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Gliclazide increases insulin receptor tyrosine phosphorylation but not p38 phosphorylation in insulin-resistant skeletal muscle cells

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Summary

Sulfonylurea drugs are used in the treatment of type 2 diabetes. The mechanism of action of sulfonylureas is to release insulin from pancreatic cells and they have been proposed to act on insulin-sensitive tissues to enhance glucose uptake. The goal of the present study was to test the hypothesis that gliclazide, a second-generation sulfonylurea, could enhance insulin signaling in insulin-resistant skeletal muscle cells. We demonstrated that gliclazide enhanced insulin-stimulated insulin receptor tyrosine phosphorylation in insulin-resistant skeletal muscle cells. Although insulin receptor substrate-1 tyrosine phosphorylation was unaffected by gliclazide treatment, phosphatidylinositol 3-kinase activity was partially restored by treatment with gliclazide. No increase in 2-deoxyglucose uptake in insulin-resistant cells by treatment with gliclazide was observed. Further investigations into the mitogen-activated protein kinase (MAPK) pathway revealed that insulin-stimulated p38 phosphorylation was impaired, as compared with extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), which were phosphorylated normally in insulin-resistant cells. Treatment with gliclazide could not restore p38 phosphorylation in insulin-resistant cells. We propose that gliclazide can regulate part of the insulin signaling in insulin-resistant skeletal muscle, and p38 could be a potential therapeutic target for glucose uptake to treat insulin resistance.

Key words: insulin resistance, skeletal muscle, insulin signaling, glucose uptake, gliclazide, p38.

Introduction

Sulfonylurea drugs are widely used for the treatment of type 2 diabetes. The primary mechanism of action of the sulfonylurea class of drugs is the acute stimulation of insulin secretion by the pancreas (Lebovitz, 1984). However, additional effects on enhanced insulin-mediated glucose uptake both *in vivo* (Bak et al., 1989; Firth et al., 1986; Kolterman et al., 1984) and *in vitro* in peripheral tissue have been reported (Cooper et al., 1990; Rogers et al., 1987; Tsiani et al., 1995), although the mechanism of action for glucose uptake is not clear. Studies have shown increased glucose uptake owing to post-receptor effects after treatment with sulfonylureas (Bak et al., 1989; Pulido et al., 1996). One study concluded that the effect of insulin is enhanced while insulin binding remains unaltered following exposure of adipose tissue to tolazamide under *in vitro* conditions (Maloff and Lockwood, 1981). Santos et al. (2000) recently reported that erythrocyte insulin receptor (IR) tyrosine kinase activity is increased in glyburide-treated patients with type 2 diabetes. The present study was designed to determine whether gliclazide, a second-generation sulfonylurea, could directly act on skeletal muscle to enhance insulin signaling.

Insulin stimulation results in the activation of two distinct pathways involved in metabolic regulation: the phosphatidylinositol 3-kinase (PI 3-kinase) pathway and the mitogenic signaling pathway [mitogen-activated protein kinase (MAPK) pathway]. The PI 3-kinase pathway (Farese, 2001) and, more recently, p38 MAPK activation have been implicated in glucose uptake (Konrad et al., 2001; Somwar et al., 2000, 2001a). Insulin has been shown to activate p38 in skeletal muscle cells (Somwar et al., 2000). In the present study, the effect of gliclazide was also studied on the IR downstream pathway and on all three MAPKs (p38 MAPK, JNK and ERK).

Materials and methods

Materials

Mouse skeletal muscle cell line C2C12 was kindly provided by Dr H. Blau, Stanford University, School of Medicine, Stanford, CA, USA and Dr J. Dhawan, CCMB, Hyderabad, India. Nutrient Mixture F-12 Ham, MCDB201 medium, bovine albumin (cell-culture grade) and protein A-agarose were obtained from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO-BRL (Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from Biological Industries (Kibbutz Beit

Haemek, Israel). Bovine insulin and SB203580 were purchased from Calbiochem, La Jolla, CA, USA. Anti-pp38 (phospho-specific p38), anti-pERK (phospho-specific extracellular-signal-regulated kinase) and anti-JNK (c-Jun N-terminal kinase) antibodies were purchased from New England Biolabs (Beverley, MA, USA). Monoclonal anti-phosphotyrosine, anti-IR-β, anti-IRS-1 (insulin receptor substrate 1), anti-pJNK, anti-p38, anti-ERK and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [γ-^{32}P]ATP was purchased from Bhaba Atomic Research Centre (Mumbai, India). 2-deoxy-D-glucose-1-^{3}H and L-α-phosphatidylinositol were purchased from Sigma. Thin-layer chromatography (TLC) plates were obtained from Merck, Dermstadt, Germany (gift from Dr K. K. Bhutani, NIPER). Other reagents were obtained from Sigma, Roche Molecular Biochemicals (Mannheim, Germany) and BioRad (Hercules, CA, USA).

