2-Decenoic acid ethyl ester possesses neurotrophin-like activities to facilitate intracellular signals and increase synapse-specific proteins in neurons cultured from embryonic rat brain

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ABSTRACT

We presently found that medium-chain fatty acids (MCFAs) with 8–12 carbons and their esters facilitated activation (phosphorylation) of mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) 1/2 of cultured embryonic cortical/hippocampal neurons. In particular, trans-2-decenoic acid ethyl ester (DAEE) had the most potent activity. Additionally, DAEE activated phosphatidylinositol 3-kinase and cAMP-response element binding protein (CREB), suggesting that DAEE generates similar intracellular signal as neurotrophins. Therefore, details of the signal elicited by DAEE were examined in comparison with those of a neurotrophin, brain-derived neurotrophic factor (BDNF). We found that 1) DAEE phosphorylated MAPK/ERK1/2 via MEK activation without the involvement of tyrosine kinases of neurotrophin Trk receptors; 2) DAEE activated CREB predominantly through MAPK/ERK1/2 activation, not through other pathways such as cAMP/protein kinase A; and 3) DAEE increased the expression of mRNAs of BDNF and neurotrophin-3 and the protein content of synapse-specific proteins such as synaptophysin, synapsin-1, and syntaxin. Based on these observations we propose that DAEE and some other derivatives of MCFAs having neurotrophin-like neurotrophic activities may become therapeutic tools for certain neurological or psychiatric disorders.

Neurotrophins are neurotrophic proteins that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. Each neurotrophin binds to a specific Trk receptor tyrosine kinase (2), which causes autophosphorylation of the Trk receptor to trigger signal transduction cascades including the pathways of mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) 1/2, phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (12). These signals then pass into the nucleus to activate transcription factors such as cAMP-response element binding protein (CREB), leading to gene expression. Although neurotrophins have been considered as regulators for neuronal survival and differentiation during development and maintenance of neuronal viability and functions in adulthood (10), they have been also confirmed to play important roles in the development, functioning, and plasticity of synapses (14). These physiological significances suggest their availability as therapeutic tools for neurological disorders, which tools have been successfully tested in various animal models of diseases. However, the clinical trials have been less successful at demonstrating therapeutic efficacy (1). Many technical and pharmacological issues remain

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to be adequately addressed. For instance, instability of the proteins and/or a lack of appropriate delivery system is problematic. To overcome these drawbacks, the development of stable and small molecules with neurotrophin-like activity may represent an alternative approach.

Recent investigations about single nucleotide polymorphism of the BDNF gene have clarified that impaired BDNF function is correlated with various neurological disorders such as Parkinson’s disease (18), Alzheimer’s disease (16), depression (23), and anxiety-related personal traits (13). Substances that mimic intracellular neurotrophin signaling may be a promising candidate for therapy of neurotrophin-sensitive neurological and psychiatric disorders. Therefore, in the present study, we searched for such molecules by using neurons cultured from the cerebral cortex/hippocampus of mouse embryos. To date there are no known biological activities of medium-chain fatty acids (MCFAs) on the central nervous system (CNS). However, Kamata et al. (11) recently reported that MCFAs with 6 to 8 straight carbon atoms induce neurite outgrowth and phosphorylate ERK1/2 of cultured rat pheochromocytoma PC12 cells, a cell line of peripheral neural crest origin. Their findings prompted us to investigate MCFAs as activators of neurons cultured from CNS. We found that MCFAs with 8–12 carbons and their methyl or ethyl esters stimulated phosphorylation of ERK1/2 of the CNS neurons. In particular, trans-2-decenoic acid ethyl ester {(E)-ethyl dec-2-enoate, DAEE} had the most potent activity, and also enhanced the phosphorylation of PI3K and CREB, suggesting that DAEE is a molecule with neurotrophin-like activities and thus a promising therapeutic tool for certain neurological and psychiatric disorders.

