Insulin activation of insulin receptor kinase in erythrocytes is not altered in non-insulin-dependent diabetes and not influenced by hyperglycemia

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Abstract

Recent studies suggest that high glucose concentrations impair insulin receptor phosphorylation and kinase activation in certain cell models. To examine whether such an effect of glucose can also be demonstrated *in vivo*, insulin receptor kinase activation was studied in erythrocytes from 11 patients with non-insulin-dependent diabetes (NIDDM), before and after reduction of hyperglycemia (from 14.6 ± 1.6 to 6.6 ± 0.5 mmol/l fasting plasma glucose within 8.6 ± 0.6 days). For the measurement of receptor kinase activation, cells were incubated with insulin (0–400 nmol/l), solubilized and insulin receptors immobilized to microwells coated with anti-insulin receptor antibody. Kinase activity towards insulin receptor substrate-1 and insulin binding were then measured in these wells. Kinase activities (expressed as amol phosphate transferred per min and per fmol insulin

binding activity) were similar before (2.4 ± 0.4 and 32.2 ± 2.0 amol/min per fmol with 0 and 400 nmol/l insulin, respectively) and after improvement of metabolic control (2.4 ± 0.5 and 32.0 ± 2.3 amol/min per fmol with 0 and 400 nmol/l insulin, respectively). Moreover, activities were also similar in 22 hyperglycemic patients with NIDDM (2.1 ± 0.3 and 35.1 ± 1.4 amol/min per fmol with 0 and 400 nmol/l insulin, respectively) compared with those in 21 non-diabetic control individuals (2.1 ± 0.3 and 34.2 ± 1.2 amol/min per fmol with 0 and 400 nmol/l insulin, respectively). We conclude that insulin activation of erythrocyte insulin receptor kinase is not impaired in NIDDM and is not influenced by hyperglycemia.

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Introduction

In patients with non-insulin-dependent diabetes mellitus (NIDDM), insulin resistance is a key metabolic abnormality, and genetic factors appear to be pivotal in its development (DeFronzo *et al.* 1992). If hyperglycemia is present, this hyperglycemia by itself contributes further to the insulin resistance. This is supported by human and animal studies that have shown that hyperglycemia can cause impaired insulin action *in vitro* and insulin resistance *in vivo* (Rosetti *et al.* 1990, Yki-Jarvinen 1992). The mechanisms that lead to the insulin resistance in NIDDM and the mechanisms by which hyperglycemia causes insulin resistance are not completely understood.

The hyperglycemia-induced insulin resistance appears to be mediated, at least in part, by an increased flux through the hexosamine biotransformation pathway (Hebert *et al.* 1996, McClain *et al.* 1996) and the accumulation of metabolites of this pathway (Hawkins *et al.* 1997). The exact mechanisms by which insulin signaling or the

function of the glucose transporter effector system are impaired are, however, not clear. Studies with cultured cells with overexpressed insulin receptors have suggested that high glucose concentrations impair insulin-stimulated insulin receptor autophosphorylation and kinase activation (Berti *et al.* 1994, Maegawa *et al.* 1995, Kroder *et al.* 1996, Pillay *et al.* 1996). Such a mechanism could contribute to hyperglycemia-induced insulin resistance in humans. Moreover, because a reversal of the glucose-induced impairment of the receptor function was observed with the thiazolidinedione troglitazone in these cells, it has been speculated that the hyperglycemia-induced impairment of receptor kinase activation could represent a target for therapeutic intervention (Kellerer *et al.* 1994).

In the present study, we investigated whether such hyperglycemia-induced alterations in insulin receptor function could also be demonstrated in hyperglycemic patients with NIDDM. An assay system was established that makes it possible to determine insulin-induced insulin receptor kinase activation in intact erythrocytes from only

2 ml of heparinized blood. With this assay, insulin receptor kinase activation was measured in patients with NIDDM, before and after therapeutic reduction of hyperglycemia. Moreover, to investigate whether NIDDM might be associated with an impaired insulin-stimulated insulin receptor kinase activation in erythrocytes independent of the hyperglycemia, receptor kinase activation was also compared between patients with NIDDM and non-diabetic control individuals.

Participants and Methods

Participants

We recruited only those individuals who, on evaluation by clinical and standard laboratory examinations, had no renal, hepatic, infectious or endocrine diseases other than diabetes. Before the volunteers entered the study, the purpose and risks of the experimental procedure were carefully explained to them, and their informed consent was obtained. The procedure was approved by the local ethics Committees and was in accordance with the Helsinki II Declaration.

