REDOX REGULATION OF NF-κB ACTIVATION AND THEREFORE OF HIV EXPRESSION

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An elaborate cell-to-cell communication network coordinates the growth, differentiation and metabolism of cells that make up multicellular organisms. Hormones, growth factors, neurotransmitters and cytokines are used as extracellular signals to effect intracellular targets, either to modify the activity of enzymes already present in the responding cells or to influence the pattern of expressed genes. The sequence of events from detection of the signal by a specific receptor in the cell membrane, transduction of the signal through the cell membrane into the cytoplasm, to the response in form of altered gene expression, is collectively called signal transduction. Many different ways to transduce signals have evolved (e.g., generation of second messengers like cAMP or inositol phosphates, activation of protein kinases, opening of ion channels) and proper regulation of these events is crucial for normal cell physiology. Here we will discuss the signal transduction pathways leading to the activation of the transcription factor NF-κB, which regulates the transcription of many immunologically important genes, including those controlled by the HIV LTR.

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N-ACETYLCYSTEINE (NAC) INHIBITS NF-κB ACTIVATION. The NF-κB transcription factor was first identified as a nuclear factor that binds to the κB enhancer of the kappa light chain (1) and was subsequently shown to greatly increase HIV transcription and replication (2). Glutathione (GSH), the primary intracellular defense against oxidative stress (3), regulates the transduction of cytokine generated signals that activate NF-κB (4-6). Diamide pretreatment depletes intracellular GSH and markedly facilitates the cytokine-stimulated NF-kB activation (4). By contrast, N-acetyl-L-cysteine (NAC), a non-toxic drug used clinically to replenish GSH and scavenge intracellular oxidants, replenishes intracellular thiols and inhibits or prevents NF-κB activation (4-6). Moreover, oxidants (e.g., hydrogen peroxide) directly activate NF-κB and NAC inhibits this activation in some cell types (5). Blocking activation of NF-κB inhibits cytokine stimulated expression of genes controlled by the HIV LTR (7) and the replication of HIV in chronic and acute infection models (8). We have used a reporter gene system, consisting of the HIV LTR fused to LacZ (which encodes β-galactosidase) to study the effects of cytokines and antioxidants on HIV transcription (7). An example from one such an experiment is given in Fig. 1, which shows that both the TNFα- and PMA- induced HIV-LTR-directed transcription is inhibited by NAC.

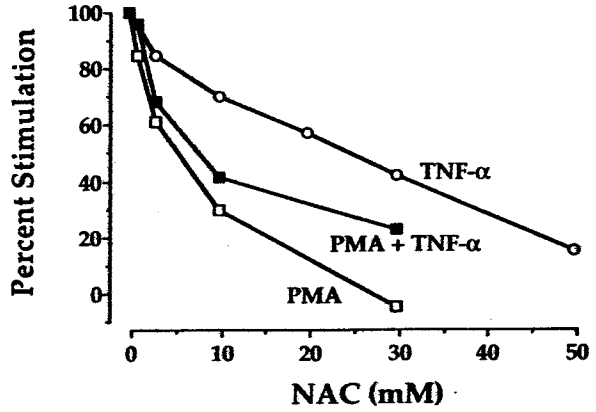

Fig. 1: NAC inhibits TNFα and PMA stimulated HIV transcription. *293 cells containing a construct consisting of the HIV LTR fused to LacZ (293.27.2 cells) were stimulated with TNFα (10 ng/ml) or PMA 20ng/ml) or both for 6 hrs in absence or presence of NAC. B-galactosidase activity was measured as a readout for HIV LTR-directed transcription. Stimulation in the absence of NAC is set as 100%. Reproduced from (7).*

Rather high concentrations of NAC are needed to inhibit HIV transcription in this model system, however in another model system we have used, acute infection of normal peripheral blood mononuclear cells (PBMC), concentrations of 300-500 μM efficiently inhibit HIV expression, as measured by the production of the viral core protein p24 (Fig. 2).

