List of Publications:

1. **Bhaskarjyoti Gogo**i, Priyajit Chatterjee, Sandip Mukherjee, Alak K Buragohain, Samir Bhattacharya, Suman Dasgupta, A polyphenol rescues lipid induced insulin resistance in skeletal muscle cells and adipocytes, *Biochemical and Biophysical Research Communication*, **452**(3), 382–388, 2014.

2. T. Borgohain, **B. Gogoi**, A.K. Buragohain, S. Dasgupta, Protective and curative effect of *Scoparia dulcis* leave extract against free fatty acid induced insulin resistance in rat L6 myotubes. *Am J Phytomed Clin Ther* **2**(7), 842-854, 2014 **.**

3. Shaswat Barua, **Bhaskarjyoti Gogoi**, Lipika Aidew, Alak Buragohain, Pronobesh Chattopadhyay, Niranjan, Karak, Sustainable resource based hyperbranched epoxy nanocomposite as an infection resistant, biodegradable, implantable muscle scaffold, *ACS Sustainable Chem. Eng.*, ***3*** (6), 1136–1144, 2015.

4. J. Upadhyay, A. Kumar, **B. Gogoi**, A.K. Buragohain, Antibacterial and hemolysis activity of polypyrrole nanotubes decorated with silver nanoparticles by an *in-situ* reduction process, *Mater Sci Eng C Mater Biol Appl.*, **1**(54), 8-13, 2015.

5. Dhaneswar Das, Parag Choudhury, Lakhyajyoti Bortahkur, **Bhaskarjyoti Gogoi**, Alak Kumar Buragohain and Swapan Kumar Dolui, Synthesis and characterization of SiO2/polyaniline/Ag core–shell particles and studies of their electrical and hemolytic properties: multifunctional core–shell particles, *RSC Adv.*, **5**, 2360-2367, 2015.

6. K. Bhattacharya, **B. Gogoi**, A. K. Buragohain, P Deb, Fe_2O_3/C nanocomposites having distinctive antioxidant activity and hemolysis prevention activity, *Material Science and Engineering C*, **42**, 595-600, 2014.

7. J. Upadhyay, A. Kumar, **B. Gogoi**, A.K. Buragohain, Biocompatibility and antioxidant activity of polypyrrole nanotubes**,** *Synthetic Metals*, **2**(189) 119– 125. 2014

8. J. Upadyay, **B. Gogoi**, A. Kumar, A.K.Buragohain, Diameter dependent antioxidant property of polypyrrole nanotubes for biomedical applications, *Materials Letter*, **102**(103), 33–35, 2013

Published in conference/ proceedings:

1. B. Gogoi, T. Borgohain, M. K. Das, A. Ramteke, S. Dasgupta, R. Mukhopadhyay, S. Bhattacharya, A. K. Buragohain, *Scoparia dulcis* Linn. prevents fatty acid induced insulin resistance in L6 muscle cells by improving the antioxidant enzyme activity ***"International conference on Molecular Signaling: Recent trends in Bioscience"***, held at NEHU, Shillong, November 20-22, 2015

2. B. Gogoi, P. Chatterjee, S. Mukherjee, A. K. Buragohain, S. Bhattacharya, S. Dasgupta, Ferulic acid, a plant derived polyphenol, prevents lipid induced PKCε activation and insulin resistance in skeletal muscle cells, ***National Seminar on "Recent Advances in Biotechnological Research in North East India: Challenges and Prospects***" held at Tezpur University, Assam, November 27- 29, 2014.

3. **Bhaskarjyoti Gogoi**, LipikaAidew, NilakshiBarua, Alak K Buragohain, *In silico* investigation of new inhibitor(s) of Protein Tyrosine Phosphatase 1B (PTP 1B) and Toll Like Receptor 4 (TLR4): the therapeutic targets of type 2 Diabetes Mellitus, ***International Symposium in Molecular Signaling***, Visva- Bharati University, Shantiniketan, Kolkata, February 18- 21 , 2013

Manuscript under preparation:

1. *Leucas aspera* leaf extract prevents lipid induced insulin resistance in L6 skeletal muscle cells by maintaining redox homeostasis.
2. Network Pharmacology based Virtual Screening of Natural Products Towards the Identification of Type 2 Diabetes Inhibitor: A novel approach

Biochemical and Biophysical Research Communications 452 (2014) 382–388

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A polyphenol rescues lipid induced insulin resistance in skeletal muscle cells and adipocytes

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ARTICLE INFO

Article history:
Received 12 August 2014
Available online 23 August 2014

Keywords:
SFA
Ferulic acid
Fetuin-A
Insulin receptor
Insulin resistance
PKCε

ABSTRACT

Skeletal muscle and adipose tissues are known to be two important insulin target sites. Therefore, lipid induced insulin resistance in these tissues greatly contributes in the development of type 2 diabetes (T2D). Ferulic acid (FRL) purified from the leaves of *Hibiscus mutabilis*, showed impressive effects in preventing saturated fatty acid (SFA) induced defects in skeletal muscle cells. Impairment of insulin signaling molecules by SFA was significantly waived by FRL. SFA markedly reduced insulin receptor β (IRβ) in skeletal muscle cells, this was affected due to the defects in high mobility group A1 (HMGA1) protein obtruded by phospho-PKCε and that adversely affects IRβ mRNA expression. FRL blocked PKCε activation and thereby permitted HMGA1 to activate IRβ promoter which improved IR expression deficiency. In high fat diet (HFD) fed diabetic rats, FRL reduced blood glucose level and enhanced lipid uptake activity of adipocytes isolated from adipose tissue. Importantly, FRL suppressed fetuin-A (FetA) gene expression, that reduced circulatory FetA level and since FetA is involved in adipose tissue inflammation, a significant attenuation of proinflammatory cytokines occurred. Collectively, FRL exhibited certain unique features for preventing lipid induced insulin resistance and therefore promises a better therapeutic choice for T2D.

1. Introduction

Insulin resistance is in the center of pathophysiology of type 2 diabetes (T2D) where loss of insulin sensitivity in target tissues silently occurs during disease progression [1,2]. Lipids are known to promote the loss of insulin sensitivity that causes insulin resistance and T2D [1,3,4]. Although how lipid, specially saturated fatty acid (SFA), produces insulin resistance is not well understood, a few recent reports indicate that lipid induced inflammation in insulin target tissues significantly contributed to the development of insulin resistance and T2D [5–7]. Interestingly, several studies have shown that lipid oversupply activates nPKCs, more specifically PKCε and PKCθ, these two nPKCs are known to be associated with lipid induced insulin resistance [8–10]. Skeletal muscle is the largest repository where more than 75% glucose is being stored, therefore insulin resistance in this tissue has a critical role in the development of T2D [11,12]. In this tissue lipid oversupply induced activation of PKCε is primarily responsible for the loss of insulin sensitivity [4,9,13–16]. In contrast, insulin resistance in adipose tissue due to excess lipid is affected through TLR4 activation [5] by fetuin-A (FetA)-lipid complex, this leads to the release of proinflammatory cytokines that affect insulin resistance [6]. Hence, it appears from the existing reports that lipid produces defects in these two important insulin target tissues through two different mechanisms, one involves the (i) activation of nPKCs in skeletal muscle cells and other through the (ii) formation of SFA-FetA-TLR4 complex in adipocytes.

In this report we have demonstrated that a polyphenol i.e., ferulic acid (FRL) isolated from the leaves of *Hibiscus mutabilis*, significantly inhibited lipid induced kinase independent activation of PKCε in the skeletal muscle cells that prevented decline of insulin receptor (IR) due to SFA induced suppression of IR gene expression. FRL targets lipid induced insulin resistance in adipocytes obtained from HFD rats by attenuating FetA expression which reduces secretion of pro-inflammatory cytokine from adipose tissue.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.079
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2. Materials and methods

2.1. Reagents and antibodies

All tissue culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, USA. Primary antibodies were purchased from Santa Cruz Biotechnology Inc., California, USA. Alkaline phosphatase conjugated respective secondary antibodies were purchased from Sigma Chemical Co., St. Louis MO, USA. [^{3}H]-2-deoxyglucose and [^{14}C]-palmitate were purchased from GE Healthcare Biosciences Ltd., Kowloon, Hong Kong. All other chemicals and reagents used were purchased from Sigma Chemical Co., St. Louis MO, USA.

