Glucose-induced Phosphorylation of the Insulin Receptor

Functional Effects and Characterization of Phosphorylation Sites

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Abstract

Elevated glucose concentrations have been reported to inhibit insulin receptor kinase activity. We studied the effects of high glucose on insulin action in Rat1 fibroblasts transfected with wild-type human insulin receptor (HIRcB) and a truncated receptor lacking the COOH-terminal 43 amino acids (ΔCT). In both cell lines, 25 mM glucose impaired receptor and insulin receptor substrate-1 phosphorylation by 34%, but IGF-1 receptor phosphorylation was unaffected. Phosphatidylinositol 3-kinase activity and bromodeoxyuridine uptake were decreased by 85 and 35%, respectively. This was reversed by coincubation with a protein kinase C (PKC) inhibitor or microinjection of a PKC inhibitor peptide. Phosphopeptide mapping revealed that high glucose or PMA led to serine/threonine phosphorylation of similar peptides. Inhibition of the microtubule-associated protein (MAP) kinase cascade by the MAP kinase kinase inhibitor PD98059 did not reverse the impaired phosphorylation.

We conclude that high glucose inhibits insulin action by inducing serine phosphorylation through a PKC-mediated mechanism at the level of the receptor at sites proximal to the COOH-terminal 43 amino acids. This effect is independent of activation of the MAP kinase cascade. Proportionately, the impairment of insulin receptor substrate-1 tyrosine phosphorylation is greater than that of the insulin receptor resulting in attenuated phosphatidylinositol 3-kinase activation and mitogenic signaling. (*J. Clin. Invest.* 1996. 97: 613–620.) Key words: insulin receptor • insulin resistance • hyperglycemia • protein kinase C • phosphorylation

Introduction

Insulin resistance is a characteristic feature of non–insulin-dependent diabetes mellitus (NIDDM)[1] (1). This results from a number of target cell defects, including decreased insulin receptor kinase activity which has been observed in skeletal muscle, adipocytes, erythrocytes, and liver tissue from NIDDM subjects (2, 3). The decreased insulin receptor kinase activity may be a secondary event in NIDDM, since the abnormality is largely reversible with weight loss and does not occur in insulin receptors obtained from cultured fibroblasts from NIDDM subjects (2). Indeed, hyperglycemia, per se, may play an important role in causing decreased insulin receptor kinase activity since in vitro exposure of cells to elevated glucose concentrations (4, 5) leads to decreased insulin receptor kinase activity. Several reports have shown that enhanced insulin receptor serine/threonine phosphorylation can impair subsequent tyrosine phosphorylation (6, 7). One possible mechanism whereby hyperglycemia would induce insulin resistance is by stimulating the activity of the serine/threonine kinase protein kinase C (PKC) (5, 8). Indeed, in vitro, hyperglycemia can cause activation and translocation of several PKC isoforms in adipocytes and fibroblasts (5, 8–11) and activation of PKC can result in serine/threonine phosphorylation of the insulin receptor at threonine 1348 as well as several serine residues (6, 7, 12–16). This increase in serine/threonine phosphorylation attenuates the insulin receptor's intrinsic tyrosine kinase activity, thus decreasing ligand-stimulated phosphorylation (6, 7). In this study, we have attempted to identify molecular mechanisms for the attenuation of insulin receptor function by hyperglycemia (17, 18). This was accomplished using Rat1 cells transfected with wild-type receptors and mutant insulin receptors (19–21) as a model system.

Methods

Cell culture and materials. The cell lines used were Rat1 fibroblasts stably transfected with and overexpressing both wild-type human insulin receptor (HIRcB) and mutant receptors lacking the distal 43 amino acids of the β subunit (19–21). These were cultured as described previously in DME/F12 containing 500 nM methotrexate. Porcine insulin was purchased from Eli Lilly & Co. (Indianapolis, IN). Enhanced chemiluminescence detection reagents were obtained from Amersham Corp. (Arlington Heights, IL). Nitrocellulose was provided by Schleicher & Schuell, Inc. (Keene, NH).

Immunoblotting. Cells were routinely cultured in 35-mm 6-well dishes as described (22) and used at subconfluence. Approximately 16–24 h before experimental manipulations, the serum-containing media were removed and replaced with fresh serum-free DME (Sigma Immunochemicals, St. Louis, MO) containing 1 mM glucose for 16 h. The appropriate amounts of glucose were then added for the

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; ΔCT, Rat1 fibroblasts transfected with a mutant human insulin receptor lacking the COOH-terminal 43 amino acids; ERK, extracellular signal regulated kinases; HIRcB, Rat1 fibroblasts transfected with wild-type human insulin receptor; IRS-1, insulin receptor substrate-1; MAP kinase, microtubule-associated protein kinase, also known as ERK; MEK, MAP kinase kinase; NIDDM, non–insulin-dependent diabetes mellitus; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TPCK, tosyl-phenyl-chloro-ketone.

