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Research report

Neuroprotection comparison of chlorogenic acid and its metabolites against mechanistically distinct cell death-inducing agents in cultured cerebellar granule neurons

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ABSTRACT

While the number of patients diagnosed with neurodegenerative disorders like Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease is increasing, there are currently no effective treatments that significantly limit the neuronal cell death underlying these diseases. Chlorogenic acid (CGA), a polyphenolic compound found in high concentration in coffee, is known to possess antioxidant and free radical scavenging activity. In this study, we investigated the neuroprotective effects of CGA and its major metabolites in primary cultures of rat cerebellar granule neurons. We show that CGA and caffeic acid displayed a dramatic protective effect against the nitric oxide donor, sodium nitroprusside. In marked contrast, ferulic acid and quinic acid had no protective effect against this nitrosative stress. While CGA and quinic acid had no protective effect against glutamate-induced cell death, caffeic acid and ferulic acid significantly protected neurons from excitotoxicity. Finally, caffeic acid was the only compound to display significant protective activity against hydrogen peroxide, proteasome inhibition, caspase-dependent intrinsic apoptosis, and endoplasmic reticulum stress. These results indicate that caffeic acid displays a much broader profile of neuroprotection against a diverse range of stressors than its parent polyphenol, CGA, or the other major metabolites, ferulic acid and quinic acid. We conclude that caffeic acid is a promising candidate for testing in pre-clinical models of neurodegeneration.

1. Introduction

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, are defined by the progression of nervous system dysfunction attributed to multiple factors including but not limited to, misfolding of proteins, mitochondrial dysfunction, excitotoxicity, and oxidative stress. Therapeutic strategies that focus on these underlying factors are promising approaches for the treatment of neurodegenerative diseases. Oxidative stress has been proposed as a common pathological mechanism of essentially all major neurodegenerative diseases, and typically results from excessive production of reactive oxygen species (ROS) consequent to mitochondrial injury or dysfunction (Carrì et al., 2015; Moon and Paek, 2015; Simoncini et al., 2015).

As a means of protecting against oxidative stress, cells are equipped with free radical scavenging defenses that prevent accumulation of ROS. In addition to enzymes that detoxify free radicals (e.g., superoxide dismutase and catalase), antioxidants are chemical substances that either directly scavenge ROS or indirectly induce the expression or activity of free radical scavenging systems that protect cells from oxidative stress. Based on their neuroprotective effects, antioxidants have been suggested as a viable approach to slow or halt the progression of neuronal cell loss in various neurodegenerative diseases. However, clinical trials with many common antioxidants (e.g., vitamin E or vitamin C) have met with limited success, indicating that utilization of additional antioxidants with diverse mechanisms of action, or targeted

Abbreviations: Bcl-2, B-cell lymphoma 2; BME, basal modified Eagle medium; BSA, bovine serum albumin; CAPE, caffeic acid phenethyl ester; CGA, chlorogenic acid; CGN, cerebellar granule neuron; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EGCG, epigallocatechin 3-gallate; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; H_2O_2, hydrogen peroxide; IFN-γ, interferon gamma; IL, interleukin; IP3, inositol trisphosphate; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor E2-related factor 2; PBS, phosphate buffered saline; ROS, reactive oxygen species; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor alpha; Glu/Gly, glutamate and glycine

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intracellular delivery of these compounds (e.g., mitochondrial-targeted antioxidants), may be necessary to reveal their full therapeutic potential (Polidori and Nelles, 2014; Jin et al. 2014). Therefore, dietary intake of natural antioxidants that can directly scavenge free radicals, as well as induce endogenous antioxidant defenses, may be one useful approach to treat neurodegenerative diseases.

Polyphenols are an abundant class of micronutrients found in many plants and are commonly taken as dietary supplements (e.g., epigallocatechin 3-gallate (EGCG) and resveratrol). They have the impressive ability to scavenge ROS and reactive nitrogen species (e.g., nitric oxide). In addition, polyphenols have been shown to modulate apoptosis, inflammation, ion channels, signal transduction, and neurotransmitter release – all processes which are implicated in a number of neurodegenerative diseases (Bhullar and Rupasinghe, 2013). Therapies that implement dietary supplementation with these compounds have demonstrated their neuroprotective potential (Bhullar and Rupasinghe, 2013; Bigford and Del Rossi, 2014; Daglia et al., 2014; Virmani et al., 2013). Although polyphenols directly scavenge ROS and nitric oxide, many studies have suggested that the antioxidant capacity of polyphenols is also dependent on their indirect activation of pathways like nuclear factor E2-related factor 2 (Nrf2)/heme oxygenase-1 (Bhullar and Rupasinghe, 2013). Others have shown that polyphenols inhibit many pathways that cause neuronal cell death through neuroinflammation, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and pro-inflammatory molecules like tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin (IL)-1β, IL-6, IL-17A, and IL-22 (Spencer et al., 2012). Polyphenols can also upregulate free radical detoxifying enzymes, including catalase, superoxide dismutase, and glutathione peroxidase (Bhullar and Rupasinghe, 2013). Finally, polyphenols have been shown to modulate the expression of pro-survival and pro-apoptotic pathway components, such as Bcl-2 and Bax (Kelsey et al., 2010).

