J Bioenerg Biomembr (2006) 38:227–232
DOI 10.1007/s10863-006-9038-x

ORIGINAL PAPER

Regulatory roles for nm23/nucleoside diphosphate kinase-like enzymes in insulin secretion from the pancreatic islet beta cell

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Received: 4 April 2006 / Accepted: 5 April 2006 / Published online: 8 September 2006
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Abstract Recent studies from multiple laboratories, including our own, provided fresh insights into the contributory roles for GTP-binding proteins (G-proteins) in glucose-stimulated insulin secretion (GSIS) from the islet β cell. However, the precise mechanisms underlying the activation of this class of signaling proteins by insulin secretagogues remain only partially understood. We recently proposed that nm23/nucleoside diphosphate kinase (NDPK) catalyzes an alternate, non-receptor-dependent activation of islet endogenous G-proteins. In further support of this proposal, we report, herein, that overexpression of wild type (WT) nm23-H1 mutant in INS cells markedly potentiated GSIS. However, an inactive mutant of nm23-H1(H118F), which is deficient in histidine kinase and NDPK activities, was considerably less effective in potentiating GSIS from these cells, suggesting that both of these activities may be relevant for the potentiating effects of nm23-H1. Potential significance of these findings in relation to contributory roles for nm23/NDPK-like enzymes in the stimulus-secretion coupling of GSIS is discussed.

Keywords nm23-H1 · Nucleoside diphosphate kinase · Histidine kinase · Pancreatic islet β cell · Insulin secretion · G-proteins

Abbreviations used: ATP: adenosine triphosphate; GK rat: Goto-Kakizaki rat; G-proteins: GTP-binding proteins; GRFs: guanine nucleotide regulatory factors; GSIS: glucose-stimulated insulin secretion; GTP: guanosine triphosphate; Mas: mastoparan; NDPK: nucleoside diphosphate kinase; PEI-TLC: polyethyleneimine-thin layer chromatography.

Introduction

The physiological process of GSIS from pancreatic β cells is felt to be mediated largely *via* the generation of soluble second messengers, such as cyclic nucleotides, hydrolytic products of phospholipases (A_2, C, and D), and adenine nucleotides (see Kowluru, 2003b for a review). However, the precise molecular and cellular mechanisms underlying GSIS remain only partially understood. It is widely accepted that after its entry into the β cell (facilitated via the glucose-transporter protein Glut-2), glucose is metabolized with a resultant increase in the intracellular ATP/ADP ratio. Such an increase in the ATP results in the closure of ATP-sensitive K^+ channels localized on the plasma membrane as a consequence of which membrane depolarization occurs. This, in turn, promotes the influx of extracellular calcium through the voltage-sensitive calcium channels. Exposure of isolated β cells to stimulatory glucose concentrations also results in mobilization of calcium from the calcium stores endogenous

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to the islet β cell. The resultant net increase in intracellular calcium has been implicated in the transport of insulin-containing secretory granules to the plasma membrane for fusion and release of insulin into the circulation (Kowluru, 2003b).

In addition to regulation of GSIS by the adenine nucleotides, several previous studies have examined the contributory roles for guanine nucleotides (i.e., GTP) in GSIS. For example, using selective inhibitors of the GTP biosynthetic pathway (e.g., mycophenolic acid), several earlier studies have suggested a permissive role for GTP in physiological insulin secretion (Metz and Kowluru, 1993 for a review). Although the exact mechanisms underlying the regulatory role (s) of GTP remain elusive, a growing body of evidence from multiple laboratories, including our own, indicates that intracellular GTP levels could partake in the activation of one (or more) G-proteins (Metz and Kowluru, 1993; Kowluru et al., 1996; Kowluru et al., 1997; Kowluru, 2003b; Li et al., 2004). In this context, at least two major groups of G-proteins have been identified in β cells. The first group consists of heterotrimeric G-proteins comprised of α, β and γ subunits. These are involved in the coupling of various receptors to their intracellular effectors, such as adenylate cyclase, phosphodiesterase, or phospholipases. The second group of G-proteins is comprised of small molecular-mass monomeric proteins, which are involved in protein sorting as well as trafficking of secretory vesicles. Accumulating body of evidence indicates that the γ subunits of trimeric as well as monomeric forms of G-proteins undergo posttranslational modifications, such as isoprenylation and carboxyl methylation, at their COOH-terminal cysteine residues. Such modification steps are felt to increase the hydrophobicity and thereby promote interaction of those proteins with their respective effector proteins (Kowluru, 2003b; Kowluru and Veluthakal, 2005).

