κ-Opioid Receptor Ligands Inhibit Cocaine-Induced HIV-1 Expression in Microglial Cells

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ABSTRACT

Cocaine abuse has been implicated as a cofactor in human immunodeficiency virus (HIV)-1-associated dementia (HAD). In this study, we tested the hypothesis that inactivation of microglial cells, the resident macrophages of the brain, to cocaine would potentiate HIV-1 expression. Because κ-opioid receptor (KOR) agonists have been shown to suppress neurochemical and neurobehavioral responses to cocaine and to inhibit HIV-1 expression in microglial cell cultures, we also postulated that KOR ligands would inhibit cocaine-induced potentiation of HIV-1 expression. Human microglial cells were infected with HIV-1 (Madi et al., 1997, Eisenstein and Hilburger, 1998; Friedman et al., 2003) and viral expression was quantified by measurement of p24 antigen in culture supernatants. Treatment of microglia with the KOR agonist trans-3,4-dichloro-N,N-(2\-[1-pyrrolidnyl]\)-benzeneacetamide methanesulfonate and 8-carboxamidocyclazocine inhibited viral expression (maximal suppression of 42 and 48%, respectively). Consistent with the hypotheses, treatment of microglia with cocaine promoted HIV-1 expression (maximal enhancement of 54%), and pretreatment of microglia with these KOR agonists as well as with the KOR-selective antagonist nor-binaltorphimine abrogated cocaine-induced potentiation of viral expression. Results of flow cytometry studies suggested that the mechanism whereby KOR ligands inhibit cocaine’s stimulatory effect on viral expression involves the suppression of cocaine-induced activation of extracellular signal-regulated kinase1/2, thereby blunting cocaine-enhanced up-regulation of the HIV-1 entry chemokine receptor CCR5. The findings of this study suggest that in addition to its neurotoxic effects, cocaine could foster development of HAD by potentiating viral expression in the brain and that this phenomenon is inhibited by KOR ligands.

Injection drug use has been recognized as a major risk factor for AIDS from the onset of the epidemic (Alcabes and Friedland, 1995). Based upon a substantial literature demonstrating that substances of abuse, such as cocaine and opiates, can both impair the function of CD4+ lymphocytes and macrophages (Klein et al., 1993, Mao et al., 1996, Baldwin et al., 1997; Eisenstein and Hilburger, 1998; Friedman et al., 2003) and potentiate the expression of HIV-1 in these cells (Peterson et al., 1990, 1992; Bagasra and Pomerantz, 1993; Nair et al., 2000, Li et al., 2002; Roth et al., 2002; Steele et al., 2003), it has been postulated that abuse of these drugs may serve as a cofactor in the progression of HIV-1 infection. The role of substances of abuse in development of HIV-1-associated dementia (HAD), one of the most devastating complications of AIDS, is supported by a growing body of epidemiological and histopathological evidence (Wang et al., 1995; Davies et al., 1997; Bell et al., 1998; Bouwan et al., 1998; Goodkin et al., 1998; Tomlinson et al., 1999), as well as by studies of molecular mechanisms whereby drugs of abuse and HIV-1 interact in causing neuronal death (Nath et al., 2002).

Both HIV-1 and substances of abuse target the central nervous system, and work in our laboratory has focused on interactions of these agents with microglia, the resident macrophages of the brain. Thought to be derived from blood monocytes, microglia are the only brain cell type that can support productive HIV-1 infection. Together with monocytes

ABBREVIATIONS: HAD, human immunodeficiency virus-1-associated dementia; HIV, human immunodeficiency virus; MOR, μ-opioid receptor; KOR, κ-opioid receptor; PBMC, peripheral blood mononuclear cell; 8-CAC, 8-carboxamidocyclazocine; U50,488, trans-3,4-dichloro-N-(2\-[1-pyrrolidnyl]\)-benzeneacetamide methanesulfonate; nor-BNI, nor-binaltorphimine; β-FNA, β-funaltrexamine; MAPK, mitogen-activated protein kinase; PD98059, 2′-amino-3′-methoxyflavone; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; Ag, antigen.
and other brain macrophages, microglia are considered to play a pivotal role in the development of HAD (Williams and Hickey, 2002). We have shown that treatment of microglia with endomorphin-1, a µ-opioid receptor (MOR) agonist, potentiates HIV-1 expression in microglial cell cultures (Peterson et al., 1999). In sharp contrast, κ-opioid receptor (KOR) agonists suppress viral replication in microglial cells (Chao et al., 1996), as well as in blood monocyte-derived macrophages (Chao et al., 2001).

