



## Research Paper

## Zinc regulates iNOS-derived nitric oxide formation in endothelial cells



Miriam M. Cortese-Krott<sup>a,b,\*</sup>, Larissa Kulakov<sup>a,c</sup>, Christian Opländer<sup>c</sup>,  
Victoria Kolb-Bachofen<sup>b</sup>, Klaus-D. Kröncke<sup>d</sup>, Christoph V. Suschek<sup>b,c</sup>

<sup>a</sup> Cardiovascular Research Laboratory, Department of Cardiology, Pneumology, and Angiology, Medical Faculty, Heinrich-Heine-University of Düsseldorf, Universitätsstr. 1, Düsseldorf D-40225, Germany

<sup>b</sup> Research Group Immunobiology, Medical Faculty, Heinrich-Heine-University of Düsseldorf, Universitätsstr. 1, Düsseldorf D-40225, Germany

<sup>c</sup> Department of Trauma and Hand Surgery, Medical Faculty, Heinrich-Heine-University of Düsseldorf, Universitätsstr. 1, Düsseldorf D-40225, Germany

<sup>d</sup> Institute of Biochemistry and Molecular Biology I, Medical Faculty, Heinrich-Heine-University of Düsseldorf, Universitätsstr. 1, Düsseldorf D-40225, Germany

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## ABSTRACT

Aberrant production of nitric oxide (NO) by inducible NO synthase (iNOS) has been implicated in the pathogenesis of endothelial dysfunction and vascular disease. Mechanisms responsible for the fine-tuning of iNOS activity in inflammation are still not fully understood. Zinc is an important structural element of NOS enzymes and is known to inhibit its catalytic activity. In this study we aimed to investigate the effects of zinc on iNOS activity and expression in endothelial cells. We found that zinc down-regulated the expression of iNOS (mRNA + protein) and decreased cytokine-mediated activation of the iNOS promoter. Zinc-mediated regulation of iNOS expression was due to inhibition of NF-κB transactivation activity, as determined by a decrease in both NF-κB-driven luciferase reporter activity and expression of NF-κB target genes, including cyclooxygenase 2 and IL-1β. However, zinc did not affect NF-κB translocation into the nucleus, as assessed by Western blot analysis of nuclear and cytoplasmic fractions. Taken together our results demonstrate that zinc limits iNOS-derived high output NO production in endothelial cells by inhibiting NF-κB-dependent iNOS expression, pointing to a role of zinc as a regulator of iNOS activity in inflammation.

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## Introduction

Endothelial dysfunction is characterized by a loss of endothelial control over vascular tone, thrombosis, and vessel wall remodeling [1]. Hallmarks of endothelial dysfunction is a decreased bioavailability of nitric oxide (NO) and increased inflammation [1]. In endothelial cells NO can be produced from L-arginine in a reaction catalyzed by the constitutive type 3 isoform of NO synthase or endothelial NOS (eNOS or NOS3; EC 1.14.13.39) [2]. Under pro-inflammatory conditions endothelial cells express also the inducible isoform of NOS (iNOS or NOS2) [2,3]. Although both NOS isoforms catalyze the same biochemical reaction, eNOS and iNOS are very different enzymes. The expression of eNOS is constitutive in endothelial cells, and produces low nanomolar levels of NO. The biochemical activity of eNOS is tightly regulated by its intracellular localization and by post-translational mechanisms, including Ca<sup>2+</sup> signaling, and phosphorylation. Instead, iNOS is not expressed

under resting non-inflammatory conditions in endothelial cells, but after its induction by pro-inflammatory stimuli iNOS produces low micromolar amounts of NO independently on Ca<sup>2+</sup> fluctuation until it is degraded by proteolytic enzymes [4]. iNOS is known to exert important regulatory and protective effects on the vasculature [2–4]. However, aberrant iNOS-derived NO production has been shown to be involved in pathological conditions, e.g. the blood pressure fall in septic shock [5], as well as in the pathogenesis of chronic inflammatory diseases, including atherosclerosis [6,7]. Although in recent years both post-translational and post-transcriptional mechanisms regulating iNOS activity have been discovered [4,8], the regulation of iNOS expression remains the main regulatory step to control iNOS activity [4,5].

Accumulating evidence indicates that iNOS-derived NO is also involved in zinc homeostasis [9–11]. It has been hypothesized that zinc released by high output NO synthesis may induce endothelial resistance against oxidative stimuli and disease [12–14], as recently reviewed [15]. It was shown that both NO donors and iNOS-derived NO induce intracellular zinc release from zinc sulfur clusters, as found in proteins such as metallothionein [9,11–14,16–21]. We and others have demonstrated that iNOS-derived NO controls zinc trafficking in endothelial cells [14,16,18,20,22]

\* Corresponding author at: Cardiovascular Research Laboratory, Department of Cardiology, Pneumology, and Angiology, Medical Faculty, Heinrich-Heine-University of Düsseldorf, Universitätsstr. 1, Düsseldorf D-40225, Germany

E-mail address: [miriam.cortese@uni-duesseldorf.de](mailto:miriam.cortese@uni-duesseldorf.de) (M.M. Cortese-Krott).

and protects endothelial cells against H<sub>2</sub>O<sub>2</sub>-mediated damage via zinc-mediated increase of cellular glutathione *de novo* synthesis [12]. Interestingly, the same pathway was activated also by exogenously added zinc [13].

Some evidence of a possible regulatory role of zinc on iNOS activity exists. Zinc is an important structural element of iNOS, holding together the two subunits via tetrahedral coordination of four cysteines, two belonging to one monomer and the other two to the second protein monomer [23]. Thus, zinc displacement from the zinc–sulfur cluster of iNOS by NO can contribute to the control of iNOS activity [24]. Moreover, it is well known that transition metals, including copper and zinc, profoundly affect NOS enzyme activity at different levels. Stuehr and Griffith have reported in a review that *in vitro* iNOS activity in activated macrophages is more than 90% inhibited by 100  $\mu$ M zinc [26]. In keratinocytes zinc supplementation was shown to decrease iNOS protein levels [27], but the molecular mechanisms responsible of zinc-mediated iNOS regulation were not further investigated.

In this study we analyzed the effects of zinc on iNOS activity in aortic endothelial cells. We could demonstrate that zinc limits high output NO production in endothelial cells by inhibiting NF- $\kappa$ B-dependent expression of iNOS. Therefore, zinc might participate to control iNOS signaling and contribute to the stabilization of the endothelium in inflammation by limiting NF- $\kappa$ B-mediated responses.

