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PINK1 DEFICIENCY-MEDIATED GLUT4 UP REGULATION IN DRUG-INDUCED TYPE 2 DIABETES

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ABSTRACT
Glucose is the primary energy substrate for cellular metabolism; however, its entry into cells is strictly regulated and requires coordinated action of glucose transporters and intracellular signaling proteins. Impairment of glucose uptake is a hallmark of type 2 diabetes mellitus (T2DM), where dysregulation of insulin signaling and GLUT4-mediated glucose transport plays a central role. Cellular signaling pathways are tightly controlled by both activators and repressors, and removal of inhibitory regulators may enhance pathway efficiency.
PTEN-induced putative kinase 1 (PINK1), a mitochondrial serine/threonine kinase, is ubiquitously expressed with high abundance in metabolically active tissues such as skeletal muscle, heart, and testes. Although PINK1 is well characterized for its role in mitochondrial quality control, its contribution to glucose metabolism remains poorly understood.
In the present study, we investigated the molecular and behavioral consequences of Pink1 gene deletion using Pink1 knockout mice. Our findings demonstrate a significant upregulation of GLUT4 expression in the absence of PINK1, suggesting that PINK1 negatively regulates GLUT4-associated glucose uptake. These results identify a previously unrecognized link between PINK1 and glucose transport regulation. However, the precise molecular mechanism underlying this regulation remains to be elucidated.
Collectively, our study highlights PINK1 as a potential modulator of glucose homeostasis and suggests that targeting PINK1-associated signaling pathways may offer new therapeutic avenues for the management of insulin resistance and type 2 diabetes.

KEYWORDS: Pink1, GLUT4, skeletal muscle, type 2 diabetes, miRNA-7019, animal behavior

1. INTRODUCTION

Diabetes mellitus is a serious metabolic disease disturbing 387 million individuals in the world. Diabetes has now touched epidemic levels and has been recognized as the 7th leading cause of expiry in the USA (1). Diabetes mellitus is a collection of metabolic disorders characterized by high glucose amounts resulting from problems in insulin excretion or insulin function (2, 3). The long-term hyperglycemia of diabetes is related with damage and dysfunction of different organs, mostly eyes, kidneys, heart, and blood vessels. The diabetes has two broad types. In type 1 diabetes, the reason is a total shortage of insulin secretion. In type 2 diabetes, the reason is a mixture of insulin resistance and an insufficient amount of insulin secretion (4). Type 2 diabetes affecting 8.3% of the adult people of the world and increasing at a shocking rate, is one of the most common diseases of current time. The major form is type 2 diabetes which accounts for approximately 90% of all diabetes diseases (5, 6). Type 2 diabetes mellitus is a multifactor disease typically related with energy misbalance (7). As a consequence of insulin resistance, irregularity of insulin production and constant pancreatic β-cell disaster leads to insulin insensitivity which is a typical component of type 2 diabetes (8, 9).

Glucose transporter 4 (GLUT4) is serves as the key insulin regulated glucose carrier protein, that transport glucose uptake in skeletal muscle, cardiac muscle and adipose tissues and heart. Where insulin stimulates the translocation to the cell surface to enhance glucose entry. (10).
The GLUT4 is a crucial regulator of postprandial glucose disposal from the circulation and overall systemic glucose balance. It is predominantly expressed in skeletal muscle, adipose tissues and cardiac muscles (11). GLUT4 receptor

perform as an key mediator for carbohydrate metabolism, they provide efficient insulin stimulated glucose uptake in peripheral tissues, where it accounts for the 90% of glucose transporters (12). The major bulk of insulin stimulated glucose dispoal occurs in skeletal muscle, which therefore exerts a major influence on systemic blood glucose control and is central to investigations of glucose metabolism (13). Uptake of glucose in skeletal muscle relies on the presence of GLUT4 in the plasma membrane, which facilitated diffusion down its concertration gradient from extracellular space into the cytosol. The overall uptake is modulated by three major points, extracellular glucose concentration, downstream glucose metabolism and the intrinsic activity (14). Two glucose transporter proteins, GLUT1 and 4, are expressed in skeletal muscles cells, GLUT4 is the key insulin regulate transporter in striated muscle, GLUT4 plays vital role in insulin mediated glucose transport by translocation to the cell surface in response to insulin which increase membrane permeability to glucose (15). The major facilitator of insulin mediated glucose transport in the muscle is GLUT4, which undergoes insulin dependent translocation to the sarcolemma causing insulin stimulations (16).
PTEN-induced Putative Kinase1 (PINK1) functions as mitochondrial serine/threonine kinase that opposes apoptosis induced by oxidative or other cellular stresses in muscle. Homozygous mutation in the PINK1 gene are a well-established cause of autosomal recessive early onset Parkinson's disease (17). PINK1 accumulates at the mitochondrial outer membrane where it phohsporylates ubiquitin and parkin to activate a fee-forward signaling cascade. The joint pathway acts as a protective quality control mechanism to uphold cellular mitochondrial health (18). PINK1 possesses a mitochondrial targeting motif that facilitates its localization to mitochondria through a cytosolic localization has been observed as well. Processing by mitochondrial protease PARL, which cleaves PINK1 within the inner membrane, Plays a pivotal role in controlling PINK1 levels and maintaining proper mitochondrial functions (19).

2. MATERIAL AND METHODS

2.1. Animals and reagents

PINK1-deficient and C57BL/6 (B6) mice were mainly selected in this study and mice were provided by Prof. Ma Ning and placed at least 6 mice per cage. Rodents were kept on a shift of 12 hours light: 12 hours dark cycle at 22°C with availability of water and food. All scientific procedures were completed according to the recommendations related to the Care and use of animals reported by National Institutes of Health (USA). Mice were designed into two different groups (control and experimental groups). Streptozotocin was purchased from Sigma Chemical Co (Sigma-Aldrich, Streptozotocin-CAS 18883-66-4- Cal biochem). Antibodies against GLUT4 (NBP1-49533) and Pink1 (BC100-494) was purchased from Novus Biologicals. β-actin were from Cell Signaling (Cell Signaling Technology, Danvers, MA).

