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Uptake and intracellular distribution of labile and total Zn(II) in C6 rat glioma cells investigated with fluorescent probes and atomic absorption

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Abstract

The uptake, intracellular distribution and cytotoxicity of high doses of extracellular zinc was investigated in C6 rat glioma cells. Net zinc uptake occurred only above certain thresholds in time and concentration, below them no alterations of the intracellular zinc level were observed. These results were obtained by measurements with the fluorescent dye Zinquin and by atomic absorption spectrometry, yielding similar results with both methods. Sequestration of zinc in intracellular vesicles was observed by fluorescence microscopy. A protective effect of vesicular sequestration is indicated, because increased levels of intracellular zinc located in vesicles did not necessarily lead to an increase in cytotoxicity. We were able to show that in C6 cells, in contrast to other cell lines, zinc that is released from proteins by the NO donor SNOC is also sequestered in vesicular structures. These zinc-carrying vesicles showed to be constitutive and are assumed to have a function in the maintainance of the cytosolic content of Zn^{2+} ions.

Abbreviations: AAS, atomic absorption spectrometry; BSA, bovine serum albumine; DMEM, Dulbeccos modified Eagle's medium; FCS, fetal calf serum; HBS, HEPES-buffered saline; PBS, phosphate-buffered saline; SNOC, S-nitrosocysteine; TSQ, N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide; Zinquin, [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy) acetic acid]

Introduction

The trace element zinc is known to be an essential part of more than 300 enzymes and a great amount of transcription factors (For reviews see Vallee & Falchuk 1993; Berg & Shi 1996).

Also a role in proliferation (Grummt *et al.* 1986), differentiation (Petrie *et al.* 1991; Schmidt & Beyersmann 1999) and apoptosis (Zalewski *et al.* 1993) has been shown for zinc. All these processes require a complex regulation of the cellular zinc content and its intracellular distribution. At present three main mechanisms are discussed to be important for zinc homeostasis. (1) Zinc can be buffered by binding mainly at cystein and histidin residues. The major zinc buffering protein is metallothionein which binds zinc as well as some other heavy metals such as cadmium (reviewed in Palmiter 1998; Fischer & Davie 1998). (2) Zinc export is mediated by plasma membrane transporters. The first mammalian zinc transporter described was ZnT-1 (Palmiter & Findley 1995). Cells overexpressing this protein showed enhanced resistance against high concentrations of extracellular zinc and exhibited lower intracellular zinc levels. (3) Zinc is sequestered by vesicular membranes. The zinc transporter ZnT-2 confers resistance to zinc by vesicular transport and ZnT-3 is thought to be a transport protein for zinc into synaptic vesicles (Palmiter *et al.* 1996a,b). The fluorescent probes Zinquin (Zalewski *et al.* 1993) and TSQ (Frederickson *et al.* 1987) are zinc specific fluorescent dyes with similar excitation and emission wavelengths and a high selectivity, chemically based on a quinoline backbone. Both dyes can easily enter the cell but Zinquin contains an ethyl ester function, that is cleaved by

unspecific intracellular esterases. This leads to a membrane impermeable molecule that is trapped inside the cell because of its charge.

It has been reported for Zinquin to form complexes both with free and loosely protein bound zinc (Coyle *et al.* 1994), so we will use the term 'available intracellular zinc' for the zinc pool detected by Zinquin. An absolute quantification of the available zinc is not possible, because the computed concentrations are extremely high (Brand & Kleinecke 1996) indicating a fluorescence enhancing effect of the cellular environment.

