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Norepinephrine Accelerates HIV Replication Via Protein Kinase A-Dependent Effects on Cytokine Production

Steve W. Cole, Yael D. Korin, John L. Fahey, and Jerome A. Zack

To explore the role of sympathetic nervous system activation in HIV pathogenesis, we examined the effect of the neuroeffector molecule norepinephrine (NE) on HIV-1 replication in quiescently infected PBMCs that were subsequently activated with Abs to CD3 and CD28. NE accelerated HIV-1 replication at concentrations ranging from $10^{-8}$ to $10^{-5}$ M. This effect could be mimicked by protein kinase A (PKA) activators (forskolin or dibutyryl-cAMP) and abrogated by $\beta$-adrenergoreceptor antagonists or the PKA inhibitor rp-cAMP, indicating transduction via the adrenoreceptor signaling pathway. NE reduced cellular activation and altered the production of several HIV-modulating cytokines: IL-10 and IFN-$\gamma$ were markedly suppressed; TNF-$\alpha$, IL-1$\beta$, IL-2, IL-4, and IL-6 were mildly suppressed; and levels of IL-12 were not significantly altered. The addition of either exogenous IFN-$\gamma$ or IL-10 abrogated the effect of NE on virus production. Thus PKA-dependent suppression of cytokine production appears to mediate the enhancement of HIV-1 replication by NE. The Journal of Immunology, 1998, 161: 610–616.

Materials and Methods

Infection, activation, and cell culture

Healthy donor PBMCs were infected with HIV-1NL4-3 (0.05 infectious units/cell) for 2 h in the presence of 10 $\mu$g/ml polybrene. Following infection, cells were washed and costimulated with Abs to CD3 (0.1 $\mu$g/ml) and CD28 (0.1 $\mu$g/ml soluble; Biodesign, Kennebunk, ME). Unstimulated control cells received no exogenous Abs. Cells were cultured for 6 to 8 days at 3 $\times$ 10$^5$/ml in RPMI 1640 supplemented with 10% (v/v) human AB serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM glutamine at 37°C in an atmosphere of 5% CO$_2$. NE (1-$\mu$g/ml; Sigma, St. Louis, MO) and other reagents were added once, at activation. Other reagents added at activation included the adenyl cyclase activator forskolin (Sigma) (28); the membrane-permeable

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cAMP analogue N6, 2'-O-dibutyl adenosine-3', 5'-cyclic monophosphate (db-cAMP; Sigma); the membrane permeable PKA antagonist adenosine-3', 5'-cyclic monophosphothioate, rp isomer (rp-cAMP; Calbiochem, La Jolla, CA) (30); human IFN-γ (R&D Systems, Minneapolis, MN), IL-2 (Genzyme, Cambridge, MA), IL-4 (Genzyme), and IL-10 (R&D Systems). To determine whether the effects of NE were mediated by β-adrenoreceptors, infected PBMCs were incubated in the presence of the receptor antagonist sotalol (ICN Biomedicals, Costa Mesa, CA) or di-propranolol HCl (ICN Biomedicals) for 1 h before activation in the presence of NE. Cell viability was assessed by trypan blue exclusion and did not differ between NE-treated and untreated cultures at 2, 4, 6, or 8 days postinfection. Cell viability was also not altered by the addition of adrenoreceptor blockers (propranolol, sotalol, phenotolamine) or PKA antagonists (rp-cAMP) to NE-treated cultures. Apoptosis rates were assessed by a flow cytometric measurement of annexin V-FITC (Brand Applications) and propidium iodide binding. Apoptosis rates did not differ across cultures of costimulated cells, cultures costimulated in the presence of NE, or NE supplemented by adrenoreceptor blockers or PKA antagonists.

