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Modulation of Intracellular Restriction Factors Contributes to Methamphetamine-Mediated Enhancement of Acquired Immune Deficiency Syndrome Virus Infection of Macrophages

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Abstract

Epidemiological studies have demonstrated that the use of methamphetamine (METH), a sympathomimetic stimulant, is particularly common among patients infected with HIV. *In vitro* studies have determined that METH enhances HIV infection of CD4⁺ T cells, monocyte-derived dendritic cells, and macrophages. In addition, animal studies have also showed that METH treatment increases brain viral load of SIV-infected monkeys and promotes HIV replication and viremia in HIV/hu-CycT1 transgenic mice. However, the mechanisms (s) of METH actions on HIV remain to be determined. In this study, we investigated the impact of METH on intracellular restriction factors against HIV and SIV. We demonstrated that METH treatment of human blood mononuclear phagocytes significantly affected the expression of anti-HIV microRNAs and several key elements (RIG-I, IRF-3/5, SOCS-2, 3 and PIAS-1, 3, X, Y) in the type I IFN pathway. The suppression of these innate restriction factors was associated with a reduced production of type I IFNs and the enhancement of HIV or SIV infection of macrophages. These findings indicate that METH use impairs intracellular innate antiviral mechanism(s) in macrophages, contributing to cell susceptibility to the acquired immune deficiency syndrome (AIDS) virus infection.

Keywords

HIV; Interferon; Intracellular restriction factors; Macrophages; METH; microRNAs; SIV; Interferon regulatory factor

INTRODUCTION

A growing body of evidence indicates an increased risk of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) infection among substance abusers. Because the USA is currently experiencing a grave epidemic of methamphetamine (METH) use as a recreational drug [1], it is becoming increasingly important to investigate the

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

association between psychotropic drug use and HIV infection. METH use is associated with risky sexual behavior, increasing the potential of users to become infected with HIV and other viruses [2]. METH-type substances can be used percutaneously and a high correlation has been reported for injection frequency and HIV transmission [3]. A strong connection between METH dependence and HIV infection has been observed for METH-dependent men who have sex with men [4–8], men who have sex with women [9] and female sex workers [10]. More importantly, METH use has been correlated with more a rapid progression to AIDS in HIV-infected people [11]. Active METH users displayed higher levels of HIV load than non-users [12], which may be attributable to reduce the effectiveness of antiretroviral therapy. The effect of METH on feline immunodeficiency virus (FIV) may be at the viral entry or viral integration into host genome level, but not at the translation level [13]. The impact of METH on HIV has also been investigated in animal studies, which demonstrated that METH treatment increases brain viral load of simian immunodeficiency virus (SIV)-infected monkeys [14] and promotes HIV production and viremia in HIV/hu-CycT1 transgenic mice [15]. HIV 'virotoxin' Tat enhanced METH-induced striatal damage in rat model and suggested that HIV-infected individuals who abuse METH may be at increased risk of basal ganglia dysfunction [14,16]. In addition, *in vitro* studies have shown that METH enhances HIV infection of CD4⁺ T cells [15], monocyte-derived dendritic cells [17], and macrophages [18].

METH has been reported to exert immunomodulatory effects [19]. To date, the immunosuppressive effects of METH have been investigated in T cells [20, 21]. METH also significantly suppressed interleukin-2 (IL-2) production by splenocytes in mice model [22]. METH exposure inhibited macrophage-mediated antiviral and cytotoxic activities and reduced their ability to produce nitric oxide (NO)/TNF- α [23]. METH treatment induced an increased percentage of CD4⁺ cells with simultaneous decreased percentages of CD8⁺ and double-positive CD4⁺ CD8⁺ in thymus [22]. Microarray analysis of human brain tissue from HIV-infected METH users showed a significant up-regulation of genes associated with inflammation [24], which may contribute to enhancement of HIV infection *in vivo* [12]. Monocytes and macrophages, as the primary sites of HIV replication, are among the first cells infected by HIV/SIV and later function as reservoirs for the virus. Although abuse of drugs such as opioids has been implicated in modulation of function of monocytes/macrophages and microglia, there is limited information about the impact of METH on the functions of monocytes/macrophages. Thus, it is of a great interest to determine if METH has the potential to increase susceptibility of macrophages to HIV infection. There is a lack of direct evidence at the cellular and molecular levels to demonstrate the mechanisms (s) of METH actions on HIV. In this study, we investigated the impact of METH on intracellular restriction factors against the AIDS virus (HIV and SIV) in macrophages.

