Delays in insulin signaling towards glucose disposal in human skeletal muscle

T Grimmsmann, K Levin¹, M M Meyer, H Beck-Nielsen¹
and H H Klein

Medizinische Klinik 1, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany

¹Diabetes Research Center, Odense University Hospital, DK-5000, Denmark

(Requests for offprints should be addressed to H H Klein; Email: klein@medinf.mu-luebeck.de)

Abstract

We explored whether the delay that occurs between a rise in plasma insulin and the increase of glucose disposal occurs before, at, or downstream of steps that are believed to be part of the insulin signaling cascade. Skeletal muscle biopsies were obtained from 16 nondiabetic subjects before, and 20 and 180 min after plasma insulin levels had been augmented in euglycemic hyperinsulinemic glucose clamps. Although plasma insulin had reached 98% of its final concentration within 10 min, insulin receptor kinase (IRK) activity, p85 associated with insulin receptor substrate-1 (IRS-1), IRS-1-associated

phosphatidylinositol 3-kinase (PI3K) activity, and Thr³⁰⁸-protein kinase B (PKB) phosphorylation in the muscle biopsies at 20 min had reached only 60, 48, 34 and 47% respectively of those at 180 min. This suggests a delay before the level of IRK and little or no delay between IRK and PKB activation. The observation that glycogen synthase activity and glucose disposal at 20 min had both only reached 25% of the respective values at 180 min suggests an additional delay downstream of the investigated signaling steps.

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Introduction

When plasma insulin concentration is increased in euglycemic hyperinsulinemic glucose clamps, the rate of activation of glucose disposal is substantially slower than the rise in plasma insulin (Prager *et al.* 1986, Freidenberg *et al.* 1994). There is evidence that this delay is more pronounced in type 2 diabetic and obese subjects compared with lean subjects (Nolan *et al.* 1994), and it may therefore be of clinical importance. At least a part of the delay appears to be due to the time needed for the transport of insulin across the endothelial barrier. Endothelial cells possess insulin receptors that bind the insulin and transport it unidirectionally into the interstitial space (Jialal *et al.* 1984, King & Johnson 1985). This process is relatively slow, and is mirrored by a delayed increase in lymph insulin concentration measured in the thoracic duct (Rasio *et al.* 1967), by lymphatic cannulation (Castillo *et al.* 1994) or microdialysis (Jansson *et al.* 1993). Moreover, activation of insulin receptor kinase (IRK) has been described to be substantially slower than the rise in plasma insulin (Freidenberg *et al.* 1994).

In the present study we investigated whether this proposed delay between plasma and lymph insulin is also reflected in a slower activation of the insulin signaling cascade, and whether additional delays occur within the

known insulin signaling cascade (Taha & Klip 1999) or downstream of these known signaling steps. Euglycemic hyperinsulinemic glucose clamps were performed in 16 nondiabetic subjects and muscle biopsies obtained before, and 20 and 180 min after the start of the insulin infusions. These biopsies were analyzed for IRK activity, association of p85-phosphatidylinositol 3-kinase subunit with insulin receptor substrate-1 (IRS-1), IRS-1-associated phosphatidylinositol 3-kinase (PI3K) activity, Thr³⁰⁸-phosphorylation of protein kinase B (PKB), and glycogen synthase activity.

Materials and Methods

Human subjects

Muscle biopsies from 16 Caucasian volunteers (for subject characteristics, see Table 1) were analyzed. None of the subjects suffered from diabetes as evaluated by an oral glucose tolerance test or any other disease as evaluated by clinical investigation and standard laboratory assessments, with the exception that two of the subjects continued to receive antihypertensive medication throughout the study. Prior to entry into the study, the purpose and risks of the experimental protocol were explained carefully to all of the volunteers and informed written consent was

Table 1 Subject characteristics: laboratory data were analysed in blood drawn under fasting conditions in the morning 2 days before the clamp. Data represent means $\pm$ S.E.M.

<i>n</i> (male/female)	16 (15/1)
Age (years)	48.6 $\pm$ 3.4
BMI (kg/m ²)	30.8 $\pm$ 0.8
Fat-free mass (kg)	59.9 $\pm$ 2.3
Fat mass (kg)	38.0 $\pm$ 2.7
Fasting insulin (pM)	46.4 $\pm$ 6.7
Fasting glucose (mM)	5.6 $\pm$ 0.1
Free fatty acids (μ M)	377.0 $\pm$ 40
Triglycerides (mM)	1.4 $\pm$ 0.2
Cholesterol (mM)	6.0 $\pm$ 0.2
HDL (mM)	1.3 $\pm$ 0.1
LDL (mM)	4.1 $\pm$ 0.2

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

obtained. The protocol was approved by the local ethical committee and was in accordance with the Helsinki II Declaration.