**HIV-1 quantification**

Virus production was quantified by ELISA for HIV-1 p24 core protein (Coulter, Maastricht, The Netherlands). p24 levels were compared across treatments by ANOVA followed by Duncan’s multiple range test for specific comparisons. Dose-response and kinetic profiles were analyzed by linear regression on log p24 values. p24 levels were graphed as the mean plus SE. To confirm the equality of viral inoculation, we also examined the proviral load at 14 h postinfection using PCR (AA55 and M667 primer sequences for the R/U5 region of the viral long terminal repeat, quantified relative to input cell number as indicated by β-globin primers LA1 and LA2) (20). Briefly, one primer of each pair was radio end-labeled, and amplification was conducted for 25 cycles in parallel with control standards consisting of linearized cloned HIV-1 DNA and known quantities of cellular DNA. Radiolabeled, amplified products were resolved on a 6% polyacrylamide gel and quantified by radioanalytic image analysis in comparison with standard curves.

**Cytokine quantification and identification of cytokine-producing cells**

ELISAs were used to measure the supernatant concentrations of human IL-1β (R&D Systems), IL-2 (Genzyme), IL-4 (Genzyme), IL-6 (Biosystems, Zwijndrecht, Belgium), IL-10 (Immunotech, Westbrook ME), TNF-α (Genzyme), IFN-γ (Biosource International, Westlake Village, CA), and IL-12 p70; capture Ab 12H4 and detection Ab C8.6 were kindly provided by G. Trinchieri, (Wistar Institute, Philadelphia, PA). Concentrations of each cytokine were determined in parallel at 0, 2, 4, and 6 days postinfection across three independent experiments and graphically expressed as the mean plus SE. Differences in cytokine production were analyzed by the Student t test. Total cytokine production was quantified as the area under the curve of the cytokine level over time (as in Fig. 3); the statistical significance of differences in total cytokine production was determined by the Student t test.

Cells producing IFN-γ were identified by flow cytometric analysis of intracellular binding of anti-cytokine Abs following a 6-h incubation in the presence of 10 μg/ml brefeldin A (following the protocol of the Ab manufacturer). The CD4+/CD8+ phenotype of cytokine-producing cells was determined by surface staining with anti-CD4 and anti-CD8 Abs (Becton Dickinson, Mountain View, CA) before intracellular cytokine staining. All flow cytometric data were acquired using a FACScan instrument (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson); dead cells and debris were excluded on the basis of forward scatter vs side scatter profiles.

**Assessment of cellular activation**

At 3 days postinfection, cellular activation was quantified by 1) the flow cytometric cell cycle stage via intracellular staining of RNA and DNA, 2) the cell surface expression of CD25 (IL-2R; expressed early in the cell cycle) and CD71 (transferrin receptor; expressed late in the cell cycle) by flow cytometry (Becton Dickinson Immunocytometry protocol), and 3) 6 h of [3H]thymidine uptake. In cell cycle staging, cells were permeabilized with saponin (0.004% w) and stained with 7-amino actinomycin D (Sigma) and pyronin Y (Calbiochem) to quantify intracellular DNA and RNA, respectively (31, 73). Cycling cells move from a 2N DNA/RNAlow state (G1/G0) through a 2N DNA/RNAhigh state (G1M) to a 4N DNA/RNAhigh state (G2/M). DNA/RNA quadrants were set on the basis of a 2N DNA/RNAlow negative control that had been established by activating PBMCs in the presence of 5 mM N-butyrate (Sigma), which blocks cell cycle progression in G1 (32). The identification of cycling cells via RNA/DNA staining was highly specific, with <1% of cells falling in the cycling quadrant in the absence of costimulation (vs >65% of costimulated cells). The results of cell cycle staging correlated closely with the expression of the CD25 and CD71 activation Ags and with [3H]thymidine uptake. Dose response relationships between NE concentration and the fraction of cycling cells were evaluated by linear regression.