periods indicated. The cells were then stimulated with various ligands as described in the figure legends. The incubation was terminated by rapid aspiration of the medium followed by the addition of 100 μl Triton X-100 lysis buffer containing phosphatase and protease inhibitors. After a 10-min incubation, the lysates were clarified by centrifugation at 10,000 *g* followed by the addition of Laemmli sample buffer containing 5% mercaptoethanol. The samples were then boiled at 100°C for 5 min and then loaded onto 7.5%T/3%C Tricine-SDS-PAGE gels. Proteins were transferred to 0.45 μm nitrocellulose using an SD Transblot (Bio Rad Laboratories, Hercules, CA). The membrane was blocked for 1 h in 3% BSA in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4, with 0.1% Tween 20) (buffer A). The blocked membranes were probed with affinity-purified rabbit antiphosphotyrosine antibody (23) (0.5–1 μg/ml) in Tris-buffered saline containing 0.1% BSA and 0.1% Tween 20 for 12 h and then washed in TBS, 0.1% Tween 20, 1 mM EDTA (buffer B) (4 × 100 ml/10 min each) and then probed with anti–rabbit peroxidase conjugate (Amersham Corp.; 1/1,000) for 1 h. The membranes were then washed extensively in buffer B as described above. Bound antiphosphotyrosine antibody was detected using anti–rabbit peroxidase and the enhanced chemiluminescence reagent according to the manufacturer's instructions and autoluminography on preflashed Kodak X-Omat AR film. Band intensities on the autoluminographs were quantified by densitometry on a Hewlett-Packard ScanJet II using Scananalysis software (Elsevier Biosoft).

The monoclonal antibody to extracellular signal regulated kinase 2 (ERK2) was purchased from Transduction Laboratories (Lexington, KY) and used to reprobe blots after stripping in 62.5 mM Tris, pH 6.7, SDS, and mercaptoethanol. The specific microtubule-associated protein (MAP) kinase kinase (MEK) inhibitor PD98059 was provided by Dr. Alan Saltiel (Parke Davis, Ann Arbor, MI) and was used as described (24) at a concentration of 10 μM.

Microinjection of the PKC inhibitor peptide. The PKC inhibitor peptide (19-31) containing the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val was purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and used in microinjection studies. Cells were cultured on acid-washed glass coverslips and grown to semiconfluence. The cells were then starved in serum-free DME with 1 mM glucose. The peptide was dissolved in microinjection buffer composed of 5 mM $NaPO_4$ and 100 mM KCl, pH 7.4. The cells were then microinjected with the PKC inhibitor peptide using glass capillary needles. Approximately 10 fl of this solution was introduced into each cell. The injection included 1×10^6 molecules of IgG. 2 h after microinjection, cells were incubated with BrdU plus various concentrations of growth factors for 16 h at 37°C. The cells were fixed with acid alcohol (90% ethanol, 5% acetic acid) for 20 min at 22°C and then incubated with mouse monoclonal anti-BrdU antibody for 1 h at 22°C. The cells were then stained by incubation with rhodamine-labeled donkey anti–mouse IgG antibody and FITC–labeled donkey anti–rabbit IgG antibody for 1 h at 22°C. After the coverslips were mounted, the cells were analyzed and photographed with an Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Microinjected cell numbers were 250–300 per coverslip. Immunofluorescent staining of the injected cells indicated that ~ 75% of the cells were successfully microinjected.

Studies were also performed using a cell-permeable inhibitor of PKC bisindolylmaleimide (GF 109203X) (25, 26) purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). This was dissolved in DMSO at a stock concentration of 1 mM and added to cells at final concentration of 1 μM. The cells were grown on coverslips and serum-starved as described above. The cells were then incubated in high glucose (25 mM) for 18 h along with the PKC inhibitor. During this period, the cells were stimulated with insulin followed by the addition of BrdU. BrdU was visualized using mouse anti-BrdU antibody and anti–mouse antibody conjugated to rhodamine as described above.

Two-dimensional phosphopeptide mapping. HIRcB cells were serum-starved for 16 h and then incubated in phosphate-free DME containing 2 mCi/ml [^{32}P]orthophosphate for 3 h as described (13). The cells were then stimulated with high glucose (25 mM) or insulin as indicated. The cells were lysed and the insulin receptor was immunoprecipitated using mAb 83-14. The washed immunoprecipitates were analyzed by SDS electrophoresis. The labeled β subunit was visualized by autoradiography, excised from the gels, and recovered by electroelution (13) in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% SDS, and 0.1% 2-mercaptoethanol for 4 h. Eluted protein was precipitated with 4 vol of acetone at −80°C for 60 min followed by centrifugation at room temperature for 10 min at 10,000 *g*. The pellet was dried and digested with 10 μg of tosyl-phenyl-chloro-ketone (TPCK)-treated trypsin (Worthington Biochemical Corp., Freehold, NJ) in 100 μl of 100 mM *N*-ethylmorpholine acetate, pH 8.2, for 24 h at 37°C. A further 10 μg of TPCK-treated trypsin was added and digestion continued for 12 h. The peptides were lyophilized, resuspended with water, and relyophilized at least three times. The ^{32}P-labeled tryptic peptides were then resuspended in 5 μl of electrophoresis buffer and spotted onto thin-layer cellulose plates. High voltage electrophoresis was performed in 1:3.5:40.5 formic acid/acetic acid/water, pH 1.9, using a Hunter thin-layer electrophoresis system (C.B.S. Scientific Co., Inc., Del Mar, CA). Plates were subjected to ascending thin-layer chromatography in the second dimension in 75:15:50:60 *n*-butanol/acetic acid/pyridine/water, dried, and then subjected to autoradiography at −80°C on preflashed X-Omat AR film.

