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Inhibition of NF-κB Activation in Human T-Cell Lines by Anetholdithiolthione

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Nuclear factor (NF)-κB is a redox sensitive cytosolic transcription factor. Redox regulation of NF-κB has been implicated in the activation of the human immuno-deficiency virus (HIV). Therefore, inhibition of NF-κB activation may be an effective strategy for acquired immunodeficiency syndrome therapy. Anetholdithiolthione (ADT, 5-[p-methoxyphenyl]-3H-1,2-dithiol-3-thione) is an antioxidant which has been used to protect against acetaminophen- and CCl_4-induced hepatotoxicity, lipid peroxidation, radiation injury, and also has been used clinically as an anti-choleretic agent. The present study examined the effect of ADT pretreatment on NF-κB activation in response to a variety of stimuli such as H_2O_2, phorbol myrisitate acetate (PMA) or tumor necrosis factor α (TNFα). PMA and TNFα induced activation of (NF)-κB in human Jurkat T-cells was partially inhibited by ADT (0.1 mM) pretreatment. ADT (0.1 mM) also inhibited H_2O_2 induced activation of the transcription factor in the peroxide sensitive human Wurzburg T-cells. Furthermore, ADT treated Wurzburg cells had significantly higher glutathione levels as compared with untreated cells. H_2O_2 induced lipid peroxidation in Wurzburg cells was remarkably inhibited by ADT pretreatment. ADT, a pro-glutathione antioxidant, was observed to be capable of modulating NF-κB activation.

Reactive oxygen species (ROS)[2] are generated during electron-transfer reactions in biological systems (1). ROS generated in amounts sufficient to overwhelm intracellular antioxidant defenses, induce oxidative lipid, protein and nucleic acid damage. However, low concentrations of intracellular ROS are also used by cells to regulate signalling and gene expression. NF-κB is a multisubunit transcription factor that is activated by a variety of primary (viruses, bacteria, stress factors) and secondary (inflammatory cytokines) pathogenic stimuli (2). This transcription factor is found in many different cell types and tissues, but has been best characterized in cells of the immune system such as pre-B-, B- and T-lymphocytes, macrophages and monocytes. Activation of NF-κB causes a rapid induction of genes encoding defense and signalling proteins. In most cells, NF-κB is present in the cytoplasm as a non-DNA binding complex composed of three subunits; a DNA-binding 48–55 kd protein (p50), a DNA-binding 65–68 kd protein (p65, also known as Rel A), and a inhibitory subunit (IκB) bound to p65 (2).

Oxidative stress is an activating stimulus for NF-κB in a number of cell types. Schreck et al. (3) showed that in human derived Jurkat T cells, NF-κB is post-translationally activated by low (μM) concentrations of hydrogen peroxide. It has also been reported that various agents that induce oxidative stress *e.g.,* tumour necrosis factor α, interleukin-1, phorbol ester, lipopolysaccharide, anti-IgM and ultraviolet light are also capable of activating NF-κB. Studies in our and other laboratories have revealed that the activation of NF-κB can be blocked by various chemically distinct antioxidants (3–6). The actual mechanism of activation, namely the series of events that

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[2] Abbreviations: HIV, human immuno-deficiency virus; ADT, anetholdithiolthione (5-[p-methoxyphenyl]-3H-1,2-dithiol-3-thione); TNFα, tumor necrosis factor α; ROS, reactive oxygen species; AIDS, acquired immunodeficiency syndrome; FCS, fetal calf serum; DMSO, dimethylsulfoxide; PMA, phorbol 12-myrisitate 13-acetate; DTT, dithiothreitol; GSH, reduced glutathione; PBS, phosphate buffered saline; TBARS, thiobarbituric acid reactive substances; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride.