Cell culture and treatment

C2C12 cells were cultured in DMEM supplemented with 15% FCS and antibiotics (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) in 5% CO_2 at 37°C. The cells were differentiated in an equal mixture of two serum-free media (MCDB201 and F-12 Ham medium) along with 0.05% bovine serum albumin (BSA) in the absence (MF) or chronic presence (MFI) of 100 nmol l^{-1} insulin for three days. The media was changed after every 12 h. Gliclazide (2 mol l^{-1}) was added during the last 24 h of differentiation, where indicated. Gliclazide was dissolved in DMSO (dimethyl sulfoxide), and control samples also received an equal amount of DMSO.

Preparation of extracts of C2C12 muscle cells for immunoblotting and immunoprecipitation

Media in the differentiated cells were changed 1 h before the start of an experiment. The cells were washed twice with Krebs-Ringer phosphate buffer [KRP; 10 mmol l^{-1} phosphate (pH 7.2), 136 mmol l^{-1} NaCl, 4.7 mmol l^{-1} KCl, 1.25 mmol l^{-1} $CaCl_2$, 1.25 mmol l^{-1} $MgSO_4$] containing 5 mmol l^{-1} glucose and 0.05% BSA. The cells were further incubated twice with KRP buffer at 37°C for 30 min. The cells were then stimulated with 100 nmol l^{-1} insulin for 5 min at 37°C or left unstimulated. The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer [50 mmol l^{-1} Hepes (pH 7.4), 150 mmol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EGTA, 10 mmol l^{-1} sodium pyrophosphate, 50 mmol l^{-1} sodium fluoride, 50 mmol l^{-1} β-glycerophosphate, 1 mmol l^{-1} Na_3VO_4, 1% Triton X-100, 2 mmol l^{-1} phenylmethylsulfonyl fluoride, 10 μg ml^{-1} each of leupeptin, aprotonin and soyabean trypsin inhibitor]. Lysis was carried out at 4°C for 30 min. Cell lysate were clarified at 16,000 ***g*** at 4°C for 15 min. 500 μg of protein was immunoprecipitated with antibody against either anti-IR-β or anti-IRS-1 with the addition of protein A-agarose. The cell lysates or immunoprecipitates were boiled with Laemmli sample buffer (Laemmli, 1970) for 5 min, resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to PVDF (polyvinylidene difluoride) membranes. The membranes were blocked and incubated with the indicated antibodies, followed by incubation with HRP-conjugated secondary antibodies. The bands were visualized using enhanced chemiluminescence. Blots were stripped in stripping buffer [62.5 mmol l^{-1} Tris-HCl (pH 6.7), 2% SDS and 100 mmol l^{-1} β-mercaptoethanol] at 50°C for 30 min and reprobed with antibodies against either IR-β or IRS-1.

PI 3-kinase assay

To study PI 3-kinase activity associated with IRS-1, 1 mg of cell lysate was used. Cell lysates were prepared after 10 min of 100 nmol l^{-1} insulin stimulation. Immunoprecipitates were washed twice with lysis buffer and twice with 10 mmol l^{-1} Tris-HCl (pH 7.5), 100 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA and 100 μmol l^{-1} Na_3VO_4. Immunoprecipitates were resuspended in 50 μl of 20 mmol l^{-1} Hepes (pH 7.5), 180 mmol l^{-1} NaCl followed by addition of 25 μl of assay buffer [28 mmol l^{-1} Hepes (pH 7.5), 50 mmol l^{-1} NaCl, 0.15% (v/v) Nonidet P40, 12.5 mmol l^{-1} $MgCl_2$, 0.4 mmol l^{-1} EGTA, 0.8 mg ml^{-1} L-α-phosphatidylinositol, 50 μmol l^{-1} [γ-^{32}P]ATP (370 Bq per assay)]. Reactions were terminated after 15 min at 30°C by the addition of 50 μl of 2 mol l^{-1} HCl followed by 160 μl of chloroform, vortexed and centrifuged briefly. 60 μl of the lower phase was applied to TLC plates. TLC plates were developed in $CHCl_3$:CH_3OH:NH_4OH:H_2O (60:47:11:5 by volume), dried and visualized by autoradiography.

2-deoxyglucose uptake

After washing with KRP buffer, cells were stimulated with 100 nmol l^{-1} insulin in KRP buffer without glucose for 15 min. 2-deoxyglucose (2-DOG) uptake (7.4 Bq in 1 μmol l^{-1} of unlabelled 2-DOG) was added and cells were incubated for 10 min. Cells were washed in ice-cold PBS three times and were solubilized in 0.1 mol l^{-1} NaOH. Protein concentration was measured in each sample by the bicinchoninic acid (BCA; Smith et al., 1985) method followed by liquid scintillation counting. The uptake measurement was made in duplicate. The results were corrected for non-specific uptake in the presence of 10 μmol l^{-1} cytochalasin B. Non-specific uptake and absorption were always <10% of the total uptake.

Densitometric analysis

Densitometric analysis of the western blots was performed using a GS-670 Imaging Densitometer (BioRad) and Molecular Analyst software (version 1.3). The relative values of the samples were determined by giving an arbitrary value of 1.0 to the control samples.

Statistical analysis

The data are expressed as means ± S.E.M. For comparison of two groups, *P*-values were calculated by two-tailed unpaired student's *t*-test. In all cases, $P<0.05$ was considered to be statistically significant.

