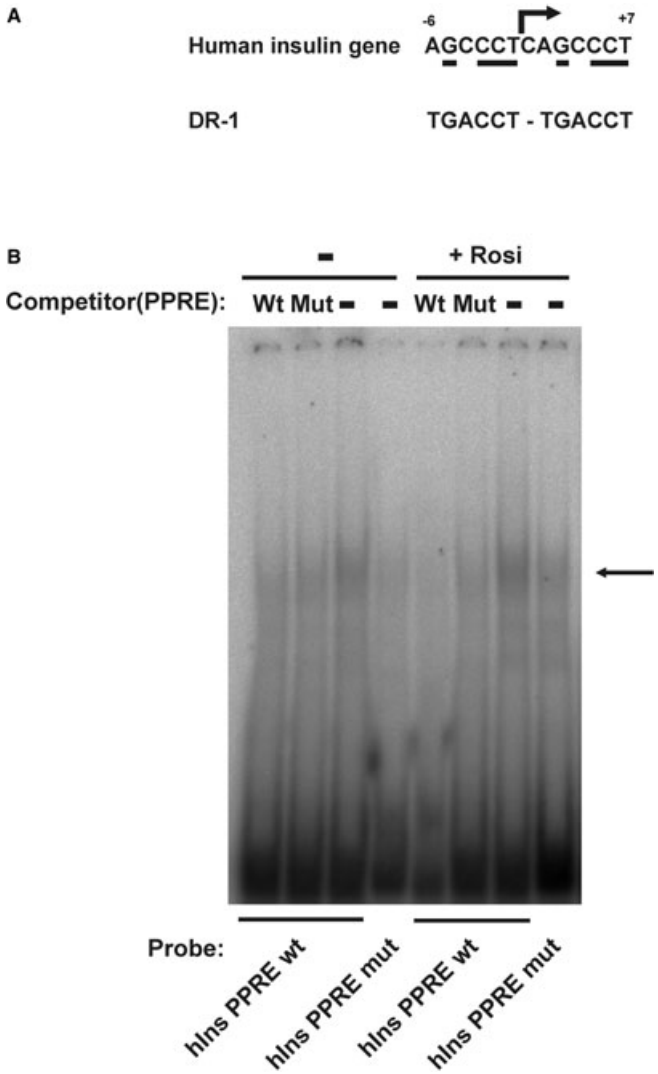

Figure 6 Protein binding to the proximal human insulin gene promoter around the transcription start site. (A) Sequence comparison between the proximal human insulin gene promoter around the transcription start site and a typical peroxisome proliferator-activated receptor γ (PPARγ) DNA binding site. PPARγ binds as a heterodimer with retinoid X receptor to response elements, which consist of a direct repeat of a hexamer half-site, spaced by one nucleotide (DR-1). The arrow indicates the transcription start site. The numbers give the first and last nucleotide relative to the transcriptional start site. The bases that match the consensus sequence are underlined. (B) Electrophoretic mobility shift assay. Islet extracts were incubated with radioactively labelled hIns PPARγ response element (PPRE) wt or hIns PPRE mut. For competition, PPRE consensus sequence (*Wt*) or its mutation (*Mut*) was used as indicated. The arrow indicates the PPARγ containing complex.

Discussion

As in adipose tissue, skeletal muscle and liver (Gervois *et al.*, 2007), PPARγ and thiazolidinediones also exert important glucose regulatory functions within pancreatic islets (Walter and Lübben, 2005; Wajchenberg, 2007). In α-cells, they inhibit glucagon gene transcription (Schinner *et al.*, 2002; Krätzner *et al.*, 2008), which may contribute to the thiazolidinediones' antidiabetic effect by reducing glucagon-dependent hepatic glucose production (Schinner *et al.*, 2002). In β-cells, PPARγ and thiazolidinediones activate the genes encoding GLUT2 (Kim *et al.*, 2000), glucokinase (Kim *et al.*, 2002) and ABCA1 cholesterol efflux transporter (Brunham *et al.*, 2007). These effects seem to allow thiazolidinediones to establish a more timely insulin secretion, as is indicated by the findings that 13 week treatment with rosiglitazone increases the ability of an oscillatory glucose infusion to programme high-frequency pulsatile insulin secretion in patients with type 2 diabetes mellitus (Juhl *et al.*, 2003), and that pioglitazone and rosiglitazone restore the first-phase insulin response to an intravenous glucose tolerance test in patients with impaired glucose tolerance and with frank type 2 diabetes mellitus (Ovalle and Bell, 2004). The present study now identifies the insulin gene as a new PPARγ target gene in pancreatic islet β-cells.

