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Garcinol promotes Neurogenesis in Rat Cortical Progenitor Cells through the Duration of Extracellular Signal-Regulated Kinase Signaling

Meng-Shih Weng,*,† Chiung-Ho Liao,‡,§ Sheng-Yung Yu,† and Jen-Kun Lin*,‡

†Department of Nutritional Science, Fu Jen Catholic University, Taipei, Taiwan, ‡Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei, Taiwan, and §Division of Drug and New Technology Product, Food and Drug Administration, Department of Health, The Executive Yuan, Taiwan

Garcinol is a polyisoprenylated benzophenone derivative found in *Garcinia indica* fruit rind and other species. The potential antioxidative and neuroprotective effects of garcinol in rat cortical astrocyte were demonstrated in our laboratory recently. Here, the effects of garcinol on the neuritogenesis process in cultured cortical progenitor cells were investigated to understand the roles of garcinol in neuronal survival and differentiation. These cells, derived from embryonic day 17 rats, differentiated into EGF-responsive neural precursor cells, would further form neurospheres. Our data exhibited garcinol induced neurite outgrowth in early developing EGF-treated neurospheres and significantly enhanced the expression of neuronal proteins, microtubule-associated protein 2 (MAP-2), and glial fibrillary acidic protein (GFAP). Furthermore, the neuronal marker, high-molecular-weight subunit of neurofilaments (NFH), was highly expressed after 5 μM garcinol treatment in neural precursor cells for 20 days. To identify the extracellular mechanism, rat cortical progenitor cells were treated garcinol and accordingly mediated the sustained activation of extracellular signal-regulated kinase (ERK) for different periods up to 20 h. In this regard, NMDA receptor-mediated calcium influx led to excitotoxic death and activated tyrosine phosphatase which limited the duration of ERK in cultured neurons. MK801, the NMDA receptor antagonist, treatment also induced the sustained phosphorylation of ERK and therefore enhanced neuronal survival. In our observation, garcinol treatment reduced growth factor deprivation-mediated cell death and nuclear import of C/EBPβ levels. Noteworthy, garcinol could promote neurite outgrowth in EGF-responsive neural precursor cells and modulate the ERK pathway in the enhancement of neuronal survival.

KEYWORDS: Garcinol; neurospheres; neurite outgrowth; ERK; C/EBPβ

INTRODUCTION

Garcinol, from the fruit rind of *Garcinia indica* and other species, is used as a garnish in curries and in some of the folk medicine in India. *Garcinia* is a rich source of secondary metabolites including xanthones, flavanoids, benzophenones, lactones, and phenolic acids. Garcinol, containing both phenolic hydroxyl groups and a β-diketone moiety, has been proven to scavenge hydroxyl radicals, superoxide anions, and methyl radicals (*1*). It has attracted considerable interest because of its health promoting properties, including antibiotic activities (*2*), antioxidant properties (*3*), and suppression of colonic aberrant crypt foci (ACF) formation (*4*). Recently, oxidative stress has been indicated as the major role in the pathogenesis of neuro-degenerative disease, such as Alzheimer's and Parkinson's disease (*5*). Blockade of oxidative stress by polyphenols are a good strategy to decrease neuro-degenerative disease. Our recent data indicated that garcinol is a scavenger of hydroxyl radicals and inhibits nitric oxide (NO) generation properties in primary astrocytes (*6*). However, the role of garcinol in neuronal survival and differentiation is still mysterious. The effects of garcinol on the survival of cortical neurons were investigated in vitro in this article.

In the mammalian central nervous system (CNS), neurons are primarily generated during the embryonic phase, while most of the glia are developed after birth. Murine embryonic cortical stem cells provide an environment that can give rise to seemingly normal neural cells in culture (*7*). The use of mature stem cell-derived transplants for the treatment of human neuronal disorders such as Alzheimer's or Parkinson's disease has been widely discussed (*8*). Meanwhile, the generation of specific neuronal population with insufficient quantities for the treatments of neuronal disorders and injuries remains a problem. At present,

*To whom correspondence should be addressed. (M.-S.W.) Department of Nutritional Science, Fu Jen Catholic University, No. 510 Jhongjheng Road, Sinjhuang City, Taipei County 24205, Taiwan. Tel: (886)-2-2905-3776. Fax: (886)-2-2902-1215. E-mail: 078670@mail.fju.edu.tw. (J.-K.L.) Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei 10018, Taiwan. Tel: (886)-2-2356-2213. Fax: (886)-2-2391-8944. E-mail: jklin@ha.mc.ntu.edu.tw.

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the most of the observed studies have used differentiated cell lines derived from teratocarcinomas or neuronal and neuroendocrine tumors (*9*), which are different from primary neuron cultures. Recently, neural precursor cells have showed responses to both epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Several studies established that neurospheres cultured in a medium with EGF alone are able to develop for up to 350 days (*10*). Alternatively, bFGF elevates the acquisition of neural precursor cells and promoted neurite outgrowth in these EGF-generated neurospheres (*11*). In the current studies, cortical progenitor cells have been isolated at the onset of neurogenesis. Initially, these cells differentiated into EGF-responsive neural precursor cells, cultivated into neurospheres, and developed into neurons and astrocytes thereafter. Neruospheres are artificially produced in the form of in vitro-generated structures of clonal origin and seem to be a major tool for studying neural stem cells in vitro (*12*). Using a variety of neural markers, such as Nestin, microtubule-associated protein 2 (MAP-2), and glial fibrillary acidic protein (GFAP), we were able to demonstrate the contents in neural tubes at early developmental stages, and the neural tube is capable of differentiating into neurons, astrocytes, and oligodendrocytes. In the meantime, axonal cytoskeleton, particularly microfilaments, and microtubules were necessary for neurite outgrowth. Cytoskeleton disruption is a key pathological change in human neurodegenerative diseases (*13*). In most mature neurons, neurofilaments comprise proteins with three subunits, neurofilament light, middle, and heavy chains (NFL, NFM, and NFH). Activation of MAPK (mitogen-activated protein kinase) cascade appears to be a common requirement for the regulation of neurite extension (*14*).

Neurotrophic factors activate the Ras−Raf−MEK [MAPK/ERKK (extracellular signal-regulated kinase kinase)] pathway and, through this pathway, produce cellular neurotrophic actions, including neurite outgrowth, regeneration, and neurogenesis (*15*). Activation of receptor-type protein tyrosine kinases by bFGF has also been shown to stimulate ERK activity in neurogenesis. Calcium-dependent transcription factors such as CCAAT/enhancer-binding protein family play an important role in coupling growth factor signal transduction to cellular differentiation in numerous developing non-neural tissues (*16*). C/EBPβ, the full-length subtype of C/EBPs, belongs to the basic region in the leucine zipper class of DNA-binding proteins. Pharmacological stimulation of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor also leads to the activation of ERK2 in both cortical and hippocampal neurons in culture (*17*, *18*). NMDA receptor-mediated influx rapidly increases nuclear C/EBPβ and induces excitotoxic death via activation of the calcium-dependent phosphatase, calcineurin (*19*).

