Biochemical and Biophysical Research Communications 376 (2008) 180–185

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reactive oxygen species activate HIV long terminal repeat via post-translational control of NF-κB

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ARTICLE INFO

Article history:
Received 18 August 2008
Available online 31 August 2008

Keywords:
Oxidative stress
HIV
LTR
NF-κB
H_2O_2

ABSTRACT

Reduction/oxidation disorder is one of the most common ailments in HIV-infected patients, and such patients are frequently left exposed to chronic oxidative stress after the generation of reactive oxygen species (ROS). Although a variety of clinical trials to inhibit HIV infection have been conducted by focusing on oxidative stress, their precise targets and reaction mechanism have remained unclear. In this study, we demonstrate that H_2O_2 treatment strongly induced HIV long terminal repeat (LTR)-driven luciferase expression in Jurkat T lymphocytes via NF-κB activation. Treatment with the SN50 peptide or the mutation of NF-κB binding site on LTR resulted in impaired LTR activity in response to ROS. H_2O_2 induced both IκB degradation and covalent modification of p65. CBP/p300-induced hyperacetylation as well as phosphorylation of p65 was implicated in ROS-mediated LTR activation. The results of our study showed that ROS-induced HIV LTR activation involves immediate early NF-κB activation at the post-translational level.

Many human immunodeficiency virus (HIV)-infected patients suffer from chronic oxidative stress resulting from an increase in plasma reactive oxygen species (ROS), a systemic reduction in the concentrations of glutathione (GSH) and thioredoxin and a disturbance of mitochondrial membrane potential [1,2]. Although the complexity inherent to the generation of oxidative stress has yet to be fully addressed, HIV infection-induced oxidative stress typically results in low levels of cysteine and GSH. Furthermore, ROS can transiently induce pro-inflammatory cytokines, including tumor necrosis factor (TNF) and Interferons, and subsequently promote further ROS generation [2]. Additionally, host reduction/oxidation (redox) balance can be disrupted by specific HIV viral proteins [3]. Among the many viral proteins, Tat, a trans-activator of viral replication by acting on the 5′ LTR, has been shown to induce the release of cytokines, thereby enhancing the intracellular levels of ROS including the pro-oxidant H_2O_2 in a variety of cell types [4,5].

Intracellular ROS involves the activation of numerous cellular genes. Among the many cellular factors, NF-κB is the best characterized ROS-triggered activating transcription factor. Until now, a broad variety of stimuli have been identified as NF-κB activators, including TNF, phorbol esters, LPS, ionizing irradiation, and invasive pathogens. Interestingly, many of the NF-κB inducers have been shown to augment ROS level [6,7]. These studies have suggested that many NF-κB inducers utilize ROS as a second messenger in activating the signal pathways. The activation cascade of IKK complex results in the ubiquitylation-dependent degradation of IκB in the cytoplasm. The transactivation potential of NF-κB in the nucleus is assured by the modification of subunits, most notably via p65 phosphorylation. Recently, accumulating evidences have demonstrated that NF-κB acetylation performs a crucial function in a variety of gene regulation [8,9]. Although the relevance of the relationship between redox condition and acetylation ratio has yet to be clearly elucidated, control of p65 acetylation has been identified as the prerequisites for the regulation of NF-κB activity [10].

Since NF-κB, which is sensitive to ROS, has been identified as a key factor in the expression of HIV structural genes, it appears likely that ROS may perform a pivotal function in LTR activation. In this study, therefore, we have attempted to determine whether the direct H_2O_2 treatment of 1G5 cells stably expressing LTR-driven luciferase can modulate the level and/or activity of NF-κB. We also attempted to determine a potential role for p65 modification in the regulation of HIV LTR promoter under oxidant conditions.

Materials and methods

Cell lines and reagents. The Jurkat T human leukemia cell line was acquired from the American Type Culture Collection. 1G5 cell line was supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institute of Health (Rockville, MD). These cells were maintained in RPMI1640 medium under standard conditions. TNF-α, Trichostatin A, and *N*-acetylcysteine were purchased from Sigma–Aldrich.

