Glutamate metabolism in HIV-infected macrophages: implications for the CNS

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Glutamate metabolism in HIV-infected macrophages: implications for the CNS. Am J Physiol Cell Physiol 291: C618–C626, 2006. First published May 10, 2006; doi:10.1152/ajpcell.00021.2006.—Central nervous system disorders are still a common complication of human immunodeficiency virus (HIV) infection and can lead to dementia and death. They are mostly the consequences of an inflammatory macrophagic activation and relate to glutamate-mediated excitotoxicity. However, recent studies also suggest neuroprotective aspects of macrophage activation through the expression of glutamate transporters and glutamine synthetase. We thus aimed to study whether HIV infection or activation of macrophages could modulate glutamate metabolism in these cells. We assessed the effect of HIV infection on glutamate transporter expression as well as on glutamate uptake by macrophages and showed that glutamate transport was partially decreased in the course of virus replication, whereas excitatory amino acid transporter-2 (EAAT-2) gene expression was dramatically increased. The consequences of HIV infection on glutamine synthetase were also measured and for the first time we show the functional expression of this key enzyme in macrophages. This expression was repressed during virus production. We then quantified EAAT-1 and EAAT-2 gene expression as well as glutamate uptake in differentially activated macrophages and show that the effects of HIV are not directly related to pro- or anti-inflammatory mediators. Finally, this study shows that glutamate transport by macrophages is less affected than what has been described in astrocytes. Macrophages may thus play a role in neuroprotection against glutamate in the infected brain, through their expression of both EAATs and glutamine synthetase. Because glutamate metabolism by activated macrophages is sensitive to both HIV infection and inflammation, it may thus be of potential interest as a therapeutic target in HIV encephalitis.

Cognitive impairment remains a common complication of late-stage human immunodeficiency virus type 1 (HIV-1) infection. Before the introduction of highly active antiretroviral therapy (HAART), most virus-infected individuals demonstrated neuropathological abnormalities at autopsy, and a quarter had had neurological manifestations of the disease including cognitive, behavioral, and motor abnormalities, ranging from discrete deficits to HIV-1-associated dementia (HAD) (25, 38). Despite the decrease in the incidence of HAD (27), the greater life expectancy of HAART-treated infected individuals suggests that its prevalence may increase. Moreover, a less severe form of neurological attack, called minor cognitive/motor disorder (MCMD), emerged since the introduction of HAART, that may be more prevalent than dementia (27). Altogether, it appears that neuroAIDS is indeed sensitive to HAART although it remains a significant independent risk factor for AIDS mortality (13).

The best histopathological correlate of HAD is the number of activated mononuclear phagocytes in the central nervous system (CNS) (26), suggesting that HAD is the consequence of indirect mechanisms involving mononuclear phagocytes rather than that of direct virus effect. It is indeed widely accepted that macrophages and microglia play a key role in HIV-induced neurotoxicity, through viral neurotoxin production and more probably through immune activation and subsequent release of neurotoxic factors (for review, see Ref. 33). The importance of macrophages in HIV-associated neuropathogenesis is further underlined by the emergence of specific monocyte subsets in the peripheral blood of patients with HAD, which express specific markers and demonstrate enhanced ability to migrate and secrete neurotoxins (19, 37, 42). Nevertheless, a paradoxical contrast exists between macrophage/microglia activation, that may be set up early in the disease at the asymptomatic pre-AIDS stage (2, 50), and the neuronal apoptosis (1) and loss (14) that occur very late (for review, see Ref. 15).