The results demonstrated that garcinol modulated the expression of both neuron- and glia-specific proteins in rat cortical precursor cells and prolonged ERK signaling. Garcinol treatment also exhibited the promotion of neuronal differentiation in primary cultures of EGF-generated neurospheres. Moreover, garcinol alone causes the sustained phosphorylation of ERK accompanied with down-regulation the cytosolic level of C/EBPβ in serum deprivation. Further study exhibited that the inhibitory effect of garcinol may block NMDA receptor signaling in the neurogenesis process.

MATERIALS AND METHODS

Chemicals and Reagents. Garcinol (purity = 95%) was purchased from Biomol/Enzo Life Sciences International, Inc. (Plymouth Meeting, PA) and was dissolved in DMSO to make a 10 mM stock solution. Epidermal growth factor (EGF), basic-fibroblast growth factor (b-FGF), PD98059, MK-801, and poly-D-lysine were purchased from Sigma Chemical (St Louis, MO). Antiphospho-ERK (Thr202/Tyr204) and anti-C/EBPβ antibodies were purchased from Cell Signal Technology (Beverly, MA). Anti-ERK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas anti-Nestin, antimicrotubule-associated protein 2 (MAP2), and antiglial fibrillary acidic protein (GFAP) were locally obtained from Millipore (Taipei, Taiwan).

Primary Cultures of Cortical Progenitors and Neurons. Primary cortical cell cultures consisting of both neuronal and glial cells were prepared from cortices of 17-day-old embryonic Wistar rats, using modifications of previously described methods (*20*). The rat embryonic cortices were dissected in ice-cold PBS and then transferred into 37 °C Neurobasal medium (Invitrogen, Taipei, Taiwan) containing 500 μM L-glutamine, 2% B27 supplement, and 1% penicillin−streptomycin (Invitrogen). This medium was supplemented with 10 ng/mL EGF in a humidified incubator equilibrated with 10% CO_2 in air at 37 °C. Cell density was 1×10^6 and 5×10^6 cells per well in 5 cm culture dishes. The stable lineage of neuronal stem cells (NSCs) as EGF-responsive colony-forming, floating neurospheres was generated and successfully prepared for 4 days in neurobasal medium containing the B27 supplement with 10 ng/mL EGF as a mitogen. Colony formation was described as an important first stage in cultured neuron cells. For subcloning at 4 days to make neurospheres, cells in suspension were plated in six-well tissue culture dishes or chamber slides precoated with poly-D-lysine. Two or 3 h after plating, progenitor cells were treated with DMSO or garcinol at a final concentration of 0.1% DMSO (v/v) in the absence/presence of 10 ng/mL EGF. Cultures were also maintained at 37 °C in a 5% CO_2 incubator. In neurons, E16-E17 mouse cortices were mechanically dissociated into a single-cell suspension in the same medium used for the progenitor cells. The cells were plated for 12 h in the same conditions as those for cortical progenitors, and then one-half of the media was removed and replaced with fresh medium. Two days later, the cells were treated and/or harvested.

Immunocytochemical Staining. For Nestin, MAP-2, GFAP, and C/EBPβ labeling, undifferentiated and differentiated cells were fixed with 4% paraformaldehyde for 5 min at 4 °C, followed by 0.01% triton-X in PBS (pH 7.4) for 5 min. The fixed cells were then incubated with the primary antibody for 45 min using the following dilutions: 1:200 for MAP-2, 1:500 for GFAP, and 1:500 for C/EBPβ. After washing with PBS, cells were incubated with the secondary antibody containing 1.5% goat serum for 30 min. For MAP-2 labeling, a 1:500 dilution of antimouse IgG-FITC (Chemicon) was used. For GFAP and C/EBPβ labeling, a 1:500 dilution of antimouse IgG-rhodamine (Chemicon) was used. Cells were visualized on a LEICA laser scanning confocal microscope.

Western Blot Analysis. Total cellular extracts were prepared using lysis buffer containing 10% glycerol, 1.0% Triton X-100, 1.0 mM $NaHPO_3$, 137 mM NaCl, 10 mM NaF, 1.0 mM EGTA, 5.0 mM EDTA, 20 mM Tris-HCl, 100 μM β-glycerophosphate, 1.0% sodium dodecyl sulfate, 1.0 mM Na_3VO_4, and protease inhibitor cocktail (Roche Applied Science, Germany). Equal amounts of total cellular protein (30−50 μg) were resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, MA, USA). Blots were revealed by chemiluminescence (ECL kits, Amersham Pharmacia Biotech). Nuclear extracts were prepared according to a modified method of Liao et al. (*3*). The total cellular lysates were suspended in hypotonic buffer (10 mM NaH_2PO_4, 10 mM NaF, 5.0 mM EDTA, 1.0 mM PMSF, 1.0% NP-40, and 5.0 mM $MgCl_2$) for 30 min on ice. The cytosolic fractions were collected from the supernatant after centrifugation at 4,000*g* for 15 min. The pellet was resuspended in hypertonic buffer (hypotonic buffer and 0.1% NaCl) for 3 h on ice. The nuclear fractions were collected by centrifugation at 14,000*g* for 30 min. The primary antibodies phospo-ERK1/2, anti-ERK1/2, and anti-C/EBPβ were used to determine the level of proteins expression. The blots were revealed by chemiluminescence (ECL kits, Amersham Pharmacia Biotech). Band intensities were quantitated by densitometry (IS-1000 Digital Imaging System).

RT-PCR. Following drug treatment, the cells were washed in ice-cold PBS, and total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction according to the manufacturer's instructions (Nippon Gene, Japan). The cDNA was prepared from the total RNA (5.0 μg) with Moloney murine leukemia virus reverse transcriptase and oligo $(dT)_{18}$ primer at 42 °C for 60 min. PCR was performed in a final volume of 25 μL, containing dNTPs (each at 200 μM), 1.0× reaction

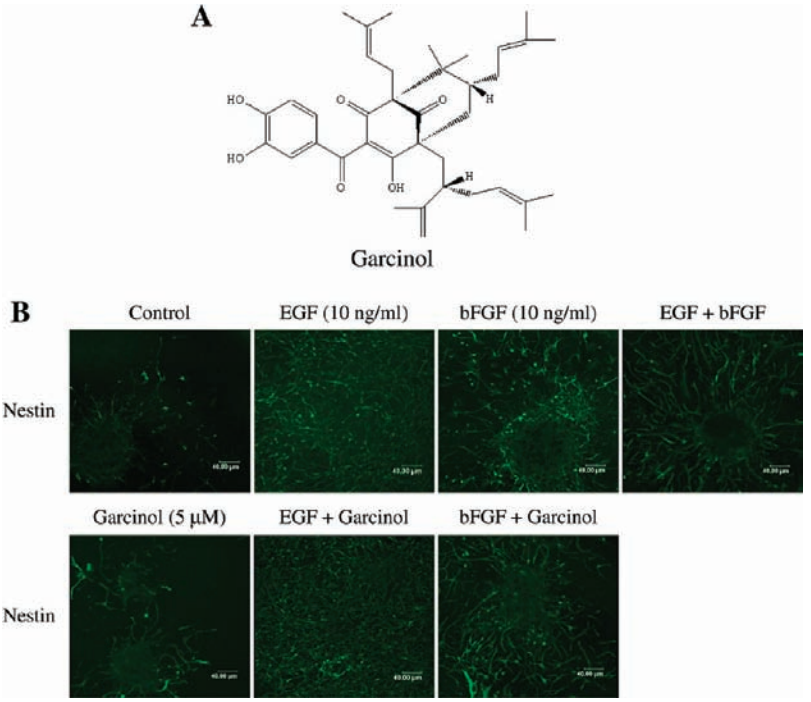

Figure 1. Garcinol enhanced neuronal survival and neurite outgrowth in EGF-generated neurospheres. (**A**) Chemical structures of garcinol. (**B**) Immunocytochemical characterization of neuron-like cells differentiated from EGF-responsive neural precursor cells. Cells from rat embryonic cortex formed neurospheres in the presence of EGF for 4 days. Then neurospheres were treated with 5 μM garcinol in the presence of 10 ng/mL EGF and/or 10 ng/mL bFGF in neurobasal media. Immunostaining for Nestin in neurospheres remained strong in the presence of EGF or bFGF with or without 5 μM garcinol for 4 days. Scale bar, 40 μm.