Transfection and plasmids. Transient transfection was conducted via the DEAE–Dextran (Sigma) method. 5–10 μg of DNA per

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doi:10.1016/j.bbrc.2008.08.114

1×10^6 cells were mixed with transfection cocktail (250 μg DEAE–Dextran/50 mM Tris, pH 7.5/ml of serum free DMEM). After 1 h, the cells were collected by spin down (1000 rpm) and then incubated continuously for 18–24 h with normal media. Expression vectors for pRK-Flag-IKKα, pRK-Flag-IKKβ, and pGST-IκBα were kindly provided by Dr. D. Goeddel, Tularik Inc. [16]. pcDNA3-CBP-Flag and pBJ5-HDAC1-Flag were generously provided by Drs. S. Impey and S. Schreiber [17].

Immunoblotting and EMSA. Immunoblotting was conducted as described previously [15]. Antibodies against p65, phospho p65 (Ser536), Acetyl-p65 (Lys310) were purchased from Santacruz (USA), and Cell Signaling Technology (USA). For EMSA, nuclear fractions of Jurkat cells were prepared in accordance with a modified version of the method previously described by Dignam [18]. DNA probe containing the consensus binding site of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was purchased from Promega and labeled with [γ-^{32}P]-ATP. For the binding assay, nuclear extracts were incubated with labeled oligonucleotides for 30 min on ice. The protein–DNA complexes were separated on 5% native polyacrylamide gel. The dried gels were subjected to autoradiography.

RT-PCR. Total cellular RNA was isolated via a cesium chloride method and was subjected to semi-quantitative RT-PCR as previously described [19]. The primers used for PCR for p65 were as follows: 5′-GTGCAGCCTCTTCGTCCT-3′ and 5′-CACTACAGACGA TCCATTC-3′ and the primers for GAPDH were 5′-CCATCACC ATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′.

Vector construction and reporter gene assay. In order to construct pLTR-Luc, the full U3 and R region (containing positions −453 to +82) were isolated with *BamHI* and *HindIII* from the pHL3 source vector. The promoter fragment was then inserted into the pGL3-Basic (Promega) vector. For the mutation of NF-κB binding sites, a site-directed mutagenesis with PCR overlap extension performed for construction of pLTR-mκB-Luc. The primers used in the mutation of the NF-κB binding site were as follows: Forward region, 5′-CGAGCTCTTACGCGTGCTA-3′; Mutation region (A) 5′-AAGT GAGCAGCGGAAAGTGAGTTG-3′; Mutation region (B), 5′-CAACTC ACTTTCCGCTGCTCACTT-3′; Reverse region, 5′-TCTTCCATGGT GGCTTTACC-3′. Primer (A) and (B) were reverse complement of each other and harbor the mutated residues of the NF-κB binding sequence. Following transfection with pLTR-Luc vectors, the grown cells were rinsed in cold PBS and lysed with cell culture lysis reagent, which is contained in the Promega luciferase assay kits. All steps of the luciferase assay were conducted in accordance with the manufacture′s recommendations. The luciferase activity and transfection efficiency of the reporter plasmid was normalized relative to the β-galactosidase activity of the co-transfected internal control plasmid, pcDNA3.1-LacZ (Invitrogen). DNA sequencing was used to confirm all modifications.

In vitro kinase assay. *In vitro* kinase assay was performed as described previously [15]. Briefly, Jurkat cells were transiently transfected with Flag epitope-tagged IKKβ, and treated with various concentration of H_2O_2 for 4 h. Aliquots of the cell lysates were immunoprecipitated with anti-Flag monoclonal antibody (Santacruz) and protein G-agarose beads. The substrate GST-IκBα was expressed in *Escherichia coli* and purified on glutathione–agarose (Pierce). *In vitro* kinase reactions were analyzed via 10% SDS–PAGE and auto-radiographed

Results

Intracellular ROS stimulates HIV LTR promoter in 1G5 cells

Oxidative stress can be mediated by several viral proteins and virus-induced cytokines. We also observed that Tat and TNF-α clearly generated intracellular ROS (data not shown). However, Tat and TNF-α have been known not only as ROS inducers but also as ROS-independent multi-signal activators. Therefore, we utilized H_2O_2 directly as a major ROS inducer in the following experiments to prevent any possible misinterpretation of ROS-independent effects of Tat or TNF-α.

