XANES spectroscopy of a single neuron from a patient with Parkinson's disease

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Chemical state of transition metals such as iron in a single neuron of the substantia nigra (SN) from a patient with Parkinson's disease (PD) was studied in this paper, using autopsy midbrain specimens including SN. Parkinson's disease is one of the major neurodegenerative diseases. The excessive accumulation of iron and its chemical states in SN neurons are related to the oxidative damage leading to neuronal cell death. X-ray absorption fine structure spectroscopy (XAFS) with SR micro beam (10 μm) was used to investigate the chemical state of iron in the SN neurons. X-ray absorption near-edge structure (XANES) spectroscopy results showed that the chemical state of iron in the neuromelanin granules within SN neurons changed from ferrous (Fe^{2+}) to ferric (Fe^{3+}) ion in the process of neuronal degeneration.

Key words: neuron, neuromelanin, chemical state, Parkinson's disease

1. Introduction

The normal functions of living organs are sustained by equilibrium of elements in a single cell level. The equilibrium and fluctuation in density of elements are associated with both physical (densities and distributions of elements), and chemical activities (chemical states). In neurodegenerative disease cases such as Alzheimer's and Parkinson's diseases, excessive accumulation of metallic elements or the possible changes in the chemical state of transition metals, such as Fe, in neurons could be related to the neuronal degeneration and cell death.

Free radicals in neurons play a key role in neuronal degeneration due to cytotoxic oxidative stress (Kondo, 1995; Jenner *et al.*, 1998). The free radicals are produced in a series of reactions, which involve transition metals such as Fe, Mn, Ti and other trace elements in a cell. Iron is an element highly abundant in neurons and one of the best-studied elements with respect to production of free radicals. The increased iron content and the loss of the melanized neurons in the SN of PD patients have been studied by many researchers (Sofic *et al*., 1991; Hirsch *et al*., 1991; Jellinger *et al*., 1992). Increased content of iron (Fe) in the SN, generates complexes through binding to neuromelanin (Kropf, *et al*., 1998; d'Ischia *et al*., 1997; Takada *et al*., 1999; Ektessabi *et al*., 1999). The Fe contained melanin complexes contribute to the formation of cytotoxic hydroxyl free radicals (·OH) in the mechanism shown in Fig. 1. Iron and other transition metals are considered to promote the generation of free radicals because they can exchange electrons and change the valence (Kienzl *et al*., 1995). Therefore, the concentration and chemical states of iron in SN neurons are matters of serious concern in Parkinson's disease studies.

The changes in composition and chemical state of elements in SN neurons in different stages of the degenerative process have not been clarified because of the limitations in spectroscopic and analytical techniques. In our previous studies we applied ion micro beam particle induced x-ray emission (PIXE), and electron probe micro analyzer (EPMA) for elemental imaging of the human tissues (Ektessabi *et al*., 1996; Ektessabi *et al*., 1998). However, techniques using electron and ion beams (Tuniz *et al*., 1990; Lidth, 1993; Thong *et al*., 1996; Vazina *et al*., 1998; Carvalho *et al*., 1998) are relatively destructive and have shortcomings in detection limit and the measurement time. The density of the elements in cells, its distribution, and the fluctuation in the density are extremely low and cannot be measured with conventional measurement techniques. The XAFS spectroscopy (Waychunas *et al*., 1983; Bianconi 1988) using a SR micro beam (Iida *et al*., 1993) can be used as a powerful tool to partially overcome such problems and to provide information on any possible relationships between neurological disorders and trace elements within neurons.

In this paper, we focused on the chemical state of iron in neuromelanin granules inside and outside neurons of the substantia nigra (SN), a part of the midbrain mainly affected by Parkinson's disease.

2. Materials and Methods

Autopsy specimens of midbrain, including SN, were obtained from a 72-year-old male patient with PD. The patient died of bronchopneumonia and ileus five and a half years after onset. The clinical diagnosis was confirmed with neuropathological hallmarks of a severe loss of dopaminergic (melanized) neurons from the SN and the presence of intraneuronal inclusions called "Lewy bodies". The specimens were fixed in 10 % formalin, embedded in paraffin, and then cut into 8 μm thick sections. Finally, the sections were mounted on a Mylar film for spectroscopy.

