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Phytochemistry 66 (2005) 2032–2037
PHYTOCHEMISTRY
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Conjugation of catechins with cysteine generates antioxidant compounds with enhanced neuroprotective activity

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Received 27 August 2004; received in revised form 8 November 2004
Available online 22 January 2005

Abstract

Antioxidant compounds derived from the conjugation of (−)-epicatechin and (−)-epicatechin 3-*O*-gallate with cysteine and cysteine derivatives protected HT-22 nerve cells (EC_{50} between 36 and 65 μM) from death triggered by glutamate while underivatized (−)-epicatechin was almost inactive (EC_{50} = 610 μM). Differences in free radical scavenging capacity (DPPH assay) could not account for the improvement in neuroprotective activity upon derivatization of (−)-epicatechin with thiols. Moreover, while the gallate-containing compounds are more efficient radical scavengers than their non-galloylated counterparts, they are only equally or less potent as neuroprotective agents. Although all of the conjugates were able to scavenge mitochondrially generated reactive oxygen species (ROS) inside the cells, the majority of their neuroprotective activity appeared to be dependent upon their ability to maintain glutathione levels. These results suggest that a mechanism other than ROS scavenging is involved in the neuroprotective action exerted by the epicatechin conjugates.

Keywords: Polyphenols; Cysteine; Antioxidants; Glutamate; Neuroprotection

1. Introduction

Flavonoids are ubiquitous plant polyphenols increasingly appreciated as chemopreventive agents against pathophysiological conditions, such as cancer, cardiovascular disease and neurodegenerative diseases (Katiyar and Mukhtar, 1997; Fremont, 2000; Fuhrman and Aviram, 2001; Yang et al., 2001; Rohdewald, 2002; Aruoma et al., 2003; Dajas et al., 2003; Murphy et al., 2003). The primary activity of plant flavonoids is believed to reside in their free radical scavenging capacity (Rice-Evans et al., 1996; Yokozawa et al., 1998). They appear to minimize the number of oxidative DNA mutations and protein modifications by scavenging harmful reactive oxygen species (ROS) (Diplock et al., 1998). Moreover, some flavonoids show other activities such as antiproliferative and pro-apoptotic effects, probably through interactions with key enzymes regulating cell cycle and apoptosis pathways (Liang et al., 1997; Ahmad et al., 1998, 2000; Hayakawa et al., 2001; Williams et al., 2004).

Oxidative stress is involved in acute and chronic neurodegenerative conditions, such as ischemia–reperfusion injuries, infectious processes (e.g., AIDS) and Alzheimer's and Parkinson's diseases (Hall and Braughler, 1989; Jenner, 1991; Beal, 1995; Sun and Chen, 1998; Halliwell, 2001; Aruoma et al., 2003). A common fea-

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doi:10.1016/j.phytochem.2004.12.003

ture of these pathologic conditions is the glutamate-stimulated entry of an excess of calcium into the cytoplasm and the generation of mitochondrially-derived reactive oxygen species (ROS) (Beal, 1992; Coyle, 1993). In nerve cells, excitotoxicity results when NMDA and non-NMDA glutamate receptors are activated by low glutamate concentrations (10–100 μM) resulting in subsequent calcium entry (Choi, 1988; Beal, 1992) and cell death. At higher concentrations (5–10 mM), glutamate exerts a different cytotoxic effect which is mediated by the inhibition of cystine uptake leading to a depletion of intracellular cysteine and glutathione (GSH) (Murphy et al., 1989). This second mechanism of glutamate toxicity is also known as oxidative glutamate toxicity, oxytosis or glutamate-mediated programmed cell death (Tan et al., 2001).

The immortalized mouse hippocampal cell line HT-22 has been developed as a model for studying glutamate-mediated programmed cell death (Davis and Maher, 1994; Tan et al., 1998a). The cells lack ionotropic glutamate receptors so all glutamate-mediated cell damage is produced by the direct depletion of GSH. This results in an exponential increase in mitochondrial ROS production to levels 50- to 100-fold higher than in control cells followed by calcium entry into the cytoplasm and cell death (Li et al., 1997a; Sagara et al., 1998). Thus, the HT-22 cells are an excellent model for testing the protective activity of flavonoids and other molecules against endogenous oxidative stress. Usually in vitro models of oxidative cell damage rely upon the external production of ROS (e.g., by hydrogen peroxide or free radical initiators such as AAPH) (Johnson and Loo, 2000; Zou et al., 2001; Cai et al., 2002) so that sometimes the efficiency of free radical scavengers is mainly a measure of their ability to compete for ROS outside the cell. In the HT-22 model, ROS are generated internally by the mitochondria so that protection of these cells measures the capacity of a putative chemopreventive agent to scavenge or prevent the formation of endogenously produced ROS. A variety of flavonoids were found to protect HT-22 cells from oxidative glutamate toxicity (Ishige et al., 2001). However, flavonoids of the catechin (flavan-3-ol) type were not protective in this system.