The early work on characterizing insulin gene transcription focused on the rat insulin I gene (Melloul *et al.*, 2002; Hay and Docherty, 2006). The early perception that human insulin promoter constructs would not function in transfected rodent cells proved to be unfounded (Melloul *et al.*, 2002; Hay and Docherty, 2006), and the human insulin promoter exhibited the expected pattern of activity in transgenic mice (Fromont-Racine *et al.*, 1990). Furthermore, it became apparent that rodent insulin promoters differ considerably from the human promoter, leading to the conclusion that extreme care should be taken when extrapolating rodent-based data to the human insulin gene (Melloul *et al.*, 2002; Hay and Docherty, 2006). In order to avoid these problems, the human insulin gene promoter was used in the present study.

In this study, we found human insulin gene transcription to be inhibited by PPARγ and thiazolidinediones in HIT β-cells. Rosiglitazone inhibited human insulin promoter activity also in normal primary pancreatic islets as revealed by the use of islets from transgenic mice carrying a human insulin promoter-luciferase transgene, which has been described before (Oetjen *et al.*, 2003a; 2007). The effect of thiazolidiones on insulin gene expression is still a matter for debate: a recent study showed no effect of 1 μmol·L^{-1} of rosiglitazone on insulin gene expression in human pancreatic islets after chronic fatty acid exposure (Vandewalle *et al.*, 2008). However, consistent with the present study, troglitazone reduced preproinsulin mRNA levels in primary islets (Bollheimer *et al.*, 2003), and PPARγ and pioglitazone decreased proinsulin biosynthesis as indicated by [^{3}H]leucine labelling in MIN6 cells (Nakamichi *et al.*, 2003).

The fact that rosiglitazone inhibited insulin gene transcription over the same range of concentrations as it stimulated, through PPARγ, the expression of a reporter gene directed by a well-defined PPARγ DNA binding site suggests that inhibition of insulin gene transcription may accompany other PPARγ-mediated effects. All findings, taken together, support the conclusion that inhibition of insulin gene transcription may be relevant both physiologically and therapeutically for the action of thiazolidinediones. Consistent with this view, the insulin content was significantly elevated in pancreatic islets from mice in which the expression of the PPARγ gene in β-cells was eliminated (βγKOmice), in spite of normal glucose and insulin levels in their sera (Rosen *et al.*, 2003).

PPARγ is well known to bind as a heterodimer with RXR to response elements in target genes and to activate transcription (Desvergne and Wahli, 1999; Natali and Ferranini, 2006). With the human insulin gene promoter, the present study provides another example that PPARγ can also inhibit gene transcription (Ricote *et al.*, 1998; Schinner *et al et al.*, 2002; Krätzner *et al.*, 2008). As has been first established for the glucocorticoid receptor (Reichardt *et al.*, 1998; Tuckermann *et al.*, 1999), nuclear receptors including PPARγ may mediate transrepression in a DNA binding-independent manner (Li *et al.*, 2000). Thus, the PPARγ/RXR heterodimer binds through protein–protein interaction to the transcription factor Pax6 and thereby represses the activity of this transcriptional activator, leading to inhibition of rat glucagon gene transcription in α-cells (Schinner *et al.*, 2002; Krätzner *et al.*, 2008). Pax6 is expressed also in β-cells (St-Onge *et al.*, 1997) and binds to the rat insulin I gene promoter at about –310 (Knepel *et al.*, 1991; Sander *et al.*, 1997). This Pax6 binding site may mediate the activation of the rat insulin I gene promoter when Pax6 is expressed in the heterologous JEG cells (this study). The rat glucagon gene promoter was more markedly activated by Pax6 (this study), most likely because it contains two Pax6 binding sites that interact synergistically (Knepel *et al.*, 1991; 1990; Sander *et al.*, 1997; Beimesche *et al.*, 1999; Grzeskowiak *et al.*, 2000). Nuclear protein binding to the rat insulin I gene at about –310 (Knepel *et al.*, 1991), later to be identified as Pax6 (Sander *et al.*, 1997; (Beimesche *et al.*, 1999), is not conserved in the human insulin gene (Yildiz *et al.*, 1996). The human promoter may also not contain other Pax6 binding sites, as is indicated by the low Pax6-induced activation of the human insulin gene promoter when compared with that of the glucagon gene promoter (this study). The finding that the inhibitory effect of rosiglitazone depends on the DNA-binding domain of PPARγ indicates that PPAR might directly bind to the promoter (this study). Mapping experiments revealed that DNA sequences between –56 and +18 may confer PPARγ responsiveness to the human insulin gene promoter. Importantly, this fragment contains a sequence motif from –6 to +7 with similarity to a typical PPARγ response element (Figure 6A), which consists of a direct repeat of hexamer half-sites, TGACCT, spaced by one nucleotide (DR-1) (Desvergne and Wahli, 1999). This raises the possibility that PPARγ may inhibit human insulin promoter activity by competing for binding to the region around the transcription start site with the general transcription machinery. Indeed, a complex of primary islet proteins bound to the sequence of the proximal promoter element, and this binding was competed for by additional incubation with a typical PPRE sequence but not to the same extent with the mutated PPRE sequence. Thus, although the mode of inhibition remains to be verified, the present study suggests that the mechanism through which PPARγ inhibits human insulin gene transcription differs from the one at the rat glucagon gene and may target the proximal insulin promoter around the transcription start site.

