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

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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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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

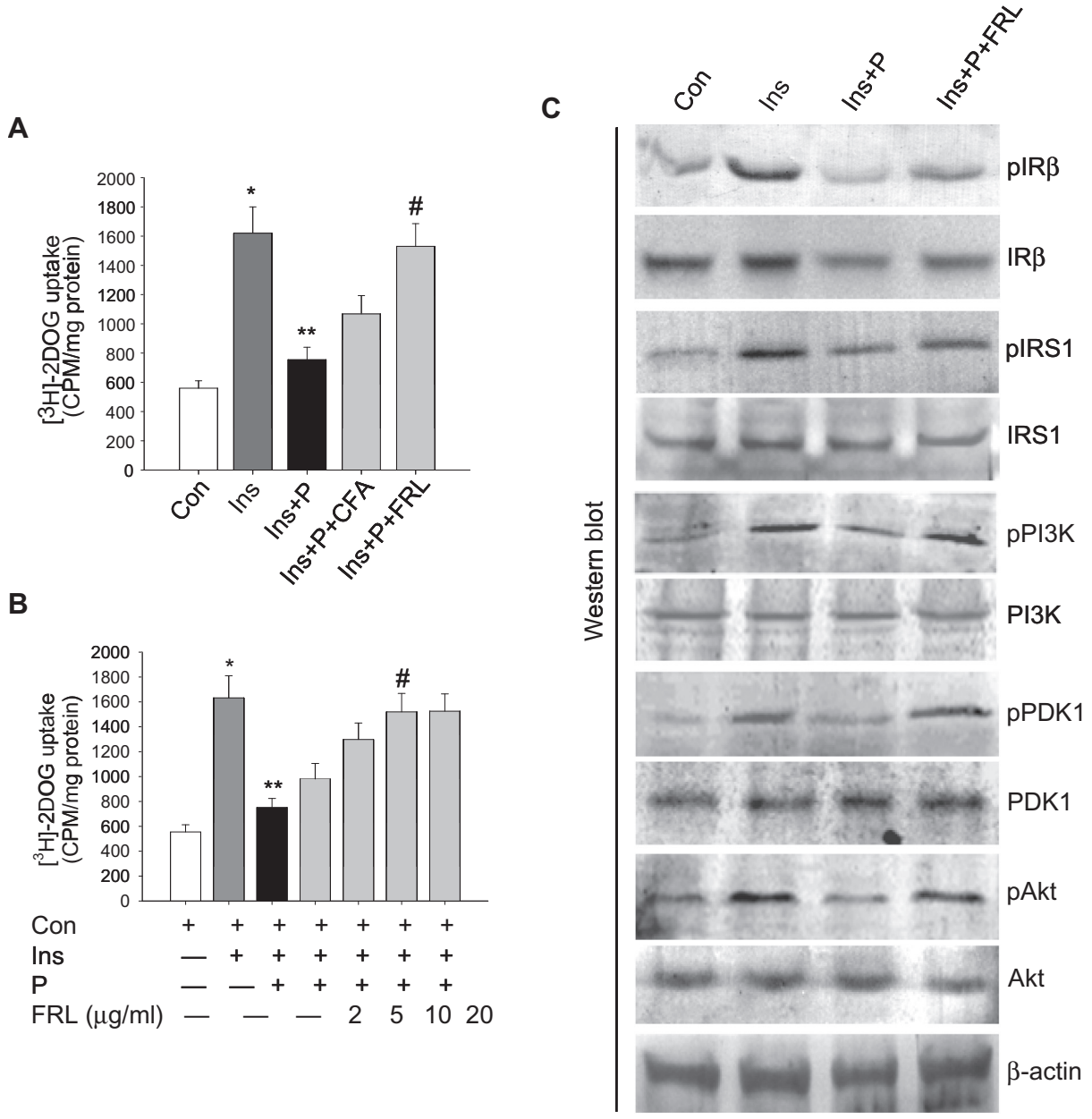

Fig. 1. Palmitate inhibition of insulin signaling pathway was prevented by FRL. (A) [^{3}H]-2DOG uptake by L6 myotubes incubated with insulin or insulin + SFA(P) in presence or absence of caffeic acid (CFA) or ferulic acid (FRL), (B) in the presence of increasing concentrations of FRL. (C) Western blot 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

