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Role of Zn^{2+} in oxidative stress caused by endotoxin challenge

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Abstract

The role of Zn^{2+} in oxidative stress during endotoxemia was investigated. In rats fed a Zn^{2+}-deficient diet (Zn^{2+} concentration of less than 1.5 mg/kg) for 8 weeks, the Zn^{2+} level in the serum was about 62% lower than that in rats fed a Zn^{2+}-adequate diet (Zn^{2+} concentration, 50 mg/kg). The Zn^{2+} level in serum 18 h after administration of endotoxin (6 mg/kg, i.p.) to Zn^{2+}-deficient diet rats was markedly lower than that of the endotoxin/Zn^{2+}-adequate diet group. Lipid peroxide formation in the liver of Zn^{2+}-deficient diet rats was markedly increased 18 h after endotoxin injection compared with that in the endotoxin/Zn^{2+}-adequate diet group. Metallothionein in the liver of endotoxin/Zn^{2+}-adequate diet rats was increased more than 17-fold by endotoxin administration, while a markedly lower level of metallothionein was observed in the endotoxin/Zn^{2+}-deficient diet group. On the other hand, treatment with $ZnSO_4$ (100 μM) significantly increased endotoxin (1 μg/ml)-induced tumor necrosis factor-α (TNF-α) production in J774A.1 cells. Our results clearly demonstrated that treatment with $ZnSO_4$ significantly inhibited the endotoxin-induced increase in intracellular Ca^{2+} level in J774A.1 cells. However, a cell membrane-permeable Zn^{2+} chelator, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, 1 μM), did not affect the endotoxin-induced TNF-α production or Ca^{2+} level in J774A.1 cells. In addition, we investigated whether Zn^{2+} can suppress nitric oxide (NO) generation and cytotoxicity in endotoxin-treated cells. Treatment with $ZnSO_4$ (50 μM) significantly inhibited endotoxin-induced NO production in J774A.1 cells, but did not affect endotoxin-induced cytotoxicity. These findings suggest that zinc may play an important role, at least in part, in the oxidative stress during endotoxemia.

Keywords: Endotoxin; Zn^{2+} level; Oxidative stress; Metallothionein; Ca^{2+}; Nitric oxide (NO)

1. Introduction

In the host, the lipopolysaccharide, endotoxin, exerts effects hemodynamically, cardiovascularly, immunologically and metabolically. Endotoxin induces various shock symptoms in the human and in animals, such as fever, hypotension, intravascular coagulation, and finally multi-organ failure. Endotoxin is believed to be initially detoxified in the reticuloendothelial system, particularly in liver Kupffer cells. These cells are among the main targets in poisoned animals. Macrophages stimulated by microorganisms or their toxins show induction of a variety of biologically active mediators known as cytokines, and tumor necrosis factor-α (TNF-α) is recognized as an important mediator in the development of endotoxicity.

The modifications induced in the apolar side residues of membrane phosphoglycerides by active oxygen generation are considered to bring about structural alterations in the membrane. Therefore, biomembranes and subcellular organelles are the major sites of lipid peroxide damage. We reported previously (Sakaguchi et al., 1981a,b) that endotoxin injection resulted in lipid peroxide formation and membrane damage in experimental animals, causing decreased levels of free radical scavengers or quenchers. Interestingly, we also suggested that TNF-α-induced oxidative stress occurs as a result of bacterial or endotoxin translocation under conditions of reduced reticuloendothelial system function in various disease states (Sakaguchi et al., 1996). In addition, we suggested that intracellular Ca^{2+} or selenium levels may participate, at least in part, in free

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radical formation in endotoxin-poisoned mice (Sakaguchi et al., 1989, 2000b; Sakaguchi and Yokota, 1995). Many studies have linked the production of nitric oxide (NO) to endotoxin-induced hypotension, vascular hyporesponsiveness and death, suggesting that excess production of NO plays an important role in the development of septic shock (Moncada et al., 1991; Titheradge, 1999). The NO radical functions efficiently as a mediator, messenger or regulator of cell function in various physiological systems and pathophysiological states. Recently, we found that it is unlikely that NO plays a significant role in the liver injury caused by free radical generation in endotoxemia (Sakaguchi et al., 2000a,b).

