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Conjugates of Catecholamines with Cysteine and GSH in Parkinson's Disease: Possible Mechanisms of Formation Involving Reactive Oxygen Species

Jeremy P. E. Spencer, Peter Jenner, *Susan E. Daniel, *Andrew J. Lees, *David C. Marsden, and Barry Halliwell

*Neurodegenerative Disease Research Centre, University of London King's College, and *Parkinson's Disease Society Brain Bank, University Department of Clinical Neurology, Institute of Neurology, London, England*

GSH = glutathione

Abstract: Oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine (DA) to generate semiquinones/quinones, oxygen radicals, and other reactive oxygen species may play a role in neuronal cell death in Parkinson's disease (PD). In particular, semiquinones/quinones can form conjugates with thiol compounds such as GSH and cysteine. Exposure of L-DOPA, DA, and other catecholamines to a system generating $O_2^{\bullet -}$ radical led to $O_2^{\bullet -}$-dependent depletion of added GSH (or cysteine), accompanied by the formation of thiol-DA or -DOPA adducts as detected by HPLC. Superoxide could additionally cause destruction of these adducts. Iron or copper ions could also promote conjugate formation between GSH or cysteine and DA and L-DOPA, especially if H_2O_2 was present. We applied HPLC to measure glutathionyl and cysteinyl conjugates of L-DOPA, DA, and 3,4-dihydroxyphenylacetic acid (DOPAC) in postmortem brain samples from PD patients and normal control subjects. Conjugates were detected in most brain areas examined, but levels were highest in the substantia nigra and putamen. In most regions, adduct levels were lower in PD, but there were significant increases in cysteinyl adducts of L-DOPA, DA, and DOPAC in PD substantia nigra, suggesting that acceleration of L-DOPA/DA oxidation occurs in PD, although we cannot say if this is a primary feature of the disease or if it is related to therapy with L-DOPA. In vitro, conjugate formation could be inhibited by the dithiol dihydrolipoate but not by its oxidised form, lipoic acid. **Key Words:** L-3,4-Dihydroxyphenylalanine—Parkinson's disease—Superoxide—5-*S*-Cysteinyldopamine—5-*S*-Cysteinyl-3,4-dihydroxyphenylalanine—Oxidative stress—α-Lipoate.
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Normal O_2-consuming metabolic processes in dopaminergic neurons [e.g., the mitochondrial electron transport chain and the oxidative deamination of dopamine (DA) by monoamine oxidase], as well as nonenzymatic autoxidation of catecholamine neurotransmitters, may result in intraneuronal formation of $O_2^{\bullet -}$ and H_2O_2 (Graham, 1978; Cohen, 1986; Gotz et al., 1994). Neurones exposed to these reactive oxygen species will be potentially at risk of damage, e.g., by the highly reactive hydroxyl radical ($^{\bullet}OH$), which can be formed from $O_2^{\bullet -}$ and H_2O_2 by Fenton chemistry (Halliwell and Gutteridge, 1989).

The nonenzymatic oxidation of catecholamines involves formation of quinone, hydroquinone, and semiquinone radical intermediates (Graham, 1978; Zhang and Dryhurst, 1993). DA may be converted to an *o*-semiquinone that, after disproportionation, gives rise to the corresponding *o*-quinone. At physiological pH, partial deprotonation of the ethylamine side-chain of DA *o*-quinone gives rise to another species that undergoes irreversible 1,4-intramolecular cyclization, a reaction that occurs through nucleophilic attack of the nitrogen atom at the 6 position of the quinone ring, to give 5,6-dihydroxyindoline (Bindoli et al., 1992; Zhang and Dryhurst, 1993). This species is oxidatively polymerised (Graham, 1978; Zhang and Dryhurst, 1993), leading to the formation of the black indolic melanin polymer. The pathway for generation of melanin is more complicated in vivo because other factors such as metal ions (Palumbo et al., 1995) and sulphydryl compounds affect the chemistry involved (Carstam et al., 1991).

The substantia nigra of rodents, primates, and humans contains lower levels of GSH than other brain regions (Perry et al., 1982). Furthermore, the levels

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Address correspondence and reprint requests to Dr. J. P. E. Spencer at Neurodegenerative Disease Centre, University of London King's College, Manresa Road, London, SW2 6LX, U.K.

Abbreviations used: DA, dopamine; DHBT-1, 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid; DHLA, dihydrolipoate; L-DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); EC, electrochemical detection; 5-HT, 5-hydroxytryptamine; HX, hypoxanthine; PD, Parkinson's disease; RP, reverse-phase; SOD, superoxide dismutase; XO, xanthine oxidase.

of GSH in the substantia nigra of patients who have died with idiopathic Parkinson's disease (PD) are decreased by ~50% compared with those of matched control subjects (Riederer et al., 1989; Jenner et al., 1992). Thus, it has been suggested that low levels of nigral GSH and consequent oxidative stress might contribute to the degeneration of dopaminergic neurons in idiopathic PD (Perry et al., 1982; Cohen, 1983, 1986). Decreased GSH levels in the substantia nigra appear to represent an early component in the pathological processes that underlie PD (Jenner et al., 1992). The decrease in nigral GSH in PD is not due to decreased activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme for biosynthesis of the tripeptide, or to alterations in GSH peroxidase, GSSG reductase, or GSH transferase activities (Kish et al., 1986; Sian et al., 1992). Exposing DOPA or DA to $O_2^{\bullet-}$ in the presence of GSH can lead to GSH depletion (Spencer et al., 1995), presumably due to reaction of GSH with DA semiquinones or quinones.

