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Focal adhesion kinase negatively regulates neuronal insulin resistance

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ABSTRACT

Focal adhesion kinase (FAK), a non-receptor protein kinase, is known to be a phosphatidyl inositol 3-kinase (PI3K) pathway activator and thus widely implicated in regulation of cell survival and cancer. In recent years FAK has also been strongly implicated as a crucial regulator of insulin resistance in peripheral tissues like skeletal muscle and liver, where decrease in its expression/activity has been shown to lead to insulin resistance. However, in the present study we report an altogether different role of FAK in regulation of insulin/PI3K signaling in neurons, the post-mitotic cells. An aberrant increase in FAK tyrosine phosphorylation was observed in insulin resistant Neuro-2a (N2A) cells. Downregulation of FAK expression utilizing RNAi mediated gene silencing in insulin resistant N2A cells completely ameliorated the impaired insulin/PI3K signaling and glucose uptake. FAK silencing in primary cortical neurons also showed marked enhancement in glucose uptake. The results thus suggest that in neurons FAK acts as a negative regulator of insulin/PI3K signaling. Interestingly, the available literature also demonstrates cell-type specific functions of FAK in neurons. FAK that is well known for its cell survival effects has been shown to be involved in neurodegeneration. Along with these previous reports, present findings highlight a novel and critical role of FAK in neurons. Moreover, as this implicates differential regulation of insulin/PI3K pathway by FAK in peripheral tissues and neuronal cells, it strongly suggests precaution while considering FAK modulators as possible therapeutics.

1. Introduction

Insulin, in addition to its varied functions in peripheral tissues, has been found to regulate various key physiological functions in brain, like energy homeostasis, food intake, reproductive endocrinology, learning, memory and also life span [1,2]. Insulin enters the brain through blood brain barrier (BBB) where it also regulates neuronal development, survival and synaptic transmission [3]. Dysfunction of the insulin signaling pathway in brain has been reported to contribute to hyperphagia, obesity and type 2 diabetes [1,4,5]. Also, currently there is widening recognition that chronic neurodegenerative disorder Alzheimer's disease (AD) is closely linked to a state of relative insulin resistance in the brain, and so AD is also termed as "type 3 diabetes" [6–9]. Despite these facts the molecular mechanism of insulin resistance in neurons, the post-mitotic functional unit of brain, remains largely unknown contrary to that in peripheral tissues like skeletal muscle, liver and adipose and thus drug development has been non-productive to treat neuronal insulin resistance and complications associated with it.

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase responsible for integrin signaling, is also known to be an activator of phosphatidyl inositol 3-kinase (PI3K) pathway and thus widely implicated in regulation of cell survival and cancer [10]. Over the past few years FAK has been established as a crucial mediator of insulin signaling in peripheral tissues [10–15]. Our laboratory has implicated an important role of FAK in the regulation of insulin resistance in peripheral tissues [12,13]. We reported that tyrosine dephosphorylation of FAK i.e. decrease in its activity, precedes the development of insulin resistance in skeletal muscle cells [12]. Downregulation of FAK expression in C2C12 skeletal muscle cells resulted in impaired insulin signaling through abrogation of activation and/or expression of several insulin signaling molecules such as insulin receptor substrate-1 (IRS-1), PI3K, Akt, protein kinase C (PKC), glycogen synthase kinase 3β (GSK-3β), etc., followed by decrease in glucose uptake [12]. Further, recently our *in vivo* study has demonstrated that inhibition of FAK expression in liver and muscle of mice result in exacerbated insulin signaling and cause hyperglycemia and hyperinsulinemia [13]. FAK-silenced animals were less glucose tolerant and have physiological and biochemical parameters similar to that of high-fat-diet (HFD)-fed insulin resistant animals [13]. Taken together, these findings strongly suggest involvement of FAK as a positive regulator of insulin signaling in peripheral tissues where decrease in its expression/activity leads to insulin resistance.

FAK is also abundantly expressed in neurons [16]. However, role of FAK in neuronal insulin signaling and resistance has not been

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deciphered yet. Interestingly, activation of FAK in brain has been implicated in the pathogenesis of AD [17,18], the disease which is increasingly being recognized as a brain-specific form of diabetes [6]. The present study was aimed to investigate the exact role of FAK in neuronal insulin resistance, if any, using an *in vitro* neuronal insulin resistant model [19] and primary cortical neurons.

2. Materials and methods

2.1. Cell culture

Mouse neuroblastoma cell line, Neuro-2a (N2A) was cultured in minimum essential medium (MEM) supplemented with 10% FBS and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in 5% CO_2 at 37 °C. Confluent cells were differentiated in an equal mixture of two serum free media (MCDB 201 and Ham's F-12 medium) in absence (MF: insulin sensitive) or in chronic presence of 100 nM insulin (MFI: insulin resistant) for 3 days in presence of 2% dimethyl sulfoxide (DMSO) with change of media after every 12 h [19]. Mouse primary cortical neurons were cultured as described previously [20].

2.2. siRNA transfection

siRNA oligonucleotides were designed and synthesized by Qiagen GmbH (Germany) to target following cDNA sequences: Nonspecific (Scrambled) siRNA, 5′ AATTCTCCGAACGTGTCACGT; FAK siRNA, F1: 5′-TGCAATGGAACGAGTATTAAA, F2: 5′-CAAGAAATAGCTGATCAAGTA. Cells were transfected with either FAK specific (F1 or F2, as specified in text) or nonspecific (scrambled) siRNA using RNAifect transfection reagent (Qiagen GmbH, Germany) in reduced serum Opti-MEM media as described previously [12].

