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Zinc is required in pyrrolidine dithiocarbamate inhibition of NF-κB activation

Chul Hoon Kim[a], Joo Hee Kim[a], Chung Y. Hsu[b], Young Soo Ahn[a,*]

[a] *Yonsei Brain Research Institute and Department of Pharmacology, Yonsei University College of Medicine, Seoul 120-752, South Korea*
[b] *Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, USA*

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Abstract Pyrrolidine dithiocarbamate (PDTC) is a potent inhibitor of nuclear factor kappa B (NF-κB) activation. PDTC inhibited basal NF-κB activity of endothelial cells. PDTC, however, failed to inhibit basal NF-κB activity after withdrawal of serum in the media, and the inhibitory effect of PDTC could be restored by addition of zinc. When various preparations of metal ion-EDTA were tested with PDTC in serum-containing media, only Zn-EDTA failed to block the inhibitory effect of PDTC. The dependence on zinc was also noted in PDTC inhibition of NF-κB stimulated by TNFα. These facts suggest that zinc is required for PDTC inhibition of NF-κB activation.

Key words: Dithiocarbamate; NF-κB; Zinc

1. Introduction

Dithiocarbamates were widely used in agriculture as insecticides and fungicides [1] and in health care for the management of alcoholism and metal intoxication [2]. Recently, dithiocarbamates have also been proposed for treating acquired immunodeficiency syndrome and cancer [3,4]. Pyrrolidine dithiocarbamate (PDTC), one of dithiocarbamates, is cytotoxic in a number of cell types [5–8], in some cases presumably due to its potent inhibitory effect on NF-κB activity [8]. NF-κB activation is responsive to reactive oxygen species (ROS) [9]. ROS scavengers such as dithiocarbamates [10], vitamin E derivatives [11] and *N*-acetyl-L-cysteine [12] prevent the NF-κB activation. PDTC inhibition of NF-κB has been attributed to its antioxidant property [13]. PDTC is also a metal chelator. Thus, removal of metal ions has been known to be an alternative mechanism of action for PDTC inhibition of NF-κB activity [10,13]. In contrast to these well-known properties of PDTC, Nobel et al. [6] showed that PDTC increased intracellular copper level. They suggested that pro-apoptotic effect of PDTC could be mediated by these redox-active copper ions. Hence, intriguing possibility that the inhibitory effect of PDTC on NF-κB may involve its interaction with metal ions remains to be confirmed. In fact, several metal ions have been shown to modulate NF-κB activity. Copper inhibits IκB phosphorylation which is an upstream event in NF-κB activation [14]. Gold, an anti-rheumatic drug, blocks DNA binding activity of NF-κB [15]. In vitro, chromium, cadmium, mercury, zinc, and arsenite also inhibit NF-κB activity [16]. However, the exact role of zinc in NF-κB activity has remained clouded by conflicting observations. Zinc was noted to be either essential for [15,17] or to exert [16,18] an inhibitory effect on NF-κB activity. None of these studies, however, addressed the role of zinc in PDTC inhibition of NF-κB activity. Results from the present study show that zinc is required for inhibitory action of PDTC on NF-κB.

2. Materials and methods

2.1. Materials

Pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma (St. Louis, MO, USA). Free base EDTA, copper-saturated EDTA (Cu-EDTA), calcium-saturated EDTA (Ca-EDTA), and zinc-saturated EDTA (Zn-EDTA) were purchased from Fluka (Buchs, Switzerland). All metal-saturated EDTAs are disodium EDTA saturated with equimolar respective metal. Double-stranded oligonucleotides containing consensus NF-κB binding sequence and its mutant form were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Poly(dIdC) was purchased from Pharmacia (Uppsala, Sweden). [γ-^{32}P]ATP was from DuPont NEN (Boston, MA, USA). Specific antisera to p50 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Cell culture products were purchased from Gibco BRL (Life Technologies, NY, USA).