Chemical degradation of human substantia nigra neuromelanin yields products that suggest that the polymer is composed of indolic residues and residues derived from 5-*S*-cysteinyl-DA (Carstam et al., 1991; Odh et al., 1994). Residues derived from 5-*S*-cysteinyl-DA may result from the facile nucleophilic addition of cysteine or GSH to the *o*-quinone intermediate (Zhang and Dryhurst, 1994, 1995*a,b*). In the latter case, 5-*S*-glutathionyl-DA is hydrolysed by peptidase enzymes to the corresponding cysteinyl conjugate (Carstam et al., 1991; Zhang and Dryhurst, 1994), and these cysteinyl derivatives have been detected in the mammalian brain tissue (Rosengren et al., 1985; Fornstedt et al., 1986, 1989). 5-*S*-Cysteinyl species may undergo further oxidation to form benzothiazine species, such as 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-1; Shen and Dryhurst, 1996; Shen et al., 1997), and recent reports have suggested that DBHT-1 can cross the outer mitochondrial membrane and irreversibly inhibit complex I (Li and Dryhurst, 1997). This raises the possibility that these compounds could be endogenous neurotoxins that may contribute to the irreversible complex I damage and cell death in substantia nigra in PD (Li and Dryhurst, 1997).

The purpose of this work was to investigate in detail how reactions of neurotransmitters and thiols could be affected by reactive oxygen species, whether 5-*S*-cysteinyl- and 5-*S*-glutathionyl conjugates of DA, L-DOPA, and 3,4-dihydroxyphenylacetic acid (DOPAC) are measurable in brain tissue, and how levels are altered in PD.

MATERIALS AND METHODS

Materials

Chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.), from the BDH Chemical Co. (Gillingham, Dorset, U.K.), and/or Aldrich (Gillingham, Dorset, U.K.). Solutions of L-DOPA and other catechols were prepared fresh in buffer at pH 7.4 when required. Superoxide dismutase (SOD; bovine erythrocyte CuZn-enzyme type S2515), mushroom tyrosinase (type T-7755), and xanthine oxidase (XO) from buttermilk (grade I; type X1875) were all obtained from Sigma.

Catecholamine-dependent GSH depletion

The concentration of GSH was established by reaction with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at pH 8.0 (Ellman, 1959). Superoxide was generated by the hypoxanthine–xanthine oxidase system. Reaction mixtures (total volume 1 ml) contained GSH or cysteine (60 μ*M*), hypoxanthine (HX; 300 μ*M*), and XO (0.05 U/ml) in 0.1 *M* sodium phosphate buffer (pH 8.0). The conditions produced sufficient $O_2^{\bullet-}$ to reduce cytochrome c at a rate of 0.118 absorbance units/min at 550 nm. Compounds were added at concentrations up to 100 μ*M*, and all reactions were initiated by adding XO and terminated by addition of DTNB (70 μ*M*). Measurement at A_{412} nm enabled calculation of the GSH remaining. The possibility of direct inhibition of XO was monitored by incubating each compound (100 μ*M*) with the enzyme and measuring uric acid production at 290 nm. Incubation of compounds with HX/XO in the absence of GSH and DTNB and measurement of absorbance changes at 412 nm were performed to investigate if a chromogen is formed when the compound reacts with superoxide. Incubation of the compound with reactants in the absence of DTNB and monitoring changes at 550 nm were carried out to check that the reaction of superoxide with compounds did not cause a decrease in the absorbance at 550 nm in the $O_2^{\bullet-}$-scavenging assay.

Synthesis of 5-*S*-glutathionyl-catecholamine and 5-*S*-cysteinyl-catecholamine conjugates

The conjugates were prepared by a method similar to that of Rosengren et al. (1985) and Agrup et al. (1983) but with some modifications. DA (0.2 g), L-DOPA (0.2 g), or DOPAC (0.2 g) was incubated in a total volume of 100 ml with GSH (0.48 g) or cysteine (0.48 g) and 75 mg of mushroom tyrosinase (2,000 U/ml) in ammonium acetate buffer (0.1 *M*; pH 5.8) for 5 h at 25°C. The reaction mixture was stirred constantly and analysed every hour by measurement of the UV absorbance and by reverse-phase (RP) HPLC (see below). Samples were freeze dried, dissolved in 10 ml of sodium acetate buffer (40 m*M*; pH 4.7), and then filtered through a 0.22-μm Centriprep particle separator (Amicon, U.K.), and 2-ml fractions were purified by preparative RP-HPLC, using a Zorbax ODS (21.2 × 250 mm; HPLC Technology, Macclesfield, Cheshire, U.K.), and eluted isocratically at a flow rate of 4 ml/min with 6% (vol/vol) methanol in 10 m*M* ammonium acetate buffer (pH 4.7). Fractions containing the glutathione and cysteine conjugates were lyophilised, dissolved in 10 ml of sodium acetate buffer, and repeatedly purified using the preparative RP-HPLC method. Glutathionyl and cysteinyl conjugates were checked for purity by UV spectroscopy (Agrup et al., 1983; Rosengren et al., 1985), by HPLC analysis, and by their ^{1}H-NMR spectra (400 MHz, D_2O, δ = 4.7 ppm) (Ploemen et al., 1994). 5-*S*-Glutathionyl-DA, 5-*S*-glutathionyl-DOPA, and 5-*S*-glutathionyl-DOPAC conjugates had a λ_{max} at 256 and 291 nm (ϵ = 1,850 M^{-1} cm^{-1}), as expected (Agrup et al., 1983; Rosengren et al., 1985). The extinction coefficient at 292 nm is 2,800 M^{-1} cm^{-1} for 5-*S*-cysteinyl-DOPA and 5-*S*-cysteinyl-DOPAC and 3,000 M^{-1} cm^{-1} for 5-*S*-cysteinyl-DA.