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Combined zinc supplementation with proinsulin C-peptide treatment decreases the inflammatory response and mortality in murine polymicrobial sepsis

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Abstract

Zinc is a trace element vital for immune function during host response to infection. The proinsulin C-peptide has been shown to exert beneficial effects through activation of the anti-inflammatory peroxisome proliferator activated receptor- γ (PPAR γ) in experimental endotoxemia. Some *in vitro* activities of C-peptide appear dependent upon the presence of zinc. We investigated the effect of zinc supplementation before onset of sepsis on the anti-inflammatory properties of C-peptide. Male C57BL/6 mice were subjected to polymicrobial sepsis by cecal ligation and puncture (CLP). Mice received zinc gluconate (1.3 mg/kg) intraperitoneally (IP) for three days before CLP. One hour after CLP, animals received C-peptide (280 nmol/kg IP) or the anti-microbial agent imipenem (25 mg/kg IP). CLP was associated with an 11% survival rate, pulmonary leukosequestration and liver injury. Molecular analysis in lungs of septic mice showed increased nuclear activation of the pro-inflammatory extracellular signal regulated kinases (ERK)1/2 and nuclear factor- κ B (NF- κ B), but decreased PPAR γ expression, when compared to sham animals. Combination of zinc supplementation with C-peptide post-treatment significantly improved survival rate (61%) similarly to antibiotic treatment (60%), ameliorated lung architecture and liver function, reduced tissue neutrophil infiltration, and increased bacterial clearance when compared with vehicle, C-peptide, or zinc treatment alone. These beneficial effects were associated with restored lung nuclear expression of PPAR γ , and reduction of pERK1/2 and NF- κ B activities in comparison to vehicle or single treatment protocols. Our data demonstrate that short-term zinc prophylaxis prior the infectious insult is a requisite for the anti-inflammatory properties of C-peptide by facilitating modulation of inflammatory pathways.

Keywords

C-peptide; extracellular signal regulated kinases 1 and 2 (ERK1/2); nuclear factor- κ B (NF- κ B); peroxisome proliferator activated receptor- γ (PPAR γ); zinc

INTRODUCTION

Sepsis is a systemic response to generalized infection characterized by hemodynamic, metabolic and inflammatory derangement. Antibiotics in combination with hemodynamic

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and respiratory support to maintain proper oxygen delivery are standard therapeutic approaches but not always efficacious to reduce the high mortality (1).

Zinc is an essential trace element, which appears to be required for the normal innate and adaptive immunity during the host response to infection. Clinical studies have reported that zinc deficiency correlates with an increased severity of illness and development of multiple organ failure in pediatric and adult patients with sepsis (2, 3). To the contrary, zinc supplementation has been demonstrated to reduce the incidence and severity of gastroenteritis and upper respiratory tract infections in children of developing countries or rural area (4). Experimental studies have proposed that a plausible molecular mechanism for the illness severity and mortality in the context of zinc deficiency is the increased activation in vital organs of the nuclear factor- κ B (NF- κ B), a transcription factor central to many pro-inflammatory and immune genes involved in sepsis (5).

The proinsulin connecting peptide (C-peptide) is a 31-amino-acid cleavage product of insulin biosynthesis that serves to link and stabilize the A- and B-chains of insulin. Our laboratory has recently demonstrated that C-peptide is a biologically active molecule with potent anti-inflammatory properties. In a murine model of endotoxic shock, C-peptide treatment reduced the systemic inflammatory response and lung injury, and improved survival (6). Little is known regarding the potential molecular mechanisms of C-peptide.

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a ligand-dependent nuclear transcription factor that has a prominent role in several biologic processes, including lipid and glucose homeostasis, and modulation of several inflammatory disorders (7). With the use of specific ligands or activators, PPAR γ has been reported to have protective effects in several preclinical models of septic shock and endotoxemia (8, 9). In addition to the binding of a specific G-protein coupled cell surface receptor, *in vitro* studies have shown that C-peptide stimulates the transcriptional activity of PPAR γ in a concentration-dependent manner (10). In animal models of endotoxic and hemorrhagic shock, we have also shown that C-peptide increases PPAR γ activation probably through an extracellular signal regulated kinases 1 and 2 (ERK1/2)-dependent pathway (6, 11, 12). Interestingly, *in vitro* studies have reported that some beneficial effects of C-peptide are observed only in the presence of adequate concentrations of zinc (13).