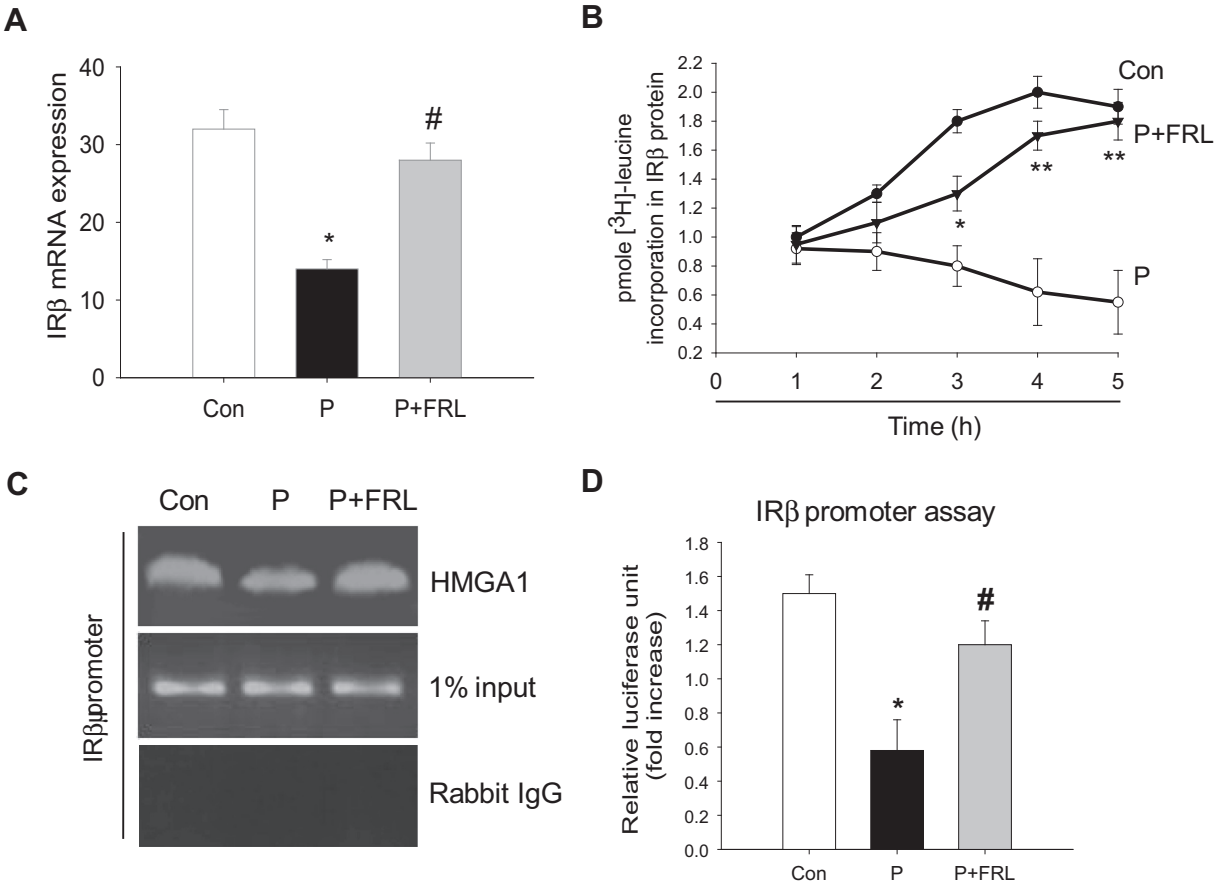

Fig. 2. FRL inhibits lipid induced inhibition of IR gene expression. (A) IRβ mRNA expression in FRL incubated L6 myotubes treated without or with SFA(P). Each value is the mean ± SEM of three independent experiments. $^{*}p < 0.01$ vs Con, $^{\#}p < 0.01$ vs SFA(P). (B) Estimation of IRβ protein synthesis 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).

