Effects of Bio Normalizer on nuclear factor-kB activation and HIV transcription

Synopsis

Acquired immunodeficiency syndrome (AIDS) results from infection of T cells with a virus called human immunodeficiency virus (HIV). Nuclear factor kappa B (NF-κB) is know to be a regulator of the activation of infected HIV leading for the virus to spread to other cells. In order for NF-κB to influence HIV, it needs to be activated. The mechanisms of how NF-κB is activated is not yet well understood, however, recent research suggests that reactive oxygen species (ROS) may be involved. If ROS cause NF-κB activation, then antioxidants which eliminate ROS should inhibit NF-κB activation. Inhibition of NF-κB activation would in turn inhibits spreading of HIV. In this project, Bio Normalizer will be tested to see if it is effective in inhibiting NF-κB and HIV activation.

Background and Rationale

Acquired immunodeficiency syndrome (AIDS) results from infection with a human immunodeficiency virus (HIV-1 or HIV-2) that eventually destroys a specific subset ($CD4^+$) of helper T lymphocytes, so that the patient ultimately succumbs to opportunistic infection and/or certain neoplasms. The long terminal repeat (LTR) region of HIV-1 proviral DNA contains two binding sites for the transcription-enhancing factor, nuclear factor kappa B (NF-κB). NF-κB is a mammalian transcriptional activator that is directly involved in the transmission of various signals from the cytoplasm to the nucleus (Baeurele, 1991). It was first isolated as a constitutive nuclear factor found in mature B cells involved in activation of immunoglobin κ light chain gene expression. NF-κB activates transcription by binding to the sequence 5'-GGGACTTTCC-3' in the κ enhancer where it presumably interacts with some components of the general transcription apparatus. Subsequent studies revealed that an inducible form of NF-κB is found in numerous cell types of both lymphoid and non-lymphoid origin, where it participates in activation of genes involved in the immune, inflammatory, or acute phase responses, such as various cytokines and surface receptors as well as viruses including HIV-1 (Osborn et al., 1989). A variety of stimuli have found to induce NF-κB activation including protein kinase activators (phorbol esters), protein synthesis inhibitors (cycloheximide, anisomycin), double-stranded RNA, lectins (phytohaemagglutinin, concanavalin A) and cytokines (interleukin-1, tumor necrosis factor α).

In cells with constitutive NF-κB activity such as mature B lymphoblasts, NF-κB is isolated as a heterodimer of 50 and 65 kDa proteins (p50p65) which possesses DNA binding and transcriptional activation capabilities. In cells which have inducible NF-κB activity, the factor is isolated in a crypic, inactive state from

the cytoplasm. It consists of p50p65 bound by an inhibitor called IκB and can be activated *in vitro* by either biochemically fractionating p50p65 from IκB or by disrupting the complex by disassociating agents such as deoxycholate (Baeuerle and Baltimore, 1988). After induction by stimuli such as treatment of cells with phorbol esters, the amount of cytoplasmic NF-κB-IκB complex is greatly reduced and the amount of nuclear NF-κB is increased. Since phorbol esters activate NF-κB, it is thought that protein kinase C phosphorylates IκB and that this modification destroys the ability of IκB to inhibit NF-κB DNA binding.

Reactive oxygen species (ROS) have recently been suggested to be involved in the signal transduction pathway which leads to the activation of NF-κB and subsequent HIV-1 activation. This was first discovered by Herzenberg and co-workers who observed that *N*-acetylcysteine (NAC) inhibited HIV LTR-directed expression of β-galactosidase enhanced by tumor necrosis factor-α (TNF-α) and phorbol ester in *in vitro* HIV model systems (Roederer et al., 1990), and subsequent findings that NAC inhibited and diamide stimulated NF-κB activation (Staal et al., 1990). NAC is known to increase the intracellular level of reduced glutathione, while diamide increases the amount of oxidized glutathione and depletes reduced glutathione. The finding that NAC blocks TNF-α induced NF-κB responses was confirmed by Mihm et al. (1991). Schreck et al. (1991) extended these findings by showing that cell exposure to H_2O_2 led the NF-κB activation.

