

Role of Rho Kinase Inhibition in the Protective Effect of Fasudil and Simvastatin Against 3-Nitropropionic Acid-Induced Striatal Neurodegeneration and Mitochondrial Dysfunction in Rats

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Abstract 3-Nitropropionic acid (3-NP)-induced neurotoxicity is an experimental model which mimics the pathology and motor abnormalities seen in Huntington's disease (HD) in human. The present investigation was directed to estimate the role of rho kinase (ROCK) inhibition in the possible protective effect of fasudil and simvastatin in 3-NP-induced striatal neurodegeneration in rats. Animals were injected s.c. with 3-NP (20 mg/kg/day) for 1 week with or without administration of fasudil (10 mg/kg/day, p.o.) or simvastatin (20 mg/kg/day, p.o.). At the end of experiment, motor and behavioral abnormalities were evaluated. Animals were then sacrificed for measurement of mitochondrial membrane potential as well as succinate dehydrogenase (SDH) and caspase-3 activities in striatum. Moreover, tumor necrosis factor-alpha (TNF- α) level and protein expressions of proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), ROCK, phosphorylated-Akt (p-Akt), endothelial and inducible nitric oxide synthase (eNOS and iNOS), Bax, and Bcl-2 were estimated. Finally, histological changes as

demonstrated by striatum injury score, glial activation, and percentage of altered mitochondria were assessed. Both fasudil and simvastatin effectively inhibited 3-NP-induced behavioral, biochemical, and histological changes through inhibition of ROCK activity. However, fasudil provided more amelioration in histological changes, mitochondrial membrane potential and SDH activity in addition to p-Akt and PGC-1 α protein expressions. The present study highlights a significant role of ROCK/p-Akt/eNOS pathway in the protective effects of fasudil and simvastatin on neurotoxicity and mitochondrial dysfunction induced by 3-NP in rats. Thus, specific inhibition of ROCK may be considered a promising new approach in the management of HD.

Keywords Fasudil · Mitochondria · Nitropropionic acid · Simvastatin · Striatum

Introduction

Huntington's disease (HD) is a neurodegenerative disorder caused by a mutation in the gene encoding the protein huntingtin. Experimental intoxication with 3-nitropropionic acid (3-NP) mimics the pathology and motor abnormalities of HD in humans including neurobehavioral, biochemical, and selective neurodegenerative changes [1, 2]. 3-NP is a fungal neurotoxin produced by *Arthrrium* fungi that grow on sugar cane, peanuts, and cheese curds [1]. 3-NP produces mitochondrial dysfunction, which is suspected to occur quite early in the course of HD and constitutes a cellular hallmark for neurodegeneration [3, 4]. The primary mechanism underlying 3-NP toxicity involves the irreversible inhibition of the activity of respiratory chain complex II via affecting succinate dehydrogenase (SDH) with subsequent prolonged energy impairments [5]. Importantly, striatal neurons are particularly dependent on

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mitochondria due to their high energy requirements, and thus 3-NP intoxication preferentially induces striatal lesions and neurodegeneration similar to those found in HD [2].

Rho kinase (ROCK) plays an important role in regulating biological events of cells, including proliferation, differentiation, and survival/death [6]. ROCK has been shown to be widely distributed in the mammalian central nervous system (CNS). Inhibitors of ROCK have been demonstrated as potential therapies that provide a beneficial management of various neurological disorders, including multiple sclerosis, Parkinson's disease, and spinal and hypoxic/ischemic injury [7, 8]. Additionally, Akt survival pathway has been identified as an important molecular mediator for the neuroprotective effects of ROCK inhibition [9]. Actually, ROCK inhibition leads to the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway which increases the production and bioavailability of endothelium-derived NO [10].

Fasudil, the first-generation of ROCK inhibitors, has been studied widely in clinical trials for treating pulmonary arterial hypertension and other cardiovascular and cerebrovascular diseases [11, 12]. It has also been shown to improve the structure and function of cardiac mitochondria from rats with type II diabetes [13]. The safety and the efficacy of fasudil in treating subarachnoid hemorrhage has been also well established clinically [14]. Likewise, fasudil has been reported to reduce neurological deficit, cerebral infarct size, and production of proinflammatory cytokines which may contribute to neuronal apoptosis in experimentally induced cerebral ischemia/reperfusion [15].

On the other side, the beneficial effects of statins have been demonstrated in several neurodegenerative conditions, including cerebral ischemia, intracerebral hemorrhage, Parkinson's disease, experimental allergic encephalomyelitis, and Alzheimer's disease [16]. The pleiotropic effects of statins include inhibition of isoprenoids synthesis which is required for post-translational modification and activation of Ras/Rho family GTPases [17]. Interestingly, the combination of lipophilicity and efficacy gives simvastatin a unique pharmacological profile compared with other statins. Simvastatin has been shown to be more effective than atorvastatin in amelioration of cerebral blood flow, edema, and blood-brain barrier permeability during the acute phase of experimental intracerebral hemorrhage [18] and thus was selected to be examined in the present study.

Although the inhibition of ROCK could be regarded as a promising avenue for therapeutic intervention in various neurological disorders, its role in 3-NP-induced striatal neurodegeneration has not yet been elucidated. Therefore, the aim of the present study was directed to assess the role of ROCK inhibition in the possible protective effect of fasudil and simvastatin against 3-NP-induced striatal neurodegeneration and mitochondrial dysfunction in rats.

Material and Methods

Animals

Male Wistar rats, weighing 170–210 g, were obtained from the animal facility of Faculty of Pharmacy, Cairo University, Egypt. Animals were housed under controlled environmental conditions at constant temperature (25 ± 2 °C) and a 12/12-h light/dark cycle. Rats were allowed standard chow diet and water ad libitum. The investigation complies with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University (Permit Number PT [1307]).

Chemicals

3-NP was purchased from Sigma-Aldrich Chemicals Co., USA. Fasudil hydrochloride and simvastatin were obtained from Alfa Aesar, USA, and MSD Pharmaceutical Company, Egypt, respectively. Other chemicals unless specified were obtained from Sigma-Aldrich Chemicals Co., USA.

Experimental Design

Rats were randomly divided into four groups. Group I ($n=10$) served as normal group. Group II ($n=12$) was injected with 3-NP (20 mg/kg/day, s.c.) for 7 days [19]. Groups III ($n=12$) and IV ($n=12$) received 3-NP as in group II and fasudil (10 mg/kg/day, p.o.) [20] or simvastatin (20 mg/kg/day, p.o.) [21], respectively. Fasudil and simvastatin were freshly prepared and daily administered to rats 1 h after 3-NP injection. The model of 3-NP-induced neurotoxicity progressed over 7 days with daily monitoring of animals.

Behavioral and Motor Studies

At the end of treatments, animals were subjected to behavioral tests with a minimum of 30-min gap between each test.

Examination of Neurological Deficit

Neurological dysfunction was tested using a scoring scale which was performed as previously described by Mittoux et al. [22] and Bantubungi et al. [23] with a score of 0–8. This test scores recumbency, dystonia of hind legs, gait abnormality, imbalance on a platform, and grasping problems. Total score of 8 points indicates maximal neurological deficit (animal showing near-death recumbency) and a score of 0 points denotes normal performance.

Open Field Test

Open field task was performed to evaluate spontaneous locomotor activity of rats. The apparatus, made of wood and covered with impermeable formica, had a 100 cm×100 cm white floor divided by black lines into 16 squares (25 cm×25 cm) and 40-cm-high white walls. Each rat was placed into the center and allowed to explore the apparatus for 5 min. Ambulation frequency (the number of squares crossed by the rat) and rearing frequency (the frequency of standing on its hind legs) were recorded [24].

