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QUARTERLY

Peroxynitrite mediated linoleic acid oxidation and tyrosine nitration in the presence of synthetic neuromelanins★✪

Krystyna Stępień✉, Adam Wilczok, Alicja Zajdel, Anna Dzierżęga-Lęcznar and Tadeusz Wilczok

Department of Molecular Biology, Biochemistry and Biopharmacy, Faculty of Pharmacy, Medical University of Silesia, Sosnowiec, Poland

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Peroxynitrite-mediated linoleic acid oxidation and tyrosine nitration were analysed in the presence of synthetic model neuromelanins: dopamine (DA) -melanin, cysteinyldopamine (CysDA) -melanin and various DA/CysDA copolymers. The presence of melanin significantly decreased the amount of 3-nitrotyrosine formed. This inhibitory effect depended on the type and concentration of melanin polymer. It was found that incorporation of CysDA-derived units into melanin attenuated its protective effect on tyrosine nitration induced by peroxynitrite. In the presence of bicarbonate, the melanins also inhibited 3-nitrotyrosine formation in a concentration dependent manner, although the extent of inhibition was lower than in the absence of bicarbonate. The tested melanins inhibited peroxynitrite-induced formation of linoleic acid hydroperoxides, both in the absence and in the presence of bicarbonate. In the presence of bicarbonate, among the oxidation products appeared 4-hydroxynonenal (HNE). CysDA-melanin inhibited the formation of HNE, while DA-melanin did not affect the aldehyde level. The results of the presented study suggest that neuromelanin can act as a natural scavenger of peroxynitrite.

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✉Corresponding author: Krystyna Stępień, Department of Molecular Biology, Biochemistry and Biopharmacy, Faculty of Pharmacy, Medical University of Silesia, Narcyzów 1, 41-200 Sosnowiec, Poland; tel: (48 32) 291 0099; fax: (48 32) 291 7496, e-mail: kstepien@farmant.slam.katowice.pl

Abbreviations: 5-S-CysDA, 5-*S*-cysteinyldopamine; 13-HPODE, 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; 13-HODE, 13(*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid; HNE, 4-hydroxy-2-nonenal; DA-melanin, dopamine-melanin; CysDA-melanin, 5-*S*-cysteinyldopamine-melanin; DTPA, diethylenetriaminepentaacetic acid.

Peroxynitrite ($ONOO^-$/ONOOH) is thought to be involved in many pathological processes associated with excessive nitric oxide (NO) production in biological systems (Beckman & Koppenol, 1996; Bartosz, 1996; Patel *et al.,* 1999; Murphy, 1999). NO reacts with the superoxide radical anion ($O_2^{\cdot-}$) to form the peroxynitrite anion at nearly the diffusion-limited rate (Huie & Padmaja, 1993). Peroxynitrite is a powerful oxidant and nitrating agent which is capable of modifying lipids (Radi *et al.,* 1991; O'Donnell *et al.,* 1999a), proteins (Ischiropoulos & Al-Mehdi, 1995; Alayash *et al.,* 1998) and DNA (Yermilov *et al.,* 1995; Douki & Cadet, 1996; Szabo *et al.,* 1996). Nitration of free and protein-bound tyrosine leading to the formation of 3-nitrotyrosine is a well established reaction of peroxynitrite (Ischiropoulos *et al.,* 1992; van der Vliet *et al.,* 1995; Ischiropoulos, 1998). It has been shown that 3-nitrotyrosine formation is catalysed by CO_2, which reacts with the peroxynitrite anion at physiological pH to form a short-lived intermediate identified as the $ONO_2CO_2^-$ adduct (nitrosoperoxycarbonate anion) (Lymar & Hurst, 1995; Lymar *et al.,* 1996; Gow *et al.,* 1996). The reaction of $ONOO^-$ with CO_2 is one of the fastest reactions known for peroxynitrite ($k = 3.0 \times 10^4$ $M^{-1} s^{-1}$) (Lymar & Hurst, 1995) and therefore can affect its reactivity towards potential biological targets. At physiological concentrations of CO_2, peroxynitrite-dependent nitration is stimulated, while oxidation is inhibited (Gow *et al.,* 1996; Berlett *et al.,* 1998; Radi *et al.,* 1999).

3-Nitrotyrosine has been suggested as a marker of peroxynitrite-mediated damage in a variety of disease states, including neurodegenerative disorders such as Alzheimer's disease (Smith *et al.,* 1997) and Parkinson's disease (Good *et al.,* 1998; Ara *et al.,* 1998). However, tyrosine nitration can also be caused by other biochemical pathways (Ischiropoulos, 1998; Pfeiffer *et al.,* 2000).

