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Development of insulin resistance and reversal by thiazolidinediones in C2C12 skeletal muscle cells☆

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Abstract

Aim/hypothesis: The aim of this study was to develop an insulin-resistant cell culture model in skeletal muscle cell line by chronic presence of insulin in serum-free medium and to determine the effect of thiazolidinediones on insulin signaling.

Methods: We differentiated C2C12 in a combination of serum-free medium in presence or absence of insulin and determined differentiation by creatine kinase activity, myogenin and MyoD expression. The development of insulin resistance was determined by tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1, phosphatidylinositol 3-kinase activity associated with insulin receptor substrate-1 and glucose uptake. We treated the cells with 50 μM of thiazolidinediones to determine the effect on these parameters.

Results: C2C12 cells were differentiated normally in the serum-free medium in the absence or presence of insulin. Chronic treatment of insulin resulted in reduced tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1; activation of phosphatidylinositol 3-kinase was impaired and insulin-stimulated glucose uptake was reduced. The treatment of insulin-resistant cells with thiazolidinediones resulted in the enhancement of insulin signaling pathway by increasing tyrosine phosphorylation of insulin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase activity and glucose uptake.

Conclusion/interpretation: These results indicate that insulin resistance can be developed in C2C12 skeletal muscle cell line. These findings implicate a direct mechanism of action of thiazolidinediones on skeletal muscle.

Keywords: Skeletal muscle; Insulin resistance; Insulin receptor; Insulin receptor substrate-1; Phosphatidylinositol 3-kinase; Thiazolidinediones

1. Introduction

The metabolic functions of insulin are impaired in type 2 diabetes mellitus due to insulin resistance of all major insulin target tissues, i.e. skeletal muscle, liver and fat [1]. Peripheral insulin resistance in skeletal muscle is a major contributor to the development of overt type 2 diabetes. Skeletal muscle accounts for more than 80% of the total insulin mediated glucose uptake [2] and the defects in the insulin action in skeletal muscle precede the clinical diagnosis of the disease [3] making it the primary target site to combat insulin resistance.

It has been well established in skeletal muscle that defects at early steps in insulin signaling cascade, including the insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI 3-kinase), leads to insulin resistance and type 2 diabetes. In skeletal muscle of nonobese type 2 diabetics, obese nondiabetic subjects and type 2 patients, IR phosphorylation is reduced [4,5]. Similarly, reduced IRS-1 phosphorylation and PI 3-kinase activity has been observed in skeletal muscle of type 2 diabetic patients [5]. Under *in vitro* conditions, it has been shown in adipocytes [6–8] and hepatoma cell lines [9,10] that insulin resistance can be induced in these cells by treatment with insulin and tumor necrosis factor-α (TNF-α). Insulin resistance has been shown to be developed in skeletal muscle cell lines [11–14]. Del Aguila *et al.* [11] have shown that insulin resistance can be developed in the insulin signaling pathway in C2C12 muscle cells by the treatment of TNF-α. However, contradictory data suggest that TNF-α possibly does not play any role in the

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Abbreviations: KRP, Kreb's Ringer phosphate; PBS, phosphate buffered saline; TZDs, thiazolidinediones; 2-DOG, 2-deoxyglucose; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase.

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development of insulin resistance in skeletal muscle cells [15,16]. In another study [12], insulin resistance was developed in C2C12 cells by treatment with palmitate, however, the investigators reported that it did not affect the tyrosine phosphorylation of IRS-1 and the tyrosine phosphorylation of IR was not reported, which are the prime candidates responsible for reduced glucose uptake in the skeletal muscle of type 2 patients [5]. In L6 skeletal muscle cells [13], insulin resistance was developed by acute exposure of amino acids, but in this study, tyrosine phosphorylation of IR and IRS-1 was normal in the resistant conditions. Recently, insulin resistance has been reported in L6 skeletal muscle cells by exposure to high glucose and insulin [14].

TZDs is a class of antidiabetic drugs, which improve insulin signaling in a variety of obese and diabetic animals as well as in human subjects by binding to PPAR-γ [17]. Pioglitazone, a TZD derivative, was shown to potentiate the IR tyrosine kinase activity in purified IRs from the skeletal muscle [18] and insulin-stimulated tyrosine phosphorylation of IR and IRS-1 in Wistar fatty rats [19]. Recently pioglitazone has been shown to restore the insulin-stimulated tyrosine phosphorylation of IR and IRS-1 in TNF-α induced insulin resistance in 3T3-L1 adipocytes [8]. However whether these drugs act directly on skeletal muscle is not understood [20].

