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Pyrrolidine Dithiocarbamate Inhibits TNF-α-Dependent Activation of NF-κB by Increasing Intracellular Copper Level in Human Aortic Smooth Muscle Cells

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Pyrrolidine dithiocarbamate (PDTC) is a metal-chelating compound that acts as antioxidant or pro-oxidant and is widely used to study redox regulation of cell function. In the present study, we investigated effects of PDTC and another antioxidant, *N*-acetyl-L-cysteine (NAC), on TNF-α-dependent activation of NF-κB in human aortic smooth muscle cells (HASMC). Treatment of the cells with TNF-α induced the activation of p65/p50 heterodimer NF-κB and increased the mRNA levels of monocyte chemoattractant protein (MCP)-1. Pretreatment with PDTC markedly suppressed the NF-κB activation and expression of MCP-1 by inhibiting IκB-α degradation. In contrast, NAC had no effect. PDTC concomitantly increased the intracellular levels of copper, and bathocuproinedisulfonic acid, a non-cell-permeable chelator of Cu^{1+}, inhibited the PDTC-induced increase in intracellular copper level and reversed the PDTC effects on IκB-α, NF-κB, and MCP-1. These results indicate that TNF-α-dependent expression of MCP-1 in HASMC is tightly regulated by NF-κB and that intracellular copper level is crucial for the TNF-α-dependent activation of NF-κB in HASMC.

Key Words: **NF-κB; TNF-α; IκB-α; pyrrolidine dithiocarbamate; *N*-acetyl-L-cysteine; human aortic smooth muscle cells; monocyte chemoattractant protein (MCP)-1; copper; bathocuproinedisulfonic acid.**

Transcription factor nuclear factor-kappa B (NF-κB) regulates a wide variety of cellular genes, including cytokines, cytokine receptors, adhesion molecules, and antiapoptotic proteins (1–3). NF-κB is a dimer consisting of the Rel family proteins such as p65 (RelA), p50, p52, c-Rel and RelB. In unstimulated cells, NF-κB is sequestered in the cytoplasm bound to an inhibitory protein IκB which includes several isoforms such as IκB-α (4) and IκB-β (5). A number of external stimuli such as tumor necrosis factor (TNF)-α induce the activation of NF-κB by promoting the phosphorylation and degradation of IκB. After the release from IκB, NF-κB translocates into the nucleus, binds to the regulatory element of the target genes, and controls their transcription.

In vascular smooth muscle cells, it has been shown that TNF-α, angiotensin II and platelet-derived growth factor induce the activation of NF-κB and the expression of monocyte chemoattractant protein-1 (MCP-1) (6, 7). MCP-1 is the main chemotactic factor involved in the migration of monocytes into vessel wall, which is a critical event leading to the development of atherosclerosis. However, little is known about the intracellular signaling pathway leading to the activation of NF-κB in the vascular smooth muscle cells.

Recently, we and others have shown, in various cell types, that the signaling pathway elicited by TNF-α involves generation of reactive oxygen species (ROS), because the NF-κB activation was inhibited by antioxidants such as *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) (8–11).

In the present study, we investigated effects of antioxidants, NAC and PDTC, on TNF-α-dependent activation of NF-κB and on expression of MCP-1 in human aortic smooth muscle cells (HASMC). It was shown that the NF-κB activation by TNF-α in HASMC is inhibited by PDTC through its action as a copper transporter, but not as an antioxidant.

MATERIALS AND METHODS

Cell culture. Primary human aortic smooth muscle cells (HASMC; Kurabo, Osaka, Japan) were grown in HuMedia-SB2 medium supplemented with 5% fetal bovine serum and rat EGF (0.5 ng/ml), rat FGF-B (2 ng/ml), insulin (5 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml). This medium contains 2.0 ng/ml of $CuSO_4 5H_2O$. The nearly confluent cells in 21-cm^2 culture dishes were

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preincubated with 80 μM pyrrolidine dithiocarbamate (PDTC, Sigma Chemical Co., St. Louis, MO) or 20 mM *N*-acetyl-L-cysteine (NAC, Sigma) alone or together with 0.8 mM bathocuproinedisulfonic acid (BCS, Sigma) for 1 h and then incubated with 100 U/ml of recombinant human TNF-α (2.5×10^3 U/μg, Asahi Chemical Industry, Tokyo, Japan) for 30 min for electrophoretic mobility shift assay and Western blot analysis or for 6 h for Northern blot analysis.

