

## Oxidative Modification of Ferritin Induced by Salsolinol, Catechol Neurotoxin

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Received August 20, 2008

Previous evidences suggest that oxidative alteration of ferritin has been linked to the pathogenesis of Parkinson disease (PD). We have investigated the modification of ferritin induced by salsolinol (SAL), endogenous neurotoxin. When ferritin was incubated with SAL, the aggregation of protein increased with the SAL concentration. SAL also led to the release of iron from ferritin in a SAL concentration-dependent manner. Free radical scavengers and iron specific chelator inhibited the SAL-mediated ferritin modification. Exposure of ferritin to SAL led to the generation of protein carbonyl compounds and the formation of dityrosine. The present results indicate that free radicals may play a role in the modification and iron releasing of ferritin by SAL. It is suggested that oxidative damage of ferritin by SAL might induce the increase of iron content in cells and subsequently led to the deleterious condition. This mechanism, in part, may provide an explanation for the deterioration of organs under neurodegenerative disorder such as PD.

**Key Words :** Parkinson disease, Salsolinol, Ferritin, Free radical

### Introduction

Although iron is required for oxidative metabolism, it can be toxic because of its ability to catalyze the generation of reactive oxygen species (ROS).<sup>1</sup> The toxicity of iron is a consequence of its ability to switch readily between two oxidation states, ferric ( $\text{Fe}^{3+}$ ) and ferrous ( $\text{Fe}^{2+}$ ), and can be largely explained by its participation in Fenton- and Haber-Weiss-type reactions.<sup>2</sup> In these reactions, a transition metal ion reduces  $\text{H}_2\text{O}_2$  to yield hydroxyl radicals which is one of the most potent oxidizing species in biological systems, and the oxidized metal ion. Hydroxyl radicals produced in iron-mediated processes can damage DNA, causing strand breakage, and chemical modification of the bases or sugar, in addition to lipid peroxidation and protein modification.<sup>3</sup>

Ferritin is a 450 kDa protein with 24 subunits forming a cavity that can store up to 4,500 atoms of ferric ion.<sup>4</sup> Ferritin assemblies comprise two functionally and genetically distinct subunit type: H (heavy) and L (light), which are present in varying ratios in different tissues. Subunits of type L contribute to the nucleation of the iron core, but lack the ferroxidase activity necessary for uptake of ferrous ( $\text{Fe}^{2+}$ ) iron. Subunits of type H possess ferroxidase activity and promote rapid uptake and oxidation of ferrous iron.<sup>5</sup>

In Parkinson disease (PD), the number of ferritin-immunoreactive microglial cells in the substantia nigra (SN) increases, with many reactive microglial cell as located in close proximity to melanin containing or degenerating neuron.<sup>6-8</sup> Ferritin cores in the SN of PD patients are reported to be denser and contain more iron than those in the SN of healthy subjects.<sup>9</sup> If iron can be released from ferritin by various exogenous and endogenous substances,<sup>10-13</sup> low molecular iron complexes may undergo redox reactions resulting in cytotoxic damage of proteins, DNA or lipids.<sup>14,15</sup>

A group of naturally occurring neurotoxic alkaloids have been detected in contain regions of mammalian brains.<sup>16</sup>

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) (SAL) found in urine of parkinsonian patients administered with L-DOPA.<sup>17</sup> In addition, increased levels of salsolinol have been detected in cerebrospinal fluid of parkinsonian patients.<sup>18</sup> Several studies indicated that SAL is toxic to dopaminergic neurons *in vitro* as well as *in vivo*. SAL is known to inhibit tyrosine hydroxylase and monoamine oxidase<sup>19</sup> as well as mitochondrial complex-I and complex-II enzyme activities.<sup>18,20,21</sup> However, the precise biochemical and molecular mechanisms underlying the oxidative stress-mediated neurotoxicity of SAL is still poorly understood. In the current study, we investigated structural modification of ferritins after exposure to SAL *in vitro*.

### Materials and Methods

**Materials.** The commercial equine spleen ferritin (Calbiochem, German) was performed by gel filtration chromatography by using Superose 6 FPLC column (Pharmacia, Sweden) for a further purification. Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydro-isoquinoline) (SAL), *N*-acetyl-L-cysteine, catalase, glutathione, 2,4-dinitrophenylhydrazine (DNPH) and deferoxamine (DFO) were purchased 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 to remove traces of transition metal ions.

