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Journal of Nutritional Biochemistry 23 (2012) 1256–1263

Journal of Nutritional Biochemistry

Comparison of intracellular zinc signals in nonadherent lymphocytes from young-adult and elderly donors: role of zinc transporters (Zip family) and proinflammatory cytokines

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Received 8 February 2011; received in revised form 8 July 2011; accepted 15 July 2011

Abstract

Intracellular zinc homeostasis is crucial in regulating the inflammatory/immune response at any age. It is tightly regulated by zinc transporters that control influx, efflux and compartmentalization of zinc within the cells. Specific methods for detecting the age-related differences in intracellular zinc signaling are poorly described. We report a novel assay induced after the *in vitro* zinc addition in peripheral blood mononuclear cells (PBMCs) and in lymphocytes from young and old donors in the absence/presence of *in vitro* zinc depletion (using EDTA). The intracellular labile zinc variations are monitored over time by flow cytometry using Fluozin-3 AM probe. The best curve fit of the data is calculated using a nonlinear regression model defined as follows: pr3/[1+Exp(−pr1−pr2*Xt)]. Pr1 depends on the initial free zinc value (time 0); pr2 describes the rate of the speed in reaching the maximum intracellular free zinc concentration; pr3 represents the maximum intracellular zinc increment (plateau curve); Xt is the time course. Age-related intracellular free zinc variations occur in PBMCs and lymphocytes incubated in EDTA-supplemented medium. The higher plateau of the curve (pr3) was observed in younger subjects. An up-regulation of Zip genes (Zip1, Zip2, Zip3), influencing zinc influx, is more pronounced in the young than old donors. Interleukin-6 and tumor necrosis factor-α overproduction was enhanced in old individuals, suggesting the presence of more marked zinc deficiency and chronic inflammation. In conclusion, the determination of intracellular zinc signals induced by *in vitro* zinc addition using logistic parameters may be useful to estimate the rate of intracellular zinc homeostasis and its role in inflammatory/immune response in aging.

Keywords: Zinc signal; Inflammation; Zinc transporters; Zinc homeostasis; Ageing

1. Introduction

Zinc ions have been primarily regarded as a static component of zinc enzymes and transcription factors [1]. Zinc modulates the expression of more than a hundred genes within the immune cells [2], and it is an essential trace element for immune function [3]. Emerging literature reports a role for zinc as an intracellular signaling molecule [4,5] affecting proinflammatory cytokine production [interleukin (IL)-6, tumor necrosis factor (TNF)-α] from human leukocytes after lipopolysaccharide (LPS) or other antigen stimulations [6] as well as T-cell proliferation induced by IL-2 stimulation [7]. Therefore, a correct intracellular zinc homeostasis is indispensable for an adequate inflammatory/immune response to external noxae [8].

Intracellular zinc homeostasis is tightly regulated by a set of specialized proteins, which control the zinc influx (Zip family proteins) [9], the zinc efflux (ZnT family) and its compartmentalization and buffering by metallothionein family [10]. Plasma zinc levels decrease in response to endotoxin (LPS) or proinflammatory cytokines due to zinc translocation into the liver [11]. LPS treatment modulates intracellular zinc homeostasis affecting the gene expression of some zinc transporter in dendritic cells [12] and in monocytes [11].

Zinc deficiency leads to an overproduction of Zip genes, most likely as a response to counterbalance the reduction of intracellular zinc [13]. Among Zip members, three highly conserved components (Zip1, Zip2, Zip3), belonging to subfamily II of the solute-carrier (Slc) 39a family, are relevant for the adaptation to dietary zinc deficiency [14]. During pulmonary inflammatory diseases, characterized by zinc deficiency [15], leukocytes display Zip1 and Zip2 overexpressions [16,17].

In aging, the zinc deficiency impairs zinc-dependent signaling with subsequent impaired immune/inflammatory response and appearance of several age-related diseases [18]. The zinc signaling is very important because, other than acting as "second messenger" for cellular proliferation and differentiation [19], it plays a pivotal role in intracellular zinc homeostasis, which is in turn relevant for healthy aging and in preventing some inflammatory age-related diseases (cardiovascular diseases and type 2 diabetes) [18,20]. An effect of *in vitro* zinc treatment on several signaling pathways has been

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doi:10.1016/j.jnutbio.2011.07.005

already described in mammalian cells [21] and, in particular, in monocytes [22]. However, specific studies investigating the changes of intracellular zinc signals in the immune cells during a zinc deficiency in relation to the age donors are not still available. Thus, the aim of the present study is to measure the variations of intracellular labile zinc during *in vitro* zinc depletion (using EDTA) and after the *in vitro* addition of extracellular zinc in peripheral blood mononuclear cells (PBMCs) and nonadherent lymphocytes from young-adult and old donors. An association with the gene expression of specific zinc transporters deputed to zinc influx, such as Zip1, Zip2, Zip3, and proinflammatory cytokines, will be analyzed.

2. Materials and methods

2.1. Subjects

This study enrolled 18 healthy young-adult volunteers (nine males and nine females, age range 30–40 years) and 27 healthy old donors (12 males and 15 females, age range 68–83 years).

Recruitment of healthy elderly volunteers was made on the basis of their clinical history and routine laboratory blood tests. They did not suffer from diabetes or any other clinical symptoms or history of cardiovascular diseases. The INRCA Hospital Ethic Committee approved the project. Informed consent was obtained from each individual in compliance with European privacy laws.

Heparinized venous peripheral blood samples were collected after an overnight fast and underwent basal biochemical laboratory determinations. Plasma was collected and frozen at −80°C until used. Peripheral blood mononuclear cells were separated by conventional density gradient centrifugation, collected, washed and immediately used for flow cytometry (FC) assessments.

