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Short communication

Biphasic effects of dithiocarbamates on the activity of nuclear factor-κB

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Abstract

Dithiocarbamates are well-known antioxidants and nuclear factor-κB (NF-κB) inhibitors. Recently, they have been characterized as zinc ionophores. Concentration-dependent biphasic effects of dithiocarbamates on NF-κB activity have been widely reported. We studied the mechanism of this phenomenon in relation to Zn^{2+} influx. Two dithiocarbamates, pyrrolidine dithiocarbamate and diethyldithiocarbamate, showed concentration-dependent biphasic effects in inhibiting NF-κB activation in cerebral endothelial cells. These unique effects of dithiocarbamates on NF-κB were tightly linked to their ability to elevate intracellular Zn^{2+} levels. At high concentrations (> 500 μM), dithiocarbamates started to lose their ability to promote Zn^{2+} influx and to inhibit NF-κB activation. These results might provide insight into the appropriate use of dithiocarbamates in various disorders.

Keywords: Cerebral endothelial cell; (Bovine); Dithiocarbamate; NF-κB (nuclear factor-κB); Zn^{2+}

1. Introduction

Dithiocarbamates are widely used as pesticides in agriculture (Hayes, 1982) and are common contact allergens (Klaassen, 1996). Carbon disulfide, a breakdown product of dithiocarbamates, is a potential neurotoxin (Johnson et al., 1998). Paradoxically, dithiocarbamates are of therapeutic use in a number of diseases, including alcoholism and metal intoxication (Thorn and Ludwig, 1962; Sunderman, 1979). Dithiocarbamates have also been proposed for the treatment of a number of diseases with seemingly unrelated causes. These include acquired immune depressive syndrome (Reisinger et al., 1990), cancer (Gandara et al., 1991), atherosclerosis (Moellering et al., 1999), endotoxic shock (Lauzurica et al., 1999) and diabetic retinopathy (Yoshida et al., 1999). The indications for dithiocarbamates in many disorders with diverse etiologies raise the possibility that this group of agents may have complex actions on cellular functions. The diverse roles of dithiocarbamates are further illustrated by their actions either as antioxidants (Schreck et al., 1992) or inhibitors of superoxide dismutase (Misra, 1979). Dithiocarbamates also exhibit metal-chelating properties (Thorn and Ludwig, 1962).

Pyrrolidine dithiocarbamate, a stable analogue of dithiocarbamate, has been extensively documented to inhibit the activation of nuclear factor-κB (NF-κB) (Schreck et al., 1992; Brennan and O'Neill, 1996; Kim et al., 1999a; Wu et al., 1996). NF-κB is a transcription factor that exerts important roles in immune function, development and cell death (Ghosh et al., 1998). Oxidative stress conferred by reactive oxygen species is a major mechanism of NF-κB activation (Schreck et al., 1992). The inhibitory effect of pyrrolidine dithiocarbamate has been attributed to its antioxidant properties (Schreck et al., 1992). However, pyrrolidine dithiocarbamate, which is considered as an antioxidant, has a biphasic effect on NF-κB activity. Pyrrolidine dithiocarbamate inhibits NF-κB activation at low (3 μM to 1 mM), but not high (300 μM to 10 mM), concentrations (Schreck et al., 1992; Galter et al., 1994; Brennan and O'Neill, 1996). Recently, we reported that pyrrolidine dithiocarbamate and diethyldithiocarbamate, another dithiocarbamate, act as zinc ionophores (Kim et al., 1999c). The inhibitory action of pyrrolidine dithiocarbamate on NF-κB activity is related to its ability to translocate extracellular Zn^{2+} to certain intracellular sites (Kim et al., 1999a,b). This novel mechanism of pyrrolidine dithiocar-

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bamate action on NF-κB activity is redox independent (Kim et al., submitted), suggesting that the antioxidant property of pyrrolidine dithiocarbamate may not be relevant to its inhibitory effect on NF-κB. In the present study, we add another piece of experimental evidence supporting the pivotal role of Zn^{2+} mobilization in the inhibition of NF-κB activity by pyrrolidine dithiocarbamate. NF-κB activity and intracellular Zn^{2+} levels are tightly coupled in the concentration-dependent biphasic effects of pyrrolidine dithiocarbamate and diethyldithiocarbamate in bovine cerebral endothelial cells.