2.2. Cell cultures

Bovine cerebral endothelial cells (BCECs) were prepared and characterized as previously described [19,20] with modification [21]. Endothelial cells of passages 4–15, which were uniformly positive for factor VIII and vimentin (>95% endothelial cells) and exhibited the characteristic bradykinin receptors, were grown to 70–80% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS before experiments. PC12 (ATCC CRL 1721) was grown in DMEM supplemented with 10% FBS and 5% horse serum.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method described by Lee et al. [22] with modification [23]. For EMSA, the following oligonucleotide with the NF-κB consensus binding sequence was used: (5′-AGTTGAGGGGACTTTCCCAGGC-3′). A mutant motif with G to C substitution (5′-AGTTGAGGCGACTTTCCCAGGC-3′) served as a control. Labeling of the oligonucleotide with [γ-^{32}P]ATP and the EMSA method have been previously detailed [23]. Reactions were started by addition of nuclear extracts and allowed for 30 min at room temperature. Samples were loaded on 4% polyacrylamide non-denaturing gel and electrophoresed for 2 h at 180 V. The dried gel was exposed to Kodak XR5 film on intensifying screen for 10–20 h at −70°C. For competition assay, an unlabeled NF-κB oligonucleotide was added in 100-fold excess. For supershift assay, 1 μl of anti-p65 or anti-p50 antibody was incubated with nuclear extract for 30 min at room temperature prior to binding reaction.

*Corresponding author. Fax: +82 (2) 313-1894.
E-mail: ahnys@yumc.yonsei.ac.kr

Abbreviations: BCECs, bovine cerebral endothelial cells; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; PDTC, pyrrolidine dithiocarbamate; NF-κB, nuclear factor kappa B; LDH, lactate dehydrogenase

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3. Results

3.1. Effect of PDTC on NF-κB activity in BCECs

Basal NF-κB activity was present in BCECs which were maintained in DMEM containing 10% FBS. To ensure that the mobility shift bands under this condition were the NF-κB/oligonucleotide complex, we used an NF-κB mutant oligonucleotide with a 'G'→'C' substitution in the NF-κB binding motif as a control. Addition of this cold NF-κB mutant oligonucleotide (100-fold in excess) did not affect the bands (p50/p65 and p50/p50) which were associated with the labeled NF-κB oligonucleotide probe; whereas the cold consensus NF-κB oligonucleotide (100-fold in excess) completely abolished these bands (Fig. 1A). AP-1 or Oct-1 consensus oligonucleotide also failed to displace the labeled NF-κB oligonucleotide probe (Fig. 1A). Incubation of nuclear extracts with antisera against NF-κB p50 or p65 subunit respectively caused a supershift of the corresponding NF-κB/oligonucleotide band (Fig. 1B). These results established the specificity of the NF-κB binding activity assay. In a time course study, PDTC (50 μM) inhibited the basal NF-κB activity almost completely in 3 h when BCECs were grown in DMEM enriched with 10% FBS (Fig. 1C). In subsequent experiments, we determined NF-κB binding activity 4 h after PDTC treatment. PDTC inhibited NF-κB activity in serum-fed BCECs in a dose-dependent manner (25–100 μM) (Fig. 1D). In the absence of serum, PDTC, in concentrations up to 1 mM, failed to alter NF-κB activity (Fig. 1E).