Glutamate, the major excitatory neurotransmitter (20), is thought to be involved in HIV-induced neurotoxicity (32, 61). A rise in extracellular glutamate concentration leads to neuronal death through hyperactivation of N-methyl-D-aspartate (NMDA) receptors, a mechanism called excitotoxicity. The clearance of extracellular glutamate is ensured by a high-affinity glutamate uptake system called X_AG− (48) involving excitatory amino acid transporters (EAAT) (for reviews, see Refs. 10 and 24). Among the five EAAT genes cloned to date, EAAT-1 and EAAT-2 are glia expressed (7, 11, 36, 46) and provide in vivo protection against glutamate toxicity (45). Astrocytes would thus play a key role in neuroprotection against glutamate in the course of HIV infection. However, glutamate uptake by astrocytes is lowered by >60% within 6 h of exposure to either HIV-1 gp120, or Tat, associated with a drop in EAAT-2 expression but not EAAT-1 (17, 35, 62). These findings strongly suggest that during infection astrocytes
would not fulfil their protective role. On the other hand our group previously described the expression of functional transporters EAAT-1 and EAAT-2 by human macrophages (43), suggesting that they may also exhibit neuroprotective properties. Another transporter expressed by macrophages is also involved in glutamate homeostasis: the cystine/glutamate antiporter. This heterodimeric transporter includes the CD98 heavy chain and the xCT light chain, which confers substrate specificity (47). It takes up extracellular cystine in exchange for intracellular glutamate through a sodium-independent transport system called x_c− (3). The rate of cystine uptake is the limiting factor for the synthesis of the major antioxidant glutathione (GSH). In the presence of high extracellular concentrations of glutamate, the cystine/glutamate antiporter functions in reverse, taking up extracellular glutamate, and leading to cysteine starvation and oxidative stress. This glutamate uptake is nevertheless less efficient than that mediated by EAATs (44). Although extracellular glutamate competes with cystine uptake, our group found that it unexpectedly increases both cystine capture and GSH synthesis by macrophages in a dose-dependent manner (44). EAAT expression in macrophages thus leads to glutamate-dependent enhancement of glutathione synthesis by providing intracellular glutamate for direct synthesis of glutathione and also for fueling the intracellular pool of glutamate and trans-stimulating the cystine/glutamate antiporter. EAAT expression by macrophages and microglia has also been described in different rat models and in simian and human AIDS (for review, see Ref. 28). Interestingly, glutamine synthetase is also expressed in cerebral macrophages and microglia in simian AIDS (9), suggesting that these cells may complete the whole glutamate-glutamine cycle in the infected brain. The first aim of this study was thus to evaluate in vitro whether HIV infection affects macrophage capacity to capture and metabolize glutamate, as it does in astrocytes, or whether HIV-infected macrophages could still exhibit a neuroprotective activity by clearing extracellular glutamate and providing substrates for GSH synthesis. In the latter case, macrophages could substitute for astrocytes in glutamate-related functions in the course of HIV infection.

The key role played by inflammation in HIV-related CNS attack also led us to investigate the influence of the cytokine microenvironment on glutamate uptake and glutamate transporter expression by macrophages. As anti-inflammatory activation of macrophage/microglia may also develop in the infected CNS, when neuronal apoptosis occurs (12, 16, 49) we aimed to evaluate the effect of macrophage activation by either pro- or anti-inflammatory stimuli on glutamate transporter expression and function. Of note, we previously described a bright EAAT expression in perineuronal microglia in HIV infection, that is totally lost when HIV encephalitis and neuronal apoptosis occur (56). In perivascular macrophages and microglial nodules, EAAT expression parallels microgliosis and is maintained in HIV encephalitis cases (56). This particular expression profile suggested that pro- and anti-inflammatory activation pathways in macrophages may differentially modulate the expression of glutamate transporters, further supporting the rationale of the present study. We assessed the effects of the anti-inflammatory cytokines IL-4 and IL-10, which can be released by infiltrating lymphocytes or macrophage/microglia, respectively. The role of PGE2 was measured because this prostaglandin mediates the anti-inflammatory switch of macrophages after phagocytosis of apoptotic cells (16). The pro-inflammatory cytokines tested are major mediators of brain inflammation, TNF-α and IFN-γ, which can be produced by infiltrating lymphocytes. Dexamethasone and LPS were used as controls for typical anti- and pro-inflammatory activation, respectively.

Using human primary monocyte-derived macrophages (MDM), we hereby show that glutamate transporters are indeed modulated by macrophage activation and infection, but in a complex scheme that is not simply related to pro- and anti-inflammatory features. Nevertheless, this suggests a real, although only partially, protective role for macrophages/microglia against glutamate toxicity in the course of HIV infection.

