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A pro-drug of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) prevents differentiated SH-SY5Y cells from toxicity induced by 6-hydroxydopamine

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Running title: Neuroprotective pro-drug of EGCG in 6-OHDA neurotoxicity

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Abstract

Regular consumption of green tea benefits people in prevention from cardiovascular disorders, obesity as well as neurodegenerative diseases. (-)-Epigallocatechin-3-gallate (EGCG) is regarded as the most biologically active catechin in green tea. However, the stability and bioavailability of EGCG are restricted. The purpose of the present study was to investigate whether a pro-drug, a fully acetylated EGCG (pEGCG), could be more effective in neuroprotection in Parkinsonism mimic cellular model. Retinoic acid (RA)-differentiated neuroblastoma SH-SY5Y cells were pretreated with different concentrations of EGCG and pEGCG for 30 mins and followed by incubation of 25 μM 6-hydroxydopamine (6-OHDA) for 24 h. We found that a broad dosage range of pEGCG (from 0.1 μM to 10 μM) could significantly reduce lactate dehydrogenase release. Likewise, 10 μM of pEGCG was effective in reducing caspase-3 activity, while EGCG at all concentrations tested in the model failed to attenuate caspase-3 activity induced by 6-OHDA. Furthermore, Western-blot analysis showed that Akt could be one of the specific signaling pathways stimulated by pEGCG in neuroprotection. It was demonstrated that 25 μM of 6-OHDA significantly suppressed the phosphorylation level of Akt. Only pEGCG at 10 μM markedly increased its phosphorylation level compared to 6-OHDA alone. Taken together, as pEGCG has higher stability and bioavailbility for further investigation, it could be a potential neuroprotective agent and our current findings may offer certain clues for optimizing its application in future.

Second only to water, tea is considered to be the most commonly consumed beverage in the world [15]. It is well known that there are many health benefits associated with the regular consumption of green tea, the unfermented leaves of the plant *Camellia sinensis* [5]. These benefits include prevention and protection in the areas of cardiovascular disorders, obesity, inflammation and against viral and bacterial infections [5].

Previous studies have demonstrated that green tea can exhibit its beneficial effects against cancer and tumor growth as an anticancer agent [15]. However, there is a recent growing trend for studying the benefits of green tea in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. It was found that individuals who consumed at least two cups of tea on a daily basis were at a decreased risk of Parkinson's disease [23]. There are also associations of green tea with Alzheimer's disease. Long term administration of green tea improved the spatial cognition and learning ability in rats and also reduced cerebral amyloids in Alzheimer transgenic mice [11, 23].

The health benefits of green tea is largely attributed to (-)-epigallocatechin-3-gallate (EGCG), the most prominent and biologically active catechin in green tea [5]. EGCG is known to be the most effective proteasome inhibitor among the polyphenols found in green tea [23]. It is the phenolic hydroxyl groups in EGCG that accounts for its anti-oxidative properties to scavenge reactive oxygen species [24]. In addition, EGCG can exert its effects on multiple cellular and molecular targets involved in the signal transduction pathways that are associated with cell death and survival including the Akt cell survival pathways [24].

However, there are several challenges associated with developing EGCG as a potential chemotherapeutic agent and even neuroprotective agent [5]. The bioavailability of EGCG in

biological systems is limited due to its poor absorption and susceptibility to metabolic transformations such as methylation, glucuronidation, sulfation and oxidative degradation. Its hydrophilic nature prevents the compound from efficiently crossing the blood-brain barrier. Furthermore, the restricted stability of EGCG in alkaline conditions (pH $\geq$ 8) leads to its narrow range of protective dosage [13].

One workable solution in overcoming the poor bioavailability and instability of EGCG is to have the hydroxyl groups replaced by acetyl groups [13]. The fully acetylated EGCG, pEGCG, can function as a pro-drug as the acetyl group can be hydrolyzed intracellularly by esterases to release EGCG inside the cell. Through this pro-drug approach, it minimizes auto-oxidation and allows for better absorption into the cells [5, 13]. It offers a means of increasing the bioavailability of EGCG in vivo [14], and enhances the anti-cancer activities in vitro and in vivo [15, 17].

The present study proposes that modifications to the natural compound EGCG via the peracetate ester of (-)-epigallocatechin-3-gallate (pEGCG) with improved the stability and bioavailability will enhance its neuroprotective ability in neurodegenerative diseases. These pro-drug modifications may lead to innovative perspectives and novel approaches when investigating the biological phenomena of natural derived compounds.

The biological effects of EGCG have already been demonstrated in cancer research by reducing and inhibiting the tumor growth in a variety of tissue types including prostate, breast, liver and lung [13-15, 17]. While the protective effects of EGCG have been investigated in different experimental models of neurodegenerative diseases [3, 18-20, 27], it is less clear whether derivative or pro-drug approach will be superior to the native EGCG. This study aims to establish potential

applications for EGCG and pEGCG in Parkinson's disease by examining the effects of EGCG and pEGCG against 6-hydroxydopamine (6-OHDA)-induced toxicity in retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells.

EGCG and 6-OHDA were purchased from Sigma (Saint Louis, USA). pEGCG was synthesized according to literature procedures [13]. Materials used for SH-SY5Y neuronal cell culture were purchased from Gibco-BRL (Invitrogen, NY, USA). Other chemicals used in this study were purchased from companies listed as follow: RA, dimethyl sulphoxide (DMSO), Triton X-100, 1,4-dithiotreitol (DTT), paraformaldehyde, protease inhibitor cocktail, phosphatase inhibitor cocktail, Tween-20, Temed, 30% acrylamide from Sigma; cytotoxicity detection kit (LDH) from Roche Diagnostics (Mannheim, Germany); colorimetric caspase-3 substrate (Ac-DEVD-pNA) from Calbiochem, Inc. (La Jolla, CA, USA); caspase-3 activity kit from Biosource (Camarillo, CA, USA); rabbit polyclonal antibodies for phospho-Akt from Cell Signaling Technology (Beverly, MA, USA); PVDF membrane and protein assay kit were from Bio-Rad (Richmond, CA, USA); Biomax X-ray film from Kodak (Tokyo, Japan); enhanced chemiluminescence (ECL) detection kit from Amersham (Buckinghamshire, UK).

The procedures for culturing SH-SY5Y cells have been described elsewhere [1-2]. In brief, SH-SY5Y cells were cultured with 10% complete medium (minimum essential medium, MEM, 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (50 μg/mL), streptomycin (50 mg/mL)) in a humidified, 5% CO_2, 37°C incubator. Forty-eight hour after seeding, serum levels of the medium were reduced to 3% with RA (10 μM) for differentiation for seven days prior to treatment. For Western-blot analysis, LDH release and caspase-3 activity assay, differentiated cells at density of 2×10^5 cells/well were cultured onto 6-well plate. Stock solutions of EGCG (1 mM)

and pEGCG (1 mM) were freshly prepared in sterilized distilled water and DMSO, respectively. Different concentrations of EGCG and pEGCG were diluted in the treatment medium. A final concentration of 6-OHDA (25 μM) was used to treat differentiated SH-SY5Y cells. Just before treatment, the culture medium was replaced with treatment medium (MEM supplemented with 3% FBS, 1% L-glutamine (2 mM), 1% penicillin (50 μg/mL) and streptomycin (50 μg/mL). Cells pre-treated with/without compounds for 30 min were exposed to 25 μM 6-OHDA for 24 h.