Rotarod Activity

Motor coordination was evaluated using the rotarod test. In brief, the rotarod apparatus consists of a rod 120 cm long and 3 cm in diameter and is subdivided into four compartments by disks 24 cm in diameter. The rod rotates at a constant speed of 25 rpm. Animals were exposed to a prior training session to acclimatize them to rotarod performance. The latency for first fall from the rod was recorded where cutoff time was 180 s. Each rat performed three separate trials with a 5 min gap, and the average time of fall was recorded [25].

Limb Withdrawal Test

This test is considered to be an important parameter to measure functional abnormalities of the hind limbs, which are indicative for the extent of striatal degeneration [26]. In this behavioral test, the animal was placed on a 20-cm-high 30 cm×30 cm Perspex platform containing four holes, two holes of 5-cm diameter for the hind limbs and two holes with a diameter of 4 cm for the forelimbs. The rat was placed on the platform by positioning first the hind limbs and then the forelimbs into the holes. The time taken by the animal to retract its first hind limb and the contra lateral hind limb was recorded. The difference between the retraction times of both hind limbs was determined. The test was performed three times with a 30-min interval, and the average values were reported.

String Test for Grip Strength

The latency to hold the grip on a horizontal wire is considered an indirect measure of grip strength [27]. The rat was allowed to hold (with the forepaws) a steel wire (2 mm in diameter and 35 cm in length), stretched horizontally at a height of 50 cm over a cushion support. The length of time for which the rat was able to hold the wire was recorded.

At the end of behavioral tests, animals were weighed and euthanized where the whole brain was quickly excised, washed with ice-cold saline and dried. Brain was then dissected and two striata were isolated and weighed. For each group,

two sets were conducted: one for biochemical investigations and the other ($n=3$) for histological examinations.

Biochemical Measurements

The first striatum was used for mitochondrial separation. Meanwhile, the second striatum was divided into two parts, one of which was homogenized in ice-cold saline to prepare 10 % homogenate for the determination of tumor necrosis factor- α (TNF- α) as well as caspase-3 activity, whereas the other part was used for western blot analysis. The protein contents of tissue homogenate and mitochondrial fractions were determined using the method of Lowry et al. [28].

Isolation of Mitochondria

Striatum was homogenized in isolation buffer using mitochondria isolation kit (Sigma-Aldrich Co., USA). The mitochondrial pellet was prepared according to manufacturer's instructions. A part of fresh mitochondrial suspension was used for estimation of mitochondrial membrane potential whereas the other part was used for assessment of SDH activity.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured in freshly isolated mitochondria using the JC-1 assay kit (Sigma, USA) according to the manufacturer's instruction. The relative fluorescence of the sample was measured at 590 nm after excitation at 490 nm using a spectrofluorophotometer (Shimadzu RF-1501, Japan). Results were expressed as JC-1 transfer rate in mitochondria (fluorescence intensity/min/100 μ g protein).

Succinate Dehydrogenase Activity

Succinate dehydrogenase activity (SDH), an indicator of complex II activity, was estimated according to the method of Sharman and Bondy [29]. SDH was assayed using phenazine methosulfate as an artificial electron acceptor from succinate and recording the increase in absorbance of ferrocytochrome-c. SDH activity was calculated as nmol succinate oxidized/min/mg protein.

Tissue Tumor Necrosis Factor-Alpha

Tissue TNF- α content was estimated using rat TNF- α ELISA kit (Elabscience Biotechnology Co., Ltd., Wuhan, China). The procedure of the used kit was performed according to the manufacturer's instructions, and the results were expressed as pg/mg protein.

Caspase-3 Activity

Tissue caspase-3 activity was estimated using caspase-3 colorimetric assay kit (R&D Systems, Inc., USA). The absorbance was read at 405 nm using a microplate reader (BioTek instruments, USA). The results were expressed as nmol p-nitroanilide (pNA)/h/mg protein.

Western Blot Analysis

Part of the striatum was homogenized in lysis buffer and quantified for protein levels using a protein assay kit (Thermo Fisher Scientific Inc., USA). Protein expression was assessed as previously described by Ahmed et al. [30] using primary antibodies against peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) and rho kinase 1 (ROCK1) from GeneTex, Inc. (Irvine, CA, USA) as well as phosphorylated-Akt (p-Akt), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), Bax, Bcl-2, and beta-actin (β -actin) from Thermo Fisher Scientific Inc. (Rockford, IL, USA). The amount of protein was quantified by densitometric analysis of the autoradiograms using a scanning laser densitometer (Biomed Instrument Inc., USA). Results were expressed as arbitrary units after normalization for β -actin protein expression.

Histological Examinations

Assessment of Striatal Damage

For light microscopic examination, part of striatum was separated, rinsed in ice-cold saline, and immediately fixed in 10 % formalin for 24 h. Specimens were processed for paraffin embedding, and 5- μ m sections were prepared. Sections were stained with hematoxylin and eosin (H&E) and examined microscopically (magnification \times 100). Images were captured and processed using Adobe Photoshop (version 8.0). Histological changes were evaluated semiquantitatively and scored from 0 to 3 based on the extent of striatal degeneration, perivascular edema, and the degree of neutrophil infiltration. The total of these scores were then recorded for each individual animal (maximum score of 9). A total of six fields were scored from each sample and averaged. Scores from different sections were then summed up to obtain an average score per field for each group [31].

Immunohistochemical Detection of Glial Cells Activation

Evaluation of astroglial alteration was carried out using paraffin-embedded tissue sections of 4- μ m thickness. To

reveal the antigens, sections were pretreated with the proteolytic enzyme proteinase K (Dako, Copenhagen, Denmark) and were then washed in phosphate-buffered saline (PBS) for 5 min. Subsequently, sections were preblocked with 5 % bovine serum albumin for 30 min and were incubated with a primary antibody against glial fibrillary acidic protein (anti-GFAP) (Dako, Copenhagen, Denmark) for 60 min at 37 °C. After washing in PBS, a secondary antibody (Dako, Copenhagen, Denmark) was applied for 60 min followed by the addition of horseradish peroxidase conjugated streptavidin for 60 min. The reaction was visualized with 3,3'-diaminobenzidine (DAB) chromagen (Dako, Copenhagen, Denmark). The slides were then counterstained with hematoxylin, mounted, and examined. GFAP immunoreactive percentage areas in individual sections were traced and measured using an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA).

Electron Microscopic Examination

Small pieces of striatum were separated, rinsed in ice-cold saline, and cut into fragments (diameter=1 mm). Fragments were rapidly fixed overnight in 2.5 % glutaraldehyde prepared in 0.1 M sodium phosphate buffer, pH 7, at 4 °C. They were postfixed in 1 % osmium tetroxide for 2 h and then dehydrated with graded series of alcohol solutions. Tissues were embedded in epoxy resin and ultrathin sections (50 nm) were mounted on copper mesh grids, and stained with uranyl acetate and lead citrate before being examined with a JEOL JEM-1400 electron microscope (Jeol Ltd., Tokyo, Japan) and photographed. Electron micrographs were taken systematically at \times 10,000 magnification. Images were analyzed by two investigators. Percentage of altered mitochondria was calculated after examining the morphology of about 200 mitochondria in different areas for each animal and average value was calculated for each group [32].

Statistical Analysis

All data obtained were presented as mean \pm SEM. Results were analyzed using one-way analysis of variance test (one-way ANOVA) followed by Tukey's multiple comparison test for all parameters except neurological scores, ambulation frequency, and rearing frequency which were done using nonparametric one-way ANOVA followed by Dunn's multiple comparison test. Statistical analysis was performed using GraphPad Prism software (version 6.04). For all the statistical tests, the level of significance was fixed at $p < 0.05$.

