Hyperglycemia Inhibits Insulin Activation of Akt/Protein Kinase B But Not Phosphatidylinositol 3-Kinase in Rat Skeletal Muscle

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Sustained hyperglycemia impairs insulin-stimulated glucose utilization in the skeletal muscle of both humans and experimental animals—a phenomenon referred to clinically as glucose toxicity. To study how this occurs, a model was developed in which hyperglycemia produces insulin resistance in vitro. Rat extensor digitorum longus muscles were preincubated for 4 h in Krebs-Henseleit solution containing glucose or glucose + insulin at various concentrations, after which insulin action was studied. Preincubation with 25 mmol/l glucose + insulin (10 mU/ml) led to a 70% decrease in the ability of insulin (10 mU/ml) to stimulate glucose incorporation into glycogen and a 30% decrease in 2-deoxyglucose (2-DG) uptake, compared with muscles incubated with 0 mmol/l glucose. Glucose incorporation into lipid and its oxidation to CO_2 were marginally diminished, if at all. The alterations of glycogen synthesis and 2-DG uptake were first evident after 1 h and were maximal after 2 h of preincubation; they were not observed in muscles preincubated with 25 mmol/l glucose + insulin for 5 min. Preincubation for 4 h with 25 mmol/l glucose in the absence of insulin produced a similar although somewhat smaller decrease in insulin-stimulated glycogen synthesis; however, it did not alter 2-DG uptake, glucose oxidation to CO_2, or incorporation into lipids. Studies of insulin signaling in the latter muscles revealed that activation of Akt/ protein kinase B (PKB) was diminished by 60%, compared with that of muscles preincubated in a glucose-free medium; whereas activation of phosphatidylinositol (PI) 3-kinase, an upstream regulator of Akt/PKB in the insulin-signaling cascade, and of mitogen-activated protein (MAP) kinase, a parallel signal, was unaffected. Immunoblots demonstrated that this was not due to a change in Akt/PKB abundance. The results indicate that hyperglycemia-induced insulin resistance can be studied in rat skeletal muscle in vitro. They suggest that impairment of insulin action in these muscles is related to inhibition of Akt/PKB by events that do not affect PI 3-kinase. *Diabetes* 48:XXX–XXX, 1999

Glucose toxicity has gained increasing recognition over the last several years as a factor contributing to both impaired insulin secretion and insulin resistance in patients with diabetes (1). Early studies of this phenomenon focused on the inhibition of insulin secretion by sustained hyperglycemia (2,3); however, it is now clear that chronic hyperglycemia also causes insulin resistance in muscle and other peripheral tissues (3–5). In a key study, Rossetti et al. (3) used an euglycemic clamp technique to demonstrate that peripheral insulin resistance in rats with moderately severe diabetes is reversed by treatment with phlorizin, an agent that restores normoglycemia by inhibiting the reabsorption of glucose by the renal tubules. In contrast, Rossetti et al. (3) found no effect of phlorizin on insulin sensitivity in control rats. Later studies confirmed these results (6,7) and established that the insulin resistance of patients with poorly controlled type 1 diabetes is reversed when their chronically elevated blood glucose levels are lowered by insulin therapy (8). Although other explanations are possible, cumulatively these findings strongly support the notion that sustained hyperglycemia, per se, can impair insulin action in muscle and possibly other tissues of both humans and experimental animals.

In an effort to determine whether glucose acts directly on the muscle cell to impair insulin action, Richter et al. (9,10) evaluated the effect of hyperglycemia on insulin-stimulated glucose uptake and glucose transport in an isolated perfused rat hindquarter. Richter et al. (9,10) found that perfusion of this muscle preparation with 25 or 12 vs. 5 or 0 mmol/l glucose for 2–5 h, especially in the presence of insulin but also in its absence, inhibited the subsequent ability of insulin to stimulate glucose uptake (40–80%) and, to a somewhat lesser extent, glucose transport (e.g., 29% in studies in which glucose uptake was diminished by 55%). Glycogen synthase activity was concurrently reduced; however, neither insulin binding to its receptor, receptor tyrosine kinase activity, nor glucose transporter number were altered. Thus, hyperglycemia of several hours' duration appears to create insulin resistance

