9. Catechins and resveratrol as protective polyphenols against beta-amyloid-induced toxicity: Possible significance to Alzheimer’s disease

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Abstract. Polyphenols have recently received particular attention because of their possible preventive role in the incidence of age-related cognitive deficits and neurological disorders. Recent evidence from epidemiological studies has shown that beverages or food enriched in polyphenols are related to a low risk of dementia such as Alzheimer’s disease (AD). These findings are in accordance with animal and cell culture studies indicating that catechins (or flavanols) and resveratrol display neuroprotective abilities or reverse cognitive deficits. Using cultured rat hippocampal neuronal cells, we investigated the neuroprotective abilities of various catechin ingredients of green tea and red wine against toxicity induced by free radicals and beta-amyloid (Aβ) peptides, in respect to the possible deleterious role of these agents in age-related neurological disorders. Our studies, along with those obtained by other groups, indicated that these beneficial effects appeared to be only partly attributable to their well-known antioxidant activities, but also to their ability to directly...
inhibit cell death produced by Aβ peptides, and to modulate intracellular signaling pathways and gene expression associated with cell death/survival as well. We summarize in this review the purported beneficial effects of polyphenols in human as well in in vitro and animal models of neurotoxicity. We will then focus on the possible mechanisms underlying their neuroprotective effects and finally discuss possible clinical implications and the possible role of polyphenols as therapeutic agents.

Abbreviations

Aβ, β-amyloid; ADDLs, Aβ-derived diffusible ligands; AD, Alzheimer’s Disease; bis-ANS: 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonate; DCF, dichlorofluorescein; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; EGb 761, ginkgo biloba extract; PKC, protein kinase C; ROS, reactive oxygen species; Th-T, Thioflavin T.

Introduction

Alzheimer’s disease (AD) is the most common form of dementia and is mainly characterized by specific types of lesions, called amyloid plaques and neurofibrillary tangles with abnormally phosphorylated tau [1]. The principal component of amyloid plaques consists of aggregated forms of amyloid (Aβ) that may play an important role in neuronal degeneration. The relative contribution of the various forms (soluble dimers, small oligomers and fibrils) of Aβ to neuronal death is still uncertain [2, 3]. Hence, the weak correlation between fibrillar Aβ accumulation and neurological dysfunctions observed in AD suggests that aggregation of Aβ fibrils is not fundamental to the neurodegenerative processes occurring in AD [2]. Moreover Aβ deposits may also develop in cognitively normal elders [2]. On the other hand, recent findings indicate that soluble monomers and oligomers of Aβ - including Aβ-derived diffusible ligands (ADDLs), may represent the most important pathologic species [3]. Preliminary analyses have revealed abundant soluble oligomers in AD patients, consistent with the notion that oligomers precede senile plaques development and may be linked to cognitive impairments [4]. Since available drugs are not able to significantly stop the progression of AD, it has been proposed that the inhibition of soluble/insoluble forms of Aβ may be an appropriate strategy to block or even reverse the progression of the disease.

There is much evidence that consumption of fruits, vegetables, green tea and red wine (in moderation) reduces the risk of developing age-related neurological disorders such as stroke, AD and Parkinson’s disease [5-9]. Polyphenols present in high amounts in fruits, vegetables and tea likely
Polyphenols and neuroprotection

contribute to their beneficial effects. In support of this hypothesis, a 5-year follow-up study reported that regular consumption of polyphenols was inversely linked to a risk of dementia [10]. Moreover, using cell cultures of transgenic mice model of AD, the tea-derived flavan-3-ol such as epigallocatechin gallate (EGCG) and the stilbene resveratrol have been reported to protect neurons from Aβ toxicity [11-19] and modulate tau pathology [20]. Moreover, moderate consumption of Cabernet Sauvignon, which contains both catechins and resveratrol and a standardized ginkgo biloba extract, a polyphenols-derived natural extract were able to attenuate deficits of spatial memory in 14-months old transgenic mice (Tg2576), that develop Aβ plaques [21,22]. Finally, a recent animal study indicated that long-term green tea administration may prevent age-related learning and memory decline by modulating the transcription factor cAMP-response element binding protein (CREB) in the hippocampus [23].