Phosphatidylinositol 3-kinase (PI3K) assays. Assays for insulin-stimulated phosphatidylinositol activity were performed as described (27) using antiphosphotyrosine immunoprecipitates. HIRcB cells were exposed to high glucose and stimulated with insulin as described above. The cells were then lysed and tyrosyl phosphorylated proteins were precipitated using antiphosphotyrosine antibody PY20 (Transduction Laboratories) overnight. 100 μl of anti–mouse agarose was added to the lysates for 1 h. The agarose was sedimented by centrifugation and then washed three times in the following buffers: (i) 50 mM Tris; 150 mM NaCl; 1% NP-40/Na_3VO_4; (ii) 100 mM Tris, pH 7.5/500 mM $LiCl_2$/100 μM Na_3VO_4; and (iii) 10 mM Tris, pH 7.5/100 mM NaCl/1 mM EDTA/100 μM Na_3VO_4. The bound immunoprecipitates were then assayed using soybean phospholipid (Sigma Immunochemicals) and [^{32}P]ATP. The phosphorylated lipids were extracted with chloroform/methanol (1:1) and then separated by ascending chromatography on *trans*-1,2-diaminocyclohexane-*N*, *N*, *N′*, *N′*–treated Silica gel 60 plates (EM Science, Gibbstown, NJ) using a methanol-chloroform-pyridine-borate buffer (27). The labeled phospholipids were visualized by autoradiography and quantified by scintillation counting of the excised spots.

Data analysis. Results were analyzed by the unpaired *t* test using Statview II (Abacus Inc., Berkeley, CA) and are representative of at least three separate experiments. Data are presented as mean±standard deviation.

Results

Attenuation of insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and the insulin receptor. Exposure of HIRcB cells to increasing concentrations of glucose ranging from 5 to 25 mM leads to a progressive decrease in IRS-1 tyrosine phosphorylation and insulin receptor tyrosine autophosphorylation as assessed by immunoblotting of whole cell lysates (Fig. 1). At 25 mM glucose, insulin-stimulated insulin receptor β-subunit tyrosine autophosphorylation was diminished by ~ 34±7% (mean±SD) (n = 4) when compared with cells incubated with 5 mM glucose, a concentration representing euglycemia. The predominant effect of high glucose appears to be on insulin-stimulated IRS-1 tyrosine phosphorylation, where even at 10 mM glucose, a 18±3% (n = 4) diminution of IRS-1 phosphorylation was apparent. At 25 mM glucose, IRS-1 phosphorylation was markedly attenuated

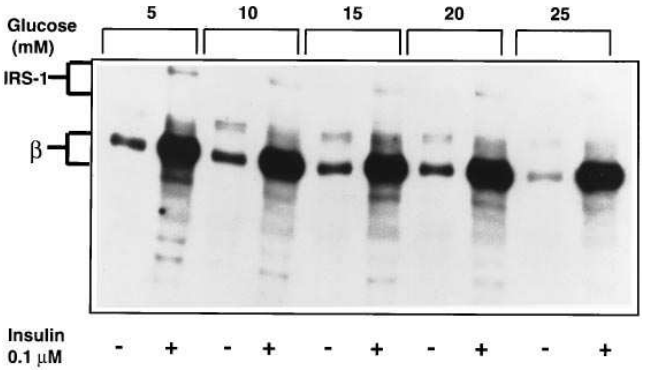

Figure 1. Dose-dependent inhibition of IRS-1 and insulin receptor β-subunit phosphorylation. HIRcB cells were serum-starved and then incubated for 1 h in the presence of the indicated concentrations of glucose. The cells were then stimulated with insulin for 5 min. The cells were lysed and the lysates were subjected to antiphosphotyrosine immunoblotting. Bound antibody was detected by chemiluminescence and an autoluminograph representative of four independent experiments is shown. β, β subunit of the insulin receptor.

(72±7%) compared with the 34% decrease in β-subunit tyrosine phosphorylation. In addition, high glucose also results in decreased tyrosine phosphorylation of certain lower molecular weight substrates of unknown identity.

We then used cells transfected with a mutant insulin receptor (ΔCT) (19–21) to define the structural features of the insulin receptor which may mediate these inhibitory effects (Fig. 2). It has been shown previously that threonine 1348 (residues are numbered according to the sequence of Ebina et al. [47]) and serine 1327 are the major sites phosphorylated in response to treatment of cells with PMA (12, 14–16). The ΔCT mutant receptor lacks the distal 43 amino acids including these residues (20). Exposure of the ΔCT cells to increasing glucose concentrations also resulted in a dose-dependent decrease in β-subunit phosphorylation (Fig. 2) as well as IRS-1 phosphorylation (Fig. 2). As observed for the wild-type receptor in HIRcB cells, the predominant effect of high glucose was to diminish IRS-1 tyrosine phosphorylation by $> 73\pm9\%$ ($n = 3$) while β-subunit phosphorylation was attenuated by $\sim 27\pm5\%$ ($n = 3$). Thus, the effect on IRS-1 tyrosine phosphorylation was proportionately greater than that on the β subunit of the insulin receptor.

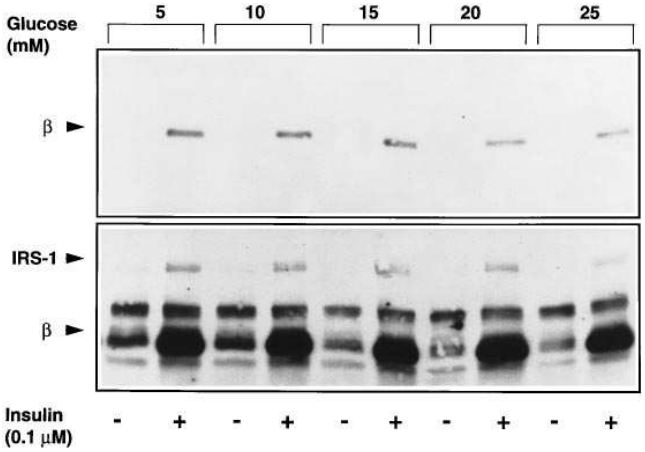

Figure 2. Dose-dependent inhibition of a COOH-terminally truncated insulin receptor β subunit. ΔCT cells overexpressing a COOH-terminally truncated insulin receptor β subunit were serum-starved and then preincubated in the indicated concentrations of glucose. The cells were then stimulated with insulin for 1 min and tyrosine phosphoproteins were analyzed by immunoblotting. A representative immunoblot from three separate experiments is shown. Two exposures are shown. The top panel was exposed for a shorter time (15 s) while the bottom panel was exposed for 1 min to detect IRS-1 phosphorylation. β, β subunit of the insulin receptor.