Flavan-3-ol conjugates with thiols have been described (Tanaka et al., 1998; Torres and Bobet, 2001; Torres et al., 2002). The derivatives are obtained by acid depolymerisation of proanthocyanidins in the presence of the thiol and they show a higher antiradical capacity than their underivatised counterparts in the DPPH assay (Torres et al., 2002).

Interestingly, the non-phenolic part of the molecule appears to influence the activity of the conjugates, particularly their capacity to penetrate biological membranes, as well as the layers of the skin (Alonso et al., 2004). Furthermore, the gallate esters of these conjugates provide even more scavenging power through their additional hydroxyl groups. We are currently investigating whether the introduction of an amino acid moiety influences the protective efficacy of flavanols against cellular oxidative stress in different experimental systems.

We report here on the neuroprotective activity of conjugates of epicatechin with cysteine and cysteine derivatives which are significantly more effective than the underivatized flavan-3-ol suggesting that the non-phenolic part of the molecule also plays a significant role in neuroprotection.

2. Results and discussion

The compounds described in this report are depicted in Fig. 1. Conjugates 2–7 derive from (−)-epicatechin (**1**) and are free radical scavengers. Compounds **2**, **4** and **6** showed an antiradical power (ARP) slightly higher than compound **1** (Table 1) as scavengers of the DPPH stable radical. As anticipated by the presence of an extra pyrogallol group, the gallate esters (compounds **3**, **5** and **7**) were more potent free radical scavengers than their non-galloylated counterparts.

We used the HT-22 cells to assess the ability of these compounds to prevent oxidative stress-induced cell death initiated by treatment with high concentrations (5 mM) of glutamate. Compounds **2**–**7** all protected the HT-22 cells while underivatized (−)-epicatechin (**1**) showed little or no protection at concentrations up to

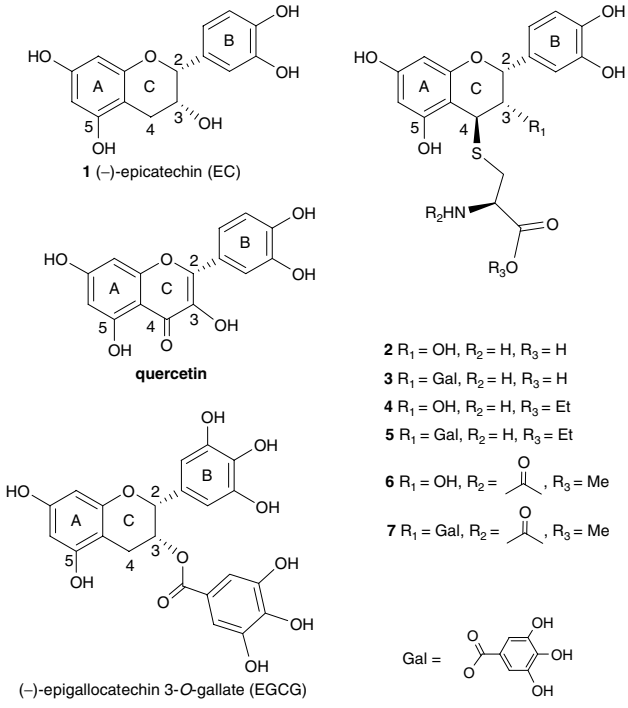

Fig. 1. Structures of natural flavonoids and the thio-conjugates.

Table 1
Protective efficiency of cysteine epicatechins against HT-22 glutamate toxicity and antiradical power in solution

Compound	EC_{50} (μM)	ARP[a,b]
1	610	6.2[c]
2	40	8.3[c]
3	65	20.0[c]
4	36	7.1
5	52	11.1
6	50	6.7
7	60	20.0
EGCG	60	21.3
Quercetin	3	10.5

[a] DPPH assay ($1/EC_{50}$).
[b] SD ($n = 3$) $\leqslant 0.4$
[c] Data from (Torres et al., 2002).