MATERIALS AND METHODS

Human monocyte isolation and differentiation. Monocytes were isolated from healthy seronegative donors by countercurrent elutriation (18). Monocyte preparations were >95% pure, as shown by flow cytometry. One hundred million monocytes were seeded into 75 cm² culture flasks in DMEM-glutamax medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated (+56°C for 30 min) fetal calf serum (Bio West, Nuaille, France) and 1% antibiotic mixture (penicillin-streptomycin-neomycin 100×, Invitrogen). Macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 and 1 ng/ml, respectively; R&D Systems, Minneapolis, MN) were included in the medium from day 0 to day 6. These relative concentrations of cytokines maintained a neutral environment with respect to activation marker expression (HLA-DR, CD163, CD206), which remained similar to that of MDM cultured in medium alone (data not shown). In these conditions, cell survival during differentiation was good. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. Blood monocytes adhered to plastic after 1 h, spontaneously detached after 48 h and retained a monocyte-like appearance for 5 days. After 3 days, monocytes were washed with phosphate-buffered saline (PBS), counted using trypan blue, and dispensed into 48-well plates (3 × 10⁵ cells/well) in M-CSF and GM-CSF-supplemented medium. On day 5 and 6, cells were washed and fresh medium lacking M-CSF or GM-CSF was added. On day 7 and 8, mature cells were stimulated with pro- or anti-inflammatory molecules, or infected.

Recombinant cytokines and biologically active substances. Recombinant human IL-4, IL-10, IFN-γ, and TNF-α were purchased from R&D Systems and used at a concentration of 10 ng/ml for stimulation. Dexamethasone (40 ng/ml) was purchased from Qualimed Laboratoires (Paris, France) and PGE2 (10 ng/ml), from Cayman Biochemicals (Ann Arbor, MI). All substances except dexamethasone contained 1 pg per ml during the stimulations. Dexamethasone was 95% pure, as shown by flow cytometry, and produced by infiltrating lymphocytes. Dexamethasone and LPS (10 ng/ml) were purchased from Sigma (St. Louis, MO). We chose to apply these concentrations for the different stimuli based on those used within the literature to activate macrophages (6, 34, 39, 41, 51, 52).

Real-time quantitative PCR. RNA was extracted using a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Total RNA was treated with 5 units of RNase-free DNase (Roche, Mannheim, Germany) for 45 min at room temperature, and DNase was then inactivated by heating for 5 min at 95°C. RNA was reverse transcribed in optimal conditions, as previously defined (4). PCR reactions were performed in the IQ SybrGreen Supermix (Bio-Rad, Hercules, CA), with PCR cycles, consisting of 15 s at 95°C, 40 s at 60°C, and 20 s at 72°C, in a iCycler apparatus (Bio-Rad). Primers were 5′-TACTCATTCAAGCAGCATCG-3′ (sense) and 5′-CCATGGTATGTTGCTTCCTAC-3′ (antisense) for...
EAAT-1 gene, 5′-CATCTTGGCTAGAGGAACC-3′ (sense) and 5′-AGATGCGAAGGAAGAAGC-3′ (antisense) for EAAT-2 gene, 5′-GACTAATGCGGACTGTCG-3′ (sense) and 5′-TTGGTGCTGAAATTTGATAGG-3′ (antisense) for GS gene, and 5′-TCGGTGAAAGGCTAGACC-3′ (sense), and 5′-TCAGCTCGAGGATGACCTTGTG-3′ (antisense) for GAPDH gene, that was used as endogenous reference to compensate for different RT efficiencies and normalize the variability in the initial quantities of total RNA, so that accurate comparison of gene expression levels could be made between the different samples.

