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Neuroglobin Modification by Reactive Quinone Species

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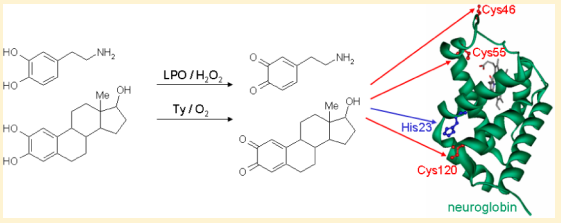
ABSTRACT: The physiological functions of neuroglobin (Ngb), the heme protein of the globin family expressed in the nervous tissue, have not yet been clarified. Besides O_2 storage and homeostasis, Ngb is thought to play a role in neuroprotection as a scavenger of toxic reactive species generated in vivo under conditions of oxidative stress. Herein, the interaction of Ngb with the quinones generated by oxidation of catecholamines (dopamine, norepinephrine) and catechol estrogens (2-hydroxyestradiol and 4-hydroxyestradiol), which have been implicated in neurodegenerative pathologies like Parkinson's and Alzheimer's diseases, has been investigated. The cytotoxicity of quinones has been ascribed to the derivatization of amino acid residues (mainly cysteine) in proteins through the formation of covalent bonds with the aromatic rings. Combined studies of tandem mass spectrometry and protein unfolding indicate the presence of quinone-promoted modifications in all of the Ngb derivatives analyzed (i.e., obtained employing either catecholamines or catechol estrogens as the source of the reactive species). Among protein residues, the highest reactivity of cysteines (Cys46, Cys55, and Cys120 in human Ngb) toward quinone species has been confirmed, and the dependence of the extent of protein modification on the method employed for catechol oxidation has been observed. When the oxidation reaction proceeds by one-electron steps, the involvement of semiquinone reactivity has been observed. The whole analysis of the data of Ngb modification suggests that the catecholamine-oxidation products can extensively modify proteins (likely by catecholamine oligomers, the compounds initially formed during the transformation of catecholamine to melanin). The modification mediated by catechol estrogens is less pronounced but strongly affects the interactions with the solvent as well as the protein stability.



INTRODUCTION

Neuroglobin (Ngb) is a heme protein expressed in the brain of rodents and humans[1] as well as fishes, amphibians, and birds,[2] and it is likely present in the nervous system of all vertebrates. Ngb has the classical globin fold,[3,4] with the peculiarity of a six-coordinate heme iron both in the ferric (met) and ferrous states, the latter being involved in O_2 (or CO or NO) binding through dissociation of the distal histidine ligand. O_2 affinity in human Ngb is regulated by the redox state of a disulfide bridge linking residues Cys46 and Cys55 that affects the six- to five-coordinate heme equilibrium: the protein with the intramolecular disulfide bond (Ngb_{S-S}) shows a 10-fold increase in oxygen affinity with respect to the reduced form (Ngb_{SH}).[5,6]

The lower affinity of the thiol form of the protein for exogenous ligands with respect to the disulfide-bridged Ngb_{S-S} was also confirmed for the met form through NO_2^- binding studies and kinetics of nitration.[7]

As with the other vertebrate nerve globins like cytoglobin,[8] globin E,[9] globin X,[10] and the α- and β-chains of Hb,[11,12] Ngb is underexpressed in neuronal tissues (~1 μM in the brain), thus suggesting some additional function(s) for this protein besides O_2 storage and supply to mitochondria. Ngb plays a role in neuronal O_2 homeostasis because its expression is involved in neuronal responses to hypoxia or ischemia.[13−17] As for the potential physiological functions of Ngb,[18] a neuroprotective activity as a sensor of the relative concentration of O_2 and NO in the tissue[19] and a role as a scavenger of toxic species generated in vivo under conditions of oxidative stress have been proposed.[20−24] At the same time, we recently reported the ability of metNgb to activate nitrite and hydrogen peroxide through a peroxidase-like mechanism, generating reactive nitrogen species (RNS), which chemically modify the protein itself.[7] Under these conditions, Ngb would act not only as a scavenger of toxic species but also as a target of the self-generated reactive species, possibly enforcing its postulated neuroprotective activity. Both Ngb pseudoenzymatic activity in response to oxidative stress conditions and its potential role as a protein target of reactive species achieve a high physiological relevance if we consider the interaction of the protein with catecholamine-oxidation products. Autoxidation of catecholamines, such as the neurotransmitters dopamine (DA) and

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norepinephrine (NE), correlate with the oxidative stress observed in various neurodegenerative pathologies, like Parkinson's disease,[25] Alzheimer's disease,[26] and amyotrophic lateral sclerosis, even though it is still unclear whether oxidative stress is a major cause or merely a consequence of the neurodegeneration.[27] The involvement of Ngb in neurodegenerative disorders, and in particular in Alzheimer's disease, is supported by the relationship between Ngb deficiency and an increased risk of Alzheimer's disease[28] and by the attenuation of both β-amyloid neurotoxicity and tau-hyperphosphorylation observed in the presence of Ngb.[29,30]

The pigmentation of dopaminergic neurons of the *substantia nigra* and noradrenergic neurons of the *locus ceruleus* is the result of autoxidation of DA and NE, respectively, to black neuromelanins.[31,32] Parkinson's and Alzheimer's diseases are characterized by progressive degeneration and depigmentation of such neurons through pathological processes including aberrant oxidation of the catecholamines (including nonenzymatic conversion of DA to its metabolite NE)[33] and cysteine-promoted diversion of the neuromelanin pathway.[34−39] Again, the generation of reactive quinone species from DA and NE (DAQ and NEQ, respectively) is involved in this process.