buffer, 2.0 μL of the above cDNA product, 50 U/mL pro Taq DNA polymerase (Promega, Madison, WI), and 4.0 μM of each primer. PCR primers for NFL, NFM, NFH, and GFAP were synthesized according to the following oligonucleotide sequences: NFL, forward primer 5′-GCTATGAAGAGGAGGTGCTGAGC-3′ and reverse primer 5′-CATGCTTCGATTTCCAGGGTC-3′; NFM, forward primer 5′-ACGGCGCTGAAGGAGATC-3′ and reverse primer 5′-GTCCAGGGCCATCTTGAC-3′; NFH, forward primer 5′-GCACAGCTCCGAGAGTACCAGG-3′ and reverse primer 5′-GTAGCTGCTGCTCCTTCCTC-3′; GFAP, forward primer 5′-ACCACAG TCCATGCCATCA-3′ and reverse primer 5′-TCCGGTACTCGTTTGACT-3′. After an initial denaturation for 5 min at 95 °C for PCR, 40 cycles of amplification (95 °C for 45 s, 60 °C for 45 s, and 72 °C for 1.5 min) were performed followed by a 10 min extension at 72 °C. All PCR products were separated by electrophoresis on a 1.0% agarose gel and visualized by ethidium bromide staining.

RESULTS

Enhancement of Neuronal Survival and Neurite Outgrowth by Growth Factors and Garcinol Cotreatment Elicited in EGF-Generated Neurospheres. Generally, the neural progenitor cells (NPCs), grown as neurospheres, showed immunoreactivity to a neural precursor cell marker, Nestin. Nestin-positive cells were identified to develop all three types of neuronal cells, neuron, microglia, and astrocytes, as distinguished by the expression of cell type-specific markers (*21*). Accordingly, the generation of EGF-responsive NPCs with an antibody against Nestin was investigated. As shown in **Figure 1B**, EGF-responsive NPCs increased during 5 μM garcinol treatment for 4 days in EGF or bFGF supplementary medium. However, garcinol treatment (5 μM) alone did not effectively raise the numbers of NPCs (**Figure 1B**).

To investigate whether garcinol possessed EGF-responsive NPC maturation and neurite outgrowth, neurospheres (EGF-responsive NPCs) were seeded on 6-cm-diameter poly-D-lysine-coated tissue culture dishes, and then neurospheres migrated and produced neurite outgrowth. The ability of these spheres to form neurites was determined by immunocytochemical staining for MAP-2, a reliable neuronal marker (**Figure 2A**). Garcinol (5 μM) added for the first 4 days increased the amount of MAP-2 protein in EGF-generated spheres (**Figure 2A**, right).

To further identify whether garcinol maintained neuritogenesis in long-term treatment, the neuronal marker MAP-2 in garcinol treated or cotreated with growth factor for 7 days was visualized by immunostaining. As demonstrated in **Figure 2B**, many of the cells in neurospheres exhibited MAP-2 protein expression upon treatment with 10 ng/mL growth factors and 5 μM garcinol for 7 days. The outgrown neurites were extended or more abundant through the affiliated addition of 5 μM garcinol to EGF-treated neurospheres than with growth factor alone. Garcinol treatment also increased GFAP-positive cells in the core of the EGF-maintained spheres (**Figure 2B**). In addition, it seemed that garcinol-treated neurospheres enhanced neurite formation in bFGF (10 ng/mL) supplementary conditions within 7 days, as indicated by MAP-2 staining (**Figure 2B**). GFAP-positive cells were also significantly increased by garcinol in the presence of bFGF. These observations suggested that MAP-2 as well as GFAP was expressed in neurospheres cultured in garcinol plus growth factor treatments. To assess the increase in MAP-2 positive cells attributed to garcinol treatment in EGF-responsive progenitor cells, the percentage of cells was subsequently quantitated by immunocytochemical staining with anti-MAP-2 and anti-GFAP antibodies. Garcinol increased the numbers of MAP-2 positive cells by 2-fold compared to that with EGF treatment alone in EGF-responsive progenitor cells (**Figure 2C**). The results implied that garcinol might increase the cell survival of GFAP-positive astrocytes and MAP-2-positive neuron cells, and were followed by the promotion of neurite outgrowth in the growth factor-supported spheres.

