Cardiac Noradrenaline Turnover and Heat Shock Protein 27 Phosphorylation in Dyskinetic Monkeys

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ABSTRACT:

Background: Autonomic dysfunction is a well-known dominant symptom in the advanced stages of Parkinson's disease. However, the role of cardiac sympathetic nerves still needs to be elucidated.

Objectives: To evaluate cardiac sympathetic response in Parkinsonian and dyskinetic monkeys.

Methods: Adult male monkeys were divided into 1 of the following 3 groups: controls, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated monkeys, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine+levodopa–treated animals. Noradrenaline, its metabolite normetanephrine, and phospho-Heat shock proten 27 (p-Hsp27) at serine 82 levels were analyzed in the left and right ventricles of the heart. Tyrosine hydroxylase immunohistochemistry was performed in the ventral mesencephalon.

Results: The results were the following: (1) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine intoxication significantly increased normetanephrine levels and decreased noradrenaline turnover in the right ventricle without changes in the left ventricle; however, (2) levodopa treatment decreased noradrenaline levels and enhanced the normetanephrine/noradrenaline ratio in parallel with a very significant increase of Hsp27 activity in both ventricles.

Conclusions: Levodopa treatment could induce protective cardiac effects through the increased Hsp27 activity. © 2019 International Parkinson and Movement Disorder Society

Key Words: Hsp27; L-dopa; NA turnover; Parkinson's disease; TH

Parkinson's disease (PD) is clinically featured by motor symptoms caused by a loss of nigrostriatal dopamine fibers.[1] Levodopa (L-dopa) treatment is the gold therapy for PD, even if dyskinesias are the most unwanted side effect.[2] Recently, the loss of cardiac postganglionic sympathetic innervation has been included as a supportive criterion for the clinical diagnosis of PD.[3] Cardiac sympathetic neuroimaging studies indicate that one of the mechanisms involved in PD dysautonomia is the loss of postganglionic noradrenergic nerves.[4,5]

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model induces Parkinsonism in humans[6] and in monkeys[7,8] and decreases myocardial concentrations of noradrenaline (NA),[9] although the mechanisms of MPTP toxicity to peripheral catecholaminergic cells are not well understood.[10]

Changes in the heat shock proteins (Hsps) seem to play a key role in stress responses as neurotoxicity.[11] Specifically, it has been demonstrated that Hsp27 binds to α-synuclein fibrils and decreases the cellular toxicity of exogenous fibrillar α-synuclein.[12] However, whether Hsp27 exerts cardioprotection against MPTP intoxication remains unknown.

The aim of our study was to evaluate the autonomic changes in MPTP or MPTP+L-dopa–treated monkeys, focusing on the cardiac sympathetic pathway. We also compared the functional and molecular responses of the left (LV) and right (RV) ventricles to MPTP intoxication and

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the possible role of Hsp27 as an endogenous cytoprotective stress response protein, eliciting cardioprotection.

Methods

Animals and Experimental Groups

A total of 12 adult male monkeys (*Macaca fascicularis*, 4–5 years old, 4–6 kg, R.C. Hartelust BV, Tilburg, the Netherlands) were divided into 1 of the following 3 different groups (n = 4/group): (1) control group, (2) MPTP-treated monkeys, (3) MPTP-treated monkeys that received daily L-dopa/Benserazide (Madopar, Hoffmann-La Roche, Mississauga, Ontario, Canada [termed L-dopa thereafter]). Studies were carried out in accordance with the Code of Ethics of the European Union Directive 2010/63/EU and were approved by the Ethics Committee of the University of Murcia.

MPTP Intoxication and Parkinsonian Ratings

Animals from groups 2 and 3 were treated with MPTP (Sigma-Aldrich, St. Louis, USA) dissolved in saline (0.3 mg/kg, intravenously for 7 months, 1 injection every 2 weeks).[13] After quarantine, the level of Parkinsonism was assessed with a previously described motor scale.[14]

L-dopa Treatment and Dyskinesia Ratings

After reaching a stable Parkinsonism, 4 animals were treated daily with 100 mg/25 mg of Madopar, orally, for 4 months until the development of dyskinesias.[15] The severity of dyskinesia was rated using a Dyskinesia Disability Scale previously described in the literature.[2,16] All monkeys in this group developed stable and moderate to severe dyskinesia.

