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Experimental Research Article

Insulin and toll-like receptor 4 interaction in the rat model of Parkinson's disease induced by lipopolysaccharide

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ABSTRACT

Introduction: Toll-like receptor (TLR) 4 is involved in neuroinflammatory processes in peripheral tissues and central nervous system. Pro-inflammatory cytokines production, due to over activation of TLR4, interfere with insulin signaling elements lead to insulin resistance. Regarding the critical roles of TLR4 and insulin in the pathogenesis of Parkinson's disease (PD), in the present study the TLR4/insulin receptor interaction was assessed in a neuroinflammation model of PD.

Methods: LPS was injected into the right striatum of male Wistar rats (20µg/rat). Insulin (2.5IU/day), insulin receptor antagonist (S961; 6.5nM/kg), or TLR4 antibody (Resatorvid (TAK242); 0.01µg/rat) were administered intracerebroventricularly (ICV) for 14 days. Insulin and TAK242 were also simultaneously injected in a distinct group. Behavioral assessments were performed using rotarod, apomorphine-induced rotation, and cylinder tests. The levels of α-synuclein, TLR4, and elements of the insulin signaling pathway were measured in the striatum.

Results: LPS impaired motor performance of the animals and increased the levels of α-synuclein and TLR4. Furthermore, it reduced mRNA levels of IRS1 and IRS2 and enhanced GSK3β mRNA and protein levels, indicating the development of insulin resistance. Treatment with insulin and TAK 242 improved motor deficits, restored insulin signaling pathway, and reduced α-synuclein and TLR4 levels.

Conclusion: The findings indicate that LPS impaired motor function, at least in part, via α-synuclein and TLR4 overexpression, leading to insulin resistance. Suppression of TLR4 and activation of insulin receptors attenuated motor deficits, suggesting that TLR4 and insulin receptors are promising therapeutic targets for PD modification.

Keywords:
Parkinson's disease
Insulin
Lipopolysaccharide
TLR4
TAK242

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Introduction

Chronic neuroinflammation is one of the main features of neurodegenerative diseases like Parkinson's disease (PD). PD is characterized by motor impairments such as bradykinesia, rigidity, resting tremor, and postural instability due to the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Heidari et al., 2022). Increase in proinflammatory and reduction of anti-inflammatory cytokines have been reported in patients with PD (Mogi et al., 1996; Qin et al., 2016).

Microglia, the immune cells of nervous system, are activated in response to inflammatory insults and produce proinflammatory cytokines including TNFα, interleukin (IL)-1β and IL-6 (Kam et al., 2020). These glial cells express high density of toll-like receptors (TLRs), which are innate immune system receptors and members of the pattern recognition receptor (PRR) family (Gorecki et al., 2021). TLRs have critical roles in recognizing pathogen-associated molecular patterns (PAMPs) expressed by microbial invaders and damage-associated molecular patterns (DAMPs) released during tissue damage (Perez-Pardo et al., 2019). TLR2 and TLR4 are two important members of this family which play roles in inflammatory processes. TLR4 is also involved in microglial activation induced by α-synuclein in PD (Heidari et al., 2022). α-Synuclein aggregates as neurotoxic oligomers in neurons, especially dopaminergic neurons in SNpc, in patients with PD leads to dopaminergic neuronal cell death. α-Synuclein also acts as an immune signaling molecule and endogenous agonist of TLR4, and activates TLR4 on microglia and astrocytes (Codolo et al., 2013; Fellner et al., 2013; Gorecki et al., 2021; Hughes et al., 2019; Rannikko et al., 2015). Lipopolysaccharide (LPS), a component of gram-negative bacteria's outer membrane, is a potent activator of TLR4 (Kim and Sears 2010). LPS activates TLR4 on microglia, leading to the production of pro-inflammatory cytokines and neuronal damage (Vargas et al., 2020). In experimental studies, LPS is used to induce a neuroinflammation model of PD, both in vivo and in vitro (Liu and Bing 2011; Tufekci et al., 2011).

Insulin and insulin receptors exist in different regions of the brain and regulate many functions including neuronal cell survival, synaptic function, and neural circuit formation (Banks et al., 2012). Insulin is important for dopaminergic neurons survival and function, and any disruption in its signaling pathway may lead to dopaminergic neurons dysfunction (Athauda and Foltynie 2016). In patients with type 2 diabetes, insulin resistance and hyperglycemia can induce dopaminergic neurons degeneration (Song and Kim 2016). It has been indicated that diabetic patients may be more susceptible to PD and have more severe movement symptoms than non-diabetic patients with PD (Mollenhauer et al., 2019; Pagano et al., 2018; Sandyk 1993). Furthermore, insulin resistance is one of the pathological hallmarks in the brain of PD patients (Athauda and Foltynie 2016). Experimental studies have shown that insulin administration protects dopaminergic neurons against 6-hydroxy dopamine (6-OHDA) and improves motor impairments (Iravanpour et al., 2021).

TLR4 and the insulin signaling pathway are closely related. Chronic activation of TLR4 increases inflammatory factors that interfere with insulin receptor and insulin receptor substrates (IRSs), resulting in insulin desensitization (Huang et al., 2017). Regarding the critical role of TLR4 and the insulin signaling pathway in the pathogenesis of PD, this study was designed to evaluate the effects of insulin, insulin receptor antagonist, and TLR4 blocker (TAK242; Resatorvid) on motor impairments in the neuroinflammation model of PD induced by LPS. Furthermore, molecular assessments were performed to elucidate the interaction of these signaling pathways.