2.2. Bioactivity driven extraction, fractionations and purification of the compounds

Fresh leaves of the *H. mutabilis* L. were collected during 2011–2013 from North-East region, India. Leaves were air dried in shade at room temperature and ground to a coarse powder (40 mesh) using a mechanical grinder. The leaf powder (2 kg) was extracted with methanol (4 L) for 48 h. The extraction and isolation procedure has been carried out on the basis of improvement of SFA (palmitate) induced impairment of insulin activity in terms of [^{3}H]-2DOG uptake by L6 myotubes. Bioactivity guided fractionation and final purification through semi preparative HPLC (XTerra™ Prep RP C18, 7.8 × 300 mm, 10 mm particle size) provided to amorphous solids, which was characterized as ferulic acid (FRL) and caffeic acid (CFA) by comparison of their spectroscopic data (mass, ^{1}H and ^{13}C NMR) with those reported previously [17,18].

2.3. Cell culture and treatments

L6 skeletal muscle cell line was procured from the National Centre for Cell Science, Pune, India and were cultured in a similar manner as described by us previously [7,9]. Briefly, L6 skeletal muscle cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37 °C. When cells reached 75% confluency, the medium was replaced with DMEM containing 2% horse serum and cultured for 24 h for its differentiation into the myotubes. Myotubes were incubated for 6 h without or with 0.75 mM palmitate (SFA) in absence or presence of insulin. For treatment with the FRL, cells were pretreated for 1 h followed by palmitate incubation. On termination of incubations, cells were harvested with trypsin–EDTA solution and cell pellets were resuspended in lysis buffer followed by centrifuged for 10 min at 10,000*g*. Protein concentrations were determined following the method of Lowry et al. [19].

2.4. Insulin resistant diabetic rat model

An insulin-resistant high-fat diet (HFD) rat model was developed by following our earlier description [20]. Briefly, the energy content of the standard diet was 15 kJ/g and the high-fat diet was 26 kJ/g. SD and HFD fed male rats were housed in group of 3–6 individuals/cage under 12 h light/dark cycle at 23 ± 2 °C (humidity 55 ± 5%) with *ad libitum* access to food and water. FRL was administered into the rat through oral gavages (0.6 mg/kg - body wt/day) at each alternative day for a period of 15 days. All animal experiments were performed following the guidelines prescribed and approved by the Visva-Bharati (A Central University) Animal Ethics Committees.

2.5. [^{3}H] 2-deoxyglucose uptake

[^{3}H]-2-deoxyglucose uptake (2DOG) was performed as previously described from our laboratory [6]. Briefly, L6-myotubes were serum starved overnight in Kreb's Ringer Phosphate (KRP) buffer supplemented with 0.2% bovine serum albumin. After relevant incubations, cells were treated with porcine-insulin (100 nM) for 30 min. Before termination of experiment, 2DOG (0.4 nmol/ml) was added to each of the incubations for 5 min. Cells were harvested with trypsin–EDTA solution, solubilized with 1% NP-40 and 2DOG uptake was measured in a Liquid Scintillation Counter (Perkin Elmer, Tri-Carb 2800TR).

2.6. Immunoblotting

Immunoblot analysis was performed by following the method described previously from this laboratory [7]. Briefly, cell lysates (60 μg of protein) were subjected to 10% SDS/PAGE and transferred on to Immobilon-P PVDF membranes (Millipore, Bedford, MA) with the help of Semi-Dry trans-blot Apparatus (Bio-Rad Trans-Blot® SD-Cell). Membranes were probed with specific primary antibodies and subsequently detected by using either ALP (alkaline phosphatase)-conjugated goat anti-rabbit IgG or rabbit anti-mouse-IgG (Sigma–Aldrich). The protein bands were visualized using 5-bromro-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

2.7. [^{3}H]Leucine incorporation study

[^{3}H]-Leucine incorporation into IRβ protein in L6 myotubes was studied by following our earlier procedure [7].

2.8. Quantitative PCR

Quantitative PCR was performed for IR-β [9] and FetA (forward: 5′-CTCACAGCCCCAACCA and reverse: 5′-CCACTCTGCTTCTGTCCT-3′) following our earlier procedure [9].

2.9. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's protocol by using a ChIP assay kit (Upstate, Temecula, CA, USA). Incubated cells were fixed with 1% formaldehyde for 10 min at 37 °C. HMGA1 antibody was used for immunoprecipitation of DNA and IR promoter specific primers (forward: 5′-AACCACCTCGAGTCACCAAAA-3′ and reverse: 5′-AGAGAGAGGGAAAGCTTGCAG-3′) were used to amplify the immunoprecipitated insulin receptor–promoter sequence. The PCR products were resolved on ethidium bromide stained 1.5% agarose gel and image was captured by Bio-Rad Gel documentation system.

2.10. Metabolic labeling

L6 skeletal muscle cells were incubated with [^{14}C]-palmitic acid (0.8 mCi/ml) at 37 °C and on termination of incubations cells was washed with PBS to remove the free label. PKCε was immunoprecipitated and subjected to electrophoresis followed by fluorography according to our earlier described method [9].

2.11. Coimmunoprecipitation

This assay was performed by following a previously described procedure [9] using HMGA1 antibody for immunoprecipitation followed by probing with p-Serine antibody.

2.12. Promoter–reporter assay

IRP-GLuc plasmid was generated by following a previously described method by us [9]. L6 myotubes were transfected for 72 h with pIRP-GLuc plasmid (0.25 mg/well) using Lipofectamine™ 2000 and luciferase activity was measured from the incubated cells in a luminometer.

2.13. Statistical analysis

All data were derived from at least three independent experiments and statistical analyses were conducted using SigmaPlot 12.0 software. Data were analyzed by one-way analysis of variance (ANOVA), where the p value indicated significance, means were compared by a post hoc multiple range test. All values were means ± SEM. A level of $p < 0.05$ was considered significant.

3. Results

3.1. SFA induced suppression of insulin activity is reversed by FRL

We purified ferulic acid (FRL) and caffeic acid (CFA) from the leaf extract of *H. mutabilis* and observed their anti-type 2 diabetic (T2D) effect on lipid induced insulin resistance *in vitro* and *in vivo* models. Incubation of skeletal muscle cells i.e. L6 myotubes, which is one of the major insulin target cells, with saturated fatty acid (SFA) greatly reduced insulin stimulated [^{3}H]-2deoxyglucose (2DOG) uptake. Palmitate (SFA) inhibitory effect on insulin stimulated glucose uptake was significantly prevented by FRL but not by CFA (Fig. 1A). Addition of increasing concentrations of FRL (2–20 μg/ml) to L6 myotube incubation showed a dose dependent improvement of insulin activity suppressed by SFA (Fig. 1B). Insulin binding to its receptor on target cell surface transduces a signal

