Involvement of Nitric Oxide in the Mitochondrial Action of Efavirenz: A Differential Effect on Neurons and Glial Cells

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The anti-human immunodeficiency virus (HIV) drug efavirenz (EFV) alters mitochondrial function in cultured neurons and glial cells. Nitric oxide (NO) is a mediator of mitochondrial dysfunction associated with HIV central nervous system symptoms. We show that EFV promotes inducible nitric oxide synthase (iNOS) expression in cultured glial cells and generated NO undermines their mitochondrial function, as inhibition of NOS partially reverses this effect. EFV inhibits mitochondrial Complex I in both neurons and glia; however, when the latter cells are treated for longer periods, other mitochondrial complexes are also affected in accordance with the increased NO production. These findings shed light on the mechanisms responsible for the frequent EFV-associated neurotoxicity.

Keywords. efavirenz; electron transport chain; HIV; mitochondria; nitric oxide; NNRTI; central nervous system.

The nonnucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) is among the most widely prescribed anti-human immunodeficiency virus (HIV) drugs. Although considered safe, the majority of patients experience a variety of neuropsychiatric side effects that can require therapy discontinuation [1]. Moreover, its long-term use has been linked to neurocognitive impairment [2]. This is of concern given that 50% of HIV(+) patients undergo a gradual cognitive deterioration

known as HIV-associated neurocognitive disorder (HAND) whose pathogenesis has been linked to the neurotoxicity of antiretrovirals [3–5]. The mechanism behind EFV-produced central nervous system (CNS) toxicity is unclear, but mounting in vivo and in vitro evidence points to an alteration of brain energy homeostasis [6, 7]. Recently, we have shown that EFV affects the bioenergetics and viability of cultured neurons and glial cells by acute mitochondrial interference. The repercussions of this action vary; compensatory mechanisms were activated in glial cells—upregulated glycolysis and augmented intracellular adenosine triphosphate (ATP) levels—but not in neurons, rendering the latter cells more vulnerable to the effects of EFV and other stressors [8]. The effects of EFV were potentiated by exogenous nitric oxide (NO), a neuromodulator and regulator of mitochondrial function. NO is also a crucial proinflammatory CNS mediator associated with the pathogenesis of different neuroinflammatory and neurodegenerative conditions. Mammalian cells generate NO as a by-product of NO synthase (NOS) activity. While neurons express neuronal NOS (nNOS), a constitutive isoform that synthesizes moderate amounts of NO, glial cells express inducible NOS (iNOS), which generates major NO amounts [9]. There is clear experimental and clinical evidence to implicate NO in the CNS symptoms of HIV. Animal studies suggest that iNOS-derived NO is a major mediator of HIV-1 gp41 neurotoxicity. More importantly, increased production of NO has been related to HIV-associated dementia, whose severity has been correlated with iNOS expression, suggesting an association with the neuroinflammatory milieu surrounding HIV infection [10–12]. The present study evaluates the role of endogenous NO in the mitochondrial effects of EFV on neurons and glial cells.

MATERIAL AND METHODS

Reagents and Treatments

Unless otherwise stated, chemicals were from Sigma-Aldrich. EFV (Sequoia Research Products) was dissolved in methanol (3 mg/mL), which was employed as a vehicle control and did not influence any of the parameters studied. To evaluate the influence of NO synthase activity, cells were treated (24 hours) with "lipopolysaccharides (LPS) cocktail," composed of *Escherichia coli* endotoxin (LPS, 1 μg/mL), interferon-γ (50 U/mL) and tumor necrosis factor-α (20 ng/mL), with or without the nonspecific NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME 50 μM, Cayman Chemical). 3-morpholinosydnonimine (SIN-1 33 μM) was employed as a positive control for peroxynitrite assessment and (Z)-1-[2-aminoethyl)-N-(2-ammonioethyl)

amino]diazen-1-ium-1,2-diolate (DETA-NO, 0.3 or 0.5 mM) as an external NO donor (Supplementary Figure 1).

Cell Culture

Cell cultures were maintained and treated as described previously [8]. All experiments were performed on human neuroblastoma (SH-SY5Y) and glioma (U-251MG) cell lines, with confirmatory analysis on primary rat cortical astrocytes and neurons (isolated in accordance with European Community guidelines for the use of animal experimental models, approved by the Ethics Committee of the University of Valencia).

