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The role of zinc in calprotectin expression in human myeloid cells

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ABSTRACT

Elevated levels of calprotectin and other inflammatory mediators have been observed in inflammatory diseases paralleling serum hypozincemia. While a role of zinc in the regulation of tumor necrosis factor α, interleukin (IL)-1β and IL-6 expression has been established, the direct interrelation of zinc and calprotectin (S100A8/S100A9 heterodimer) expression is so far missing. In the present study, we analyzed mRNA and protein levels of S100A8 and S100A9 in monocytic Mono Mac (MM)1 and early myeloid THP-1 and U937 cells to elucidate the effect of zinc deficiency on their expression. We could depict that zinc deficiency alone enhances mRNA and protein expression of calprotectin in myeloid cells, independently from maturity stage. Moreover, pre-existing zinc deficiency augmented lipopolysaccharide (LPS)-induced calprotectin expression in $CD14^+$ MM1, but not in $CD14^-$ U937 or $CD14^-$ THP-1 cells. Zinc deficiency and LPS seem therefore to activate different intracellular pathways. Our findings suggest that zinc does not only regulate the activity of calprotectin but also its expression by human myeloid cells.

1. Introduction

A balanced zinc homeostasis is crucial for an appropriate immune response [1]. Zinc deficiency caused by infections, malnutrition, age or diseases may therefore lead to complex immune impairments [2–5]. In the elderly, zinc deficiency was associated with constantly elevated inflammatory parameters [6] and increasing rates of infectious diseases with high mortality therefrom [5,7]. Generally, severe and chronic serum hypozincemia are accompanied by an increase of pro-inflammatory mediators like tumor necrosis factor α, interleukin (IL)-1β, IL-6 and calprotectin [5,8–10].

Calprotectin is a heterodimeric complex belonging to the S100 family, consisting of the subunits S100A8 and S100A9. It can influence zinc metabolism by chelating zinc in the presence of calcium. Amongst other roles, it thereby deprives bacteria of zinc, conveying antimicrobial properties [11]. Predominantly it is expressed by cells of the innate immune system including neutrophil granulocytes and monocytes [12].

Hallmarks of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, cystic fibrosis or sepsis are elevated levels of calprotectin in serum paralleled by serum hypozincemia [8,9,13,14]. A prior report links zinc deficiency to the upregulation of calprotectin [15]. The precise association of zinc with calprotectin expression especially in an inflammatory context has not been investigated in detail so far, but is important to understand to develop new treatment strategies for these diseases.

Hence, the aim of this study was to investigate the influence of zinc homeostasis on calprotectin expression in human myeloid cells of different maturity stages.

2. Methods and materials

2.1. Cell culture

MM1, THP-1 and U937 cells were cultivated at 37 °C, humidified atmosphere and 5% CO_2. Culture medium for U937 was RPMI 1640 (Sigma-Aldrich, München, Germany) with 10% heat-inactivated, low-endotoxin FCS (PAA, Cölbe, Germany), 100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich).

MM1 cells were cultured in U937 medium where 1% non-essential-amino acids (NEAA, Lonza, Köln, Germany) and 1% sodium pyruvate (Lonza) were added.

To culture THP-1 cells, 2.5 µl β-Mercaptoethanol (Merck, Darmstadt, Germany) were added per 500 ml of U937 medium. For zinc-deficient medium, zinc adequate control medium with 10% FCS was treated with CHELEX 100 ion exchange resin (Sigma-Aldrich) for 1 h at 20 °C and was subsequently reconstituted with 500 µM $CaCl_2$

Abbreviations: AAS, atomic absorption spectrometry; IL, interleukin; LPS, lipopolysaccharide; MT, metallothionein; MM1, Mono Mac 1; TNF, tumor necrosis factor; TPEN, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine

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(Sigma-Aldrich) and 400 µM $MgCl_2$ (Merck).

The metal composition of RPMI 1640 medium with 10% FCS prior and after treatment with CHELEX resin can be found in Mayer et al. [16].

The cells were pre-cultivated for 24 h either in zinc adequate control medium, in zinc deficient medium (CHELEX) as described above, or in zinc reconstituted CHELEX-medium (CHELEX + Zn) with ZnSO4 ($ZnSO_4 \times 7$ H_2O, Merck) to yield a final concentration of 8 µM. Subsequently lipopolysaccharide (LPS), *E. coli* serotype O111:B4 (Sigma-Aldrich) was added for another 24 h treatment as indicated in the figure legends.

2.2. Measurement of free intracellular zinc with ZinPyr-1

Free intracellular zinc was measured using ZinPyr-1 (10 µM, Santa Cruz Biotechnology, Heidelberg, Germany). The mean fluorescence signal was detected by FACSCalibur (BD Biosciences, Heidelberg, Germany) using Cellquest software 3.0.

The formula $[Zn] = K_D \times [(F - F_{min})/(F_{max} - F)]$ was used to calculate the concentration of free labile intracellular zinc. In doing so, the dissociation constant (K_D) was 0.7 nM for the Zn/ZinPyr-1 complex [17]. The maximal (F_{max}) and minimal (F_{min}) mean fluorescence signals were generated by addition of zinc (100 µM) combined with pyrithione (50 µM) (Sigma-Aldrich) or N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (50 µM) (TPEN, Sigma-Aldrich), respectively [18].

2.3. ELISA

Supernatants were harvested after stimulation, stored at −20 °C until measurement, and only thawed once for cytokine detection. IL-6 protein production was quantified using OptEIA assays (BD Biosciences) according to the manufacturer's instructions.