Neuromelanin, a dark pigment that deposits with age in the cytoplasm of nigrostriatal dopaminergic neurons, originates from the oxidative pathway of dopamine (DA) metabolism in the human brain (Graham, 1978; Marsden, 1983). The synthesis of neuromelanin is initiated by the oxidation of DA to its *o*-quinone, which can undergo intramolecular cyclization and further oxidation to indoles (Graham, 1978; Costa *et al.,* 1992), or can react with the thiol group of cysteine to give cysteinyl conjugates, mainly 5-*S*-cysteinyldopamine (5-S-CysDA) (Tse *et al.,* 1976; Ito *et al.,* 1986; Zhang & Dryhurst, 1995). These conjugates are easily oxidized (more easily than DA) to form benzothiazine derivatives (Shen & Dryhurst, 1996). Degradative studies of neuromelanin isolated from human *substantia nigra* have demonstrated that the pigment is a copolymer of eumelanin and phaeomelanin, and consists of indole-type and benzothiazine-type units (Carstam *et al.,* 1991; Odh *et al.,* 1994). It has been postulated that neuromelanin formation is an autoxidative process catalysed by iron and other transition metals (Rodgers & Curzon, 1975; Fornstedt *et al.,* 1989; D'Ischia & Prota, 1997), although enzymes such as prostaglandin H synthase (Hastings, 1995), lipoxygenase (Rosei *et al.,* 1994) and peroxidase (Okun, 1997) are capable of oxidizing DA to DA-quinone *in vitro*. The ability of peroxynitrite to promote DA oxidation has been also demonstrated (Kerry & Rice-Evans, 1999; LaVoie & Hastings, 1999).

Recently, we have shown that synthetic DA-melanin inhibited peroxynitrite-mediated nitration of free tyrosine, loss of tryptophan residues in bovine serum albumin and Ca^{2+}-ATPase inactivation (Stępień *et al.,* 2000). In this work we investigated the effect of synthetic neuromelanins derived from DA and 5-S-CysDA on peroxynitrite-induced tyrosine nitration and linoleic acid oxidation.

MATERIALS AND METHODS

Chemicals. Mushroom tyrosinase (6680 U/mg of solid; EC 1.14.18.1), linoleic acid

(99% by capillary GC), 13(*S*)-hydroperoxy-(9*Z*, 11*E*)-octadecadienoic acid (13-HPODE), 13(*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid (13-HODE), 4-hydroxy-2-nonenal (HNE) were purchased from Sigma-Aldrich Co. All the other reagents were also obtained from Sigma-Aldrich Co. and were of analytical grade. Solvents of HPLC grade and type I deionized water (Barnstead, NANOpure) were used.

Preparation of 5-S-CysDA and synthetic neuromelanins. 5-*S*-Cysteinyldopamine was obtained by tyrosinase-catalysed oxidation of dopamine in the presence of cysteine, and purified by ion-exchange chromatography (Dowex 50Wx2) according to Ito *et al.* (1986). Synthetic models of neuromelanin were prepared by oxidative polymerization of dopamine (DA-melanin) or 5-*S*-cysteinyldopamine (CysDA-melanin), and by copolymerization of both precursors at molar ratios 3 : 1 (DA/CysDA(3 : 1)-melanin) and 1 : 1 (DA/CysDA(1 : 1)-melanin). Solutions of the melanin precursors (total concentration 5 mM) in phosphate buffer (50 mM, pH 7.4) were incubated at 37°C for 48 h. The insoluble melanins formed during such oxidation were separated by centrifugation (3000 × ***g***), washed with water and stored as aqueous suspensions of known concentrations. Elemental analysis of the melanin samples was performed by the Center for Molecular and Macromolecular Research of the Polish Academy of Sciences in Łódź (Poland).

Synthesis of peroxynitrite. Peroxynitrite was synthesised according to the method of Uppu and Pryor (1996), using a 2 M H_2O_2 solution in NaOH (100 ml, pH 13) and isoamyl nitrite (0.2 mol). The aqueous phase that contained peroxynitrite was separated after centrifugation and washed with dichloromethane (6 × 100 ml) to remove the contaminating isoamyl alcohol and traces of isoamyl nitrite. The unreacted H_2O_2 was removed by passing the aqueous phase through a column of MnO_2 (25 g) prewashed with water (20 ml) and 0.5 M NaOH (20 ml). A stock solution of peroxynitrite was stored frozen at –80°C. The peroxynitrite concentration was determined spectrophotometrically at 302 nm using a molar absorption coefficient of 1700 $M^{-1}cm^{-1}$ (Bohle *et al.,* 1996) before each experiment, and appropriate amounts of peroxynitrite solution were added to reaction mixtures. A drop of peroxynitrite was placed on the test tube wall and the samples were immediately vortexed for 30 s. In control experiments, decomposed peroxynitrite was used (Stępień *et al.,* 2000).

