Neurochemistry International 61 (2012) 119–127

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Daidzein has neuroprotective effects through ligand-binding-independent PPARγ activation

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ARTICLE INFO

Article history:
Received 29 November 2011
Received in revised form 27 March 2012
Accepted 6 April 2012
Available online 11 April 2012

Keywords:
PPARγ
Phytoestrogens
Stroke

ABSTRACT

Phytoestrogens are a group of plant-derived compounds that include mainly isoflavones like daidzein. Phytoestrogens prevent neuronal damage and improve outcome in experimental stroke; however, the mechanisms of this neuroprotective action have not been fully elucidated. In this context, it has been postulated that phytoestrogens might activate the peroxisome proliferator-activated receptor-γ (PPARγ), which exerts neuroprotective effects in several settings. The aim of this study was to determine whether the phytoestrogen daidzein elicits beneficial actions in neuronal cells by mechanisms involving activation of PPARγ. Our results show that daidzein (0.05–5 μM) decreases cell death induced by exposure to oxygen–glucose deprivation (OGD) from rat cortical neurons and that improves synaptic function, in terms of increased synaptic vesicle recycling at nerve terminals, being both effects inhibited by the PPARγ antagonist T0070907 (1 μM). In addition, this phytoestrogen activated PPARγ in neuronal cultures, as shown by an increase in PPARγ transcriptional activity. Interestingly, these effects were not due to binding to the receptor ligand site, as shown by a TR-FRET PPARγ competitive binding assay. Conversely, daidzein increased PPARγ nuclear protein levels and decreased cytosolic ones, suggesting nuclear translocation. We have used the receptor antagonist (RE) fulvestrant to study the neuroprotective participation of daidzein via estrogen receptor and at least in our model, we have discarded this pathway. These results demonstrate that the phytoestrogen daidzein has cytoprotective properties in neurons, which are due to an increase in PPARγ activity not mediated by direct binding to the receptor ligand-binding domain but likely due to post-translational modifications affecting its subcellular location and not depending to the RE and it is not additive with the agonist rosiglitazone.

1. Introduction

Most phytoestrogens found in typical human diets can be categorized into two primary classes, isoflavones and lignans, being genistein and daidzein two of the major isoflavones. Prior studies have reported neuroprotective actions of daidzein and genistein, although the mechanisms are not yet fully elucidated. *In vitro*, daidzein and genistein protect primary neurons from β-amyloid toxicity (Zhao et al., 2002; Zeng et al., 2004) and from thapsigargin-induced apoptosis (Linford and Dorsa, 2002). In addition, both molecules have been reported to be neuroprotective against glutamate excitotoxicity and oxygen–glucose deprivation (OGD) in cultured neurons (Schreihofer and Redmond, 2009). *In vivo*, a high soy diet reduces stroke injury in male rats following transient middle cerebral artery occlusion (MCAO) (Burguete et al., 2006; Ma et al., 2010a,b) and in female rats with permanent (Schreihofer et al., 2005) and transitory MCAO (Lovekamp-Swan et al., 2007a,b; Ma et al., 2010a,b). Genistein, daidzein and its metabolite equol are also neuroprotective after cerebral ischemia in rodents (Trieu and Uckun, 1999; Mizutani et al., 2000; Ma et al., 2010a,b; Castello-Ruiz et al., 2011).

In general, many of these effects have been attributed to binding to estrogen receptors (ERs) (for rev. see Turner et al. (2007)). Furthermore, other mechanisms have been reported to explain

Abbreviations: CNS, central nervous system; CV, coefficient of variation; DIV, days *in vitro*; ERs, estrogens receptors; FITC, fluorescein isothiocyanate; LBD, ligand binding domain; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; OGD, oxygen–glucose deprivation; PPARγ, peroxisome proliferator-activated receptor-γ; RSG, rosiglitazone; TR-FRET, Time-Resolved Fluorescence Resonance Energy Transfer.