As the models developed, so far, to study insulin resistance in skeletal muscle are inadequate, the main aim of this study was to develop a model of insulin resistance in C2C12 mouse myoblasts in the chronic presence of insulin under serum-free conditions and test the effect of TZDs on the resistance. We provide evidences in favor of the development of insulin resistance in C2C12 skeletal muscle cells. We also demonstrate that TZDs can act directly on the insulin-resistant skeletal muscle to improve insulin signaling.

2. Materials and methods

2.1. Materials

Mouse skeletal muscle cell line, C2C12 was kindly provided by H. Blau, Stanford University, School of Medicine and J. Dhawan, CCMB. Nutrient Mixture F-12 Ham, MCDB201 medium, bovine albumin (cell culture grade) and protein A-agarose were from Sigma. Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), was purchased from GIBCO BRL. Fetal calf serum (FCS) was purchased from Biological Industries. Bovine insulin was purchased from Calbiochem. Monoclonal antiphosphotyrosine, anti-IR-β, anti-IRS-1, anti-MyoD, antimyogenin and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. [γ-^{32}P]ATP was purchased from Bhaba Atomic Research Centre. 2-Deoxy-D-glucose-1-^{3}H was purchased from Sigma Chemical Company. Mouse monoclonal antibody against myosin heavy chain (MHC) was a gift from J. Dhawan, CCMB. TLC plates were from Merck (gift from K.K. Bhutani, NIPER). Other reagents were obtained from Sigma Chemical Company, Roche Molecular Biochemicals, and Bio-Rad.

2.2. Cell culture and treatment

C2C12 cells were cultured in DMEM supplemented with 15% fetal calf serum and antibiotics (Penicillin 100 IU/mL, Streptomycin 100 μg/mL) in 5% CO_2 at 37°. The cells were differentiated in an equal mixture of two serum-free media (MCDB201 and Nutrient Mixture F-12 Ham medium) along with 0.05% BSA in the absence (MF) or chronic presence of 100 nM insulin (MFI) for 3 days. The media was changed after every 12 hr. C2C12 cells were also differentiated under low serum conditions in the presence of 2% horse serum. Pioglitazone or rosiglitazone was added during last 24 hr of differentiation where indicated. Pioglitazone and rosiglitazone were dissolved in DMSO. DMSO was added to the control samples.

2.3. Creatine kinase (CK) activity

The CK activity was determined as described previously [21].

2.4. Immunofluorescence microscopy

Immunofluorescence studies with MyoD were carried out as described previously [21].

2.5. Preparation of extracts of C2C12 muscle cells for immunoblotting and immunoprecipitation

Media in the differentiated cells was changed 1 hr before the start of experiment. The cells were washed twice with the KRP: 10 mM phosphate (pH 7.2), 136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$) containing 5 mM glucose and 0.05% BSA. The cells were further incubated twice with KRP buffer at 37° for 30 min. After that the cells were stimulated with 100 nM of insulin for 5 min at 37° or left unstimulated. The cells were washed with ice-cold PBS and lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL each of leupeptin, aprotonin and soyabean trypsin inhibitor). The estimation of protein concentration was performed by protein assay kit based on bicinchoninic acid (BCA) method using bovine serum albumin as standard protein. Five hundred micrograms of protein was immunoprecipitated with antibody against either anti-IR-β or anti-IRS-1 with the addition of protein A-agarose.

2.6. PI 3-kinase assay

PI 3-kinase activity associated with IRS-1 was measured as described previously [12].

2.7. 2-DOG uptake

After washing with KRP buffer, cells were stimulated with 100 nM insulin in KRP buffer without glucose for 15 min 2-DOG uptake (0.2 μCi/mL in 1 μM of unlabelled 2-deoxyglucose) was added and cells were incubated for 10 min. Cells were washed in ice-cold PBS three times and solubilized in 0.1N NaOH. Protein concentration was measured in each sample by BCA method followed by liquid scintillation counting. The uptake measurement was made in duplicates. The results were corrected for non-specific uptake in the presence of 10 μM of cytochalasin B. Nonspecific uptake and absorption were always less than 10% of the total uptake.

2.8. Densitometric analyses

Densitometric analysis of the western blots were done by using a GS-670 Imaging Densitometer (Bio-Rad) and Molecular Analyst software (version 1.3). Giving an arbitrary value of 1.0 to the control samples, the relative values of the samples were determined.

2.9. Statistical analyses

The data are expressed as mean ± SEM. 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.

