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Mechanisms of HIV-1 Inhibition by the Lipid Mediator N-Arachidonoyldopamine\textsuperscript{1}

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Several linear fatty acid dopamines (N-acyldopamines) have been identified recently in the brain. Among them, N-arachidonoyldopamine (NADA) is an endogenous lipid mediator sharing endocannabinoid and endovanilloid biological activities. We have reported previously that NADA exerts some of its biological activities through inhibition of the NF-κB pathway and, because this transcription factor plays a key role in HIV-1-long terminal repeat (LTR) \textit{trans} activation, we have evaluated the anti-HIV-1 activity of NADA. In this study, we show that NADA inhibits vesicular stomatitis virus-pseudotyped HIV-1 infection in the human leukemia T cell line Jurkat, in primary T cells, and in the human astrocytic cell line U373-MG. Other endocannabinoids such as anandamide, 2-arachidonoylglycerol, and noladin ether did not show inhibitory activity in the HIV-1 replication assays. The anti-HIV-1 activity of NADA was independent of known cannabinoid and vanilloid receptor activation. In addition, NADA did not affect reverse transcription and integration steps of the viral cycle, and its inhibitory effect was additive with that of the reverse transcriptase inhibitor azidothymidine. NADA inhibited both TNF-α and HIV-1 \textit{trans} activator protein-induced HIV-1-LTR activation. We also show that NADA counteracts the TNF-α-mediated \textit{trans} activation capacity of the p65 NF-κB subunit without affecting its physical association to the HIV-1-LTR promoter. Moreover, NADA inhibited the p65 transcriptional activity by specifically targeting the phosphorylation of this NF-κB subunit at Ser\textsuperscript{526}. These findings provide new mechanistic insights into the biological activities of NADA, and highlight the potential of lipid mediators for the management of AIDS. \textit{The Journal of Immunology}, 2005, 175: 3990–3999.

Endocannabinoids are a class of lipid mediators found in several tissues and based on a polyunsaturated fatty acid amide or ester motifs (1). Five major endocannabinoids have been described to date, anandamide (AEA)\textsuperscript{3}, virodhamine, N-arachidonoyldopamine, 2-arachidonoylglycerol (2-AG), and the ester of 2-AG, noladin, which differ from each other in the binding affinity and activation of the known endocannabinoid receptors, cannabinoid receptor type 1 (CB1) and type 2 (CB2) (2, 3). More recently, four long-chain, linear fatty acid dopamines (N-acyldopamines) have been identified in the brain. One member of this family of lipids, N-arachidonoyldopamine (NADA), was shown to activate both the CB1 and the transient receptor potential channel vanilloid subfamily member 1 (TRPV1) (4, 5). Over the past few years, there has been a growing awareness that the endocannabinoid system is very complex. Indeed, its inherent complexity is further increased by the cross talk between CB1 and TRPV1 signaling pathways, and by the fact that CB- and TRPV1-independent biological activities have been demonstrated for the archetypal endocannabinoids AEA and 2-AG (6, 7) and for the N-acyldopamine NADA (8).

Endocannabinoids have been implicated in a multitude of processes, such as immune response, cell survival and apoptosis, and neuronal signaling (2, 9). In addition, emerging evidence suggests that endocannabinoids could play an important role in HIV-1 pathogenesis. For instance, enhanced AEA degradation in vivo has been associated with neuronal apoptosis induced by the HIV-1 gp120 in the rat neocortex (10). Moreover, the endocannabinoid system prevents HIV-1 \textit{trans} activator protein (Tat)-induced cytotoxicity in rat glioma cells (11).

The CNS is a major target for HIV-1 infection. HIV-1 enters the CNS soon after systemic infection and persists there for life. HIV-1 is thought to infect the brain by the infiltration of infected monocytes, which then differentiate into macrophages and spread the virus to other cells such as microglia, astrocytes, and oligodendrocytes (12). HIV-1 might also enter the brain in infected CD4\textsuperscript{+} T cells, although the contribution of these cells to the pool of replicating virus in the brain is still unclear (13).

HIV-1 replication is regulated by multiple events occurring at different steps of the viral life cycle. After fusion-mediated entry within host cells, uncoating, reverse transcription of the RNA genome, and nuclear entry of the preintegration complex, the proviral DNA is integrated into the host cell genome (13). The postintegration phase of the viral cycle preferentially is tightly regulated by interplay between a combination of distinct viral proteins and cellular transcription factors acting at the long terminal repeat (LTR) region of the viral genome. The HIV-1-LTR promoter is ~640 nt

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\textsuperscript{3}Abbreviations used in this paper: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; ATPK, arachidonyl trithromethylketone; AZT, azidothymidine; CB, cannabinoid receptor; ChIP, chromatin immunoprecipitation; FAAH, fatty acid amide hydrolase; HAART, highly active antiretroviral therapy; IKK, IκB kinase; LTR, long terminal repeat; NADA, N-arachidonoyldopamine; PKC, protein kinase C; PKR, protein kinase R-activated death domain; Tat, \textit{trans} activator protein; TRPV1, transient receptor potential channel vanilloid subfamily member 1; VSV, vesicular stomatitis virus.

