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Morphine and HIV-Tat Increase Microglial Free Radical Production and Oxidative Stress: Possible Role in Cytokine Regulation

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Abstract

Opiate abuse alters the progression of HIV and may increase the risk of neuroAIDS. As neuroAIDS is associated with altered microglial reactivity, the combined effects of HIV-Tat and morphine were determined in cultured microglia. Specifically, experiments determined the effects of Tat and morphine on microglial free radical production and oxidative stress, and on cytokine release. Data show that combined Tat and morphine cause early and synergistic increases in reactive oxygen species, with concomitant increases in protein oxidation. Furthermore, combined Tat and morphine, but not Tat or morphine alone, cause reversible decreases in proteasome activity. The effects of morphine on free radical production and oxidative stress are prevented by pretreatment with naloxone, illustrating the important role of opioid receptor activation in these phenomena. While Tat is well-known to induce cytokine release from cultured microglia, morphine decreases Tat-induced release of the cytokines TNF α and IL-6, as well as the chemokine MCP-1. Finally, experiments using the reversible proteasome inhibitor MG115 show that temporary, non-cytotoxic decreases in proteasome activity increase protein oxidation and decrease TNF α , IL-6, and MCP-1 release from microglia. Taken together, these data suggest that oxidative stress and proteasome inhibition may be involved in the immunomodulatory properties of opioid receptor activation in microglia.

Keywords

cytokines; inflammation; opiates; proteasome; redox signaling

INTRODUCTION

Over 1 million people in the United States may be infected with HIV, and of those, 30-40% could eventually suffer from some manifestation of neuroAIDS (Grant et al. 2005), (Anthony et al. 2005). NeuroAIDS encompasses a spectrum of clinical neurologic disorders caused by HIV-induced damage and/or inflammation in the nervous system, and includes sensory neuropathies, myelopathies, behavioral/cognitive perturbations, and overt HIV dementia (McArthur et al. 2005), (Grant et al. 2005). Although the pathophysiological mechanisms

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underlying these neurologic syndromes are not understood, epidemiological data indicate that HIV infection, while a necessary precipitant for neuroAIDS, is not always sufficient to cause neuroAIDS. Altered immune reactions, particularly macrophage and microglial activation, are pathophysiological phenomena common to neuroAIDS syndromes (Glass et al. 1995), (Gray et al. 2001), (Tyor et al. 1995). The products of macrophage/microglial activation, including pro-inflammatory cytokines, excitotoxins, and reactive oxygen species (ROS), may play a significant role in the neuronal damage and dysfunction associated with neuroAIDS (Nelson et al. 2002), (Streit et al. 1999), (D'Aversa et al. 2005). However, microglia are also important players in beneficial local immune responses (Aloisi 2001), (Ling et al. 2001), (Streit et al. 2008), and the mechanisms underlying the phenotypical and functional transformation of these cells are still unclear. Interestingly, data suggest that ROS production, which is a characteristic feature of microglial activation both in vitro and in vivo, may control intracellular inflammatory signaling and thus regulate local immunology (Pawate et al. 2004), (Qin et al. 2004), (Droge 2002). For instance, microglial ROS have been specifically implicated in activation of NF κ B and synthesis of TNF α (Kaul and Forman 1996), which are proposed to be important mediators of HIV-related neuronal damage (Westendorp et al. 1995). Likewise, oxidative burst activity has also been shown to regulate microglial release of glutamate (Barger et al. 2007) and to participate in microglial-mediated neurotoxicity in experimental models of both Alzheimer's disease (Jana and Pahan 2004), and Parkinson's disease (Gao et al. 2003). Conversely, oxidative burst activity has been shown in some circumstances to limit inflammatory signaling in immune cells (Marikovsky et al. 2003), (Keenan et al. 2005), (Komatsu et al. 2006). Thus, oxidative reactions may potentially be an important regulator of CNS inflammation and local immunity, but this phenomenon has not been well studied in terms of HIV and neuroAIDS.

While it is still not understood why only selected AIDS patients develop neuroAIDS, it has been suggested that HIV infected individuals who also abuse opiate-based drugs may have an accelerated rate of overall AIDS progression (Donahoe and Vlahov 1998), (Bell et al. 2002), (Kapadia et al. 2005), (Lucas et al. 2006). Furthermore, opiate-addicted individuals, which may account for nearly one-third of HIV patients (UNAIDS 2006), have a higher incidence of dementia and may have more severe neurocognitive and pathological abnormalities (Bell et al. 1996), (Davies et al. 1997), (Nath et al. 2002). It is not yet clear how opiate abuse exacerbates neuroAIDS, but brains from opiate-abusing HIV patients appear to have increased numbers of macrophages/microglia (Bell et al. 2002), (Anthony et al. 2005) which may alter local inflammatory and/or immune reactions. Opioid receptors are present on both astrocytes and microglia (Ruzicka et al. 1995), (Chao et al. 1996), (Chao et al. 1997), (Stiene-Martin et al. 1998), and activation of opioid receptors by agonists such as morphine has been shown to dramatically alter glial immune responses (Rojavin et al. 1993), (Chao et al. 1997), (El-Hage et al. 2005), (Mahajan et al. 2005), (El-Hage et al. 2006). Overall, these data suggest that opiates could facilitate HIV-associated neurological dysfunction via alterations in local brain anti-viral responses. However, opiate-HIV interactions are still very poorly understood and the degree to which opiate abuse *per se* contributes to the progression to neuroAIDS is controversial (Donahoe 2001), (Barr et al. 2003), (Everall 2004). Thus, to better understand how opioid receptor stimulation might alter HIV-induced immune signaling in microglia, the modulatory effects of morphine and HIV-Tat on microglial ROS production, oxidative stress, and inflammatory cytokine release were measured in cultured microglial cells. Specifically, following exposure to Tat in the presence and absence of morphine, oxygen free radical production was measured in N9 microglial cells and in primary rodent microglia using fluorescent and luminescent dyes. Additionally, oxidative stress was measured by quantifying the time-dependent accumulation of protein carbonyls and the degree of proteasome inhibition. Finally, the role of proteasome inhibition in cytokine regulation was determined by measuring cytokine release from microglia following application of combinations of Tat, morphine, and/or the reversible proteasome inhibitor MG115.