Various hypotheses have been proposed to explain this inhibitory action on Aβ-associated events. Ono et al. (2009) have shown that various polyphenols were able to inhibit amyloid fibrils and destabilize fibrillized forms of Aβ [24], suggesting that they could be viewed as therapeutic agents for the treatment of Aβ-associated diseases [17,25]. Moreover, resveratrol, promoted the intracellular degradation of Aβ by a proteasome-dependent and secretases-independent mechanism [26] whereas EGCG may modulate the nonamyloidogenic γ-secretase proteolytic pathway [15].

We review here the interaction of polyphenols with soluble and insoluble forms of Aβ peptides, and their neuroprotective abilities. We then discuss the identification and characterization of specific binding sites for polyphenols in the rat brain and their possible relevance to the neuroprotective action of these molecules.

Materials and methods

Mixed hippocampal cell cultures and experimental treatments

Hippocampal cell cultures were prepared from E19 fetuses obtained from Sprague-Dawley rats. Animal care was according to protocols and guidelines of the McGill University Animal Care Committee and the Canadian Council for Animal Care. Mixed (glial/neuronal) hippocampal cells were obtained as described in detail elsewhere [12].

Measurement of cell survival/death was performed as described in detail elsewhere, using MTT and Sytox® green assays, respectively [12]. Briefly, 6-day old cells were exposed to fresh solutions of either Aβ25-35 (25µM) or Aβ1-42 (15µM) for 24 hours, in the presence or absence of different drugs.
Measurement of soluble and insoluble forms of Aβ

4, 4′-dianilino-1,1′-binaphthyl-5,5′-disulphonate (bis-ANS) is a fluorescent probe that has been shown to evaluate amounts of soluble forms of Aβ [27]. Briefly, the physiological fragment Aβ1–42 (15µM) was incubated for 30 min at room temperature in the presence of different polyphenols. Bis-ANS fluorescence (excitation = 360 nm, emission = 485 nm) was then measured by dilution of 100µl aliquots to a final volume of 300 µl with citrate buffer (30 mM, pH 2.4) containing bis-ANS (25µM), using a fluorescence multi-well plate reader (Bio-Tek Instruments® Inc.). The thioflavin T (Th-T) fluorescence method was performed to determine amyloid fibril formation, as previously described [17]. Briefly, a fresh solution of Aβ1–42 (15µM) was incubated at 37°C for 24 h in phosphate-buffered saline (pH 7.4). After incubation, a 100µl aliquot of solution was added to a final volume of 300 µl of phosphate buffer (50mM, pH 6.0) containing 5 µM Th-T in the presence of different drugs. Th-T fluorescence was evaluated using a fluorescence multi-well plate reader (excitation and emission wavelengths of 450 and 485 nm, respectively).

Binding assays and receptor autoradiography

In brief, binding assays were initiated by adding membrane preparations in a solution of Krebs containing [3H]-resveratrol, and competitors as described earlier [28]. Saturation experiments were performed at room temperature in the presence of increasing concentrations of [3H]-resveratrol, whereas competition binding experiments were performed in the presence of 20 nM [3H]-resveratrol and various competitors (10⁻¹⁰ to 10⁻⁴ M). Non-specific binding was determined in the presence of 100 µM resveratrol [28]. Quantitative receptor autoradiography was performed as described previously [29].

Results

Catechins gallate esters protected hippocampal cells against Aβ–induced toxicity

The neurotoxic effect of Aβ25-35 was reduced, in a dose-dependent manner by a treatment with green tea extract (5-25 µg/ml), as well as a black tea extract (5 µg/ml) which contains 80% of condensed products of catechins. The neuroprotective action of green and black tea extracts was shared by the most abundant green tea flavan-3-ols gallate esters, also called catechins gallate esters, known as epigallocatechin gallate [EGCG], and to a lesser
extent by epicatechin gallate (ECG), which represents approximately 5% of the total extract [30]. In contrast, non-gallate forms of catechins such as epicatechin (EC) and epigallocatechin (EGC) were ineffective.

