A Study of the Oxidation-Induced Conformational and Functional Changes in Neuroserpin

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ABSTRACT

Background: Neuroserpin, a member of the Serine Proteinase Inhibitor (Serpin) superfamily, is known to be a neuroprotective factor in the focal ischemic stroke followed by reducing the microglial activation. Neuroserpin is a protein rich of methionine residues that can scavenge the free radical species which may increase its neuroprotective effect. On the other hand, the oxidative modifications of the amino acid residues in neuroserpin may lead to changes in its conformation and function. In this study, it was investigated the changes in the conformation and the function of the oxidized neuroserpin. **Methods:** Neuroserpin expressed in *E. coli*, BL21 or M15 harboring plasmid pQE81L containing neuroserpin cDNA. Expressed neuroserpin was purified by resin sulfopropyl A50 precharged with 0.1 M NiSO$_4$ under denaturing condition. Neuroserpin was oxidized under oxidative stress condition in the presence of different concentration of hydrogen peroxide. The oxidation of neuroserpin was conveniently detected by a carbonyl content assay using 2, 4 dinitrophenylhydrazine. Changes in tertiary structure of neuroserpin were monitored by spectrofluorimeter to study the alteration of intrinsic fluorescence and also fluorescence of 8-anilinonaphthalin-1 sulfonic acid (ANS) in native and oxidized form of neuroserpin. **Results:** Total expressed neuroserpin was estimated 4-5 mg/lit in 2XYT culture media. SDS-PAGE analysis of purified neuroserpin showed a single band which reflects the efficiency of the resin SP A50 for purification of the proteins containing 6×His tag. Carbonyl content of oxidized and native neuroserpin was estimated 12.3 ± 0.3 and 0.45 ± 0.05, respectively. The inhibitory activity of oxidized neuroserpin decreased up to 40-60% as compared with native form of neuroserpin. Intrinsic fluorescence and also the emission of ANS bind to the hydrophobic region of the protein altered from 380 to 85 and in the case of ANS from 105 to 150 in oxidized and native form of neuroserpin, respectively. **Conclusion:** The decreased intrinsic fluorescence intensity, an enhancement in the fluorescence of ANS, and loss of the inhibitory activity up to 40-60% in neuroserpin, all suggested a conformational modification in the protein under the oxidative stress condition. Remaining the inhibitory activity of neuroserpin reflects that the protein tolerates the oxidative stress condition effectively. *Iran. Biomed. J. 11 (1): 41-46, 2007*

INTRODUCTION

Neuroserpin is a member of the Serine Proteinase Inhibitor (Serpin) superfamily [1, 2]. It is expressed in the neurons of the central and peripheral nervous systems in adults and during development [3]. The serpins are a medically and biologically important family of proteins, which includes alpha 1 antitrypsin, antithrombin III, plasminogen activator inhibitor 1, C1 inhibitor, alpha 1 antichymotrypsin, neuroserpin, and many others [4]. Like all the other members of the serpin superfamily, this protein has a structure consisting of three β-sheets, nine α-helices, and a reactive center loop, which in inhibitory serpins contain the residues that directly interact with protease substrates [5]. It