In view of the physiological role of zinc in immune regulation and the potential effects of C-peptide in inflammation, the purpose of our study was to investigate whether zinc supplementation before the onset of sepsis would potentiate the anti-inflammatory efficacy of C-peptide in a clinically relevant model of peritonitis and polymicrobial sepsis by cecal ligation and puncture (CLP). Also, purpose of our study was to provide insights into the mechanisms of action of C-peptide on pro-inflammatory signaling pathways. Our data demonstrate that C-peptide exerts beneficial effects in sepsis when there is an adequate supplementation of zinc.

MATERIALS AND METHODS

Murine model of polymicrobial sepsis

The investigation conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and commenced with the approval of the Institutional Animal Care and Use Committee. Male C57BL/6 mice (20–25 g body weight, 8–12 weeks old, Charles River Laboratories, Wilmington MA) were anesthetized with pentobarbital (40 mg/kg) intraperitoneally (IP). Polymicrobial sepsis was induced by CLP as previously described (14). After opening the abdomen, the cecum was exteriorized and ligated by a 6-0 silk ligature at its base without obstructing intestinal continuity. The cecum was punctured twice

with a 22-gauge needle and squeezed to allow fecal material to be excreted in small amount. The cecum was then returned into the peritoneal cavity and the abdominal incision was closed with a 6-0 silk ligature suture. Six groups of mice were used in the experiment. The first group (Control, $n = 16$) of mice was sacrificed before any surgical intervention. The second group (Sham, $n = 20$) had only laparotomy surgery with cecum externalization, but the cecum was neither ligated nor punctured. The third group (CLP + Vehicle, $n = 36$) underwent CLP surgery and was treated with vehicle (0.001% acetic acid, 10 ml/kg IP). The fourth group (CLP + C-peptide, $n = 36$) underwent CLP surgery and was treated with C-peptide (280 nmol/kg in 0.001% acetic acid IP) 1 h after CLP. The fifth group (CLP + zinc, $n = 36$) received daily injections of zinc gluconate (1.3 mg/kg IP) for three consecutive days prior CLP surgery. The sixth group (CLP + zinc/C-peptide, $n = 36$) received daily IP injections of zinc gluconate (1.3 mg/kg) for 3 days before surgery followed by C-peptide treatment 1 h after CLP surgery. After the procedure, mice were fluid resuscitated with 0.6 ml normal saline injected subcutaneously to replace the fluid and blood loss during operation. In time-course studies, mice of the different treatment groups ($n = 4-8$) were sacrificed at 3, 6, 18 h after CLP. Blood and plasma samples, peritoneal fluid, broncho-alveolar lavage fluid (BALF), lung, liver and spleen were collected for histological and biochemical studies described below. In survival studies, two more groups were added in the experimental design in order to test the efficacy of antibiotics (CLP + Imipenem and CLP + zinc/C-peptide + Imipenem). Imipenem (25 mg/kg) was administered IP as a single dose at 1 h after CLP surgery. For these studies, mice ($n = 9-19$ in each group) were awakened and allowed free access to water and food, and survival was monitored for 6 days.

Bacterial colony counts

Bacterial clearance was indirectly assessed by counting bacterial colonies in blood and peritoneal fluid, and homogenized samples of spleen, liver, and lung at 18 h after CLP surgery. Serial dilutions of samples were cultured on 5% sheep blood trypticase soy agar plates (BBL Stacker Plate TSA II, BD Biosciences, Sparks, MD) and incubated at 37°C in aerobic conditions for 24 h. Colony forming units (CFU) were counted and log-transformed to obtain a normal distribution. Results were expressed as log CFU per milliliter for blood, log CFU per gram for tissues, and log CFU per mouse for peritoneal lavage fluid.

Histopathology analysis

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated by four independent observers unaware of the experimental protocol. Specifically, lung injury was analyzed by a semiquantitative score as previously reported (6) based on the following histological features: a) alveolar congestion; b) hemorrhage; c) infiltration or aggregation of neutrophils in airspace or vessel wall; and d) thickness of alveolar wall/hyaline membrane formation. Each feature was graded from 0 to 4 (i.e., absent, mild, moderate, or severe). The four variables were summed to represent the lung injury score (total score, 0–16).

