5.1 Introduction

Insulin resistance is in the centre of pathophysiology of type 2 diabetes mellitus (T2DM) 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 causing insulin resistance and T2DM[1,3,4]. Although the underlying mechanism by which lipid specially saturated free fatty acid (FFA) cause insulin resistance is not well understood, a few recent reports indicate that lipid induced inflammation in insulin target tissues significantly contribute to the development of insulin resistance and T2DM[5,6,7]. As already mentioned in the previous chapter, an oversupply of lipid activates nPKCs, more specifically PKCε and PKCθ which are thought to play imperative role in lipid induced insulin resistance in skeletal muscle cell and activated PKCε also attenuates the IR gene transcription in presence of FFA[8,9,10].

In contrast, insulin resistance in adipose tissue due to excess lipid is affected through TLR4 activation by fetuin-A (FetA)-lipid complex, and leads to release of proinflammatory cytokines resulting in insulin resistance [5,6]. Hence, it appears from the existing reports that lipid produces defects in these two important insulin target tissues primarily through two different mechanisms by involving the activation of (i) nPKCs in muscle cells and (ii) FetA-TLR4 for adipose tissue.

In this study, it was investigated using both *in vitro* and *in vivo* model, how ferulic acid (FRL), a polyphenol isolated from the leaves of *Hibiscus mutabilis* L., significantly protected downregulation of insulin receptor in the skeletal muscle cells by inhibiting lipid induced kinase independent activation of PKCε and decreased proinflammatory cytokine release in adipocytes

5.2 Materials and methods

5.2.1 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 [^{3}H]-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.

5.2.2 Bioactivity driven extraction, fractionations and purification of the compounds

Fresh leaves of the *Hibiscus mutabilis* Linn. were collected during 2011-2013 from the North-East region of 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 had been carried out on the basis of improvement of FFA (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 (XTerraTM Prep RP C18, 7.8x300mm, 10mm paricle size) was provided to amorphous solids. These were characterized as ferulic acid (FRL) and caffeic acid (CFE) by comparison of their spectroscopic data (mass, ^{1}H- and ^{13}C-NMR) with those reported previously[11,12].

5.2.3 Cell culture and treatments

L6 skeletal muscle cells and 3T3L1 adipocytes were procured from the National Centre for Cell Science, Pune, India and were cultured in a similar manner as described by us previously[7,9]. Confluent cells were incubated for 6 h without or with 0.75 mM palmitate (FFA). For treatment with the FRL, cells were pretreated for 1 h followed by palmitate incubation. On termination of incubations, the 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. 1951[13].

5.2.4 Insulin resistant diabetic rat model

An insulin-resistant high-fat diet (HFD) rat model was developed by following our earlier description[14]. Briefly, the energy content of the standard diet was 15 kJ/g and the high-fat diet was 26 kJ/g. SD (Standard diet) and HFD fed male rats were housed in a group of 3 individuals/cage under 12 h light/dark cycle

at 23±2 ºC (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 every alternate 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.

5.2.5 [^{3}H] 2-deoxyglucose uptake

To perform [^{3}H]-2-deoxyglucose uptake (2DOG) assay 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)[15].

5.2.6 Immunoblotting

Immunoblot analysis was performed by following the method described previously by Chatterjee, P., et. al.[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 phosphatease)-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).

5.2.7 [^{3}H] Leucine Incorporation Study

To determine the rate of IRβ protein synthesis [^{3}H] Leucine Incorporation into IRβ protein in L6 myotubes was studied by following our earlier procedure[16]. Briefly, L6 myotubes were serum-starved using 0.2% BSA containing Krebs–Ringer phosphate (KRP) buffer. The serum starved L6 myotubes were incubated without or with palmitate or with palmitate plus actinomycin D in presence of 10

μCi/ml [^{3}H] leucine. The medium was collected, and IRβ was pulled down using anti- IRβ antibody. Radioactive count was measured in a liquid scintillation counter (PerkinElmer Tri-Carb 2800TR).

5.2.8 RNA extraction and Quantitative PCR

Total RNA from different incubations were extracted using TRI Reagent (Sigma-Aldrich) following the manufacturer protocol. cDNA synthesis of each RNA samples were performed using Revert Aid™ first strand cDNA synthesis kit (Fermentas Life Sciences, Hanover, MD). Quantitative PCR was performed to quantify gene expression of IR-β and Fetuin A (FetA) using gene specific primer. Gapdh was also amplified simultaneously in separate reactions and the C_t value was normalised using corresponding Gapdh control.

