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Green tea catechins potentiate the neuritogenic action of brain-derived neurotrophic factor: Role of 67-kDa laminin receptor and hydrogen peroxide

Usha Gundimeda [a], Thomas H. McNeill [a], Tiffany K. Fan [a], Ronald Deng [a], David Rayudu [a], Zachary Chen [a], Enrique Cadenas [b], Rayudu Gopalakrishna [a,*]

[a] Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA
[b] Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA

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ABSTRACT

Delivery of optimal amounts of brain-derived neurotrophic factor (BDNF) to regions of the brain affected by neurodegenerative diseases is a daunting task. Using natural products with neuroprotective properties, such as green tea polyphenols, would be a highly useful complementary approach for inexpensive long-term treatment of these diseases. In this study, we used PC12(TrkB) cells which ectopically express TrkB, a high affinity receptor for BDNF. They differentiate and induce neurite outgrowth in response to BDNF. Using this model, we show for the first time that treatment with extremely low concentrations (<0.1 μg/ml) of unfractionated green tea polyphenols (GTPP) and low concentrations (<0.5 μM) of their active ingredient, epigallocatechin-3-gallate (EGCG), potentiated the neuritogenic ability of a low concentration (2 ng/ml) of BDNF. A synergistic interaction was observed between GTPP constituents, where epigallocatechin and epicatechin, both individually lacking this activity, promoted the action of EGCG. GTPP-induced potentiation of BDNF action required the cell-surface associated 67 kDa laminin receptor (67LR) to which EGCG binds with high affinity. A cell-permeable catalase abolished GTPP/EGCG-induced potentiation of BDNF action, suggesting the possible involvement of H_2O_2 in the potentiation. Consistently, exogenous sublethal concentrations of H_2O_2, added as a bolus dose (5 μM) or more effectively through a steady-state generation (1 μM), potentiated BDNF action. Collectively, these results suggest that EGCG, dependent on 67LR and H_2O_2, potentiates the neuritogenic action of BDNF. Intriguingly, this effect requires only submicromolar concentrations of EGCG. This is significant as extremely low concentrations of polyphenols are believed to reach the brain after drinking green tea.

1. Introduction

Brain-derived neurotrophic factor (BDNF) promotes neuronal differentiation, survival, axonal growth, and synaptic plasticity [1,2]. Its actions are mediated by its transmembrane receptor TrkB [1,2]. BDNF is widely expressed in the CNS and its levels are low in affected regions of the brain in a variety of neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's [3]. Furthermore, its depletion has been implicated in psychiatric disorders, particularly chronic depression [4]. Therefore, BDNF delivery to the brain is crucial in treating various neuropsychiatric disorders.

When BDNF is administered systemically, due to its macromolecular nature, it poorly crosses the blood–brain barrier [5]. Moreover, it has a short half-life in the blood circulation [5]. These limitations in delivering BDNF to the brain led to the exploration of small molecules such as G-protein-coupled ligands, which effectively transactivate TrkB receptor in the absence of BDNF [6]. Furthermore, some natural products, such as 7,8-dihydroxyflavone and deoxygedunin (flavonoid), were shown to directly activate TrkB and protect neurons in a BDNF-independent manner [7,8]. A complementary approach is to potentiate the action of limited amounts of BDNF present in affected regions of the brain.

Abbreviations: BDNF, brain-derived neurotrophic factor; GTPP, green tea polyphenols; EGCG, (−)-epigallocatechin-3-gallate; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EC, (−) epicatechin; 67LR, 67-kDa laminin receptor; ROS, reactive oxygen species; NGF, nerve growth factor; NAC, N-acetyl-L-cysteine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

* Corresponding author. Fax: +1 323 442 1771.
E-mail address: rgopalak@usc.edu (R. Gopalakrishna).

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Green tea may be well suited for inexpensive long-term treatment of the aforementioned neurological disorders. Its constituent polyphenols have neuroprotective and neurorescuing properties at low micromolar or submicromolar concentrations as elegantly shown by others [9]. A typical cup of green tea contains approximately 150 mg of water-soluble green tea polyphenols (GTPP), commonly known as catechins. GTPP has (−)-epigallocatechin-3-gallate (EGCG), the major bioactive polyphenol. It also has other polyphenols: (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−) epicatechin (EC).

