Pyrrrolidine Dithiocarbamate Inhibits the Production of Interleukin-6, Interleukin-8, and Granulocyte-Macrophage Colony-Stimulating Factor by Human Endothelial Cells in Response to Inflammatory Mediators: Modulation of NF-κB and AP-1 Transcription Factors Activity

By Cecilia Muñoz, Dora Pascual-Salcedo, María del Carmen Castellanos, Arantxazu Alfranca, Julián Aragónes, Alicia Vara, Juan Miguel Redondo, and Manuel O. de Landázuri

Endothelial cells (EC) play a key role in the inflammatory response, both by the production of proinflammatory cytokines and by their interaction with leukocytes. Molecular genetic analysis has demonstrated that functional NF-κB sites are involved in the transcription of interleukin-6 (IL-6), IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes in response to inflammatory mediators. Thus, we have explored the effect of two inhibitors of the NF-κB activation, pyrrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC), on the production of these cytokines by EC. Both PDTC and NAC inhibited, in a dose-dependent manner, the synthesis of IL-6, IL-8, and GM-CSF induced by tumor necrosis factor (TNF-α) or bacterial lipopolysaccharides (LPS) in human umbilical vein endothelial cells (HUVEC). PDTC appeared to prevent IL-6, IL-8, and GM-CSF gene transcription, as it blocked the induction of specific mRNA by TNF-α or LPS. The TNF-α-mediated transcriptional activation of a chloramphenicol acetyltransferase (CAT) plasmid containing three copies of the −72 κB binding site from the IL-6 promoter was abrogated by PDTC. According to transfection experiments, electrophoretic mobility shift assays (EMSA) demonstrated that the antioxidant prevented the induction of NF-κB DNA-binding activity by TNF-α. Under the same conditions, PDTC by itself or in combination with TNF-α, enhanced the DNA-binding activity of AP-1, as well as c-fos and c-jun mRNA levels. Altogether, these results indicate that the antioxidant PDTC specifically inhibits the transcription of IL-6, IL-8, and GM-CSF genes through the inhibition of the NF-κB activation, while increasing the expression of AP-1. Our data make evident the anti-inflammatory and immunoregulatory potential of the pharmacological inhibition of the NF-κB activation. In addition, PDTC and related molecules may be a useful tool to explore the expression of genes involved in the inflammatory response.

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From the Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid; Servicio de Inmunología, Hospital La Paz, Madrid; and Centro de Biología Molecular-CSIC-UAM, Madrid, Spain.

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Address reprint requests to Manuel O. de Landázuri, MD, Servicio de Inmunología, Hospital de la Princesa, C/Diego de León, 62, Madrid, 28006 Spain.

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MATERIALS AND METHODS

Endothelial cells. Human umbilical vein endothelial cells (HUVEC) were isolated from normal human umbilical cord veins. The umbilical vein was cannulated and incubated with 1% collagenase for 15 minutes at 37°C. Following removal of collagenase, cells were pooled and established as primary cultures in medium 199 (GIBCO, Gaithersburg, MD) containing 20% fetal calf serum (FCS). HUVEC were serially passaged and maintained using medium 199 supplemented with 20% FCS, 50 µg/mL endothelial cell growth factor and 100 µg/mL heparin in tissue culture flasks precoated with 0.5% gelatin. Cells were used within the first six passages.

Reagents and antibodies. TNF-α (3.2 x 10^7 U/mg) was purchased from Wicher (Vienna, Austria). LPS (Escherichia coli 055:B5), PDTC, and NAC were purchased from Sigma Chemical Co (St Louis, MO). Undetectable levels of endotoxin were found in cultured supernatants of resting and cytokine-stimulated cells by using a limulus amebocyte lysate assay kit (Whittaker, Wavelskire, MD).

489 IgG1 monoclonal antibody (MoAb) (anti-VCAM-1) was generously provided by Dr J. Harlan (University of Washington, Seattle, WA). P3X63 IgG1 myeloma culture supernatant was used as negative control.