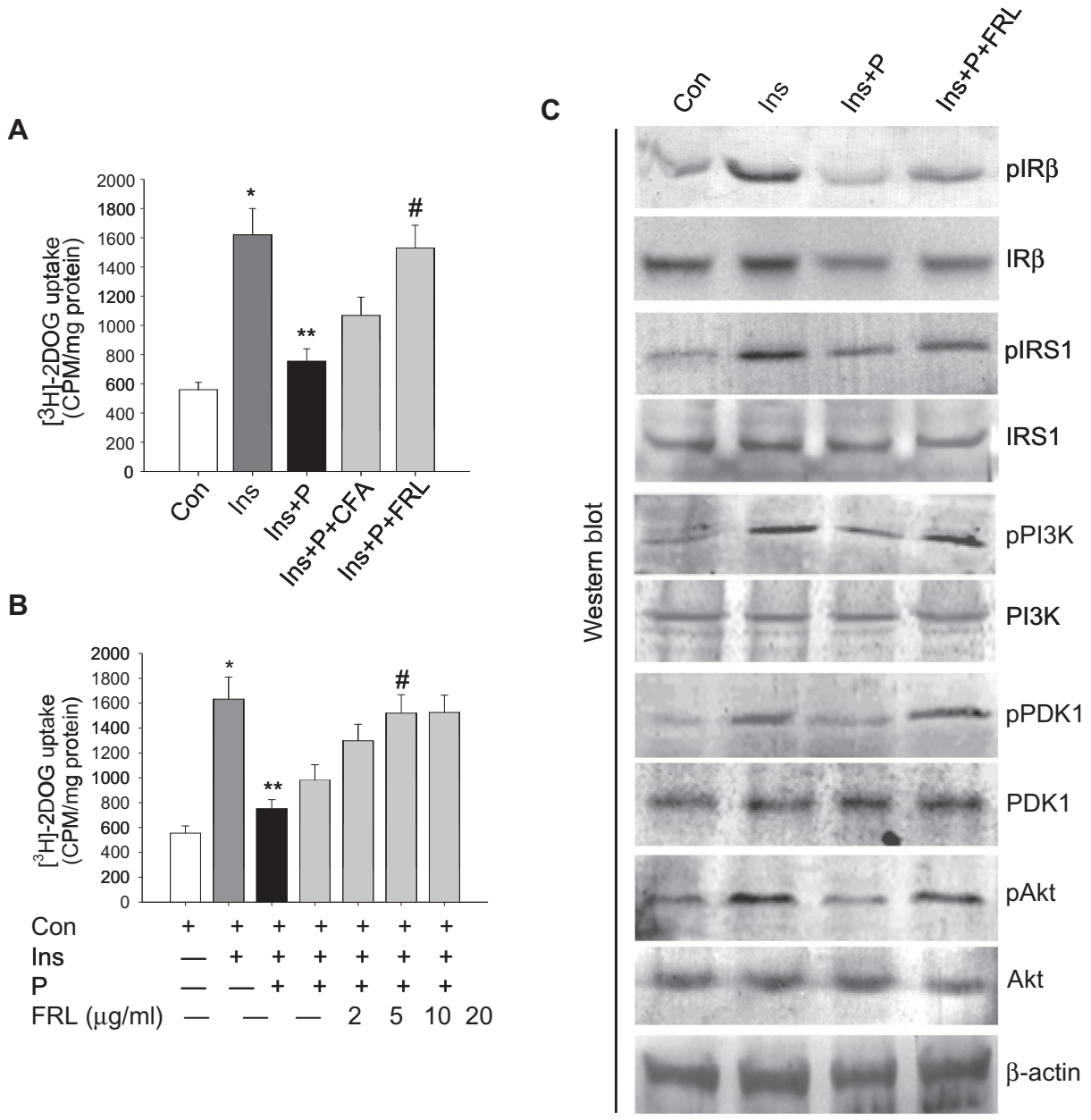

Fig. 1. Palmitate inhibition of insulin signaling pathway was prevented by FRL. (A) [^{3}H]-2DOG uptake by L6 myotubes incubated with insulin or insulin + SFA(P) in presence or absence of caffeic acid (CFA) or ferulic acid (FRL), (B) in the presence of increasing concentrations of FRL. (C) Western blot showing insulin stimulated phosphorylation of IRβ, IRS1, PI3K, PDK1 and Akt in L6 myotubes incubated with insulin or insulin + SFA(P) or insulin + SFA(P) + FRL. Each value is the mean ± SEM of three independent experiments, $^*p < 0.001$ vs Con, $^{**}p < 0.01$ vs Ins, $^{\#}p < 0.001$ vs Ins + SFA(P).

cascade which is initiated with insulin receptor tyrosine kinase phosphorylation and ultimately to protein kinase B or Akt through several signaling molecules. It could be seen from Fig. 1C that all these signaling molecules were activated by insulin and addition of FRL markedly reduced SFA induced inhibition. These results indicate FRL protection to lipid induced insulin resistance.

One of the interesting points revealed in these experiments was the depletion of insulin receptor (IR) protein that coincided with the inhibition of IR-tyrosine kinase activation by SFA. On investigating this further, we found that SFA suppressed IR gene expression and protein synthesis and both of these could be intervened by FRL (Fig. 2A and B). To observe the mechanism of SFA's inhibition of IR gene expression, we monitored the binding of high mobility group A1 (HMGA1) protein, an architectural transcription factor of IR gene [21], to IR promoter and IR-promoter activation, both declined due to SFA. Interestingly, FRL significantly waived these inhibitory effects of SFA (Fig. 2C and D).

3.2. FRL protection to SFA's inhibitory effect on IR expression in skeletal muscle cells

SFA's inhibitory effect on IR expression is mediated through the kinase independent phosphorylation of PKCε (pPKCε), this then migrates to the nuclear region and phosphorylates HMGA1 that retards its migration to IR promoter which compromised IR expression [9]. Our results show that FRL attenuated palmitoylation of PKCε thus decreased its kinase independent phosphorylation (Fig. 3A). This was also evident from subdued pPKCε mobilization from cytosol to nuclear region (Fig. 3B), and that significantly reduced HMGA1 phosphorylation (Fig. 3C). These findings indicate that FRL rescues repression of IR gene expression through the inhibition of PKCε palmitoylation, that reduces its phosphorylation and migration to nuclear region thus prevents impairment of HMGA1.

3.3. FRL prevents lipid induced disruption of adipocyte function

Recent reports on adipose tissue insulin resistance due to lipid oversupply identified two important regulators, one is Toll-like receptor 4 (TLR4) and another is fetuin-A (FetA) which are involved in lipid induced adipocyte inflammation. In high-fat diet (HFD) fed mice, TLR4 and FetA association stimulate the production of pro-inflammatory cytokines through NF-κB activation and that causes insulin resistance. It has been reported that TLR4 or FetA KO mice are protected from high-fat diet induced insulin resistance [5,6] suggesting that FetA and/or TLR4 are required to implement insulin resistance. In the present investigation, we used HFD fed type 2 diabetic rat model, where oral administration of FRL reduced elevated circulatory glucose nearly to control level (Fig. 4A and B). FRL also recovered lipid uptake ability of adipocytes collected from HFD rats (Fig. 4C). When we examined FetA level in HFD rat, a high level of it could be observed in comparison to standard diet (SD) fed rats. Oral administration of FRL in HFD rats significantly reduced FetA circulatory level (Fig. 4D) and its

