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Acrolein, A Reactive Product of Lipid Peroxidation, Induces Oxidative Modification of Cytochrome *c*

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Acrolein (ACR) is a well-known carbonyl toxin produced by lipid peroxidation of polyunsaturated fatty acids, which is involved in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD). In Alzheimer's brain, ACR was found to be elevated in hippocampus and temporal cortex where oxidative stress is high. In this study, we evaluated oxidative modification of cytochrome *c* occurring after incubation with ACR. When cytochrome *c* was incubated with ACR, protein aggregation increased in a dose-dependent manner. The formation of carbonyl compounds and the release of iron were obtained in ACR-treated cytochrome *c*. Reactive oxygen species scavengers and iron specific chelator inhibited the ACR-mediated cytochrome *c* modification and carbonyl compound formation. Our data demonstrate that oxidative damage of cytochrome *c* by ACR might induce disruption of cyotochrome *c* structure and iron mishandling as a contributing factor to the pathology of AD.

Key Words : Acrolein, Cytochrome *c*, Reactive oxygen species, Iron

Introduction

Acrolein (ACR) occurs in the environment as a ubiquitous pollutant that is generated as a by-product of overheated organic materials. ACR formed *in vivo*, through iron-catalyzed oxidation of arachidonic and docosahexanoic acids, exhibits facile reactivity with various biological macromolecules, including proteins and phospholipids, has the potential to inhibit many enzymes, and quickly depletes cellular glutathione levels.[1] ACR reacts preferentially with cysteine, lysine and histidine residues *via* Michael-type addition reactions preserving the aldehyde functionally on the modified protein.[2] Previous studies have shown that ACR led to the modification and inactivation of several enzymes.[2-4] ACR-modified proteins can be seen immunochemically in several diseases,[5-7] suggesting that may be useful diagnostic markers.

Iron is an essential ion for life, playing a central role in many metabolic processes.[8] The most important property of free iron is its capacity to be reversibly oxidized and reduced, but at the same time this makes it a highly prooxidant molecule. In this regard, iron is able to generate powerful reactive oxygen species (ROS).[9,10] 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.[11,12] 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 Parkinson's disease (PD) and Alzheimer's disease (AD).[13,14]

Cytochrome *c* is a well-known globular heme protein, which transfers electron from cytochrome *c* reductase to cytochrome *c* oxidase in the respiratory chain in mitochondria.[15,16] It also plays a key role in apoptosis, where it is released to the cytosol when permeabilization of the mitochondrial outer membrane occurs.[17,18] When cytochrome *c* was heated at 75 °C for 12 h, it forms amyloid fibrils that are observed in other proteins including those related to neurodegenerative diseases.[19-21] Due to this connection to neurodegenerative diseases, the mechanism of the protein structural change has been studied intensively,[22,23] and oligomeric proteins have gained interest as initial intermediates.[24,25]

Previous studies have been reported that cytochrome *c* participates in two other biological processes, apoptosis and oxidative stress.[26,27] Apoptosis, also referred to as programmed cell death, is a key to development and is linked to human diseases, such as cancer and neurodegeneration.[28,29] Mitochondria are critical for meeting the high energy demands of the brain, but they also generate the majority of intracellular ROS, which can cause oxidative damage to important cellular structures. Several studies have found that increases in ROS and oxidative stress are involved in AD.[30,31]

In the current study, the effects of ACR on the oxidative modification of cytochrome *c* were assessed. The results demonstrated that ACR induced cytochrome *c* aggregation *via* ROS generation. ACR-mediated cytochrome *c* modification, in turn, resulted in the release of iron from the protein.