The Sytox® green assay revealed that the extent of neuronal death was increased in the presence of Aβ₁₋₄₂, as compared to the vehicle-treated control group. The green (25 µg/ml) and black (5 µg/ml) tea extracts were able to completely block cell death produced by Aβ₁₋₄₂, as did EGCG (10 µM) and the ginkgo biloba extract EGb 761 (100 µg/ml), a well-known standard natural extract prescribed for the treatment of cognitive disorders in AD patients [31]. Finally gallic acid and tannic acid - a polymer of gallic acid molecules also displayed neuroprotective action, data summarized in Table 1.

**Resveratrol protected cells against Aβ-induced neurotoxicity**

Treatment with 20 µM Aβ peptides (Aβ₂₅₋₃₅ or Aβ₁₋₄₂) caused nearly 40% cell death and that was dose-dependently reduced in the presence of resveratrol (15–40 µM), with a maximal effect at 25 µM. A pre-treatment with the PKC inhibitor, GF 109203X, but not MAP kinase (PD98059) and PI3 kinase (LY294002) inhibitors significantly blocked the neuroprotective action of resveratrol against Aβ₂₅₋₃₅-induced neurotoxicity. Moreover, the role of the PKC-δ isoform was confirmed by Western blot that resveratrol induced the phosphorylation of PKC and abolished the inhibitory effect of Aβ₂₅₋₃₅ on phosphorylation of PKC-δ at the same range of concentrations, it protected hippocampal neurons. Among other stilbenes tested in our model, piceatannol, a natural resveratrol analog, was the most potent, whereas the other stilbenes known as trans-4-stilbenemethanol, transtilbene and diethylstilbestrol were ineffective (Table 1).

**Effects of polyphenols on soluble/insoluble forms of Aβ**

We investigated the effect of catechins and stilbenes on both soluble and insoluble forms of Aβ₁₋₄₂ using the bis-ANS and Th-T fluorescence assays, respectively. The bis-ANS assay revealed that EGCG and piceatannol reduced Aβ₁₋₄₂-induced increased fluorescence, whereas resveratrol did not modify bis-ANS fluorescence. An incubation with Aβ₁₋₄₂ increased Th-T fluorescence by 30-75 fold relative to control; the effect was diminished in the presence of neuroprotective polyphenols including EGCG, resveratrol and piceatannol. In contrast, EC and EGC were ineffective. Results are summarized in Table 2.
**Table 1.** Summary of the effects of catechins, phenolic acids and stilbene derivatives against toxicity induced by Aβ peptides (Aβ_{25-35}, Aβ_{1-42}) in rat hippocampal cell cultures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neuroprotection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green tea extract</td>
<td>+</td>
</tr>
<tr>
<td>Black tea extract</td>
<td>+</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>+</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>+</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>-</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>-</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>+</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>+</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>+</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>+</td>
</tr>
<tr>
<td>Trans-4-stilbenemethanol</td>
<td>-</td>
</tr>
<tr>
<td>Transtilbene</td>
<td>-</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>-</td>
</tr>
</tbody>
</table>

Taken from [11, 12, 19, 26]

**Table 2.** Summary of the effects of catechins, phenolic acids and stilbene derivatives on soluble and fibrillar forms of Aβ_{1-42}.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble forms of Aβ_{1-42}</th>
<th>Fibrillar forms of Aβ_{1-42}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green tea extract</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Black tea extract</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Trans</em>-4-stilbenemethanol</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Transtilbene</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

ND: not determined

Taken from [12, 19, 26]
Identification of [3H]-resveratrol binding sites

We next identified and characterized [3H]-resveratrol binding sites in the rat brain subcellular fractions. Significant [3H]-resveratrol binding was detected in plasma membrane, and to a lesser extent in nuclear fraction. Binding to the PII fraction was significantly reduced by pretreatment with trypsin or boiling, suggesting that specific [3H]-resveratrol binding sites are particularly abundant in the plasma membrane. Scatchard transformation of isotherm saturation binding experiments suggested that [3H]-resveratrol specifically binds to a single class of sites, with an apparent affinity of 220 ± 80 nM in the PII fraction [28].

