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A caffeic acid-ferulic acid hybrid compound attenuates lipopolysaccharide-mediated inflammation in BV2 and RAW264.7 cells

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ABSTRACT

In the present study, we synthesized and evaluated the anti-inflammatory effects of the two component hybrids, caffeic acid (CA)-ferulic acid (FA), FA-Tryptamine (Trm), CA-Piperonyl Triazol (PT) and FA-PT. Of these five hybrids, CA-FA had the most potent inhibitory effect on butyrylcholinesterase (BuChE) activity. The CA containing hybrids, CA-FA, CA-Trm, and CA-PT, dose-dependently inhibited LPS-induced nitric oxide (NO) generation in BV2 cells, whereas FA-PT, FA-Trm, CA, FA, Trm, and PT did not. Although CA-FA, CA-Trm and CA-PT had similar inhibitory effects on LPS-induced NO generation, CA-FA best protected BV2 cells from LPS-induced cell death. CA-FA, but not CA or FA, dose-dependently inhibited LPS-induced up-regulations of NO synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expressions in BV2 and RAW264.7 cells. Furthermore, CA-FA inhibited LPS-induced iNOS, COX-2, interleukin-6, and interleukin-1β mRNA expressions in BV2 cells. CA-FA also inhibited the LPS-induced phosphorylations of STAT3, Akt, and IκB and selectively inhibited LPS-induced NF-κB activation. Overall, our data suggest that CA-FA has BuChE inhibitory effects and down-regulates inflammatory responses by inhibiting NF-κB, which indicates CA-FA be viewed as a potential therapeutic agent for the treatment of inflammatory diseases of the peripheral system and central nervous systems.

1. Introduction

Polyphenols are universally distributed in foods (fruit, vegetables, and beverages) and in plants [1], and phenolic acids are one of the most abundant polyphenols in food and beverages. Phenolic acids are easily absorbed in the intestine, circulate in human plasma as conjugated forms (mainly as glucuronate and sulfate derivatives), and are well metabolized in man [2–5]. Notably, dietary phenolic acids have many therapeutic properties including antioxidant activity [6]. Caffeic acid (3,4-dihydroxycinnamic acid, CA) and ferulic acid (hydroxycinnamic acid, FA) are representative members of the naturally occurring hydroxycinnamic acid class of phenolic acids. CA has been extensively investigated in various physiological and pathological processes. For example, it has been shown to suppress bacterial lipopolysaccharide (LPS)-induced inflammation in primary bovine mammary epithelial cells and macrophage cells [7,8]. FA also has various biological effects, which include anti-oxidation and anti-inflammatory effects [9], and has been proposed as a potential therapeutic for free radical-induced diseases such as diabetes, cancers, Alzheimer's disease, and cardiovascular diseases [9–12]. Recent research interest in the therapeutic applications of polyphenols has resulted in a search for combinations of agents that are more effective and less toxic than their constituent parts at therapeutic doses.

Hybrid drugs, that is, drugs produced by covalently linking known existing pharmacophores, are being investigated with a view toward improving treatment efficiencies, though hybridization achieved using a metabolically stable linker might be expected to result in pharmacologically distinct entities rather than drugs displaying a combination of the pharmacologic properties of their

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constituent parts [13]. Hybridization can synergize, amplify, modify, or abrogate the effects of individual components [14]. We previously reported that a hybrid composed of covalently linked acetyl-protected CA and piperonyl piperazine (PP) inhibited LPS-induced inflammation by inhibiting NF-κB in RAW264.7 cells at concentrations substantially lower than those required for inhibition by CA or PP [15]. In the present study, our intension was to design anti-inflammatory CA containing hybrids. We found a CA-FA hybrid exhibited stronger anti-inflammatory effects than those expected based on the effects of CA or FA alone in the BV2 microglial and RAW264.7 macrophage cell-lines.

2. Materials and methods

2.1. Reagents

Except where otherwise noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Synthesis of hybrid compounds

CA-FA: Acetylated caffeic acid (ACA) and linker 2-(2-aminoethoxy) ethanol were coupled with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-Hydroxysuccinimide (NHS) to obtain linker-acetyl CA. Activated AcFA treated with SOCl2 was coupled with linker-acetyl CA in the presence of DMAP to give rise to CA-FA.

CA-Trm: ACA and propargyl amine were coupled with EDC and 4-Dimethylaminopyridine (DMAP) to obtain propargylated ACA. Tryptamine (Trm) azide was synthesized by converting the amine group of Trm to an azide group. The two intermediates, AC alkyne and Trm azide, were coupled using a click-reaction using a copper catalyst to synthesize a CA-Trm using triazole as a linker.

FA-Trm: Acetylated ferulic acid (FA) and propargyl amine were coupled with EDC and DMAP to obtain propargylated acetyl FA. The two intermediates, FA alkyne and Trm azide, were coupled by a click-reaction using a copper catalyst to synthesize a FA-Trm using a triazole as a linker.

CA-PT: Piperonyl azide was obtained by reacting piperonyl bromide with NaN_3, which was followed by a click reaction with propargylated ACA to obtain CA-PT.