EGCG acts as an antioxidant as well as a prooxidant in vitro [10,11]. However, these actions require 10–100 μM concentrations of EGCG, which are two orders of magnitude higher than the concentration reached in the plasma (<1 μM) after drinking green tea. Schaffer and Halliwell have critically discussed the fact that antioxidant actions of polyphenols are unlikely to occur in the brain, where extremely low concentrations of polyphenols are reached [12]. EGCG may carry out other types of actions due to its high affinity binding to some cellular proteins [13]. Tachibana and his associates have identified a high affinity binding of EGCG (K_d of 40 nM) to 67-kDa laminin receptor (67LR), a nonintegrin-type cell-surface associated protein [14]. Although 67LR is present in various regions of the brain, its role in neuronal regulation is unknown. Our recent studies revealed that the binding of EGCG to the 67LR leads to activation of NADPH-oxidase and subsequent generation of H_2O_2, which preconditions neuronal cells against death induced by oxygen-glucose deprivation [15].

We hypothesized that GTPP may potentiate BDNF action to induce neuritogenesis. We tested this hypothesis using PC12(TrkB) cells, which ectopically express TrkB and induce neurite outgrowth in response to BDNF. Here, we show for the first time that GTPP and EGCG potentiate the neuritogenic action of BDNF. Furthermore, we show that they mediate this potentiation by a mechanism presumably involving 67LR and H_2O_2.

2. Materials and methods

2.1. Materials

BDNF was obtained from Prospec. Purified GTPP constituents (EGCG, ECG, EGC, and EC), catalase-polyethylene glycol (PEG), horseradish peroxidase, glucose oxidase from *Aspergillus niger*, N-acetyl-L-cysteine, and H_2O_2 were from Sigma. Amplex UltraRed was from Molecular Probes. Anti-67LR-(MluC5) mouse monoclonal antibody and mouse IgM were from Santa Cruz Biotechnology. Decaffeinated extract of GTPP, which was standardized to contain 97% polyphenols and nearly 70% catechins, was obtained from Pharmanex. The typical preparation contained the following polyphenols expressed as percentage of original weight of GTPP preparation: EGCG (36%), ECG (15%), EC (7%), and EGC (3%) [16].

2.2. Cell culture and treatments

PC12(TrkB) cell line ectopically expressing TrkB was a kind gift from Dr. Moses Chao (New York University School of Medicine, New York). We referred to this cell line as PC12(TrkB). Stocks of PC12(TrkB) cells were grown on collagen-coated flasks in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum, 50 units/ml penicillin, 0.05 mg/ml streptomycin, 200 μg/ml G418. EGCG and other constituent polyphenols were dissolved in dimethylsulfoxide and the aliquots of samples were stored at −80 °C to minimize their autooxidation.

2.3. Quantitation of neurite outgrowth

PC12(TrkB) cells were seeded at a low density (1500 per well) on collagen-coated 96-well plates in RPMI medium supplemented with serum and antibiotics but without G418. After 1 day, the medium was replaced with serum-free medium and treated with BDNF, GTPP, or EGCG for 3 days. The cells were then scored for the presence of neurites as described previously and neurite outgrowth measurements were expressed as a percentage of the total cell number [17].

2.4. Steady-state generation of H_2O_2

For steady-state generation of H_2O_2, we used glucose oxidase, which utilizes glucose in the medium to produce H_2O_2. We employed elegant methodology developed by Antunes and his associates for the steady state generation of H_2O_2, except the reaction was scaled down to 96-well plates [18]. PC12(TrkB) cells were grown in phenol red-free RPMI medium and glucose oxidase was added. At various intervals of time, 5–10 μl of medium were taken into a 96-well plate, and 100 μl of reagent solution containing 50 μM Amplex UltraRed and 0.1 unit/ml of horseradish peroxidase in 50 mM sodium phosphate buffer (pH 7.4) were added. After incubation for 10 min in the dark, the fluorescence was measured by excitation at 530 nm and emission at 590 nm using SpectraMax M2e fluorescence microplate reader (Molecular Devices).

2.5. Cytotoxicity assay

Cytotoxicity was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method [19]. Cell death was determined by lactate dehydrogenase (LDH) assay [15].

2.6. Statistical analysis

Data are expressed as the mean ± SE and analyzed using one-way analysis of variance, followed by post hoc Scheffe's test. $p < 0.05$ was considered statistically significant. Statistical analyses were performed with StatView software.

3. Results

PC12 cells (wild type) do not express TrkB and therefore, BDNF cannot induce neurite outgrowth in these cells. However, BDNF can induce neuritogenesis in PC12(TrkB) cells ectopically expressing TrkB. Since EGCG and GTPP are unstable in the culture medium [20], we added them once daily to the medium. Unless, otherwise mentioned, GTPP was used at a concentration of 0.1 μg/ml, whereas EGCG was used at a concentration of 0.5 μM. BDNF at 2 ng/ml was used as the low concentration, and BDNF at 50 ng/ml was used as the high concentration.

