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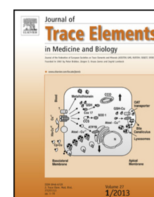

Copper brain protein protection against free radical-induced neuronal death: Survival ratio in SH-SY5Y neuroblastoma cell cultures

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ABSTRACT

In Creutzfeldt Jakob, Alzheimer and Parkinson diseases, copper metalloproteins such as prion, amyloid protein precursor and α-synuclein are able to protect against free radicals by reduction from cupric Cu^{+2} to cupreous Cu^{+}. In these pathologies, a regional copper (Cu) brain decrease correlated with an iron, zinc or manganese (Mn) increase has previously been observed, leading to local neuronal death and abnormal deposition of these metalloproteins in β-sheet structures. In this study we demonstrate the protective effect of Cu metalloproteins against deleterious free-radical effects. With neuroblastoma SH-SY5Y cell cultures, we show that bovine brain prion protein in Cu but not Mn form prevents free radical-induced neuronal death. The survival ratio of SH-SY5Y cells has been measured after UV irradiation (free radical production), when the incubating medium is supplemented with bovine brain homogenate in native, Cu or Mn forms. This ratio, about 28% without any addition or with bovine brain protein added in Mn form, increases by as much as 54.73% with addition to the culture medium of native bovine brain protein and by as much as 95.95% if the addition is carried out in cupric form. This protective effect of brain copper protein against free radical-induced neuronal death has been confirmed with Inductively Coupled Plasma Mass Spectrometry Mn and Cu measurement in bovine brain homogenates: respectively lower than detection limit and 9.01 μg/g dry weight for native form; lower than detection limit and 825.85 μg/g dry weight for Cu-supplemented form and 1.75 and 68.1 μg/g dry weight in Mn-supplemented brain homogenate.

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1. Introduction

In Creutzfeldt Jakob (CJD) and Alzheimer's diseases (AD), it has been shown that copper (Cu) bound to prion protein or amyloid protein precursor (APP) is able to protect against the deleterious effects of free radicals by valence reduction from Cu^{+2} to Cu^{+} [1,2]. In Parkinson (PD) and Lewy Body (LBD) diseases, Cu can bind with α-synuclein [3] even though this metal has been suspected in protein misfolding [4,5]. In these pathologies, a regional brain Cu decrease is observed with abnormal deposition of brain copper proteins in proteinase K-resistant β-sheet conformations. A manganese (Mn) increase has been described in human or animal transmissible spongiform encephalopathies [6,7].

Subsequent to a local brain Cu substitution by bivalent cations (Fe^{++}, Zn^{++}, Mn^{++}), we have recently proposed a free radical mechanism aimed at explaining CJD, AD, PD and LBD diseases [8]. Since they are in their lower oxidation degree +2, these bivalent cations cannot be further reduced. Free radicals, by accumulation in the cellular medium, will then induce local neuronal death and also, following a radical chain process, generate D-amino acid in metalloprotein sequences leading to their deposition as β-sheet structures. Deloncle et al. [9] have shown with bovine brain homogenates that in prion protein, Cu could be substituted for transition metals such as Mn in reductive medium, the substitution being reversible on return to oxidative conditions.

In order to demonstrate the protective effect of Cu metalloproteins against free radicals' deleterious effects, we wish to show

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with neuroblastoma SH-SY 5Y cell cultures that bovine brain prion protein in Cu but not Mn form prevents free radical-induced neuronal death. The survival ratio of SH-SY 5Y cells is measured after UV-induced free radical irradiation when the incubating medium is supplemented with bovine brain homogenate in native form or with Cu or Mn.

2. Materials and methods

2.1. Chemicals

Nitric acid suprapure® and chemicals of analytical grade for preparation of bovine brain homogenates were purchased from Merck. All chemicals for Cu and Mn (ICP-MS) measurements and Triton X100 were of suprapure quality and purchased from Fisher Scientific (Illkirch, France), Rhodium from SCP Sciences (Villebon sur Yvette, France)

For cell cultures, chemicals were of analytical grade and the quantity of total proteins was measured with the Quant-it® protein assay, all purchased from Gibco Invitrogen laboratories.

2.2. Instrumentation

UVA irradiation was performed at 372 nm with a SOL2 apparatus (Hönle, Plannegg, Germany).

ICP-MS: Instrument parameters using a NexION®3OOX ICPMS spectrometer (PerkinElmer®, Inc., Shelton, CT, USA), are in agreement with Li et al. [10].

2.3. Sample preparation of bovine brain obex homogenate

Bovine brain homogenates were prepared, from 300 bovine brain obex negative to Prionics test [11]. Protein purification was carried out by trichloroacetic defecation before division into three identical fractions. The first fraction of non-treated bovine obex homogenate was submitted to dialysis at +4 °C in phosphate buffer saline (pH 7.2) on an Amicon ultrafiltration apparatus equipped with a Millipore cellulose regenerated membrane allowing filtration retention for molecules over 3000 kDa. Filtration was carried on until Cu or Mn was found in the dialysate under the limit of detection (LOD) by ICP MS.