Tyrosine nitration. Peroxynitrite (final concentration 1 mM) was added to solutions of tyrosine (1 mM) in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and various amounts of model melanins (50–800 μg/ml). In a separate set of experiments, the reaction medium was supplemented with sodium bicarbonate (20 mM). Bicarbonate was introduced 2 min before the addition of peroxynitrite (pH of the solution increased to 7.6). Incubations were carried out for 30 min at room temperature. The formation of 3-nitrotyrosine was quantified by HPLC and expressed as a percent of the control incubated without melanin.

Linoleic acid oxidation. The reaction mixtures contained linoleic acid (1 mM) dispersed in sodium phosphate buffer (0.1 M, pH 7.4), DTPA (0.1 mM), various concentrations of DA-melanin or CysDA-melanin (10–200 μg/ml) and 0 or 20 mM of sodium bicarbonate. The oxidation was initiated by adding peroxynitrite to a final concentration of 4 mM. After 2 h of incubation at room temperature, each sample was diluted with methanol (1 : 1, v/v) and directly analysed by HPLC.

HPLC analysis. Reverse-phase HPLC was performed with a Hewlett-Packard model 1050 liquid chromatograph equipped with a HP 1100 diode array detector and interfaced to a HPLC ChemStation (HP). Each sample was filtered (filter Millex GV13, pore diameter 0.22 μm; Millipore) to remove melanin and then the reaction products were separated on an Eurospher 100 C18 column (particle size

5 μm, 250 × 4 mm; Knauer) at 35°C. 3-Nitrotyrosine and tyrosine were eluted isocratically with 50 mM KH_2PO_4 (pH 3) and methanol (92:8) and detected at 276 nm (tyrosine was eluted at 4.6 min and 3-nitrotyrosine at 12.4 min). Linoleic acid oxidation products were separated using water (A) and acetonitryle (B) containing 0.2% phosphoric acid and the following gradient programme: 0 min 50% A: 50% B, 2 min 50% A:50% B, 16 min 17% A: 83% B and 17 min 100% B. HNE, 13-HODE and four linoleic acid hydroperoxides isomers were identified by matching their retention times and UV-Vis spectra with respective standards. The observed retention times were 5.2 min, 19.1 min, 21.1 min, 21.9 min, 24.2 min and 25.5 min, respectively. The amounts of 3-nitrotyrosine, 13-HODE and HNE were calculated with respect to the appropriate calibration curves. Quantifications of linoleic acid hydroperoxides were made using 13-HPODE as a standard.

The results are expressed as mean values ± S.D. calculated from three or more independent experiments.

RESULTS

Elemental analysis revealed that CysDA-melanin, DA/CysDA(1:1)-melanin and DA/CysDA(3:1)-melanin contained 11.4%, 9.1% and 5.8% sulphur, respectively. These data indicate that phaeomelanin-type units derived from 5-S-CysDA have been indeed incorporated into the melanin polymers in different proportions.

To determine the effect of synthetic neuromelanins on tyrosine nitration mediated by peroxynitrite, formation of 3-nitrotyrosine was measured by HPLC. In the absence of melanin, the yield of 3-nitrotyrosine was 95 ± 6 μM (n = 12) after the addition of 1 mM $ONOO^-$. The presence of melanin in the reaction medium significantly decreased the amount of 3-nitrotyrosine formed. As shown in Fig. 1, this inhibitory effect depended on the type and concentration of melanin polymer. At low concentrations (50 and 100 μg/ml), all the melanins tested had no effect on peroxynitrite-mediated nitration of tyrosine but a further increase in melanin concentrations caused a gradual decrease in the yield of 3-nitrotyrosine. DA-melanin was the most efficient inhibitor of 3-nitrotyrosine formation, while CysDA-melanin and DA/CysDA (1:1)-melanin were the least effective and their inhibitory effects were virtually the same at any concentration employed. Addition of 20 mM bicarbonate, prior to peroxynitrite, enhanced the level of 3-nitrotyrosine in controls about 1.5-fold (132 ± 7 μM of 3-nitrotyrosine; n = 12). In the presence of bicarbonate, the melanins inhibited 3-nitrotyrosine formation in a concentration dependent manner, although the extent of inhibition was lower than in the absence of bicarbonate (Fig. 1).