Results

Effect of Fasudil and Simvastatin on 3-NP-Induced Changes in Mortality as Well as Body and Striata Weights

3-NP induced an increase in mortality (33.33 %) together with a significant decrease in final body and striata weights. Fasudil and simvastatin significantly decreased mortality to 8.33 and 16.67 %, respectively. Moreover, both treatments completely alleviated changes in final body and striata weights (Table 1).

Effect of Fasudil and Simvastatin on 3-NP-Induced Behavioral and Motor Changes

3-NP intoxication caused behavioral and motor abnormalities as demonstrated using neurological score as well as open field, rotarod performance, limb withdrawal and string strength tests. Both fasudil and simvastatin treatments significantly ameliorated the aforementioned behavioral and motor changes (Table 1).

Effect of Fasudil and Simvastatin on 3-NP-Induced Changes in SDH Activity, Mitochondrial Membrane Potential, and PGC-1 α Protein Expression

3-NP-treated group showed severe mitochondrial dysfunction as demonstrated by the significant decrease in SDH activity, mitochondrial membrane potential, and PGC-1 α protein expression. Treatment with fasudil caused a significant increase in SDH activity and reverted mitochondrial membrane potential and PGC-1 α protein expression back to normal values. Meanwhile, simvastatin-treated group showed only a significant increase in mitochondrial membrane potential and PGC-1 α protein expression as compared to 3-NP group (Fig. 1).

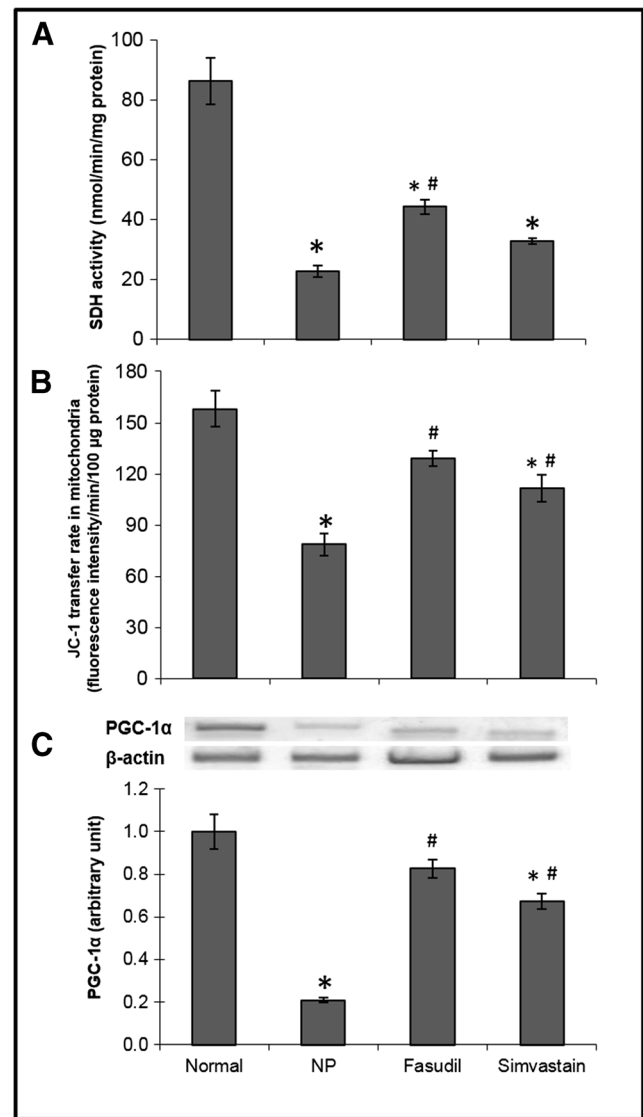


Fig. 1 Effect of fasudil and simvastatin on 3-NP-induced changes in SDH activity (A), mitochondrial membrane potential (B), and PGC-1 α protein expression (C). Each value represents the mean of five to eight experiments \pm SEM. * p <0.05 vs. normal, # p <0.05 vs. NP

Table 1 Effect of Fasudil and Simvastatin on 3-NP induced changes in mortality as well as body and striata weights, neurological score, open field, rotarod, limb withdrawal and string tests

Groups	Mortality %	Final body weight (g)	Striata weight (g)	Neurological score	Open field test		Rotarod test (s)	Limb withdrawal test (s)	String test (s)
					Ambulation frequency	Rearing frequency			
Normal	0 %	204.29 \pm 3.50	0.131 \pm 0.007	0.00 \pm 0.00	86.2 \pm 9.65	17.8 \pm 1.53	178.50 \pm 1.15	1.00 \pm 0.00	81.17 \pm 6.91
NP	33.33 %	176.57 \pm 3.71*	0.094 \pm 0.008*	5.38 \pm 0.71*	11.57 \pm 2.79*	2.14 \pm 0.88*	19.86 \pm 4.63*	7.63 \pm 1.03*	10.13 \pm 1.97*
Fasudil	8.33 %	197.75 \pm 7.12 [#]	0.118 \pm 0.006 [#]	0.38 \pm 0.18 [#]	53.00 \pm 9.18 [#]	8.71 \pm 1.89 [#]	100.25 \pm 17.95 [#]	1.37 \pm 0.26 [#]	41.44 \pm 5.98 [#]
Simvastatin	16.67 %	203.50 \pm 3.77 [#]	0.126 \pm 0.007 [#]	0.88 \pm 0.29 [#]	48.66 \pm 3.79 [#]	6.33 \pm 1.15 [#]	123.43 \pm 20.11 [#]	1.62 \pm 0.32 [#]	79.43 \pm 9.53 [#]

Each value represents the mean of 8-11 experiments \pm S.E.M. * p <0.05 vs. normal, [#] p <0.05 vs. NP

Effect of Fasudil and Simvastatin on 3-NP-Induced Changes in ROCK, p-Akt, and eNOS Protein Expressions

Treatment with 3-NP caused a marked increase in ROCK protein expression and a significant decrease in both p-Akt and eNOS protein expressions. Simvastatin treatment significantly decreased ROCK protein expression whereas it significantly increased both p-Akt and eNOS protein expressions. On the other hand, fasudil succeeded to provide more amelioration regarding p-Akt protein expression (Fig. 2).