**Results**

**NE accelerates HIV-1 replication**

To examine the effects of NE on HIV-1 replication, p24 levels were assessed at 2, 4, 6, and 8 days postinfection (Fig. 1A). As expected, costimulating HIV-1, IL-1β, IL-4, IL-10, IL-12, and IFN-γ costimulated PBMCs with Abs to CD3 and CD28 markedly increased viral replication relative to unstimulated cultures. Identical costimulation in the presence of 10 μM NE led to a significant additional increase in HIV replication. This effect emerged reliably across 15 independent experiments, with NE increasing day 6 p24 levels to an average of 337% of costimulated control levels (p < 0.0001). In some donors, NE increased day 6 p24 levels by >11-fold. The effect of NE on HIV-1 replication was dose-dependent, with day 6 p24 levels increasing approximately twofold with each log increase in NE concentration over the range 10−5–10−3 M (Fig. 1B) (p = 0.002). These differences emerged despite the fact that a PCR analysis of the HIV-1 proviral load confirmed equivalent initial viral inoculation (no difference in proviral penetration at 14 h postinfection; data not shown). PCR analysis corroborated p24 ELISA results in documenting increased proviral spread in NE-treated cultures at 6 to 8 days postinfection (data not shown).
In the classical NE signaling pathway, ligation of the cell surface adrenoreceptor-adenylyl cyclase-cAMP-PKA signaling pathway is capable of accelerating HIV replication (Table I), reducing p24 concentrations to levels that were statistically indistinguishable from costimulated control cultures (70.7% to 65.2%). Thus, the activation of the PKA signaling cascade via the cell surface β-adrenoreceptor appears to mediate the effects of NE on HIV-1 replication.

Effect of NE on HIV-1 replication is not mediated by increased cellular activation

Cellular activation is critical to efficient HIV-1 replication (20, 21). NE and other cAMP-inducing agents partially suppress cellular activation in uninfected PBMCs (9), but their effect on cellular activation in HIV-infected lymphocytes is unknown. A flow cytometric assessment of cell cycling by analysis of intracellular DNA and RNA indicated that NE decreased cellular activation in HIV-1-infected PBMCs (Fig. 2). These effects were dose-dependent, with the fraction of cycling cells declining by an average of 15.3% per log increase in NE concentration over the range 10⁻⁷ to 10⁻⁵ M (p < 0.0001 across four experiments). Similar effects emerged in flow cytometric analyses of the early CD25 and the late CD71 activation Ags and in measurements of 6-h [³H]thymidine uptake (data not shown). Thus the effect of NE on HIV-1 replication does not appear to be mediated by increased cellular activation in HIV-infected cultures.

**NE alters cytokine production profiles**

Previous research indicates that NE can suppress the production of IL-2 and IFN-γ by activated (HIV-uninfected) PBMCs and mouse Th1 clones while sparing or enhancing IL-4 production by activated PBMCs and Th2 clones (13, 33). The effects of NE on cytokine production in HIV-infected PBMCs are unknown. However, effects on cytokine production could play a role in altered viral replication, since HIV-1 production can be suppressed by several immunoregulatory cytokines (e.g., IL-10, and IL-12) and enhanced by several other proinflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β) (34–36).

Consistent with the partial suppression of cellular activation by NE, ELISAs of supernatant cytokine concentrations showed a moderate suppressive effect of NE on the production of IL-2 and IL-4 (Fig. 3). The total production of each cytokine over time (area under curve) was reduced by 49% and 64% for IL-2 and IL-4, respectively, with neither suppression reaching statistical significance (p = 0.32 and 0.10, respectively). NE similarly suppressed the proinflammatory cytokines IL-1β (by 48% over time, p = 0.017), IL-6 (48%, p = 0.008), and TNF-α (41%, p = 0.052) (Fig. 3). However, the most pronounced

**Table I. Effects of PKA-inducing and-blocking agents on HIV replication**

<table>
<thead>
<tr>
<th>Agent</th>
<th>p24 at 6 Days Postinfection</th>
<th>Difference from Costimulated Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE (10⁻⁵ M)</td>
<td>337.3% (62.5)</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>NE + β-adrenoreceptor blocker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sotalol (10⁻⁵ M)</td>
<td>100.4% (16.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Propranolol (10⁻⁵ M)</td>
<td>87.9% (8.7)</td>
<td>NS</td>
</tr>
<tr>
<td>NE + α-adrenoreceptor blocker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolamine (10⁻⁵ M)</td>
<td>327.6% (70.7)</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>PKA-activating agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forskolin (10⁻⁵ M)</td>
<td>283.7% (184.7)</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>db-cAMP (10⁻⁴ M)</td>
<td>358.4% (291.8)</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>NE + PKA inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rp-cAMP (10⁻⁵ M)</td>
<td>109.7% (6.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*PKA inducers or inhibitors were added postinfection and prior to CD3/CD28 costimulation. Data represent the mean (SE) percent change in p24 levels relative to costimulated controls (equaling 100%) at 6 days postinfection. NS, not significant.