Although the exact physiological activator of HIV-1 is not yet determined, tumor necrosis factor α (TNF-α) appears to play an important role. A considerable amount of reports point to the generation of ROS in tumor necrosis factor α (TNF α) responses (Matthews et al., 1987; Zimmerman et al., 1989; Yamauchi et al., 1989). Furthermore, TNF α has been found to induce manganous superoxide dismutase in some cells, and its implications as a possible mechanism of cellular resistance to cytotoxicity have been suggested (Wong and Goeddel, 1988; Wong et al., 1989). Thus, these findings establish a signal transduction scheme as follows:

TNF α --> ROS --> NFκB --> expression of HIV-1.

Given that ROS are involved in the signal transduction mechanisms for HIV activation, it is logical to investigate a possibility for the therapeutic use of antioxidant in preventing HIV activation. Indeed, earlier evidence of the involvement of ROS in NF-κB and HIV activation has come from experiments using a pharmacological strategy eliminating ROS by antioxidants (i.e. *N*-acetylcysteine) as noted above. Thus, the investigation for the use of antioxidant for the treatment of AIDS has already started to emerge.

In our laboratry, we have observed the inhibition of NF-κB activation by various antioxidants including α-lipoic acid (Suzuki et al., 1992), dihydrolipoic acid, tetranorlipoic acid, bisnorlipoic acid (Suzuki and Packer, 1993a), vitamin E acetate, vitamin E succinate, pentamethyl hydroxychromane (Suzuki and Packer, 1993b) and nitecapone.

Although some of antioxidants have been shown to block the activation and one of ROS has been found to directly activate NF-κB, the exact mechanisms of TNFα-induced NF-κB activation are far from being understood. Schreck et al. (1992) recently reported that NF-κB activation induced by TNFα, interleukin 1β (IL-1) or phorbol 12-myristate 13-acetate (PMA) was not blocked by overexpressing manganous supeoroxide dismutase (MnSOD), and authors concluded that superoxide anions are not required for NF-κB activation. This conclusion is, however, rather difficult to accept given that MnSOD is a mitochodrial enzyme which probably has a little influence on the NF-κB activation processes occuring in the cytosolic compartment. Such a question forced to further test this conclusion for validity in our laboratory. We thus examined the role of superoxide anion in the activation of NF-κB induced by TNF-α, IL-1β or PMA by incorporating Cu,Zn SOD into the cytosolic compartment by liposome encapsulation of SOD. Our results show a consistant results with overexpression study by Schreck et al. (1992) that SOD does not block the NF-κB activation. These results suggest that either supeoxide anions are not directly involved in the activation of NF-κB or hydrogen peroxide is equally effective. Further studies will be needed to further analyze the intracellular compartmentalization of the involvement of ROS in NF-κB activation in order to effectively search for potent antioxidants which may be used as AIDS therapeutics.

Significance

Currently used antiviral agents against HIV such as reverse transcriptase inhibitors exhibit some toxicity and the use of high concentrations is not desirable. Less toxic, natural compounds which are capable of affecting the HIV lifecycle are attractive in that they can be used to support the actions of more aggressive antiviral agents without a risk of toxicity.

Specific Aims

The goal of this project is to elucidate whether Bio Normalizer inhibits the activation of NF-κB and subsequently the HIV LTR-directed gene expression.

Proposed Experiments

Experimental Protocols

1. Determination of whether Bio Normalizer inhibits TNF-α induced NF-κB activation

Jurkat T cells will be plated in individual wells of a 24-well Falcon plates at a density of 1 x 10^6 cells per well in 1.0 ml. The next day, TNF-α (25 ng/ml) will be added and cells will be incubated for 4 hr in an atmosphere of 5% CO_2 in air humidified at 37°C followed by nuclear extraction for EMSA.

In order to examine the effects of Bio Normalizer, various concentrations will be added 2 hours before the addition of TNF-α.

2. Determination of synergistic effect of Bio Normalizer with other types of antioxidants

Bio Normalizer, if it is determined to be effective in inhibiting TNF-α induced NF-κB activation, will be examined to see if its effect would be synergistically improved by other types of antioxidants such as ascorbate, α-tocopherol, and *N*-acetylcysteine on NF-κB activation.

3. Verification of the inhibitory capabilities of Bio Normalizer and effective combinations of antioxidants on HIV activation.

A theory, that inhibitors of TNF-α induced activation of NF-κB in turn inhibit TNF-α induced HIV activation, will be verified by using a HIV model system which will quantitatively measure the HIV LTR-directed gene expression of a reporter enzyme.

