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Prostaglandin H Synthetase-Mediated Metabolism of Dopamine: Implication for Parkinson's Disease

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Abstract: Differences in prostaglandin H synthetase (PHS) activity in the substantia nigra of age- and postmortem interval–matched parkinsonian, Alzheimer's, and normal control brain tissue were assessed. Prostaglandin E_2 (PGE_2, an index of PHS activity) was higher in substantia nigra of parkinsonian brain tissue than Alzheimer's or control tissue. Incubation of substantia nigra slices with arachidonic acid (AA) increased PGE_2 synthesis. Dopamine stimulated PHS synthesis of PGE_2. [^{3}H]Dopamine was activated by PHS to electrophilic intermediate(s) that covalently bound to DNA, microtubulin protein, bovine serum albumin, and sulfhydryl reagents. When AA was replaced by hydrogen peroxide, PHS/H_2O_2-supported binding proceeded at rates similar to those observed with PHS/AA. Indomethacin and aspirin inhibited AA-mediated cooxidation of dopamine but not H_2O_2-mediated metabolism. PHS-mediated metabolism of dopamine was not affected by monoamine oxidase inhibitors. Substrate requirements and effects of specific inhibitors suggest cooxidation of dopamine is mediated by the hydroperoxidase activity of PHS. ^{32}P-postlabeling was used to detect dopamine-DNA adducts. PHS/AA activation of dopamine in the presence of DNA resulted in the formation of five dopamine-DNA adducts, i.e., 23, 43, 114, 70, and 270 amol/μg DNA. DNA adduct formation was PHS, AA, and dopamine dependent. PHS catalyzed cooxidation of dopamine in dopaminergic neuronal degeneration is discussed. **Key Words:** Arachidonic acid—Prostaglandins—Dopamine—Cooxidation—Neurons—Parkinson's disease.
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Lipid peroxidation and associated processes have been linked to a variety of disorders and pathological conditions, including Parkinson's disease (PD), Alzheimer's disease (AD), and ischemia–reperfusion (Horton et al., 1987; Minotti and Aust, 1987; Dexter et al., 1989; Olanow, 1990; Adams and Odunze, 1991; Jesberger, 1991; Strong et al., 1993). The CNS is exquisitely sensitive to peroxidative damage because it is rich in oxidizable substrates such as polyunsaturated fatty acids and catecholamines (Halliwell and Gutteridge, 1988). The arachidonic acid (AA; 20:4; 5,8,11,14-eicosatetraenoic acid) is an abundant lipid, incorporated mostly into phosphatidylcholine of cell membrane phospholipids (Rosenbaum et al., 1989). The release of free AA serves as the rate-limiting reaction in the AA cascade that results in the formation of biologically active eicosanoids (Wolfe, 1982; Rosenbaum et al., 1989). A wide range of stimuli including trauma, hypoxia, and ischemia initiate AA release (Wolfe et al., 1989). Hydrogen peroxide (H_2O_2), a by-product of catecholamine/monoamine oxidase and aerobic cellular metabolism, is a strong stimulator of AA release and prostaglandin synthesis in intact cells (Polgar and Taylor, 1980). Lipid hydroperoxides, serotonin, and noradrenaline stimulate prostaglandin synthesis in the CNS by mobilizing AA (Horrobin, 1978; Hemler et al., 1979). The catecholamines serotonin and noradrenaline have been shown to stimulate prostaglandin synthesis by serving as reducing cofactors (Sih et al., 1970; Eling et al., 1990). AA-derived hydroperoxides result from the action of PHS and lipoxygenase (Wolfe, 1982; Smith et al., 1991). PHS is a bifunctional enzyme containing cyclooxygenase and peroxidase activity (Eling et al., 1990), and the two

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Abbreviations used: AA, arachidonic acid; AD, Alzheimer's disease; BSA, bovine serum albumin; FAB, fast atom bombardment; HRP, horseradish peroxidase; LC/MS, liquid chromatography/mass spectrometry; MAO, monoamine oxidase; PD, Parkinson's disease; PGD_2, PGE_2, $PGF_{2\alpha}$, prostaglandins D_2, E_2, $F_{2\alpha}$, respectively; PHS, prostaglandin H synthetase; PMI, postmortem interval; SN, substantia nigra.

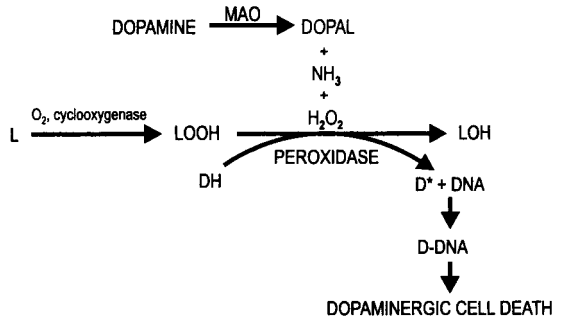

FIG. 1. Schematic representation of PHS-mediated activation of dopamine. L, lipid, AA; LOOH, 15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGG_2); LOH, 15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGH_2); DH, reducing cosubstrate (dopamine); D*, one- or two-electron oxidized metabolite of dopamine (*o*-semiquinone or *o*-quinone).

enzyme activities reside in one enzyme but can be studied separately (Zenser et al., 1980; Eling et al., 1990). The oxygenase aspect of this bifunctional enzyme is inhibited by aspirin and indomethacin (Eling et al., 1990). PHS activity is found in astrocytes, a major source of monoamine oxidase (MAO), in brain blood vessels and in primary neuronal cultures (Keller et al., 1985; Brooks et al., 1989; Minn et al., 1991).