The influence of DA-melanin and CysDA-melanin on the oxidation of linoleic acid by peroxynitrite was investigated. Treatment of linoleic acid with peroxynitrite led to the formation of isomeric hydroperoxides with *cis, trans* 13-HPODE and *cis, trans* 9-HPODE being the major products. The tested melanins inhibited formation of HPODEs, and the inhibition extent, similar for both melanins, depended on their concentration (Fig. 2A). When amounts of 13-HODE were analysed, only a little increase was observed for CysDA-melanin (maximally 1 ± 0.1 μM HODE for 200 μg/ml CysDA-melanin), while the effect of DA-melanin did not look significant. In the presence of bicarbonate, in control samples, the observed levels of linoleic acid hydroperoxides were about two times lower than in the absence of bicarbonate (Fig. 2). The addition of DA-melanin and CysDA-melanin caused a further decrease of HPODE concentration while the amounts of HODE remained almost unchanged. In experiments carried out with added bicarbonate, 4-hydroxy-2-nonenal, which was absent in the samples without bicarbonate, appeared among the analysed oxidation products. DA-melanin did not affect

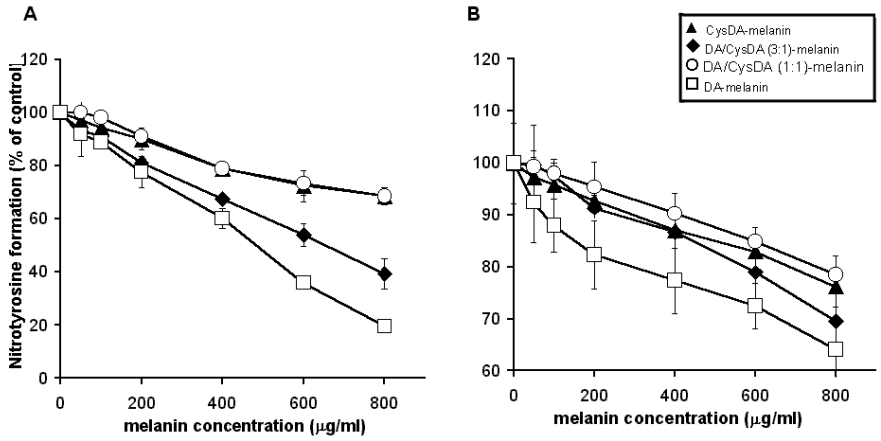

Figure 1. Effect of melanins on peroxynitrite-mediated 3-nitrotyrosine formation.

Peroxynitrite (final concentration 1 mM) was added to solutions of tyrosine (1 mM) in phosphate buffer (100 mM, pH 7.4) containing indicated concentrations of melanins in the absence (A) or in the presence (B) of 20 mM sodium bicarbonate.

the HNE level at all, whereas CysDA-melanin showed an inhibiting effect (Fig. 3).

DISCUSSION

Recently, we have demonstrated the ability of DA-melanin to protect against nitrating action of peroxynitrite (Stępień *et al.,* 2000). In this study, the effect of model neuromelanins derived from CysDA and from DA/CysDA mixtures on peroxynitrite-mediated tyrosine nitration was investigated and compared to the effect of DA-melanin. It was found that all the melanins tested significantly inhibited the formation of 3-nitrotyrosine but DA-melanin was a more efficient inhibitor than CysDA-melanin and DA/CysDA-melanins. This indicates that incorporation of the phaeomelanin component into the eumelanin polymer attenuates its protective effect on tyrosine nitration induced by peroxynitrite. In the presence of 20 mM bicarbonate (which is in equilibrium with approximately 1 mM CO_2) stimulation of tyrosine nitration in the control was observed; this is in agreement with the data of other authors (Lymar *et al.,* 1996; Lehnig, 1999). The observed stimulation depends only on the formation of the highly reactive peroxynitrite-CO_2 adduct and does not depend on the pH change caused by bicarbonate addition. As shown by Lymar *et al.* (1996), the CO_2-catalysed 3-nitrotyrosine formation is not influenced by pH in the range of 5–8. Addition of the melanins diminished the levels of 3-nitrotyrosine formed, indicating their ability to protect against the nitrating action of peroxynitrite-CO_2 adduct.