3. Results

3.1. Differentiation of C2C12 myoblasts under serum-free conditions

A combination of serum-free medium was selected based on its ability to support the differentiation of C2C12 myoblasts into myotubes in the absence or chronic presence of 100 nM of insulin. MF medium (equivalent mixture of MCDB201 and Nutrient Mixture F-12 Ham) was used for differentiation of the myoblasts in absence (MF) or presence of 100 nM of insulin (MFI) for 3 days. The proper differentiation of the cells in MF and MFI was determined microscopically by the formation of myotubes (Fig. 1A), biochemically by the creatine kinase activity (Fig. 1B), by immunofluorescence studies of MyoD (Fig. 1C) and by the expression of myogenin as shown by immunoblotting (Fig. 1D) against the normal differentiation of C2C12 cells in 2% horse serum containing medium (control). Data shows appropriate differentiation of C2C12 cells in MF and MFI as compared to control.

3.2. Effect of chronic presence of insulin on tyrosine phosphorylation of IR-β and IRS-1

We then determined whether the chronic presence of insulin in the cells differentiated in MFI causes any resistance to insulin. First, immunoprecipitated IR-β from MF and MFI cells was immunoblotted with antibodies against phosphotyrosine. The results (Fig. 2A) demonstrate that chronic presence of insulin in MFI caused 58% (Fig. 2E) reduction in the insulin-stimulated tyrosine phosphorylation of IR-β as compared to MF samples. The blot on stripping and immunoblotting with IR-β showed 80% reduction in the expression levels in cells grown in MFI (Fig. 2B) as compared to MF samples. To determine the similar effect immediate downstream of IR-β, IRS-1 was immunoprecipitated, as IRS-1 plays major role in insulin signaling in the skeletal muscle [22]. The immunoprecipitated IRS-1 on immunoblotting with antiphosphotyrosine antibody showed 37% (Fig. 2C and F) reduction in insulin-stimulated tyrosine phosphorylation in MFI as compared to MF cells. However chronic presence of insulin did not cause any reduction in the expression levels of IRS-1 (Fig. 2D). These results indicated that chronic presence of insulin in combination of serum-free medium (MCDB201 and Nutrient Mixture F-12 Ham) causes insulin resistance in C2C12 skeletal muscle cell line.

3.3. Effect of pioglitazone and rosiglitazone on tyrosine phosphorylation of IR-β and IRS-1

Pioglitazone has been shown to have insulin-sensitizing effects on 3T3-L1 adipocytes by enhancing the tyrosine phosphorylation of IR-β and IRS-1 [8]. Therefore, to determine whether TZDs can reverse the down-regulation of insulin signaling at the levels of IR-β, the cells were treated during last 24 hr of differentiation with various concentrations of pioglitazone (Fig. 3A) and rosiglitazone (data not shown) from 10 to 65 μM, and immunoprecipitated IR-β samples were immunoblotted with antiphosphotyrosine antibody. Fifty micromolar concentration of both pioglitazone (Fig. 3C) and rosiglitazone (data not shown) showed maximum enhancing effect on tyrosine phosphorylation of IR-β in MFI samples stimulated with insulin. Immunoprecipitation of IR-β was carried out with MF and MFI samples treated with or without 50 μM of pioglitazone and the samples were immunoblotted with antiphosphotyrosine antibody (Fig. 4A). The results indicated that pioglitazone was able to enhance the tyrosine phosphorylation of IR-β under resistant conditions by 151% (Fig. 4A and C) as compared to untreated MFI cells stimulated with insulin. Pioglitazone did not alter the expression of IR-β (Fig. 4B and D). Similarly, pioglitazone was able to enhance the insulin-stimulated tyrosine phos-

