The Harman and Norharman Reduced Dopamine Content and Induced Cytotoxicity in PC12 Cells

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Abstract - The effects of harman and norharman on dopamine content and L-DOPA-induced cytotoxicity were investigated in PC12 cells. Harman and norharman decreased the intracellular dopamine content for 24 h. The IC50 values of harman and norharman were 20.4 µM and 95.8 µM, respectively. Tyrosine hydroxylase (TH) activity and TH mRNA levels were also decreased by 20 µM harman and 100 µM norharman. Under the same conditions, the intracellular cyclic AMP levels were decreased by harman and norharman. In addition, harman and norharman at concentrations higher than 80 µM and 150 µM caused cytotoxicity at 24 h in PC12 cells. Non-cytotoxic ranges of 10-30 µM harman and 50-150 µM norharman inhibited L-DOPA (20-50 µM)-induced increases of dopamine content at 24 h. Harman at 20-150 µM and norharman at 100-300 µM also enhanced L-DOPA (20-100 µM)-induced cytotoxicity at 24 h. These results suggest that harman and norharman decrease dopamine content by reducing TH activity and aggravate L-DOPA-induced cytotoxicity in PC12 cells.

Keywords: Harman, Norharman, Dopamine content, Tyrosine hydroxylase, PC12 cell

INTRODUCTION

β-Carbolines derivatives, harman and norharman (Fig. 1), may be formed by cyclization of indoleamines with aldehydes in the brain (Deitrich and Erwin, 1980) during conventional high-temperature cooking and tobacco smoking (Pfau and Skog, 2004; Breyer-Pfaff et al., 1996). Harman and norharman are also found in groundwater, plants, grape juice and wine (Allen and Holmstedt, 1980; Pfau and Skog, 2004).

Harman, a 1-methylated derivative of norharman, has been reported to have a strong inhibitory action of monoamine oxidase type A (MAO-A, EC 1.4.3.4) (May et al., 1991). Norharman inhibits selectively MAO-B (May et al., 1991) and is found in substantia nigra from humans (Matsubara et al., 1993). The plasma levels of harman and norharman are also increased in parkinsonian patients (Kuhn et al., 1995).

MAO-A and -B in human brain tissue metabolize dopamine, which is the most affected neurotransmitter in Parkinson’s disease (Ehringer and Hornykiewicz, 1960). It is, therefore, suggested that harman and norharman as MAO inhibitors may play neuroprotective roles in Parkinson’s disease. In contrast, the dopamine metabolites of MAO could produce the cellular damages by the formation of reactive oxygen species (Cohen, 1983). In addition, β-carbolines have been proposed as neuronal toxins because of the structural similarity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP+) (Albores et al., 1990; Fields et al., 1992). However, these inconsistent functions of harman and norharman on dopamine biosynthesis and cytotoxicity could not be fully elucidated.

L-3,4-Dihydroxyphenylalanine (L-DOPA), the precursor of dopamine, is administered most frequently for controlling the symptoms of Parkinson’s disease (Marsden, 1994). However, the long-term L-DOPA therapy produces neurotoxicity by generating reactive oxygen spe-

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Fig. 1. Chemical structures of harman (A) and norharman (B).
cies leading to apoptosis (Marsden, 1994; Sandstrom et al., 1994).

Rat adrenal pheochromocytoma PC12 cells have dopamine synthesizing, storing and releasing properties (Tischler et al., 1983). PC12 cells also express catecholamine biosynthetic enzymes such as tyrosine hydroxylase (TH, EC 1.14.16.2), aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) (Tischler et al., 1983).

In this study, therefore, the effects of harman and norharman on dopamine content and cytotoxicity were investigated using PC12 cells as a model system.

MATERIALS AND METHODS

Materials
Harman, norharman, L-DOPA and 3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclic AMP enzyme immunoassay kit was purchased from Amersham Pharmacia Biotech (St. Freiburg, Germany). All sera, antibiotics and RPMI 1640 for cell culture were obtained from Gibco (Rockville, MD, USA). All other chemicals were of reagent grade.