## Materials and methods

### Chemicals

Non-fat dry milk was purchased from BioRad (Munich, Germany), materials for western blot (NuPAGE LDS sample buffer, NuPAGE reducing agent, NuPAGE 10% Bis-Tris pre-cast gels) from Invitrogen GmbH (Karlsruhe, Germany), Ponceaus S from SERVA Electrophoresis GmbH (Heidelberg, Germany), Hybond P transfer membrane from Amersham Biosciences (Munich, Germany), mouse monoclonal anti-human  $\beta$ -actin, horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum and HRP-conjugated goat anti-rabbit antiserum from BD biosciences (Erembodegem, Belgium), pro-inflammatory cytokines (PeproTech INC, Rocky Hill, USA), and L-N<sup>5</sup>-(1-iminoethyl)-ornithine-dihydrochloride (L-NIO) from Alexis Biochemicals (Lörrach, Germany). Unless otherwise specified, chemicals were purchased from Sigma (Deisenhofen, Germany), cell culture plastics from Greiner (Frickenhausen, Germany) and cell culture material from PAA (Pasing, Austria).

### Isolation and culture of rat aorta endothelial cells

Rat aorta endothelial cells were isolated by outgrowth from aortic rings, expressed the phenotype vWF<sup>high</sup> O<sub>x</sub>43<sup>high</sup> eNOS<sup>high</sup> and were cultured as described [13,28].

### Culture of A549/8 iNOS cells

The human alveolar epithelium-like A549/8 cells stably transfected with a 16 kB fragment of the human iNOS promoter in front of a luciferase reporter gene and a neomycin resistance gene was a kind gift of Prof. Dr. H. Kleinert (Institute of Pharmacology, Johannes Gutenberg-University of Mainz) [29]. Cells were grown in RPMI 1640/10% FCS supplemented with 1 mg/ml Geneticin G418. Experiments were performed in the absence of G418.

### Zinc determination

The zinc concentrations of cell culture media, sera and buffers were determined by flame atomic adsorption spectrometry as described previously [13]. We found that FBS contained  $24.98 \pm 2.00 \mu$ M zinc and the complete medium  $2.28 \pm 0.32 \mu$ M zinc. Increase in intracellular “free” zinc concentration after addition of zinc to culture media was measured by loading with Zinquin as described [13].

### Measurement of iNOS derived NO production

Activity of recombinant bovine iNOS (155.5 U/ml) was determined at room temperature in a NOS reaction buffer at pH 7.4 containing 50 mM HEPES, 0.15 mM NADPH, 1 mM arginine, 1 mM magnesium acetate, 18  $\mu$ M tetrahydrobiopterin, and 180  $\mu$ M DTT in the presence or absence of zinc. The nitrite concentration was measured in aliquots of the reaction mixture collected at different time points by chemiluminescence by using triiodide reduction in CLD 60 (Echophysics GmbH, München, Germany), as described [30]. The results corresponding to 10 min incubations are shown. Nitrite accumulation in culture supernatants during 24 h of incubation with pro-inflammatory cytokines (as indicated in the figure legend) in the absence or presence of the NOS inhibitor L-NIO was determined using the diazotization reaction (Griess assay), as described previously [28]. The nitrite concentrations in the cell culture supernatant were normalized against the number of live cells as assessed by neutral red staining [13].

### Treatments with pro-inflammatory cytokines and zinc

Endothelial cells ( $5 \times 10^4$  cells/well) were cultured in 12-well plates for 48 h and then incubated for 12 h with a mix of pro-inflammatory cytokines (1000 U/ml TNF $\alpha$ +400 U/ml IL-1 $\beta$ +200 U/ml INF $\gamma$ ) to induce the expression of the inducible nitric oxide synthase (iNOS) and high output NO synthesis. NO synthesis was inhibited by addition of the specific NOS-inhibitor L-NIO (500  $\mu$ M). Zinc (50 mM stock in 0.9% NaCl) was diluted in culture medium to the indicated concentrations [12,13].