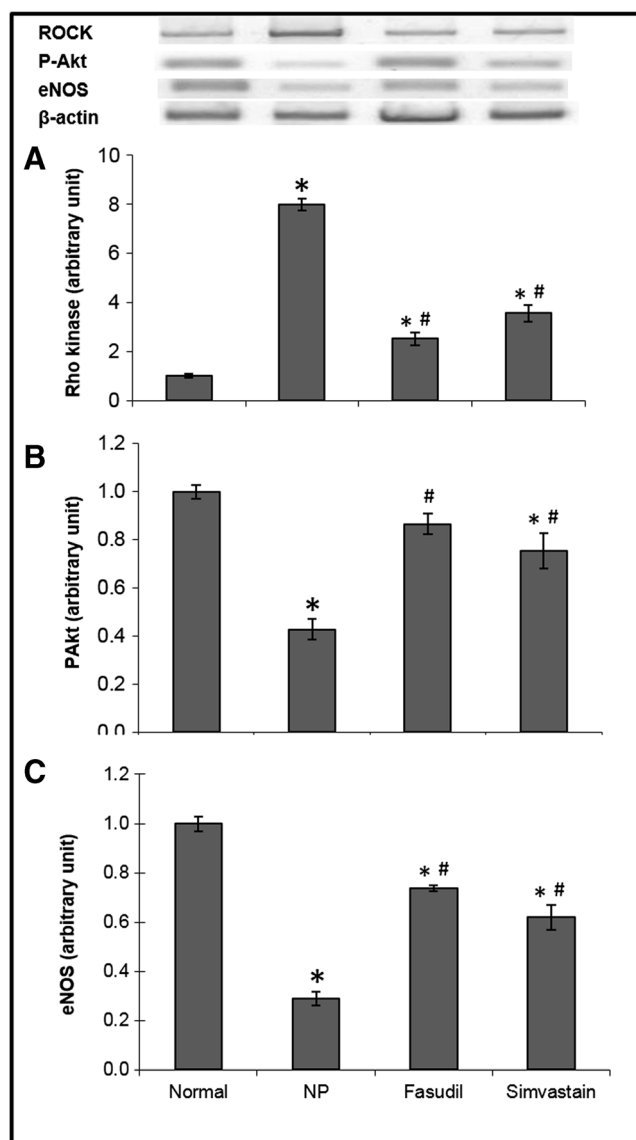


Fig. 2 Effect of fasudil and simvastatin on 3-NP-induced changes in ROCK (A), p-Akt (B), and eNOS (C) protein expressions. Each value represents the mean of five to eight experiments \pm SEM. * $p < 0.05$ vs. normal, # $p < 0.05$ vs. NP

Effect of Fasudil and Simvastatin on 3-NP-Induced Changes in iNOS Protein Expression and TNF- α Level

3-NP induced a state of inflammation as demonstrated by the significant elevation of iNOS protein expression and TNF- α level. Both fasudil and simvastatin succeeded to normalize iNOS protein expression and TNF- α level (Fig. 3).

Effect of Fasudil and Simvastatin on 3-NP-Induced Changes in Caspase-3 Activity as Well as Bax and Bcl-2 Protein Expressions

The previously mentioned state of inflammation in 3-NP group was associated with elevation of apoptotic markers as demonstrated by the significant increase in caspase-3 activity and Bax protein expression together with the significant decrease in Bcl2 protein expression. Both fasudil and simvastatin normalized caspase-3 activity and Bax protein expression. On the other hand, Bcl-2 protein expression was significantly reduced with simvastatin treatment and completely normalized with fasudil treatment (Fig. 4).

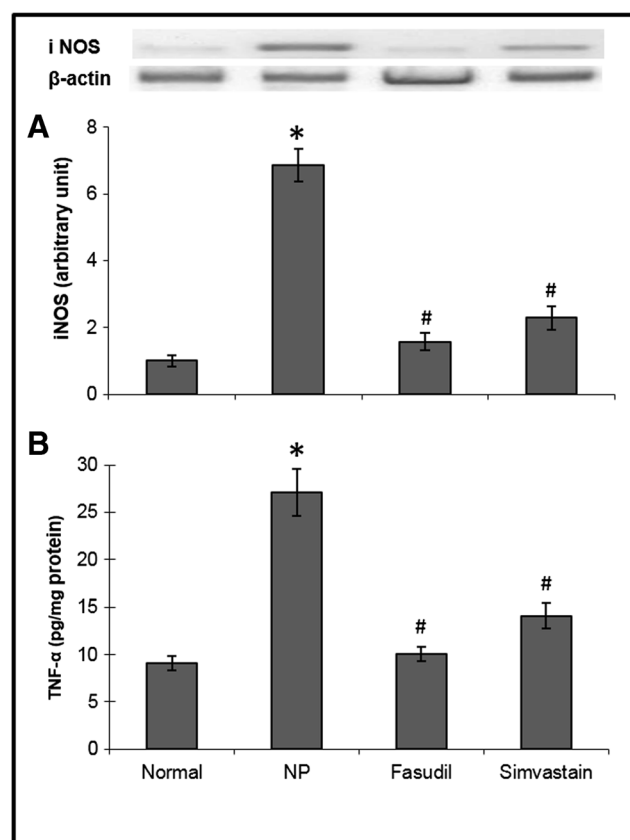


Fig. 3 Effect of fasudil and simvastatin on 3-NP induced changes in iNOS protein expression (A) and TNF- α level (B). Each value represents the mean of five to eight experiments \pm SEM. * $p < 0.05$ vs. normal, # $p < 0.05$ vs. NP

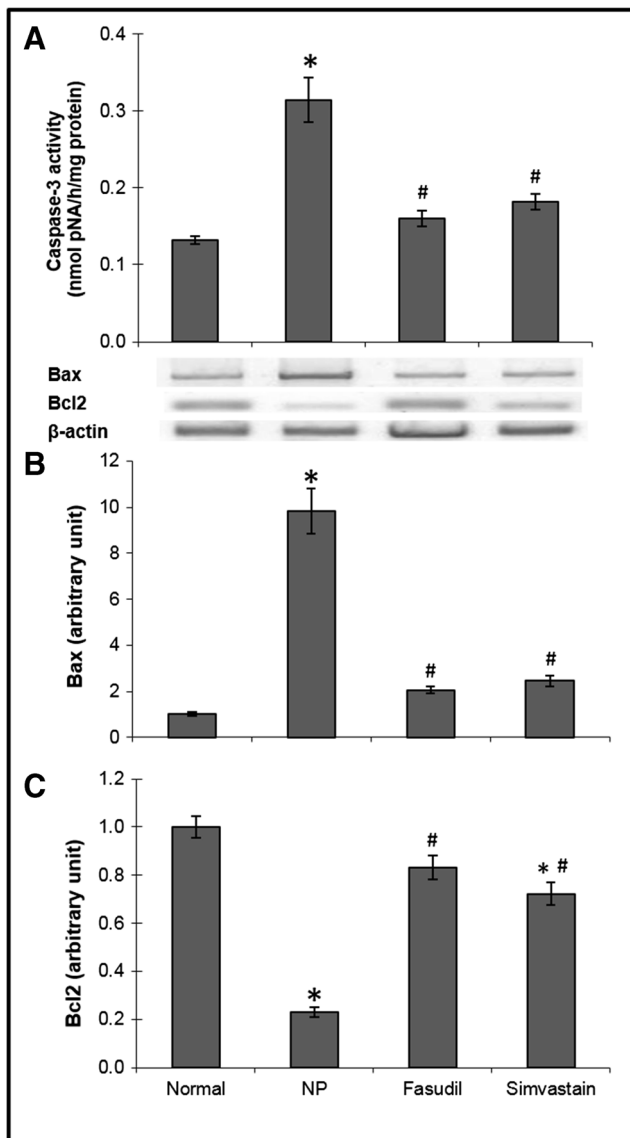


Fig. 4 Effect of Fasudil and Simvastatin on 3-NP-induced changes in caspase-3 activity (A) as well as Bax (B) and Bcl-2 (C) protein expressions. Each value represents the mean of 5–8 experiments \pm S.E.M. * $p < 0.05$ vs. normal, # $p < 0.05$ vs. NP

Effect of Fasudil and Simvastatin on 3-NP-Induced Histological Changes

3-NP group showed a marked elevation of striatum injury score as evidenced by remarkable neuronal degeneration and edema. However, neutrophil infiltration was unremarkable. Extensive astroglial activation was also demonstrated by a marked increase in GFAP immunoreactivity. Additionally, 3-NP induced considerable mitochondrial damage as revealed by the significant increase in percentage of altered mitochondria. Treatment with simvastatin significantly ameliorated striatum injury score and astroglial activation as well as percentage of altered mitochondria compared to 3-NP group. On the

other hand, fasudil treatment more or less normalized the aforementioned parameters (Fig. 5).