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plays a neuroprotective role in focal ischemic stroke by reducing microglial activation after a stroke via complex formation with tissue-type plasminogen activator (tPA); however, it does not appear to inactivate substantial amounts of urokinase plasminogen activator (uPA) by complex formation [6]. Complex formation and inhibition assays have demonstrated that it is a potent inhibitor of tPA, uPA and plasmin [7, 8]. It acts as a suicide inhibitor by forming an extremely stable acyl complex with its target proteases. Neurodegenerative disease with intraneuronal inclusions containing neuroserpin has also been described [9]. Two point mutations of neuroserpin were discovered in two families suffering from presenile dementia [10]. In both families, the affected individuals underwent progressive neuronal degeneration due to the formation of inclusion bodies composed of polymerized uncleaved mutant neuroserpin in the cerebral cortex [9]. Under oxidative stress conditions, chemical transformations of amino acid residues in proteins can lead to loss of specific protein functions [11-14]. There is considerable evidence consistent with the importance of oxidative stress in the pathology of Alzheimer's disease [15] and other neurodegenerative brain disorders such as Huntington's disease, prion disorders, Parkinson's disease, frontotemporal dementia in which protein aggregation is observed. What the relationships might be among protein aggregation, oxidative stress and neurodegeneration remain unclear [11]. We hypothesized neuroserpin, which contains twenty methionine residues, considerably scavenge free radical species and may play a neuroprotective role in the oxidative stress condition. In this study, we investigated the modifications in the structure and the function of the neuroserpin oxidized with H_2O_2. We applied the spectrofluorimetry for the evaluation of changes in the intrinsic fluorescence intensity and the surface hydrophobicity, together with the inhibitory activity of the protein compared to the native form. In this study we examined the oxidation of neuroserpin to obtain insights into the effect of oxidative stress condition on the structure and inhibitory activity of neuroserpin.

MATERIALS AND METHODS

Peptone, yeast extract, Tris-HCl, imidazole, TEMED, PEG8000, single chain tPA were purchased from Sigma (USA). NaCl, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KCl, $MgCl_2$, urea,

dinitrophenylhydrazine (DNPH), 8-anilino-naphthalin-1 sulfonic acid, H_2O_2, EDTA, TCA, ethanol, ethyl acetate, formic acid, coommassie blue G250 and R250, ammonium persulfate, acrylamide, bis-acrylamide, BSA, $NiSO_4$, triton-X100 and isopropyl-1-thio-β-D-galacto-pyranoside (IPTG) were purchased from Merck, (Germany), H-D-isoleusyl-prolyl-arginine-*p*-nitro-aniline-dihydrochloride (S2288) from chromogenix, Japan and sulfopropyl A50 resin from Pharmacia (Sweden).

Expression and purification of the recombinant human neuroserpin. Human neuroserpin was expressed in *E. coli* with a stretch of six-histidine tag fused to the N-terminal of the protein. For the expression, a colony of *E. coli* strains BL21 or M15 harboring the expression plasmid (PQE81L) was precultured overnight at 37°C in 50 ml of Luria-Bertani medium containing 50 μg/ml ampicillin and 25 μg/ml kanamycin. 2XYT medium (500 ml) containing 50 μg/ml ampicillin and 25 μg/ml kanamycin was incubated with the precultured grown in a shaking incubator at 37°C, and induced with 2 or 1 mM IPTG at an OD_{550} of 0.5-1. The bacteria were harvested by centrifugation 4 h after the induction, resuspended in buffer A (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 7.8) and disrupted by sonication. The inclusion bodies were washed 3 times by the inclusion body washing buffer (buffer A + 0.5% (v/v) Triton X-100) and one more time with buffer A before solubilization in the inclusion body solubilization buffer (50 mM Tris-HCl, 8M Urea, pH 8.0). Then they were loaded onto a sulfopropyl A50 resin, a metal chelating resin, precharged with 0.1 M $NiSO_4$. After extensive washing with the loading buffer (50 mM Tris-HCl, 8M Urea, 20 mM Imidazole, pH 8.0), the protein adhered to the metal chelate resin was eluted with the elution buffer (50 mM Tris-HCl, 8M Urea, pH 8, 200 mM Imidazole or EDTA). The eluted protein was refolded overnight, drop by drop in refolding buffer (20 mM NaH_2PO_4, 100 mM NaCl, pH 7.8) at 4°C, ultra-filtered to remove any excess substances, and stored at -70°C [16]. Purified neuroserpin migrated as a single band on SDS-PAGE 10% [17]. Protein concentration of neuroserpin was estimated by Bradford assay using Coommassie Blue G250 [18].