2.3. Preparation of cell lysates for immunoblotting

N2A cells were transfected with siRNA as indicated and allowed to differentiate under MF or MFI conditions for 3 days. Cells were then incubated with or without insulin (100nM) for 30 min [21] and lysed in cell lysis buffer as described previously [12]. Equal concentrations of all the samples were subjected to SDS-PAGE followed by western immunoblotting. The immunoblots were quantified using Quantity One 1-D analysis software as described previously [12].

2.4. Glucose uptake assay

Glucose uptake assays were performed by a modified method of Scheele *et al.* [22] using [^{3}H]2-deoxyglucose (2-DOG).

2.5. Phosphatidyl inositol 3-kinase (PI3K) activity

PI3K activity associated with IRS-1 was determined with PI3K ELISA Kit (Echelon Biosciences Inc., SaltLake City, UT, USA) according to the manufacturer's instructions and as reported previously [12].

2.6. Statistical analysis

All the experiments were performed a minimum of three times and a representative result is shown. Data are expressed as mean ± SE. Significance of difference between two groups (*P*-values) was calculated by two-tailed unpaired student's *t*-test. In all the cases $P<0.05$ was considered to be statistically significant, with $P<0.05$ was given *, while $P<0.01$ was given **.

2.7. Materials

Minimum Essential Media (MEM), Nutrient Mixture F-12 Ham, MCDB 201 medium, bovine albumin (cell culture grade), [^{3}H]2-deoxyglucose were purchased from Sigma Chemical Company (MO, USA). Fetal bovine serum (FBS) was from Biological Industries (Kibbutz Beit, Haemek, Israel). Anti-phospho Akt (Ser473), Anti-phospho Akt (Thr 308), Anti-Akt, Anti-phospho PKCζ (Thr410/403), Anti-PKCζ, Anti-phospho GSK3β, Anti-GSK3β and Anti-GLUT4 antibodies were from Cell Signaling Technology (MA, USA). Anti-FAK antibody was from Upstate Biotechnology (NY, USA). Anti-phosphotyrosine, Anti-IRβ, Anti-IRS1 and Anti-Pyk2 antibodies were from Santa Cruz Biotechnology (CA, USA). Anti- α-Tubulin was from Neomarkers (CA, USA). Bovine insulin was purchased from Calbiochem (CA, USA). Nitrocellulose membranes were procured from Bio-Rad Laboratories (Hercules, CA, USA). All the other reagents unless attributed specifically were from Sigma Chemical Company (MO) or Amresco (OH, USA).

3. Results

3.1. Effect of neuronal insulin resistance on FAK tyrosine phosphorylation

To determine the role of FAK in neuronal insulin resistance, if any, neuro-2a (N2A) cells were differentiated under insulin-sensitive (MF) or insulin-resistant (MFI) conditions (Supplementary Fig. 1) [19] and FAK tyrosine phosphorylation under these conditions was examined. Cells were lysed, immunoprecipitated with anti-FAK and subjected to western immunoblotting with anti-phosphotyrosine or anti-FAK antibodies. As compared to MF, we observed an aberrant increase (~ 500%) in tyrosine phosphorylation of FAK under MFI conditions (Fig. 1, $p<0.01$), without any change in its protein level (Fig. 1), suggesting activation of FAK under neuronal insulin resistant conditions. These findings emphasize the possible involvement of FAK in neuronal insulin resistance.

3.2. FAK silencing using RNA interference (RNAi)

To ascertain the role of FAK in neuronal insulin resistance, we employed RNAi to suppress endogenous FAK levels in N2A cells.

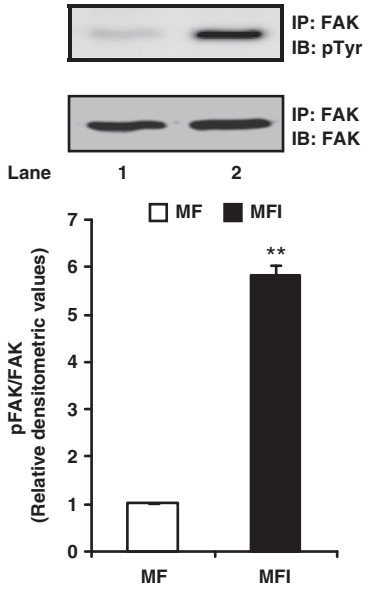

Fig. 1. Effect of neuronal insulin resistance on FAK tyrosine phosphorylation. N2A cells were differentiated in serum free medium in the absence of (MF) or chronic presence of 100 nM insulin (MFI) for 3 days. Protein extracts (500 μg) were immunoprecipitated with anti-FAK antibody, subjected to western immunoblotting and probed with anti-phospho-tyrosine or anti-FAK antibody. Bars represent relative densitometric values of pFAK after normalizing with FAK expression (pFAK/FAK). Experiments were repeated thrice and a representative result is shown. Values are mean ± SE. **$P<0.01$ compared with lane 1. Open bars, MF; filled bars, MFI. IP, Immunoprecipitated; IB, Immunoblotted.