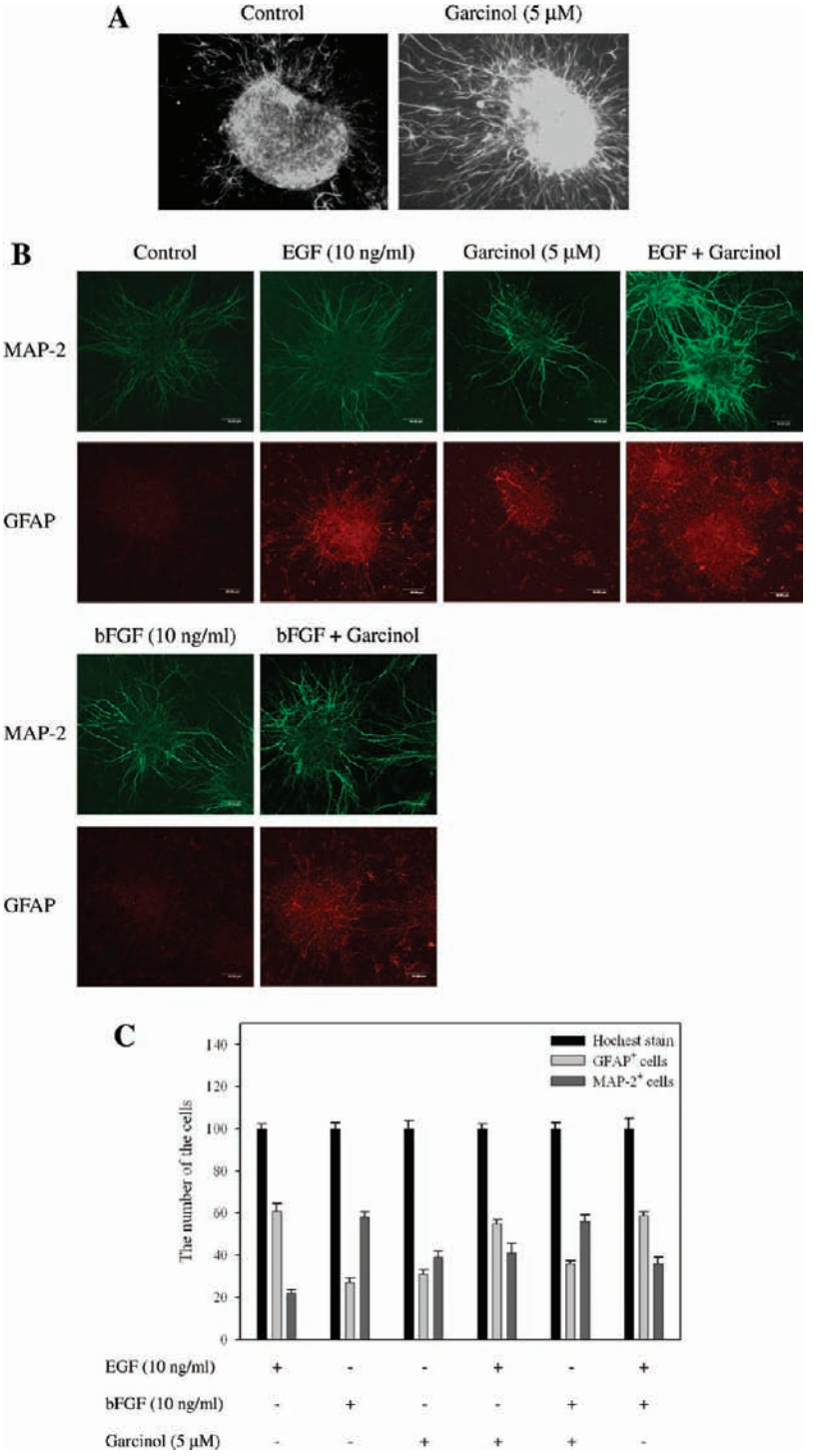

Figure 2. Immunocytochemical characterization of garcinol induced neuron-like cell differentiation from embryonic cortical progenitor cells. Cells were aggregated in neurobasal medium to form neurospheres in the presence of 10 ng/mL EGF and bFGF for 4 days. Neurospheres were subsequently plated in neurobasal medium containing 10 ng/mL growth factors plus B27. (**A**) Garcinol-induced EGF-responsive neuron precursor cell neurite outgrowth was detected by immunocytochemical staining for microtubule-associated protein MAP-2 as described in Material and Methods. Scale bar, 40 μm. (**B**) Garcinol enhanced EGF-responsive neurosphere neurite expression. Each pair of panels represent the same field, with the upper panel and lower panel being immunostaining for neuron and glia cell marker, MAP-2 and GFAP, respectively. Scale bar, 40 μm. Results shown are from a representative experiment and are reproduced in three other identical experiments. (**C**) Garcinol enhanced the viability of MAP-2 positive cells in EGF-responsive neuron precursor cells. After 4 DIV in neurosphere cultures, the generation of neuronal, glial cells was analyzed. For each individual experiment, at least 10 neurospheres were analyzed. Cells were coimmunostained with MAP-2 and GFAP antibodies, and the nuclei were identified by Hoechst33342 staining. The number of immunoreactive cells was counted three times within a microscopic visual field with a magnification of 100× in each well on the plate.

Expressions of Neuronal and Glial Proteins for Neuronal Differentiation Increased by Garcinol in EGF-Responsive Cortical Progenitor Cells. To further establish the role of garcinol in neurite outgrowth, cortical progenitor cells, isolated from the E16-E17 mouse cortex, were used. After the generation of an EGF-responsive neuronsphere, the single cells were prepared by dispersed from

