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SYNERGISTIC NEUROPROTECTION BY EPICATECHIN AND QUERCETIN: ACTIVATION OF CONVERGENT MITOCHONDRIAL SIGNALING PATHWAYS

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Abstract—In view of evidence that increased consumption of epicatechin (E) and quercetin (Q) may reduce the risk of stroke, we have measured the effects of combining E and Q on mitochondrial function and neuronal survival following oxygen–glucose deprivation (OGD). Relative to cortical neuron cultures pretreated (24 h) with either E or Q (0.1–10 μM), E + Q synergistically attenuated OGD-induced neuronal cell death. E, Q and E + Q (0.3 μM) increased spare respiratory capacity but only E + Q (0.3 μM) preserved this crucial parameter of neuronal mitochondrial function after OGD. These improvements were accompanied by corresponding increases in cyclic AMP response element binding protein (CREB) phosphorylation and the expression of CREB-target genes that promote neuronal survival (Bcl-2) and mitochondrial biogenesis (PGC-1α). Consistent with these findings, E + Q (0.1 and 1.0 μM) elevated mitochondrial gene expression (MT-ND2 and MT-ATP6) to a greater extent than E or Q after OGD. Q (0.3–3.0 μM), but not E (3.0 μM), elevated cytosolic calcium (Ca^{2+}) spikes and the mitochondrial membrane potential. Conversely, E and E + Q (0.1 and 0.3 μM), but not Q (0.1 and 0.3 μM), activated protein kinase B (Akt). Nitric oxide synthase (NOS) inhibition with L-N^G-nitroarginine methyl ester (1.0 μM) blocked neuroprotection by E (0.3 μM) or Q (1.0 μM). Oral administration of E + Q (75 mg/kg; once daily for 5 days) reduced hypoxic–ischemic brain injury. These findings suggest E and Q activate Akt- and Ca^{2+}-mediated signaling pathways that converge on NOS and CREB resulting in synergistic improvements in neuronal mitochondrial performance that confer profound protection against ischemic injury.

Key words: neuroprotection, flavonoid, mitochondria, cAMP response element-binding protein, protein kinase B, nitric oxide synthase.

INTRODUCTION

Flavonoids are a diverse group of polyphenolic compounds comprised of approximately 9000 members (Williams and Grayer, 2004) that have received considerable attention for the prevention and treatment of stroke (Cherubini et al., 2008). These compounds have excellent safety (Ross and Kasum, 2002; Harwood et al., 2007; Prasain et al., 2010), efficacy in a wide variety of models for ischemic stroke (Simonyi et al., 2005; Gutierrez-Merino et al., 2011) and when consumed in the form of a flavonoid-enriched diet may reduce the risk of stroke (Joshipura et al., 1999; He et al., 2006). Flavonols (quercetin) and flavan-3-ols (epicatechin), abundant in apple peel, are implicated in the protective effects of a flavonoid-enriched diet against stroke (Mursu et al., 2008; Mizrahi et al., 2009; Hollman et al., 2010; Cassidy et al., 2012).

We have proposed that these flavonoid sub-types activate distinct signal transduction pathways that

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Abbreviations: 7-AAD, aminoactinomycin; ANT, adenine nucleotide translocator ; CI, combination index; CREB, cyclic AMP response element binding protein; DMSO, dimethyl sulfoxide; E, epicatechin; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone ; HBSS, hanks balanced salt solution; HI, hypoxia/ischemia; L-NAME, L-N^G-nitroarginine methyl ester; MCU, mitochondrial calcium uniporter ; OCR, oxygen consumption rate; OGD, oxygen–glucose deprivation; PBS, phosphate-buffered saline; PE, phycoerythrin; PI3K, phosphatidylinositol-3-kinase; Q, quercetin; SRC, spare respiratory capacity; TG, thapsigargin; TMRM, tetramethylrhodamine methylester; TTC, triphenyltetrazolium chloride.

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converge on the mitochondrion, resulting in synergistic improvements in respiratory performance that confer profound resistance to ischemic brain injury (Jones et al., 2012). In keeping with this hypothesis, oral administration of the flavonoid-enriched fraction AF4 (isolated from the peel of Northern Spy apples) improved mitochondrial function and reduced motor deficits, pro-inflammatory cytokine expression and damage in the central nervous system (CNS) of mice subjected to an experimental stroke or experimental autoimmune encephalomyelitis (Keddy et al., 2012; Warford et al., 2014). Epicatechin (E) and quercetin (Q) account for over 80% of the total phenolic content of AF4 (Keddy et al., 2012). Cyanidin is a minor flavonoid component that, together with chlorogenic acid, comprises most of the remaining phenolic content of AF4. Each of these four compounds exhibit anti-oxidant, anti-inflammatory and neuroprotective properties in a broad array of models for ischemic brain injury (Simonyi et al., 2005; Gutierrez-Merino et al., 2011; Wang et al., 2011; Kim et al., 2012). However, the effects of combining these compounds on neuronal mitochondrial function and resistance to ischemic brain damage have not been systematically studied.

