

## HIV-1 Tat Protein-dependent Cytotoxicity is Attenuated by 15-deoxy-Delta<sup>12,14</sup>-Prostaglandin J2 in Rat Hippocampal Slices: Involvement of the ERK1/2 Signaling Pathway

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15-deoxy-delta<sup>12,14</sup> prostaglandin J2 (15d-PGJ2) may hold promise in treatment of the pathologies associated with human immunodeficiency virus (HIV) infection of the central nervous system. However, its precise role and neuroprotective mechanism in the hippocampus remain poorly understood. In the present study, rat hippocampal slices were stimulated with HIV-1 Tat protein to investigate the protective role of 15d-PGJ2 on the hippocampal cytotoxicity. Full-length HIV-1 Tat protein (Tat1-86), but neither its Tat32-62 nor Tat30-86 fragment, significantly induced cytotoxicity in the hippocampus, the brain region most commonly damaged in HIV-associated dementia. This Tat-induced cytotoxicity was associated with inactivation of MEK/extracellular signal-regulated kinase (ERK) signaling pathway. In contrast, Tat1-86 did not alter Wnt signaling pathway necessary for cell survival. Pretreatment of slices with 15d-PGJ2 markedly reduced Tat-driven cytotoxicity. Interestingly, this reduction was accompanied by suppression of ERK inactivation in response to Tat. Moreover, the inhibition of the MEK/ERK pathway with SL327 enhanced the Tat-induced cytotoxicity, confirming the ERK-dependent mechanism of Tat-driven cytotoxicity. Collectively, these data demonstrate that the protective action of 15d-PGJ2 against the hippocampal cytotoxicity upon Tat stimulation is exerted through suppression of Tat-mediated ERK1/2 inactivation.

**Key Words:** Hippocampus, HIV-1 Tat, Cytotoxicity, 15d-PGJ2, ERK, Wnt

### INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) infection can cause HIV-associated neurocognitive disorders (HAND) (1, 2), which is clinically characterized by cognitive, motor, and behavioral impairments. HIV-associated dementia (HAD) is the most severe form of HAND. Despite the availability

to HIV sufferers of combination active antiretroviral therapy (CART)/highly active antiretroviral therapy (HAART), prevalence of HANDs is continued to rise. Therefore, an understanding of the mechanisms through which HIV-1 induces the neuronal damage in the hippocampus, one of the brain regions most commonly damaged in HAD, is critical for the development of novel therapies aimed at reducing the neurotoxicity in patients suffering from HAND

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in the neuroCART era (3).

HIV-1 Tat is the product of two exons and act as a transactivator protein contributing to the induction of viral and cellular genes (4). Tat, a viral nonstructural protein of 86-104 amino acids in length, is secreted actively from HIV-infected T cells and monocytes/macrophages, and has neurotoxic properties (5). Secreted Tat also possesses proinflammatory attributes, inducing cytokine/chemokine production in microglia/macrophages and astrocytes via the activation of several signaling pathways (6~8). Tat is elevated in patients with known central nervous system (CNS) pathology, suggesting a possible role in the progression of HAD (4, 9). Interestingly, it has also shown that inhibition of Tat activity is associated with expression of protein kinase R that has a strong HIV-1 antiviral activity (10). However, the intracellular mechanism via which Tat triggers the neurotoxicity in the hippocampus remains to be clearly demonstrated.

A natural peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) ligand, 15-deoxy-delta<sup>12,14</sup>-prostaglandin J2 (15d-PGJ2), has been associated with reduced risk of cardiovascular disease, cancer, diabetes, and Alzheimer's disease. Moreover, recent evidence suggests that 15d-PGJ2 may exert a protective effect on the CNS under pathological conditions, as well as antiviral effects (11). Thus, current research interests for the treatment of neurodegenerative diseases are focused primarily on PPAR $\gamma$  agonists such as 15d-PGJ2, which is being clinically evaluated for potential improvement of cognition (12, 13). Recently, only minimal information is available regarding the effects of 15d-PGJ2 on HIV-1 Tat-induced cytotoxicity in the hippocampus, as well as the possible molecular pathways impinged upon by this process, despite of the intense research conducted to characterize the various biological activities of 15d-PGJ2.

