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Formoterol attenuated mitochondrial dysfunction in rotenone-induced Parkinson's disease in a rat model: Role of PINK-1/PARKIN and PI3K/Akt/CREB/BDNF/TrKB axis

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ABSTRACT

β2-adrenoreceptors (β2AR have been identified recently as regulators of the α-synuclein gene (SNCA), one of the key milieus endorsed in injury of dopamine neurons in Parkinson's disease (PD). Accumulation of α-synuclein leads to mitochondrial dysfunction *via* downregulation of mitophagy proteins (PINK-1 and PARKIN) and inhibition of mitochondria biogenesis (PGC-1α) along with an increase in the master inflammatory regulator NF-κB p65 production that provokes neurodegeneration and diminishes neuroprotective signaling pathway (PI3k/Akt/CREB/BDNF). Recently, formoterol exhibited a promising neuroprotective effect against neurodegenerative conditions associated with brain inflammation. Therefore, the present investigation aims to unveil the possible neuroprotective activity of formoterol, β2AR agonist, against rotenone-induced PD in rats. Rats received rotenone (1.5 mg/kg; s.c.) every other day for 3 weeks and cured with formoterol (25 μg/kg/day; i.p.) 1 hr. after rotenone administration, starting from day 11. Formoterol treatment succeeded in upregulating β2-adrenoreceptor expression in PD rats and preserving the function and integrity of dopaminergic neurons as witnessed by enhancement of muscular performance in tests, open field, grip strength-meter, and Rotarod, besides the increment in substantia nigra and striatal tyrosine hydroxylase immunoexpression. In parallel, formoterol boosted mitophagy by activation of PINK1 and PARKIN and preserved mitochondrial membrane potential. Additionally, formoterol stimulated the neuro-survival signaling axis *via* stimulation of PI3k/pS473-Akt/pS133-CREB/BDNF cascade to attenuate neuronal loss. Noteworthy formoterol reduces neuro-inflammatory status by decreasing NFκBp65 immunoexpression and TNF-α content. Finally, formoterol's potential as a stimulant therapy of mitophagy *via* the PINK1/PARKIN axis and regulation of mitochondrial biogenesis by increasing PGC-1α to maintain mitochondrial homeostasis along with stimulation of PI3k/Akt/CREB/BDNF axis.

1. Introduction

Parkinson's disease (PD) is described as a degenerative neurological disorder that progresses over time, leading to the loss of dopaminergic neurons in a specific brain region called the substantia nigra pars compacta (SNpc), resulting in a decline of dopamine (DA) levels [1,2]. A Plethora of events is incriminated in the pathophysiology of PD, such as mitochondrial impairment, neuroinflammation, and microglial activation leading to α-synuclein (α-syn) accumulation, the pathological features of PD [3]. Noteworthy, α-syn is a small, highly abundant, and

Abbreviations: Akt, Protein kinase B; BDNF, Brain-Derived Neurotrophic Factor; cAMP, Cyclic adenosine monophosphate; CREB, cAMP response element-binding; DA, dopamine; H&E, Hematoxylin and eosin; MAO-B, Monoamine oxidase B; SIRT1, NAD-dependent protein deacetylase sirtuin 1; NF-κB, The nuclear factor kappa B; OFT, Open Field Test; PD, Parkinson's disease; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Pi3k, Phosphoinositide 3-kinases; PINK-1, PTEN-induced kinase 1; PKA, protein kinase A; PP2A, Protein phosphatase 2A; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TrKB, Tropomyosin receptor kinase B; α-syn, α-synuclein; β2AR, β2 adrenoceptor.

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highly conserved presynaptic protein that orchestrates DA homeostasis and preserves normal neuronal function [1]. Indeed, overexpression of α-syn is associated with many neurodegenerative diseases [4], as it inhibits cAMP response element-binding (CREB) translocation activation and reduces brain-derived neurotrophic factor (BDNF) secretion [5,6]. Moreover, the deposition of α-syn results in mitochondrial fragmentation, reduction in mitochondrial membrane potential, and mitophagy impairment [7]. Mitophagy is one of the mechanisms that selectively eliminates defective mitochondria to maintain mitochondrial homeostasis and neuronal health [8]. Accumulating evidence showed that PINK1/PARKIN axis is the major signaling pathway in mitophagy, and impairment of the latter axis results in the mounting of dysfunctional mitochondria, leading to neuronal loss and neurodegeneration [9]. Remarkably, mitochondrial impairments such as inhibition of complex I activity have been perceived in post-mortem brains of sporadic PD patients, signifying the essential role of mitochondria in PD pathophysiology [10].

Formoterol is a long-acting selective β2 adrenoreceptor β2AR agonist for bronchodilation in chronic obstructive pulmonary disease and asthma [11]. Interestingly, Rasheed et al. reported that formoterol has blood–brain barrier crossing liability and can mitigate memory impairment and cognitive decline observed in sporadic Alzheimer's disease (AD) in mice *via* targeting central β2ARs [12]. Furthermore, the administration of long-acting β2AR-agonists exhibited a neuroprotective effect *via* triggering neurotrophic growth factor production and astrocyte activation in numerous *in-vivo* models of neuronal loss [13]. Also, β2ARs are expressed in microglial cells, and their activation protects against microglial-induced neuroinflammation [14]. Consequently, the current work aimed to assess the potential therapeutic use of formoterol, long-acting β2AR-agonists, against rotenone-induced neurotoxicity with emphasis on neuroprotective cascade PI3K/Akt/CREB/BDNF and PINK1/PARKIN axis.

2. Material and methods

2.1. Ethics statement

The experimental procedures were reviewed and approved by "the Research Ethics Committee, faculty of pharmacy, Cairo University with a Permit Number" (2903). The Ethics standards complied with "the Guide for the Care and Use of Laboratory (NIH Publication No. 85-23, revised 1996)". All attempts were employed to lessen animal suffering.

2.2. Animals

Forty-eight male Wistar rats, aged as adults and weighing around 180 ± 20 g, were acquired from "the National Research Centre (NRC, Egypt)". They were housed at the animal facility located in the Faculty of Pharmacy, Cairo University, where the conditions were maintained at ambient conditions, with a 12-hour light/dark cycle. Before commencing the experimental procedures, the rats were allowed a period of 7 days for acclimatization. Throughout the study, the rats had unrestricted access to chow-pellets and drinking water, and all neuropsychological assessments were conducted in a laboratory with soundproofing.

2.3. Experimental design

Rats were chosen in random order and assigned into four groups of 12 rats each, and the study lasted for a total of 21 days. Group I received dimethyl sulfoxide 10 % (0.2 ml/kg; s.c.) on alternate days, besides normal saline (1 ml/kg; i.p.) beginning from day 11 to be designed as the control group. Group II received formoterol (25 μg/k/day; i.p.; Novartis, Cairo, Egypt) dissolved in 0.9 % saline starting from day 11, intended to be a drug-control group. Groups III and IV received rotenone (1.5 mg/kg; s.c.; Sigma-Aldrich, MO, USA) dissolved in dimethyl sulfoxide 10 % on alternate days [15,16]. In parallel, group IV was treated with formoterol (25 μg/k/day; i.p.) 1 hr. after rotenone injection starting from day 11 [12,17].

After 24 hrs. subsequently to last rotenone and formoterol administration, the animal's motor capabilities were evaluated using the open field, Rotarod, and grip strength tests. Then, rats were euthanized by cervical decapitation under ketamine/xylazine anesthesia [18] and classified into 3 subsets; 1st subset (n = 6) was used for ELISA assessment, 2nd subset (n = 3) was utilized for Western blot evaluation, and the final selection (n = 3) was chosen for histopathology, and immunehistochemical analysis of tyrosine hydroxylase (TH) in SNpc and striatum. The following diagram summarizes the timeline for treatment and behavioral test.

2.4. Motor performance assessment

2.4.1. Open field test

The open field apparatus consists of a woody box (80 × 80 × 40 cm) with bright red sidewalls and black, flat flooring separated into four by-four squares with white lines. Each animal was carefully placed in the box's middle and given five minutes to explore the arena. Between each animal tested, the floor and walls were scrubbed to eliminate any possible odor impact [19]. ANY-maze software "version 7.1, Stoelting Co, Illinois, USA" was utilized to analyze the total distance covered, mean speed, immobility time, and frequency of rearing for each rat.

