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Antioxidants Inhibit Stimulation of HIV Transcription

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ABSTRACT

In studies presented here, we demonstrate that antioxidants regulate NF-κB activation and signal transduction pathways leading to HIV expression. We show (1) that *N*-acetyl-L-cysteine (NAC), an antioxidant and an efficient glutathione (GSH) precursor, inhibits NF-κB activation and HIV expression under conditions in which GSH is depleted and NAC cannot be converted to GSH, (2) that the D-stereoisomer of NAC and a wide variety of chemically unrelated antioxidants also inhibit NF-κB activation and/or transcription directed by the HIV LTR, and (3) that depletion of GSH, the principal intracellular antioxidant, augments HIV production in an acute infection model. Taken together, these findings suggest direct antioxidant action as the mechanism for inhibition of HIV transcription by NAC. They also confirm that GSH, acting in its capacity as an antioxidant, regulates HIV expression and that exogenous antioxidants can potentiate this regulation.

INTRODUCTION

Studies from several laboratories indicate that the signal transduction pathways leading to NF-κB activation and HIV production are redox regulated.[1–7] *N*-Acetylcysteine (NAC), a well-known antioxidant used to replenish intracellular glutathione (GSH) *in vivo* and in cells cultured *in vitro*,[8–10] has been shown to inhibit cytokine-stimulated HIV transcription and replication in a variety of model systems.[1–5] For example, NAC inhibits stimulation of transcription directed by the HIV long terminal repeat (LTR) in reporter gene systems and inhibits cytokine-stimulated HIV production both in acutely infected peripheral blood mononuclear cells (PMBCs)[1] and in chronically infected cell lines.[5] Furthermore, NAC inhibits the activation of the transcription factor Nuclear Factor-κB (NF-κB), which is essential for the stimulated expression of genes controlled by the HIV LTR.[3,6,7] Finally, studies by Baeuerle and colleagues[7] and confirmed in our laboratory (M.T. Anderson, manuscript in preparation), demonstrate that hydrogen peroxide specifically activates NF-κB in certain cell lines and thus clearly implicate oxidants in signal transduction leading to transcriptional activation of the HIV LTR.

Normally, GSH serves as the major scavenger for intracellular oxidants in addition to playing a variety of other roles in cellular metabolism (reviewed in Ref. 11). Because NAC is a GSH precursor, the inhibition of HIV transcription by NAC could be caused by its conversion into GSH, which subsequently acts as a scavenger. Alternatively, NAC could act directly as an antioxidant and not by increasing the availability of GSH. Here we demonstrate that NAC inhibits HIV LTR-directed transcription under conditions in which GSH biosynthesis is inhibited. Moreover, we show that the D-stereoisomer of NAC, which is not a GSH precursor, is as effective as the L-enantiomer. Thus, by two independent assays, we demonstrate that the property of NAC as an antioxidant is responsible for the inhibition of cytokine-induced HIV transcription.

We also show, in accordance with this model of direct reduction (i.e., without conversion into GSH), that a wide variety of antioxidants other than NAC can inhibit transcription directed by the HIV LTR. Furthermore, we show that depletion of GSH augments HIV expression in an acute infection model. These results confirm that antioxidants in general, and GSH in particular, regulate HIV expression.

MATERIALS AND METHODS

Cells. Derivation of the HIV-*lacZ* reporter cell line, 293.27.2, has been described.[1] Human peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density centrifu-

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gation from whole-blood buffy coats purchased from the Stanford Blood Bank. The 293.27.2 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% (v/v) FCS and 5% (v/v) horse serum, L-glutamine, penicillin, and streptomycin.