Zn^{2+} is an essential trace mineral required for normal growth, protein metabolism, the function of over 200 zinc metalloenzymes, membrane integrity, gene expression, wound healing, and immune function. One of the characteristic phenomena associated with endotoxin administration is the rapid and significant decrease of serum Zn^{2+} concentration as found in patients with various infectious diseases and malignancy during endotoxemia (Gaetke et al., 1997; Philcox et al., 1995). On the other hand, the cysteine-rich metal-binding protein, metallothionein has been ascribed broad functions in the regulation of Zn^{2+} and copper homeostasis, participation in the acute-phase response, detoxification of heavy metals and scavenging of free radicals. In addition, metallothionein is known to be associated with resistance to oxidative stress (Nath et al., 2000; Bray and Better, 1990). As induction of hepatic metallothionein occurs at the onset of inflammation (Bremmer and Beattie, 1990), it has been suggested that the resulting increased supply of exchangeable Zn^{2+} may facilitate the many enzymic processes necessary for mounting the acute-phase response. Therefore, in the present study, we designed the following experiments to investigate the role of Zn^{2+} in oxidative stress caused by endotoxin challenge.

2. Materials and methods

2.1. Chemicals

N,*N*,*N*′,*N*′-Tetrakis (2-pyridylmethyl) ethylenediamine, TPEN) and fluo 3-AM were obtained from Wako (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) and dimethyl sulfoxide were obtained from Nacalai Tesque, (Kyoto, Japan).

2.2. Animals and treatment

Male Wistar rats, 3 weeks old and weighing 38–50 g, were purchased from Japan SLC (Hamamatsu, Japan) and maintained on a Zn^{2+}-supplemented or a Zn^{2+}-deficient diet (Oriental Yeast, Tokyo, Japan) at the Tohoku Pharmaceutical University Experimental Animal Center for 8 weeks before the experiment. They were housed in an air-conditioned room at 23 ± 1 °C with a 12-h light/dark cycle. According to the manufacturer's specifications, the Zn^{2+}-deficient diet contains less than 1.5 mg/kg of total Zn^{2+} and is comprised of dextrose (63.7%), egg albumin (20%), corn oil (5%), mineral mixture (3.13%), vitamin mixture (1.7%), and cellulose (2%). A Zn^{2+}-adequate diet of the same composition supplemented with 50 mg/kg Zn^{2+} as zinc chloride was given to the Zn^{2+}-supplemented group. *Salmonella typhimurium* lipopolysaccharide (endotoxin), which was obtained from Difco Laboratories (Detroit, MI, USA), was used throughout this study. During the experiment, the animals were given water ad libitum. The rats were divided into four groups, A (control), B, C and D. Groups A and B were given the Zn^{2+}-adequate diet and C and D groups were fed the Zn^{2+}-deficient diet. Group A and D rats were injected with 0.2 ml of 0.9% saline alone; Group B and C rats were injected intraperitoneally with 6 mg/kg of endotoxin.

2.3. Estimations of lipid peroxide, metallothionein in the liver and Zn^{2+} in serum

The rats were anesthesized with sodium pentobarbital (50 mg/kg, i.p.) before isolation of the liver for measurements of lipid peroxide, metallothionein and serum Zn^{2+} levels. Lipid peroxide levels in the liver were measured according to the method of Ohkawa et al. (1979). The liver was homogenized with 0.15 M KCl solution to obtain a 10% homogenate. After addition of 8.1% sodium dodecyl sulfate, 20% acetate buffer (pH 3.5) and 0.8% thiobarbituric acid, the solution was heated at 95 °C for 1 h, and the reaction product was assayed spectrophotometrically at 531 nm. Lipid peroxide levels are expressed as nmol of malondialdehyde formed per gram of wet liver. The remainder of the liver was retained for metallothionein assay (Eaton and Toal, 1982; Philcox et al., 1995). The liver was homogenized in four volumes of 0.01 M Tris/HCl buffer (pH 8.2) solution, and then centrifuged at $20,000 \times g$ for 30 min. The supernatant was separated, heat-treated at 95 °C for 2 min and centrifuged at $14,000 \times g$ for 5 min prior to Cd^{2+}-heme assay of Zn^{2+}-metallothionein. The serum Zn^{2+} level was determined by atomic absorption spectroscopy after the samples were decomposed with HNO_3.

2.4. Cell lines and culture conditions

The murine monocyte–macrophage cell line, J774A.1, was obtained from Japan Cancer Research Resources Bank (Tokyo, Japan). The cells were maintained continuously in 75 cm^2 plastic culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with penicillin (100 U/ml, Meiji Seika, Tokyo, Japan), streptomycin (100 μg/ml, Meiji Seika), and 10% fetal bovine serum (FBS; RH

Biosciences, Lenexa, KS, USA). The cells were incubated at 37 °C in a 5% CO_2/air environment.

