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INSULIN DECREASES THE GLYCOGEN SYNTHASE KINASE-3α mRNA LEVELS BY ALTERING ITS STABILITY IN STREPTOZOTOCIN-INDUCED DIABETIC RAT LIVER

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The chronic effect of insulin on the expression of the glycogen synthase kinase-3α gene in streptozotocin-induced diabetic rat liver is examined. The mRNA levels of glycogen synthase kinase-3α were increased (143 % of normal levels) in diabetic livers and these were normalized by insulin supplementation to the diabetic animals. Neither diabetes nor insulin supplementation to diabetic rats altered the transcription rate of glycogen synthase kinase-3α. However, diabetes caused an increase in the half-life of glycogen synthase kinase-3α mRNA from 5 h in normal hepatocytes to 8 h in diabetic ones. Insulin supplementation to the incubation medium of diabetic hepatocytes decreased the half-life of glycogen synthase kinase-3α mRNA to a level comparable with normal values. This study suggests that the chronic effect of insulin decreases the levels of glycogen synthase kinase-3α mRNA by altering its stability.

Glycogen synthase kinase-3 is one of the several protein kinases that phosphorylates and inactivates glycogen synthase, a key enzyme for glycogen biosynthesis (1). The protein kinase is identical to Factor A, an activator of the ATP-Mg-dependent protein phosphatase-1 (2,3). Glycogen synthase kinase-3 is involved in phosphorylation of many substrates such as type II regulatory subunit of protein kinase A (4), inhibitor-2 of protein phosphatase-1 (5), ATP-citrate lyase (6), the eukaryotic initiation factor 2B (7), microtubule associated tau protein (8), transcription factors (c-jun, c-myb, c-myc and CREB) (9-11) and also in the regulation of several developmental processes in *Drosophila* (11). Woodgett has identified two GSK-3 isoforms, termed α and β, from molecular cloning of rat brain library (12).

Insulin stimulates glycogen synthesis in muscle by causing the dephosphorylation and activation of glycogen synthase (13). The intravenous administration of insulin removes phosphate from serine residues, commonly referred as phosphorylation site 3, of glycogen synthase (13). The Site 3 is phosphorylated and dephosphorylated by glycogen synthase kinase-3 and protein phosphatase-1, respectively (1,13-15). These studies have suggested that the activation of glycogen synthase by insulin could be mediated by the inhibition of glycogen synthase kinase-3 and/or activation of protein phosphatase-1. Similar results on phosphorylation

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and inactivation of glycogen synthase by glycogen synthase kinase-3 have also been observed in liver tissue (16,17). Recently, evidence has accumulated that insulin decreases the activity of glycogen synthase kinase-3 in rat adipocytes (18), CHO.T cells (7), and L6 cells (19). The inhibition of glycogen synthase kinase-3 has also been shown by IGF-1, EGF, serum and phorbol esters (19-22). These studies have further revealed that insulin and growth factors inhibit glycogen synthase kinase-3 through the regulation of the ribosomal protein S6 kinase/MAP kinase pathway (19-22).

Insulin is involved in both the acute and the chronic regulation of metabolic processes (23,24). Recent studies have established the acute effect of insulin on the inhibition of glycogen synthase kinase-3 activity involving ribosomal protein S6 kinase/MAP kinase pathway. The chronic effect of insulin on glycogen synthase kinase-3 has not been examined so far and is the subject of this investigation. We have studied the chronic effect of insulin on the hepatic expression of glycogen synthase kinase-3α isoform in streptozotocin-induced diabetic rats (insulin-deficient) and compared them with normal or insulin-treated diabetic animals. Our results suggest that the chronic effect of insulin causes a reduction in the glycogen synthase kinase-3α mRNA levels by decreasing its stability.

EXPERIMENTAL PROCEDURES

Materials: TRIzol, fetal calf serum, DMEM and William's E medium were purchased from Gibco BRL. Random primer labeling kit, salmon sperm DNA and Nuctrap push columns for radiolabeled probe purification were supplied by Stratagene. Oligo-(dT) cellulose spin columns were from 5 prime --> 3 prime, Inc. and [α-^{32}P]dCTP and [α-^{32}P]UTP were obtained from DuPont. Insulin was obtained from Eli Lilly Co. RNA synthesis inhibitor 5,6-dichloro-1-β-D ribofuranosyl benzimidazole, creatine phosphokinase, creatinine phosphate, CTP, UTP, tRNA, streptozotocin, Swim's S-77 medium and all other chemicals used in this investigation were purchased from Sigma Chemical Co. Sustained release insulin implants (Linplant) were purchased from Linshin Canada Inc. cDNA probes for glycogen synthase kinase-3α and phosphoenolpyruvate carboxykinase were generously provided by Dr. J.R. Woodgett (Ontario Cancer Centre, Toronto, Canada) (12) and Dr. W. J. Roesler of this Department, respectively.