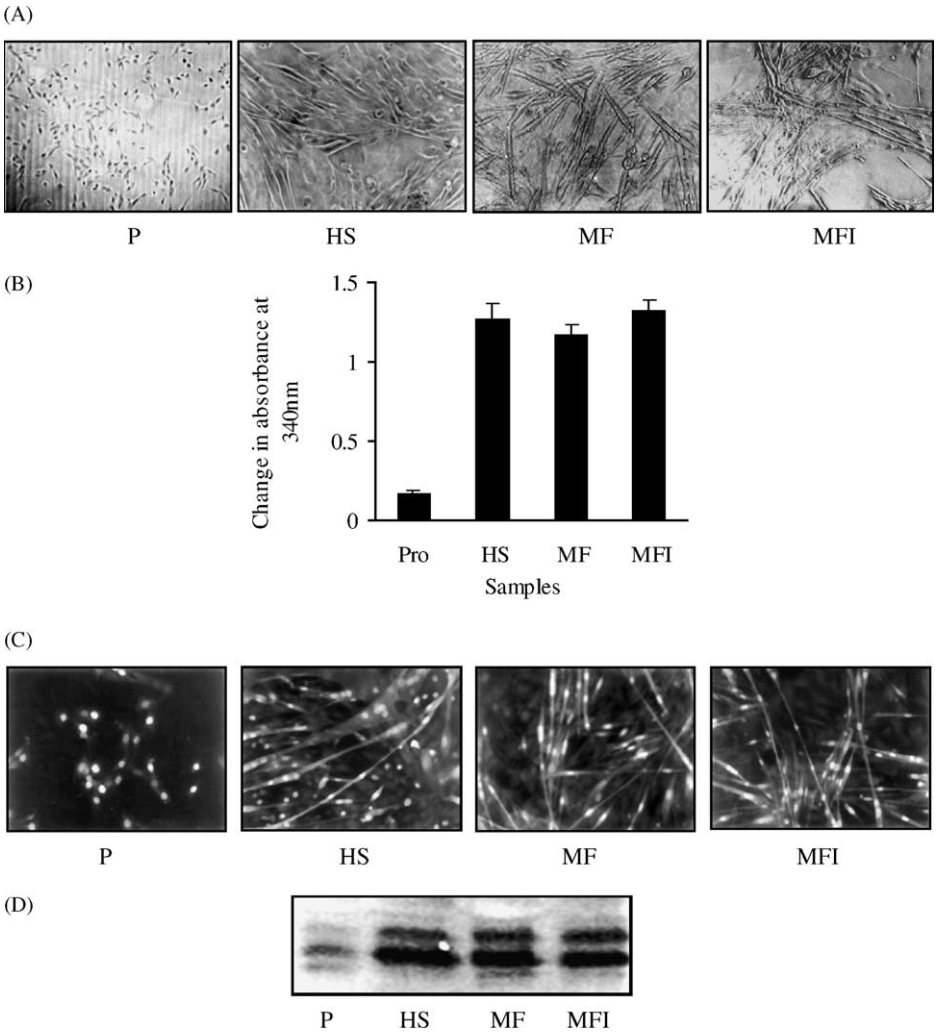

Fig. 1. Determination of characteristics of differentiation in C2C12 mouse skeletal muscle cells cultured under various conditions. (A) Phase contrast microscopy of C2C12 cells: proliferated (denoted as P), differentiated in 2% horse serum (denoted as HS), in MF (equal mixture of MCDB201 and Nutrient Mixture F-12 Ham), and MF in chronic presence of 100 nM of insulin (denoted as MFI). (B) Creatine kinase activity in the proliferated and differentiated cells. (C) MyoD expression in proliferated and differentiated cells by indirect immunofluorescence microscopy using anti-MyoD antibodies. (D) Western immunoblot analysis of myogenin in proliferated and differentiated cells. All the experiments were performed three times and one representative data has been shown.

phorylation of IRS-1 under the resistant conditions by 46% (Fig. 4E and G) as compared to untreated MFI cells stimulated with insulin without having any effect on the expression of IRS-1 (Fig. 4F and H).

Recently it has been shown that troglitazone and darglitazone are much potent sensitizer than rosiglitazone in L6 skeletal muscle cells [23] although rosiglitazone has more binding affinity towards its receptor, PPAR-γ [24]. To determine whether there is any difference in the efficacy of pioglitazone and rosiglitazone in the skeletal muscle under our conditions, cells were treated with rosiglitazone as described for the pioglitazone treatment. The results obtained were comparable to that of pioglitazone treatment (data not shown). Data suggest that there is no significant difference in the efficacy of two TZDs evaluated in insulin-resistant skeletal muscle model in terms of enhancement of tyrosine phosphorylation of IR-β and IRS-1. Treatment with 50 μM of pioglitazone or rosiglitazone did not affect myogenesis as seen microscopically by the formation of myotubes, biochemically by measuring the change in creatine kinase activity, and by western blotting to determine the expression of MyoD, myogenin and myosin heavy chain (data not shown).

3.4. Effect of pioglitazone and rosiglitazone on PI 3-kinase activity

PI 3-kinase plays a key role in insulin-stimulated glucose uptake in insulin-responsive tissues [25,26]. Therefore we determined whether the chronic presence of insulin had any inhibitory effect on the PI 3-kinase activity, and if any, the effect of TZDs on it. Insulin-stimulated PI 3-kinase activity associated with IRS-1 was drastically reduced in MFI as compared to MF. Insulin stimulation (Fig. 5A and B) caused only 30% increase in PI 3-kinase activity in MFI samples as compared to 280% increase in MF

