

## The Reaction of Salsolinol with Ferritin Induces DNA Strand Breakage

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Iron released from ferritin may trigger oxidative stress leading to progressive degeneration of brains from patients with neurodegenerative diseases. Previous studies have shown that oxidative damage of proteins and DNA was induced by catechol neurotoxin such as salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline). In the present study, we have investigated oxidative damage of DNA induced by the reaction of salsolinol with ferritin. When DNA was incubated with ferritin and salsolinol, DNA strand breakage increased in a time-dependent manner. Hydroxyl radical scavengers, such as azide, mannitol and dimethyl sulfoxide, effectively inhibited the salsolinol/ferritin system-mediated DNA cleavage, whereas Cu,Zn-superoxide dismutase did not suppress DNA cleavage. Catalase significantly inhibited the salsolinol/ferritin system-mediated DNA cleavage. Iron specific chelator, deferoxamine, also inhibited DNA cleavage. Spectrophotometric study using a color reagent showed that the release of iron from salsolinol-treated ferritin was increased in a time dependent manner. These results suggest that DNA strand breakage is mediated in the reaction of salsolinol with ferritin *via* the generation of hydroxyl radicals by the Fenton-like reaction of free iron ions released from oxidatively damaged ferritin.

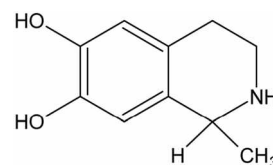
**Key Words :** Salsolinol, Ferritin, DNA, Hydroxyl radical

### Introduction

Ferritin plays an essential role in iron detoxification and acts as a large reservoir for iron in bioavailable form. The protein has a shell-like structure composed 24 similar and identical subunits encapsulating a hydrous ferric oxide mineral core, the storage form of iron.<sup>1-4</sup> As many as 4500 irons can be accommodated within a ferritin assembly.<sup>5</sup> The ferritin monomer exists in several isoforms, which differ in their amino-acid sequences and molecular sizes. Two forms have been identified in human tissues, heavy chain (H-chain; ~21 kDa) and light chain (L-chain; ~19 kDa), with ratios ranging from 2:22 to 20:4.<sup>6</sup> H-rich ferritins catalyze the oxidation of iron, while L-rich ferritins promote the nucleation and storage of iron.

Iron is the most abundant heavy metal in the human body, especially in the brain. Iron is necessary for normal neuronal function, as it is an essential cofactor for many enzymes. However, iron progressively accumulates with aging in some regions of the brain up to high levels associated with oxidative stress, which can promote neurodegeneration.<sup>7,8</sup> Previous studies have demonstrated that iron released from ferritin and its associated toxicity is a risk factor for age at onset of neurodegenerative diseases such as Parkinson disease (PD) and Alzheimer disease (AD).<sup>9,10</sup>

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) (Fig. 1), a dopamine-derived endogenously synthesized compound, revealed its involvement in the progression of disease characterized by dysfunction of dopaminergic neurons, as in the case of PD.<sup>11</sup> Salsolinol increased the production of reactive oxygen species (ROS) and significantly decreased glutathione levels in SH-SY5Y cells.<sup>12,13</sup> ROS may play an important role in several pathological



Salsolinol

**Figure 1.** The chemical structure of salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline).

conditions of the central nervous system, where the directly injured tissue, and where their formation may also be a consequence of tissue injury. ROS produces tissue damage through multiple mechanisms and can worsen acute neurodegenerative disorders including PD. It has been reported that salsolinol in conjunction with ferric ion undergoes redox cycling to produce ROS such as hydroxyl radicals that cause DNA strand scission and cell death.<sup>14</sup> Recently, we have shown that salsolinol led to the modification of ferritin.<sup>15</sup> In this context, we hypothesize that ferritin could be a source of iron and oxidative stress that might trigger the salsolinol-mediated DNA damage. Although cytotoxicities of salsolinol have been extensively investigated, DNA damage induced by the reaction of salsolinol with ferritin has not been reported.

In this study, we examined the DNA cleavage caused by salsolinol and ferritin. Our results indicate that salsolinol in the presence of ferritin can cleave DNA through a mechanism that involves hydroxyl radical.

