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NOS-mediated morphological and molecular modifications in rats infused with Aβ (1-40), as a model of Alzheimer's disease, in response to a new lipophilic molecular combination codrug-1

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ABSTRACT

Alzheimer's disease is a neurodegenerative pathology due to the presence of β-amyloid plaques at brain level and hippocampus level and associated with the loss of memory speech and learning. At the basis of these effects lie molecular mechanisms which include nitric oxide metabolic pathway, whose involvement in the occurrence of morphological modifications related to such neurodegenerative process is suggested. Current evidences show that the non-steroidal anti-inflammatory drug ibuprofen posses a protective effect against the development of the disease, substantially delaying its onset; furthermore (R)-α-lipoic acid seems to have an antioxidant ameliorating effect on disease progression. Starting from these data, a new lipophilic codrug 1, obtained by joining an antioxidant molecule with an NSAID, has been previously synthesized. Our aim has been to investigate the possible therapeutical effects of codrug 1, compared to ibuprofen, on the molecular events at the basis of behavioural and morphological modifications occurring in Aβ (1-40) infused rat brains. Ibuprofen and codrug 1 seem to protect the subject against memory performance impairment and against behavioural detriment, induced by administration of Aβ (1-40) peptide. Such evidences are supported by morphological and biochemical findings showing Aβ (1-40) to determine cell disorganization, increased number of β-amyloid plaques and capillary vessels dilatation in parallel to increased total and specific NOS activity and to apoptosis occurrence, partly prevented by ibuprofen, more broadly by codrug 1. Such results underline the involvement of nitric oxide metabolic pathway in the events related to the onset of this pathology and suggest codrug 1 as a useful tool to protect the brain against cognitive and behavioural dysfunction, by reducing β-amyloid plaques formation and by inhibiting NOS signalling pathway and apoptosis occurrence.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative pathology associated with the loss of cognitive abilities such as memory, speech and computing, determining the dementia in the elderly (Parihar and Hemmani, 2004). Even though a certain diagnosis of such disorder can be performed only by autopsy, which has revealed cortical atrophy, increased size of ventricles associated to the presence of neurofibrillary tangles (NFTs) and of β-amyloid plaques (AP) (Zetterberg et al., 2010; Bateman et al., 2007), a number of possible mechanisms, including oxidative stress, exocytotoxicity, energy depletion, inflammation and apoptosis, which trigger the amyloid deposition and thus the cause of neuronal death, have been considered (Leslie, 2002; Torreilles and Touchan, 2002). Obviously at the basis of these effects lie molecular mechanisms which, determining morphological modifications, lead to the occurrence of such neurodegenerative process. Among the mechanisms proposed intracellular calcium accumulation, reactive oxygen species (ROS) and nitric oxide (NO) production, inflammatory or autoimmune processes, which lead to apoptosis occurrence, are

Abbreviations: AD, Alzheimer's disease; AP, amyloid plaques; APP, amyloid precursor protein; BBB, blood–brain barrier; CNS, central nervous system; COX, cyclooxygenase; DAB, diaminobenzidine chromogen; IBU, ibuprofen; LA, R-(α)-lipoic acid; NFTs, neurofibrillary tangles; NGS, normal goat serum; NO, nitric oxide; NOS, nitric oxide synthase; NSAIDs, non-steroidal anti-inflammatory drugs; RAM, arm radial maze; RME, reference memory errors; RNS, reactive nitrogen species; ROS, reactive oxygen species; TUNEL, terminal-deoxynucleotidyl-transferase-mediated dUTP nick end labeling.

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included (Smith et al., 1996; Multhaup et al., 1997; Drouet et al., 2000; Vajda, 2002). The central nervous system (CNS) is not only specially vulnerable to oxidative stress, which, determining the intracellular production of ROS and reactive nitrogen species (RNS) in the brain, induces DNA damage and initiates the cell death program, but also shows a decreased capability of repairing the oxidative damage and the accumulation of DNA errors considered the main factors in the progression of neuronal loss in AD. Both intracellularly and extracellularly generated ROS and RNS can activate the NO signalling pathway (Fialkow et al., 2007), which in the last years has been recognized to play a key role in the response of the CNS (Pepicelli et al., 2004). In neurons, NO is mainly formed by the calcium-dependent activation of neuronal nitric oxide synthase (NOS) (Gow and Ischiropoulos, 2001), although the endothelial isoform of this enzyme (eNOS), involved in the control of blood pressure, vascular remodelling and angiogenesis (Shaul, 2002), can be functionally expressed. Lastly inducible NOS (iNOS) is expressed in the brain both in astrocytes and neurons. NO, by interacting with mitochondria, is considered a major messenger molecule in mammals involved in blood vessel dilatation, immune function and neurotransmission in the brain and in the peripheral nervous system (Torreilles, 2001). In fact elevated NO concentrations can interact with superoxide anion, generated by mitochondria or by other mechanisms, leading to the formation of peroxynitrite and its over-production may cause neuronal energy compromise, leading to neurodegeneration (Moncada and Bolanos, 2006). In addition, over-activation of NOS enzymes leads to the production of nitrated protein aggregates highly toxic to neurons, promoting, in turn, neurodegeneration (Chung and David, 2010).

