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Research article

Eupatolide, isolated from *Liriodendron tulipifera,* sensitizes TNF-mediated dual modes of apoptosis and necroptosis by disrupting RIPK1 ubiquitination

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ABSTRACT

Ubiquitination of RIPK1 plays an essential role in the recruitment of the IKK complex, an upstream component of pro-survival NF-κB. It also limits TNF-induced programmed cell death by inhibiting the spatial transition from TNFR1-associated complex-I to RIPK1-dependent death-inducing complex-II or necrosome. Thus, the targeted disruption of RIPK1 ubiquitination, which induces RIPK1-dependent cell death, has proven to be a useful strategy for improving the therapeutic efficacy of TNF. In this study, we found that eupatolide, isolated from *Liriodendron tulipifera,* is a potent activator of the cytotoxic potential of RIPK1 by disrupting the ubiquitination of RIPK1 upon TNFR1 ligation. Analysis of events upstream of NF-κB signaling revealed that eupatolide inhibited IKKβ-mediated NF-κB activation while having no effect on IKKα-mediated non-canonical NF-κB activation. Pretreatment with eupatolide drastically interfered with RIPK1 recruitment to the TNFR1 complex-I by disrupting RIPK1 ubiquitination. Moreover, eupatolide was sufficient to upregulate the activation of RIPK1, facilitating the TNF-mediated dual modes of apoptosis and necroptosis. Thus, we propose a novel mechanism by which eupatolide activates the cytotoxic potential of RIPK1 at the TNFR1 level and provides a promising anti-cancer therapeutic approach to overcome TNF resistance.

1. Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine that plays an important role in diverse cellular events, including inflammation, cell proliferation and programmed cell death (PCD), in the form of apoptosis and necroptosis, depending on the cellular context [1–3]. TNF receptor 1 (TNFR1) ligation induces the formation of the proximal TNFR1 signaling complex, called complex-I, by recruiting TNFR1-associated death domain protein (TRADD) and receptor-interacting protein kinase 1 (RIPK1) [4–6]. RIPK1

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subsequently undergoes ubiquitin (Ub) modification by recruiting several E3 Ub ligases, including TNF receptor-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis proteins. (cIAPs) [7–9]. The ubiquitination of RIPK1 plays a key role in regulating the dynamic assembly of complex-I with the nuclear factor-kappa B (NF-κB) upstream kinase, known as the IκB kinase (IKK) complex. This assembly drives the transcriptional induction of NF-κB-mediated anti-apoptotic genes such as B-cell lymphoma-2, A20, and cellular FLICE-inhibitory proteins, which promote cell survival [10–12]. Alternatively, de-ubiquitination of RIPK1 leads to its dissociation from the plasma membrane-bound complex-I and promotes the formation of a pro-apoptotic cytoplasmic complex, referred to as complex-II, by associating with Fas-associated death domain protein (FADD) and caspase-8, ultimately resulting in the activation of downstream caspases and ulminating in cell death through apoptosis [13,14]. In the absence of apoptosis, deubiquitinated RIPK1 interacts with RIPK3 and mixed lineage kinase domain-like protein (MLKL) to form a necrosome (also referred to as the complex-IIb), which triggers necroptosis as an alternative mode of PCD [15–18]. Thus, it is now believed that the ubiquitination status of RIPK1 plays an essential role in determining the cytotoxic potential of TNF, regulating the dual modes of apoptosis and necroptosis. Furthermore, upstream kinases of NF-κB, such as IKKs and transforming growth factor β activated kinase (TAK1) have been reported to phosphorylate ubiquitinated RIPK1 in complex-1, suppressing RIPK1-dependent PCD by preventing the assembly of complex-II [19–24]. Thus, the development of bioactive compounds that target RIPK1 ubiquitination represents a novel strategy to enhance the therapeutic efficacy of TNF in cancer biology.

Sesquiterpene lactones comprise many active plant constituents that exhibit biological properties, including anti-inflammatory and anticancer effects [25–27]. In a previous phytochemical study conducted in *Liriodendron tulipifera (L. tulipifera*), we isolated several types of anti-NF-κB compounds, including aporphine alkaloids, sesquiterpenoids and flavonoids [28]. As part of our ongoing search for bioactive compounds that can modulate RIPK1 ubiquitination upstream of IKKβ, we identified that eupatolide, a sesquiterpene lactone isolated from *L. tulipifera,* interferes with TNF-induced RIPK1 ubiquitination in complex-I, thereby inhibiting the canonical IKK/NF-κB signaling pathway. We found that eupatolide induces an RIPK1-dependent dual mode of apoptosis and necroptosis upon TNFR1 ligation. Such properties of eupatolide may not only increase the therapeutic efficacy of TNF but also provide an effective strategy to overcome apoptosis resistance by shifting the fate of cells from apoptosis to necroptosis.

2. Materials and methods

2.1. Chemicals and antibodies

All commercial antibodies were purchased from the following resources: anti-phospho-p65 Ser (#3033), anti-phospho-RIPK1 Ser (#65746, #31122), anti-phospho-IKKα/β (#2697), anti-caspase-8 (#9746), anti-caspase-3 (#9662), anti-RIPK3 (#13526), anti-IκBα (#9242), anti-phospho-MLKL (#37333) antibodies were from Cell Signaling Technology (Danvers, MA, USA); anti-NF-κB p52/p100 (sc-7386), anti-TRAF2 (sc-7346), anti-IKKγ (sc-8330), anti-p65 (sc-8008) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-actin (A2228) antibody, doxorubicin(44583), etoposide (341205), cycloheximide (C7698) were from Sigma-Aldrich (St, Louis, MO, USA); anti-RIPK1 (610459), anti-PARP (556362) antibodies were from BD Biosciences Pharmingen (San Diego, CA, USA); anti-IKKβ (05–535) antibodies were from EMD Millipore (Burlington, MA, USA); anti-phospho-MLKL (ab187091), anti-MLKL (ab184718), anti-TNFR1 (ab19139), antibodies were from Abcam (Cambridge, MA, USA); anti-caspase-8 (ALX-804-447) antibody and SuperFasLigand (ALX-522-010-3055) were from Enzo Life Science (Ann Arbor, MI, USA); recombinant TNF-α (410-MT), LIGHT (664-LI), TWEAK (1090-TW-025) were from R&D system (Minneapolis, MN, USA); pan-caspase inhibitor (z-VAD-fmk) (218759), necrostatin-1 (Nec-1) (480065), were from Calbiochem (San Diego, CA, USA); Protein G-Sepharose (17061801) was purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA).

