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Short Communication

Induction of heat-shock protein 70 by prostaglandin A_1 inhibits HIV-1 Vif-mediated degradation of APOBEC3G

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ABSTRACT

Previous studies have demonstrated that cyclopentenone prostaglandins (cyPGs) inhibit human immunodeficiency virus type 1 (HIV-1) replication in various cell types. This antiviral activity has been associated with the induction of heat-shock protein 70 (HSP70) in infected cells. We investigated a new role of prostaglandin A_1 (PGA_1) in the replication of HIV-1 in non-permissive cells. Because overexpression of HSP70 blocks the viral infectivity factor (Vif)-mediated degradation of APOBEC3G (A3G) via the ubiquitin–proteasome pathway, we examined the effects of PGA_1 on A3G and HIV-1 replication. The induction of HSP70 synthesis by PGA_1 blocked Vif-mediated A3G degradation and enhanced the incorporation of A3G into both wild-type and Vif-deficient viruses. Furthermore, we determined the viral titer of HIV-1 particles produced from PGA_1-treated 293T cells. The induction of HSP70 synthesis by PGA_1 significantly reduced the viral titer in the presence of A3G. Additionally, the p24 Gag antigen levels were dramatically reduced in non-permissive cells treated once or repeatedly with PGA_1. Thus, we showed that PGA_1 inhibits HIV-1 replication, at least in part, by blocking Vif-mediated A3G degradation.

HIV-1 infections and the resulting acquired immunodeficiency syndrome (AIDS) remain global challenges due to the absence of a protective vaccine (Barouch, 2008; Robb, 2008) and the rapid selection of viral variants that are resistant to all currently available antiviral therapies. Development of new and highly active antiviral agents would greatly facilitate effective clinical management of HIV-1 infections and could delay the onset of AIDS.

Recent advances in our understanding of the intracellular immunity conferred by the host cytidine deaminase APOBEC3G (A3G) (Harris et al., 2003; Sheehy et al., 2002, 2003; Zhang et al., 2003), and the mechanism by which the virally encoded viral infectivity factor (Vif) protein induces the proteasomal degradation of A3G (Iwatani et al., 2007; Kobayashi et al., 2005; Mehle et al., 2004; Mercenne et al., 2010; Stopak et al., 2003; Yu et al., 2003, 2004) provides fresh opportunities for the development of novel antiviral treatments. Interestingly, the interactions between Vif and A3G that overcome this host defense mechanism are structurally distinct, and they provide two potential targets for antiviral drug development (Nathans et al., 2008; Zuo et al., 2012). To develop potential novel therapeutic strategies that exploit the antiviral function of A3G, we have investigated the role of heat shock protein 70 (HSP70) in A3G function (Sugiyama et al., 2011). Overexpression of HSP70 blocked the Vif-mediated degradation of A3G via the ubiquitin–proteasome pathway, rendering the viral particles non-infectious. In addition, siRNA-targeted knockdown of HSP70 expression enhanced the Vif-mediated degradation of A3G. Co-immunoprecipitation experiments revealed that HSP70 overexpression inhibited A3G binding to Vif. Thus, we provided evidence for an A3G-dependent host protein-mediated suppression of HIV-1 replication.

PGA_1 inhibits the replication of a wide variety of DNA and RNA viruses, including paramyxoviruses, picornaviruses, togaviruses, poxviruses, herpesviruses, rhabdoviruses, and retroviruses, in different mammalian cell types (Ankel et al., 1985; Carattoli et al., 2000; D'Onofrio et al., 1990; Hirayama et al., 2006; Santoro, 1980, 1982, 1983, 1988, 1997; Yamamoto et al., 1987). The antiviral activity of PGA_1 may be due to an α,β-unsaturated carbonyl group in the cyclopentenone ring structure, which has been associated with the synthesis of heat-shock proteins, especially HSP70 (Amici and Santoro, 1991; Santoro et al., 1989). HSP70 could play a role in the antiviral activity of PGA_1. Furthermore, PGA_1 inhibits HIV-1 replication by different mechanisms, including the

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regulation of important cellular transcription factors such as nuclear factor-κB (NF-κB) (Rossi et al., 1997) and proliferator-activated receptor γ (PPAR γ) (Hayes et al., 2002).