Among the therapeutic strategies set up on the basis of the knowledge of the molecular mechanisms of AD pathogenesis, the most used is the non-steroidal anti-inflammatory drug (NSAIDs) ibuprofen (IBU), which seems to protect against the development of the disease substantially delaying its onset through inhibition of pro-amyloidogenic factors (Davis, 2002; Vlad et al., 2008), even though it has a marginal efficiency in crossing the blood–brain barrier (BBB). Weggen et al., investigating the effect of various NSAIDs on the production of Aβ (1-42) in cell culture, reported that not all NSAIDs affected its production, noting that it seemed not to be mediated by inhibition of cyclooxygenase (COX) activity, the principal pharmacological target of NSAIDs (Weggen et al., 2001). In particular, these investigations have found that IBU and indomethacin reduced Aβ (1-42) production, while naproxen and aspirin did not have the same effect. The proposed mechanism for this activity is an allosteric modulation of γ secretase activity, the enzyme that mediates the final proteolytic cleavage of amyloid precursor protein (APP), which liberates β-amyloid (1-42) peptide (Miners et al., 2008; Leuchtenberger et al., 2006; Hirohata et al., 2005). These NSAIDs exhibit a window modulation where β-amyloid (1-42) production is greatly reduced without inhibition of Notch receptors, the key factor of mechanism-based side effects associated with γ-secretase inhibitors treatment.

Also (R)-α-lipoic acid (LA) has been used in trials to prevent AD, basing on its antioxidant ameliorating effect on the progression of the disease (Reljanovic et al., 1999). Starting from these evidences in Di Stefano's Lab a lipophilic molecular combination (codrug 1), obtained by joining an antioxidant molecule with an NSAID, has been synthesized (Sozio et al., 2010). This new codrug seemed to protect against behavioural detriment induced by simultaneous administration of β-amyloid (1-40) protein.

Thus our aim has been to investigate the molecular events which are at the basis of behavioural and morphological modifications occurring in Aβ (1-40) infused AD rat model (Bateman et al., 2007) and the possible therapeutical effects of IBU, used in clinical trials to prevent AD (Pignatello et al., 2006, 2008), compared to the effects of newly synthesized codrug 1, potential antagonist of deleterious structural and cognitive effects of such pathology (Packer et al., 1997; Wang et al., 2001; Di Stefano et al., 2010). In addition, in human AD patients, different molecular species of Aβ peptide have been identified. It appears that the form (1-42) is the predominant one deposited during the initial stages of plaque formation whereas Aβ (1-40) is the predominant species in advanced stages of the disease, which are related to severe cognitive decline. In our protocol we have selected the molecular species (1-40) because it has a higher affinity to form amyloid fibrils in rats, and its neurodegenerative effect has been evidenced more pronounced within the CA1 subfield of the hippocampus than Aβ (1-42) (Nag et al., 1999). CA areas are filled of densely packed pyramidal cells similar to those formed in neo-cortex (Miguel-Hidalgo and Cacabelos, 1998).

2. Materials and methods

2.1. Animals

Male Wistar rats ($n=42$) (Harlan, UD, Italy), that weighed 200–225 g at the beginning of the experiments, have been used. The animals have been individually housed in a room on a 12:12-h light/dark cycle (lights off at 7:00 A.M.) at constant temperature (20–22 °C) and humidity (45–55%). Rats have been offered food pellets (4RF; Mucedola, Settimo Milanese, Italy) and tap water ad libitum. All procedures have been conducted in adherence to the European Community Council Directive for Care and Use of Laboratory Animals and in accordance with Local Ethical Committee.

2.2. Drug preparation and administration

Aβ (1-40) peptide (Bachem, Switzerland) has been dissolved in sterile saline with 35% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid. IBU and codrug 1 have been both solubilized in sterile saline containing 20% (v/v) DMSO and daily administered in parallel to different animals subcutaneously (s.c.) for 28 days at a dose of 5 mg/kg and 10 mg/kg, respectively. A vehicle solution (vehicle for subcutaneous injections) prepared with sterile saline containing 20% (v/v) DMSO or a sterile saline alone has been also administered s.c. for 28 days at a dose volume of 250 μL/kg as IBU (Fujisawa et al., 2005) and codrug 1 (Sozio et al., 2010). The trial has been performed for 2 months after which both cognitive and morphological tests have been made.

2.3. Surgical procedure

The rats have been anesthetized with a mixture of zolazepam and tiletamine (10 mg/kg i.p.) (Zoletil 100, Italmed, Italy). Continuous infusion of Aβ peptide (1-40) solution (4.6 nmol/rat at a final volume of 200 μL) or the vehicle alone has been delivered for 28 days by attachment of an infusion kit connected to an osmotic pump (Alzet model 2004, Charles River, Italy). The infusion kit has been implanted into the right cerebral ventricle (1.0 mm posterior to the bregma, 1.8 mm lateral to the midline, and 3.5 mm ventral to the surface of the skull), according to the brain atlas of Paxinos and Watson (Paxinos and Watson, 1997). Aβ peptide (1-40) cerebrospinal infusion and s.c. drug treatments have been delivered over the same period of time.

2.4. Behavioural training

The behavioural test has been performed 1 month after the last day of Aβ peptide (1-40) infusion corresponding to 2 months elapsed from the implantation of the infusion kit.

The 8-arm radial maze (RAM) had a platform (diameter 30 cm), 8 enclosed arms (51 cm long × 11 cm × 11 cm high) separated from the central platform with guillotine doors, and a small compartment located at the end of each arm containing fresh food in order to saturate each arm with food odours. The animals have been food-deprived (maintained at 85–90% of free-feeding body weight). The task itself was a simplified version of Stepanichev's RAM task (Stepanichev et al., 2006)