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Effect of Comorbidities on HIV-1 Pathogenesis in Monocytic Cells

Abstract

The advent of antiretroviral therapy (ART) has substantially increased the life span of people living with HIV-1 (PLWH). However, the presence of comorbid conditions such as high prevalence of drug abuse and coinfections have deteriorated the health outcomes in PLWH and progression of HIV-1 infection to AIDS. In this study, we will be focusing on the independent effect of two major comorbidities: cigarette smoking and human papilloma virus (HPV) coinfection on HIV-1 replication and pathogenesis in monocytic cells.

The prevalence of smoking is approximately 3-times higher in PLWH compared to the general population. Scientific evidences suggest that smoking aggravates HIV-1 pathogenesis and leads to decreased responses to ART. However, the exact mechanism by which smoking enhances HIV-1 replication is still not clear. The Kumar group has demonstrated the role of nicotine, at least in part, on HIV-1 replication in monocytic cells via cytochrome P450 (CYP)-mediated oxidative stress pathway. They have also shown the role of cigarette smoke condensate (CSC), which contains numerous organic compounds such as polyaryl hydrocarbons (PAHs), on cytotoxicity and HIV-1 replication in monocytic cells. Therefore, the goal in this study was to identify active ingredient of CSC, especially PAHs, which is responsible for cytotoxicity and HIV-1 replication in monocytic cells, and their underlying mechanism. PAHs such as benzo(a)pyrene (BaP), naphthalene (NPh), phenanthrene (Phe), benzo(a)antharacene (BeA), and benzo(b)fluoranthene (BeF) are known carcinogens present in cigarette smoke. As a first part of our study, we examined the relative effect of these constituents on the cytotoxicity of monocytic cells and the possible mechanism of PAH-mediated cytotoxicity. We examined the acute (6-24 hours) and chronic (7 days) effects of these PAHs on the expression of cytochromes P450 (CYPs), oxidative stress, and cytotoxicity. The treated cells were examined for mRNA and protein levels of CYPs (1A1 and 3A4) and antioxidants enzymes (AOEs) superoxide dismutase-1 (SOD1) and catalase. Further, we assessed the levels of reactive oxygen species (ROS), caspase-3 cleavage activity, and cell viability. We performed these experiments in U937 cell lines and/or primary monocytic cells. Of the five PAHs tested, after chronic treatment only BaP (100 nM) showed a significant increase in the expression of CYP1A1, AOEs (SOD1 and catalase), ROS generation, caspase-3 cleavage activity, and cytotoxicity. However, acute treatment with BaP showed only an increase in the mRNA expression of CYP1A1. These results suggested that of the five PAHs tested, BaP is the major contributor to the toxic effect of PAHs in monocytic cells, which is likely to occur through CYP and oxidative stress pathways.

Secondly, we investigated a molecular mechanism that would explain BaPinduced HIV-1 replication. We hypothesized that CYP-mediated BaP metabolism generates ROS, and the resultant oxidative stress aggravates HIV-1 replication. As expected, we observed ~4-fold increase in HIV-1 replication in differentiated U1 (HIV-1- infected U937) monocytic cell lines and human primary macrophages after chronic BaP exposure. We also observed ~30-fold increase in the expression of CYP1A1 at mRNA level, ~2-fold increase in its enzymatic activity, as well as, an elevated ROS level and cytotoxicity in U1 cells. The knock-down of the CYP1A1 gene using siRNA and treatment with selective CYP inhibitors and antioxidants significantly reduced HIV-1 replication. Further, we observed a nuclear translocation of NF-κB subunits (p50 and p65) after chronic BaP exposure, which was reduced by treatment with siRNA/ inhibitors of CYP and antioxidants. Suppression of NF-κB pathway using specific NF-κB inhibitors also significantly reduced HIV-1 replication. Together, our results suggest that BaP enhances the HIV-1 replication in macrophages by CYP-mediated oxidative stress pathway via NF-κB signaling cascade.

Thirdly, we explored the underlying mechanism by which HPV increases HIV-1 pathogenesis. HPV infection is one of the major factors that contribute to a reduced suppression of the virus in HIV-1 patients. There is a high prevalence of comorbidity of HIV-1 and HPV (which leads to cervical cancer) among HIV-1-infected population. We proposed that exosomes secreted from HPV-infected cervical

cancer cells exacerbate HIV-1 replication in HIV-1-infected macrophages. To test the hypothesis, we treated U1 cells (HIV-1-infected monocytic cell line) with the cell culture supernatant (CCS) obtained from caski cells (HPV-infected cervical cancer cells). We observed an ~2-fold increase in HIV-1 replication in the treated U1 cell. We also observed a significant increase in the expression of CYPs (CYP 1A1 and 2A6) at the mRNA level. However, we did not observe any significant change in the expression of CYPs as well as antioxidants enzymes (catalase, PRDX6) at the protein level. Furthermore, we isolated exosomes from the caski cell culture supernatant and observed the presence of CYPs (1A1, 2A6), SOD1 and HPV protein HPV16 E6 in caski exosomes. The exosomes derived from caski cells (CCS-Exo) significantly reduced cytotoxicity, while it increased HIV-1 replication in U1 cells. Treatment of antioxidant such as resveratrol, CYP1A1 inhibitor (ellipticine) and CYP2A6 inhibitor (Tryptamine) including chemodietary agents such as curcumin (20 µM) and curcubitacin-D (0.1 µM), significantly reduced the CCS and CCS-Exo mediated HIV-1 replication in U1 cells. These results suggest the role of specific CYP-induced oxidative stress pathway in HIV-1 replication. Altogether, we demonstrated that cervical cancer cells exacerbate HIV-1 replication in monocytic cells via transferring oxidative stress factors such as CYPs and HPV oncoproteins through exosomes. We also showed that the viral replication undergoes via a CYP-mediated oxidative stress pathway and it can be reduced by treatment of chemodietary agents like curcumin and Cucurbitacin-D. The present study therefore, illustrates that comorbidities such as smoking and HPV coinfection contribute to HIV-1 replication and pathogenesis in monocytic cells via a CYP-mediated oxidative stress pathway. The study also provides scientific rationale for the development of novel therapeutic treatment for PLWHs with these comorbidities by targeting CYP and specific oxidative stress pathway.

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Effect of Comorbidities on HIV-1 Pathogenesis in Monocytic Cells

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Sabina Ranjit
December 2018

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DEDICATION

I dedicate this dissertation to my beloved parents, Mr. Krishna Prasad Ranjitkar and Mrs. Sanu Maiya Ranjit, for their unconditional love and support. Thank you for believing in me and encouraging me to achieve my dreams.

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ABSTRACT

The advent of antiretroviral therapy (ART) has substantially increased the life span of people living with HIV-1 (PLWH). However, the presence of comorbid conditions such as high prevalence of drug abuse and coinfections have deteriorated the health outcomes in PLWH and progression of HIV-1 infection to AIDS. In this study, we will be focusing on the independent effect of two major comorbidities: cigarette smoking and human papilloma virus (HPV) coinfection on HIV-1 replication and pathogenesis in monocytic cells.

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level, ~2-fold increase in its enzymatic activity, as well as, an elevated ROS level and cytotoxicity in U1 cells. The knock-down of the CYP1A1 gene using siRNA and treatment with selective CYP inhibitors and antioxidants significantly reduced HIV-1 replication. Further, we observed a nuclear translocation of NF-κB subunits (p50 and p65) after chronic BaP exposure, which was reduced by treatment with siRNA/inhibitors of CYP and antioxidants. Suppression of NF-κB pathway using specific NF-κB inhibitors also significantly reduced HIV-1 replication. Together, our results suggest that BaP enhances the HIV-1 replication in macrophages by CYP-mediated oxidative stress pathway via NF-κB signaling cascade.

Thirdly, we explored the underlying mechanism by which HPV increases HIV-1 pathogenesis. HPV infection is one of the major factors that contribute to a reduced suppression of the virus in HIV-1 patients. There is a high prevalence of comorbidity of HIV-1 and HPV (which leads to cervical cancer) among HIV-1-infected population. We proposed that exosomes secreted from HPV-infected cervical cancer cells exacerbate HIV-1 replication in HIV-1-infected macrophages. To test the hypothesis, we treated U1 cells (HIV-1-infected monocytic cell line) with the cell culture supernatant (CCS) obtained from caski cells (HPV-infected cervical cancer cells). We observed an ~2-fold increase in HIV-1 replication in the treated U1 cell. We also observed a significant increase in the expression of CYPs (CYP 1A1 and 2A6) at the mRNA level. However, we did not observe any significant change in the expression of CYPs as well as antioxidants enzymes (catalase, PRDX6) at the protein level. Furthermore, we isolated exosomes from the caski cell culture supernatant and observed the presence of CYPs (1A1, 2A6), SOD1 and HPV protein HPV16 E6 in caski exosomes. The exosomes derived from caski cells (CCS-Exo) significantly reduced cytotoxicity, while it increased HIV-1 replication in U1 cells. Treatment of antioxidant such as resveratrol, CYP1A1 inhibitor (ellipticine) and CYP2A6 inhibitor (Tryptamine) including chemodietary agents such as curcumin (20 μM) and curcubitacin-D (0.1 μM), significantly reduced the CCS and CCS-Exo mediated HIV-1 replication in U1 cells. These results suggest the role of specific CYP-induced oxidative stress pathway in HIV-1 replication. Altogether, we demonstrated that cervical cancer cells exacerbate HIV-1 replication in monocytic cells via transferring oxidative stress factors such as CYPs and HPV oncoproteins through exosomes. We also showed that the viral replication undergoes via a CYP-mediated oxidative stress pathway and it can be reduced by treatment of chemodietary agents like curcumin and Cucurbitacin-D.

The present study therefore, illustrates that comorbidities such as smoking and HPV coinfection contribute to HIV-1 replication and pathogenesis in monocytic cells via a CYP-mediated oxidative stress pathway. The study also provides scientific rationale for the development of novel therapeutic treatment for PLWHs with these comorbidities by targeting CYP and specific oxidative stress pathway.

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LIST OF ABBREVIATIONS

AHR	Aryl Hydrocarbon Receptor
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
APOBEC	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like
Arnt	AHR Nuclear Translocator
AOE	Antioxidant Enzymes
ASK1	Apoptosis Signal-regulating Kinase 1
BaP	Benzo(a)pyrene
BeA	Benzo(a)antharacene
BeF	Benzo(b)fluoranthene
BHT	Butylatedhydroxy Toluene
BHA	Butylatedhydroxy Anisole
CCS	Cell Culture Supernatant from Caski Cells
CCS-Exo	Exosomes Derived from Caski Cell Culture Supernatant
CCR5	C-C Chemokine Receptor Type 5
CD4/8	Cluster of Differentiation 4/8
CDC	Center for Disease Control and Prevention
C/EBPβ	CCAAT Enhancer Binding Protein β
CSC	Cigarette Smoke Condensate
CXCR4	C-X-C Chemokine Receptor Type 4
CYP	Cytochrome P450
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
Epi	Ellipticine
ESCRT	Endosomal Sorting Complex Required for Transport
gp120	Glycoprotein 120
HIV-1	Human Immunodeficiency Virus-1
HPV	Human Papilloma Virus
HSIL	High-grade Squamous Epithelial Lesions
IL1-β	Interleukin 1-beta
IKK-16	IkB Kinase 16
IARC	International Agency for Research on Cancer
LTR	Long Terminal Repeat
MAPK	Mitogen-Activated Protein Kinases
MHC I	Major Histocompatibility Complex
MIP-1a	Macrophage Inflammatory Protein
Nef	Negative Regulatory Factor
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NMDA	N-Methyl-D-Aspartate
NPh	Naphthalene
Nrf-2	Nuclear Factor Erythroid 2- related Factor
NRTI	Nucleoside Reverse Transcriptase Inhibitor

NNRTI	Non- Nucleoside Reverse Transcriptase Inhibitor
PAH	Polyaryl Hydrocarbon
PBMC	Peripheral Blood Mononuclear Cells
PLWH	People Living with HIV-1
PLWHA	People Living with HIV-1/AIDS
Phe	Phenanthrene
PI	Protease Inhibitors
PIC	Preintegration Complex
Pin	Pinnostilbene
P-TEFb	Positive Transcription Elongation Factor b
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
Res	Resveretrol
RT	Reverse Transcriptase
ROS	Reactive Oxygen Species
SOD1/2	Superoxide Dismutase 1/2
SiRNA	Small Interfering RNA
TAC	Total Antioxidant Capacity
TAR	Transactivation Response
Tat	Transactivator of Transcription
TB	Tuberculosis
TNF-β	Tumor Necrosis Factor-beta
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	Viral Infectivity Factor
Vpr	Viral Protein R
Vpu	Viral Protein U
Vit C	Vitamin C
Vit E	Vitamin E
XRE	Xenobiotic Responsive Element

CHAPTER 1. INTRODUCTION*

HIV-1 Prevalence and Epidemiology

According to the surveillance report by Center for Disease Control and Prevention (CDC), 36.7 million people are currently living with HIV-1 worldwide and about the same number of people (35 million) have died since the start of the epidemic. The Joint United Nations Program on HIV-1/AIDS (UNAIDS) published a report in 2017, which revealed that the global prevalence of HIV-1 is 0.8% in adults. The report also revealed that, approximately 69.4% of the people living with HIV/AIDS (PLWHA) reside in the Sub-Saharan Africa, 13.8% in Asia and Pacific, 5.7% in West and Central Europe and North America, 5.7% in Latin America and Caribbean, 4.3% in East Europe and Central Asia, and 0.6% in Middle East and North Africa [1]. In the United States alone, there were around 1.1 million PLWHA with 38,500 new infections and 6465 deaths due to HIV infection in the year 2015 (CDC report). However, the deaths resulting from HIV-1 infection dropped by 45% between 2005 and 2015 (UNAIDS 2015 report). The decrease in HIV-1-related deaths may be attributed to the advancements in antiretroviral therapy (ART). It was reported that 62% of the PLWH received ART, 48% retained continuous treatment and 49% achieved viral suppression in 2014 [2]. Although, there was a substantial decrease in deaths due to HIV-1, the rate of incidence of HIV-1 decreased only by 8% from year 2010 to 2015. The incidence rate was observed highest among males (IR= 24.1), persons aged 25-34 (IR=31.3) and black/African Americans (IR=49%). The introduction of ART has indeed elongated the life span of PLWHA, however, they are now vulnerable to various HIV-1-associated comorbidities like cancer, tuberculosis and HIV-associated neurodegenerative diseases (HAND). The burden of cancer (especially non-Hodgkin's lymphoma, Kaposi's sarcoma, anal cancer and lung cancer) has substantially increased in PLWHA by about 50% in 2010 [3]. Cancer-related deaths in PLWHA have also increased by 8% within a decade from 2000 to 2010 [4]. Similarly, in a study conducted by Sactor et al. (2016), they observed that the frequency of HAND increased in PLWHA by 6% from 2009 to 2012 [5].

HIV-1 Biology

HIV-1 Structure

HIV-1 is a primate lentivirus that belongs to a family of retrovirus. Retroviruses are capable to reverse-transcribe their RNA into DNA and incorporate the viral DNA into

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* Portions of chapter 1 modified with permission. Ranjit, S., Kumar, S. *Recent advances in cancer outcomes in HIV-positive smokers.* F1000 Faculty Rev, 2018. 718 (doi:10.12688/f1000research.12068.1)

the host genome, which is an important mechanism of sustenance for the viruses belonging to this category Lentivirus is a type of retroviruses with longer incubation period. HIV-1 is spherical in shape with a diameter of approximately 100 nm. The virus comprises of two single-stranded RNA enclosed within a cone shaped capsid made of p24 proteins. The RNA comprises of three essential genes (gap, pol and env), two regulatory genes (tat and rev) and four accessory genes (vif, vpr, vpu, and nef) that encodes for altogether 15 proteins. Within the capsid, there are other proteins and enzymes such as reverse transcriptase, integrase, protease, vif vpr, nef, and p7, which are crucial for viral development. Surrounding the capsid is a matrix made of p17 protein, whose integrity is maintained by bilayered phospholipid membrane. The phospholipid membrane, which forms the viral envelope is embedded with 72 Env knobs, each comprised of trimer of gp41 topped with trimer of gp120. The gp41 trimer form a stem of Env protein, which is embedded into the lipid membrane, while gp120 forms a cap that protrudes out of the membrane [6].

HIV Life Cycle

Viral entry. HIV-1 entry to the host cells is initiated by binding of viral envelope protein, gp120 with CD4 receptors on the surface of the host cell membrane. Binding of gp120 with CD4 receptor changes the conformation of gp120, which facilitates its binding to host cell co-receptors such as CCR5 and CXCR4. This interaction exposes the gp41 subunit of the viral Env protein, which causes fusion of viral envelop and host cell membrane. The viral matrix and capsid are then phosphorylated by cellular kinases (e.g. MAPK kinases), which uncoats the capsid and releases its contents including the two single-stranded RNA, into the host cytoplasm [7]. HIV-1 enters the host cell through CCR5 coreceptors during the early stages of viral infection, while during the later stages of developing immunodeficiency, the virus tends to enter via CXCR4 coreceptors [8]. HIV-1 also enters macrophages through endocytic pathway in a CD4 and CCR5-independent manner, especially via clathrin-mediated micropinocytosis. However, the viruses that enter the host cells via endocytosis have lesser infectivity, which is most likely due to the inactivation of viral particles within the endosomes and lysosomes [9]. Another mechanism of HIV-1 entry is via DC-SIGN (a C-type lectin expressed in the surface of dendritic cells), independent of CD4 receptor. Dendritic cells are present in peripheral mucosal tissues and have a major function as antigen- presenting cells in lymphoid organs. HIV-1 gp120 proteins binds to DC-SIGN lectins on the immatured dendritic cells. Upon maturation, the dendritic cells transport the DC-SIGN-bound viruses to the T-cells in the lymphatic organs [8].

Reverse transcription. Shortly after the virus fuses to the host plasma membrane, there occurs the formation of reverse transcription complex into the host cytoplasm, which comprises two single-stranded viral RNA, cellular lysine transfer RNA (tRNA Lys), reverse transcriptase (RT), integrase, capsid and cellular proteins and viral matrix. RT enzymes comprise of two components: DNA polymerase and RNase H. DNA polymerase uses cellular nucleotides to synthesize DNA strands. Cellular tRNA Lys binds to the primer binding site near the 5’ terminal end of the viral RNA genome and

serves as a primer for the synthesis of (-)-strand DNA by adding complementary nucleotides along the 5' end of the viral RNA genome. RNase H degrades sections of RNA from the RNA/DNA duplex, except for the purine-rich segments. These purine-rich segments serve as primers for the synthesis of (+)-strand DNA using tRNA Lys bound (-)-strand DNA as a template [10]. After the formation of linear double stranded DNA, it forms a preintegration complex (PIC) with viral proteins (integrase, matrix, Vpr and capsid), which is translocated into the host nucleus via microtubule network [11].

Viral integration and transcription. Within the host nucleus, the preintegration complex is integrated into the host DNA by viral protein, integrase, preferentially near the DNAse-hypersensitive sites [12]. As the host cell is unable to distinguish between the proviral DNA and the DNA of its own, the host nuclear enzymes transcribe proviral DNA into viral messenger and genomic RNA. The LTR region of the proviral DNA contains binding sites for host cell nuclear transcription factors such as nuclear factor - kappa B (NF-κB) and nuclear factor of activated T cells (NFAT) and RNA polymerase II. NF-κB and NFAT are present in the cytoplasm in their inactive state, which translocate into the nucleus after activation by ROS, various cytokines or antigens. Binding of these transcription factors and RNA polymerase II at the proviral LTR region leads to the initiation of viral DNA transcription, forming short HIV-1 transcripts with RNA stem loop called transactivation response (TAR) element. The elongation of these short transcripts is facilitated by the binding of a viral transactivator protein, Tat and positive transcription elongation factor b (P-TEFb) at the TAR element. The complex of Tat and P-TEFb activates RNA polymerase II to elongate the viral transcripts. The viral mRNA and genomic RNA thus formed are exported from the host nucleus to the host cytoplasm.

Viral translation. Within the cytoplasm, the cellular enzymes translate the viral mRNA into viral proteins. Unspliced transcripts are used to synthesize Gag and Gag-Pro-Pol proteins and spliced transcripts are used to synthesize Env, Nef, Tat, and Vpr. Larger polypeptides are cleaved by viral protein, protease into small functional proteins. Viral proteins such as Tat and Rev have functions in the transcription of proviral DNA within the nucleus, while viral Nef and Vpr alter the cellular environment for efficient viral replication. Nef downregulates the expression of CD4 and MHC I determinants on the surface of the infected cells, thereby protecting them from being engulfed by CD8 cytotoxic T cells. Nef also inhibits p53-mediated apoptosis of the infected cells [13] and promotes viral morphogenesis, budding, and infectivity [14]. During the budding stage, vif protects the virion from the antiviral activity of a cellular protein, APOBEC through polyubiquitination and proteosomal degradation [15].

Viral assembly and budding. All viral structural proteins can be derived from three polyproteins: Gag, Gag-Pro-Pol, and Env. Viral assembly occurs by clustering of ~1500-2000 Gag proteins, ~200 Gag-Pol-Pro, and 2 copies of viral RNA genome at the inner surface of the plasma membrane. Env proteins synthesized at the rough endoplasmic reticulum also translocate towards the viral budding site of the plasma membrane and package on the surface of the Gag after budding of the virion. The immature virion buds from the plasma membrane by hijacking the host cell endosomal sorting complex required for transport (ESCRT) [16]. After budding, the viral protease

cleaves Gag and Gag-Pol-Pro proteins into functional proteins of a mature, infectious virion [17, 18]. The schematic diagram for the HIV-1 life cycle and antiretroviral drugs that act on its various stages has been shown in **Figure 1-1**.

Viral latency. HIV-1 particles can reside within the cells in a dormant state without replicating, which is termed as viral latency. Viral latency may occur either via failure of PIC integration into the host gene or inhibition of the proviral DNA transcription. PIC integration may be interrupted if the nuclear transport of the complex is inhibited or if a weak PIC complex is synthesized due to poor reserve transcription [19]. Unintegrated PICs are often observed in viral reservoirs such as macrophages and they are capable of transcribing viral genes such as Nef and Tat [20]. If PIC is able to reach the nucleus, but binds to transcriptionally inactive heterochromatins, proviral DNA transcription does not occur [19]. Some cells lack transcription factors (NFκB, NFAT) and viral proteins (Tat), which impedes the proviral DNA transcription within them. While some cells, for example, monocytes contain high levels of anti-HIV-1 miRNA (miRNA-382, miRNA-150, miRNA-28, miRNA-223) that also inhibit the proviral DNA transcription [21].

HIV Treatment Strategies

HIV-1 mainly targets the immune cells with CD4 receptors such as T lymphocytes, monocytes, macrophages and dendritic cells [22]. After infection, the virus replicates within the cells leading to progressive loss of $CD4^+$ T cells and immunosuppression. Over the time, the immune deficiency develops characteristic opportunistic infections and/or cancerous conditions called Acquired Immunodeficiency Syndrome (AIDS). With the introduction of antiretroviral therapy (ART), HIV-1 is no longer a life-threating disease but a chronic manageable disease. According to the recent guidelines by the U.S. department of health and human services, ART treatment for HIV-1 is recommended for all individuals with HIV-1, regardless of $CD4^+$ cell counts [23]. ART does not cure or eradicate HIV-1, but suppresses the viral replication and minimizes the risk of viral transmission. There are 5 different classes of antiretroviral (ART) drugs approved by Food and Drug Administration (FDA) that are currently available commercially: 1. Nucleoside reverse transcriptase inhibitors (NRTIs) 2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) 3. Protease inhibitors (PIs) 4. Entry inhibitors, and 5. Integrase inhibitors [24].

NRTIs are the first FDA approved ARTs for HIV-1 treatment. As they have structural similarity to the cellular nucleosides, they compete with these natural nucleosides to make up viral DNA. Since, they do not have a 3'-hydroxyl group at the sugar moiety, the drugs targeting this hinder the formation of 3, 5'-phosphodiester bond between a NRTI and incoming 5'-nucleosides, thus interrupting the formation of viral DNA chain. On the other hand, NNRTIs binds to the noncatalytic allosteric site of the reverse transcriptase enzyme, which brings about a conformational change in its substrate binding site, leading to its reduced polymerase activity. NRTIs are administered as prodrugs, which require activation by cellular kinases to mediate its action, while

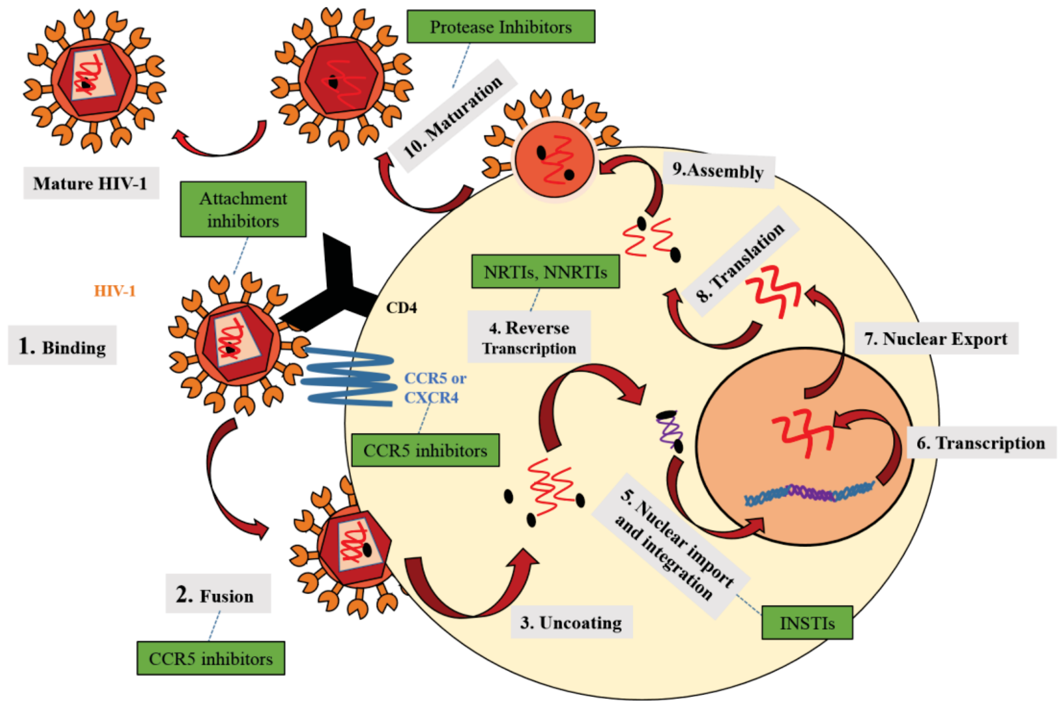

Figure 1-1. **HIV-1 life cycle and antiretroviral drugs that act on its various stages.**

NNRTIs are administered in their active forms. NRTIs and NNRTIs include 50% of the commercially available ART drugs [25]. Integrase inhibitors acts by inhibiting the HIV-1 DNA strand transfer and integration into the cellular DNA. Protease inhibitors interfere with viral particle synthesis by blocking proteolysis of essential viral polyproteins, gag and gag-pol. Entry inhibitors are relatively new class of ART drugs that are subclassified into Fusion inhibitors, CCR5 antagonists, and post attachment inhibitors. By binding to the viral envelop proteins, fusion inhibitors block the formation of six helical bundle structure, which provides the force necessary for fusion pore formation in the cell membrane. CCR5 antagonists block viral entry by binding to the allosteric sites of CCR5 coreceptors thereby, changing its structural conformation such that the HIV-1 envelop protein, gp41 cannot recognize the binding site [26]. Post attachment inhibitors act after gp120 binds to CD4 receptors. Binding of these inhibitors changes the conformation of gp120-bound CD4 receptors such that it impedes the interaction of the later with the CCR5 and CXCR4 coreceptors, which is an important step for viral entry into the host cells [27]. Pharmacokinetic enhancer drugs such as cobicistat and ritonavir are also used in combination with other ART drugs. Although ritonavir has some antiviral activity, cobicistat does not have antiviral activity but it boosts the activity of other antiviral drugs (such as PIs and elvitegravir) by inhibiting CYP3A4 enzymes, which metabolize majority of the antiretroviral drugs [28].

Drug Abuse

Drug abuse is a major problem among PLWHA with approximately three-fold higher prevalence compared to the general population [29]. A study done by the NIH during 2005-2009 revealed that about one-third of the HIV-1 population were current drug users or alcoholics and 24% of them received medical treatment for drug abuse [30]. A cross sectional analysis study from the data obtained from 16 studies conducted at 14 sites across United States revealed that 47.7% of PLWHA are involved in alcohol use and 38.1% involved in illicit drug use. The report also suggested that 22.1% of the participants used more than one drugs of abuse [31]. Another report published by Gamarel et al. (2016) suggests that out of 2216 PLWHA they surveyed, 32.9% frequently use tobacco, 27.5% use marijuana, 21.3% use alcohol, and 22.5% use other illicit drugs [32]. These drugs directly affect the pathogenesis of HIV-1/AIDS by enhancing immunosuppression or by activating cellular signaling pathways that enhance the viral replication [33]. As an indirect effect, substance abuse alters the mood, behavior, and capacity of judgement and decision making in PLWHA. This behavioral change indulges them in activities such as sharing needles, unprotected sex, and sex with multiple partners, which highly increase the risk of exposure to blood and other fluids of infected individuals and therefore HIV-1 transmission. The report by the NIH also showed that 16% of HIV-1 transmission occurred through intravenous drug use and the remaining 84% via sexual contact, especially men having sex with men (MSM). Drug abuse also causes non-adherence to ART and drug-drug interactions resulting in reduced efficacy and increased toxicity of ART [34, 35]. Moreover, PLWHA are usually involved in the use of more than one drugs of abuse [35] , which directly act on central nervous system, causing neuroinflammation and neurotoxicity. The neurotoxic effects from multiple drug

use further accentuates the neuronal damage caused by HIV-1 and increase risk of HIV-1-associated neurodegenerative diseases.

Smoking

The frequency of tobacco smoking is more than twice as high among PLWHA as it is in the general population [36]. Due to this increased prevalence, the deleterious effects of smoking on treatment outcomes for PLWHA have been investigated in several studies [37, 38]. While smoking in the general population is known to enhance the risk of cancers and various respiratory and cardiovascular diseases, PLWHA face an even greater likelihood of developing life-threatening conditions related to tobacco use [39, 40]. Additionally, an analysis of patient populations provided with sufficient access to ART and quality healthcare has demonstrated that HIV-1-infected smokers display the highest rate of all-cause mortality compared to other HIV-1-infected individuals and population controls [41]. The explanation about how tobacco plays role in HIV-1 replication and pathogenesis will be discussed in detail in later sections.

Alcohol

The prevalence of alcohol abuse among PLWHA is 2-4 times higher than the general population [42]. Approximately 40-50% PLWHA are reported to have used alcohol, among which ~28.8% and ~10% are involved in harmful and hazardous drinking habits respectively [43-46]. High prevalence of heavy alcohol drinking is observed more among PLWHA who use one or more kinds of illicit drugs but the opposite has been observed in case of patients on ART with low CD4 counts [44, 47]. PLWH involved in hazardous drinking are 35% less likely to show adherence to ART, and the viral suppression among these patients is also 25% lesser than their non-drinking counter parts [44]. Alcohol also enhances the metabolism of ART drugs (such as PIs and NNRTIs) by upregulating the expression of drug metabolizing enzyme CYP3A4. The enhanced metabolism prevents ART to attain optimal concentrations at the target tissues and therefore reduces their efficacy [29]. Alcohol induces oxidative stress by upregulating the expression of CYP2E1, NAPDH oxidase, chemokines, and cytokines [29, 48-50] in hepatic cells, monocytes/macrophages, astrocytes, and neurons. The alcohol-induced oxidative stress causes liver damage and aggravates other comorbid conditions (like Hepatitis B and C infections, hypertension, and diabetes) in PLWH. Within the brain, the oxidative stress compromises the integrity of the blood-brain-barrier, making the brain tissues more vulnerable to neurotoxic effects of HIV-1 and other toxic substances. The alcohol-induced oxidative stress adds to the effect of HIV-1-induced oxidative stress, which further aggravate viral replication and apoptosis in brain cells. By enhancing the expression of various pro-inflammatory cytokines and chemokines within the brain tissues, alcohol promotes neuroinflammation and further elevate ROS generation. Alcohol exposure is also reported to cause glutamate toxicity in neurons through overstimulation of NMDA receptors [51]. Moreover, alcohol exposure is shown to potentiate the neurotoxic effects of HIV-1 proteins such as Tat, Nef, Vpr and gp120 [52,

53]. Several clinical studies have shown that alcohol use has both additive and interactive effects on mediating neuropathological conditions in HIV-1 patients, including HAND [54, 55].

Marijuana

Marijuana is the most commonly used illicit drug among PLWHA. Recent studies conducted on a cohort of HIV-1-infected patients receiving ART report that approximately 20-30% of the infected population are addicted to marijuana [32, 56-59]. A study conducted by Fogarty et al. (2007) reported that ~60% of 408 HIV-1 infected patients in their study used marijuana; 55.7% of which reported to use it for recreational purpose, while 44.3% used marijuana for both recreational and medicinal purpose [60]. The effect of marijuana on adherence to ART and viral suppression in PLWH under care, is a disputed subject due to the mixed findings obtained in different cohorts of PLWH depending upon its use as illicit, medicinal, or recreational purpose. Some studies have revealed that non-adherence to ART is frequently observed in PLWH who are addicted to marijuana [61, 62]. In contrast, some studies suggest that use of marijuana can have a positive effect on ART adherence through management of HIV symptoms and side effects of ART like anxiety, depression, fatigue, nausea, diarrhea, and peripheral neuropathy [58, 59, 63]. Interestingly, a recent study has revealed no significant association between the high-intensity marijuana use and ART adherence, suggesting that its use can rather be clinically beneficial for HIV symptom management [64]. Similarly, there is also a discrepancy on whether marijuana use affect the viral suppression in PLWH, with different studies showing contrast results [57, 61, 65]. However, a significant decrease in neurocognitive performance, learning, memory, and poor mental quality of life have been observed in PLWH who use marijuana in a moderate to heavy range, compared to those who used lightly or none [65-68]. Moreover, higher impact on learning and memory impairment have been observed among PLWH with early onset of marijuana (regular use prior to age 18) compared to the non-marijuana users or those with late onset of marijuana (regular use at 18 or later) [69]. Use of marijuana also leads to behavioral changes like involving in unprotected sex, which increases the probability of HIV-1 transmission [70].