In this study, we report that PGA_1 inhibits the Vif-mediated degradation of A3G in human cells. Induction of HSP70 is associated with inhibition of Vif-dependent polyubiquitination of A3G, which results in selective inhibition of Vif-mediated A3G degradation.

We used a tetrazolium-based MTS assay to determine the viability of 293T cells in the presence of PGA_1 (Supplementary Mate-

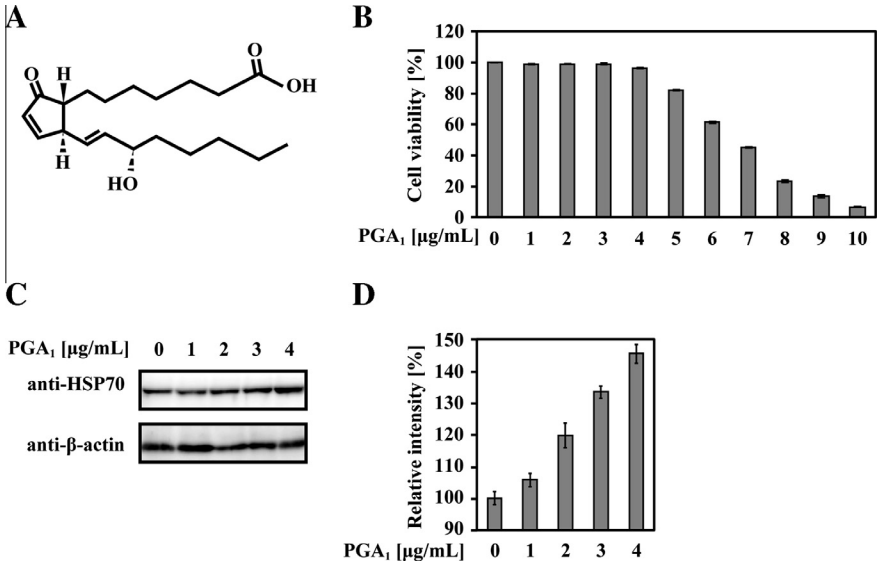

Fig. 1. Induction of HSP70 synthesis by PGA_1. (A) Structure of cyclopentenone prostaglandin A_1 (PGA_1). (B) The cytotoxic effect of PGA_1 (Biomol GmbH) in 293T cells, shown as the percentage reduction of viable cell numbers as assessed in a tetrazolium-based MTS assay. The results are representative of three independent experiments, and error bars show the standard deviations of the means. (C) 293T cells (5×10^5) were treated with 1–4 μg/mL PGA_1. After 24 h, the cell lysates were harvested, and samples containing the same amounts of protein were separated by SDS–PAGE and processed for immunoblot analysis using an anti-HSP70 monoclonal antibody. (D) The relative intensity of HSP70 bands was determined by densitometry. Results are representative of three independent experiments, and error bars show the standard deviations of the means.