General toxicity was assessed by LDH assay. The procedures were according to the methods published elsewhere [1, 4, 26, 28]. In brief, 46 μL of culture medium was collected after treatment, and equal volume of reaction solution mixed with them in 96-well plate. The reaction took place in a dark environment for 30 min prior to measurement. Changes in absorbance were measured at 492 nm by a multiplate reader (Labsystem). Results were expressed as fold of control.

Apoptosis was determined by caspase-3-like activity assay. The procedures of caspase-3-like activity assay have been described elsewhere [12, 21, 28]. In brief, cellular proteins were harvested in lysis buffer after treatment. Proteins were separated by centrifugation at 20,000 *g* for 30 min at 4°C. Supernatant was collected and protein concentration was determined by protein assay kit (Bio-Rad). Equal amount of protein from each sample were incubated with caspase-3 substrate (Ac-DEVD-pNA) for 2 h in dark at 37°C. The caspase-3-like activity was determined by measuring the absorbance at 405 nm of the yellow product (pNA) cleaved from the substrate. Specific activity (s.a., unit = pmol/min/mg) were calculated and reported in text. Results were expressed as fold of control.

Procedures of Western-blot were described elsewhere [1-2, 4]. After treatment, SH-SY5Y cells were harvested in ice-cold lysis buffer containing Tris (10 mM, pH 7.4), NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), $Na_4P_2O_7$ (20 mM), Na_3VO_4 (2 mM), Triton X-100 (1%), glycerol (10%), SDS (0.1%), deoxycholate (0.5%). Phenylmethylsulfonyl fluoride (1 mM), protease inhibitor cocktail, and phosphatase inhibitor cocktail were added. Protein extracts (50 μg) were separated in 10% SDS-PAGE gel and then transferred onto a PVDF membrane. The membrane was blocked by 5% non-fat dry milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. It was then incubated with rabbit anti-phosphorylated Akt at serine 473 (1:1000) for 4 h at room temperature. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for 45 min at room temperature. Bands were visualized on a Biomax X-ray film (Kodak) using an enhanced chemiluminescence (ECL) kit.

The results are expressed by mean ± SEM from at least three independent experiments. For statistical comparisons, quantitative data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey test according to the statistical program SigmaStat® (Jandel Scientific, Chicago, IL, USA). $P \leq 0.05$ was regarded as significant.

The chemical structure of EGCG and pEGCG was illustrated in Fig. 1. All hydroxyl groups in EGCG (Fig. 1A) are acetylated in order to generate the more biologically stable pEGCG (Fig. 1B).

We at first examined the morphology of cells after different treatments (Fig. 2). SH-SY5Y cells exposed to 6-OHDA showed dying neurons with shrinkage cell body (Fig. 2B). One could

found this type of shrinkage cell body even the cells were exposed to EGCG (Fig. 2C). However, cell morphology was preserved very well when the cells were treated with pEGCG (Fig. 2D).

Increased LDH level reflects decreased cell viability and increased general cytotoxicity. We examined the effects of EGCG on preventing LDH release in differentiated SH-SY5Y cells by exposing cells to 6-OHDA. The data were reported as fold of control.

As shown by Fig. 3A, LDH activity increased 1.7 fold compared to control after the application of 25 μM of 6-OHDA. Among all concentrations used in this study, only 10 μM of EGCG slightly but significantly decreased the LDH release induced by 6-OHDA.

Measurement of caspase-3 specific activity indicated cellular apoptosis induced by 6-OHDA. After 7 days treatment of RA, differentiated SH-SY5Y cells were pre-treated with different concentrations of EGCG (from 0.1 μM to 10 μM) for 30 min, followed by 24 h incubation of 25 μM 6-OHDA. The activity was reported as fold of control.

The results (Fig. 3B) showed that application of 6-OHDA alone led to more than two-fold increase in caspase-3-like activity compared to control. However, all concentrations of EGCG (from 0.1 to 10 μM) could not markedly attenuate caspase-3-like activity induced by 6-OHDA.

Consistent with previous assays, 3-fold of LDH release was induced by exposing to 6-OHDA in differentiated SH-SY5Y cells. As demonstrated in Fig. 4A, pre-treatments of all concentrations (from 0.1 to 10 μM) of pEGCG markedly and significantly reduced the LDH release triggered by 6-OHDA. Furthermore, the reduction in released LDH was in a dose-dependent

manner, with the higher concentration of pEGCG leading to lower LDH release. A broad range of effective dosages was shown by pEGCG in reducing LDH release in differentiated SH-SY5Y cells compared to EGCG.

To illustrate and compare the effects of pEGCG with the natural compound EGCG on caspase-3-like activity in differentiated SH-SY5Y cells, cells were pre-treated with varying concentrations of pEGCG (from 0.1 to 10 μM), followed by exposure to 6-OHDA for 24 h. The data was reported as fold of control.

As illustrated in Fig. 4B, 6-OHDA at 25 μM increased caspase-3-like activity to 2.3 fold of control. There was a significant reduction of caspase-3-like activity by pre-application of 10 μM pEGCG. However, EGCG (from 0.1 to 10 μM) at all dosages used in the experiments could not attenuate 6-OHDA-triggered caspase-3-like activity.

Akt maintains cell survival by inhibiting apoptosis via regulating the phosphorylation and expression of proteins that are involved in the apoptotic cascade [22]. An inactivation of Akt would therefore lead to cell death by apoptosis [22]. It has been reported that EGCG affects multiple cellular and molecular targets involved in signal transduction pathways that are associated with cell death and survival such as the Akt pathway [6, 22, 24].

Previous studies have shown that EGCG treatment inhibits the Akt signaling pathway and increases phospho-Akt immunoreactivity, however, limited experimentation have been done with pEGCG and its effects on Akt and phospho-Akt levels [22, 24].

By Western-blot analysis shown in Fig. 5, 25 μM of 6-OHDA significantly suppressed the phosphorylation level of Akt, which could not be increased by application of EGCG from 0.1 μM to 10 μM. However, pEGCG at 10 μM exerted specific stimulation to Akt signaling, markedly increasing its phosphorylation level compared to 6-OHDA alone. Furthermore, pEGCG (0.1 μM to 10 μM) itself could markedly upregulate active Akt, in which similar effect was not observed in EGCG (0.1 μM to 10 μM) alone group.

In green tea, EGCG is the most abundant and active catechin [15]. It is generally accepted that EGCG contributes considerably to the beneficial health effects of green tea. However, EGCG is unstable under physiological conditions, especially those being neutral or alkaline, thus contributing to its poor bioavailability [13, 23]. In addition, the hydroxyl groups of EGCG are susceptible to metabolic methylation, glucuronidation and sulphate formation [15].

By chemically replacing the hydroxyls of EGCG with acetyl groups, the molecular stability is improved [13]. Furthermore, the less hydrophilic pEGCG may be better absorbed by the cells followed by hydrolysis of the acetate moiety to the hydroxyl group by intracellular esterases, resulting in increased bioavailability [13-15]. These improvements were confirmed when previous reports showed that cancer cells with pEGCG treatment had lowered levels of proteasome chymotrypsin-like activity, higher growth suppression and enhanced apoptosis as compared to the control cells treated with naturally occurring EGCG [15]. Our study investigated these beneficial and stability effects of pEGCG that were found in cancer for similar applications in neurodegenerative diseases.

