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Pterostilbene reverses palmitic acid mediated insulin resistance in HepG2 cells by reducing oxidative stress and triglyceride accumulation

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Abstract

Insulin resistance (IR) is known to precede onset of type 2 diabetes and increased oxidative stress appears to be a deleterious factor leading to IR. In this study, we evaluated ability of Pterostilbene (PTS), a methoxylated analog of Resveratrol and a known antioxidant, to reverse palmitic acid (PA)-mediated IR in HepG2 cells. PTS prevented ROS formation and subsequent oxidative lipid damage by reducing the expression of NADPH Oxidase 3 (NOX3) in PA treated HepG2 cells. Hepatic glucose production was used as a measure of IR and PTS reversed PA-mediated increase in hepatic glucose production by reducing expression of genes coding for gluconeogenic enzymes namely glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PC); and their transcription factors cAMP response element binding protein (CREB) and fork head class box O (FOXO1) along with its co-activator peroxisome proliferator-activated receptor gamma co-activator-1 α (PGC1 α). PTS reversed PA-mediated activation of c-jun N-terminal kinase (JNK), which in turn altered insulin signaling pathway by phosphorylating IRS-1 at Ser 307, leading to inhibition of phosphorylation of Akt and GSK3 β . PTS also reduced PA-mediated lipid accumulation by reducing expression of transcription factors SREBP1c and PPAR α . SREBP1c activates genes involved in fatty acid and triglyceride synthesis while PPAR α activates CPT1, a rate limiting enzyme for controlling entry and oxidation of fatty acids into mitochondria. PTS, however, did not influence PA uptake confirmed by using BODIPY-labeled fluorescent C16 fatty acid analog. Thus, our data provides a possible mechanistic explanation for reversal of PA-mediated IR in HepG2 cells.

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Contributions

SAM and JDA performed the cellular studies and wrote the manuscript. NKM and SSK performed the quantitative lipidomics experiments. SSG and JDA designed and supervised the study. SSG and SSK edited the manuscript.

Conflict of interests

The authors declare no conflict of interest.

Keywords

Insulin resistance; Palmitic acid; oxidative stress; pterostilbene; gluconeogenesis; reactive oxygen species

Introduction

Fatty acids are major components of biological cell membranes and play important physiological roles in energy storage, membrane structure, intracellular signaling and transcriptional regulation of genes. However, chronic exposure to fatty acids can lead to severe pathophysiological conditions such as obesity and metabolic syndrome by depositing excess fat in non-adipose tissues such as liver [1]. It has been shown that diets rich in saturated fatty acids induce a greater increase in liver fat and insulin resistance (IR) compared to unsaturated fatty acids [2,3]. Liver is the primary organ regulating glucose and lipid homeostasis and long-term exposure to saturated free fatty acids (FFA) such as palmitic acid (PA) has been shown to induce hepatic IR [4].

IR is a state in which response to insulin of liver, muscle cells and adipocytes is altered significantly [5]. In muscle and fat cells, IR reduces glucose uptake, while in liver cells it results in reduced glycogen synthesis, storage and failure to suppress glucose production in response to insulin. IR is associated with several disorders including type 2 diabetes, cardiovascular dysfunction, polycystic ovarian syndrome, fatty liver, etc. It is difficult to elucidate origin of IR, given that a diverse set of risk factors are associated with it. However, reactive oxygen species (ROS) has been linked to development of IR [6]. Although at physiological concentration ROS is known to regulate the intermediates involved in insulin signaling cascade, excess ROS generated under conditions such as nutrient excess leads to oxidative damage prior to the development of IR both *in vivo* and *in vitro* [4,7,8].

Oxidative stress, due to excess ROS, is known to alter insulin signaling by activating c-Jun N-terminal kinase (JNK), a stress kinase. Stressors like cytokines and FFAs, implicated in type 2 diabetes, are reported to interfere with the physiological action of insulin in liver, and muscle cells by activating JNK [9–11]. PA-mediated increase in mitochondrial ROS and NADPH Oxidase 3 (NOX3) derived ROS activates JNK which in turn leads to IR [2]. This was further confirmed by suppressing JNK pathway which led to decreased expression of gluconeogenic enzymes and endogenous glucose production in obese diabetic mice [4]. Thus, therapeutic approaches ameliorating ROS production and subsequently reverting IR by decreasing gluconeogenesis in liver are warranted.