5.2.9 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 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.

5.2.10 Metabolic labeling

L6 skeletal muscle cells were incubated with 0.8 mCi/ml [^{14}C]-palmitic acid at 37°C and on termination of incubation, the cells were washed with PBS to remove the free label. PKCε was immunoprecipitated and subjected to electrophoresis followed by fluorography according to the method described by Dasgupta, S., et al, 2011[9]. Since cysteine residues are the probable site of palmitoylation, the CSS-palm palmitoylation algorithm was used to predict the cysteine residues within the entire coding sequence of PKCε. It was set to the highest cutoff in CSS-palm algorithms.

5.2.11 Co-immunoprecipitation

The Co-immunoprecipitation assay was performed by following the method described by Dasgupta, et al., 2011[9]. Briefly, 2 μg of anti-HMGA1 antibody was incubated with 200 μg of nuclear protein at 4 °C for overnight. Protein A Sepharose (50 μl) was then added to each tube and incubated at 4 °C for 2 hrs. After the termination of incubation the tubes were allowed to centrifuge at 10,000g. The immunocomplexed HMGA1 was then resuspended in 500 μl PBS containing 0.1% CHAPS and washed thoroughly. The immunoprecipitates were boiled in SDS sample buffer and run in 10% SDS-PAGE. The resolved proteins were then transferred to the PVDF membrane (Millipore, Bedford, MA) followed by immunoblotting with p-Serine antibody.

5.2.12 Promoter-reporter assay

IRP-GLuc plasmid was generated by following the method described by Dasgupta, S. et al., 2011[9]. Briefly, by taking phINSRP-1 as a template and using the following primers (forward: 5′-GGGGGAATTCGGCCATTGCACTCCA-3′ and reverse: 5′-AATTGGATCCTGCGGGAGCGCGGGG-3′), the insulin receptor promoter sequence was amplified by PCR. The PCR products were allowed to digest with *Eco*RI/*Bam*HI restriction enzymes and cloned into the pGLuc vector to yield pIRP-GLuc and were confirmed by sequencing. L6 muscle cells were transfected for 72 h with pIRP-GLuc plasmid (0.25 mg/well) using Lipofectamine^{TM} 2000 and the luciferase activity was measured in a luminometer from the media of the incubated cells.

5.2 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.

5.3 Results

5.3.1 FFA induced suppression of insulin activity is reversed by FRL

Ferulic acid (FRL) and caffeic acid (CFA) were purified from the leaf extract of *H. mutabilis* L. and their anti T2D effect on lipid induced insulin resistance were observed in *in vitro* and *in vivo* models. Incubation of skeletal muscle cells i.e. L6 myotubes, one of the major insulin target cells with free fatty acid (FFA), greatly reduced insulin stimulated [^{3}H]-2deoxyglucose (2DOG) uptake. Palmitate (FFA) inhibitory effect on insulin stimulated glucose uptake was significantly prevented by FRL but not by CFA (Fig. 5.1A). Addition of increasing concentrations of FRL (2–20 µg/ml) to L6 myotube incubation showed a dose dependent improvement of insulin activity suppressed by FFA (Fig. 5.1B). Insulin binding to its receptor on target cell surface transduces a signal 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. 5.1C that all these signaling molecules were activated by insulin, and addition of FRL markedly reduced FFA 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 FFA.

