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Genistein and daidzein prevent low potassium-dependent apoptosis of cerebellar granule cells

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ABSTRACT

We have investigated the ability of certain dietary flavonoids, known to exert beneficial effects on the central nervous system, to affect neuronal apoptosis. We used cerebellar granule cells undergoing apoptosis due to potassium deprivation in a serum-free medium in either the absence or presence of the flavonoids genistein and daidzein, which are present in soy, and of catechin and epicatechin, which are present in cocoa. These compounds were used in a blood dietary concentration range. We found that genistein and daidzein, but not catechin and epicatechin, prevented apoptosis, with cell survival measured 24 h after the induction of apoptosis being higher than that of the same cells incubated in flavonoid free medium (80% and 40%, respectively); there was no effect in control cells. A detailed investigation of the effect of these compounds on certain mitochondrial events that occur in cells *en route* to apoptosis showed that genistein and daidzein prevented the impairment of glucose oxidation and mitochondrial coupling, reduced cytochrome c release, and prevented both impairment of the adenine nucleotide translocator and opening of the mitochondrial permeability transition pore. Interestingly, genistein and daidzein were found to reduce the levels of reactive oxygen species, which are elevated in cerebellar granule cell apoptosis. These findings strongly suggest that the prevention of apoptosis depends mainly on the antioxidant properties of genistein and daidzein. This could lead to the development of a flavonoid-based therapy in neuropathies.

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1. Introduction

Deregulated apoptotic mechanisms have been implicated in many pathologic conditions, including AIDS, infectious diseases, inflammation, cancer, heart failure, osteoporosis, stroke and trauma. In particular, the process is involved in many human neurological disorders, including Alzheimer's, Parkinson's and Huntington's diseases and amyotrophic lateral sclerosis [1]. It is intriguing that key events/components in the cellular regulation of apoptosis have been identified and thus may be targeted by therapeutic strategies [2]. In this regard, targeting apoptosis with dietary bioactive agents could be a therapeutic approach to either prevent apoptosis in those diseases that have an apoptotic origin [3] or induce apoptosis, for instance in cancer cells. To better investigate whether and how some dietary compounds can affect apoptosis, the availability of an experimental system is needed in which a dissection of the steps that lead to cell death has already been done.

We have already shown how apoptosis of cerebellar granule cells (CGCs) occurs when they are deprived of extracellular K^+ (SK5 cells) (for Ref., see [4–9]). In particular, we investigated the role of mitochondria in this process. Briefly, in early apoptosis (0–3 h after induction) the rate of glucose oxidation by CGCs decreases [5], mitochondria are subjected to time-dependent uncoupling [5] and elevated production of reactive oxygen species (ROS) occurs [6]. Cytochrome *c* (cyt *c*) is released from the mitochondria while still coupled [6,7] and an increase in the ATP level occurs [8]. In late apoptosis (3–8 h after induction), an alteration of the adenine nucleotide translocator (ANT) occurs, with ANT becoming a

Abbreviations: Act D, actinomycin D; ANT, adenine nucleotide translocator; Ap5A, P_1,P_5-di(adenosine-5′)penta-phosphate; ASC, ascorbate; ATP D.S., ATP detecting system; ATR, atractyloside; BME, basal medium Eagle; CE, catechin; CGC, cerebellar granule cell; CN^-, potassium cyanide; CsA, cyclosporine A; Cyt *c*, cytochrome *c*; DIV, days *in vitro*; DMSO, dimethyl sulfoxide; DZN, daidzein; DZN-S-K5 cells, S-K5 cells treated with daidzein; EC, epicatechin; Fe^{3+}-cyt *c*, ferricytochrome *c*; Fe^{2+}-cyt *c*, ferrocytochrome *c*; GEN, genistein; GEN-S-K5 cells, S-K5 cells treated with genistein; G6PD, glucose-6-phosphate dehydrogenase; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; mPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; PTE, pterine; RCR, respiratory control ratio; S.D., standard deviation; S-K25 cells, control cells; S-K5 cells, apoptotic cells; *x*h S-K5 cells, apoptotic cells *x*h after the induction of apoptosis; SOD, superoxide dismutase; SUCC, succinate; XO, xanthine oxidase.

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component of the mitochondrial permeability transition pore (mPTP); the latter is dispensable for occurrence of apoptosis [9]. On the assumption that neuropathies are a result of neuronal apoptosis, the identification of compounds able to protect neurons against apoptosis is highly desirable.

Given that the soy flavonoids have been claimed to improve human memory and neuro-cognitive performance, there is reason to suspect that this might be due to their ability to protect neurons against stress-induced injury, perhaps preventing apoptosis [10,11]. However, although the biological processes modulated by flavonoids, and especially by the isoflavone genistein (GEN), have been extensively studied, there is no clear understanding of the cellular and molecular mechanisms of action involved [12]. Flavonoids may target mitochondria in apoptosis, given that they have been reported to impair mitochondrial ATPase [13], to modulate the mPTP [14,15] and interact with other mitochondria-associated pro-apoptotic factors such as DIABLO/smac [16,17].

