An in Vitro Model of Morphine Withdrawal Manifests the Enhancing Effect on Human Immunodeficiency Virus Infection of Human T Lymphocytes through the Induction of Substance P

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Opioid withdrawal is a crucial and recurring event during the course of opioid abuse that has a negative impact on the immune system. In this study, we investigated whether abrupt withdrawal (AW) or precipitated withdrawal (PW) potentiates human immunodeficiency virus (HIV) infection of human T lymphocytes. AW and PW enhanced HIV infection of peripheral blood lymphocytes and T-cell lines (Jurkat and CEMX174). In addition, both AW and PW induced HIV replication in a latently HIV-infected human T-cell line (J1.1). The enhancing effect of AW and PW was associated with the induction of neuropeptide substance P in both peripheral blood lymphocytes and the T-cell lines. The substance P receptor antagonist, CP-96,345, not only blocked AW- or PW-induced HIV replication but also abrogated AW- or PW-induced HIV replication in T cells. These findings provide a cellular mechanism that supports the notion that opioids have a co-factor role in promoting HIV infection of the immune cells. (Am J Pathol 2006, 169: 1663–1670; DOI: 10.2353/ajpath.2006.060358)}

Because as many as 96% of opiate abusers use injection as the primary route of administration, opiate abuse contributes significantly to human immunodeficiency virus (HIV) transmission. Recent data from the Center for Disease Control (CDC) indicated that more than 27% of total AIDS cases in the United States were associated with injection drug use. Although it is known that injection drug use of heroin contributes significantly to HIV transmission, there is limited information available regarding whether opiate use such as heroin increases HIV replication and promotes HIV disease progression in HIV-infected opiate abusers or enhances susceptibility to HIV infection in HIV-seronegative individuals exposed to opiates such as heroin. Clinical and epidemiological evidence from early, pre-AIDS studies supports the concept that drug abuse, such as opiates, is a co-factor in the pathogenesis of HIV disease. Several lines of evidence have also shown that opioids are involved in HIV infection of different cell systems. Opioids alter cytokine production and cell trafficking, enhancing susceptibility of the immune cells to HIV infection. Morphine enhances HIV replication in chronically infected promonocytes co-cultured with human brain cells. Although general immunosuppression contributes to HIV infection and replication, the cellular mechanism(s) by which opiates effectively enhance HIV infection of immune cells remain to be determined. There are several potential mechanisms by which opiates could effectively enhance HIV infection. One is direct action through lymphoid opioid receptor on viral replication. Another would be indirect action through the modulation of other HIV-enhancing factors.

There is substantial evidence supporting the existence of a complex, bi-directional link between the central nervous system and the immune system in HIV infection. Both opioids and neuropeptide substance P (SP) play
important roles as modulators of neuroimmunoregulation and are involved in modulation of the immune system and HIV infection. SP and its receptor have been implicated in opioid dependence and withdrawal. Opioid withdrawal is a crucial and recurring event during the course of opioid abuse. The phenomenon of opioid tolerance is well established; once tolerance develops, termination of the drug by withdrawal (drug cessation) or precipitated withdrawal (PW) (administration of an opioid antagonist with or without drug cessation) can lead to an abstinence syndrome indicating a state of physical dependence, which is among the defining characteristics of opioid addiction. Although opiate addicts may not suffer immune dysfunction when getting the drug on a regular basis, opioid withdrawal has a negative impact on the immune system, thus having a co-factor role in promoting viral infections. Drug withdrawal adversely affects the immune function of macrophages, perhaps making the addict more susceptible to infections. We recently demonstrated that morphine withdrawal enhances hepatitis C virus replicon expression. Donahoe and colleagues have reported that precipitated morphine withdrawal increases viral load in SIV-infected monkeys. Our previous studies have shown that morphine treatment enhances HIV R5 strain infection of macrophages and microglia. In the present study, we examined the effect of morphine withdrawal on HIV infection of human T lymphocytes. We also investigated the role of SP in morphine withdrawal-mediated enhancement of HIV infection of T lymphocytes.

**Materials and Methods**

**Cell Isolation and Cell Lines**

Peripheral blood was obtained from six healthy adult donors without any known history of drug abuse. Informed consent was obtained, and the Institutional Research Board of our institution approved this study. Heparinized blood samples were identified as HIV-negative by anonymous testing with the enzyme-linked immunosorbent assay method (Coulter Immunology, Hialeah, FL). Peripheral blood lymphocytes (PBLs) were prepared as previously reported and maintained in a culture of RPMI 1640 medium containing 10% fetal calf serum and 1 μg/mL of phytohemagglutinin-P (PHA-P) for 72 hours. Cells were then transferred to a T-25 flask (5 × 10⁶ cells/flask) and treated with interleukin-2 (50 ng/mL). CEMX174, 1G5 cell lines, and HIV-infected J1.1 cell line were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health, Bethesda, MD. 1G5 is a Jurkat T-cell line that harbors two copies of a stably transfected plasmid containing the luciferase reporter gene downstream of the HIV long terminal repeat (LTR). J1.1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell viability was assessed using trypan blue dye exclusion. In all cases, limulus amebocyte lysate assay demonstrated that the media and reagents were endotoxin-free.

