RESEARCH ARTICLE

Role of PKCζ translocation in the development of type 2 diabetes in rats following continuous glucose infusion

Jing-fang Zhang[1,2]
Jing-ping Ou Yang[1]
Guang-hao Wang[1,3]
Zhengyuan Xia[4]
Sheng Zhong Duan[5]
Yong Wu[6]*

[1]*Department of Pathophysiology, Medical College, Wuhan University, Wuhan, Hubei, China*

[2]*Medical School, Jingchu University of Technology, Jingmen, Hubei, China*

[3]*Department of Internal Medicine, The Second People's Hospital of Jingmen City, Jingmen, Hubei, China*

[4]*Department of Anesthesiology, Research Centre of Heart, Brain, Hormone and Healthy Aging, University of Hong Kong, Hong Kong SAR, China*

[5]*Nephrology Division, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA*

[6]*Department of Biochemistry, University of California, Riverside, CA, USA*

*Correspondence to: Yong Wu, Department of Biochemistry, University of California, 900 University Ave., Riverside, CA 92521, USA.
E-mail: yong.wu@ucr.edu

Abstract

Aim   We investigated the molecular mechanisms of hyperglycaemia-induced insulin resistance and type 2 diabetes in rats receiving a continuous glucose infusion (GI).

Methods   Female Wistar rats were infused with either 2.8 mol/L glucose or saline (2 mL/h) for durations varying from 0 to 15 days. Blood samples were analysed daily to determine glucose and insulin dynamics. Subsets of animals were sacrificed and solues muscles were extracted for determination of protein expression, subcellular location, and activities of insulin-signalling proteins.

Results   Rats accommodated this systemic glucose oversupply and developed insulin resistance on day 5 (normoglycaemia/hyperinsulinaemia) and type 2 diabetes on day 15 (hyperglycaemia/normoinsulinaemia). The effect of GI on protein kinase Cζ (PKCζ) activity was independent of changes in phosphatidylinositol 3-kinase activity, and occurred in parallel with an increase in PDK1 activity. Activated PKCζ was mainly located in the cytosol after 5 days of GI that was coincident with the translocation of GLUT4 to the plasma membrane, and normoglycaemia. After 15 days of GI, PKCζ translocated from the cytosol to the plasma membrane with a concomitant decrease in PDK1 activity. This caused an increase in the association between PKCζ and PKB and a decrease in PDK1–PKB reactions at the plasma membrane, leading to reduced PKB activity. The activity of PKCζ *per se* was also compromised. The PKCζ and PKB activity reduction and the blunted insulin-stimulated GLUT4 translocation eventually led to hyperglycaemia and diabetes.

Conclusion   Translocation of PKCζ may play a central role in the development of type 2 diabetes. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords   insulin resistance; type 2 diabetes; phosphatidylinositol-dependent kinase 1; protein kinase B; protein kinase C ζ; translocation

Introduction

Insulin resistance is a major pathogenic stage in the development of type 2 diabetes [1]. In humans and rodents, skeletal muscle is the most important tissue for the maintenance of balanced postprandial glucose homeostasis: about 80% of insulin-stimulated glucose uptake is accounted for by muscle [2,3].

Insulin increases glucose uptake in muscle by eliciting translocation of the protein GLUT4 from an intracellular storage site to both the plasma membrane (PM) and the transverse (T)-tubules through a complex signalling cascade [4,5]. Impaired GLUT4 translocation is linked to reduced glucose utilization in skeletal muscle of insulin resistant and type 2 diabetic humans [6,7]. Considerable work has been done to elucidate the molecular mechanisms regulating GLUT4 translocation. The general consensus is that activation of phosphatidylinositol 3-kinase (PI 3-kinase) via its interaction with insulin receptor substrates (IRSs) [8] plays an essential role [9,10]. In addition, downstream of PI 3-kinase, two serine/threonine kinases, namely PKB/Akt and atypical protein kinase C (PKC) ζ/λ have been implicated as major transducer proteins of glucose transport in response to insulin in different cell models [11,12]. However, it is now clear that GLUT4 translocation can be activated by different agents by a mechanism not involving the proximal steps of the insulin-signalling cascade. For example, bypassing the initial insulin signal transduction steps, hyperosmolarity can stimulate GLUT4 translocation and glucose transport through activation of a common convergent signal transduction step downstream of and/or in parallel to PI 3-Kinase/Akt [13,14].

Hyperglycaemia is a major factor contributing to the development of peripheral insulin resistance in patients with both type 1 and type 2 diabetes [15]. Restoration of glycaemia normalizes insulin action on glucose transport in isolated skeletal muscle from type 2 diabetic subjects [16], and in animal models of diabetes [17]. Thus, hyperglycaemia appears to have a profound impact on insulin action through a desensitization of the insulin-signalling transduction, but how it impairs insulin action is largely unknown. Studies [18,19] show that hyperglycaemia impairs insulin sensitivity and insulin-stimulated phosphorylation/activation of Akt/PKB in the liver of Zucker diabetic fatty rats. Also insulin-induced Akt/PKB activation is reduced in skeletal muscles incubated in medium containing glucose at high concentration, without alteration of PI 3-kinase activity [20]. Furthermore, insulin-induced Akt/PKB activation is reported to be decreased in skeletal muscles of the diabetic Goto-Kakizaki rat [17,21]. Thus, the reduced activation of Akt/PKB is a candidate for the defects leading to insulin resistance caused by hyperglycaemia in both the liver and the skeletal muscle.

Interestingly, hyperglycaemia, just like hyperosmolarity, in addition to its inhibiting effect on PKB and GLUT4 translocation, could stimulate glucose uptake directly. Studies in diabetic patients revealed that hyperglycaemia leads to increased glucose uptake in skeletal muscle in a glucose concentration-dependent manner [22], but the underlying mechanism is poorly understood. Studies on cultured myotubes and isolated rat skeletal muscle tissues have shown that short-term hyperglycaemia is an insulin-independent inducer of GLUT4 translocation [23] that involves PKC activation. Moreover, studies [23,24] show that enhanced PKC activity is involved in mediating

insulin resistance. Taken together, these *in vitro* studies indicate that acute increases in glucose activate PKC, which on one hand induces insulin-independent glucose uptake and, on the other hand, could mediate insulin resistance.

The focus of the current study is on two members of the AGC kinase super family: PKB/Akt and PKCζ. PKCζ is the major atypical PKC in skeletal muscles and adipose tissues of humans, monkeys, and rats. It is considered as a negative regulator of PKB [25] and is required for insulin-stimulated glucose transport. To study the role of PKB and PKCζ in the development of insulin resistance and diabetes *in vivo*, we used the glucose-infused rat model originally developed by Leahy *et al.* [26]. In this model, continuous systemic glucose oversupply induces insulin resistance (normoglycaemia/hyperinsulinaemia) and then overt type 2 diabetes (hyperglycaemia/normoinsulinaemia). This model mimics the pathobiochemical changes in humans with nutrient oversupply and subsequent insulin resistance associated with increased lipid accumulation in adipose tissue and skeletal muscle. Using this model we were able to observe in the hindlimb muscle dynamic changes in insulin-signalling molecules such as PDK1, PKCζ, PKB, and GLUT4 activities as well as their localization in the development of insulin resistance and type 2 diabetes.

