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Zn^{2+} inhibits nitric oxide formation in response to lipopolysaccharides: Implication in its anti-inflammatory activity

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Abstract

There is compelling evidence to indicate an anti-inflammatory action of Zn^{2+}. Most inflammatory diseases are associated with an increase of the inducible form of nitric oxide (NO) synthase. Additionally, inflammatory mediators such as histamine or bradykinin stimulate the constitutive NO synthase. Thus, the present study was undertaken to investigate whether Zn^{2+} inhibits production of inducible NO synthase and/or constitutive NO synthase activity to produce NO. Lipopolysaccharide, 5 mg/kg i.v., administered to Zn^{2+}-deficient (ZD) rats, rats supplemented with Zn^{2+} sulfate (ZG), 10 mg/kg s.c., or controls resulted in a significant reduction of their serum Zn^{2+}. The levels of N^G-nitro-L-arginine methylester (L-NAME)-sensitive cyclic GMP (cGMP) in aortas isolated from ZD or ZG were significantly lower than those obtained from control animals. Zinc (100–150 μM) produced a dose-dependent inhibition of lipopolysaccharide or interleukin-1β-induced NO formation in isolated rat aortic smooth muscle cells. Compared to cyclohexamide or actinomycin-D, the time course of inhibition of NO formation by 150 μM Zn^{2+} did not suggest an effect of Zn^{2+} on inducible NO synthase protein synthesis. Moreover, Zn^{2+} (150 μM) significantly reduced the rate of conversion of [^{3}H]arginine to [^{3}H]citrulline in lung homogenates from lipopolysaccharide-treated rats. Incubation of rat aortic smooth muscle cells and bovine pulmonary artery endothelial cell co-cultures with Zn^{2+} (150 μM) caused a significant reduction in basal and bradykinin- or A-23187-induced formation of cGMP. Thus, our results indicate that Zn^{2+} is capable of inhibiting lipopolysaccharide- or interleukin-1β-induced NO formation as well as NO formation by constitutive NO synthase basally or in response to bradykinin or A-23187, and may explain the reported anti-inflammatory activity of Zn^{2+}. © 1998 Elsevier Science B.V.

Keywords: Zn^{2+}; Inflammation; Nitric oxide $(NO)_i$; Nitric oxide (NO) synthase; Endotoxin; Lipopolysaccharide

1. Introduction

Zinc is an essential metal which participates in a wide range of basic cellular functions in eukaryotic cells. It binds structural proteins and acts as a cofactor for metalloenzymes such as DNA polymerase, collagenase, angiotensin converting enzyme, superoxide dismutase, etc. (Vallee, 1983). Endogenous Zn^{2+} is also involved in both human and experimental inflammation (Oliva et al., 1987). Low levels of serum Zn^{2+} have been observed in most rheumatic diseases (Kennedy et al., 1975; Sevensen et al., 1985) and Zn^{2+} supplementation was found to inhibit the increase in vascular permeability and paw edema induced by phospholipase A_2 (Ferrer and Moreno, 1991). In acute and chronic rat models of inflammation, Zn^{2+} administration enhanced the activity of indomethacin and diclofenac (Abou-Mohamed et al., 1995a). Similarly, in humans, oral administration of Zn^{2+} improved joint swelling, morning stiffness and walking times in patients with rheumatoid arthritis (Simkin, 1976).

Nitric oxide (NO) is a labile molecule that is formed from L-arginine by NO synthase. Thus far, three distinct isoforms of NO synthase, representing three distinct gene products, have been isolated and purified. Two of the enzyme type are constitutively present and they are localized in the endothelium and in the central and peripheral neurons (Bredt et al., 1991; Moncada et al., 1991). The amounts of NO produced by these two enzymes are relatively small and participate in cellular signaling. However, a third isoform of NO synthase that can be induced by immunologic and inflammatory stimuli known as the inducible NO synthase has also been identified (Förstermann et al., 1991; Hevel et al., 1991). Such induction results in a sustained production of NO. NO participates in various pathophysiological processes, including inflammation. Be-

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cause NO is a vasodilator, it increases blood flow resulting in heat, redness and edema associated with inflammation in several tissues (Hughes et al., 1990; Mulligan et al., 1991; Ialenti et al., 1992). On the other hand, NO inhibits the adhesion of circulating neutrophils to the endothelium (Kubes et al., 1991).

Nevertheless, inflammatory diseases such as graft vs host disease (Langrehr et al., 1992), insulitis (Kolb and Kolb-Bachofer, 1992), arthritis (McCartney-Francis et al., 1993) and carrageenan-induced paw edema (Ialenti et al., 1992) are associated with production of inducible NO synthase, thus implicating NO in chronic inflammation. Indeed, NO increases the formation of cyclooxygenase products which are major mediators of inflammation (Salvemini et al., 1993).