Results

Effect of gliclazide on tyrosine phosphorylation of IR-β

A 4 mmol l^{-1} concentration of gliclazide has been shown to enhance glucose uptake in L6 cells (Tsiani et al., 1995). To determine whether gliclazide can enhance insulin signaling in C2C12 skeletal muscle cells, MF (differentiated in the absence of insulin) and MFI (differentiated in the chronic presence of insulin) myotube cells were treated with various concentrations of gliclazide (0.02 mmol l^{-1}, 0.2 mmol l^{-1} or 2 mmol l^{-1}) during the last day of differentiation. Cell lysates were prepared and used to immunoprecipitate IR-β. Samples were run on SDS-PAGE and immunoblotted against phosphotyrosine. Insulin-stimulated tyrosine phosphorylation of chronically insulin-treated cells (MFI) was impaired as compared with control cells (MF) (Fig. 1A). Neither 0.02 mmol l^{-1} nor 0.2 mmol l^{-1} gliclazide had any effect on the tyrosine phosphorylation of IR; however, 2 mmol l^{-1} gliclazide stimulated tyrosine phosphorylation of IR-β maximally (data not shown). Therefore, a 2 mmol l^{-1} concentration was used for further experiments. Concentrations of gliclazide above 2 mmol l^{-1} resulted in cell lifting and death. 2 mmol l^{-1} gliclazide treatment resulted in 53% stimulation in tyrosine phosphorylation of IR under the resistant conditions (MFI) as compared with untreated MFI cells, and a 35% increase in tyrosine phosphorylation of IR was observed in MF cells as compared with untreated MF cells (Fig. 1C). Chronic insulin treatment resulted in downregulation of IR-β expression by 80%; however, gliclazide did not affect the expression of IR-β (Fig. 1B). Inoue et al. (1996) reported that chronic insulin treatment leads to a 90% reduction in IR-β expression. Data suggest that gliclazide enhances IR tyrosine phosphorylation in insulin-resistant skeletal muscle.

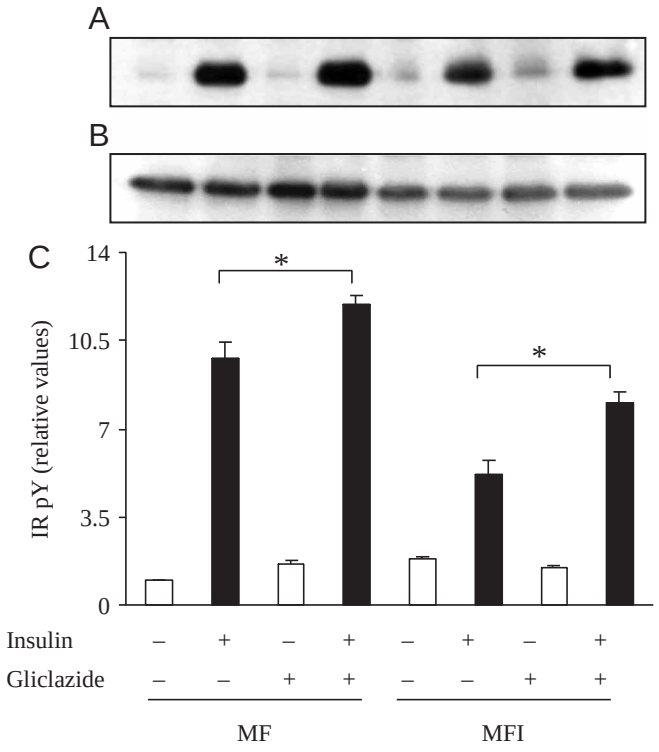

Fig. 1. Effect of the chronic presence of insulin and gliclazide on tyrosine phosphorylation of insulin receptor β (IR-β) in C2C12 myotubes. Gliclazide (2 mmol l^{-1}) was added during the last day of differentiation for 24 h to C2C12 cells differentiated in the absence (MF) or in the chronic presence (MFI) of insulin. The cells were then stimulated with 100 nmol l^{-1} insulin for 5 min and lysed. Cell lysate was immunoprecipitated (IP) with antibodies against IR-β and western immunoblotted (IB) with anti-phosphotyrosine (pTyr) antibody (A). The blots were stripped and reprobed with IR-β (B). Experiments were repeated three times and representative blots are shown. Phosphorylation levels of IR (C) were quantified by densitometry and expressed relative to MF (control) samples. Error bars represent the S.E.M. of three independent experiments (*P<0.05).

Effect of gliclazide on tyrosine phosphorylation of IRS-1

As insulin-stimulated IR tyrosine phosphorylation was increased by gliclazide, we determined tyrosine phosphorylation of IRS-1, as IRS-1 is the major molecule of IR signaling in skeletal muscle (Cusi et al., 2000). MF and MFI cells were treated with 2 mmol l^{-1} gliclazide and then lysed and immunoprecipitated with anti-IRS-1 antibody followed by