It is well established (see Kimura et al., 2003 for a review) that nm23/NDPK-like enzymes catalyze the transfer of terminal phosphates from nucleoside triphosphates (e.g., ATP) to nucleoside diphosphates (e.g., GDP) to yield their respective nucleoside triphosphates (e.g., GTP). Emerging experimental evidence (Kimura et al., 2003; Kowluru, 2003b) indicates that, in addition to the generation of nucleoside triphosphates, nm23/NDPK plays key roles in the direct activation of certain G-proteins (e.g., trimeric as well as monomeric) as well as phosphorylation and/or regulation of several key enzymes of intermediary metabolism (e.g., ATP citrate lyase, aldolase, pyruvate kinase, glucose-6-phosphatase, and succinyl thiokinase). Although multiple regulatory roles have been suggested for NDPK, one of the unique roles of this enzyme is its ability to promote the synthesis of GTP and the subsequent activation of specific G-proteins. The latter is thought to occur via chaneling of GTP to the "vicinity" of candidate G proteins for their functional activation. It has also been shown that NDPK mediates transphosphorylation of GDP bound to G-proteins (inactive conformation) to their GTP-bound (active conformation) of G-proteins (Cuello et al., 2003; Hippe et al., 2003; Kimura et al., 2003; Kowluru and Metz, 1994; Kowluru et al., 1995; Kowluru, 2003b).

In light of the above suggestions of potential contributory roles for nm23/NDPK-like enzymes in G-protein activation, and as a logical extension to our previously published evidence, we quantitated, herein, GSIS in INS cells following overexpression of a histidine kinase and NDP kinase-deficient mutant. Our findings from these studies suggest a critical regulatory role(s) for NDPK in GSIS (see below).

Materials and methods

Materials

[8-^{3}H]GDP (9.7 Ci/mmol) was purchased from NEN-DuPont (Boston, MA). Nucleoside di- and triphosphates and their non-hydrolyzable analogs (lithium or sodium salts) were obtained from Boehringer Mannheim (Indianapolis, IN). PEI-cellulose TLC plates were purchased from E.M. Separations (Gibbstown, NJ). The rat insulin ELISA kit was purchased from American Laboratory Products Co (Windham, NH). Effectene reagent kit was purchased from Qiagen (Valencia, CA). EGTA, DTT, leupeptin and pepstatin were obtained from Sigma Chemicals (St. Louis, MO). All other reagents were of analytical grade.

Transfection of WT and H118 mutants of nm23-H1 and quantitation of GSIS

INS-1 cells (kindly provided by Dr. Chris Newgard, Duke University School of Medicine, Durham, NC) were subcultured at 70–80% confluence and transfected using Effectene transfection kit, with a maximum 0.2 μg of either WT or histidine kinase/NDP kinase activity-deficient mutant of nm23H1 according to manufacturer's instructions. Six hours after transfection cells were cultured overnight in the presence of 5 mM glucose and 2.5% fetal bovine serum. After preincubation in the presence of 3 mM glucose for another 1 h, cells were incubated in the presence of low (5 mM) or high (25 mM) glucose, for 45 min at 37°C. The supernatant was then removed, centrifuged at 300 g for 10 min, and the amount of insulin released was quantitated by ELISA as we described previously in (Amin et al., 2003).

NDPK catalytic activity determinations

Following respective incubations, control and transfected INS cells were homogenized in a buffer consisting of 230 mM mannitol, 70 mM sucrose, 5 mM Hepes buffer, pH