Materials and Methods

Animals

Male Wistar rats (220-250g) were used in this study under the standard cycle of 12 hours of light/12 hours of darkness and a temperature of 23±2 °C. The rats had free access to water and food *ad libitum*. The experiments were performed at the same time during the day (light phase) to avoid circadian variations in animals. All procedures were followed the guidelines of the National Institutes of Health for the care and use of laboratory animals (8th edition, 2011) and approved by the Animal Research Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1400.676).

Drugs

LPS (Sigma-Aldrich, USA) was dissolved in sterile 0.9% normal saline (NS) (20µg/2µl /rat) (Hunter et al., 2007; Shukuri et al., 2021). Insulin and S961, a high-af-

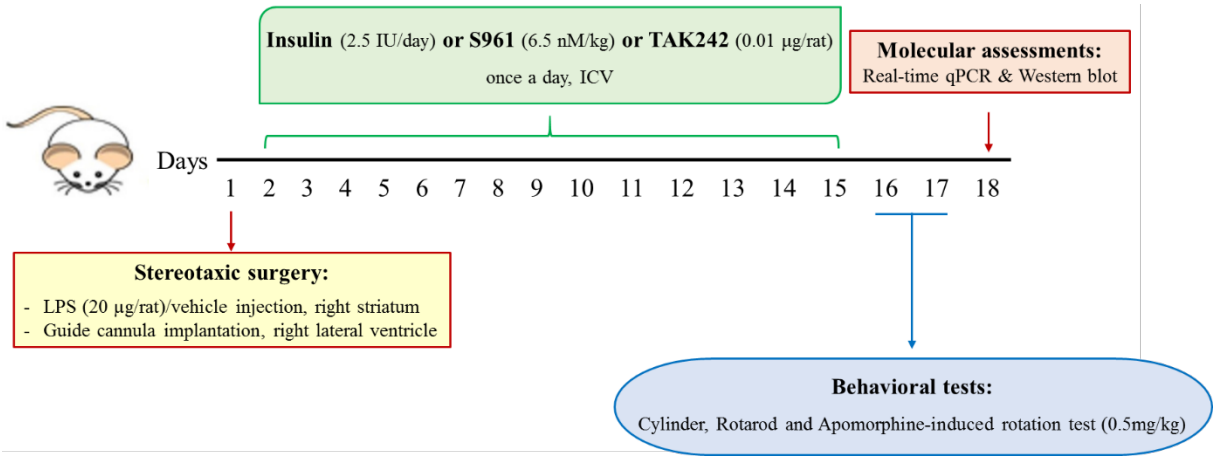

FIGURE 1. Experimental time-line.

finity biosynthetic insulin receptor antagonist, were prepared from Novo-Nordisk, Denmark. These two drugs were dissolved in NS and administered ICV (insulin: 2.5 IU/day (Balakumar et al., 2013), S961: 6.5nM/kg (Knudsen et al., 2012; Vikram and Jena 2010)). TAK-242 (*resatorvid*) (InvivoGen, USA), a small-molecule inhibitor of TLR4, was dissolved in dimethyl sulphoxide (DMSO) (0.01µ/rat, ICV) (Suzuki et al., 2012).

Stereotaxic surgery

The rats were anesthetized with ketamine/xylazine (80/20mg/kg, intraperitoneally) and placed on a stereotaxic instrument (Stoelting, USA). LPS was injected (20µg/2µl/rat) into the right striatum (AP: +1; ML: +3; DV: -5). The rats in the sham group received the same volume of vehicle (2µl of sterile 0.9% NS). A guide cannula was implanted in the right lateral ventricle (AP: -0.75, ML: +1.7, DV: -4) for intracerebroventricular (ICV) administration of drugs.

Experimental groups

The rats were randomly divided to the following groups (n=10):

1) Sham: NS in right striatum
2) LPS: LPS in right striatum
3) LPS+NS: LPS in right striatum + NS (ICV)
4) LPS+DMSO: LPS in right striatum + DMSO (ICV)
5) LPS+Insulin: LPS in right striatum + insulin
6) LPS+S961: LPS in right striatum + S961
7) LPS+TAK242: LPS in right striatum + TAK242
8) LPS+TAK242+insulin: LPS in right striatum + TAK242/insulin

All treatments were administered for 14 consecutive days. Then, the rats were subjected to behavioral tests, and were sacrificed for striatum extraction for molecular assessments (n=3). The experiment's time-line is presented in Figure 1.

Behavioral tests

Rotarod test

Rotarod test is commonly used to assess motor coordination and balance (Martinez 2019; Moon et al., 2018). This test contains of two consecutive days of training, with constant speed (10 rpm) on the first day, and escalating speed (from 5 to 20 rpm in 5 minutes) on the second day. On the test day, each rat performed five trials with escalating speed (5-40 rpm) and a 5-minute cut off time. The average of five trials was recorded as the final score.

Apomorphine-induced rotation test

Apomorphine was dissolved in NS with 0.2 mg/ml ascorbic acid and subcutaneously injected (0.5 mg/kg). All rotations were recorded for 40 minutes. The net contralateral rotations were then calculated (contralateral - ipsilateral) (da Conceição et al., 2010).

Cylinder test

The cylinder test is a common method to evaluate motor-sensory function in animal models of PD (Moon et al., 2018). Due to the nigrostriatal lesion, the use of the contralateral forelimb is reduced (Martinez 2019). In this test, the rat was placed in a glass cylinder for 5 minutes, and the total number of the paw touches (right, left,

TABLE 1: Primer sequences use for qPCR.

