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The transcription factor HNF1α induces expression of angiotensin-converting enzyme 2 (ACE2) in pancreatic islets from evolutionarily conserved promoter motifs

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ABSTRACT

Pancreatic angiotensin-converting enzyme 2 (ACE2) has previously been shown to be critical for maintaining glycemia and β-cell function. Efforts to maintain or increase ACE2 expression in pancreatic β-cells might therefore have therapeutic potential for treating diabetes. In our study, we investigated the transcriptional role of hepatocyte nuclear factor 1α (HNF1α) and hepatocyte nuclear factor 1β (HNF1β) in induction of ACE2 expression in insulin-secreting cells. A deficient allele of HNF1α or HNF1β causes maturity-onset diabetes of the young (MODY) types 3 and 5, respectively, in humans. We found that ACE2 is primarily transcribed from the proximal part of the ACE2 promoter in the pancreas. In the proximal part of the human ACE2 promoter, we further identified three functional HNF1 binding sites, as they have binding affinity for HNF1α and HNF1β and are required for induction of promoter activity by HNF1β in insulinoma cells. These three sites are well-conserved among mammalian species. Both HNF1α and HNF1β induce expression of ACE2 mRNA and lead to elevated levels of ACE2 protein and ACE2 enzymatic activity in insulinoma cells. Furthermore, HNF1α dose-dependently increases ACE2 expression in primary pancreatic islet cells. We conclude that HNF1α can induce the expression of ACE2 in pancreatic islet cells via evolutionarily conserved HNF1 binding sites in the ACE2 promoter. Potential therapeutics aimed at counteracting functional HNF1α depletion in diabetes and MODY3 will thus have ACE2 induction in pancreatic islets as a likely beneficial effect.

1. Introduction

The carboxypeptidase angiotensin-converting enzyme 2 (ACE2) that hydrolyzes the octapeptide angiotensin (Ang)-II to the heptapeptide Ang-(1–7) has an important glycemia-protective role. ACE2 knockout mice have impaired glucose tolerance and impaired insulin secretion in response to glucose [1]. Conversely, ACE2 gene therapy delivered to the pancreas improves glycemia and β-cell function in the *db/db* mouse [2], a commonly-used genetic model of obesity-induced diabetes. We have recently demonstrated that deleterious effects of Ang-II on glycemia and beta-cell function of wild-type mice in the absence of obesity can likewise be countered by pancreatic ACE2 gene therapy [3]. Remarkably, these improvements occur with small, less than 3-fold, changes in pancreatic ACE2 activity. Procedures for increasing ACE2 expression might therefore have potential therapeutic value for treatment or prevention of diabetes.

Hepatocyte nuclear factor 1α (HNF1α) and hepatocyte nuclear factor 1β (HNF1 β) are homeodomain transcription factors for which haplo-insufficiency in humans causes MODY3 and MODY5, respectively [4]. HNF1β was previously shown to induce ACE2 promoter activity and ACE2 mRNA expression in a human embryonic kidney cell line [5]. This report led us to investigate whether HNF1α and HNF1β can induce ACE2 in pancreatic β-cells. In this study, we describe the structure of the human ACE2 gene promoter and demonstrate that there are three functional, evolutionarily conserved motifs in the proximal part of the promoter, capable of binding both HNF1α and HNF1β. Both transcription factors induce expression of ACE2 mRNA, leading to elevated levels of ACE2 protein and ACE2 enzymatic activity in insulinoma cells. Finally, we show that overexpression of HNF1α dose-dependently increases ACE2 expression in primary cells from pancreatic islets.

2. Materials and methods

2.1. Cell lines

The rat insulinoma cell line 832/13 [6] was a kind gift from Dr. Christopher B. Newgard, Duke University Medical Center, Durham,

Abbreviations: ACE2, angiotensin-converting enzyme 2; HNF1α, hepatocyte nuclear factor 1α; HNF1β, hepatocyte nuclear factor 1β; MODY, maturity-onset diabetes of the young; Ang, angiotensin; MOI, multiplicity of infection; eGFP, enhanced green fluorescent protein; RLU, relative light units; DPT, distal promoter transcripts; PPT, proximal promoter transcripts; Mca-APK(Dnp), 7-(Methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-Dinitrophenyl)-OH; RAS, renin-angiotensin system; C_t, cycle-threshold

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NC and maintained as previously described [7]. Mouse βTC3 cells were maintained in DMEM with 4.5 g/l glucose and supplemented with 15% horse serum, 2.5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human embryonic kidney (HEK) 293T cells (ATCC® CRL-11268) were maintained in DMEM with 4.5 g/l glucose and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.2. Tissues and islets collection

C57BL/6J mice were used with protocols approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center. Immediately following anesthesia and euthanasia by decapitation, the whole brain, kidney, heart and lung were isolated, snap-frozen, and stored at −80 °C until RNA isolation. Whole pancreas was isolated and immediately stored in RNAlater® (Ambion/Life Technologies, Grand Island, NY) at −20 °C to minimize RNA degradation during subsequent RNA isolation. Islets were isolated from collagenase-treated pancreata from 25 euthanized female mice, as described [8]. Alzet® micro-osmotic pumps (model 1004, Durect Corporation, Cupertino, CA) containing vehicle (saline) or Ang-II at a flow rate of 600 ng/kg/min were implanted subcutaneously in a subset of mice 15 days before islet isolation. Each islet preparation was from a single mouse. Islets were handpicked and treated with trypsin and repeated pipetting to separate cells. Each resulting suspension of primary islet cells was split into wells of a 12-well plate in 1 ml of islet medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) or, for insulin secretion, into wells of a 48-well plate in 300 μl of islet medium. Assuming an average of 100 cells per islet, the cells were infected with adenovirus at multiplicities of infection (MOI) of 4, 20, or 100 and incubated for up to 48 h. Human islets from normoglycemic individuals were purchased from the National Disease Research Interchange (NDRI), Philadelphia, PA.