2.5. Measurement of Ca^{2+} content in J774A.1 cells

For measurement of the intracellular Ca^{2+} level, cells were loaded with 1 μM fluo 3 (Ca^{2+} probe) in HEPES buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4). After a loading period of 30 min, excess dye was removed by centrifugation, the cells were resuspended in fresh buffer and the cell-associated fluo 3 fluorescence was measured by flow cytometry (FACScan, Becton Dickinson, Tokyo, Japan) (Rijkers et al.,1990).

2.6. TNF-α assay in J774A.1 cells

The J774A.1 macrophage cells (2×10^5/ml) were cultured in 96-well flat-bottomed plates with endotoxin (1 μg/ml). TNF-α production in J774A.1 cells was measured after 2 h. To assess the effects of 100 μM $ZnSO_4$ and 1 μM TPEN, the agents were added to the cells 15 min before treatment with endotoxin. TNF-α production in the culture supernatant was measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cytoscreen™ Mouse TNF-α, Biosource International, Camarillo, CA, USA). Optical density at 450 nm was measured with an Immuno-Mini NJ-2300 ELISA analyzer (Inter Med, Tokyo, Japan). TNF-α concentrations were calculated by comparison with standard solutions of TNF-α.

2.7. Measurement of nitrite production as an assay of nitric oxide release in J774A.1 cells

Nitric oxide production by J774A.1 cells was assayed by measurement of the accumulation of nitrite in the culture medium, using the Griess reaction (Green et al., 1982). J774A.1 cells were removed from culture flasks by vigorous pipetting and centrifuged, then resuspended in medium at a concentration of 5×10^5 cell/ml. Cells (1×10^6/ml) were plated in 24-well culture plates (Falcon) and incubated with medium containing endotoxin and/or $ZnSO_4$ at 37 °C in 5% CO_2 in air for up to 24 h. Briefly, aliquots of 100 μl of culture supernatant from control cells or cells stimulated with endotoxin were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethlenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. Absorbance was measured at 540 nm with an Immuno-MiniNJ-2300 ELISA analyzer (Inter Med). The nitrite concentration in the medium was determined with sodium nitrite as a standard.

2.8. Cytotoxicity assay

Cell number was determined by Trypan blue staining or counting using a Coulter model ZI (Coulter, Hialeah, FL, USA). J774A.1 cells were seeded at a density of 3×10^4/ml in 96-well, flat-bottom plates and incubated with RPMI-1640 medium containing 10% FBS and antibiotics in the presence or absence of drugs at 37 °C in an atmosphere of 5% CO_2 in air. After a 48-h incubation, cytotoxicity was assessed by estimation of the viability of J774A.1 cells by MTT-based colorimetric assay (Heo et al., 1990). After completion of drug treatment, 10 μl of MTT (5 mg/ml) solution was added to each well followed by 100 μl of complete medium. After incubation for 4 h at 37 °C, the MTT solution was removed. The incorporated formazan crystals in viable cells were solubilized with 100 μl of dimethyl sulfoxide. The absorbance at 540 nm was determined with an Immuno-Mini NJ-2300 ELISA analyzer (Inter Med). Cytotoxicity was calculated by subtracting from one the ratio of mean absorbance value for treated cells over mean absorbance value for untreated cells.

2.9. Statistical analysis

All data were expressed as means ± S.E.M. Statistical significance was determined with the Student–Newman–Keuls multiple comparison procedure applied after a one-way analysis of variance (ANOVA) (P value of less than 0.05 was considered significant).

3. Results

3.1. Changes in Zn^{2+} levels in the serum of rats given Zn^{2+}-deficient diet 18 h after endotoxin administration

The acute phase host response to stress, trauma, inflammation, and infection includes a marked decrease in the serum Zn^{2+} level. Therefore, in this study, we fed rats a diet deficient in Zn^{2+} to examine the role of this element in oxidative stress caused by endotoxin. Fig. 1 shows the Zn^{2+} content in serum of Zn^{2+}-deficient rats at the end of 8 weeks of the experimental period. In rats fed with Zn^{2+}-deficient diet, the Zn^{2+} level in serum was about 62% lower than that in the Zn^{2+}-adequate diet group. The serum Zn^{2+} level 18 h after administration of endotoxin (6 mg/kg, i.p.) to Zn^{2+}-deficient diet rats was markedly lower than that in the endotoxin/Zn^{2+}-adequate diet group. The following experiment was, therefore, performed to examine the role of Zn^{2+} in oxidative stress caused by endotoxin administration, using rats fed the Zn^{2+}-deficient diet.