Erythrocyte insulin receptor kinase and binding activities

Between 0730 h and 0830 h after the participants had undergone an overnight fast and before they had taken any medication, 20 ml blood were directly drawn into a vial that contained heparin (final concentration 3.5 U/ml). Aliquots (2 ml) of the blood were then transferred to 5 ml polyethylene vials (Nunc, Wiesbaden, Germany) and preincubated for 5 min in a rotary waterbath (37 °C, 70 r.p.m.). Insulin (0–400 nmol/l final concentration) was then added and incubations continued for 15 min. The blood was then rapidly transferred to glass tubes for manual tissue grinding (Braun, Melsungen, Germany) that were filled with 1000 µl ice-cold buffer (1% Triton X-100, 2.5 mmol/l phenylmethylsulfonylfluoride, 800 trypsin inhibitor units/ml aprotinin, 8 mmol/l EDTA, 8 mg/ml bacitracin, 2.5 mg/ml benzamidin, 2.5 µg/ml pepstatin, 2.5 µg/ml leupeptin, 160 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 0.2 mmol/l sodium vanadate, 2 mmol/l dichloroacetic acid, and 20 mmol/l HEPES, pH 7.4), and the cells quickly broken by the investigator manually moving the pestle up and down 30 times. Samples were kept on ice for an additional 20 min, centrifuged for 15 min at 100 000 *g* to remove insoluble material and stored at –80 °C. Insulin receptor kinase activity with recombinant insulin receptor substrate (IRS)-1 (Upstate Biotechnology Incorporated, New York, NY) as substrate and binding activity were then measured essentially as previously described (Klein *et al.* 1999). Kinase activity was expressed as amol phosphate transferred to IRS-1 per min and per fmol insulin binding activity. Briefly, samples

were thawed, twice concentrated by vacuum centrifugation, and added for 16 h to microwells coated with monoclonal anti-insulin receptor antibody. Wells were then washed and phosphorylation reactions performed with the immobilized insulin receptors by adding a mixture containing 0.05% Triton X-100, 60 mmol/l NaCl, 1.5 mmol/l KCl, 0.6 mmol/l CaCl₂, 0.06 mmol/l sodium vanadate, 12 mmol/l HEPES, 5 mmol/l MnCl₂, 10 mmol/l MgCl₂, 500 µmol/l CTP, 0.3 µmol/l ³²P-ATP (100–200 Ci/mmol) and 2.3 µg/ml recombinant IRS-1, pH 7.4, for 120 min at 4 °C (phosphate incorporation into IRS-1 was linear with time during this period). Reactions were stopped by adding 15 µl of a solution that contained phosphatase and kinase inhibitors (final concentrations: 160 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 0.2 mmol/l sodium vanadate, 2 mmol/l dichloroacetic acid, 8 mmol/l EDTA and 20 mmol/l HEPES, pH 7.4), and the supernatant transferred to anti-phosphotyrosine antibody-coated microwells. After 16 h at 4 °C, wells were washed and the bound IRS-1 detached by adding a solution of 2% SDS for 30 min (room temperature). The radioactivity in the collected SDS solution was determined by β-counting. [¹²⁵I-Tyr-A¹⁴]Monoiodoinsulin binding to immobilized insulin receptors was measured in the wells as described previously (Klein *et al.* 1999). Non-specific insulin binding determined in the presence of 3.5 µmol/l unlabeled insulin was <0.5%. Insulin binding activity was defined as the amount of specifically bound insulin at a concentration of 8.7 nmol/l (Klein *et al.* 1995). Experiments with leukocyte filters (RC 50 KCE, Pall-Newquay, Cornwall, UK) showed that almost complete removal of the leukocytes from the blood did not alter measured binding or kinase activities expressed per erythrocyte, indicating that the contribution of the leukocytes to the measured kinase and binding activities was negligible (data not shown). Although the measurements were performed in whole blood, the results therefore reflect, almost exclusively, erythrocyte receptor binding and kinase activities.