The measurements were carried out in the beam line 39XU at SPring-8 synchrotron radiation facilities, Japan. The electron energy was 8 GeV and maximum electron current was 70 mA in the storage ring. The synchrotron radiation from an undulator was monochromatized by a Si (111) double crystal monochromator. The incident SR beam was restricted by a set of x-y slits and a pinhole of 10 μm on the front side of the sample. The beam size on the sample was 9.6 μm in diameter, and was measured using a Cu grid. A Si (Li) detector was mounted at 90 degrees to the incident beam to minimize the contribution of scattered x-rays to the fluorescence signals.

During measurement of the elemental distributions, the incident x-ray energy was 7.2 keV, which was about 90 eV higher than the iron K-edge. The scanning area was 500 × 240 μm^2 and was divided into 50 × 24 pixels. The data were collected for 8 seconds on each measurement point.

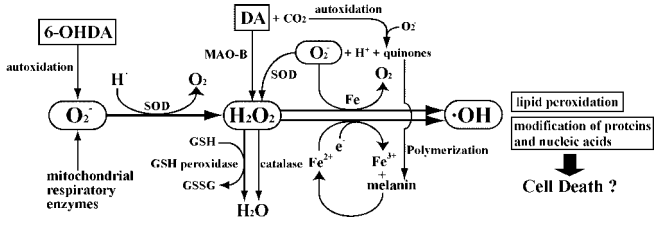

Figure 1
Reaction cycle for the generation of cytotoxic free radicals, hydrogen peroxide and hydroxyl radical (·OH). (6-OHDA: 6-hydroxydopamine, DA: dopamine, SOD: superoxide dismutase, MAO-B: monoamine oxidase B, GSH: glutathione, GSSG: oxidized glutathione)

To obtain the relative concentration of the constituent elements inside and outside neurons, fluorescence x-ray spectra were also obtained at several points of interest in the scanning area. The beam size was 9.6 μm in diameter and the x-ray energy was 7.2 keV. The measurement time at each point was 100 seconds.

XANES spectra of the iron K-edge, were measured at several points on the sample. The beam size was 9.6 μm in diameter. The energy scan step was 0.5 eV. The measurement time was between 20 and 100 seconds per step in different experiments.

Since the content of elements in the samples used in this study is very low, fluorescence x-ray spectra was used to obtain the XANES spectra. In addition, absorption spectra of the iron K-edge iron oxide powders (FeO and Fe_2O_3) were analyzed as reference. The absorption coefficients were calculated from the incident (I_0) and transmission intensity (I). The measurement time was one second per step.

3. Experimental Results

3.1. *Elemental Imaging of Neuron*

X-ray fluorescence imaging of iron distribution in the SN tissues is shown in Fig. 2a. A microscopic photograph of the scanning area is shown in Fig. 2b. Iron was highly accumulated in neuromelanin granules within a dying neuron (*n1*), one of glial cells (*g3*) and an arteriosclerotic small vessel (*v*). Iron had accumulated in the neuromelanin aggregates (*a1*) or scattered particles (*a2*) released from dead neurons and in some glial cells (*g1, g2*), with a higher concentration than in the neuromelanin granules within surviving neurons (*n2, n3*), which were apparently normal.

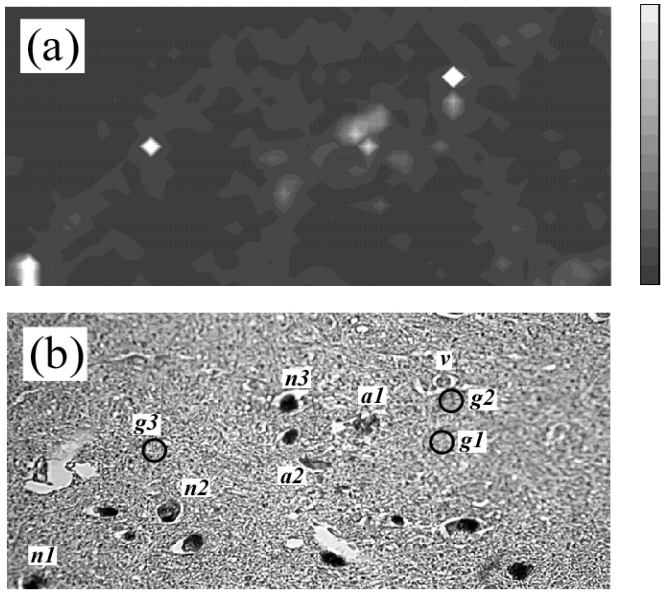

Figure 2
Imaging of a tissue in substantia nigra of a patient afflicted with Parkinson's disease. (a) XRF imaging of iron. The scale on the right-hand side of the map shows the level of the fluorescence x-ray intensity. The range of intensity (counts) is 0 ~ 1000. (b) Microscopic photograph of the scanning area of the sample. Marks *n1, n2* and *n3* show neuromelanin granules within surviving neurons, mark *a1* and *a2* show neuromelanin aggregates released from dead neurons, marks *g1, g2* and *g3* indicate nuclei of glial cells, and mark *v* indicates a blood vessel diagnosed as arteriosclerosis. The scanning area was 500×240 μm^2.