2.4.2. Rotarod

Animals were evaluated for their motion balance and coordination using a rotarod device (Diameter 3 cm diameter, Height 90 cm height, and Speed 20 rpm). Prior to experimentation, each rat underwent six training trials on 3 days, and rats that were maintained on the rotating rod for five minutes were nominated to run the study. After finalizing the open field assessments, the test was repeated, and the time for rats to fall was recorded as fall-off latency [20].

2.4.3. Test for grip strength

Rats were suspended by their forepaws on a steel wire (Diameter 2 mm, Length 35 cm, and Height 50 cm). Each rat's ability to hold the wire for the specified time was evaluated. The test was repeated 3 times, and the average was considered [21].

2.5. Striatal processing

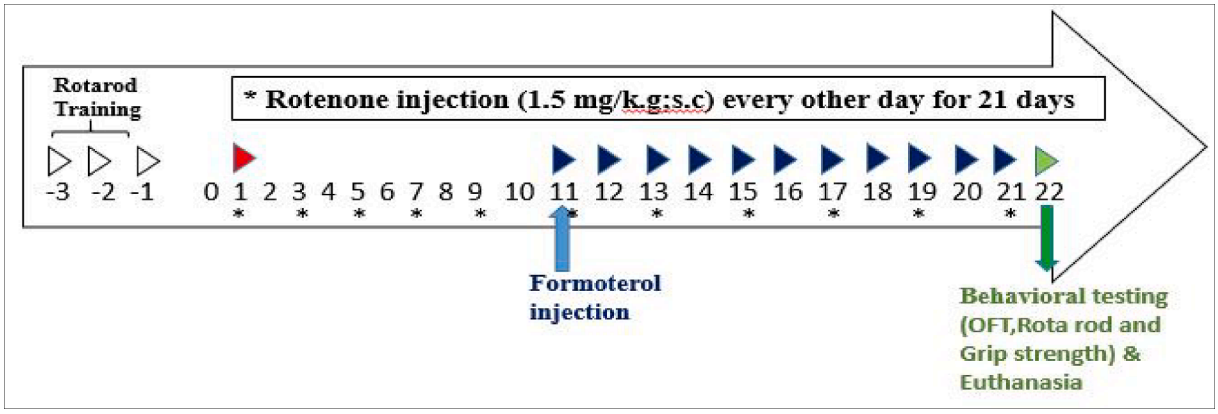
After the behavioral tests were completed, rats were sacrificed, the brains were removed instantly, and striata were isolated immediately from every brain, flash-frozen in the liquid nitrogen, and kept at −80 °C.

2.5.1. Quantification of striatal p-Akt, p-CREB, as well as striatal PI3K, DA, PGC1α, TNF-α and BDNF

Striata were homogenized in phosphate buffer saline (PBS, pH 7.4), and rat MyBioSource ELISA kits (CA, USA) were utilized for the determination of striatal p-Akt (Cat#; MBS1600201) and p-CREB (Cat#; MBS7255484), PI3K (Cat#; MBS260381), DA (Cat#; MBS7214676), Peroxisome proliferator-activated receptor gamma coactivator 1 alpha PGC1α (Cat#; MBS1600213), tumor necrosis factor-alpha TNF-α (Cat#; MBS2507393), and BDNF (Cat#; MBS824814). The experimental procedures were done in accordance with the manufacturer's instructions, and the results were referred to protein content using Bradford assay [22].

2.5.2. Western blot analysis of striatal α-synuclein, parkin, PINK 1 and β2-adrenoceptors

Striata were homogenized in a protease and phosphatase inhibitor-containing RIPA "Radio immunoprecipitation assay" buffer. After protein measurement using Bradford protein assay (BCA) kit (SK3041; BIO BASIC INC., Ontario, Canada), 20 μg protein of each sample was



Timeline for experimental design and behavioral testing

separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene (PVDF) membranes. Afterward, the membranes were blocked with 5 % bovine serum albumin (BSA) for 1 h at room temperature and incubated with primary antibodies (ThermoFisher Scientific, MA, USA) against alpha-synuclein (1:3000; cat#: PA5-85791), β2 adrenoreceptor (1:1000; cat#: MA5-32570), parkin (0.5 μg/mL; cat#: PA1-751), and PINK1 (1:50; cat#: PA5-23072), and β-actin (1:1000; Cat#PA5-85271) overnight, on a roller shaker, at 4 °C. Later, the membranes were probed with horseradish peroxidase-conjugated goat antirabbit immunoglobulin (Novus Biologicals, Colorado, USA) for 1 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence (ECL) system (Clarity™ Western ECL substrate- BIO-RAD, CA, USA), and the protein was quantified by densitometric analysis using a scanning laser densitometer (GS-800 system, Bio-Rad, CA, USA). The results were presented as arbitrary units after normalization for β-actin protein expression.

2.5.3. Mitochondrial isolation and mitochondrial membrane potential measurement

The striatum was homogenized using a mitochondria isolation kit (Sigma-Aldrich, MO, USA) and the resulting mitochondrial pellet was prepared in accordance with manufacturer instructions. A fresh fraction of mitochondrial suspension was used to estimate the mitochondrial membrane potential using the JC-1 test kit (Sigma-Aldrich, MO, USA) in accordance with the manufacturer's instructions. The sample's relative fluorescence at 590 nm was assessed using a spectrofluorophotometer (Shimadzu RF-1501) after excitation at 490 nm. The findings were presented in terms of the rate of JC-1 transfer in mitochondria [23].

2.6. Histopathological examination

Three rats from each group's brains were fixed for 24 h in 10 % buffered formalin. Samples were rinsed, dehydrated using successive alcohol dilutions, clarified in xylene, then embedded in paraffin at 56 oC for 24 h in a hot air oven. Hematoxylin and eosin (H&E)-stained sections of 4–5 μm were produced and examined under a light microscope [24]. The severity of the identified histopathological lesions was assessed using a modified 5-point scale scoring method (0–4). According to that grading system, alterations are classified as none, minimal (1–10 %), mild (30 %), moderate (50 %) and severe (>50 %) [25].

2.7. Immunohistochemical examination

Tyrosine hydroxylase (TH) and phosphorylated Nuclear factor kappa B (NF-κB p65) expression were studied in brain sections that were preserved in paraffin. The samples were exposed to primary monoclonal antibodies against TH and p-NF-κB p65 (Abcam, Cambridge, MA, USA) at dilutions of 1:150 and 1:200, respectively, for 60 min at 37 °C. Subsequently, the sections were washed with PPBS and incubated for another 60 min at 37 °C with biotinylated secondary antibodies (Dako, Carpenteria, CA, USA), followed by Avidin DH and biotinylated horseradish peroxidase H complex application according to the instructions of the "Vectastain ABC peroxidase kit (Vector Laboratories Inc., Burlingame, CA, USA). After a PBS wash, the reaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB Substrate Kit, Vector Laboratories Inc., Burlingame, CA, USA). Hematoxylin was used to counterstain the sections, which were then dehydrated, cleared in xylene, and cover-slipped for examination under a light microscope. Furthermore, the optical density from seven randomly selected high power microscopic fields (at 40x objective magnification, the area for each microscopic field was 18.8913Sqmm) in each section was measured and averaged using image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA) to quantify the positive brown color of the expression of Tyrosine Hydroxylase and NF-B. All histopathological evaluations were completed by a skilled researcher who was blinded to the identity of the samples to eliminate bias [26].

2.8. Statistical analysis

The results were analyzed and displayed as means ± standard deviation (SD) using "GraphPad Prism® software (Version 6, CA, USA)". Statistical significance was determined using a "one-way analysis of variance (ANOVA) test, followed by Tukey's Multiple Comparison test," except for rearing frequency and histopathological scoring, where the Kruskal-Wallis Test followed by Dunn's Multiple Comparison test was applied. The data for rearing frequency were presented as median (maximum-minimum). Pearson's correlation analysis was also used to examine the correlation between β2AR expression and α-synuclein content. The threshold for statistical significance was set at a p-value below 0.05.

3. Results

Rats treated with formoterol only (Gp 2) showed no significant difference from the control group (Gp 1) in all parameters. Hence, comparisons were referred to the control group (Gp1) only.