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2-DG, 2-deoxyglucose; EDL, extensor digitorum longus; IRS, insulin receptor substrate; KHS, Krebs-Henseleit solution; MAP, mitogen-activated protein; PH, pleckstrin homology; PI, phosphatidylinositol; PKB, protein kinase B; TBST, Tris-buffered saline with Tween.

in muscle by a mechanism that operates *1*) distal to the insulin receptor and *2*) does not involve a change in glucose transporter abundance.

To gain further insight into the signaling events altered by hyperglycemia, studies were performed with an incubated rat extensor digitorum longus (EDL) muscle preparation. The results reveal that within 2 h, hyperglycemia selectively alters insulin-stimulated glucose disposition and, in some instances, glucose transport in this model, much as it does in the perfused hindquarter. They also reveal that these effects of hyperglycemia are associated with impairment of insulin activation of Akt/protein kinase B (PKB), despite apparently normal activation of PI 3-kinase.

RESEARCH DESIGN AND METHODS

Experimental animals. Male Sprague-Dawley rats weighing 50-65 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). They were maintained on a 12-h light/dark cycle in a temperature-controlled (19–21°C) room and were allowed free access to water and standard rat diet until 18–20 h before being killed when food was withdrawn.

Incubations. On the experimental day, rats were anesthetized with sodium pentobarbital (6 mg/100 g body wt, i.p.) and EDL muscles isolated as described by Maizels et al. (11). The muscles were attached to stainless steel clips and incubated in Krebs-Henseleit solution (KHS) containing 6 mmol/l glucose for 20 min and then washed in glucose-free KHS for 2 min. After this, they were preincubated for periods ranging between 5 min and 4 h in KHS containing 0 or 25 mmol/l glucose or 25 mmol/l glucose + 10 mU/ml insulin as indicated in RESULTS. After the preincubation, muscles were washed in fresh glucose-free KHS for 2 min and then transferred to KHS containing 6 mmol/l glucose, insulin (10 mU/ml), and other additions as described below and in Table 1. For determination of glucose transport and disposition, muscles were incubated for 20 min, after which they were quickly removed from the medium, blotted, frozen in liquid N_2, and stored at –80°C until analyzed. For assessment of signaling, muscles were incubated for 5 min in the presence or absence of insulin and were processed as described below. All incubations were at 37°C in medium continuously gassed with 95% O_2/5% CO_2.

Glucose transport and disposition. Glucose transport was assessed on the basis of 2-[1,2-^{3}H]deoxy-D-glucose uptake and glucose disposition from the incorporation of [U^{14}C]glucose into lipid and glycogen and its oxidation to CO_2 as described previously (12).

PI 3-kinase and Akt/PKB immunoprecipitation and assays. Immediately after a 5-min incubation in the presence or absence of insulin (10 mU/ml), muscles were weighed and homogenized in 1 ml of ice-cold buffer containing 250 mmol/l sucrose, 20 mmol/l Tris (pH 7.4), 10 mmol/l NaF, 5 mmol/l EDTA, 2 mmol/l Na_3VO_4, 2 mmol/l NH_4MbO_4, 1 mmol/l β-glycerolphosphate, 1 mmol/l DTT, 1 μmol/l microcystin-LR (Calbiochem-Novabiochem, La Jolla, CA), and 10 μl/ml Protease Inhibitor Cocktail P8340 (Sigma Chemical, St. Louis, MO). Igepal CA630 (Sigma) was added to each sample to achieve a final concentration of 1% (by volume) and the samples solubilized for 1.5 h at 4°C. The resultant lysate was centrifuged (12,000*g* for 5 min) and the supernatant incubated with a specific antibody preincubated with Protein A/G plus Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Incubations were for 2 h with an anti-Akt/PKB1 antibody, which crossreacts with Akt/PKB2 (13), or overnight with either antiphosphotyrosine (Upstate Biotechnology [UBI], Lake Placid, NY) or anti–insulin receptor substrate (IRS)-1 (UBI) antibody. Beads were collected by brief (1–3 s) centrifugation and washed repeatedly as indicated below.