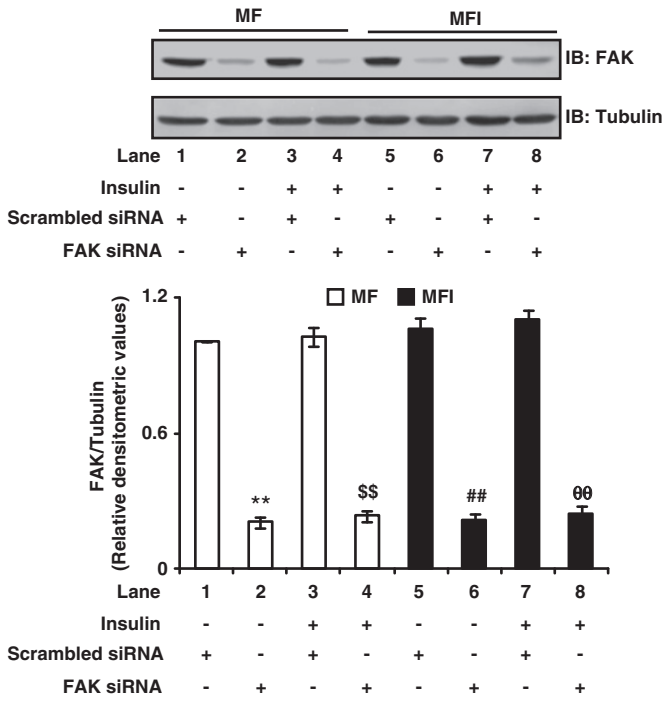

Fig. 2. Level of FAK expression in FAK-siRNA transfected MF-MFI differentiated N2A cells. Scrambled or FAK-specific siRNA transfected N2A cells were differentiated in absence (MF) or chronic presence of insulin (MFI) for 3 days and stimulated with or without insulin (100nM) for 30 min. Cell lysates were subjected to western immunoblotting and probed with anti-FAK or anti-Tubulin antibodies. Bar represents relative densitometric values of FAK expression after normalizing with tubulin expression (FAK/Tubulin). Experiments were repeated thrice and a representative result is shown. Values are mean ± SE. $^{**}P<0.01$ compared with lane 1; $^{\$\$}P<0.01$ compared with lane 3; $^{\#\#}P<0.01$ compared with lane 5; $^{\theta\theta}P<0.01$ compared with lane 7. Open bars, MF; filled bars, MFI. IB, Immunoblotted.

FAK-specific siRNA duplexes (denoted as F1 in materials and methods) showed a dose-dependent silencing of FAK, with maximal silencing at 150 pmol (data not shown). FAK-specific siRNA (150 pmol) demonstrated significant FAK downregulation (82.0 ± 0.05%) after normalizing it with similar concentration of scrambled siRNA (to determine non-specific silencing) (Supplementary Fig. 2), without affecting cellular morphology and viability as seen by phase contrast microscopy (Supplementary Fig. 3) and MTT assay (data not shown). We next studied the effect of FAK specific or non-specific (scrambled) siRNA transfection on FAK expression under MF and MFI conditions, with or without insulin stimulation. Data showed that the level of FAK in FAK-siRNA-transfected cells remained downregulated (~80%) under all the conditions as compared to scrambled-siRNA transfected cells (Fig. 2).

3.3. Effect of FAK downregulation on impaired glucose uptake under neuronal insulin resistance

To assess the functional contribution of altered FAK protein level in the pathogenesis of neuronal insulin resistance, glucose uptake was measured in FAK silenced N2A cells differentiated under insulin sensitive (MF) and insulin resistant (MFI) conditions, with or without insulin stimulation. Insulin-stimulated glucose uptake of chronically insulin treated cells (MFI) was completely impaired as compared to control cells (MF) (Fig. 3, lane 5 and lane 7 vs. lane 1 and lane 3). FAK silencing under the MFI condition resulted in marked enhancement (71.6 ± 0.05%) of insulin-stimulated glucose uptake (Fig. 3, lane 8 vs. lane 7, $p<0.01$), resulting in comparable glucose uptake to that observed under insulin-sensitive (MF) conditions (Fig. 3, lane

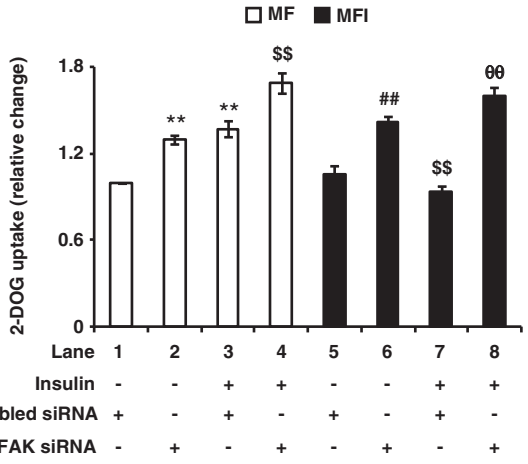

Fig. 3. Effect of FAK downregulation on impaired glucose uptake under neuronal insulin resistance. Scrambled or FAK-specific siRNA transfected N2A cells were differentiated in absence (MF) or chronic presence of insulin (MFI) for 3 days and stimulated with or without insulin (100 nM) for 30 min. Uptake of 2-deoxyglucose (2DOG) was measured in 40 μg cell lysates. Uptake of each sample was measured in duplicate. Bar represents relative change in 2-DOG uptake. All the experiments were repeated thrice and a representative result is shown. Values are mean ± SE. $^{**}P<0.01$ compared with lane 1; $^{\$\$}P<0.01$ compared with lane 3; $^{\#\#}P<0.01$ compared with lane 5; $^{\theta\theta}P<0.01$ compared with lane 7. Open bars, MF; filled bars, MFI.

