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Altered Expression of HES-1, BETA2/NeuroD, and PDX-1 Is Involved in Impaired Insulin Synthesis Induced by Glucocorticoids in HIT-T15 Cells

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Expression of the insulin gene is highly specific to pancreatic beta cells and is upregulated mainly by PDX-1 and BETA2/NeuroD depending on the extracellular glucose concentration. However, its downregulation has not been well studied. Reporter gene analyses using pancreatic HIT-T15 cells revealed that the glucose-dependent insulin promoter activity was blocked by glucocorticoids, dexamethasone (DEX) and hydrocortisone, in a dose-dependent manner. After the addition of DEX (20 nM) to HIT-T15 cells, a decrease of insulin mRNA was observed at 12–24 h, followed by a decline of insulin protein at 48 h. Expressions of PDX-1 and BETA2/NeuroD decreased within 2 h. HES-1, a potent negative regulator of bHLH-type transcription factors, was found to be expressed in HIT-T15 cells, and its expression was increased 6 h after the addition of DEX. Overexpression of HES-1 suppressed the insulin promoter activity in a dose-dependent manner. These results suggest that glucocorticoids impair insulin synthesis in HIT-T15 cells by decreasing PDX-1 and BETA2/NeuroD and that enhancement of HES-1 expression is involved in this regulation.

Key Words: **pancreatic beta cells; insulin; glucocorticoids; dexamethasone; PDX-1; BETA2/NeuroD; HES-1; transcription factor.**

Abbreviations used: DEX, dexamethasone; PDX-1, pancreatic duodenal homeobox-1; BETA2, beta cell E-box transcription factor 2; HES-1, hairy and enhancer of split-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bHLH, basic helix–loop–helix; GR, glucocorticoid receptor; D-MEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PLAP, placental alkaline phosphatase; RT-PCR, reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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The development of type 2 diabetes is associated with an impaired insulin secretion by pancreatic beta cells that occurs together with peripheral insulin resistance, and excessive hepatic glucose production (1–3). The insulin secretory defects are often accompanied by a decrease of insulin synthesis, as well as a decrease in the number of mature beta cells, probably due to the poor regenerating ability of beta cells (4–6). However, the mode of beta cell dysfunction in type 2 diabetes has not yet been well characterized.

Excessive signaling of glucocorticoids is believed to be involved in the pathogenesis of diabetes, since glucocorticoids induce insulin resistance, and increase hepatic glucose production by stimulating gluconeogenesis (7, 8). Additionally, evidence has been accumulated that glucocorticoids can also induce dysfunction of beta cells. They directly inhibit insulin release from, and modulate differentiation of, beta cells (9–11). Inhibition of insulin synthesis by glucocorticoids at the level of transcription was recently reported (12, 13). Therefore, glucocorticoids should be a useful tool for the study of beta cell dysfunction.

Expression of the insulin gene is highly specific to beta cells, and depends on the extracellular glucose concentration (14, 15). It is controlled by specific transcription factors, including PDX-1 and BETA2/NeuroD (16), which also play essential roles in differentiation and regeneration of beta cells (17–21). Glucocorticoids are known to inhibit the expression of PDX-1 (12), but their effects on other transcription factors which regulate insulin gene expression have not been studied.

Our aim in this study is to investigate the transcription factors involved in beta cell dysfunction. In this report, we show that expression of not only PDX-1, but also BETA2/NeuroD and HES-1 was changed by glucocorticoids in a pancreatic beta cell line, HIT-T15. Prior to the decrease in expression of insulin, expression of PDX-1 and BETA2/NeuroD decreased and

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HES-1 expression increased. Since HES-1 is known to be a negative regulator of transcription, and overexpression of HES-1 suppresses the insulin promoter activity in HIT-T15 cells, all these changes appear to contribute to impairment of insulin synthesis in beta cells.