Materials and Methods

Materials. Horse cytochrome *c*, ACR, 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). Precast gels of 4-20% Tris/SDS-PAGE were purchased from KOMABIOTECH

(Korea). 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.[32] The modification of cytochrome *c* was induced *via* incubation with ACR 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 (cut-off: 3,000 dalton) (Sartorius Stedim Biotech, Goettingen, Gerrman) and were centrifuged for 1 h at 10,000 × g to remove the ACR. The mixture was then washed with Chelex 100-treated water and centrifuged for 1 h at the same speed to remove any remaining ACR. This procedure was repeated four times, after which the washed proteins were dried in a freeze-dryer and dissolved with 10 mM potassium phosphate buffer (pH 7.4). ROS scavenger-induced protection against ACR-mediated cytochrome *c* modification was induced by the 5 min of preincubation of the protein with ROS scavengers at room temperature, after which the mixture was reacted with ACR for 24 h at 37 °C. The unreacted reagent was then washed through a Vivaspin ultrafiltration spin column (Sartorius Stedim Biotech, Goettingen, Gerrman).

Analysis of Cytochrome *c* Modification. 15 μL of samples were treated with 5 μL of 4 sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenol blue) and were then boiled for 10 min at 100 °C prior to electrophoresis. Each sample were analyzed with a precast 4-20% Tris/SDS-PAGE (KOMABIOTECH) according to the procedure provided by the manufacturer. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Detection of Carbonyl Compounds. The carbonyl contents of the proteins were determined *via* spectrophotometric assays, as described elsewhere.[33] Both native and oxidized proteins were incubated with 10 mM 2,4-dinitrophenyl hydrazine (DNPH) in 2.5 M HCl for 1 h at room temperature. After incubation, 20% TCA was added to the samples, and the tubes were left in an ice bucket for 10 min and then centrifuged for 5 min with a tabletop centrifuge to collect the protein precipitates, after which the supernatants were discarded. Another wash was performed using 10% TCA, and the protein pellets were mechanically broken using a pipette tip. Finally, the pellets were washed 3 times with ethanol-ethyl acetate (1:1) (v/v) to remove any free DNPH. The final precipitates were dissolved in 2 mL of 6 M guanidine hydrochloride solution and left for 10 min at 37 °C with general vortex mixing. Carbonyl contents were calculated from the absorbance (370 nm) with an absorption coefficient ε of 22,000 M^{-1} cm^{-1}.

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*, various concentrations of ACR in a total volume of 0.5 ml. The reaction was initiated by the addition of ACR and was incubated for 24 h at 37 °C. After incubation, the mixtures were placed into a Vivaspin ultrafiltration spin column and were then centrifuged at 10,000 × g for 1 h. The colorimetric reagent was added to the filtrate prior to analysis by UV/VIS spectrophotometer (Shimadzu, UV-1601) at 535 nm. The final concentrations of the color reagents 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.

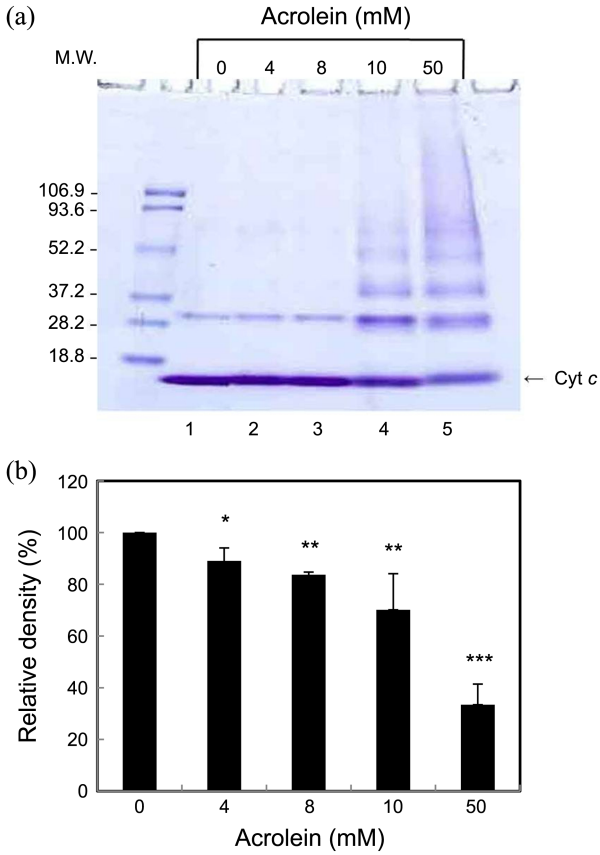

Figure 1. Oxidative modification of cytochrome *c* after incubation with ACR. 20 μM cytochrome *c* was incubated with various concentrations of ACR in 10 mM phosphate buffer (pH 7.4) at 37 °C for 24 h. Reactions were stopped by freezing at −80 °C. (a) The pattern of protein bands was analyzed *via* SDS-PAGE. The positions of the molecular weight markers (kDa) are indicated on the left. (b) Relative staining intensity of SDS-PAGE gel was analyzed by densitometric scanning. Asterisk (*), double asterisks (**) and triple asterisks (***) denote statistical significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Results and Discussion

