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Regulation of Multidrug Resistance Protein 1 by Tumor Necrosis Factor α in Cultured Glial Cells: Involvement of Nuclear Factor-κB and c-Jun N-Terminal Kinase Signaling Pathways

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ABSTRACT

Pharmacotherapy of brain HIV-1 infection may be limited by ABC transporters [i.e., P-glycoprotein (P-gp), multidrug resistance protein 1 (Mrp1)] that export antiretroviral drugs from HIV-1 brain cellular targets (i.e., astrocytes, microglia). Using an in vitro astrocyte model of an HIV-1 associated inflammatory response, our laboratory has shown that cytokines [i.e., tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6], which are secreted in response to HIV-1 envelope glycoprotein gp120 exposure, can decrease P-gp functional expression; however, it is unknown whether these same cytokines can alter expression and/or activity of other ABC transporters (i.e., Mrp1). In primary cultures of rat astrocytes, Mrp1 expression was increased by TNF-α (2.7-fold) but was not altered by IL-1β or IL-6. Cellular retention of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, an Mrp substrate, was reduced in TNF-α-treated astrocytes, suggesting increased Mrp-mediated transport. Pharmacologic inhibition of nuclear factor-κB (NF-κB) signaling with SN50 prevented both TNF-α release and Mrp1 expression changes in astrocytes triggered with gp120; however, SN50 did not attenuate Mrp1 expression in cells triggered with TNF-α. In contrast, Mrp1 functional expression was not altered in the presence of gp120 or TNF-α when astrocyte cultures were pretreated with 1,9-pyrazoloanthrone (SP600125), an established c-Jun N-terminal kinase (JNK) inhibitor. SP600125 did not affect TNF-α release from cultured astrocytes triggered with gp120. Mrp1 mRNA expression was increased after treatment with gp120 (1.6-fold) or TNF-α (1.7-fold), suggesting altered *Mrp1* gene transcription. These data suggest that gp120 and TNF-α can up-regulate Mrp1 expression in cultured astrocytes. Furthermore, our results imply that both NF-κB and JNK signaling are involved in Mrp1 regulation during an HIV-1 associated inflammatory response.

Astrocytes, the most numerous cell type in the brain, perform multiple functions required for CNS homeostasis. During HIV-1 infection of the brain, astrocytes are known to participate in the immune response via release of proinflammatory cytokines (Speth et al., 2005). Increased cytokine secretion (i.e., TNF-α, IL-1β, IL-6) during brain HIV-1 infection is well established and may be triggered by soluble viral proteins (i.e., HIV-1 envelope glycoprotein gp120) (Kaul et al., 2005). Studies in cultured glial cells suggest that gp120 binding to chemokine receptors (i.e., CXCR4, CCR5) may mediate this inflammatory response (Ronaldson et al., 2008). In vitro, our laboratory has shown that proinflammatory cytokine release is increased in cultured rat astrocytes treated with gp120 via a CCR5-dependent mechanism (Ronaldson and Bendayan, 2006).

Although advances in HIV-1 pharmacotherapy have efficiently reduced systemic viral load, HIV-associated neurologic disease remains a significant cause of morbidity and mortality in patients infected with HIV-1 (McArthur et al., 2003). These neurologic complications may be associated

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ABBREVIATIONS: CNS, central nervous system; TNF-α, tumor necrosis factor; IL, interleukin; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP/Mrp, multidrug resistance protein; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; PSC833, valspodar; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BAY 11-7082, (*E*)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile; SP600125, anthra[1–9*cd*]pyrazol-6(2*H*)-one; SAPK, stress-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; AM, acetoxymethyl ester.

with poor CNS permeation of antiretroviral compounds, a phenomenon that may be attributed to expression of ATP-binding cassette (ABC) efflux transporters [i.e., P-glycoprotein (P-gp), multidrug resistance proteins (MRPs in humans; Mrps in rodents)] at brain barrier sites (i.e., blood-brain barrier, blood-cerebrospinal fluid barrier) and in brain cellular targets of HIV-1 (i.e., microglia, astrocytes). MRP1/Mrp1, a 190-kDa membrane protein, extrudes from cells many organic anions as well as their glutathione, glucuronide, and sulfate conjugates (Ronaldson et al., 2008). Although MRP1/Mrp1 is primarily associated with efflux of anticancer drugs, antiretroviral agents (i.e., HIV-1 protease inhibitors) are also known substrates of this transporter (Williams et al., 2002; Dallas et al., 2004). Mrp1 expression has been identified in several brain cellular compartments, including brain capillary endothelial cells (Miller et al., 2000), choroid plexus epithelial cells (Wijnholds et al., 2000), and glial cells (Dallas et al., 2003; Ronaldson and Bendayan, 2008). In the context of HIV-1 infection, expression levels of MRP1/Mrp1 remain controversial. Although studies in peripheral blood mononuclear cells isolated from patients infected with HIV-1 showed no difference in MRP1 expression compared with cells from healthy persons (Meaden et al., 2001), another study has shown higher MRP1 expression levels in response to HIV-1 infection (Turriziani et al., 2008). The high variability in the data can be, in part, explained by differences in therapeutic regimens because some antiretroviral drugs are known to alter expression of membrane transporters (Ronaldson et al., 2008; Zastre et al., 2009).

Cytokine secretion (i.e., TNF-α, IL-1β, IL-6) in response to infection or cell stress may alter MRP1/Mrp1 functional activity. Using a human hepatoma cell line (HepG2), IL-1β and IL-6 treatment resulted in an increase in MRP1 mRNA expression and transport activity (Lee and Piquette-Miller, 2003). Studies in Sprague-Dawley rats have demonstrated that treatment with lipopolysaccharide (LPS), a bacterial endotoxin that stimulates cytokine release, enhances hepatic Mrp1 mRNA expression, suggesting involvement of cytokines in regulating Mrp1 expression (Cherrington et al., 2004). In contrast, studies in human monocyte-derived macrophages reported that gp120-induced production and secretion of TNF-α and IL-6 are not correlated to altered expression of MRP1 (Jorajuria et al., 2004).

Cellular exposure to HIV-1 virions, HIV-1 viral proteins, and/or HIV-1 cytokines is associated with activation of intracellular signaling systems such as nuclear factor-κB (NF-κB) (Kim et al., 2005) and the mitogen-activated protein kinase (MAPK) pathway (Ghorpade et al., 2003; Hayashi et al., 2005, 2006). In addition, both NF-κB and components of the MAPK pathway [i.e., c-Jun N-terminal kinases (JNKs)] have been implicated in the regulation of ABC transporters such as P-gp (Zhou et al., 2006; Bauer et al., 2007; Hartz et al., 2008) and Mrp1 (Hayashi et al., 2006). No published reports have demonstrated involvement of either pathway in the regulation of Mrp1 in glial cells exposed to HIV-1 gp120 and/or cytokines.

Our laboratory has reported increased functional expression of Mrp1 in response to oxidative stress in cultured rat astrocytes treated with gp120 (Ronaldson and Bendayan, 2008). Furthermore, we have also shown that gp120 treatment can induce secretion of TNF-α, IL-1β, and IL-6 from these astrocyte cultures (Ronaldson and Bendayan, 2006). It is unknown whether cytokines can regulate Mrp1 expression in glial cells and, if so, which intracellular signaling pathways might be involved. In the present study, we have 1) evaluated Mrp1 functional expression in cultured rat astrocytes triggered with TNF-α, IL-1β, and IL-6 and 2) investigated the role of NF-κB and JNKs in the regulation of Mrp1 expression in cultured astrocytes exposed to HIV-$1_{96ZM651}$ gp120 or cytokines.

Materials and Methods

Materials. HIV-$1_{96ZM651}$ gp120 full-length protein (derived from subtype C, R5-tropic HIV-1) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). PSC833 (i.e., valspodar) was a generous gift from Novartis Pharma (Basel, Switzerland). The rat monoclonal MRP1 antibody MRPr1 was obtained from Kamiya Biomedical Company (Seattle, WA). 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), acetoxymethyl ester and free acid, were purchased from Invitrogen (Mississauga, ON, Canada). MK571 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). The cell-permeant NF-κB inhibitory peptide SN50 and the pharmacologic NF-κB inhibitor BAY 11-7082 were purchased from EMD Biosciences Inc. (La Jolla, CA). Rat recombinant TNF-α, the anthrapyrazolone JNK inhibitor SP600125, and the murine monoclonal actin antibody AC-40 were obtained from Sigma-Aldrich (Oakville, ON, Canada). Rat recombinant IL-1β, rat recombinant IL-6, the murine monoclonal TNF-α neutralizing antibody, and the murine monoclonal IL-1β neutralizing antibody were purchased from Millipore Bioscience Research Reagents (Temecula, CA). The rat monoclonal IL-6 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN). The rabbit polyclonal total JNK/SAPK antibody and the rabbit polyclonal phosphorylated JNK/SAPK antibody were purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture. Primary cultures of rat astrocytes were prepared as we described previously (Ronaldson et al., 2004; Ronaldson and Bendayan, 2006, 2008). All procedures were carried out in accordance with the University of Toronto Animal Care Committee and the Province of Ontario Animals for Research Act. Postnatal (1–3-day-old) Wistar rats (Charles River Laboratories, St. Constant, QC, Canada) were killed by cervical dislocation, and whole brains were isolated. Cerebral cortices were dissected and subjected to enzymatic digestion for 30 min in serum-free minimum essential medium containing 2.0 mg/ml porcine pancreatic trypsin (Sigma-Aldrich) and 0.005% DNase I (Roche Applied Science, Laval, QC, Canada). Tissue was mechanically disaggregated using a cell dissociation kit (Sigma-Aldrich) to yield a mixed glial cell suspension. The cell suspension was then centrifuged for 10 min at 100*g* and resuspended in fresh culture medium consisting of minimum essential medium supplemented with 5% horse serum, 5% fetal bovine serum, and 50 μg/ml gentamicin. The cells were plated on 75-cm^2 polystyrene tissue culture flasks (Sarstedt, St. Leonard, QC, Canada) and incubated in fresh medium at 37°C, 5% CO_2, and 95% air overnight for 7 to 10 days. The cells were then placed on an orbital shaker at 120 rpm for 6 h to remove contaminating oligodendrocytes, microglia, progenitor cells and neurons. The cells were harvested with 0.1% trypsin/EDTA in Hanks' balanced salt solution and plated at a density of 5×10^4 cells/well on 48-well polystyrene plates (BD Biosciences, Franklin Lakes, NJ). The astrocytic nature of isolated cells and culture purity were previously assessed by morphologic analysis and by immunostaining for standard biochemical markers (i.e., glial fibrillary acidic protein) (Ronaldson et al., 2004).

