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Multiple Kinases Regulate *mafA* Expression in the Pancreatic Beta Cell Line MIN6*

Nathan L. Vanderford, Jamie E. L. Cantrell, Gabriel J. Popa, and Sabire Özcan

From the Department of Molecular and Cellular Biochemistry, University of Kentucky, College of Medicine, Lexington, KY 40536

Abstract

MafA is a basic leucine zipper transcription factor expressed within the beta cells of the pancreas and is required to maintain normal glucose homeostasis as it is involved in various aspects of beta cell biology. MafA protein levels are known to increase in response to high glucose through mechanisms that have yet to be fully characterized. We investigated whether discrete intracellular signaling events control *mafA* expression. We found that the general kinase inhibitor staurosporine induces *mafA* expression without altering the stability of the protein. Inhibition of the MAP-kinase JNK mimics the effects of staurosporine on the expression of *mafA*. Calmodulin kinase and calcium signaling are also important in stimulating *mafA* expression by high glucose. However, staurosporine, JNK, and calmodulin kinase have different effects on the induction of *insulin* expression. These data reveal that MafA levels are tightly controlled by the coordinated action of multiple kinase pathways.

Keywords

Diabetes; Glucose; Beta Cells; Insulin; Transcription; MafA; JNK; CaMK

INTRODUCTION

Insulin gene transcription is regulated by many *cis*-acting elements within the 350 base pair promoter region of the *insulin* gene. The glucose-dependent induction of *insulin* transcription is mainly driven by the A3, E1, and C1 elements of the *insulin* promoter [1–3], which are recognized by the transcription factors Pdx-1, NeuroD1, and MafA (formerly known as RIPE-3b1), respectively [1–4]. MafA is a major activator of *insulin* transcription [5] and consistent with this, *insulin* transcript levels are reduced in the *mafA* knockout mice [6]. Knockdown of MafA by siRNA also decreases insulin promoter activity and *insulin* mRNA levels [5,7]. In addition to its importance in *insulin* transcription, MafA is also required for proper insulin secretion and maintenance of islet/beta cell integrity [6]. *mafA* knockout mice develop diabetes due to comprised beta cell function [6].

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Address correspondence to: Sabire Özcan, Department of Molecular and Cellular Biochemistry, University of Kentucky, College of Medicine, 741 South Limestone Street, Lexington, KY 40536, USA. Tel: 859 257-4821; Fax: 859 257-2283; E-mail address: sozcan@uky.edu.

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mafA mRNA and protein levels are increased in high glucose-grown beta cells [5,8,9] via increased flux through the hexosamine biosynthetic pathway and O-linked glycosylation [10, 11]. MafA protein is also constitutively phosphorylated by glycogen synthase kinase (GSK) leading to its ubiquitination and subsequent proteasomal degradation [12,13].

Glucose metabolism stimulates several signal transduction events within beta cells, including those that rely on calcium influx [14]. Intracellular calcium metabolism activates multiple kinases including calmodulin kinase (CaMK) [15–17], the extracellular signal-regulated protein kinase 1/2 (ERK1/2) [18], and protein kinase C (PKC) [16,17]. The signaling pathways associated with these kinases coordinate a variety of events including both insulin secretion and *insulin* transcription [15–19].

In this report, we investigated the role of kinases in the regulation of *mafA* expression. We provide evidence that *mafA* expression increases in response to the general kinase inhibitor staurosporine in a glucose-independent manner. This suggests that a kinase-dependent signaling event suppresses *mafA* expression irrespective of glucose concentration. The inhibition of the JNK MAP-kinase pathway mimics the effects induced by staurosporine on *mafA* expression. We also show that calmodulin kinase and calcium signaling influence the expression of *mafA*. These data reveal new pathways by which beta cells ultimately control the total levels of MafA.

MATERIALS AND METHODS

Chemicals

D-glucose, EGTA, and nifedipine were obtained from Sigma (St. Louis, MO). SP600125, JNK inhibitor V, staurosporine, and ionomycin were obtained from Calbiochem (San Diego, CA). Cell culture media were obtained from Atlanta Biologicals (Lawrenceville, GA).

Cell culture

Mouse insulinoma 6 (MIN6) cells of passages 20–30 were cultured in Dulbecco's modified Eagle media (DMEM) containing 25 mM glucose, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 2 mM glutamine, and 100 μ M β -mercaptoethanol [20].