Postmortem Macaque Tissue

At 4 hours after the last L-dopa administration, the animals were sacrificed by sodium pentobarbital overdose (150 mg/kg, intravenously). The hearts and brains were immediately removed. The hearts were longitudinally dissected to RV and LV and were immediately frozen with dry ice and then stored at −80° C for high-performance liquid chromatography and Western blot analysis. Brains were blocked and postfixated 5 days in 4% paraformaldehyde. They were cryoprotected in a 30% sucrose solution in 0.01 mol/L phosphate buffered saline (PBS) until processing. The brains were sliced into 40 μm-thick coronal sections along the rostral axis with a freezing microtome (Leica, Wetzlar, Germany) and collected in 0.125 mol/L PBS containing 0.05% sodium azide and were stored at −20°C until their subsequent analysis. A free-floating immunohistochemistry technique was used for histological analysis of the brain tissue sections.

Immunohistochemistry

Tissue sections containing ventral mesencephalon (substantia nigra pars compacta [SNpc] and ventral tegmental area) were washed in bidistilled water and 0.01 mol/L PBS to remove the cryoprotectant solution and incubated in PBS with 0.02% hydrogen peroxide for endogenous peroxidase inhibition. The sections were blocked, incubated overnight with the primary antibody in PBS (Supplementary Table 1), rinsed in PBS, and incubated for 30 minutes in PBS containing the secondary antibody. Subsequently, they were revealed with a 3,3'-diaminobenzidine substrate kit (Vector Laboratories, Peterborough, United Kingdom) previously incubated with a Vector avidin–biotin complex (1:200, Vectastain Elite ABC kit, Vector Laboratories). The next day, sections were thionine counterstained and coverslipped using DPX (Sigma-Aldrich, St. Louis, USA).

Stereological Count

The absolute number of tyrosine hydroxylase immunoreactive (TH-ir) neurons in the SNpc and ventral tegmental area of all animals was obtained in each region by the application of the optical fractionator.[17] All measurements were performed using an interactive computer system consisting of a Zeiss (Oberkochen, Germany) Axioskop optical microscope equipped with a digital camera (AxioCam HRc; Zeiss).

Western Blot

Western blot analysis was performed for phospho-Hsp27 protein in the LV and RV; 50 μg of protein/lane was loaded on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoresed, and transferred onto a polyvinylidene fluoride membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The blots were incubated at 4°C with the primary antibodies diluted in tris-buffered saline + 0.5% triton X-100 + bovine serum albumin listed in Supplementary Table 1. The membranes were then incubated with the appropriate secondary antibodies (Supplementary Table 1). Immunoreactivity was detected with ECL Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized by a Typhoon 9410 (GE Healthcare). Quantification of the phospho-Hsp27 (27 kDa) band immunoreactivity was carried out by optical densitometry and expressed as relative units (%) to the controls (AlphaImager, Nucliber, Madrid). Antitotal Hsp27 was used as loading control.

High-Performance Liquid Chromatography Analysis

NA and NMN were determined by high-performance liquid chromatography with electrochemical detection.

The mobile phase (pH 4.3) consisted of a 95:5 (v/v) mixture of water and methanol with sodium acetate (50 mmol/L), citric acid (20 mmol/L), l-octyl-sodium sulfonate (3.75 mmol/L), di-n-butylamine (1 mmol/L), and ethylenediaminetetraacetic acid (0.135 mmol/L). The flow rate was 0.9 ml/min, and chromatographic data were analyzed with Millenium 2010 Chromatography Manager Equipment (Millipore, MA, USA). The content of NA and NMN in the RV and LV was expressed as ng/g wet weight of tissue.

Statistical and Data Analysis

The animals were assigned to the different groups randomly, and the data collection and analysis were done blindly. Data were analyzed using 1-way analysis of variance followed by the Bonferroni post hoc test. Two-sided *P* values of less than 0.05 were considered significant. All statistics were performed with GraphPad Prism software (GraphPad [San Diego, CA] Prism version 5.0).

Results

Stereological Counts of Dopaminergic Neurons

The number of TH-ir neurons in the SNpc declined in parallel along the time of MPTP monkey's treatment in groups 2 and 3. As expected, a marked and significant ($P < 0.01$) loss of dopaminergic neurons was observed in the SNpc of all MPTP-treated monkeys (~75% neuronal loss; Fig. 1A), including a significant negative correlation between the disability motor scale and the total TH+ neurons ($r^2 = -0.84$, $P = 0.006$; Fig. 1C). However, no significant differences were observed between the L-dopa and MPTP groups, indicating that L-dopa does not alter the survival of dopaminergic cells in the SNpc. The loss of TH-ir neurons in the ventral tegmental area was modest in