8 vs. lane 3). Under the basal MFI condition also FAK silencing led to significant enhancement (33.3 ± 0.04%) of glucose uptake (Fig. 3, lane 6 vs. lane 5, $p<0.01$). Silencing FAK under insulin-sensitive conditions (MF) led to additional increases in glucose uptake as

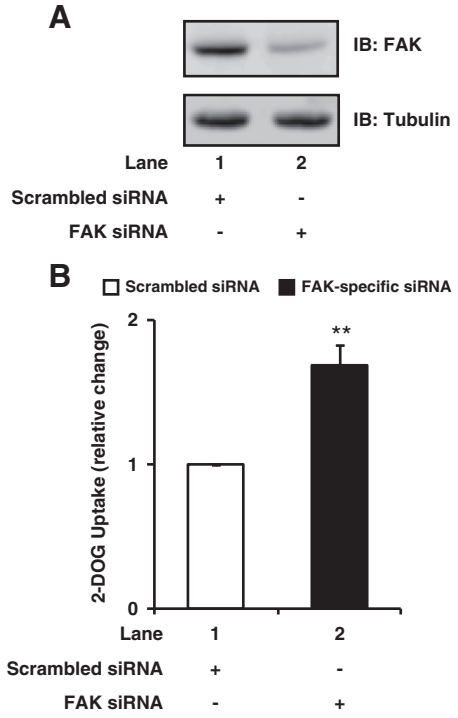

Fig. 4. Effect of downregulation of FAK on glucose uptake in mouse primary cortical neurons. Primary cortical neurons were transfected with scrambled or FAK-specific siRNA. (A) Cell lysates were subjected to western immunoblotting and probed with anti-FAK or anti-Tubulin antibodies (B) Uptake of 2-deoxyglucose (2DOG) was measured in 40 μg cell lysates. Uptake of each sample was measured in duplicate. Bar represents relative change in 2-DOG uptake. Experiments were repeated thrice. Values are mean ± SE. $^{**}P<0.01$ compared with lane 1. Open bars, Scrambled siRNA transfected primary neurons; filled bars, FAK-specific siRNA transfected primary neurons. IB, Immunoblotted.

compared to respective scrambled-siRNA transfected controls (Fig. 3, $p<0.01$). The results were further confirmed by silencing FAK using another FAK-specific siRNA (denoted as F2 in materials and methods) in N2A cells differentiated under MF and MFI conditions, with or without insulin stimulation. It also demonstrated nearly same increase in glucose uptake (data not shown). The results thus suggest that FAK silencing has insulin-sensitizing effects in neuronal cells and it could effectively ameliorate the impaired glucose uptake in insulin resistant neuronal cells.

3.4. Effect of FAK downregulation on glucose uptake in primary cortical neurons

To further examine the functional relevance of FAK in regulating neuronal insulin signaling, we measured glucose uptake in FAK silenced mouse primary cortical neurons. Consistent with the results obtained in N2A cells, FAK silencing in primary cortical neurons (Fig. 4A) showed marked enhancement ($68.9 \pm 0.62\%$) in glucose uptake, as compared to scrambled-siRNA transfected primary neurons