Western blotting

Western blotting was performed as described previously [10]. Proteins were separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose membranes (Osmonics Inc.). Membranes were blocked for 1 hour in 1X TTBS (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton-X 100) supplemented with 5% Carnation non-fat dry milk. After blocking, membranes were incubated overnight at 4°C with either MafA (Calbiochem), TATA-binding protein, TBP, CaMKII or phospho-CaMKII (Santa Cruz Biotechnology Inc.) antibody. Membranes were washed four times for 10 minutes in TTBS and subsequently incubated for 1 hour at room temperature with a secondary horseradish peroxidase-conjugated antibody and proteins were visualized by the ECL chemiluminescent detection system (Amersham Biosciences).

Real-time qRT-PCR

Real-time qRT-PCR analysis was performed as previously described [10]. Briefly, RNA was isolated from MIN6 cells using the RNeasy[®] Mini Kit (Qiagen). First-strand cDNA synthesis was performed using the Enhanced Avian HS RT-PCR Kit (Sigma), after treatment with DNase I (Sigma) to remove contaminating genomic DNA. Real-time amplification of the cDNA was performed using the Brilliant SYBR Green QPCR Master Mix (Stratagene). The oligonucleotide primers used in this study are listed in Supplemental Table 1. Real-time PCR

reactions were performed on an Mx4000 instrument (Stratagene) and the data were analyzed using the $2^{-\Delta\Delta C_t}$ method as we have previously described [10].

RESULTS

Staurosporine Induces *mafA* Expression

To test whether MafA levels are regulated by a cellular kinase(s), we treated high or low glucose-grown MIN6 beta cells with the general protein kinase inhibitor staurosporine. The mouse insulinoma MIN6 cell line is a widely used model system to study glucose metabolism and signaling pathways regulating insulin production and beta-cell function. MIN6 cells are responsive to physiological concentration of glucose and display glucose induced insulin secretion and insulin transcription [20]. Incubation of MIN6 cells with staurosporine dramatically increased MafA levels in a glucose-independent manner without altering the levels of TBP (Fig. 1A). Real-time RT-PCR analysis revealed that the increase in MafA protein levels after staurosporine treatment is due to increased *mafA* mRNA levels. We found that *mafA* mRNA levels were about 2-fold increased in high glucose incubated MIN6 cells as compared to low glucose alone (Fig. 1B). Staurosporine induced *mafA* expression nearly 4-fold above that within cells treated with low glucose alone (Fig. 1B). Treatment of MIN6 cells with the protein phosphatase inhibitor okadaic acid blocked the induction of MafA protein expression by high glucose (Supplemental Fig. 1). Overall, these data indicate that a phosphorylation event(s) suppresses *mafA* expression in both low and high glucose incubated MIN6 cells.

Since MafA has been shown to be degraded via the proteasome [12,13], we next tested whether staurosporine also prevents MafA degradation. For this, we treated MIN6 cells with low glucose and cycloheximide and with or without staurosporine. While the addition of staurosporine to low glucose incubated MIN6 cells drastically increased MafA protein levels, it did not prevent the degradation of MafA in the presence of cycloheximide (Fig. 1C). Proteasome inhibition even in the presence of cycloheximide prevents MafA degradation (data not shown) as previously reported [12,13]. These data suggest that while staurosporine induces *mafA* expression, it has no effect on MafA protein stability.

Since MafA is a major regulator of *insulin* expression [5,8,9,21–24], we next tested whether staurosporine treatment causes induction of *insulin* transcription. As illustrated in Fig. 1D, *insulin I* or *insulin II* mRNA levels in MIN6 cells treated with 1 mM glucose plus staurosporine were similar to low glucose only grown cells. However, as expected, 25 mM glucose induced both *insulin I* and *insulin II* expression (Fig. 1D). These data are consistent with previous work, demonstrating that staurosporine inhibits insulin promoter activity [25].

Inhibition of JNK Induces *mafA* Expression

After screening many kinase inhibitors, we found that only the inhibition of JNK mimicked the effect staurosporine produced on *mafA* expression. To test whether JNK regulates MafA levels, we treated MIN6 cells with the JNK inhibitor SP600125. As shown in Supplemental Fig. 2A, phosphorylation of JNK was inhibited by treatment with SP600125. MafA protein levels increased in both high and low glucose-grown MIN6 cells treated with SP600125 (Fig. 2A). Similar effects on MafA expression levels were observed when a different JNK inhibitor was used (Supplemental Fig. 2B). Real-time RT-PCR analysis indicated that low glucose-grown MIN6 cells treated with SP600125 expressed about 2-fold more *mafA* than cells incubated in low glucose alone (Fig. 2B). Furthermore, like staurosporine, JNK inhibition did not alter the stability of MafA after the addition of cycloheximide, suggesting that JNK does not regulate MafA degradation (Fig. 2C). Overall, these data are consistent with the idea that JNK has an inhibitory effect on the expression of *mafA*.

