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Thiol Antioxidant Reversal of Pyrrolidine Dithiocarbamate-Induced Reciprocal Regulation of AP-1 and NF-κB

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Pyrrolidine dithiocarbamate (PDTC) has been shown to have unique reciprocal activities in activating AP-1 and inhibiting NF-κB, two oxidative stress-sensitive transcription factors. The opposing effects of PDTC on these two transcription factors have been attributed to its thiol antioxidant properties. In the present study, PDTC activation of AP-1, like its inhibition of NF-κB, in bovine cerebral endothelial cells (BCECs) was zinc-dependent, consistent with the contention that PDTC acts as a zinc ionophore and the apparent reciprocal actions of PDTC are mediated by zinc. Unlike PDTC, other thiols and non-thiol antioxidants did not activate AP-1 on their own. Thiol, but not non-thiol, antioxidants reversed PDTC actions on AP-1 and NF-κB. PDTC reduced the intracellular glutathione content, and depletion of the cellular glutathione store by buthionine sulfoximine (BSO) further augmented PDTC actions on AP-1 and NF-κB. N-acetylcysteine (NAC), a thiol antioxidant, reversed PDTC actions even after irreversible depletion of the cellular glutathione store by BSO. These findings together suggest that thiol antioxidant reversal of PDTC actions on AP-1 and NF-κB is independent of their established roles in scavenging oxygen free radicals or repleting the cellular glutathione content. The results in the present and earlier studies suggest that thiol antioxidants are likely to act as metal chelators that buffer zinc mediation of the reciprocal actions of PDTC on AP-1 and NF-κB.

Key words: Endothelial cells / Oxidative stress / Zinc.

Introduction

Dithiocarbamates are widely used in agriculture as insecticides and fungicides (Hayes, 1982) and in health care for the management of alcoholism and heavy metal poisoning (Thorn and Ludwig, 1962). Dithiocarbamates have been used in the treatment of acquired immune deficiency syndrome (Reisinger *et al.*, 1990) and cancer (Gandara *et al.*, 1991) and proposed as an adjunct regimen to enhance the therapeutic effect of other anticancer drugs (Chinery *et al.*, 1997; Bach *et al.*, 2000). Dithiocarbamates have also been applied to prevent atherosclerosis (Somers *et al.*, 2000). In addition to its various clinical applications, PDTC, a stable pyrrolidine analog of dithiocarbamate, has been used widely as a tool in studying the regulation of gene transcription. As a low molecular weight thiol compound, PDTC effects have been largely ascribed to its antioxidant property (Schreck *et al.*, 1992; Meyer *et al.*, 1993; Chinery *et al.*, 1997; Bach *et al.*, 2000; Somers *et al.*, 2000).

Activator protein-1 (AP-1) and nuclear factor κB (NF-κB) are key transcription factors involved in the transactivation of pro-inflammatory genes (Baud and Karin, 2001). These two transcription factors have also been implicated in signaling events involving apoptosis, proliferation, and other cellular functions (Cato and Wade, 1996). The activation of both AP-1 and NF-κB is tightly linked to the redox state (Sen and Packer, 1996). Despite its implied role as a thiol antioxidant, PDTC paradoxically exhibits opposing effects on AP-1 and NF-κB (Meyer *et al.*, 1993; Galter *et al.*, 1994; Schenk *et al.*, 1994). Since oxidative stress has been associated with the activation of both NF-κB and AP-1, the apparent conflicting effects of PDTC, a thiol antioxidant, on these two transcription factors demand further exploration. Thiol antioxidants are important regulators of AP-1 and NF-κB (Sen and Packer, 1996). However, unlike other thiol antioxidants, PDTC may have a mixed role on oxidative stress. A possible pro-oxidant action of PDTC has also been suggested, based on the observations of PDTC-induced pro-oxidative shift of cellular glutathione balance (Nobel *et al.*, 1995; Brennan and O'Neill, 1996; Haddad *et al.*, 2000; Kim *et al.*, 2001). Glutathione is a major determinant of the cellular redox state. Changes in the ratio of the oxidized form of glutathione, GSSG, and the reduced form, GSH, affects the transcriptional activities of redox-sensitive transcription factors such as AP-1 and NF-κB (Galter *et al.*, 1994; Brennan and O'Neill, 1996; Klatt *et al.*, 1999; Haddad *et al.*, 2000; Rahman, 2000). Another explanation for the pro-oxidant action of PDTC is its copper-ionophore action (Nobel *et al.*, 1995; Verhaegh *et al.*, 1997; Iseki *et al.*, 2000). Copper, as a redox-active metal, is also a major determinant of the redox state that involves the cellular thiol content.

