Involvement of Protein Kinase C β2 in c-myc Induction by High Glucose in Pancreatic β-Cells*

Received for publication, October 5, 2001, and in revised form, November 13, 2001
Published, JBC Papers in Press, November 19, 2001, DOI 10.1074/jbc.M109647200

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The expression of the basic helix-loop-helix transcription factor c-Myc is induced in pancreatic islets of several different diabetic model animals and is possibly involved in suppression of the insulin gene transcription. In this study, we found that activity of protein kinase C is increased by high glucose, preceding the induction of c-myc expression and that PKC β2 specifically regulates c-myc expression in pancreatic β-cells. Since PKC α, β2, δ, ε, and ζ were expressed in rat pancreatic islets, we prepared each wild type (WT) and dominant negative type (DN) PKC isoform (α, β2, δ, ε, and ζ)-expressing adenovirus to examine the effect of each PKC isoform on c-myc expression. In isolated rat pancreatic islets, adenovirus-mediated overexpression of WT PKC β2, but not other PKC isoforms, markedly increased c-myc expression. Moreover, c-myc induction by high glucose was suppressed by adenovirus-mediated overexpression of DN PKC β2 but not by other DN PKC isoforms. Finally, adenovirus-mediated overexpression of WT PKC β2, but not of other PKC isoforms, leads to suppression of the insulin gene transcription in pancreatic islets. These results suggest that at least some of the reduction of insulin gene transcription found in the diabetic state is mediated by PKC β2 regulation of c-myc expression.

It is well known that hyperglycemia contributes to the deterioration in pancreatic β-cell function found in diabetes (1), this being characterized by phenotypic changes that include a decline of the insulin gene transcription and degranulation of β-cells (2–5). These adverse effects of chronic hyperglycemia have been called glucose toxicity. To explain the molecular basis of these phenomena, we have postulated that the basic helix-loop-helix transcription factor c-Myc (6–8) inhibits insulin gene transcription. In the presence of hyperglycemia, expression of many β-cell-associated genes is decreased, but c-myc expression is induced. This increase of c-myc mRNA has been found in the partial pancreatectomy model of diabetes and in rats made hyperglycemic with glucose clamps, with the change in c-myc expression correlating with graded levels of hyperglycemia (9, 10). It has been reported that marked c-Myc activation in β-cells of transgenic mice leads to extensive β-cell apoptosis and diabetes (8). Furthermore, we found recently that adenovirus-mediated c-Myc overexpression suppresses the insulin gene transcription by inhibiting NeuroD/BETA2-mediated transcriptional activation (11). Thus, we feel that hyperglycemia-mediated c-myc induction is likely to be involved in β-cell dysfunction found in diabetes. However, the mechanisms responsible for the regulation of c-myc expression in β-cells are unknown.

Protein kinase C (PKC)1 is an important mediator of signal transduction in response to several cellular signals (12, 13), and in the diabetic state it has been found to be activated in a variety of tissues including aorta, heart, retina, and glomerular cells (13–17). Moreover, some PKC isoforms are activated by hyperglycemia and associated with vascular abnormalities in cardiovascular, retinal, and renal tissues. There are multiple PKC isoforms including the classical PKCs (α, β1, β2, and γ), novel PKCs (δ, ε, and θ), and atypical PKCs (ζ and λ). Among these isoforms, PKC β is thought to be mainly involved in development of diabetic complications. Transgenic mice overexpressing PKC β in myocardium developed cardiac hypertrophy and failure (18), supporting the hypothesis that PKC β activation can contribute to myocardial dysfunction. Furthermore, various other diabetic complications found in diabetes such as microvascular or renal dysfunction in diabetic rats were ameliorated by treatment with a PKC β inhibitor (19, 20). Hyperglycemia-induced oxidative stress may explain some of the adverse effects of PKC β, since antioxidant treatment can inhibit PKC activation and reverse glucose-induced vascular dysfunction (15). Various PKC isoforms have been identified in pancreatic islets, and PKC has been postulated to play a role in glucose-stimulated insulin secretion (21–23).

In this study, we show that PKC β2 is involved in c-myc induction by high glucose in pancreatic β-cells.