Stimulation of cells. 293.27.2 cells were plated in individual wells of 96-well Costor (Cambridge, MA) plates at a density of 5000 cells/well in 0.2 ml of medium. The next day, phorbol myristate acetate (PMA; Sigma, St. Louis, MO), tumor necrosis factor α (human rTNF-α; Cetus, Emeryville, CA), L-NAC (Aldrich, Milwaukee, WI), D-NAC (kindly provided by Dr. A. Tunek, Astra Draco, Lund, Sweden), or *N*-acetylserine (NAS; Sigma) were added to the desired concentrations from stock solutions. The enantiomeric purity of L- and D-NAC was confirmed by measurement of optical rotation: $[\alpha]^{25}$ for D-NAC = -4.6 ± 0.3 and $[\alpha]^{25}$ for L-NAC = $+ 4.4 \pm 0.3$. Stimulations were for 6 or 8 hr at 37°C,5% CO_2. In experiments with buthionine sulfoximine (BSO; Sigma), cells were pretreated with BSO (100 μM) for 2 days with daily refeeding (including BSO), and received BSO at the start of the stimulations as well. Experiments with the panel of antioxidants were performed as with NAC. Glutathione, glutathione disulfide (GSSG), L-cysteine, L-cystine, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), sodium diethyldithiocarbamate (DDTC), nordihydroguaiaretic acid (NDGA), vitamin E succinate, and sodium ascorbate were all from Sigma. L-2-Oxothiazolidine-4-carboxylic acid (OTC) was from Clintec Nutrition. All stocks were neutralized to pH 7.0 prior to use.

Measurement of β-galactosidase activity. The 4-methylumbelliferyl β-D-galactoside (MUG) assay of β-galactosidase was carried out as described.[12]

Nuclear protein extracts and electrophoretic mobility shift assays. Nuclear protein extracts were made essentially as described,[6,13] with some modifications to optimize conditions for our cells. Cells (10^7) were harvested after stimulation for 2 hr, centrifuged (10 min, 1200 rpm, 4°C), and washed in 1 ml of ice-cold Tris-buffered saline. All subsequent steps were done in the cold room and on ice. Cells were pelleted and washed once in 0.4 ml of buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 0.1 mM EDTA (ethylenediaminetetraacetic acid) 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], supplemented with protease inhibitors and incubated on ice for 17 min. Then 25 μl of a 10% Nonidet P-40 (NP-40) solution was added and cells were vigorously mixed and centrifuged. Pelleted nuclei were resuspended in buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 3 mM DTT, 0.1 mM PMSF, 10% (v/v) glycerol] and gently mixed for 30 min, after which samples were centrifuged for 10 min at 14,000 rpm to obtain clear supernatants containing the nuclear proteins. Protein samples were stored at −70°C. Electrophoretic mobility shift assays (EMSAs) were done as described[14] with 1.0 μg of nuclear extract and labelled κB probes under experimentally determined optimal binding conditions (final salt concentration, 70 mM; DTT concentration, 5 mM; 0.2 μg dIdC). Samples were run on 4.5% acrylamide gels and bands were visualized by autoradiography and quantitated by direct counting of radioactivity with a radioanalytic imaging system (Ambis Systems, Inc).

Monitoring of HIV infection. Peripheral blood mononuclear cells were maintained in complete RPMI-1640 medium [supplemented with 20% heat-inactivated fetal calf serum (FCS), glutamine, and antibiotics]. The PMBCs (3×10^6/ml) were stimulated for 3 days with 2 mg of phytohemagglutinin (PHA)/ml and 5% purified human interleukin 2 (IL-2; Pharmacia, Piscataway, NJ) in complete medium. Cells were resuspended at 1×10^6/ml in complete RPMI with IL-2, and with or without 100 μM BSO. After 3 days cells were pelleted, and infected by incubation for 45 min at 37°C with the supernatant from TNF-α-stimulated ACH-2 cells and 3 μg of Polybrene/ml. Cells were then washed twice with RPMI and resuspended at 1×10^6/ml in complete RPMI with IL-2 and supplemented with TNF-α (10 ng/ml), PMA (20 ng/ml), BSO (100 μM), and/or the indicated concentrations of NAC or OTC. After 48 hr, cells were pelleted, counted, and resuspended at 1×10^6/ml in medium supplemented identically for continued culture. Twenty-four hours later, aliquots of the medium were collected for p24 measurements by enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, Abbott Park, IL).