The present study was undertaken to explore the differential role of PDTC *versus* other thiols antioxidants in an attempt to delineate the mechanism of the reciprocal actions of PDTC on AP-1 and NF-κB. In our previous studies, NF-κB inhibition by PDTC could be attributed to its zinc, but not copper, ionophore action (Kim *et al.*, 1999a,c); therfore, PDTC effects on AP-1 may be mediated by zinc. We also explore how thiol antioxidants may interact with zinc in the reversal of PDTC actions on AP-1 and NF-κB.

Results

Effects of Thiol and Non-Thiol Antioxidants on AP-1 and NF-κB Activities

PDTC increased the activity of AP-1 in BCECs in a time-dependent manner for up to 12 h, peaking at 6 h (Figure 1A). The specificity of this assay was confirmed by the ability of antibodies against c-Jun and c-Fos, the AP-1 subunits, to block AP-1 binding activity (Figure 1B). Since either anti-c-Jun or anti-c-Fos antibody could blocked AP-1 binding, AP-1 formed in BCECs in response to PDTC treatment is likely to be dominated by heterodimers consisting of both Fos and Jun family proteins. Nonspecific antibodies that do not react with AP-1 subunits could not abolish the signal. AP-1 binding was also blocked with a 100-fold excess of the unlabeled oligonucleotide, but not by the same amount of a mutant oligonucleotide (CA→TG substitution in the AP-1 binding motif; data not shown). Ca-EDTA, but not Zn-EDTA, completely blocked the effect of PDTC on AP-1 activation, suggesting that zinc is involved in PDTC-induced AP-1 activation (Figure 1C). The differential effects of Ca-EDTA and Zn-EDTA shown here are similar to those on PDTC-induced NF-κB inhibition (Kim *et al.*, 1999a). Thiol antioxidants and non-thiol antioxidants were compared with PDTC for their effects on AP-1 activity in BCECs (Figure 2A). No activation of AP-1 was noted in cells treated with either a thiol or non-thiol antioxidant alone (Figure 2A). This finding suggests that PDTC action is different from other antioxidants. We have previously shown that NAC and dihydrolipoic acid reverse the inhibitory effects of PDTC on NF-κB activity in a dose-dependent manner (Kim *et al*, 1999a). Thiols such as NAC and L-cys-

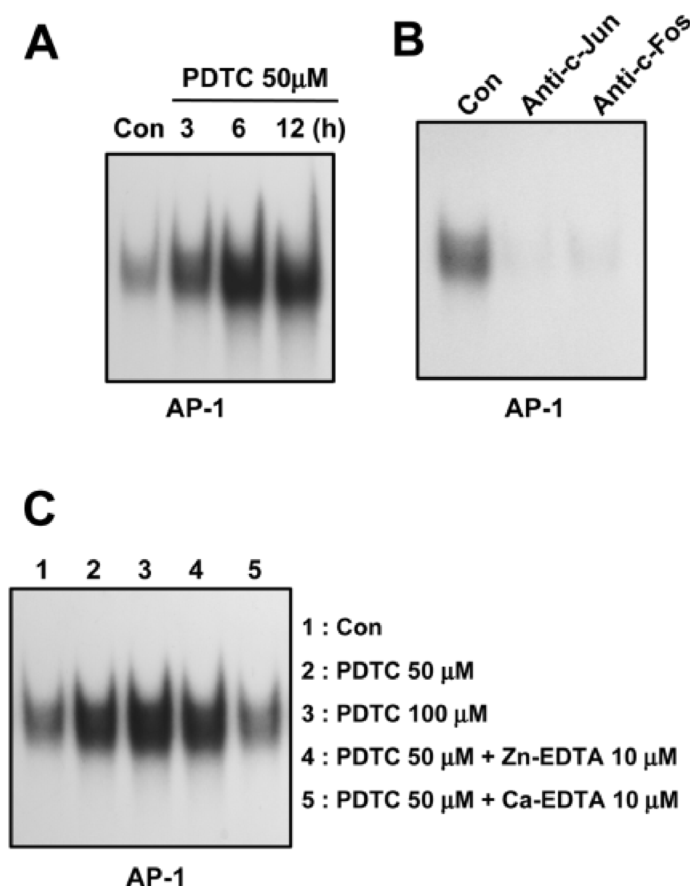

Fig. 1 Effect of PDTC on AP-1 Activity.
(A) BCECs were treated with PDTC (50 μM) for the indicated time periods. Control (Con) represents basal AP-1 activity of BCECs grown in DMEM containing 10% FBS. (B) Nuclear extracts were incubated with anti-c-Jun or anti-c-Fos IgG for 30 min before radio-labeled AP-1 consensus oligonucleotide was added to the binding tube. (C) Ca-EDTA or Zn-EDTA was added with PDTC to BCECs grown in DMEM containing 10% FBS.