Research design and methods

Materials

Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were obtained from Biovision (Palo Alto, CA, USA) and the apparatus used was from Bio-Rad (Richmond, CA, USA). Protein A/G-Sepharose, Tris, NP-40, and nitrocellulosemembranes were obtained from Sigma Chemical Co. (St Louis, MO, USA). Monoclonal anti-Akt/PKB antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-rat carboxy-terminal IRS-1 antibody (Clone 8–63) was from NeoMarkers (Fremont, CA, USA). Anti-PKCζ antibody and the antibody to the p85 Subunit of PI-3-Kinase (αp85) was from Upstate Biotechnology (Lake Placid, NY, USA). The monoclonal antibody to PDK1 was from Transduction Laboratories (Lexington, UK). The monoclonal anti-rat GLUT4 antibody was from R&D Systems, Inc. (Minneapolis, AL, USA). Myristoylated PKCζ pseudosubstrate peptide inhibitor was from Biomol (Plymouth Meeting, PA, USA) and SH-6 from Kamiya Biomedical Co. (Seattle, WA, USA). Enhanced Chemiluminescence detection reagents were from KPL (Gaithersburg, MA, USA). (γ-^{32}P)ATP was from Amersham (Aylesbury, UK). The Perfuser syringe pump was purchased from Perfusor B. Braun (Melsungen, Germany). All other chemicals used were of the highest analytical grade.

Animals

The Institutional Animal Experimentation Ethics Committee approved all surgical procedures performed in this study, which followed established principles of laboratory animal care (NIH publication no. 85-23, revised 1985). Female Wistar rats weighing about 280 g were purchased from the Experimental Animal Center of Wuhan University and were kept at 22 °C with a 12 : 12 h light-dark cycle and a relative humidity of 55–60% during the whole experimental period. The rats had free access to water and standard chow pellet diet.

Prolonged glucose infusion into conscious rats

Glucose infusion (GI) was performed as previously described [27,28]. The age-matched rats undergoing the infusion studies were anesthetized with Ketamine (125 mg/100 g body weight, i.p.). Surgery was performed under sterile conditions. A 1-cm long incision was made between the sternum and the mandible; the right external jugular vein was then exposed by blunt dissection and dissected free of the surrounding connective tissue. The jugular vein was ligated cranially and the vein was incised distal to the ligature. A catheter filled with 50 IU heparin in 0.9% saline was introduced into the vein so that its tip laid just cranial to the right atrium. The catheter was then anchored by double ligation. The free end of the catheter was routed dorsally subcutaneous to the back of the neck. A second incision was then made and the catheter was externalized and fixed using an anchoring device. The incision was then sutured. The externalized catheter was routed through a flexible spring tether to a swivel and connected by an oscillating arm to a Perfusor syringe pump. The rats were housed singly in a metabolic cage and allowed to recover for 48 h after which GI (2.8 mol/L glucose) was started at a rate of 2 mL/h (group GR) *versus* 77 mmol/L saline infusion at 2 mL/h for controls (group C). Glucose and saline infusions were carried out at the same time. Blood samples were taken from the tail vein and glucose and insulin concentrations were determined as described [27].

Muscle isolation and preparation

After 5 or 15 days of continuous glucose or saline infusion, experiments were terminated by disconnecting the syringe pump. Rats were then immediately removed from the cage and then anesthetized by pentobarbital (100 mg/kg i.p.). The hind limb muscles were quickly excised before and 15 min after insulin injection (5 U/kg), dissected free of connective tissue and fat and chopped into small pieces. This step was carried out on ice. Chopped muscles were immediately frozen in liquid nitrogen and subsequently stored at −70 °C until further preparation.

2-deoxyglucose uptake assay

To investigate effects of PKB or PKCζ inhibition on glucose transport in skeletal muscle of glucose-infused rats after five days, we employed enzyme inhibition studies. Hindlimb soleus skeletal muscles were removed, blotted on gauze, and transferred to 25-mL flasks containing 2 mL of Krebs–Henseleit buffer (KHB) with 0.1% bovine serum albumen, 32 mM mannitol, and 8 mM glucose. The incubation studies of this muscle have been extensively described [29]. The flasks were incubated in a shaking water bath maintained at 30 °C for 1 h, and were continuously gassed with 95% O_2/5% CO_2. Muscles were initially incubated, respectively, in the presence or absence of myristoylated PKCζ peptide inhibitor (PSζ) (40 µM) or SH-6 (5 M), an inhibitor of Akt (PKB) for 1 h prior to incubation under basal condition or stimulation with insulin (13 nM) for 10 min. The muscles were then transferred to flasks containing 2 mL of KHB with 0.1% bovine serum albumen, 40 mM mannitol, 2 mM pyruvate, and the same additions as in the previous incubation. The flasks were incubated for 10 min at 30 °C to wash out glucose and the gas phase in the flasks was maintained at 95% O_2/5% CO_2. Following the above incubations, the muscles were rinsed by incubation in KHB – 0.1% bovine serum albumen – 40 mM mannitol with or without insulin for 10 min and blotted and transferred to flasks containing 1.5 mL of KHB with 1 mM 2-deoxy-[1,2-^{3}H] glucose (1.5 mCi/mmol), and 39 mM [1-^{14}C] mannitol (8 µCi/mmol) and the same additions as in the previous incubation. The flasks were incubated at 30 °C for 20 min and continuously gassed with 95%O_2/5%CO_2. Thereafter the muscles were frozen and stored at −80 °C until processed for measurement of 2-deoxyglucose (2-DOG) transport. Frozen muscles were dissolved and then processed, and the extracellular space and intracellular 2-DOG concentrations were determined as described previously [30]. Glucose transport activity is expressed as µmol/20 min/mL intracellular water.

Skeletal muscle subcellular fractionation

Cytosol and membrane fractions were separated as described [31]. Briefly, frozen samples were homogenized in a 10 : 1 volume to weight ratio of homogenization buffer [20 mM Tris pH 7.5, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethyleneglycol bis(2-aminoethyl-ether)teraacetic acid (EGTA), 25 µg/mL aprotinin, 25 µg/mL leupeptin, 10 mM NaF, 10% glycerol, 5 mM $MgCl_2$, 0.5 mM dithiothreitol] on ice using a glass on glass homogenizer. The sample was centrifuged for 20 min at 160 000 *g* in an ultracentrifuge. The supernatant was collected as the cytosolic fraction. Half the original volume of homogenization buffer was added to the pellet, with 0.5% Triton X-100. The sample was further homogenized on ice for 10–15 s and incubated on ice for 30 min. The homogenate was then centrifuged

at 160 000 *g* for 20 min. The resulting supernatant was collected as the PM fraction.