3.2. Effects of Zn^{2+}-deficient diet on lipid peroxide (A) and metallothionein (B) levels in the liver of endotoxin-injected rats

Previously (Sakaguchi et al., 1981a,b), we observed that lipid peroxidation by free radicals generated in endotoxin-treated mice was markedly increased after 18–24 h and also that levels of free radical scavengers or quenchers

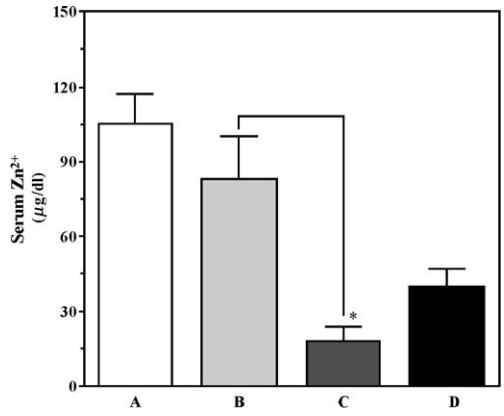

Fig. 1. Changes in Zn^{2+} level in the serum of rats fed the Zn^{2+}-deficient diet 18 h after endotoxin challenge. Rats were fed the Zn^{2+}-deficient diet for 8 weeks, and were then injected with endotoxin (6 mg/kg, i.p.). A: Zn^{2+}-adequate diet (Control); B: endotoxin/Zn^{2+}-adequate diet; C: endotoxin/Zn^{2+}-deficient diet; D: Zn^{2+}-deficient diet. Data are results from five experiments, and each value represents the mean ± S.E.M. for five rats per group. * $P<0.05$, compared to endotoxin/Zn^{2+}-adequate diet group.

were decreased in the liver 18 h after endotoxin administration. On the other hand, metallothionein has been thought to be involved as a free radical scavenger in cooperation with established biomolecules that act as antioxidants, such as the reduced form of glutathione (Thornalley and Vasak, 1985). Therefore, we examined the changes in lipid peroxide formation and metallothionein levels in the liver in Zn^{2+}-deficient rats 18 h after endotoxin (6 mg/kg, i.p.) challenge. As shown in Fig. 2A, the levels of lipid peroxide in endotoxin/Zn^{2+}-deficient rats were markedly higher than those in the endotoxin/Zn^{2+}-adequate group. On the other hand, Zn^{2+} deficiency is known to lower the basal metallothionein level in the rat liver. The induction of metallothionein in Zn^{2+}-adequate rats was increased more than 17-fold by endotoxin administration. However, the level of induction in endotoxin/Zn^{2+}-deficient rats was markedly lower than that in the endotoxin/Zn^{2+}-adequate diet group (Fig. 2B). These observations suggested a role of Zn^{2+} in endotoxin-induced lipid peroxide formation.

3.3. Effect of Zn^{2+} and its chelator on intracellular Ca^{2+} content in endotoxin-treated J774A.1 cells

As noted above, these findings suggested that Zn^{2+} concentration was a factor in oxidative stress in the liver during endotoxemia. Therefore, we designed the following experiments to clarify the role of intracellular Zn^{2+} in endotoxin-induced oxidative stress in J774A.1 cells. We used flow cytometry to measure intracellular Ca^{2+} content in J774A.1 cells treated with endotoxin and Zn^{2+} or its chelator, TPEN. As shown in Fig. 3, the treatment with endotoxin (10 μg/ml) had a significant enhancement effect on intracellular Ca^{2+} content in J774A.1 cells, while $ZnSO_4$ (100 μM) treatment significantly inhibited the endotoxin-induced increase in intracellular Ca^{2+} content. However, treatment with TPEN (1 μM) did not significantly affect the levels of intracellular Ca^{2+} produced by endotoxin treatment. The results of our previous studies (Sakaguchi et al., 1989; Sakaguchi and Yokota, 1995) suggested that intracellular Ca^{2+} may participate in free radical formation in endotoxemic mice. It is, therefore, of interest that Zn^{2+} clearly prevented the endotoxin-induced increase in cellular Ca^{2+} content.

3.4. Effects of Zn^{2+} on nitric oxide production (A) and cytotoxicity (B) in endotoxin-treated J774A.1 cells

The results of the above experiments suggested a role of Zn^{2+} in oxidative stress during endotoxemia. The NO radical functions efficiently as a mediator, or a regulator,