ACR is a highly reactive α,β-unsaturated aldehyde, and humans are exposed to this compound in multiple situation.[36] We first investigated whether the structure of cytochrome *c* was affected by ACR. When cytochrome *c* was incubated with ACR at 37 °C, the frequency of protein aggregation increased (Fig. 1(a)) and the intensity of the original band decreased (Fig. 1(b)) in a dose dependent manner. The participation of ROS in the modification of cytochrome *c* by ACR was studied by examining the inhibition of ROS. The modification of cytochrome *c* was shown to be effectively suppressed in the presence of azide, formate, mannitol, *N*-acetyl-L-cysteine and glutathione (Fig. 2(a) and (b)). In the present experiments, very much higher concentrations of ACR were employed (10-50 mM). Whilst such values might be physiological questionable, they were necessary to demonstrate clear protein aggregation that were used to provoke protein damage in a relatively short period of time.

Metal catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl groups into the amino acid residues of proteins.[37] All cellular components are vulnerable to oxidative stress. Carbonyl group production was shown to be a useful biomarker for protein modification by ROS.[38] Such an oxidative modification is an indicator of oxidative stress and may be significant in several physiological and pathological processes.[39,40] We determined the levels of carbonyl group in ACR-mediated cytochrome *c* aggregates. The reactions were carried out with cytochrome *c* and various concentration of ACR for 24 h at 37 °C. As the concentration of ACR was increased, the formation of carbonyl group was increased (Fig. 3(a)). We investigated the effects of ROS scavengers on the formation of carbonyl compounds. Azide, formate, mannitol, *N*-acetyl-L-cysteine and glutathione inhibited the formation of carbonyl group