Pterostilbene (PTS), a methoxylated analogue of Resveratrol, has gained importance in the last decade owing to its increased lipophilicity, enhanced bio-availability and biological activity such as anti-oxidant, anti-inflammatory, anti-cancer and anti-diabetic [12]. Satheesh and Pari [13] demonstrated that PTS significantly lowered the levels of triglycerides, phospholipids, FFAs and total cholesterol in serum, liver and kidney of streptozotocin diabetic rats. PTS treatment in diabetic mice led to decreased blood glucose levels through improvement in insulin secretion [14]. Although anti-diabetic activity of PTS is known, the mechanism by which PTS reverses PA-induced IR state in HepG2 cells has not yet been

worked out. In this study, we have evaluated ability of PTS to modulate insulin-signaling pathway by regulating the activation of signaling intermediates and the expression of gluconeogenic enzymes along with their key regulators.

Materials and Methods

Chemicals

PA, PTS, insulin, sodium lactate and sodium pyruvate were purchased from Sigma-Aldrich (USA). Antibodies against phosphorylated IRS-1(Ser 307), total IRS-1, phosphorylated Akt (Ser 473), total Akt, phosphorylated glycogen synthase kinase 3 β (GSK3 β) (Ser 9), total GSK3 β , phosphorylated FOXO1(Ser 256), β -Actin, phosphorylated JNK(Thr 183/Tyr 185) and total JNK were purchased from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA, USA). Other chemicals were procured from one of the following companies: SRL (Mumbai, India), BDH (Mumbai, India), Hi-media (Mumbai, India) or Merck (Darmstadt, Germany).

Cell Culture

Human hepatocarcinoma HepG2 cells were purchased from National Centre for Cell Sciences (NCCS), Pune. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μ g/mL). Cells were grown in a humidified atmosphere containing 5% (v/v) CO₂ at 37°C.

Preparation of PA

PA-bovine serum albumin (BSA) conjugate was prepared through soaping of PA with sodium hydroxide (NaOH) and mixing with BSA. Briefly, 100 mM solution of PA in 0.1 N NaOH was incubated at 70°C for 30 mins. 50 μ L of 100 mM PA was added to 950 μ L of 10% BSA at 55°C, vortexed for 30 sec and incubated further for 15 mins at 55°C. Final working solution contained 5 mM PA.

Cell Viability assay

HepG2 cells were plated at a density of 8×10^3 cells/well in a 96-well plate and treated with various concentrations of PA (0.25, 0.5, 0.75 and 1.0 mM) and PTS (1.5, 10 and 20 μ M) for 16 hrs. MTT solution was added to each well to a final concentration of 0.5 mg/mL. After 3hrs of exposure at 37°C, formazan crystals formed were dissolved in lysis buffer (10% SDS in 0.01N HCl) and absorbance was measured at 570 nm.

Treatment of cells with PA and PTS

For all experiments, cultured HepG2 cells at around 80% confluency were treated with 0, 5 or 10 μ M PTS for 4 hrs. Medium was then removed and cells were incubated with 0.5 or 1 mM PA for 16 hrs.

ROS measurement

HepG2 cells were seeded in a 96 well plate at a density of 8×10^3 cells/well and were incubated at 37°C. Cells were pretreated with 5 or 10 μ M PTS followed by incubation with 0.5 or 1 mM PA for 16 hrs. Cells were then loaded with 10 μ M dichlorodihydro-fluorescein diacetate (DCFH-DA) or 5 μ M Mitosox Red for 20 mins, washed and fluorescence was read on Fluostar spectrofluorometer (BMG Labtech, USA) at an excitation/emission wavelength of 488/525 nm and 510/580 nm for DCFH-DA and Mitosox, respectively.

Oil red O and fatty acid uptake assay

To determine intracellular lipid accumulation, Oil Red O assay was performed. After treatment, HepG2 cells were washed twice with 1X PBS and fixed with 4% (w/v) formaldehyde for 15 mins. After fixation, cells were washed twice and stained with Oil Red O stain for 20 mins at room temperature. Cells were washed again with 1XPBS and images were taken by inverted microscope (Olympus Opto systems Pvt. Ltd., Delhi, India).

For fatty acid uptake assay, after 4hrs pretreatment with PTS, HepG2 cells were incubated with BODIPY-PA for 30 mins. After incubation cells were washed with 1X PBS and fluorescence was read on FLUOstar spectrofluorimeter (BMG Labtech, Cary, NC, USA) at an excitation/emission wavelength of 500/515 nm.