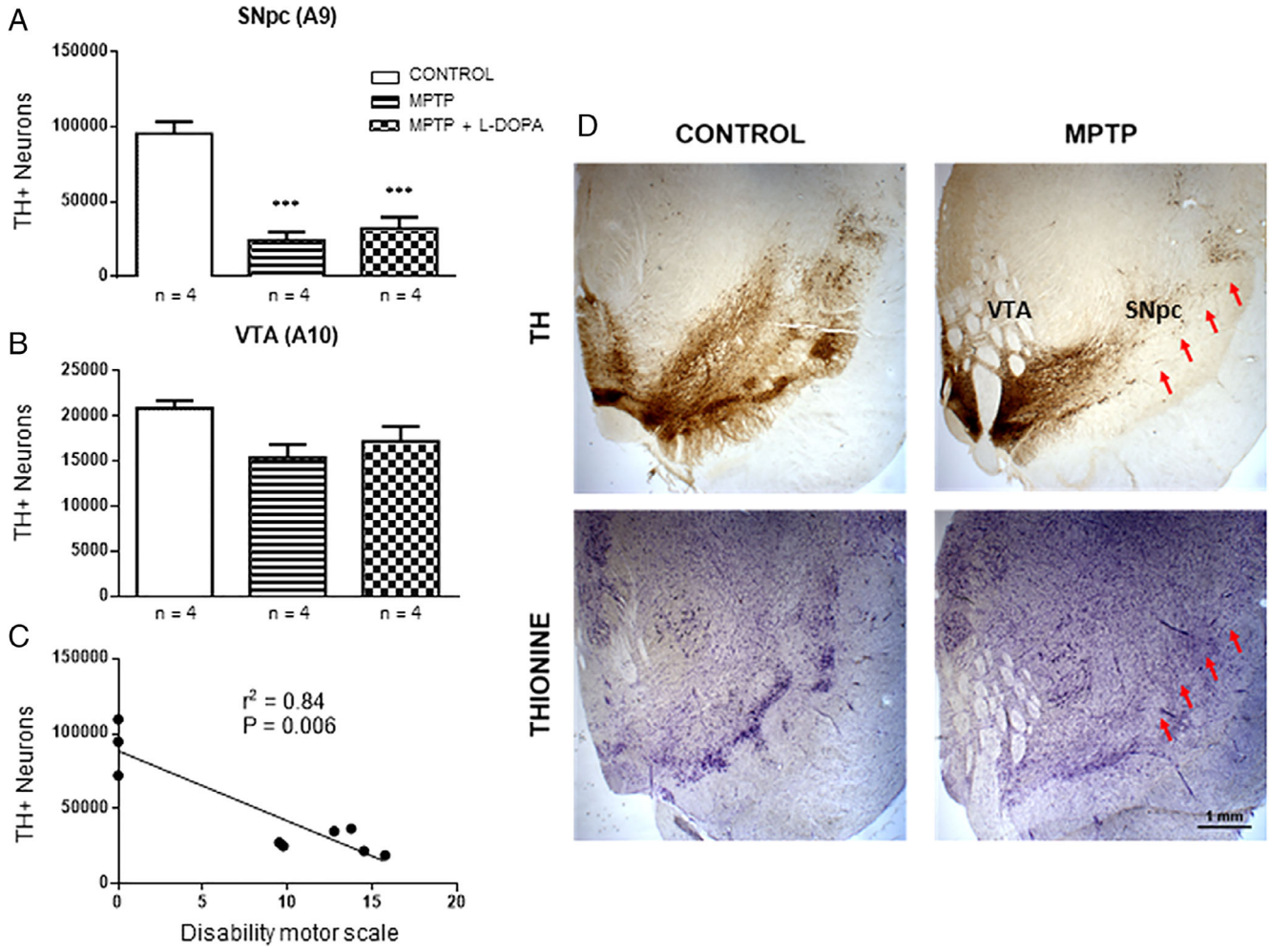

FIG. 1. Effects of MPTP and MPTP+L-dopa on the number of TH+ neurons. (**A**) Histograms showing the effect of MPTP and L-dopa administration on the total number of TH-immunoreactive- (ir) cells remaining in the substantia nigra pars compacta and (**B**) ventral tegmental area. (**C**) Correlation between TH+ neurons in the substantia nigra pars compacta and the disability motor score (parkinsonian ratings). (**D**) Above: Loss of dopaminergic neurons in MPTP-treated monkeys compared with controls. Representative sections showing TH-positive neurons in the substantia nigra pars compacta compared with the control. Below: Thionine staining. Histogram bars represent mean ± standard error of the mean. Symbols indicate significant differences between groups. ***$P < 0.001$ compared with the control group. L-dopa, levodopa; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH, tyrosine hydroxylase. [Color figure can be viewed at wileyonlinelibrary.com]