2.2. KG-1a and lymphocyte culture condition

KG-1a cell line was used in order to optimize the assay. The cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell cultures (1×10^6/ml) were prepared in Iscove's modified Dulbecco's medium (ATCC Manassas, VA, USA) supplemented with 20% of fetal bovine serum (FBS) (GIBCO), 100 U/ml penicillin and streptomycin (Invitrogen, MI, Italy). In order to obtain nonadherent lymphocytes from PBMCs of young and old donors, lymphocytes were isolated through the incubation of PBMCs in tissue culture plates with RPMI-1640 medium (GIBCO) supplemented with 100 U/ml penicillin and streptomycin, 1% glutamine and 10% heat-inactivated FBS (37°C; pH 7.4; 5% CO_2). "Zinc-free" supplemented RPMI-1640 medium (zinc concentration less than 1 ppb) was used by treating the medium with 5% Chelex 100 (Sigma-Aldrich, Milan, Italy). Plates were then washed with warm medium to recover nonadherent lymphocytes, while adherent monocytes were removed. Nonadherent lymphocytes (2×10^6/ml) were incubated for 24 h (37°C and 5% CO_2) in "zinc free" RPMI-1640 medium in the presence of EDTA (0.5 mM) in order to obtain zinc depletion.

Although EDTA is not a zinc-selective chelator, we have used this compound in order to create an extracellular zinc deprivation, as suggested by Lavoie et al. [23] and by Kumar and Satchidanandam [24]. Moreover, the medium was previously treated with Chelex-100, which is the most powerful and widely used chelating resin to remove all divalent cations including zinc [25]. Therefore, the addition of EDTA induces a deeper zinc depletion not impugned by other metals.

At the end of 24 h of culture, cells were recovered, washed two times with phosphate-buffered saline (PBS) and counted at microscopy in a hemocytometer chamber. The cell viability (more than 90%) was determined by propidium iodide.

2.3. Time-dependent measurement of intracellular labile zinc

Time-dependent measurement of intracellular labile zinc was tested in KG-1a cells in fresh PBMCs and nonadherent lymphocytes. The method has been primarily optimized in KG-1a cells in triplicate experiments using three different concentrations of Fluozin-3 AM probe (0.1 μM, 1 μM, 10 μM) (Invitrogen, S. Giuliano Milanese, Italy) as well as of zinc sulphate (10 μM, 20 μM, 100 μM). A total of 3×10^6/ml cells were incubated in free zinc HEPES buffer (Neurobiotex, Galveston, TX, USA) for 30 min′ at 37°C and 5% CO_2 atmosphere with the three Fluozin-3 AM concentrations reported above. After incubation, the cells were washed two times in HEPES buffer. The pellet was then resuspended in HEPES and subdivided in three aliquots.

The first aliquot (2×10^6/ml) was used to measure the fluorescence variations over time by FC after the *in vitro* zinc addition (10 μM, 20 μM, 100 μM). The mean fluorescence intensity was recorded for 30 min (plateau phase). The second aliquot (5×10^5/ml) was used to measure the intracellular free zinc (iFZn) at time 0 following the methods developed by Malavolta et al. [26] and Haase et al. [27]. After the basal fluorescence acquisition (at time 0), 75 μM of zinc sulphate and 50 μM of the zinc ionophore 2-mercaptopyridine-1-oxide sodium salt (pyrithione) (Sigma-Aldrich, Milan, Italy) were added in order to measure the maximum fluorescence intensity (MFImax) [26].

The third aliquot (5×10^5/ml) was employed to test the minimum fluorescence intensity (MFImin) by means of the addition of *N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine (Sigma-Aldrich, Milan, Italy) at the concentration of 50 μM [26].

The concentration of intracellular labile zinc (iFZn) at time 0 and during each minute of data acquisition during the time course was calculated from the mean fluorescence (MFI) as the following [26,27]:

$$(\text{iFZn}) = \text{Kd} \times [(\text{MFI} - \text{MFImin}) / (\text{MFImax} - \text{MFI})],$$

where Kd is the constant of dissociation of Fluozin-3 AM probe.

A nonlinear regression model was used to calculate the best curve representing the time-dependent variations of intracellular labile zinc, with a goodness of fit coefficient R^2=0.99.

Following the logistic nonlinear regression modelling by Ramsay and Silverman [28], the applied three-parametric logistic function was:

$$\text{Pr3} / [1 + \text{Exp}(-\text{pr1} - \text{pr2}*\text{Xt})](a),$$

where *=multiplication sign and Xt=time in minute.

Pr3 is the maximum asymptote of the curve representing the maximum zinc increment within the cells; pr2 is proportional to the slope of the curve describing the rate of the speed to reach the maximum intracellular free zinc concentration; pr1 is the slope at time 0 related to the initial intracellular free zinc concentration (for further explanations, see Box 1).

2.4. Detection of apoptosis by FC

In order to choose the best zinc concentration for the time course analysis and, at the same time, avoid zinc toxicity, apoptosis was tested in PBMCs. A total of 2×10^6/ml of cells were incubated for 30 min (the last of the time course) with different doses of zinc sulphate (10, 20 and 100 μM) in PBS buffer. Briefly, cells were washed twice with cold PBS and once with binding buffer [140 mM NaCl/10 mM HEPES (pH 7.4)/25 mM $CaCl_2$], resuspended in 200 μl of binding buffer and stained with 2.5 μl of fluorescein isothiocyanate-labeled Annexin-V (Annexin-V-FITC) and 5 μl of propidium iodide. After 10 min of incubation, the cells were immediately analyzed by FC. For each analysis, 10 000 cells were recorded.