PI 3-kinase activity

Muscle pieces were immediately homogenized in a $10\times$ volume of ice-cold *buffer A* (in mM: 50 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 137 NaCl, 1 MgCl$_2$, 1 CaCl$_2$, 10 Na$_4$P$_2$O$_7$, 50 NaF, 2 EDTA, 40β-glycerophosphate, 2 Na$_3$VO$_4$, 0.2 phenylmethylsulfonyl fluoride (PMSF), and 1% NP-40, 10% glycerol, and 2 µg/mL aprotinin) and centrifuged at 15 000 *g* at 4 °C for 30 min. Protein concentration was determined using a commercial assay kit (Bio-Rad). The supernatants containing equal amounts of protein were immunoprecipitated overnight at 4 °C with anti-IRS-1 antibody or anti-phosphotyrosine antibody coupled to protein A Sepharose. The immunoprecipitates were collected and washed. PI 3-kinase activities in the immunoprecipitates were assayed as described [32].

PDK1 activity

A fragment of frozen muscle (25–30 mg) was homogenized in 500 µL of ice-cold buffer containing 50 mM Tris (pH 8.0), 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na$_4$P$_2$O$_7$, 10 mM β-glycerophosphate, 1 mM sodium vanadate, µM microcystin, 20 mM β-mercaptoethanol. Samples were solubilized by continuous stirring for 40 min at 4 °C and centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was immunoprecipitated for 2 h at 4 °C with anti-PDK1 antibody coupled to protein A/G Sepharose. The immune complex was washed and PDK1 activity was determined as described [33]. Radioactivity was counted using a Packard Tri-Card 2000 Ca liquid scintillation counter.

PKCζ activity

The remaining fragment of muscle (25–30 mg) was homogenized in 600 µL of ice-cold buffer containing 20 mM of Tris/HCl (pH 7.5), 250 mM sucrose, 1.2 mM EGTA, 20 mM β-mercaptoethanol, 1 mM PMSF, 5 µg/mL leupeptin, 5 µg/mL aprotinin, 1 mM Na$_3$VO$_4$, 1 mM Na$_4$P$_2$O$_7$, 1 mM NaF, 1% Triton-X 100, 0.5% nonidet, and 2 µg/mL pepstatin. Samples were solubilized by continuous stirring for 30 min and centrifuged at 3000 *g* for 15 min at 4 °C. The supernatant was immunoprecipitated with anti-PKCζ antibody coupled to protein A/G Sepharose. The immune complex was washed and suspended in *Buffer B* [50 mM Tris/HCl (pH 7.5), 5 mM MgCl$_2$, 100 µM Na$_3$VO$_4$, 100 µM Na$_4$P$_2$O$_7$, 1 mM NaF, and 100 µM phenylmethylsulfonyl fluoride]. Suspensions were then incubated for 8 min at 30 °C in 100 µL of *Buffer B* containing 3–5 µCi of [γ-^{32}P]ATP, 50 µM ATP, 4 µg of phosphatidylserine, and 40 µM [^{159}Ser]PKC-ε (AA153–164)-NH2 (Upstate

Biotechnology, Inc.), a preferred substrate for PKC-ζ. Reactions were stopped by addition of 10 mL of 5% acetic acid. PKCζ activity was determined as described [34].

Akt/PKB activity

PKB activity was measured as described by Kurowski *et al.* [20]. Muscles were homogenized in a $10\times$ volume of ice-cold *buffer C* (in mM: 20 Tris pH 7.5, 250 sucrose, 10 NaF, 5 EDTA, 1 β-glycerophosphate, 2 Na$_3$VO$_4$, 0.2 PMSF, 0.2 NH$_4$MbO$_4$, 1 dithiothreitol, with 1% NP-40 and 2 µg/mL aprotinin) and centrifuged as for *buffer A* (described above). The supernatants were immunoprecipitated overnight at 4 °C with anti-Akt/PKB antibody. The immunoprecipitates were collected on protein A Sepharose and washed. They were then incubated for 30 min at 25 °C in 40 µL of a reaction mix consisting of kinase buffer to which had been added 100 µM (γ-^{32}P) ATP (10 µCi/Sample) and 30 µM crosstide (Upstate Biotechnology). The reaction was stopped by spotting 5 µL of the mix on P81 phosphocellulose paper (Whatman) followed by 3×15 min washing with 1% H$_3$PO$_4$. The papers were dried, and the radioactivity was determined by exposure to BAS 2000 film (Fuji-Film, Tokyo, Japan). To investigate the inhibitory effects of PKCζ on PKB activity in skeletal muscle PM of glucose-infused rats after 5 or 15 days, PM fraction were incubated with 25 µM PSζ for an hour and PKB activity was measured as aforementioned.

Protein kinase expression and translocation

Thirty microgram aliquots from the cytosolic and particulate fraction were used in the determination of PKCζ expression and translocation. Aliquots (30–50 µg) of homogenate prepared for the PI 3-kinase assay were used for the determination of PI 3-kinase-p85, PDK1, PKCζ and PKB concentrations. Proteins were subjected to western blots using enhanced chemiluminescence - Plus, as described previously [35]. The intensity (area $\times$ density) of the individual bands on Western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area.

PDK1–PKB reaction and PKCζ–PKB association in the PM

Immunoprecipitation was used for the isolation of PDK1 and PKCζ. The protein concentrations of the membrane fractions (separated as described above) were measured and diluted with buffer to 10 µg protein/µL. Then 100 µL of the latter dilution were added to 10 µL Protein A Sepharose and 10 µL Sepharose and mixed. Anti-PDK1 or -PKCζ-antibodies (5 µg) were added and incubated overnight at 4 °C. Beads were then centrifuged at

500 rpm for 1 min. The pellet was washed three times and resuspended in 20 μL washing buffer. Eventually, Laemmli buffer was added and cups were heated at 98 °C for 10 min. Supernatants were then quantitatively transferred to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5 or 10% acrylamide), separated and detected as described above. PDK1–PKB reactions and PKCζ–PKB associations were determined using the anti-Akt/PKB -antibody.

GLUT$_4$ translocation in skeletal muscle

After 5 d or 15 d of GI, GLUT$_4$ translocation in skeletal muscle was measured using the method of Douen *et al.* [36].

Statistical analysis

All data are expressed as means ± standard error of the mean. Data were analysed using unpaired Student's *t* tests for the comparison of mean values. Data are also expressed as percentages of control animals. $p < 0.05$ was considered statistically significant.