For measurement of Akt/PKB activity, beads containing the anti-Akt/PKB immunoprecipitates were washed three times with buffer A, which contained 25 mmol/l Hepes (pH 7.5), 10% glycerol, 0.1% Triton X-100, 0.5 mol/l NaCl, 0.1% bovine serum albumin (fatty acid free), 50 mmol/l NaF, 10 mmol/l β-glycerolphosphate, 1 mmol/l Na_3VO_4, and 1 mmol/l DTT, and then were washed twice with buffer B, which contained 50 mmol/l Tris (pH 7.5), 12 mmol/l $MgCl_2$, 10 mmol/l β-glycerolphosphate, 1 mmol/l Na_3VO_4, and 1 mmol/l DTT. They were then incubated for 10 min at 30°C in 40 μl of a reaction mix consisting of buffer B, to which had been added 100 μmol/l [γ-^{32}P]ATP (10 μCi/sample) and 30 μmol/l crosstide (UBI). The reaction was stopped by spotting 25 μl of the mix on P81 phosphocellulose paper (Whatman, Clifton, NJ) and washing the papers three times for 15 min in 1% H_3PO_4. The papers were dried and radioactivity determined by Cerenkov counting. In some instances, the blocking peptide was added to the cell lysate before immunoprecipitation with anti-Akt/PKB antibody. Under these conditions, basal Akt/PKB activity was diminished by 50%, and insulin-stimulated activity was abolished.

For Western blotting of Akt/PKB, 20–25 μg of crude muscle homogenate was added to 2X Laemmi sample buffer, boiled for 5 min, and subjected to SDS-PAGE. Resolved samples were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) that were rinsed in Tris-buffered saline with Tween (TBST) (pH 7.5) and blocked in 5% nonfat dry milk in TBST overnight at 4°C. The membranes were blotted with either anti-Akt antibody (13), anti-Akt antibody after preincubation with blocking peptide (RPHFPQFSYSASGTA), in a 1/10 ab/peptide ratio (vol/vol) or anti–phospho-Akt antibody (Ser-473 Antibody Kit, New England Biolabs, Beverly, MA) for 3 h. They were then rinsed in TBST, incubated in horseradish peroxidase-coupled goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) for 50 min. The resolved bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL) and quantified by densitometry.

For measurement of PI 3-kinase activity, anti-phosphotyrosine and anti–IRS-1 immunoprecipitates were prepared and activity assayed as described by Chen et al. (14) with phosphatidylinositol used as the substrate. Mitogen-activated protein (MAP) kinase was assayed using a phospho-MAP kinase antibody (New England Biolabs, Beverly, MA) that recognizes the active phosphorylated forms of extracellular signal-regulated kinase (ERK1 and ERK2) on an immunoblot (15). Detection of the resolved bands was as with Akt/PKB.

Statistical analysis. Values are expressed as means ± SE. Statistical differences between groups were determined by analysis of variance, followed by the Student-Neuman-Keuls multiple comparison test, or by Student's *t* test with Bonferroni modification.

RESULTS

Effects of preincubation with insulin and glucose on subsequent insulin-stimulated glucose transport and disposition. As shown in Table 1, insulin-stimulated glucose transport, as assessed by 2-DG uptake, was diminished by ~30% when the EDL was incubated for 4 h with a medium containing 25 mmol/l glucose and 10 mU/ml insulin, compared with a medium devoid of these additions. In parallel experiments, insulin-stimulated glucose incorporation into glycogen was decreased by 70%. Glucose incorporation into total lipid and its oxidation to CO_2 tended to be diminished to the same extent as 2-DG uptake, although only the decrease in glucose incorporation into lipid was statistically significant.

