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Sodium Salicylate Protects Against Rotenone-Induced Parkinsonism in Rats

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ABSTRACT Complex I deficiency culminating in oxidative stress is proposed as one of the upstream mechanisms of nigral neuronal death in Parkinson's disease. We investigated whether sodium salicylate, an active metabolite of aspirin, could afford protection against rotenone-induced oxidative stress, neuronal degeneration, and behavioral dysfunction in rats, because it has the potential to accept a molecule each of hydroxyl radical ($^{\bullet}$OH) at the third or fifth position of its benzyl ring. Rotenone caused dose-dependent increase in $^{\bullet}$OH in isolated mitochondria from the cerebral cortex and time- (24–48h) and dose-dependent (0.1–100 μM) increase in the substantia nigra and the striatum, ipsilateral to the side of rotenone infusion. Administration of sodium salicylate at 12-h intervals for 4 days showed dose-dependent (50–100 mg/kg, i.p) reductions in the levels of $^{\bullet}$OH in the nigra on the fifth day. These animals showed significant attenuation in rotenone-induced loss in striatal dopamine levels, number of nigral dopaminergic neurons, reduced and oxidized glutathione levels, and complex I activity loss, but superoxide dismutase activity was increased further. Amphetamine- or apomorphine-induced ipsilateral rotations in rotenone-treated rats were significantly reduced in rats treated with sodium salicylate. Our results indicate a direct role of $^{\bullet}$OH in mediating nigral neuronal death by rotenone and confirm the neuroprotective potential of salicylate in a rodent model of parkinsonism. **Synapse 67:502–514, 2013.** © 2013 Wiley Periodicals, Inc.

INTRODUCTION

Sodium salicylate (SA) is an active metabolite of aspirin, a widely used nonsteroidal anti-inflammatory drug (NSAID). This molecule possesses analgesic, antipyretic, anti-inflammatory, and antioxidant properties, which arise out of its inhibitory effects on cyclooxygenase (COX). The anti-inflammatory action and the potency of acetyl salicylic acid or aspirin lies in its ability to inhibit COX, but the ability of SA to bring out anti-inflammatory effect without inhibiting prostaglandin synthesis is reported (Asanuma and Miyazaki, 2006; Grilli et al., 1996; Sairam et al., 2003). The protective mechanism of action of aspirin in prevention of stroke is due to irreversible inhibition of the enzyme COX-1 by acetylation in platelets, resulting in blockade of thromboxane A_2 production (Patrono, 1994), whereas SA is a weak inhibitor of COX-1 (Insel, 1996). Similar to its acetylation effect on COX-1, aspirin can acetylate and thus inhibit prostaglandin H synthase, whereas SA, which lack acetyl group, is ineffective in inhibiting the prostaglandin H synthase, nevertheless, rendering anti-inflammatory action (Weissmann, 1991).

Both aspirin and SA are known free radical scavengers (Kataoka et al., 1997; Maharaj et al., 2004). SA has been used as a hydroxyl radical ($^{\bullet}$OH) trapping agent in in vivo (Cao et al., 1988; Floyd et al.,

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1986; Grootveld and Halliwell, 1986; Mohanakumar et al., 1998; Thomas et al., 2000) or in vitro studies (Floyd et al., 1984; Sairam et al., 2003). SA, due to its capability to trap $^{\bullet}OH$ and get itself transformed into dihydroxy benzoic acids (DHBA), has been shown to block 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced $^{\bullet}OH$ formation and subsequently striatal dopamine (DA) and reduced glutathione (GSH) depletion in mice or rats (Aubin et al., 1998; Ferger et al., 1998; Mohanakumar et al., 2000). The protective effect of aspirin or SA is demonstrated in 6-hydroxydopamine (6-OHDA) (Carrasco and Werner, 2002; Gören et al., 2009) and 1-methyl-4-phenyl pyridinium (MPP^+) (Sairam et al., 2003; Tasaki et al., 2010) models of Parkinson's disease (PD) too. A solitary study failed to show any effect of aspirin or other NSAID on rotenone-induced dopaminergic neurotoxicity (Tasaki et al., 2010). Most of these studies have endorsed the $^{\bullet}OH$ scavenging property of SA for its neuroprotective effect.

In the present study, SA has been investigated for its in vitro and in vivo $^{\bullet}OH$ scavenging activity, changes in brain oxidative stress indicators, and dopaminergic neurotoxicity following intranigral infusion of rotenone. The study reasoned that if rotenone's neurotoxic action is exerted via the production of reactive oxygen species (ROS), then SA should provide neuroprotection in this model of PD too.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats from the Institute's colony were maintained under standard conditions of 12-h light/dark cycles, $22 \pm 1^{\circ}C$ temperature and $60 \pm 5\%$ humidity, and provided free access to food and water. All animal experiments were carried out in accordance with the CPCSEA national guidelines on the proper care and use of animals in laboratory research in India (Animal Welfare Division, Ministry of Environment and Forests, Govt. of India) and were approved by the statutory institutional animal ethics committee.

Abbreviations

•OH	hydroxyl radical
6-OHDA	6-hydroxydopamine
COX	cyclooxygenase
DA	dopamine
DHBA	dihydroxybenzoic acid
GSH	reduced glutathione
GSSG	oxidized glutathione
HPLC	high performance liquid chromatography
MPP^+	1-methyl-4-phenyl pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NSAID	nonsteroidal anti-inflammatory drug
PD	Parkinson's disease
ROS	reactive oxygen species
SA	sodium salicylate
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase

Drugs and chemicals

Rotenone, DA, 2,3- and 2,5-DHBA, Triton-X100, sodium azide, O-pthaldialdehyde, 3,3′-diaminobenzidine, H_2O_2, *N*-ethylmaleimide, and pyrogallol were procured from Sigma Chemicals (St. Louis, MO). Chloral hydrate was from Fluka, Germany. Anti-tyrosine hydroxylase (TH) antibody and horseradish peroxidase-conjugated secondary antibody were purchased from Chemicon (Temecula, CA). GSH was purchased from Calbiochem (San Diego, CA). SA was obtained from Himedia, India. Dimethyl sulfoxide, polyethylene glycol, GSSG, and other reagents were of analytical grade, procured locally. For high-performance liquid chromatography (HPLC), double distilled water was filtered and deionized using Milli-Q system (Waters, Milford, MA).

Drug treatment

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed on a stereotaxic frame (Stoelting, IL). Rotenone dissolved in dimethyl sulfoxide : polyethylene glycol (1:1) was infused (1 μL) into the right substantia nigra pars compacta (SNpc) at a flow rate of 0.2 μL/min using a Worker Bee and Syringe Pump (BAS, West Lafayette). The stereotaxic coordinates were $L = 0.24$; AP = 0.30; and DV = 0.78, from the lambda point. One microliter of vehicle was infused into the left SNpc. In three sham control animals, one side received vehicle and the other side received normal saline. A total of 125 rats were used for the entire study. This included naive rats used for isolation of mitochondria, as well as rotenone alone, SA alone, and rotenone + SA-treated groups.

