Background: Mitochondrial dysfunction is one of the major hallmarks of Parkinson’s disease (PD). Recently, angiotensin II type 1 and type 2 receptors (AT1R, AT2R) were reported to be present on the mitochondrial membrane. Both are crucial players in the brain renin-angiotensin system (RAS). Current evidence indicates that blockade of brain AT1R protects dopaminergic neurons in PD. Methods: Thus, the current study was aimed to explore the effects of Telmisartan (Tel), a selective AT1R blocker, on mitochondrial function and neuronal motor functions during the beam walk in MPTP-treated mice. Tel attenuated the increased levels of AT1R, eNOS, and iNOS in MPTP-treated mice. Results: Pretreatment with Tel significantly protected motor functions during the beam walk in MPTP-treated mice. Tel attenuated the increased levels of AT1R, α-syn, and inflammatory markers such as inducible nitric oxide synthase (iNOS) and ionized calcium-binding adapter molecule 1 (IBA1) in MPTP-treated mice. Moreover, Tel preserved the expression of AT2R, tyrosine hydroxylase (TH), p-Akt/Akt, and p-GSK3 α/β pathway. Conclusion: These results indicate that Tel protects mitochondrial function and gait in a mouse model of PD by modulating the Akt/GSK3β/PGC1α pathway.

Keywords: Parkinson’s disease; renin-angiotensin system; mitochondria; telmisartan; MPTP; Adenosine triphosphate (ATP)

1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease and is characterized by aggregation of alpha-synuclein (α-Syn) in the brain, principally in the substantia nigra pars compacta (SNPc) and striatal regions [1,2]. α-Syn is the major component of Lewy bodies (LBs), which are one of the characteristic pathological features of PD. Levodopa, a dopamine precursor, is currently the drug of choice in the treatment of PD. However, its long-term use is associated with decreased efficacy and side-effects such as dyskinesia and motor dysfunction [3,4]. Furthermore, the current therapeutic regimens including carbidiopride/levodopa, dopamine agonists, catechol O-methyltransferase (COMT) inhibitors, and anticholinergics provide only limited improvement in patient quality of life and survival due to their reduced efficacy after long-term usage. Therefore, the identification of other potential disease target(s) and the development of new therapeutic strategies are urgently needed for PD.

The major causes of PD pathogenesis are mitochondrial dysfunction, neuroinflammation, and α-Syn aggregation [5–7]. Recently, González-Rodríguez et al. [8] reported that loss or disruption of mitochondrial complex I in the dopaminergic neurons of mice was sufficient to cause progressive, human-like Parkinsonism. Earlier reports suggested that cytosolic and mitochondrial deposition of aggregated α-Syn promoted the fragmentation of mitochondria, which was then followed by mitochondrial dysfunction [5,9,10]. Hence, the overexpression of α-Syn alters mitochondrial fusion/fission dynamics, thereby contributing to mitochondrial dysfunction and the pathogenesis of PD [11–13].
The brain renin-angiotensin system (RAS) plays a crucial role in the pathogenesis of Parkinsonism. Angiotensin II (All), the most important effector peptide in RAS, exerts its effect via the All type 1 receptor (AT1R) and the All type 2 receptor (AT2R) [14,15]. In physiological conditions, activation of AT2R counteracts the function of AT1R [16]. In PD, the upregulation of AT1R expression contributes to neuroinflammation and apoptosis, leading to dopaminergic cell death. In our earlier study using a mouse model of PD, we reported that blockade of AT1R with a selective AT1R antagonist, Telmisartan (Tel), resulted in neuroprotection [15]. An interesting report by Valenzuela et al. [17] (2016) revealed the presence of AT1R and AT2R on the mitochondrial membrane of dopaminergic neurons, thus demonstrating a link between RAS and mitochondrial function. There is now accumulating evidence that PD is associated with alterations in mitochondrial dynamics and in the biogenesis of proteins such as mitofusin protein 1 (MFN1) and Peroxisome proliferator-activated receptor-gamma coactivator-α (PGC1α) [18]. Recently, we also demonstrated that AT1R blockade by Tel protects the expression of mitochondrial-specific genes (PINK1, Parkin, and PARK7) in the brain tissue of a mouse PD model, as well as locomotor and gait function [6]. However, the underlying mechanisms behind restoration of mitochondrial proteins/functions in PD models are still poorly understood and require further research. The aim of the current study was therefore to investigate the signaling mechanisms by which Tel protects mitochondrial function, gait activity, and neuronal cells from death in a mouse PD model.