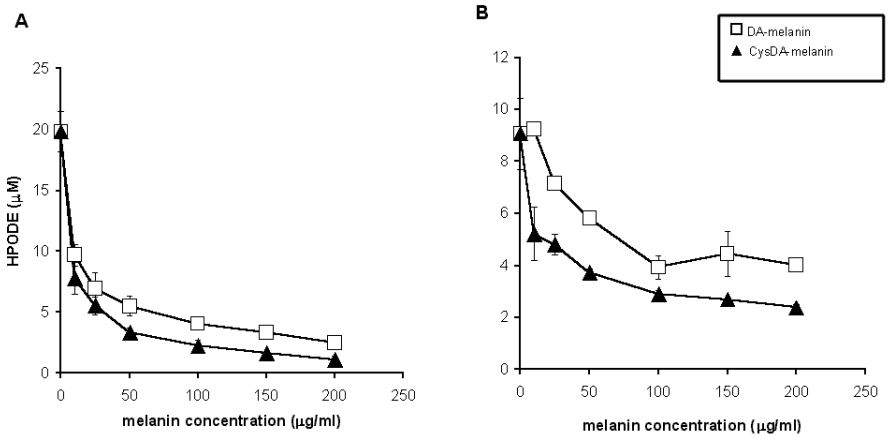

Figure 2. Effect of melanins on peroxynitrite-mediated linoleic acid hydroperoxides formation.

Peroxynitrite (final concentration 4 mM) was added to solutions of linoleic acid (1 mM) in phosphate buffer (100 mM, pH 7.4) containing indicated concentrations of melanins in the absence (A) or in the presence (B) of 20 mM sodium bicarbonate.

Study of the effect of the melanins on peroxynitrite-induced oxidation of linoleic acid demonstrated that both DA-melanin and CysDA-melanin significantly decreased the

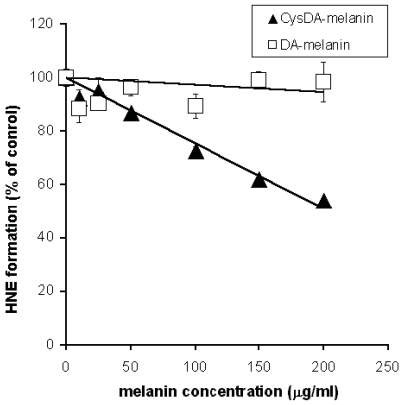

Figure 3. Decrease of 4-hydroxynonenal induced by melanins during peroxynitrite/CO_2-mediated linoleic acid oxidation.

Peroxynitrite (final concentration 4 mM) was added to solutions of linoleic acid (1 mM) in phosphate buffer (100 mM, pH 7.4) containing indicated concentration of melanins in the presence of 20 mM sodium bicarbonate.

yield of linoleic acid hydroperoxides formed. As melanins are capable of reducing linoleic acid hydroperoxides to its hydroxyl derivatives (Stępień *et al.,* 1998; Wilczok *et al.,* 1999), the formation of 13-HODE was analysed simultaneously with HPODEs after treatment of linoleic acid with peroxynitrite in the presence of the melanins. A little increase in the amount of 13-HODE formed was observed only for CysDA-melanin at the highest concentration applied. These experiments showed that DA-melanin and CysDA-melanin indeed inhibited linoleic acid oxidation induced by peroxynitrite. When the peroxynitrite-mediated oxidation of linoleic acid was carried out in the presence of bicarbonate, the levels of HPODEs were attenuated and HNE was detected among the oxidation products. This aldehyde was not found after the reaction of linoleic acid with peroxynitrite in the absence of bicarbonate. HNE is the secondary oxidation product derived from omega-6- polyunsaturated fatty acids such as 20:4 and 18:2 (Comporti, 1998). This suggests that the peroxynitrite- CO_2 adduct is able to decompose linoleic acid hydroperoxides. CysDA-melanin inhibited the formation of HNE, while DA-melanin did not affect the aldehyde level. The results indicate that CysDA-melanin is a more effective inhibitor of peroxynitrite/CO_2-mediated linoleic acid oxidation than DA-melanin.

O'Donnell *et al.* (1999a; 1999b) have shown that treatment of linoleic acid with peroxynitrite leads to the formation of nitrated linoleate species (LNO_2/LONO), in addition to HPODE and HODE. In our study, we did not detect any linoleic acid nitration products. In our experiment, peroxynitrite was introduced to linoleic acid by bolus addition and products of the reaction were analysed without extraction, which requires acidification of samples and may cause secondary reactions. In the studies described by O'Donnell *et al.* (1999a; 1999b), the diluted solution of peroxynitrite in 0.1 M NaOH was infused at a constant rate over 15 min into the linoleate emulsion and an equal volume of 0.1 M HCl was simultaneously infused to keep a constant pH value. These authors noted that "relative yields of oxidized *vs* nitrated products are likely to vary depending on the administration method chosen".