Total cell count and protein analysis of bronchoalveolar lavage fluid (BALF)

BALF was collected after cannulation of the trachea. Lungs were lavaged with 1.0 mL Hanks balanced salt solution without calcium or magnesium. The lavage fluid was centrifuged at $300 \times g$ for 10 minutes, and the supernatant was removed. The cell pellet was resuspended in 1 mL of 10% fetal bovine serum in phosphate buffered saline. Total cell numbers were counted on a hemocytometer and were expressed as cells/mL BALF. Total protein in the supernatant was determined by the Bradford method.

Lung wet-to-dry weight ratio

The left lung was excised, washed in phosphate buffered saline, gently dried using blotting paper, and weighed. The tissue was dried at 60°C for 72 h and reweighed. The change in the ratio of wet weight to dry weight was used as an indicator of lung edema formation.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation in lung and liver, as previously described (8). Tissues were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0) and centrifuged for 30 minutes at $4000 \times g$ at 4°C. An aliquot of the supernatant was allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and hydrogen peroxide (0.1 mM). The rate of change in absorbance was measured by spectrophotometry at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μ mol of hydrogen peroxide/min at 37°C and expressed in units per 100 mg weight of tissue.

Assessment of liver injury

Plasma levels of alanine aminotransferase (ALT) was measured as a marker of liver injury according to standard enzymatic assay (Sekisui Diagnostic, Charlottetown, Prince Edward Island, Canada).

Plasma levels of cytokines

Plasma levels of macrophage inflammatory protein-1 α (MIP-1 α) and tumor necrosis factor- α (TNF α) were evaluated by a commercially available multiplex array system (Linco-Research, St. Charles, MO) using the protocols recommended by the manufacturer. Plasma levels of interleukin 6 (IL-6) were evaluated by commercially available solid-phase enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN).

Nuclear protein extraction

Lungs were homogenized with a Polytron homogenizer in a buffer containing 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 2 mM EDTA, 5 mM NaN₃, 10 mM β -mercaptoethanol, 20 μ M leupeptin, 0.15 μ M pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate, and 0.4 nM microcystin. The homogenates were centrifuged ($1,000 \times g$ for 10 min), the supernatants were collected (cytosol extract), and the pellets were solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris HCl at pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride). The lysates were centrifuged ($15,000 \times g$ for 30 min, 4°C) and supernatant (nuclear extract) was collected.

Western blot analysis

Expression of PPAR γ , ERK1/2, phosphorylated form of ERK1/2 (pERK1/2) and β -actin was determined by immunoblot analyses using specific primary antibodies. Immunoreaction was visualized by chemiluminescence and exposed to a photographic film. Densitometric analysis of blots was performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

NF- κ B DNA binding activity

NF- κ B DNA binding activity was analyzed by a TransAM Transcription Factor assay kit specific for the activated form of p65 (Active Motif, Carlsbad CA) using the NF- κ B consensus site (5'-GGGACTTCC-3') according to the manufacturer's protocol.

Materials

Proinsulin C-peptide was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in 0.001% acetic acid solution. The primary antibodies directed at p-ERK1/2, ERK1/2, PPAR γ , β -actin and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Imipenem was obtained from Merck & Co, Inc. (Whitehouse Station, NJ). All other chemicals were obtained from Sigma-Aldrich.

Statistical analysis

Statistical analysis was performed using SigmaStat for Windows Version 3.10 (SysStat Software, San Jose, CA). Data in figures and text are expressed means $\pm$ SEM or median with 25th and 75th percentile of n observations ($n = 4$ –19 animals for each group). The results were examined by analysis of variance followed by the Bonferroni's correction post hoc t test. Statistical analysis of damage scores was performed using the Mann-Whitney Rank Sum test; when normality and equal variance passed, data were further analyzed by t test. The Kaplan-Meier log-rank analysis was used to compare differences in survival rates. P values less than 0.05 were considered significant.