The differences in insulin-stimulated glucose transport and incorporation into glycogen between the two groups were evident after 1 h of preincubation and were maximal at 2 h (Fig. 1). In contrast, no differences between the groups were observed after 5 min of preincubation, indicating that the observed changes were not an artifact caused by the presence of residual glucose.

Effect of preincubation with 25 mmol/l glucose in the absence of insulin on insulin-stimulated glucose transport and disposition. Insulin-stimulated glucose incorporation into glycogen was diminished by 50% in muscles preincubated for 4 h with 25 vs. 0 mmol/l glucose, even in the absence of insulin (Table 2). However, in contrast to the findings in muscles preincubated with insulin, insulin-stimulated 2-DG uptake was not diminished. In addition, neither glucose incorporation into total lipid nor its oxidation to CO_2 was decreased.

Effect of preincubation with 25 mmol/l glucose on activation of PI 3-kinase, Akt/PKB, and MAP kinase (ERK-2) by insulin. To study the effect of hyperglycemia on insulin signaling, muscles were preincubated for 4 h in the presence of 0 or 25 mmol/l glucose, washed (see METHODS), and then incubated for 5 min in fresh media containing 6 mmol/l glucose and 0 or 10 mU/ml insulin. As shown in Table 3, in EDL preincubated without glucose added to the medium, insulin caused a 3.5-fold increase in Akt/PKB activity (see Fig. 2 for absolute group data), and a 2.7-fold increase in MAP kinase activity. Insulin also increased PI 3-kinase activity threefold in anti-phosphotyrosine immunoprecipitates and more

TABLE 1
The effect of 4 h of preincubation with media devoid of insulin and glucose or containing 25 mmol/l glucose and 10 mU/ml insulin on the subsequent insulin-stimulated glucose uptake and disposition in rat EDL muscle

	Preincubation conditions	
	0 mmol/l glucose	25 mmol/l glucose + 10 mU/ml insulin
	$nmol \cdot g^{-1} \cdot ms \cdot min^{-1}$	
2-DG uptake	189 ± 9 (5)	125 ± 6† (17)
Glucose to glycogen	50 ± 8 (11)	15 ± 2† (17)
Glucose to total lipids	3 ± 0.3 (11)	2 ± 0.3* (17)
Glucose to CO_2	5 ± 0.7 (6)	4 ± 0.4 (4)

Data are means ± SE (number of observations). After preincubation, muscles were washed and then incubated for 20 min in media containing 10 mU/ml insulin, 6 mmol/l glucose, and either 2-[1,2-^{3}H]deoxy-D-glucose and [^{14}C]mannitol (uptake) or [^{14}C]glucose. Significantly different from muscles preincubated in the absence of added glucose and insulin: *P < 0.05; †P < 0.0001.

than fivefold in anti–IRS-1 immunoprecipitates (Table 3). Similar results were found in anti–IRS-2 immunoprecipitates (data not shown).

Preincubation with 25 mmol/l glucose differentially affected the signaling events activated by insulin. In muscles preincubated with 25 mmol/l glucose, the increase in Akt/PKB activity was ~40% less than that in the muscles preincubated with 0 mmol/l glucose (Table 3, Fig. 2). In contrast, the increases in PI 3-kinase and MAP kinase activity caused by insulin were the same. Immunoblots revealed that the antibody used to precipitate Akt/PKB recognized a doublet in the 60-kDa range (Fig. 3*B*) and that the lower band diminished in intensity in muscles incubated with insulin. Studies with antibody to phosphorylated Akt/PKB suggested that this was due to a gel shift attributable to phosphorylation. Elimination of the lower band by incubation with a specific blocking peptide (see METHODS) confirmed that it was Akt/PKB (Fig. 3A). Figure 3*B* also demonstrates the absence of gross differences in Akt/PKB abundance between muscles preincubated with 0 vs. 25 mmol/l glucose.