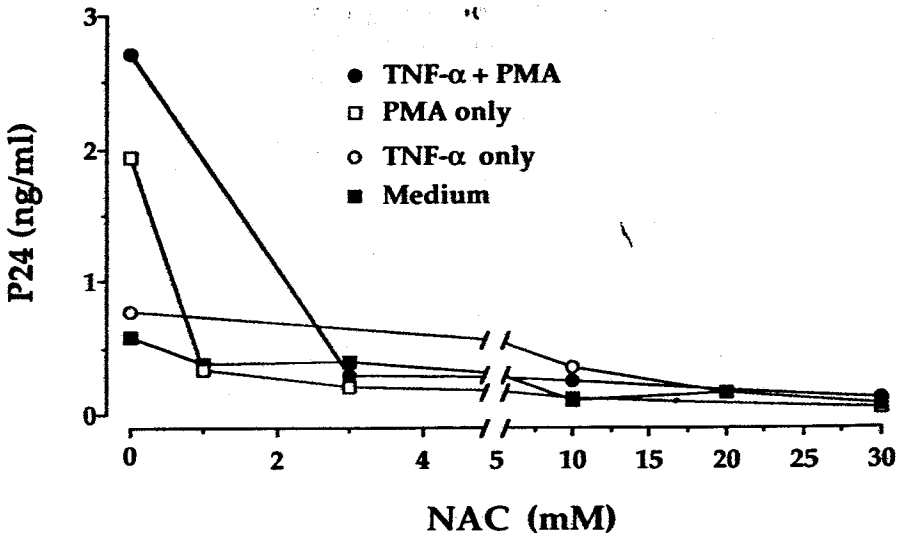

Fig. 2: NAC inhibits HIV expression in an actue infection model. *Normal, uninfected PBMC were infected with HIV in vitro. Cells were stimulated with TNFα and/or PMA and viral expression was assayed by measuring the production of the viral core protein p24 by ELISA. Reproduced from (7).*

Recently, we have extended these findings, showing that NAC inhibits NF-κB activation and HIV LTR-directed transcription under conditions where GSH is depleted and NAC cannot be converted to GSH (9). In addition, the D-stereoisomer of NAC, which is not a GSH precursor, and a wide variety of chemically unrelated antioxidants also inhibit NF-κB activation and/or transcription directed by the HIV LTR (9). Taken together, this suggests direct antioxidant action as the mechanism for inhibition of HIV transcription by NAC. In this study, we also demonstrated

that lowering GSH, the principal intracellular antioxidant, augments HIV production in the acute infection model of PBMC (9). From these findings we conclude that GSH, acting in its capacity as an antioxidant, regulates HIV expression and that exogenous antioxidants can potentiate this regulation.

These findings, which implicate the production of oxidative species in signal transduction of inflammatory cytokines, may have profound clinical implications. HIV-infected individuals have lower GSH levels in plasma (10), lung epithelial lining fluid (11), and intracellularly in CD4 and CD8 T cells (12). Since oral administration of NAC restores GSH levels in these individuals, we have suggested that NAC (a non-toxic and widely used drug approved for other purposes), be tested as a therapeutic agent for HIV infection. We have reviewed the possible use of NAC as a therapeutic agent in HIV-disease extensively elsewhere (13, 14).

OXIDATIVE ACTIVATION OF NF-κB. Taken together with 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 (5), 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 possibilities can now be ruled out. Schreck et al. recently reported that the antioxidant pyrrolidine dithiocarbamate (PDTC) strongly inhibits NF-kB activation and HIV transcription but does not interfere with nuclear uptake of NF-κB or with the release of IκB (15). In addition, we have shown that NAC does not interfere with *in vitro* binding of NF-κB to its DNA site, and if binding reactions are done under suboptimal (< 5 mM) DTT concentrations, NAC actually increases *in vitro* binding to the DNA. These findings locate the redox-sensitive step within the actual signal transduction pathway leading to release of IκB from NF-κB.

Consistent with this we have recently obtained evidence that other oxidative stimuli can also activate NF-κB (16). Thus, in a T cell line that responds to H_2O_2 by the induction of NF-κB other oxidative stimuli like UV-irradiation or the oxidative DNA-damaging agent mitomycin C also induce NF-κB. Interestingly, a T cell line that does not respond to H_2O_2 stimulation by the induction of NF-κB also fails to respond to UV-irradiation and mitomycin C.

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 (17-19). In these reports, oxidants decrease *in vitro* binding to the κB site and reducing agents (e.g. DTT, thioredoxin) increase this binding. Significantly, Yodoi's group identified cysteine residue 62 in the NF-κB p50 subunit as a likely candidate-site for the redox effects on *in vitro* DNA binding (19). However, we have shown the opposite for induction of NF-κB in intact cells, i.e., oxidants stimulate NF-κB activation and reducing agents block this stimulation. In other words, in intact cells a whole signal transduction cascade needs to be induced to activate NF-κB and some steps of this signal transduction cascade are redox-sensitive. 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. Recent data from Orrenius' group, showing a three-fold higher GSH concentration in the nucleus as compared to the cytoplasm (20), give some support for this notion.