2.2. Extraction of eupatolide from L. tulipifera

In previous chemical investigation of *L. tulipifera*, we extracted and isolated eupatolide, a sesquiterpene lactone [28]. In brief, the fraction LT2-3 (481.5 mg) was separated using prep HPLC (a gradient of acetonitrile:water from 40:60 to 100:0 over 60 min) with a C18 column (Hector, 250 × 21 mm) to yield eupatolide (24.2 mg, $t_R = 51.9$ min). Eupatolide: white crystalline powder; $[\alpha]_D^{22}$ +69.5 ($c = 0.10$, MeOH); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.14 (1H d, $J = 3.40$ Hz, H-13a), 5.68 (1H, d, $J = 3.40$ Hz, H-13b), 5.13 (1H, dd, $J =$ 10.00, 7.86 Hz, H-6), 5.06 (1H, d, $J = 4.22$ Hz, -OH), 4.82 (1H, d, $J = 10.00$ Hz, H-5), 4.76 (1H, dd, $J = 11.69$, 3.12 Hz, H-1), 4.47 (1H, m, H-8), 2.87 (1H, dd, $J = 7.86$, 3.56 Hz, H-7), 2.47 (1H, overlapped, H-9a), 2.32 (1H, dd, $J = 11.69$, 5.17 Hz, H-2a), 2.26 (1H, m, H-3b), 2.20 (1H, dd, $J = 13.37$, 2.18 Hz, H-9b), 2.10 (1H, m, H-2b), 2.00 (1H, m, H-3a), 1.63 (3H, s, H_3-14), 1.57 (3H, s, H_3-15); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 169.9 (C-12), 140.8 (C-4), 139.0 (C-11), 136.1 (C-1), 128.2 (C-5), 127.8 (C-10), 120.1 (C-13), 74.8 (C-6), 70.4 (C-8), 52.7 (C-7), 47.1 (C-9), 38.8 (C-3), 25.7 (C-2), 19.5 (C-15), 17.0 (C-14); EIMS m/z 248 $[M]^+$ (Supplementary Fig. S1).

2.3. Structure determination of eupatolide

Eupatolide was determined by the analysis of NMR and MS spectroscopic data. Inspection of the 1D NMR spectra revealed characteristic signals for a sesquiterpene lactone. The presence of the α-methylene-γ-lactone group was confirmed by the ^{1}H NMR resonances at δ 6.14 (1H, d, $J = 3.40$ Hz), 5.68 (1H, d, $J = 3.40$ Hz) and ^{13}C NMR resonances at δ 169.9, 139.0, 120.1. Additionally, two olefin protons at δ 4.82 (1H, d, $J = 10.00$ Hz), 4.76 (1H, dd, $J = 11.69$, 3.12 Hz), two oxygenated methines at 5.13 (1H, dd, $J = 10.00$, 7.86 Hz), 4.47 (1H, m), one methine at δ 2.87 (1H, dd, $J = 7.86$, 3.56 Hz), three methylenes at δ 2.47–2.00 (6H), and two methyls at δ

1.63 (3H, s), 1.57 (3H, s) were observed (Supplementary Fig. S2). On the basis of the NMR spectroscopic data analysis and the comparison of the data with those previously published [29], the structure was identified as eupatolide (Supplementary Fig. S3).

2.4. Cell culture

Wild-type (WT) and RIPK1 deficient (RIPK1 knockout (KO)) mouse embryonic fibroblasts (MEFs) were kindly donated by Dr. Zheng-gang Liu (National Institute of Health, Bethesda, MD, USA). A human cervical cancer epithelial cell line, HeLa were purchased from the American Type Culture Collection (Bethesda, MD, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and antibiotics (100 U/mL penicillin and 100 μg/ml streptomycin), and maintained in a humidified incubator at 37 °C in 5% CO2 atmosphere.

2.5. Transfection and luciferase reporter assay

WT MEFs (0.3×10^6 cells/well) were seeded in a 6-well plate and transfected with the plasmids (1 μg of p2xNF-κB-Luc and 0.2 of μg pRSV-β-galactosidase) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen Life Technologies, CA, USA). After 24 h, cells were pretreated with eupatolide (0, 2.5, 5, 10, and 20 μM in dimethyl sulfoxide (DMSO)) for 30 min and then treated with TNF (15 ng/mL in phosphate-buffered saline (PBS)) for 10 h. Luciferase activities were measured using a luciferase assay kit (Promega, Madison, CA, USA). Briefly, cells were harvested and lysed in 100 μL of lysis reagent. The cell lysate (20 μL) was mixed with 100 μL of luciferin, and luminescence was measured using a microplate reader. NF-κB luciferase activity was normalized relative to the β-galactosidase activity of each sample.

2.6. Confocal imaging analysis

WT MEFs (0.3×10^6 cells/well) were seeded in chamber slides for 1 d before the experiment. Cells were pretreated with either 0.001% DMSO or eupatolide (10 μM) for 30 min and then treated with TNF (15 ng/mL) for 1 h. Cells were washed in cold PBS and permeabilized with 0.2% Triton X-100 for 10 min. Subsequently, cells were incubated with rabbit anti-p65 antibody at a dilution 1:400 in blocking buffer (PBS supplemented with 1% bovine serum albumin) for 1 h. After washing, the cells were incubated with a phycoerythrin-conjugated secondary anti-goat antibody (1:400 dilution) for 30 min. The slides were embedded in Fluoromount-G and assessed using fluorescence microscopy (EVOS M5000, Thermo Fisher Scientific, USA).