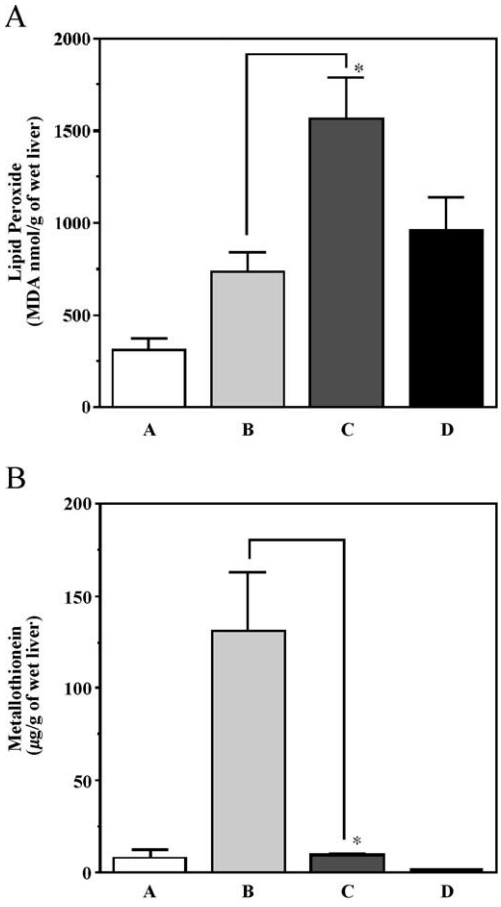

Fig. 2. Effects of Zn^{2+}-deficient diet on lipid peroxide (A) and metallothionein (B) levels in liver of rats 18 h after endotoxin challenge. Rats were fed the Zn^{2+}-deficient diet for 8 weeks, and were then injected with endotoxin (6 mg/kg, i.p.). Lipid peroxide and metallothionein levels were determined as described in Materials and methods. A: Zn^{2+}-adequate diet (Control); B: endotoxin/Zn^{2+}-adequate diet; C: endotoxin/Zn^{2+}-deficient diet; D: Zn^{2+}-deficient diet. Data are results from five experiments, and each value represents the mean ± S.E.M. for five rats per group. * $P<0.05$, compared to endotoxin/Zn^{2+}-adequate diet group.

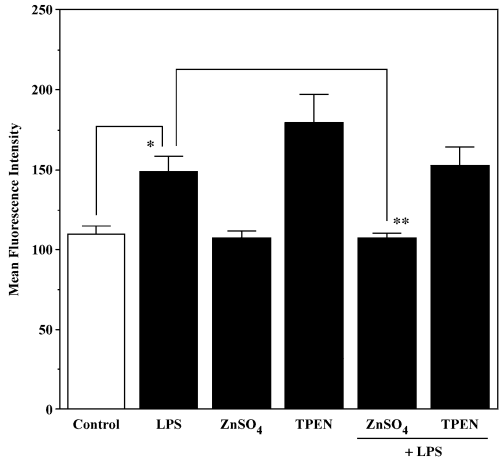

Fig. 3. Effects of $ZnSO_4$ and TPEN on enhancement of intracellular Ca^{2+} in J774A.1 cells stimulated with endotoxin. J774A.1 cells (1×10^6/ml) were loaded with 1 μM fluo 3 for 30 min. Cells were incubated with endotoxin (10 μg/ml) in the presence or absence of 100 μM $ZnSO_4$ and 1 μM TPEN for 1 h and the mean fluorescence intensity of fluo 3 of 10^4 cells was measured with FACScan. Data represent the means ± S.E.M. from three independent experiments performed in triplicate. * $P<0.05$, compared to untreated cells. ** $P<0.05$, compared to cells treated with endotoxin alone.

of cell function in a number of physiological systems and pathophysiological states. Therefore, we designed the following experiments to investigate whether Zn^{2+} is involved in NO production and cytotoxicity in endotoxin-treated J774A.1 cells. J774A.1 cells were incubated with endotoxin (1 μg/ml) plus interferon-γ (100 U/ml) and $ZnSO_4$ (10–50 μM) at 37 °C for up 24 h. The amount of stable nitrite (NO_2^-), the end product of NO production by activated macrophages under various conditions, was determined by the Griess method (Green et al., 1982). As shown in Fig. 4A, J774A.1 cells, activated in the presence of endotoxin alone, produced a high level of NO_2^-. However, over the range of 10 μM $ZnSO_4$, the level following the incubation of cells in the presence of endotoxin was slightly lower than that in cells treated with endotoxin alone. In contrast, treatment with $ZnSO_4$ (25–50 μM) showed a significant inhibitory effect on endotoxin-activated NO production in J774A.1 cells. However, cell viability in the presence of endotoxin (10 μg/ml) was 54% and was not affected by addition of Zn^{2+} (10–50 μM) in endotoxin-treated J774A.1 cells (Fig. 4B). It is of interest that endotoxin-induced NO production was inhibited by Zn^{2+}, as Zn^{2+} showed no protective effect on the cytotoxicity of endotoxemia.