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cause the dissociation of IκB from the p50-p65-IκB complex is still unclear. It appears that reactive oxygen species may play a messenger function in the activation of the factor. Once activated, the p50-p65 dimer is rapidly translocated to the nucleus where it binds as a *trans*-acting factor to the consensus sequence 5′-GGGRNN(YYC)C-3′ (where R = any of the purine nucleotides *i.e.*, A or G;N = any of the four nucleotides A, T, G, or C; Y = any of the pyrimidine nucleotides *i.e.*, T or C; fragment in parenthesis may or may not be present in the binding site) in the κ enhancer. Binding of the activated factor initiates transcription. Redox regulation of (NF)-κB has been implicated in the activation of a HIV, the virus believed to cause AIDS (5–9).

A number of thiol antioxidants are known to have therapeutic properties as protectors against chemo- and radio-injuries (10, 11). Anetholdithiolthione (ADT, Fig. 1) has been used since 1947 as an anticholeretic agent without any clinically adverse side-effects (11). Dithiolthiones have been established as anticarcinogens (10,12–14), effective inhibitors of lipid peroxidation (15, 16), and protective agents against hepatotoxicity and radiation injury (17). Early works have shown that cabbage, brussel sprouts and other cruciferous vegetables contain dithiolthiones (18, 19). In the present study we have sought to determine if ADT can inhibit the activation of the redox sensitive transcription factor NF-κB.

MATERIALS AND METHODS

Cell culture. Human lymphoma Jurkat T-cells were obtained from American Type Culture Collection (ATCC, Bethesda). Wurzburg cells, a clone of Jurkat T-cells known to be peroxide sensitive with respect to (NF)-κB activation, developed by Dr. Patrick Baeuerle (Frieburg, Germany) were a kind gift of Dr. Leonard Herzenberg of Stanford University, CA. Both cells were grown in RPMI 1640 medium supplemented with 10% FCS, 1% (w/v) penicillin-streptomycin, 1% sodium pyruvate and 1% L-glutamine (University of California, San Francisco Cell Culture Facility) in humidified air containing 5% CO_2.

Nuclear extraction. Cultured cells were harvested, centrifuged at 125 *g* for 10 min, and synchronized by resuspending in 1% FCS growth medium either with 0.1 mM ADT (from 0.1 M stock solution in DMSO; ADT was kindly provided by Solvay Pharma, L.T.M. laboratories, France) or 0.1% (v/v) DMSO at a density of $\sim 1.25–1.5 \times 10^6$ cells per ml. Cells were seeded in a 24-well plate with 1.5 ml cell suspension per well. After 18 h of incubation, cells were challenged with either H_2O_2 (Sigma, MO), PMA (Sigma, MO) or TNFα (Genentech Inc., CA).

Nuclear extracts were prepared from $\sim 2 \times 10^6$ cells as previously described (6, 20) with slight modifications. Briefly, cells were harvested, pelleted and washed twice in 1 ml of ice-cold PBS, and centrifuged for 15 sec at 14,000 rpm in an Eppendorf-Brinkman centrifuge at 4°C. Cells were pelleted and washed once in 0.6 ml of buffer A [10 mM HEPES, pH 7.8 containing 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_2EDTA, 0.1 mM PMSF, 5 μg/ml antipain and 5 μl/ml leupeptin] and incubated on ice for 15 min. To the ice-cold suspension, 38 μl of 10% Nonidet P-40 solution was added, and cells were vigorously mixed for 15 sec and then centrifuged for 30 sec at 16000 *g*. Pelleted nuclei were resuspended in 60 μl of buffer C [50 mM HEPES, pH 7.8 containing 50 mM KCl, 300 mM NaCl, 0.1 mM Na_2EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% (v/v) glycerol], mixed for 20 min and centrifuged for 5 min at 16000 *g* at 4°C. The supernatant containing the nuclear proteins was harvested, protein concentration determined and stored at −80°C until EMSA was performed.