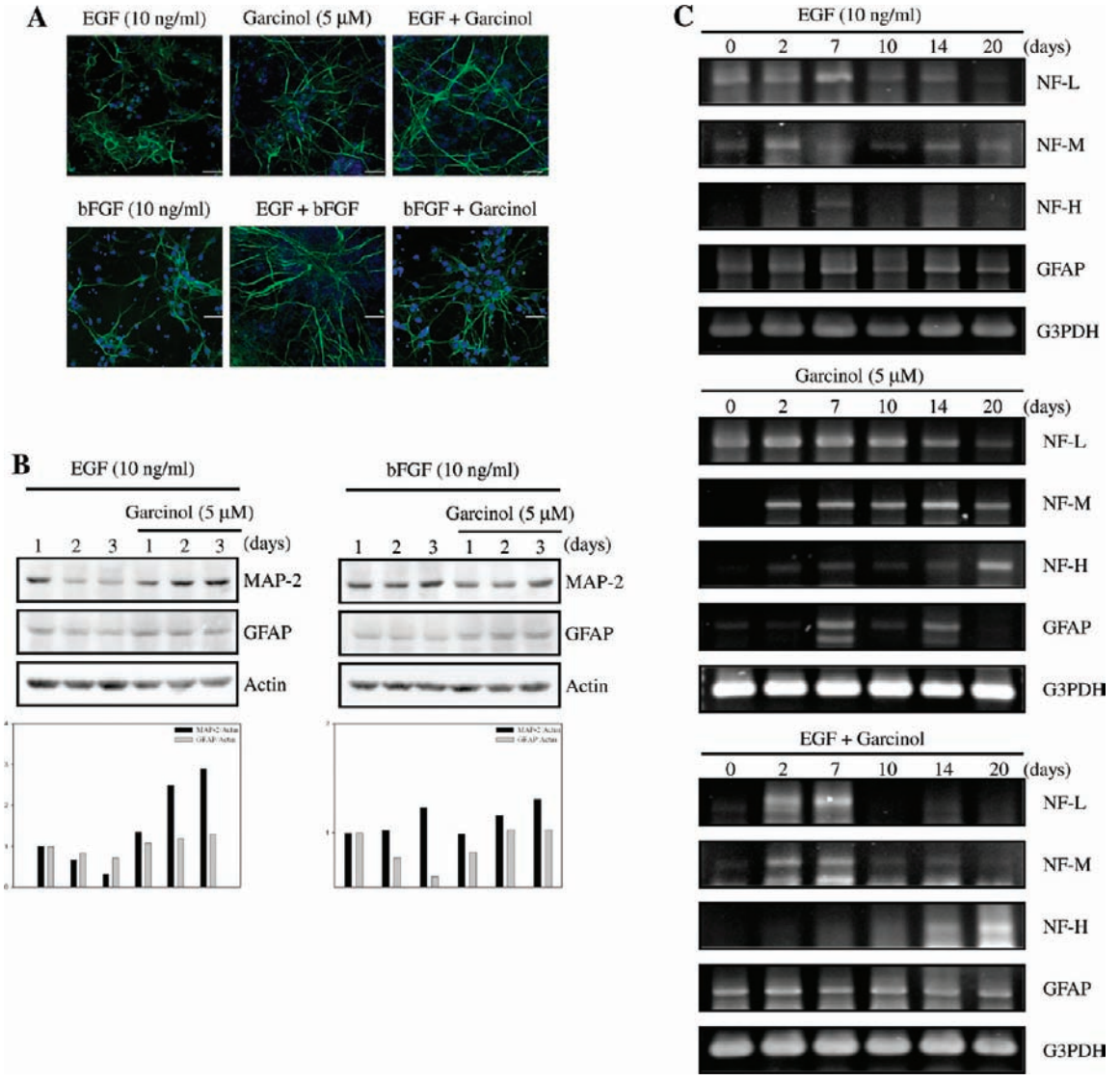

Figure 3. Generation of neurons from cortical progenitor cells induced by garcinol. The EGF-responsive cortical progenitor cells were cultured with EGF or bFGF (10 ng/mL) in the absence or presence of garcinol (5 μM) in neurobasal medium containing the supplement B27. Growth factor-induced neurospheres dispersed to single cells and treated with growth and/or garcinol for 7 days. (**A**) Immunostaining for MAP-2 in progenitors remaining in growth factors with or without garcinol for 7 days. The developing neurites were stained by MAP-2 antibody, and Hoechst labeling showed all of the nuclei in the field. Scale bar, 40 μm. (**B**) Western blot analyses for MAP-2 and GFAP proteins in EGF-responsive progenitor cells remaining in growth factors alone or treated with 5 μM garcinol at various time points (1, 2, 3 DIV). The relative level was calculated as a ratio of MAP-2 or GFAP to actin protein levels. (**C**) EGF-responsive progenitor cells were treated with 5 μM garcinol and/or EGF from 6 h to 20 days. Total RNAs were subjected to RT-PCR with NFL, NFM, NFH, GFAP, and G3PDH as the internal control. The PCR products were resolved in 1% agarose gel. Each experiment was performed at least three times.

the neuronsphere and further cultured in the presence of garcinol and/or growth factor for 7 days. In three days after seeding, dissociated cortical neurons adhered to the coverslip substrate and elaborated slender neurites. Analyses of the morphological and cytoskeletal characteristics of developing cortical neurons were performed at 7 DIV (day in vitro) using an antibody with specificity for MAP-2 of microtubules (**Figure 3A**). Following 7 days of exposure to garcinol together with growth factors, the majority of cells developed extended neurites (**Figure 3A**). In the same experiment, the reduction of neurite-bearing cortical neurons in the treatment with EGF (10 ng/mL) alone was detected, whereas neurite outgrowth was also exhibited in these cells during EGF and bFGF (10 ng/mL) cotreatment. As shown in **Figure 3B**, the differences in expression of neuronal and glial proteins were perceived by immunoblotting analyses with anti-MAP-2 and anti-GFAP antibodies. Under growth factors and garcinol cotreatment, a significant time-dependent increase in the amount of MAP-2 protein for 3 days, compared with EGF only, was defined. However, the expression of MAP-2 protein did not show significant differences between bFGF alone and cotreatment with garcinol (**Figure 3B**). To further evaluate whether cotreatment with garcinol and EGF could prolong MAP-2 protein expression, a striking increase in the amounts of MAP-2 and a slight increase in GFAP protein under garcinol and EGF cotreatment were noted during a cultivation period between 1 and 10 DIV (data not shown).

Cotreatment with garcinol and EGF induced the high expression of neuron marker protein MAP-2 rather than EGF treatment alone. Thus, the expression of neurofilaments by RT-PCR analysis to verify the effects of garcinol in neuronal differentiation was explored. The light-molecular-weight (NFL) and middle-molecular-weight (NFM) neurofilaments of fully differentiated and myelinated axons were usually detectable during initial neurite outgrowth although the high-molecular-weight subunit (NFH) was massively expressed at a much later developmental

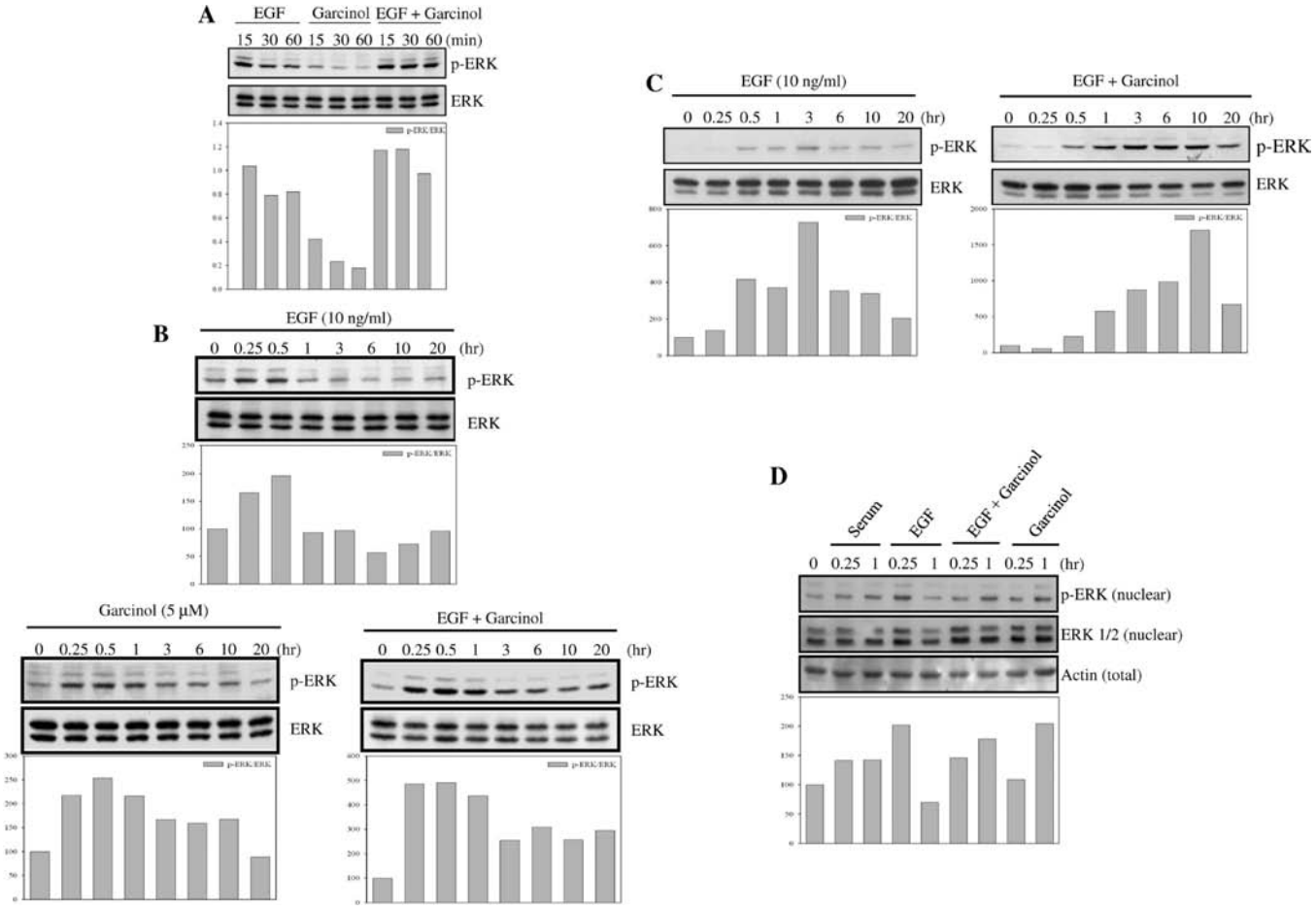

Figure 4. Garcinol induced ERK activation in EGF-responsive cortical progenitor cells. (**A**) The EGF-responsive cortical progenitor cells were treated with EGF (10 ng/mL) in the absence or presence of garcinol (5 μM) at indicated time points (15, 30, and 60 min), and total cell lysate was subjected to Western blot analysis using antiphospho-ERK1/2 antibody. (**B**) The EGF-responsive cortical progenitor cells were treated with EGF (10 ng/mL) in the absence or presence of garcinol (5 μM) for different lengths of time, ranging from 15 min to 20 h as indicated. Immunoblotting was performed on total cell lysates using antiphospho-ERK1/2 antibodies and subsequently with antibodies against ERK1/2. (**C**) EGF-responsive progenitor cells were also incubated with 20 μM PD98059, an inhibitor for MEK phosphorylation, in the presence of garcinol and/or EGF and analyzed by immunobloting with phospho-ERK1/2 antibody. (**D**) The nuclear levels of phospho-ERK rose in the presence of serum or treatment with garcinol and/or EGF in cortical progenitor cells for 15 min and 1 h. Nuclear fractions were performed to detect nuclear phospho-ERK levels by immunostaining with phospho-ERK antibodies.