FA-PT: Propargylated acetyl ferulic acid (FA) and piperonyl azide were coupled by a click-reaction using a copper catalyst to synthesize FA-PT using triazole as a linker.

2.3. Cholinesterase assay

The acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities of hybrid compounds were determined using the Ellman method as previously described [16].

2.4. Cell cultures

Murine BV2 microglial cells have been used as a model for *in vitro* studies of activated microglia cells. BV2 and RAW264.7 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Hyclone, Logan, Utah), streptomycin and penicillin in a 5% CO_2 atmosphere.

2.5. Cell viability

Cell viabilities were measured using an MTT assay, as described previously [15]. In brief, cells were prepared into the wells of 24-well plates. MTT solution (50 μl) was added, and cells were incubated in the dark for 4 h at 37 °C. The formazan crystals produced were then dissolved in a DMSO:EtOH (1:1 vol/vol) solution, and absorbances at 595 nm were read using a microplate reader. Results are expressed as percentages of untreated controls.

2.6. Nitrite measurement

Nitrite level, an index of NO production, was measured in cultured cell supernatants using the Griess method [15]. In brief, cells were stimulated with LPS (100 ng/ml) and/or control or hybrid compounds. Nitrite accumulations in culture media were measured by adding equal volumes of Griess reagent (1% sulphanilamide, 0.1% naphthylenediamine 5% phosphoric acid) and culture medium. Optical densities were measured at 550 nm (OD 550) using a microplate reader. A standard curve was generated using a concentration ladder of sodium nitrite (10–100 μM) in culture medium.

2.7. RT-PCR and quantitative real-time PCR

Total RNA from BV2 cells was extracted with TRIzol™ (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA using SuperScript II. PCR was performed using specific mouse primers as described below. Gene expressions values were expressed with respect to the housekeeping gene GAPDH. PCR was conducted in 15 μl of reaction mixture containing 1.5 mM magnesium chloride ($MgCl_2$), 250 μM deoxy-nucleoside triphosphate, 1.25 units Taq DNA polymerase, 10 pmol of primer, and 25 ng of DNA templates. PCR products were electrophoresed in 1% agarose gel in Tris/Borate/EDTA (TBE) buffer. Gels were observed and photographed under UV light. mRNA expressions were quantified by measuring the incorporation of fluorescent SYBR green into double-stranded DNA (iCycleriQ, Bio-Rad). Relative mRNA levels were calculated from sample PCR profiles using the threshold cycle (Ct) method. To correct for total cDNA differences, Ct values of endogenous controls (input DNA) were subtracted from those of samples.

PCR primers used in this study.

	Forward Primer	Reverse Primer
iNOS	ACTTCCGAGTGTGGAACTCG	TGGCTACTTCCTCCAGGATG
COX-2	GCTGTACAAGCAGTGGCAAA	GTCTGGAGTGGGAGGCACT
IL-1β	GGAGAAGCTGT GGCAGCTA	GCTGATGTACCAGTTGGGGA
IL-6	CCGGAGAGGAGACTTCACAG	TGGTCTTGGTCCTTAGCCAC
TNF-α	GACCCTCACACTCAGATCAT	TTGAAGAGAACCTGGGAGTA
GAPDH	TCATTGACCTCAACTACATGGT	CTAAG CAGTTGGTGGTGCAG

2.8. Immunoblotting

Total cell protein was prepared by lysing cells in buffer (10 mM Tris, 140 mM NaCl, 1% Triton, 0.5% SDS and protease inhibitors, pH 8.0). Prepared protein samples (20–40 μg) were separated by SDS-PAGE and transferred to Hybond™-ECL™ nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA), except antibodies against iNOS (BD Biosciences, San Jose, CA), COX-2 (Cayman Chemicals, Ann Arbor, Mi), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA), ERK1/2 (Cell Signaling Technology), *p*-JNK (Invitrogen), JNK (Cell Signaling Technology), p-P38 (Cell Signaling Technology). Membranes were incubated with antibodies overnight at 4 °C. After washing with TBST (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.6), membranes were incubated in a buffer containing HRP-conjugated secondary antibodies (1:10,000 dilution in TBST) for 1 h at room temperature.

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Protein bands were detected using an ECL detection reagent (Amersham Biosciences).