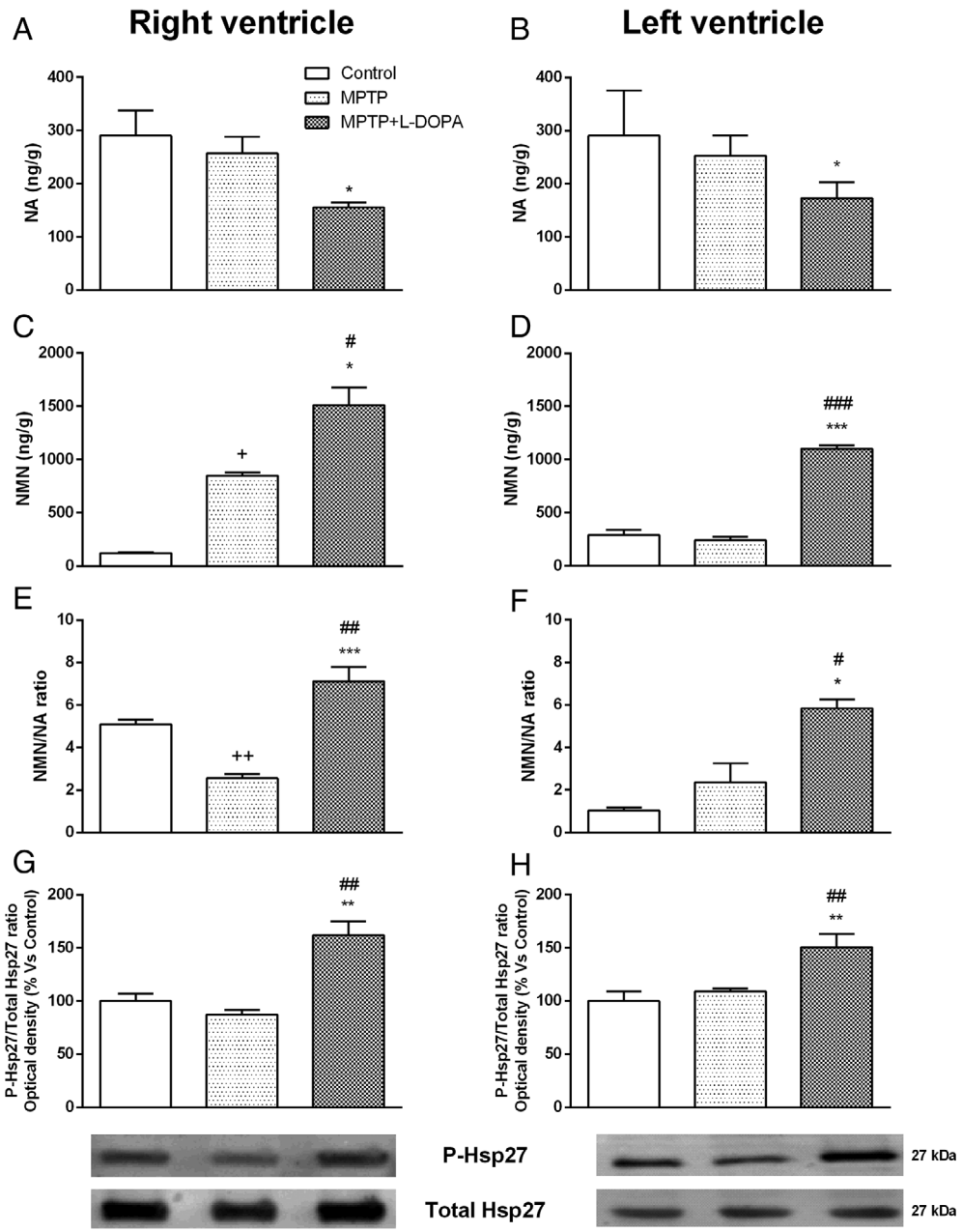

FIG. 2. Effects of MPTP and MPTP+L-dopa on NA turnover in the right and left ventricles: (**A,B**) NA concentrations (ng/g); (**C,D**) NMN concentrations (ng/g); (**E,F**) NMN/NA ratio in controls, MPTP-treated monkeys, and MPTP+L-dopa groups. NA turnover was significantly increased in both ventricles in MPTP+L-dopa–treated monkeys compared with both MPTP-intoxicated monkeys and control animals. (**G,H**) P-Hsp27/total Hsp27 ratio (optical density % vs. control) in controls, MPTP-treated monkeys, and MPTP+L-dopa groups. Hsp27 activity was significantly increased in the right and left ventricles in MPTP+L-dopa–treated animals versus both controls and MPTP-treated monkeys. Histogram bars represent mean ± standard error of the mean (+$P < 0.05$, ++$P < 0.01$ = comparing MPTP-treated monkeys with controls; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ = comparing L-dopa–treated animals with controls; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ = comparing L-dopa–treated animals with MPTP-treated monkeys). Hsp27, heat shock protein 27; L-dopa, levodopa; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NA, noradrenaline; NMN, normetanephrine; P-Hsp27, phosphor-heat shock protein 27.

all MPTP-treated monkeys (~25% neuronal loss and remains almost unchanged in L-dopa group; Fig. 1B).