DISCUSSION

The central finding of this study was that preincubation with a hyperglycemic medium diminishes the ability of insulin to stimulate Akt/PKB activity in an incubated EDL muscle. Furthermore, it does so despite the absence of an apparent alteration in the activation of PI 3-kinase, an upstream regulator of Akt/PKB in the insulin-signaling cascade, and MAP kinase, a parallel signal. To carry out these investigations, we developed a model in which incubation of muscle in a hyperglycemic media induces insulin resistance. Our experimental design was patterned after that of Richter et al. (9,10) who observed inhibition of insulin-stimulated glucose transport in rat hindquarters preperfused for several hours with high concentrations of glucose (12 and 25 vs. 0 and 5 mmol/l) + insulin. In keeping with their findings, we observed that preincubation of the EDL for 4 h in a medium containing 25 mmol/l glucose + insulin versus no glucose and insulin caused an inhibition of insulin-stimulated glucose transport of 30%. Glucose incorporation into glycogen was even more inhibited (70%), whereas its incorporation into lipid and oxidation to CO_2 was diminished to the same extent as 2-DG uptake, if at all. Thus, the effects of hyperglycemia appear to be relatively selective for glucose transport and glycogen synthesis. Glycogen synthesis was not specifically measured in the hindquarter studies; however, hyperglycemia was shown to inhibit glycogen synthase activation by insulin (9).

Because preincubation with insulin would make evaluation of subsequent signaling changes induced by a second dose of insulin more complex to interpret, insulin signaling was only compared in the second model evaluated in the hindquarter studies—muscles preincubated with 25 vs. 0 mmol/l glucose in the absence of added insulin. Hansen et al. (9) observed qualitatively similar effects on insulin action in these muscles as they did in muscles preperfused with insulin + glucose. In

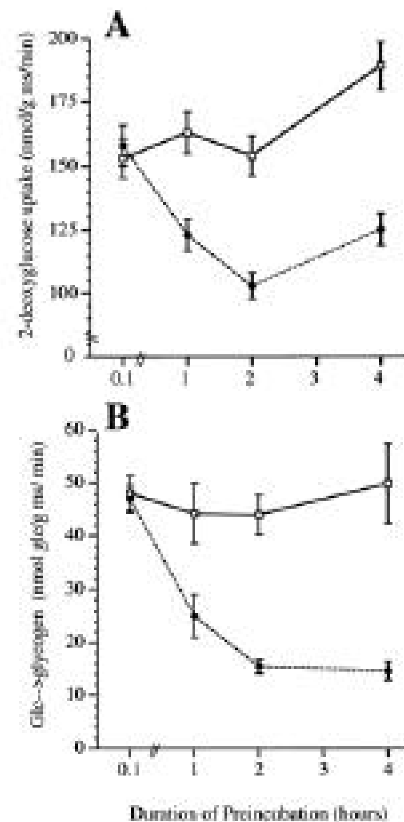

FIG. 1. Effect of preincubation for various times in media containing 0 mmol/l glucose (□) or 25 mmol/l glucose + insulin (●) on subsequent insulin-stimulated 2-DG uptake (*A*) and [^{14}C]glucose incorporation into glycogen (*B*) in the EDL. Results are means ± SE. See footnote to Table 1 for details.

TABLE 2
The effect of 4 h of preincubation in media containing 0 or 25 mmol/l glucose on the subsequent stimulation of 2-deoxyglucose uptake and [^{14}C]glucose disposition by insulin in the rat EDL muscle

	Preincubation conditions	
	0 mmol/l glucose	25 mmol/l glucose
	$nmol \cdot g^{-1} \cdot ms \cdot min^{-1}$	
2-DG uptake	169 ± 13 (10)	167 ± 9 (10)
Glucose to glycogen	42 ± 3 (10)	22 ± 2* (10)
Glucose to total lipids	2 ± 0.1 (10)	2 ± 0.1 (10)
Glucose to CO_2	4 ± 0.3 (10)	4 ± 0.3 (9)

Data are means ± SE (number of observations). See legend to Table 1 for details of incubation. *Significantly different from muscles preincubated in the absence of added glucose ($P < 0.0001$).

contrast, we observed no inhibition of insulin-stimulated 2-DG uptake in this model. However, we found that insulin-stimulated glucose incorporation into glycogen was diminished by 50%. As in the presence of insulin, the effect of hyperglycemia on glycogen synthesis was selective, since glucose incorporation into lipid and its oxidation to CO_2 were not specifically depressed. Thus, preincubation of the EDL in a medium containing 25 mmol/l glucose clearly leads to an impairment of insulin action, albeit the pattern of impairment was not identical to that observed when the preincubation medium also contained insulin.