3.1. GTPP potentiates BDNF-induced neurite outgrowth

A low concentration (2 ng/ml) of BDNF induced neurite outgrowth in PC12(TrkB) cells to only a limited extent (Fig. 1A and B). GTPP (0.05–5 μg/ml) alone by itself did not induce neurite outgrowth in the cells. However, when GTPP was added to the low concentration of BDNF, it significantly increased the neuritogenic ability of BDNF. This potentiation was optimal at 0.05–0.2 μg/ml of GTPP (Fig. 1C). GTPP enhanced neuritogenic ability of the low concentration of BDNF to a level that is seen with the high concentration of BDNF (50 ng/ml) alone. However, adding a limited amount of GTPP (0.1 μg/ml) to the high concentration of BDNF did not further enhance its neuritogenic ability. Furthermore,

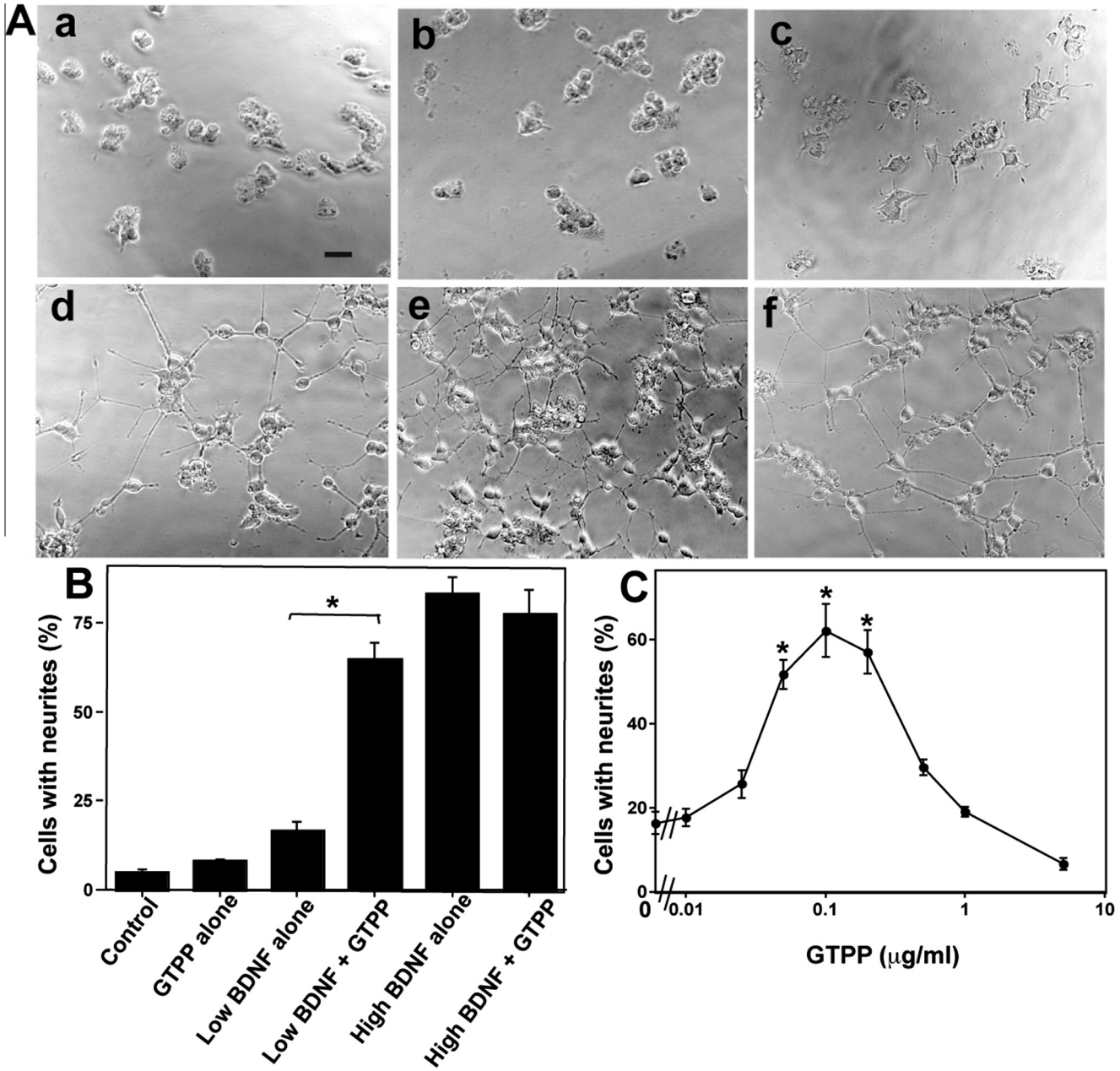

Fig. 1. GTPP potentiates BDNF-induced neuritogenesis. (A) Morphological changes in PC12(TrkB) cells treated with the following: a, control; b, GTPP (0.1 μg/ml) alone; c, low concentration of BDNF (2 ng/ml) alone; d, low concentration of BDNF along with GTPP; e, high concentration of BDNF (50 ng/ml) alone; f, high concentration of BDNF along with GTPP. Cells were treated with the indicated agents in 96-well plates and morphological changes were photographed after 3 days. Scale bar = 100 μm. (B) Quantitative analysis of GTPP potentiation of BDNF action. Cells with neurites were counted and expressed as the percentage of the total cell number. (C) Dose–response curve of GTPP potentiation of BDNF-induced neurite outgrowth. PC12(TrkB) cells were incubated with a low concentration of BDNF (2 ng/ml) along with the indicated concentrations of GTPP for 3 days and neurite outgrowth was measured. Each value is the mean ± SE obtained from three experiments. Statistically different values ($^*p < 0.01$).

