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Ephedrine enhances HIV-1 reactivation from latency through elevating tumor necrosis factor receptor II (TNFRII) expression

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ABSTRACT

HIV-1 persists during antiretroviral therapy (ART) due to long-lived and proliferating latently-infected host cells, with the outcome being an incomplete cure. The latently-infected cells, or reservoir cells, are transcriptionally absent and invisible to the immune response. Elimination of latency is one strategy in activating virus production, making it visible to immune clearance. We previously showed that Ephedrae herba reactivated HIV-1 from latency. In this study, we used ephedrine, a major component of Ephedra herba, to reactivate HIV-1 from latency. The results showed that ephedrine enhances HIV-1 reactivation in the presence of TNFα. Combination treatment demonstrates a synergistic effect of HIV-1 reactivation compared to TNFα alone. Ephedrine treatment shows a higher TNFRII expression level, which is related to increased HIV-1 reactivation. However, the mechanism of ephedrine in HIV-1 reactivation is still unclear, and may be related to TNFRII receptor expression. Our results indicate that ephedrine enhances HIV-1 reactivation from latency in combination with TNFα treatment. This new reagent could be a promising latency reversal agent (LRA).

1. Introduction

HIV-1 provirus persists in reservoir cells such as resting memory CD4 T cells and macrophage-monocyte lineages [1]. HIV-1 provirus integrates into the genomic DNA of reservoir cells but transcriptionally silences them [2, 3]. The current combined antiretroviral therapy (cART) does not offer clearance of reservoir cells, while interruption of cART results in a rapid HIV-1 rebound from latency [4, 5, 6, 7]. Clearance of both HIV-1 and reservoir cells is still the ideal curative treatment for HIV-1 infection [8]. Approaches to eradicate reservoir cells require a Shock-and-Kill strategy that activates HIV-1 replication from latency [2, 9, 10]. Inducing the expression of latent provirus using this strategy can elevate the expression of latent HIV-1 provirus, and HIV-1 latent infected cells can be killed by the host immune system and virus-induced apoptosis [9].

Numerous studies regarding latency reversal agents (LRAs) such as histone deacetylase (HDAC) inhibitors, methylation inhibitors, and cytokines were successful in reactivating HIV-1 from latency *in vitro* [11, 12, 13, 14]. However, current LRAs are unable to diminish the reservoir size [15]. Thus, the development of new or more selective and effective LRAs would increase the chances of eradicating HIV-1 reservoirs [16]. Our previous report revealed that Ephedrae herba, one of the components of the traditional Japanese herbal medicine Mao-to, enhanced HIV-1 reactivation from a latent-infected promonocytic cell line, U1 [17]. U1 cell line derived from U937, is the chronically HIV-1 infected cell line and one of well-characterized models of HIV-1 latency [18]. The cells contain two copies of integrated proviruses [19]. One of the *tat* cDNAs has the starting codon mutation (ATG→ACG) that changes methionine to a threonine amino acid. Another *tat* cDNA harbors H13L (CAT→CTT) mutation that changes a histidine residue at position 13 to a leucine [20]. Nevertheless, cytokine stimulation can induce HIV-1 reactivation in U1 cells.

In this work, we studied the effects and mechanism of ephedrine, the main component of Ephedrae herba, on the reactivation of HIV-1 from latency. We found that ephedrine reactivates HIV-1 from latency by combination with TNFα. In addition, we found that ephedrine increases TNFRII expression, which causes HIV-1 reactivation with TNFα in U1 cells.

2. Materials and methods

2.1. Cell culture and treatment

Latent HIV-1 infected U1 cells (derived from U937 cells) were supplied by the AIDS Research and Reference Reagent Program, Division of

