Regulation of Multidrug Resistance Protein 1 (Mrp1) by Tumor Necrosis Factor Alpha (TNF-α) in Cultured Glial Cells: Involvement of Nuclear Factor-κB (NF-κB) and c-Jun N-terminal Kinase (JNK) Signaling Pathways

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Abbreviations List: ABC, ATP-binding cassette; BCECF, 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; HBSS, Hank’s balanced salt solution; IκB, inhibitor of κB kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MRP, Multidrug Resistance Proteins; NF-κB, nuclear factor-κB; P-gp, P-glycoprotein; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; TBS-T, Tris-buffered saline containing Tween-20.
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., TNF-α, 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 if 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 BCECF, an Mrp substrate, was reduced in TNF-α treated astrocytes, suggesting increased Mrp-mediated transport. Pharmacological inhibition of 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 pre-treated with SP600125, an established 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 regulation of Mrp1 during an HIV-1 associated inflammatory response.
Introduction

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 neurological disease remains a significant cause of morbidity and mortality in HIV-1 patients (McArthur et al. 2003). These neurological complications may be associated 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-CSF 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. While studies in peripheral blood mononuclear cells isolated from HIV-1 infected patients showed no difference in MRP1 expression as compared to healthy individuals (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 were not correlated to altered expression of MRP1 (Jorajuria et al., 2004).

Cellular exposure to HIV-1 virions, HIV-1 viral proteins and/or 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; Hayashi et al., 2006). Additionally, 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). Currently, there are no published reports demonstrating the involvement of either pathway in the regulation of Mrp1 in glial cells exposed to HIV-1 gp120 and/or cytokines.

Recently, 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 if cytokines can regulate Mrp1 expression in glial cells and, if cytokines are capable of altering Mrp1 expression, which intracellular signaling pathways may be involved. In the present study, we have i) evaluated Mrp1 functional expression in cultured rat astrocytes triggered with TNF-α, IL-1β, and IL-6 and ii) investigated the role of NF-κB and JNKs in the regulation of Mrp1 expression in cultured astrocytes exposed to HIV-196ZM651 gp120 or cytokines.

Materials and Methods

Materials: HIV-196ZM651 gp120 full length protein (derived from subtype C, R5-tropic HIV-1) was obtained from the National Institute of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS (DAIDS), NIAID, NIH (Bethesda, MD). PSC833 (i.e., valspodar) was a generous gift from Novartis Pharma (Basel, Switzerland). The rat monoclonal MRPI 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 Inc. (Plymouth Meeting, PA). The cell-permeable NF-κB inhibitory peptide SN50 and the
pharmacological NF-κB inhibitor (E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (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 Chemicon Inc. (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 previously described by our laboratory (Ronaldson et al., 2004; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 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. Briefly, postnatal (1-3 day old) Wistar rats (Charles River Laboratories, St Constant, PQ, Canada) were killed by cervical dislocation and whole brains 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, PQ, 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 cm² polystyrene tissue culture flasks (Sarstedt, St. Leonard, PQ, Canada) and
incubated in fresh medium at 37°C, 5% CO2 and 95% air overnight for 7-10 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 Hank’s Balanced Salt Solution (HBSS) and plated at a density of 5 x 10^4 cells/well on 48-well polystyrene plates (Becton-Dickinson, Franklin Lakes, NJ). The astrocytic nature of isolated cells and culture purity were previously assessed by morphological 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 cm² tissue culture flasks at 37°C in 5% CO2 and 95% air. Cultures were maintained in Dulbecco’s modified Eagle’s medium (4 mM L-glutamine; 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 cm² tissue culture flasks. At the beginning of each experiment, culture medium was aspirated and fresh culture medium containing 1.0 nM HIV-196ZM651 gp120. HIV-196ZM651 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 HIV-1 infected patients, concentrations ranging between 12 ng/ml 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 nM to approximately 1.0 nM. All experiments were conducted at 37°C in 5% CO₂ and 95% air. Control (i.e., untreated) cultures were comprised of 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 pre-treated with 1 µM SN50, 5 µM BAY 11-7082 or 20 µM SP600125 respectively for 30 min prior to HIV-1₉₆ző₆₅₁ 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 ng/ml or 10 ng/ml TNF-α, 0.4 ng/ml or 10 ng/ml IL-1β, or 0.3 ng/ml or 10 ng/ml IL-6. These proinflammatory cytokines were selected since their expression is increased during HIV-1 associated immunological 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-1₉₆zzo₆₅₁ gp120 as determined by ELISA (Ronaldson and Bendayan, 2006) while 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 5% horse serum, 5% fetal bovine serum containing culture medium 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 pre-treated with 1 µM SN50 or 20 µM SP600125 respectively for 30 min prior to 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-1₉₆zzo₆₅₁ gp120 were conducted by aspirating culture medium and replacing it with fresh medium containing the cytokine neutralizing antibody and 1.0 nM HIV-1₉₆zzo₆₅₁ 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 since 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 (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM EGTA, 1% (v/v) Nonident P-40, 0.25% (m/v) sodium deoxycholate, 0.1% (m/v) SDS, 200 μM phenylmethylsulfonyl fluoride, 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 Bradford’s protein assay.

