Short Communication: Methamphetamine Treatment Increases *in Vitro* and *in Vivo* HIV Replication

Sima Shelly Toussi,^{1,*} Aviva Joseph,^{2,*} Jian Hua Zheng,² Monica Dutta,²
Laura Santambrogio,³ and Harris Goldstein^{1,2}

Abstract

To delineate the mechanistic basis for the epidemiological association between methamphetamine use and accelerated progression to AIDS, we evaluated the direct *in vitro* and *in vivo* effects of methamphetamine on HIV-1 replication. Methamphetamine administration significantly increased HIV-1 production by both HIV-infected monocytes and CD4 T lymphocytes *in vitro*. In addition, *in vivo* methamphetamine treatment increased HIV production and viremia in mice transgenic for a replication-competent HIV provirus and human cyclinT1. Methamphetamine activated transcription of the HIV long terminal repeat (LTR) regulatory region, was associated with nuclear translocation of NF- κ B. Our results provide further insights into the mechanisms by which methamphetamine accelerates disease course in HIV-infected individuals.

SINCE ITS EMERGENCE IN THE EARLY 1980s, methamphetamine (meth) abuse has progressively increased to become, second to marijuana, the most frequently used illicit drug in the United States; meth abuse is particularly prevalent among HIV-infected individuals and those at high risk of acquiring HIV infection.¹ As a result of the association of meth use with high-risk sexual behavior, increased HIV transmission, and the development of antiretroviral resistance, meth use plays an important role in driving the course of the HIV epidemic in the United States.^{2–4} Although epidemiological studies have linked meth use with increased HIV-1 transmission, it is not yet established whether meth has direct *in vivo* effects on HIV replication and subsequent progression to AIDS. In the current study we examined the *in vitro* and *in vivo* capacity of meth to increase HIV production by HIV-infected CD4 T cells and macrophages. For the *in vivo* studies, we used our well-characterized JR-CSF/hu-CycT1 HIV transgenic mouse model, which is populated with T lymphocytes and monocytes that produce infectious HIV-1 and develops plasma viremia.^{5,6} We also identified a new mechanism by which meth enhances HIV production, by increasing transcription of the HIV long terminal repeat (LTR) and provided further evidence that meth induces nuclear translocation of NF- κ B, a known stimulator of tumor necrosis factor (TNF)- α secretion that potentially induces HIV replication in macrophages.

To determine the effect of meth on HIV production, human macrophages were infected with HIV-1_{ADA} and then treated

with dosages of meth pharmacologically comparable to meth serum levels reported in meth abusers. Intravenous, intranasal, anal, or oral administration of meth by occasional users at doses that usually range from 250 to 500 mg or by chronic abusers at doses of up to 1 g can generate meth levels in the spleen ranging from 100 to 400 μ M after a single dose and 240 to 1144 μ M after binge administration.⁷

Human peripheral blood mononuclear cells (PBMCs) were purified from leukocyte packs by Ficoll-Hypaque density gradient centrifugation. Monocytes (>95% pure) isolated by CD14 magnetic bead sorting according to the manufacturer's protocol (Miltenyi Biotec Inc., Auburn, CA) were activated in complete media [RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; 10% v/v), penicillin (100 U/ml), streptomycin (10 μ g/ml), HEPES buffer (10 mM), and glutamine (2 mM)] by GM-CSF (30 ng/ml, Peprotech, Rocky Hills, NJ). The next day the cells were infected with a monocyte-tropic strain of HIV-1 (HIV_{ADA}) by spinning down the cells and incubating the cell pellet with HIV_{ADA} for 3 h at 37°C. Cells were washed and cultured in 12-well plates (1 $\times$ 10⁶ cells/well) in quadruplicate with fresh GM-CSF (30 ng/ml) added every other day. At day 4 postinfection, cells were either left untreated or treated daily with varying doses of meth: 10 μ M, 50 μ M, 100 μ M, or 150 μ M. The p24 antigen content of the supernatant was measured 8 days later using an ELISA assay as described.⁶ Meth significantly increased HIV production by the infected monocytes in a dose-responsive manner as indicated by increased p24 antigen production,

¹Department of Pediatrics, ²Department of Microbiology and Immunology, and ³Department of Pathology, Albert Einstein College of Medicine, Bronx, New York 10461.