The aim of the present study is to investigate the in vivo and in vitro effects of Zn^{2+} on inducible NO synthase production and/or activity which may underly the anti-inflammatory activity of Zn^{2+}.

2. Materials and methods

2.1. Drugs and chemicals

The following materials were used: $ZnSO_4$ (Fisher Scientific, Fair Lawn, NJ), interleukin 1-β (Mannheim-Gmbh, Germany), F12 and Dulbeco's modified Eagle medium (GIBCO Laboratories, Grand Island, NY), fetal bovine serum (Hyclone Laboratories, Logan, UT), tetrahydro-L-biopterin (RBI, Natick, MA), ^{125}I and L-[2,3-^{3}H]arginine (Du Pont, NEN, Boston, MA), and protein binding dye (Bio-Rad, Richmond, CA). *E. coli* lipopolysaccharide serotype (0127:B8), N^{G}-nitro L-arginine methylester, isobutyl methyl xanthine, bovine serum albumin, EGTA, pepstatin A, leuopeptine, bestatin, phenylsulfonylfluoride, Earle's salt, glutamine, penicillin, streptomycin and sodium nitroprusside were purchased from Sigma Chemical, St Louis, MO.

2.2. In vivo and ex vivo experiments

Male Sprague–Dawley rats were housed individually in stainless steel cages. Animals were put under 12 h light-dark periods at a controlled temperature. The rats were fed a standard diet (Harlan Teklad, WI) for 5 days after which they were randomly distributed into three experimental groups; ZD, ZG and controls. The ZD group received a Zn^{2+}-deficient diet ad libitum (Harlan Teklad, WI) and distilled deionized water for 6 weeks. The ZG and control groups were given standard diet ad libitum. During the last 5 days prior to lipopolysaccharide administration (see below), the ZG group received 10 mg/kg/day $ZnSO_4$ s.c. Six weeks after starting the experiment, under pentobarbital anesthesia (55 mg/kg i.p.), 6 animals from each group were equipped with a jugular vein catheter for lipopolysaccharide administration and blood withdrawal.

One to two days later, experiments were performed in conscious rats. Blood samples were taken and lipopolysaccharide, 5 mg/kg, was given i.v. to all groups. Six hours after lipopolysaccharide administration, another blood sample was taken via cardiac puncture under ether anesthesia. Rats were sacrificed and the thoracic aorta was excised, cleaned and cut into equal segments, approximately 3 mm long. All aortic segments were incubated at 37°C in 1 ml of Earl's balanced salt solution. One segment (from each rat) was incubated in the presence of N^{G}-nitro L-arginine methylester (L-NAME), final concentration of 0.5 mM, and a second segment was incubated only in buffer. Twenty minutes later, the phosphodiesterase inhibitor, isobutyl methyl xanthine (IBMX), final concentration 0.5 mM, was added. After 15 min, segments were transferred into tubes containing 500 μl of 0.1 N HCl to extract cGMP. After 1 h of incubation with HCl at room temperature, segments were removed and weighed. The extract was analyzed for cyclic GMP (cGMP) by radioimmunoassay, as previously described, in the Gammaflo automated radioimmunoassay system (Papapetropoulos et al., 1994).

Blood samples were kept at room temperature for 30 min and the serum was separated and stored at −70°C. Serum levels of Zn^{2+} were determined by the method of Carter (1974).

2.3. Vascular smooth muscle cells

2.3.1. Cell culture

Rat aortic smooth muscle cells were isolated from male rats (325–350 g) and cultured using previously published procedures (Papapetropoulos et al., 1994). Cells were positively identified as smooth muscle cells by indirect immunofluorescent staining for α-actin, using a mouse anti-α-actin antibody and anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate.

2.3.2. Protocol I

Rat aortic smooth muscle cells between passages 1–5 were incubated in growth medium containing lipopolysaccharide; l μg/ml or interleukin-lβ; 10 U/ml for 4 h. In similar groups of cells, 10–150 μM (final concentration) of $ZnSO_4$ was added simultaneously with lipopolysaccharide or interleukin-lβ. After a 4-h incubation period, samples of the incubation medium were taken for determination of Zn^{2+} content. Cells were washed with Earle's balanced salt solution and 1 ml of 1 mM IBMX and l mM L-arginine were added. Fifteen minutes later, medium was rapidly aspirated, and 500 μl of 0.1 N HCl was then added to each well to stop the reactions and to extract cGMP. Thirty minutes later, the HCl extract was collected and cell remnants removed from the wells by adding hot 1.0 N NaOH and scraping the well with a rubber policeman. The HCl extract was analyzed for cGMP by radioimmunoassay and the NaOH-solubilized cell remnants were used for protein determination.