2.2. Induction of type 2 diabetes

2.2.1. Prepare animals

To induce type 2 diabetes in wild type and Pink1 knockout mice, we were divided the 2 months old female mice in to two cages (WT and KO groups). Each cage contains 6 mice with availability of food and water. Firstly, we were provided continuously two months high fat diet.

2.2.2. Treat animals with STZ

Weighed 30mg drug streptozotocin into a 1.5 ml microcentrifuge tubes and shelter the tubes with aluminum foil. To dissolved 0.52g of citric acid (solution A) in 25ml distilled water. Then dissolved 0.73g of sodium citrate (solution B) in 25ml distilled water. Take 1.3ml of solution A and 1.7ml of solution B with the total volume of 3ml. Instantly before the injection, dissolved 30mg STZ in 3ml of (A+B) solution to a final concentration of 10 mg/ml. On experimental day we were removed food for 1-5 hours before inject STZ. Return the all mouse in to their separate cages and deliver usual food and 10% of sucrose water. Then we injected a STZ solution intraperitoneally continuous five days at a dose a dose of 40mg/kg to make them diabetic. Then we were sacrifice mice and get skeletal muscle to perform molecular biology experiments.

2.3. RNA Extraction

To obtain the high quality of RNA is the foundation for several molecular biology researches. The extraction of total RNA by using Trizol (Invitrogen Life Technologies) is a most common method in microarray biological experiments. Tissue homogenization at room temperature (15–25 °C), as stated in the manufacturer's procedure, generates that heat may enhance RNase activity and affects RNA quality.

2.4. cDNA formation and quantitative real time PCR

Reverse transcription formation of complementary DNA from messenger RNA were carried out by using random primers. Afterward, 7500 Real-Time PCR (AB Applied Biosystems, Mannheim, Germany) were used to perform a quantitative real-time polymerase chain reaction (to check mRNA level) the protocol have been taken from (20) . The beta-actin was used for inner crossing standardization. The mRNA level of each transcript was normalized to the beta-actin mRNA level found in the similar sample. The resultant stabilized level of Pink1 KO samples were formed as the fold change versus with the corresponding normalized values of the wild type skeletal muscle samples.

The primer sequences were given as below: Pink1:

Forward: 5′-TTGCAATGCCGCTGTGTATG-3′, Reverse: 5′-TGGAGGAACCTGCCGAGATA-3′; GLUT4: Forward: 5′-CCTCTACATCATCCGGAACC-3′, Reverse: 5′-ACATTGGACGCTCTCTCTCC-3′; β-actin: Forward: 5′-GTCAACGGATTTGGTCTGTATT-3′, Reverse: 5′-AGTCTTCTGGGTGGCAGTGAT-3′. miRNA-7019-3p: Forward: 5′-TATCACCTTGGCCGCCTCTC-3′, Reverse: 5′-CGTATCCAGTGCGTGTCGTG-3′; U6: Forward: 5′-GCTTCGGCAGCACATATACTAAAAT-3′, Reverse: 5′-CGCTTCACGAATTTGCGTGTCAT-3′.
U6 is a housekeeping gene (small nuclear RNA) used to control the miRNA expression in qPCR.

2.5. Protein Extraction

The method of protein extraction was performed on ice, in order to prevent the degradation of proteins or other contamination. Reagents used in this protein extraction were, RIPA and Protease Inhibitor (EDTA). To set up the lysis buffer for protein extraction, add 100ul of PI to 10mL of RIPA Buffer and mix well. To extract a protein from skeletal muscle tissue, homogenize a tissue in pestle and mortar with the help liquid nitrogen to freeze a tissue to avoid protein degradation.

2.6. Determination of protein concentration

Protein amounts were detected in skeletal muscle tissue samples after the solubilization with 0.1M sodium hydroxide (NaOH). A protein amount was detected as explained by using bovine serum albumin (BSA) as ordinary. Reagents were used in protein determination are as follows: BCA, Cu, BSA, RIPA and Protein samples.

2.7. Western Blot

The total protein was extracted from skeletal muscle tissue and is quickly crushed in liquid Nitrogen (temp: -196 ^{0}C). The resultant precipitate was altered into cold radio-immunoprecipitation examine lysis buffer comprising phenylmethyl sulfonyl fluoride (1:100 dilution) for 40 minutes. Afterward, the different samples remained centrifuged at 4°C and 12,000 rpm for 15 minutes. The upper portion called Supernatants were shifted into a new tube, and the quantities of protein was detected by performing the bicinchoninic acid method (Boster Biological Technology). All protein samples were diluted to the same concentration. A 40 µg of samples were loaded on 10% of sodium dodecyl sulfate polyacrylamide gel.
To prevent non-specific binding, a 5% dry and non-lipid milk in tris buffered saline Tween 20 (TBST) was performed for blocking. After about 2 hours, the membranes remained incubated with the primary antibodies to GLUT4 (1:1000 dilution), Pink1 (Wanleibio, 1:1000 dilution), or beta-actin (Thermo Fisher Scientific, 1:2000) overnight at 4°C. Afterward wash the membranes for three times with TBST; a membrane was incubated with anti-rabbit secondary antibody G- horseradish peroxidase (1:5000 dilution). After that supplementary washing with TBST, the protein bands were seen by performing chemiluminescence. Visualized bands were measured by Image J (NIH, Bethesda, MD).