In order to determine the effect of oxidative stress on HIV gene expression, the 1G5 cell line, a derivative of Jurkat cells harboring a stable LTR-luciferase reporter gene, was employed for the monitor-

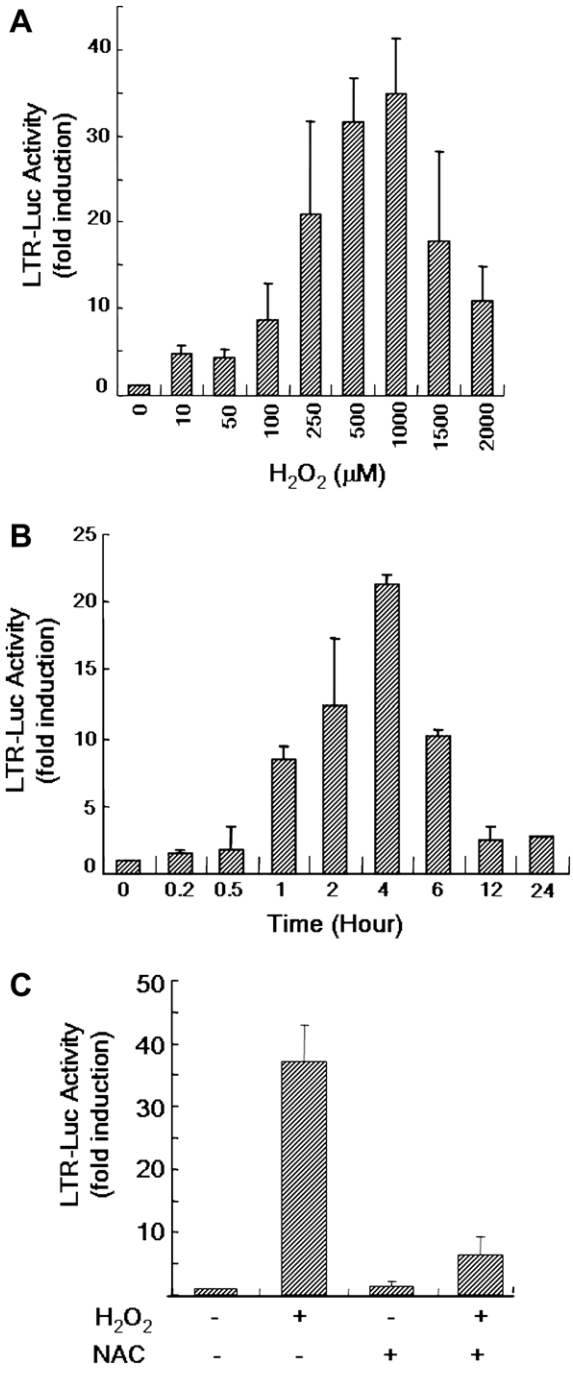

Fig. 1. H_2O_2-mediated induction of HIV LTR-dependent gene expression. (A) 1G5 cells were treated for 4 h with the indicated concentration of H_2O_2, and the luciferase activity was analyzed. (B) 1G5 cells were treated with exogenous H_2O_2 (0.5 mM) for the indicated time periods and the luciferase activity was analyzed. (C) 1G5 cells were pretreated with 10 mM of NAC for 2 h, and then incubated with 0.5 mM of H_2O_2 for 4 h. Every luciferase activity was analyzed with 100 μg of proteins from the total cell lysates. All bar represents means ± SE of triplicate samples from three independent experiments.

ing of LTR activity in response to ROS. As the concentration of H_2O_2 increased, the maximum luciferase activity (46-fold) occurred at 1 mM, and then declined gradually (Fig. 1A). The exposure duration (4 h) yielding the maximum activity was determined by the time course experiments (Fig. 1B). In order to confirm the influences of ROS on the LTR activation, *N*-acetyl-L-cysteine (NAC) was used as an antagonist since NAC is a well known thiol-antioxidant. Cells were pretreated with NAC and incubated with H_2O_2. As is shown in Fig. 1C, H_2O_2-induced luciferase activation was reduced markedly by NAC. Therefore, these inhibitory effects of NAC imply that H_2O_2 can evoke LTR activation via a unique oxidative stimulation mechanism, thereby allowing for accelerated viral genome expression.