### Materials and Methods

**Materials.** Salsolinol, catalase, sodium azide, dimethyl

sulfoxide (DMSO), deferoxamine (DFO), and ethidium bromide were purchased from Sigma. Ferritin and Cu,Zn-superoxide dismutase (SOD) were purchased from Calbiochem. The commercial equine spleen ferritin was subjected to gel filtration chromatography using Superose 6 FPLC column (Pharmacia, Sweden) for further purification. pUC19 plasmid DNA was prepared and purified from *E. coli* cultures by using QIAGEN plasmid kit (Santa Clarita, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad. All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

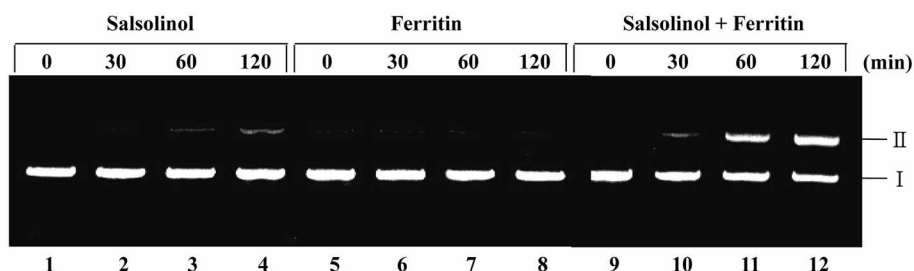
**Analysis of DNA strand breakage.** Supercoiled plasmid pUC19 DNA (0.5-1.0  $\mu\text{g}$ ) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 2 h at 37 °C with different concentrations of salsolinol and ferritin in a total volume of 20  $\mu\text{L}$ . The reaction was stopped at -80 °C. The loading buffer (0.25% bromophenolblue, 40% sucrose) was added and samples were analyzed by electrophoresis in 0.8% agarose in TBE buffer (2 mM EDTA, 89 mM boric acid and 89 mM Tris at pH 8.3). The gel was stained with ethidium bromide. Bands of DNA were detected and photographed under UV light in a dark room.

**Determination of free iron concentration.** The concentration of iron ions released from oxidatively damaged ferritin was determined by using a bathophenanthroline sulfonate in the method described previously.<sup>16</sup> The reaction mixture contained ferritin (1 mg/mL) and salsolinol and 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 mL. The reaction was incubated for various periods at 37 °C. After incubation, the mixture was 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 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).

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

## Results and Discussion

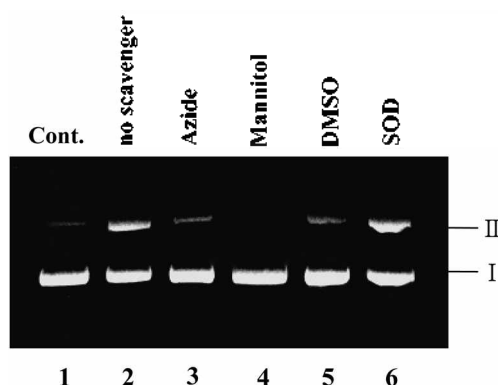
Untreated DNA showed a major band corresponding to



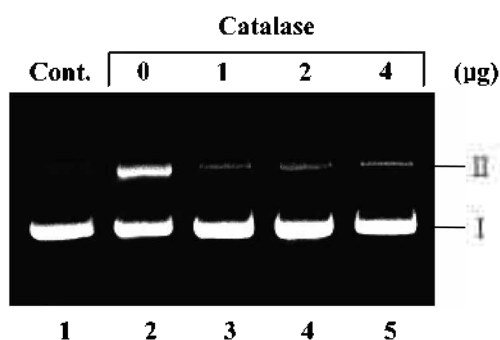
**Figure 2.** DNA cleavage after incubation with salsolinol and ferritin. pUC 19 DNA (1  $\mu\text{g}$ ) was incubated with salsolinol and ferritin in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C during various incubation periods. The reaction mixture contained 50  $\mu\text{M}$  salsolinol alone (lane 2-4), 0.1  $\mu\text{M}$  ferritin alone (lane 6-8), 50  $\mu\text{M}$  salsolinol and 0.1  $\mu\text{M}$  ferritin (lane 10-12). Reaction was stopped by -80 °C deep freezing. Loading buffer was added to the samples and analyzed by electrophoresis in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.

the supercoiled form (form I) and a minor band corresponding to nicked circular form (form II) (Fig. 2, lane 1, 5 and 9). Plasmid DNA remained intact after incubation with 50  $\mu\text{M}$  salsolinol (Fig. 2, lane 1-4) or 0.1  $\mu\text{M}$  ferritin (Fig. 2, lane 5-8) alone. However, when DNA was incubated in a mixture of salsolinol and ferritin, the DNA damage occurred during the initial 30 min of incubation and increased up to 120 min (Fig. 2, lane 9-12). This indicates that both ferritin and salsolinol were required for the production of strand breaks in DNA. When plasmid DNA was incubated with salsolinol alone for 60 min, DNA was slightly cleaved. Salsolinol is participated to cause oxidative stress in cells by generating ROS during autooxidation at the catechol moiety. It has been reported that salsolinol generates ROS through redox cycling that can be facilitated by the transition metal ion, such as iron.<sup>14</sup> The present result suggested that ferritin might facilitate the salsolinol-induced DNA damage.