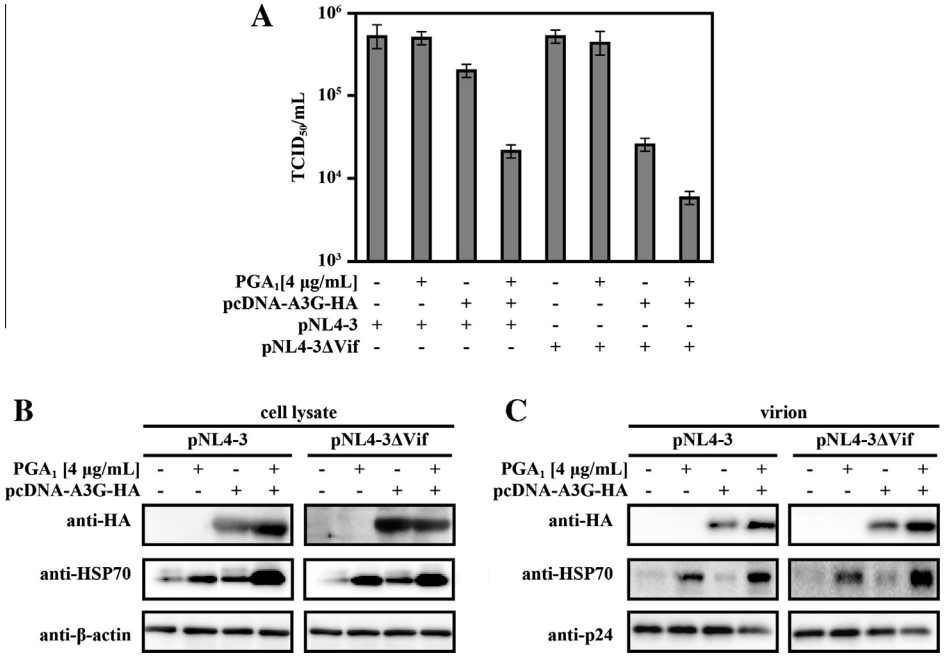

Fig. 2. Induction of HSP70 synthesis by PGA_1 regulates HIV-1 infectivity in an A3G-dependent manner. (A) 293T cells (5×10^5) were treated with 4 μg/mL PGA_1 (+) or control diluents (−) for 4 h. The cells were co-transfected with 0.1 μg of pNL4-3 or pNL4-3-delta-Vif and 1.0 μg of pcDNA-A3G-HA or pcDNA3.1 (empty plasmid) using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, the supernatants were harvested, and the amount of each virus was normalized to the equivalent level of p24 Gag antigen. MAGI cells (1×10^4) were infected with each virus (corresponding to 5 ng of p24 Gag antigen), and infected cells were stained with X-Gal 2 days later. The 50% tissue culture infective dose ($TCID_{50}$) was determined for the last dilution of each virus that was capable of infecting the cells. The results are representative of three independent experiments, and error bars show the standard deviations of the means. (B and C) Each stock of cell lysate or virus in Figure 2A was subjected to western blotting and then analyzed with the indicated antibody.

rials and Methods). Dose-dependent cytotoxicity was not observed upon application of PGA_1 (99% at concentrations ranging between 1 and 4 μg/mL) (Fig. 1B). Next, we investigated whether PGA_1 affects HSP70 synthesis. 293T cells were treated with PGA_1 (1–4 μg/mL), and the level of HSP70 protein was examined by western blot. PGA_1 treatment significantly increased HSP70 expression in 293T cells (Fig. 1C and D). Furthermore, we examined whether the induction of HSP70 synthesis by PGA_1 influenced A3G function. Either pNL4-3 or pNL4-3-delta-Vif was transfected into 293T cells along with either pcDNA3.1 (empty plasmid) or pcDNA-A3G-HA in the absence or presence of PGA_1. Viral infectivity was measured using the MAGI assay (Supplementary Materials and Methods). As shown in Figure 2A, the PGA_1-treated 293T cells clearly suppressed the infectivity of wild-type HIV-1 in the presence of A3G. In the absence of A3G, PGA_1 did not affect the infectivity of wild-type HIV-1. As expected, the induction of HSP70 by PGA_1 in A3G-HA-transfected 293T cells led to an inhibition of the infectivity of Vif-deficient HIV-1 (Fig. 2A). Moreover, HSP70 induction had no effect on the infectivity of the Vif-deficient HIV-1 particles produced by mock-transfected 293T cells.