In human brain, regional distribution of prostaglandins has shown the presence of prostaglandin D_2 (PGD_2), PGE_2, and $PGF_{2\alpha}$ in the substantia nigra (SN), caudate nucleus, and putamen, areas affected in PD (Abdel-Halim et al., 1980; Ogorochi et al., 1984). Prostaglandin H synthetase (PHS)-mediated lipid peroxidation requires ferrous iron and oxygen for maximal enzyme activity (Rao et al., 1978). Increased free iron in the SN, especially the pars reticulata (Sofic et al., 1991), and generation of large amounts of H_2O_2 from dopamine turnover/metabolism (compensatory mechanism) by MAO have been reported in PD (Spina and Cohen, 1989). Moreover, catechols have been shown to release AA and serve as reducing cosubstrate for the production of prostaglandins (Sih et al., 1970; Markey et al., 1987; Eling et al., 1990). These observations, combined with the intracellular concentration of dopamine (millimolar) reported within the neurons of the nigrostriatal tract (Hedqvist, 1977), suggest that cooxidative metabolism of dopamine by PHS to reactive intermediates and subsequent covalent adduct with tissue nucleophiles may represent another possible mechanism underlying the neuronal degeneration seen in PD (Fig. 1). Evidence for the involvement of the PHS peroxidase activity in human diseases has been reported (Moldeus et al., 1982; Andersson et al., 1983; Mattammal et al., 1987).

In the present study, we have assessed PHS activity in the SN of parkinsonian, AD, AD/PD, and age- and postmortem interval (PMI)-matched control brain tissue by measuring PGE_2, one of the major prostaglandins produced by PHS in SN. Irreversible covalent binding of dopamine metabolite(s) to bovine serum albumin (BSA), microtubulin protein, and DNA, as well as the trapping of reactive metabolite(s) with nucleophiles such as *N*-acetylcysteine and glutathione, were used as an index of metabolic activation mediated by PHS/AA and PHS/H_2O_2. In addition, the ^{32}P-postlabeling method was used to evaluate the covalent adduct(s) resulting from the cooxidative metabolism of dopamine by PHS. Studies were also performed using various peroxidatic systems as a mechanistic framework in understanding the bioactivation of dopamine catalyzed by PHS.

MATERIALS AND METHODS

Materials

Purified tubulin protein (2 mg/ml) was a gift from Dr. John J. Correia, University of Mississippi Medical Center (Jackson, MS, U.S.A.). Purified PHS (ram seminal vesicle, 0.25 mg/ml, 51,000 U/mg) was purchased from Oxford Biomedical Research, Inc. (Oxford, MI, U.S.A.). AA was purchased from Nu-Check Inc. (Elysian, MN, U.S.A.). Hemoglobin, indomethacin, acetylsalicylic acid (aspirin), and horseradish peroxidase (HRP; type VI, 250 U/mg, RZ = 3.0), lactoperoxidase (bovine milk, 80 U/mg, A_{412}/A_{280} = 0.92), myeloperoxidase (human leukocytes, 50 U/mg), dopamine hydrochloride, 30% H_2O_2, 2,4,5-trihydroxyphenethylamine hydrobromide, potassium ferricyanide, GSH, calf thymus DNA (type 1) and *N*-acetylcysteine, micrococcal endonuclease (grade VI), and potato apyrase (grade 1) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [*ring*-2,5,6-^{3}H]Dopamine (25.8 Ci/mmol) was purchased from NEN Research Products (Boston, MA, U.S.A.). Carrier-free [^{32}P]phosphate (4,500 Ci/mmol) was purchased from ICN (Irvine, CA, U.S.A.). HPLC-grade methanol and 6-ml C-18 reverse-phase Bakerbond solid-phase extraction columns (1,000 mg adsorbent) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). SKF-525A, deprenyl, clorgyline, and pargyline were purchased from Research Biochemicals International (Natick, MA, U.S.A.). Nuclease P_1 and spleen exonuclease were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN, U.S.A.). T_4 polynucleotide kinase was from PL-Biochemicals. Polyethylenimine-cellulose plates were purchased from Brinkmann Instruments (Westbury, NY, U.S.A.).

Source of postmortem brain tissue. Postmortem human brain tissue samples, pathologically identified as normal control, PD, AD, AD/PD, were obtained from the St. Louis University brain bank and Brain Tissue Resource Center, McLean Hospital (Belmont, MA, U.S.A.). The pathology in the SN from AD/PD patients was identical to PD. Brain tissue samples (SN) examined in the present study were from four AD/PD cases who were not on L-DOPA therapy (age, 81.3 ± 1.9 years; PMI, 7.7 ± 2.1 h); four PD cases who were on L-DOPA therapy (age, 78 ± 3.2 years; PMI, 10.3 ± 3 h); four pure controls (age, 78 ± 2.8 years; PMI, 8.5 ± 3.2 h); and three AD cases (age, 76 ± 3.1 years; PMI, 9.3 ± 3 h). At autopsy, SN tissue was removed, quick-frozen in liquid nitrogen, and stored at −135°C.

HPLC and mass spectrometry

HPLC purifications were performed using two Shimadzu (Tokyo, Japan) LC-6A pumps, an LC-6A controller, and a

25 cm × 4.6 mm (i.d.), 5-μm Supelcosil C-18 column (Supelco Inc., Bellefonte, PA, U.S.A.). The eluting solvent was methanol/water (10:90, vol/vol), and the flow rate was 1.0 ml/min.

Thermospray liquid chromatography/mass spectrometry (LC/MS) was performed on a Vestec (Houston, TX, U.S.A.) 210N mass spectrometer. Fast atom bombardment (FAB) mass spectra were acquired using a VG-ZAB (VG Analytical, Cheshire, U.K.) double-focusing mass spectrometer. Glycerol was used as the matrix, and ionization was performed by a xenon gun at 8 keV.