MATERIALS AND METHODS

Cell Culture

Peripheral blood was purchased from the Center for AIDS Research at the University of Pennsylvania. The protocol used to isolate blood from donors, purify the blood components, and distribute this material to the investigators was approved by the Institutional Review Board of the Center for AIDS Research. These blood samples were screened for all normal blood-borne pathogens and certified to be pathogen free. Monocytes were purified from peripheral blood of three healthy adult donors according to our previously described technique [25]. Freshly isolated monocytes were cultured in 48-well culture plates at a density of 2.5×10^5 cells/well in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum. Macrophages refer to 7-day-cultured monocytes *in vitro*.

Methamphetamine Treatment

METH was obtained from Sigma (St Louis, MO). Seven-day-cultured macrophages (2.5×10^5 cells/well) were treated with or without METH at different concentrations (100 and 250 μ M) for different time points (3, 6, and 24 h). The concentrations of METH used were based on previous dose response studies (100 and 250 μ M) that produced a maximum biological response without causing toxicity to the target cells and also were based on published *in vitro* studies [18,26,27]. These concentrations are similar to the levels found in blood, urine or tissue samples of METH users that range from 2 μ M to 600 μ M [28–31]. There were no cytotoxic effects of METH treatment on macrophages at concentrations of 1000 μ M or lower as demonstrated by the CellTiter 96® Aqueous Assay (Promega, Madison, WI) (Table 1).

Infection of Macrophages with HIV Bal Strain or SIV Delta_{B670} Strain

HIV Bal strain and SIV Delta_{B670} strain were obtained from the AIDS Research and Reference Reagent Program (NIH, Bethesda, MD). Macrophages were infected with equal amounts of cell-free HIV Bal (p24 20 ng/10⁶ cells) or SIV Delta_{B670} (p28 20 ng/10⁶ cells) for 2 h at 37°C after 24 h of treatment with or without METH. The cells were then washed three times with Dulbecco's modified Eagle's medium to remove any unabsorbed virus, and fresh media containing METH were added to the cell cultures. The final wash was tested for HIV/SIV reverse transcriptase (RT) activity and shown to be free of residual inocula. Untreated cells served as a control. Culture supernatants were collected for HIV/SIV RT activity assay at days 9, 12, 15 and 18 after virus infection.

HIV/SIV RT Assay

HIV and SIV RT activity was determined based on the technique [32] with modifications [33]. In brief, 10 μ l of culture supernatants from macrophages infected with or without HIV/SIV was added to a cocktail containing poly(A), oligo(dT) (Amersham Biosciences, Inc., Piscataway, NJ), MgCl₂, and [32P]dTTP (Amersham Biosciences, Inc.) and incubated for 20 h at 37°C. Then, 30 μ l of the cocktail was spotted onto DE81 paper (Whatman International Ltd, England), dried and washed five times with $2 \times$ saline-sodium citrate buffer and once with 95% ethanol. The filter paper was then air-dried. Radioactivity was counted in a liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA).

microRNA Extraction and Quantification

Total cellular RNA, including microRNA (miRNA, miR), was extracted from cells using miRNeasy Mini Kit from QIAGEN (Valencia, CA). Total RNA (1 μ g) was reverse-transcribed with miScript Reverse Transcription Kit from QIAGEN. The real-time RT PCR for the quantification of a subset of miRNAs (miRNA-28, miRNA-125b, miRNA-150, miRNA-198, miRNA-223, and miRNA-382) was carried out with miScript Primer Assays and miScript SYBR Green PCR Kit from QIAGEN as described [34,35].

RNA Extraction and Real-Time RT-PCR

Total RNA from macrophages was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described [36]. Total RNA (1 μ g) was subjected to reverse transcription using the RT system (Promega, Madison, WI) with random primers for 1 h at 42°C. The reaction was terminated by incubating the reaction mixture at 99°C for 5 min, and the mixture was then kept at 4°C. The resulting cDNA was then used as a template for real-time PCR quantification. Real-time PCR was performed with 1/10 of the cDNA with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) as previously described [37]. The amplified products were visualized and analyzed using the software MyiQ provided with the thermocycler (iCycler iQ real time PCR detection system; Bio-Rad Laboratories).

The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and sequences will be available upon request. The cDNA was amplified by PCR and the products were measured using SYBR green I (Bio-Rad Laboratories, Inc., Hercules, CA). The data was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented as the change in induction relative to that of untreated control cells.

Statistical Analysis

Student's t-test was used to evaluate the significance of difference between groups, and multiple comparisons were performed by regression analysis and one-way analysis of variance. Statistical analyses were performed with Graphpad InStat Statistical Software (Graphpad Software Inc., San Diego, CA), and all data are presented as mean $\pm$ SD. Statistical significance was defined as $P < 0.05$.