ROS-induced activation of HIV LTR via post-translational control of NF-κB

The regulation of HIV gene expression occurs principally on the U3 region of the 5′ LTR, in which various cellular transcription factors are implicated [11,12]. Among many factors, NF-κB is one of the most sensitive transcription factors in response to oxidative stress. Therefore, we attempted to determine whether ROS-sensitive NF-κB could be functionally involved in LTR activation. In order to address this question, we conducted EMSA to obtain independent evidence implicating of NF-κB (Fig. 2A and B) and showed a gradual increase in DNA binding with H_2O_2-dose-dependent-manner. Maximal binding of NF-κB to

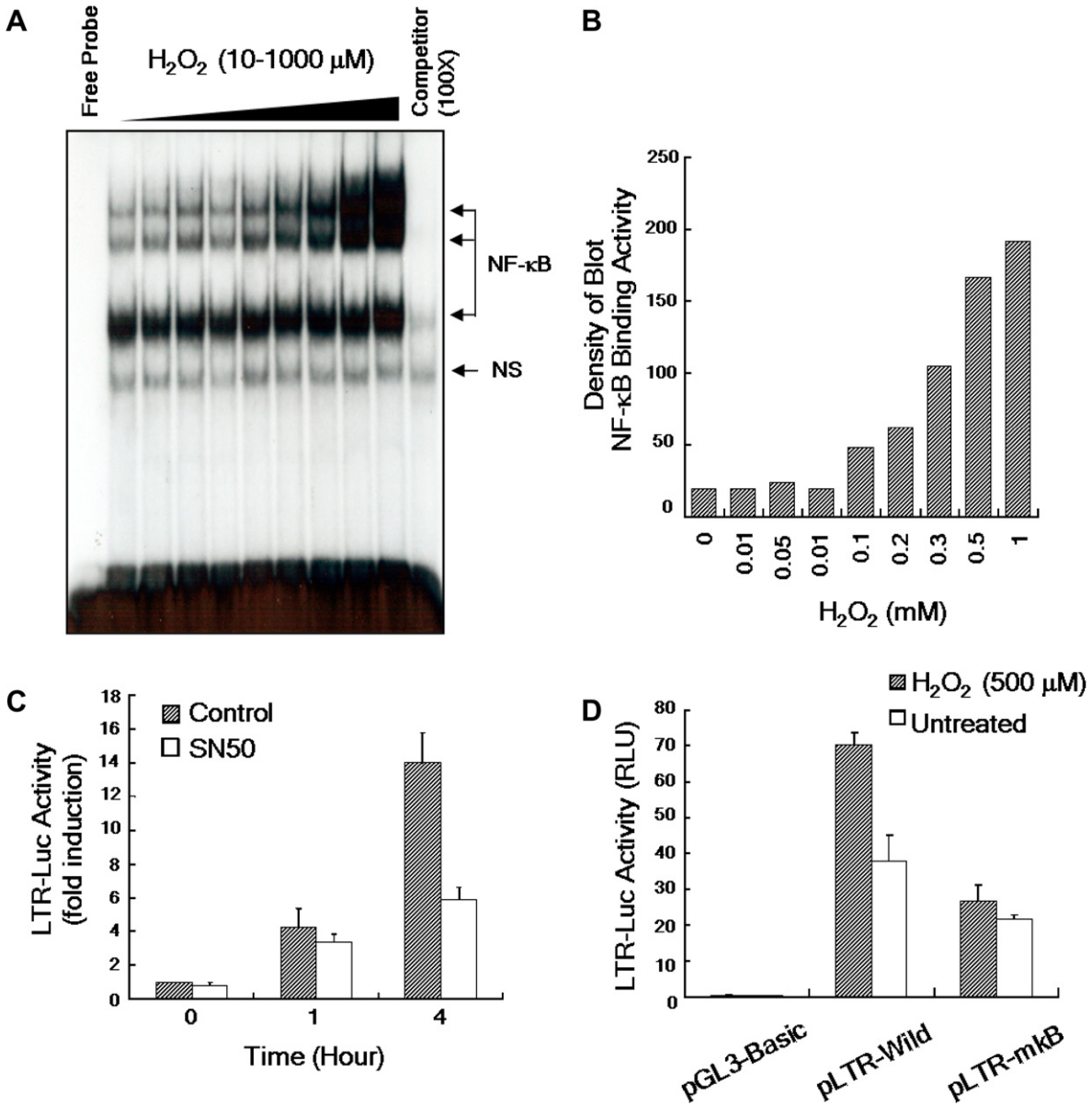

Fig. 2. A critical role of NF-κB in H_2O_2-induced LTR activation. (A) DNA binding activity of NF-κB was evaluated with EMSA. Jurkat cells were treated with various concentration of exogenous H_2O_2 (10 μM–1 mM) for 4 h. Nuclear extracts (7 μg) were incubated with [γ-^{32}P] ATP labeled NF-κB probe and separated on 5% polyacrylamide gel. A 100-fold molar excess of the unlabeled NF-κB probe was used as a competitor. NS stands for non-specific bindings. (B) Average intensity of bands detected in EMSA was graphed via densitometric imaging analysis by the NIH Image J Program. (C) 1G5 cells (5×10^5/ml) were incubated with a specific NF-κB inhibitory peptide, SN50 (50 μg/ml) for 1 h. After inhibitor pretreatment, the cells were incubated with H_2O_2 (0.5 mM) for the indicated times. LTR activity was determined with luciferase activity. (D) Effects of NF-κB binding site mutations on H_2O_2-induced LTR-driven gene expression. Three micrograms of pGL3-Basic, pLTR-Luc, and pLTR-mkB-Luc were transfected to Jurkat cells. After 18 h, H_2O_2 (0.5 mM) was treated for 4 h and luciferase activity was determined. Transfection efficiency was normalized with β-galactosidase activity from co-transfected pcDNA3.1/LacZ (0.5 μg). All point bar represents means ± SE of triplicate samples from three or four different experiments.