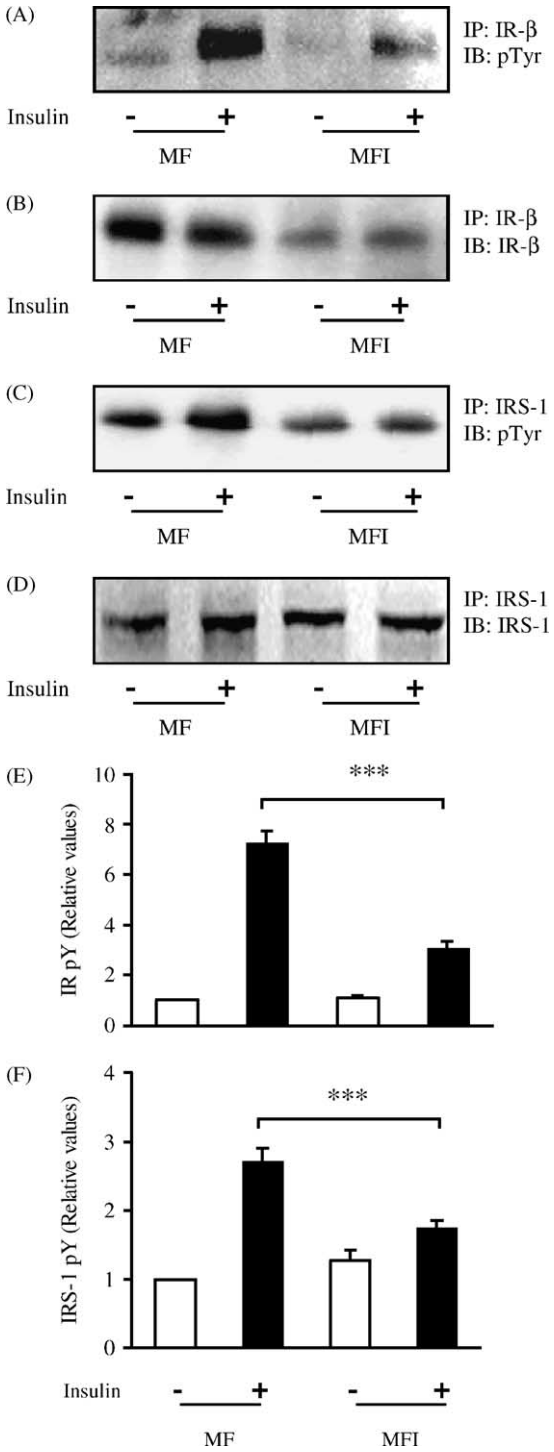

Fig. 2. Effect of insulin stimulation on C2C12 myotubes differentiated in absence (MF) or in chronic presence of insulin (MFI): cells cultured in either MF or MFI were stimulated with 100 nM of insulin for 5 min and lysed. Cell lysates were immunoprecipitated with antibodies to IR-β (A) or IRS-1 (C) and western immunoblotted (IB) with antiphosphotyrosine antibody. The blots were stripped and reprobed with IR-β (B) and IRS-1 (D). Representative blots are shown from six experiments. Phosphorylation levels of IR and IRS-1 (E and F) were quantified by densitometry and expressed relative to MF (control) samples. Error bars represent SEM of six independent experiments (***, $P < 0.001$).

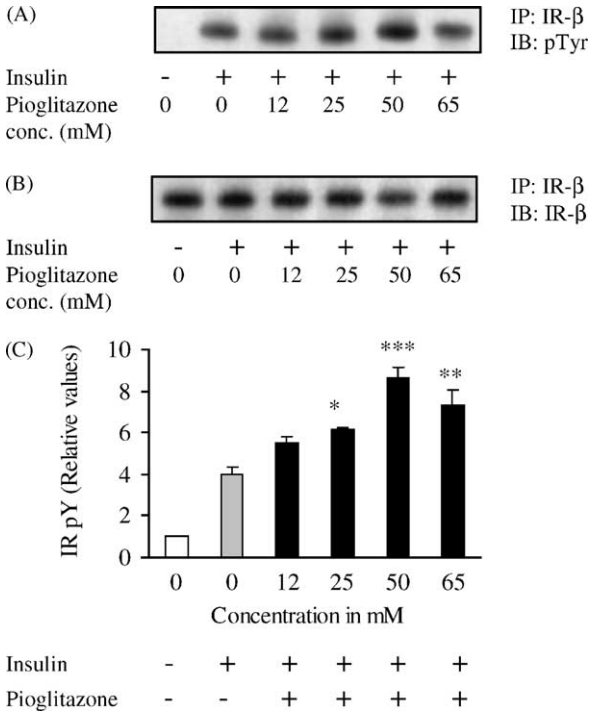

Fig. 3. Effect of concentration of pioglitazone on tyrosine phosphorylation of IR-β in MFI samples: pioglitazone was added at concentration varying between 10 and 65 μM during the last day of differentiation in resistant myotubes (MFI). Cells were stimulated with 100 nM of insulin for 5 min and lysed. Cell lysates were immunoprecipitated with antibodies against IR-β and western immunoblotted with antiphosphotyrosine (A) or anti-IR-β (B). Experiments were repeated thrice and representative blots are shown. Phosphorylation levels of IR-β (C) were quantified by densitometry and expressed relative to control (MFI cells without insulin stimulation) samples. Error bars represent SEM of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. MFI cells stimulated with insulin).

samples against the noninsulin-stimulated control samples. Pioglitazone was able to restore the PI 3-kinase activity associated with IRS-1 in insulin-resistant cells (Fig. 5A and B), showing 170% increase, as compared to drug untreated MFI samples stimulated with insulin. Similar results were observed with rosiglitazone (data not shown).