Electrophoretic mobility shift assay (EMSA). Procedure for preparation of nuclear and cytosolic extracts was described previously (10). Protein concentration was determined by a microassay kit (Bio-Rad, Hercules, CA) using the bovine serum albumin as a standard. Nuclear extracts (10 μg of protein) were used for EMSA. The detailed procedure for EMSA was also described previously (12). The κBwt oligonucleotides (5′-TCGAGCAGAGGGGACTTTCCGAGAG-3′ and 5′-TCGACTCTCGGAAAGTCCCCTCTGC-3′) containing a canonical NF-κB binding site (underlined), and AP-1 oligonucleotides (5′-CGCTTGATGAGTCAGCCGGAA-3′ and 5′-TTCCGGCTGACTCATCAAGCG-3′) containing a AP-1 binding site (underlined) were annealed, labeled by Klenow enzyme in the presence of [^{32}P]dCTP, and used as probes. To identify the NF-κB subunits, supershift analysis was performed using antibodies directed against p50, p52, p65, c-Rel, and Rel B (Santa Cruz Biotechnology, Santa Cruz, CA). They were added to the binding reaction mixture before addition of the labeled probe and incubated for 1 h at 4°C. Samples were analyzed by 4% polyacrylamide gel electrophoresis. The gels were then dried and autoradiographed.

Northern blot analysis. Total RNA was extracted from HASMC by the method of Chomczynski and Sacchi (13). Northern blot and hybridization were carried out as described previously (14). In brief, total RNA (15 μg) was fractionated in 0.8% agarose gels, and then was transferred onto a nylon membrane (Gene Screen Plus; New England Nuclear, Boston, MA). Heat-denatured cDNA probes were labeled with [^{32}P]dCTP using random primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Preparation of cDNAs for monocyte chemoattractant protein (MCP)-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were described previously (9, 15). After hybridization and wash, the membrane was subjected to image analyzer (BAS 2000; Fuji Photo Film Co. Ltd., Tokyo, Japan) to quantify radioactivities of bands. The membrane was then autoradiographed.

Western blot analysis. The detailed procedure was described previously (16). In brief, cytosolic extracts (40 μg of protein) and standards for molecular weight were fractionated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, and electroblotted onto a Hybond-C super membrane (Amersham Corp., Arlington Heights, IL). A part of membrane with molecular weight marker was separated and stained with Coomassie Brilliant Blue R (Sigma). The remaining membrane was soaked overnight in a blocking buffer [phosphate-buffered saline (PBS) containing 5% low-fat dried milk powder (Snowbrand Milk Products Co. Ltd., Tokyo, Japan)]. Then, the membrane was incubated for 1 h with an antibody against either IκB-α (Santa Cruz) diluted at 1:1000 with the blocking buffer. After washing three times with PBS containing 0.1% Tween-20, the membrane was incubated for 1 h with anti-rabbit-IgG goat IgG conjugated with alkaline phosphatase (Zymed, San Francisco, CA) diluted at 1:2000 with PBS containing 0.1% Tween 20. After washing three times, it was incubated in a color development solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP Tablet, Boehringer Mannheim).

Determination of intracellular concentration of copper. HASMC were preincubated with 80 μM PDTC or 20 mM NAC alone or together with 0.8 mM BCS for 1 h and then incubated with TNF-α for 1 h. After the cells were washed several times, the whole cell extracts were prepared as described previously (12). Copper contents were measured using a Hitachi Z6100 atomic absorption spectrophotometer (Hitachi), and corrected by protein contents.