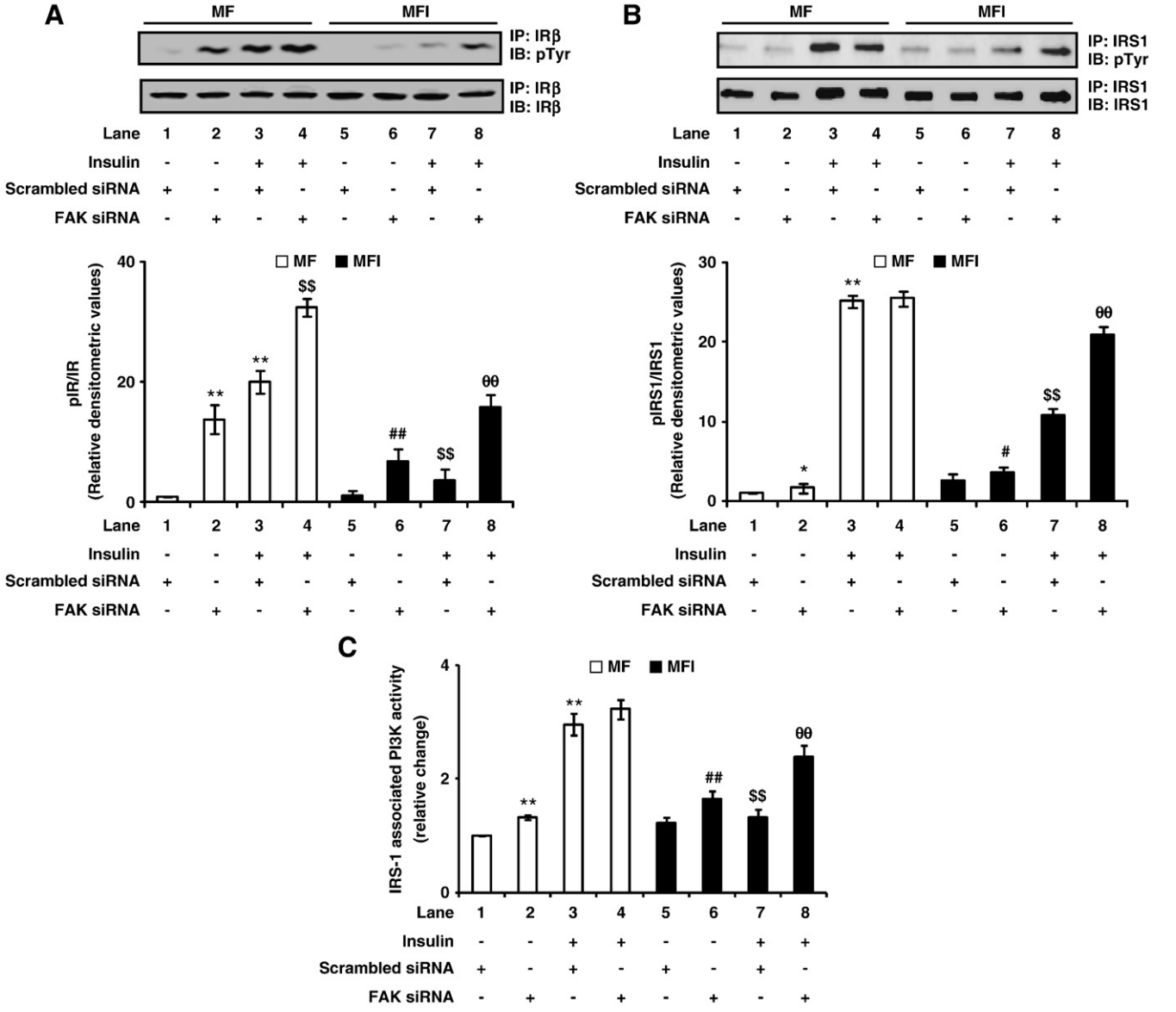

Fig. 5. Effect of FAK downregulation on impaired insulin signaling under neuronal insulin resistance. Scrambled or FAK-specific siRNA transfected N2A cells were differentiated in absence (MF) or chronic presence of insulin (MFI) for 3 days and stimulated with or without insulin (100 nM) for 30 min. (A) Cell lysates (500 μg) were immunoprecipitated with anti-IRβ antibody, subjected to western immunoblotting and probed with anti-phosphotyrosine or anti-IRβ antibodies. Bar represents relative densitometric values of pIRβ after normalizing with IRβ expression (pIRβ/IRβ). (B) Cell lysates (500 μg) were immunoprecipitated with anti-IRS1 antibody, subjected to western immunoblotting and probed with anti-phospho-tyrosine or anti- IRS1 antibodies. Bar represents relative densitometric values of pIRS1 after normalizing with IRS1 expression (pIRS1/IRS1). (C) Cell lysates (500 μg) were immunoprecipitated with anti-IRS1 antibody and PI3K activity was measured. Bar represents relative change in IRS1 associated PI3K activity. (D) Cell lysates were subjected to western immunoblotting and probed with anti-phospho Akt (Ser473) or anti-Akt antibodies. Bar represents relative densitometric values of pAkt (Ser473) after normalizing with Akt expression [pAkt(Ser473)/Akt]. (E) Cell lysates were subjected to western immunoblotting and probed with anti-phospho GSK3β (Ser9) or anti-GSK3β antibodies. Bar represents relative densitometric values of pGSK3β (Ser9) after normalizing with GSK3β expression (pGSK3β/GSK3β). (F) Cell lysates were subjected to western immunoblotting and probed with anti-phospho PKCζ or anti-Tubulin antibodies. Bar represents relative densitometric values of pPKCζ after normalizing with tubulin expression (pPKCζ /Tubulin). (G) The low-density microsome (LDM) and plasma fractions (PM) were isolated, subjected to western immunoblotting and probed with anti-GLUT4 or anti-Tubulin antibodies. Bar represents relative densitometric values of GLUT4 expression in LDM and PM fractions. All the experiments were repeated thrice and a representative result is shown. Values are mean ± SE. $^{**}P<0.01$ compared with lane 1; $^{*}P<0.05$ compared with lane 1; $^{\$\$}P<0.01$ compared with lane 3; $^{\#\#}P<0.01$ compared with lane 5; $^{\#}P<0.05$ compared with lane 5; $^{\theta\theta}P<0.01$ compared with lane 7. Open bars, MF; filled bars, MFI. IP, Immunoprecipitated; IB, Immunoblotted.