Because JNK inhibition induced *mafA* expression similar to staurosporine treatment, we next tested whether JNK inhibition had any effect on the expression of *insulin* in low glucose-grown cells. In contrast to staurosporine treatment, both *insulin I* and *insulin II* mRNA levels were increased in MIN6 cells treated with 1 mM glucose plus SP600125 (Fig. 2D). Therefore, the expression of *mafA* correlates with an increase in *insulin* expression in cells treated with the JNK inhibitor SP600125. These data are consistent with previous reports indicating JNK suppresses *insulin* expression [26,27].

Calmodulin kinase and calcium signaling regulate *mafA* expression on high glucose

Staurosporine is known to inhibit many kinases, including calmodulin kinase (CaMK). Our screen for various kinase inhibitors revealed a role for CaMK in regulating MafA levels. We treated MIN6 cells with the CaMK inhibitor KN-93, which blocks CaMKII activation (Supplemental Fig. 3A). Treatment of MIN6 cells with KN-93 lead to a decline in MafA protein levels in high glucose-grown cells (Fig. 3A), while there was no effect on MafA levels on low glucose treated MIN6 cells (Supplemental Fig. 3B). This suggests that CaMK activity is required to induce *mafA* expression under high glucose conditions. Real-time RT-PCR analysis revealed that KN-93 decreased *mafA* expression below that observed in MIN6 cells grown in low glucose alone (Fig. 3B). These data are consistent with the idea that CaMK plays a role in mediating the high glucose-dependent expression of *mafA*.

Glucose metabolism is known to induce an increase in intracellular calcium levels leading to CaMK activation [15,28]. Thus, we next determined whether calcium levels may affect *mafA* expression. We treated high glucose-grown MIN6 cells with the calcium channel blocker nifedipine or with the calcium chelating agent EGTA. Both nifedipine and EGTA blunted, but did not completely inhibit MafA protein expression induced by high glucose (Fig. 3C). However, the calcium ionophore ionomycin led to an increase in MafA levels in low glucose-grown MIN6 cells (Fig. 3D). These data strongly suggests that CaMK and calcium signaling mediate the high glucose-dependent expression of *mafA* in beta cells.

CaMK inhibition decreases insulin expression

Calcium signaling and CaMK is known to regulate insulin secretion and *insulin* transcription [15–17,28–30]. To establish a correlation between the effects of KN-93 on *mafA* expression and the involvement of CaMK in *insulin* expression, we measured *insulin* mRNA levels in high glucose-grown MIN6 cells treated with KN-93. Consistent with previous work [30–32], the high glucose-dependent expression of *insulin I* and *insulin II* were blunted, but not completely inhibited by the inhibition of CaMK (Fig. 4). These data indicate that calcium signaling and CaMK affect both *mafA* and *insulin* expression.

DISCUSSION

Glucose metabolism induces *mafA* expression via flux through the hexosamine biosynthetic pathway followed by an unknown O-linked glycosylation event [10]. MafA protein levels are also controlled through ubiquitin-mediated proteasomal degradation after phosphorylation of MafA by GSK [12,13]. Other signaling events controlling MafA levels are unknown.

Here we demonstrate that the kinase inhibitor staurosporine increases *mafA* expression independent of glucose concentration. Staurosporine has previously been shown to inhibit *insulin* transcription and insulin secretion [25,33–35]. Consistent with these data, we observed that despite the fact that staurosporine induced *mafA* expression in low glucose-grown MIN6 cells, *insulin* expression was not increased. This is likely because staurosporine inhibits many kinases that may be required for proper *insulin* expression.

Staurosporine inhibits the expression of many genes including several transcription factors, such as c-Jun [36]. It is possible that staurosporine blocks the expression of a transcriptional regulator(s) that normally suppresses *mafA* expression, thereby increasing expression of *mafA*. On the other hand, staurosporine also activates some transcription factors like NF- κ B. As such, staurosporine increases the synthesis of some NF- κ B target genes [37,38]. Therefore, perhaps staurosporine activates a transcription factor necessary for inducing *mafA* expression.