Animals: Male Sprague-Dawley rats (200-250 g body weight) were divided into three groups of 7 animals each: normal control, streptozotocin-induced diabetic and insulin-treated streptozotocin-induced diabetic. The latter two groups of rats were made diabetic by a single ip injection of streptozotocin (60 mg/kg BW) dissolved in 100 mM citrate (pH 4.5) containing 150 mM NaCl (25-27). Control rats were injected with citrate buffer alone. Diabetic rats were divided into two groups one week after streptozotocin injection and insulin implants were subcutaneously inserted in one group of diabetic rats. Normal control and diabetic animals were also similarly sham treated. After three weeks, all rats were killed between 0900 and 1000 h in fed condition. Blood samples collected by orbital sinus bleeding were centrifuged at 3000 g for 10 min and the plasma samples were stored at -80 °C for glucose and insulin estimation. Liver samples were processed immediately for total RNA and nuclei isolation.

Isolation of total RNA and northern blot analysis: Total RNA was isolated from liver using TRIzol reagent (28). Poly (A)$^+$ RNA was separated from total RNA by using oligo (dT) cellulose spin columns. Poly (A)$^+$ RNA (2 μg) was fractionated in 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the gel was soaked in 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate) for 1 h and was blotted onto Hybond N+ membrane. The filter was then baked for 2 h at 80 °C in a vacuum oven. Prehybridization was performed for 6 h at 42 °C in 50% formamide, 5 X Denhardt's solution (0.1% each of Ficoll, polyvinylpyrrolidone, BSA), 5 X SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 0.1% SDS and 200 μg/ml sonicated salmon sperm DNA. Glycogen synthase kinase-3α cDNA was labeled with [α-^{32}P]dCTP using random

labeling kit. Rat brain GSK-3α cDNA insert of 2.2 kb size in plasmid Bluescript SK was digested with Hind 3 and 1.6kb fragment thus produced was used for probing. Chicken brain β-actin cDNA, 2 kb Pst fragment, was used as a control for probing. Hybridization was carried out at 42 °C for 16 h. The membranes were subsequently washed thrice for 10 min each in 200 ml 2 X SSC, 0.1% SDS at 28 °C, followed by two times for 30 min in 200 ml of 0.1 X SSC and 0.1% SDS at 50 °C. The hybridized filters were exposed to Kodak X-OMAT x-ray film with an intensifying screen at -80 °C. Quantification of mRNA was accomplished by densitometric scanning of autoradiograms with the use of an LKB 2202 Ultroscan laser densitometer.

Nuclear transcription assay: Nuclei were isolated from the liver tissue essentially as described by Chauhan and Dakshinamurti (29) with the following minor modifications. One ml of homogenate was mixed with 2 ml of 2.3 M sucrose in 50 mM Tris/HCl, pH 7.5 containing 25 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothritol, 1 mM EGTA, 0.14 mM spermidine and 0.1 mM PMSF by inversion in a Backman Ti 50 centrifuge tube. The mixture was then underlaid by 1.0 ml of 2.3 M sucrose in the above buffer with a syringe. After centrifugation for 30 min at 39000 rpm in a Beckman Ti 50 rotor at 4 °C, the nuclear pellet was taken up in the storage buffer containing 50 mM Tris/HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 50% glycerol and 0.1 mM PMSF and stored at -80 °C. The nuclear run-on assay was carried out according to Chauhan and Dakshinamurti (29) except that the incubation temperature was 30 °C. Labeled transcripts were isolated with the use of TRIzol reagent as described by Chomczynski and Sacchi (28) . After adding 50 μg of tRNA as carrier RNA, the samples were homogenized in 4 volumes of TRIzol reagent by passing through a 22 gauge needle several times. RNA was precipitated with isopropyl alcohol and the pelleted RNA was solubilized in hybridization solution.