2.9. Streptavidin-agarose pull-down assay

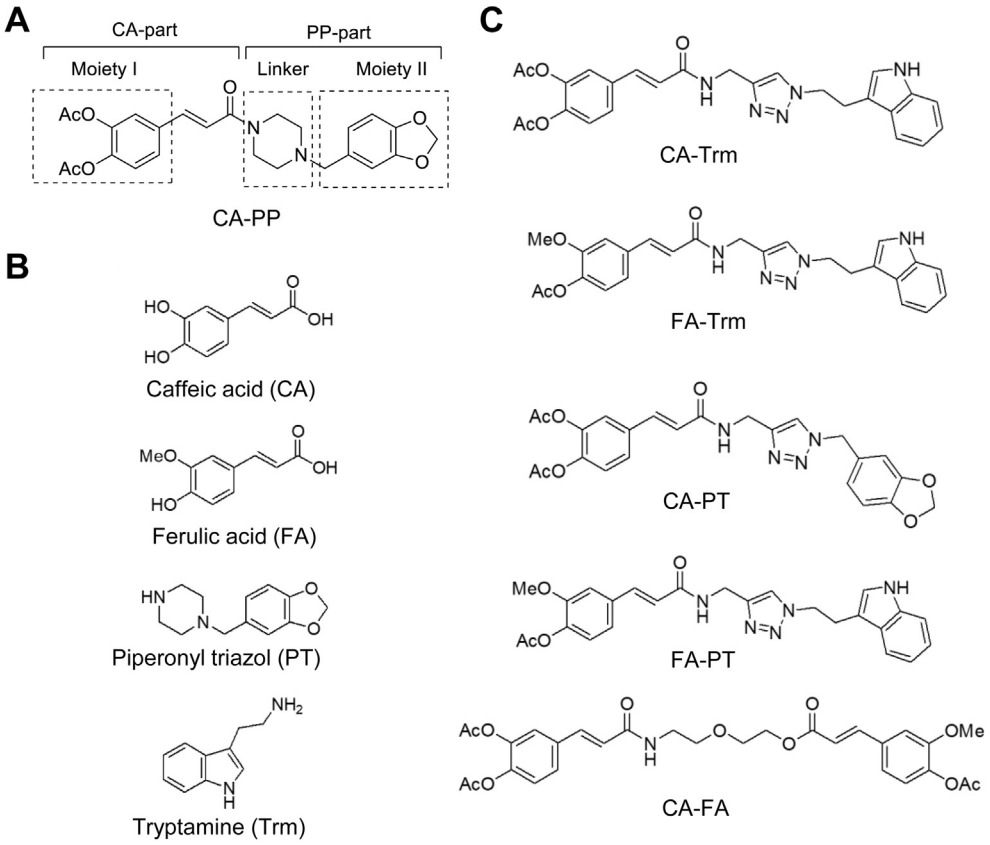
Biotin pull-down assays were performed as described previously, with minor modifications [17]. The procedure allows for quantitative binding of transactivators or molecules of interest to a specific probe, that is, a 20-nucleotide sequence containing the NF-κB binding site (5′-GCTAGGGGGATTTTCCCTCT-3′) at position −957/-977 of iNOS promoter. Two complementary DNAs were synthesized and biotinylated by Bioneer Corporation (Korea) and annealed to generate a double-stranded probe. Binding assays were performed by incubating 500 μg of nuclear protein extracts with 2 μg of biotinylated DNA probe and 25 μl of streptavidin-conjugated agarose beads for 1 h. DNA-protein complexes were analyzed by Western blotting using the indicated antibodies.

2.10. Transfection and the NF-κB reporter gene assay

NF-κB reporter (Clontech, Mountain View, CA) contained three copies of the κB-binding sequence fused to the firefly luciferase gene. Briefly, RAW 264.7 cells in 12-well plates (5 x 10^5/well) were transfected with Lipofectamine Plus. Luciferase activity was assayed 24 h after transfection. Transfection efficiencies were normalized by cotransfected β-galactosidase plasmid. Bioluminescence was measured using a luminometer (TD-20/20).

2.11. Electrophoretic mobility shift assay (EMSA

Nuclear protein extracts were prepared as described previously [15] A double-stranded DNA oligonucleotide probe containing the consensus NF-κB binding site (Promega, Madison, WI) was labeled by polynucleotide kinase (New England Biolabs, Beverly, MA) and purified using Sephadex G-25 spin columns. Aliquots of nuclear



D

Compounds	IC_{50} for AChE (μM)	IC_{50} for BuChE (μM)
HBU-47	>400	>400
CA-FA	>400	10.4±0.9
CA-Trm	>450	>450
FA-Trm	>450	41.79 ±1.30
CA-PT	>450	>300
FA-PT	>450	69.6±1.04
galantamine	0.64±0.1	8.4±0.1

Fig. 1. The chemical structures and BuChE activities of CA-Trm, FA-Trm, CA-PT, FA-PT, and CA-FA. The structures of CA-PP (A), CA, FA, PT, and Trm (B) are shown. Hybrid compounds, CA-Trm, FA-Trm, CA-PT and CA-FA were synthesized from CA, FA, PT or Trm as described in Materials and Methods (C). The AChE and BuChE inhibitory activities of hybrids were measured (D).

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proteins (20 μg) were incubated with labeled oligonucleotides in binding buffer (50 mM KCl, 12.5 mM HEPES pH 7.6, 6.25 mM $MgCl_2$, 0.05 mM EDTA, 0.5% Nonidet P-40, 0.5 mM DTT, 5% glycerol and 2 μg poly-[dI-dC]) for 30 min on ice. Samples were then electrophoresed on 4.5% polyacrylamide gel containing 2% glycerol at 200 V for 1.5 h at room temperature. Gels were then dried and exposed to x-ray film (Kodak, Rochester, NY) using an intensifying screen at −80 °C.