The results suggest that one site at which hyperglycemia impairs insulin signaling is at Akt/PKB. Akt/PKB is a 60-kDa serine/threonine kinase, which contains a pleckstrin homology (PH) domain, and has been shown to be functionally located downstream of PI 3-kinase (13,16–20). Three Akt/PKB isoforms have been described; however, to date most studies have focused on Akt/PKB1. Akt/PKB is activated by insulin and a number of growth factors including platelet-derived growth factor, epidermal growth factor, and basic fibroblast growth factor (13,16,20). This activation requires that Akt/PKB be phosphorylated on two key amino acid residues, Thr 308 and Ser 473 (18,19). The threonine is phosphorylated by a 3-phosphoinositide–dependent protein kinase commonly referred to as PDK1, and the serine, by a still undefined enzyme, sometimes is referred to as PDK2 (19). Both of these kinases, like Akt/PKB, contain PH regions. Current thinking holds that activation of PI 3-kinase sets in motion Akt/PKB activation by generating PI 3,4,5 P3 and possibly PI 3,4 P2 in the plasma membrane and that this secondarily causes the three PH domain–containing enzymes, (i.e., Akt/PKB, PDK1, and PDK2) to come together and interact (18,19,21). In keeping with this view, recent studies have shown that activation of Akt/PKB by insulin in human embryonic kidney 293 cells is associated with an increase in its localization in the plasma membrane (22)

The finding that prior hyperglycemia inhibits insulin activation of Akt/PKB in skeletal muscle (Fig. 2) is perhaps not unexpected, since studies with cultured cells and primary adipocytes have implicated Akt/PKB in the mediation of a variety of insulin's metabolic actions, including glucose (see below) and type A amino acid transport and glycogen and protein synthesis (18,20). Also, impaired activation of Akt/PKB by insulin has been observed in insulin-resistant muscle of diabetic rodents (7,23) and humans (24). The most surprising and perhaps novel observation of this study was that the impaired insulin activation of Akt/PKB induced by hyperglycemia did not appear to involve PI 3-kinase. Thus, preincubation with 25 mmol/l glucose led to a 40% decrease in insulin-stimulated Akt/PKB activation, compared with that of muscles incubated with 0 mmol/l glucose, but it had no effect on PI 3-kinase activation. A very similar observation recently has been made by Song et al. (23), who found an impaired activation of Akt/PKB, but not PI 3-kinase, by insulin in EDL muscles of diabetic, insulin-resistant GK rats. Furthermore, treatment of these rats with phlorizin, which diminished their hyperglycemia without increasing plasma insulin, reversed this inhibition. Thus, data from two very different models suggest that hyperglycemia-induced insulin resistance correlates with inhibition of Akt/PKB activation in the apparent absence of an impairment of PI 3-kinase activation. A similar occurrence has recently been reported in 3T3 L1 adipocytes incubated with the short-chain ceramide analog, C_2-ceramide (25).