The participation of free radicals in the DNA damage by the salsolinol/ferritin system was studied by examining the protective effect of radical scavengers. When plasmid DNA was incubated with salsolinol and ferritin in the presence of azide, mannitol and DMSO at 37 °C for 2 h, all scavengers



**Figure 3.** Effect of radical scavengers on the DNA cleavage by the reaction of salsolinol with ferritin. pUC 19 DNA was incubated with 50  $\mu\text{M}$  salsolinol and 0.1  $\mu\text{M}$  ferritin in potassium phosphate buffer (pH 7.4) at 37 °C for 2 h, in the absence and the presence of 200 mM scavengers or SOD (1 mg/mL) at 37 °C. Lane 1, plasmid DNA alone; lane 2, no addition of scavenger; lane 3, azide; lane 4, mannitol; lane 5, DMSO; lane 6, SOD. Agarose gel electrophoresis was performed in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.

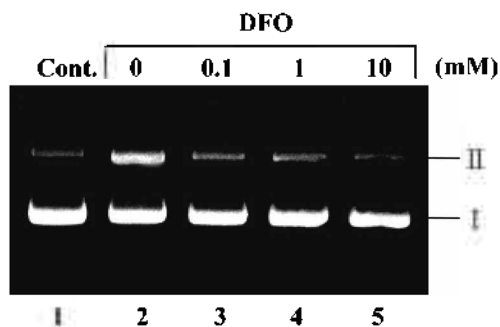


**Figure 4.** Effect of catalase on the DNA cleavage by the reaction of salsolinol with ferritin. pUC 19 DNA was incubated with salsolinol and ferritin in potassium phosphate buffer (pH 7.4) at 37 °C for 2 h in various concentrations of catalase. Lane 1, plasmid DNA alone; lane 2, no addition of catalase; lane 3, 1 µg catalase; lane 4, 2 µg catalase; lane 5, 4 µg catalase. Agarose gel electrophoresis was performed in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.

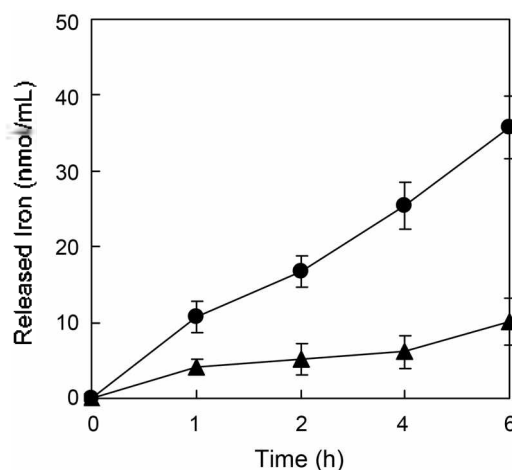
significantly prevented DNA cleavage (Fig. 3, lane 3-5). The sugar alcohol mannitol, azide and DMSO are often used hydroxyl radical scavengers in laboratory experiments, but these scavengers can react with other species of oxygen radicals.<sup>17</sup> In the present study, Cu,Zn-SOD did not decrease DNA strand breakage (Fig. 3, lane 6), while catalase effectively inhibited the salsolinol/ferritin-mediated DNA cleavage (Fig. 4). The result indicates that the hydroxyl radical and hydrogen peroxide participate in the mechanism of strand break produced by salsolinol and ferritin.

