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Calcium/calmodulin-dependent protein kinase IV involvement in the pathophysiology of glucotoxicity in rat pancreatic β-cells

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Abstract

Glucotoxicity is a critical component of the pathophysiology of type 2 diabetes mellitus; however, the molecular mechanisms of glucotoxicity are still not fully understood. We have attempted to determine the protein kinases involved in glucotoxicity in pancreatic β-cells by the use of a new technique. Using Multi-PK antibodies, which are capable of detecting a wide variety of protein kinases, we analyzed the protein kinase that correlated with insulin synthesis in INS-1 cells under glucotoxic conditions. When expression patterns of protein kinases in INS-1 cells were analyzed by Western blotting with Multi-PK antibodies, a kinase of 63 kd was significantly reduced concomitant with the decrease of insulin secretion under glucotoxic conditions. To identify the 63-kd kinase, we used a unique 2-dimensional gel electrophoretic technique and MicroRotofor (Bio-Rad Laboratories, Tokyo, Japan) electrophoresis. From the molecular size of a native kinase/cyanogen bromide fragment and pI value, the 63-kd protein kinase was deduced to be CaMKIV. This was confirmed by Western blotting analysis using anti-CaMKIV antibodies. The decreased CaMKIV levels under glucotoxic conditions recovered to original levels after changing the medium to a normal glucose concentration. Recombinant CaMKIV was degraded in a Ca^{2+}-dependent manner by incubation with cell lysates from INS-1 cells under glucotoxic conditions, and degradation was protected by calpain inhibitor. Furthermore, CaMKIV was reduced in the pancreatic islets of diabetic Otsuka Long-Evans Tokushima fatty rats, whereas that of nondiabetic Long-Evans Tokushima Otsuka rats was not. This study suggests that the abnormal regulation of CaMKIV is a component of β-cell dysfunction caused by high glucose.

1. Introduction

The maintenance of blood glucose concentrations within narrow limits is of critical physiologic importance in mammals. Elevated glucose concentration stimulates the transcription of insulin and several other genes that regulate glucose homeostasis [1]. Transient elevation of extracellular glucose promotes pancreatic β-cell function and survival [2], whereas chronic elevation of glucose has the opposite effect, impairing β-cell function [3,4]. The deleterious effects of chronically elevated glucose are referred to as *glucotoxicity*. Glucotoxicity is a critical component of the pathophysiology in type 2 diabetes mellitus because it impairs both the actions of insulin on peripheral tissues and the secretion of insulin by β-cells. Impairment of insulin secretion contributes to further glucose elevation and results in glucotoxicity. This self-perpetuating forward feedback mechanism accelerates the progression of diabetes and the appearance of diabetic complications [5]. One of the hallmarks of such glucotoxicity is reduced insulin gene expression caused by decreased insulin promoter activity [6-8]. Pancreatic β-cells play a critical role in the pathogenesis of both type 1 and type 2 diabetes mellitus. Loss of pancreatic β-cell is mainly the result of apoptosis and is enhanced by

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hyperglycemia [9]. Although a number of transcription factors such as pancreas duodenum homeobox–1 and CCAAT/enhancer-binding protein–β have been shown to be implicated in the toxic effects of glucose and β-cell apoptosis [10-13], the molecular mechanisms of this glucotoxicity are still not fully understood.

Protein kinases are known to play pivotal roles in various cellular functions through the regulation of signaling pathways [14]. Therefore, it is important to know the expression profiles of all the protein kinases in cells and tissues to elucidate their functional roles. In previous research, we detected a wide variety of protein kinases through the production and use of unique monoclonal antibodies directed to a highly conserved region (subdomain VIB) of protein kinases (Multi-PK antibodies) [15,16]. It is therefore evident that these antibodies can serve as powerful detection tools for multiple types of protein kinases by Western blotting. MicroRotofor (Bio-Rad Laboratories, Tokyo, Japan)/sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis combined with Multi-PK antibodies is also a useful technique for the efficient separation of proteins, especially when analyzing the variety of protein kinases expressed in cells and tissues [17]. In addition, identification of protein kinases in cells and tissues through analysis of cyanogen bromide (CNBr) fragments containing subdomain VIB of protein kinases using 2-dimensional (2D) PAGE with the Multi-PK antibody has been reported [18].

In this study, we attempted to determine the protein kinases involved in glucotoxicity in INS-1 cells, a rat insulinoma cell line, by the new techniques described above. As a result, we were able to identify the protein kinase Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV as a potential kinase mediating glucotoxicity.

2. Materials and methods

2.1. Materials

Recombinant rat CaMKIV was expressed in *Escherichia coli* BL21 (DE3) and purified as described previously [16]. Multi-PK antibodies (M8C and YK34) were obtained from 2 hybridoma cell lines established as described previously [15,16].