FDA has approved Δ9-tetrahydrocannabinol (Δ9-THC), a major psychoactive component in marijuana, for the treatment of anorexia in PLWH. Clinical studies have also shown that its administration have minimal or very less effect on the viral load, $CD4^+$ and $CD8^+$ cell counts and protease inhibitor levels over a short-term treatment [71]. Chronic exposure of Δ9-THC in SIV-infected rhesus macaques showed that Δ9-THC does not enhance viral load or morbidity but attenuates the SIV progression through reduction of viral levels and inflammation [72]. However, the effect of Δ9-THC in SIV-infected rhesus macaques is sex-dependent. In case of female rhesus macaques, Δ9-THC did not show any reduction in viral load or $CD4^+/CD8^+$ ratio; instead, it increased the expression of CXCR4 in $CD4^+/CD8^+$ lymphocytes prior to SIV inoculation [73]. With these mixed results, it is not clear if the use of marijuana can actually attenuate HIV-1

progression in human, which highlights the need of further clinical investigation in this regard.

Cocaine

After Marijuana, cocaine is the second most used illicit drugs among PLWH with 8-15% PLWH involved in cocaine abuse [31, 74, 75]. Unlike marijuana, cocaine use has a negative impact on ART adherence. The adherence to ART is observed to be ~3-fold lower in cocaine users compared to non-users [76]. In addition, decreased ART response, increased viral load and $CD4^+$ T cell depletion, and increased progression of AIDS have also been observed in PLWH who used cocaine [77, 78]. Furthermore, patients under ART are more prone to show increased cocaine toxicity through cocaine-ART interactions. Since, ART drugs such as PIs and NNRTIs are inducers/inhibitors of CYP3A4, they alter the CY3A4-mediated metabolism of cocaine. Enhanced formation of norcocaine, a hepatotoxic metabolite of cocaine leads to severe liver injury [34].

Cocaine abuse directly impacts HIV-1 replication in various immune cells by enhancing the viral entry into the host cells through upregulation of CCR5 and CXCR4 coreceptors and downregulation of chemokines (RANTES, MIP-1a and MIP-1b) that suppress HIV-1 entry into the host cell membrane [79, 80]. Cocaine also induces viral replication by enhancing the integration of PIC into host cell DNA [81]. Studies have also shown that cocaine negatively modulates anti-HIV miRNAs (such as miRNA-125b), which suppress HIV-1 replication in monocyte-derived dendritic cells by inhibiting the translation of HIV-1 transcripts [82]. In addition to its role in viral replication, cocaine exposure has also shown to cause apoptosis of $CD4^+$ cells by inducing oxidative stress and mitochondrial depolarization [83].

Moreover, cocaine also plays a major role in the progression of HAND in PLWH. Higher extent of neurocognitive impairment and neurodegeneration, and faster progression to NeuroAIDS have been observed in PLWH who use cocaine compared to the non-drug users [84]. By compromising the integrity of BBB, cocaine increases the viral invasion into the brain tissues. More importantly, cocaine exposure not only enhances viral replication in macrophages and microglia but also in astrocytes, which are normally resistance to viral replication [85]. Cocaine also forms platelet-monocyte complex in the blood, which can easily cross the BBB and cause neuroinflammation. In addition, cocaine activates viral proteins such as Tat and gp120, which causes neurotoxicity by inducing oxidative stress, loss of mitochondrial potential, and neuronal apoptosis [86, 87]

Coinfections in HIV-1

As HIV-1 weakens the immune system in HIV-1-infected individuals, they are susceptible to infections by various pathogens, including bacteria, fungus, protozoa, and virus. The coinfection with these pathogens is also common in HIV-1 patients who are on

ART because ART does not completely restore the immune response to normal levels. The most common types of coinfections as reported by National Institute of health (NIH) are tuberculosis, hepatitis B and C, and human papilloma virus (HPV) coinfections [88].

Tuberculosis (TB)

According to epidemiological data reported by WHO, the prevalence of TB is about 16-27 times higher among PLWH compared to general population [89]. The report also indicated TB to be the most common coinfection in PLWH and one of the major causes of HIV-1-related deaths [90]. The risk of TB is observed to be high among PLWH with higher viral load, lower $CD4^+$ cell counts, and prior history of TB, however, the risk is observed to be lower in patients who are under ART [91]. $CD4^+$ T cells protect against M. *tuberculosis* by releasing proinflammatory cytokines; macrophages engulf M. *tuberculosis* by phagocytosis and autophagy; $CD8^+$ T cells inactivate TB antigen by lysing the antigen-presenting cells [92]. The number of these cells, (especially $CD4^+$ T cells which are major cells for protecting against M. *tuberculosis*) are reduced in PLWH, which increase the risk and pathogenesis of TB in this population compared to the general population. Further, HIV-1 proteins such as Nef inhibits apoptotic response of macrophages against M. *tuberculosis* [93, 94].

HIV-1 viral load in lung tissues has been observed to be increased by 4-fold compared to the plasma viral load in PLWH coinfected with TB [95]. Similarly, macrophage cell lines (U937 and THP-1) coinfected with HIV-1 and M. *tuberculosis* also exhibited ~3-fold increase in viral load compared to their counterparts without TB infection [96]. Innate and adaptive immune response to M. *tuberculosis* release different proinflammatory cytokines (IL1-β, IL-6 and TNF-β). These cytokines stimulate the nuclear transcription factors that cause the activation of HIV-1 LTR, thereby enhancing the transcription of HIV-1 genome [97-99]. Activated lymphocytes are shown to increase HIV-1 replication in HIV-1/TB coinfected alveolar macrophages through downregulation of CCAAT enhancer binding protein β (C/EBPβ) and upregulation of NF-κB in infected macrophages. These are nuclear transcription factors that inhibit and promote activation of HIV-1 long terminal repeat (LTR), respectively [97]. Innate immune response to TB has also been shown to increase the gene and protein expression of another nuclear transcription factor, nuclear factor of activated T cells 5 (NFAT 5), which also act on HIV-1 LTR leading to enhanced HIV-1 gene transcription [98]. Furthermore, release of cytokines in response to TB causes accumulation of T cells, which are major targets for HIV-1 infection [100, 101]

Hepatitis B and C

Coinfection with hepatitis B virus (HBV) and hepatitis C virus (HCV) are common in PLWH. The prevalence of hepatitis B and C are 10-times and 6-times, respectively, higher in PLWH compared to the general population [102]. However, CDC reported that the incidence of HCV is approximately 3-times higher than hepatitis B

among PLWH in USA [103]. The higher prevalence of HCV and HBV coinfection in PLWH is perhaps due to the common mode of transmission for these viruses through sharing of infected needles and sexual contact. However, HCV is more prevalent among PLWH who use intravenous drugs (72-95%) compared to those who are MSM (1-12%) or heterosexuals (9-27%) [102]. With the depletion of $CD4^+$ T cells in HIV-1 infection, there is further loss of HCV specific $CD4^+$ and $CD8^+$ T cell responses in HIV-1/hepatitis coinfection, which provides a favorable environment for HCV replication and pathogenesis [104, 105]. Further, HIV-1 envelop proteins such as gp120 directly enhance HCV replication by binding to CCR5 and CXCR4 coreceptors in hepatocytes and subsequently by upregulating cytokines like TGF-β1, which have a stimulatory effect on HCV infection [106].

The effect of HIV-1/hepatitis virus coinfection in the progression of HIV-1 disease is not clear due to the conflicting evidences presented by different studies. Several studies conducted before ART era reported that HIV-1/hepatitis coinfection does not impact the progression of HIV-1 disease [107-110]. A study done in 9802 European HIV-1 cohorts revealed that HIV-1/HBV coinfection did significantly increase the liver-related mortalities, but it did not have any effect on the progression of HIV-1/AIDS [111]. Another study done in a cohort of 328 HIV-1 patients under antiretroviral therapy also indicated no association of HCV on the mortality observed in PLWH [108]. In contrast, many studies conducted in ART era reported increased morbidity and mortality in HIV-1/hepatitis coinfected patients compared to patients infected with HIV-1 alone [112, 113]. For example, a study conducted in 3111 Swiss HIV-1 cohorts under potent ART showed that HCV coinfection increased the clinical progression to AIDS and death rate in HIV-1/HCV coinfected patients, possibly via impaired CD4-cell recovery [112]. HIV-1 envelop proteins and the use of ART are also associated with severe hepatotoxicity, which could have increased the risk of hepatitis infections and subsequent morbidity and mortality in the HIV-1/hepatitis coinfected patients [106, 114]. Further, several studies have suggested the role of HBV protein X (pX) as transcriptional transactivator that lead to HIV-1 replication in $CD4^+$ T cells. HBV pX does not directly bind to HIV-1 LTR, but it activates HIV-1 transcription by stimulating the signaling pathways that activate transcription factors involved in HIV-1 transcription (NF-κB, NFAAT) or by interacting with the basal transcriptional machinery [115].

Human Papilloma Virus (HPV)

Infection with low-risk HPV strains (type 6 and 11) only causes benign warts and condylomas, whereas infection with high risk HPV strains (type 16 and 18) leads to malignant diseases. The incidence of HPV among PLWHA is 1.5-times higher compared to general population. Both HPV 16 and 18 genotypes, which cause cervical cancer, are highly prevalent among PLWHA, with the predominance of the later genotype [116]. The incidence rate high risk HPV infection of cervix is approximately 3-times higher in HIV-1-positive woman compared to HIV-1-negative women. Similarly, the progression of HPV to high-grade squamous epithelial lesions (HSIL) was also ~2.55 times higher among HIV-1-positive women compared to HIV-1-negative women [117]. Several

studies showed that progression of invasive cervical cancer in these patients increased with the decrease in $CD4^+$ cell counts, suggesting a strong association of immunosuppression in the progression of the cancer [118-120]. Furthermore, many studies suggested that HIV-1 infection promotes HPV acquisition either by disrupting the mucosal epithelium or weakening the mucosal immune system, which facilitates HPV entry [121]. In addition to increased HPV acquisition, increased reactivation of latent HPV has also been observed in HIV-1-positive women [122]. HIV-1 proteins like Tat has also been reported to enhance transcription of HPV genome, leading to increased HPV replication [123].

On the other hand, several studies have indicated higher risk of HIV-1 acquisition in HPV-infected patients compared to uninfected individuals [124-126]. A study conducted by Averbach et al. (2010) indicated that HPV-infected women were 2.4-times more susceptible to HIV-1 infection compared to uninfected women [126]. Furthermore, another study suggested the involvement of specific strains of HPV, especially oncogenic HPV Type 16 and 18 in increasing HIV-1 acquisition among PLWHA [127]. However, the actual mechanism by which HPV induces the progression of HIV-1 disease is still unclear. HPV 16 oncoprotein E7 downregulates E-cadherin, an adhesion molecule in the genital epithelial layer [128]. HPV could possibly increase HIV-1 acquisition by interfering with the expression of E-cadherin. Immunological response to HPV accumulates $CD4^+$ T cells at the site of infection, which are primary targets for HIV-1 infection [129]. More than 10-fold increase in the level of the cytokines (IL-6, IL-8, TNF-α, MIP-1α) has also been observed during HPV infection [130]. Several of these cytokines including IL-6, IL-8, and TNF-α are shown to increase the expression of HIV-1 in host immune cells [131-133]. Massive oxidative stress occurs during HPV infection. HPV 16 oncoproteins (E6 and E7) and HPV 18 E2 proteins are known to cause chronic oxidative stress by a NADPH oxidase pathway or by increasing the mitochondrial ROS, respectively [134]. This increase in ROS could possibly activate ROS-sensitive nuclear transcription factors such as NF-κB, leading to the enhanced expression of HIV-1.

CHAPTER 2. INTERACTION OF HIV-1 WITH TOBACCO SMOKING AND HPV INFECTION*

Tobacco Smoking

Tobacco Prevalence in HIV-1 Patients

In a cross-sectional survey conducted on 419,945 patients with HIV-1 in the US in 2015, 42.4% were current smokers, 20.3% were former smokers, and 37.3% were non-smokers [36]. According to the study, the prevalence of smoking among patients with HIV-1 (42.4%) was more than two times higher than the uninfected US adult population (20.6%). A similar trend of smoking prevalence was observed in a study that surveyed HIV-1-infected patients in New York in 2016, which found the association of smoking with poor health outcomes among PLWHA [135]. A study conducted in Ontario, Canada, in 2008 reported that 39.3% of the patients with HIV-1 were smokers, which declined by 1.6% by 2014. However, the rate of smoking remained higher among HIV-1 population than the general population [136]. About 33% of patients with HIV-1 in a study conducted on South Africans were also found to be smokers [136]. Furthermore, the prevalence of smoking is much higher among male HIV-1 patients who have sex with men (MSM) than it is among uninfected men [137-140]. A meta-analysis conducted by Jin et al. on Chinese PLWHA reported the prevalence of smoking to be 41.1% among the study population [141]. **Table 2-1.** summarizes the prevalence of smoking among patients with HIV-1 in different countries (most of the patients are from the US) from 2014 to 2017 [142-144], showing that smoking is highly prevalent in HIV-1-infected populations.

Effect of Tobacco on HIV-1 Pathogenesis

Combustion of cigarette smoke constituents generates large quantity of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite, which are circulated through the blood to various tissues [145]. Excessive

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* Portions of chapter 2 modified with permission. Ranjit, S., Kumar, S. *Recent advances in cancer outcomes in HIV-positive smokers.* F1000 Faculty Rev*,* 2018. 718 (doi:10.12688/f1000research.12068.1)

* Portions of chapter 2 modified with permission. Ranjit, S., Patters, B.J., Gerth, K.A., Haque, S., Choudhary, S., Kumar, S. *Potential neuroprotective role of astroglial exosomes against smoking-induced oxidative stress and HIV-1 replication in the central nervous system*. Expert Opinion on Therapeutic Targets, 2018. **22** (8) (doi.org/10.1080/14728222.2018.1501473)

Table 2-1. Prevalence of smoking among HIV-1-infected population.

Authors	Date of publication	Location	HIV-1 patients (n)	HIV-1 smokers (%)
Mdodo et al. [36]	2015	United States	419,945	42.4
Hile et al. [135]	2016	United States	14,713	40
Bekele et al. [136]	2017	Canada	4473	39.3
Akhtar-Khaleel et al. [138]	2016	United States	6577	36.9
Pacek et al. [142]	2014	United States	358	75
Pollack et al. [144]	2017	Vietnam	636	36.3
Murrison et al. [146]	2016	South Africa	146	33
Brath et al. [143]	2016	Austria and Germany	447	49.4

ROS causes oxidative stress that leads to oxidation of vital cellular components such as DNA, RNA, proteins, and lipids causing cytotoxicity and cell death. Furthermore, ROS also triggers the expression of pro-inflammatory cytokines and chemokines by activating the redox-sensitive nuclear transcription factor, NF-κB [147]. The surge of pro-inflammatory cytokines and chemokines causes the recruitment of leukocytes, primarily monocytes and neutrophils, to the inflamed vascular endothelial cells [148]. Free radicals, elastase, and collagenase released from leukocytes, especially neutrophils, cause endothelial injury contributing to the development of atherosclerotic plaques. Oxidative stress induced by smoking and the subsequent complex pro-inflammatory cascade and atherosclerosis is linked to the pathogenesis of various chronic cardiovascular and respiratory disorders in smokers. As HIV-1 also causes oxidative stress [149-151], the oxidative damage is more pronounced in HIV-1 smokers, which increases the severity of the smoking-induced chronic diseases in PLWH. Higher incidence and severe forms of respiratory infections have been observed in HIV-1 smokers compared to HIV-1 non-smokers and uninfected smokers [152]. The meta-analysis study done by Marshall et al. (2009) suggested that there was high incidence of respiratory infections and non-AIDS-related mortality in HIV-1 smokers compared to HIV-1 non-smokers [140]. HIV-1 smokers mainly suffered death due to smoking-induced chronic diseases like stroke, asthma, COPD, coronary artery disease, peripheral vascular diseases, or cancers of organs like oropharynx, larynx, esophagus, lungs, cervix, stomach, bladder or kidney [37].

Studies have shown that smoking enhances HIV-1 replication in immune cells [153, 154]. Recent studies from Kumar's lab demonstrated higher nicotine metabolism, HIV-1 viral load, ROS level and oxidative damage in HIV-1-positive smokers relative to HIV-1-positive non-smokers and uninfected smokers [153, 155]. Similar increase in HIV-1 viral load and oxidative stress have been reported *in vitro* in cigarette smoke condensate (CSC)-treated HIV-1-infected monocytic cells (U1 cells) or HIV-1-infected human primary cells [154]. We also observed significant increase in CYP enzyme expression: CYP2A6 and CYP3A4 in HIV-1 patients and HIV-1 smokers respectively; CYP1A1 and CYP1B1 in HIV-1-infected monocytic cells. When we inhibited the activity of these CYPs using specific CYP inhibitors or by specific siRNA knockdown, we observed a reduction in oxidative stress and viral load in CSC-treated cells, indicating the role of CYP enzymes in smoking-mediated oxidative stress and HIV-1 replication. Similarly, other studies have also revealed that CYP enzymes metabolize polyaromatic hydrocarbons in cigarette smoke into toxic metabolites that enhances HIV-1 gene expression by forming DNA adducts [156]. Another study showed that, tobacco enhances HIV-1 infectivity by up-regulating the expression of genes involved in viral replication (e.g. CD59 and TAX1BP1) as well as by down-regulating the genes that modulate cellular defense (e.g. TXNIP and PHGPX) and antigen presentation (e.g. MHC) [157].

Effect of Tobacco on HIV-1 Treatment Outcomes

Several studies have indicated that cigarette smoke decreases the response to ART [158, 159]. However, it is not clear how tobacco use in PLWH impact ART efficacy. A

study done by Feldman et al. (2006) demonstrated that there is higher rate of mortality (53%) and morbidity (AIDS-defining cancer = 36%) among PLWH on ART who smoked compared to those who did not smoke [158]. The study findings emphasize non-adherence to ART as a possible cause for decreased response to ART among HIV-1 smokers. Another report by Miguez-Burbano et al. (2003) also showed that the immune and virologic response to ART decreased by 40% in HIV-1 smokers compared to HIV-1 non-smokers [160]. This study suggested that tobacco use may reduce ART efficacy by interfering with the metabolism of ART drugs. Tobacco induces the expression of various CYP enzymes such as CYP 1A1, 1B1, 2A6, and 3A4 in different cell types including monocytes and macrophages [153, 154]. CYP3A4, which is a major drug metabolizing enzyme, metabolizes different ART drugs especially, NNRTIs and PIs [161]. Induction of CYP3A4 by tobacco may result in the enhanced metabolism of ARTs and consequently its reduced efficacy [159]. On the other hand, inhibition of CYPs by tobacco can also lead to decreased metabolism of the drugs, resulting in drug toxicity. Nicotine, an addictive substance in tobacco, also alter the expression of drug efflux transporters such as P-gp, MRP, and BCRP in the intestinal cells [162]. Alteration in the expression of these efflux transporters can have a direct impact on the absorption of orally administered ART drugs. As ART drugs are taken in combination, the drug-drug interactions with concomitant tobacco use can be even more complex.

HPV Coinfection

HPV Prevalence in HIV-1-infected Patients

The prevalence of any kinds of HPV infection is approximately 20-25% higher in PLWH compared to general population [163, 164]. A survey conducted among African women with or without HIV-1 reported that the prevalence of any kind of HPV infection was about 72.2% in HIV-1-positive women, which was about 25% higher than the prevalence in uninfected women (47%) [164]. Similarly, another study conducted on Italian women by Tartaglia et al. (2017) revealed that 48% of HIV-1-positive women had HPV compared to 28% of HIV-1-negative women [163].

High risk HPV that causes malignant diseases are more common compared to low risk HPV among HIV-1-infected individuals [165, 166]. High risk HPV type 16 and 18 are responsible for most of the cervical carcinoma in general population [166]. **Table 2-2**. shows the standard incidence ratio (SIR) for HPV-induced cervical cancer among HIV-1-positive women all over the world, mainly in USA. The data in the table suggests that the average SIR for cervical cancer in HIV-1-positive women compared to uninfected women is 4.7, which has remained fairly constant for almost the last two decades. Several studies have mentioned that the higher incidence and severity of cervical cancer in HIV-1-positive women is associated with the decrease in the rate of $CD4^{+}$ cell counts [119].

Joshi et al. (2014) reported that, the most common HPV genotypes causing cervical intraepithelial neoplasia (CIN) stages 2 and 3 among Indian HIV-1-positve

Table 2-2. Standard incidence ratio (SIR) for HPV-induced cervical cancer among women living with HIV-1 worldwide.

Authors	Year	Location	SIR (per 100,000)
Massad et al. [172]	2017	United States	3.31
Chen et al. [173]	2015	Taiwan	4.01
Abraham et al. [119]	2013	North America	3
Massad et al. [174]	2010	United States	1.32
Chaturvedi et al. [175]	2009	United States	8.9
Engels et al. [176]	2006	United States	4.2
Clifford et al. [177]	2005	Global	8
Frisch et al. [178]	2000	United States	5.3

women were type 16 (58.5%), type 31 (22.6%), and type 18 and 68a (11.3%) [167]. Similarly, a study conducted in HIV-1-infected women with CIN (stages 2 and 3) living in Botswana, indicated the presence of either HPV 16 or 18 or both in 51% of the participants [168]. However, Clifford et al. (2016) indicated higher prevalence of HPV types 16 (42.5%), 18 (22.2%), 45 (14.4%), and 35 (7.1%) in HIV-1-positive women with invasive cervical cancer [166]. **Table 2-3.** demonstrates data from 9 different studies showing HPV genotypes observed in HIV-1-positive women with different stages of cervical cancer, living at different geographic locations. According to the data tabulated in Table 2-3, infection with multiple types of HPV are involved in the progression of cervical cancer. Infection with HPV type 16 and 18 has been observed commonly in HIV-1-positive women with ICC. In case of the HIV-1 patients with CIN, infection with HPV type 16 and other high-risk HPV such as 31, 35, 45, 52, and 58 are also commonly observed.

Effect of HPV on HIV-1 Pathogenesis

Several studies have indicated higher risk of HIV-1 acquisition in HPV-infected patients compared to uninfected individuals [124-126]. HPV-infected women are 2.4-times more susceptible to HIV-1 infection compared to uninfected women [126]. The actual mechanism involving HPV-mediated HIV-1 acquisition is still not clear. HPV 16 oncoprotein E7 downregulates E-cadherin, an adhesion molecule in the genital epithelial layer [128]. HPV could possibly increase HIV-1 acquisition by increasing the permeability of the genital linings through interference with E-cadherin. Persistent HPV infections lead to preinvasive lesions and invasive cervical cancer. Large number of $CD4^+$ and $CD8^+$ T cells have been observed in the stromal infiltrates from invasive cervical carcinoma as well as from preinvasive lesions [169]. Accumulation of $CD4^+$ and $CD8^+$ T cells, which are major targets of HIV-1 infection, could increase the risk of HIV-1 acquisition. Some studies have also shown the association of non-persistent HPV infections with the increased risk of HIV-1 acquisition [127]. The immune response for HPV clearance could also aggravate HIV-1 acquisition.

High levels of HIV-1 RNA has also been detected in the plasma of HIV-1-infected women with high risk HPV cervical infection [170]. In an in vitro study done by Gage et al. (2000), there was a significant increase in viral load in U1 monocytic cells after treatment with supernatant collected from the cultured cervical biopsies and cervical cancer cell lines [171]. The study also suggested that cytokines released in response to HPV infection, especially IL-6, may be responsible for increased HIV-1 replication. More than 10-fold increase in the level of the cytokines (IL-6, IL-8, TNF-α, MIP-1α) has also been observed during HPV infection [130]. Several of these cytokines including IL-6, IL-8, and TNF-α are shown to increase the expression of HIV-1 in host immune cells [131-133].

Massive oxidative stress occurs during HPV infection. HPV 16 oncoproteins (E6 and E7) are known to cause chronic oxidative stress by a NADPH oxidase pathway [134]. The oxidative stress caused by these HPV oncoproteins is shown to cause genomic instability

Table 2-3. HPV genotypes observed in HIV-1-postive women with cervical cancer.

Authors	Year	Location	HPV types	Stage of HPV infection
Badial et al. [165]	2018	Brazil	56 (17%) and 16 (15.3%)	
Ortiz et al. [179]	2017	Puerto Rico	52 (17.6%), 58 (11.0%), 59 (11.0%), 16 (9.9%) and 18 (9.9%)	
Menon et al. [180]	2016	Kenya	16 (37%) and 18 (24%)	ICC
Clifford et al. [181]	2016	Africa	16 (42.5%), 18 (22.2%), 45 (14.4%) and 35 (7.1%)	ICC
Joshi et al. [167]	2014	India	16 (58.5%), 31 (22.6%) and 18 & 68a (11.3%)	CIN stage 2 and 3
McKenzie et al. [166]	2014	United States	16 (45%), 35 (40%) , 45 (40%)	Cervical dysplasia
Marchetti et al. [182]	2013	Milan	16 (19%), 31 & 52 (11%) and 18 (4%)	
Ramogola-masire et al. [168]	2011	Bostwana	16 and/or 18 (51%)	CIN stage 2 and 3
Sahasrabudhhe et al. [183]	2007	Zambia	52 (37.2%), 58 (24.1%), 53 (20.7%), 16 (17.2%) and 18 (13.1%)	high-grade squamous intraepithelial lesions

and DNA damage in infected cells. The oxidative DNA damage caused by HPV may enhance integration of HIV-1 DNA into the host DNA. Similarly, HPV 16 and 18 protein E2, which controls the HPV transcription, also contribute to ROS generation in mitochondria [184]. HPV 16 and 18 proteins E6, E1, and E2 are known to decrease the level and activity of several antioxidant enzymes and molecules like superoxide dismutase 2 (SOD2), catalase, and glutathione (GSH) [185, 186]. Significant increase in thioredoxin reductase and GST has also been reported in HPV 16 -infected dysplastic and neoplastic tissue samples [187]. Both, increase or decrease in the level of antioxidants is an indicative of the ongoing oxidative stress in the cells.

Cytokines like IL-6, IL-8, and TNF-α have the ability to stimulate nuclear transcription factors that regulate the transcription of HIV-1 DNA. TNF-α increases HIV-1 expression by facilitating the nuclear translocation of NF-κB, a nuclear transcription factor that binds to HIV-1 LTR and activate HIV-1 transcription [188]. IL-6 independently increases expression of HIV-1 proteins, facilitates viral transcription by enhancing RT activity, and in synergy with TNF-α it also helps to enhance the viral transcription [189]. Similarly, ROS such as superoxide and hydrogen peroxide are also known to activate NF-κB and cause reactivation of latent HIV-1 [190, 191]. Schreck et al. (1991) demonstrated that treatment of micromolar concentrations of hydrogen peroxide in Jurkat T cells enhances the nuclear translocation of NF-κB as well as binding of NF-κB to the HIV-1 LTR, leading to activation of the latent HIV-1 [192]. Another study done by Piette et al. (1994) also suggested the role of ROS as secondary messengers in activation of latent HIV-1 in monocytes and lymphocytes through activation of signaling pathways regulating HIV-1 transcription [190].

Effect of Antiretroviral Therapy on HPV

Studies suggest that PLWHA on ART show more rapid HPV clearance and higher rate of regression of cervical dysplasia and invasive cervical cancer compared to their counterparts who are not on ART [193]. ART is beneficial in clearing most of the oncogenic HPV, however some studies have mentioned that ART is not effective enough to clear HPV 16 and 18, which are the major strains causing cervical cancer [194]. A study by Clifford et al. (2016) showed that HIV-1-positive women receiving ART for > 2 years showed high regression of cervical intraepithelial neoplasia compared to those who never received ART [195]. Another study by Konopnicki et al. (2013) also reported that ART significantly controlled high risk HPV, and it lowered the risk of even HPV 16 and 18 [196]. These studies suggested a strong role of ART on HPV clearance and alleviation of cervical lesions through restoration of immune response. A meta-analysis done by Kelly et al. (2017) with 31 studies, also concluded that patients on ART, showing sustained HIV-1 suppression and stable high $CD4^+$ cell count, have reduced incidence and progression of cervical lesions and cancer [197]. However, another meta-analysis study by Cobucci et al. (2015) suggested the opposite, i.e. higher risk of cervical cancer with ART treatment [198]. Most of these studies were performed in early ART era, when ART was initiated at lower nadir $CD4^+$ cell count. In these studies, ART could not have caused the complete immune restoration in the patients, which could be a possible reason

for the persistent HPV infection and cervical cancer progression [197]. Unlike smoking, HPV coinfection in HIV-1-infected patients do not have an adverse effect on ART prevalence and efficacy.

CHAPTER 3. SPECIFIC AIMS OF THE STUDY

Scientific Premise

Antiretroviral therapy has prolonged the life span of PLWHA. However, the quality of life of this population has not improved much, because of the presence of comorbidities. Since HIV-1 causes immunosuppression, PLWHA are highly prone to opportunistic infections by various pathogens [199]. Furthermore, high prevalence of smoking, alcohol, and substance drug abuse has also been reported in this population [32]. PLWHA with these comorbidities are observed to have higher progression of HIV-1 disease despite ART use, mainly because of their impact on ART adherence, HIV-1 acquisition, and multiplication. In this study, we will be focusing mainly on smoking and HPV coinfection as comorbid factors, because of their high prevalence and high impact on HIV-1 replication in PLWHA.

The prevalence of tobacco smoking is 2-times higher in PLWHA compared to the general population [36], and is known to exacerbate HIV-1 pathogenesis [153, 154]. In vivo and in vitro studies have demonstrated that tobacco smoking is associated with decreased immune responses [200], increased inflammation [201] and oxidative stress [202, 203], and increased occurrence of opportunistic infections [160, 204]. In particular, tobacco smoking is known to accelerate HIV-1 replication in monocytes and macrophages, which are viral sanctuary sites where low concentrations of ART drugs fail to eliminate the virus [154, 205] . HIV-1-infected macrophages can also infiltrate the brain and spread the virus to perivascular macrophages, leading to the development of neuro-AIDS. Previous studies have demonstrated that cigarette smoke condensate induces HIV-1 replication in monocytic cells via a CYP-mediated oxidative stress pathway [154]. Similarly, other studies have also revealed that CYP enzymes metabolize polyaromatic hydrocarbons in cigarette smoke into toxic metabolites that enhance HIV-1 gene expression by forming DNA adducts [156].

On the other hand, higher risk and prevalence of HPV infections that lead to cervical cancer, has also been reported in HIV-1-positive women [163, 164]. Presence of HPV coinfection in HIV-1-positive women has also been shown to exacerbate HIV-1 pathogenesis [124-126]. HPV-infected women are 2.4-times more susceptible to HIV-1 infection compared to uninfected women [126]. However, it is not clear how smoking or HPV coinfection in PLWHA enhance HIV-1 replication and pathogenesis. In the present study we will investigate the molecular mechanism by which smoking or HPV-infection induce HIV-1 replication in monocytic cells, particularly focusing on CYP and oxidative stress pathway.

In this study, we have used three different types of cell lines: U937, U1 and Caski Cells. U937 cells are monocytic cells derived from the histolytic lymphoma of a 37-year-old male patient and U1 cells are U937 cells chronically infected with HIV-1. As U1 cells comprise of HIV-1 incorporated into their genome but the cells are not capable of producing a new virus, they represent an ideal model for cells latently infected with HIV-

1. Further, monocytes/monocyte-derived macrophages are one of the major targets and reservoirs for HIV-1 infection [206]. Once infected, these cells can travel through bloodstream and spread the virus in other organ systems such as brain [207]. Therefore, it is imperative to study HIV-1 replication in monocytes and monocyte-derived macrophages. We used U1 monocyte-derived macrophages, instead of U1 monocytes for the experimental purpose because monocytes lack cellular machinery required for HIV-1 transcription [208, 209]. Monocytes express high levels of transcriptionally inactive NF-κB p50 homodimers, which are replaced by transcriptionally active NF-κB p50/p65 or p50/RelB as they differentiate into macrophages after stimulation by PMA [208]. Monocytes also lack cellular cofactors for HIV-1 Tat-mediated transactivation of viral LTR promotor, which is critical for HIV-1 transcription. However, the cofactors essential for HIV-1 Tat transactivation are abundantly expressed in monocyte-derived macrophages [209]. To confirm the results from the U937 and U1 cells, we used infected/uninfected human primary monocytes and macrophages. We also used Caski cells which are cervical cancer cells with integrated HPV 16 and 18 genomes. Although, there are other HPV-infected cell lines (e.g. Siha, hela, C33A), we particularly employed Caski cells because these cells have higher p53 expression, lower antioxidant genes expression, and higher extent of oxidative stress compared to other cell lines [210].

Hypothesis

We propose that smoking and HPV infection independently increase HIV-1 replication in monocytic cells latently infected with HIV-1, via Cytochrome p450 (CYP)-mediated oxidative stress pathways. Cigarette smoke constituents especially polyaryl hydrocarbons (PAH) enhance the expression of CYPs (CYP 1A1, 1A2, 1B1), which in turn metabolize PAHs into various water-soluble metabolites. During the metabolism of PAHs, CYPs generate large amount of reactive oxygen species (ROS). The ROS thus generated acts as secondary messengers for activating signaling pathways that regulate HIV-1 replication. On the other hand, HPV-infected cervical cancer cells also contribute to increased oxidative stress in monocytic cells latently infected with HIV-1, by encapsulating oxidative stress factors, especially CYPs into exosomes and transporting them into the HIV-1-infected monocytic cells. These exosomes release the CYPs into the infected monocytic cells, which aids the endogenous CYPs to generate more ROS. The excess of ROS leads to oxidative stress and HIV-1 replication via a similar mechanism as explained in case of smoking. Hypothesis of the project has been illustrated in **Figure 3-1**.