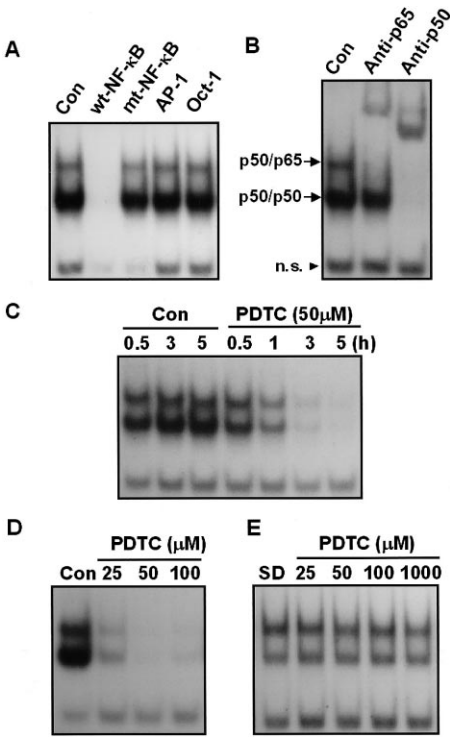

Fig. 1. Effect of PDTC on NF-κB activation in BCECs. Nuclear extracts were obtained 4 h after PDTC or vehicle treatment followed by EMSA. Basal NF-κB activity was present in BCECs grown in DMEM containing 10% FBS (Con). A: Competition for NF-κB binding with cold nucleotides (wild-type (wt-) or mutant (mt-) NF-κB, AP-1 or Oct-1) which were in excess of 100-fold. B: Supershift with anti-p65 or anti-p50 IgG. Arrowhead denotes the non-specific band (n.s.). C: A time course of NF-κB inhibition by PDTC (50 μM). D: Dose-dependent inhibition of NF-κB by PDTC in DMEM containing 10% FBS. E: Lack of PDTC effect on NF-κB activity in serum-deprived BCECs.

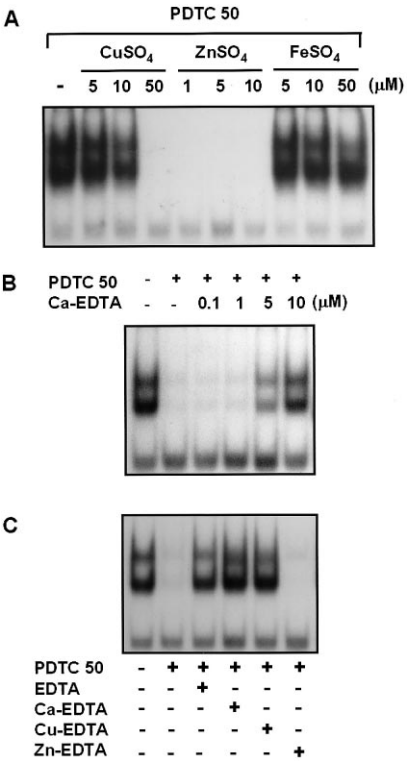

Fig. 2. Effects of selected metals on PDTC inhibition of NF-κB activity. A: BCECs were treated with various concentrations of $CuSO_4$, $FeSO_4$ or $ZnSO_4$, plus PDTC (50 μM) for 4 h after serum withdrawal. B: PDTC was co-administered with the indicated concentrations of Ca-EDTA to BCECs grown in the DMEM containing 10% FBS. C: Free base, Ca-, Cu- or Zn-EDTA (each 10 μM) and PDTC (50 μM) were added together to BCECs grown in DMEM containing 10% FBS.

3.2. Effect of metal ions on PDTC action

To determine whether selected factor(s) in serum might be required for PDTC action, we investigated selected metal ions including iron, copper and zinc which are likely to affect NF-κB binding activity. BCECs deprived of serum were exposed to PDTC with or without one of the selected metal ions for 4 h. Each metal was prepared freshly just prior to experiment. $FeSO_4$ failed to restore the inhibitory effect of PDTC on NF-κB activity (Fig. 2A). $ZnSO_4$, at concentrations as low as 1 μM when co-administered with PDTC, completely blocked NF-κB activity under the serum-deprived condition (Fig. 2A). $CuSO_4$ showed a similar effect as $ZnSO_4$, but at a much higher concentration (50 μM). If a specific metal ion (zinc or copper) is responsible for the lack of PDTC effect in the absence of serum, chelating the selected metal ion(s) in serum should block PDTC inhibition of NF-κB activity. This approach was tested using Ca-, Cu- and Zn-EDTA. Each of these metal ion-EDTA complexes chelates metal ions other than the particular ion that forms the complex with EDTA. Thus, Zn-EDTA chelates metal ions other than Zn. When various preparations of metal ion-EDTA were compared simultaneously, the biological effect of a certain metal ion may be distinguished from the non-effective ones [24]. PDTC, at a concentration of 50 μM, was added with one of the three metal ion-EDTA preparations for 4 h under se-