2.5. Real-time reverse-transcription polymerase chain reaction (PCR)

Total RNA was isolated from fresh lymphocytes using the kit SV Total RNA Isolation System (Promega, Madison, WI, USA) and quantified by NanoDrop spectrophotometer. The quality of RNA samples was validated by denatured agarose gel electrophoresis according to the integrity of 28S and 18S rRNAs.

cDNA synthesis from total RNA was performed using i-Script reverse transcriptase (Bio-Rad, Hercules, CA, USA) according to the manufacturer's guidelines. The resulting cDNA was subjected to real-time PCR assay to detect the expression levels of β-actin housekeeping gene as well as SLC39A1 (Zip1), SLC39A2 (Zip2), SLC39A3 (Zip3), IL-6 and TNF-α genes.

Primers were designed using the software Beacon Designer 3.0 (Bio-Rad, Hercules, CA, USA). The primers used were as follows:

β-Actin forward 5′-GGATAGCACAGCCTGGATAG-3′,
reverse 5′-GCGAGAAGATGACCCAGATC-3′;
SLC39A1 forward 5′-GCCTAAAGGGAGGGGTAAGCG-3′,
reverse 5′-AGTGGGCGGGGACAGACC-3′;
SLC39A2 forward 5′ GTTTGCCCTGTTGGCTCTCA-3′,
reverse 5′-ATCAATCTGGAACCATTTGAAGC-3′;
SLC39A3: forward 5′-GTTTCTGGCCACGTGCTT-3′,
reverse 5′-AGGCTCAGGACCTTCTGGA-3′;
IL-6: forward 5′-CAAATTCGGTACATCCTCGACG-3′;
reverse 5′-TTTTCAGCCATCTTTGGAAGGTT-3′;
TNF-α: forward 5′-AGGCGGTGCTTGTTCCTCA-3′
reverse 5′-GTTCGAGAAGATGATCTGACTGCC-3′

Conditions for amplification were as follows:
β-Actin: 50 repeats of 95°C for 15 s, 62°C for 30 s; 81 repeats of 55°C for 10 s.
SLC39A1 and *SLC39A2*: 50 repeats of 95°C for 15 s, 60°C for 30 s; 81 repeats of 55°C for 10 s.
SLC39A3: 50 repeats of 95°C for 15 s, 61°C for 30 s; 81 repeats of 55°C for 10 s.
IL-6: 45 repeats of 95° for 15 s, 58°C for 30 s; 81 repeats of 55°C for 10 s.
TNF-α: 45 repeats of 95° for 15 s, 60°C for 30 s; 81 repeats of 55°C for 10 s.

One microgram of cDNA was amplified in a total volume of 25 μl containing iQ SYBR GREEN SUPERMIX (Bio-Rad, Hercules, CA, USA) on a BioRad iQ5 optical real-time PCR (Bio-Rad, Hercules, CA, USA), employing a primer concentration of 200 nM for all genes studied, with the exception of β-actin (150 nM). Assays for each transcript were carried out in duplicate, and PCR amplification was repeated twice. An internal control, XpressRef human universal reference total RNA (SuperArray Bioscience Corporation, Frederick, MD, USA), was included for each experiment. Any inefficiencies in RNA input or reverse transcription were corrected by normalization to the housekeeping gene.

Box 1
Interpretation of logistic parameters pr1, pr2, pr3

Considering the following equation:

Y=pr3/[1+Exp(−pr1−pr2*Xt)],

we try to establish the meaning of pr1, pr2 and pr3 parameters.

pr1 determination at time zero:

Y0=pr3/[1+Exp(−pr1−pr2*X0)],

where X0=0 and Y0=free $zinc_{t0}$ ($iFZn_{t0}$),

then

${}_{f}Zn_{t0}$=pr3/[1+Exp(−pr1)].

Elaborating the function, we obtain:

pr1=−log(pr3/$iFZn_{t0}$−1).

The absolute value of pr1 will be proportional to pr3/free $zinc_{t0}$ ratio.

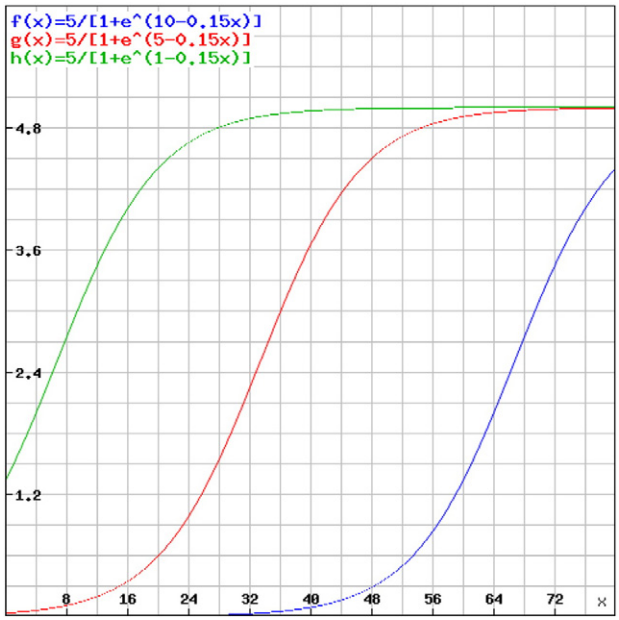
On the hypothesis that pr3 value is constant, pr1 depends exclusively on the initial free zinc value as shown from the following graphical representations of the three logistic functions where pr2 and pr3 are fixed while pr1 values are different [Pr1=−10 (blue); pr1=−5 (red); pr1=−1 (green)]:

Y=5/[1+e^(10−0.15×)] (blue)

Y=5/[1+e^(5−0.15×)] (red)

Y=5/[1+e^(1−0.15×)] (green)

A



pr2 determination at time zero:

After the calculation of the first derivative of the logistic function at time, zero we obtain:

pr2=($iFZnmax/iFZn_{t0}$)[m/($iFZnmax/iFZn_{t0}$)],

where m is the slope of the curve at t_0.