For immunoblotting, 1 μg or 25 μg 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 (TBS-T) and 5% (m/v) dry skim milk powder. Following six washes (5 min each) with TBS-T, 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 194-360 of human MRP1 and its epitope was subsequently localized to amino acids 238-247 (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-S-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). Following 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<sub>96ZM651</sub> gp120 (1.0 nM) or TNF-α (10 ng/ml) for 6 h, 12 h 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'-GCCGTAGTGCTTCAGCTT-3') were designed using Primer Express 3 software (Applied Biosystems) and were validated for specificity and efficacy using BioTaq universal rat normal tissue cDNA (BioTaq Inc., Gaithersburg, MD). Quantitative PCR (qPCR) 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 CT method (ΔΔCT). Results were expressed as mean ± SD of at least three separate experiments.

**ELISA Analysis:** 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<sub>GZ651</sub> 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 as pg/ml. 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<sub>96ZM651</sub> gp120 or with TNF-α (0.5 or 10 ng/ml) and grown on 24-well polystyrene plates (Becton-Dickinson) at an approximate density of 8 x 10<sup>4</sup> cells/well. Cells were washed and incubated at 37°C for 30 min in HBSS, 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 permeable ester BCECF-AM (5 μM) in the presence or absence of SP600125 (20 μM). Since 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 ice-cold PBS. The cells were then solubilized with 200 μl 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 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 (nmol/mg 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 ± SD 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 to be 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 were involved in the regulation of other ABC transporters that are expressed in astrocytes. Therefore, we explored the role of these cytokines in the 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 did not observe any 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).

In order 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**

In order 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). Additionally, 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 ng/ml TNF-α 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 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 if NF-κB signaling can regulate Mrp1 expression. Therefore, we investigated the possible involvement of NF-κB in the regulation 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-196ZM651 gp120 but was unchanged in cultures treated with denatured (i.e., heat-inactivated) HIV-196ZM651 gp120 (Fig 4A). These data imply that a cellular response specific for the native conformation of HIV-196ZM651 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-196ZM651 gp120 samples. Immunoblot analysis of primary cultures of rat astrocytes triggered with 1.0 nM HIV-196ZM651 gp120 in the presence and absence of SN50, a cell-permeable NF-κB inhibitory peptide, was performed. SN50 has been previously shown 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-196ZM651 gp120, Mrp1 expression was increased by 2.5-fold; however, Mrp1 protein expression was unchanged in cultures treated with HIV-196ZM651 gp120 and 1.0 µM SN50 at the time points examined (6, 12, or 24 h) (Fig 4B). In order 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 pharmacological 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-196ZM651 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.

Since 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-196ZM651 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-196ZM651 gp120 for 6, 12, and 24 h (table 1). In contrast, TNF-α release in astrocyte cultures treated with 1.0 nM HIV-196ZM651 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-196ZM651 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-196ZM651 gp120; however, these data were unable to discern if NF-κB is directly involved in the regulation of Mrp1 itself. In order to address this question, we pre-treated our astrocyte cultures with 1.0 µM SN50 followed by exposure to 0.5 ng/ml 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 following exposure to HIV-196ZM651 gp120.

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). Additionally, 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-196ZM651 gp120. Immunoblot analysis of cultured astrocytes triggered with 1.0 nM HIV-196ZM651 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 IC50 values in the range of 40-90 nM (Bennett et al., 2001). Furthermore, SP600125 displays greater than 300-fold selectivity for JNK over related MAPKs (i.e., ERK1 and p38 MAPK) and 10-100-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). In our hands, 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-196ZM651 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.

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-196ZM651 gp120 in the presence of SP600125. TNF-α release in astrocyte cultures treated with 1.0 nM HIV-196ZM651 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-196ZM651 gp120 alone (table 1). These observations imply that JNK signaling is likely not involved in the release of TNF-α from primary cultures of rat astrocytes triggered with HIV-196ZM651 gp120.

In order to determine if 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 ng/ml 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 ng/ml TNF-α 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.

In order to investigate if pharmacological inhibition of JNK isoforms resulted in altered Mrp-mediated transport activity, we measured cellular retention of BCECF in cortical astrocyte monolayers triggered with HIV-196ZM651 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-196ZM651 gp120 or 10 ng/ml TNF-α for 24 h. In cultures treated with the JNK inhibitor, SP600125 (20 µM) was added 30 min prior to HIV-196ZM651 gp120 or TNF-α exposure. In cultured rat astrocytes treated with 1.0 nM HIV-196ZM651 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-196ZM651 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 following exposure to HIV-1 viral proteins or proinflammatory cytokines.