To further explore the mechanisms whereby high glucose inhibited insulin-stimulated insulin receptor function, we studied IGF-1–stimulated IGF-1 receptor and IRS-1 phosphorylation in HIRcB cells, which have high endogenous levels of IGF-1 receptors (Fig. 3). In these cells, autophosphorylation of the IGF-1 receptor β subunit is readily apparent as a tyrosine phosphoprotein of 100 kD when cells are stimulated with IGF-1 (Fig. 3). In addition, IGF-1–stimulated phosphorylation of IRS-1 is readily apparent. Preincubation of cells in 25 mM glucose did not affect IGF-1–stimulated β-subunit phosphorylation or IGF-1–stimulated IRS-1 phosphorylation.

Analysis of sites phosphorylated in response to high glucose. Although hyperglycemia leads to activation of PKC and concomitant decreased insulin receptor kinase activity, it is possible that the effects of high glucose occur through mechanisms independent of PKC. To address this question, we performed two-dimensional tryptic phosphopeptide analysis of the insulin receptor β subunits from cells exposed to high glucose, PMA, and insulin (Fig. 4). The cells were labeled with [^{32}P]orthophosphate and then stimulated. PMA increased phosphorylation of the insulin receptor by 3-fold above basal, while glucose-stimulated phosphorylation by 1.5-fold above basal (Fig. 4). The ^{32}P-labeled bands were then excised and digested exhaustively, and the resulting peptides were analyzed. This revealed that the pattern of glucose-stimulated serine/threonine phosphorylation was similar to that induced by PMA. High glucose stimulated the phosphorylation of the receptor on four distinct peptides, designated T, S1, S2, and S3. The major phosphopeptide recovered from high glucose–treated cells was peptide S1, while peptides T, S1, and S3 were phosphorylated to a lesser extent. PMA stimulated the phosphorylation of the receptor on phosphopeptides which migrated with a mobility similar to those observed under high glucose conditions, although there were differences in the extent of labeling of individual phosphopeptides, particularly peptides T, S2, and S3 which showed comparable amounts of ^{32}P incorporation after PMA treatment, to peptide S1.

Effects of high glucose on PI3K activity (Fig. 5). Activation of PKC in response to phorbol esters has been shown to inhibit insulin-stimulated PI3K activity (28–30). We assessed PI3K activity in antiphosphotyrosine immunoprecipitates of cells stim-

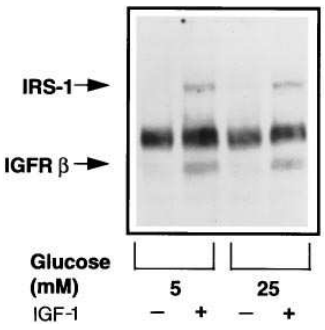

Figure 3. Effects of glucose on IGF-1–stimulated phosphorylation. HIRcB cells were serum-starved and exposed to 5 or 25 mM glucose for 1 h. The cells were then stimulated with 0.1 μM IGF-1 and the whole cell lysates were analyzed for tyrosine phosphoproteins by antiphosphotyrosine immunoblotting and autoluminography.