RESULTS

Effect of zinc supplementation with C-peptide treatment on survival

After CLP, vehicle-treated mice exhibited symptoms of sepsis including diarrhea, ruffled fur, and decreased motor activity and early high mortality within 48 h; only 11% of animals were still alive at 6 days after CLP. A single dose of C-peptide (280 nmol/kg, IP), given as post-treatment at 1 h after CLP, did not improve survival (6%). Prophylactic supplementation with zinc alone increased survival percentage to 32%, although not statistically significant when compared to the vehicle-treated group ($P=0.218$). Interestingly, when zinc supplementation was combined with C-peptide post-treatment, animals exhibited a significant survival advantage (61%) when compared with C-peptide or vehicle treatment alone. Antibiotic treatment with imipenem also significantly increased survival rate (60%) when compared to vehicle treatment alone. The addition of imipenem to the combination of zinc supplementation and C-peptide treatment further improved survival (67%), although not statistically significant when compared to the combination protocol of zinc and C-peptide (Fig. 1).

Effect of zinc supplementation with C-peptide treatment on bacterial clearance

To examine the effect of combined zinc supplementation with C-peptide treatment on host defense response to eliminate bacterial pathogens, we counted bacterial CFU in blood, peritoneal fluid, lung, spleen and liver. Bacterial colonies were found in all these compartments at 18 h after CLP in vehicle-treated mice. The numbers of colonies in mice receiving zinc prophylaxis or C-peptide treatment alone were similar to those found in vehicle-treated mice. Combined zinc supplementation with C-peptide treatment reduced CFU counts at 18 h after CLP compared with all other treatment groups, suggesting that the combined therapeutic approach modulates the ability of the host to clear bacterial infection (Fig. 2).

Effect of zinc supplementation with C-peptide treatment on lung injury

In the sham group, laparotomy alone caused some mild cellular infiltration in the alveolar space at 18 h after the surgical procedure when compared to the normal histology of control naïve mice. At 18 h after CLP, vehicle-treated mice exhibited a severe lung damage, which was characterized by extravasations of red cells and accumulation of inflammatory cells into the air spaces, as determined by histological examination (Fig. 3A–F) and score injury (Fig.

3G). Combined zinc supplementation with C-peptide treatment ameliorated lung injury when compared with vehicle-treated mice; whereas there was no histological amelioration in the lungs of mice treated with C-peptide or zinc prophylaxis alone (Fig. 3H). In agreement with this pathological analysis, vehicle-treated mice exhibited significantly higher levels of leukocyte in the BALF when compared with sham animals. Combined zinc supplementation with C-peptide treatment lowered infiltrating leukocytes in the BALF. At this time point after CLP, there was no notable pulmonary edema, as total protein levels in BALF and wet weight to dry weight ratio of lung tissue were not significantly different among groups (data not shown).

Effect of zinc supplementation with C-peptide treatment on organ neutrophil infiltration and liver damage

To further confirm accumulation of inflammatory cells in the lung, we measured MPO activity as a specific index of lung neutrophil infiltration. We also measured MPO activity in the liver as indicative of systemic inflammation involving other major organs. Laparotomy alone in sham animals caused an early significant infiltration of neutrophils in liver (at 3 and 6 h) and in the lung (at 6 h) when compared to baseline values of control mice (Fig. 4). In the lung, MPO significantly increased at 3 h and continued to rise at 6 h when compared to sham mice, while starting to decline at 18 h after CLP (Fig. 4A). Combined zinc supplementation with C-peptide treatment reduced lung MPO activity at all time points when compared with vehicle treatment; whereas zinc prophylaxis or C-peptide treatment alone afforded a partial significant but not consistent reduction of neutrophil infiltration at 6 or 18 h, respectively (Fig. 4A). In the liver, MPO significantly increased at 3 h when compared to sham mice and started to decline at 6 and 18 h after CLP (Fig. 4B). Combined zinc supplementation with C-peptide treatment significantly reduced liver MPO activity when compared with vehicle-treated mice as well as C-peptide treatment or zinc prophylaxis alone at 3 h after CLP (Fig. 4B). At 18 h after CLP, a similar protective effect was observed in the liver when animals were given zinc supplementation or C-peptide alone, or the combination of the two treatments (Fig. 4B). Consistently with liver neutrophil infiltration reduction, the combination of zinc supplementation with C-peptide treatment also reduced the elevation of plasma ALT activity, suggesting an amelioration of liver function at 18 h after CLP (Fig. 5).