RESULTS

METH Enhances AIDS Virus Infection of Macrophages

We first determined the effect of METH on AIDS virus (HIV and SIV) infection of macrophages. The addition of METH to the cultures resulted in an increase in HIV RT activity (Fig. 1A). Similarly, METH treatment enhanced SIV Delta_{B670} replication (Fig. 1B). These effects of METH on HIV or SIV were time- and dose-dependent (Fig. 1).

METH Inhibits Anti-HIV miRNA Expression in Macrophages

Our recent study [34] demonstrated that freshly isolated monocytes from human blood expressed significantly higher levels of the cellular anti-HIV miRNAs (miRNA-28, miRNA-150, miRNA-223, and miRNA-382) than donor-matched macrophages. These miRNAs play a key role in suppressing HIV replication in monocytes and macrophages [34]. Thus, we examined whether METH has the ability to suppress these anti-HIV miRNA expression in macrophages. We observed that the expression of four anti-HIV miRNAs (miRNA-28, miRNA-150, miRNA-223 and miRNA-382) in macrophages treated with METH was lower than that in untreated cells (Fig. 2). The highest inhibition by METH was observed at a concentration of 250 μ M (Fig. 2). In addition, we found that METH treatment could inhibit miRNA-198 (a newly identified anti-HIV miRNA [38]) expression in macrophages (Fig. 2B). In contrast, METH treatment of macrophages had little effect on the expression of miRNA-125b (Fig. 2A).

METH Downregulates Type I Interferon (IFN) Expression

IFNs play a key role in host cell innate immunity against viral infections, including HIV/SIV. We thus examined whether METH has the ability to inhibit intracellular IFN gene expression in macrophages or not. We found that METH treatment significantly suppressed the expression of IFN- α (Fig. 3A), and IFN- β (Fig. 3B) in macrophages.

METH Suppresses RIG-I, IFN Regulatory Factors and APOBEC3

Since IFN regulatory factors (IRFs) have a crucial role in the regulation of IFNs [39–41], we examined whether METH treatment has the ability to modulate IRF expression. METH treatment of macrophages decreased the expression of IRF3 and IRF-5 (Fig. 4), but had little effect on IRF-7 expression (Fig. 4). As retinoic acid inducible gene I (RIG-I) plays a key role in IFN-mediated innate immunity against viral infections, we also examined the effect of METH on RIG-I expression in macrophages. As shown in figure, METH inhibited RIG-I expression in macrophages. In addition, we examined whether METH has the ability to inhibit apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) gene expression in macrophages, as several members of APOBEC3 family have the ability

to restrict HIV or SIV replication [42,43]. METH-treated macrophages expressed lower levels of several members (3C, 3G, and 3F) of APOBEC3 family than untreated macrophages (Fig. 5A–C). In contrast, METH had little effect on APOBEC3H expression (Fig. 5D).

METH Induces Suppressor of Cytokine Signaling (SOCS) and Protein Inhibitors of Activated STAT (PIAS)

To further explore the mechanism(s) involved in the METH action on HIV and IFN signaling pathway, we investigated the effects of METH on the negative regulatory factors of IFN pathway. SOCS and PIAS are two major families of negative regulators in signaling transduction induced by cytokines [44,45]. SOCS members form a classical negative feedback loop with key actions involving in inhibition of the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signaling cascade, while PIASs are specific inhibitors of STAT signaling. As demonstrated in figure, METH treatment of macrophages induced the expression of SOCS-2, 3 (Fig. 6A, B) and PIAS-1, 3, X, Y in macrophages (Fig. 6C–F), and had little effect of SOCS-1 (data not shown).

DISCUSSION

Epidemiological studies indicate that ~ 4.9% of Americans have tried METH at least once in their life [46]. METH addiction is prevalent among individuals with HIV infection. Although it has been reported that METH use is associated with HIV disease progression, much remains to be learned about the mechanisms of METH-mediated broad influence on host immunity related to control of HIV replication. In this study, we showed that METH significantly inhibited anti-HIV miRNA (Fig. 2) and endogenous type I IFN (IFN- α/β) expression (Fig. 3), which was associated with increased susceptibility of macrophages to HIV and SIV infection and enhanced virus replication (Fig. 1). We were particularly interested in miR-28, miR-150, miR-223, and miR-382, as these miRNAs can target highly conserved regions of HIV, present in all HIV clades [47]. These miRNAs are highly expressed in resting CD4⁺ T cells [48] and monocytes [34]. The levels of these miRNAs correlate with susceptibility of monocytes and macrophages to HIV infection [34]. Thus, the demonstration of inhibition of these miRNAs by METH provides a plausible mechanism for METH-mediated enhancement of HIV infection of macrophages. This METH-mediated inhibition of anti-HIV miRNAs was specific, as the expression of miR-125b (one of five identified anti-HIV miRNAs), was not affected by METH (Fig. 2A). Furthermore, we found that METH treatment of macrophages could inhibit miRNA-198 (the newly identified anti-HIV miRNA [38]) expression (Fig. 2B).