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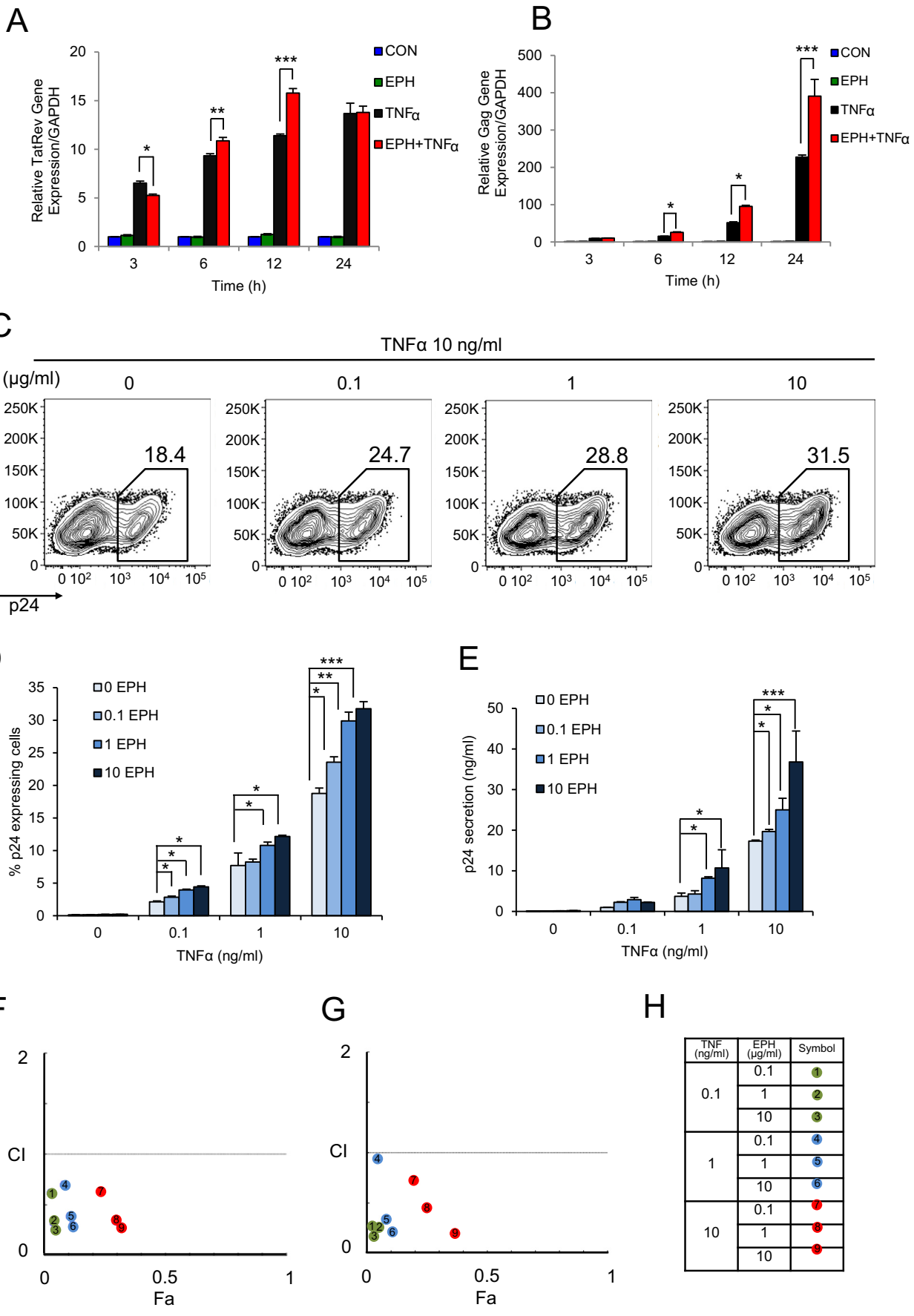

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Fig. 1. Ephedrine treatment enhances HIV-1 Reactivation in mRNA and protein level. Ephedrine treatment enhances HIV-1 TatRev (A) and Gag (B) mRNA expression. (C) Ephedrine treatment enhances HIV-1 p24 expression in dose dependent manner. Ephedrine treatment enhances the level of p24 expressing cells (D) and p24 secretion (E). One-way analysis of variance (ANOVA) with the Dunnett's multiple-comparison test was used to analyze the data. Differences at *P < 0.05, **P < 0.01, ***P < 0.001 were considered significant. The data represents from triplicate experiments. Synergistic effects on p24 intracellular expression (F) and p24-secretion (G) with ephedrine and combination treatment. (H) Concentrations of combination treatment in this study. Drug interactions between ephedrine and TNFα were assessed using a combination index (CI) calculated with CompuSyn software (ComboSyn, Inc. NJ, USA.), where CI < 1, CI = 1, and CI > 1 indicated synergistic, additive, and antagonistic effects, respectively.