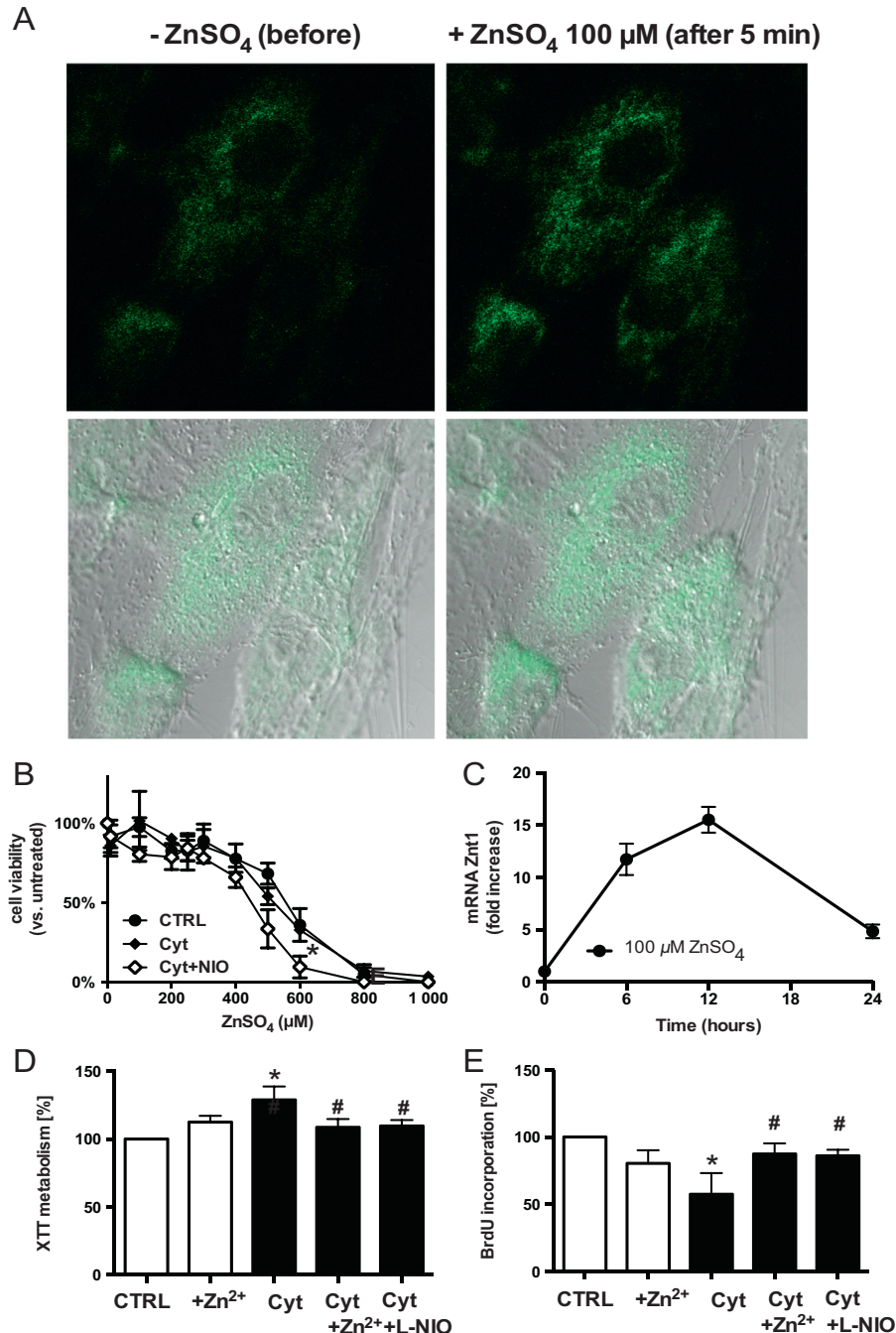
The effects of iNOS-derived NO  $\pm$  zinc on cell viability was assessed with three different methods. Cell integrity was assessed by neutral red staining, as described [12,13]. The amount of cells accumulating neutral red into their lysosomes (viable cells) is expressed as percentage of live cells vs. untreated cells. The effects of the treatments on mitochondrial dehydrogenase activities was measured by analyzing the reduction of a tetrazolium salt to formazan [31]. Sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT; Roche Diagnostic GmbH, Mannheim, Germany) was used following the manufacturer's instructions. Briefly, 300  $\mu$ l of a solution containing XTT was added to the culture medium and incubated for 4 h at 37 °C, 5% CO<sub>2</sub>. Absorption was measured in triplicates at 450 nm in a FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany). Cell proliferation was assessed as DNA synthesis by analyzing the incorporation of 5-bromo-2'-deoxyuridine with a commercial kit (BrdU Cell Proliferation ELISA, ZytoMed). Briefly, endothelial cells ( $10^4$  cells/well) were cultured in 96-well plates for 24 h, and then treated in quadruplicates as indicated. Six hours after the beginning of the treatments 20  $\mu$ l of a solution containing BrdU (provided in the kit) was added to the culture medium and incubated for 18 h. Cells were then fixed and stained with an anti-BrdU mouse monoclonal antibody (1:200) for 1 h at RT. The signal was detected using a horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000) and tetra-methylbenzidine as a substrate. XTT was added to the culture medium and incubated for

4 h at 37 °C, 5% CO<sub>2</sub>. Absorption was measured at 450 nm in a FLUOstar OPTIMA.

#### RNA interference, RNA extraction and real time RT-PCR

Endothelial cells ( $1 \times 10^5$  cells/well) were grown in a 6-well plate for 48 h, treated for 24 h as indicated and then lysed with 350  $\mu$ l RLT-lysis buffer (Qiagen, Hilden, Germany). For gene silencing experiments  $10^5$  cells were transfected with 3 nM silencing RNA (siRNA) and 4.5  $\mu$ l of HiPerfect transfection reagent (Qiagen)

following the manufacturer's instruction (see also [29]). After 48 h, the culture medium was changed and the cells were treated for 24 h with pro-inflammatory cytokines at the concentrations indicated or left untreated. The siRNA sequence targeting *RelA* (p65) was purchased from Qiagen (Rn-RelA-2-HPsiRNA). Silencing was considered successful when *RelA* (p65) mRNA was 2-fold decreased as compared to cells treated with the transfection reagent only (mock control). The silencing sequence with the maximal efficiency has been selected by comparing silencing efficiency, i.e. a decrease in mRNA expression of Rel A after transfection as



**Fig. 1.** Non-toxic zinc concentrations increases intracellular free zinc, induces the expression of Znt1 and rescues endothelial cells from cytokines-dependent inhibition of cell proliferation. (A) Increases in intracellular “free” zinc concentration 5 min after addition of 100  $\mu$ M zinc (right panels) as compared to the untreated control (left panel) as assessed by loading cells with zinquin. (B) Toxicity of zinc treatment in the presence or absence of pro-inflammatory cytokines (Cyt=IL-1 $\beta$ , TNF-alpha, IFN-gamma), or of the NOS inhibitor L-NIO, as assessed by neutral red staining. (C) Kinetics of zinc-mediated increases in *Znt1* mRNA levels. (D) Cytokines increase metabolic activity of the cells as measured by conversion of XTT in the supernatant of the cells after 4 h of incubation. Both zinc and NOS inhibition abolish this effect. (E) Treatment with pro-inflammatory cytokines decreases cell proliferation, as assessed by a decrease in BrdU accumulation within 24 h. Addition of zinc or inhibition of NOS activity by L-NIO abolish the effect of the cytokines.