Under the fluorescence microscope cells incubated with Zinquin and TSQ show cytosolic but not nuclear fluorescence, although it is known that a considerable amount of the cellular zinc is located in the nucleus. This is presumably due to the tight zinc binding of nuclear proteins, so that this zinc is not available for the dyes. Vesicular zinc has been visualized in some regions of the brain, the mossy fiber boutons, with TSQ (Frederickson *et al.* 1989) and with Zinquin (Zalewski *et al.* 1993; Palmiter *et al.* 1996a,b). Treatment of cells with NO leads to the formation of nitrosothiols causing the release of cystein bound zinc. Investigations of the effects of NO on labile zinc with various cell lines using Zinquin showed an increased cytosolic fluorescence and intense nuclear staining but no vesicular sequestration (Berendji *et al.* 1997).

It has been reported that Zinquin may increase the zinc uptake of some cell lines (Palmiter *et al.* 1996 a), so we decided to load the cells with Zinquin after the $ZnCl_2$ incubations were finished, to exclude such an influence.

In the present study we investigate the uptake of zinc in C6 rat glioma cells depending on time and extracellular zinc concentration with two different methods, fluorimetrically and by AAS. Furthermore the intracellular localization and the resulting toxicity of zinc uptake is studied.

Materials and methods

Material

All tissue culture reagents were purchased from GIBCO (Eggenstein, Germany), petri dishes, multiwell plates and eight well Lab-Tec chamber slides were obtained from Nunc (Wiesbaden, Germany). Zinquin ethyl ester was from Alexis (Grünberg, Germany), Zinquin free acid from Luminis (Adelaide, Australia), TSQ from Teflabs (Austin, USA) and HNO_3 (suprapur) from MERCK (Darmstadt, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

Cell culture

C6 rat glioma cells (Benda *et al.* 1968) were grown in DMEM high Glu supplemented with 5% fetal bovine serum, 100 units/ml Penicillin and 100 μg/ml Streptomycin in 92 mm culture dishes in a humidified atmosphere at 37 °C and 10% CO_2. The cell line was kindly provided by Prof. Dr. D. Leibfritz, University of Bremen.

Neutral red assay

For the viability tests we used a modification of the procedure from Babich *et al.* 1986. Cells were plated (1.5×10^5 cells per well) on a 24 multiwell dish. After 24 h the cells were treated with zinc in concentrations from 0 to 450 μM for 1, 2 and 3 h respectively, washed with PBS once and supplemented with 1 ml medium. After additional 24 h cells were incubated with 16 μg/ml neutral red under normal culture conditions for 3 hours. Cells were lysed in ethanol/H_2O/acetic acid 50:49:1 and the absorption of the dye was measured at 540 nm in a Milton Roy Spectronic 1201 spectrophotometer.

Atomic absorption spectrometry

For the determination of the total cellular zinc content 2×10^7 cells were incubated with different zinc concentrations for 3 h, washed with PBS twice, detached in 1 ml of PBS and 50 μl were taken to determine the protein content of the sample using Bicinchoninic Acid as described by Smith *et al.* (1985) with an incubation time of 30 min at 60 °C. The remaining cell suspension was centrifuged and the pellet was treated with 100 μl 33% H_2O_2 and 100 μl 65% HNO_3 (suprapur) at 85 °C overnight. Samples were dissolved in 1 ml 0,2% HNO_3 and the zinc concentration was determined using a Perkin Elmer 2380 atomic absorption spectrometer with a HGA-400 graphite furnace under argon gas. All solutions were prepared from Milli-Q water and all plastic and glass wares were previously treated with 0,2% HNO_3 to avoid contaminations with extraneous zinc ions.