SA was dissolved in distilled water and administered intraperitoneally (50 and 100 mg/kg, i.p.) 1 h after rotenone/vehicle infusion. The animals were treated twice daily for the next 3 days and sacrificed by decapitation on the fifth day for all the experiments except for the behavior analyses and DA measurement. Behavioral studies were performed on 14th and 16th day post-rotenone infusion, and these rats were sacrificed on 18th day to perform DA measurement using HPLC-electrochemistry (EC). The animals were treated with SA twice daily based on our and others observations where SA has been used for neuroprotective studies in MPP^+ model in rats (Ferger et al., 1999; Sairam et al., 2003). Furthermore, our previous study with rotenone showed 47% DA depletion on 5th day that only modestly increased (52%) by 30th day (Saravanan et al., 2005) indicating most of the DA-depletion is occurring at acute to subacute period necessitating an earlier SA treatment. In addition, we have recently shown that in rotenone-induced neurodegeneration in rats, nitric oxide production, and subsequent microglial activation

happen only after 96 h after the neurotoxin, and containing these events with nitric oxide synthase inhibitors with strong antioxidant capability could effectively arrest the whole cascade of events and protect the dopaminergic neurons against cell death (Madathil et al., 2013).

$^{\bullet}$OH generation in mitochondrial P_2 fraction

Mitochondrial P_2 fraction (sub-mitochondrial particles) was obtained from the cerebral cortex of normal rats (Saravanan et al., 2007). The tissue was homogenized using a glass-Teflon homogenizer in 0.32*M* sucrose in cold 10 m*M* potassium phosphate buffer, pH 7.2. The supernatant arising out of centrifugation at 1,000*g* for 10 min at 4°C in a Sorvall centrifuge was again centrifuged at 10,000*g* for 30 min, and washed twice in the same volume of the ice-cold potassium phosphate buffer. The pellet thus obtained was suspended in the same volume of buffer and kept overnight at −20°C. Freeze-thawed mitochondrial P_2 fraction that releases submitochondrial particles were incubated with different doses of rotenone (100 n*M*−10 m*M*) and SA (0.75 m*M*) for 30 min. The reaction was stopped by the addition of ice-cold $HClO_4$ (0.1*N*) containing 0.01% EDTA and the supernatant resulting from centrifugation in the cold at 10,000*g* for 10 min was directly injected into the HPLC system for the assay of 2,3- and 2,5-DHBA as described earlier (Thomas et al., 2000). Protein concentration was measured following the method of Lowry et al. (1951).

Studies on the generation of $^{\bullet}$OH adducts in vivo

Measurement of $^{\bullet}$OH was done following the method of Thomas et al. (2000). To evaluate acute production of $^{\bullet}$OH, rotenone (2, 6, and 12 μg in 1 μL) or vehicle-treated animals were administered 100 mg/kg SA (as the $^{\bullet}$OH trap), 90 min before sacrifice, and sacrificed at 2, 24, and 48 h after rotenone administration. For studying the subacute effects of SA, the animals that received SA intraperitoneally at 12-h intervals for 4 days were treated with 100 mg/kg of SA 90 min (as the $^{\bullet}$OH trap) before sacrifice on the fifth day. Levels of 2,3- and 2,5-DHBA were analyzed using HPLC-EC.

Measurement of $^{\bullet}$OH adducts and DA

The left and right substantia nigra (SN) and striata were micropunched separately, weighed, and processed for the analyses of DA and 2,3- and 2,5-DHBA levels (Thomas et al., 2008). The tissues were sonicated in ice-cold 0.1*M* $HClO_4$ containing 0.01% EDTA (100 μL per mg wet weight). The supernatant (10,000*g* for 5 min) was injected (10 μL) into the HPLC system (Merck Hitachi, Germany) equipped with LaChrome L-3500A amperometric detector (Merck Hitachi, Germany) and C_{18}, ion pair, analytical column (4.6 mm × 250 mm; Ultrasphere IP; Beckman), with a particle size of 5 μm and pore size of 80 Å. The flow rate was 0.7 mL/min and the electrodetection was performed at 0.74 V. The composition of the mobile phase was 8.65 m*M* heptane sulfonic acid, 0.27 m*M* EDTA, 13% acetonitrile, 0.43% triethylamine, and 0.22% phosphoric acid.

Determination of GSH and GSSG levels

Micropunched SN was weighed and homogenized in 0.1*M* potassium phosphate buffer, pH 7.8, using a glass-Teflon homogenizer. The tissue homogenates were centrifuged at 100,000*g* for 60 min at 4°C using a Beckman ultracentrifuge. The supernatant corresponding to the cytosolic fraction was collected and kept at −20°C until analysis. GSH was measured fluorimetrically following the method as described earlier (Thomas et al., 2000), and GSSG was measured according to Hissin and Hilf (1976), as adapted by Saravanan et al. (2007). GSH and GSSG were estimated in the cytosolic fraction after deproteination with ice-cold 0.1*M* H_3PO4 (10% vol/vol), using *O*-pthaldialdehyde condensation reaction to yield a fluorescent product. For GSSG assay, the deproteinized sample was incubated with 40 m*M* *N*-ethylmaleimide for 30 min. The supernatant was buffered with 1.8 mL of 0.1*M* sodium phosphate buffer, pH 8.0, and 0.1 mL of 0.1% *O*-pthaldialdehyde in methanol was added with thorough mixing in the case of GSH and 1.8 mL of 1*N* NaOH, pH 11, and 0.1 mL of 0.1% *O*-pthaldialdehyde in methanol for GSSG. After a 20-min reaction period at room temperature, the incubation mixture was transferred to quartz cuvette and the fluorescence at 423 nm resulting from activation at 337 nm was read in a Hitachi F-4010 spectrofluorimeter. A standard curve was prepared using commercially obtained GSH and GSSG.

Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) was analyzed in the cytosolic fractions of SNpc and the striatum, using the method as described earlier (Saravanan et al., 2007). Pyrogallol in presence of 10 m*M* EDTA auto-oxidizes rapidly in the alkaline solution (50 m*M* Tris-HCl; pH 8.2) and develops a yellow color, which can be measured spectrophotometrically at 420 nm. SOD inhibits the autooxidation of pyrogallol. The assay mixture contained 0.2 m*M* sublimed pyrogallol, 1 m*M* EDTA, and 50 m*M* tris-HCl buffer, pH 8.2. Pyrogallol auto-oxidation was monitored at 420 nm for 3 min in a Hitachi U-2000 spectrophotometer with or without the enzyme protein. The inhibition of pyrogallol oxidation was linear with the activity of the enzyme present. Fifty percent inhibition per milligram protein per minute is taken as one unit of the specific enzyme activity.

Measurement catalase activity

Catalase activity was measured in the cytosolic fractions as described earlier (Saravanan et al.,

2007). Briefly, SN and the striatum were homogenized in 0.1 m*M* phosphate buffer and centrifuged at 100,000*g* for 1 h at 4°C, and the supernatant was collected for the assay. Sample was mixed with 30 m*M* H_2O_2 and the absorbance at 240 nm was measured for 30 s. The enzyme activity was represented as change in absorbance per minute per milligram of protein.

Measurement mitochondrial complex I activity

Complex I activity was assayed as described earlier (Saravanan et al., 2005). Nigral area was micropunched from fresh frozen sections and sonicated in 0.1*M* potassium phosphate buffer, pH 7.8. After a short spin for 20 s at 600*g*, the supernatant was collected and assayed for complex I activity. The specific enzyme activity is expressed as nanomoles of NADH oxidized per minute per milligram of protein (extinction coefficient of NADH is taken as 6.2 mM^{-1} cm^{-1} at 340 nm).