MATERIALS AND METHODS

Isolation and Culture of Rat Pancreatic Islets—Islets were isolated from pancreases of 200–250-g male Sprague-Dawley rats (Taconic Farms, Germantown, NY) with collagenase digestion. The common bile duct was cannulated and injected with 6 ml of cold M199 medium containing 1.5 mg/ml collagenase (Roche Molecular Biochemicals). The

1 The abbreviations used are: PKC, protein kinase C; WT, wild type; DN, dominant negative type; JNK, c-Jun N-terminal kinase; GFP, green fluorescent protein; pfu, plaque-forming unit; TPA, 12-O-tetradecanoylphorbol-13-acetate; MAPK, mitogen-activated protein kinase.

This paper is available online at http://www.jbc.org

¶ This work was supported in part by National Institutes of Health Grants DK-35449 and EY-5110 and by an important group of private donors. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a fellowship and grant from the Japan Society for the Promotion of Science.

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islets were separated on a Histopaque 1077 (Sigma) density gradient. The washed islets were picked individually under a dissecting microscope and cultured in RPMI medium (supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO₂ at 37°C. All animal procedures were approved by the Animal Care Committee of the Joslin Diabetes Center.

Preparation of Recombinant Adenoviruses Expressing Wild Type (WT) and Dominant Negative Type (DN) PKC Isoforms (PKCα, β, γ, δ, ε, η, ζ, and θ)—Recombinant adenoviruses expressing WT and DN PKC isoforms were prepared using the AdEasy system (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center) (23). The coding region of each WT and DN PKC isoform (α, β, γ, δ, ε, ζ, η, and θ) were inserted into the adenoviral backbone plasmid, pAdEasy-1, which was introduced into Escherichia coli BL21 (DE3) cells with electroporation (2,500 V, 200 μF). Then the resultant plasmids were retransformed into E. coli XL-Gold Ultracompetent cells (Stratagene, La Jolla, CA). The plasmids were linearized with PacI and then transfected into the adenovirus packaging cell line 293 using LipofectAMINE (Invitrogen). Ten days after transfection, cell lysates were obtained from the 293 cells. The cell lysates were added to 293 cells again, and when more of the cells were killed by the adenovirus infection and detached, cell lysates were again obtained. (This process was repeated three times.) Control adenovirus transfection, cell lysates were obtained from the 293 cells. The cell lysates were retransformed into E. coli using LipofectAMINE (Invitrogen). Forty-eight hours after the transfections, cell lysates from islets were incubated with [γ-32P]ATP and PKC biotinylated protein peptide substrate buffer at 30°C for 5 min and then spotted on SAM biotin capture membrane (Promega). After washing with 2×NaCl and 2×NaCl in 1% H₃PO₄, PKC activity was examined with a scintillation counter.

Gene Transfection and Luciferase Assays—βTC1 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin sulfate in a humidified atmosphere of 5% CO₂ at 37°C. A rat c-myc promoter-reporter (luciferase) plasmid (1.0 mg) containing 377-bp 5'-flanking sequences of the c-myc promoter region (25) or a rat insulin II promoter-reporter plasmid (1.0 mg) containing 1033-bp 5'-flanking sequences of the rat insulin II promoter region (26) was co-transfected with 1.0 μg of WT (or DN) PKC isoform expression plasmid (or the empty vectors) and 0.5 μg of pSV-β-galactosidase control vector (Promega) using the LipofectAMINE reagent (Life Technologies). Forty-eight hours after the transfections, cells were harvested for luciferase and β-galactosidase assays. Preparations of cellular extracts were assayed using a luciferase assay system (Promega). β-galactosidase assays were performed with a β-galactosidase enzyme assay system (Promega). The luciferase results were normalized with respect to transfection efficiency assessed from the results of the β-galactosidase assays.

Statistical Analysis—All results are presented as mean ± S.E. of three or four independent experiments. Statistical analysis was performed using the unpaired Student’s t-test.