In this study, pEGCG demonstrated more effective prevention from the toxicity induced by 6-OHDA in differentiated SH-SY5Y cells than EGCG. It was found that EGCG at 10 μM was able to decrease the LDH release that was initially induced by 6-OHDA toxicity, but EGCG at all concentrations applied in this study failed to suppress the caspase-3-like activity induced by 6-OHDA. While our study demonstrated that a broad dosage range of pEGCG (from 0.1 to 10 μM) could significantly reduce LDH release, 10 μM of pEGCG turned out to be the most effective in reducing caspase-3-like activity in current cellular model.

In addition, this study also examined the potential cell signaling through which pEGCG exerts its effects. Previous studies in cancer research have suggested that EGCG inhibits the Akt signaling pathway [24-25]. As the Akt pathway is associated with cell proliferation and survival via the inhibition of apoptosis and the down-regulation of the proteins involved in the apoptotic cascade, the inhibitory effect of EGCG on Akt signaling pathway is beneficial in suppressing tumor growth [22, 24]. These studies suggest that EGCG contributes to cell survival via the Akt pathway. Although EGCG has been reported to down-regulate the Akt pathway, leading to apoptosis in cancer cells [24-25], other reports have demonstrated the anti-apoptotic effect of EGCG via the upregulation of PI3k/Akt pathway in neuronal cells [7-8], suggesting that modulation of the Akt pathway by EGCG is cell dependent.

Previously, we have demonstrated the role of Akt in protecting our culture model from 6-OHDA-challenge [2], with limited information about the neuroprotective effect of pEGCG on the Akt signaling pathway, we compared between EGCG and pEGCG in our culture model. In our study we found that after differentiating the cells with RA and applying 6-OHDA, pEGCG at 10 μM increased the expression of phospho-Akt as shown in Fig. 5. Under the same conditions, EGCG

had very little effect on up-regulating the level of phospho-Akt. Furthermore, pEGCG *per se* showed remarkably increase of phosphorylated Akt, suggesting that Akt may be a specific signaling for pEGCG to exert its neuroprotective effects in differentiated SH-SY5Y cells.

The neuroprotective, anti-oxidative and anti-apoptotic ability of EGCG has been widely studied in neuroscience research [7-8, 11, 16]. Whether the superior effect of pEGCG compared to EGCG is merely on its higher stability and bioavailability, or it undergoes its unique protective mechanisms, requires more experimental data to support. From the literature, pEGCG, but not EGCG, has been demonstrated to be a proteasome inhibitor, indicating the possibility for pEGCG to function as a new compound in cancer research [9]. Here we report that Akt may be another unique pathway in pEGCG-mediated neuroprotection in PD research.

Overall, it was found that the modifications of EGCG via pEGCG allowed the green tea polyphenol to be more effective than its natural precursor EGCG. The effective range of pEGCG in suppressing LDH release and caspase-3-like activity was broad compared with that of EGCG. The effects may be attributed to its higher stability and higher bioavailability as well as differentiation state in current cellular model, but more importantly via the up-regulation of the Akt pathway. Directions for further research would include determining the protective effects of EGCG and pEGCG against various types of stresses such as MPP^+-induced toxicity, compare the underlying protective mechanisms and confirm the enhanced bioactivity of pEGCG in future animal experiments. Taken together, pEGCG has the potential to act as protective agents in neurological degenerative disorders such as Parkinson's disease, and the current experimental findings are of great importance for optimizing potential applications of EGCG as neuroprotective agents.

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Figure Legends

Fig. 1. (A) Chemical structure of EGCG. (B) Chemical structure of fully acetylated EGCG (pEGCG).

Fig. 2. Morphology of differentiated neuroblastoma SH-SY5Y cells after (A) vehicle control, (B) 6-OHDA at 25 μM, (C) 6-OHDA + EGCG at 10μM, and (D) 6-OHDA + pEGCG at 10 μM for 24 h. Arrow indicates cells with shrinkage and round up in 6-OHDA and 6-OHDA + EGCG groups, but not in 6-OHDA + pEGCG group.

Fig. 3. Neuroprotective effects of EGCG on LDH release and caspase-3 activity in differentiated SH-SY5Y cells. Differentiated neurons were pre-treated with EGCG (0.1 – 10 μM) for 30 min, followed by incubation with 6-OHDA (25 μM) for 24 h. After treatment, LDH release assay (A) and caspase-3 activity assay (B) were performed. Results are expressed as mean ± SEM from at least three independent experiments. $^{*}P < 0.05$ vs. the group treated with 6-OHDA only by one-way ANOVA for multiple comparison and Tukey test as *post hoc* test.

Fig. 4. pEGCG markedly reduced LDH release and caspase-3 activity induced by 6-OHDA in differentiated SH-SY5Y cells. Neurons were differentiated by RA for 7 days, prior to incubating 6-OHDA (25 μM) for 24 h, pEGCG (0.1 – 10 μM) were applied for 30 min. After incubation, LDH release assay (A) and colorimetric caspase-3-like activity assay (B) were performed. Results are expressed as mean ± SEM from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. the group treated with 6-OHDA only by one-way ANOVA for multiple comparison and Tukey test as *post hoc* test.

Fig. 5. Effects of EGCG and pEGCG on phospho-Akt level in differentiated SH-SY5Y cells. In differentiated SH-SY5Y cells, EGCG (0.1 - 10 μM) and pEGCG (0.1 - 10 μM) were pre-treated for 30 min, followed by exposure to 25 μM 6-OHDA for 4 h. Proteins extracted were subjected to Western-blot analysis to detect the level of phospho-Akt and total Akt (loading control). Results are expressed from at least three independent experiments.

Marked Revision

A pro-drug of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) prevents differentiated SH-SY5Y cells from toxicity induced by 6-hydroxydopamine

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Abstract

Regular consumption of green tea benefits people in prevention from cardiovascular disorders, obesity as well as neurodegenerative diseases. (-)-Epigallocatechin-3-gallate (EGCG) is regarded as the most biologically active catechin in green tea. However, the stability and bioavailability of EGCG are restricted. The purpose of the present study was to investigate whether a pro-drug, a fully acetylated EGCG (pEGCG), could be more effective in neuroprotection in Parkinsonism mimic cellular model. Retinoic acid (RA)-differentiated neuroblastoma SH-SY5Y cells were pretreated with different concentrations of EGCG and pEGCG for 30 mins and followed by incubation of 25 μM 6-hydroxydopamine (6-OHDA) for 24 h. We found that a broad dosage range of pEGCG (from 0.1 μM to 10 μM) could significantly reduce lactate dehydrogenase release. Likewise, 10 μM of pEGCG was effective in reducing caspase-3 activity, while EGCG at all concentrations tested in the model failed to attenuate caspase-3 activity induced by 6-OHDA. Furthermore, Western-blot analysis showed that Akt could be one of the specific signaling pathways stimulated by pEGCG in neuroprotection. It was demonstrated that 25 μM of 6-OHDA significantly suppressed the phosphorylation level of Akt. Only pEGCG at 10 μM markedly increased its phosphorylation level compared to 6-OHDA alone. Taken together, as pEGCG has higher stability and bioavailbility for further investigation, it could be a potential neuroprotective agent and our current findings may offer certain clues for optimizing its application in future.

Second only to water, tea is considered to be the most commonly consumed beverage in the world [15]. It is well known that there are many health benefits associated with the regular consumption of green tea, the unfermented leaves of the plant *Camellia sinensis* [5]. These benefits include prevention and protection in the areas of cardiovascular disorders, obesity, inflammation and against viral and bacterial infections [5].