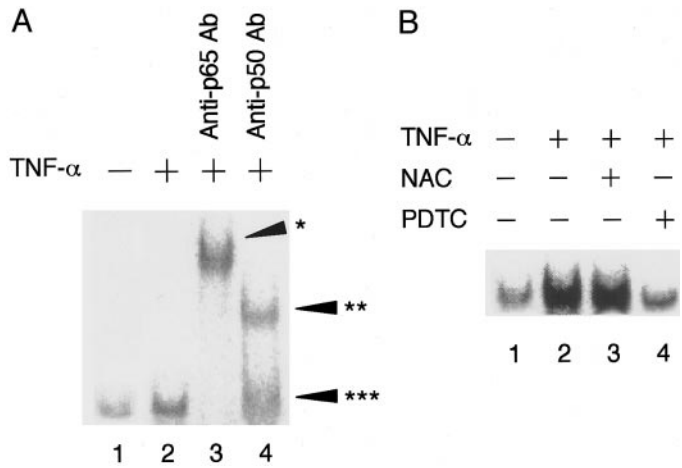

FIG. 1. Effects of PDTC and NAC on TNF-α-dependent activation of NF-κB in HASMC. (A) HASMC cells were incubated with 100 U/ml TNF-α for 30 min. Nuclear extracts were subjected to EMSA using κBwt oligonucleotide as a probe, and supershift analysis was performed using anti-p65 and anti-p50 antibodies. (B) The cells were preincubated with 20 mM NAC or 80 μM PDTC for 1 h, and then incubated with TNF-α. The nuclear extracts were used for EMSA.

Statistical analysis. Statistical analysis was carried out by using one-way ANOVA followed by Fisher's protected least significant difference (PLSD) analysis. The *P* value less than 0.05 is considered significant.

RESULTS

Primary human aortic smooth muscle cells (HASMC) were treated with or without TNF-α for 30 min, and the activation of NF-κB was studied by EMSA using the nuclear extracts (Fig. 1A). Slight DNA-binding activity was observed before TNF-α treatment (lane 1), indicating constitutive, low level of activation of NF-κB in the cells. Treatment of the cells with TNF-α markedly induced its activation (lane 2).

To characterize the subunit of NF-κB, supershift analysis was performed. Anti-p65 antibody supershifted the NF-κB/DNA complex (single asterisk in lane 3). Anti-p50 antibody also supershifted the complex (double asterisk in lane 4), but some complex was only slightly shifted from its original position by the antibody (triple asterisk in lane 4). The antibodies directed against p52, c-Rel and Rel B, and preimmune rabbit serum did not affect the mobility of the complex (data not shown), indicating that the complex consists of p65/p50 heterodimer NF-κB.

We then examined effect of antioxidants on the activation of NF-κB by TNF-α. HASMC were treated with 20 mM NAC or 80 μM PDTC, because these doses were shown to be sufficient for the inhibition of TNF-α-dependent activation of NF-κB (9–11). Interestingly, as shown in Fig. 1B in lane 3, preincubation of 20 mM NAC had no effect on the TNF-α-dependent activation of NF-κB in HASMC. In contrast, preincubation of 80

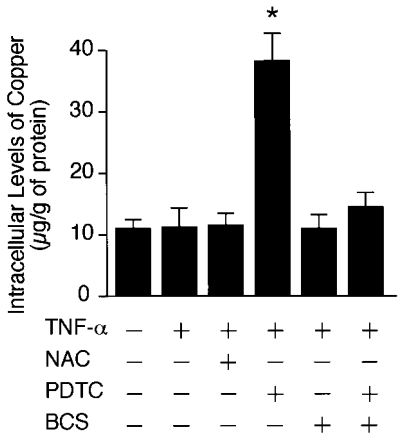

FIG. 2. Effects of TNF-α, NAC, PDTC, and BCS on intracellular levels of copper. The cells were pretreated with 20 mM NAC or 80 μM PDTC alone or in combination with 0.8 mM BCS, followed by 1-h incubation of 100 U/ml TNF-α. Whole cell lysates were used for determination of copper contents. Experiment was performed in quadruplicate flasks. Data were corrected by total protein contents, and expressed as μg/g of protein (mean ± SD, $n = 4$). *$P < 0.05$ vs the value in the presence of TNF-α alone.