**Protein modification.** Protein concentration was determined by the BCA method.<sup>22</sup> Modification of ferritin (1 mg/mL) was carried out by incubation with SAL in potassium phosphate buffer (pH 7.4) at 37 °C. After incubation of the reaction mixtures, the mixtures were then placed into Microcon filter (Amicon) and centrifuged at 13,000 rpm for 1 h to remove SAL. The mixture was then washed with Chelex 100 treated water and centrifuged for 1 h at same speed to further remove SAL. This was repeated four times.

The filtrate was dried by freeze dryer and dissolved with 10 mM potassium phosphate buffer (pH 7.4). The protection by free radical scavengers against SAL-mediated ferritin modification was performed by preincubation of the enzyme in the presence of free radical scavengers for 5 min at room temperature and the reaction of mixture with SAL for 6 h at 37 °C. The unreacted reagent was washed by using Microcon filter (Amicon).

**Analysis of ferritin modification.** The samples were treated with 7  $\mu$ L of 4 X concentrated sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20%  $\beta$ -mercaptoethanol, 0.01% bromophenolblue) and were boiled at 100 °C for 10 min before electrophoresis. Each sample was subjected to SDS-PAGE as described by Laemmli,<sup>23</sup> using a 18% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

**Determination of free iron ions concentration.** The concentration of iron ions released from oxidatively damaged ferritin was determined by using a bathophenanthroline sulfonate in the method described previously.<sup>24</sup> The reaction mixture contained ferritin (1 mg/mL), various concentrations of SAL and 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 mL. The reaction was initiated by addition of SAL and incubated for 6 h at 37 °C. After the incubation, the mixture were then placed into an Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetric reagent was added into the filtrate for analyzed by a uv/vis spectrophotometer (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).

**Determination of protein carbonyl compound.** The carbonyl content of proteins was determined by spectrophotometric assay as described elsewhere.<sup>25</sup> Both native and oxidized protein were incubated with 10 mM 2,4-DNPH in 2.5 M HCl at room temperature for 1 h. After incubation, 20% TCA(w/v) solution was added to the sample and the tubes were left in ice bucket for 10 min and centrifuged for 5 min in a tabletop centrifuge to collect the protein precipitates. The supernatants were discarded. Next another wash was performed using 10% TCA, and protein pellets were broken mechanically with the aid of pipette tip. Finally, the pellets were washed 3 times with ethanol-ethyl acetate (1:1) (v/v) to remove the free DNPH. The final precipitates were dissolved in 2 mL of 6 M guanidine hydrochloride solution and were left for 10 min at 37 °C with general vortex mixing. Carbonyl content was calculated from the absorbance (370 nm) using an absorption coefficient  $\epsilon$  of 22,000  $M^{-1} \text{ cm}^{-1}$ .

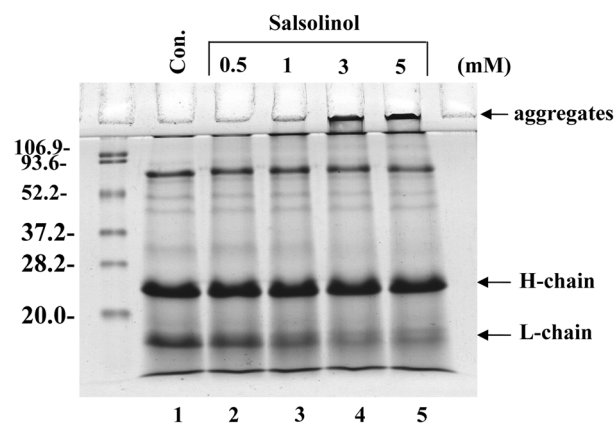
**Detection of *o,o'*-dityrosine.** The reactions for the detection of *o,o'*-dityrosine were carried with ferritin (1 mg/mL) and SAL in 10 mM potassium phosphate (pH 7.4). The fluorescence emission spectrum of the sample was then monitored in the 340-500 region (excitation, 325 nm) using Spectrofluorometer SMF 25 (Bio-Tek Instruments).