2.2. Cell culture

The INS-1 cells used in this study were derived from a rat insulinoma cell line that was developed and propagated at the Division of Biochimie Clinique (courtesy of Dr CB Wollheim, Geneva, Switzerland). The INS-1 cells were cultured in 5% CO_2/95% air at 37°C in RPMI 1640 media (Gibco, Tokyo, Japan) containing 2.8, 5.6, 11.2, and 22.4 mmol/L glucose and supplemented with 10% heat-inactivated fetal bovine serum (bio west, Canada origin; Roche Diagnostics, Basel, Switzerland) and 50 μmol/L 2-mercaptoethanol for at least 7 days (unless otherwise stated).

2.3. Protein determination, SDS-PAGE, and Western blotting

Proteins were determined by the method of Bensadoun and Weinstein [19] using bovine serum albumin as a standard. The SDS-PAGE was performed essentially according to the method of Laemmli [20] on slab gels consisting of a 10% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes. Immunoreactive protein bands were detected using Multi-PK antibodies, anti-CaMKIV antibodies (BD Transduction Laboratories, Lexington, KY, USA), anti-serum and glucocorticoid-regulated kinase 1 (SGK-1) antibodies (Cell Signaling Technology, Danvers, MA, USA), or anti–His_6-tag antibodies (Invitrogen, San Diego, CA, USA) at dilutions of 1:200, 1:1000, 1:1000, and 1:1000. This was followed by a secondary application of goat anti-mouse immunoglobulin G + A + M (ICN Pharmaceuticals, Costa Mesa, CA, USA) and goat anti-rabbit immunoglobulin G (Pierce Biotechnology, Rockford, IL), which had been conjugated with horseradish peroxidase, at a dilution of 1:1000 in accordance with a method described previously [15].

2.4. Insulin enzyme-linked immunosorbent assay

After exposure for indicated days, INS-1 cells were rinsed twice with phosphate-buffered saline, followed by preincubation in Krebs-Ringer bicarbonate HEPES buffer (KRBH buffer: 115 mmol/L NaCl, 24 mmol/L $NaHCO_3$, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 25 mmol/L HEPES, 0.5% BSA, pH 7.4) containing 3 mmol/L glucose at 37°C for 30 minutes. After aspiration of the buffer, INS-1 cells were incubated in fresh KRBH buffer supplemented with 22.4 mmol/L glucose at 37°C for 20 minutes. The insulin secreted into the medium was collected and determined using a commercially available sandwich-type enzyme-linked immunosorbent assay (ELISA) (Shibayagi, Tokyo, Japan). As previously reported, this ELISA is sensitive to 1 ng/mL insulin, has an intraassay coefficient of variation of less than 0.5%, and has an interassay coefficient of variation of less than 10% [21].

2.5. Analysis of CNBr fragments of INS-1 cells by 2D-PAGE using the Multi-PK antibody

Approximately 200 μg of cell lysates from INS-1 cells exposed to 11.2 mmol/L glucose was digested by CNBr, analyzed by 2D-PAGE, and followed by Western blotting using a Multi-PK antibody as described previously [18].

2.6. MicroRotofor/SDS-PAGE

Approximately 2.5 mg of cell lysates from INS-1 cells exposed to 11.2 mmol/L glucose was separated by Micro-Rotofor/SDS-PAGE according to the method described previously [17].

2.7 In vitro digestion of recombinant CaMKIV by INS-1 cell lysates

Recombinant CaMKIV (5 μg) was digested with 125 μg of cell lysates from INS-1 cells exposed to 11.2 mmol/L or 22.4 mmol/L in 250 μL of Tris-buffered saline (50 mmol/L Tris-HCl at a pH 7.5, 100 mmol/L NaCl) in the presence or absence of 1 mmol/L $CaCl_2$ and/or 50 nmol/L calpain inhibitor III (Calbiochem, San Diego, CA, USA) at 37°C for 4 or 8 hours. The digestion was halted by the addition of SDS sample buffer.

2.8. Transfection of INS-1 cells and luciferase reporter gene assay

The reporter gene used in our studies was kindly provided by Dr Roland Stein (Vanderbilt University Medical Center, Nashville, TN). The wild type (−238 WT-LUC) contained the rat insulin-2 gene sequences, which spanned the region from −238 to 2 base pairs and were linked to the luciferase reporter gene as previously described [22]. Purified reporter plasmid was transfected into INS-1 cells and incubated for 48 hours using a conventional cationic liposome transfection method (Lipofectamine 2000, Invitrogen). Both the complementary DNA of constitutively active CaMKIV (CaMKIV-c, 305 HMDT to DEDD) and a kinase-dead mutant of CaMKIV (CaMKIV-kd, 305 HMDT to DEDD, K71E) were constructed as described previously [23,24]. Transfected cells were harvested, and β-galactosidase activity was measured in an aliquot of the cytoplasmic fraction. After correcting all assays for β-galactosidase activity, the total amounts of protein per reaction proved to be identical [25]. Twenty-microliter aliquots were taken from the luciferase assay in accordance with the manufacturer's instructions (ToyoInk, Tokyo, Japan).