The authors have full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Metabolic effects of continuous GI

Continuous GI for 10 days induced transient hyperglycaemia and persistent hyperinsulinaemia (Figure 1A). Hyperglycaemia peaked after 24 h of GI. Despite further continuous GI, plasma glucose concentrations returned to normal values on the fifth day compared with controls. Plasma glucose concentrations in rats receiving continuous GI was high relative to that in the control rats on the tenth day, but the difference did not reach statistic significance. Serum insulin concentration increased ∼20-fold after one day of GI, declined thereafter but remained significantly elevated up to the tenth day as compared to the control. Saline infusion affected neither plasma glucose nor insulin concentrations in control rats (Figure 1A). Serum insulin concentration recovered to normal and the plasma glucose increased in glucose-infused rats by the fifteenth day. Thus in this model the rats accommodated to systemic glucose oversupply and developed insulin resistance before the tenth day and type 2 diabetes by the fifteenth day. Because the metabolism of the rats changed from normoglycaemic/hyperinsulinaemic on day 5 to hyperglycaemic/normoinsulinaemic on day 15, subsequent studies were focused on these two time points. Variations of body weight were presented in Figure 1B. There was a general tendency for the animals to gain weight with increasing length of infusion of either type,

this was significant on day 5 of infusion. The animals gradually lost weight after day 5. The body weight in glucose-infused rats significantly reduced by day 15 as compared with day 0 of the same group or compared with the saline-infused controls at day 15.

In vivo effects of GI on PI 3-kinase expression and activity

Earlier studies with this model show that continuous GI induces increased glucose metabolism and insulin resistance in rat skeletal muscle [37–39]. Here, protein levels of the downstream signalling proteins IRS-1, PI 3-kinase-p85 was not affected, and no significant
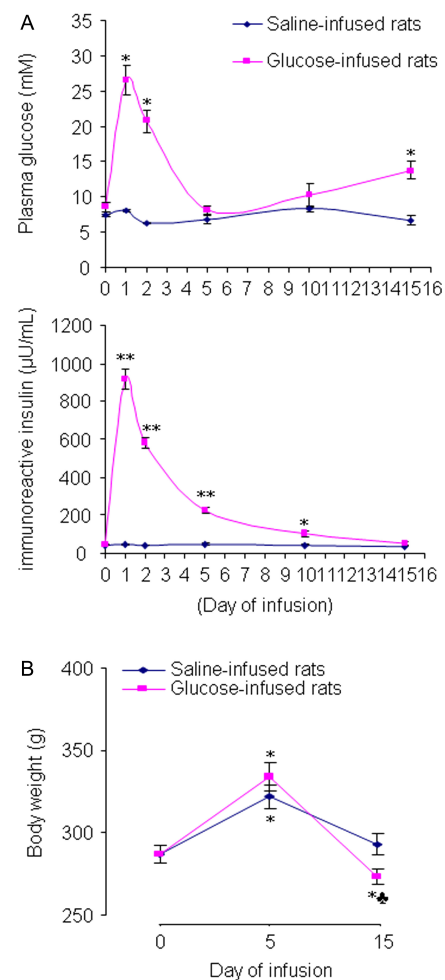
Figure 1. Effect of glucose infusion on plasma glucose, insulin concentrations and body weight. (A) The rats were treated with glucose infusion with the indicated time as described in Methods. Glucose and saline infusions were carried out at the same time. Blood samples were taken from the tail vein and glucose and insulin concentrations were determined as described in Methods. Data representing results of six independent experiments are expressed as means ± standard error of the mean. * $p < 0.05$; ** $p < 0.01$ glucose infusion *verus* corresponding control rats. (B) Values are shown as means ± standard error of the mean. * $p < 0.05$ *verus* Day 0; ♣ $p < 0.05$ *verus* saline-infused animals. This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

increases in basal IRS-1-associated PI 3-kinase activity and total phosphotyrosine-dependent PI 3-kinase activity were found in glucose-infused rats (Figure 2). Compared with control rats, rats exhibited a reduced response to insulin after either 5 or 15 days of GI. After insulin stimulation the IRS-1-associated PI 3-kinase activity and total phosphotyrosine-dependent PI 3-kinase activity of glucose-infused rats after 5 days and especially after 15 days were reduced significantly compared with control animals. Because activation of PI 3-kinase has been shown to be an essential step in insulin-dependent stimulation of glucose transport, these data show that the insulin resistance in this model can be traced down to the molecular level.

In vivo effects of GI on PDK1 protein production and activity

GI for five days significantly increased basal PDK1 activity about twofold (Figure 2D, E), but did not affect PDK1 protein expression. However, PDK1 activity was significantly lower in glucose-infused rats on day 15 than on day 5. Continuous saline infusion did not alter PDK1 activity or expression in control rats.

In vivo effects of GI on overall PKCζ protein levels and activity

To delineate the mechanism by which continuous changes in the extracellular glucose concentration might contribute directly to insulin resistance and diabetes, we determined PKCζ protein levels and activity in rat skeletal muscle after 5 and 15 days of GI. GI did not affect PKCζ protein levels (Figure 3A), but significantly increased basal PKCζ activity about twofold (Figure 3B) after 5 days. The PKCζ activity significantly decreased and was lower than that in the control rats at day 15 of continued GI. To elucidate which fraction is responsible for the increased PKCζ activity at day 5 infusion, we detected PKCζ activity in PM and cytosolic fraction. As indicated in Figure 3C, five days of GI resulted in dramatic increase of PKCζ activity in the cytosolic instead of PM
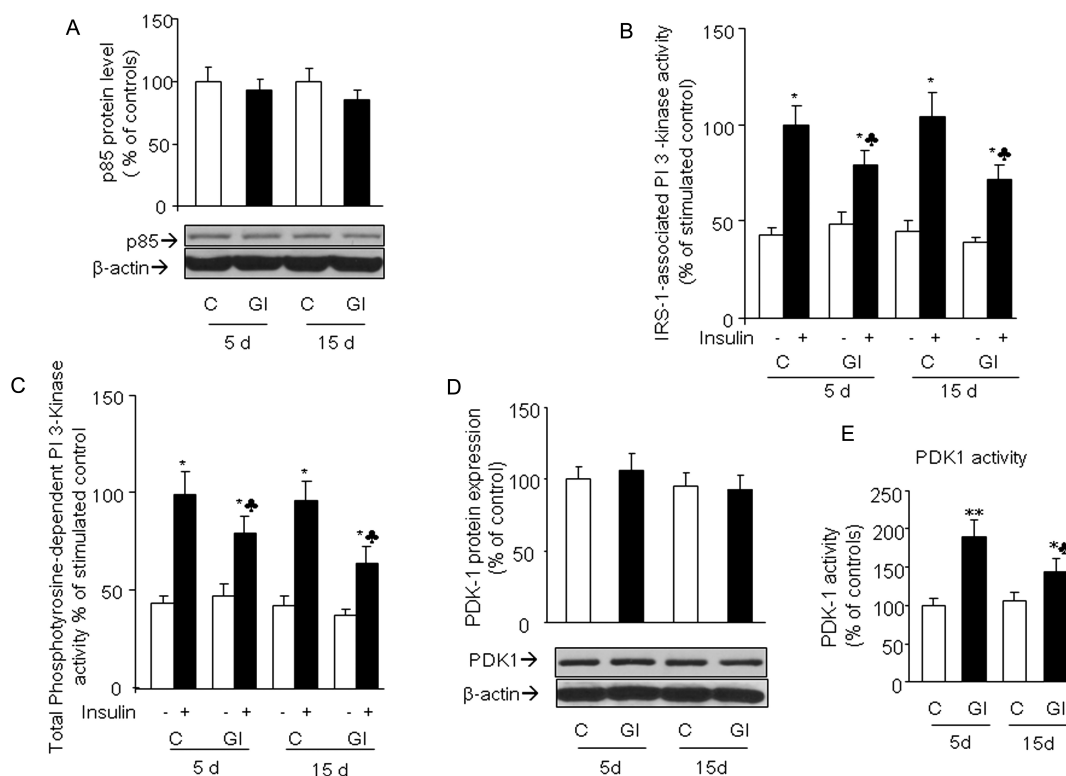
Figure 2. Total p85, phosphatidylinositol 3-kinase (PI 3-kinase) activity associated with insulin receptor substrate (IRS)-1 and total PI 3-kinase activity, PDK1 protein and activity in the skeletal muscle of control and glucose-infused rats. Homogenates were prepared from glucose-infused (GI) and control (C) rats and western blotting was performed to detect the p85 and PDK1. Protein, immunoprecipitated with anti-IRS-1 or anti-phosphotyrosine antibody, was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with anti-p85 antibody and determination of IRS-1-dependent PI 3-kinase activity and total PI 3-kinase activity. The other protein was immunoprecipitated with anti-PDK-1 antibody and subjected to determination of PDK1 activity as described in the Materials and Methods. (A) Representative immunoblot analysis showing p85 content (top) and quantification (bottom) of p85 protein levels by scanning densitometry. (B) PI 3-kinase activity associated with IRS-1. (C) Total PI 3-kinase activity. Data shown are means ± standard error of the means of four independent experiments. Values are expressed as percentages of controls. * $P < 0.05$ basal *verus* insulin-stimulated conditions; ♣, $p < 0.05$ *verus* corresponding control rats. (D) Representative immunoblot analysis showing the presence of PDK1 (top) and quantification (bottom) of PDK1 protein levels by scanning densitometry. (E) PDK1 activity. Data shown are means ± standard error of the means of four independent experiments. Values are expressed as percentages of controls. * $p < 0.05$; ** $p < 0.01$ *verus* controls, ♣, $p < 0.05$ *verus* GI on day 5