MATERIALS AND METHODS

Cell culture. A clonal pancreatic beta cell line, HIT-T15, derived from SV-40 transfected Syrian hamster pancreatic islets (22) was purchased from American Type Culture Collection (ATCC, Rockville, MD). The glucose dependency of insulin synthesis in HIT-T15 cells gradually decreases when the cells are cultured in a relatively high concentration (11≈25 mM) of glucose (23). This change is thought to be caused by so-called glucose toxicity or oxidative stress (24, 25). Therefore, we cultured HIT-T15 cells in modified D-MEM with a low concentration of glucose and an antioxidative concentration of 2-mercaptoethanol, that is D-MEM without glucose (Gibco BRL, Grand Island, NY), supplemented with 10% FCS (Cat. SJ-01767, Lot. S09100, Sanko Junyaku, Tokyo, Japan), 100 U/ml penicillin-streptomycin (Gibco), 2 mM MEM sodium pyruvate (Gibco), 0.1 mM MEM non-essential amino acids (Gibco) and 55 nM 2-mercaptoethanol (Gibco). The medium contains about 0.5 mM glucose derived from FCS. The doubling time of HIT-T15 cells in the conditioned medium is 48 h. The insulin synthesis and its glucose dependency in the cells were maintained for at least 3 months.

RNA isolation and cDNA synthesis. HIT-T15 cells were plated at a density of $1.0 \times 10^5 \sim 1.5 \times 10^5$ cells/cm^2 in 6-well plates or 25-cm^2 flasks and were grown for 1 or 2 days. Then, the cells were transferred into D-MEM containing 10% FCS, 1 mM MEM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 5.5 mM glucose and either 0.2% ethanol or 20 nM DEX (Sigma, St. Louis, MO)/0.2% ethanol for various periods of time. At the same time, each cell culture was washed with PBS (−) (Nissui Seiyaku, Tokyo, Japan) twice and mixed in TRIzol Reagent (Gibco). Total RNA was isolated according to the manufacturer's instructions and 3 μg of total RNA was synthesized by the use of SuperScript II reverse transcriptase (Gibco). After cDNA synthesis, the reaction mixture was diluted with 40 μl of dH_2O.

Probe preparation and Northern blot analysis. cDNA (2 μl) as a template was mixed with 48 μl of Expand High Fidelity PCR solution (Roche Diagnostics GmbH, Mannheim, Germany) and PCR was carried out with GeneAmp 9600 (Perkin–Elmer, Palo Alto, CA). Primer sequences were 5′-CCTGCCCAGGCTTTTGTCA-3′ and 5′-GGTGCAGCACTGATCCACAATG-3′ (insulin), 5′-CAGCTAGATAGGGAGGGAATGATG-3′ and 5′-CTAAA-GAGGGGAAGAATCTAGGGG-3′ (PDX-1), 5′-GCAAAGGTTTGTCCCAGC-3′ and 5′-ACGTGGAAGACGTGGGAG-3′ (BETA2/NeuroD), 5′-GCTGGTGCTGATAACAGCGGAATC-3′ and 5′-TCAGTTCCGCCACGGCCTCCACA-TG-3′ (HES-1), and 5′-GACCCCTTCATTGACCTCAACTAC-3′ and 5′-GGCCATGAGGTCCACCACCCTGTT-3′ (GAPDH). Possible primers were synthesized based on published sequences for rat, mouse, human or hamster. PCR conditions were 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, with a final extension for 5 min at 72°C. PCR products were subcloned into pT7Blue (R) T-vector (Novagen, Madison, WI) and plasmids were purified with a QIAprep Spin Miniprep kit (Qiagen, Chatsworth, CA) and verified by sequencing. The nucleotide sequence for each plasmid was determined with a Dye Primer Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA), using −21M13 and M13Rev primers with an ABI Prism 377 DNA Sequencer (Applied Biosystems). The PCR products for insulin, PDX-1, BETA2/NeuroD and GAPDH were purified with a QIAquick gel extraction kit (Qiagen) and used as probes. The plasmid for HES-1 was digested with *Pst*I/*Eco*RI and the fragment containing the carboxyl-terminal domain was used as a probe after having been purified on GenElute Agarose Spin Columns (Sigma). Total RNA (2–40 μg) was electrophoresed on a 16.7% formaldehyde (Sigma)/20 mM Mops buffer (Wako Junyaku, Osaka, Japan)/1.5 or 2% agarose gel (Gibco) and then transferred to GeneScreen Plus (NEN Life Science, Boston, MA). The probe was labeled with [α-^{32}P]dCTP using a BcaBEST Labeling kit (Takara, Kyoto, Japan). Northern blot hybridization was conducted at 65°C for 3–16 h in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) with the labeled probe. The membrane filters were then washed in 2× SSC/0.05% SDS at 50°C for 15 min, and 0.1× SSC/0.1% SDS at 60°C for 15 min twice. The filters were exposed to an imaging plate for 3–24 h, and the band intensity was quantified by a Bioimaging analyzer, BAS 2000 system (Fuji Photofilm, Tokyo, Japan). The integrity and the amount of RNA loaded in each lane were verified from the GAPDH bands.