2.7. Cell viability assay

WT and RIPK1 KO MEFs (0.3×10^6 cells/well) were seeded in a 6-well plate and cultured in DMEM before treatment. For assessment of the sensitizing effect of eupatolide on death receptor- or genotoxic drug-mediated apoptotic cell death, WT MEFs were pretreated with either 0.001% DMSO or eupatolide (10 μM) for 30 min and then treated with TNF (15 ng/mL), Fas L (100 ng/mL), etoposide (20 μM), and doxorubicin (5 μM) for 24 h. Furthermore, for assessing the sensitizing effect of eupatolide on the RIPK1-dependent necroptosis, WT and RIPK1 KO MEFs were pretreated with either 0.001% DMSO or necrostatin-1 (Nec-1; 50 μM) for 30 min, and then treated with TNF (15 ng/mL) or in combination with eupatolide (10 μM) and z-VAD-fmk (z-VAD; 20 μM) for 24 h. Cell viability assay was conducted using Cell Titer-glo Luminescent Cell Viability Assay kit (Promega Co. USA), which measures cell viability based on adenosine triphosphate levels present in live cells. Luminescence measurements were performed at excitation/emission wavelengths of 560/590 nm using a microplate reader (Infinite 200pro, Tecan, Switzerland). Images were captured using an inverted microscope (Nikon, TE200, Japan).

2.8. Immunoblot analysis and immunoprecipitation

To evaluate the effect of eupatolide on the canonical and non-canonical NF-κB activation, WT MEFs and HeLa cells were pretreated with either 0.001% DMSO or eupatolide (10 μM) for 30 min and then treated with TNF (15 ng/mL) for up to 30 min, TWEAK (100 ng/mL) up to 4 h, and LIGHT (50 ng/mL) for up to 9 h. To evaluate the effect of eupatolide on the recruitment of ubiquitinated RIPK1 into TNFR1, WT MEFs were pretreated with either 0.001% DMSO or eupatolide (10 μM) for 30 min and then treated with TNF (15 ng/ml) for either 5 min or 10 min. To evaluate the impact of eupatolide on the RIPK1-dependent caspase activation, WT MEFs were treated with eupatolide (10 μM) and TNF (15 ng/mL) for up to 36 h. Subsequently, WT and RIPK1 KO MEFs were pretreated with Nec-1 (50 μM) for 30 min, followed by TNF (15 ng/mL) in combination with eupatolide (10 μM), Smac Mimetic-164 (100 nM), and cycloheximide (10 μg/mL) for 24 h. To evaluate the impact of eupatolide on the RIPK1-dependent RIPK3 and MLKL phosphorylation, WT and RIPK1 KO MEFs were pretreated with Nec-1 (50 μM) for 30 min and then treated with TNF (15 ng/mL) in combination with eupatolide (10 μM) and z-VAD (20 μM) for up to 6 h. To analyze the necrosome formation, WT MEFs were treated with TNF (15 ng/mL) in combination with eupatolide (10 μM) and z-VAD (20 μM) for either 4 or 6 h. After treatment, the cells were collected and lysed in M2 buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate and 1 μg/mL leupeptin). Fifty micrograms of cell lysates were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoprecipitation, lysates were mixed and incubated with an antibody and protein A-agarose or G-sepharose beads overnight at 4 °C. The beads were washed three times with M2 buffer, and the bound proteins were resolved using SDS-PAGE for immunoblotting. The blots were visualized using enhanced