Clinical Significance and Impact

The proposed project will help to find the mechanism by which comorbidities such as, smoking and HPV coinfection enhance HIV-1 replication in monocytic cells latently infected with HIV-1. Through the elucidation of the mechanism, the project will increase scientific understanding and help improve clinical practice in HIV-1 patients with tobacco addiction or HPV coinfection, by providing knowledge of the contribution

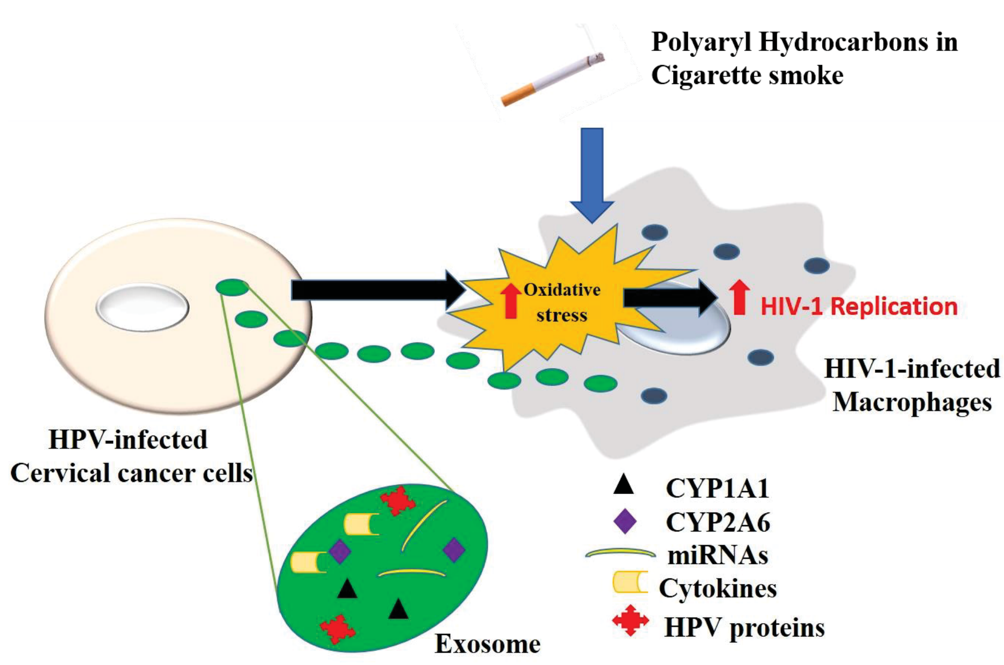

Figure 3-1. Hypothesis: Polyaryl hydrocarbons in cigarette smoke and HPV-infected cervical cancer cells exacerbate HIV-1 replication via CYP-mediated oxidative stress pathway.

of monocytic and/or exosomal CYP enzymes in tobacco/HPV-mediated HIV-1 pathogenesis. The project would impact the treatment of HIV-1 in patients with these comorbidities, by providing a target for novel therapeutic interventions, adjustment of drug regimens, and potential application of exosomes as therapeutic carriers. This project would also provide new knowledge for exosomal biology thus impacting the understanding of other diseases and drugs of abuse.

Objectives

Our objectives are 1). To identify specific PAHs in cigarette smoke that cause oxidative stress and cytotoxicity in monocytic cells and show the potential role of CYPs in PAH-mediated cytotoxicity; 2). To demonstrate that PAHs cause HIV-1 replication in monocytic cells latently infected with HIV-1 via CYP-mediated oxidative stress pathway; 3). To show that exosomes from HPV-infected cervical cancer cells transfer oxidative stress factors, especially CYPs to the monocytic cells latently infected with HIV-1 and thereby induce HIV-1 replication via CYP-mediated oxidative stress pathway. Based on our objectives, we designed 3 aims for the study, which are described below:

Aim 1: Study of Contribution of CYP Enzymes in PAH-enhanced Cytotoxicity in Monocytic Cells.

We examined the acute (3-9 hours) and chronic (7 days) effects of five different carcinogenic PAHs such as Benzo(a)pyrene (BaP), Phenanthrene (Phe), Naphthalene (NPh), Benzo(a)fluoranthene (BeF), and Benzo(a)anthracene (BeA) on cytotoxicity in U937 monocytic cells. We also monitored ROS level, expression of CYPs (CYP1A1, CYP3A4) and antioxidant enzymes (AOEs: SOD1 and catalase) at mRNA and protein level and caspase-3 activity after PAH exposure and showed an association of the expression of CYPs and oxidative stress with PAH-induced cytotoxicity. Out of the five compounds tested, chronic exposure of BaP induced the highest level of cytotoxicity in U937 cells, which were associate with increased expression of CYP and oxidative stress, suggesting the potential role of CYPs in BaP-induced toxicity. We also confirmed these results in human primary macrophages.

Aim 2: Study of Contribution of CYP Enzymes in BaP-enhanced HIV-1 Replication in Monocytic Cells Latently Infected with HIV-1.

We examined if chronic exposure of BaP would increase HIV-1 viral load in U1 cells. As expected, we observed a significant increase in p24 levels in the supernatant of BaP-treated U1 cells, as measured by p24 ELISA. These results were also confirmed in HIV-1-infected human primary macrophages. In order to see the involvement of CYP-mediated oxidative stress in BaP-induced HIV-1 replication, we monitored ROS level and expression of CYPs (CYP1A1, CYP3A4) and AOEs (SOD1, Catalase) at mRNA and protein level. Furthermore, we established the role of CYPs and oxidative stress pathway

in BaP-induced HIV-1 replication in U1 cells, by knocking-down the CYP1A1 gene using siRNA and treatment with selective CYP inhibitors and antioxidants. We also demonstrated that BaP-induced oxidative stress leads to the nuclear translocation of NF-κB subunits (NF-κB p50 and p65), which was reduced by treatment with siRNA and antioxidants/CYP inhibitors. Treatment of specific NF-κB inhibitors (IKK-16 and SC-540) significantly decreased HIV-1 replication in BaP-exposed U1 cells, indicating the specific role of NF-κB pathway. Altogether, our results suggested that BaP enhances HIV-1 replication in U1 cells by activation of NF-κB through ROS generated by CYP-mediated pathway.

Aim 3: Study of Contribution of Exosomes Derived from HPV-infected Cervical Cancer Cells in Enhancing HIV-1 Replication in U1 cells.

Initially, we examined whether supernatant from Caski cells can induce HIV-1 replication in U1 cells. We also monitored ROS level, total antioxidant capacity, cytotoxicity, DNA damage and expression of CYPs (CYP1A1, CYP2A6), and AOEs (SOD1, SOD2, catalase, PRDX6) after treatment of U1 cells with Caski cell culture supernatant (CCS). Our results suggested the association of CYPs and oxidative stress with CCS-induced HIV-1 replication. Next, we were interested to study if exosomes derived from CCS were responsible for the CCS-induced HIV-1 replication. We isolated exosomes from CCS using exosome isolation kit and characterized them by the methods described earlier [211]. We observed the presence of specific exosomal markers (CD63, CD81), CYPs (CYP1A1, CY2A6), AOEs (SOD1), and HPV-16 oncoprotein E6 in the CCS-derived exosomes (CCS-Exo). We also demonstrated that CCS-Exo diffused into U1 cells and induced oxidative stress and HIV-1 replication. By using specific CYP inhibitors (ellipticine and tryptamine) and antioxidant (resveratrol), we confirmed that both CCS and CCS-Exo induced HIV-1 replication in U1 cells via a CYP-mediated oxidative stress pathway. Furthermore, we also showed that treatment of chemo-dietary agents such as curcumin and cucurbitacin-D reduced HIV-1 replication in CCS and CCS-Exo-treated U1 cells. Overall, our results suggested the role of HPV infection on HIV-1 replication via exosome containing oxidative stress factors.

CHAPTER 4. EFFECT OF POLYARYL HYDROCARBONS ON CYTOTOXICITY IN MONOCYTIC CELLS*

Introduction

According to the International Agency for Research on Cancer (IARC), there are around 5,300 chemicals identified in mainstream cigarette smoke, among which seventy are classified as carcinogens [212, 213]. The IARC monograph program has listed several categories of chemical compounds that are potential carcinogens in cigarettes, including polyaryl hydrocarbons (PAHs), N-nitrosamines, aldehydes, phenols, volatile hydrocarbons, and other organic and inorganic compounds. Since the identification of cigarette constituents in 1950s, several studies have been conducted with regard to its carcinogenicity in different human organs. Tobacco-specific nitrosamines and PAHs are the most studied cigarette carcinogens. Tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) are reported to cause lung cancer and oral cavity cancer [214]. PAHs are associated with several cancers such as skin, lung, oral, and breast cancer [215-218]. Among the hundreds of PAHs present in the cigarette smoke, benzo(a)pyrene (BaP) is the most extensively studied PAH due to its known carcinogenic effects. Naphthalene (NPh) and phenanthrene (Phe) have relatively low carcinogenicity, but they contribute highly to the total PAH yield [219].

Cytochrome P450 (CYP) enzymes metabolize PAHs successively into epoxides and diol-epoxides [220]. These metabolites cause cellular damage either by forming DNA and protein adducts or by generating reactive oxygen species (ROS) [221, 222]. BaP is metabolized by CYP1A1 into a carcinogen, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE), via a series of metabolic reactions. BPDE is reported to form DNA adducts which cause mutations in DNA, ultimately leading to carcinogenesis in lung and skin epithelial cells [223]. BaP is also reported to cause apoptosis in ovarian follicular cells via a CYP-mediated pathway [224]. Several reports in literature suggest the role of aryl hydrocarbon receptor-mediated CYP-induction and the subsequent oxidative stress in various forms of cancer and cardiac toxicity [225, 226]. Some studies reveal that BaP causes carcinogenic effects by inducing CYP1A1 expression through binding of p-53 to promoter region of CYP1A1 [227]. Rapid formation of [D10] r-1,t-2,3,c-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ([D10] PheT), a carcinogenic diol metabolite of Phe, occurs in smokers [228]. Oxidative stress, DNA damage, and cell toxicity were observed following treatment with NPh in cultured J774A.1 macrophages [229].

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Previous studies from our lab have shown that nicotine, a major constituent of tobacco, causes oxidative stress in U937-derived macrophages through a CYP2A6-mediated nicotine metabolic pathway [230]. Our in vitro data was validated by an in vivo study in which HIV-1-positive smokers displayed a higher rate of nicotine metabolism by CYP2A6 than HIV-1-negative smokers [155]. HIV-1-positive smokers displayed a higher rate of nicotine metabolism by CYP2A6 than HIV-1-negative smokers [155]. The results from these studies were consistent with the findings from another study, in which there was an increase in viral load, cytokines, and oxidative stress, likely through CYP pathway, in the plasma and monocytes of HIV-1-infected smokers compared to HIV-1-infected nonsmokers [153]. As the ultimate goal of the aforementioned studies was to explore the role of CYP enzymes in smoking-induced oxidative stress and HIV-1 pathogenesis, the experiments were conducted in monocytes/macrophages. Monocytes/ macrophages are one of the cellular targets for HIV-1 and they also serve as important viral reservoirs [231]. Smoking may enhance the infiltration of the infected monocytes/macrophages into the brain and further infect the microglia and astrocytes, ultimately leading to NeuroAIDS. However, it is still not clear whether other cigarette components besides nicotine are also responsible for CYP-mediated oxidative stress and HIV-1 replication in monocytes/macrophages. Therefore, the current study was designed to first examine the relative contribution of five PAHs; BaP, NPh, Phe, BeA, and BeF, on the regulation of CYP enzymes, oxidative stress, and cytotoxicity in U937 monocytic cells followed by primary macrophages.

Materials and Methods

Cell Culture and Treatment

U937 monocyte cells. The U937 monocytic cell line used for the study was obtained from ATCC (Manassas, VA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Sigma Aldrich, St. Louis, MO), which included 1% gentamycin (Mediatech Inc. Manassas, VA), L-glutamine (Fischer Scientific, PA), and sodium bicarbonate (Fischer Scientific, PA). To assess the acute effect of PAHs (1 µM NPh, 1 µM Phe, and 100 nM BaP) at 3, 6, and 9 hours, 0.8 million cells/well were seeded in a 12-well plate. The cells were incubated overnight at 37°C in an incubator with 5% CO_2 prior to treatment with PAHs. Following treatment, cells were collected at the designated time points. To assess the chronic effect of PAHs (100 nM NPh, 100 nM Phe, 100 nM BeA, 100 nM BeF, (5, 25, and 100) nM BaP), 0.1 million cells were seeded per well in a 6-well plate. The cells were treated with PAHs after 30 minutes of incubation. The cells were treated every 12 hours for 7 days, with addition of 250 µl of fresh media during each treatment to maintain the concentrations of PAHs. DMSO treated cells served as control for both the acute and chronic treatment paradigms.

Primary macrophages. Blood from interstate blood bank Inc. (Memphis, TN) was diluted with phosphate-buffered saline (PBS, Life Technologies, NY), layered on Ficoll (Mediatech Inc. Manassas, VA) and centrifuged at 400g for 30 minutes. The white

ring of peripheral blood mononuclear cells (PBMCs) formed in between the plasma and Ficoll layers were carefully isolated. The PBMCs were washed with PBS several times to ensure the removal of Ficoll. The cells were incubated with ammonium-chloride-potassium (ACK) lysing buffer (Life Technologies, NY) at 4ºC for 15 minutes to lyse and remove any red blood cells, if present. The clear pellets of PBMCs were then cultured in RPMI media with human serum and macrophage colony-stimulating factor for macrophage differentiation. The differentiated macrophages were treated with BaP (100 nM) for 6 days, every 24 hours with an addition of 500 µl fresh media after each treatment.

Isolation of DNA, RNA, and Protein

DNA, RNA, and protein were isolated from the lysed cells using Allprep DNA/RNA/Protein QIAGEN Kit (Valencia, CA) using the supplier's protocol. RNA and DNA were quantified using Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Fischer Scientific, Rockford, IL) by measuring their absorbance at 260 nm. The protein was quantified using the BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTPCR)

Quantitative RTPCR was performed to measure the relative mRNA fold expression of the CYPs 1A1, 3A4 and the antioxidant enzymes (AOEs) superoxide dismutase 1 (SOD1) and catalase in U937 cells upon exposure to PAHs. Purified RNA (120 ng) was reverse transcribed to cDNA using a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA). The cDNA was amplified in a Step-One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression kit (Applied Biosystems, Foster City, CA). The following probes from Applied Bioscience were used for the Q-RTPCR reaction: CYP1A1 (Hs01054794_m1), CYP3A4 (Hs00430021_ml), SOD1 (Hs00533490_ml) and catalase (Hs00156308_ml). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA fold expression of the genes, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control.

Western Blotting

To determine the expression of proteins of interest, 30 µg of proteins in 5% SDS were separated on a polyacrylamide gel (4% stacking, 10% resolving gel) at 150 V for 70 minutes. The proteins from the gel were transferred to a polyvinylidene fluoride membrane at 0.35 Amp for 90 minutes. The transferred blots were blocked with 5-10 ml of Li-Cor blocking buffer (LI-COR Biosciences, Lincon, NE) for 1 hour and incubated overnight with primary antibodies (GAPDH Rabbit Mab, 1:2000 dilution, Cell Signaling Technology, Danvers, MA; CYP1A1 rabbit Mab, 1:200 dilution, Abcam, Cambridge, MA; CYP3A4 Mouse Mab. 1:200 dilution, Santa Cruz Biotechnology. Inc. Dallas, TX;

SOD1 Mouse Mab, 1:1500 dilution, Santa Cruz Biotechnology. Inc. Dallas, TX; Catalase Mouse Mab, 1:1200 dilution, Santa Cruz Biotechnology. Inc. Dallas, TX) at 4°C. After subsequent washing, the blots were incubated with corresponding secondary antibodies (1:10000 dilution, Goat anti-Mouse Mab, LI-COR Biosciences, Lincon, NE; 1:10000 dilution, Goat anti-Rabbit Mab, LI-COR Biosciences, Lincon, NE) for 1 hour at room temperature. The blots were scanned with Li-Cor Scanner (LI-COR Biosciences, Lincon, NE) and the densitometry data obtained from Image Studio Lite version 4.0 were used to calculate the fold expression of the proteins. GAPDH was used as an internal loading control to normalize the expression of sample proteins.

Measurement of Reactive Oxygen Species (ROS) and Cell Viability

ROS and cell viability were quantified by flow cytometry using the fluorescence dye 5-(and-6)-chloromethyl 2',7'- dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Life Technologies, Oregon, USA) and Ghost Dye (Tonbo Biosciences, San Diego, CA), respectively. The treated cells were thoroughly washed with PBS and resuspended in 1 mL of PBS containing 2% FBS supplemented with 2-5 μl of CM-H2DCFDA and 1 μl of the Ghost dye. The cells were then incubated at room temperature in the dark for 30 minutes and subsequently washed and resuspended in 300 μl of PBS containing 2% FBS. Dichlorodihydrofluorescein (DCF) emission at 525 ± 20 nm, which is proportional to the ROS generated in the cells, and emission of Ghost dye at 780 nm, which is proportional to the cell viability, were detected by flow cytometry (BD Biosciences, San Jose, CA). The data were analyzed by using the BD FACS software version 8.

XTT Assay

Cell viability was also measured using the XTT assay kit (Cell Signaling Technology Inc., Danvers, MA). The PAH-treated cells (0.12 million) were suspended in 200 μl of phenol red-free media. XTT detection solution was made by mixing electron coupling solution and XTT reagent in the ratio of 1:50. Fifty microliters of the XTT detection solution was added to each well of the 96-well plate containing 200 μl of the cell suspension and the plate was incubated at 37°C for three hours. The absorbance measured at 450 nm represented cell viability.

Caspase-3 Activity Assay

Caspase-3 activity was measured using Caspase-3 colorimetric assay kit (BioVision, Inc., Milpitas, CA). Cells (1-5 millions) obtained after the treatments were lysed and protein was extracted. About 100 μg of the protein was diluted to a final volume of 50 μl in cell lysis buffer. Each protein sample was added to 50 μl of 2X reaction buffer (containing 10 mM DTT) and 5 μl of 4 mM DEVD-pNA substrate and the reaction mixture was incubated at 37°C for 1 hour. Absorbance of the samples was measured at 405 nm.

Statistical Analysis

For the statistical analysis of the data obtained from RTPCR, Western blot, cell viability assay, ROS level, and caspase-3 activity assay, mean ± SEM was calculated and One-way ANOVA was applied to calculate p values. A p value of ≤ 0.05 was considered significant.

Results

Effect of Acute Treatment of BaP, NPh, and Phe on CYP and AOE Expression, ROS and Cell Viability in U937 Cells

Initially, we monitored the effect of the acute exposure of BaP, NPh, and Phe in U937 cells. The cells were treated with BaP (100 nM), NPh (1 μM), and Phe (1 μM) for 3, 6, and 9 hours. There was no significant change in ROS and cell viability in cells treated with the PAHs for 3 and 6 hours (**Figure 4-1A-B**). However, a significant decrease ($p \leq 0.05$) of ~10% in ROS was observed with all the three compounds after 9 hours of exposure (**Figure 4-1A**). The cell death at 9 hours was not particularly different from the control. The small decrease in ROS would not have been sufficient enough to cause a significant impact on cell viability. In general, there was minimal to no effect of acute exposure of BaP, NPh, and Phe on oxidative stress and cytotoxicity in U937 cells.

Furthermore, there was no significant effect of the acute exposure of the PAHs over the induction of CYPs (**Figures 4-2** and **4-3**) and AOEs (**Figures 4-4** and **4-5**). Only BaP exposure showed a significant ascending trend in the mRNA expression of CYP1A1 over time (~10 fold increase at 6 hours to ~30 fold increase at 24 hours, $p \leq 0.05$), as well as with increasing concentration (**Figure 4-2A**). However, there was no significant increase in the expression of CYP1A1 protein with either 100 nM or 1 μM BaP treatment. None of the PAHs showed significant change in either the mRNA and protein expression levels of CYP3A4, except for the treatment with Phe (1 μM), which showed ~3 fold increase in CYP3A4 expression at mRNA level at 12 hours ($p \leq 0.05$) (**Figure 4-3C**). At 24 hours, we observed a significant increase in the SOD1 mRNA expression with (1 μM) BaP (~3 fold, $p \leq 0.05$) (**Figure 4-4A**). None of these compounds showed significant alteration in the protein expression levels of SOD1. Similarly, no significant change in the mRNA and protein expression levels of catalase was observed with any of the acute PAH treatments (**Figure 4-5**).

Effect of Chronic Treatment of BaP, NPh, and Phe on ROS and Cell Viability

The cells were treated with three different concentrations of BaP (5 nM, 25 nM, and 100 nM), 100 nM NPh, and 100 nM Phe for seven days to examine the chronic effect of the PAHs on ROS generation and cell viability. A significant concentration-dependent

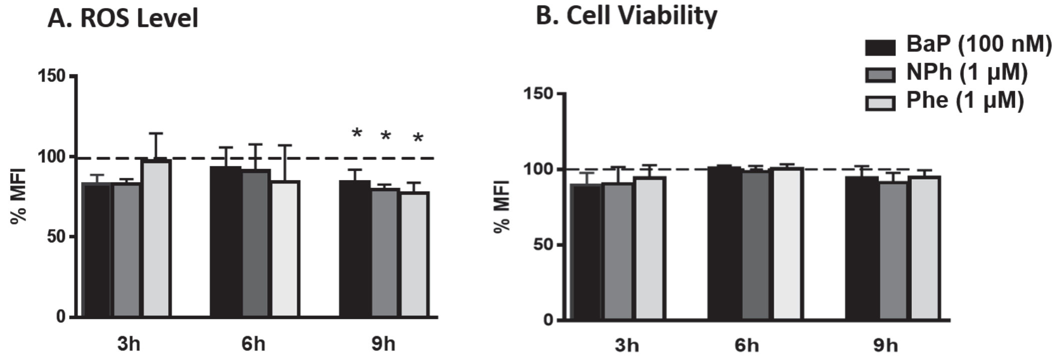

Figure 4-1. Effect of acute treatment of BaP, NPh, and Phe on ROS level (A) and cell viability (B) in U937 cells.
The U937 cells were treated with 100 nM BaP, 1 μM NPh, and 1 μM Phe for 3, 6, and 9 hours. ROS level and cell viability were measured using flow cytometry. Measured values at every time point were normalized to control that was set at 100%. X-axis and Y-axis correspond to time points and % of mean fluorescent intensity (%MFI), respectively. The data are presented as a mean ± SEM of three independent experiments. * represents $p \leq 0.05$ compared with the control group.

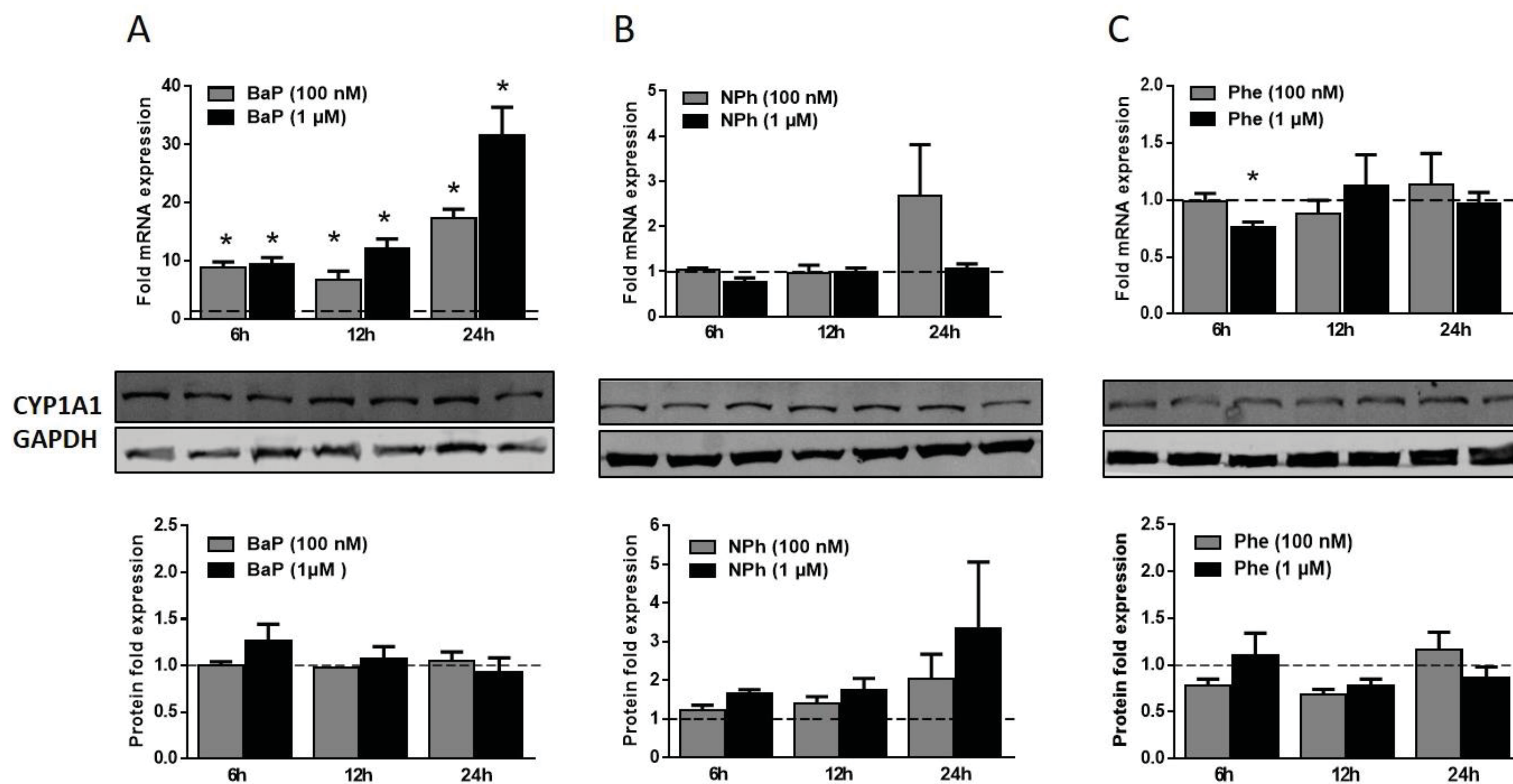

Figure 4-2. Effect of acute treatment of BaP (A), NPh (B), and Phe (C) on mRNA and protein expression of CYP1A1 in U937 cells.

The U937 cells were treated with 100 nM and 1 μM of BaP, NPh, and Phe for 6, 12 and 24 hours. The mRNA fold expressions were calculated using qRT-PCR and the protein fold expressions were measured by Western blots, and normalized with control (DMSO treated cells) whose expression was set at 1-fold. GAPDH was used as an endogenous control. Blots are representative of at least three independent experiments. The data are presented as a mean ± SEM of three independent experiments. * represents $p \leq 0.05$, compared with the control group.

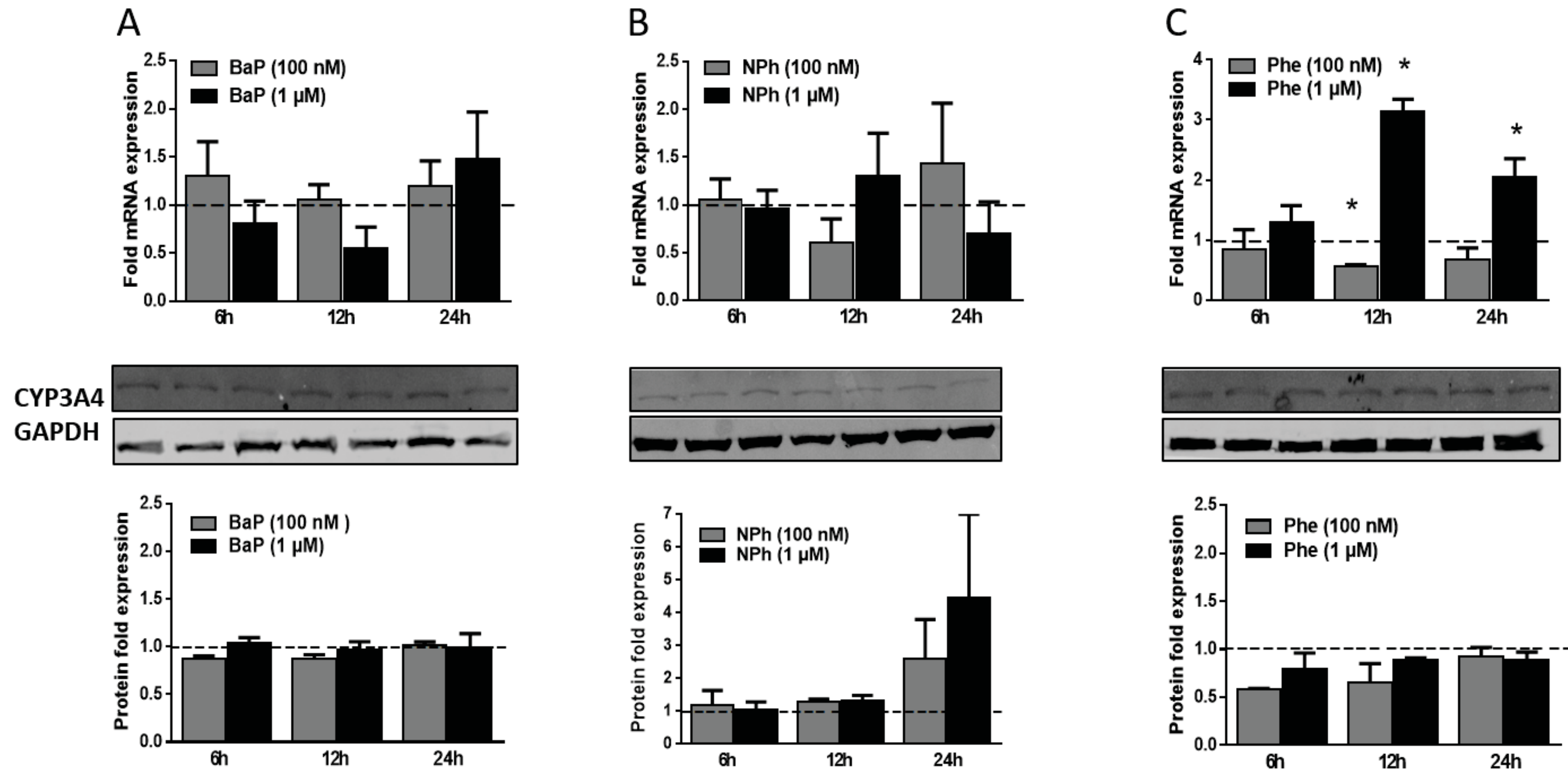

Figure 4-3. Effect of acute treatment of BaP (A), NPh (B), and Phe (C) on mRNA and protein expression of CYP3A4 in U937 cells.

The U937 cells were treated with 100 nM and 1 µM of BaP, NPh, and Phe for 6, 12 and 24 hours. The mRNA fold expressions were calculated using qRT-PCR and the protein fold expressions were quantified by Western blots, and normalized with control (DMSO treated cells) whose expression was set at 1-fold. GAPDH was used as an endogenous control. Blots are representative of at least three independent experiments. The data are presented as a mean ± SEM of three independent experiments. * represents $p \leq 0.05$, compared with the control group.

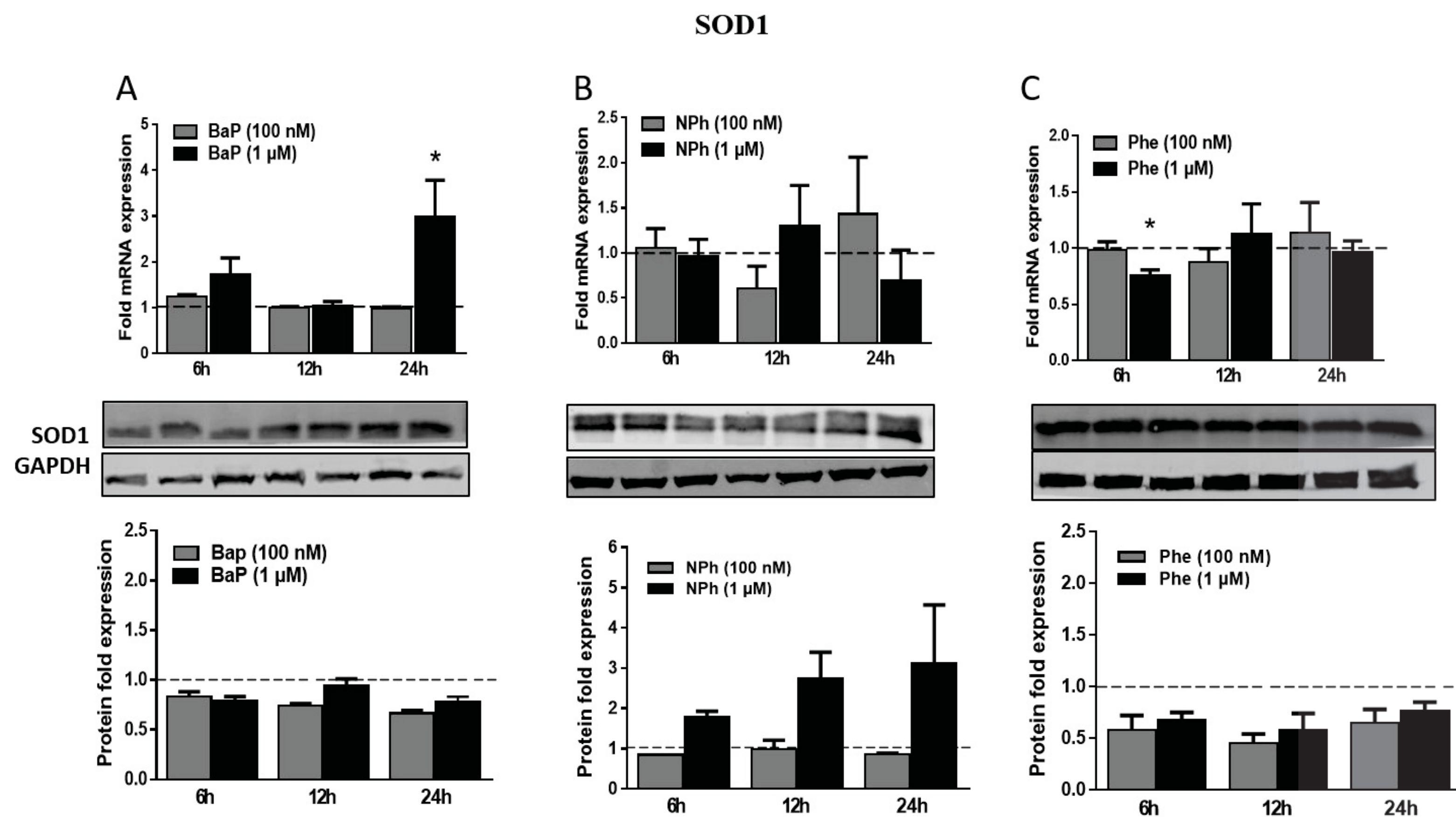

Figure 4-4. Effect of acute treatment of BaP (A), NPh (B), and Phe (C) on mRNA and protein expression of SOD1 in U937 cells.

The U937 cells were treated with 100 nM and 1 μM of BaP, NPh, and Phe for 6, 12 and 24 hours. The mRNA fold expressions were calculated using qRT-PCR and the protein fold expressions were quantified by Western blots, and normalized with control (DMSO treated cells) whose expression was set at 1-fold. GAPDH was used as an endogenous control. Blots are representative of at least three independent experiments. The data are presented as a mean ± SEM of three independent experiments. * represents $p \leq 0.05$, compared with the control group.