Incubation conditions for PGE_2 synthesis by human brain tissue (SN)

Age- and PMI-matched SN tissue samples weighing 81–132 mg were sliced into 500-μm sections and placed in cold (4°C) Krebs–Ringer bicarbonate buffer solution containing (m*M*) NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, Na-EDTA 0.026, and glucose 11.1, bubbled with O_2 (95%)/Co_2 (5%) for 20 min before use. All brain tissue samples were preincubated for 10 min at 37°C with or without inhibitor in 2 ml of buffer. The supernatant was removed and replaced with 2 ml of fresh buffer for a 30-min incubation at 37°C with or without 20 μ*M* sodium arachidonate. After 30 min, the supernatant and the tissue were separated. Each was added to 8 ml of cold (−30°C) methanol (final methanol concentration, 80%). The tissue was homogenized, on ice, for 1 min with a Polytron tissue homogenizer. The supernatant and tissue homogenate were stored at −15°C for 18 h to precipitate proteins. Proteins and insoluble material were then removed by centrifugation at 1,800 *g* for 30 min at 4°C. The clear methanolic extract was evaporated to dryness under vacuum at 30°C in a Speed-Vac concentrator (Savant). Samples for eicosanoid assay were solubilized in 0.1 *M* phosphate buffer, pH 7.4, containing 400 m*M* NaCl, 1.0 m*M* EDTA, 0.1% BSA, and 0.01% sodium azide (assay buffer).

Measurement of immunoreactive PGE_2

Enzyme immunoassay of PGE_2 was performed as described previously (Pradelles et al., 1985). Enzymatic tracers consisted of authentic prostaglandin covalently linked to purified acetylcholinesterase. The assay was performed in 96-well microtiter plates precoated with goat anti-rabbit IgG (2 μg/well), as described (Westcott et al., 1986; Stephenson et al., 1990, 1992). Before use, the plates were washed with 10 m*M* phosphate buffer, pH 7.4, containing 0.05% Tween 20 (wash buffer). The assay was performed in a total volume of 150 μl. In brief, 50 μl of acetylcholinesterase-conjugated eicosanoid tracer, 50 μl of antiserum directed against PGE_2 (Advanced Magnetics), and 50 μl of a standard or sample in buffer were combined and incubated at 25°C for 18–20 h. After washing the plate three times with wash buffer, 200 μl of Ellman's reagent [consisting of 2 μg/ml acetylthiocholine iodide and 2.15 μg/ml 5,5′-dithiobis(2-nitrobenzoic acid) in 10 m*M* potassium phosphate buffer, pH 7.4] was dispensed into each well. The reduction of 5,5′-dithiobis(2-nitrobenzoic acid) was monitored at 405 nm using a Bio-Tek (model EL-309) enzyme immunoassay plate reader. Plates were read when absorbance for the well containing zero standard (B_0) exceeded 0.200 absorbance units. Each sample was assayed in duplicate and a standard curve was generated for each assay. Sample eicosanoid concentrations were determined by comparison to a log–logit transformation of the standard curve (Pradelles et al., 1985; Stephenson et al., 1990). Values were reported as prostaglandin/mg wet weight of brain tissue (see Table 1).

Determination of the effect of dopamine on PGE_2 synthesis

The standard reaction mixture contained 0.01 mg/ml PHS, 1.2 μ*M* hemoglobin, 0.1 *M* Tris buffer (pH 7.2), and increasing concentrations of dopamine (5–150 μ*M*) in a total volume of 0.5 ml. Reactions were started by the addition of 100 μ*M* AA. After 3 min at 37°C, 0.5 ml of cold methanol (−20°C) was added and the reaction mixture was quick-frozen and stored at −130°C until assayed. PGE_2 synthesis was measured as described above and is shown in Fig. 2.

Absorption spectroscopy of dopamine oxidation

When PHS-mediated dopamine oxidation was assessed, the reaction was initiated by the addition of 2 μl of ethanolic solution of 100 μ*M* AA. When HRP, myeloperoxidase, lactoperoxidase, or chloroperoxidase was used as the activating system, the reaction was started by the addition of 100 μ*M* H_2O_2 (5 μl). The absorption spectrum of dopamine was recorded first. The absorption spectrum of the oxidation mixture was then recorded in the overlay mode 3.0 min after the addition of the substrate. A double-beam recording spectrophotometer (UV-160A, Shimadzu Scientific Instruments, Inc., Columbia, MD, U.S.A.) was used to scan the spectra between 600 and 220 nm (see Fig. 3).

Preparation of authentic 5,6-dihydroxyindole

To a stirred solution of 2,4,5-trihydroxyphenethylamine hydrobromide (0.5 g) in water was added a solution of potassium ferricyanide (1.0 g) and sodium hydrogen carbonate (1.0 g) dissolved in 5 ml of water over a period of 5 min. After 24 h, the reaction mixture was lyophilized, and the residue was extracted with ethyl acetate (6 × 2 ml). Ethyl acetate was removed under nitrogen and the residue was sublimed at 150°C/0.01 mm. High-resolution mass measurement of the trimethylsilyl derivative of the product gave correct elemental composition: calculated, $C_{14}H_{23}NO_2Si_2$, 293.1285; found, 293.1281.

Isolation of PHS-mediated oxidation product(s) of dopamine

PHS (0.1 mg) was suspended in 5 ml of 0.1 *M* Tris buffer, pH 7.2, containing 6.0 μ*M* hemoglobin and 1.0 m*M* dopamine. After a 2-min preincubation at room temperature, AA (500 μ*M*) was added and the reaction was allowed to proceed at 37°C. After 5 min, the reaction mixture was lyophilized, and the residue was dissolved in 2 ml of nitrogen-purged water. Tandem-column chromatography with Bakerbond C-18 column and elution with nitrogen-purged water (6 ml, 1.0 ml/min) gave a light-reddish eluent and a deep purple material(s) adsorbed onto the column. The eluent fractions that showed absorption maxima at 305 and 475 nm (3 ml, fractions 3–5) were combined, lyophilized, and redissolved in 1.0 ml of nitrogen-purged methanol/water (30:70, vol/vol). One-half of this sample was analyzed by thermospray LC/MS. The major product peak eluting at 5 min showed $[M + H]^+$ ion at m/z 150. Two minor components were also observed but not identified

Identification of *N*-acetylcysteine conjugates

To the other half of the sample prepared above in methanol/water, 500 μ*M* *N*-acetylcysteine was added, and the mixture was stirred under nitrogen. Immediate decolorization of the solution resulted. After 0.5 h the light-yellow solution

was lyophilized, redissolved in 0.5 ml of nitrogen-purged water, and purified by HPLC. A major peak eluting at 11 min was collected for mass spectral analysis.