MATERIALS AND METHODS

Preparation of MCFAs. We tested the following saturated or unsaturated MCFAs with 8 to 12 straight carbons and their methyl or ethyl esters: octanoic acid (8:0; which means the numbers of carbons: unsaturated bond), decanoic acid (10:0), trans-2-decenoic acid (10:1), trans-3-decenoic acid (10:1), trans-9-decenoic acid (10:1), trans-10-undecenoic acid (11:1), dodecanoic acid (12:0) and their methyl or ethyl esters. The acids were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The methyl or ethyl ester of each MCFA was prepared as follows: the acid (0.01 mol) was treated with thionyl chloride (SOCl₂, 10 mL) under reflux for 3 h, and the excess of SOCl₂ was distilled. Methyl or ethyl alcohol (0.01 mol) in tetrahydrofuran (20 mL) was added to the carbonyl chloride. The reaction mixture was boiled for 2 h and then poured into 1N-hydrochloric solution (50 mL) after cooling. The solution was extracted with ethyl acetate (EtOAc), and the EtOAc solution after concentration was purified with silica gel chromatography using a solution mixed with 2 volume of hexane and 1 volume of EtOAc as eluent to obtain the desired ester.

Primary cultures of central neurons. The neocortices and hippocampi of 17-day-old embryonic Wistar rats were dissected out, treated with phosphate-buffered saline (PBS) containing 0.25 % trypsin, 10 mM glucose, and DNAase (6 μg/mL; Sigma, St. Louis, MO), and mechanically dissociated. Following centrifugation (900 × g, 3 min), the cell pellet was resuspended in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo) containing 5% fetal bovine serum (FBS); and the resuspended cells were plated in 35-mm culture dishes (10⁵ cells/cm²) precoated with poly DL-ornithine (Sigma-Aldrich, St. Louis, MO). After a 24-h culture period, the medium was changed to B27-Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine; and the cells were then cultured for 3 days.

Immunoblot analysis. Cell proteins were extracted with a lysis buffer: 20 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, 10 μg/mL apro- tinin, 10 μg/mL leupeptin, 1 mM orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Cell protein (5 μg each) was subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel was transferred to a PVDF membrane, and the membrane was reacted with rabbit IgG antibody against protein or phospho-protein of ERK1/2, CREB or Akt (Cell Signaling, Beverly, MA) followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Promega, Madison, WI). Enzyme activity was visualized by using nitro blue tetrazolium/bromochloroindolyl phosphate color substrate. Immunoreactive bands were analyzed by using image J analysis software (NIH, USA).

Reverse transcription polymerase chain reaction (PT-PCR). PT-PCR was used to evaluate mRNA levels of neurotrophins and β-actin, as described previously (8). Total RNA was prepared by using TRIsol (Invitrogen). First-strand cDNA was synthe-
sized with PowerScript™ Reverse Transcriptase (Clontech, Palo Alto, CA). The synthesized cDNA was amplified as follows: for NGF, BDNF, and NT-3, denaturation at 94°C, 45 s; annealing at 61°C, 45 s; extension at 72°C, 30 s; and for β-actin, denaturation at 94°C, 45 s; annealing at 57°C, 45 s; and extension at 72°C, 45 s. The cycle was repeated 31–34 times for neurotrophins, and 28 times for β-actin. The amounts of all PCR products were within the range of linear increase. PCR products were electrophoresed and stained with ethidium bromide. The intensity of the bands was analyzed by using image analysis software (NIH, USA). DNA sequences of the PCR products were analyzed, and their identity was confirmed. The primers and the product sizes were as follow: up-primer 5'-GGCAAGTCAGCTCTTTGGTAGCTTCC-3' and down-primer 5'-GGGGATCCTCCACCCACCTCCAC-3' for NGF (product size: 376 bases); up-primer 5'-CCCCAGGGCAGGTTGAGAGG-3' and down-primer 5'-CCCGCCAGACATGCCACTG-3' for BDNF (product size: 350); up-primer 5'-TGCCATCAGAGGCAACAGCA-3' and down-primer 5'-CCTCTTTGTAGCCTTCC-3' and down-primer 5'-CTCTTTGTAGCCTTCCACATAA-3' for NT-3 (product size: 346); and up-primer 5'-GTGGCCGCTCTTAACCACAA-3' and down-primer 5'-TCACGCAGAT-3' for β-actin (product size: 542).