Catalase

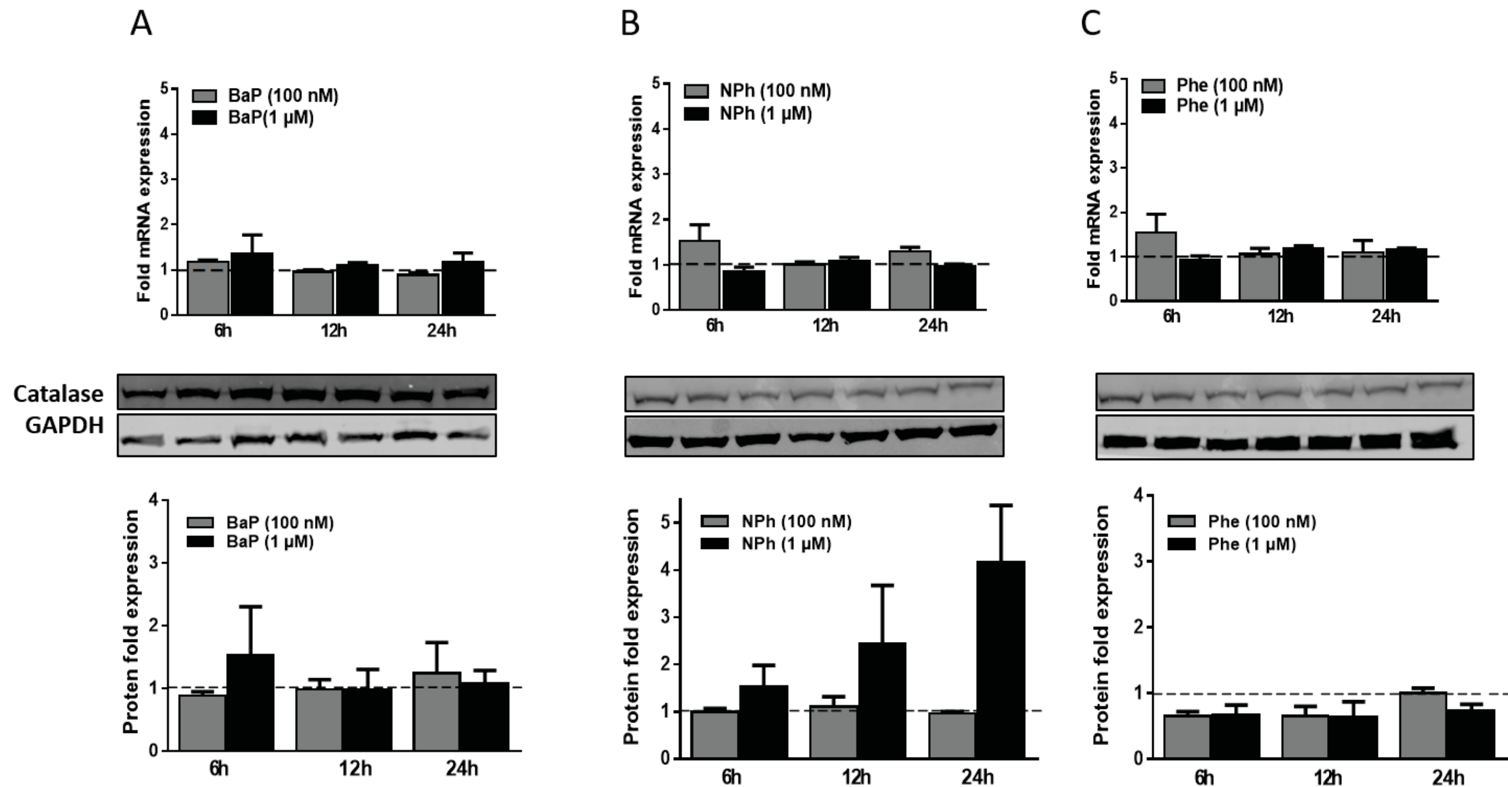

Figure 4-5. Effect of acute treatment of BaP (A), NPh (B), and Phe (C) on mRNA and protein expression of catalase in U937 cells.

The U937 cells were treated with 100 nM and 1 µM of BaP, NPh, and Phe for 6, 12 and 24 hours. The mRNA fold expressions were calculated using qRT-PCR and the protein fold expressions were quantified by Western blots, and normalized with control (DMSO treated cells) whose expression was set at 1-fold. GAPDH was used as an endogenous control. Blots are representative of at least three independent experiments. The data are presented as a mean ± SEM of three independent experiments. * represents $p \leq 0.05$, compared with the control

increase in ROS was observed with the chronic treatment of 25 nM BaP (~1.5 fold, $p \leq 0.05$) and 100 nM BaP (~ 2.5 fold, $p \leq 0.05$) (**Figure 4-6A-B**). However, there was no significant alteration in the ROS levels with 5 nM BaP. Similarly, no significant change in ROS was observed with NPh and Phe treatments. A statistically significant ($p \leq 0.05$) decrease in cell viability (~60%) was observed with 100 nM BaP treatment. Lower concentrations (5 nM and 25 nM) of BaP, as well as, the 100 nM NPh and Phe treatments, however, did not have any effect on cell toxicity (**Figure 4-7B**). Similar decrease in cell viability with 100 nM BaP treatment was observed using XTT cell viability assay (~40% decrease in cell viability, **Figure 4-7C**). **Figure 4-7A** show the graphical representation of cell viability, measured via flow cytometry.

Effect of Chronic Treatment of BaP, NPh, and Phe on Caspase-3 Activity

In order to delineate the mechanism of cytotoxicity after chronic treatment of PAHs (BaP, NPh, and Phe), we monitored the caspase-3 activity. We observed a significant increase in caspase-3 activity with 100 nM BaP treatment (~2 fold, $p \leq 0.05$, **Figure 4-6C**). However, there was no effect on caspase-3 activity with lesser concentrations of BaP (5 nM and 25 nM) or 100 nM of NPh or Phe.

Effect of Chronic Treatment of BaP, NPh, and Phe on the Expression of CYPs at mRNA and Protein Levels

PAHs are metabolized by various CYP enzymes, mainly CYPs 1A1, 1B1, and to a lesser extent by CYPs 1A2, 2C9, 3A4, and 2C19 into reactive metabolites and produce ROS that cause DNA damage [232]. Therefore, to examine the effect of these compounds on expressions of CYPs 1A1 and 3A4 that are prominently present in U937 cells, we treated the cells separately with 100 nM of each compound for seven days and measured the mRNA and protein levels of CYP1A1 and CYP3A4 (**Figure 4-8**). With BaP treatment, we observed ~15 folds increase ($p \leq 0.05$) in the CYP1A1 mRNA expression (**Figure 4-8A**) and ~3 folds increase in CYP3A4 mRNA expression (**Figure 4-8B**). However, the CYP1A1 and 3A4 protein expression levels quantified from the Western blots did not correspond to the respective changes in mRNA expression levels. NPh and Phe did not show much change in the induction of either CYPs. Interestingly, there was ~2.5 fold ($p \leq 0.05$) increase in the protein expression of CYP1A1 in the cells treated with NPh (**Figure 4-8A**, lower panel).

Effect of Chronic Treatment of BaP, NPh, and Phe on the Expression of AOEs at the mRNA and Protein Levels

PAHs are likely to induce expression of AOEs to combat PAH-induced oxidative stress in U937 cells. To determine whether these compounds alter the expression of AOEs, we measured the mRNA and protein levels of the major AOEs SOD1 and catalase (**Figure 4-9**). Chronic (seven days) exposure of 100 nM BaP upregulated the mRNA

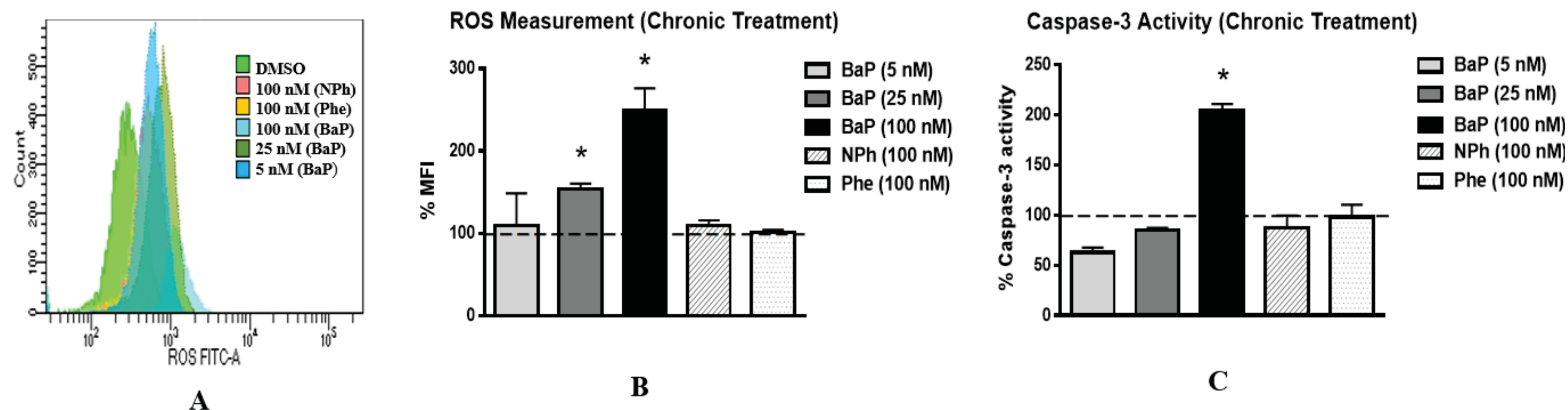

Figure 4-6. Effect of chronic (7 days) treatment of BaP, NPh, and Phe on reactive oxygen species (ROS) level (A-B) and caspase-3 activity (C) in U937 cells.
The U937 cells were treated with (5, 25 and 100) nM BaP, 100 nM NPh, and 100 nM Phe for seven days. ROS level and caspase-3 activity were calculated and normalized with control that was set as 100%. The data is presented as mean ± SEM of the three independent experiments. * represents $p \leq 0.05$, compared with the control group.

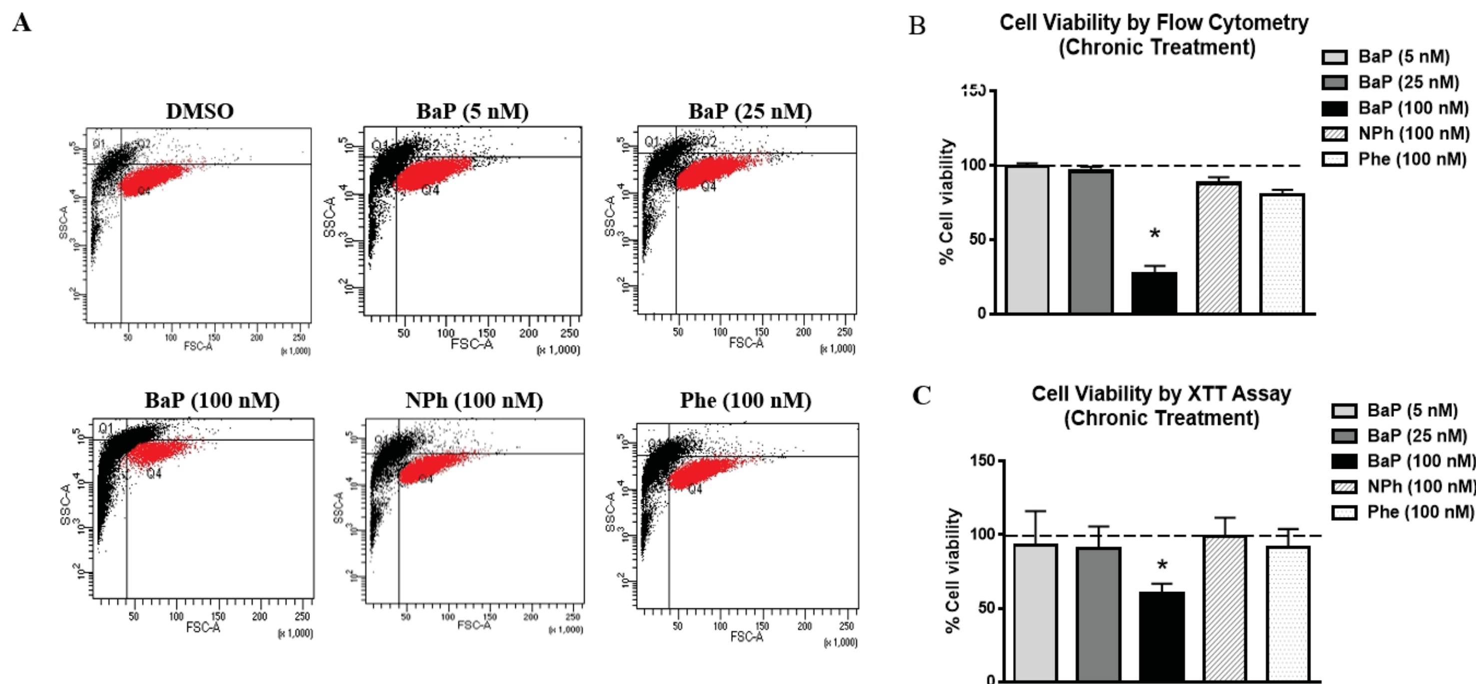

Figure 4-7. Cell viability upon chronic treatment by BaP, NPh, and Phe measured by flow cytometry (A-B) and XTT assay (C).

The U937 cells were treated with (5, 25 and 100) nM BaP, 100 nM NPh, and 100 nM Phe for seven days. Cell viability was calculated using flow cytometry and XTT assay, and normalized with control that was set as 100%. The data is presented as mean ± SEM of the three independent experiments. * Represents $p \leq 0.05$, compared with the control group.

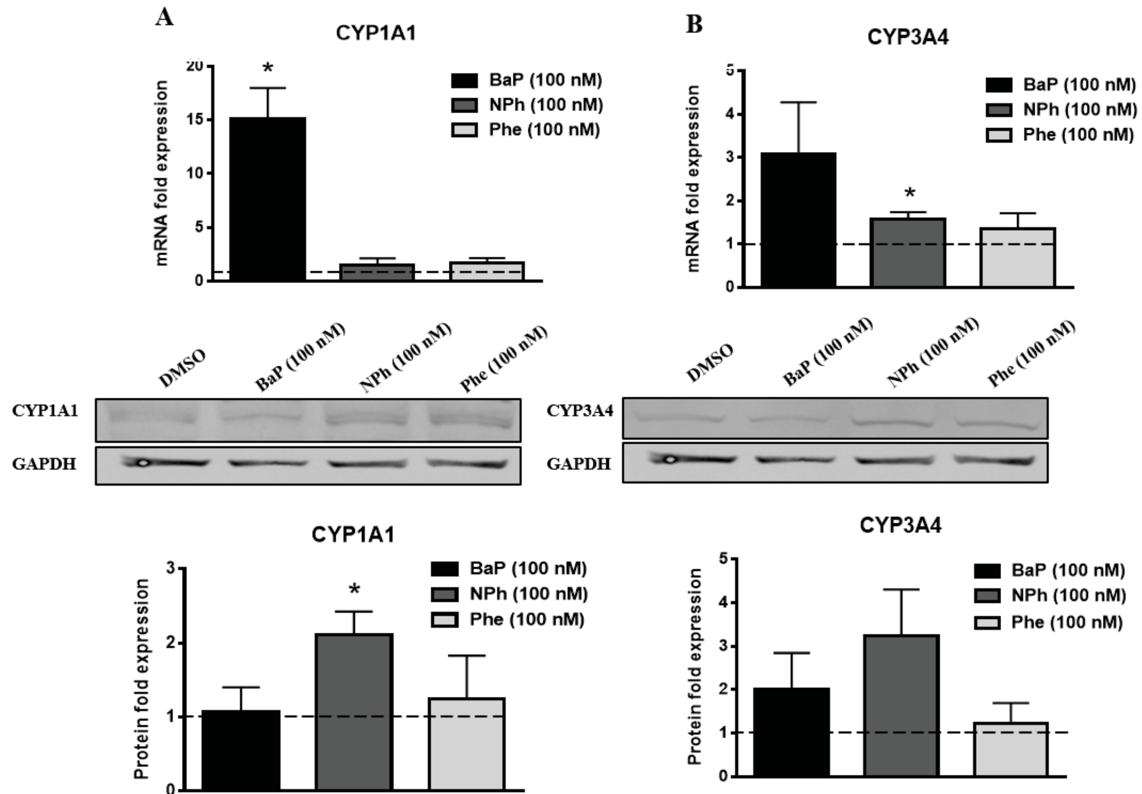

Figure 4-8. Effect of chronic (7 days) treatment of BaP, NPh, and Phe on mRNA and protein expression of CYP1A1 (A) and CYP3A4 (B) in U937 cells.
The U937 cells were treated with 100 nM BaP, 100 nM NPh, and 100 nM Phe for seven days. The mRNA fold expressions were calculated using qRT-PCR and normalized with control (DMSO treated cells) whose expression was set at 1-fold. GAPDH was used as an endogenous control. The protein fold expressions were quantified by Western blot and normalized with control that was set to 1-fold at every time point. Blots are representative of at least three independent experiments. The data are presented as a mean ± SEM of three independent experiments. *represents $p \leq 0.05$, compared with the control group.

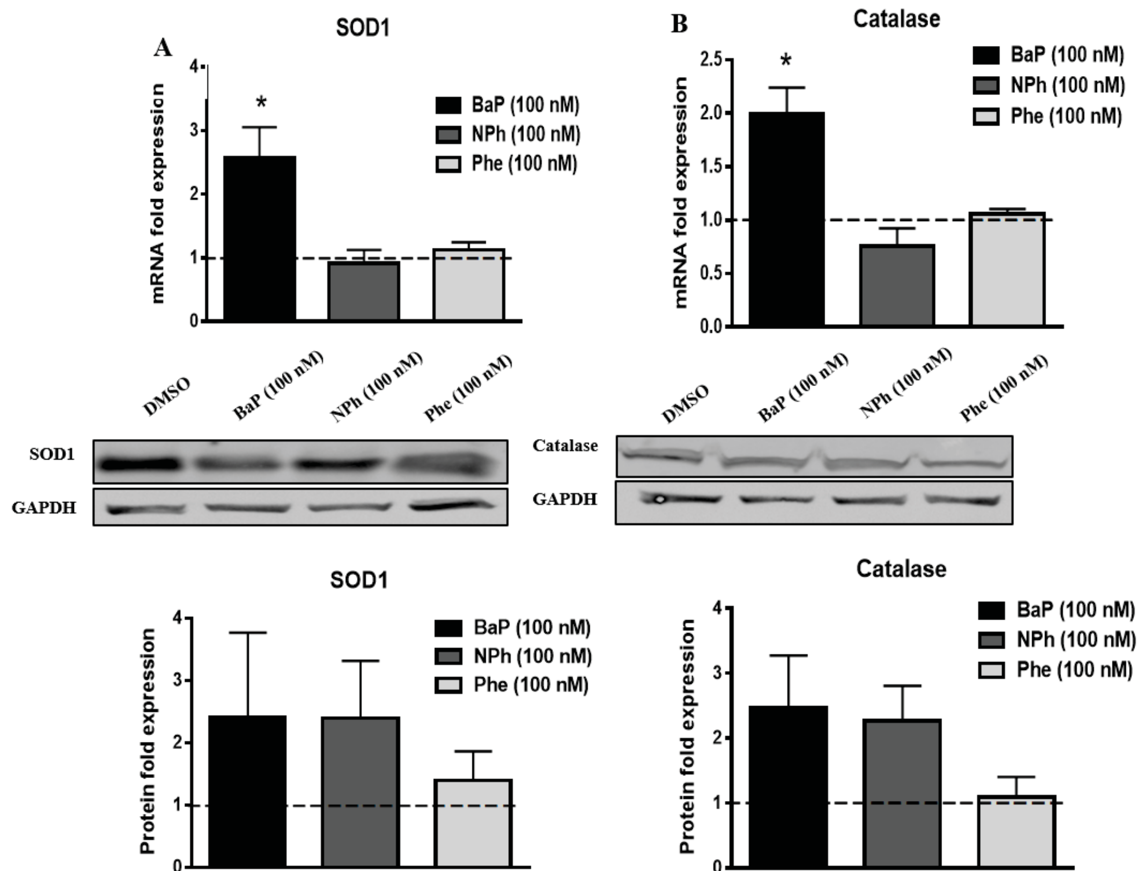

Figure 4-9. Effect of chronic (7 days) treatment of BaP, NPh, and Phe on mRNA and protein expression of AOEs SOD1 (A) and catalase (B) in U937 cells.
The U937 cells were treated with 100 nM BaP, 100 nM NPh, and 100 nM Phe for seven days. The mRNA fold expressions were calculated using qRT-PCR and normalized with control (DMSO treated cells) whose expression was set at 1-fold. GAPDH was used as an endogenous control. The protein fold expressions were quantified by Western blot and normalized with control (DMSO treated cells) whose expression was set at 1 fold. Blots are representative of at least three independent experiments. The data are presented as a mean ± SEM of three independent experiments. *represents $p \leq 0.05$, compared with the control group.

expression of both SOD1 (~2.5 folds, $p \leq 0.05$, **Figure 4-9A**) and catalase (~2 folds, $p \leq 0.05$, **Figure 4-9B**).The corresponding protein expression levels of both AOEs were also upregulated by similar proportions: SOD1 (~2.5 fold) and catalase (~2.5 fold) but the data were not statistically significant (**Figure 4-9A-B**, lower panel). Further, treatment with NPh or Phe did not significantly alter the mRNA or protein expression of AOEs.

Effect of Chronic Treatment of BeA and BeF on ROS, Cell Viability, and Caspase-3 Activity

In addition to three PAHs (BaP, NPh, and Phe), BeA and BeF are other PAHs that have carcinogenic potential. Therefore, we also studied these compounds with regard to oxidative stress and cytotoxicity. The cells were treated with 100 nM of BeA or BeF for seven days and were examined for ROS level and cell viability. Cell viability data from XTT assay showed approximately 10-13% cell death when treated with 100 nM of BeA or BeF for seven days (**Figure 4-10A**). A significant decrease in ROS level was observed with the chronic treatment of BeA 100 nM (~35%, $p \leq 0.05$) as well as BeF 100 nM (~23%, $p \leq 0.05$) (**Figure 4-10B**). There was no significant change in the caspase-3 activity with chronic treatment of both the compounds (**Figure 4-10C**). Overall, treatment with BeA and BeF had relatively less effect on the oxidative stress and cell viability of U937 compared to BaP.

Effect of Chronic Treatment of BaP on CYP Expression, ROS and Cell Viability in Primary Macrophages

Of the five compounds, only exposure with BaP (100 nM) resulted in significant increased expression of CYPs and AOEs, ROS and cell death in U937 cells. So, we exposed the primary macrophages with 100 nM BaP to verify our observations in U937 cells. When we treated the primary macrophages with BaP 100 nM for seven days, we observed ~25% cell death accompanied by ~30% increase in ROS and ~10 and ~ 3 fold ($p \leq 0.05$) increase in CYP 1A1 and 3A4 respectively (**Figure 4-11A-D**). These results confirm that BaP exposure leads to cytotoxicity in monocytes/macrophages via CYP-mediated oxidative stress pathway.

Discussion

Carcinogenic effects of smoking have been widely studied in various organs, especially lungs and liver [233, 234]. Several studies have also reported CYP-mediated PAH toxicity in different organs [26, 27]. However, relatively less information is available on the effects of smoking constituents on the blood cells such as monocytes and lymphocytes. A recent study conducted in our lab has shown that nicotine causes oxidative stress in monocytic cells U937 cells through a CYP-mediated pathway [230].

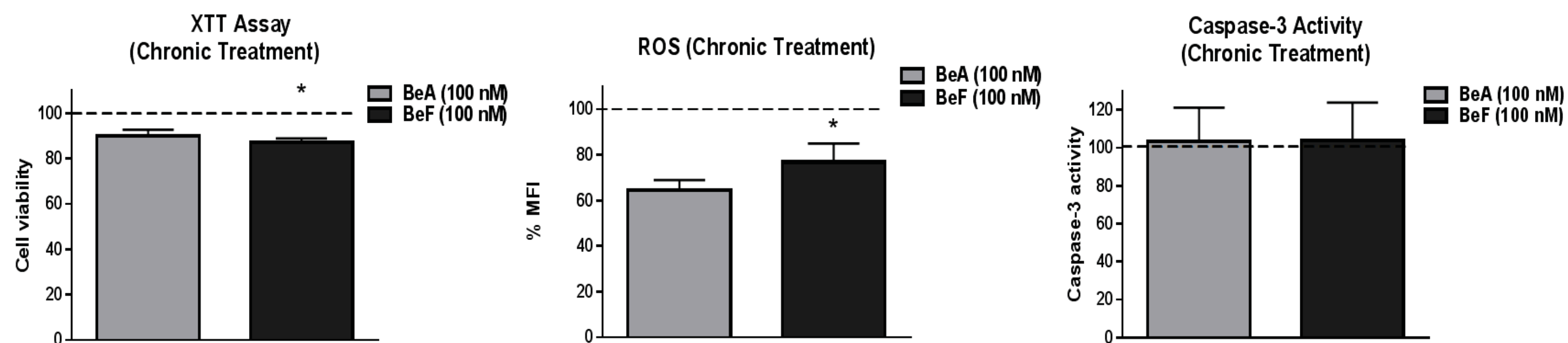

Figure 4-10. Cell viability (A), ROS level (B) and caspase-3 activity (C) upon chronic treatment by BeA and BeF in U937 cells.

The U937 cells were treated with 100 nM BeA and 100 nM BeF for seven days. ROS level, cell viability, and caspase-3 activity were measured and normalized with control that was set as 100%. The data is presented as mean ± SEM of the three independent experiments. * represents $p \leq 0.05$, compared with the control group.

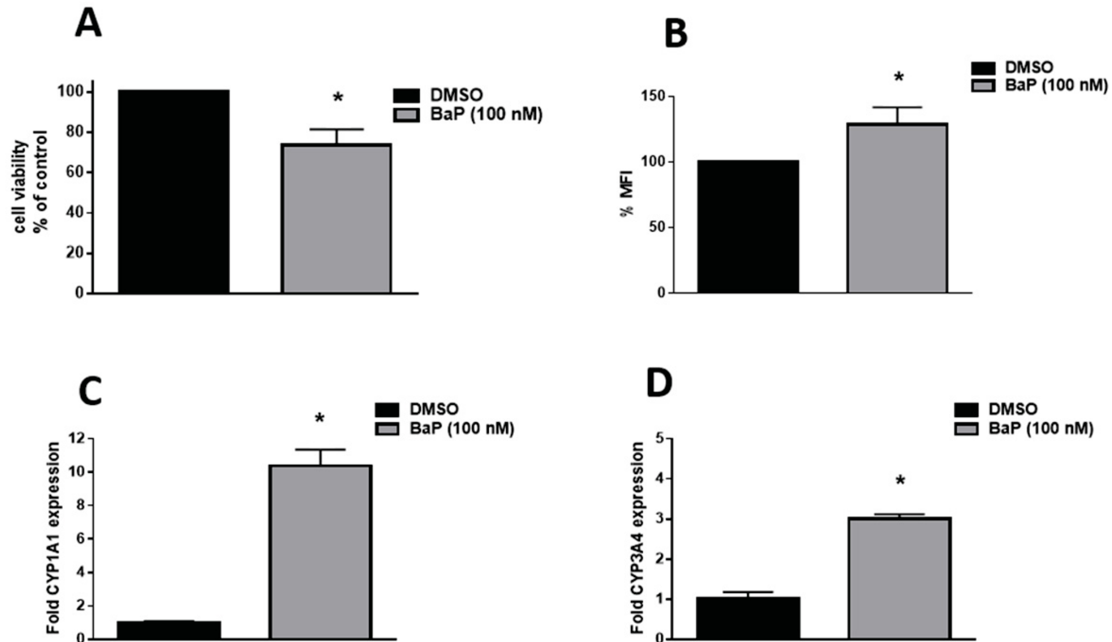

Figure 4-11. Cell viability (A), ROS level (B) and mRNA expression level of CYP1A1 (C) and CYP3A4 (D) upon chronic treatment by BaP in primary macrophages.

The differentiated macrophages obtained from healthy donors were treated with BaP (100 nM) for 7 days. Cell viability and ROS were calculated and normalized with control that was set as 100%. The mRNA expression of treated cells were normalized with control (DMSO treated cells) whose expression was set at 1-fold. The results for cell viability were confirmed using 3 different donors. Due to inadequate number of primary cells, ROS and CYPs expression level were examined in only one donor. The data is presented as mean ± SEM of the three replicates for ROS and two replicates for CYP expression levels. * represents $p \leq 0.05$, compared with the control group.

However, there is no report on the effect of PAHs and relative contribution of different PAHs on oxidative stress and cytotoxicity, and underlying mechanism in the monocytic cells. The present study is the first report of relative contribution of five PAHs (BaP, NPh, Phe, BeA, and BeF) on the expression of CYPs and AOEs, induction of ROS, and cytotoxicity in U937 monocytic cells. The results from this study support the existing literatures that among the tested PAHs, BaP is the most harmful compound, which causes oxidative stress and subsequent cytotoxicity, at least in part through CYP pathway.

Zhu et al. (2014) have shown that BaP (5 μM) significantly increases the expressions of CYP1A1 and CYP1B1, ROS level, and cytotoxicity in lung epithelial cells (BEAS-2B cells) after 24 hour treatment [235]. In this paper, we examined the effect of both acute (6-24 hours) and chronic (7 days) exposure of PAHs in U937 cells. We initially treated the cells with 100 nM and 1 μM of each of the PAHs for acute study. These concentrations are very near to the physiological concentrations of PAHs that caused toxicity in different cell lines [235, 236]. For the chronic treatment, the lower concentration (100 nM) was preferred because higher concentration (1 μM) resulted in immense cell death when exposed for a prolonged period. There are very few reports that account for the toxicity of chronic BaP exposure in human cells.The reports of chronic BaP exposure in animal model suggest its association with neurotoxicity [237], DNA damage [238], carcinogenicity, and cytotoxicity [239]. Most of the studies of BaP toxicity have been conducted at higher concentration for an acute period. In this context, our experimental design to study the acute as well as chronic effects of near physiological concentrations of PAHs on monocytic cells is pragmatic because it closely simulates the effects of tobacco on these cells in vivo.

We have used U937 cells derived from histiocytic lymphoma tissues, which have functionally deficient p53 tumor suppressor gene due to gene mutation [240]. The phenomenon of apoptosis is apparently ceased in these cell lines, causing massive cell proliferation. However, apoptosis may occur in U937 cells when triggered by various stress factors such as ROS, via a p53-independent mechanism. There are also reports that associate p53 with BaP-induced CYP1A1 expression, which occur via p53 binding to a p53RE in the CYP1A1 regulatory region [241]. Some studies suggest that p53 upregulates the gene associated with antioxidant activity and thereby prevent the genome from oxidative damage by ROS [242]. Due to the lack of functional p53 in U937 cells, induction of CYP1A1 and antioxidants by BaP occur via a p53 independent pathway, probably via Nf-κB-mediated pathway. Another possible pathway for induction of CYPs could be via binding of BaP to arylhydrocarbon receptor (AhR) and the subsequent gene activation by constitution of the Arnt protein-BaP complex with XRE responsive element [243].

With acute treatment of three PAHs BaP, NPh, and Phe, we did not observe any significant change in the expressions of CYPs and AOEs, ROS level, or cell viability. So, we exposed the cells for seven days with 100 nM of each of the compounds to monitor their chronic effect. Out of five PAHs (BaP, NPh, Phe, BeA, and BeF), only BaP (100 nM) significantly increased the expressions of CYPs and AOEs, generation of ROS, and cytotoxicity in U937 cells. The prolonged exposure of BaP to the cells probably caused

the accumulation of ROS, via CYP-pathway. Elevated ROS then contributed to the oxidative stress and cytotoxicity. An increased level of AOE expression with chronic BaP treatment is also consistent with the finding that there was an increase in the ROS level.

BaP is considered a prototype compound for PAH carcinogenicity. IARC has listed BaP as a group I human carcinogen, while NPh, Phe, BeA, and BeF have been classified as compounds possibly carcinogenic to humans (IARC 2004). BaP-induced carcinogenesis has been studied extensively for more than five decades. Previous studies reveal that CYP metabolites of BaP form DNA adducts that inactivate the tumor suppressor p53 in human epithelial cells and bronchial epithelial cells [233]. Kucab et al. (2015) has also suggested the role of BaP and its metabolites in p53 mutagenesis, which is a common pathway observed in almost half of human cancers [244]. However, the role of BaP and other PAHs with respect to oxidative stress and cytotoxicity in monocytic cells is not known. In this context, evaluation of the role of PAHs and their relative contributions to the expressions of CYPs, induction of oxidative stress, and cytotoxicity further validates the literature that BaP is toxic to many cells including monocytic cells.

BaP induces the expression of several CYPs including 1A1 and 1B1 in different cells [245-247]. Since the basal expression of CYP1B1 in U937 cells is very low [248], we examined the effect of PAHs on the expression of CYP1A1 only. Cytotoxicity induced by CYP1A1 metabolites of BaP has been reported in different cellular systems: lung cells [249], porcine urinary bladder epithelial cells [250], and bone marrow cells [251]. CYP1A1 metabolizes BaP into BPDE which forms DNA adducts leading to genotoxicity and carcinogenesis. Some studies also suggest that ROS generated from BaP via CYP pathway causes oxidative stress that lead to cytotoxicity. In the present study, CYP1A1 mRNA expression was consistently upregulated (~15 fold) following the acute and chronic treatment of BaP (100 nM) in U937 cells. However, the CYP1A1 protein levels were not consistent with the mRNA expression levels. Similar contradiction was reported in earlier studies [252], the reason for which is not clear. However, it is possible that CYP1A1 is relatively less stable upon extraction or there may be post-translational modification of the protein. The metabolites obtained from the CYP1A1-mediated oxidation of BaP may be responsible for the elevated ROS level and subsequent oxidative stress and cytotoxicity. Further investigation is required to confirm our hypothesis that CYP1A1 metabolizes BaP into ROS-generating metabolites that cause cytotoxicity in U937 cells. This can be done either by knocking down CYP1A1 gene or by using a selective inhibitor of CYP1A1 and treating with BaP to determine if ROS generation and cytotoxicity still occur. The chronic exposure to NPh (100 nM) showed ~ 2 fold increase in CY1A1 protein expression but there was no significant effect on AOE expression, ROS, cell viability and caspase-3 activity. Although, we observed increase in CYP1A1 protein expression to some extent, the induced protein may not have been sufficient enough to metabolize the compound.