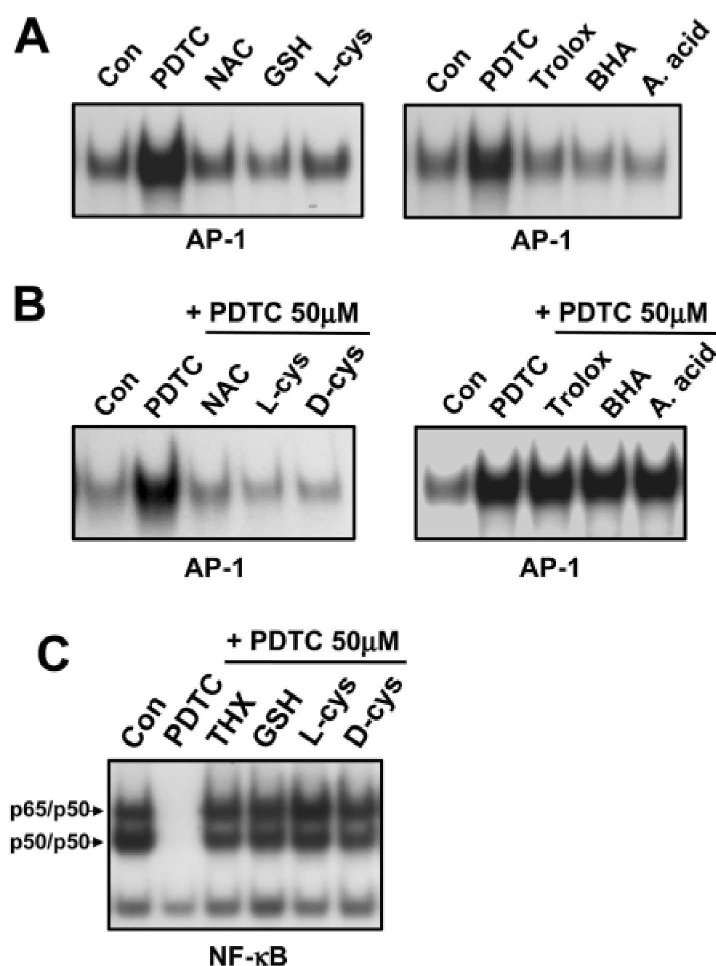

Fig. 2 Effects of Thiols and Non-Thiol Antioxidants on AP-1 and NF-κB Binding Activities in the Absence or Presence of PDTC. (A) Antioxidant effects in the absence of PDTC. BCECs were treated with thiols, NAC (1 mM), GSH (1 mM) or L-cysteine ('L-cys', 1 mM) (left panel) or non-thiol antioxidant, trolox (1 mM), BHA (1 mM) or ascorbic acid ('A. acid', 1 mM) (right panel) for 4 h before EMSA for AP-1 was performed. PDTC was not added to antioxidants treatment in these experiments. A parallel experiment with PDTC alone was performed for comparison (lane 2 of each panel). (B) Antioxidant effects in the presence of PDTC. BCECs were treated with thiols, NAC (1 mM), L-cysteine ('L-cys', 1 mM) or D-cysteine ('D-cys', 1 mM) (left panel) for 4 h or the non-thiol antioxidants trolox (1 mM), BHA (1 mM) or ascorbic acid ('A. acid', 1 mM) (right panel) before EMSA for AP-1 was conducted. PDTC (50 μM) was added with the antioxidants in these experiments. (C) BCECs were treated with thioredoxin (THX; 10 μg/ml), GSH (1 mM), L-cys (1 mM) or D-cys (1 mM) plus PDTC (50 μM) for the investigation of change of NF-κB activity. In all cases, BCECs were treated with the agent(s) for 4 h.