Although several research groups have shown that cocaine potentiates HIV-1 expression in peripheral blood mononuclear cell (PBMC) cultures (Peterson et al., 1992; Bagasra and Pomerantz, 1993; Nair et al., 2000; Roth et al., 2002) by a mechanism that seems to involve up-regulation of the HIV-1 entry chemokine coreceptor CCR5 (Nair et al., 2000), little or nothing is known about the effects of cocaine on HIV-1 expression in microglial cells. Thus, we hypothesized in the present study that cocaine would potentiate viral expression in microglial cells. On the basis of neurobiological and behavioral studies in several animal species, the KOR system has been implicated in the reinforcing effects of cocaine abuse (Kreek, 1996; Shippenberg and Rea, 1997; Bharj and Hickey, 2002). We have shown that treatment of microglia with cocaine (Kreek, 1996; Shippenberg and Rea, 1997; Bharj and Hickey, 2002) was chosen for this study based upon its potential for treatment of cocaine dependence (Bidlack et al., 2002).

**Materials and Methods**

**Reagents.** The KOR-selective agonist U50,488 was a gift of Upjohn Co. (Kalamazoo, MI), and the KOR-selective antagonist nor-binaltorphimine (nor-BNI) was purchased from Tocris Cookson Inc. (Ellisville, MO). The cyclohexadine derivative 8-CAC, which is an agonist at KOR and MOR, was kindly provided by Dr. M. P. Wentland (Rensselaer Polytechnic Institute, Troy, NY). All other reagents were purchased from the indicated sources: cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO), the MOR-selective antagonist β-fumalrexamine (β-FNA) (Sigma-Aldrich); antibodies to CD68 (a marker for microglial cells) (BD PharMingen, San Diego, CA) and glial fibrillary acidic protein (an astrocyte marker) (DAKO, Carpineteria, CA); antibodies to CCR5 (BD PharMingen); the mitogen-activated protein kinase (MAPK) inhibitor PD98059 (an inhibitor of extracellular signal-regulated kinase (ERK)1/2 phosphorylation) (Calbiochem, San Diego, CA); cell proliferation assay kit (Cell Proliferation ELISA bromodeoxyuridine kit) (Roche Diagnostics, Indianapolis, IN); and fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT).

**Microglial Cell Cultures.** Human fetal brain tissue was obtained from 16- to 22-week-old aborted fetuses under a protocol approved by the Human Subjects Research Committee at our institution. The procedure for isolating purified human fetal microglial cells has been described previously (Chao et al., 1996). Briefly, brain tissues were dissociated after 30-min trypsinization (0.25%) and plated into 75-cm² Falcon culture flasks in DMEM containing 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The medium was replenished 4 days later with DMEM containing 10% FBS without antibiotics, and microglial cells were harvested after 10 to 14 days in culture. By immunocytochemistry, microglial cells were >99% CD68 positive with <1% other glial cells (stained with anti-glia fibrillary acid protein) and were >98% viable by trypan blue exclusion criteria. Microglial cells were added to 48-well culture plates at densities of 1 × 10⁶ cells/well.

**Drug Treatment and Assessment of Viral Expression.** After treatment with test compounds at indicated concentrations and time intervals, the monocytotropic HIV-1L929 strain (provided by the National Institutes of Health AIDS Research and Reference Reagent Program) was added to microglial cells at a multiplicity of infection of 0.02. After 24-h absorption with HIV-1 at 37°C, cells were washed three times with DMEM and then culture medium (DMEM, 10% FBS) containing indicated concentrations of test compounds was added. On day 7 postinfection, microglial cell culture supernatants were collected from wells in triplicate for HIV-1 p24 antigen (Ag) assay. Viral expression was quantified by measuring HIV-1 p24 Ag levels in culture supernatants using an enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, IL), as described previously (Chao et al., 1996). A standard dilution curve derived from known amounts of p24 Ag was used to quantitate the Ag levels in culture supernatants. The sensitivity of this assay is 30 pg/ml.