TABLE 3
The effect of 4 h of preincubation in a media containing 0 or 25 mmol/l glucose on the subsequent activation of PI 3-kinase and Akt/PKB by insulin in rat EDL muscle

	Preincubation conditions	
	0 mmol/l glucose	25 mmol/l glucose
	% Activation by insulin	
PI 3-Kinase (*a*)	302 ± 41 (10)	341 ± 83 (12)
PI 3-Kinase (*b*)	559 ± 127 (5)	527 ± 94 (5)
Akt/PKB	352 ± 42 (9)	214 ± 43* (10)
ERK2	269 ± 91 (4)	315 ± 135 (5)

Data are means ± SE (number of observations) and are expressed as percent of activity determined in contralateral muscle incubated in the absence of insulin. Experimental conditions were as described in Table 2, except that incubations were for only 5 min. The symbol (*a*) indicates that PI 3-kinase was immunoprecipitated with anti-phosphotyrosine antibody and (*b*) with anti–IRS-1 antibody. Akt/PKB was immunoprecipitated with an antibody that recognizes Akt/PKB2 as well as Akt/PKB1. PI 3-kinase and Akt/PKB activities were determined by using standard kinase assays and ERK2 by immunoblotting with a phospho-antibody. See METHODS for details. *Significantly different from muscles preincubated in the absence of added glucose ($P < 0.05$).

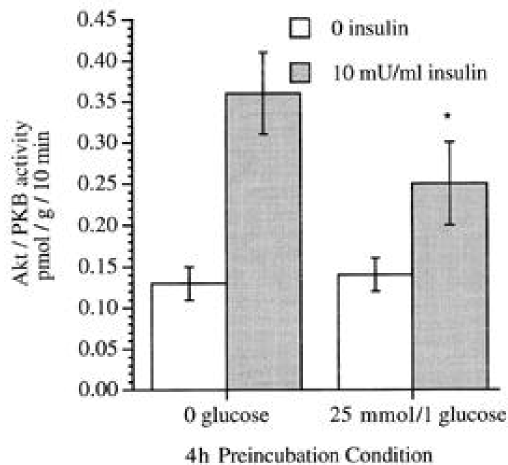

FIG. 2. Effect of preincubation with media containing 0 or 25 mmol/l glucose on Akt/PKB activity in the presence or absence of insulin in rat EDL muscle. Results are means ± SEM. *Significantly different (P < 0.05) vs. insulin-stimulated muscle preincubated in the absence of added glucose.

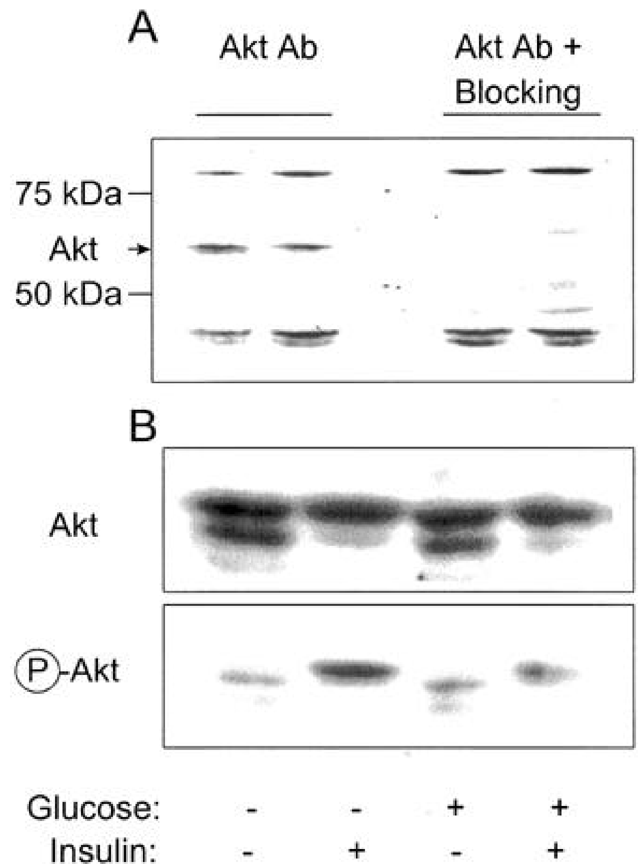

FIG. 3. Immunoblots of Akt/PKB in rat muscle lysates. *A*: Demonstration that blocking peptide eliminates bands identified as Akt/PKB (60 kDa) with the anti-Akt antibody used for immunoprecipitation (8% SDS-PAGE). Blots are for muscles incubated with 0 mmol/l glucose without (*left lane*) or with (*right lane*) insulin in each condition. *B*: Blot showing equal abundance of Akt/PKB in lysates of muscle preincubated with 0 and 25 mmol/l glucose (10% SDS) (top panel). Immunoblot with antibody to phosphorylated Akt/PKB (P-Akt) showing that insulin causes a gel shift due to phosphorylation (bottom panel). The blots shown are representative of one experiment that was repeated four times.