Viruses, infection, and quantification of HIV-1 replication. We used the macrophage-tropic HIV-1 reference strain Ba-L (21). This virus was amplified in vitro using only human PHA-P-activated umbilical blood mononuclear cells. Clarified cell-free umbilical blood mononuclear cell supernatants were ultracentrifuged at 360,000 g for 10 min at 4°C just before virus use, to eliminate soluble factors such as cytokines and avoid nonspecific modulations. MDMs were infected at a multiplicity of infection (MOI) of 0.1 at day 7–8 of culture. At day 2 post infection (PI), cells were thoroughly washed to remove residual virus. Supernatant was collected twice weekly until day 25 PI, and stored at −20°C to measure the reverse transcriptase (RT) activity by using a commercial kit (RetroSys; Innovan, Lund, Sweden).

Glutamate uptake. Glutamate uptake was determined using MDM seeded in 48-well plates. The uptake medium was composed of (in mM) 137 NaCl, 0.7 K2HPO4, 1 CaCl2, 1 MgCl2, 5 glucose, and 10 HEPES (pH 7.4). We assessed Na+ dependence by replacing the NaCl with 137 mM choline chloride (Sigma). Cells were washed with 1 ml of PBS and incubated for 20 min at 37°C in 200-μl uptake medium, with ionic modifications if necessary. The medium was aspirated and replaced with 100-μl uptake medium (with ionic modifications or inhibitors if necessary) containing 1-[2,3-3H]glutamic acid (62 Ci/mmol; Amersham Biosciences, Piscataway, NJ). For glutamate concentration above 50 mM, [3H]glutamate-specific activity was reduced by a factor of 100 or 200 by diluting in unlabeled glutamate (Sigma). Uptake was stopped after 5 min by removal of medium and by being washed twice with 1 ml of PBS. Cells were then lysed with 130 μl of 100 mM NaOH. The radioactivity of 60 μl of lysate was determined by liquid scintillation counting.

In a series of preliminary experiments, we tested whether protein content quantification in lysates from differently activated macrophages was or was not suitable for assessing MDM number. This method gave the same results as two other cell quantification methods: cell counting with trypan blue exclusion (average number of viable macrophages in one ×4 magnification field), and methylthiazolyl diphenyl tetrazolium (MTT) colorimetric assay (data not shown). To correlate glutamate uptake to a fixed number of cells, we thus evaluated the quantity of cells per well by quantifying the lysate protein content.

The protein content of 60 μl of cell lysates was determined with the use of a commercial kit (MicroBC assay, Uptima; Interchim, Montluçon, France). All experiments were performed in triplicate. Glutamate uptake is expressed as a percentage of control MDM uptake activity.

Western blot analysis. MDM from 12 replicate culture wells were lysed at 4°C in 50 μl of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100] with protease inhibitor (Roche). Pooled lysates were then centrifuged for 2 min at 12,000 g to remove DNA, followed by a 2-h centrifugation at 4°C and 12,000 g to pellet proteins. The pellet was resuspended in 50 μl of 20 mM Tris pH 8 buffer containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, and 1% Triton X-100. Laemmli buffer containing 100 μM 2-mercaptoethanol was then added, and the samples were boiled for 5 min before being subjected to SDS-PAGE in a 12% acrylamide gel (200 V, 90 min). The separated proteins were transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham, Uppsala, Sweden) (100 V, 90 min). Membranes were blocked (3 h, RT) with PBS containing 0.1% Tween 20 and 5% BSA and incubated overnight at 4°C with anti-EAAT-1 or anti-EAAT-2 mouse monoclonal (1:500; Novoceastra, Newcastle-upon-Tyne, UK) and anti-actin mouse monoclonal (1:2,000 Calbiochem, San Diego, CA) antibodies. Membranes were then washed three times for 10 min in Tris-buffered saline-Tween 20 (TBS-T) and incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse antibody (Zymed Laboratories, San Francisco, CA). After two washes in TBS-T and one in TBS, the proteins were subsequently detected using the “ECL Plus” Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Signal intensity on the film was quantified with densitometric analysis software (NIH Image version 1.2; W. Rasband, National Institutes of Health, Bethesda, MD), and the ratio between EAAT and actin signals probed on the same membrane calculated.