The rabbit antisera against Fos family (RR26/8) and Jun family (636/6) were kindly provided by Dr Rodrigo Bravo (Bristol Myers Squibb, Princeton, NJ).

Culture conditions and enzyme-linked immunosorbent assays (ELISA). HUVEC were grown to confluence on 24-well tissue culture plates. Medium was removed and replaced with fresh medium containing 10% FCS with or without 50 µmol/L PDTC or 25 mmol/L NAC for 1 hour. Then, 25 ng/mL TNF-α or 50 ng/mL LPS were added either to the untreated or PDTC/NAC-pretreated cells without removing the antioxidants. After incubation for 6 or 24 hours at 37°C, supernatant was collected and tested for IL-6, IL-8, and GM-CSF release. HUVEC monolayer was then washed and cells were analyzed for cell surface antigen expression by flow cytometry (see below). Cell viability by staining with fluorochrome propidium iodide was stimulated to rule out the possibility that some of the inhibitory effects of PDTC and NAC were due to toxicity. Under the experimental conditions and in the range of PDTC and NAC concentrations used, cell viability was always higher than 90% (data not shown). In addition, when PDTC-treated HUVEC (in which IL-6 induction and VCAM-1 upregulation mediated by TNF-α were inhibited) were washed and further cultured with fresh medium for an additional period of 24 hours, the restimulation of the cells with TNF-α resulted in an induction of IL-6/VCAM-1 indistinguishable from that observed in the cells cultured in parallel that were not exposed to PDTC (data not shown).

IL-6 concentration was measured by a sandwich ELISA in which CLB-IL-6/16 anti–IL-6 MoAb and an affinity purified sheep anti–IL-6 polyclonal antibody (a kind gift of Dr L.A. Aarden, University of Amsterdam, Amsterdam, The Netherlands) were used for the capture and detection of IL-6, respectively. The assay was essentially performed as described, except for the amplification step that was replaced by incubation with streptavidine-horseradish peroxidase complexes. IL-8 and GM-CSF production was determined using commercially available kits (Quantikine, R&D Systems, Minneapolis, MN) according to manufacturer’s instructions.

Flow cytometry. After collecting supernatant to measure cytokine production, HUVEC were removed from tissue culture plates. Cells were washed with phosphate-buffered saline (PBS) and incubated at 4°C for 30 minutes with 1:5000 diluted 489 MoAb ascites in PBS buffer, followed by washing and labeling with an fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab)2 anti-mouse IgG (Dakopatts, Glostrup, Denmark). Samples were analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA).

RNA isolation and Northern blot analysis. Total cellular RNA was isolated by using the guanidium thiocyanate/ CsCl method, as previously described. HUVEC were either untreated or pretreated for 1 hour with PDTC, and then 25 ng/mL TNF-α or 50 ng/mL LPS were added to the cells without removing PDTC. After 4 hours of incubation, HUVEC were washed with PBS and lysed with 4 mol/L guanidium thiocyanate. The cell lysate was layered on a cushion of 5.7 mol/L CsCl and ultracentrifuged at 35,000 rpm for 18 hours. Purified RNA from each sample (20 µg per lane) was denatured, electrophoresed through a 1% formaldehyde agarose gel, and blotted onto a nitrocellulose membrane that was hybridized with 32P-labeled IL-8 cDNA (kindly provided by Dr J. Oppenheim, NCI-FRDC, Frederick, MD), and sequentially probed either with IL-6 and GM-CSF cDNA (kindly provided by Dr J.M. Alvaro Gracia, Hospital de la Princesa, Madrid, Spain) or c-fos and c-jun cDNA (a kind gift of Dr P. Angel, D.K.Z., Heidelberg, Germany).

Plasmids and transient transfection assays. The NF-κB–dependent Cat reporter plasmid pI-6CAT59/IREx3 contains three copies of the –72 kb motif of the IL-6 gene inserted upstream to position 59 of the pI-6CAT 59, which itself is transcriptionally inactive, and was a kind gift from Dr K. Yamamoto (Kanazawa University, Kanazawa, Japan). Plasmids containing the wild-type or the mutant –72 kb sequence placed in front of the thymidine kinase promoter and CAT structural gene have previously been described and kindly provided by Drs J. Vilcek (New York University Medical Center, New York, NY) and E. Benveniste (University of Alabama at Birmingham, Birmingham, AL).