Glucose production assay

HepG2 cells were cultured in a 6-well plate at a density of 1×10^6 cells/well. Cells were pretreated with 5 or 10 μ M PTS and then exposed to 0.5 or 1mM PA for 16 hrs. After treatment, cells were washed with 1X PBS and incubated in phenol red, glucose-free DMEM containing gluconeogenic substrates (20 mM sodium lactate and 2 mM sodium pyruvate) for 5 hrs. During the last 1 hr of incubation 100 nM insulin was added to the medium. This medium was then collected and used for glucose estimation by glucose assay kit (BioVision, Milpitas, CA, USA) [15]. Readings were normalized to the total protein content determined from the cell lysate by Bradford's reagent [16]. Similar experiments were carried out using two other cell lines namely; HepaRG (human hepatic progenitor cell line) and S103 (a sub-clone of HUH cell line).

TBARS analysis

TBARS formed during the decomposition of lipid hydro peroxides were measured by following the standard protocol of [17]. After pretreatment of cells with either 5 or 10 μ M PTS, they were incubated with 0.5 or 1mM PA for 16 hrs. Cells were harvested and lysed with 100 μ L lysis buffer (20 mM Tris-Cl pH 7.8, 1 mM EDTA and 0.01% Triton X-100). 500 μ L TBA was added to the cell lysate and incubated at 95°C in water bath for 20 mins. Centrifugation was done at 10000 rpm at 4°C for 10 mins, supernatant was removed and read at 535 nm using ELISA reader (ThermoFisher Scientific, USA).

Lipid extraction and quantitation of triglycerides and oxidized phospholipids by LC-MS/MS

All MS lipid quantitation standards were purchased from Avanti Polar Lipids Inc., USA. Cells were washed with PBS (x 3 times), and transferred into a glass vial using 1 mL PBS. 3

mL of 2:1 (v/v) chloroform (CHCl_3): methanol (MeOH) with the internal standard mix (1 nmol each of 17:1 free fatty acid (FFA), 51:1 triglyceride (TAG), 100 pmol each of 17:0-20:4 phosphatidylserine (PS), 17:0-20:4 phosphatidylcholine (PC), 17:0-20:4 phosphatidylethanolamine (PE), and 17:0-20:4 phosphatidic acid (PhA)) was added, and the mixture was vigorously vortexed. The two phases were separated by centrifugation at 2800g for 5 mins. The organic phase (bottom) was removed, 50 μL of formic acid was added to acidify the aqueous homogenate (to enhance extraction of phospholipids, including oxidized phospholipids), and CHCl_3 was added to make up 4 mL volume. The mixture was vortexed, and separated using centrifugation described above. Both the organic extracts were pooled, and dried under a stream of N_2 . The lipidome was re-solubilized in 200 μL of 2:1 (v/v) CHCl_3 : MeOH, and 20 μL was used for the targeted LC-MS/MS analysis [18,19]. All the lipid species analysed in this study were quantified using the multiple reaction monitoring high resolution (MRM-HR) method on a Sciex X500R QTOF mass spectrometer fitted with anExion UHPLC series quaternary pump. All data was acquired and analyzed using the SciexOS software. The LC separation was achieved using a Gemini 5U C-18 column (Phenomenex, 5 μm , 50 x 4.6 mm) coupled to a Gemini guard column (Phenomenex, 4 x 3 mm, Phenomenex security cartridge). The LC solvents were: For positive mode: buffer A: 95:5 (v/v) H_2O : MeOH + 0.1% formic acid + 10 mM ammonium formate; and buffer B: 60:35:5 (v/v) isopropanol (IPA): MeOH: H_2O + 0.1% formic acid + 10 mM ammonium formate, For Negative mode: buffer A: 95:5 (v/v) H_2O : MeOH + 0.1% ammonium hydroxide; and buffer B: 60:35:5 (v/v) IPA: MeOH: H_2O + 0.1% ammonium hydroxide. All the MS based lipid estimations was performed using an electrospray ion source, using the following MS parameters: ion source = turbo spray, collision gas = medium, curtain gas = 20 L/min, ion spray voltage = 4500 V, temperature = 550 $^{\circ}\text{C}$. A typical LC-run consisted of 55 mins, with the following solvent run sequence post injection: 0.3 mL/min 0% buffer B for 5 mins, 0.5 mL/min 0% buffer B for 5 mins, 0.5 mL/min linear gradient of buffer B from 0 – 100% over 25 mins, 0.5 mL/min of 100% buffer B for 10 mins, and re-equilibration with 0.5 mL/min of 0% buffer B for 10 mins. A detailed list of all the species targeted in this MRM-HR study, describing the precursor parent ion mass and adduct, the product ion targeted can be found in Supplementary Table S1. All the endogenous lipid species were quantified by measuring the area under the curve in comparison to the respective internal standard, and then normalizing to the protein content. All oxidized phospholipids detected were normalized to the corresponding unoxidized phospholipid internal standard. All the data is represented as mean $\pm$ s.e.m. of 4 biological replicates per treatment group (Supplementary Table S1).