NF-κB ACTIVATION IS CONTROLLED BY TYROSINE PHOSPHORYLATION AND REDUCTION/OXIDATION. Elsewhere, we show that tyrosine phosphorylation in T lymphocytes is under redox control because of the redox sensitivity of phosphotyrosine phosphatases (PTP) (21). Thus, the GSH depleting agent and oxidant diamide, increases tyrosine phosphorylation in both Jurkat T cells and in primary lymphocytes (PBL) (21). When PBL are treated with buthionine sulfoximine (BSO), an inhibitor of GSH synthesis which specifically lowers GSH levels, tyrosine phosphorylation by a wide variety of agents is enhanced, demonstrating that GSH plays a direct role in the regulation of a signal transduction pathway (tyrosine phosphorylation). Using *in vitro* tyrosine kinase (TK) and PTP assays, we could provide evidence that the redox sensitive step is located at the PTP, not the TK (21).

Here we show that TKs are involved in the activation of NF-κB. Certain forms of IκB contain consensus tyrosine phosphorylation sites (22), making it conceivable that the NF-

κB/IκB complex can be activated by tyrosine phosphorylation. Since tyrosine phosphorylation is redox-regulated (e.g., oxidants like diamide increase phosphorylation, antioxidants and reducing agents decrease it), we investigated the involvement of TKs in the activation of NF-κB. Using the TK inhibitor Herbimycin A, we found that both the PMA and TNFα induction of NF-κB is completely inhibited in the presence of Herbimycin (Fig. 3a). The induction of NF-κB by IL-1 and in hydrogen peroxide-responsive cell lines by H_2O_2 is also blocked by Herbimycin (Anderson and Staal, manuscript in preparation). This implicates the involvement of TKs in the induction of NF-κB.

PMA is believed to activate PKC which may directly phosphorylate IkB on serine and threonine residues; however, the PMA induction of NF-κB is also inhibited by Herbimycin A. To assure that Herbimycin A does not inhibit all signal transduction pathways involving protein kinases, we investigated the induction of AP-1 (fos/jun), which is the classical transcription factor induced by phorbol esters (23). Probing the same extracts as we used for NF-κB with an AP-1 probe, we found that the PMA induction of AP-1 is not inhibited by Herbimycin (Fig. 3b). TNFα did not efficiently induce AP-1 in Jurkat T cells. Taken together, this shows that Herbimycin A selectively inhibits the induction of NF-κB in Jurkat cells. In contrast, it has recently been reported that the induction of NF-κB by PMA is not inhibited by Herbimycin (but the IL-1 induction is) in the pre B cell line 70Z/3 (24). This may be because of differences between B and T cells in the signal transduction leading to NF-κB activation.

A MODEL FOR THE REDOX REGULATION OF NF-κB ACTIVATION. The finding that NF-κB activation can be controlled by tyrosine phosphorylation provides a molecular mechanism for the redox-sensitivity of its activation. For instance, TNFa-induced NF-κB activation is partly inhibited by both the antioxidant NAC and the TK inhibitor Herbimycin. Indeed, TNFα increases tyrosine phosphorylation in Jurkat T cells and this phosphorylation is partly blocked by NAC (21). Baeuerle and colleagues have shown that NAC inhibits the activation of NF-κB by a wide variety of stimuli (5). Similarly, Herbimycin A inhibits the TNFα-, IL-1-, H_2O_2-, and PMA- induced NF-κB activation. (Fig. 3 and data not shown). PMA activates PKC, which *in vitro* phosphorylates IκB, however many different protein