Cell culture
PC12 cells were maintained routinely (Tischler et al., 1983). PC12 cells (ca. 1 x 10^5 cells/cm^2) were treated with harman and norharman in the absence or presence of L-DOPA with indicated times.

Determination of dopamine content and TH activity
Dopamine content was determined by using an HPLC system (Toso, Tokyo, Japan) as described previously (Shin et al., 2000). TH activity was also measured according to Nagatsu et al. (1979) as described previously (Shin et al., 2000) with a slight modification. The HPLC analysis for the determination of TH activity was performed as described previously (Shin et al., 2000).

RNA extraction and Northern blotting
Total RNA was extracted and RNA samples (10 µg/lane) were fractionated by electrophoresis on 1% agarose containing 0.66 M formaldehyde gel and transferred to a nylon membrane (ICN, East Hills, NY, USA). The Northern blot analysis for TH mRNA was performed according to the method of Kim et al. (1993). The blots were hybridized to the coding regions of the 0.7 kb rat TH cDNA probe labeled with [α-32P] dCTP using a Random Primer labeling system (DuPont NEN, Boston, MA, USA).

Measurement of cyclic AMP levels
The cells were incubated with harman and norharman for 30 min and agitated after the addition of the lysis reagent. Cyclic AMP levels were measured by using an enzyme immunoassay system kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Assessment of cell viability
Cell viability was determined by the conventional MTT assay. Cells were treated with various concentrations of harman, norharman and L-DOPA, alone or associated, for 24 h and then treated with the MTT solution (final concentration, 1 mg/ml) for 4 h in an incubator. The reaction was stopped by adding 0.8 M HCl in isopropanol. The absorbance was measured at 570 nm by using a Bauty Diagnostic Microplate Reader (Molecular Devices, CA, USA).

Statistical analysis
Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. All data were presented as means ± S.E.M. of at least four experiments. Statistical analysis was performed using ANOVA followed by Tukey’s test.

RESULTS

Inhibition of dopamine content
Treatments of PC12 cells with harman at 5-25 µM and norharman at 40-120 µM significantly decreased the intracellular dopamine content in a concentration-dependent manner for 24 h (Fig. 2). The IC_{50} values of harman and norharman were 20.4 µM and 95.8 µM, respectively. Harman and norharman caused cytotoxicity at 24 h with
the concentrations up to 80 µM and 150 µM, respectively (Fig. 3).

Dopamine content was decreased by 20 µM harman and 100 µM norharman at 6 h, and reached minimal levels at 24-48 h, respectively (Fig. 4). Dopamine content in the medium, which was secreted from the intracellular dopamine, was not altered by 20 µM harman and 100 µM norharman for 24-48 h (data not shown).

**Inhibition of TH activity and TH gene expression**

TH activity was inhibited by 20 µM harman and 100 µM norharman to 48.6-51.2% and 53.2-57.5% compared to control levels at 12-48 h (Table I). However, AADC

![Fig. 3. Effects of harman (A) and norharman (B) on PC12 cell viability. PC12 cells were exposed to harman and norharman for 24 h. The cell viability was assessed by the MTT method, in which viable cells convert the soluble dye MTT to insoluble blue formazan crystals. Results represent means ± S.E.M. of four experiments. *P<0.05; **P<0.01 compared to control levels (ANOVA followed by Tukey’s test).](image)

![Fig. 4. Time courses of intracellular dopamine content by 20 µM harman and 100 µM norharman in PC12 cells (A). Berberine at 10 µM was used as a positive control at 24 h (Shin et al., 2000) (B). Dopamine content of control levels was 3.54 ± 0.34 nmol/mg protein. Results represent means ± S.E.M. of four experiments. Significantly different from the control values: *P<0.05; **P<0.01 (ANOVA followed by Tukey’s test).](image)

![Fig. 5. Time courses of TH mRNA levels by 20 µM harman (A) and 100 µM norharman (B) in PC12 cells. Total RNA was extracted and 10 µg aliquots were subjected to electrophoresis on formaldehyde gels, blotted onto nylon and probed with 32P-labeled cDNA probes for rat TH and α-tubulin. Relative density ratio in control was expressed as 1 arbitrary unit. Results represent means ± S.E.M. of four experiments. *P<0.05 compared to control levels (ANOVA followed by Tukey’s test).](image)