HIV-1-LTR-CAT Reporter cells will be plated in individual wells of a 96-well plates at a density of 2,000 cells per well in 0.2 ml (96-well plate). The next day, TNF-α (10 ng/ml) will be added and cells will be incubated for 8 hr in an atmosphere of 5% CO_2 in air humidified at 37°C. Chloramphenicol acetyltransferase (CAT) assays will be performed. Bio Normalizer and combinations of antioxidants which are determined to be effective in inhibiting TNF-α induced NF-κB activation will be added at various concentrations.

Materials and Methods

1. Cell lines and cell culture

Jurkat T (human lymphoma) cells will be grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% (w/v) penicillin/streptomycin, 1% sodium pyruvate and 1% glutamine in an atmosphere of 5% CO_2 in air humidified at 37°C.

2. Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts will be prepared from 1 x 10^6 cells essentially as described by Staal et al. (1990) with slight modifications. Cells will be harvested and centrifuged for 10 min at 1,200 rpm, washed in 1 ml of ice-cold phosphate-buffered saline (PBS), and centrifuged for 15 sec at 14,000 rpm at 4°C in an Eppendorf Brinkman-5412 centrifuge. Cells will be pelleted and washed once in 0.4 ml of buffer A [10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml antipain and 5 μg/ml leupeptin] and will be incubated on ice for 15 min. 25 μl of a 10% Nondiet P-40 solution will be added, and cells will be vigorously mixed for 15 sec and then centrifuged for 30 sec at 14,000 rpm.

Pelleted nuclei will be suspended in 50 µl of buffer C [50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% (v/v) glycerol], mixed for 20 min, and centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant containing the nuclear proteins will be harvested, protein concentration determined and stored at -80°C.

For EMSA, binding reaction mixtures (20 µl) will contain 1 to 5 µg protein of nuclear extract, 1 µg poly(dI-dC), 5-10000 cpm ^{32}P-labeled probe in binding buffer [50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 2% (v/v) glycerol and 10 mM Tris-HCl (pH 7.5)]. Samples will be analyzed on native 6% polyacrylamide gels followed by autoradiography. NF-κB probe will be labelled with [α-^{32}P]dATP using Klenow Fragment and purified using a NAP-5 column (Pharmacia) in 100 mM NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5).

3. Transfections and Chloramphenicol Acetyltransferase (CAT) Assays

The wild-type transfections will be performed by the DEAE-dextran method. HIV-1-LTR-CAT will contain HIV-1 sequence from -453 to +80 from the transcription start site of the viral genome upstream of a CAT reporter gene. 1 x 10^7 cells will be washed with PBS and resuspended in Tris-buffered saline containing 200 µg/ml DEAE-dextran and 15 µg/ml of plasmid DNA in a total volume of 1 ml. 20 hrs after transfection, aliquots from batchwise-transfected cells will be stimulated with CS and incubated for 9 hrs. Cell extracts will be prepared by three cycles of freeze-thawing and protein concentrations determined.

To determine the CAT activity, a reaction mix of 150 µl containing 20 mM acetyl CoA, 0.3 µCi ^{14}C-chloramphenicol and 60 µg of protein will be incubated for 4 hrs at 37°C. Chloramphenicol acetylation will be analyzed by thin-layer chromatography followed by autoradiography and liquid scentillation counting.

Summary

Acquired immunodeficiency syndrome (AIDS) results from infection with a human immunodeficiency virus (HIV). The long terminal repeat (LTR) region of HIV proviral DNA contains binding sites for nuclear factor kappa B (NF-κB). This transcriptional activator, which normally regulates genes involved in the immune, inflammatory, or acute phase responses, appears to regulate HIV activation. Action of NF-κB requires activation which is thought to occur by dissociation of inhibitory subunit which is controlled by phosphorylation. Recent findings suggest an involvement of reactive oxygen species (ROS) in signal transduction pathways leading to NF-κB activation. The present proposal is based on an idea that antioxidants which eliminate ROS would block the activation of NF-κB and subsequently HIV, and antioxidants can be used as therapeutic agents for AIDS. In this particular study, a free radical scavenger, Bio Normalizer will be tested for its capability in inhibiting NF-κB and HIV activation. Cultured Jurkat T cells will be stimulated by tumor necrosis factor α in the presence or absence of

Bio Normalizer. NF-κB activation will be detected by electrophoretic mobility shift assay whereas HIV activation will be monitored by chloramphenicol acetyltransferase assay of HIV-1-LTR-transfected cells. These studies will determine whether Bio Normalizer is effective in AIDS therapeutics at the level of NF-κB inhibition.

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