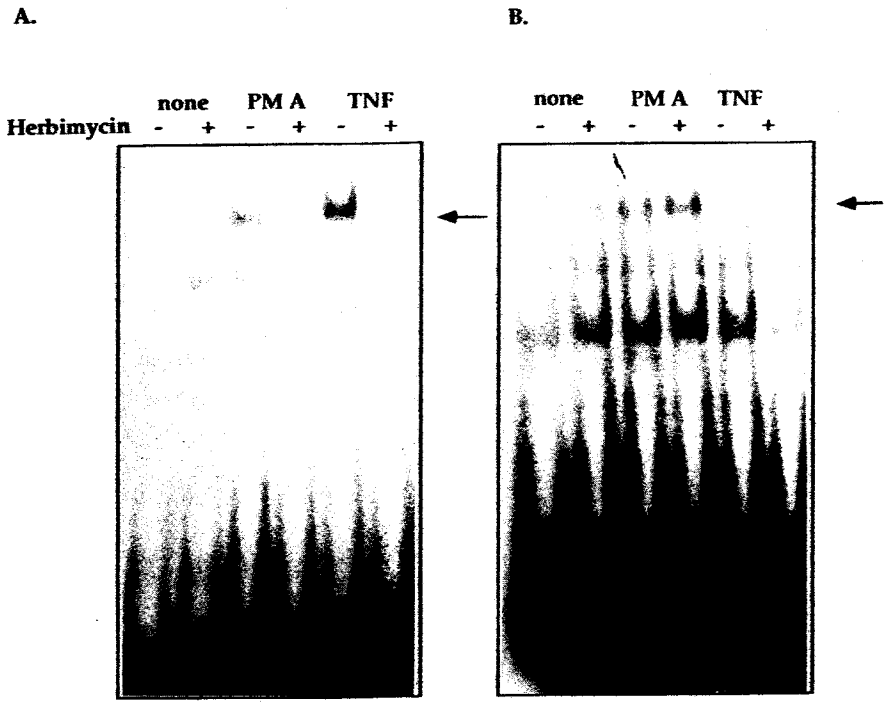

Fig. 3: Herbimycin A inhibits NF-κB activation but not PA-1 activation in Jurkat T cells. *Jurkat T cells were pretreated with TK inhibitor Herbimycin A and stimulated with TNFα or PMA. Nuclear protein extracts were prepared and activation of transcription factors NF-κB and AP-1 was analyzed by gel retardation assay. The arrow indicates the inducible upper band in both figures. (a) Induction of NF-κB in Jurkat T cells. (b) Induction of AP-1. The same nuclear protein extracts used under (a) were probed with an AP-1 probe.*

kinases do so *in vitro* (25). From our results, we conclude that in intact cells PKC is not directly involved in NF-κB/IκB phosphorylation, but PKC may in turn activate a TK that subsequently phosphorylates IκB. Support for this notion comes from studies by Schreck et al., demonstrating that the antioxidant pyrrolidine dithiocarbamate does not inhibit AP-1 induction by PMA, but does inhibit NF-κB activation (15).

We therefore propose a model in which some stimuli (e.g., cytokine-receptors directly, phorbol esters via PKC) activate a TK, which phosphorylates IκB and then activates NF-κB by dissociation of the complex. This induction is redox-sensitive because the net tyrosine phosphorylation of the IκB substrate is the result of a balance between the actions of TKs and PTPs, and PTPs are redox-sensitive. Thus oxidants (diamide) inhibit the action of PTPs and indeed augment NF-κB dependent transcription (4), whereas reducing agents and antioxidants (NAC, DTT, GSH) enhance phosphatase activity and thus decrease both IκB phosphorylation and NF-κB activation. Alternatively, a step more upstream from the IκB phosphorylation may involve tyrosine phosphorylation and therefore may be redox-controlled.

We cannot exclude the possibility that other stimuli phosphorylate some forms of IκB on serine/threonine residues and activate NF-κB in this way. This may be true for forms of IκB that do not have a tyrosine phosphorylation site. Direct evidence for phosphorylation of IκB on tyrosine residues (or other amino acids for that matter) may be difficult to obtain experimentally, because IκB is rapidly degraded after phosphorylation (26, 27).

In conclusion, in this report we have shown that the signal transduction pathways leading to the activation of NF-κB are regulated by reduction/oxidation. We propose a model in which tyrosine phosphorylation is required at a crucial step in the signal transduction pathway of NF-κB possibly the phosphorylation of IκB. Because PTPs are redox-regulated, the induction of NF-κB is redox controlled as well. We anticipate that a fine interplay between redox type mechanisms and tyrosine phosphorylation exists in many signal transduction pathways (not only those leading to NF-κB) and in cell growth and differentiation.

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Discussion

Q. Nakashima: I'm very much interested in the tyrosine phosphorylation promotion by diamide. But I wonder if the inhibition of the phosphatase activity is the only mechanism of the tyrosine phosphorylation promotion. Actually, we and Dr. Yodoi independently, using different systems, have observed tyrosine phosphorylation promotion by other kinds of thioreactive substances. Have you compared the action of diamide with the action of LiCl as another inhibitor of tyrosine phosphatase?