2.8. Immunofluorescence staining

After the skeletal muscle collection, the sample was blotted to eliminate extra blood and other noticeable fats were removed. Some portion of sample was fixed in Tissue Tek OCT chemical (Sakura) and instantly freeze in liquid nitrogen. The sample was then moved to an aluminum cryotube for storage at -80°C. For the immunofluorescence experiment frozen tissue samples were cryosection by performing a microtome inside a cryostat (Bright Instrument Company Limited, Huntingdon, UK) to a width of 10µm into microscope slides. WT and Pink1 KO sections to be compared with each other and slides for each part were formed in triplicate way. Slides were than stained within 60 minutes of sectioning or freeze at -20°C. The experiment of all slides was treated in similar way. The stored slides were gone at room temperature for about 30 minutes before staining. The following antibodies were used in immunofluorescence experiments. Primary antibody Pink1 (dilution 1:100; Novus biotechnology) and GLUT4 primary antibody (dilution 1:100; Novus biotechnology) at 4 ^{0}C overnight. One the second day secondary antibodies (1:400 dilution) were incubated for 60 minutes in the dark environment and then 4′,6-diamidino-2-phenyl-indole remained added for 5 minutes. Immunofluorescence staining was visualized by fluorescence microscopy.

2.9. Behavioral methods

2.9.1. Treadmill exhaustion test

Muscle diseases typically lead to variations in skeletal muscle function. In vivo tests that can estimate muscle working ability are therefore of significant value as primary phenotypic screening. Here, we explain how to use the treadmill test, which measures exercise ability of mice. In the treadmill exhaustion test, the mice were enforced to run over a conveyor belt by slowly growing speed (21).

2.9.2. Limb grip strength assay

The limb grip strength test by grip strength meter uses a horizontal grip–that is gripped by the mouse–to check the maximum power that is essential to make the mouse release it. Limb grip strength test measure the muscular strength

of mice. These behavioral tests can be easily performed to check muscle performance under different conditions, such as after the therapeutic interventions, or to check the important functions of specific genes on muscle physiopathology (21).

2.9.3. To check balance by rotarod machine

The initial trials in the scientific history of mouse behavior were made by Colin Stewart, a Ph.D. scholar at Clark University in the United States. He evaluates the movement of mouse by locating into a drum and calculated the how many number of rounds it twisted (22). In latest few years, the rotarod has been used to study modifications among inbred strains gene knockout and transgenic mice, effects of drugs, regaining from brain injuries and animal studies for human disease (23). Debits in motor functions are devastating aspects in disorders disturbing neurological, neuromuscular and musculoskeletal systems (24).

2.10. Body Weight and Blood Glucose level

We were selected 2 month's old 6 WT and 6 Pink1 KO mice and divided into two separate cages with free access of water and food. Firstly, checked the body weight of mice. After the induction the type 2 diabetes we found that body weight of a mice was decreased and blood glucose level was increased. Body weight and glucose level is given below of WT and Pink1 KO mice.

Table 1. Body weight and blood glucose level of type 2 diabetic WT mice.

WT mice		
S.No	Body Weight (Grams)	Glucose levels (Mmol/L)
1	26.3	13.7
2	23.1	16.5
3	22.4	27.7
4	20.1	27.2
5	20.1	17.7
6	19.4	19.5

Pink1 KO mice

Table 2. Body weight and blood glucose level of type 2 diabetic Pink1 KO mice.

S.No	Body Weight (Grams)	Glucose levels (Mmol/L)
1	23.4	21
2	20.5	14.4
3	16.2	17.3
4	14.7	23.1
5	14.1	22
6	14.9	15.8

2.11. Statistical analysis

For all mouse model experiments, we prepared at least three animals in one group, and the results are indicated as the mean±s.e.m. For the assessments of 2 groups of values, the statistical study of the data was achieved by the Student's t-test. The bio statistical analysis was performed by using the Graph Pad Prism 6 software. In all cases, $P<0.05$ was considered significant.

3. RESULTS

3.1. Pink1 expression in skeletal muscle in type 2 diabetes

The association between Parkinson's disorder and PINK1 mutations and the idea that Parkinson's disease individuals may have weakened glucose tolerance, there is a chance that reduce in PINK1 may characterize a direct relation between neurodegeneration and type 2 diabetes, although one operating by physical laziness and insulin resistance in skeletal muscle. Upcoming studies should discover the function of PINK1 protein in glucose metabolism as well as potential epigenetic pathways that could clarify the silencing of the PINK1 locus and hence the relation between neurodegeneration and type 2 diabetes (25). After the induction of type 2 diabetes in WT mice we were checked Pink1 expression by qRT-PCR and western blot. Our results suggested that the Pink1 mRNA and protein expression both were decreased in WT diabetes as compared to WT control mice (Fig. 1a and 1b).

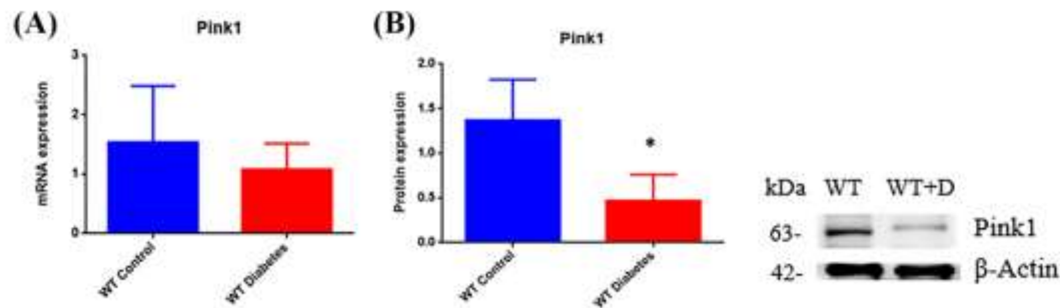

Figure 1. Pink1 expression is decreased in type 2 diabetes. (a)

Quantitative real time PCR against Pink1 in WT control and WT diabetes mice skeletal muscle samples; beta-actin was used as a loading control. Values are means ± S.D. (n=5). Data analysed by students t-test revealed a non-significantly decreased mRNA expression versus WT diabetes, (**b**) Western blot against Pink1 of WT diabetes has significantly decreased Pink1 protein expression versus the corresponding WT control mice (n=3). Only representative western blot bands shown in this figure. The mean ± s.e.m. values of the mRNA data were measured from the fold change of β-Actin normalized transcript abundance in the WT control versus the WT diabetes samples. *P < 0.05

3.2. GLUT4 expression in skeletal muscle in type 2 diabetes

To investigate the result of type 2 diabetes on GLUT4 expression, we found that GLUT4 expression is reduced in WT diabetes as compared to WT control at both mRNA and protein level (Fig. 2a and 2b). We were also performed immunofluorescence to check GLUT4 content in WT versus WT diabetes skeletal muscle tissue; WT diabetes tissue has low GLUT4 content as compared to WT (Fig. 2c). GLUT4 mRNA level is also decreased in Pink1 KO diabetes as compared to Pink1 KO mice (Fig. 2d). These results suggested GLUT4 expression is reduced in type 2 diabetes (both in WT diabetes and Pink1 KO diabetes).

