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Extracellular HIV-Tat Induces Cyclooxygenase-2 in Glial Cells through Activation of Nuclear Factor of Activated T Cells

Almudena Blanco,* Susana Álvarez, † Manuel Fresno, † and María Ángeles Muñoz-Fernández‡*

Both the HIV-1 protein Tat and cyclooxygenase-2 (COX-2) have been involved in the neuropathogenesis associated with HIV-1 infection. However, the relationship among them has not been addressed. Here, we found that extracellular Tat was able to induce COX-2 mRNA and protein expression and PGE₂ synthesis in astrocytoma cell lines and primary human astrocytes. Moreover, Tat induced COX-2 promoter transcription. Deletion of NF-κB sites of the promoter did not diminish Tat-dependent transcription. Interestingly, Tat did not induce NF-κB activity, suggesting that NF-κB was not necessary to control COX-2 transcription induced by Tat. In contrast, deletion or mutation of the NFAT and/or AP-1 site abrogated COX-2 induction by Tat. Moreover, Tat induced transcription of NFAT- and AP-1-dependent reporter genes. Transfection of a dominant negative c-Jun mutant protein, TAM-67, or of a dominant negative version of NFAT, efficiently blocked the induction of COX-2 promoter by Tat, confirming the requirement of both transcription factors. Moreover, Tat induced NFAT translocation to the nucleus and binding to the distal site of the COX-2 promoter. The importance of NFAT and AP-1 in COX-2 induction and PGE₂ synthesis by Tat was corroborated by using pharmacological inhibitors of the NFAT, ERK, and JNK pathways. In summary, our results indicate that HIV-1 Tat was able to induce COX-2 and PGE₂ synthesis in astrocytic cells through an NFAT/AP-1-dependent mechanism. The Journal of Immunology, 2008, 180: 530–540.

The pathogenesis of HAD is complex and not fully understood. Direct infection of some brain cells as well as direct or indirect action of viral proteins have been proposed to play a role in neuropathogenesis (4–6). The major target cells for productive HIV-1 infection in the CNS are microglia and brain macrophages, the replication of HIV-1 in astrocytes and especially in neurons still being a controversial issue (7). Thus, entry and replication are inefficient in astrocytes (8), whereas neurons are not generally infected by HIV-1. Moreover, brain damage is induced by soluble factors including viral proteins, proinflammatory cytokines, or chemokines released by HIV-1-infected macrophages/microglial cells (9). Astroglia are the major glial cells in the CNS, and reactive astroglia have been found in large numbers in the brains of HAD patients.

The HIV trans activator protein is an 86- to 101-aa protein that plays many roles in HIV infection, including the enhancement of the transcription of viral RNA nonstructural protein, and due to its trans-activating properties is essential for viral replication (10). Tat has been proposed as a potential contributor to HAD (11). Thus, Tat is actively secreted by infected cells of HAD patients (12, 13), including HIV-infected astrocytes (14). Elevated Tat mRNA levels have been found in the brain of HIV-1-infected individuals with neurological alterations (15, 16), and anti-Tat Abs are frequently detected in the serum of HAD patients (17). Moreover, a single intraventricular injection of Tat leads to macrophage infiltration, progressive glial activation, and neuronal death, pathologies that are also observed in HAD brains (18). In addition, transgenic mice expressing Tat in astrocytes also showed neurological symptoms similar to those associated with brain HIV-1 infection in humans (19).

Both extracellular and intracellular Tat may cause neurotoxicity by several mechanisms that interfere with the normal functioning of neuronal and glial cells (reviewed in Ref. 20). Extracellular Tat may induce neurotoxicity by induction of oxidative stress, mitochondrial injury, and/or initiation of inflammatory cascades (19). Tat may also induce neurotoxicity through the release of MCP-1, favoring the migration of inflammatory cells (21, 22). There are
synergistic effects of Tat with other pathogenetically relevant factors in HAD such as the HIV-1 envelope protein gp120 (23). Tat can directly interact with the cell membrane of neurons and depolarize them as well as to increase intracellular Ca\(^{2+}\) levels, generating of reactive oxygen species and eventually apoptosis of neurons. In addition, Tat was shown to prevent long-term potentiation in organotypic hippocampal slices of rats (24).

In astrocytes, Tat induces production of various inflammatory mediators, particularly TNF, and this promotes brain inflammation (25, 26). Tat was also found to activate expression of inducible NO synthase and increase production of NO (27). In addition, Tat increases the production of reactive oxygen species and eventually apoptosis of neurons (26). Tat was also found to activate expression of inducible NO synthase and increase production of NO (27). In addition, Tat increases the production of reactive oxygen species and eventually apoptosis of neurons (26). Tat was also found to activate expression of inducible NO synthase and increase production of NO (27).