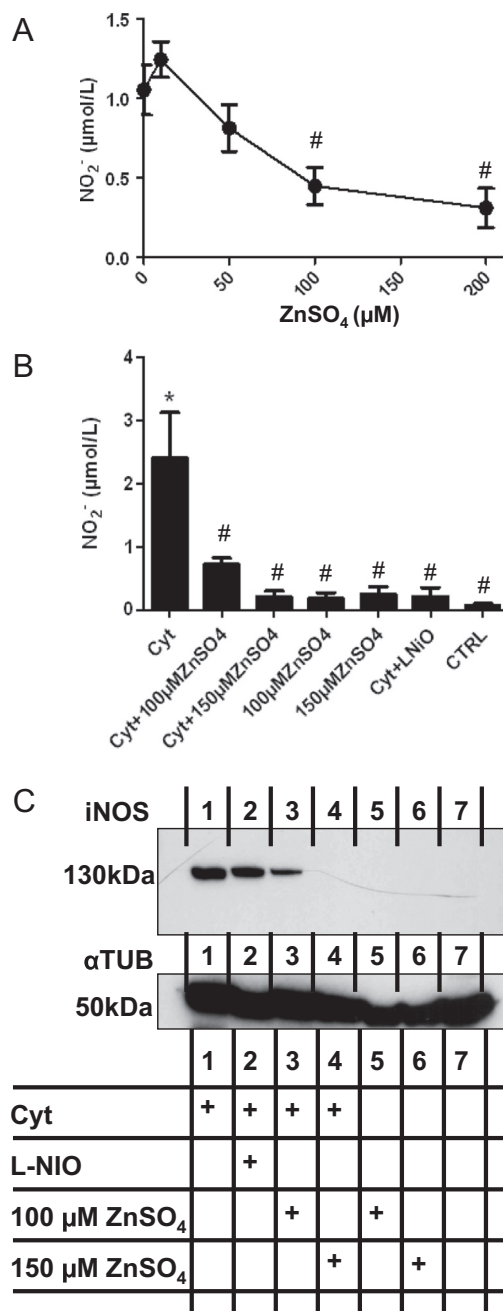
compared to the mock control (cells treated with transfection reagent only). Other sequences tested and not selected were Rn-RelA-1-HPsiRNA, Rn-RG2:727889\_2\_HPsiRNA, and Rn-RG2:727889\_3\_HPsiRNA. As a control for unspecific effects, a non-silencing (NS) siRNA sequence was used (Qiagen). The positive control was an siRNA sequence directed against rat *Gapdh* mRNA (Dharmacon), which has been used also for determining the best conditions for transfection. The silencing efficiency was verified in each experiment by measuring the expression of *Gapdh* and *RelA* (*p65*) mRNA. RNA was isolated by using RNeasy mini kit (Qiagen) following the manufacturer's instruction. The RNA (0.5 µg) was then reversely transcribed in MyCycler Personal Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany) using a QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. The cDNA (5 ng) or control RNA was used as a template for real time PCR performed in triplicate, using SYBR GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, Karlsruhe, Germany), or TaqMan Universal PCR Master Mix in ABI PRISM 7900 (Applied Biosystems) and 18 sr RNA as a house-keeping gene. Primers were purchased from Applied Biosystems: transporter 1 (*Znt1*) (NM\_022853, assay Rn00575737-m1), and 18S rRNA (Eukaryotic 18S rRNA, endogenous control VIC/TAMRA primer limited); or from Qiagen: *RelA* (*p65*) (QT01580012), inducible NO synthase, nitric oxide type 2 (*Nos2*) (QT00068740), interleukin 1beta (*il1b*) QT00181657 cyclooxygenase 2 (*Cox2*, official name: prostaglandin-endoperoxide synthase 2, *Ptgs2*, QT00192934), and 18SrRNA (Hs-RRN18S-1-SG). Primer specificity was verified by melting curve analysis. Results were analyzed as described [13].

#### Preparation of total and nuclear protein extracts, and western blot analysis

Total protein lysate was prepared as previously described [12,13] from endothelial cells grown in a 6-well plate and treated for 24 h with pro-inflammatory cytokines ± zinc, as indicated in the figure legends. Nuclear protein extracts were obtained from endothelial cells grown in 100 mm dishes (10<sup>7</sup> cells/plate) for 48 h and then treated for 30 min with pro-inflammatory cytokines ± zinc as indicated. Nuclei were extracted with Nuclear extract kit (Active Motif, Carlsbad, USA) following the manufacturer's protocol. Protein concentration was determined by the Lowry assay (DC Protein Assay, Bio-Rad). Total cell lysates (30 µg) and nuclear cells extracts (10 µg) were denatured and were loaded together with protein markers (Magic Mark, Invitrogen) on a 10% NuPAGE Bis-Tris pre-cast gel following the manufacture's protocols. The proteins were transferred on PVDF membrane Hybond P (Amersham Biosciences, GE Healthcare, Freiburg, Germany). Staining was performed as described [13] with mouse anti-NOS2 or mouse anti *RelA* (*p65*) antibodies (BD Biosciences, Heidelberg, Germany) diluted 1:1000 or anti-human-actin (1:5000) and then incubated with HRP-conjugated goat anti-mouse antibody diluted 1:5000 in blocking buffer. The bands were visualized by autoradiography on Hyperfilm ECL (Amersham Biosciences) using SuperSignal west Pico Chemiluminescent Substrate (Pierce, Thermo Lifescience, Bonn, Germany).

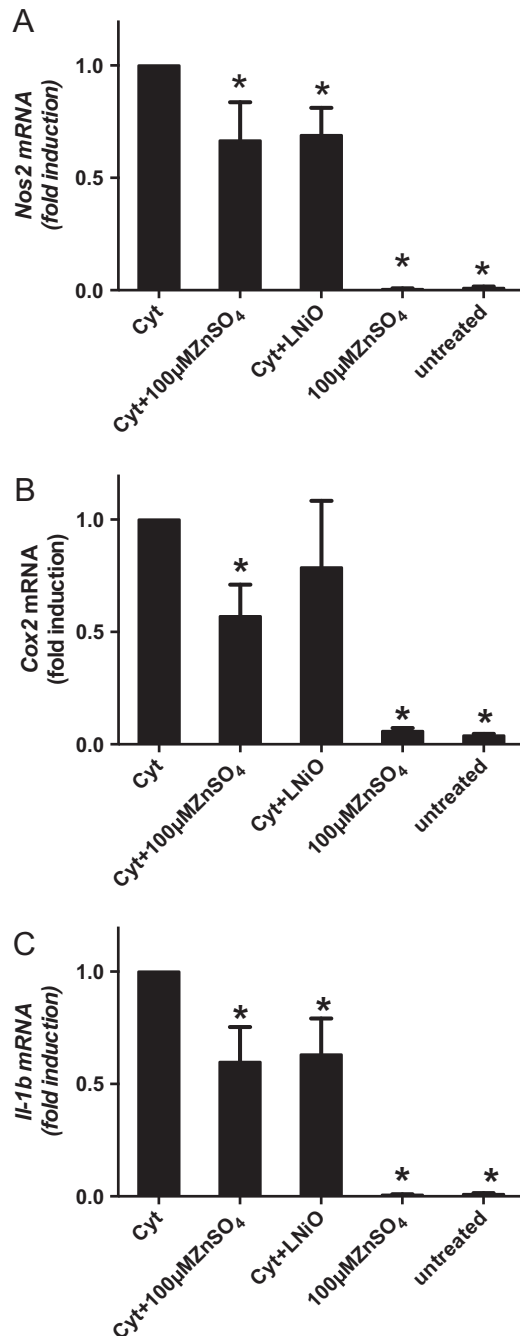
#### Determination NF-κB-dependent gene expression activation and iNOS promoter activity