In order to investigate the effect of Zn^{2+} on guanylyl cyclase activity, rat aortic smooth muscle cells were incubated with the different concentrations of Zn^{2+} (10–400 μM) for 4 h. Cells were washed with Earle's balanced salt solution and incubated for 15 min with 10 μM sodium nitroprusside in the presence of 1 mM IBMX.

2.3.3. Protocol II

In another series of experiments, rat aortic smooth muscle cells were incubated with Earle's balanced salt solution containing lipopolysaccharide (1 μg/ml). Zinc (150 μM), actinomycin-D (5 μg/ml) or cyclohexamide (10 μM) was added 60–210 min after the time of lipopolysaccharide addition. Four hours after lipopolysaccharide addition, cells were washed with Earle's balanced salt solution and incubated for 15 min with 1 mM IBMX and 1 mM L-arginine. Preliminary experiments indicated that levels of inducible NO synthase (measured indirectly by cGMP) are maximal 4 h after lipopolysaccharide administration. Cell content of cGMP was determined as above. In another experiment, Zn^{2+} was added at 240 min relative to lipopolysaccharide and the incubation period was extended to 6 h.

2.3.4. Protocol III

In order to examine the effects of Zn^{2+} on constitutive NO synthase activity, rat aortic smooth muscle cells were co-cultured with bovine pulmonary artery endothelial cells for 24 h (Papapetropoulos et al., 1994). The co-culture was incubated with Zn^{2+} (150 μM) for 2 h, washed with Earle's balanced salt solution and exposed for 15 min to IBMX (1 mM) in the presence and the absence of bradykinin (8.5 μM) or A-23187 (0.85 μM). Cell content of cGMP was determined as in protocol I.

2.4. Measurement of toxicity

To quantify cytotoxic effects of Zn^{2+}, cells were incubated with 0, 150 or 400 μM Zn^{2+} for 4 h before evaluating ^{51}Cr release, trypan blue exclusion or cell respiration. Cells were loaded overnight with $Na_2^{51}CrO_4$ (1 μCi/ml). The following day cells were washed twice to remove extracellular ^{51}Cr and incubated with or without Zn^{2+} for 4 h. A sample of medium was taken and the radioactivity was measured. Cell injury index was calculated as $100 \times (A - B)/(C - B)$, where A is chromium release from cells treated with Zn^{2+}, B is spontaneous chromium release from control cells and C is total chromium released after lysing the cells with 0.1% Triton X-100. To determine cell viability, cells were treated in the presence or absence of Zn^{2+} for 4 h trypsinized using a trypsin-EDTA (0.05% and 0.02% w/v, respectively) solution for 10 min and incubated with 0.15% trypan blue in Earle's balanced salt solution. Cell respiration was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide to formazan. After the treatment with Zn^{2+}, cells were incubated with MTT (0.2 mg/ml) for 30 min at 37°C. Medium was then aspirated and cells solubilized with dimethylsulfoxide. Absorbance at 550 nm was used to measure MTT conversion to formazan.

2.5. Effect of Zn^{2+} on inducible nitric oxide synthase activity

Control conscious rats were treated with 5 mg/kg of lipopolysaccharide, i.v. Six hours later, the animals were killed by decapitation and their lungs were removed, washed with Earle's balanced salt solution and frozen under liquid nitrogen until use. Frozen lungs were homogenized on ice using a polytron homogenizer (Polytron, Brinkmann Instruments, Westbury, NY) in a buffer composed of: Tris–HCl, 50 mM; EDTA, 0.1 mM; EGTA, 0.1 mM; pH 7.4. The buffer also contained the following protease inhibitors: 1 μM pepstatin A, 2 μM leupeptine, 1 μM bestatin and 1 mM phenylsulfonylfluoride. The homogenate was centrifuged at 1000 rpm for 10 min and the supernatant was used for the assay. Conversion of [^{3}H]arginine to [^{3}H]citrulline was measured by the method of Bredt and Snyder (1990) as described by Abou-Mohamed et al. (1995b). Briefly, 100 μl of lung homogenates were incubated in a buffer (Tris–HCl, 50 mM; EDTA, 0.1 mM; EGTA, 0.1 mM, pH 7.4) containing the cofactors (100 nM calmodulin, 1 mM NADPH and 3 μM tetrahydrobiopterin) and substrate 10 μM L-arginine combined with 0.6 μCi L-[2,3-^{3}H]arginine. The incubation mixture also contained 1mM L-citrulline to minimize any conversion of the formed [^{3}H]citrulline back to [^{3}H]arginine. The reaction was performed in the presence and the absence of 150 μM Zn^{2+} at 37°C for 15 min. In some experiments, Ca^{2+} and/or calmodulin were omitted from the reaction mixture. The reaction was terminated by adding 1 ml of ice-cold buffer (Tris HCl, 20 mM; EGTA, 2 mM and EDTA, 2 mM, pH 5.5). Reaction mixtures were applied to Dowex 50W-8 (Na^+ form) columns and the eluted [^{3}H]citrulline activity was determined by scintillation counting (Beckman LS 7500).