RESULTS

Induction of c-myc Gene Expression in Rat Pancreatic Islets Cultured in a High Glucose Concentration—When primary rat islets were cultured in a high glucose concentration, c-myc mRNA expression was induced in a dose- and time-dependent manner (Fig. 1, A and B). These results are consistent with previous results showing that c-myc expression is induced after partial pancreatectomy model of diabetes and in rats maintained with a hyperglycemic clamp (9, 10). Since the diabetic state is associated with PKC activation (13–20) and the production of reactive oxygen species in several cell types (27–31), the involvement of PKC and oxidative stress in c-myc induction was studied. As shown in Fig. 1C, induction of c-myc gene expression by high glucose was totally suppressed by a PKC-specific inhibitor, GF109203X (5 μM), whereas the antioxidant N-acetyl-l-cysteine (10 mM) showed only a trend toward a suppressive effect on the c-myc induction. Furthermore, as shown in Fig. 1D, c-myc expression was directly induced by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). Also, c-myc induction by TPA or high glucose was expressed by TPA pretreatment, a condition that is known to down-regulate PKC. These results implicate PKC in the regulation of c-myc induction by high glucose in pancreatic islets.

Expression of PKC Isoforms and Activation of PKC Enzyme Activity by High Glucose in Rat Pancreatic Islets—To determine which PKC isoform might be involved in c-myc induction by high glucose, the presence of several PKC isoforms was evaluated in islets using Western blot analysis. As shown in Fig. 2A, PKC α, β, δ, ε, ζ, and θ were expressed in islets, but PKC γ, η, ζ, and θ could not be detected. Concerning the PKC isoforms that were not detected in islets, the antibodies of the chemicals were as shown in Fig. 2A, PKC β1, γ, θ, and...
PKC β2 Regulates c-myc Expression in Pancreatic β-Cells

Glucose for 3 days (Fig. 2), expression by high glucose in isolated rat islets. Isolated islets were incubated in high glucose in isolated rat pancreatic islets. As shown in Fig. 2, PKC activation by high glucose in rat islets. Isolated islets were cultured in 5–30 mM glucose for 3 days (B) or 20 mM glucose for 0–3 days (C), and then PKC activity was examined. -Fold increase of total PKC activity is expressed as mean ± S.E. in bar graphs (n = 4).

β-cell-derived cell line βTC1 after transient overexpression of PKC isoforms (α, β2, δ, ε, and ζ). As shown in Fig. 3A, c-myc promoter activity was increased by overexpression of WT PKC β2 but not by other PKC isoforms (α, δ, ε, and ζ), JNK, or p38 mitogen-activated protein kinase (MAPK). In contrast, as shown in Fig. 3B, c-myc promoter activity was decreased by overexpression of DN PKC β2 but not by other DN PKC isoforms (α, δ, ε, and ζ), DN JNK, or p38 MAPK inhibitor SB203580. These results suggest that PKC β2, but not PKC α, δ, ε, or ζ, is likely to be involved in c-myc induction by high glucose.

Effects of Adenovirus-mediated Overexpression of WT and DN PKC Isoforms (α, β2, δ, ε, and ζ) on the c-myc Gene Transcription in Isolated Rat Pancreatic Islets—To examine the effect of each PKC isoform on c-myc gene transcription in pancreatic islets, we prepared adenoviruses containing the PKC isoforms found in rat islets (α, β2, δ, ε, and ζ). Pancreatic islets freshly isolated from Sprague-Dawley rats were infected with adenoviruses containing each PKC isoform (Ad-PKC α, β2, δ, ε, and ζ) or control adenovirus (Ad-GFP). Fig. 4A depicts representative islets 3 days after infection with Ad-PKC β2. The -fold increase of total PKC activity 3 days after infection with each Ad-PKC isoform (α, β2, δ, and ζ) using 200 islets was 5.5 ± 0.6, 4.6 ± 0.5, 5.7 ± 0.3, 4.8 ± 0.5, and 4.6 ± 0.6, respectively (n = 3), with that after infection with Ad-GFP being arbitrarily set at 1, indicating that the effects of each PKC isoform overexpression on total PKC activity are comparable. To evaluate the possible involvement of each PKC isoform in c-myc induction, the effects of adenovirus-mediated overexpression of each PKC isoform on c-myc gene expression were examined. As shown in Fig. 4B, 3 days after the infection, total RNA was isolated and used for RT-PCR followed by densitometric analyses. The amount of c-myc mRNA was markedly increased by adenovirus-mediated overexpression of PKC β2, whereas no increase was observed after overexpression of other PKC isoforms (α, δ, ε, and ζ). These