The human cervical carcinoma cell line stably transfected with human MRP1 (MRP1-HeLa) was kindly provided by Dr. Susan Cole (Queen's University, Kingston, ON, Canada). Cells were grown as monolayers on 75-cm^2 tissue culture flasks at 37°C in 5% CO_2 and

95% air. Cultures were maintained in Dulbecco's modified Eagle's medium (4 mM L-glutamine and 25 mM D-glucose) supplemented with 400 μg/ml G418 and 10% fetal bovine serum. Confluent cultures were subcultured with 0.25% trypsin-EDTA and were used as a positive control for Western blotting experiments.

gp120/Cytokine Treatments. All treatments were performed on monolayers of primary cultures of rat astrocytes grown in 75-cm^2 tissue culture flasks. At the beginning of each experiment, culture medium was aspirated and fresh culture medium containing 1.0 nM HIV-$1_{96ZM651}$ gp120. HIV-$1_{96ZM651}$ gp120 is R5-tropic (also known as macrophage-tropic) and is derived from a subtype C viral isolate. R5-tropic viruses are the most prevalent strains of HIV-1 in the brain (Gabuzda and Wang, 2000). In patients infected with HIV-1, concentrations ranging between 12 and 92 ng/ml have been reported to be released in serum (Oh et al., 1992). These serum concentrations correspond to a molar concentration range of 0.1 to ~1.0 nM. All experiments were conducted at 37°C in 5% CO_2 and 95% air. Control (i.e., untreated) cultures comprised untreated cells in fresh culture medium. For experiments examining the involvement of NF-κB or JNK on the regulation of Mrp1 in gp120-treated cells, cultures were pretreated with 1 μM SN50, 5 μM BAY 11-7082, or 20 μM SP600125 for 30 min before HIV-$1_{96ZM651}$ gp120 exposure. At 6, 12, and 24 h, the cells were collected and prepared for immunoblot analysis as described below.

Cytokine exposure experiments were initiated by aspirating the culture medium and adding fresh medium containing 0.5 or 10 ng/ml TNF-α, 0.4 or 10 ng/ml IL-1β, or 0.3 or 10 ng/ml IL-6. These proinflammatory cytokines were selected because their expression is increased during HIV-1-associated immunologic responses in the brain (Kaul et al., 2005). The lower concentration of each cytokine was selected based on the maximum level of TNF-α, IL-1β, or IL-6 secreted from primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 as determined by ELISA (Ronaldson and Bendayan, 2006), whereas the higher cytokine concentration (i.e., 10 ng/ml) is widely reported in the literature to induce a profound inflammatory response in vitro. Untreated cells in culture medium containing 5% horse serum and 5% fetal bovine serum were used as control. For experiments examining the involvement of NF-κB or JNKs on the regulation of Mrp1 in cells exposed to TNF-α, cultures were pretreated with 1 μM SN50 or 20 μM SP600125, respectively, for 30 min before triggering with TNF-α. At 6, 12, and 24 h, the medium was aspirated and the cells were collected for immunoblot analysis.

Treatment of primary cultures of rat astrocytes with cytokine-neutralizing antibodies and HIV-$1_{96ZM651}$ gp120 were conducted by aspirating culture medium and replacing it with fresh medium containing the cytokine-neutralizing antibody and 1.0 nM HIV-$1_{96ZM651}$ gp120. At 6, 12, and 24 h, the medium was aspirated and the cells were collected for immunoblot analysis. Concentrations for the neutralizing antibodies were determined from cytokine activity curves provided by the manufacturer. For these experiments, the following concentrations were selected because they were shown to completely neutralize the biological activity of their respective cytokine: 0.2 μg/ml TNF-α neutralizing antibody, 0.5 μg/ml IL-1β neutralizing antibody, and 0.5 μg/ml IL-6 neutralizing antibody.

Immunoblot Analysis. Whole-cell lysates from primary cultures of rat astrocytes and HeLa-MRP1 cells were prepared by exposing the cells to 1.0 ml of modified radioimmunoprecipitation assay buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0 mM EGTA, 1% (v/v) Nonidet P-40, 0.25% (m/v) sodium deoxycholate, 0.1% (m/v) SDS, 200 μM phenylmethylsulfonyl fluoride, and 0.1% protease inhibitor cocktail (Sigma-Aldrich)]. The cells were then gently rocked for 15 min at 4°C to allow lysis to occur. Cell suspensions were collected and centrifuged at 3000*g* for 15 min at 4°C to remove cellular debris. Supernatants were then collected for immunoblot analysis. Protein concentration of the cell lysates was determined using the Bradford protein assay (Bradford, 1976).

For immunoblotting, 1, 25, or 50-μg aliquots of cell lysates were mixed in Laemmli buffer and resolved on a 10% SDS-polyacrylamide gel. The gel was then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. Protein transfer was verified by Ponceau S staining. The membranes were blocked overnight at 4°C in Tris-buffered saline (15 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% (v/v) Tween 20 and 5% (m/v) nonfat dry milk powder. After six washes (5 min each) with Tris-buffered saline/Tween 20, the membrane was incubated with the appropriate primary antibody for 4 h at room temperature. MRP1/Mrp1 protein expression was assessed using the monoclonal MRPr1 antibody, which was raised against a bacterial fusion protein containing amino acids 194 to 360 of human MRP1, and its epitope was subsequently localized to amino acids 238 to 247 (Hipfner et al., 1999). MRPr1 does not cross-react with P-gp or MRP2–6 (Hipfner et al., 1999; Scheffer et al., 2000). Total and phosphorylated JNK protein expression was determined using the polyclonal total JNK/SAPK antibody and the polyclonal phosphorylated JNK/SAPK antibody, respectively. The polyclonal total JNK/SAPK antibody was produced by the immunization of rabbits with a glutathione transferase/human JNK2 fusion protein (Product Data Sheet, Cell Signaling Technology, 2008). The polyclonal phosphorylated JNK/SAPK antibody was produced by immunizing the animals with a fusion protein corresponding to the amino acids surrounding threonine 183 and tyrosine 185 of human JNK and is specific for JNK isoforms that are phosphorylated at these residues (Product Data Sheet, Cell Signaling Technology, 2008). Actin expression was detected using the monoclonal AC-40 antibody, which recognizes a conserved C-terminal epitope on all actin isoforms (Product Data Sheet, Sigma-Aldrich Canada, 2005). After a second wash, the membranes were incubated for 1.5 h in the presence of anti-mouse (Serotec Inc., Raleigh, NC), anti-rat (Sigma-Aldrich), or anti-rabbit (Sigma-Aldrich) horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) in 5% milk at room temperature. Protein bands were detected by enhanced chemiluminescence and exposed to X-ray film for 1 min. The MRP1-HeLa cell line was used as a positive control for MRP1/Mrp1.

Quantitative PCR. Total RNA was extracted from confluent monolayers of primary cultures of rat astrocytes treated with either HIV-$1_{96ZM651}$ gp120 (1.0 nM) or TNF-α (10 ng/ml) for 6, 12, or 24 h using TRIzol reagent (Invitrogen). Extracted RNA was treated with amplification grade DNase I (Invitrogen) to remove contaminating genomic DNA. The concentration of RNA in each sample was quantified spectrophotometrically by measuring UV absorbance at 260 nm. The high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA) was used to synthesize first-strand cDNA. Primer pairs for the rat *Mrp1* gene (5′-AGAAGGAATGTGTTAAGTCGAGGAA-3′ and 5′-CCTTAGGCTTGGTGGGATCTT-3′) and the rat *Cyclophilin B* gene (housekeeping gene; 5′-GGAGATGGCACAGGAGGAA-′3 and 5′-GCCCGTAGTGCTTCAGCTT-3′) were designed with the use of Primer Express 3 software (Applied Biosystems) and validated for specificity and efficacy by using BioTaq universal rat normal tissue cDNA (BioTaq Inc., Gaithersburg, MD). Quantitative PCR was performed using SYBR Green Master Mix (Applied Biosystems) on an ABI 7900HT Fast Real-time PCR System (Applied Biosystems). The quantity of the target gene (i.e., *Mrp1*) was normalized to Cyclophilin B using the comparative C_T method ($\Delta\Delta C_T$). Results were expressed as mean ± S.D. of at least three separate experiments.

ELISA. An ultrasensitive ELISA kit for detection of rat TNF-α (Pierce Biotechnology, Rockford, IL) was used to measure secretion of cytokines from primary cultures of rat astrocytes treated with HIV-$1_{96ZM651}$ gp120 in the presence or absence of SN50. Standard curves for TNF-α (0–2500 pg/ml) were generated using purified recombinant rat TNF-α, and the assay was performed according to manufacturer's instructions. Absorbance was read at 450 nm using a SpectraMax Plus384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentration of secreted TNF-α was expressed in picograms per milliliter. All experiments reflect eight separate measurements obtained from different cell cultures on different days.

Functional Studies. These studies were performed on confluent monolayers of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 or with TNF-α (0.5 or 10 ng/ml) and grown on 24-well polystyrene plates (BD Biosciences) at an approximate density of 8×10^4 cells/well. Cells were washed and incubated at 37°C for 30 min in Hanks' balanced salt solution, pH 7.4, containing 10 mM HEPES and 0.01% bovine serum albumin. The cells were then incubated for the desired time with the cell-permeant ester BCECF-AM (5 μM) in the presence