Gene	Forward Primers (5′3′)	Revers Primers (5′3′)
α-synuclein	CCAACATATAGGCTGGAGTG	TAGCCATCCACAGACACACC
TLR4	GTGGGTCAAGGACCAGAAAA	GGCTACCACAAGCACACTGA
IRS1	AGGTTTTCCCCTCCTAGCAA	GCTGAGATCGAAACATGCAA
IRS2	GGCTCACCAGTTTTCTGCTC	GTAGAATTGCTCCCGTTGGA
GSK3β	TCGGCTCTCTCCTTCCATTA	CCCTCATCCCTGTACCTCAA
β-actin	TAGGGTCCATTGGTGGAAAC	TGCCGATAGTGATGACCTGA

or both) on the cylinder wall were counted. Finally, the percentage of using contralateral hand was calculated.

RNA isolation and qPCR Protocol

Total RNA was extracted from the striatum using the Total RNA Isolation System (Qiagen, USA), according to the manufacturer's instruction. RNA concentration was evaluated using a NanodropTM spectrophotometer (Nanodrop; Thermo Fisher Scientific, Wilmington, DE, USA). Then, total RNA (1μg) was used for cDNA synthesis by RevertAid™ First Strand cDNA Synthesis kit (Qiagen, USA). The expression of target genes was quantitatively measured using SYBR Green Real-Time PCR Master Mix reagents in the ABI system (USA). Relative expression of α-synuclein, TLR4, IRS1, IRS2 and GSK3β was calculated by the $2^{-\Delta\Delta CT}$ method, and β-actin was used as the house-keeping gene. Primers sequences used for qPCR are presented in Table 1.

Western blotting

A Potter-Elvehjem tissue grinder (Sigma, St Louis, Missouri, USA) with chilled tris-buffered saline with tween (TBST) (20mM Tris, pH=7.5; 0.75M NaCl; 2mM 2-mercaptoethanol) and 10 μl/ml protease inhibitor cocktail (Sigma) was used to homogenize the striatum in a centrifuge (23,000 g) at 4 ˚C for 45 minutes. Protein concentration was measured using a bicinchoninic acid protein assay kit (Sigma-Aldrich) with bovine serum albumin (BSA). In the next stage, 25 μg of total protein was loaded on sodium dodecyl sulphate polyacrylamide gels for electrophoresis and transferred to polyvinylidene difluoride membranes (MSI, Westborough, Massachusetts, USA). BSA blocking buffer was used to block non-specific binding sites for 60 minutes. The membranes were incubated with rabbit anti-α-synuclein (1:1000, ab52168, Abcam), anti-TLR4 (1:1000, ab22048, Abcam), anti-IRS1 (1:1000, ab52167, Abcam), anti-IRS2 (1:1000, ab134101, Abcam), anti-GSK3β (1:1000, ab2602, Abcam), and anti-β-actin (1:1000, ab20272, Abcam) antibodies at 4 ˚C overnight. After washing with TBST, the membranes were incubated with horseradish-peroxidase-conjugated rabbit anti-mouse secondary antibody (1:2000, ab6728, Abcam) at room temperature for 30 minutes. Enhanced chemiluminescent substrate (ChemiGlow; Alpha Innotech, San Leandro, California, USA) and chemiluminescent imaging system (FluorChem 5500; Alpha Innotech) were used to visualize the immunoreactive bands. Quantification of the band's density was performed using ImageJ software.

Statistical analysis

Data were analyzed using the 16th version of SPSS. One-way ANOVA with Tukey's post hoc tests was used to compare behavioral data, gene expression, and protein levels between groups. Statistically significant level was set at $P<0.05$. Data are reported as mean± standard error of the mean (SEM).

Results

Insulin significantly improved motor impairment induced by LPS

In all behavioral tests, insulin attenuated motor impairments induced by LPS (Figure 2). the rotarod test was used to evaluate balance and motor coordination. Statistical analysis indicated that the time spent on the rotarod was different between groups [$F_{(7, 72)} = 12.59$, $P<0.001$]. LPS reduced the latency time to fall from the rotarod compared to sham ($P<0.001$), and insulin significantly increased it ($P<0.01$ compared to LPS), and restored motor coordination to the control level ($P>0.05$ in comparison to sham). An improvement in motor co-