adding very high concentrations (1–5 μg/ml) of GTPP not only failed to potentiate the action of the low concentration of BDNF, but also decreased the neuritogenic ability of the high concentration of BDNF. It is possible that oxidation products of GTPP might have caused cytotoxicity to these cells.

3.2. Potentiation of BDNF action by individual polyphenols and their synergistic interaction

Four major polyphenols present in the GTPP, namely two galloylated polyphenols (EGCG and ECG) and two nongalloylated polyphenols (EGC and EC), were tested in pure form for their ability to potentiate neuritogenic action of BDNF (Fig. 2A). EGCG at concentrations of 0.25–1 μM potentiated neuritogenic ability of low concentrations of BDNF (Fig. 2B). However, EGCG concentrations above 5 μM were cytotoxic to the cells. The other galloylated polyphenol, ECG also potentiated BDNF action to induce neurite outgrowth, but to a lower extent than EGCG (Fig. 2C). Conversely, nongalloylated polyphenols EGC and EC did not potentiate BDNF-induced neurite outgrowth. This suggests that the galloylated group in the polyphenolic compounds tested may be required to potentiate neuritogenic action of BDNF.

The amount of pure EGCG (0.5 μM or 0.23 μg/ml) required for the optimal potentiation of BDNF to induce neuritogenesis was nearly 6-fold higher than that of EGCG (0.036 μg/ml) present in the amount of GTPP extract (0.1 μg/ml) needed for optimal