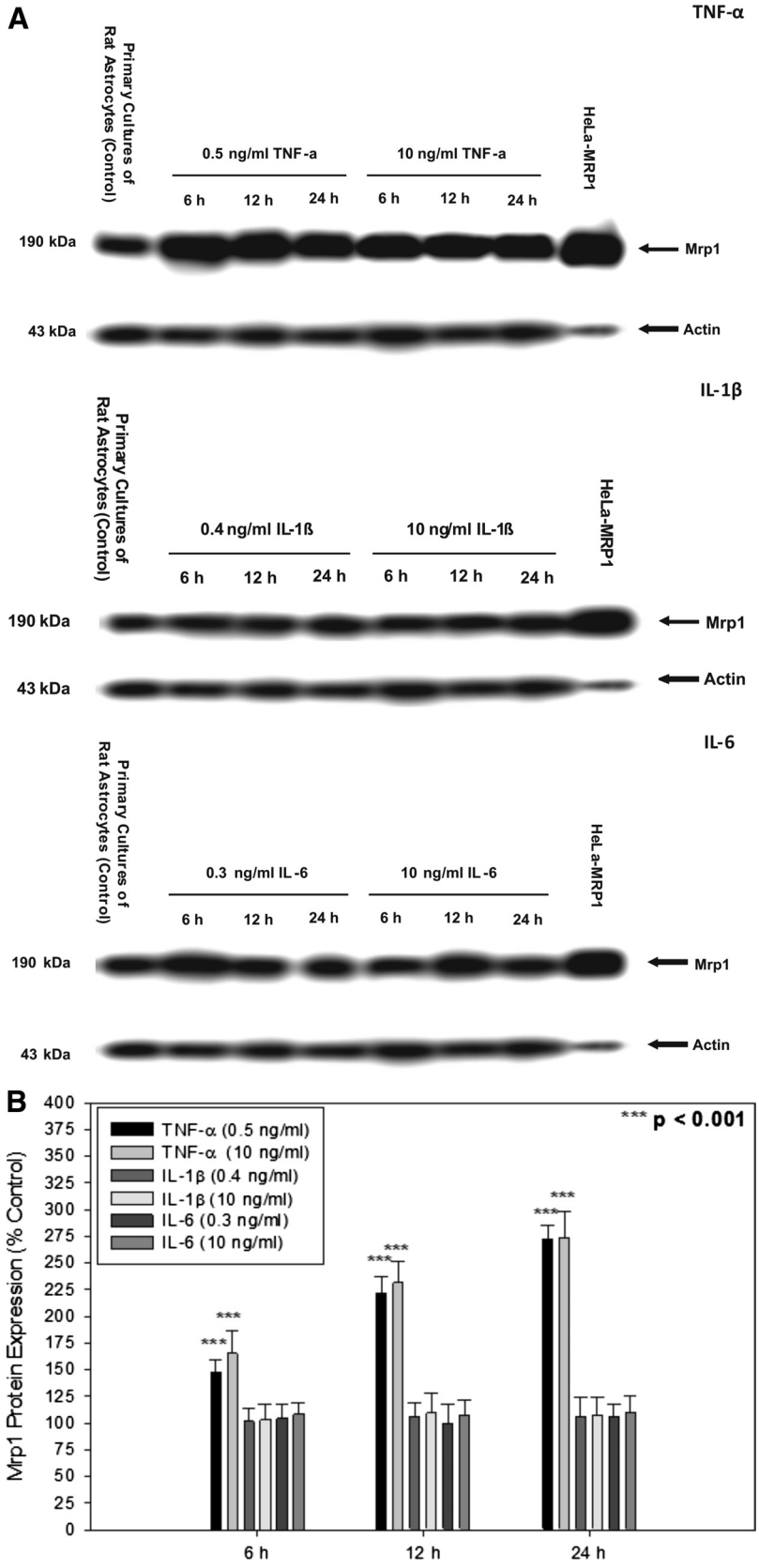
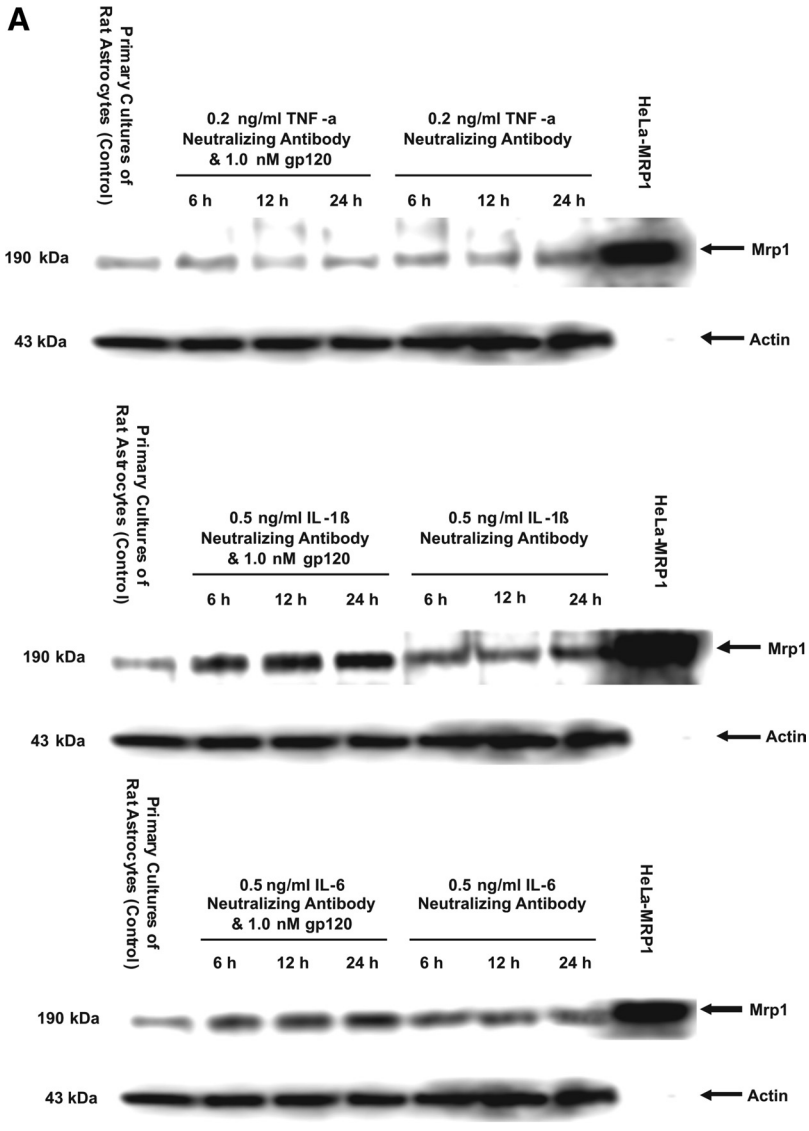


Fig. 1. Effect of cytokines on Mrp1 protein expression in primary cultures of rat astrocytes. A, primary cultures of rat astrocytes were treated with TNF-α (0.5 or 10 ng/ml), IL-1β (0.4 or 10 ng/ml), and IL-6 (0.3 or 10 ng/ml) for 6, 12, and 24 h, and Mrp1 expression was assessed by immunoblot analysis. Crude membrane preparations of primary cultures of rat astrocytes (25 μg) and MRP1-HeLa cells (1 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The blots were incubated with the monoclonal MRP1/Mrp1 antibody MRPr1 (1:500 dilution). Equal sample loading was confirmed by the detection of actin using the monoclonal antibody AC40 (1:500 dilution). Primary cultures of rat astrocytes not exposed to cytokines were used as a control. B, densitometric analysis of Mrp1 protein in cultured rat astrocytes treated with TNF-α, IL-1β, or IL-6. Results (percentage of control) are expressed as mean $\pm$ S.D. of three separate experiments. Asterisks indicate data points that are significantly different from control.

or absence of SP600125 (20 μM). Because BCECF-AM is a known P-gp substrate (Bachmeier et al., 2004), all incubations were performed in the presence of 1.0 μM PSC833, an established P-gp inhibitor. At the end of each time point, the incubation medium was aspirated, and the reaction was terminated with 1000 μl of ice-cold PBS. The cells were then solubilized with 200 μl of 1% Triton-X-100 for 30 min. BCECF cellular retention was measured using a fluorescent assay plate reader at an excitation wavelength of 505 nm and an emission wavelength of 535 nm. All samples were corrected for background fluorescence. Cellular BCECF content was standardized



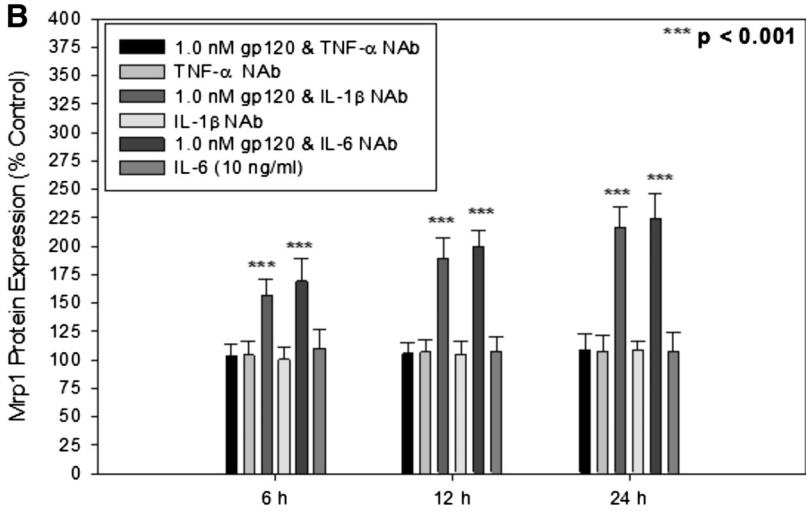


Fig. 2. Effect of cytokine-neutralizing antibodies on Mrp1 protein expression in primary cultures of rat astrocytes treated with HIV-$1_{96ZM651}$ gp120. A, primary cultures of rat astrocytes were treated with neutralizing antibodies for TNF-α (0.2 ng/ml), IL-1β (0.5 ng/ml), and IL-6 (0.5 ng/ml) in the presence or absence of 1.0 nM HIV-$1_{96ZM651}$ gp120 for 6, 12, and 24 h, and Mrp1 expression was assessed by immunoblot analysis. Crude membrane preparations of primary cultures of rat astrocytes (25 μg) and MRP1-HeLa cells (1 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The blots were incubated with the monoclonal MRP1/Mrp1 antibody MRPr1 (1:500 dilution). Equal sample loading was confirmed by the detection of actin using the monoclonal antibody AC40 (1:500 dilution). Primary cultures of rat astrocytes not exposed to HIV-$1_{96ZM651}$ gp120 or to the cytokine neutralizing antibodies were used as a control. B, densitometric analysis of Mrp1 protein in cultured rat astrocytes treated with HIV-$1_{96ZM651}$ gp120 and various cytokine-neutralizing antibodies. Results (percentage of control) are expressed as mean $\pm$ S.D. of three separate experiments. Asterisks indicate data points that are significantly different from control. NAb, neutralizing antibody.

to cellular protein content (mg/ml) determined by the Bradford colorimetric method using bovine serum albumin (Sigma-Aldrich) as the standard. Cellular retention of BCECF was expressed as nanomoles per milligram of protein (nanomoles per milligram of protein).

Data Analysis. Each set of experiments was repeated at least three times in cells pertaining to different isolations. In an individual experiment, each data point represents quadruplicate trials. Results are reported as a mean ± S.D. from at least three separate experiments. To determine significance of transport inhibition, Student's *t* test was used for unpaired experimental data. For multiple comparisons, the test of repeated measures ANOVA and the post hoc multiple-comparison Bonferroni *t* test were used. A value of $p < 0.05$ was considered statistically significant.