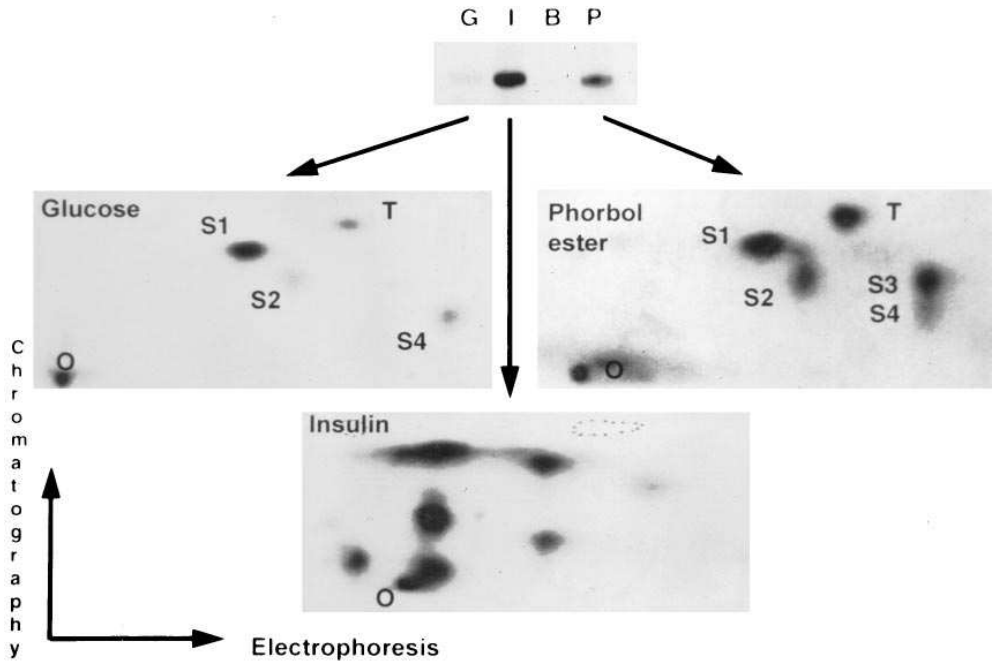

Figure 4. Two-dimensional tryptic phosphopeptide mapping of insulin receptor phosphorylation. Serum-starved HIRcB cells were labeled with [^{32}P]orthophosphate for 3 h and incubated with 25 mM glucose (1 h) or phorbol ester (1 μM, 1 h) or insulin (0.1 μM, 5 min) as indicated. The receptors were immunoprecipitated using anti–human insulin receptor mAb 83-14 and subjected to SDS gel electrophoresis and autoradiography (*top panel*). The receptor bands were excised and digested exhaustively with TPCK-trypsin. The resulting phosphopeptides were separated by thin-layer high voltage electrophoresis and chromatography. The peptides were then visualized by autoradiography at −70°C. The autoradiographs are representative of three independent experiments. *G*, 25 mM glucose; *I*, insulin; *B*, basal unstimulated cells; *P*, PMA, 1 μM.

ulated with insulin before and after they were preincubated in high glucose medium. In cells exposed to 25 mM glucose for 1 h, insulin-stimulated PI3K activity, as detected by the presence of phosphorylated inositol, was decreased by 85±4% (mean±SD) (n = 4) (Fig. 5). To determine whether this hyperglycemia-induced inhibition of insulin signaling was mediated through a PKC-dependent mechanism, a sample of cells was coincubated with the inhibitor of PKC, bisindolylmaleimide GF109203X at a concentration of 1 μM. As seen in the middle three lanes of Fig. 5, the inhibitory effects of high glucose were completely prevented by this agent.

Effects of high glucose on insulin-stimulated mitogenesis (Fig. 6). To examine the effects of high glucose on biologic signaling, insulin-induced DNA synthesis (BrdU) uptake was determined. Cells were exposed to high glucose for 1 h before the addition of insulin and BrdU for 18 h. High glucose decreased the uptake of BrdU in glucose-exposed cells by 35%. Coincubation of the cells with a PKC inhibitor (Fig. 6 *A*) reversed the inhibition of mitogenesis caused by incubation in high glucose.

To further explore the role of PKC in this inhibitory mechanism, we used a relatively specific pseudosubstrate peptide inhibitor of PKC. Since this peptide is not permeable into cells, we used the technique of single-cell microinjection in order to introduce the reagent into the cell interior where it would have access to PKC. As seen in Fig. 6 *B*, microinjection of the PKC inhibitor peptide largely prevented the effect of hyperglycemia to attenuate insulin-stimulated DNA synthesis.

Role of MAP kinase activation in the effects of high glucose. Stimulation of cells with PMA results in insulin receptor phosphorylation on additional sites compared with those phosphorylated by purified PKC when added to purified insulin receptor (14–16, 20, 31). This is taken to indicate that other serine/threonine kinases, in addition to PKC, are activated in intact cells. Activation of PKC also results in activation of the Raf kinase–MAP kinase kinase–MAP kinase cascade (32–34). The MAP kinases (ERK1 and ERK2) are serine/threonine kinases which could phosphorylate the insulin receptor and/or IRS-1 in a negative feedback manner leading to impaired insulin-stimulated tyrosine phosphorylation of the receptor and its endogenous substrates. To examine the role of the MAP kinase cascade, we used a specific inhibitor of MEK, PD98059 (24), in cells exposed to both normal and high glucose concentrations (Fig. 7). Activation of ERK2 in response to insulin was assessed by decreased mobility (gel-shift) on SDS gel electrophoresis resulting from phosphorylation by activated MEK (Fig. 7). Addition of the MEK inhibitor PD98059 prevented the insulin-induced decrease in electrophoretic mobility and activation of ERK2. Tyrosine phosphorylation of whole cell ly-

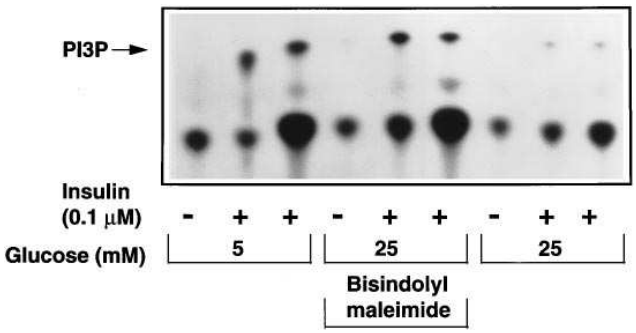

Figure 5. Glucose-induced inhibition of PI3K activity. Serum-starved HIRcB cells were incubated in high and low glucose for 1 h and then stimulated with insulin for 5 min. Some groups of cells were also coincubated with a specific PKC inhibitor, bisindolylmaleimide, at 1 μM. Tyrosyl phosphoproteins were then immunoprecipitated using antiphosphotyrosine antibodies and the immunoprecipitates were analyzed for PI3K activity using phosphatidylinositol and thin-layer chromatography. The thin-layer chromatography plates were then subjected to autoradiography at −70°C. The autoradiograph represents four independent experiments.