Protein Extraction and Western Blot Analysis of nNOS and iNOS

Treatments (6 and 24 hours), whole-cell protein extraction, and Western blot (WB) were performed as described elsewhere [8]. Primary antibodies: mouse monoclonal anti-iNOS (1:1000; BD Biosciences) and anti-nNOS (1:2500; BD Biosciences), and anti-actin rabbit polyclonal (1:1000; Sigma-Aldrich). Secondary antibodies: horseradish peroxidase–conjugated goat anti-mouse (1:2000) and anti-rabbit immunoglobin-G (1:5000), both from Thermo Scientific.

Fluorescence Microscopy and Static Cytometry

Fluorescence microscopy was performed using an Olympus IX81 microscope with "ScanR" static cytometry software version 2.03.2. U-251MG (3×10^4 cells/cm^2), SH-SY5Y (1.5×10^4 cells/cm^2), primary neurons (2.5×10^5 cells/cm^2), and astrocytes (4×10^4 cells/cm^2) were treated (48-well plates, 6 or 24 hours), and Hoechst 33342 (1 μM) to mark nuclei and specific fluorochromes (2.5 μM except for DHR123, 25 μM) added for the last 30 minutes. Subsequently, cells were Hank's balanced salt solution (HBSS)–washed and 16–20 live-cell images/well recorded. Intracellular NO and peroxynitrite (ONOO$^-$), mitochondrial superoxide, and mitochondrial membrane potential ($\Delta\Psi_m$) were assessed using diaminorhodamine-4M AM (DAR-4M AM), dihydrorhodamine 123 (DHR123), both from Sigma Aldrich, and MitoSOX and tetramethylrhodamine methyl ester (TMRM), both from Molecular Probes, Invitrogen respectively.

Electrochemical Measurement of Oxygen Consumption

After 6 hours treatment, cells (4×10^6) were placed in a gas-tight chamber (1 mL HBSS, 37°C) and their oxygen (O$_2$) consumption monitored with a Clark-type O$_2$ electrode (Rank Brothers) with the Duo.18 data acquisition device (WPI).

Spectrophotometric Analysis of the Activity of the Oxidative Phosphorylation Complexes

Cell homogenates (1.5×10^6 U-251MG and 3.0×10^6 SH-SY5Y) were obtained with 0.2 mL SETH buffer (250 mM sucrose, 2 mM ethylenediaminetetraacetic acid, 10 mM Tris-HCl, 100 U/L heparin, pH 7.4) after sonication (ice-cold water, 3×10 seconds with 30 seconds rests) and centrifugation (8000 g, 10 minutes).

Protein concentration was determined by the Bradford method. Reduced nicotinamide adenine dinucleotide (NADH): coenzyme Q$_1$ oxidoreductase activity (CI) was evaluated according to the rate of NADH-dependent coenzyme Q$_1$ reduction (340 nm). Succinate dehydrogenase activity (CII) was assessed according to the decrease in absorbance due to the reduction of 2,6-dichlorophenolindophenol (600 nm). Cytochrome c reduction from decylubiquinone (DBH$_2$) (550 nm) was calculated to evaluate ubiquinol: cytochrome c oxidoreductase activity (CIII) and cytochrome c oxidase activity (CIV) was assessed by calculating the decrease in absorbance due to the oxidation of previously reduced (with sodium borohydride) cytochrome c (550 nm). Absorbance was detected using TECAN infinite M200 spectrophotometer.

Lactate Determination

Lactate in the extracellular medium was evaluated using the Lactate Assay Kit (BioVision) and a Multiskan plate reader spectrophotometer (Thermo Labsystems) [8].

Statistics and Data Representation

Data (% of control—untreated cells considered 100%) were analyzed (GraphPad Prism v.5 software) with 1-way ANOVA followed by a Newman-Keuls post hoc test (control conditions independently analyzed by Student t-test). All measurements were performed in duplicate, repeated at least 3 times and data represented as mean ± standard error of margin of these independent experiments.

RESULTS

EFV-induced NOS Activation in Glial Cells

Cells were treated with EFV (10 or 25 μM) or an LPS cocktail (a mixture of proinflammatory molecules that induce iNOS) for 6 or 24 hours. WB analysis revealed that EFV increased the expression of iNOS in glial cells (U-251MG), at 25 μM. Expression levels were similar at 6 and 24 hours, and reflected those induced by the LPS cocktail (24 hours). Neither EFV nor LPS cocktail activated nNOS in neurons (SH-SY5Y) (Figure 1A). Assessment of NO by fluorescence microscopy confirmed the results of iNOS expression, as EFV (10 and 25 μM) increased NO levels in glial cells but not in neurons. This effect was confirmed in primary cortical rat cell cultures, which ruled out the possibility of it being due to the cancerous nature of the cell lines employed (Figure 1B).