Results

Effect of Cytokines on Mrp1 Protein Expression. Our laboratory has previously reported increased cytokine secretion (i.e., TNF-α, IL-1β, IL-6) in primary cultures of rat astrocytes exposed to HIV-$1_{96ZM651}$ gp120 (Ronaldson and Bendayan, 2006). In addition, we demonstrated that cellular exposure to these cytokines decreased functional expression of the ABC transporter P-gp (Ronaldson and Bendayan, 2006); however, it was unknown whether TNF-α, IL-1β, or IL-6 was involved in the regulation of other ABC transporters that are expressed in astrocytes. Therefore, we explored the role of these cytokines in regulation of Mrp1 protein expression (Fig. 1A). Mrp1 protein was detected using the monoclonal MRPr1 antibody, which has been shown to react with both human MRP1 and rat Mrp1 (Dallas et al., 2003). As expected, in the MRP1-HeLa cell line (the positive control), a single band was observed at approximately 190 kDa, a size previously reported for MRP1/Mrp1 (Hipfner et al., 1999). Mrp1 protein expression was increased up to 2.7-fold by 24 h in primary cultures of rat astrocytes treated with TNF-α but was not altered in cultures treated with either IL-1β or IL-6 (Fig. 1B). Appropriate loading of each sample was confirmed by detection of a single band at approximately 43 kDa, which corresponds to actin. We observed no change in actin protein expression in response to TNF-α, IL-1β, or IL-6 treatment at any of the time points examined (data not shown).

To confirm the involvement of these cytokines in altering Mrp1 expression, we measured Mrp1 protein expression in cultured rat astrocytes treated with HIV-$1_{96ZM651}$ gp120 and various cytokine-neutralizing antibodies. In the presence of cytokine-neutralizing antibodies only, Mrp1 protein expression was not significantly altered in our rat astrocyte cultures (Fig. 2). We observed no change in Mrp1 expression when cultures were treated with HIV-$1_{96ZM651}$ gp120 and the TNF-α-neutralizing antibody (Fig. 2A-B). In contrast, Mrp1 protein expression was significantly increased in primary cultures of rat astrocytes treated with HIV-$1_{96ZM651}$ gp120 and the IL-1β- or the IL-6-neutralizing antibody. These data suggest that TNF-α, but not IL-1β or IL-6, is involved in up-regulation of Mrp1 protein expression.

Functional Studies. To investigate whether increased Mrp1 mRNA and protein expression in response to TNF-α exposure resulted in altered Mrp-mediated transport activity, we measured cellular retention of BCECF, a fluorescein derivative and established Mrp1, Mrp2, Mrp4, and ABCG2 substrate (Bachmeier et al., 2004). Mrp2 expression was not detected in our primary cultures of rat astrocytes, and Mrp4 expression was not altered in response to either HIV-$1_{96ZM651}$ gp120 treatment (Ronaldson and Bendayan, 2008) or TNF-α exposure (data not shown). In addition, a previous study by our laboratory demonstrated that ABCG2 was expressed but not capable of efflux transport in primary cultures of rat astrocytes (Lee et al., 2007). Therefore, we hypothesized that any change in BCECF cellular retention would most likely correspond to an alteration in Mrp1-mediated transport activity. For these experiments, cells were grown as monolayers and incubated in the presence or absence of 0.5 or 10 ng/ml TNF-α for 24 h. The time course of BCECF (5 μM) cellular retention at 37°C (Fig. 3) showed increasing accumulation until approximately 15 min. At this point, BCECF cellular retention decreases for the duration of the experiment, suggesting the presence of an active efflux process for this fluorescent substrate. In cultured rat astrocytes treated with 0.5 ng/ml TNF-α for 24 h, BCECF cellular retention was significantly decreased up to 2.4-fold (Fig. 3), suggesting an increase in Mrp1 functional activity. BCECF cellular retention was also decreased up to

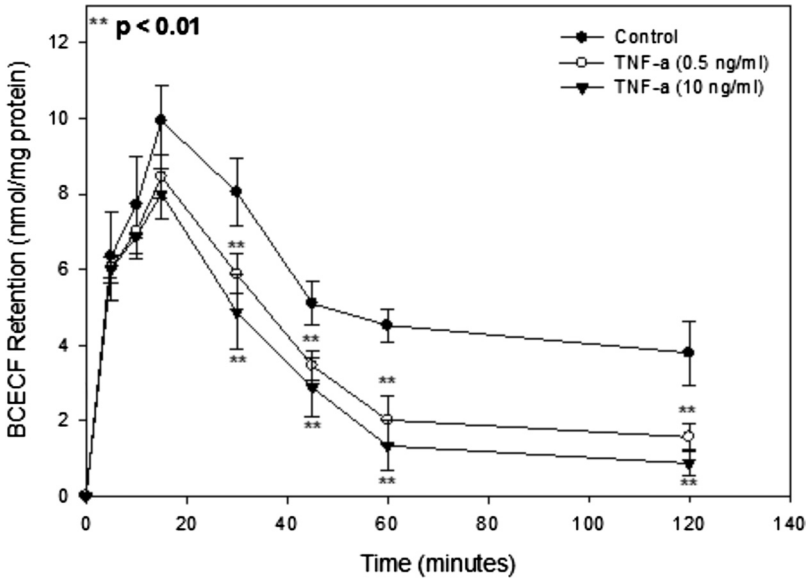


Fig. 3. Effect of 24 h TNF-α exposure on the cellular retention of BCECF, a fluorescent Mrp substrate, by cortical rat astrocyte monolayers. BCECF (5 μM) accumulation was measured at 37°C in the presence of 0.5 or 10 ng/ml TNF-α. Results are expressed as mean ± S.D. of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control.

4.4-fold in cultures treated with 10 ng/ml TNF-α, implying that TNF-α increases Mrp1 functional activity in a concentration-dependent manner.

Role of NF-κB on Cytokine Release and Mrp1 Protein Expression. NF-κB is a redox-regulated transcription factor that is known to be activated in cultured cells triggered by gp120 (Saha and Pahan, 2007). In addition, NF-κB-mediated signaling has been implicated in the regulation of ABC transporters such as P-gp in rat brain capillaries (Bauer et al., 2007); however, it is currently unknown whether NF-κB signaling can regulate Mrp1 expression. Therefore, we investigated the possible involvement of NF-κB in the regulation

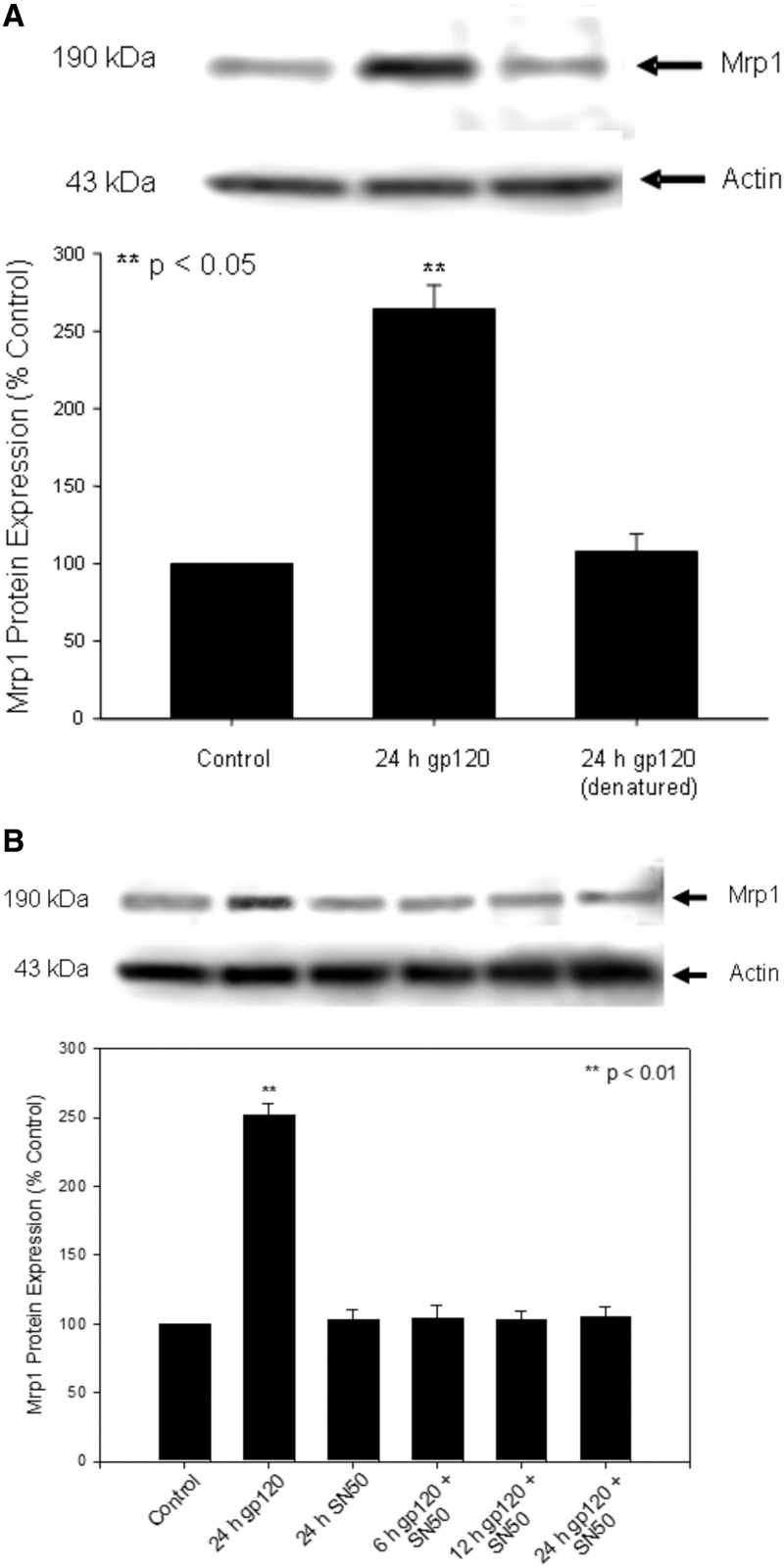


Fig. 4. Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SN50, a peptidic NF-κB inhibitor. A, immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 and with denatured HIV-$1_{96ZM651}$ gp120. Whole-cell lysates (25 μg) from primary cultures of rat astrocytes were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution), and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± S.D. of three separate experiments. Asterisks represent data points that are significantly different from control. B, immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 in the presence of 1.0 μM SN50, a cell-permeant NF-κB inhibitory peptide. Whole-cell lysates (25 μg) from primary cultures of rat astrocytes were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. MRP1/Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution), and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± S.D. of three separate experiments. Asterisks represent data points that are significantly different from control.

of Mrp1 protein expression in primary cultures of rat astrocytes triggered with gp120. Control experiments showed that Mrp1 protein expression was increased in cultured astrocytes triggered with 1.0 nM HIV-$1_{96ZM651}$ gp120 but was unchanged in cultures treated with denatured (i.e., heat-inactivated) HIV-$1_{96ZM651}$ gp120 (Fig. 4A). These data imply that a cellular response specific for the native conformation of HIV-$1_{96ZM651}$ gp120 is required for enhancement of Mrp1 expression. Furthermore, these results also indicate that altered Mrp1 expression was not associated with low-level endotoxin contamination in recombinant HIV-$1_{96ZM651}$ gp120 samples. Immunoblot analysis of primary cultures of rat astrocytes triggered with 1.0 nM HIV-$1_{96ZM651}$ gp120 in the presence and absence of SN50, a cell-permeant NF-κB inhibitory peptide, was performed. SN50 has been shown previously to specifically inhibit NF-κB nuclear translocation at a concentration of 10 μM or less (Lin et al., 1995), thus rendering it a good pharmacologic inhibitor of NF-κB-mediated signaling processes. Using trypan blue exclusion, we observed that cell viability was not compromised by exposure to SN50 at concentrations up to 10 μM (data not shown). In cells triggered with HIV-$1_{96ZM651}$ gp120, Mrp1 expression was increased by 2.5-fold; however, Mrp1 protein expression was unchanged in cultures treated with HIV-$1_{96ZM651}$ gp120 and 1.0 μM SN50 at the time points examined (6, 12, or 24 h) (Fig. 4B). To confirm the involvement of NF-κB signaling in the regulation of Mrp1 protein expression, we also conducted experiments in the presence and absence of BAY 11-7082, an established pharmacologic NF-κB inhibitor. Similar to our results with SN50, Mrp1 protein expression was not significantly different from control in rat astrocyte cultures triggered with HIV-$1_{96ZM651}$ gp120 (24 h) in the presence of 5.0 μM BAY 11-7082 (Fig. 5). Appropriate loading of each sample was confirmed by the detection of a single band at approximately 43 kDa, which corresponds to actin. Overall, these data provide evidence for involvement of NF-κB signaling in the regulation of Mrp1 expression in primary cultures of rat astrocytes triggered with HIV-1 viral envelope proteins.