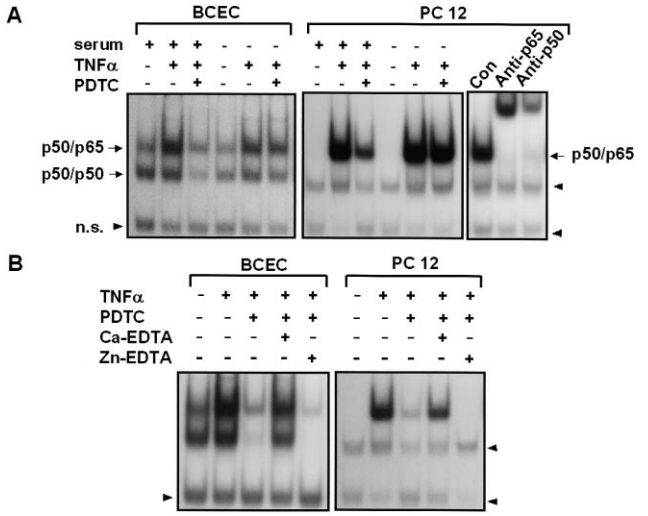

Fig. 3. Effect of PDTC on TNFα activation of NF-κB activity. A: BCECs were treated with TNFα (5 ng/ml) for 2 h in the presence or absence of 10% FBS in the medium with or without PDTC (50 μM) (left). PC12 cells were preincubated with PDTC (50 μM) for 30 min, followed by TNFα (5 ng/ml) treatment for 30 min in the presence or absence of 10% FBS (middle). Supershift assay was done with anti-p65 or anti-p50 IgG (right). B: BCECs were treated with TNFα (5 ng/ml). PDTC (25 μM) with or without Ca-EDTA (20 μM) or Zn-EDTA (20 μM) was co-administered with TNFα for 3 h in DMEM containing 10% FBS. PC12 cells were preincubated with PDTC (50 μM) plus Ca-EDTA (20 μM) or Zn-EDTA (20 μM), followed by treatment with TNFα (5 ng/ml). Arrowheads denote non-specific bands.

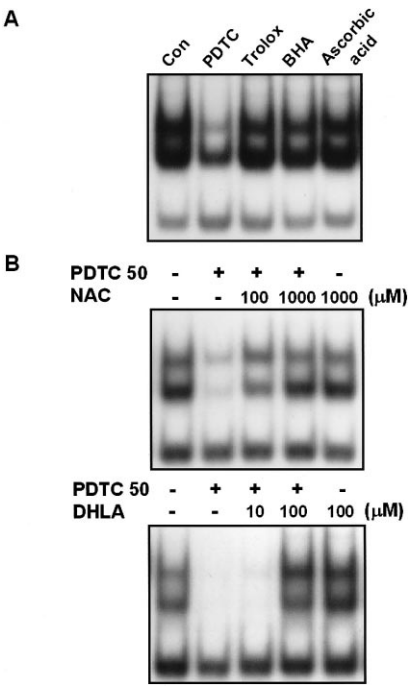

Fig. 4. Effect of thiol or non-thiol antioxidant on NF-κB activity and PDTC action. BCECs were treated with antioxidants or antioxidants plus PDTC in serum-containing media. A: BCECs were treated with non-thiol antioxidants, trolox (1 mM), BHA (500 μM) or ascorbic acid (1 mM), for 4 h. Second lane represents inhibition of NF-κB by 4 h treatment of 50 μM PDTC. B: BCECs were treated with thiol antioxidants or PDTC (50 μM) plus thiol antioxidants for 4 h.