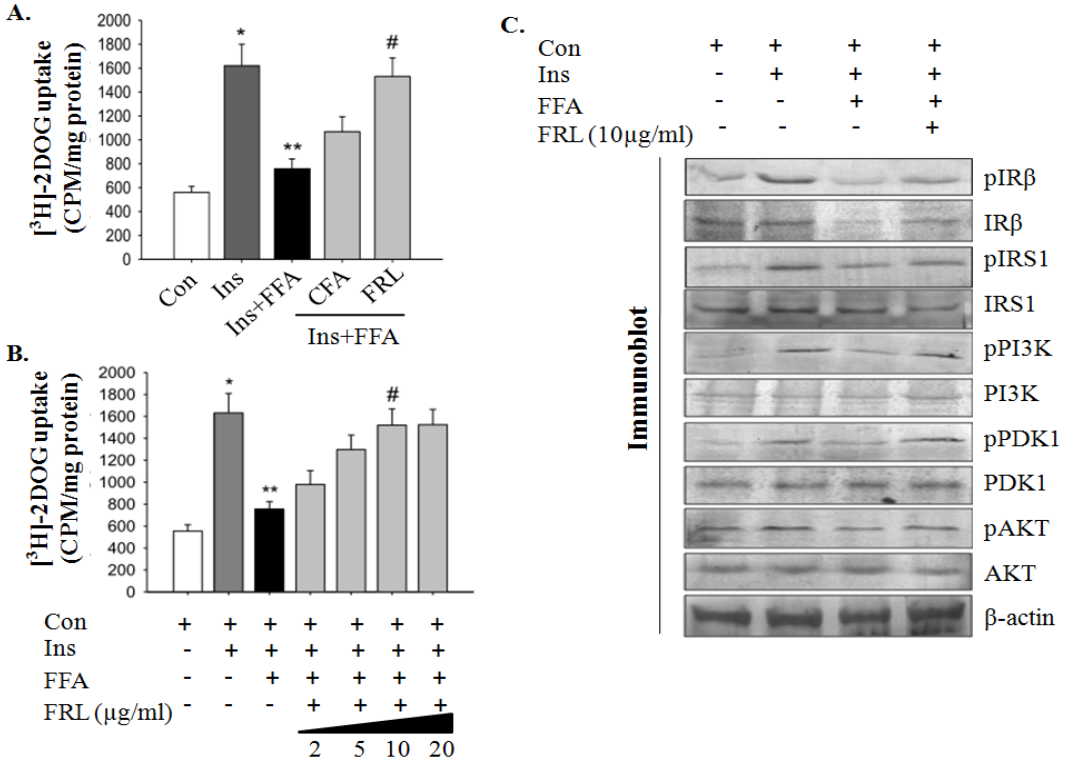

Figure 5.1: Palmitate inhibition of insulin signaling pathway was prevented by FRL. (A) [3H]-2DOG uptake by L6 myotubes incubated with insulin or insulin + FFA(P) in presence or absence of caffeic acid (CFA) or ferulic acid (FRL), (B) in the presence of increasing concentrations of FRL. (C) Western blot showing insulin stimulated phosphorylation of IRb, IRS1, PI3K, PDK1 and Akt in L6 myotubes incubated with insulin or insulin + FFA(P) or insulin + FFA(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 + FFA(P).

On investigating this further, it was found that FFA suppressed insulin receptor (IR) gene expression and protein synthesis, and both of these could be intervened by FRL (Fig. 5.2A and B). To observe the mechanism of FFA's inhibition of IR gene expression, the binding of high mobility group A1 (HMGA1) protein, an architectural transcription factor of IR gene was monitored [17], to IR promoter and IR-promoter activation, both declined due to FFA. Interestingly, FRL significantly waived these inhibitory effects of FFA (Fig. 5.2 C and D).

