Salsolinol, a catechol neurotoxin, induces oxidative modification of cytochrome c

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INTRODUCTION

The catechol isoquinoline derivatives are endogenous compounds that are present in the mammalian brain (1), and are also detected in the urine of patients suffering from Parkinson’s disease (PD) who have been treated with L-DOPA (2). The representative catechol isoquinoline compound is referred to as salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydro-isoquinoline). Increased salsolinol levels are detected in the brain of PD patients (3, 4). Previous studies have shown that salsolinol is toxic to dopaminergic neurons under both in vitro and in vivo conditions. It has also been reported that salsolinol in conjugation with iron undergoes redox cycling to generate reactive oxygen species (ROS), most notably hydroxyl radicals, which induce DNA strand scission and cell death (5).

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Iron is the most abundant heavy metal in the human body, and is particularly abundant in the brain. Although iron required for oxidative metabolism, it can also be toxic, owing its ability to catalyze ROS generation (6). Iron accumulates progressively with aging in some regions of the brain, often to the high levels which are associated with oxidative stress; this process has been previously shown to promote neurodegeneration (7, 8). The iron released from cytochrome c, and its associated toxicity, has also been implicated as a risk factor for age of onset for certain neurodegenerative diseases, namely PD and Alzheimer’s disease (AD) (9, 10).

Cytochrome c is well known as the penultimate electron transport protein of the eukaryotic mitochondrial chain (11). The conformational, posttranslational attachment of a heme moiety to the nuclear-encoded apoprotein confers the native structure (12). The heme is necessary for the protein to attain its proper three-dimensional folding pattern (13). In the mitochondria, cytochrome c transfers an electron from cytochrome c reductase to cytochrome c oxidase. These electron transfer reactions are mediated by a conserved cytochrome c-binding epitope (14, 15).

Recent discoveries implicate this protein in two other biological processes, apoptosis and oxidative stress (16, 17). Apoptosis, also referred to as programmed cell death, is a key to development and is linked to human diseases, such as cancer and neurodegeneration (18, 19). The cellular damage induced by oxidative stress has also been associated with several diseases, including Parkinson’s disease (PD). More specifically, it has been shown that cytochrome c is co-localized with α-synuclein aggregates in Lewy bodies, the pathological hallmarks of PD (15). It has also been shown that cytochrome c catalyzes the H2O2-induced aggregation of α-synuclein (17).

Although all of these observations are archetypical results in vitro, it is not yet clearly understood how free radicals are generated by the effect of cytochrome c, or which initial and cascading radical species are responsible for these damages in vivo.

In the current study, the effects of salsolinol on the oxidative modification of cytochrome c were assessed. The results demonstrated that salsolinol induced cytochrome c aggregation via free radical generation. Salsolinol-mediated cytochrome c modification, in turn, resulted in the release of iron from the protein.
RESULTS AND DISCUSSION

All cellular components are vulnerable to oxidative stress. The damage that oxidative stress causes to proteins includes side chain modification and main chain fragmentation. One of the biological markers for oxidative damage in proteins is the accumulation of carbonyl compounds (20). In the present study, salsolinol led to the oligomerization of cytochrome c (Fig. 1A), and the formation of carbonyl compound (Fig. 1B). These phenomena were increased in a salsolinol concentration-dependent manner. Metal catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl groups into the amino acid residues of proteins (21). The autoxidation of salsolinol induced ROS formation that oxidizes amino acid residues at or near that cation-binding site, which then introduces carbonyl groups. Such an oxidative modification is an indicator of oxidative stress and may be significant in several physiological and pathological processes (22, 23). The participation of ROS in the oligomerization of cytochrome c by salsolinol was studied by examining the protective effects of ROS scavengers. When cytochrome c was incubated with salsolinol in the presence of ROS scavengers, azide, formate, glutathione and N-acetyl-cysteine prevented the oligomerization of cytochrome c (Fig. 2A). These scavengers also prevented the formation of carbonyl compounds (Fig. 2B). The results suggest that ROS might play a critical role in the mechanism of salsolinol-mediated cytochrome c modification.