Discussion

3-NP-induced neurotoxicity is an experimental model which induces severe striatal damage over several days. It is used as an experimental model of neurodegeneration, which mimics some of the pathology seen in HD in human [33]. The aim of the present work was to address the role of ROCK inhibition in the possible protective effect of fasudil and simvastatin in 3-NP-induced striatal neurodegeneration and mitochondrial dysfunction in rats.

In the current study, 3-NP induced a significant increase in mortality together with a significant decrease in final body and striata weights. The decrease in body weight could be related to anorexia and decreased food intake associated with motor deficit in 3-NP group [31]. Following 3-NP intoxication, behavioral and motor abnormalities were also evidenced using neurological score as well as open field, rotarod performance, limb withdrawal and string strength tests. Earlier studies have demonstrated that 3-NP treatment produced significant reduction in spontaneous locomotor activity, loss of grip strength, and increase in retraction time in limb withdrawal tests [34, 35], indicating striatal degeneration and motor impairment [26]. Furthermore, the current findings are in tune with the report of Kumar et al. [36] which showed that 3-NP administration reduced the locomotor and rotarod activities in rats, suggesting that the effects of 3-NP most probably mimic the late stages of HD-like behavior. Deficiencies in behavioral and motor activities could be related to excessive generation of free radicals and increased brain protein oxidation [37] that might also contribute to the onset of symptoms associated with HD and other movement disorders, such as dystonias and Parkinsonism [38]. Importantly, neurological disorders and the decrease in striata weight, observed herein, were correlated with the histological changes evidenced by increased striatum injury score and marked astroglial activation that in turn could provide the underlying structural basis for the neurological deficits.

3-NP-treated group showed severe mitochondrial dysfunction as manifested by the significant decrease in SDH activity, mitochondrial membrane potential, and PGC-1 α protein expression. These biochemical alterations are consistent with mitochondrial ultrastructural changes as evidenced by significant increase in percentage of altered mitochondria. Previously, 3-NP has been demonstrated to produce an early loss of the mitochondrial membrane potential using cultured neurons [39]. 3-NP, being an irreversible inhibitor of SDH, the principal component of mitochondrial complex II, impairs energy production leading to neurodegeneration. Interestingly, striatal neurons are highly sensitive to impairment in energy metabolism which could explain the link between mitochondrial

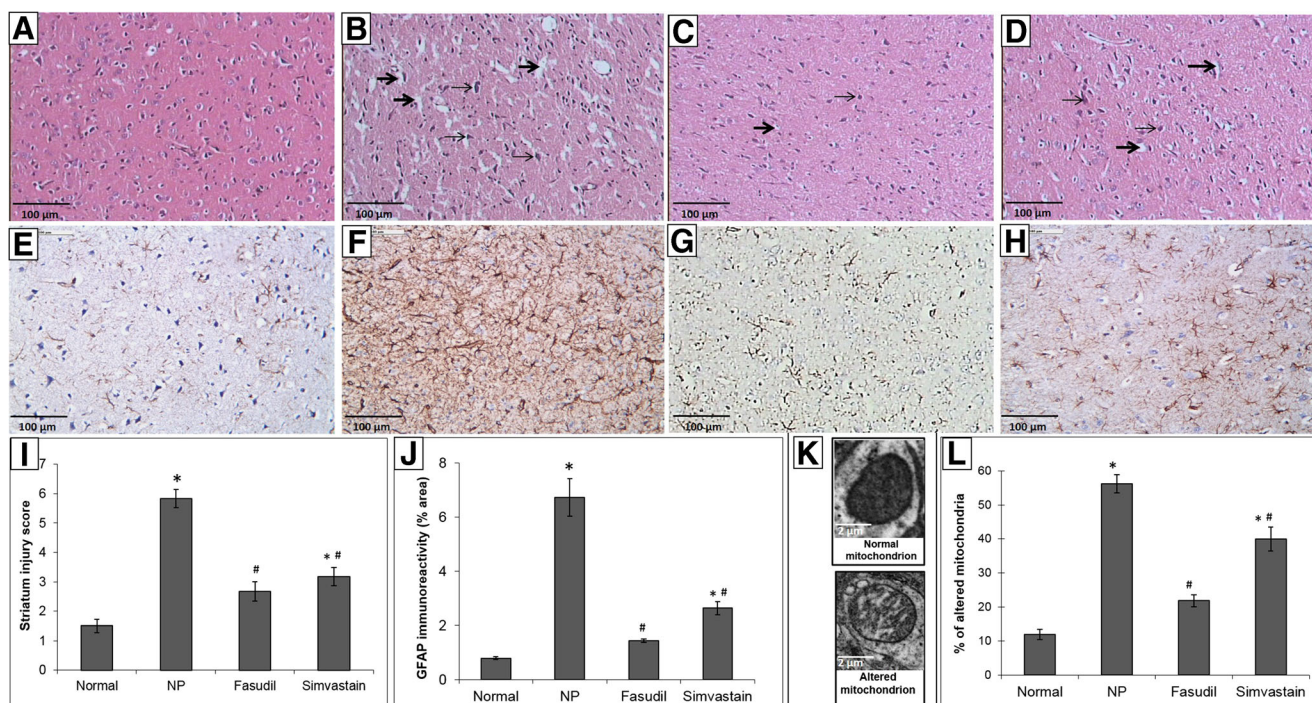


Fig. 5 Effect of fasudil and simvastatin on 3-NP-induced histological changes. **A–D** Specimens stained with H&E $\times 100$. **A** Normal group showed no neuronal loss and neuropil with unremarkable changes. **B** NP group showed extensive neuronal degeneration (*thin short arrow*), marked perivascular and perineuronal edema (*thick short arrow*), and glial proliferation. **C** Fasudil group showed minimal neuronal degeneration (*thin short arrow*) and focal edema (*thick short arrow*). **D** Simvastatin group showed moderate neuronal degeneration (*thin short arrow*) and evident edema (*thick short arrow*). **E–H** Specimens stained with GFAP $\times 100$. **E** Normal group showed normal GFAP immunostaining of the

inactivated astrocytes. **F** NP group showed marked increase in reactivity (strong and diffused brown GFAP immunostaining) of the activated proliferated plump astrocytes. **G** Fasudil group showed mild increase in reactivity of the activated proliferated astrocytes. **H** Simvastatin group showed moderate increase in reactivity of the activated proliferated astrocytes. **I** Striatum injury score. **J** Image analysis of GFAP immunoreactivity (% area). **K** Normal and altered mitochondria. **L** Percentage (%) of altered mitochondria. Each value represents the mean results of three animals \pm SEM. * $p < 0.05$ vs. normal, # $p < 0.05$ vs. NP

defects and the preferential vulnerability of the striatum to acute poisoning with mitochondrial toxins (cyanide, sodium azide, and 3NP) in experimental and clinical studies [40].

Furthermore, reduction of PGC-1 α protein expression and disruption of mitochondrial transmembrane potential in 3-NP group could indicate an impairment of mitochondrial function that precedes the initiation of apoptosis. PGC-1 α plays a central role in regulating the expression of mitochondrial genes involved in a wide variety of biological responses, including mitochondrial biogenesis in brain tissues [41]. Formerly, positive correlations between PGC-1 α and mitochondrial bioenergetics in neurodegenerative progression were documented in experimental and clinical studies [42–44].