Therefore, pr2, which is proportional to the slope of the curve, describes the rate to reach the maximum free zinc concentration iFZnmax.

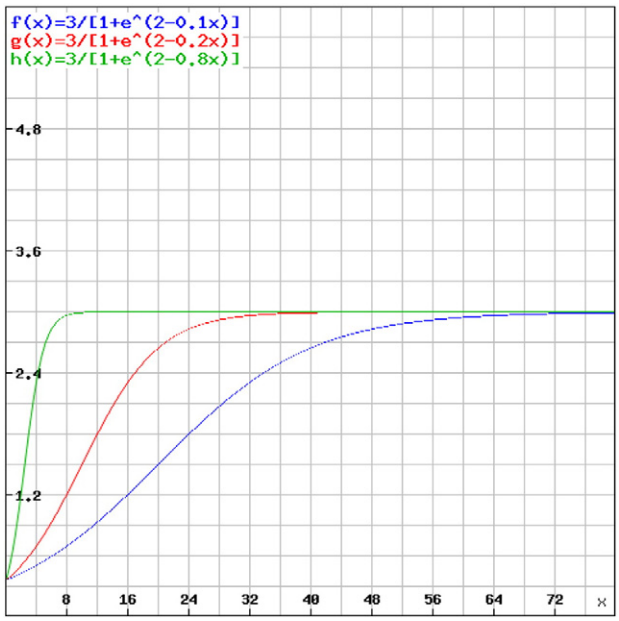
For an easier understanding, we report below a graphical representation of the three logistic functions where pr1 and pr3 are fixed while pr2 values are different [pr2=0.1 (blue); pr2=0.2 (red); pr3=0.8, (green)].

Y=3/[1+e^(2−0.1×)] (blue)

Y=3/[1+e^(2−0.2×)] (red)

Y=3/[1+e^(2−0.8×)] (green)

B



pr3 determination at time ∞:

In this case,

$Y\infty$=pr3/[1+Exp(−pr1−pr2*$X\infty$)].

For time ∞

$Y\infty$=pr3 represents the maximum asymptote of the curve that is the maximum intracellular zinc increment in the cells.

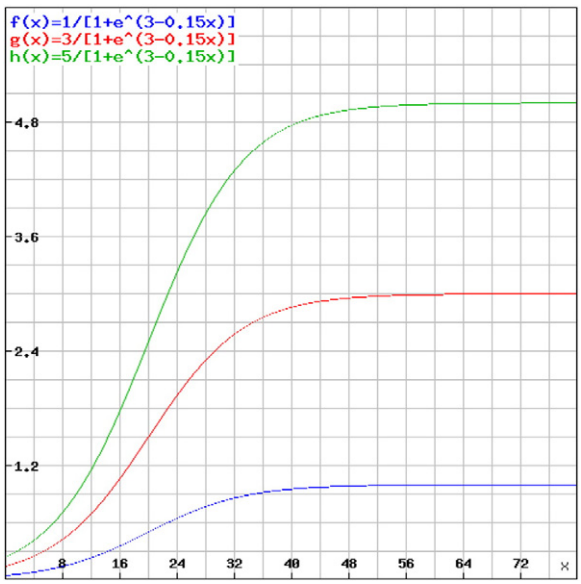
For an easier understanding, we report below a graphical representation of three logistic functions where pr1 and pr2 are fixed while pr3 values are different [pr3=1, (blue); pr3=3, (red); pr3=5, (green)].

y=1/[1+e^(3−0.15×)] (blue)

y=3/[1+e^(3−0.15×)] (red)

y=5/[1+e^(3−0.15×)] (green)

C



Relative amounts of SLC39A1, SLC39A2, SLC39A3, IL-6 and TNF-α mRNAs were calculated based on standard curves prepared by a serial dilution of control cDNA. The fold changes were calculated using the comparative CT method [ΔΔCt (cycle threshold)] [29]. The mRNA levels of the genes studied were normalized to the β-ACTIN mRNA housekeeping gene levels as endogenous reference.

2.6. Statistical analysis

Data were analyzed with SPSS/Win program (version 15.0; SPSS Inc., Chicago, IL, USA). Differences among groups were compared using analysis of variance followed by post hoc tests or by Mann–Whitney *U* test. Differences between control culture and EDTA treatment were evaluated by Wilcoxon matched-pairs test. The time-dependent variations of intracellular zinc were fitted with the three-parameter logistic model using XLSTAT Pro 7.5.2. The goodness-of-fit coefficients, including the R^2 (coefficient of determination) and the sum of square of errors, were calculated by XLSTAT. Pearson or Spearman correlations among variables were also performed.

3. Results

3.1. Optimization of the experimental conditions for the determination of the time-dependent intracellular labile zinc variations in KG-1a cell line

Applying the three-parametric logistic function [see *(a)* in Materials and Methods] to estimate the changes in time-dependent intracellular labile zinc, the maximum Zn increment (pr3) varied in relation to the zinc sulphate and Fluozin-3 AM probe concentrations (Fig. 1A, B, C). The probe concentration of 0.1 μM showed a lower sensibility as compared to 1 and 10 μM. In particular, reduced pr3 parameter was observed using Fluozin-3 AM (0.1 μM) with respect to the other two concentrations (1 μM, 10 μM) in relation to each concentration of zinc sulphate added (Table 1; $P<.001$). Significant changes in pr2 parameter were observed with Fluozin-3 AM (0.1 μM) only using the higher zinc concentration (100 μM) ($P<.05$ post hoc test). Conversely, Fluozin-3 AM (1 μM and 10 μM) showed sensitive variations of pr1, pr2 and pr3 parameters in relation to the amounts of zinc sulphate added (Table 1). Since no differences in pr3 or pr2 parameters were found between Fluozin-3 AM 1 μM or 10 μM (Table 1), the dose of Fluozin-3 AM 1 μM was used for the experiments in PBMCs and nonadherent lymphocytes.