teine prevented the activation of AP-1 by PDTC in the present study (Figure 2B, left), suggesting that this thiol-reversible event applies to AP-1 as well as NF-κB. NAC may exert dual actions: serving as an oxygen free radical scavenger or an exogenous source of glutathione. L-cysteine is the rate-limiting substrate for the synthesis of glutathione. However, extracellular D-cysteine that cannot replenish intracellular glutathione store also reversed PDTC effects on AP-1 (Figure 2B, left). For PDTC-induced NF-κB inhibition, thiols that do not increase the intracellular glutathione content, such as thioredoxin and D-cysteine, were also capable of reversing PDTC action on NF-κB activation (Figure 2C). Thus, it is unlikely that the effect of NAC on PDTC actions on AP-1 and NF-κB is related to an increase in the cellular glutathione content. To further explore whether a free radical generating activity of PDTC is involved in its reciprocal actions on AP-1 and NF-κB, non-thiol antioxidants were also tested. Trolox, ascorbic acid and butylated hydroxyanisole (BHA) failed to reverse PDTC activation of AP-1 in BCECs (Figure 2B, right). Similar results on the inability of non-thiol antioxidants to reverse PDTC inhibition of NF-κB have been obtained earlier (Kim *et al*, 1999a). The finding that non-thiol antioxidants could not reverse the PDTC actions on AP-1 and NF-κB activities excludes the possibility that free radicals are involved in the reciprocal regulation of AP-1 and NF-κB by PDTC.

Effect of PDTC on Cellular GSH Content

PDTC may serve as a pro-oxidant because of its reduction of the cellular GSH content (Nobel *et al.*, 1995; Burkitt *et al.*, 1998). Therefore, the cellular content of the reduced form of glutathione (GSH) was measured after

PDTC treatment using the glutathione S-transferase (GST)-based glutathione assay (Arttamangkul, 1999). PDTC reduced the GSH level in a concentration-dependent fashion (Figure 3). NAC restored or even increased the GSH content in the presence of PDTC (Figure 4A). Other thiols including GSH, L-cysteine and D-cysteine also reversed the reduction of GSH by PDTC (Figure 4B). However, non-thiol antioxidants including trolox, BHA, ascorbic acid and catechin failed to reverse PDTC reduction of the cellular GSH content (Figure 4C), suggesting that GSH depletion was unlikely due to its consumption in free radical scavenging. Twenty μM of EDTA, which effectively reversed PDTC inhibition of NF-κB in a previous study (Kim *et al*, 1999a), completely prevented the decrease of GSH levels caused by PDTC (Figure 4B; first line from the right). This observation suggests that zinc influx caused by PDTC treatment is likely involved in the reduction of the intracellular GSH content.

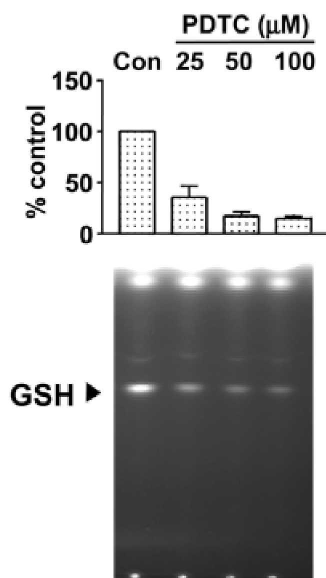

Fig. 3 Effect of PDTC on GSH Content.
BCECs were treated with PDTC at indicated concentrations for 4 h. Intensities of bands corresponding to GSH were quantified by densitometry and are presented as percentages of control value. Value of each bar represents the mean ± SEM of GSH contents obtained from 4 independent experiments. The control (Con) represents the GSH content of parallel BCEC plates without PDTC treatment.

Effect of Glutathione-Depleting Agent on the Modulation of AP-1 and NF-κB Activities

We also explored the PDTC effects in the regulation of AP-1 and NF-κB activities upon glutathione depletion to further investigate whether the cellular glutathione content is a determinant of PDTC actions. To deplete the cellular glutathione store, BCECs were treated with buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthase. Near complete depletion of the cellular glutathione pool was achieved as early as 10 h after BSO treatment (Figure 5A). The binding activity of AP-1 (Figure 5B, upper panel) or NF-κB (middle panel) was not altered by BSO at a concentration (1 mM) that completely depleted GSH (Figure 5B, lower panel).