Previous studies have demonstrated that green tea can exhibit its beneficial effects against cancer and tumor growth as an anticancer agent [15]. However, there is a recent growing trend for studying the benefits of green tea in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. It was found that individuals who consumed at least two cups of tea on a daily basis were at a decreased risk of Parkinson's disease [23]. There are also associations of green tea with Alzheimer's disease. Long term administration of green tea improved the spatial cognition and learning ability in rats and also reduced cerebral amyloids in Alzheimer transgenic mice [11, 23].

The health benefits of green tea is largely attributed to (-)-epigallocatechin-3-gallate (EGCG), the most prominent and biologically active catechin in green tea [5]. EGCG is known to be the most effective proteasome inhibitor among the polyphenols found in green tea [23]. It is the phenolic hydroxyl groups in EGCG that accounts for its anti-oxidative properties to scavenge reactive oxygen species [24]. In addition, EGCG can exert its effects on multiple cellular and molecular targets involved in the signal transduction pathways that are associated with cell death and survival including the Akt cell survival pathways [24].

However, there are several challenges associated with developing EGCG as a potential chemotherapeutic agent and even neuroprotective agent [5]. The bioavailability of EGCG in

biological systems is limited due to its poor absorption and susceptibility to metabolic transformations such as methylation, glucuronidation, sulfation and oxidative degradation. Its hydrophilic nature prevents the compound from efficiently crossing the blood-brain barrier. Furthermore, the restricted stability of EGCG in alkaline conditions (pH $\geq$ 8) leads to its narrow range of protective dosage [13].

One workable solution in overcoming the poor bioavailability and instability of EGCG is to have the hydroxyl groups replaced by acetyl groups [13]. The fully acetylated EGCG, pEGCG, can function as a pro-drug as the acetyl group can be hydrolyzed intracellularly by esterases to release EGCG inside the cell. Through this pro-drug approach, it minimizes auto-oxidation and allows for better absorption into the cells [5, 13]. It offers a means of increasing the bioavailability of EGCG in vivo [14], and enhances the anti-cancer activities in vitro and in vivo [15, 17].

The present study proposes that modifications to the natural compound EGCG via the peracetate ester of (-)-epigallocatechin-3-gallate (pEGCG) with improved the stability and bioavailability will enhance its neuroprotective ability in neurodegenerative diseases. These pro-drug modifications may lead to innovative perspectives and novel approaches when investigating the biological phenomena of natural derived compounds.

The biological effects of EGCG have already been demonstrated in cancer research by reducing and inhibiting the tumor growth in a variety of tissue types including prostate, breast, liver and lung [13-15, 17]. While the protective effects of EGCG have been investigated in different experimental models of neurodegenerative diseases [3, 18-20, 27], it is less clear whether derivative or pro-drug approach will be superior to the native EGCG. This study aims to establish potential

applications for EGCG and pEGCG in Parkinson's disease by examining the effects of EGCG and pEGCG against 6-hydroxydopamine (6-OHDA)-induced toxicity in retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells.

EGCG and 6-OHDA were purchased from Sigma (Saint Louis, USA). pEGCG was synthesized according to literature procedures [13]. Materials used for SH-SY5Y neuronal cell culture were purchased from Gibco-BRL (Invitrogen, NY, USA). Other chemicals used in this study were purchased from companies listed as follow: RA, dimethyl sulphoxide (DMSO), Triton X-100, 1,4-dithiotreitol (DTT), paraformaldehyde, protease inhibitor cocktail, phosphatase inhibitor cocktail, Tween-20, Temed, 30% acrylamide from Sigma; cytotoxicity detection kit (LDH) from Roche Diagnostics (Mannheim, Germany); colorimetric caspase-3 substrate (Ac-DEVD-pNA) from Calbiochem, Inc. (La Jolla, CA, USA); caspase-3 activity kit from Biosource (Camarillo, CA, USA); rabbit polyclonal antibodies for phospho-Akt from Cell Signaling Technology (Beverly, MA, USA); PVDF membrane and protein assay kit were from Bio-Rad (Richmond, CA, USA); Biomax X-ray film from Kodak (Tokyo, Japan); enhanced chemiluminescence (ECL) detection kit from Amersham (Buckinghamshire, UK).

The procedures for culturing SH-SY5Y cells have been described elsewhere [1-2]. In brief, SH-SY5Y cells were cultured with 10% complete medium (minimum essential medium, MEM, 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (50 μg/mL), streptomycin (50 mg/mL)) in a humidified, 5% CO_2, 37°C incubator. Forty-eight hour after seeding, serum levels of the medium were reduced to 3% with RA (10 μM) for differentiation for seven days prior to treatment. For Western-blot analysis, LDH release and caspase-3 activity assay, differentiated cells at density of 2×10^5 cells/well were cultured onto 6-well plate. Stock solutions of EGCG (1 mM)

and pEGCG (1 mM) were freshly prepared in sterilized distilled water and DMSO, respectively. Different concentrations of EGCG and pEGCG were diluted in the treatment medium. A final concentration of 6-OHDA (25 μM) was used to treat differentiated SH-SY5Y cells. Just before treatment, the culture medium was replaced with treatment medium (MEM supplemented with 3% FBS, 1% L-glutamine (2 mM), 1% penicillin (50 μg/mL) and streptomycin (50 μg/mL). Cells pre-treated with/without compounds for 30 min were exposed to 25 μM 6-OHDA for 24 h.

General toxicity was assessed by LDH assay. The procedures were according to the methods published elsewhere [1, 4, 26, 28]. In brief, 46 μL of culture medium was collected after treatment, and equal volume of reaction solution mixed with them in 96-well plate. The reaction took place in a dark environment for 30 min prior to measurement. Changes in absorbance were measured at 492 nm by a multiplate reader (Labsystem). Results were expressed as fold of control.

Apoptosis was determined by caspase-3-like activity assay. The procedures of caspase-3-like activity assay have been described elsewhere [12, 21, 28]. In brief, cellular proteins were harvested in lysis buffer after treatment. Proteins were separated by centrifugation at 20,000 *g* for 30 min at 4°C. Supernatant was collected and protein concentration was determined by protein assay kit (Bio-Rad). Equal amount of protein from each sample were incubated with caspase-3 substrate (Ac-DEVD-pNA) for 2 h in dark at 37°C. The caspase-3-like activity was determined by measuring the absorbance at 405 nm of the yellow product (pNA) cleaved from the substrate. Specific activity (s.a., unit = pmol/min/mg) were calculated and reported in text. Results were expressed as fold of control.

Procedures of Western-blot were described elsewhere [1-2, 4]. After treatment, SH-SY5Y cells were harvested in ice-cold lysis buffer containing Tris (10 mM, pH 7.4), NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), $Na_4P_2O_7$ (20 mM), Na_3VO_4 (2 mM), Triton X-100 (1%), glycerol (10%), SDS (0.1%), deoxycholate (0.5%). Phenylmethylsulfonyl fluoride (1 mM), protease inhibitor cocktail, and phosphatase inhibitor cocktail were added. Protein extracts (50 µg) were separated in 10% SDS-PAGE gel and then transferred onto a PVDF membrane. The membrane was blocked by 5% non-fat dry milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. It was then incubated with rabbit anti-phosphorylated Akt at serine 473 (1:1000) for 4 h at room temperature. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for 45 min at room temperature. Bands were visualized on a Biomax X-ray film (Kodak) using an enhanced chemiluminescence (ECL) kit.