Amphetamine- or apomorphine-induced rotations

Rats unilaterally infused with vehicle or rotenone and that received SA regularly for 4 days were placed in perspex transparent spherical cages (45 cm diameter, 40 cm deep) on the 14th and 16th day for measuring rotations following *d*-amphetamine (5 mg/kg; i.p.) or apomorphine (1 mg/kg; s.c.) injections, respectively. The number of rotations (360°, in short axis) ipsilateral and contralateral to the side of infusion were counted and recorded from the initiation of rotational bias till the rotations vanished completely (Sindhu et al., 2005).

Immunohistochemistry and quantification

Animals were perfused intracardialy with ice-cold 0.1*M* phosphate buffered saline (PBS) followed by cold 4% (wt/vol) paraformaldehyde in 0.1*M* PBS. Animals were decapitated and the brains were postfixed in 4% paraformaldehyde in PBS at 4°C for 3 h. The fixed brains were immersed in 30% sucrose in 0.1*M* PBS overnight. Twenty-micrometer thick striatal sections were cut on a cryostat (Leica) and were collected free floating in PBS and processed for immunohistochemistry. Sections were incubated with H_2O_2 (1%) for 15 min for utilizing endogenous peroxidase activity and blocked with 4% normal goat serum, 0.2% Triton X-100, and 0.02% sodium azide prepared in Tris-buffered saline (TBS), pH 7.4 for 30 min. Sections were incubated for 24 h with primary antibody (anti-rabbit TH polyclonal; 1:1000) in TBS, which contained 2% normal goat serum, 0.2% Triton X-100, and 0.02% sodium azide. After washing with 1% normal goat serum prepared in TBS pH 7.4, the sections were incubated in HRP-conjugated secondary goat anti-rabbit IgG (1: 1000) in 1.5% normal goat serum for 45 min. Visualization was performed by incubation in 3,3′-diaminobenzidine for 2–10 min. Sections were washed in TBS and mounted on gelatin-coated glass slides and mounted in glycerine jelly. Images were were viewed under a Zeiss Stemi 2000-C (Carl Zeiss, Germany) microscope using bright field optics. The images were captured with a Canon single reflector camera, developed, and printed. The photomicrographs were scanned at 300 dpi resolution and transferred as digital images to the Adobe Illustrator software for the production of final illustrations. Images (magnification, 20X) from SN region were used for TH-neuronal counts and from striatal region for TH-intensity measurement (3 sections/rat and 3–5 rats/treatment group). Using Image Pro Plus software (MediaCybernetics) SN region was traced carefully avoiding ventral tegmental area and the tyrosine-positive perikarya were counted. Limits for intensity and object size were applied to avoid interferences from background staining and fibers, respectively. Striatal intensity was measured using image master 1D elite version 3.01 software (Pharmacia Biotech).

Statistical analysis

Statistical analysis was performed using the Sigmastat-version 3 software. The rotational and neurochemical data were statistically evaluated for significance using one-way ANOVA followed by Newman Keuls post hoc analysis. Results are given as mean ± SEM. Values of $P \leq 0.05$ were considered significant.

RESULTS

Effect of rotenone on $^{\bullet}$OH generation in mitochondria

The $^{\bullet}$OH production was measured by salicylate hydroxylation procedure, wherein the measure of $^{\bullet}$OH adducts of salicylate, 2,3-DHBA, and 2,5-DHBA were taken as the levels of $^{\bullet}$OH formed. Rotenone caused a dose-dependent production of 2,3- and 2,5-DHBA formed several minutes following incubation up to a dose of 100 μ*M* with mitochondria prepared from naive rat cortex, beyond which there appeared an inhibition (Fig. 1).

Acute effects of rotenone on $^{\bullet}$OH generation in the brain in vivo

Rotenone administration into SNpc caused a significant generation of $^{\bullet}$OH not only in the region of injection but also in the terminal region, the striatum, ipsilateral to the side of infusion (Fig. 2). Interestingly, this effect was seen only in the levels of 2,3,-DHBA, the more reliable indicator of $^{\bullet}$OH production in tissues, in vivo (Halliwell et al., 1991). The effect was dose dependent for 2–12 μg rotenone in 1 μL administered into SNpc at 48 h following rotenone

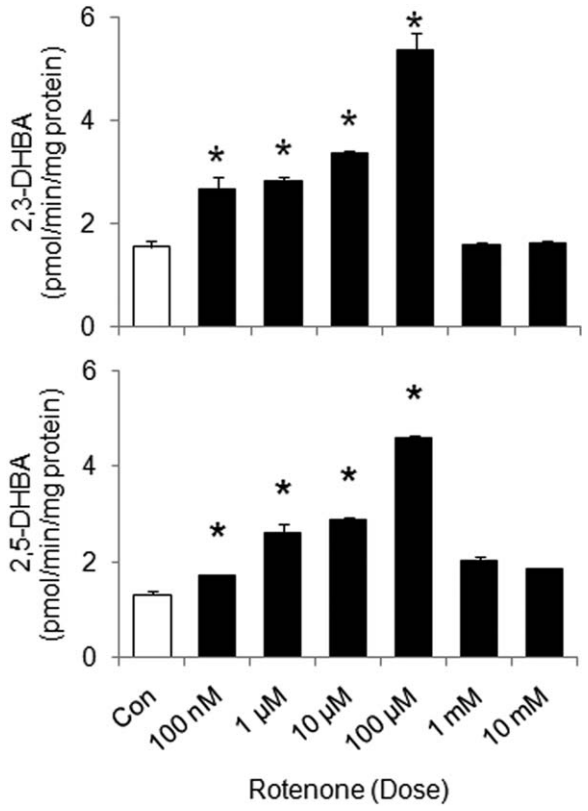

Fig. 1. Rotenone causes dose-dependent production of •OH in the mitochondria. Mitochondrial P_2 fractions isolated from normal rat cortices were incubated with rotenone (0.01 μ*M*−10 m*M*) and salicylic acid for 30 min, and the resulting •OH adducts 2,3-DHBA and 2,5-DHBA were assayed using HPLC-electrochemical procedure. The results are depicted as nmol of DHBA formed per min per mg of protein. n = 3 independent experiments; $^*P \leq 0.05$.

infusion (Fig. 2). The •OH formation was time dependent and 2,3-DHBA generation was observed from 2 to 48 h in both the ipsilateral SNpc and striatum (Fig. 3). The level of 2,5-DHBA was increased only in ipsilateral SNpc (Figs. 2 and 3).

Subacute treatment of SA on the levels of •OH in SN and striatum

To evaluate the levels of •OH production in SNpc and striata of animals that received subacute administration of SA at 12 h intervals for 4 days, these animals were sacrificed on the fifth day, 90 min following 100 mg/kg (i.p.) SA administration. Interestingly, we found no change in the levels of either 2,3-DHBA or 2,5-DHBA concentrations in the SN or striata of ipsi- or contralateral sides in rotenone alone-treated animals (Fig. 4). However, these values (2,3-DHBA = 0.42 ± 0.05 and 0.54 ± 0.05 pmol/mg tissue in contra- and ipsilateral SN, respectively; Fig. 4) were significantly ($P \leq 0.05$; Student's *t* test) lower than the levels of 2,3-DHBA in SN of animals analyzed 2 h (2,3-DHBA = 0.73 ± 0.1 and 1.1 ± 0.1 pmol/mg tissue in SN in contra- and ipsilateral sides, respectively; Fig. 3) or 48 h (2,3-DHBA = 0.91 ± 0.1 and 2.54 ± 0.74 pmol/mg tissue in SN in contra- and ipsilateral sides, respectively; Fig. 3) after 6 μg of rotenone intranigral infusion. This was true for 2,5-DHBA values of ipsilateral side only (contralateral, 3.13 ± 0.1, and ipsilateral, 3.60 ± 0.12; Fig. 4, compared with contralateral, 3.11 ± 0.06, and ipsilateral, 4.42 ± 0.59, at 2 h; and 3.69 ± 0.39 contralateral and 13.99 ± 1.90 ipsilateral SN at 48 h, respectively; Fig. 3). However, the SN, but not the striata, of both the ipsi- and contralateral sides of animals that received 50 or 100 mg/kg every day during the 4 days showed significantly lower levels of 2,3-DHBA (Fig. 4). Levels of 2,3-DHBA was not altered in the striatum in these animals. Significant differences were found not only between the rotenone + SA-treated rats but also between the contralateral and ipsilateral sides (Fig. 4).