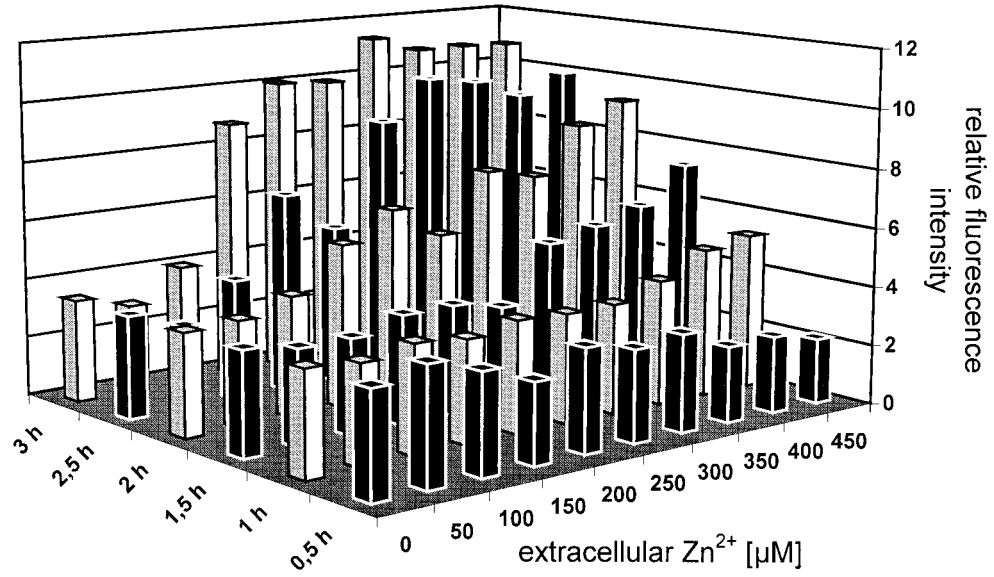

Figure 1. Dependence of zinc uptake by C6 cells on time and extracellular concentration of $ZnCl_2$. Zinquin was used to estimate the relative intracellular levels of available zinc ions. Measurements were carried out as described in the methods section. Data represent the averages of six independent measurements, and the relative standard deviation was $+/-0{,}85$ relative fluorescence intensity units.

Fluorescence measurements

Cells were transferred to 96 multiwell plates (5×10^5/cells/250 μl/well). After 24 h the confluent cells were incubated as described in the text. Subsequently cells were washed with HBS and loaded with Zinquin (25 μM in HBS-buffer containing 0,3% BSA) for 30 min at 37 °C or TSQ (30 μM in HBS-buffer containing 0,3% BSA) for 15 min at room temperature. After the loading the cells were washed twice and 100 μl HBS were added. The resulting fluorescence was measured at excitation/emission wavelengths of 364/485 nm (for both dyes) in a Perkin Elmer LS 50 B luminescence spectrometer.

Release of protein bound intracellular zinc

A 100 mM stock solution of SNOC (S-nitrosocysteine) was prepared not more than two minutes before the incubation was started following the procedure of Kröncke & Kolb-Bachofen (1999). This solution was directly added to the culture medium to achieve the desired concentrations.

N-ethylmaleimide was used as a 100 mM stock solution in ethanol. After the dye loading cells were treated with 1 mM of N-ethylmaleimide in HBS for 1, 5 and 10 min.

To exclude a possible fluorescence increasing effect the fluorescence of a 2.5 μM solution of Zinqin (free acid) in HBS before and after the addition of 10 mM SNOC or 1 mM N-ethylmaleimide, respectively, was investigated. Also the effect of these two substances on the autofluorescence of a suspension of 3×10^6 cells in 2 ml HBS was tested.

Fluorescence microscopy

C6 cells were grown to confluence on 8 well Lab-Tec chamber slides and incubated as indicated in the text. Loading with Zinquin or TSQ was carried out as described under fluorescence measurements. Photographs were taken at a 400 fold magnification on a Zeiss Axioskop 50 Typ B using a 365/420 nm filter set with a beamsplitter at 395 nm (Zeiss filter set 09).

Results

Correlation between intra- and extracellular zinc levels

To analyze the change in intracellular available Zn^{2+} dependent on extracellular zinc concentration, the fluorescent zinc-specific probe Zinquin was employed. Figure 1 demonstrates the time and concentration dependent increase in intracellular available Zn^{2+}. Although zinc-specific fluorescence intensified with increasing extracellular zinc concentration and incubation time, there were no proportional relations.