HUVEC were plated at near confluence in 35 mm culture dishes and 24 hours later were transfected with 2.5 µg of plasmid DNA per ml by using a standard calcium phosphate technique. Briefly, HUVEC were incubated with precipitated DNA for 6 hours, washed with PBS, and cultured with fresh complete 199 medium for 18 hours. Then, transfected cells were exposed to different stimuli for 12 hours, and CAT activity was measured according to instructions of a CAT ELISA kit (Boehringer Manheim, Mannheim, Germany).

Electrophoretic mobility shift assay (EMSA). Small scale nuclear extracts were prepared from HUVEC as described with some modifications. Briefly, cells were washed once with PBS and twice with buffer A. The cell pellet was then resuspended (105 cells/100 µL) in buffer A (0.1% Nonidet P-40, and incubated for 10 minutes. Lysed cells were microcentrifuged for 20 minutes, supernatant was discarded, and nuclear pellet was extracted with 3 volumes of buffer C in the presence of D-L-Dithiotreitol (DTT) and protease inhibitors. Nuclear pellet volume was estimated to correct the final KCI concentration to 400 mmol/L. After 30 minutes of incubation, nuclei were microcentrifuged for 30 to 45 minutes, and the supernatant was diluted with 4 volumes of buffer D modified to contain 50 mmol/L KCl instead of 100 mmol/L. EMSAs were performed as described. Briefly, 4 µg of nuclear protein were incubated for 10 minutes at 4°C in a total volume of 12.5 µL containing 1 µg of pol(dI-dC) DNA carrier and 5.5 µL of binding buffer, with or without 10-fold molar excess of unlabeled oligonucleotide. For serologic characterization of the nuclear complexes, 1 µL of each polyclonal antisera against Fos and Jun families was preincubated with the nuclear extracts at 4°C for 30 minutes before the addition of the probe. Then, 1 x 105 cpm (105 cpm/µg) of annealed 32P-labeled oligonucleotides were added and incubated at room temperature for 20 minutes. Samples were subjected to electrophoresis on a 4% polyacrylamide gel. The sequences of the synthetic oligonucleotides used as probes in EMSAs were as follows (factor binding sites are underlined):

AP-1: 5'-GCCTTGATGATCGCCGGA-3';

αB: 5'-GATCCCTTGGGGCTCCTTCCAAGGGATTTCTCCG-3'.

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RESULTS

Endothelial cells play a key role in the inflammatory response. These cells synthesize several chemotactic and pro-inflammatory cytokines in response to both physiological (C5a, IL-1, TNF-α) or nonphysiological (LPS) stimuli. We have explored the effect of PDTC and NAC, two inhibitors of the NF-κB activation, on the synthesis of several cytokines by EC.

The incubation of HUVEC with TNF-α or LPS during 24 hours resulted in a strong enhancement of IL-6, IL-8, and GM-CSF secretion that was detected as early as 6 hours after treatment (Fig 1A, and data not shown). When cells were preincubated for 60 minutes with 50 μmol/L PDTC, there was a marked downregulation of the induction of cytokine release mediated by TNF-α or LPS (Fig 1A). Similar results were obtained with NAC (Fig 1A). Dose-response experiments showed that the inhibitory effect of PDTC on cytokine synthesis was detectable at a dose of 20 μmol/L, with a maximal suppression at doses of 50 μmol/L PDTC (Fig 1B). This inhibitory effect of the antioxidants was not due to cell toxicity, since in the range of PDTC and NAC concentrations used, the cell viability was always higher than 90% (see Materials and Methods).