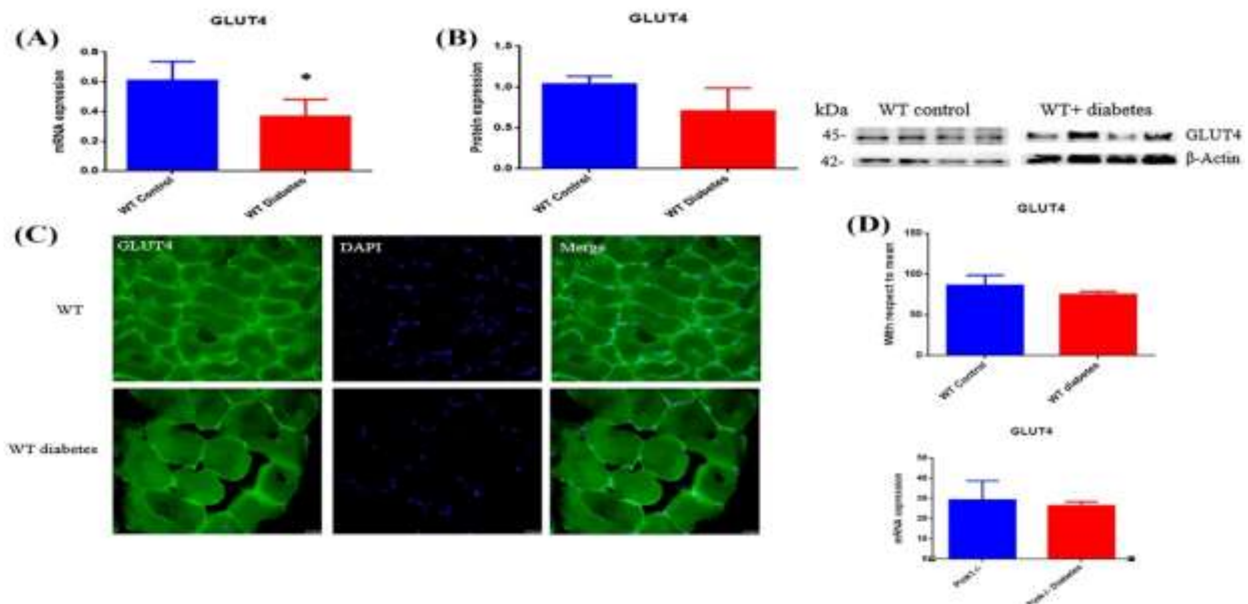

Figure 2. GLUT4 expression is decreased in type 2 diabetes. (a)

Quantitative real time PCR against GLUT4 in WT control and WT diabetes mice skeletal muscle samples; beta-actin was performed as a loading control. Values are means ± S.D. (n=3-4). Data analysed by students t-test revealed a decreased significant effect versus WT control, (**b**) Western blot against GLUT4 of WT diabetes has non-significantly decreased protein expression versus the corresponding WT control mice (n=4), **(c)** Immunofluorescence against GLUT4 of WT diabetic mice has non-significantly decreased expression versus the WT control mice (n=3). (d) Pink1 KO diabetes has non-significantly decreased GLUT4 mRNA level as compared to Pink1 KO mice (n=6). The mean

± s.e.m. values of the mRNA data were considered from the fold change of each β-Actin normalized transcript abundance in the WT control samples versus the WT diabetes samples. *P < 0.05

3.3. Expression of miRNA-7019 in Pink1 KO mouse

We realized there is miRNA-7019 is present in Pink1 KO mice. To make sure there is absence pink1 mRNA expression in Pink1 KO mice, we were performed qPCR of WT and Pink1 KO brain samples. We find that the pink1 mRNA expression is significantly decreased in Pink1 KO samples as compared to WT brain samples (Fig. 3a). To confirm the presence of miRNA-7019 we were performed quantitative real time PCR of wild type and Pink1 KO brain samples. We found that Pink1 KO samples has non significantly increased miRNA-7019 expression as compared to WT samples (Fig. 3b). U6 is a housekeeping gene (small nuclear RNA) used to control the miRNA-7019 expression in qPCR.