Assay for insulin contents. Insulin and proinsulin contents were measured with an ELISA kit for insulin (Morinaga Seikagaku, Yokohama, Japan) according to the manufacturer's instruction. HIT-T15 cells were washed with PBS (−) twice and were mixed in PBS (−) containing 2% Triton X-100 (Nacalai tesque, Kyoto, Japan), 0.04% Tween 20 (Bio-Rad, Hercules, CA), and 0.02% BSA (Sigma). The mixtures were diluted 100-fold and used for ELISA. Wavelengths of 490 nm was used for measurement of each sample with a TermoMax microplate reader (Molecular Device M-Tmax System, Wako) and values were calculated from the results obtained with standard insulin solutions.

Plasmid preparation and DNA transfection. A fragment of 358 bp (−358 to −1) (26, 27) from rat insulin I promoter containing a *Hin*dIII site or *Xba*I site was generated by PCR using primers 5′-GATCTAGAT-ACCAGGTCCCCAACAACTG-3′ and 5′-TTAAG-CTTGGGAGTTACTGGGTCTCCACT-3′. cDNA from AR42J cells was used as the template. PCR conditions were 34 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C, with a final extension for 7 min at 72°C. The fragment subcloned into the pT7Blue (R)T vector was digested with *Xba*I/*Hin*dIII and inserted into the sites of the polylinker region of a plasmid having placental alkaline phosphatase (PLAP) cDNA as a reporter gene (28) (pr-Ins-PLAP). HIT-T15 cell cultures (5 ml) were plated at a density of $0.5 \times 10^5 \sim 1 \times 10^5$ cells/cm^2 in 25-cm^2 flask. One or 2 days later, 5 μg of pr-Ins-PLAP was combined with 15 μl of FuGENE 6 (Roche) and transfected into HIT-T15 cells. Stable transformants were selected in the presence of 600 μg/ml G418 (Calbiochem–Novabiochem, San Diego, CA) and one was used in each experiment.

Plasmid preparation and transient cotransfection. A fragment containing the coding region of HES-1 from HIT-T15 cells was generated by PCR with 5% DMSO using primers 5′-GCTGG-TGCTGATAACAGCGGAATC-3′ and 5′-TCAGTTCCGCCACGGCC-TCCACATG-3′. PCR conditions were 35 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C, with a final extension for 5 min at 72°C. The fragment subcloned into the pT7Blue (R)T vector was digested with *Eco*RI and inserted into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) in the sense and antisense directions (HES-1-pcDNA3.1 and AHES-1-pcDNA3.1). HIT-T15 cell cultures (2 ml) were plated at a density of $0.5 \times 10^5 \sim 1 \times 10^5$ cells/cm^2 in 6-well plates. One or 2 days later, a total of 1 μg of HES-1-pcDNA3.1 and pcDNA3.1(+) or a total of 1 μg of AHES-1-pcDNA3.1 and pcDNA3.1(+), and 1 μg of pr-Ins-PLAP were combined with 6 l of FuGENE 6 and transfected into HIT-T15 cells. Then, 3–4 h later, 5 ml of fresh medium containing 7.7 mM glucose was added and the cells were incubated for 24–48 h.