**Replicates.** Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

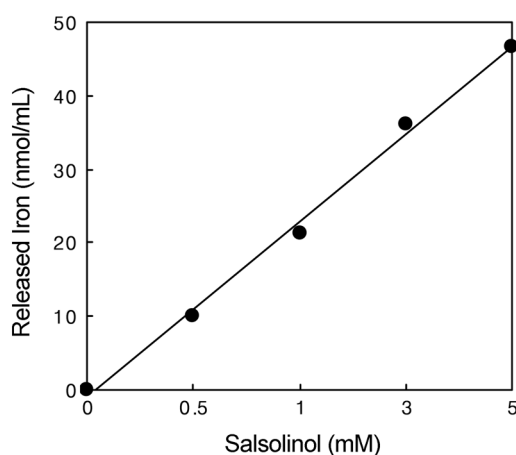
## Results

To determine if the SAL-mediated modification of ferritin is associated with either protein aggregation or fragmentation, the reaction mixtures were subjected to SDS-PAGE analysis. As shown in Figure 1, there was the SAL concentration-dependent increase in the formation of ferritin aggregates. SDS-PAGE showed a gradual decrease in the intensity of the band of ferritin L-chain in the SAL concentration-dependent manner, whereas H-chain remained intact after incubation with various concentrations of SAL. During incubation of ferritin with SAL, the release of iron from ferritin was gradually increased as a concentration of SAL (Fig. 2). The results suggested that the modification of ferritin L-chain by SAL was associated with the release of iron from the protein.

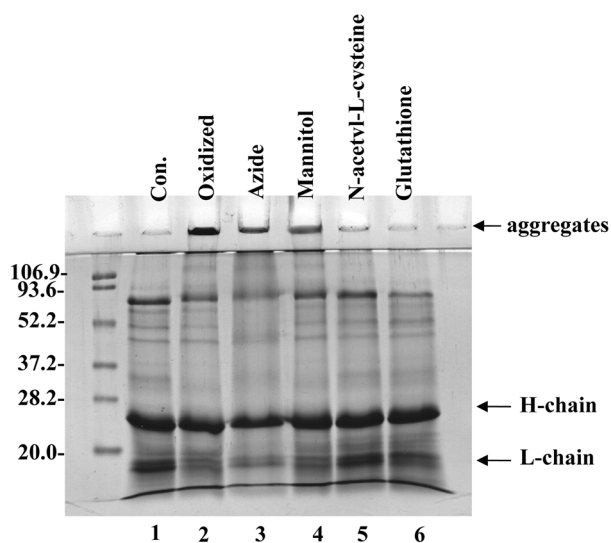
The participation of free radicals in the modification of ferritin by SAL was studied examining the inhibition of free



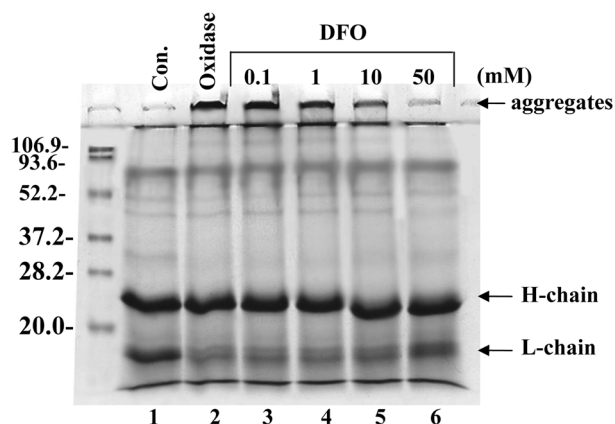
**Figure 1.** Modification of ferritin by SAL. Ferritin (1 mg/mL) was incubated in 10 mM phosphate buffer (pH 7.4) at 37 °C for 6 h under various conditions. Lane 1, ferritin control; lane 2-5, with 0.5, 1, 3 and 5 mM SAL. The positions of molecular weight markers (kDa) are indicated on the left.