**HIV Strains**

Based on the different use of the major HIV co-receptors (CCR5 and CXCR4), HIV isolates used in this study are referred to as R5, X4, or R5X4 strains. The T-cell-tropic X4 strain (NL4-3) and dual-tropic R5X4 strain (89.6) were obtained from the Center for AIDS Research at the University of Pennsylvania, School of Medicine.

**Morphine Abrupt Withdrawal (AW) and Precipitated Withdrawal (PW)**

PHA-stimulated (72 hours) PBLs, J-MOR5.1, and CEMX174 cells were incubated with or without morphine (10⁻⁶ mol/L) for 4 days and then subjected to AW or PW (blocking opioid receptors by naloxone after morphine cessation) for 24 hours. At the time of AW or PW, the cells were incubated with or without CP-96,345 or CP-96,344 (inactive enantiomer of CP-96,344) generously provided by Pfizer Central Research (Groton, CT). CP-96,345 and CP-96,344 were dissolved in fast-performance liquid chromatography-grade water at the concentration of 10⁻³ mol/L, filtered through a 0.22-μm filter (Millipore, Billerica, MA), and stored at −70°C.
AW: Day 0 → Day 4 → Day 5 → Day 14
Morphine Treatment

HIV

PW: Day 0 → Day 4 → Day 5 → Day 14
Morphine Treatment

PW

Figure 1. Experimental design to study the effect of morphine withdrawal on HIV infection of human T lymphocytes. PHA-stimulated (72 hours) PBLs, J-MOR5.1, and CEMX174 cells were incubated with or without morphine (10^-8 mol/L) for 4 days and then subjected to AW or PW for 24 hours. At the time of AW or PW, the cells were incubated with or without CP-96,345 or CP-96,344 (inactive enantiomer of CP-96,345). For AW, we removed morphine from the cell cultures by washing the cells three times with plain RPMI. For PW, the cells were treated with naloxone (10^-7 mol/L) immediately after AW. The cells undergoing AW or PW as described above were infected with equal amounts (HIV p24, 20 ng/10^6 cells) of cell-free HIV strains (NL4-3 or 89.6). The cells were then washed three times with plain RPMI to remove unabsorbed HIV 24 hours after infection. Supernatants were collected from HIV-infected cell cultures for HIV RT activity assay at day 9 after infection (day 14 after morphine treatment).

HIV Infection of T Cells

The cells (PBLs, J-MOR5.1, and CEMX174) that underwent AW or PW as described above were infected with equal amounts (HIV p24, 20 ng/10^6 cells) of cell-free HIV strains (NL4-3 or 89.6) for 24 hours. The cells were then washed three times with plain RPMI to remove unabsorbed HIV 24 hours after infection. Supernatants were collected from HIV-infected cell cultures for HIV RT activity assay at day 9 after infection. Selection of the day 9 time point is based on our earlier study showing that HIV RT activity in the infected PBL cultures is not detectable until day 6 or day 9 (dependent on donors). The time line for morphine treatment, withdrawal, and infection is illustrated in Figure 1.

RT and Luciferase Assays

HIV RT activity was determined based on a modified technique of Wiley and colleagues. In brief, 10 μl of collected culture supernatants were added to a cocktail containing poly(A), oligo(dT) (Pharmacia Inc., Piscataway, NJ), MgCl2, and [32P]dTTP (Amersham Corp., Arlington Heights, IL) and incubated for 20 hours at 37°C. Then 30 μl of the cocktail was spotted onto DE81 paper, dried, and washed five times with 2x saline-sodium citrate buffer and once with 95% ethanol. The filter paper was then air-dried. Radioactivity was counted in a liquid scintillation counter (Packard Instrument Inc., Piscataway, NJ). HIV RT activity in the infected PBL cultures is not detectable until day 6 or day 9 (dependent on donors). The time line for morphine treatment, withdrawal, and infection is illustrated in Figure 1.