EFV-induced NO Interferes with Mitochondrial Function and Bioenergetics in Glial Cells

We evaluated 3 characteristic parameters (O$_2$ consumption, mitochondrial reactive oxygen species [ROS] generation, and $\Delta\Psi_m$) in the presence of the nonselective NOS inhibitor, L-NAME (50 μM). Inhibition of NO synthesis alone did not modify the respiration rate in either cell type (data not shown). Glial
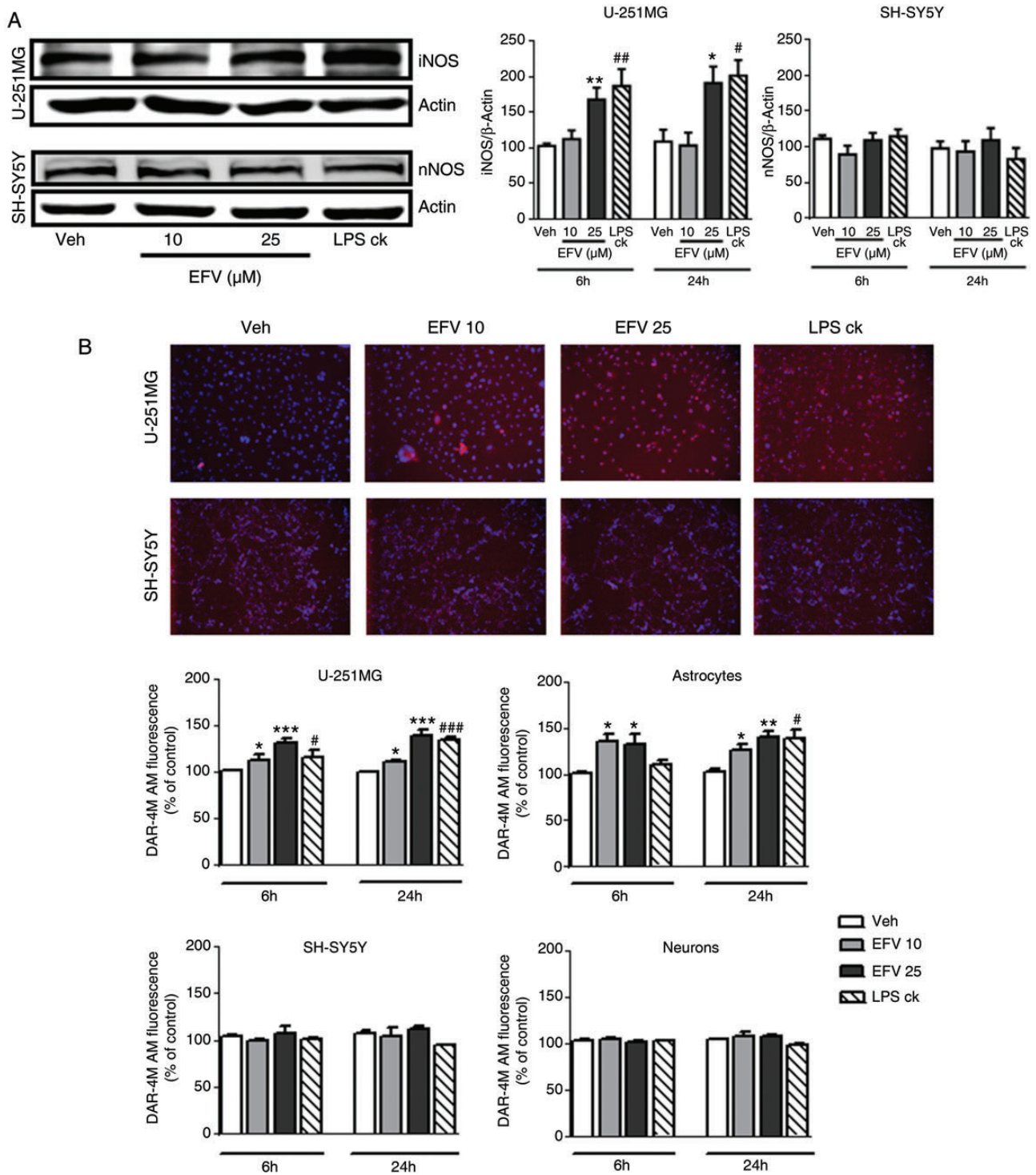
Figure 1. Effect of efavirenz (EFV) on nitric oxide (NO) production. Human cell lines U-251MG (glioblastoma) and SH-SY5Y (neuroblastoma) and primary cultures of rat cortical astrocytes and neurons were exposed to EFV (10 or 25 μM) or lipopolysaccharide (LPS) cocktail for 6 or 24 hours. *A*, inducible nitric oxide synthase (iNOS) (U-251MG) and neuronal nitric oxide synthase (nNOS) (SH-SY5Y) expression were analyzed by Western blot. Representative images (24 hours) and summary of the densitometry results of several independent experiments are shown (6- and 24-hour treatment, expressed as mean ± standard error of the mean (SEM), n = 3–4). *B*, Intracellular NO content (U-251MG, SH-SY5Y, and primary cell cultures) was assessed by fluorescence microscopy using the fluorochrome DAR-4M AM. Representative life-cell images (10×) (U-251MG and SH-SY5Y) showing nuclei (in blue, stained with Hoechst 33342) and the red fluorescent signal coming from NO (24 hours-treatment), and summary of the quantification