Elaidic Acid Potentiates Extracellular ATP-Induced Apoptosis via the P2X,ROS-ASK1-p38 Axis in Microglial Cell Lines

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trans-Fatty acids (TFAs) are unsaturated fatty acids with at least one carbon–carbon double bond in trans configuration. TFA consumption has been epidemiologically associated with neurodegenerative diseases (NDs) including Alzheimer’s disease. However, the underlying mechanisms of TFA-related NDs remain unknown. Here, we show a novel microglial signaling pathway that induces inflammation and cell death, which is dramatically enhanced by elaidic acid (EA), the most abundant TFA derived from food. We found that extracellular ATP, one of the damage-associated molecular patterns (DAMPs) leaked from injured cells, induced activation of the apoptosis signal-regulating kinase 1 (ASK1)-p38 pathway, which is one of the major stress-responsive mitogen-activated protein (MAP) kinase signaling pathways, and subsequent caspase-3 cleavage and DNA ladder formation (hallmarks of apoptosis) in mouse microglial cell lines including BV2 and MG6 cells. Furthermore, we found that in these microglial cell lines, EA, but not its cis isomer oleic acid, facilitated extracellular ATP-induced ASK1/p38 activation and apoptosis, which was suppressed by pharmacological inhibition of either p38, reactive oxygen species (ROS) generation, P2X purinoceptor 7 (P2X7), or Ca2+/calmodulin-dependent kinase II (CaMKII). These results demonstrate that in microglial cells, extracellular ATP induces activation of the ASK1-p38 MAP kinase pathway and ultimately apoptosis downstream of P2X7 receptor and ROS generation, and that EA promotes ATP-induced apoptosis through CaMKII-dependent hyperactivation of the ASK1-p38 pathway, in the same manner as in macrophages. Our study may provide an insight into the pathogenesis of NDs associated with TFAs.

Key words trans-fatty acid; microglia; extracellular ATP; apoptosis; apoptosis signal-regulating kinase 1; neurodegenerative disease

INTRODUCTION

trans-Fatty acids (TFAs) are unsaturated fatty acids that possess at least one carbon–carbon double bond. Among TFAs, elaidic acid (EA, C18:1 9) is the most abundant TFA in processed foods, produced mainly through partial hydrogenation of fish and vegetable oils containing oleic acid (OA, C18:1 9), a cis isomer of EA. Compelling epidemiological studies have linked consumption of TFAs, particularly EA, with various disorders, such as cardiovascular diseases (CVDs), metabolic syndrome, systemic inflammation. TFA intake has been also associated with neurodegenerative diseases (NDs) such as Alzheimer’s disease by multiple lines of epidemiological and animal model evidence. Previous biological studies have revealed the underlying mechanisms of the TFA-related disorders including atherosclerosis. However, so far, very few studies have focused on those of neurodegenerative disorders, which remains to be elucidated.

We have recently uncovered a novel toxicity mechanism of TFAs, triggered by a damage-associated molecular pattern (DAMP), extracellular ATP, which is known as a potent pro-inflammatory factor leaked from injured cells. Once extracellular ATP binds to the P2X purinoceptor 7 (P2X7), as a ligand, P2X7 induces non-selective cation flux including Ca2+ influx, which provokes reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated reactive oxygen species (ROS) generation and subsequent activation of the apoptosis signal-regulating kinase 1 (ASK1)-p38 mitogen-activated protein (MAP) kinase pathway, eventually leading to apoptosis. We demonstrated that EA, but not OA as its cis isomer, augments extracellular ATP-induced Ca2+/calmodulin-dependent kinase II (CaMKII) activation, and thereby facilitates activation of the ASK1-p38 MAP kinase pathway and ultimately apoptosis. Since accumulation of apoptotic macrophages in atherosclerotic lesions is a major cause of atherosclerosis progression, these findings can well explain the pathological mechanism of TFA-related CVDs.