**Assessment of Microglial Cell CCR5.** The effects of cocaine and KOR ligands on microglial cell expression of the chemokine receptor CCR5 was examined by flow cytometry. Microglial cells (3 × 10⁶/0.5 ml medium) were incubated for 30 min in Teflon-coated vials in the absence or presence of U50,488, S-CAC, or nor-BNI before treatment with cocaine for 24 h. Cells were then double-stained with fluorescein isothiocyanate-labeled anti-CCR5 antibody and phycoerythrin-labeled anti-CD68 followed by flow cytometry analysis. Data are expressed as the percentage of microglial cells positive for CCR5 and CD68 using the EPICSr XL software provided by the manufacturer (Beckman Coulter Inc., Miami, FL).

**Assessment of Intracellular Signaling.** Activation of the ERK1/2 MAPK intracellular signaling pathway was assessed by flow cytometry using a previously described method (Chow et al., 2001) modified for our purposes. Microglial cells (3 × 10⁶/0.5 ml of medium) were incubated in Teflon-coated vials for 30 min in the absence or presence of nor-BNI, U50,488, or PD98059 before treatment with cocaine. Cells were then fixed in 2% paraformaldehyde (10 min; 37°C) and permeabilized in 98% methanol (30 min on ice). After washing twice, cells were stained with Alexa Fluor-488-conjugated phospho-ERK1/2 antibodies for 60 min at room temperature. Data are expressed as percentage of cells positive for phosphorylated ERK1/2.

**Statistical Analyses.** For comparison of means of two groups, Student’s t test was used; for comparison of means of multiple groups, analysis of variance was performed by Sheffé F-test. For analyses in which the effects of multiple drug concentrations and treatment times were compared, a mixed effects repeated measures model was used that accounts for within-person correlations and intrinsic differences among individuals. The Tukey method was applied to adjust for multiple comparisons, and the Mixed Procedure in SAS version 8.2 was used to perform these analyses.

**Results**

**Effects of Cocaine and KOR Ligands on HIV-1 Expression.** To compare the effects of cocaine and KOR ligands on HIV-1 expression, a concentration- and time-response study was carried out with cocaine and the KOR agonists U50,488 and S-CAC in which these compounds were added to microglial cell cultures before or at the time of infection with the monocytotropic (R5) isolate HIV-1L929 (Fig. 1). As hypothesized, cocaine was found to stimulate HIV-1 expression with maximal potentiation above untreated control cells of 54% (10⁻⁸ M, 24-h pretreatment) (Fig. 1A). As previously
reported (Chao et al., 1996), U50,488 inhibited viral expression with maximal suppression of 42% (10^{-8} M, 24-h pretreatment) (Fig. 1B). Similar inhibition of HIV-1 expression was observed with the KOR agonist 8-CAC (48%; 10^{-6} M, 24-h pretreatment), although when it was added at the time of viral infection of microglia, 8-CAC (10^{-6} M), but not U50,488, significantly inhibited viral expression (Fig. 1C).

Based upon these findings, all subsequent studies of HIV-1 expression were performed using a drug concentration of 10^{-8} M and a pretreatment interval of 24 h.

Previously, we have shown that U50,488-mediated suppression of HIV-1 replication in microglial cells can be blocked by the KOR-selective antagonist nor-BNI (Chao et al., 1996). Because 8-CAC is an agonist at KOR and MOR, we investigated whether its suppressive effect involves KOR or MOR. As is shown in Fig. 2, pretreatment of microglia with an equimolar concentration of the KOR-selective antagonist nor-BNI, which had no activity by itself, completely abrogated the suppressive activity of 8-CAC (10^{-8} M), whereas the MOR-selective antagonist β-FNA had no effect on 8-CAC-
mediated inhibition of viral expression. Thus, it seems that 8-CAC's inhibitory effect on HIV-1 expression involves a KOR-related mechanism.