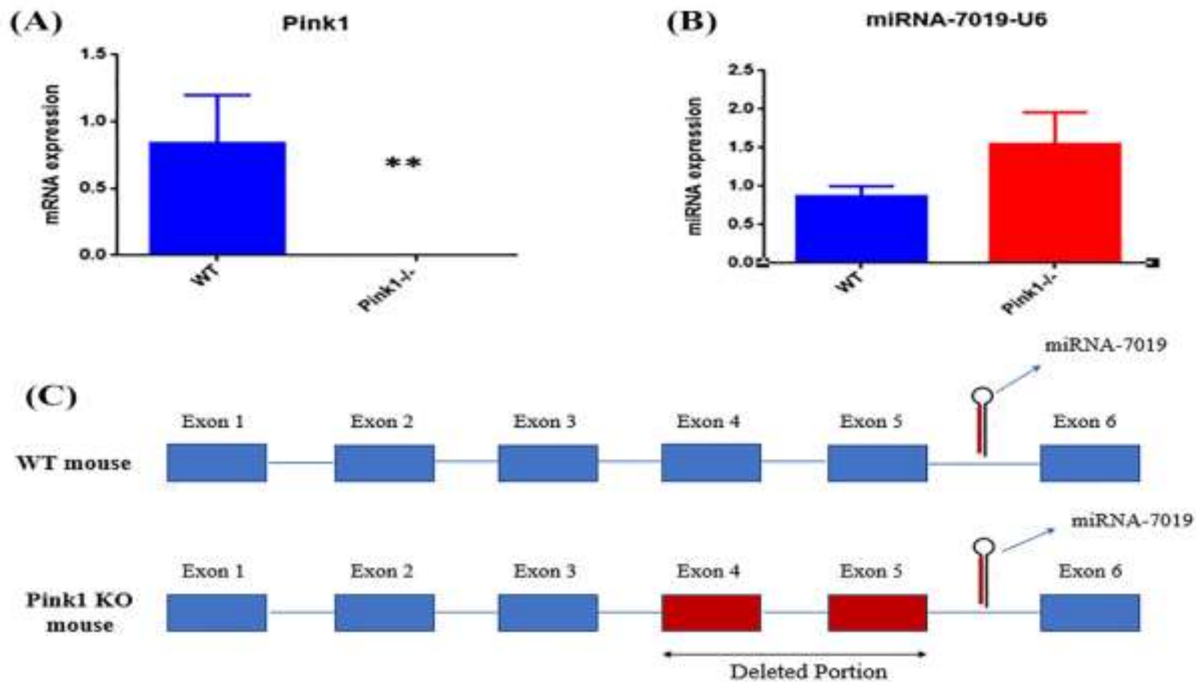

Figure 3. Presence of miRNA-7019 in Pink1 KO mice. (**a**)

Quantitative real time PCR against Pink1 in WT and Pink1 KO mice brain samples; beta-actin was used as an internal control. Values are means ± S.D. (n=3-4). Data analysed by students t-test revealed a significant effect versus WT samples, (**b**) Pink1 KO mice has non significantly increased miRNA-7019 expression versus the corresponding WT mice. The mean ± s.e.m. values of mRNA data were measured from the fold change of each beta-Actin normalized transcript abundance in the Pink1 KO samples versus the WT samples. **(c)** Imagination picture of a wild type and Pink1 knockout mouse shows the presence of miRNA-7019 in both Pink1 genes, miRNA-7019 is present on the 5th intron of Pink1 gene. There is no effect of deletion of 4 and 5 exons of Pink1 gene on miRNA-7019. **P < 0.01

3.4. Blood glucose level in WT and Pink1 KO mouse

Continuous 2 months high fat diet and a drug Streptozotocin were used. After the 8 days of an injected drug the WT and Pink1 KO mouse becomes diabetic. A Pink1 knockout diabetic mouse has non significantly decreased blood glucose level as compared to wild type diabetic mice (Fig. 4c).

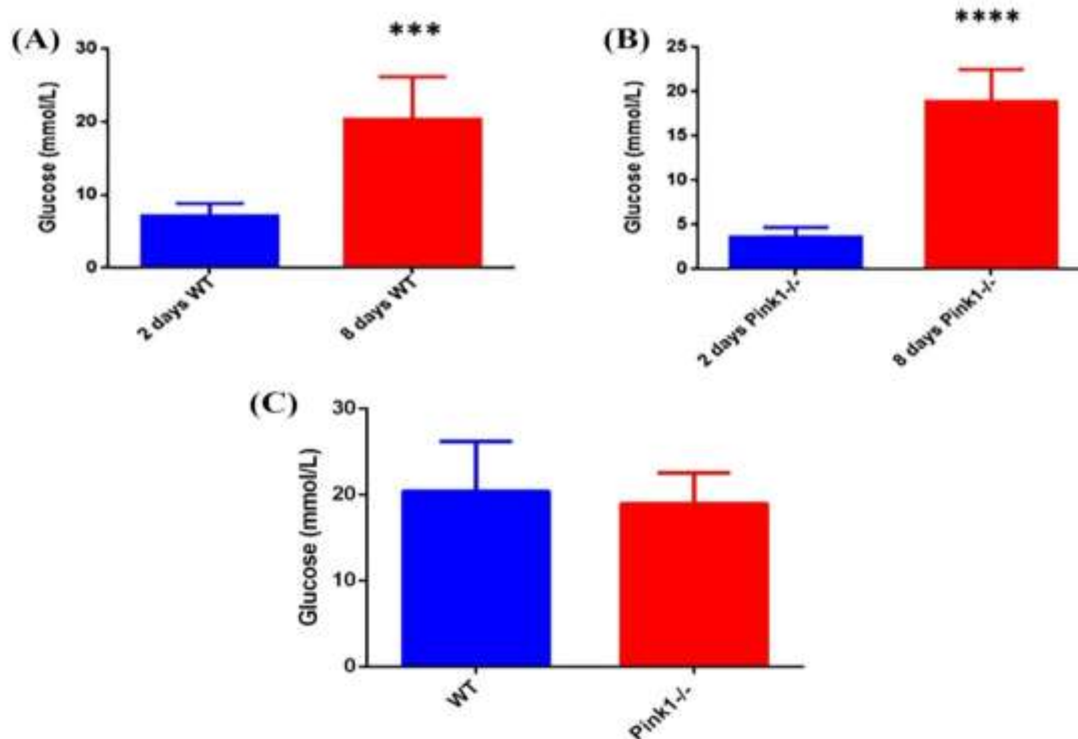

Figure 4. After 8 days of streptozotocin increases the blood glucose level (mmol/L) in Wild type and Pink1 knockout mice.

(a) Values are means ± S.D. (n=6). Data analysed by students t-test revealed an increased significant effect versus 2 days WT mice, (**b**) After 8 days Pink1 KO mice has significantly increased blood glucose level versus the corresponding 2 days Pink1 KO mice (n=6). **(c)** After the induction of type 2 diabetes the Pink1 KO diabetic mice has low blood glucose level versus Wild type diabetic mice (n=6). ***P<0.0003, ****P< 0.00001, P<0.61

3.5. GLUT4 expression in WT and KO mouse skeletal muscle

Some previous reports suggest that the deletion of Pink1 causes a metabolic change that helps glucose uptake and glycolysis (18). To investigate whether the Pink1 KO mice regulates GLUT4 expression of a gene, we were used real time PCR and western blot of different mice groups. Our results suggest that the GLUT4 mRNA and protein level both are increased in Pink1 KO mice as compared to WT mice (Fig. 5a and 5b). Immunofluorescence against GLUT4 of Pink1 KO mice has higher expression as compared to WT mice (Fig. 5c).