EMSAs were performed essentially as described earlier (21). Binding reaction mixtures (20 μl) containing 5–10 μg protein of nuclear extract, 1 μg poly(dI-dC)(Pharmacia), ^{32}P-labeled probe (NF-κB consensus oligonucleotide), 50 mM NaCl, 0.2 mM Na_2EDTA, 0.5 mM DTT, 2% (v/v) glycerol and 10 mM Tris-HCl (pH 7.5) were incubated for 20 min at 37°C. Proteins were separated by electrophoresis in a native 6% polyacrylamide gel using a Tris-borate running buffer (12.5 mM Tris-borate containing 0.25 mM Na_2EDTA, pH 8.0), followed by autoradiography. (NF)-κB probe (Promega Inc., WI) was labeled with [γ-^{32}P]dATP (ICN Biomedicals, CA) using T4 polynucleotide kinase (Promega Inc., WI). The labelled probe was purified using a NAP-5 column (Pharmacia, Stockholm, Sweden) equilibriated with 10 mM Tis-HCl (pH 7.5) containing 100 mM NaCl and 1 mM EDTA.

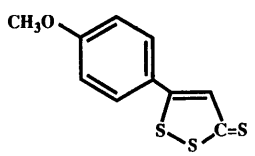

FIG. 1. Structure of anetholdithiolthione (ADT).

Glutathione HPLC-electrochemical assay. Cell GSH content was determined using a HPLC-electrochemical method described previously (22). Cell suspensions (1.25–1.5 × 10^6/ml, 13 ml) were washed with 1 ml ice-cold PBS, pelleted at 125 *g* and resuspended in 1 ml 0.1 M monochloroacetic acid. The acid extract was centrifuged (16000 *g* × 5 min) and the supernatant was used for the determination. GSH was separated using a Nova-Pak C_{18} column (particle size 4 μm, pore size 60Å, 5 mm × 100 mm; Millipore, MA) and detected by a dual mercury-gold electrode (BAS, IN). The pellet of the acid-extract was resuspended in 1 M NaOH and protein content was determined using the Biorad D_C protein assay kit (Biorad, CA).

Lipid peroxidation. Wurzburg cells (1.25–1.5 × 10^6/ml; grown in 1% FCS growth medium) were treated with 100 μM ADT for 18 h. Cells were then challenged with 250 μM H_2O_2. At 0.5, 2, 4, and 6 hours after the addition of H_2O_2, 12 ml aliquots of the cell suspension were collected. Cell suspensions were washed with 1 ml PBS, pelleted at 125 *g* and then resuspended in 100μl of 300 mM glycine buffer (pH 3.6). TBARS was quantitated by measuring absorbances at 532 and 650 nm using acid-hydrolysed 1,1,3,3-tetramethoxypropane as a standard (23).

RESULTS

Treatment of Jurkat T-cells with PMA (100 nM) or TNFα (50 ng/ml) resulted in the activation of NF-κB. Activated NF-κB in the nuclear protein extract of cells was observed to bind with the labelled NF-κB consensus oligonucleotide. Retardation in the electrophoretic migration of the DNA-protein complex in a non-denaturing gel is evident in Fig. 2. The autoradiograph illustrates inhibition of PMA- and TNFα-induced (NF)-κB activation in 0.1 mM ADT pre-treated cells (Fig. 2). At this concentration, ADT dissolved in DMSO or DMSO alone (0.1%, v/v) did not have any effect on cell viability as tested by the trypan blue exclusion test (results not shown). The effect of ADT was tested at various (0.05 − 0.5 mM) concentrations. Higher concentrations of ADT added to cell suspension were observed to be poorly soluble in the aqueous suspension and some crystals of the compound were visible under a 10 × inverted microscope. H_2O_2 (0.25 mM) treatment resulted in a marked activation of NF-κB in Wurzburg cells (Fig. 3). H_2O_2 induced activation of NF-κB in Wurzburg cells was totally prevented in cells that were pre-treated with 0.1 mM ADT for 18 h (Fig. 3). In a separate experiment, HPLC-electrochemical measurements revealed that pre-treatment of the Wurzburg cells with 0.1 mM ADT for 18 hours significantly elevates intracellular GSH levels (Fig. 4).