Effects of Parkinsonism and L-dopa Treatment on NA Turnover and Phospho-Hsp27 Expression

NA levels significantly decreased in the MPTP+L-dopa–treated monkeys with respect to the controls in both ventricles ($P < 0.05$; Fig. 2A,B). When compared with the control group, NMN concentration was significantly increased in the RV of MPTP-treated monkeys without changes in LV ($P < 0.05$), and in both the RV ($P < 0.05$) and LV ($P < 0.001$) of the MPTP+L-dopa group (Fig. 2C,D). In contrast, in the MPTP-treated monkeys, the NMN/NA ratio was significantly decreased ($P < 0.01$) in RV with respect to controls, with no significant changes in LV. However, L-dopa treatment

significantly increased the NMN/NA ratio (NA turnover) in both RV and LV when compared with the MPTP-intoxicated monkeys ($P < 0.001$ and $P < 0.05$, respectively) and with controls ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2E,F).

We also examined phospho-Hsp27 at Ser82, which is highly expressed in the heart.[18] MPTP treatment did not modify the phospho-Hsp27/total Hsp27 ratio in the RV nor LV. However, this ratio was significantly increased after MPTP+L-dopa treatment versus the MPTP group ($P < 0.01$) and control group ($P < 0.01$) in both ventricles (Fig. 2G,H).

Discussion

Cardiovascular alterations have been consistently reported in PD patients and in experimental models. Specifically, the systemic administration of MPTP reduces cardiac NA in rodents.[19] These results are consistent with our study, which demonstrated a significant decrease of NA turnover in the RV in MPTP-treated monkeys.

Recent findings correlate PD and several apoptotic markers (the overexpression of p53 and active caspase-3 in the cardiac muscle) and the underexpression of the β-adrenergic receptor, which can potentially promote the cardiac dysfunction observed in PD patients.[20] Clinical and pathological studies have provided strong evidence of the involvement of cardiac sympathetic nerves in PD patients.[21-24] Our results demonstrate noradrenergic sympathetic loss (significant decrease in NA turnover in RV) in MPTP-treated monkeys. According to these results, it has been demonstrated that MPTP administration in nonhuman primates decreases TH-ir fibers in cardiac tissue and the peripheral catecholamine system,[25,26] and additionally, MPTP intoxication induced the depletion of cardiac NA in both mice[9] and rats.[19]

Regarding the differences observed in the concentrations of NMN in the RV versus LV (higher concentration in RV than in LV) they may be a result of the different amount and distribution of sympathetic loss in PD (higher in LV than in RV, with maximal sympathetic loss in the apex and in the inferior and lateral walls of the LV).[27] However, L-dopa treatment increases NA turnover, suggesting that the cardiac sympathetic pathways improved in both ventricles when compared with non-L-dopa parkinsonian monkeys. Nevertheless, some studies showed a controversy regarding the effect of L-dopa in cardiovascular autonomic function. L-dopa could ameliorate abnormal heart rate variability, and not exacerbate orthostatic hypotension,[28-32] but other evidence has demonstrated that L-dopa lowered blood pressure and even enhanced orthostatic hypotension.[33-35]

In addition, we show an enhancement of the phospho-Hsp27/total Hsp27 ratio in parallel with the increased NA turnover, indicating an increased activity of this protein in both ventricles in dyskinetic monkeys. Hsp27 could induce cardioprotection, acting as a molecular chaperone and in the phosphorylation-dependent stabilization of actin,[36] but no studies have directly evaluated the toxic or protective cardiac effects of L-dopa. In our study, L-dopa increased Hsp27 levels, both total and phosphorylated, which might induce cardioprotection.[36]

In conclusion, our results indicate cardiac impairments in MPTP animals, possibly as a result of decreased cardiac sympathetic pathways (but further investigations are required to determine the origin of these changes), and we show for the first time that L-dopa increases both NA turnover and the activity of Hsp27 in both ventricles. ●

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.