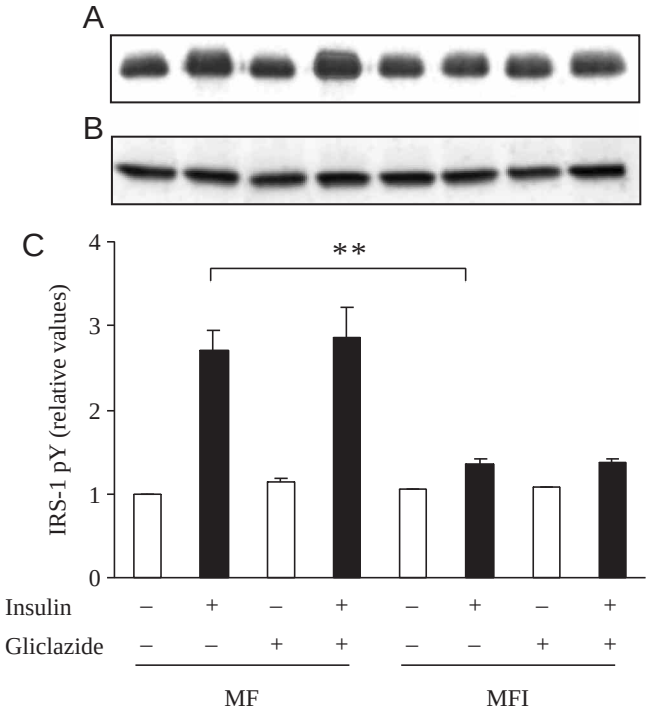

Fig. 2. Effect of the chronic presence of insulin and gliclazide on tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) in C2C12 myotubes. Samples were treated as described in Fig. 1. Cell lysate was immunoprecipitated (IP) with antibodies against IRS-1 and western immunoblotted (IB) with anti-phosphotyrosine (pTyr) antibody (A). The blots were stripped and reprobed with IRS-1 (B). Experiments were repeated three times and representative blots are shown. Phosphorylation levels of IRS-1 (C) were quantified by densitometry and expressed relative to MF (controls, differentiated in the absence of insulin) samples. Error bars represent the S.E.M. of three independent experiments (**P<0.01). MFI, cells differentiated in the chronic presence of insulin.

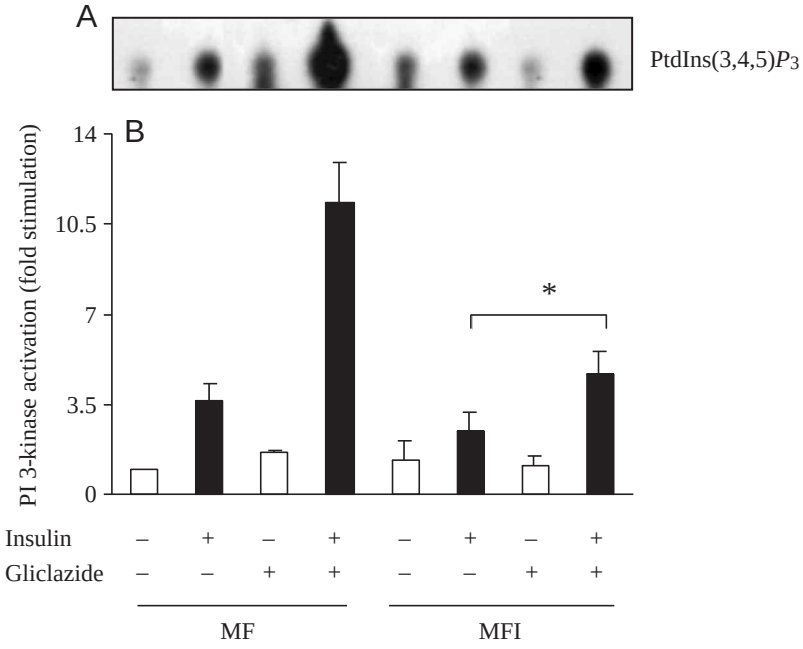

Fig 3. Effect of the chronic presence of insulin and gliclazide on phosphatidylinositol 3-kinase (PI 3-kinase) activity in C2C12 myotubes. C2C12 cells, differentiated in the absence (MF) or in the chronic presence (MFI) of insulin, were washed with Krebs-Ringer phosphate (KRP) buffer as described in Materials and methods, followed by stimulation with insulin (100 nmol l^{-1}) for 10 min. PI 3-kinase activity in anti-IRS-1 (insulin receptor substrate 1) immunoprecipitates was measured as described under Materials and methods. Cells were treated with 2 mmol l^{-1} gliclazide during the last 24 h of differentiation. A representative autoradiogram for three independent experiments is shown (A). The relative density of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] spots (B) was quantified by densitometry and expressed relative to control samples (unstimulated insulin samples). Error bars represent the S.E.M. of three independent experiments (*P<0.05).

immunoblotting with phosphotyrosine. Insulin-stimulated IRS-1 phosphorylation was impaired in MFI cells as compared with MF cells (Fig. 2A). Treatment with gliclazide did not increase IRS-1 tyrosine phosphorylation (Fig. 2C) in sensitive (MF) or in insulin-resistant skeletal muscle cells (MFI). Expression of IRS-1 was unaffected by treatment with the chronic presence of either insulin or gliclazide (Fig. 2B).

