Curcuminoids from *Curcuma longa* L. (Zingiberaceae) that protect PC12 rat pheochromocytoma and normal human umbilical vein endothelial cells from βA(1–42) insult

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Abstract

β-Amyloid (βA) induced oxidative stress is a well-established pathway of neuronal cell death in Alzheimer’s disease. From turmeric, *Curcuma longa* L. (Zingiberaceae), three curcuminoids, curcumin, demethoxycurcumin, and bisdemethoxycurcumin, were found to protect PC12 rat pheochromocytoma and normal human umbilical vein endothelial (HUVEC) cells from βA(1–42) insult, as measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction assay. ED₅₀ values of curcumin, demethoxycurcumin, and bisdemethoxycurcumin toward PC12 and HUVEC cells were 7.1 ± 0.3, 4.7 ± 0.1, 3.5 ± 0.2 μg/ml and 6.8 ± 0.4, 4.2 ± 0.3, and 3.0 ± 0.3 μg/ml, respectively. These compounds were better antioxidants than α-tocopherol as determined by DPPH radical trapping experiment. α-Tocopherol did not protect the cells from βA(1–42) insult even at > 50 μg/ml concentration. The results suggest that these compounds may be protecting the cells from βA(1–42) insult through antioxidant pathway. © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: Natural antioxidant; Turmeric; Curcuminoid; Alzheimer disease; β; A(1–42); PC12 cells; HUVEC cells; Protection

Alzheimer’s disease (AD) is the most common cause of progressive cognitive dysfunction that affects approximately four million Americans, causing more than 100 000 deaths each year with a total annual cost approaching $70 billion [2]. Recently, β-Amyloid (βA)-induced oxidative stress to neuronal cells was found to be one of the major causes of AD pathology. Thus, modulation of βA insult has been speculated to be an important therapeutic approach to control the onset of AD. Because of the involvement of βA-induced oxidative stress in the etiology of AD, one of the current pharmacological approaches related to AD preventive and neuroprotective interventions include antioxidant therapy [6,20]. As a result of strong interest to discover compounds with βA-toxicity-modulating property and antioxidative effect, as a chemopreventive measure against AD, we were interested in the isolation of such agents from medicinal plants. To our surprise, plants have attracted relatively a little attention as a potentially valuable resource for drug discovery against AD. Only *Ginkgo biloba* L. [8,14] and *Huperzia serrata* (Thunb. ex Murray) Trevis. [1,18] have been extensively investigated as natural therapeutic agents for the treatment of AD patients.

From turmeric (*Curcuma longa* L., Zingiberaceae), we have isolated three curcuminoids, including curcumin, that protected PC12 and HUVEC cells from βA(1–42) insult. Turmeric has been used as curry spice and as a well-known constituent of Indonesian traditional medicine [13]. Curcumin, the major constituent in turmeric, has been known as a natural antioxidant with antitumor activity [16], an inhibitor of arachidonic acid metabolism [3], and a good anti-inflammatory agent [12]. Currently, it is being evaluated as a chemopreventive agent by the National Cancer Institute [5]. Along with curcumin, demethoxycurcumin and bisdemethoxycurcumin were isolated using bioassay-guided fractionation scheme.

Ground turmeric (1 kg) was extracted with methanol (2 × 2000 ml) and sequentially partitioned with petroleum ether, ethyl acetate, chloroform, and butanol. The chloroform and ethyl acetate fractions were found to effectively protect PC12 and HUVEC cells from βA(1–42) insult at
viability was undermined. This was found to cause profound cell damage, such that, cell spike formation (Fig. 3).

showed extensive MTT formazan granules in the cytoplasm (2000 cells per ml) were incubated with A(1–42). A(1–42) insult was shown to exert direct toxic effects on neurons and inhibit the neurite outgrowth in vitro in a dose dependent manner [15]. Although A(1–42) did not appear to cause cell death at low concentration (1–5 μg/ml), it was found to cause profound cell damage, such that, cell viability was undermined. This A(1–42)-induced cell viability reduction was determined by observing the protective interventions, we evaluated the antioxidant properties of these curcuminoids using 1,1-diphenyl-2-picrylhydrazyl (DPPH)-generated radical trapping experiment. DPPH is known to generate stable free radicals in aqueous or ethanolic solution. It is well-known that the antioxidant potency of compounds through free radical reduction assay. The data points were obtained in triplets on three different dates. From the slope, ED50 values of A(1–42) on PC12 and HUVEC cells were obtained. Data are mean ± SEM from nine determinations. 
P< 0.05 (Student’s t-test).