To demonstrate whether HSP70 induction by PGA_1 affects the packaging of A3G into virions, cell lysates and viruses were analyzed for A3G expression by western blotting. The 293T cells treated with PGA_1 showed a significant increase in the amount of intracellular and wild-type virion-associated A3G (Fig. 2B and C). Interestingly, HSP70 induction by PGA_1 enhanced the level of A3G packaging in Vif-deficient virions but had no effect on intracellular A3G and viral release (Fig. 2B and C). These results indicate that HSP70 induction by PGA_1 blocked Vif-mediated A3G degradation and enhanced the incorporation of A3G into both wild-type and Vif-deficient virions. (Sugiyama et al., 2011).

Importantly, HSP70 had no effect on the expression of A3G in 293T cells transfected with the Vif-deleted HIV-1 proviral plasmid (Fig. 2B). Our results suggest that HSP70 induction by PGA_1 may inhibit the degradation of A3G by Vif. To further investigate whether our findings have physiological relevance in cells expressing endogenous A3G, we used H9 cells. First, we investigated whether PGA_1 affects HSP70 synthesis in H9 cells. The cells were treated with PGA_1 (4 μg/mL), and the level of HSP70 protein was examined by western blot. PGA_1 treatment significantly increased the amount of HSP70 in uninfected H9 cells (Fig. 3A), but there was no difference in A3G expression after PGA_1 treatment (Fig. 3A). H9 cells were challenged with NL4-3 or NL4-3-delta-Vif viruses, which corresponded to 5 ng of p24 Gag antigen. After incubation at 37 °C for 4 h, the cells were washed three times in PBS and treated with PGA_1 (4 μg/mL). For some cultures, PGA_1 treatment was repeated at 48 h post-infection. After 96 h, the cell lysates were harvested and analyzed by western blotting. When H9 cells were infected with NL4-3, the level of endogenous A3G and HSP70 were enhanced by a single PGA_1 treatment (Fig. 3B, compare lanes 1 and 2). Repeated PGA_1 treatments had no effect on the level of endogenous A3G and HSP70 compared with a single PGA_1 treatment (Fig. 3B, lane 2 [a single PGA_1 treatment] and lane 3 [repeated PGA_1 treatments]). In contrast, in NL4-3-delta-Vif-infected H9 cells, PGA_1 did not have a significant effect on the level of endogenous A3G (Fig. 3D, compare lanes 1 and 2 [a single PGA_1 treatment]