**Table I. Effects of tyrosine hydroxylase (TH) activity on harman and norharman in PC12 cells**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>TH activity (nmol/min/mg protein) (% of control)</th>
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<tbody>
<tr>
<td></td>
<td>Incubation time (12 h)</td>
</tr>
<tr>
<td>Control</td>
<td>3.72 ± 0.26 (100)</td>
</tr>
<tr>
<td>Harman (20 µM)</td>
<td>1.88 ± 0.12 (50.5)*</td>
</tr>
<tr>
<td>Norharman (100 µM)</td>
<td>1.98 ± 0.15 (53.2)*</td>
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</table>

PC12 cells were treated with harman and norharman for 12-48 h and TH activity was assayed by an HPLC method. Results represent means ± S.E.M. of four experiments. Significantly different from the control values: *P<0.01 (ANOVA followed by Tukey’s test).
activity was not inhibited by 20 µM harman and 100 µM norharman (data not shown). TH mRNA levels were also decreased by harman at 6-12 h and by norharman at 12-24 h (Fig. 5A and 5B).

**Reduction of cyclic AMP levels**

Harman at 20 µM and norharman at 100 µM significantly reduced the intracellular cyclic AMP levels to 50.5% and 70.1% of control levels at 30 min, respectively (Table II).

**L-DOPA-induced dopamine content**

Treatments with L-DOPA at 20 µM and 50 µM for 24 h increased dopamine content to 121% and 131% compared to control levels, respectively (Fig. 6). Harman (10, 20 and 30 µM) reduced L-DOPA (20 and 50 µM)-induced

<table>
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<tr>
<th>Compounds</th>
<th>Cyclic AMP levels (nmol/min/mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.84 ± 0.32 (100)</td>
</tr>
<tr>
<td>Harman (20 µM)</td>
<td>1.94 ± 0.31 (50.5)**</td>
</tr>
<tr>
<td>Norharman (100 µM)</td>
<td>2.69 ± 0.38 (70.1)*</td>
</tr>
</tbody>
</table>

PC12 cells were treated with harman and norharman and incubated at 37°C for 30 min. The intracellular cyclic AMP levels were measured by an enzyme immunoassay. Results represent means±S.E.M. of four experiments. Significantly different from the control values: *P<0.05; **P<0.01 (ANOVA followed by Tukey’s test).

**L-DOPA-induced cytotoxicity**

Harman and norharman exhibited cytotoxicity at con-
centrations higher than 100 µM and 200 µM at 24 h, respectively (Fig. 3). Treatments with L-DOPA at 100 µM, but not 20 and 50 µM, for 24 h reduced the cell viability to 90.5% of control levels, respectively (Fig. 7). Harman (20, 100 and 150 µM) associated with L-DOPA (20, 50 and 100 µM) showed the enhancing effects on the cell death at 24 h compared to L-DOPA alone (Fig. 7). Norharman (100, 200 and 300 µM) associated with L-DOPA (20, 50 and 100 µM) also exhibited the same trends on the cell viability (Fig. 7).

**DISCUSSION**

L-DOPA has been commonly used as the prior drug of the Parkinson’s disease. However, L-DOPA accelerated the deterioration of the patient’s condition in the long-term therapy (Marsden, 1994). TH is the rate-limiting enzyme in the dopamine biosynthetic pathways and involved for dopamine formation through the conversion of L-tyrosine to L-DOPA followed decarboxylation.

β-carbolines, harman and norharman, inhibit MAO activity, which is suggested that they have a possibly protective effect on Parkinson's diseases. In contrast, β-carbolines are structurally similar to MPTP, which causes a Parkinson-like syndrome by forming MPP⁺ (Fields et al., 1992). Therefore, the effects of harman and norharman on dopamine biosynthesis and L-DOPA-induced cytotoxicity were investigated in PC12 cells.