With regard to the best zinc dose to use for the time-dependent intracellular labile zinc fluctuations, taking into account that 10 μM of zinc determines a low increment of labile zinc (0.955 vs. 1.931) (see pr3, Table 1), the choice was between 20 and 100 μM of zinc. Considering that high doses of zinc provoke immune apoptosis [30], initial experiments in testing apoptosis were carried out. The higher concentration of zinc (100 μM) induced significant increments in the percentage of apoptotic cells (79% ± 10%) with respect to 20 μM of zinc (5.5% ± 2.2%) ($P<.05$). Following these data, 20 μM of zinc, which is in turn considered a physiological dose [18], represents the best one to be used in the next experiments with reduced toxic effects on the cell viability. Thus, 20 μM of zinc sulphate and 1 μM of Fluozin-3 AM probe were chosen for the assessment of the time-dependent intracellular labile zinc signaling.

3.2. Time-dependent intracellular labile zinc signals in PBMCs and nonadherent lymphocytes from young and elderly donors

The intracellular free zinc ion availability (iFZn) is age dependent. A significant decrement of iFZn was observed in elderly subjects when compared to young-adult donors (0.16 vs. 0.32; $P<.01$) at time 0 (Table 2). The addition of zinc sulphate (20 μM) provoked a different course in intracellular zinc signaling between young-adult and old donors. Using the three parameters of the logistic function [see *(a)* in Materials and Methods], the maximum intracellular Zn increment (pr3) was higher in young-adult individuals than elderly ones ($P<.01$) (Table 2). Significant age-related differences were also found for pr2 ($P<.01$), whereas no differences were observed for pr1, with a trend however to be higher in young-adult people (Table 2). The kinetic curves and the iFZn mean increments over time up to the plateau region in PBMCs from young and elderly donors are reported in Fig. 2, showing a dichotomy between the two kinetic curves from 5 min up to 30 min with a plateau at 30 min.

The intracellular labile zinc (iFZn) in nonadherent lymphocytes, after 24 h of incubation in control medium, as well as pr1 and pr3 parameters were higher in young donors than elderly ones (Table 3; $P<.01$, $P<.05$).

Using a zinc-depleted medium containing EDTA, the iFZn was strongly reduced in lymphocytes from elderly donors when compared to those from young ones ($P<.01$) (Table 3). Decreased pr3 and pr1 parameters were observed in old donors with respect to young ones ($P<.05$; Table 3). The comparisons between control and EDTA medium (Wilcoxon signed-ranks test) within the same class of subjects showed reduced iFZn levels in EDTA-treated lymphocytes from young donors (Table 3; $P<.05$). Pr1 and pr3 showed only a trend to diminish in both classes of age (Table 3). Significant positive correlations were observed between iFZn and pr1 ($r=0.45$, $P<.05$) and between iFZn and pr3 ($r=0.60$, $P<.01$) (Spearman correlations calculated in the whole sample in both zinc-depleted and control medium).

3.3. Gene expression of Zip transporters and proinflammatory cytokines in nonadherent lymphocytes from young-adult and elderly donors before and after incubation with EDTA

Ten elderly and 10 young subjects (five males and five females for each age group) were randomly chosen for the relative gene expression study (fold changes). The fold changes were calculated as the ratio between the expression of each gene in lymphocytes incubated in control and EDTA medium. Zinc transporters (Zip1, Zip2, Zip3) showed higher fold changes in lymphocytes from young donors than old ones (Fig. 3, $P<.05$). Zip2 was the most up-regulated (Fig. 3). Conversely, IL-6 and TNF-α showed higher fold changes in lymphocytes from elderly subjects than those from young ones (Fig. 3, $P<.05$). However, using Wilcoxon matched-pairs test, the gene expression of zinc transporters Zip1, Zip2 and Zip3 was up-regulated in EDTA-treated lymphocytes from young and old subjects with respect to control medium ($P<.01$). Using the same statistical analysis, increased IL-6 and TNF-α mRNA levels occurred in both young and old subjects after the *in vitro* Zn deprivation ($P<.05$ and $P<.01$, respectively) (data not shown).

Positive correlations existed between pr3 and Zip2 or Zip3 ($r=0.45$, $P<.05$ and $r=0.42$, $P<.05$, respectively) (Spearman correlations in the whole sample before and after EDTA treatment). No differences in the gene expression profile in relation to the gender were found (data not shown).

4. Discussion

The mobilization of intracellular free zinc, induced by antigen stimulation, is implied in the signal transduction of several immune cells, activating the inflammatory/immune response [7]. Zinc deficiency is a common event in elderly people. This phenomenon may lead to impaired immune function with subsequent increased susceptibility to infections and other age-degenerative diseases [31]. However, no knowledge exists to date on the measurement of intracellular zinc signals in lymphocytes in relation to the age of donors.