2.12. Statistical analysis

The analysis was conducted using the unpaired Student's *t*-test. Results are presented as the means±SDs of three separate experiments. Statistical significance was accepted for p values < 0.05.

3. Results

3.1. Acetylcholinesterase (AChE) and butyrylcholinesterase (buChE) activities of CA-FA, CA-Trm, CA-PT, FA-PT, and FA-Trm

We previously showed that the caffeic acid (CA) and 1-piperonylpiperazine (PP) (CA-PP) hybrid displays anti-inflammatory effects in RAW264.7 macrophage cells (Fig. 1A) [15]. We generated derivatives of CA-PP using different combinations of CA and ferulic acid (FA), tryptamine (Trm), or piperonyl triazole (PT) (Fig. 1B). CA-PT was produced by changing the piperazine of the linker in CA-PP to triazole (linker modification); FA-PT was produced by converting CA to FA in CA-PT (the moiety I modification of CA-PT in Fig. 1A); CA-Trm was synthesized by converting the piperonyl structure in CA-PT to Trm (moiety II modification of CA-PP); FA-Trm was generated by replacing CA to FA in CA-Trm (moiety I modification of CA-PP); and CA-FA was produced by linking CA and FA using 2-(2-aminoethoxy) ethanol as a linker (Fig. 1C).

Since many phenolic compounds and their derivatives have cholinesterase inhibitory effects [18], we measured AChE and BuChE activities of hybrid compounds and compared these with those of galantamin (a well-described cholinesterase inhibitor) [19] (Fig. 1D). FA-containing hybrid molecules, that is, CA-FA, FA-Trm, and FA-PT all demonstrated BuChE inhibition but CA-FA was the most effective (IC50 = 10.4 + 0.9 μM for BuChE).