In this context, we sought to probe the effect of 15d-PGJ2 on Tat-induced cytotoxicity and its underlying signaling pathways in the hippocampus. Our data demonstrate that Tat-induced cytotoxicity in the hippocampus is at least exerted through interfering extracellular signal-regulated kinase-1/2 (ERK1/2) phosphorylation as the consequence of Tat treatment. SL327, the ERK inhibitor, which eliminated

ERK1/2 phosphorylation, significantly enhanced the cytotoxicity in the Tat-exposed hippocampus. Furthermore, our data provide the first solid evidence that 15d-PGJ2 possesses a strong ability to reduce hippocampal cytotoxicity upon HIV-1 Tat stimulation and that the protective mechanism of 15d-PGJ2 is coupled to hippocampal repression of Tat-mediated inactivation of the ERK1/2 pathway.

## MATERIALS AND METHODS

### Materials

Rabbit polyclonal antibodies for ERK1/2 and phospho-ERK1/2 (Thr<sup>185</sup>/Tyr<sup>189</sup>) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish-peroxidase conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson Immuno Research (West Grove, PA, USA). 15d-PGJ2 and SL327 were purchased from Calbiochem (La Jolla, CA, USA). Anti- $\beta$ -catenin, anti-GSK-3 $\beta$  and anti-phosphorylated GSK-3 $\beta$  were obtained from BD Biosciences. Anti  $\beta$ -actin antibody and other chemicals were acquired from Sigma (St. Louis, MO, USA).

### Preparation of Tat

The HIV-1 Tat fragments Tat32-62 and Tat30-86 were purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Biologically active recombinant HIV-1 Tat1-86 of (HIV-1<sub>IIIb</sub>) was obtained from Advanced Bioscience Laboratories, Inc. (Kensington, MD, USA). A stock concentration of 50  $\mu$ M Tat peptides was made up fresh each time in distilled H<sub>2</sub>O to add to slice culture medium. Heat-inactivated Tat1-86 (Tat1-86<sub>hi</sub>), which was prepared by incubation at 85°C for 1 h, was used to show the specificity of Tat1-86-mediated effect.

### Hippocampal slice cultures

Hippocampal slice cultures were prepared from 10-day-old Sprague-Dawley rat pups (Harlan, Indianapolis, IN, USA) as previously described (14, 15). All experimental procedures were performed using protocols approved by the Animal Care Committee of the Ewha Medical Research Institute. In hippocampal slices, cells, synapses, and neuronal

connectivity mature in parallel with their counterparts *in vivo* (16). Thus, these preparations are deemed appropriate for studies of prolonged pharmacological treatment and recovery without blood flow, which would be difficult to perform in an intact animal system.

### Experimental treatments

Treatments were initiated at 10 days post-dissection. All reagents were added to serum-free medium (no horse serum) equilibrated at 37°C in 5% CO<sub>2</sub> prior to addition to the slices. Tat was added to the slice cultures as previously described (14, 15). To determine the effects of specific inhibition of Tat-induced responses, cells were pretreated with 15d-PGJ2 for 1 h before further stimulation with Tat1-86 (1 µM) for 3 days. Vehicles were prepared identically to experimental treatments but did not contain Tat. Vehicle alone exerted no detectable effects on cell viability. At the indicated times after treatment initiation, the slices were rinsed twice in phosphate-buffered saline (PBS), then harvested via the removal of Millicell membrane inserts after sample freezing on dry ice. They were then processed for immunoblotting as described below.

### Mitochondrial dehydrogenate activity assay

Cell viability was assayed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method as previously described (15). Only viable slices are able to reduce MTT into a formazan product. After 3 days treatment of slice culture with Tat or Tat fragments, MTT was added to the medium (1 mg/ml) and incubated for 4 h at 37°C. The medium was removed and the slices were quickly removed from the membrane and completely diluted in 600 µl of 1 N HCl:isopropyl alcohol (1:24) and incubated for 30 min at room temperature with shaking. After centrifugation, the relative formazan concentration in each supernatant was measured by determination of the absorbance at 570 nm in a microplate reader.

### Polyacrylamide gel electrophoresis and immunoblotting

Immunoblotting analysis was conducted as recently described (14, 15). Equal quantities of sample proteins of

whole cell extracts were separated by molecular weight on 10% SDS polyacrylamide gels and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were probed with primary antibody diluted in 1% milk and incubated overnight at 4°C. Signal was acquired with enhanced chemiluminescence after incubation with horseradish-peroxidase conjugated secondary antibodies (Jackson). Densitometric values were normalized versus β-actin.