Euglycemic hyperinsulinemic glucose clamps

Subjects were admitted to the clinical research center at 0800 h and intravenous catheters inserted for infusion of insulin and glucose into the antecubital vein. Blood samples for plasma glucose analyses were drawn from a catheter of the dorsal wrist vein of the opposite arm. During the study, the subjects remained in a supine position with the hand designated for collection of blood samples placed and maintained in a heated Plexiglas box for arterialization of venous blood (McGuire *et al.* 1976). After a 30-min relaxation period, at time zero, a surface-adjusted priming dose of 3-³H glucose was given (14 μ Ci/m²; DuPont-New England Nuclear, Boston, MA, USA) followed by a continuous infusion of 3-³H glucose (0.14 μ Ci/min per m²), which was continued throughout the study periods. After a 120-min basal tracer equilibration period, insulin (Actrapid, Novo-Nordisk, Bagsvaerd, Denmark) was infused at a rate of 40 mU/m² per min for 180 min and euglycemia (5.9 $\pm$ 0.1 mM) maintained using a variable infusion of 18% glucose. In order to keep plasma specific activity constant at the basal level during the clamp, 3-³H glucose was added to the glucose infusate (HOT-GINF) as previously described (Hother-Nielsen *et al.* 1995). The glucose disposal rate was calculated from the plasma concentrations of tritiated glucose and plasma glucose using Steele's non-steady-state equations (Steele 1959). In these calculations, the distribution volume of glucose was taken as 200 ml/kg body weight and the pool fraction as 0.65. Body composition was estimated with the bioimpedance method using the formula of Kushner (Kushner & Schoeller 1986). Plasma

glucose was measured at bedside on a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA, USA) by the glucose oxidase method. Plasma insulin blood samples were immediately centrifuged at 4 °C and stored at -20 °C for later analysis by two-site time-resolved immunofluorimetric assay. Triglyceride was measured by an enzymatic colorimetric method, as was free fatty acid (Wako Chemicals, Neuss, Germany).

Muscle biopsies

Percutaneous *M. vastus lateralis* biopsies were taken under local anesthesia (2% lidocaine without epinephrine) 20 cm above the knee using the Bergstrom method (Bergstrom 1975) at the end of the basal period (*t*₀), after 20 min (*t*₂₀), and again 3 h (*t*₁₈₀) after the beginning of the insulin infusion. Biopsies were immediately frozen and for subsequent analytical procedures approximately 50 μ g frozen tissue was homogenized at 4 °C in 500 μ l solubilization buffer (8 mM EDTA, 20 mM Hepes, 1% IGEPAL, 530 mM NaF, 30 mM sodium pyrophosphate, 7 mM sodium vanadate, 2 mM dichloroacetic acid, 9 mM PMSF, 21 mM benzamidine, 5 μ M leupeptin, 4 μ M pepstatin, and 10 μ g/ μ l aprotinin, pH 7.4). After 20 min, detergent-insoluble material was removed by centrifugation (12 000 g, 20 min, 4 °C) and the protein concentration measured using protein-dye binding assay (BioRad, Munich, Germany).

IRK and binding activities

These were measured essentially as published (Klein *et al.* 1993, Krützfeldt *et al.* 2000). Muscle sample lysates (300 μ g protein) were added to microwells coated with anti-insulin receptor antibody (ATCC, Rockville, MD, USA) for 16 h at 4 °C. The wells were washed and receptor-mediated ³²P incorporation into recombinant IRS-1 (17 nM; Upstate Biotechnology Inc., New York, NY, USA) measured at 120 nM ³²P-ATP. [¹²⁵I-Tyr-A¹⁴]-Monoiodoinsulin (Amersham-Pharmacia, Freiburg, Germany) binding to immobilized insulin receptors was also measured in the wells as described (Klein *et al.* 1993). Insulin binding capacity was defined as the amount of specifically bound insulin at a concentration of 8.7 nM (Klein *et al.* 1995). IRK activity was expressed as amol phosphate transferred into IRS-1 per min per fmol insulin binding capacity.