λ were clearly detected in HeLa (human epithelioid carcinoma cell), 3611RF (Raf-1 transformed rat fibroblast), MOLT-4 (human acute lymphoblastic leukemia cell), and HeLa cells, respectively. It should be noted that since different antibodies were used to detect each PKC isoform, the expression levels cannot be accurately compared. Also, to examine whether PKC activity is increased by high glucose in pancreatic islets, we measured total PKC activity in islets after exposure to high glucose. As shown in Fig. 2, B and C, PKC enzyme activity in isolated islets was increased by high glucose in a dose- and time-dependent manner.

Effects of Overexpression of WT and DN PKC Isoforms (α, β2, δ, ε, and ζ) on the c-myc Promoter Activity in βTC1 Cells—To examine the effects of PKC isoform overexpression on c-myc gene transcription, we examined c-myc promoter activity in...
results suggest that PKC β2 specifically regulates c-myc expression in pancreatic islets. Also, the c-myc induction by PKC β2 overexpression (fold increase: 3.4 ± 0.3) was blocked by a PKC-specific inhibitor GF109203X (5 μM) (1.3 ± 0.1), indicating that the PKC activity transduced by the virus is responsible for the effect on c-myc expression. To further examine the involvement of each PKC isoform in c-myc expression by high glucose, we examined the effect of overexpression of each DN PKC isoform on c-myc expression by high glucose. As shown in Fig. 4C, c-myc induction by high glucose was suppressed by Ad-DN PKC β2 but not by other Ad-DN PKC isoforms (α, δ, ε, and ζ). To obtain further insight into the specificity of the PKC pathway, it was found that c-myc expression by high glucose was not influenced by infection of Ad-DN JNK or the p38 MAPK inhibitor SB203580. These results suggest that PKC β2 is specifically involved in c-myc induction by high glucose in pancreatic islets.

**Effects of Adenovirus-mediated Overexpression of PKC Isoforms (α, β2, δ, ε, and ζ) on the Insulin Gene Transcription in Pancreatic Islets**—Since PKC β2 specifically induces c-myc expression (Figs. 3A and 4B) and we found recently that adenovirus-mediated c-Myc overexpression suppresses the insulin gene transcription (11), we examined the effects of PKC β2 overexpression on the insulin gene transcription. To evaluate the possible involvement of each PKC isoform in insulin gene transcription, we examined the effects of adenovirus-mediated overexpression of each PKC isoform on insulin gene expression. As shown in Fig. 5A, 3 days after the infection, total RNA was isolated and used for RT-PCR followed by densitometric analysis. The amount of insulin mRNA was decreased by adenovirus-mediated overexpression of PKC β2 but not by other PKC isoforms (α, δ, ε, and ζ). To examine the effects of PKC isoform overexpression on insulin promoter activity, we examined insulin promoter activity in βTC1 cells after transient overexpression of PKC isoforms (α, β2, δ, ε, and ζ). As shown in Fig. 5B, insulin promoter activity was decreased by overexpression of PKC β2 but not by other PKC isoforms (α, δ, ε, and ζ) in βTC1 cells. These results indicate that PKC β2 specifically regulates c-myc expression in pancreatic islets and further strengthen the hypothesis that the induction of c-Myc expres-
In the present study, we have found that c-myc expression is induced by high glucose levels and that this induction was suppressed by the PKC-specific inhibitor GF109203X or TPA, a condition known to inhibit PKC activity (Fig. 1). In contrast, c-myc gene expression was directly induced by acute exposure to TPA. Thus, these data suggest that high glucose levels induce c-myc gene expression in a PKC-dependent manner, which fits with previous studies showing that PKC is activated under diabetic conditions (13-20). The PKC isoforms α, β2, δ, ε, and ζ were found to be present in rat pancreatic islets, and PKC activity was increased by high glucose concentrations (Fig. 2). Furthermore, it was found that adenovirus-mediated overexpression of PKC β2, but not PKC α, δ, ε, or ζ, induced c-myc gene transcription (Figs. 3A and 4B) and that induction of c-myc gene transcription by high glucose levels was suppressed with overexpression of DN PKC β2, but not by DN PKC α, δ, ε, or ζ (Fig. 4C).