CYP3A4 is a major drug metabolizing enzyme that metabolizes about 50% of the xenobiotics and is present in relatively high abundance compared to other CYPs, not only in hepatic cells, but also in U937 monocytic cells [252]. BaP-induced CYP3A4 upregulation at mRNA level has been observed in HepG2 liver cells and HEK-293

kidney cells [253]. Kumagai et. al also suggested that BaP enhances CYP3A4 gene expression via PXR activation in liver cells [254]. Furthermore, they suggested the possibility that the metabolites obtained through CYP1A1 metabolism could act as CYP3A4 inducers. It is important to study the expression level of CYP3A4 in context of BaP metabolism and toxicity. CYP3A4 does not metabolize the PAH parent compounds but it does convert dihydrodiols into diol epoxides. However, the rate of conversion is slower than that observed through CYP1A1 metabolism [232]. In the present study, we did not observe any significant change in CYP3A4 expression at mRNA and protein levels after acute or chronic treatment with BaP. Since the overall CYP3A4 expression was low, CYP1A1 metabolites also did not seem to contribute to CYP3A4 induction.

Cells are equipped with an antioxidant defense mechanism to counteract the oxidative stress resulting from elevated ROS [255]. Oxidative stress is alleviated either by endogenous antioxidants such as reduced glutathione (GSH) or by adaptive defense mechanism through induction of genes encoding AOEs [256]. The induction of genes encoding AOEs are regulated by nuclear factor erythroid 2- related factor (Nrf-2) signaling pathway [257]. Any perturbations in the AOE defense system that decrease the AOE expression may aid elevation in ROS level. Higher incidence of oxidative stress-induced carcinogenesis is reported in Nrf-2 knockout mice that are treated with BaP. Furthermore, if the level of ROS exceeds the threshold, the AOEs may not be able to overcome the resulting oxidative insult [153, 258]. SODs and catalase are major AOEs in majority of the cell lines. In case of acute treatment of BaP, we observed a slight decrease in ROS which may be attributed to the protective activity of the endogenous AOEs. We anticipated higher levels of ROS and AOEs with chronic treatment of the PAHs, but we observed the expected result only with BaP-treated cells. The elevation in gene transcription of SOD1 and catalase over the course of seven-day treatment with BaP may not be sufficient enough to alleviate the high ROS level. Rather, an increase in the expression level of these AOEs are the indication that there is oxidative stress in the system.

In addition to BaP-mediated mutagenesis and carcinogenesis through DNA adduct formation, there are also reports of apoptosis induced by BaP metabolites in different human cell lines: Daudi B cells [259], H460 lung cancer cells [260], hepatoma HepG2 cells [261] and endometrial cancer RL95-2 cells [262], the latter two being directly associated with CYP-mediated metabolism of BaP. ROS triggers apoptosis by enhancing the permeability of the mitochondrial outer membrane and thereby leaking out pro-apoptotic proteins [263]. Pro-apoptotic proteins aid in the activation of caspases, cysteine proteases that cause cellular degradation. We observed apoptosis in U937 cells via caspase-3 dependent pathway, with chronic treatment of BaP. Our data suggests the involvement of CYP in the metabolism of BaP and generation of ROS with chronic treatment of BaP.

Conclusions

Therefore, we conclude that BaP causes apoptosis in U937 cells through a caspase-3-dependent pathway. BaP-induced cytotoxicity, in turn, is mediated by ROS that is likely to be generated through CYP-mediated metabolism of BaP. Our previous in vitro and ex vivo studies have suggested the role of nicotine metabolism via CYP2A6 in generating oxidative stress and HIV-1 replication in monocytic cells. The current study has established the fact that, in addition to nicotine, BaP is the major PAHs that is responsible for inducing oxidative stress, apoptosis, and cytotoxicity, perhaps through the CYP pathway in monocytic cells. The confirmation of increase in CYP expression, ROS generation and cytotoxicity mediated by chronic treatment of BaP in primary macrophages has further strengthened our findings from U937 monocytic cells. We speculate that BaP-induced oxidative stress via CYP metabolism would enhance HIV-1 replication. In fact, our study with cigarette smoke condensate (CSC), which contains nicotine and PAHs, has demonstrated an increase in HIV-1 replication in primary human monocyte-derived macrophages [153]. This is also based on the fact that BaP induced greater levels of ROS (>150%) compared to nicotine (15-20%) [230]. Taken together these findings indicate that BaP is likely the most active compound in cigarette smoke, and may generate oxidative stress leading to cytotoxicity and increased HIV-1 replication in monocytic cells. However, this has yet to be fully demonstrated using HIV-infected U937 and human primary monocytic cells.

CHAPTER 5. BENZO(a)PYRENE ENHANCES HIV-1 REPLICATION IN MONOCYTIC CELLS *

Introduction

The association of cigarette smoking and HIV-1 pathogenesis has been demonstrated by multiple studies in the past two decades [153, 154, 157, 159, 264, 265]. Smoking increases HIV-1 infectivity and viral load, and it lowers the $CD4^{+}$ T cell counts in HIV-1 patients, with a subsequent increase in immunosuppression [135, 153]. Smoking also decreases the response to antiretroviral therapy (ART) by approximately 40% in HIV-1 patients [160], which further accentuates the hazards of smoking on HIV-1 pathogenesis. However, little is known about the mechanisms underlying smoking-induced HIV-1 replication. A recent study has shown that cigarette smoke condensate (CSC) induces CYP expression and oxidative stress in HIV-1-infected monocyte-derived macrophages, and the findings are consistent with increased oxidative stress, nicotine metabolism and HIV-1 replication in HIV-infected individuals who smoke [154]. Another study has revealed that the toxic metabolites released through the CYP-mediated metabolism of cigarette smoke constituents enhance HIV-1 gene expression through DNA adduct formation [156]. Aqueous tobacco smoke extract is also known to enhance the upregulation of genes that enhance HIV-1 infection, but downregulate the expression of other genes that promote cell survival and antigen presentation [157].

Of the 5300 compounds that are present in CSC, polyaryl hydrocarbons (PAHs) are a class of carcinogenic compounds that are implicated by several studies for their potential to induce oxidative stress [266-268]. Benzo(a)pyrene (BaP) is a prototype PAH, which has been widely studied for its carcinogenicity, genotoxicity, and mutagenicity [269-272]. BaP is also known to induce CYP enzymes, especially CYP1A isoforms, which can have a direct impact on the biological disposition of various drugs [253, 273]. BaP is metabolically activated by CYP 1A1/1B1 enzymes into epoxide intermediates, which are further metabolized by CYPs or epoxide hydrolase into carcinogenic diol products [220, 274]. As a result of CYP-mediated BaP metabolism, excessive reactive oxygen species (ROS) are generated, leading to oxidative stress [275, 276]. Oxidative stress further leads to oxidative DNA damage, lipid peroxidation, and the oxidation of several proteins, ultimately causing cytotoxicity and cell death [277, 278]. Recently, we have demonstrated that exposure of BaP causes the induction of CYPs and a subsequent increase in oxidative stress and cytotoxicity in U937 monocytic cells [273]. Oxidative stress is implicated in enhanced replication of HIV-1 via the activation of redox sensitive nuclear transcription factor Kappa- B (NF-κB) [192, 279-281]. Various

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stress factors regulate the NF-κB pathway resulting in the transcription of over hundreds of genes that regulate inflammation, immune response, cell proliferation, growth, and survival [282-284]. NF-κB is activated by a number of triggers such as viral proteins and drugs of abuse, leading to the expression of various cytokines, chemokines, and CYPs [282, 285-287]. Interestingly, most of the stress factors use ROS as a secondary messenger to modulate NF-κB activity [288]. In an inactive state, NF-κB proteins are localized in the cytoplasm by forming a complex with inhibitors of NF-κB proteins (IKB) proteins. ROS triggers the activation of the IKB kinase complex that facilitates the ubiquitination of IKB proteins, thereby releasing the NF-κB proteins into the nucleus [289]. Within the nucleus, the activated NF-κB proteins induce the transcription of HIV-1 structural genes by binding to the enhancer region of long terminal repeat (LTR) on HIV-1 DNA, that contains NF-κB binding sites [290]. Several reports have emphasized the role of ROS in the activation of NF-κB and its subsequent impact on HIV-1 gene transcription [281, 291]. However, whether smoking/tobacco mediated oxidative stress via CYP pathways causes the nuclear trafficking of NF-κB and resultant HIV-1 replication, is yet to be examined. In the current study, we examined the potential role of CYP-mediated oxidative stress and subsequent HIV-1 replication via the NF-κB pathway by an important tobacco constituent, BaP, in HIV-1-infected macrophages. We used macrophages in this study because they are a secondary target of HIV-1 infection and a major viral reservoir where it is difficult to effectively suppress the virus with antiretroviral agents [292, 293]. Moreover, HIV-1-infected macrophages cross the blood-brain-barrier (BBB) and infect CNS cells such as perivascular macrophages, microglia, and to some extent astrocytes, which eventually cause HIV-1-associated neurocognitive disorders [294, 295].

Materials and Methods

Cell Culture and Treatment

U1 cells. U1 cells, which are U937 cells chronically infected with HIV-1, were obtained from the NIH AIDS Reagent Program (Germantown, MD). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media containing 10% Fetal bovine serum (FBS) and penicillin. To differentiate the cells into macrophages, 0.8 million cells were seeded in 1.5 ml of media containing 100 nM phorbol 12-myristate 13-acetate (PMA) in each well of a 6-well plate. After 3 days, the media containing PMA and non-adherent cells was removed and the differentiated cells were washed with phosphate buffer saline (PBS). The cells were topped with fresh 1 ml media and treated with BaP (1 μM) every 24 hours for 3 days for acute treatment. An additional 0.5 ml of media was added and BaP concentration was maintained constant at every treatment. In order to monitor the chronic (7 days) effect of BaP, we initially treated U1 cells with BaP (10-100 nM) for 7 days and later differentiated them into macrophages.

Human primary macrophages infected with HIV-1. Human primary macrophages were derived from the whole blood of healthy individuals using a method described in Chapter 4. The macrophages were treated with polybrene (2 μg/ml) and IL-2

(interleukin-2, 10 ng/μl) and infected with HIV-Ada strain (20 ng/1 million cells/ml media). The infected cells were then treated with BaP (100 nM) every 24 hours for 7 days.

Viral Load

The viral load of HIV-1 was determined by assessing the level of p24 antigen in the supernatant collected from the treated U1 cells and primary macrophages. We used the HIV-1 p24 Antigen ELISA kit (Zeptometrix Corporation, Buffalo, NY) for this purpose. The kit is comprised of monoclonal antibody- coated microwells, which specifically bind the HIV-1 p24 antigen in the added samples. The captured antigen was incubated with biotin conjugated human anti-HIV-1 antibody at 37°C for 1 hr, followed by incubation with enzyme, streptavidin-peroxidase and tetramethylbenzidine substrate at 37°C and room temperature/dark, respectively, for 30 minutes each. The reaction of the enzyme with the substrate developed a blue color, the absorbance of which was measured at 450 nm to determine the p24 level. The optical density of the samples was compared against the standard curve.

Isolation of RNA and Protein

RNA was isolated using RNeasy Mini kit (250) (QIAGEN, Germantown, MD), following the manufacturer's protocol. The extracted RNA was quantified using Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific) at 260 nm. To isolate the protein from the treated cells, 100 μl of RIPA buffer was added to the cell pellet. The cell suspension was sonicated for 30 seconds with pulse set at 4 and centrifuged at 13000 rpm for 5 minutes. The supernatant containing the protein was collected and protein quantification was done by using the BCA protein assay kit (Thermo Fischer Scientific).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTPCR)

The relative mRNA fold expression level of CYPs (CYP1A1 and CYP3A4) and AOEs (Catalase, SOD1) after chronic BaP treatment was monitored using quantitative RTPCR as described in Chapter 4.

Western Blotting

We determined the protein expression level of CYPs (CYP1A1 and CYP3A4) and AOEs (Catalase, SOD1) after chronic BaP treatment in U1 cells using Western blotting as described in Chapter 4. In addition to CYPs and AOEs, we also monitored the expression of NFκB subunits, p50 and p65 in the cytoplasmic and nuclear fractions of protein. We used β-lamin as a loading control for the nuclear protein fractions. We used

NFκB p50 Rabbit Mab (1:200 dilution), NFκB p65 Mouse Mab (1:200), β-lamin Rabbit Mab (1:400 dilution) from Santa Cruz Biotechnology. Inc. Dallas, TX.

Measurement of ROS Level

We quantified ROS generated after acute (3 days) treatment of BaP on U1 cells using the NovoCyte flow cytometer (ACEA, Biosciences Inc., San Diego, CA). We followed the method as described in Chapter 4 for the measurement of ROS level in the cells. The data were analyzed by using the NovoExpress software.

7-Ethoxy-Resorufin-O-Deethylase (EROD) Assay

We used the EROD assay to determine the activity of CYP1A1 after the chronic treatment of BaP. Briefly, the treated cells were washed with PBS and suspended in 900 µl 0.1 M Hepes buffer, pH 7.4. The cell suspension was then sonicated for 10 seconds, 3 times, with pulse on and off. The cell extract (90 µl) thus obtained was added with 1 µl of substrate, 7-ethoxyresorufin (100 µM) and incubated at 37º C for 10 minutes. Later, 10 µl NADPH (10 mM) was added to the reaction mixture and incubated for another 15 minutes. The reaction was stopped by using 75 µl fluorescamine solution in acetonitrile (150 µg/ml). When the cell extracts were incubated with the substrate and NADPH, the CYP1A1 enzyme present in the cells reacted with the substrate resulting in the formation of a fluorescence product, resorufin. The fluorescence thus generated was measured at excitation/emission wavelengths of 535/590 nm. The CYP1A1 activity was calculated by measuring the amount of fluorescence generated per unit time. The quantity of resorufin in the samples was calculated by comparing it with the standard calibration curve prepared by using 0-100 pmol of resorufin standard.

Apoptotic DNA Damage

We used the Apoptag® Iso Dual Florescence Apoptosis Detection kit (Millipore Sigma, Burlington, Massachusetts) to determine apoptotic DNA damage after BaP treatment in U1 cells. The assay was carried out using the manufacturer's protocol.

Statistical Analysis

All the data are presented as Mean ± SEM of at least three independent experiments. The mean value obtained for the control group was normalized to 100% or 1-fold, to which the treated cells were compared as a % or fold of control, respectively. Student's t-test or one- or two-way ANOVA were used to calculate the statistical differences ($p \leq 0.05$) between the control and the treated groups, where applicable. All the statistical analyses were performed using GraphPad Prism 7 (San Diego, CA).

Results

BaP Induces HIV-1 Replication in U1 Cells and HIV-1-infected Human Primary Macrophages

Chronic exposure of BaP (100 nM for 7 days) showed an approximately 4-fold increase in HIV-1 replication in U1 cells (**Figure 5-1A**). However, a 10-fold lower concentration of BaP (10 nM) did not have any significant effect on the viral replication. We also confirmed this result in HIV-1-infected human primary macrophages, in which, BaP (100 nM) showed an approximately 2-fold increase in HIV-1 replication (**Figure 5-1B**). Furthermore, we examined apoptotic DNA fragmentation in HIV-1-infected human primary macrophages after 3 days exposure of BaP (100 nM). The fluorescent images revealed an increased apoptotic DNA fragmentation with DNase Type I ends in cells treated with BaP (100 nM) compared to that of the control (**Figure 5-1C**). DNA fragmentation with DNase Type II ends were not visible in both the control and the treated cells. The results suggest that BaP induces DNA fragmentation during the early phase of apoptosis, within the nucleus of the treated cells. Upon validating the results of the U1 cells in human primary macrophages, we performed the subsequent experiments that examined the underlying mechanism in U1 cells.

BaP Induces the Expression of CYP1A1

The expression of CYP1A1 and CYP3A4, which are the major BaP-metabolizing CYPs, were examined in U1 cells exposed to BaP (100 nM) for 7 days. The chronic exposure of BaP showed an approximately 30-fold increase in the mRNA expression of CYP1A1 in U1 cells (**Figure 5-2A**), but failed to show any significant expression of CYP1A1 at the protein level (**Figure 5-2B**). The chronic exposure of BaP (100 nM) increased the enzymatic activity of CYP1A1 by approximately 2-fold (**Figure 5-2C**). On the other hand, we did not observe any significant change in the expression of CYP3A4 at both the mRNA and protein levels (**Figure 5-2D-E**).

BaP Does Not Alter the Expression of AOEs

The induction of CYPs could metabolize BaP and increase ROS. However, the ROS could subsequently be neutralized by the induction of antioxidant enzymes (AOEs). Therefore, we examined the induction of two of the most important and general AOEs; superoxide dismutase 1 (SOD1) and catalase by 100 nM BaP. Our results demonstrated no significant change in the mRNA and protein expression levels of both SOD1 and catalase (**Figure 5-3A-D**).

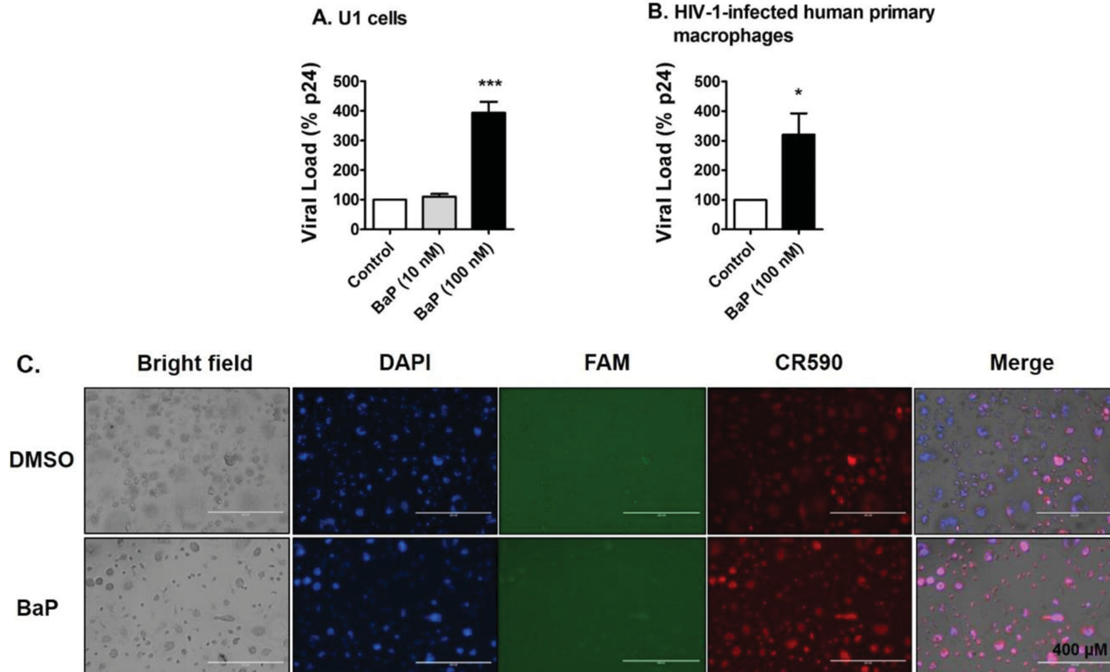


Figure 5-1. Chronic treatment of BaP induces HIV-1 replication and apoptotic DNA damage in HIV-1-infected macrophages.

(**A**)**.** The U1 cells were treated with 10 nM and 100 nM BaP for seven days. After the BaP treatment, the U1 cells were stimulated with 100 nM of Phorbol 12-myristate 13-acetate (PMA) to produce HIV-1. Supernatants were collected after two days of differentiation, which were used for the p24 ELISA assay to assess the viral load. The chronic (7 days) treatment of BaP (100 nM) significantly increased the viral replication in U1 cells, while the 10-fold lower concentration did not have any significant effect. The data is displayed as mean ± SEM (n= 6), calculated as a percentage of the control. (**B**). HIV-1-infected human primary macrophages were treated with BaP (100 nM) for 3 days. The supernatant was collected thereafter and used for the p24 ELISA assay to assess the viral load. The acute (3 days) treatment of BaP (100 nM) significantly increased the viral replication in HIV-1-infected macrophages. The data is displayed as mean ± SEM (n= 4), calculated as percentage of the control. The statistical significance was calculated at $p \leq 0.05$ (*), where *** represents $p \leq 0.0005$, compared with the control group. (**C**)**.** The apoptotic DNA damage assay was performed on the treated cells. DAPI, FAM and CR590 stained nucleus (blue), apoptotic DNA damage with DNase Type II ends (green) and Type I ends (red) respectively. A higher signal for CR590 is visible in the fluorescent images, indicating apoptotic DNA fragmentation with DNase Type I ends in the infected human primary macrophages after BaP (100 nM) exposure for 3 days. DNA fragmentation with DNase Type II ends (green) was not visible in either the control or the treated cells. Therefore, the images indicate that BaP (100 nM) induces DNA fragmentation during the early phase of apoptosis in the HIV-1-infected human primary macrophage

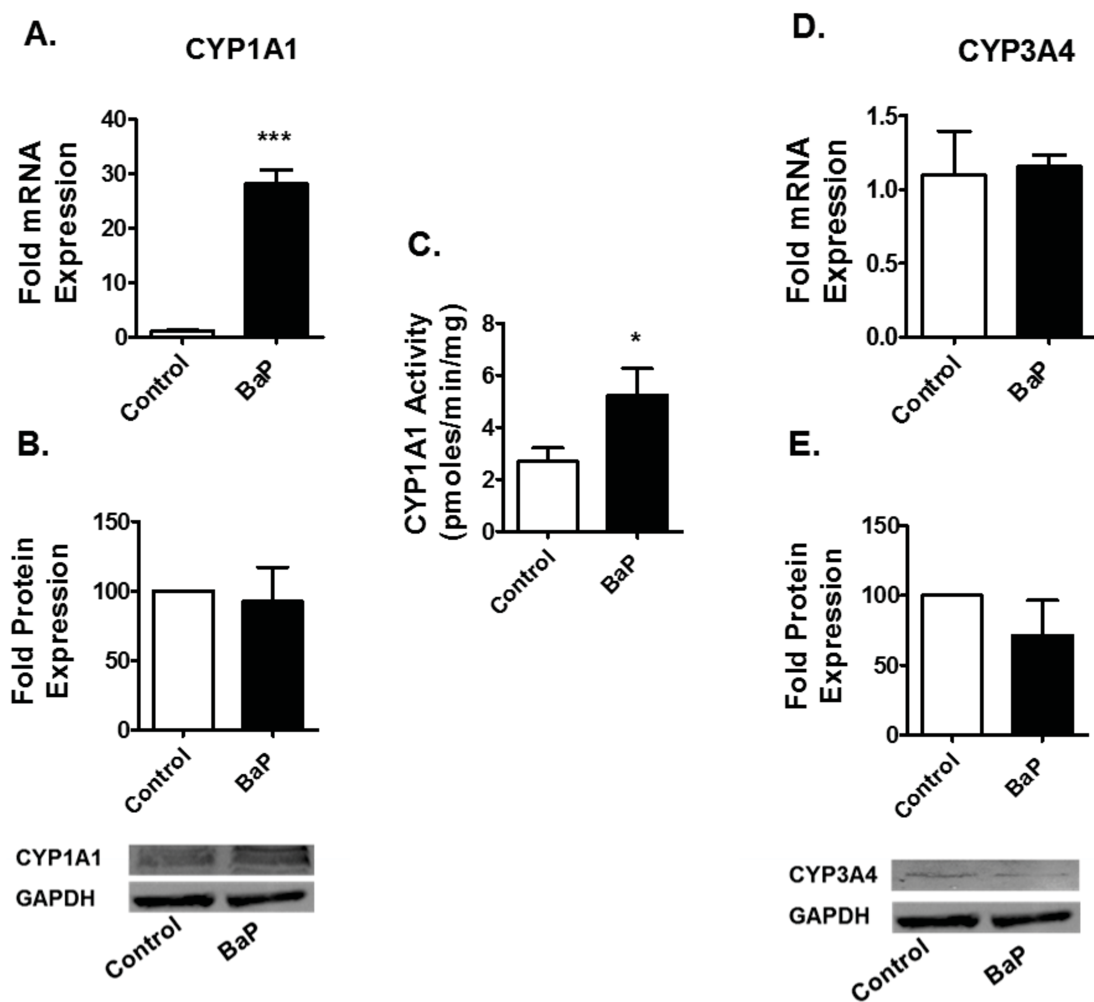


Figure 5-2. Chronic treatment of BaP induces expression of CYPs in U1 cells. The U1 cells were treated with 100 nM BaP for seven days. We measured the mRNA expression (**A, D**) and protein expression (**B, E**) of CYPs (1A1, 3A4) using RTPCR and western blotting, respectively. Chronic exposure of BaP (100 nM) significantly induced the expression of CYP1A1 at the mRNA level, but not at the protein level. Therefore, we measured activity of CYP1A1 using the EROD assay (**C**). Chronic BaP treatment increased CYP1A1 activity by approximately 2-fold. However, there was no significant change in the expression of CYP3A4 at both the mRNA and protein levels. The data are displayed as mean ± SEM of at least three independent experiments ($n \geq 3$). The mRNA/protein expression of the treated cells are normalized to control cells, whose expression was set at 1-fold. GAPDH was used as an endogenous control and loading control for RTPCR and western blotting, respectively. The statistical significance was calculated at $p \leq 0.05$ (*) compared with the control group. The blots are representative of at least three independent experiments.

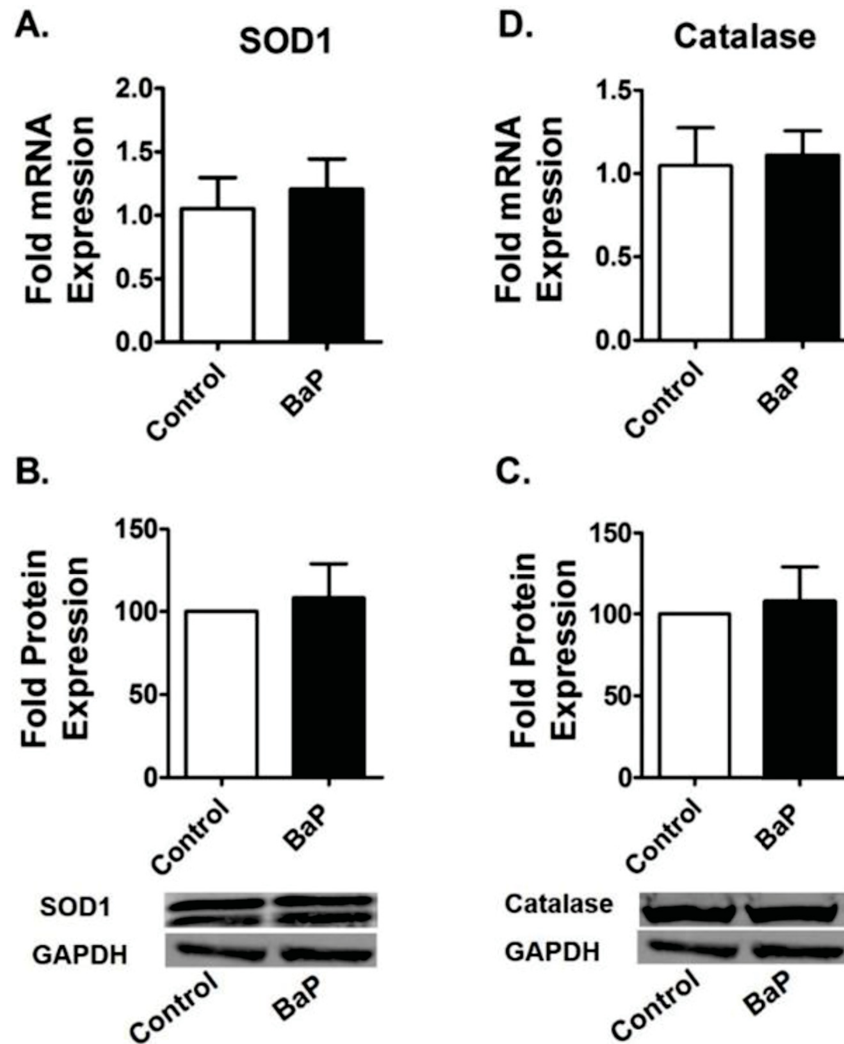


Figure 5-3. Chronic treatments of BaP have no significant effect on the expression of AOEs in U1 cells.

The U1 cells were treated with 100 nM BaP for seven days. We measured the mRNA expression (**A, D**) and protein expression (**B, E**) of AOEs (SOD1 and Catalase) using RTPCR and western blotting, respectively. There was no significant change in the expression of SOD1 and catalase at both the mRNA and protein levels after chronic treatment of BaP (100 nM). The data are displayed as mean ± SEM of at least three independent experiments ($n \geq 3$). The mRNA/protein expression of treated cells are normalized to control cells, whose expression was set at 1-fold. GAPDH was used as an endogenous control and loading control for RTPCR and western blotting, respectively. The statistical significance was calculated at $p \leq 0.05$ (*) compared with the control group. The blots are representative of at least three independent experiments.

Role of CYP1A1 on BaP-induced ROS Generation

Treatment of BaP (1 µM) in U1 cells for 3 days increased the generation of ROS by 30% (**Figure 5-4A-B**). In this and the subsequent experiments we used 1 µM BaP for 3 days to induce oxidative stress and/or HIV-1 replication, because chemical inhibitors and antioxidants are toxic to the cells when treated for 7 days. Further, we pre-treated the U1 cells with different antioxidants or CYP inhibitors prior to BaP exposure to determine whether these compounds reduce the BaP-induced ROS generation. We used vitamin C (100 µM), vitamin E (100 µM), resveratrol (50 µM), a resveratrol analog, pinostilbene (2 µM) as antioxidants, and a selective CYP1A1 inhibitor, ellipticine (1 µM) for the study at the concentrations previously shown to be effective [296, 297]. Vitamin C and ellipticine significantly reduced the BaP-induced production of ROS (**Figure 5-4A-B**). However, there was no significant change in BaP-induced ROS after treatment with vitamin E, resveratrol, and pinostilbene. **Figure 5-4C** shows the graphical representation of the results obtained in **Figure 5-4A-B**.

Role of CYP1A1 and Oxidative Stress Pathways on BaP-induced HIV-1 Replication

The U1 cells treated with BaP (1 µM) for 3 days showed approximately 70% increase in HIV-1 replication (**Figure 5-5A)**. Treatment with antioxidants, vitamin C and E (100 µM each) and, resveratrol (50 µM) rescued the viral replication caused by BaP (1 µM) (**Figure 5-5A**). In addition, treatment with the CYP1A1 inhibitor, ellipticine (1 µM) also exhibited a reduction in viral load in BaP-treated U1 cells (**Figure 5-5B**). To further validate our results, we knocked down the CYP1A1 gene in the U1 cells, using a siRNA silencing technique prior to BaP treatment. The viral load significantly decreased after silencing the CYP1A1 gene (**Figure 5-5C**), which further confirms a role of CYP1A1 on BaP-induced HIV-1 replication.

Nuclear Translocation of NF-κB Subunits upon BaP Exposure

There was no prominent change in the expression level of NF-κB p50 and p65 in the cytoplasmic fraction after chronic (**Figure 5-6A**, left panel) and acute (**Figure 5-6B**, left panel) treatment of BaP (100 nM) and BaP (1µM), respectively. Interestingly, there was an increase in the expression of both the NF-κB proteins, especially p65, in the nuclear fraction after acute and chronic BaP exposures compared to the control (**Figure 5-6A-B**, right panel).