**FIGURE 2.** Effect of NE on cellular activation in HIV-1-infected PBMCs. Cell cycle stage was determined by flow cytometric assessment of intracellular DNA (vertical axis) and RNA (horizontal axis). Costimulation increased the fraction of cycling lymphocytes (i.e., those outside the G₀/G₁A quadrant that are identified by arresting costimulated cells at G₁A with 5 mM N-butylate) from a mean of 0.3% (A) to 65.2% (B). Identical costimulation in the presence of 10 μM NE reduced the cycling fraction to 44.5% (C) across four experiments (p < 0.01).
FIGURE 3. Effect of NE on cytokine production. Cytokine levels were determined by ELISA at 0, 2, 4, 6, and 8 days following the infection of quiescent PBMCs and subsequent costimulation in the presence (10 μM) (●) or absence (○) of NE. NE suppressed the total production of IFN-γ and IL-10 by 96% (p = 0.006) and 94% (p = 0.014), respectively. NE moderately suppressed the total production of TNF-α, IL-6, and IL-1β (p < .05) and did not significantly alter the total production of IL-2, IL-4, or IL-12 (all p > .10). The statistical significance of comparisons at specific time points is indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

Discussion

In the present study, we find that the SNS neuroeffector molecule NE can accelerate HIV-1 replication up to 11-fold in acutely infected PBMCs. The effect of NE on viral replication is transduced via the β-adrenoreceptor-adenyl cyclase-cAMP-PKA signaling cascade and is mediated by a pronounced suppression of IFN-γ and IL-10. The present results confirm previous observations that cAMP can increase HIV-1 replication (26, 27) and identify a basis for those effects in the suppression of HIV-limiting cytokines. Consistent with its partial suppression of cellular activation, NE also modestly suppressed the production of several proinflammatory cytokines that are known to increase HIV-1 replication (IL-1β, IL-6, and TNF-α). However, any replication-retarding effects of reduced cellular activation or proinflammatory cytokine production appear to be offset by the replication-enhancing effects of suppressing IFN-γ and IL-10. The effects of NE on viral replication were dose-dependent over a physiologically relevant range (3, 5), raising the possibility that the activation of SNS neurons innervating lymphoid tissue could conceivably influence HIV disease pathogenesis.

The simultaneous suppression of both IFN-γ and IL-10 was necessary for accelerated HIV-1 replication. Replacement of either cytokine alone abrogated the effects of NE. In humans, both IFN-γ and IL-10 regulate cellular immune responses (37–40). However, altered cellular immunity is not likely to explain the present data, because viral replication differences emerged over periods of time that were too short to support the development of an acquired immune response in vitro (41–43). Both IFN-γ and IL-10 are produced at high rates throughout HIV infection (44–48). Both of these cytokines also regulate HIV-1 replication (35, 49–53), although the mechanisms by which they do so remain incompletely understood (54–57). In the present studies, virus production kinetics lagged behind IFN-γ and IL-10 expression kinetics, even though these cytokines were shown to mediate the effects of NE on viral replication. Because the HIV life cycle takes ~2 days under optimal conditions, effects on virus production can lag up to two days due to the time required for viral replication to reach detectable levels.

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days behind any potential cytokine influences. In addition, cytokine alterations may influence the viral life cycle via downstream events that require further time to develop. The identification of such downstream mediators represents an important focus for further research.