2.4. Flow cytometry

Cells were stained with phycoerythrin (PE)-conjugated CD14 monoclonal antibody (mAbs; BD Biosciences) or with the isotypic control PE-conjugated immunoglobulin IgG2bκ mAb (BD Biosciences) for 15 min. Washed cells were then analyzed in a FACSCalibur (BD Biosciences) using Cellquest software 3.0.

2.5. Atomic absorption spectrometry (AAS)

Cells were washed with washing buffer (0.9% sodium chloride (NaCl, Merck), 10 mM ethylenediaminetetraacetic acid (EDTA, Merck) and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, AppliChem, Darmstadt, Germany)) twice and scraped into ice-cold water. Subsequently, cells were hydrolyzed in 200 µl HNO_3 (> 69.5%, for trace analyses, Sigma-Aldrich) for 2 h at 80 °C. Cellular zinc concentration was measured by flame atomic absorption spectrometry (AAS, Perkin Elmer, Baesweiler, Germany) using a standard curve made from AAS grade zinc standard solution. Absorbance values were normalized to cell counts.

2.6. Reverse transcription and real-time PCR

RNA was isolated using the NucleoSpin® RNA Kit (Macherey-Nagel, Düren, Germany) and cDNA was acquired through use of qScript™ cDNA Synthesis Kit (Quanta Biosciences, Darmstadt, Germany) according to the manufacturers´ protocols. The primers for S100A8 and S100A9 [15] and the primers for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [19] were used as previously described. All samples were run using a Step-1 plus (Applied Biosystems, Darmstadt, Germany) with the following parameters: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for S100A8 and GAPDH and 61 °C for S100A9 in duplicate, using Power SYBR Green PCR Master Mix (Applied Biosystems). Fold-changes were calculated through the ΔΔ C_T method.

2.7. Cell extracts and Western blotting

A total of 1×10^6 cells were lysed in 100 µl lysis buffer (0.5 M Tris–HCl (pH 6.8) (Carl Roth, Karlsruhe, Germany), 2% (w/v) sodium dodecyl sulfate (Merck), 1 mM sodium vanadate (Sigma-Aldrich), 26.6% glycerol (Carl Roth), 1% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue (Fluka, Buchs, Switzerland)). The cells were sonicated for 15 s and then boiled for 3 min at 95 °C.

An equivalent of 2×10^5 cells per lane was separated on 15% polyacrylamide (S100A9 and S100A8) or 10% polyacrylamide gels (β-actin) at 150 V and blotted to nitrocellulose membranes. Homogeneous loading of membranes was confirmed with Ponceau S staining (Sigma-Aldrich). Following destaining, membranes were blocked with tris buffered saline (TBS, 20 mM Tris-HCl (pH 7.6, Carl Roth), 136 mM NaCl (Merck)) including 0.1% (v/v) Tween 20 (AppliChem, Darmstadt, Germany)) and 3% bovine serum albumin (Fluka) and incubated overnight with the primary antibodies against S100A9, S100A8 (Santa Cruz Biotechnology, Heidelberg, Germany) or β-actin (Cell Signaling Technology, Frankfurt am Main, Germany) at 1:200 dilutions in TBS-T containing 5% bovine serum albumin. Membranes were then incubated with horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody and HRP-coupled anti-biotin antibody for detection of biotin-labeled molecular weight (MW) standard (anti-biotin 1:1000, anti-rabbit 1:2000; Cell Signaling Technology) for 2 h. This was followed by detection with LumiGlo reagent (Cell Signaling Technology) on a LAS-3000 (Fujifilm Lifescience, Düsseldorf, Germany).

2.8. Statistical analysis

Statistical significance of the results was analyzed by GraphPad Prism software version 5 (GraphPad software, La Jolla, CA, USA). In the case of Gaussian distribution, as tested by D'Agestino's K-square test of normality, student's t-test for unpaired samples was performed. Without this assumption, Mann-Whitney-*U* test was performed for unpaired samples and Wilcoxon signed rank test was performed for paired samples. p-values ≤ 0.05 were regarded statistically significant and are illustrated in the figures by *, p-values ≤ 0.01 are shown by ** and p-values ≤ 0.001 are shown by ***.

3. Results

3.1. Zinc homeostasis of MM1 cells during zinc deficiency and after LPS-stimulation

Initially, we confirmed that pre-incubation in CHELEX-treated medium decreased intracellular free zinc levels in MM1 cells (Fig. 1A), to verify our zinc deficiency model [15,16]. Intracellular free zinc was decreased significantly in MM1 cells by 58.2% to a mean of 0.028 nM (Fig. 1A) incubated in CHELEX-treated medium compared with the zinc adequate controls. However, intracellular zinc levels of zinc adequate controls and zinc deficient MM1 cells were not affected significantly by LPS stimulation for 24 h, compared with the matching unstimulated control. In order to eliminate artefacts from CHELEX resins, we used a second control sample, which comprised of CHELEX-treated medium, replenished with zinc to yield a final concentration of 8 µM zinc, comparable to regular zinc adequate medium. No significant difference was detected between MM1 cells incubated in regular zinc adequate control medium or in zinc replenished CHELEX-treated medium (Fig. 1B). Due to simplification, only one control group, i.e. cells cultured in regular medium, was used consequentially during further experiments.

To support our data for the intracellular free zinc measurements, we used atomic absorption spectrometry to determine total zinc levels in