A relevant feature of the cytotoxicity of quinones consists in their reactivity toward the nucleophilic side chain of many amino acidic residues, primarily cysteines but also histidines. Cysteine is in fact the only amino acid that competes with intracyclization of the amino group in DAQ or NEQ.[34,40−44] Modification of proteins through quinone linkages can induce changes in their structural and functional properties, including the tendency to aggregation and precipitation, with pathological implications.[45−50] Actually, the accumulation of aberrant or misfolded proteins, protofibril formation, and deposits of Lewy bodies in the brain is one important feature in Parkinson's disease.[51] In this context, we recently reported that the modification of myoglobin (Mb)[52] by DAQ residues initially occurs at specific amino acid residues whose reactivity depends on their surface exposure and on the local environment in the protein. Most importantly, when the derivatization becomes extensive and involves DA oligomers, it has dramatic effects on Mb stability, producing protein unfolding and incorporation into insoluble melanic precipitates.[52]

Here, we report on the effects of modification of human Ngb by reactive quinone species generated by oxidation of various catechols. Besides the catecholamines DA and NE, we also employed catechol estrogens (CEs) as source of reactive quinones. Our interest in the reactivity of these molecules stems from their potential involvement as oxidative metabolites of the female steroid hormone 17β-estradiol in neurodegenerative diseases,[53] but they have also a recognized role in carcinogenesis.[54,55] It should be noted that estrogens not only affect reproductive organs but also play important roles in all male and female physiology, including areas of the brain that are not primarily involved in reproduction.[56−58] Recent data indicate that Ngb is part of the 17β-estradiol signaling mechanism that is activated to protect against H_2O_2-induced apoptosis,[59] validating the role suggested for Ngb in the brain as an antiapoptotic neuroprotective globin.[60,61] In our study, CEs have been chosen as source of quinone species not only for their physiological relevance but also for their high steric bulkiness that, along with the absence of the competing internal cyclization (typical of DAQ and NEQ alkylamine side chains),[62] should increase the lifetime of the quinone active species in solution. The results presented herein aim at elucidating the role played by Ngb in neurodegeneration in relation to protein modifications upon reaction with catecholamine- or catechol estrogen-derived reactive quinone species.

EXPERIMENTAL PROCEDURES

Reagents. All buffer solutions were prepared using deionized Milli-Q water. Hydrogen peroxide (30% solution), DA hydrochloride, NE, N-acetyl-L-Cys, guanidinium chloride, trypsin, and mushroom tyrosinase (Ty) were obtained from Sigma-Aldrich (St. Louis, MO). All reagents were purchased at the best grade available.

The preparation of catechol estrogens 2-OH-ED and 4-OH-ED was carried out following a literature method.[63] Solid *o*-iodoxybenzoic acid (2.5 equiv) was added to a solution of estradiol (200 mg) in $CHCl_3$/MeOH 3:2 v/v (40 mL) at 25 °C. A yellow-to-orange color developed, and the mixture was stirred for 24 h. Methanolic $NaBH_4$ (15 mg in 1 mL) was then added at 25 °C under vigorous stirring until the color disappeared. After mild acidification with acetic acid (200−500 μL) to remove excess $NaBH_4$, the mixture was washed five times with equal volumes of a saturated NaCl solution containing 10% sodium dithionite buffered at pH 7.0 with sodium phosphate. Evaporation of the organic layer eventually furnished the desired products, which could be separated by preparative TLC (benzene/ethyl acetate/acetic acid 1:1:0.01) on silica.

The concentration of hydrogen peroxide solutions was controlled by monitoring the formation of the ABTS radical cation according to a standard enzymatic method.[64] Lactoperoxidases (LPO) was purified from bovine milk as previously described.[65] The reduced and oxidized forms of Ngb (Ngb_{SH} and Ngb_{S-S}, respectively) were obtained from recombinant human Ngb as previously reported.[7,66] In all of the experiments, Ngb_{SH} and Ngb_{S-S} were utilized in their met form. All spectrophotometric measurements were performed on a Hewlett-Packard HP 8452A diode array spectrophotometer (Palo Alto, CA).

Kinetic Study of H_2O_2 Activation by Ngb. The kinetic experiments were carried out in 200 mM sodium phosphate buffer (pH 7.5) using a quartz cuvette with path length of 1 cm, thermostatted at 25.0 ± 0.1 °C, and equipped with a magnetic stirrer. The reaction was started by the addition of variable volumes of the hydrogen peroxide solution (final $[H_2O_2]$ = 0.006−0.6 M) to the initial solution containing the protein, Ngb_{S-S} (1.0×10^{-6} M), and the substrate DA (0.05 M) and was followed during the initial 10−15 s by monitoring the absorbance change at 476 nm (the λ_{max} of dopaminochrome). The conversion of the rate data from absorbance per second into molar per second was done using the extinction coefficient of dopaminochrome at 476 nm, ε = 3300 M^{-1} cm^{-1}.[67] The kinetic constant for the reaction of Ngb with hydrogen peroxide (k_1) was obtained by fitting the plots of the experimental rates at different peroxide concentrations to the equation considering the activation of peroxide by Ngb as the rate-limiting step:

$$\text{rate} = \frac{k_1[H_2O_2]}{1 + \frac{[H_2O_2]}{K_M}} + b$$

The reaction rates observed for the noncatalytic reaction (i.e., in the absence of the protein or without hydrogen peroxide) are about 1 order of magnitude lower with respect to the catalytic reaction. Absorbance variations of the Soret band upon hydrogen peroxide addition account for the nonzero value of the *b* parameter.

Analysis of Cys-NE conjugates. The product mixtures derived from the reaction of N-acetyl-L-Cys with the NE-oxidation products generated by LPO/H_2O_2 or Ty/O_2 were obtained by allowing the reaction to proceed for 10 min at room temperature in 200 mM sodium phosphate buffer (pH 7.5). The concentration of the reactants were as follows: (a) for the LPO/H_2O_2 catalytic system, [N-acetyl-L-Cys] = 30 mM, [NE] = 10 mM, [LPO] = 2×10^{-7} M, and $[H_2O_2]$ = 10 mM (divided into five aliquots added every 5 min) and (b) for the Ty/O_2 catalytic system, [N-acetyl-L-Cys] = 30 mM, [NE] = 10 mM, and [Ty] = 2×10^{-8} M (divided into five aliquots added every 5 min). The products were analyzed by HPLC using a Jasco MD-1510 instrument (Easton, MD) with spectrophotometric diode array

detection equipped with a Discovery BIO Wide Pore C8 reverse-phase semipreparative column (5 μm, 250 × 10 mm) (Supelco, Sigma-Aldrich). Elution was carried out starting with 0.1% trifluoroacetic acid in water for 5 min followed by a linear gradient, in 30 min, to 10% acetonitrile in water containing 0.1% trifluoroacetic acid. The flow rate was 4 mL/min. Spectrophotometric detection of the eluate was performed in the range from 200 to 600 nm (Figure 2). The characterization of the products eluted from HPLC was achieved by NMR and MS analysis and compared with literature data.[38]

Tandem MS Analysis of Protein Fragments Obtained from Ngb Derivatives. Samples of modified Ngb_{SH} and Ngb_{S-S} were prepared by adding to the protein solutions (6.0×10^{-5} M in 50 mM Tris-HCl/0.5 mM EDTA buffer, pH 7.5) 1.0 mM catechol (DA or NE from 40 mM stock solutions in the same buffer and 2-OH-ED or 4-OH-ED from 40 mM stock solutions in methanol) and the following reagents: (a) for the LPO/H_2O_2 catalytic system, 8.0×10^{-8} M LPO and 0.3 mM H_2O_2 and (b) for the Ty/O_2 catalytic system, 2.0×10^{-8} M Ty. The solutions of catechols, H_2O_2, and Ty were divided into five aliquots and added every 5 min; after the last addition, the proteins were allowed to react at room temperature for 10 min. As blank experiments, Ngb_{SH} and Ngb_{S-S} were treated as above but in the absence of catechols.

