Biphasic effect of PDTC on the regulation of HIV1-LTR gene expression

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Abstract

NF-κB plays a key role to activate HIV provirus after HIV is reverse transcripted and integrated into host DNA. Reactive oxygen intermediate (ROI) is thought to be common regulator of NF-κB and activation of NF-κB is inhibited by anti-oxidant. Pyrrolidine dithiocarbamate (PDTC) is well studied anti-oxidant and has been shown to inhibit HIV1-LTR gene expression and NF-κB activation. In human promonocytic U937 cells, PDTC inhibited CAT protein response driven by HIV1-LTR gene to TPA higher than 2 ng/ml. However, PDTC augmented CAT protein without or with TPA less than 1 ng/ml. This biphasic effect of PDTC was also observed with the transfection of the plasmid which has only κB biding sites, suggesting kB biding sites are necessary for both augmentative and inhibitory effect of PDTC. Electromobility shift assay showed PDTC always inhibited NF-κB when it is induced by TPA, and no NF-κB induction was observed with PDTC ± TPA less than 1 ng/ml. This suggested NF-κB is important for the inhibitory effect of PDTC, not for augmentative effect. PDTC did not inhibit the HIV1-LTR gene expression stimulated by TNFα, and did not inhibit NF-κB induced by TNF. TPA and TNFα may use different pathways to regulate NF-κB and anti-oxidant, PDTC inhibited TPA pathway, but not TNFα pathway.

Introduction

A major regulation of human immunodeficiency virus (HIV) long terminal repeat (LTR) activation followed by viral transcription is mediated by nuclear factor kB (NF-κB) (1, 2). NF-κB is a multisubunit transcription factor that can rapidly activate the expression of target genes through an interaction with κB sequence elements. It normally regulates the expression of various T cell genes involved in growth, including interleukin-2 and interleukin-2 receptor α-subunit (日3-7). NFκB binds to and activate the duplicated κB enhancer element present in the U3 region of the HIV-1 proviral LTR (8). NF-κB was initially identified as a heterodimer of 50 kD protein (p50) and 65 kD protein (p65).that was bound in the cytoplasm to a retention protein called IκB and maintained inactive (9, 10). With various stimulation such as phorbol ester (11, 12), tumor necrosis factor-α (TNFα) (13-15), and interleukin-1 (IL-1) (13)., IκB is phosphorylated and proteolysed (16-18). The common denominator to proteolyse IκB is thought to be reactive oxygen intermediates (ROI) (19). An involvement of ROI was from the results that showed the depletion of glutathion levels (20) and release of H_2O_2 and O_2^- when cells are stimulated with TNF, phorbol ester, phorbol or IL-1. And also H_2O_2 itself activated the NF-κB and HIV1-LTR gene expression (21).

Pyrrolidine dithiocarbamate (PDTC) is an antioxidant and known to inhibit NF-κB activation by 12-myristate 13-acetate (TPA) or TPA + PHA (19, 21).

In human promonocytic cell line U937 is one of the cell lines to be studied in HIV research, because monocyte is one of target cells. To explore the HIV1-LTR gene regulation,, plasmid construct which has HIV1-LTR and reporter gene chrolamphenicol acetyl transferase (CAT) was used in this study. CAT response to TPA is great in U937 and small change is detectable. Although PDTC inhibited the CAT protein responded to TPA more than 2 ng/ml, when focusing on the gene regulation with TPA less than 1 ng/ml, this is more physiological stimulation, PDTC enhanced the LTR gene expression. It is obvious that with TPA more than 4 ng/ml TPA, NF-κB plays a

role to switch on the LTR gene expression and this NF-κB is suppressed by PDTC. This biphasic activation and suppression of HIV1-LTR activity is unique with PDTC. The signal transduction pathways modulated by ROI and PDTC are defined in this model system.

Materials and Methods

Cell culture and Treatments.