Effect of SA on antioxidant enzymes and complex I activity in SN

Rotenone caused a significant increase in cytosolic SOD activity of SNpc (Fig. 5A) but no effect on the enzyme activity in the striatum (Fig. 5B). SA treatment (100 mg/kg, i.p.) for 4 days caused a further significant increase in the enzyme activity in SNpc (Fig. 5A) and in the striatum (Fig. 5B). Catalase activity was measured in the cytosolic fraction of SNpc. Rotenone treatment caused a significant increase in catalase activity in SNpc, which was reversed by subacute treatment of SA (100 mg/kg) (Fig. 5C). Mitochondrial complex I activity was significantly inhibited in the ipsilateral SNpc that received rotenone 5 days previously (Fig. 5D). SA treatment for 4 days at 12 h intervals helped to recover the loss in complex I activity in the ipsilateral SN (Fig. 5D).

Effect of rotenone and SA on the contents of GSH and GSSG of SN

GSH content in the SN infused with 1 μL of vehicle was not different from saline infused side on fifth day. SA injections in these vehicle-treated animals showed no change in the content of GSH or GSSG. Rotenone infusion caused about 55% reduction in GSH (Fig. 6A) and 35% in GSSG (Fig. 6B) compared with the contralateral side. Administration of SA (50 or 100 mg/kg, i.p.) caused a significant recovery in the levels of GSH and GSSG, which was dose dependent (Figs. 6A and 6B).

Intranigral infusion of vehicle or saline caused no difference in the striatal biogenic amine levels in sham control animals. Unilateral intranigral infusion of rotenone at a dose of 6 μg caused significant DA depletion in the ipsilateral striatum by the 18th day when compared with the striatum contralateral to the side of infusion (45 ± 2%). SA treatment alone did not change the DA levels. While SA at 50 mg/kg

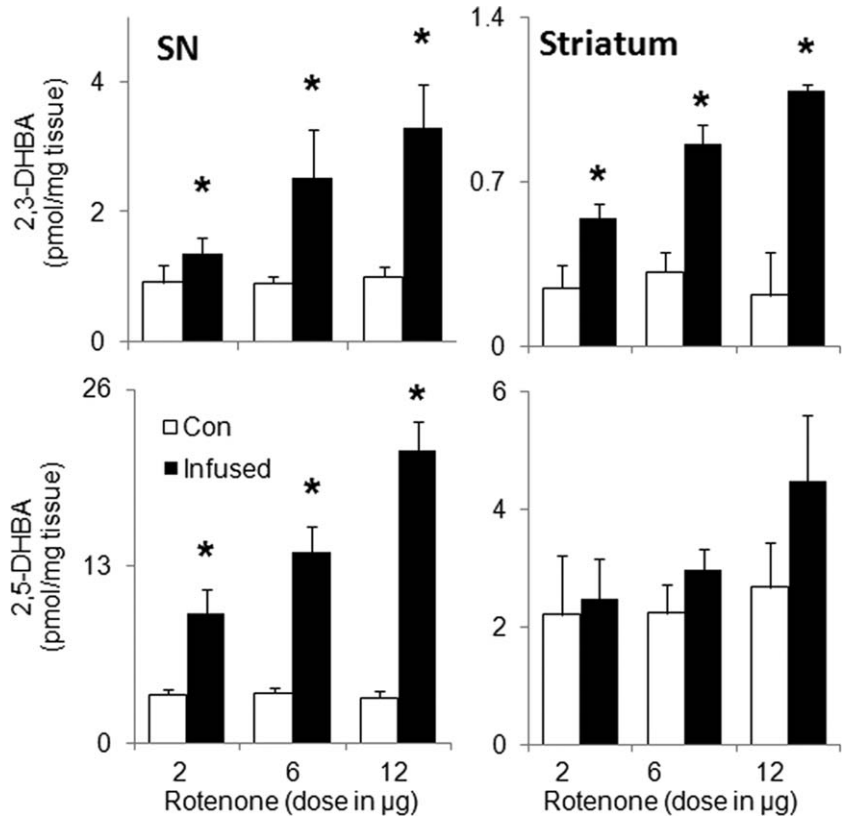

Fig. 2. Rotenone causes dose-dependent production of •OH in the SN. Adult male Sprague-Dawley rats were intranigrally infused with rotenone (2, 6, 12 µg in 1 µL/per rat) and SA (100 mg/kg, i.p.) was administered 90 min prior to sacrifice as an •OH trap on the second day (48 h). The •OH adducts of salicylate, 2,3- and 2,5-DHBA, formed were measured in contralateral (empty bars, white) and ipsilateral (filled bars, black) SN and striatum employing a sensitive HPLC-electrochemical detection procedure. Results are expressed as pmol/mg tissue and are depicted as mean ± SEM. *$P \leq 0.05$ compared with contralateral side (n = 5/treatment).

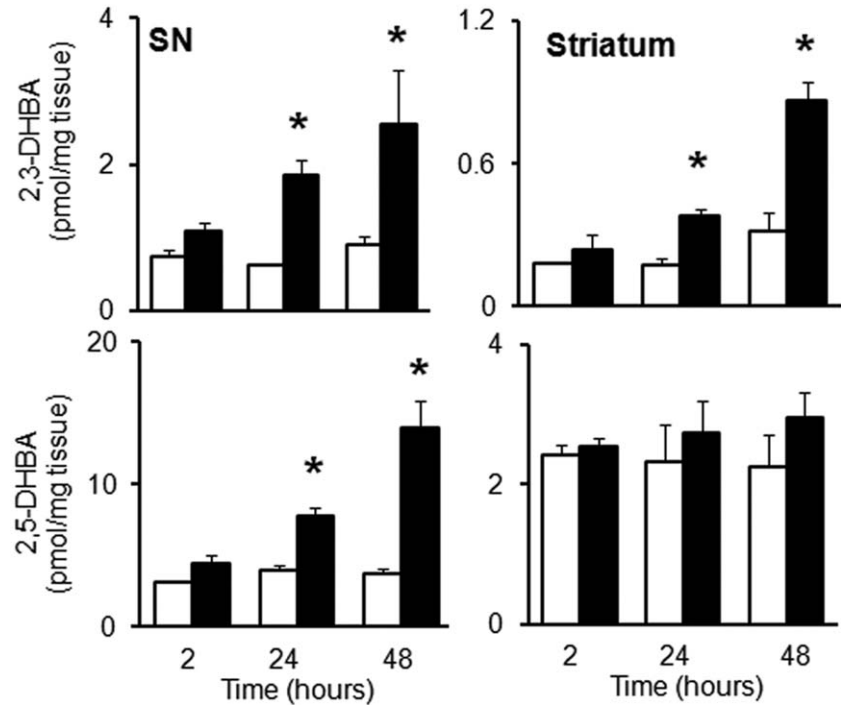

Fig. 3. Rotenone-induced •OH production is time dependent in the SN. Legends to this figure are as explained in Figure 2, except that the animals were sacrificed at various intervals, at 2, 24, and 48 h following 6 µg/rat of rotenone in 1 µL infusion volume. The •OH adducts of salicylate were measured separately in contralateral (empty bars, white) and ipsilateral (filled bars, black) sides. Results are expressed as pmol/mg tissue and are depicted as mean ± SEM. *$P \leq 0.05$ compared with contralateral side (n = 3 or 4/time point).

