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Time-course correlation of early toxic events in three models of striatal damage: Modulation by proteases inhibition

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ABSTRACT

Metabolic alterations in the nervous system can be produced at early stages of toxicity and are linked with oxidative stress, energy depletion and death signaling. Proteases activation is responsible for triggering deadly cascades during cell damage in toxic models. In this study we evaluated the early time-course of toxic events (oxidative damage to lipids, mitochondrial dysfunction and LDH leakage, all at 1, 3 and 6 h) in rat striatal slices exposed to quinolinic acid (QUIN, 100 μM) as an excitotoxic/pro-oxidant model, 3-nitropropionic acid (3-NP, 1 mM) as an inhibitor of mitochondrial succinate dehydrogenase, and a combined model produced by the co-administration of these two toxins at subtoxic concentrations (21 and 166 μM for QUIN and 3-NP, respectively). In order to further characterize a possible causality of caspases or calpains on the toxic mechanisms produced in these models, the broad calpain inhibitor IC1 (50 μM), and the pan-caspase inhibitor Z-VAD (100 μM) were tested. Lipid peroxidation (LP) was increased at all times and in all models evaluated. Both IC1 and Z-VAD exerted significant protection against LP in all models and at all times evaluated. Mitochondrial dysfunction (MD) was consistently affected by all toxic models at 3 and 6 h, but was mostly affected by 3-NP and QUIN at 1 h. IC1 differentially protected the slices against 3-NP and QUIN at 1 h and against QUIN at 3 h, while Z-VAD exhibited positive actions against QUIN and 3-NP at all times tested, and against their combination at 3 and 6 h. LDH leakage was enhanced at 1 and 3 h in all toxic models, but this effect was evident only for 3-NP + QUIN and 3-NP at 6 h. IC1 protected against LDH leakage at 1 h in 3-NP + QUIN and 3-NP models, at 3 h in all toxic models, and at 6 h in 3-NP + QUIN and 3-NP models. In turn, Z-VAD protected at 1 and 6 h in all models tested, and at 3 h in the combined and QUIN models. Our results suggest differential chronologic and mechanistic patterns, depending on the toxic insult. Although LP, MD and membrane cell rupture are shared by the three models, the occurrence of each event seems to obey to a selective recruitment of damaging signals, including a differential activation of proteases in time. Proteases activation is likely to be an up-stream event influencing oxidative stress and mitochondrial dysfunction in these toxic models.

1. Introduction

The administration of quinolinic acid (QUIN) or 3-nitropropionic acid (3-NP) to rodents and non-human primates produce useful experimental models of Huntington's disease (HD) and other neurodegenerative disorders. QUIN is a known agonist of the N-methyl-D-aspartate receptor (NMDAr) and typically produces excitotoxic damage (Beal et al., 1986; Schwarcz, 1983; Stone, 1993, 2001). In addition, the toxin has been involved in the pathogenesis of different neurological disorders such as Alzheimer's disease, AIDS-dementia complex, hepatic encephalopathy and Huntington's disease itself (Sardar et al., 1995; Heyes et al., 1996; Walsh et al., 2002; Guidetti et al., 2004; Guillemin et al., 2005). Although its toxic effect on neuronal populations has been mostly attributed to excitotoxic damage, it has also been demonstrated to be responsible for progressive mitochondrial alteration, resulting in

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inhibition of complexes II, III and II–III at the electron transport chain, with the consequent reduction of ATP levels and further neurodegeneration (Bordelon et al., 1997; Ribeiro et al., 2006; Schuck et al., 2007). Oxidative stress has been demonstrated to be an integral part of the mechanism of QUIN toxicity (Santamaría and Rios, 1993; Behan et al., 1999; Santamaría et al., 2001), not only implying NMDAr-dependent and -independent reactive oxygen and nitrogen species (ROS and RNS) formation, but also inducing major alterations in endogenous antioxidant systems and capacity (Rodríguez-Martínez et al., 2000; Leipnitz et al., 2005).

On the other hand, 3-NP is a mitochondrial micotoxin capable of irreversibly inhibiting succinate dehydrogenase (SDH) (Complex II), thereby causing prolonged energy impairment. The hypothesis of indirect or "secondary" excitotoxicity suggests that 3-NP-induced striatal degeneration is due in first place to depletion in ATP levels produced by a deficit in energy metabolism, further leading to membrane depolarization and sustained voltage-gated NMDAr activation by primary alteration of membrane Na^+, K^+-ATPases (Novelli et al., 1988; Albin and Greenamyre, 1992; Beal et al., 1993). Under these conditions, even low levels of extracellular glutamate may produce neuronal necrotic death (Behrens et al., 1995; Pang and Geddes, 1997). Altogether, these findings have served to suggest that a cascade of toxic events related with enhanced Ca^{2+} influx – via NMDAr overactivation – may play a relevant role for cell damage following a toxic insult that involves deficient energy metabolism and excitotoxicity, but the question still remains on whether alternative toxic mechanisms might be contributing to the toxic patterns elicited by these models.