2.6. Protein determination

The NaOH-solubilized samples were centrifuged at 2000 rpm for 5 min at room temperature. The protein content of the supernatant was measured by the Bradford method (Bradford, 1976). Sample aliquots were combined with the protein binding dye and optical density was determined at 630 nm using a multiwell plate reader (Dynatech Laboratories). Bovine albumin, fraction V was used as a standard.

2.7. Statistical analysis

Data are presented as mean ± S.E.M. of the indicated number of observations (n) and the difference between

groups was assessed using, when appropriate, unpaired *t*-test or analysis of variance followed by Student-Newman-Keuls post hoc test. A probability value less than 0.05 was considered to be statistically significant.

3. Results

3.1. In vivo and ex vivo studies

Five days supplementation with Zn^{2+} produced a significant increase in Zn^{2+} serum level of ZG rats, relative to controls (Fig. 1). Conversely, 6 weeks of exposure to Zn^{2+}-deficient diet reduced serum Zn^{2+} levels in ZD rats, relative to controls (Fig. 1). Lipopolysaccharide (5 mg/kg) administered to ZD, ZG or control rats caused a significant reduction in their serum Zn^{2+} levels. Such reduction was more profound in the ZG rats than in the other groups (Fig. 1).

As expected, 6 h after lipopolysaccharide administration, the level of cGMP in isolated aortic rings of control rats increased about 3-fold as compared to those from non-lipopolysaccharide-treated controls. This elevation of cGMP was not blocked by 2.7 mM of EGTA (data not shown). In aortic rings isolated from rats given Zn^{2+}-deficient diet for 6 weeks, as well as those supplemented with 10 mg/kg $ZnSO_4$ for 5 days, levels of L-NAME-sensitive cGMP were significantly lower than those obtained from control animals (Fig. 2).

3.2. In vitro studies

Incubation of rat aortic smooth muscle cells with lipopolysaccharide (1 μg/ml) or interleukin-1β (10 U/ml) for 4 h resulted in a significant increase of inducible NO synthase as indicated by the rise in cGMP levels from 13.3 ± 0.5 to 642.7 ± 15.4 and 102 ± 4.7 pmol/mg prot./15 min for lipopolysaccharide and interleukin-1β, respectively.

Co-incubation of these cells with Zn^{2+} (10–150 μM) and lipopolysaccharide or interleukin-1β resulted in a significant reduction in cGMP levels with maximal inhibition at 150 μM Zn^{2+} (Fig. 3). Incubation of rat aortic smooth muscle cells with 150 μM Zn^{2+} did not significantly inhibit the basal or sodium nitroprusside-stimulated guanylyl cyclase activity (Fig. 4). In fact, it significantly

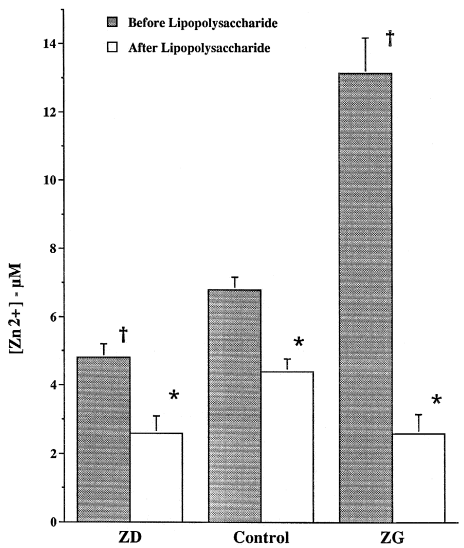

Fig. 1. Effects of lipopolysaccharide (5 mg/kg, i.v.) on serum [Zn^{2+}] in Zn^{2+}-deficient (ZD), control and Zn^{2+}-supplemented (10 mg/kg/day for 5 days) (ZG) rats. Results are expressed as the mean of 6 observations $\pm$S.E.M. * Significantly different from before lipopolysaccharide. † Significantly different from control rats.

