Mobilization of iron from cellular ferritin by ascorbic acid in neuroblastoma SK-N-SH cells: an EPR study

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Abstract The mobilization of iron from intracellular ferritin by ascorbic acid has been analysed in situ by electron paramagentic resonance (EPR) spectroscopy. EPR enables a distinction between ferritins and other Fe^{3+}-binding cellular components. The ordered iron core of ferritin gives rise to a resonance signal which can be observed only at temperatures above 50 K. In the present study we clearly demonstrate that ascorbic acid is capable of mobilizing iron from ferritin in the cellular system by reduction of the ferric ion core in neuroblastoma SK-N-SH cells. This mechanism may open new ways in the therapy of this hardly curable tumor in stage IV, especially in combination with some cytostatic drugs.

Key words: Neuroblastoma; Iron release; Ferritin; Ascorbic acid; Electron paramagnetic resonance

1. Introduction

Elevated ferritin levels in the serum of cancer patients are commonly used as a tumour marker to get informations about the size, mass and stage of the tumour. This may be true for a diversity of cancers like head and neck [1], lung [2], liver [3,4], breast [5], bladder [6] and renal cancer [7] as well as for teratoblastoma [8] and Morbus Hodgkin [9]. The serum ferritin level is also known to be a tumour marker in neuroblastoma patients [10]. In addition, as it could be demonstrated for other tumour cells, neuroblastoma tissue contains high amounts of ferritin intracellularly [11,12].

Intracellular ferritin may either function as an iron storage protein to support iron dependent enzymatic activity or as an iron scavenger to prevent oxidative damage [13]. If iron is released unspecifically from the ferritin core it would catalyze – like any other form of adventitious cellular iron – the formation of hydroxyl radicals via Haber-Weiss-Fenton reaction cycles [14]. The hydroxyl radical gives rise to a radical reaction chain inducing, e.g., DNA strand breaks [15,16] and generating lipid peroxidation products [17]. Such a process eventually could lead to cell death.

Based on in vitro experiments it has been suggested that the release of iron from ferritin in vivo could be achieved by a variety of reductants including the catecholamine metabolite 6-OHDA [18], reactive oxgen species like superoxide anion [19,20], nitric oxide [20,21] and substances normally active as antioxidants like glutathione [22,23] and ascorbate [22].

Ascorbate is particularily interesting because of its hypothesized pro-oxidative activity in neuroblastoma tissue originating from the potentiating effect of ascorbate on commonly used antitumourigenic agents like fluorouracil or bleomycin [24]. Further work demonstrated that ascorbic acid induces DNA strand breaks in neuroblastoma cells [25,26] and tissues [27]. These effects were especially pronounced in the presence of small amounts of H_2O_2 and transition metals like iron [28,29]. Therefore, oxidative damage in neuroblastoma cells may be supported by an initial reductive release of iron from the large ferritin pool [11,12] and by high amounts of intracellularly produced H_2O_2 [26,30]. Both, ferritin [11,12] and H_2O_2 [26,30], are abundant in neuroblastoma cells at high concentrations. In addition, ascorbate can be accumulated in these cells reaching intracellular concentrations in the millimolar range [31]. Because neuroblastoma cells exhibit crucial prerequisites for ascorbic acid to act as a pro-oxidant this compound might be of aid in the treatment of that tumour. We have focused our interest on ascorbate because it acts either as an antioxidant in most normal tissues or as a pro-oxidant in neuroblastoma cells under certain conditions.

So far, merely limited experimental evidence was found for the assumed pro-oxidative activity of ascorbic acid. In vitro, iron release was demonstrated by several assay systems like the ferrozine or bathophenanthroline assay [22,23]. Recently, we could get some hints that iron release by ascorbic acid is also possible in a cellular system using ^{59}Fe-labeled ferritin. However, this was only practical in iron pretreated cells. Using the electron paramagnetic resonance (EPR) technique, we are now able to characterize ferritin iron clusters and changes in their size.

2. Materials and methods

2.1. Cell culture

The cell line SK-N-SH [32] was procured from the American Type Culture Collection, USA. Cells were cultured in 5% CO_2 supplemented air in RPMI 1640 medium containing 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 10% foetal calf serum (Biochrom, Germany). For EPR spectroscopy, 400×10^6 cells were suspended in 50 ml culture medium and incubated with or without 1 mM ascorbate (Sigma, Germany) for 4 h at 37°C with sequential agitation. Afterwards, 1 mM H_2O_2 (Merck, Germany) or, as a control, PBS (Biochrom) was added and incubated for further 10 min. Thereafter, cells were washed twice with ice-cold PBS. Part of the cell pellet was used for protein and iron determination (see section 2.3). The residual cell material was diluted with 100 µl PBS. The cell number/ml of this suspension was

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Abbreviations: EPR, electron paramagnetic resonance; PBS, phosphate-buffered saline; au, arbitrary units.