PC12 rat pheochromocytoma cells were obtained from the American Type Culture Collection (ATCC). HUVEC normal umbilical human vein endothelial cells were obtained from Clonetics (San Diego, CA). Cells were routinely cultured on a polystyrene-coated Corning tissue culture plate (Corning, New York, NY, USA). PC12 cells were grown in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM), 10% horse serum, 5% fetal calf serum, and 1% penicillin/streptomycin. HUVEC cells were grown in EGM-2 Bullet Kit (Clonetics, San Diego, CA, USA). For the bioassay using A(1–42), 100 μL of exponentially growing PC12 and HUVEC cells (2000 and 500 cells per ml, respectively) were plated in 96-well tissue culture plates. A different number of cells per ml were used for the experiments because of the cell size difference between PC12 and HUVEC cells. A(1–42) was purchased from Bachem California (Torrance, CA, USA).

A(1–42) was shown to exert direct toxic effects on neurons and inhibit the neurite outgrowth in vitro in a dose dependent manner [15]. Although A(1–42) did not appear to cause cell death at low concentration (1–5 μg/ml), it was found to cause profound cell damage, such that, cell viability was undermined. This A(1–42)-induced cell viability reduction was determined by observing the amount of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction [11,17]. The change in cell viability was colorimetrically measured at 570 nm, after an incubation of cells in MTT solution (100 μg/ml) for 2 h at 37°C, 100 μL Lysing buffer was added and incubated overnight at 37°C. The optical density of the resulting solutions was colorimetrically determined at 570 nm using ELISA microplate reader. The ED50 values of the compounds were accordingly determined. The compounds’ ability to protect HUVEC cells (500 cells per ml) from A(1–42) insult was similarly evaluated using 2 μg/ml of A(1–42).

Since A-mediated oxidative stress has been suggested as one of the major cause of neuronal cell death in the etiology of AD and antioxidant therapy is one of the current pharmacological approaches related to AD preventive and neuroprotective interventions, we evaluated the antioxidant properties of these curcuminoids using 1,1-diphenyl-2-picrylhydrazyl (DPPH)-generated radical trapping experiment. DPPH is known to generate stable free radicals in aqueous or ethanolic solution. It is well-known that the antioxidant potency of compounds through free radical reduction assay. The data points were obtained in triplets on three different dates. From the slope, ED50 values of A(1–42) on PC12 and HUVEC cells were obtained. Data are mean ± SEM from nine determinations. 
P< 0.05 (Student’s t-test).

The compounds’ ability to protect PC12 and HUVEC cells from A(1–42) insult was investigated. PC12 cells (2000 cells per ml) were incubated with A(1–42) (1.0 μg/ml, prepared from a stock solution, 1.0 mg/ml in DMSO) and the test compounds at various concentrations (10, 2, 0.4, and 0.08 μg/ml) in a 96-well culture plate for 24 h. The compounds’ ability to protect PC12 cells from A(1–42) insult was determined by measuring the cell’s potential to reduce MTT against positive control (1% DMSO only) and negative control (1.0 μg/ml A(1–42) in 1% DMSO without the presence of the test compounds). After an incubation of cells in MTT solution (100 μL per well, 1 mg/ml) for 2 h at 37°C, 100 μL Lysing buffer was added and incubated overnight at 37°C. The optical density of the resulting solutions was colorimetrically determined at 570 nm using ELISA microplate reader. The ED50 values of the compounds were accordingly determined. The compounds’ ability to protect HUVEC cells (500 cells per ml) from A(1–42) insult was similarly evaluated using 2 μg/ml of A(1–42).

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scavenging can be evaluated by observing the optical density change of DPPH radicals in a solution at 515 nm [19]. The curcuminoids were prepared in various concentrations (200, 20, 2.0, 0.2 mg/ml) by serial dilution of stock solution (5.0 mg/ml). Reaction mixtures containing the test sample (dissolved in DMSO) and 300 μM DPPH ethanolic solution in 96-well microtiter plates were incubated at 37°C for 30 min and absorbance was measured at 515 nm as previously described by others [19]. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated positive control group. IC\textsubscript{50} values were determined from percent inhibition by the sample treatment. IC\textsubscript{50} values denote the concentration of sample that was required to scavenge 50% of DPPH free radicals. Both demethoxycurcumin and bisdemethoxycurcumin showed strong antioxidant properties, trapping the DPPH generated radicals as efficiently as a well-known strong antioxidant curcumin (Table 1).