For the tandem MS analysis of protein modifications, the Ngb_{SH} and Ngb_{S-S} derivatives (and, for comparison purposes, the unmodified proteins) were transformed into the apo-proteins by the standard hydrochloric acid/2-butanone method,[68] dialyzed against 20 mM ammonium bicarbonate buffer (pH 8.0), and subsequently hydrolyzed by trypsin (1:50 w/w with respect to the protein) at 37 °C for 4 h. Prior to HPLC−MS/MS analysis, the samples were incubated with 5.0 mM DTT for 15 min at 37 °C to prevent coupling of the Cys-containing peptides during digestion. The reduction of the disulfide bonds was necessary for the quantification of unreacted Cys residues in Ngb derivatives.

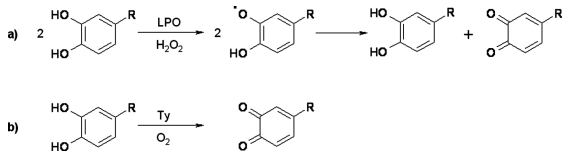
Electrospray ionization MS spectra were acquired using an LCQ ADV MAX ion trap mass spectrometer equipped with an ESI ion source and controlled by Xcalibur software 1.3 (Thermo-Finnigan, San Jose, CA). ESI experiments were carried out in positive ion mode under the following constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, capillary temperature 210 °C, and tube lens voltage 55 V. The system was run in automated LC−MS/MS mode and using a Surveyor HPLC system (Thermo Finnigan) equipped with a Jupiter 4u Proteo column (Phenomenex, Torrance, CA), 4 μm, 150 × 2.0 mm. The elution was performed using 0.1% HCOOH in distilled water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B), with a flow rate of 0.2 mL/min; elution started with 98% solvent A for 5 min followed by a linear gradient from 98 to 55% A in 65 min. MS/MS spectra obtained by collision-induced dissociation were performed with an isolation width of 2 Th (m/z), and the activation amplitude was around 35% of the ejection-radio-frequency amplitude of the instrument. For the analysis of protein fragments derived from Ngb derivatives, the mass spectrometer was set such that one full MS scan was followed by zoomscan and MS/MS scan on the most intense ion from the MS spectrum. To identify the modified residues, the acquired MS/MS spectra were automatically searched against protein database for human Ngb using the SEQUEST algorithm incorporated into Bioworks 3.1 (Thermo Finnigan).

Guanidinium Chloride Denaturation Assay. The stability to denaturation of the Ngb_{SH}-DA, Ngb_{SH}-NE, Ngb_{SH}-2-OH-ED, and Ngb_{SH}-4-OH-ED derivatives, which were obtained by reaction of the protein with the catecholamine or CE in the presence of the LPO/H_2O_2 catalytic system (as reported in the previous section) and, for comparison purposes, of the unmodified protein and the protein modified in the absence of catechols, were determined in 50 mM Tris-HCl/0.5 mM EDTA buffer (pH 6.0) at 20 °C. The absorbance variation of the Soret band of the proteins (about 4 μM) was monitored upon addition of increasing amounts (up to 4.5 M final concentration) of a 8 M guanidinium chloride solution (in the same buffer at the same pH). Data were corrected for dilution by the denaturant addition. The unfolding parameters (guanidinium concentration at 50% of protein unfolding, D_0, and the slope of the sigmoid curve, d') and the thermodynamic parameters ($\Delta G°$ and $-m$) for Ngb denaturation were evaluated from absorbance changes as a function of guanidinium chloride concentration according to a standard method.[69]

RESULTS

Ngb Modification by Catecholamine-Derived Reactive Species. To analyze the reactivity of quinone species toward protein targets of physiological relevance, Ngb derivatives were initially obtained upon reaction of the protein, either in its reduced (i.e., Ngb_{SH}) or oxidized (i.e., Ngb_{S-S}) form, with catecholamine-derived reactive species. In experiments carried out in parallel, DA and NE were employed as source of the quinone reactive species; these were generated by two types of enzymatic oxidation: (i) by LPO in the presence of hydrogen peroxide (LPO/H_2O_2 system), which promotes substrate oxidation through one-electron steps (Scheme 1a),[70]

Scheme 1. Mechanism of Enzymatic Catechol Oxidation Promoted by LPO in the Presence of H_2O_2 (One-Electron Oxidation, Reaction a) and by Ty in the Presence of O_2 (Two-Electron Oxidation, Reaction b)



and (ii) by Ty in the presence of molecular oxygen (Ty/O_2 system), which promotes substrate oxidation in a single two-electron process (Scheme 1b).[71] In both cases, oxidation of the catecholamine (DA or NE) produces the corresponding quinone (DAQ or NEQ, respectively), but with LPO, the product derives from dismutation of a semiquinone species ($DA^{\bullet}$ or $NE^{\bullet}$).[52] Besides mimicking a physiological inflammatory response, the oxidizing system LPO/H_2O_2 is worthy of interest because heme peroxidases with activity similar to LPO have been proposed to be involved in the oxidation of proteins and lipids that is primarily responsible for neurodegeneration.[72] However, because Ty was reported to be expressed in human brain tissues,[73] the quinones production through the single two-electron oxidation process mediated by this enzyme is also likely to occur in vivo. LPO and Ty were chosen also because of their narrow active site that allows the approach of small molecules (such as the catechols employed here) but prevents the direct reaction with Ngb; to control this point, blank experiments were performed by reacting Ngb_{SH} or Ngb_{S-S} with these enzymatic systems in the absence of catechols.