2. Materials and methods

2.1. Materials

Pyrrolidine dithiocarbamate and diethyldithiocarbamate were purchased from Sigma (St. Louis, MO, USA). Double-stranded oligonucleotide containing consensus NF-κB binding sequence was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mag-fura-2 and *N*-(6-methoxy-8-quinolyl)-*para*-toluenesulfonamide (TSQ) were from Molecular Probes (Eugene, OR, USA).

2.2. Cell cultures

Bovine cerebral endothelial cells were prepared and characterized as previously described (Xu et al., 1997; Kim et al., 1999a,b,c). Endothelial cells of passages 4–15, which were uniformly positive for factor VIII and vimentin (> 95% endothelial cells) and which exhibited the characteristic bradykinin receptors, were grown to 70–80% confluence in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum before experiments.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method described in previous studies (Xu et al., 1997). For EMSA, the following oligonucleotide with the NF-κB consensus binding sequence was used: (5′-AGTTGAGGGGACTTTCCCAGGC-3′). Labeling of the oligonucleotide with γ-^{32}P-ATP and the EMSA method have been previously described (Xu et al., 1997; Kim et al., 1999a). Nuclear fractions of equal protein content (4–6 μg) were used in each assay. The reaction mixture in a final volume of 20 μl contained 2 μg polydeoxyinosinic–deoxycytidylic acid, 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol and 0.0175 pmol ^{32}P-labeled DNA probe. Reactions were started by the addition of nuclear extracts and they were allowed to proceed 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 an intensifying screen for 10–20 h at −70°C.

2.4. Intracellular $[Zn^{2+}]_i$ measurement

Changes in intracellular $[Zn^{2+}]_i$ in individual cells were measured according to the method used in previous studies (Kim et al., 1999b). Briefly, bovine cerebral endothelial cells were loaded with 3 μM of mag-fura-2 in a HEPES-buffered solution containing (in mM) NaCl 110, KCl 4.5, NaH_2PO_4 1, $MgSO_4$ 1, HEPES–Na 5, HEPES 5, $NaHCO_3$ 25 and D-glucose 10, for 30 min at 37°C. $[Zn^{2+}]_i$ was measured by real-time spectrofluorometry (Photon Technology International, Brunswick, NJ, USA) with excitation at 340 and 380 nm, and emission at 510 nm. The average Zn^{2+} signal from all bovine cerebral endothelial cells in each well was also measured using TSQ dye, as previously described (Kim et al., 1999c). Bovine cerebral endothelial cells grown in a 12-well plate were incubated for 3 h after the addition of pyrrolidine dithiocarbamate. TSQ (25 μM) was then added to the medium. After a 10-min incubation, cells were washed three times to remove extracellular TSQ. Endothelial cells were then lysed with 0.5% Triton X-100. After centrifugation, TSQ fluorescence in the supernatant containing 50 μg protein was measured in a spectrofluorophotometer (SLM instrument, Urbana, IL, USA) at excitation 365 nm and emission 480 nm.

3. Results

Bovine cerebral endothelial cells in 10% fetal calf serum showed a basal level of NF-κB activation (Fig. 1A,B). The NF-κB bands have been previously confirmed by competition and supershift assays (Kim et al., 1999a). Pyrrolidine dithiocarbamate and diethyldithiocarbamate were dissolved in distilled water in a concentration of 100 mM. In a control experiment, the same volume of distilled water was added instead of pyrrolidine dithiocarbamate or diethyldithiocarbamate. Pyrrolidine dithiocarbamate and