As a control experiment, (±)-α-tocopherol (vitamin-E), a well-known antioxidant currently under investigation to treat AD patients, was tested along with the curcuminoids. It was found that vitamin-E did not protect the cells from βA(1–42) insult at concentrations >50 μg/ml under the same experimental condition. The antioxidant activity of vitamin-E using DPPH generated radical trapping experiment was 115.0 ± 7.0. The results demonstrated that these curcuminoids are not only better antioxidants but also protect cells from βA(1–42) insult more effectively than vitamin-E (Table 1).

In response to a report on synergistic interaction between estrogens and reduced glutathione (GSH), an intracellular antioxidant that results in neuroprotection [4], a possible involvement of these curcuminoids in such mechanism was evaluated using PC12 cells. PC12 cells were treated with βA(1–42) in the presence of the test compounds and reduced GSH. The procedure used for this experiment was

Table 1
Antioxidant activity and PC12 and HUVEC cell protective property of curcuminoids and (±)-α-tocopherol against βA(1–42) insult

<table>
<thead>
<tr>
<th>Name</th>
<th>Antioxidant activity (IC\textsubscript{50} (μg/ml))</th>
<th>PC12 ED\textsubscript{50} (μg/ml)</th>
<th>HUVEC ED\textsubscript{50} (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>28.2 ± 0.5</td>
<td>7.1 ± 0.3</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>Demethoxycurcumin</td>
<td>32.5 ± 0.4</td>
<td>4.7 ± 0.1</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Bisdemethoxycurcumin</td>
<td>36.2 ± 0.6</td>
<td>3.5 ± 0.2</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>(±)-α-Tocopherol</td>
<td>115 ± 7.0</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

a The tests were performed in triplets on three different dates. Data are mean ± SEM from nine determinations. P < 0.05 (student’s t-test).
concentrations cytotoxicity against PC12 and HUVEC cells at compounds (10\(^{-3}\) M) found in the literature [4]. The addition of reduced GSH did not show any change in the compounds’ ability to protect cells from βA(1–42) insult.

In the present study, we identified potentially valuable antioxidants that protect cells from βA insult from a medicinal plant, C. longa (turmeric). Three curcuminoid antioxidants from C. longa were found to effectively protect PC12 and HUVEC cells from βA(1–42) insult at ED\(_{50}\) = 3–7 μg/ml (Table 1). These compounds did not show any cytotoxicity against PC12 and HUVEC cells at concentrations > 50 μg/ml.

Fig. 4 shows colorimetrically determined results of MTT reduction assay on PC12 and HUVEC cells, treated with test compounds (10 μg/ml) in the presence of βA(1–42) at 5.0 μg/ml, respectively. The results show complete protection of the cells from βA(1–42) insult by the compounds at 10 μg/ml level.

MTT is a monotetrazolium salt that can be intracellularly reduced to purple-colored formazan and is widely used for measuring cell viability [11,17]. Although it was widely assumed that MTT is reduced by active mitochondria, recently it was suggested that MTT is taken up by cells through endocytosis and reduced by a N-methylmaleimide-sensitive flavin oxidase [9,10]. MTT reduction assay showed that βA(1–42) profoundly attenuate the viability of PC12 and HUVEC cells, resulting in MTT formazan spike formation. The negative control cells (βA(1–42) treatment without the presence of the test compounds) showed extensive MTT formazan spike formation. Meanwhile, the positive control cells (1% DMSO only treatment) showed extensive formation of MTT formazan granules in the cytoplasm, a sign of healthy viable cells. Cells treated with both βA(1–42) and the compounds showed extensive formation of MTT formazan granules without MTT formazan spike generation in the cytoplasm, demonstrating that the cells were effectively protected by the curcuminoids from βA(1–42) insult (Fig. 3).

βA-mediated oxidative stress has been suggested as one of the major cause of neuronal cell death in the etiology of AD. All three curcuminoids were found to possess strong antioxidant properties, effectively trapping the DPPH generated radicals, suggesting that these compounds may be protecting cells from βA(1–42) insult through antioxidant pathway. However, the possibility of other pathways involved in protecting cells from βA(1–42) insult can not be disputed since the presence of reduced form of GSH did not effect the activity of the compounds in protecting the cells from βA(1–42) insult. Curcumin was recently reported to inhibit NFκB activation in neuroblastoma cells to attenuate or abolish βA-induced apoptotic cell death [7].

In conclusion, we identified three potentially valuable curcuminoid antioxidants from a medicinal plant, C. longa (turmeric), that protect PC12 and HUVEC cells from βA(1–42) insult. Our results suggest that turmeric may be a potentially valuable source of natural therapeutic agents for the treatment of AD patients. We are in a process of developing these antioxidants into such therapeutic agents.

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