RESULTS

NAC inhibits HIV LTR-directed transcription, whereas NAS does not

Using a reporter cell system in which the HIV LTR directs expression of β-galactosidase, we have demonstrated previously that TNF-α- and/or PMA-induced HIV transcription is effectively inhibited by NAC (see Ref. 1; similar data from different experiments are presented in Fig. 1A and Fig. 3).

Because rather high (10–30 mM) concentrations of either D- or L-NAC are required to inhibit expression of the HIV LTR-I *lacZ* construct in the 293.27.2 cell line, we used *N*-acetylserine (NAS) to control for nonspecific inhibitory effects, for instance, effects of osmolarity. *N*-Acetylserine differs from NAC only in that it contains a hydroxyl group instead of a sulfhydryl group. As shown in Fig. 1A&B, NAC inhibits the TNFα- and/or PMA-induced activation of the HIV LTR, whereas the same concentrations of NAS show no inhibition. In addition, 30 mM OTC, a non-reducing cysteine precursor, also had no inhibitory effect. Thus, the inhibition is specifically brought about by the reducing thiol group of NAC rather than by nonspecific effects due to the presence of a high concentration of reagent.

NAC inhibits HIV LTR-directed transcription when GSH synthesis is blocked

N-Acetylcysteine is a GSH precursor[10,15] and could potentially inhibit HIV LTR-directed transcription by raising (or by preventing a decrease of) intracellular GSH. However, NAC is an antioxidant by itself and it could act as a direct reductant and free radical scavenger.[8] To try to distinguish between these possibilities, we carried out two different types of experiments. In the first experimental approach we utilized BSO, an inhibitor of γ-glutamylcysteine synthetase, one of the key enzymes in GSH biosynthesis.[16] Buthionine sulfoximine is frequently used to lower GSH levels in cells, but here we used it to ensure that NAC (or cysteine after deacetylation) will not be converted into GSH. The BSO-treated cells (100 μM, 48 hr) also have lower

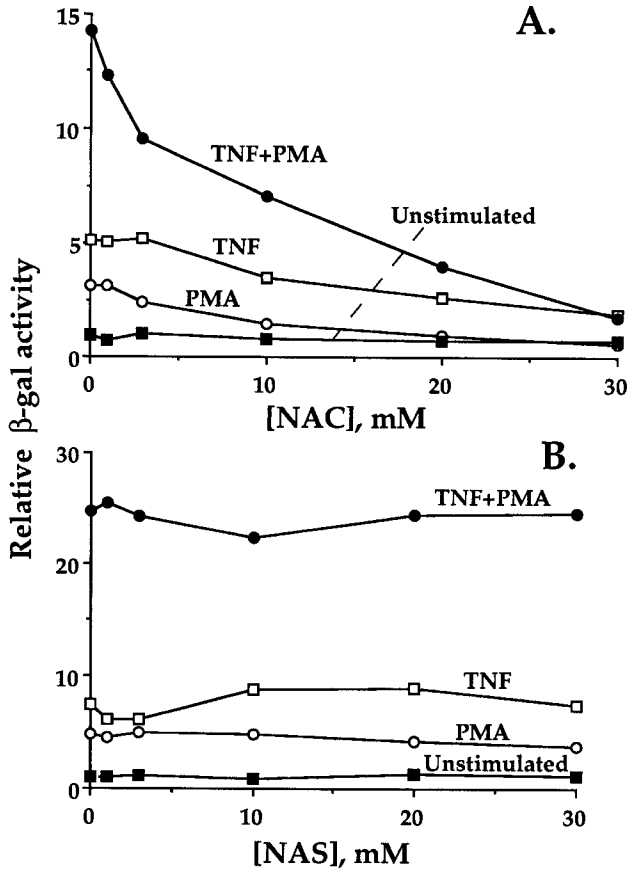

FIG. 1. NAC inhibits HIV LTR-directed transcription, but NAS does not. 293.27.2 cells, containing a construct in which the HIV LTR directs β-galactosidase expression, were stimulated as described in Materials and Methods. All stimulations were for 6 hr. Titration curves for NAC (A) and NAS (B) were established, showing that NAS does not inhibit HIV LTR-directed transcription in this reporter gene system, whereas NAC shows a concentration-dependent inhibition.