RESULTS AND DISCUSSION

Novel activity of MCFAs and their derivatives: facilitated phosphorylation of ERK1/2 in cultured central neurons

ERK1/2, which is one of the subgroups of MAPK (9) and widely expressed in neurons, is activated by glutamate receptors (24) and receptor tyrosine kinases (29). Activation of the ERK1/2 pathway facilitates the phosphorylation of CREB, a neurotrophin-regulated transcription factor involved in numerous functions in the adult brain including learning and memory ability (17). In fact, impairment of the Ras/ERK1/2 pathway inhibits dendritic spine development/maintenance, promotes synaptic depression rather than long-term potentiation, and abolishes learning and memory ability (20).

The ability of MCFAs and their methyl or ethyl esters to elicit phosphorylation of ERK1/2 in neurons cultured from CNS was examined. All compounds were more or less stimulatory; however, the potency of the activity varied with the compounds: 1) the activity of the fatty acids was strengthened by methyl, and much more by ethyl, esterification; 2) the MCFAs with 10 carbons were more potent than those with 8, 11, or 12 carbons; 3) decenoic acids with 10 carbons and an unsaturated bond (10:1) at the 2-position had higher activity than those with a double bond at the 9- or 3-position. Namely, trans-2-decenoic acid ethyl ester (DAEE, see Fig. 1A) was the most potent of the compounds tested. Therefore, we used it for further study. The amount of neuronal phosphorylated ERK1/2 (pERK1/2) elicited by DAEE increased in a DAEE concentration-dependent manner up to 300 μg/mL, with significant activation occurring at a concentration of 1 μg/mL or higher (Fig. 1B). The increase started at 15 min and lasted until 12 h after the addition of DAEE to the cultures (Fig. 1C).

To date, knowledge about the physiological activities of MCFAs on the CNS is quite limited. Kamata et al. (11) showed that MCFAs with 6 to 8 straight carbon atoms induce neurite outgrowth of cultured PC12 cells in the absence of NGF. Among the MCFAs they tested, octanoic acid (8:0) had the greatest activity; and p38MAPK and ERK1/2 were activated by it prior to neurite outgrowth. However, we found that octanoic acid (8:0) only weakly phosphorylated ERK1/2 in primary cultures of CNS neurons (data not shown). Although Kamata et al. (11) never examined the esters of MCFAs, the discrepancy between their results and ours is probably due to the difference in the cells used for evaluation.

Neurotrophin-like activity to facilitate intracellular signals

We next evaluated simultaneously the effect of trans-2-decenoic acid (DA) and DAEE on the phosphorylation of ERK1/2, CREB, and Akt, the last mentioned being a signal transduction molecule of the PI3K pathway (Fig. 1D). DAEE significantly enhanced the phosphorylation of CREB and Akt in addition to that of ERK1/2. Although DA had a tendency to stimulate the phosphorylation of both CREB and Akt, the effects were not significant; whereas it did significantly phosphorylate ERK1/2. Ethyl-esterification of DA, resulting in DAEE, strengthened the activity to stimulate the phosphorylation of ERK1/2, CREB and Akt. These results suggest that DAEE is a neurotrophin-like neurotrophic molecule, which prompted us to compare the intracellular signaling by DAEE with that by neurotrophins.

First, we examined whether Trk family tyrosine kinase receptors were involved in the DAEE actions. A selective inhibitor of tyrosine kinases of Trk receptors, K252a, significantly suppressed BDNF-induced ERK1/2 phosphorylation, but not the DAEE-induced one (Fig. 2A). Second, the effect of the
MEK (MAPK kinase) inhibitor U0126 was examined. The drug clearly suppressed both DAEE- and BDNF-enhanced phosphorylation of ERK1/2 (Fig. 2B), demonstrating that DAEE phosphorylated ERK1/2 through MEK activation without involvement of Trk receptor tyrosine kinases.