RNA Extraction and Reverse Transcription

Total cellular RNA was extracted from PBLs, J-MOR5.1, and CEMX174 using Tri-reagent (Molecular Research Center, Cincinnati, OH). In brief, the total cellular RNA was extracted by a single step, guanidium thiocyanate-phenol-chloroform extraction. After centrifugation at 13,000 x g for 15 minutes at 4°C, the RNA-containing aqueous phase was collected and precipitated in isopropanol. The RNA precipitates were then washed once in 75% ethanol and resuspended in 30 μl of RNase-free water. Total RNA (1 μg) was subjected to reverse transcription using the reverse transcription system (Promega, Madison, WI) with specific primers (anti-sense) for 1 hour at 42°C. The reaction was terminated by incubating the reaction mixture at 99°C for 5 minutes and then kept at 4°C. The resulting cDNA was ready to serve as a template for polymerase chain reaction (PCR) amplification.

Statistical Analysis

Where appropriate, data were expressed as mean ± SD. For comparison of the mean of the two groups (AW- or PW-treated versus untreated control cells), statistical significance was assessed by analysis of variance with the appropriate post hoc test. Calculations were performed with the use of Stata Statistical Software (StataCorp., College Station, TX). Statistical significance was defined as P < 0.05.

Results

AW or PW Enhances Acute HIV Infection of T Cells

We first investigated whether AW and PW enhanced HIV infection of PBLs and the T-cell lines (J-MOR5.1, CEMX174). In comparison to control cells, the increased HIV RT activity was observed in both PBLs and the T-cell lines (J-MOR5.1, CEMX174) undergoing AW (Figure 2). Although there are differential effects of AW or PW on HIV infection of PBLs from different donors, PBLs from five of six donors showed enhanced susceptibility to HIV infection in response to AW or PW (Figure 2A). Naloxone alone had no effect on HIV replication in these cells (data not shown). In addition, although we did not observe morphine withdrawal- and/or HIV infection-induced cytotoxicity in J-MOR5.1 and CEMX174 cell cultures, we observed that there was HIV-induced PBL death (presumably CD4+ T-cell death) in the cultures. However, there were no differences in the HIV-caused cell...
death between the PBL cultures undergoing morphine withdrawal and control cultures (data not shown).

**AW or PW Induces HIV Replication in Latently Infected T Cells and Activates HIV LTR**

We then examined whether AW or PW activates HIV replication in J1.1 cells, a latently infected T-cell line. A significant increase in HIV RT activity in J1.1 cells was observed at 48 hours after AW or PW (Figure 3A). To investigate the mechanism(s) responsible for AW- or PW-mediated up-regulation of HIV replication, we examined whether AW or PW has a stimulatory effect on HIV promoter LTR. HIV-LTR promoter activity was increased in 1G5 cells undergoing AW or PW (Figure 3B). Naloxone alone had no effect on HIV activation in both J1.1 cells and 1G5 cells (data not shown).

**The Role of the SP and NK-1R in AW- or PW-Mediated Action**

Using PBLs isolated from six different donors, we demonstrated that AW or PW induced SP mRNA expression in PBLs by as much as 20-fold, although there is variability in SP mRNA expression in PBLs from different donors (Figure 4A). A significant increase of SP mRNA expression in both J-MOR5.1 and CEMX174 cells was also observed at 24 hours after AW or PW (Figure 4B). The role of SP in enhancing HIV infection of T cells was confirmed in the experiments showing that exogenous SP, in a dose-dependent manner, significantly enhanced
HIV infection of PBLs and CEMX174 cells (Figure 5), and the NK-1R antagonist (CP-96,345) inhibited exogenous SP-mediated enhancement of HIV infection of PBLs and CEMX174 cells (Figure 5).

**CP-96,345 Blocks AW- and PW-Induced SP Expression and HIV Replication in PBLs**

The biological interaction of morphine with the SP-NK-1R pathway in T cells was further examined in experiments showing that CP-96,345 abrogated the effects of AW or PW on SP expression in PBLs (Figure 6A). To further determine the role of endogenous SP in AW- or PW-mediated HIV enhancement, we examined whether CP-96,345 antagonizes the effect of AW or PW. CP-96,345 inhibited AW- or PW-induced HIV replication in PBLs (Figure 6B) whereas its inactive enantiomer CP-96,344 had no effect.