Plasmid DNA (4 μg) was applied onto Hybond N+ membrane using a slot blot manifold and baked for 2 h at 80 °C. The membranes were prehybridized for 6 h at 50 °C in 50% formamide, 5 X SSPE, 5 X Denhardt's solution, 0.2% SDS and 250 μg/ml tRNA. Hybridization was carried out with the corresponding labeled transcripts in the same prehybridization solution (1.0 ml final volume) at 45 °C for 72 h. The membranes were washed twice for 30 min in 2 X SSC, 0.1% SDS at 30 °C and thrice in 0.1 X SSC and 0.1% SDS at 55 °C and exposed to Kodak X-OMAT x-ray film with intensifying screen at - 80 °C. Densitometric scanning of autoradiograms was carried out with the use of an LKB 2202 Ultroscan laser densitometer. The densitometric values of the experimental groups were expressed as a percentage of the normal group. The amount of radioactivity in bands were further confirmed by cutting out the appropriate membrane bands and counting in a scintillation counter. The results by densitometry and scintillation counting showed identical patterns (results not shown). Non-specific hybridization of the respective probes to pBluescript SK or pBR 322 DNA was negligible.

Cell culture and stability of mRNA: The procedures for isolation of hepatocytes from normal and diabetic rats and maintenance of monolayer culture were as described (30). Briefly, cell suspensions obtained by perfusing livers with collagenase were washed three times with William's E medium and the final cell pellets were resuspended in DMEM containing 10% new born calf serum. Cells (5×10^6) were seeded onto collagen coated dishes (diameter, 100 mm). After 4 h, the medium was changed to serum-free DMEM containing 5.5 mM glucose, 0.2% BSA and 100 nM dexamethasone. After 16 h, the medium was replaced with fresh serum-free medium containing glucose, BSA and dexamethasone with the RNA synthesis inhibitor 5,6-dichloro-1-β-D ribofuranosyl benzimidazole(50 μg/ml) in the presence and absence of insulin (100 nM). Isolation of total RNA, poly $(A)^+$ and northern blot analysis was carried out as mentioned earlier.

Plasma analysis: Plasma glucose was determined according to the method described by Raabo and Terkildsen (31). Plasma insulin was measured by the double-antibody method (32). Statistical analysis was carried out by the Student's *t*--test.

RESULTS

The diabetic rats used in this study had approximately a five-fold increase in plasma glucose (diabetic, 31.4 ± 1.9 mM and normal, 6.8 ± 0.4 mM) and 80% deccrease in plasma insulin (diabetic, 75 ± 8 pM and normal, 424 ± 35 pM) levels. The administration of insulin to the diabetic animals normalized both of these values (plasma glucose, 6.0 ± 0.5 mM and plasma insulin, 530 ± 45 pM) and were comparable to normal rats.

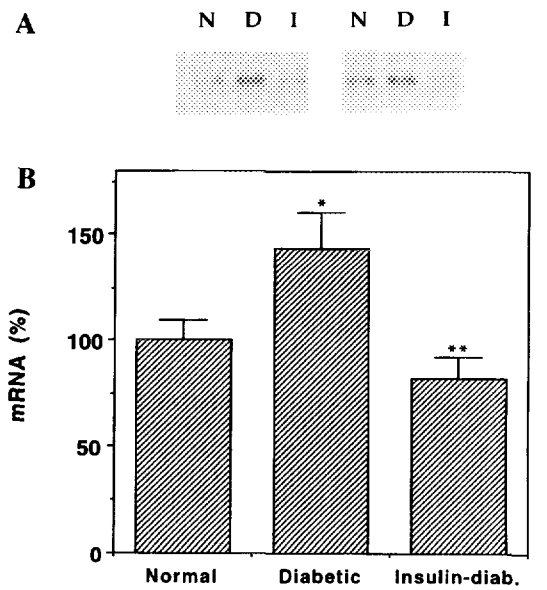

Fig. 1. (A) Northern blot analysis and (B) quantitation of the relative levels of glycogen synthase kinase-3α mRNA in livers of normal (N), streptozotocin-induced diabetic (D) and insulin-treated diabetic (I or insulin-diab.) rats. Quantitation was carried out with respect to equal amounts of β-actin in each group. Comparisons were made by Student's *t* -test. * P<0.05, vs normal rats. **P<0.01, vs diabetic rats.