We also considered the possibility of employing Ngb as a catalyst for promoting self-modifying reactions of a peroxidase type (in the presence of H_2O_2, Scheme 1a) because of the Ngb active site similarities with Mb and Hb that were previously reported to promote this type of reaction in their ferric form.[74−77] Although in a previous study we reported that the peroxidase-like activity of Ngb with H_2O_2 toward phenolic substrates was negligible,[7] here the catalytic oxidation of DA indeed has been observed, albeit with low efficiency. In this Ngb-promoted catechol oxidation, the slow step is the activation of hydrogen peroxide: for the reaction of Ngb_{S-S} with H_2O_2 (described in the Experimental Procedures), we estimated a kinetic constant of 3.4 ± 0.4 M^{-1} s^{-1}, compared with a rate constant of 700 M^{-1} s^{-1} for the reaction of horse

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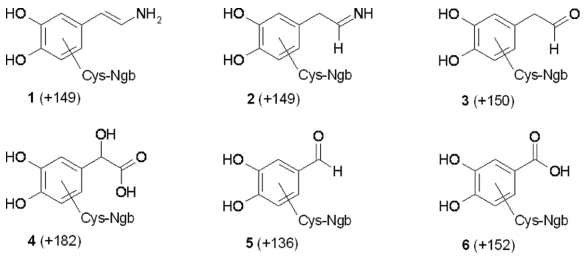
heart Mb with H_2O_2.[78] This suggests that Ngb is able to activate H_2O_2 only in the presence of high peroxide concentration and that the generated protein active species has a redox potential compatible with the oxidation of catecholic substrates (with $E°$(semiquinone/catechol) ~0−0.5 V vs standard hydrogen electrode) but insufficient to oxidize phenolic substrates (with $E°$($PhO^•$/PhOH) ~0.8−1.0 V vs standard hydrogen electrode),[79] as is typical of peroxidases.[80] This very low peroxidase activity of Ngb precludes the possibility of obtaining appreciable protein modifications in the absence of an external catalyst like LPO or Ty. Therefore, because the self-modification induced by Ngb modification in the absence of LPO or Ty was always found to be negligible, we employed the LPO/H_2O_2/catecholamine and Ty/O_2/catecholamine reactive systems for generating the quinone reactive species.

In the experiments of Ngb modification induced by the enzymatic systems, the Ngb solutions progressively darkened, turning from reddish-brown to dark brown or black, especially in the experiments carried out with systems generating DAQ reactive species. Nevertheless, no formation of precipitates of melanized protein was detectable, even forcing the reaction conditions (e.g., by increasing the reagent concentrations and/or prolonging the reaction time). This is consistent with the formation of soluble melanin-protein conjugates; a similar behavior has been recently reported in the case of DA melanization in the presence of albumin.[81]

The modifications induced on Ngb by the reactions with catecholamines were analyzed in separate experiments: the apo-Ngb derivatives were subjected to proteolysis by trypsin, and the protein fragments were searched for specific amino acidic derivatizations by LC−MS/MS analysis. However, the consequences of Ngb modification on protein stability were analyzed by performing unfolding studies of the holo-Ngb derivatives in the presence of guanidinium chloride as the electrostatic denaturant.

Tandem MS Analysis of Ngb-DA and Ngb-NE Derivatives. The nucleophilic reactivity of amino acid side chains toward quinone species is in the order Cys > His > Lys.[40,43] Usually, in the presence of free Cys, as with Ngb_{SH} (that contains three cysteine residues Cys46, Cys55, and Cys120), the reactivity of the other protein residues becomes negligible.[40] Consequently, our initial attempts were oriented to the identification of Cys derivatization by DA and NE molecules, either as monomers or oligomers (up to five catecholamine units attached to each Cys residue). In the case of NE, the enamine-aldehyde rearrangement of the aliphatic chain (compounds **1**−**3** in Scheme 2) and Cys adducts with the reported products of NE oxidation promoted by horseradish peroxidase/H_2O_2[32] (compounds **4**−**6** in Scheme 2) were also considered.

Scheme 2. Structure of Potential Cys-NE Adducts Searched in Tandem MS Analysis of Ngb Derivatives[a]



[a]For each adduct, the mass increment (in amu) of the modified Cys residue is indicated.

Employing DA as the source of the quinone species and either LPO/H_2O_2 or Ty/O_2 as oxidants, we obtained Ngb_{SH} and Ngb_{S-S} derivatives (Ngb_{SH}-DA and Ngb_{S-S}-DA, respectively) that did not allow for the detection of appreciable Cys modification upon LC−MS/MS analysis of their tryptic digests. However, in the case of NE, the tandem MS analysis of protein derivatives (Ngb_{SH}-NE and Ngb_{S-S}-NE) allowed the identification of the Cys120 residue in Ngb_{SH} as the site of chemical modification by the quinone species generated only in the presence of LPO/H_2O_2. The amount of derivatization was estimated to be about 5%, and the measured mass increment of 147 amu is compatible with the formation of a covalent bond between the quinone of NE in its enamine/imine form (obtained upon oxidation of compounds **1** or **2**) to the Cys thiol group (Figure 1). It is worth noting that the extent of Mb

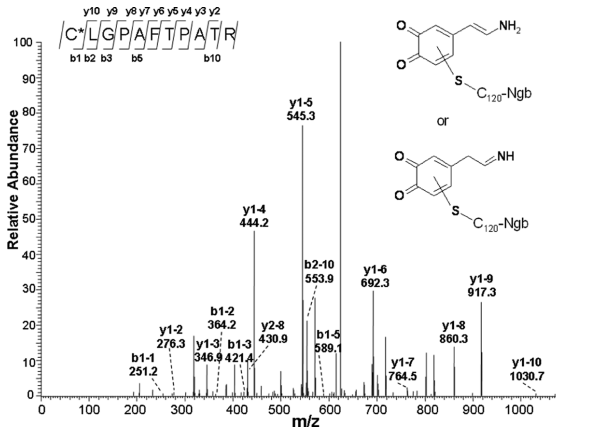


Figure 1. MS/MS spectrum of the m/z 641.3 peak assigned to the 120−130 peptide of Ngb_{SH} in a double-charged state containing the adduct of Cys120 with the quinone of NE in its enamine/imine form (peptide mass of 1281.3 amu, corresponding to a mass increase of 147 amu with respect to the unmodified peptide). The assignment of the y and b ion series (either in mono- or double-charged states) is shown. Above the spectrum, the sequence of the 120−130 peptide is shown with an asterisk on the modified residue and with the summary of the y and b ions found in the spectrum.

modification by the DAQ species depended also on the enzymes used to promote DA oxidation,[52] possibly indicating a different reactivity of Cys residues in the one-electron- and two-electron-oxidation pathways.