PI3K activity

Solubilized muscle (260 μ g protein) was added to microwells coated with anti-IRS-1 antibody (Upstate Biotechnology Inc.) that had been prepared as described (Krützfeldt *et al.* 2000). After 16 h at 4 °C, wells were washed and 10 μ g phosphatidylinositol (Sigma, Deisenhofen, Germany) in 30 μ l 10 mM Tris, 100 mM

NaCl, 1 mM EDTA, and 15 mM MgCl₂, pH 7.5 added. The phosphorylation reaction was started by the addition of 5 µl of a solution that contained 720 µM ³²P-ATP (12.5 µCi/mmol; NEN, Dreieich, Germany). After 1 h at room temperature the reaction was stopped by the addition of 20 µl 25% HCl and 95 µl of methanol-chloroform (1:1). The organic phase was extracted and applied to a silica gel thin-layer chromatography plate. Phosphatidylinositol-3 phosphorylation was then determined using a phosphoimager.

Glycogen synthase activity

Extraction of muscle samples and assays for glycogen synthase were performed as previously described (Vaag *et al.* 1992). Briefly, glycogen synthase activity was assayed in the presence of a near physiological concentration of glucose-6-phosphate (G6P; 0.1 mM) and in the presence of 10 mM G6P to determine maximal enzyme activity. The substrate concentration of uridine diphosphate glucose (UDPG) was 0.31 mM and glycogen synthase activity was expressed as nmol of UDPG incorporated into glycogen per min per µg extract protein. Fractional velocities (FV) were calculated as the ratio between glycogen synthase activities assayed at 0.1 mM and 10 mM G6P (FV 0.1).

Immunoblots

For the determination of the amount of IRS-1 and IRS-1-associated p85, muscle sample lysates (620 µg protein) were incubated with anti-IRS-1 C-terminal antibody (2 µg; Upstate Biotechnology Inc.) coupled to protein G-agarose (Pierce, Rockford, USA). The agarose was washed, and bound proteins separated by SDS-PAGE. In order to express the IRS-1-associated amount of p85 as percentage of its total amount, complete muscle lysates (25 µg protein) were separated on the same gels. Proteins were transferred to PVDF membranes or nitrocellulose (Millipore, Bedford, MA, USA) for IRS-1 or p85 blots respectively. Membranes were blocked overnight with PBS that contained 5% skim milk. Proteins were then detected with anti-IRS-1 or anti-p85 antibodies (both Upstate Biotechnology Inc.). PKB phosphorylation was detected by immunoblotting of muscle sample lysates (155 µg protein) with an antibody against phosphorylated Thr³⁰⁸-PKB (BioLabs, Beverly, MT, USA).

Data analysis

Arbitrary units (AU) represent the ratio between the densitometric value measured at the respective time point (t_0 , t_{20} , t_{180}) and the mean of the densitometric values measured at all three time points of the same experiment. Statistical comparisons were carried out by one-way repeated measurements ANOVA and the

Figure 1 Insulin levels and glucose disposal rates (GDR). Euglycemic hyperinsulinemic clamps were performed in 16 obese volunteers. Shown are insulin levels ● and glucose disposal rates ▲. Results represent means $\pm$ S.E.M.

Student–Newman–Keul *post hoc* test. Differences between the time points were considered as statistically significant if P was <0.05 .

Results

After the start of the insulin infusion, plasma insulin levels increased rapidly and reached 99% of the value at t_{180} within 10 min (Fig. 1). In contrast, the increase in glucose disposal was slow, and at t_{20} only 25% of the increase at t_{180} had been reached (Fig. 1). To explore where this delay between plasma insulin levels and stimulation of glucose disposal occurs, the activation or phosphorylation of downstream signaling proteins was investigated (Fig. 2). IRK activity increased 4.2-fold within 180 min after the insulin infusion had been started. Compared with the quick increase of the plasma insulin concentration, IRK activation was slower. After 20 min the increase in IRK activity had reached only 60% of the increase after 180 min ($P < 0.05$). Our data therefore show that a delay occurs between the increase in plasma insulin levels and IRK activation. On the other hand, the increase of IRK activity within the first 20 min was more pronounced ($\sim 60\%$) than the increase in glucose disposal ($\sim 25\%$) in that time period. This indicates that a delay occurred not only between the increase in plasma insulin and IRK activation but also between IRK activation and the increase in glucose disposal.

For insulin-induced association of p85 with IRS-1, PI3K activation, and Thr³⁰⁸ phosphorylation of PKB 48, 34 or 47% respectively of the increases at t_{180} had been reached at t_{20} (Fig. 2B–D). This indicates that, if at all, only slight delays occurred between IRK activation and these downstream signaling steps, and suggests that a major part of the delay observed between IRK activation and the increase in glucose disposal occurs downstream of the investigated signaling steps.