Because gp120 treatment is known to stimulate cytokine release (Ronaldson and Bendayan, 2006), we investigated the role of NF-κB in TNF-α secretion in primary cultures of rat astrocytes triggered with gp120. Secretion of TNF-α was measured in rat astrocyte cultures treated with HIV-$1_{96ZM651}$ gp120 and SN50. Ultrasensitive ELISA analysis demonstrated increased TNF-α protein expression ($p < 0.01$) in cell culture supernatants from primary cultures of rat astrocytes triggered with 1.0 nM HIV-$1_{96ZM651}$ gp120 for 6, 12, and 24 h (Table 1). In contrast, TNF-α release in astrocyte

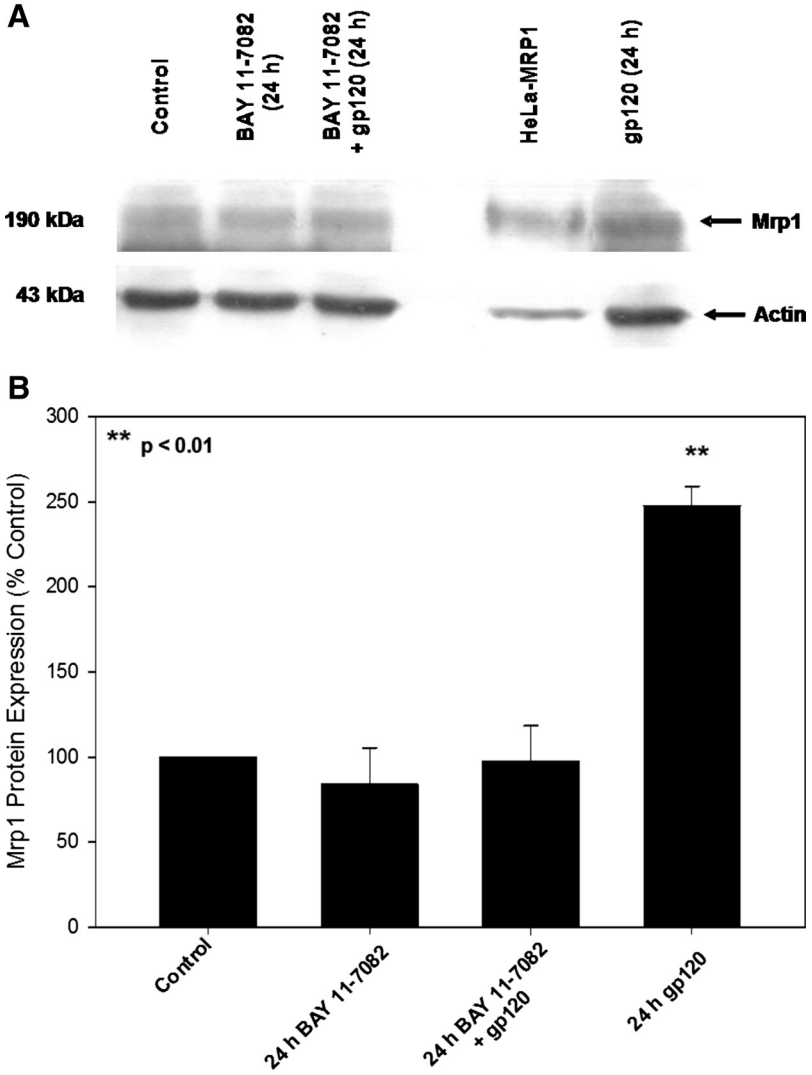


Fig. 5. Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and BAY 11-7082, a pharmacologic NF-κB inhibitor. A, immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 in the presence and absence of BAY 11-7082 (5 μM). Whole-cell lysates (50 μg) from primary cultures of rat astrocytes were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Whole-cell lysate from HeLa-MRP1 cells (1 μg) was used as a positive control. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution). B, densitometric analysis of Mrp1 expression in primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 in the presence and absence of 5 μM BAY 11-7082. Results are expressed as mean $\pm$ S.D. of three separate experiments. Asterisks represent data points that are significantly different from control.

cultures treated with 1.0 nM HIV-$1_{96ZM651}$ gp120 in the presence of 1.0 μM SN50 was below the detection limit of the assay (i.e., less than 15 pg/ml). Previous work by our laboratory has shown that basal levels of TNF-α in our primary cultures of rat astrocytes are below the detection limit of the assay (Ronaldson and Bendayan, 2006). These observations suggest that TNF-α secretion from primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 is mediated by an NF-κB-dependent mechanism.

The above results imply that NF-κB-mediated signaling is involved in release of TNF-α in cultured astrocytes triggered with HIV-$1_{96ZM651}$ gp120; however, these data were unable to allow us to discern whether NF-κB is directly involved in the regulation of Mrp1 itself. To address this question, we pretreated our astrocyte cultures with 1.0 μM SN50 followed by exposure to 0.5 or 10 ng/ml TNF-α. Immunoblot analysis showed increased expression of Mrp1 in astrocyte cultures treated with 1.0 μM SN50 and 0.5 ng/ml TNF-α (2.4-fold) or 10 ng/ml TNF-α (2.6-fold) (Fig. 6), suggesting that NF-κB does not directly regulate Mrp1 expression. Appropriate loading of each sample was confirmed by detection of a single band at approximately 43 kDa, which corresponds to actin. Taken together, these data indicate that NF-κB signaling processes are indirectly involved in the regulation of Mrp1 protein expression by triggering release of cytokines (i.e., TNF-α) in glial cells after exposure to HIV-$1_{96ZM651}$ gp120.

TABLE 1

ELISA analysis of TNF-α secretion in cultured astrocytes treated with HIV-$1_{96ZM651}$ gp120

Results are expressed as mean ± S.D. of eight separate measurements obtained from different cultures on different days. Statistical comparisons are between cultures treated with HIV-$1_{96ZM651}$ gp120 alone and cultures triggered with HIV-$1_{96ZM651}$ gp120 plus inhibitor.

Time of Exposure	HIV-$1_{96ZM651}$ gp120 (1.0 nM)	HIV-$1_{96ZM651}$ gp120 (1.0 nM) + SN50 (1.0 μM)	HIV-$1_{96ZM651}$ gp120 (1.0 nM) + SP600125 (20 μM)
		pg/ml	
6 h	583.08 ± 22.58	B.D.L**	570.43 ± 49.28
12 h	483.90 ± 32.81	B.D.L**	490.54 ± 36.43
24 h	384.71 ± 34.28	B.D.L.**	369.56 ± 33.29

B.D.L., below detection limit of the assay. The limit of detection for the rat TNF-α ELISA used in this study was 15 pg/ml.

** $P < 0.01$.

Role of JNKs on Mrp1 Functional Expression. Components of the MAPK pathway, such as the JNKs, have also been shown to be activated in response to HIV-1 viral proteins and/or inflammation (Hayashi et al., 2006; Chen and Thorner, 2007). In addition, JNK isoforms may be involved in regulation of ABC membrane transporters (Hayashi et al., 2006; Hartz et al., 2008). Therefore, we investigated the involvement of JNKs in regulation of Mrp1 protein expression in primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120. Immunoblot analysis of cultured astrocytes triggered with 1.0 nM HIV-$1_{96ZM651}$ gp120 in the presence or absence of 20 μM SP600125, an established JNK inhibitor, was performed. SP600125 is known to reversibly inhibit JNK signaling with IC_{50} values in the range of 40 to 90 nM (Bennett et al., 2001). Furthermore, SP600125 displays greater than 300-fold selectivity for JNK over related MAPKs (i.e., extracellular signal-regulated kinase 1 and p38 MAPK) and 10- to 100-fold greater selectivity over other intracellular kinases (Bennett et al., 2001). Using the trypan blue exclusion method, we observed that cell viability was not altered in the presence of 20 μM SP600125 (data not shown). We demonstrated that 20 μM SP600125 decreased total JNK phosphorylation in primary cultures of rat astrocytes triggered with TNF-α to a level that was not significantly different from control untreated cells (data not shown). Mrp1 protein expression was unchanged in cultures treated with HIV-$1_{96ZM651}$ gp120 and SP600125 at the time points examined (6, 12, or 24 h) (Fig. 7), suggesting that JNKs may be involved in the regulation of this ABC transporter. Appropriate loading of each sample was confirmed by the detection of a single band at approximately 43 kDa, which corresponds to actin.