of the mean fluorescence of 4 independent experiments (mean ± SEM, n = 4) after 6 and 24 hours of treatment. Data were expressed as comparison with those of untreated cells (considered 100%) and analyzed by a 1-way analysis of variance (ANOVA) multiple comparison test followed by Newman–Keuls test; significance versus vehicle (Veh) *$P < .05$, **$P < .01$, ***$P < .001$. Data for LPS cocktail were independently analyzed by Student *t* test, #$P < .05$, ##$P < .01$, ###$P < .001$.
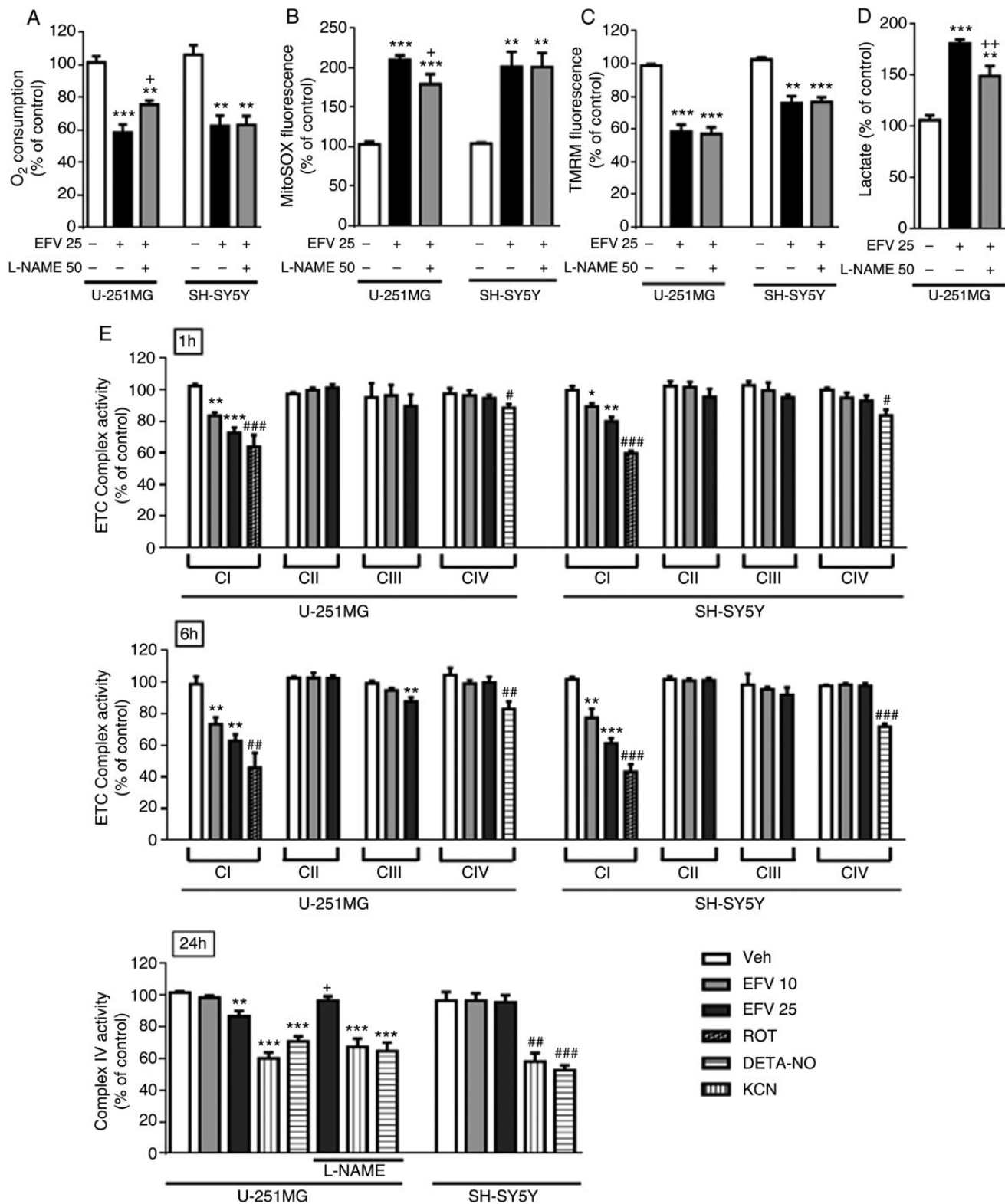
Figure 2. Interference of nitric oxide (NO) with mitochondrial function and bioenergetics. U-251MG (glioblastoma) and SH-SY5Y (neuroblastoma) cells were exposed to efavirenz (EFV) (10 or 25 μM) or a cotreatment of 25 μM EFV + 50 μM LNG-nitroarginine methyl ester (L-NAME) for 6 or 24 hours: *A*, O_2 consumption in intact cells (Clark-type O_2 electrode); quantification of data showing the rate of O_2 consumption in the absence (vehicle [Veh]) and presence of EFV or EFV+L-NAME after 6 hours treatment. L-NAME alone did not have any effect on O_2 consumption (not shown). *B*, Determination of superoxide production (mean MitoSOX fluorescence) after 6 hours treatment. *C*, Determination of mitochondrial membrane potential (relative TMRM fluorescence) after 6 hours treatment. *D*, Analysis of extracellular lactate levels in U-251MG cells after 24 hours treatment. *E*, Impact of EFV on the mitochondrial electron