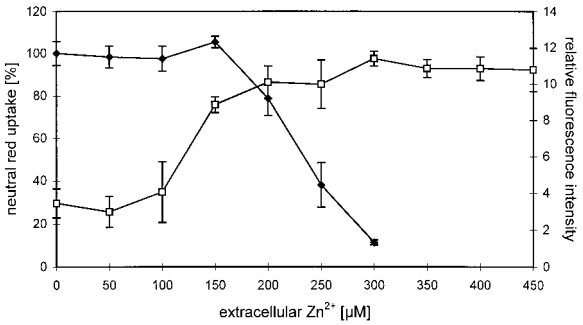

Figure 2. Viability (solid symbols) and intracellular zinc levels (open symbols) of C6 cells dependent on extracellular $ZnCl_2$ concentration after three hours of incubation. Viability was determined by the Neutral Red assay as described in the methods section; relative intracellular zinc levels were estimated by Zinquin fluorescence data from Figure 1. The data are means from eight viability and six fluorescence measurements, respectively, with the standard deviations shown.

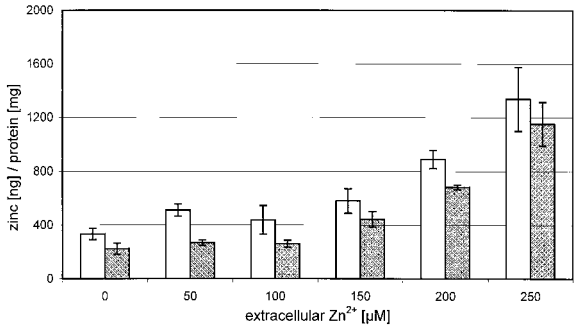

Figure 3. Total cellular zinc content of C6 cells dependent on extracellular $ZnCl_2$ concentration after three hours of incubation. Open bars show results obtained immediately, filled bars represent data obtained after additional 30 minutes in incubation buffer. Experimental details are given in the methods section. Data are means of four independent experiments with the standard deviations shown.

Instead, there were thresholds in concentration and time below which no significant increase in intracellular available Zn^{2+} levels occurred. Concentrations of extracellular zinc below 150 μM or times below 60 minutes caused no measurable increase in available intracellular zinc. It took 90 min at 150 μM and 60 min at 300 μM to cause an increase in intracellular zinc.

Toxicity and intracellular level of available Zn^{2+}

To investigate the relation between intracellular available zinc and cytotoxicity, the Neutral Red assay was used. Figure 2 shows that a three h incubation with extracellular $ZnCl_2$ above 100 μM led to an increase in intracellular free zinc levels which reached a plateau at 200 μM extracellular $ZnCl_2$.

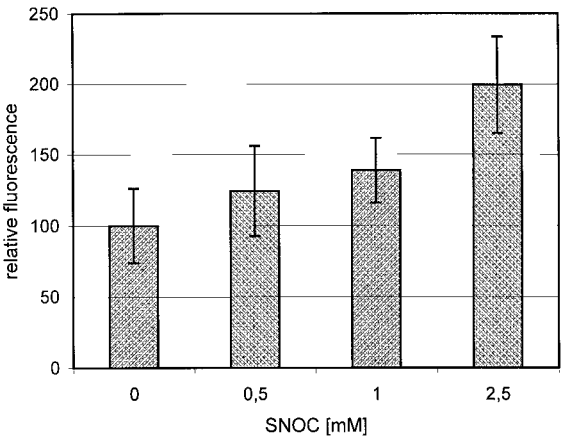

Figure 4. Effect of treatment with S-nitrosocysteine (SNOC) on available intracellular zinc levels in C6 cells. Cells were incubated with the SNOC concentrations shown for one hour at 37 °C and loaded with TSQ for 30 minutes at 4 °C. Intracellular available zinc was estimated from the fluorescence of the zinc-TSQ complex. The fluorescence of control cells loaded with TSQ was set as 100. Data are given as means of six measurements. For further experimental details, see the methods section.