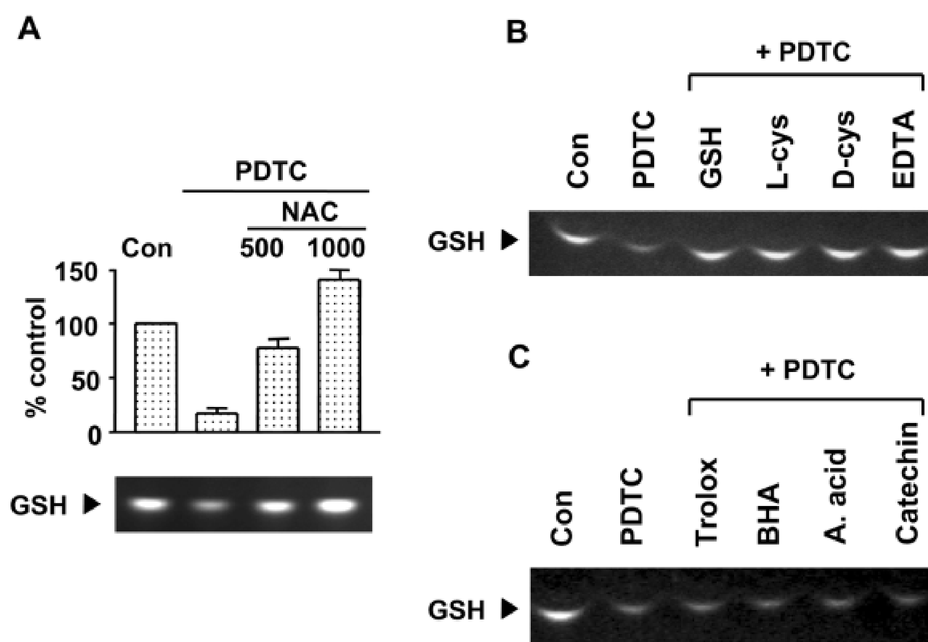

Fig. 4 Effects of Thiol and Non-Thiol Antioxidants on PDTC Reduction of GSH Content.
(A) BCECs were treated with PDTC (50 μM) with or without NAC (500 or 1000 μM). The value of each bar represents the mean ± SEM of GSH contents obtained from four independent experiments. The control (Con) represents the GSH content of parallel BCEC plates without PDTC treatment. (B) BCECs were treated with PDTC (50 μM) with and without GSH (1 mM), L-cysteine ('L-cys', 1 mM), D-cysteine ('D-cys', 1 mM) and EDTA (20 μM). (C) BCECs were treated PDTC (50 μM) with or without trolox (1 mM), BHA (1 mM), ascorbic acid (1 mM) or catechin (1 mM). In all cases, GSH content was determined 4 h after treatment.

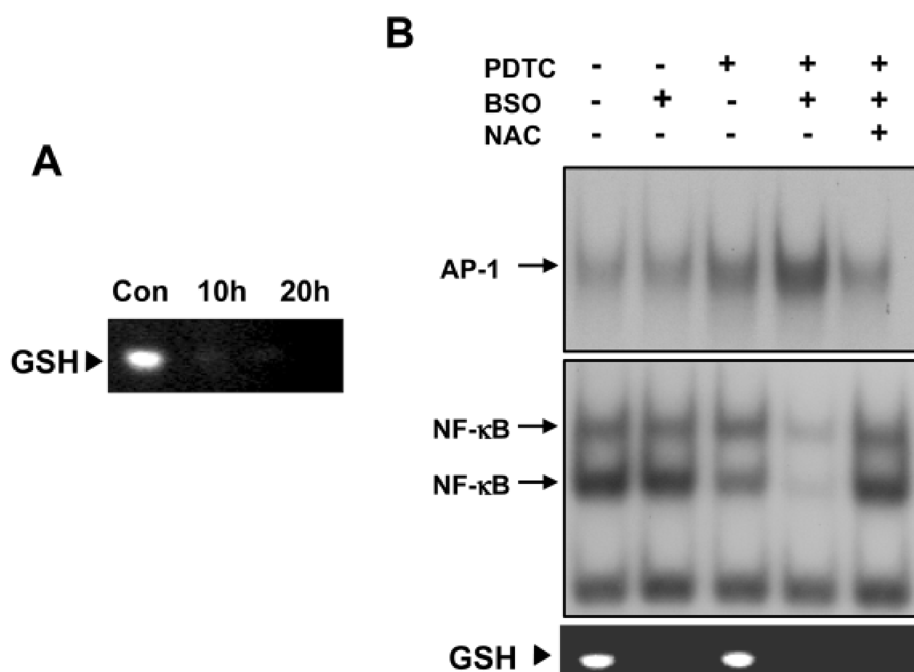

Fig. 5 Effects of Glutathione Depletion on PDTC and Thiol Modulation of AP-1 and NF-κB Activities.
(A) BSO (1 mM) was added for 10 h and 20 h. The control (Con) represents the GSH content of parallel BCEC plates without BSO. (B) After 20 h preincubation in BSO (1 mM), PDTC (25 μM) or NAC (1 mM) were added separately or in combination. Changes in AP-1 activity (upper panel), NF-κB activity (middle panel) and GSH content (lower panel) were measured at 4 h after treatment with PDTC or NAC.