Results

Reduction of plasma glucose and erythrocyte insulin receptor kinase activity

The effect of a therapeutic reduction in hyperglycemia was studied in 11 patients (four women, seven men) with NIDDM (ages 59 ± 4 years, body mass index (BMI) 27.7 ± 1.7 kg/m², glycated hemoglobin (HbA1c) 13.2 ± 0.3%). As shown in Fig. 1, mean daily preprandial plasma glucose concentrations (measured at 1100 h, 1700 h and 0700 h) were decreased from 17.6 ± 1 to 6.6 ± 0.4 mmol/l (*P* < 0.001, paired *t*-test) in 8.6 ± 0.6 days. Plasma insulin concentrations were 122 ± 34 and 91 ± 17 mU/ml (not significant; data not shown) when blood was drawn for first and second insulin receptor

Figure 1 Effects of the therapeutic intervention on plasma glucose concentrations. Eleven patients with NIDDM and hyperglycemia were admitted to hospital. On the first morning after admission (day 1), blood for receptor kinase measurements was drawn as described in Methods. Hyperglycemia was treated with diet alone ($n=1$), initiation of sulfonylurea ($n=2$), initiation of insulin therapy ($n=5$), or adaptation of previous insulin treatment ($n=3$). Blood for receptor kinase measurements was obtained again after 8.6 ± 0.6 days. Preprandial plasma glucose concentrations are shown for 1100 h (Δ), 1700 h ($\circ$) and 0700 h ($\square$, $\blacksquare$) on the first 3 days after admission and on the 3 days before the second kinase measurement. Data represent means $\pm$ s.e. $\blacksquare$, Time points when blood was drawn for the measurements of receptor kinase and insulin binding activities.

kinase activity measurements, respectively. The reduction in plasma glucose concentrations was not associated with an alteration of the insulin binding activity that was extracted from the erythrocytes (Fig. 2A) or an altered efficiency of 400 nmol/l insulin to stimulate erythrocyte insulin receptor kinase activity (Fig. 2B). A maximal

stimulation of the insulin receptor kinase in erythrocytes was produced by 400 nmol/l insulin (data not shown). To explore whether differences in insulin receptor kinase activation before and after the reduction in glucose concentrations might have existed at submaximal insulin concentrations, concentration–response curves were measured in eight of the 11 patients (Fig. 2C). Similar concentration–response curves were obtained, indicating that plasma glucose concentrations also had no effect on receptor kinase activation at submaximal insulin concentrations.

Figure 2 Effects of the therapeutic intervention on insulin binding and insulin receptor kinase activities in erythrocytes. Aliquots of the blood samples obtained from the 11 patients before ($\square$) or after (hatched bars) reduction of plasma glucose concentrations were incubated for 15 min with 0 or 100 nmol/l insulin (A, B) or with increasing insulin concentrations (C) as described in Methods. The cells were then solubilized, insulin receptors immunoprecipitated to anti-insulin receptor antibody-coated microwells, and insulin receptor kinase and binding activities measured. (A) Insulin binding activity: amount of insulin that bound to immobilized insulin receptors from 2×10^8 erythrocytes in the presence of 8.7 nmol/l insulin. Data represent means $\pm$ s.e. (bars) or individual data (circles); lines connect values obtained in the same individual before and after reduction in plasma glucose. (B) Insulin receptor kinase activity: insulin receptor-mediated incorporation of phosphate (P) into recombinant IRS-1 per min and per fmol insulin binding activity of the receptors. Presentation of data as in (A). (C) Concentration–response curves for insulin activation of receptor kinase: effect of the indicated insulin concentrations on insulin receptor kinase activity, expressed as percentage of the maximal insulin effect (400 nmol/l insulin). Concentration–response curves were measured only in eight of the 11 patients; data represent means $\pm$ s.e.

Figure 3 Activation of insulin receptor kinase in erythrocytes from patients with (hatched bars) or without (□) NIDDM. Blood from 22 individuals with NIDDM and 21 non-diabetic control individuals was incubated in the presence or absence of 400 nmol/l insulin. Insulin receptor kinase activity was then measured as described. Means $\pm$ S.E.

Insulin activation of insulin receptor kinase in erythrocytes from individuals with and without NIDDM

Insulin activation of insulin receptor kinase was also compared in 22 patients (12 women, 10 men) with NIDDM (ages 64 ± 3 years, BMI 27.9 ± 1 kg/m², HbA1c $9.8 \pm 0.6\%$, fasting plasma glucose (FPG) 9.8 ± 0.6 mmol/l, fasting serum insulin 84 ± 9 pmol/l) and 21 non-diabetic control individuals (nine women, 12 men; ages 63 ± 3 years, BMI 27.5 ± 1 kg/m², HbA1c $6.3 \pm 0.1\%$, FPG 4.6 ± 0.1 mmol/l, fasting serum insulin 43 ± 4 pmol/l). No differences were found between the two groups (Fig. 3). The insulin binding activity that was extracted from the erythrocytes was also not different (volunteers without diabetes: 0.79 ± 0.06 and 0.84 ± 0.07 fmol insulin binding activity per 2×10^8 erythrocytes in erythrocytes stimulated with 0 or 400 nmol/l insulin respectively; patients with diabetes: 0.77 ± 0.05 and 0.82 ± 0.06 fmol insulin binding activity per 2×10^8 erythrocytes in erythrocytes stimulated with 0 or 400 nmol/l insulin respectively).