We first compared the effects of E, Q, cyanidin and chlorogenic acid, either alone, or in combination, on the survival of primary cultures of mouse cortical neurons exposed to an *in vitro* model of stroke (oxygen–glucose deprivation (OGD)). These studies showed that combining E and Q (E + Q) synergistically reduced the death of cultured neurons exposed to a lethal period of OGD. Cortical neurons were not protected against OGD by cyanidin or chlorogenic acid, nor did these compounds enhance neuroprotection by E + Q suggesting that E and Q are primarily responsible for neuroprotective effects of AF4. Since mitochondrial collapse is considered to be a pivotal event in ischemic brain damage (Lin and Beal, 2006; Jones et al., 2012; Sanderson et al., 2013), we next compared the effects of E, Q and E + Q on key aspects of mitochondrial function and examined the signaling mechanisms that mediate neuroprotection. Findings from these studies suggest that the activation of protein kinase B (Akt) and the elevation of cytosolic Ca^{2+} concentrations $[Ca^{2+}]_c$ by E and Q, respectively, synergistically improve key aspects of neuronal mitochondrial function, conferring marked resistance to ischemic damage. Oral administration of E + Q reduced brain damage in a hypoxic–ischemic injury model, suggesting that combined treatment with these flavonoids may have therapeutic utility for stroke.

EXPERIMENTAL PROCEDURES

Primary cortical neuron cultures

Embryonic brains were aseptically removed from timed pregnant CD1 mice at E16 and placed in Neurobasal medium containing 10% fetal bovine serum (FBS) on ice. The meninges were then peeled back and the cortices transferred to 35-mm petri dishes containing Hanks Balanced salt solution (HBSS; Invitrogen) at 4 °C. Cortices were pooled and treated with 2 ml of Stem-Pro® Accutase® cell dissociation reagent. The cortices were then triturated with a fire polished pipette 3–4 times and allowed to incubate for 15 min (min) at 37 °C. This procedure was repeated a second time. Protease activity was inhibited by the addition of 1 ml FBS and the cortical tissue was transferred to a 15-ml falcon tube containing 3 ml of neurobasal mix (Neurobasal 500 ml + B27 + 500 μM glutamine + 20 μg/ml gentamicin) at 4 °C. The solution was then triturated 3–4 more times and left to settle. The supernatant was then collected and passed through a 40 μ filter. The neurons were then centrifuged at 300×*g* for 7 min and resuspended in neurobasal mix, counted and plated for experiments. Media changes were 60% of the total volume and occurred on the fourth day and every third day thereafter.

OGD

Primary cortical neuron cultures were used after 8–10 days *in vitro* for all OGD testing. Glucose-free balanced salt solution (GBSS) was prepared by bubbling nitrogen through the solution to remove oxygen. The cultures were then washed twice with oxygen free HBSS and placed in air-tight containers that were purged with a gas mixture of 90% nitrogen, 5% carbon dioxide and 5% hydrogen for 5 min. Next, the air-tight containers holding the cultures were placed in a 37 °C incubator for 2 h. OGD was terminated by aspirating the HBSS and replacing it with the original media. Cells were then harvested for future experiments at the indicated time points. Cultures were pretreated with flavonoid for 24 h before OGD, but not during OGD. Immediately after OGD, flavonoids were re-added to the cell cultures.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay

Cell viability was assessed using an MTT (Sigma–Aldrich) assay performed in 48-well plates that each contained about 150,000 neurons. Briefly, 60 μl of 10 mg/ml MTT (dissolved in no phenol red neurobasal) was added to each well. After incubation at 37 °C for 1.5 h, 5% CO_2, the supernatant was removed and the formazan crystal was then dissolved in 200 μl/well of dimethyl sulfoxide (DMSO, Sigma). The absorbance of each well was then measured at 592 nm with a plate reader (ELx 800, BioTek). The absorbance of blank wells (200 μl DMSO) was subtracted from the absorbance of all of the other wells. The net absorbance of untreated no-OGD control wells was defined as 100% cell viability.

Fluorescent assisted cell sorting (FACS)

Primary cortical neurons were used day 10–14 *in vitro* for FACS analysis using a FACSAria III (BD Biosciences, San Jose, CA, USA). OGD was performed as previously described (Keddy et al., 2012). Briefly, cells were washed in chilled HBSS containing 25 μg/ml DNAse followed by trypsin (2.5%) treatment for 5 min. The plates were then

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