We herein report a new assay to detect the time-dependent intracellular labile zinc increments after *in vitro* zinc addition (20 μM) in both fresh PBMCs and cultured lymphocytes (in control and *in vitro* zinc-depleted medium) from young and old donors. A zinc fluorophore probe (FluoZin-3) has been used. Logistic function parameters were also defined by using XLSTAT. The main advantage of our assay,

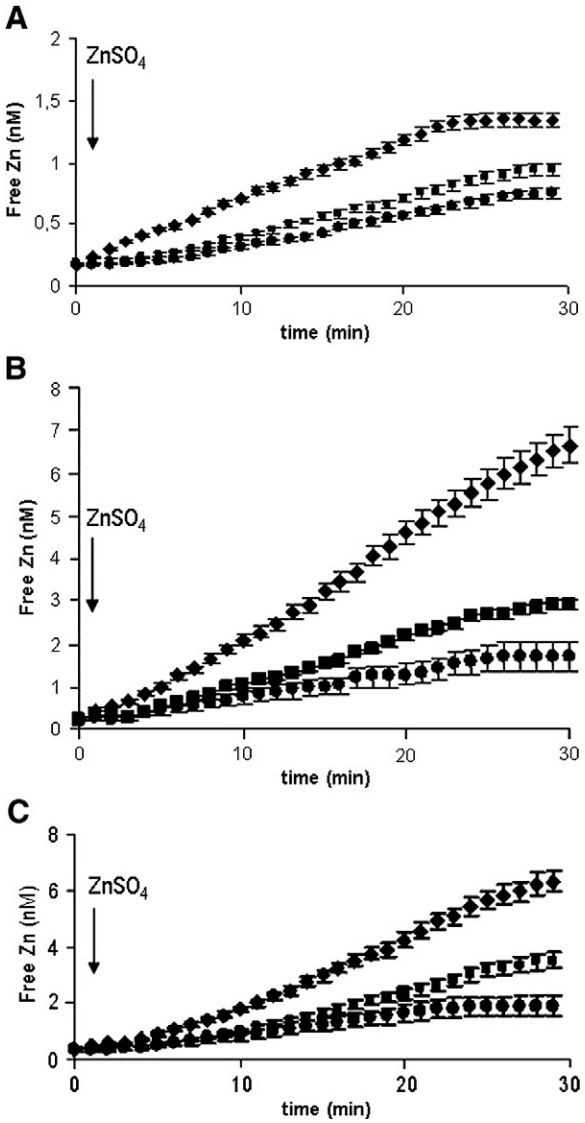


Fig. 1. (A, B, C) Optimization of the method to detect the time-dependent increments of intracellular labile zinc in KG-1a cells. The experiments were performed in triplicate using three different concentrations of the zinc fluorescent probe Fluozin-3 AM [0.1 μM (A), 1 μM (B), 10 μM (C)] and three different concentrations of zinc sulphate (10 μM, ●; 20 μM, ▪; 100 μM, ♦). Data expressed as mean±S.D.

Table 1
Logistic parameters of the intracellular zinc signal curve using three different Fluozin-3 AM and zinc concentrations

Concentrations		Zn 10 μM	Zn 20 μM	Zn 100 μM	*P* value
Fluozin-3 AM 0.1 μM	Pr1	−1.754 ± 0.062	−1.844± 0.055	−1.629 ± 0.064	*P*<.001
	Pr2	0.107 ± 0.006	0.114 ± 0.001	0.159 ± 0.002	*P*<.001
	Pr3	0.955 ± 0.122	1.194 ± 0.133	1.440 ± 0.152	*P*<.05
Fluozin-3 AM 1 μM	Pr1	−1.939 ± 0.051 *	−2.385 ± 0.036 *	−2.562 ± 0.019 *	*P*<.001
	Pr2	0.138 ± 0.001 †	0.152 ± 0.001 †	0.155 ± 0.002	*P*<.001
	Pr3	1.931 ± 0.186	3.655 ± 0.253 ‡	7.457 ± 0.328 ‡	*P*<.001
Fluozin-3 AM 10 μM	Pr1	−1.957 ± 0.045 *	−2.542 ± 0.034 *	−2.739 ± 0.018 *	*P*<.001
	Pr2	0.139 ± 0.004 †	0.146 ± 0.005 †	0.156 ± 0.001	*P*<.01
	Pr3	2.072 ± 0.154 ‡	4.168 ± 0.303 ‡	7.645 ± 0.294 ‡	*P*<.001

Data expressed as mean±S.D.
* *P*<.01 as compared to pr1 value of Fluozin-3 AM 0.1 μM to other probe concentrations.
† *P*<.05 as compared to pr2 value of Fluozin-3 AM 0.1 μM to other probe concentrations.
‡ *P*<.001 as compared to pr3 value of Fluozin-3 AM 0.1 μM to other probe concentrations.

Table 2
Logistic parameters of intracellular zinc signal curve and free zinc concentration (iFZn) in PBMCs from young and elderly donors

Parameters	Young-adult donors	Healthy old donors
iFZn (nM)	0.32 ± 0.10 *	0.16 ± 0.08
pr1	−1.95 ± 0.66	−1.88 ± 0.82
pr2	0.29 ± 0.11 *	0.18 ± 0.06
pr3	3.03 ± 2.30 *	1.22 ± 1.02

Data expressed as mean±S.D.
* *P*<.01 as compared to healthy old donors.