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long and has binding sites for many cellular transcription factors and a cis-activating stem-loop RNA structure called trans activation response element (TAR) (14, 15), which represents the main binding site for the HIV-1 regulatory protein, Tat (16–18). Through interaction with TAR, Tat increases the efficiency of transactivation of HIV-1 (19–21). The initial phase of HIV-1 transactivation is regulated by the interaction of cellular transcription factors with binding sites present within the core promoter region of the HIV-1 LTR (15, 22). This region contains three tandem Sp1 binding sites and two κB elements located upstream of the TATA box that have been shown to play a key role in HIV-1 replication (23–25). The basal level of HIV-1 gene transcription allows the accumulation of HIV-1 Tat protein that in turn binds to the TAR region, enhances the trans activation activity of cellular transcription factors, and promotes HIV-1 replication very efficiently.

NF-κB is a family of transcription factors involved in the control of a variety of cellular processes, such as immune and inflammatory responses, development, cellular growth, apoptosis, and HIV-1 gene expression (26, 27). NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65, p52, RelB, and c-rel subunits that interact with a family of IkB proteins (26). Phosphorylation of IkBe at Ser276 and Ser36 is a key step involved in the activation of NF-κB complexes. This event is mediated by IkB kinases (IKKs) (28), which are formed by a high m.w. complex (IkB kinase complex) containing at least two kinase subunits (IKKα and IKKβ), and the associated modulatory protein NF-κB essential modulator (IKKe) (26, 27). In addition to the control of NF-κB activity exerted at the nuclear translocation level, there is increasing evidence for another complex level of regulation that is mediated by direct phosphorylation and acetylation of nuclear NF-κB subunits (29). For instance, following TNF-α stimulation, p65 can be phosphorylated at Ser529 and Ser536 by casein kinase II (30, 31). IL-1 can also mediate p65 phosphorylation at Ser529 through casein kinase II and IKK, respectively (30, 31). IL-1 can also mediate p65 phosphorylation at Ser529 through casein kinase II (32). In addition, Ser11, Ser276, and Ser468 have also been described to be phosphorylated by protein kinase C (PKC) (33, 34). Protein kinase A/mitogen- and stress-activated kinase 1, and glycogen synthase kinase-3β, respectively (33–36).

We and others have reported that the endocannabinoids system may regulate the immune response by targeting inducible transcription factors (7, 8, 37, 38). We also showed that NADA is a potent immunosuppressive compound in vitro, by inhibiting a common step in the signaling pathways that activate NF-κB, AP-1, and NF-AT transcription factor in T cells (8). Because NF-κB plays an important role in HIV-1 gene transcription, we sought to investigate the possible role of the endocannabinoid system in HIV-1 replication with the aim of elucidating the molecular events involved in the modulation of HIV-1 by this endogenous system.

**Materials and Methods**

**Cell lines and reagents**

The human leukemia Jurkat cell line was cultured in RPMI 1640 medium (Invitrogen Life Technologies), containing 10% heat-inactivated FBS, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml), and was maintained at 37°C in a 5% CO₂ humidified atmosphere and splinted twice per week. The 5.1 cell line (obtained from A. Israel, Institute Pasteur, Paris, France) is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-1-LTR promoter and was maintained in complete medium supplemented with G418 (200 μg/ml). The U373-MG astrocyte cell line was maintained in DMEM (Invitrogen Life Technologies) supplemented with FBS and antibiotics at 37°C in a 5% CO₂ humidified atmosphere and splinted when confluent. The endocannabinoids NADA, AEA, and 2-AG, and the fatty acid amide hydrolase (FAAH) inhibitor arachidonyl trifluoromethyl ketone (ATFMMK) were purchased from Alexis. The CB1 inhibitor SR144716A was from Sanofi Recherche, and 2-archidonoylglycerol ether (noladin) was synthesized, as described before (39). The anti-IκBα mAb 10B was a gift from R. Hay (Centre of Biomolecular Sciences, St. Andrews, Scotland), the anti-p65 antisera 1262 and 1207 were kindly provided by A. Israel (Institut Pasteur, Paris, France); the polyclonal Abs anti-phospho-p65 (Ser536), anti-phospho-p65 (Ser276), and anti-phospho-p65 (Ser468) were from New England Biolabs; and the mouse monoclonal anti-HDAC1 (H11) was purchased from Santa Cruz Biotechnology. All other reagents not cited above or later were purchased from Sigma-Aldrich.