500 μM (Table 1). Surprisingly, compounds **3**, **5** and **7**, which were more potent free radical scavengers in solution, were slightly less effective than compounds **2**, **4** and **6** against oxidative glutamate toxicity suggesting that something besides their free radical scavenging power was playing a role in their neuroprotective activity in this model. The HT-22 cells, as well as primary cortical neurons lacking ionotropic glutamate receptors, die when exposed to high concentrations of glutamate by a process which involves, sequentially, a decrease in intracellular GSH, new protein synthesis, caspase activation, ROS production by mitochondria, lipoxygenase (LOX) activation, guanylate cyclase activation, and finally, the influx of Ca^{2+} via a cGMP-gated channel (Li et al., 1997a,b; Tan et al., 1998a,b). Three mechanisms have been described for the neuroprotective activity of flavonoids and other polyphenols in the HT-22 cells exposed to glutamate (Ishige et al., 2001): mitochondrial ROS scavenging, maintenance of intracellular GSH levels and inhibition of calcium entry via a cGMP-gated Ca^{2+} channel.

Direct mitochondrial ROS scavenging activity is exerted by the polyhydroxylated flavonoids. In overall agreement with structure–activity relationship studies of the free radical scavenging capacity of flavonoids (Bors et al., 1990; Rice-Evans et al., 1996), several structural features were shown to be important for the protective effect of flavonoids against glutamate-mediated programmed cell death (Ishige et al., 2001) including the presence of a hydroxyl group on C-3 and a 2–3 double bond in conjugation with a C-4-ketone function. The formation of hydrogen bonds between the ketonic oxygen and the hydroxyls at C-3 and C-5 may have some influence on the scavenging power as well (Bors et al., 1990). Catechins do not contain either the unsaturation or the ketone function on C-4 and this is the reason why compounds such as (−)-epicatechin (**1**) are less potent scavengers than flavonols such as quercetin (Fig. 1, Table 1). Some catechins such as (−)-epicatechin 3-*O*-gallate (ECG) and (−)-epigallocatechin 3-*O*-gallate (EGCG) (Fig. 1, Table 1) compensate for this by the presence of more hydroxyl groups as well as the ester function at C-3 (Rice-Evans et al., 1996; Bors et al., 2000; Bors et al., 2001). However, the free radical scavenging power does not appear to explain the neuroprotective activity of the thio-conjugates (Table 1). First, non-galloylated compounds **2**, **4** and **6** were more efficient neuroprotective agents than EGCG while being much less efficient free radical scavengers and second, the gallate-containing derivatives (compounds **3**, **5** and **7**) were not more effective than their non-galloylated counterparts. Moreover, studies designed to look at the effects of the conjugates on ROS production by the HT-22 cells (data not shown) indicated that although the conjugates were able to somewhat decrease ROS levels when added after the addition of glutamate, this did not appear to be the main mechanism behind their protective effect.

Interestingly, all of the cysteine conjugates were very effective at maintaining GSH levels in the presence of oxidative stress while (−)-epicatechin (**1**) was not (Fig. 2(a)). Furthermore, the ability of the conjugates to maintain GSH levels appeared to be critical for protection since their effectiveness was significantly reduced in the presence of buthionine sulfoximine, an inhibitor of glutamate cysteine ligase, the rate limiting enzyme in GSH biosynthesis (Fig. 2(b)). Other flavonoids and polyphenols not of the flavan-3-ol type were shown to inhibit oxidative glutamate toxicity by this mechanism (Ishige et al., 2001). These include flavonols such as quercetin and fisetin, hydroxycinnamic acid derivatives such as methyl caffeate and gallate esters such as propyl gallate but not catechins. Thus, the thio-derivatization on C-4 appears to dramatically alter the effects of epicatechin on GSH metabolism. The presence of a sulphur atom on C-4, which could allow for the formation of hydrogen bonds with the hydroxyls at either C-5 or C-3, may play a role in the ability of the epicatechin conjugates to maintain GSH levels in a manner similar to quercetin. The latter would be more efficient than the conjugates (3 vs. 36–65 μM) at protecting the HT-22 cells from oxidative glutamate toxicity due to its additional very effective scavenging of ROS. Alternatively, the thio-conjugates might be acting by a mechanism distinct from quercetin to maintain GSH levels. We are currently investigating this point in our laboratories.

Flavonoid hydrophobicity has also been proposed to play an important role in HT-22 cell protection from oxidative stress (Ishige et al., 2001). In view of the chemical structure of the compounds active at inhibiting cGMP-stimulated calcium entry, hydrophobicity may be most crucial at this late step. However, in our hands, the hydrophilic compounds **2**–**5** were as effective at neuroprotection as the more hydrophobic compounds **6**–**7** suggesting that it is unlikely that they protect by blocking calcium influx.