Microglia are tissue resident macrophages in the central nervous system (CNS), derived from hematopoietic myeloid lineage. In response to endogenous and exogenous stimuli, including DAMPs (e.g. extracellular ATP), reactive oxygen species, and endotoxins (e.g. lipopolysaccharide (LPS)), microglia induce inflammatory responses and cell death as do macrophages, which contributes to pathogenesis of various NDs, such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS). Based on our previous findings, it can be assumed that accumulation of TFAs may promote extracellular ATP-induced microglial inflam-
matory signaling and cell death, and thereby exacerbate NDs. To date, extracellular ATP-induced apoptosis via the P2X₇-ASK1-p38 axis has been observed only in macrophages. However, it remains to be determined whether the apoptotic signaling axis is conserved and can be facilitated by TFAs among other types of cells, particularly in microglia.

In this study, we showed that extracellular ATP induces apoptosis in mouse microglial BV2 and MG6 cells, via the P2X₇-ROS-ASK1-p38 axis. We further showed that EA promotes ATP-induced activation of the ASK1-p38 MAP kinase pathway in a manner dependent on CaMKII, and ultimately apoptosis in BV2 cells. These results demonstrate a significant role of extracellular ATP in inducing microglial inflammation and cell death, which may account for the pathogenesis and progression of NDs associated with TFA intake.

MATERIALS AND METHODS

Reagents KN-93 was purchased from either Sigma (St. Louis, MO, U.S.A.) or Wako (Osaka, Japan). Coomassie brilliant blue G-250 (CBB) was purchased from Fluka (Everett, WA, U.S.A.). SB203580 (SB) was purchased from Santa Cruz (Dallas, TX, U.S.A.). ATP, SP600125 (SP), N-acetylcysteine (NAC) and Propyl gallate (PG) were purchased from Wako.

Cell Culture BV2 and MG6 cells were obtained from ATCC and RIKEN, respectively, and cultured in Dulbecco’s Modified Eagle Medium (Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (Sigma) and 1% penicillin–streptomycin solution (Nacalai Tesque) in 5% CO₂ at 37 °C.

Preparation and Treatment of Fatty Acids EA (Sigma) and OA (Nacalai Tesque) were prepared as described previously. Briefly, fatty acids were dissolved in 0.1 N NaOH at 70 °C, and then conjugated with fatty acid-free BSA (Wako, pH 7.4) at 55 °C for 10 min to make 5 mM BSA-conjugated fatty acid stock solutions containing 10% BSA. Cells were treated with various concentrations of BSA-conjugated fatty acids by diluting stock solutions in medium without fetal bovine serum (final BSA concentration was set to 1%).

Immunoblot Analysis Cells were lysed in ice-cold lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, 10% Glycerol, and 1% protease and phosphatase inhibitor cocktail (Nacalai Tesque). After centrifugation, the cell lysates were treated with various concentrations of BSA-conjugated fatty acids by diluting stock solutions in medium without fetal bovine serum (final BSA concentration was set to 1%).

DNA Fragmentation Assay DNA fragmentation assay was performed as described previously. Briefly, stimulated cells were collected and suspended with lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100), and the cell lysates were incubated at room temperature for 10 min, followed by centrifugation at 12000 × g for 10 min. The supernatants were incubated with 0.2 mg/mL proteinase K and 0.1 mg/mL ribonuclease (RNase) A for 1 h at 42 °C, purified with phenol/chloroform extraction and ethanol precipitation, and separated on an agarose gel.

RESULTS

Extracellular ATP Induces Apoptosis in Microglial Cell Lines To address whether extracellular ATP has an ability to induce apoptosis in microglial cells, we treated mouse microglial BV2 and MG6 cells with ATP. We found that ATP treatment dramatically decreased cell viability in a dose-dependent manner in both cell lines (Figs. 1A, B). Extracellular ATP induces apoptosis via the ASK1-p38 MAP kinase pathway. Immunoblot analysis showed that ATP treatment induced phosphorylation of ASK1 and p38, which clearly indicates their activation in response to ATP (Fig. 1C). Apoptosis is a programmed cell death that requires caspase-3 activation via cleavage by upstream caspases, and subsequent nuclease activation causing DNA ladder formation. Therefore, caspase-3 activation and DNA ladder formation are widely used as typical markers for apoptosis. We indeed observed that ATP treatment caused caspase-3 cleavage (Fig. 1D) and DNA ladder formation (Fig. 1E). These results suggest that extracellular ATP induced apoptotic cell death in microglial cell lines, BV2 and MG6.

