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Glucose-induced insulin resistance of skeletal-muscle glucose transport and uptake

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The ability of glucose and insulin to modify insulin-stimulated glucose transport and uptake was investigated in perfused skeletal muscle. Here we report that perfusion of isolated rat hindlimbs for 5 h with 12 mM-glucose and 20000 μunits of insulin/ml leads to marked, rapidly developing, impairment of insulin action on muscle glucose transport and uptake. Thus maximal insulin-stimulated glucose uptake at 12 mM-glucose decreased from 34.8 ± 1.9 to 11.5 ± 1.1 μmol/h per g (mean ± S.E.M., $n = 10$) during 5 h perfusion. This decrease in glucose uptake was accompanied by a similar change in muscle glucose transport as measured by uptake of 3-*O*-[^{14}C]-methylglucose. Simultaneously, muscle glycogen stores increased to 2–3.5 times initial values, depending on fibre type. Perfusion for 5 h in the presence of glucose but in the absence of insulin decreased subsequent insulin action on glucose uptake by 80 % of the effect of glucose with insulin, but without an increase in muscle glycogen concentration. Perfusion for 5 h with insulin but without glucose, and with subsequent addition of glucose back to the perfusate, revealed glucose uptake and transport similar to initial values obtained in the presence of glucose and insulin. The data indicate that exposure to a moderately increased glucose concentration (12 mM) leads to rapidly developing resistance of skeletal-muscle glucose transport and uptake to maximal insulin stimulation. The effect of glucose is enhanced by simultaneous insulin exposure, whereas exposure for 5 h to insulin itself does not cause measurable resistance to maximal insulin stimulation.

INTRODUCTION

Hyperinsulinaemia and insulin resistance are common features of obesity (Kolterman *et al.*, 1980) and of some patients with type II (non-insulin-dependent) diabetes who also experience hyperglycaemia (Wajngot *et al.*, 1982). Since the major part of a glucose challenge is thought to be taken up by skeletal muscle (DeFronzo *et al.*, 1985; James *et al.*, 1985*b*), this tissue is usually considered the most important in determining whole-body insulin sensitivity. Indeed, insulin resistance of skeletal muscle in patients with type II diabetes has been reported (DeFronzo *et al.*, 1985). However, whether hyperinsulinaemia is a cause rather than an effect of insulin resistance in skeletal muscle remains unclear (Czech *et al.*, 1978; Rizza *et al.*, 1985; Wardzala *et al.*, 1985; Young *et al.*, 1986). For instance, after 2 weeks chronic hyperinsulinaemia *in vivo*, insulin sensitivity of incubated rat muscle was slightly increased (Wardzala *et al.*, 1985), and incubation of muscle for 5 h with submaximal insulin concentrations has been reported to increase insulin action (Young *et al.*, 1986). On the other hand, it has been reported that in the rat *in vivo* (Rossetti *et al.*, 1987), and in type I diabetes (Yki-Järvinen *et al.*, 1987) hyperglycaemia itself may cause insulin resistance on the whole-body level, possibly reflecting insulin resistance of skeletal muscle.

The present experiments were carried out to elucidate whether skeletal-muscle membrane transport and uptake of glucose develop insulin resistance by exposure to high concentrations of insulin and/or glucose. Isolated rat hindlimbs were perfused for up to 5 h with a perfusate containing either insulin (20000 units/ml) plus glucose (11–13 mM), glucose alone or insulin alone, and maximally insulin-stimulated glucose transport and uptake was then measured. The data indicate that a modestly increased glucose concentration (11–13 mM) rapidly causes development of resistance of glucose transport and uptake to maximally stimulating insulin concentrations in muscle. The effect is enhanced by simultaneous exposure to insulin, but is not caused by insulin itself.