H_2O_2 challenge resulted in marked lipid peroxidation in the Wurzburg cells. H_2O_2 was added to the cell suspension at 0 h. Individual data points plotted in the figure illustrate that TBARS content in challenged cell extracts was highest 2 h after the challenge. Wurzburg cells pre-incubated for 18 h in 0.1 mM ADT were remarkably protected against oxidant-induced lipid damage (Fig. 5).

DISCUSSION

PMA and TNFα are known to activate (NF)-κB in a antioxidant-inhibitable manner. Several studies have also shown that subtle changes in intracellular redox status may cause such activation

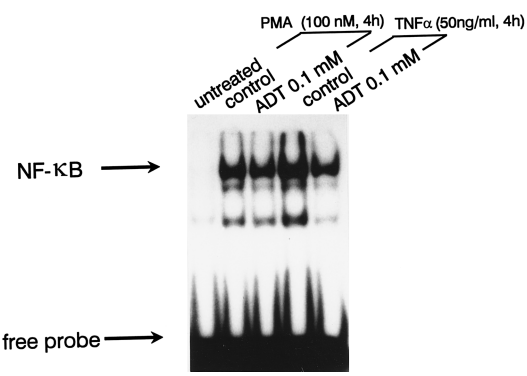

FIG. 2. Effect of ADT pretreatment on PMA- and TNFα-induced NF-κB activation in human Jurkat T-cells. *Cells were pretreated for 18 h with 0.1 mM ADT.*

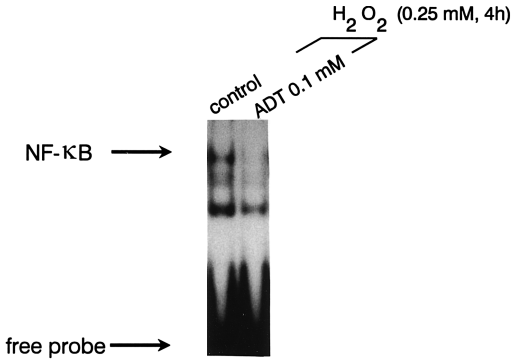

FIG. 3. Effect of ADT pretreatment on hydrogen peroxide-induced NF-κB activation in human Wurzburg cells. Cells were pretreated for 18 h with 0.1 mM ADT.

(2–6,21). ADT pretreatment partially inhibited (NF)-κB activation in Jurkat cells in response to PMA or TNFα treatment (Fig. 2). The Wurzburg clone of Jurkat T-cells is responsive to H_2O_2 with respect to NF-κB activation (24), and activation of (NF)-κB in these cells was prevented as a result of ADT pretreatment. Thus, ADT appears to be particularly effective in suppressing the activation of (NF)-κB caused by a direct oxidant challenge (Fig. 3). This observation is consistent with studies indicating that dithiolthiones, including ADT, have marked antioxidant properties (10–14, Fig. 5). Antioxidants are known to be capable of regulating NF-κB activation (6, 21), and the observed inhibitory effect of ADT on such activation may be because of the antioxidant properties of the drug. Our observation that ADT treatment elevates intracellular GSH content (Fig. 4) and markedly protects against lipid peroxidation (Fig. 5) is consistent with a previous report that female mice fed with diet supplemented with 0.1% ADT had higher hepatic glutathione content, increased hepatic glutathione reductase and glutathione S-transferase activities, and increased protection against hepatic DNA damage and lipid peroxidation (16). Experimentally, ADT has been shown to protect mice against acute acetaminophen- and CCl_4-induced hepatotoxicity (17), and has also been characterized as a potent inhibitor (IC_{50} $\sim\mu$M) of lipid peroxidation induced in rat liver microsomes (15). ADT inhibited ethane exhalation and prevented mortality of mice intoxicated with 0.5 g/kg acetaminophen (15). ADT and its analogue oltipraz have been shown to protect benzo[*a*]pyrene administered mice against the development of forestomach tumours and pulmonary adenomas (14.). These dithiolthiones have also been shown to protect against aflatoxin tumorigenecity in rats