PLAP assay. HIT-T15 cells expressing pr-Ins-PLAP were cultured in D-MEM containing 5.5 mM glucose with DEX, hydrocortisone (Wako) or RU-486 (mifepristone) (Sigma). At the indicated times, the culture supernatant was drawn from each sample and the PLAP activity was determined with the use of a chemiluminescent substrate (CDP-*Star* ready-to-use with sapphire-II, Tropix, Bedford, MA). Samples were heated at 65 for 20 min to inactivate nonspecific alkaline phosphatase in the medium derived from FCS, and 10-μl aliquots of heat-treated samples were mixed with 50 μl of assay buffer (0.28 M Na_2CO_3–$NaHCO_3$, pH 10.0, containing 8.0 mM

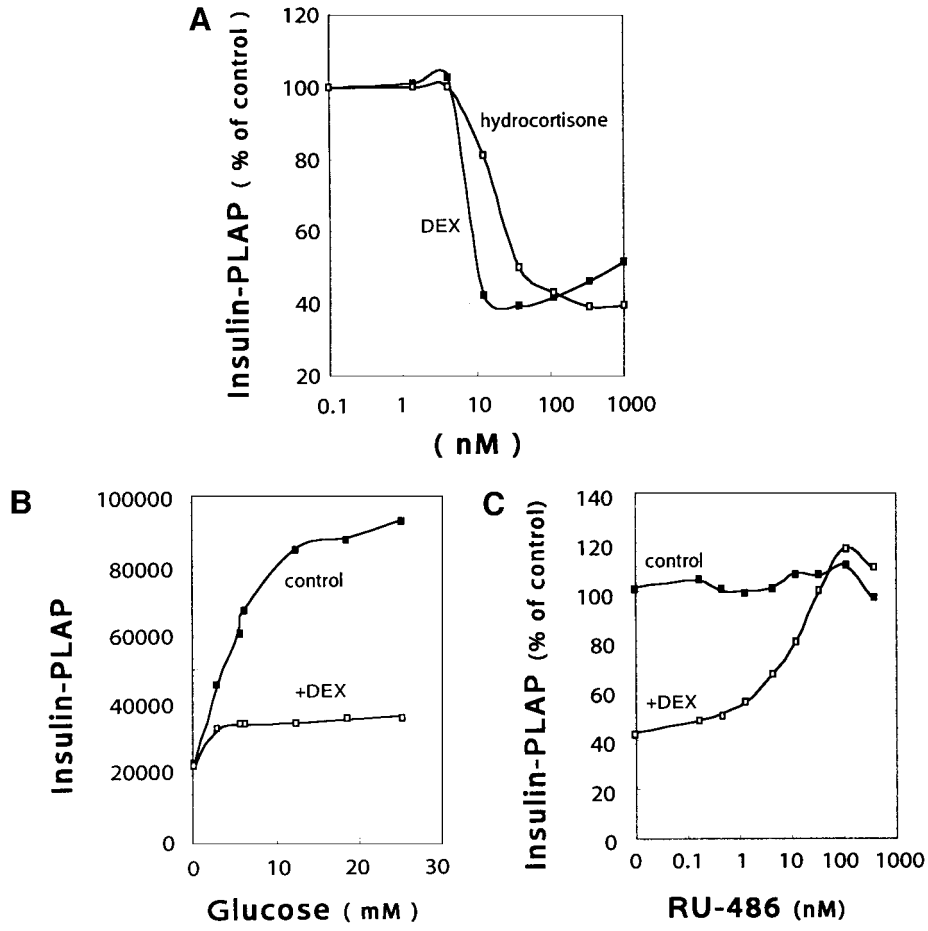

FIG. 1. The inhibitory effect of glucocorticoids on the insulin promoter activity in HIT-T15 cells. (A) Cells were cultured with 5.5 mM glucose and various concentrations of hydrocortisone or DEX. (B) Cells were cultured with DEX (20 nM) in the presence of various concentrations of glucose. (C) Cells were cultured with 5.5 mM glucose, DEX (20 nM) and various concentrations of RU-486. PLAP activity in the conditioned medium was measured. PLAP activity in the conditioned medium without hormones was used as a control.

$MgSO_4$), and then 50 μl of chemiluminescent substrate was added. The mixtures were incubated at room temperature for 30 min, and the steady-state chemiluminescence was measured with a microplate luminometer (Wallac Eg & G, Turku, Finland).