Role of NF-κB Pathway in BaP-induced HIV-1 Replication

Treatment of BaP (1 µM)-exposed U1 cells with NF-κB inhibitors such as IKK-16 (**Figure 5-7A**) or SC-514 (**Figure 5-7B**) for 3 days, significantly reduced HIV-1 replication in BaP-exposed U1 cells, suggesting that the viral replication occurred via the NF-κB signaling pathway. We also monitored the translocation of NF-κB p65, the major

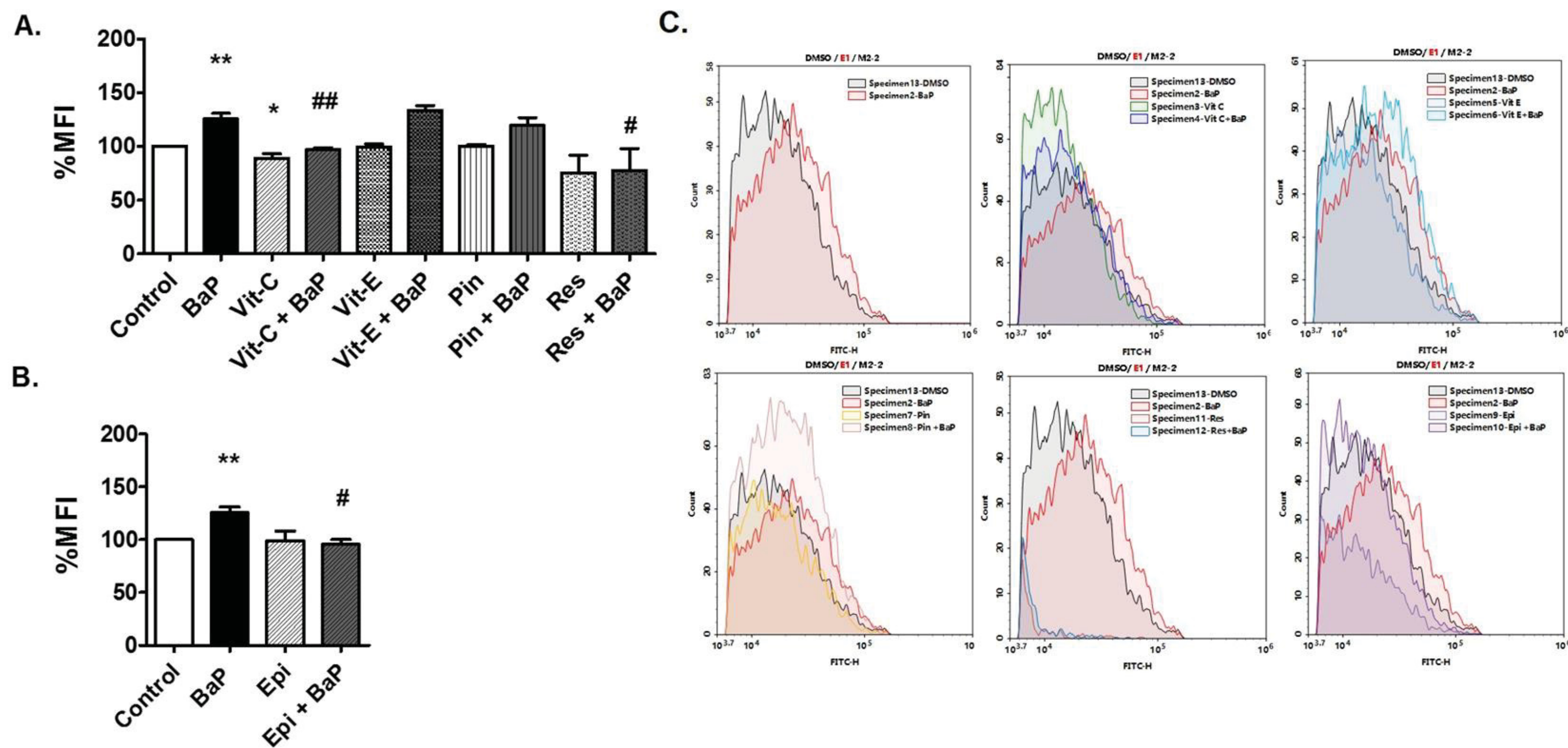


Figure 5-4. Treatment of antioxidants and CYP1A1 inhibitors reduce ROS in U1 cells due to BaP exposure.
U1 cells were concomitantly treated with BaP (1µM) and antioxidants [vitamin C (100 µM), vitamin E (100 µM), pinostilbene (2 µM), and resveratrol (50 µM)] (**A**) or a CYP1A1 inhibitor [ellipticine (1 µM)] (**B**). Treated cells were stained with CM-DCFDA dye and the fluorescence emitted was measured using flow cytometry at excitation/ emission of 495/519 nm. Treatment of BaP (1 µM) significantly increased ROS in U1 cells, which was rescued by the treatment of vitamin C, and ellipticine. The data were obtained from the mean of at least three independent experiments. * and ** Represents $p \leq 0.05$ and $p \leq 0.005$ respectively, compared with the control group while # and ## represent $p \leq 0.05$ and $p \leq 0.005$, respectively, compared to the BaP-treated groups. **Figure C**. shows the graphical representation of mean fluorescence intensity due to the treatmen

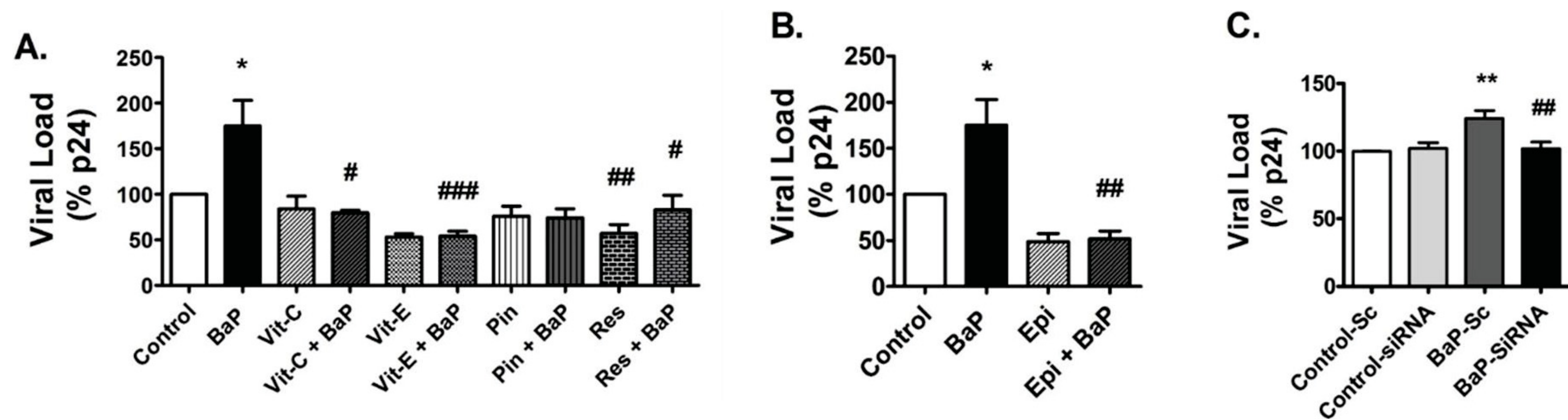


Figure 5-5. Treatment of antioxidants and CYP1A1 inhibitors reduce HIV-1 replication in U1 cells due to BaP exposure.
U1 cells were concomitantly treated with BaP (1μM) and antioxidants [vitamin C (100 μM) and vitamin E (100 μM), pinostilbene (2 μM), and resveratrol (50 μM)] (**A**) or CYP1A1 inhibitor epiliptine (1 μM)] (**B**) for 3 days. Prior to BaP treatment, the CYP1A1 gene was knocked down in the U1 cells using siRNA specific to CYP1A1 (**C**). The cells were then treated with BaP (100 nM) for 3 days. After the treatment, supernatants were collected to determine the viral load using the p24 ELISA assay. HIV-1 replication significantly increased with 3-days exposure of BaP (1 μM), which was rescued by all the antioxidants (vitamin C and E, and resveratrol) as well as the CYP1A1 inhibitor, epilliptine. The knock-down of the CYP1A1 gene also rescued HIV-1 replication in BaP-exposed U1 cells. The data were obtained from the mean of at least three independent experiments. * and ** Represents $p \leq 0.05$ and $p \leq 0.005$ compared with the control group while #, ## and ### represents $p \leq 0.05$, $p \leq 0.005$ and $p \leq 0.0005$, respectively, compared to the BaP-treated groups.

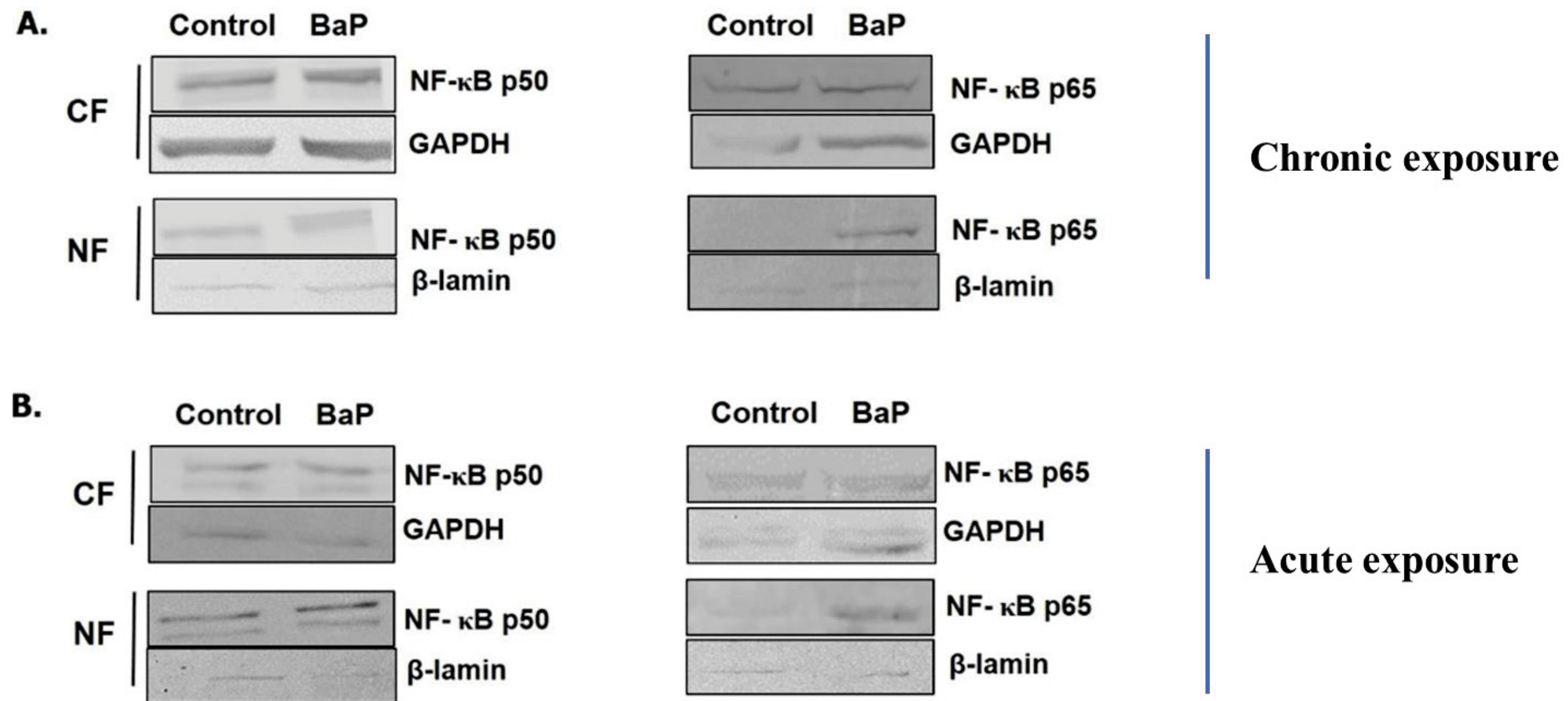


Figure 5-6. Translocation of NF-κB subunits from cytoplasm to nucleus upon BaP exposure.
U1 cells were treated with BaP 100 nm (**A**) and 1 μM (**B**) for 7 days and 3 days, respectively. After the treatment, proteins from the cytoplasm and nucleus were extracted from the cells. Western blot was run to determine the expression of the NF-κB p50 and p65 subunits in the proteins in cytosolic fraction (CF) and nuclear fraction (NF). GAPDH and β-lamin were used as loading controls for the cytoplasmic and nuclear proteins, respectively. The blots are representative of at least three independent experiments. There is not much difference in the expression of NF-κB p50 and p65 between the control and the BaP-treated cells. However, there is a clear increase in the expression of both the subunits in the nuclear fraction of acutely or chronically BaP-treated cells compared to the control group.

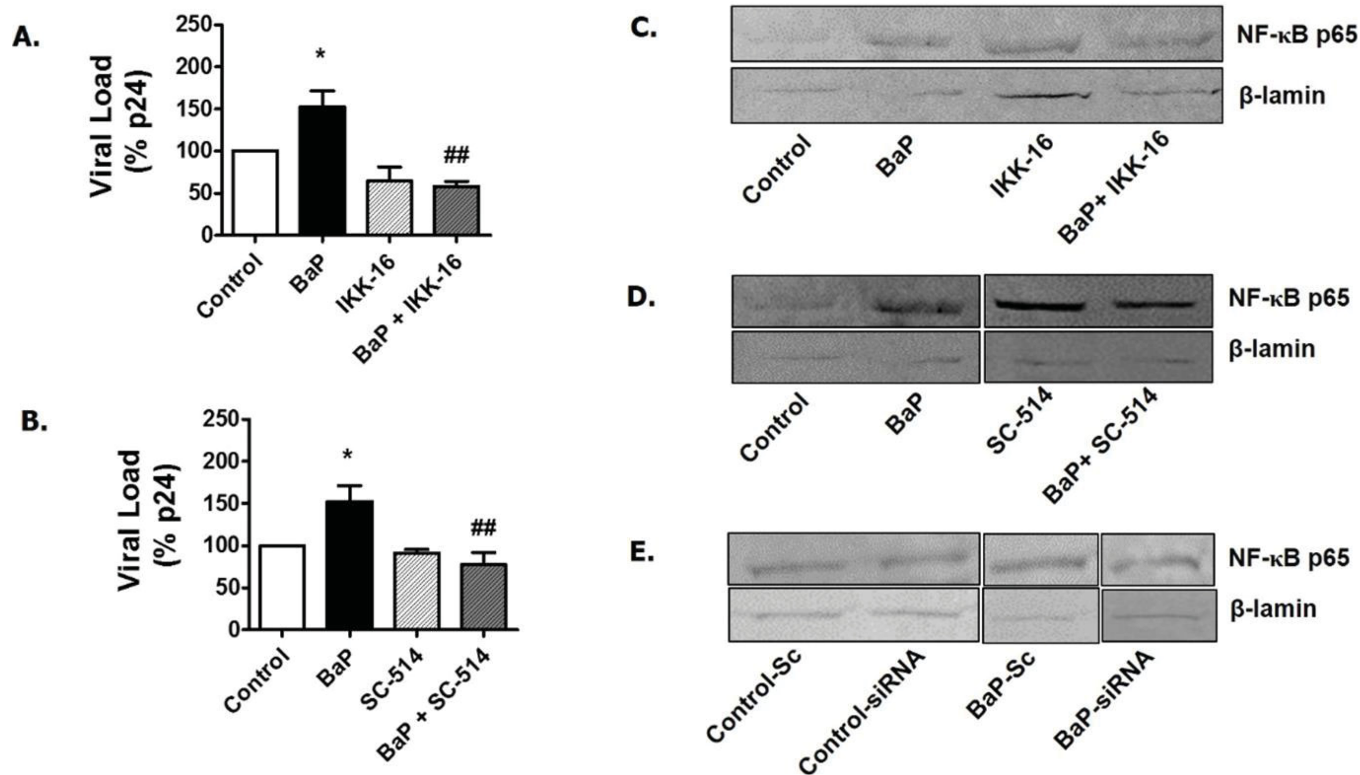


Figure 5-7. Treatment of NFκ-B inhibitors reduce HIV-1 replication in U1 cells due to BaP exposure.
U1 cells were concomitantly treated with BaP (1 μM) and NFκ-B inhibitors, IKK-16 (400 nM) (**A**), and SC-515 (10 μM) (**B**) for 3 days. After the treatment, supernatants were collected to determine the viral load using the p24 ELISA assay. HIV-1 replication due to BaP (1 μM) exposure was significantly rescued by NFκ-B inhibitors, IKK-16 (400 nM) and SC-514 (10 μM). * Represents $p \leq 0.05$ compared with the control group while ## represents $p \leq 0.005$ compared to the BaP-treated groups. Western blots were run using the nuclear fraction proteins obtained from the BaP-exposed cells treated with IKK-16 (**C**), SC-524 (**D**) or siRNA CYP1A1 (**E**) to determine the expression of NFκ-B p65 subunits. The blots indicate that treatment with both the NFκ-B inhibitors and CYP1A1 siRNA reduced the expression of NFκ-B p65 in the nuclear fraction protein of the BaP-treated cells compared to the control. The blots presented are representative of at least three different experiments.

DNA-binding subunit of the NF-κB protein into the nucleus, after treatment with NF-κB inhibitors, IKK-16 (**Figure 5-7C**) and SC-514 (**Figure 5-7D**) and after siRNA silencing of CYP1A1 (**Figure 5-7E**). The results showed that the knock-down of CYP1A1 in BaP-treated cells reduces the translocation of the NF-κB p65 unit into the nucleus. As expected, the expression of the NF-κB p65 protein was also observed to be lower in the nucleus of BaP-treated cells, after treatment with IKK-16 and SC-514

Discussion

Several reports suggest that cigarette smoke exposure is associated with increased HIV-1 replication and infectivity [140, 157, 211]. However, the mechanism of smoking induced HIV-1 replication is poorly understood, except for the fact that oxidative stress is a possible mechanism for enhanced viral load [153, 154]. The current report reveals a novel mechanism for BaP-mediated HIV-1 replication in monocyte-derived macrophages in vitro. In this study, we have demonstrated that oxidative stress generated by the CYP1A1-mediated metabolism of BaP, triggers the redox-sensitive transcription factor, NF-κB that leads to the amplification of HIV-1. This is the first evidence of the involvement of a novel CYP-mediated oxidative stress pathway in tobacco-mediated HIV-1 replication via NF-κB in macrophages (**Figure 5-8**)

Recently, we have shown that the exposure of CSC increases HIV-1 replication, oxidative stress, and induction of CYP enzymes in U937 and/or U1 macrophages [154]. We have also shown the involvement of CYP2A6 in nicotine metabolism and oxidative stress in U937 cells [230]. Further, our study using ex vivo samples from HIV-1-infected individuals who smoke also demonstrated an increase in HIV-1 replication, oxidation stress, and nicotine metabolism [153]. Taken together, these studies suggest an association of CYP-mediated oxidative stress with HIV-1 replication in tobacco smokers. Comparison of oxidative stress levels after nicotine and CSC exposure revealed that the induction of ROS by CSC is much higher than ROS induction by nicotine [230]. Therefore, we recently studied the effect of BaP, an important PAH component of CSC, in U937 cells and observed that BaP increases CYP1A1 expression, ROS levels, and cytotoxicity [273]. The excessive ROS production by BaP likely disturbed the redox homeostasis, causing oxidative stress, which resulted in cytotoxicity in U937 cells [298].

In the present study, we examined whether BaP induces HIV-1 replication in HIV-1-infected macrophages via the CYP-induced oxidative stress pathway. To demonstrate this, first we studied the effect of BaP in HIV-1 replication in U1 cells and our findings suggested that chronic exposure of BaP (100 nM) increases HIV-1 replication in these cells. We have also confirmed this result in HIV-1-infected human primary macrophages. Furthermore, BaP exposure induced DNA fragmentation during the early phase of apoptosis. Secondly, the expression of CYP1A1 and CYP3A4, which are the major BaP-metabolizing CYPs, were examined in U1 cells after BaP (100 nM) exposure for 7 days. The results showed an increase in the mRNA expression of CYP1A1 by BaP which is consistent with the previous study findings [220, 273, 299, 300]. BaP induces the expression of CYPs in cells by activating a nuclear receptor, aryl

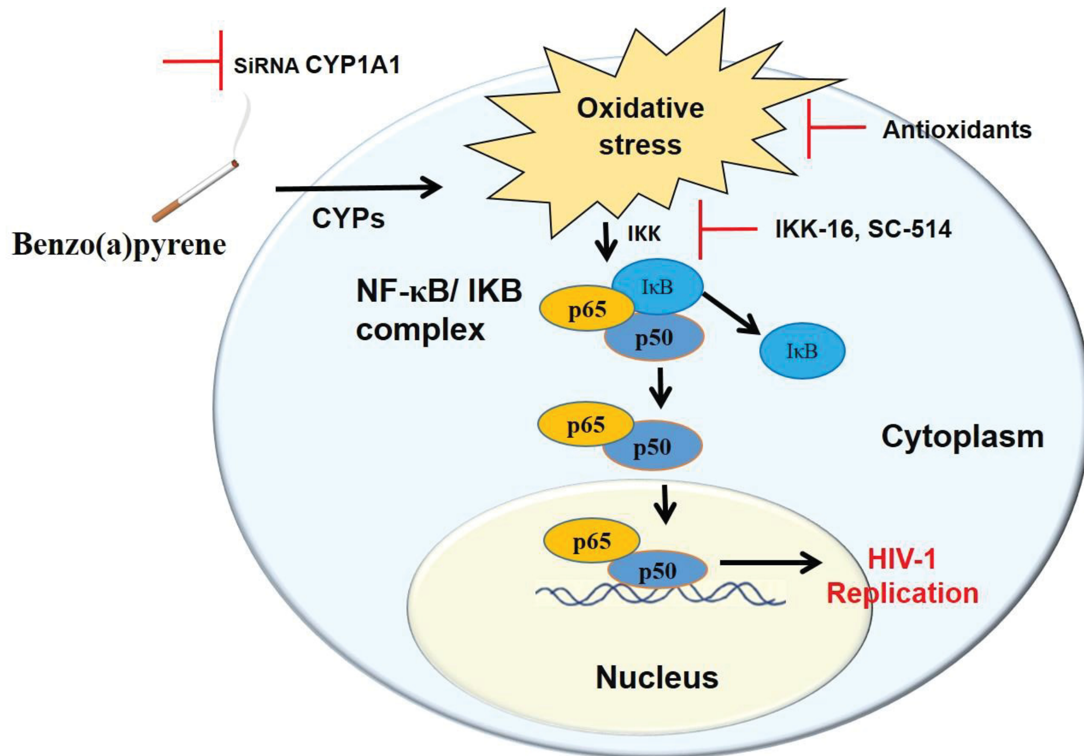


Figure 5-8. Schematic diagram showing the mechanism of BaP-induced -1-1 replication in macrophages.

Cigarette smoke constituents such as benzo(a)pyrene (BaP) induces expression of CYP1A1 in HIV-1-infected cells. CYP1A1 in turn metabolizes BaP into various BaP-metabolites, during which large amounts of reactive oxygen species (ROS) are generated. The resulting oxidative stress induces HIV-1 replication in the cells via a NF-κB pathway. In an inactive state, NF-κB (p65 and p50 subunits) forms a complex with an inhibitor of NF-κB proteins (IκB), which inhibits its translocation from the cytoplasm to the nucleus. Oxidative stress due to BaP triggers the activation of IκB kinase (IKK) that facilitates the ubiquitination of IκB proteins, thereby releasing the NF-κB subunits into the nucleus. Within the nucleus, the NF-κB proteins bind to specific DNA regions and trigger the expression of HIV-1 structural genes. Knock-down of CYP1A1 by siRNA specific to CYP1A1 and inhibition of the NF-κB pathway using specific NF-κB inhibitors such as IKK-16 and SC-514 significantly reduced HIV-1 replication in HIV-1-infected macrophages.

hydrocarbon receptor (AHR) [251]. The binding of BaP dissociates AHR from the AHR-heat shock protein 90 complex, which facilitates AHR translocation into the nucleus. Inside the nucleus, AHR binds to DNA with the help of AHR nuclear translocator (ARNT) and activates the transcription of CYP1A1, CYP1A2 and CYP1B1 [301, 302]. This is a well-known pathway and therefore a similar mechanism likely occurs in inducing CYP1A1 by BaP in macrophages presented in this study. However, as seen before, we did not observe any significant expression of CYP1A1 at the protein level, perhaps due to post translational modifications of the protein or instability of the protein after extraction. Therefore, we determined its enzymatic activity using the Erod assay [303]. The chronic exposure of BaP (100 nM) increased the enzymatic activity of CYP1A1 by approximately 2-fold. On the other hand, we did not observe any significant change in the expression of CYP3A4 at both the mRNA and protein levels. However, BaP induced the expression of CYP3A4 in human primary monocytes [273]. This discrepancy could be due to the use of two different, uninfected human primary monocytes and HIV-1-infected (U1), monocytic cell lines.

Thirdly, it is well known that CYPs generate ROS while metabolizing a wide range of substrates via coupling and auto oxidation reactions [304], and our results are in agreement with previous reports [276, 305], showing that acute treatment of BaP (1 μM) in U1 cells for 3 days increase the ROS levels. Further, we treated the U1 cells with different antioxidants or CYP inhibitors prior to BaP exposure to determine whether these compounds reduced the BaP-induced ROS generation. We chose a shorter duration of time and a higher BaP concentration to induce ROS in this experiment, because chronic treatment of antioxidants or CYP inhibitors along with BaP causes cytotoxicity. We used vitamin C, vitamin E, resveratrol, a resveratrol analog, pinostilbene as antioxidants, and a selective CYP1A1 inhibitor ellipticine to see if these antioxidants can block the BaP-induced oxidative stress. In addition to their antioxidant effects, resveratrol and pinostilbene are also known to inhibit CYP1A1 [297]. Vitamin C and ellipticine significantly reduced the BaP-induced production of ROS. The results suggest that BaP-induced ROS occurs via CYP1A1-mediated metabolic activation of BaP.

Cells express AOEs to neutralize the excessive accumulation of ROS generated by various stress factors [278, 306]. BaP is known to induce the expression of AOEs by the activation of the nuclear Nrf2 (nuclear factor erythroid 2-related factor) pathway [307]. BaP downregulates the activity of the Nrf2 inhibitory protein, kelch-like ECH-associated protein 1(Keap1), which prevents the proteasomal degradation of Nrf2 and promotes its translocation to the nucleus, where it binds to the enhancer ARE in DNA, leading to the transcription of the AOEs [307, 308]. Therefore, we examined the induction of two of the most important and general AOEs; superoxide dismutase 1 (SOD1) and catalase in U1 cells after BaP exposure. There was no significant change in the mRNA and protein expression levels of both SOD1 and catalase. Although the basal AOEs may have played their part in scavenging ROS, the persistent increase in oxidative stress after BaP exposure suggests that their antioxidant capacity was not sufficient. Furthermore, these results suggest that the inability of BaP to enhance AOE expression could cause an increased generation of CYP-induced ROS by BaP.

Next, we checked whether oxidative stress induced through the CYP1A1-mediated metabolism of BaP increased HIV-1 replication in HIV-1-infected macrophages. The treatment of U1 cells with BaP showed an approximately 70% increase in HIV-1 replication, which was significantly reduced by the treatment of antioxidants such as vitamin C and E, and resveratrol. In addition, treatment with the CYP1A1 inhibitor, ellipticine, also exhibited a reduction in the viral load of BaP-treated U1 cells. Taken together, these results suggest that BaP induces HIV-1 replication in U1 cells via the CYP-mediated oxidative stress pathway. We knocked down the CYP1A1 gene in the U1 cells, using the siRNA silencing technique prior to BaP treatment to further confirm our results. The reduction in HIV-1 replication after siRNA silencing of the CYP1A1 gene, further strengthens the role of CYP1A1 in BaP-induced HIV-1 replication.

Upon demonstrating that BaP enhances HIV-1 replication in U1 cells via the CYP-mediated oxidative stress pathway, our next goal was to identify the molecular mechanism of oxidative stress-induced viral replication upon BaP exposure. Several studies have suggested that the NF-κB pathway triggered by ROS has a role in HIV-1 replication [192, 280]. Therefore, we monitored the translocation of NF-κB subunits from the cytoplasm into the nucleus after chronic treatment of BaP in U1 cells. Since the differentiated macrophages specifically express the transcriptionally active NF-κB p65-p50 heterodimer [309], and this heterodimer is specifically expressed during NF- κB-mediated HIV-1 transcription [310], we monitored the protein expression of these NF-κB subunits in both the cytoplasm and the nucleus. We observed that BaP increases the expression of both of the NF-κB proteins, especially p65, in the nuclear fraction after acute and chronic BaP exposure. Based on these findings, we suggest that ROS generated through CYP1A1-mediated metabolism of BaP could trigger the NF-κB pathway, which eventually perpetuates viral transcription. Furthermore, we confirmed the involvement of the NF-κB pathway in BaP-induced HIV-1 replication by treating BaP-exposed U1 cells with NF-κB inhibitors such as IKK-16 and SC-514. Both of the compounds inhibit NF-κB activity by selectively acting on IKK-β, an isoform of IKK protein [311, 312]. In addition, SC-514 decreases the import of p65 into the nucleus and expedites the export of p65 from the nucleus, as well as inhibiting the phosphorylation and transactivation of p65 [313]. Treatment of IKK-16 or SC-514 significantly reduced HIV-1 replication in BaP-exposed U1 cells, which provides strong evidence that the viral replication occurred via the NF-κB signaling pathway. To further verify our results, we monitored the translocation of NF-κB p65, the major DNA-binding subunit of the NF-κB protein into the nucleus, after treating the cells with the NF-κB inhibitors, IKK-16 and SC-514 and after siRNA silencing of CYP1A1. The knock-down of CYP1A1 in BaP-treated cells reduced the translocation of the NF-κB p65 unit into the nucleus, which shows an association of CYP1A1 with NF-κB-mediated viral replication. As expected, the expression of the NF-κB p65 protein was also observed to be lower in the nucleus of BaP-treated cells, after treatment with IKK-16 and SC-514, which further confirms that BaP-mediated HIV-1 replication occurs via the NF-κB signaling pathway. Our findings reconfirm the role of ROS in the activation of NF-κB and its subsequent impact on HIV-1 gene transcription [147, 314].

In addition to the ROS generated via the CYP-mediated metabolism of BaP, there are other factors such as HIV-1 proteins whose contribution to ROS production cannot be ruled out. HIV-1 proteins such as tat, gp120, Nef, and Vpr are known to cause oxidative stress in the infected cells [150, 151, 286, 315, 316]. These HIV-1 proteins generate ROS via different mechanisms: tat via upregulation of spermine oxidases [317] or by activating the NADPH oxidase pathways [318]; gp120 via upregulation of CYP2E1, NADPH oxidases [286], and proline oxidase [319]; Vpr via interaction with adenine nucleotide translocator or NADPH oxidases 70; and Nef via direct interaction with NADPH oxidases [320]. Moreover, some of these viral proteins, such as tat and nef, are also known to enhance HIV-1 replication by interacting directly or indirectly on the LTR of the viral DNA [321].

Conclusion

In conclusion, the present study suggests NF-κB activation through ROS generated via CYP1A1-mediated activation/metabolism of BaP as a novel pathway to explain smoking-mediated HIV-1 replication in monocytes-derived macrophages. This study has clinical relevance because the outcomes obtained from this study provide potential targets such as CYPs, oxidative stress, and NF-κB signaling pathways, for developing novel interventions to improve treatment strategies for HIV-1-infected smokers. We have demonstrated that antioxidants such as vitamin C and E, resveratrol, and pinostilbene, and CYP1A1 inhibitors namely ellipticine are capable of neutralizing the oxidative stress induced by BaP and subsequent viral load. These antioxidants and CYP1A1 inhibitors, which are chemodietary agents, have the potential to effectively control the viral replication in HIV-1-infected individuals who smoke tobacco. Furthermore, reduction of the viral load in these cells by targeting CYPs and the oxidative stress pathway may be beneficial in treating HIV-1-infected CNS cells.

CHAPTER 6. EXOSOMES FROM HUMAN PAPILLOMA VIRUS-INFECTED CERVICAL CANCER CELLS ENHANCE HIV-1 REPLICATION IN MACROPHAGES

Introduction

HIV-1-infected women have a high prevalence of human papilloma virus (HPV), particularly type 16 and 18 infection that causes cervical cancer [322, 323]. Epidemiological data suggests that the incidence of cervical cancer in HIV-1-infected women is approximately 5- to 10-times higher compared to the uninfected women [324]. Cervical cancer has also been categorized into AIDS-defining cancer because of its specific higher prevalence in HIV-1-infected patients [325]. HIV-1 increases HPV infectivity by disrupting the tight junctions surrounding the epithelial cells. HIV-1 further enhances the ability of HPV to develop precancerous lesions and ultimately cervical cancer by promoting immunosuppression and upregulation of HPV oncogene [324]. However, whether HPV infection can have a reciprocal effect on HIV-1 pathogenesis is largely unknown. Therefore, there is a need to examine the biological interactions among HPV and HIV-1 to find a better preventive and treatment strategies to reduce HIV-1 pathogenesis in HIV-1/HPV coinfected patients

Evidences from previous reports show that cervical cancer cells constantly undergo oxidative stress [210, 326, 327]. The clinical samples from the cervical cancer patients have revealed higher ROS level, higher oxidative damage, and lower level of antioxidants, compared to the samples from healthy individuals [328-330]. In vitro experiments have also suggested a high level of ROS, profound downregulation in the genes associated with antioxidant proteins such as superoxide dismutase 1 (SOD1), SOD2, SOD3, peroxiredoxin 1 (PRDX1), PRDX2, glutathione S-synthetase (GSS), and glutathione peroxidase 6 (GPX6), and high presence of Poly [ADP-ribose] polymerase 1 (PARP1), a marker of oxidative stress-induced DNA damage in cervical carcinoma-derived Caski cells [210]. HPV oncoproteins E6 and E7 stimulate the degradation of tumor-suppressor p53 protein [331], causing uncontrolled cell growth. p53 protein, which primarily has a function in apoptosis and genomic stability, is also reported to have an antioxidant role [332]. Downregulation of p53 suppresses its antioxidant potential and renders the cells vulnerable to oxidative damage. Furthermore, the expression of HPV E6 and E7 oncoproteins alone is sufficient to cause oxidative stress. Marullo et al. (2015) have demonstrated that HPV E6 and E7 proteins generate chronic oxidative response in the host cells via NADPH oxidase 2 (NOX2) activation [134]. E6 expression also downregulates SOD2 and glutathione peroxidase [134]. The suppression of antioxidant activity of these antioxidants could be another mechanism by which HPV generates ROS in the host cells.

Similar to other cells, HPV-infected cervical cells also secrete small nanosized extracellular vesicles (30-150 nm in size) called exosomes [333, 334]. Exosomes originate from the invagination of the lumen of the early endosomes. As the endosomes reach the later phase of their development, these invaginations bud off forming hundreds

of intraluminal vesicles. These vesicles are released into the extracellular matrix of the cells through fusion into the cell membrane [335]. Exosomes carry wide variety of cellular proteins, lipids, mRNA, miRNA, and DNA from their originating cells to other cells via blood circulation. The role of exosomes in intracellular communication and transport has been well supported by many studies [336]. In HPV-infected cells, the virus hijacks the host exosomal system, thereby modulating both the contents and amount of exosomes released from HPV-infected cervical cells [334, 337]. The exosomes from the HPV-infected cervical cells are known to transport various cellular factors to the recipient cells [337, 338]. It is possible that HPV-infected cells may be releasing oxidative stress factors into exosomes and transferring them to other cells via blood stream.

Oxidative stress is known to enhance the frequency of integration and replication of HPV genome [327, 339, 340]. ROS enhance HPV DNA integration by making a space for the viral genome to integrate into the host DNA through DNA damage in the host cells [339]. Oxidative DNA damage and the HPV oncoproteins E6 and E7 enhance the viral amplification by activating the viral transcriptional and replication proteins, E1 and E2 [327]. Oxidative stress-induced viral replication has also been observed in HIV-1 infection [149, 154]. We recently demonstrated that oxidative stress generated via CYP-mediated metabolism of benzo(a)pyrene (BaP) triggered HIV-1 replication in monocytes and macrophages [341]. We showed that ROS thus generated, activated the nuclear transcription factor NF-κB, thereby inducing transcription of the HIV-1. In the current study, we have introduced exosomes as a mediator for intracellular communication between HPV-infected cervical cancer cells and macrophages latently infected with HIV-1. Our findings demonstrate that cervical cancer cells release exosomes containing oxidative stress factors into HIV-1-infected macrophages, where they induce HIV-1 replication.

Materials and Methods

Cell Culture and Treatment

Caski cells. Caski cells are human cervical cancer cell lines containing about 600 copies of HPV-16 genome per cell as well as sequences related to HPV-18 (ATCC). We cultured the Caski cells in Roswell Park Memorial Institute (RPMI) 1640 media containing 10% Fetal bovine serum (FBS) and penicillin. We seeded about 2.5 million cells in a T-75 flask, cultured them for 3 days and collected the culture supernatant on the 4th day. For exosome isolation, we cultured the Caski cells in an RPMI media containing 10% exosome-free FBS and penicillin.

U1 cells. We cultured U1 cells as described in Chapter 5 and treated them with CCS and CCS-Exo for 3-4 days.

Exosome Isolation and Characterization

We isolated exosomes from Caski cell culture media using ultracentrifugation method and Invitrogen-Total Exosome Isolation (from cell culture media) kit (Life Technologies, NY). For ultracentrifugation method, we first centrifuged at a lower speed 300g followed by 2,000g for 10 minutes each, which removed live cells and dead cells respectively. Further we centrifuged at 10,000g for 30 minutes to remove cellular debris and microvesicles. Finally, we obtained exosomal pellet after centrifugation at 100,000-200,000g for 70 minutes [342]. We followed the manufacturer's protocol to isolate exosomes using Invitrogen-Total Exosome Isolation kit. The cell culture supernatant was centrifuged at 2000g for 30 minutes to remove any cellular debris present. Total exosome isolation reagent was added to the cell culture supernatant in a 1:2 ratio and the mixture was incubated overnight at 2°–8 °C. Next day, the mixture was centrifuged at 10,000g, 2°–8 °C for 1 hour, to obtain the exosomal pellets. Although ultracentrifugation method yields exosomes of high purity, it is tedious and consumes >10-times media. The exosomes we obtained from Invitrogen-Total Exosome Isolation kit had purity almost equivalent to that obtained from the ultracentrifugation and the method takes less effort and reagent. Therefore, we used Invitrogen-Total Exosome Isolation kit for isolating exosomes for all our experiments. Western blotting was performed using antibodies against specific exosomal markers such as CD63 and CD81 to confirm the identity of exosomes. The exosomes were further characterized by measuring their size using zeta sizer as described previously.