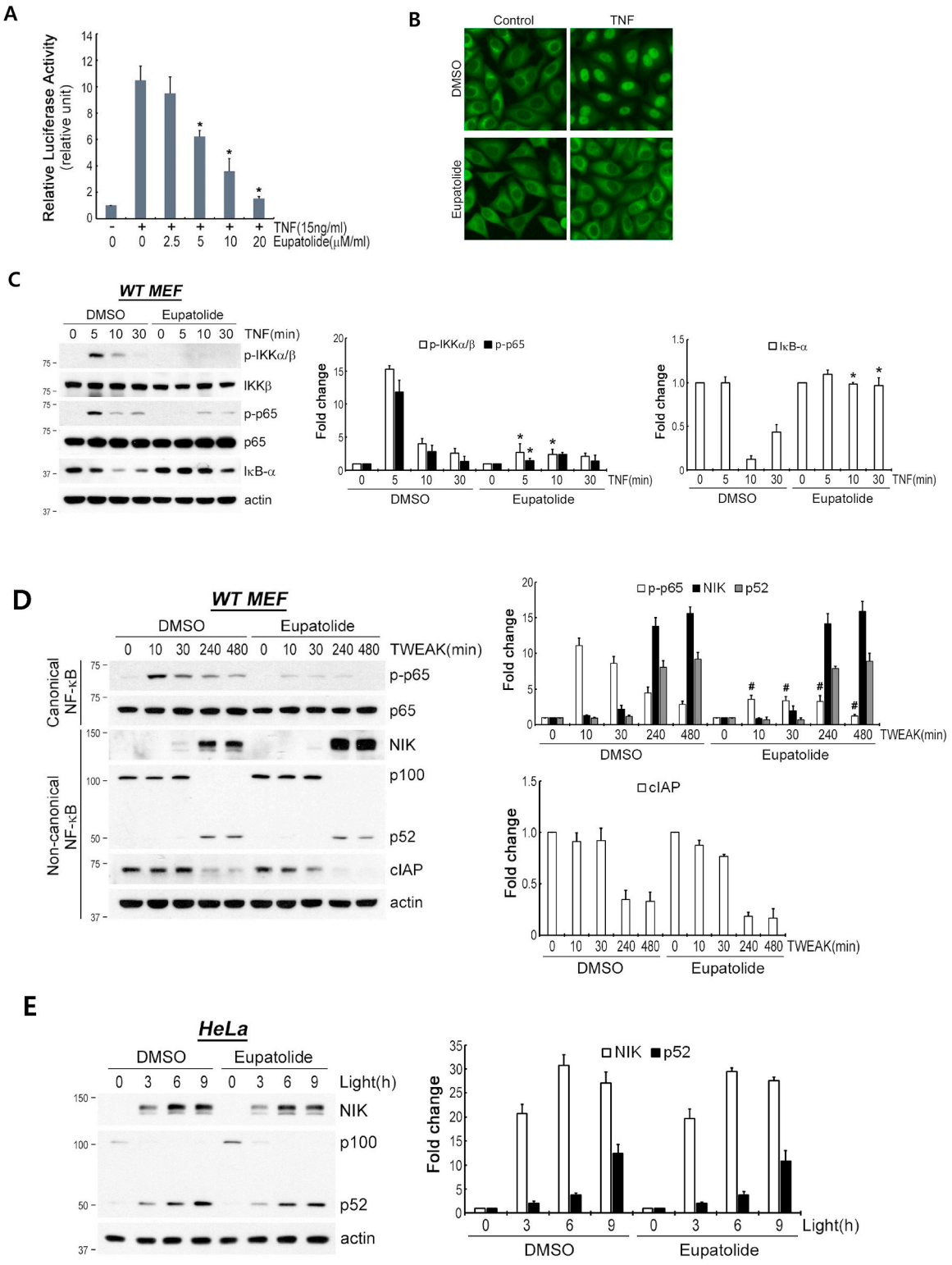

Fig. 1. Eupatolide suppresses the canonical but not the non-canonical NF-κB activation upon TNFR1 ligation. (A) WT MEFs were transfected with expression plasmids of p2xNF-κB-Luc and RSV-β-gal for 24 h. Cells were then pretreated with the indicated concentrations of eupatolide and then treated with TNF (15 ng/mL) for 10 h. Luciferase assay was performed as described in Materials and Methods. Each column shows the mean ± SE of three independent experiments. *$p < 0.05$, compared with TNF only-treated group. (B) WT MEFs were pretreated with eupatolide (10 μM), followed by TNF (15 ng/mL) for 1 h, and subcellular localization of p65 was analyzed by immunofluorescence confocal microscopy with a rabbit anti-p65

antibody (green). (C, D, E) WT MEFs and Hela cells were pretreated with eupatolide (10 μM), followed by 15 ng/mL of TNF (C), 100 ng/mL of TWEAK (D) and 50 ng/mL of LIGHT (E) for the indicated times. Whole cell lysates from each sample were separated by 10% SDS-PAGE, and immunoblotting was performed with the indicated antibodies (left). The relative densities of the indicated proteins were obtained by densitometry analysis (right). *$p < 0.05$, compared with TNF only-treated group. #$p < 0.05$, compared with TWEAK only-treated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

chemiluminescence staining (Biomax, Seoul, Korea).

2.9. Statistical analysis

The quantitative data are presented as the as mean ± SE from at least three separate experiments, each performed in triplicate. The difference between groups were analyzed using Student's *t*-test, with $P < 0.05$ considered statistically significant. Statistical analyses were conducted using SPSS software ver. 13.0 (SPSS, Inc, Chicago, IL, USA).

3. Results

3.1. Eupatolide interferes with the IKKβ-mediated canonical but not the IKKα-mediated non-canonical NF-κB signaling pathway

To test the inhibitory efficacy of eupatolide on the canonical NF-κB activation pathway, we first examined the effect of eupatolide on the TNF-induced transcriptional activation of NF-κB. As shown in Fig. 1A, pretreatment of MEFs with eupatolide significantly suppressed TNF-induced NF-κB luciferase activity in a dose-dependent manner. No cell death was observed when the cells were treated with eupatolide alone at the concentrations used. Consistently, TNF treatment led to a nuclear translocation of p65 within 15 min, whereas pretreatment with eupatolide completely prevented TNF-induced p65 nuclear translocation (Fig. 1B). To gain further insight into the molecular mechanism underlying anti-NF-κB activity by eupatolide, the involvement of the NF-κB signaling cascades was systematically monitored. In a kinetic analysis, treatment of MEFs with TNF induced rapid phosphorylation of p65 and the degradation of IκBα, and such effects were counteracted by eupatolide (Fig. 1C. 3rd and 5th panels). Furthermore, eupatolide almost completely inhibited TNF-induced IKKα/β phosphorylation (Fig. 1C top panel), suggesting that the anti-NF-κB effects of eupatolide are IKKα or IKKβ dependent. TWEAK initiates both IKKβ-mediated canonical and IKKα-mediated non-canonical pathways, activating NF-κB [30].