RESULTS

Glucocorticoid Reduced the Expression of Insulin in HIT-T15 Cells

There is evidence that glucocorticoids are physiological inhibitors of insulin synthesis at the level of transcription (12, 13). We confirmed the inhibitory effect of glucocorticoids, hydrocortisone and DEX, on insulin promoter activity in pancreatic HIT-T15 cells, which expressed a PLAP reporter gene containing nucleotides −358 to −1 of the 5′ flanking region of the rat insulin I gene (pr-Ins-PLAP). This region is thought to be sufficient for both beta cell-specific and glucose-dependent transcription of the gene (27, 28). As shown in Fig. 1A, hydrocortisone and DEX strongly inhibited insulin promoter activity in a dose-dependent manner. The glucose dependency of the insulin promoter activity almost disappeared, showing that glucocorticoids blocked the glucose-dependent insulin transcription (Fig. 1B). RU-486 (mifepristone), which is a glucocorticoid receptor (GR) antagonist, completely blocked the inhibitory effect of DEX (29), indicating that the effect is mediated by a GR-dependent signal (Fig. 1C). Similar results were obtained using HIT-T15 cells which transiently expressed a pr-Ins-PLAP (data not shown).

We then studied the changes of mRNA and protein levels of endogenous insulin in HIT-T15 cells treated with DEX. Northern blot analysis revealed that insulin mRNA was decreased to 81% at 12 h, and to less than 35% at 24 h after treatment with DEX (20 nM) (Fig. 2A). ELISA analysis showed that the protein level of insulin in HIT-T15 cells was maintained for at least 12 h, but had decreased to 12% at 48 h (Fig. 2B). During this time, insulin secretion from the cells was rather inhibited, indicating that the decrease of insulin content was not due to the enhancement of insulin

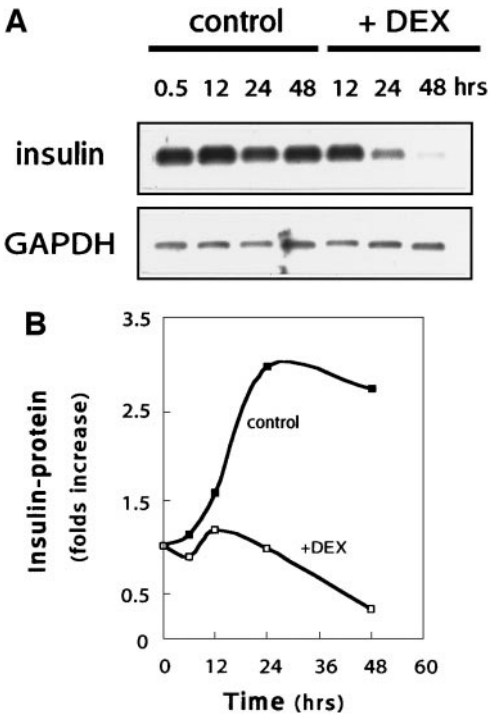

FIG. 2. Change of insulin in HIT-T15 cells treated with DEX. HIT-T15 cells were cultured with DEX (20 nM) for the indicated period in D-MEM containing 5.5 mM glucose. (A) Total RNA of the cells was extracted and mRNA of insulin was analyzed by northern blotting. (B) Proteins were extracted and the insulin content was measured by ELISA.

secretion from the cells. These results showed that DEX inhibited insulin gene expression, resulting in a decrease of biosynthesis and storage of insulin in HIT-T15 cells.

Change of Transcription Factors in HIT-T15 Cells Treated with DEX

Insulin gene expression is controlled by several transcription factors, two major ones being PDX-1 and BETA2/NeuroD. Therefore we studied the expression of these factors in HIT-T15 cells treated with DEX. As shown in Fig. 3, the expression of PDX-1 decreased to 65% and that of BETA2/NeuroD mRNA to 67% after 2-h treatment. The depression of both mRNAs continued for at least 48 h (data not shown).

In nerve cells, the expression and activity of BETA2/NeuroD and other bHLH transcription factors are negatively regulated by HES-1 (30). Since HES-1 is also known to be expressed in the developing pancreas in mouse (31), we studied the expression of HES-1 in HIT-T15 cells. As shown in Fig. 3, HES-1 was actually expressed in the cells, and its expression was increased after 6-h treatment with DEX. The increase of HES-1 expression was confirmed in at least three independent experiments, although we could not calculate the increase ratio precisely owing to the weak expression of HES-1 in nontreated HIT-T15 cells. HES-5, another HES family gene (32), was found to be expressed in HIT-T15 cells, but its expression was not changed by DEX (data not shown).