Measurement of antioxidant enzymes

After treatment, cells were harvested and pellet was resuspended in thrice the volume of protein extraction buffer (20 mM Tris-Cl pH 7.8, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF and 1X Protease Inhibitor Cocktail). Cells were sonicated and centrifuged at 10000 rpm for 10 mins. Supernatant was collected and used to quantitate activity of antioxidant enzymes namely catalase (CAT), and Glutathione peroxidase (GPx) using standard protocols [20,21].

RNA isolation and quantitative real-time PCR

RNA was isolated using Trizol reagent and cDNA was prepared using verso cDNA synthesis Kit (Thermo scientific, USA). qRT-PCR was performed on Step One Plus RT-PCR system (ABI, USA) using SYBR green. Sequences of all gene specific primers are listed in Supplementary Table S2. PCR products were normalized to the housekeeping gene GAPDH and fold-change in expression was calculated by using 2^{-C_t} method.

Western blot analysis

Protein extracted from HepG2 cells after treatment was quantitated using Bradford's micro estimation assay. 60 µg protein was loaded in each well and electrophoresed on SDS-PAGE. Proteins were electro-transferred to PVDF membrane and the membrane was blocked with BSA/non-fat dry milk and probed for different primary antibodies, namely pIRS-1, tIRS-1, pAkt, pGSK3β, pJNK, pFOXO1, tAkt, tGSK3β, tJNK and β-actin (Sigma-Aldrich, USA) overnight. Membrane was then incubated with appropriate secondary antibody tagged with horseradish peroxidase. Signal was detected by chemiluminescence using luminol as a substrate and imaging was carried out using ChemiDoc Imaging System (BioRad, USA).

Statistical analyses

All experiments were repeated at least five times with reproducible results. The data presented are an average of these replicates. One-way analysis of variance (ANOVA) test associated with the Tukey's test was used to determine the statistical significance of differences among experimental groups with one variable and Two-way ANOVA was used to determine the statistical significance of differences among experimental groups with two variables. All LC-MS/MS data is represented as mean ± s.e.m of 4 biological replicates. A P value of less than 0.05 was considered statistically significant. All the statistical analyses were done using SPSS 19.0 (SPSS, Chicago, IL, USA) and Past version 3 software [22].

Results

PTS protects HepG2 cells from PA induced cytotoxicity

HepG2 cells treated with different concentrations of PTS did not show any cell toxicity (Fig. 1A), while the % cell viability of HepG2 cells exposed to different concentrations of PA decreased in a dosedependent manner (Fig.1B). In HepG2 cells treated with 0.5 mM PA, the cell viability was 72.0±3.7 % that further reduced to 40.0±3.3 % at 1 mM PA. Pretreatment with PTS protected cells from PA induced cytotoxicity (Fig.1C).

PTS reduces lipid accumulation in HepG2 cells

Lipid accumulation in PA treated HepG2 cells was assessed by Oil Red Ostaining and cells were observed under inverted microscope. Cells treated with PA showed accumulation of lipid droplets which was significantly reduced in presence of PTS (Fig. 2A). Since lipids in HepG2 cells are stored mostly in the form of triglycerides, characterization of triglycerides was also done by MRM-HR based targeted LC-MS/MS profiling [18] and we observed that PA containing triglyceride levels significantly increased in PA treated cells and were reduced in presence of PTS. Interestingly, a few oleic acid containing triglycerides also exhibited a

similar trend, albeit not to the same extent in magnitude to the PA containing triglycerides (Fig. 2B).

In order to examine if PTS affected fatty acid uptake, cells were treated with BODIPY-labeled fluorescent PA analog alone and/or in combination with PTS. Results indicated that PTS did not influence uptake of PA in HepG2 cells (Fig. 2C). Further, to understand the mechanism by which PTS reduced lipid accumulation in PA treated cells, expression of markers of FFA metabolism namely, Sterol regulatory element-binding protein (SREBP1c), Carnitine palmitoyl transferase1 (CPT1), a mitochondrial transporter for PA and its transcription factor Peroxisome proliferator-activated receptor alpha (PPAR α) were evaluated. In HepG2 cells treated with 0.5 or 1 mM PA, expression of both CPT1 and PPAR α was found to be significantly increased compared to untreated control cells (Fig. 2D-F). In cells treated with PTS prior to PA exposure, expression of both CPT1 and PPAR α was reduced. PTS alone did not have any effect on the expression CPT1 and PPAR α . SREBP1c, a transcription factor involved in activating genes involved in fatty acid metabolism, increased in PA treated cells compared to control untreated HepG2 cells. This effect was inhibited in PTS treated cells.