In addition to Cys residues, we also considered the nucleophilic reactivity of His and Lys side chains in Ngb toward the catecholamine-derived quinone species, but no specific amino acid modification with DA or NE was detected.

Regarding the position of attack of the Cys thiol group to the catechol ring, the HPLC separation and NMR/MS characterization of NE/N-acetyl-Cys conjugates obtained with LPO/H_2O_2 show both the 2-S-cysteinyl-NE and the 5-S-cysteinyl-NE isomers as the major products (Figure 2, top). Conversely, in the Ty-mediated conjugation of NE with N-acetyl-Cys, we obtained the 5-S-cysteinyl conjugate of NE as the predominant product (Figure 2, bottom).

Ngb Modification by CE-Derived Reactive Species. We employed two different CEs as the source of reactive quinone species: the compounds 2-hydroxyestradiol (2-OH-ED) and 4-hydroxyestradiol (4-OH-ED) (Scheme 3) were oxidized to their quinone by either LPO/H_2O_2 or Ty/O_2 in the presence of Ngb, both in its oxidized form (yielding the Ngb_{S-S}-2-OH-ED

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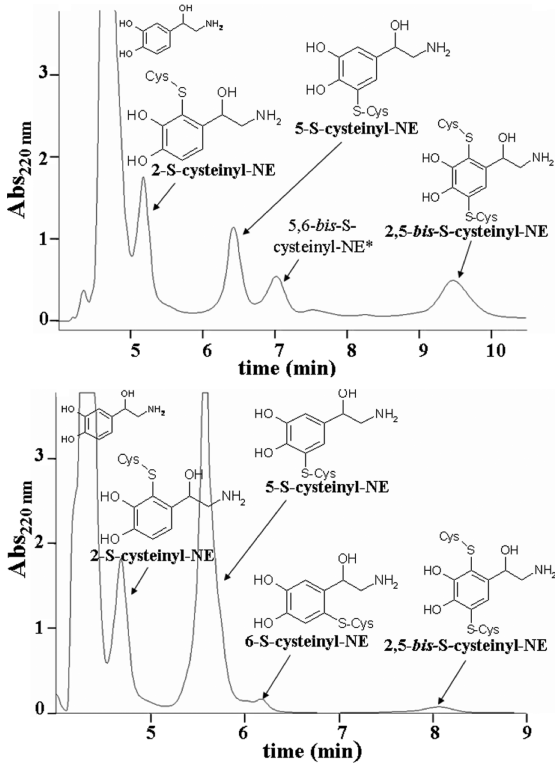
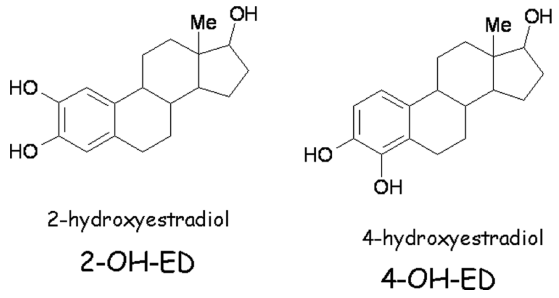


Figure 2. HPLC elution profile, with absorbance reading at 220 nm, for the catalyzed reaction of N-acetyl-cysteine (30 mM) with NE (10 mM) in the presence of LPO (2×10^{-7} M)/H_2O_2 (10 mM) (top) and Ty (2×10^{-8} M) (bottom) in 200 mM sodium phosphate buffer (pH 7.5) at room temperature. The assignment of the peaks, achieved by NMR and MS characterization of the products eluted from HPLC (consistent with literature data),[38] is shown. The asterisk (*) indicates that this product has not been fully characterized.

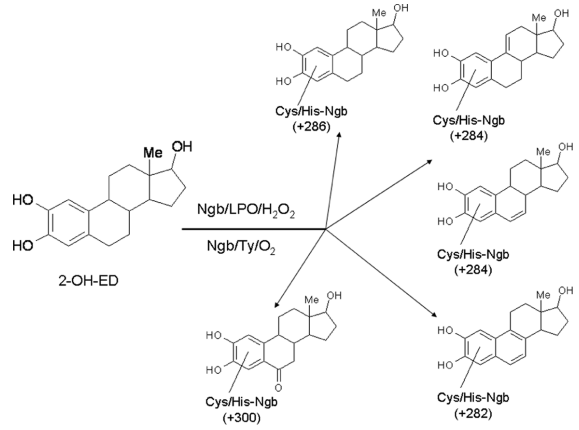
Scheme 3. Structure of Catecholestrogens



and Ngb_{S-S}-4-OH-ED derivatives) or reduced form (yielding the Ngb_{SH}-2-OH-ED and Ngb_{SH}-4-OH-ED derivatives). Like the Ngb-catecholamine derivatives described above, the Ngb-catechol estrogen derivatives did not give rise to precipitates. Moreover, in this case, the solutions did not darken. The characterization of the Ngb_{S-S}-2-OH-ED, Ngb_{S-S}-4-OH-ED, Ngb_{SH}-2-OH-ED, and Ngb_{SH}-4-OH-ED derivatives was carried out by tandem MS studies and by unfolding studies.

Tandem MS Analysis of Ngb-CE Derivatives. The high complexity of the estradiol skeleton introduces a wide spectrum of CE oxidation products, besides the simple quinone or semiquinone species, that can be obtained by enzymatic oxidation. Therefore, the LC−MS/MS data obtained from the analysis of Ngb_{S-S} and Ngb_{SH} modified by 2-OH-ED-derived reactive species were searched for Cys and/or His modification by each of the characterized products of Ty-catalyzed oxidation of 2-OH-ED[82] (in both their catechol or quinone form), according to Scheme 4. The covalent bond of

Scheme 4. Structure of Potential Cys-2-OH-ED or His-2-OH-ED Adducts Searched in Tandem MS Analysis of Ngb Derivatives[a]



[a]For each adduct, the mass increment (in amu) of the modified protein residue has been reported.

two units of CE oxidation products to each nucleophilic residue of Ngb was also considered. The amount of derivatization of all of the Ngb free Cys residues (i.e., Cys46, Cys55, and Cys120 in Ngb_{SH} and Cys120 in Ngb_{S-S}) and of the His23 residue, obtained by comparing the areas of the peaks corresponding to the derivatized peptides with those of the corresponding peptides in the starting proteins in the chromatograms with extracted ion current, are summarized in Table 1.