MATERIALS AND METHODS

Hindquarters of 200–220 g fed male Wistar rats were surgically prepared for perfusion as described by Ruderman *et al.* (1971). The initial perfusate (300 ml) consisted of Krebs–Henseleit solution, 5 % (w/v) bovine albumin (Cohn fraction V; Sigma, St. Louis, MO, U.S.A.) that had been dialysed twice against 10 vol. of Krebs–Henseleit solution, 1–2-day-old washed bovine erythrocytes at a haematocrit of 30 %, approx. 1 mM-lactate originating from the erythrocytes, and 0.15 mM-pyruvate. The perfusate was gassed with O_2/CO_2 (49:1), giving pO_2 about 400 mmHg, pCO_2 about 40 mmHg and pH 7.4–7.3, measured with a Radiometer ABL 30 acid–base laboratory instrument (Radiometer, Copenhagen, Denmark). The first 25 ml of perfusate that passed through the hindquarter was discarded, whereupon the perfusate was recirculated at a flow rate of 12.5 ml/min. The hindquarter was pre-perfused for 20 min, whereupon the actual experimental period began. Three perfusion protocols were employed. (1) Glucose (15 mM) and pig insulin (kindly given by NOVO Industries, Copenhagen,

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Denmark; 20000 μunits/ml) were present in the initial perfusate. After the pre-perfusion period (20 min), perfusate glucose concentration was 13–13.5 mM. Hindquarters were perfused for 0 or 5 h after the pre-perfusion period. Arterial and venous perfusate glucose was measured after 1 h with a YSI 23A rapid glucose analyser (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). At 15 min into the next hour of perfusion, glucose was added to the perfusate to restore the arterial glucose concentration to 13 mM. In this way, arterial glucose concentrations were kept within approx. 13–11 mM, occasionally reaching 10 mM. To ensure the presence of maximum insulin concentrations throughout the perfusion period, insulin in an amount sufficient to raise perfusate concentrations by 10000 μunits/ml was added to the perfusate after 3 h of perfusion. (2) Glucose at 14 mM but no insulin was present in the initial perfusate. After the pre-perfusion period, perfusate glucose concentration was 13–13.5 mM. As in protocol (1), perfusate glucose was kept between 13 and 11 mM by adding glucose to the perfusate every hour. After perfusion for 4 h 40 min insulin sufficient to give a perfusate concentration of 20000 μunits/ml was added, and the perfusion was terminated 20 min later. (3) No glucose, but insulin at 20000 μunits/ml, was present in the initial perfusate and for 4 h 40 min after the pre-perfusion period. Glucose was then added in an amount sufficient to raise perfusate glucose concentrations to 15 mM. Thus 20 min later, when the perfusions were terminated, perfusate glucose concentrations were 13–13.5 mM.

At the end of the perfusion, muscle samples were obtained. The superficial part of the gastrocnemius muscle, which consists mainly of fast-twitch white fibres (Armstrong & Phelps, 1984), was freeze-clamped *in situ*. Then the soleus muscle, which consists mainly of slow-twitch red fibres (Armstrong & Phelps, 1984), was reflected and clamped. Finally, a portion of the deep part of the medial head of the gastrocnemius, consisting mainly of fast-twitch red fibres (Armstrong & Phelps, 1984), was cut out and clamped. Muscles were stored at −80 °C until analysed.

For measurement of muscle membrane glucose transport, 10–20 μCi of 3-*O*-[U-^{14}C]methyl-D-glucose (New England Nuclear, Boston, MA, U.S.A.), along with 20 μCi of [^{3}H]mannitol (New England Nuclear) and unlabelled mannitol to give a perfusate concentration of 1 mM, was added so that the hindquarter was exposed to the radioisotopes for 7 min. Uptake of 3-*O*-methylglucose in individual muscles was determined in $HClO_4$ extracts and corrected for label in the extracellular space determined by the ^{3}H radioactivity from mannitol. To calculate glucose transport, it was assumed that 3-*O*-[^{14}C]methylglucose is transported across the cell membrane at the same velocity as glucose, and consequently the 'specific radioactivity' of 3-*O*-methylglucose was calculated as:

$$\frac{^{14}\text{C c.p.m./ml of cell-free perfusate}}{\text{[glucose] in cell-free perfusate}}$$