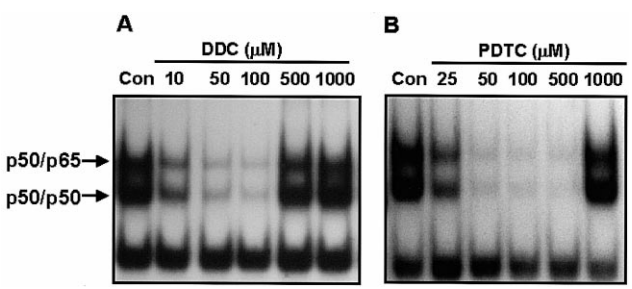

Fig. 1. Concentration-dependent effects of diethyldithiocarbamate and pyrrolidine dithiocarbamate on NF-κB binding activity. Bovine cerebral endothelial cells were treated with diethyldithiocarbamate (A) or pyrrolidine dithiocarbamate (B) at indicated concentrations for 3 h. Controls (Con) represent basal levels of NF-κB activity in bovine cerebral endothelial cells grown in DMEM containing 10% fetal calf serum.

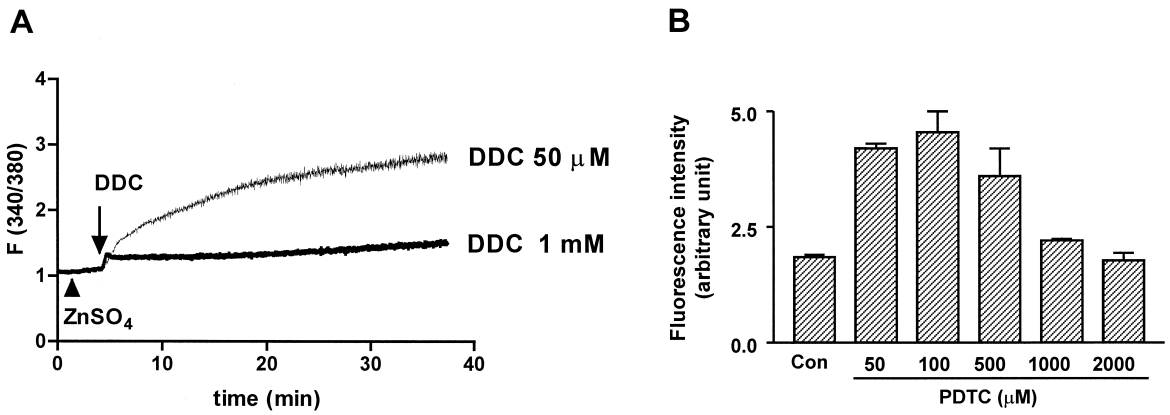

Fig. 2. Effects of diethyldithiocarbamate and pyrrolidine dithiocarbamate on intracellular Zn^{2+} levels of bovine cerebral endothelial cells. After 3 min perfusion with 1 μM of $ZnSO_4$ in HEPES-buffered solution, diethyldithiocarbamate was added to the perfusate for 37 min (A). Pyrrolidine dithiocarbamate was added to bovine cerebral endothelial cells in DMEM containing 10% fetal calf serum. The intracellular TSQ fluorescence was measured by spectrofluorophotometer at 3 h after treatment (B).