**Discussion**

Our earlier studies showed that morphine, the principal metabolite of heroin, enhanced HIV infection of macrophages and microglia. Others also showed that morphine has a co-factor role in facilitating HIV infection of the immune cells. There have been no studies, however, to examine the impact of complete morphine removal (AW) or PW (addition of naloxone to the cell cultures immediately after removal of morphine) on HIV infection of T lymphocytes. The common outcomes of repetitive use of opiate are tolerance, physical dependence, and abstinence syndrome. Naloxone-precipitated opioid withdrawal is also a validated model for testing medications to treat opiate withdrawal. In this study, we pretreated the T cells with morphine for 4 days to induce a tolerant/dependent state, followed by AW or PW. Although this model system does not reflect the in vivo situation in which opiate withdrawal occurs repetitively during the course of opiate abuse, this in vitro system should provide direct evidence and initial evidence about the effect of AW on the T cells in the context of HIV infection. Using this cell system, we examined the effect of AW or PW on HIV infection, a common infection among injecting opioid users. We demonstrated that both AW and PW significantly enhanced HIV infection of human T lymphocytes. In addition, AW and PW activated HIV replication in latently infected T cells. Although the mechanism(s) underlying the morphine action on HIV remain to be determined, we showed that the enhancing effect of AW or PW on HIV was associated with the induction of SP mRNA, an important proinflammatory mediator.
neuropeptide that has an important role in modulating the immune system. The neuropeptide SP, the most extensively studied member of the tachykinin family, is a modulator of neuroimmunoregulation. Both in vitro and in vivo studies have clearly indicated that SP is involved in the physiological changes accompanying opioid withdrawal.9,10,13 SP levels were altered during opiate dependence after withdrawal. Morley and colleagues10 found that SP levels in the brain were elevated during long-term morphine treatment and attenuated after an injection of naloxone. Blockade of the SP-prefering receptor (neurokinin 1 receptor, NK-1R) induced a decrease in the expression of naloxone-precipitated morphine withdrawal syndrome in rats.13 Murtra and colleagues14 reported a loss of the rewarding properties of morphine in mice with a genetic disruption of the SP receptor NK-1R. Our observation that CP-96,345 sup-

pressed AW- or PW-induced SP expression in the T cells (Figure 6) is in agreement with the in vivo observation that CP-96,345 inhibited SP production and morphine withdrawal response in guinea pigs.15 Our earlier study also showed that CP-96,345 down-regulated SP mRNA expression in human mononuclear phagocytes.45

Our earlier study showed that there is SP and NK-1R expression in human immune cells,46,47 and morphine induced SP expression in immune cells, including T cells.48 We also reported that SP enhances HIV expression in macrophages49 and latently infected T lymphocytes.50 Thus, we hypothesized that AW or PW induces SP expression in T cells, through which it enhances HIV replication. The role of SP in AW- or PW-mediated HIV infection of T cells was evidenced by the following observations: 1) AW and PW induced endogenous SP expression in the T cells, 2) exogenous SP enhanced HIV infection of T cells, and 3) the SP receptor antagonist (CP-96,345) not only abolished AW- or PW-induced endogenous SP expression but also antagonized AW- and PW-mediated enhancement of HIV infection. The effect of CP-96,345 is specific because its inactive enantiomer (CP-96,344) had little effect on AW- or PW-induced SP and HIV expression in T cells (Figure 6). The role of SP in the morphine action is also supported by our earlier findings that human T lymphocytes express SP and SP receptors47 and that SP enhanced HIV infection of macrophages49 and activated HIV replication in chronically infected immune cells.50 The interaction between SP and HIV in the immune cells is bi-directional because HIV infection and activation also induced SP expression in human immune cells.8,51 SP released from HIV-infected immune cells, in return, may enhance HIV infection by directly facilitating virus replication and/or by indirectly affecting HIV proliferation through induction of inflammatory cytokines, such as, interleukin-1, interleukin-6, and TNF-α, which are HIV-enhancing cytokines.52,53 SP modulates expression of these cytokines through activation of nuclear factor (NF)-κB,54 which is supported by our earlier study showing that SP activates NF-κB promoter in human T cells.55 In addition, treatment with SP enhanced HIV-LTR activation in the immune cells.56 These collective findings strongly indicate that AW- or PW-induced endogenous SP in T cells is likely to be one of the mechanisms responsible for the morphine withdrawal action on HIV infection. Although SP may play a major role in morphine withdrawal-mediated HIV replication, other factors such as norepinephrine are also involved in the withdrawal action in vivo. For example, a recent study showed that norepinephrine is widely implicated in opiate withdrawal.57 Interestingly, norepinephrine accelerates HIV replication in quiescently infected peripheral blood mononuclear cells that were subsequently activated with antibodies to CD3 and CD28.58 These collective data suggest that morphine withdrawal-mediated HIV enhancement is complex and is involved in multiple factors in the microenvironment.

Taken together, we and others have demonstrated that there are biological as well as pathological interactions between opiates and SP in both the central nervous system and immune systems. The interaction of opioids...
and SP in human T lymphocytes is likely to have a role in the immunopathogenesis of HIV disease among opiate abusers. Because SP is an important modulator of immune regulation and a possible co-factor in HIV infection of immune cells, morphine withdrawal-induced SP is likely to have a critical role in morphine withdrawal-mediated enhancement of HIV infection of the human immune system. Future investigations are necessary to understand molecular mechanism(s) involved in both the in vitro and in vivo interactions between opioids and SP in the human immune system in the context of HIV infection.

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References

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