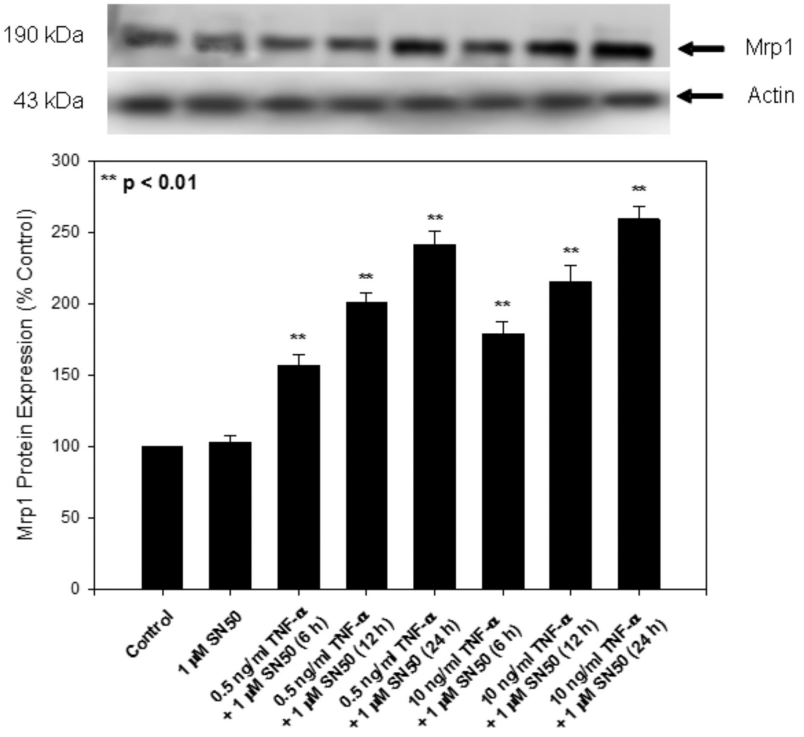


Fig. 6. Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SN50, a peptidic NF-κB inhibitor. Immunoblot analysis of primary cultures of rat astrocytes triggered with TNF-α (0.5 or 10 ng/ml) in the presence of 1.0 μM SN50. Whole-cell lysates from primary cultures of rat astrocytes (25 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution), and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± S.D. of three separate experiments. Asterisks represent data points that are significantly different from control.

To determine the role of JNKs in the regulation of cytokine release, we measured the secretion of TNF-α in primary cultures of rat astrocytes treated with HIV-$1_{96ZM651}$ gp120 in the presence of SP600125. TNF-α release in astrocyte cultures treated with 1.0 nM HIV-$1_{96ZM651}$ gp120 in the presence of 20 μM SP600125 was not statistically different ($p > 0.05$) from TNF-α secretion in astrocyte cultures treated with 1.0 nM HIV-$1_{96ZM651}$ gp120 alone (Table 1). These observations imply that JNK signaling is not involved in the release of TNF-α from primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120.

To determine whether JNK signaling was directly involved in regulation of Mrp1 protein expression, we pretreated our rat astrocyte cultures with 20 μM SP600125 followed by exposure to 0.5 or 10 ng/ml TNF-α. Control experiments in the absence of SP600125 demonstrated increased Mrp1 protein expression in primary cultures of rat astrocytes exposed to 0.5 ng/ml TNF-α (2.5-fold) or 10 ng/ml TNF-α (2.6-fold) (Fig. 8A). In contrast, no change in protein expression of Mrp1 was observed in astrocyte cultures treated with 20 μM SP600125 and 0.5 or 10 ng/ml TNF-α (Fig. 8B). Appropriate loading of each sample was confirmed by detection of a single band at approximately 43 kDa, which corresponds to actin.

To investigate whether pharmacologic inhibition of JNK isoforms resulted in altered Mrp-mediated transport activity, we measured cellular retention of BCECF in cortical astrocyte monolayers triggered with HIV-$1_{96ZM651}$ gp120 or TNF-α in the presence or absence of SP600125. For these experiments, cells were grown as monolayers and incubated with 1.0 nM HIV-$1_{96ZM651}$ gp120 or 10 ng/ml TNF-α for 24 h. In cultures treated with the JNK inhibitor, SP600125 (20 μM) was added 30 min before HIV-$1_{96ZM651}$ gp120 or TNF-α exposure. In cultured rat astrocytes treated with 1.0 nM HIV-$1_{96ZM651}$ gp120 or 10 ng/ml TNF-α, BCECF cellular retention was significantly decreased up to 2.4-fold (Fig. 9). In contrast, BCECF cellular retention was not altered in rat astrocyte cultures treated with SP600125 and HIV-$1_{96ZM651}$ gp120 or with SP600125 and TNF-α. Control experiments demonstrated that 20 μM SP600125 itself did not affect cellular retention of BCECF (data not shown). Taken together, these data indicate that inhibition of JNK signaling processes attenuates the increase in Mrp1 functional expression observed in glial cells after exposure to HIV-1 viral proteins or proinflammatory cytokines.

Effect of HIV-$1_{96ZM651}$ gp120 and TNF-α on Mrp1 mRNA Expression. Because we observed increased protein expression of Mrp1 in cultured rat astrocytes triggered with gp120 or TNF-α, we sought to evaluate Mrp1 mRNA expression in cultured astrocytes exposed to these same mediators. Quantitative PCR analysis was used to measure the expression of Mrp1 mRNA in primary cultures of rat astrocytes treated with HIV-$1_{96ZM651}$ gp120 or TNF-α. Mrp1 mRNA was significantly increased (1.6-fold) in cultured astrocytes triggered with 1.0 nM HIV-$1_{96ZM651}$ gp120 for 6 h; however, Mrp1 expression was not altered in primary cultures of rat astrocytes exposed to HIV-$1_{96ZM651}$ gp120 for 12 or 24 h (Fig. 10A). Likewise, Mrp1 mRNA expression was increased in cells treated with 10 ng/ml TNF-α for 6 h (1.7-fold) but no change in Mrp1 expression was observed in primary cultures of rat astrocytes triggered with TNF-α for 12 h or 24 h (Fig. 10B). Taken together, these data suggest that increased Mrp1 protein expression in cultured rat astrocytes triggered with either gp120 or TNF-α may result, at least in part, from increased expression of Mrp1 mRNA.

Discussion

ABC transporters (i.e., P-gp, Mrp1) are important determinants of xenobiotic permeation across brain barriers and brain parenchyma cellular compartments (i.e., astrocytes, microglia) (Ronaldson et al., 2008). This is particularly significant for treatment of HIV-1 infection because antiretroviral agents (i.e., HIV-1 protease inhibitors) are known substrates for P-gp and/or Mrp1 (Williams et al., 2002; Dallas et al., 2004; Ronaldson and Bendayan, 2006), a factor that may limit the ability of these drugs to attain efficacious CNS

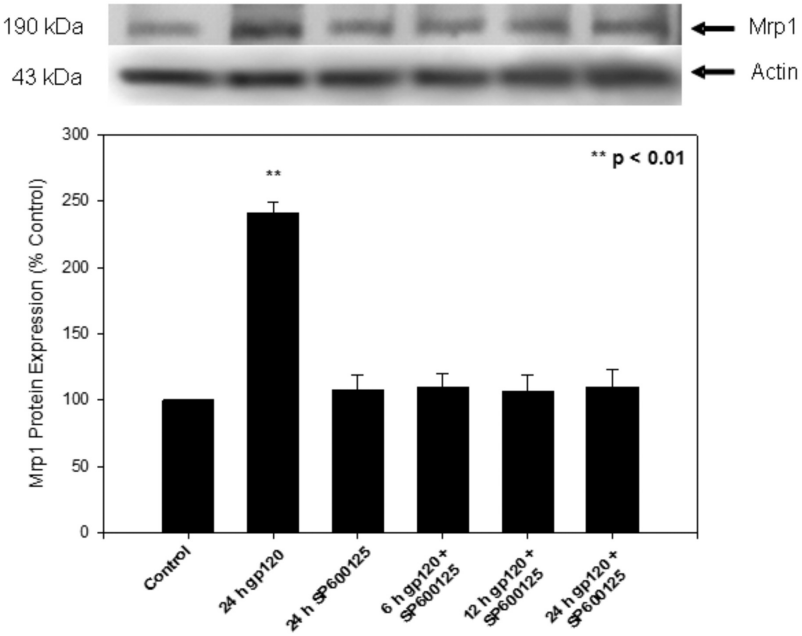


Fig. 7. Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SP600125, a pharmacologic JNK inhibitor. Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-$1_{96ZM651}$ gp120 in the presence of 20 μM SP600125. Whole-cell lysates from primary cultures of rat astrocytes (25 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean $\pm$ S.D. of three separate experiments. Asterisks represent data points that are significantly different from control.

concentrations. Until recently, ABC transporter functional expression had only been characterized in nonpathologic (i.e., healthy) astrocyte cultures (Ronaldson et al., 2004). To elucidate the role of brain pathologies on ABC transporter expression and/or activity, we implemented an in vitro model of an HIV-1 associated inflammatory response by triggering

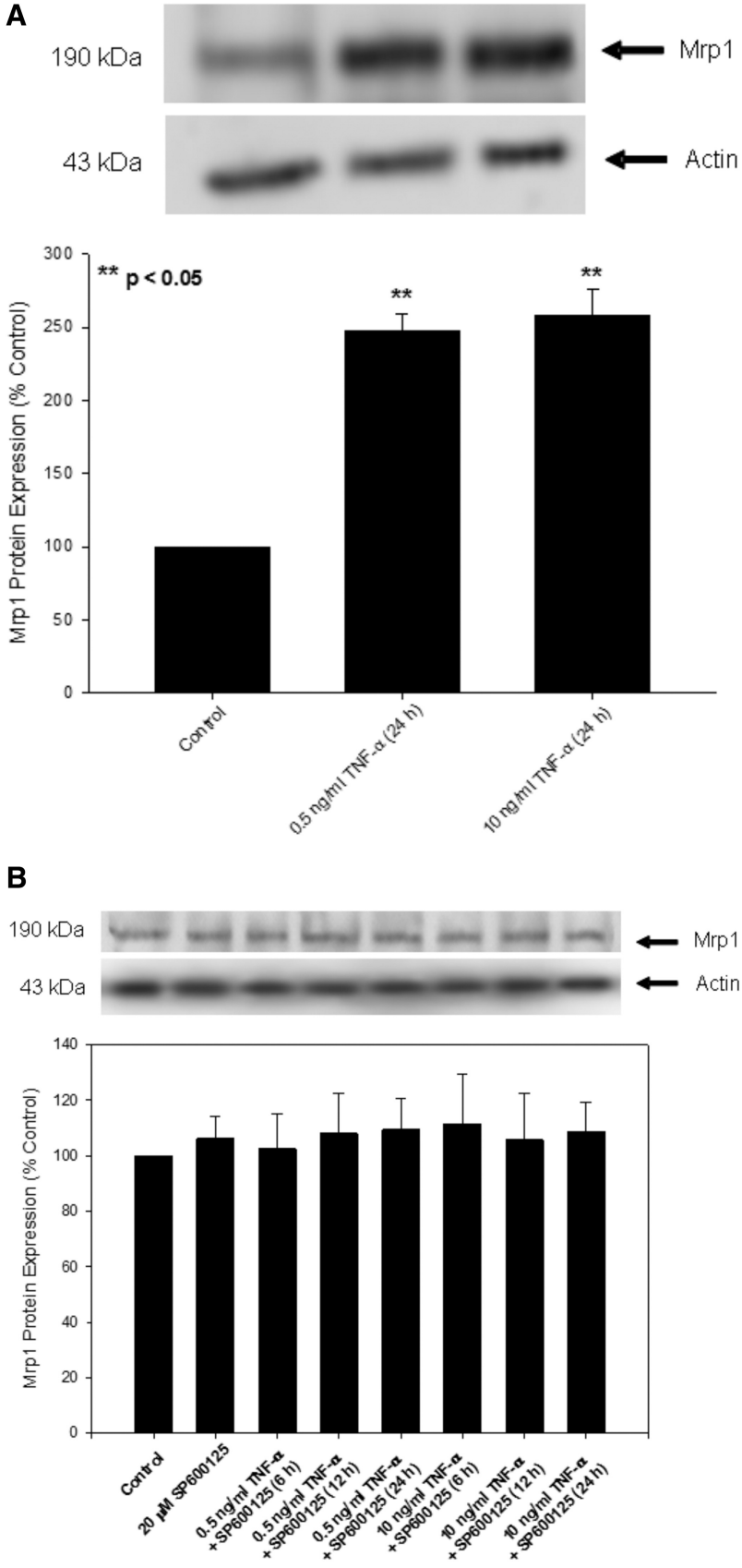


Fig. 8. Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SP600125, a pharmacologic JNK inhibitor. A, immunoblot analysis of primary cultures of rat astrocytes triggered with TNF-α (0.5 ng/ml or 10 ng/ml). Whole-cell lysates from primary cultures of rat astrocytes (25 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution), and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean $\pm$ S.D. of three separate experiments. Asterisks represent data points that are significantly different from control. B, immunoblot analysis of primary cultures of rat astrocytes triggered with TNF-α (0.5 or 10 ng/ml) in the presence of 20 μM SP600125. Whole-cell lysates from primary cultures of rat astrocytes (25 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution), and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean $\pm$ S.D. of three separate experiments. Asterisks represent data points that are significantly different from control.