fraction, suggesting that PKCζ is primarily activated in the cytosol after glucose challenge.

Effects of PSζ or SH-6 on 2-DOG uptake in skeletal muscle of glucose-infused rats after 5 days

As shown in Figure 3D, the basal 2-DOG uptake dramatically elevated (approximately threefold) and maximally insulin-stimulated rates of 2-DOG uptake was impaired in 5 days of glucose-infused rats compared with saline-infused rats. To gain more insight into the mechanism of glucose transport in insulin-resistant state, we measured 2-DOG uptake in skeletal muscle pretreated with myristoylated PKCζ peptide inhibitor (PSζ) or SH-6, a specific inhibitor of Akt/PKB in 5 days of glucose-infused rats. 2-DOG uptake in skeletal muscle was reduced by 78% and 66% ($p < 0.05$), respectively, in PSζ-treated basal and insulin-stimulated groups compared with their corresponding non-treated groups. However, 2-DOG uptake was reduced by 19% and 18% ($p < 0.05$),

respectively, in SH-6-treated basal and insulin-stimulated groups compared with their corresponding non-treated groups. Combination of PSζ and SH-6 treatment caused a dramatic reduction of 2-DOG uptake, to a level of 10% of the values in non-treated groups both in basal and insulin-stimulated muscle (Figure 3D). These data suggest that PKCζ may be mainly responsible for glucose transport in skeletal muscle in insulin-resistant state. In addition, as indicated in Figure 3D, 2-DOG uptake was dramatically impaired at day 15 of GI relative to day 5.

The translocation of PKCζ

High glucose is known to activate PKC isoforms, which inhibit insulin signalling and stimulate GLUT4 translocation. Therefore, the cellular localization of the PKCζ isoform was studied to assess possible activation under these experimental metabolic conditions. An increase in PKCζ protein content was seen in the cytosolic fractions after five days of GI, although it did not reach statistical significance (Figure 4). However, the activated
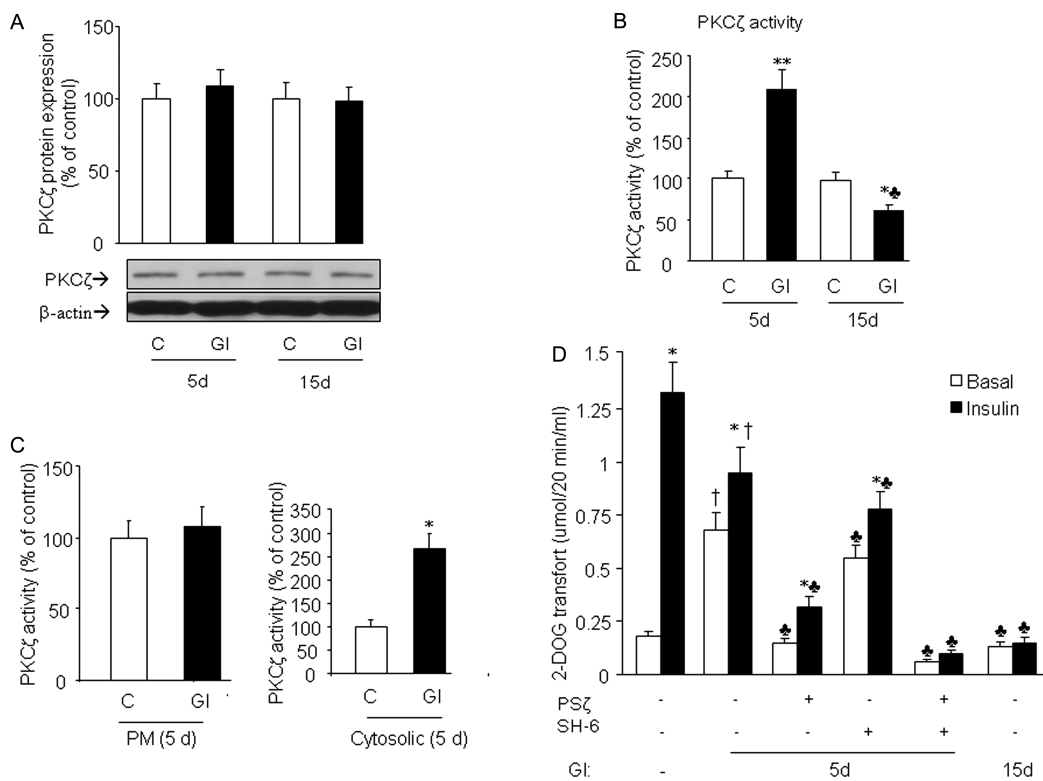