The purple material that was adsorbed onto the Bakerbond C-18 column was eluted with 5 m*M* *N*-acetylcysteine dissolved in methanol/water (10:90, vol/vol). The faint yellow eluent was collected, lyophilized, redissolved in water (0.5 ml), and purified by HPLC. A product eluting at 17 min was collected for mass spectral analysis.

High-resolution mass measurement by peak matching of the protonated molecular ion in the FAB mass spectra of these conjugates gave the following compositions: for the peak eluting at 11 min (see Fig. 4), $C_{13}H_{15}N_2O_3S$ (calculated, 311.0702; found, 311.0705), for the peak eluting at 17 min, $C_{13}H_{19}N_2O_5S$ (calculated, 315.1015; found, 315.1019). The *N*-acetylcysteine conjugate prepared from mushroom tyrosinase and dopamine (Rosengren et al., 1985) showed elemental composition and chromatographic properties identical to that of the product eluting at 17 min obtained from PHS/AA-mediated cooxidation.

Determination of covalent binding of [2,5,6-^{3}H]dopamine metabolite(s) to BSA, tubulin protein, and DNA

Incubations were performed for 3 min, at 37°C, in 0.1 *M* Tris buffer, pH 7.2, and contained 1.0 mg/ml BSA, microtubulin protein, or purified DNA, in a total volume of 0.5 ml.

PHS/AA-mediated binding. The complete reaction mixture contained 50 μM (0.25 μCi) [^{3}H]dopamine, 0.01 mg/ml of purified PHS, and 1.2 μM hemoglobin. The reaction was started by the addition of 100 μM AA (see Table 2).

PHS/H_2O_2-mediated binding. Incubation mixtures were as described above. The reaction was started by the addition of 100 μM H_2O_2 (see Table 3).

Peroxidase/H_2O_2-mediated binding. The reaction mixture contained 5 μg/ml HRP, myeloperoxidase, or lactoperoxidase. The reaction was started by the addition of 100 μM H_2O_2 (see Table 4).

Blanks were obtained by incubating without the enzyme at 37°C. Blank values obtained are subtracted from the values obtained for the complete reaction mixture. Inhibitors (dissolved in deionized water), when used, were added to the enzyme in buffer and incubated at room temperature for 2 min before initiation of the reaction.

Binding of dopamine metabolites to DNA, BSA, and tubulin protein was determined as described previously (Mattammal et al., 1981). In brief, the reaction was terminated by addition of ethyl acetate saturated with Tris buffer (pH 6.0) and extracted with the same solvent system until the radioactivity reached background values. For protein binding, the aqueous phase was treated with an equal volume of 10% trichloroacetic acid and centrifuged (15 min at 3,000 *g*). The pellet was first washed with 5% trichloroacetic acid (2 × 0.5 ml), then with ice-cold ethanol (2 × 1.0 ml), and dissolved in 400 μl of 0.1 *M* sodium hydroxide. Radioactivity in the aqueous phase was determined by liquid scintillation spectrometry.

For the assessment of DNA binding, after removal of ethyl acetate with a stream of nitrogen, 400 μl of 10% sodium dodecyl sulfate and protease K (0.5 mg) were added, and the mixture was incubated at 37°C for 30 min. Water-saturated phenol (1 ml) and water-saturated chloroform (1 ml) were added, and the mixture was shaken vigorously. After centrifugation (2,000 g, 20 min), the aqueous layer was transferred to a new test tube and the macromolecules were precipitated by the addition of 5% potassium acetate in ethanol. After centrifugation (2,000 *g*, 30 min), the supernatant was discarded. The DNA pellet was dissolved in water (1 ml), reprecipitated, and redissolved in water (400 μl). Radioactivity in the aqueous phase was determined by liquid scintillation spectrometry.

^{32}P-postlabeling analysis of DNA-dopamine adduct(s)

Control or adducted DNA was prepared from PHS/AA or HRP/H_2O_2 incubations as described above and was hydrolyzed to deoxynucleoside 3′-monophosphates by incubating 5 μg of DNA with 5 μg each of micrococcal nuclease and spleen diphosphoesterase in 15 μl of 10 m*M* sodium succinate (pH 6.0)/5 m*M* $CaCl_2$, at 37°C for 5 h. Nuclease P_1 was added to the digest and the incubation was continued at 37°C for 60 min. The mixture was evaporated in a Speed Vac concentrator (Savant Instruments) and the residue was dissolved in 10 μl of water. To this solution was added 5 μl of a solution containing 30 m*M* Tris-HCl (pH 9.5)/10 m*M* $MgCl_2$/10 m*M* dithiothreitol/10 m*M* spermidine/0.1 μl of T_4 polynucleotide kinase and [^{32}P]ATP (100 μCi, 22 pmol at 4,500 Ci/mmol). The mixture was incubated at room temperature for 40 min, converting the modified nucleotides into the ^{32}P-labeled 3′,5′-dpNps (3′,5′-deoxynucleoside bisphosphates). Potato apyrase (2 U) was then added and the incubation was continued for 45 min at 37°C. Labeled adducts were separated by a three-directional polyethylenimine-cellulose TLC system (Gupta et al., 1982) using the following solvents: D1, 1.7 *M* sodium phosphate, pH 6.8; D3, 4.5 *M* lithium formate/7 *M* urea, pH 3.5; D4, 0.8 *M* lithium chloride/0.5 *M* Tris-HCl/7 *M* urea, pH 8.0. The ^{32}P-labeled adducts were located by autoradiography using Kodak X-Omat AR or XAR-5 films. Adduct image acquisition and analysis were performed with an AMBIS densitometer (AMBIS, Inc., San Diego, CA, U.S.A.). Adduct levels were calculated (Gupta, 1985) by relative adduct labeling and were then translated into attomoles per microgram of DNA.