2. Materials and Methodology

2.1 Chemicals and Reagents

Telmisartan (Product No. T2861) and Probenecid (Product No. P1975) were purchased from Tokyo Chemical Industry (TCI) Private Limited (Tamil Nadu, India). MPTP hydrochloride (Cat. No.: HY-15608) was obtained from MedChemExpress (Middlesex County, NJ, USA). The specific primary antibodies used in this study were: anti-β-actin [1:1000 dilution, Cat# 13E5, Cell Signaling Technology (CST), Danvers, MA, USA], anti-GSK3β (1:1000, Cat# ab131356, Abcam, Cambridge, United Kingdom), anti-Phospho-(Ser9)-GSK3β (1:1000, Cat #sc-373800, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-iNOS (1:1000, Cat# D6B6S, CST), anti-Akt1 (1:1000, Cat #BT-AP00347, Bioassay Technology Laboratory (BT-Lab) Birmingham, United Kingdom), anti-Phospho-(S473)-Akt (1:1000, Cat #BT-PHS00006, BT-Lab), anti-α-Synuclein (1:1000, Cat #sc-12767, Santa Cruz), anti-Mfn1 (1:1000, Cat #sc-166644, Santa Cruz), anti-AT1R (1:1000, Cat #sc-515884, Santa Cruz), anti-AT2R (1:1000, Cat #sc-518054, Santa Cruz), anti-Bax (1:1000, Cat #2772, CST) and anti-PGC1α (1:1000, Cat #NPB3-08971, Novus Biological India Private Limited, Pune, India). The secondary antibodies used were anti-rabbit (1:2000, Cat #7074, CST) and antimouse (1:2000, Cat# sc-516102, Santa Cruz). The Adenosine triphosphate Enzyme-linked Immunosorbent Assay (ATP ELISA) kit was purchased from Thermo Fischer (Catalog number: A22066, Waltham, MA, USA). All other chemicals and reagents used in this study were analytical grade.

2.2 Animal Husbandry and Ethics Approval

Young male C57BL/6J mice (18–22 g body weight) were procured from Adita Bioscience (Tumkur, Karnataka, India). The animals were housed in polypropylene cages in the good laboratory practice standard central animal facility at JSS Academy of Higher Education And Research (JSS AHER, Mysuru, India). Animals were acclimatized for 7 days in an experimental room at a controlled temperature of 22 ± 3 °C, 40–65% relative humidity, and 12-h light/12-h dark cycles. This study was approved by the Institutional Animal Ethics Committee (IAEC) (JSS AHER, Mysuru, India) (approval number: JSSAHER/CPT/IAEC/016/2020).

2.3 Experimental Design and Treatments

Following acclimatization, mice were trained for the beam walk test (Fig. 1). The animals were divided into 5 groups (n = 8 per group). Group A (vehicle) was administered with 0.5% carboxymethylcellulose (CMC) by oral gavage + saline i.p. Group B was given 0.5% CMC (vehicle) by oral gavage + MPTP [intraperitoneally at 250 mg/kg b.wt. (in 10 divided injections, each 25 mg/kg b.wt. at 3.5 days interval] + Probencid 250 mg/kg i.p.) MPTP; positive control, and Groups C and D were given Tel at 3 and 10 mg/kg, by oral gavage, respectively, up to 35 days + MPTP (Vehicle or Tel was administered 1 hr before the first injection of MPTP and thereafter daily once for 35 days). Group E (probencid control) received 250 mg/kg i.p. in the study Probencid was used here to reduce renal excretion of MPTP and its metabolites, because its usage is recommended for experimental protocols of chronic PD [20]. A separate group for Tel alone was not included, since our earlier study showed that 10 mg/kg Tel did not cause any toxicity [15]. Mice were evaluated for motor functions using a beam walk every 10th day. On the 35th day, mice were euthanized using excess CO2, and mouse brains were collected and quickly frozen. Brain tissue was kept at −80 °C until the preparation of extracts for biochemical and Western blot analyses. SNP regions (∼Bregma –3.16 mm, interaural 0.64 mm) were identified using the Paxinos and Franklin mouse brain atlas [21].
2.4 Gait Function Analysis

Beam Walk Experiment

Prior to MPTP administration, mice were pre-trained for the beam walk test by allowing them to traverse the narrow 100 cm length runway with a dark escape box at the other end [22]. An aversive stimulus was created by using a bright light (100 lux) placed above the beam to motivate the walk. Mice were allowed a maximum of 60 seconds for the travel. The observer for the experiment was blinded to the identity of the treatment groups.