Figure 2 IRK activity, p85 associated with IRS-1, PI3K activity, Thr³⁰⁸-phosphorylation of PKB and glycogen synthase activity. Muscle biopsies obtained at the indicated times after the initiation of the insulin infusion were solubilized, and parameters measured as described. (A) IRK activity determined as amol P transferred per min and per fmol insulin binding activity; (B) amount of p85 precipitated with anti-IRS-1 antibody as a percentage of the total p85; (C) IRS-1-associated PI3K activity (AU = arbitrary units); (D) Thr³⁰⁸-phosphorylation of PKB; (E) glycogen synthase activity expressed as fractional velocity (FV 0.1) as described. Data represent means $\pm$ s.e.m., $n=12-16$. * $P<0.05$ compared with corresponding basal; +, $P<0.05$ compared with corresponding 20-min value.

Glycogen synthase is an important enzyme involved in non-oxidative glucose metabolism and, similar to the activation of glucose uptake, its activation is thought to be mediated, at least in part, by the PI3K/PKB pathway. At t_{20} only 25% of the increase in glycogen synthase activity at t_{180} was reached (Fig. 2E), which was analogous to the

findings for glucose disposal (Fig. 1). This indicates that activation of glycogen synthase was similarly delayed compared with the investigated signaling parameters as was glucose disposal.

Insulin stimulation did not alter the insulin-binding capacity, or the amounts of IRS-1 and p85, that were

extracted per μg muscle protein. It also did not affect glycogen synthase activity at 10 mM G6P, indicating that the amount of the enzyme did not change (data not shown).

Discussion

The stimulation of glucose disposal in response to an increase of the plasma insulin level has been found to be substantially delayed in previous studies (Prager *et al.* 1986, Freidenberg *et al.* 1994) and the present study, and it has been proposed that differences in this delay could play a role in the pathogenesis of type 2 diabetes (Ciaraldi *et al.* 1991, Nolan *et al.* 1994). Here we describe that a part of this delay occurs already between the increase in plasma insulin and the activation of IRK. This delay is most likely almost exclusively due to the time needed for the trans-capillary transport of insulin since in isolated adipocytes (Klein *et al.* 1986) or cultured human muscle cells (Krützfeldt *et al.* 2000) insulin-stimulated IRK activation occurs within seconds and almost parallels receptor occupancy. The finding of a delayed IRK activation is therefore consistent with previous studies that showed delayed increases in lymphatic insulin concentration compared with plasma insulin concentration (Rasio *et al.* 1967, Yang *et al.* 1989, Jansson *et al.* 1993, Castillo *et al.* 1994). It is also consistent with previous data where the time course of IRK activation was measured in human skeletal muscle (Freidenberg *et al.* 1994, Wojtaszewski *et al.* 1997, 2000). Compared with our study, Freidenberg *et al.* (1994) observed sufficient IRK activation only at plasma insulin concentrations >2000 pM, and delays observed at such high insulin concentrations may not reflect the physiological *in vivo* situation. Wojtaszewski *et al.* (1997, 2000) also found that IRK activity tended to increase further after steady-state insulin levels had been reached; this was, however, not significant. Our present study is therefore consistent with these previous studies and adds the information that IRK activation at insulin levels within, or close to, the physiological range is in fact significantly slower than the rise in plasma insulin levels.

Although IRK activation was delayed compared with the rise in plasma insulin levels, its activation was substantially more rapid than the stimulation of glucose disposal and the activation of glycogen synthase, suggesting that further delays occur downstream of the IRK. Association of p85 with IRS-1, IRS-1-associated PI3K activity, and Thr³⁰⁸-phosphorylation of PKB were, however, if at all, only slightly delayed compared with the activation of IRK, which suggests that the major delay between IRK activation and the effects of insulin on glucose uptake and glycogen synthesis occurs downstream from PI3K and/or PKB. The notion that a slight delay might occur between IRK and PI3K would be consistent with findings in human muscle strips where IRK activity tended to reach