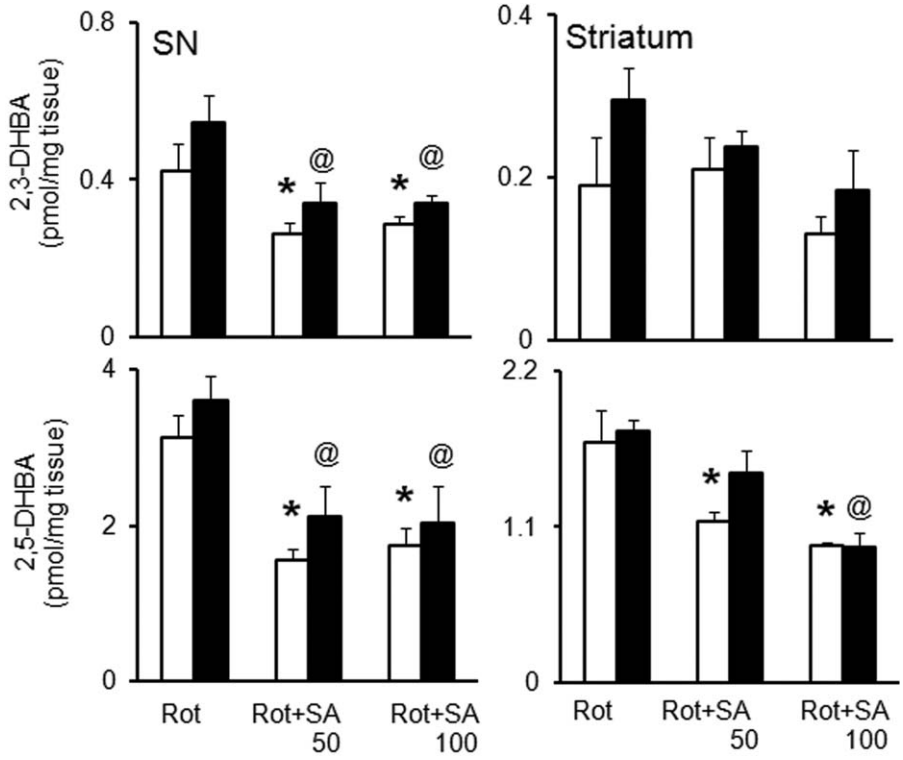

Fig. 4. Subchronic administration of SA on rotenone-induced •OH generation. Adult male Sprague-Dawley rats were intranigrally infused with rotenone (Rot) (6 μg in 1 μL/per rat). SA was administered (50 and 100 mg/kg, i.p.) at 12-h interval for 4 days, and the animals were sacrificed on the fifth day. SA was once again administered (100 mg/kg, i.p.) to serve as •OH trap 90 min before sacrifice. The •OH adducts of salicylate, 2,3- and 2,5-DHBA, formed were measured, and the results are expressed as pmol/mg tissue and mean ± SEM. Empty bars represent contralateral side and filled bars represent ipsilateral side. $^{*}P \leq 0.05$ when compared with contralateral side in the rotenone only group, $^{@}P \leq 0.05$ when compared with rotenone-infused side of rotenone only group (n = 5/treatment).

doses could render only partial protection against the rotenone-induced DA depletion, a dose of SA at 100 mg/kg offered almost complete protection against rotenone-induced striatal DA depletion (Fig. 6C).

Rotations following administration of dopaminergic drugs in rats

Animals unilaterally infused with rotenone and treated with amphetamine or apomorphine caused ipsilateral rotations when tested on the 14th day and 16th day, respectively. Amphetamine administration caused rotations that lasted for more than 4 h, and the number of rotations reached about 75/10 min by 30 min, which returned to nil by 4 h (Fig. 7A). Apomorphine caused rotary response for about an hour, which reached a zenith by the first 10 min itself (Fig. 7C). The total number of rotations were about 650 for the whole duration of response in amphetamine-treated rats (Fig. 7B), and about 325 following apomorphine administration (Fig. 7D), both of which were found to be significantly lower in animals that received SA during the previous 4 days (Figs. 7B and 7D). The duration of rotary response was unaffected, but the numbers of rotation were significantly reduced in animals that received SA for 4 days at 12 h intervals (Figs. 7A–7D).

TH-immunoreactive cells in the SNpc and terminals in the striatum

Strongly stained TH-positive neurons were seen in the control SNpc (Fig. 8A), which was reduced in the rotenone-treated SNpc (Fig. 8B). SA-treated (100 mg/kg for 4 days, at 12 h intervals) animal's SNpc showed more intense TH immunostaining that was comparable with vehicle-infused SN (Fig. 8C). The mean number of TH-positive neurons in one of the three sections, from a minimum of three rats per group of the SNpc of vehicle treated, contralateral to the side of rotenone infused, ipsilateral to the side of rotenone treated, and 4 days SA-treated contralateral and ipsilateral to the side of rotenone-infused sides were 190 ± 42.6, 192 ± 19.5, 78.0 ± 15.9, 214.7 ± 27.7, and 145 ± 27.9, respectively. Neuronal counts from SN region showed only 36% of neurons survived after rotenone treatment (Fig. 8G), but SA treatment significantly reduced the loss of nigral neurons and raised the value of surviving neurons to 86% (Fig. 8G). Striatal TH-immunoreactive fiber intensity