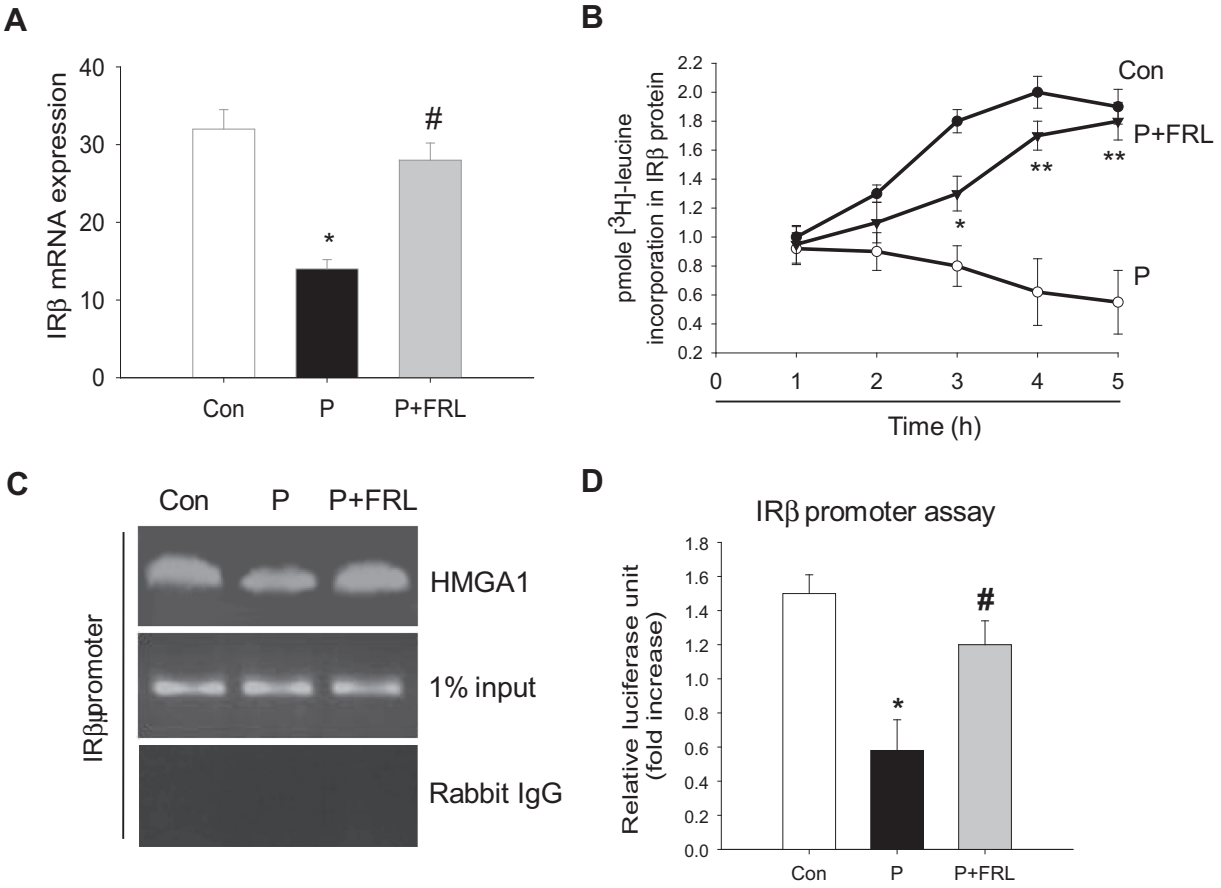

Fig. 2. FRL inhibits lipid induced inhibition of IR gene expression. (A) IRβ mRNA expression in FRL incubated L6 myotubes treated without or with SFA(P). Each value is the mean ± SEM of three independent experiments. $^{*}p < 0.01$ vs Con, $^{\#}p < 0.01$ vs SFA(P). (B) Estimation of IRβ protein synthesis in L6 myotubes incubated with [^{3}H]-leucine (10 μCi/ml) in the presence or absence of SFA(P) or SFA(P) + FRL. Each value is the mean ± SEM of three independent experiments. $^{*}p < 0.05$ vs SFA(P), $^{**}p < 0.01$ vs SFA(P). (C) ChIP assay showing HMGA1 binding to IR promoter and (D) the determination of relative IR-luciferase activity in L6 myotubes incubated without or with SFA(P) or SFA(P) + FRL. Each value is the mean ± SEM of three independent experiments. $^{*}p < 0.01$ vs Con, $^{\#}p < 0.05$ vs SFA(P).

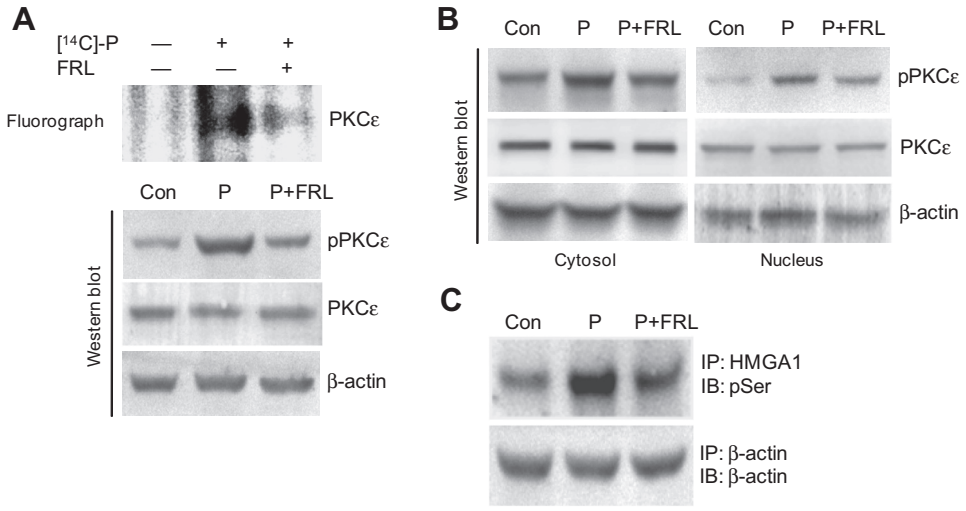

Fig. 3. FRL attenuates HMGA1 phosphorylation through the inhibition of PKCε activation due to palmitoylation. (A) Fluorograph showing [^{14}C]-palmitate incorporation into PKCε in L6 myotubes in the absence or presence of FRL (upper panel) and Western blot exhibits pPKCε and PKCε in total cell lysate (lower panel). (B) Cytosolic and nuclear pPKCε or PKCε in L6 myotubes incubated without or with SFA(P) or SFA(P) + FRL. (C) IP-IB assay showing phosphorylated HMGA1 level in L6 myotubes incubated without or with SFA(P) or SFA(P) + FRL.

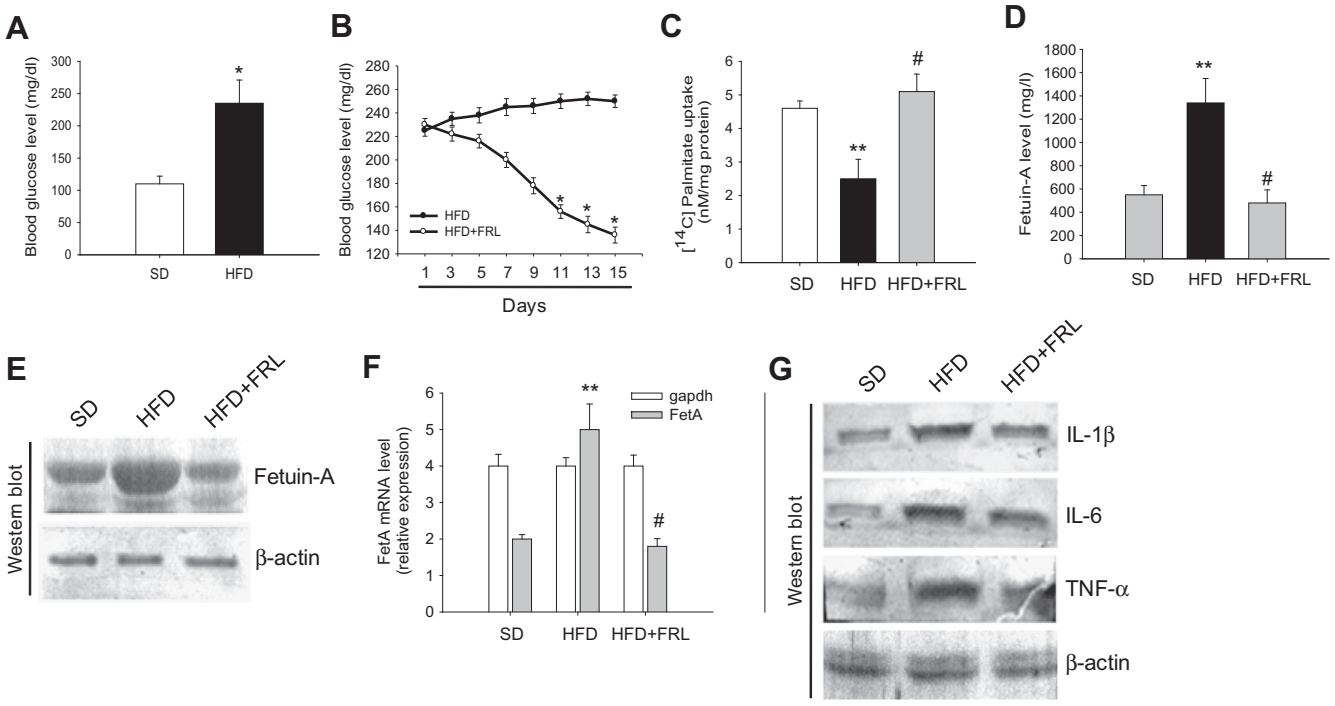

Fig. 4. FRL effects on HFD-fed diabetic rats. (A) Blood glucose level in SD- or HFD-fed rats; (B) blood glucose level in response to FRL administration for 15 d in HFD-fed rats. (C) [^{14}C]-palmitate uptake in the adipocytes isolated from SD or HFD or HFD + FRL treated rats. (D) Circulatory FetA level in SD or HFD or HFD + FRL rats and (E) Western blot of adipocyte FetA in the same group of rats. (F) Adipocyte FetA mRNA expression in SD or HFD or HFD + FRL rats. (G) Immunoblot of proinflammatory cytokines from the plasma samples of SD or HFD or HFD + FRL rats. Each value is the mean ± SEM of three independent experiments and each experiment had six SD or HFD or HFD + FRL rats. $^{*}p < 0.01$ vs HFD, $^{**}p < 0.01$ vs SD, $^{\#}p < 0.01$ vs HFD.

expression in adipocytes (Fig. 4E). Interestingly, FRL decreased FetA in adipose tissue by suppressing its gene expression (Fig. 4F). FetA is required to activate TLR4 mediated increase of pro-inflammatory cytokines that produces insulin resistance [6]. FRL inhibition of FetA significantly attenuated excess of pro-inflammatory cytokine productions in HFD rats (Fig. 4G). These results imply that FRL could be a therapeutic choice for insulin resistance and type 2 diabetes.