7.4 containing 1 mM each of EGTA and DTT, and 2.5 μg/ml leupeptin and pepstatin. NDPK activity was assayed according to a procedure we described earlier (Kowluru et al., 2002) by quantitating the formation of [^{3}H]GTPγS from [^{3}H]GDP in the presence of unlabeled ATPγS. ATPγS was used instead of ATP in order to prevent rapid hydrolysis of "newly-formed" GTP by the GTPase activity intrinsic to G-proteins endogenous to β cells. By using ATPγS and GDP, the amount of GTPγS formed (which is non-hydrolyzable) can be quantitated readily by scintillation spectrometry. In brief, the reaction mixture (total volume of 50 μl) consisted of 20 mM Tris-HCl, pH 7.5, containing 3 mM DTT, [^{3}H]GDP (1 μCi/tube), and INS cell lysate protein. The reaction was initiated by the addition of unlabeled ATPγS (200 μM) and was continued for 5 min. It was terminated by the addition of 10 μl of ice-cold 30 mM Na-EDTA (pH 7.4) and a mixture of unlabeled GDP and GTPγS (1 mM final concentration) as carrier nucleotides. The tubes were immediately plunged into an ice bath. An aliquot of the reaction mixture (typically, 10 μl) was applied to PEI-TLC plates, and then nucleotides were separated using 0.75 M K_2HPO_4, pH 3.4, as we described in (Kowluru et al., 2002). Nucleotides were identified under a UV light (Mineralight UVS-1; UV Products, San Gabriel CA) using authentic standards. The radioactivity associated with each spot was measured by scintillation spectrometry.

Other assays and statistical analysis of experimental data

Protein concentration in cell lysates was quantitated according to Bradford using BSA as a standard. The statistical significance of the differences between the experimental conditions was determined by Student's *t*-test. p values < 0.05 were considered significant.

Results

Original studies from our laboratory have characterized NDPK activity in normal rat and human islets as well as clonal β cell preparations (Kowluru and Metz, 1994). More recent studies have identified at least three isoforms of NDPK in the pancreatic β cell. They include nm23-H1, a predominantly membrane-associated form of NDPK, and the nm23-H2 isoform appears to be localized in both membranous as well as the soluble compartments (Kowluru, 2001; Kowluru et al., 2002). In addition, a mitochondrial isoform of NDPK (nm23-H4) has been identified in the islet β cell (Kowluru et al., 2002). Based on our findings on identity and subcellular distribution of various isoforms of NDPK, we surmised that the relay of high-energy phosphates as a consequence of protein histidine phosphorylation (e.g., mediated by nm23/NDPK-like enzymes) constitutes an important non-receptor-mediated activation of specific G-proteins (and other key proteins relevant to nutrient metabolism) by physiological stimuli such as glucose (Kowluru, 2001; Kowluru, 2002; Kowluru, 2004). However, convincing evidence in support of this postulate is still lacking. Along these lines, we obtained the following evidence to suggest regulatory roles for nm23/NDPK-like enzymes in GSIS from the islet β cell.

First, we observed positive modulatory effects of long-chain fatty acids, which have been implicated in GSIS (Kowluru, 2004). The phosphoenzyme formation of NDP kinase (e.g., autophosphorylation of H-118 residue) was stimulated by various fatty acids in the following rank order: linoleic acid > arachidonic acid > oleic acid > palmitic acid = stearic acid = control. Furthermore, the catalytic activity of NDP kinase was stimulated by these fatty acids in the rank order of: oleic acid > arachidonic acid > linoleic acid > palmitic acid = stearic acid = control. Arachidonic acid methyl ester, an inactive analog of arachidonic acid, did not significantly affect either the phosphoenzyme formation or the catalytic activity of NDP kinase. Together, these findings identify additional loci (e.g., NDPK) at which unsaturated, but not saturated, fatty acids could exert their intracellular effects leading to exocytotic secretion of insulin.

We obtained further evidence to suggest that overexpression of wild type (WT) nm23-H1 mutant (Ma et al., 2004) in insulin-secreting INS cells markedly potentiated GSIS. However, an inactive mutant of nm23-H1[H118F], which is deficient in histidine kinase and NDPK activities (Ma et al., 2004), was considerably less effective in potentiating GSIS from these cells, suggesting that both of these activities may be relevant for the potentiating effects of nm23-H1 (Fig. 1; panels A and B) on GSIS. It must be pointed out that overexpression of the WT mutant of nm23-H1 had no significant effects on basal insulin secretion seen in the presence of 5 mM glucose (not shown). Together, these findings are suggestive of regulatory roles for nm23/NDPK-like enzymes in GSIS.