### Statistical analysis

Differences among groups were evaluated for statistical significance using one-way ANOVA with a Student's *t* test. Data were expressed as the mean ± SEM of the indicated numbers of experimental replicates. Null hypotheses of no difference were rejected if *p*-values were less than 0.05.

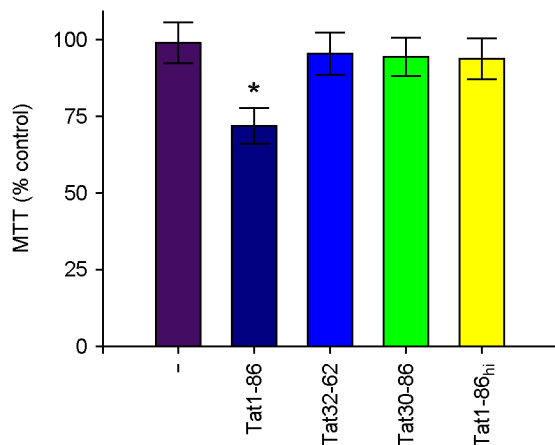
## RESULTS

### Tat1-86 induced the cytotoxicity in the hippocampus

Our recent study demonstrated that 20 h exposure to 1 µM Tat1-86 did not induce cell toxicity in cultured rat hippocampal slices as demonstrated by LDH release assay, a marker of tissue necrosis and MTT reduction assay, a marker for a decline of cell viability (14). However, longer exposure of hippocampal slices to 1 µM Tat1-86 for 3 days clearly induced a decline of cell viability as measured by the MTT reduction assay as shown in Fig. 1. However, Tat fragments, Tat32-62 and Tat30-86, did not induce cell toxicity under the same experimental conditions. Heat-inactivated Tat1-86 showed little cytotoxicity, confirming the specificity of biologically active recombinant Tat1-86 protein effect in the hippocampus.

### Tat decreased the ERK1/2 signaling pathway in the hippocampus

Critical roles of Mitogen-activated Protein Kinases (MAPK) have been recently implicated in inflammation (17). Furthermore, our earlier study clearly demonstrated that ERK1/2 activation mediated Aβ oligomer-induced neurotoxicity in rat organotypic hippocampal slice cultures

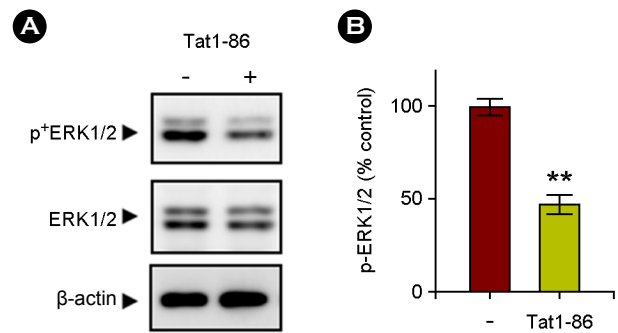


**Figure 1.** Influences of Tat on cell toxicity of hippocampal slices. Hippocampal slices were exposed to 1  $\mu$ M of Tat1-86, Tat fragments, or heat inactivated Tat1-86 (Tat1-86<sub>hi</sub>) as indicated for 3 days. MTT reduction activity in Tat-treated slices were assayed as described in materials and methods. Values are expressed as the means  $\pm$  SEM of triplicate experiments. \*,  $p < 0.05$  versus vehicle-treated samples.

(14). In order to further describe the potential involvement of ERK signaling in the hippocampus undergoing Tat-induced cytotoxicity, we attempted to determine whether ERK signal transduction was altered. As shown in Fig. 2, the stimulation of hippocampal slices by Tat1-86 for 3 days resulted in a prominent decrease in ERK1/2 phosphorylation (Fig. 2A and B). This finding indicates that longer exposure of hippocampal slices to Tat1-86 significantly inhibited the activation of the ERK1/2 signaling pathway.