Overexpression of HES-1 in HIT-T15 Cells

To address whether HES-1 could be involved in the regulation of insulin gene expression in HIT-T15 cells, we cotransfected HES-1 cDNA and pr-Ins-PLAP into the cells. Northern blot analysis confirmed that the exogenous HES-1 cDNA was efficiently expressed in HIT-T15 cells (Fig. 4B), and the cDNA repressed insulin-PLAP activity in a dose-dependent manner. AHES-1 cDNA, however, which has an antisense sequence to HES-1, showed no inhibitory effect (Fig. 4A). These results suggested that increased HES-1 could negatively regulate the insulin gene expression in HIT-T15 cells.

DISCUSSION

Here we have shown that glucocorticoids reduced the mRNA and protein levels of insulin in pancreatic HIT-T15 cells, and in advance of these changes, the PDX-1 and BETA2/NeuroD mRNA levels decreased, and the HES-1 mRNA level increased in the cells. It was reported that glucocorticoids suppress insulin gene expression by reducing the PDX-1 expression in pancre-

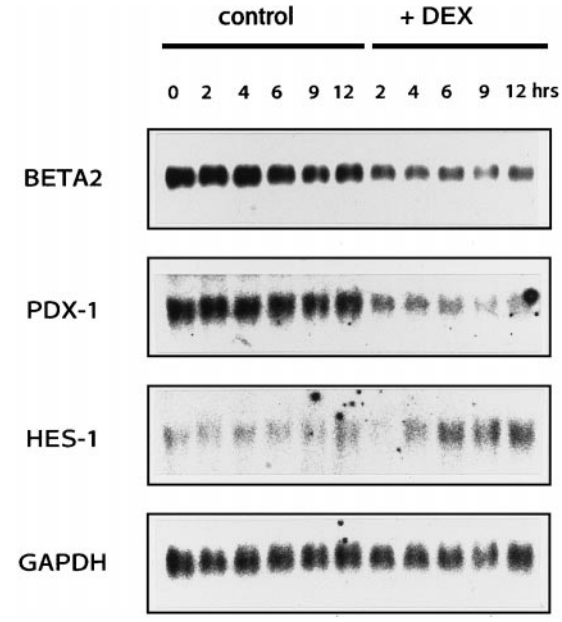

FIG. 3. Change of transcription factors in HIT-T15 cells treated with DEX. HIT-T15 cells were cultured with DEX (20 nM) for the indicated time period in D-MEM containing 5.5 mM glucose. Total RNA of the cells was extracted and mRNAs of PDX-1, BETA2/NeuroD, HES-1 and GAPDH were analyzed by Northern blotting.

