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Spinal RyR2 pathway regulated by the RNA-binding protein HuD induces pain hypersensitivity in antiretroviral neuropathy

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ABSTRACT

The antiretroviral toxic neuropathy, a distal sensory polyneuropathy associated with antiretroviral treatment, is a frequently occurring neurological complication during treatment of patients with AIDS and often leads to discontinuation of antiretroviral therapy. The mechanisms by which antiretroviral drugs contribute to the development of neuropathic pain are not known. Using drugs that reduce intracellular calcium ions (Ca^{2+}), we investigated the hypothesis that altered cytosolic Ca^{2+} concentration contributes to the 2′,3′-dideoxycytidine (ddC)-evoked painful neuropathy. Administration of ddC induced mechanical and cold allodynia, which were abolished by intrathecal administration of TMB-8, a blocker of Ca^{2+} release from intracellular stores, and by ryanodine, a RyR antagonist. Treatment with the IP3R antagonist heparin prevented mechanical allodynia with no effect on thermal response. To further clarify the pathway involved, we investigated the role of HuD, a RNA binding protein involved in neuronal function. HuD silencing reverted both mechanical and cold allodynia inducing, a phenotype comparable to that of ryanodine-exposed mice. HuD binding to the RyR2 mRNA, the most abundant RyR isoform in the spinal cord, was demonstrated and RyR2 silencing prevented the ddC-induced neuropathic pain. A positive regulation of gene expression on CaMKIIα by HuD was also observed, but sequestration of CaMKIIα had no effect on ddC-induced allodynia. The present findings identify a spinal RyR2 pathway activated in response to ddC administration, involving the binding activity on RyR2 mRNA by HuD. We propose the modulation of the RyR2 pathway as a therapeutic perspective in the management of antiretroviral painful neuropathy.

Introduction

The introduction of combination antiretroviral therapy (cART) dramatically reduced the morbidity and mortality associated with HIV among patients who have access to treatment (Mocroft et al., 2003). Life expectancy with HIV in well-resourced countries is now estimated to be up to two-thirds that of the general population (Lohse et al., 2007). While the incidence of most neurological complications of HIV has fallen with the introduction of effective therapy (Mocroft et al., 2003), rates of HIV-associated distal sensory neuropathy (HIV-SN) have been rising since the first effective antiretroviral drugs were developed (Bacellar et al., 1994; Ellis et al., 2010).

HIV-SN is comprised of at least two clinically indistinguishable, and often coexisting, neuropathies: a distal sensory polyneuropathy associated with HIV disease itself (HIV-DSP), and a distal sensory polyneuropathy associated with antiretroviral treatment, antiretroviral toxic neuropathy (HIV-ATN) (Kamerman et al., 2012; Kokotis et al., 2013). HIV-DSP was recognized early in the HIV pandemic (Lipkin et al., 1985) and is associated with advanced HIV disease (Smyth et al., 2007; Barohn et al., 1993). HIV-ATN was initially observed following the introduction of certain nucleoside reverse transcriptase inhibitors (NRTIs), and patients receiving NRTI therapy develop a distal symmetric small fiber 'dying back' axonal neuropathy (Dalakas, 2001; Simpson, 2002). Neuropathic pain associated with the use of NRTIs is clinically quite common with a greater prevalence than that reported for other common types of peripheral neuropathy (Morgello et al., 2004; Maritz et al., 2010). Unfortunately, analgesics used in other forms of neuropathic pain have proven ineffective for painful HIV-SN (Phillips et al., 2010), leading the HIV-SN associated pain to become a major impact on quality of life in otherwise largely healthy individuals. There is an urgent need to better understand the pathogenesis of HIV-SN, to identify and implement effective pain management strategies.

Abbreviations: aODN, antisense oligonucleotide; CaMKII, Ca^{2+}-calmodulin-dependent kinase II; ddC, 2′,3′-dideoxycitidine; dODN, degenerate oligonucleotide; ELAV protein, embryonic lethal abnormal vision protein; HIV-SN, HIV-associated sensory neuropathy; i.p., intraperitoneal; IP3Rs , inositol 1,4,5-trisphosphate receptors; i.t., intrathecal; NRTIs , nucleoside reverse transcriptase inhibitors; PKC, protein kinase C; RBP, RNA binding protein; RyRs , ryanodine receptors; SDH, spinal dorsal horn.