3.5. Influence of Zn^{2+} or its chelator on TNF-α production in endotoxin-activated J774A.1 cells

Zn^{2+} is known to be involved in the regulation of immune functions, and its level in the serum is low in endotoxemia. In addition, immune function is markedly attenuated in endotoxemia. On the other hand, TNF-α, a macrophage-derived cytokine inducible by endotoxin, has frequently been reported to cause a shock syndrome similar to that caused by endotoxin, and has been suggested to be one of the major mediators of shock (Fiers, 1991). Therefore, we examined the effects of Zn^{2+} or its chelator on TNF-α production by endotoxin-activated J774A.1 cells. J774A.1 cells were incubated with endotoxin (1 μg/ml) and $ZnSO_4$ (100 μM) or TPEN (1 μM). TNF-α levels in cells were analyzed after 2-h treatment, using a standard ELISA system. As shown in Fig. 5, when cells were treated with endotoxin, the TNF-α level was markedly increased, while treatment with $ZnSO_4$ (100 μM) significantly enhanced the endotoxin-activated TNF-α production. Treatment with the Zn^{2+} chelator, TPEN (1 μM), however, was ineffective to enhance TNF-α production in endotoxin-activated J774A.1 cells. These results suggested that Zn^{2+} concentration may

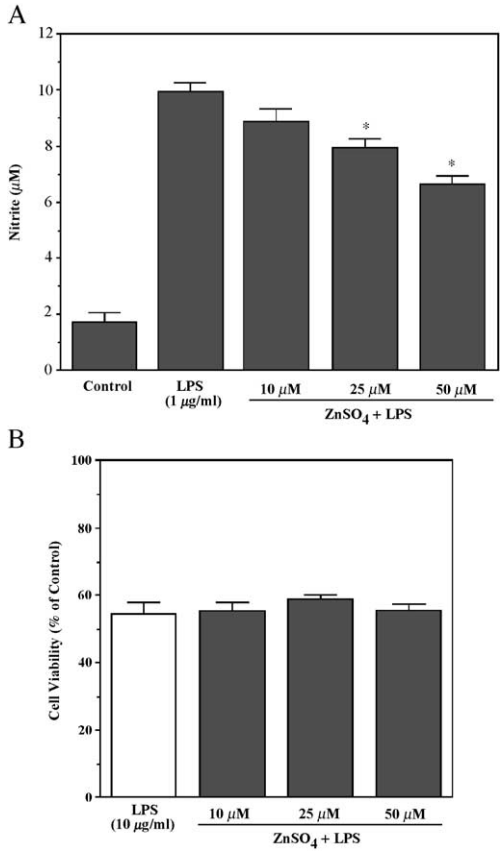

Fig. 4. Effects of $ZnSO_4$ on nitric oxide production (A) and cytotoxicity (B) induced by endotoxin in J774A.1 cells. (A) Cells were incubated with endotoxin (1 μg/ml) plus interferon-γ (100 U/ml) in the presence or absence of $ZnSO_4$ (10–50 μM). Nitrite was measured in culture supernatants using the Griess reagent. Data represent the means ± S.E.M. from three independent experiments performed in triplicate. * $P<0.05$, compared to cells treated with endotoxin/interferon-γ alone. (B) Cells were incubated with endotoxin (10 μg/ml) in the presence or absence of $ZnSO_4$ (10–50 μM). Cell viability was assessed by the MTT assay as in Materials and methods. The surviving fraction was determined by dividing the absorbance of treated cells by that of control cells. Data represent the means ± S.E.M. from three independent experiments performed in triplicate.

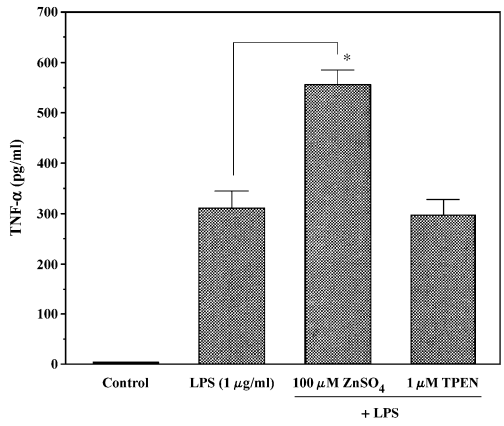

Fig. 5. Effects of $ZnSO_4$ and TPEN on endotoxin-induced TNF-α production in J774A.1 cells. Cells (2×10^5/ml) were incubated with endotoxin (1 μg/ml) for 2 h. $ZnSO_4$ (100 μM) or TPEN (1 μM) was added to the cells 15 min before treatment with endotoxin. TNF-α production in the culture supernatant was measured by ELISA. Data represent the means ± S.E.M. from three independent experiments performed in triplicate. $*P < 0.05$, compared to cells treated with endotoxin alone.

contribute to the extent of the TNF-α release triggered by endotoxin.