diethyldithiocarbamate inhibited NF-κB activation in a concentration-dependent manner at lower concentrations (Fig. 1A,B). However, both pyrrolidine dithiocarbamate and diethyldithiocarbamate failed to inhibit basal NF-κB activation at higher concentrations (500 μM and 1 mM for diethyldithiocarbamate and 1 mM for pyrrolidine dithiocarbamate) (Fig. 1A,B). Because we previously demonstrated that the presence of extracellular Zn^{2+} was required for pyrrolidine dithiocarbamate inhibition of NF-κB activation (Kim et al., 1999a), we measured the changes in intracellular Zn^{2+} levels after dithiocarbamate treatment in cerebral endothelial cells to determine whether the biphasic effects of dithiocarbamates are related to their action on Zn^{2+} transport. Mag-fura-2 was used to monitor the intracellular Zn^{2+} levels in cerebral endothelial cells treated with diethyldithiocarbamate, because the signal generated by mag-fura-2 proved to be Zn^{2+}-specific in a previous study (Kim et al., 1999b). At 50 μM, diethyldithiocarbamate inhibited NF-κB activation (Fig. 1A) and also increased the intracellular fluorescence intensity of mag-fura-2 in 37 min (Fig. 2A). However, at 1 mM, diethyldithiocarbamate failed to inhibit NF-κB activation (Fig. 1A) and was also without effect on the mag-fura-2 signal in the same time frame (Fig. 2A). Similar findings were noted in studies of the concentration-dependent effects of pyrrolidine dithiocarbamate on NF-κB (Fig. 1B), using TSQ as the fluorescent probe to monitor intracellular Zn^{2+} levels (Fig. 2B).

4. Discussion

We found that the dithiocarbamates pyrrolidine dithiocarbamate and diethyldithiocarbamate showed concentration-dependent biphasic effects in inhibiting NF-κB activation in cerebral endothelial cells, with high concentrations failing to cause inhibition. Similar concentration-dependent effects of pyrrolidine dithiocarbamate have been reported previously by others. Pyrrolidine dithiocarbamate failed to affect NF-κB activation by tumor necrosis factor-α (TNF-α) (Schreck et al., 1992) or interleukin-1 (Brennan and O'Neill, 1996) at high concentrations (300 μM to 10 mM), while it inhibited NF-κB activity at lower concentrations (3 μM to 1 mM). The mechanism of the unusual effects of pyrrolidine dithiocarbamate in this regard has not been addressed except by Galter et al. (1994). They suggested that high concentrations of pyrrolidine dithiocarbamate might saturate the monooxygenase which converts pyrrolidine dithiocarbamate to sulphenic acid. Sulphenic acid may then counteract the pyrrolidine dithiocarbamate effects on NF-κB. However, this hypothesis has not yet been confirmed experimentally.

We previously showed that extracellular Zn^{2+} was required for pyrrolidine dithiocarbamate inhibition of NF-κB activation (Kim et al., 1999a), and in this study, we found that the ability of pyrrolidine dithiocarbamate to inhibit NF-κB was always associated with its ability to increase intracellular Zn^{2+} levels (Figs. 1B and 2B). These findings indicate that at high concentrations (1 mM or higher) pyrrolidine dithiocarbamate is not capable of mobilizing Zn^{2+} into the intracellular sites to exert its inhibitory effect on NF-κB. The pyrrolidine dithiocarbamate studies were also carried out with another Zn^{2+}-specific dye, TSQ. This fluorescent probe offers the advantage of measuring average fluorescence intensity for the total population of bovine cerebral endothelial cells in each well (Kim et al., 1999c). The application of two probes to monitor the effects of two different dithiocarbamates on NF-κB inhibition and Zn^{2+} influx, with both resulting in a similar conclusion, strengthens the contention that Zn^{2+} indeed mediates the inhibitory effects of dithiocarbamates on NF-κB. The findings also raise the possibility of a new mechanism of action that enables a drug to show a concentration-dependent biphasic effect.

Similar concentration-dependent biphasic effects of pyrrolidine dithiocarbamate on cell death in relation to

mitogen-activated protein kinase activation and NF-κB activation have been reported for PC12 cells (Chung et al., 2000). In bovine cerebral endothelial cells, the same profile of dual effects on cell death was observed (data not shown). These findings indicate that the concentration-dependent biphasic effects of dithiocarbamates are not cell-type specific and are not unique to NF-κB regulation. This means that the various effects of dithiocarbamates are biphasic. We have observed that the biphasic dose–response curves of dithiocarbamates shifted to the left or right in different cell types (unpublished data). This information is also particularly important when dithiocarbamate compounds are used in clinical situations in which plasma drug levels may be affected by various factors, such as the route of administration, dosing schedule, drug absorption, distribution, and metabolism.

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