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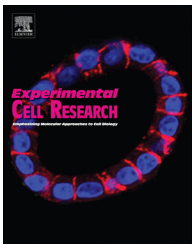

Research Article

The phosphorylation status and cytoskeletal remodeling of striatal astrocytes treated with quinolinic acid

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ABSTRACT

Quinolinic acid (QUIN) is a glutamate agonist which markedly enhances the vulnerability of neural cells to excitotoxicity. QUIN is produced from the amino acid tryptophan through the kynurenine pathway (KP). Dysregulation of this pathway is associated with neurodegenerative conditions. In this study we treated striatal astrocytes in culture with QUIN and assayed the endogenous phosphorylating system associated with glial fibrillary acidic protein (GFAP) and vimentin as well as cytoskeletal remodeling. After 24 h incubation with 100 μM QUIN, cells were exposed to ^{32}P-orthophosphate and/or protein kinase A (PKA), protein kinase dependent of Ca^{2+}/calmodulin II (PKCaMII) or protein kinase C (PKC) inhibitors, H89 (20 μM), KN93 (10 μM) and staurosporin (10 nM), respectively. Results showed that hyperphosphorylation was abrogated by PKA and PKC inhibitors but not by the PKCaMII inhibitor. The specific antagonists to ionotropic NMDA and non-NMDA (50 μM DL-AP5 and CNQX, respectively) glutamate receptors as well as to metabotropic glutamate receptor (mGLUR; 50 μM MCPG), mGLUR1 (100 μM MPEP) and mGLUR5 (10 μM 4C3HPG) prevented the hyperphosphorylation provoked by QUIN. Also, intra and extracellular Ca^{2+} quelators (1 mM EGTA; 10 μM BAPTA-AM, respectively) prevented QUIN-mediated effect, while Ca^{2+} influx through voltage-dependent Ca^{2+} channel type L (L-VDCC) (blocker: 10 μM verapamil) is not implicated in this effect. Morphological analysis showed dramatically altered actin cytoskeleton with concomitant change of morphology to fusiform and/or flattened cells with retracted cytoplasm and disruption of the GFAP meshwork, supporting misregulation of actin cytoskeleton. Both hyperphosphorylation and cytoskeletal remodeling were reversed 24 h after QUIN removal. Astrocytes are highly plastic cells and the vulnerability of astrocyte cytoskeleton may have important implications for understanding the neurotoxicity of QUIN in neurodegenerative disorders.

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Introduction

Kynurenine pathway (KP) catabolises the essential amino acid L-tryptophan to nicotinamide adenine dinucleotide. KP also leads to the production of several neuroactive metabolites, of which the NMDA receptor agonist, quinolinic acid (QUIN) is likely to be more important in terms of biological activity [1]. Dysregulation of this pathway is associated with neurodegenerative conditions, such as Huntington's disease [2], Alzheimer's disease (AD) [3,4] and other neurological disorders, as well as with psychiatric diseases such as depression and schizophrenia [5].

QUIN is produced and released by infiltrating macrophages and activated microglia, the very cells that are prominent during neuroinflammation. QUIN acts as an agonist of the *N*-methyl-D-aspartate (NMDA) receptor and as such is considered to be a brain endogenous excitotoxin [6]. The primary mechanism exerted by QUIN in the central nervous system (CNS) has been largely related with the overactivation of NMDA receptors and increased cytosolic Ca^{2+} concentrations, followed by mitochondrial dysfunction, cytochrome *c* release, ATP exhaustion, free radical formation and oxidative damage [6,7].

In this context, Rahman et al. [8] described a NMDA-mediated role of QUIN in the AD pathology through promotion of tau phosphorylation, since QUIN in pathophysiological concentrations is co-localized with hyperphosphorylated tau within cortical neurons in AD brain. Accordingly, we have previously described QUIN-elicited hyperphosphorylation of glial fibrillary acidic protein (GFAP) in astrocytes and neurofilaments (NF) in neurons, achieved by rat intrastriatal QUIN injection. NMDA-mediated Ca^{2+} events and oxidative stress were able to be related to the altered hyperphosphorylation of these cytoskeletal proteins and could represent an early step in the pathophysiological cascade of deleterious events exerted by QUIN in rat striatum [9]. Moreover, studies of signaling mechanisms involved in the disruption of the cytoskeletal homeostasis were performed in striatal slices acutely exposed to an excitotoxic concentration of QUIN (100 μM). In astrocytes, the action of QUIN was mainly due to increased Ca^{2+} influx through NMDA and L-type voltage-dependent Ca^{2+} channels (L-VDCC). In neurons, QUIN acted through metabotropic glutamate receptor (mGluR) activation and influx of Ca^{2+} through NMDA receptors and L-VDCC, as well as Ca^{2+} release from intracellular stores. These mechanisms set off a cascade of events including activation of cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase (PKCaMII) and protein kinase C (PKC), which phosphorylate head domain sites on GFAP in astrocytes [10].