**Effects of KOR Ligands on Cocaine-Induced Potentiation of HIV-1 Expression.** To test the second hypothesis of this study, i.e., that KOR ligands would inhibit cocaine-induced potentiation of HIV-1 expression in microglial cells, microglia were treated with U50,488, 8-CAC, or nor-BNI before exposure to cocaine. Cocaine stimulated HIV-1 expression, and the KOR agonists U50,488 and 8-CAC by themselves suppressed viral expression, whereas the KOR antagonist nor-BNI by itself had no activity (Fig. 3). Consistent with the second hypothesis of this study, pretreatment with equimolar concentrations of each of these KOR ligands inhibited cocaine-induced potentiation of HIV-1 expression (Fig. 3).

**Effects of KOR Ligands on Cocaine-induced CCR5 Up-Regulation.** Based upon the report of other investigators (Nair et al., 2000) that the stimulatory effect of cocaine on HIV-1 expression in PBMCs is associated with up-regulation of the viral entry chemokine coreceptor CCR5, we next investigated whether cocaine would also induce up-regulation of CCR5 in microglial cells, and if so, whether KOR ligands would inhibit this phenomenon. As shown in Fig. 4, treatment of microglial cells with cocaine increased CCR5 expression from 8% of untreated (control) microglia to 47% of cocaine-treated cells. Also, pretreatment of microglia with U50,488, 8-CAC, or nor-BNI before cocaine exposure abrogated the cocaine-induced up-regulation of CCR5 (Fig. 4). These findings suggest that the mechanism whereby KOR ligands inhibit cocaine-induced potentiation of HIV-1 expression could involve suppression of cocaine-induced up-regulation of CCR5.

**Effect of KOR Ligands on Cocaine-induced ERK1/2 Activation.** Although the inhibitory effects of the KOR agonists on cocaine could be explained most simply by a cancellation of cocaine's stimulatory effect on the expression of HIV-1 (Fig. 3) and CCR5 (Fig. 4), the finding that the antag-
onist nor-BNI also blocked the potentiating effects of cocaine suggested that binding of a KOR ligand to microglial cells is sufficient to inhibit an intracellular signaling pathway involved in these cocaine-induced responses. Other investigators have demonstrated that cocaine-rewarding properties involve activation of the ERK intracellular signaling cascade throughout the mouse striatum (Valijent et al., 2000). Thus, we were interested to know whether cocaine also activates this intracellular signaling pathway in microglia, and if so, whether nor-BNI would inhibit activation of this MAPK system. Using flow cytometry to measure signal transduction, we found that treatment of microglia with cocaine (10^-8 M) was associated with activation of ERK1/2 (percentage of cells positive for phosphorylated ERK1/2: mean ± S.E.M., 14 ± 2 cocaine-treated versus 3 ± 1 control cells; n = 3 experiments, p < 0.01). Next, we used this same assay system to investigate whether pretreatment of microglia with nor-BNI would inhibit cocaine-induced ERK1/2 activation. For this experiment, nor-BNI was compared with PD98059, a specific inhibitor of ERK1/2 activation. As shown in Fig. 5, both nor-BNI and PD98059 significantly suppressed cocaine-induced ERK1/2 phosphorylation. Similar results were obtained when the effects of the KOR agonist U50,488 was studied (percentage of cells positive for phosphorylated ERK1/2: mean ± S.E.M. unstimulated cells, 2.6 ± 0.4; U50,488-treated cells, 2.6 ± 0.5; cocaine-treated cells, 10.4 ± 1.5; U50,488 plus cocaine-treated cells, 5.4 ± 0.7; n = 3 experiments, p < 0.01 cocaine versus unstimulated, p < 0.05 cocaine versus U50,488 plus cocaine-treated cells). Together, these results suggest that binding of a KOR ligand to microglial cell KOR interferes with cocaine-induced activation of ERK1/2 and that this mechanism could play a role in KOR ligand-mediated suppression of cocaine-induced up-regulation of CCR5 and HIV-1 entry.

Effect of Inhibition of ERK1/2 Activation on HIV-1 Expression. If KOR ligand-mediated inhibition of cocaine-induced ERK1/2 activation is involved in the mechanism whereby KOR ligands inhibit cocaine-enhanced HIV-1 expression, then the specific ERK1/2 inhibitor PD98059 would be expected also to suppress viral expression. To determine whether this is the case, microglial cells were treated with PD98059 before stimulation with cocaine. As shown in Fig. 6, PD98059 significantly suppressed HIV-1 expression and inhibited cocaine-enhanced viral expression.