2.9 Protocol for diabetic animals

We used Otsuka Long-Evans Tokushima fatty (OLETF) rats, an animal model of type 2 diabetes mellitus. Four-week-old male Long-Evans Tokushima Otsuka (LETO) rats (n = 5) and OLETF rats (n = 5) were supplied by Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All rats were kept in specific pathogen-free conditions at Kagawa University Animal Center in rooms that were controlled for both temperature and light (on from 8:00 AM to 8:00 PM). The experiments were approved by the Kagawa University Institutional Animal Care and Use Committee. The rats had free access to tap water and a diet of standard chow (Clea, Tokyo, Japan). We periodically measured blood glucose and insulin under fasting condition (9:00-10:00 AM). At 80 weeks of age, all rats were killed under fasting condition. Pancreatic islets were isolated at that time as described previously [26]. Western blotting was carried out using either anti-CaMKIV antibody or β-actin (Sigma, St Louis, MO) as previously described [27]. Serum glucose concentrations were determined by the glucose oxidase method using a glucose kit as described previously [28]. The levels of immunoreactive insulin were quantified using a commercially available sandwich-type ELISA.

2.10. Others

Crude extracts of rat brain were prepared in essentially the same manner as the method described previously [15]. The immunoreactive bands were quantified using the computer software application Scion Image (Scion Corporation, Frederick, MD, USA).

3. Results

3.1. Detection of protein kinases by Multi-PK antibodies

In type 2 diabetes mellitus, glucotoxicity is an important aspect of disease progression. However, pathogenic mechanisms of glucotoxicity are poorly understood. Earlier studies have reported that many protein kinases are involved in insulin secretion [29,30], but the relationships between kinase expression and insulin secretion in glucotoxic conditions have never been properly elucidated. To detect the changes in expression of protein kinases, we conducted Western blotting analyses of cell extracts from INS-1 cells exposed to different glucose concentrations using Multi-PK antibodies. As shown in Fig. 1A, more than 5 immunoreactive protein bands were detected in INS-1

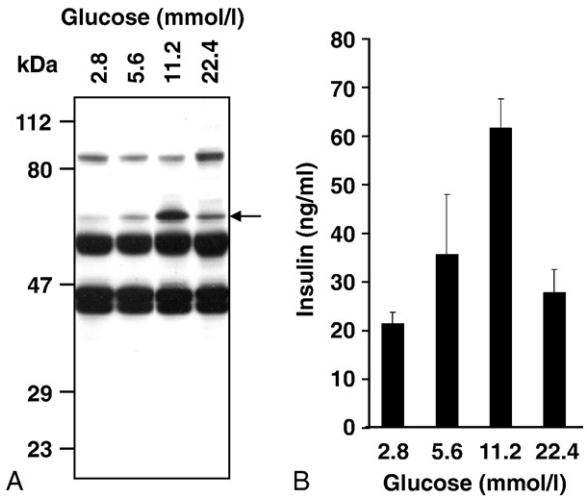

Fig. 1. The expression level of 63-kd protein kinase correlates with insulin secretion. A, The expression levels of protein kinases in INS-1 cells incubated with several concentrations of glucose were determined by Western blotting using the M8C antibody. INS-1 cells were incubated for 7 days with RPMI 1640 medium supplemented with 10% fetal bovine serum and indicated amounts of glucose. Cell lysate proteins (20 μg) were subjected to SDS-PAGE followed by Western blotting using the M8C antibody. Arrow indicates the 63-kd protein band that significantly changed in its expression level. B, Effect of glucose concentration on basal insulin secretion. INS-1 cells were incubated with the indicated concentrations of glucose. Insulin secretion was detected by sandwich-type ELISA, and each data point shows the mean ± SE (n = 3) of separate experiments.

cells exposed to different glucose concentrations by Western blotting with M8C antibodies. Our results revealed that the expression level of the 63-kd protein had changed in parallel with that of insulin secretion (Fig. 1B), whereas the other immunoreactive bands, including 45 and 60 kd, had not changed (Fig. 1A). Basal insulin secretion of INS-1 cells increased at a concentration of 11.2 mmol/L glucose, but decreased markedly at 22.4 mmol/L glucose (Fig. 1B), indicating that chronic high glucose–induced glucotoxicity results in impaired insulin secretion. Conversely, the expression of tyrosine kinases did not change with glucose concentrations when tyrosine kinase-specific Multi-PK antibody (YK34) was used instead of M8C in Western blotting (data not shown).