To further explore the mechanisms involved in this suppressive effect, we performed Northern blot analysis on EC stimulated with TNF-α or LPS in the presence of PDTC. We found undetectable or very low levels of IL-6, IL-8,
and GM-CSF mRNA in unstimulated HUVEC (Fig 2). The exposure of these cells to TNF-α or LPS during 4 hours resulted in a strong upregulation of IL-6, IL-8, and GM-CSF transcripts, an effect that was almost completely abrogated when HUVEC were simultaneously treated with PDTC (Fig 2). The specificity of this inhibition was examined by studying the effect of this dithiocarbamate on the expression of c-jun and c-fos mRNA levels, as it has been previously demonstrated that the transcription of these genes is induced by PDTC in HeLa cells. Treatment of HUVEC with the antioxidant resulted in an accumulation of c-jun and c-fos mRNA levels that were further augmented when EC were cotreated with PDTC and TNF-α (Fig 3A and data not shown). The induction of c-jun mRNA by PDTC was detectable after 30 minutes, reached a maximum at approximately 2 hours, and persisted for long periods of time (Fig 3A and data not shown). In the presence of TNF-α and PDTC, there was a further augmentation of the c-jun mRNA levels under conditions in which the induction of IL-8 mRNA was completely inhibited. The induction of c-fos mRNA by PDTC began to increase at 30 minutes, reached a maximum at 60 minutes, and decreased after 3 hours, but cotreatment with PDTC and TNF-α resulted in a longer lasting induction of c-fos mRNA (data not shown). Since c-jun is an early responsive gene (the previous experiments were performed at 3 and 5 hours after activation), we also studied the effect of PDTC on a gene that is induced later in the endothelial cell activation response. As shown in Fig 3B, 20 hours of treatment with PDTC did not affect the TNF-α-mediated ICAM-1 mRNA induction, whereas it inhibited the induction of IL-
8 mRNA. Similar results were obtained when ICAM-1 cell surface expression was analyzed by cytofluorometry (data not shown).

To investigate if the inhibitory effect of PDTC was mediated by an alteration in the stability of the mRNA of such cytokines, we examined the effect of the antioxidant on the half-life of IL-8 mRNA. HUVEC were treated with the transcription inhibitor actinomycin D, and the decrease in the level of IL-8 mRNA was analyzed by Northern blot analysis, showing that TNF-α prolonged the half-life of IL-8 mRNA from approximately 5 hours to far greater than 6 hours (Fig 4). When cells were cotreated with PDTC plus TNF-α, a further increase in IL-8 mRNA half-life was detected (Fig 4), thus indicating that the inhibitory effects of PDTC were not due to alteration on the mRNA stability.

Deletional and mutational analyses have demonstrated that the transcription factor NF-κB is involved in the activa-
DITHIOCARBAMATES REGULATION OF CYTOKINE PRODUCTION

A

![Graph showing transcriptional activity](image)

Fig 5. Inhibitory effect of PDTC on the TNF-α transcription activation of the IL-6 promoter. HUVEC were transfected with either pBLCAT3 and pIL-6CAT59/LREx3 or wild-type IL-6 κB and mutant IL-6 κB. Following transfection, cells were pretreated or not with 50 μmol/L PDTC for 1 hour and then exposed or not to 25 ng/ml TNF-α for 12 hours. CAT activities are expressed as fold activation over the basal CAT activity of the promoterless plasmid pBLCAT3 or the mutant IL-6 κB, which was set as a value of 1. Data are expressed as the mean ± SD (n = 3).

B

![Graph showing transcriptional activity](image)

In agreement with the transfection experiments, EMSAs showed that the basal levels of NF-κB DNA-binding activity detected on nuclear extracts from unstimulated HUVEC were significantly increased after exposure of the cells to TNF-α (Fig 6). The enhancement of NF-κB binding activity induced by TNF-α was prevented by the treatment of HUVEC with PDTC (Fig 6). Since the initial experiments indicate that PDTC induced significant mRNA levels of c-jun and c-fos, we also investigated the presence of AP-1 protein complexes with DNA-binding activity in the nuclear extracts. The treatment of HUVEC with 50 or 100 μmol/L PDTC for 4 hours resulted in a strong enhancement of the DNA-binding activity of AP-1 that was further increased in nuclear extracts from HUVEC treated simultaneously with TNF-α and PDTC (Fig 6). Supershift experiments using antisera against Jun and Fos demonstrated that both antisera completely prevented the formation of specific complexes indicating the presence of members of Fos and Jun families of transcription factors in the complex (data not shown). These results indicate that PDTC activates AP-1 DNA-binding activity in human EC, whereas under identical conditions, it inhibits the induction NF-κB complexes mediated by TNF-α.