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Ca^{2+} is a key regulator of major cellular processes. Its cytosolic concentration is determined mainly by extracellular Ca^{2+} influx, release of Ca^{2+} from internal stores, and mitochondrial uptake. An increase in cytosolic Ca^{2+} concentration mediates a wide range of neuronal functions including membrane excitability, neurotransmitter release, synaptic plasticity, gene expression, and excitotoxicity (Berridge et al., 2000). Abnormal neuronal Ca^{2+} homeostasis has been implicated in numerous diseases of the nervous system, including neuropathic pain and diabetic polyneuropathy (Fernyhough and Calcutt, 2010), and blockers of voltage-gated Ca^{2+} channels, such as gabapentin and pregabalin, represent effective treatments for neuropathic pain (Chen and Mao 2013; Pexton et al., 2011). Even if the role of altered cytosolic Ca^{2+} concentration has been investigated in trauma- and chemotherapy-induced neuropathic pain, little is known on its involvement in antiretroviral neuropathy. The aim of the present study is to determine the role of intracellular calcium modulation in an animal model of antiretroviral neuropathy.

To better elucidate the signaling pathway involved in the antiretroviral toxic neuropathy, we focused on the ELAV proteins, a family of RNA-binding proteins (RBPs) binding to U-rich and AU-rich elements prevalently in the 3′-untranslated regions (UTRs) of target mRNAs, enhancing their stability and/or allowing increased protein production. In mammals, there are four known members of the ELAV family including the ubiquitously expressed HuR and the neuron-specific HuB, HuC and HuD (nELAV proteins). nELAV proteins are highly enriched in neurons and promote the trafficking and translation of specific mRNAs involved in neuronal differentiation, axonal outgrowth and synaptic plasticity (Pascale et al., 2008; Hinman and Lou 2008). Out of the neuronal ELAV proteins, HuD is emerging as a central master regulator of neuronal development and plasticity. In animal models, HuD knockout has been associated to anomalies in nerve development and functional motor defects (Akamatsu et al., 2005). Furthermore, expression of HuD is increased in the DRG and sciatic nerve in an animal model of trauma-induced neuropathic pain (Anderson et al., 2003, Yoo et al., 2013) and, recently, we found it overexpressed at spinal and supraspinal levels in antiretroviral neuropathy (Sanna et al., 2014a, 2014b). On these bases, we investigated the role of HuD in the signaling pathway involved in antiretroviral neuropathy.

Materials and methods

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 behavioral 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 24 November 1986 (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE (McGrath et al., 2010).

Behavioral testing

Mechanical allodynia

Mechanical allodynia was measured by using Dynamic Plantar Anesthesiometer (Ugo Basile). The mice were placed in individual Plexiglas cubicles (8.5 cm L 3,4 cm H 3,4 cm) on a wire mesh platform and allowed to acclimate for approximately 1 h, during which exploratory and grooming activity ended. After that, the mechanical stimulus was delivered to the plantar surface of the hind paw of the mouse from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronic ascending force (0–5 g in 35 s). When the animal withdrew its hind paw, the mechanical stimulus was automatically withdrawn and the force recorded to the nearest 0.1 g. Nociceptive response for mechanical sensitivity was expressed as mechanical paw withdrawal threshold. Each mouse served as its own control, the responses being measured both before and after ddC administration. PWT was quantified by an observer blinded to the treatment.

Cold allodynia

For assessment of cold allodynia, mice were placed on a cold plate that is maintained at a temperature of 4 ± 0.1 °C. Reaction times (s) were measured with a stopwatch before and 1, 2, 4, 5, 7 and 10 days after administration of ddC. The time between placements of a mouse on the plate and licking or lifting of a hind paw was measured with a digital timer. An arbitrary cut-off time of 60 s was adopted.

Locomotor activity

The locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 5 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous mobility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. Experiments were performed 3 days after administration of ddC.

Western blot analysis

The lumbar spinal cord (L4–L6 segment) and DRG were removed 3 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 Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9000 ×*g* for 15 min at 4 °C, and the low speed pellet was discarded. The supernatant (whole cell lysate) was centrifuged at 100,000 ×*g* for 60 min at 4 °C. The resulting supernatant was the cytosol fraction. Protein concentration was quantified using the Bradford's method (protein assay kit, Bio-Rad Laboratories, Milan, Italy). Homogenates (10–20 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 HuD (1:1000); CaMKIIα (1:1000); glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (1:5000) (Santa Cruz Biotechnology Inc., CA, USA). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:5000) and left for 1 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). Exposition and developing time used was standardized for all the blots. Optical density measurements were performed by dividing the intensity of the bands by the intensity of the housekeeping protein GAPDH at each time point. Measurements in control samples were assigned a relative value of 100%.

Immunofluorescence

Animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). Lumbar spinal cords and DRG were removed, postfixed in 4% paraformaldehyde (2 h at 4 °C) and