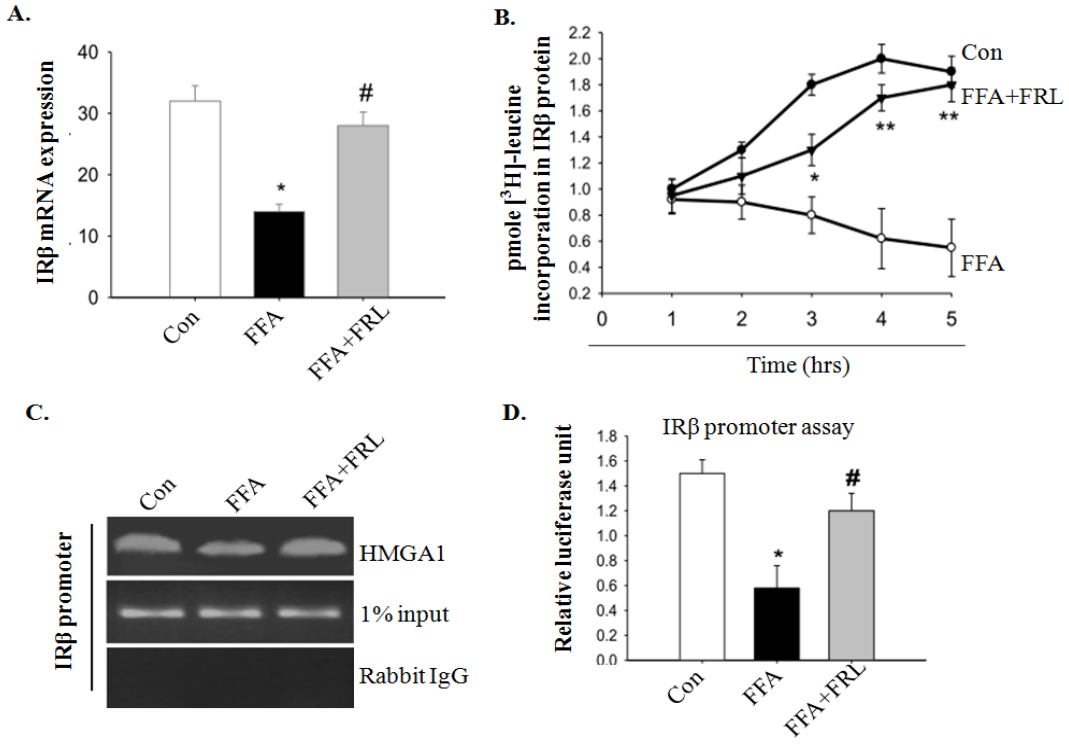

Figure 5.2: FRL inhibits lipid induced inhibition of IR gene expression. (A) IRb mRNA expression in FRL incubated L6 myotubes treated without or with FFA (P). Each value is the mean ± SEM of three independent experiments. *p < 0.01 vs Con, #p < 0.01 vs FFA(P). (B) Estimation of IRβ protein synthesis in L6 myotubes incubated with [^{3}H]-leucine (10 lCi/ml) in the presence or absence of FFA (P) or FFA (P) + FRL. Each value is the mean ± SEM of three independent experiments. *p < 0.05 vs FFA (P), **p < 0.01 vs FFA (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 FFA (P) or FFA(P) + FRL. Each value is the mean ± SEM of three independent experiments. *p < 0.01 vs Con, #p < 0.05 vs FFA (P).

5.3.2 FRL protection to FFA's inhibitory effect on IR expression in skeletal muscle cells

FFA'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]. The results show that FRL attenuated palmitoylation of PKCε thus decreasing its kinase independent phosphorylation (Fig. 5.3A). This was also evident from subdued pPKCε mobilization from cytosol to nuclear region (Fig. 5.3B), and that has significantly reduced HMGA1 phosphorylation (Fig. 5.3C). These findings indicate that FRL rescues repression of IR gene expression through the inhibition of PKCε palmitoylation. This in turn reduces its

phosphorylation and migration to nuclear region thereby preventing impairment of HMGA1.