Treatment with fasudil or simvastatin showed significant decrease in mortality together with significant improvement in body and striata weights as well as behavioral and motor abnormalities indicating amelioration of 3-NP-induced neurotoxicity and neurological deficit. In an experimental model of Parkinson's disease, fasudil treatment exhibited a marked improvement in motor performance which was correlated with inhibition of ROCK activity [45]. Blocking the ROCK pathway has been reported to markedly inhibit the polyQ

protein aggregation and decrease its toxicity in *Drosophila* model of HD [9]. On the other hand, simvastatin was previously shown to protect striatal neurons and to enhance motor functions in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice [46].

Concerning mitochondrial function, treatment with fasudil caused a significant increase in SDH activity and reverted mitochondrial membrane potential and PGC-1 α protein expression back to normal values. Meanwhile, simvastatin-treated group showed only a significant increase in mitochondrial membrane potential and PGC-1 α protein expression. Fasudil, a selective ROCK inhibitor, has previously increased SDH activity, improved the structures of cardiac mitochondria, and inhibited the dissipation of mitochondrial transmembrane potential in rats with type 2 diabetes [13]. Similarly, simvastatin attenuated oxidant-induced mitochondrial dysfunction in cardiac myocytes through a “mitohormesis mechanism” involving the preservation of mitochondrial membrane [47].

ROCK may play a crucial role in regulating biological events of cells, including proliferation, differentiation, and survival/death [6]. Inhibition of ROCK activation has been

shown to promote axonal regeneration and neuron survival in various *in vivo* and *in vitro* studies, thereby considered as an efficacious approach for generating neuroprotection in several neurological disorders [7]. Herein, 3 NP-induced ROCK activation was significantly suppressed following fasudil and simvastatin administration. Beforehand, experimental spinal cord injury was associated with increase in ROCK activity which was markedly attenuated by fasudil treatment [48]. It is worthy to note that the potent ROCK inhibition by fasudil could be mediated via competition for ATP binding site in the ROCK catalytic domain [49]. On the other hand, the effect of simvastatin could be attributed to pleiotropic effects of statins via reduction of isoprenoids. Blocking the isoprenylation of Rho family GTPases (i.e., RhoA) causes its accumulation in the cytosol and hence its inactivation, which prevents ROCK signaling [17].

Akt survival pathway has been identified as an important molecular mediator for neuroprotective effects of ROCK inhibition [45]. In the present study, inhibition of ROCK by fasudil or simvastatin increased Akt phosphorylation and up-regulated eNOS protein expression. In harmony, Tönges et al. [45] have stated that the neuroprotective effect of fasudil was mediated via the activation of Akt signaling after ROCK inhibition, implying its vital role in the regulation of both neuronal survival and integrity in a mouse model of Parkinson's disease. Similarly, atorvastatin, as an example of statins, was reported to induce the phosphorylation of Akt in cultured primary cortical neurons promoting neurogenesis and angiogenesis after an ischemic insult [50]. Progressive alterations of Akt have been reported to occur during neuronal dysfunction or prior neurodegeneration [51]. Interestingly, Akt activation in HD has been shown to increase the phosphorylation of mutant huntingtin and abolish its toxic effects [52, 53].

It is worthy to note that ROCK activation, as seen in 3-NP-treated group, not only downregulates eNOS expression by decreasing its mRNA stability but it also inhibits eNOS phosphorylation and activity [54]. Alternatively, ROCK inhibition leads to rapid phosphorylation and activation of Akt via PI3K, which in turn activates eNOS and increases NO production. Moreover, the neuroprotective effects of ROCK inhibitors are absent in eNOS knockout mice, indicating the critical role of eNOS in mediating the beneficial effects of ROCK inhibition [17]. In this context, several studies have documented that both fasudil and statins, by inactivating ROCK, lead to increased Akt phosphorylation, eNOS expression and activity [10, 55].

Despite the important role of NO in neurophysiological function, NO in high concentration can act paradoxically as a neurotoxin primarily due to its oxidative properties and its ability to produce peroxynitrite, a highly destructive reactive oxygen species [56]. High levels of peroxynitrite lead to neuronal cell death by causing typical free radical damages and energy depletion secondary to mitochondrial impairment. Activation of iNOS and peroxynitrite in response to a number of

proinflammatory mediators has been implicated in a number of CNS disorders, such as cerebral ischemia, Alzheimer's disease, and HD, contributing to neuronal death [57–59].

In the present study, decrease in eNOS protein expression was associated with significant increase in both iNOS protein expression and TNF- α level in 3-NP group. Downregulated eNOS protein expression by ROCK activation plays a key role in the activation of the inflammatory cascade due to decreased endothelium-derived NO which facilitates increased neutrophil adhesion [60]. However, the preservation of eNOS by ROCK inhibition, as demonstrated herein by fasudil and simvastatin, may block early leukocytes adhesion and consequently diminish the local production of proinflammatory cytokines.

Fasudil, the only ROCK inhibitor used clinically, has well established anti-inflammatory and immunomodulatory benefits [12]. The administration of fasudil protected against ischemia-induced neuronal cell loss in mice via reducing the proinflammatory factors such as interleukin-1 beta (IL-1 β), IL-6, and TNF- α [6]. Statins were also shown to exert anti-inflammatory effects by regulating proinflammatory molecules in astrocytes and macrophages [61]. This effect was proposed to be due to nuclear factor kappa B inhibition as a direct consequence of ROCK inhibition and p-Akt activation which would lead to reduction of proinflammatory cytokines secretion [62].

Furthermore, statin, as previously mentioned, could attenuate ROCK activation and the subsequent inflammatory response in CNS via lowering of isoprenoids [17]. Owing to their antioxidant and anti-inflammatory effects, statins could block not only ROS-mediated brain damage but also the release of proinflammatory cytokines as well as NO synthesis [63]. Thus, statins increase the beneficial NO production by eNOS, while reducing NO overproduction by iNOS.

This encountered state of inflammation in 3-NP group was associated with increased apoptotic machinery as evidenced by the significant increase in caspase-3 activity and Bax protein expressions together with the significant decrease in Bcl-2 protein expression. ROCK activation may act as an upstream event that is involved in the neuronal apoptosis. Importantly, ROCK-induced caspase activation is initiated by an early ROCK-dependent increase in Bax expression. Bax upregulation appears to be sufficient to shift the balance in Bcl-2 protein expression, induce mitochondrial permeabilization, initiate activation of caspase-3, and ultimately induce cell death [64]. Moreover, iNOS overexpression and increased TNF- α level, as shown in this study, may contribute to neuronal apoptosis. iNOS upregulation causes reduction in mitochondrial membrane potential and strongly induces neuronal apoptosis via NO-induced activation of caspase-3 leading to apoptotic cell death [65, 66]. Concurrently, proinflammatory cytokines such as TNF- α act directly on neurons to induce apoptosis [67].

The current data showed a significant amelioration of apoptotic markers following both fasudil and simvastatin

treatments. Fasudil has been previously demonstrated to alleviate neuronal apoptosis and caspase-3 activation with improvement of neurological deterioration in brain ischemic injury through ROCK/Akt pathway [68]. ROCK cleavage is an essential step for apoptosis given that pharmacological inhibition of its kinase activity effectively abrogates apoptosis in a number of cell types [69]. Moreover, the increase of Akt activity by ROCK inhibition may contribute to the antiapoptotic effect of fasudil. Activated Akt promotes cell survival and suppresses apoptosis by phosphorylation and inhibition of several downstream substrates [70]. It is worthy to note that the anti-inflammatory effect of fasudil may also help to explain its potent antiapoptotic effect.