In contrast, recent results support a role of cyclooxygenase-2 (COX-2) in HAD. This hypothesis is based on evidence that COX-2 inhibitors prevented apoptotic death produced by HIV gp120 injection in the rat neocortex (29). Besides, enhanced levels of PGE\(_2\), the major product of COX-2, have been detected in cerebrospinal fluid in patients with HAD; these enhanced levels were associated with severity of dementia (30). Moreover, COX-2-positive macrophages infiltrating the brain of patients with HIV-1 encephalitis were described (31). There is also increasing evidence that COX-2 is involved in the pathogenesis of other neurological disorders associated with inflammation, including ischemic brain injury or Alzheimer’s disease (reviewed in Ref. 32).

COX is the key enzyme in the conversion of arachidonic acid to prostaglandins. Two isoenzymes, COX-1 and COX-2 (33, 34), have been shown to be expressed in mammalian tissues; however, they have distinct functions in cell proliferation and differentiation. Thus, COX-1 is thought to be constitutively expressed in most tissues, and it is related to prostanoid synthesis in housekeeping cellular functions, whereas COX-2 is induced by several stimuli associated with cellular activation and inflammation and is thought to be responsible for the increased production of prostaglandins in pathological processes. In the brain, COX-2 is constitutively expressed in selected neurons like human cortical and hippocampal ones, and it is regulated by synaptic activity (35).

The regulation of COX-2 synthesis occurs at both transcriptional and posttranscriptional levels (reviewed in Ref. 34). Depending on the cell type and the stimuli used, different transcription factors play a role in COX-2 induction. For example, in macrophages NF-κB (36), NF-IL6/CCAAT enhancer-binding protein (37), and cAMP response element-binding proteins are required for COX-2 transcriptional induction in response to variety of stimuli. In contrast, NFAT participates in COX-2 induction in T cells and vascular endothelial cells (38, 39), and activation of components of the AP-1 transcription factor complex such as fos/jun proteins has been reported to be important for the activation of the transcription of the COX-2 gene in several cell types (38, 40).

The NFAT family of transcription factors consists of four classical members: NFAT1 (NFATc1); NFAT2 (NFATc2); NFAT3 (NFATc3); and NFAT4 (NFATc4); and NFAT1 and NFAT2 being preferentially expressed in peripheral lymphoid tissues (41). NFAT is usually present in the cytoplasm of cells in a hyperphosphorylated state that present it to enter into the nucleus. A calcium increase activates the phosphatase calcineurin (Cn), which dephosphorylates NFAT, inducing its translocation to the nucleus. The activity of Cn (and therefore NFAT activation) can be inhibited by the immunosuppressive drug cyclosporin A (CsA) (41).

In contrast, activation of AP-1 transcription factor composed mostly of fos/jun dimers is achieved via one or several of the MAPK pathways: ERK; JNK; and the p38 stress-activated protein kinase pathway (42). Several reports have described the ability of p38 MAPK to augment COX-2 expression in human neuroblastoma cells, suggesting that p38 MAPK may be involved in neuronal expression of COX-2 (43, 44).

In the current study, we examined the effect of extracellular Tat on COX-2 expression in astroglial cells and the molecular mechanism involved in this induction. Our results indicate that Tat induces COX-2 expression at the transcriptional level by inducing both AP-1 and NFAT activities.

### Materials and Methods

#### Cell culture and treatments

The astrocytoma human cell lines U-87 and U-373 were routinely grown in DMEM (Invitrogen Life Technologies) containing 10% heat-inactivated FCS, 1% penicillin/streptomycin, and 2 mM L-glutamine (ICN Pharmaceuticals) at 37°C in a humidified atmosphere of 5% CO\(_2\). Confluent monolayers were passaged routinely by trypsinization. Normal human astrocytes (NHA) were purchased from Cambrex (CC-2565; NHA). NHA cells were cultured by plating the cells at a density of 15,000 cells/cm\(^2\) in expansion medium (AGM Bullet Kit media; Cambrex) according to the manufacturer’s protocol. The cells were incubated at 37°C with 5% humidified CO\(_2\) and allowed to reach confluency. Confluent monolayers were routinely passaged after trypsinization. HIV-1 Tat recombinant prepared as described was generously donated by Dr. C. Suñé (National Centre for Biotechnology, Madrid, Spain; Ref. 45). Tat preparations used were analyzed for endotoxin and found to be endotoxin free (<25 pg/ml).

The ERK-1 kinase inhibitor PD98059, the specific JNK inhibitor SP600125 as well as CsA, as inhibitor of Cn, were obtained from Santa Cruz Biotechnology. Inhibitors were tested in a concentration range chosen according to published results to allow specific inhibition of the each enzyme. None of the inhibitors, at the indicated concentrations, affected the viability of the cells. LPS from *Escherichia coli* was from Sigma-Aldrich. COX-2 Ab was obtained from Cayman Chemical. Tat Ab was obtained from AIDS repository agent.