Trace metals such as copper and iron which are present in biological systems may interact with hydrogen peroxide, to damage biological molecules including DNA.<sup>18-23</sup> In cultured cells, raising the level of iron in the culture medium leads to increases in steady-state levels of oxidative DNA damage.<sup>24</sup> Previous studies have suggested that iron ions could stimulate the Fenton-like reaction to produce hydroxyl radicals, which mediates DNA strand breakage.<sup>25,26</sup> We have investigated the participation of iron ions in the salsolinol/ferritin-mediated DNA strand breaks. When DNA reacted with salsolinol and ferritin in the presence of iron specific chelator, DFO, DNA strand breakage was significantly inhibited (Fig. 5). The results indicated that iron ions were involved in the DNA cleavage by the salsolinol/ferritin system

H-ferritin subunits have an active ferroxidase site and occur in multiple forms, in humans, animals, plants and bacteria. L-ferritin subunits have a degenerate ferroxidase site and the gene duplication to encode L-ferritin subunits is found only in vertebrate animals. It was shown that the co-presence of both subunits in hybrids make the molecule more efficient for iron incorporation, because of the combined action of the ferroxidase centers on H-chains and of the effective nucleation centers on L-chains.<sup>27</sup> X-ray crystallographic data of the mouse L-chain showed that a metal-binding site on the cavity surface, involving residues Glu57, Glu60, Glu61, and Glu64, and in the hydrophilic 3-fold channel involving His118, Glu134, Asp131 and His136.<sup>28</sup> In



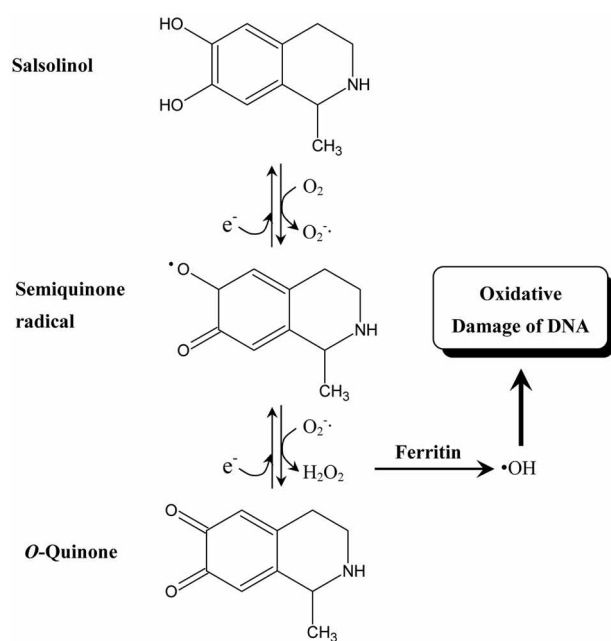
**Figure 5.** Effect of iron chelator on the DNA cleavage by the reaction of salsolinol with ferritin. pUC 19 DNA was incubated with salsolinol and ferritin in potassium phosphate buffer (pH 7.4) at 37 °C for 2 h in various concentrations of DFO. Lane 1, plasmid DNA alone; lane 2, no addition of DFO; lane 3, 0.1 mM DFO; lane 3, 1 mM DFO; lane 4, 10 mM DFO. Agarose gel electrophoresis was performed in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.



**Figure 6.** Iron release in the salsolinol and ferritin system. 2 µM Ferritin was incubated with (●) or without (▲) 1 mM salsolinol in 10 mM phosphate buffer at pH 7.4. After incubation, the mixture was then placed into Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetric reagent was added into the filtrate for 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). Data represent the means ± S.D. ( $n = 4-5$ ).

the present study, the incubation of ferritin with salsolinol led to the release of iron from the protein (Fig. 6). Thus, it is suggested that iron binding sites were modified during the reaction of ferritin with salsolinol. Consequently, iron became almost free from the ligand and was released from the oxidatively-damaged protein.

It has been reported that the induction of DNA damage by salsolinol and iron ion might be due to the generations of hydrogen peroxide and hydroxyl radicals.<sup>14</sup> Interestingly, ferritin iron is increased in discrete brain regions in different neurodegenerative disorders; in PD, it is characteristically increased in the substantia nigra.<sup>29-32</sup> Therefore, the present results suggested that early oxidation of salsolinol is partici-



**Figure 7.** Proposed scheme for the salsolinol and ferritin system that lead to oxidative damage of DNA.

participated in the releasing of iron ions from ferritin and the generation of hydrogen peroxide. The subsequent interaction of free iron ions and hydrogen peroxide in the Fenton-like reaction will yield hydroxyl radicals. Our evidence that catalase and iron chelator prevented the DNA cleavage induced by the salsolinol/ferritin system supports this mechanism.

In conclusion, the present results indicated that DNA cleavage was induced by the reaction of salsolinol/ferritin involving hydroxyl radicals that were generated by the Fenton-like reaction (Fig. 7). Our study suggested that DNA damage by the salsolinol/ferritin system could be relatively favored in diseases where ferritin concentration is elevated such as PD and other neurodegenerative disorders.

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