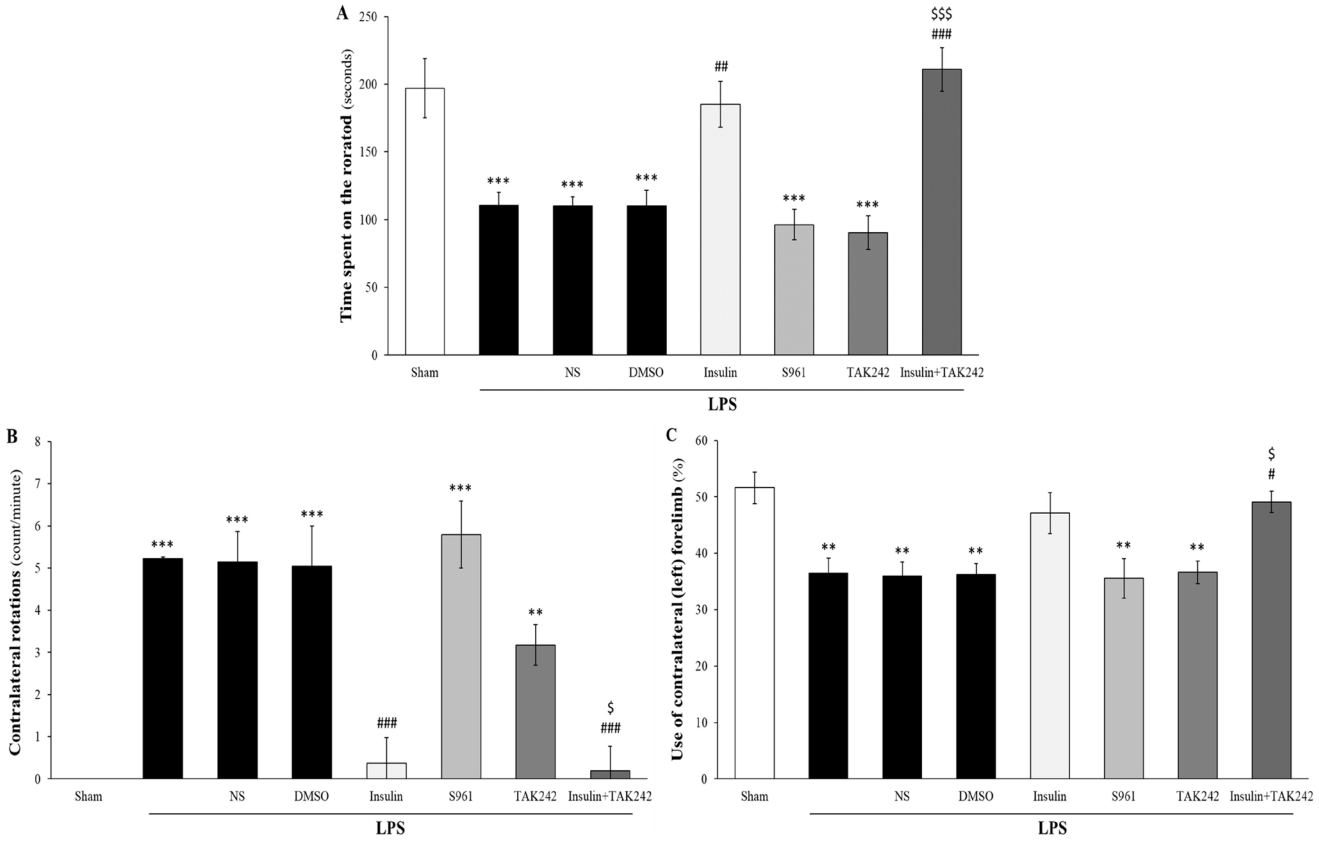

FIGURE 2. Insulin treatment improved motor impairments induced by LPS.
LPS administration in rats induced motor deficits in rotarod (A), apomorphine-induced rotation (B) and cylinder (C) tests. Insulin, alone and with TAK242, could improve these impairments. S961 and TAK242 had no effects on motor performance. Data are indicated as mean ± SEM (n=10).
P<0.01,*P<0.001 vs. Sham
#P<0.05, ##P<0.01, ###P<0.001 vs. LPS
$P<0.05, $$$P<0.001 vs. LPS+TAK242

ordination and balance was also observed in animals treated by insulin+TAK242 (P<0.001 compared to LPS and TAK242). However, S961 and TAK242 could not improve motor coordination (P<0.001 vs. sham and insulin, and P>0.05 vs. LPS) (Figure 2A).

As shown in Figure 2B, a significant increase in contralateral rotations induced by apomorphine was seen in LPS-treated animals compared to sham [$F_{(7, 72)} = 17.34$, P<0.001]. Insulin significantly reduced apomorphine-induced rotations, alone or in combination with TAK242, in comparison to LPS (P<0.001). However, S961 and TAK242 could not decrease contralateral rotations compared to LPS (P>0.05). In these groups, there was a significant increase in contralateral rotations compared to sham (P<0.001 and P<0.01, respectively) (Figure 2B).

LPS administration also decreased the use of contralateral forelimb in the cylinder test [$F_{(7, 72)} = 6.57$, P<0.001], and insulin partially improved forelimb asymmetry (P>0.05 vs. sham), which was not statistically significant compared to LPS. This improvement was also observed in the insulin+TAK242 group compared to LPS (P<0.05). However, there was no improvement in the S961 and TAK242 groups (Figure 2C). The findings of behavioral tests proposed that TLR4 inhibition simultaneous with insulin receptor activation is more effective in attenuating motor deficits than TLR4 suppression alone.

Insulin and TAK242 attenuated LPS-induced increase in α-synuclein and TLR4

α-synuclein and TLR4 levels in the striatum were evaluated by qPCR and western blot techniques (Figure 3). Statistical analysis indicated that LPS significantly increased α-synuclein mRNA [$F_{(5, 12)} = 688.711$, P<0.001]. Insulin, TAK242, and insulin+TAK242 decreased it compared to LPS (P<0.001). However, in