#### Plasmid constructs

The different COX-2 promoter luciferase constructs (P2: 1900 to +104), P2-431 (−327 to +104), P2-274 (−170 to +104), P2-192 (−88 to +104), P2-150 (−46 to +104), and P2-274 pNFAT-mut, P2-274 dNFAT-mut as well as the P2-274 COX-2 d&pNFAT-mut were originally described by Iniguez et al. (38). The pNF-κB-luc reporter plasmid contains a trimer of the NF-κB-binding motif of the H-2k gene upstream of the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p-73col-luc reporter plasmid contains the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p-73col-luc reporter plasmid contains the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p-73col-luc reporter plasmid contains the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p-73col-luc reporter plasmid contains the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p-73col-luc reporter plasmid contains the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p-73col-luc reporter plasmid contains the TK minimal promoter and the luciferase reporter gene. The pNFAT-luc reporter plasmid was a gift from Dr. G. R. Crabtree. It contains three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter.
determined by using a luciferase assay kit (Promega) with a luminometer
1450 Microbeta Luminescence Counter. Protein contents were measured
using the bicinchoninic acid method (BCA Protein Assay Kit from Pierce)
according to the manufacturer’s instructions. Transfection experiments were
performed in either duplicate or triplicate. Data are expressed as the mean of
the determinations in relative luciferase units
or as fold induction with
respect to untreated cells of at least three independent experiments.

PGE$_2$ detection

U-87 cells and human astrocytes were exposed to Tat in the presence or absence of different inhibitors. Cell supernatants were collected after 16 h
of stimulation and measured by PGE$_2$ EIA Kit-monoclonal (Cayman
Chemical) following the manufacturer’s instructions.

Western blot analysis

Human primary astrocytes and U-87 cells were exposed to Tat in presence or absence of CsA, PD98059 and SP600125 inhibitors where indicated. Cells were washed with PBS and lysed. Protein from each sample (20 μg) was subjected to SDS-PAGE on a 7.5% gel and then transferred onto a polyvinylidene difluoride membrane (Millipore) by humidified transference blotting. The membrane was blocked overnight at 4°C using Rotiblock (Roth) and for another hour at room temperature

FIGURE 1. COX-2 mRNA and protein expression and PGE$_2$ release in human astrocytes after Tat stimulation. A, COX-2 mRNA induction Cells were exposed to Tat (50 ng/ml) and analyzed 16 h after treatment by RT-PCR. Purified total RNA was reverse transcribed and amplified with COX-2-specific primers. B, COX-2 mRNA quantification by real-time PCR. Cells stimulated or not with Tat (50 ng/ml) were collected 16 h poststimulation. RT-PCR and amplification were performed using 200 ng of total RNA and a Light-Cycler RT-PCR kit. Results are the mean ± SD of three independent experiments. C, COX-2 protein induction in human astrocytes. Cell lysates from untreated control cells or cells treated with the indicated amounts of protein for 16 h were separated by SDS-PAGE on a 7.5% acrylamide gel, blotted, and incubated with Abs against COX-2 or tubulin as control (Ct). D, COX-2 protein cellular expression by immunofluorescence. U-87 cells were stained with an anti-COX-2 polyclonal Ab in control untreated cells (left) and after overnight exposure to 50 ng/ml Tat (right). Both optical fields contained the same number of cells. Fluorescence images and Western blot data are representative of three independent experiments. E, PGE$_2$ release in the supernatant of human astrocytes treated with Tat. Primary astrocytes as well as U-87 cells were treated with Tat alone (50 ng/ml) or in combination with the specific inhibitor of COX-2, NS398, for 16 h. The supernatants were collected, and PGE$_2$ was measured by ELISA. Data are representative of at least three independent experiments. C, Control. *, Statistically different from controls ($p < 0.05$).
before incubation with rabbit anti-human COX-2 mAb (Cayman Chemical) at 1/500 dilution.

To detect NFAT nuclear translocation in both U-87 cells and primary human astrocytes, nuclear and cytosolic protein extracts were obtained using Nuclear/Cytosolic fractionation kit (MBL International). After SDS-PAGE and blotting as in Western blot analysis, cells were incubated with anti-NFAT1 672 serum (gift from Dr. J. M. Redondo, Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain) from 1 h (1/1000 dilution; Ref. 38). After extensive washing (three times for 15 min each in Tris-buffered saline containing 0.1% Tween 20), proteins were detected with peroxide-coupled goat anti-rabbit IgG (Amersham-Pharmacia Biotech; 1/10,000) using ECL reagents (Amersham-Pharmacia Biotech). All Western blot experiments were conducted at least three times.