with respect to the one published by Haase et al. [6], is the detection of the fluorescence by FC. In this way, the double staining with Fluozin-3 AM and propidium iodide allows to read the zinc signals only in viable gated cells. This double staining is crucial especially when the cells are incubated in EDTA medium which may induce a proapoptotic effect. One possible disadvantage of this assay is linked to the crossbeam compensation with undesirable overlapping of the fluorescence signals in multicolor analysis. The aim of the present manuscript is to apply this new method for testing the variations of intracellular labile zinc during zinc deficiency in immune cells. Although significant variations in the number of lymphocyte subpopulations occur with advancing age [32], intracellular zinc fluctuations might be independent of the cellular immune changes related to age because zinc affects the function of different lymphocyte subpopulations at any age [8]. It has been reported that FluoZin-3 probe labels mainly the lysosomal compartment of T-cells [6]. Moreover, FluoZin-3 fluorescence increased in the vesicles and not in the cytoplasm when extracellular zinc was added, suggesting a rapid sequestration of the additional zinc within these organelles [7]. The FluoZin-3 probe is also useful to test the increased free zinc in response to specific stimuli by *Escherichia coli* in monocytes and granulocytes [6]. In the present study, using the FluoZin-3 probe, an increment of free zinc was observed in lymphocytes after the external *in vitro* zinc addition. Such a finding suggests a possible employment of FluoZin-3 to check the external zinc uptake in lymphocytes followed by an intracellular translocation into lysosomes, where zinc is stored and then released under specific stimuli (IL-2, LPS) for a prompt inflammatory/immune response [6,7]. Intracellular free zinc concentration (iFZn) decreased in elderly donors as compared to young ones, confirming the impaired zinc status in aging [33]. Using the logistic nonlinear regression modeling [28], which displays the best fitting with our time course data, age-related differences in intracellular zinc increments were observed in PBMCs with significant

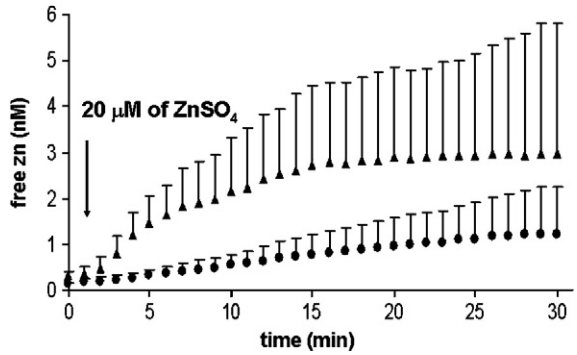


Fig. 2. Kinetic curves of time-dependent increments of intracellular labile zinc in PBMCs from *n*=27 old (●) and *n*=18 young-adult (▲) donors. Fresh PBMCs were loaded with the FluoZin-3 probe (1 μM). Free zinc increments were measured by FC assay for 30 min after zinc sulphate addition (20 μM). The free zinc levels at the time 0 are reported. Data expressed as mean±S.D.

Table 3
Logistic function parameters of zinc intracellular zinc signal curve and free zinc concentration (iFZn) in lymphocytes from young and elderly donors in control and EDTA medium after 24 h of culture

Parameters	Control medium		EDTA medium	
	Young-adult donors	Old donors	Young-adult donors	Old donors
iFZn (nM)	0.36±0.10	0.13 ± 0.06†	0.28 ±0.06‡	0.09±0.05†
pr1	−0.98±0.40	−1.66±0.69†	−1.21±0.78	−1.99±0.97*
pr2	0.15±0.04	0.16±0.03	0.16 ±0.08	0.16±0.04
pr3	1.41 ±0.49	0.92±0.53*	1.20 ± 0.46	0.76±0.32*

Data expressed as mean±S.D.
* $P<.05$ as compared to young donors by Mann–Whitney U test.
† $P<.01$ as compared to young donors by Mann–Whitney U test.
‡ $P<.05$ as compared between control medium and EDTA by Wilcoxon signed-ranks test.

differences of the logistic parameters (pr2, pr3), which, respectively, represent the rate of the speed to reach the plateau curve and the maximum intracellular zinc increment.

The impaired intracellular zinc signals in elderly donors may be due to a reduced uptake caused by decreased Zip-1 and Zip-2 gene expression in PBMCs, as it occurs with advancing age [34]. Noteworthy, lymphocytes from young subjects incubated under zinc-deficient conditions (EDTA for 24 h) showed higher iFZn and the maximum intracellular zinc increment (pr3) than lymphocytes from old donors. Concomitantly, an up-regulation of Zip genes was observed in lymphocytes from young and old subjects, but the induction was more pronounced in young ones, explaining in part the higher capacity of intracellular zinc uptake observed in the young. The presence of significant correlations among the logistic parameter pr3 and Zip-2 or Zip-3 gene expressions is in line with this interpretation. However, despite the induction of Zip transporters, iFZn in lymphocytes from elderly donors is not modified after zinc deprivation as compared to control medium, whereas decrements occur in young donors (Table 3). This last finding might indicate a primary mobilization of zinc from lysosomes to the cytosol in order to compensate for the stress condition provoked by EDTA. Although ZnT-1 expression was not herein tested, a possible alternative explanation of our results might be an attenuation of zinc influx by ZnT-1 via the LTCC pathway [35]. Anyway, regardless of the mechanism involved in regulating the intracellular zinc fluctuations after EDTA zinc deprivation, it is well known that Zip transporters (Zip-1, Zip-2, Zip-3) play a relevant role in intracellular zinc homeostasis during zinc deficiency, as shown in dietary zinc deficiency [14] and in inflammatory pulmonary diseases, characterized by Zip-2 overexpression [17] coupled with decreased plasma zinc levels [15]. In agreement with this last finding and with another study by Cousins et al. [13], our results show that Zip-2 is the most sensitive zinc transporter to *in vitro* zinc depletion and that it is strongly increased in lymphocytes from young subjects, confirming and further suggesting the key role played by Zip-2 in regulating the zinc homeostasis in immune cells. On the other hand, Zip-2 is prevalently expressed in immature dendritic cells [36], which are crucial in the regulation of T-cell immune response [37]. In addition, a novel Zip-2 polymorphism in the coding region was associated in severe carotid stenosis in the elderly [38], denoting an implication of Zip-2 also in atherosclerosis development that is in turn a pathology characterized by zinc deficiency and chronic inflammation [39]. However, the induction of Zip genes during *in vitro* zinc deprivation is less evident in elderly than in young donors (Fig. 3). Taking into account that zinc restriction increases plasma membrane levels of Zip-1 and Zip-3 by decreasing their rates of endocytosis [40] as well as Zip-2 expression [13], the reduced intracellular zinc increments in lymphocytes from the elderly exposed to zinc depletion might depend on a less efficient membrane localization of these proteins or be related to an impaired activity of these zinc transporters with advancing age. Such an assumption may be supported by studies showing that targeted deletion of mouse Zip1, Zip2 or Zip3 did not cause overt signs of zinc deficiency but led to an impaired ability of adaptation to zinc deficiency [36,41]. During aging, the reduced ability to adapt to zinc deficiency, rather than clinical sign of zinc deficiency, is an usual event named “marginal zinc deficiency” [42]. Therefore, a decreased activity of the zinc transporters coupled to reduced intracellular zinc increments in lymphocytes from elderly donors might be justified. An intriguing point is an overexpression of IL-6 and TNF-α mRNA provoked by zinc depletion in lymphocytes from both elderly and young donors, which is more marked in old age. Although these phenomena are usual events during zinc deficiency through the tyrosine pathways [4] or NF-kappaB transcriptional factor [43] activation, IL-6 and TNF-α mRNA levels are higher in elderly donors but these levels are not well compensated by an efficient intracellular free zinc increment, as it occurs in young donors. As a final result, the persistently reduced intracellular free zinc might limit the anti-inflammatory effect of the zinc finger protein A20 [44] and, at the same time, might increase the NF-kappaB DNA binding activity [43,45], contributing to fuel the proinflammatory cytokine production. By contrast, the higher increments of intracellular free zinc and Zip-1 in young subjects could limit the proinflammatory cytokine production because Zip-1 overexpression has an inhibitory effect on NF-kappaB-dependent pathways [46]. Therefore, a marginal zinc deficiency in lymphocytes from elderly people might favor the chronic inflammatory phenomena leading to an impaired immune response to noxious antigens, as reported in naïve CD4 T-cells from elderly individuals [47].