Quantitative autoradiographic studies revealed that specific [3H]-resveratrol binding sites are the most abundant in the choroid plexus and subfornical organ, and to a lesser extent in other regions such as the hippocampal formation and the cortex [28]. We evaluated the ability of various analogs of resveratrol and catechins to compete for specific [3H]-resveratrol binding in PII fraction. Interestingly, EGCG and (-)-epicatechin gallate (ECG) are most potent to compete for specific [3H]resveratrol binding with $K_i$ values of 45 and 25 nM, respectively, whereas resveratrol was found to be less potent ($K_i = 102$ nM). In contrast, the non-neuroprotective polyphenols including EC, EGC, trans-4-stilbenemethanol and diethylstilbestrol were inactive ($K_i > 10000$ nM). Most importantly, the apparent affinities of a series of analogs of resveratrol and catechins for [3H]resveratrol binding correlated well ($r = 0.74$) with their neuroprotective abilities against Aβ25-35-induced toxicity in primary hippocampal cells, suggesting their functional relevance [28].

Discussion

We have demonstrated that resveratrol and catechins gallate esters derived from fruits, vegetables and beverages (e.g. red wine, green tea) protect hippocampal cells against the neurotoxic action of Aβ. These data are in accordance with previous findings reporting neuroprotective action of polyphenols derived from grape seeds and tea extracts in various in vitro and animal models of toxicity [11-19]. It also suggests that regular consumption of polyphenols may attenuate the accumulation of Aβ peptides that contributes to the process of neurodegeneration occurring in AD [1-3].

Resveratrol, an active stilbene from grapes, was shown to concentration-dependently protect against Aβ-induced toxicity in cultured hippocampal neurons. The mechanism(s) involved in the neuroprotective effects of resveratrol likely include PKC as evidenced by the inhibitory action of the potent PKC antagonist GF 109203X and the stimulation of the phosphorylation
of this enzyme by resveratrol, suggesting that the PKC pathway plays a major role in the neuroprotective properties of resveratrol in our model. Other mechanisms may also be involved since resveratrol was shown to promote proteasome-dependent intracellular Aβ degradation [26] and inhibit Aβ fibrils formation and amyloid plaques [18,32], in accordance with our findings.

The strong neuroprotective effects of tea extracts against Aβ toxicity are likely to be explained by the presence of catechins found as monomers and dimers (i.e. theaflavins) in green and black teas respectively [30]. Among catechins tested here, EGCG, and to a lesser extent ECG, displayed strong neuroprotective activities, in accordance with previous studies [15, 16]. Similar protective effects were also observed with theaflavins that are almost exclusively present in black tea [30]. These findings suggest that neuroprotective activities of catechin gallate esters depend on the esterification of the pyran hydroxyl group of catechins by gallic acid – a phenolic acid present in tea and red wine. In support of this hypothesis, gallic acid and tannic acid – a polymer of gallic acid significantly blocked both toxicity and fibrils formation produced by Aβ peptides [17].

We subsequently investigated the ability of these polyphenols to modulate the formation of soluble and fibril forms of Aβ1-42. Globally, polyphenols with neuroprotective actions tend to inhibit Aβ fibrils and to a lesser extent, soluble forms of Aβ, whereas non-gallate forms of catechins were ineffective. These data agree with previous findings reporting anti-amyloidogenic and fibril-destabilizing activities of these polyphenols [25, 33]. Among them, EGCG appeared to be the most potent polyphenol and was able to inhibit Aβ-derived diffusible ligands (ADDLs), suggested to mediate the neurotoxic effects of Aβ1-42 [3]. Taken together, these data suggest that the neuroprotective action against Aβ-induced neurotoxicity may be due, at least in part, to their inhibitory action on Aβ fibrils/oligomers formation.

Finally, binding and autoradiographic studies revealed the existence of specific polyphenols binding sites in the rat brain, in particular in the choroid plexus. Structure-activity data support the hypothesis that these specific binding sites may be responsible for the neuroprotective actions of polyphenols.

In summary, our results demonstrate that the neuroprotective action of catechins gallate esters and resveratrol is partly due to their interaction with intracellular kinases and their inhibitory action on Aβ oligomers and/or fibrils formation. We have also shown the presence of specific [3H]-resveratrol binding sites at the level of the plasma membrane in the rat brain. These findings support the hypothesis that regular intake of polyphenols derived
from red wine, tea, fruits and vegetables may delay or even prevent age-related neurological disorders such as Alzheimer’s disease, and suggest that polyphenols may be considered as possible neuroprotective agents.

Acknowledgements

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References