**Plasmids**

The KBF-Luc contains three copies of the MHC enhancer κB site upstream of the conalbumin promoter, followed by the luciferase gene (40). The vector pNL4-3.Luc.R’E’ from N. Landau (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD), as described previously (41), contains the firefly luciferase gene inserted into the pNL4-3 nef gene. Two frameshifts (5‘ Env and Vpr aa 26) render this clone Env- and Vpr- (42). The pC6.2-C-N1/κB expression vector contains the VSV G protein coding sequence and was obtained from F. Arenzana-Seisdedos (Institute Pasteur, Paris, France), and the pcDNA3-Tat expression vector and the pXP1-LTRwt reporter plasmid have been previously described (42, 43). The GST-Tat plasmid was kindly provided by A. P. Rice ( Baylor College of Medicine, Houston, TX). The Gal4-Luc reporter plasmid includes five Gal4 DNA binding sites fused to the luciferase gene (44). The Gal4-p65 vector containing the Gal4-F1 (regulating the Gal4 binding site upstream of the human the gal4 DNA binding domain and the Gal4-VP16 plasmid were obtained from M. L. Schmitz (University of Bern, Bern, Switzerland) and have been previously described (44).

**Production of VSV-pseudotyped HIV-1 recombinant virus**

High titer VSV-pseudotyped HIV-1 recombinant virus stocks were produced in 293T cells by cotransfection of pNL4-3.Luc.R’E’ together with pcDNA3.VSV, VSV encoding the VSV G protein and the calcium phosphate transfection system, as described before (45). Supernatants, containing virus stocks, were harvested 48 h posttransfection, were centrifuged 5 min at 500 × g to remove cell debris, and stored at −80°C until use. Cell-free viral stock was tested using an enzyme-linked immunosassay for Ag HIV-p24 detection (Innotest HIV Ag mAb; Innogenetics). Cultures were infected at a dose of 200 ng of HIV-1 gag p24 protein.

**VSV-pseudotyped HIV-1 infection assays**

Jurkat cells (10⁶/ml), U373-MG cells seeded the day before infection (10⁶/ml), or staphylococcal enterotoxin B (SEB)-activated PBMCs (10⁹/ml) were pretreated with the compounds for 30 min. After pretreatment, cells were inoculated with virus stocks (200 ng of p24), and 24 h later cells were washed twice in PBS and lysed in 25 mM Tris-phosphate, pH 7.8, 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100, and 7% glycerol for 15 min at room temperature. Then the lysates were spun down and the supernatants used to measure luciferase activity using an Autolumat LB 9510 (Berthold Technologies), following the instructions of the luciferase assay kit (Promega). The results are represented as the percentage of activation (considering the infected and untreated cells 100% activation). Results represent mean ± SD of four different experiments. Statistical analysis was performed using ANOVA, followed by the Student-Newman-Keuls method, with values of p < 0.05 considered to be significant.

**Isolation of human peripheral mononuclear cells and cytotoxicity assays**

Human PBMCs, from healthy HIV-1-seronegative donors, were isolated by centrifugation of venous blood on Ficoll-Hypaque density gradients (Amersham Biosciences). PBMCs (2.5 × 10⁶/ml) were treated with SEB (1 μg/ml) for 72 h and then collected and used for recombinant virus infection assays. For cytotoxicity analysis, NADA-treated and untreated HIV-1-infected cells were incubated for 1 min at room temperature in the presence of propidium iodide (10 μg/ml), as previously described (46). After incubation, cells were immediately analyzed by flow cytometry.

**Semiquantitative PCR analysis**

Reverse transcriptase products were detected, as previously described (46). Briefly, Jurkat cells or U373-MG were infected with VSV-pseudotyped
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recombinant virus for 24 h, as indicated, and total DNA was extracted with QIAamp DNA minikit (Qagen) and quantified by UV spectrophotometry at 260 nm. PCR was performed with the following oligonucleotides to amplify short retrotranscribed product (amplon size: 140 bp): R/U5 (forward), 5'-GGCTAAGTGGAGGACACCTG-3'; R/U5 (reverse), 5'-CTGTAAGTTGCTTTACACGTACG-3' and to amplify long reverse transcription product (amplon size: 200 bp): R/U5 (forward), LTR/igg (reverse), 5'-CTGCTCTCGGAGAAGCTCAGTCTG-3'. As a control, genomic DNA was amplified with β-actin amplification, and PCR products were electrophoresed on a 2% (w/v) agarose gel.

Analysis of HIV-1-integrated DNA by nase PCR

Genomic DNA from VSV-pseudotyped HIV-1-infected cells and control cells was extracted and quantified, as described above. The detection of HIV-1-LTR integrated into the cell genome was performed, as previously described (46). The first PCR was conducted in hot start conditions by using primers Alu-LTR 5'-5'TCCAGACCTCAAGGGCTTGAGG-3' from conserved human Alu sequences and Alu-LTR 3'-from conserved HIV-1-LTR sequences (5'-TTTCAGGTCCTGGTCCGGGCCTCA-3'). Following the initial PCR, a second nested PCR amplification was conducted by using an aliquot equivalent to 1/25 of the first PCR. The second PCR was performed by semiquantitative PCR analysis with primers R/U5 (forward) and R/U5 (reverse) (described above). As a control, genomic DNA was subjected to β-actin amplification and used to normalize the nested PCR products, which were electrophoresed on a 2% (w/v) agarose gel. The PCR bands were quantified using a Versadoc 3000 imaging system (Bio-Rad), and the data were presented as fold induction over untreated cells (in the case of R/U5) or over untreated cells (in the case of β-actin).