DNA occurred at 1 mM of H_2O_2, and was increased by 10.5-fold as compared to the untreated control, which indicating the implication of NF-κB in ROS-mediated LTR activation. We further verified the involvement of NF-κB in the LTR activation using a specific NF-κB inhibitor, SN50 peptides. After the pretreatment with SN50, the cells were incubated with 0.5 mM of H_2O_2 for 4 h. Luciferase assay showed that SN50 reduced luciferase activity by 56% when compared with the controls (Fig. 2C). For further verification of role of NF-κB in LTR activation, a mutant form of LTR-luciferase vector, pLTR-mκB-Luc was constructed. Two conserved NF-κB binding sites located proximally to the TATA box on the U3 region were mutated. Twenty hours after transfection with the wild-type and mutant forms of vectors, the cells were incubated for 4 h with H_2O_2. As shown in Fig. 2D, the mutation of NF-κB sites resulted in the reduction of luciferase activity by 60% as compared to that of the wild-type vector. These results indicate that NF-κB performs a crucial function in ROS-mediated LTR activation.

Both IκB degradation and p65 phosphorylation are implicated in ROS-induced LTR activation

Considering that NF-κB activation was responsible for the LTR activation, we attempted to determine whether H_2O_2 directly activated IKKs, which were immediate upstream components of the classical pathway for NF-κB activation. We conducted *in vitro* kinase assays to determine the activity of IKKs in response to H_2O_2. H_2O_2-treated cell lysates were immunoprecipitated with antibody against Flag-IKKβ, and reacted with GST-IκBα substrates (Fig. 3A). As the concentration of H_2O_2 increased, the degree of IκBα phosphorylation increased. It appeared that H_2O_2 activated IKKβ in a dose-dependent manner, thereby increasing IκB phosphorylation.