There are plural signal pathways for the activation of CREB. To determine which pathway was responsible for the phosphorylation of CREB elicited by DAEE, we examined the effect of U0126 or PKA inhibitor H89 on the phosphorylation. U0126 almost completely suppressed the basal-level and DAEE-induced phosphorylation as well as the BDNF-dependent one (Fig. 3A). H89 reduced the forskolin-induced phosphorylation, but did not influence the DAEE-induced one (Fig. 3B). Furthermore, the DAEE-enhanced phosphorylation of Akt was inhibited by LY294002, an inhibitor of PI3K, similarly as the BDNF-induced one (Fig. 3C). These results indicate that DAEE predominantly induced the phosphorylation of CREB through MEK-dependent ERK1/2 activation, not through other pathways such as cAMP/PKA, and phosphorylated Akt via PI3K as did neurotrophins. Like BDNF, DAEE did not affect P38MAPK or calcium-activated calmodulin kinase II and IV (data not shown). A growing body of evidence has demonstrated that the ERK1/2-dependent phosphorylation of CREB is involved in the regula-

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**Fig. 1** Chemical structure of DAEE (A), dose-dependent (B) and time-dependent ERK1/2 phosphorylation elicited by DAEE (C), and the effects of DAEE and DA on the phosphorylation of ERK1/2, CREB, and Akt (D). The degree of ERK1/2 phosphorylation in the cultured neurons was analyzed after treatment of the cells with the indicated concentrations of DAEE for 30 min (B), or with DAEE (100 μg/mL) for the indicated times (C). Neurons were treated with DAEE or DA (100 μg/mL) for 6 h, and the levels of phosphorylated ERK1/2, CREB, and Akt were evaluated (D). The values (ratios of the intensity of the band of phospho-protein/the intensity of the protein band) are expressed as the mean ± SE (n = 4), and indicated as fold-increase of the value for the vehicle-treated control neurons (B, C, D). The significance of the difference between a value for DA or DAEE and the value for the vehicle-treated control neurons was determined by one-way ANOVA with Tukey's test as *P < 0.05, **P < 0.01 or ***P < 0.001.
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tion of functions such as learning/memory and the formation of long-term potentiation (6, 20). Therefore, DAEE may be expected to regulate neuronal and/or synaptic functions of the CNS via regulation of the expression of various CREB-driven genes.

Gene expression of BDNF and NT-3

The influence of DAEE on the gene expression of neurotrophins including NGF, BDNF, and NT-3, was evaluated next. DAEE facilitated markedly the expression of BDNF mRNA and slightly but significantly that of NT-3 mRNA but not that of NGF mRNA (Fig. 4A). BDNF gene is known to be up-regulated by CREB activation (6, 25). However, NT-3 gene expression is not up-regulated by Ca²⁺ entry, suggesting that it is under CREB-independent regulation. In fact, both specificity protein (Sp)3 and Sp4 are transcription factors that bind to the NT-3 promoter (25). NGF transcription is regulated via an activating protein 1 (AP-1) binding site in the first intron of the NGF gene (4), suggesting NGF to be regulated CREB-independently. These observations confirm DAEE-mediated CREB-dependent BDNF gene expression and lack of CREB-independent NGF gene expression in response to DAEE. However, facilitated expression of the NT-3 gene is not explainable at present. Transcription factor Sp3 and/or Sp4 may be involved in the downstream of signal transduction by DAEE.

Expression of synapse-specific proteins

By Western blot analysis we next estimated the expression levels of synaptic proteins such as synapsin I, a non-integral membrane protein tightly associated with synaptic vesicles; synaptophysin, an integral membrane protein of the synaptic vesicle membrane (5); and syntaxin, located in the presynaptic plasma membrane (21) in cultured cortical neurons, and found that all of these proteins were increased in expression after a 6-h exposure to DAEE (Fig. 4B). In each experiment, samples from the same experimental conditions were run in triplicate, and multiple proteins were simultaneously measured on the same immunoblots. The same experiment was repeated 3–5 times, using samples from independent culture preparations. It is likely that DAEE facilitates synaptogenesis and/or functional synaptic development via enhanced expression and/or suppressed degradation of these synaptic proteins.