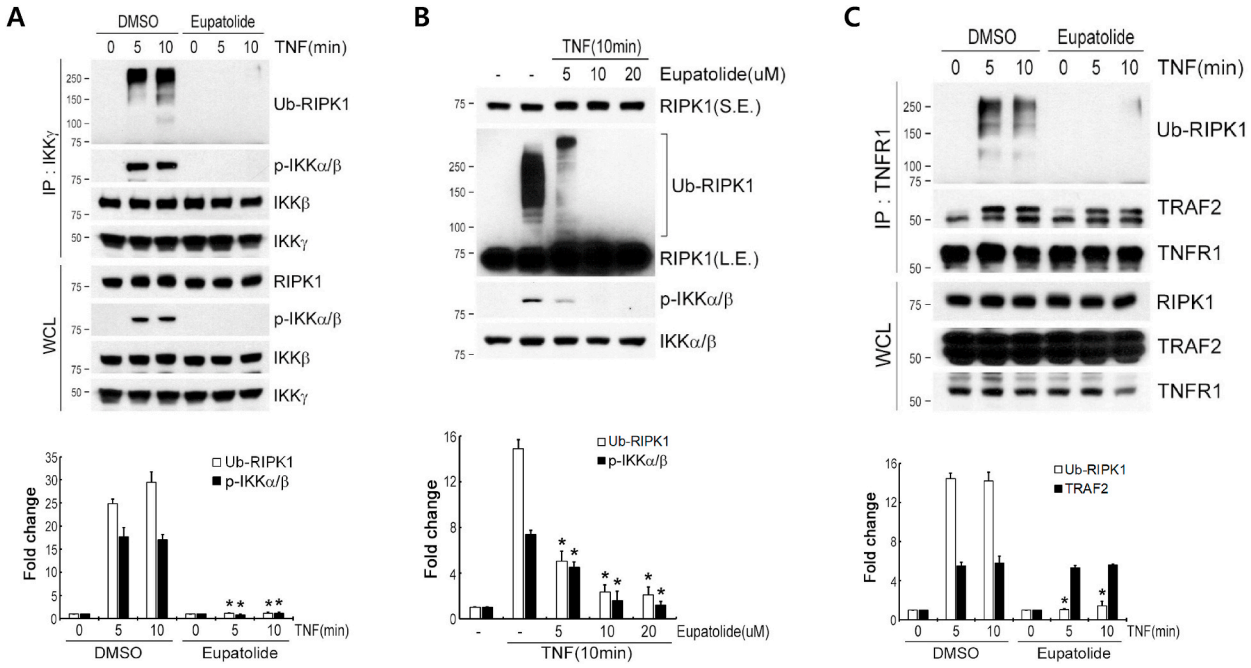

Fig. 2. Eupatolide disrupts the interaction between ubiquitinated RIPK1 and IKK-γ in the proximal TNFR1 signaling complex. (A, C) WT MEFs were pretreated with eupatolide (10 μM), followed by TNF (15 ng/mL) for the indicated times. Cell extracts from each sample were subjected to immunoprecipitation with anti-IKKγ (A) and anti-TNFR1 (C) antibodies. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. A total of 1 % of the cell extract volume (WCL) from each sample was used as input control. (B) WT MEFs were pretreated with the indicated concentrations of eupatolide and then treated with TNF (15 ng/mL) for 10 min. Whole cell lysates from each sample were immunoblotted with the indicated antibodies (top). Densitometry analysis of the bands from the relevant proteins was performed (bottom). *$p < 0.05$, compared with TNF only-treated group.

To compare the inhibitory effects of eupatolide on IKKα or IKKβ, we examined whether eupatolide affects NF-κB activation triggered by TWEAK. Consistently, eupatolide drastically inhibited TWEAK-induced p65 phosphorylation, which requires the catalytic activity of IKKβ (Fig. 1D, top panel). In contrast, neither the processing of p100 to p52 nor the degradation of cIAP1/2 mediated by NIK/IKKα was affected in eupatolide-pretreated cells (Fig. 1D, 3rd to 5th panels), suggesting that eupatolide selectively inhibits the catalytic activity of IKKβ. To confirm this hypothesis, we further examined whether eupatolide affects non-canonical NF-κB activation by LIGHT, a member of the TNF superfamily known to require the catalytic activity of IKKα but not IKKβ in specific cell types, including HeLa cells [31,32]. Consistently, IKKα-mediated non-canonical NF-κB activation elicited by LIGHT was not affected by eupatolide pretreatment, as evident by the processing of p100 to p52 and the NIK accumulation, confirming the selective inhibitory effect of eupatolide on IKKβ (Fig. 1E).

3.2. Eupatolide inhibits the interaction of IKKs with polyubiquitinated RIPK1 in the proximal TNFR1 signaling complex

To gain insight into the molecular mechanisms underlying the anti-IKKβ property of eupatolide, we next examined whether eupatolide affects the interaction between RIPK1 and IKK-γ, which is an essential step for TNF-induced NF-κB activation at the upstream level IKKα/β [33.34]. To address this issue, MEFs were treated with TNF for different durations, and the cell extracts were immunoprecipitated with an anti-IKKγ antibody. Treatment of cells with TNF led to the immediate interaction of IKK-γ with polyubiquitinated RIPK1, and this TNF-induced association was completely disrupted by eupatolide pretreatment (Fig. 2A, top panel). Consistently, the phosphorylation of IKKα/β in the TNF-treated immunoprecipitates was abolished in the eupatolide-pretreated samples, even though the same amount of IKK complex was precipitated in each sample (Fig. 2A, second and third panels). The level of polyubiquitinated RIPK1 induced by TNF was significantly decreased in the whole cell lysates of eupatolide-pretreated samples in a dose-dependent manner without altering the level of unmodified RIPK1 (Fig. 2B). These observations suggest that the disrupted polyubiquitination of RIPK1 by eupatolide affects the interaction of RIPK1 with IKK-γ in response to TNF, subsequently inhibiting the phosphorylation of IKKα/β.

Given that the interaction between RIPK1 and IKKs occurs in the proximal TNFR1 complex-I, including RIPK1 and adaptor molecules such as TRADD and TRAF2 [33,34], we subsequently examined whether eupatolide affects the recruitment of polyubiquitinated RIPK1 to the TNFR1 complex. As expected, treatment of MEFs with TNF led to the transient recruitment of polyubiquitinated RIPK1 to TNFR1, and this enhanced RIPK1 recruitment upon TNF treatment was completely abolished in eupatolide-pretreated MEFs (Fig. 2C, top panel). Of note, such inhibitory effect by eupatolide was specific to the RIPK1 recruitment, based on the results that TNF-induced