The results are expressed by mean ± SEM from at least three independent experiments. For statistical comparisons, quantitative data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey test according to the statistical program SigmaStat® (Jandel Scientific, Chicago, IL, USA). $P \leq 0.05$ was regarded as significant.

The chemical structure of EGCG and pEGCG was illustrated in Fig. 1. All hydroxyl groups in EGCG (Fig. 1A) are acetylated in order to generate the more biologically stable pEGCG (Fig. 1B).

We at first examined the morphology of cells after different treatments (Fig. 2). SH-SY5Y cells exposed to 6-OHDA showed dying neurons with shrinkage cell body (Fig. 2B). One could

found this type of shrinkage cell body even the cells were exposed to EGCG (Fig. 2C). However, cell morphology was preserved very well when the cells were treated with pEGCG (Fig. 2D).

Increased LDH level reflects decreased cell viability and increased general cytotoxicity. We examined the effects of EGCG on preventing LDH release in differentiated SH-SY5Y cells by exposing cells to 6-OHDA. The data were reported as fold of control.

As shown by Fig. 3A, LDH activity increased 1.7 fold compared to control after the application of 25 μM of 6-OHDA. Among all concentrations used in this study, only 10 μM of EGCG slightly but significantly decreased the LDH release induced by 6-OHDA.

Measurement of caspase-3 specific activity indicated cellular apoptosis induced by 6-OHDA. After 7 days treatment of RA, differentiated SH-SY5Y cells were pre-treated with different concentrations of EGCG (from 0.1 μM to 10 μM) for 30 min, followed by 24 h incubation of 25 μM 6-OHDA. The activity was reported as fold of control.

The results (Fig. 3B) showed that application of 6-OHDA alone led to more than two-fold increase in caspase-3-like activity compared to control. However, all concentrations of EGCG (from 0.1 to 10 μM) could not markedly attenuate caspase-3-like activity induced by 6-OHDA.

Consistent with previous assays, 3-fold of LDH release was induced by exposing to 6-OHDA in differentiated SH-SY5Y cells. As demonstrated in Fig. 4A, pre-treatments of all concentrations (from 0.1 to 10 μM) of pEGCG markedly and significantly reduced the LDH release triggered by 6-OHDA. Furthermore, the reduction in released LDH was in a dose-dependent

manner, with the higher concentration of pEGCG leading to lower LDH release. A broad range of effective dosages was shown by pEGCG in reducing LDH release in differentiated SH-SY5Y cells compared to EGCG.

To illustrate and compare the effects of pEGCG with the natural compound EGCG on caspase-3-like activity in differentiated SH-SY5Y cells, cells were pre-treated with varying concentrations of pEGCG (from 0.1 to 10 μM), followed by exposure to 6-OHDA for 24 h. The data was reported as fold of control.

As illustrated in Fig. 4B, 6-OHDA at 25 μM increased caspase-3-like activity to 2.3 fold of control. There was a significant reduction of caspase-3-like activity by pre-application of 10 μM pEGCG. However, EGCG (from 0.1 to 10 μM) at all dosages used in the experiments could not attenuate 6-OHDA-triggered caspase-3-like activity.

Akt maintains cell survival by inhibiting apoptosis via regulating the phosphorylation and expression of proteins that are involved in the apoptotic cascade [22]. An inactivation of Akt would therefore lead to cell death by apoptosis [22]. It has been reported that EGCG affects multiple cellular and molecular targets involved in signal transduction pathways that are associated with cell death and survival such as the Akt pathway [6, 22, 24].

Previous studies have shown that EGCG treatment inhibits the Akt signaling pathway and increases phospho-Akt immunoreactivity, however, limited experimentation have been done with pEGCG and its effects on Akt and phospho-Akt levels [22, 24].

By Western-blot analysis shown in Fig. 5, 25 μM of 6-OHDA significantly suppressed the phosphorylation level of Akt, which could not be increased by application of EGCG from 0.1 μM to 10 μM. However, pEGCG at 10 μM exerted specific stimulation to Akt signaling, markedly increasing its phosphorylation level compared to 6-OHDA alone. Furthermore, pEGCG (0.1 μM to 10 μM) itself could markedly upregulate active Akt, in which similar effect was not observed in EGCG (0.1 μM to 10 μM) alone group.

In green tea, EGCG is the most abundant and active catechin [15]. It is generally accepted that EGCG contributes considerably to the beneficial health effects of green tea. However, EGCG is unstable under physiological conditions, especially those being neutral or alkaline, thus contributing to its poor bioavailability [13, 23]. In addition, the hydroxyl groups of EGCG are susceptible to metabolic methylation, glucuronidation and sulphate formation [15].

By chemically replacing the hydroxyls of EGCG with acetyl groups, the molecular stability is improved [13]. Furthermore, the less hydrophilic pEGCG may be better absorbed by the cells followed by hydrolysis of the acetate moiety to the hydroxyl group by intracellular esterases, resulting in increased bioavailability [13-15]. These improvements were confirmed when previous reports showed that cancer cells with pEGCG treatment had lowered levels of proteasome chymotrypsin-like activity, higher growth suppression and enhanced apoptosis as compared to the control cells treated with naturally occurring EGCG [15]. Our study investigated these beneficial and stability effects of pEGCG that were found in cancer for similar applications in neurodegenerative diseases.

In this study, pEGCG demonstrated more effective prevention from the toxicity induced by 6-OHDA in differentiated SH-SY5Y cells than EGCG. It was found that EGCG at 10 μM was able to decrease the LDH release that was initially induced by 6-OHDA toxicity, but EGCG at all concentrations applied in this study failed to suppress the caspase-3-like activity induced by 6-OHDA. While our study demonstrated that a broad dosage range of pEGCG (from 0.1 to 10 μM) could significantly reduce LDH release, 10 μM of pEGCG turned out to be the most effective in reducing caspase-3-like activity in current cellular model.

In addition, this study also examined the potential cell signaling through which pEGCG exerts its effects. Previous studies in cancer research have suggested that EGCG inhibits the Akt signaling pathway [24-25]. As the Akt pathway is associated with cell proliferation and survival via the inhibition of apoptosis and the down-regulation of the proteins involved in the apoptotic cascade, the inhibitory effect of EGCG on Akt signaling pathway is beneficial in suppressing tumor growth [22, 24]. These studies suggest that EGCG contributes to cell survival via the Akt pathway. Although EGCG has been reported to down-regulate the Akt pathway, leading to apoptosis in cancer cells [24-25], other reports have demonstrated the anti-apoptotic effect of EGCG via the upregulation of PI3k/Akt pathway in neuronal cells [7-8], suggesting that modulation of the Akt pathway by EGCG is cell dependent.

Previously, we have demonstrated the role of Akt in protecting our culture model from 6-OHDA-challenge [2], with limited information about the neuroprotective effect of pEGCG on the Akt signaling pathway, we compared between EGCG and pEGCG in our culture model. In our study we found that after differentiating the cells with RA and applying 6-OHDA, pEGCG at 10 μM increased the expression of phospho-Akt as shown in Fig. 5. Under the same conditions, EGCG

had very little effect on up-regulating the level of phospho-Akt. Furthermore, pEGCG *per se* showed remarkably increase of phosphorylated Akt, suggesting that Akt may be a specific signaling for pEGCG to exert its neuroprotective effects in differentiated SH-SY5Y cells.