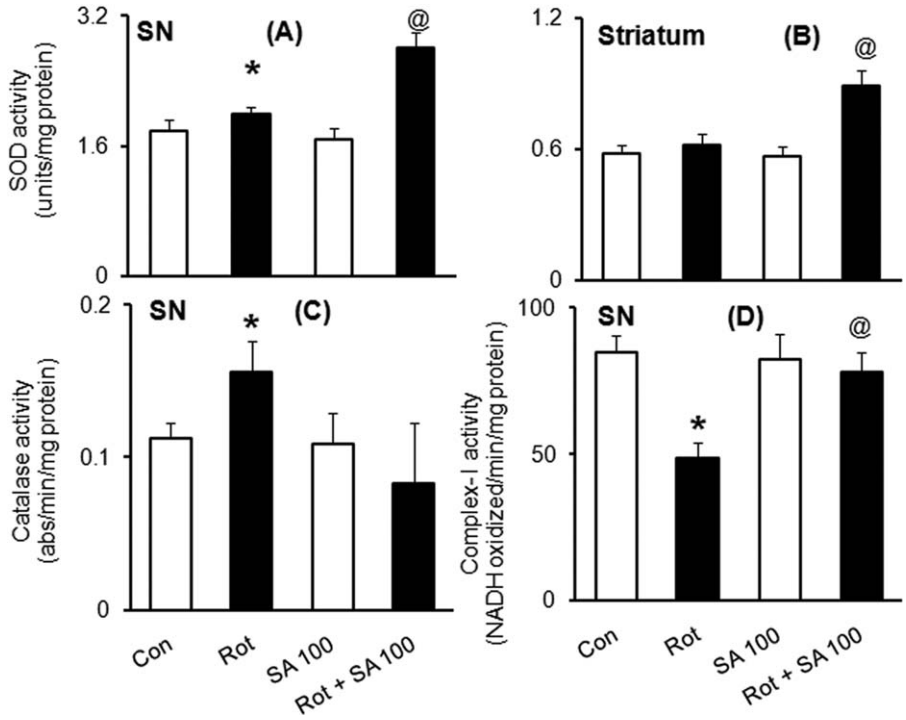

Fig. 5. Effect of SA on antioxidant enzymes and mitochondrial complex I activity in substantia nigra. Rotenone (6 μg in 1 μL) was infused into the rat SN, and the rats were treated with SA (100 mg/kg, i.p.) every 12 h for 4 days and sacrificed on the fifth day. SN and the striatum were dissected out, and the cytosolic fractions were prepared and assayed for SOD activity (**A**,**B**). Only SN was assayed for catalase activity (**C**). Mitochondrial electron transport chain complex I activity was assayed in the crude homogenate of SN (**D**). $^{*}P \leq 0.05$, significantly different when compared with control (Con) side, which received vehicle. $^{@}P \leq 0.05$, significantly different when compared with rotenone group. Data represent mean ± SEM (n = 4/group/assay for SOD and catalase; n = 3/group for complex I activity).

showed a highly significant decrease in the staining intensity in the striatum, ipsilateral to the side of rotenone infusion (Figs. 8E and 8H) when compared with the contralateral striatum (Figs. 8D and 8H). SA treatment (100 mg/kg, i.p) for 4 days caused a significant increase in the staining intensity of the terminals, which was similar to the striatum contralateral to the side of rotenone infusion (Figs. 8F and 8H).

DISCUSSION

NSAIDs possess analgesic, antipyretic, anti-inflammatory, and antioxidant properties, which arise out of their inhibitory effects on COX and nitric oxide synthase effects, potent free radical scavenging potency, and modulatory actions on cytokine synthesis. Epidemiological studies have indicated NSAIDs to be useful in reducing risk of PD pathophysiology in several populations. It has been suggested that NSAIDs may have protective role in PD based on a population-based study in which less than 300 samples were involved (Wahner et al., 2007). In a couple of other studies ibuprofen, but not aspirin, other NSAIDs, or acetaminophen, has been found to be associated with lower risk for PD (Chen et al., 2005; Gao et al., 2011). A critical survey on the published data has also revealed that there is protective effects of nonaspirin NSAIDs and ibuprofen, but not with aspirin or acetaminophen on the risk of PD (Gagne and Power, 2010), and such association has been suggested to be gender specific, with higher risk in women, but lower risk in men for developing the disease (Hernán et al., 2006). On the other hand, there are serious considerations contrary to the above contention, since several epidemiological investigations suggested otherwise. No indication of association of use of aspirin, ibuprofen, or nonaspirin NSAIDs with risk of PD was found in studies conducted in Seattle (Ton et al., 2006), in Netherlands on more than 6000 participants over a period of 9 years (Bornebroek et al., 2007), in British Columbia using a cohort of elderly people for a period of 5 years (Etminan et al., 2008), or in a huge Danish population (Manthripragada et al., 2011). While an inverse association was found of smoking and caffeine intake, no association was found with use of NSAIDs and development of PD in a family-based study (Hancock et al., 2007).

Clinical epidemiological reports being thus, the present study assumes importance, since this has

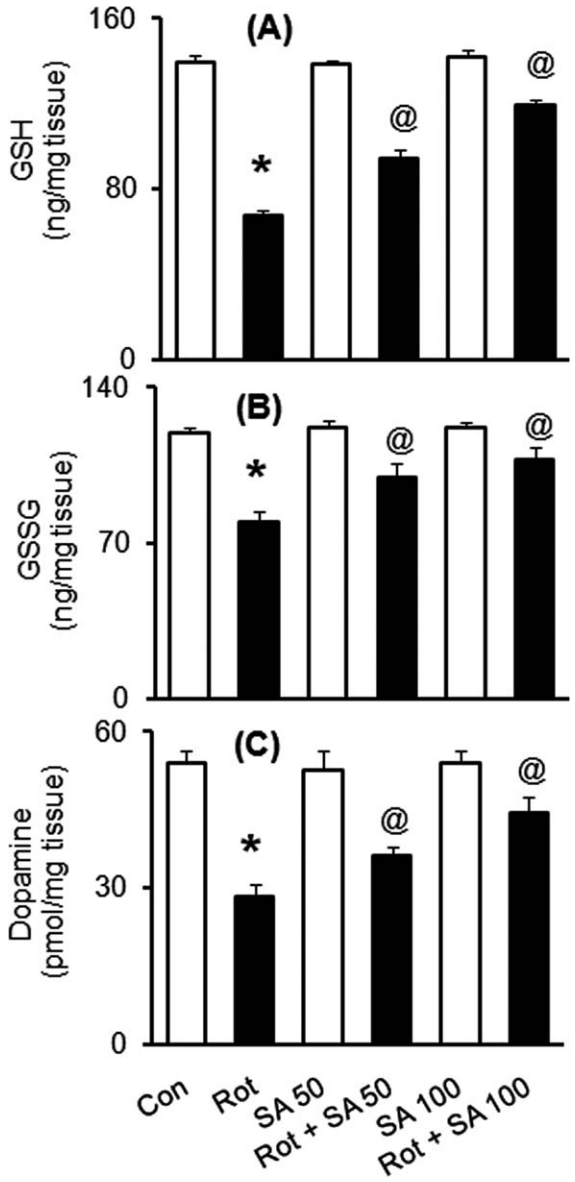

Fig. 6. SA attenuates rotenone-induced nigral glutathione and striatal dopamine levels. Ipsilateral and contralateral SN or striatum were micropunched separately. SN tissues were assayed for (**A**) GSH and (**B**) GSSG contents, and the striata were assayed for (**C**) dopamine levels employing HPLC-electrochemistry. Animals were sacrificed on the fifth day for GSH and GSSG analysis, and on the 18th day for striatal dopamine level measurements. Some of these animals received SA (50 or 100 mg/kg; i.p.) for 4 days at 12-h intervals following 6 μg in 1 μL rotenone infusion into the SN (Rot + SA groups). $^{*}P \leq 0.05$, significant when compared with the contralateral vehicle infused side; $^{@}P \leq 0.05$ significantly different from the lower dose group. Data presented as mean ± SEM (n = 5 in each group for GSH studies). DA (n = 3–6) measured in rats that were subjected to behavior.

been conducted in a very relevant animal model of PD, which exhibits not only pathological resemblances to human idiopathic PD following systemic administration (Betarbet et al., 2000; Sherer et al., 2003) but also is progressive in nature, when administered intracranially (Saravanan et al., 2005). The most important finding arising out of the present study is the dose- and time-dependent production of $^{\bullet}$OH soon after inhibition of mitochondrial complex I activity in the SN by rotenone, and the great potency of SA to effectively scavenge these highly reactive molecules. Rotenone has been shown to cause increased ROS production in cultured tobacco cells (van der Merwe and Dubery, 2006). Not only complex I inhibition in the mitochondria due to rotenone (Madathil et al., 2013; Saravanan et al., 2007) but also knockout of *Ndufs4* gene in dopaminergic cells, which compromise this enzyme function (Choi et al., 2011), causes ROS generation. Our study clearly indicated that the 2,3- and 2,5-DHBA contents in the SN were significantly less by the fifth day compared with 2, 24, or 48 h following rotenone. This necessarily means that the initial ROS production during the initial days may trigger other related activities, such as generation of reactive nitrogen species as shown recently by us (Madathil et al., 2013), and $^{\bullet}$OH production may cease to exist by 5 days, as seen from the present study.