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determined and 400 μl of it were filled in EPR tubes and frozen in liquid nitrogen until measurement.

2.2. EPR measurement

Measurements were made at 10 K, 73 K and 160 K at X-band (9.4 GHz) using a cw-EPR spectrometer (Bruker ER200D) equipped with a helium-flow cryostat (Oxford Instruments ESR910). The data acquisition system, based on a personal computer, is our own development. The experimental spectra were numerically double-integrated. Prior to integration the experimental baselines were adjusted for offset and linear slope in the integration intervalls by means of computer graphics. Based on a test series, we estimated an error of 20%.

2.3. Determination of iron and protein concentrations

The protein content of the samples was determined using the bicinchoninic acid reagent (Pierce, USA) following the instructions of the manufacturer. The iron content of SK-N-SH cells was determined according to Sofic et al. [33]. In brief, 70×10^6 cells were resuspended in PBS and boiled for 10 min. The ice-chilled solutions were acidified, treated with 20 mM dithiothreitol (Serva, Germany) and 2 mg/ml ferrozine (Serva, Germany) and the absorbance was measured at 562 nm in a Ultrospect 4050 photometer from LKB Biochrom, Germany. Ferritin containing 12% iron (Serva, Germany) was used as a control. Iron concentrations were calculated using the extinction coefficient of 27,900 $M^{-1}\cdot cm^{-1}$.

3. Results

Electron paramagnetic resonance spectrometry (EPR) was used to analyse ferritin-bound iron in neuroblastoma SK-N-SH cells. Three independent sets of samples were prepared from different cultures yielding qualitatively the same EPR spectra but differing in protein and iron concentrations. Fig. 1 displays EPR spectra of neuroblastoma cells recorded at various temperatures. The iron concentration of the sample was 49 μM and the protein content 133 mg/ml. At 15 K (Fig. 1A), two spectral features can be observed at $g=4.3$ and around $g=2$. The signal at $g=4.3$ corresponds to ferric iron in a rhombic enviroment. Rhombic iron was found in all biological systems we have analysed so far: bacteria, fungi and animal cell lines. However, without a more detailed knowledge of the particular system, this signal cannot be attributed to a specific compound. Double integration of this signal gave a relative area of 160 arbitrary units (au). Around $g=2$ several signals are found (relative area after double integration: 174 au). We assume that these signals reflect a variety of iron and Cu compounds. One of the components probably represents an iron sulfur protein. At 10 K (data not shown) there is a weak signal at $g=6$ reflecting an ferric iron compound of axial symmetry. This signal is typical for cytochromes. At 15 K this signal disappears.

Ferritins, however, cannot be detected in EPR at low temperatures. In case, ferric iron particles are antiferromagnetically ordered (as in the iron core of ferritin), their EPR signals will broaden and decrease in intensity below the Neél (ordering) temperature with decreasing temperature. The linewidth, DH, is given by:

$$\Delta H = AKV\exp(KV/k_BT) \quad (1)$$

where A is a constant dependent upon the core size, K is the magnetic anisotropy constant per unit volume, V is the volume of the particle, k_B is the Boltzmann constant, and T the temperature [34,35].

Therefore, we have analysed the sample also at 73 K (Fig. 1B) and 160 K (Fig. 1C). The intensities of the EPR signals at $g=4.3$ (area after double integration: 90 au) and $g=2$ decrease with increasing temperature as expected. However, an additional feature is visible at 73 K, a very broad derivative signal stretching over a field range of 350 mT in this measurement (double integration yields an area of 4018 au). At 160 K this feature is even more pronounced and dominates the spectrum (4636 au) indicating the existence of antiferromagnetically ordered ferric iron in small particles. Therefore, we attribute this component to ferritin. The linewidth ΔH of 47.6 mT at 160 K is close to that of isolated horse spleen ferritin.

In Fig. 2, EPR spectra of neuroblastoma cells are shown which were treated 4 h with 1 mM ascorbic acid. The iron concentration of the sample was 42 μM and the protein content was 127 mg/ml. At 15 K (Fig. 2A), again the same signals near $g=4.3$ and $g=2$ can be detected as in Fig. 1A. The same holds for the appearance of a broad signal at 73 K (double integration yields an area of 1656 au). However, this broad signal narrows considerably at 160 K (2040 au). The line width ΔH of 25.8 mT is almost half as broad as that of the untreated cell sample shown in Fig. 1C. According to Eqn. 1 [35] and assuming the same anisotropy constant for sample 1 and 2 we attribute the decreased linewidth to a smaller core size of ferritins. This change of core size is a consequence of ascorbate treatment. Hence, ascorbate is capable of mobilizing iron from ferritin. Moreover, the relative area of the double integrated signals of ascorbate treated cells compared with that of the control displays the same trend (50% drop).