Figure 3. The active PKCζ, which is mainly located in cytosol after glucose challenge, is responsible for glucose transport in skeletal muscle in insulin-resistant state. Homogenates were prepared from glucose infused (GI) and control (C) rats and western blotting was performed to detect the PKCζ protein. Protein was immunoprecipitated with anti-PKCζ antibody coupled to protein A/G Sepharose and subjected to determination of PKCζ activity as described in the Materials and Methods. (A) Representative immunoblot analysis showing PKCζ presence (top) and quantification (bottom) of PKCζ protein levels by scanning densitometry. (B) PKCζ activity. Data shown are means ± standard error of the means of four independent experiments. Values are expressed as percentages of controls. * $p < 0.05$; ** $p < 0.01$ *verus* corresponding controls, ♣, $p < 0.05$ *verus* GI on day 5. (C) PKCζ activity in plasma membrane and cytosolic fraction. $n = 3$; * $p < 0.05$ *verus* control group. (D) Effects of PSζ or SH-6 on 2-DOG uptake in skeletal muscle of glucose-infused rats. The soleus muscles were removed and incubated in the presence or absence of myristoylated PKCζ peptide inhibitor (PSζ) (40 μM) or SH-6 (5 M), an inhibitor of Akt (PKB) for 1 h prior to incubation under basal conditions or stimulation with insulin (13 nM). 2-deoxyglucose uptake was measured as described in 'Materials and Methods.' Values are the means ± standard error of the means of four independent experiments. *, $p < 0.05$ basal *verus* insulin-stimulated conditions; ♣, $p < 0.05$ *verus* non-treated groups on day 5; †, $p < 0.05$ *verus* corresponding non-GI groups
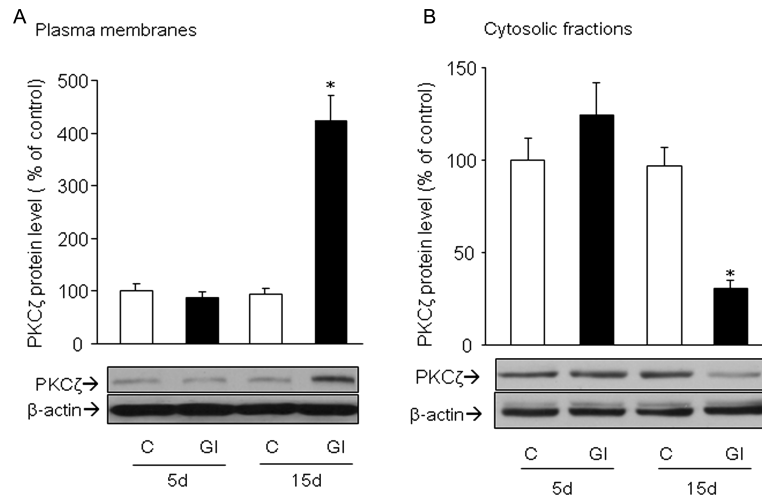
Figure 4. Effect of glucose infusion on the translocation of PKCζ in rat skeletal muscle. Hind limb skeletal muscles were prepared from glucose-infused or saline-infused (C) rats and the plasma membrane and cytosol fractions were obtained as described in the Materials and Methods. Representative western blots indicating PKCζ contents in plasma membrane (A) and cytosol fractions (B) are shown from a set of four independent experiments. The upper panels: Quantification of PKCζ protein levels in plasma membranes and cytosol fractions by scanning densitometry. Data are shown as means ± standard error of the means. Values are expressed as percentages of corresponding control values. * $p < 0.05$ *verus* corresponding control rats

PKCζ by GI is mainly located in the cytosol after five days of GI. Thereafter, PKCζ was enriched in the PM after 15 days of GI and the corresponding cytosolic fractions were depleted (Figure 4). Quantitatively, GI caused more than fourfold enrichment of PKCζ in the PM. Thus, continuous GI led to translocation of PKCζ from the cytosol to the PM.

In vivo effects of GI on Akt/PKB content and activity

The protein levels of Akt/PKB did not differ between the groups (Figure 5A). No significant increase in basal PKB activity was found in glucose-infused rats after

five days, whereas it was reduced to about 44% of controls after 15 days (Figure 5B). In control rats, insulin administration resulted in a noticeable increase in PKB activity (Figure 5B). GI after 5 days and 15 days resulted in reduced response to insulin, and the activity of Akt/PKB after insulin stimulation was decreased by 22% and 56% ($p < 0.05$), respectively, compared with values measured in control rats.

In vivo effects of GI on PDK1–PKB reaction and PKCζ–PKB association at PM

Binding studies have shown that PKB associates *in vitro* with the α, δ, and ζ isoforms of PKC [40]. We performed
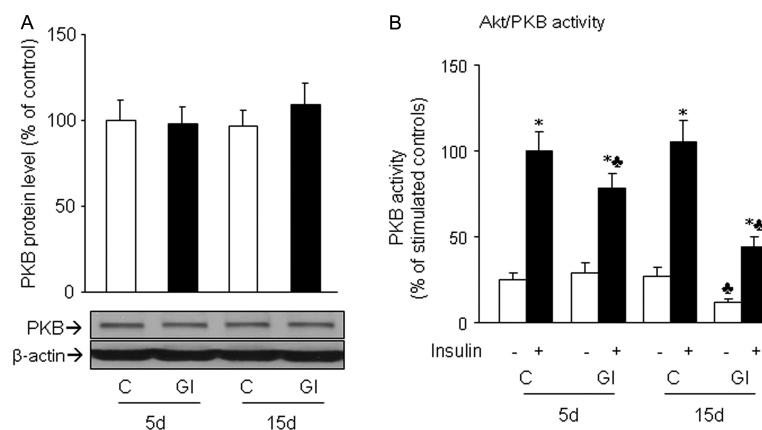
Figure 5. *In vivo* effects of GI on Akt/PKB protein content and activity in skeletal muscle of Wistar rats. The total lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with anti-Akt/PKB antibody (A). An equal amount of muscle protein was immunoprecipitated with anti-Akt/PKB followed by determination of Akt/PKB activity (B). (A) representative immunoblot analysis showing Akt/PKB presence (bottom) and quantification (top) of Akt/PKB protein levels by scanning densitometry. (B) Akt/PKB activity in skeletal muscle. Data are shown as means ± standard error of the means of three independent experiments. Values are expressed as percentages of controls. *, $p < 0.05$ basal *verus* insulin-stimulated conditions; ♣, $p < 0.05$ *verus* corresponding control rats

co-immunoprecipitation studies using PM fractions of skeletal muscle to investigate the possible interactions of PKCζ as well as PDK1 with PKB *in vivo*. Endogenous PDK1 and PKCζ were immunoprecipitated from membrane fractions. PKB was detected in the PKCζ and PDK1 immunoprecipitates using western blotting (Figure 6A, B). The amounts of PDK1–PKB reactions and PKCζ–PKB associations at PM were not different between the control rats and glucose-infused rats after five days of GI. Interestingly, GI for 15 days led to a significant threefold increase in PKCζ–PKB association at the PM, whereas at the same time, the PDK1–PKB reaction was reduced to approximate 40% of the controls (Figure 6A, B). These results clearly demonstrate that the association between PKCζ and PKB is negatively correlated with PDK1–PKB reactions at the PM. We propose that this demonstrates competitive inhibition.