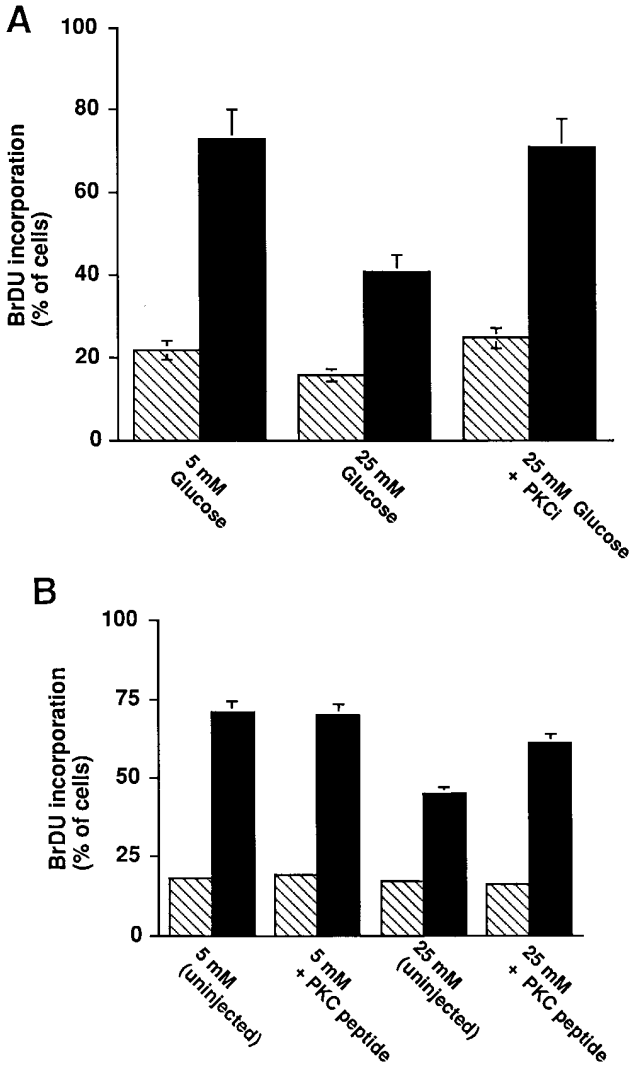

Figure 6. Effects of glucose on mitogenesis. (*A*) HIRcB cells were maintained in the absence of serum for 36 h before exposure to high glucose medium. After preincubation for 1 h, the cells were then stimulated with insulin followed by the addition of BrdU for 18 h. The cells were then washed, fixed and stained using anti-BrdU antibodies, and quantified for positive staining. Some groups of cells were incubated with the PKC inhibitor (*PKCi*) bisindolylmaleimide GF109203X (1 μM). (*B*) In similar experiments as described in *A*, a pseudosubstrate PKC inhibitor peptide (*PKC peptide*) was introduced into the cells by microinjection before the addition of high glucose and insulin. The cells were then stained for BrdU and counted. Results for both are presented as mean±SEM ($n = 4$). *Hatched bars*, basal; *filled bars*, insulin.

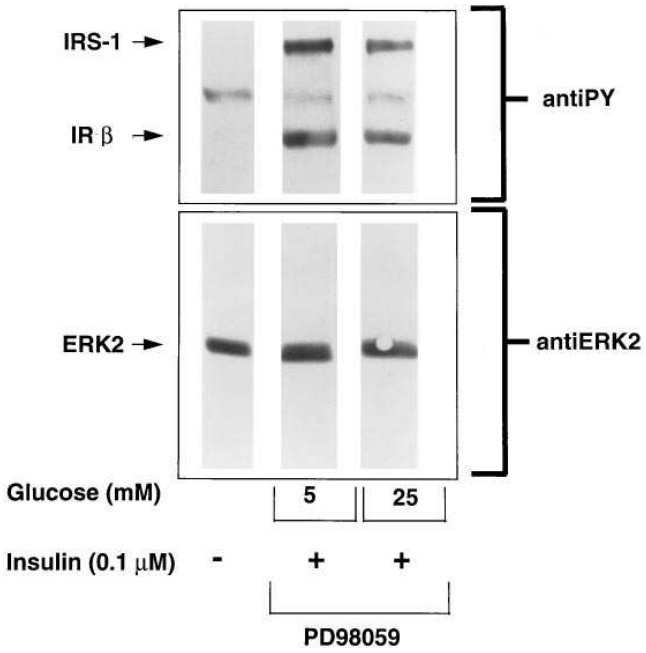

Figure 7. Effect of PD98059 and inhibition of the MAP kinase cascade. HIRcB cells were serum-starved and treated with high and low glucose medium before insulin stimulation. A highly specific inhibitor of MEK (PD98059) was added for 1 h at a concentration of 10 μM where indicated. The cell lysates were then analyzed by antiphosphotyrosine immunoblotting (*top panel*). The nitrocellulose blot was then stripped and reprobed using an anti-ERK2 antibody (*bottom panel*). A representative autoluminograph is shown.

sates was assessed by antiphosphotyrosine immunoblotting. Incubation of cells in 25 mM glucose inhibited insulin receptor β-subunit tyrosine phosphorylation (Fig. 7) and this was not prevented by preincubation with PD98059 which resulted in complete inhibition of ERK2 phosphorylation and activation.