4. Discussion

Despite the remarkable progress in intensive care medicine, sepsis and shock continue to be major clinical problems in intensive care units. Septic shock may be associated with a toxic state initiated by the stimulation of monocytes by bacterial toxins such as endotoxin, which is released into the bloodstream. On the other hand, TNF-α is considered to be a major early mediator in the systemic inflammatory response syndrome observed during Gram-negative sepsis (Vassalli, 1992). This mediator is responsible, at least in part, for a number of pathophysiological responses in the liver, including acute phase response, hyperlipidemia, free oxygen radical formation, fibrogenesis and choleostasis (Fiers, 1991; Camussi et al., 1991). Zn^{2+} is an essential trace element for several metabolic processes. For example, Zn^{2+} is believed to be associated with a protective action on liver cell activity and possible prevention of cellular damage caused by oxidative stress (Bray and Better, 1990). On the other hand, administration of endotoxin to experimental animals initiates the acute phase response and produces marked decreases in serum Zn^{2+} levels (Pekarek and Beisel, 1969; Philcox et al., 1995). Therefore, the present study was performed to clarify the role of Zn^{2+} in oxidative stress, which occurs under conditions of tissue ischemia during endotoxemia, using a Zn^{2+}-deficient rat model in vivo and the Zn^{2+} chelator, TPEN, in J774A.1 cells in vitro.

In this experiment, feeding the Zn^{2+}-deficient diet resulted in reduction of the serum Zn^{2+} level to about 62% of that in the Zn^{2+}-adequate diet group at 8 weeks (Fig. 1). Under these conditions, in the Zn^{2+}-deficient diet group, endotoxin challenge (6 mg/kg, i.p.) enhanced lipid peroxidation in the liver compared with the Zn^{2+}-adequate diet group given endotoxin, suggesting that more biomembrane damage occurred in the poisoned Zn^{2+}-deficient diet rats (Fig. 2A). Oxygen-derived free radicals generated during reperfusion after ischemia or hypoxia, or by activated neutrophils, are mediators of tissue injury. Free radical injury occurs via lipid peroxidation in a variety of disease processes including shock (McCord, 1985). In support of our findings, previous studies (Sakaguchi et al., 1981a,b) indicated that lipid peroxidation by free radicals occurs under conditions of ischemia, probably induced by disseminated travascular coagulation in endotoxin-poisoned mice. On the other hand, metallothionein is a low molecular weight (approximately Ms = 7000) cysteine-rich protein present in various animal and plant species. This protein, in addition to playing a detoxifying role against harmful heavy metals such as cadmium and mercury as well as a homeostatic role for essential metals such as copper and zinc, has been suggested to act as a free radical scavenger in cooperation with established biomolecules that act as antioxidants, such as the reduced form of GSH (Thornalley and Vasak, 1985). As shown in Fig. 2B, the results of the present study clearly demonstrated that endotoxin (6 mg/kg, i.p.) can effectively produce metallothionein in the liver in the Zn^{2+}-adequate diet group, while in the Zn^{2+}-deficient diet group, endotoxin challenge showed no effect on production of metallothionein. Zn^{2+} deficiency enhances the cytotoxic effect of endotoxin on bone marrow (Nakajima and Suzuki, 1996). Zn^{2+} deficiency is thought to be associated with endotoxin-induced lethality. In fact, some investigators have reported inhibition of the lethality of endotoxin in Zn^{2+}-treated animals (Unoshima et al., 2001) and a protective role of Zn^{2+} against endotoxin-associated mediators (Abou-Mohamed et al., 1998). It is, therefore, of interest that endotoxin-induced metallothionein in the liver is closely related to lipid peroxide formation and the concentration of Zn^{2+} in endotoxemia. It seems that the endogenous Zn^{2+} level may contribute to the extent of oxidative stress caused by endotoxin challenge.

Ca^{2+} has important roles in many cellular functions. Previously (Sakaguchi et al.,1993; Sakaguchi and Yokota, 1995), we suggested that the increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the liver cytoplasm may partially explain various endotoxin-induced metabolic disorders. In addition, in support of our findings, previous studies showed that intracellular Ca^{2+} may play an important role in free radical formation during endotoxemia (Sakaguchi et al., 1989; Sakaguchi and Yokota, 1995). For example, it was found that endotoxin-induced lipid peroxide formation in the mouse liver was markedly inhibited by administration of the Ca^{2+} channel blocker, verapamil, or by feeding a Ca^{2+}-deficient diet. In the present study, we analyzed whether the endotoxin-induced increase in intracellular Ca^{2+} level could be prevented by Zn^{2+}. As shown in Fig. 3, the elevation of the intracellular Ca^{2+} level in J774A.1 cells