MATERIALS AND METHODS

Preparation of Tat

Recombinant Tat 1-72 was produced and purified as described previously (Chen et al. 1997). Briefly, the Tat gene encoding the first 72 amino acids of HIV-1BRU (obtained from Dr. Richard Graynor, through the NIH AIDS repository) was inserted into an *Escherichia Coli* vector Pin Point Xa-2 (Promega, Madison, WI). Biotinylated Tat was purified on a column of soft release avidin resin, cleaved from the fusion protein using factor Xa, eluted and desalted using a PD10 column, and treated with Pierce Detoxi-Gel (Pierce). A reticulocyte amoebocyte lysate assay was used to assay for trace endotoxin (Associates of Cape Cod, Inc.; sensitivity < 1 pg/ml), and any preparations that contained detectable levels of endotoxin were discarded. The protein concentrations of the resultant preparations were determined, and the solutions were aliquoted, lyophilized, and stored at -80°C.

Cell culture and treatment

N9 murine microglial cells (kindly provided by Dr. Paola Ricciardi-Castagnoli, Consiglio Nazionale Delle Ricerche, Milano, Italy) were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL, Gaithersburg, MD) supplemented with 5% heat-inactivated FBS (Sigma, St. Louis MO). All experiments were conducted on cells at 70-80% confluence in phenol red-free, serum-free medium.

Primary microglia were isolated from mixed glial cultures derived from neonatal Sprague-Dawley rat pups as described previously (Bruce-Keller et al. 1999). Briefly, mixed glial cultures were generated from the brain tissues (cerebral cortices without meninges) and maintained in Modified Eagle Medium (Gibco BRL) with 10% fetal bovine serum. After 7-10 days *in vitro*, microglia were isolated from confluent mixed glial cultures by differential panning (45 min at 200 rpm on an orbital shaker). Cells were pelleted and subcultured at 1×10^5 cells/ml, and used within 24 h after subculturing. Such cultures generally contain primarily microglia with very few type I or II astrocytes (less than 5% of total cells).

Analyses of ROS production

The intracellular formation of ROS was detected by using the fluorescent probes DHE, DCFDA, and DHR-123, which can reflect the presence of a variety of reactive oxygen species (reviewed in (Halliwell and Whiteman 2004)). Briefly, cells were plated into 96 well plates at 1×10^5 /ml, and switched to serum-free medium the day after plating for 12-18 hrs before treatment. Cells were then loaded with dyes (10 μ M, made fresh before each use) for 45 min at 37°C, then rinsed with HBSS and exposed to morphine and/or Tat for 45 min, and then analyzed using fluorescent plate reader with the following settings: DHE: λ_{ex} =488 nm, λ_{em} =567 nm; DCFDA: λ_{ex} =488 nm, λ_{em} =525 nm; and DHR-123: λ_{ex} =500 nm, λ_{em} =536 nm. In experiments in which naloxone was used to assess the role of opioid receptors in the effects of morphine, 3 μ M naloxone was applied 60 min before morphine. In each experiment, the background fluorescence was accounted for by subtracting out the fluorescence intensity recorded in control wells that contained no cells. Additionally, the NADPH-dependent production of superoxide in N9 cells was measured by documenting MCLA luminescence. Briefly, following treatment, cells were lysed by 3 freeze-thaw cycles and then brought up in protease inhibitor-containing PBS at 4°C. Samples (10-25 μ g total protein) were incubated with 5 μ M MCLA and 100 μ M NADPH. NADPH oxidase activity was measured immediately using a luminescence plate reader at 37°C. To verify the amount of luminescence specifically caused by NADPH oxidase, background levels of luminescence (generated by measuring luminescence in the presence of 1 μ M of the NADPH oxidase inhibitor diphenylene iodonium) were subtracted from each sample.

Protein oxidation

Protein oxidation was determined by dot blot analysis using a commercially available OxyBlot protein carbonyl detection system (Chemicon), following the manufacturers' instruction as described previously (Keller et al. 2005). This assay is based on the immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenyl hydrazine. All samples were performed in triplicate, and blot images were scanned and densitometrically analyzed using NIH image software.