Transient transfections and luciferase assays

Jurkat cells were transfected with the indicated plasmids using lipofectamine (Invitrogen Life Technologies), according to the manufacturer's recommendations. Twenty-four hours posttransfection, cells were preincubated for 30 min with NADA, as indicated. Cells were then washed twice with cold PBS, and chromatin preparation was set aside and designated as input fraction. To reduce the nonspecific background, the chromatin solution was preincubated with salmon sperm DNA, BSA, and protein A agarose beads (50% slurry) rocking for 1 h at 4°C, followed by centrifugation. The supernatant was used as the input sample for immunoprecipitation with the antibodies of interest.

Western blots

Jurkat cells (2 × 10^6/ml) were treated with the agonists in complete medium, as indicated. Cells were then washed twice with cold PBS, and proteins from cytoplasmic/nuclear fraction or whole cell extracts were extracted in lysis buffer A (20 mM HEPES, pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 1 mM PMSF, 10 μM leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM NaF, 1 mM NaVO₃, and 0.5% Nonidet P-40) and the chromatin was end labeled with [γ-32P]ATP. The binding reaction mixture contained 3 μM of nuclear extract, 0.5 μg of poly(dI-dC), 20 mM HEPES, pH 7.0, 70 mM NaCl, 2 mM DTT, 0.01% Nonidet P-40, 100 μg/ml BSA, 4% Ficoll, and 100,000 cpm of end-labeled DNA fragments in a total volume of 20 μl. After 30-min incubation at 30°C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris-base, 89 mM boric acid, and 2 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to x-ray film at ~80°C. For the Tat-TAR-binding assay, RNA probe containing the 5' bulge of TAR (47) was end labeled with [γ-32P]ATP and incubated with 20 nM rGST-Tat protein in EMSA buffer for 30 min at 4°C in the presence or absence of NADA, and RNA-protein complexes were separated by 6% nondenaturing polyacrylamide gels, dried, and exposed x-ray film at ~80°C.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed, as described previously (48), with minor modifications. Briefly, 5.1 cells were treated with TNF-α for 15 min in the presence or absence of NADA. After treatment, the cells were cross-linked by 1% formaldehyde for 10 min, followed by quenching with 125 mM glycine. The cells were washed twice with chilled PBS, and the cell pellets were washed with cold PBS containing 0.5 mM PMSF, 10 μM leupeptin, 10 μg/ml aprotinin, and 1 μg/ml leupeptin, and the pelleted was resuspended in 0.2 ml radioimmunoprecipitation lysis buffer (0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 0.2 mM NaVO₃ supplemented with protease inhibitors) and incubated on ice for 10 min. The cell lysate was sonicated to shear chromatin into lengths of ~1500 bp. Supernatant and the sheared chromatin were diluted 3-fold in radioimmunoprecipitation lysis buffer. An aliquot (20 μl) of the chromatin preparation was set aside and designated as input fraction. To reduce the nonspecific background, the chromatin solution was preincubated with salmon sperm DNA, BSA, and protein A agarose beads (50% slurry) rocking for 1 h at 4°C, followed by centrifugation. The supernatant was used as the input sample for immunoprecipitation with the antibodies of interest.

Results

NADA inhibits HIV-1 replication

We have shown recently that NADA inhibits IL-2 gene transcription by targeting a common pathway that regulates NF-AT, NF-κB, and AP-1 transcription factors (8). Because NF-κB positively regulates LTR-dependent HIV-1 transcription, we were interested in studying the anti-HIV-1 effect of the most relevant endocannabinoids described to date in transformed and primary cells. First, we infected Jurkat cells with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope, which bypasses the natural mode of HIV-1 entry into these cells that support robust HIV-1 replication (46, 49). Upon integration into host chromosomes, this recombinant virus expresses the firefly luciferase gene, and consequently, luciferase activity in infected cells correlates with the rate of viral replication. Thus, high luciferase activity levels were detected 24 h after cellular infection with the VSV-pseudotyped HIV-1 clone,
and pretreatment of Jurkat cells 30 min prior infection with increasing doses of NADA resulted in a dose-dependent inhibition of luciferase activity with an IC50 below 2.5 μM and reaching a complete inhibition at 10 μM (Fig. 1A). In contrast, AEA, 2-AG, or noladin, at the doses tested, showed no inhibitory activity on HIV-1 replication. These results suggest that the effect observed is restricted to NADA and is independent of the natural mode of fusion of HIV-1 to the host cell. The inhibitory effect of NADA on HIV-1 replication was not restricted to lymphoid cells because we found similar effects in the astrocytoma cell line U373-MG, which has been used previously as a model of HIV-1 replication in astrocytes (49) (Fig. 1B). Moreover, NADA also showed a potent anti-HIV-1 activity in human primary T cells acutely infected with this HIV-1 clone (Fig. 2). The anti-HIV-1 effect of NADA was comparable to the reverse transcriptase inhibitor azidothymidine (AZT) (Fig. 1A) and was not due to cytotoxicity because cell viability assays did not reveal a significant decrease in the percentage of cell viability both in Jurkat cells (data not shown) and in primary T cells (Fig. 2) treated for 24 h with increasing concentrations of this compound.