3.1. Formoterol mitigated rotenone-induced motor alterations in rats

Rats injected with rotenone (Rot.) exhibited an obvious drop in muscular and motor performance confirmed by a noticeable reduction in the total distance traveled, mean speed, and frequency of rearing by (66, 88, and 77 %), correspondingly, together with escalation in immobility time by 81 %, relative to control rat group. On the contrary, the administration of formoterol enhanced muscular outcomes as

proven by striking elevation in the distance traveled (70 %), mean speed (3.2-folds), and the rearing frequency (3.5-folds), combined with a drop in the immobility time by 20 % as relative with Rot. group (Fig. 1). In collaboration with these findings, Rot. group declined fall-off latency and grip time (38 % and 47 %, respectively) in rotarod and grip strength test, whereas treatment with formoterol increased the aforementioned parameters (1.9 and 1.6-folds) respectively, relative to the insult (Fig. 2).

3.2. Formoterol protected SNpc and striatal dopaminergic neurons in rotenone-induced PD in rats

The Substantia nigra and striatum of control, as well as the drug control groups' rats, revealed normal histological structures (Figs. 3a and 4a). Meanwhile examination of the substantia nigra and striatum of Parkinson's disease model rats showed marked deleterious effects on both. Regarding the substantia nigra, there was extensive neuronal degeneration, necrosis, and marked substantial neuronal loss (Fig. 3c). A marked loss of the melanin pigment from most of the substantial neurons was a conspicuous finding (Fig. 3d). The necrotic neurons appeared either with nuclear pyknosis or without any nuclear structure, ghost-like with marked neuronophagia. Some of the vacuolated neurons showed the presence of intraneuronal Lewy inclusion body, which appeared as spherical eosinophilic hyalinized bodies. Variable degrees of vacuolation of the neuropil were also observed in addition to congested blood vessels in the vicinity (Fig. 3e). The substantia nigra of the treated group showed marked neuronal changes improvement, and most of the substantial neurons showed normal dark colored cytoplasm with mild loss of the melanin pigment (Fig. 3f). Mild degenerative changes were noticed with mild perineuronal edema. Concerning the striatum, Parkinson's disease model rats showed marked swelling and vacuolation of the striatal neurons with many necrotic cells that appeared with pyknotic nuclei or without any nuclear structure accompanied with neuronophagia. Apoptotic bodies were also noticed as eosinophilic bodies surrounded by a halo with neuronophagia (Fig. 4c). The striatum of some cases showed marked necrosis and loss of the striatal neurons with vacuolation of the neuropil (Fig. 4d). The striatal neurons of the treated group revealed a good degree of restorative effect, only mild to moderate neuronal degenerative changes with few scattered neuronophagia was noticed (Fig. 4e). Moreover, Fig. 3g and 4f presented the scoring of variously observed histopathological lesions in different experimental groups.

The similar outline was monitored in immunohistochemical analysis of TH, the principal step in dopamine biosynthesis, as illustrated in Fig. 5. Relative to the control group, the rotenone group displayed a clear drop in SNpc and striatal TH immunoexpression by 71 and 70 %. Inversely, formoterol administration protected dopaminergic neurons and boosted SNpc and striatal TH immunoexpression to 1.9 and 1.6-folds, respectively, as compared to the insult.

3.3. Formoterol enhanced neuroprotective signaling cascade in rotenone-induced PD in rats

The PI3K/Akt/CREB/BDNF axis performs a fundamental role in neuroprotection against neurodegenerative diseases as it promotes neuronal plasticity and neuronal survival. In the present investigation, rotenone suppressed striatal PI3K, p-Akt, p-CREB, BDNF, and DA contents by 60, 74, 68, 51, and 59 %, respectively, relative to the control group. Oppositely, formoterol treatment succeeded in antagonizing the aforementioned parameters and boosting striatal PI3K/p-Akt/p-CREB/BDNF contents (1.2, 2.3, 2.5 and 0.9-folds, respectively) together with DA content (1.1-folds) as compared to PD-rats (Fig. 6).

3.4. Formoterol repressed neuroinflammation in rotenone-induced PD in rats

PD rats showed a marked increase in immunoreactivity of SNpc and striatal NF-κB p65, the parent active inflammatory transcriptional factor, to reach 3 and 4-fold, respectively, relative to the control group. On

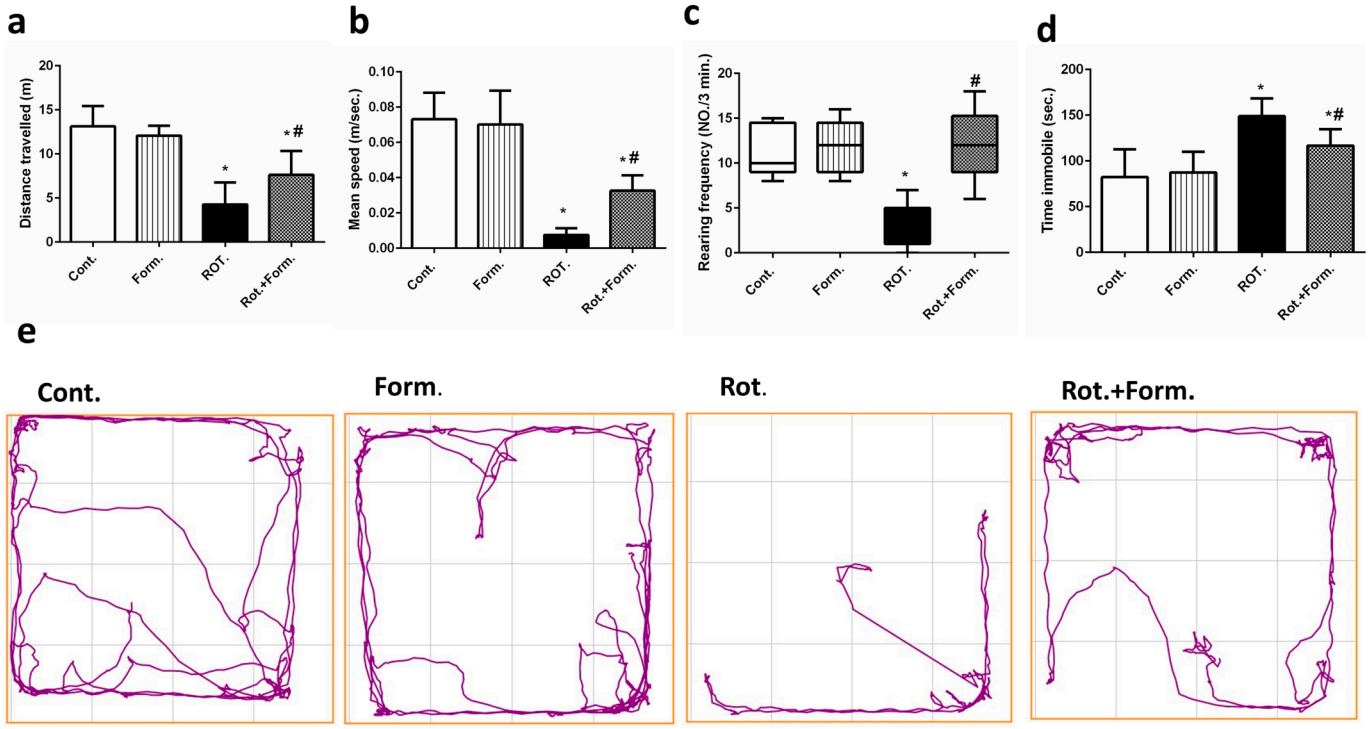


Fig. 1. Effect of formoterol on total distance traveled in meters (a), the mean speed (b), the rearing frequency (c), and immobility time (d) in open field test. Panel (e) shows descriptive track plots of rats during the open field test. Parametric data are presented as mean ± SD of 12 rats per group, using one-way ANOVA followed by Tukey's post hoc test. Non-parametric data are presented as boxplots with median (max–min) and 25th and 75th percentile values using Kruskel–Wallis test followed by Dunn's as a post hoc test; $p < 0.05$, * vs. cont., # vs Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