Data analysis

Data are expressed as means ± SEM. Statistical significance of differences between group means was assessed by one-way analysis of variance. Differences between individual means were assessed by Bonferroni *t* tests, using the Instat Statistical Package (GraphPad Software, Inc., San Diego, CA, U.S.A.).

RESULTS

PGE_2 synthesis by SN tissue

Addition of AA to age- and PMI-matched control, AD, and parkinsonian SN brain tissue slices showed a significant increase ($p < 0.001$) in the synthesis of PGE_2 compared with basal values (Table 1). The AA-stimulated synthesis of PGE_2 was higher in parkinsonian brain tissue compared with that of control, AD, or AD/PD SN brain tissue ($p < 0.05$). When the SN slices were preincubated with the cyclooxygenase inhibitor indomethacin, the AA-stimulated PGE_2 synthesis was inhibited. Because the ratio of prostaglandins formed within a tissue may change when there is a change in the predominant cell type making up the tissue or when selective induction/inhibition of a spe-

TABLE 1. *PGE_2 synthesis by SN from PD, AD, AD/PD, and normal brain tissue and the effect of AA and the cyclooxygenase inhibitor indomethacin*

Conditions	PGE_2 (pg/mg of wet tissue weight)			
	PD (n = 4)	AD (n = 3)	AD/PD (n = 4)	Normal control (n = 3)
Basal	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
Tissue + AA (100 μM)	5.5 ± 0.2[a,b]	3.0 ± 0.1	4.7 ± 0.2[a,b]	2.5 ± 0.1
Tissue + indomethacin + AA (100 μM)	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0

Age- and PMI-matched brain tissue samples weighing 81–132 mg sliced into 500 μm sections were used. When inhibitor was used, tissue slices were preincubated for 10 min at 25°C with inhibitor in 2 ml of buffer. PGE_2 synthesis was determined as described in the experimental section. Data represent means ± SEM of independent determinations on the number of samples indicated, performed in duplicate.

[a] Statistically different from normal control, $p < 0.01$.

[b] Statistically different from AD, $p < 0.01$.

cific prostaglandin isomerease or synthase occurs. To account for this, we have also measured the metabolite 6-keto-$PGF_{1\alpha}$, the nonenzymatic hydrolysis product of PGI_2 in some samples. This value was also increased as seen with PGE_2 measurements (data not shown).

Effect of dopamine on PGE_2 synthesis

Addition of increasing concentrations of dopamine to the incubation mixture containing PHS fortified with AA resulted in pronounced PGE_2 synthesis (Fig. 2). PGE_2 synthesis was dependent on the presence of PHS and AA and was inhibited by heat treatment or the cyclooxygenase inhibitors, indomethacin and aspirin. The PGE_2 synthesis was linear for 5 min and optimal rates were obtained at 50 μM dopamine, 100 μM AA, 1.2 μM hemoglobin, and 0.01 mg/ml of PHS, and 3 min.

Absorption spectroscopy

The UV-visible absorption spectrum resulting from the cooxidation of dopamine during AA-mediated PGE_2 synthesis is shown in Fig. 3. The addition of AA to the incubation mixture resulted in the formation of a red color with a broad absorption between 450 and 500 nm and another absorption at 303 nm. The intensity of the color was directly proportional to dopamine, AA, and enzyme concentration.

The absorption spectra of the oxidation mixture obtained when HRP/H_2O_2, myeloperoxidase/H_2O_2, or lactoperoxidase/H_2O_2 was used as the activating system was identical to that obtained with the PHS/AA system (data not shown). Addition of H_2O_2 to the incubation mixture resulted in the very rapid formation of a red color. The intensity of the color was directly proportional to enzyme, dopamine, and H_2O_2 concentration. Both the peroxidase/H_2O_2 and PHS/AA systems oxidized dopamine to colored products that have identical absorption characteristics.

Identification of cooxidation product(s) of dopamine

Large scale incubations with PHS were performed. The thermospray mass spectrum of a major product in the combined (fractions 3–5) aqueous fractions showed absorption maxima at 305 and 475 nm. The mass spectrum showed an $[M + H]^+$ ion at m/z 150, which corresponds to a dopaminochrome (dopaminechrome, 2,3-dihydro-1*H*-indole-5,6-quinone) or its di-

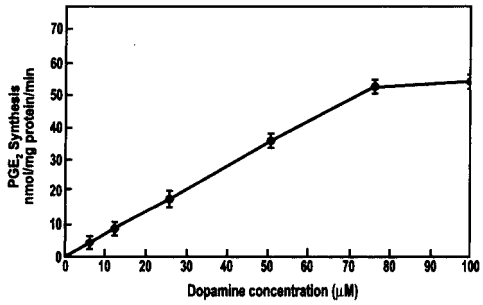

FIG. 2. Effect of various concentrations of dopamine on PGE_2 synthesis. The reaction mixture contained 0.01 mg/ml PHS, 1.2 μM hemoglobin, and various concentrations of dopamine. PGE_2 synthesis was measured as described in the experimental section. Data represent means ± SEM of three separate experiments performed in duplicate.

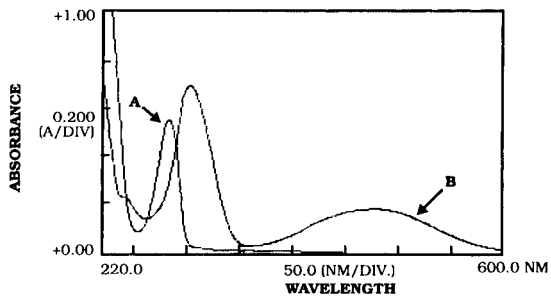

FIG. 3. A: Absorption spectrum of 50 μM dopamine. B: Absorption spectrum of oxidation mixture containing 50 μM dopamine, 1.2 μM hemoglobin, and 0.01 mg/ml PHS. Cooxidation of dopamine was initiated by the addition of AA and absorption spectrum was recorded in the overlay mode 3 min after the addition of the substrate.