The peroxynitrite anion is relatively unreactive, however, protonation or addition of CO_2 generates a reactive species that can either interact with target compounds or undergo isomerization to NO_3^- ion (Uppu *et al.,* 1996). Currently proposed mechanisms of peroxynitrite reactivity involve the spontaneous decomposition of peroxynitrous acid (HOONO) with the generation of $^{\cdot}OH$ and $NO_2^{\cdot}$ radicals (Richeson *et al.,* 1998; Hodges & Ingold, 1999; Coddington, 1999). Carbon dioxide enhances the rate of decay of peroxynitrite to form $ONOOCO_2^-$ which can be a source of $CO_3^{\cdot-}$ and $NO_2^{\cdot}$ (Goldstein & Czapski, 1998; 1999). The stimulating effect of CO_2 on tyrosine nitration induced by peroxynitrite is explained by the participation of $CO_3^{\cdot-}$ in

one-electron oxidation of tyrosine, leading to tyrosyl radical (Zhang *et al.,* 1997; Lehnig 1999).

Melanins are known to be able to scavenge the hydroxyl radicals (Sarna *et al.,* 1986). Interactions of synthetic eu- and phaeo-melanins derived from 3,4-dihydroxyphenylalanine with strong oxidising radicals such as $NO_2^{\cdot}$ were described (Różanowska *et al.,* 1999). The observed inhibition of 3-nitrotyrosine formation by all the melanins tested, both in the absence and presence of added bicarbonate, suggests that the model neuromelanins are capable of scavenging $NO_2^{\cdot}$ radicals.

The results of the presented study suggest that neuromelanin can act as natural scavenger of peroxynitrite. As peroxynitrite is proposed to be a mediator of neurotoxic processes associated with Parkinson's disease, a protective effect of neuromelanin against peroxynitrite may be of physiological importance.

REFERENCES

Alayash, A.I., Ryan, B.A. & Cashon, R.E. (1998) Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins. *Arch. Biochem. Biophys.* **349**, 65–73.

Ara, J., Przedborski, S., Naini, A.B., Jackson-Lewis, D., Trifiletti, R.R., Horwitz, H. & Ischiropoulos, H. (1998) Inactivation of tyrosine hydroxylase by nitrating following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7659–7663.

Bartosz, G. (1996) Peroxynitrite: Mediator of the toxic action of nitric oxide. *Acta Biochim. Polon.* **43**, 645–660.

Beckman, J.S. & Koppenol, W.H. (1996) Nitric oxide, superoxide and peroxynitrite: The good, the bad and the ugly. *Am. J. Physiol. Cell Physiol.* **271**, C1424–C1437.

Berlett, B.S., Levine, R.L. & Stadtman, E.R. (1998) Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase; both modifications mimic effects of adenylylation. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2784–2789.

Bohle, D.S., Glassbrenner, P.A. & Hansert, B. (1996) Syntheses of pure tetramethylammonium peroxynitrite. *Methods Enzymol.* **269**, 302–311.

Carstam, R., Brinck, C., Hindemith-Augustsson, A., Rorsman, H. & Rosengren, E. (1991) The neuromelanin of the human substantia nigra. *Biochim. Biophys. Acta* **1097**, 152–160.

Coddington, J.W., Hurst, J.K. & Lymar, V. (1999) Hydroxyl radical formation during peroxynitrous acid decomposition. *J. Am. Chem. Soc.* **121**, 2438–2443.

Comporti, M. (1998) Lipid peroxidation and biogenic aldehydes: From the identification of 4-hydroxynonenal to further achievements in biopathology. *Free Radical Res.* **28**, 623–635.

Costa, C., Bertazzo, A., Allegri, G., Toffano, G., Curcuruto, O. & Traldi, P. (1992) Melanin biosynthesis from dopamine. II. A mass spectrometric and collisional spectroscopic investigation. *Pigment Cell Res.* **5**, 122–131.

D'Ischia, M. & Prota, G. (1997) Biosynthesis, structure, and function of neuromelanin and its relation to Parkinson's disease: A critical update. *Pigment Cell Res.* **10**, 370–376.

Douki, T. & Cadet, J. (1996) Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radical Res.* **24**, 369–380.

Fornstedt, B., Brun, A., Rosengren, E. & Carlsson, A. (1989) The apparent autoxidation rate of catechols in dopamine-rich regions of human brains increases with the degree of depigmentation of substantia nigra. *J. Neural Transm.* **1**, 279–295.

