Pharmacological Research 111 (2016) 23–33

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Increase of neurofilament-H protein in sensory neurons in antiretroviral neuropathy: Evidence for a neuroprotective response mediated by the RNA-binding protein HuD

M.D. Sanna[a], D. Peroni[b], T. Mello[c], C. Ghelardini[a], A. Quattrone[b], N. Galeotti[a,*]

[a] Laboratory of Neuropsychopharmacology, Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), Section of Pharmacology, University of Florence, Florence, Italy
[b] Laboratory of Translational Genomics, Centre for Integrative Biology, University of Trento, Trento, Italy
[c] Department of Biomedical, Experimental and Clinical Sciences, University of Florence, Florence, Italy

ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form 6 May 2016
Accepted 25 May 2016
Available online 26 May 2016

Keywords:
Antiretroviral
Neuropathy
HuD
BDNF
Neurofilament

ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) are key components of HIV/AIDS treatment to reduce viral load. However, antiretroviral toxic neuropathy has become a common peripheral neuropathy among HIV/AIDS patients leading to discontinuation of antiretroviral therapy, for which the underlying pathogenesis is uncertain. This study examines the role of neurofilament (NF) proteins in the spinal dorsal horn, DRG and sciatic nerve after NRTI neurotoxicity in mice treated with zalcitabine (2′,3′-dideoxycitidine; ddC). ddC administration up-regulated NF-M and pNF-H proteins with no effect on NF-L. The increase of pNF-H levels was counteracted by the silencing of HuD, an RNA binding protein involved in neuronal development and differentiation. Sciatic nerve sections of ddC exposed mice showed an increased axonal caliber, concomitantly to a pNF-H up-regulation. Both events were prevented by HuD silencing. pNF-H and HuD colocalize in DRG and spinal dorsal horn axons. However, the capability of HuD to bind NF mRNA was not demonstrated, indicating the presence of an indirect mechanism of control of NF expression by HuD. RNA immunoprecipitation experiments showed the capability of HuD to bind the BDNF mRNA and the administration of an anti-BDNF antibody prevented pNF-H increase. These data indicate the presence of a HuD – BDNF – NF-H pathway activated as a regenerative response to the axonal damage induced by ddC treatment to counteract the antiretroviral neurotoxicity. Since analgesics clinically used to treat neuropathic pain are ineffective on antiretroviral neuropathy, a neuroregenerative strategy might represent a new therapeutic opportunity to counteract neurotoxicity and avoid discontinuation or abandon of NRTI therapy.

1. Introduction

AIDS, a disease previously associated with rapid death, has become a chronic illness in the industrialized world where patients with HIV infection are surviving significant durations [1–3]. The introduction of nucleoside reverse transcriptase inhibitors (NRTIs) in combinations with other antiretrovirals, the combination antiretroviral therapy, dramatically reduced the morbidity and mortality associated with HIV among patients who have access to treatment [4]. Despite the initial positive impact of NRTIs, therapeutic experience revealed serious side effects. The use of NRTIs such as zalcitabine, didanosine, and stavudine is strongly associated with the development of a painful sensory neuropathy [5–8]. Furthermore, between 30% and 60% of individuals infected with HIV develop HIV-associated sensory neuropathy, a peripheral sensory neuropathy that is frequently painful and it is further aggravated by antiretroviral therapy [8], leading to discontinuation or abandon of NRTI treatment.

It may be reasonable to consider that as the AIDS epidemic continues and as survival with HIV infection is prolonged by treatment with combined antiretroviral therapy, long-term side effects of NRTIs may become increasingly common. There is,

Abbreviations: aODN, antisense oligonucleotide; BDNF, brain derived neurotrophic factor; ddC, 2′,3′-dideoxycitidine; DMSO, dimethylsulfoxide; DRG, dorsal root ganglia; i.p., intraperitoneal; i.t., intrathecal; LFB, luxol fast blue; NF-L, neurofilament light; NF-M, neurofilament medium; NF-H, neurofilament heavy; NRTI, nucleoside reverse transcriptase inhibitors; PKC, protein kinase C; SDH, spinal dorsal horn.

* Corresponding author at: Dept. Neurofarba, Viale G. Pieraccini 6, I-50139 Florence, Italy.
E-mail address: nicoletta.galeotti@unifi.it (N. Galeotti).

http://dx.doi.org/10.1016/j.phrs.2016.05.026
1043-6618/

therefore, an increasing need to elucidate the mechanism underpinning antiretroviral neurotoxicity and recovery processes in order to prevent sensory neuropathies and to maintain in the therapy this class of highly active antiretroviral drugs.