Effect of zinc supplementation with C-peptide treatment on plasma cytokine levels

The plasma concentration of the pro-inflammatory TNF α and MIP-1 α increased significantly at 6 and 18 h after CLP in vehicle-treated mice when compared to control or sham animals (Fig. 6). Treatment with C-peptide alone did not affect the increase in cytokine levels at any time. Interestingly, zinc supplementation alone or in combination with C-peptide treatment significantly reduced the early increase of TNF α and MIP-1 α at 6 h, but not at 18 h, after CLP when compared to vehicle treatment (Figs. 6A and B). A substantial increase of the pro-inflammatory IL-6 was also found in vehicle treated-mice at 6 and 18 h after CLP when compared with control or sham mice (Fig. 6C). Zinc prophylaxis or C-peptide treatment alone did not affect plasma levels of IL-6. However, combined zinc supplementation with C-peptide treatment significantly reduced the circulating levels of IL-6 at 18 h after CLP (Fig. 6C).

Effect of zinc supplementation with C-peptide treatment on nuclear activation of ERK1/2 and expression of PPAR γ

To investigate the mechanism of action of the combined treatment, we evaluated the lung nuclear activation of pERK1/2, an important mitogen-activated protein kinase that has been postulated to have a pathological role in sepsis also by mediating PPAR γ degradation (15). At Western blot analysis, expression of the active pERK1/2 slightly increased in lung

nuclear extracts 18 h after laparotomy (sham) and was significantly enhanced by CLP procedure when compared to baseline expression of control normal mice. This event paralleled with a marked downregulation of PPAR γ nuclear expression (Fig. 7). Zinc supplementation or C-peptide treatment alone partially reduced the expression of active pERK1/2, but did not enhance the nuclear availability of PPAR γ at 18 h after CLP. Combination of zinc supplementation with C-peptide further inhibited ERK1/2 activation and restored PPAR γ expression in the lung similar to the sham animals without infection (Fig. 7).

Effect of zinc supplementation with C-peptide treatment on activity of NF- κ B

To further identify the molecular changes associated with lung inflammation we analyzed the kinetics profile of NF- κ B. The p65 NF- κ B activity rapidly rose at 3 h, was maximally elevated at 6 h, while declined at 18 h after CLP in the lung of vehicle-treated mice. Zinc supplementation or C-peptide alone did not affect NF- κ B activity. On the contrary, combination of zinc supplementation with C-peptide completely inhibited NF- κ B activity, which exhibited kinetics similar to the sham group (Fig. 8).

DISCUSSION

In our study we have demonstrated that short-term prophylaxis with zinc prior to infection restored the anti-inflammatory effects of the PPAR γ activator C-peptide, and significantly improved survival of mice with polymicrobial sepsis. This improvement in sepsis-induced mortality was associated with a significantly reduction of the inflammatory response, reduction of neutrophil infiltration in lung and liver, and enhancement of the bacterial clearance.

It has been well established that zinc homeostasis is essential for proper immune function and zinc deficiency itself influences both adaptive and innate responses resulting in an increased susceptibility for infections (2–4). These clinical observations have been supported by experimental studies in models of polymicrobial infections. Knoell and coauthors demonstrated that zinc deficient diet enhanced the mortality rate of mice subjected to polymicrobial sepsis when compared to mice with normal diet (16). Zinc-deficient mice also exhibited significantly higher systemic inflammation and severe organ injury than normal fed animals (16). However, even in the absence of pre-existing diet deficiency, there is also evidence that sepsis and other acute critically ill conditions are associated with perturbations in zinc metabolism. For instance, it has been shown that serum zinc concentration rapidly declined in patients after emergency abdominal surgery (17) or in healthy adult volunteers subjected to endotoxin administration (18). The low systemic concentration appeared without recognizable body losses or altered affinity to serum albumin and may be secondary to changes in internal redistribution of the metal (17, 18). Low plasma zinc concentrations are found in pediatric critically ill patients and correlate with known biomarkers of inflammation (C-reactive protein and IL-6) and degree of organ failure (2), and with lymphopenia (19). Interestingly, a genomic analysis from whole blood-derived RNA revealed that pediatric patients with septic shock exhibited a persistent downregulation of genes related to zinc homeostasis (20). In adult patients, the acute decline of systemic zinc levels was more pronounced in septic patients than in noninfected critically ill patients and correlated with high severity of illness and cardiovascular dysfunction (3). The critical role of the labile pool of zinc is further supported by experimental studies demonstrating that zinc supplementation may mitigate the acute phase response and the systemic inflammation (5, 16), and may decrease mortality during infection (21). Interestingly, pilot experiments conducted in our laboratories have suggested that administration of zinc after the sepsis challenge does not affect survival in mice subjected to peritonitis (21). However, in contrast to these previous reports demonstrating beneficial