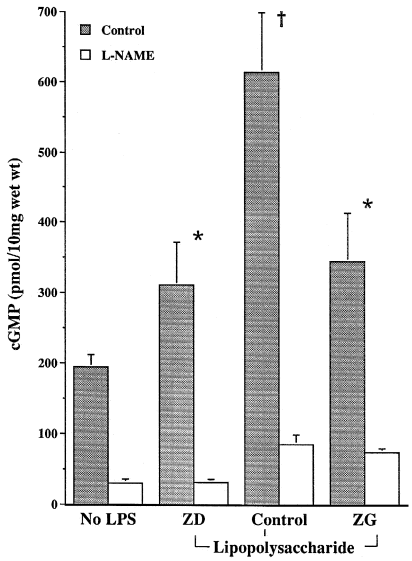

Fig. 2. Effect of lipopolysaccharide (LPS), 5 mg/kg, i.v., on the basal accumulation of cGMP, and its inhibition by L-NAME, in aortic rings isolated from Zn^{2+}-deficient (ZD), control or Zn^{2+}-supplemented (ZG) rats. Results are expressed as the mean of 6 observations $\pm$S.E.M. * Significantly different from control. † Significantly different from non-lipopolysaccharide-treated rats.

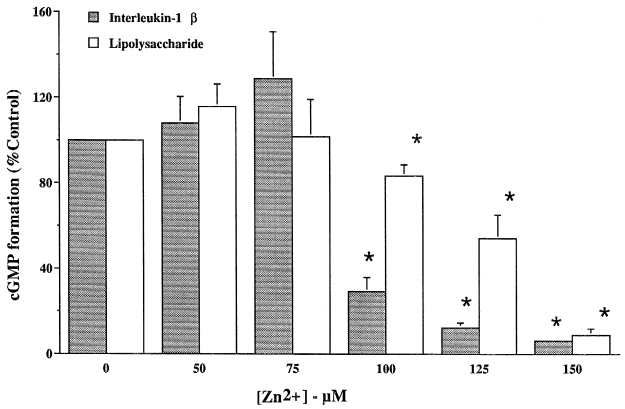

Fig. 3. Effect of different concentrations of Zn^{2+} on lipopolysaccharide (1 μg/ml) or interleukin-1β (10 U/ml)-induced cGMP formation in isolated rat aortic smooth muscle cells. Results are expressed as the mean of 6–8 observations $\pm$S.E.M. * Significantly different from control.

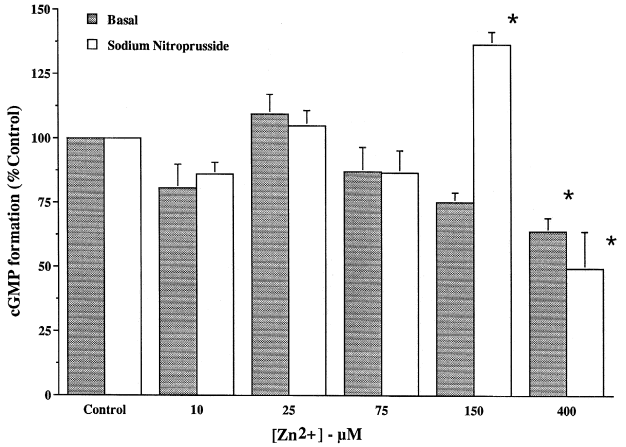

Fig. 4. Effect of different concentrations of Zn^{2+} on the basal and sodium nitroprusside-stimulated soluble guanylyl cyclase activity. Results are expressed as the mean of 6–8 observations ± S.E.M. * Significantly different from control.