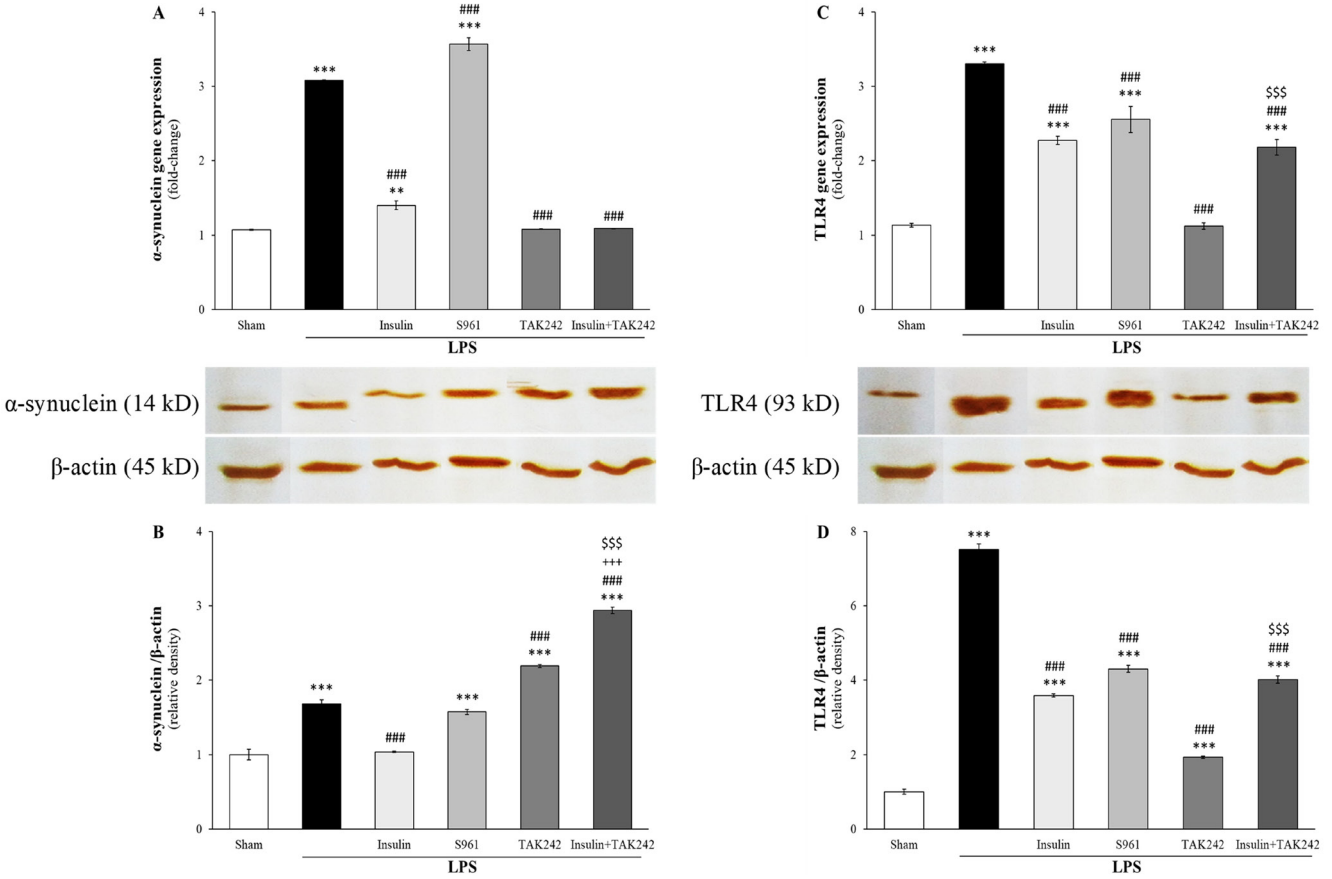

FIGURE 3. The effect of insulin, S961 and TAK242 on α-synuclein and TLR4.
The mRNA and protein levels of α-synuclein (A, B) and TLR4 (C, D) were significantly increased by LPS. Insulin and TAK242 could decreased both α-synuclein and TLR4. S961 decreased only TLR4 mRNA and protein levels. Data are shown as mean ± SEM (n=3).
P<0.01,*P<0.001 vs. Sham
###P<0.001 vs. LPS
+++P<0.001 vs. LPS+Insulin
$$$P<0.001 vs. LPS+TAK242

S961-treated animals, α-synuclein expression was significantly higher than sham and LPS (P<0.001) (Figure 3A). α-Synuclein protein expression was also elevated by LPS [$F_{(5, 12)}$ = 270.434, P<0.001] and significantly attenuated by insulin (P<0.001). Treatment with S961, TAK242, and insulin+TAK242 could not reduce α-synuclein protein expression (P<0.001 compared to both sham and LPS) (Figure 3B).

LPS injection significantly enhanced the mRNA [$F_{(5, 12)}$ = 91.113, P<0.001] and protein [$F_{(5, 12)}$ = 593.77, P<0.001] levels of TLR4. All treatments attenuated it at both mRNA and protein levels (P<0.001 in comparison to LPS). However, there was a significant increase in all treatments compared to sham (P<0.001) (Figure 3C-D).

IRS1 and IRS2 genes expressions were reduced by LPS and enhanced by insulin

Changes in the mRNA and protein levels of IRS1 and 2 are shown in Figure 4. ANOVA analysis indicated that IRS1 gene expression was decreased by LPS [$F_{(5, 12)}$ = 731.758, P<0.001], while insulin and insulin+TAK242 significantly increased it compared to both sham and LPS (P<0.001). S961 and TAK242 did not enhance IRS1 expression compared to LPS (P>0.05) (Figure 4A). IRS1 protein level was elevated in all experimental groups, with the exception of S961, compared to sham [$F_{(5, 12)}$ = 143.057, P<0.001]. Moreover, IRS1 protein was significantly increased in insulin, TAK242 and insulin+TAK242 groups compared to LPS (P<0.001) (Figure 4B).