Incubation with up to 150 μM $ZnCl_2$ for three h caused no significant toxicity, although these conditions led to an increase in intracellular available zinc levels. In the range between 200 and 300 μM extracellular $ZnCl_2$, a steep decline in cell viability was observed in spite of no further increase in the measurable intracellular zinc level. Toxicity measurements at the shorter incubation times of one and two h showed that there was no detectable toxic effect of extracellular zinc at all concentrations investigated (data not shown). Hence, the level of available zinc inside the C6 cells as such is not directly related to cytotoxicity.

Uptake and total cellular zinc content

The increase in cellular Zinquin fluorescence reached a plateau at a 200 μM medium $ZnCl_2$ concentration. To answer the question whether zinc uptake was limited by a protective cellular mechanism or whether the fluorescent probe was simply saturated, the total cellular zinc content was determined by atomic absorption spectroscopy. C6 cells were treated with additional $ZnCl_2$ concentrations from 0 to 250 μM for three h in normal culture medium. Figure 3 (open bars) shows that the total zinc content of cells did not significantly increase at the lower medium concentrations up to 100 μM. This observation corresponds to the lack of increase in intracellular available zinc in this range. Hence, the cells seem to possess mechanisms which

protect against extracellular zinc up to 100 μM under these conditions. In contrast, total cellular zinc still increased between 150 and 250 μM medium zinc concentrations, i.e., a range where no corresponding increase in the Zinquin fluorescence occurred.

As mentioned in the introduction, Zinquin seems to influence the uptake of zinc, so we chose to load the cells with the fluorescent probe after the zinc treatment. To investigate the amount of zinc lost in these 30 min in incubation buffer, the cellular zinc content was determined by atomic absorption spectroscopy. Cells were incubated with medium $ZnCl_2$ as described above and subsequently held for 30 min in incubation buffer. Figure 3 (filled bars) shows that at all extracellular zinc concentrations employed, the incubation caused a loss of total cellular zinc that averaged at 177 ng zinc per mg of protein. Interestingly, these losses were not correlated with the total cellular zinc contents, but had a constant value.

Mobilization of intracellular bound Zn^{2+} by nitric oxide

S-Nitrosocysteine (SNOC) is a compound that spontaneously releases nitric oxide after its preparation. Nitric oxide reacts with thiol groups like cysteine side chains forming nitrosothiols. In the case of zinc ions bound to thiol groups, SNOC causes the release of protein-bound zinc. We investigated the effect of different concentrations of SNOC on the available intracellular zinc in C6 cells in relation to an untreated control. Cells were incubated with SNOC at various concentrations for one hour and subsequently labelled with TSQ, a zinc-specific fluorescent probe chemically related to Zinquin. Figure 4 shows that the intracellular available zinc increases in a manner dependent on the concentration of SNOC. 2.5 mM of this compound caused a doubling of the control value of the fluorescence caused by the zinc-TSQ complex. In further controls, no increase in TSQ fluorescence was found if the dye was treated with SNOC, and there was no change in the autofluorescence of C6 cells caused by SNOC. Hence, it is concluded that nitric oxide mobilizes protein-bound zinc ions resembling the amount of the available zinc in untreated control cells.

Intracellular localization of Zn^{2+} and its modulation by nitric oxide.

The biological functions of cellular zinc are believed to be mediated by the intracellular localization of this metal. Hence, we studied the intracellular distribution of zinc with fluorescent probes. Figure 5 shows epifluorescence photomicrographs of C6 cells treated with various concentrations of $ZnCl_2$ for three h and subsequently loaded with Zinquin. At all subtoxic zinc concentrations applied the fluorescence was localized in the cytoplasm and no detectable fluorescence was observed in the nuclei of the cells. Whereas the cytoplasmic distribution of Zinquin fluorescence was homogeneous at the lower external zinc concentrations up to 100 μM, at 150 μM $ZnCl_2$ zinc fluorescence started to appear in punctuate structures interpreted to be vesicles indicating a sequestration of the additional zinc. At 300 μM $ZnCl_2$, which represents a cytotoxic concentration, there is a breakdown in intracellular zinc distribution leading to a brighter cytoplasm and the disappearance of the difference to the nuclear staining.

