The inhibitory effect of 2-deoxyglucose on insulin receptor autophosphorylation does not depend on known serine phosphorylation sites or other conserved serine residues of the receptor β-subunit

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Abstract Hyperglycemia induces insulin resistance in diabetic patients. It is known that supraphysiological levels of D-glucose or 2-deoxyglucose inhibit the insulin receptor and it is speculated that this effect is mediated by serine phosphorylation of the insulin receptor β-subunit and other proteins of the insulin signaling chain. To test this hypothesis we prepared point mutations of the human insulin receptor where serine was exchanged to alanine at 16 different positions, either at known phosphorylation sites or at positions which are conserved in different tyrosine kinase receptors. These receptor constructs were expressed in HEK 293 cells and the effect of 2-deoxyglucose (25 mM) on insulin (100 nM) induced receptor autophosphorylation was studied. 2-Deoxyglucose consistently inhibits insulin stimulated autophosphorylation of all constructs to the same degree as observed in wild-type human insulin receptor. The data suggest that none of the chosen serine positions are involved in 2-deoxyglucose induced receptor inhibition.

Key words: Insulin receptor inhibition; 2-Deoxyglucose; Serine phosphorylation

1. Introduction

Hyperglycemia contributes to the pathogenesis of insulin resistance both in NIDDM and IDDM patients [1,2]. The underlying mechanism appears to involve both a modulation of insulin signaling at the level of the insulin receptor as well as a modulation of signaling events downstream of the receptor [3–5]. Effects of high glucose concentrations on the tyrosine kinase activity of the insulin receptor have been demonstrated in a number of different cell types including freshly isolated rat adipocytes as well as rat-1 fibroblasts overexpressing HIR [1,3,6]. We have earlier used these cultured cell lines to characterize the mechanism which is relevant for hyperglycemia induced receptor inhibition [7,8] and found evidence that a glucose induced activation of protein kinase C is involved. Other investigators have suggested that activation of tyrosine phosphatases may be important in hyperglycemia induced receptor inhibition [9]. According to studies in HEK 293 cells the PKC isoforms α, β1, β2 and θ are able to inhibit the insulin receptor [10] but the exact mechanism remains unclear. A phosphorylation of the receptor β-subunit [11,12] and the insulin receptor substrate-1 [10] at serine residues might be involved in this effect.

The aim of the present study was to obtain direct evidence for a role of specific serine residues in the receptor for glucose induced receptor inhibition. Attempts to find 2-deoxyglucose stimulated serine phosphorylation sites in the receptor using ^{32}P-labeled cells were so far unsuccessful. As an alternative approach to define relevant serine residues we have used insulin receptor constructs with either a 43 amino acid deletion at the C-terminal tail of the β-subunit or a 12 amino acid deletion at the juxtamembrane region of the insulin receptor to define the role of potential serine phosphorylation sites in these regions for glucose and 2-deoxyglucose dependent receptor inhibition [8]. These deletions, which eliminate the candidate sites at positions 955/956 and 962/964 [13] as well as the candidate sites 1308/09 and 1315, were efficiently inhibited by hyperglycemic incubation conditions excluding a function of these serine residues in hyperglycemia dependent receptor inhibition. Now we prepared point mutations of the human insulin receptor in which serine was exchanged to alanine at 16 different positions and transiently expressed these constructs in HEK 293 cells. The candidate sites were chosen either because they are known serine phosphorylation sites including 1023/25 [14], 1293/94, [15,16] and 1309 (with the neighboring serine 1308) [17] or because they are conserved in different receptor tyrosine kinases (HIR, hIGF-1 receptor, hIRR and dIR). As outlined by Danielsen et al. [18], these positions include 962, 994, 1037, 1055, 1074/78, 1177/78/82, 1202, 1263 and 1267. Among these Ser-1178 and Ser-1182 are also conserved in other receptor tyrosine kinases, i.e. the EGF-R, PDGF-R and FGF receptors [19]. In addition we used other closely related serine mutants (HIR-1192, HIR-1258, HIR-1275) and the juxtamembrane deleted receptor (HIR-ΔJM). HEK 293 cells transiently overexpressing the respective constructs were exposed to 25 mM 2-deoxyglucose and the effect on insulin stimulated HIR autophosphorylation was determined.