4. Discussion

In this report we have demonstrated that two major insulin target cells, skeletal muscle cells and adipocytes, where lipid induced defects causes insulin resistance and type 2 diabetes (T2D), could be effectively rescued by a polyphenol i.e. ferulic acid (FRL), isolated from a plant source. Interesting part of our observations includes two separate modes of adversities in skeletal muscle cells

and adipocytes affected by lipid may lead to insulin resistance and T2D. Both these pathways, although fundamentally different, are remarkably intervened by FRL. In skeletal muscle cells SFA impairs insulin activity through kinase independent phosphorylation of PKCε, pPKCε then migrates to nuclear region and phosphorylates HMGA1. pHMGA1 preferentially interacts with positively charged histones that cause an increase of its residential time in the heterochromatin region, thus inhibits its occupation of IR promoter (9,22). This adversely affects IRβ gene expression. We found this to be a major pathway of SFA induced defects in skeletal muscle cells because substantial amount of IR decreased due to SFA [9,13]. In fact, deficiency of IR in diabetic patients has also been previously reported [21–24]. On the other hand, impairment of adipose tissue due to lipid oversupply is related to TLR4 activation [5] which is mediated through FetA [6]. TLR4-KO or FetA-KO mice are resistant to HFD induced insulin resistance, indicating that TLR4 and FetA are necessary to implement lipid induced insulin resistance [5,25]. On this background, it is indeed interesting to find that FRL protects lipid induced insulin resistance in skeletal muscle cells and adipocytes by targeting different pathways. In skeletal muscle, FRL subdues kinase independent activation of PKCε, this is a very significant effect because PKCε does not have any NLS and its phosphorylation permits recognition by F-actin which in turn chaperoned it to the nuclear region where it phosphorylates HMGA1 that blocks its migration to IR promoter. This has been shown to markedly reduce IR expression [9].

Therapeutic choice to deal with lipid induced insulin resistance and T2D is extremely limited. On this background, the thiazolidinedione (TZD) class of drugs exhibited improvement of insulin sensitivity through the activation of peroxisome proliferator-activated receptor gamma ($PPAR_{\gamma}$). Number of genes in adipocytes that promote FFA entry and decreases excess FFA release from adipocytes are regulated by $PPAR_{\gamma}$ [26–28]. TZDs success in clinical practice has been aborted because of their adverse side effects such as development of edema, congestive heart failure [29,30] and decrease in hemoglobin and hematocrit values [31].

FRL's effect in the amelioration of Type 1 or insulin dependent diabetes have been reported [32,33], where the models are related to decrease in insulin availability that occurs due to pancreatic β-cell destruction. But this affects only 3–5% of diabetic patients. In contrast, we worked with T2D model where the disease occurs due to insulin resistance. This is the epidemic disease, 90–97% patients are T2D. Few reports also indicated that FRL possesses antioxidant role [34,35] and since oxidative stress is associated with insulin resistance, this effect of FRL is also significant. Here we report about the amelioration of T2D by FRL which targets recently reported new domains of T2D [5,6,9]. Our *in vivo* experiments with nutritionally induced diabetic rats demonstrate that FRL could influence the improvement of glycemic level within 15 days when orally administered for 8 days. In addition, FRL suppressed FetA expression in adipose tissue of HFD rats and since FetA is associated with adipose tissue inflammation, FRL attenuation of FetA significantly decreased pro-inflammatory cytokines which are responsible for insulin resistance and T2D. Importantly, FRL has better bioavailability than other dietary flavones [36]. In conclusion, FRL demonstrates impressive role on the amelioration of skeletal muscle and adipose tissue insulin resistance and is therefore expected to be a good therapeutic choice for T2D.

Acknowledgments

This research was financially supported by the grants from the Department of Biotechnology, Government of India (Ref. No. BT/56/NE/TBP/2010). We thank the National Centre for Cell Science, Pune, India, for providing L6 skeletal muscle cell line and Prof. Graeme Bell (University of Chicago, Illinois, USA) for the gift of human insulin receptor promoter plasmid construct phINSRP-1. S.B. thanks the National Academy of Science, India for his NASI Senior Scientist position. The authors appreciate the use of facilities as extended by the Head, Department of Zoology, Visva-Bharati (A Central University), Santiniketan, W. Bengal; and the Head, Department of Molecular Biology and Biotechnology, Tezpur University, Assam, India. We also thank Animal Ethics Committee of Visva-Bharati for permitting the research with SD and HFD fed rats. The technical help from Mr. Hemanta Jadav and Mr. Kali Charan Nayek of Visva-Bharati is greatly appreciated.

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Original Article

Protective and Curative Effect of *Scoparia dulcis* Leave Extract Against Free Fatty Acid Induced Insulin Resistance in Rat L6 Myotubes

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ABSTRACT

Objective: The objective of the present study was to investigate the insulin sensitizing effect of *Scoparia dulcis* (L) leave extract (SDE) against free fatty acid (FFA) induced insulin resistance in an *in vitro* L6 myotubes.
Materials and Methods: The rat L6 skeletal muscle cells were differentiated to myotubes by treating it with DMEM containing 2% horse serum for 12h. For generating an *in vitro* cellular model of insulin resistant condition, L6 myotubes were treated with FFA (palmitate) for 4h. Insulin resistant L6 myotubes were either pre- or post treated for 1h with SDE and its insulin sensitivity activity was assessed by measuring cellular glucose uptake and the activation status of insulin signalling pathway molecules.
Results: SDE significantly stimulated glucose uptake in L6 myotubes in a dose-dependent manner with maximal effect at 50µg/ml. To investigate the underlying mechanism of effect SDE, we examined the expression and activity of insulin signalling pathway molecules. We found that the SDE treatment notably increased insulin signalling pathway by inducing activatory phosphorylation status of IRS-1 and Akt without altering expression levels of these proteins. The comparative analysis revealed that SDE is more potent than known insulin sensitizer, pioglitazone.
Conclusion: These results suggest that induction of insulin signalling pathway and increased glucose uptake activity of SDE will aid in the treatment of insulin resistance and type 2 diabetes. Future studies on the isolation of bioactive components and a detailed investigation in the animal model of insulin resistance may be promising to find out a novel type 2 diabetes drug.

Keywords: FFA, Insulin resistance, L6 myotube, Glucose uptake,

Scoparia dulcis.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease and around 95% of diabetic patients are diagnosed with type 2 diabetes (T2D)[1]. Menacing increase of T2D is one of the major cause of morbidity and mortality of human beings worldwide and its increased annual rate indicates insufficiency of existing therapeutic options. The pathology of this disease is characterized by the presence of insulin resistance where target cells fail to respond normal circulatory level of insulin and that leads to the development of hyperglycemia[2]. Hence loss of insulin sensitivity in target tissues is the early sign in the progression of this disease which precedes and predicts the development of T2D.