#### Tat did not dysregulate the Wnt/ $\beta$ -catenin signaling pathway in the hippocampus

$\beta$ -catenin is a multifunctional protein, which serves as a cell signaling component in the Wnt pathway for cell survival and as a structural element in the cell adhesion involved in neuroplasticity and cognition (18). GSK-3 $\beta$  is a critical component of the multimolecular complex responsible for targeting  $\beta$ -catenin to proteasome in the Wnt pathway (19). Recent study reported that HIV-1 Tat exerted down regulation of  $\beta$ -catenin signaling in human astrocytes (20). To examine the relevance of Wnt pathway and the interaction between  $\beta$ -catenin and GSK-3 $\beta$  associated with Tat-induced cytotoxicity in the hippocampus, the effect of

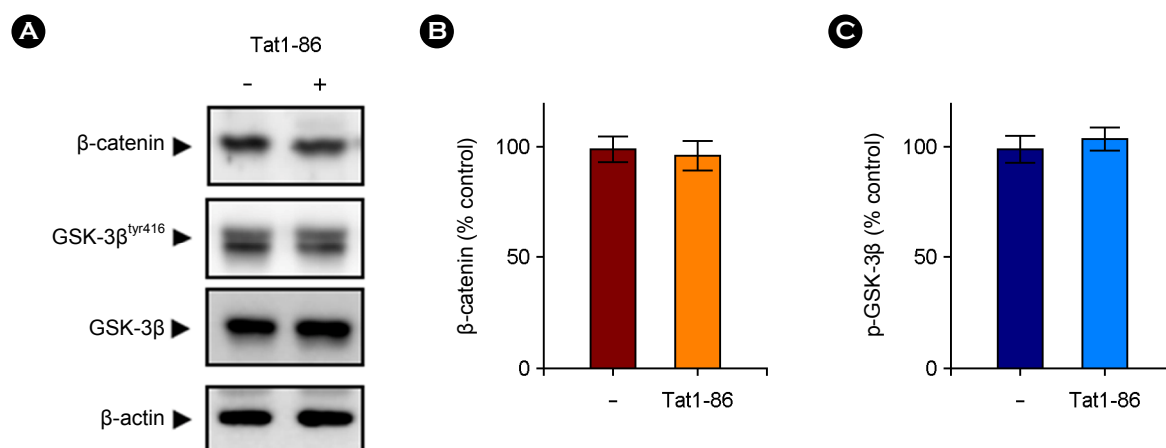


**Figure 2.** Tat1-86 decreased the activation of ERK1/2 signaling pathway. The slices were treated for 3 days with Tat1-86 (1  $\mu$ M) or vehicle only. (A) Immunoblots showing the Tat-induced inactivation of the ERK1/2 signaling pathway in hippocampal slices. Total lysates were analyzed via immunoblotting with phospho-antibody against the activated form of ERK1/2 or anti-total ERK1/2 evidencing decreased ERK1/2 phosphorylation. The blots were stripped and developed with anti- $\beta$ -actin for equal protein loading. The data represent three independent experiments. (B) A normalized densitometric quantification of ERK1/2 activity against- $\beta$ -actin for equal protein loading. The results of triplicate experiments are expressed as the means  $\pm$  SEM. \*\*,  $p < 0.01$  versus vehicle-treated samples.

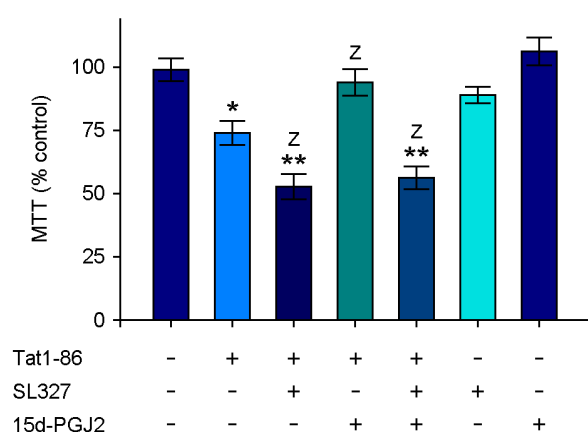
Tat1-86 on levels of  $\beta$ -catenin and GSK-3 $\beta$  were analyzed under the same experimental conditions. As shown in Fig. 3, Tat1-86 did not induce a decrease in  $\beta$ -catenin level (Fig. 3A and C). Furthermore, the levels of either GSK-3 $\beta$  or the phosphorylated form of active GSK-3 $\beta$  were not altered by Tat1-86 stimulation (Fig. 3A and B). These findings together suggest that Tat treatment did not evoke a dysregulation of Wnt signaling pathway in the hippocampus undergoing Tat1-86-induced cytotoxicity.