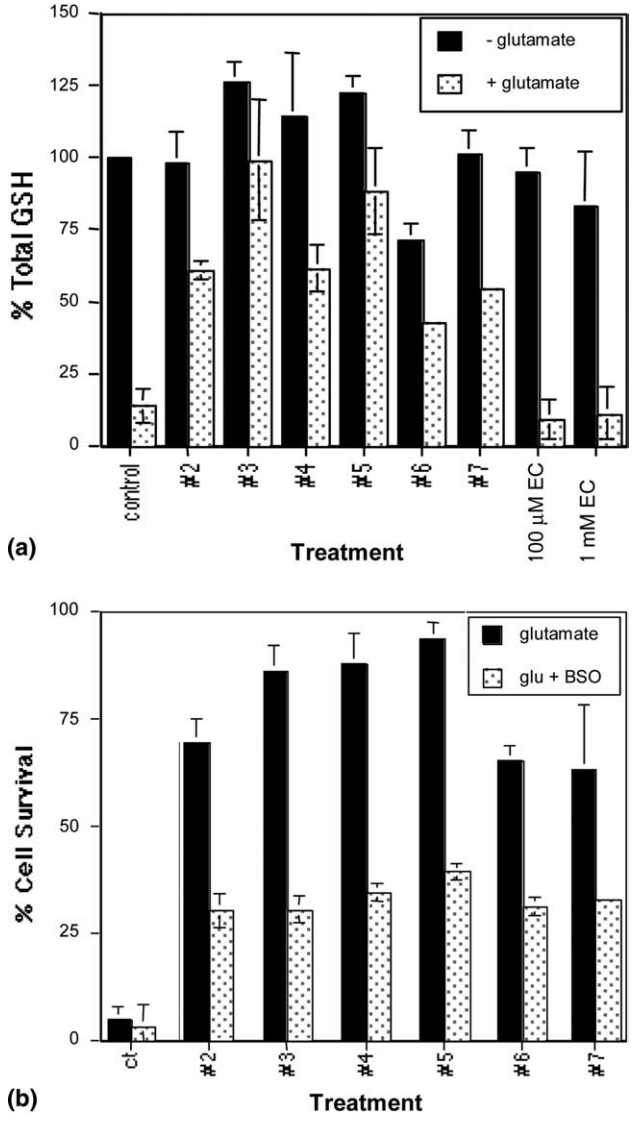

Fig. 2. (a) Maintenance of GSH levels by epicatechin thio-conjuagtes. HT-22 cells were treated with the indicated epicatechin conjugates (compounds **2**, **4**, **6** at 50 μM and compounds **3**, **5** and **7** at 75 μM) in the absence or presence of 5 mM glutamate for 8 h and cellular levels of total glutathione (GSH) were determined as described in Section 4. The GSH level of the control sample was taken as 100% (56.8 ± 11 nmoles/mg protein). The results are the average of duplicate determinations ±SD from three independent experiments. (b) BSO reduces protection by thio-conjugates of epicatechin. HT-22 cells were treated with 5 mM glutamate or 5 mM glutamate + 1 mM BSO (glu/BSO) alone or in the presence of 50 μM compounds **2**, **4** and **6** or 75 μM compounds **3**, **5** and **7**. % survival was measured 24 h later by the MTT assay. Results are the average of quadruplicate determinations ±SD from two independent experiments.

In summary, the conjugation of inactive flavonoids of the flavan-3-ol (catechin) type with cysteine and its derivatives yielded compounds which could protect nerve cells from oxidative stress-induced death. The conjugates appear to act predominantly through their ability to maintain GSH levels in the presence of oxidative stress with the additional advantage that they may be safer than other active flavonoids and polyphenols. In fact, all of the polyphenols described to date which are active at maintaining GSH levels exhibit other activities which make them potentially harmful to cells. For instance, quercetin may be a prooxidant and cytotoxic under certain circumstances (Metodiewa et al., 1999; Dangles et al., 2000). Moreover, both quercetin and gallates induce cell cycle arrest and apoptosis, perhaps through the inhibition of kinases and other key enzymes in the cell mitotic machinery (Liang et al., 1997; Richter et al., 1999). We have tested the epicatechin thio-conjugates for antiproliferative activity, cell cycle arrest and apoptosis induction in a number of cell lines including colon carcinoma and melanoma cells (Torres et al., 2002 and data not shown). While the gallate esters did show antiproliferative and pro-apoptotic activity, the non-galloylated compounds were barely active or inactive in these assays. Thus, the non-galloylated conjugates (compounds **2**, **4** and **6**) appear to be both active and safe.