μM PDTC markedly suppressed the activation (lane 4). This observation is against a hypothesis that generation of ROS is involved in the TNF-α-dependent signaling cascade leading to the NF-κB activation in HASMC.

It has been recently reported that, in addition to ROS-scavenging action, PDTC can bind and transport external copper ions into cells (17, 18). We thus measured the intracellular copper levels in HASMC which were treated with PDTC. We also studied the effect of bathocuproinedisulfonic acid (BCS) that is a non-cell-permeable chelator of Cu^{1+} (19) and thereby can inhibit PDTC-dependent transport of copper. As shown in Fig. 2, treatment of HASMC with 80 μM PDTC resulted in a marked increase in the intracellular concentration of copper. In contrast, TNF-α and NAC had no effect on the level. Pretreatment with 0.8 mM BCS completely prevented the PDTC-dependent increase in the intracellular copper level. BCS alone did not alter the level.

We next examined whether this elevation of intracellular copper content affects the TNF-α-dependent activation of NF-κB. As shown in Fig. 3, when the cells were treated with PDTC together with 0.8 mM BCS, the suppression of NF-κB activation by PDTC (lane 4) was almost completely abrogated (lane 6). BCS alone did not affect the TNF-α-dependent activation of NF-κB (lane 5). In contrast, AP-1 binding activity was not affected by TNF-α, PDTC or BCS, indicating specific effect of PDTC and BCS on NF-κB activation.

Effects of TNF-α, PDTC, NAC and BCS on expression of MCP-1 mRNA in HASMC are shown in Fig. 4. TNF-α markedly increased the MCP-1 mRNA levels fourfold. NAC did not affect the TNF-α-dependent induction of MCP-1 mRNA. However, PDTC completely

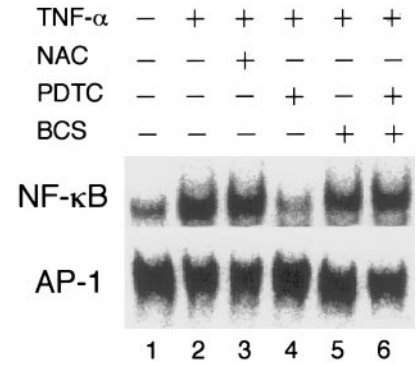

FIG. 3. Effects of PDTC and BCS on TNF-α-dependent activation of NF-κB. The cells were pretreated in the same way as described in the legend to Fig. 2, followed by 30-min incubation of 100 U/ml TNF-α. Nuclear extracts were subjected to EMSA using κBwt or AP-1 oligonucleotides as probes.

suppressed the induction, and this PDTC effect was reversed by BCS. BCS alone did not affect the expression of MCP-1 mRNA. In contrast, GAPDH mRNA levels were not altered by TNF-α, PDTC or BCS. Taken together, these results suggest that inhibitory effect of PDTC on TNF-α-dependent activation of NF-κB and induction of MCP-1 is due to the copper-transporting action of PDTC.

We then studied effects of NAC, PDTC and BCS on TNF-α-dependent degradation of IκB-α. As shown in