AIDS, National Institute of Allergy and Infectious Diseases, NIH (Rockville, MD). Cells were cultured at 37 °C in 5% CO_2 in RPMI 1640 (Gibco by Life Technologies, NY) supplemented with 10% fetal bovine serum (Thermo scientific, UT). Human TNFα (Peprotech INC, NJ) and ephedrine (Dainihon Sumitomo Pharmaceuticals co. ltd, Osaka, Japan) were used to treat U1 cells.

2.2. Viability assay

The viability of ephedrine-treated U1 cells was measured by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Sigma-Aldrich, MO). Briefly, 2×10^4 cells were incubated in a 96-well plate in the presence of various concentrations of ephedrine in a final volume of 100 μl for 24 and 48 h at 37 °C. Subsequently, MTT solution was added to each well to give a final concentration of 0.5 mg/ml. After 4 h of additional incubation, 100 μl of 0.04 N HCl was added to dissolve the formazan crystals. Finally, double absorbances at 595/630 nm were measured by an ELISA plate reader (Bio-Rad, CA). Values were normalized to non-treatment (cell control) samples. Percentage cell viabilities compared with controls were calculated using the following formula:

$$\%\ \text{cell viability} = 100 \times \frac{(\text{OD595} - \text{OD630})\ \text{experiment}}{(\text{OD595} - \text{OD630})\ \text{cell control}}$$

2.3. Quantitative real time PCR

Total RNA was isolated from cells using RNAiso Plus (TaKaRa Bio, Kusatsu, Japan). Extracted RNA was reversed transcribed to cDNA (PrimeScript RT Master Mix, Takara Bio). Real-time PCR was carried out with Fast SYBR green master mix (Applied Biosystems, CA) to detect the level of HIV-1 Gag and Tat-Rev mRNA expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization. The *CT* values of each gene and GAPDH were measured, and the relative expression values ($2^{-\Delta\Delta CT}$) were determined. The primers used in this study are as follows: Tat-Rev (5′-ATGGCAGGAAGAAGCGGAG-3′, 5′-ATTCCTTCGGGCCTGTCG-3′), Gag (5′-TGTGGAAAATCTCTAGCAGTGG-3′, 5′-CGCTCTCGCACCCATCTC-3′), GAPDH (5′-CGGGAAGCTTGTGATCAATGG-3′, 5′-GGCAGTGATGGCATGGACTG-3′)

2.4. Flow cytometry analysis

To detect intracellular p24, cells were fixed with 1% paraformaldehyde for 20 min at room temperature. Cells were then permeabilized in 0.1% saponin containing PBS for 10 min, and stained with FITC-conjugated anti-HIV-1 p24 mAb (Beckman Coulter, CA) for 30 min on ice. For TNFRI and TNFRII receptor staining, cells were stained with anti-CD120a-PE (clone W15099A, Biolegend, CA) and anti-CD120b-APC (clone 3G7A02, Biolegend) for 30 min on ice, then washed and fixed as described above. Cells were analyzed by LSR II flow cytometry (BD Bioscience, CA). Data were analyzed with FlowJo version 9.9 software (Tree Star, CA).

2.5. Enzyme linked immunosorbant assay (ELISA)

The amounts of HIV-1 p24 antigen were determined using an HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corp., NY) according to the manufacturer's instruction [18].

2.6. Western blot

U1 cells (5×10^6) were seeded into 10 cm for 2 dishes, treated with TNFα and/or ephedrine, and nuclear protein was recovered using Schreiber's methods as described elsewhere [21]. Briefly, cells were collected and washed with cold PBS. Then, cold buffer A (10 mM HEPES KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 0.1% NP-40, 0.5 mM DTT) was added and mixed with cells following incubation on ice for 10 min, vortex 10s, and centrifugation. The supernatant was collected as cytoplasmic protein. The pellet was then washed twice with cold buffer A to eliminate cytoplasmic contamination. Then, 70 μl of buffer C (50 mM HEPES KOH pH 7.9, 10% glycerol, 420 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) was added to the pellet and sonicated 10s 10 times. Next, the nuclei were incubated on ice 3 h. The nuclei were centrifuged and supernatant was collected to achieve nuclear protein. NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, 1mM EDTA) was used in case of whole cell lysate extraction. The amount of protein was measured by Bradford assay. The protein (20 μg for cytoplasmic protein/whole cell lysate, and 40 μg for nuclear protein) was loaded on to 10% or 12% SDS-PAGE and subsequently transferred onto a PVDF membrane. The antibodies used in this study included anti γ-tubulin (C-20), anti-p65 (F-6), anti-actin (C-2), and anti-lamin B1 (A-11) (Santa Cruz Biotechnology Inc.). The anti-p–NF–kappaB p65 (93H1) is from Cell Signaling Technology Inc. Protein signals were detected by ImageQuant Biomolecular Imager (GE Life Sciences, Uppsala, Sweden).