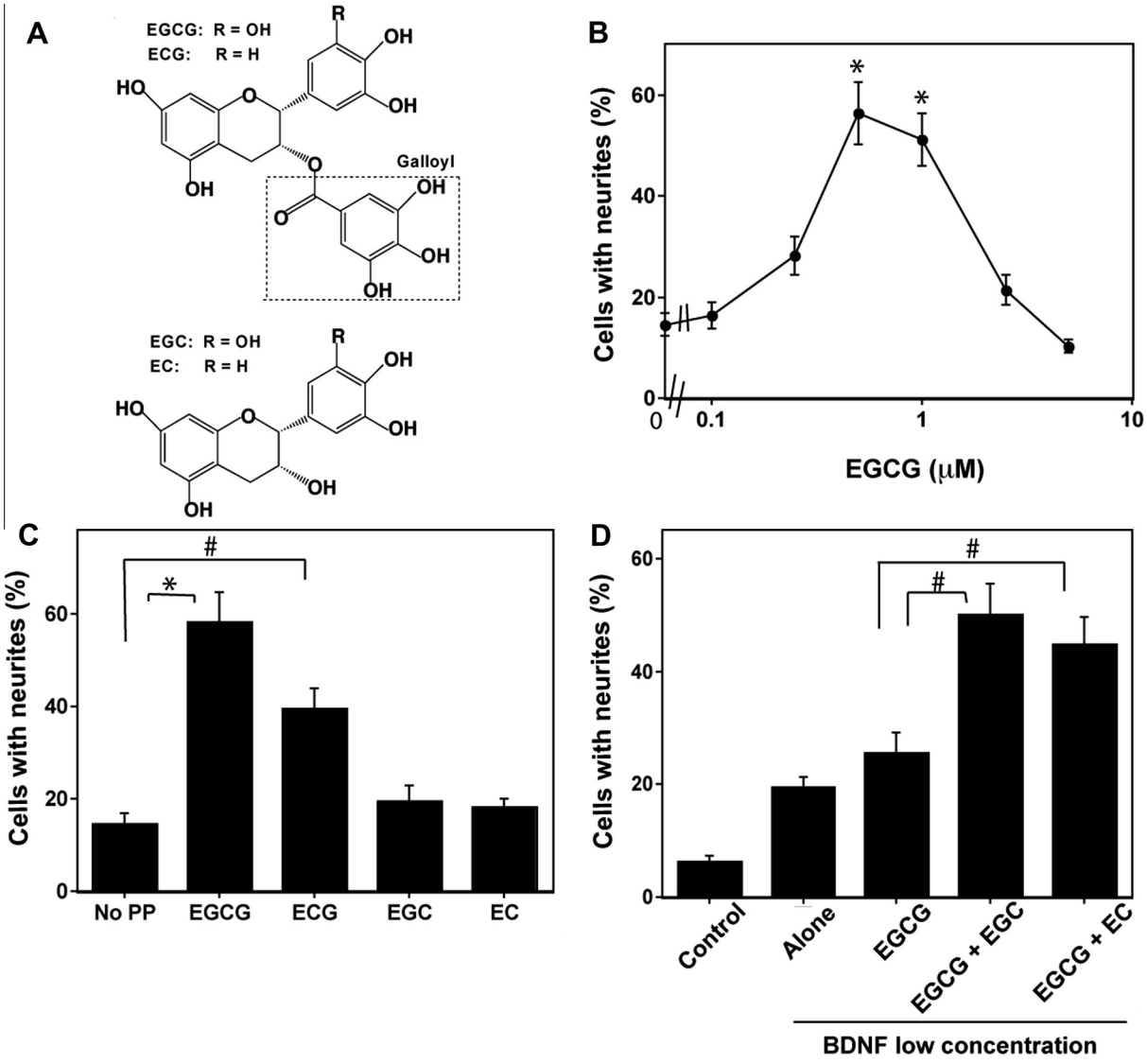

Fig. 2. Effect of various polyphenols present in GTPP on BDNF-induced neurite outgrowth. (A) Structures of galloylated and nongalloylated polyphenols present in GTPP. Galloyl structure with most potent activity is shown in dashed lines (B) Dose–response curve of EGCG potentiation of BDNF-induced neurite outgrowth. PC12(TrkB) cells were incubated with a low concentration of BDNF (2 ng/ml) along with the indicated concentrations of EGCG and neurite outgrowth was determined after 3 days. (C) Potentiation of BDNF-induced neurite outgrowth by individual polyphenols present in GTPP. Cells were incubated for 3 days with a low concentration of BDNF without a polyphenol (No PP) or with 0.5 μM concentration of the indicated polyphenols, and then neurite outgrowth was measured. (D) Synergistic interaction among polyphenols present in GTPP. Neurite outgrowth was induced in PC12(TrkB) cells by incubating with a low concentration of BDNF along with suboptimal concentration of EGCG (0.1 μM) to potentiate BDNF action. EGC (0.5 μM) and EC (0.5 μM) were included to determine their synergism with EGCG. Each value is the mean ± SE obtained from three experiments. Statistically different values ($^{*}p < 0.01$; $^{\#}p < 0.05$).

potentiation of BDNF-induced neurite outgrowth. Therefore, we determined whether the polyphenols EGC and EC present in GTPP, which lack the ability to potentiate BDNF-induced neurite outgrowth on their own, could synergistically promote the action of EGCG. When EGCG was used at a suboptimal concentration (0.1 μM) to potentiate BDNF action, both EGC and EC enhanced the ability of EGCG to potentiate the action of BDNF, thereby suggesting a synergistic interaction (Fig. 2D).

3.3. Role of cell-surface associated 67LR in GTPP-induced potentiation of BDNF action

Initially, PC12(TrkB) cells were preincubated with 67LR-blocking antibodies at room temperature for 1 h. Cells were continuously exposed to these antibodies throughout the incubations with GTPP, EGCG, and BDNF. Under these conditions, 67LR-blocking antibodies did not inhibit neurite outgrowth induced by high concentrations of BDNF (50 ng/ml). However, they substantially inhibited GTPP, EGCG, and ECG to potentiate neuritogenesis induced by the low concentration (2 ng/ml) of BDNF (Fig. 3A). The control IgM did not block the effect of these polyphenols. This suggests that the actions of these polyphenols, at least in part, are mediated through cell-surface 67LR.

3.4. Possible role of H_2O_2 in GTPP or EGCG-induced potentiation of BDNF action

N-Acetyl-L-cysteine (NAC) inhibited EGCG-induced potentiation of BDNF action (Fig. 3B). However, it also blocked neurite outgrowth induced by the high concentration of BDNF, suggesting its effect may be nonspecific. Therefore, we used cell-permeable PEG-catalase to determine whether H_2O_2 is involved in the aforementioned action of GTPP/EGCG. PEG-catalase, but not heat-inactivated PEG-catalase (control), substantially blocked the ability of