Proteasome activity

Analyses of proteasome activity were conducted by monitoring the rates of cleavage for peptides specific for individual proteasome activities as described previously (Keller et al. 2002), (Ding et al. 2003). Briefly, cell lysates were collected and aliquots (1 µg/µl protein) were generated in proteasome activity buffer (10 mmol/l Tris-HCl (pH 7.8), 1 mmol/l EDTA, 0.5 mmol/l dithiothreitol, and 5 mmol/l MgCl₂). The fluorogenic peptide suc-Leucine-Leucine-Valine-Tyrosine-MCA (Bachem; Torrance, CA) was used to test the chymotrypsin-like activity of the proteasome. A standard curve of free 7-amido-4-methylcoumarin (MCA) was utilized for quantification of proteasome activity, and fluorescence was monitored at 340 nm excitation and 440 nm emission and the rate of activity was determined over the course of 1 hr. As an additional means to determine background, additional samples in each experiment were treated with the proteasome inhibitor MG262 (1 µM) for 30 min prior to the addition of proteasome substrates.

ELISA

Levels of IL-6, MCP-1, and TNFα in cell culture medium were measured by ELISA as described previously (Bruce-Keller et al. 2001). In each case, a dose-response and time-course curve was generated, and the threshold sensitivity was typically 1pg/ml.

Cytotoxicity indices

Cell viability in primary microglia was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) conversion as described previously (Ding and Keller 2001), (Ding et al. 2002), (Keller et al. 1998). Briefly, after treatment *in vitro*, cells were exposed to 0.5 mg/ml MTT in serum-free and phenol red-free medium for 1-4 h at 37°C. Following exposure, medium was aspirated, and formazan precipitates were extracted by addition of 100 µl dimethylsulfoxide. The amount of formazan formation was analyzed within an hour by determination of optical density at 570 nm with reference wavelength of 630 nm on a spectrophotometric UV/VIS plate reader (Molecular Devices, Sunnyvale, CA). At least eight cultures were utilized for each data point.

N9 cells were assayed for apoptosis using a modified version of a well-established protocol (Reid et al. 1996), (Laakko et al. 2002) using dual staining for live cells with the DNA stain Hoechst 33342 (Sigma) and Merocyanin 540 (Molecular Probes), which binds to the outer membrane of cells and readily fluoresces in the highly disordered membranes of apoptotic cells. Briefly, cells were suspended in 0.9 ml phenol-red free IMDM-2% FBS to which 100 µl of 50 µg/ml Hoechst 33342 was added and incubated for 30 min at 37°C. Cells were then washed and resuspended in 100 µl medium, after which Merocyanin 540 (0.5 µg/ml final concentration) was added to each tube and incubated at room temperature in the dark for 20 min. Cells were then washed again, and cell suspensions were stored on ice until data acquisition on a MoFlo cytometer with the argon laser set at 488 nM to detect MC540 and the UV laser excitation at 351 nM to detect Hoechst from 3×10^4 N9 cells from each sample.

Statistical analyses

All data were analyzed using one-way or two-way ANOVA, followed by Bonferroni's post-hoc analysis, respectively, to determine statistical significance; *p* values < 0.05 were designated as statistically significant, and are indicated in the text as *, **, or *** corresponding to *p* values < 0.05, < 0.01, or < 0.001, respectively.

RESULTS

1. Morphine and Tat increase ROS production in microglia

Previous reports have shown that HIV-Tat can induce or augment free radical production in cultured cells (Westendorp et al. 1995), (Polazzi et al. 1999), (Sheng et al. 2000), (Price et al. 2006) in a manner that is specific to the recombinant Tat protein and not based on endotoxin contaminants (Bruce-Keller et al. 2001). As opioid receptor stimulation has been shown to increase ROS in cultured cells (Wang et al. 2005), initial experiments were designed to evaluate if morphine modulates HIV-Tat-induced ROS production in microglia. Cultured N9 microglial cells were grown and preloaded with the ROS-sensitive dyes DHE, DCFDA, and DHR-123 in serum-free conditions as described in Methods. These dyes are relatively nonselective probes that react with ROS in the cytosolic, nuclear, mitochondrial compartments (Halliwell and Whiteman 2004). Following dye-loading and washing, the cells were exposed to increasing doses of HIV-Tat 1-72 in the presence or absence of 500 nM morphine sulfate for 45 min in serum-free medium, after which dye oxidation was measured as described in Methods. Data confirm the ability of Tat to dose-dependently increase ROS production as measured by both DHE and DCFDA (Fig. 1A and 1B). Conversely, morphine did not affect the oxidation of any dye when applied alone (Fig 1A, 1B, and 1C). However, morphine co-treatment did significantly and synergistically increase Tat-induced oxidation of both DHE and DCFDA, and combined Tat/morphine treatment also induced significant increases in the oxidation of DHR-123 (Fig 1A, 1B, and 1C). Finally, pretreatment of N9 cells with 3 μ M naloxone completely prevented the effects of morphine, but not Tat, on ROS production (Fig 1A, 1B, and 1C), indicating that the effects of morphine were opioid receptor-dependent.