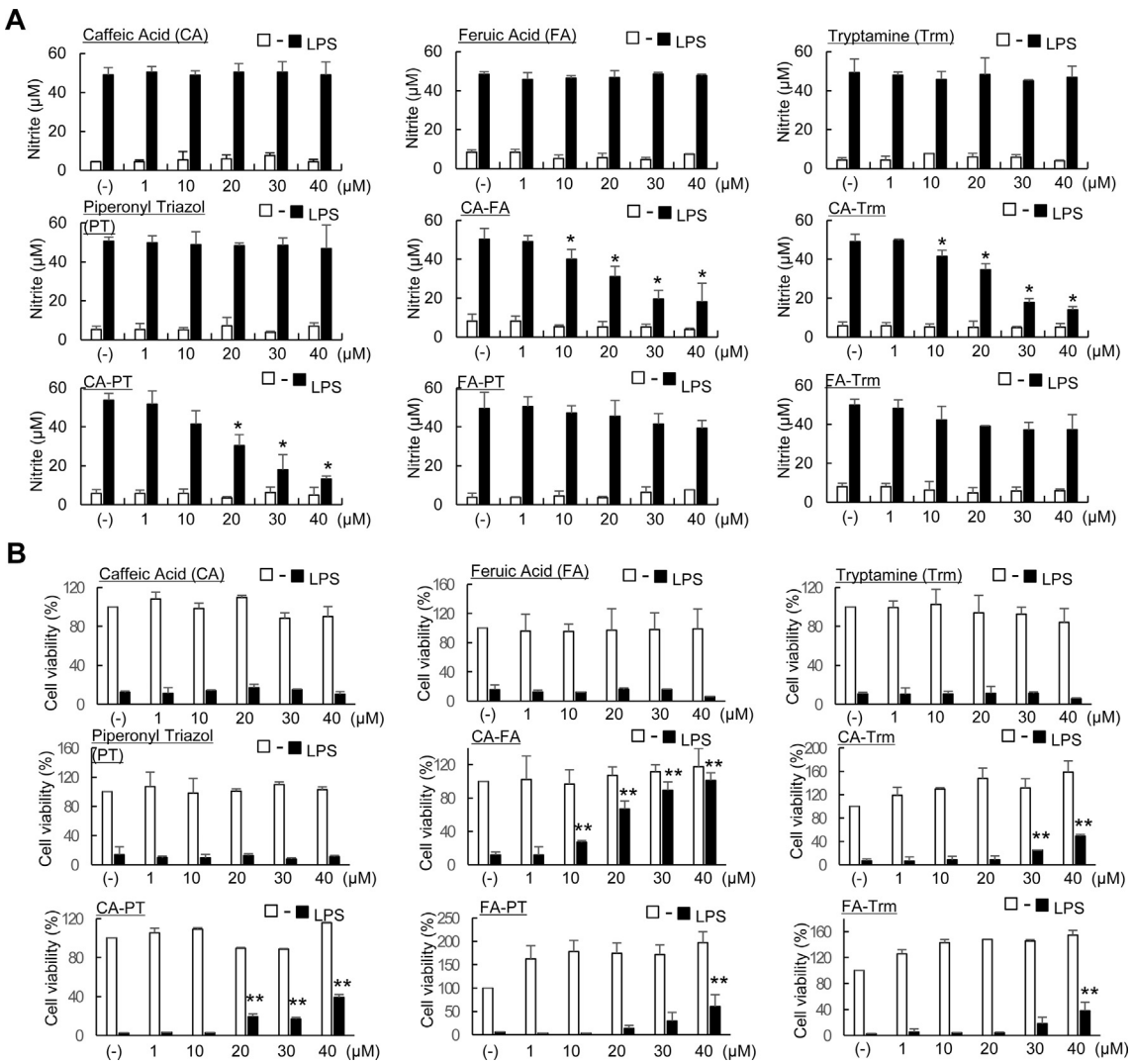

Fig. 2. LPS-induced nitrite production and cytotoxicities of CA-Trm, FA-Trm, CA-PT, FA-PT, and CA-FA and of their component compounds in BV2 cells. BV2 cells were pre-treated with the indicated concentrations of CA, FA, PT, Trm, CA-Trm, FA-Trm, CA-PT, or CA-FA for 2 h and then stimulated with 0.1 μg/ml LPS. Nitrite levels in the culture medium were measured after 24 h of LPS treatment (A). Cell viabilities were determined using MTT assays, and results are expressed as percentages versus untreated controls (B). *represents significantly different from the untreated control. **represents significantly different from LPS-treated BV2 cells.

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3.2. The effects of CA, FA, Trm, PT and their hybrids on LPS-induced NO generation and cytotoxicity in BV2 cells

We compared the effects of hybrid compounds on LPS-induced nitrite production in BV2 cells. Cells were treated with the indicated concentrations of hybrids or control compounds, that is, CA, FA, Trm, or PT, for 2 h prior to LPS (100 ng/ml) stimulation for 24 h. LPS-induced nitrite production was dose-dependently suppressed by CA containing hybrids (CA-FA, CA-Trm, and CA-PT) but not by CA, FA, Trm, PT, FA-PT or FA-Trm (Fig. 2A). CA-FA, CA-Trm, and CA-PT at 40 μM also suppressed LPS-induced NO production in RAW264.7 cells (data not shown).

The cytoprotective effects of hybrids in the presence of LPS were examined in BV2 cells. Treatments with hybrids or control compounds did not significantly affect cell viability or proliferation at the all concentrations tested, excepting FA-PT and FA-Trm, which increased cell proliferation (Fig. 2B). LPS caused cell death after activation and CA-FA strongly and dose-dependently suppressed LPS-induced BV2 cell death (Fig. 2B). CA-Trm and CA-PP minimally suppressed LPS-induced cell death at concentrations of 30 and 40 μM and 20, 30, and 40 μM, respectively.

3.3. CA-FA inhibited the upregulation of pro-inflammatory molecules at the protein and mRNA levels

BV2 or RAW264.7 cells were treated with indicated concentrations of CA-FA, CA, or FA for 2 h prior to LPS stimulation (100 ng/mL). LPS-induced the protein expressions of iNOS and COX-2 in 24 h and these increases were dose-dependently suppressed by CA-FA in BV2 cells, whereas in RAW 264.7 cells, CA-FA inhibited LPS-induced iNOS expression but not COX-2 expression (Fig. 3A). At the same concentrations, CA and FA failed to inhibit the LPS-induced up-regulations of iNOS and COX-2 in BV2 and RAW264.7 cells.

Morphological examinations conducted under an optical microscope indicated that LPS induced BV2 cell death in 24 h. CA-FA pretreatment at 30 μM significantly protected against LPS-induced cell death (Fig. 3B), but CA or FA failed to do so. Furthermore, LPS increased the mRNA expressions of iNOS, COX-2, IL-6, IL-1β, and TNF-α in 6 and 24 h in BV2 cells and CA-FA, but not CA or FA, inhibited the LPS-induced expressions of iNOS, COX-2, IL-6, and IL-1β at 6 and 24 h (Fig. 3C and D).

3.4. CA-FA suppressed the LPS-induced phosphorylations of STAT3, Akt, and IκBα and NF-κB activation.

We next examined the effect of CA-FA on the activations of MAPKs, STAT3, and Akt, that is, the signaling mediators involved in the regulation of pro-inflammatory molecules in response to LPS, in RAW264.7 cells. Neither CA-FA, CA, nor FA influenced the LPS-induced phosphorylations of ERK1/2, p38, or JNK after 30 min, 2 h, or 6 h of LPS-stimulation (Fig. 4A). However, CA-FA, but not CA or FA, significantly inhibited the LPS-induced phosphorylations of STAT3 and Akt at 2 h and 6 h, respectively (Fig. 4A). LPS-induced IκBα degradation was not significantly affected by CA-FA, CA, or FA, whereas CA-FA significantly inhibited LPS-induced increases in IκBα phosphorylation (Fig. 4B). In addition, the LPS-mediated activation of NF-κB luciferase reporter activity was markedly suppressed by CA-FA but not by CA or FA (Fig. 4C). The inhibitory