It is now well established that free fatty acids (FFAs) are major player in promoting the loss of insulin sensitivity causing insulin resistance and T2D[3-5]. Several reports demonstrated that increased plasma FFA level contributes to the development of insulin resistance[3,4], while lowering of its level in diabetic subjects improves insulin sensitivity[6]. Incubation of skeletal muscle cells with FFA decreased insulin-stimulated glucose uptake[3]. Reports in this line suggest that FFA disrupts insulin signaling pathway in many facets in insulin target tissues and thus can adversely affect insulin resistance[7,8]. Insulin stimulated glucose uptake is mediated through the activation of insulin signalling pathway that leads to the translocation of glucose transporter, Glut4, from the interior to the cell surface[9]. Any defect in this pathway triggers the development of insulin resistance, which leads to the progression of future T2D. Therefore, amount of insulin stimulated glucose uptake into the peripheral tissues, particularly in skeletal muscle where 75% of postprandial glucose is deposited[10], is an important way to assess insulin sensitivity. Although insulin sensitizers thiazolidinedione group of drugs are effective in improving insulin sensitivity[11-13], however different adverse side effects limits their long term use[14-16]. Hence, the demand for new anti-diabetic compounds continues.

Medicinal plants constitute a common alternative treatment for T2D in many parts of the world[17-21] and that is the reason for thousands of years, plants and their derivatives are being used for treatment of T2D. We have selected *Scoparia dulcis* Linn. (Family - Scrophulariaceae), commonly known as sweet broom weed, in our investigation which has been used traditionally by the ayurvedic practitioners for the treatment of diabetes mellitus[22]. It is an erect perennial herb with serrated leaves and many small white flowers and mainly found in the tropical and subtropical regions. Its anti-diabetic activity has been explored in streptozotocin (STZ) treated animal model of type 1 diabetes (T1D)[23-25] which do not address the pathophysiology of insulin resistance and T2D. These studies only focused the glucose lowering effects of this plant; however their potential mechanism(s) of action have not been clearly elucidated at the molecular level. Since, insulin stimulated glucose uptake in skeletal muscle cells is important for maintaining glucose homeostasis and a decrease in the glucose uptake is one of the key signs of insulin resistance and T2D, the present study was conducted to evaluate the insulin sensitizing effect of *Scoparia dulcis* plant on skeletal muscle glucose uptake and insulin signalling pathway molecule activation.

MATERIALS AND METHODS

Reagents and antibodies

All cell culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, USA. Anti-IRS-1 (anti-rabbit), anti-pIRS-1 (Tyr-989, anti-goat), anti-pAkt (Ser 473, anti-rabbit), anti-pAkt (Thr-308, anti-rabbit), anti-Akt (anti-rabbit) and anti-β actin (anti-rabbit) antibodies were purchased from Santa Cruz Biotechnology Inc., California, USA. Alkaline phosphatase conjugated respective secondary antibodies were purchased from Sigma Chemical Co., St. Louis MO, USA. Glucose uptake cell-based assay kit was purchased from Cayman Chemical Company, Ann Arbor, MI, USA. All other chemicals and reagents used were either purchased from Himedia, Mumbai, India or Sigma Chemical Co., St. Louis MO, USA.

Collection of plant leaves and preparation of extract

The *Scoparia dulcis* plant was collected from Tezpur, Assam, India in 2013 and was authenticated by a competent plant taxonomist, Dr. Dipanwita Banik, CSIR-North East Institute of Science & Technology (NEIST), Jorhat, Assam, India and a voucher specimen was preserved in our laboratory. For preparation of extract, the leaves were washed, shade dried and crushed in a grinder and then mixed with ethanol and kept at 150rpm in an orbital shaker for 24h. The mixture was then filtered through Whatman No.1 filter paper and the filtrate was condensed in rota evaporator and was used for the present investigation.

Cell culture

L6 skeletal muscle cell line was procured from the National Centre for Cell Science, Pune, India and Prof. Samir Bhattacharya, Visva - Bharati University, West Bengal, India and was cultured in a similar manner as described by us previously[26]. Briefly, L6 skeletal muscle cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100U/ml) and streptomycin (100µg/ml) in a humidified 5% CO_2 atmosphere at 37°C. Approximately $1x10^6$ cells were plated in each well of the 6-well culture plate and when cells reached 75% confluency the medium was changed to DMEM supplemented with 2% horse serum. After 24h of culture in this medium all the cells were differentiated to myotubes which was used in our experiments.

Cell treatments

Palmitate (FFA) was conjugated with FFA-free bovine serum albumin following the method as described previously[27]. Briefly, palmitate was dissolved in ethanol and diluted 1:100 in 1% FBS-DMEM containing 5% (w/v) bovine serum albumin. Confluent L6 myotubes were incubated for 4h without or with 0.75 mM palmitate. For treatment with the plant extract, cells were either pre- or post-treated for 1h. On termination of incubations, cells were washed twice with ice-cold calcium-magnesium free PBS and harvested with trypsin (0.25%)–EDTA (0.5mM). Cell pellets were resuspended in lysis buffer, vortexed in every 10mins for 30mins and centrifuged for 10min at 10,000rpm at 4°C. Protein concentrations were determined following the method of Lowry *et al* (1951)[28].

Glucose uptake assay

2-NBDG (2-deoxy-2-[(7-nitro-2, 1, 3-benzoxadiazol-4-yl) amino]-D glucose) uptake assay was performed by following a previously described method[29]. Briefly, L6 myotubes ($1x10^6$ cells/ml) were serum starved overnight in glucose free DMEM supplemented with 2% FBS and then treated

without or with plant extract for 1h followed by 4h palmitate (0.75mM) incubation. Cells were then treated with or without 100nM of porcine insulin for 30 min and 10 min prior to the termination of experiment 2-NBDG (100µg/ml) was added to each of the incubations. Cellular uptake of 2-NBDG was measured using a fluorometer at excitation and emission wavelengths of 485 and 535 nm, respectively.

Immunoblotting

Immunoblot analysis was performed by following the method described previously[26]. Briefly, cell lysates (60µg protein) were subjected to 10% SDS/PAGE and transferred on to Immobilon-P PVDF membranes (Millipore, Bedford, MA) with the help of Semi-Dry trans-blot apparatus (GE Healthcare). Membranes were first blocked with 10% non-fat dried milk in TBST buffer followed by probing with primary antibodies (pAkt^{Ser473}, pAkt^{Thr308}, Akt, pIRS-1^{Tyr989}, IRS-1) and subsequently detected using ALP (alkaline phosphatease)-conjugated goat anti-rabbit IgG or mouse anti-goat IgG. The protein bands were visualized using 5-bromro 4-chloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

MTT Assay

The MTT assay was performed by following the method described previously[30]. In brief, L6 myotubes ($1x10^5$ cells/ml) were seeded into 96 well microtiter plates. The next day, medium replaced with DMEM containing SDE at various concentrations and incubated for 24h. After 24h, media was removed and replaced with complete media. MTT (20µl of 5 mg/ml in PBS) was added to each well of the plates and incubated further for 5h. The formazan crystals formed were dissolved in 100µl of acidic isopropanol (0.04 M HCl in absolute isopropanol) after aspirating the medium and incubated further for 30min at 37ºC. Cytotoxicity was measured spectrophotometrically at 570 nm with a microplate reader. Absorbance values were blanked against acidic isopropanol and the absorbance of cells exposed to medium only (without any treatment) were taken as 100% cell viability (control).

Statistical analysis

All data were derived from at least three independent experiments and statistical analyses were conducted using Sigma Plot 8.0 software. Data were analyzed by one-way analysis of variance (ANOVA), where the F value indicated significance, means were compared by a post hoc multiple range test. All values were means ± SEM. A level of $p<0.05$ was considered significant.

RESULTS

Effect of SDE on FFA induced insulin insensitivity

For searching anti-diabetic activity from medicinal plants of North-East India, we have selected *Scoparia dulcis* plant (Figure 1) based on the traditional knowledge in its use against diabetes mellitus. In order to observe the insulin sensitizing effect, *Scoparia dulcis* plant extract (SDE, 100µg/ml) was either pre- or post- incubated for 1h in L6 myotubes treated with FFA (palmitate, 0.75mM) for 4h in absence or presence of insulin (100nM) and 2-NBDG (100µg/ml). Insulin effected 3-fold induction of 2-NBDG uptake in comparison to control cells while FFA significantly inhibited insulin-stimulated 2-NBDG uptake. Both pre- and post-treatment of SDE showed considerable induction of glucose uptake in presence of FFA suggesting that its effect may be due to the suppression of FFA inhibition (Figure 2). SDE pre-incubation showed greater activity in preventing FFA induced reduction of insulin stimulated glucose

uptake by L6 myotubes in comparison to post-treatment (Figure 2). Since loss of insulin sensitivity by FFA that leads to insulin resistance is the major problem in T2D to be addressed, our assay system reflects the validity of such determination.