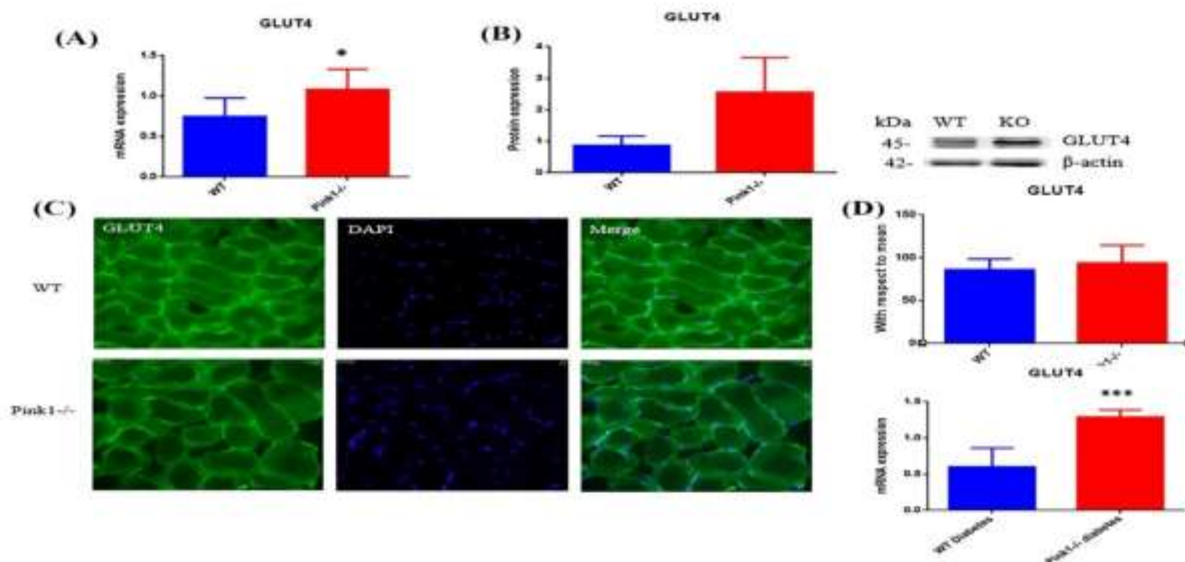

Figure 5. Pink1 KO upregulates GLUT4 expression in skeletal muscle.

(a) Quantitative real Time PCR against GLUT4 in WT and Pink1 KO mice samples; beta-actin was used as an internal control. Values are means ± S.D. (n=6). Data analysed by students t-test revealed an increased significant effect versus WT control, (**b**) Western blot against GLUT4 of Pink1 KO has non-significantly increased protein expression versus the corresponding WT control mice (n=3). Only representative western blot bands shown in this figure, **(c)** Immunofluorescence against GLUT4 of Pink1 KO mice has non-significantly increased expression versus the WT control mice (n=3). **(d)** Pink1 KO diabetes has significantly increased GLUT4 mRNA expression as compared to WT diabetic mice (n=6). The mean ± S.D. values of mRNA data were measured from the fold change of each beta-Actin normalized transcript abundance in the Pink1 KO versus the WT control. *P < 0.05, ***P<0.01

3.6. Pink1 KO mouse has higher Grip strength and low balance ability

PINK1 KO mice exposed decreased spontaneous locomotors activity at three to six months of age as evaluated by the number of steps, rears, and landings in the cylinder test. They also acquired longer than wild-type mice to turn and climb down a pole, a test of locomotors ability (26). To study the behavioral functions of a WT and Pink1 KO mice, we were selected three types of behavioral methods, grip strength test, balance of a mice on rotarod machine and treadmill exhaustion test. Our research platform is Pink1 KO mice, so we covered both the molecular and behavioral features of knockout mice, because genetic composition of any organism also affects its behavior. Pink1 KO mice has significantly (P<0.05) increased grip strength as compared to WT mice (Fig 5a), although treadmill exercise non-significantly (P>0.05) increased versus WT mice. Balance (rotarod machine at 25 rpm) of a WT mouse has significantly (P<0.05) increased as compared to Pink1 KO mice.