rum-enriched condition (DMEM containing 10% FBS) in which PDTC has been shown above to inhibit basal NF-κB activity. The inhibitory effect of PDTC on NF-κB activity under this condition was abolished by Ca-EDTA in a dose-dependent manner (Fig. 2B). At 10 μM, Ca-EDTA almost completely blocked the inhibitory effect of PDTC (Fig. 2B and C). Free base EDTA and Cu-EDTA, at a concentration of 10 μM, had a similar effect (Fig. 2C). However, Zn-EDTA failed to block PDTC inhibition of basal NF-κB activity at this concentration (Fig. 2C). None of the metal ion-EDTA preparations alone had any effect on the basal NF-κB activity (data not shown).

3.3. Effect of PDTC on TNFα-stimulated NF-κB activation

Treatment of BCECs with TNFα (5 ng/ml) for 3 h resulted in an increase in NF-κB activity. TNFα activation of NF-κB was evident with or without serum in the medium (Fig. 3A). However, PDTC (25 μM) inhibition of NF-κB activity induced by TNFα (5 ng/ml) was detected only in the presence of serum (Fig. 3A, left panel, the left three lanes). The same concentration of PDTC failed to inhibit NF-κB activation by TNFα upon serum withdrawal (Fig. 3A, left panel, the right three lanes). In PC12 cells, minimal basal NF-κB activity was detected. TNFα (5 ng/ml) increased NF-κB activation within 30 min with or without serum (Fig. 3A, middle panel). The p65 and p50 subunits were present in the shifted complexes (Fig. 3A, right panel). PDTC (50 μM) inhibited TNFα-stimulated NF-κB activity in PC12 cells in the presence of serum (Fig. 3A, middle panel, the left three lanes). PDTC was without inhibitory action upon serum withdrawal (Fig. 3A, middle panel, the right three lanes). The ability of PDTC to inhibit TNFα (5 ng/ml) activation of NF-κB was abolished by Ca-EDTA (20 μM), but not Zn-EDTA (20 μM), in BCECs (Fig. 3B, left panel) and PC12 cells (Fig. 3B, right panel). Other forms of EDTA such as free base EDTA or Cu-EDTA also blocked PDTC inhibition of TNFα-stimulated NF-κB activation in both BCECs and PC12 cells (data not shown).

3.4. Effect of thiol or non-thiol antioxidants on NF-κB activity and PDTC action

We tested the effects of thiol or non-thiol antioxidants on basal NF-κB activities of endothelial cells. Nuclear extracts of BCECs were obtained after 4 h of treatments of antioxidants or PDTC plus antioxidants. Non-thiol antioxidants such as trolox (1 mM), butylated hydroxyanisole (BHA; 500 μM) and ascorbic acid (1 mM) and thiol antioxidants such as dihydrolipoic acid (DHLA) and *N*-acetyl-L-cysteine (NAC) themselves did not change the basal NF-κB activation (Fig. 4A, first lanes from right of upper and lower panels of Fig. 4B). However, thiol antioxidants prevented the inhibitory action of PDTC (Fig. 4B). In contrast, non-thiol antioxidants did not affect the NF-κB inhibition by PDTC (data not shown).

4. Discussion

In the present study, PDTC inhibited basal NF-κB activity in BCECs under serum-enriched conditions. However, it did not alter NF-κB activation significantly when serum was absent in media. It was reasonable to speculate that certain factor(s) in serum might be required for the inhibitory effect of PDTC on NF-κB activity. Recently, selected metal ions have emerged as important regulators of NF-κB activity