IRS2 mRNA and protein levels were also statistically different between groups [$F_{(5, 12)}$ = 142.819, P<0.001 and $F_{(5, 12)}$ = 325.258, P<0.001, respectively] (Figure 4C, D). IRS2 gene expression was decreased by LPS (P<0.01), while insulin and insulin+TAK242 increased

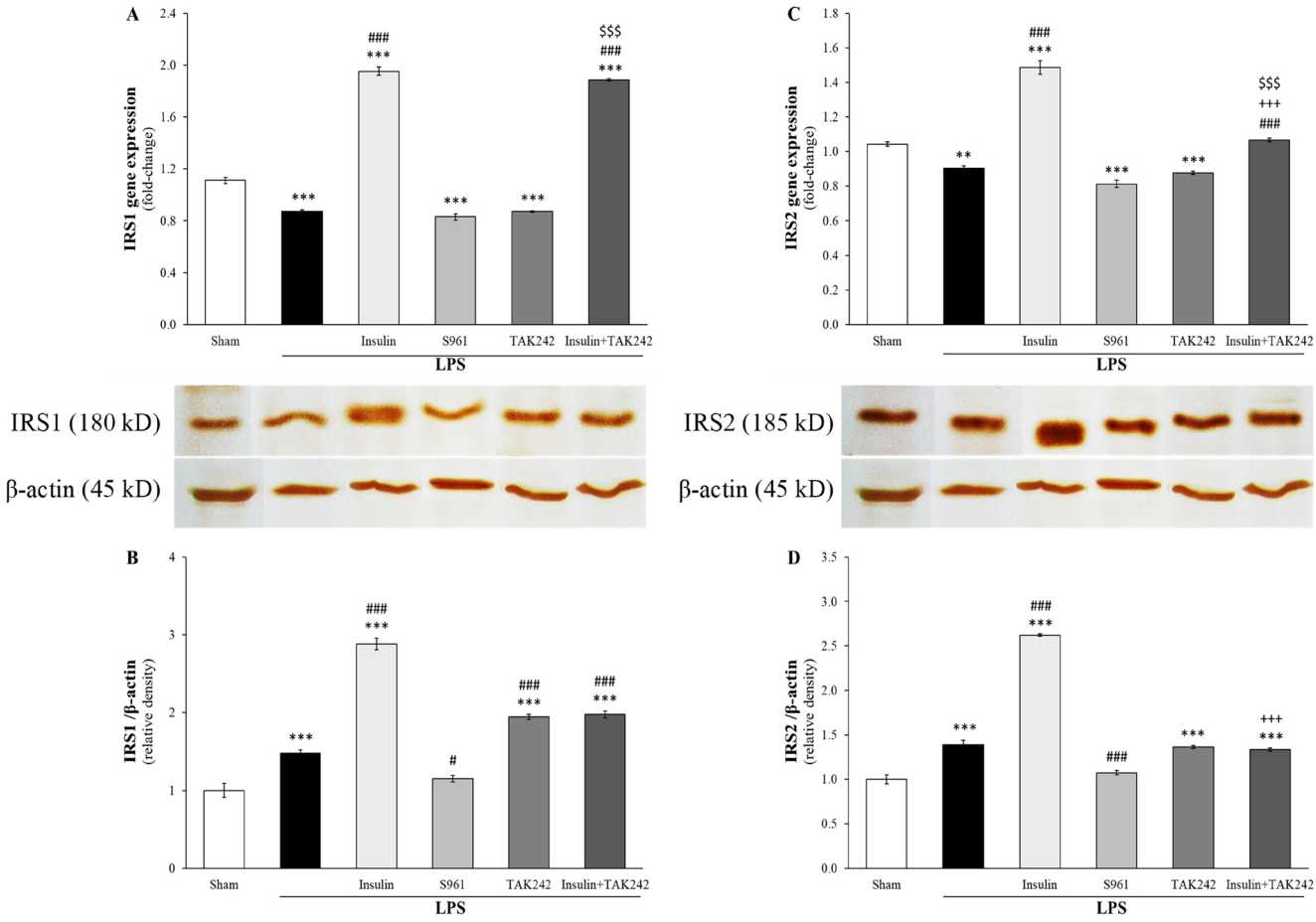

FIGURE 4. Changes in IRS1 and 2 following LPS injection and insulin treatment.
LPS administration reduced the mRNA and increased protein of IRS1 (A, B) and IRS2 (C, D). Insulin could increase IRS1 and 2 at both mRNA and protein levels. TAK242 increased the mRNA of IRS1 and 2, and the protein of IRS1. Data are shown as mean ± SEM (n=3).
P<0.01,*P<0.001 vs. Sham
#P<0.05, ###P<0.001 vs. LPS
+++P<0.001 vs. LPS+Insulin
$$$P<0.001 vs. LPS+TAK242

it in comparison to LPS (P<0.001). IRS2 gene expression was also higher in insulin treated animals compared to sham (P<0.001). S961 and TAK242 could not change it (P>0.05 compared to LPS) (Figure 4C). IRS2 protein, similar to IRS1, was increased in all groups compared to sham, with the exception of S961 (P<0.001) (Figure 4D).