To measure the effects of zinc on cytokine-dependent κB responsive element activation in endothelial cells, endothelial cells were transfected with a reporter vector pGL4.32[Luc2P/NF-κB-RE/Hygro] with 5 NF-κB responsive elements controlling the expression of a luciferase (Promega, Mannheim, Germany). Endothelial cells (10<sup>5</sup> cells/well) were seeded on to a 6-well plate and grown for 48 h, and then transfected with 2 µg plasmid,



**Fig. 2.** Zinc inhibits iNOS expression and activity in endothelial cells. (A) Inhibition of recombinant iNOS activity as assessed by measuring the accumulation of nitrite into the reaction buffer after 10 min of incubation with the indicated concentrations of zinc by reductive chemiluminescence. # *T*-test *p* < 0.05 vs. control without zinc. (B) Inhibition of cytokine-induced accumulation of nitrite into the supernatant of endothelial cells by zinc as assessed after 24 h of incubation. ANOVA *p* < 0.001; \* *p* < 0.05 vs. CTRL (untreated); # *p* < 0.05 vs. Cyt-treated cells. (C) Inhibition of cytokine-induced iNOS protein expression by zinc as assessed by western blot analysis. Staining of α-tubulin was used as a loading control.

0.2 µg Renilla luciferase expression vector (pRL3-SV40), and 8 µl lipofectin (Invitrogen). 24 h after transfection cells were treated with pro-inflammatory cytokines ± zinc for 6 h and then lysed in 150 µl of a lysis buffer (reporter gene assay lysis buffer, Roche Diagnostic GmbH), centrifuged at 13,000g for 2 min at 4 °C in Universal 30RF (Hettich Zentrifugen), quick frozen in liquid N<sub>2</sub>, and stored at −80 °C until luciferase assays was performed.



**Fig 3.** Zinc inhibits cytokine-induced expression of pro-inflammatory genes. Zinc supplementation of cytokine-treated cells for 24 h decreased the mRNA levels of (A) Nos2, (B) IL-1 $\beta$ , (C) Cox2 mRNA as assessed by real time RT-PCR. \*  $p < 0.05$  vs. Cyt-treated cells.

To specifically determine the effects of zinc on cytokines-dependent transactivation of the iNOS promoter, A549/8 iNOS cells were treated for 6 h as indicated and then lysed as described above.

Luciferase assay was carried out in a luciferase assay buffer (30 mM tricine, 0.1 mM EDTA, 15 mM MgSO<sub>4</sub>, 10 mM DTT) supplemented with 0.27 mM coenzyme-A (Applichem, Darmstadt, Germany), 0.53 mM ATP and with 0.5 mM of the substrate D-luciferine (Applichem). Cell lysate (20  $\mu$ l) was assayed in double for luminescence using a microtiter plate reader with automatic

injection (FLUOstar OPTIMA, BMG). The light units (LU) of the luciferase assay were normalized by protein content and were calculated as relative luciferase activity given in percent as compared to the controls (= 100%).

#### Statistical analysis

Real-time PCR data were processed as described [13]. Data are reported as mean  $\pm$  SEM. For statistical analysis, we used ANOVA followed by an appropriate *post hoc* multiple comparison test (Tukey or Student's *T* test).

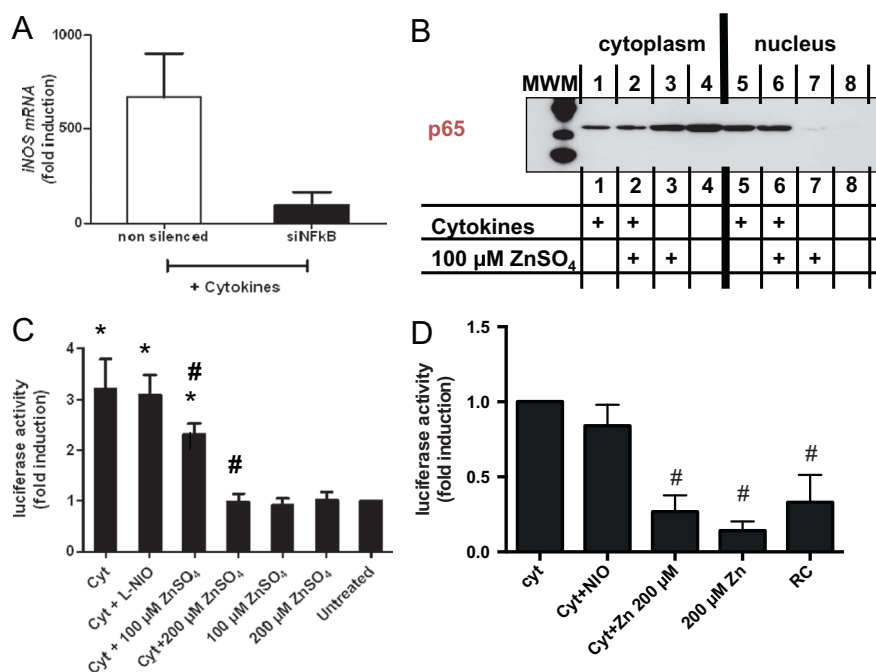
## Results

### Zinc reverses iNOS activity-dependent inhibition of endothelial cell proliferation

We aimed to study whether zinc might affect iNOS activity in endothelial cells. The expression of iNOS was induced by treating primary rat aorta endothelial cells with a mixture of pro-inflammatory cytokines (IL-1 $\beta$ +INF $\gamma$ +TNF $\alpha$ ), which leads to a significant accumulation of nitrite in the supernatant found after 6–8 h of incubation (Supplementary Fig. S1). Accumulation of nitrite was fully inhibited by treatment with 500  $\mu$ M of the specific NOS inhibitor L-NIO (Supplementary Fig. S1).

To increase intracellular “free” zinc concentrations, a zinc solution obtained by dissolving ZnSO<sub>4</sub> in NaCl 0.9% was added directly to the culture medium (containing  $2.28 \pm 0.32$   $\mu$ M zinc). This treatment induced a concentration-dependent increase in the intracellular fluorescence intensity of the zinc-specific fluorophore Zinquin, which could be observed immediately (< 30 s) after addition of zinc to the medium [13]. Fig. 1A shows the increases of Zinquin fluorescence before and 5 min after adding 100  $\mu$ M zinc. After the addition of 100  $\mu$ M ZnSO<sub>4</sub>, intracellular Zinquin fluorescence increased approximately 3 folds. As a result of an increase in “free” bioavailable zinc, the expression of zinc transporter 1 (ZnT-1) (Fig. 1C) and metallothionein (MT1a) [13] is induced in the endothelial cells.