On the other hand, in recent reviews GEN was shown to have a variety of effects, including binding to estrogen receptors, antioxidative activity, the capacity to increase cellular reduced glutathione and effects on other physiological functions (for Ref., see [18]). In particular, GEN was reported to be a potent inhibitor of tyrosine kinase (TK, E.C.2.7.10) in tumor tissues [19,20] and of xanthine oxidase (XO, E.C.1.2.3.2) [21]. Recently, protection by GEN of the rat brain synaptosome and cultured hippocampal neurons from insult induced by β-amyloid peptide Aβ25–35 (for Ref. see [18]) was found. Indeed, GEN was shown to be either pro-apoptotic or anti-apoptotic depending both on its concentration and the cell system [14,22–24]. Similarly, the GEN analogue daidzein (DZN), which does not inhibit TK [25], can inhibit D-gal-induced apoptosis via the Bcl-2/Bax apoptotic pathway [26] and may be a potential medical candidate for neurodegeneration therapy. DZN exhibits an antioxidant activity [27] and shows other biological properties, including estrogen-like and estrogenin-dependent effects (for Ref. see [28]).

Herein we report investigations on the effect of GEN and DZN on the steps outlined above in the process by which CGCs undergo apoptosis as a result of potassium deprivation. We found that GEN and DZN, the latter with a lower efficiency, when used in the "dietary concentration range" (up to 10 μM) (for Refs. see [29–31]), can prevent apoptosis from occurring in a manner consistent with their antioxidant activity. This provides them with a role as potential drugs in neurodegenerative disease therapy. In contrast with [32,33], we found that GEN does not impair xanthine oxidase, at least in CGCs.

2. Materials and methods

2.1. Reagents

Tissue culture medium and fetal calf serum were purchased from GIBCO (Grand Island, NY) and tissue culture dishes were from NUNC (Taastrup, Denmark). All enzymes and biochemicals were from Sigma Chemicals Co. (St. Louis, MO, USA).

All procedures involving the use of animals were performed in compliance with relevant laws and institutional guidelines. The animals were anesthetized and insensitive to pain throughout the procedure.

2.2. Cell cultures

Primary cultures of CGCs were obtained from dissociated cerebella of 7-day-old Wistar rats as in Levi et al. [34]. Cells were plated in basal medium Eagle (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicin on dishes coated with poly-L-lysine. Cells were plated at 2×10^6 per 35 mm dish, 6×10^6 per 60 mm dish, or 15×10^6 per 90 mm dish. Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells.

2.3. Induction of apoptosis

Primary neuronal cultures at 6–7 days in vitro (DIV), which showed typical neuronal morphologies with healthy cell bodies and intact processes, were used for the experiments. Apoptosis was induced as in D'Mello et al. [35]: cells were washed twice and switched to serum-free BME (**S-**) containing 5 mM KCl supplemented with 2 mM glutamine and 100 μg/ml gentamicin. In some experiments, a variety of compounds (including the flavonoids) were also added at the induction time at the indicated concentrations selected to avoid any possible interference with cell viability. The flavonoids were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C in the dark, with the final DMSO concentration kept below 0.1%. The drug exposure was terminated by removing the flavonoid-containing media, followed by washing twice and replacing with fresh media. Sister cultures prepared under the same conditions were used in each experiment. Control cells (without or with DMSO) were treated identically but maintained in serum-free BME medium supplemented with 25 mM KCl for the indicated times; they are referred to as S-K25 cells. Apoptotic cells are referred to as S-K5 cells, and to indicate the different times × after apoptosis induction, they are referred to as xtime-S-K5.

The occurrence of apoptosis was checked, as in [6,35], by measuring DNA laddering and prevention of death due to the addition of actinomycin D (Act D). The occurrence of necrosis was assessed, as in [6], by checking the release of L-lactate dehydrogenase and the ability of MK801 to prevent CGC death.

2.4. Assessment of CGC survival

As advised in [36,37], cell survival was quantified by counting the number of intact nuclei using a hemacytometer after lysing the cells in detergent-containing solution as in [38,39]. Each measurement was performed in triplicate and reported as the mean ± SEM. The data are expressed as the percentage of intact nuclei in the control cultures at each time point.

2.5. DNA fragmentation analysis

Fragmentation of DNA was performed as in [6,40]. Briefly, CGCs (6×10^6) were plated in poly L-lysine-coated 60 mm tissue culture dishes, collected with cold phosphate-buffered saline (PBS, pH 7.2) and, after removal of the medium and washing once with cold PBS, the cells were centrifuged at $3500 \times g$ for 5 min. The pellet was lysed in 10 mM Tris–HCl, 10 mM EDTA, and 0.2% Triton X-100 (pH 7.5). After 30 min on ice, the lysates were centrifuged at $17000 \times g$ for 10 min at 4 °C. The supernatant was digested with proteinase K and then extracted twice with phenol–chloroform/isoamyl alcohol (24:1). The aqueous phase, containing soluble DNA, was recovered and nucleic acids were precipitated with sodium acetate and ethanol overnight. The pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). After digestion with RNase A (50 ng/mL at 37 °C for 30 min), the sample was subjected to electrophoresis in a 1.8% agarose gel and visualized by ethidium bromide staining. Soluble DNA from equal numbers of cells was loaded in each lane.

2.6. Cell suspension and homogenate preparations

Before each experiment, the culture medium was removed and the plated CGCs were washed with phosphate-buffered saline

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