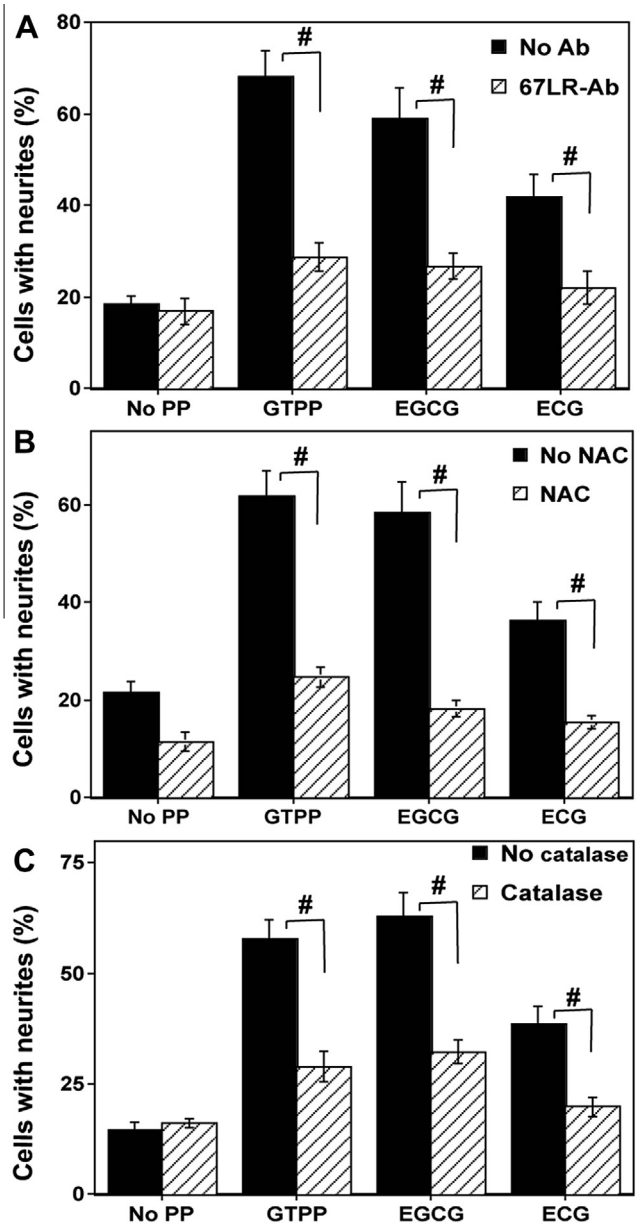

Fig. 3. 67LR-blocking antibody (A) and NAC (B) and cell-permeable PEG-catalase (C) inhibit GTPP, EGCG, and ECG to potentiate BDNF-induced neurite outgrowth. PC12(TrkB) cells in 96-well plates were treated with a low concentration of BDNF (2 ng/ml) along with GTPP (0.1 μg/ml), EGCG (0.5 μM), or ECG (0.5 μM) to induce neurite outgrowth. 67LR-blocking antibody (1 μg/ml), NAC (2.5 mM), or PEG-catalase (125 units/ml) was also included to block the effects of these polyphenols. Each value is the mean ± SE obtained from three experiments. Statistically different values ($^{\#}p < 0.05$).

GTPP, EGCG, or ECG to potentiate the neuritogenic action of BDNF at low concentrations (Fig. 3C). However, PEG-catalase did not inhibit neurite outgrowth induced by the high concentration of BDNF tested. This suggests a causal role of H_2O_2 in the GTPP-induced potentiation of BDNF action.

3.5. Exogenous H_2O_2 mimics GTPP/EGCG in potentiating the action of BDNF

We determined whether exogenous H_2O_2 could mimic GTPP/EGCG in potentiating the action of the low concentration of BDNF. Initially, the cytotoxicity of H_2O_2 to PC12(TrkB) cells was determined by incubating these cells with bolus doses of H_2O_2 (1–50 μM). H_2O_2 above 5 μM was clearly cytotoxic to these cells as determined by a decrease in MTT reduction (Fig. 4A). Cell death was also observed as revealed by a release of LDH (data not shown). However, LDH was inactivated by high concentrations of H_2O_2. Nonetheless, H_2O_2 at sharply 5 μM concentration potentiated the neuritogenic ability of low concentration of BDNF, but to a lower extent than that achieved with GTPP or EGCG (Fig. 4B). Since a high concentration of H_2O_2 is toxic to these cells, it is difficult to achieve optimal potentiation of BDNF action.

To overcome problems associated with use of a bolus dose of H_2O_2, we employed a steady-state generation of H_2O_2. By adjusting glucose oxidase levels in the medium, a steady-state generation of H_2O_2 was achieved. A steady-state generation of H_2O_2 at concentrations below 1 μM produced only limited cytotoxicity (Fig 4C). At concentrations as low as 0.8–1 μM, H_2O_2 potentiated the neuritogenic ability of BDNF comparable to the extent observed with GTPP/EGCG (Fig. 4D). Furthermore, this potentiating effect was highly reproducible than that observed with an addition of bolus dose of 5 μM H_2O_2. Intriguingly, 67LR-blocking antibodies did not inhibit the ability of H_2O_2 to potentiate BDNF action.