So far, the effects of BDNF on the expression of synaptic proteins have been investigated in vitro. The expression of synaptophysin increases within 6 h after BDNF treatment without affecting the levels of syntaxin and synapsin I in cultured hippocampal slices of postnatal 7-day-old rats (26). Another report demonstrated that 1-day exposure of postnatal rat cortical neurons to BDNF up-regulates the expression of synapsin I and synaptophysin, but not that of syntaxin, through the PLC-γ and Ras/ERK1/2
BDNF seems to differentially increase the amount of certain proteins per synaptic vesicle. DAEE up-regulated the expression of not only synaptophysin and synapsin-1 like BDNF, but also stimulated that of syntaxin, unlike BDNF. Although the reason why the response of syntaxin was different between BDNF and DAEE is unknown at present, it may be due to the difference in cell samples. That is, DAEE was presently tested by using embryonic cortical/hippocampal neurons; but in the experiments with BDNF, brain slices of postnatal rodents were used (15, 26). Although DAEE-induced BDNF might be partly involved in this regulation, it is likely that the ERK1/2 pathway directly activated by DAEE may play central roles. Thus we
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postulate that DAEE can regulate the expression of synaptic proteins similarly as BDNF. These results suggest that DAEE is a BDNF-like or neurotrophin-like molecule with respect to intracellular signaling including ERK1/2-mediated CREB activation and PI3K-activated Akt phosphorylation followed by gene expression of BDNF and NT-3 and up-regulation of expression of synaptic proteins.

**DAEE may be a promising candidate as an antidepressant drug**

Medium-chain triglycerides (MCTs), constituents of coconut and palm kernel oils, are composed of predominantly glycerol esters of MCFAs and have a favorable safety profile for the treatment of various disorders (27). MCTs of coconut oil contain 67% octanoic acid (8:0), 27% decanoic acid (10:0), and 6% other fatty acids, but it is not clear whether 2-decanoic acid (10:1; DA) is included. Therefore, the safety profile of MCTs does not necessarily guarantee the safety of DA and its ester form DAEE. However, the safety of DA applies roughly to that of decanoic acid, the saturated form of DA, because 1) both fatty acids are similarly oxidized by β-oxidation processes in mitochondria, and 2) DA is generated as an intermediate metabolite in the process of the β-oxidation of safety long-chain fatty acids (22).

Rate of penetration of straight-chain saturated monocarboxilic acids into brain increased with chain length and was virtually complete at lengths greater than that of hexanoic acid (6:0), however, smaller monocarboxilic acids cross the blood-brain barrier (BBB) relatively slowly by means of a saturable and stereospecific transport mechanism (19, 28). Alternatively, nuclear magnetic resonance spectroscopy of the extract of whole brain from rats infused with 13C-labeled octanoic acid clarified that octanoic acid is avidly incorporated and metabolized by brain (3), demonstrating that MCFAs offer a readily available non-carbohydrate fuel source in brain. These observations suggest that MCFAs are rather stable in blood stream and extracellular space until incorporation by the cells.

Neurotransmitter- or neurotrophin-regulated intracellular signaling in the hippocampus is hypothesized to contribute to depression and antidepressant efficacy (7). For instance, chronic stress selectively reduces the level of pERK1/2 in the hippocampal dentate gyrus, which reduction is followed by increased immobility and decreased response in an operant conditioning task of motivation (7). Furthermore, the ERK1/2 pathway is thought to be involved in fear extinction mechanisms, which are aberrant in anxiety disorders and posttraumatic stress disorder (20). Antidepressants may restore the hippocampal pERK1/2 level after a stress-related insult, and reveal a novel role for neurotrophins in depressive-like symptomology. However, delayed actions and substantial side effects are major drawbacks of current antidepressant drugs, which may be avoided by DAEE and its derivatives; because they would be expected to rapidly pass through the BBB and directly transduce signals like BDNF in the brain without generation of unexpected neuronal transmissions that cause side effects. In fact, our recent results showed that intraperitoneally administered DAEE facilitated phosphorylation of ERK1/2 in the hippocampus and cerebral cortex of normal adult rats (A. Makino et al., unpublished results), suggesting the possibility that DAEE is a promising candidate for antidepressant. Further studies using animal models of depression are necessary for precise elucidation of the action mechanism and clarification of the pharmacokinetics of DAEE. Also, our present results may provide a new insight into the action of the derivatives of unsaturated MCFAs on the brain.

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