3.2. Identification of protein kinase that correlates with insulin secretion

To identify the 63-kd protein kinase, we used techniques that used Multi-PK antibodies to get information on the kinase's molecular mass, the molecular mass of subdomain VIB fragments, and the kinase's isoelectric point (pI) [16,17]. The lysate resulting from INS-1 cells cultured in 11.2 mmol/L glucose was separated by SDS-PAGE in the first dimension and digested by CNBr in gel. After digestion, the gel strip was separated by SDS-PAGE in the second dimension. This was followed by detection of subdomain VIB fragments of protein kinases by Western blotting using Multi-PK antibodies. Subdomain VIB fragments of the 63-kd protein kinase were detected by Western blotting using Multi-PK antibodies in the first dimension, whereas a 19-kd immunoreactive spot was detected in the second dimension (Fig. 2A). In addition, when INS-1 cell lysates were separated by MicroRotofor/SDS-PAGE and immunodetected with Multi-PK antibodies, the pI of the 63-kd protein kinase was estimated to be approximately pH 5 (Fig. 2B). Taken as a whole, these results suggest that the molecular mass of the native enzyme was approximately 63-kd, whereas the mass of the subdomain VIB-containing fragment was approximately 19-kd, and that the pI of the 63-kd protein kinase was approximately pH 5. On the basis of the aforementioned results, the Swiss-Prot database of the TagIdent program (http://kr.expasy.org/tools/tagident.html) was searched with the following assumptions: A native protein with a molecular weight between 48 000 and 72 000, a subdomain VIB containing a fragment with a molecular weight between 15 200 and 22 800, and a pI value between 4 and 6. The database search revealed that only 1 Ser/Thr protein kinase, CaMKIV, met the above criteria. The molecular weight of rat CaMKIV was calculated to be 53 150, and the expected molecular weight of its CNBr-cleaved fragment-containing subdomain VIB was 21 480. The pI value of CaMKIV was estimated to be 4.8 on the basis of the sequence data. When searched on the basis of both molecular weights of a native protein and a subdomain VIB-containing fragment, another protein kinase, SGK-1, was found to meet the above criteria. Molecular weights of the native form and CNBr-cleaved subdomain VIB fragment of SGK-1 were calculated to be 48 927 and 16 929, respectively. However, pI value of SGK-1 was estimated to