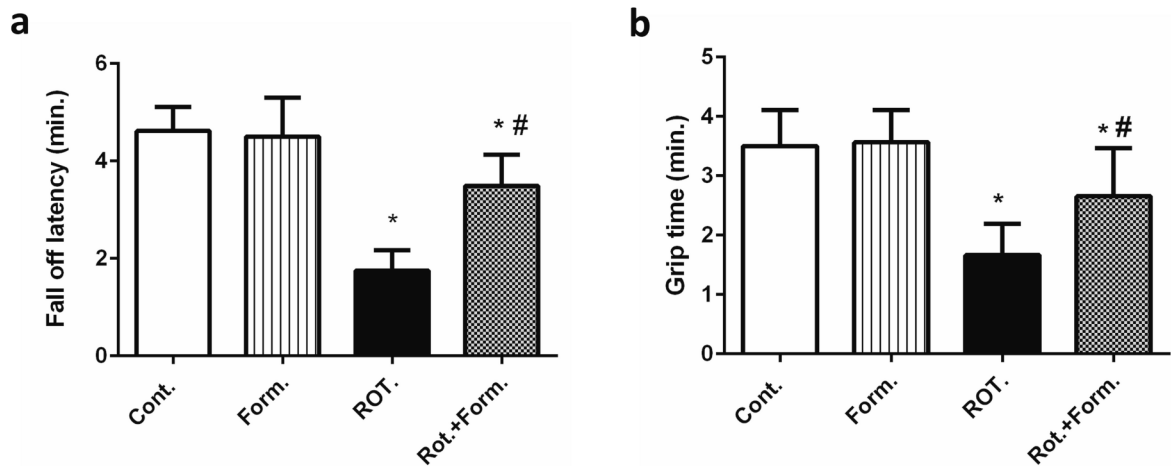


Fig. 2. Effect of formoterol on fall-off latency in Rotarod (a) and grip time in grip strength (b). Data are presented as mean ± SD of 12 rats per group, using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$, * vs. cont., # vs. Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

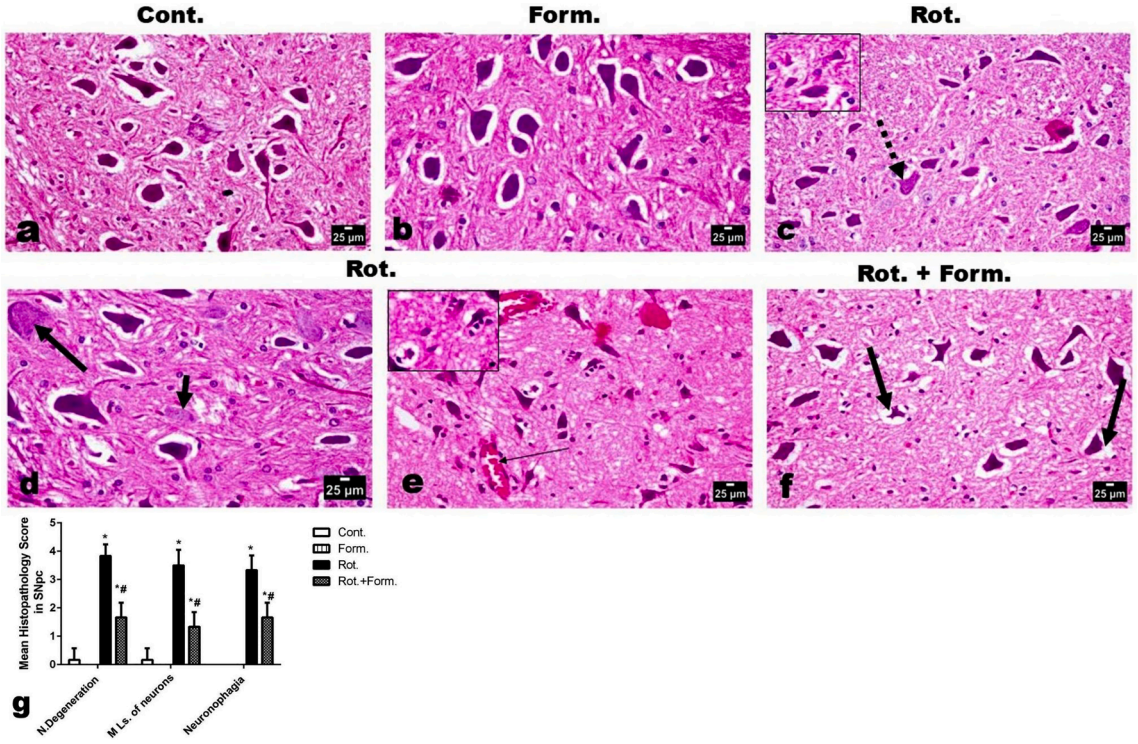


Fig. 3. Effect of formoterol on substantia nigra pars compacta (SNpc) histopathological alterations in rotenone rats. Photomicrographs represent H&E staining of SNpc from (a) control group, (b) formoterol control rats, (c–e) Rot. group showing neuronal degeneration (dotted arrow), necrosis (arrow), and marked substantial neuronal loss, marked loss of the melanin pigment from most of the substantial neurons, peripheral chromatolysis (arrow), and ghost-like necrotic neurons (dotted arrow), marked neuronophagia (arrow) and dilated vessels (dotted arrow) and (f) Form-treated group showed marked neuronal changes' improvement with mild degenerative changes with mild perineuronal edema (arrow). (g) Mean histopathology scores were calculated. Data are presented as the mean ± SD of each group and were analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test; $p < 0.05$, * vs. cont., # vs Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

the opposite side, treatment with formoterol decreased SNpc and striatal NF-κB p65 immunoreactivity to 33 and 25 %, respectively, as compared to the insult (Fig. 7). Furthermore, (Fig. 8) illustrated the inflammatory mediator TNF-α level, which was dramatically increased in the striatal tissues of Rot. group to 4-folds, as compared to control rats. Contrariwise, formoterol administration significantly decreased TNF-α level to 40 % compared to the untreated ones.

3.5. Formoterol reduced oxidative stress and boosted striatal mitophagy in rotenone-induced PD in rats

Rotenone administration provokes an oxidative stress status as observed by a marked reduction in PGC-1α content (30 %), a principal transcriptional coactivator in regulating mitochondrial biogenesis. The reduction in PGC-1α is associated with defective mitophagy as monitored by a reduction in Parkin and PINK-1 protein expression by 70 and 65 %, respectively, along with a marked reduction in mitochondrial membrane potential by 50 % relative to the control group. Inversely,

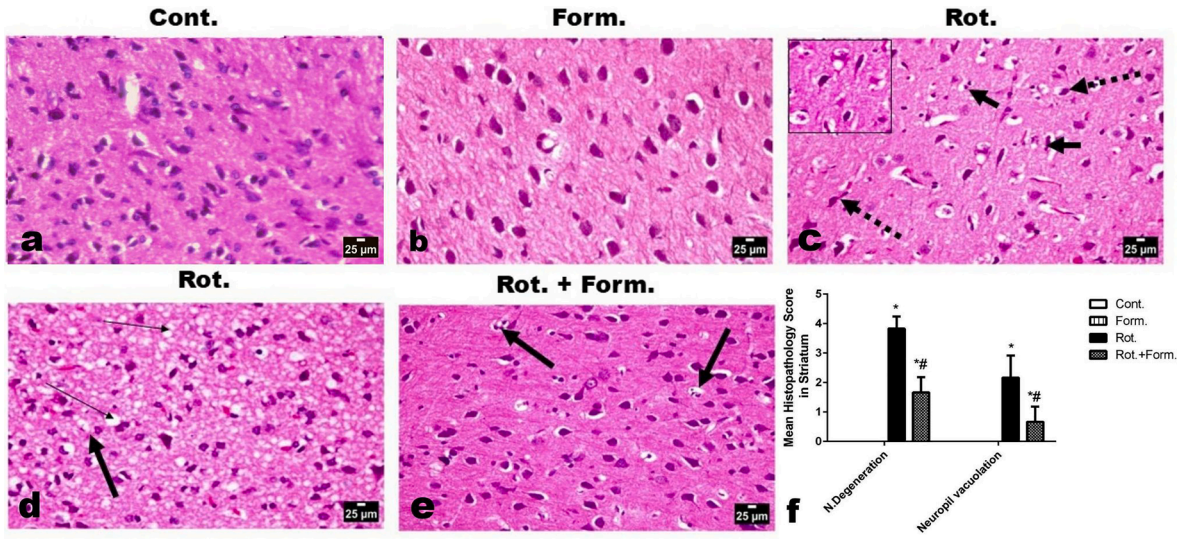


Fig. 4. Effect of formoterol on striatal histopathological alterations in rotenone rats. Photomicrographs represent H&E staining of the striatum from (a) control, (b) formoterol control rats, (c-d) Rot. group showing vacuolation (arrow) of the striatal neurons with many necrotic cells (dotted arrow) and neuronophagia (short arrow), apoptotic bodies (arrow) and vacuolation of the neuropil (dotted arrow) and (e) Form-treated rat showing only mild to moderate neuronal degenerative changes with few scattered neuronophagia (arrow). (f) Mean histopathology scores were calculated. Data are presented as the mean ± SD of each group and were analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test; $p < 0.05$, * vs. cont., # vs Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