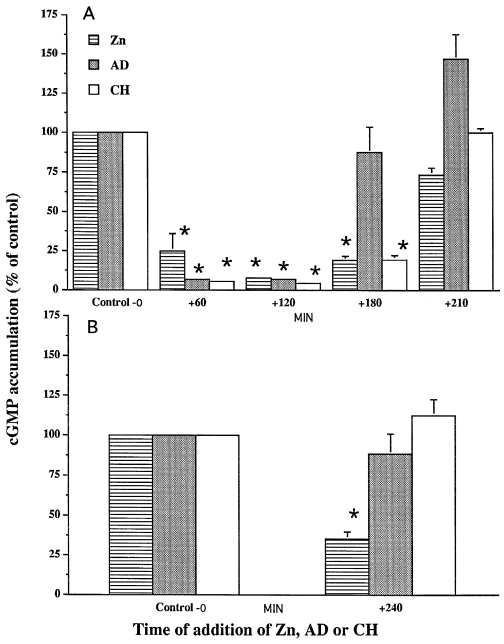

Fig. 5. (A) Effect of 150 μM [Zn^{2+}], cyclohexamide (CH) and Actinomycin-D (AD) on lipopolysaccharide-induced cGMP formation ($n = 6$). Zinc, CH and AD were added at the indicated time points relative to 4 h incubation with lipopolysaccharide. (B) When Zn^{2+} was added 240 min after lipopolysaccharide administration and inducible nitric oxide synthase activity was measured 6 h after lipopolysaccharide. * Significantly different from control.

enhanced sodium nitroprusside-stimulated guanylyl cyclase activity. However, 400 μM Zn^{2+} reduced both basal and sodium nitroprusside-stimulated activity of guanylyl cyclase. Based on these data, Zn^{2+} concentration of 150 μM was used in the subsequent experiments. Table 1 demonstrates that not all the Zn^{2+} added to the cultured cells gained access into the cell. Thus, only 8.2, 17.6, 25.6, 35 and 48 nmoles of Zn^{2+} were taken by cells out of the amount added: 50, 75, 100, 125 and 150 nmoles in 1 ml of bathing medium, respectively.

Zinc inhibited lipopolysaccharide-induced cGMP accumulation over the 4 h incubation period when added to the cell culture 60, 120 and 180 min after the addition of lipopolysaccharide (Fig. 5A). The transcription and protein synthesis inhibitors, actinomycin-D and cyclohexamide, respectively, acted in a fashion similar to Zn^{2+} with the exception that actinomycin-D added 180 min after lipopolysaccharide did not prevent the rise in cGMP levels due to lipopolysaccharide. When these inhibitors were added after 210 min, they did not affect cGMP accumulation (Fig. 5A). However, when the incubation period with lipopolysaccharide was extended to 6 h, Zn^{2+} significantly inhibited cGMP formation when added 240 min relative to lipopolysaccharide in contrast to actinomycin-D and cyclohexamide (Fig. 5B).

To determine if the inhibition of NO production by Zn^{2+} was not due to cytotoxicity, several measures of toxicity were examined. Exposure of cells for 4 h to 150 μM Zn^{2+} reduced cellular respiration to 76 ± 2% of control, increased trypan blue exclusion from 0 to 6 ± 2% but did not alter ^{51}Cr release. However, exposure to 400 μM Zn^{2+} significantly increased ^{51}Cr release by 56 ± 3% of the releasable radioactivity. In addition, in cells treated with 150 μM Zn^{2+} no change in the amount of protein per well was noticed.

In experiments designed to directly measure inducible NO synthase activity in lung-homogenates from lipopolysaccharide-treated rats, Zn^{2+} (150 μM) significantly reduced the rate of conversion of [^{3}H]arginine into [^{3}H]citrulline (Fig. 6). Such reduction was seen regardless of the presence or absence of Ca^{2+} and/or calmodulin.

In order to examine the effect of Zn^{2+} on constitutive nitric oxide synthase activity, rat aortic smooth muscle cells and bovine pulmonary artery endothelial cells co-cul-

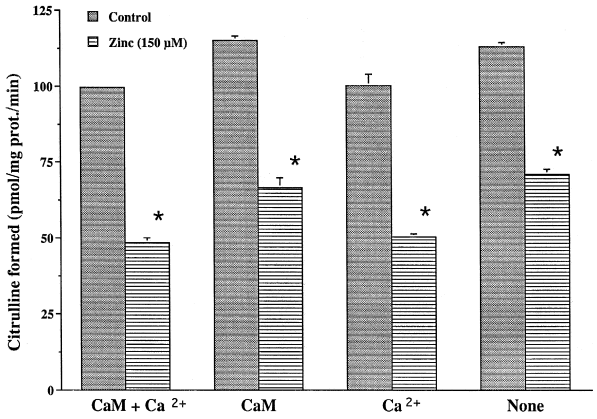

Fig. 6. Effect of 150 μM [Zn^{2+}] on the activity of inducible NO synthase in lung homogenate from lipopolysaccharide-treated rats in the absence and the presence of Ca^{2+} and/or calmodulin (CaM). Results are expressed as the mean of 6–8 observations ± S.E.M. * Significantly different from control.