EA Promotes Extracellular ATP-Induced Apoptosis in Microglial Cell Lines We have recently shown that EA promotes extracellular ATP-induced apoptosis in a macrophage cell line RAW264.7. To investigate whether the proapoptotic effect of EA is also observed in microglial cell lines, we first checked whether EA itself has a cytotoxic effect on BV2 and MG6 cells. As shown in Figs. 2A and B, EA treatment did not affect cell viability up to 200 µM, neither as OA treatment, indicating that EA has no cytotoxic effect on these cell lines at least at 200 µM. We then pretreated BV2 and MG6 cells with 200 µM EA or OA, treated with various concentrations of ATP, and subjected to survival assay. We found that pretreatment of EA, but not OA, dramatically decreased cell viability in BV2 cells (Fig. 2C), whereas modestly but significantly decreased it in MG6 cells (Fig. 2D) in response to ATP. Moreover, when ATP concentration was constant, cell viability was declined by EA pretreatment in a concentration-dependent manner both in BV2 and MG6 cells (Figs. 2E, F). In the presence of EA, extracellular ATP-induced caspase-3 cleavage, an apoptosis marker, was apparently increased (Fig. 2G). These results suggest that EA, but not OA as its cis isomer, specifically promotes extracellular ATP-induced apoptosis in microglial cell lines.

EA Facilitates p38 Activation via the ATP-P2X₇-ROS Axis Once extracellular ATP binds to its receptor P2X₇, p38 MAP kinase is activated downstream of ASK1 in response to ROS generated by NADPH oxidase, ultimately leading to the induction of apoptosis. Immunoblot analysis showed that ATP-induced p38 phosphorylation was clearly increased.
by pretreatment of EA, but not that of OA, in BV6 microglial cells (Fig. 3A). In addition, cell viability analysis showed that EA-dependent cell death enhancement was reversed by p38 inhibitor SB, but not by JNK inhibitor SP (Fig. 3B). These results suggest that EA enhances extracellular ATP-induced p38 activation and subsequent cell death in a microglial cell line, BV2. We have previously shown that ATP-induced pro-apoptotic action of EA strongly depends on ROS generation downstream of P2X7 receptor, due to the critical role of ROS as an activator of ASK1.\textsuperscript{10} Indeed, EA-mediated increase in ATP-induced p38 activation and cell death was suppressed in the presence of ROS scavengers including PG and NAC (Figs. 3C, D), supporting the requirement of ROS for p38 activation. We also observed that P2X\textsubscript{7} inhibitor CBB canceled ATP-induced cell death, indicating that the observed apoptosis depends on the ATP receptor P2X\textsubscript{7} (Fig. 3D). These data collectively suggest that EA facilitates extracellular ATP-induced p38 activation through P2X\textsubscript{7}-dependent ROS generation in BV2 cells.

**CaMKII-Dependent ASK1/p38 Hyperactivation Involves Pro-apoptotic Function of EA** To investigate the involvement of ASK1, which is activated in response to ROS generation downstream of P2X\textsubscript{7} and serves as MAP kinase kinase (MAP3K) upstream of p38 MAP kinase,\textsuperscript{11} we assessed phosphorylation status of ASK1 by immunoblot analysis. As shown in Fig. 4A, extracellular ATP-induced ASK1 phosphorylation was increased with EA pretreatment, as was p38 phosphorylation. We previously showed that EA enhances ATP-induced ASK1 activation in a manner dependent on CaMKII, which can be activated by Ca\textsuperscript{2+} influx \textit{via} P2X\textsubscript{7} and acts as an ASK1 activator.\textsuperscript{22,23} We observed that a widely used CaMKII inhibitor KN-93 suppressed EA-mediated promotion of ASK1 activation (Fig. 4B), p38 activation (Fig. 4C) and cell death (Fig. 4D), which were induced by ATP. These results suggest that CaMKII-dependent ASK1 hyperactivation contributes to extracellular ATP-induced pro-apoptotic signaling mediated by EA in BV2 cells.