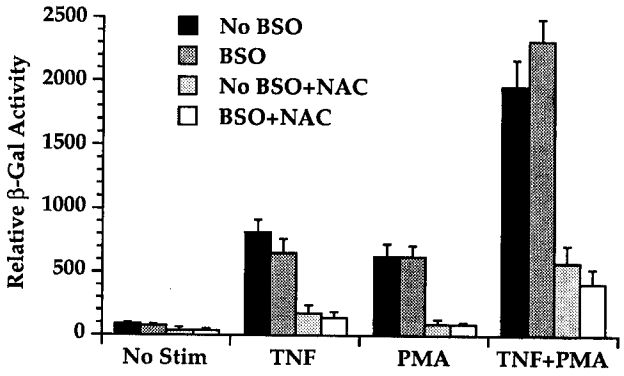

FIG. 2. NAC inhibits HIV LTR-directed transcription as measured by β-galactosidase in the presence of BSO. BSO inhibits biosynthesis of glutathione and lowers GSH levels in cells. Cells were pretreated with BSO (100 μM) for 2 days (see Materials and Methods). When 293.27.2 cells are cultured under conditions in which GSH synthesis is impossible, NAC still is inhibitory for HIV transcription. Error bars indicate the standard deviation of four replicates for each condition.

GSH levels (about 10% of normal, data not shown), showing that BSO is indeed active under these conditions. As shown in Fig. 2, NAC inhibits stimulation of the reporter construct in both BSO-treated cells and the parallel cultures that are untreated. The degree of inhibition by NAC is essentially the same with or without BSO. Thus, when *de novo* GSH biosynthesis is inhibited, NAC still inhibits stimulation of HIV LTR-directed transcription, suggesting that NAC works as a direct antioxidant under these conditions and not through conversion into GSH.

D-NAC inhibits HIV transcription to the same degree as L-NAC

In the second type of experiment we used the D-stereoisomer of NAC (D-NAC, *N*-acetyl-D-cysteine). This stereoisomer, which cannot be converted into GSH because the enzymes that are involved in GSH biosynthesis use only L-amino acids, has been used by others to address the same question (direct antioxidant or GSH precursor) in different experiments, for example, in studies using NAC as an antidote against acetaminophen-induced liver toxicity.[17] In those studies, the D-stereoisomer was ineffective, showing that conversion of L-NAC to GSH was required for protection. In contrast, we find that D-NAC and L-NAC both inhibit HIV LTR-directed transcription, and essentially to the same degree (Fig. 3). In fact, D-NAC is slightly more inhibitory, possibly because enzymes that metabolize L-NAC attack the D-form much less efficiently,[18] and thus higher levels of D-NAC are maintained. In conclusion, these experiments show in two independent ways that NAC can act as a direct antioxidant to inhibit HIV LTR-directed transcription in this reporter gene system.

Other antioxidants also inhibit HIV LTR-directed transcription

Because NAC apparently works as a direct antioxidant in the inhibition of HIV transcription, we determined whether other antioxidants could also inhibit HIV LTR-directed transcription in our β-galactosidase reporter gene system. Table 1 shows that L-cysteine and glutathione (GSH), but not their oxidized disulfides L-cystine and GSSG, inhibit HIV transcription. Other antioxidants that do not contain a sulfhydryl group, such as BHA, NDGA, vitamin E succinate, and reduced vitamin C (ascorbate), also inhibit the cytokine-stimulated HIV transcription. Inhibition of HIV transcription by ascorbate has also been reported by Harakeh and Jariwalla,[19] who have further demonstrated synergism between NAC and ascorbate *in vitro*. The BHA-related compound BHT did not inhibit HIV transcription. Both BHA and ascorbate are cytotoxic at concentrations 5- to 10-fold higher than their IC_{50} (50% inhibitory concentration). The thiol compounds were not found to be cytotoxic, but are cytostatic at concentrations higher than the IC_{50} for both TNF-α and PMA.