Discussion

Typically in most cells, the transduction of extracellular signals involves binding of a ligand to its respective receptor, often followed by the activation of one (or more) G-proteins leading to the activation of corresponding effector proteins. The pancreatic β cell is unusual in that, glucose, the most potent physiological agonist for insulin secretion, lacks an extracellular receptor. Instead, as stated above, events consequent to glucose metabolism promote insulin secretion *via* the generation and/or altered distribution of diffusible second messengers, such as ions, cyclic nucleotides, and biologically-active lipids. Changes in calcium

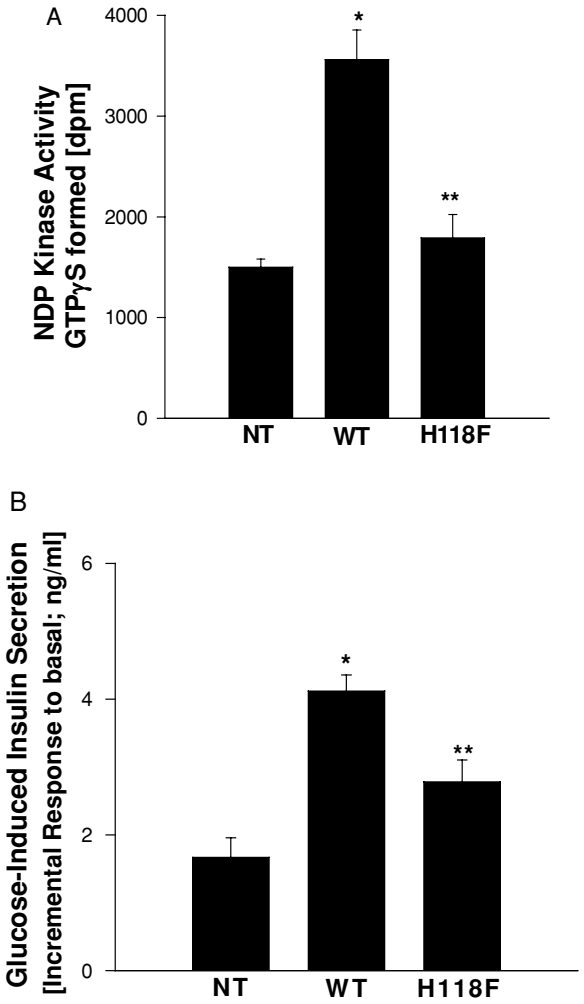

Fig. 1 *Effects of over-expression of WT or NDPK/histidine kinase-deficient mutant of nm23-H1 on NDPK activity (Panel A) and GSIS (Panel B) from insulin-secreting INS-1 cells*: *Panel A*: INS cells were transfected with either WT or NDPK/histidine kinase-deficient mutant (H118F) of nm23-H1 using Effectene reagent. NDPK activity was quantitated (Kowluru and Metz, 1994) in lysates from non-transfected (NT)], WT or H118F mutant transfected cells. Data are expressed as the amount of [^{3}H]GTPγS formed from [^{3}H]GDP and ATP. Data are mean ±SEM from two independent experiments carried out in triplicates. *represents $p = 0.001$ vs NT and ** represents no significant difference from NT. *Panel B*: INS cells were transfected with either WT or NDPK/histidine kinase-deficient nm23-H1 mutant as described above. GSIS from non-transfected (NT) or nm23 mutant transfected cells was quantitated under static incubation conditions in the presence of 25 mM glucose for 45 min. The amount of insulin released was quantitated by ELISA as in (Amin et al., 2003). Data, which are mean ± SEM from four individual measurements, are expressed as incremental response to basal (5 mM) glucose. * represents $p = 0.01$ vs NT; and ** represents $p = 0.05$ vs WT or NT

concentration not only initiate insulin secretion but also regulate various enzymes (e.g., protein kinases), thereby facilitating insulin secretion. In addition to calcium-dependent protein kinase(s), several other kinases, including calmodulin-, cyclic nucleotide-, phospholipid-dependent protein kinases, tyrosine kinases, and mitogen-activated protein kinases have been implicated in GSIS in β cells. The majority of these kinases mediate phosphorylation of endogenous β cell proteins using ATP as the phosphoryl donor. In addition to these, we have also described localization of GTP-specific histidine kinases which we have implicated in the activation of trimeric G-proteins (Kowluru, 2002). It is in this context, we believe that nm23/NDPK-like enzymes could contribute toward GSIS in the pancreatic β cell (see below).