effects of zinc pretreatment (5, 16, 21), in our study zinc prophylaxis alone reduced some indices of the systemic inflammation but was not efficacious in promoting bacterial clearance or in providing a significant survival advantage. Differently from these previous studies, it is important to note that we adopted a highly lethal model of sepsis. Taken together these data suggest that the immunomodulatory properties of zinc may depend on the severity of the disease. Furthermore, a few studies have also suggested a link between zinc levels and changes in endogenous bacteria. For example, microbiota in the gastrointestinal tract appear to contribute to reduce the quantity of zinc in chicks (22). Furthermore, feeding zinc and inorganic copper to pigs decreased fecal microbiota diversity (23). Additional studies are, therefore, needed to assess whether zinc supplementation and/or C-peptide treatment may also affect microbiome in the setting of sepsis.

Interestingly, in our current model of sepsis, the combination of zinc prophylaxis with C-peptide treatment had a similar survival benefit as the treatment with the broad-spectrum antibiotic imipenem. Thus, these findings further confirmed the potential anti-microbial effect of C-peptide when combined with zinc supplementation. Although we observed a further increase in survival benefit when imipenem was associated with the combination protocol of zinc and C-peptide, this increase was not statistically significant when compared to the antibiotic alone. In this regard, we must note that in our model treatments were given as single bolus on the first day of surgery only, a protocol that does not mimic the clinical regimens of several days.

Several clinical reports have provided evidence that C-peptide exerts anti-inflammatory properties in type I diabetes patients and may have beneficial effects in preventing vascular complications, nephropathy and neuropathy in an insulin-independent manner (24, 25). Previous studies from our group have further confirmed that C-peptide has anti-inflammatory activity in preclinical models of cardiovascular shock and, when administered as post-treatment at supra-physiologic concentrations, ameliorates lung injury and survival in mice subjected to endotoxic shock and ameliorates multiple organ failure in rats subjected to hemorrhagic shock (6, 11, 12). Similar protective effects of C-peptide treatment have been reported in experimental models of myocardial ischemia-reperfusion (26) and endothelial dysfunction (27) in non-diabetic animals. There are also studies suggesting that C-peptide as well as zinc supplementation may directly affect cytokine production. For example, C-peptide significantly reduced IL-6 production from LPS-stimulated monocytes (28); whereas zinc sulfate reduced IL-6 production from immuno-stimulated airway smooth muscle cells (29). However, in contrast to these previous reports, in our current model of severe septic shock, post-treatment with C-peptide alone did not exert any beneficial effects. Interestingly, the anti-inflammatory properties of C-peptide were restored when mice were subjected to prophylaxis with zinc for three days. In this combined therapeutic regimen, C-peptide was capable of significantly improving survival when given after the development of CLP-induced sepsis and to reduce multiple organ injury, thus implicating that zinc is an important requisite for the activity of C-peptide.

It appears that while the combination of the zinc prophylaxis and C-peptide therapy had an anti-inflammatory effect by reducing cytokine production and tissue neutrophil recruitment, it did not jeopardize the immune function of mice and their capability of clearing bacteria in blood, peritoneal fluid, lung and spleen. Although our previous report demonstrated that *in vivo* zinc supplementation might improve phagocytosis and intracellular killing of peritoneal macrophages of septic mice (21), in our current model of sepsis zinc supplementation alone was totally ineffective on bacterial clearance. Thus, our study is also the first report suggesting that C-peptide acquires immunomodulatory properties after a short-term pretreatment with zinc.