The hepatic expression of glycogen synthase kinase-3α in the various groups of rats is shown in Fig. 1. Glycogen synthase kinase-3α mRNA levels were significantly increased (43%, P<0.01) in diabetic rats as compared to the normal animals. Insulin treatment of the diabetic rats decreased glycogen synthase kinase-3α mRNA levels to a value slightly lower than normal animals. These results suggest the possible involvement of insulin at either the transcription or the mRNA stability level. The rates of transcription for glycogen synthase kinase-3α, phosphoenol-pyruvate carboxykinase and β-actin genes are presented in Fig. 2. The transcription of the rat liver phosphoenolpyruvate carboxykinase gene was used as an internal standard because of its well established negative regulation by insulin (33). The transcription of the β-actin gene was included as an internal control because it is not influenced by the diabetic state or by insulin administration. The transcription of the phosphoenolpyruvate carboxykinase gene increased in diabetic animals and was normalized after the insulin treatment. The transcription rate of the glycogen synthase kinase-3α gene was, in general, slightly higher (~10 %) in the diabetic rats as compared to the normal or the insulin-treated diabetic animals. However, the values were not statistically different among the various groups of animals. Non-specific hybridization of the respective probes to pBluescript SK or pBR 322 DNA was negligible (results not shown).

These results suggested that insulin might regulate the mRNA levels of glycogen synthase kinase-3α at the post-transcriptional level. In order to test this possibility, we examined the stability of glycogen synthase kinase-3α mRNA in isolated hepatocytes from normal and diabetic rats in the presence of 5,6-dichloro-1-β-D ribofuranosyl benzimidazole, an inhibitor of RNA synthesis. As shown in Fig. 3, the rate of degradation of glycogen synthase kinase-3α mRNA

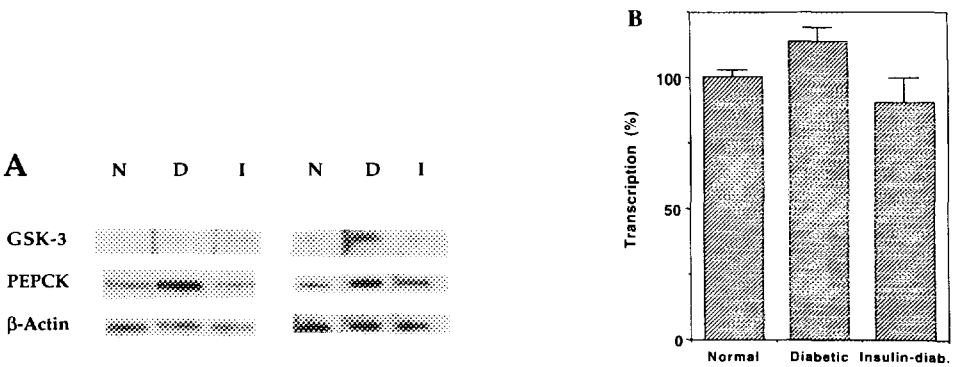

Fig. 2. (A) Nuclear run-on analysis and (B) quantitation of the relative levels of glycogen synthase kinase-3α transcription from livers of normal (N), streptozotocin-induced diabetic (D) and insulin-treated diabetic (I or Insulin -diab.) rats. Quantitation was carried out with respect to equal amounts of β-actin in each group. No significant difference was observed between any group as determined by Student's *t* -test.

was slower in diabetic hepatocytes as compared to the normal hepatocytes; the half lives of glycogen synthase kinase-3α mRNA in normal and diabetic hepatocytes were 5 h and 8 h, respectively. The presence of insulin in the medium of diabetic hepatocytes decreased the glycogen synthase kinase-3α mRNA stability by decreasing its half-life from 8 h to 5 h, a value similar to that observed in normal hepatocytes.