Compatible with our overall hypothesis are at least four published reports which provided support for non-receptor-dependent activation of G-proteins involving protein histidine phosphorylation and high-energy phosphate transfer. First, Cuello et al. demonstrated (Cuello et al., 2003) activation of trimeric G-proteins by a high-energy phosphate transfer from the histidine-phosphorylated NDPK to the β subunit of trimeric G-proteins. Using bovine retinal and brain preparations, these investigators observed that the B isoform of NDPK forms complexes with the $\beta\gamma$ subunits of trimeric G-proteins and contributes to the activation of the respective G-protein by increasing the high-energy phosphate transfer from a transiently phosphorylated His^{266} in the β subunit to the GDP bound to the α subunit, to yield an active conformation. In the second study, Hippe et al. demonstrated the existence of a complex between NDPK (B isoform) and the $\beta\gamma$ complex of trimeric G-proteins, and they implicated a role for NDPK in the phosphorylation of the β subunit, which is then transferred to the GDP bound to the α-subunit, resulting in its active, GTP-bound conformation (Hippe et al., 2003). Third, we reported similar regulatory mechanisms involving histidine phosphorylation of β subunit, and subsequent transfer of that high-energy phosphate for the activation of trimeric G-proteins in the insulin-containing secretory granule fractions isolated from normal islets as well as clonal β cell preparations (Kowluru et al., 1996). Lastly, evidence is also presented (Marciniak and Edwardson, 1996) to suggest physical association of NDPK with pancreatic zymogen granules, and implicated this enzyme in the generation of GTP to facilitate optimal fusion of granules with the plasma membrane for exocytosis of the granular contents. Together, it is plausible that nm23/NDPK-like enzymes that we characterized in the islet β cell could subserve the function of histidine phosphorylation of key proteins, leading to the generation of appropriate signals necessary for physiological insulin secretion, including fusion of insulin-laden secretory granules with the plasma membrane.

Recently, we also reported significant abnormalities in ATP- or GTP-mediated histidine phosphorylation of nm23/NDPK in islets derived from the Goto-Kakizaki (GK) rat, a model for type 2 diabetes (Metz et al., 1999; Kowluru, 2003a). Furthermore, we provided evidence for a marked reduction in the activities of ATP- or GTP-sensitive (histone IV-phosphorylating) histidine kinases in the GK rat islets

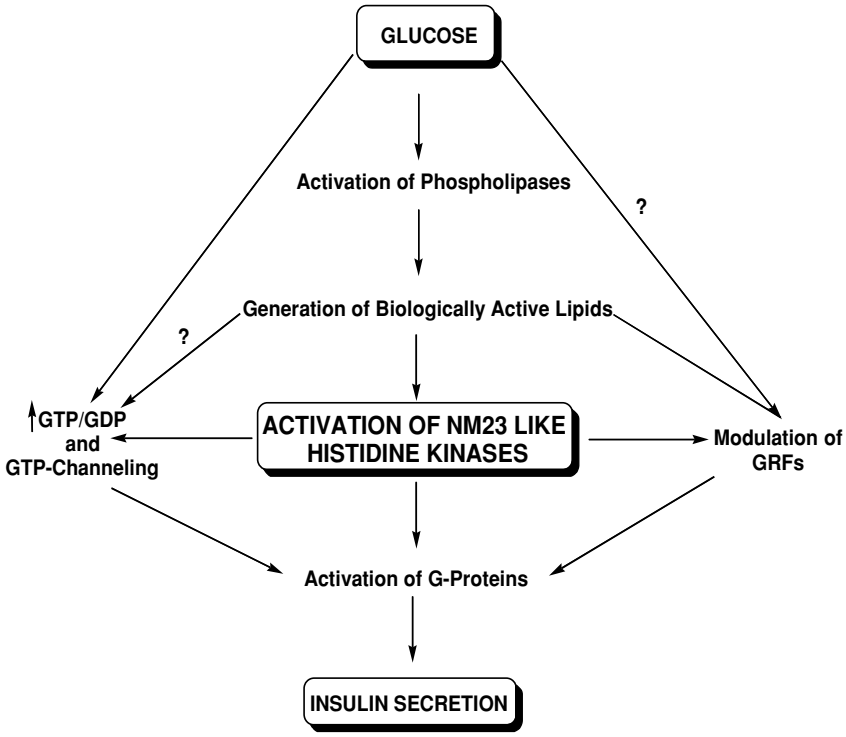

Fig. 2 *Our proposed model to suggest novel roles for nm23/NDPK-like enzymes in GSIS from the islet β cell*: Based on our current knowledge, we propose that signaling mechanisms leading to GSIS involves intermediacy of nm23/NDPK-like enzymes. We propose that these enzymes regulate several steps in islet metabolism, including activation of both trimeric as well as monomeric G-proteins, which are necessary for GSIS (see text). Significant amount of work is still needed in terms of potential identity of the isoforms of nm23/NDPK which are under the fine control of glucose metabolism. At least from our preliminary data (Fig. 1), it appears that nm23-H1 could subserve some of such regulatory functions. GRFs = guanine nucleotide regulatory factors