To investigate whether the protein-bound zinc follows the same pattern of intracellular sequestration, cells were incubated with SNOC prior to analysis with the fluorescent probe TSQ. Figure 6 demonstrates that the zinc that was mobilized by nitric oxide from protein-bound states also accumulated within vesicular structures. These results were obtained with both fluorescent zinc-specific probes, TSQ and Zinquin (not shown).

To investigate the time needed to induce vesicular sequestration following intracellular mobilization of zinc by thiol reactive agents, 1 mM of N-ethylmaleimide was added to cells preincubated with Zinquin. It was found that an incubation time of 5 min was sufficient to cause the appearance of vesicular structures.

Discussion

Our studies on zinc uptake by C6 cells demonstrate the existence of a regulation system that maintains the cellular zinc level even in the presence of high extracellular concentrations of this metal. However, if the extracellular treatment with zinc exceeds certain thresholds in time and concentration this regulation is not effective any more, the intracellular level of available zinc increases.

Below the threshold of extracellular zinc (e.g., up to 150 μM for three h) the fluorescence of Zinquin was evenly distributed throughout the cytosol, with nearly no nuclear localization. At higher zinc concentrations we found an increased level in intracellular Zn^{2+} which has been observed fluorimetrically

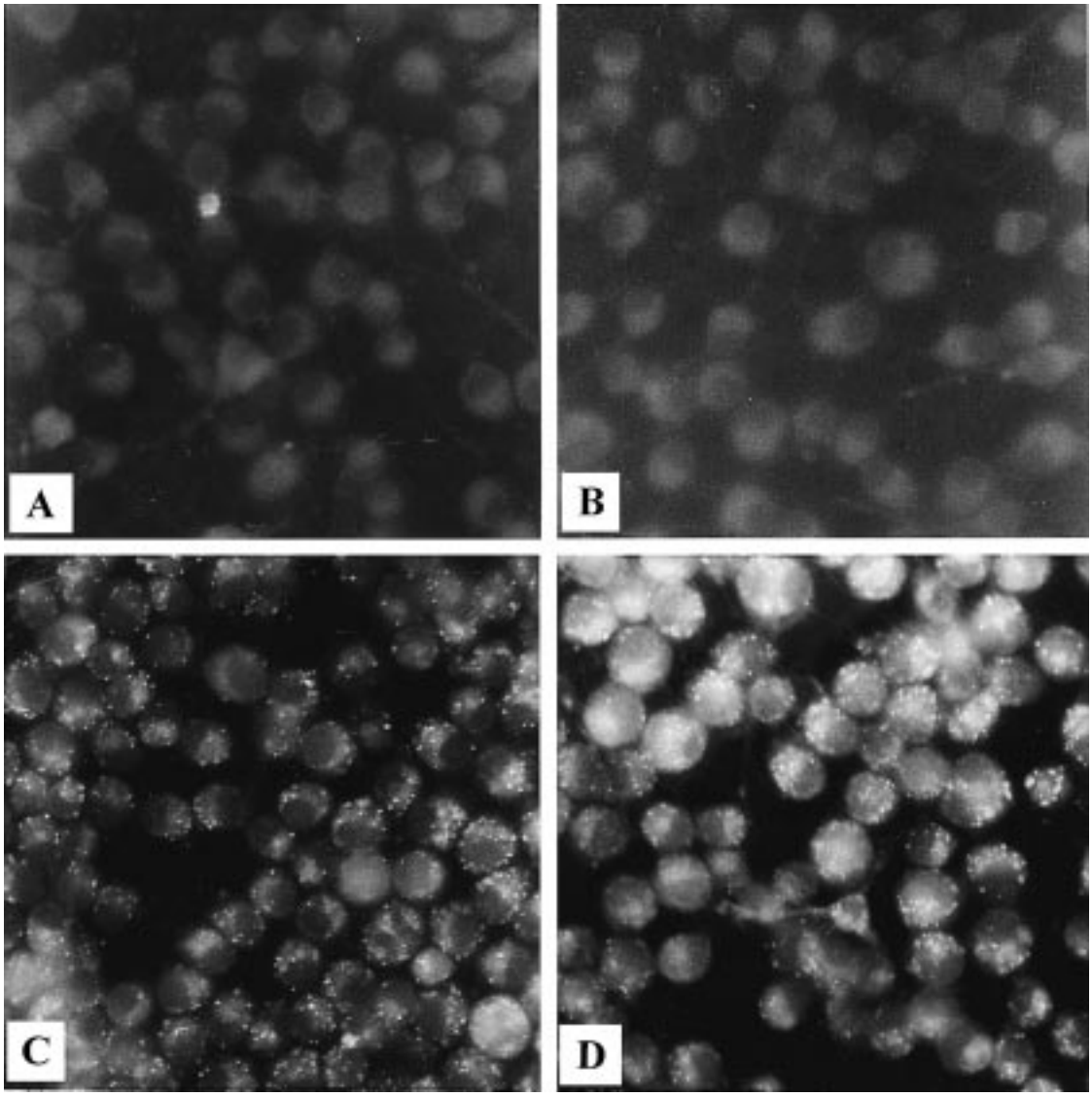