Effect of gliclazide on PI 3-kinase activity

Stimulation of cells with insulin causes activation of PI 3-kinase, which results in glucose uptake. PI 3-kinase has been shown to play an important role in insulin-stimulated glucose uptake in insulin-responsive tissues (Farese, 2001). Therefore, we determined whether gliclazide could affect PI 3-kinase activity. Insulin-stimulated PI 3-kinase activity was severely reduced in MFI cells as compared with MF cells (Fig. 3A). The treatment of cells with 2 mmol l^{-1} gliclazide was able to restore IRS-1-associated PI 3-kinase activity, and a 95% increase in activity was observed in MFI cells as compared with untreated insulin-stimulated MFI cells. A 210% increase in drug-treated MF cells stimulated with insulin was observed as compared with untreated insulin-stimulated MF cells (Fig. 3B). These data suggest that gliclazide can restore PI 3-kinase activity of insulin-resistant myotubes to the untreated insulin-stimulated sensitive conditions, although there was no effect on IRS-1 tyrosine phosphorylation.

Effect of gliclazide on 2-DOG uptake

Gliclazide has been shown to enhance glucose uptake in L6 skeletal muscle cells (Tsiani et al., 1995). So, the effect of the chronic presence of insulin and gliclazide was tested on basal and insulin-stimulated glucose uptake in MF and MFI cells. Insulin stimulated a 20% increase in 2-DOG uptake in control samples (MF); however, we could not observe any increase in 2-DOG uptake in the C2C12 cells chronically treated with insulin (MFI) (Fig. 4). Treatment with gliclazide could not restore the insulin-mediated 2-DOG uptake in resistant cells. However, the MF cells maintained sensitivity to the insulin after treatment with gliclazide.

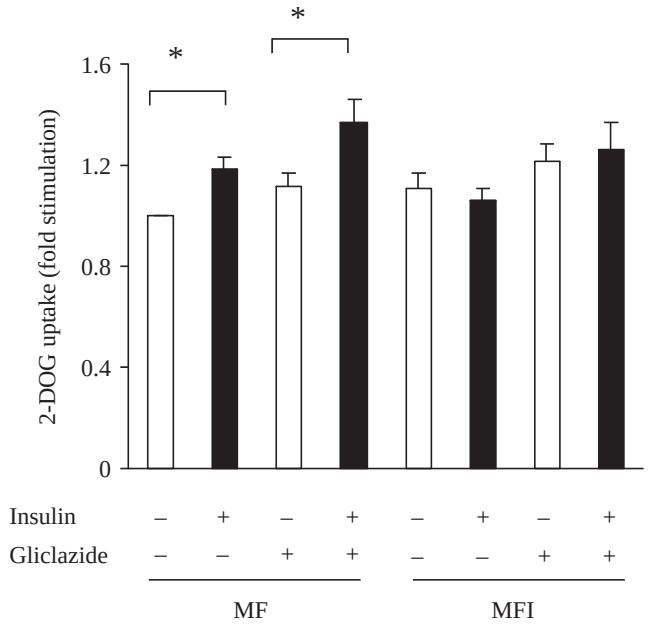

Fig. 4. Effect of the chronic presence of insulin and gliclazide on 2-deoxyglucose (2-DOG) uptake in C2C12 myotubes. C2C12 cells, differentiated in the absence (MF) or in the chronic presence (MFI) of insulin, were washed with Krebs-Ringer phosphate (KRP) buffer as described in Materials and methods, followed by stimulation with insulin (100 nmol l^{-1}) for 15 min. [^{3}H]2-DOG uptake was measured as described in Materials and methods. Cells were treated with 2 mmol l^{-1} gliclazide during the last 24 h of differentiation. Error bars represent the S.E.M. of four independent experiments (*P<0.05).

Effect of gliclazide on MAPK phosphorylation in insulin-resistant skeletal muscle

So far, glucose uptake by insulin has been shown to be mediated by two different pathways: PI 3-kinase is the pathway that has been widely implicated in glucose uptake (Farese, 2001; Cefalu, 2001), and, more recently, the p38 pathway (Konrad et al., 2001; Somwar et al., 2000, 2001a) has also been shown to play a role in glucose uptake. Although gliclazide was able to increase PI 3-kinase activity in MFI cells, we could not observe any increase in insulin-stimulated glucose uptake in resistant cells after gliclazide treatment. Therefore, we determined the effect of gliclazide on p38 activation and related MAP kinases to find out whether they play any role in insulin resistance. The results (Fig. 5A,C,D,F) show that insulin stimulation increased ERK and JNK activation in both MF and MFI cells. The insulin-stimulated activation of p38 was impaired in MFI cells, whereas p38 was activated by insulin in MF cells (Fig. 5G,I). Treatment with gliclazide could not restore the activation of p38 in MFI cells; however, insulin was able to activate p38 in MF cells. Gliclazide treatment resulted in an increase in JNK activation; however, the increase was not statistically significant. The expression of all three