2.7. Statistical analysis

Significance of differences was determined using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test or Student's *t*-test (Graphpad Prism Ver 6, CA). *P < 0.05, **P < 0.01, and ***P < 0.001 were defined as significant. Drug interactions between ephedrine and TNFα were assessed using a combination index (CI) calculated with CompuSyn software (ComboSyn, Inc. NJ, USA.) [22], where CI < 1, CI = 1, and CI > 1 indicated synergistic, additive, and antagonistic effects, respectively.

3. Results and discussion

3.1. Ephedrine enhances HIV-1 reactivation by combined treatment with TNFα

Persistent integration of HIV-1 provirus in host genomic DNA results in silencing of HIV-1 transcription and protein expression. The induction of HIV-1 provirus transcription is the platform for HIV-1 reactivation by LRA treatment [23]. TNFα is well-known to reactivate HIV-1 from latency through the NF-κB signaling pathway [24]. Nuclear translocation of p65 was observed to be a major pathway of HIV-1 replication [25]. We examined the HIV-1 mRNA expression with ephedrine (10 μg/ml) and/or (TNFα 10 ng/ml). The concentration of ephedrine in this study did not affect cytotoxicity (Supplementary Fig. 1). As shown in Fig. 1A, TNFα treatment caused a time-dependent increase of Tat-Rev mRNA expression. Ephedrine and TNFα in combination caused a significant increase in Tat-Rev mRNA expression at 6 and 12 h post-treatment. Tat-Rev is an HIV-1 regulatory gene, and is expressed at an early phase of HIV-1 gene transcription [26, 27]. In contrast to Tat-Rev, Gag is expressed late [26];

a gradual rise of Gag mRNA expression was observed following TNFα treatment. Similarly, there was a significant increase in Gag mRNA expression following the combination treatment compared to TNFα treatment alone at 6, 12, and 24 h (Fig. 1B).

Enhanced HIV-1 provirus transcription leads to an increase in HIV-1 protein expression. We analyzed the levels of HIV-1 p24 Gag intracellular protein and p24 Gag secretion after treatment with TNFα and/or ephedrine. A dose-dependent increase in HIV-1 reactivation was observed with different concentrations of ephedrine (Fig. 1C). Combination treatment with ephedrine at 0.1, 1, and 10 μg/ml and TNFα revealed 24.7%, 28.8%, and 31.5% of p24-expressing cells, respectively, whereas TNFα treatment alone gave 18.4% of p24-expressing cells. Next, we analyzed the p24-expressing cells and secretion of p24 with varying concentrations of TNFα and ephedrine. The results showed significant and dose-dependent increases in p24-expressing cells and secretion of p24 protein (Fig. 1D and E). We analyzed the synergistic effects using CompuSyn software based on the Chou-Talalay Method [22]. The combination index (CI) value indicates the effect of combination treatment, and is divided into synergism (CI $<$ 1), additive effect (CI $=$ 1) and antagonism (CI $>$ 1). Fig. 1F and G show that the combination of TNFα and ephedrine had a synergistic effect on HIV-1 reactivation. As shown in Table 1, a synergistic effect was evident for higher doses of ephedrine. Taken together, the enhancement of HIV-1 reactivation was increased by ephedrine, and the enhancement level was dependent on the ephedrine concentration.