Similar experiments were undertaken in cultured primary microglia to verify that the effects of Tat and morphine on ROS production exist in primary microglia as well as in immortalized cell lines. Primary rat microglia were isolated from mixed glial cultures, plated into 96-well plates, and loaded with the ROS-sensitive dyes DCFDA and DHR-123 as described in Methods. In place of DHE, which is generally used to indicate the presence of superoxide anion, the superoxide-dependent oxidation of MCLA was measured in primary microglia. Data show that, as in N9 cells, 50 nM Tat increased the production of ROS as measured by MCLA and DCFDA, but not DHR-123 (Fig 1D, 1E, and 1F). Moreover, morphine significantly and synergistically increased Tat-induced ROS production as measured by oxidation of MCLA, DCF-DA, and DHR-123, but did not affect dye oxidation when applied alone (Fig 1D, 1E, and 1F). Finally, pretreatment of microglia with naloxone completely prevented the effects of morphine, but not Tat, on ROS production (Fig 1D, 1E, and 1F), again confirming that the effects of morphine in microglia were opioid receptor dependent.

2. Morphine increases Tat-induced oxidative stress in microglia

To determine if morphine-induced ROS were associated with measurable oxidative stress in microglia, overall protein oxidation was measured by quantifying the accumulation of protein carbonyls. Proteins are carbonylated by direct oxidation of amino-acid side chains, and carbonyls can also be formed by protein glycation and/or by the binding of reactive aldehydes (Halliwell and Whiteman 2004). The accumulation of protein carbonyls is routinely measured in laboratory settings by derivatization of samples with 2,4-dinitrophenyl hydrazine followed by immuno-based detection of the attached DNP moieties (Keller et al. 2005), (Smith et al.

1998). Therefore, this system was used to document the temporal profile of protein oxidation in N9 cells following treatment with Tat in the presence or absence of morphine as described in Methods. Data show that exposure to either 50 nM Tat or 500 nM morphine alone did not result in measurable increases in protein oxidation over 24 hrs of exposure (Fig. 2A). However, combined Tat/morphine treatment caused significant but reversible increases in protein carbonylation after 3 and 6 hrs of exposure (Fig. 2A). Experiments were then repeated in primary microglia, in which the same time-dependent, reversible pattern of protein oxidation following combined Tat/morphine exposure was observed (data not shown). To then determine the role of opioid receptors in the effects of morphine on protein carbonylation, selected primary microglia were pretreated with 3 μ M naloxone before a 6-hr application of Tat and morphine. Data show that 6 hrs of combined Tat and morphine, but not Tat or morphine alone, increased protein carbonylation in primary microglia (Fig. 2B). Moreover, while naloxone alone did not affect basal protein carbonylation, pretreatment with naloxone completely prevented the effects of combined Tat/morphine on protein oxidation (Fig. 2B), indicating that morphine-induced increases in oxidative stress are opioid receptor-dependent.

The disposition of intracellular oxidized proteins is generally mediated by the proteolytic activity of the multi-catalytic proteasome (Grune et al. 1996), (Ding et al. 2003). Furthermore, the proteasome has been highlighted as a key intracellular system that might regulate inflammatory signaling in immune cells (Hayashi and Faustman 2000), (Goldberg et al. 2002), (Borissenko and Groll 2007). As increases in protein oxidation following Tat and morphine exposure were reversible in microglia, the temporal profile of proteasome activity in N9 cells following treatment with Tat in the presence or absence of morphine was measured as described in Methods. Data show that exposure to 500 nM morphine did not result in measurable alterations to chymotrypsin-like activity in N9 microglia over 24 hrs of exposure (Fig. 3A). However, the application of 50 nM Tat caused a reversible decrease in proteasome activity at 1 hr after exposure (Fig. 3A). Moreover, combined Tat/morphine caused significant but reversible decreases in proteasome activity after 1 and 3 hrs of exposure that were no longer significantly different from control after 8 hrs of exposure (Fig. 3A). Experiments in primary microglia were then designed to verify that similar effects were observable in primary cells, and to determine the role of opioid receptors in the effects of morphine. Data show that a 1-hr exposure to 50 nM Tat caused a moderate but significant decrease in chymotrypsin-like activity in primary microglia, while morphine alone did not affect proteasome activity in primary microglia (Fig. 3B). However, combined Tat and morphine caused synergistic and significant decreases in proteasome activity after 1 hr of exposure (Fig. 3B). Finally, while naloxone alone did not affect proteasome activity in control or Tat-treated microglia, pretreatment with naloxone completely prevented the effects of combined Tat/morphine on protein oxidation (Fig. 3B), indicating that morphine-induced decreases in proteasome activity are opioid receptor-dependent.