Oxothiazolidine-4-carboxylic acid is not an antioxidant, but is a GSH precursor, because it is converted intracellularly to L-cysteine through the action of 5-oxoprolinase.[20] Cysteine is subsequently used to synthesize GSH. This compound did not inhibit HIV transcription in the reporter gene model (Table 1);

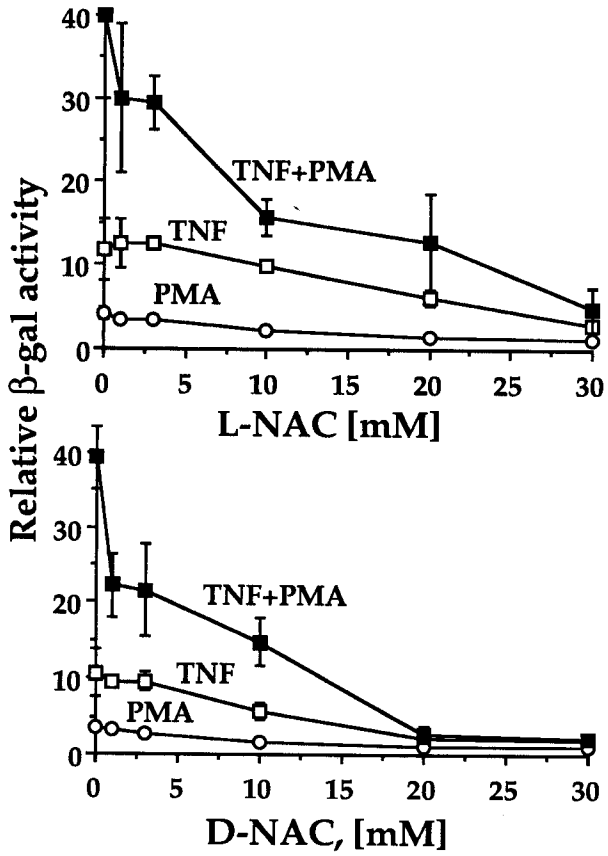

FIG. 3. L-NAC (*top*) and D-NAC (*bottom*) both inhibit HIV LTR-directed transcription measured as β-galactosidase activity. The D-stereoisomer of NAC cannot be used for GSH biosynthesis, but still inhibits HIV transcription to the same degree as the L-stereoisomer. For experimental details see text. Error bars indicate the standard deviation of five replicates for each point.

apparently the 5-oxoprolinase activity is too low to provide enough cysteine (within the time span of the assay), which then could act as an antioxidant.

We also tested DDTC (Ditiocarb sodium, Imuthiol), a non-thiol-containing antioxidant with strong metal-chelating properties that has been used in clinical trials for HIV infection.[21] We observed strong inhibition of HIV transcription at low concentrations (IC_{50}, ~10 μM); however, even slightly higher concentrations were toxic to the cells. In conclusion, we show that a wide variety of antioxidants (sulfhydryl-containing compounds, nonsulfur antioxidants, and metal chelators) are capable of inhibiting HIV transcription *in vitro*. This supports the assertion that, in this model system, GSH synthesis is not required for inhibition by NAC.

Both L-NAC and D-NAC inhibit NF-κB activation

We and others have reported previously that L-NAC can inhibit the cytokine-induced activation of the cellular transcription factor NF-κB.[3,6,7] This transcription factor is used by HIV to markedly up-regulate transcription of its genes and to enhance viral replication.[22] Because D-NAC inhibits HIV transcription in a β-galactosidase reporter gene system (as described above), we tested if D-NAC can also inhibit activation of the NF-κB transcription factor. Nuclear protein extracts were prepared from 293.27.2 cells stimulated with TNF-α, PMA, or both in the presence or absence of 30 mM L- or D-NAC and gel retardation assays were done. Both enantiomers effectively inhibited activation of NF-κB, and both are more effective in inhibiting PMA-induced activation than TNF-induced activation (Fig. 4). The inhibition of NF-κB activation by both stereoisomers is in agreement with data from the HIV LTR–reporter gene experiments and provides a partial explanation in terms of a molecular mechanism for the observed inhibition of HIV transcription in that system.