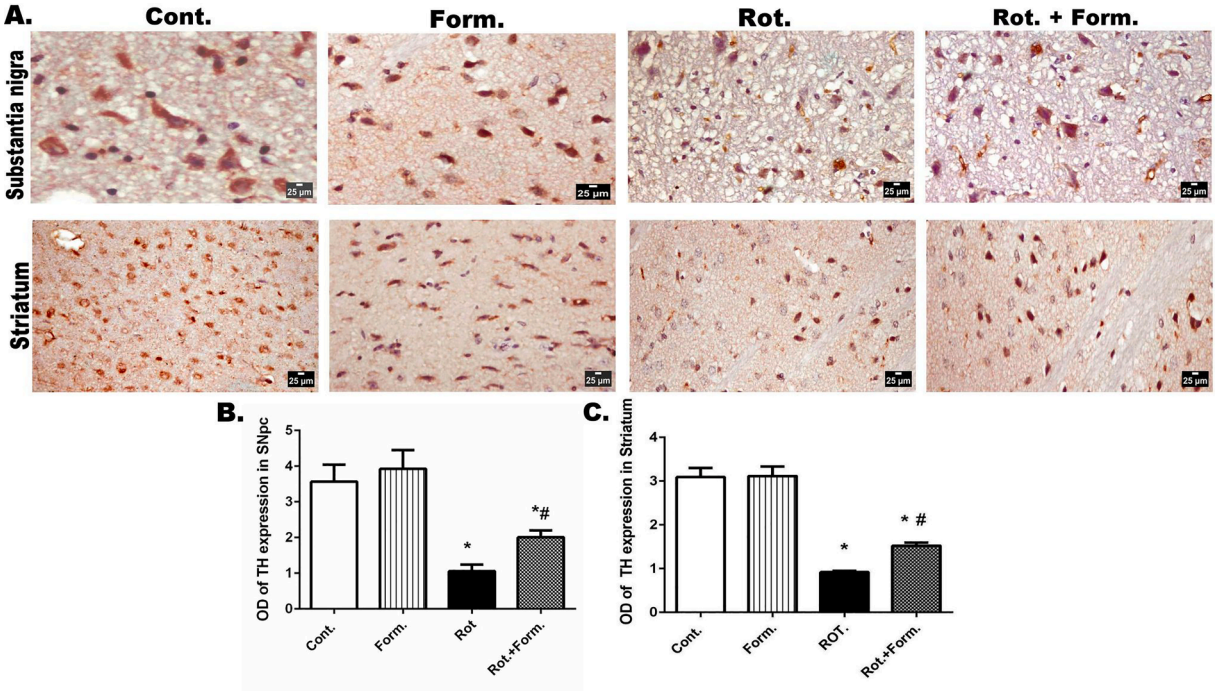


Fig. 5. Effect of formoterol on TH immunoreactivity (A) in SNpc as well as striatum in rotenone-induced alteration. Panel B and C represent the optical density of the positive (brown color) TH expression in SNpc and striatum of all experimental groups. Each bar represents mean ± SD (n = 3 rats per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$, * vs. cont., # vs. Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

formoterol administration reduced oxidative stress status as witnessed by an increase in PGC-1α content (2.6-folds) and mitochondrial membrane potential by 47 %, together with marked improvement in mitophagy biomarkers as endorsed by the upsurge in Parkin and PINK-1 protein expression (2.6 and 2.3-folds) to remove the defective abnormal protein aggregates and further elucidate formoterol impact on mitochondrial activity. (Fig. 9).

3.6. Formoterol halted striatal α-syn accumulation via upregulation of β2AR in rotenone-induced PD in rats

The defective mitophagy observed in PD-rats is coupled by the accumulation of abnormal protein aggregates, α-synuclein, to reach 6-fold above their normal values together with downregulation of β2AR protein expression to 40 %, relative to the control group. In contrast, formoterol treatment succeeded in reversing the aforementioned parameters. Moreover, an opposite correlation was detected between β2AR protein expression and α-synuclein accumulation ($r = -0.99$, $p =$

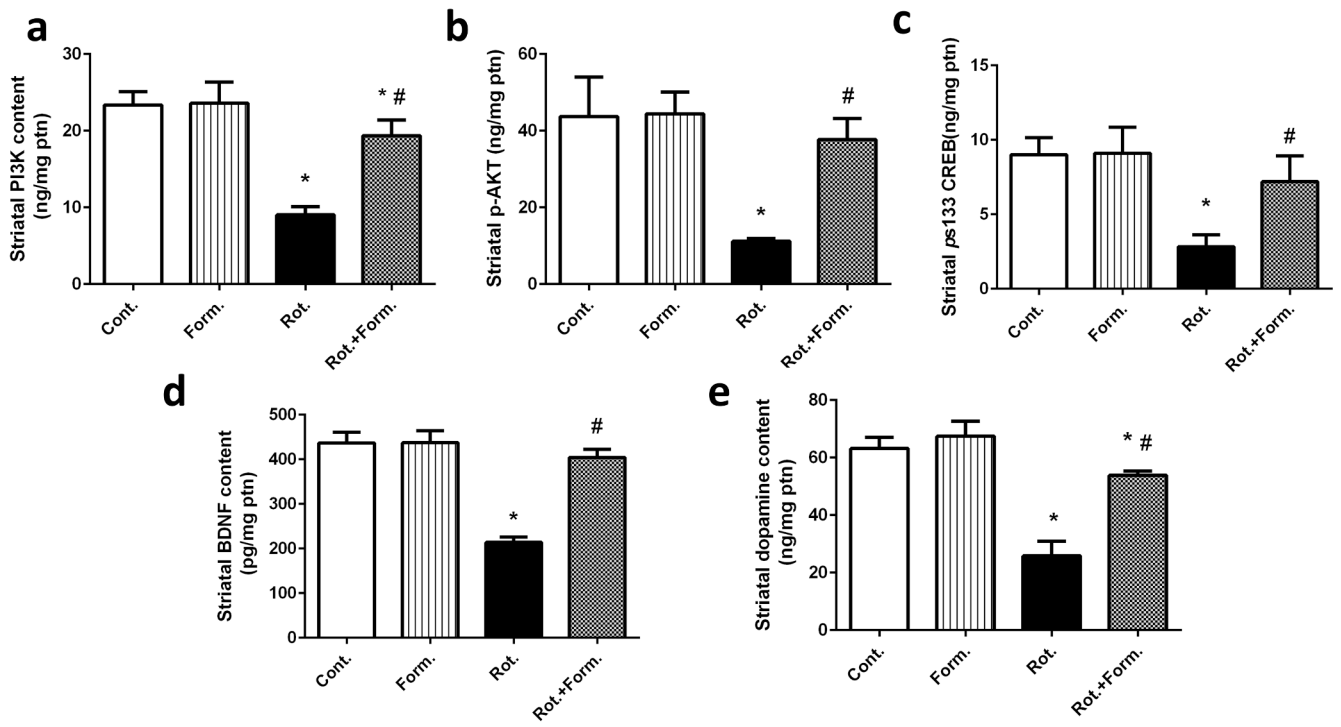


Fig. 6. Effect of formoterol on striatal PI3k (a), *p*-Akt (b), *p*-CREB (c), BDNF (d), and DA (e) contents in rotenone rats. Each bar represents mean ± SD (n = 6 rats per group); statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$, * vs. cont., # vs Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