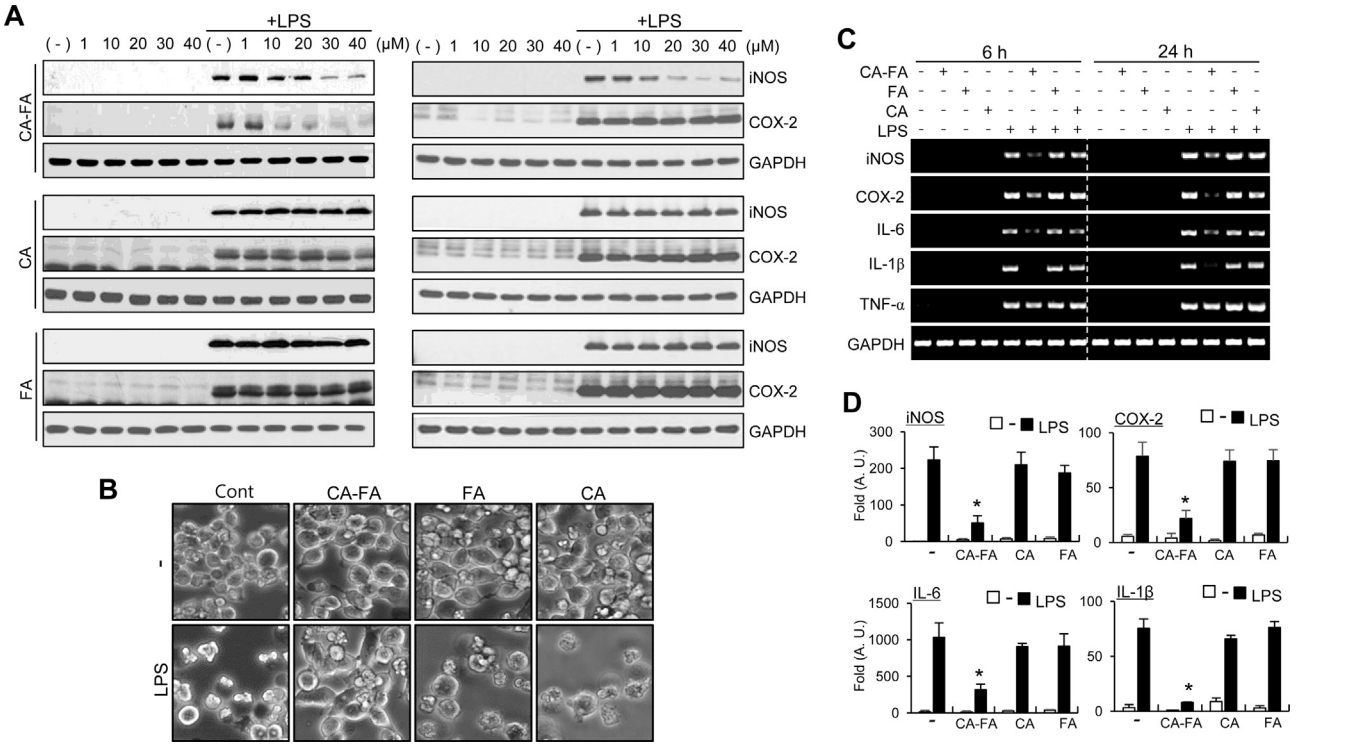

Fig. 3. Inhibition of the LPS-induced inductions pro-inflammatory molecules by CA-FA. (A) BV2 (left panel) or RAW264.7 (right panel) cells were pre-treated with the indicated concentrations of CA-FA, CA, or FA for 2 h and then treated with 0.1 μg/mL LPS for 24 h iNOS, COX-2 and GAPDH protein levels were measured by Western blotting. (B ~ D) BV2 cells were pre-treated with 30 μM of CA-FA, CA, or FA for 2 h and then stimulated with 0.1 μg/ml LPS. Phenotypes of control, CA-FA, CA, or FA treated cells, treatment with or without LPS were observed under a phase-contrast microscope after 24 h of LPS treatment (B). Total RNA was prepared at 24h, and the mRNA levels of iNOS, COX-2, IL-6, TNF-α, and IL-1β were determined by RT-PCR. GAPDH mRNA served as a control (C). mRNA levels of iNOS, COX-2, IL-1β, and IL-6 were measured by quantitative real-time PCR (D). *represents significantly reduced vs. LPS-treated cells. Western blots and RT-PCR results are representative of 3 independent experiments.