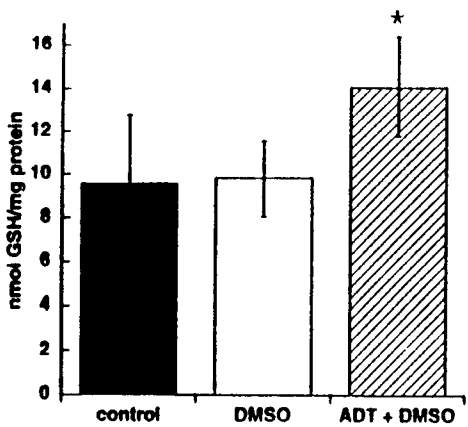

FIG. 4. ADT treatment increased glutathione content of human Wurzburg T-cells. Data are means ± SD. *$p < 0.02$. Cells were pretreated for 18 h with 0.1 mM ADT.

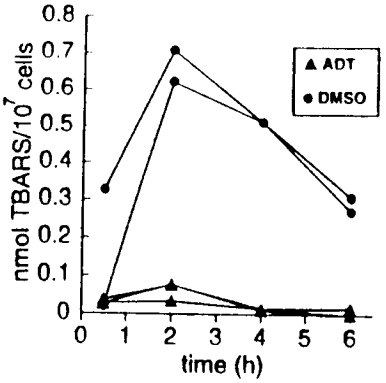

FIG. 5. Protective effect of ADT against H_2O_2 induced lipid peroxidation in Wurzburg cells. H_2O_2 (0.25 mM) treatment was done at 0 h. Kinetics of lipid peroxidation (individual data) is illustrated. Cells were pretreated for 18 h with 0.1 mM ADT.

(10). ADT protects against singlet oxygen-induced damage to rat lenses *in vitro* (25). Inhibition of lipid peroxidation by ADT could be as a result of intracellular GSH elevation as well as direct chain breaking antioxidant properties of the dithiolthione.

Redox regulation of (NF)-κB appears to be a crucial factor in the activation of human immunodeficiency virus replication and the pathogenesis of AIDS (26). The long-term repeat region of HIV-1 proviral DNA contains two binding sites for (NF)-κB (7, 8). Blocking the activation of HIV transcription using antioxidant (NF)-κB inhibitors is indeed an attractive therapeutic strategy (5, 6, 9). The effectiveness of thiols *e.g.* N-acetylcysteine, (6, 27), lipoic acid (5), 2-mercaptoethanol, glutathione, and dithiocarbamates (4, 28) has been reported earlier. ADT prevents the activation of (NF)-κB, and unlike most thiols mentioned above, is lipophilic and acts as a potent inhibitor of lipid peroxidation. (NF)-κB mediates the action of many substances important in immune responses including proinflammatory cytokines, acute phase proteins, cell adhesion molecules, and lymphocyte mitogens (2). Secreted by many cells of the immune system, most of these substances sense a wide range of pathogenic signals and cause inflammation. Thus, ADT as a modulator of (NF)-κB function may act as a potent immunoregulatory agent.

Talalay et al. (29) reported that dithiolthiones also induce quinone reductases, and may thus inhibit carcinogenesis. Dithiolthiones, compounds known to occur naturally in cruciferous vegetables, appear to be potent in upregulating intracellular glutathione level, inhibiting lipid peroxidation damage, and inhibiting the activation of the transcription factor (NF)-κB. These properties lend further support to the potential usefulness of ADT as a therapeutic agent.

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