3. Morphine decreases Tat-induced cytokine and chemokine release from microglia

To next determine if opiate-based drugs might alter cell-to-cell inflammatory signaling from microglia in the context of HIV infection, the effects of morphine on Tat-induced cytokine release was measured. N9 cells were treated with increasing doses of Tat for 24 hrs, and data show that Tat caused significant, dose-dependent increases in the release of the cytokines TNF α and IL-6, and the chemokine MCP-1 from N9 cells (Fig. 4A, 4B, and 4C). However, co-treatment with 500 nM morphine significantly attenuated Tat-induced release of all 3 inflammatory mediators (Fig. 4A, 4B, and 4C). Preliminary dose-response experiments indicated that while 100 nM, 250 nM, 500 nM, and 1 μ M morphine were all effective in lowering Tat-induced cytokine release, 500 nM was the most effective dose (data not shown). To document the role of opioid receptors in morphine-regulated cytokine and chemokine release, selected cells were pretreated with 3 μ M naloxone before morphine application. Data

show that while morphine-induced decreases in TNF release were reversed in part by naloxone (Fig 4A), naloxone treatment alone was rather effective at suppressing Tat-induced cytokine release (Fig. 4A, 4B, and 4C). One possible explanation for this unexpected finding is that endogenous opioid peptides released from microglia might act through autocrine feedback mechanisms to affect cytokine release over a 24-hr application of Tat. Especially with stress or infection, primary monocyte-derived macrophages can express Met- and Leu- enkephalins, which are preferentially δ opioid receptor agonists (Przewlock et al. 1992), (Ovadia et al. 1996), (Machelska and Stein 2006) and also proopiomelanocortin, a preferential μ opioid receptor agonist (Lolait et al. 1986), (Mousa et al. 2004) genes. Even prodynorphin gene products, such as dynorphin A and B, have recently been reported to be expressed in U-937 macrophage cells (Sun et al. 2006).

To confirm that the effects of morphine on Tat-induced cytokine/chemokine release were not related to the use of immortalized cell lines, primary rat microglia were grown as described in Methods, and treated with Tat and/or morphine for 24 hrs. Data show that Tat induced the release of the cytokines TNF α and IL-6, and the chemokine MCP-1 from N9 cells (Fig. 4D, 4E, and 4F) and verified that co-treatment with 500 nM morphine significantly decreased Tat-induced release of all three inflammatory mediators from primary microglia (Fig. 4D, 4E, and 4F). Finally, as was observed in N9 cells, naloxone treatment generally suppressed Tat-induced cytokine release (Fig. 4D, 4E, and 4F).

4. Proteasome inhibition increases oxidative stress and decreases cytokine and chemokine release from microglia

As proteasome inhibition has been associated with attenuated inflammatory responses in antigen-presenting cells (Kalogeris et al. 1999), (Nencioni et al. 2006), (Ortiz-Lazareno et al. 2008), experiments were designed to determine what relevance the temporary decreases in proteasome activity might have on subsequent cytokine release following Tat application. To this end, primary rodent microglia were treated with MG115 in the presence or absence of Tat. MG115 is a potent, reversible proteasome inhibitor that specifically inhibits the chymotrypsin-like activity of the proteasome (Fu et al. 2002), (Ding et al. 2006). Preliminary experiments confirmed that 1 μ M MG115 treatment caused significant (~40%) decreases in chymotrypsin-like proteasome activity in primary microglia at 3 and 6 hrs, returning to baseline after 24 hrs (data not shown). To next determine the effects temporary proteasome inhibition on microglial protein oxidation, the time course of protein carbonyl accumulation was determined in microglia following MG115 exposure. Data show that MG115 caused highly significant increases in protein carbonylation after 3 hrs of exposure, with levels peaking at 12 hrs and returning to baseline after 24 hrs (Fig. 5A). It was then determined if MG115 could modulate Tat-induced cytokine release in a manner similar to morphine. Primary microglia were thus exposed to 10 nM Tat in the presence or absence of 1 μ M MG115 for 24 hrs in serum-free medium, after which the accumulation of the cytokines TNF α and IL-6, and the chemokine MCP-1 in the media was documented. ELISA data show that co-treatment with 1 μ M MG115 significantly reduced Tat-induced release of all 3 inflammatory mediators (Fig. 5B, 5C, and 5D).

The final set of experiments was designed to document the effects of Tat, morphine, and/or MG115 on microglial viability to determine if the decreased cytokine release following Tat/morphine or Tat/MG115 application was secondary to a decrease in cell number. To determine if combined Tat and morphine could induce apoptosis in N9 cells, cells were exposed to increasing doses of Tat in the presence or absence of morphine for 24 hrs in serum-free medium, after which apoptosis was determined using flow cytometry as described in Methods. Data show that no measurable increases in apoptosis could be detected in N9 cells following exposure to Tat either in alone or with morphine (Fig. 6A). Conversely, 24 hrs of 100 U/ml

IFN, known to cause apoptosis in microglia (Badie et al. 2000), (Takeuchi et al. 2006), elicited a significant, near 3-fold increase the apoptosis in N9 cells (Fig. 6A). To likewise confirm that the survival of primary microglia was not compromised by exposure to Tat, morphine, or MG115, primary microglia were isolated as described in Methods, and were exposed to 50 nM Tat, 500 nM morphine, or 1 μ M MG115 alone or in combination for 24 hrs in serum-free medium. Microglial viability was then measured using MTT as described in Methods, using combined ferrous chloride and ascorbic acid as a positive control. While iron/ascorbate did induce significant decreases in MTT conversion in primary microglia, microglial survival was completely unaffected by the various combinations of Tat, Morphine, and/or MG115 (Fig. 6B).