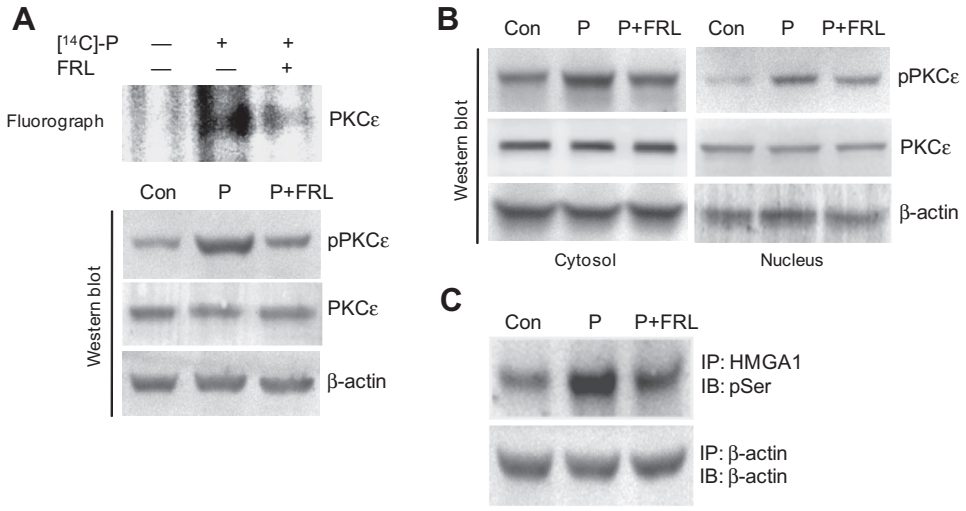

Fig. 3. FRL attenuates HMGA1 phosphorylation through the inhibition of PKCε activation due to palmitoylation. (A) Fluorograph showing [^{14}C]-palmitate incorporation into PKCε in L6 myotubes in the absence or presence of FRL (upper panel) and Western blot exhibits pPKCε and PKCε in total cell lysate (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.

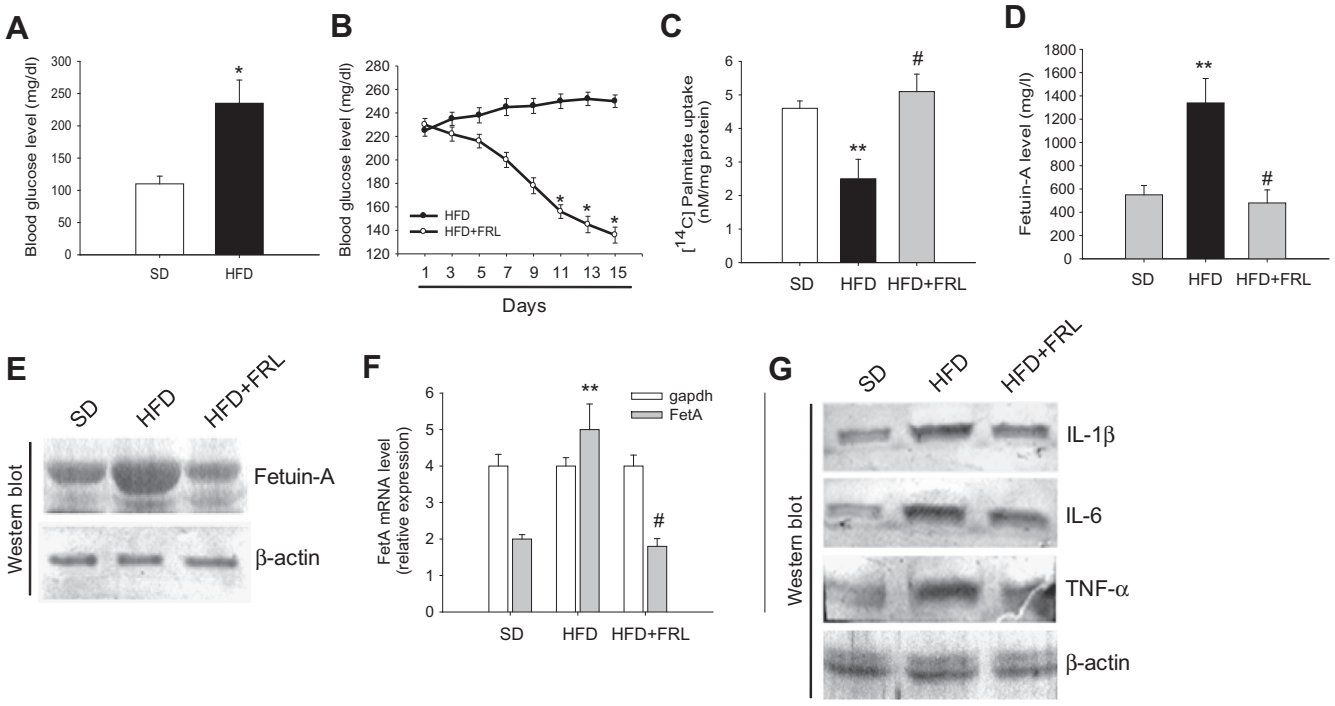

Fig. 4. FRL effects on HFD-fed diabetic rats. (A) Blood glucose level in SD- or HFD-fed rats; (B) blood glucose level in response to FRL administration for 15 d in HFD-fed rats. (C) [^{14}C]-palmitate uptake in the adipocytes isolated from SD or HFD or HFD + FRL treated rats. (D) Circulatory FetA level 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

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