U937 and Jurkat T cell lines were grown in RPMI 1640 supplemented with 10 % FBS, 2mM L-glutamine (Gibco BRL, Life Technology Inc., Grand Island, N.Y.),100 units of penicillin and 100 mg of streptomycin (Sigma Co., St. Louis, M.O.). TPA was purchased from Sigma Co., and dissolved in dimethyl sulfoxide (DMSO), TNFα from Cellular Products Inc. Buffalo, N.Y., and PDTC, Dipyridyl and N-acetyl L-cysteine (NAC) from Sigma Co.

Plasmid Constract, Transfection and CAT ELISA Assay

(NOTE: I DON'T KNOW THE ORIGIN OF HIV-CAT and mHIV-CAT)

4 x kB-CAT is tandemly repeated κB binding sites were inserted into pSP-CAT containing SV40 promoter (22).

$4x10^7$ cells were washed in PBS and resuspend in serum free RPMI 1640 and put in the gene pulsar cuvette (Bio-Rad, Hercules, CA) and 10 μg of plasmid were electroporated with the condition of 250 μF and 300 volts with gene pulsar (Bio-Rad). 10 min after electroporation, cells were aliquoted into 24 wells plate. 24 h later, cells were treated with PDTC or NAC for 1 h, then stimulated with TPA or TNFα for 24 h. Cell extracts were prepared by three cycles of freeze-thawing and amounts of protein were determined by the method of Bradford (Bio-Rad). CAT protein amounts were determined by CAT ELISA (Boheringer Manheim, Indianapolis, IN).

Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides used to detect the DNA binding activities of NFκB was: GATCCAGAGGGGACTTTCCAAGAGG, (binding site is

underlined). The strands created 5'-overhanging ends, which allowed labeling by the Klenow polymerase (Amersham, Arlington Heights, IL) using α-[P^{32}] dCTP (Amersham) and unlabeled other dNTPs. The labeled dsDNA probe was purified on push columns (Strategene Co., La Jolla, CA).

Nuclear extracts were prepared from cultured cells as described (23), with some modification. Cells were harvested and pelleted and resuspended in 1.5 ml cold PBS. The cell suspensions were then transfered to microfuge tube. All subsequent steps were done on ice. Cells were pelleted for 10 sec and resuspended in 400 µl cold Buffer A (10mM HEPES-KOH ph 7.9 at 4°C, 1.5 mM MgCl, 10mM KCl, 0,5 mM dithiothreitol, 0.2 mM PMSF). The cells were allowed to swell on ice for 10 min. and then centrifuged for 10 sec, and the supernatant fraction is discarded. The pellet was resuspended in 40 µl of cold buffer C (20 mM HEPES-KOH pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol 0.2 mM PMSF) and incubated on ice for 20 min for high salt extraction. Cellular debris is removed by centrifugation for 5 min at 4 °C and supernatant fraction is stored at -70°C. Binding reactions contains 10 µg of nuclear extract and P^{32} labeled DNA probe in 100mM NaCl, 0.1 % NP40, 1mM DTT, 1mg/ml BSA and 0.1mg/ml dI/dC. Reactions were started by the addition of nuclear extracts and incubated in room temperature for 30 min. Samples were analyzed on native 4 % polyacrylamide gels. Dried gels were exposed to film with intensifying screens.at -70°C.

Results

PDTC suppressed CAT response to TPA in U937 cells.

Gene expression of HIV1-LTR was observed through the reporter gene expression, CAT protein driven by HIV1-LTR. Fig. 1 shows the CAT protein response to TPA and effect of PDTC. With TPA, CAT protein level increased with up to 6 ng/ml and it seemed to reach plateau at 6 ng/ml. PDTC 50 µM weakly suppressed and 100 µM and 200 µM PDTC strongly suppressed CAT response. With PDTC 100 µM or 200 µM, CAT protein level did not increase with more than

1 ng/ml TPA. And with 1 ng/ml TPA, PDTC barely suppressed CAT response to TPA. It seemed that the inhibitory effect of PDTC is remarkable with more than 1 ng/ml TPA. This led us to focus on the PDTC effect on CAT response to low dose TPA.