Table 1. Modification of Ngb Residues by 2-OH-ED Reactive Species[a]

		modified residues (%)			
protein target	enzymatic system	Cys46	Cys55	Cys120	His23
Ngb_{SH}	LPO/H_2O_2	19	52	14	2
	Ty/O_2	23	41	8	0
Ngb_{S-S}	LPO/H_2O_2	Cys residues involved in the S−S bond		8	<1
	Ty/O_2			5	<1

[a]Amount of derivatization (%) of Cys and His residues in Ngb_{SH} and Ngb_{S-S} derivatives (6.0×10^{-5} M) was obtained by protein modification in the presence of either LPO (8.0×10^{-8} M)/H_2O_2 (0.3 mM)/2-OH-ED (1.0 mM) or Ty (2.0×10^{-8} M)/O_2/2-OH-ED (1.0 mM). All reactions were performed in 50 mM Tris-HCl/0.5 mM EDTA buffer (pH 7.5).

Tandem MS data are compatible with mass increments of either 284 or 282 amu for Cys55, Cys120, and His23 in the modified peptides, corresponding to the covalent bond of the catechols 9,11-dehydro-2-OH-ED and 6,7-dehydro-2-OH-ED (+ 284 amu) or the corresponding quinones (+ 282 amu). The last mass increment could also correspond to the modification of Ngb by 6,7,8,9-dehydro-2-OH-ED, but on the basis of the reported distribution of 2-OH-ED oxidation products, this derivatization is unlikely.[82] As an example, the representative MS/MS spectrum of the 120−130 peptide of the Ngb_{SH} derivative obtained upon reaction with LPO/H_2O_2/2-OH-ED, modified at the Cys120 residue with a mass increment of 284 amu, is shown in Figure 3.

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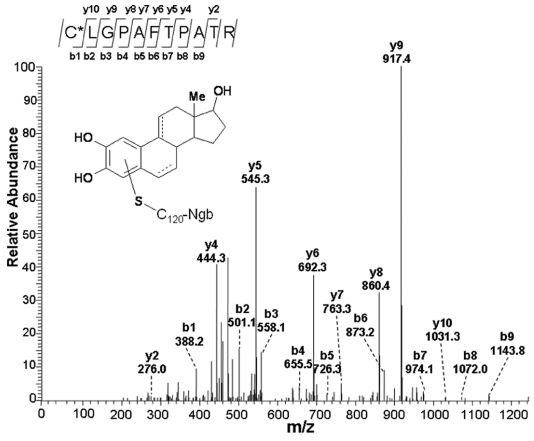

Figure 3. MS/MS spectrum of the m/z 709.9 peak assigned to the 120−130 peptide of Ngb_{SH} in a double-charged state containing the adduct of Cys120 with 9,11-dehydro-2-OH-ED or 6,7-dehydro-2-OH-ED (peptide mass of 1418.3 amu, corresponding to a mass increase of 284 amu with respect to the unmodified peptide). The assignment of the y and b ion series is shown. Above the spectrum, the sequence of the 120−130 peptide is shown with an asterisk on the modified residue and with the summary of the y and b ions found in the spectrum.

Regarding Cys46, the mass increment of 298 amu corresponds to the addition of the quinone of 6-oxo-2-OH-ED, which together with 6,7- and 9,11-dehydro-2-OH-ED represents the main Ty-catalyzed oxidation product of 2-OH-ED.[82]

The regiochemistry of nucleophilic attack by the Cys thiol on the quinone (or semiquinone) ring depends on the system generating the reactive species. The one-electron oxidation of 2-OH-ED by LPO in the presence of GSH has been reported to generate the C4-isomer as the most abundant conjugate, whereas the C1 position is the preferred site for direct nonenzymatic coupling of the quinone with Cys, N-Ac-Cys, or GSH.[83,84] The latter regioselectivity is probably the one preferred by Ty-catalyzed formation of protein adducts because this enzyme directly generates the quinone by oxidation of 2-OH-ED (Scheme 1b).

The analysis of the data reported in Table 1 shows a more extensive modification of Ngb_{SH}, the most physiologically relevant form of Ngb,[66] with respect to Ngb_{S-S}. Cys46 and Cys55, the most accessible in Ngb_{SH}, are subjected to modification the most.

Cys46 and Cys55 are the only residues that have been identified as a target of 4-OH-ED reactive species. Table 2 reports the percent of derivatization of Cys residues in Ngb_{SH}. Regarding the structure of the adducts between Cys residues and 4-OH-ED, the C2 position of the catechol ring has been indicated as the only site of thiol binding,[83,84] and the mass increments of 284/282 amu for Cys55 and 298 amu for Cys46 are compatible with the derivatization of the former residue by 9,11-dehydro-4-OH-ED (possibly oxidized to quinone) and the latter residue by the quinone of 6-oxo-4-OH-ED, similar to the above-mentioned modification of the same residues by 2-OH-ED-derived reactive species. 9,11-Dehydro-4-OH-ED was reported as the major product of Ty-catalyzed oxidation of 4-OH-ED.[82]

Table 2. Modification of Ngb Residues by 4-OH-ED Reactive Species[a]

		modified residues (%)			
protein target	enzymatic system	Cys46	Cys55	Cys120	His23
Ngb_{SH}	LPO/H_2O_2	0	28	0	0
	Ty/O_2	20	37	0	0

[a]Amount of modification (%) of Cys residues in Ngb_{SH} (3.0×10^{-5} M) were obtained by reaction with LPO (8.0×10^{-8} M)/H_2O_2 (0.3 mM)/4-OH-ED (1.0 mM) and Ty (2.0×10^{-8} M)/O_2/4-OH-ED (1.0 mM). All reactions were performed in 50 mM Tris-HCl/0.5 mM EDTA buffer (pH 7.5).

The data shown in Tables 1 and 2 are in agreement with the lower oxidizability of 4-OH-ED with respect to 2-OH-ED.[85] These tables show that the enzymatic system LPO/H_2O_2 is most active in the modification with 2-OH-ED, whereas Ty/O_2 shows comparable values with the two CEs.