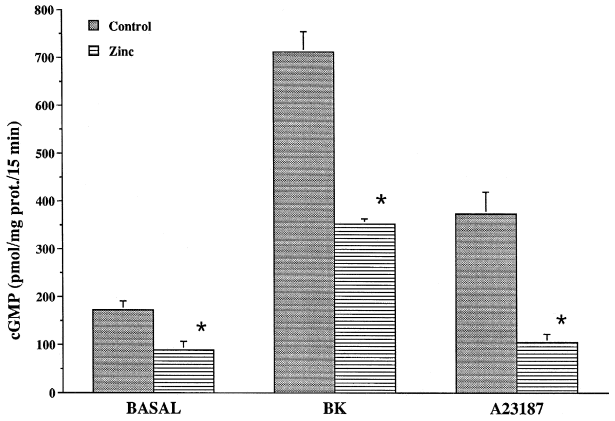

Fig. 7. Effect of 150 μM [Zn^{2+}] on basal, bradykinin (BK)- and A23187-stimulated constitutive nitric oxide synthase-mediated cGMP formation. Results are expressed as the mean of 6 observations ± S.E.M. * Significantly different from basal values.

tures were incubated with 150 μM Zn^{2+} for 2 h. Under these conditions, Zn^{2+} caused a significant reduction in basal and bradykinin- or A-23187-induced stimulation of cGMP formation (Fig. 7).

4. Discussion

In the present study, we investigated the effects of Zn^{2+} supplementation and Zn^{2+} deficiency on the induction of NO synthase. Moreover, the possible underlying mechanisms of this effect were studied in vitro. Administration of lipopolysaccharide significantly reduced serum levels of Zn^{2+} in ZD, control, or ZG rats. Similar results have been observed in inflammatory, trauma related-processes and with in vivo administration of interleukin-1β (Sobocinski et al., 1977), lipopolysaccharide (DiSilvestro and Cousins, 1984) or glucocorticoids (Dinarello, 1984). It is assumed that the effect of lipopolysaccharide and interleukin-1β is mediated by the resultant increase in plasma levels of glucocorticoid hormones (Dinarello, 1988). These changes in serum Zn^{2+} levels were found to be accompanied by an increase in the level of metallothioneins in the liver (DiSilvestro and Cousins, 1984). Metallothioneins may serve as a regulatory system, i.e. different metallothioneins could carry Zn^{2+} to certain cellular components to regulate different Zn^{2+} requiring enzymes (Karin, 1985). Additionally, metallothioneins are strong scavengers of free radicals (Thornalley and Vasak, 1985; Greenstock et al., 1987); thus, they may protect against the massive formation of free radicals by macrophages and neutrophils upon activation by lipopolysaccharide, interleukin-1β or interferon-γ.

In vivo administration of Zn^{2+} significantly suppressed the formation of L-NAME-sensitive cGMP in response to lipopolysaccharide. However, in ZD animals, the levels of cGMP were also significantly lower than in the control group. Presumably, under conditions of Zn^{2+} deficiency, there is an increase in the free radical pools either due to a reduction in tissue content of metallothioneins and superoxide dismutase activity (Carrico and Deutsch, 1970). Free radicals are capable of scavenging and inactivating NO to form peroxynitrite anion (Beckman et al., 1990); thus in ZD rats, the enhanced NO formation may not be reflected as an increase in cGMP.

Incubation of rat aortic smooth muscle cells with Zn^{2+} (100–150 μM) reduced the formation of cGMP in response to lipopolysaccharide or interleukin-1β in a concentration-dependent fashion. In these experiments, although high concentrations of Zn^{2+} were used, only 16–32% of the added Zn^{2+} were taken by the cells. The reason relatively high concentrations of Zn^{2}, compared to the plasma levels, are required to inhibit cGMP formation in response to lipopolysaccharide in isolated cells is not clear. It is unlikely that cytotoxicity contributed to the effects of Zn^{2+} on NO production since responses of sCG to SNP remained unaffected.