3.5. Effect of pioglitazone and rosiglitazone on 2-deoxyglucose uptake

Insulin-stimulated glucose transport in skeletal muscle of noninsulin-dependent diabetes mellitus (NIDDM) has been shown to be down-regulated [27]. So, the effect of chronic presence of insulin on the basal and insulin-stimulated glucose uptake was tested in MF and MFI in the absence or presence of TZDs. 2-DOG uptake was impaired in the C2C12 cells chronically treated with insulin (MFI) (Fig. 6) as there was no increase in 2-DOG uptake as compared to 20% increase in samples those were not chronically treated with insulin (MF) ($P < 0.01$). The treatment with pioglitazone resulted in enhancement of insulin-stimulated 2-DOG uptake in insulin-resistant cells (MFI) by 17% ($P < 0.05$) as compared to drug untreated MFI samples

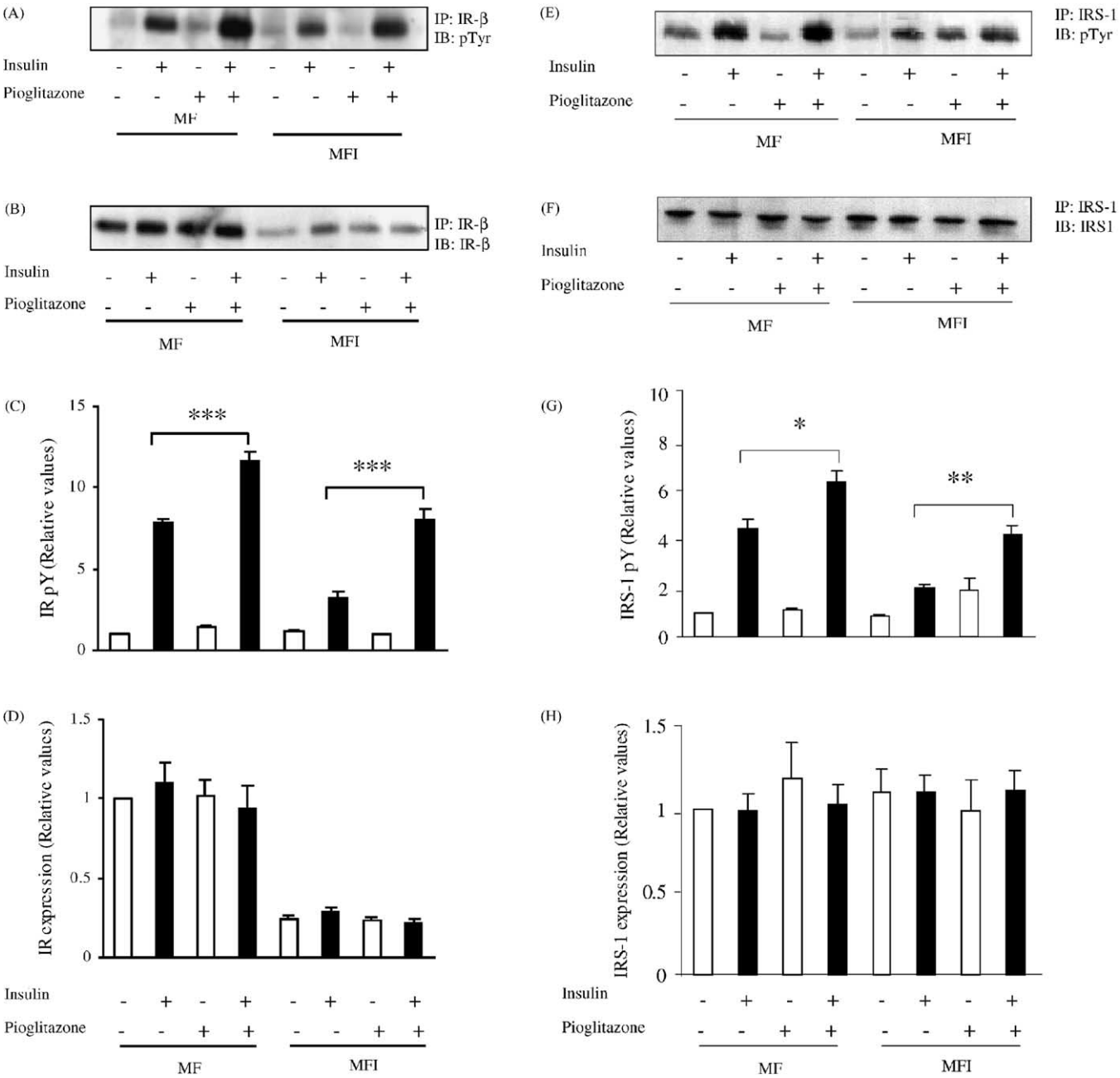

Fig. 4. Effect of pioglitazone on tyrosine phosphorylation of IR-β and IRS-1: pioglitazone (50 μM) was added during the last day of differentiation for 24 hr. Then, the cells were stimulated with 100 nM of insulin for 5 min and lysed. Cell lysate were immunoprecipitated with antibodies against IR-β (A) or IRS-1 (E) and western immunoblotted (IB) with antiphosphotyrosine antibody. The blots were stripped and reprobed with IR-β (B) and IRS-1 (F). Experiments were repeated thrice and representative blots are shown. Phosphorylation (C and G) and expression levels (D and H) of IR-β and IRS-1 were quantified by densitometry and expressed relative to MF (control) samples. Error bars represent SEM of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

stimulated with insulin. However, there was no significant difference in 2-DOG uptake in TZDs treated or untreated samples in absence of insulin stimulation. Similar results were observed with rosiglitazone (data not shown).