DISCUSSION

Data in this manuscript document the specific and biologically significant effects of morphine on the inflammatory properties of HIV-Tat on cultured microglia. Specifically, data show that morphine significantly and synergistically increases Tat-induced ROS production in cultured N9 and primary microglia. Furthermore, morphine and Tat combined but not alone cause significant but reversible increases in protein oxidation and decreases in proteasome activity in microglia. Additionally, while Tat is well known to elicit the release of neuroactive cytokines from cultured monocytic cells (Bruce-Keller et al. 2001), data in this manuscript demonstrates that Tat-induced cytokine and chemokine production is significantly attenuated by morphine co-treatment. Finally, data show that temporary, subtoxic proteasome inhibition is sufficient to increase Tat-induced protein oxidation, and to decrease Tat-induced cytokine/chemokine release. Collectively, these data support a specific and central role for the proteasome in regulating microglial inflammatory signaling, and suggest that morphine-induced increases in oxidative stress may participate in the typically suppressive effects of morphine on immune responses. Thus, this study also suggests that morphine-associated increases in microglial oxidative stress could disrupt microglial responses to HIV proteins, leading to deficits in local anti-viral CNS responses which could potentially facilitate the development of neuroAIDS.

Opioid receptors are divided into three major classes of seven transmembrane, G protein-coupled receptors, and mu, delta, and kappa opioid receptors are present on microglia (Chao et al. 1996), (Chao et al. 1997), (Turchan-Cholewo et al. 2008). Furthermore, activation of opioid receptors by agonists such as morphine has been shown to dramatically alter glial immune responses (Rojavin et al. 1993), (Chao et al. 1997), (El-Hage et al. 2005), (Mahajan et al. 2005), (El-Hage et al. 2006). Data in this manuscript illustrate a suppressive effect of morphine on microglial cytokine release, which is in general agreement with a number of studies showing that the use or abuse of morphine can have adverse effects on immune function (McCarthy et al. 2001), (Walker 2003), (Wang et al. 2005). For example, several studies have shown that exposure to opioids can compromise immune responses to bacterial infection and lead to immunosuppression (Risdaal et al. 1998), (MacFarlane et al. 2000), (Bhaskaran et al. 2001), (Ocasio et al. 2004), (Roy et al. 2006). With specific reference to CNS immunity, treatment of microglial cells with specific opioid ligands has been shown to alter their phagocytic (Peterson et al. 1998), chemotactic (Chao et al. 1997), and free radical producing (Hu et al. 1998), (Liu et al. 2002) properties. As microglia are the major immunocompetent cells in the brain, these data suggest that morphine-mediated suppression of microglial immune responses could predispose the brain to infection. Thus, a potential mechanism whereby opiates increase the neurological complications of HIV could be through the facilitation of brain-resident opportunistic infections, which could aggravate the progression of AIDS (Quang-Cantagrel et al. 2001). Conversely, morphine exposure has also been shown to directly facilitate HIV infection and accelerate HIV replication (Peterson et al. 1990), (Peterson et al. 1994), (Wang et al. 2006), (Kumar et al. 2006). Thus, activation of opioid receptors in the brain of AIDS patients could precipitate neurological abnormalities via increases in CNS levels of HIV, and/or increases in opportunistic infections. Interestingly, previous reports from our labs show

that co-application of Tat prevents morphine-induced downregulation of cell surface mu opioid receptors in microglia (Turchan-Cholewo et al. 2008). These data suggest that the presence of Tat could exacerbate MOR-based signaling by effectively increasing cell surface receptors. Thus, if the typical actions of morphine in the brain include suppression of innate anti-bacterial and anti-viral microglial immunity, and if Tat acts to increase the availability of surface opioid receptors to morphine, then these data raise the possibility that the combination of Tat and morphine could synergistically accelerate the already detrimental actions of morphine.

While the exact mechanism(s) of morphine-mediated immunosuppression is not known, morphine has been reported to increase oxidative stress in immune cells (Chao et al. 1994), (Bhat et al. 2004) which is in agreement with data reported in this paper. This is interesting in light of the many reports that suggest that ROS production in microglia triggers inflammatory signaling (Pawate et al. 2004), (Qin et al. 2004), (Droge 2002). However, this relationship is not completely clear as many reports also document that oxidative stress can blunt inflammatory signaling in immune cells (Marikovsky et al. 2003), (Keenan et al. 2005), (Komatsu et al. 2006), particularly in response to high concentrations of ROS (Li and Engelhardt 2006). Thus, it is possible that the relationship between ROS production and inflammatory signaling in microglia may follow an inverse U-shaped curve. Furthermore, data in this manuscript suggest that the proteasome may be a point of intersection between intracellular oxidative stress and transcellular inflammatory signaling. More specifically, the present data suggest that oxidative stress-induced proteasome inhibition may represent a threshold of sorts, and that unimpeded activity of the proteasome is necessary for cytokine/chemokine induction. In support of this scenario, published reports have documented that the proteasome is necessary for the physiologic maintenance of protein pools in activated microglia, and is specifically responsible for the disposition of proteins oxidized during activation (Mehlase et al. 2000), (Ullrich et al. 2001). Furthermore, proteasome inhibition, which can occur in association with oxidative stress (Ding and Keller 2001), (Ding et al. 2003), is well known to inhibit inflammatory signaling (Fine et al. 1999), (Sappington and Calkins 2006). Thus, it seems quite logical to propose that proteasome inhibition might mediate the immunosuppressive effects of combined Tat and morphine. This theory is also supported by the large body of literature documenting that Tat itself is capable of directly interacting with the proteasome in antigen presenting cells (Apcher et al. 2003). For instance, Tat has been shown to decrease antigen processing by inhibiting proteasome activity, modulating proteasome subunit composition, and by preventing binding of the 20S proteasome with the PA28 cap (Huang et al. 2002), (Gavioli et al. 2004), (Remoli et al. 2006). Interestingly, morphine has also been shown to induce alterations in ubiquitin binding and on proteasome activity and subunit composition in vitro (Moulédous et al. 2005), (Rambhia et al. 2005), (Yang et al. 2008). More specifically, data demonstrate that morphine directly decreases proteasome activity neuroblastoma cells, and also decreases expression of the “immunoproteasome” subunit LMP7 in response to inflammatory stimulation (Rambhia et al. 2005). Thus, it is possible that the immunomodulatory properties of morphine could be based in part on direct actions on the proteasome, rather than or in addition to indirect effects caused by oxidative stress.