The neuronal cytoskeleton is composed of three interconnected structures: the actin microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs). Neurofilaments (NF) are the major IFs present in adult neurons and their expression is restricted to neuronal cell types. Neurons express differentially several IF proteins depending on their developing stage or their localization in the nervous system: nestin (200 kDa), three NF subunits called NF-L (light, 68 kDa), NF-M (medium, 160 kDa) and NF-H (heavy, 205 kDa), α-internexin (66 kDa), peripherin (57 kDa) and synemin (41 kDa) [9–12]. The main role recognized for NFs is to increase the axonal caliber of myelinated axons and consequently their conduction velocity. They also contribute to the dynamic properties of the axonal cytoskeleton during neuronal differentiation, axon outgrowth, regeneration and guidance [13]. Perturbations of their metabolism and organization are frequently associated with various neurodegenerative diseases [14]. Recently, an altered expression of NFs was also observed in peripheral neuropathies. Loss of pNF-H immunoreactivity was shown to correspond with the neurotoxicity of platinum derivatives [15,16]. Vincristine generates painful neuropathy by causing peripheral nerve abnormalities, including disorganization of axonal constitutive elements, such as NF-H [17]. Axotomy is followed by a strong down-regulation of NF mRNAs and proteins in the PNS [18,19] and CNS [20,21], leading to reduced levels of axonally transported NF in injured neurons [22,23]. However, the role of NF in antiretroviral-induced neurotoxicity is still not known.

The elucidation of the processes involved in nerve fibre dysfunction through the NRTI therapy is confounded in HIV-infected individuals by concomitant viral-induced nerve damage. We, therefore, used an animal model of pure NRTI-induced neuropathy. In the current study, we investigated the role of NF protein levels in dorsal root ganglia (DRG), spinal cord and sciatic nerve tissue in the response to antiretroviral toxic agents. To better elucidate the role of cytoskeletal proteins, the intracellular pathway involved in the modulation of NF expression was also investigated.

2. Materials and methods

2.1. Animals

Male CD1 mice (20–22 g) from the Harlan Laboratories (Bresso, Italy) breeding farm were used. Mice were randomly assigned to standard cages, with four to five animals per cage. The cages were placed in the experimental room 24 h before behavioural test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee of the University of Florence, Italy, under license from the Italian Department of Health and in compliance with the European Communities Council Directive of 22 September 2010 (2010/63/EU). All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals [24].

2.2. Animal treatment

2.2.1. Drug administration

Zalcitabine (2′,3′-dideoxycitidine; ddC) (Sigma, Milan, Italy) solution was freshly prepared in saline (0.9% NaCl) the day of the experiment. ddC- and saline-treated groups were given a one-time intraperitoneal (i.p.) injection of ddC (25 mg/kg) or saline, respectively. Experiments were performed on day 1 (absence of neuropathic pain), 3 and 7 (presence of neuropathic pain and neuronal damage).

The protein kinase C (PKC) blocker calphostin C (0.2 μg per mouse) (Calbiochem, Milan, Italy) was dissolved in 0.5% DMSO and administered intrathecally (i.t.), as previously described [25], 1 h before testing. Anti-BDNF was administered i.t. (1:100) 1 h before testing.

Doses and administration schedules were chosen on the basis of dose-response and time-course experiments performed in our laboratory.

2.2.2. Antisense oligonucleotide administration

Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were obtained from Tib Molbiol (Genoa, Italy). The antisense ODN (aODN) against PKCγ was the following: 5′-A*C*GAAGTCCGGGTTCACA*T*A-3′ where the asterisks indicate the phosphorotioate phosphate groups, its action was previously characterized by in vitro and in vivo experiments in our laboratory [26]. The aODN against HuD was the following: 5′-G*T*TCTGGAGCCTCATC*T*T-3′ where the asterisks indicate the phosphorotioate phosphate groups, its action was previously characterized by in vitro [27] and in vivo [28] experiments in our laboratory. A 18 and a 20mer fully degenerate ODNs (dODNs), where each base was randomly G, or C, or A, or T, were used as control treatment for anti-HuD and anti-PKCγ, respectively. aODNs and dODNs were preincubated at 37 °C for 30 min with an artificial cationic lipid (13 μM *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate, DOTAP, Sigma, Milan, Italy), to enhance both uptake and stability, before administration. To achieve the HuD protein knockdown, mice received a single i.t. injection every 24 h on day 1–3 for a total of 3 injections, as previously reported [27]. The spinal cord and DRG were removed on day 3, 12 h after the last injection, and on day 7. Sciatic nerve samples were removed on day 21 and, to achieve the HuD protein knockdown at this time point, anti-HuD was administered every 5 days for 4 times.

2.3. Western blot analysis

The lumbar spinal cord and DRG were removed 1, 3 and 7 days after ddC administration. Samples were homogenized in an homogenization buffer containing 25 mM Tris-HCl pH = 7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPP, 4 mM pNFF, 1 mM Na3VO4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9000g for 15 min at 4 °C, the low speed pellet was discarded. Protein concentration was quantified using the BiCinchoninc Acid (BCA; Sigma-Aldrich, Italy) assay.

Membrane homogenates (10–50 μg) were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes (90 min at 120 V) using standard procedures. Membranes were blocked in PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 120 min. Following washes, blots were incubated overnight at 4 °C with specific antibodies against neurofilament L (NF-L, 1:1000), neurofilament M (NF-M, 1:1000), neurofilament H phosphorylated in Ser 1 (pNF-H, 1:1000), BDNF (1:50) HuD (1: 1000); PKCγ (1:1000); glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (1:5000) (Santa Cruz Biotechnology Inc, CA, USA). After having being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with horseradish peroxidase-conjugated secondary antisera (1:5000) and left for 2 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using an enhanced chemiluminescence detection system (Pierce, Milan, Italy). The exposition and developing time used were standardized for all the