Effects of PSζ on PKB activity in skeletal muscle PM of glucose-infused rats

To confirm whether or not PKCζ inhibited PKB activity after its membrane translocation, PM fraction of glucose-infused rats after 5 or 15 days were isolated and incubated with 25 µM PSζ for an hour and PKB activity was measured. The specific PKCζ inhibitor, PSζ, was used to selectively inhibit PKCζ activity. PKB activity in cytosol fraction could not be detected. PSζ did not alter PKB activity in PM of saline-infused rats (data not shown). Basal and insulin-induced PKB activity in PM was significantly lower in glucose-infused rats on day 15 than on day 5. As expected, basal and insulin-induced PKB activity in PM was considerably increased after PSζ treatment in glucose-infused rats on day 15 when compared with non-treated groups (Figure 6C). It is logical to speculate that the inhibition of PKB activity might, at least partly, attribute to PKCζ in PM. Taken together, these data show for the first time in skeletal muscle that PKCζ can inhibit PKB after translocation to PM.

In vivo effects of GI on GLUT4 translocation in skeletal muscle

GLUT4 protein levels were similar between control and glucose-infused rats (data not shown). GI for 5 days significantly increased GLUT4 protein levels in the PM but decreased the amount of GLUT4 protein in the low-density microsome fractions (Figure 7A, B), suggesting that glucose oversupply alone could contribute to increase in basal GLUT4 translocation at day 5. The insulin-stimulated translocation of GLUT4 from the low-density microsome to the PM fractions in skeletal muscle was blunted progressively from 5 days to 15 days of GI (Figure 7A, B).

Discussion

Hyperglycaemia impaires insulin signalling, but the underlying mechanism is not fully understood [41]. To address the molecular mechanisms underlying glucose-induced insulin resistance and diabetes, we employed the glucose-infused rat model. Inadequate pancreatic β-cell function is an essential component of all forms of diabetes. In our animal model, rats accommodating systemic glucose oversupply developed insulin resistance after 5 and 15 days, which can be traced down to the molecular level. Compared with control rats, rats exhibited a reduced response to insulin after either 5 or 15 days of GI. The insulin-stimulated IRS-1-associated PI 3-kinase activity and total phosphotyrosine-dependent PI 3-kinase activity (Figure 2B, C), insulin-stimulated DOG-uptake (Figure 3D), insulin-stimulated PKB activity (Figure 5B) and insulin-stimulated GLUT4 PM-expression (Figure 7B) were reduced significantly compared with control animals, clearly indicating insulin resistance at the level of muscle. To maintain glucose homeostasis, β-cells increase insulin secretion and release, and the resulting enhanced insulin concentrations can compensate for insulin resistance. With the development of insulin resistance, β-cell exhaustion occurs by depletion of the readily releasable pool of intracellular insulin following prolonged exposure to a secretagogue [42]. After prolonged exposure to elevated glucose, pancreatic β-cell function is progressively and irreversibly impaired by chronic hyperglycaemia and over secretion. This detrimental effect is referred to as 'glucotoxicity' [43]. At this point, β-cells decompensate and reduce insulin secretion, so hyperglycaemia and overt diabetes emerge, which was shown in our animal model after 15 days of GI (hyperglycaemia/normoinsulinaemia). In addition to inducing functional changes, chronic hyperglycaemia can also decrease β-cell mass by inducing apoptosis [44].

A novel mechanistic finding of the current study is that the normalization of blood glucose concentration in insulin resistance state was mediated at least partially by an increased translocation of the GLUT4 protein to the PM of skeletal muscle. The insulin-signalling transduction on day 5, as assessed by the determination of protein amounts and basal activities of PI 3-kinase and PKB, was not further increased when compared to control rats. PI 3-kinase activity and PKB/Akt activity after stimulation with exogenous insulin were impaired in skeletal muscle of glucose-infused rats after five days. Furthermore, the effect of glucose on PKCζ was independent of changes in PI 3-kinase activity, and occurred in parallel with an increase in PDK1 activity. Our findings are consistent with an earlier report of Steiler *et al.* that basal PKCζ activity was increased under hyperglycaemic conditions in Goto-Kakizaki and Wistar rats [45]. This finding of increased PKCζ activity was confirmed *in vitro* in isolated soleus muscle exposed to high extracellular glucose, and occurred concomitant with an increase in
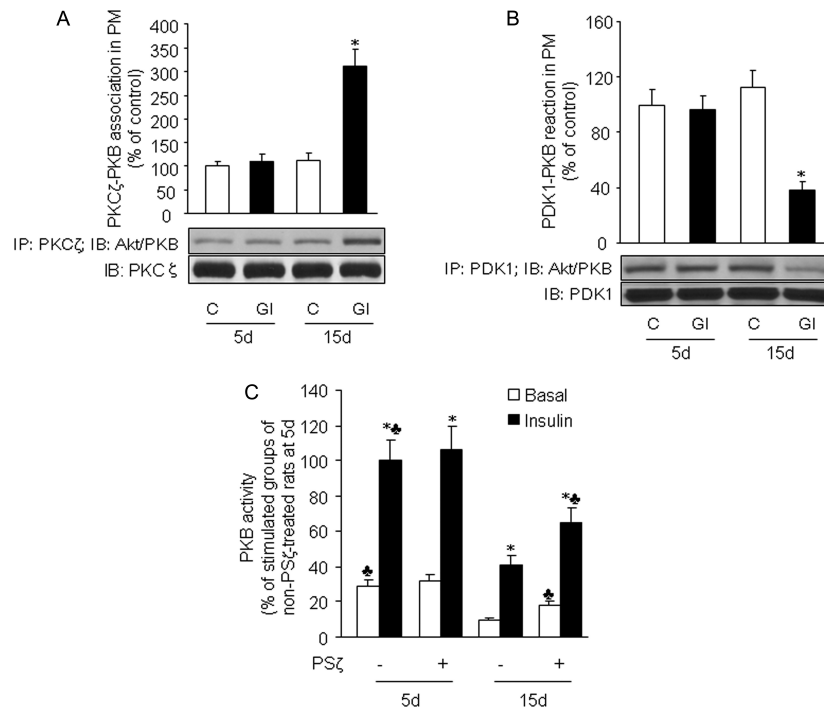
Figure 6. The translocation of PKCζ to plasma membrane after 15 days of GI increases PKCζ-PKB association and inhibits PKB activity. The membrane fractions of skeletal muscle were prepared and incubated with antibodies against PDK1 or PKCζ as described in the Materials and Methods. Immunoprecipitates were analysed for the presence of PKB by western blotting using anti-Akt/PKB monoclonal antibody. (A) Representative immunoblot analysis showing PKCζ–PKB association (top) and quantification (bottom) of PKCζ–PKB associated protein levels by scanning densitometry. (B) Representative immunoblot analysis indicating PDK1–PKB reactions (top) and quantification (bottom) of PDK1–PKB associated protein levels by scanning densitometry. Data shown are means ± standard error of the means of three independent experiments. Values are expressed as percentages of controls. ** $p < 0.01$ *verus* corresponding control rats. C, Effects of PSζ on PKB activity in skeletal muscle plasma membrane of glucose-infused rats. Plasma membrane fraction of glucose-infused rats after five or 15 days were isolated and incubated with 25 μM PSζ for an hour and PKB activity was measured as described in 'Materials and Methods.' Data shown are means ± standard error of the means of four independent experiments. Values are expressed as percentages of insulin-stimulated groups of non-PSζ-treated plasma membrane of glucose-infused rats after 5 days. *, $p < 0.05$ basal *verus* insulin-stimulated conditions; ♣, $p < 0.05$ *verus* non-PSζ-treated groups of glucose-infused rats after 15 days