#### Tat-dependent cytotoxicity was reduced by 15d-PGJ2 in the hippocampus

To test whether the endogenous PPAR $\gamma$  ligand 15d-PGJ2 inhibits the HIV-1 Tat-induced cytotoxicity in the hippocampus to impair cognitive function, we performed MTT reduction assays. 15d-PGJ2 significantly suppressed HIV-1 Tat-driven cytotoxicity in rat hippocampal slices (Fig. 4), but exerted minimal effects when administered alone. To characterize the ERK signaling pathway relevant to the observed Tat-induced cytotoxicity in the hippocampus, we evaluated the result of the blockage of ERK pathway with pharmacological agent, SL327 which is known to selectively



**Figure 3.** Effects of Tat1-86 on the Wnt signaling pathway in the hippocampus. (A) Western blotting was used to measure the proteolysis of  $\beta$ -catenin and GSK-3 $\beta$  using total lysates of hippocampal slices treated for 3 days with Tat1-86 (1  $\mu$ M) or vehicle only as described for Fig. 2. Representative western blots showed that  $\beta$ -catenin level was not changed after Tat treatment for 3 days. Similarly, levels of the active form of GSK-3 $\beta$  (phosphorylated GSK-3 $\beta^{\text{tyr216}}$ ) and non-phosphorylated GSK-3 $\beta$  were not altered. (B and C) Quantitative analysis of A, showing the levels of  $\beta$ -catenin (B) and GSK-3 $\beta$  (C) normalized to  $\beta$ -actin. Numbers represent the means  $\pm$  SEM ( $n=3$ ). Representative gels from three experiments are shown.



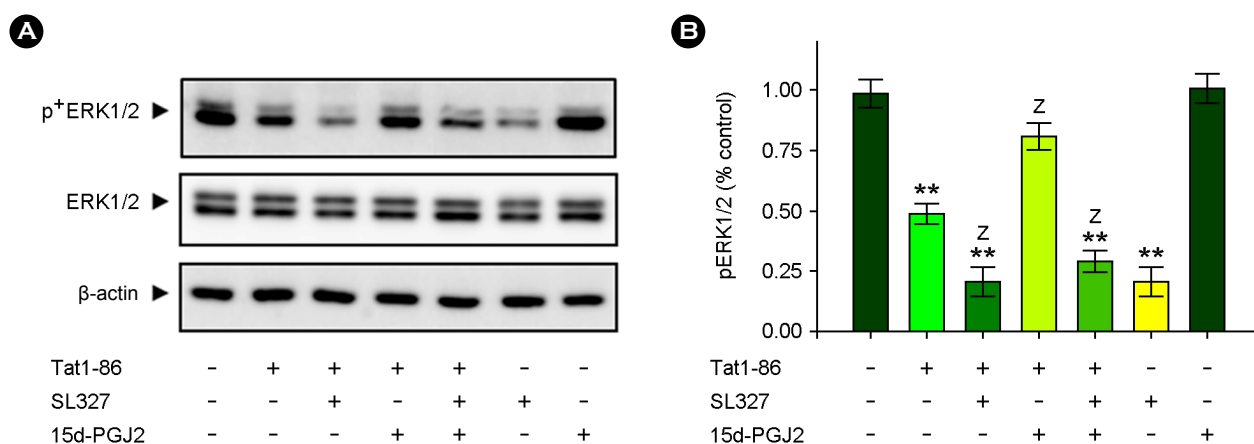
**Figure 4.** 15d-PGJ2 attenuated Tat-dependent cytotoxicity in the hippocampus. Hippocampal slices were pretreated with 15d-PGJ2 (5  $\mu$ M) and/or SL327 (10  $\mu$ M) for 1 h and then stimulated with Tat1-86 (1  $\mu$ M) for 3 days. MTT reduction activity in Tat-treated slices was measured as described in Fig. 1. Values are expressed as the means  $\pm$  SEM ( $n=5$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus vehicle-treated samples; Z,  $p < 0.05$  versus Tat-treated samples.

block the activity of MAPK kinase (MEK1/2), the activator of ERKs (ERK1/2). In contrast to 15d-PGJ2, SL327, which is capable of crossing the blood-brain barrier, increased the Tat-induced cytotoxicity while protective effect of 15d-PGJ2 was significantly decreased by SL327. These results together indicated that 15d-PGJ2 possesses a pronounced ability to

reduce the hippocampal cytotoxicity upon Tat stimulation in the hippocampus and confirmed that the Tat-induced cytotoxicity is associated with decreased ERK1/2 signaling pathway.