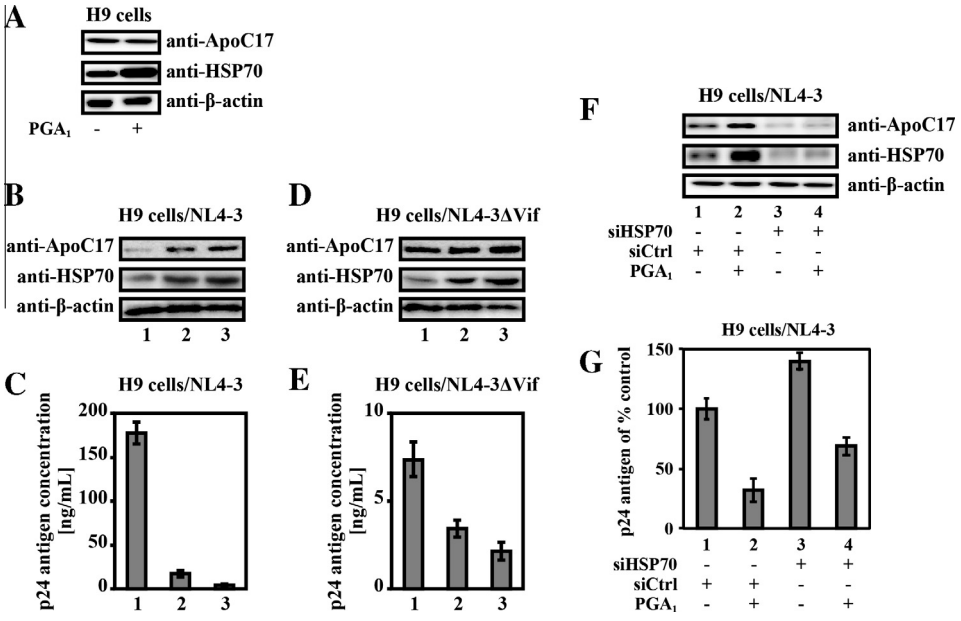

Fig. 3. PGA_1 affects the level of endogenous A3G expression in non-permissive T cells expressing HIV-1 Vif. (A) HSP70 synthesis and A3G expression by PGA_1. H9 cells (5×10^5) were treated with 4 μg/mL PGA_1. After 48 h, the cell lysates were harvested, and samples containing the same amounts of protein were separated by SDS–PAGE and processed for immunoblot analysis using anti-HSP70 and anti-ApoC17 monoclonal antibodies. (B-E) H9 cells (1×10^6) were challenged with wild-type (NL4-3) or Vif-defective (NL4-3-delta-Vif) virus particles (corresponding to 5 ng of p24 Gag antigen). After incubation at 37 °C for 4 h, the cells were washed three times in PBS and then treated with 4 μg/mL PGA_1. For some cultures, PGA_1 treatment was repeated at 48 h post-infection. After 96 h, the cell lysates were harvested and analyzed by western blotting with the indicated antibodies (B, D). Lane 1: no PGA_1 treatment; lane 2: a single PGA_1 treatment; lane 3: repeated PGA_1 treatments. (C, E) p24 Gag antigen levels in supernatants were determined after 96 h using an ELISA-based system. The amount of p24 Gag antigen was quantified with LUMIPULSE®*f* (forte), a fully automated chemiluminescent enzyme immunoassay (CLEIA) system (Fujirebio) (Sakai et al., 1999). Lane 1: no PGA_1 treatment; lane 2: a single PGA_1 treatment; lane 3: repeated PGA_1 treatments. The results are representative of three independent experiments, and error bars show the standard deviations of the means. (F, G) H9 cells (1×10^6) were transfected with 100 nM control siRNA (siCtrl) or 100 nM HSP70-specific siRNA (siHSP70) using Lipofectamine 2000 for 4 h. The cells were then challenged with NL4-3 (corresponding to 5 ng of p24 Gag antigen). After incubation at 37 °C for 4 h, the cells were washed three times in PBS and treated with PGA_1 (4 μg/mL). After 48 h, the cell lysates were harvested and analyzed by western blotting with the indicated antibodies (F). Lane 1, 3: no PGA_1 treatment; lane 2, 4: PGA_1 treatment. (G) p24 Gag antigen levels in supernatants were determined after 48 h using the CLEIA system (LUMIPULSE®*f*). Lane 1, 3: no PGA_1 treatment; lane 2, 4: PGA_1 treatment. The vertical axis of the inset represents the percentage concentration of p24 Gag antigen compared with the control (lane 1). The results are representative of three independent experiments, and error bars show the standard deviations of the means.