Insulin and TAK242 attenuated GSK3β mRNA and protein levels following LPS

Statistical analysis indicated a significant difference between groups in mRNA [$F_{(5, 12)} = 215.127$, P<0.001] and protein [$F_{(5, 12)} = 387.765$, P<0.001] levels of GSK3β (Figure 5). GSK3β gene expression was elevated by LPS (P<0.001). Insulin, TAK242, and insulin+TAK242 significantly decreased it compared to LPS (P<0.001). GSK3β gene expression reached control levels in insulin and insulin+TAK242 treated animals (P>0.05), however, it was still higher in TAK242 group compared to sham (P<0.001). S961 not only could not reduce the GSK3β mRNA but also significantly increased it compared to both sham and LPS (P<0.001) (Figure 5A). GSK3β protein expression was increased by LPS (P<0.001) and attenuated by insulin, S961, and TAK242 (P<0.001). In animals receiving insulin+TAK242, GSK3β protein expression was significantly higher than sham and LPS (P<0.001) (Figure 5B).

Discussion

The findings of the present study showed that LPS impaired motor performance through an increase in α-synuclein and TLR4, and insulin signaling dysfunction in the striatum, all of which improved by insulin, alone and with TAK24. Administration of S961 had no effect on

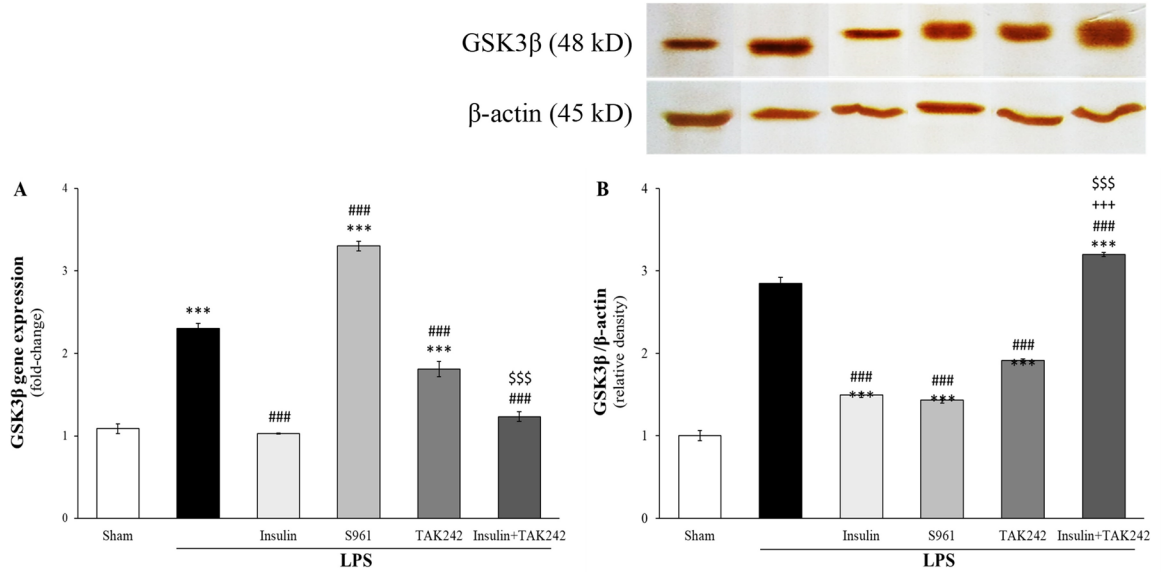

FIGURE 5. The effect of insulin, S961 and TAK242 on GSK3β following LPS injection.
LPS increased the mRNA (A) and protein (B) of GSK3β. Insulin and TAK242 could significantly attenuate both of them. S961 could decrease only the protein of GSK3β. Data are expressed as mean ± SEM (n=3).
***p<0.001 vs. Sham
###p<0.001 vs. LPS
+++p<0.001 vs. LPS+Insulin
$$$p<0.001 vs. LPS+TAK242

these behavioral and molecular deficits, confirming the important role of insulin signaling pathways in LPS-induced PD pathology.

LPS is widely used to study the neuroinflammation model of PD, both in vitro and in vivo. In animals, LPS can be injected into the striatum, SN or globus pallidus, or be systemically administered by an intraperitoneal injection (Liu and Bing 2011). It has been demonstrated that systemic inflammation induced by LPS is correlated with neuroinflammation, neurodegeneration, and the development of PD (Oliynyk et al., 2021). LPS is a potent agonist for TLR4 on microglial cells, and therefore produces some pathological features of PD, like dopaminergic neurons loss in SNpc, by extensive microglial activation and releasing pro-inflammatory mediators (Liu and Bing 2011). Astrocytes also highly express TLR4 and are activated after exposure to LPS (La Vitola et al., 2021). In vitro studies have shown that LPS treatment increases the expression of TLR4 at both mRNA and protein levels in primary murine and rat astrocytes cells (Bowman et al., 2003; Li et al., 2016), and lithium reduces astrocyte activation through inhibition of TLR4 expression (Li et al., 2016). An in vitro study has indicated that LPS treatment increases TLR4 gene expression, and inflammatory factors like IL-1β, IL-6 and TNF as well. Insulin-like growth factor-I (IGF-I) treatment has been shown to reduce LPS-induced TLR4 overexpression (Bellini et al., 2011). Exogenous IGF-I and its gene delivery to primary astrocytes from the mouse cerebral cortex could decrease TLR4 expression and eventually counteract LPS-induced neuroinflammation (Bellini et al., 2011). Consistent with these studies, we also indicated overexpression of TLR4 in the striatum of rats receiving LPS, confirming the induction of neuroinflammation, which was attenuated by insulin.