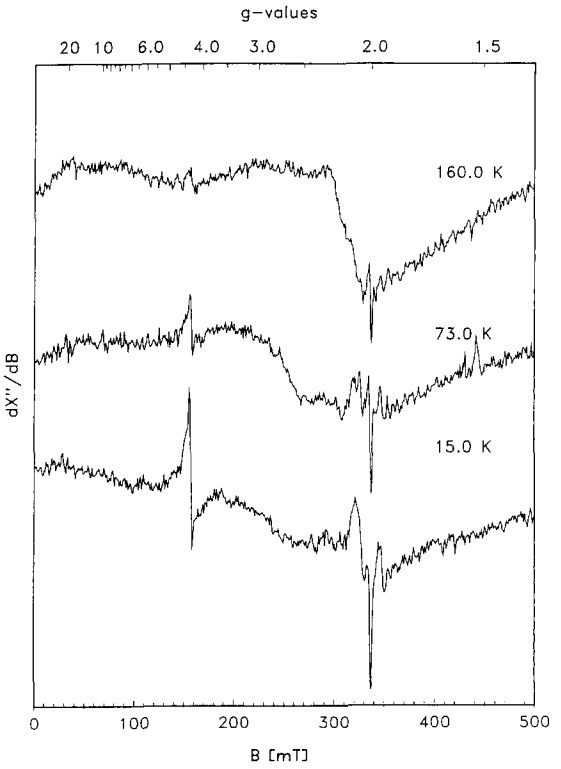

Fig. 1. EPR spectra of neuroblastoma SK-N-SH cells mesured at 15 K (microwave frequency = 9.428 GHz; microwave power = 2.0 mW/20 dB; modulation frequency = 100 kHz; modulation amplitude = 1 mT. If not stated otherwise the EPR spectra in Figs. 2 and 3 were measured under the same conditions), 73 K (microwave frequency = 9.426 GHz), and 160 K (microwave frequency = 9.428 GHz). The broad signal observed at 160 K is attributed to ferritin (double integration–spin density = 4636 arbitrary units).

In Fig. 3 the EPR spectra of neuroblastoma cells are depicted which were treated 4 h with 1 mM ascorbic acid and then for additional 10 min with 1 mM H_2O_2. The iron concentration of the sample was 26 μM and the protein content was 60 mg/ml indicating the cell density was approximately half of that in sample 1 and 2. Consequently, only weak signals near $g=4.3$ and $g=2$ could be detected at 15 K (Fig. 3A). Again a very broad signal is observed at 73 K (area of 1529 au) and 160 K (area of 1917 au). At 160 K the signal width of 46 mT is similar to that of the untreated control thus indicating a core size which is larger than that which corresponds to the linewidth shown in Fig. 2C. At a first glance, the calculated spectral area of ascorbate/H_2O_2 treated cells at 160 K is much too low to fit this model. However, taking into account the smaller number of cells in this sample three, the relative area should be corrected to approximately 3800 au which is within experimental error the same value as that obtained for untreated cells.

4. Discussion

Iron is an essential metal in all biological systems serving as a cofactor of many enzymes like ribonucleases, iron–sulfur proteins and cytochromes. However, iron in its ferrous form may also be cytotoxic because of its reaction with H_2O_2 yielding various reactive oxygen species [36–38]. These naturally occurring oxygen metabolites are believed to have a dual role in the cellular metabolism: they are either involved in regulatory processes like the activation of NF-κ-B [39,40]