Trace metals such as iron and copper, which are present in a variety of biological systems, may interact with active oxygen species, ionizing radiation or microwave radiation, to damage macromolecules (24-26). In some biological cells, the cleavage of metalloproteins by oxidative damage may lead to increases in the levels of metal ions (27). The participation of iron in the salsolinol-mediated cytochrome c modification was investigated by an examination of the protective effects of the iron chelator, deferoxamine (DFX). When cytochrome c was incubated with salsolinol in the presence of DFX, the oligomerization of cytochrome c (Fig. 3A) and carbonyl compound for-
Modification of cytochrome c by salsolinol

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Fig. 4. Modification of amino acid residues in cytochrome c by salsolinol. Cytochrome c was incubated in 10 mM potassium phosphate buffer (pH 7.4) at 37°C with the following conditions: 20 μM cytochrome c alone (dark bar); 20 μM cytochrome c plus 0.5 mM salsolinol (gray bar). After incubation for 24 h, the amino acid composition of acid and alkaline hydrolysates was determined, as described in Materials and Methods.
recent report has also shown that salsolinol increases ROS generation and effects a significant reduction in glutathione levels in SH-SYSY cells (30).

In conclusion, the findings of the current study demonstrate that salsolinol induces the oxidative modification of cytochrome c and subsequent iron release, and that this phenomenon involves free radical generation. Salsolinol-mediated cytochrome c modification may be associated with the pathogenesis of PD, as well as related disorders.

MATERIALS AND METHODS

Materials
Horse cytochrome c, salsolinol, azide, formate, N-acetyl-L-cysteine, glutathione, trichloroacetic acid (TCA) and deferoxamine (DFX) were obtained from Sigma (St. Louis, MO, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA, USA). All solutions were treated with Chelex 100 resin in order to remove any traces of transition metal ions.

Protein oxidative modification
Protein concentrations were determined using the BCA method (31). 20 μM cytochrome c modification was induced via incubation with salsolinol in phosphate buffer (pH 7.4) at 37°C. After the incubation of the reaction mixtures, the mixtures were placed onto a Vivaspin ultrafiltration spin column (Sartorius Stedim Biotech, Goettingen, Germany) and were centrifuged for 1 h at 13,000 rpm to remove the salsolinol. The mixture was then washed with Chelex 100-treated water and centrifuged for 1 h at the same speed to remove any remaining salsolinol. This procedure was repeated four times, after which the filtrates were dried in a freeze-dryer and dissolved with 10 mM potassium phosphate buffer (pH 7.4). The free radical scavenger-induced protection against salsolinol-mediated cytochrome c modification was induced by the 5 min of preincubation of the enzyme with free radical scavengers at room temperature, after which the mixture was re-acted with salsolinol for 24 h at 37°C. The unreacted reagent was then washed through a Microcon filter (Amicon).  

Determination of free iron ions concentration
The concentration of iron ions released from oxidatively damaged cytochrome c was determined by using bathophenanthroline sulfonate in the method described previously (34). The reaction mixture contained 20 μM cytochrome c, 0.5 mM salsolinol in a total volume of 0.5 ml. The reaction was initiated by the addition of salsolinol and was incubated for various periods of time at 37°C. After incubation, the mixtures were placed into a Vivaspin ultrafiltration spin column (Sartorius Stedim Biotech, Goettingen, Germany) and were then centrifuged at 13,000 rpm for 1 h. The colorimetric reagent was added to the filtrate prior to analysis by UV/VIS spectrophotometry (Shimadzu, UV-1601) at 535 nm. The final concentrations of the color reagent were 1% ascorbate, 0.02% bathophenanthroline sulfonate and 1% acetic acid-acetate buffer (pH 4.5).

Amino acid analysis
Aliquots of modified and native cytochrome c were hydrolyzed at 110°C for 24 h after the addition of 6 N HCl. Since acid hydrolysis destroys tryptophan, the tryptophan content of oxidized and native cytochrome c preparations was determined by alkaline hydrolysis, as described previously (35). The amino acid content of acid and alkaline hydrolysates was determined by HPLC separation of their phenylisothiocyanate-derivatives by using a Pico-tag free amino acid analysis column and a 996 photodiode array detector (Waters, USA).

Statistical analysis
Values are expressed as the means ± S.D of three to five separate experiments. The statistical differences between the means were determined by the Student t-test.

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