PTS inhibits PA mediated ROS production in HepG2 cells

PA on metabolism is known to generate ROS [2]. In HepG2 cells treated with PA, total ROS was measured using DCFH-DA. At both concentrations of PA intracellular ROS significantly increased to a value of 1.32 ± 0.01 and 1.89 ± 0.15 relative fluorescence unit (RFU) at 0.5 and 1 mM PA, respectively, compared to untreated control cells. Pretreatment with PTS inhibited PA-induced ROS production (Fig. 3A). Mitochondrial ROS was also checked using Mitosox Red, which increased to a value of 1.65 ± 0.03 and 2.76 ± 0.01 RFU at 0.5 and 1 mM PA, respectively, compared to untreated control cells. Pretreatment with PTS inhibited PA-induced mitochondrial ROS production significantly (Fig.3A). Since NOX3 is the source of cellular ROS in PA treated cells [4], we examined ability of PTS to inhibit expression of NOX3 in presence of PA. NOX3 expression was found to be elevated in PA treated cells and pretreatment with PTS inhibited PA-mediated increase in the expression of NOX3 (Fig.3B).

PA-mediated increased ROS production results in oxidation of lipids, which can be measured in terms of TBARS. We observed significant increase in TBARS in 0.5 and 1 mM PA treated cells (888 ± 88 and 1034 ± 23 nmole TBARS/mg protein, respectively) compared to untreated control cells (583 ± 23 nmole TBARS/mg protein). This increase in TBARS was restored when cells were pretreated with PTS. PTS alone did not change concentration of TBARS in HepG2 cells (Fig.3C). Further, we characterized the oxidized phospholipids in HepG2 cells under treatment conditions using MRM-HR based LC-MS/MS profiling. Our lipid extraction methods specifically afforded the enrichment of two types of oxidatively damaged phospholipids. These included the ox-phospholipids (+16 mass addition) and hy-phospholipids (+18 mass addition) formed due to ROS damage. We found significantly elevated levels of such oxidatively damaged phospholipids from the phosphatidylserine (PS), phosphatidic acid (PhA), and phosphatidylethanolamine (PE) class of phospholipids in PA treated cells (Fig. 3D). This effect was found to be significantly reduced in cells pretreated

with PTS. Concentration of oxidatively damaged phospholipids in PTS treated cells was almost comparable to those in control untreated cells.

PTS reduces gene expression of antioxidant enzymes in PA treated cells

In control untreated cells, CAT and GPx activities were 1.38 ± 0.09 and 0.17 ± 0.01 units/mg protein, respectively. Cells exposed to 0.5 or 1 mM PA significantly increased activity of CAT to 2.75 ± 0.29 and 6.98 ± 1.58 units/mg protein, respectively (Fig. 4A), and GPx to 0.21 ± 0.01 units/mg protein and 0.35 ± 0.02 units/mg protein, respectively (Fig. 4B). In cells pretreated with PTS significant reduction in activity of CAT and GPx was observed and PTS alone did not show any effect on activities of these enzymes. Expression of genes for antioxidant enzymes was also quantitated by qRT-PCR. We indeed observed significant increase in the expression of CAT and GPx in PA treated cells (Fig. 4C-D) which was reversed in cells treated with PTS prior to PA exposure.

PTS reduces PA-induced IR in HepG2 cells

Glucose output in control HepG2 cells was 17 ± 2 nmol/mg protein, which reduced to 5 ± 1 nmol/mg protein in response to insulin indicating insulin responsiveness of these cells (Fig. 5A). Exposure to 0.5 mM PA increased glucose output in response to insulin to 140 ± 2 nmol/mg protein and further in presence of 1 mM PA it increased to 220 ± 6 nmol/mg protein indicating development of IR state. Interestingly in cells treated with PTS prior to exposure with PA, glucose output in response to insulin was significantly reduced. Treatment with PTS alone did not alter insulin sensitivity in HepG2 cells. To confirm that this effect of PTS on glucose production could be attributed to its antioxidant potential, we used NAC, a known antioxidant. We indeed found that at 1 mM concentration NAC significantly reduced glucose output in insulin resistant cells. Upto 1 mM concentration, NAC did not have any cytotoxic effect on HepG2 cells and also inhibited ROS production in PA treated cells (Supplementary Fig. S1). To check whether effect of PTS in reducing PA-induced IR is not specific to HepG2 cells, we used two more cell lines namely, HepaRG and S10-3. HepaRG cells are often used as an alternative to ex vivo cultured primary human hepatocytes and S10-3 is a subclone of human hepatoma Huh7 cells. In both these cell lines we found increased glucose production at 1 mM concentration of PA which was significantly reduced in presence of PTS as was found in HepG2 cells (Supplementary Fig.S2).