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Abbreviations: dIR, *Drosophila* insulin receptor; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; HEK, human embryonic kidney; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; hIGF-1, human insulin like growth factor-1; HIR, human insulin receptor; hIRR, human insulin related receptor; IDDM, insulin dependent diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus; PDGF, platelet derived growth factor; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Materials

Cell culture reagents and fetal calf serum were purchased from Gibco (Eggenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Porcine insulin, aprotinin, phenylmethylsulfonyl fluoride, Na_3VO_4 and Triton X-100 were from Sigma (Munich, Germany). The reagents for SDS-PAGE and Western blotting were obtained from Roth (Karlsruhe, Germany) and Bio-Rad (Munich, Germany). Nitrocellulose was from Schleicher and Schuell (Dassel, Germany). All other reagents were of the best grade commercially available. Visualization of immunocomplexes after Western blotting was performed with the non-radioactive enhanced chemiluminescence system (ECL) and the Hyperfilm-ECL from Amersham Buchler (Braunschweig, Germany).

The cDNAs for the wild-type receptor (HIR-wt) and the insulin receptor with a juxtamembrane deletion (HIR-ΔJM) were gifts from Axel Ullrich (Max-Planck-Institute, Martinsried, Germany). The monoclonal mouse antibody against phosphorylated tyrosine residues (αPY) were from Leinco Technology Inc. (Ballwin, USA).

2.2. Preparation of serine to alanine point mutants of HIR

All point mutations were prepared by the method of Kunkel [20] in a CMV promoter based expression vector [21] containing the cDNA sequence for the wild-type human insulin receptor. The mutagenic oligos were purchased from Boehringer Mannheim (Mannheim, Germany). Introduced point mutations were verified by DNA sequencing using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, USA).

2.3. Transient expression of HIR mutants

Human embryonic kidney fibroblast 293 cells (ATCC CRL 1573) were grown in Dulbecco's MEM/nutrient mix F12 medium supplemented with 10% fetal calf serum. A total of 4 μg plasmid DNA was transfected per semiconfluent 35 mm diameter dish according to the protocol of Chen and Okayama [22]. Briefly, cells were grown in 6 well dishes at a density of 10^5 cells per well in 2 ml of medium. A total of 4 μg supercoiled plasmid DNA was mixed with 0.25 M $CaCl_2$ in a final volume of 0.1 ml. To this was added equal amount of 2× transfection buffer (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) and after incubation for 10 min at room temperature the mixture was given dropwise to the cells. After incubation for 16 h at 37°C and 3% CO_2 the cells were serum starved for 24 h in DMEM (1000 mg/l glucose) containing 2 mM glutamine.

2.4. Stimulation and cell lysis

According to the experiments serum starved cells were preincubated with or without 25 mM 2-deoxyglucose for 25 min before stimulation with 100 nM insulin for 5 min at 37°C and lysed in 0.2 ml ice-cold lysis buffer (50 μM HEPES pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin). Cleared crude cell lysates (20 min/12 000×*g*) were analyzed on 7.5% SDS-PAGE (40 μl per lane).

2.5. Western blotting

Separated proteins were transferred to nitrocellulose membranes by semi-dry electroblotting (transfer buffer: 48 mM Tris-HCl pH 7.5; 0.004% SDS; 39 mM glycine; 20% methanol). After transfer, the membranes were blocked with NET-G buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100 and 0.25% gelatine, pH 7.4) for 1 h. Subsequently filters were incubated with the first antibody (αPY, αHIR-β) overnight at 4°C. The membranes were washed four times with NET buffer before being incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. Visualization of immunocomplexes was performed by enhanced chemiluminescence (ECL).