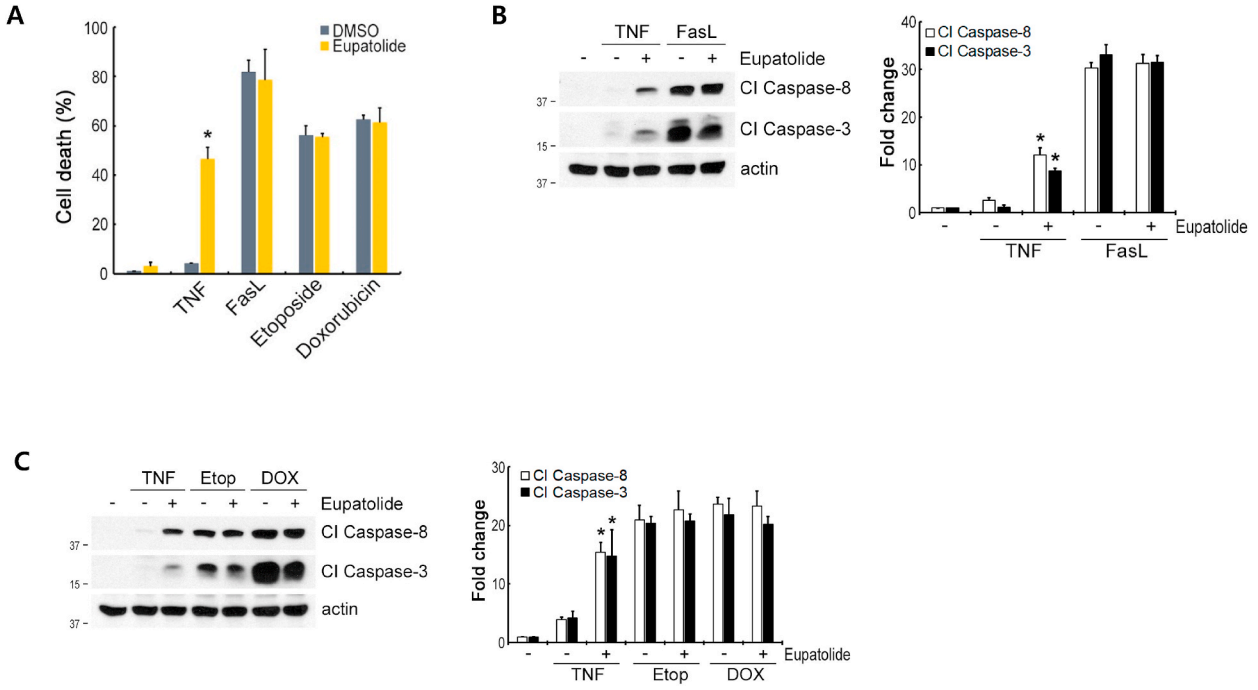

Fig. 3. Eupatolide exclusively enhances apoptotic cell death by TNF, but not by FasL and DNA damaging agents. WT MEFs were pretreated with eupatolide (10 μM), followed by 15 ng/mL of TNF, 100 ng/mL of FasL, 20 μM of etoposide and 5 μM of doxorubicin for 24 h. (A) Cell death was quantified by using Cell Titer-glo Luminescent cell viability assay as described in Materials and Methods. The data represents mean ± SE of three independent experiments. *$p < 0.05$, compared with TNF only-treated group. (B, C) Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). *$p < 0.05$, compared with TNF only-treated group.