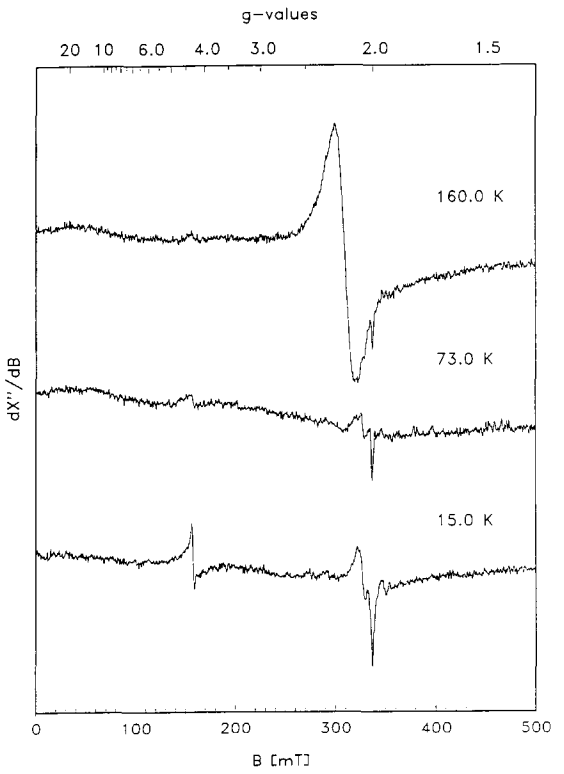

Fig. 2. EPR spectra of neuroblastoma SK-N-SH cells treated for 4 h with 1 mM ascorbic acid measured at 15 K (microwave frequency = 9.428 GHz), 73 K (microwave frequency = 9.429 GHz), and 160 K (microwave frequency = 9.429 GHz). The ferritin signal (spin density = 2040 arbitrary units) observed at 160 K is much sharper than in Fig. 1.

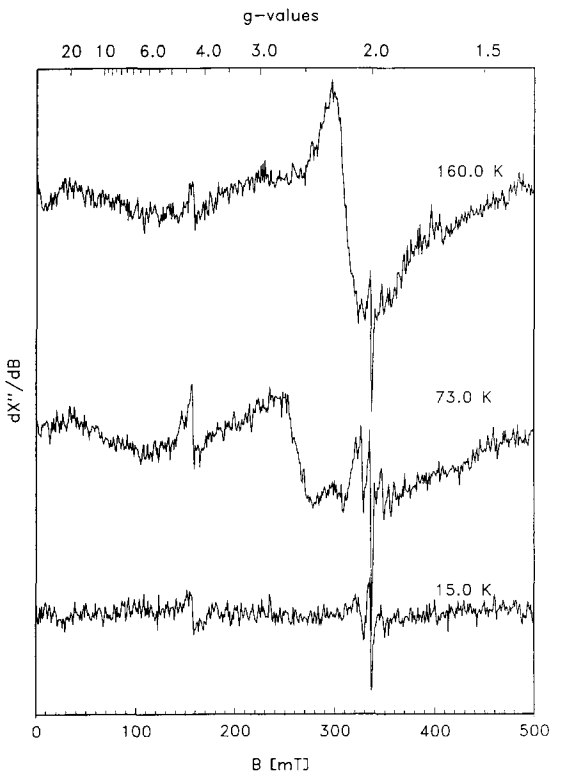

Fig. 3. EPR spectra of neuroblastoma SK-N-SH cells treated for 4 h with 1 mM ascorbic acid and subsequently for 10 min with 1 mM H_2O_2. Spectra were measured at 15 K (microwave frequency = 9.432 GHz; microwave power = 20 μW/20 dB); 73 K (microwave frequency = 9.428 GHz); and 160 K (microwave frequency = 9.428 GHz); spin density of the ferritin signal at 160 K = 3800 arbitrary units (corrected value, see text).

and the Bcl-2 protein preventing apoptosis [41], or they may induce oxidative damage to cell membranes or nucleic acids [42]. Normally, iron is stored in ferritin to prevent cell injury [13]. However, several reducing substances, among them ascorbic acid, are able to release iron from ferritin which was shown in in vitro assays [20,22].

In the present study we show that ascorbic acid decreases the size of the iron core of ferritin in situ which is reflected by a decrease of linewidth ΔH and also by drop of the relative area of the double integrated signal. These findings could indicate that a significant portion of iron in ascorbate treated cells is EPR-silent which might be attributed to the formation of high-spin Fe^{2+}. After additional treatment with H_2O_2 both, linewidth ΔH and area increase again. This could reflect the formation of large-size precipitates of ferric hydroxide originating from reoxidized ferrous iron. If this is true the observed line shape is then a superposition of (modified) ferritin and of ferric hydroxide precipitates.

The mechanism by which ascorbic acid releases iron from the ferritin core remains, however, unclear. Reduction of Fe^{3+} to Fe^{2+} in ferritin requires an agent exhibing a reduction potential lower than −230 mV at pH 7 [20]. The reduction potential of ascorbic acid is +166 mV [43]. Therefore ascorbic acid itself may not be the active releasing agent. However, ascorbic acid rapidly oxidizes in aqueous solution in the presence of oxygen and traces of metal ions [43]. One known intermediate of the oxygen/ascorbic acid reaction is the superoxide anion which can release iron from ferritin ($E^{\circ\prime} = -330$ mV, [20]).

The results presented in this in situ EPR study provide evidence for the hypothesis that ascorbic acid can induce iron release from ferritin. Since neuroblastoma cells contain elevated iron levels (stored in ferritin) and produce high amounts of H_2O_2 [26,30], conditions for pro-oxidative cell injury can be generated by application of ascorbic acid. This may open new ways in cancer therapy of this hardly curable tumor in stage IV, especially in combination with some cytostatic drugs [24].

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