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Antiretroviral agents increase NO production in gp120/IFNγ-stimulated cultures of rat microglia via an arginase-dependent mechanism

Lucia Lisi, Antonella Tramutola, Pierluigi Navarra *, Cinzia Dello Russo

Institute of Pharmacology, Catholic University Medical School, L.go F Vito 1, 00168, Rome, Italy

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ABSTRACT

In the present study we carried out a screening of different Antiretroviral drugs (ARVs) for their potential pro-inflammatory effects on microglial cells. Efavirenz, neviparine, darunavir and atazanavir increased nitric oxide (NO) production in microglial cells activated with Gp120CN54 and interferon-γ. The stimulatory effect on NO production appeared to be mediated by inhibition of arginase (ARG) I activity. Consistently the ARG inhibitor, N_{ω}-hydroxy-nor-arginine, mimicked the effects of ARVs. Take together these data suggest that ARG is an additional molecular target of different ARVs, whose inhibition can contribute to their pharmacological activity as well as explain the neurotoxic potential.

1. Introduction

The introduction of the Highly Active Antiretroviral Therapy (HAART), i.e. a combination of different drugs from three or more classes of antiretroviral drugs (ARVs), has significantly increased life expectancy of HIV seropositive patients, reducing both morbidity and mortality (May and Ingle, 2011). However, approximately 50% of HIV infected patients show signs and symptoms of neurological complications (Clifford, 2008). Even though some studies highlight a positive effect of HAART initiation on neurocognitive functions (Cysique et al., 2011), it has been shown that neurocognitive impairments often progress during the clinical course of the disease (Grovit-Ferbas and Harris-White, 2010) or persist despite the induction of peripheral viral suppression (Joska et al, 2010). HAART has significantly reduced the incidence of the most severe form of AIDS related dementia, whereas mild cognitive impairment is still affecting the majority of HIV infected patients (Cysique and Brew, 2009). Moreover, since an increasing proportion of HIV patients treated with HAART shows a prolonged life expectancy, it is foreseen that the incidence of such neurological complications will significantly raise with time (Valcour et al, 2004). The occurrence of these complications may result from a combination of factors such as reduced effectiveness of HAART in the central nervous system (CNS) reservoir, concurrent illnesses, use of stimulants, adverse effects associated with drug treatments, including ARVs (Valcour et al, 2011). Many ARVs display a low CNS penetration effectiveness (CPE) score (Letendre et al., 2008), i.e. reduced ability to cross the blood brain barrier (BBB) thus reaching subtherapeutic concentrations in the brain and maintaining a residual viral replication (Liner et al., 2010). In this regards, therapeutic regimens that include high CPE ARVs (>3) and an earlier initiation of HAART ($CD4^+$ counts > 350 cell/mm^3) may have neuroprotective effects (Smurzynski et al., 2011). However, caution should be used while adopting these therapeutic strategies, in consideration of some evidence showing the neurotoxic potential of ARVs (Marra et al., 2009; Robertson et al., 2010).

The molecular mechanisms underlying ARV neurotoxicity are not completely elucidated. Most of the available evidence suggests that the main mechanism of neuronal injury during HIV infection is indirect, through toxins, cytokines, reactive oxygen species (ROS) and nitric oxide (NO) released by activated glial cells in response to residual viral replication. In particular, NO derived from activated microglia appears to be a crucial mediator of neuronal injury in several neurological disease, including HIV infection (Saha and Pahan, 2006). Exaggerated NO production exerts cytotoxic effects, leading to protein modifications, glutathione depletion, DNA damage, and inactivation of the mitochondrial respiration chain (Bogdan et al., 2000). NO is produced from L-arginine in the presence of O2 by different NO synthase (NOS) isoforms. During inflammation, high amounts of NO are produced following the up-regulation of inducible NOS isoform (NOS2) in inflammatory activated cells. Once induced, NOS2 leads to sustain NO production, which is terminated by enzyme degradation, depletion of substrates, or cell death (MacMicking et al., 1997). Therefore, the availability of intracellular L-arginine is an important rate-limiting factor for NO production (Mori, 2007). L-Arginine is a substrate for the biosynthesis of proteins, creatinine and agmatine; it is mainly metabolized into