It is important to note that while the presence of activated microglia and macrophages has been repeatedly documented in neuroAIDS, exact cause and effect relationships are not fully understood. While microglial-mediated neuronal injury was not specifically addressed in this manuscript, there are several mechanisms whereby increased oxidative stress in microglia could preferentially evoke neuronal injury. A compelling and novel theory involves the manipulation of extracellular glutamate by activated microglia. While microglia express glutamate transporters (Nakajima et al. 2001), these cells also release significant levels of glutamate via a membrane associated cystine-glutamate anti-porter system (Xc) whose expression is significantly upregulated in activated cells (Piani et al. 1992). Indeed, as

upregulation of the Xc antiporter in immunocompetent cells is thought to be a self-protective mechanism to increase intracellular cysteine and glutathione so that the cells are not damaged by their own oxidative burst, it can be proposed that any stimulus that increases microglial ROS production and oxidative stress will also increase glutamate release. In support of this potential scenario, a series of elegant reports have indeed shown that microglial activation in general, and free radical production in particular, is associated with the release of neurotoxic levels of glutamate (Barger and Basile 2001), (Barger et al. 2007). Alternatively, although not addressed in this manuscript, it is possible that significant aspects of HIV-induced brain inflammation originate not just from microglia, but also from astrocytes, which have been shown to respond to opiates with increased inflammatory signaling (El-Hage et al. 2005), (Mahajan et al. 2005). Finally, different doses or treatment regimens (specifically pretreatment) of morphine may differentially modulate microglial inflammatory responses, both alone and in the presence of astrocytes. Thus, ongoing studies are under development to resolve these important temporal and spatial complexities of opiate-mediated actions on the various cell types in the CNS.

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Abbreviations

DHE, dihydroethidium; DCFDA, 2',7'-dichlorofluorescein diacetate; DHR-123, dihydrorhodamine 123; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IMDM, Iscove's Modified Dulbecco's Medium; MCLA, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo [1,2- α]pyrazin-3-one; RLU, relative light unit; ROS, reactive oxygen species..

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Figure 1. Effects of morphine on Tat-induced ROS formation in microglia

Cultured N9 cells (N9: **A, B, and C**) or cultured primary rat microglia (PMG: **D, E, and F**) were grown as described in Methods, and loaded with DHE (**A**), DCF-DA (**B and E**), and DHR-123 (**C and F**) for 45 min. Cells were then rinsed and treated with Tat (10 -50nM) in the presence or absence of 500 nM morphine and/or 3 μ M naloxone (added 60 min before morphine) for 1 hr, after which fluorescence was read using a plate reader. 10 μ M PMA (45 min) was used as positive control in N9 cells. To measure NADPH oxidase in primary microglia (**D**), cultured cells were treated for 45 min with Tat in the presence or absence of 500 nM morphine and/or 3 μ M naloxone. Cells were then harvested, lysed, and NADPH oxidase activity was measured. Data were obtained from 3 separate experiments with 6-8 samples per

group in each experiment, and were analyzed by 1-way ANOVA, and ++ and +++ indicate statistically significant increases ($p > 0.01$ and $p > 0.001$, respectively) in ROS production in Tat-treated cells compared with control treated cells. * and *** indicate statistically significant differences ($p > 0.05$, and $p > 0.001$, respectively) in ROS cytokine levels between Tat-treated and Tat/morphine-treated cells, while # and ### indicate statistically significant differences ($p > 0.05$, and $p > 0.001$, respectively) between Tat/morphine- and Tat/morphine/naloxone-treated cells.