**COX-2 mRNA detection**

The mRNA from 10⁵ cells was isolated using oligodeoxythymidylate-coated magnetic beads and subsequent reverse transcription (PolyAtract Series 9600; mRNA isolation and cDNA synthesis system; Promega), according to the manufacturer’s instructions. PCR was conducted in an automatic Thermal Cycler (PerkinElmer GeneAmp PCR system 9600).

Real-time PCR using 200 ng of total RNA was performed using a Roche Light Cycler PCR instrument and Light-Cycler RT-PCR kit (Roche PerkinElmer). The standard used to make the control curve was the commercial plasmid pCRTM II (Invitrogen Life Technologies) where a fragment of the COX-2 promoter was inserted. The number of molecules of the plasmid was quantified measuring the quantity of DNA in a spectrophotometer. Because the weight of the standard (plasmid p;us sequence of COX-2) was known, the exact number of molecules can be extrapolated (43).

**EMSAs**

Nuclear extracts from U-87 cells unstimulated or stimulated with 50 ng/ml Tat were prepared as described previously (38). Briefly, cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 1 mM DTT, 0.5 mM PMSF, 10 mM Na₂MoO₄, 1 μg/ml pepstatin, and leupeptin and aprotime at 2 μg/ml each). After 15 min on ice, cells were lysed with 0.6% (v/v) Nonidet P-40. The nuclear pellet was extracted with 50 μl of buffer C (20 mM HEPES (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 mM Na₂MoO₄, 1 μg/ml pepstatin, and leupeptin and aprotime at 4 μg/ml each). Nuclear extracts were collected and stored at −80°C. Protein concentration was determined with a bicinchoninic acid protein assay kit.

Synthetic oligonucleotides specific for the proximal site of the COX-2 promoter NFAT were annealed and labeled with the Klenow fragment of the DNA polymerase I using DCTP (38). Nuclear extracts (3–5 μg) were incubated with 1 mg/ml polyoxymethylene-polyoxycytidylicate in DNA binding buffer (2% (v/v) polyvinyl ethanol, 2.5% (v/v) glycerol, 10 mM Tris, pH 8, 0.5 mM EDTA, 0.5 mM DTT) on ice for 10 min. Then 100,000 cpm of ³²P-labeled double-stranded oligonucleotides were added and incubated at room temperature for 40 min. In competition experiments, a 20-fold molar excess of unlabeled oligonucleotides was added to the binding reaction mixture before the probe. DNA-protein complexes...
were resolved by PAGE on a 4% nondenaturing gel and exposed to autoradiography.

Results

Tat protein up-regulates COX-2 expression, promoter activity, and PGE$_2$ release in astrocytic cells

We first evaluated whether extracellular Tat induces COX-2. For this U-87 astrocytoma cells were treated with recombinant HIV-1 Tat and COX-2 mRNA expression was analyzed. As shown in Fig. 1A, U-87 cells did not express any detectable COX-2 mRNA by RT-PCR and Tat (50 ng/ml) strongly induced COX-2 gene expression. Those results were confirmed by quantitative RT-PCR, in which COX-2 mRNA basal levels were observed due to the highest sensitivity of this technique. Nonetheless, an increase of ~9-fold in COX-2 mRNA was observed in the presence of Tat (Fig. 1B).

We next analyzed COX-2 protein in normal human primary astrocytes as well as in U-87 cells. Although some COX-2 protein was already detectable by Western blot, especially in unstimulated cells, in U-87 cells stimulation of both cell types with Tat induced
an increase in the synthesis of COX-2 protein (Fig. 1C). In U-87 cells, the induction was clearly detected especially 16 h posttreatment. We also analyzed COX-2 protein expression at the individual cell level by immunofluorescence analysis using a COX-2 mAb in a culture of U-87 cells treated with Tat (Fig. 1D). In agreement with the above data, COX-2 immunofluorescence was not detected in most of the unstimulated cells. However, COX-2 protein expression strongly increased after treatment, with 50 ng/ml Tat being expressed in all cells in the culture and with more intensity. This higher COX-2 protein expression correlates with a strong increase in PGE₂ released to the supernatant which was completely inhibited in the presence of the COX-2-specific inhibitor NS398 (Ref. 34 and Fig. 1E). Similar increase in PGE₂ production was also detected in the supernatants of primary astrocytes after Tat stimulation (Fig. 1F).