For analytical purposes, SA has been widely used to trap $^{\bullet}$OH and to measure the formation of this ROS in in vitro cell-free system (Floyd et al., 1984; Madathil et al., 2013; Mohanakumar et al., 1998, 2000), in ex vivo system using tissue homogenates or isolated mitochondrial preparations (Karuppagounder et al., 2013; Madathil et al., 2013; Sairam et al., 2003; Saravanan et al., 2007), and in in vivo system (Chiueh et al., 1994; Obata, 2002; Thomas et al., 2000, 2008). The $^{\bullet}$OH adducts formed, 2,3- and 2,5-DHBA, are nontoxic and thus use of SA in in vivo could serve dual function; to inactivate the cytotoxic $^{\bullet}$OH formed in situ and to convert it into an excretable nontoxic product. Although levels of $^{\bullet}$OH adducts measured were similar between contralateral and ipsilateral sides of rotenone-only groups, it was significantly reduced in SA-treated rats 5 days post-infusion indicating strong antioxidant potential of subchronic SA treatment. Rotenone-induced $^{\bullet}$OH generation was not evident at this time point suggesting the initial days as the upstream of the neurotoxic event that culminates in neurodegeneration, since availability of SA in the brain in subacutely SA-treated animals efficiently converted $^{\bullet}$OH to non toxic DHBA, preventing action of the cytotoxic $^{\bullet}$OH and DA neurotoxicity. Similar to the observations made in our study, an analog of SA, 3,4,5-amino salicylic acid, has been shown to reverse Mn-induced reductions in mitochondrial complex I activity, membrane potential, and ROS in the dopaminergic cell line, SK-M-NC (Yoon et al., 2011). Similarly, SA has been shown to block MPTP- (Aubin et al., 1998; Ferger et al., 1998; Mohanakumar et al., 2000), MPP^{+}- (Carrasco and Werner, 2002; Maharaj et al., 2004; Sairam et al.,

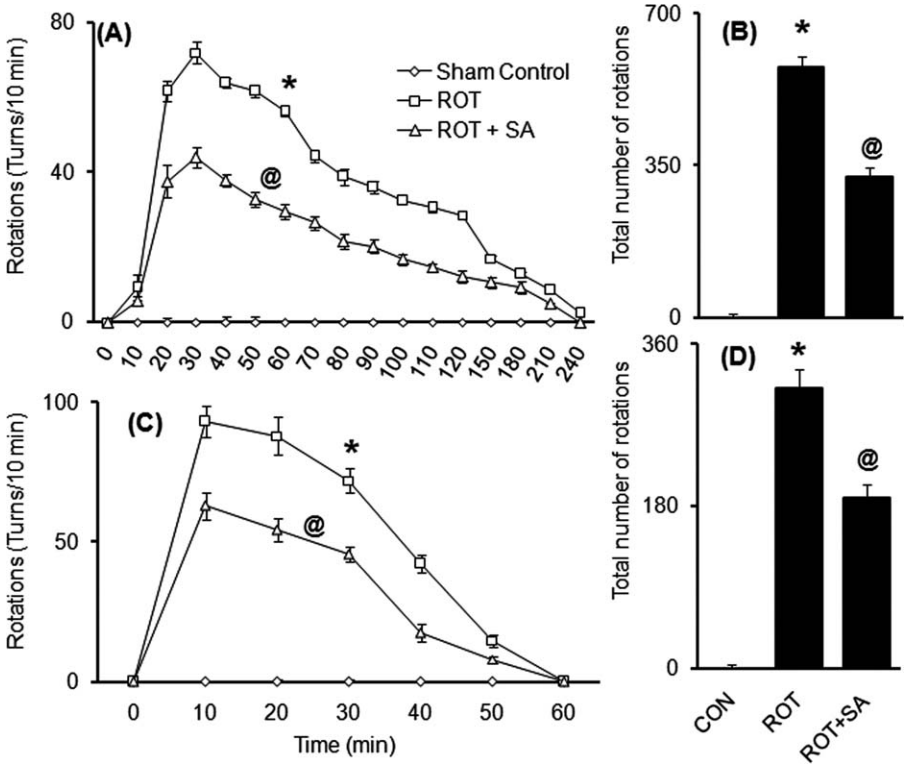

Fig. 7. SA attenuates the rotational behavior following apomorphine or amphetamine in hemiparkinsonian rats. Adult male Sprague-Dawley rats intranigrally infused with rotenone (6 μg in 1 μL) were administered vehicle or SA (100 mg/kg; i.p.) at 12-h intervals for 4 days. Animals were injected with (**A**,**B**) amphetamine (5 mg/kg; i.p.) on the 14th day and (**C**,**D**) apomorphine (1 mg/kg; s.c.) on the 16th day, and resulting stereotypic unilateral rotations (ipsilateral) were recorded until it lasted. Results are expressed as number of rotations/10 min for a period of 4 h for amphetamine (A), for 1 h for apomorphine (C) and total number of rotations during 4 h (B) and 1 h (D), respectively. Data are represented as mean ± SEM. $^{*}P \leq 0.05$ when compared with sham control animals; $^{@}P \leq 0.05$ when compared with rotenone-infused animals (n = sham control 3; rotenone only 5 and rotenone + SA 6).

2003;) and 6-OHDA- (Carrasco and Werner, 2002) induced neurotoxicity.

The present study also demonstrated a significant potency of SA to effectively block rotenone-induced striatal DA depletion, GSH and GSSG loss in the SN, and its ability to protect against dopaminergic neuronal damage in SNpc. GSH is one of the most abundant intracellular thiols in the central nervous system and acts as a major cellular antioxidant. GSH, which is synthesized in the cytoplasm, is transported into the mitochondria, the major intracellular source of ROS. The major role of this tripeptide in the brain is to scavenge $^{\bullet}$OH and form GSSH, which can react with "SH" group of proteins to form protein-glutathione mixed disulphides, which have a longer half-life than GSSG (Brigelius et al., 1983). This could be the reason for the loss of GSH without corresponding increase in levels of GSSG in SN as observed in the present study.