stage in neuronal cell differentiation (*22*). EGF-responsive cortical progenitor cells were treated with 5 μM garcinol for up to 20 days in the presence of 10 ng/mL EGF. At the indicated time points, total cellular RNA was isolated and followed by reverse transcription PCR to identify the neurofilaments. As expected, the results showed that cotreatment with both garcinol and EGF enhanced NFH expression during 20 days postplating (**Figure 3C**, lane 6). Moreover, examination of garcinol induction of the expression of neuron-specific cytoskeletal elements (NFL and NFM), expressed shortly after the initiation of axonal outgrowth, was also implemented. Within 2 days of garcinol treatment, noteworthy numbers of NFL transcripts were established by RT-PCR (**Figure 3C**, lane 2). All garcinol-treated EGF-responsive cortical progenitors revealed the expressions of NFL and NFM at high levels throughout the experimental 10-day period (**Figure 3C**). The mRNA levels of NFH were substantially increased by 5 μM garcinol treatment in the presence of EGF at 14 days postseeding. In addition, expressions of GFAP were also determined by RT-PCR in differentiation progress of the neuronal precursors to glial lineage. GFAP-positive cells could be identified in the presence of EGF (10 ng/mL) within 20 days by RT-PCR (**Figure 3C**). These findings suggested that garcinol contributed to neurite maturation and astrocytic survival up to 20 days in EGF-responsive cortical progenitors with the media containing 10 ng/mL of EGF.

Sustained Activation of ERK1/2 Signaling Triggered by Garcinol Treatment in EGF-Responsive Cortical Progenitor Cells. Activation of extracellular signal-regulated kinases (ERK) is required for cortical progenitor cell survival (*14*) and cell differentiation (*15*). To determine the pathway activated by garcinol treatment, the phosphorylation level of ERK in garcinol treated cortical progenitor cells was studied at time points ranging from 15 min to 1 h. ERK activation was monitored using immunoblotting analysis with an antibody against phospho-ERK 1 and 2 isoforms (**Figure 4A**). The studies showed that maximum stimulation of ERK phosphorylation after 10 ng/mL EGF treatment was reached at 15 min, whereas the phosphorylation of ERK remained the same when EGF and garcinol (5 μM) cotreatment was kept to 1 h. To explore the sustained activation of ERK, EGF-responsive cortical progenitor cells were treated with EGF and/or garcinol up to 20 h. The outcome in **Figure 4B** appeared to be the cotreatment with EGF and garcinol resulting in increased levels of phospho-ERK for 20 h. In contrast, the activation of ERK was reduced at 1 h in cultures supplemented with EGF alone. Afterward, PD98059, an inhibitor of MEKK, was used to identify the role of active ERK in the garcinol-mediated neurite

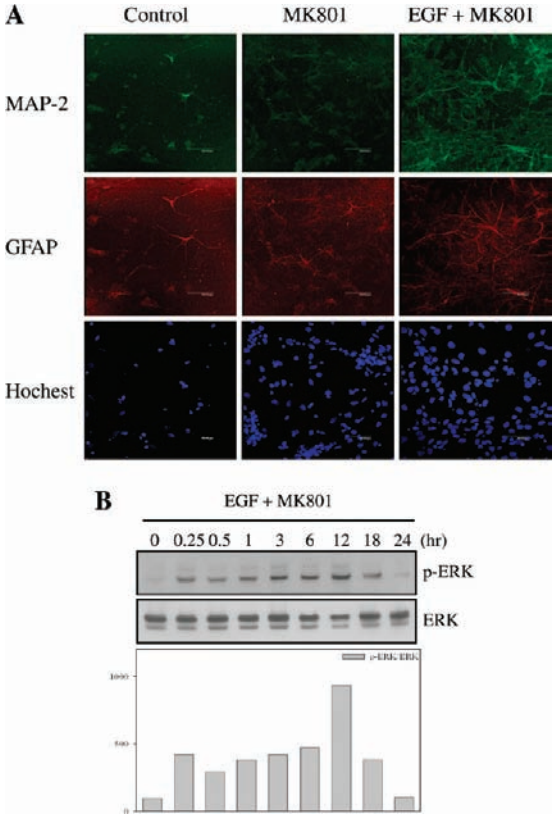

Figure 5. Effects of NMDA receptor on neurite outgrowth in serum withdrawal, EGF-responsive neuron precursor cells, and EGF-induced ERK activation. (**A**) EGF-responsive cortical progenitor cell extended neurites when NMDA receptor antagonist MK801 was added in the absence of serum for 4 days, and the neurites were stained by MAP-2 and GFAP antibodies. Data represents the fields from three independent experiments. (**B**) MK801 (10 ng/mL) prolonged the phosphorylation of ERK in the presence of EGF during 0−24 h. Blots were probed by phospho-ERK1/2 antibody. Each experiment was performed at least three times.

maturation process. The phosphorylation of ERK was barely detected in the presence of 20 μM PD98059 in EGF-treated cells within 20 h (**Figure 4C**). However, ERK exhibited time-dependent phosphorylation in response to garcinol and EGF cotreatment in the presence of PD98059 from 1 to 10 h of the experimental period and was sustained up to 20 h. The results also demonstrated that garcinol was able to activate ERK in the presence of EGF for prolonged periods. Additionally, the nuclear fraction of phosphorylated ERK after treatment with garcinol and/or EGF was examined. By immunoblotting analysis, the protein levels exhibited the upregulation of phospho-ERK in the nucleus in response to garcinol for 1 h (**Figure 4D**). The levels of nuclear phospho-ERK decreased after 1 h addition of EGF (**Figure 4D**, lane 4 and 5). However, the nuclear localization of phospho-ERK was increased in garcinol and EGF cotreated group for 1 h (**Figure 5C**, lane 6 and 7). The consequences suggested that prolonged localization of phospho-ERK in the nucleus might contribute to garcinol-improved EGF-responsive NPC survival and neurite outgrowth.