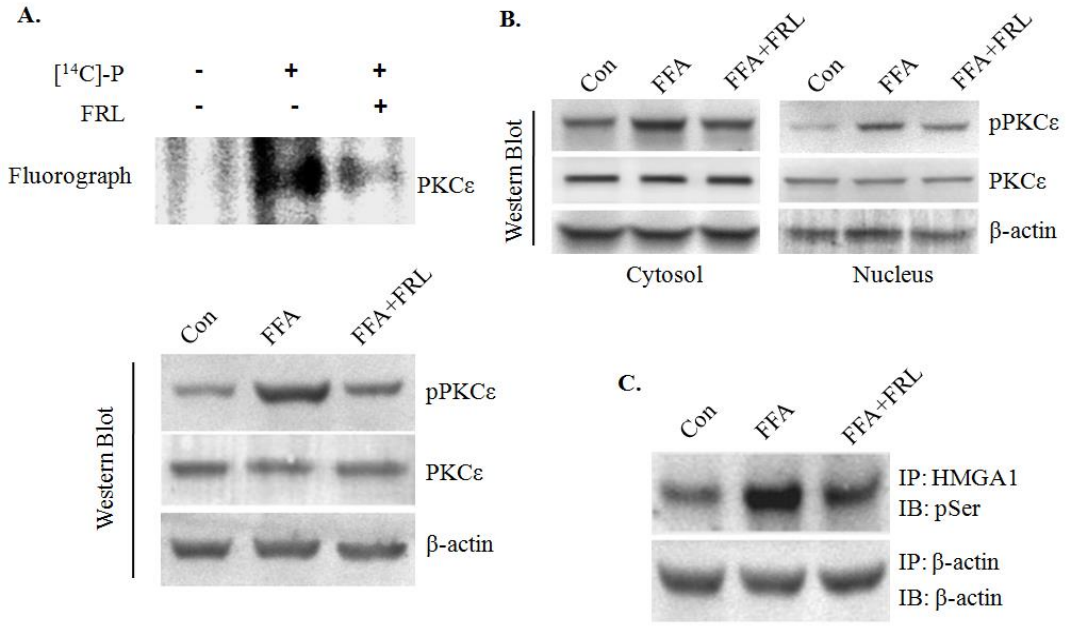

Figure 5.3: FRL attenuates HMGA1 phosphorylation through the inhibition of PKCε activation due to palmitoylation. (A) Fluorograph showing [^{14}C]-palmitate incorporation into PKCε in L6 myotubes in the absence or presence of FRL (upper panel) and Western blot exhibits pPKCε and PKCε in total cell lysate (lower panel). (B) Cytosolic and nuclear pPKCε or PKCε in L6 myotubes incubated without or with FFA (P) or FFA (P) + FRL. (C) IP-IB assay showing phosphorylated HMGA1 level in L6 myotubes incubated without or with FFA (P) or FFA (P) + FRL.

5.3.3 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, which causes insulin resistance. It has been reported that TLR4 or FetA knockout 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, HFD fed type 2 diabetic rat model was used, where oral administration of FRL reduced elevated circulatory glucose nearly to control level (Fig. 5.4A and B). FRL also recovered lipid uptake ability of adipocytes collected from HFD rats (Fig. 5.4C).

When FetA level in HFD rat was examined, a high level of it could be observed in comparison to SD fed rats. Oral administration of FRL in HFD rats significantly reduced FetA circulatory level (Fig. 5.4D) and its expression in adipocytes (Fig. 5.4E). Interestingly, FRL decreased FetA in adipose tissue by suppressing its gene expression (Fig. 5.4F). FetA is required to activateTLR4 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. 5.4G). These results imply that FRL could be a therapeutic choice for insulin resistance and T2DM.