3.2. NF-κB is not major mechanism of ephedrine in HIV-1 reactivation

A previous report revealed that Ephedrae herba activates HIV-1 replication from latency through the activation of p65 nuclear translocation [17]. The NF-κB signaling pathway is important for regulating HIV-1 expression. NF-κB is activated (phosphorylated) with TNFα stimulation, translocates into nucleus, and induces HIV-1 replication. Therefore, we examined the expression of the total p65 and phosphorylated NF-κB (p-p65) in whole cell lysate, cytoplasm, and nucleus. After 4 h of the treatment with ephedrine (10 μg/ml) and TNFα (10 ng/ml), we examined the level of phosphorylated p65 (p-p65) and p65 in whole cell lysate, cytoplasmic protein, and nuclear protein. The results demonstrated that p-p65 in whole cell lysate increased with TNFα single treatment but not with ephedrine. Nonetheless, combination treatment with TNFα and ephedrine did not show the increase of p-p65 expression compared with TNFα single treatment in whole cell lysate. The p65 in cytoplasmic protein reduced with TNFα treatment and combination treatment, but the p-p65 did not change. Nuclear p65 and p-p65 increased in the reciprocal level with TNFα single treatment compared to combination treatment (Fig. 2A). Moreover, the data showed similar level of nuclear p65 at earlier time points for combination treatment compared to TNFα treatment (Fig. 2B). These results indicate that ephedrine single treatment does not activate NF-κB pathway and ephedrine treatment has no synergistic effect with TNFα.

Table 1
Combination index (CI) of ephedrine and TNFα treatment.

Concentration		CI (FACS)	CI (ELISA)	Meaning
Ephedrine	TNFα			
0.1	0.1	0.61959	0.25567	Slightly synergistic
	1	0.70017	0.93681	
	10	0.63418	0.72613	
1	0.1	0.32081	0.17339	Synergistic
	1	0.39133	0.33392	
	10	0.33717	0.45588	
10	0.1	0.25408	0.26157	Strongly synergistic
	1	0.30110	0.21287	
	10	0.28433	0.19717	

The combination index (CI) was analyzed using CompuSyn software based on the Chou-Talalay Method (See materials and methods).