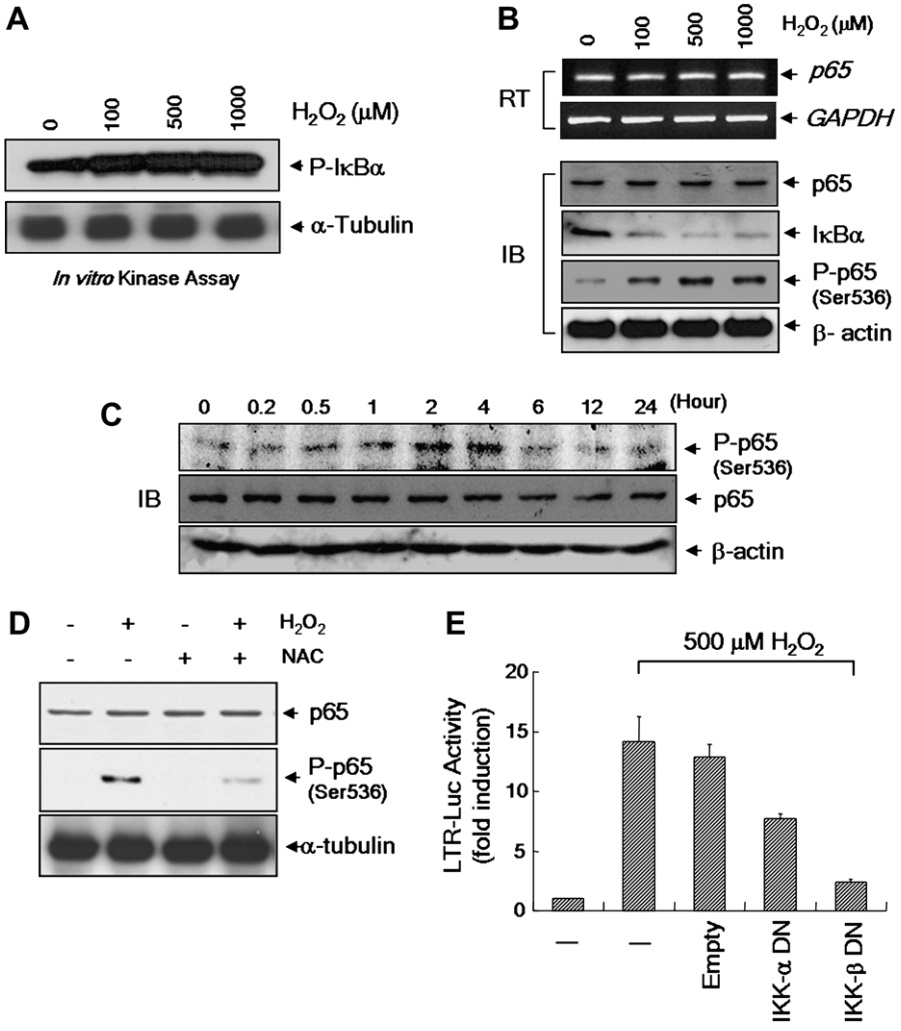

Fig. 3. H_2O_2 utilizes the classical NF-κB activation mechanism for LTR activation. (A) *In vitro* kinase assay for IKKβ was conducted as described in Materials and methods. Cells were treated with the indicated concentration of H_2O_2. Immunoprecipitated IKKβ was subjected to *in vitro* kinase assay by reacting with wild-type GST-IκBα proteins. As an internal control, the expression level of α-tubulin was detected from the total lysates. P-IκBα indicates phosphorylated IκBα. (B) 1G5 cells (6×10^5/ml) were treated with H_2O_2 with the indicated concentration for 4 h. After treatment, total RNA and lysates were prepared as described in Materials and methods. For RT-PCR experiments (RT), cDNA was amplified with p65 primers via PCR. GAPDH expression was used as an internal control. Twenty micrograms of total lysates were also analyzed via immunoblotting (IB). Proteins were separated on 10% SDS–polyacrylamide gel and immunoblotted with p65, IκB, and phospho-p65 (Ser536) antibodies. P-p65 (Ser536) indicates phosphorylated p65 (Ser536). β-actin expression was detected for normalization. (C) Under the same condition as (B), protein expression and its phosphorylation level were analyzed via immunoblotting using 20 μg of total lysates after incubation with 0.5 mM of H_2O_2 for the indicated time. All samples were separated on 10% SDS–polyacrylamide gel and detected with antibodies against phospho-p65 or p65. β-actin level was detected as an internal control. (D) 1G5 cells (5×10^5 cells/ml) were pretreated with 10 mM of NAC for 2 h, and then incubated with 0.5 mM of H_2O_2 for 4 h. Proteins (20 μg) from the total cell lysates were separated on 10% SDS–polyacrylamide gel. The expression and phosphorylation level of p65 protein were detected with each corresponding antibody. (E) 1G5 cells (4×10^5/ml) were transfected with the dominant negative form of IKKα and IKKβ expression vectors (1 μg) and co-transfected with pcDNA3.1/LacZ (0.2 μg) for transfection normalization. Non-transfected cell were treated with transfection reagent for the same time. After 18 h, the cells were treated with H_2O_2 (0.5 mM) for 4 h. Luciferase activities were counted with a luminescence meter. The results are expressed as means ± SE of triplicate samples, and represent two individual experiments.