Oxidation of neuroserpin and carbonyl content Assay. Purified neuroserpin was incubated in the reaction mixture buffer (50 mM potassium phosphate, 100 mM potassium chloride, 1 mM

magnesium chloride, 10 mM H_2O_2, pH 7.0) at room temperature for 2 h [11]. The concentration of H_2O_2 was measured at 240 nm (ε: 39.4 $\pm$ 0.2 $M^{-1}cm^{-1}$). Oxidized neuroserpin was reacted with 10 mM DNPH in 2 M HCl at room temperature for 1 h. The derivatives were sequentially extracted with 10% (v/v) trichloroacetic acid and consequently treated for three times with ethanol/ethyl acetate, 1:1 (v/v). The resulting precipitate was dissolved in 8 M urea and 50% formic acid. It was centrifuged at 16000 ×g for 5 min to remove any trace of insoluble material and its carbonyl was measured spectrophotometrically at 366 nm. The reference absorbtivity of 21.0 $mM^{-1}cm^{-1}$ for aliphatic hydrazones was used [19-21].

Inhibition of proteinase amidolytic activity. Single-chain tPA from human melanoma cell culture and recombinant neuroserpin and its oxidized form were preincubated at 25°C for 5 min in Amidolytic assay buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.2% (w/v) BSA, and 0.1% (w/v) polyethylene glycol 8000). Oxidation reaction was quenched by the addition of catalase, and samples were assayed for inhibitory activity against tPA. The chromogenic protease substrate H-D-isoleucyl-prolyl-arginine-*p*-nitroaniline-dihydrochloride was then added to a concentration of 1 mM. Samples were further incubated at 25°C and the velocities of amidolytic liberation of *p*-nitroaniline were determined by measuring the absorbance at 405 nm [22].

Intrinsic fluorescence intensity and surface hydrophobicity. All fluorimetric studies were performed on a Shimadzu spectrofluorimeter with a 0.5-ml cuvette of 1 cm path length and excitation and emission bandwidths of 5-10 nm. Emission spectra were recorded between 295-425, and 400-600 nm, (excitation wavelength: 280, and 340 nm) for intrinsic fluorescence intensity and ANS binding to the hydrophobic area of the protein, respectively. Neuroserpin (30 µg) in 1 ml reaction mixture buffer (50 mM potassium phosphate, 100 mM potassium chloride, 1 mM magnesium chloride, 10 mM H_2O_2, pH 7.0) was studied for changes in fluorescence intensity in addition to a 30 mM ANS to study the surface hydrophobicity. All experiments were performed at room temperature in 10 min intervals until the emissions became constant [23]. Results were reported as a mean of duplicate assays.

RESULTS

Expression, purification and refolding of recombinant human neuroserpin. The human neuroserpin was expressed in *E. coli* and purified by SP A50 resin precharged with $NiSO_4$. Purified neuroserpin, about 45 KDa, migrated as a single band on SDS-PAGE 10% as shown in Figure 1. Refolding of purified neuroserpin was performed in the refolding buffer and its inhibitory activity assay confirmed that neuroserpin was refolded.
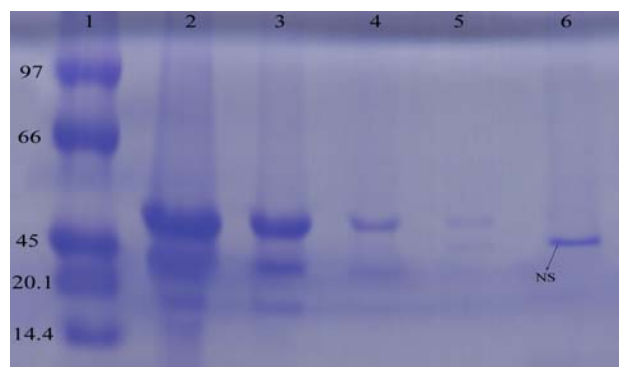

Fig. 1. Electrophoretic pattern (10% SDS-PAGE) of purified of neuroserpin from the inclusion bodies. From left; lane 1: molecular weight marker, lane 2-5: samples washed from the column, lane 6: purified neuroserpin.