*Both authors contributed equally.

with a dose of 150 μ M of MA inducing a 4-fold rise in p24 antigen production (Fig. 1A). Little to no toxicity was observed in the meth-treated macrophages as described for meth-treated dendritic cells.⁷

We extended these studies to examine the effect of meth on HIV production by primary CD4⁺ T cells. Human CD4 T cells (>95% purity) were isolated by CD4 magnetic bead sorting from the PBMCs depleted of monocytes as described above. The highly purified primary human CD4 T cells were then washed and activated overnight in complete media with added phytohemagglutinin (5 μ g/ml) and interleukin (IL)-2. The following day, CD4 T cells were infected as described above with HIV-1_{JR-CSF}, washed, and then cultured in 12-well plates (1×10^6 cells/well). On day 4 postinfection the cells were either left untreated or treated daily with meth (100 μ M) and the effect of meth on HIV production was evaluated as described above. After human CD4 T cells were infected with HIV_{JR-CSF} ($n = 3$ independent experiments), meth treatment induced a significant 2.6-fold increase ($p = 0.03$) in p24 antigen production.

We hypothesized that one mechanism contributing to meth upregulation of HIV-1 production was meth-induced activa-

tion of the LTR. We examined this possibility using the TZM-bl reporter cell line that permits the determination of the capacity of factors to initiate HIV LTR activation and induce HIV replication. The TZM-bl indicator cell line,⁸ obtained through the AIDS Research and Reference Reagent Program, is a HeLa cell line engineered to express high levels of CD4 and CCR5 and to detect HIV LTR activation with HIV LTR-driven β -galactosidase and luciferase reporter cassettes. TZM-bl cells (0.1×10^6 /cells/well) were added to 24-well microtiter plates in 1 ml of complete media and allowed to adhere overnight at 37°C. The cells were then either left untreated, or treated with varying doses of meth (10 μ M, 50 μ M, 100 μ M, or 150 μ M) each day for 2 days. On day 3, cells were collected by trypsinization and luciferase activity was measured using a luciferase assay system kit (Promega). As shown in Fig. 1B, meth treatment increased luciferase activity in TZM-bl cells in a dose-response manner that paralleled the increased p24 production observed for meth-treated macrophages.

Induction of nuclear translocation of NF- κ B, a nuclear transcription factor that binds to the HIV LTR and plays a critical role in activating HIV transcription by infected cells, is a common pathway utilized by multiple factors to stimulate

FIG. 1. Meth administration increases HIV production in human monocytes and activates the HIV LTR. (A) Eight days after infection and exposure to the indicated concentrations of meth, HIV production was measured by determining the concentration of p24 antigen in the culture supernatant. Cells were cultured in triplicate and the experiment was repeated three times. Data shown represent the mean $\pm$ standard error (SEM) of fold increase in p24 production induced by the indicated dose of meth from three independent experiments. (B) The TZM-bl reporter cell line was exposed to the indicated dose of meth for 2 days and then luciferase activity was measured. Data shown represent the mean $\pm$ SEM of fold increase in luciferase activity induced by the indicated dose of meth from two independent experiments. The Student's t test for unpaired data was used for all statistical comparisons made. Significance was assigned to p values of <0.05 . (C) Monocytes were exposed to the indicated concentration of meth, and 2 h later nuclear extracts were prepared and analyzed for NF- κ B p65. Nuclear extracts from LPS-stimulated monocytes cultured and processed in parallel are included as positive controls. Results are presented as specific absorbance (450 nm) of the ELISA assay and are means for two independent experiments with SEM shown.

HIV production by infected T cells and macrophages.⁹ Consequently, we investigated whether meth induces nuclear translocation of the NF- κ B transcription factor in primary monocytes. Highly purified primary monocytes were isolated by immunomagnetic sorting, resuspended in complete RPMI media, and incubated for 2 h with meth at concentrations ranging from 0 to 150 μ M or with LPS (100 ng/ml), a well-described inducer of NF- κ B nuclear translocation in monocytes. Following incubation, the monocytes were washed and nuclear extracts were isolated using the Nuclear Extract kit (Active Motif, Carlsbad, CA) and nuclear protein was quantified using the Bradford reagent (Sigma, St. Louis, MO). NF- κ B (p65) levels in the nuclear extract were quantified using the TransAM NF- κ B p65 ELISA kit (Active Motif) according to the method provided by the manufacturer. Specific absorbance of samples was measured at 450 nm and values were normalized to a protein concentration of 2.5 mg/ml. As shown in Fig. 1C, meth treatment induced nuclear translocation of NF- κ B in a dose-responsive manner that paralleled both the meth-induced increased p24 production observed for meth-treated macrophages as well as meth-stimulated increased luciferase activity in TMZ-bi cells.