The anti-HIV-1 effect of NADA is not reverted by CB1 antagonism or FAAH inhibition

NADA was first identified as a hybrid endocannabinoid/endovanilloid molecule with high affinity for TRPV1 and CB1, and negligible affinity for CB2 (4, 5). However, Jurkat cells do not express either CB1 (7) or TRPV1 (our unpublished results) receptors, whereas CB1, but not TRPV-1, is expressed in the U373-MG cell line (50) (data not shown). Thus, to study whether NADA exerted its inhibitory activity in both cell lines through different mechanisms, Jurkat and U373-MG cells were preincubated with the CB1 antagonist SR141716A for 30 min before virus inoculation, and 24 h later the luciferase activity was measured in the cell lysates. As depicted in Fig. 3, the inhibitory effect of NADA was not prevented by the presence of the CB1 antagonist in either Jurkat (Fig. 3A) or U373-MG cells (Fig. 3B). Although NADA has been described not to be a good substrate for FAAH (4), we also studied the effect of the FAAH inhibitor ATFMK on NADA-mediated anti-HIV-1 activity. As expected, ATFMK did not reverse the inhibitory activity of this lipid mediator in any of the tested cell lines. On the contrary, we observed that ATFMK by itself was able to inhibit HIV-1 replication, although this effect was only significant in Jurkat cells. Altogether, our results strongly suggest that the inhibitory activity of NADA was independent of the known endocannabinoid receptors.

NADA does not affect HIV-1 reverse transcription or integration steps

Although the VSV-pseudotyped HIV-1 clone bypasses the natural mode of HIV-1 entry into mammalian cells, it does need the reverse transcription and integration steps. Therefore, we studied
whether these steps were affected by NADA. First, semiquantitative PCR was performed to amplify HIV-1 strong-stop (R/U5) and full-length (LTR/gag) reverse transcriptase products, which represent early and late reverse transcriptase products, respectively. As expected, AZT inhibited the amplification of the full-length (LTR-Gag); however, NADA did not decrease the amount of either R/U5 or LTR/gag products obtained following Jurkat cells or U373-MG infection with VSV-pseudotyped HIV-1 (Fig. 4A). Next, we investigated the effect of NADA in HIV-1 integration; the DNA of infected cells was extracted and subjected to a first round of Alu-PCR, followed by nested PCR using internal LTR primers, as described in Materials and Methods, and β-actin was also amplified and used to normalize the amount of integrated HIV-1. Fig. 4B shows that NADA did not affect HIV-1 integration in either Jurkat or U373-MG acutely infected with the VSV-pseudotyped HIV-1 proviral clone. To further confirm the lack of effect of NADA on viral reverse transcription and integration steps, several functional luciferase-based analyses were performed. First, Jurkat cells were infected with the HIV-1 recombinant clone, and 12 h later (time at which reverse transcription and integration have occurred), AZT (10 μM) or increasing concentrations of NADA were added for an additional 24-h period of time, and finally, the luciferase activity in cell extracts was measured, and the results represented as fold induction ± SD compared with nontreated infected cells.

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** Effects of SR141716A and ATFMK on HIV-1 inhibition by NADA. Jurkat cells (A) or U373-MG cells (B) were pretreated with NADA in the presence or absence of either the CB1 antagonist SR141716A (5 μM) or the FAAH inhibitor ATFMK (5 μM) and then infected with VSV-pseudotyped pNL4-3.Luc.R-E' for an additional 24 h. Luciferase activity in cell extracts was measured, and the results represented as fold induction ± SD compared with nontreated infected cells.