Dose dependent effect of SDE on insulin sensitivity

In order to observe the dose dependent effect of SDE, L6 myotubes were pre- or post- incubated for 1h with varied concentration of SDE (25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml). Cells were incubated with FFA (palmitate) for 4h without or with insulin in presence of 2-NBDG. Both pre- and post- incubation of SDE showed dose dependent suppression of FFA inhibition, peak of which was observed at 50µg/ml (Figure 3).

Effect of SDE on FFA induced impairment of insulin signaling

It is well known that stimulation of insulin signaling pathway leads to the glucose uptake in insulin target cells. Binding of insulin to its receptor causes phosphorylation of tyrosine residues that activates the signaling pathway. Phosphorylated insulin receptor tyrosine kinase recruits and phosphorylates insulin receptor substrate-1 (IRS-1) and amplifies the signal which leads to the activation of a key kinase, Akt/PKB, that regulates the movement of glucose transporter 4 (Glut4) from cytosol to the plasma membrane. Activation of Akt depends on the phosphorylation of its Thr308 and Ser473 residues which plays a critical role in insulin stimulated glucose uptake in skeletal muscle and adipose tissue. Several reports indicate that FFA mitigates insulin-stimulated glucose uptake in skeletal muscle cells through the deactivation of IRS-1 and Akt. To observe the IRS-1 and Akt phosphorylation status in response to SDE is therefore a highly relevant assay system to assess insulin activity. In order to observe whether FFA induced impairment of insulin signaling pathway could be prevented or waived by SDE, we incubated L6 myotubes without (Con) or with insulin (Ins) or insulin plus FFA (Ins + FFA) in absence or presence of pre- or post- SDE treatment. Insulin signaling was augmented by insulin as showing increased level of IRS-1 and Akt phosphorylation while addition of FFA notably attenuated this stimulation. SDE addition reversed FFA inhibitory effect on insulin signaling as enhanced level of phosphorylated IRS-1 and Akt were observed in FFA incubated pre- and post-SDE treated cells (Figure 4).

Comparison of SDE versus pioglitazone on insulin sensitizing effect

It is now well established that thiazolidinedione (TZD) group of drugs including pioglitazone, rosiglitazone showed significant insulin sensitizer effect on adipocytes, but so far, it has not been clarified whether TZD can induce insulin-sensitizing effect on skeletal muscle cells. Although TZDs are clinically very effective, but prolong treatment is found to be associated with serious side effects including congestive heart failure, bladder cancer, fluid retention, peripheral edema and weight gain. To compare the insulin sensitizing activity of SDE and TZD, we incubated L6 myotubes with either SDE (50µg/ml) or TZD (pioglitazone, 10µmol/l) for 1h and then treated without or with FFA (palmitate) for 4h in absence or presence of insulin and evaluated the effect of SDE and TZD on Akt phosphorylation in L6 myotubes. SDE showed significantly greater stimulation on insulin sensitivity than that seen with TZD as indicated by the level of Akt phosphorylation (Figure 5).

Cytotoxic Effect of SDE on L6 myotubes

To observe the cytotoxic effect of SDE, the L6 myotubes were treated for 24h with various concentrations of SDE. MTT result indicates that L6 myotubes incubated with various concentrations of SDE were safe and did not show any toxic effect on cell viability in comparison to control (Figure 6). This result suggests that it could be a viable option for future drug development against insulin resistance and T2D.

DISCUSSION

Free fatty acids (FFAs) play a key role in producing insulin resistance and type 2 diabetes (T2D). Although this disease is threatening the global health and spreading fast, there is yet no suitable medicine that addresses the disease properly. Lipid induced insulin resistance is in the center of pathogenesis of T2D and this problem remains yet unattended so far the available drugs are concerned. Insulin loses its sensitivity in T2D patients due to higher circulatory level of FFA[3-5] and removal of FFA from circulation improves insulin sensitivity[6]. Only thiazolidinediones (TZD) class of antidiabetic drugs can augment greater uptake of FFA into the adipocyte by activating its molecular target peroxisome proliferator-activated receptor gamma (PPARγ) and thereby preventing insulin resistance[31-34]. Recent clinical trial data suggest that long term use of TZD produces several adverse side effects such as congestive heart failure and development of edema[14-16]. Since there is no other drug that targets improvement of insulin sensitivity caused by FFA, requirement of alternative therapeutic choice becomes imminent.

Studies conducted over last several years have shown that plant based therapies have potential to provide relief in T2D without producing harmful side effects[17-21]. To find a viable alternative, we made an extensive search of plant extracts that address the problems of insulin resistance and T2D. Based on the traditional knowledge, we selected *Scoparia dulcis* leaf extract (SDE) in our investigation and observed strong insulin sentitizing activity. Although there are some reports on SDE's antihyperglycemic effect in STZ-induced T1D[23-25] but its effect on insulin resistance and T2D has not been explored. We have shown that both pre- and post- treatment of SDE improves insulin sensitivity in skeletal muscle cells which is lost due to FFA. SDE treatment in L6 myotube did not produce any toxic effect on cell viability. Taken together, SDE would be a potential therapeutic choice to deal with the problem of insulin insensitivity and T2D.

CONCLUSION

The present study showed therapeutic potential of *Scoparia dulcis* leave extract against insulin resistance which support its use in the traditional medicine for the treatment of T2D. Further interaction studies between the plant extract and insulin signalling pathway molecules are needed to identify the precise site of action. Since the ethanolic extract of *Scoparia dulcis* leaves could induce strong insulin sensitivity in skeletal muscle cells, the isolation and a detailed investigation with its bioactive components in different animal models of T2D may be promising to find out novel T2D drugs.

ACKNOWLEDGEMENTS

We thank to National Centre for Cell Science, Pune, India and Prof. Samir Bhattacharya, Visva-Bharati University, West Bengal, India for providing rat L6 skeletal muscle cell line. The authors are grateful to the Head, Department of MBBT, Tezpur University, Assam for extending all facilities for this present investigation. The authors are also thankful to Dr. D. Banik,

CSIR-NEIST, Jorhat, Assam, India, for the authentication of plant and the DBT, New Delhi, India, for providing financial support to Ms. T. Borgohain as a part of her M.Sc project work.

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Figure 1. Plant of *Scoparia dulcis* and its leaves used for extract preparation

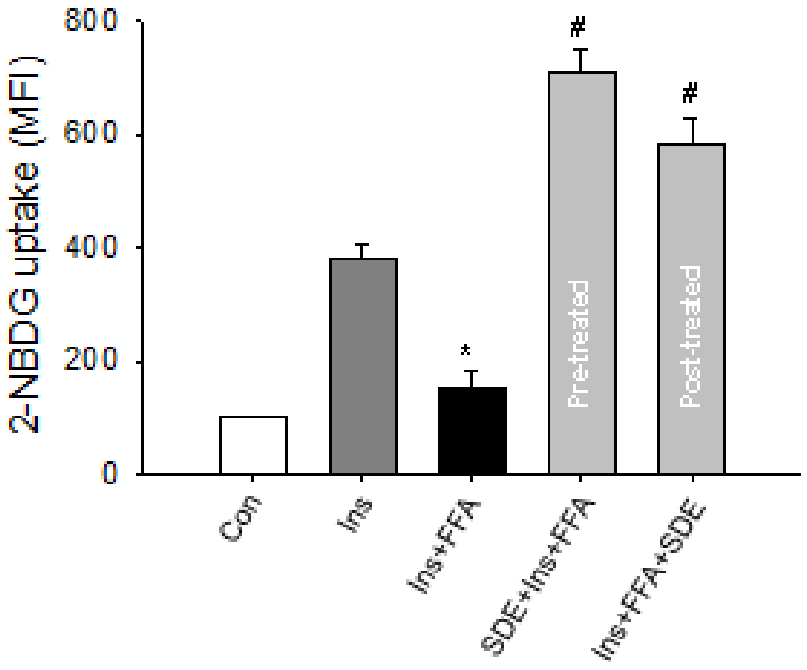

Figure 2. Preventive and curative effect of *Scoparia dulcis* plant extract (SDE) on insulin stimulated 2-NBDG uptake by L6 myotubes in response to the indicated conditions

Each value is the Mean ± SEM of three individual experiments. *p<0.01 vs. Ins, #p<0.001 vs. Ins + FFA.