The precise mechanisms by which zinc may modulate the anti-inflammatory properties of C-peptide remain to be elucidated. *In vitro* studies have demonstrated that C-peptide activity is increased when it forms a complex with a metal, such as iron, chromium or zinc (13, 30). In red blood cells, for example, the ability of C-peptide to promote ATP release from erythrocytes or to promote glucose uptake in endothelial cells increases when incubated with zinc (13). Additional studies in erythrocytes have demonstrated that a glutamic acid residue (Glu27) is important in zinc-mediated C-peptide activity, as Glu27 mutant peptides exerted a significant decrease in ATP release when compared to wild-type C-peptide (30). The effect of zinc on potentiating or restoring the C-peptide activity may extend to other molecular mechanisms. *In vitro* and *in vivo* studies have reported that the cyto-protective effects of C-peptide are secondary to activation of PPAR γ (6, 10–12), a nuclear receptor that regulates the expression of several genes involved in inflammation, by either controlling directly the transcription process or by indirectly inhibiting the activation of NF- κ B (7, 8). We have previously demonstrated that PPAR γ dysfunction, as determined by downregulation of tissue protein expression and decreased DNA binding, is an important pathologic factor of the systemic inflammatory response and development of multiple organ failure, and that treatment with PPAR γ ligands may afford protective effects in experimental models of sepsis (8, 9). It is known that the specificity and polarity of DNA binding of PPAR γ depend at least in part on the zinc finger domains (31). Therefore, zinc may be important in PPAR γ signaling. *In vitro* studies in porcine endothelial cells have shown that zinc deficiency results, indeed, in reduced PPAR γ expression, loss of its DNA binding and failure of PPAR γ agonists to downregulate NF- κ B activation and the inflammatory response (32). A link between zinc and anti-inflammatory properties has been reported for PPAR α as well (32, 33). In human monocytic leukemia THP1 and endothelial cells zinc supplementation resulted in increased PPAR α protein expression and inhibition of TNF α release and adhesion molecule expression (33). Consistent with these previous studies, we found that combined treatment of zinc supplementation and C-peptide restored lung nuclear expression of PPAR γ , which was associated with a remarkable inhibition of NF- κ B activity and subsequent downregulation of the NF- κ B dependent pro-inflammatory cytokines, TNF- α and IL-6, and the chemokine MIP-1 α . To further define the anti-inflammatory molecular mechanisms of zinc supplementation of C-peptide we also investigated the role of ERK1/2, which have been implicated in the regulation of pro-inflammatory transcription factors and in the pathogenetic events of sepsis (15). *In vitro* studies in cultured cells have also suggested that ERK1/2 activation may result into PPAR γ inactivation, as the kinases induce PPAR γ phosphorylation, thus triggering the nuclear receptor for protein degradation (34). Furthermore, we have previously demonstrated that C-peptide inhibits the activation ERK1/2 and this may contribute to the anti-inflammatory effects in endotoxic and hemorrhagic shock *in vivo* (6, 11, 12). In our current study, zinc prophylaxis further potentiated the inhibitory effect of C-peptide on ERK1/2 phosphorylation. Taken together, our data clearly indicate that zinc is required for the modulation of the several molecular targets of C-peptide.

In summary, our data demonstrate the efficacy of a combined novel approach of zinc prophylaxis and C-peptide treatment for experimental sepsis. Since in the setting of intensive care units sepsis is a common secondary complication in critically ill patients, prophylactic strategies are currently being investigated in clinical studies as means to reduce morbidity and mortality of sepsis (35). Therefore, our data also propose that supplementation of zinc may serve as an adjunctive prophylactic strategy for patients who are at high risk of infections. From a pharmacological perspective, our data also suggest that normalization of zinc metabolism may be required for proper drug efficacy, especially for compounds that are metal activated and compounds that target zinc-dependent pathways.

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FIG. 1. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on survival in mice subjected to cecal ligation and puncture (CLP)

Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) and Imipenem (25 mg/kg IP) were administered at 1 h after CLP. Log-rank curve of survival rate is shown. * $P < 0.05$ versus vehicle or C-peptide treatment alone.