PDTC, at a concentration of 25 μM, partially activated AP-1 (Figure 5B, upper panel) and suppressed NF-κB (Figure 5B, middle panel) activity. AP-1 activation was greatly enhanced and NF-κB activity completed abolished at the same concentration of PDTC (25 μM) where GSH was depleted by BSO treatment (Figure 5B, lower panel). These combined effects of BSO and PDTC on AP-1 (Figure 5B, upper panel) and NF-κB (Figure 5B, middle panel) could be fully reversed by NAC. Consistent with the known effect of BSO to irreversibly inhibit γ-glutamylcysteine synthase, addition of thiol antioxidants such as NAC after 20 h treatment of BSO could not restore the cellular GSH level (Figure 5B, lower panel). This confirms that reversal of PDTC action by NAC is not related to its well known role as a precursor for glutathione synthesis in a number of oxidative stress paradigms. The results presented here suggest that the thiol antioxidant, such as NAC, counteract PDTC actions in a manner which is independent of their expected roles in repleting the cellular glutathione content. An alternative mechanism of action for the thiol effect on PDTC remains to be identified.

Effects of Zinc on AP-1 and NF-κB Activities and the GSH Content in BCECs

We have previously shown that PDTC acts as a zinc ionophore (Kim *et al*, 1999b), and that its inhibitory effect on NF-κB activity is mediated by an increase in intracellular zinc levels (Kim *et al*, 1999a). In the present study, we also examined the effects of zinc itself on AP-1 and NF-κB activity and the cellular GSH content. Similar to PDTC (Figure 2), $ZnSO_4$ increased AP-1 (Figure 6A, upper panel) and decreased NF-κB (Figure 6A, middle panel) activities. $ZnSO_4$ also decreased GSH content in a dose-dependent manner (Figure 6A, lower panel). NAC reversed $ZnSO_4$ effects on AP-1 and NF-κB activities and the GSH content (Figure 6B) while trolox did not (Figure 6C). These results suggest that differential effects of thiols and non-thiol antioxidants are related to their interaction with zinc ions that were mobilized by PDTC.

Discussion

Both AP-1 and NF-κB are oxidative stress-sensitive transcription factors (Meyer *et al.*, 1993). The reciprocal action of PDTC in activating AP-1 and suppressing NF-κB activity is unique and raises the possibility that the mechanism of these PDTC actions may be related to its known pro-oxidant effects by acting as copper ionophore (Nobel *et al.*, 1995; Verhaegh *et al.*, 1997; Iseki *et al.*, 2000). However, in the present study we showed that thiol antioxidants inhibited the effect of PDTC on AP-1 and NF-κB, while non-thiol antioxidants were without effect. These findings make it unlikely that the reciprocal actions of PDTC on AP-1 and NF-κB can be ascribed to its pro-oxidant effect.

The cellular glutathione store is a major determinant of the redox state. Being sensitive to oxidative stress, it is not surprising to note that AP-1 and NF-κB activities are affected by the cellular glutathione levels (Staal *et al*, 1990; Galter *et al*, 1994; Mihm *et al*, 1995). Perturbation