The toxicity of the treatment was tested by treating endothelial cells with different concentrations of ZnSO<sub>4</sub> in the absence or in the presence of cytokines, or the NOS-inhibitor L-NIO (Fig. 1B). Under the experimental conditions chosen the toxicity of 24 h incubation with zinc in cytokine-treated cells was not different from untreated cells (half maximal lethal dose (LD<sub>50</sub>) =  $547 \pm 85$   $\mu$ M with zinc vs. LD<sub>50</sub> =  $579 \pm 91$   $\mu$ M without zinc). To test the effects of zinc supplementation on metabolic activity and proliferation of cytokine-treated cells, we measured the mitochondrial dehydrogenase catalyzed reduction [31] of XTT into formazan (Fig. 1D) and the incorporation of BrdU into the DNA (Fig. 1E), respectively. We found that cytokines increased metabolic activity of the cells by about 20% (Fig. 1D, Cyt), but decreased cell proliferation by about 40% (Fig. 1E, Cyt) as compared to untreated cells (Fig. 1D, E, CTRL). These effects were inhibited by adding the specific NOS inhibitor L-NIO (500  $\mu$ M; Fig. 1D, E, Cyt+L-NIO), indicating that NOS-derived NO production is responsible for the cytokine-induced effects. Addition of non-toxic zinc concentrations to cytokine treated cells exerted the same effects as NOS inhibition on the effects induced by cytokines on cell proliferation and mitochondrial dehydrogenase activity, respectively (Fig. 1D, E, 100  $\mu$ M ZnSO<sub>4</sub>).



**Fig 4.** Zinc affects NF-κB-dependent activation of the iNOS promoter without affecting NF-κB translocation into the nucleus. (A) NF-κB is the dominant transcription factor regulating cytokine-induced expression of iNOS under the experimental conditions used as assessed by knocking down the expression of NF-κB via RNA interference. T-test  $p < 0.05$  (B) zinc does not affect cytokine-induced translocation of NF-κB into the cell nucleus as assessed 30 min after addition of cytokines (representative of 3 independent experiments). (C) Zinc decreases cytokines induced NF-κB activation, as assessed 6 h after addition of cytokines by using an NF-κB luciferase reporter construct transfected in endothelial cells. ANOVA  $p < 0.0001$ ; \*  $p < 0.05$  vs. untreated; #  $p < 0.05$  vs. Cyt-treated cells. (D) Zinc inhibits cytokine-induced iNOS promoter activity as assessed 6 h after addition of cytokines in A549-iNOS reporter cells. 1-Way ANOVA  $p = 0.0135$ , # T-test  $p < 0.05$  vs. Cyt-treated cells.

#### Zinc inhibits iNOS activity and decreases cytokine-induced iNOS expression in endothelial cells

Next we aimed to analyze whether zinc might affect iNOS-dependent NO production in endothelial cells. We found that concentrations  $> 50 \mu\text{M}$  zinc affect the catalytic activity of recombinant iNOS *in vitro*, as tested by measuring the accumulation of nitrite in the reaction buffer by chemiluminescence analysis (Fig. 2A). In endothelial cells, addition of zinc decreased nitrite accumulation in the supernatant of cytokine-treated endothelial cells as measured after 24 h of incubation (Fig. 2B). We found that zinc added together with cytokines shown in Fig. 2B, or 6 h after the treatment with cytokines (not shown) strongly decreased iNOS expression as shown by Western blotting (Fig. 2C).

#### Zinc inhibits iNOS expression by affecting NF-κB-dependent gene transcription

To verify whether these effects were mainly dependent on zinc-mediated inhibition of iNOS transcription, iNos (*Nos2*) mRNA levels were measured by real-time RT-PCR. Cytokines increased the levels of *Nos2* mRNA already after 2–4 h of incubation (not shown), reaching the maximum after 6–8 h (Fig. 3A shows the results after 6 hours of incubation). In the presence of zinc, *Nos2* mRNA levels strongly decreased. Similarly, the expression of two NF-κB-dependent inflammatory genes *Cox2* (Fig. 3B) and *IL-1β* (Fig. 3C) was strongly affected by zinc, as assessed after 6 h of incubation. Interestingly, inhibition of iNOS during cytokines exposure results in about the same effect on mRNA for iNOS, COX2, *IL-1β* as zinc exposure.

Next, we studied whether NF-κB was involved in zinc-mediated inhibition of iNOS expression. We knocked down the expression of the NF-κB (p50/p65) by transfecting endothelial cells with an siRNA directed against p65, which decreased mRNA levels of p65 to a minimum of 90%. Cytokine-induced iNOS expression in the

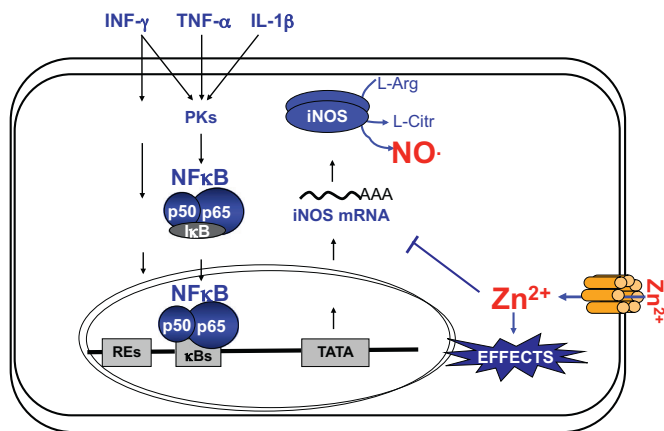
absence of NF-κB was decreased by almost 99% (Fig. 4A), confirming that NF-κB is the dominant transcription factor involved in cytokine-induced iNOS expression in our cells. To analyze whether zinc affects NF-κB translocation into the nucleus, the presence of p65 in the cytoplasmic as well as the in the nuclear fraction of endothelial cells was assessed by western blot analysis (Fig. 4B). Treatment with zinc did not affect the cytokine-induced translocation of p65 into the nucleus. In fact, the amount of p65 found in the nucleus of cytokine-treated cells after a 1 h-incubation with cytokines+zinc is not different as found in cells treated with cytokines only (Fig. 4B).