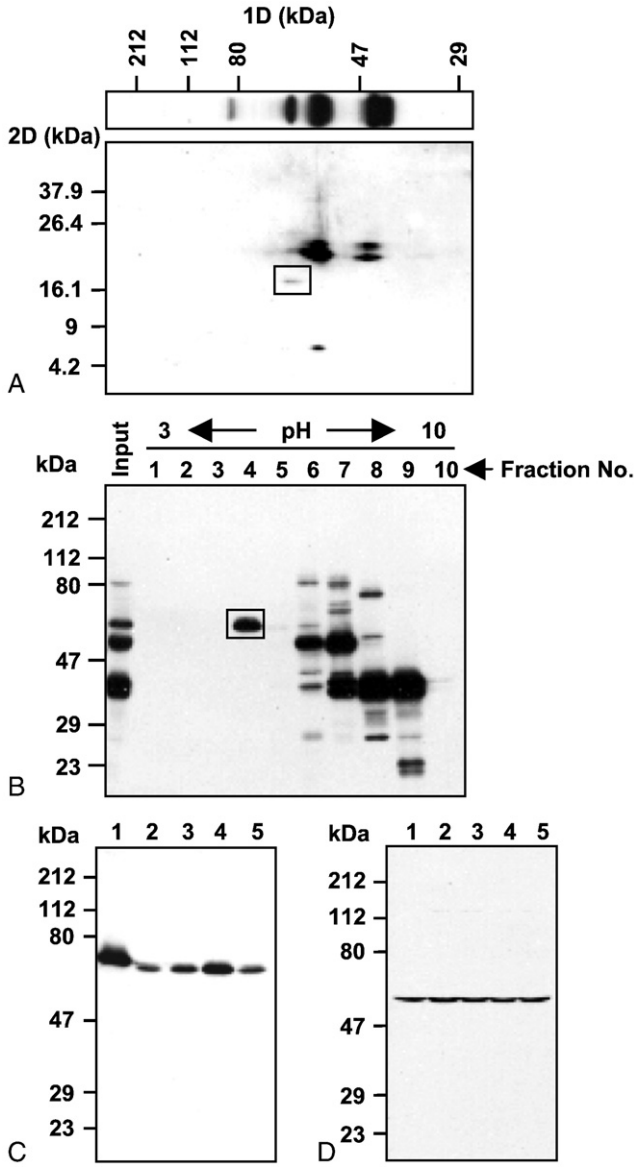

Fig. 2. Identification of protein kinase that correlates with insulin secretion. A, Cell lysate proteins (200 μg) from INS-1 cells incubated with 11.2 mmol/L glucose were separated on SDS-PAGE, and the molecular mass of subdomain VIB-containing CNBr fragment of the 63-kd protein kinase was determined by Western blotting after separation by 2D-PAGE. Details are described in "Materials and methods." The square indicates the immunoreactive spot from the 63-kd protein kinase fragment. B, Isoelectric point of protein kinase was examined by MicroRotofor/SDS-PAGE analysis of INS-1 cell lysate proteins as described in "Materials and methods." The 63-kd protein kinase is indicated by a square. Detection of CaMKIV (C) and SGK-1 (D) in INS-1 cells by Western blotting using specific antibodies. Approximately 20 μg of cell lysate proteins from INS-1 cells exposed to 2.8 (lane 2), 5.6 (lane 3), 11.2 (lane 4), and 22.4 (lane 5) mmol/L glucose was analyzed by SDS-PAGE followed by Western blotting. CaMKIV (C, 50 ng) and rat brain extract (D, 20 μg) were applied on lane 1 as a positive control.

be 8.7, and this value was out of pI range between 4 and 6. When combined, these pieces of information suggest that CaMKIV is the most probable candidate for the 63-kd protein kinase. This possibility was further confirmed by Western blotting with CaMKIV-specific antibodies. As shown in Fig. 2C, the expression level of CaMKIV in INS-1 cells in each concentration of glucose was correlated with insulin secretion (Fig. 1B), whereas expression of SGK-1, which has a similar molecular weight with CaMKIV, was not changed when exposed to the same conditions (Fig. 2D). These results imply that CaMKIV expression could be regulated by glucose concentration in INS-1 cells.

3.3. Protein level of CaMKIV recovered in INS-1 cells under glucotoxic conditions

The deleterious effects of chronically elevated glucose are referred to as *glucotoxicity* in type 2 diabetes mellitus. It is known that, in patients with type 2 diabetes mellitus, impairment of insulin secretion is partially reversed after elimination of the glucotoxic conditions [5]. In our study, we examined whether the reduction of CaMKIV expression under glucotoxic conditions recovered after incubation with the regular medium containing 11.2 mmol/L glucose. As shown in Fig. 3, protein levels of CaMKIV were gradually decreased over a 7-day incubation period with 22.4 mmol/L glucose; and it completely recovered by the end of an additional 3-day incubation period with 11.2 mmol/L glucose.

3.4. CaMKIV was digested by calpain in vitro

A previous report indicated that nuclear calpain regulates Ca^{2+}-dependent signaling via proteolysis of nuclear CaMKIV in cultured neurons [31]. To further examine how high-glucose conditions might suppress the protein levels of CaMKIV, we looked at the potential involvement of calpain activation in glucotoxicity. Therefore, we tested whether recombinant CaMKIV was degraded by incubation with lysates of INS-1 cells exposed to 11.2 or 22.4 mmol/L glucose in the presence or absence of $CaCl_2$ and/or calpain inhibitor III. As expected, CaMKIV was digested by incubation with high glucose–exposed INS-1 cell lysates in the presence of $CaCl_2$, whereas this digestion was completely blocked by calpain inhibitor III (Fig. 4). The results suggested that high-glucose stress induces calpain expression, leading to CaMKIV digestion.

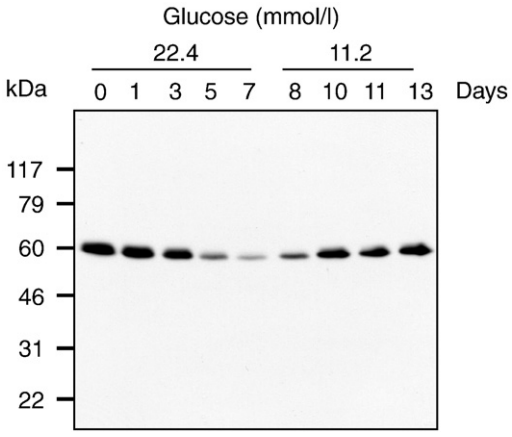

Fig. 3. Effect of glucose concentration on CaMKIV expression. INS-1 cells were incubated with 22.4 mmol/L glucose for 7 days and then incubated for an additional 7 days with 11.2 mmol/L glucose. The cell lysate proteins (20 μg) were harvested at the indicated days and analyzed by SDS-PAGE followed by Western blotting with the anti-CaMKIV antibody.