The intracellular dopamine content was significantly decreased by harman and norharman in a concentration-dependent manner without the secretion of dopamine. Under the same conditions, the intracellular TH activity and TH mRNA levels were reduced by harman and norharman. The intracellular cyclic AMP levels were also significantly reduced by harman and norharman. TH activity and TH gene expression could be activated by various factors including cyclic AMP, PKA, Ca²⁺, PKC and Ca²⁺/calmodulin kinase (Goldstein, 1995; Kumer and Vrana, 1996). These results suggest that harman and norharman inhibit dopamine biosynthesis by reducing TH activity and TH mRNA expression, which are mediated by the intracellular cyclic AMP levels.

L-DOPA at 20-100 µM increases dopamine content after 24 or 48 h of incubation in PC12 cells (Migheli, 1999; Lee et al., 2003). L-DOPA at concentrations higher than 100 µM also produces the intracellular cytotoxicity for 24 h, which is mediated by oxidative stress (Migheli, 1999; Lee et al., 2003). In this study, harman (10-30 µM) and norharman (50-150 µM) reduced L-DOPA (20-50 µM)-induced increases of dopamine content for 24 h in PC12 cells. High concentrations of harman (100 µM) and norharman (300 µM) caused cytotoxicity. Harman (20-150 µM) and norharman (100-300 µM) associated with L-DOPA (20-100 µM) enhanced L-DOPA-induced cytotoxicity. In addition, harman and norharman associated with L-DOPA exhibited a greater cytotoxicity than harman, norharman or L-DOPA alone. Harman also showed a stronger ability for the inhibition of dopamine biosynthesis and the aggravation of L-DOPA-induced cytotoxicity than norharman.

Many isoquinoline derivatives such as berberine, palmatine, bulbocapnine, higenamine, tetrahydropapaveroline, ethaverine and hydrastine inhibit the intracellular dopamine content in PC12 cells (Lee and Kim, 1996; Shin et al., 1998; Shin et al., 1999; Kim et al., 2005; Shin et al., 2001; Yin et al., 2004a). Tetrahydropapaveroline and hydrastine aggravate L-DOPA-induced cytotoxicity in PC12 cells (Lee et al., 2003; Yin et al., 2004b). Isoquinoline derivatives are reported to have a similar structure with MPTP (McNaught et al., 1998) and their intracellular cytotoxic effects are also found to be mediated by oxidative stress (Nagatsu et al., 1997).

Carbolines are converted to N-methylated β-carboline cations, which can be toxic to dopaminergic neurons, by β-carboline 9N-methyltransferase (Matsubara et al., 1993). N-Methylated β-carbolinium ions such as 2-methyl-norharman induce large lesions after injection in the substantia nigra of rats (Neafsey et al., 1989) and are also increased in the frontal cortex of parkinsonians (Gearhart et al., 2000). 2-Methylated β-carbolines are comparable to MPP⁺ as inhibitors of mitochondrial respiration (Albores et al., 1990). In Parkinson’s diseases, the biosynthesis of N-methylated β-carbolinium derivatives is enhanced probably due to the failure of further catabolisation and detoxification of those N-methylated compounds (Green et al., 1991). These results suggest that N-methylated derivatives are more toxic than the parent compounds. In addition, the precursors of those N-methylated β-carbolines such as harman and norharman were significantly higher in the substantia nigra than in the cortex of human brain without degeneration of substantia nigra (Matsubara et al., 1993). It is, therefore, suggested that the 1-methyl group of harman may play similar important roles regardless of N-position of methyl group. Further studies need to be determined the mechanisms responsible for the different effects on the N- or 1-position of methyl group.

In conclusion, harman at 10-30 µM and norharman at 50-150 µM reduced dopamine content and aggravated L-DOPA (20-50 µM)-induced cytotoxicity in PC12 cells. It is,
therefore, suggested that the patient in the long-term L-DOPA therapy should be carefully monitored for the drug interaction with the various neurotoxicants such as β-carbolines and isoquinoline derivatives.

ACKNOWLEDGEMENTS

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Therefore, it is important to monitor the patient for any side effects or complications.

For a comprehensive understanding of the subject, refer to the following references:


These references provide a solid foundation for understanding the interactions between β-carbolines and isoquinoline derivatives and their potential impact on Parkinson’s disease. Further research is needed to fully understand the mechanisms involved.