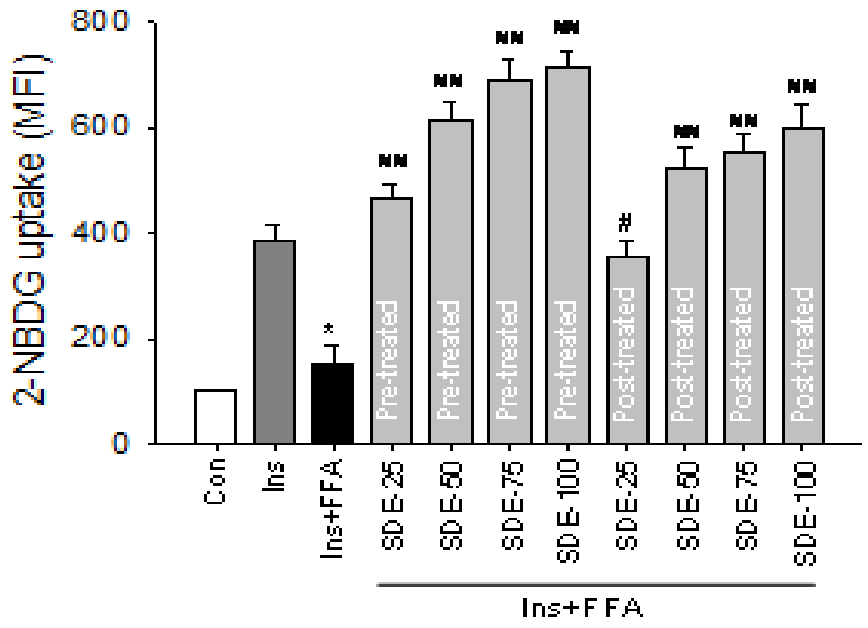

Figure 3. Dose dependent effect of *Scoparia dulcis* plant extract (SDE) on insulin stimulated 2-NBDG uptake by L6 myotubes in response to the indicated conditions

Each value is the Mean ± SEM of three individual experiments. *p<0.01 vs. Ins, #p<0.01, ##p<0.001 vs. Ins + FFA.

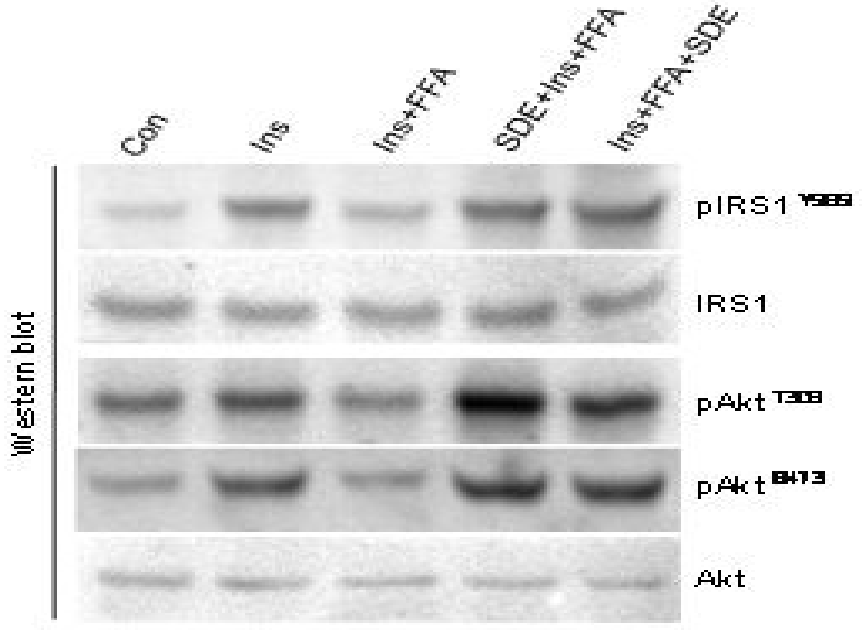

Figure 4. Effect of *Scoparia dulcis* plant extract (SDE) on IRS-1 and Akt phosphorylation. Western blot showing pIRS-1 (Tyr-989) and pAkt (Thr-308 and Ser-473) abundance in L6 myotubes incubated under the indicated conditions. Total IRS-1 and Akt were used as loading control

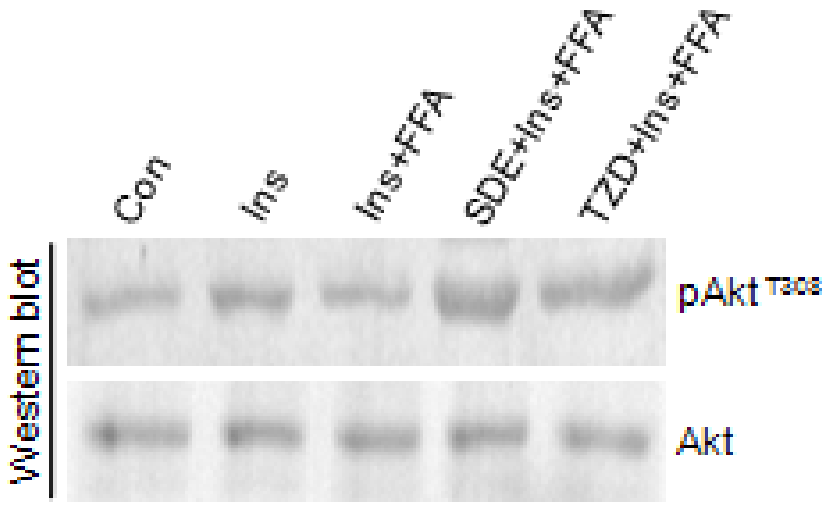

Figure 5. Akt activation status in *Scoparia dulcis* plant extract (SDE) and Pioglitazone treated L6 myotubes. Western blot showing phosphorylated and total Akt level in L6 myotubes incubated under the indicated conditions

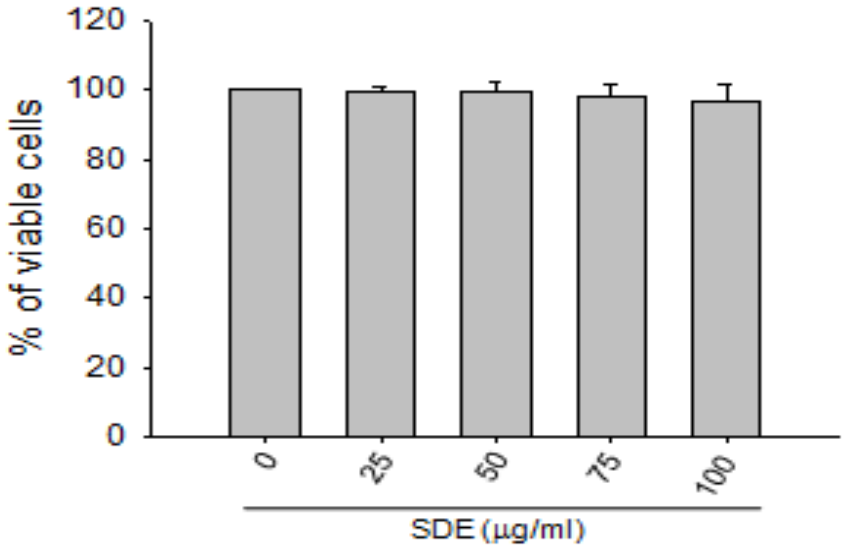

Figure 6. % of viable cells in response to indicated concentrations of *Scoparia dulcis* plant extract (SDE) treated L6 myotubes

Each value is the Mean ± SEM of three individual experiments.