On the other side, Hunt et al. [71] have reported that statins treatment activated the antiapoptotic signaling pathways causing a reduction in caspase-3 expression and thereby promoting neuroprotection. Expression of the prosurvival molecule, Bcl-2, was also shown to be upregulated in neurons upon *in vitro* and *in vivo* treatments with simvastatin [71, 72]. As with fasudil, Akt pathway seems to play a crucial role in the antiapoptotic effect of simvastatin in the present study.

In conclusion, this study revealed for the first time the role of ROCK/p-Akt/eNOS pathway in the protective effects of fasudil and simvastatin in 3-NP-induced neurotoxicity and mitochondrial dysfunction in rats. This protection was manifested by amelioration of behavioral, biochemical and histological changes. Inhibition of ROCK activity led to the maintenance of Akt activity, with subsequent amelioration of mitochondrial function, eNOS and iNOS protein expressions as well as inflammatory and apoptotic markers. Thus, specific inhibition of ROCK may be considered a promising new approach in the management of HD.

References

- Ludolph AC, He F, Spencer PS et al (1991) 3-Nitropropionic acid exogenous animal neurotoxin and possible human striatal toxin. *Can J Neurol Sci* 18:492–498
- Brouillet E, Jacquard C, Bizat N et al (2005) 3-Nitropropionic acid: a mitochondrial toxin to uncover physiopathological mechanisms underlying striatal degeneration in Huntington's disease. *J Neurochem* 95:1521–1540
- Rosenstock TR, Duarte AI, Rego AC (2010) Mitochondrial-associated metabolic changes and neurodegeneration in Huntington's disease - from clinical features to the bench. *Curr Drug Targets* 11:1218–1236
- Brouillet E (2014) The 3-NP model of striatal neurodegeneration. *Curr Protoc Neurosci* 67:9.48.1–9.48.14
- Borlongan CV, Nishino H, Sanberg PR (1997) Systemic, but not intraparenchymal, administration of 3-nitropropionic acid mimics the neuropathology of Huntington's disease: a speculative explanation. *Neurosci Res* 29:185–189
- Ding J, Li QY, Wang X et al (2010) Fasudil protects hippocampal neurons against hypoxia-reoxygenation injury by suppressing microglial inflammatory responses in mice. *J Neurochem* 114(6):1619–1629
- Mueller BK, Mack H, Teusch N (2005) Rho kinase, a promising drug target for neurological disorders. *Nat Rev Drug Discov* 4:387–398
- Labandeira-Garcia JL, Rodríguez-Perez AI, Villar-Cheda B et al. (2014) Rho Kinase and Dopaminergic Degeneration: a promising therapeutic target for parkinson's disease. *Neuroscientist* [Epub ahead of print]
- Pollitt SK, Pallos J, Shao J et al (2003) A rapid cellular FRET assay of polyglutamine aggregation identifies a novel inhibitor. *Neuron* 40:685–694
- Wolfrum S, Dendorfer A, Rikitake Y et al (2004) Inhibition of Rho-kinase leads to rapid activation of phosphatidylinositol 3-kinase/protein kinase Akt and cardiovascular protection. *Arterioscler Thromb Vasc Biol* 24:1842–1847
- Omeis I, Jayson NA, Murali R et al (2008) Treatment of cerebral vasospasm with biocompatible controlled release systems for intracranial drug delivery. *Neurosurgery* 63(6):1011–1019
- Olson MF (2008) Applications for ROCK kinase inhibition. *Curr Opin Cell Biol* 20(2):242–248
- Guo R, Liu B, Zhou S et al (2013) The protective effect of fasudil on the structure and function of cardiac mitochondria from rats with type 2 diabetes induced by streptozotocin with a high-fat diet is mediated by the attenuation of oxidative stress. *Biomed Res Int* 2013:430791
- Zhao J, Zhou D, Guo J et al (2011) Fasudil Aneurysmal Subarachnoid Hemorrhage Study Group. Efficacy and safety of fasudil in patients with subarachnoid hemorrhage: final results of a randomized trial of fasudil versus nimodipine. *Neurol Med Chir (Tokyo)* 51(10):679–683
- Li Q, Huang XJ, He W et al (2009) Neuroprotective potential of fasudil mesylate in brain ischemia-reperfusion injury of rats. *Cell Mol Neurobiol* 29:169–180
- Van der Most PJ, Dolga AM, Nijholt IM et al (2009) Statins: mechanisms of neuroprotection. *Prog Neurobiol* 88(1):64–75
- Rikitake Y, Liao JK (2005) Rho GTPases, statins, and nitric oxide. *Circ Res* 97(12):1232–1235
- Yang D, Knight RA, Han Y et al (2013) Statins protect the blood brain barrier acutely after experimental intracerebral hemorrhage. *J Behav Brain Sci* 3(1):100–106
- Beal MF, Brouillet E, Jenkins BG et al (1993) Neurochemical and Histologic characterization of Striatal excitotoxin lesions produced by mitochondrial toxin 3-nitropropionic acid. *J Neurosci* 13:4181–4192
- Jiang BH, Tawara S, Abe K et al (2007) Acute vasodilator effect of fasudil, a Rho-kinase inhibitor, in monocrotaline-induced pulmonary hypertension in rats. *J Cardiovasc Pharmacol* 49(2):85–89
- Zhao H, Ji Z, Tang D et al (2013) Role of autophagy in early brain injury after subarachnoid hemorrhage in rats. *Mol Biol Rep* 40(2):819–827
- Mittoux V, Ouary S, Monville C et al (2002) Corticostriatopallidal neuroprotection by adenovirus-mediated Ciliary Neurotrophic factor gene transfer in a rat model of progressive Striatal degeneration. *J Neurosci* 22:4478–4486
- Bantubungi K, Jacquard C, Greco A et al (2005) Minocycline in phenotypic models of Huntington's disease. *Neurobiol Dis* 18:206–217
- Moreira EL, Rial D, Duarte FS et al (2010) Central nervous system activity of the proanthocyanidin-rich fraction obtained from croton celtidifolius in rats. *J Pharm Pharmacol* 62:1061–1068
- Avila DS, Colle D, Gubert P et al (2010) A possible neuroprotective action of a vinylic telluride against Mn-induced neurotoxicity. *Toxicol Sci* 115:194–201
- Vis JC, Verbeek MM, De Waal RMW et al (1999) 3-nitropropionic acid induces a spectrum of Huntington's disease-like neuropathology in rat striatum. *Neuropathol Appl Neurobiol* 25:513–521