3.5 CaMKIV up-regulates insulin gene expression in INS-1 cells under glucotoxic condition

In a previous study, we showed that CaMKIV activity played an important role in glucose-stimulated insulin secretion in INS-1 cells under normal conditions [27]. To

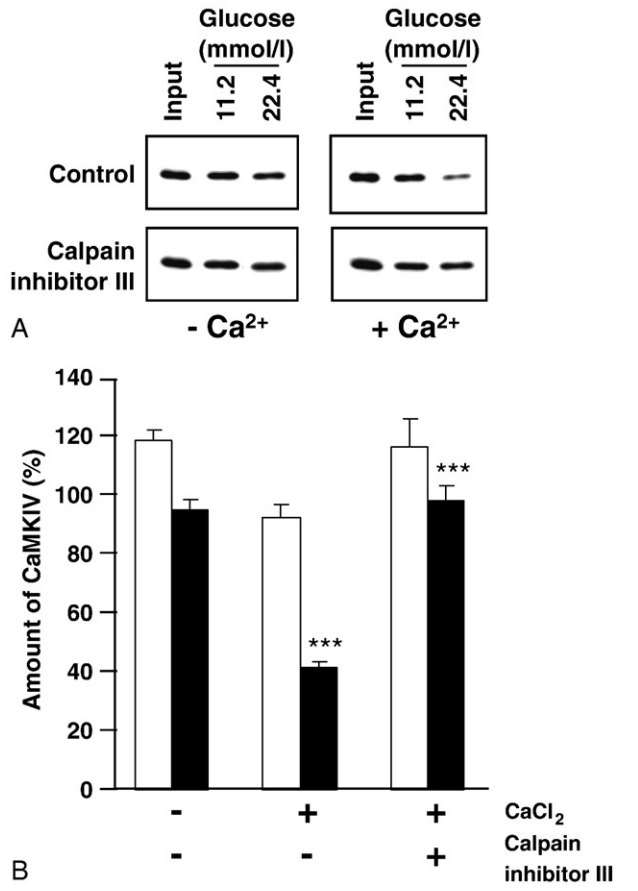

Fig. 4. Ca^{2+}-dependent degradation of CaMKIV by calpain. A, INS-1 cells were cultured in RPMI 1640 medium containing 11.2 or 22.4 mmol/L glucose for 7 days. Recombinant CaMKIV was digested by lysate proteins from INS-1 cells in the presence or absence of 1 mmol/L $CaCl_2$ and/or 50 nmol/L calpain inhibitor III at 37°C for 4 hours. Remaining CaMKIV was detected by Western blotting using the anti–His_6-tag antibody. B, Recombinant CaMKIV digested by lysate proteins of INS-1 cells incubated with 11.2 mmol/L (open bars) or 22.4 mmol/L (closed bars) glucose at 37°C for 8 hours as in panel A. The remaining CaMKIV was visualized by Western blotting and quantified by Scion Image. Each data point shows the mean ± SE (n = 3) of separate experiments. ***Significant difference ($P < .001$) from absence of $CaCl_2$ and presence of $CaCl_2$ and calpain inhibitor III.

examine the potential role of CaMKIV activity in glucotoxicity, we transfected INS-1 cells under glucotoxic conditions with −238 WT-LUC plus CaMKIV-c or CaMKIV-kd to determine whether these proteins affected insulin gene transcription. Results showed that CaMKIV-c stimulated a 4-fold increase in insulin promoter activity in INS-1 cells under glucotoxic conditions (Fig. 5). In contrast, transfection of CaMKIV-kd did not activate the promoter pINS-LUC (Fig. 5).

3.6. CaMKIV expression is decreased in the pancreatic islets of OLETF rats

Next, we examined whether CaMKIV expression is down-regulated in an animal model of diabetes. The expression of CaMKIV in the pancreatic islets of the 2 rat types was examined by Western blot analysis. Fasting serum glucose and insulin concentrations in 80-week-old OLETF or LETO rats were as follows: OLETF, 16.1 ± 1.3 mmol/L and 94 ± 15.4 pmol/L; LETO, 6.2 ± 0.5 mmol/L and 144.8 ± 19.2 pmol/L. As expected, CaMKIV protein expression in the pancreatic islets of 80-week-old OLETF rats was significantly decreased in comparison with that of 80-week-old LETO rats (Fig. 6).