2.5 Western Blotting

The SNPc region from either side of the mouse brain was isolated and used for Western immunoblot analyses. The isolated brain tissues were homogenized using Radioimmunoprecipitation Assay (RIPA) lysis buffer (Cat #786-490, G-Biosciences, St. Louis, MO, USA) and a protease and phosphatase inhibitor cocktail (MP Biomedicals, Santa Ana, CA, USA), incubated for 30 min on ice, and then centrifuged (15,000 g at 4 °C for 10 min) to obtain individual tissue lysates. Tissue lysate supernatants were collected and used for measuring the protein concentration with a BCA Protein Assay Kit (Cat #23225, ThermoFisher, Rockford, IL, USA). Aliquots of 40 µg protein were mixed with sodium dodecyl sulfate (SDS)-sample loading buffer containing bromophenol blue and β-mercaptoethanol [SDS (CAS RN: 151-21-3), B-mercaptoethanol (CAS RN: 60-24-2), Sample Loading Buffer (B6104), from Tokyo Chemical Industry (TCI) Private Limited (Tamil Nadu, India)]. Electrophoresis using 10% SDS-polyacrylamide gels was performed to separate the proteins, which were then electrically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin, (BSA, 3%; Bio-Rad Laboratories) was used to block nonspecific binding sites, and the membranes were rinsed three times with Tris-buffered saline for 5 min each. Each membrane was then incubated with a specific primary antibody at 4 °C for 8–10 h and then rinsed 3 times for 30 min each with TBST (1% Tween20 + Tris Buffered Saline). Next, each membrane was incubated with the appropriate secondary antibody (HRP-conjugated anti-mouse IgG or anti-rabbit IgG) at room temperature for 2 hours and then rinsed 3 times with TBST for 30 min each. Visualization and imaging of the target protein bands were conducted using Clarity Max Western ECL Substrate (Cat# 1705062, Bio-Rad) for 10 min. Band intensities were quantified by ImageJ software (Version 1.54, National Institutes of Health, Bethesda, MD, USA).

2.6 ELISA Assay

The Adenosine triphosphate (ATP) content of SNPc tissues was measured using an Enzyme-linked Immunosorbent Assay (ELISA) kit as recommended by the manufacturer (Catalogue number: A22066, Invitrogen™, Waltham, MA, USA) and a luminometer (TECAN/SPARK 10M, Männedorf, Switzerland).
2.7 Statistical Analysis

Experimental data (mean differences) were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, with a $p$-value $\leq 0.05$ considered to represent statistical significance. GraphPad Prism 6.1 software (San Diego, CA, USA) was used for statistical analysis. Final values were expressed as the mean $\pm$ standard error of the mean (SEM).

3. Results

3.1 Effect of Tel on Gait Function in PD mice

Chronic MPTP + probenecid administration caused motor dysfunction and decreased the body weight of mice in the MPTP control group. However, treatment with Tel protected the normal body weight of MPTP mice (Body weight data is given in Supplementary Materials).

3.2 Beam Walk

Mice in the MPTP group took significantly longer to reach the goal (dark escape box at the end of the runway) on all of the locomotor test days [11th day ($p < 0.01$); 22nd and 32nd days (each $p < 0.001$)] compared to vehicle-treated mice (Fig. 2). Tel treatment significantly decreased the time taken to reach the goal on the 22nd and 32nd days of the experiment ($p < 0.05$ and $p < 0.001$).

3.3 Effect of Tel on ATP Content in PD Mouse Brains

The ATP content in SNpc regions of the mouse brains was determined with an ELISA kit. ATP concentration was significantly lower in the MPTP group (group B) compared to the vehicle (group A) and Probenecid (group E) control groups. However, mice treated with Tel (3 and 10 mg/kg) showed a significantly higher ATP content compared to MPTP-treated mice (Fig. 3).