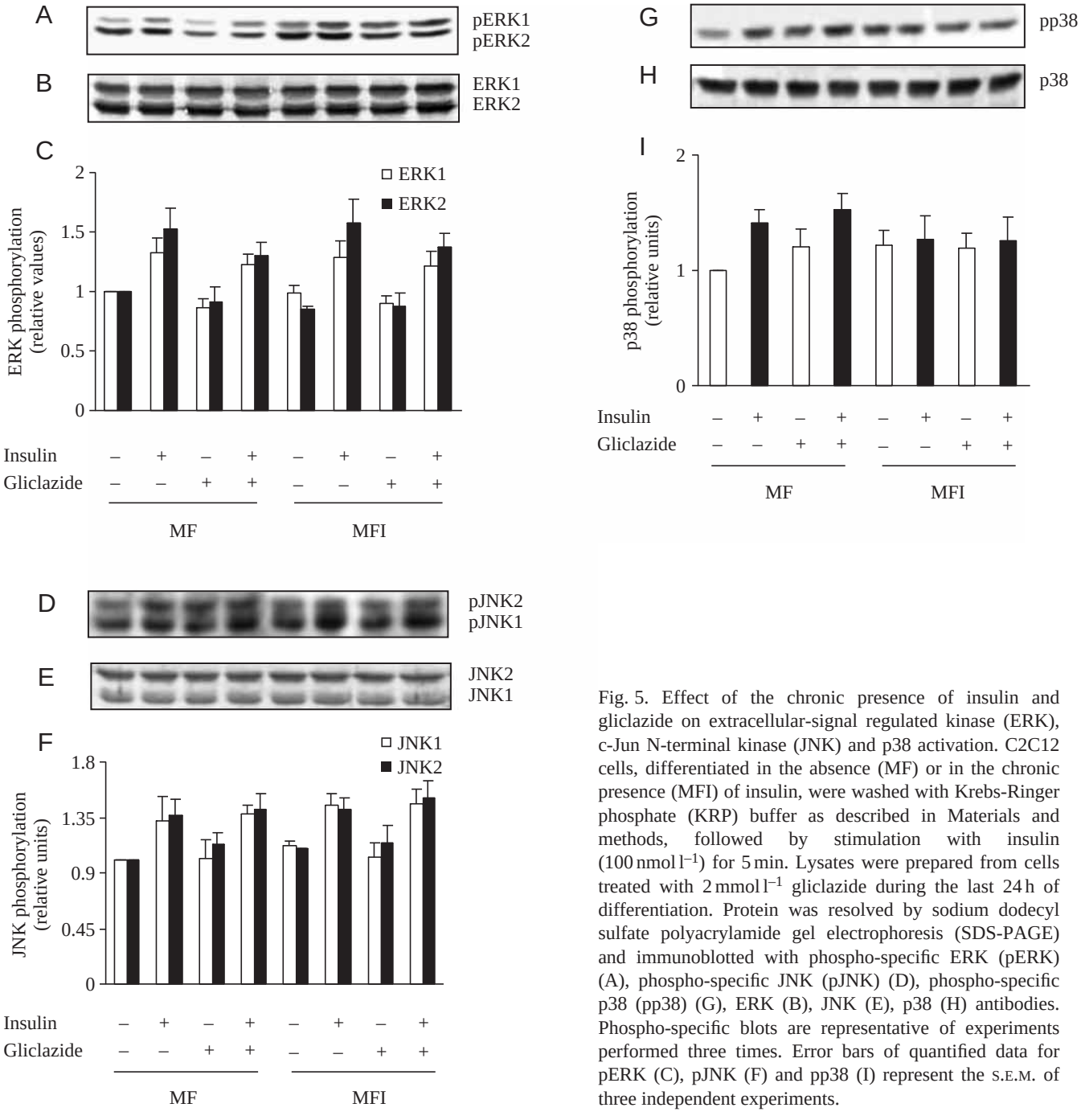

Fig. 5. Effect of the chronic presence of insulin and gliclazide on extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 activation. C2C12 cells, differentiated in the absence (MF) or in the chronic presence (MFI) of insulin, were washed with Krebs-Ringer phosphate (KRP) buffer as described in Materials and methods, followed by stimulation with insulin ($100\,nmol\,l^{-1}$) for 5 min. Lysates were prepared from cells treated with $2\,mmol\,l^{-1}$ gliclazide during the last 24 h of differentiation. Protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with phospho-specific ERK (pERK) (A), phospho-specific JNK (pJNK) (D), phospho-specific p38 (pp38) (G), ERK (B), JNK (E), p38 (H) antibodies. Phospho-specific blots are representative of experiments performed three times. Error bars of quantified data for pERK (C), pJNK (F) and pp38 (I) represent the S.E.M. of three independent experiments.