cells cotreated with EFV+L-NAME (6 hours) manifested a smaller decrease in the O_2 consumption than those treated with EFV alone (Figure 2A), whereas no such differences were observed in neurons. Mitochondrial superoxide generation revealed similar results (Figure 2B), showing that as the decrease in O_2 consumption, the enhanced ROS generation in EFV-treated glial cells was partially due to the increased NO levels. However, no $\Delta\psi_m$ recovery was observed in either neurons or glial cells when treated with L-NAME (Figure 2C), thus indicating that the EFV-induced reduction of $\Delta\psi_m$ was NO-independent. Moreover, the stimulatory effect of EFV on glycolysis in glial cells [8] was also partially reversed when NO synthesis was impaired, suggesting NO participation in this bioenergetic action of EFV (Figure 2D).

EFV Specifically Alters Mitochondrial Electron Transport Chain: Effect on CI Activity and NO Interference

Recently, EFV has been shown to compromise mitochondrial respiration in both glial cells and neurons [8]. In the present study, 1-hour treatment with EFV produced a major and concentration-dependent decrease in CI activity in glial cells similar to the CI pharmacological inhibitor rotenone (ROT, 10 µM). This effect was enhanced when treatment was extended to 6 hours; furthermore, a modest, but statistically significant, decrease of CIII activity was detected (Figure 2E). EFV-exposed neurons also manifested a concentration- and time-dependent (1 and 6 hours) decrease in CI activity, but without alterations with respect to CIII (Figure 2E). As expected, NO interfered with CIV, as shown by the fact that exogenous NO (DETA-NO, 1 and 6 hours) diminished CIV activity in a time-dependent manner in both neurons and glial cells. Twenty-four-hour EFV exposure led to a significant decrease in CIV activity in glial cells but not in neurons, which is in line with our results regarding EFV-induced NO generation in glial cells only. The participation of NO was confirmed by the finding that the inhibitory effect of EFV on CIV was prevented when glial cells were coincubated with EFV (25 µM) + L-NAME (50 µM). We found no increase of intracellular peroxynitrite levels in glial cells at any of the time points studied (Supplementary Figure 2). Inside live cells, DHR123 is oxidized to the fluorescent product rhodamine 123, which due to its cationic properties, localizes in mitochondria. However, the nonfluorescent compound DHR123 is not selectively targeted to the mitochondrial compartment; therefore, although DHR123 detects changes in intracellular peroxynitrite content, it may be not so sensitive to detect small changes occurring intramitochondrially.