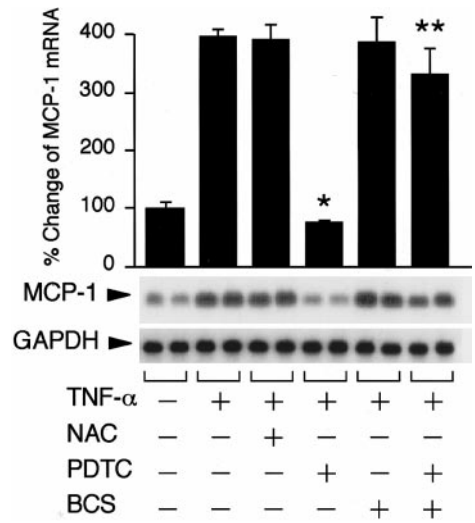

FIG. 4. Effects of TNF-α, NAC, PDTC, and BCS on MCP-1 expression in HASMC. The cells were pretreated as described in the legend to Fig. 2, followed by 6-h incubation of 100 U/ml TNF-α. Total RNA was subjected to Northern blot analysis using MCP-1 and GAPDH cDNA as probes. Experiment was performed in duplicate flasks. Similar results were obtained from a separate experiment. Radioactivities of the bands were measured by BAS 2000 system, normalized by the GAPDH mRNA levels, and expressed as percentage of the level of control, nontreated cells ($n = 4$, mean ± SD). *$P <$ 0.05 vs the level in the presence of TNF-α alone. **$P < 0.05$ vs the level in the presence of TNF-α and PDTC.

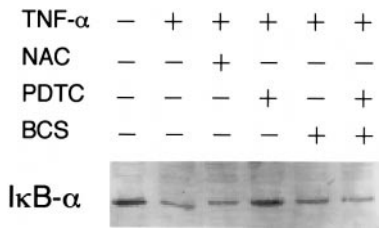

FIG. 5. Effects of NAC, PDTC and BCS on TNF-α-dependent degradation of IκB-α. The cells were pretreated as described in the legend to Fig. 2, followed by 30-min incubation of 100 U/ml TNF-α. Cytosolic extracts (40 μg of protein) were subjected to Western blot analysis using anti-IκB-α antibody.

Fig. 5, Western blot analysis revealed that TNF-α-dependent degradation of IκB-α was inhibited by PDTC, and this PDTC effect was reversed by BCS. In contrast, NAC had no effect on TNF-α-dependent degradation of IκB-α.

DISCUSSION

PDTC and NAC have been widely used as antioxidative compounds to study redox regulation of intracellular signaling cascade and of cell function. The present study demonstrated that the inhibition of TNF-α-dependent NF-κB activation and MCP-1 expression by PDTC is associated with elevation of intracellular copper levels in HASMC. Furthermore, it was shown that this inhibition is due to the increase in Cu^{1+} in the cells, because BCS, a non-cell-permeable chelator of Cu^{1+}, prevented the PDTC action. To our knowledge, this is the first demonstration that PDTC-dependent inhibition of NF-κB activation is reversed by blocking the influx of copper. ROS-scavenging, antioxidative action of PDTC may not be important for the inhibition in HASMC, because the antioxidant NAC had no effect on the NF-κB activation.

Copper is an essential transition metal which plays an important role in the function of various transcription factors and enzymes. Intracellular copper exists in several oxidation status, which can change from one redox status to another under physiological conditions. This redox cycling is considered to be responsible for physiological and toxic potential of this metal.

Several studies reported the effect of copper on NF-κB binding and activation. Yang *et al.* showed that treatment with 500 μM $CuCl_2$ of the nuclear extracts prepared from human primary lymphocytes did not alter the NF-κB binding (20), suggesting that copper does not directly affects the DNA-binding of NF-κB. Satake *et al.* demonstrated that treatment of Jurkat T cells with 500 μM copper inhibited the phosphorylation of IκB-α and the activation of NF-κB induced by TNF-α (21), indicating that copper prevents the signaling pathway at some step prior to IκB-α phosphorylation. Consistent with the latter report, the present study also demonstrated that PDTC inhibited TNF-α-dependent degradation of IκB-α, and that BCS reversed the PDTC effect. These observations strongly suggested that the inhibitory effect of PDTC on NF-κB activation in HASMC is mainly due to the prevention of nuclear translocation of NF-κB.

MCP-1 is the major chemotactic factor involved in the recruitment of monocytes into vessel wall. The present study showed that TNF-α-dependent expression of MCP-1 in HASMC is tightly regulated by NF-κB, since the prevention of NF-κB activation correlated well with the suppression of MCP-1 mRNA levels. This result is compatible with the recent report that MCP-1 induction coincides with NF-κB activation in the atherosclerotic vessels in a rabbit model (22).

In conclusion, the present study indicates that the copper-transporting action is another important property of PDTC for its inhibitory effect on NF-κB activation, and that the intracellular level of copper is crucial for the TNF-α-dependent activation of NF-κB in HASMC.

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