Discussion

To our knowledge this study has demonstrated for the first time that cocaine potentiates the expression of HIV-1 in microglial cells. This finding is consistent with previous reports that cocaine fosters replication of HIV-1 in PBMC (Peterson et al., 1992; Bagasra and Pomerantz, 1993; Nair et al., 2000; Roth et al., 2002) and is compatible with the prevailing view that microglia are derived from blood monocytes. Our finding that cocaine-induced potentiation of viral expression in microglia was associated with up-regulation of the chemokine coreceptor CCR5 is consistent with studies of cocaine-treated PBMCs (Nair et al., 2000), which interestingly also has been proposed as the mechanism whereby opiates promote HIV-1 infection of mononuclear phagocytes (Li et al., 2002; Steele et al., 2003). The chemokine receptor CCR5 is used by R5 isolates, such as HIV-1SF162, for entry into macrophages. The demonstration that the KOR ligand 8-CAC suppressed viral expression adds support to the notion that KOR agonists inhibit HIV-1 expression in microglial cells (Chao et al., 1996). Microglial cells have been reported to express both KOR (Chao et al., 1996) and MOR (Chao et al., 1997), and the observation that the KOR-selec-
tive antagonist nor-BNI blocked 8-CAC’s inhibitory property suggested that 8-CAC was operating through KOR rather than MOR sites on microglial cells.

Of greatest interest to us in this study was the finding that KOR ligands interfered with the stimulatory action of cocaine on microglia, cells that are found in numbers equivalent to neurons in the brain and that serve as the intrinsic immune system of the brain. The rationale underlying the hypothesis that KOR ligands would have such an inhibitory effect on cocaine was based upon results of neurobiological and behavioral studies indicating that KOR ligands can suppress cocaine-seeking behavior (Kreek, 1996; Shippenberg and Rea, 1997; Bhargava and Cao, 1998; Collins et al., 2001a,b; Chen et al., 2002; Kreek et al., 2002).

The finding that the KOR-selective antagonist nor-BNI, which by itself had no effect on viral expression, was as potent as the agonists U50,488 and 8-CAC in blocking cocaine-induced up-regulation of CCR5 and HIV-1 expression was surprising to us. Although the basis for this phenomenon is incompletely understood, this observation suggested to us that binding of a KOR ligand to microglial cells is sufficient to inhibit an intracellular signaling pathway involved in cocaine-induced up-regulation of CCR5. Support for this hypothesis was provided by experiments demonstrating that treatment of microglia with either nor-BNI or U50,488 significantly inhibited cocaine-induced activation of ERK1/2 in a manner similar to PD98059, a specific inhibitor of ERK1/2 activation. Moreover, in support of the hypothesis that KOR ligand-mediated inhibition of cocaine-induced ERK1/2 activation is related to their inhibitory effects on cocaine-enhanced viral expression, we found that PD98059 also suppressed HIV expression in microglia, as has been previously...
reported by others (Si et al., 2002) and completely abrogated cocaine’s enhancing effect on viral expression.

The finding that an opioid antagonist can alter the response of cells of the immune system to cocaine is not unprecedented, because we previously showed that the nonselective opioid receptor antagonist naloxone blocked cocaine-induced suppression of superoxide production by blood monocytes (Chao et al., 1991). Also, nor-BNI has been reported by some investigators to decrease the acquisition of cocaine self-administration behavior in rodents (Kuzmin et al., 1998).

The clinical significance of the findings in this study are unknown, but they suggest that cocaine abuse could contribute to development of HAD not only by its synergistic interaction with the neurotoxic HIV-1 proteins gp120 and Tat (Nath et al., 2002) but also by potentiating viral expression in the brain. Together with previous studies of U50,488 in microglial cell (Chao et al., 1996), monocyte-derived macrophages (Chao et al., 2001), and CD4+ lymphocyte (Peterson et al., 2001; Lokensgard et al., 2002) cultures, the findings in this study extend the observation that KOR agonists inhibit HIV-1 expression to 8-CAC, a compound with potential for the treatment of cocaine addiction (Bidlack et al., 2002).

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References


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