To determine if meth increases *in vivo* HIV production, we used our JR-CSF/hu-CycT1 mouse model. JR-CSF/hu-CycT1 transgenic mice express the HIV-1_{JR-CSF} provirus under the control of the endogenous HIV-1 LTR, as well as the human cyclin T1 gene regulated by the CD4 promoter, enabling support of Tat transactivation of the HIV LTR in the mouse CD4⁺ T cells and myeloid-lineage cells.⁶ JR-CSF/hu-CycT1 transgenic mice develop plasma viremia and are populated with T lymphocytes and monocytes that produce infectious HIV-1.^{6,10} All animal studies were approved by the Institutional Animal Care and Use Committee and were consistent with the guidelines for the care and use of laboratory animals.

JR-CSF/hu-CycT1 mouse monocytes (>95% CD11b⁺) were isolated from the bone marrow by immunomagnetic sorting as described.⁶ Monocytes were added to each well (1 $\times$ 10⁶ monocytes/well) of a 12-well plate in Complete media with added recombinant mouse GM-CSF (20 ng/ml, BioSource, Camarillo, CA). Three days after plating, cells were either left untreated, or treated with varying doses of meth (10 μ M, 50 μ M, 100 μ M, or 150 μ M) daily. Culture supernatant was collected at day 8 and the p24 antigen concentration was quantified by ELISA assay. Treatment of JR-CSF/hu-CycT1 mouse bone marrow-derived macrophages with meth increased HIV production significantly at the 100 μ M and 150 μ M meth doses (Fig. 2A).

To examine the *in vivo* effect of meth on HIV production, we treated JR-CSF/hu-CycT1 mice (10–12 weeks old) by ip injection three times each week for 2 weeks (five mice in each group) either with phosphate-buffered saline (PBS) or with meth at a constant dose (5 mg/kg) or an escalating doses that increased from 5 mg/kg for the first three doses to 6 mg/kg for dose #4, 6.5 mg/kg for dose #5, and 7 mg/kg for dose #6. Escalating meth doses were used because there was a high mortality rate in mice treated with six meth doses of 7 mg/kg. These levels of meth correlate with doses consumed by some meth abusers that can range up to 3–4 g of meth ingested over a 6-day interval.⁷ After 2 weeks of treatment the mice were sacrificed, and p24 antigen content in their spleens was quantified as described.⁶ In comparison to splenocytes from control JR-CSF/hu-CycT1 mice, the p24 antigen content in the

splenocytes of meth-treated mice was increased significantly by treatment with 5 mg/kg meth ($p=0.03$) and with an escalating dosage of 5–7 mg/kg ($p=0.005$) by 2.5-fold and nearly 18-fold, respectively (Fig. 2B).

The impact of meth exposure on HIV production *in vivo* was further evaluated by determining if meth treatment increased plasma HIV RNA levels, which were measured using the Versant HIV-1 RNA 3.0 bDNA assay (Siemens, Tarrytown, NY). Only minimal changes were observed in plasma HIV RNA levels after 2 weeks of treatment with PBS. In contrast, administration of meth for 2 weeks significantly increased ($p<0.023$) HIV viremia by almost 6-fold from baseline plasma HIV RNA levels prior to meth treatment (Fig. 2C).

HIV-infected patients who use meth are more likely to have increased cognitive impairments, decreased compliance with antiretroviral regimens, and higher viral loads.³ Furthermore, *in vitro* studies have shown that meth has immunomodulatory effects on the host defense.^{7,11} These observations provide some rationale for the poorer outcomes observed in HIV-infected patients using meth. In the current study we extend those studies by demonstrating that meth increases HIV production *in vitro* and *in vivo*, which may contribute to the association between meth use and accelerated HIV disease progression. A recent study reported that meth induced an almost 2-fold increase in reverse transcriptase activity in HIV-infected primary human macrophages while upregulating CCR5 expression and reducing interferon (IFN)- α production.¹²