The pathways through which high glucose levels stimulate PKC activity in pancreatic islets are unknown. One possibility is reactive oxygen species (27), which are produced in various tissues in the diabetic state and are suspected to be involved in the development of diabetic complications (28-31). Several studies have provided evidence that oxidative stress is involved in the β-cell dysfunction found in diabetes (32-35), and other studies have shown that PKC is activated by oxidative stress as well as by high glucose per se (13, 30, 38). However, in experiments using the antioxidant N-acetyl-l-cysteine, we were unable to show that reactive oxygen species are involved in the glucose-induced increase in c-myc expression (Fig. 1C). We also asked if the stress-related kinases such as JNK and p38 MAPK (27, 36, 37) were involved in glucose-mediated c-myc induction, but induction of c-myc promoter activity and mRNA levels in βTC1 cells and primary islets was not suppressed by overexpression of DN JNK or p38 MAPK inhibitor (Figs. 3B and 4C), suggesting a lack of involvement of these pathways. From these studies we have not been able to identify the mechanisms through which PKC β2 is activated by high glucose levels, but dicacylglycerol, which is known to activate PKC in many other systems (13), must be considered as a strong candidate.

As shown in this study, PKC β2 specifically induces c-myc expression (Figs. 3A and 4B) and suppresses the insulin gene transcription (Fig. 5). Also, we found recently that adenovirus-mediated c-Myc overexpression suppresses the insulin gene transcription (11), which supports the concept that PKC β2-mediated c-Myc induction leads to suppression of insulin gene transcription. However, we must acknowledge the possibility that the effect of PKC β2 on insulin gene transcription is not totally exerted through c-Myc induction; several other factors could be induced or activated by PKC β2 and exert a suppressive effect on insulin gene transcription. Also, since several PKC isoforms are detected in rat pancreatic islets (Fig. 2A), we do not know that the increase of total PKC activity with high glucose levels (Fig. 2, B and C) is accounted for specifically by PKC β2. Nonetheless, we feel that strong circumstantial evidence is provided to support a link between high glucose concentrations, activation of PKC β2, induction of c-myc expression, and subsequent inhibition of insulin gene transcription. At present, it seems safe to conclude that the PKC β2-c-Myc axis is at least one of the candidate pathways that lead to suppression of insulin gene transcription.

In considering the inhibitory influences of PKC β2 and c-Myc upon insulin gene expression, an obvious question is why high glucose acting through PKC β2 and c-Myc does not lead to inhibition of insulin gene expression more rapidly. It is known that glucose has a positive influence upon the insulin promoter (26, 39), although islets cultured in high glucose concentrations for several days have only modest elevations in insulin mRNA (34). Also, it is known that the transcription factor PDX-1 is activated after acute exposure to high glucose levels, which should lead to enhancement of insulin promoter activity (40-42). Thus, we assume that the inhibitory effect of increased
PKC β2 Regulates c-myc Expression in Pancreatic β-Cells

C-Myc levels is counterbalanced by activation of PDX-1, which may obscure the effect of c-Myc. In contrast, with chronic exposure to hyperglycemia, as occurs in the diabetic state, the expression and activity of PDX-1 are decreased (9, 32, 33), which may allow the influence of c-Myc to become more prominent, thereby contributing to the process of glucose toxicity (9–11).

In conclusion, PKC β2 regulates c-myc expression, leading to suppression of the insulin gene transcription, and overexpression of DN PKC β2 inhibits c-myc induction by high glucose in pancreatic β-cells. These results imply that the PKC β2-c-Myc pathway is responsible for some of the β-cell glucose toxicity found in diabetes.

Acknowledgments—We thank Dr. Bert Vogelstein (Johns Hopkins Oncology Center) for kindly providing the AdEasy system and Dr. Linda Z. Penn (Ontario Cancer Institute) for c-myc promoter-luciferase fusion plasmid.

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*J. Biol. Chem.* 2002, 277:3680-3685.

doi: 10.1074/jbc.M109647200 originally published online November 19, 2001

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