4. Discussion

This is the first report illustrating the ability of GTPP/EGCG to potentiate the neuritogenic action of BDNF in vitro. Conventional wisdom is that green tea polyphenols act as antioxidants to counteract oxidative stress, which is known to cause various neurodegenerative disorders and neuronal injuries. While this is an important mechanism, it requires concentrations of polyphenols substantially higher than those readily bioavailable in the body, especially in the brain where they are expected to be present in nanomolar range after green tea consumption [12]. Our current study is strikingly different from the antioxidant paradigm and supports a novel mechanism involving a specific receptor (67LR) and endogenous H_2O_2 in the GTPP-induced potentiation of the neuritogenic action of BDNF. This potentiation has implication that EGCG may enhance the neuritogenic activity of low levels of BDNF present in affected regions of the brain in neurodegenerative diseases. However, it does not enhance the action of high levels of BDNF present in other regions of the brain. This has significance since previous studies have shown that indiscriminate 'flooding' of the CNS with neurotrophic factors will probably leads to various side effects, including epileptic activity [3].

Previously several studies identified a variety of synthetic agents and natural products that potentiate NGF action to induce neurite outgrowth [15,21,22]. Most of these studies employed wild type PC12 cells having TrkA, which is a high affinity receptor for NGF. PC12 cells as well as many other in vitro established neuronal-like cells, such as SH-SY5Y and HT22, have no TrkB, the high affinity receptor for BDNF. Identification of agents that potentiate the action of BDNF to induce neurite outgrowth, therefore, became difficult. Alternatively, PC12(TrkB) cell line, ectopically expressing TrkB, provides an excellent model to identify various agents that potentiate BDNF action and to understand mechanisms of such potentiation.

The concentration of GTPP (0.1 μg/ml) and EGCG (0.5 μM) required for optimal potentiation of BDNF action to induce neurite outgrowth are extremely lower than those used for antioxidant effects and to induce apoptosis in cancer cells, which requires 10–200 μM EGCG. Since EGCG undergoes rapid auto-oxidation in cell culture medium, the actual concentration of unmodified EGCG present in the medium may even be lower than what we added, probably in the nanomolar range. EGCG reaches a peak plasma concentration of 0.2 μM in individuals after drinking two cups of