DISCUSSION

The production of IL-6, IL-8, and GM-CSF is increased at sites of inflammation. Both IL-8 and GM-CSF are chemotactic cytokines involved in leukocyte trafficking. In a first step, the cytokine is retained on endothelium in solid phase and acts as an immobilized ligand for trapping a specific type of leukocyte. Subsequent binding of chemokines (ie, IL-8) or cytokines (ie, GM-CSF) to leukocytes results in an activation of leukocyte integrins, and the cells become firmly adhered to the endothelium. After strong adhesion to EC, the leukocyte migrates toward the inflammed tissue under the influence of a chemotactic gradient.

On the other hand, IL-6 promotes the proliferation of vascular smooth muscle cells and increases the permeability of EC events that are involved in the onset and development of conditions such as vasculitis and atherosclerosis. Given the pathologic relevance of the phenomena considered above, the cytokine production by EC is presumably a crucial target in the therapy for different inflammatory processes.

In this regard, we demonstrate that PDTC and NAC molecules inhibit the production and mRNA expression of cytokines (IL-6, IL-8, GM-CSF) induced by proinflammatory stimuli (TNF-α, LPS) in human EC. PDTC inhibits IL-6 production of IL-6, IL-8, and GM-CSF genes by proinflammatory mediators. To further investigate if PDTC selectively inhibited transcriptional activation of these genes, HUVEC were transiently transfected with a reporter construct containing three copies of the −72 κB site of the IL-6 5' flanking region placed upstream of the homologous minimal promoter. As shown in Fig 5A, the TNF-α stimulatory effect was inhibited by the antioxidant, indicating that the main effect of PDTC is at the transcriptional level. To confirm that NK-κB activation is required for the induction of these genes in HUVEC, we used CAT reporter constructs containing the wild-type or the mutant −72 κB sequence of the IL-6 gene. With the wild-type IL-6 κB construct, the transcriptional activity of CAT reporter plasmid was significantly increased after treatment with TNF-α (Fig 5B). However, on transfection of the mutant κB sequence, no transcription of the CAT gene was detected with TNF-α. In addition, the transcriptional activity of the wild-type κB plasmid induced by TNF-α was inhibited by PDTC, thus indicating that this antioxidant exerted its effect by inhibiting the activation of NF-κB.
promoter-driven CAT activity induced by TNF-α and does not destabilize IL-8 mRNA, suggesting that this antioxidant inhibits cytokine production mainly at the transcriptional level. CAT assays performed with wild-type, mutant, and trimerized −72 κB sequence of the IL-6 gene, together with EMSAs, in which a reduction of the TNF-α–induced NF-κB DNA-binding activity was detected, showed that the nuclear factor NF-κB is a crucial element to confer inducibility by TNF-α and inhibition by PDTC. These results confirm the molecular evidence that cytokine- or LPS-induced activation of the promoters of these cytokines may critically depend on NF-κB activation in HUVEC. In this regard, it is important to point out that the activation of NF-κB is indispensable for IL-6 and IL-8 genes activation in any type of cell examined. In addition, molecular analysis of IL-6 and IL-8 suppression by different agents such as glucocorticoids, estrogens, IFN-γ, cyclosporin, or FK506 showed that all of them affected the activity of NF-κB, thereby suppressing the transcription of these cytokines. Our results strongly suggest that this is also the case for PDTC. Various studies have proposed that, although NF-κB is the most crucial factor for IL-6 and IL-8 gene transcription, cooperation with other transcription factor, NF-IL6, is necessary for the activation of these genes, as demonstrated by mutation of either NF-κB or NF-IL6 binding sites and cotransfection experiments with expression vectors of NF-IL6 and NF-κB subunits. Hence, it will be also interesting to define the effects of PDTC on the activity of NF-IL6.