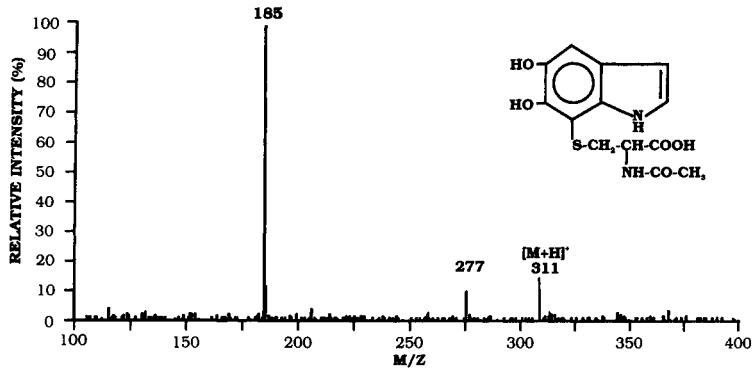

FIG. 4. FAB mass spectrum of the product isolated from the reaction of 2,3-dihydro-1*H*-indole-5,6-dione and *N*-acetylcysteine (calculated for $[M + H]^+$: $C_{13}H_{15}N_2O_3$, 311.0702; found: 311.0705). Peak at *m/z* 185 corresponds to glycerol.

hydroxyindole isomer. Absorptions in the regions of 300–303 nm and 473–480 nm have been attributed to 2-hydroxy-1,4-benzoquinone, 1,2-benzoquinone, and 2,3-dihydro-2-carboxy-1*H*-indole-5,6-dione (Swan, 1976; Graham, 1978). An authentic sample of the dihydroxyindole prepared showed only one absorption peak at 300 nm. These data suggest that the isolated product is most likely the dopaminochrome. The identity of the product was further established by reaction with *N*-acetylcysteine. The elemental composition (experimental section) of the conjugate was consistent with the proposed structure of the oxidation product as a dopaminochrome. The structure of the conjugate is shown in Fig. 4.

Elution of the adsorbed pink quinone metabolite from the chromatographic column with *N*-acetylcysteine gave a different conjugate. This conjugate showed identical elemental composition and chromatographic properties to those of the conjugate prepared from mushroom tyrosinase (Rosengren et al., 1985). Therefore, this conjugate was identified as 5-*S*-(*N*-acetylcysteinyl)-3,4-dihydroxyphenethylamine.

Covalent binding of dopamine metabolites to DNA, microtubulin, and BSA protein

PHS, in the presence of AA, catalyzed the rapid irreversible binding of [^{3}H]dopamine to DNA, microtubulin protein, and BSA. The reaction was linear for 5 min. In the absence of AA, or when boiled microsomes were used, no binding could be detected (Table 2). Incubations under nitrogen atmosphere ($-O_2$) reduced the binding reactions by 96% as did indomethacin, aspirin, ethoxyquin, and GSH. However, the MAO inhibitors pargyline, clorgyline, deprenyl, or SKF-525A, a cytochrome P-450 inhibitor, had no effect on macromolecular binding. Addition of 50 μM AA resulted in significant binding, whereas optimal rates were obtained at an AA concentration of 100 μM (data not shown).

To study the role of the peroxidase function of PHS, AA was replaced by H_2O_2. The latter catalyzed the irreversible binding of dopamine (Table 3) to DNA at rates comparable with AA. The cyclooxygenase inhibitors indomethacin or aspirin did not have any effect on binding. This demonstrates that dopamine activation to electrophilic products results from the peroxidase activity of PHS.

To investigate further the peroxidatic activation of dopamine, different peroxidases were used as activating systems. Addition of H_2O_2 to the incubation mixture containing HRP resulted in irreversible binding when DNA, microtubulin protein, and BSA were used as the acceptor macromolecules (Table 4). Oxygen was not required for the reaction, but the absence of peroxidase of H_2O_2 decreased the binding reaction by 85–90%. Addition of GSH to the incubations inhibited the binding. Of great importance for the peroxidase/H_2O_2 activation is the amount of enzyme used in the experiments. In all cases, increased irreversible binding was observed from 0.04 to 1 μg of peroxidase/ml with 100 μM H_2O_2 (data not shown).

To determine if the radioactive incorporation of dopamine into DNA resulted in specific DNA adducts, ^{32}P-postlabeling analysis was performed. Control and dopamine-adducted DNA was analyzed (Fig. 5). Complete reaction mixtures incubated in the absence of PHS (Fig. 5A) did not contain detectable adducts. In contrast, when PHS was present, five separate adducts were observed. Quantitative analysis indicated that the amounts of adducts 1–5 corresponded to 23, 43, 114, 70, and 270 amol/μg DNA, respectively. Adduct formation was not detected in the absence of dopamine or AA.

DISCUSSION

The results in this study show that PHS activity is present in SN obtained from parkinsonian brain tissue. The PHS activity in SN can be stimulated by AA and the activity is inhibited by the cyclooxygenase inhibitor indomethacin. PGE_2 synthesis, an index of PHS activity, was higher ($p < 0.001$) in SN of parkinsonian brain tissue when compared with that of age- and PMI-matched Alzheimer's and control SN tissue (Table 1). This difference in prostaglandin synthesis may have important implications for SN cell function, because the eicosanoids and their metabolism in brain have been shown to influence cerebral nutrient blood flow (Pickard, 1981), potentiate the action of excitatory

TABLE 2. *Effect of various test agents on AA-mediated cooxidation of dopamine by PHS: binding of [2,5,6-^{3}H]dopamine metabolite(s) to DNA, tubulin protein, and BSA*