induced by endotoxin (1 μg/ml) was significantly inhibited by treatment with $ZnSO_4$ (100 μM), while treatment with the Zn^{2+} chelator, TPEN (1 μM), did not significantly inhibit the increase in intracellular Ca^{2+} level. It is, therefore, possible that Zn^{2+} may modulate, not only oxidative stress, but also cellular function through Ca^{2+} mobilization in endotoxemia. On the other hand, the immune function is known to be markedly attenuated in endotoxemia. Zn^{2+} is involved in the regulation of cellular function and maintenance of immune function, and its level in the serum is low in endotoxemia (Fig. 1). Interestingly, we found that treatment with $ZnSO_4$ (100 μM) significantly enhanced endotoxin (1 μg/ml)-induced TNF-α production in J774A.1 cells. However, treatment with Zn^{2+} chelator, TPEN (1 μM), did not affect the endotoxin-induced TNF-α production in J774A.1 cells (Fig. 5). Zn^{2+} acts synergistically with endotoxin to bring about cytokine induction in leukocytes, even at substimulative concentrations (Driessen et al. 1995). The synergism is caused by a direct interaction of Zn^{2+} with endotoxin, resulting in the conversion of endotoxin into a less fluid, but biologically more active, phase (Wellinghausen et al., 1996). Since endotoxin is an important pathogenic factor in sepsis, pharmacological Zn^{2+} supplementation in patients with systemic Gram-negative infection may have devastating consequences. In contrast, the induction of stress proteins by prophylactic Zn^{2+} administration can diminish the inflammatory response after subsequent endotoxin stimulation in a porcine sepsis model (Klosterhalfen et al. 1996). Therefore, Zn^{2+} may be helpful in the prophylactic treatment of patients at high risk of sepsis, but be unsuitable for the treatment of already septic patients.

Endothelium-derived NO plays a role in the physiological regulation of vascular tone and blood pressure. In addition, NO has been implicated in the pathogenesis of vascular injury, hypotension and shock induced by endotoxin or TNF-α. The present results demonstrated a clear inhibitory effect of $ZnSO_4$ (10–50 μM) on the production of NO from endotoxin (1 μg/ml)-activated J774A.1 cells (Fig. 4A). It seems that Zn^{2+} had a protective effect against endotoxin-induced shock symptoms through its ability to inhibit NO production. On the other hand, NO can combine with superoxide radicals in vitro to produce peroxynitrite, a potentially cytotoxic substance. This radical has been shown to oxidize sulfhydryl groups and peroxidize membrane lipids (Radi et al., 1991). In addition, Asahi et al. (1995) reported that glutathione peroxidase was inactivated by endogenous NO and that this mechanism may at least in part explain the cytotoxic effects of NO on cells and NO-induced apoptotic cell death. Thus, it is possible that the preventive effect of zinc on NO production in endotoxemia is due to protection against endotoxin-induced cell death. However, as shown in Fig. 4B, $ZnSO_4$ (10–50 μM) did not affect endotoxin-induced cytotoxicity in J774A.1 cells. MacMicking et al. (1995) reported that in mice genetically deficient in inducible NO synthase (iNOS), both iNOS-dependent and -independent routes exist for endotoxin-induced hypotension and death. Recently, we suggested (Sakaguchi et al., 2000a) that NO is not crucial for lipid peroxide formation during endotoxemia. In addition, we reported that the non-selective iNOS inhibitor, N^G-nitro-L-arginine-methyl ester, and the relatively selective iNOS inhibitor, aminoguanidine, may not protect against the cytotoxicity of endotoxin in J774A.1 cells (Sakaguchi et al., 2000b, 2001). The observations shown in Fig. 4A,B are consistent with this conclusion.

In conclusion, our series of studies (Sakaguchi et al., 1981a,b, 1989, 1996, 2000b; Sakaguchi and Yokota, 1995) demonstrated that oxidative stress caused by endotoxin can reduce the levels of scavengers or quenchers of free radicals. In these previous studies, we had suggested that its endotoxin formation was regulated by TNF-α production (Sakaguchi et al., 1996), Ca^{2+} mobilization (Sakaguchi et al.,1989; Sakaguchi and Yokota, 1995) or selenium level (Sakaguchi et al., 2000b). Therefore, although the Zn^{2+} level may improve the regulation of cellular or immune function in septic shock, the findings described above suggested that oxidative stress caused by endotoxin may be due, at least in part, to changes in Zn^{2+} regulation during endotoxemia.

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