We further found that treatment with zinc decreased cytokine-induced transcriptional activation of an NF-κB-reporter plasmid, which was transiently transfected in the endothelial cells (Fig. 4C). To further confirm that zinc inhibits transactivation of iNOS promoter, we repeated these experiments by using a reporter cell line transfected with 1.3 kb of a human iNOS promoter in front of a luciferase reporter gene. We found that zinc decreased the cytokine-induced luciferase activity also in this cell line (Fig. 4D). Zinc itself did not affect luciferase basal activity or the viability of A549 cells as tested by neutral red assay (not shown).

Taken together these data show that zinc inhibits iNOS expression and activity via inhibition NF-κB-dependent transactivation of the iNOS promoter.

#### Discussion

In this study we found that an increase in intracellular “free” zinc concentration by addition of exogenous zinc results in a decreased NF-κB-dependent activation of iNOS expression in endothelial cells, thereby limiting high output NO production and cell activation, while promoting cell proliferation under pro-inflammatory conditions. Thus we found that 1. addition of non-toxic zinc concentrations to cultured primary endothelial cells



**Fig. 5.** Potential feedback regulation of iNOS expression by zinc in endothelial cells. Pro-inflammatory cytokines induce iNOS expression. iNOS-derived NO induces zinc release from zinc clusters of intracellular proteins. We here show that zinc decreases NF-κB mediated iNOS expression.

increased intracellular “free” zinc concentrations as demonstrated by increased Zinquin fluorescence, and increases in mRNA levels of the zinc responsive gene *Znt1*, 2. similar to NOS inhibition, zinc reverses cytokine-dependent inhibition of endothelial cell proliferation, 3. zinc decreases cytokines-induced iNOS expression and high out NO synthesis, and 4. zinc decreases NF-κB-mediated transactivation of iNOS promoter. Taken together these results show that zinc limits iNOS-derived high-output NO production in endothelial cells by inhibiting NF-κB-mediated transactivation of iNOS promoter, pointing to a role of zinc as a regulator of iNOS activation in inflammation in endothelial cells.

#### Zinc-mediated inhibition of iNOS activity

Although in recent years both post-transcriptional as well as post-translational mechanisms regulating iNOS activity have been discovered [4], the regulation of iNOS expression remains the main regulatory step to control iNOS activity. Usually iNOS synthesizes NO continuously until the enzyme becomes degraded. We here found that zinc strongly decreases cytokine-induced iNOS expression (mRNA + protein) as well as NOS-dependent nitrite accumulation in the culture medium of the endothelial cells. Similar results were previously obtained with mouse keratinocytes [27], but the molecular mechanisms involved were not further investigated. As shown here, zinc directly inhibits iNOS catalytic activity in a concentration-dependent fashion ( $EC_{50} \approx 75 \mu\text{M}$ ), but would require local intracellular zinc concentrations of  $> 50 \mu\text{M}$ . The inhibitory effects exerted by zinc on NOS catalytic activity are well known [25,26], and other divalent transition metals like copper exert similar effects at micromolar concentrations. Total cellular zinc concentrations are in the range of a few hundred micromolar, and it is distributed within sub-cellular compartments. However, the affinity of zinc for cytosolic zinc proteins is in the picomolar range, suggesting that the availability of free zinc ions within the cytoplasm is quite low [11]. In lysosomes and zinosomes the concentration of zinc may reach millimolar concentrations. Since iNOS is mainly localized within the cytoplasmic compartment of the cell, we may exclude a direct effect of zinc ions on the enzyme itself. Therefore, we conclude that the effects of zinc supplementation on iNOS activity within the endothelial cells are mainly dependent on the modulation of iNOS expression.

By using a reporter cell line stably transfected with the human iNOS promoter, we found that zinc supplementation directly inhibits the cytokine-mediated activation of the iNOS promoter. The sequence of human, rat and mouse iNOS promoters show a

high grade of homology. The transcription factor NF-κB seems to be a central target for activators or inhibitors of iNOS expression in all three species. Pro-inflammatory stimuli including LPS, IL-1β, TNFα, in combination with INFγ, have been shown to induce iNOS expression in different cell types via activation of NF-κB [4]. By silencing the expression of p65, we found a 99% reduction of cytokine-induced up-regulation of iNOS, confirming that in our system NF-κB plays a central role in cytokine-mediated induction of iNOS expression. The rat promoter contains an upstream (−965 to −956 bp) and downstream (−107 to −98) NF-κB site, both of which are important for iNOS induction. In the rat promoter a third NF-κB site located at −901 to −892 bp with an opposite orientation, also defined as “the reverse NF-κB site”, is important for IL-1β-/INFγ-induced promoter activity [32].

We here show that zinc-mediated down regulation of iNOS expression is due to inhibition of NF-κB transactivation activity, and not NF-κB activation/translocation into the nucleus. The active transcription factor, which typically is the p50/p65 heterodimer [33], is released from an inhibitory cytosolic complex and translocates into the nucleus to bind to κB-responsive elements upstream of its target genes. Inhibition of NF-κB can occur by direct capture of NF-κB via protein–protein interactions [34], inhibition of NF-κB phosphorylation [35], inhibition of nuclear NF-κB-translocation [36], inhibition of NF-κB transactivation activity [37], or by increasing the expression of i-κB, the specific inhibitor of NF-κB [38].

We found that zinc inhibited NF-κB transactivation activity in cytokine-treated endothelial cells, as assessed by transient transfection of a κB-luciferase construct. While inhibition of the NF-κB signaling by zinc was shown in different tissues and cells, the mechanisms involved are cell/tissue specific and unknown for many cell types [39,40]. Zinc was shown to inhibit NF-κB activation/translocation in mononuclear cells [35,41], and other cell types [35,42,43] and to reduce the levels of activated NF-κB in diabetic CD1 mice [44]. Severe zinc deficiency was shown to inhibit the translocation and transactivation capacity of NF-κB in neuronal cells and rat testes [45,46]. Zinc chelation has been shown to increase LPS-induced iNOS expression in RAW 264.7 cells [47]. In endothelial cells it was shown that zinc affects the expression of pro-inflammatory cytokines [48,49], while zinc deficiency exerts a permissive effect on the expression of inflammatory genes and exacerbates chronic inflammation in endothelial cells [48,49]. However, to the best of our knowledge this is the first time that zinc was shown to regulate iNOS expression via inhibition of NF-κB transactivation in primary endothelial cells. Future studies should be undertaken to investigate the molecular mechanisms responsible for zinc-mediated inhibition of transactivation activity of NF-κB into the nucleus.