In order to further investigate the mechanism(s) responsible for the action of METH, we examined the effect of METH on the expression of IRFs, the key regulator of type I IFNs [49]. IRFs not only recognize the elements in the IFN promoter to modulate the expression of type I IFN genes selectively, but also regulate the IFN-stimulated response element (ISRE) in some of the IFN-stimulated genes (ISGs), leading to induction of an antiviral state [50,51]. Among IRFs, IRF-3, IRF-5 and IRF-7 are the key regulators of type I IFN gene expression induced by viruses [52]. IRF-3 is the master regulator of type I IFN-dependent immune response, as it not only induces IFN- α expression, but also activates many antiviral ISGs [49,53]. Therefore, the suppression of IRF-3 and IRF-5 expression (Fig. 4) in macrophages by METH treatment explained the inhibitory effect of METH on type I IFN expression. We also examined the expression of RIG-I, the key sensor to recognize viral infections and activate IFN signaling pathway [54]. A recent study showed that purified genomic RNA from HIV induced a RIG-I-dependent type I IFN response [55]. Thus, the suppression of RIG-I expression by METH (Fig. 4) should impair intracellular type I IFN-mediated innate immunity, providing a favorable environment for viral replication.

APOBEC3 family members are cellular cytidine deaminases that have the ability to inhibit the mobility of HIV [42,43]. Among the APOBEC3 family members, APOBEC3G and APOBEC3F have been identified to have the ability to restrict HIV replication in both CD4⁺ T cells and macrophages [56–59]. APOBEC3G can either edit the newly synthesized viral DNA or have an inhibitory effect through lethal editing of nascent reverse transcripts of the HIV life cycle [60–62]. APOBEC3F also encodes an antiretroviral protein that is selectively packaged into HIV virions and profoundly inhibits HIV infectivity [63]. APOBEC3B and APOBEC3C have been shown to act as the potent inhibitors of SIV replication [43]. Thus, the suppression of several key members of APOBEC3 family in macrophages by METH (Fig. 5A–C) provides additional justification for the enhancing effect of METH action on HIV or SIV infection and replication.

To further explore the mechanisms involved in METH-mediated enhancement of the AIDS virus infection of macrophages, we attempted to determine whether METH modulates the expression of the negative regulators of the JAK-STAT signaling pathway, the major pathway for type I IFN-mediated signaling and activation of gene expression [64]. The binding of IFNs to their specific receptors activate JAK-STAT pathway, which regulates the expression of immune system genes [65]. The activation of the JAK-SAT pathway is controlled by both positive and negative regulators. Among the negative regulator family members, SOCS-1 and SOCS-3 have been shown to inhibit IFN-mediated antiviral activities [66] and IFN signaling [67,68]. Members of the SOCS family play a key role in the inhibition of the JAK-STAT signaling cascade [45]. In addition, the members of PIAS are special inhibitors of STATs, as they have the ability to inhibit STAT-mediated gene activation by blocking the DNA binding activity of STATs [69,70]. The antiviral activity of IFN- γ or IFN- β was significantly increased in PIAS1^{-/-} cells [71]. Furthermore, PIAS1^{-/-} mice displayed increased protection against bacterial infection and showed enhanced antiviral response against vesicular stomatitis virus (VSV) infection [71]. We observed that METH treatment not only induced the expression of SOCS-2 and SOCS-3 (Fig. 6A, B), but also enhanced the expression of PIAS-1, PIAS-3, PIAS-X and PIAS-Y (Fig. 6C–F).

Taken together, our study provides compelling experimental evidence that METH enhances AIDS virus replication in macrophages through the modulation of multiple factors in IFN signaling pathway at both cellular and molecular levels. Although additional mechanisms might also be involved in the METH action on AIDS virus, to suppress the expression of endogenous IFNs and IFN-inducible antiviral genes should account for much of METH-mediated HIV or SIV enhancement in macrophages. Because METH exerts profound and detrimental effects on host cell innate immunity that has a critical role in restricting HIV or SIV replication in macrophages, it is likely that METH abuse is a cofactor in facilitating HIV disease progression.