![FIGURE 4.](http://www.jimmunol.org/)

**FIGURE 4.** Effect of NADA on HIV-1 reverse transcription and integration steps. A. Semiquantitative PCR was performed on DNA extracted from Jurkat or U373-MG cells 24 h postinfection with VSV-pseudotyped pNL4-3.Luc.R-E' (200 ng of p24) virus, following treatment with 5 μM NADA or 10 μM AZT. Primers were used to amplify the HIV-1 reverse transcriptase short products R/U5 (top panel) and long products LTR/gag (middle panel) as well as the β-actin (bottom panel) as a control. B. Detection of integrated DNA by using Alu-LTR PCR. Genomic DNA from Jurkat or U373-MG cells treated as in A was extracted and subjected to PCR by using the primers Alu-LTR-3' and Alu-LTR-5'. An aliquot of the first PCR product was subjected to the second round of PCR by using nested HIV-1-LTR-specific primers (R/U5 (forward) and R/U5 (reverse), and the products were visualized by agarose gel electrophoresis (upper panel). The results were normalized with the products of β-actin PCR performed using the same genomic DNA as template.
FIGURE 5. The inhibitory activity of NADA requires HIV-1 integration. A, Jurkat cells (10⁶/ml) were infected with VSV-pseudotyped pNL4-3.Luc.R’E’ (200 ng of p24) virus, and 12 h postinfection the cells were washed and treated with AZT (10 μM) or increasing concentrations of NADA for an additional 24 h (Inset). In parallel, cells were pretreated with the same concentrations of NADA and AZT and infected with recombinant virus for 24 h (Inset). Luciferase activity was measured and represented as percentage of activation compared with nontreated infected cells. Additive effect of NADA and AZT. B, Jurkat cells were pretreated with AZT, NADA, or a combination of both for 30 min, and then infected with recombinant virus for 24 h. Luciferase activity in cell extracts was determined, and results represented as percentage of activation ± SD compared with nontreated infected cells (100% activation).

pXP1LTRwt and pcDNA3-Tat, and 24 posttransfection the transfected cells were treated with increasing concentrations of NADA for an additional period of 6 h, after which luciferase activity was measured. As shown in Fig. 6A, the HIV-1 Tat protein increased the luciferase expression driven by the HIV-1-LTR (near 20-fold induction), and this effect was inhibited by NADA with an IC₅₀ close to 2.5 μM. It is well known that Tat/TAR binding is a critical step for the potent Tat-induced HIV-1-LTR transactivation. Therefore, we studied the effects of NADA on the in vitro binding activities of the protein complexes bound to TAR using the HIV-1 Tat recombinant protein. We found that NADA did not interfere with the ability of Tat to bind the TAR element, indicating that the inhibitory activity of NADA on Tat-induced HIV-1-LTR transactivation was not mediated by a disruption of Tat/TAR complexes (Fig. 6A, inset). Because HIV-1 Tat directly binds to the NF-κB enhancer sequence (52) and regulates HIV-1-LTR transactivation by acting on NF-κB-responsive elements (53), we studied the effects of NADA on the NF-κB-dependent regulation of the HIV-1-LTR promoter in 5.1 cell line, which contains the luciferase gene driven by the HIV-1-LTR promoter that is highly responsive to TNF-α through the NF-κB pathway. The 5.1 cells were preincubated with increasing concentrations of NADA and then stimulated with TNF-α (5 ng/ml) for 6 h, and luciferase activity was measured. As depicted in Fig. 6B, pretreatment with NADA resulted in a dose-dependent inhibition of TNF-α-induced LTR activation, extending our previous results showing that the NF-κB pathway is involved in the inhibitory mechanism of NADA (8). To investigate directly the effects of NADA on TNF-α-induced NF-κB activation, we transfected Jurkat cells with a κB luciferase reporter plasmid and we found that NADA effectively inhibited the transcriptional activity of this promoter in a dose-dependent manner (Fig. 6C). Similar results were obtained in the U373-MG cell line (data not shown).

NADA inhibits TNF-α-induced nuclear phosphorylation of p65

The signaling pathways that activate NF-κB include a complex activation of regulatory kinases resulting in the phosphorylation and degradation of the IκB proteins and nuclear translocation of NF-κB (26). In addition to this pathway, a second level of NF-κB activation involves the phosphorylation of the p65 and the subsequent stimulation of NF-κB trans activation (29). Thus, to investigate the level at which NADA exerted its inhibitory effect on NF-κB activation, we stimulated Jurkat cells with TNF-α for different times in the presence or absence of NADA (2.5 μM), and proteins from nuclear extracts were analyzed for NF-κB DNA-binding activity by EMSA. The kinetic experiments revealed a
clear increase in NF-κB binding to DNA after 5 min of TNF-α treatment was maintained through the time of stimulation and was not affected by NADA pretreatment (Fig. 7A). The DNA-binding specificity was studied by supershift experiments with specific anti-p50 and anti-p65 (RelA) Abs and by cold competition experiments with unlabeled competitors, and the heterodimer p50/p65 was identified as the main complex in this cell line (7). To further confirm that NADA does not interfere with the binding of p65 to DNA in vivo, we performed ChIP assays using a mix of anti-p65 Abs (anti-N- and anti-C-terminal rabbit polyclonal Abs). As shown in Fig. 7B, in TNF-α-stimulated Jurkat cells, p65 is associated with the HIV-1-LTR promoter, but not with the protein kinase regulated by RNA (PKR) promoter that does not contain NF-κB binding sites (48), and, as expected, NADA pretreatment did not interfere with the p65 association to the HIV-1-LTR promoter.