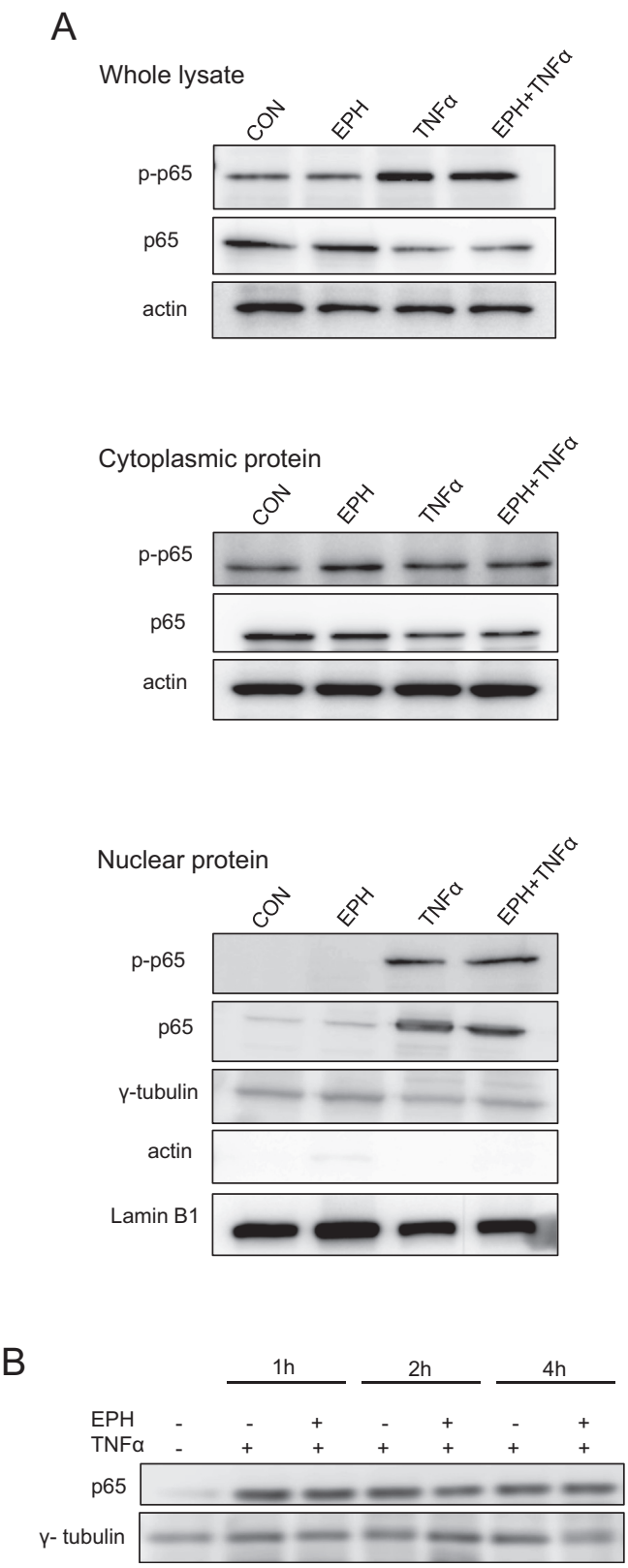

Fig. 2. NF-κB is not the major mechanism of ephedrine on HIV-1 reactivation. (A) U1 cells were treated with ephedrine (10 μg/ml) and TNFα (10 ng/ml) for 4 h. The whole/cytoplasmic/nuclear protein was collected and Western blotting was performed. (B) U1 cells were treated with ephedrine (10 μg/ml) and TNFα (10 ng/ml) for 1, 2, and 4 h. The nuclear protein was collected and Western blotting was performed. Full size images are shown in supplementary materials.

3.3. Tumor necrosis factor receptor II (TNFRII) mediates the partial effect of ephedrine on HIV-1 reactivation from latency

It has been shown that tumor necrosis factor receptor II (TNFRII) is increased by epinephrine treatment in monocytes [28], and the level of TNFR expression on the cell surface affects the level of HIV-1 reactivation by TNFα treatment [29]. Since epinephrine and ephedrine share an identical skeleton of β-phenylethylamine and are similar in overall structure [30], we hypothesized that ephedrine treatment might elevate TNFR expression and raise TNFα sensitivity, resulting in an enhancement of HIV-1 reactivation by combination treatment with TNFα. TNFα is a ligand of TNFRI and TNFRII [31]. TNFRI expression is ubiquitous, while that of TNFRII is limited in immune cells such as monocytes and lymphocytes. The TNFRI cytoplasmic tail contains a death domain and directly correlates to cell death. In contrast, TNFRII does not contain a death domain and modulates cell survival [32, 33].

We measured the levels of TNFRI/II expression on U1 cells. At 6 h post-treatment, ephedrine did not affect the level of TNFRI expression. TNFRI expression was dramatically reduced by TNFα treatment, but the combination treatment did not alter this (Fig. 3A and B). In contrast, a significantly higher level of TNFRII expression was observed 6 h after treatment with ephedrine alone. TNFRII expression was dramatically suppressed by TNFα treatment, but was recovered by the combination treatment (Fig. 3C and D). The time courses of TNFRI/II expression by ephedrine and/or TNFα are shown in Fig. 3E and F. As shown in Fig. 3F, ephedrine treatment for 6, 9, and 12 h caused significant increases in TNFRII expression compared to non-treated cells. The combination treatment significantly recovered the level of TNFRII expression