4. Discussion

Glucose is the major physiologic regulator of insulin gene expression. However, prolonged elevation of glucose concentrations contributes to β-cell dysfunction in type 2 diabetes mellitus, partly because it inhibits insulin gene expression [6,7,11]. Indeed, in this study, we observed that chronic exposure of INS-1 cells to a high glucose concentration decreased insulin synthesis in a time-dependent manner. The suppression of insulin gene expression by glucotoxicity in β-cells involves several transcription factors and different pathophysiologic mechanisms. These mechanisms include the translocation of pancreas duodenum homeobox–1 (also known as *STF-1*, *IPF-1*, and *IDX-1*) from the nucleus to the cytoplasm [10], the proteasomal degradation of MafA (RIPE3b1) [11], and the induction of transcription factors such as CCAAT/enhancer-binding protein–β [12], all of which act as negative regulators of insulin gene transcription [13]. However, the signal transduction pathways are complex and not well understood. In this study, we reported that both the kinase activity and expression level of CaMKIV might be involved in the mechanisms of glucotoxicity.

A variety of cellular signalings in response to extracellular stimuli are known to be mediated by second messengers, modulator proteins, and protein kinases. In particular, protein kinases are believed to play pivotal roles in various signaling pathways [14]. In this study, therefore, we investigated the expression of the protein kinases in high-glucose–treated INS-1 cells by Western blotting using unique monoclonal antibodies, which we designated as *Multi-PK antibodies* [15]. Multi-PK antibodies were generated by immunizing a synthetic peptide corresponding to a highly conserved region (subdomain VIB) of protein kinases, so as to detect a wide variety of protein kinases in cells and tissues. We found that INS-1 cells incubated with high glucose concentration decreased insulin secretion. Our research further indicated that one of the protein bands

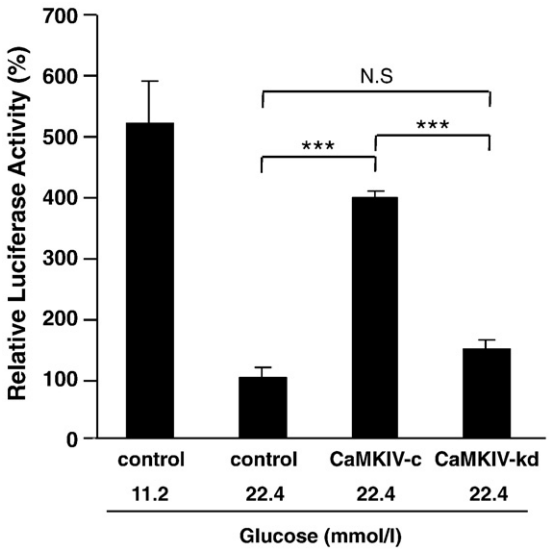

Fig. 5. Stimulation of insulin gene expression by CaMKIV under glucotoxic conditions. CaMKIV activated insulin promoter activity in INS-1 cells under glucotoxic conditions. The cells were cotransfected with −238 WT-LUC in combination with empty vector and CaMKIV-c or CaMKIV-kd expression vectors. The results are expressed as relative luciferase activity compared with control cells arbitrarily set at 100. Each data point shows the mean ± SE (n = 3) of separate transfections. ***Significant difference ($P < .001$) from the control.

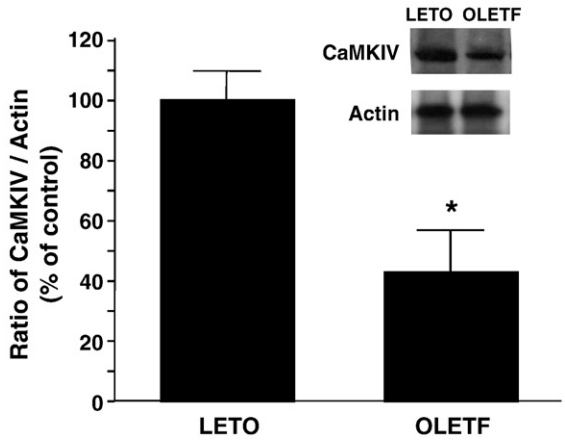

Fig. 6. Low-level expression of CaMKIV in the pancreatic islets of OLETF rats. Islets were isolated from 80-week-old male OLETF and LETO rats. CaMKIV and β-actin in the pancreatic islets of OLETF and LETO rats were detected by Western blotting. The results are shown as increasing ratios of CaMKIV expression to β-actin relative to the ratio in LETO rats. Data are the means ± SE of 5 separate measurements. *$P < .05$ compared with the equivalent LETO rat values.

reactive to the Multi-PK antibodies was changed with insulin secretion in INS-1 cells. By analyzing CNBr fragments of protein kinases via 2D-PAGE using Multi-PK antibodies, we showed that CaMKIV was decreased in INS-1 cells under glucotoxic conditions. In addition, protein levels of CaMKIV were lower in the pancreatic islets of diabetic OLETF rats than in the islets of the nondiabetic counterpart rats. The diabetic OLETF rat islets also expressed less insulin in a high-glucose condition. Collectively, these data suggest that CaMKIV could play a role in mediating the β-cell dysfunction that arises from the metabolic stress conferred by chronic hyperglycemia.