2.3. Insulin secretion

After pre-incubation in KRBH buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 2% w/v BSA) with 2.8 mM glucose, primary islet cells were incubated for 1 h in KRBH with 2.8 or 25 mM glucose. The solution was recovered for determination of secreted insulin by an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL). The cells were washed with Dulbecco's PBS and lysed in Laemmli buffer for subsequent determination of protein concentration.

2.4. Plasmids and adenovirus

A plasmid, Rc mth HNF1beta, for CMV-driven expression of myc-tagged HNF1β [9] was a kind gift from Dr. G. Ryffel, Universitätsklinikum Essen, Germany. A control plasmid, called pRcNhe, was generated by replacing the HNF1β coding sequence between HindIII and XbaI sites with the sequence CCTGAGCTAGC. An expression plasmid for human HNF1α (cat. no. SC300093) and a corresponding empty control plasmid pCVM6-XL5 were purchased from OriGene Technologies, Inc., Rockville, MD. Mouse −470/−1, −2139/−915 and human −454/−1, −1699/−887, and −1699/−1 ACE2 promoter sequences (numbers indicate bases upstream of the start codon) were cloned into pGL3-Basic between MluI and XhoI sites. Putative HNF1 binding sites in the human ACE2 promoter were mutated by replacing bases −1195/−1184, −340/−335, −319/−314 and −249/−244 with NotI, ApaI, SmaI, and XhoI restriction sites, respectively. Amplicons of distal and proximal ACE2 promoter region transcripts identical in sequences to bases 155–267 of sequence NM_001130513.1 and 13–119 of sequence NM_027286.4, respectively, were cloned into the pCR®II-TOPO® plasmid (Invitrogen/Life technologies). The HNF1α coding sequence from the HNF1α expression plasmid was released by EcoRI digestion and cloned into the EcoRI site of plasmid pAd5CMVmscpA which generated an entry clone for subsequent adenovirus production at the Gene Transfer Vector Core at the University of Iowa. The resulting adenovirus, Ad-HNF1α, also expresses enhanced green fluorescent protein (eGFP) from the RSV promoter. A control adenovirus Ad-GFP for CMV-driven expression of eGFP was purchased from the Gene Transfer Vector Core at the University of Iowa.

2.5. Transfection

Cells were seeded in multi-well plates or tissue culture flasks the day before transfection. Cells were transfected with Lipofectamine 2000 in medium DMEM without serum and glucose, as previously described [7,10]. After incubation for 4 h, the transfection medium was replaced with normal maintenance medium. Cells were incubated for an additional 20 h for nuclear extraction and ChIP and for 44 h for the other assays. Experiments with luciferase reporters were done with 12-well plates, where each well was treated with 1 μg firefly luciferase reporter, 1 μg HNFβ expression plasmid or control plasmid pRcNhe, and 0.25 μg of Renilla luciferase expression plasmid phRL-TK from Promega (control for transfection efficiency). Relative light units (RLU) were calculated as the ratio of firefly and Renilla luciferase activities. Each figure summarizes results of 4 transfection experiments with each treatment given to duplicate wells. Experiments for isolation of RNA, protein, or ACE2 activity were done with cells grown in 6-well plates where each well was treated with 2 μg expression plasmid. For isolation of nuclear extracts, cells grown in 6-well plates were transfected with 2.5 μg expression plasmid per well. For ChIP, cells in a 75 cm^2 tissue culture flask were transfected with 20 μg of the HNF1β expression plasmid.

2.6. Quantitative RT-PCR

RNA from pancreas and cell lines was isolated with TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH). RNA from islet cells was isolated with RNeasy® Mini Kit (Qiagen, Valencia, CA). RNA from brain, kidney, heart and lung was isolated with Illustra RNAspin Mini kit (GE Healthcare, Buckinghamshire, UK). RNA for quantification of ACE2 distal and proximal promoter transcripts (DPT and PPT) was treated with TURBO DNA-free (Life Technologies). DPT and PPT were measured with the Taqman RNA-to-C_T 1-Step Kit (Life Technologies) and Taqman MGB probes with 6-carboxyfluorescein at the 5′ end. All other mRNAs were measured with the Power SYBR Green RNA-to-C_T 1-Step Kit (Life Technologies) and normalized to the concentration of β-actin mRNA. Primer and probe sequences are listed in Table 1. Quantifications of mRNA from cell lines were done using relative standards that were dilution series of RNA from insulinoma cells overexpressing HNF1α or HNF1β. Quantification of mRNA from islet cells was based on values of $\Delta C_t = C_t$ for target mRNA $- C_t$ for β-actin mRNA, where C_t is the cycle-threshold parameter for amplification curves in the real-time RT-PCR assays. The assays were conducted on an Applied Biosystems ABIPRISM 7900HT Sequence Detection System.

2.7. Protein concentration

The protein content of samples was measured with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

2.8. Western blotting

Western blotting of whole cell lysates was conducted as previously described [7]. Antibodies used were anti-ACE2 (sc-20998), anti-HNF1 (sc-8986), and anti-HNF1β (sc-7411) from Santa Cruz Biotechnology, Dallas, TX, and anti-γ-tubulin (T5192) from Sigma-Aldrich, St. Louis, MO. Secondary antibodies were HRP-conjugated anti-rabbit IgG