The neuroprotective, anti-oxidative and anti-apoptotic ability of EGCG has been widely studied in neuroscience research [7-8, 11, 16]. Whether the superior effect of pEGCG compared to EGCG is merely on its higher stability and bioavailability, or it undergoes its unique protective mechanisms, requires more experimental data to support. From the literature, pEGCG, but not EGCG, has been demonstrated to be a proteasome inhibitor, indicating the possibility for pEGCG to function as a new compound in cancer research [9]. Here we report that Akt may be another unique pathway in pEGCG-mediated neuroprotection in PD research.

Overall, it was found that the modifications of EGCG via pEGCG allowed the green tea polyphenol to be more effective than its natural precursor EGCG. The effective range of pEGCG in suppressing LDH release and caspase-3-like activity was broad compared with that of EGCG. The effects may be attributed to its higher stability and higher bioavailability as well as differentiation state in current cellular model, but more importantly via the up-regulation of the Akt pathway. Directions for further research would include determining the protective effects of EGCG and pEGCG against various types of stresses such as MPP^+-induced toxicity, compare the underlying protective mechanisms and confirm the enhanced bioactivity of pEGCG in future animal experiments. Taken together, pEGCG has the potential to act as protective agents in neurological degenerative disorders such as Parkinson's disease, and the current experimental findings are of great importance for optimizing potential applications of EGCG as neuroprotective agents.

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Figure Legends

Fig. 1. (A) Chemical structure of EGCG. (B) Chemical structure of fully acetylated EGCG (pEGCG).

Fig. 2. Morphology of differentiated neuroblastoma SH-SY5Y cells after (A) vehicle control, (B) 6-OHDA at 25 μM, (C) 6-OHDA + EGCG at 10μM, and (D) 6-OHDA + pEGCG at 10 μM for 24 h. Arrow indicates cells with shrinkage and round up in 6-OHDA and 6-OHDA + EGCG groups, but not in 6-OHDA + pEGCG group.

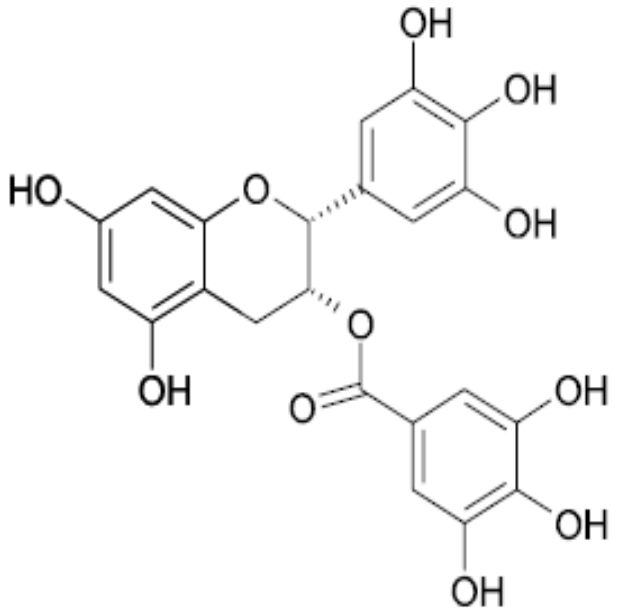
Fig. 3. Neuroprotective effects of EGCG on LDH release and caspase-3 activity in differentiated SH-SY5Y cells. Differentiated neurons were pre-treated with EGCG (0.1 – 10 μM) for 30 min, followed by incubation with 6-OHDA (25 μM) for 24 h. After treatment, LDH release assay (A) and caspase-3 activity assay (B) were performed. Results are expressed as mean ± SEM from at least three independent experiments. $^{*}P < 0.05$ vs. the group treated with 6-OHDA only by one-way ANOVA for multiple comparison and Tukey test as *post hoc* test.

Fig. 4. pEGCG markedly reduced LDH release and caspase-3 activity induced by 6-OHDA in differentiated SH-SY5Y cells. Neurons were differentiated by RA for 7 days, prior to incubating 6-OHDA (25 μM) for 24 h, pEGCG (0.1 – 10 μM) were applied for 30 min. After incubation, LDH release assay (A) and colorimetric caspase-3-like activity assay (B) were performed. Results are expressed as mean ± SEM from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. the group treated with 6-OHDA only by one-way ANOVA for multiple comparison and Tukey test as *post hoc* test.

Fig. 5. Effects of EGCG and pEGCG on phospho-Akt level in differentiated SH-SY5Y cells. In differentiated SH-SY5Y cells, EGCG (0.1 - 10 μM) and pEGCG (0.1 - 10 μM) were pre-treated for 30 min, followed by exposure to 25 μM 6-OHDA for 4 h. Proteins extracted were subjected to Western-blot analysis to detect the level of phospho-Akt and total Akt (loading control). Results are expressed from at least three independent experiments.

Figure

(A)



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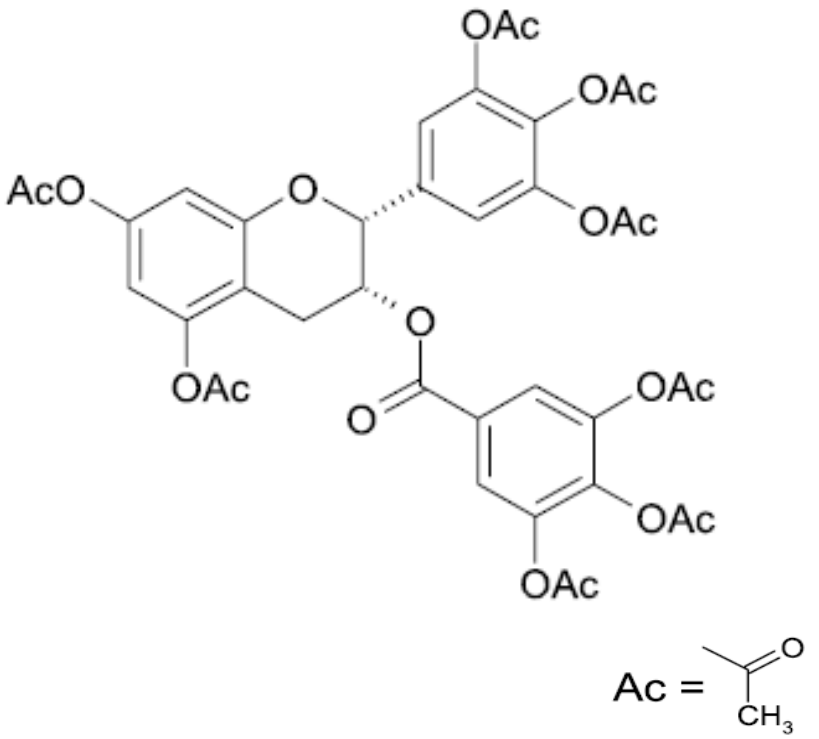