Discussion

The molecular mechanism for hyperglycemia-induced insulin resistance is unclear. Findings in cultured cells with overexpressed insulin receptors (rat embryo fibroblasts (Berti *et al.* 1994, Kellerer *et al.* 1994, Maegawa *et al.* 1995, Pillay *et al.* 1996) or NIH3T3-cells (Kroder *et al.*

1996)) have suggested that an impaired insulin-stimulated insulin receptor kinase activation may cause or contribute to the effect of glucose on insulin sensitivity. In tissues of hyperglycemic patients with NIDDM such a mechanism could cause or worsen insulin resistance, and it has been suggested that the hyperglycemia-induced impairment of receptor kinase activation could be a target for therapeutic intervention (Kellerer *et al.* 1994). The initial goal of the present study was therefore to establish a simple assay that allows evaluation of such secondary alterations in insulin-induced receptor kinase activation in patients. With the technique described in the present report, only 20 ml of blood is required to obtain a complete concentration-response curve, and this is particularly advantageous if multiple measurements at different time points are required. Insulin activation of the receptor kinase occurs *in situ* in the intact erythrocytes which, compared with most previous studies in which receptors were insulin-stimulated in a cell-free system (Comi *et al.* 1987, Santos *et al.* 1994, Sowers *et al.* 1995), represents a more physiological situation. Moreover, this *in situ* stimulation can be performed in the complete heparinized blood, without the need of prior erythrocyte isolation. It is therefore likely that *in vivo* conditions are better maintained than, for example, in adipocytes, in which the isolation procedure takes approximately 1 h. Finally, compared with previous studies by our own group (Klein *et al.* 1995, 1997), we now used recombinant IRS-1 as a physiological substrate, instead of a synthetic polymer.

Were an effect of glucose on insulin-stimulated receptor kinase activation such as is described in cultured cells with overexpressed insulin receptors (Berti *et al.* 1994, Kellerer *et al.* 1994, Maegawa *et al.* 1995, Kroder *et al.* 1996, Pillay *et al.* 1996) also to exist in erythrocytes of hyperglycemic patients with NIDDM, a reduction in pathologically increased plasma glucose concentrations should result in a reversal of this effect. Therapeutic reduction of hyperglycemia in patients with NIDDM did not, however, result in a detectable alteration in the effect of insulin on insulin receptor kinase activation in their erythrocytes. Moreover, erythrocyte insulin receptor kinase activation was not different between hyperglycemic patients with NIDDM and euglycemic non-diabetic control individuals. These data suggest that the effects of glucose on receptor kinase activation observed in cultured cell models may not be present under '*in vivo*' conditions, at least not in the presence of mean preprandial serum glucose concentrations less than 18 mmol/l and/or not in all tissues.

A potential explanation for the observation of an effect of glucose in cultured cell systems, but not in our study, could be that glucose concentrations (25 or 27 mmol/l) used in the studies with the cultured cells (Berti *et al.* 1994, Kellerer *et al.* 1994, Maegawa *et al.* 1995, Kroder *et al.* 1996, Pillay *et al.* 1996) were greater than the initial mean daily preprandial serum glucose concentrations in the hyperglycemic patients in our study

(17.6 ± 1.0 mmol/l). Only in one of these studies (Pillay *et al.* 1996) were measurements also performed in the presence of lower glucose concentrations, and no effects of glucose on receptor kinase activation were apparent with concentrations of 20 mmol/l or less. To explore the possibility that greater glucose concentrations than those reached in our *in vivo* study might affect insulin receptor kinase activation in erythrocytes, we incubated heparinized blood *in vitro* for 3 h at 37 °C with 27 or 4 mmol/l glucose. No glucose-induced impairment of insulin-stimulated receptor kinase was observed (data not shown), suggesting either that the exposure to glucose was too short, or that insulin receptor kinase activity, in contrast to the cultured cell systems mentioned above, is not responsive to glucose.