The second fraction was set up under oxidative conditions by addition of 5 mM sodium persulfate and supplemented with 10^{-4} M Cu sulfate. This persulfate concentration was verified as oxidative in the homogenate by precipitating copper as blue cupric hydroxide. This second fraction was dialysed as described above. The third fraction was set up under reductive conditions by addition of 5 mM hydrogen peroxide. These conditions were verified as reductive in the homogenate by precipitating Cu as brown cuprous hydroxide. This third fraction was then supplemented by manganese sulfate (10^{-4} M), and submitted to dialysis like the first fraction. In order to obtain fluid mixtures suitable for further uses, all three resulting dialysed bovine brain homogenates were diluted up to 80% PBS and pH adjusted to 7.00.

2.4. SH-SY 5Y neuroblastoma cell culture

The human SH-SY5Y neuroblastoma cell line(ATCC®) was cultured in a Memmert incubator (+37 °C, 5% CO_2, hygrometry 80%) and was initially allowed to grow in a single 75 cm^2 flask (NUNC™) before seeding evenly into eight 25 cm^2 flasks (NUNC™). They were then allowed to grow until cell confluence (3×10^6 cells/flask) before differentiation with retinoic acid (10^{-5} M in sterile DMSO). The culture change was carried on every two days. In order to avoid a possible effect of retinoic acid with regard to free radicals on bovine brain obex homogenate, whole nutritive medium was withdrawn from the eight flasks and replaced with 4 ml nutritive medium. The cell cultures were then supplemented with the different Obex suspensions containing 120 μg protein/ml for a total 5 ml volume. Flasks 1 and 2 were supplemented with 1 ml nutritive medium more; flasks 3 and 4 with 1 ml diluted native bovine brain homogenate; flasks 5 and 6 with 1 ml diluted copper bovine brain homogenate; flasks 7 and 8 with 1 ml diluted manganese bovine brain homogenate.

2.5. Irradiation process

Flasks with even numbers were submitted under slight stirring to a 5 min UV irradiation with a SOL2 apparatus under 14 mW/cm^2. Odd-numbered flasks were not submitted to irradiation. After UVA irradiation, the nutritive mediums of all the flasks were removed and rinsed twice with 5 ml PBS. They were then filled with 15 ml of nutritive medium supplemented with retinoic acid (10^{-5} M in sterile DMSO) and allowing for 24 more hours of cell growth before numeration of the surviving cells.

Numeration of the surviving cells was carried on as follows: The medium was withdrawn and the flask rinsed twice with 5 ml PBS before addition of 2 ml trypsin EDTA and 8 ml nutritive culture medium. Centrifugation was then carried out at +4 °C for 5 min at 1500 rpm. The supernatant was removed and replaced with 1 ml of nutritive medium. The resulting cell suspension in nutritive medium was diluted 1:10 with 0.2% PBS Blue Trypan for numeration with a Malassez cell.

2.6. ICP-MS copper and manganese analysis

Samples of 1 ml of the diluted bovine brain homogenates native, or subjected to Cu or Mn supplementation, were digested according to the method of van Ginkel et al. [12] as modified for dry-weight samples. After homogenization, mineralized samples were diluted 1/100 in a mixed solution of nitric acid (1%) and Rh (100 μg/L). Standards and reference material were prepared following the same procedure. Aqueous standard solutions prepared daily from mono-element standard of 1 g/L were used for calibration. As the differences of the calibration slopes of samples and acid-based standards were less than 10%, the external calibration mode (linear thru zero) was used. Concentrations of the elements were as follows: blank, 5, 10, 20, 50 and 100 μg/L for Cu and blank, 2, 5, 10, 20 and 50 μg/L for Mn. To compensate for possible instrumental drift and matrix effects, all samples and solutions contained of Rh and 0.5% of Triton X100 (1% in ultrapure water). Peak area mode and 2 s data acquisition time and three replicates were used for measurement.

2.7. Quality assurance and quality control

For assessment of the accuracy and precision of the concentration of Cu and Mn determined in mineralized samples, a certified reference material NIST 1640a (National Institute of Standards & Technology, USA) was used.

2.8. Statistical analysis

Results were analyzed using GraphPad Prism® software. Comparisons between groups were performed by ANOVA followed by Newman-Keuls' test. The level of significance was set at $p < 0.05$.

3. Results and discussion

In Table 1, the survival percentages of neuroblastoma cells after 5 min of UVA irradiation are indicated. The cell suspensions supplemented with 20% diluted Obex-Cu in cupric form present a

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