To further analyze the level at which NADA inhibits the NF-κB pathway, we stimulated Jurkat cells with TNF-α in the presence or absence of NADA (2.5 μM) for the indicated times, and total, cytoplasmic, and nuclear cell extracts were subjected to immunoblot assays to study TNF-α-mediated IκBα phosphorylation and degradation and p65 phosphorylation (Ser536). In Fig. 8A, it is shown that TNF-α-induced phosphorylation and degradation of IκBα were not affected by the presence of NADA. In contrast, late (15 min), but not early (5 min), p65 phosphorylation in response to TNF-α was clearly inhibited by NADA. Because p65 phosphorylation has been shown to occur both in cytoplasmic and nuclear compartments (54), we investigated the localization at which NADA inhibits p65 phosphorylation (Ser536), and we found that cytoplasmic p65 phosphorylation was not affected by NADA in TNF-α-stimulated Jurkat cells. On the contrary, nuclear p65 phosphorylation was clearly inhibited by the same treatment with this endocannabinoid (Fig. 8A). Interestingly, the inhibitory effect of NADA on TNF-α-mediated p65 phosphorylation was restricted to Ser536, because the phosphorylations of Ser276 and Ser468 were not affected by NADA (Fig. 8B). Finally, we studied whether NADA inhibits directly p65-transcriptional activity using the Gal4-p65/Gal4-Luc system that has the advantage that the Gal4 trans activator fusion protein is exclusively nuclear, and thus is regulated independently of IκB (44). The results presented in Fig. 8C revealed that transcriptional activity of Gal4-p65 was clearly inhibited by NADA in a concentration-dependent manner. NADA also inhibited the luciferase activity induced by the fusion protein Gal4-VP16, but to a much lesser extent.

Discussion

The CNS is susceptible to infection by retroviruses of various species and by members of the lentivirus family such as the HIV-1. Therefore, HIV-1-infected individuals develop a range of cognitive symptoms, motor disturbances, behavioral changes, reduced concentration, headache, and peripheral neuropathies, which are collectively known as HIV-associated dementia (12, 55). Before the advent of highly active antiretroviral therapy (HAART), ~20–30% of individuals infected with HIV-1 developed HIV-associated dementia (56), and despite the clinical and immunological improvement of patients treated with HAART, the occurrence of HIV-1-related neuropathology in postmortem tissue has not changed (57). Because the antiviral drugs used in HAART have limited access to the brain, it is likely that the current antiviral therapy reduces the neurological symptoms by indirect means such as the reduction of peripheral viremia and the low occurrence of opportunistic infections due to the partial reconstitution of the immune response.

It is known that the main target for HIV-1 infection in the brain is the microglia compartment. However, both in vitro and in vivo studies have unequivocally demonstrated that astrocytes are also infected by the HIV-1 (58–60). Although the proportion of HIV-1-infected astrocytes is very low (61), these cells constitute the most abundant cell type in the brain, and these infected cells may be a significant factor in HIV-1-mediated astrogliosis and neuro-pathogenesis (59). Moreover, astrocytes may provide a viral reservoir with capacity for reactivation. Although it is thought that HIV-1 replication is very limited in these type of cells (62), more recent evidence demonstrated that there is no cell-type restriction for HIV-1 replication in primary astrocytes (49), and under certain proinflammatory conditions latent HIV-1 infection in astrocytes can be reactivated (63). The results presented in this work further confirm that HIV-1 can replicate in the astrocytoma cell line U373-MG (64), and this replication was selectively inhibited by NADA and not by other endocannabinoids. Therefore, as a working hypothesis, we can argue that NADA may exert a neuroprotective role in HIV-1-infected brains by acting at least at two levels: 1) through a CB1-independent pathway by inhibiting the transcriptional regulation of integrated HIV-1; and 2) by exerting CB1-mediated neuroprotective and anti-inflammatory actions. Endocannabinoids are selectively and transiently elevated in specific brain areas during several CNS pathological conditions and are

FIGURE 7. NADA does not affect in vitro (EMSA) and in vivo (ChIP) association of p65 to DNA in TNF-α-stimulated T cells. A, Jurkat cells were incubated with NADA for 30 min and then treated with TNF-α for the indicated times. Nuclear extracts were obtained, and their NF-κB-DNA-binding activity was evaluated by EMSA. B, ChIP analysis was performed in TNF-α-stimulated 5.1 cells in the presence or absence of NADA (2.5 μM) using an Ab against p65. Nested PCR using LTR- and PKR-specific primers, as described above, was performed with immunoprecipitated DNA. DNA isolated from input chromatin was amplified as a control.
believed to be produced as a CB1-dependent self-defense mechanism after brain insults. In this sense, experimental evidence indicates that endocannabinoids may protect neurons from toxic insults such as glutaminergic overstimulation, ischemia, excess of NO generation, and oxidative stress through a CB1 receptor pathway (2, 65). Interestingly, CB1 activation inhibits HIV-1 Tat enhancement of NO production in glioma cells (11), and gp120-induced neuronal apoptosis is attenuated by methyl-arachidonoyl fluorophosphate, an inhibitor of the AEA-hydrolyzing enzyme FAAH (10).