Oxidation of neuroserpin and carbonyl content assay. To determine whether protein oxidation occurred during the sample preparation, a sample of native neuroserpin also was assayed. The carbonyl content for the oxidized and native forms of neuroserpin was estimated at 12.3 $\pm$ 0.3 and 0.45 $\pm$ 0.05 nmol/mg proteins, respectively.

Inhibition of proteinase amidolytic activity. The inhibitory activity of neuroserpin to inhibit tPA decreased at 40-60% after oxidation in the reaction mixture buffer (Fig. 2). The inhibitory activity of native neuroserpin considered as 100% of the total activity.

Intrinsic fluorescence intensity and surface hydrophobicity. Fluorescence intensity of neuroserpin decreased by time from 380 (just before and after adding 10 mM H_2O_2) to 85 (110 min after starting the reaction) as shown in Figure 3. Exposure of hydrophobic area of the neuroserpin during the oxidation by ANS led to an increase in ANS emission from 105 to 150 resulted from the modification of amino acid residues in neuroserpin in oxidative stress conditions (Fig. 4).
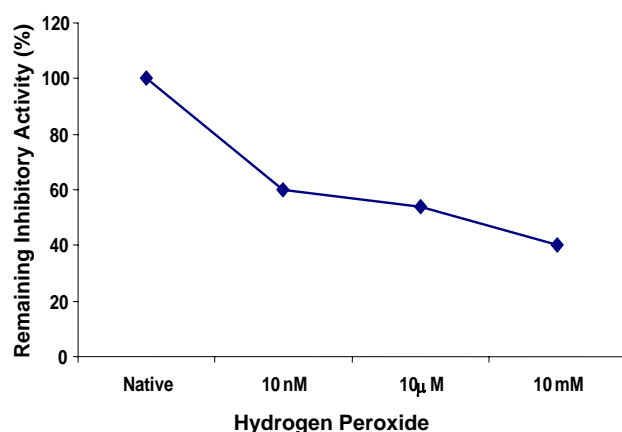
Fig. 2. Effect of the oxidative stress condition on the inhibitory activity of neuroserpin in reaction mixture buffer containing different concentration of hydrogen peroxide. The samples were assayed for inhibitory activity against tPA.

DISCUSSION

The oxidative stress attracts considerable interests in aging and age-related neurodegenerative diseases [14] and is probably one of the mechanisms involved in neuronal damage induced ischemia-reperfusion. The antioxidant activity of plasma may
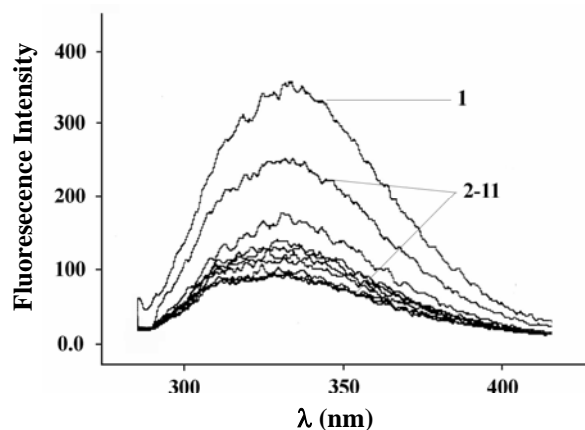

Fig. 3. Intrinsic fluorescence intensity of the native and the oxidized neuroserpin (excitation wavelength: 280 nm, and emission between 295-420 nm). 1, native form of neuroserpin; 2-11, neuroserpin under oxidative stress condition in reaction mixture buffer containing 10 mM H_2O_2 monitored in 10 min intervals.

be an important factor providing protection from neurological damage caused by the stroke-associated oxidative stress [24]. Neuroserpin, a natural serine

proteinase inhibitor in nervous system, which contains 20 methionine residues, increases after stroke and plays a neuroprotective role, because of inhibition of tPA, uPA, and plasmin after ischemic stroke [25, 26].