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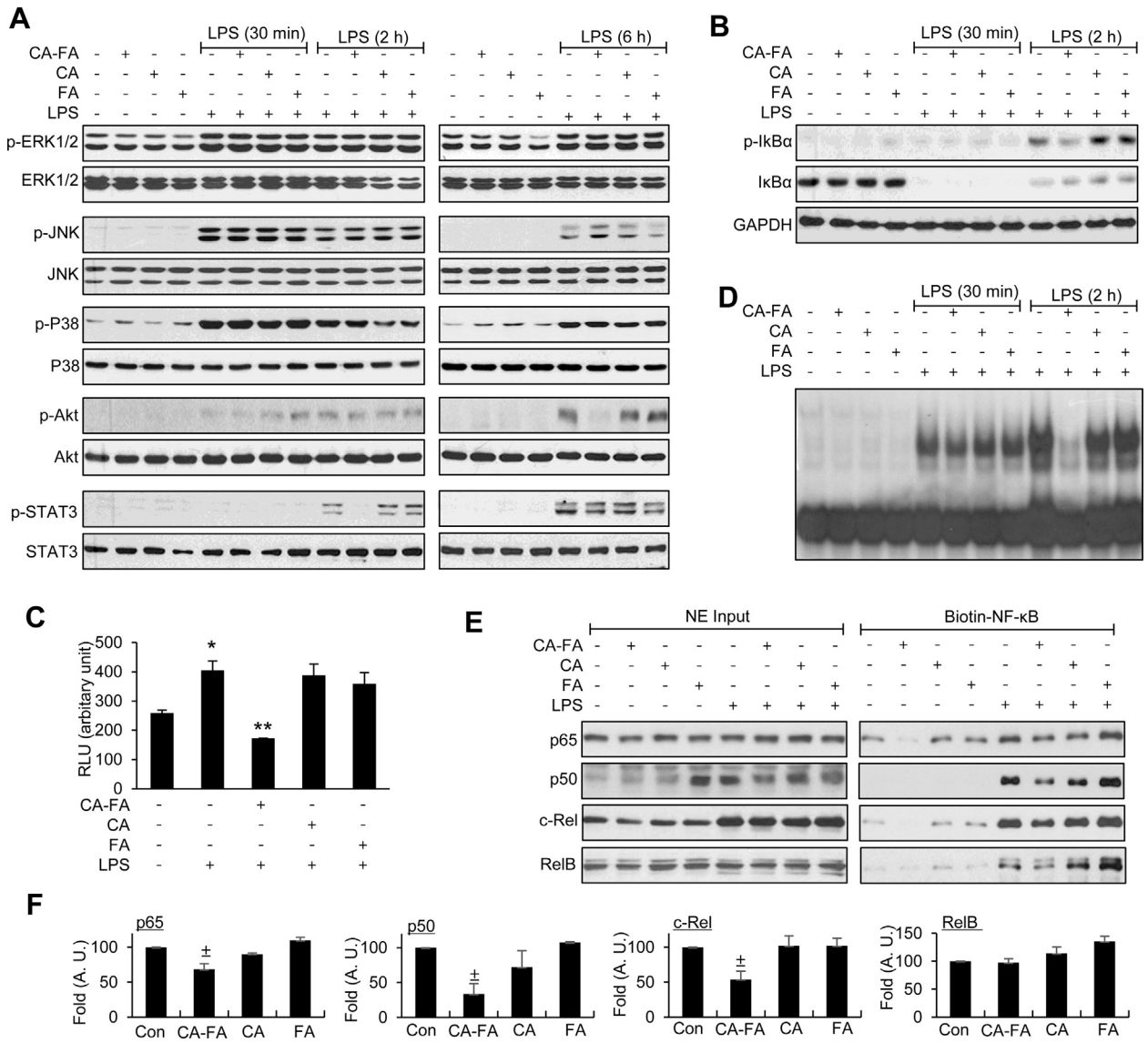

Fig. 4. Inhibitions of LPS-induced Akt and STAT3 phosphorylations and NF-κB activation by CA-FA. (A) RAW264.7 cells were pre-treated with 30 μM of CA-FA, CA, or FA for 2 h and then stimulated with 0.1 μg/ml LPS. Total cell lysates were prepared after 30 min, 2 h, or 6 h of LPS treatment, and levels of unphosphorylated- and phosphorylated-ERK1/2, -p38, -JNK, Akt, -STAT3 were evaluated by Western blotting using specific antibodies. (B) RAW264.7 cells were pre-treated with 30 μM of CA-FA, CA, or FA and stimulated with 0.1 μg/ml LPS. Phospho-IκBα and IκBα levels were assessed by Western blotting after 30 min or 2 h of LPS treatment. (C) RAW264.7 cells were transfected with a NF-κB-luciferase reporter plasmid, treated with CA-FA, CA, or FA (30 μM) and/or LPS (0.1 mg/ml), and luciferase activities (relative light units) were measured 24 h of LPS treatment. (D ~ F) RAW264.7 cells were pre-treated with CA-FA, CA, or FA for 2 h and then with 0.1 μg/mL LPS. Nuclear extracts were prepared after 30 min or 2h of LPS treatment and DNA binding to a ^{32}P-labeled NF-κB probe was measured using an electrophoretic mobility shift assay (D). Nuclear extracts were prepared after 24 h of LPS treatment. The binding of proteins to the iNOS promoter-derived, biotinylated NF-κB probe was assessed using streptavidin-agarose pull-down assays, and this was followed by Western blotting for p65, c-Rel, p50, and RelB (E). Graphs represent densitometric quantification of p65, c-Rel, p50, and RelB (F). Nuclear extract (NE) input represents 2% of nuclear extract used for biotin–NF–κB pull-down assay. Experiments are representative of 3 independent experiments. *denotes significantly different from untreated controls. **represents significantly reduced as compared with LPS-treated cells.±denotes significantly decreased from untreated controls.