In insulin sensitive cells, insulin-mediated phosphorylation of IRS-1 leads to phosphorylation of its downstream targets namely Akt, FOXO1 and GSK3 β . PA-mediated increase in both mitochondrial and NOX3-derived- ROS promotes JNK activation resulting in phosphorylation of IRS-1 at Ser 307. This in turn leads to impaired phosphorylation of Akt, GSK3 β and FOXO1 [4]. In our study PTS treatment resulted in inhibition of PA-mediated increase in both JNK and IRS-1(Ser 307) phosphorylation. In cells treated with NAC, a known antioxidant, similar effects were observed (Fig.5B-C). To confirm that JNK acts as signaling intermediate in the cascade linking PA exposure with hepatic IR, we further incubated HepG2 cells with JNK inhibitor SP600125 (10 and 20 μ M) for 30 mins in presence of PA. Inhibition of JNK (Fig. 5D), led to reduced glucose output in PA treated HepG2 cells (Fig. 5E). These results suggest that PTS inhibited PA-induced hepatic IR through JNK pathway.

PTS inhibits expression of gluconeogenic enzymes in PA treated HepG2 cells

Akt mediated phosphorylation of FOXO1 in response to insulin excludes it from nucleus resulting in decreased expression of key rate limiting enzymes in gluconeogenesis namely glucose-6-phosphatase(G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase(PC). Indeed, insulin treatment to control untreated cells led to a significant increase in FOXO1 phosphorylation (Fig. 5B) which in turn led to decreased expression of gluconeogenic enzymes. In PA treated HepG2 cells expression of all three enzymes was found to be significantly increased in response to insulin compared to untreated control cells (Fig.6A-C). This effect of PA was reversed in cells pretreated with PTS. Expression of gluconeogenic enzymes in turn is regulated by transcription factors such as cAMP response element binding protein (CREB) and FOXO1 along with its co-activator PGC1 α . We indeed found elevated expression levels of CREB, FOXO1 and PGC1 α in PA treated cells (Fig. 6D-F) which was reversed in cells pretreated with PTS. Treatment with PTS alone did not have any effect on the expression of both gluconeogenic enzymes and their transcription factors. Gluconeogenesis is additionally regulated by transcription factor HNF4- α and therefore, its expression was checked and we found that in presence of PA, expression of HNF4 α was significantly increased (Fig. 6G) and this was reversed in presence of PTS as was found in CREB, FOXO1 and PGC1 α .

Discussion

Incidence of type 2 diabetes, characterized by hyperglycemia, is increasing at an alarming rate throughout the world. IR, a condition where insulin responsive cells fail to respond to insulin, is known to precede the development of diabetes and is commonly found in unaffected individuals years before hyperglycemia sets in. In hepatic cells, in response to insulin gluconeogenesis is inhibited. However, in IR state, the strength of insulin signaling starting from its interaction with receptor to its final action of inhibiting gluconeogenesis is attenuated leading to uncontrolled hepatic glucose output causing hyperglycemia. Increased gluconeogenesis in liver of diabetic patients is considered as a major contributor to fasting hyperglycemia [22–24]. Therefore, it becomes relevant to develop therapeutic approaches targeting reversal of IR which can prevent progression of diabetes. Despite several diverse set of risk factors being linked to IR such as nutrient overload, obesity, inflammation, polycystic ovarian syndrome, etc. a common unifying link between them is excess ROS generation. Houstis et al [6] demonstrated both the association and causation related with ROS induced IR in 3T3-L1 pre-adipocytes. IR in fact is considered a cellular antioxidant defense mechanism to cope up with increased ROS and that scavenging ROS can reverse IR state in muscle cells and adipocytes was demonstrated by Hoehn et al [8]. Therefore, this justifies use of antioxidants as an alternative approach for reversing IR.

