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Determining the oxidation states of manganese in NT2 cells and cultured astrocytes

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Abstract

Excessive brain manganese (Mn) can produce a syndrome called "manganism", which correlates with loss of striatal dopamine and cell death in the striatum and globus pallidus. The prevalent hypothesis for the cause of this syndrome has been oxidation of cell components by the strong oxidizing agent, Mn^{3+}, either formed by oxidation of intracellular Mn^{2+} or transported into the cell as Mn^{3+}. We have recently used X-ray absorption near edge structure spectroscopy (XANES) to determine the oxidation states of manganese complexes in brain and liver mitochondria and in nerve growth factor (NGF)-induced and non-induced PC12 cells. No evidence was found for stabilization or accumulation of Mn^{3+} complexes because of oxidation of Mn^{2+} by reactive oxygen species in these tissues. Here we extend these studies of manganese oxidation state to cells of brain origin, human neuroteratocarcinoma (NT2) cells and primary cultures of rat astrocytes. Again we find no evidence for stabilization or accumulation of any Mn^{3+} complex derived from oxidation of Mn^{2+} under a range of conditions.

Keywords: Mn oxidation states; Mn toxicity; XANES spectroscopy; NT2 cells; Astrocytes

1. Introduction

While manganese (Mn) is essential for the function of pyruvate carboxylase, arginase, glutamine synthase, and mitochondrial superoxide dismutase [17], excessive brain manganese can induce a neurological syndrome known as manganism, resulting in clinical rigidity and/or dystonia [6]. This syndrome is associated with loss of dopamine in the striatum and death of neurons in the striatum and globus pallidus [30] and it shares many features with idiopathic Parkinson's disease.

The oxidation states of Mn most commonly encountered by living cells and tissue are Mn^{2+}, Mn^{3+} and Mn^{4+}. Mn^{2+} is stable in aqueous solution up to a pH of around 7.5, while Mn^{3+} is unstable in aqueous solution at pH's above around 2 unless stabilized by formation of stable complexes with any of a wide variety of anions. Mn^{4+} is usually encountered in insoluble complexes and particulate matter, the most common form being MnO_2. Mn^{4+} has not been observed in living cells of animal origin. Mixtures of soluble Mn^{2+} and Mn^{4+} salts such as MnO_2 can lead to the formation of Mn^{3+} which can be stabilized by complexation or acid pH. Mn^{2+} can be sequestered by most types of cells and there is suggestive evidence that Mn^{3+} may also be sequestered by cells [3,4,11]. Within the cell, Mn^{2+} can be readily sequestered by mitochondria via the Ca^{2+} uniporter [13,14,20,22,26,28,34]. The mitochondrial electron transport chain produces more superoxide radical than any other source in the cell [12], and

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reactive oxygen species (ROS) can be produced both inside and outside the mitochondrion [38], and at other locations within the cell [9]. Mn^{2+} can be oxidized by superoxide radical to Mn^{3+} and Mn^{3+} pyrophosphate can oxidize dopamine, and its precursor, L-dopa [2]. These observations and ideas have led to the hypothesis that the cell damage underlying Mn toxicity is caused by oxidation of cell components by Mn^{3+} [2,16]. The two most likely ways through which Mn^{3+} could get into the cell are: (1) through transport of a Mn^{3+} complex into the cell, or (2) through sequestration of Mn^{2+} followed by oxidation of Mn^{2+} by ROS. Until recently there was no evidence for either the presence or absence of Mn^{3+} complexes in brain cells or brain mitochondria.

We have recently used X-ray absorption near edge structure (XANES) spectroscopy to assess the Mn^{2+} and Mn^{3+} complexes inside brain mitochondria and both nerve growth factor (NGF)-induced and non-induced PC12 cells [18,19,21]. The use of XANES spectroscopy for determination of the oxidation state of complexes is well established both theoretically and experimentally [7,39,42], and XANES and related techniques have been used to determine the oxidation states of specific Mn complexes in photosystem II [10,15,25,31,46] and in Mn catalase complexes [31,43]. Much of the methodology of the current work was developed in the applications of XANES and related techniques to these studies of photosystem II and Mn catalase.

