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Thioredoxin interacting protein mediates lipid-induced impairment of glucose uptake in skeletal muscle

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ABSTRACT

Insulin resistance (IR) is an important determinant of type-2 diabetes mellitus (T2DM). Free fatty acids (FFAs) induce IR by various mechanisms. A surfeit of circulating FFA leads to intra-myocellular lipid accumulation that induces mitochondrial ROS generation and worsens IR. However, the molecular mechanisms behind are unclear. We identified thioredoxin interacting protein (TxNIP), which is over-expressed in T2DM, to be a promoter of ROS-induced IR. We observed upregulation of TxNIP upon palmitate treatment in skeletal muscle cells that led to ROS generation and Glut-4 downregulation resulting in impaired glucose-uptake. FFA-induced overexpression of TxNIP gene was mediated through the activation of its bona-fide trans activator, ChREBP. Further, Palmitate-induced impairment in AMPK-SIRT-1 pathway resulted in overexpression of ChREBP. While Fenofibrate, abrogated PA-induced TxNIP expression and ROS generation in skeletal muscle cells, Saroglitazar, a dual PPARα/γ-agonist, not only inhibited PA-induced TXNIP expression but also led to greater improvement in glucose uptake. Taken together, TxNIP appears to be an important factor in FFA-induced ROS generation and IR in skeletal muscle cells, which can be modulated for the management of this complex disorder.

1. Introduction

Type-2 Diabetes (T2DM) has emerged as a global epidemic and health burden because of its multiple, long term macro and micro vascular complications. Excess circulating FFA promotes oxidative stress, an important risk factor for IR and cardiovascular disease [1]. Multiple factors i.e., changes in mitochondrial respiration, ER-stress and inflammation are major contributors of FFA-induced IR. ROS is an important mediator in the development of hypertension, atherosclerosis, IR and T2DM [2]. The imbalance between ROS generation and detoxification leads to oxidative stress [3]. Several antioxidant enzyme systems like thioredoxin (Trx), Mn-SOD, and catalase have evolved to protect cells against oxidative damage [4].

Thioredoxin interacting protein (TxNIP) was identified as a binding partner for thioredoxin [5]. This binding and intermolecular disulphide bond formation causes inhibition of thioredoxin function, leading to oxidative stress [6] that impairs the expression of thioredoxin-dependent proteins like PTEN and ASK-1 [7]. Recent evidence suggests the involvement of TxNIP in the pathogenesis of diabetes. While TxNIP overexpression suppresses basal and insulin mediated glucose uptake, TxNIP knockdown increases peripheral glucose uptake [8]. Stable transfection of human TxNIP led to apoptosis of β-cells through activation of Bax, Bcl2 and caspase-3 [9]. Mice with subdued TxNIP expression are resistant to streptozotocin-induced diabetes, suggesting a protective effect of TxNIP inhibition on β-cells [10]. The transcription of TxNIP gene is regulated by ChREBP in a glucose-dependent manner; promoter analysis shows tandem binding sites for ChoRE and FOXO family transcription factors [11].

Although, the role of TxNIP in the generation of oxidative stress followed by T2DM is documented, the detailed mechanism of its regulation by TxNIP remains unclear. Hence, understanding the precise role and mechanism of TxNIP-induced oxidative stress

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might provide potential therapeutic strategies to ameliorate T2DM. Therefore, the present study was designed to delineate the role of TxNIP in FFA-induced oxidative stress leading to IR followed by impaired glucose uptake in PA-treated C2C12 mouse skeletal muscle cells.

2. Materials and methods

2.1. Cell culture

Mouse skeletal muscle cell lines (C2C12) were obtained from ATCC, maintained in DMEM high-glucose supplemented with 10% FBS, 100U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C under 5%CO_2 atmosphere. For biochemical study, the cells were grown on six-well plates at a density of 2.5–3 × 10^4 cells/well in growth medium. After, cells had reached ~80–90% confluence; differentiation was induced by replacing the growth medium with DMEM supplemented with 2% calf serum (differentiation medium). The differentiation medium was changed every 24 h, and the myotubes were used for subsequent experiments. N-Acetyl-L-cysteine (NAC), Fenofibrate were obtained from Sigma-Aldrich and Saroglitazar is a gift from Zydus Research Centre, Ahmedabad, India.

2.2. Preparation of fatty acid containing media and treatment

Fatty acid containing media used for the treatment was prepared as described in our previous report [12]. The dose of PA was selected on the basis of previous reports [13] and thereafter standardized at our laboratory.