The above results suggested that extracellular Tat was able to induce COX-2 at the transcriptional level, because COX-2 mRNA was barely detected in unstimulated cultures. To further address this, we transfected U-87 cells with the COX-2 promoter containing a region spanning from −1796 to +104 bp relative to the transcription start site of the human COX-2 gene in the luciferase reporter plasmid pXp2 (P2-1900) that contains all the sites previously shown to be required for COX-2 induction in response to stimulation (32, 38, 49). The transfected cells were stimulated for 16 h, with the viral protein at different doses, and then luciferase activity was measured. P2-1900 COX-2 promoter activity was strongly up-regulated in U-87 cells after treatment with HIV-1 Tat, especially at the dose of 50 ng/ml (an average of >2-fold in six independent experiments; Fig. 2A). Higher doses of Tat were less effective. This type of effect has been described for other activities of extracellular Tat with a narrow window of concentrations (12). To determine whether the effect we observed was directly due of Tat protein, and not to a putative contaminant of the recombinant protein, we performed the transfection experiments in the presence of a mAb anti-Tat before stimulation with viral protein. As shown in Fig. 2B, the COX-2 induction was completely abrogated in the presence of anti-Tat. In addition, it has been described that extracellular Tat can be taken up by cells including astrocytes and perform its effects intracellularly (27, 50); therefore, we wished to know whether the effect observed in U-87 cells could be mediated by Tat internalization. For this, we studied the effect of recombinant Tat on LTR-luc transcriptional activity which is the most sensitive readout of intracellular Tat activity. However, this does not seem the case in our cultures because activation of the HIV-LTR was not enhanced in U-87 cells transiently transfected with LTR-luc plasmid and treated with extracellular Tat, whereas the same cells transiently cotransfected with CMV-TAT plasmid strongly up-regulated LTR-dependent transcription (Fig. 2C).

This result indicated that Tat was acting from outside of the cells. To gain further insight into the type of receptor used by Tat, we used pertussis toxin (PTx), which blocks signaling through some G protein-coupled receptors. As shown in Fig. 2D, COX-2 promoter induction by Tat was absolutely abrogated by PTx, suggesting that this kind of receptor could regulate second messenger activities through the Goα - GTP-binding proteins (51, 52).

To identify the region in the COX-2 promoter responsible for Tat-mediated activation, U-87 cells were transfected with different deletions of the COX-2 promoter (P2-431, P2-274, P2-192, and P2-150). Although the first deletion (up to −327 bp in P2-431) that eliminated the distal NF-κB site reduced the inducibility of COX-2 promoter activity by Tat, this effect was not statistically significant. Similar results were observed by deleting up to −170 bp in the P2-274. This deletion eliminated both NF-κB binding sites. The P2-274 promoter contained 2 NFAT binding sites. Thus, we used next the P2-192 that deleted the distal NFAT site. Tat protein increased the transcription driven by P2-432, P2-274, and P2-192 promoter constructs similarly, ~2-fold (Fig. 3A). However, deletion of the composed proximal NFAT/AP-1 site in P2-150 completely and significantly abrogated Tat inducibility. To confirm the data obtained in the U-87 cell line, we conducted transfection assays with another astroglial cell line, U-373. Tat stimulated in this cell line P2-1900, the full length COX-2 promoter, and P2-274 which maintains NFAT binding sites to similar extent that in U-87 cells, suggesting again the main role of NFAT in this induction (data not shown).

To confirm the role that both NFAT and AP-1 sites play in COX-2 induction in response to Tat, mutations were introduced into each of these sites in the contest of the P2-274 promoter. Thus, transient transfections with the P2-274 promoter construct containing dNFAT, pNFAT, or AP-1 sites mutated...
showed that induction of transcriptional activity by Tat was not affected by mutation of the distal NFAT site in agreement with deletion analysis. In contrast, pNFAT and AP-1 mutated plasmids presented a complete loss in the inducibility by Tat (Fig. 3B). Those results clearly indicated that the pNFAT/AP-1 site significantly contributes to the activation of the human COX-2 promoter in U-87 cells by Tat.

Involvement of NFAT and AP-1, but not NF-κB, in Tat-induced regulation of COX-2 transcription

Depending on the cellular type, many transcription factors can regulate COX-2 expression (32, 38, 49). The above results with the promoter studies suggested that NF-κB/H9260 was not required for Tat induction of COX-2 in these cells; in contrast, NFAT/AP-1 activation induced by extracellular Tat is required for COX-2 promoter activation. To corroborate those results, we first tested whether extracellular Tat induced NFAT-dependent reporter activity. In U-87 cells cotransfected with a NFAT-dependent luciferase reporter plasmid, Tat caused a marked increase of ~2-fold on average of a NFAT activity compared with the untreated control (Fig. 4A). To confirm the role of NFAT on COX-2 induction by Tat, we cotransfected the P2-1900 COX-2 promoter construct together with a plasmid encoding a version of NFAT (dnNFAT) that lacks the trans activation domain that acts as dominant negative. Overexpression of this dominant negative version of NFAT (dnNFAT) completely inhibited the P2-1900 COX-2 promoter-driven transcription induced by Tat (Fig. 4A).