(Kowluru, 2003a). Potential significance of these findings is unclear at this time. We speculate that such a defect in NDPK activation in the diabetic islet could contribute toward defective G-protein activation that we reported in the diabetic GK rat islet (16). Such a proposal is also based on our original observations to demonstrate a complete restoration of insulin secretory abnormality in the GK islet by mastoparan (Mas), which is a global activator of G-proteins (Kowluru, 2003b) and an activator of NDPK (Klinker and Seifert, 1995; Kowluru et al., 1995). We demonstrated that Mas, but not its inactive analog, Mas-17, markedly augmented insulin secretion from the GK rat islet. These findings suggest that the abnormalities in GSIS in the GK islet could result from defects in the activation of a Mas-and nm23/NDPK-regulatable G-protein(s). Together, on the basis of these observations, we postulate that alterations in protein histidine phosphorylation could contribute toward insulin-secretory abnormalities demonstrable in the diabetic islet. Additional studies are underway to further identify potential regulatory factors that could contribute toward insulin secretory abnormalities in this animal model.

Conclusions and future directions

Published evidence from our laboratory indicates localization of at least three forms of nm23/NDPK (e.g., nm23-H1, nm23-H2 and nm23-H4) in the islet β cell (Kowluru et al., 2002). We also described localization of other novel histidine kinases in the islet, and have demonstrated regulation of endogenous G-protein activation by these kinases (Kowluru, 2003b). It appears that insulinotropic fatty acids modulate the functional activation of these enzymes in a structure-specific manner (Kowluru, 2004). On the basis of our current understanding of the biochemical properties and physiological regulation of nm23/NDPK in the islet β cell, we propose a model for potential contributory roles of NDPK in GSIS, specifically at the level of activation of G-proteins (Fig. 2). We propose that glucose-induced increase in the GTP/GDP ratio may in part be due to the activation of NDPK, which generates GTP via transphosphorylation of GDP from ATP. This increase in GTP concentrations favors either an increase in the GTP/GDP exchange on a relevant G-protein(s) or chaneling of GTP to candidate G-protein(s), culminating in their activation. It is also likely that glucose-derived second messengers (e.g., biologically-active lipids) could directly activate NDPK-like enzymes, which, in turn, may regulate G-protein functional activation. Lastly, it is possible that glucose-generated biologically-active lipids promote a unique cross-talk between various guanine nucleotide regulatory factors (GRFs; e.g. Tiam 1) and NDPK-like enzymes (Kimura et al., 2003; Otsuki et al., 2001; Palacios et al., 2002) in the modulation of specific G-proteins (e.g., Rac1 and Cdc42) that are essential for GSIS. In conclusion, we emphasize that our current knowledge on nm23/NDPK-like histidine kinase-mediated regulation of hormone secretion is still very rudimentary. We hope that this article will form the basis for additional investigations in this area, specifically in furthering our current understanding of the nature

of nm23/NDPK defects in the diabetic islet for the development of novel therapeutics for the prevention and onset of diabetes. These might include, but not limited to, the development of potent, but specific stimulators of nm23/NDPK (e.g., Mas-like compounds), with a potential for reversal of insulin secretory defects in diabetics. These studies are currently underway in our laboratory.

Acknowledgements This research was supported by grants (to AK) from the Medical Research Service of the Department of Veterans Affairs, the National Institute of Health (DK-56005) and the American Diabetes Association. This research is also supported by financial support from the NIH (CA 83237) to DMK. AK is the recipient of Research Career Scientist Award from the Department of Veterans Affairs, and Charles H. Gershenson Distinguished Faculty Fellowship from Wayne State University. AK also thanks the former and current members of the Kowluru laboratory who have contributed significantly to the islet β cell nm23/NDPK-related projects.

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