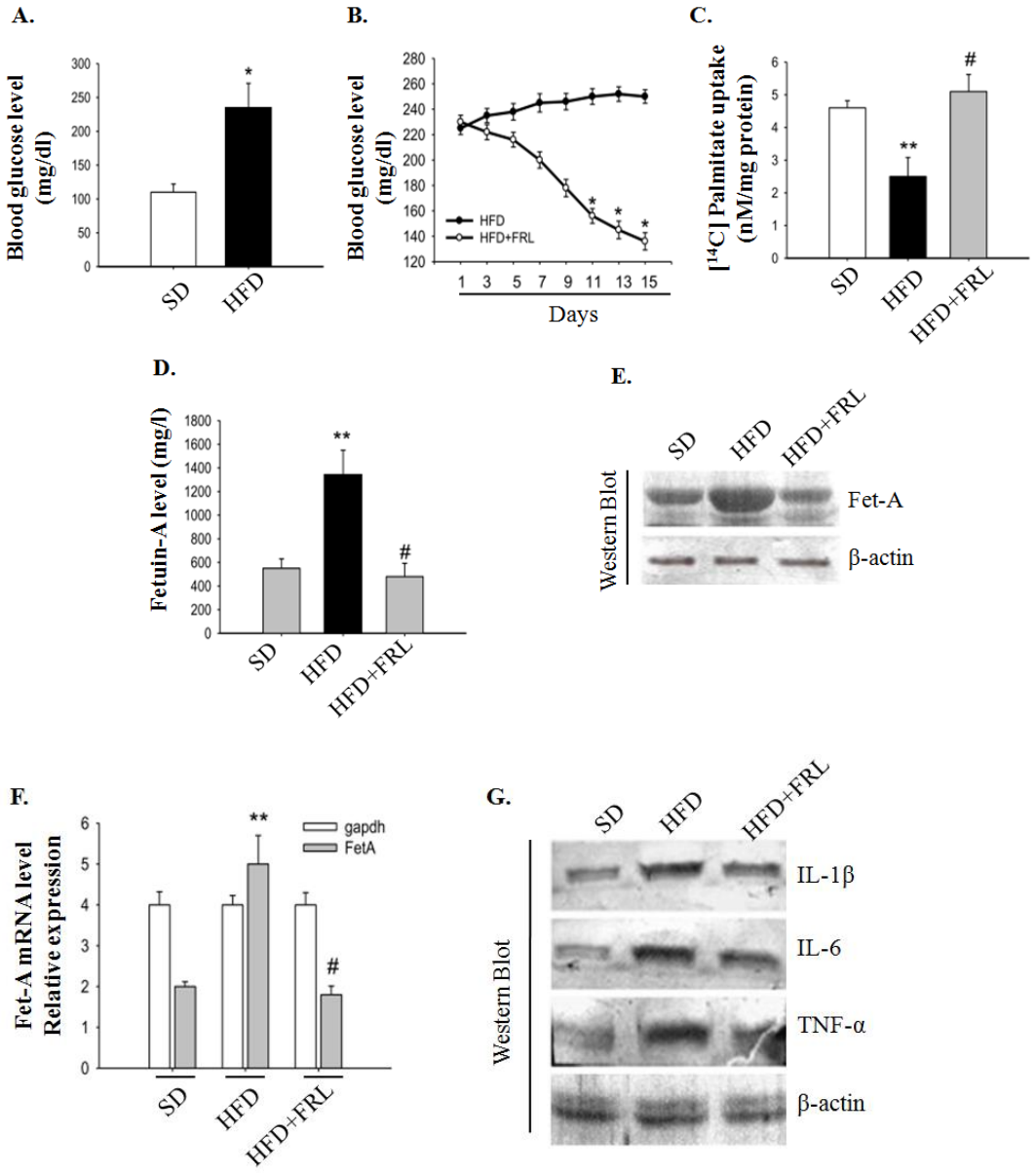

Figure 5.4: FRL effects on HFD-fed diabetic rats. (A) Blood glucose level in SD- or HFD-fed rats; (B) blood glucose level in response to FRL administration for 15 d in HFD-fed rats. (C) [^{14}C]-palmitate uptake in the adipocytes isolated from SD or HFD or HFD + FRL treated rats. (D) Circulatory FetA 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.

5.4 Discussion

In this report it has been demonstrated that two major insulin target cells, skeletal muscle cells and adipocytes, where lipid induced defects cause insulin resistance and T2D, could be effectively rescued by a polyphenol i.e. ferulic acid (FRL) that has been isolated from a plant source. It must be noted that these observations which include 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 FFA 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[9,18]. This adversely affects IRβ gene expression. This was found to be a major pathway of FFA induced defects in skeletal muscle cells because substantial amount of IR decreased due to FFA[9,19]. In fact, deficiency of IR in diabetic patients has also been previously reported[17,18,20,21]. On the other hand, impairment of adipose tissue due to lipid oversupply is related to TLR4 activation which is mediated through FetA[5,6]. 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, 22]. 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 (PPARγ). Numbers of genes in adipocytes that promote FFA entry and decreases excess FFA release from adipocytes are regulated by PPARγ[23,24,25]. TZDs success in clinical practice has been aborted because of their adverse side effects such as development of edema, congestive heart failure[26,27] and decrease in hemoglobin and hematocrit values[28]. FRL's effect in the amelioration of Type 1 or insulin dependent diabetes have been reported[29,30], where the models are related to decrease in insulin availability that occurs due to pancreatic b-cell destruction. But this affects only 3–5% of diabetic patients. In contrast, this investigation was performed with T2DM model where the disease occurs due to insulin resistance. This is the epidemic disease, 90–97% patients are T2DM. Few reports also indicated that FRL possesses antioxidant role[31,32] and since oxidative stress is associated with insulin resistance, this effect of FRL is also significant. Here it is reported about the amelioration of T2DM by FRL which targets recently reported new domains of T2D[5,6,9]. The *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 T2DM. Importantly, FRL has better bioavailability than other dietary flavones[33]. 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 T2DM.

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