GFAP is the main intermediate filament (IF) protein expressed in mature astrocytes, where it is thought to help maintaining mechanical strength, and the shape of cells. However, recent evidence has shown that GFAP plays a role in a variety of additional astrocyte functions, such as cell motility/migration, cell proliferation, glutamate homeostasis, neurite outgrowth and injury/protection [11].

Because of their multiple roles into the cell, cytoskeletal protein components are among the main targets modified in response to extracellular signals that ultimately determine cell morphology and physiological role [12]. Consequently, it is not surprising that IFs are likely to be targeted in several genetically determined protein misfolding/aggregation diseases [13–15] as well as by a variety of pathogens [16] and toxins [17–20].

IF proteins are known to be phosphorylated on their head and tail domains and the dynamics of their phosphorylation/dephosphorylation plays a major role in regulating the structural organization and function of IFs in a cell- and tissue-specific manner [21]. Amino-terminal phosphorylation plays a major role in regulating the assembly/disassembly equilibrium of type III IFs (GFAP, vimentin) as well as of low and medium molecular weight NF proteins (NF-L and NF-M, respectively) [22]. In vivo and ex vivo studies from our group and from others demonstrated that the phosphate groups on the amino-terminal head domain on GFAP, vimentin and NF-L are added by the second messenger-dependent protein kinases PKA, PKCaMII and PKC [9,10,22–24]. Phosphorylation of Ser-8, Thr-7, Ser-13 and Ser-38 in the N-terminal region (head domain) of GFAP [25,26] causes disassembly of the IFs and conversely, dephosphorylation (by protein phosphatases) restores their ability to polymerize [27]. GFAP phosphorylation is possibly a key factor in astrocytes, since cell uses phosphorylation/dephosphorylation levels to regulate the dynamic of the polymerization/depolymerization of these proteins promoting cell survival and physiological roles.

Astrocytes are involved in a wide range of CNS pathologies, including trauma, [28] ischaemia [29], and neurodegeneration [30]. Alterations of the functionality of glial cells, including changes in morphology and proliferative activity, are a common feature of pathologies [31], and during brain inflammation associated with HD, astrocytes are activated leading to their cellular hypertrophy and/or proliferation [28]. Also, astrocytes in culture have been long time considered a useful model for evaluating neurotoxic-induced injury [32]. In addition, we have previously described that the cells change both their morphology and phosphorylation status of GFAP and/or vimentin, in response to metabolites or toxins [18,33].

Therefore, considering the pivotal role of cytoskeletal remodeling and the relevance of astrocyte plasticity in several pathologies that affect CNS, the aim of the present work was to study the effects of QUIN in concentrations previously described as neurotoxic to astrocytes and neurons in acute slices [9,10], on the cytoskeleton of primary astrocytes. We searched for the implications of glutamate mechanisms and Ca^{2+} levels on GFAP and vimentin phosphorylation and their link with actin cytoskeletal remodeling.

Material and methods

Phalloidin-fluorescein, anti-GFAP (clone GA-5), monoclonal anti-vimentin (clone Vim 13.2), anti-mouse IgG (whole molecule), anti-mouse IgG (whole molecule)–FITC, F(ab0)2 fragment-Cy3, quinolinic acid (QUIN), benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide and material for cell culture were obtained from Sigma (St. Louis, MO, USA). Polyclonal anti-GFAP was from DAKO. 40, 60-diamidino-2-phenylindole (DAPI) was from Calbiochem (La Jolla, CA, USA). β-actin was from Cell Signaling (Boston, MA, USA). [^{32}P]-orthophosphate was purchased from CNEN, São Paulo, Brazil. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), fungizone and penicillin/streptomicin were purchased from Gibco BRL (Carslbad, CA, USA). All other chemicals were of analytical grade.

Animals

Pregnant Wistar rats (200–250 g) were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22 °C) colony room, with food and water at libitum and animals were observed on gestational day 22.