DISCUSSION

Our results demonstrate that clinically relevant concentrations of EFV induce the expression of iNOS in glial cells and that NO thus generated participates in the inhibitory mitochondrial effects of EFV. It is well known that NO interferes with mitochondrial function and that the extent of this effect depends on the NO amount. Moderate quantities, like those continuously generated by nNOS, modulate cell respiration by competing with O_2 at CIV of the mitochondrial electron transport chain (ETC) [13]. Larger amounts, like those produced following the expression of iNOS by inflammatory mediators, result in persistent damage of CIV and other ETC complexes, in which the role of NO is complex [13]. The present results show that incubation with EFV for a short period (1 hour) reduces mitochondrial O_2 consumption in both glial cells and neurons in what seems to be the consequence of a direct and immediate inhibitory effect on CI. This finding is in line with previously published evidence of such an effect in isolated rat mitochondria [14]. Longer periods further enhance CI inhibition in both glial cells and neurons; however, in glial cells, other mitochondrial complexes—CIII and CIV—were also affected (at 6 and 24 hours, respectively), an effect that coincides with the timeframe of increased NO production in these cells only. Indeed, this later effect of EFV on CIV involves the participation of NO as shown by its prevention upon nonselective NOS inhibition. Moreover, such inhibition partially reversed EFV actions on mitochondrial respiration and superoxide generation in glial cells, but not in neurons, which clearly implies a specific iNOS involvement in the actions of this anti-HIV drug in glial cells. The present results, which expand our previous findings [8], are partially in accordance with 1 report on EFV-treated mice describing inhibition of CIV activity in different brain areas [15]; however, in this work CI was not altered, which makes further studies of this mitochondrial effect necessary.

We also demonstrate that iNOS-derived NO enhances the upregulation of glycolysis that follows EFV-induced mitochondrial interference in glial cells. There are important differences

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Figure 2 continued. transport chain (ETC): individual analysis of ETC complex activity. U-251MG and SH-SY5Y cells were exposed to EFV (10 and 25 µM) for 1 and 6 hours and the individual activity of complexes I–IV was studied. Complex IV activity was also assessed at 24 hours of incubation. Rotenone (ROT) 10 µM was employed as a specific inhibitor of coenzyme Q1 oxidoreductase activity (CI), and potassium cyanide (KCN) 200 µM and (Z)-1-[2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) 0.5 mM (U-251MG) or 0.3 mM (SH-SY5Y) were used as inhibitors of cytochrome c oxidase activity (CIV). In all panels of this figure, data were compared to those of untreated cells (considered 100%), represented as mean ± standard error of the mean (SEM) of several independent experiments (n = 3–5) and analyzed by a 1-way analysis of variance (ANOVA) multiple comparison test followed by Newman–Keuls test, significance versus vehicle *P < .05, **P < .01, ***P < .001. Data for Rotenone, KCN, and DETA-NO were independently analyzed by means of a Student t test; #P < .05, ##P < .01, ###P < .001. +P < .05 and ++P < .01 represent the significance of the data obtained with EFV 25 µM versus EFV 25 µM [image] AME (Student t test).

between the bioenergetics of glial cells and neurons. The former are highly glycolytic and capable of maintaining ATP levels, making them more resistant than neurons to mitochondrial insults and bioenergetic stress. Importantly, a scenario of glial cell survival and neuronal degeneration with mitochondrial dysfunction has been associated with neurocognitive disorders, including HIV-associated dementia. Finally, our results, which implicate NO in the mitochondrial effects of EFV, shed some light on the mechanisms of the CNS side effects of this drug. It is plausible that EFV-induced NO modulates the interplay between brain cells, as NO generated within glial cells may act on adjacent neurons and influence their mitochondria and other cellular pathways. It could also be argued that some of the neuropsychiatric symptoms that appear early after EFV therapy initiation sometimes overlap with a degree of neuroinflammation, and that EFV, alone or in combination with the NO produced by glial cells, could exacerbate this situation. Given that HIV treatment is for life, further research is vital to evaluate whether EFV-induced NO plays a role in the long-term development of the neuroinflammatory CNS symptoms of HIV, including HAND.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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