**Effect of HIV-196ZM651 gp120 and TNF-α on Mrp1 mRNA Expression**

Since 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-196ZM651 gp120
or TNF-α. Mrp1 mRNA was significantly increased (1.6-fold) in cultured astrocytes triggered with 1.0 nM HIV-196ZM651 gp120 for 6 h; however, Mrp1 expression was not altered in primary cultures of rat astrocytes exposed to HIV-196ZM651 gp120 for 12 h or 24 h (Fig 10A). Similarly, 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 concentrations. Until recently, ABC transporter functional expression had only been characterized in non-pathological (i.e., healthy) astrocyte cultures (Ronaldson et al., 2004). In order 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 cultured astrocytes with HIV-196ZM651 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 RT-PCR and ELISA respectively (Ronaldson and Bendayan, 2006).
Previous \textit{in vitro} and \textit{in vivo} studies have shown that cytokines (i.e., TNF-\(\alpha\), IL-1\(\beta\), IL-6) can alter Mrp1 expression (Lee and Piquette-Miller, 2003; Cherrington et al., 2004). In the context of HIV-1 associated inflammation, Jorajuria and colleagues reported increased TNF-\(\alpha\) and IL-6 production and increased expression of \textit{MRP1} mRNA in human monocyte-derived macrophages infected with HIV-1 BaL, an R5-tropic viral strain (Jorajuria et al., 2004). Using Spearman’s rank correlation test, these researchers concluded that \textit{MRP1} mRNA expression was not directly correlated with TNF-\(\alpha\) or IL-6 production (Jorajuria et al., 2004); however, a causal relationship between cytokine secretion and altered \textit{MRP1} mRNA levels was not established. In our study, we have directly triggered primary cultures of rat astrocytes with proinflammatory cytokines. While we observed no change in Mrp1 expression in cultured astrocytes triggered with IL-1\(\beta\) or IL-6, Mrp1 expression was increased in the presence of TNF-\(\alpha\) (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\textsubscript{96ZM651} gp120 in the presence of TNF-\(\alpha\), IL-1\(\beta\) or IL-6 neutralizing antibodies. Our results indicate that Mrp1 expression was not altered in the presence of TNF-\(\alpha\) neutralizing antibody but was significantly increased when IL-1\(\beta\) or IL-6 neutralizing antibodies were utilized. These data confirm that TNF-\(\alpha\) 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-\(\alpha\) and IL-1\(\beta\) (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.

In order to determine if 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. Since these transporters were either not expressed (i.e., Mrp2), nor affected by HIV-196ZM651 gp120 or TNF-α treatment (i.e., Mrp4) or not 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 attributed 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). Since 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 may also be involved in Mrp1 regulation. In the present study, we show that increased
Mrp1 expression induced by HIV-1<sub>96ZM651</sub> 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 AP-1, NFAT, STAT1, 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 NFAT at a concentration that was 5.6-fold lower than used by Torgerson and colleagues (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<sub>96ZM651</sub> gp120 in the presence and absence of SN50. Indeed, pre-treatment 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 (IκB) kinase β demonstrated a similar increase in hepatic Mrp1 mRNA levels as compared to 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 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 previously shown 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. Pharmacological inhibition of JNK signaling with SP600125 prevented up-regulation of Mrp1 expression in HIV-196ZM651 gp120 triggered astrocyte cultures. Pre-treatment with SP600125 attenuated up-regulation of Mrp1 in response to TNF-α exposure; however, SP600125 had no effect on HIV-196ZM651 gp120 induced release of TNF-α from rat astrocyte cultures. Furthermore, SP600125 prevented the increase in cellular BCECF efflux in cultures treated with HIV-196ZM651 gp120 or TNF-α. Our data corroborates the work of Hayashi and colleagues (2006) and implies 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 upregulation 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). Since we did not observe a change in Mrp1 protein expression in cultured astrocytes treated with HIV-196ZM651 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 shows increased Mrp1 mRNA expression in cultured astrocytes triggered with HIV-196ZM651 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). Additionally, 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-196ZM651 gp120 and/or proinflammatory cytokines.

In addition to inflammatory processes, oxidative stress is also involved in HIV-1 associated pathologies 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
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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 (Caccamo et al., 2005) and JNK MAPK (Chen et al., 2008). Additionally, 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). Clearly, the cellular response to HIV-1 viral proteins such as gp120 is 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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References


Footnotes

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Table 1: ELISA Analysis of TNF-α Secretion in Cultured Astrocytes Treated with HIV-196ZM651 gp120.