We have previously shown that uptake of 3-*O*-methylglucose is linear at least up to an intracellular water concentration of 30% of the extracellular water concentration (Richter *et al.*, 1985). This limit was not exceeded in the present experiments. Concentrations of ATP and phosphocreatine were determined by standard enzymic methods (Lowry & Passonneau, 1972). Muscle mass of the hindquarter was estimated to be one-sixth of body weight (Ruderman *et al.*, 1971). Glucose uptake was calculated as arteriovenous differences multiplied by the flow. Differences between groups were statistically analysed by the unpaired *t* test. Differences were considered significant if P values < 0.05 were obtained.

RESULTS

Exposure of isolated perfused rat hindlimbs to a supramaximal insulin concentration and perfusate glucose concentrations at 11–13 mM caused a gradual, marked and significant decrease in glucose uptake over the 5 h perfusion period (Table 1). After 5 h perfusion, glucose uptake was still approx. 2.5–3 times higher than insulin unstimulated uptake (Table 1), but only one-third of the 0 h value (Table 1). Simultaneously, muscle glycogen concentrations increased to roughly 2, 3 and 3.5 times initial values in muscles representing fast-twitch white, slow-twitch red and fast-twitch red fibres respectively (Table 2). The decrease in glucose uptake with time was not due to deterioration of the hindquarter preparation, since concentrations of ATP and phosphocreatine in all three muscle fibre types after 5 h of perfusion were identical with values obtained at the beginning of perfusion (results not shown).

To elucidate whether the combined effect of glucose and insulin was due to glucose, to insulin, or to both, perfusions were carried out in the presence of 11–13 mM-glucose and no insulin for the first 280 min. Then insulin was added to reach a perfusate concentration of 20000 μunits/ml and the perfusion was terminated

Table 1. Glucose uptake in rat hindlimbs at various perfusion times with different perfusate compositions

Rat hindlimbs were pre-perfused for 20 min, at which time 0 min samples were taken. The perfusate contained glucose at 11–13 mM and either insulin at 20000 μunits/ml (+glucose, +insulin) or no insulin (+glucose, −insulin) until 280 min, at which point insulin at 20000 μunits/ml was added, or 20000 μunits of insulin/ml but no glucose until 280 min, at which point glucose was added to 15 mM (−glucose, +insulin). In the last group the glucose concentration at 300 min was 13–13.5 mM. Glucose uptake was calculated as arteriovenous concentration differences multiplied by the perfusate flow rate (12.5 ml/min) divided by muscle mass (one-sixth of the total rat weight). Values are means ± S.E.M. for n rats (N.M., not measurable); *$P < 0.05$ compared with 300 min value in +glucose, +insulin perfusions.

Perfusion time (min)	Glucose uptake (μmol/h per g)		
	+Glucose, +insulin ($n = 10$)	+Glucose, −insulin ($n = 8$)	−Glucose, +insulin ($n = 8$)
0	34.8 ± 1.9	4.95 ± 0.91	N.M
60	21.3 ± 1.0	4.16 ± 0.85	N.M
120	16.8 ± 1.0	3.74 ± 0.55	N.M
180	16.3 ± 1.1	5.00 ± 0.74	N.M
240	14.1 ± 0.7	5.54 ± 0.57	N.M
300	11.5 ± 1.1	15.5 ± 1.3*	35.3 ± 2.8*

Table 2. Glucose transport and muscle glycogen concentrations in individual muscle fibre types of perfused rat hindlimbs