Interestingly, at this time there is no full characterization available in the literature in regard to the early temporal course of toxic events following the co-administration of these toxins. Some valuable approaches of toxic facilitation describe cellular alterations generated by the administration of functional glutamate analogues + electron chain transport inhibitors (i.e., NMDA, AMPA or glutamate itself plus malonate, or QUIN + malonate) (Greene and Greenamyre, 1995; Bazzett et al., 1996); however, the model of toxic facilitation induced by these two specific toxins is particularly relevant as a potential phenotypic model of HD given the metabolic proximity of the kynurenine pathway and its metabolites (including QUIN) to this disorder (Guidetti et al., 2004; revised in Pérez-De La Cruz et al., 2007), as well as the capacity of 3-NP to resemble the metabolic deficiencies observed in HD in animal models (Ramaswamy et al., 2007; Túnez and Santamaría, 2009). In addition, a recent characterization of the role of intracellular Ca^{2+} in nerve endings exposed to these toxins when administered separately or together, revealed that this cation, when released from intracellular sources, is mostly responsible of producing oxidative damage to synaptosomes (Pérez-De La Cruz et al., 2008). Since Ca^{2+} can also be responsible for deadly signaling associated with proteases (mostly calpains), an immediate interest on evaluating the time-course of toxic events linked to activation of proteases in these models emerged.

Caspases and calpains are cysteine proteases with important roles in execution of cell death processes. Indeed, the intracellular calcium deregulation from endoplasmic reticulum seems to be related with excitotoxicity (Fernandes et al., 2008) and proteases activation (Mattson, 2003). In addition, calpains are implicated in disease and injury models where intracellular calcium is a feature of necrotic cell death. In turn, calcium-activated calpain can cleave target proteins which can directly or indirectly lead to cell rupture (Harwood et al., 2005). Moreover, calpains are known to cleave several important apoptosis modulators and to cross-talk with caspase cascades (Harwood et al., 2005). In this regard, it has been shown that the stimulation of NMDAr in cultured cortical neurons induces death by apoptotic or necrotic mechanisms, depending on the intensity of the insult (Bonfoco et al., 1995). Furthermore, the role of caspases and calpains has been only moderately investigated in both the QUIN and 3-NP toxic models. For instance, it was established by Qin et al. (2000) that a caspase-3-like protease contributes to the QUIN-induced apoptosis of striatal neurons via NF-κB activation. In addition, the caspase-1 contribution to cell death in the QUIN model has also been described (Cao et al., 2005). Only few reports more on this topic are available (Pérez-Navarro et al., 2005). In regard to calpains, the potential use of calpain inhibitors to ameliorate the excitotoxic neuronal damage has been described by Higuchi et al. (2005). Regarding the 3-NP model, it has been shown that activation of caspase 3, calpains and LDH leakage under *in vitro* and *in vivo* conditions occur in this model, where calpains were the major effectors for neuronal death (Almeida et al., 2004; Nasr et al., 2003; Bizat et al., 2003). Other studies have shown that activation of caspases and/or calpins is an event entirely dependent on the experimental conditions (dosage, time of exposure, etc.) employed in the 3-NP toxic model (Almeida et al., 2004; Nasr et al., 2003). In 2006, Jacquard et al. evaluated the potentiation of QUIN-induced cell death by 3-NP, that was associated with increased calpain activity and massive calpain-mediated cleavage of several postsynaptic proteins, suggesting major neuronal Ca^{2+} deregulation in the striatum. However, to our knowledge, no further approaches on the role of calpains and caspases in these models, and their toxic implications, have been described. Therefore, in order to provide further and complementary information on the toxic mechanisms elicited by proteases activation in the neurotoxic models described above, in this study we evaluated the early time-course of toxic responses, cell damage and death (characterized as oxidative damage to membrane lipids, mitochondrial dysfunction and lactate dehydrogenase leakage, respectively) in an integral biological preparation – striatal slices – in direct relation to the activation of caspases and calpains, and their pharmacological modulation by broad-spectrum inhibitors. Our results revealed a differential pattern of toxicity exerted in time, as well as a differential role of proteases, for each model tested.

2. Experimental procedures

2.1. Animals

Striatal slices were obtained from the brains of male Wistar rats (280–320 g) provided by the vivarium of the Instituto Nacional de Neurología y Neurocirugía (Mexico City). A total of 48 rats were employed throughout the study. Before they were assigned to the experiments, animals were housed five-per cage in polycarbonate cages and provided with standard commercial rat diet (Laboratory rodent diet 5001, PMI Feeds Inc., Richmond, IN, USA) and water *ad libitum*. Housing room was maintained under constant conditions of temperature (25 ± 3 °C), humidity (50 ± 10%) and lighting (12 h light/dark cycles, light on at 7:00 a.m.). To obtain the brains, rats were anesthetized with ether and immediately killed by decapitation. Brains were then immersed into an ice-cooled buffer-HEPES solution (pH 7.0) and collected for further processing. All procedures with animals were carried out according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and the local guidelines on the ethical use of animals from the Health Ministry of Mexico. All efforts were made to minimize animal suffering (pain and discomfort).

2.2. Reagents

3-NP, QUIN, thiobarbituric acid (TBA), malonaldehyde (MDA), 3-[4,5-dimethylthiazol]-2,5diphenyl tetrazolium bromide (MTT), Inhibitor of Calpain-1 (IC1, N-acetyl-Leu-Leu-norleucine 1), β-NADH and piruvate were all obtained from Sigma Chemical Company (St. Louis, MO, USA). The pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) was from Calbiochem (CA, USA). All other reagents were reactive grade and obtained from known commercial suppliers. Solutions were prepared using deionized water obtained from a Milli-RQ purifier system from Millipore (Billerica, MA, USA).

2.3. Preparation and incubation of striatal slices

Striatal slices (200–300 nm of thickness) were collected fresh from both hemispheres of rat brains using a tissue chopper, and immediately transferred into an ice-cooled HEPES medium (pH 7.4, 5 °C) containing NaCl 0.1 M + NaH_2PO_4