Next, we investigated the unique molecular mechanism of NF-κB in ROS-induced LTR activation by analyzing its gene expression level and post-translational modification. Jurkat cells were treated with various concentrations of exogenous H_2O_2, after which total RNA and proteins were extracted. Fig. 3B shows little significant impact of ROS on both the transcription and translation of p65. However, IκBα was efficiently degraded when exposed to H_2O_2. Simultaneously, when total proteins were analyzed using anti-phospho-p65 (Ser536), the phosphorylation level increased steadily in a dose-dependent manner. This result implies that the increase in NF-κB activity result from the post-translational modification. Furthermore, we attempted to study the kinetics of NF-κB activation in a time-dependent manner. With increases in the exposure time to H_2O_2, the degree of p65 phosphorylation increased gradually and achieved a maximum at 4 h, and then diminished thereafter (Fig. 3C). The induction pattern was consistent with that of a previous luciferase assay (Fig. 1B). As shown in Fig. 3D, the level of p65 protein expression was not affected by H_2O_2 even in the presence of NAC. However, p65 phosphorylation was significantly induced by H_2O_2, but was inhibited by NAC. Finally, we evaluated the result of H_2O_2-induced modification of NF-κB in LTR promoter strength. To do this, dominant negative mutants of IKKs were transfected into 1G5 cells to investigate their effect on LTR-driven luciferase reporter gene expression. Fig. 3E showed that the transient expression of IKKβ dominant negative more profoundly suppressed H_2O_2-induced LTR activation, whereas the IKKα dominant negative mutant was less effective. These data clearly suggest that ROS stimulates LTR promoter activity via an induced increase in the phosphorylation level of NF-κB p65, and that IKKβ is crucial for this regulation.

Acetylation of p65 contributes to ROS-induced LTR activation

We showed that H_2O_2 affected NF-κB activity principally via the IKK-mediated signaling cascade. However, we could not dismiss the possibility that there might be other modification in addition to phosphorylation. Interestingly, recent reports indicated that p65 acetylation is a crucial in various gene expressional contexts [13]. They have demonstrated that p300/CBP HAT acetylates p65 at Lys^{218}, Lys^{221}, and Lys^{310} [14]. The acetylated p65 is associated with both weak interactivity with IκBα and increased transcriptional activity. Reversibly, HDACs subsequently deacetylate p65 and induce a downregulation of NF-κB activity. Therefore, we examined whether p65 acetylation may be implicated in ROS-induced LTR activation. After Jurkat cells were treated with H_2O_2, immunoprecipitation was conducted with CBP/p300 antibody and p65 antibody. As is shown in Fig. 4A, the increase in CBP-p65 interaction suggested the possibility of p65 acetylation and a potential role for HAT in response to ROS. Additionally, the results of immunoblotting analysis demonstrated that ROS increased endogenous levels of acetyl-p65 (Lys^{310}) (Fig. 4B). For a further investigation of the molecular relevance between p65 acetylation and redox status, we employed an expression vector for HDAC, and Trichostatin A (TSA), a specific HDAC inhibitor. When HDAC was transiently expressed, LTR activity was reduced in the H_2O_2-treated cells, but restored efficiently as the result of TSA treatment (Fig. 4C, compare lanes 5 and 7). These results indicated that HAT might be involved in H_2O_2-induced LTR activation via p65 acetylation, which may contribute to increase in NF-κB-mediated LTR activation in addition to phosphorylation.