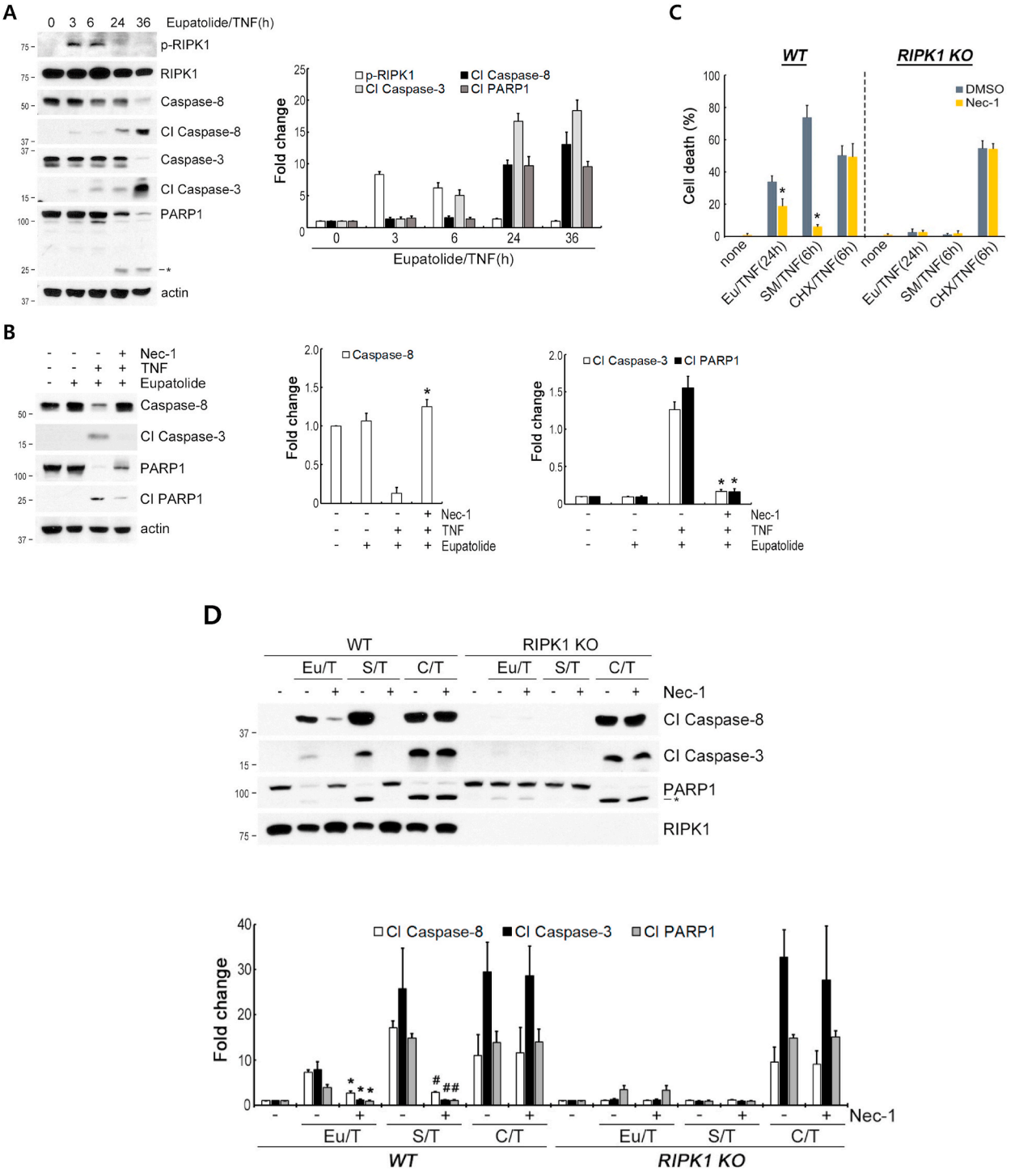

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Fig. 4. Sensitization of TNF-mediated apoptosis by eupatolide requires RIPK1. (A) WT MEFs were treated with TNF (15 ng/mL) and eupatolide (10 μM) for the indicated times. (B) WT MEFs were untreated or pretreated with Nec-1 (50 μM) for 30 min and then treated with 15 ng/mL of TNF or in combination with eupatolide (10 μM) for 24 h. Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). *$p < 0.05$, compared with Eupatolide/TNF-treated group. (C, D) WT and RIPK1 KO MEFs were untreated or pretreated with Nec-1 (50 μM) for 30 min, followed by 15 ng/mL of TNF with the various combinations with the indicated compounds (10 μM eupatolide; 100 nM SM-164; 10 μg/mL cycloheximide) for the indicated times, respectively. (C) Cell death was quantified as in Fig. 3A. The data represents mean ± SE of three independent experiments. *$p < 0.05$, compared with DMSO-treated group. (D) Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (top), and densitometry analysis of the bands from the relevant proteins was performed (bottom). *$p < 0.05$, compared with Eupatolide/TNF-treated group. #$p < 0.05$, compared with SM-164/TNF-treated group.

TRAF2 recruitment was not affected by the eupatolide pretreatment (Fig. 2C, second panel). Thus, these data support the notion that the anti-IKK activity of eupatolide may be achieved by limiting the recruitment of polyubiquitinated RIPK1 to TNFR1 complex-I, which in turn prevents subsequent interactions between RIPK1 and IKK-γ.

3.3. Eupatolide exclusively sensitizes TNF-mediated apoptotic cell death

NF-κB-mediated expression of pro-survival genes functions as a protective mechanism against death receptor (DR)-mediated extrinsic apoptosis [35,36]. Thus, we evaluated whether eupatolide sensitizes cells to apoptosis mediated by different DR ligands, including TNF and Fas ligand (FasL). As expected, MEFs were largely resistant to TNF and eupatolide treatment alone. However, pretreatment with eupatolide drastically enhanced TNF-mediated cytotoxicity with significant apoptotic features, as evidenced by the activation process of the caspase cascade, including those of caspase-8 and the resultant cleavage of caspase-3 (Fig. 3A and B). In contrast, eupatolide failed to sensitize the cells to apoptosis mediated by FasL (Fig. 3A and B), suggesting that the sensitizing effect of eupatolide against DR-mediated apoptosis is specific to TNF. Furthermore, neither the degree of cell death nor the cleavage of caspase-8 and caspase-3 induced by genotoxic stresses, including etoposide and doxorubicin, were largely affected by pretreatment with eupatolide (Fig. 3A and C). These results suggest that the enhancing potency of eupatolide toward cytotoxicity is not only specific against TNFR1 among the various death receptors but also does not regulate the intrinsic apoptotic pathway.

3.4. Sensitization of TNF-mediated apoptosis by eupatolide requires RIPK1 phosphorylation

Recent studies have revealed that IKKβ functions as a negative regulator of TNF-induced apoptosis by reducing the kinase activity of RIPK1 at the level of the death-inducing signaling complex (DISC, also known as complex-II) [23,34]. Based on the ability of eupatolide to suppress TNF-induced IKKβ phosphorylation, we further evaluated the possibility that eupatolide could enhance the cytotoxic potential of RIPK1. Kinetic analysis showed that RIPK1 was phosphorylated in MEFs, peaking at 3–6 h after TNF/eupatolide treatment, which was subsequently accompanied by the cleavage of caspase-8/3 and poly (ADP-ribose) polymerase (Fig. 4A). Importantly, pretreatment of MEFs with a RIPK1 inhibitor, necrostatin-1 (Nec-1), significantly abrogated TNF/eupatolide-induced apoptosis, as evidenced by cell viability and the caspase-8 cascade activation (Fig. 4B and C, left panel). These results suggest that the enhanced RIPK1 phosphorylation by eupatolide triggers downstream apoptotic cell death signaling upon TNFR1 ligation.

To directly explore whether RIPK1 or its phosphorylation is responsible for the upregulation of apoptosis, we compared the cytotoxic efficacy of eupatolide/TNF between WT and RIPK1 KO MEFs. We found that the extent of cell death and the cleavage of caspase-8/-3 induced by TNF/cycloheximide (CHX) in RIPK1−/− MEFs was comparable with that of WT MEFs (Fig. 4C and D). Furthermore, TNF/CHX-induced apoptosis was not affected by Nec-1 in either WT and RIPK1 KO MEFs (Fig. 4C and D), confirming the previously established notion that RIPK1 is not involved in TNF/CHX-induced apoptosis [22,37]. In contrast, cell death and caspase-8/-3 activation by co-treatment with eupatolide or Smac Mimetic-164 and TNF-were significantly prevented by Nec-1 in WT MEFs MEFs (Fig. 4C and D, left panels), and this apoptotic cell death was completely abolished in RIPK1 KO MEFs (Fig. 4C and D, right panels). These results suggest that eupatolide-induced RIPK1 phosphorylation is critical for enhancing TNF-induced apoptosis upstream caspase-8.

3.5. Blockade of RIPK1-dependent apoptosis by TNF/eupatolide triggers a shift to the necroptotic mode of cell death

Given that RIPK1 phosphorylation plays an essential role in engaging the necroptotic cell death machinery via RIPK3 or MLKL in the absence of apoptosis, we examined the effect of the pan-caspase inhibitor z-VAD on TNF-/eupatolide-induced cell death in MEFs. Consistent with this concept, we found that the extent of cell death induced by TNF/eupatolide was markedly increased by z-VAD pretreatment, which was completely prevented by Nec-1 in WT MEFs (Fig. 5A, left panel; Fig. B, top panel). Enhanced cell death induced by the necroptotic trigger (TNF/eupatolide/z-VAD) was completely abrogated in RIPK1 KO MEFs (Fig. 5A, right panel; Fig. B, bottom panel). These results suggest that, upon apoptotic pathway blockade, eupatolide could shift to the necroptotic mode of cell death in an RIPK1-dependent manner.