2.3. RNA isolation and cDNA preparation

Total RNA was isolated from C2C12 cells using Trizol reagent and its quantity/quality was verified. The cDNA synthesis was carried out by using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer protocol.

2.4. Real time quantitative PCR

Real-time PCR was carried out by ABI 7500 Real Time PCR System with Power SYBR® Green (ABI) dye at the melting temperature of the corresponding genes with the primer concentration of 5 pmol each forward and reverse. 18s rRNA was used as endogenous control for each gene. The oligonucleotide primers used in the reactions have been listed in Table 1, supplied as Supporting Information.

2.5. Western blot

C2C12 cells were lysed in buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA) supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM PMSF, 1 μg/ml trypsin inhibitor) and 1%NP-40. It was then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected, resolved on a SDS-PAGE and subjected to immunoblotting with antibodies including, anti-TXNIP, anti-ChREBP, anti-SIRT1 and anti-α-Tubulin (Santa Cruz, CA; 1:1000 dilution), anti p-AMPK, anti-Glut-4, anti-GAPDH and anti-IRS-1 (Cell Signalling Technology; all 1:1000 dilution).

2.6. Measurement of ROS and superoxide (o_2^-) production

The peroxide-sensitive fluorescent probes H2DCFDA and MitoSOX™ red (Molecular Probes) were used to assess the generation of intracellular ROS. Briefly, After experimental treatment, the cells were harvested and re-suspended in 0.5 mL of serum-free medium and incubated with 20 μmol/L either with H2DCF-DA for 30min or with MitoSOX™ for 15 min at 37 °C at dark. The fluorescence activity was recorded on BD FACSVers.

2.7. siRNA transfection in C2C12

C2C12 myotubes in six-well plate were transfected with 40 nM siRNA directed against TxNIP or ChREBP according to the manufacturer protocol using RNAimax (ThermoFisher Scientific, USA).

2.8. Glucose uptake by fluorescent 2-NBDG assay

The uptake of glucose into C2C12 cells is determined by "Glucose uptake cell-based assay kit" (Caymann Chemicals, MI, USA) according to manufacturer protocol. Then the amount of 2-NBDG taken up by the cells was measured at 485/535 nm wavelength (excitation/emission) using fluorescent ELISA reader (Synergy H1 Multi-Mode Reader, BioTek, WI, USA).

2.9. Oxygen consumption rate (OCR) estimation by extra cellular flux analyser

Cellular oxygen consumption was measured by XF^e24 Analyser (Seahorse Bioscience). Briefly, C2C12 myotubes were transfected with TxNIP-siRNA. At the end of transfection, differentiation media was replaced with XF assay media supplemented with 2.5 mM glucose, sodium pyruvate and L-Glutamine as indicated in the vendor's manual and the cells were incubated in non-CO_2 incubator for 1hr. Mitochondrial function was assessed by XF Mitostress assay kit (Seahorse Bioscience). The cartridge was loaded with oligomycin (2 μM) in injection port A, FCCP (2 μM) in port B and Rotenone + Antimycin A in port C. Then, the OCR and ECAR were measured using the flux analyser according to the manufacturer's protocol. Basal respiration was calculated as the difference between the OCR obtained before the addition of oligomycin and after the addition of rotenone and Antimycin-A (non-mitochondrial respiration). Maximal respiration as the difference between OCR obtained following the addition of FCCP and non-mitochondrial respiration. Proton linked respiration as the difference between FCCP-induced respiration and non-mitochondrial respiration, whereas ATP-linked respiration as the difference between basal respiration and proton leak.

2.10. GLUT-4 cell membrane translocation assay

Glut-4 translocation to the cell membrane was measured by FACS as described previously [14]. Briefly, TxNIP KD cells were treated with 750 μMPA for 16 h. At the end, the cells were washed with PBS and incubated with Glut-4 antibody in dark for 30min (Santa Cruz, cat. sc-53566), which was conjugated for 10min with Alexa flour-488 secondary antibody. At the same time, the cells in the respected wells were treated with 100 nm insulin and then were fixed with 1% para formaldehyde (PFA) for 20min. The cells were then harvested, washed twice with PBS and re-suspended in PBS containing 1% PFA then analysed by FACS.

2.11. Statistical analysis

All data were expressed as the mean ± S.E.M of at least three independent experiments. The statistical significance between the groups was calculated by two-tailed Student's t-test. $^*P < 0.05$, $^{**}P < 0.01$ were considered to be significant. Results shown are combined mean ± S.E.M of three independent experiments.

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