3.4 Effects of Tel on the Expression of Key Signaling Proteins in the Mouse Brain

Protein-Protein Interactions

Protein-protein interactions (PPI) were analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/). Ten nodes of predicted protein interactions were built based on genomic context analysis. The PPI enrichment value ($p$-value: $2.81 \times 10^{-9}$) indicated the proteins were biologically connected, as described previously [23] (Fig. 4). The expected number of edges was 4 large domains, which can expand to a final number of 21 edges, with an average node degree of 4.2 and an average local clustering coefficient of 0.715. A primary interaction was found between AT1R (AGTR1) and Akt1, which was in turn connected with GSK3B (GSK3β), iNOS, IBA1 (Ionized calcium binding adaptor molecule 1, activated glial cell marker), Bax (BCL2 Associated X Protein, an apoptosis marker), $\alpha$-Syn (SNCA), TH (tyrosine hydroxylase as a marker of PD),
SNPc region homogenates were diluted 10-fold and processed as per the kit instructions. Luminescence was read at ~560 nm using a luminometer. One-way ANOVA and Tukey’s multiple comparison tests were applied. *** denotes a p-value < 0.001 compared to the vehicle control. ### denotes a p-value < 0.001 compared to the MPTP group. SNPc, substantia nigra pars compacta; ATP, Adenosine triphosphate; ELISA, Enzyme-linked Immunosorbent Assay; ANOVA, Analysis of Variance.

**Fig. 3.** Quantification of ATP content using a commercial ELISA kit. To further validate the PPI data, the expression levels of specific proteins were analyzed by Western blot analysis. Chronic MPTP injections significantly upregulated the expression of AT1R (p < 0.05), α-Syn (p < 0.01), iNOS (trend for increase), IBA-1 (p < 0.05) and Bax (p < 0.001) in MPTP-treated mouse brains compared to the vehicle control group (Fig. 5). On the other hand, MPTP treatment downregulated the expression of AT2R (a trend for decrease), Tyrosine Hydroxylase (TH) (p < 0.05), Mitofusin 1 (MFN1) (p < 0.001), p-Akt/Akt (p < 0.01), p-GSK3β (Ser-9)/GSK3β (p < 0.001) and PPARG Coactivator 1 Alpha (PGC1α) (p < 0.01) in the MPTP group compared to the vehicle control group.

**Fig. 4.** Protein-protein interaction networks (STRING Database). To further validate the PPI data, the expression levels of specific proteins were analyzed by Western blot analysis. Chronic MPTP injections significantly upregulated the expression of AT1R (p < 0.05), α-Syn (p < 0.01), iNOS (trend for increase), IBA-1 (p < 0.05) and Bax (p < 0.001) in MPTP-treated mouse brains compared to the vehicle control group (Fig. 5). On the other hand, MPTP treatment downregulated the expression of AT2R (a trend for decrease), Tyrosine Hydroxylase (TH) (p < 0.05), Mitofusin 1 (MFN1) (p < 0.001), p-Akt/Akt (p < 0.01), p-GSK3β (Ser-9)/GSK3β (p < 0.001) and PPARG Coactivator 1 Alpha (PGC1α) (p < 0.01) in the MPTP group compared to the vehicle control group.

**Fig. 4.** Protein-protein interaction networks (STRING Database). The lines between the protein connections represent the types of interactions with other proteins with different color. These are based on experimentally proven or predicted results from other available databases. STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; AGTR1 (AT1R), Angiotensin II Receptor Type 1; AKT1, AKT Serine/Threonine Kinase 1; iNOS, inducible nitric oxide synthase; BAX, BCL2 Associated X Protein; GSK3B, Glycogen Synthase Kinase 3 Beta; IBA1, Ionized calcium binding adaptor molecule 1; TH, Tyrosine Hydroxylase; SNCA, Synuclein Alpha; PGC1A, PPARG Coactivator 1 Alpha; MFN1, Mitofusin 1.

PGC1α (PPARGC1A, a mitochondrial biogenesis marker) and mitochondrial fusion protein 1 (MFN1). Interactions of GSK3β with α-Syn, PGC1α and Bax were also observed (Fig. 4).

**4. Discussion**

The findings of this study suggest the mechanism by which Tel, a selective AT1R blocker, can protect mitochondrial function, gait function, and neuronal apoptosis in a mouse model of PD. Moreover, the current study validated the STRING database PPI associated with the renin-angiotensin system in PD (Fig. 4). MPTP treatment in the present study was observed to upregulate α-Syn expression,
consistent with earlier reports [24,25]. α-Syn inhibits TH by activating protein phosphatase-2A. In turn, the inactivation of TH results in dopamine depletion and apoptosis of dopaminergic cells [26]. Consistent with our previous results using an MPTP-treated mouse model, MPTP caused severe gait impairment and loss of body weight compared to the vehicle control group [6,27]. The present results also showed that Tel treatment inhibits α-Syn expression in the MPTP-exposed mouse model, as well as protecting mitochondrial and gait functions. Earlier studies from various authors including ourselves found a correlation between decreased α-Syn expression and improved motor and gait function, which was further linked to increased dopamine turnover [15,28,29].