regulation of NF-κB by CA-FA was further examined using electrophoretic mobility shift assays (EMSAs), and CA-FA, but not CA or FA, was found to significantly reduce the LPS-induced DNA binding of NF-κB (Fig. 4D). Mouse iNOS promoter contains two putative NF-κB binding sites, and the distal binding site (−957/-977) is important for LPS response. Thus, we measured the binding activities of NF-κB/Rel family proteins (p65, p50, c-Rel and RelB) to a biotinylated oligonucleotide corresponding to the distal NF-κB site of iNOS promoter. LPS was found to increase the DNA bindings of p65, p50, c-Rel, and RelB after 6 h of treatment, and CA-FA inhibited the DNA bindings of p65, p50, and c-Rel, whereas CA or FA had no effect (Fig. 4E and F).

4. Discussion

Covalently joined chemical entities often display new or more potent activities than their components [20]. In this study, we compared the effects of CA-FA, CA-Trm, CA-PT, FA-PT, or FA-Trm hybrids on the inflammatory responses of BV2 microglial and RAW264.7 macrophage cells. CA-FA, CA-Trm, and CA-PT potently inhibited LPS-induced NO generation in BV2 cells whereas at the same concentration CA was ineffective, though CA has been shown to inhibit the LPS-induced activation of NF-κB in primary bovine mammary epithelial cells [7]. In the present study, CA (at ≤ 100 μM) had no inhibitory effect on LPS-induced NO generation in these two

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cell lines (data not shown). In our previous work, an acetyl-CA (ACA) and 1-piperonylpiperazine (PP) hybrid had an anti-inflammatory effect in RAW264.7 cells, and the same concentrations ACA and PP were ineffective. However, CA-PP was cytotoxic at $\geq$ 30 μM in BV2 and RAW264.7 cells [15]. The present study was undertaken to design more potent combinations of nontoxic anti-inflammatory CA hybrids effective in microglia and macrophages. Although CA-Trm and CA-PT were similar to CA-FA in terms of LPS-induced NO generation, they were either less effective or ineffective at protecting BV2 cells from LPS-induced cell death. We and others have previously shown over-activation by LPS causes the apoptosis of microglia and macrophages [21–23]. Although NO is known to be a major mediator of apoptosis in activated microglia, various other inflammatory molecules contribute to microglial activation-induced death [21]. Therefore, we found CA-FA offers greater promise as an anti-inflammatory than CA-Trm of CA-PT. Furthermore, since CA and FA are often found in coffee, fruit, and vegetables, and both have antioxidant and anti-inflammatory effects, the therapeutic effect of CA-FA is potentially close to those of natural plants. The ability to CA-FA to inhibit BuChE may be important, as plasma and/or tissue levels and the activities of BuChE and AChE are closely associated with systemic inflammation and because cholinesterase inhibitors have been proposed to be potential anti-inflammatories [24,25]. However, our results show that the abilities of hybrid compounds to inhibit BuChE and their anti-inflammatory effects are not necessarily correlated. Although the functional effects of BuChE inhibition by CA-FA remain to be elucidated, our findings suggest that CA-FA has potential use for the treatment of neurodegeneration because it inhibits inflammation and suppresses BuChE activity.

MAPKs and NF-κB are important regulators of inflammatory genes, including iNOS and COX-2 [26]. In the present study, CA-FA suppressed LPS-induced NF-κB activation but not LPS-induced MAPK activation, which suggests that the inhibition of NF-κB activity by CA-FA is independent of MAPKs. Instead, CA-FA inhibited the LPS-induced activations of Akt and STAT3. The Akt pathway is actively involved in the regulation of NF-κB via the phosphorylations of IKKα and IκB [27], and we observed CA-FA inhibited LPS-induced IκB phosphorylation. For functional crosstalk between STAT3 and NF-κB, CA-FA may inhibit STAT3 to regulate the NF-κB pathway or CA-FA may suppress STAT3 activation by inhibiting NF-κB. The results we obtained in a previous study indicated STAT3 is a downstream target of NF-κB and regulates iNOS transcription in RAW264.7 cells [15], which suggests CA-FA inhibited STAT3 by suppressing NF-κB in BV2 or RAW264.7 cells.

In summary, the present study demonstrates a two-component hybrid of the well-described phenolic acids CA and FA, which are commonly found in fruits, vegetables, and beverages, acts as a BuChE inhibitor and anti-inflammatory in our LPS-induced BV2 and RAW264.7 cell models of inflammation. We propose that CA-FA may be further investigated as a potential treatment with minimal side effects for neurodegenerative and other inflammation-related diseases.

Declaration of interests

The authors declare no competing interests.

Conflicts of interest

The authors have no conflict of interest to declare.

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