Elevated levels of oxidized protein are present in animals and cell culture following their exposure to various conditions of oxidative stress. Exposure of animals or cell cultures to either hyperoxia, forced exercise, ischemia-reperfusion, rapid correction of hyponatremia, paraquat toxicity, magnesium deficiency, ozone, neutrophil activation, cigarette smoking, x-radiation, chronic alcohol treatment, or mixed function oxidation systems leads to an increase in the level of oxidized protein [27]. Methionine oxidation is significantly important both to the biological functioning of proteins [28, 29] and to the stability of proteins produced for therapeutic purposes [30, 31].
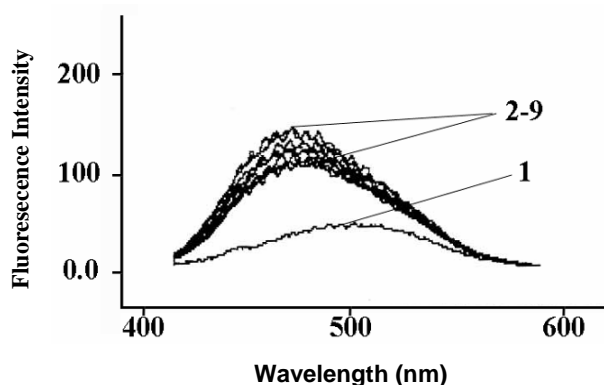

Fig. 4. Changes in ANS fluorescence intensity (FI) of oxidized neuroserpin. 1: Fluorescence intensity of ANS binds to the hydrophobic region of the native neuroserpin, 2-9: Fluorescence intensity of ANS binds to the hydrophobic region of the oxidized neuroserpin in the presence of 10 mili M hydrogen peroxide.

In this study, the relationship between the structural and functional properties of neuroserpin and its oxidation by hydrogen peroxide was analyzed by different methods [9, 16]. High amount of carbonyl content, 12.3 ± 0.3 nmol/mg proteins, showed that the oxidation of neuroserpin was effectively carried out in the reaction mixture buffer containing H_2O_2. Because of high amount of methionine residues in neuroserpin, it is predictably susceptible to oxidation. Changes of the methionine residues to the methionine sulfoxide resulted in considerable changes in the tertiary structures as confirmed by fluorimetric study, the intrinsic

fluorescence of neuroserpin and ANS emission bind to the hydrophobic region of the protein. In our experiments, decrease in fluorescence intensities during oxidation of neuroserpin showed some sort of compactness or higher exposure of aromatic residues in oxidized neuroserpin. But the data from the study of ANS fluorescence confirmed that the oxidation of neuroserpin led to exposure of neuroserpin hydrophobic groups to water due to oxidation. Reduction in inhibitory activity of neuroserpin showed that the oxidative stress condition also had a strong effect on its inhibitory activity. But the remaining of the inhibitory activity of neuroserpin revealed that the exposure of the hydrophobic region of the protein was limited and denaturation of neuroserpin was not complete due to oxidation. In other word, the protein tolerates oxidative stress to some extent. Griffiths and Cooney [30] have shown that recombinant human alpha 1-antitrypsin, the other member of the serpin superfamily, loses its activity up to 80% in the reaction mixture buffer containing 10 mM H_2O_2 at 25°C and pH 7.0 for 90 min. Taggart *et al.* [31] have shown that the oxidation of either Met351 or Met358 in alpha 1-antitrypsin results in a loss of biological activity against human neutrophil elastase. Based on the data from fluorimetric studies, changes in the intrinsic fluorescence intensity and the surface hydrophobicity along with the loss of inhibitory activity of the oxidized neuroserpin show that although the structure and function of neuroserpin modified under oxidative stress condition considerably but neuroserpin tolerated the oxidative stress condition effectively.

ACKNOWLEDGEMENTS

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