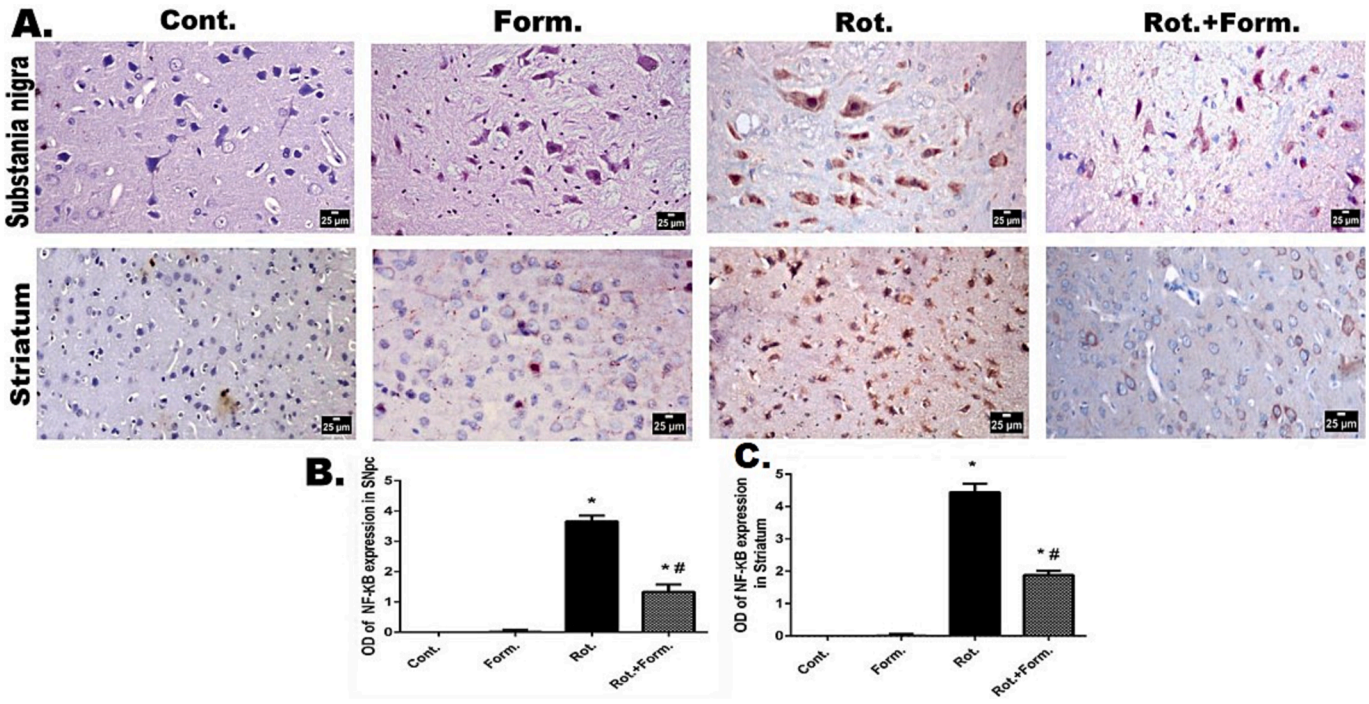


Fig. 7. Effect of formoterol on NF-κB immunoreactivity (A) in SNpc as well as striatum in rotenone-induced alteration. Panel B and C represent the optical density of the positive (brown color) NF-κB expression in SNpc and striatum of all experimental groups. Each bar represents mean ± SD (n = 3 rats per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$, * vs cont., # vs. Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

0.0003), referring to the potential neuroprotective role of β2AR in PD (Fig. 10).

4. Discussion

This study provides the initial evidence supporting the neuronal protection benefits of a β2 adrenergic receptor (β2AR) agonist, formoterol, against rotenone-induced neurotoxicity in rats that is mediated

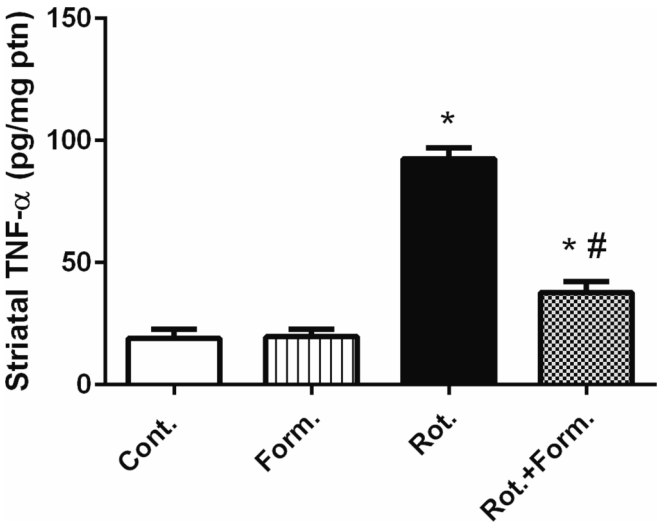


Fig. 8. Effect of formoterol on striatal TNF-α contents in rotenone rats. Each bar represents mean ± SD (n = 6 rats per group); statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$, * vs. cont., # vs Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

in part *via* activation of the PINK-1/Parkin axis that facilitates mitophagy, and restored mitochondrial membrane potential to stabilize the mitochondrial function and thus inhibited alpha-synuclein accumulation. In parallel, formoterol triggered neuro-survival cascade, PI3k/Akt/CREB, the latter dimerize to transcribe BDNF. Additionally, CREB increases TH immunoexpression, the late determining step for dopamine (DA) biosynthesis, with subsequent increase in DA content. Moreover, formoterol suppresses neuroinflammation *via* decreasing NF-kB p65 immunoreactivity in both the substantia nigra and striatum as well as striatal TNF-α content. The positive outcomes were evident in the behavioral assessments, demonstrating enhancements in muscle coordination and motor performance alongside a decrease in Parkinson's disease symptoms.

In the present research, formoterol's neuroprotective properties were investigated under conditions simulating a clinical scenario. The drug was administered after the manifestation of PD symptoms, as observed herein and in previous observations [27]. Administering prompt pharmacotherapy after a reduction in motor function could significantly preserve the dopaminergic neurons of rats with Parkinson's disease (PD) [28]. Significantly, continuous exposure to rotenone, which inhibits complex I, worsens the loss of dopaminergic neurons in the nigrostriatal pathway, leading to motor impairment and muscular incoordination. This is evident through a decrease in mean speed, the total distance traveled, and rearing frequency in the open field test, as well as an increase in immobility time. In addition, a reduction in fall-off latency in the rotarod test and grip time in the grip strength test was observed. Conversely, treatment with formoterol, a long-acting β2AR agonist, preserved dopaminergic neurons and results in improved motor performance and muscle coordination. O'Neill et al. demonstrated that formoterol treatment alleviated LPS (lipopolysaccharide)-induced skilled motor deficits in rats when administered intranigrally [29]. In parallel, formoterol amended histopathological alterations and mitigated striatal and nigrostriatal neuronal loss in PD rats.

Indeed, formoterol and salmeterol, long acting β2-AR agonist, display a higher lipophilicity and have a higher affinity, selectivity, and potency than most short-acting β2-AR agonists at β2 adrenoreceptor [30]. Of these factors, lipophilicity is one of the most important factor that determines the amount of drug crossing blood brain barrier, permitting formoterol to reach CNS and exerting its neuroprotective effect against neuroinflammation [29]. In the same context, Peterson et al. [31] studied the effects of both short-acting β2AR agonist, salbutamol, and a variety of long-acting β2AR agonists, including formoterol