TABLE 1. INHIBITION OF HIV LONG TERMINAL REPEAT-DIRECTED TRANSCRIPTION BY ANTIOXIDANTS[a]

Compound	*Toxic level*[b]	*Approximate IC_{50} for stimulation*		
		TNF-α	*PMA*	*TNF-α + PMA*
NAC (L- and D-)	>50 mM	15 mM	7 mM	15 mM
L-Cysteine	20 mM	15 mM	7 mM	15 mM
L-Cystine10 mM	10 mM		No inhibition	
GSH	>50 mM	20 mM	20 mM	10 mM
GSSG	10 mM	*	*	*
DDTC	0.02 mM	0.10 mM	0.010 mM	0.010 mM
NDGA	0.30 mM	0.1 mM	0.005 mM	0.1 mM
Vitamin E succinate	0.25 mM	0.1 mM	0.025 mM	0.1 mM
Ascorbate (vitamin C)	6 mM	5 mM	3 mM	3 mM
BHA	1 mM	0.1 mM	0.050 mM	0.05 mM
BHT	1 mM	*	*	*
OTC	>30 mM	*	*	*

[a] 293.27.2 cells were stimulated with TNF-α (10 ng/ml), PMA (20 ng/ml), or both as described in Methods. A range of concentrations of the above compounds was tested and the concentration that gave 50% inhibition (IC_{50}) of HIV transcription was determined from the titration curves. Compounds that showed no inhibition were tested for concentrations up to 30 mM (GSSG, L-cystine, OTC) or until cytotoxic levels were reached (millimolar range for BHT).

[b] Toxic levels were defined as viability < 90% after a 24 hr incubation period with the compound.

*No inhibition

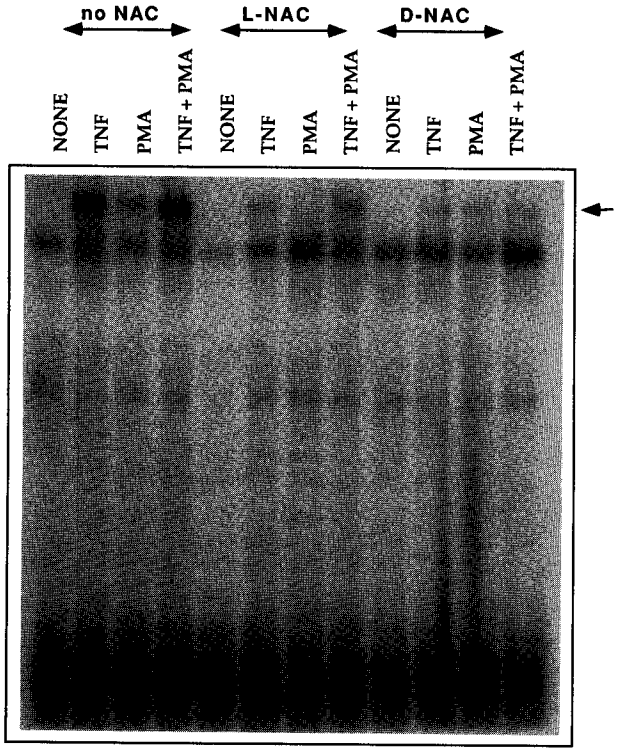

FIG. 4. D-NAC inhibits NF-κB activation to the same degree as L-NAC. Nuclear protein extracts were prepared from 293.27.2 cells after stimulation for 2 hr in the presence or absence of D-NAC or L-NAC. Retardation assays were done as described in Materials and Methods. The arrow indicates the inducible NF-κB band; free probe runs at the bottom of the gel.

Acute, in vitro HIV infection is inhibited by both D-NAC and L-NAC

Results described above establish the efficacy of D-NAC in model systems for HIV infection. We have demonstrated earlier that L-NAC inhibits HIV infection of normal (HIV^-) PBMCs at low (<1 mM) concentrations. As shown in Table 2, D-NAC is as effective as L-NAC in inhibiting viral replication after acute infection *in vitro*. In fact, a 1 mM concentration of either stereoisomer inhibits, virtually completely, viral production in this experiment. Data from other experiments with L-NAC are given in Table 3, showing that 1 mM L-NAC inhibits viral replication by ~90%. In contrast, the GSH prodrug OTC, even at a 10 mM concentration, only partially inhibits HIV replication (Table 3), again demonstrating the importance of antioxidant properties for the compounds that efficiently inhibit HIV replication and transcription.