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http://dx.doi.org/10.1016/j.neuint.2012.04.007

the neuroprotective effects of these molecules, and include inhibition of tyrosine kinase (for genistein) (Akiyama et al., 1987), DNA topoisomerase (Kulling and Metzler, 1997) and aromatase function (Adlercreutz et al., 1993), as well as antioxidant effects (Mitchell et al., 1998; Ma et al., 2010a,b). Also, phytoestrogens have been proposed to act as "calcium antagonists" producing cerebral arteries relaxation and increasing cerebral blood flow (Salom et al., 2001,2007; Torregrosa et al., 2003). Phytoestrogens are also anti-inflammatory and could act by reducing edema and secondary damage associated with vascular inflammation following stroke (Chacko et al., 2005; Lee and Lee, 2008).

In other way, phytoestrogens have been shown to influence cell survival, growth and neuroplasticity (McEwen, 2001). Phytoestrogens increase the expression of choline acetyltransferase and neurotrophins in the frontal cortex and hippocampus (Pan et al., 1999a,b). Daidzein acts as a transcriptional inducer of Arg1 producing regeneration in CNS (Ma et al., 2010a,b). Ischemia reduces the number of presynaptic vesicles in the axon boutons, as indexed by synaptophysin intensity (Sun et al., 2008). Interestingly, genistein has been shown to increase the expression of the synaptic vesicle protein synaptophysin (Chindewa et al., 2007), which may reflect an increase in synaptic vesicle formation and recycling (Calakos and Scheller, 1994) or an increase in the number of synapses (Eastwood et al., 1994). This effect has been found also with the PPARγ agonist rosiglitazone, which increases synaptophysin levels after cerebral hypoperfusion (Sayan-Ozacmak et al., 2011). Thus, the neuroprotective effect of phytoestrogens could be also due to an effect on synaptic function.

Interestingly, it has been proposed that phytoestrogens might act through nuclear receptors other than ERs. In this context, genistein and daidzein have been reported to act as PPARγ ligands (Dang et al., 2003; Shen et al., 2006) and to stimulate PPARγ-dependent gene transcription in macrophages (Mezei et al., 2003).

PPARγ is a ligand-dependent nuclear transcription factor belonging to the nuclear hormone receptor superfamily, involved in the expression of genes related to metabolic processes, such as lipid and glucose homeostasis, but also responsible for important anti-inflammatory effects (rev. in Willson et al. (2001), Berger and Moller (2002), Heneka et al. (1999), and Tontonoz and Spiegelman (2008)). PPARγ is activated by several molecules that include the anti-diabetic glitazones (Yki-Jarvinen, 2004) and endogenous mediators such as cyclopentenone prostaglandins (Straus and Glass, 2001). Importantly, several groups including ours have demonstrated the neuroprotective effects of PPARγ agonists in experimental models of cerebral ischemia (Shimazu et al., 2005; Sundararajan et al., 2005; Pereira et al., 2005, 2006; Zhao et al., 2006).

The aim of the present study was to determine whether the phytoestrogen daidzein elicits beneficial actions in neurons by mechanisms involving activation of PPARγ. To this aim we have explored the effect of daidzein on cell viability after oxygen and glucose deprivation (OGD) in cultured rat neuronal cells and on neuronal synaptic function by measuring the exo-endocytotic cycle of synaptic vesicles.

2. Methods

2.1. Primary cultures of pure cortical neurons and cerebellar granule cells

All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (EU directives 86/609/CEE, 2003/65/CE and RD 1201/2005). A special effort was made to reduce the number of animals used in the study and to provide them with the most comfortable conditions possible. Pure cortical neuronal cultures were performed as described (Romera et al., 2004). Studies were performed at 9–10 days *in vitro* (DIV), time at which the cultures consisted of 94 ± 6% neurons, as determined by flow cytometry (Romera et al., 2004). Cerebellar granule cells were performed as described (Jurado et al., 2003).