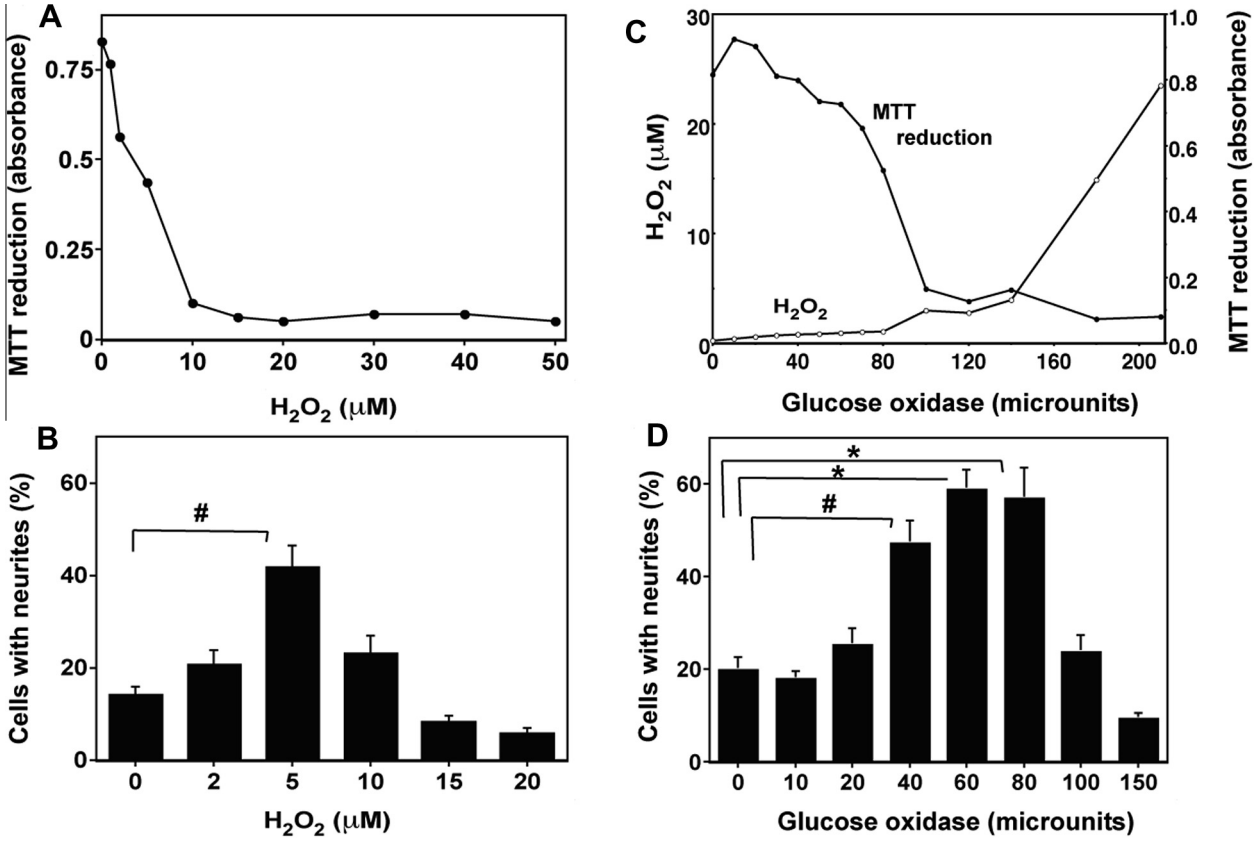

Fig. 4. Exogenous H_2O_2 potentiates BDNF-induced neurite outgrowth. (A) Cytotoxicity of bolus addition of H_2O_2. PC12(TrkB) cells grown in 96-well plates were treated with H_2O_2 added as a bolus dose. After 24 h, 10 μl of medium were used for quantitation of H_2O_2. Cytotoxicity was measured with MTT reduction assay. The absorbance of formazan formed was read at 550 nm. (B) Potentiation of BDNF-induced neurite outgrowth by H_2O_2 added as a bolus dose. Cells were treated with a low concentration of BDNF (2 ng/ml) and indicated concentrations of H_2O_2 and after 3 days the neurite outgrowth was measured. (C) Steady-state generation of H_2O_2 and cytotoxicity. Cells were treated with the indicated units of glucose oxidase, and generation of H_2O_2 and cytotoxicity were measured after 24 h. (D) Steady-state generation of H_2O_2 by glucose oxidase reaction potentiates BDNF-induced neurite outgrowth. Each value is the mean obtained from three experiments. Statistically different values ($^{*}p < 0.01$; $^{\#}p < 0.05$).

green tea [23]. It reaches as high as 0.4–0.8 μM in the plasma after oral administration of 800 mg EGCG, which is found to be safe in humans [24]. Due to its hydrophilic nature, EGCG is expected to cross blood–brain barrier inefficiently. In one study, upon administration of 500 mg GTPP/kg body weight to rats, only a limited amount of EGCG (0.5 nmol/g) reached the brain [25]. This may be due to the fact that bioavailability of green tea polyphenols in the rats is several folds lower than that in the mice and humans [26]. In another study involving the oral administration of radiolabeled EGCG to mice, an appreciable amount of radioactivity was detected in the brain and found to increase upon readministration [27]. Therefore, it is possible that the concentrations of EGCG will be at least in nanomolar range after green tea consumption by humans and it may be sufficient for potentiating the action BDNF.

The current study demonstrates the role of sublethal levels of H_2O_2 in GTPP/EGCG-mediated potentiation of BDNF action. Previously, we have shown that the ability of GTPP to induce intracellular ROS, especially H_2O_2 is dependent on 67LR and NADPH oxidase [15]. Consistently, exogenous sublethal concentrations of H_2O_2, either as bolus dose (5 μM) or more effectively through a steady-state generation (approximately 1 μM), potentiated BDNF action. High concentrations of H_2O_2 produce damaging effects in neuronal injuries and neurodegenerative diseases [28]. Contrarily, sublethal levels of H_2O_2 can act as a second messenger [29]. H_2O_2 has been shown to induce redox signaling by inactivating some protein tyrosine phosphatases which have highly redox-sensitive cysteine residue in their catalytic site [30]. Inactivation of specific protein tyrosine phosphatases that dephosphorylates intracellular domain of TrkB could enhance the extent and duration of tyrosine phosphorylation of TrkB. This may result in the increase in activation of downstream pathways, such as Akt, extracellular signal-regulated kinase, and protein kinase C, which may enhance the neuritogenic ability of low concentrations of BDNF.

In summary, this study suggests that EGCG, through a synergistic interaction with other polyphenols present in GTPP, acts via its high-affinity target 67LR and potentiates the neuritogenic action of BDNF. This EGCG-induced potentiation is mediated by sublethal levels of endogenous H_2O_2 and is mimicked by exogenous H_2O_2. Although these promising results are obtained with a novel in vitro model, additional studies with primary neurons, relevant animal models, and clinical trials are required to better assess the efficacy of GTPP to potentiate various actions of BDNF.

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