Coffee, the second most consumed drink in the world, has been shown to have protective effects from diverse inflammatory diseases associated with oxidative stress (Andersen et al., 2006). Coffee consists of many antioxidant compounds including its main element caffeine, polyphenols like chlorogenic acid (CGA), and volatile aromatic compounds (Gonthier et al., 2003; Ludwig et al., 2014; Monente et al., 2015). Several studies have shown that oxidative stress biomarkers are inversely correlated with the consumption of coffee (Hori et al., 2014; Kempf et al., 2010; Yoshida et al., 2008). Furthermore, coffee consumption enhances antioxidant capacity in vivo (Corrêa et al., 2012). In the context of neurodegeneration, several studies have documented that coffee intake is associated with a reduced risk of developing Parkinson's disease (Ross et al., 2000; Sääksjärvi et al., 2008; Costa et al., 2010).

Chlorogenic acid is a major polyphenolic component in many plants and is abundant in coffee - particularly in green coffee beans that contain from 5% to 12% CGA by weight (Farah and Donangelo, 2006; Farah et al., 2008). It is an ester of trans-cinnamic acids (including caffeic acid, ferulic acid, *p*-coumaric acid) and quinic acid (Clifford, 1999). Chlorogenic acid has diverse health benefits such as cardioprotective effects, lipid peroxidation inhibitory activity, anti-tumor effects, and antioxidant activity (Namba and Matsuse, 2002; Wan et al., 2013). Chlorogenic acid is extensively metabolized in vivo and several of its major metabolites which have been identified in the plasma and urine of rats fed CGA include caffeic acid and ferulic acid, as well as hippuric acid which is apparently derived from metabolism of quinic acid by gut microflora (Gonthier et al., 2003).

Caffeic acid is a component and metabolite of CGA that also has antioxidant properties, a function dependent on its chemical structure (Rice-Evans et al., 1996). The neuroprotective effects of CGA and caffeic acid have been revealed through in vitro studies using rat brain, where both polyphenolic compounds have been shown to inhibit acetylcholinesterase activity and lipid peroxidation induced by various pro-oxidants, suggesting a possible beneficial effect for Alzheimer's disease (Oboh et al., 2013). Caffeic acid has also been shown to reduce intracellular ROS produced by H_2O_2 and protects neuronal cells in vitro from this oxidative insult (Jeong et al., 2011).

Ferulic acid, a component of green coffee beans and another metabolite of CGA, has also been found to be an effective free radical scavenger. This antioxidant action is related to its phenolic hydroxyl group that donates electrons in order to render free radicals inert (Srinivasan et al., 2007). In addition to its intrinsic free radical scavenging activity, in vivo studies have established that administration of ferulic acid inhibits astrocyte activation induced by beta-amyloid peptide in mice (Cho et al., 2005).

The neuroprotective effects of quinic acid, another metabolite of CGA, have been investigated through in vitro studies. Although not typically regarded as an antioxidant, various chemical derivatives of quinic acid have been shown to inhibit the toxicity induced by beta-amyloid peptide in PC12 cells (Hur et al., 2001).

In order to investigate the neuroprotective effects of CGA and some of its metabolites against a number of diverse mechanisms that contribute to neurodegenerative diseases, we pre-incubated cerebellar granule neurons (CGNs) with CGA, caffeic acid, ferulic acid, or quinic acid (Fig. 1), and then exposed them to different stressors that result in neuronal cell death. Cell viability was measured by MTT assay, immunocytochemistry was used to visualize the microtubule network, and Hoechst stain was used to visualize nuclear morphology. The results demonstrate that CGA, caffeic acid, and ferulic acid each show some neuroprotective effects; however, caffeic acid displays a much broader neuroprotective profile against a diverse range of neurotoxic stressors than CGA or ferulic acid. Based on its broad neuroprotective profile, caffeic acid is a viable therapeutic candidate for testing in preclinical models of neurodegeneration.

2. Results

2.1. CGA and caffeic acid protect CGNs from nitrosative stress induced by the nitric oxide donor, sodium nitroprusside (SNP)

In an initial series of experiments, CGNs were incubated with CGA (10 μM), caffeic acid (50 μM), ferulic acid (50 μM), or quinic acid (10 μM) for 24 h, and then assessed for nuclear morphology by Hoechst staining and overall morphology by bright field microscopy. In comparison to untreated control CGNs, none of these compounds had any adverse effects on CGN nuclear or cellular morphology at the concentrations tested (Fig. 2).

Next CGNs were pre-incubated with CGA, caffeic acid, ferulic acid, or quinic acid at the concentrations described above for 24 h, and were subsequently treated in the presence or absence of SNP (50 μM) for an additional 16–18 h. To assess neuronal injury induced by SNP in CGNs, we first examined the integrity of the microtubule network and nuclear morphology. Compared to control cells, SNP caused a dramatic disassembly of the microtubule network in CGNs as shown by immunostaining for β-tubulin (Fig. 3A). CGA and caffeic acid each substantially protected the microtubule cytoskeleton from damage induced by SNP. Nuclei were considered as apoptotic if fragmented or condensed as assessed by Hoechst staining. De-colorized (black & white) Hoechst staining images are shown in Fig. 3A and enlarged (2.5X) in Fig. 3B to clearly show differences in nuclear size and morphology. Control cells displayed large intact nuclei. In contrast, SNP- treated CGNs showed very small and condensed nuclei (Fig. 3B). However,

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