Denaturation of Ngb-DA, Ngb-NE, and Ngb-CE Derivatives. To observe the effects of Ngb modification by catecholamine or CE-derived reactive species on protein stability, unfolding studies in the presence of guanidinium chloride as the denaturant agent were performed on a set of representative Ngb derivatives. The unfolding studies focused on the protein derivatives with higher physiological relevance (i.e., Ngb_{SH}) and, regarding the enzymatic system, the one (LPO/H_2O_2) that better mimics the one-electron radical oxidations that occur under oxidative stress conditions. Absorbance variations of the Soret band of native Ngb_{SH} and Ngb_{SH} derivatives upon addition of increasing amounts of the denaturant agent (Figure 4, left) were fitted according to a standard method,[69,86] which yields the unfolding parameters D_0 (guanidinium concentration at 50% unfolding of the protein) and d' (connected to the shape of the sigmoid curve) reported in Table 3. Subsequently, from the linear plot of the free energy of unfolding at each guanidinium concentration, ΔG°_{obs}, versus [guanidinium] (Figure 4, right), the thermodynamic parameters ΔG° (free energy change for the conversion of native to unfolded protein in the absence of denaturant) and $-m$ (related to the protein−water interaction) (Table 3) were determined from the extrapolation to 0 denaturant concentration and from the slope of the line, respectively.[69,86]

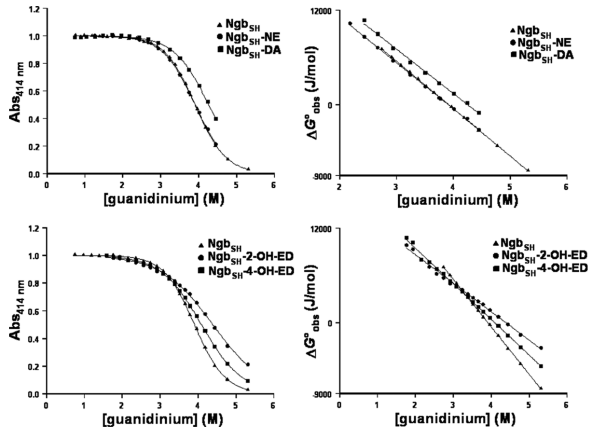

Figure 4. On the left, absorbance variation of the Soret band of Ngb_{SH}-catecholamine (top) and Ngb_{SH}-catechol estrogen (bottom) derivatives, with respect to native Ngb_{SH}, on guanidinium concentration. On the right, dependence of the free energy of unfolding (calculated at each denaturant concentration) of Ngb_{SH}-catecholamine (top) and Ngb_{SH}-catechol estrogen (bottom) derivatives, with respect to native Ngb_{SH}, on guanidinium concentration. All data were obtained in 50 mM Tris-HCl/0.5 mM EDTA buffer (pH 6.0) at 20 °C.

Table 3. Guanidinium-Induced Unfolding of Ngb_{SH} Derivatives[a]

protein	D_0 (M)	d' (M)	$\Delta G°$ (J/mol)	$-m$ (J/mol M)
Ngb_{SH}	3.91 ± 0.01	0.40 ± 0.01	23 550 ± 100	6020 ± 30
Ngb_{SH}-DA	4.27 ± 0.09	0.46 ± 0.03	24 000 ± 500	5640 ± 130
Ngb_{SH}-NE	3.91 ± 0.03	0.43 ± 0.01	22 860 ± 180	5850 ± 50
Ngb_{SH}-2-OH-ED	4.39 ± 0.06	0.68 ± 0.03	15 900 ± 200	3620 ± 60
Ngb_{SH}-4-OH-ED	4.09 ± 0.01	0.54 ± 0.01	18 500 ± 200	4520 ± 60

[a]Unfolding parameters of native and modified Ngb_{SH} were obtained from the analysis of data shown in Figure 4.

Figure 4 (left) and Table 3 indicate that catecholamine- and catechol estrogen-modified Ngb_{SH} derivatives maintain high stability to guanidinium (a D_0 parameter similar or even slightly increased with respect to that of native Ngb_{SH}) but unfold over a broader range of denaturant concentrations (giving flatter sigmoid curves, corresponding to larger d' values with respect to that of native Ngb_{SH}). The increase of the d' parameter both in Ngb_{SH}-DA/NE and particularly in Ngb_{SH}-CE derivatives indicate the presence of mixtures of differently modified proteins, each characterized by a different stability profile against the denaturant.

In addition, the thermodynamic parameters obtained from denaturation assays indicate that the reactions of Ngb_{SH} with catecholamine- or catechol estrogen-derived quinone species affect the stability of the protein, lowering the solvent-exposed surface area (related to $-m$) and, in the case of Ngb_{SH}-CE derivatives, lowering the unfolding free energy change ($\Delta G°$). Again, the most considerable effect on Ngb stability is induced by CE derivatization of Ngb_{SH} residues.

The denaturation study has been performed also on the Ngb_{SH} derivative obtained upon treatment of the protein with the LPO/H_2O_2 system in the absence of catechols. The curve obtained is comparable, within the experimental error, with that of native Ngb_{SH} (data not shown), confirming that the changes in the denaturation parameters in Ngb_{SH}-DA/NE and Ngb_{SH}-CE are mediated by the catechols.

DISCUSSION

The physiological function of Ngb, in particular its role in pathological conditions, is still under debate.[87,88] The present study indicates that in the presence of reactive quinone species, like those generated in vivo during neurodegenerative disorders,[25,26,35] Ngb undergoes modification through the formation of covalent bonds with the catechols. The high, with respect to physiological values, cathecol concentrations employed here were due to the need to force the modification that are very slow in vivo to occur (the Parkinson's and Alzheimer's diseases are typical of the third age). The identification of specific sites of protein modification by tandem MS analysis allows for the elucidation of the relative propensity of protein residues to react with the quinone species. However, tandem MS analysis is unable to reveal the complete pattern of amino acid modifications, likely because of the inefficiency of proteolytic digestion of the modified protein, even under denaturing conditions. The limits of this approach have been noted in other cases where the modification hinders the site of attack by the proteolytic enzymes.[81] In the present study, the guanidinium denaturation assays clearly indicate the presence of quinone-promoted modifications in all of the Ngb derivatives, including those (i.e., Ngb_{SH}-DA and Ngb_{SH}-NE) for which MS analysis could not recognize the presence of amino acid−catechol adducts. These two analytical approaches thus provide complementary results.