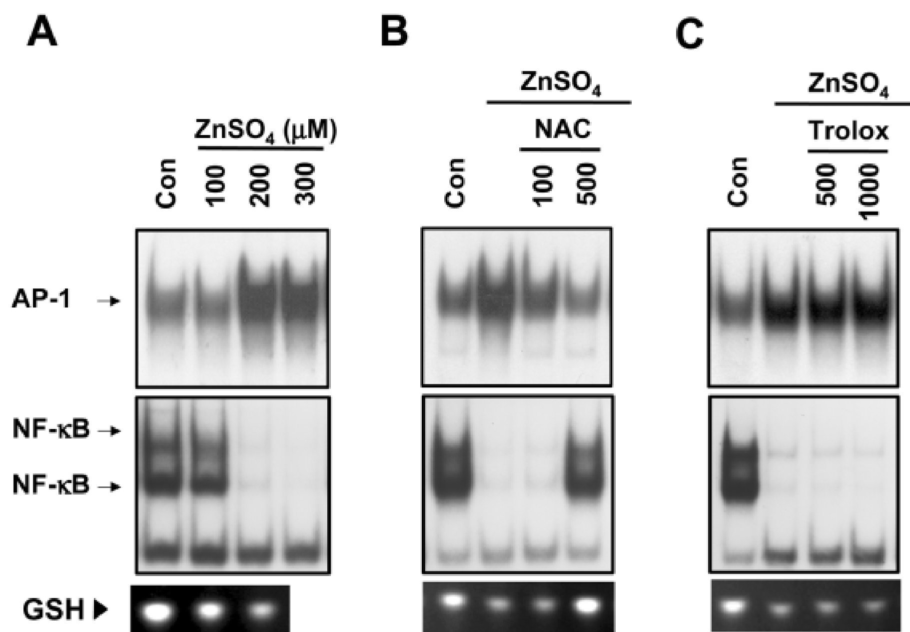

Fig. 6 Effects of Zinc Ions on AP-1 and NF-κB Activities and GSH Content.
(A) BCECs were treated with $ZnSO_4$ at the concentrations indicated. AP-1 activity (upper panel), NF-κB activity (middle panel) or GSH content (lower panel) was measured at 4 h after zinc treatment. (B) $ZnSO_4$ (200 μM) was added with NAC (1 mM) for 4 h. (C) $ZnSO_4$ (200 μM) was added with trolox (1 mM) for 4 h.

of cellular glutathione status has been shown to have profound effects on AP-1 (Klatt *et al.*, 1999; Rahman, 2000) and NF-κB activity (Galter *et al.*, 1994; Brennan and O'Neill, 1996; Haddad *et al.*, 2000). We, therefore, explored whether PDTC-induced reduction of the cellular GSH store could be the underlying mechanism of PDTC action in BCECs. PDTC decreased the GSH content in BCECs in a concentration-dependent manner. Since thiol antioxidants may replenish the GSH pool, it seemed that their reversal of the PDTC actions on AP-1 and NF-κB could be attributed to the repletion of the cellular glutathione store. This contention, however, is not supported by the results derived from experiments using BSO. The experiments were designed to test the effects of PDTC and thiol antioxidants in a glutathione-depleted state. Similar to the report by Sato *et al.* (1995), BSO pretreatment for 10 h or longer reduced GSH below detectable levels in BCECs. NAC, a precursor for glutathione synthesis, failed to restore the cellular GSH content in the presence of BSO, an irreversible inhibitor of γ-glutamylcysteine synthase. However, NAC was effective in reversing PDTC activation of AP-1 and inhibition of NF-κB. These findings suggest that restoring the cellular GSH content by thiol antioxidant is not required for the reversal of PDTC actions on AP-1 and NF-κB. This contention is supported by another set of data showing that extracellular thiols, whether they are glutathione precursors or not, are capable of reversing PDTC actions on AP-1 and NF-κB. However, a possible regulatory role of the cellular glutathione content on PDTC actions remains a possibility. Depletion of cellular glutathione store appears to enhance PDTC actions, resulting in the activation of AP-1 and inhibition of NF-κB to a larger extent.

If the ability of thiol antioxidants to reverse PDTC effects on AP-1 and NF-κB is independent of their established roles in scavenging oxygen free radicals or repleting the cellular glutathione store, an alternative mechanism of action remains to be delineated. Results from the present and previous studies (Kim *et al.*, 1999a,b) raise the possibility that thiol antioxidants may act as zinc chelators. We have demonstrated here that zinc mediates the reciprocal action of PDTC on AP-1 and NF-κB. This contention is based on the following observations: (i) PDTC treatment enhances zinc influx into BCECs (Kim *et al.*, 1999b); (ii) activation of AP-1 and inhibition of NF-κB could be blocked by chelation of zinc with Ca^{2+}-EDTA, but not Zn^{2+}-EDTA; (iii) $ZnSO_4$ mimicked PDTC action in activating AP-1 and inhibiting NF-κB; (iv) $ZnSO_4$ depleted the cellular GSH store in a manner that could be reversed by thiol, but not non-thiol antioxidants, and (v) thiol, but not non-thiol, antioxidants were capable of reversing zinc activation of AP-1 and inhibition of NF-κB.