PDTC augmented CAT response to TPA less than 1 ng/ml.

Fig. 2a showed CAT response to TPA with or without PDTC. With less than 1 ng/ml TPA, PDTC did not inhibit the CAT protein, on the contrary, it augmented the CAT protein response to TPA. Even without TPA, PDTC itself increased CAT protein; PDTC can turn on the HIV-1 LTR gene. With TPA 1 ng/ml, PDTC started to inhibit CAT response to TPA. These results suggested PDTC has biphasic effect on TPA stimulation, i.e. augmentation on TPA less than 1 ng/ml, and suppression on TPA more than 1 ng/ml. This suggested that there might be two distinct signal pathway to turn on HIV1-LTR gene expression, one is activated with TPA less than 1 ng/ml and augmented by PDTC and the other is activated with TPA more than 1 ng/ml and suppressed by PDTC.

Next we observed where in LTR is responsible to the augmentation and suppression by PDTC. 4 x κB-CAT plasmid which has only κB binding sites as an enhancer was transfected and the effect of PDTC with or without PDTC was observed (Fig. 2b). CAT protein response was comparable to that with whole HIV1-LTR and PDTC had both augmentative and suppressive effects as well as with whole LTR-CAT. The pattern mimicked well to that with LTR-CAT. This meant κB binding sites are essential for both augmentative and suppressive effect of PDTC. Adding that we observed the effect of PDTC on HIV1-LTR (mutant κB)-CAT which had mutant κB binding region. The mutation of κB biding sites abolished both augmentative and suppressive effects by PDTC, showing another evidence that κB binding sites are essential for biphasic effect of PDTC.

Ion chelator dipyridyl and anti-oxidant sulphydril compound NAC did not enhanced the CAT response to TPA.

PDTC has two properties; one is heavy metal chelator and the other is anti-oxidants. To see this unique augmentation by PDTC is

due to the metal chelating function or common among other anti-oxidants, cells were treated with iron chelator, dipyridyl and another anti-oxidant, NAC. Although suppression of CAT response to TPA by dipyridyl is slight, it did not enhance the CAT response even with TPA less than 1 ng/ml (Fig. 3a). That was also the case with NAC (Fig. 3b). NAC inhibited the gene expression, however, it did not augment CAT response. So this biphasic effect is unique with PDTC.

NFkB is induced with TPA more than 4 ng/ml and inhibited by PDTC

It has been shown that NF-κB is induced by TPA and this induction is inhibited by PDTC. EMSA showed NF-κB DNA binding was observed with TPA more than 4 ng/ml (Fig. 4). Less than TPA 2 ng/ml, NF-κB binding was not observed even with long exposure. This is the case in Jurkat T cells . NF-κB was always inhibited by PDTC once it is induced (Fig. 5 a, b). This is no surprise and this explains the inhibitory effect of PDTC on TPA. PDTC inhibited TPA by inhibiting the activation of NFκB.

Next question is what is responsible for the augmentation by PDTC. Is it possible that same NF-κB is induced by TPA less than 1 ng/ml TPA and augmented by PDTC and inhibited by PDTC when induced strongly with TPA more than 1 ng/ml? Despite a vigorous effort to detect NF-κB induction by PDTC with or without TPA less than 1 ng/ml, NF-κB was not observed in EMSA. So there was no proof that NFκB is responsible for the augmentation of CAT response to TPA less than 1 ng/ml.

PDTC also enhanced the CAT response to TNF in U937

To examine the augmentation of CAT protein is specific to TPA or not, cells were stimulated with another oxidative stress inducer, TNF. The gene expression level stimulated with TNF was lower than that stimulated with TPA. The level of CAT protein was maximum and reached plateau with 1.0 ng/ml TNF. PDTC augmented the CAT protein response to TNF no matter what the TNF dose was (Fig. 6).