cultured astrocytes with HIV-$1_{96ZM651}$ gp120 (Ronaldson and Bendayan, 2006). This model was characterized by increased production and secretion of proinflammatory cytokines (i.e., TNF-α, IL-1β, IL-6) as determined by semiquantitative reversed transcription PCR and ELISA, respectively (Ronaldson and Bendayan, 2006).

Previous in vitro and in vivo studies have shown that cytokines (i.e., TNF-α, IL-1β, IL-6) can alter Mrp1 expression (Lee and Piquette-Miller, 2003; Cherrington et al., 2004). In the context of HIV-1-associated inflammation, Jorajuria et al. (2004) reported increased TNF-α and IL-6 production and increased expression of MRP1 mRNA in human monocyte-derived macrophages infected with HIV-1 BaL, an R5-tropic viral strain. With the use of Spearman's rank correlation test, these researchers concluded that MRP1 mRNA expression was not directly correlated with TNF-α or IL-6 production (Jorajuria et al., 2004); however, a causal relationship between cytokine secretion and altered MRP1 mRNA levels was not established. In our study, we have directly triggered primary cultures of rat astrocytes with proinflammatory cytokines. Although we observed no change in Mrp1 expression in cultured astrocytes triggered with IL-1β or IL-6, Mrp1 expression was increased in the presence of TNF-α (2.7-fold). We further examined the role of these cytokines on Mrp1 protein expression by treating primary cultures of rat astrocytes with HIV-$1_{96ZM651}$ gp120 in the presence of TNF-α, IL-1β, or IL-6 neutralizing antibodies. Our results indicate that Mrp1 expression was not altered in the presence of TNF-α neutralizing antibody but was significantly increased when IL-1β or IL-6 neutralizing antibodies were used. These data confirm that TNF-α is prominently involved in up-regulation of Mrp1 expression in our astrocyte cultures. Taken together with our previous publication (Ronaldson and Bendayan, 2006), these results provide evidence for the complex manner by which cytokines regulate ABC transporter expression. With respect to P-gp, we observed decreased expression mediated by IL-6 but increased expression mediated by TNF-α and IL-1β (Ronaldson and Bendayan, 2006), suggesting that multiple cytokine signaling pathways are involved in regulation of P-gp expression. In the present study, we demonstrate that Mrp1 is increased by TNF-α, but not by IL-1β or IL-6, suggesting that Mrp1 expression is regulated by a TNF-α mediated pathway during an inflammatory response.

To determine whether increased Mrp1 protein expression correlated with enhanced activity, we used BCECF, an established Mrp substrate (Bachmeier et al., 2004). An important consideration is that BCECF is also a substrate for Mrp2, Mrp4 and ABCG2. Because these transporters were neither expressed (i.e., Mrp2), affected by HIV-$1_{96ZM651}$ gp120 or TNF-α treatment (i.e., Mrp4), nor functional (i.e., ABCG2) in our primary cultures of rat astrocytes (Lee et al., 2007; Ronaldson and Bendayan, 2008), we are able to conclude that any difference in BCECF efflux is most likely attributable to changes in Mrp1 activity. Our studies showed that TNF-α treatment reduced BCECF cellular retention in a concentration-dependent manner, which implies an increase in Mrp-mediated transport. These data are particularly intriguing in light of our previous study, which showed a significant decrease in P-gp functional expression in the same in vitro model (Ronaldson and Bendayan, 2006). Therefore, we propose that Mrp1 may play an enhanced role in antiretroviral drug transport during HIV-1 associated inflammatory responses. Changes in Mrp1 functional expression may be particularly relevant for HIV-1 protease inhibitors, which are substrates for MRP1/Mrp1 (Williams et al., 2002; Dallas et al., 2004).

Intracellular signaling mechanisms responsible for gp120 effects in glial cells have not been clearly identified. Previous studies have indicated that NF-κB-mediated signaling pathways are activated in response to gp120 exposure in cultured rat astrocytes (Saha and Pahan, 2007). Because it has been shown that NF-κB activation is associated with changes in expression of other ABC transporters, such as P-gp (Hayashi et al., 2005; Bauer et al., 2007), we hypothesized that NF-κB

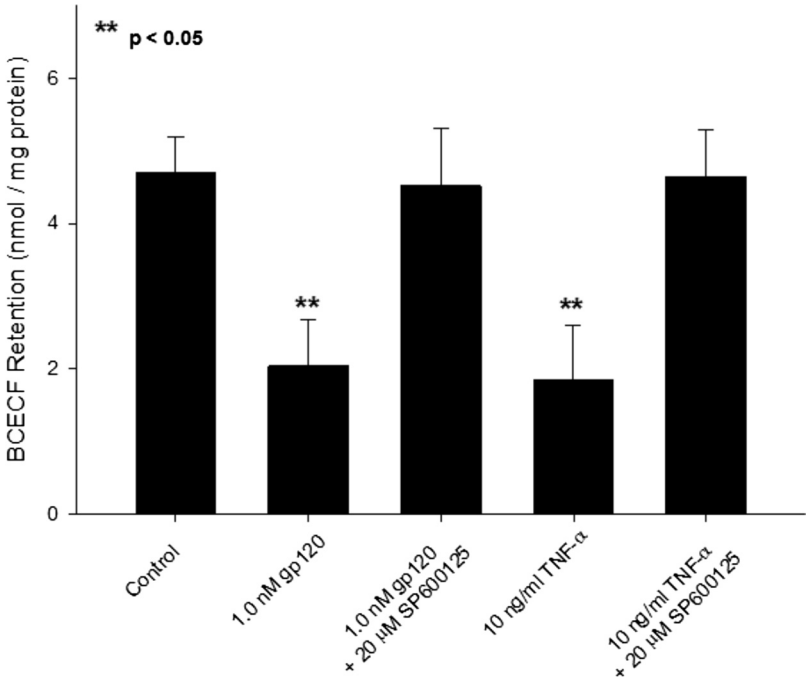


Fig. 9. Effect of 24 h gp120 or TNF-α exposure on the cellular retention of BCECF, a fluorescent Mrp substrate, by cortical rat astrocyte monolayers. BCECF (5 μM) accumulation was measured at 37°C in cultured astrocytes treated with 1.0 nM HIV-$1_{96ZM651}$ gp120 or 10 ng/ml TNF-α in the presence and absence of SP600125, a specific pharmacologic JNK inhibitor. Results are expressed as mean ± S.D. of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control.

may also be involved in Mrp1 regulation. In the present study, we show that increased Mrp1 expression induced by HIV-$1_{96ZM651}$ gp120 was attenuated by SN50, an NF-κB inhibitory peptide. Although SN50 was used primarily as an inhibitor of NF-κB nuclear import, it may also affect nuclear translocation of other transcription factors. Using an immortalized human T-lymphocyte cell line, SN50 (210 μg/ml; 75 μM) was shown to inhibit nuclear import of multiple transcription factors, including activator protein 1 (AP-1), nuclear factor of activated T cells, signal transducer and activator of transcription 1, and NF-κB (Torgerson et al., 1998). In contrast, studies in primary cultures of human peripheral blood-derived T-lymphocytes demonstrated that SN50 had no effect on nuclear translocation of AP-1 or nuclear factor of activated T cells at a concentration that was 5.6-fold lower than used by Kolenko et al. (1999), suggesting that cross-talk with other signaling pathways occurs only at high concentrations of SN50. This corroborates data obtained in a murine fibroblast cell line (3T3), which showed that SN50 specifically inhibited NF-κB nuclear translocation at concentrations less than 10 μM (Lin et al., 1995). We used a much lower concentration of SN50 than any of these studies (i.e., 2.8 μg/ml; 1 μM), suggesting that our results reflect an inhibition of NF-κB with little contribution from other signaling pathways.