Rat hindlimbs were pre-perfused for 20 min, at which time 0 h values were determined, or perfused for 5 h after pre-perfusion. The perfusate contained glucose at 11–13 mM and either insulin at 20000 μunits/ml (+glucose, +insulin) or no insulin (+glucose, −insulin) until 280 min, at which point insulin at 20000 μunits/ml was added, or 20000 μunits of insulin/ml but no glucose until 280 min, at which point glucose was added to 15 mM (−glucose, +insulin). Glucose transport was calculated from uptake of trace 3-*O*-[^{14}C]methylglucose in individual muscle fibre types by using the plasma glucose concentration for calculation of the 'specific radioactivity' of 3-*O*-[^{14}C]methylglucose. Uptake of 3-*O*-[^{14}C]methylglucose was corrected for label in the extracellular space, determined with [^{3}H]mannitol. Hindlimbs were in all experiments exposed to the radioisotopes for the last 7 min of perfusion. Samples for determination of muscle glycogen concentration were taken at the end of the perfusions. Values are means ± S.E.M., with the numbers of determinations in parentheses: *$P < 0.05$ compared with (+glucose, +insulin, 0 h) values; †$P < 0.1$ compared to (+glucose, +insulin, 0 h) values.

Muscle	+Glucose, +insulin 0 h	+Glucose, +insulin 5 h	+Glucose, −insulin 5 h	−Glucose, +insulin 5 h
	Glucose transport (μmol/h per g)			
Fast-twitch white	12.7 ± 2.0 (7)	3.6 ± 1.1* (5)	2.3 ± 0.6* (7)	8.7 ± 1.7 (8)
Fast-twitch red	34.2 ± 1.3 (7)	9.5 ± 1.6* (5)	8.5 ± 1.6* (8)	28.2 ± 3.0 (8)
Slow-twitch red	29.4 ± 3.1 (7)	14.2 ± 4.8* (5)	21.1 ± 2.7† (8)	38.8 ± 3.5 (8)
	Glycogen (μmol/g)			
Fast-twitch white	37.3 ± 2.0 (10)	69.3 ± 5.8* (9)	40.3 ± 1.6 (8)	40.9 ± 4.9 (8)
Fast-twitch red	36.9 ± 2.8 (10)	137.4 ± 10.1* (9)	40.5 ± 1.7 (8)	46.8 ± 4.2† (8)
Slow-twitch red	27.7 ± 2.2 (10)	80.0 ± 5.4* (9)	24.6 ± 1.7 (8)	33.5 ± 5.3 (8)

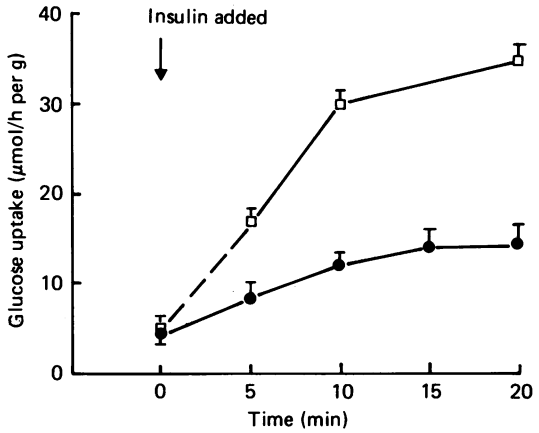

Fig. 1. Time course of effect of insulin on glucose uptake in perfused hindlimbs

Insulin was added to give a concentration of 20000 μunits/ml; ●, perfusions ($n = 4$) in which glucose at 11–13 mM but no insulin was present for the first 280 min after pre-perfusion and with subsequent addition (0 min in the figure) of insulin; □, perfusions ($n = 8$) in which the effect of insulin was measured in the pre-perfusion period, i.e. at the onset of perfusion. Since no measurements were done right at the beginning of the perfusion in these hindlimbs, owing to the absence of steady state at this time, a broken line has been drawn in the Figure to indicate that the 0 min value is hypothetical in these perfusions. The 0 min value used is the value measured after 20 min pre-perfusion in the absence of insulin (Table 1). Values are means ± S.E.M.