In EMSA no obvious NF-κB inhibition by PDTC was observed when U937 cells were stimulated with TNF in both U937 and Jurkat T cells (Fig. 7 a, b).

Discussion

PDTC has biphasic effect on LTR gene expression

NF-κB has been studied in great in detail because it is thought to be the main regulatory factor to turn on HIV1- LTR gene at the proviral stage. NF-κB is composed of three different subunits, p65, p50 and IκB. There are a lot of evidences that reactive oxygen intermediates (ROI) is involved in activating NF-κB by releasing IκB from p65 and p50 complex (22, 24-27). IκB is proteolysed when it is exposed to ROI and allow p65 and p50 complex enter into the nucleus and bind to κB binding site followed by transactivation of HIV gene expression (16-18). PDTC inhibits the proteolysis of IκB and inhibits NF-κB activation and LTR gene expression.

In U937 cells, PDTC also inhibited NF-κB and LTR gene expression when cells are stimulated with high dose TPA. It was not the case when the cells were stimulated with TPA less than 1 ng/ml. Most of the former studies used high dose TPA; 10-50 ng/ml or more. One of the reasons is to see the LTR gene expression in several cell lines including Jurkat cells, 10-50 ng/ml TPA is necessary. In our laboratory, only 3-5 fold induction of LTR gene expression was observed with CAT-ELISA (data not shown).

In U937 cells, even with 0.5 ng/ml of TPA, CAT protein increased to 20-30 fold of control. This allowed to observe a small change of the gene expression with various kinds of stimulators and inhibitors. PDTC itself turned on LTR gene expression and also augmented the gene expression by TPA less than 1 ng/ml TPA, EMSA showed the inhibitory effect by PDTC on TPA stimulation is due to the inhibition of NF-κB. It is difficult to understand that same NFκB is involved in both augmentation and inhibition by PDTC. This finding led us to an idea that there are two distinct pathways; one which is turned on with low dose TPA and augmented by PDTC and one which is turned on with high dose TPA and inhibited by PDTC.

PDTC has two properties; a chelating activity for heavy metals and an antioxidative activity of its dithiocarbamate group. The

metal chelators such as desferroxamine and o-phenanthroline are known to be potent inhibitors of NF-κB activation (28-32). These metal chelators can complex Fe^{3+} and other ions that is involved in the production of hydroxyradicals from H2O2. Dipyridil is an ion chelator. Dipyridil showed slight inhibition on LTR gene expression by TPA, but augmentation was not observed with dipyridil. This suggested the augmentation of LTR gene was not characteristic in iron-chelators. N-acetyl cysteine (NAC) is sulphydril compound also known to block NF-κB induction upon TPA or TNF (16,17, 33-35). NAC also inhibited LTR gene expression with TPA from low dose to high dose. This biphasic effect on LTR gene expression is unique in PDTC.

κB binding site is necessary for both augmentation and inhibition of PDTC

Next question is where in LTR is responsible for the augmentation and inhibition by PDTC. With 4 x kB-CAT, the effect of TPA mimicked to LTR-CAT, i.e. augmentation with low dose TPA and inhibition with high dose TPA. With mutant κB LTR-CAT, both augmentation and inhibition were abolished. These two results suggested that kB binding sites in LTR is responsible for both augmentation and inhibition. It is possible that a molecule which

PDTC only enhanced TNF effect

PDTC showed biphasic effect on LTR gene expression depend on TPA dosage. It is not the case on TNF stimulation. The gene expression level stimulated by TNF was lower than that stimulated by TPA and it reached plateau at 1 ng/ml TNF. Gene expression level detected by CAT ELISA is always enhanced by TNF in U937. NFkB induction shown by EMSA also different when the cells were stimulated with TNF and with TPA. With TPA stimulation, NF-κB is not detected under 2 ng/ml TPA, and with more than 4 ng/ml TPA, it is detected and inhibited by PDTC in both U937 cells and Jurkat cells. This inhibition by PDTC is stronger in Jurkat cells. With TNF stimulation, NFkB is induced with 0.1 ng/ml and NF-κB was barely inhibited by PDTC.

