Actin cytoskeleton disruption is an early event upon exposure of cerebellar granule neurons to SIN-1-induced oxidative stress

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Summary

In this work we have studied the alterations of the actin cytoskeleton in cultured cerebellar granule neurons during exposure to the peroxynitrite-releasing agent SIN-1 for less than 2 hours. Actin polymerization state was assessed by fluorescence microscopy ratio images using double labelling for actin filaments (phallacidin) and monomers (DNase-I). In addition, agonists and antagonists of L-type Ca^{2+} channels and NMDA receptors were used in order to find out whether these compounds were able to attenuate or potentiate the effects of oxidative stress on the perturbation of the actin cytoskeleton. The results reveal that a flux of peroxynitrite as low as 0.5 μM/min during 1h is sufficient to promote alterations of actin dynamics leading to partial actin cytoskeleton disruption and suggest that this is an early event linked to cytosolic calcium concentration changes.

Introduction

Cytoskeleton damage is a frequent feature in neuronal cell death and one of the early events in oxidant-induced cell injury [1, 2]. Calcium, on the other hand, plays important roles in regulating dynamic functions of the cell cytoskeleton. For example, alterations of the actin polymerization state in response to Ca^{2+} influx have been shown to stabilize intracellular calcium levels protecting neurons against excitotocixity [3]. In a previous work of our laboratory it was demonstrated that L-type Ca^{2+} channels are a primary target of SIN-1-induced extracelular nitrosative/oxidative stress in cerebellar granule neurons (CGN) being inactivated by chronic exposure to fluxes of peroxynitrite ($ONOO^-$) of 0.5-1 μM/min, whereas higher concentrations are required to induce an increase of intracellular Ca^{2+} concentration eliciting necrotic cell death [4]. In the present work we have studied the modulation of the actin cytoskeleton in CGN exposed to SIN-1 concentrations which produce a peak of peroxynitrite between 1 and 20

μM/min for less than 2 hours and addressed the possible role of Ca^{2+} dynamics in the observed changes.

Materials and Methods

CGN were obtained from dissociated cerebella of 7 days old Wistar rats as described previously [4]. Immunodetection was done following standard protocols for immunocytochemistry with cell cultures after fixation with 2% paraformaldehyde. Actin polymerization state was assessed by ratio images after background subtraction obtained in fluorescence microscopy using double labelling: Bodipy-FL phallacidin for actin filaments and DNase-I-Alexa594 for monomeric actin. Pixels quantification was done assuming just the extensions by the selection of areas excluding somas. Alterations of Ca^{2+} concentration were measured by loading CGN with fura-2-AM after which fluorescence microscopy images were acquired with a Hisca CCD camara (Hamamatsu) mounted on an epifluorescence Nikon Diaphot 300 microscope. Quantitative analysis of the fluorescence intensity was done using the Argus/Hisca software, as indicated in a previous work [4]. All the results were confirmed with, at least, three different CGN preparations and in duplicate with each preparation ($n \geq 6$).

Results and Discussion

Previous studies of our laboratory demonstrated that SIN-1 produces a biphasic kinetics on the steady-state free cytosolic Ca^{2+} concentration in cultured CGN and therefore we decided to perform a screening of different SIN-1 concentrations (0.1, 0.25 and 0.5 mM) for short-term exposure (30 min or 1h). Alterations of the actin polymerization state in CGN upon SIN-1 treatment (Figure 1A and 1B) were assessed by the ratio between DNase-I-red and phallacidin-green fluorescence intensities, which stain respectively for actin monomers (G-actin) and actin filaments (F-actin). At 1 hour after treatment with 0.1mM SIN-1 it can be observed an increase of the ratio G-/F-actin staining meaning that actin dynamics has been altered towards the non-polymerized form. Nevertheless, as SIN-1 concentration and time of exposure increase this effect is cancelled and for the higher SIN-1 concentration used (0.5mM) the ratio G-/F-actin staining is lower than the control indicative of an F-actin network growth. This biphasic behaviour of the actin cytoskeleton dynamics appears to correlate well with the changes on cytosolic Ca^{2+} concentration produced by CGN exposure to SIN-1, i.e., a decrease of the actin polymerization state is observed for SIN-1 concentrations (average peroxynitrite fluxes lower than 0.5μM/min up to 1 hour) inducing a decrease of the cytosolic Ca^{2+} concentration whereas an increase of the actin polymerization state is

obtained for higher SIN-1 concentrations (average peroxynitrite fluxes up to 20μM/min for 1 hour) that produce an increase of cytosolic Ca^{2+} concentration at longer exposure times.
The changes of the cytosolic Ca^{2+} concentration induced by CGN exposure to the lower SIN-1 concentrations have been previously attributed to the inactivation by peroxynitrite of L-type voltage operated Ca^{2+}-channels [4]. In view of this, we addressed the effect of SIN-1 on the perturbation of both actin (Figure 1A and 1B) and cytosolic Ca^{2+} dynamics (Figure 1C) by an L-type Ca^{2+} channel agonist (FPL-64176) in the absence or presence of an NMDA-receptor antagonist (MK801). Incubation of CGN with 50μM FPL produced a rapid and high rise in cytosolic Ca^{2+} concentration giving rise to a marked decrease of the ratio G-/F-actin. However, MK801 is able to partially revert the effect of FPL re-establishing the ratio G-/F-actin to values close to the control. This is in agreement with previous reports [5] and suggests that regulation of actin dynamics involves mainly Ca^{2+} influx through NMDA-receptors. Incubation with SIN-1 for 30 min before FPL and FPL+MK801 treatments resulted in a partial inhibition and almost blockade of the cytosolic Ca^{2+} rise, respectively. In the same way, SIN-1 partially prevented the decrease of G-/F-actin ratio induced by FPL and the effect was found to be dose dependent up to 0.5mM. Altogether these results suggest that disruption of the actin cytoskeleton is an early event upon exposure of CGN to SIN-1-induced oxidative stress linked to cytosolic Ca^{2+} concentration changes.

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Figure captions

Figure 1. Alterations of the actin cytoskeleton and cytosolic Ca^{2+} dynamics in CGN during exposure to the peroxynitrite-releasing agent SIN-1 in the absence or presence of an L-type Ca^{2+} channel agonist (FPL) and an NMDA-receptor antagonist (MK801). A) Fluorescence microscopy images of CGN with double labelling for F-actin (BODIPY-Phallacidin) and G-actin (DNase-I-Alexa594) and the corresponding merge image. B) Red/Green fluorescence ratio obtained by pixel quantification over the extensions in labelled CGN. C) Intracellular Ca^{2+} changes measured by ratio fluorescence images obtained with excitation filters of 340 and 380 nm and emission at 510 nm.