A. Leonard Herzenberg: We have not used LiCl, we have used BSO to decrease glutathione levels. We've used some other compounds which would give rise to cystine. Studies are actually rather recent and we don't have the whole story characterized.

Q. Nakashima: We once used LiCl for inhibiting the tyrosine phosphatase activity. But the activity was very limited as compared to the very strong action of other oxidizing agents. We are using $HgCl_2$, and actually we have got about 100 times more tyrosine phosphorylation than is induced by other compounds.

A. Leonard Herzenberg: Well, as was shown by Klausner, phenylarsine oxide couples dithiols very nicely and inactivates them. It can also act with monothiols and a compound that Gitler has made which is IAIT, basically a phenyltyrosine analog with an iodine on it, so it can be iodinated. That will react with dithols. People talk mostly about kinase stimulation and its inhibition, so I think this is an area worth exploring some more.

Q. Klausner: I think it's important as redox regulation appears to be of great interest in lots of systems, to be very careful about what one means by redox regulation. One has to distinguish redox regulation, especially via the glutathione system, with the ability to affect the activity of proteins by thioreactive drugs. Diamide really is not a redox reactive reagent. It forms by chemical modification of a sulphanyl hydrozine intermediate, which is extremely unstable in water, and decays into a sulfone irreversibly, unless there are vicinal or nearby sulfhydryls, and then it actually chemically

catalyzes disulphide conversion. It has no relationship necessarily with the glutathione system. Redox is very complex. The oxidation potential of particular thiols will determine their reactivity to particular types of redox buffers such as glutathione, which is a relatively weak one. Phenylarsine oxide binds to vicinal sulfhydryls, but is not a signature, nor is diamide, of redox regulation. I think it's much more important to think about these things as attacking critical sulfhydryls with very tight binding affinities, or in the case of diamide, as a covalent sulphanyl hydrazine bond. One needs to dissect out these drugs and inhibitors, keeping in mind that there are fundamental differences between redox regulation and the effect of reactive oxygen species or reduced oxygen intermediates, and the ability to affect protein function by actually modifying critical cysteines, as with CD 45 and some other phosphatases. It's a complex problem, and it's important to not lump all thio reactive reagents into a concept of redox regulation.

A. Leonard Herzenberg: It is very complex. Dr. Yodoi and others have shown *in vitro* that you can use thioredoxin to have the opposite effects on NF-κB activation. Of course, you need DTT, you need a reducing environment for the activity of NF-κB to bind to DNA *in vitro*. That does not say anything about what happens *in vivo*, in terms of the signal transduction mechanism. To try and figure out the signals, what are the elements in signal transduction, is only a beginning. But I think it is interesting to note that nobody thought before in terms of redox regulating transcription factors or regulating gene expression. The first paper I saw was Tom Curran's, showing that, if you inactivate or mutate a cystine, you would not get a Fos/Jun interaction. I welcome your participation in this area. We should have a clarifying discussion, to determine what are good criteria for seeing what gene expression systems are regulated by reduction and oxidation.

Q. Virelizier: Dr. Naomi Wakasugi during her stay in our laboratory has made, in collaboration with Dr. R. T. Hay (St Andrews, Scotland) the following observation, recently reported in Nucleic Acids Research. Recombinant human Thioredoxin (from Dr. J. Yodoi) increases the binding of recombinant NF-κB p50 protein to the HIV enhancer, through an effect on a disulphide bond involving cysteine 62. Furthermore, transfection of a thioredoxin-expressing vector upregulates the activity of the HIV-LTR, but not that of a κB-deleted LTR construct. This opens up the intriguing possibility that trasncription of the Thioredoxin gene, induced in normal T lymphocytes during activation (unpublished), participates in the control of NF-κB activity through post-translational effects on p50. How this phenomenon relates to the modification of the redox equilibrium of the cells induced by either NAC or free radical scavengers such as BHA (N. Israël *et al*, J. Immunol., 1992) is not understood. Thioredoxin is a reducing molecule induced and produced physiologically. It may thus be too simplistic to assume that oxidation

increases and reduction decreases NF-κB activity. The reverse situation must be envisaged, at least in the nucleus.

A. Leonard Herzenberg: These results are, of course, opposite in interpretation to what we have seen. We find oxidants increase HIV transcription through increased NF-κB activation. If transfected, thioredoxin increases NF-κB activation, perhaps because the large amount of thioredoxin interacts with NF-κB proteins. The reduction provided *in vitro* (in the DNA binding assay) may cause more gel shifting. Are your gel shifting assays done with adequate DTT? If not, this could be the explanation.