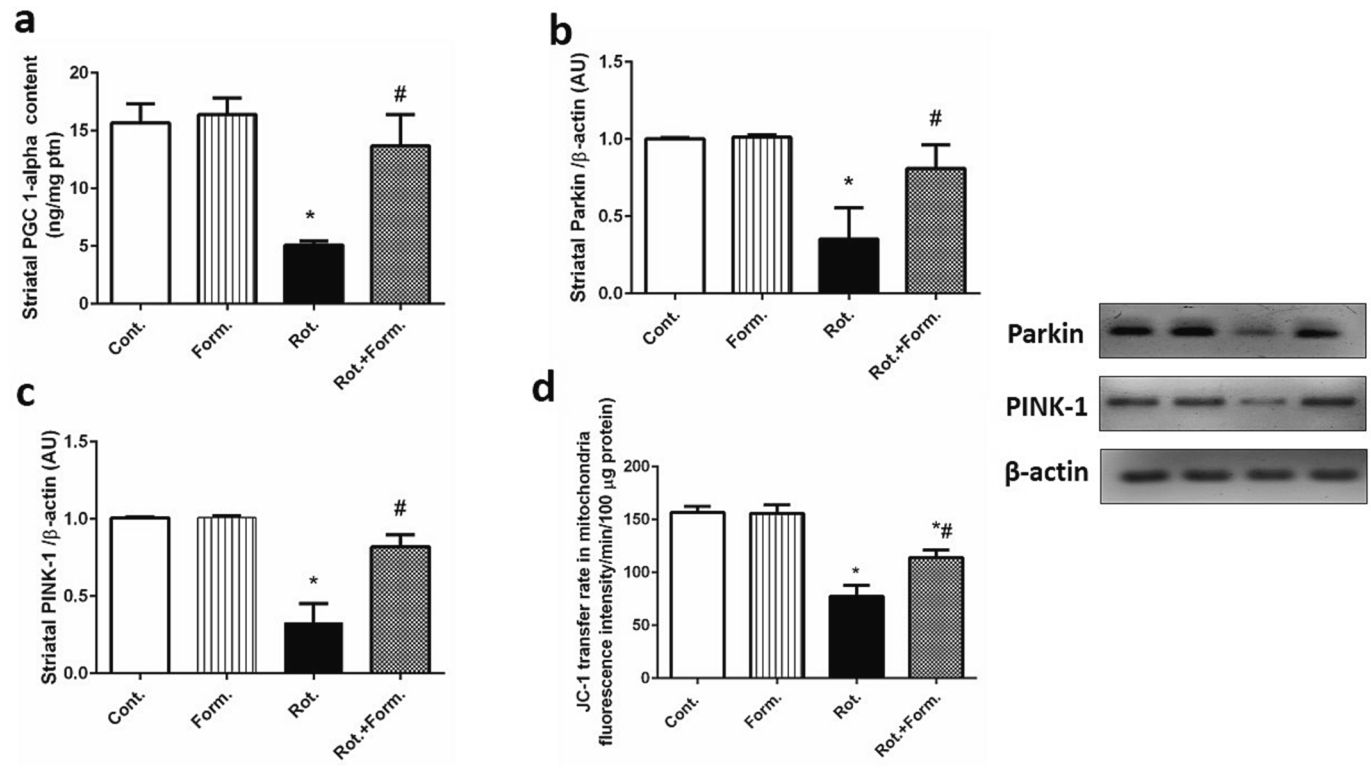


Fig. 9. Effect of formoterol on striatal PGC1-alpha (a) as well as protein expression of parkin (b), PINK-1 (c), and mitochondrial membrane potential (d) in rotenone rats. Each bar represents mean ± SD (n = 3–6 rats per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$, * vs. cont., # vs. Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

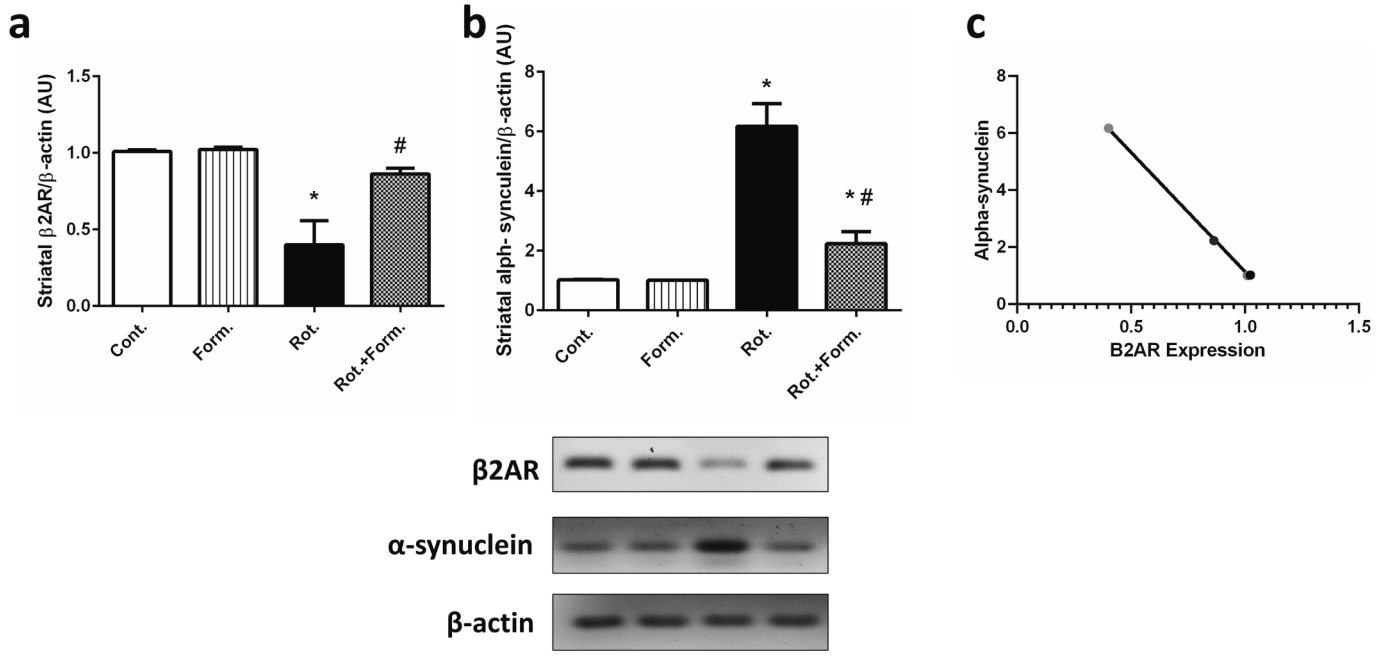


Fig. 10. Effect of formoterol on the protein expressions of beta 2 (β-2) adrenergic receptor (a) and alpha-synuclein (b) as well as the correlation between β-2AR and α-synuclein (c) in rotenone rats. Each bar represents mean ± SD (n = 3 rats per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Correlation between β-2AR expression and α-synuclein was established using Pearson's correlation test; $p < 0.05$, * vs. cont., # vs. Rot. Cont.: control; Form.: formoterol; Rot.: Rotenone.

or salmeterol, on the survival of DA neurons after induction of inflammation in different disease models of Parkinson's disease (PD). Noteworthy, the results stated that short acting β2AR agonists exhibit neuroprotection but higher doses are required, in addition long-acting β2AR agonists, such as salmeterol, have more pronounced anti-inflammatory effects to attenuate the progressive loss of DA neurons and improve motor activity in patients [31].

Interestingly, formoterol increased tyrosine hydroxylase (TH) immunoexpression, the rate-determining step for DA production, in SNpc and striatum that justifies the improved motor activity witnessed herein. Similarly, treatment with formoterol attenuated LPS-induced loss of TH+ dopaminergic neurons in the substantia nigra and striatum [29]. Furthermore, the use of β2AR agonist, salbutamol, was associated with reduced risk for developing PD. In opposition, the use of β2AR antagonist was coupled with an increased risk of PD, as reported previously [32].

Mitochondrial homeostasis is a dynamic process regulated by interdependent pathways, including mitophagy, mitochondrial biogenesis, mitochondrial fission, and fusion [33,34]. These dynamical mechanisms facilitate cellular adaptation to energy demands change, nutrient availability, and oxidative stress [35,36]. Mitophagy is a crucial process for the selective elimination of damaged mitochondria and maintenance of healthy mitochondria. Mitophagy plays a key role in neuronal health, and defective mitophagy has been implicated in the pathophysiology of PD and coupled with accelerated neuronal loss [37,38]. Therefore, mitochondrial quality control and removal of damaged mitochondria via mitophagy are vital cellular mechanisms to maintain homeostasis [39]. Rotenone neurotoxin inhibits complex I of the electron transport chain, provoking reactive oxygen species production with subsequent reduction of brain ATP creating an oxidative stress status [40]. Thus, one of the early responses to combat oxidative stress and ROS production is to induce mitophagy [41]. PTEN-induced kinase 1 (PINK1) and Parkin RBR E3 ubiquitin-protein ligase (PARKIN) play a chief role in mitophagy [42,43] as well as mitochondrial mobility and size. PINK1 accumulates at the outer mitochondrial membrane (OMM) as a result of a decrease in mitochondrial membrane potential triggered by dysfunction/damage; this is coupled by PARKIN recruitment from the cytosol to the OMM to endorse mitophagy, resulting in mitochondrial degradation. Significantly, the dysfunction of the PINK1/PARKIN signaling pathway is connected with the development of neurodegenerative disorders such as AD, PD, and glaucoma [44,45].

Furthermore, peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) plays a critical role as a transcription factor in controlling both mitochondrial biogenesis and respiration [46]. PGC-1α promotes the antioxidant activity of ROS-defensive enzymes and mitigates oxidative stress status [47,48]. On the other hand, overexpression of PGC-1α in cerebellar neurons increases mitochondrial density and protects the neurons against mutant synuclein A53T-α induced neural degeneration.