Figure 2. Effects of Tat and morphine on protein oxidation in microglia

(A) N9 microglia were exposed to Tat in the presence or absence of morphine for 3, 5, and 24 hrs. Cells were then harvested, lysed, and the accumulation of protein carbonyls was measured by dot blot as described in the Methods. Data were analyzed by 2-way ANOVA, and *** indicates statistically significant increases ($p < 0.001$) in protein carbonyls in cells treated with combined Tat and morphine compared to control cells. (B) Primary rodent microglia were treated with 50 nM Tat for 4 h, and additional cells were treated with Tat in the presence of 500 nM morphine or 3 μ M naloxone and morphine. Cells were then harvested, lysed, and the accumulation of protein carbonyls was measured by dot blot. Data were obtained from 4 separate experiments with 6 samples per group in each experiment, and were analyzed by 1-

way ANOVA, and *** indicates statistically significant increases ($p < 0.001$) in protein carbonyls in cells treated with combined Tat and morphine compared to cells treated only with Tat, while # indicates the statistically significant decrease ($p < 0.05$) in protein oxidation in cells treated with combined Tat, morphine and naloxone compared to cells treated with just Tat and morphine.

Figure 3. Effects of Tat and morphine on proteasome activity in microglia

(A) N9 microglia were exposed to Tat in the presence or absence of morphine for 1, 3, 8, and 24 hrs. Cells were then harvested, lysed, and chymotrypsin-like proteasome activity was measured enzymatically. Data were obtained from 4 separate experiments with 6 samples per group in each experiment, and were analyzed by 2-way ANOVA, and *, ++, and *** indicate statistically significant decreases ($p > 0.05$, $p > 0.01$, and $p > 0.001$, respectively) in chymotrypsin activity in Tat and Tat-morphine treated cells compared to control cells. (B) Primary rodent microglia were treated with 50 nM Tat for 1 h, and additional cells were treated with Tat and/or 500 nM morphine and/or 3 μ M naloxone. Cells were then harvested, lysed, and chymotrypsin-like proteasome activity was measured enzymatically. Data were obtained

from 2 separate experiments with 5 samples per group in each experiment, and were analyzed by 1-way ANOVA, and + and *** indicate statistically significant decreases ($p>0.05$ and $p>0.001$, respectively) in chymotrypsin-like proteolytic activity in cells treated with Tat or combined Tat and morphine compared to control cells, while # indicates the statistically significant increase ($p<0.05$) in proteasome activity in cells treated with combined Tat, morphine and naloxone compared to cells treated with just Tat and morphine.

Figure 4. Effects of morphine on Tat-induced cytokine release from microglia
 Cultured N9 cells (N9: **A, B, and C**) or cultured primary rat microglia (PMG: **D, E, and F**) were treated with Tat (10 -50nM) in the presence or absence of 500 nM morphine and/or 3 μ M naloxone apocynin for 24 h, after which the medium was harvested, centrifuged, and analyzed for levels of TNF α (**A and D**), IL-6 (**B and E**), and MCP-1 (**C and F**). Data were obtained from 5 separate experiments with 4-6 samples per group in each experiment, and were analyzed by 1-way ANOVA, and *** indicates statistically significant increases ($p > 0.001$) in cytokine/chemokine release from Tat-treated cells compared to control treated cells. +++ indicates statistically significant decreases ($p > 0.001$) in cytokine release from cells treated with 50 nM Tat or morphine combined with 50 nM Tat, while # indicates the statistically significant

difference ($p>0.05$) between TNF release from N9 cells treated with combined Tat/morphine compared to Tat/morphine/naloxone-treated cells.

Figure 5. Effects of proteasome inhibition on protein oxidation and on Tat-induced cytokine/chemokine release from primary microglia

(A) Primary rodent microglia were treated with 1 μ M MG115 Tat for increasing amounts of time in serum-free medium, after which they were harvested, lysed, and the accumulation of protein carbonyls was measured by dot blot. Data were obtained from 3 separate experiments with 4 samples per group in each experiment, and were analyzed by 1-way ANOVA, and *** indicates statistically significant increases ($p < 0.001$) in protein carbonyls in cells treated with MG115 compared to control cells. Alternatively, primary microglia were treated with 50 nM Tat, 1 μ M MG115, or combined Tat/MG115 for 24 hrs, after which the medium was harvested, centrifuged, and analyzed for levels of TNF α (B), IL-6 (C), and MCP-1 (D). Data were obtained from 2 separate experiments with 6 samples per group in each experiment, and were analyzed by ANOVA, and *** indicates statistically significant increases ($p < 0.001$) in cytokine/chemokine release from cells treated with Tat as compared to vehicle-treated (control) microglia. # indicates statistically significant decreases ($p < 0.05$) in Tat-induced cytokine/chemokine release from primary microglia treated with Tat in the presence of MG115.

Figure 6. Effects of Tat, morphine, and MG115 on microglial survival

(A) N9 microglia were exposed to increasing doses of Tat in the presence or absence of 500 nM morphine for 24 hrs. 100 U/ml IFN γ was used as a positive control. Cells were then harvested, and analyzed for apoptosis using flow cytometry. (B) Primary rodent microglia were treated with 50 nM Tat, 500 nM morphine, or 1 μ M MG115, either alone or in combination, for 24 hrs. Combined ferrous sulfate and ascorbic acid was used as a positive control. Cells were then treated with 0.5 mg/ml MTT for 2 hrs, after which the medium was aspirated, the formazan precipitates dissolved in DMSO, and the amount of formazan conversion analyzed at 570 nm.