Our results extend those findings by demonstrating that meth also enhances HIV production by the primary target of HIV infection, human CD4 T cells, and that meth also increased *in vivo* HIV production. We also identified an additional mechanism for the meth-induced increase in HIV production, direct activation of the HIV LTR. HIV replication is tightly controlled at the transcriptional level and is induced by the binding of cellular transcription factors to a variety of cis-acting DNA sequences in the HIV LTR.¹³ Binding of NF- κ B to the LTR initiates efficient formation of elongated HIV-1 transcripts and the *de novo* synthesis of Tat, which subsequently promotes highly efficient RNA Pol II elongation.¹⁴ NF- κ B production is induced by meth in human brain endothelial cells that stimulates increased secretion of TNF- α ,¹⁵ a potent inducer of HIV-1 replication in macrophages.¹⁶ We extended these findings by demonstrating that meth also induced nuclear translocation of NF- κ B in primary monocytes. Taken together, these observations indicate that meth may stimulate HIV production by inducing cellular transcription factors such as NF- κ B that activate the HIV LTR.

The physiological relevance of these *in vitro* observations was indicated by our demonstration that meth stimulated *in vivo* HIV production in our JR-CSF/hu-CycT1 mouse model. These double transgenic mice are populated with CD4-expressing T cells and macrophages that express a full-length HIV provirus regulated by the endogenous HIV LTR and produce human hu-CycT1 that enables mouse cells to support Tat-mediated HIV production.^{5,6} Because the JR-CSF/hu-CycT1 mouse cells are not infectible with HIV, the increased HIV production by these cells that we observed is solely due to an effect of meth on the postintegration stages of HIV replication.⁶ Meth increased *in vitro* HIV replication in JR-CSF/hu-CycT1 monocytes. Treatment of JR-CSF/

FIG. 2. Meth administration increases *in vitro* and *in vivo* HIV production by JR-CSF mouse leukocytes. **(A)** Purified bone marrow-derived monocytes from JR-CSF/hu-CycT1 mice were cultured in quadruplicate in the presence of GM-CSF and the indicated dose of meth. After 8 days, HIV-1 production was measured by determining p24 antigen concentration present in the culture supernatant. Data shown represent the mean $\pm$ SEM of fold increase in p24 values in JR-CSF/huCycT1 murine monocytes from two independent experiments. **(B)** JR-CSF/hu-CycT1 mice were treated with PBS or meth at a dose of 5 mg/kg for six doses, or at a dosage range of 5–7 mg/kg for 2 weeks (administered as 5 mg/kg for the first week, then 6.0, 6.5, and 7.0 mg/kg for the last three doses). HIV p24 antigen production in lysed splenocytes harvested after treatment was quantified as described. Data shown represent the mean $\pm$ SEM of fold increase in p24 antigen of the spleen of meth-treated mice compared to PBS-treated mice. There were five mice in each group and three independent experiments were performed. **(C)** JR-CSF/hu-CycT1 mice were treated with either PBS or meth (5 mg/kg for six doses over 2 weeks). HIV RNA copy number in mouse serum was quantified using the Versant HIV-1 RNA 3.0 assay (bDNA). Data shown represent the mean $\pm$ SEM of HIV RNA (copies/ml) initially and after completion of the indicated treatment. There were five mice in each group and the experiment was performed twice.

hu-CycT1 mice with meth for 2 weeks significantly induced *in vivo* HIV production as evidenced by higher p24 antigen production and markedly increased HIV viremia in the MA-treated mice compared to the untreated mice. Meth may increase *in vivo* HIV production by directly activating the LTR as we demonstrated to enhance HIV transcription.

Meth use is becoming increasingly prevalent, and is disproportionately used in those with HIV infection or in high-risk groups, in particular men who have sex with men.¹ Results presented in the current study demonstrate that meth use in this population may further compromise the health of this population by increasing HIV replication and precipitating progression to AIDS. These findings underscore the importance of having providers question and counsel their patients on substance abuse, particularly the use of meth, and to monitor their compliance with HAART.

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Disclosure Statement

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Address correspondence to:

Harris Goldstein

Albert Einstein College of Medicine

Forchheimer Building, Room 408

1300 Morris Park Avenue

Bronx, New York 10461

E-mail: hgoldste@aecom.yu.edu