There are several lines of evidence that the effects of glucose on the insulin receptors described in the cultured cell systems are mediated, at least in part, by protein kinase C (PKC). High glucose concentrations have been shown to lead to PKC activation (Lee *et al.* 1989) and the translocation of certain PKC isoforms (e.g. PKC α (Berti *et al.* 1994)), potentially by mechanisms related (Filippis *et al.* 1997) or not related (Laybutt *et al.* 1999) to an increased flux through the hexosamine biosynthetic pathway. This PKC activation and/or translocation may result in an increased serine phosphorylation of the insulin receptor and/or IRS-1 which, in turn, has been linked to impaired receptor kinase activation and downstream signaling (Bollag *et al.* 1986, Takayama *et al.* 1988, Hotamisligil *et al.* 1996). Erythrocytes contain PKC that can be stimulated by phorbol-myristate 13-acetate, and it has also been shown that the PKC α isoform can be activated and translocated to the plasma membrane in erythrocytes (Fathallah *et al.* 1997). Another proposed mechanism for the glucose-mediated reduction in insulin receptor kinase activation in rat embryo fibroblasts with overexpressed insulin receptors involves alterations in phosphotyrosine phosphatase activity (Maegawa *et al.* 1995). Phosphotyrosine phosphatase-1B (PTP-1B) appears to have a major role in the dephosphorylation of the insulin receptor and IRS-1 (Goldstein *et al.* 2000), and erythrocytes contain a phosphotyrosine phosphatase that immunoprecipitates with monoclonal antibodies against this phosphatase (Zipser & Kosower 1996), and therefore most probably represents PTP-1B. It is nevertheless possible that erythrocytes lack certain components that are present in the cultured cell systems and that are necessary for the effect of glucose on receptor kinase activity or its reversal.

As the cultured rat embryo fibroblasts or NIH3T3-cells with overexpressed receptors (Berti *et al.* 1994, Kellerer *et al.* 1994, Maegawa *et al.* 1995, Kroder *et al.* 1996, Pillay *et al.* 1996) also do not represent typical insulin target tissues, it can also be questioned whether such components are operative in more relevant tissues such as adipocytes, liver or skeletal muscle. In fact, no effects of glucose on

insulin-induced *in situ* insulin receptor autophosphorylation or receptor kinase activity were observed in cultured rat adipocytes (Lima *et al.* 1991). Likewise, no improvement of the effect of insulin on receptor autophosphorylation was observed in liver insulin receptors from hyperglycemic rats when hyperglycemia was reduced from 27 to 6 mmol/l by phlorizin treatment (Considine *et al.* 1995), and in a recent study a hyperglycemia-induced reduction of protein kinase B (PKB) but not phosphatidylinositol 3-kinase, activity was observed in rat skeletal muscle – suggesting that the preceding steps of insulin signaling such as receptor kinase activation were not altered (Kurowski *et al.* 1999). In contrast to the findings of that study, Kim *et al.* (1999) and Patti *et al.* (1999) found normal PKB activation and reduced phosphatidylinositol 3'-kinase activation in rat skeletal muscle when they stimulated the hexosamine pathway with glucosamine instead of glucose. With respect to our data it is, however, important to note that insulin-stimulated insulin receptor phosphorylation was not affected in either of the above studies, despite the glucosamine-induced reduction in insulin-stimulated phosphatidylinositol 3'-kinase activity. Our finding that hyperglycemia did not affect insulin-stimulated insulin receptor kinase activation in human erythrocytes is therefore consistent with previous studies in more relevant target tissues in animals.

Our present observation that insulin receptor kinase activation is not impaired in erythrocytes from patients with NIDDM compared with those from non-diabetic control individuals is in contrast to the findings of studies in which reduced insulin receptor autophosphorylation and/or kinase activity was measured after insulin stimulation of erythrocyte receptors in a cell-free system (Comi *et al.* 1987, Santos *et al.* 1994). It is, however, consistent with data reported by Hagino *et al.* (1994) who, similarly to our study procedure, stimulated the intact erythrocytes with insulin, and found no difference in receptor autophosphorylation between patients with NIDDM and non-diabetic controls. Our finding that *in situ* insulin activation of receptor kinase was not different in erythrocytes from those with and without NIDDM is also consistent with data on human skeletal muscle obtained by our group (Klein *et al.* 1995) and others (Krook *et al.* 1999).

In summary, our data indicate that mean daily preprandial glucose concentrations of approximately 18 mmol/l do not impair insulin receptor kinase activation in erythrocytes from patients with NIDDM. The assay described can therefore not be used to monitor hyperglycemia-induced changes in insulin signaling and insulin sensitivity in patients. Moreover, our data in the human erythrocyte model confirm the findings of other studies in animal models that suggest that the hyperglycemia-induced insulin resistance appears to be mainly caused by mechanisms affecting insulin signaling steps downstream of the insulin receptor kinase. To our

knowledge, no similar study in patients has so far been performed with other, more relevant tissues.

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