The results of these earlier XANES studies of the oxidation states of Mn complexes in brain mitochondria and neuron-like cells was that no intramitochondrial or intracellular Mn^{3+} spectra were seen except for small amounts of spectra very similar to those of Mn superoxide dismutase, which would be expected to show both Mn^{2+} and Mn^{3+} character since the function of this enzyme requires it to cycle between these oxidation states [19,21]. There was no spectroscopic evidence for formation or accumulation of Mn^{3+} complexes with time even under conditions of increased oxidative stress.

While these past studies focused on brain mitochondria and on PC12 cells (a rat adrenal medulla pheochromocytoma cell line) which develop a neuron-like phenotype when treated with nerve growth factor, no studies were carried out on cells of neuronal or brain origin. Mn^{3+} damage to the central nervous system (CNS) could be mediated either through damage to neurons or astrocytes, which are necessary to support the function of neurons. Consequently, we have used XANES spectroscopy to investigate the possible formation of Mn^{3+} complexes in a cell line originally of neuronal origin, human neuroteratocarcinoma cells (NT2), and in primary cultures of rat astrocytes. These studies focus on the question of whether any of the Mn sequestered by the cell as Mn^{2+} is subsequently oxidized to Mn^{3+} and stabilized within the cell as a Mn^{3+} complex.

Any Mn^{3+} found in brain cells must either have been transported into the cells as Mn^{3+} or formed by oxidation of Mn^{2+} to Mn^{3+} in the cells. XANES could be used in a study of the transport of Mn^{3+} into neuronal cells or astrocytes; however, before the results of such a study could be interpreted, it would be necessary to determine whether or not Mn^{3+} complexes were formed in the cell because of oxidation of Mn^{2+}. Therefore, studies such as those described here are also necessary for the interpretation of any results using XANES to test whether Mn^{3+} can be transported into neuronal or astrocytic cells.

2. Materials and methods

2.1. Media and cell preparation

Reagents used were from Sigma or Aldrich (Sigma–Aldrich Fine Chemicals, St. Louis, MO) unless otherwise indicated. Human neuroteratocarcinoma cells (NT2), obtained from Stratagene, were incubated in "NT2" medium (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, pH 7.2). The cells were incubated in T-75 flasks in 12 ml of NT2 medium in a 37 °C, high humidity, 5% CO_2 incubator. Upon confluence (usually 2–2.5 days), cells were trypsinized and subcultured at a ratio of 1:6.

Astrocytic cultures from cerebral cortices of newborn (1 day old) Sprague–Dawley rats were established as previously described in detail [1]. Briefly, after removal of the meninges, the cerebral cortices were digested with bacterial neutral protease (dispase) and astrocytes recovered by repeated removal of dissociated cells. Twenty-four hours after the initial plating in poly-D-lysine coated Corning 35 mm dishes, the media were changed to preserve adhering astrocytes, and remove the neurons and oligodendrocytes. The cultures were maintained at 37 °C in a 5% CO_2 incubator for 4–6 weeks in minimal essential medium (MEM) with Earle's salts supplemented with 10% heat-inactivated horse serum, 100 U/ml streptomycin, and 0.25 μg/ml Fungizone. The media were changed twice per week. These monolayers, surface-adhering cultures were >95% positive for the astrocyte marker, glial fibrillary acidic protein (GFAP). These cells became confluent following 3–4 weeks of incubation.

2.2. Mn^{2+} uptake

For studies of Mn^{2+} uptake and associated cell protein measurements and for making XANES samples, NT2 cells were released from a 95% confluent T-75 flask or 100 mm Petri dish by addition of 3.5 ml of a trypsin (0.05% trypsin by weight)/ethylene diamine tetraacetate (EDTA) (2 gm/100 ml) solution. Once released, the NT2 cells or primary astrocyte cultures were diluted 37-fold with NT2 medium or with MEM, respectively. 10.4 ml of the cell suspension was added to each well of the 24 wells in 4 six-well dishes. After 24 h for cell attachment, various concentrations of sterile $MnCl_2$ were added and the cells incubated for varying periods of time. The medium containing sterile $MnCl_2$ was changed