Quantification of glutamine synthetase activity. All solutions were maintained at 4°C. Cell cultures were washed with PBS, and the cells were then scraped with the use of a rubber policeman into 75 μl of ultrapure water. The cell lysates were sonicated at 4°C with a 750-W ultrasonic processor (5 pulses of 5 s at maximum power; VibraCell, Sonic, Inc., Newtown, CT), aliquoted, and frozen at −20°C. The measurement of GS activity was performed in 96-well plates, by the addition of 50 μl of cell extract to 75 μl of a reaction mixture containing 0.1 M imidazole-HCl buffer (pH 7.2), 50 mM glutamate, 20 mM adenosine triphosphate, 1 mM β-mercaptoethanol, 40 mM MgCl2, and 100 mM hydroxylamine hydrochloride (Sigma). After a 60-min incubation at 37°C, the reaction was stopped by the addition of 150 μl/well of stop solution containing 0.37 M FeCl3, 0.2M trichloroacetic acid (Sigma), and 0.67 M HCl. After 30 min at 4°C and 5 min at room temperature, the amount of γ-glutamyl hydroxamate formed in the reaction was read at 540 nm using a Microplate autoreader (model EL311; Biotek Instruments, Winooski, VT) and assessed against a standard curve of known concentrations of γ-glutamyl hydroxamate. Specific activity was normalized against protein content in cell samples (MicroBC assay).

RESULTS

HIV replication upregulates EAAT-2 gene expression in macrophages with poor effect on protein level. EAAT-1, EAAT-2, and xCT gene expression levels were quantified for 3 wk in the course of HIV infection. As shown in Fig. 1A, EAAT-1 and xCT gene expression levels were not modulated whatever the replication level. In contrast, EAAT-2 gene expression was upregulated by >20-fold when HIV replication reached its maximum level. Moreover, EAAT-2 gene upregulation strictly paralleled the level of HIV replication, indicating either that the EAAT-2 gene linearly responds to HIV stimulation or that both events respond to the very same induction cascade. Densitometric analysis of the Western blot suggests that, relative to actin, EAAT-1 protein level decreased with time and was not modulated by infection, while EAAT-2 was stable over time and repeatedly higher in HIV replicating cells. Nevertheless, this effect was of low amplitude (Fig. 1B) and has no statistical value as we did not perform replicate wells to assess intraexperiment signal variation. Contrasting with its gene expression level, EAAT-2 protein level was thus poorly modulated (Fig. 1B) suggesting either a block at the posttranscriptional level or an increased rate of transporter degradation.

Glutamate uptake by macrophages is decreased in the course of HIV replication. The discrepancy between EAAT-2 expression at the gene and protein levels led us to assess whether glutamate transport is affected or not in HIV-infected MDM. Glutamate uptake was assessed twice weekly over a 24-day post infection period. As shown on Fig. 2, uptake tended to decrease by day 7–10 PI, an effect that was significant

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at day 14 PI, when a high virus replication occurred. The reduction in glutamate uptake by infected vs. control MDM reached 35%. HIV replication in macrophages thus reduces their capacity to clear extracellular glutamate, but this effect is moderate. Of note, this reduction cannot be attributed to the sodium-independent xCT transport system, which was not affected and accounted for ~10% of the total glutamate uptake (Fig. 2).

Glutamine synthetase is expressed in MDM. Besides EAAT, glutamine synthetase is a critical effector in the trophic glutamate-glutamine cycle. To verify whether the whole cycle could occur in MDM, we tested the expression of the GS gene and the presence of GS activity in MDM. As shown in Fig. 3, both GS gene expression and enzyme activity were detectable throughout the monocyte to macrophage differentiation process.

Glutamine synthetase is transcriptionally repressed in the course of HIV replication. Inhibition of glutamate uptake may disturb the dynamic pool of intracellular glutamate, and thus alter substrate availability for the glutamine synthetase. We accordingly assessed whether HIV replication, that decreased EAAT functionality, may also affect GS gene expression and activity levels in macrophages. As shown in Fig. 4, GS gene expression was repressed by ~50% by day 17 PI. GS activity in cell lysates was also repressed in the same proportion, suggesting a transcriptional repression.