Figure 5. Intracellular distribution of zinc in C6 cells treated with $ZnCl_2$ for three hours and subsequently loaded with Zinquin (25 μM for 30 minutes at 37 °C. $ZnCl_2$ concentrations were 0 μM (A), 100 μM (B), 150 μM (C), 300 μM (D).

(available zinc) and by AAS (total zinc). Under the fluorescence microscope a bright punctuate fluorescence became appareant, indicating a sequestration of the additional zinc in vesicles which tentatively are named 'zincosomes'. The level of cytosolic and nuclear fluorescence obviously remained unchanged.

Cell viability tests with the neutral red assay showed that under these conditions when significant amounts of zinc had entered the cells there were no toxic effects observable. This result shows, that an increased amount of intracellular zinc which is located in vesicels is not cytotoxic. At even higher levels of extracellular zinc (starting at 200 μM for 3 h), the cells lost viability coming along with a cytosolic and nuclear Zinquin fluorescence increase, indicating a breakdown of the cellular control of zinc distribution. These results are in agreement with the properties of the mammalian zinc transporter protein ZnT-2 which is assumed to prevent the toxicity of zinc by vesicular sequestration (Palmiter *et al.* 1996a).

We found a saturation of Zinquin fluorescence in cells that were incubated with more than 150 μM medium zinc for three h, leading to a plateau in fluorescence, while AAS still showed an increase in total