The activation of GM-CSF gene expression in response to different extracellular stimuli is somewhat more complex, and the activation of GM-CSF promoter requires the interaction of NF-κB with other transcription factor families. Therefore, the final effect of PDTC on the activity of this promoter would require a more detailed analysis.

To control for possible nonspecific effects of PDTC on EC gene expression, we studied the effect of the antioxidant on the expression of genes whose transcription is apparently independent of NF-κB. We found that PDTC by itself or in cooperation with TNF-α lead to an increase in the mRNA levels of c-jun and c-fos, whereas it inhibited the TNF-α–mediated inducibility of IL-6, IL-8, and GM-CSF. Moreover, PDTC did not affect the induction of ICAM-1 mRNA by TNF-α after 20 hours of coculture. Finally, DNA-binding activities of AP-1 was augmented by PDTC or PDTC/TNF-α cotreatment, whereas under the same conditions, the antioxidant inhibited the induction of NF-κB DNA-binding activity mediated by TNF-α, thus adding molecular evidence for the specificity of the inhibitory effect of PDTC. Collectively, these data suggest that NF-κB activation can represent a common and necessary step for the transcription of several genes involved in the establishment of the inflammatory response. As a consequence, the inhibition of the NF-κB pathway by dithiocarbamates may constitute a single and critical target for therapeutic intervention in those pathologies associated with abnormal cytokine secretion. In this regard, it has been described that the progression of HIV-infected patients to acquired immune deficiency syndrome (AIDS) is clearly related to the overproduction of cytokines, a phenomenon derived from the uncontrolled activation of lymphocytes seen in these patients. Thus, the beneficial effect of
dithiocarbamates reported in HIV+ individuals could be related to the downregulation of cytokine synthesis induced by these drugs.

NF-κB activation takes place following the phosphorylation of the inhibitory subunit IκB-α that may serve as the signal for its subsequent proteolytic degradation by the 26S proteasome. In this regard, pharmacological inhibitors of either IκB-α phosphorylation or proteasome activity have been demonstrated to inhibit the activation of NF-κB and subsequently the transcription of EC adhesion molecules genes dependent on functional κB sites. Similarly, PDTC is able to abrogate cytokine- or LPS-induced transcription of these same adhesion molecules and tissue factor activity in EC, as well as cytokine production by monocytes. The mechanism of action of PDTC has not yet been well defined, but probably involves the inhibition of reactive oxygen intermediates (ROI) generation that would result in the activation of an IκB-α kinase, or alternatively, in the activation of a process involved in the phosphorylation of the inhibitor of NF-κB. Casein kinase II has been recently suggested as the IκB-α kinase.

Regarding the transcription factor AP-1, the results from EMSA and Northern blot experiments suggest that it may function as an antioxidant responsive transcription factor in human EC, as it has been demonstrated in other cell systems. The enhanced DNA-binding activity of AP-1 might contribute to repress cytokine gene activation mediated by NF-κB. In this regard, Fos and Jun have been shown to interact with NF-IL6 and repress transcription activation by this factor. As stated above, NF-IL6 activation is also necessary for the activation of IL-6 and IL-8 genes and its inhibition could account, together with the inhibition of NF-κB activation, for the inhibitory effect of the antioxidant on the transcription of these cytokines. It will be interesting to determine the overall effect of PDTC on the transcription and subsequent expression of different genes on EC, as well as other cells.

In addition to the therapeutic potential in autoimmune and inflammatory diseases, dithiocarbamates can represent an invaluable tool to investigate the role of NF-κB and AP-1 transcription factors in the expression of genes associated with the inflammatory response in human EC.

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C Munoz, D Pascual-Salcedo, MC Castellanos, A Alfranca, J Aragones, A Vara, MJ Redondo and MO de Landazuri