**DISCUSSION**

In this study, we have shown that extracellular ATP induces apoptosis in microglial cell lines MG6 and BV2. Although it has been reported that extracellular ATP induces apoptosis in microglia,\textsuperscript{24,25} this study for the first time revealed the involvement of the P2X\textsubscript{7}-ROS-ASK1-p38 axis in ATP-induced microglial apoptosis. P2X\textsubscript{7} receptor is expressed throughout...
Fig. 2. EA Promotes Extracellular ATP-Induced Apoptosis in Microglial Cell Lines
(A, B) BV2 cells (A) and MG6 cells (B) were treated with the indicated concentrations of OA or EA for 26h, and subjected to cell survival assay. Data shown are the mean ± S.D. (C, D) BV2 cells (C) and MG6 cells (D) were pretreated with 200 µM OA or EA for 14h, and then treated with the indicated concentrations of ATP for 6h, subjected to cell survival assay. Data shown are the mean ± S.D. Significant differences were determined by one-way ANOVA, followed by Tukey-Kramer test; **p < 0.01; ***p < 0.001 (versus control). (E, F) BV2 cells (E) and MG6 cells (F) were pretreated with the indicated concentrations of OA or EA for 14h, and then treated with ATP at 1.5 mM (E) or 2 mM (F) for 6h, subjected to cell survival assay. Data shown are the mean ± S.D. Significant differences were determined by one-way ANOVA, followed by Tukey-Kramer test; ***p < 0.001 (versus OA). (G) BV2 cells were pretreated with or without 200 µM EA for 14h, and then treated with 1.5 mM ATP for 6h, subjected to immunoblotting with the indicated antibodies.
the brain, including neurons and microglia. In response to a low (i.e., physiological) concentration of extracellular ATP, P2X7 receptor plays significant roles in neuronal axonal growth, regulation of presynaptic neurotransmitter release, and glial activation. In a pathological condition, ATP is actively released from neurons and microglia, or leaked from injured cells, which dramatically increases extracellular ATP concentration. Consequently, strong and sustained activation of P2X7 receptor is triggered, resulting in the formation of non-selective large pores, besides that of the typical non-selective cationic channels, which induces various neuroinflammatory events, such as ROS production, inflammasome activation, pro-inflammatory cytokine release, and apoptosis in neurons and microglia. Accumulating evidence has shown a critical link of P2X7 receptor with a variety of NDs, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and Multiple Sclerosis. Importantly, ASK1 has been also associated with all the NDs described above. ASK1 is a member of the MAP3K family proteins that selectively activates the JNK/p38 MAP kinase pathways in response to various types of stresses, including oxidative stress, ER stress, Ca2+ influx, and pro-inflammatory factors (e.g. cytokines, pathogens and DAMPs), which induces cell death and inflammation. Accumulating in vitro and in vivo evidence has shown that in a variety of ND models, ASK1 is activated and induces neuronal cell death and inflammation, which can be suppressed by its genetic or pharmacological inhibition. Thus, extracellular ATP-induced microglial apoptosis mediated by the P2X7-ROS-ASK1-p38 axis may possibly have a significant contribution to NDs, which should be addressed in the future studies.