Increase in EGF-Responsive Progenitor Cell Survival and Prolonged ERK Activation by Treatment with NMDA Receptor Antagonist MK801. *N*-Methyl-D-asparate receptor blockade has also demonstrated the enhancement of the differentiation into the phenotypes of mature neurons (*23*) and influenced neuron cell survival (*19*). NMDA receptor antagonist MK801 was used to examine whether the NMDA receptor is involved in the regulation EGF-responsive progenitor cell survival and differentiation. Interestingly, cotreatment with MK801 and EGF boosted MAP-2-positive and GFAP-positive cell survival in primary cultured EGF-responsive progenitor cells (**Figure 5A**). In the meantime, EGF-responsive progenitor cells were cotreated with MK801 plus EGF followed by immunoblotting analysis to explore MK801 increased neuron cell survival and neurite outgrowth related with ERK activation. Treatment with EGF and MK801 (10 μM) resulted in a significant duration of phospho-ERK for time points ranging from 15 min to 18 h (**Figure 5B**) consistent with garcinol mediated sustained activation of ERK (**Figure 4D**). Accordingly, garcinol-prolonged activation of ERK might derive from the NMDA receptor blockade as MK801.

Reducing the Nuclear C/EBPβ Level Elevated Neuronal Survival and EGF-Induced Cortical Neurogenesis. Previously, Marshall and his co-workers demonstrated that the loss of growth factor support or strongly stimulating NMDA receptors rapidly increased nuclear import of C/EBPβ and induced subsequent cell death (*19*). Garcinol maintained EGF-responsive progenitor cell survival and prolonged ERK activation (**Figures 2** and **4**). Furthermore, the effects of garcinol through the regulated C/EBPβ pathway in EGF-responsive NPCs were examined. The EGF-responsive NPCs were serum deprived or treated with 5 μM garcinol and/or EGF in serum withdrawal conditions for 15 min or 1 h, and then the nuclear extracts were detected by immunoblotting analysis with anti-C/EBPβ antibody (**Figure 6A**). The nuclear levels of C/EBPβ were detected after 15 min of serum deprivation and persisted up to 1 h. However, the nuclear levels of C/EBPβ remained low in the cells maintained in serum-free media supplemented with garcinol and EGF for 1 h. (**Figure 6A**). Meanwhile, immunostaining by C/EBPβ antibody also showed that the increase in C/EBPβ nuclear localization by serum deprivation for 30 min and garcinol and/or EGF treatment blocked serum deprivation-induced nuclear localization of C/EBPβ (**Figure 6B**). These results suggested that garcinol maintained EGF-responsive NPC survival in a serum deprivation system through the decrease C/EBPβ nuclear translocation.

NMDA Receptor Antagonist MK801 Regulated Nuclear Localization of C/EBPβ and Survival in EGF-Responsive NPCs. Subsequently, excessive NMDA receptor activation triggers neuron cell death by acute excitotoxic insults and chronic neurodegenerative disease (*24*). The findings showed that the treatment with MK801 could maintain EGF-responsive NPC survival and prolonged ERK activation up to 18 h (**Figure 5**). The relationships between serum deprivation-induced nuclear localization of C/EBPβ and NMDA receptor were investigated. NMDA receptor antagonist MK801 was selected to treat the EGF-responsive NPCs and immunostained with C/EBPβ. The findings showed that serum deprivation-induced nuclear C/EBPβ localization was blocked by MK801 treatment and cotreatment with MK801 and EGF. Moreover, the observation indicated that cotreatment with MK801 and EGF reversed serum deprivation-reduced neuronal survival. The nuclear levels of C/EBPβ were detected in the presence of MK801 and/or EGF. The results revealed that serum deprivation-induced C/EBPβ nuclear protein was blocked in the presence of MK801 (**Figure 6D**). Collectively, our findings indicated that nuclear translocation of C/EBPβ might be an important mechanism by the facts of garcinol controlled neuritogenesis and survival in developing cortical progenitor cells through the blockade of NMDA receptor to inhibit C/EBPβ translocation.

DISCUSSION

In this study, our findings provide evidence that stem cell-like neurospheres were well induced by garcinol to exert neuronal

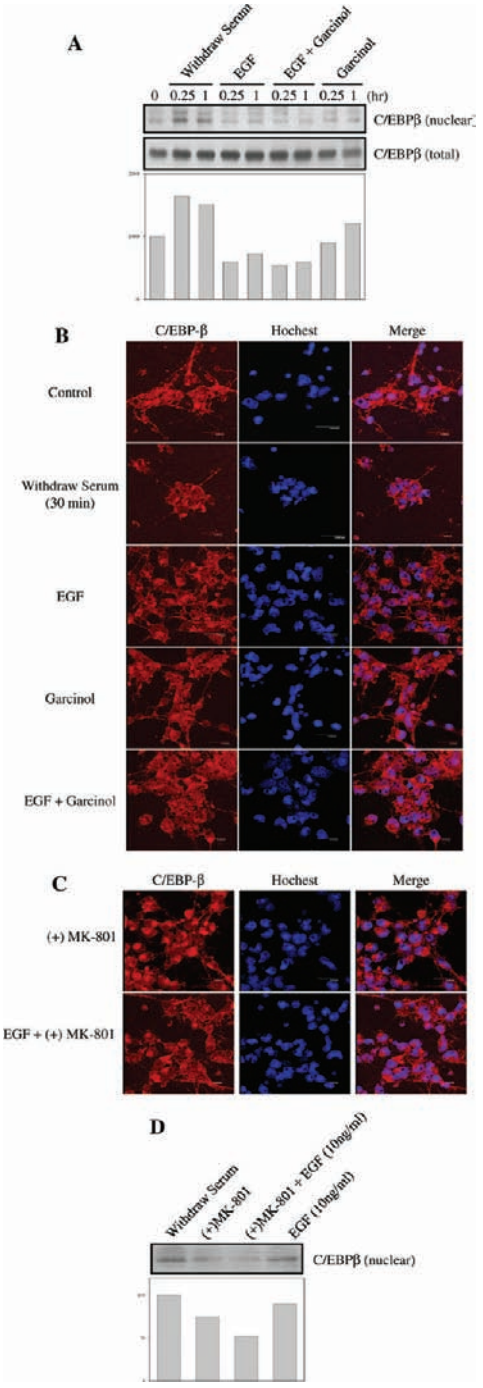

Figure 6. Role of C/EBPβ in garcinol-enhanced EGF-responsive neuron progenitor cell neurite outgrowth. (**A**) The EGF-responsive cortical progenitor cells were serum-deprived or treated with EGF (10 ng/mL) in the absence or presence of garcinol (5 μM) under serum-free conditions for the time points as indicated. Total cell lysates and the nuclear C/EBPβ levels were immunoblotted with the C/EBPβ antibody. (**B**) The EGF-responsive progenitor cells were stained by C/EBPβ antibody in the treatments described in **A**. Hoechst labeling showed all of the nuclei in the field. Scale bar, 40 μm. Data were reproduced in three separate identical experiments. (**C**) NMDA receptor antagonist, MK801, reduced nuclear localization of C/EBPβ. Cells were treated with MK801 in the presence or absence of EGF (10 ng/mL) and then immunostaining with C/EBPβ was performed as described in Material and Methods. Hoechst labeling showed all of the nuclei in the field. Scale bar, 40 μm. (**D**) EGF-responsive progenitor cells were serum-deprived or treated with MK801 (10 μM) in the presence of EGF for 60 min. Cell lysates were subjected to Western blot analysis using the antibody against C/EBPβ protein.