3. Conclusions

The thio-conjugates of epicatechin are active in protecting HT-22 nerve cells from oxidative stress-induced death. Maintenance of GSH levels rather than scavenging of mitochondrial ROS appears to be the main mechanism behind their protective effect. This is supported by direct evidence and by the results on mitochondrial ROS scavenging. Since the galloylated conjugates and other flavonoids known to be protective by the same mechanism may have other, potentially harmful, effects such as prooxidation by redox cycling and induction of apoptosis, the non-galloylated thio-conjugates (compounds **2**, **4** and **6**) are good candidates for safe neuroprotective agents. Thus, the results presented here have important implications for neuroprotection strategies aimed at promoting the maintenance of endogenous antioxidant mechanisms with no interaction with the cell replication machinery.

4. Experimental

4.1. Materials

The thio-conjugates used in this study were prepared from grape *(Vitis vinifera)* pomace, essentially as described before (Torres and Bobet, 2001; Torres et al., 2002). Briefly, the grape polymeric procyanidins were submitted to acid cleavage in the presence of the corresponding thiol containing compound and the resulting conjugates purified by ion exchange and/or reversed-phase high-resolution liquid chromatography. (−)-Epicatechin, (−)-epigallocatechin 3-*O*-gallate and quercetin

were from Sigma Chemical CO, (Saint Louis, MO, USA).

4.2. Free radical scavenging activity in solution (DPPH assay)

The free radical scavenging activity was evaluated by the DPPH method (Blois, 1958; Brand-Williams et al., 1995). The samples (0.1 ml) were added to aliquots (3.9 ml) of a solution made up with 4.8 mg DPPH in MeOH (200 ml) and the mixture incubated for 1 h at room temperature. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517\,nm} = 11{,}345 \times C_{DPPH}$ as determined by linear regression. The initial concentration of the scavengers ranged from 1 to 30 μM. The results were plotted as the degree of absorbance disappearance at 517 nm ($(1 - A/A_0) \times 100$) against μmoles of sample divided by the initial μmoles of DPPH. Each point was acquired in triplicate. A dose–response curve was obtained for every product. The results were expressed as the efficient dose ED_{50} given as μmoles of product able to consume half the amount of free radical divided by μmoles of initial DPPH. The inverse of this value ($1/ED_{50}$) is referred to as antiradical power (ARP) in the text.

4.3. Cell culture and viability assays

The immortalized mouse hippocampal cell line HT-22 was used to assay neuroprotection. Cells were maintained at 37 °C in Dulbecco's modified minimal essential medium/10% fetal calf serum and passaged by trypsinization. Cell viability was routinely assayed at 37 °C using the MTT assay (Hansen et al., 1989). For this assay, cells were plated into 96-well plates at 5×10^3 cells/well in complete medium and 24 h later the experimental agents were added. The ability of the cells to reduce MTT was assayed 24 h after the addition of the experimental agents, exactly as described (Davis and Maher, 1994). Controls employing wells without cells and cells without glutamate were used to determine the effects of agents upon the assay chemistry or cell viability, respectively. In all cases, the cells were examined under phase contrast microscopy prior to the addition of MTT to visually assess the degree of cell death.

4.4. Total intracellular GSH/GSSG

Cells were washed twice with ice-cold PBS, collected by scraping, and lysed with 3% sulfosalicylic acid. Lysates were incubated on ice for 10 min and supernatants were collected after centrifugation in an Eppendorf microfuge. Upon neutralization of the supernatant with triethanolamine, the concentration of total glutathione (reduced and oxidized) was determined by the method of (Tietze, 1969) with modifications (Ishige et al., 2001). Briefly, a neutralized supernatant from above (25 μl) was mixed with 175 μl of a reaction mixture containing 143 mM sodium phosphate (pH 7.5), 6.3 mM Na_4EDTA, 6 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 0.25 mg/ml NADPH. The reaction was started by adding 1 U/ml of glutathione reductase. Color development was monitored at 405 nm in kinetic mode with a microplate reader. Pure GSH was used to obtain a standard curve. The protein content of each sample was determined using the BCA Protein Assay kit from Pierce (Rockford, IL) with BSA as a standard.

Acknowledgements

Financial support (Research Grant PPQ2003-06602-C04-01, and doctoral fellowship to C.L.) from the Spanish Ministry of Science and Technology is acknowledged. P.M. was supported by the National Institutes of Health (NS28121) and the Mericos-TSRI Neurobiology and Vision Science program.

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