The much higher reactivity of Cys residues with respect to the other protein residues toward quinone species has been confirmed here for Ngb. Cys modification depended on the enzymatic system used for catechol oxidation. In fact, a similar efficiency of LPO with respect to Ty was observed in Cys modification by 2-OH-ED reactive species; however, in the case of 4-OH-ED, the effect of Ty was higher than that of LPO (Tables 1 and 2). Because Ty directly generates quinones by two-electron oxidation of CEs, the greater reactivity of 4-OH-ED- compared to 2-OH-ED-derived quinones toward Cys residues is in agreement with literature results.[83] Referring to the one-electron-oxidation pathway promoted by LPO, the involvement of CE semiquinones, besides quinones, has been suggested in the coupling reaction.[83] In addition, the known different regiochemistry of Cys-2-OH-ED conjugates obtained by direct coupling of the quinone with Cys with respect to those obtained enzymatically with LPO endorse the idea of semiquinone reactivity in peroxidase-promoted pathways.[83,84]

Among protein residues, besides Cys residues, His side chains are also known to exert nucleophilic reactivity toward quinone species. Different from the other globins, in which His residues are widely represented,[68] in the sequence of human Ngb only three His are present (His23, His96, and His64). Considering that His96 and His64 are buried in the Ngb active site and are involved in either iron coordination (i.e., His96 and His64) or stabilization of heme axial ligands (His64), it is not surprising that tandem MS analysis revealed that only His23 was modified in Ngb by quinone reactive species (specifically, those derived from oxidation of 2-OH-ED, as reported in Table 1). Although solvent exposure of this residue[3] is compatible with its modification by a bulky molecule like CE, the low extent of modification observed can be ascribed to the reduced reactivity of His residues with respect to Cys side chains. In this regard, it is worth noting that Cys120, the only free Cys residue in both Ngb_{SH} and Ngb_{S-S}, is located in the proximity of His23, with which it competes for quinones capture.

Ngb modifications by the monomeric catechol residues identified by tandem MS analysis most likely represent the initial stage of a multistep pathway leading further to protein modifications by catechol oligomers. This is an important aspect because extensive modifications of Ngb residues could mask the recognition sites to the proteolytic enzymes, thus preventing fragmentation of the protein and the complete detection of the modification sites with tandem MS.[81] In the case of extended modification in vivo, both the stability and physiological properties of Ngb will be compromised, with the possibility of activation of undesired reactivity. Because Ngb exhibits some (albeit low) pseudoperoxidase activity, aggregates of Ngb could catalyze the slow but irreversible degeneration observed in parkinsonian brains over long periods of time.

The unfolding experiments reported here provide significant information regarding Ngb derivatives at an intermediate stage of modification. Besides confirming the formation of protein−catechol conjugates in all of the Ngb derivatives, the denaturation assays show that modification of polar residues

(i.e., Cys and His) make Ngb less sensitive to a hydrophilic denaturant like guanidinium.

The present study allows for the comparison of the effects of catecholamine and catechol-estrogen oxidation products toward the protein target Ngb. Tandem MS and unfolding studies show the ability of DAQ/NEQ to extensively modify Ngb, yielding protein derivatives that are difficult to characterize completely using MS techniques. In spite of the modification with oligomers, the solvent exposure of the Ngb-DA and Ngb-NE derivatives inferred from unfolding experiments is not changed as dramatically as it is in the case of Ngb-CE derivatives because of the higher polarity of DA and NE moieties as compared to the lipophilic CEs. However, the altered unfolding parameters obtained for Ngb-CE derivatives (even with few derivatized Ngb residues) can be accounted for by considering the bulkiness and hydrophobicity of CE moieties. The large steric hindrance in CEs and the absence of a nucleophilic amino substituent, present in catecholamine's aliphatic chains, prevents further reactions of the derivatized residue. The protein modifications by CE residues are easy to identify by tandem MS analyses and dramatically alter the protein−solvent interactions, thereby compromising protein stability.

The reversible oxidation of Cys thiols to disulfide has been extensively studied, revealing that it functions in redox signaling pathways and, along with reactive oxygen and nitrogen species, affects oxidative stress response.[89] In this context, the Cys-modification reactions investigated here are of particular relevance. Actually, the Cys-modified protein could be considered as the product of a neuroprotective function exerted by Ngb by means of a self-targeting from the reactive quinone species. In fact, it is worth noting that under oxidative stress conditions, together with the increase of quinone and semiquinone species, Ngb levels also increase as a result of the 17β-estradiol signaling mechanism.[90,91] However, the physiological structure and properties of Ngb are compromised by the catechol modification reactions, with a further possibility for the resulting Ngb derivatives to promote pseudoenzymatic peroxidase reactions.

The present study has shown the propensity of specific amino acid residues (mainly cysteine) in Ngb to react with catechol-derived reactive species, with the extension of protein modification depending upon both the catechol source and the system generating the quinone (or semiquinone) species. The different effect produced by catecholamine with respect to CE protein modifications is worthy of interest. Small and polar catechol molecules with nucleophilic groups, like DA or NE, can modify proteins through their oligomers, eventually leading to substantial modification of protein properties. However, protein modification mediated by bulky and lipophilic molecules like CEs, although less pronounced/extended than the previous one, strongly compromise protein stability through alteration of its interactions with solvent.

Our studies suggest that the quinone-promoted modification of amino acid residues and the consequent impairment of protein functions may have physiological relevance, in particular under pathological conditions. Because the high reactivity toward quinones is probably common to all exposed Cys residues in proteins (as well as for the SH group of glutathione), there is likely an efficient trapping system for these reactive species in vivo. However, over a period of years, the quinones that are not quenched generate modified proteins that can accumulate because of their increased resistance to proteolysis.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

2-OH-ED, 2-hydroxyestradiol; 4-OH-ED, 4-hydroxyestradiol; CE, catechol estrogen; DA, dopamine; DA$^{\bullet}$, DA semiquinone species; DAQ, reactive quinone species from DA; LPO, lactoperoxidase; Mb, myoglobin; metMb, ferricMb; NE, norepinephrine; NE$^{\bullet}$, NE semiquinone species; NEQ, reactive quinone species from NE; Ngb, neuroglobin; Ngb_{SH}, reduced form of Ngb without the intramolecular Cys46−Cys55 disulfide bond; Ngb_{S-S}, oxidized form of Ngb with the intramolecular Cys46−-Cys55 disulfide bond; Ty, tyrosinase

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