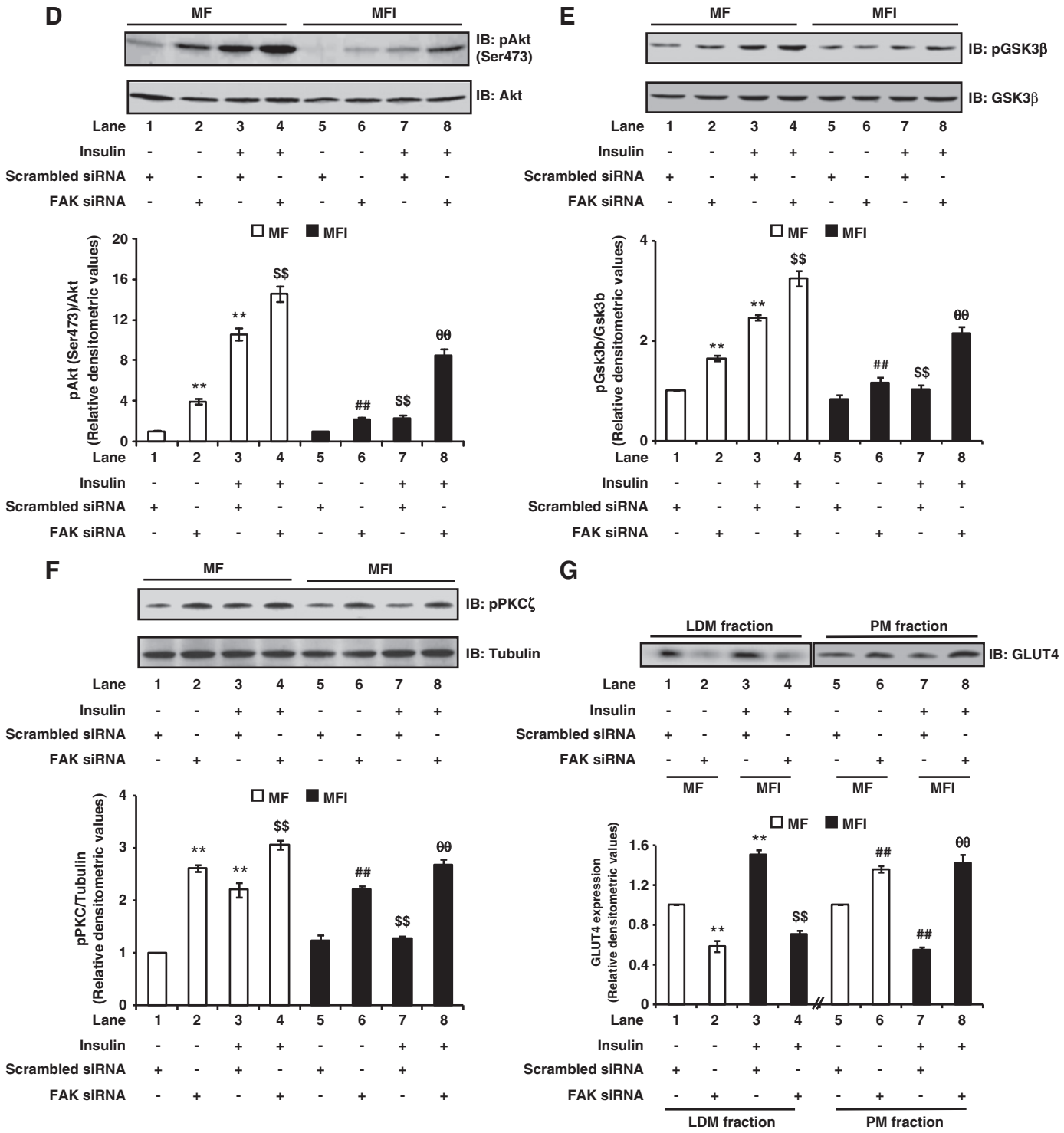

Fig. 5 (*continued*).

(Fig. 4B, $p<0.01$), emphasizing a novel role of FAK as a negative regulator of neuronal insulin signaling.

3.5. Effect of FAK downregulation on impaired insulin signaling under neuronal insulin resistance

We next examined the potential molecular mechanism that might underlie the protection from neuronal insulin resistance conferred by downregulation of FAK levels, by testing insulin signal transduction cascade in FAK-specific or scrambled siRNA transfected N2A cells differentiated under insulin sensitive (MF) and insulin resistant (MFI) conditions, with or without insulin stimulation.

A marked reduction ($93.1 \pm 2.1\%$) in insulin-stimulated tyrosine phosphorylation of IRβ under MFI condition was observed (Fig. 5A, lane 5 and lane 7 vs. lane 1 and lane 3). FAK silencing under MFI condition resulted in ~450% increase in insulin-stimulated tyrosine

phosphorylation of IRβ (Fig. 5A, lane 8 vs. lane 7, $p<0.01$), thereby greatly improving the impaired IRβ tyrosine phosphorylation (Fig. 5A, lane 8 vs. lane 3). Under insulin sensitive condition (MF) FAK silencing showed an increment of 58.3 ± 2.0% in insulin-stimulated tyrosine phosphorylation of IRβ, as compared to scrambled-siRNA transfected cells stimulated with insulin (Fig. 5A, lane 4 vs. lane 3, $p<0.01$). In accordance to glucose uptake data, FAK silencing alone was able to enhance tyrosine phosphorylation of IRβ as was evident by its increment of 284.7 ± 0.60% and 718.3 ± 1.2% at the basal level, in cells differentiated under MF and MFI condition, respectively, as compared to respective scrambled-siRNA transfected controls (Fig. 5A, lane 2 vs. lane 1 and lane 6 vs. lane 5, respectively, $p<0.01$). Expression of IRβ was unaltered due to FAK silencing (Fig. 5A).

The impaired insulin-stimulated IRS1 tyrosine phosphorylation observed under MFI condition (Fig. 5B) was also effectively ameliorated by FAK silencing, as evident by an increase of 92.7 ± 0.9% in insulin-stimulated tyrosine phosphorylation of IRS1 as compared to scrambled-siRNA transfected MFI cells stimulated with insulin (Fig. 5B, lane 8 vs. lane 7, $p<0.01$). This led to comparable IRS1 tyrosine phosphorylation to that observed under insulin-sensitive (MF) conditions (Fig. 5B, lane 8 vs. lane 3). However, FAK silencing under MF condition did not show any further increment in its insulin-stimulated tyrosine phosphorylation as compared to scrambled-siRNA transfected insulin-stimulated MF control (Fig. 5B, lane 4 vs. lane 3), probably due to the saturable activation of IRS1. FAK silencing increased the basal IRS1 tyrosine phosphorylation by 72.5 ± 0.62% and 44.0 ± 0.73%, in cells differentiated under MF and MFI condition, respectively, as compared to respective scrambled-siRNA transfected controls (Fig. 5B, $p<0.05$). Expression of IRS1 was unaltered by FAK silencing (Fig. 5B).

Parallel to impaired tyrosine phosphorylation of IRS1, an impaired insulin-stimulated IRS1-associated PI3K activity was observed under MFI condition (Fig. 5C). Moreover, FAK silencing under MFI condition significantly restored (increased by 80.2 ± 0.21%) its activity to that of insulin-stimulated MF control (Fig. 5C, lane 8 vs. lane 3). FAK silencing under MF condition did not cause any significant increment in insulin-stimulated PI3K activity associated to IRS1, as compared to scrambled-siRNA transfected insulin-stimulated MF control (Fig. 5C). Silencing of FAK increased the basal IRS1-associated PI3K activity by 33.0 ± 0.04% and 35.2 ± 0.14%, in cells differentiated under MF and MFI conditions, respectively, as compared to respective scrambled-siRNA transfected controls (Fig. 5C, $p<0.01$).