* Corresponding author. Tel.: +39 0630154367; fax: +39 06 233 235 103.
E-mail addresses: lucia.lisi@rm.unicatt.it (L. Lisi), antonella.tramutola@gmail.com (A. Tramutola), pnavarra@rm.unicatt.it (P. Navarra), cinzia.dellorusso@rm.unicatt.it (C. Dello Russo).

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NO and L-citrulline by NOS, and into urea and L-ornithine by Arginase I (ARG). It has been shown that ARG negatively regulates NOS activity by reduction of L-arginine availability in murine macrophages (Munder et al., 1999), and that this enzymatic pathway can be a crucial regulator of several immune functions (Bronte and Zanovello, 2005). Increased expression of ARG is found in a variety of infectious diseases, including tuberculosis (Zea et al., 2006) and leishmaniasis (Kropf et al., 2005). A highly significant inverse correlation exists between $CD4^+$ T cell counts and the level of ARG activity in peripheral blood mononuclear cells from naïve HIV-seropositive patients (Cloke et al., 2010a,b). However, such correlation is abrogated by HAART (Cloke et al., 2010a,b), thus supporting the hypothesis that ARVs may directly modulate ARG activity and thus NO production in inflammatory cells. Therefore, in the present study we carried out a screening of different ARVs for their potential of regulating NOS2 activity and expression in microglial cells, investigating the complex interplay between NOS2 and ARG activity.

2. Methods

2.1. Materials

Cell culture reagents [Dulbecco's modified Eagle's medium (DMEM), DMEM-F12 and Fetal calf serum (FCS)] were from Invitrogen Corporation (Paisley, Scotland). Antibiotics were from Biochrom AG (Berlin, Germany). The recombinant glycoproteins, $gp120_{CN54}$, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MA, USA). The rat recombinant pro-inflammatory cytokine, IFNγ, was purchased by Endogen (Pierce Biotechnology, Rockford, IL, USA). N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and L-arginine were purchased by Sigma-Aldrich (St. Louis, MO, USA). The ARG inhibitor, N_ω-hydroxy-nor-arginine (nor-NOHA) was purchased by Cayman Chemical (Ann Arbor, MI, USA). All ARVs as pure substances were provided by courtesy of the relevant marketing authorization holders.

2.2. Cell cultures

Primary enriched cultures of rat microglia cells, were prepared as previously described (Dello Russo et al., 2004). Briefly, microglial cells were detached from the astrocyte monolayer by gentle shaking. The cells were plated in 96-well plates at a density of 3×10^5 cells/cm^2 using 100 μL/well DMEM-F12 containing 10% FCS and antibiotics. Under these conditions, the cultures were 95–98% CD11b positive (Fig. 1). For immunocytofluorescent characterization of rat microglial cell cultures, cells were plated on coated glass coverslips (Nunc™ Lab-Tek™ II Chamber Slide™ System), kept in culture overnight and stained with mouse monoclonal primary antibody anti-rat CD11b (Serotec, Oxford, UK), diluted (1:250) in PBS-w containing 0.1% BSA and 0.2% Triton X100. Microglial activation was induced by incubating cells with 10 nM $gp120_{CN54}$ and 10 IU/ml IFNγ (gp120/IFNγ) (Lisi et al., 2012). Selected ARVs were tested in the therapeutic concentration ranges (Table 1).

2.3. Cell viability

At the end of each experiment microglial viability was assessed by measurement of the released lactate dehydrogenase (LDH), using the CytoTox-96 kit from Promega (Madison, WI), according to the manufacturer's instructions.