3. Results

Fig. 1 shows representative autoradiograms of insulin receptor autophosphorylation in HEK 293 cells. Cells transfected with HIR-wt and different receptor mutants were stimulated with or without 100 nM insulin for 5 min in the presence or absence of 25 mM 2-deoxyglucose (2-DG). 2-DG was added to the cells 25 min prior to insulin. Receptor autophosphorylation was determined in immunoblots with phosphotyrosine antibodies (αPY), the expression level of insulin receptors was controlled by immunoblots with antibodies against the C-terminal end of the insulin receptor β-subunit (αHIR-β). One can see that 2-DG induces a consistent and reproducible receptor inhibition in HEK 293 cells. In contrast to this observation the glucose induced receptor inhibition found in rat adipocytes and rat-1 fibroblasts overexpressing HIR was not reproducibly found in HEK 293 cells. Preincubation of the cells with 25 mM glucose instead of 2-DG

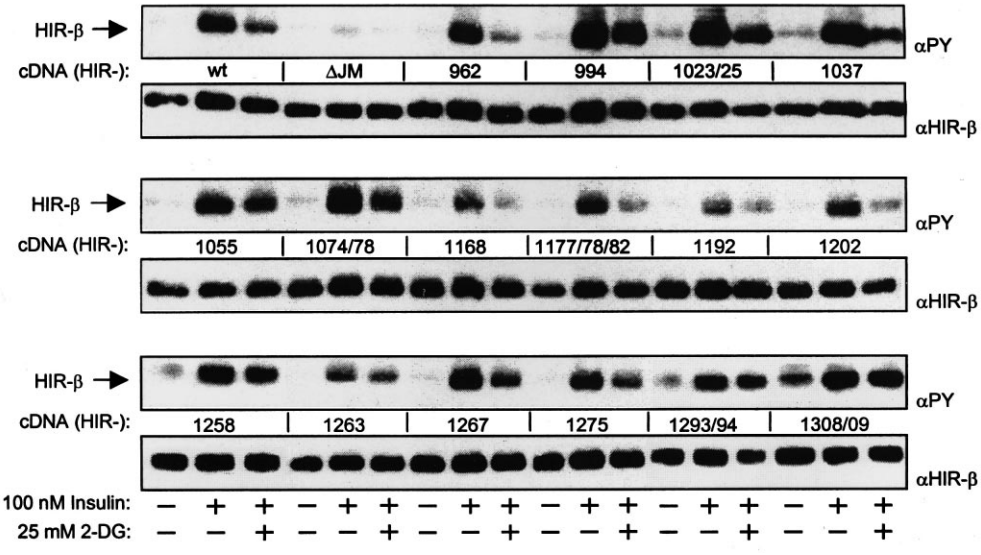

Fig. 1. Insulin induced receptor autophosphorylation and the effect of 2-DG. Whole cell lysates were prepared from HEK 293 fibroblast. Cells overexpressing the wild-type receptor or the mutant receptors were incubated with 25 mM 2-DG for 25 min and subsequently stimulated with 100 nM insulin at 37°C for 5 min. Proteins were separated by SDS-PAGE; Western blots were probed with antibodies against phosphotyrosine residues (αPY) or insulin receptor (αHIR-β). Representative immunoblots are shown. These results were reproduced in five different experiments.