#### 15d-PGJ2 inhibits Tat-induced cytotoxicity through ERK1/2 activation in the hippocampus

Numerous studies have shown that 15d-PGJ2 can modulate mitogen-activated protein (MAP) kinase pathways independent of PPAR $\gamma$ . In an effort to further define the molecular target that mediates 15d-PGJ2's protective action against the hippocampal cytotoxicity provoked by Tat1-86, we evaluated the effect of this compound on a decreased ERK1/2 phosphorylation in response to Tat stimulation for 3 days. 15d-PGJ2 significantly rescued Tat-induced ERK1/2 inactivation (Fig. 5A and B). However, SL327, which increased the observed Tat-induced toxicity, further decreased ERK1/2 phosphorylation in the Tat-stimulated slices (Fig. 5A and B). In addition, 15d-PGJ2-mediated rescue of Tat-induced ERK1/2 inactivation was decreased by SL327-treatment. Collectively, these findings verified that Tat-mediated cytotoxicity involves the specific inactivation of ERK1/2 kinase and that reactivation of the ERK1/2 pathway participates in the 15d-PGJ2-mediated protection against



**Figure 5.** 15d-PGJ2 exerted its protective action against Tat-induced cytotoxicity through ERK1/2 activation. Hippocampal slices were pretreated with 15d-PGJ2 (5  $\mu$ M) and/or SL327 (10  $\mu$ M) for 1 h and then stimulated with Tat1-86 (1  $\mu$ M) for 3 days. Total lysates were analyzed via immunoblotting as described in Fig. 2. (A) Western blots showed that 15d-PGJ2 suppressed Tat-induced inactivation of the ERK1/2 pathway in hippocampal slices. (B) A normalized densitometric quantification of ERK1/2 activity against  $\beta$ -actin for equal protein loading. The results of experiments are expressed as the means  $\pm$  SEM (n=5). \*\*,  $p < 0.01$  versus vehicle-treated samples; Z,  $p < 0.05$  versus Tat-treated samples.

Tat-induced cytotoxicity in the hippocampus.

## DISCUSSION

This study clearly demonstrates that longer exposure of full length of Tat for 3 days elicited the cytotoxicity in the hippocampus, which is primarily linked to cognitive function, and that this Tat-induced cytotoxicity is at least exerted through interfering ERK1/2 phosphorylation as the consequence of Tat treatment. Furthermore, SL327 that reduced ERK1/2 phosphorylation significantly enhanced cytotoxicity in the Tat-exposed hippocampus. Importantly, our data clearly demonstrated that 15d-PGJ2 possesses a profound ability to reduce HIV-1 Tat-mediated cytotoxicity in the hippocampus and that the mechanism for the protective effect of 15d-PGJ2 is coupled to repression of inactivation of the ERK1/2 pathway elicited by Tat in the hippocampus, the brain region most commonly damaged in HIV-associated dementia.

Infection with HIV-1 causes degeneration of neurons leading to motor and cognitive dysfunction in AIDS patients. One of the key viral regulatory proteins, Tat, which is released by infected cells, can be taken up by various uninfected cells including neurons, and induces cell injury

and death by dysregulating several biological events. HIV-1 Tat has been shown to induce neurotoxicity in rat fetal primary neuronal cell cultures prepared from hippocampus, midbrain, or cortex (21). Indeed, extracellular Tat is known to induce caspase activation in primary cultures of rat fetal neurons (22, 23). Importantly, our recent study showed that full-length bioactive Tat1-86 or Tat fragments such as Tat32-62 and Tat30-86, all of which evidence neurotoxic properties (24, 25), elicited no direct neurotoxicity in the hippocampal slices, which treated for 20 h as recently reported (15). However, longer exposure to Tat1-86 for 3 days significantly induced hippocampal cytotoxicity as measured by MTT reduction assay under the experimental conditions utilized herein. This observation is in part consistent with a previous study (26). Furthermore, our findings also indicate that HIV-1 Tat protein itself can induce the cytotoxicity in the hippocampus prior to leukocyte recruitment based on the finding that the hippocampal slice culture system is an appropriate system for the elimination of peripheral leukocyte infiltration (16).