Discussion

Hyperglycemia is a predominant pathophysiological feature of diabetes and is causally linked to many of the complications of long-standing, uncontrolled diabetes (2, 3). In humans with NIDDM and in animal models of diabetes, hyperglycemia leads to secondary insulin resistance in vivo as evidenced by the fact that insulin resistance worsens as one goes from the prediabetic stage to the development of overt NIDDM (2, 3). Experimental hyperglycemia leads to reversible insulin resistance in animals and in humans (3). Consistent with this is the partial reversal of insulin resistance in NIDDM (1, 2) when glucose levels are brought down to euglycemic values. In cells in culture, high glucose levels stimulate PKC (5, 10) and activation of PKC results in serine/threonine phosphorylation of the insulin receptor (13, 14, 16, 31). The insulin receptor can also be phosphorylated directly by PKC in vitro (6, 35). Attenuation of insulin receptor autophosphorylation and tyrosine kinase activity has been reported when cells are exposed to supraphysiological levels of glucose representative of those found in poorly controlled diabetes mellitus (5, 8). This effect was blocked by PKC inhibitors (5, 8). Therefore, in the current study, we assessed the molecular basis for these effects in detail using transfected Rat1 cells which overexpressed wild-type and mutant human insulin receptors (19–21).

In the HIRcB cells which overexpress the wild-type human insulin receptor, our results indicate that in terms of total tyrosine phosphorylation of proteins, the predominant effect of high glucose is an attenuation of insulin-stimulated IRS-1 tyrosine phosphorylation with a less marked effect on the receptor's insulin-stimulated tyrosine phosphorylation state. Interestingly, high glucose led to decreased basal insulin receptor phosphorylation which was of a similar magnitude to the effect on IRS-1 phosphorylation. The mechanism for this effect

might also involve serine/threonine phosphorylation. This is consistent with previous studies in Chinese hamster ovary cells overexpressing both the human insulin receptor and PKC where, despite the high level of stoichiometric serine/threonine phosphorylation, the major effect of PKC activation was on IRS-1 tyrosine phosphorylation (28, 36). One possible mechanism whereby this may occur is through serine phosphorylation of the juxtamembrane domain of the insulin receptor. Serines 967/968 of the insulin receptor are phosphorylated in intact cells. These serines are located in close proximity to tyrosine 972 contained in the NPXY motif (37). This region has been shown to interact with IRS-1 and SHC (38, 39) through their phosphotyrosine binding domains (39) which, unlike SH2 domains, require conservation of residues NH_2-terminal to the phosphorylated tyrosine for binding (37). Therefore, it is possible that phosphorylation of serines 967/968 could modulate this interaction, decreasing IRS-1 tyrosine phosphorylation. The activation of PI3K in response to insulin is largely dependent on IRS-1 phosphorylation, and decreased IRS-1 phosphorylation would lead to decreased PI3K activation. Our data are consistent with this scenario, and this would explain how extensive inhibition of downstream signaling occurs in the presence of a modest decrease in the phosphotyrosine content of the receptor.

Furthermore, IRS-1 contains numerous serine/threonine phosphorylation sites (40) and is substantially serine/threonine phosphorylated in the basal state. It is also conceivable that increased serine/threonine phosphorylation of IRS-1 would block its subsequent tyrosine phosphorylation and/or affect its docking with SH2 domain–containing molecules, including the p85 subunit of PI3K.

Another mechanism may involve the tyrosine phosphatase PTP1B particularly as prolonged exposure to high glucose increases the expression and activity of PTP1B (4). However, these effects may occur only after several days of exposure to high glucose (4) and may not explain the short-term effects of high glucose which have been addressed in the present study.

In intact cells, serine 1327 and threonine 1348 of the human insulin receptor have been identified as stoichiometrically the major phosphorylation sites in response to phorbol ester treatment (12, 14–16). We reasoned that if the inhibitory effects of glucose were directly due to phosphorylation at these sites, then removal of these sites would block the attenuation of insulin receptor kinase activity and would result in a receptor refractory to inhibition. We tested this hypothesis directly using a COOH-terminally truncated insulin receptor (20, 21) lacking the distal 43 amino acids of the β subunit which contain these COOH-terminal serine and threonine phosphorylation sites. The results indicate that high glucose inhibits the effects of insulin stimulation to the same extent in the wild-type and the mutant receptor even when serine 1327 and threonine 1348 are removed, and this is consistent with the recent work of Mosthaf et al. (41). These authors examined the effects of high glucose on the tyrosine autophosphorylation state of the receptor when the distal COOH terminus was removed and found that the high glucose–induced inhibition was still present. From those data and ours, we can draw the conclusion that glucose exerts its effects at other sites on the receptor which do not involve the COOH-terminal 43 amino acids of the receptor β subunit. One such mechanism is by phosphorylation at sites in the insulin receptor cytoplasmic domain proximal to serine 1327 and threonine 1348. Recently, using skeletal muscle tissue from patients with NIDDM, Kellerer et al. (42) examined the phosphorylation state of serine 1327 and threonine 1348 with antiphosphopeptide antibodies (14) and found that this was unaltered. It has been demonstrated previously that the insulin receptor is phosphorylated at multiple serine sites (13, 15, 16). In addition, we have now demonstrated multi-site phosphorylation in response to high glucose. Several other serine phosphorylation sites, in addition to serine 1327 and threonine 1348, have been identified. These include serines 967/968, serine 1035, and serine 1305/1306 (43, 44). The juxtamembrane serines 967/968 do not appear to regulate tyrosine autophosphorylation (41) although they may regulate protein–protein interactions. Phosphorylation at the other sites may regulate the intrinsic tyrosine kinase activity of the receptor and its autophosphorylation per se. However, site-directed mutagenesis of serines 1305/1306 does not affect insulin-stimulated tyrosine kinase activity (45). In HIRcB cells, IGF-1–stimulated tyrosine phosphorylation was not affected by incubation in high glucose. One explanation may be that the activity of the IGF-1 receptor is regulated in a different manner from that of the insulin receptor. Indeed, direct comparison of the insulin receptor and the IGF-1 receptor phosphorylation sites in intact cells and the respective amino acid sequences reveals a number of differences (20). The sites of serine/threonine phosphorylation in the IGF-1 and insulin receptors are not the same (36, 46, 47) since PMA treatment of cells overexpressing IGF-1 receptor results in serine phosphorylation on a single major phosphopeptide (13) which is not conserved in the insulin receptor.