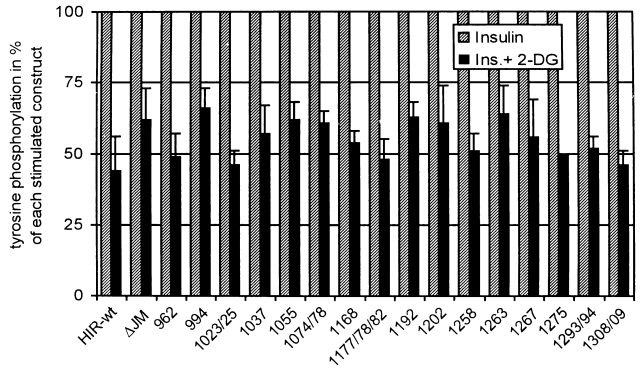

Fig. 2. Statistical analysis of insulin induced receptor autophosphorylation and the effect of 2-DG. Insulin stimulated and 2-DG preincubated levels of receptor autophosphorylation were measured by densitometric analysis of five independent experiments. The value of the each stimulated receptor was taken as 100% (white bars). The black bars show the reduced level of the receptor autophosphorylation after preincubation with 2-DG ($n = 5$, ± S.E.M.).

caused inconsistent effects (data not shown). While in some experiments glucose inhibits HIR-wt and the different mutants, in other experiments an even stimulatory effect is observed. The cellular conditions which determine whether glucose is stimulatory or inhibitory are at present not clear.

Basal, insulin stimulated and 2-DG inhibited autophosphorylation of the insulin receptor were quantified by scanning densitometry, shown in Fig. 2. Receptor autophosphorylation as determined by immunoblotting with PY antibody was normalized for equal amounts of receptors based on the immunoblots with the receptor antibody. The basal and insulin stimulated autophosphorylation was not significantly altered by most of the point mutations. Exceptions were found only with the mutations at 994, 1023/25 and 1178 as reported earlier [23]. To quantify the inhibitory effect of 2-DG we set the insulin stimulated receptor autophosphorylation of each construct at 100%. We observed a reduction in the receptor autophosphorylation of all constructs not significantly different from that observed in HIR-wt ($n = 5$, ± S.E.M.).

4. Discussion

Our earlier studies in rat-1 fibroblasts overexpressing human insulin receptor had suggested that the hyperglycemia induced inhibition of the insulin receptor kinase involves protein kinase-C activation. We had also speculated that this effect requires serine phosphorylation of the insulin receptor β-subunit. The evidence for this hypothesis is based on the observation that different protein kinase-C inhibitors were able to prevent the effect of hyperglycemia [6]. Furthermore, a phosphoamino acid analysis of the receptor β-subunit has shown a small increase of serine phosphorylation of the receptor β-subunits in cells incubated in the presence of high glucose levels [7]. While we could further substantiate the inhibitory role of specific PKC isoforms [10], the hypothesis of a direct phosphorylation of the receptor β-subunit could not be further proven. As a first approach to find serine sites in the insulin receptor we had earlier eliminated a number of candidate sites using insulin receptor constructs with C-terminal and juxtamembrane deletions which were stably overexpressed in rat-1 fibroblasts [8]. In the present paper we continued this approach by preparing systematically a number of further HIR mutants eliminating known phosphorylation sites of the receptor β-subunit and all conserved serine residues which were thought to be candidates. In HEK 293 cells a transient overexpression of different constructs is possible, therefore this cell system is suitable to screen rapidly the function of these HIR constructs. Under our experimental conditions this cell system reacts to glucose not in the same way as was the case in adipocytes and rat-1 fibroblasts [3,6]. In contrast, the effects of 2-deoxyglucose are the same as observed in rat-1 fibroblasts. The reason for this cell specific difference is at present unclear. Unpublished data by Mosthaf et al. suggest that the expression level of the insulin receptor might be crucial. However, it is also possible that the cellular effects caused by glucose and 2-deoxyglucose might involve different mechanisms or might require different metabolic situations of the cells.

The present data exclude a crucial function of all the chosen serine positions in the inhibitory effect of 2-deoxyglucose. Liu and colleagues have defined the serine 1023/25 as a phosphorylation site for PKC-α [14]. The present data suggest that this site might indeed be an inhibitory receptor serine residue as a somewhat elevated insulin stimulated autophosphorylation is found. However, this potentially inhibitory phosphorylation site seems not to be required for the 2-deoxyglucose dependent receptor inhibition. Slightly increased basal and insulin stimulated receptor phosphorylation was also seen with the 994 mutant. Serine 994 might therefore as well be an inhibitory serine phosphorylation site which is however also not required for the 2-deoxyglucose induced receptor inhibition. Taken together with the negative results of the ^{32}P labeling experiments the hypothesis of serine phosphorylation of the receptor as mediator of hyperglycemia and PKC dependent receptor inhibition could not be proven. Recent data suggest rather that PKC dependent serine phosphorylation occurs at IRS-1 [10]. It is however unclear how this mediates receptor inhibition.