DISCUSSION

Studies in various laboratories have demonstrated that insulin is a major player in the acute and the chronic regulation of enzymes involved in glucose homeostasis (23,24). The acute regulation occurs through hormone-mediated changes in enzyme activities, generally by the phosphorylation/dephosphorylation of key enzymes (23). The chronic effects of insulin are

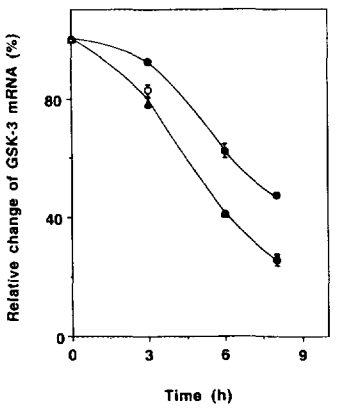

Fig. 3. Glycogen synthase kinase-3α mRNA degradation in hepatocytes prepared from normal and streptozotocin-induced diabetic rats. Zero time point in each group was taken as 100 %. Each point is the mean ± S.E. for three hepatocyte preparations in each group. The glycogen synthase kinase-3α mRNA levels in diabetic hepatocytes (incubated without insulin, ●) were significantly lower ($p<0.01$) at all time points between 3 and 8 h as compared to the mRNA levels in normal (○) and diabetic (incubated in the presence of insulin, ▲) hepatocytes .

through changes in the rates of enzyme synthesis (24). For example, insulin exerts both the acute and the chronic effects in the activation of key glycoglytic enzymes (bio-degradative pathway) and in the inhibition of key enzymes of gluconeogenesis (biosynthetic pathway) (23). Recently, it has been shown that insulin acutely inhibits the activity of glycogen synthase kinase-3, the enzyme responsible for the conversion of an active-form of glycogen synthase into an inactive-form of glycogen synthase (7,18-22). Furthermore, with the use of wortmannin (an inhibitor of phosphatidylinositol 3-kinase) and rapamycin (inhibitor for the activation of p70 S6 kinase), it has been established that the acute effect of insulin on glycogen synthase kinase-3 is mediated through the MAP kinase pathway involving the activation of p90 S6 kinase and not the other p70 S6 kinase isoform (19,21). In the present study, we have shown that diabetes causes an increase in levels of the glycogen synthase kinase-3α mRNA and these levels are normalized by insulin supplementation to diabetic animals (Fig. 1) This effect is due to an alteration in the stability of the glycogen synthase kinase-3α mRNA (Fig. 3). Both mechanisms of regulation i.e. the acute and the chronic will, therefore, result in the decreased physiological activity of glycogen synthase kinase-3. Because glycogen synthase kinase-3 is involved in the inhibition of glycogen synthase, the decrease in the activity of glycogen synthase kinase-3 by insulin complements the previous findings on the regulation of key enzymes of metabolic pathways of glucose homeostasis.

The diabetic condition which leads to decreased circulating insulin levels also results in significant increase in glucagon levels (26,27). Insulin replacement in diabetic rats which produces normalization of the glycogen synthase kinase-3α mRNA levels, also causes a decrease in glucagon levels. It is, therefore, reasonable to think that glucagon might also contribute to glycogen synthase kinase-3α gene expression. However, insulin caused a decrease in glycogen synthase kinase-3α mRNA stability in hepatocyte culture in which no manipulation of glucagon levels was carried out. This suggests that insulin is primarily responsible for this metabolic response. The future studies will determine whether glucagon (and/or glucocorticoids) also plays any role in expression of the glycogen synthase kinase-3α gene.

Glucagon (or cAMP) and insulin play important roles in the regulation of hepatic gene expression both at the transcription and/or post transcription level (23,24,34,35). Both of these compounds regulate the synthesis of many mRNAs of glucose metabolizing enzymes. The rate of mRNA degradation also plays an important role in controlling the level of gene expression (36) and it is very well known that the stabilities of many of these mRNAs are altered in response to these hormones. For example, the synthesis as well as the stability of the phosphoenolpyruvate carboxykinase mRNA is positively regulated by cAMP and that these effects are reversed by insulin (24). Although significant progress has been made in understanding the regulation of transcription, only fragmentory information is available on the regulation of mRNA stability. Specific sequences within the translated, 5' non-translated and 3' non-translated regions seem to function as cis-acting elements in regulating mRNA stability. The trans-acting cytoplasmic proteins bind to these cis-acting sequences and change the secondary structures of the mRNA molecules, thereby increasing its accessibility for nuclease attack. Although it is known that type I diabetes produces a lower plasma insulin (and higher glucagon) level and alters the expression of hepatic genes under the regulation of these hormones, the exact mechanism by which insulin and/or glucagon regulate the translatability and stability of mRNA is yet to be examined.

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