Another significant observation of the study is the reversal in the inhibition of mitochondrial complex I activity caused by rotenone, in animals that were treated with several doses of SA. This is surprising, since rotenone is an irreversible inhibitor of complex I. The SN of PD patients also show complex I and GSH deficit (Jenner, 1993; Mizuno et al., 1989; Schapira et al., 1989). There is a site of electron leak upstream of the rotenone binding site in complex I (Hensley et al., 1998), and thus mild genetic or acquired defects of complex I that are insufficient to affect respiration may nonetheless lead to chronic upregulation of ROS production (Greenamyre et al., 2001). Thus, rotenone, a selective complex I inhibitor, causes an inhibition of the respiratory chain enzymes and an increase in ROS production leading to nigral neurodegeneration. In a transgenic model with doxocycline-inducible monoamine oxidase-B inhibition, which has been shown to have a temporal sequence of events as oxidative stress, complex I inhibition, and SNpc neuronal death, interference with lipophilic, synthetic SOD/catalase mimetic antioxidants such as EUK-189 has caused blockade of complex-I inhibition resulting in neuronal rescue (Siddiqui et al., 2010). In the present study, SOD activity was significantly upregulated by rotenone on fifth day in nigral region and the SA treatment significantly

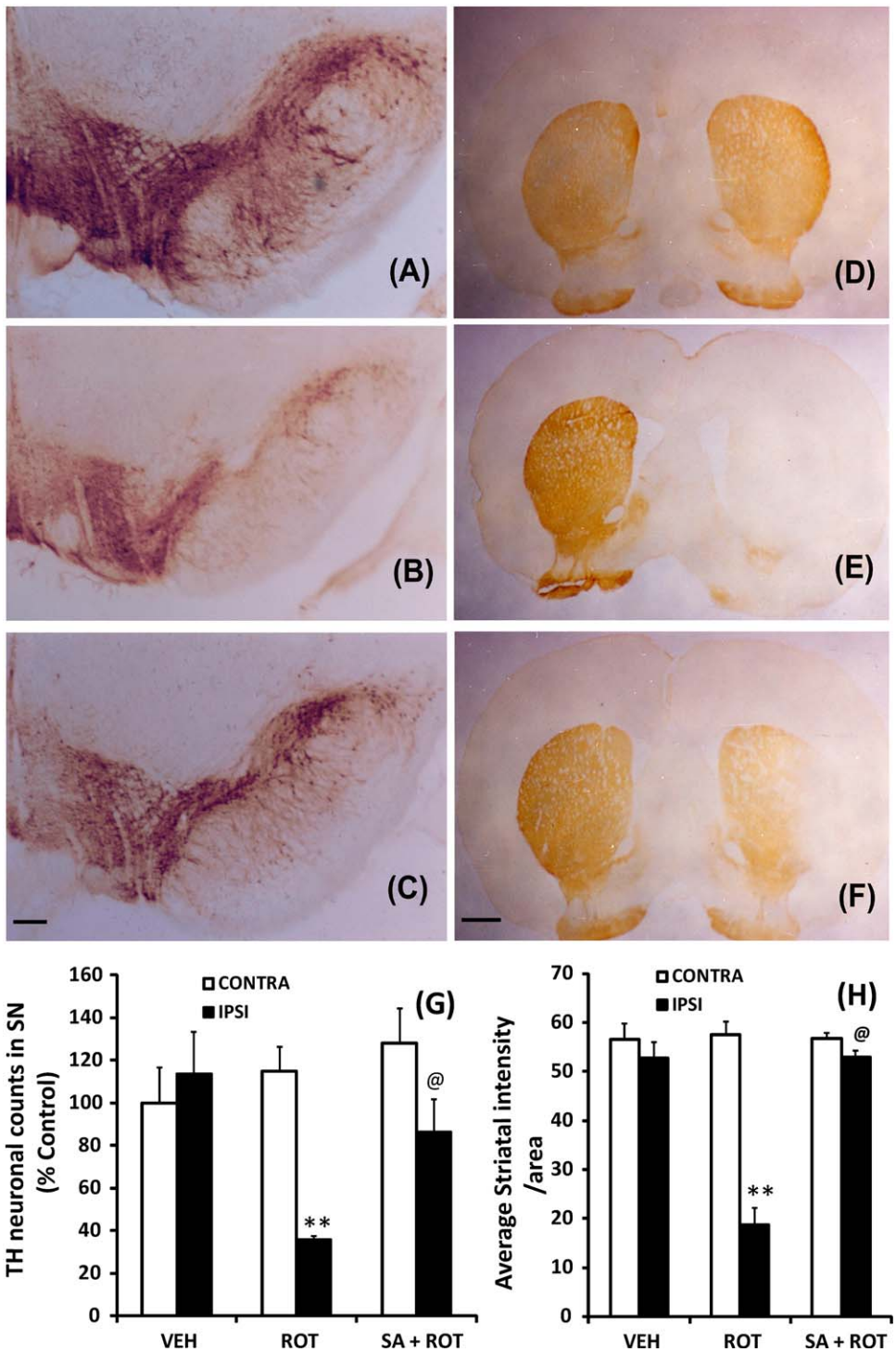

Fig. 8. Effect of rotenone on TH immunopositive neurons in SN and terminals in the striatum. Male Sprague-Dawley rats intranigrally infused with rotenone (6 μg in 1 μL) were administered vehicle or SA (100 mg/kg; i.p.) at 12-h intervals for 4 days. On fifth day, the animals were transcardially perfused, brains were fixed with 4% PFA, and the brain sections (20 μm) passing through SN or the striatum were processed for TH-immunostaining. Representative photographs shown in the left panel illustrate ipsilateral SN from animals that are (**A**) control, (**B**) rotenone infused, and (**C**) rotenone and SA treated, and the representative photographs shown in right panel are from ipsilateral and contralateral striata of animals treated with (**D**) vehicle, (**E**) rotenone, and (**F**) rotenone and SA. **G**: TH-positive neurons from SNpc were counted using image Pro plus software and are represented as percentage of contralateral side. The mean number of neurons in the contralateral side was 192.3 ± 19.5 per section. **H**: Striatal TH-intensity was quantified using image master 1D elite version 3.01 software (Pharmacia Biotech) and shown as intensity/unit area. $^{**}P \leq 0.05$, significant when compared with the contralateral vehicle infused side. $^{@}P \leq 0.05$, significant when compared with the rotenone-treated animals, ipsilateral side. Data presented are mean ± SEM (n = 3 in each group). Scale bars: A–C, 200 μm; E–F, 1 mm.

enhanced the activity in SN region. The protective effect of SA as observed in the present study could be due to the upregulation of SOD, which is the important antioxidant molecule in mitochondria, especially in the absence of such effects in the activity of catalase whose activity was brought to control value by SA treatment.

Chronic systemic administration of SA dose-dependently attenuated rotenone-induced striatal DA depletion in the striatum. Apparently, subacute SA treatment effectively scavenged the cytotoxic $^{\bullet}OH$ and blocked the neurotoxic events that followed, resulting in neuroprotection. The recovery in striatal DA and the TH-positive cells in the SNpc and the terminals in the striatum also explain the significant amelioration of amphetamine- and apomorphine-induced rotary behavior in animals treated with SA. Apomorphine has been shown to cause ipsilateral rotations but not contralateral rotations following intranigral infusion of rotenone (Sindhu et al., 2005), probably due to damage of SN pars reticulata region (Sindhu et al., 2006). Interestingly, the effect of SA is found to be significantly better in behavioral recovery than observed following deprenyl treatment in the rotenone model of PD (Saravanan et al., 2006) and may account for the better $^{\bullet}OH$ scavenging effect of SA. It has been suggested that prostaglandin H synthase is responsible for the oxidation of DA as well as formation of dopamine-quinone and neuromelanin in SNpc (Asanuma and Miyazaki, 2006; Mattammal et al., 1995). Inhibitors of nonspecific COX inhibitor aspirin and specific COX-2 inhibitor meloxicam have been shown to block 6-OHDA- or MPTP-induced neurodegeneration (Gören et al., 2009; Tasaki et al., 2010; Teismann and Ferger, 2001). Furthermore, per-oral administration of nitric oxide-donating NSAIDs effectively counteracted MPTP- (L'Episcopo et al., 2010) and lipopolysaccharide-induced (L'Episcopo et al., 2011) dopaminergic neurotoxicity, motor impairment, and microglia activation in young and aged mice. Thus, understanding the wide spectrum pharmacological activities of SA will help for developing therapeutic strategies in PD.

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