What is different between TNF stimulation and TPA stimulation. Protein kinase C (PKC) is directly induced by TPA (36), and it has been shown that PKC inhibitor blocked TPA effect on NF-κB activation (37). PKC is also induced in some cell lines but not always (38,39). In Jurkat T cells and U937 cells, PKC is activated by TNFα, however NF-κB is induced by TNF without PKC (40-42). Another difference between TPA stimulation and TNF stimulation is that although TNF activates PKC, TNF does not increase intracellular calcium level in U937 cells (43). It is conceivable that calcium is necessary to inhibit LTR gene expression by PDTC.

NF-κB is activated by both TPA and TNF. Reactive oxygen intermediate (ROI) is thought to be common modulator to regulate NF-κB activation and this activation is thought to be inhibited by anti-oxidant. Here we showed one example that TPA and TNF use different pathways to regulate NF-κB and anti-oxidant, PDTC inhibited TPA pathway, but not TNFα pathway. Further investigations are necessary to clarify what is responsible to the LTR gene induction by PDTC. and the difference between the stimulation by TPA and TNF.

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Figure Legends

Fig. 1. PDTC inhibited CAT protein response driven by HIV1-LTR to TPA more than 1 ng/ml.
PDTC inhibited CAT response to TPA in dose dependent manner between 1-10 ng/ml TPA. With PDTC 100 mM and 200 mM, CAT protein dod not increase a lot from the level at 1 ng/ ml TPA. It is not clear PDTC inhibited the gene expression at the TPA concentration 1 ng/ml.

Fig. 2. PDTC augmented CAT response to TPA less than 1 ng/ml.
A) When cells are treated with TPA less than 1 ng/ml, PDTC augmented CAT response to TPA. Even without TPA
B) This augmentation and inhibition by PDTC with or without TPA is also observed with 4xκB-CAT. This data showed that κB binding sites are necessary for both inhibitory and augmentative effect of PDTC.

Fig. 3. Other antioxidants, NAC and dipyridyl only inhibit CAT response to TPA and do not augment.
A) The suppression of CAT response to TPA by iron chelator dipyridyl is weak. No enhancement of the CAT response to TPA was observed from 0 to 10 ng/ml. B) NAC inhibited the gene expression, however, it did not augment CAT response. So this biphasic effect is unique with PDTC.

Fig. 4. NF-κB binding is detected with TPA more than 4 ng/ml in U937 cells.
EMSA showed NF-κB DNA binding was observed with TPA more than 4 ng/ml. Less than TPA 2 ng/ml, NF-κB binding was not observed even with long exposure.

Fig. 5. PDTC effect on NF-κB activated by TPA. This is the case in Jurkat T cells . A) NF-κB was induced with 5 ng/ml and 10 ng/ml TPA. When NF-κB is induced by TPA, PDTC inhibited NF-κB. NF-κB is not induced by PDTC with or without TPA. B) NF-kB is also induced

with TPA more than 4 ng/ml TPA in Jurkat T cells and induced NF-κB is inhibited by PDTC.

Fig. 5. PDTC augmented the CAT response to TNF.
The gene expression level stimulated with TNF was lower than that stimulated with TPA. The level of CAT protein was maximum and reached plateau with 1.0 ng/ml TNF. PDTC augmented the CAT protein response to TNF at the concentrations from 0-6.25 ng/ml.

Fig. 6. In EMSA no obvious NF-κB inhibition by PDTC was observed when U937 cells were stimulated with TNF in both U937 (A) and Jurkat T cells (B).