References

[1] DeFronzo, R.A., Bonadonna, R. and Ferrannini, E. (1992) Diabetes Care 15, 318–368.
[2] Warram, J.H., Martin, B.H., Krolewski, A.S., Soeldner, J.S. and Kahn, C.R. (1990) Ann. Intern. Med. 113, 909–915.
[3] Häring, H.U. (1991) Diabetologia 34, 848–861.
[4] White, M.F. and Kahn, C.R. (1994) J. Biol. Chem. 269, 1–4.
[5] Kahn, C.R. (1994) Diabetes 43, 1066–1084.
[6] Müller, H.K., Kellerer, M., Ermel, B., Mühlhöfer, A., Obermaier-Kusser, B., Vogt, B. and Häring, H.U. (1991) Diabetes 40, 1440–1448.
[7] Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Mushack, J., Seffer, E., Seedorf, K. and Haring, H.U. (1994) J. Biol. Chem. 269, 3381–3386.
[8] Mosthaf, L., Berti, L., Kellerer, M., Mushak, J., Seffer, E., Bossenmaier, B., Coghlan, M., Siddle, K., Ullrich, A. and Häring, H.U. (1995) Eur. J. Biochem. 227, 787–791.
[9] Maegawa, H., Ide, R., Hasegawa, M., Ugi, S., Egawa, K., Iwanishi, M., Kikkawa, R., Shigeta, Y. and Kashiwagi, A. (1995) J. Biol. Chem. 270, 7724–7730.
[10] Kellerer, M., Mushack, J., Seffer, E., Mischak, H., Ullrich, S.A. and Häring, H.U. (1998) Diabetologia 41, 833–838.
[11] Pillay, T.S., Whittaker, J., Lammers, R., Ullrich, A. and Siddle, K. (1991) FEBS Lett. 288, 206–211.
[12] Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D. and Koshland, D.E. (1986) Proc. Natl. Acad. Sci. USA 83, 5822–5824.

[13] Liu, F. and Roth, R.A. (1994) Biochem. J. 298, 471–477.
[14] Liu, F. and Roth, R.A. (1994) FEBS Lett. 352, 389–392.
[15] Lewis, R.E., Wu, P.G., MacDonald, R.G. and Czech, M.P. (1990) J. Biol. Chem. 265, 947–954.
[16] Tavare, J.M., Zhang, B., Ellis, L. and Roth, R.A. (1991) J. Biol. Chem. 266, 21804–21809.
[17] Al Hasani, H., Eisermann, B., Tennagels, N., Magg, C., Passlack, W., Koenen, M., Muller-Wieland, D., Meyer, H.E. and Klein, H.W. (1997) FEBS Lett. 400, 65–70.
[18] Danielsen, A.G., Liu, F., Hosomi, Y., Shii, K. and Roth, R.A. (1995) J. Biol. Chem. 270, 21600–21605.
[19] Hubbard, S.R., Wei, L., Ellis, L. and Hendrickson, W.A. (1994) Nature 372, 746–754.
[20] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[21] Gorman, C.M., Gies, D., McGray, G. and Huang, M. (1989) Virology 171, 377–385.
[22] Chen, C. and Okayama, H. (1987) Mol. Cell Biol. 7, 2745–2752.
[23] Strack, V., Stoyanov, B., Bossenmaier, B., Mosthaf, L., Kellerer, M. and Häring, H.U. (1997) Biochem. Biophys. Res. Commun. 239, 235–239.