The current work found that Tel prevented the increase in expression of AT1R, while at least partially preventing AT2R in MPTP-exposed mice. In a rat model of insulin resistance, inhibition of AT1R was shown to cause neuroprotection via the Akt-mediated pathway in dorsal root ganglion (DRG) neurons [30]. MPTP treatment decreased the active form of Akt (phosphorylated at Ser-473) and downregulated p-GSK3β (Ser-9, inactive form), which in turn triggered apoptosis [31]. In the present study, Tel significantly increased Ser-473 phosphorylation of Akt (activation), and upregulated inactive p-GSK3β (Ser-9), both of which correlated with decreased Bax expression [32]. Hence, we propose that Tel-mediated anti-apoptosis in PD mouse brains is likely to be caused by modulation of the Akt/GSK3β pathway.

Cytosolic GSK3β phosphorylates α-Syn, leading to its aggregation and neuronal accumulation in PD [33]. On the other hand, the increase in non-phosphorylated GSK3β (active form) stimulates autophagic clearance of aggregated α-Syn in neurons [34]. Hence, the decrease in α-Syn in Tel-treated mice could be mediated via the Akt-GSK3β pathway [35–37]. In addition, GSK3β promotes the inflammatory response by activating microglia [38], which was evidenced by the upregulation of inflammatory markers such as IBA1 and iNOS (non-significantly) in MPTP-treated mice [39,40]. Pretreatment with Tel inhibited IBA1 and iNOS expression, which confirms the functional link between AT1R and GSKβ in PD.

The Akt and GSK3β proteins located in the mitochondria are highly active compared to the cytosolic counterparts [41]. Upregulation of GSK3β (active form) resulted in aberrant mitochondrial function, which has been
Fig. 6. Tel protects mitochondrial function in the brain of a mouse model of PD. MPTP is converted to MPP+ in glial cells by MAO (monoamine oxidase). AT1R and AT2R are present on the mitochondrial membrane surface. AT1R performs the opposite function to AT2R, and its blockade is neuroprotective. The expression of AT1R in PD is elevated compared to AT2R. AT1R activation inhibits p-Akt, triggers the aggregation of α-Syn, and stimulates apoptosis by activating p-Akt/GSK3β located in the mitochondria. Additionally, AT1R activation inhibits mitochondrial biogenesis by suppressing the expression of PGC1α. However, blockade of AT1R by Tel reverses all of these negative effects by regulating the Akt/GSK3β/PGC1α pathway. MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+, 1-methyl-4-phenylpyridinium; PD, Parkinson’s disease; TEL, Telmisartan.

5. Conclusion

In conclusion, the present study showed that Tel upregulates p-GSK3β (Ser 9, inactive form) and PGC1α to preserve mitochondrial biogenesis and bioenergetics in MPTP-treated mice (Fig. 6). The current results are consistent with our earlier findings. Moreover, they provide additional evidence regarding the signaling mechanisms by which Tel protects neuronal cells through the preservation of mitochondrial and motor functions. Therefore, the present results suggest that Tel could be a potential candidate for improving the management of PD, at least as an adjunct therapeutic agent.

Abbreviations

α-Syn, alpha-synuclein; CMC, Carboxymethylcellulose; COMT, Catechol O-methyltransferase; IAEC, Institutional Animal Ethics Committee; MFN1, mitofusin protein 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson’s disease; RAS, renin-angiotensin system; SNPC, Substantia nigra pars compacta; Tel, Telmisartan; TH, tyrosine hydroxylase.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

BR: conceptualized and designed the study, Performed, Data acquisition, analysis, and Manuscript writ-
ing; ST, PGN and AS: Data acquisition, analysis, and Manuscript Editing; AMM, PP and BJS: Data analysis and manuscript editing; SBC: conceptualized and designed the study, Manuscript Editing, and supervision. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The current study was approved by the Institutional Animal Ethics Committee (IAEC) (JSS AHER, Mysuru, India) (Approval number: JSSAHER/CPT/IAEC/016/2020).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2302029.

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