Pro- and anti-inflammatory activation modulate the expression of glutamate transporters and glutamate uptake. To assess the role of the inflammatory environment on both the expression of transporters and on glutamate uptake, mature macrophages were treated for 3 days with different pro- and anti-inflammatory agents. Then, real-time PCR for EAAT-1, EAAT-2, and xCT genes, as well as glutamate uptake were performed. EAAT-1 gene expression was on the whole poorly modulated by the tested stimuli (Fig. 5), except for dexamethasone that increased it 5-fold. Pro-inflammatory cytokines and LPS induced a weak 2-fold upregulation of EAAT-1 that did not test significant (P = 0.27; 0.12; 0.12 for IFN-γ; TNF-α and LPS, respectively, t-test).

EAAT-2 was much more responsive to inflammation-related stimuli than EAAT-1 (Fig. 5). PGE2, which does not polarize cells towards a clear pro- or anti-inflammatory phenotype (41), as well as LPS, upregulated EAAT-2 mRNA by about 20- and
consequences for neurons through the release of pro-inflammatory cytokines, viral proteins, and other neurotoxins, including glutamate (for review, see Ref. 33). Although these features are undeniable and well documented, the demonstration by our group of efficient glutamate transport by human macrophages (43) led us to investigate whether macrophage activation could also entail protective aspects with regard to glutamate metabolism. In this report, we first augment our previous findings and demonstrate that MDM also express glutamate synthetase at the gene and enzymatic activity levels. Together with EAAT, GS is a critical effector of the trophic and protective glutamate-glutamine cycle. Nevertheless, whether this coexpression of EAAT and GS in activated macrophages leads to the provision of the trophic amino acid glutamine to neighboring neurons remains to be determined, as we assessed neither glutamine secretion nor the expression of system N or ASCT2 transporters that mediate glutamine efflux from astrocytes (5, 8, 29). In this study, we showed that glutamate transport is less repressed by HIV in macrophages than what was described by others in astrocytes. Indeed, Wang et al. (58) observed a >60% reduction in glutamate uptake by HIV-infected or gp120-stimulated astrocytes. On the other hand, macrophage uptake capacity was only partially lowered throughout a 25-day infection kinetic study, and this decrease appeared only when high replication occurred, suggesting that virus contact per se may not allow such an effect. This relative insensitivity of macrophages to HIV replication suggests that these cells may, at least partially, fill in the defect of astrocytic glutamate uptake in the infected brain.

At the gene expression level, HIV replication in macrophages induced a striking increase in EAAT-2 gene expression. The physiological significance of this EAAT-2 mRNA overexpression observed in the course of infection remains to be elucidated, bearing in mind that it was followed by a poor increase in protein content, and even a diminished transporter function. The dissociation between gene expression and trans-

Fig. 3. Glutamine synthetase is expressed in MDM. Glutamine synthetase gene expression and enzyme activity were assessed during monocyte to macrophage differentiation. A: real time quantitative RT-PCR reveals a constitutive expression of the GS gene, with a tenfold induction at day 1 compared with monocytes, followed by a return to basal level. B: GS activity in cell extracts gradually increases during MDM differentiation. For both RT-PCR and activity, results are expressed as means ± SE of 3 independent experiments.

The role of macrophages in CNS infection by HIV has so far been restricted to pro-inflammatory features with deleterious con-