[14–16]. We explored the possibility that metals ions that are known to be present in serum could be the factor(s) required for PDTC action. Among copper, zinc and iron, copper and zinc restored the inhibitory effect of PDTC on NF-κB under serum deprivation. As reported previously, PDTC increased intracellular copper level in several cell types [6,25]. The mechanism was suggested as that PDTC formed lipophilic complex with copper ion [6]. Thus, metal ion that could be available for PDTC action would not be necessarily free from. However, in BCEC examined in the present study, the concentrations of copper (50 μM or above) required for PDTC action were much higher than the concentration of copper that is expected to be normally present in serum. In contrast, zinc restored PDTC action in serum-deprived cells at concentrations as low as 1 μM, which was equivalent to the degree of inhibition by the same concentration of PDTC in serum condition. To verify that zinc is the dominant metal ion that is essential for the inhibitory action of PDTC, various preparations of metal ion-EDTA complex were tested. Cu-EDTA or Zn-EDTA can chelate metal ions in serum-containing media without lowering the concentration of respective metal ion that is the constituent of the metal ion-EDTA complex [24]. Thus, it is possible to determine the relative potency of these metal ions in their possible contribution to PDTC inhibition of NF-κB activity. In BCECs, NF-κB inhibition by PDTC could be completely abolished by free base EDTA, Ca-EDTA or Cu-EDTA. However, Zn-EDTA failed to block the inhibitory effect of PDTC. Moreover, under selected experimental paradigms, Zn-EDTA actually potentiated the inhibitory effect of PDTC on NF-κB probably because of the release of zinc ion from the Zn-EDTA complex (Fig. 3B). It is plausible that zinc is an essential element in the PDTC inhibition of NF-κB activation. The notion that zinc is the serum factor that is required for PDTC inhibition of NF-κB activity could also be extended to PDTC inhibition of TNFα-induced NF-κB activation. We explored this scheme of zinc-mediated PDTC inhibition of NF-κB in other cell types. We found that zinc was also required in PDTC inhibition of NF-κB activity in PC12. In NIH/3T3, Hep 3B and HeLa cell, this paradigm was also applicable (unpublished data).

We have previously shown that PDTC was cytotoxic to BCECs by mediation of zinc ion [26]. Since NF-κB is known to promote cell viability [27–30], it would be entertained that PDTC-induced cell death may be related to its inhibition of NF-κB activity. Other studies also reported that PDTC induced apoptosis in B-lymphoma cell [8] and rendered T-lymphoma cell susceptible to apoptosis [31] by suppression of basal NF-κB activities. In BCECs, NF-κB activity was completely inhibited by PDTC in 3 h in the present study which was followed by apparent cell death after 6 h in our previous study [26]. Future study using experimental design for selective inhibition of NF-κB will support a cell death scheme in which PDTC induction of BCEC death is secondary to zinc-mediated inhibition of NF-κB activity.

PDTC has been extensively used as a NF-κB inhibitor. The molecular basis for the action of PDTC has been claimed to be due to its antioxidant or metal chelator activity [10,13]. The dithiocarboxyl group on PDTC is expected to exert antioxidant effect [13]. Previously we showed that PDTC increased the intracellular zinc that was recruited from extracellular source [26]. In the present study, the inhibitory effect of PDTC on NF-κB activity was abolished when extracellular metal ions were chelated by membrane non-permeable chelators. Furthermore, thiol or non-thiol antioxidants did not affect NF-κB activation in BCECs. It is noteworthy that thiol antioxidants in contrary to non-thiol antioxidant, when co-administered with PDTC, rather abolished the inhibitory action of PDTC. This seems to be a result of direct interaction of zinc with thiol moiety [32]. Thus, at least in the cell types we studied, NF-κB inhibition by PDTC is unlikely to be due to simple chelation of metal ions or direct scavenging of reactive oxygen species by certain functional moiety of PDTC. The fact that PDTC is a far more potent NF-κB inhibitor as compared to most antioxidants, also suggests that interaction with zinc, but not the antioxidative effect, is the likely mechanism of PDTC effects on NF-κB activity. This study also implies that zinc would be an important negative regulator of NF-κB in certain physiologic or pathologic condition.

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