Furthermore, we have shown that EA, the most abundant TFA in foods, but not OA as its cis isomer, facilitates extracellular ATP-induced activation of the ASK1-p38 MAP kinase pathway, and thereby promotes apoptosis in microglial cell lines, as well as in a macrophage-like cell line RAW264.7. Mechanistically, CaMKII, an upstream kinase of ASK1 that promotes its activation, is involved in the EA-mediated enhancement of p38 activation and cell death in BV2 cells (Figs. 4B–D), in the same manner as in RAW264.7 cells. ROS also contributes to pro-apoptotic actions of EA (Figs. 3C, D). However, we have previously demonstrated that EA does not affect the amount of ROS generation induced by the ATP/P2X7 receptor signaling, suggesting that ROS generation is necessary for but not directly engaged in the potentiation of apoptotic signaling mediated by EA. We have previously shown that EA augments extracellular ATP-induced CaMKII phosphorylation at threonine (Thr)-286, an autophosphory-
lation site that assists CaMKII activation. However, the mechanism by which EA enhances CaMKII phosphorylation still needs to be clarified. Pro-apoptotic effect of EA is apparently larger in BV2 cells than in MG6 cells (Figs. 2C–F). BV2 and MG6 cell lines were originally immortalized by virus-mediated transduction of rapidly accelerated fibrosarcoma (v-raf)/v-myc and c-myc, respectively. v-Raf-transduced BV2 might have higher potency for CaMKII activation than MG6 without v-Raf, since CaMKII and Raf-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK MAPK pathway have been reported to interact each other, and thereby promote activation in a cooperative fashion. Regarding the TFA amount in human bodies, plasma concentration of EA is approx. 10 µM and that of the total TFAs is approx. 40 µM. Although there has been no evidence of the actual TFA amount in human brain, a previous report demonstrated that the TFA proportion in normal diet-fed rat brain is 0.18%. Considering that rat brain contains approx. 30 mg/g brain total fatty acids, TFA concentration in rat brain is estimated to be 200 µM. Therefore, the EA concentration utilized in this study (25–200 µM) would be physiologically relevant, and we thus set its concentration as 200 µM in most of the experiments. To date, the disease etiology of TFA-related NDs is totally unknown. Epidemiologic evidence has indicated that TFA intake increases plasma low-density lipoprotein cholesterol while decreases high-density lipoprotein cholesterol, which most likely contributes to the pathogenesis of CVDs. TFAs may exacerbate NDs via dysregulation of lipoprotein metabolism, since CVDs have been implicated with NDs such as Alzheimer’s disease. Intriguingly, however, a most recent cohort study showed that serum EA levels are significantly associated with all-cause dementia and Alzheimer’s disease, but not with vascular dementia. Interestingly, two recent studies demonstrated more direct neurodegenerative mechanisms of EA. EA is less protective than OA and docosahexaenoic acid from the cytotoxicity of 7-ketocholesterol, an oxidized form cholesterol that is increased in plasma and cerebrospinal fluid of ND patients, in BV2 cells. EA promotes apoptosis via elevation of ROS generation and ER stress in SH-SY5Y neuroblastoma cell line. Moreover, importantly, our study uncovered a neurodegenerative mechanism of EA that accelerates extracellular ATP-induced microglial pro-inflammatory signaling and apoptosis. Thus, our findings provide an insight into pathogenetic mechanisms of NDs, which may lead to the development of novel prevention and therapeutic strategies for NDs associated with TFAs.

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Fig. 4. EA Enhances Extracellular ATP-Induced ASK1 Activation in a Manner Dependent on CaMKII

(A) BV2 cells were pretreated with 200 µM EA for 14 h, and then treated with 1.5 mM ATP for the indicated time periods, subjected to immunoblotting with the indicated antibodies. (B, C) BV2 cells were pretreated with or without 200 µM EA for 14 h, treated with a CaMKII inhibitor KN-93 for the last 30 min, and then stimulated with 1.5 mM ATP for the indicated time periods, subjected to immunoblot analysis with the indicated antibodies. (D) BV2 cells were pretreated with or without 200 µM EA for 14 h, treated with a CaMKII inhibitor KN-93 for the last 30 min, and then stimulated with 1.5 mM ATP for 6 h, subjected to cell survival assay. Data shown are the mean ± S.D. Significant differences were determined by one-way ANOVA, followed by Tukey–Kramer test; ***p < 0.001.
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**Conflict of Interest** The authors declare no conflict of interest.

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