PI3K activates several PIP3-dependent serine/threonine kinases; one of them is Akt, which gets activated by its phosphorylation at Ser473. Insulin-induced Akt phosphorylation was found to be impaired under MFI condition (Fig. 5D). FAK silencing markedly increased (~280%) insulin-stimulated Akt phosphorylation at Ser473 under insulin- resistant conditions (MFI) as compared to scrambled-siRNA transfected MFI cells stimulated with insulin (Fig. 5D, lane 8 vs. lane 7, $p<0.01$), thereby significantly ameliorating its impaired phosphorylation (Fig. 5D). Under insulin sensitive condition (MF), FAK silencing further increased insulin-stimulated Akt phosphorylation by 37.8 ± 0.79% (Fig. 5D, $p<0.01$). FAK silencing also increased the basal Akt phosphorylation by 293 ± 0.26% and 131 ± 0.24%, in cells differentiated under MF and MFI conditions, respectively, as compared to respective scrambled-siRNA transfected controls (Fig. 5D, $p<0.01$). Expression of Akt was unaltered by FAK downregulation (Fig. 5D).

One of the most prevalent downstream targets of Akt is GSK3β which gets inhibited by Akt mediated phosphorylation at serine 9. The inhibition of GSK3β activity is a key regulatory mechanism for activation of glycogen synthase (GS) leading to glycogen synthesis. Silencing FAK also resulted in marked amelioration of insulin-stimulated GSK3β phosphorylation at Ser9 (increased by 109.2 ± 0.13%) under the insulin-resistant conditions (MFI) as compared to scrambled-siRNA transfected MFI cells stimulated with insulin (Fig. 5E, lane 8 vs. lane 7, $p<0.01$). The extent of insulin-stimulated inhibition of GSK3β was close to that observed in insulin sensitive cells (MF) (Fig. 5E, lane 8 vs. lane 3, $p<0.01$). Under MF, FAK silencing further increased the insulin-stimulated GSK3β phosphorylation by 31.9 ± 0.14% (Fig. 5E, $p<0.01$). It increased the basal phosphorylation of GSK3β by 65.0 ± 0.06% and 39.8 ± 0.10%, in cells differentiated under MF and MFI condition, respectively, compared to respective scrambled-siRNA transfected controls (Fig. 5E, $p<0.01$). The data therefore suggests anabolism between enhanced glucose uptake and glycogen synthesis as a result of increased GSK3β phosphorylation due to FAK silencing.

Previously PKC has been demonstrated to regulate FAK-mediated glucose uptake in skeletal muscle [12]. Thus effect of FAK silencing on PKCζ activation was determined in N2A cells differentiated under MF and MFI condition. Insulin-induced PKCζ activation was found to be impaired under MFI condition (Fig. 5F). FAK silencing led to complete restoration (increased by 122.3 ± 0.10%) of the impaired insulin-stimulated PKCζ phosphorylation under MFI condition (Fig. 5F, lane 8 vs. lane 7, $p<0.01$). Also, FAK downregulation under insulin sensitive (MF) conditions led to marked increase in PKCζ phosphorylation (Fig. 5F).

GLUT4, an insulin responsive glucose transporter in neuronal cells [23], translocation to plasma membrane was found to be impaired under insulin resistant (MFI) condition as evident by GLUT4 increased levels in cytosolic (LDM) (Fig. 5G, lane 3 vs. lane 1, $p<0.01$) and decreased level in the plasma membrane (PM) fractions (Fig. 5G, lane 7 vs. lane 5, $p<0.01$) under insulin-stimulated MFI condition. Silencing FAK under MFI condition resulted in marked increase (160.2 ± 0.08%) in insulin-stimulated GLUT4 translocation to plasma membrane (Fig. 5G, lane 8 vs. lane 7, $p<0.01$), indicating that FAK negatively regulates GLUT4 translocation. GLUT4 expression was same under all conditions (data not shown). Data thus demonstrate that FAK regulates glucose uptake under neuronal insulin resistance by differentially regulating the translocation of GLUT4 molecules.

Overall, these results indicate that FAK negatively regulates neuronal insulin resistance by suppressing activation of IR, IRS1, PI3K, Akt, PKC, GLUT4 translocation and glucose uptake and its downregulation could effectively ameliorate neuronal insulin resistance. This is in contrast to its role in regulation of insulin signaling in peripheral tissues.

4. Discussion

The present study provides evidences that show a direct correlation between FAK activation and impairment of insulin actions in neuronal cells. The study demonstrates a novel role for FAK as a negative regulator of the insulin/PI3K signalling pathway. The conclusion is based on the following findings: (1) an increase in FAK activity was observed in insulin resistant N2A cells, (2) downregulation of FAK expression in insulin resistant N2A cells effectively ameliorated impaired insulin/PI3K signaling and glucose uptake, and (3) downregulation of FAK expression in mouse primary cortical neurons led to marked increase in glucose uptake.