FIG. 2. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on bacterial clearance in mice subjected to cecal ligation and puncture (CLP)
 Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery. Tissue samples were harvested at 18 h after CLP surgery. Colony forming units (CFU) were counted and log-transformed to obtain a normal distribution. Data ($n = 5$ mice for each group) were expressed as log CFU per milliliter for blood, log CFU per gram for tissues, and log CFU per mouse for peritoneal lavage fluid. * $P < 0.05$ versus vehicle, zinc supplementation or C-peptide treatment alone.

FIG. 3. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on lung injury at 18 h after cecum ligation and perforation (CLP)

Representative histology of lung sections stained with hematoxylin and eosin (A-F, magnification $\times 400$). (A) Normal lung of a control mouse (*Control*). (B) Lung after sham surgery (*Sham*). (C) Lung of mouse receiving vehicle treatment after CLP (*CLP+Vehicle*). (D) Lung of mouse receiving C-peptide treatment after CLP (*CLP+C-peptide*). (E) Lung of mouse receiving zinc supplementation before CLP (*CLP+Zinc*). (F) Lung of mouse receiving zinc supplementation before and C-peptide treatment after CLP (*CLP+Zinc/C-peptide*). (G) Histopathologic scores of lung sections ($n = 5$ mice for each group). Lung injury was scored from 0 (no damage) to 16 (maximum damage). Box plots represent 25th percentile, median, and 75th percentile; error bars define 10th and 90th percentiles. (H) Total leukocyte count in broncho-aveolar lavage fluid. Data are mean $\pm$ SEM of 8–12

animals for each group. Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery.
* $P<0.05$ vs. control mice; # $P<0.05$ vehicle-treated mice subjected to CLP (*Vehicle*).

FIG. 4. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on lung and liver neutrophil infiltration

Myeloperoxidase (MPO) activity in the lung (A) and in the liver (B) at 3, 6 and 18 h after cecum ligation and puncture (CLP). Data are mean $\pm$ SEM of 4–6 animals for each group. Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery. * P <0.05 versus control mice; # P <0.05 versus vehicle-treated mice subjected to CLP; ‡ P <0.05 versus zinc supplementation or C-peptide treatment alone.

FIG. 5. Effect of combination of zinc supplementation and C-peptide on liver function
 Plasma alanine aminotransferase (ALT) levels at 18 h after CLP. Data are mean $\pm$ SEM of 14–16 animals for each group. Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery. * P <0.05 versus sham mice at 18 h after surgery; # P <0.05 versus vehicle-treated mice subjected to CLP.

FIG. 6. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on plasma levels of cytokines

(A) Tumor necrosis factor (TNF)- α , (B) macrophage inflammatory protein (MIP)-1 α , and (C) interleukin (IL)-6 at 6 and 18 h after cecum ligation and puncture (CLP). Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery. Data are mean $\pm$ SEM of 4–8 animals for each group. * $P < 0.05$ versus control mice; # $P < 0.05$ versus vehicle-treated mice subjected to CLP.

FIG. 7. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on nuclear expression of peroxisome proliferator activated receptor (PPAR) γ , extracellular signal regulated kinases (ERK)1/2 and phosphorylated ERK1/2 (pERK1/2) in the lung

(A) Representative radiographs of Western blot analyses of pERK1/2, total ERK1/2 and PPAR γ expression in nuclear extracts. Expression of β -actin was used as loading control protein. (B) Image analysis of pERK1/2 and PPAR γ expression determined by densitometry of 4 animals for each group. Fold increase was calculated versus control value set to 1.0. Lung samples were harvested at 18 h CLP. Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery. Box plots represent 25th percentile, median, and 75th percentile; error bars define 10th and 90th percentiles. * P <0.05 vs. control mice; # P <0.05 vehicle-treated mice subjected to CLP.

FIG. 8. Effect of zinc supplementation, C-peptide or combination of zinc supplementation and C-peptide on activity of the p65 subunit of NF-κB in lung nuclear extracts at 3, 6 and 18 h after cecum ligation and puncture (CLP)

Zinc-gluconate (1.3 mg/kg/day IP) was administered for three days before CLP. C-peptide (280 nmol/kg IP) was administered at 1 h after CLP surgery. Data are mean $\pm$ SEM of 4–6 animals for each group and are expressed as optical density units. * P <0.05 versus control mice; # P <0.05 versus vehicle-treated mice subjected to CLP.