4. Discussion

It is well known that myoblasts can be differentiated into myotubes when they are switched from high serum medium to low serum medium. Recent studies have provided evidences that C2C12 skeletal muscle cells can be differentiated in serum-free medium [28–30]. It has been reported that addition of insulin is required to differentiate C2C12 myoblasts in serum-free medium [28,29]. In our culture media, C2C12 cells were differentiated normally in MF in the presence as well as in the absence of insulin. However, another widely used skeletal muscle cell line, L6E9, could not be differentiated under serum-free conditions in absence of insulin, as also reported by Lawson

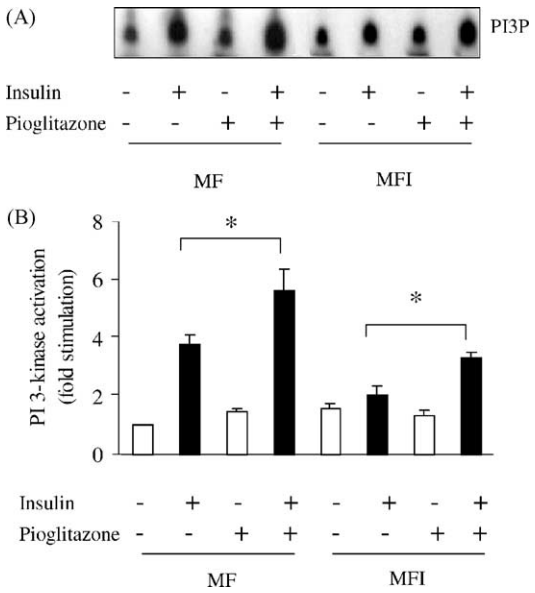

Fig. 5. Effect of chronic presence of insulin and pioglitazone on PI 3-kinase activity: C2C12 cells, differentiated in MF or MFI, were washed with KRP buffer as described in Section 2, followed by stimulation with insulin (100 nM) for 10 min. PI 3-kinase activity in anti-IRS-1 immunoprecipitates was measured. Cells were treated with 50 μM pioglitazone for 24 hr. PI3P refers to PI 3-phosphate. A representative autoradiogram from three independent experiments is shown. Relative density against control (MF) of PI3P spots (B) was quantified by densitometry and error bars represent SEM of three independent experiments (∗, $P < 0.01$).

and Purslow [30] and Pinset *et al*. [31]. Although insulin-stimulated glucose uptake is lower in C2C12 cells as compared to L6 cells, but C2C12 cells express more PPAR-γ receptor as compared to L6 cells [32,33], it was pertinent to choose C2C12 cells as a model to study the effects of TZDs on skeletal muscle cells under *in vitro* conditions.