#### Zinc and cell signaling: in vitro evidence and limitations

Because zinc ion fluctuations occur at such low concentrations and zinc interacts strongly with proteins, released zinc ions are now considered as potent intracellular signals [9,11]. Picomolar to low nanomolar concentrations of zinc ions inhibit enzymes involved in energy metabolism, signaling and mitochondrial respiration [50–54]. Here we found that zinc ion fluctuation in cytokine-treated cells induced by administration of non-toxic zinc concentrations, reduced the activation of the cells [55] and rescued their proliferation capability, leading to inhibition of iNOS expression and activity. Changes in intracellular free zinc concentrations and zinc ion fluctuations were induced here by exogenous administration of zinc ions to the culture medium, as demonstrated by analyzing increases in intracellular Zinquin fluorescence, and in the expression of zinc-responsive genes, including metallothionein-1 (MT-1) and zinc transporter-1 (ZnT1) [13,16].



Metallothioneins and zinc transporters contribute to rapidly “buffer” high intracellular zinc concentrations either by binding zinc (e.g. MT-1), or by transporting zinc outside of the cell (e.g. ZnT1) or into sub-cellular compartments, like lysosomes and zincosomes (e.g. via ZnT2 and/or other zinc transporters) [56], which also explains why cells are resistant to micromolar levels of zinc.

Should we consider the changes of intracellular “free” zinc, which are induced by adding micromolar concentrations of zinc to the culture media, physiological or superphysiological? Are the responses to these zinc fluctuations relevant for *in vivo* biology and/or pharmacology of zinc? The concentrations of zinc needed to increase the intracellular zinc concentrations in our endothelial culture system [12,13] are 5–20 fold higher as the zinc concentrations found in serum of healthy persons (about 12–15  $\mu\text{mol/l}$ ; 78–98 mg/dl) [57]. The effects of zinc supplementation to cultured cells are strongly dependent on the cell type under consideration, but also on the composition of the cell culture media, which may contain micromolar levels of zinc and millimolar concentrations of zinc-complexing molecules/chelators [58]. In our medium, we measured a total zinc concentrations of  $2.28 \pm 0.32 \mu\text{M}$ , as assessed in 3 different media preparations. As pointed out by Bozym et al. [58], in media containing 5–10% v/v serum, albumin is present at a concentration of 0.3 mmol/l, and therefore will bind a significant portion of the available zinc with relatively high affinity ( $K_D = 0.1 \mu\text{mol/l}$ ). The “free” zinc concentration is typically two to three orders of magnitude below the total added zinc, such that adding  $\sim 100 \mu\text{mol/l}$  total zinc typically resulted in  $\sim 100 \text{ nmol/l}$  free zinc ( $pZn = 7$ ) being present, as assessed by different fluorimetric methods [58]. This means that in serum containing media the zinc levels are functionally buffered [58]. This is very similar to the *in vivo* situation where zinc is transported in the serum bound principally to albumin (70%),  $\alpha$ -2-macroglobulin (18%), and other proteins such as transferrin or ceruloplasmin. A very small amount (i.e., 0.01%) is complexed with amino acids, especially histidine and cysteine [57,58]. As also pointed out by Bozym et al., care is particularly necessary if cells are cultured in serum-free media, since zinc buffering ligands may be inadequate. The same could be said if zinc chelators – like pyrithione – is used to transport zinc inside the cells. Both zinc-depleted media or chelators decrease the effective doses of zinc used for experiments, but may also interfere with intracellular zinc equilibrium and with zinc signaling. The equilibrium between buffering and fluctuation of zinc ions will determine the borderline between physiology and pathophysiology [11].

Although we carefully characterized our model for: 1. concentration of zinc in media and sera; 2. increases in intracellular “free” zinc concentrations; 3. bioactivity of zinc, as shown by increases in the expression of the zinc responsive gene *Znt1*; 3. lack of overall toxicity, metabolic activity or proliferation, we cannot provide direct evidence that the effects exerted by zinc *in vitro* in this and other similar studies are also relevant for *in vivo* biology. This is one of the main limitations of cell culture models. The role of zinc in iNOS regulation in the endothelium should be addressed in future study in animal models, and in humans.

#### Are iNOS and zinc signaling pathways interdependent?

We and others have shown that iNOS-derived NO can nitrosate metallothionein [59] and thereby induce zinc release. Zinc released by NO may exert signaling functions leading to both cytoprotection as shown in endothelial cells by us [12,14] or in the lung [17,19], as well as to neurotoxicity in the brain [60], depending on the cellular compartment. By considering zinc-mediated inhibition of iNOS, it is tempting to speculate that zinc released by iNOS-derived NO may be part of the termination signals needed for the control of acute inflammatory reactions.

Thus, under pro-inflammatory conditions, NF- $\kappa$ B activation leads to endothelial cell activation corresponding to the expression of inflammatory genes, including pro-inflammatory cytokines and iNOS, and contribute to the propagation of the inflammatory response. In activated endothelial cells expression of iNOS is induced and long-term high-output NO synthesis follows. iNOS-derived NO may then induce zinc release from zinc–sulfur clusters of intracellular proteins, and NF- $\kappa$ B might be a target of NO-mediated zinc redistribution [40,61]. If this would be the case, the NO-mediated intracellular zinc release may contribute to limit an excessive propagation of the inflammatory response by inhibiting or limiting endothelial cell activation. At present, this feedback loop connecting iNOS-mediated zinc release, and zinc mediated inhibition of iNOS expression should be considered a speculative observation, which should be addressed experimentally in future.

#### Summary and conclusions

To summarize, we here have shown that zinc ions control iNOS expression and activity in endothelial cells via inhibition of NF- $\kappa$ B transactivation activity (Fig. 5). Interestingly, it has been shown that not only zinc supplementation inhibits NF- $\kappa$ B activation, but also that zinc deficiency exerts a permissive effect on the expression of inflammatory genes and exacerbates chronic inflammation [48,49]. Therefore, zinc signals and the maintenance of an adequate zinc status in the endothelium may be an important clinical strategy to prevent the development of endothelial dysfunction and cardiovascular diseases.

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#### Appendix A. Supplementary material

Supplementary material for this article can be found online at <http://dx.doi.org/10.1016/j.redox.2014.06.011>.

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