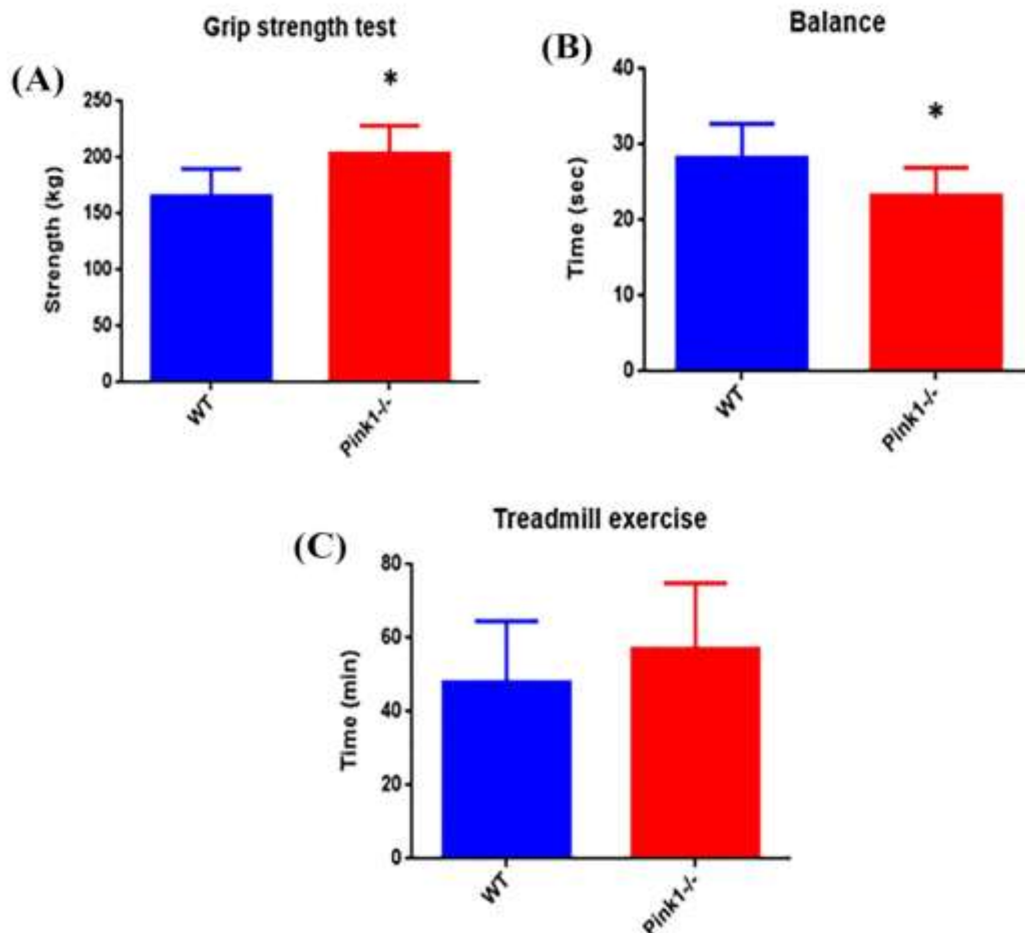

Figure 6. Pink1 KO mouse has higher grip strength and low balance ability.

(a) Data analysed by students t-test revealed that Pink1 KO mice has increased significant grip strength versus WT mice (n=6), **(b)** WT mice has increased significant balance ability versus Pink1 KO mice (n=8), **(c)** Pink1 KO mice has non-significantly increased treadmill exhaustion test versus the WT mice (n=6). *P<0.05

3.7. GLUT4 expression in skeletal muscle after treadmill activity

Physical activity plays a key role in the treatment and prevention of type 2 diabetes, has been noticeable acute and chronic effects on the maintenance of glucose uptake and on metabolism. Exercise stimulates the insulin signaling

mechanism, increasing GLUT4 gene expression and translocation in to the cell surface membrane (27). To evaluate the effect of exercise on the mice, we were divided the wild type mice into two groups. A single session of exercise about 30 minutes has been performed with the help of treadmill exercise machine. We found that the GLUT4 protein was increased in WT exercise group as compared to WT control group.

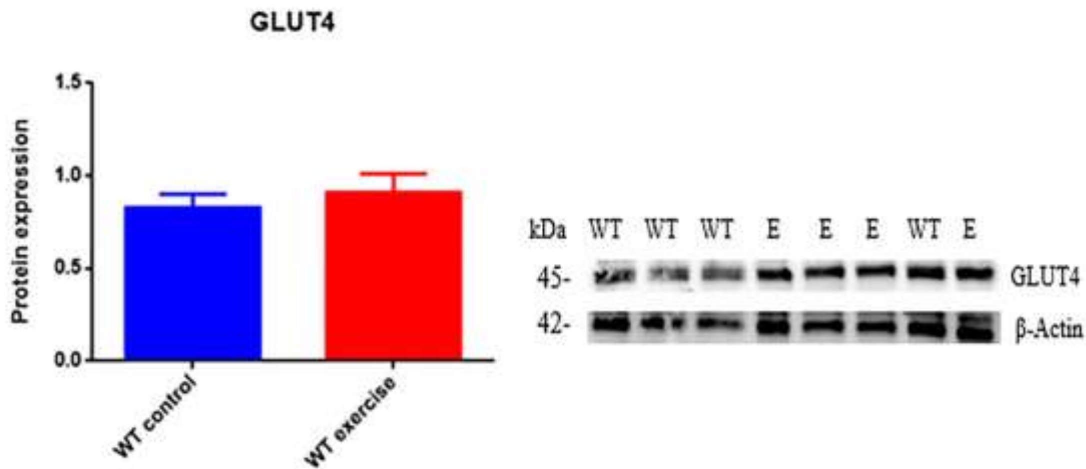

Figure 7. Exercise increased GLUT4 protein expression in skeletal muscle.

Western blot against GLUT4 in WT control and WT exercise mice skeletal muscle samples; beta-actin was used as an internal control. Values are means ± S.D. (n=4). Data analysed by students t-test revealed a non-significantly ($P>0.05$) increased GLUT4 protein expression versus WT control mice. E symbol represents exercise mouse group.

DISCUSSION

The human genome encodes thousands of genes, yet epigenetic processes enable specific genes to control expression or functions of others via mechanism that do not alter DNA structures. PINK1 exemplifies a multifunctional gene tied to pathology, while pathogenic mutations cause Parkinson's disease, emerging evidences connects PINK1 alterations such as reduced expression in type 2 diabetes skeletal muscle or hyperglycemia induced mitophagy defects to the pathophysiology of type 2 diabetes mellitus and potentially other metabolic diseases (28). The PINK1 gene encodes a mitochondrial serine/threonine kinase, and biallelic loss of function alterations in this gene results in autosomal recessive Parkinson disease. In vitro experiments have shown that PINK1 localizes to the outer membrane of depolarized mitochondria, phosphorylating both ubiquitin and parkin to facilitate parkin recruitment and activation. The resulting ubiquitination cascade promotes mitophagy the targeted autophagic degradation of malfunctioning mithochondria ensuring mitochondrial quality control (29).