Our results demonstrate that NADA also inhibits HIV-1 replication in SEB-activated peripheral primary T cells, PBMCs, and in the Jurkat T cell line by targeting transcriptional regulation once the proviral DNA is integrated into the host cell genome. The role of CD4+ T cells to spread HIV-1 into the CNS is less evident than for macrophages, but CD8+ T cells are consistently observed in the brain of HIV-1-infected patients (66). Moreover, in certain circumstances, CD8+ T cells can coexpress the CD4 molecule at the cell surface, and therefore, can also be infected by the HIV-1 (67). Thus, NADA may inhibit not only the viral replication in T cells, but also T cell activation (8), and therefore limits the neurotoxicity induced by CTLs.

Although NADA may exert biological activities through CB1-dependent and -independent pathways, the anti-HIV-1 activities reported in this work are clearly independent of CB1 receptor activation. Similar results were obtained in Jurkat and in U373-MG cells that express distinct types of CB receptors (7, 68). Moreover, none of the cell types used in our study expresses the TPRV1, another known cell surface receptor for NADA (4). Thus, it is likely that the inhibitory activity of NADA on the transcriptional regulation of the HIV-1 could be mediated by novel pathway. This yet to be identified pathway could be a novel endocannabinoid receptor, or alternatively, NADA may enter into the cells, and once inside the cells, this compound, or a metabolite generated by an enzyme other than FAAH, might interact with a specific component of the signaling cascade, leading to the inhibition of p65 transcriptional activity.

We found that NADA inhibited both Tat and TNF-α-induced LTR-dependent transcription, without affecting Tat/TAR binding. Moreover, in transient transfections with KBF-Luc plasmid, we found that NADA inhibits TNF-α-mediated NF-κB activation. Altogether, these results suggest that NADA may target a common pathway shared by Tat and TNF-α signaling pathways leading to NF-κB activation. Because NADA did not affect the in vivo association of p65 to DNA, the pathway inhibited should rely on the status of DNA-bound p65, which involves acetylation and phosphorylation. Interestingly NADA inhibits nuclear phosphorylation of p65 at Ser536 without affecting Ser468 or Ser276 phosphorylation, and these results suggest that kinase(s) responsible for nuclear phosphorylation of Ser536 is the main target for the inhibitory activity of NADA; however, the existence of additional phosphorylation sites affected by NADA cannot be ruled out. For instance, NADA also inhibited the Tat-induced p65 transcriptional independently of Ser276, Ser279, and Ser536 phosphorylation (R. Sancho and E. Muñoz, unpublished results), and it has been shown that p65 is phosphorylated at Ser311 in response to TNF-α by PKCζ, this phosphorylation step being essential for NF-κB transcriptional activation (33). Ser311 phosphorylation mediates contact of p65 with activators, corepressors, or components of the basal transcriptional machinery controlling the phosphorylation status of p65, and thus its transcriptional potential (33). Interestingly, it has been described that HIV-1 Tat induces PKCζ activation in PC12 cells (69), and therefore, it is possible that NADA...
may target PKCζ and hence inhibit p65 phosphorylation. We are currently investigating this possibility.

We observed in our experiments that NADA inhibited the HIV-1 LTR promoter (LTR-Luc) more efficiently than the artificial promoter containing only three NF-κB binding (KB-F-luc), and therefore, we cannot rule out that NADA may interfere with the transcriptional activity of transcription factors other than NF-κB. In contrast, it has been shown that p65 can also stimulate elongation from the HIV-1 LTR (70), and it is possible that NADA inhibits both the transcriptional and elongation activities of the p65 NF-κB subunit; this could explain the fact that NADA is a more potent inhibitor of NF-κB in the context of the full HIV-1 LTR promoter.

In addition to the CB1 and TRPV1-independent effects described in this work for NADA, it is also possible that, under pathological conditions, this compound may have an entourage effect, i.e., inhibits the hydrolysis and/or the uptake of AEA, with an overall increase of its bioavailability. In this sense, it has been suggested that lipid mediators simultaneously synthesized and released can have synergistic effects not only by heightening the potency of the major bioactive compound, but also by targeting distinct signaling pathways. This process could be specially relevant in the neuropathogenesis of AIDS, because some endocannabinoids can be neuroprotective by acting in a CB-dependent way. Therefore, we may target PKCζ and hence inhibit p65 phosphorylation. We are currently investigating this possibility.

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