**Figure 2.** Release of iron from ferritin by SAL. After the reactions of ferritin with 0.5, 1, 3 and 5 mM SAL, the colorimetric reagent was added to the reaction mixtures. The iron contents were measured by a UV/vis spectrophotometer at 535 nm. Data represent the means  $\pm$  S.D. ( $n = 3-5$ ).



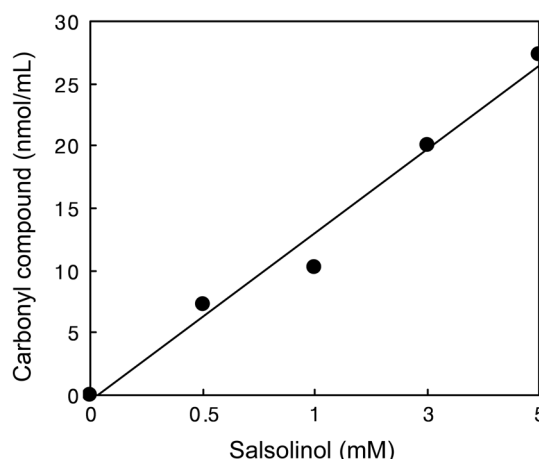
**Figure 3.** Effect of free radical scavengers on the modification of ferritin by SAL. Ferritin (1 mg/mL) was incubated with 5 mM SAL in 10 mM phosphate buffer (pH 7.4) at 37 °C for 6 h in the presence of free radical scavengers. Lane 1, ferritin control; lane 2, oxidized ferritin (without free radical scavenger); lane 3, 200 mM azide; lane 4, 200 mM mannitol; lane 5, 20 mM *N*-acetyl-L-cysteine; lane 6, 20 mM glutathione.



**Figure 4.** Effect of iron chelator on the modification of ferritin by SAL. Ferritin (1 mg/mL) was incubated with 5 mM SAL in 10 mM phosphate buffer (pH 7.4) at 37 °C for 6 h in the presence of iron chelator. Lane 1, ferritin control; lane 2, oxidized ferritin (without chelator); lane 3-5, 0.1, 1, 10 and 50 mM DFO.

radical scavengers during the reaction of ferritin with SAL. The modification of ferritin was slightly suppressed in the presence of azide and mannitol, whereas the protein modification was effectively inhibited by *N*-acetyl-L-cysteine and glutathione (Fig. 3). These results suggest that free radicals may be involved in the modification of ferritin and the release of iron from the protein. The released irons may amplify free radicals *via* transitional metal-catalyzed reaction (Fenton reaction). Evidence that iron chelator, DFO, protected the protein against SAL-mediated ferritin modification supported this mechanism (Fig. 4).

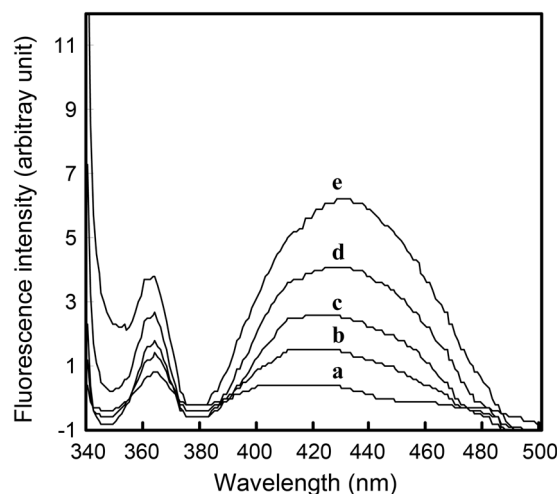
With the discovery that protein oxidation leads to the conversion of some amino acid residues to carbonyl deriva-



**Figure 5.** Determination of carbonyl compounds after the incubation of ferritin with SAL. Ferritin (1 mg/mL) was incubated with various concentration of SAL in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C for 6 h. Reaction mixtures were derivatized DNPH as described under Materials and Methods.

tives,<sup>26</sup> the presence of protein carbonyls has been widely used as a measure of oxidative damage.<sup>27</sup> The carbonyl content of protein can be measured using a phenylhydrazine formation reaction. The method for detecting carbonyl-containing proteins employs derivatization with 2,4-DNPH followed by analysis with spectrophotometer.<sup>25</sup> Result obtained from the spectrophotometric analysis of SAL-treated ferritin was shown in Figure 5. The generation of carbonyl compound was increased in a SAL-concentration dependent manner.