Intracellular Ca^{2+} plays an important role in β-cell function. The ion is the key intracellular mediator of glucose-stimulated insulin secretion [32] and directly affects the biosynthesis of insulin [33]. In addition, Ca^{2+} also enhances transcription of the human insulin gene. This effect of Ca^{2+} is completely blocked by exposure to a CaMK inhibitor [34] and, thus, suggests that the actions of Ca^{2+} on the insulin gene are mediated in part by CaMKs. These kinases belong to a diverse group of enzymes that participate in many cellular responses and are activated by increasing concentrations of intracellular Ca^{2+}. There are 2 multifunctional CaMKs—named *CaMKI* and *CaMKIV*—that are activated by an upstream CaMK kinase (CaMKK) through phosphorylation of a threonine residue within the active loop of the protein. This phosphorylation strongly up-regulates the catalytic activity of both enzymes [35]. Numerous studies have demonstrated that the CaMKK/CaMKIV cascade is present and functional in various cell types, such as the Jurkat cell, cultured hippocampal neurons, and transfected COS-7 cells [36]. We showed that both pancreatic β-cells and the insulin-secreting cell line, INS-1, have this signal cascade. CaMKIV, which has significant nuclear localization [37], phosphorylates transcription factors such as cAMP-responsive element binding protein and serum response factor [38]. Several studies have shown that CaMKIV can mediate transcriptional stimulation through cAMP-responsive element binding protein phosphorylation [39,40]. Because the CaMK inhibitor blocked Ca^{2+} stimulation of insulin gene transcription [34], this finding suggests the participation of CaMKs in glucose control of the gene. In addition, previous studies showed that activity of the activating transcription factor 2, which binds to the human insulin gene for cAMP-responsive elements, is enhanced by CaMKIV [41]. Previously, we reported that the CaMKK/CaMKIV cascade stimulated insulin gene transcription in response to glucose stimulation [27]. We generated recombinant adenoviruses carrying the CaMKIV-c. Insulin messenger RNA was significantly up-regulated by the expression in the presence of CaMKIV-c, whereas β-actin was not. In support of this finding, the insulin secretion from the adenovirus-infected INS-1 cells was 4-fold higher in the presence of CaMKIV-c. In this study, we found that the decreased expression of CaMKIV might be one of the mechanisms for glucotoxicity, suggesting that the activation of the CaMKIV cascade may play an important role in insulin biosynthesis in pancreatic β-cells.

Calpains are a family of Ca^{2+}-dependent, neutral cysteine proteases involved in a number of signaling processes via limited proteolysis of substrate proteins. The 2 major isoforms, μ- and m-calpain, are ubiquitously expressed in vivo [42]. A mutation of the gene-encoding calpain-10 (*CAPN10*) has recently been associated with an increased prevalence of type 2 diabetes mellitus in humans [43], highlighting the importance of calpains in the regulation of fundamental signaling pathways in diabetes. In keeping with observations for human *CAPN10*, a previous study observed a number of different isoforms of *CAPN10* in INS-1 cells, presumably a result of differential splicing [43]. The presence of multiple isoforms gives rise to the clear possibility of multiple calpain actions based either upon differential isoform activation and/or localization. The situation becomes more complex when one takes into account that not only are there multiple isoforms of *CAPN10*, there are also other calpain species (and indeed other cysteine proteases) present in β-cells [44]. Exposure of INS-1 cells to high-glucose environment stimulated *CAPN10* gene expression with a concomitant increase in calpain activity [45]. Tremper-Wells and Vallano [31] reported that nuclear calpain regulates Ca^{2+}-dependent signaling via proteolysis of nuclear CaMKIV in cultured neurons. In this study, we showed that recombinant CaMKIV was degraded by lysates of INS-1 cells under glucotoxic conditions and that the degradation was dependent on Ca^{2+}. This degradation was completely blocked by a calpain inhibitor. These results suggested that calpain activity in INS-1 cells under glucotoxic conditions was higher than calpain activity in INS-1 cells under normal conditions. Although this appears to be a logical explanation for the decrease of CaMKIV protein levels in glucotoxic conditions, further investigation will be needed to clarify the role of calpains on glucotoxicity.

In conclusion, this study suggests that β-cell dysfunction induced by chronic high-glucose exposure is mediated by the abnormal regulation of CaMKIV. Our findings imply that the CaMKIV pathway up-regulated insulin gene expression under glucose stimulation. To more clearly elucidate the role that CaMKIV plays in the development of β-cell dysfunction, it would be useful to generate animals in which CaMKIV in pancreatic β-cells is conditionally knocked out.

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