	Inhibitor concentration (μM)	nmol/mg of protein/min		
		BSA	Tubulin	DNA (nmol/mg)
Complete system[a]		10.3 ± 0.1	11.2 ± 0.1	5.6 ± 0.2
−PHS		ND	ND	ND
−AA		ND	ND	ND
−Hemoglobin (1.2 μM)		ND	ND	ND
$-O_2$		ND	ND	ND
Aspirin	100	ND	ND	ND
Indomethacin	100	0.9 ± 0.2	1.0 ± 0.1	0.6 ± 0.1
SKF-525A	500	10.5 ± 0.2	10.9 ± 0.2	5.8 ± 0.1
Pargyline	100	10.9 ± 0.1	11.5 ± 0.4	5.1 ± 0.1
Clorgyline	100	10.5 ± 0.1	11.8 ± 0.4	5.2 ± 0.1
Deprenyl	100	10.0 ± 0.4	11.7 ± 0.5	5.7 ± 0.1

[a] Complete system consisted of 50 μM [2,5,6-^{3}H]dopamine, 1.0 mg/ml of DNA, tubulin, or BSA, 0.01 mg/ml PHS, 1.2 μM hemoglobin, and 100 μM AA in a total volume of 0.5 ml. The reaction was started by the addition of AA, and the incubations were performed for 3 min. All inhibitors were preincubated for 2 min at 25°C before the addition of AA. Data represent means ± SEM of three to four separate experiments performed in duplicate. ND, not detected, corresponds to blank values.

amino acids, disrupt intracellular Ca^{2+} homeostasis, and alter neuronal metabolism (Hedqvist, 1977; Abdel-Halim et al., 1980; Wolfe et al., 1989). Another important observation is the PHS-dependent activation of dopamine in the presence of H_2O_2. PHS/H_2O_2-mediated activation and irreversible binding of [^{3}H]-dopamine metabolite(s) to microtubulin protein, BSA, and DNA was comparable with that of PHS/AA-mediated activation of dopamine. In both cases, the peroxidase-mediated activation of dopamine was not inhibited by MAO inhibitors pargyline, clorgyline, or deprenyl. Our study thus demonstrates that intracellular availability of H_2O_2 in the neuronal cell body may be of physiological importance in vivo, because H_2O_2 serves as a substrate for the peroxidase activity of PHS, or stimulates the release of free AA.

The present study also demonstrates that dopamine, like other catecholamines, stimulates PGE_2 synthesis (Fig. 2) and is an efficient reducing cosubstrate for prostaglandin synthesis. At the lowest concentration of dopamine tested (5 μM), stimulation of PGE_2 synthesis was observed. Thus, increased availability of free AA from various stimuli (Wolfe et al., 1989), or large amounts of H_2O_2 produced (compensatory mechanism), represents an increased prostaglandin formation/cooxidation of dopamine and a corresponding increase in DNA or protein binding. The lack or decreased amount of detoxifying enzymes observed in PD (Olanow, 1990) may enhance the macromolecular binding resulting in neuronal degeneration.

PHS peroxidase appears to activate dopamine by a mechanism similar to that of other peroxidase/H_2O_2

TABLE 3. *Effect of various test agents on prostaglandin hydroperoxidase/H_2O_2-mediated cooxidation of [2,5,6-^{3}H]dopamine: binding of dopamine metabolite(s) to DNA, tubulin protein, and BSA*

	Inhibitor concentration (μM)	nmol/mg/min		
		BSA	Tubulin	DNA (nmol/mg)
Complete system[a]		5.5 ± 0.6	7.0 ± 0.2	4.9 ± 0.2
Heat-treated PHS		ND	ND	ND
−PHS		ND	ND	ND
$-H_2O_2$		ND	ND	ND
$-O_2$		5.7 ± 0.5	6.9 ± 0.2	4.6 ± 0.3
Indomethacin	100	5.2 ± 0.3	6.6 ± 0.2	5.0 ± 0.2
Deprenyl	100	6.2 ± 0.1	7.1 ± 0.3	4.9 ± 0.1
Pargyline	100	6.3 ± 0.2	7.2 ± 0.2	4.8 ± 0.4

[a] Complete system consisted of 50 μM [2,5,6-^{3}H]dopamine, 1.0 mg/ml of DNA, tubulin, or BSA, 0.01 mg/ml purified PHS, and 100 μM H_2O_2 in a total volume of 0.5 ml. The reaction was started by the addition of hydrogen peroxide, and the incubations were performed for 3 min. All inhibitors were preincubated for 2 min at 25°C before the addition of hydrogen peroxide. Data represent means ± SEM of three to four separate experiments performed in duplicate. ND, not detected, corresponds to blank values.

TABLE 4. *Effect of various inhibitors on HRP/H_2O_2-mediated cooxidation of [2,5,6-^{3}H]dopamine: binding of dopamine metabolites to DNA, tubulin protein, and BSA*

	Inhibitor concentration (μM)	nmol/mg of protein/min		
		BSA	Tubulin	DNA (nmol/mg)
Complete system[a]		5.1 ± 0.1	6.4 ± 0.2	5.8 ± 0.2
−HRP + H_2O_2		ND	ND	ND
−H_2O_2		ND	ND	ND
Pargyline	100	6.8 ± 0.1	7.1 ± 0.2	6.3 ± 0.2
Chlorgyline	100	6.9 ± 0.1	7.2 ± 0.2	6.1 ± 0.2
Deprenyl	100	7.2 ± 0.2	7.7 ± 0.2	6.3 ± 0.2

[a] Complete system consisted of 50 μM [2,5,6-^{3}H]dopamine, 1.0 mg/ml of either BSA or tubulin protein or DNA, 3 units of HRP, 100 μM H_2O_2, in a total volume of 0.5 ml. All inhibitors were preincubated for 2 min at 25°C. The reaction was started by the addition of H_2O_2 and incubations were performed for 3 min at 37°C. Data represent means ± SEM of three separate experiments performed in duplicate. ND, not detected, corresponds to blank values.