It has been reported that *o,o'*-dityrosine crosslink formation between two tyrosine residues might play a part in the formation of oxidative covalent protein crosslink.<sup>28</sup> We have investigated the formation of *o,o'*-dityrosine during the SAL-mediated ferritin aggregation by measuring fluores-



**Figure 6.** Fluorescence spectra of SAL-mediated ferritin modification. The fluorescence spectra of the formation of dityrosine was observed when ferritin (1 mg/mL) was incubated with SAL. (a) ferritin control (b) ferritin + 0.5 mM SAL (c) ferritin + 1 mM SAL (d) ferritin + 3 mM SAL (e) ferritin + 5 mM SAL.

science emission spectrum between 340 and 500 nm with an excitation at 325 nm. The reactions were carried out with ferritin in various concentrations of SAL for 6 h at 37 °C. As the concentration of SAL increased, the formation of *o,o'*-dityrosine crosslink was increased (Fig. 6). Oxidative protein crosslink is produced through several means such as direct interactions between carbon-centered radical derivatives of amino acids. Our result suggested that the tyrosine-tyrosine crosslink formation might participate in the SAL-mediated ferritin aggregation.

### Discussion

Alterations in the homeostasis/metabolism of iron may involve in the pathogenesis of neurodegenerative disease. Iron released from ferritin may trigger oxidative stress leading to progressive neurodegeneration of substantia nigra resulting in PD.<sup>29</sup> It has been reported that SAL induced DNA strand breaks in PC12 cells and neurons in the presence of copper or iron.<sup>30,31</sup> The present study investigated the potential role of neurotoxin, SAL, in the modification of ferritin. Our result showed that the aggregation of ferritin was induced by SAL and free radical scavengers inhibited the SAL-mediated ferritin modification. The toxicity of SAL may be augmented by its ROS-generating function in neurodegenerative disorder. Recent report has been revealed that SAL increased the production of ROS and significantly decreased glutathione levels in SH-SY5Y cells.<sup>32</sup> Thus, it was suggested that free radicals might be involved in the modification of ferritin by SAL.

Trace metal such as iron and copper, which are variously present in biological systems, were generated hydroxyl radicals through Fenton reaction and then led to damages of macromolecules.<sup>33-36</sup> The cleavage of the metalloproteins by oxidative damage may lead to increases in the levels of metal ions in some biological cells.<sup>37</sup> It has been reported that iron concentration was significantly increased in parkinsonian brain.<sup>38,39</sup> These reports suggested that iron catalyzed oxidative reaction might contribute to the pathogenesis of PD. The results of the present *in vitro* study demonstrate that SAL cause release of iron from ferritin and suggest that it may occur *in vivo*. Since DFO is a strong iron chelator, it may be possible that some small amounts of loosely bound ferritin-iron are easily released by SAL. These results may explain at least one of the reasons underlying selective vulnerability of parkinsonian brain to iron mediated oxidative damage.

All cellular components are vulnerable to oxidative stress. The damage that oxidative stress causes to proteins includes side chain modification and main chain fragmentation. Two biological markers for oxidative damage in proteins are the accumulation of carbonyl compounds and the accumulation of dityrosine.<sup>40</sup> In the present study, SAL led to the carbonyl formation and dityrosine in ferritin. Metal catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl groups into the amino acid residues of proteins.<sup>28</sup> The reaction of ferritin with SAL generates a free

radical that oxidizes amino acid residues at or near that cation-binding site, which then introduces carbonyl groups. Dityrosine is also a useful marker for protein oxidation<sup>41-43</sup> and is found in amino acid hydrolysates of brain tissue affected by neurodegenerative disorder.<sup>44</sup> Such oxidative modifications are the indicator of oxidative stress and may be significant in several physiological and pathological processes.<sup>45,46</sup>

In conclusion, the present results suggested that the modification of ferritin and iron release from the protein were induced by SAL, involving free radical generation. Iron released from ferritin may lead to progressive neurodegeneration of brain through oxidative stress. Therefore, the SAL-mediated ferritin modification might be associated with the pathogenesis of PD and related disorders.

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