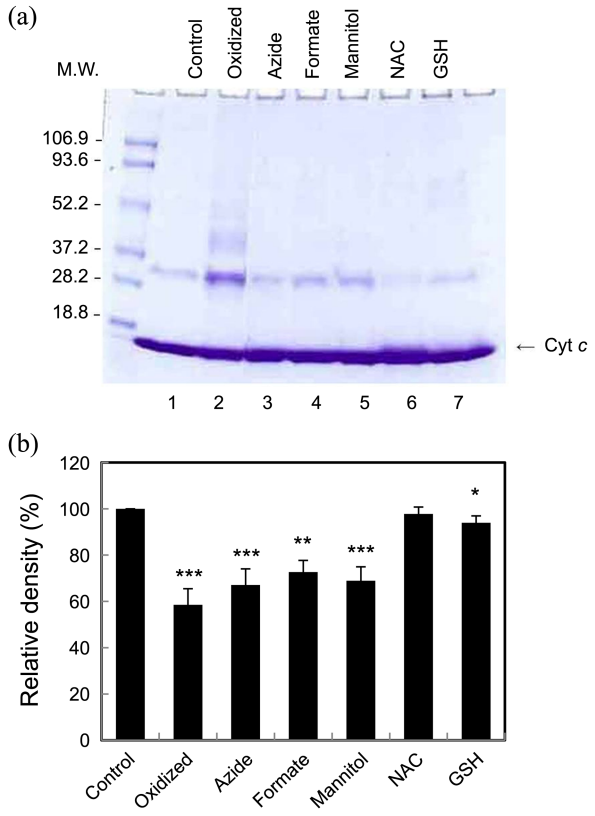

Figure 2. The effect of ROS scavengers on ACR-mediated cytochrome *c* aggregation. 20 μM cytochrome *c* was incubated with 10 mM ACR in 10 mM phosphate buffer (pH 7.4) at 37 °C for 24 h in the presence of ROS scavengers. (a) The pattern of protein bands was analyzed *via* SDS-PAGE. Lane 1, cytochrome *c* control; lane 2, oxidized cytochrome *c* (without ROS scavenger); lane 3, 100 mM azide; lane 4, 100 mM formate; lane 5, 100 mM mannitol; lane 6, 20 mM *N*-acetyl cysteine (NAC); lane 7, 20 mM glutathione (GSH). (b) Relative staining intensity of SDS-PAGE gel was analyzed by densitometric scanning. The positions of the molecular weight markers (kDa) are indicated on the left. Asterisk (*), double asterisks (**) and triple asterisks (***) denote statistical significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

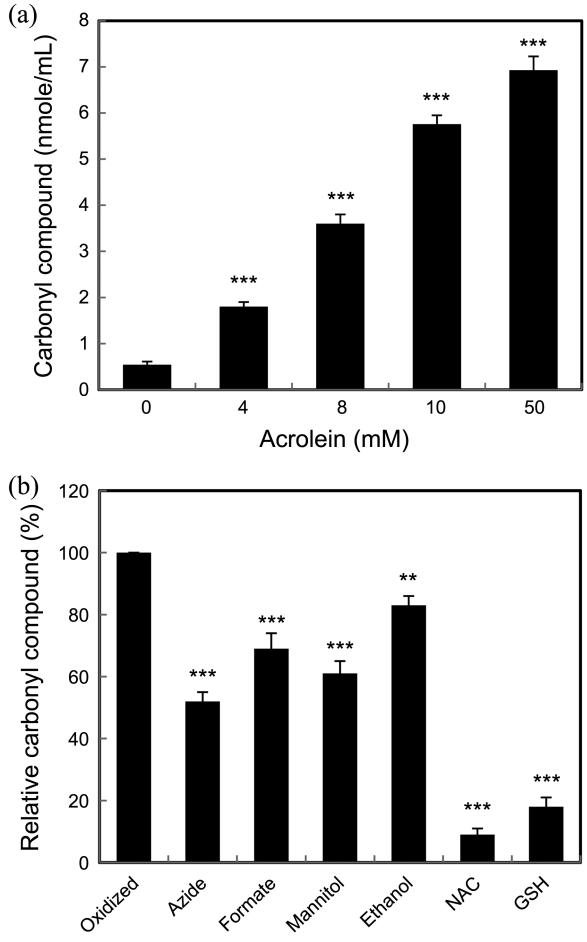

Figure 3. Formation of carbonyl group during ACR-mediated cytochrome *c* modification and the effects of ROS scavengers on carbonyl group formation. (a) 20 μM cytochrome *c* was incubated with various concentrations of ACR in 10 mM phosphate buffer (pH 7.4) at 37 °C for 24 h. (b) 20 μM cytochrome *c* was incubated with 10 mM ACR without or with ROS scavengers. Reaction mixtures were reacted with 2,4-DNPH and then the derivatives were measured using spectrophotometry. Double asterisks (**) and triple asterisks (***) denote statistical significance at $p < 0.01$ and $p < 0.001$ respectively.

(Fig. 3(b)). The results suggest that ROS might play a critical role in the mechanism of ACR-mediated cytochrome *c* modification.

Trace metals such as iron and copper, which are present in a variety of biological systems, may interact with ROS, ionizing radiation or microwave radiation, to damage macromolecules.[41-43] In some biological cells, the cleavage of metalloproteins by oxidative damage may lead to increases in the levels of metal ions.[44] The participation of iron in the ACR-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 ACR in the presence of DFX, the oligomerization of cytochrome *c* (Fig. 4(a)) and carbonyl group formation (Fig. 4(b)) were inhibited in a DFX concentration-dependent manner. The reaction of cytochrome *c* with ACR resulted in a dose-dependent increase in the release of free iron ions (Fig. 5). Through Fenton-like reactions, hydroxyl radicals can be formed from H_2O_2 in the presence of a transition metal ion, such as iron or copper. Therefore, iron ions released from acrolein-damaged cytochrome *c* may lead