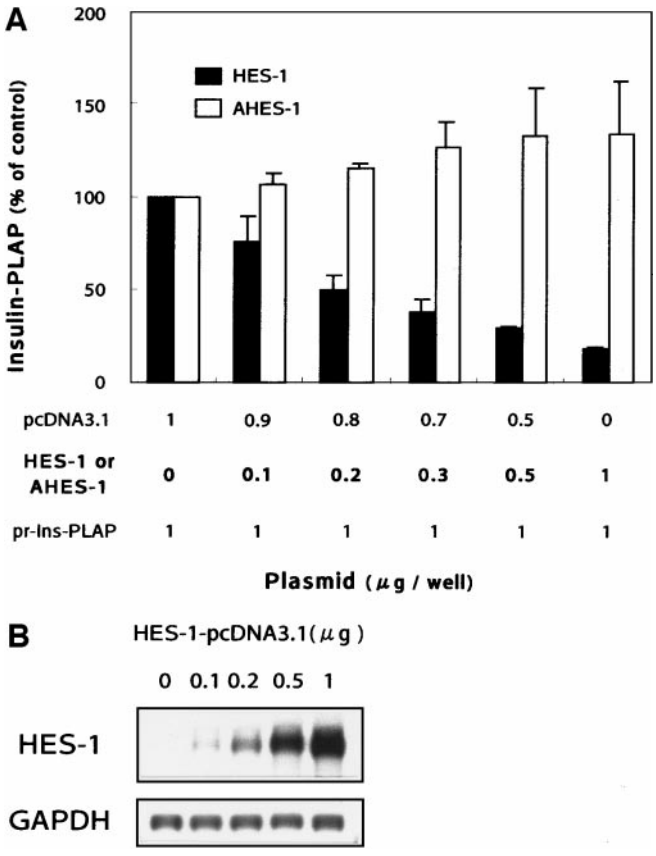

FIG. 4. PLAP activity in HIT-T15 cells cotransfected with HES-1-pcDNA3.1 and pr-Ins-PLAP. (A) HIT-T15 cells cotransfected with various amounts of HES-1-pcDNA3.1 and pr-Ins-PLAP (1 μg) were cultured for 48 h in D-MEM containing 5.5 mM glucose, and each PLAP activity was measured. Values are means ± SD of the relative activities in at least three independent experiments. Antisense HES-1 is abbreviated as AHES-1. (B) Total RNA of the cells was extracted and mRNAs for HES-1 and GAPDH were analyzed by Northern blotting.

atic beta cells (12). However, a decrease of BETA2/NeuroD and an increase of HES-1 in HIT-T15 cells induced by glucocorticoids have not previously been reported.

Upon DEX treatment of the cells, the glucose dependency of the insulin promoter activity almost disappeared. This phenomenon might be related to the decreased expression of PDX-1 and BETA2/NeuroD, since both transcription factors are essential to the glucose dependency of insulin gene expression (14–16). We also showed that HES-1 might be involved in the downregulation of insulin gene expression by glucocorticoids. HES-1 was originally found in nerve cells and was demonstrated to downregulate the differentiation of nerve cells by inhibiting both the expression and activities of bHLH-type transcription factors, including BETA2/NeuroD (33). HES-1 is also expressed in pancreatic cells and plays an important role in cell differentiation (31, 34). This prompted us to study the expression of HES-1 in HIT-T15 cells. HES-1 is expressed in HIT-T15 cells and its expression was elevated by treatment with glucocorticoids. Overexpression of HES-1 cDNA repressed insulin promoter activity, suggesting that the increase of HES-1 might be involved in the downregulation of insulin gene expression, in addition to the decreases of PDX-1 and BETA2/NeuroD.

HES-1 itself has a bHLH structure and can repress gene expression via two mechanisms; by directly binding to the N-box sequences (CACNAG) of target genes, or by preventing other bHLH activators from binding to their E-box sequences (CANNTG) (33). Since typical N-box sequences are present in the promoter regions of insulin genes in rat, mouse and human (27, 35, 36), it is possible that HES-1 could directly inhibit insulin gene expression. It is not clear whether HES-1 inhibits the expression or activities of other transcription factors. Figure 3 shows that PDX-1 and BETA2/NeuroD were decreased in advance of the elevation of HES-1, implying that HES-1 is not a trigger for the rapid decrease of PDX-1 and BETA2/NeuroD. Since insulin mRNA decreased gradually over 24 h, HES-1 might have a role in maintaining the repression of insulin gene expression. Further study is required to understand the cascade of changes in HIT-T15 cells caused by DEX, as well as the role of HES-1.

Recent studies of knockout mice showed that PDX-1, BETA2/NeuroD and HES-1 are all essential factors for the differentiation of pancreatic endocrine cells, including beta cells (37, 20, 34). PDX-1 and BETA2/NeuroD are stimulators, whereas HES-1 is an inhibitor of differentiation being correlated to the regulation of the insulin gene expression. The evidence implies that glucocorticoids may regulate the differentiation of pancreatic endocrine cells by altering the balance of these transcription factors. It was reported that glucocorticoids can modulate the development of the embryonic pancreas (10). Pancreatic AR42J cells have the potential to differentiate into both endocrine-like and exocrine-like cells, and glucocorticoids stimulated differentiation to the latter (38).

Impaired abilities of insulin gene expression and differentiation are typical features of beta cells in type 2 diabetes. Thus, studies of the effect of glucocorticoids on transcription factors which regulate both insulin gene expression and differentiation of beta cells should be important in elucidating and eventually overcoming the beta cell dysfunction in type 2 diabetes.

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