Fig. 4. Glutamine synthetase gene expression and activity are repressed in the course of HIV replication. Mature macrophages were infected with HIV-1/ Ba-L (MOI = 0.1) at day 0. Glutamine synthetase gene expression and enzyme activity were measured on days 3, 7, 14, 17, and 21 PI. RT activity was similar to the ones shown in Figs. 1 and 2. GS gene expression decreased by ~50% beginning at day 14 PI, whereas the GS activity showed a nonsignificant (NS) increase at day 14 PI, followed by a 50% decrease thereafter. For both RT-PCR and activity, results are expressed as means ± SE of 3 independent experiments. *P < 0.05, Student’s unpaired t-test.
porter function may relate to posttranscriptional and/or transporter trafficking events, as already shown in astrocytes where, for example, AMPA-kainate receptor activation upregulates GLAST (the murine EAAT-1) activity without affecting its mRNA level (22). Interestingly, the presence of functional AMPA-kainate receptors has also been demonstrated on brain macrophages, suggesting that they may also respond to extracellular glutamate (40). We thus tested whether infected macrophages may regulate their glutamate uptake capacity in response to an excess of extracellular glutamate. To this end, we assayed glutamate uptake 12 h after treating both uninfected and infected macrophages with 10 mM glutamate. This stimulation had no effect (data not shown), suggesting that other regulatory pathways are responsible for the gap between EAAT-2 gene expression, its protein content, and its function. Vanoni et al. (57) showed in a mouse model of amyotrophic lateral sclerosis that the murine counterpart of EAAT-2, GLT-1, exhibits increased internalization and degradation through aberrant oxidative stress, whereas the other glutamate transporters are not affected. If active in our system, such a mechanism would explain the discrepancy between EAAT-2 gene expression, its protein amount, and its function. The establishment of an oxidative stress in HIV replicating macrophages is strongly supported by different studies. First, Yeh et al. (59) reported that HIV-1-infected MDM produce significantly less cysteine than uninfected ones, following TNF-α stimulation. This indicates that HIV replication consumes cysteine. Cysteine is the limiting amino acid precursor for GSH synthesis, and its decreased availability in infected MDM suggests that HIV replication may alter the GSH synthesis pathway. Concordant with this idea, Mialocq et al. (38a) showed that HIV replication is indeed associated with a significant decrease in intracellular GSH concentration, as well as transcriptional modulation of enzymes involved in the regulation of intracellular GSH levels, leading to oxidative stress. The involvement of oxidative stress in EAAT-2 expression is still a matter of debate (53), but one can suggest that HIV replication in MDM might account for a decrease in EAAT-2 expression at the cell membrane, as suggested by amyotrophic lateral sclerosis data (57). In this hypothesis, the strong overexpression of EAAT-2 mRNA that we observed during HIV infection, leading to slight increase in protein content, might be a compensatory mechanism to counterbalance this degradation. Pulse-chase experiments to measure EAAT-2 protein turnover would answer this question. In addition to transporter degradation, mediators of oxidative stress also inhibit EAAT function through a sulfhydryl sensor based mechanism (54). Finally, regardless of whether it acts through the degradation of transporter protein or through the regulation of its function, the HIV-induced oxidative stress is likely to be responsible for the observed repression of glutamate uptake.

Another mechanism that may account for the overexpression of the EAAT-2 gene involves the cystine/glutamate antiporter.

Fig. 5. Effect of pro- and anti-inflammatory stimuli on transporter gene expression and glutamate uptake. Mature macrophages were treated for 3 days with 10 ng/ml [except for 40 ng/ml dexamethasone (Dex)] of each molecule. Gene expression and glutamate uptake capacity were assessed on replicate wells of the same cells. Data are expressed as percentage of untreated cells and are means ± SE of 3 independent experiments. *P < 0.05, Student’s unpaired t-test.
Because the need for cysteine increases during HIV replication, and because cystine is the most abundant extracellular precursor of cysteine, the xc− system would act to replenish the cysteine pool. By uptaking cystine, this system releases glutamate, a consequence of which is a drop in the glutamate gradient that would stimulate the EAAT system via an over-expression of EAAT-2. The sodium-independent cystine/glutamate antiporter transport capacity was not modified throughout HIV infection, and its role in cystine uptake would thus not be altered. Nevertheless, it is known from previous studies (44) that intricate interactions between the xc− and XAG− transport systems regulate GSH synthesis in MDM. The observed perturbations of the EAAT (XAG−) expression and function may thus arise from normal transport systems interaction rather than from a defect of the cystine/glutamate antiporter (xc−). The interactions between these transport systems, and their perturbation by HIV infection and oxidative stress are schematized in Fig. 6.

Whatever the reason for the drop in glutamate transport, one important consequence of this drop may be the decrease in glutamine synthetase expression. This 50% decrease occurred at both the transcriptional and enzyme activity levels. It seemingly corresponds to a transcriptional repression in response to a disturbed glutamate pool. From this point of view, we must keep in mind that the glutamate pool, although abundant, is dynamically regulated and can easily be deprived (for review, see Ref. 28).