Regulation of the PINK1 locus has been associated with neurodegenerative disease in earlier studies but the recent findings also demonstrate alterations in its expression during type 2 diabetes and obesity states. These observations raise the possibilities that PINK1 plays a significant role in cellular glucose metabolism, perhaps by influencing mitochondrial bioenergetics, mitophagy and downstream insulin responsiveness in peripheral tissues (25). After the development of type 2 diabetes our present study suggested that the Pink1 gene expression is reduced in type 2 diabetes as compared to WT control at both mRNA and protein level, but the mechanism of reduced Pink1 gene expression in type 2 diabetes is still not clear.

Mature miRNAs are short about 18-25 nucleotide long RNAs that play a key role in post transcriptional gene silencing. Through base pairing with target sites in mRNAs, they can trigger mRNA degradation or inhibit protein translation by interfering with ribosomal machinery (30). In Our previous Study we investigated the effect of gene loss in Pink1 KO mouse the introns 4th and 5th were deleted and we observed that Glut 4 is still regulated by something (31). We realized there is still a presence of miRNA-7019 in the intron region of a Pink1 KO mouse after the deletion of a 4th and 5th exon of a gene. After performing the quantitative real time PCR, we found that miRNA-7019 is present in the 5th intron of a Pink1 KO mouse.

Previous research indicated that loss of PINK1 triggers metabolic reprogramming of glucose pathway through HIF1-alpha resulting in upregulated glycolysis and promotion of cell proliferations. In Pink-1 deficient cell and tissues, HIF1-alpha dependent enchancement of intracellular glucose metabolism appears essential for maintaining elevated rates of cell growth and division (18). On the other side the loss of pink1 in pancreatic beta cells increases basal insulin secretion and regulates the glucose levels in mice (17). Based on the evidence linking PINK1 loss to altered glucose metabolism, we hypothesized that PINK1 deficiency may affect GLUT4 expression. GLUT4 remains the principal

mediator of stimulated glucose transport in skeletal muscle, adipose tissue and heart. Exerting a dominant influence on the maintenance of euglycemia and whole body metabolic equilibrium (32). Our present studies suggested that Pink1 knockout mouse up regulates GLUT4 expression in skeletal muscle at both mRNA and protein level. After the induction of type 2 diabetes in WT and Pink1 knock mouse, our results suggested that Pink1 knockout diabetic mouse has twice GLUT4 expression as compared to WT diabetic mouse at mRNA level, although non-significant increase of GLUT4 expression at protein level. The GLUT4 and Pink1 expression is reduced in type 2 diabetes but the mechanism of action is still not clear. On the other hand, how Pink1 knockout up regulates GLUT4 expression, an in vitro study is required to know the mechanism of action.

In response to exercise, GLUT4 is recruited to the cell surface of skeletal muscle fibers in an insulin dependent fashion in both humans and experimental animals, reinforcing the role of physical activity as a keystone treatment for metabolic diseases characterized by impaired glucose handling. Acute exercise induced increases in GLUT4 at the sarcolemma are largely explained by accelerated translocation from the intracellular compartments, with a little evidence for enhanced transcription during single bouts. Chronic exercise training, by contrast, increases resting (Basal) GLUT4 protein abundance through adaptive changes in expression (33). Studies in human and mouse models have demonstrated that regular aerobic exercise training increases skeletal muscle GLUT4 protien levels by 20-70%. Such upregulation is believed to potentiate acute muscle glucose disposal, leading to greater responsiveness to both insulin and exercise induced contractions (34). Our present study also suggested that after acute exercise (about 30 minutes) the GLUT4 protein expression has been increased in exercised mouse group as compared to sedentary mouse groups.

Our research interest was based on Pink1 KO mouse, so we were covered both the molecular and behavioral features of a mouse. Genetic make-up or genetics also affects the behavior of a mouse or human. The 99% of mouse and human genome is same so that’s why we were used mouse for behavioral studies. In behavioral studies we were divided mouse in to two groups WT and Pink1 KO groups. There are three kinds of behavioral functions has been evaluated grip strength, balance and treadmill exhaustion abilities of a mouse. We found that the Pink1 KO mouse has significantly increased grip strength ability as compared to WT mouse. On the other hand, Pink1 KO mouse has significantly decreased balance ability as compared to WT group. Pink1 KO mouse has non-significantly increased treadmill exhaustion ability as compared to WT mouse group. Pink1 knockout mouse regulates physical activity, because KO mouse upregulates GLUT4 expression. It is very clear that the GLUT4 is involved in transportation of glucose into the muscle cells. If glucose is not transported the energy is not formed and physical activity will become reduced.

CONCLUSION

Knockout mouse are used to know the gene function, usually by exploring the consequences of the absence of the gene. In Pink1 knockout mouse the 4th and 5th exon positions are removed from the current gene. The deletion of these two exons from Pink1 gene cannot affect the activity of miRNA-7019, because this miRNA is still present in the 5th intron of the Pink1 gene. So, this Knockout mouse can be used in research studies. We found that the Pink1 and GLUT4 expression was decreased in diabetic mouse model. Still we are unfamiliar that why Pink1 and GLUT4 dysexpression is takes place in type 2 diabetes. An In vitro research study is essential to discover the process of reduced expression of these two genes in type 2 diabetes. After our present research, these results are concluded that the deletion of Pink1 gene has improved the GLUT4 expression inside the cells and decreases the risk of type 2 diabetes. Higher the GLUT4 level has reduces the chances of type 2 diabetes in animals.

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Authors Contribution:

G.M were responsible for first phase of data collection and ethical approval.
A. M contributed in Data collection and analysis.
S.T Critically reviewed in the manuscript and revisions.
S.M were critically analyzed the results and manuscript development
S.H Contributed in data collection and manuscript development
N.H.M contributed in statistical and submission process
G.S.S Review the manuscript and help in critical analysis
M. A. A contributed in overall manuscript development and guideline and reviews.

Conflict of interest

Author shows no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request. Due to privacy and ethical restrictions related to participant confidentiality, the data are not publicly available.

Author Approval Statement
All authors have read and approved the final version of the manuscript. The authors confirm that they meet the criteria for authorship as outlined in this document and take full responsibility for the content of the manuscript. Each author believes that the manuscript represents honest and original work.

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