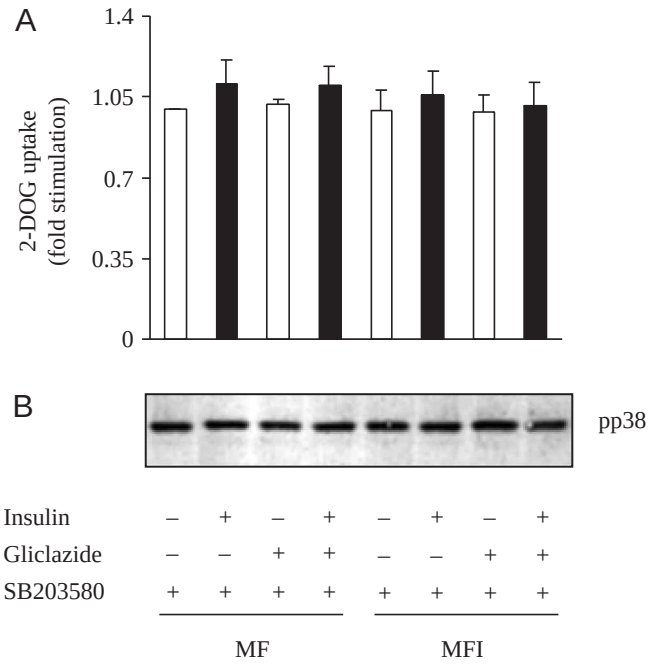

Fig. 6. Effect of SB203580 on 2-deoxyglucose (2-DOG) uptake and p38 activation. C2C12 cells, differentiated in the absence (MF) or in the chronic presence (MFI) of insulin, were incubated in Krebs-Ringer phosphate (KRP) buffer for 30 min followed by another incubation of 30 min in the presence of 10 μmol l^{-1} SB203580. Cells were stimulated with 100 nmol l^{-1} insulin in the presence of SB203580, followed by 2-DOG uptake for 10 min (A) or immunoblot analysis of p38 activation (B). Cells were treated with 2 mmol l^{-1} gliclazide during the last 24 h of differentiation. Error bars represent the S.E.M. of three independent experiments.

MAP kinases was unaffected by the chronic presence of insulin and by treatment with gliclazide (Fig. 5B,E,H). Data suggest the role of p38 in the uptake of glucose.

Effect of SB203580 on 2-DOG uptake and p38 activation

SB203580, a specific inhibitor of p38 MAPK, has been shown to reduce insulin-stimulated glucose uptake in L6 myotubes and 3T3-L1 adipocytes in culture (Sweeney et al., 1999). To determine whether activation of p38 plays a role in glucose transport in C2C12 skeletal muscle cells under MF and MFI conditions, glucose uptake was determined in the presence of SB203580. The results (Fig. 6A) show that there was an insignificant increase in insulin-stimulated 2-DOG uptake in MF and MFI samples by pretreatment with SB203580 as compared with a significant increase (20%, $P<0.05$, $N=4$) observed in MF cells in the absence of the p38 inhibitor (Fig. 4). Insulin-stimulated 2-DOG uptake was also insignificant, even when the samples were treated with gliclazide in the presence of the p38 inhibitor during insulin stimulation. These results implicate the potential role of p38 in glucose uptake in skeletal muscle cells. Results in Fig 6B clearly demonstrate that 10 μmol l^{-1} SB203580 completely blocks the activation of p38 by insulin.

Discussion

The results of the present study show that gliclazide has a direct effect on insulin-resistant skeletal muscle to enhance insulin signaling. Previous reports have observed that sulfonylureas stimulate glucose uptake in skeletal muscle as well as in adipocytes in insulin-sensitive cells (Cooper et al., 1990; Rogers et al., 1987; Maloff and Lockwood, 1981). In the present study, we have observed the effects of gliclazide on sensitive and insulin-resistant cells. The treatment of cells with gliclazide resulted in increased tyrosine phosphorylation of IR. To the best of our knowledge, this is the first study in which a sulfonylurea (gliclazide) has been shown to increase IR tyrosine phosphorylation under *in vitro* conditions in insulin-resistant skeletal muscle. Recently, it was reported that erythrocyte IR tyrosine kinase activity is increased in glyburide-treated patients with type 2 diabetes (Santos et al., 2000). This result, together with the study by Santos et al. (2000), suggests that sulfonylureas might play an important role in the regulation of IR activity.

IRS-1 is the major signal-transducing molecule in the IR pathway in skeletal muscle (Cusi et al., 2000), and it has been shown that IRS-2 has no effect on insulin-dependent glucose uptake in skeletal muscle (Higaki et al., 1999). Our study on the effect of gliclazide on IRS-1 reveals that there is no significant difference in insulin-stimulated tyrosine phosphorylation of IRS-1 with gliclazide treatment in either insulin-sensitive or insulin-resistant myotubes. In our study, levels of IRS-1 expression remained unaltered. Some of the studies suggest that insulin also downregulates the expression of IRS-1 (Ricort et al., 1995; Zhande et al., 2002); however, insulin did not affect IRS-1 expression in 3T3-L1 preadipocytes and mouse embryo fibroblasts (Rui et al., 2001). In our study, PI 3-kinase activity associated with IRS-1 was enhanced by gliclazide treatment, although IRS-1 phosphorylation was unchanged. IRS-1 contains over 20 putative tyrosine and 30 potential serine/threonine sites (Myers and White, 1996). Changes in the level of phosphorylation at any of these sites could potentially alter the ability of IRS-1 to bind and to activate PI 3-kinase. Protein kinase B (PKB/Akt), which acts downstream of PI 3-kinase, phosphorylates IRS-1 at serine residues within the pTyr-binding domain of IRS-1 and protects it from the rapid actions of protein tyrosine phosphatases and maintains the Tyr-phosphorylated active conformation of IRS-1, thus acting as a positive regulator (Zick, 2001). Another downstream molecule of PI 3-kinase, PKCζ, negatively regulates IRS function by inhibiting the ability of IRS-1 to undergo insulin-stimulated tyrosine phosphorylation (Zick, 2001). It has also been shown that PI 3-kinase is a dual-specificity enzyme containing an intrinsic serine kinase activity that phosphorylates Ser608 of the p85 subunit. The phosphorylation on this site of the enzyme inhibits its enzyme activity (Dhand et al., 1994). It has also been demonstrated that tyrosine phosphorylation of the p85 subunit of PI 3-kinase may regulate the activity of the enzyme or alter the affinity of its SH2 domain for phosphotyrosine residues (Hayashi et al., 1992, 1993). Therefore, it is apparent that a number of factors may modify the regulation of PI 3-kinase in a complex manner,