Moreover, an augmented susceptibility to MPTP neuronal loss of dopamine neurons in nigra region was monitored in PGC-1α Knockout mice, referring to a pivotal contribution of PGC-1α in neuronal protection. Thus, boosting PGC-1α content significantly protected neural cells from ROS and permanent cell damage [23,49]. In addition, reduced PGC-1 expression increases α-synuclein accumulation and decreases the Akt/GSK-3 signaling cascade in neural cells [50]. Indeed, PGC-1α has been confirmed to be dramatically downregulated in PD patients. This result evoked a correlation between mitochondrial biogenesis and PD [51,52]. Taken together that, a reduction in mitochondrial membrane potential has been associated with impaired mitophagy and elevating the levels of ROS and consequently causing oxidative stress [23].

In current work, formoterol attenuated mitochondrial dysfunction *via* increasing mitophagy as witnessed by increased PINK-1 and PARKIN protein expression, as well as enhancing mitochondrial biogenesis as observed by increased PGC-1α and mitochondrial membrane potential by maintaining mitochondrial membrane permeability; this result is in harmony with the previous study [53,54]. Indeed, a firm association has been suggested between PINK-1, PARKIN, and SNCA, as PARKIN can block α-synuclein clustering and neurofibrillary tangles via its initiation of protein phosphatase-2 (PP2A) that refers to a substance or entity that has the capability to remove phosphate groups from pSer129 SNCA. As a result, it reduces the creation of harmful oligomers and ultimately

prevents the formation of Lewy bodies and neurofibrillary tangles [53,55,56]. Furthermore, SNCA can undergo nitration facilitated by monoamine oxidase B (MAO-B). However, PARKIN inhibits the transcription and expression of MAO-B, resulting in reduced nitrated synuclein levels and a decrease in the formation of oligomers. Additionally, PARKIN regulates the expression of PGC-1 α indirectly, which is attributed to the ability of PARKIN to degrade the parkin interacting substrate (PARIS), which usually inhibits the activity of PGC-1α [57]. Indeed, treatment with formoterol enhances mitophagy and mitochondrial biogenesis along with the reduction of α-synuclein accumulation. In recent findings, it has been documented that β2AR plays a role in controlling the expression of SNCA protein, which is the primary component of Lewy bodies found in PD brains [32,58]. Accumulation of α-synuclein, the pathological feature of PD, results in microglial activation, production of pro-inflammatory cytokines, and neurodegeneration [59,60]. Treatment with formoterol decreased α-synuclein protein expression and decreased the risk of developing PD. These effects could be attributed to the activation of mitophagy that clears out defective mitochondria as a result of complex I inhibitor rotenone. In the same context, Mittal et al. [32] reported that treatment with a selective β2AR agonist, clenbuterol, reduced the relative expression of human SNCA mRNA levels via inhibiting acetylation or activating deacetylation of H3K27 a promoter of SNCA transcription on both SNCA promoter and enhancer sites. [31,32] as Acetylation of histone proteins results in a loose chromatin state which allows for transcriptional activation, whereas histone deacetylation results in a tight compact chromatin structure which suppresses transcriptional activity [61]. Therefore, treatment with β2AR agonist formoterol may be a potential therapeutic agent via stimulation of the PINK-1/PARKIN/PGC-1α axis and reducing the burden of accumulated α-synuclein in PD rats.

β2AR is a G protein-coupled receptor expressed post-synaptically on neurons and microglial and astrocytic cells [62]. Activation of β2AR stimulates adenylyl cyclase that elevates intracellular cAMP level, leading to activation of protein kinases A (PKA), which entails cAMP-response element binding protein (CREB) phosphorylation. The latter acts as a pleiotropic factor that aids in the transcription of BDNF and its receptor, TrKB [63]. Increased BDNF amplified neurogenesis [64] and stimulated/phosphorylated TrKB receptor in a positive feed-forward cycle to restimulate/phosphorylate phosphatidyl inositol-3 kinase (PI3K) and protein kinase B (Akt) [65]. Indeed, stimulation of PI3K/Akt signaling cascade can promote mitophagy via PINK-1 and PARKIN activation, as reported herein and previously [66], along with inhibition of α-synuclein aggregation that ultimately ameliorates ROT-induced PD symptoms [67]. In the current study, formoterol enhanced PI3K/Akt activation that entails CREB phosphorylation at serine 133. The phosphorylated CREB is a key player in multiple intracellular processes involved in the neuroprotection against several neurodegenerative disorders [68]. The p-CREB may account for the elevation of PGC-1α and BDNF as reported herein and earlier [69,70]. Firstly, p-CREB plays a chief role in neurotrophin-mediated neuronal survival via transcription of BDNF [71] and its receptor TrKB [72] to restimulate PI3K/Akt signaling cascade, as stated previously [73]. Secondly, the PGC-1α gene possesses a cAMP-responsive element (CRE) site for CREB; thus, increased p-CREB reported can be the reason for increased PGC-1α expression, as observed earlier [74,75]. Thirdly, p-CREB is known to directly activate a significant number of target genes that possess the CRE motif "TGACGTCA," including the TH gene [76]. Furthermore, additional evidence has been presented to support this claim based on relative bioinformatics analysis, the presence of the consensus CRE at the promoter region of the TH gene makes CREB has the greatest potential to be employed for the transcription of TH [77]. Indeed, p-CREB stimulates the transcription of BDNF [78] and its receptor TrKB [79]. Increased BDNF endorses neurogenesis and phosphorylates TrKB to act in a positive feed forward loop to re-stimulate PI3K/Akt signal, as reported herein and previously [80]. Noteworthy, activation of PI3K/Akt signaling cascade directly involved in the regulation of TH expression [81]; this finding supports Chen et al. [77] Who indicated that active CREB promotes TH transcription to synthesize DA, which protects nerve terminals in the striatum from degeneration and prevents nigrostriatal dopamine depletion, hence enhancing motor activity [29], as proved herein. Furthermore, p-CREB (phosphorylated CREB) provokes de novo synthesis of the NF-κB inhibitory protein (IκBα) and hinders its phosphorylation. As a result, it stabilizes the levels of IκBα in the cytosol, effectively preventing NF-κB from translocating into the nucleus. This, in turn, leads to the inhibition of NF-κB activity and subsequently reduces the expression of pro-inflammatory cytokines [82,83]. Qian and colleagues documented that the activation of β2AR generates anti-inflammatory and neuroprotective consequences in the CNS. Additionally, it impedes the loss of dopamine neurons caused by LPS both in laboratory settings (in vitro) and within living organisms (in vivo) [84]. In the same context, pretreatment with clenbuterol, β2AR agonist, suppresses NF-κB activity and mitigates the expression of pro-inflammatory molecules such as Interleukin- (IL)-1β, interferon-(IFN)-γ, and inducible NOS in the kainic acid model of excitotoxicity [85]. In the current study, formoterol, a β2AR agonist, stimulates the protein expression/activation of β2AR with subsequent reduction of NF-κB immunoreactivity and neuroinflammatory status. Taken together these findings fit in line with the theory that β2AR exerts a biphasic neuroprotective role in the brain via the inhibition of pro-inflammatory mediator release and stimulating growth factor production from glial cells, as shown herein that stimulation of CNS β2AR with formoterol suppresses NfκB activity and effectively rescinded striatal inflammation via inhibiting TNF-α.

In conclusion, activation of β2AR attenuated rotenone-induced neurotoxicity *via* stimulation of the PINK-1/PARKIN axis along with enhancement of mitochondrial biogenesis to remove α-synuclein aggregates. Moreover, β2AR activated neuronal survival pathway PI3K/Akt/CREB/BDNF, in addition to increment in TH immunoexpression, the most crucial step for DA biosynthesis that accounts for improving motor function.

Ethics declarations

The protocols used in this study were approved by the Ethics Committee for Animal Experimentation at the Faculty of Pharmacy, Cairo University [PT number (2903)].

CRediT authorship contribution statement

Haneen Y. Khidr: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing - original draft. **Noha F. Hassan:** Conceptualization, Supervision, Writing - review & editing. **S.S. Abdelrahman:** Data curation, Supervision, Writing - review & editing. **Mona R. El-Ansary:** Data curation, Supervision, Writing - review & editing. **Mohammed F. El-Yamany:** Supervision, Writing - review & editing. **Mostafa A. Rabie:** Conceptualization, Methodology, Visualization, Supervision, Data curation, Formal analysis, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.

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