Similarly, to determine the possible implication of AP-1 in the regulation of COX-2 promoter by Tat, we tested Tat ability to induce AP-1-dependent transcription. Extracellular Tat induced a significant increase in AP-1-dependent reporter expression of ~2-fold (Fig. 4B). Next, we analyzed the effect of a dominant negative version of c-Jun, the main component of the AP-1 complex, TAM 67, which lacks the trans activation domain, in the induction of COX-2 promoter. TAM 67 overexpression, completely suppressed the transcriptional activity of COX-2 promoter induced by Tat (Fig. 4B), indicating that AP-1 transcription factor is also required to induction of the COX-2 gene in U-87 cells by Tat.

The results obtained with the promoter deletions seem to discard the involvement of NF-κB in COX-2 regulation. To discard any effect of Tat on NF-κB, we tested the effect of viral
protein on a reporter gene under control of NF-κB sites in U-87 cells. In contrast to the effect observed in NFAT and AP-1, Tat did not induce transcription of the NF-κB reporter whereas LPS, used as a positive control, induced the NF-κB-dependent reporter activity (Fig. 4C). This confirms that NF-κB does not participate in Tat-mediated COX-2 induction. This also further discards that some endotoxin present in the Tat preparation used as responsible for the observed effects. Similar results were observed with another astrocytoma cell line, U-373 (data not shown).

**Effect of pharmacological inhibitor of AP-1 and NFAT in the Tat-induced regulation of COX-2 expression**

All of our results suggested the cooperation of AP-1 and NFAT in COX-2 induction by Tat in U-87 cells. To corroborate this and to gain some insights of the signal transduction pathways involved, we tested the effects of pharmacological inhibitors of several pathways in the induction of COX-2 promoter transcription. U-87 cells transfected with the COX-2 promoter were pretreated with PD98059 an inhibitor of ERK-activating kinase, or the JNK inhibitor SP600125 before Tat stimulation. Both inhibitors decreased COX-2 induction by Tat (Fig. 5A). Moreover, the immunosuppressive drug CsA, which inhibits Cn activity and consequently blocks NFAT activity, inhibited COX-2 promoter stimulation induced by Tat protein. To further corroborate this, we then tested the effect of those inhibitors in the induction of COX-2 protein synthesis by Tat. In agreement with promoter results, pretreatment with all inhibitors tested, markedly suppressed induction of COX-2 protein after Tat stimulation in U-87 cells (Fig. 5B).

COX-2-induction by Tat in normal human astrocytes was also inhibited by CsA (data not shown). To check the involvement of Cn/NFAT pathway on PGE$_2$ induction in primary astrocytes, we measured PGE$_2$ release in human astrocytes cells treated with Tat alone or in combination with CsA. As shown in Fig. 5C, the increase of PGE$_2$ release induced by Tat in stimulated normal human astrocytes was absolutely reduced to control levels by the addition of CsA. Those results suggest the involvement of Cn/NFAT pathway in the COX-2-dependent PGE$_2$ production by primary astrocytes.

**Tat induces NFAT activation in astrocytes**

Next we studied whether Tat induces NFAT activation. For this, we performed EMSAs with nuclear extracts of U-87 cells using DNA probes of NFAT/AP-1-proximal site. Stimulation of U-87 cells with extracellular Tat resulted in NFAT activation detected by the presence of a retarded complex able to bind to the NFAT/AP-1 consensus-binding oligonucleotide in nuclear extracts (Fig. 6A) this complex was already detectable after 5 min of Tat stimulation (50 ng/ml), and peaked at 10 min. Competition assay with 20-fold excess of the unlabeled probe completely abolished the complex formed with labeled probe, suggesting that the complex is specific. Moreover, the formation of the complex was prevented by incubation with an anti-NFAT Ab (not shown).

To confirm that extracellular Tat activates NFAT, we tested whether Tat induces the dephosphorylation of NFAT which is required for nuclear translocation and activation; for this, we performed Western blot assays with fractionated cellular extracts of U-87 cells. Untreated cells presented two bands specifically recognized by the anti-NFAT Ab. The upper one likely corresponds to the highly phosphorylated inactive NFAT that have lower mobility in SDS-PAGE and the lower one, the active dephosphorylated NFAT. Treatment of U-87 cells with extracellular Tat induced a significant loss in the amount of the phosphorylated NFAT. This effect was detected at 5 min and peaked at detected at 10–15 min, representing a decrease of 50–60% on average (depending of the experiment) when the stain of the bands was quantified in contrast by densitometer scanning (Fig. 6B). Unstimulated U-87 cells contain some dephosphorylated NFAT in the nucleus, but 5 min after Tat stimulation an increase on nuclear NFAT can be detected although 30 min after stimulation NFAT nuclear levels almost disappeared. Blotting with anti-tubulin Ab was used to check the amount of protein. Those results nicely correlate with EMSA assaying, indicating that extracellular Tat induces NFAT dephosphorylation and subsequent translocation to the nucleus. Moreover, similar results were obtained in human primary astrocytes. Thus, NFAT was absent from the nucleus of those cells, but after Tat treatment NFAT was detected, with a peak 10 min after stimulation that decreased thereafter (Fig. 6C).