Viral Load

We collected the supernatant from U1 cells treated with Caski cell supernatant or Caski cell-derived exosomes. HIV-1 viral p24 antigen level was measured in the U1 cell culture supernatant to access the viral load, using HIV-1 p24 Antigen ELISA kit (Zeptometrix Corporation, Buffalo, NY). We followed the manufacturer's protocol to perform the ELISA. The principle behind the p24 ELISA assay has been explained in Chapter 5.

Measurement of ROS

ROS was measured in U1 cells after CCS or CCS-Exo treatment. ROS was measured in the treated cells using the method described in Chapter 4. We used NovoCyte flow cytometer (ACEA, Biosciences Inc., San Diego, CA) to measure ROS and NovoExpress software to analyze the data. The background fluorescence signal due to unlabeled cells was reduced and only the live healthy patch of cells was gated to calculate the mean fluorescence intensity of the dye.

Total Antioxidant Capacity

We measured antioxidant capacity of the CCS or CCS-exo treated U1 cells using Total Antioxidant Capacity Assay Kit (Bio Vision, Milpitas, CA). The assay was performed according to the manufacturer's protocol and as described previously [211].

Cytotoxicity

We used Pierce TM LDH cytotoxicity assay kit (Thermo Scientific, Rockford, IL) to measure the cytotoxicity in U1 cells after the exposure of Caski cell exosomes. We followed the manufacturer's protocol to conduct the assay. We performed the assay on culture supernatant obtained after the treatment. Dead cells release LDH enzymes from the cytosol into the culture supernatant through their damaged plasma membrane. The measure of LDH in the culture supernatant is proportional to the cytotoxicity suffered by the cells. The LDH kit is comprised of LDH reaction mixture containing lactate, NAD^+, diaphrose, and terazolium salt. A catalytic reaction between the LDH in the medium and the reaction mixture generates a red color product, formazan, whose absorbance at 490 nm is directly proportional to the amount of LDH.

Apoptotic DNA Damage

We observed the DNA damage of the CCS or CCS-Exo treated U1 cells under fluorescent microscope using the Apoptag® Iso Dual Fluorescence Apoptosis Detection kit (Millipore Sigma, Burlington, Massachusetts). We followed the manufacturer's protocol to perform the assay.

RNA and Protein Isolation

We used the method described in Chapter 5 to isolate RNA and protein from the cells.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTPCR)

We used RTPCR to calculate the relative mRNA fold expression level of CYPs and AOEs in U1 cells after treatment of Caski supernatant/Caski-derived exosomes. The mRNA expression was measured following the method described as earlier in Chapter 4. We used the following specific TaqMan TM probes (CYP1A1 (Hs01054794_m1), CYP2A6 (Hs00430021_m1), SOD1 (Hs00533490_m1), SOD2 (Hs00167309_m1), catalase (Hs00156308_m1), and PRDX6 (Hs00705355_s1)) and GAPDH as an endogenous control.

Western Blotting

We used Western blotting to identify the presence of CYPs, antioxidants, and HPV proteins in Caski-derived exosomes and to calculate the relative protein fold expression level of CYPs and AOEs in U1 cells upon treatment with Caski supernatant/Caski-derived exosomes. We performed western blotting as described in Chapter 4. We used the following primary antibodies: GAPDH Rabbit Mab, 1:2000 dilution, Cell Signaling Technology, Danvers, MA; CYP1A1 rabbit Mab, 1:200 dilution; CYP2A6 Mouse Mab, 1:200 dilution, Abcam, Cambridge, MA; SOD1 Mouse Mab, 1:1500 dilution; SOD2 Mouse Mab, 1:500 dilution; Catalase Mouse Mab, 1:1200 dilution, Santa Cruz Biotechnology. Inc., Dallas, TX; PRDX6 Rabbit Mab, 1:500 dilution, LifeSpan Biosciences, Inc., Seattle, WA; CD63, Rabbit Pab, 1:200 dilution, Proteintech Group, Inc Rosemont, IL). GAPDH and CD63 were used as loading controls for cellular and exosomal proteins, respectively.

Exosome Uptake

The CCS-Exo were labelled with GFP protein using Exo-GlowTM Exosome Labeling Kits (System Biosciences, CA) and as described previously [211]. Exosomal pellet containing ~ 42 µg of protein in 500 µl of 1X PBS was incubated with 50 µl of 10X Exo-Green dye at 37ºC for 30 minutes. The labelling reaction was stopped by adding 100 µl of ExoQuick-TC, provided along with the kit. The labelled CCS-Exo were exposed to U1 cells for 6 hours and their uptake was monitored under fluorescent microscope and flow cytometry.

Statistical Analysis

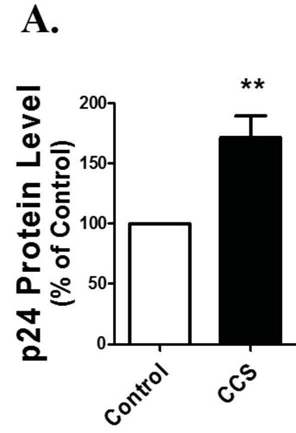
Statistical analyses were performed using Graphpad Prism 7.0 (La Jolla, CA). All data are presented as Mean ± SEM of 3-5 independent experiments. Student's t-test or one-way ANOVA were used to calculate the statistical differences between the control and the treated groups, where appropriate. Significant difference was considered at $p<0.05$.

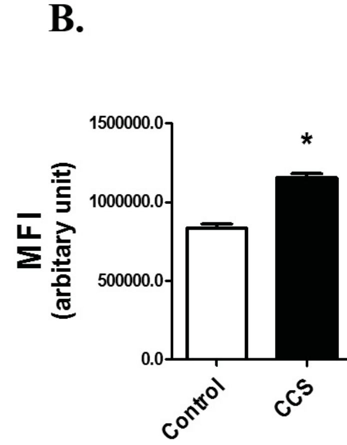
Results

Cell Culture Supernatant from Caski Cells Enhances Oxidative Stress and Viral Load in U1 Cells

The exposure of CCS for 4 days significantly (p-value = 0.0084, n=5) increased the viral load in U1 cells by approximately 1.7-fold compared to the control (untreated cells) (**Figure 6-1A**). ROS acts as a secondary messenger for inducing HIV-1 replication in cells latently infected with HIV-1 [281]. To determine whether the viral replication is

Figure 6-1. Caski cell culture supernatant (CCS) increases HIV-1 replication and oxidative stress in U1 macrophages.
(A). U1 macrophages were treated with 250 µl of CCS every 24 hours for 4 days. p24 ELISA was performed on the supernatant obtained from the treatment to measure the viral load. To minimize high standard deviation of the mean due to variability in absorbance values in different experiments, we converted the control absorbance values to 100% and normalized the values of treated groups to % of the control. (B). ROS was measured in U1 macrophages after the 4 days exposure of CCS. The treated cells were incubated with H_2DCFDA, the fluorescence of which was monitored at maximum excitation and emission spectra of 495 nm and 529 nm respectively, using flow cytometer. (C). Figure C shows the graphical representation of ROS increase in CCS-treated U1 cells (red graph) versus control cells (grey graph). X-axis represents mean fluorescence intensity (MFI), showing ROS level. (D). Total antioxidant capacity of the cells were measured in CCS-treated cells using Total antioxidant capacity assay kit. The values on Y-axis represents the total amount of reduced Cu^+ in nmol/µl, which quantitatively gives the measure of antioxidant capacity of the cells. (E). Cytotoxicity after CCS exposure was measured using Pierce TM LDH cytotoxicity assay kit. The values on Y-axis represent the absorbance values of formazan dye at 490 nm, which gives the measure of cytotoxicity. The mean absorbance is obtained by subtracting the background absorbance at 680 nm. All the data were obtained from the mean of at least three independent experiments with the error bars representing standard error of mean. Significant difference was considered at $p<0.05$. *, **, *** represents $p<0.05$, $p<0.005$ and $p<0.0005$ respectively. (F). Apoptotic DNA damage was examined using Apoptag® Iso Dual Fluorescence Apoptosis Detection kit. DAPI, FAM and CR590 dyes were used to stain the nucleus, DNase II and I-type of DNA breaks respectively.


A.
p24 Protein Level
(% of Control)
**
200
150
100
50
0
Control
CCS


B.
MFI
(arbitary unit)
*
1500000.0
1000000.0
500000.0
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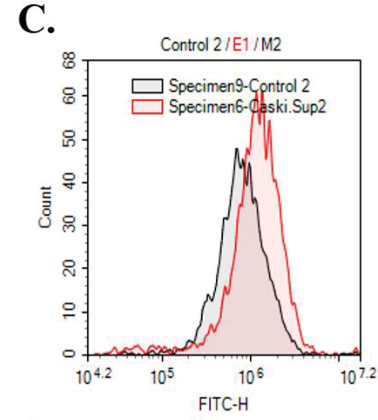
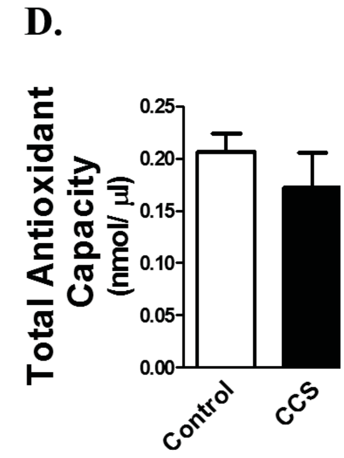
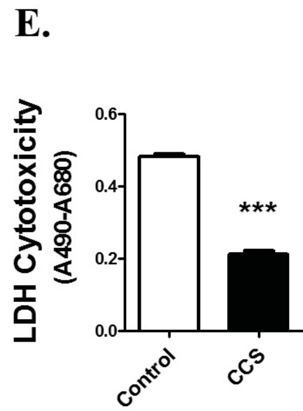
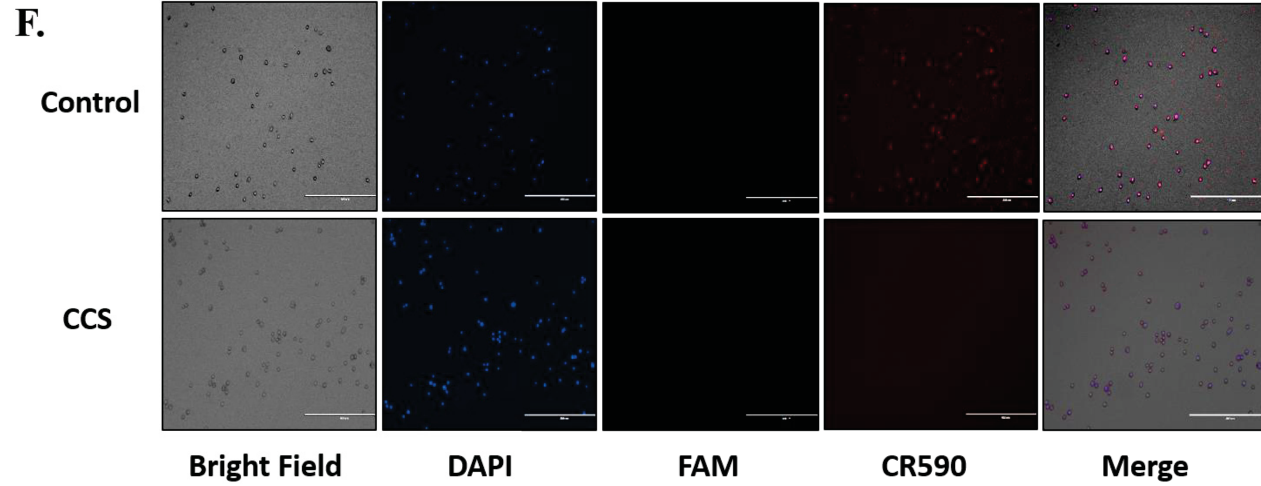
Control
CCS


C.
Control 2 / E1 / M2
Specimen9-Control 2
Specimen6-Caski.Sup2
Count
FITC-H


D.
Total Antioxidant
Capacity
(nmol/ µl)
0.25
0.20
0.15
0.10
0.05
0.00
Control
CCS


E.
LDH Cytotoxicity
(A490-A680)
***
0.6
0.4
0.2
0.0
Control
CCS


F.
Control
CCS
Bright Field
DAPI
FAM
CR590
Merge

associated with oxidative stress, we measured the levels of ROS in the CCS-treated U1 cells. Our results showed that 4 days exposure of CCS induces ROS by ~1.25-fold (p-value = 0.0169, n=3) in U1 cells (**Figure 6-1B**). **Figure 6-1C** shows the graphical representation of the ROS measurements shown in **Figure 6-1B**. The graph on **Figure 6-1C** is also consistent with the result in **Figure 6-1B**. During the oxidative stress, cells employ antioxidant enzymes and proteins to neutralize the excess ROS, which may eventually wear away the total antioxidant capacity of the cells. Therefore, we monitored antioxidant capacity of U1 cells after 4 days of CCS treatment using total antioxidant capacity (TAC) assay kit. The TAC assay kit comprises of Cu^{2+}, which is reduced by antioxidant molecules/proteins in the cell supernatant into Cu^{+}. Although not significant, the data presented in **Figure 6-1D** shows a trend in decrease in the total antioxidant capacity of the CCS-treated cells compared to the control.

Next, we were interested to examine whether the excessive ROS induces cellular toxicity and DNA damage after CCS treatment. The cytotoxicity depicted in **Figure 6-1E** was performed using LDH assay. Interestingly, the 4 days treatment of CCS significantly (p-value <0.0001, n=6) decreased the cytotoxicity in U1 cells by ~ 60% (**Figure 6-1E**). To further confirm this result, we monitored apoptotic DNA fragmentation in the CSC-treated U1 cells. **Figure 6-1F** shows the fluorescent images of control and CCS-treated U1 cells labelled with DAPI, FAM, and CR590 dyes, which were used to stain the nucleus, DNA fragmentation with DNase Type II, and Type I ends, respectively. DNA fragmentation with Type I ends, commonly observed in most cells, occurs within the nucleus, which is an indicative of apoptosis by self-driven cell disassembly. In contrast, DNA fragmentation with Type II ends occurs in lysosomes of the phagocytes, where they eliminate the remains of apoptotic bodies [343]. The merged panel of **Figure 6-1F** shows reduced fluorescence intensity for CR590 in CCS-treated U1 cells compared to the control, which suggests that treatment of CCS protects U1 cells from DNase I type of DNA break. We did not observe any signal with FAM dye in both the control and treated cells, which suggests the absence of DNase II type of DNA break.

Cell Culture Supernatant from Caski Cells Induces CYP Expression in U1 Cells

CYP enzymes are known to generate ROS as byproducts, while metabolizing various endogenous and exogenous substances within the cells. Therefore, we monitored the mRNA and protein expression of CYPs (CYP 1A1 and 2A6) in U1 cells after 4 days of exposure of CCS. We particularly examined the expression of CYP 1A1 and 2A6, because these CYPs are significantly expressed in U1 cells. The 4 days exposure of CCS significantly increased the expression of CYP1A1 at the mRNA level (p-value = 0.0367, n =3) (**Figure 6-2A**) and but not at the protein level (**Figure 6-2B**). The treatment also increased the protein expression of CYP2A6 in U1 cells significantly (p- value = 0.0647, n = 3) (**Figure 6-2D**). However, there was no significant change in the expression of CYP2A6 at the mRNA level.

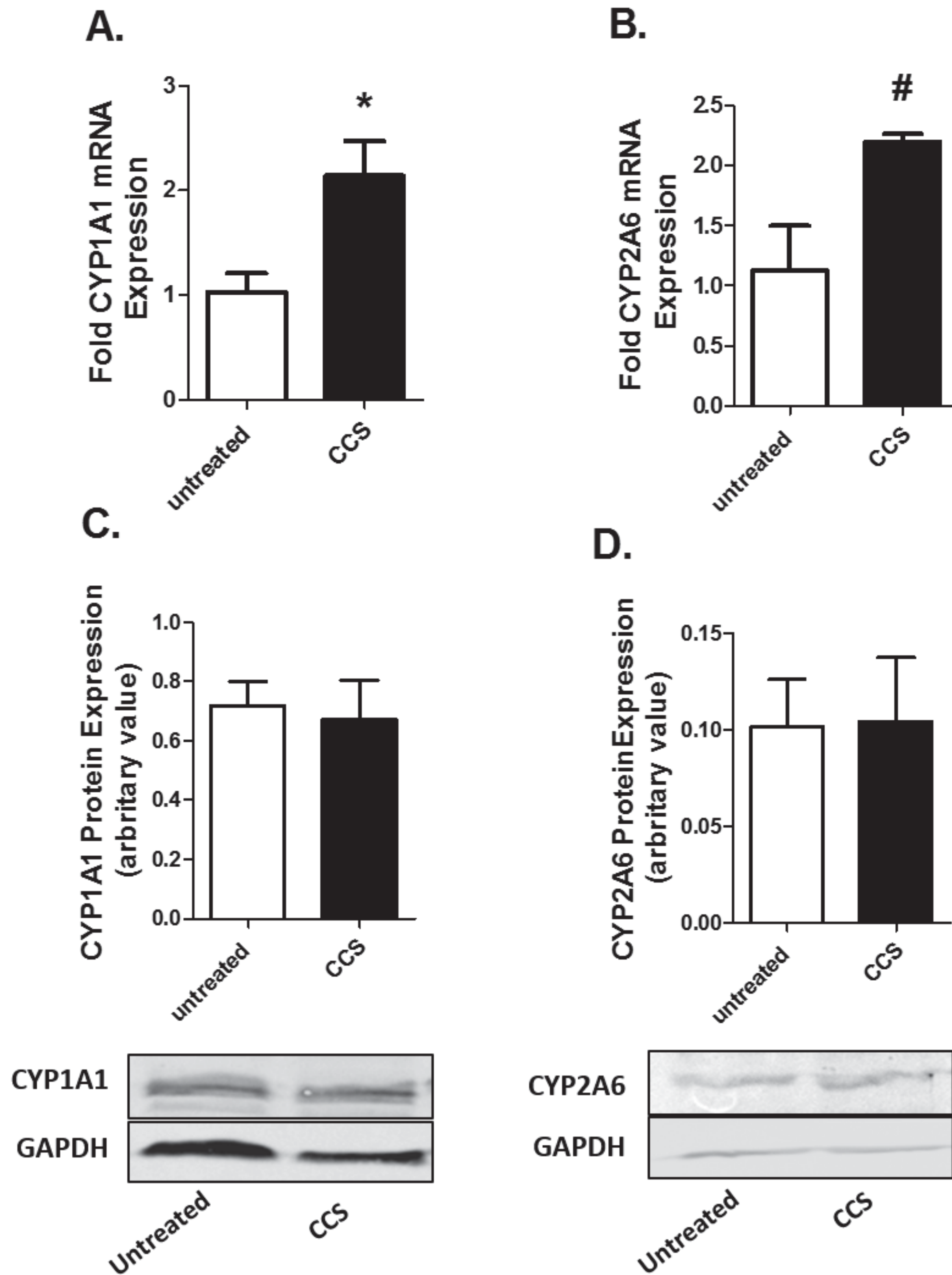


Figure 6-2. Caski cell culture supernatant (CCS) enhances CYP expression in U1 macrophages.

U1 macrophages were treated with 250 µl of CCS every 24 hours for 4 days. Expression of CYP1A1 and CYP2A6 at mRNA (**A** and **B**) and protein (**C** and **D**) level were monitored in the treated cells. Data were obtained from the mean of at least three independent experiments with the error bars representing standard error of mean. Significant difference was considered at $p<0.05$, which is represented by *.

Cell Culture Supernatant from Caski Cells Have No Significant Effect on AOEs Expression in U1 Cells

AOE expression is expected to rise at the time of oxidative stress as a cellular response to combat the resulting oxidative damage. Therefore, we monitored the mRNA and protein expressions of major AOEs (SOD1, SOD2, catalase, and PRDX6) in U1 cells after 4 days exposure of CCS. The 4 days exposure of CCS did not have any significant effect on the expression of the AOEs at both the mRNA as well as protein level (**Figure 6-3**).

U1 Cells Uptake CCS-derived Exosomes Containing Oxidative Stress Factors

Since exosomes are one of the major factors that are secreted in the media from a variety of cells and are gaining attention in intercellular communication, we isolated and characterized exosomes secreted from the Caski cells. Recently, we reported isolation and characterizations of exosomes from the media of monocytic cells [211], and in this study we essentially used similar techniques. We characterized these exosomes with CD63 and CD81 (commonly used exosomal marker) in the CCS-derived exosomes (CCS-Exo) (**Figure 6-4A**) and size (~100 nm) (data not shown). Our western blot images also showed the expression of CYP enzymes (CYP 1A1, 1B1 and 2A6), SOD1 and HPV 16 oncoprotein E6 in CCS-derived exosomes (**Figure 6-4A**). To show that U1 cells uptake these exosomes, we first labelled the exosomes with GFP and monitored its uptake by U1 cells under fluorescent microscope and flow cytometer after 6 hours. The merged panel of **Figure 6-4B** shows higher GFP fluorescence in CCS-Exo treated U1 cells, compared to the untreated cells, indicating the exosome uptake by U1 cells. We further verified this result by measuring the GFP fluorescence using flow cytometer. In **Figure 6-4C**, we can see that the red graph (CCS-Exo treated U1 cells) shifts towards the far right compared to the grey graph (untreated U1 cells), indicating higher mean fluorescence intensity for the former and hence the uptake of CCS-Exo by U1 cells. Our observation is consistent with our previous study, where we observed uptake of U937 monocytes-derived exosomes by U1 cells in 6 hours [211].

CCS-derived Exosomes Enhance Oxidative Stress and Viral Load in U1 Cells

To examine if the exosomes in the CCS were responsible for the increase in viral load in U1 cells, we treated the CCS-Exo to the U1 cells. U1 cells were treated with exosomes isolated from 1ml of CCS (equivalent to ~ 42 µg protein) and cultured for 4 days. Exposure of the CCS-Exo for 4 days significantly (p-value = 0.0007, n = 6) increased the viral load by approximately 1.3-fold (**Figure 6-5A**). CCS-Exo treatment significantly (p-value = 0.0236, n = 3) increased the ROS level in U1 cells (**Figure 6-5B-C**). Although the data was not statistically significant, we observed a decreasing trend in the total antioxidant capacity of the U1 cells after CCS-Exo treatment (**Figure 6-5D**). The increase in ROS and decrease in total antioxidant capacity both are indicative of

Figure 6-3. Caski cell culture supernatant (CCS) alters antioxidant enzyme (AOE) expression in U1 macrophages.

U1 macrophages were treated with 250 µl of CCS every 24 hours for 4 days. Expression of SOD1, SOD2, catalase and PRDX6 were monitored at mRNA (**A**-**D**) and protein (**E**-**H**) levels in the treated cells. Data were obtained from the mean of at least three independent experiments with the error bars representing standard error of mean. Significant difference was considered at $p<0.05$, which is represented by *

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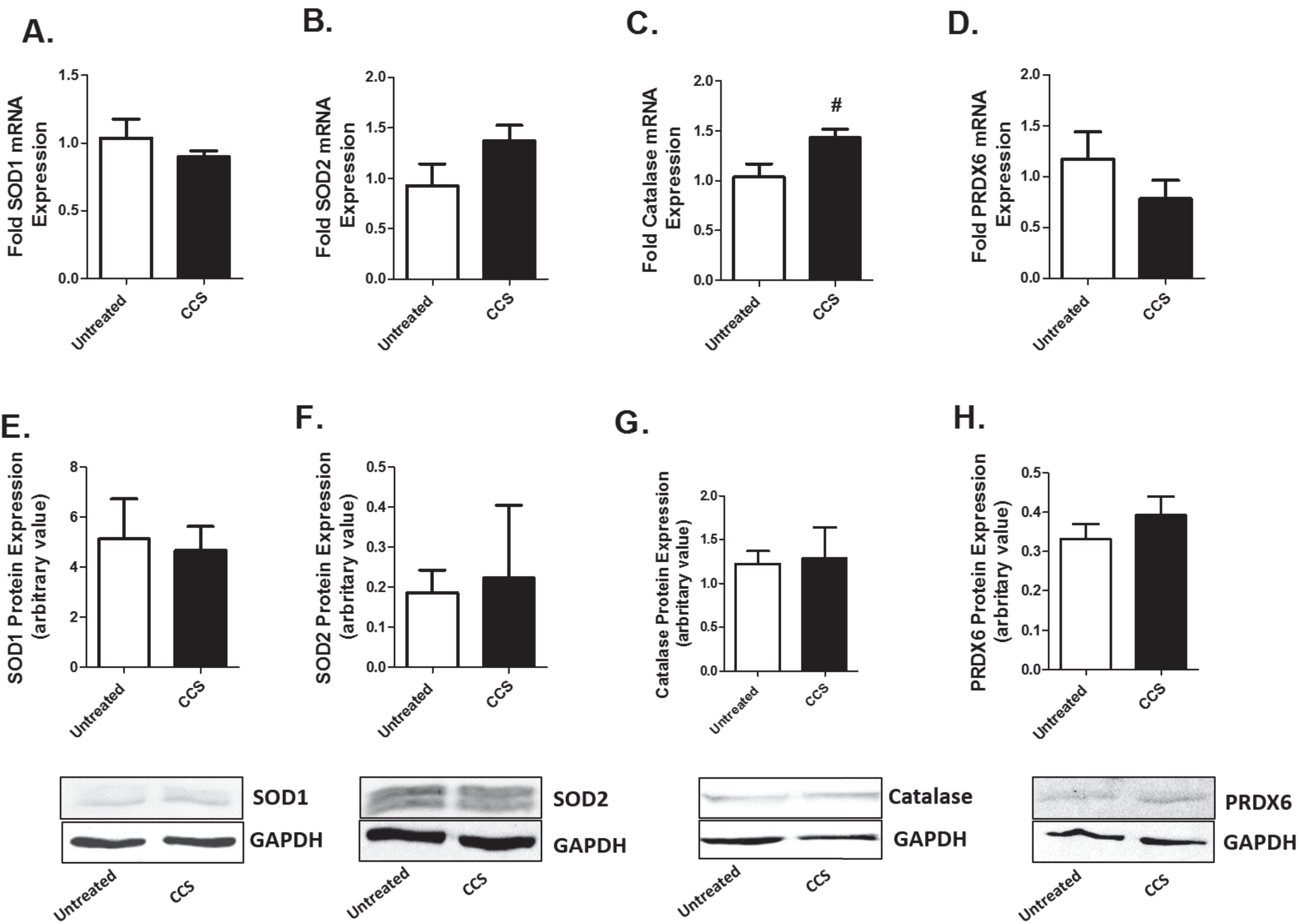

A.
B.
C.
D.
Fold SOD1 mRNA Expression
Fold SOD2 mRNA Expression
Fold Catalase mRNA Expression
Fold PRDX6 mRNA Expression
#
Untreated
CCS
E.
F.
G.
H.
SOD1 Protein Expression (arbitrary value)
SOD2 Protein Expression (arbitrary value)
Catalase Protein Expression (arbitrary value)
PRDX6 Protein Expression (arbitrary value)
SOD1
SOD2
Catalase
PRDX6
GAPDH

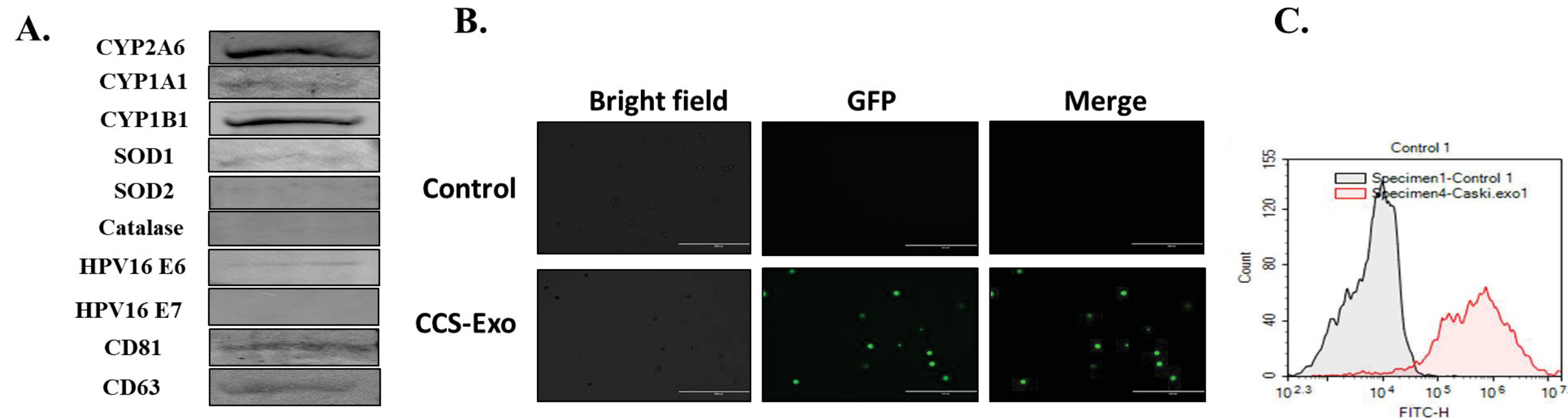
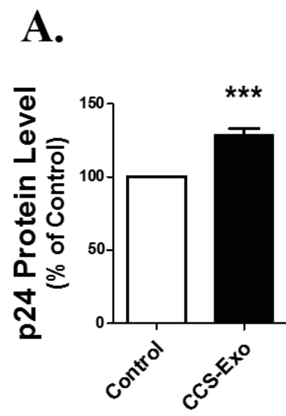
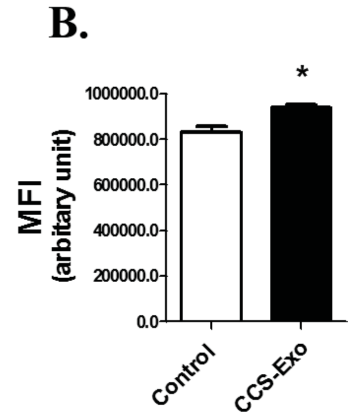


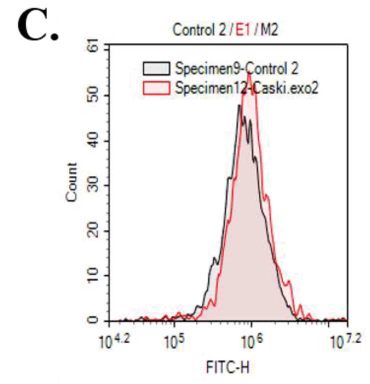
Figure 6-4. U1 macrophages uptake CCS-derived exosomes (CCS-Exo) that contain oxidative stress factors. (**A**). The exosomal protein obtained from CCS-derived exosomes (CCS-Exo) were examined for the expression of exosomal marker, CD63, CYPs (1A1, 1B1, 2A6, 3A4) and AOEs (SOD1, SOD2, catalase, PRDX6) and HPV 16 oncoproteins (E6). Data not shown for the proteins which were absent. (**B**). To monitor the exosomes uptake by U1 cells, we labeled the CCS-Exo with GFP and treated the labelled exosomes to U1 cells. After 6 hours of incubation, we monitored the fluorescent intensity of GFP under fluorescent microscope. (**C**). The GFP fluorescence was further quantified using flow cytometry.

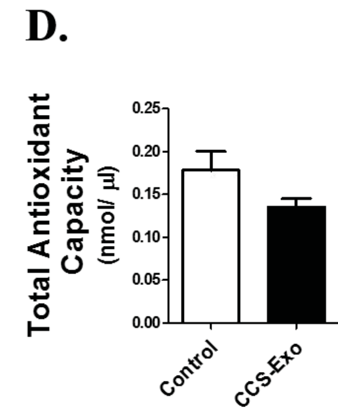
Figure 6-5. CCS-derived exosomes (CCS-Exo) increases HIV-1 replication and oxidative stress in U1 macrophages.

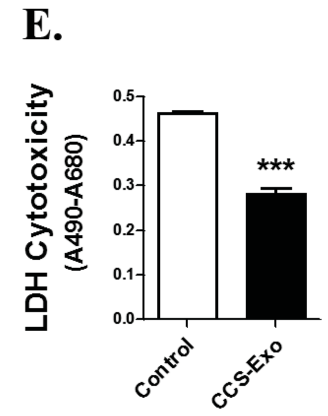
(**A**). U1 macrophages were exposed with exosomes derived from 1 ml of CCS (~ 42 µg of exosomal protein) for 4 days. p24 ELISA was performed on the supernatant obtained from the treatment to measure the viral load. To minimize high standard deviation of the mean due to variability in absorbance values from different experiments, we converted the control absorbance values to 100 % and normalized the values of treated groups to % of the control. (**B**). ROS was measured in U1 macrophages after the 4 days exposure of CCS-Exo. The treated cells were incubated with H_2DCFDA, the fluorescence of which was monitored at maximum excitation and emission spectra of 495 nm and 529 nm respectively, using flow cytometer. (**C**). Figure C shows the graphical representation of ROS increase in CCS-Exo-treated U1 cells (red graph) versus control cells (grey graph). X-axis represents mean fluorescence intensity (MFI), showing ROS level. (**D**). Total antioxidant capacity of the cells was measured in CCS-treated cells using Total antioxidant capacity assay kit. The values on Y-axis represents the total amount of reduced Cu^+ in nmol/µl, which quantitatively gives the measure of antioxidant capacity of the cells. (**E**). Cytotoxicity after CCS exposure was measured using Pierce TM LDH cytotoxicity assay kit. The values on Y-axis represent the absorbance values of formazan dye at 490 nm, which gives the measure of cytotoxicity. The mean absorbance is obtained by subtracting the background absorbance at 680 nm. All the data were obtained from the mean of at least three independent experiments with the error bars representing standard error of mean. Significant difference was considered at $p<0.05$. *, **, *** represents $p<0.05$, $p<0.005$ and $p<0.0005$ respectively. (**F**). Apoptotic DNA damage was examined using Apoptag® Iso Dual Fluorescence Apoptosis Detection kit. DAPI, FAM and CR590 dyes were used to stain the nucleus, DNase II and I-type of DNA breaks respectively.