2.4. Nitrite assay

NO production was assessed indirectly by measuring nitrite accumulation in the incubation media by the Griess reaction (Lisi et al., 2012). In the absence of stimuli, basal levels of nitrites were below the detection limit of the assay (≥6.25 μM $NaNO_2$).

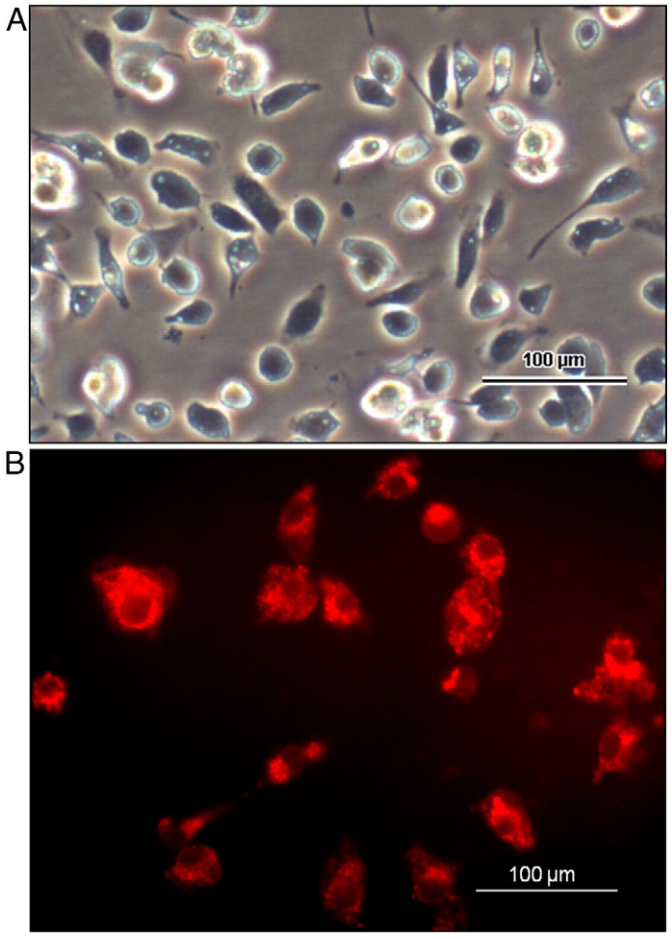

Fig. 1. Primary cultures of microglia cells. A) Morphology of a primary culture of rat microglia cells was observed under phase-contrast microscopy. B) Cells were stained with antibodies against the rat CD11b surface antigen.

2.5. DCF assay

Reactive free radicals were measured by using 2′,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Invitrogen). At the end of the experiment, the incubation medium was replaced by balanced salt solution [BSS, 124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, 20 mM Hepes, 0.3 mM $CaCl_2(H_2O)_2$] containing when indicated the NO production inhibitor, L-NAME and cells were incubated for 30 min at 37 °C. At the end of this pre-incubation period, 20 μM H_2DCF-DA was added to the cells, which were incubated for additional 45 min at 37 °C. The fluorescence signal due to H2DCF-DA oxidation within the cells was quantified using a spectrophotometric

Table 1
Drugs and dose-range administrated.

Class of drugs	Drug name	CSF concentration (nM)	Dose-range (nM)	CPE[a]
NRTI	Abacavir (ABC)	0.447	0.1–1	4
	Tenofovir (TNV)	0.018	0.01–0.1	2
PI	Atazanavir (ATV)	0.013	0.01–0.1	3
	Darunavir (DRV)	0.058	0.01–0.1	3
	Indinavir (IDV)	0.285	0.1–1	4
	Lopinavir (LPV)	0.028	0.01–0.1	3
	Ritonavir (RIT)	0.032	0.01–0.1	2
NNRTI	Efavirenz (EFV)	0.035	0.03–1	3
	Nevirapine (NVP)	3.500	30–1000	4

[a] CPE (central nervous system penetration effectiveness): 1(none), 2 (low), 3 (intermediate) and 4 (high) (Yilmaz et al., 2012).