Goldstein, S. & Czapski, G. (1998) Formation of peroxynitrate from the reaction of peroxynitrite with CO_2: Evidence for carbonate radical production. *J. Am. Chem. Soc.* **120**, 3458–3463.

Goldstein, S. & Czapski, G. (1999) Viscosity effects on the reaction of peroxynitrite with CO_2: Evidence for radical formation in a solvent cage. *J. Am. Chem. Soc.* **121**, 2444–2447.

Good, P.F., Hsu, A., Werner, P., Perl, P.D. & Olanow, C.W. (1998) Protein nitration in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **57**, 338–342.

Gow, A., Duran, S., Thom, S.R. & Ischiropoulos, H. (1996) Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch. Biochem. Biophys.* **333**, 42–48.

Graham, D.G. (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol. Pharmacol.* **14**, 633–643.

Hastings, T.G. (1995) Enzymatic oxidation of dopamine: The role of prostaglandin H synthase. *J. Neurochem.* **64**, 919–924.

Hodges, G.R. & Ingold, K.U. (1999) Cage-escape of geminate radical pairs can produce peroxynitrate from peroxynitrite under a wide variety of experimental conditions. *J. Am. Chem. Soc.* **121**, 10695–10701.

Huie, R.E. & Padmaja, S. (1993) The reaction of NO with superoxide. *Free Radical Res. Commun.* **18**, 195–199.

Ischiropoulos, H. & Al-Mehdi, A.B. (1995) Peroxynitrite-mediated oxidative protein modification. *FEBS Lett.* **364**, 279–282.

Ischiropoulos, H. (1998) Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356**, 1–11.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J.S., Smith, C.D. & Beckman, J.S. (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298**, 431–437.

Ito, S., Fujita, K., Yoshioka, M., Sienko, D. & Nagatsu, T. (1986) Identification of 5-*S*- and 2-*S*-cysteinyldopamine and 5-*S*-glutathionyldopamine formed from dopamine by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **375**, 134–140.

Kerry, N. & Rice-Evans, C. (1999) Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationships. *J. Neurochem.* **73**, 247–253.

LaVoie, M. & Hastings, T.G. (1999) Peroxynitrite- and nitrite-induced oxidation of dopamine: Implications for nitric oxide in dopaminergic cell loss. *J. Neurochem.* **73**, 2546–2554.

Lehnig, M. (1999) Radical mechanisms of the decomposition of peroxynitrite and the peroxynitrite-CO_2 adduct and of reactions with L-tyrosine and related compounds as studied by ^{15}N chemically induced dynamic nuclear polarization. *Arch. Biochem. Biophys.* **368**, 303–318.

Lymar, S.V. & Hurst, J.K. (1995) Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J. Am. Chem. Soc.* **117**, 8867–8868.

Lymar, S.V., Jiang, Q. & Hurst, J.K. (1996) Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **35**, 7855–7861.

Marsden, C.D. (1983) Neuromelanin and Parkinson's disease. *J. Neural Transm.* (Suppl.) **19**, 121–141.

Murphy, M.P. (1999) Nitric oxide and cell death. *Biochim. Biophys. Acta* **1411**, 401–414.

O'Donnell, V.B., Eiserich, J.P., Chumley, P.H., Jablonsky, M.J., Krishna, N.R., Kirk, M., Barnes, S., Darley-Usmar, V.M. & Freeman, B.A. (1999a) Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12**, 83–92.

O'Donnell, V.B., Eiserich, J.P., Bloodsworth, A., Chumley, P.H., Kirk, M., Barnes, S., Darley-Usmar, V.M. & Freeman, B.A. (1999b) Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. *Methods Enzymol.* **301**, 454–470.

Odh, G., Carstam, R., Paulson, J., Wittbjer, A., Rosengren, E. & Rorsman, H. (1994) Neuromelanin of the human substantia nigra: A

mixed-type melanin. *J. Neurochem.* **62**, 2030–2036.

Okun, M.R. (1997) The role of peroxidase in neuromelanin synthesis: A review. *Physiol. Chem. Phys. Med. NMR* **29**, 15–22.

Patel, R.P., McAndrew, J., Sellak, H., White, R., Jo, H., Freeman, B.A. & Darley-Usmar, V.M. (1999) Biological aspects of reactive nitrogen species. *Biochim. Biophys. Acta* **1411**, 385–400.