Fig. 1 Chao *et al.*, 2009

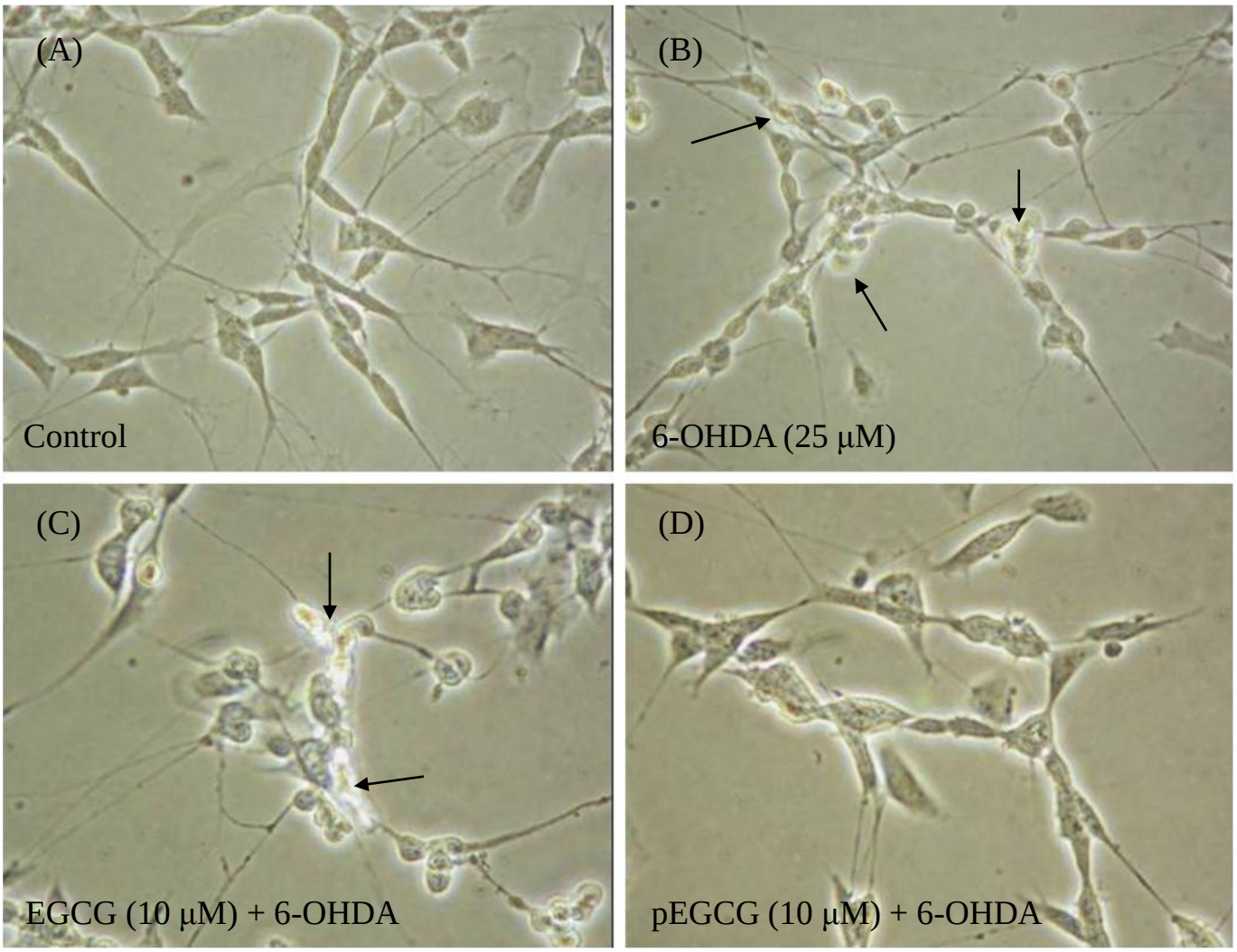

Fig. 2 Chao *et al.*, 2009

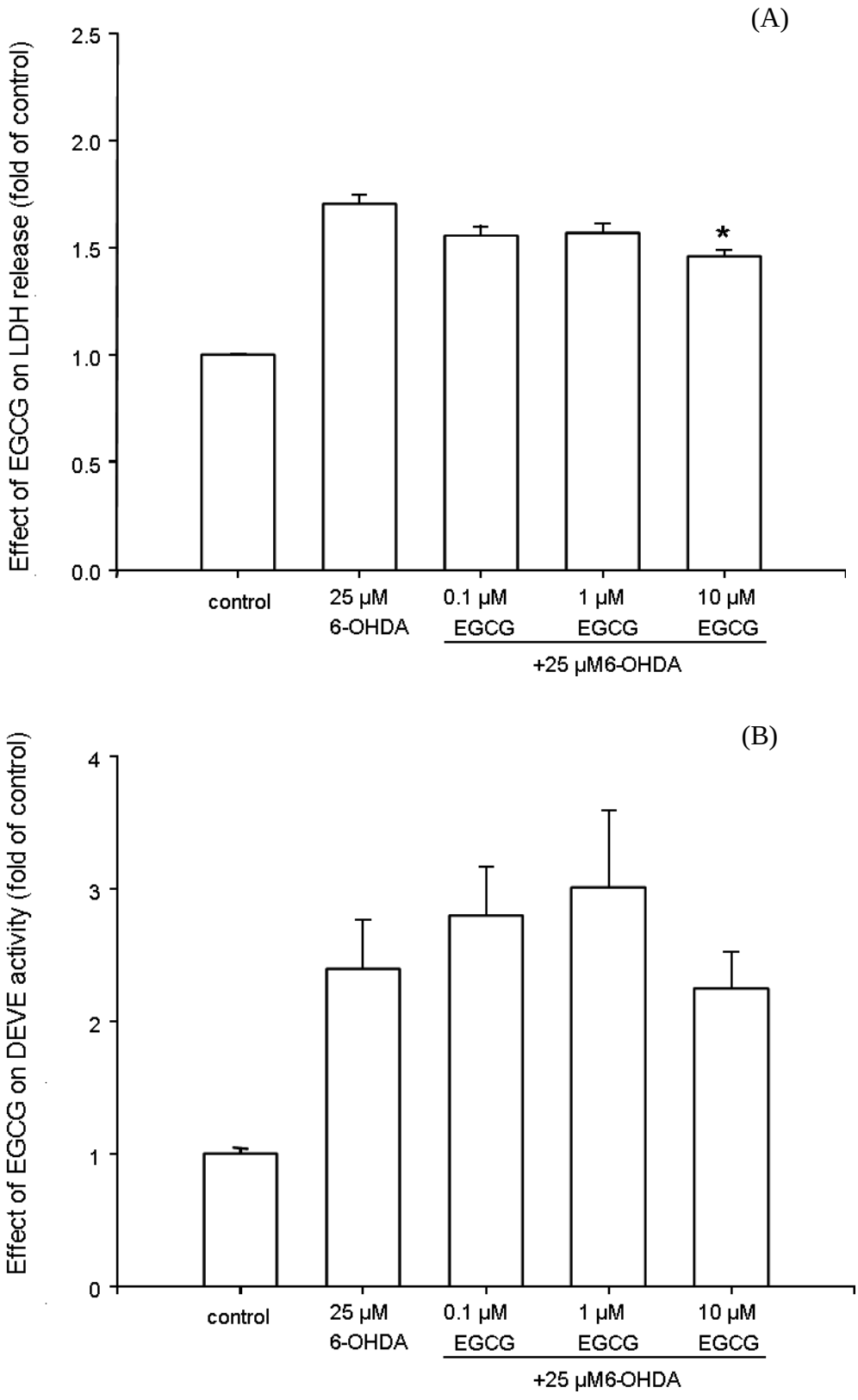