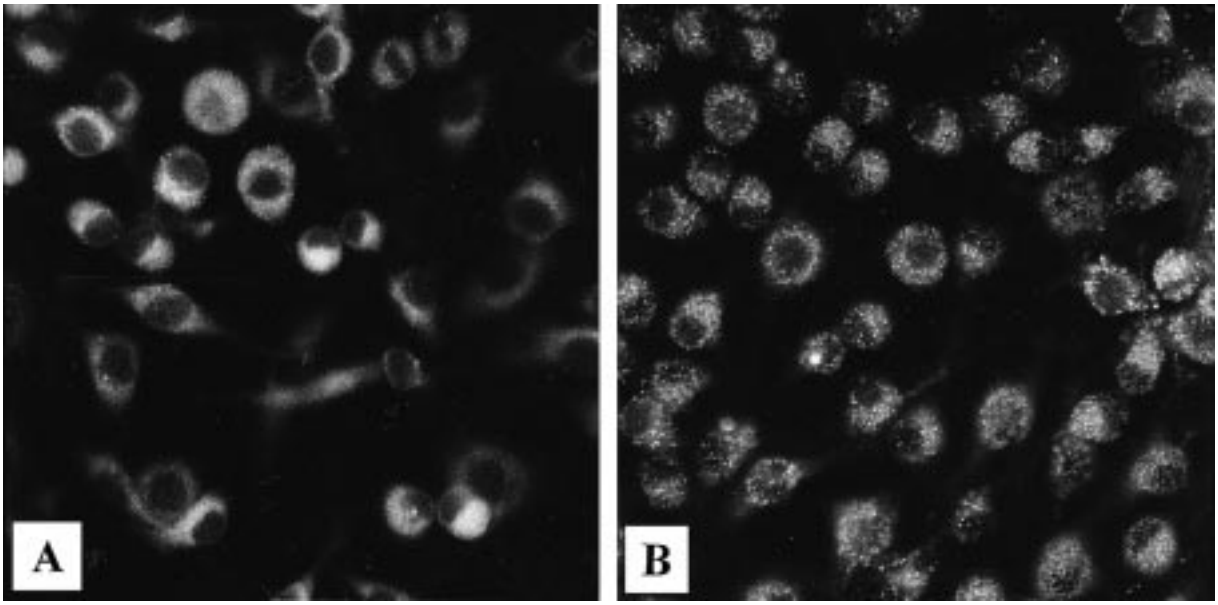

Figure 6. Effects of nitric oxide on the intracellular localization of zinc in C6 cells. A.) Cells incubated with TSQ only. B.) Cells incubated with 10 mM SNOC for one hour at 37 °C and subsequently with 30 μM of the fluorescent zinc probe TSQ for 15 minutes at room temperature.

cellular zinc content. This is no major drawback for the use of this dye, because under these conditions the cells were already severely damaged, and physiological changes in the available zinc level are still in the range that can be sensed with Zinquin. The amount of zinc lost during the 30 min incubation time was independent of the total cellular zinc content. Thus, we postulate that extracellular zinc associated to the plasma membrane has been removed by binding to bovine serum albumine.

If intracellular zinc is mobilized by the reaction of the NO-donor SNOC with zinc binding cysteine sulfurs, the fluorescence of the zinc complex of the probe TSQ increases. As in the case of excessive zinc taken up from the medium, the zinc mobilized by nitric oxide is mainly found in intracellular spots indicating accumulation in vesicles. No significant nuclear fluorescence of TSQ could be found. On the other hand, vesicular structures were also found in untreated control cells but to a much lesser extent. The appearance of vesicles after only five minutes incubation with N-ethylmaleimide shows that zinc vesicles are constitutive because a *de novo* synthesis of the transporter protein induced by increased levels of available intracellular zinc could not have taken place in this time. So we postulate that these 'zincosomes' play a role in zinc homeostasis as well as in zinc detoxification by regulating the available cytosolic Zn^{2+} level.

With some other cell lines a nuclear accumulation of zinc-Zinquin fluorescence after treatment with NO donors has been observed (Berendji *et al.* 1997). For L929 fibroblasts this effect has been successfully reproduced in our lab (result not shown). The absence of nuclear TSQ and Zinquin Fluorescence in C6 cells under the same conditions indicates a more effective control of intracellular available zinc in this cell line.

Several zinc transporters, the possible tools for such a regulation, have been recently identified (reviewed by McMahon & Cousins 1998). Zinc-storing vesicles, the 'zincosomes', may have a similar significance for the control of availability of zinc for regulatory zinc proteins, e.g., zinc finger transcription factors, as calcisomes do have for the control of the intracellular free Ca^{2+} concentration.

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