2.2. Exposure of cultures to oxygen–glucose deprivation (OGD)

Oxygen–glucose deprivation was performed as described (Hurtado et al., 2005). Culture medium was replaced by a solution containing (in mM): NaCl (130), KCl (5.4), $CaCl_2$ (1.8), $NaHCO_3$ (26), $MgCl_2$ (0.8), NaH_2PO4 (1.18) and 2% HS bubbled with 95%N_2/5% CO_2 for OGD cells (OGD solution). OGD cells were transferred to an anaerobic chamber (Forma Scientific, Hucoa Erloss, Spain) containing a gas mixture of 95% N_2/5% CO_2 and humidified at 37 °C, and maintained at a constant pressure of 0.15 bars. Time of exposure to OGD was 150 min. In some experiments, daidzein (0.05, 0.5 and 5 μM), in the absence or presence of the specific PPARγ antagonist T0070907 (1 μM, Calbiochem, Merck), was included 24 h before, during and after OGD. In other sets of experiments rosiglitazone (RSG, 1 μM, Alexis Biochemical) was included as a *bona fide* PPARγ agonist. And in other sets of experiments, the fulvestrant (ICI 182,780; 100 nM, Sigma) has been included as estrogen receptors antagonist alone or mixed with daidzein 5 μM. OGD was terminated by replacing the exposure medium with oxygenated MEM containing 0.6% glucose, 0.029% glutamine, 50 I.U./ml penicillin, 50 μg/ml streptomycin, 10% HS (reperfusion medium) and returned to the normoxic incubator. Control cultures in a solution identical to OGD solution but containing glucose (33 mM; control solution) were kept in the normoxic incubator for the same time period as the OGD, and then incubation solution was replaced with reperfusion buffer and cultures were returned to the normoxic incubator until the end of the experiment. Culture medium and cells were collected at times indicated for LDH (lactate dehydrogenase) determination.

2.3. Cell viability

As a marker of necrotic tissue damage, LDH activity released from damaged cells was determined. Culture medium and lysate from 1% Triton X-100 in PBS were collected 24 h after the OGD period. LDH activity was measured spectrophotometrically at 340 nm by following the oxidation of NADH (decrease in absorbance) in the presence of pyruvate (Koh and Choi, 1987) using a Thermomax micro-plate reader (Molecular Devices, Palo Alto, CA).

LDH was measured in at least six different wells per experiment, and the experiment was repeated at least three times. LDH release was calculated as percentage of total LDH and expressed as percentage of OGD-induced LDH. Basal LDH was 6 ± 1%.

2.4. Synaptic bouton functionality (FM1–43)

We studied the effect of daidzein on synaptic bouton maturation and analyzing endo-exocytosis as a measure of synaptic bouton function. The strategy is based on the uptake and unloading of the styryl dye FM1–43 in 7 DIV rat cerebellar granule cells plated on coverslips as previously described (Jurado et al., 2003). Cells were incubated for 10 min in calcium-free and low potassium buffer containing (in mM): NaCl (140), KCl (5), $NaHCO_3$ (5), NaH_2PO_4 (1.2), $MgCl_2$ (1), glucose (10), HEPES (10), pH 7.4. They were then incubated with 10 μM FM1–43 dye (Invitrogen) in high potassium buffer containing (in mM): Hepes–NaOH (10), NaCl (95), KCl (50), $MgCl_2$ (1), $NaHCO_3$ (5), NaH_2PO_4 (1.2), $CaCl_2$ (1.33), glucose (10), pH 7.4 for 5 min, followed by a 10-min wash by perfusion with the calcium-free low potassium buffer to remove the surface-bound dye. Images were acquired on a Nikon microscope equipped with a Nikon 60 × 1.3 (NA) oil-immersion objective and iXon^{EM} + DU-888 (Andor Technology) at a rate of 1 frame every 2 s.