20 min later. As indicated in Table 1, the maximum insulin response in these perfusions was markedly lower than the response at 0 min in the perfusions containing insulin and glucose from the start. Thus perfusions with glucose alone for 5 h caused marked attenuation of the maximum insulin response, almost to the same extent as with glucose plus insulin (Table 1). In four of these perfusions the time course of insulin-stimulated glucose uptake was assessed and compared with insulin action in hindlimbs exposed to insulin in the pre-perfusion period. As shown in Fig. 1, the decreased insulin response after perfusion with glucose for 280 min was not due to time lag for achieving maximum insulin response, since the response was maximal already 15 min after addition of insulin (Fig. 1) and the data in Table 1 were obtained 20 min after insulin addition. Furthermore, Fig. 1 clearly shows the much larger insulin response in rats exposed to insulin at the onset of perfusions compared with rats pre-perfused for 280 min in the absence of insulin but in the presence of 11–13 mM-glucose. In contrast, when glucose but not insulin was omitted from the perfusate, with subsequent addition of glucose to the perfusate after 280 min perfusion, the maximum insulin effect on glucose uptake was not decreased at all (Table 1). These data clearly indicate that it is glucose and not insulin that causes the attenuation of the insulin response of glucose uptake. Of note is also the observation that glucose uptake was constant for the first 280 min of perfusion in the absence of insulin (Table 1 and Fig. 1).

To determine whether changes in glucose uptake were accompanied by changes in muscle membrane permeability to glucose, glucose transport as reflected by uptake of the non-metabolizable glucose analogue 3-*O*-[^{14}C]methyl-D-glucose was measured in the various groups of hindlimbs. The time-dependent decrease in insulin-stimulated glucose uptake with glucose plus insulin, and with glucose alone, was associated with marked decreases in insulin-stimulated glucose transport in all muscle fibre types (Table 2). It should be noted not only that the absolute responses of the different muscle

fibre types were quite dissimilar, but also that qualitatively their responses were similar (Table 2).

Muscle glycogen concentrations did not increase significantly during perfusions in the groups with only insulin or glucose present (Table 2).

Arterial perfusate pH in perfusions with insulin and glucose was 7.35 ± 0.02, 7.30 ± 0.02 and 7.27 ± 0.01 at 0, 1 and 5 h respectively. In hindlimbs perfused with only glucose or insulin for the first 280 min, after which insulin or glucose, respectively, was added to the perfusate, arterial pH values were 7.30 ± 0.01 and 7.35 ± 0.01 respectively in the two groups after 5 h of perfusion.

DISCUSSION

Exposure of isolated perfused rat hindlimbs to 12 mM-glucose for 5 h markedly attenuated the response of glucose transport and uptake to subsequent maximal insulin stimulation. The effect of glucose was enhanced by simultaneous exposure to insulin, whereas exposure to insulin itself did not attenuate its stimulatory effect on subsequent glucose transport and uptake. Thus isolated perfused muscle develops severe insulin resistance of glucose transport and uptake after only 5 h of exposure to a moderately high (12 mM) glucose concentration. These findings agree with findings in diabetic patients (Yki-Järvinen *et al.*, 1987) and in rats (Rossetti *et al.*, 1987) in which exposure to hyperglycaemia leads to insulin resistance on the whole-body level. Our findings for the first time identify muscle as a tissue in which insulin action is remarkably sensitive to increased glucose concentrations.

After 5 h perfusion, insulin resistance was slightly more pronounced after exposure to both insulin and glucose than after exposure to glucose alone. However, since no time course of the effect of glucose alone was carried out, it cannot be determined from the present study whether insulin resistance possibly developed faster in the presence of glucose plus insulin compared with glucose alone.