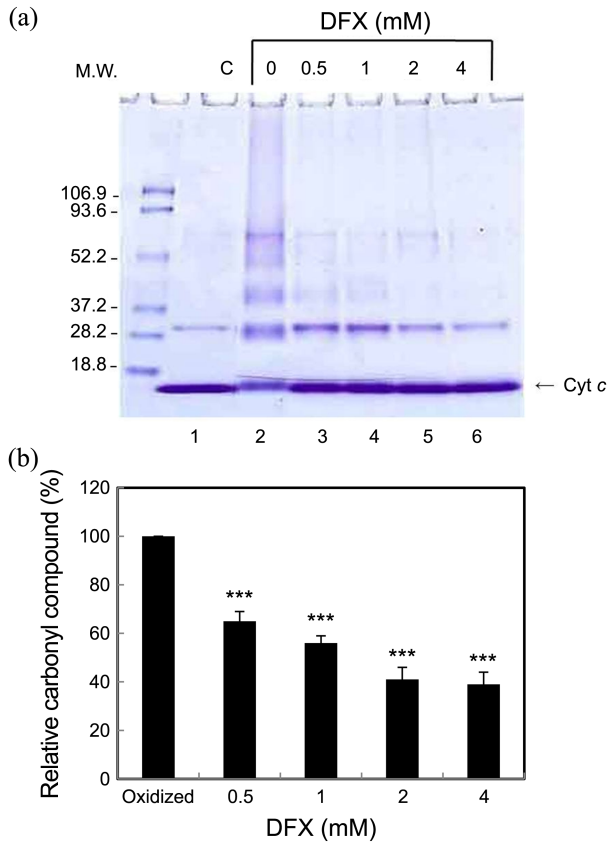

Figure 4. Effects of iron chelator on ACR-mediated cytochrome *c* aggregation and carbonyl group formation. 20 μM cytochrome *c* was incubated with 50 mM ACR in 10 mM phosphate buffer (pH 7.4) at 37 °C for 24 h in the presence of DFX. (a) The pattern of protein bands was analyzed *via* SDS-PAGE. Lane 1, cytochrome *c* control; lane 2, oxidized cytochrome *c* (without DFX); lane 3, 0.5 mM DFX; lane 4, 1 mM DFX; lane 5, 2 mM DFX; lane 6, 4 mM DFX. (b) The formation of carbonyl group was determined by spectrophotometry. Triple asterisk (***) denotes statistical significance at $p < 0.001$.

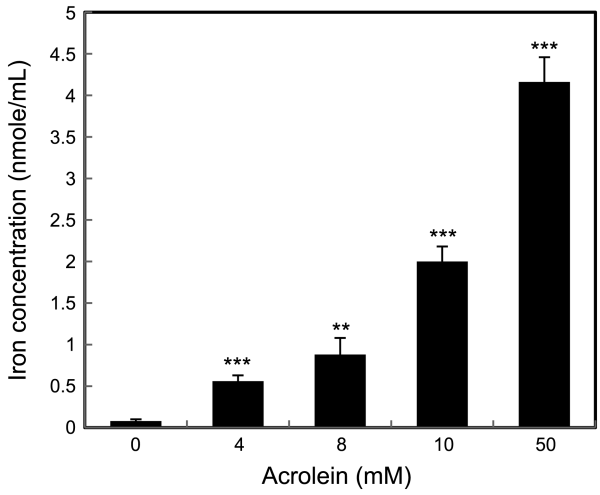

Figure 5. The release of iron from cytochrome *c* during the reaction between cytochrome *c* and ACR. The reaction mixture contained 20 μM cytochrome *c* and various concentrations of ACR in 10 mM phosphate buffer at pH 7.4 for 24 h. The free iron ions were determined with a colorimetric reagent using bathophenanthroline sulfonate. Double asterisks (**) and triple asterisks (***) denote statistical significance at $p < 0.01$ and $p < 0.001$ respectively.

to a pro-oxidant condition in cells.