NF-κB activation is associated with production/secretion of cytokines such as TNF-α (Filipov et al., 2005). Therefore, we examined TNF-α release from primary cultures of rat astrocytes exposed to HIV-$1_{96ZM651}$ gp120 in the presence and absence of SN50. Indeed, pretreatment with SN50 reduced TNF-α secretion to levels that were below ELISA detection limits (i.e., less than 15 ng/ml), suggesting involvement of NF-κB. When we treated our cultures with TNF-α in the presence of SN50, we observed a significant increase in Mrp1 protein expression, implying that NF-κB does not directly regulate Mrp1 expression. A recent study in LPS-treated mice deficient in inhibitor of κB kinase kinase β demonstrated a similar increase in hepatic Mrp1 mRNA levels compared with LPS-treated wild-type mice (Lickteig et al., 2007). LPS treatment has been shown to induce the cellular release of proinflammatory cytokines that can alter the expression of ABC transporters, including Mrp1 (Cherrington

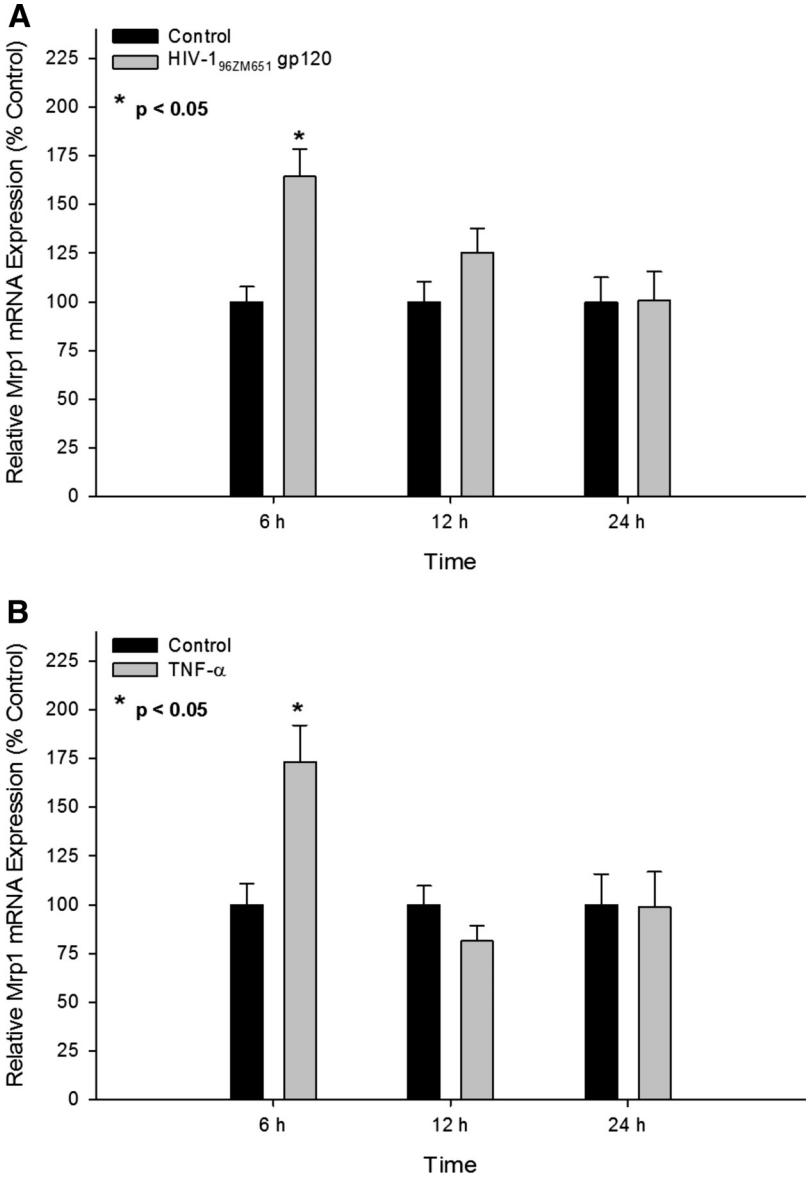


Fig. 10. Effect of gp120 or TNF-α exposure on Mrp1 mRNA expression in primary cultures of rat astrocytes. Primary cultures of rat astrocytes were triggered with 1.0 nM HIV-$1_{96ZM651}$ gp120 (A) or 10 ng/ml TNF-α (B) for 6, 12, or 24 h. Mrp1 mRNA expression was measured using quantitative PCR analysis. Results (percentage of control) are expressed as mean ± S.D. of four separate experiments. Asterisks indicate data points that are significantly different from control.

et al., 2004). Taken together with our present study, these data suggest that NF-κB activity is involved in the regulation of Mrp1 expression only by enhancing the release of TNF-α.

The cellular response to gp120 and/or cytokines involves a multiplicity of signaling pathways in addition to NF-κB. It has been shown previously that both gp120 and TNF-α can activate the MAPK pathway, in particular the JNKs (Barbin et al., 2001; Bodner et al., 2004). Other HIV-1 proteins (i.e., Tat) have been shown to up-regulate Mrp1 expression in primary cultures of murine astrocytes via a JNK-dependent mechanism (Hayashi et al., 2006). Therefore, we investigated the role of the JNK pathway on regulation of Mrp1 expression in cultured astrocytes exposed to gp120 and/or TNF-α. Pharmacologic inhibition of JNK signaling with SP600125 prevented up-regulation of Mrp1 expression in HIV-$1_{96ZM651}$ gp120 triggered astrocyte cultures. Pretreatment with SP600125 attenuated up-regulation of Mrp1 in response to TNF-α exposure; however, SP600125 had no effect on HIV-$1_{96ZM651}$ gp120 induced release of TNF-α from rat astrocyte cultures. Furthermore, SP600125 prevented the increase in cellular BCECF efflux in cultures treated with HIV-$1_{96ZM651}$ gp120 or TNF-α. Our data corroborate the work of Hayashi et al. (2006) and imply that JNKs are involved, in part, in regulation of Mrp1 functional expression in glial cells exposed to HIV-1 viral proteins and/or inflammatory mediators (Fig. 11). Specifically, our results indicate that JNK phosphorylation and up-regulation of Mrp1 occurs subsequent to NF-κB-mediated TNF-α release. Studies in human macrophages and microglia have demonstrated that JNK phosphorylation may also occur in response to gp120 binding to CCR5 (Yi et al., 2004). Because we did not observe a change in Mrp1 protein expression in cultured astrocytes treated with HIV-$1_{96ZM651}$ gp120 and the TNF-α-neutralizing antibody, we can conclude that JNK phosphorylation resulting from the gp120-CCR5 interaction was not a confounding factor in our study.

Our data show increased Mrp1 mRNA expression in cultured astrocytes triggered with HIV-$1_{96ZM651}$ gp120 or with TNF-α, suggesting that an HIV-1 inflammatory response may, in part, alter transcription of the *Mrp1* gene. MAPK signaling cascades (i.e., JNK) are complex and may affect the expression of ABC transporter genes by the recruitment of transcription factors (i.e., AP-1, c-Jun) (Shinoda et al., 2005; Zhou et al., 2006; Hartz et al., 2008). Using chromatin immunoprecipitation, increased c-Jun binding to the *MRP1* promoter was observed in human small-cell lung cancer cell lines treated with the anticancer drug doxorubicin (Shinoda et al., 2005). In addition, this study showed that SP600125 inhibited c-Jun binding, confirming the involvement of JNK signaling in *MRP1* regulation. Although conducted in cancerous human cell culture systems, the hypotheses proposed in this study can be tested in healthy rodent cell culture systems because similar signaling pathways and/or transcription factors are expressed in both models. Some of these similarities include expression of JNK/AP-1 (Nair et al., 2008), JNK/c-Jun (Shinoda et al., 2005), and Nrf2 (Song et al., 2009). Furthermore, human *MRP1* and rat *Mrp1* are both regulated by a highly conserved 100-nucleotide sequence in the promoter region, suggesting that regulatory mechanisms for both genes may be structurally and functionally similar (Muredda et al., 2003). Nonetheless, studies are required to determine specific JNK-associated transcription factors involved in regulation of *Mrp1* during cellular exposure to HIV-$1_{96ZM651}$ gp120 and/or proinflammatory cytokines.

In addition to inflammatory processes, oxidative stress is also involved in HIV-1-associated pathologic conditions in the CNS. Our group has recently shown that gp120 treatment can lead to an oxidative stress response in primary cultures of rat astrocytes characterized by increased free radical production and increased oxidation of intracellular glutathione (Ronaldson and Bendayan, 2008). Our study demonstrated, for the first time, that gp120-induced oxidative stress increases Mrp1 functional expression in cultured glial cells (Ronaldson and Bendayan, 2008). Oxidative stress responses in astrocytes are associated with activation of several intracellular signaling mechanisms, including NF-κB

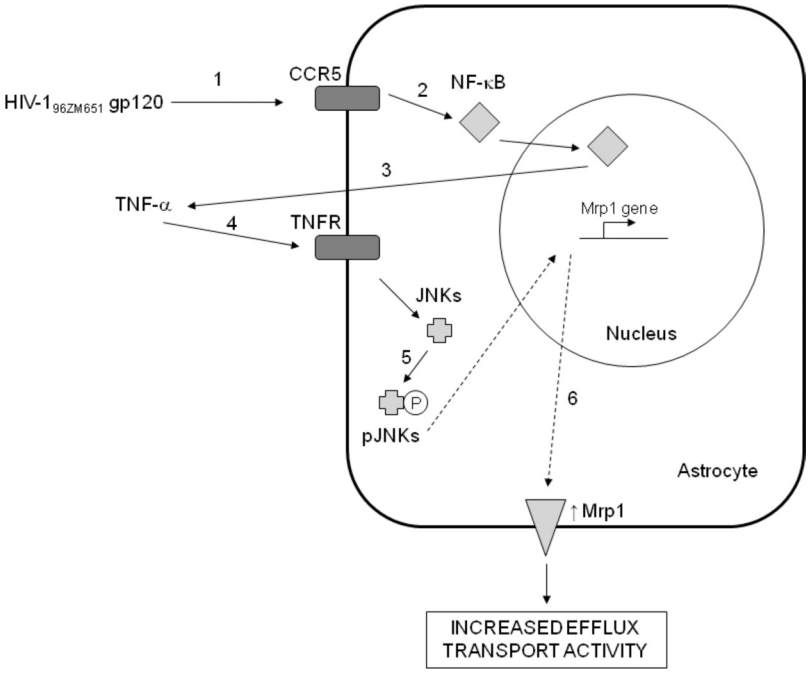


Fig. 11. Proposed mechanism of NF-κB and JNK signaling in glial cells during an HIV-1 associated inflammatory response. Brain HIV-1 infection is characterized by the presence of HIV-1 viral proteins such as gp120 within the brain parenchyma. In our model, R5-tropic gp120 (i.e., HIV-$1_{96ZM651}$ gp120) directly binds to specific chemokine receptors (i.e., CCR5) expressed at the astrocyte cell surface (1). In turn, this activates NF-κB mediated signaling (2), leading to increased production and secretion of proinflammatory cytokines including TNF-α (3). Once secreted, TNF-α may bind to its receptor (TNFRs) that are expressed at the plasma membrane of astrocytes (4). The activation of TNFRs leads to increased phosphorylation (i.e., activation) of JNK isoforms (5). Our data show that these signaling events can lead to increased mRNA and protein expression of ABC membrane transporters such as Mrp1 (6). The end result of this signaling mechanism is an increase in Mrp1 transport activity. Overall, these data may point to a greater role for Mrp1 in antiretroviral drug resistance during an HIV-1 associated inflammatory response.

(Caccamo et al., 2005) and JNK MAPK (Chen et al., 2008). In addition, Nrf2 signaling is also known to be activated in response to oxidative stress and may be involved in the regulation of ABC transporters such as MRP1/Mrp1 (Hayashi et al., 2003; Song et al., 2009). The cellular response to HIV-1 viral proteins such as gp120 is clearly complex and involves multiple pathophysiologic responses (i.e., inflammation, oxidative stress). Future studies will delineate those cellular signaling processes that are activated by proinflammatory cytokines and those that are induced by oxidative stress in an effort to clarify mechanisms of HIV-associated pathophysiologic processes as well as novel strategies for the treatment of brain HIV-1 infection.

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