PTS, a methoxylated analogue of Resveratrol, is a natural compound known for its antioxidant activity [25] among several other pharmacological properties such as, anti-cancer, anti-inflammatory, anti-obesity, etc. PTS is one of the major active constituent in *Pterocarpus marsupium*, an Indian medicinal plant, known for reducing blood glucose level in newly-diagnosed diabetic patients [26]. Extract of *P. marsupium* effectively reduced serum glucose level and prevented hypertriglyceridemia, hyperglycemia and IR in fructose-

fed [27,28] and obesogenic diet-fed [29] diabetic rat models. Although ability of PTS to revert IR in diabetic models has been demonstrated, the mechanism by which PTS reduces gluconeogenesis is not completely defined.

In this study, we checked the mechanism by which PTS significantly reduces hepatic gluconeogenesis in insulin resistant HepG2 cells. The rate of gluconeogenesis is governed by activities of three regulatory enzymes namely PEPCK, PC and G6Pase [30]. In PA treated cells hepatic glucose production increased concomitantly with expression of gluconeogenic enzymes. Cells treated with PTS prior to PA treatment, hepatic glucose production was significantly reduced due to lowered expression of gluconeogenic enzymes. Earlier, PTS has been shown to reduce hepatic glucose production in streptozotocin and nicotinamide induced diabetic rats, by regulating the expression of gluconeogenic enzymes [31]. Expression of these gluconeogenic enzymes is in turn modulated by their transcription factors. CREB directly activates transcription of PEPCK, G6Pase and PC by binding to cAMP response elements (CRE) in their promoters whereas HNF-4 α and co-activator PGC1 α regulate the expression of gluconeogenic enzymes by binding to HNF-4 α binding cis elements in their promoters [32–34]. Additionally FOXO1, another transcription factor, binds to insulin response elements (IRE) along with its coactivator PGC1 α in promoters of G6Pase and PEPCK and enhances their expression [35]. In our study we found that in presence of PTS expression of CREB, FOXO1, PGC1 α and HNF-4 α (Fig. 6) were down regulated. AMPK is already shown to be an upstream regulator of both CREB and FOXO1 [36]. Recently PTS is also reported to activate AMPK and reduce gluconeogenesis in H4IIE cells [37]. Thus, down regulation of CREB and FOXO1, responsible for inhibition of gluconeogenesis could possibly be through activated AMPK. In our study, PTS treatment also inhibited expression of these transcription factors significantly, thereby down regulating expression of gluconeogenic enzymes (Fig. 6).

Since most of the metabolic stressors alter insulin signaling by generating stress response, we looked at phosphorylation status of stress kinase JNK. JNK is activated in response to oxidative stress and inhibition of JNK activity has been shown to improve insulin sensitivity in mice [38,39]. JNK activation leads to inhibitory phosphorylation of IRS-1 at ser307, thereby further reducing insulin dependent PI3K-Akt signaling. Akt phosphorylation in response to insulin is crucial for phosphorylation of its downstream signaling intermediates, GSK3 β and FOXO1. On one hand, Akt phosphorylation inactivates GSK3 β which then promotes glycogenesis by keeping glycogen synthase in its active state; and on the other hand, Akt phosphorylated FOXO1, on its translocation from nucleus to cytoplasm reduces expression of gluconeogenic enzymes [40]. We found that ROS generated due to exposure to excess PA led to activation of JNK which altered insulin signalling by phosphorylating IRS-1 at Ser 307 in HepG2 cells (Fig. 5B). PTS prevented PA-mediated JNK activation which resulted in insulin-dependent activation of Akt and subsequent regulation of gluconeogenesis (Fig. 5B). This was further confirmed by using JNK inhibitor (SP600125) in whose presence both PA-induced JNK activation and gluconeogenesis was inhibited (Fig. 5D-E).

In cells treated with PA excess ROS is generated by activation of NOX3 which induces expression of antioxidant enzymes. In our study we indeed found increased expression and

activity of antioxidant enzymes in PA treated cells (Fig. 4). PTS treatment to PA exposed cells reduced ROS production and subsequently reduced both the expression and activity of CAT and GPx. PTS has been reported previously to scavenge/inhibit ROS formation both in *in vitro* [24,41] and *in vivo* [28,42] studies. Excess ROS that escapes the cellular antioxidant defense machinery causes oxidative damage to biomolecules especially membrane lipids [43]. This was reflected in increased oxidation of phospholipids in cells treated with PA, and PTS treatment prevented the oxidative damage to these phospholipids.