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ABBREVIATIONS

METH	Methamphetamine
AIDS	Acquired immune deficiency syndrome
HIV	Human immunodeficiency virus
SIV	Simian immunodeficiency virus
miRNA	microRNA

FIV	Feline immunodeficiency virus
IL-2	Interleukin-2
NO	Nitric oxide
DMEM	Dulbecco modified eagle medium
RT	Reverse transcriptase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IFN	Interferon
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
RIG-I	Retinoic acid inducible gene I
IRF	IFN regulatory factor
SOCS	Suppressor of cytokine signaling
JAK	Janus Kinase
STAT	Signal Transducer and Activator of Transcription
PIAS	Protein inhibitors of activated STAT
ISRE	IFN-stimulated response element

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Fig. (1). Effect of METH on AIDS virus (HIV-1/SIV) replication in human macrophages Seven-day-cultured macrophages were incubated with or without METH at indicated concentrations for 24 h prior to (A) HIV-1 (Bal) or (B) SIV (B670) infection. Replication kinetics of HIV-1 Bal or SIV_{delta} B670 in cell culture were measured by RT activity in culture supernatants. Culture supernatants were collected at the indicated time points post-infection. The data shown are the mean of triplicate cultures, representative of three experiments using cells from three different donors (*, $p < 0.05$, **, $p < 0.01$, METH vs control).

Fig. (2). Effect of METH on anti-HIV miRNA expression of human macrophages
Seven-day-cultured macrophages were cultured with or without METH at indicated concentrations for 24h. Total cellular RNA extracted from cell cultures was subjected to realtime RT-PCR for miRNA expression: (A) miR-28, miR-125b, miR-150 and (B) miR-223, miR-382, miR-198. The data shown are the mean of triplicate cultures, representative of three experiments using cells from three different donors (*, $p < 0.05$, **, $p < 0.01$, METH vs control).

Fig. (3). Effect of METH on endogenous IFN-alpha and IFN-beta expression of human macrophages

Seven-day-cultured macrophages were cultured with or without METH at indicated concentrations for 3h or 6h. Total cellular RNA extracted from cell cultures was subjected to realtime RT-PCR for expression of IFN-alpha (**A**), and IFN-beta (**B**). The data shown are the mean of triplicate cultures, representative of three experiments using cells from three different donors (*, $p < 0.05$, **, $p < 0.01$, METH vs control).

Fig. (4). Effect of METH on IRF-3/5/7 and RIG-I expression of human macrophages
 Seven-day-cultured macrophages were cultured with or without METH at indicated concentrations for 6h. Total cellular RNA extracted from cell cultures was subjected to realtime RT-PCR for expression of IRF-3, IRF-5, IRF-7 and RIG-I. The data shown are the mean of triplicate cultures, representative of three experiments using cells from three different donors (*, $p < 0.05$, **, $p < 0.01$, METH vs control).

Fig. (5). Effect of METH on APOBEC3C/G/F/B expression of human macrophages

Seven-day-cultured macrophages were cultured with or without METH at indicated concentrations for 6h. Total cellular RNA extracted from cell cultures was subjected to realtime RT-PCR for expression of APOBEC3C (A), APOBEC3G (B), APOBEC3F (C) and APOBEC3B (D). The data shown are the mean of triplicate cultures, representative of three experiments using cells from three different donors (*, $p < 0.05$, **, $p < 0.01$, METH vs control).

Fig. (6). Effect of METH on suppressor of cytokine signaling (SOCS)-2/3 and protein inhibitors of activated STAT (PIAS)-1/3/X/Y expression of human macrophages

Seven-day-cultured macrophages were cultured with or without METH at indicated concentrations for 6h. Total cellular RNA extracted from cell cultures was subjected to realtime RT-PCR for expression of SOCS-2 (A), SOCS-3 (B), PIAS-1 (C), and PIAS-3 (D), PIAS-X (E), and PIAS-Y (F). The data shown are the mean of triplicate cultures, representative of three experiments using cells from three different donors (*, $p < 0.05$, **, $p < 0.01$, METH vs control).

Table 1

Cytotoxicity Effect of Methamphetamine

Treatment	Control	METH 1 μ M	METH 10 μ M	METH 100 μ M	METH 250 μ M	METH 500 μ M	METH 1000 μ M	METH 2500 μ M
MTS (O.D.)								
Mean	2.149	2.095	2.01	2.172	2.083	2.365	1.967	0.814
Std. Deviation	0.426	0.239	0.291	0.033	0.308	0.054	0.138	0.347
Significant	ns	ns	ns	ns	ns	ns	ns	**