**Discussion**

Although the introduction of HAART has greatly reduced the severity of HAD, still a great number of HIV-1-infected individuals manifest a plethora of CNS diseases unrelated to opportunistic infections, including HAD (1–3). Moreover, the underlying mechanism of HIV-1-mediated neuropathogenesis is still unclear and remains the subject of active research. In HAD, chronic inflammation of the brain results in the production and secretion of a variety of cytokines, chemokines, and growth factors from activated and infected cells. Astrocytes play a key role regulating inflammation in the CNS and neuronal survival. In patients with HAD, alterations in astrocytic function are induced by viral proteins and macrophage products (4–6). The viral protein Tat has been proposed as a mediator of HAD. Thus, Tat is secreted by different cellular types of CNS, mainly astrocytes and microglia, and it can act in an autocrine or paracrine manner in neighboring cells altering their normal functioning directly through its activity in neurons and astrocytes or indirectly through the induction of a proinflammatory milieu (reviewed in Refs. 20 and 53).

In contrast, recent results point to COX-2 as possible mediator of HIV-1-induced neuronal disturbances. Thus, enhanced levels of PGE$_2$, the major product of COX-2, have been detected in cerebrospinal fluid in patients with HAD and were associated with severity of dementia (30). Interestingly, gp120, another HIV-1 protein involved in HAD, has been found to induce COX-2 in neuroblastoma cells (43) and aggravate excitotoxic conditions by impairing astrocyte uptake of glutamate via COX-derived products resulting in calcium overload and neuronal dysfunction or death (20, 54, 55). Besides, intracerebroventricular gp120 injection enhanced the expression of COX-2 and apoptotic death in the brain neocortex of rats was prevented by COX-2 inhibitors (29, 56).

Thus, both, the HIV-1 protein Tat and COX-2 have been involved in the neuropathogenesis associated to HIV-1-infection. However, the relationship among them has not been addressed. In this work, we have investigated the effect of extracellular Tat on astrocytes and its relationship with COX-2, and we found that Tat induces COX-2 in U-87 and U-373 cell lines and in primary normal human astrocytes. Moreover, this activation correlates in all instances with PGE$_2$ production in the supernatants of cultures.

The effect of Tat observed was not due to a contaminant of the recombinant Tat preparation used, as endotoxin, because anti-Tat Abs abrogated the effect of Tat. Besides, LPS and Tat do not have the same effects on COX-2 in U-87 cells. Extracellular Tat can be rapidly internalized by astrocytes and perform its effects (27, 50). However, in our work, HIV-LTR-dependent transcription, which is the most sensitive readout on intracellular Tat activity, was not enhanced in U-87 cells treated with extracellular Tat, suggesting that Tat was acting from outside the cells likely interacting with a
surface receptor of the cell. More importantly, we found that treatment with PTx could abrogate the transcriptional activity of COX-2 promoter. This fact points to a heterotrimeric G protein as transducer of Tat-activity COX-2 in astrocytes. Tat has been shown to interact with some G-coupled protein receptors. as chemokine receptors (57), and several reports have shown that some of the activities of extracellular Tat including neurotoxic ones in astrocytes are blocked by PTx (58, 59). The characterization of the putative Tat receptor deserves further studies.

Regulation of COX-2 transcription is a paradigm of a complex regulation that varies not only with the cell type but also with the stimuli. Thus, COX-2 has been shown to be regulated by IFN-regulatory factors, NF-kB, NF-IL6, CREB, AP-1, Ets, PEA-1, and NFAT transcription factors among others (32, 38, 39). Previous studies have shown that NF-kB regulates neuronal COX-2 gene expression (43, 60). We have found that another HIV-1 protein, gp120, also involved in HAD, induces COX-2 transcription in neuroblastoma (43) and U-87 cells (61). However, those effects were mediated via an NF-kB-dependent mechanism. The human COX-2 gene contains two NF-kB-binding sequences, and mutational and EMSA analysis conferred the regulatory NF-kB activity by gp120 to the distal NF-kB site in the human COX-2 promoter (43), whereas the basal COX-2 transcription activity was controlled by the distal site (60) in neuronal cells.