survival and neurite outgrowth. Garcinol-treated neurospheres possessed the biologically EGF-responsive properties, resulting in undifferentiated cortical progenitor cells, which could be differentiated to neuronal and glial cells in high percentages. In the EGF-treated cultures, garcinol considerably enhanced the expression of microtubule-associated proteins (MAP-2) and promoted neurite outgrowth (**Figures 1B** and **2**). Moreover, the induction of EGF and garcinol-treatment was intensively investigated to explore the activated signaling cascades on stimulation. The results showed that the phosphorylation of ERK1/2 lasted up to 1 h in the presence of garcinol for the EGF-treated neurospheres. The activations by both ERK1/2 and C/EBP pathways had been demonstrated on cortical progenitors forming neural cells in the presence of bFGF and EGF. Typically, the C/EBPβ protein was also widely distributed in the cortical progenitor cells and involved in cellular survival (*17*). Nuclear C/EBPβ levels plummeted under serum-withdrawal conditions that induced excitotoxic death (**Figure 6B**). Our data indicated that garcinol maintained the cytosolic levels of C/EBPβ against serum-deprivation induced cell death (**Figure 6A** and **B**). The evidence exhibited that garcinol was a potent neuroprotective agent and enhanced neuritogenesis in cortical progenitor cells.

Cultured embryonic cortical progenitor cells will mimic the temporal differentiation pattern observed in vivo, initially producing neurons and then developing to glia (*7*, *25*). Single embryonic and cortical stem cells can proliferate in response to exogenous EGF or bFGF to give rise to clonal colonies of undifferentiated neural precursor cells resulting in floating spheres (*11*). However, recent studies display that cortical progenitors differentiate into astrocytes and/or oligodendrocyte in addition to inhibiting neuron survival after culturing in vitro (*26*). In this study, garcinol was demonstrated to elevate neuronal survival and effectively induce neurite outgrowth in EGF-generated neurospheres. These cultured cells expressed MAP-2 protein in a composition similar to that of newly developed axons and dendrites in primary central neuron cultures. Furthermore, garcinol-treated EGF-responsive NPCs expressed the light-molecular-weight (68-kDa) neurofilament subunit NFL, which was the characteristic of initial neurite outgrowth (**Figure 4**). In the consecutive 14-day exposure to garcinol together with EGF, the mRNA levels of heavy (200-kDa) neurofilament subunit NFH were detected, which hitherto was thought to express much later in neuronal development. It was likely that garcinol exerted the features of neural maturation. In addition, the GFAP protein was also expressed in the presence of garcinol cultured with EGF or bFGF (**Figure 4D**). The freshly developed astrocytes might secrete a neurotrophic factor, which assured the survival and maturation of the neurons (*27*). These conclusions support our discoveries that garcinol influenced neuronal maturation in EGF-generated cortical progenitor cells.

Garcinol exerted a similar anti-inflammatory effect and might be neuroprotective against brain injury in our previous findings (*6*). Continually, garcinol also triggered neurite outgrowth in the presence of EGF and enhanced neuroprotection when growth factors were withdrawn in this observation. A critical issue in understanding the mechanism of neuronal maturation was the response of stem cells to extracellular signals. Encinas and coworkers had demonstrated that BDNF promotes cell survival and neurite outgrowth, but only neurite outgrowth rather than cell survival was blocked by MAP kinase inhibitor PD98059 (*28*). Additionally, bFGF was shown to activate ERK in embryonic chick retinal neurons (*14*), and BMP4, bone morphogenic protein 4, induced neural differentiation of neural stem cells (NSCs) grown in a medium containing bFGF by the activation of the Ras-ERK pathway (*29*). These combined conclusions show that ERK activation is one point of convergence for signaling pathways generated

by a variety of axon growth inducers and neural differentiation. It also has been observed that the genes for ERK are highly expressed in cortical progenitors, and at the protein level, both ERK1 and ERK2 are widely but differently expressed in various regions of the rat brain (*30*). On stimulation with EGF and/or garcinol, our data showed that the major tyrosine-phosphorylated protein was ERK. Furthermore, garcinol triggered rapid activation of ERK1/2 and a return to basal levels after 20 h in EGF-maintained cortical progenitor cells. It therefore seemed plausible that the late and prolonged activation of ERK1/2 by garcinol could be responsible for neurite differentiation. Interestingly, MEK inhibitor PD98059 completely blocked the garcinol-induced phosphorylation of ERK1/2 for 30 min but less than 1 h (**Figure 4C**). However, PD98059 did not initiate a complete block of garcinol-promoted neurite outgrowth. All of the collected data suggested that ERK signaling was an important pathway in the signal transduction modulated by garcinol rather than through the rapid activation of MEK/ERK cascade.

Glutamate/glycine activated the *N*-methyl-D-aspartate (NMDA) receptor located in the central nervous system and plays an important role during neuronal damage. Pathological activation of NMDA receptor is a key mechanism of neuronal death following acute excitotoxic trauma such as brain ischemia and hypoxia (*24*). The NMDA receptor-mediated regulation of ERK activity modulates transcription and neuronal functions, such as protection against neuronal cell death, and promotes the generation of new neuron (*23*). Moreover, MK801, the NMDA receptor antagonist, modulated the phosphorylation of ERK in the presence of EGF. Blocking NMDA receptor activity rescues neurons from excitotoxic death and decreases nuclear C/EBPβ levels, while NMDA receptor stimulation promotes death and elevates nuclear C/EBPβ levels (*19*). C/EBPβ expression increased in the hippocampus during excitotoxic injury, and C/EBPβ deficiency was resistant to excitotoxic-induced cell death (*17*). Conclusively, C/EBPβ has a crucial role in the neuronal survival in response to excitotoxic insult. In this study, MK801 treatment exhibited blocking of the NMDA receptor and reduced nuclear C/EBPβ levels (**Figure 6**). Take together, the results indicated that the levels soared under serum-free conditions inducing excitotoxic death, while garcinol elevated neuronal survival, maturation, sustained the phosphorylation of ERK cascades, and reduced nuclear C/EBPβ levels. Garcinol treatment mediated the maturation of newly generated neurons which acquired NMDA receptor blockade to expedite the construction of ERK phosphorylation cascades.

Previously, Liao et al. have confirmed that garcinol is a potent antioxidant from a natural source (*3*). Several studies indicate natural products that display the potential for neuroprotective effects and induction of neuritogenesis, such as the terpenoid fraction of *Ginkgo biloba* extract and flavonoids (*31*,*32*). Garcinol has been demonstrated as the scavenger of hydroxyl radicals and displays the ability to inhibit nitric oxide (NO) generation, in addition to decreasing the protein level of inducible nitric oxide synthase (iNOS) in primary astrocytes (*6*). The present data suggest additional properties of garcinol in neuroprotection and neurite outgrowth of cortical progenitor cells. It has been found that the duration of ERK activation contributed to the regulation of neural maturation by garcinol-treatment. To compare with the structures of MK801 and ginkgolides, garcinol contains the functional group of isoprenyl and is expected to have similar effects on neuroprotection through the modulation of the nuclear localization of C/EBPβ. In this study, our findings suggested that garcinol enhanced neuronal survival and regulated the potential effects of neuritogenesis.

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