The link between Akt/PKB and insulin-stimulated glucose transport is largely based on studies in which Akt/PKB was overexpressed in adipocytes and muscle cells (20,26,27). In the present study, the inhibition of Akt/PKB activation by insulin was associated with inhibition of glucose incorporation into glycogen, but not 2-DG uptake (glucose transport) in muscles preincubated with 25 mmol/l glucose. A similar dissociation between insulin-stimulated glucose transport and Akt/PKB activation has also been noted in GK rats (23). In addition, expression of a dominant negative mutant of Akt/PKB in adipocytes and Chinese hamster ovary (CHO) cells (28) has recently been shown to inhibit the stimulation of protein synthesis, but not glucose transport by insulin. Thus, the link between Akt/PKB activation by insulin and its ability to stimulate glucose transport remains open to question.

One can only speculate as to how hyperglycemia causes insulin activation of Akt/PKB to become defective in the face of normal activation of PI 3-kinase. Possible mechanisms include inhibition of PDK1 or the still unidentified serine kinase (PDK2), a defect in intracellular PI 3-kinase translocation not reflected by IRS or antiphosphotyrosine immunoprecipitable activity of a whole-cell lysate, or direct modification of Akt/PKB by a kinase or phosphatase activated by hyperglycemia. A possible factor in these events could be protein kinase C (PKC), which has been shown to be activated in a number of cells in which incubation in a hyperglycemic medium leads to insulin resistance (29,30) (see below). In keeping with this notion, alterations in the distribution of specific PKC isoforms ε and θ have been observed in muscle of a variety of insulin-resistant rodents (31, 32), including rats in which insulin resistance was caused by chronically infusing glucose (33). Also, PKC activation by phorbol esters has been shown to inhibit insulin activation of Akt/PKB in cultured adipocytes; although, the precise site at which PKC acted was not determined (16).

Of note are prior studies of the signaling changes associated with hyperglycemia-induced insulin resistance carried out in cultured adipocytes (24 h) by Muller et al. (29) and in fibroblasts by Pillay et al. (30) and Berti et al. (34). In all of these investigations, insulin receptor tyrosine kinase was found to be diminished as was PI 3-kinase activity, where measured (30). Phosphorylation of the insulin receptor (29,30) and IRS-1 (30) by protein kinase C appeared to play a causal role. Whether the proximal site of inhibition of insulin signaling differed in these studies from that observed here because of the cell types used remains to be determined. Also requiring evaluation is whether the changes observed here are simply the earliest alterations in the insulin-signaling cascade caused by hyperglycemia. If so, changes in upstream events might be observed in muscles preincubated with insulin as well as a high concentration of glucose or simply in a hyperglycemic medium for a longer time.

Finally, the present study only compared the effects of preincubation with 0 vs. 25 mmol/l glucose on insulin signaling; therefore, it remains to be determined whether the effects of glucose occur predominantly when its concentration is raised from 0 to 5 mmol/l or from the latter to the 25 mmol/l glucose concentration used here. Experiments with the perfused hindquarter (9,10) suggest that the inhibitory effect of glucose on insulin action occurs in both concentration ranges.

In conclusion, the results indicate that incubation of a rat EDL muscle with 25 mmol/l glucose vs. 0 mmol/l glucose for

2 h or more leads to impaired stimulation of glucose incorporation into glycogen. They suggest that the inhibition of insulin action in this situation could involve Akt/PKB and that it occurs in the face of apparently normal PI 3-kinase activation.

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