Our study, as well as others (23, 30), emphasizes the preponderant role of EAAT-2 in glutamate transport adaptation to changes in the external conditions. Indeed, EAAT-1 was not modulated at the mRNA or the protein level. As this transporter may account for ~70% of glutamate uptake by MDM (43), its refractory state to external stimuli may contribute to the moderate modulation of glutamate uptake we observed.

Beside the effects of HIV replication, our data also clearly show that EAAT gene expression and function can be modulated by either pro- or anti-inflammatory stimuli. Again, EAAT-2 was the most responsive to stimulations, suggesting that it may fulfil a predominant role in maintaining glutamate homeostasis through rapid responses. EAAT-mediated glutamate uptake by MDM is not simply linked to inflammatory activation because both pro- and anti-inflammatory molecules can increase it, suggesting that inflammation per se is not the key regulator for these transporters. Although activation can induce high EAAT overexpression at the mRNA level, modulation of glutamate uptake level in macrophages is of lower magnitude, suggesting an intricate control through finely tuned post-transcriptional regulation of the different transporters. However, modulation of glutamate transport grossly fits gene expression patterns. Moreover, the amplitude of the variations we observed are in the same range as those induced by other recognized EAAT regulation mediators such as polyunsaturated fatty acids (55, 60). This suggests that the observed effects may indeed have relevant functional consequences.

PGE2, which production by macrophage/microglia is markedly increased after phagocytosis of apoptotic cells (12, 16), strongly upregulated EAAT-2 expression and slightly increased glutamate uptake in our conditions. This feature may be a component of the protective action of PGE2 in different neurological diseases (12). Likewise, LPS also induced MDM to overexpress EAAT-2 mRNA, and increased glutamate transport. Such induction may relate to the need to maintain the glutamate concentration gradient in conditions where microbiocidal functions of macrophages entail a demand for GSH synthesis, and thus cystine uptake and glutamate secretion (44). These data suggest that extracellular glutamate concentration may be strictly regulated in the vicinity of macrophages through modulation of EAAT expression and function.

Taken together, our data suggest that HIV replication in MDM disturbs the balance between glutamate uptake and secretion, and leads to EAAT-2 gene induction. The significance of this EAAT-2 gene induction still requires further investigation to be fully understood. This may nevertheless participate in the important uncoupling that is observed between macrophage/microglia activation throughout the in-
fected brain, with its well documented neurotoxic consequences, and neuronal apoptosis that occurs late in this process. Different effectors of macrophage activation seemingly also modulate these equilibriums. Nevertheless, the fact that only TNF-α and LPS are able to significantly enhance glutamate uptake supports the idea that the inflammation that occurs within the CNS during HIV infection, that is mainly mediated by TNF-α (for review, see Ref. 33), plays a major role in glutamate-mediated neurotoxicity. Indeed, TNF-α induces the production and release of nitric oxide (31) that generate an oxidative stress likely compensated by an increase in glutathione synthesis via cystine uptake and glutamate release through the cystine/glutamate antiporter. Thus compensatory mechanisms involving EAAT must also be set up in response to inflammatory mediators and not only in response to HIV replication. The effect of LPS on glutamate uptake leads to similar mechanisms during bacterial infection.

In conclusion, our results strongly support the hypothesis that modulation of glutamate transport and glutamine synthetase in activated macrophages responds to the natural functions of macrophages that necessitate adapting GSH metabolism during defense against microorganisms. On the other hand, this protective profile that includes EAAT and GS expression in macropahes is altered upon HIV replication, although this effect is probably less important than the HIV-induced depression of the same functions in astrocytes. The fine interactions between, on the one hand, the protective glutamate-glutamine cycle and the GSH metabolic pathways in macrophages/microglia and astrocytes, and, on the other hand, the level of HIV replication and the subsequent inflammatory activation features may be critical to the local and temporal outcome of the neuron survival/death decision.

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