Our data provide the first solid evidence that the specific inactivation of MEK-mediated ERK1/2 kinase performed a central function in the hippocampal cytotoxicity in response to Tat stimulation. Recent studies reported that

neuroprotection of CCL2 against HIV-1 Tat toxicity in rat primary midbrain neurons (27) was dependent on ERK activation and that ERK activation was also critical for PDGF-mediated protection against Tat toxicity in SH-SY5Y cells or in mice (28, 29) although a direct suppression of ERK phosphorylation by Tat was not seen. These observations along with our finding that SL327 significantly enhanced the cytotoxicity in the Tat-exposed hippocampus support a critical role of Tat-mediated ERK inactivation in induction of hippocampal cytotoxicity. Conflicting reports dispute the Tat-mediated activation of ERK signaling (15, 30). Such discrepancies may be attributable to different tissues, different concentrations of Tat and preparations of Tat, or different treatment schemes. It is interesting to note that SL327 alone had a little effect, which might suggest multiple signaling pathways involved. Further experiments also remain to clarify the effect of 15d-PGJ2 on SL327-mediated ERK inactivation.

Consistently, our study also indicates that the cyclopentanone prostaglandin 15d-PGJ2, a natural PPAR $\gamma$  ligand, possesses specific capacity to reduce hippocampal cytotoxicity upon HIV-1 Tat stimulation, and that it likewise reduces Tat-dependent cytotoxicity in the hippocampus through activation of the ERK1/2 signaling pathway. This observation is in good agreement with recent reports demonstrating that 15d-PGJ2 can induce ERK activation (31, 32). It has been shown that not all of 15d-PGJ2's effects in the body are due to PPAR $\gamma$  activation. For instance, 15d-PGJ2 can regulate the expression of numerous molecules, including HO-1, cytokines, and caspases, independent of PPAR $\gamma$  (33, 34), and may signal through several PPAR $\gamma$ -independent pathways, among them that of MAPK (33, 35, 36). Indeed, our study verified that reactivation of the ERK1/2 MAP-kinase pathway participates in the 15d-PGJ2-mediated protection against Tat-induced cytotoxicity in the hippocampus. This observation was strongly supported by the report demonstrating that independent of its PPAR $\gamma$  activity, 15d-PGJ2 protected RPE cells from oxidative stress via ERK1/2 activation (37). Yet another study reported that 15d-PGJ2 upregulated vascular endothelial growth factor in MCF-7 cells via ERK1/2 activation independent of

PPAR $\gamma$  (38). These findings altogether suggest pleiotropic effects of 15d-PGJ2 independent of its PPAR $\gamma$  activity via ERK1/2 activation in various cell types. Interestingly, our study showed that partial rescue of Tat-induced ERK-inactivation by 15d-PGJ2 led to almost complete inhibition of the Tat-dependent cytotoxicity. This strongly suggests the multiple mechanisms underlying 15d-PGJ2-mediated protection against Tat in the hippocampus. Given that 15d-PGJ2 covalently modifies Tat and inhibits its transcriptional activity (11), modified Tat by 15d-PGJ2 could be less toxic, leading to partial reduction of Tat-induced cytotoxicity in the hippocampus although further study remains to examine this possibility.

In conclusion, our data demonstrate that Tat can suppress the ERK1/2 pathway to augment the cytotoxicity in the hippocampus. Our data also indicate the existence of a protective mechanism whereby 15d-PGJ2 activates the ERK1/2 pathway, at least in part reducing hippocampal Tat-induced cytotoxicity. Thus, our findings imply that a therapeutic strategy to enhance an endogenous level of 15d-PGJ2 to reduce Tat-dependent cytotoxicity in the hippocampus through sustained activation of ERK1/2 pathway is likely to be beneficial to HIV-infected patients in the neuroCART era for delaying development of HAND/HAD. However, the relevance of our findings in the *in vivo* context remains to be definitively elucidated.

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