including protein–protein interactions and alterations in its phosphorylation state. The treatment of gliclazide may modulate any one or all of these factors to potentiate the activation of the enzyme. The potentiation can also occur in a completely novel way still to be identified. Earlier, it was reported that PI 3-kinase activity was enhanced by thiazolidinediones in Chinese hamster ovary (CHO) cells overexpressing human insulin receptor without having any effect on the tyrosine phosphorylation of IRS-1 (Zhang et al., 1994).

Treatment with gliclazide could not restore glucose uptake in insulin-resistant myotubes despite the fact that PI 3-kinase activity was enhanced by gliclazide treatment. It has been reported that, although PI 3-kinase activity is required for glucose uptake, there is at least one additional pathway involved in glucose uptake as the activation of PI 3-kinase by platelet-derived growth factor (PDGF) or interleukin 4 or through engagement of integrin receptors does not stimulate glucose transport (Saltiel, 2001). In addition, two naturally occurring insulin receptor mutations were fully capable of activating PI 3-kinase yet were unable to mediate insulin action (Krook et al., 1997). Moreover, addition of a phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] analog had no effect on glucose transport, and treatment of adipocytes with wortmannin, insulin and the PtdIns(3,4,5)P_3 analog resulted in enhanced glucose uptake (Jiang et al., 1998). The other pathway employed by the IR could be p38 activation, as recent studies have demonstrated the requirement of p38 activation for glucose uptake (Konrad et al., 2001; Somwar et al., 2000, 2001a). Earlier studies on sulfonylurea on insulin-sensitive cells have indicated the enhanced basal as well as insulin-stimulated glucose uptake by adipocytes and muscle cells (Cooper et al., 1990; Rogers et al., 1987; Maloff and Lockwood, 1981). Although there was an increase in the basal glucose uptake in insulin-resistant cells by gliclazide treatment in our study, this did not reach significant levels.

The effect on ERK observed in the present study is in line with the study by Kahn and co-workers (Cusi et al., 2000), where it was shown that the ERK pathway is unaffected in skeletal muscle of type 2 diabetic subjects. White and co-workers have implicated the role of JNK in insulin resistance caused by tumor necrosis factor α (TNF-α) in Chinese hamster ovary cells overexpressing human insulin receptor (Aguirre et al., 2000). In C2C12 skeletal muscle cells, there was no difference in JNK activity in insulin-resistant myotubes induced by the chronic presence of insulin. In the present study, activation of p38 was impaired in insulin-resistant cells. The treatment of cells with gliclazide could not restore p38 activation by insulin in insulin-resistant cells. These data are in accord with the data obtained on glucose uptake. Another antihyperglycemic drug, α-lipoic acid, has been shown to enhance glucose uptake by increasing the activation of p38 (Konrad, 2001). Recently, it has also been shown that inhibition of p38 activity by the p38 inhibitor SB203580 results in the blockage of glucose uptake by insulin in rat skeletal muscle (Somwar et al., 2000). Moreover, it has been shown that protein-synthesis inhibitors, such as anisomycin, which activates p38 and JNK, can stimulate glucose uptake (Clancy et al., 1991) and that GLUT-4 (glucose transporter 4) translocation and glucose transport are differentially regulated by PI 3-kinase inhibition, possibly through the activation of the p38 MAPK pathway (Somwar et al., 2001b). At a lower concentration of wortmannin (10 nmol l^{-1}), GLUT-4 translocation and Akt activity were unaffected, but glucose transport and p38 MAPK kinase activity were shown to be impaired, implicating the role of p38 activation in glucose transport (Somwar et al., 2001b). In our study, the activation of p38 by insulin and corresponding stimulation of glucose uptake was blocked by SB203580, implicating the potential role of p38 in glucose uptake. Although we have observed an increase in insulin-stimulated tyrosine phosphorylation of IR and PI 3-kinase activity in resistant cells by treatment with gliclazide, we did not observe the corresponding increase in glucose uptake, as was the case with the insulin-stimulated p38 activation. Based on these data, we propose the role of p38 in glucose uptake.

In conclusion, we show that gliclazide can enhance insulin signaling in skeletal muscle by increasing tyrosine phosphorylation of IR and PI 3-kinase activity. Gliclazide treatment could not restore insulin-stimulated IRS-1 phosphorylation, glucose uptake and p38 activation. We conclude that p38 plays an important role in glucose uptake and can be a potential therapeutic target for anti-diabetic drugs to enhance glucose uptake. Further studies in this regard are needed to find the exact role of p38 in glucose uptake and insulin resistance in type 2 diabetic subjects.

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