FAK has been known to be a PI3K activator and thus also widely implicated in regulation of cell survival pathways and cancer [24]. Previous findings in peripheral tissues like skeletal muscle and liver strongly suggested involvement of FAK as a positive regulator of insulin signaling where decrease in its expression/activity leads to insulin resistance by abrogation of activation and/or expression of several insulin signaling molecules, followed by decrease in glucose uptake [11–13]. However, here an altogether different role of FAK in regulation of insulin resistance in neurons is being reported. FAK is unexpectedly found to be involved in impairment of insulin signaling and glucose metabolism in neurons. The involved molecular mechanisms of FAK-mediated negative regulation of neuronal insulin

resistance include impairment of IRβ, IRS1, PI3K, PKCζ and Akt activation, followed by impaired GLUT4 translocation to the plasma membrane and thus resulting in impaired glucose uptake. The mechanism also involved activation of GSK3β, implying negative regulation of glycogen synthesis by FAK in neurons. In hepatocytes and skeletal muscle FAK has been implicated in positive regulation of glycogen synthesis [10,11]. Moreover, a significant decrease of FAK tyrosine phosphorylation has been reported in insulin resistant C2C12 skeletal muscle, which has been shown to precede development of insulin resistance in these cells [12]. A similar decrease has also been reported in skeletal muscles from insulin-resistant Sprague–Dawley rats fed on HFD diet [12]. Decrease in FAK activity has also been reported to precede TNF-α mediated insulin resistance in liver [25]. In adipocytes also FAK has been found to positively regulate glucose uptake [26]. Thus FAK-mediated negative regulation of insulin resistance appears to be specific to neurons.

The FAK that is expressed in neurons has been reported to be quite different from those expressed in non-neuronal cells in various aspects (described below); though the functional consequences of these differences are not much known: (1) FAK exists in various spliced isoforms that were conserved during evolution and are exclusively or preferentially found in the CNS [27–30]. Several of the splice variants predict the insertion of short peptides in the coding sequence, a fact that accounts for the higher apparent molecular weight of FAK in brain than in other tissues [29,31]. Neurons mostly express FAK +6,7 isoform (number indicates the length of peptide in amino acids encoded by additional exons) [32], (2) The presence of these additional exons has been shown to dramatically increase the autophosphorylation of FAK in unstimulated transfected COS7 cells as compared to isoform without these additional exons (i.e. non-neuronal isoform) [27,29,32]. Though it is important to note that autophosphorylation of this neuronal isoform of FAK is tightly regulated *in vivo* and increases dramatically only in response to some extracellular messengers or stress [31,33,34]. The contrast between the levels of phosphorylation of neuronal isoform of FAK in transfected non-neuronal cells (COS7) and in nervous system suggests that specific regulatory mechanisms controling FAK activation exists in neurons [32], (3) FAK is expressed at very high levels in the brain but in contrast to non-neural cells, FAK is not restricted to focal adhesion contacts only. FAK is distributed throughout the neuron including the cell body, dendritic tree, and axon [16,31,35]. This suggests that FAK has different mechanisms for subcellular localization in neural cells and as a consequence has novel targets [35], (4) In neurons which mostly expresses FAK +6,7 isoform, phosphorylation of Tyr^{397} (a critical residue for activation and function of FAK) by Src-family kinases, which normally lead to FAK activation and thus triggering several signaling cascades, may not be possible [32]; pointing towards an additional difference with the ubiquitous isoforms, (5) FAK has been associated with protective (anti-apoptotic) signaling [36,37]; however, in neurons its activation has been shown to lead to cell death [17]. Neurons have been classically recognized as post-mitotic cells, and it is suggested that activated FAK may mediate cell cycle activation thereby leading to neurodegeneration [18].

These observations raise the question of specific functions of neuronal FAK. Our present findings demonstrates that in contrast to peripheral tissues (where FAK acts as a positive regulator of insulin/PI3K signaling), FAK in neurons acts as a negative regulator of insulin/PI3K signaling where increase in its activity precedes development of neuronal insulin resistance. It is to be noted that the increased phosphorylation of FAK under neuronal insulin resistance is demonstrated in N2A cells. It would be interesting and more supporting to test the effect in primary neurons, which has not been undertaken in this study. A separate experimental program has been initiated to generate insulin resistance in primary neurons to address various important questions along with this issue. We anticipate that this cell type specific function of FAK is due to the particular isoform exclusively expressed in neurons which might be in some way relevant to the neuronal physiology (like its post-mitotic nature). Future studies with various splice variants of FAK would be interesting in this regard. Interestingly, similar to our observations an aberrant increase in FAK phosphorylation is observed in chronic neurodegenerative disorder, Alzheimer's disease (AD) [17,18,38,39]. There is widening recognition that neuronal insulin resistance acts as the mediator of AD. AD is being referred as brain-specific form of diabetes and even termed as “type 3 diabetes” [6,8,9]. Our finding that FAK in neurons inhibits insulin/PI3K pathway i.e. the route which is reported to be the vital promoter of cell survival, supports the observation of FAK's involvement in AD-type neurodegeneration and highlights a crucial role of FAK in linking the two pathophysiological states.

The highly FAK related protein, Pyk2/RAFTK, is known to compensate for loss of FAK function in some cell types [40]. Pyk2/RAFTK is highly expressed in the brain and in particular, the hippocampus. However, in contrast to the non-neuronal cell types the regulation of these two related kinases (FAK and Pyk2) appear to be very distinct in the neuronal systems [27,33,41], suggesting that these two homologous tyrosine kinases have different functions in the nervous system [27]. This differential regulation of FAK and Pyk2 in nervous system has been suggested to be due to their localization to different cellular compartments and to their different modes of activation [27,41]. We also observed no significant change in the levels of Pyk2 with FAK downregulation as compared to the conrol siRNA transfected cells (Supplementary Fig. 4).

Summing up, the present findings provide a “neuronal cell-type specific” participating molecular mechanism of insulin resistance, where activation of FAK results in impairment of insulin/PI3K signaling and glucose metabolism. Importantly, based on the positive role of FAK in regulating peripheral insulin resistance, chemical entities that act as activator of FAK has been previously proposed as therapeutic strategy for pathophysiological condition of insulin resistance; however, present findings suggest a note of caution. Such modulators should be designed in such a way that it should not impart neuronal insulin resistance and complications associated with it, including AD.

Statement of conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbadis.2012.02.011.

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