<table>
<thead>
<tr>
<th>Time of Exposure</th>
<th>HIV-196ZM651 gp120 (1.0 nM)</th>
<th>HIV-196ZM651 gp120 (1.0 nM) + SN50 (1.0 µM)</th>
<th>HIV-196ZM651 gp120 (1.0 nM) + SP600125 (20 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>583.08 ± 22.58</td>
<td>BDL **</td>
<td>570.43 ± 49.28</td>
</tr>
<tr>
<td>12 h</td>
<td>483.90 ± 32.81</td>
<td>BDL **</td>
<td>490.54 ± 36.43</td>
</tr>
<tr>
<td>24 h</td>
<td>384.71 ± 34.28</td>
<td>BDL **</td>
<td>369.56 ± 33.29</td>
</tr>
</tbody>
</table>

** p < 0.01; Results are expressed as mean ± SD of eight separate measurements obtained from different cultures on different days. Statistical comparisons are between cultures treated with HIV-196ZM651 gp120 alone and cultures triggered with HIV-196ZM651 gp120 plus inhibitor; Cytokine values are expressed as pg/ml; BDL= Values that are below detection limit of the assay. The limit of detection for the rat TNF-α ELISA used in this study is 15 pg/ml.
Figure Legends

**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 ng/ml; 10 ng/ml), IL-1β (0.4 ng/ml; 10 ng/ml), and IL-6 (0.3 ng/ml; 10 ng/ml) for 6 h, 12 h, 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 (% control) are expressed as mean ± SD of three separate experiments. Asterisks indicate data points that are significantly different from control.

**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 h, 12 h, 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 (% control) are expressed as mean ± SD of three separate experiments. Asterisks indicate data points that are significantly different from control. NAb = Neutralizing Antibody.

**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 ng/ml or 10 ng/ml TNF-α. Results are expressed as mean ± SD 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.

**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 ± SD 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 permeable 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 ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.

Fig. 5. Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and BAY 11-7082, a pharmacological NF-κB inhibitor. A: Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-196ZM651 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-196ZM651 gp120 in the presence and absence of 5 μM BAY 11-7082. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.

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 ng/ml 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 ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.

**Fig. 7. Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SP600125, a pharmacological JNK inhibitor.** Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-1gag 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 ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.

**Fig. 8. Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SP600125, a pharmacological 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 ± SD 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 ng/ml 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 ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.

**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-196ZM651 gp120 or 10 ng/ml TNF-α in the presence and absence of SP600125, a specific pharmacological JNK inhibitor. Results are expressed as mean ± SD 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.

**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-196ZM651 gp120 (A) or 10 ng/ml TNF-α (B) for 6 h, 12 h, or 24 h. Mrp1 mRNA expression was measured using quantitative PCR analysis. Results (% control) are expressed as mean ± SD of four separate experiments. Asterisks indicate data points that are significantly different from control.

**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-196ZM651 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.
Figure 1B

Graph showing Mrp1 Protein Expression (% Control) over time (6 h, 12 h, 24 h) for different concentrations of TNF-α (0.5 ng/ml and 10 ng/ml), IL-1β (0.4 ng/ml and 10 ng/ml), and IL-6 (0.3 ng/ml and 10 ng/ml). The graph includes error bars for each condition, with significant differences indicated by *** p < 0.001.
Figure 3
**Figure 4B**

The image shows a protein gel with molecular weight markers and bands labeled as Mrp1 and Actin. The gel also includes a bar graph representing Mrp1 protein expression in different conditions:

- Control
- 24 h gp120
- 24 h SN50
- 6 h gp120 SN50
- 12 h gp120 SN50
- 24 h gp120 SN50

The graph indicates a significant increase in Mrp1 expression in the 24 h gp120 condition compared to other treatments, as denoted by **p < 0.01**.
Figure 5B

B.

** p < 0.01

Mrp1 Protein Expression (% Control)

Control  24 h BAY 11-7082  24 h BAY 11-7082 + gp120  24 h gp120

**
Figure 6

The image shows a Western blot analysis with Mrp1 and Actin as markers. The graph represents the Mrp1 protein expression (as % of control) under different conditions:

- Control
- 1 mM SN50
- 0.5 ng/ml TNF-α
- 1 mM SN50 (6 h) + 0.5 ng/ml TNF-α
- 0.5 ng/ml TNF-α (12 h)
- 1 mM SN50 (24 h) + 10 ng/ml TNF-α
- 10 ng/ml TNF-α (6 h)
- 10 ng/ml TNF-α (12 h)
- 1 mM SN50 (24 h)

The graph includes error bars indicating variability. The significance level is marked with **p < 0.01.
Figure 9

** p < 0.05
Figure 10B

* $p < 0.05$
**Figure 11**

Diagram showing the pathway of HIV-1 gp120 interaction with TNF-α, TNFR, NF-κB, JNKs, and Mrp1 gene expression, leading to increased efflux transport activity.