Thiol antioxidants, such as NAC, may antagonize PDTC actions on AP-1 and NF-κB not by directly interacting with PDTC but by providing a buffering capacity to neutralize a rising intracellular zinc concentration. We have observed that overexpression of metallothionein, a major metal-binding protein in cells, decreased the susceptibility to PDTC activation of AP-1 and inhibition of NF-κB in HeLa cells (Kim *et al.*, unpublished data). Sulfhydryl groups of endogenous GSH and thiols have a high affinity for metal ions (Wang and Ballatori, 1998).

Glutathione has been used in the treatment of metal poisoning. Following metal exposure, glutathione is the first line of defense because it binds metal faster than metallothionein (Singhal *et al*, 1987). These findings raise the possibility that the mechanism of action of thiol antioxidants on reversing the reciprocal actions of PDTC on AP-1 and NF-κB is probably derived from their metal-chelating property.

In summary, results from the present and earlier studies (Kim *et al.*, 1999a;b) suggest a potential role for zinc in the regulation of transcription factors, such as AP-1 and NF-κB. PDTC activation of AP-1 and inhibition of NF-κB in BCECs can be attributed to its zinc ionophore action. The ability of thiols to counteract PDTC effects is likely related to their zinc-buffering action but not to scavenging free radicals or repleting the cellular glutathione store.

Materials and Methods

Materials

The chemicals and supplies used in the present study were of the highest grade available with the sources indicated at the end of each list: pyrrolidine dithiocarbamate (PDTC), N-acetyl-L-cysteine (NAC), L-cysteine, D-cysteine, L-buthionine-(S,R)-sulfoximine (BSO), reduced glutathione (GSH), ascorbic acid, thioredoxin, butylated hydroxyanisole (BHA) and ethylenediaminetetraacetic acid (EDTA): Sigma, St. Louis, USA; Trolox: Aldrich Chemicals, Milwaukee, USA; (+)-catechin: Calbiochem, La Jolla, USA; double stranded oligonucleotides containing the NF-κB binding sequence and AP-1 binding sequence: Promega, Madison,USA; Poly(dIdC): Pharmacia, Uppsala, Sweden; 5-(pentafluorobenzoylamino) fluorescein (PFB-F): Molecular Probes, Eugene, USA; Cell culture products: Gibco-BRL Life Technologies, Rockville, USA.

Cell Culture

Bovine cerebral endothelial cells (BCECs) were prepared and characterized as previously described (Kim *et al*, 1999b). BCECs 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 DMEM containing 10% FBS prior to use in experiments (Xu *et al.*, 1998).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared according to the method described previously (Kim *et al*, 1999a). For EMSA, the oligonucleotides with the NF-κBs consensus binding sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') and AP-1 consensus binding sequence (5'-CGCTTGATGACTCAGCCGGAA-3') were used. 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 poly(dIdC), 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 0.0175 pmol [γ-^{32}P]-labeled DNA probe. Reactions were started by the addition of nuclear extracts and were incubated for 30 min at room temperature. Samples were loaded on a 4% non-denaturing polyacrylamide gel and electrophoresed for 2 h at 180V. The dried gel was exposed to Kodak XR5 film on an intensifying screen for 10 – 20 h at – 70 °C.

Measurement of Reduced Glutathione (GSH)

GSH was measured according to the technical bulletin for glutathione assay system (Molecular Probes, Inc.). This assay is based on the selectivity of glutathione S-transferase (GST) for GSH (Arttamangkul *et al.*, 1999). We used this assay to overcome possible non-specific detection of intracellular sulfhydryl groups other than GSH by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the relatively time-consuming processes of HPLC detection. Cells were washed twice with PBS and lysed with lysis buffer containing 1% Triton X-100. After centrifugation at 20 000 *g* for 15 min at 4 °C, GSH in the supernatant was derivatized by adding fluorescent GST substrate, 5-(pentafluorobenzoylamino) fluorescein (PFB-F) and GST for 30 min at 37 °C. After the reaction, the mixture was spotted on a thin layer chromatography (TLC) plate. Spots of reaction mixture were separated for 4 h in 60% butanol/20% methanol/20% distilled water TLC solvent. After drying, fluorescence band was obtained in Fuji LAS1000 luminescent image analyzer (Tokyo, Japan). Bands were analyzed quantitatively in an MCID imaging system (Imaging Research, Ontario, Canada).

Acknowledgments

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