TABLE 2. *N*-ACETYL-D-CYSTEINE AND *N*-ACETYL-L-CYSTEINE INHIBIT HIV REPLICATION IN VITRO[a]

Stimulation condition	*No antioxidant p24 production*	*L-NAC*[b]	*D-NAC*[b]
No stimulation	40	<0.1	<0.1
TNF-α	60	<0.1	<0.1
TNF-α + PMA	107	<0.1	<0.1
BSO	57	<0.1	<0.1
TNF-α + BSO	117	<0.1	<0.1
TNF-α + PMA + BSO	131	<0.1	<0.1

[a] Normal (HIV^-) PBMCs were infected *in vitro* with HIV as described in Materials and Methods. Inhibition measured as p24 production (in nanograms per milliliter).
[b] ImM of both D-NAC and L-NAC was added.

TABLE 3. INHIBITION OF STIMULATED VIRAL REPLICATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS BY *N*-ACETYLCYSTEINE AND L-2-OXOTHIAZOLIDINE-4-CARBOXYLIC ACID[a]

Treatment	*Concentration (mM)*	*HIV production (% of untreated)* *Expt. 1*	*Expt. 2*	*Expt. 3*
NAC	1	15	13	8
OTC	1	74	88	nd
	3	62	nd	nd
	10	46	75	43
	30	5	nd	nd

[a] Peripheral blood mononuclear cells were prepared and infected as in Materials and Methods, and stimulated with TNF (10 ng/ml) and PMA (20 ng/ml) with or without the indicated concentrations of NAC or OTC. The amount of p24 produced in the presence of NAC or OTC is shown as a percentage of that produced without either drug. nd, not done.

GSH depletion enhances HIV production

Because HIV-infected individuals have low levels of intracellular GSH in T cells,[23] we tested the effect of lowering GSH levels in normal PBMCs on acute viral infection. When the cells are cultured in the presence of BSO (100 μM), intracellular GSH levels are lowered to about 60% of normal under these conditions (data not shown). Under all conditions, cell viability and growth were identical. Data in Table 2 show that the viral production is enhanced in cells with lower GSH levels (as compared to cells with normal GSH levels). This enhancement is observed under stimulated and unstimulated conditions. This suggests that the low GSH levels seen *in vivo* (in patients) may contribute to a higher rate of HIV replication.

DISCUSSION

Studies presented here demonstrate that the cytokine-stimulated signal transduction pathway leading to NF-κB activation and HIV transcription is redox regulated. In previous studies, we have shown that the transduction of these signals is inhibited by NAC, an antioxidant that is efficiently converted to GSH and used medically to replenish GSH. The key regulatory role suggested for GSH by these findings is confirmed here by studies that show that depletion of GSH increases HIV expression in PBMCs. In addition, studies presented here demonstrate that NAC works as a direct antioxidant to inhibit HIV transcription and replication. A number of other antioxidants with different chemical structures also inhibit stimulated HIV transcription (in a reporter gene system).

The most likely way that L-NAC could be useful for treatment *in vivo* is through restoration of GSH levels. HIV-infected people have lower plasma and intracellular GSH (reviewed in Ref. 24), and NAC may restore this deficiency. High GSH levels may keep the virus in a silent state (by blocking inappropriate NF-κB activation) and prolong clinical latency. The potential use of L-NAC as a therapeutic agent for HIV infection has been reviewed elsewhere.[24–27] Because D-NAC is not a GSH precursor, we do not regard it as a potential therapeutic agent for HIV disease.