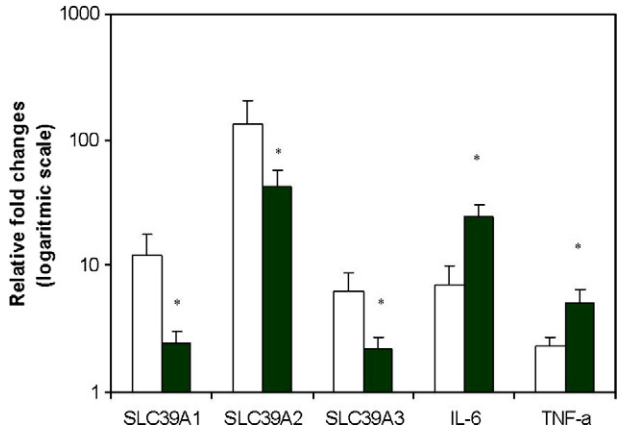


Fig. 3. Relative gene expression (fold change) for zinc transporters (Zip1, Zip2, Zip3), IL-6 and TNF-α in nonadherent lymphocytes from young (□) and old (•) donors before (control medium) and after EDTA treatment (0.5 mM) for 24 h. The fold changes were calculated as the ratio of the gene expressions of lymphocytes incubated in EDTA or control medium using the comparative CT method. Data expressed as mean±S.E.M. $n=10$ donors/group. *$P<.05$ as compared to young-adult donors.

Emerging studies on the relevance of intracellular zinc homeostasis and zinc transporters in the development of different types of cancer [48–51] suggest further potential applications of this method in the study of carcinogenesis or in the cancer prognosis. Noteworthy, zinc and zinc transporters play an important role in the molecular pathogenesis of prostate cancer; in particular, Zip-1, Zip-2 and Zip-3 are down-regulated in prostate cancer with a loss of the capability to accumulate zinc in the malignant cells. This a defense mechanism of tumor cells because zinc induces G2/M phase arrest and apoptosis in prostate cancer cells [52]. Therefore, Zip-1, Zip-2 and Zip-3 are suggested as tumor suppressor genes and zinc as a tumor suppressor agent [53]. Another zinc transporter, Zip-10, has been associated with invasive metastatic breast cancer [54]. It has been demonstrated that the migratory activity of metastatic breast cancer cells was inhibited

by knockdown of Zip10 expression and Zn chelation. Importantly, analysis of clinical samples showed that breast cancers with lymph node metastases expressed significantly higher levels of the Zn transporter Zip10 than those without lymph node metastases [54]. Thus, altered intracellular Zn homeostasis due to changes in Zn transporter expression may be a key factor that determines tumor malignancy. The study of the role played by these specific zinc transporters in cancer using our assay may be useful to suggest new therapies in cancer patients (zinc associated with conventional chemotherapy, drugs affecting zinc homeostasis or zinc-chelating agents).

In conclusion, we have established a method to measure the time-dependent intracellular free zinc signaling after *in vitro* zinc addition through the determination of three logistic parameters (pr1, pr2 and pr3). This method may be a useful tool to estimate the rate of intracellular zinc homeostasis and its role in inflammatory/immune response in aging or other age-related diseases in which intracellular zinc homeostasis is involved, such as cancer, cardiovascular diseases and diabetes type 2 [20,38,52,53]. However, it does not exclude that age-related immune cell variations, especially in very old age, may play a role. Further investigations in age-related different lymphocyte subpopulations are in progress.

Acknowledgments

Supported by INRCA and CariVerona Foundation (Italy).

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