maximal levels slightly earlier than IRS-1-associated PI3K activity (Goodyear *et al.* 1995, Krook *et al.* 2000). With respect to the temporal relationship between PI3K activation and PKB phosphorylation in skeletal muscle, data in the literature are fairly contradictory. In isolated rat soleus muscle, PI3K activity was reached within 3 min whereas PKB phosphorylation needed 20 min to reach maximal levels (Song *et al.* 1999). A slight delay between PI3K activation and PKB phosphorylation was also found in cultured human muscle cells (Krützfeldt *et al.* 2000); the approximate 2-min delay may, however, not be detectable in an *in vivo* situation. In contrast, studies in isolated human skeletal muscle strips have revealed a similar or even slightly faster PKB phosphorylation (maximal after ≈ 8 min (Zierath *et al.* 2000)) than PI3K activation (maximal after 8–15 min (Krook *et al.* 2000)). Finally, in an investigation with skeletal muscle biopsies that were obtained during hyperinsulinemic euglycemic glucose clamps as in our study, a substantially more rapid Ser⁴⁷³-PKB phosphorylation and glycogen synthase activation (Wojtaszewski *et al.* 2000) than PI3K activation was found (Wojtaszewski *et al.* 1997). Wojtaszewski *et al.* concluded from their data that a limited activation of PI3K may be sufficient to maximally stimulate PKB or that mechanisms alternative to PI3K may regulate insulin stimulation of PKB. Such hypotheses are not required for the interpretation of our data that, most consistent with the data obtained with the isolated human skeletal muscle strips, show an almost parallel activation of PI3K and phosphorylation of PKB. With respect to the discrepancy between Wojtaszewski *et al.*'s data and ours it is, however, possible that, due to a different study design, insulin-stimulated PI3K activity levels were higher in their study and therefore, in contrast to our study, exceeded a level required for maximal PKB phosphorylation. In this context, it may also be of importance that our subjects were more obese than those studied by Wojtaszewski *et al.* In any case, our data indicate that, even if a slight delay in insulin signaling had occurred between IRK and PI3K or PKB, this would not account for the delay that we observed between IRK activation and the stimulation of glucose disposal and glycogen synthase. Our data therefore suggest that the major delay between IRK activation and the insulin effects on glucose uptake and glycogen synthesis occurs downstream from PI3K and/or PKB. Moreover, the fact that the increase in glucose disposal and the covalent activation of glycogen synthase, as measured in our study, were similarly delayed suggests that downstream signaling towards these parameters might involve common signaling steps that are responsible for both delays.

At first sight, the concept that an additional delay occurs downstream of PI3K and PKB activation appears to be in partial contrast to data by Freidenberg *et al.* (1994) and Nolan *et al.* (1994), who showed comparable or even faster increases of glucose disposal compared with IRK

activation. It must be considered, however, that plasma insulin concentrations during the clamps were considerably higher (≈ 1800 pM) in these studies than in our study (≈ 400 pM). It is likely that at high insulin concentrations a delay that occurs between plasma insulin levels and IRK activation does not cause a similar delay in the activation of glucose disposal. This is because submaximal IRK activities already maximally stimulate glucose disposal ('spare kinase' (Freidenberg *et al.* 1991)), and therefore the level of kinase activity needed to activate glucose disposal is reached more quickly at higher insulin concentrations (Freidenberg *et al.* 1994). Consistent with this view, activation of glucose disposal, expressed as percentage of its maximal response, occurs more rapidly at higher insulin concentrations (Prager *et al.* 1986), and glucose disposal was activated even faster than IRK in the study by Freidenberg *et al.* (1994). Taking these findings together, it is therefore conceivable that at the high insulin concentrations used in the studies by Freidenberg *et al.* (1994) and Nolan *et al.* (1994) the delay between plasma insulin levels and IRK activation contributed only little to the overall delay between plasma insulin levels and stimulation of glucose disposal, and that this latter delay was in fact caused predominantly by delays downstream of IRK activation.

The notion that there is a delay between IRK activation and stimulation of glucose disposal in human skeletal muscle is also consistent with findings in isolated human adipocytes. In these cells, complete activation of the insulin receptor occurred within seconds (Brillon *et al.* 1988), but half-maximal glucose uptake was only reached after 9 or 15 min in adipocytes from lean or obese subjects respectively (Molina *et al.* 1989, Ciaraldi *et al.* 1991). Moreover, since an even more pronounced delay was observed in isolated adipocytes from type 2 diabetic subjects (half-maximal effect at 23 min), a pathophysiological role for this delay has been suggested (Ciaraldi *et al.* 1991).

In summary, our data suggest that the delay between the increase in plasma insulin and stimulation of glucose disposal is caused by the time needed for insulin to reach the interstitial space, and an additional delay downstream of IRK activation. Little or nothing of this delay appears to occur between the activation of IRK and the activation of PI3K or Thr³⁰⁸-phosphorylation of PKB. The fact that the increases in glucose disposal and the covalent activation of glycogen synthase were similarly delayed compared with the measured insulin signaling parameters may suggest that these delays occur at shared downstream signaling steps.

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