The inhibition of the MAP-kinase JNK mimics the effects of staurosporine on *mafA* expression while the inhibition of other MAP-kinases like ERK do not affect the expression of *mafA* (data not shown). There are several putative binding sites for JNK-sensitive transcription factors such as c-Jun within the *mafA* promoter (data not shown). Beyond the expression of *mafA*, JNK also plays a role in inhibiting *insulin* expression under oxidative stress conditions [26, 27,39]. We found that the inhibition of JNK promotes *insulin* expression in low glucose-grown beta cells, suggesting a role for JNK in the suppression of *insulin* expression under low glucose conditions.

We have also shown that KN-93, nifedipine, and EGTA blocked the glucose-dependent increase in *mafA* expression, while the calcium ionophore ionomycin induces *mafA* expression in low glucose-grown beta cells. This suggests that CaMK and calcium signaling are important for the induction of *mafA* by high glucose. CaMKs are known to regulate gene expression via the activation of the cAMP response element-binding protein (CREB). CREB regulates the expression of many genes via cAMP response elements (CREs) within target gene promoters [40,41]. There are potential binding sites for CREB and for several other calcium-sensitive transcription factors within the *mafA* promoter (data not shown).

In summary, we report that the JNK MAP-kinase pathway suppresses *mafA* expression irrespective of glucose concentration. CaMK and calcium signaling also play a role in high glucose-dependent expression of *mafA*. Future studies will address the exact molecular mechanisms by which JNK and CamK regulate *mafA* expression and determine how this information can be utilized to control the levels and/or activity of MafA in beta cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Staurosporine induces *mafA*, but not *insulin* expression

A, MIN6 cells were cultured in the presence of 25 mM or 1 mM glucose +/- 100 nM staurosporine overnight (16 to 18 hours) and MafA and TBP protein levels were analyzed by Western blotting (n > 3). B, *mafA* mRNA levels, in MIN6 cells cultured overnight on 25 mM, 1 mM, or 1 mM glucose plus 100 nM staurosporine, were analyzed by real-time RT-PCR (n=4). C, Western blot analysis of the stability of MafA and TBP in MIN6 cells incubated with 25 mM glucose +/- 40 μ M cycloheximide (CHX), 1 mM glucose, and 1 mM glucose and 100 nM staurosporine +/- 40 μ M cycloheximide (n > 4). D, Real-time RT-PCR analysis of *insulin* mRNA levels in MIN6 cells cultured in 25 mM, 1 mM, or 1 mM glucose plus 100 nM staurosporine overnight (n=4).

Figure 2. Inhibition of JNK induces *mafA* and *insulin* expression

A, MIN6 cells were cultured in the presence of 25 mM or 1 mM glucose +/- 20 μ M SP600125 overnight. MafA and TBP protein levels were analyzed by Western blotting (n=3). B, MIN6 cells were cultured in 1 mM glucose, 1 mM glucose plus 20 μ M SP600125, or 25 mM glucose overnight and real-time RT-PCR was utilized to measure *mafA* mRNA levels (n=3). C, Western blot analysis MafA and TBP protein levels in MIN6 cells incubated with 25 mM glucose +/- 40 μ M cycloheximide (CHX), 1 mM glucose, and 1 mM glucose and 20 μ M SP600125 +/- 40 μ M cycloheximide (n=3). D, Real-time RT-PCR analysis of *insulin* expression in MIN6 cells incubated with 25 mM glucose, 1 mM glucose, or 1 mM glucose plus 20 μ M SP600125 overnight (n=3).

Figure 3. CaMK and calcium signaling regulate *mafA* expression

A, MIN6 cells were cultured in the presence of 25 mM glucose +/- 10 μ M of the CaMK inhibitor KN-93 or 1 mM glucose overnight and MafA and TBP protein levels were analyzed by Western blotting (n > 3). B, MIN6 cells were cultured in 1 mM glucose, 25 mM glucose +/- 10 μ M KN-93 overnight and *mafA* mRNA levels were measured by real-time RT-PCR (n=4). C, MIN6 cells were cultured overnight in 25 mM glucose, 25 mM glucose plus 10 μ M nifedipine, 25 mM glucose plus 1 mM EGTA, or 1 mM glucose. MafA and TBP protein levels were analyzed by Western blotting. D, After overnight incubation of MIN6 cells in 25 mM glucose or 1 mM glucose +/- 1 μ M ionomycin, Western blotting was utilized to measure MafA and TBP levels.

Figure 4. Inhibition of CaMK suppresses *insulin* expression

MIN6 cells were cultured in 1 mM glucose or 25 mM glucose $\pm$ 10 μ M KN-93 overnight and *insulin* levels were measured by real-time RT-PCR. The data shown are averages of three to four measurements $\pm$ standard deviations.