Exposure to excess PA causes increased β -oxidation in mitochondria, leading to cellular FFA storage in form of triglycerides. We therefore checked whether PTS modulates expression of key proteins involved in lipid metabolism namely SREBP1c, which is involved in FFA biosynthesis, CPT1 and PPAR α , involved in FFA transport (Fig. 2D-F). PA exposure increased accumulation of triglycerides in HepG2 cells, which was evident from Oil Red O staining (Fig. 2A). Treatment with PA also led to increased expression of SREBP1c, CPT1 and PPAR α . PTS treatment not only reduced expression of SREBP1c, thereby reducing lipid accumulation but also reduced expression of CPT1 and its transcription factor PPAR α , causing decreased rate of mitochondrial β -oxidation. Thus in our study, PTS at the concentrations used decreased lipid accumulation by reducing the expression of enzymes involved in both transport and oxidation of lipids. PTS has been known to activate expression of PPAR α , which leads to utilization of excess fats by channelizing it through β -oxidation [44]. In our study, we found increased expression of PPAR α in cells treated with PTS alone; however this effect was not significant.

To summarize (Fig. 7), PTS reversed PA-mediated IR in HepG2 cells by decreasing the expression of gluconeogenic enzymes (G6pase, PEPCK and PC). This effect can be attributed to its ability to reduce lipid accumulation and also to its antioxidant property which prevents NOX-3 mediated ROS accumulation and thereby reduces oxidative damage to lipids in PA treated HepG2 cells. PTS did not affect FFA uptake in HepG2 cells but regulated triglyceride accumulation and FFA metabolism. However, further *in vivo* studies are required to confirm the ability of PTS to inhibit gluconeogenesis which may help to conduct clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effect of different concentrations of (A) PTS and (B) PA on HepG2 cell viability. (C) PTS inhibited cytotoxic effect in PA treated HepG2 cells. Values are presented as mean $\pm$ s.e.m for 5 biological replicates per group and * indicates $p < 0.05$ versus control; # indicates $p < 0.05$ versus PA treated cells.

Fig. 2.

PTS reduced lipid accumulation in PA treated HepG2 cells. (A) Intracellular lipid accumulation was analyzed by Oil Red O staining. (B) Triglyceride characterization by LC-MS/MS analysis. (C) Fatty acid uptake by Bodipy under treatment conditions. Expression levels of (D) SREBP, (E) CPT1 and (F) PPAR α . Values are expressed as mean $\pm$ s.e.m for 5 biological replicates where * indicates $p < 0.05$ versus control; # indicates $p < 0.05$ versus PA treated cells.

Fig. 3. (A) PTS inhibited cellular and mitochondrial ROS generation in PA treated cells. (B) PTS reduced expression of NOX3. PTS inhibited (C) lipid peroxidation and (D) reduced oxidized phospholipids levels in PA treated HepG2 cells. Values are expressed as mean $\pm$ s.e.m for 5 biological replicates, where * indicates $p < 0.05$ versus control and # indicates $p < 0.05$ versus PA treated cells.

Fig. 4. PTS reduced activity of (A) CAT and (B) GPx, and the expression of (C) CAT and (D) GPx. Values are expressed as mean $\pm$ s.e.m for 5 biological replicates where * indicates $p < 0.05$ versus control; # indicates $p < 0.05$ versus PA treated cells.

Fig. 5.

(A) PTS reduced glucose output in PA treated HepG2 cells. (B) PTS significantly inhibited IRS1 phosphorylation(Ser 307) and restored phosphorylation of Akt, GSK3β, JNK and FOXO1 at 5 and 10 μM concentrations. (C) Protein band intensities were quantified by densitometric analysis. (D) Inhibition of JNK phosphorylation and (E) reduced glucose output in presence of JNK inhibitor (SP600125). Values are expressed as mean ± s.e.m for 5 biological replicates where # indicates p<0.05 versus control; \$ indicates p<0.05 versus insulin treated control cells; * indicates p<0.05 versus PA treated cells.

Fig. 6.

PTS induced changes in the expression of gluconeogenic enzymes in PA treated HepG2 cells. (A) G6Pase, (B) PEPCK, (C) PC, (D) FOXO, (E) CREB, (F) PGC1α, and (G) HNF-4α. Values are expressed as mean $\pm$ s.e.m (n=4), where # indicates $p < 0.05$ versus control; \$ indicates $p < 0.05$ versus insulin treated control cells; * indicates $p < 0.05$ versus PA treated cells.

Fig.7.

Pterostilbene (PTS) prevented ROS formation and reversed palmitic acid (PA) mediated increase in hepatic glucose production by reducing expression of genes coding for gluconeogenic enzymes and their transcription factors. PA mediated activation of c-jun N-terminal kinase (JNK) was also reversed by PTS, which in turn altered insulin signaling pathway by phosphorylating Akt and GSK3β. Triglyceride levels and expression of SREBP1c, CPT1 and PPARα were also found to be reduced in presence of PTS.