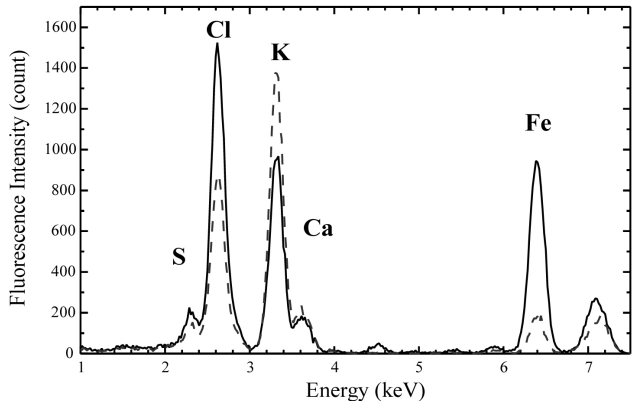

Figure 3
Typical x-ray fluorescence spectra obtained from substantia nigra of a patient with Parkinson's disease. The solid curve was measured in neuromelanin aggregates from a dead neuron (*a1* in Fig. 2) and the dotted curve was neuropil (outside of nerve cells). The incident x-ray energy was 7.2 keV.

X-ray fluorescence spectra were measured at several points on the scanning area. Two typical spectra are shown in Fig. 3. The solid-curve is a spectrum collected at the neuromelanin aggregates released from a dead neuron (*a1*) and the dashed-curve in a spectrum obtained from neuropil. Both spectra had a very high signal/background ratio. From these spectra, relative elemental concentrations were obtained at each measurement point.

3.2. *Chemical State Analysis of Iron in the Neuron*

Figure 4 shows typical micro-XANES spectra of the iron K-edge in neuromelanin granules within a surviving neuron (a), in neuromelanin aggregates released from a dead neuron (b). The spectra of the reference materials (FeO and Fe_2O_3 powder) are shown as spectra (c) and (d), respectively. No remarkable peak appears at the energies higher than that of the absorption edge of the spectra of the PD sample. The pre-edge peak at the energies lower than the absorption edge of the spectra of the PD sample is attributed to the 1s $\rightarrow$ 3d transition that is excluded by dipole selection rules. The pre-edge peak indicates that the centrosymmetry around the iron site was broken *(Waychunas, 1983)*.

The energy of the absorption edge, where the absorption coefficient is at half-height of the absorption jump, is the minimum energy necessary to excite the inner shell electron to the outer empty level. The absorption energies shift slightly (chemical shift) with variation of the effective atomic charge. The chemical shift of the XANES spectra is shown in Fig. 5. The vertical axis of the graph shows the chemical shifts from the absorption edge of iron in FeO (Fe^{2+}). Judging from the chemical shifts of the spectra, the valence of the accumulated iron changed significantly, and the changes are corresponding to differences in the histological observation. Iron in the neuropil is mainly ferrous form (Fe^{2+}), binding to iron regulatory proteins, e.g., transferrin and ferritin, in the glial cells (Connor *et al*., 1990; Connor, *et al*., 1992). Iron binding to neuromelanin has changed from a ferrous (Fe^{2+}) to ferric (Fe^{3+}) form inside (a) and outside (b) neurons.

In a wider area of the SN tissues, XANES spectrum was measured to investigate the integrated value of chemical states of iron (Fig. 6), using a beam of 300×300 μm^2. The measurement

area consisted of a neuropil and 6 surviving neurons. The XANES spectrum shows that the iron in the SN tissue is mainly in ferrous form (Fe^{2+}). The result supports that chemical changes, from a ferrous (Fe^{2+}) to ferric (Fe^{3+}) form, occurred in the neuromelanin aggregates released from the dead neurons.

4. Conclusions

In this study, we demonstrated iron distribution and chemical state in substantia nigra tissues of a patient with Parkinson's disease, in a single cell level. A SR microbeam with high brilliance made it possible to analyze very low content of iron and other trace elements in single neurons without isolating or purifying procedures. The XANES spectroscopy results show that chemical states of iron binding to neuromelanin may be related to the stage in the degenerative process of SN neurons.