The suppression of IFN-γ by NE appears to occur at the level of the cytokine-producing cell itself (primarily CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes), because the production of IL-12, the major exogenous trigger for IFN-γ production (58–60), was not altered. A direct effect on the cytokine-producing cell is consistent with the presence of a cAMP response element in the IFN-γ promoter (61–63) as well as with the NE-induced suppression of IFN-γ production in isolated murine T cell clones (13). Direct suppressive effects of NE on human IL-10 production have not been demonstrated previously, but the IL-10 promoter does contain a cAMP-linked β<sub>2</sub>-adrenoreceptors on both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes provides a structural basis for direct effects of NE on cytokine-producing cells (8, 13).

The simultaneous suppression of both IFN-γ and IL-10 by NE is consistent with an emerging body of evidence suggesting that the innervation of lymphoid tissue by SNS may function as a natural mechanism for selectively suppressing cellular immune responses while sparing or enhancing other aspects of immune system function (e.g., humoral immune responses) (33, 65–69). As such, analyzing the mechanisms by which the SNS alters lymphocyte function may lead to novel strategies for selectively manipulating immune responses in HIV infection and other disease settings.

**FIGURE 4.** Effect of NE on IFN-γ production by CD4<sup>+</sup> and CD8<sup>+</sup> cells. Cytokine-producing lymphocytes were identified by intracellular staining with anti-cytokine Abs at 3 days following the infection of quiescent PBMCs and subsequent costimulation. **A**, Relative to costimulated controls (open histogram) 10 μM NE (solid histogram) significantly reduced the fraction of CD4<sup>+</sup> lymphocytes expressing IFN-γ. **B**, NE similarly reduced IFN-γ expression in CD8<sup>+</sup> lymphocytes. Data are representative of three experiments in which NE reduced the fraction of CD4<sup>+</sup> cells staining positive for IFN-γ by an average of 68% (*p* = 0.016) and reduced the fraction of CD8<sup>+</sup> cells staining positive for IFN-γ by an average of 66% (*p* = 0.023). Because histogram comparisons can confound alterations in cytokine production with alterations in cell size, we used anti-IFN-γ (FL-2) and an irrelevant, isotype-matched control Ab (FL-1) to spatially separate the effects on cell size from effects on cytokine induction. Relative to unstimulated cells (**C**), costimulation markedly increased IFN-γ expression (**D**). Identical costimulation in the presence 10 μM NE (**E**) suppressed the fraction of lymphocytes staining positive for IFN-γ by an average of 82% across three experiments (*p* = 0.006). The higher suppression rate in the later analyses stems from the greater number of costimulated cells identified as IFN-γ<sup>+</sup>; this increased identification is the result of the capacity of two-color analyses to distinguish between small IFN-γ<sup>+</sup> and nonspecific staining of large cells.

**FIGURE 5.** Effect of exogenous IL-10 or IFN-γ on NE-induced acceleration in HIV-1 replication. Quiescently infected PBMCs were costimulated in the presence of 10 μM NE or NE plus the indicated cytokines (500 pg/ml human IFN-γ, IL-10, or IL-2; 50 pg/ml human rIL-4). Data represent the mean fold increase in supernatant p24 levels at 6 days postinfection relative to costimulated controls; the statistical significance of comparisons at specific time points is indicated as **p < 0.01.
Our interest in the effects of SNS activation on the basic HIV life cycle led us to examine a model of CD4+ T cell infection in the absence of acquired immune responses (e.g., CD8 antiviral factors or HIV-specific CTLs) (41–43). Basic viral replication rates play a critical role in driving HIV pathogenesis and long-term disease progression (70). The present data indicate that the products of SNS activation can alter HIV-1 replication in activated PBMCs. Such effects underscore the critical role of immunomodulatory factors in altering HIV replication (35) and raise the possibility that the course of HIV could conceivably be affected by factors associated with SNS activation (e.g., physical or psychological stress) (71, 72). Such results also suggest that it may be productive to address immunoregulatory influences from outside the Ag-immune system axis in attempts to suppress HIV replication over the extended periods of time that are necessary for sustained clinical benefit.

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