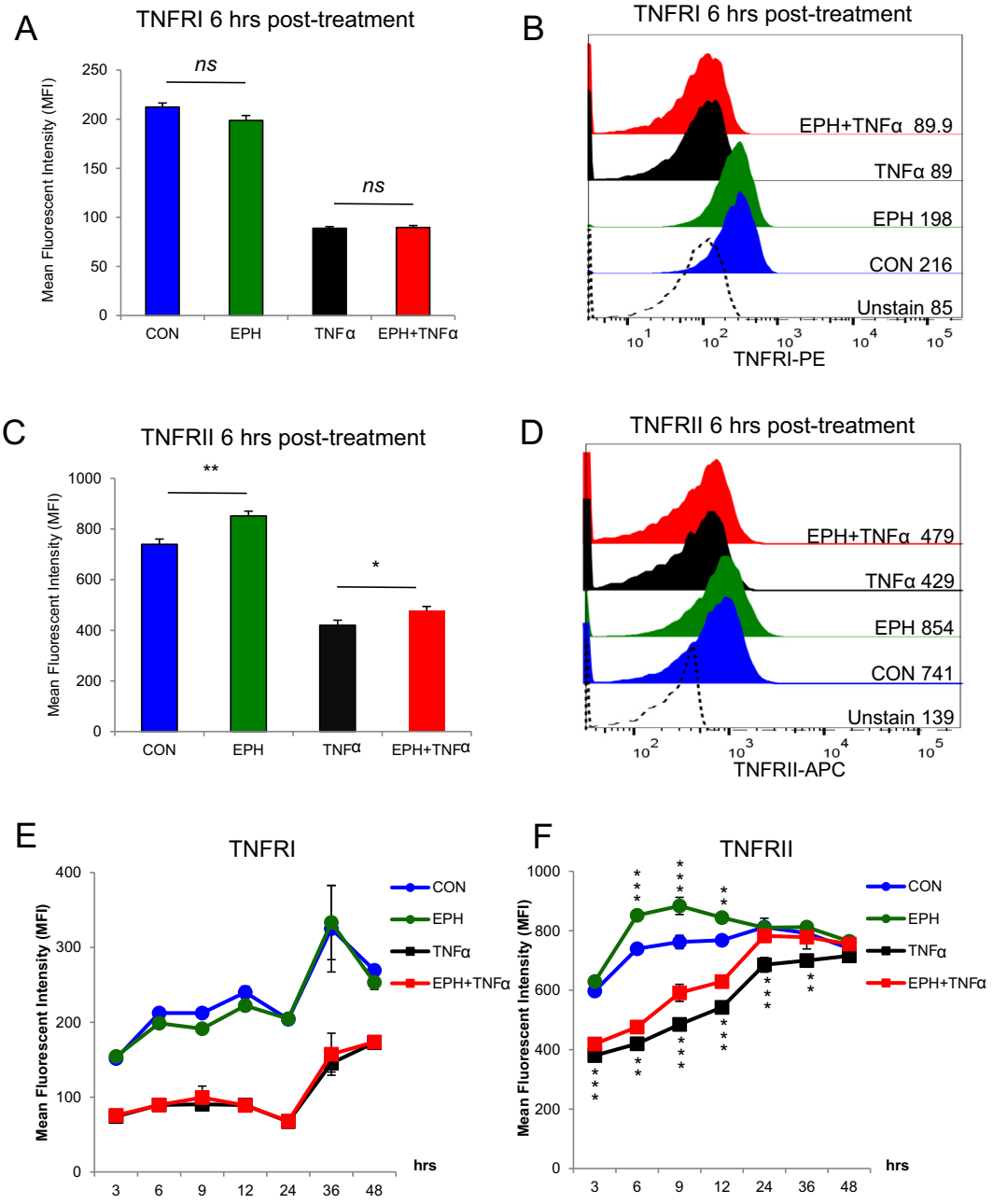

Fig. 3. Ephedrine enhances the expression of tumor necrosis factor receptor II (TNFRII). (A,B) TNFRI expression at 6 h post treatment. (C,D) TNFRII expression at 6 h post treatment. (E) Time course of TNFRI expression at indicated times post treatment. (F) Time course of TNFRII expression at indicated times post treatment. One-way analysis of variance (ANOVA) with the Dunnett's multiplecomparison test was used to analyze the data. Differences at *P < 0.05, **P < 0.01, ***P < 0.001 were considered significant. The data represents from triplicate experiments.

compared to TNFα single treatment. These results reveal that ephedrine increases TNFRII expression.

In a previous study, we showed that Ephedrae herba treatment enhances HIV-1 reactivation in U1 cells, and that combined treatment with TNFα showed a strong synergy in HIV-1 reactivation with activation of NF-κB [17]. Since ephedrine is one of the major components of Ephedrae herba, we expected ephedrine to have strong synergistic effects with TNFα. However, in contrast to the result with Ephedrae herba treatment, we did not observe synergistic activation of NF-κB in the present study. This is because a different component of Ephedrae herba is responsible for NF-κB stimulation [34].

In this study, we used U1 cells as HIV-1 latent infection model. U1 cells contain two copies of integrated provirus with *tat* mutation, and produce defective HIV-1 with the stimulation of pro inflammatory cytokines such as TNFα and IL-6 [35], and PMA [19]. Thus, U1 cells have been used as HIV latency model of myeloid lineage. Further study is needed using primary myeloid lineages to confirm synergistic effects.

This study revealed that ephedrine demonstrates a synergistic effect with TNFα treatment. The synergistic effect enhanced the TNFα-dependent reactivation of HIV-1 from latently-infected U1 cells by increasing the surface expression of TNFRII, resulting in an increase of TNFα sensitivity. Finally, the combination treatment demonstrates higher HIV-1 gene and protein expression resulting in an induction of higher HIV-1 reactivation from U1 cells. Our study provided the information that HIV-1 reactivation relates to TNFRII expression.

Declarations

Author contribution statement

Jutatip Panaampon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Eriko Kudo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ryusho Kariya: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Seiji Okada: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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