In order to determine the target site against the ACR on the protein, cytochrome *c* which had been reacted with ACR was examined by amino acid analysis following acid and alkaline hydrolysis of the modified proteins. Cytochrome *c* exposure to ACR produced particularly losses of aspartate, glutamate, histidine, threonine and lysine residues (Fig. 6). Presumably, the primary, secondary and tertiary structure of a protein greatly influences the reactivity of each amino acid. The amino acid losses presented in Figure 6 would be

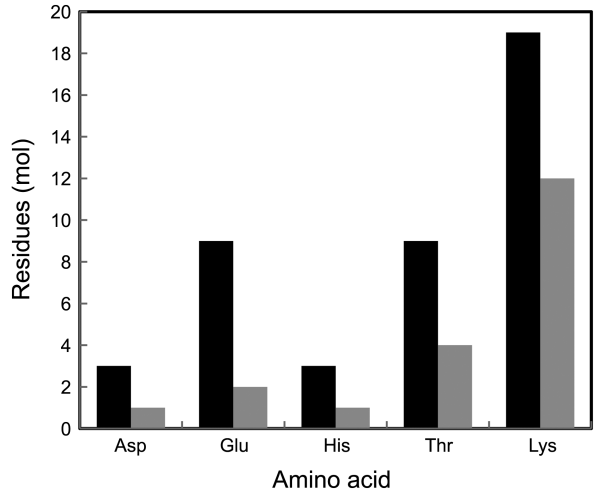

Figure 6. Modification of amino acid residues in cytochrome *c* by ACR. Cytochrome *c* was incubated in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C with the following conditions: 20 mM cytochrome *c* alone (dark bar); 20 mM cytochrome *c* plus 10 mM ACR (gray bar). After incubation for 24 h, the amino acid composition of acid and alkaline hydrolysates was determined, as described in Materials and Methods.

expected to have a dramatic effect on cellular structure and function. It has been reported that ROS-dependent protein modifications led to a loss of lysine residues. A loss of lysine residues may have been due to formation of Schiff bases through a direct reaction between the lysine amino group and ROS.[45] It has been reported that hypochlorite-mediated aggregation of low-density lipoprotein is caused by cross-linking of apoproteins through Schiff base formation.[46] Thus, the lysine modification was the major cause for the ACR-mediated cytochrome *c* aggregation.

Cytochrome *c* consists of 105 amino acid residues including six β-turns and five α-helices. The three conserved helices form a basket around the heme group.[47,48] The four flexible regions of cytochrome *c* called loop-1(20-35 residues), loop-2(36-59 residues), loop-3(70-85 residues), and C-treminal helix regions are denoted based on Singh *et al.*.[49] The 3D structure has CXXCH motif (Cys14, Cys17, and His18) to bind with heme group.[50] The reaction of cytochrome *c* with H_2O_2 induced the formation of a bound hydroxyl radical (Fe^{3+}-•OH) by a site-specific reaction at the heme and subsequently led to heme destruction.[51] Consequently, iron became almost free form the ligand, and was released from the oxidatively-damaged protein. Taken together the present findings suggest that ACR-mediated cytochrome *c* oligomerization might be due to oxidative damage resulting from ROS generated by a combination of the oxidation of ACR and the Fenton-like reaction of free iron ions released from oxidatively-damaged cytochrome *c*. Recent studies suggested an important neurotoxic role of ACR-induced oxidative stress in the development of AD.[52-54] Iron released from cytochrome *c* may trigger oxidative stress, which can result in the progressive neurodegeneration of the substantia nigra and may ultimately result in PD.[55] The Aβ peptide will coordinate first row transition metals iron, copper, and zinc to induce Aβ aggregation and in the case of iron and copper generate ROS. Therefore, the interactions of Aβ with transition metals potentially explain two of the prominent pathological features of AD; Aβ deposition and oxidative stress.[56] Therefore, the toxicity of ACR may be augmented by its ROS-generating activity in neurodegenerative disorders.

In summary, we report that ACR induced the oxidative modification of cytochrome *c* and subsequent iron release, and that this phenomenon involves ROS generation. ACR-mediated cytochrome *c* modification may be associated with the pathogenesis of AD, as well as related disorders.

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