systems (Markey et al., 1987; Eling et al., 1990). Dopamine undergoes a classical two-electron oxidation to product(s) that show a characteristic absorption spectrum that has been reported for quinoidal chromophores (Swan, 1976; Graham, 1978). Identification of 2,3-dihydro-1*H*-indole-5,6-dione, 5-*S*-[*N*-acetylcysteinyl]-3,4-dihydroxyphenethylamine, and 7-*S*-(*N*-acetylcysteinyl)-5,6-dihydroxyindole conjugates support the presence of quinone metabolites in PHS/AA-mediated metabolism of dopamine. Formation of dopamine-*o*-quinone in SN during PHS synthesis, even at picogram levels, could have serious neuronal functional significance arising from the inhibition of sulfhydryl enzymes. GSH oxidation to GSSG (Eling et al., 1990; O'Brien, 1991), or GSH conjugate formation (GSH depletion/oxidative stress) will result in changes in the GSH/GSSG ratio in the mitochondria or cytoplasm (O'Brien, 1991; DiMonte et al., 1992; Jenner et al., 1992). Furthermore, when the GSH/GSSG ratio is low, phospholipase A_2 is activated (Horton et al., 1987; O'Brien, 1991), leading to AA release (substrate for PHS). Impairment of the glutathione pathway has been suggested as a leading cause of dopaminergic neuronal degeneration in PD (DiMonte et al., 1992). This is the first report showing that formation of electrophilic dopamine-quinone(s) metabolites can be generated in SN by an enzyme system present in SN (PHS). The peroxidase component of this enzyme activates dopamine to electrophilic products and the peroxidase activity is not inhibited by MAO inhibitors.

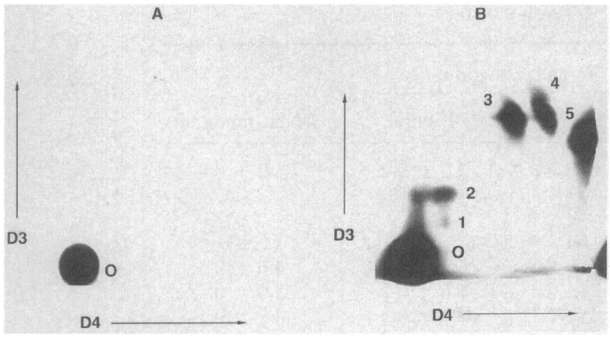

FIG. 5. PHS-dependent formation of dopamine-DNA adducts. Autoradiograms of ^{32}P-postlabeled DNA. Complete reaction mixtures (Table 2) containing 0.05 m*M* dopamine were incubated in the absence (**A**) or presence (**B**) of PHS for 3 min. The numbers refer to different DNA adducts. Plates were exposed for 3 min at −20°C.

Cytoskeletal integrity is important in the maintenance of neuronal function. In the present study, we have also provided evidence to show that PHS/AA-mediated metabolism of dopamine results in electrophilic metabolites that form covalent adducts with tissue nucleophiles such as DNA (Reddy et al., 1984; Levay et al., 1993) and microtubulin protein to exert their cytotoxic effect. A rate of DNA repair lower than the rate of damage could result in the accumulation of errors and loss of cellular function. Covalent binding to microtubulin protein may lead to defective assembly/disassembly of microtubules within the cells and structural changes of the neuronal cytoskeleton. These effects may lead to loss of vital neuronal function.

Identification of dopaminochrome from the PHS-mediated cooxidation of dopamine is another aspect that has biological significance. Dopaminochrome has been suggested as the active alkylating agent that disrupts catecholamine metabolism by irreversibly deactivating catechol-*O*-methyltransferase (Borchardt et al., 1976). This metabolite probably arises from oxidation of 6-hydroxydopamine (Tse et al., 1975; Swan, 1976) formed in the PHS/AA oxidation of dopamine. The isolation of oxygenated products during peroxidatic oxidation has been reported. For example, isolation of tyrosine from the oxidation of phenylalanine by myeloperoxidase/H_2O_2 (Fujimoto et al., 1991) and 2-nitrofluorene from the oxidation of 2-aminofluorene by HRP/H_2O_2 has been reported (Boyd et al., 1983). The formation of dopaminochrome and 5,6-dihydroxyindole from oxidation of 6-hydroxydopamine has been

reported (Swan, 1976; Liang et al., 1977; Graham, 1978). However, it should be mentioned that the dihydroxyindole was not a substrate for PHS/AA-mediated cooxidation. This is based on the fact that no covalent binding with DNA or tubulin protein was observed when [^{3}H]dihydroxyindole was used as the substrate with PHS. Moreover, no change in absorption spectrum of 5,6-dihydroxyindole or chromophore absorbing at 475–480 nm was seen when oxidation was performed spectrophotometrically. Thus, the formation of dopaminochrome from dihydroxyindole does not appear to occur. These data suggest that the neurotoxin 6-hydroxydopamine may be formed in the PHS-mediated cooxidation of dopamine.

In the present study, we have provided evidence to show that PHS activity is present in SN and that this activity is significantly higher in parkinsonian brain tissue compared with age- and PMI-matched control and AD, and AD/PD. Dopamine is a reducing cosubstrate for the peroxidase activity of PHS. Dopamine is cooxidized during peroxidatic metabolism to reactive intermediates that bind to sulfhydryl reagents, DNA, and proteins (BSA and microtubulin). PHS/H_2O_2 also elicited covalent binding of dopamine to macromolecules. Using ^{32}P-postlabeling, specific DNA adducts were detected after PHS-mediated metabolism of dopamine. Adduct formation did not involve cytochrome P-450 or MAO, because inhibitors of these enzymes had no effect on dopamine metabolism. The data presented in the present study support our hypothesis that cooxidative metabolism of dopamine by PHS results in the formation and accumulation of dopamine-DNA adducts in SN. The formation of dopamine-DNA adducts in SN may be responsible for the selective vulnerability of the dopamine-containing neurons. This is hypothesized as a contributing event (''accelerator'') in the development of PD (Fig. 1). ^{32}P-postlabeling demonstrated specific dopamine-DNA adducts that can now be assessed in the SN of parkinsonian brain.

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