Pfeiffer, S., Schmidt, K. & Mayer, B. (2000) Dityrosine formation outcompetes tyrosine nitration at low steady-state concentrations of peroxynitrite. Implications for tyrosine modification by nitric oxide/superoxide *in vivo*. *J. Biol. Chem.* **275**, 6346–6352.

Radi, R., Beckman, J.S., Bush, K.M. & Freeman, A. (1991) Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288**, 481–487.

Radi, R., Denicola, A. & Freeman, B.A. (1999) Peroxynitrite reactions with carbon dioxide-bicarbonate. *Methods Enzymol.* **301**, 353–367.

Richeson, C.E., Mulder, P. Bowry, V.W. & Ingold, K.U. (1998) The complex chemistry of peroxynitrite decomposition: New insights. *J. Am. Chem. Soc.* **120**, 7211–7219.

Rodgers, A.D. & Curzon, G. (1975) Melanin formation by human brain *in vitro*. *J. Neurochem.* **24**, 1123–1129.

Rosei, M.A., Blarzino, C., Foppoli, C., Mosca, L. & Coccia, R. (1994) Lipoxygenase-catalyzed oxidation of catecholamines. *Biochem. Biophys. Res. Commun.* **200**, 344–350.

Różanowska, M., Sarna, T., Land, E.J. & Truscott, T.G. (1999) Free radical scavenging properties of melanin. Interaction of eu- and pheo-melanin models with reducing and oxidizing radicals. *Free Radical Biol. Med.* **26**, 518–525.

Sarna, T., Pilas, B., Land, E.J. & Truscott, T.G. (1986) Interaction of radical from water radiolysis with melanin. *Biochim. Biophys. Acta* **883**, 162–167.

Shen, X.-M. & Dryhurst, G. (1996) Further insights into the influence of L-cysteine on the oxidation chemistry of dopamine: Reaction pathways of potential relevance to Parkinson's disease. *Chem. Res. Toxicol.* **9**, 751–763.

Smith, M.A., Richey-Harris, P.L., Sayre, L.M., Beckman, J.S. & Perry, G. (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17**, 2653–2657.

Stępień, K., Zajdel, A., Wilczok, A., Wilczok, T., Grzelak, A., Mateja, A., Soszyński, M. & Bartosz, G. (2000) Dopamine-melanin protects against tyrosine nitration, tryptophan oxidation and Ca^{2+}-ATPase inactivation induced by peroxynitrite. *Biochim. Biophys. Acta* **1523**, 189–195.

Stępień, K., Zajdel, A., Świerczek, G., Wilczok, A. & Wilczok, T. (1998) Reduction of 13-hydroperoxy-9,11-octadecadienoic acid by dopamine-melanin. *Biochem. Biophys. Res. Commun.* **244**, 781–784.

Szabo, C., Zingarelli, B., O'Connor, M. & Salzaman, A.L. (1996) DNA strand breakage, activation of poly(ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1753–1758.

Tse, D.C.S., McCreery, R.L. & Adams, R.N. (1976) Potential oxidative pathways of brain catecholamines. *J. Med. Chem.* **19**, 37–40.

Uppu, R.M. & Pryor, W.A. (1996) Synthesis of peroxynitrite in a two-phase system using isoamyl nitrite and hydrogen peroxide. *Anal. Biochem.* **236**, 242–249.

Uppu, R.M., Squadrito, G.L. & Pryor, W.A. (1996) Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327**, 335–343.

van der Vliet, A., Eiserich, J.P., O'Neill, C.A., Halliwell, B. & Cross, C.E. (1995) Tyrosine modification by reactive nitrogen species: A closer look. *Arch. Biochem. Biophys.* **319**, 341–349.

Wilczok, T., Stepien, K., Dzierzega-Lecznar, A., Zajdel, A. & Wilczok, A. (1999) Model neuromelanins as antioxidative agents during

lipid peroxidation. *Neurotoxicity Res*. **1**, 141–147.

Yermilov, V., Rubio, J., Becchi, M., Friesen, M.D., Pignatelli, B. & Ohshima, H. (1995) Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. *Carcinogenesis* **16**, 2045–2050.

Zhang, F. & Dryhurst, G. (1995) Reactions of cysteine and cysteinyl derivatives with dopamine-*o*-quinone and further insights into the oxidation chemistry of 5-*S*-cysteinyldopamine: Potential relevance to idiopathic Parkinson's disease. *Bioorg. Chem*. **23**, 193–216.

Zhang, H., Squadrito, G.L. & Pryor, W.A. (1997) The mechanism of the peroxynitrite-carbon dioxide reaction probed using tyrosinase. *Nitric Oxide* **1**, 301–307.