Insulin through IRS1/PI3K/Akt pathway regulates microglial activation and pro-inflammatory cytokines production (Yang et al., 2017). Microglial cells, in response to inflammatory insults, act as a neuroprotective mechanism and prevent neuronal damage. However, in pathological conditions, excessive and long-term microglial activation leads to releasing the proinflammatory cytokines, called chronic neuroinflammation (Kim and Joh 2006), which interfere with insulin signaling elements, such as IRSs (Copps and White 2012; Kim and Feldman 2012). Proinflammatory cytokines decrease IRS interactions with insulin receptors through serine phosphorylation IRSs, reduce insulin sensitivity, and cause insulin resistance (Copps and White 2012; Kim and Feldman 2012).

IRS1 and 2 are highly expressed in different regions of the brain and regulate GSK3β activity, which is involved in regulating many cellular processes such as protein synthesis, cell survival, and metabolism (Speed et al., 2011). GSK3β overactivation initiates several intracellular signaling cascades which promote apoptotic cell death (Ghasemi et al., 2013). GSK3β plays an important role in the pathogenesis of neurodegenerative diseases, and inhibition of its activity can be considered as a therapeutic strategy in reducing the pathology and severity of PD (Duka et al., 2009; Li et al., 2014). Insulin exerts some of its neuroprotective effects through inactivating GSK3β by phosphorylation on Ser9, which is mediated by IRS/PI3K/Akt signaling pathway. Therefore, in pathological conditions correlated with chronic neuroinflammation, insulin resistance occurs, which is characterized by a decrease in insulin receptor and IRSs, concomitant with increased activity of GSK3β.

Several studies have indicated a link between insulin resistance and brain dysfunction (Ma et al., 2015; Masciejczyk et al., 2019). Dopaminergic neurons, in particular, have a high energy demand, which may partly explain their increased sensitivity to hyperglycemia (Lv et al., 2021). Insulin receptors are expressed in the substantia nigra, and the development of insulin resistance reduces the insulin-dependent release of dopamine (Akhtar and Sah 2020). It has been shown that chronic insulin resistance due to a high-fat diet disrupts nigrostriatal function by reducing the release and clearance of dopamine (Vijiaratnam et al., 2021). There is evidence that insulin signaling pathways are disrupted in PD. Insulin receptors are reduced in the striatum of PD patients, accompanied by decrease of the release and clearance of dopamine (Morris et al., 2011). It has been also indicated that insulin resistance due to a high-fat diet in mice leads to more severe motor deficits induced by 6-OHDA than control mice, suggesting that insulin resistance increases the risk of PD pathology (Sharma and Taliyan 2018). Clinical studies have shown that uncontrolled diabetes acts as a risk factor for developing PD (Ou et al., 2021). Insulin resistance can also induce iron deposition in dopaminergic neurons, which lead to the production of highly reactive radicals and neuronal dysfunction (Pignalosa et al., 2021). Consistent with this evidence, our findings revealed that LPS impaired the motor function of the animals, at least in part, due to the induction of insulin resistance. Moreover, there was also overexpression of TLR4 following LPS injection, proposing the interaction of TLR4 and insulin signaling pathway and the role of TLR4 in LPS-induced insulin resistance.

α-Synuclein aggregation and accumulation in the brain, especially in dopaminergic neurons, is the main pathological hallmark of PD. This presynaptic protein is involved in many physiological processes including synaptic transmission, neurotransmitter release, and mitochondrial function (Bendor et al., 2013). However, when aggregating in the oligomeric form, it becomes toxic and causes mitochondrial dysfunction via interaction with the respiratory chain complexes (Chinta et al., 2010). Besides, α-synuclein is also involved in neuroinflammatory processes through activation of TLR4 on microglia and astrocytes (Codolo et al., 2013; Fellner et al., 2013; Gorecki et al., 2021; Hughes et al., 2019; Rannikko et al., 2015). Previous studies have shown that LPS induces overexpression of α-synuclein via overactivation of microglial cells (Niu et al., 2020). It has been shown that chronic microglial overactivation due to overexpression of α-synuclein leads to motor impairments in mice (Drouin-Ouellet et al., 2015). Here, we observed overexpression of α-synuclein in the striatum of animals that received LPS concomitant with overexpression of TLR4. Administration of insulin and TLR4 blocker (TAK242) could decrease them. Insulin has been indicated to activate autophagy through PI3K/Akt/mTOR pathway, and promote degradation of accumulated toxic proteins like α-synuclein (Heras-Sandoval et al., 2014). In the present study, α-synuclein protein was attenuated following insulin treatment, while S961 could not reduce α-synuclein. This proposes that α-synuclein clearance might be mediated by mTOR inhibition via IRS/ PI3K/Akt pathway. In parallel with these findings, during an in vitro study insulin could reduce α-synuclein in PC12 cells due to treatment with MPP^+ (Ramalingam and Kim 2017). Furthermore, other studies have previously reported that rapamycin, an inhibitor of mTORC1, decreased α-synuclein aggregation and prevented dopaminergic neuron loss (Sarkar et al., 2007; Tain et al., 2009).

Conclusion

Generally, the present study indicated that LPS impaired motor behaviors in animal models through neuroinflammation, overexpression of α-synuclein, and

insulin resistance. Insulin administration and TLR4 suppression by TAK242 could overcome LPS-induced deficits at both behavioral and molecular levels, proposing the modulation of TLR4 and insulin receptor as a promising therapeutic approach to improve PD-related problems.

Acknowledgements

There is no grant for this project.

Conflict of interest

The authors have no competing interests to declare.

Ethics approval

This study was approved by the Animal Research Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1400.676).

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