The mechanism behind the effect of glucose is not clear. Although in perfusions with insulin plus glucose muscle glycogen concentrations increase 2–3.5-fold, there was no significant increase in muscle glycogen concentrations in hindlimbs perfused with glucose alone (Table 2). These findings therefore indicate that the decrease in glucose transport found in the present study is not necessarily directly linked to an increase in muscle glycogen concentration. Rather, the effect seems to depend on changes in glucose transporter function and/or number elicited by the mere exposure to high glucose concentrations. The explanation for the more pronounced insulin resistance after exposure to both glucose and insulin than after glucose alone might then be that insulin makes more transporters available for interaction with glucose. The molecular nature of this phenomenon remains to be resolved, however. Experiments with cultured adipocytes suggest that long-term incubation with insulin and glucose causes insulin resistance by impairing translocation of glucose transporters from an intracellular storage site to the plasma membrane (Garvey *et al.*, 1987). In this context it is also noteworthy that glucose starvation of 3T3 adipocytes (Van Putten & Krans, 1985) and mouse fibroblasts (Haspel *et al.*, 1986) leads to increased glucose transport and synthesis of new glucose transporters (Haspel *et al.*, 1986), which can be reversed by glucose feeding (Van Putten & Krans, 1985; Haspel *et al.*, 1986).

The notion that glucose availability also in muscle regulates basal (non-insulin-stimulated) glucose transport has been suggested by studies in incubated soleus muscle. High glucose concentrations caused down-regulation of basal glucose transport (Sasson *et al.*, 1987). This is in contrast with our findings, in which glucose uptake in the absence of insulin was stable for 280 min (Table 1, Fig. 1). In other words, in perfused skeletal muscle exposure to 12 mM-glucose for 5 h induces insulin resistance, but does not cause down-regulation of basal glucose transport. It has also been reported that in the epitrochlearis muscle incubation with a submaximal insulin concentration in 1 mM- and 8 mM-glucose for 5 h progressively increased glucose transport (Young *et al.*, 1986). This increase was partially prevented by higher glucose concentrations; however, at no time did high glucose concentrations actually cause insulin resistance. The difference between these studies in incubated muscle and our study may reflect basic differences in the models used; we believe that perfused muscle is a more physiological system than incubated muscle.

Previous studies suggest that prolonged exposure of peripheral tissues to high insulin concentrations may increase (James *et al.*, 1985*b*; Wardzala *et al.*, 1985; Young *et al.*, 1986) as well as decrease (Rizza *et al.*, 1985; Garvey *et al.*, 1986; James *et al.*, 1985*a*) their response to insulin. Insulin sensitivity of incubated skeletal muscle *in vitro* after hyperinsulinaemia *in vivo* has been found to increase slightly after 2 weeks and to return to control values after 6 weeks of insulin infusion (Wardzala *et al.*, 1985). However, in that study the rats were chronically hypoglycaemic. Our study clearly demonstrates that insulin-induced insulin resistance of skeletal muscle occurs rapidly only when insulin exposure is associated with simultaneous glucose exposure. The critical concentration of glucose necessary to induce insulin resistance remains to be determined, and it is also possible that the critical concentration may vary from tissue to tissue.

It might be speculated that during 5 h of perfusion with insulin and glucose perfusate pH could decrease, owing to lactate release into the recirculating perfusate, and a decrease in arterial perfusate pH might then account for the decrease in glucose transport and uptake. However, changes in arterial perfusate pH were small during perfusions, decreasing by only 0.08 pH unit over the 5 h perfusion with insulin and glucose. It is unlikely that a fall in pH of this magnitude could significantly affect the rate of glucose transport in muscle, since in human erythrocytes glucose transport rate is unaffected by changes in pH of 0.1 unit (Brahm, 1983). Furthermore, in perfused rat hindlimbs basal glucose uptake was unaffected by changes in perfusate pH from 7.4 to 7.1 (Spriet *et al.*, 1985).

The present results were obtained *in vitro*, but studies in diabetic patients (Yki-Järvinen *et al.*, 1987) and in rats *in vivo* (Rossetti *et al.*, 1987) support our conclusions. The finding that hyperglycaemia itself rather than hyperinsulinaemia itself induces insulin resistance in skeletal muscle may explain why intense insulin treatment resulting in normoglycaemia in type II diabetic patients results in improved insulin actin (Foley *et al.*, 1972; Scarlett *et al.*, 1983).

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