Direct analysis of insulin receptor phosphorylation by two-dimensional tryptic phosphopeptide mapping revealed that high glucose and PMA stimulated the phosphorylation of residues contained in similar phosphopeptides. This is the first demonstration that the effects of high glucose and PMA on insulin receptor phosphorylation are similar and validates the assumption that the mechanisms of insulin resistance induced by both stimuli are directly comparable. The extent of phosphorylation of individual phosphopeptides was somewhat different and may reflect the contribution of individual PKC isoforms (48, 49) in the overall spectrum of PKC activation. Each stimulus may have slightly different effects on individual PKC isoforms, and furthermore, PMA in this context is used at pharmacological concentrations. One phosphorylation site, serine 1035 (44) is located near lysine 1030, a residue critical for binding ATP (50, 51). High glucose–dependent phosphorylation of this residue may exert a direct effect on the affinity of the receptor for ATP. Indeed, it has been reported that phorbol esters regulate the affinity of the insulin receptor for ATP (52).

Some of the serine/threonine sites on the insulin receptor phosphorylated in vitro are identical to those phosphorylated in intact cells (13, 31). However, additional sites appear to be phosphorylated in intact cells, raising the possibility that additional serine kinases phosphorylate the receptor in vivo (12, 13, 15, 31, 53). Phorbol esters and glucose may also activate serine/threonine kinases in the Raf/MEK/MAP kinase pathway (33) and activation of these kinases could result in retrograde or "back" phosphorylation of the insulin receptor and IRS-1 and, hence, may be responsible for the inhibitory effects. While there is no precedent to indicate phosphorylation of the insulin receptor by MEK or MAP kinases in high glucose, the similarity between the effects of phorbol esters and high glucose suggested that this may be possible. We tested

this hypothesis using a synthetic highly specific inhibitor of the MEK kinase, PD98059 (24). Although this resulted in complete inhibition of MAP kinase activation, it did not prevent impaired tyrosine phosphorylation of the insulin receptor. However, it does not rule out direct phosphorylation of the insulin receptor by these kinases, but it does indicate that the effects of PKC activation on the insulin receptor can be dissociated from that of the MAP kinases.

To explore the role of PKC in this hyperglycemia-induced decreased receptor kinase activity, we have used several different PKC inhibitors and, in each case, showed that they prevent this effect. A problem with many chemical inhibitors is that they may not be completely specific for the target enzyme in question. Consequently, we used a peptide inhibitor of PKC, which acts as a pseudosubstrate for this enzyme, preventing PKC from phosphorylating its natural targets. While peptide inhibitors may be highly specific, a significant problem in biologic studies is that peptides do not cross cell surfaces and, therefore, conventional experimental methods do not allow them to gain access to the cell interior. To overcome this problem, we used the method of single-cell microinjection in which individual, living HIRcB cells were microinjected with the pseudosubstrate PKC inhibitor. Within the cell, this peptide should competitively inhibit PKC activity, and, consistent with this, we found that microinjection of the PKC inhibitor largely prevented the hyperglycemia-induced attenuation of insulin signaling. This peptide is subject to intracellular proteolysis and since DNA synthesis is not measured until many hours after insulin stimulation, the incomplete inhibition observed after microinjection of the peptide is most likely due to degradation within the cell. Taken in aggregate, our studies argue strongly that PKC is at least one effector mediating hyperglycemia-induced cellular insulin resistance.

In summary, we have shown that hyperglycemia can cause cellular insulin resistance by inhibition of insulin receptor autophosphorylation/kinase activity accompanied by a more pronounced effect to impair IRS-1 phosphorylation. Inhibition of these upstream elements in the insulin signaling cascade is associated with striking attenuation of more distal insulin-induced events, such as activation of PI3K and stimulation of DNA synthesis. The effect is relatively specific for the insulin receptor, since IGF-1 receptor function was not impaired in the same cells. In addition, studies with a mutant insulin receptor indicate that residues in the COOH-terminal 43 amino acids are not involved in this phenomenon. Together, these results suggest that unique structural features of the insulin receptor proximal to the COOH terminus and not represented in the IGF-1 receptor provide the molecular mechanism for this effect. Hyperglycemia is known to activate various PKC isoforms, and our studies reveal that PKC is at least one mediator of hyperglycemia-mediated cellular insulin resistance. It is now well recognized that in vivo hyperglycemia can cause insulin resistance, or exacerbate an underlying state of insulin resistance, such as in NIDDM. As such, the current studies provide further understanding into the cellular mechanisms underlying this form of glucotoxicity. The use of the HIRcB model has certain limitations and is less physiologic compared with the use of animal models or diabetic human tissue. However, coupled with the single-cell microinjection method and the other techniques used in this study, this cell system provides a useful means to identify the molecular and cellular consequences of in vitro hyperglycemia. Similar studies are not easily performed in animals or on human tissues. The information derived from this cell culture model should provide a rational basis for the design of experiments in vivo. A thorough understanding of the basic mechanisms of glucose-induced insulin resistance could provide new therapeutic targets for treatment of this metabolic abnormality.

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