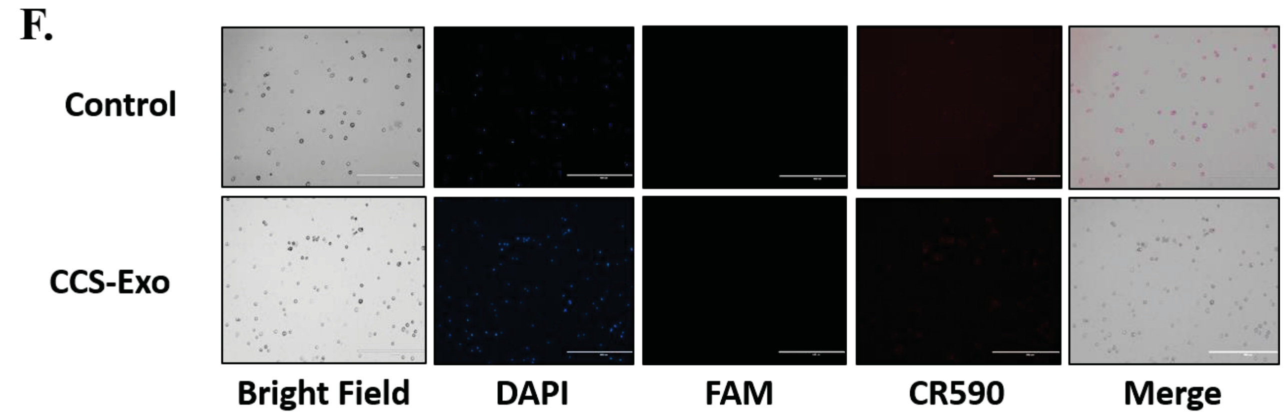

A.
p24 Protein Level
(% of Control)
***
150
100
50
0
Control
CCS-Exo


B.
MFI
(arbitary unit)
*
1000000.0
800000.0
600000.0
400000.0
200000.0
0.0
Control
CCS-Exo


C.
Control 2 / E1 / M2
Specimen9-Control 2
Specimen12-Caski.exo2
Count
FITC-H


D.
Total Antioxidant Capacity
(nmol/ µl)
0.25
0.20
0.15
0.10
0.05
0.00
Control
CCS-Exo


E.
LDH Cytotoxicity
(A490-A680)
***
0.5
0.4
0.3
0.2
0.1
0.0
Control
CCS-Exo


F.
Control
CCS-Exo
Bright Field
DAPI
FAM
CR590
Merge

oxidative stress. Next, we monitored the cytotoxicity and DNA damage in CCS- Exo treated U1 cells. Interestingly, we observed ~ 40% decrease (p-value < 0.0001, n = 6) in cytotoxicity (**Figure 6-5E**) in the treated U1 cells. Similarly, the merged panel of **Figure 6-5F** shows lower CR590 fluorescence for the treated cells than for the control, indicating lower extent of DNA damage in the treated cells. These results are in agreement with the results from CCS-treated U1 cells.

Treatment of Antioxidants, CYP-inhibitors, and Chemodietary Agents Reduce Viral Load in Caski Exosomes-treated U1 Cells

To confirm that HIV-1 replication is occurring via a CYP-mediated oxidative stress pathway, we treated the CCS-Exo exposed U1 cells with an antioxidant, resveratrol, CYP1A1 inhibitor, ellipticine, and CYP2A6 inhibitor, tryptamine. These inhibitors are relatively selective for the individual CYPs. U1 cells were exposed to exosomes isolated from 1 ml of CCS (comprising of ~ 42 μg of exosomal protein) for 4 days. To the CCS-Exo-treated U1 cells resveratrol (25 μM), ellipticine (Epi, 1 μM), and Tryptamine (Tryp, 20 μM) were added every 24 hours for 4 days. 500 μl of exosome-free media was added after each treatment to ensure the healthy growth of the cells. Treatment of resveratrol (p-value < 0.0001, n =3), ellipticine (p-value < 0.0001, n =3), or tryptamine (p-value < 0.0001, n =3) significantly reduced the viral load in CCS-Exo exposed U1 cells, suggesting the role of CYP 1A1 and 2A6, and oxidative stress on viral load increase in U1 cells (**Figure 6-6A-B**).

Chemodietary agents such as curcumin and cucurbitacin-D are known to reduce cancer progression [344-346], as well as HIV-1 replication [347, 348] through their antioxidant potential. We treated Curcumin (20 μM) and Cucurbitacin-D (0.1μM) to the CCS or CCS-Exo-treated U1 cells for 4 days, every 24 hours, with an assumption that they will combat the oxidative stress caused by CCS or CCS-Exo and hence reduce the viral replication in U1 cells. As expected, treatment of curcumin (p-value <0.0001, n=7) and cucurbitacin-D (p-value <0.001, n= 5) significantly reduced the viral load in CCS-treated cells (**Figure 6-6C**). We also observed significant decrease in viral load in CCS-Exo-treated U1 cells with curcumin (p-value <0.01, n=3) or Cucurbitacin-D (p-value <0.001, n=3) treatment (**Figure 6-6D**).

Discussion

There is a high risk and prevalence of HPV co-infection in HIV-1-infected individuals, which causes severe forms of cervical cancer. HIV-1 modulates the host cell microenvironment for HPV invasion, through destruction of tight junctions between the cells and immunosuppression [324]. However, it is unknown if there exists a cross-talk between HPV and HIV-1-infected cells. The current study highlights exosomes as a means of intracellular communication between the two cells. Here, we demonstrate for the first time that HPV-infected cervical cancer cells transfer oxidative stress factors

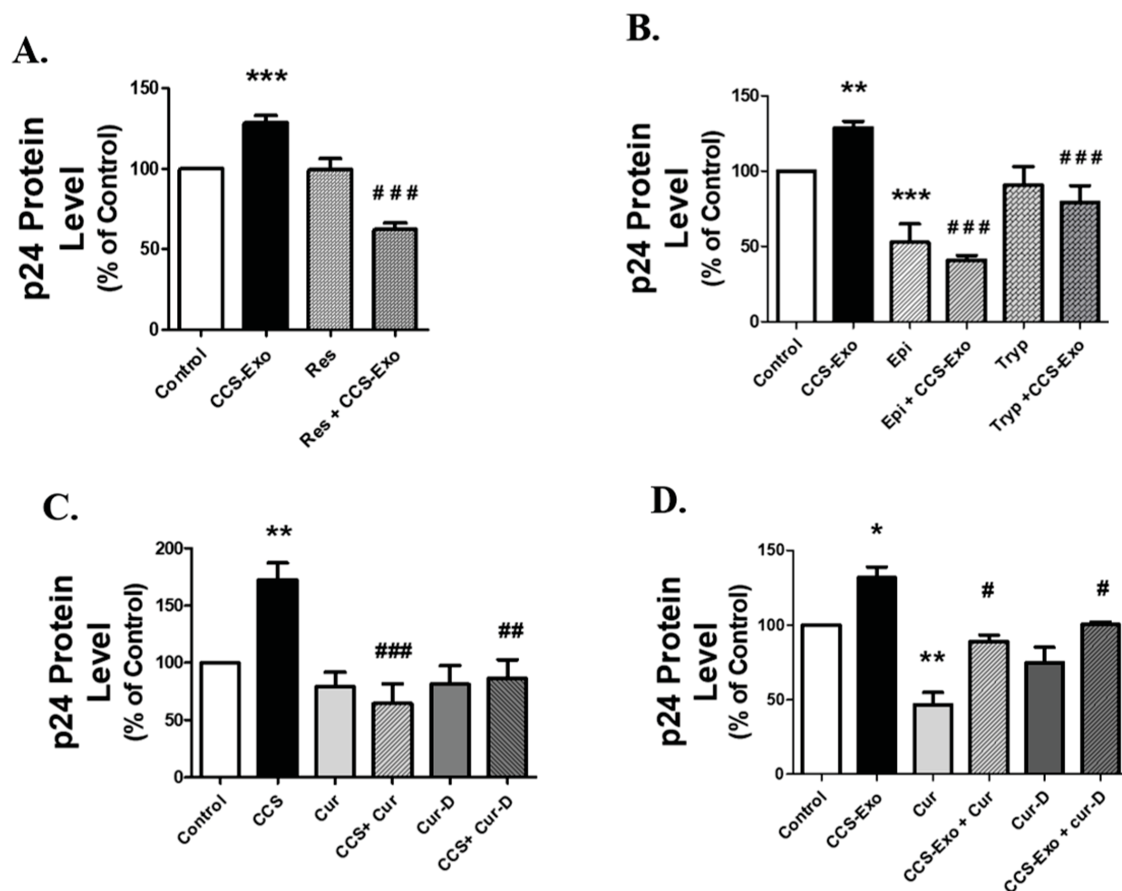


Figure 6-6. Treatment of antioxidant, CYP inhibitors, and chemodietary agents reduce CCS or CCS-derived exosomes (CCS-Exo)-induced HIV-1 replication in U1 macrophages.

To confirm the role of CYP-induced oxidative stress in CCS-Exo-treated U1 cells, we treated U1 macrophages with antioxidant, resveratrol (**A**. Res, 25 μM), CYP1A1 inhibitor, ellipticine (**B**. Epi 1 μM) and CYP2A6 inhibitor, Tryptamine (**B**. Tryp, 20 μM). We also treated the U1 cells exposed to CCS (**C**) or CCS-Exo (**D**) with curcumin (cur, 20 μM) and cucurbitacin-D (Cur-D, 0.1μM). All the data were obtained from the mean of at least three independent experiments with the error bars representing standard error of mean. Significant difference was considered at $p<0.05$. *, **, *** represents $p<0.05$, $p<0.005$ and $p<0.0005$ respectively, when compared to the control. #, ##, ### represents $p<0.05$, $p<0.005$ and $p<0.0005$ respectively, when compared CCS or CCS-Exo groups.

(such as CYPs and HPV proteins) via exosomes to the recipient HIV-1-infeceted monocytic cells to induce HIV-1 replication. The exosomal CYPs (CYP 1A1 and 2A6) exert an additive effect on the basal CYPs of the recipient cells. The cumulative CYPs promote the metabolism of endogenous substances, thereby generating massive ROS, which eventually exacerbate the HIV-1 replication in the recipient cells (**Figure 6-7**). Our study also suggests a treatment strategy for HIV-1-infected patients who are also infected with HPV by using chemodietary agents.

In the current study, we have shown the interaction between HPV and HIV-1 infected cells using Caski cells and U1 cells, which are widely used cell models for HPV and latent HIV-1 infection. To determine the interaction between the Caski cells and U1 cells, we initially exposed the U1 cells with Caski cell culture supernatant (CCS). Exposure of CCS to U1 cells significantly increased oxidative stress and HIV-1 replication in U1 cells. Initially, we made an assumption that Caski cells released oxidative stress factors into the cell culture media, which when transferred to the U1 cells, induced oxidative stress and viral replication in U1 cells. Later, we were interested to investigate if Caski cells were communicating with the U1 cells via exosomes in the CCS. Therefore, we isolated exosomes from CCS and exposed them to U1 cells. Interestingly, exposure of CCS-derived exosomes (CCS-Exo) also revealed higher viral load and ROS level.

We mostly used the exosome isolation kit method for isolating exosomes from the CCS. Initially, we tried both ultracentrifugation and exosome isolation kit method to isolate exosomes. As we did not observe a significant difference in the yield and purity of the exosomes isolated from these methods, we opted to use the kit method because it is easy to use, consumes less time and is cost-effective. The presence of specific exosome markers such as CD63 and CD81 in the exosomal pellet obtained from CCS justifies its identity as exosomes. Moreover, in our previous work we have used extensive characterization methods to confirm the identity of exosomes from cell culture media [211]. Our results from transmission electron microscope and zetasizer have shown that the extracellular vesicles we isolated have size around 100 nm, which is about the size of the exosomes; we also observed acetylcholinesterase activity in the isolated exosomes and the presence of several exosomal marker proteins such as alix, CD63, CD81 and Tsg101 in the exosomes.

ROS can activate cells latently infected with HIV-1 to produce new viruses via activation of nuclear transcription factor, NF-κB [281, 341, 349]. ROS induces translocation of cytosolic NF-κB subunits into the nucleus, where it binds to the core enhancer region of HIV-1 long terminal repeat (LTR) to induce HIV-1 transcription. ROS generated through CCS/CCS-derived exosomes could induce HIV-1 replication in the U1 cells via similar mechanism. Furthermore, redox state of the monocytes/macrophages are also reported to regulate the expression of C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4), which are major coreceptors for HIV-1 entry into monocytes/macrophages [350, 351]. ROS elevation through CCS/CCS-derived exosomes could facilitate the viral entry and hence increased the viral load within the U1 cells via overexpression of these chemokine

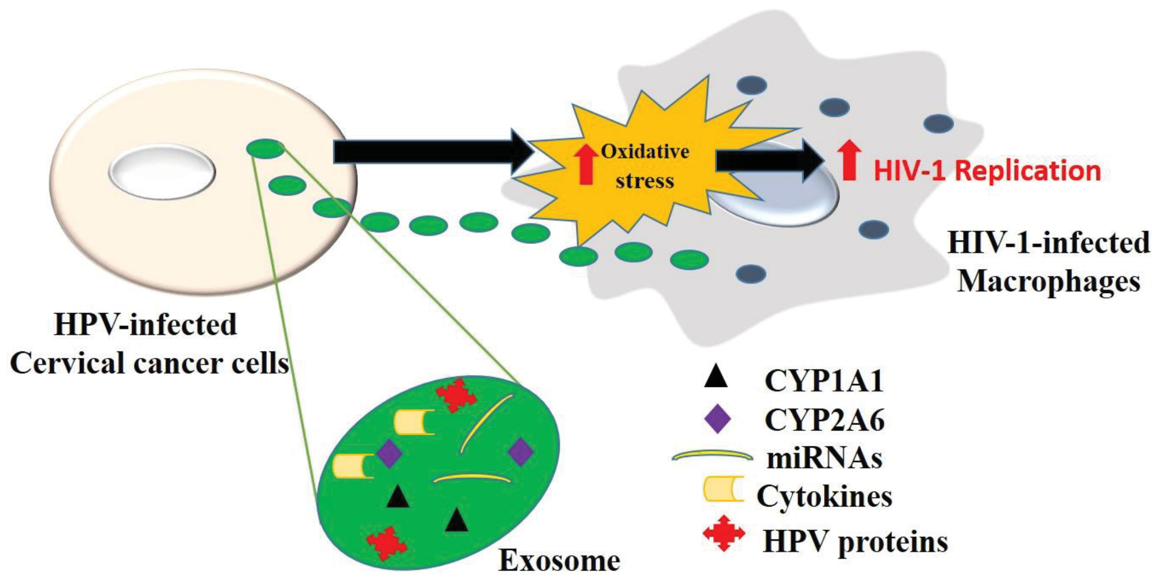


Figure 6-7. Proposed model for HPV-HIV-1 interaction via exosomes.
Exosomes from cervical cancer cells carry oxidative stress factors that exacerbates HIV-1 replication in HIV-1-infected macrophages. As a result of constant oxidative stress, cervical cancer cells package oxidative stress factors like CYP enzymes (CYP 1A1, 2A6, 1B1), miRNAs, cytokines, HPV oncoproteins etc. into exosomes and transfer them to cells, e.g. macrophages at distant sites. The exosomes then release the oxidative stress factors in recipient cells, which further enhances ROS production in the cells. The ROS thus generated contributes as a secondary messenger to trigger HIV-1 in macrophages.

receptors. Treatment of antioxidants such as glutathione, glutathione ester, and N-acetyl-L-cysteine has been reported to suppress HIV-1 expression in U1 cells, which strongly supports the involvement of oxidative stress in HIV-1 expression in macrophages [352]. Our findings are consistent with the previous reports and they further elaborate CCS/CCS-derived exosomes as a novel source of ROS for HIV-1 replication in macrophages.

We observed that exposure of CCS/CCS-derived exosome decreased the cell death and DNA damage in U1 cells, despite the oxidative stress. Under the stressed condition, the cells initially attempt to protect themselves from the insult by activating signaling pathways that promote cell survival. In case they are unable to overcome the insult, they activate death signaling pathways [353]. In the current context, treatment of CCS/CCS-derived exosome is likely to activate cell survival pathways such as antioxidant defense system, heat shock and unfolded protein response, and DNA damage repair to rescue the U1 cells from the oxidative insult [353]. Furthermore, most of the cancer cells, including cervical cancer are known to overexpress and release anti-apoptotic factors like survivin, which arrest apoptosis by inhibiting caspase activation [354, 355]. It could be possible that protective mechanism is mediated by anti-apoptotic factors like survivin in the CCS-derived exosomes. In addition, HIV-1 protein Nef, is known to protect HIV-1-infected host cells by inhibiting apoptosis signal-regulating kinase 1 (ASK1) thus preventing Fas and TNF-α-mediated apoptotic cell death [356]. HIV-1 could be promoting the cell survival in the treated U1 cells, so as to frame a favorable environment for its multiplication.

Next, we were interested in finding what oxidative stress factors are present in CCS/CCS-derived exosomes, and what mechanistic pathway they trigger to induce oxidative stress and consequently HIV-1 replication in U1 cells. Overexpression of CYPs are observed in several cancers, including cervical cancer [357] and CYPs are known to generate excessive ROS as a byproduct during the phase-I metabolism of various endogenous and exogenous substrates [304]. Taking this fact into consideration, we examined the effect of CCS in the expression of CYPs in U1 cells. We particularly examined the expression of CYP1A1 and CYP2A6 because these enzymes are expressed in monocytic cells. In addition to metabolize many endogenous compounds, CYP1A1 and CYP2A6 are the major enzymes for metabolizing polyaryl hydrocarbons and nicotine present in the cigarette smoke respectively [155, 273]. As expected, we observed significant increase in the protein expression of both the CYPs, 1A1 and 2A6 in U1 cells after CCS exposure. The concurrent upregulation of CYPs and elevation in ROS levels signified that the oxidative stress in the U1 cells could be occurring via a CYP-mediated pathway. We did not observe any change in the expression of antioxidant enzymes after CCS exposure. However, there was a decrease in the total antioxidant capacity of the cells after CCS or CCS-mediated exosome treatment.

In this study, we observed increase in the expression of CYPs at the mRNA level but not at the protein level. We have observed this kind of discrepancy in our previous studies as well [154]. As mRNA is translated into protein, it is theoretically believed that, there is a correlation between the expression of gene at the mRNA and the protein level

but in practical, it is not necessary that mRNA expression changes would reflect similar changes in the protein expression [358-360]. The reason for decrease in expression of the proteins, despite their increased mRNA expression could be due to various transcriptional and post-translational modifications, differential stability of mRNAs vs. proteins, or due to interference by different miRNAs.

Next, we verified the presence of CYPs 1A1, 2A6 and 1B1 in the CCS-derived exosomes, which further strengthens our assumption about involvement of CYP-pathway in CCS-induced oxidative stress. To further confirm the role of CYPs in oxidative stress-induced HIV-1 replication, we treated the U1 cells exposed to CCS-derived exosomes with resveratrol (an antioxidant), ellipticine (CYP1A1 inhibitor) and Tryptamine (CYP2A6 inhibitor). Treatment of these antioxidant and CYP inhibitors significantly reduced the viral load in the treated U1 cells. (role of CYP from CCS-derived exosomes using siRNA) Overall, our findings suggested that Caski cells transfer CYPs to the U1 cells via exosomes. Upon reaching the U1 cells, these exosomes release the CYPs into the cytosol where they induce oxidative stress and subsequently HIV-1 replication. Our observations are in agreement with our previous reports, where we have observed higher expression of CYPs, oxidative stress, and HIV-1 viral load in the plasma samples of HIV-1 smokers, which shows the association of CYP-mediated oxidative stress pathway in HIV-1 replication [153]. Recently, we have confirmed this association in vitro in U1 cells where we demonstrated, CYP1A1 metabolizes benzo(a)pyrene (a harmful carcinogen in cigarette smoke), causing massive production of ROS and subsequently trigger HIV-1 replication in the U1 cells [341].

Apart from CYPs, we also observed the presence of other oxidative stress-inducing factors such as HPV type16 oncoprotein E6 in the CCS-derived exosomes. HPV16 E6 and E7 oncoproteins are known to cause chronic oxidative stress in HPV-infected and uninfected cells via NOX2 activation [134]. Besides, CCS-derived exosomes may also contain various cytokines and miRNAs that may trigger HIV-1 replication in U1 macrophages. Cytokines such as, TNF-α triggers HIV-1 gene transcription through activation of NF-κB; IL-6 increases the expression of viral proteins and RT; IL-6 together with TNF-α have a synergistic effect on HIV-1 replication [189]. Micro RNA (miRNA) 34a and miRNA 181 are reported to enhance HIV-1 viral load by inhibiting the cellular restriction factors (e.g., p21, TASK, and SAMHD1), which inhibits different stages of HIV-1 life cycle [361, 362]. Since, these miRNAs are also expressed in cervical cancer cells, it is possible that they are being transported to the host cells via exosomes, where they contribute to HIV-1 replication.

Curcumin and cucurbitacin-D are shown to inhibit cancer growth in cervix by inducing apoptosis and arresting cell cycle [344-346]. In addition to the anti-cancer property, curcumin and cucurbitacin have also shown to suppress HIV-1 pathogenesis through its antioxidant potential [347, 348]. Curcumin is shown to reduce HIV-1 transcription by inhibiting the HIV-1 protein, Tat-mediated LTR promotor transactivation [347]. Considering their potential antioxidant and antiviral properties, we examined the preventive effect of these agents to HIV-1 replication induced by CCS/CCS-derived exosomes. As expected, treatment of both the compounds significantly reduced HIV-1

viral load induced by CCS or CCS-derived exosomes in U1 cells. Curcumin and cucurbitacin-D are known to scavenge hydroxyl and superoxide ions at higher concentration [363]. It is therefore possible that these compounds are inhibiting the viral replication by lowering the ROS level. Furthermore, the use of curcumin and Cucurbitacin-D as antioxidants to block the viral replication is clinically safer as these natural antioxidants are less carcinogenic compared to synthetic antioxidants, such as butylatedhydroxy toluene (BHT) and butylatedhydroxy anisole (BHA). Furthermore, the use of these chemodietary agents in HIV-1 patients with cervical cancer is more beneficial because of their dual effect on reducing cancer progression and HIV-1 replication.

Conclusion

In conclusion, we have demonstrated that cervical cancer cells exacerbate HIV-1 replication in monocytic cells via transferring oxidative stress factors such as CYPs and HPV oncoproteins through exosomes. We have also shown that the viral replication undergoes via a CYP-mediated oxidative stress pathway and it can be reduced by treatment of chemodietary agents like curcumin and Cucurbitacin-D. The present study therefore, provides scientific rationale for the development of novel therapeutic treatment for HIV-1 infected women with cervical cancer.

CHAPTER 7. CONCLUSIONS AND FUTURE DIRECTIONS

With the introduction of ART since 1995, the number of AIDS-related deaths have significantly decreased and consequently, the number of PLWHA has increased [364]. With the increase in life-span, numerous pathological complications including cancers of various organs and tissues, neurological disorders and various opportunistic infections have been observed in PLWHA. ART has been able to control plasma HIV-1 levels and improve $CD4^{+}$ cell counts to large extent, however, complete eradication of HIV-1 has not been achieved. One of the major hindrances to cure HIV-1 is the rapid formation of latent HIV-1 reservoirs in memory T cells, monocytes, and macrophages. The presence of these viral reservoirs is observed even in PLWHA on ART for lifelong. The latent HIV-1 in these reservoirs are activated to produce new viruses in presence of stress factors like cytokines and [133]infections [365] and exposure to various drugs of abuse [366]. Most of these stress factors use ROS as a secondary messenger to stimulate the nuclear transcription factors, NF-κB and NFAT, which enhance the transcription of latent HIV-1 genome [149].

There is a high prevalence of drug abuse, especially tobacco smoking among PLWHA. Several components in cigarette smoke such as nicotine and polyaryl hydrocarbons are known to be metabolized by CYP enzymes through phase I metabolism. During the metabolism, CYPs generate ROS as intermediate products through various coupling and auto oxidation reactions. Therefore in project I, we initially proposed that ROS generated through CYP-mediated metabolism of cigarette smoke constituents, especially BaP, triggers HIV-1 replication in viral reservoirs such as monocytes and macrophages. Furthermore, PLWHA also suffer from coinfection by various pathogens due to immunosuppression by HIV-1. Among the various coinfections, high-risk HPV coinfection by genotype 16 and 18 that causes cervical cancer is highly prevalent in PLWHA. Infections by pathogens such as syphilis [367], mycobacterium tuberculosis [365], and hepatitis [368] have been shown to induce HIV-1 replication. Therefore in project II, we proposed that HPV may also trigger latent HIV-1 reactivation in infected monocytes/macrophages via a mechanism similar to that proposed in project I. Here, we hypothesized that, HPV-infected cells transport exosomes containing oxidative stress factors, especially CYPs to monocytes/macrophages latently infected with HIV-1. The CYPs from the exosomes are released into the infected macrophages and monocytes where they add to the effect of the cellular CYPs to mediate oxidative stress and ultimately HIV-1 replication.

As expected, our results indicated that, out of the five compounds tested, chronic exposure (7 days) of BaP significantly increased oxidative stress and cytotoxicity in U937 monocytic cells and human primary monocytic cells. Acute exposure of BaP did not cause any significant increase in oxidative stress and cytotoxicity in U937 cells, probably because of the protective effect from the basal antioxidant enzymes. Chronic exposure of BaP also induced the mRNA expression of CYPs (CYP1A1 and CYP3A4) and AOEs (SOD1 and catalase) in the monocytic cells, which indicated the possible involvement of CYPs and oxidative stress pathway in BaP-induced cytotoxicity. Further,

the increased caspase-3 activity after chronic BaP exposure suggested that BaP may be causing cytotoxicity possibly via a caspase-3-dependent apoptotic pathway. Therefore, we demonstrated that chronic treatment of BaP induces cytotoxicity in monocytes, possibly through generation of ROS via a CYP pathway.

We also demonstrated that BaP induced oxidative stress and HIV-1 replication in U1 cells as well as HIV-1-infected human primary macrophages. Treatment of antioxidants and CYP1A1 inhibitor significantly decreased the oxidative stress and viral replication induced by BaP, indicating that BaP-induced HIV-1 replication occurs via a CYP-mediated oxidative stress pathway. Further, we also showed that ROS generated via CYP metabolism of BaP facilitates the nuclear translocation of NF-κB subunits, leading to enhanced HIV-1 transcription. We also confirmed the role of NF-κB pathway using specific NF-κB inhibitors. Our results give an insight of a novel mechanism for smoking-induced HIV-1 replication, that CYP-mediated metabolism of BaP generates ROS, which triggers NF-κB pathway to enhance the transcription of HIV-1 genome.

Next, we observed that, exosomes derived from HPV-infected caski cells package oxidative stress factors (like CYPs, HPV oncoprotein and antioxidant enzymes) and diffuse into the U1 cells. We also observed that the treatment with these exosomes enhance oxidative stress and HIV-1 replication in U1 cells. Antioxidants and specific CYP inhibitors significantly decreased exosomes-induced HIV-1 replication in U1 cells. This confirms, at least in part, the role of oxidative stress and CYPs in the exosome-mediated HIV-1 replication. Our results demonstrate that HPV-infected cells induce HIV-1 replication in monocytic cells latently infected with HIV-1 via exosomes containing CYPs. These CYPs along with the cellular CYPs may be generating ROS during the metabolism of various endogenous and exogenous substrates within the cells, thereby resulting oxidative stress and ultimately HIV-1 replication.

Overall, our study illustrates that comorbidities like smoking or HPV coinfection contribute to HIV-1 replication and pathogenesis in monocytic cells, at least in part, via a CYP-mediated oxidative stress pathway. This study is clinically relevant as it provides the basis for potentially novel therapeutic targets like CYPs and oxidative stress. Since there are no guidelines to titrate ART regimens in smokers, the outcomes from the present study will also help in optimizing ART regimens for HIV-1-positive smokers. Identification of the role of CYP enzymes in tobacco/HPV-mediated HIV-1 pathogenesis would not only help to adjust the ART regimen, but would also help to design a better treatment strategy for HIV-1-positive patients with tobacco addiction or HPV-coinfection. CYP enzymes are an important target for drug development to combat many disease conditions, such as cancer (CYP1A1, 1A2, 1B1, 2A13) [369, 370], cardiovascular disease (CYP1A, 1A2, 1B1) [371], inflammation and pain (CYP4A) [372], hypertension [373], pulmonary disease (CYP1B1) [374], and HIV-1 (CYP3A4) [153]. In the present study, we demonstrated that antioxidants (resveratrol, vitamin E and C) and CYP inhibitors (ellipticine and tryptamine) reduce HIV-1 viral load in vitro in BaP or HPV-exposed U1 cells. These compounds can further be optimized and formulated into potent and clinically safe drug molecules to alleviate disease progression in PLWHA who are either smokers or have HPV coinfection. Furthermore, we have

shown that chemo-dietary agents such as curcumin and cucurbitacin-D reduce HPV-induced HIV-1 replication in monocytic cells. Curcumin and cucurbitacin-D have been shown to have a significant impact on alleviating cervical cancer [344]. The identification of these compounds provides a novel insight for the design and development of new drugs, or their use as adjuvant therapy to alleviate both HIV-1 and HPV infection in PLWHA.

Moreover, these antioxidants, CYP inhibitors, and chemo-dietary agents can be synthetically loaded into exosomes and delivered to the target tissues. Exosomes are non-immunogenic and biocompatible; they also have target specificity, a long circulating half-life, and no associated toxicological problems. These features make exosomes a promising drug delivery system, compared to synthetically constructed nano-carriers [375]. Recently, exosomes derived from brain cells have been shown to be an effective drug carrier to deliver anticancer drugs across the BBB for the treatment of brain cancer in a zebrafish model [376]. In another study, dendritic cells were engineered to produce exosomes that expressed exosomal membrane proteins fused to the neuron-specific RVG peptide. The RVG-targeted exosomes were capable of delivering siRNA targeted to BACE1, a therapeutic target of Alzheimer's disease, to brain tissues of mice, resulting in successful BACE1 knockdown [377]. Furthermore, exosomes loaded with antioxidant agents such as curcumin have also been reported to have a relatively improved distribution in brain tissues and reduce loss of neurons, glia, and brain endothelia after ischemia-reperfusion injury [378]. The exosomes loaded with antioxidants or CYP inhibitors or chemo-dietary agents can also be therapeutically beneficial to reduce the oxidative stress-induced HIV-1 replication in monocytic cells of HIV-1-infected smokers as well as HIV-1 patients with HPV co-infection.

HIV-1-infected monocytes and macrophages circulating in the bloodstream can spread HIV-1 infection in the CNS once they cross the BBB [379]. These cells further activate astrocytes and microglia to produce various inflammatory chemokines and cytokines. The release of cytokines, chemokines, viral particles, and ROS causes oxidative stress and inflammation within the brain, ultimately leading to HIV-1-associated neurological disorders (HAND) [380]. Currently available ART drugs have very low bioavailability at the brain tissues as their diffusion to the CNS is limited by BBB [381]. The viral reservoirs and the infected cells within the brain are therefore protected from ART, which further makes it difficult to attain the complete eradication of HIV-1. As exosomes can easily cross the BBB, the delivery of antioxidant/ART-loaded exosomes can be an efficient therapeutic strategy to reduce the viral load in brain cells by lowering ROS level. The use of exosomes as a therapeutic carrier to deliver ART in the brain can also help to reduce HAND, which has been a rising problem among PLWHA in the ART era.

In addition to smoking, individuals infected with HIV-1 often use various other drugs of abuse such as cocaine, methamphetamine, marijuana, and alcohol, which result in similar oxidative damage. The concomitant use of these drugs is likely to further exacerbate oxidative stress and viral load in PLWHA. Thus our strategy discussed above that contain CYP inhibitors/antioxidants/chemo-dietary agents loaded-exosomes may

serve to alleviate oxidative damage due to abuse of these poly-drugs in different cells and tissues. Furthermore, these exosomes may also have therapeutic benefits in other neuropathological conditions associated with oxidative stress such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis.

The International Agency for research on Cancer (IARC) has classified smoking as one of the risk factors for cervical cancer [212]. Several studies indicated that cigarette smoking enhances the progression of HPV infection into cervical lesions and cancer [381]. A meta-analysis study conducted by the International Collaboration of Epidemiological Studies of Cervical Cancer on 23 different studies concluded that the relative risk is 1.6 times higher for a current smoker to develop HPV infection into squamous cell carcinoma, compared to a non-smoker [382]. Cigarette smoke constituents such as nicotine and benzo(a)pyrene are found to be present in the mucus of cervical cancer patients [204]. Tobacco has also been shown to enhance the transcription of HPV oncoproteins E6 and E7 that reduce p53 levels and activity, enhance DNA damage and mutation, thereby leading to cervical cancer [383]. Nicotine, BaP, and their metabolites are known to form DNA and protein adducts that induce carcinogenesis in cervix [384]. Moreover, BaP has also been shown to increase HPV synthesis in cervical cancer cells [385]. In the present study, we have shown how smoking and HPV independently impact HIV-1 replication in monocytic cells. As smoking enhances HPV pathogenesis and progression to cervical cancer, these factors together may further have an additive or synergistic effect on the HIV-1 replication in monocytic cells. Therefore, in future, our lab will be investigating the combined effect of smoking and HPV coinfection on HIV-1 replication in monocytic cells.

Numerous CYPs are upregulated in cervical cancer cells including the CYPs that metabolize cigarette smoke constituents, especially polyaryl hydrocarbons (CYP1A1/1A2/1B1) [386]. Exposure of polyaryl hydrocarbons, especially BaP, may enhance oxidative stress in cervical cancer cells via CYP pathway. The packaging of oxidative stress factors into the exosomes, as well as the release of the exosomes may be altered after BaP exposure into the cervical cancer cells. In the current project, we have only demonstrated the role of CYPs in the exosomes. We will be investigating other oxidative stress factors in the exosomes that are responsible for reactivating latent HIV-1 in monocytic cells, using proteomic and microarray analysis. Based on their known contribution in reactivating latent HIV-1, we speculate that cytokines (IL-6, TNF-α) and miRNAs (34a, 181) may have a role in activating HIV-1 replication in monocytic cells. We also observed the presence of HPV oncoprotein E6 in the exosomes derived from HPV-infected cervical cancer cells, which adds the possibility that these oncoproteins may also have a role in generating oxidative stress in the recipient cells. We propose that the exosomes derived from cervical cancer cells, exposed to BaP may have a greater potential to reactivate latent HIV-1 in monocytic cells than the exosomes from cervical cancer cells alone. Overall, this new project will demonstrate how smoking and HPV coinfection together impact reactivation of latent HIV-1 in viral reservoirs such as monocytes.

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