Tat has been described to activate NF-kB transcription factor in various cell types. Moreover, Tat-related neurotoxicity is mediated to a great extent by the production of the cytokines TNF, IL-6, IL-8, and IL-10 in glial cells. This cytokine production is likely maintained by a positive feedback loop of Tat-induced NF-kB activation (62, 63). Other results have indicated that intracellular Tat induced inducible NO synthase in astrocytes by a mechanism mediated by NF-kB. However, this effect was partially blocked by anti-Tat Abs, indicating that Tat is released by astrocytes acting in an autocrine/paracrine manner (27). Thus, it was not entirely clear whether those effects were mediated by extracellular or intracellular Tat.

Our results clearly indicate that NF-kB is not involved in Tat induction of COX-2 promoter in two astrocytoma cell lines. This was demonstrated by the inducibility of COX-2 promoter with deleted NF-kB sites and more importantly by the lack of induction of NF-kB by Tat in U-87 and U-373 cells. The apparent discrepancies in the ability of Tat to induce NF-kB may lie in the different cell types used or more likely due to differences in extracellular vs intracellular actions, because the effect we observed was clearly due to extracellular Tat.

In contrast, we found that Tat induction of COX-2 in U-87 cells requires AP-1 and NFAT activation. This was shown by the complete inhibition of Tat-induced COX-2 promoter transcription by a dominant negative version of c-Jun as well as the overexpression of a dominant negative version of NFAT. Moreover, mutation of the pNFAT or the AP-1 sites present in the COX-2 promoter site abrogates Tat inducibility. Those results were confirmed by the use of specific pharmacological inhibitors in that COX-2 transcription and protein expression was completely inhibited by the Cn inhibitor CsA, as well as by JNK or ERK pathways inhibitors. Tat has been shown to induce AP-1 activation in U-87 cells (64). Moreover, in primary astrocytes, NFAT seems also to be involved in COX-2 induction and PGE2 production because CsA can decrease PGE2 levels when stimulated with Tat protein.

More interestingly, our results show for the first time that Tat activates NFAT. This was demonstrated by the fact that extracellular Tat induces translocation of NFAT to the nucleus in U-87 cells and primary astrocytes. Moreover, we demonstrated an enhanced binding of NFAT to the pNFAT-proximal DNA probe of the COX-2 promoter by EMSA assays after Tat stimulation. Activation of NFAT was transient, being maximal at 10 min post-stimulation and decreasing thereafter. Extracellular Tat also induces a NFAT-dependent reporter, an effect that was blocked by CsA, an inhibitor of the Cn/NFAT pathway. To our knowledge, this is the first report indicating that extracellular Tat induces NFAT in any cell type.

In this regard, there are few reports analyzing the role of Cn/NFAT pathway in astrocytes. However, both Cn (65) and NFAT4 (66) expression has been described in astrocytes. NFAT4 is activated by glutamate-mediated Ca2+ increase (66), and Cn signaling mediated survival of reactive astrocytes (65). In neurons, Tat activates PLC (67), increases levels of inositol 1,4,5-triphosphate, releases calcium from inositol 1,4,5-triphosphate-sensitive endoplasmic reticulum internal stores, and increases activity of the protein kinase C isoforms α, -ε, and β (68). A similar mechanism may be operating in U-87 cells, being responsible for the Cn/NFAT activation that we observed with Tat.

In contrast, COX-2 expression has been previously observed in astrocytes, and COX-2 mediates purinergic P2 receptor-induced astrogliosis (69) a feature of HAD. Because Tat interacts with astrocytes resulting in gliosis (18, 70, 71), it is tempting to speculate that Tat induces this activation through COX-2-derived prostandins. In this sense, it is worth mentioning that PGE2 acts in an autocrine manner to alter astrocyte morphology and function (72, 73). Thus, it is possible that some of those effects of Tat are mediated through COX-2 induction. Experiments are in progress in our laboratory to elucidate this. Those results together with the ones described here indicate COX-2 as a mediator of Tat-induced reactive gliosis in HAD. This is supported by a report appearing while our manuscript was being written, showing that in mice Tat injection induces brain inflammation as well as COX-2 expression in the brain (74). More interestingly, the inflammation induced by Tat was attenuated by treatment of those mice with COX-2-specific inhibitors.

In summary, we have demonstrated that extracellular HIV-1 Tat protein promotes COX-2 induction in astrocytic cells through a mechanism mediated by NFAT and AP-1 transcription factors. Thus, targeting these cellular signaling pathways may be beneficial in controlling inflammatory neurological sequel of HIV infection.

Disclosures
The authors have no financial conflict of interest.

References