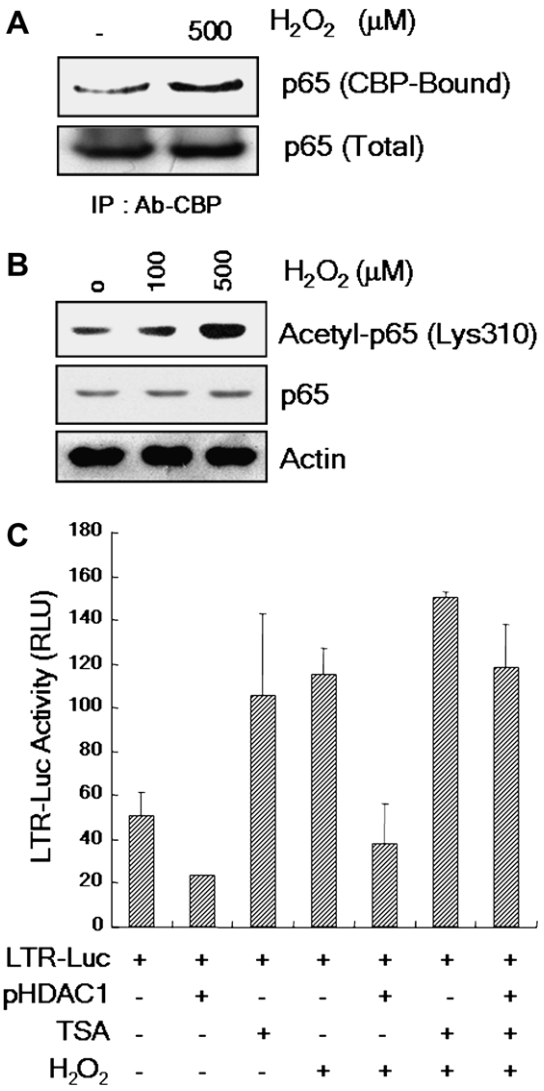

Fig. 4. Association of histone acetyl transferase (HAT) with H_2O_2-induced LTR activation. (A) After Jurkat cells were treated with H_2O_2 (0.5 mM) for 2 h, nuclear extracts were prepared. Immunoprecipitation was conducted with anti-CBP/p300 antibody for 12 h, and then visualized with anti-p65 antibody. (B) Jurkat cells were incubated with 100 and 500 μM of H_2O_2 for 4 h. Immunoblotting analysis to detect acetyl-p65 (Lys310) and p65 was conducted with 30 μg of total lysates. (C) Two micrograms of pLTR-Luc and 3 μg of pHDAC vectors were transfected for 18 h into Jurkat cells. The transfection efficiency was normalized with β-galactosidase activity from co-transfected pcDNA3.1/LacZ (0.5 μg). After transfection, the cells were treated with either H_2O_2 (0.5 mM) for 2 h or TSA (20 nM) for 5 h. Luciferase activities were analyzed with 100 μg of total lysates as described.

Discussion

It has been demonstrated that HIV infection-induced oxidative stress is mediated by several HIV viral proteins and cellular cytokines. Among many HIV viral proteins, Tat enhances the intracellular levels of ROS including the pro-oxidant H_2O_2 in a variety of cell types [4,5]. In this study, we showed that H_2O_2 treatment concomitantly evoked an extremely high level of HIV LTR-driven gene expression. This H_2O_2-induced LTR activation may explain the explosive increase in viral replication after the middle stage of infection, during which a great deal of oxidative stress occurred [2]. The induced oxidative stress would subsequently promote further ROS generation, and eventually result in disease progression by causing serious immune system damages and cellular abnormalities in the host cells. Therefore, we have employed a derivative of the Jurkat T cell line, 1G5, which stably expresses LTR-driven luciferase, and explored the primary target of ROS-induced LTR activation, as well as its putative molecular reaction mechanism.

As the gene regulation occurred principally on the U3 region of the 5′ LTR, we were readily able to narrow down the binding sites

of NF-κB as a factor responsive to ROS, and to determine its specificity using an NF-κB inhibitor and site-directed mutagenesis. In the EMSA assay, NF-κB was strongly activated when the cells were treated with H_2O_2. However, H_2O_2 did not alter p65 expression level, but stimulated p65 phosphorylation. Both IKKα and IKKβ dominant negatives mutants profoundly suppressed H_2O_2-induced LTR activation. This result was consistent with recent studies demonstrating that both IKKα and IKKβ are indispensable with regard to p65 phosphorylation [16]. Indeed, *in vitro* kinase assays showed that IκBα phosphorylation increased with increasing doses of H_2O_2. However, the fold induction ratio of p65 phosphorylation and IκBα phosphorylation were unexpectedly less than those of LTR-luciferase activity. There appeared to be at least one additional positive regulator for NF-κB modification in the ROS-induced LTR activation. This assumption compelled us to study the histone acetylation system. As a result, we showed that ROS increased interaction between CBP/p300 HAT and p65 (Fig. 4A). This data indicates a potential role for HAT in the regulation of p65 acetylation under oxidant conditions [9]. However, more study will be required in order to determine whether a ROS-mediated increase in acetyl-p65 is induced by the inhibition of endogenous HDACs by H_2O_2. Consequently, we can conclude that ROS induces LTR activation, and that the LTR activation is regulated delicately by post-translational modification of p65, including phosphorylation and acetylation. Although the AP-1 transcription factor is connected to LTR transcription [11], we are able to rule out its possible association with ROS, as 1G5 cells do not harbor the entire LTR promoter containing all of the AP-1 sites. However, its functional involvement in the ROS-induced LTR activation remains to be investigated.

Our results may contribute to our understanding of the manner in which NF-κB is regulated by oxidative stress and the effect of chronic oxidative stress to HIV-infected patients. The results of this study may also shed some light on researches into the development of therapeutic strategies for HIV-mediated AIDS diseases.

Acknowledgments

The authors gratefully thank Dr. D. Goeddel for providing wild-type and dominant negative IKKs vectors, Dr. S. Impey for pcDNA3-CBP-Flag and Dr. S. Schreiber for pBJ5-HDAC-Flag vector. This work was supported by a Korea university grant.

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