One patient with PD was investigated in detail. There were 12 surviving neurons and 2 neuromelanin aggregates released from dead neurons in the analyzed area of the sample.

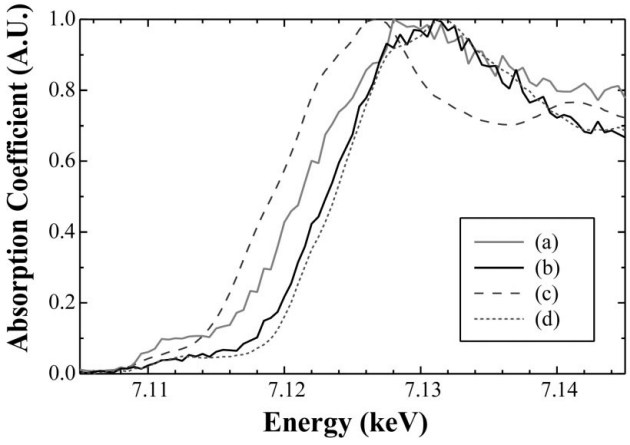

Figure 4
Iron K-edge micro-XANES spectra measured in neuromelanin granules within a surviving neuron (a) and in neuromelanin aggregates from a dead neuron (b). Curves (c) and (d) show the XANES spectra of FeO and Fe_2O_3 powder, respectively, as reference materials.

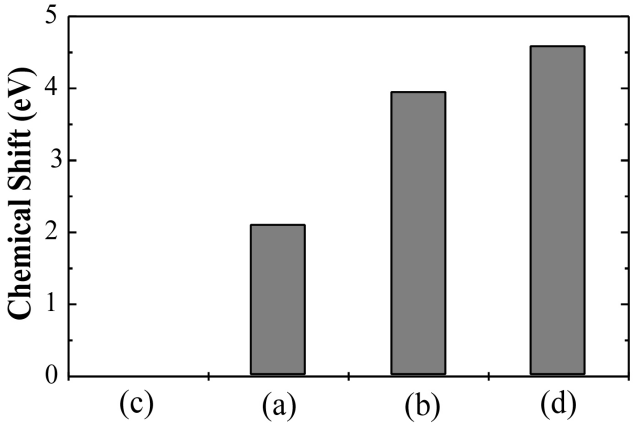

Figure 5
Chemical shifts of the XANES spectra from a patient with PD and reference materials. The measuring points are the same as those in Fig. 4. The vertical axis shows the chemical shifts from the absorption edge of the FeO. (a)Neuromelanin granules within a surviving neuron (b) neuromelanin aggregates from a dead neuron. Curves (c) and (d) show the XANES spectra of FeO and Fe_2O_3 powder, respectively.

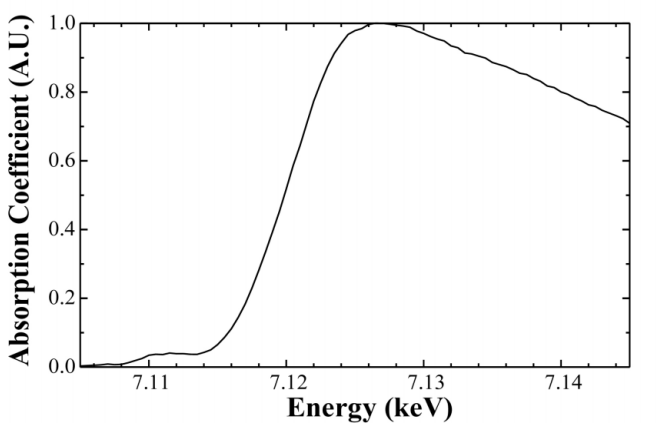

Figure 6
Iron K-edge micro-XANES spetrum measured in a wide area of SN tissues. The beam size was $300 \times 300\ \mu m^2$.

The XANES spectroscopy results indicate that iron binding to neuromelanin has changed from a ferrous (Fe^{2+}) to ferric (Fe^{3+}), probably in the neurodegenerative process. This observation is in good agreement with the results reported previously that iron-melanin interaction may be involved in the degenerative process of neurons, as the complex is a source of cytotoxic hydroxyl free radicals (·OH) (Orrenius *et al*., 1992).

Here, one should note that the chemical state of the wide area could not represent the chemical state of iron in a single cell. This emphasizes the importance of using micro beam XANES analysis in investigations related to a single neuron.

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