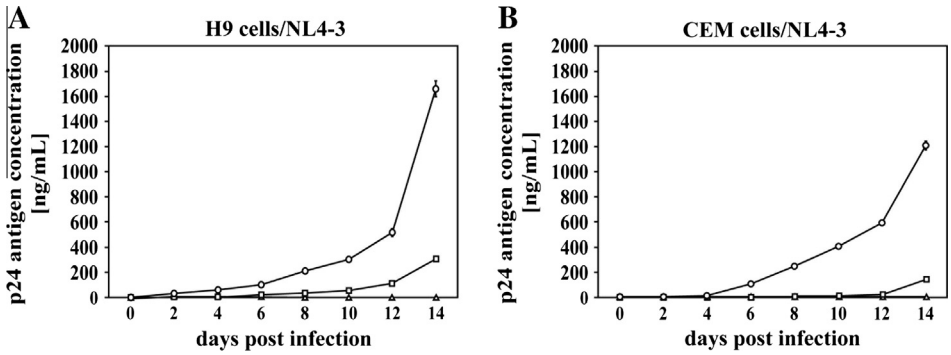

Fig. 4. PGA_1 suppresses long-term HIV-1 replication in non-permissive T cells. (A) H9 cells (1×10^6) or (B) CEM cells (1×10^6) were challenged with NL-4-3 (corresponding to 5 ng of p24 Gag antigen). After incubation at 37 °C for 4 h, the cells were washed three times in PBS and then treated with 4 μg/mL PGA_1 on day 0 or every 2 days for 14 days. To monitor HIV-1 production, the p24 Gag antigen concentration in supernatants was measured using the CLEIA system (LUMIPULSE®*f*). Single PGA_1 treatment (white squares), repeated PGA_1 treatments (white triangles), and control (white circle). The results are representative of three independent experiments, and error bars show the standard deviations of the means.

with lane 3 [repeated PGA_1 treatments]) but did affect the virion packaging of A3G (see Fig. 2C). The PGA_1-mediated anti-HIV-1 effect was examined by measuring p24 Gag antigen levels after 96 h. p24 Gag antigen levels were dramatically reduced in cells treated once or repeatedly with PGA_1 compared with the control when cells were infected with NL4-3 (Fig. 3C). In addition, inhibitory effect of PGA_1 reduced in H9 cells infected with Vif-deficient HIV-1 (Fig. 3E). There was no significant decrease in cell viability between treatment groups, as assessed by a tetrazolium-based MTS assay (data not shown).

To further investigate the influence of HSP70 silencing on PGA_1-mediated anti-HIV-1 effects, H9 cells were transfected with control siRNA (siCtrl) or HSP70-specific siRNA (siHSP70) (Supplementary Materials and Methods) and were then challenged with NL4-3 (corresponding to 5 ng of p24 Gag antigen). After incubation at 37 °C for 4 h, the cells were washed three times in PBS and treated with PGA_1 (4 μg/mL). As shown Fig. 3F, the knockdown of HSP70 expression in PGA_1-untreated H9 cells significantly decreased the amount of A3G (compare lanes 1 and 3), whereas the p24 Gag antigen levels were slightly increased (Fig. 3G, compare lanes 1 and 3). We think the enhancement of p24 Gag antigen expression is due to Vif-mediated degradation of endogenous A3G by the knockdown of HSP70. However, the knockdown of HSP70 in PGA_1-treated H9 cells (Fig. 3G, lane 4) decreased the amount of p24 Gag antigen compared with HSP70 knockdown in PGA_1-untreated H9 cells (Fig. 3G, lane 3). This effect may occur by the regulation of two important cellular transcription factors: nuclear factor-κB (NF-κB) (Rossi et al., 1997) and proliferator-activated receptor γ (PPAR γ) (Hayes et al., 2002). However, anti-HIV-1 effects of PGA_1 were constantly more prominent in H9 cells transfected with siCtrl (about 70% inhibition) than with siHSP70 (about 50% inhibition). These results suggest that the induction of HSP70 by PGA_1 suppressed Vif-mediated degradation of endogenous A3G in non-permissive cells.

Finally, we examined the effect of PGA_1 on HIV-1 replication over time. When H9 or CEM cells were infected with NL4-3 and then treated with PGA_1 (4 μg/mL), the levels of p24 Gag antigen were reduced through day 6 (H9 cells) or 8 (CEM cells) but had increased slightly by day 8 (H9 cells) or 10 (CEM cells) (Fig. 4A and B, white squares). However, when PGA_1 (4 μg/mL) was added to the cells at 2-day intervals for 14 days (white triangles), the significant reduction in p24 Gag antigen was sustained through day 14.

In conclusion, we found that the induction of HSP70 synthesis by PGA_1 blocks Vif-mediated A3G degradation and enhances the incorporation of A3G into both wild-type and Vif-deficient virions. PGA_1 inhibits HIV-1 replication, at least in part, by blocking Vif-mediated A3G degradation. These results suggest that a new mechanism used by prostaglandins may aid in the search for novel anti-retroviral drugs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.06.017.

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