Exposure of rat aortic smooth muscle cells to the transcriptional inhibitor, actinomycin-D prevented the lipopolysaccharide-induced increase in cGMP formation even when added 120 min after lipopolysaccharide. However, when it was added 180 min after lipopolysaccharide, it failed to inhibit production suggesting that, at that time, mRNA synthesis was completed. Similar to cyclohexamide, Zn^{2+} inhibited the lipopolysaccharide-induced increase in cGMP formation, even when added 180 min after lipopolysaccharide, but not at 210 min. This finding suggests that Zn^{2+} may act via inhibition of either protein synthesis (similar to cyclohexamide) or inducible nitric oxide synthase activity. However, when cells were incubated with lipopolysaccharide for 6 h (instead of 4 h), Zn^{2+} inhibited inducible nitric oxide synthase-mediated increase in cGMP levels when added 240 min relative to lipopolysaccharide. A possible explanation of the lack of effectiveness of Zn^{2+}, when inducible nitric oxide synthase activity was assessed 30 min after Zn^{2+} addition, is that this time period may not be enough for Zn^{2+} to get into the cell in a concentration high enough to inhibit inducible NO synthase activity. Taken together, these data indicate that Zn^{2+} may inhibit NO formation via inhibition of inducible NO synthase activity rather than inhibition of the induction process. To further investigate the mechanism by which Zn^{2+} inhibits NO formation, the rate of conversion of [^{3}H]arginine into [^{3}H]citrulline was examined in the absence and the presence of 150 μM of Zn^{2+}. The rate of citrulline formation was reduced to approximately 50% of the control value when 150 μM of Zn^{2+} were added to the reaction mixture. These data clearly demonstrate an inhibitory effect of Zn^{2+} on the activity of inducible NO synthase.

How does Zn^{2+} inhibit NO formation? Evidence is accumulating that Zn^{2+} is an inhibitor of calcium-activated calmodulin function which might explain the reciprocal actions of calcium and Zn^{2+} (Brewer, 1980). It seems that

Zn^{2+} has a high affinity for calcium-activated calmodulin complexed to protein (Donnelly, 1978). For inducible NO synthase, although its activity is not calcium or calmodulin dependent (Yui et al., 1991), its expression may be calcium dependent (Hauschildt et al., 1990). In contrast to constitutive NO synthase, inducible NO synthase from macrophages has a tightly bound calmodulin which can be considered as an enzyme subunit (Cho et al., 1992). Thus, we hypothesize that Zn^{2+} binds to the complexed calmodulin, thereby modifying its association with inducible NO synthase leading to destabilization of the complex and/or inhibition of the enzyme activity. Similarly, Zn^{2+} may inhibit constitutive NO synthase activity by interfering with calcium-activated calmodulin function.

An alternative explanation is related to the well known NADPH dependency of both forms of nitric oxide synthase (Bredt and Snyder, 1990). The NADPH is not only necessary for the electron transfer during NO formation, but also for the activity of a NADPH-dependent dihydropteridin reductase which recycles tetrahydrobiopterin during NO synthesis (Kwon et al., 1990). Interestingly, it has been reported that Zn^{2+} inhibits the NADPH-dependent cytochrome P-450 (Hammermueller et al., 1986). Knowing that nitric oxide synthase and NADPH-cytochrome P-450 are similar in their dependence on NADPH and share a sequence homology in some binding domains (Snyder and Bredt, 1991), it is not unreasonable to propose that Zn^{2+} inhibits NO synthase activity in an NADPH-dependent manner. A third possibility is that Zn^{2+} may bind to glutathione and other thiols to decrease nitric oxide synthase activity. Thiols are important for non-enzymatic regeneration of tetrahydrobiopterin (Kaufman, 1959; Bublitz, 1977). Indeed, the inhibitory activity of Zn^{2+} on NO production, in vitro, was prevented by dithiothreitol (unpublished observation).

Recent data indicate involvement of NO in the development of different types of inflammation as evidenced by the observation in arthritis, inflamed synovial tissues expressed augmented levels of induced NO synthase mRNA and administration of NO synthase inhibitors suppressed the development of the disease (McCartney-Francis et al., 1993). Moreover, some of the pro-inflammatory activities of interleukin-1, -2, and -6, tumor necrosis factor, interferon-γ and lipopolysaccharide are mediated by the stimulation of macrophage NO production (Ding et al., 1988). Nitric oxide stimulates prostaglandin synthesis (Warren et al., 1992; Salvemini et al., 1994). In carrageenan-induced edema, for example, inflammation involves an early phase of histamine, 5-hydroxytryptamine and bradykinin release followed by a late phase that involves the release of arachidonic acid metabolites (Di Rosa et al., 1971; Di Rosa and Willoughby, 1971). Thus, a possible mechanism for the anti-inflammatory activity of Zn^{2+} is due to its ability to inhibit histamine, 5-hydroxytryptamine and bradykinin-stimulated NO release via constitutive NO synthase and inhibit inducible nitric oxide synthase activity.

In conclusion, our results indicate that Zn^{2+} is capable of inhibiting NO formation, by inducible NO synthase in response to lipopolysaccharide or interleukin-1β. Additionally, Zn^{2+} inhibits NO formation by constitutive NO synthase in response to bradykinin or A-23187. These results may help to explain the reported anti-inflammatory activity of Zn^{2}.

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