Fig. 3 Chao *et al.*, 2009

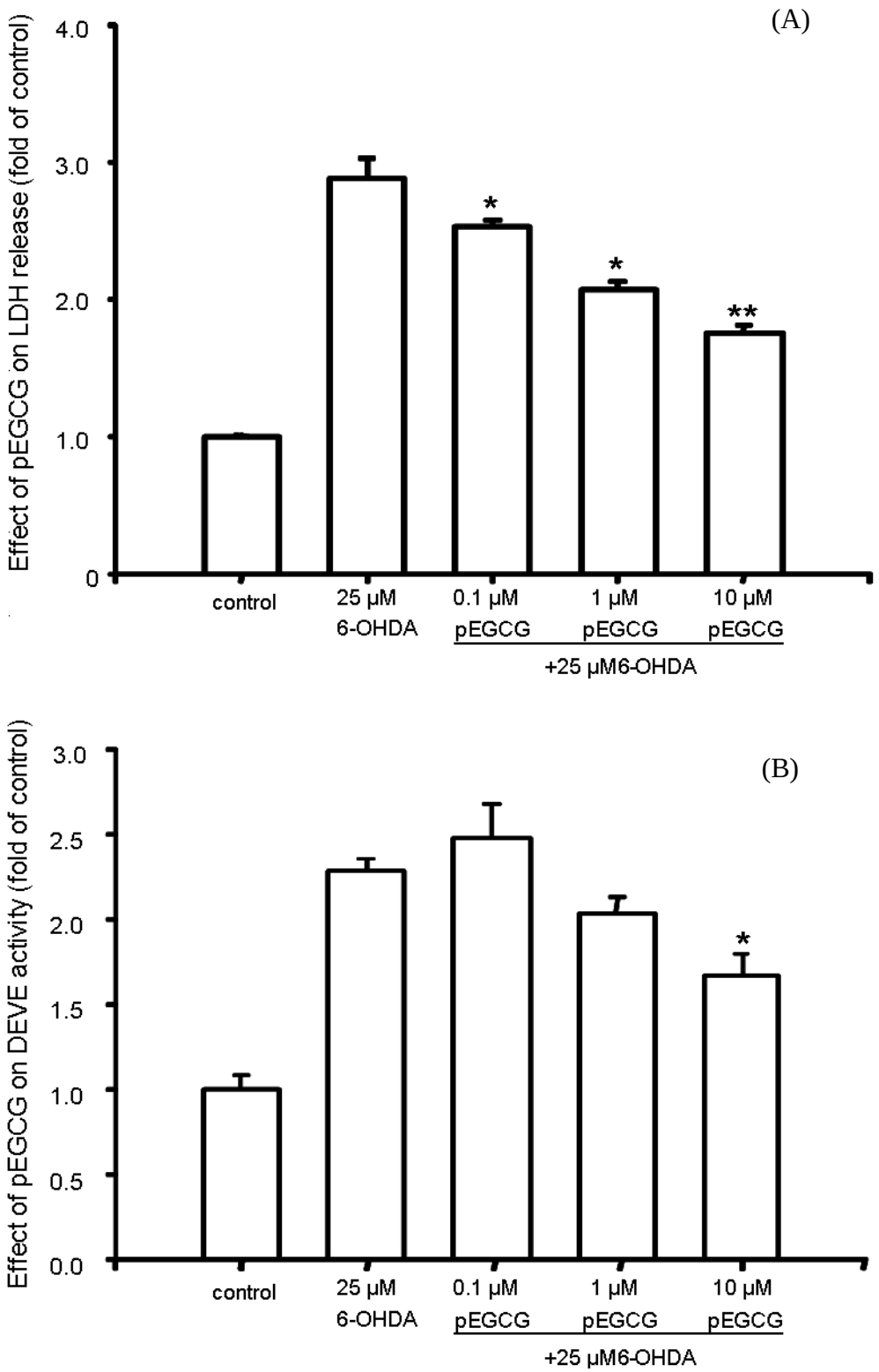

Fig. 4 Chao *et al.*, 2009

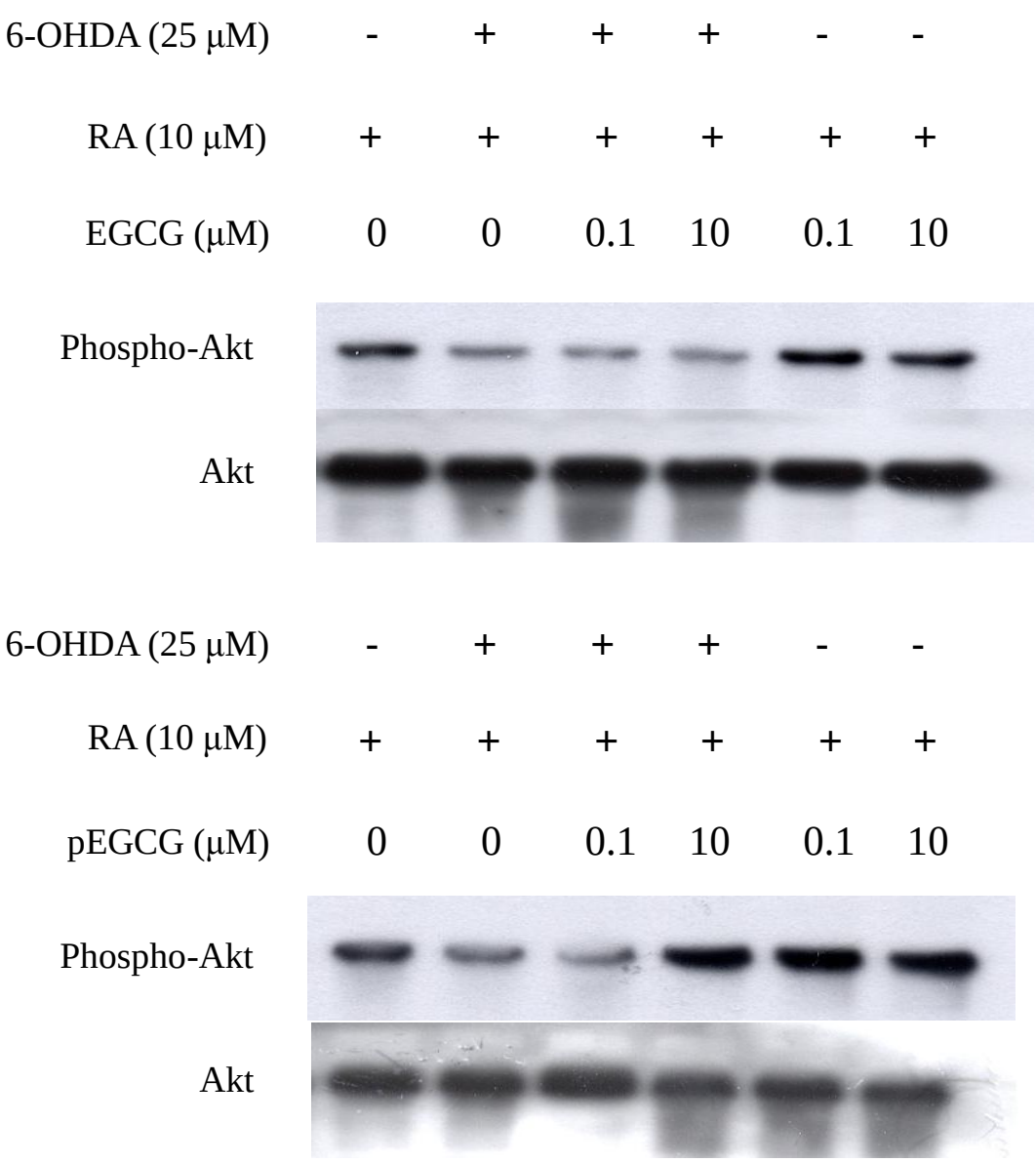

Fig. 5 Chao *et al.*, 2009