27. Shear DA, Dong J, Gundy CD et al (1998) Comparison of Intrastratial injections of quinolinic acid and 3-nitropropionic acid for use in animal models of Huntington's disease. *Prog Neuro-Psychopharmacol Biol Psychiatry* 22:1217–1240
28. Lowry OH, Rosebrough NJ, Farr AL et al (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
29. Sharman EH, Bondy SC (2001) Effects of age and dietary antioxidants on cerebral electron transport chain activity. *Neurobiol Aging* 22:629–634
30. Ahmed LA, Shehata NI, Abdelkader NF et al (2014) Tempol, a superoxide dismutase mimetic agent, ameliorates cisplatin-induced nephrotoxicity through alleviation of mitochondrial dysfunction in mice. *PLoS One* 9(10), e108889
31. Woodruff TM, Crane JW, Proctor LM et al (2006) Therapeutic activity of C5a receptor antagonists in a rat model of neurodegeneration. *FASEB J* 20(9):1407–1417
32. Vega-Núñez E, Alvarez AM, Menéndez-Hurtado A et al (1997) Neuronal mitochondrial morphology and transmembrane potential are severely altered by hypothyroidism during rat brain development. *Endocrinology* 138(9):3771–3778
33. Ouay S, Bizat N, Altaïrac S et al (2000) Major strain differences in response to chronic systemic administration of the mitochondrial toxin 3-nitropropionic acid in rats: implications for neuroprotection studies. *Neuroscience* 97:521–530
34. Shear DA, Haik KL, Dunbar GL (2000) Creatine reduces 3-nitropropionic-acid-induced cognitive and motor abnormalities in rats. *Neuroreport* 11(9):1833–1837
35. Keene CD, Rodríguez CM, Eich T et al (2001) A bile acid protects against motor and cognitive deficits and reduces Striatal degeneration in the 3-nitropropionic acid model of Huntington's disease. *Exp Neurol* 171(2):351–360
36. Kumar P, Kalonia H, Kumar A (2011) Role of LOX/COX pathways in 3-nitropropionic acid-induced Huntington's disease-like symptoms in rats: protective effect of licoferone. *Br J Pharmacol* 164(2):644–654
37. Forster MJ, Dubey A, Dawson KM et al (1996) Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. *Proc Natl Acad Sci U S A* 93(10):4765–4769
38. Mandavilli BS, Boldogh I, Van Houten B (2005) 3-nitropropionic acid-induced hydrogen peroxide, mitochondrial DNA damage, and cell death are attenuated by Bcl-2 overexpression in PC12 cells. *Brain Res Mol Brain Res* 133(2):215–223
39. Maciel EN, Kowaltowski AJ, Schwalm FD et al (2004) Mitochondrial permeability transition in neuronal damage promoted by Ca^{2+} and respiratory chain complex II inhibition. *J Neurochem* 90:1025–1035
40. Wani TA, Al-Omara MA, Zargarb S (2011) Huntington disease: current advances in pathogenesis and recent therapeutic strategies. *Int J Pharm Sci Drug Res* 3(2):69–79
41. Liang H, Ward WF (2006) PGC-1 alpha: a key regulator of energy metabolism. *Adv Physiol Educ* 30:145–151
42. Cui L, Jeong H, Borovecki F et al (2006) Transcriptional repression of PGC-1alpha by mutant huntingtin leads to mitochondrial dysfunction and neurodegeneration. *Cell* 127:59–69
43. Weydt P, Pineda VV, Torrence AE et al (2006) Thermoregulatory and metabolic defects in Huntington's disease transgenic mice implicate PGC 1alpha in Huntington's disease neurodegeneration. *Cell Metab* 4:349–362
44. Yadav A, Agarwal S, Tiwari SK et al (2014) Mitochondria: prospective targets for neuroprotection in Parkinson's disease. *Curr Pharm Des* 20(35):5558–5573
45. Tönges L, Frank T, Tatenhorst L et al (2012) Inhibition of rho kinase enhances survival of dopaminergic neurons and attenuates axonal loss in a mouse model of Parkinson's disease. *Brain* 135: 3355–3370
46. Selley ML (2005) Simvastatin prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- induced striatal dopamine depletion and protein tyrosine nitration in mice. *Brain Res* 1037:1–6
47. Bouitbir J, Charles AL, Echaniz-Laguna A et al (2012) Opposite effects of statins on mitochondria of cardiac and skeletal muscles: a 'mitohormesis' mechanism involving reactive oxygen species and PGC-1. *Eur Heart J* 33(11):1397–1407
48. Impellizzeri D, Mazzon E, Paterniti I et al (2012) Effect of fasudil, a selective inhibitor of Rho kinase activity, in the secondary injury associated with the experimental model of spinal cord trauma. *J Pharmacol Exp Ther* 343(1):21–33
49. Narumiya S, Ishizaki T, Uehata M (2000) Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol* 325: 273–284
50. Zacco A, Togo J, Spence K et al (2003) 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors protect cortical neurons from excitotoxicity. *J Neurosci* 23:11104–11111
51. Colin E, Regulier E, Perrin V et al (2005) Akt is altered in an animal model of Huntington's disease and in patients. *Eur J Neurosci* 21: 1478–1488
52. Humbert S, Bryson EA, Cordelieres FP et al (2002) The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves huntingtin phosphorylation by Akt. *Dev Cell* 2:831–837
53. Rangone H, Pardo R, Colin E et al (2005) Phosphorylation of arfaptin 2 at Ser260 by Akt inhibits polyQ-huntingtin-induced toxicity by rescuing proteasome impairment. *J Biol Chem* 280: 22021–22028
54. Laufs U, Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* 273(37):24266–24271
55. Endres M, Laufs U, Huang Z et al (1998) Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 95:8880–8885
56. Beckman JS, Chen J, Ischiropoulos H et al (1994) Oxidative chemistry of peroxynitrite. *Methods Enzymol* 233:229–240
57. Vodovotz Y, Lucia MS, Flanders KC et al (1996) Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. *J Exp Med* 184:1425–1433
58. Iadecola C (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci* 20:132–139
59. Hanna DM, Tadros MG, Khalifa AE (2015) ADIOL protects against 3-NP-induced neurotoxicity in rats: Possible impact of its anti-oxidant, anti-inflammatory and anti-apoptotic actions. *Prog Neuropsychopharmacol Biol Psychiatry* 60:36–51
60. Harbrecht BG, Wu B, Watkins SC et al (1997) Inhibition of nitric oxide synthesis during severe shock but not after resuscitation increases hepatic injury and neutrophil accumulation in hemorrhaged rats. *Shock* 8:415–421
61. Menge T, Hartung HP, Stuve O (2005) Statins—a cure-all for the brain? *Nat Rev Neurosci* 6:325–331
62. He Y, Xu H, Liang L et al (2008) Antiinflammatory effect of Rho kinase blockade via inhibition of NF-kappaB activation in rheumatoid arthritis. *Arthritis Rheum* 58:3366–3376
63. Cordle A, Landreth G (2005) 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors attenuate β -amyloid-induced microglial inflammatory responses. *J Neurosci* 25: 299–307
64. Del Re DP, Miyamoto S, Brown JH (2007) RhoA/Rho kinase up-regulate Bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. *J Biol Chem* 282(11):8069–8078

65. Nomura Y (2004) Neuronal apoptosis and protection: effects of nitric oxide and endoplasmic reticulum-related proteins. *Biol Pharm Bull* 27:961–963
66. Sen N, Hara MR, Kornberg MD et al (2008) Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Nat Cell Biol* 10:866–873
67. McCoy MK, Tansey MG (2008) TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *J Neuroinflammation* 5:45
68. Wu J, Li J, Hu H et al (2012) Rho-kinase inhibitor, fasudil, prevents neuronal apoptosis via the Akt activation and PTEN inactivation in the ischemic penumbra of rat brain. *Cell Mol Neurobiol* 32(7):1187–1197
69. Street CA, Bryan BA (2011) Rho kinase proteins—pleiotropic modulators of cell survival and apoptosis. *Anticancer Res* 31(11):3645–3657
70. Kelly S, Zhao H, Hua Sun G et al (2004) Glycogen synthase kinase-3 beta inhibitor Chir025 reduces neuronal death resulting from oxygen–glucose deprivation, glutamate excitotoxicity, and cerebral ischemia. *Exp Neurol* 188:378–386
71. Hunt WT, Salins PB, Anderson CM et al (2010) Neuroprotective role of statins in Alzheimer’s disease: anti-apoptotic signaling. *Open Neurosci J* 4:13–22
72. Franke C, Noldner M, Abdel-Kader R et al (2007) Bcl-2 upregulation and neuroprotection in guinea pig brain following chronic simvastatin treatment. *Neurobiol Dis* 25:438–445