N-Acetylcysteine (and other antioxidants) could have an intracellular or extracellular mode of action. Because D-NAC is deacetylated at a much slower rate than L-NAC,[18] the intracellular action of D-NAC is likely through D-NAC itself, whereas L-NAC probably partially acts as L-cysteine. (Note that L-NAC, D-NAC, and L-cysteine have identical IC_{50} values; see Table 1). These findings confirm and extend the observations from Droge's laboratory that GSH may not be the sole low molecular weight thiol that can modulate HIV expression.[3] We cannot exclude the possibility that L-NAC and D-NAC act extracellularly to facilitate equally the uptake for cystine from the medium into the cells. Precedence for such a mechanism comes from studies by Issels *et al.*,[28] who examined the uptake of [^{35}S]cystine and GSH synthesis in CHO cells. On adding either cysteamine or L-NAC they found increases in radioactivity in the cells and increases in GSH synthesis, supporting the conclusion that NAC and cysteamine acted as delivery systems for cystine from the medium outside the cells into the cells. However, because L-NAC is readily taken up by cells and converted into cysteine,[29] an intracellular mechanism seems more likely, especially because other chemically distinct antioxidants that will not facilitate cystine uptake also inhibit HIV transcription.

Taken together with earlier data from Baeuerle's group that hydrogen peroxide can directly activate NF-κB and that NAC can block NF-κB induction by a wide variety of agents,[7] there is a substantial body of evidence implicating a redox-sensitive step in the signal transduction leading to NF-κB activation and HIV transcription. The molecular nature of such step(s) is as yet unknown, although several can now be ruled out. Schreck et al.[30] reported that the antioxidant pyrrolidine dithiocarbamate (PDTC) strongly inhibits NF-κB activation and HIV transcription but does not interfere with nuclear uptake of NF-κB or with the release of Inhibitor of NF-κB (IκB). In addition, it has been shown that NAC does not interfere with *in vitro* binding of NF-κB to its DNA site.[7] These findings locate the redoxsensitive step within the actual signal transduction pathway leading to release of IκB from NF-κB. Consistent with this hypothesis, preliminary data indicate that there may be a redox-regulated tyrosine phosphorylation step in the transduction of certain signals leading to NF-κB induction (Staal *et al.*, manuscript in preparation).

These findings might be viewed as inconsistent with evidence from three reports demonstrating redox effects on NF-κB binding to DNA *in vitro* (as opposed to regulation of NF-κB activation in intact cells).[31–33] In these reports, oxidants decrease *in vitro* binding to the κB site and reducing agents increase this binding. However, we have shown the opposite for *induction* of NF-κB in intact cells, that is, oxidants stimulate NF-κB activation and reducing agents block this stimulation. This difference can be understood by recognizing that the mechanisms that increase NF-κB binding activity *in vitro* are probably unrelated to the mechanisms that increase the amount of activated NF-κB in the cell. Alternatively, the *in vitro*-binding studies may have relevance to the actual situation within the cell, if one assumes that different redox environments exist in the cytoplasm and the nucleus. Data from Orrenius's group, showing a three-fold higher GSH concentration in the nucleus as compared to the cytoplasm,[34] give some support for this notion.

Transcription factors other than NF-κB can be regulated by redox mechanisms as well. Curran's group has described that the DNA binding of Fos and Jun is redox regulated.[35,36] Reduction of a conserved cysteine residue in the DNA-binding domain of Fos and Jun is required for AP-1 binding. A nuclear redox factor, named Ref-1, has been identified[37] and cloned[38] and is probably responsible for reduction of this cysteine residue. Intriguingly, Ref-1 can also stimulate the DNA-binding activity of NF-κB, as well as other transcription factors (Myb, CREB, ATF-1, ATF-2).[38]

Oxidative stress [e.g., hydrogen peroxide and ultraviolet (UV) irradiation], induces activation of c-*fos* and c-*jun* in intact cells.[39,40] Thus redox regulation of AP-1 is similar to redox regulation of NF-κB, in that oxidation can induce or activate the transcription factor, whereas reduction seems to be required for DNA binding, at least *in vitro*. This again illustrates the complexities of redox regulation, especially in intact cells in which different signal transduction pathways, redox control systems, and cell compartments all contribute to the final readout. In addition to NF-κB and AP-1, the DNA-binding activity of certain other transcription factors, including OxyR,[40] TFIIIC,[41] and some steroid receptors,[42,43] is redox regulated. Thus redox mechanisms may provide a means for control of signal transduction pathways and posttranslational regulation of transcription factor function.

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