Thiazolidinediones efficiently improve glycaemic control and may reduce the risk of death from any cause in type 2 diabetic patients, with significant side effects (Dormandy *et al.*, 2005; Lago *et al.*, 2007; Lincoff *et al.*, 2007; Nissen and Wolski, 2007; Singh *et al.*, 2007). Inhibition of insulin gene transcription appears to be detrimental to these patients, who already suffer from insulin deficiency, and to be in opposition to several lines of evidence suggesting that treatment with thiazolidinediones may preserve and even improve β-cell function (Walter and Lübben, 2005; Wajchenberg, 2007). However, repression by thiazolidinediones of the insulin gene may in fact be in keeping with known thiazolidinedione effects and even be beneficial for the patients. Consequently, inhibition of insulin gene transcription is in line with and may contribute to the reductions in fasting plasma insulin levels found in most clinical trials performed with rosiglitazone, pioglitazone or troglitazone in patients with type 2 diabetes mellitus (Walter and Lübben, 2005; Wajchenberg, 2007). These reductions have so far been attributed solely to the increase in insulin sensitivity and the decrease in blood glucose concentrations (Walter and Lübben, 2005; Wajchenberg, 2007). Furthermore, insulin gene repression may protect β-cells from some of the damage induced by chronic overstimulation. Firm genetic and other lines of evidence indicate that β-cells may be especially sensitive to adverse effects of perturbed endoplasmic reticulum function (Wajchenberg, 2007). Glucose-mediated stimulation of proinsulin biosynthesis promotes some endoplasmic reticulum stress because it imposes a load on the folding and protein processing machinery of the endoplasmic reticulum. In hypersecretory states, such as insulin resistance, glucose intolerance and frank diabetes mellitus, proinsulin and other client proteins are translocated into the lumen of the endoplasmic reticulum in excess of the folding capacity of the organelle, inducing a state of severe endoplasmic reticulum stress with induction of programmed cell death. This mechanism may well contribute to the decline in β-cell function and mass in the insulin-resistant patient (Wajchenberg, 2007). Inhibition of insulin gene transcription by thiazolidinediones, we presume, decreases protein flux through the endoplasmic reticulum of the β-cell and thus reduces endoplasmic reticulum stress. Evidence of endoplasmic reticulum stress such as the accumulation of electron-dense material in the endoplasmic reticulum and distorted organelle morphology in diabetic islets are indeed reduced by thiazolidinedione treatment (Diani *et al.*, 1984; 2004; Walter and Lübben, 2005; Wajchenberg, 2007). Similarly, induction of β-cell rest by K_{ATP}-channel openers has been shown to improve β-cell function (Ritzel *et al.*, 2004). In conclusion, in this study we show an inhibition of insulin gene transcription by thiazolidinediones. Our results suggest that this inhibition is mediated by the proximal promoter region.

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Statement of conflicts of interest

The authors state no conflict of interest.

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