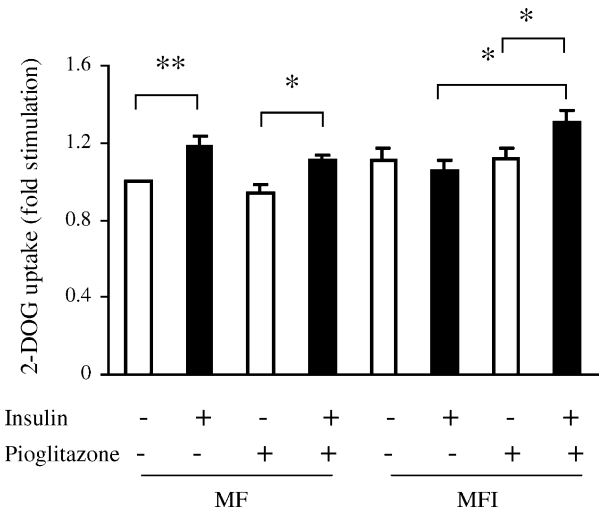

Fig. 6. Effect of chronic presence of insulin and pioglitazone on 2-DOG uptake: C2C12 cells, differentiated in MF or MFI, were washed with KRP buffer as described in Section 2, followed by stimulation with insulin (100 nM) for 15 min. [^{3}H]2-DOG uptake was measured. Cells were treated with 50 μM pioglitazone for 24 hr. Mean ± SEM for four independent experiments are shown (∗, $P < 0.05$; ∗∗, $P < 0.01$).

In L6 myotubes a model of insulin resistance has been developed by sustained exposure of 24 hr with high glucose and insulin [14]; however, the model was not validated with clinically used insulin sensitizers to determine whether it is responsive to these drugs. Skeletal muscle models has also been developed in C2C12 skeletal muscle cell line by TNF-α treatment [11] and by palmitate treatment [12] and in L6 skeletal muscle cells by chronic exposure to amino acids [13]. Recently, in L6 rat skeletal muscle cell line, it was shown that TNF-α does not induce insulin resistance [15]. Insulin resistance, developed by palmitate treatment in C2C12 myoblasts, does not show any difference in the tyrosine phosphorylation of IRS-1 [12]. IRS-1 is the principal molecule involved in insulin signal transduction in skeletal muscle [22] and its tyrosine phosphorylation is impaired in type 2 diabetic patients [5]. Moreover, tyrosine phosphorylation status of IR-β was not reported in the above model of insulin resistance [12]. In another model of insulin resistance developed in L6 cells by chronic exposure to amino acids is also not promising as the proximal steps in the insulin signaling cascade did not exhibit any impairment in the tyrosine phosphorylation of IR-β and IRS-1 [13].

Chronic presence of insulin resulted in the severe reduction in the expression of IR-β as reported in the literature [34]. The reduction in the tyrosine phosphorylation of IR in our study could be due to either the reduction in the tyrosine kinase activity or reduction in the amount of IR or for both of the reasons. In our study, levels of IRS-1 remained unchanged. Some of the studies suggest that insulin also down-regulates the expression of IRS-1 [6,14], however, it did not affect the IRS-1 expression in 3T3-L1 preadipocytes and mouse embryo fibroblasts [35].

Increased tyrosine phosphorylation of IR observed by TZDs treatment in our study suggests that the reduction of tyrosine phosphorylation of IR in MFI samples could be due to the reduction in tyrosine kinase activity of IR, as TZDs did not affect the expression of IR. In an *in vivo* study, Hayakawa *et al*. [19] reported that in skeletal muscle of Wistar fatty rats, pioglitazone increased IR and IRS-1 tyrosine phosphorylation. But, another *in vivo* study also reported that the effect in the skeletal muscle by TZDs could be due to the secondary mechanisms by reduction in the level of free fatty acids, leptin and resistin, secreted by adipocytes, which causes insulin-resistance in skeletal muscle [20]. We have observed a direct effect of TZDs in a skeletal muscle cell line under *in vitro* cultured conditions. In our study TZDs were able to restore IRS-1 associated PI 3-kinase activity. We have shown that 2-DOG uptake was reduced in the resistant cells, which was up-regulated by TZDs. Our study reflects that pioglitazone and rosiglitazone can directly act on skeletal muscle and can improve tyrosine phosphorylation of IR and IRS-1 and by enhancing PI 3-kinase activity. To the best of our knowledge, this is the first *in vitro* report whereby it is

shown that TZDs can improve proximal steps of insulin signaling in insulin-resistant skeletal muscle and indicate a possible mechanism of action.

This model could be of importance as it can be used to screen the potential antidiabetic agents directed against insulin resistance in skeletal muscle. There are good animal models available for screening of antidiabetic agents. However, screening large number of compounds using animals would be very expensive. Moreover, the variability among the animals makes it harder to compare the results. It also has considerations of regulatory aspects of animal ethics. So an *in vitro* model in skeletal muscle is highly relevant. The model might be important to delineate further defects in the insulin signaling in the skeletal muscle and to elucidate the mechanism of action of TZDs in skeletal muscle.

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