PDK1 activity. Their study also authenticated that acute hyperglycaemia leads to a parallel increase in PDK-1 and PKCζ phosphorylation/activity via a PI 3-kinase-PKB/Akt-independent mechanism. Taken together, these data suggest that the concentration of glycaemia may directly regulate PKCζ activity in skeletal muscle. In the present study, when PKCζ was inhibited using a specific inhibitor, PSζ, basal and insulin-induced 2-DOG uptake in skeletal muscle of glucose-infused rats after 5 days were reduced by 78% and 66%, respectively. Thus, it is mainly PKCζ rather than PKB that leads to increases in translocation of GLUT4 to the PM of skeletal muscle, which maintains normoglycaemia in the insulin-resistant state.

PDK1 is a downstream target of PI 3-kinase that was first described as an activator of PKB/Akt [46] and was later shown to activate members of the AGC kinase superfamily including PKCζ [47]. However, glucose increases activity of PKCζ rather than PKB/Akt indicating the existence of a divergence in the effects of PDK1 on these downstream substrates in the insulin-resistant state. The intracellular location of the temporary complex formed by PDK1 and its substrates can determine substrate specificity. Studies in 3T3 L1 adipocytes, in which wild type or PM-targeted

PDK1 are overexpressed, show that full activation of PKB/Akt requires PDK1 translocation to the PM, whereas, PKCζ can be fully activated independently of insulin by overproduction of wild-type cytosolic PDK1 [48]. Thus, differences in the intracellular compartmentalization of PDK1 may provide a mechanism for these divergent effects on PKB/Akt and PKCζ.

PKCζ was originally discovered as a unique PKC isotype [49]. To date in mammals, it is classified into the atypical PKC subfamily, based on its structural similarity to PKC λ/ι. Here we have shown that activation of PDK1 and PKCζ can be regulated by changes in the extracellular glucose concentration. Intriguingly, the activated form of PKCζ is found in the cytosol after five days of GI. These results provide proof of concept for the first time that when activated, PKCζ mainly retains in the cytosol rather than immediately translocates. Given that 5 day of GI increases PKCζ activity and glucose uptake, which is coincident with an increased GLUT4 translocation, we speculate that the activated PKCζ contributes to the translocation of GLUT4 to PM and maintenance of normoglycaemia. Taken together, this striking activation of PKCζ in the cytosol seems to serve as a compensatory mechanism for maintaining glucose homeostasis in the
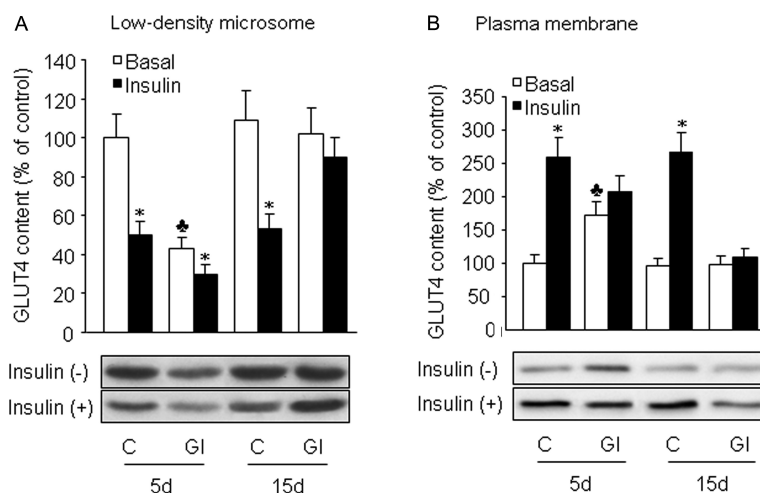
Figure 7. Effects of glucose-infusion on GLUT4 translocation in skeletal muscle tissues from Wistar rats. The plasma membrane and low-density microsome fractions isolated from skeletal muscles of saline-infused (C) or glucose-infused (GI) rats were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with anti-GLUT4 antibody. Representative immunoblot analyses showing GLUT4 protein levels (top) and quantified data (bottom) of GLUT4 protein in low-density microsome (A) and plasma membrane (B) fractions. Results are expressed as means ± standard error of the means of three independent experiments. Values are expressed as percentages of controls. * $p < 0.01$ verus basal groups; ♣ $p < 0.05$ verus control rats

insulin-resistant state. The insulin-signalling pathway on day 15 of GI, assessed by determination of the activities of PDK1, PKCζ, and PKB as well as translocation of GLUT4 to the PM, was decreased compared with those seen on day 5. A particularly notable aspect of the present studies was the translocation of PKCζ from the cytosol to PMs on day 15. With this move, the association of PKCζ with PKB is increased, accompanied by reduced reactions between PDK1 and PKB in the membrane and inhibition of PKB activity. Our results suggest that PKB activity was impaired by PKCζ in PM (Figure 6). The combination of PKCζ as well as PDK1 with PKB in PM demonstrates competitive inhibition. Therefore, we can not exclude the possibility that the inhibition of PKB activity is partly due to the decrease in reactions between PDK1 and PKB in the PM. In addition, the activity of PKCζ per se was compromised because of the increased association between PKCζ and PKB. Whether PKCζ is directly inhibited by PKB at the PM remains to be determined. The reduced activity of PKCζ and PKB leads to markedly blunted translocation of GLUT4 from low-density microsome to PM fractions in the skeletal muscle and eventual hyperglycaemia in rats with overt type 2 diabetes. Thus, it seems logical to assume that, when translocating to the PM, PKCζ seems to serve primarily as a negative feedback inhibitor of glucose transport and maintain intracellular glucose homeostasis under normal physiological conditions. However, once the translocation is excessively activated, hyperglycaemia and overt diabetes will emerge. Thus, the long-term consequence of changed PDK1 and PKCζ activity/localization should be considered in the context of diabetes mellitus.

In summary, we provide evidence that translocation of PKCζ in skeletal muscle may play a vital role in the development of type 2 diabetes.

Acknowledgements

Competing interests

None declared.

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