To confirm the mode of cell death caused by treatment with TNF/eupatolide/z-VAD, we monitored the phosphorylation of RIPK1 and MLKL as markers of TNF-mediated necroptosis. Immunoblotting analysis showed a marked increase in RIPK1 phosphorylation in response to TNF/eupatolide/z-VAD treatment, which was simultaneously accompanied by enhanced MLKL phosphorylation in WT MEFs (Fig. 5C, left panel). In contrast, treatment of RIPK1 KO MEFs with TNF/eupatolide/z-VAD failed to induce MLKL

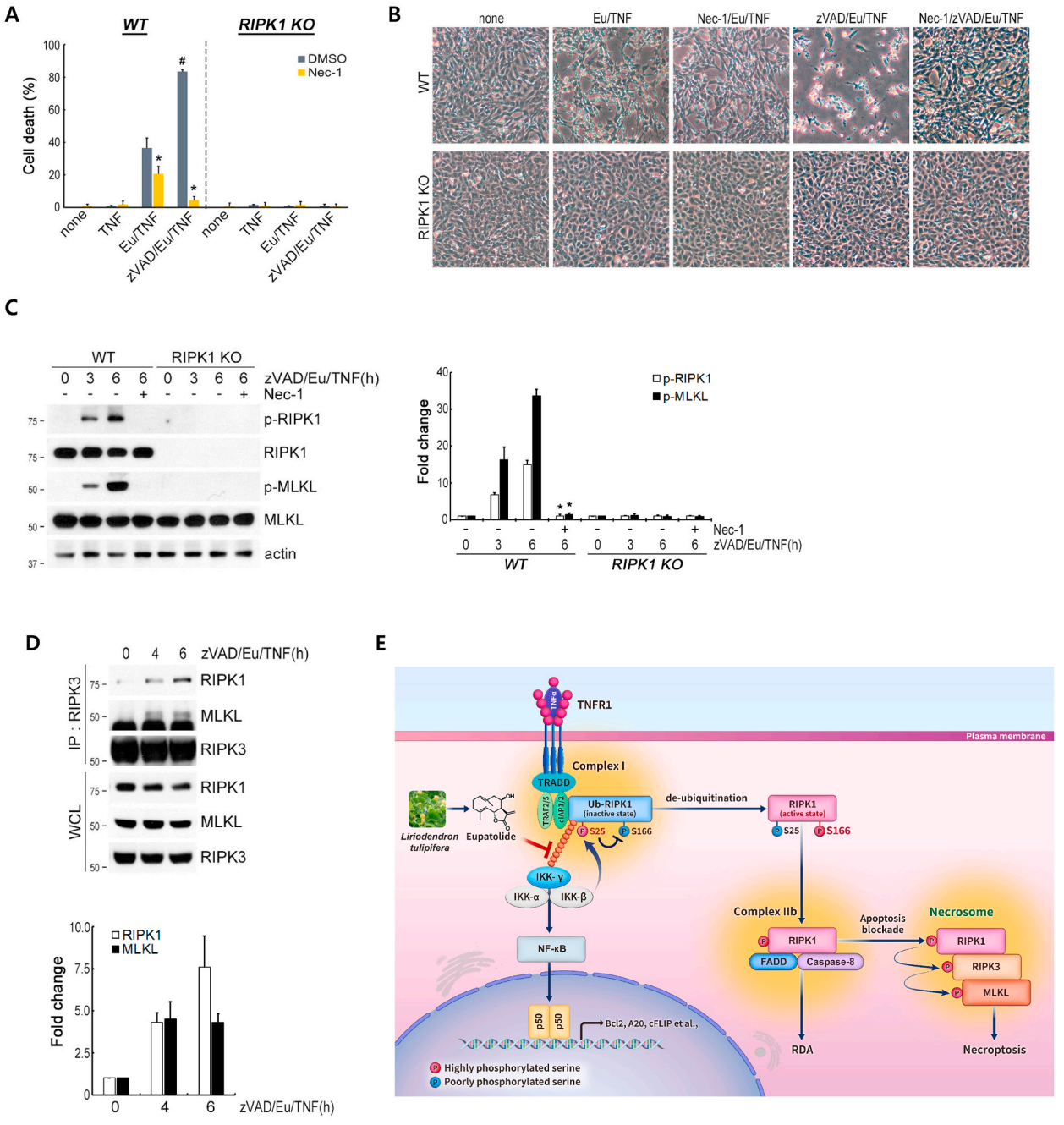

Fig. 5. Eupatolide facilitates RIPK1-dependent necroptosis by necrosome formation under caspase-blocked conditions. (A, B) WT and RIPK1 KO MEFs were untreated or pretreated with Nec-1 (50 μM) for 30 min, and the treated with 15 ng/mL of TNF or in combination with eupatolide (10 μM) and z-VAD-fmk (20 μM) for 24 h. (A) Cell death was quantified as in Fig. 3A. The data represents mean ± SE of three independent experiments. $^{*}p < 0.05$, compared with DMSO-treated group. $^{\#}p < 0.05$, compared with Eu/TNF-treated group. (B) Cells were visualized using an inverted microscope. (C) WT and RIPK1 KO MEFs were untreated or pretreated with Nec-1 (50 μM) for 30 min, and then treated with the indicated combination of compounds (15 ng/mL TNF; 10 μM eupatolide; 20 μM z-VAD-fmk) for the indicated times. Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). $^{*}p < 0.05$, compared with zVAD/Eu/TNF-treated group. (D) WT MEFs were treated with the indicated combination of compounds (15 ng/mL TNF; 10 μM eupatolide; 20 μM z-VAD-fmk) for the indicated times. Cell extracts from each sample were subjected to immunoprecipitation with anti-RIPK3 antibody and immunoprecipitates were analyzed by immunoblotting with indicated antibodies (left). A total of 1 % of the cell extract volume (WCL) from each sample was used as input control. Densitometry analysis of the bands from the relevant proteins was performed (right). (E) Potential mechanisms by which eupatolide enhances TNF-driven apoptosis and necroptosis by the disruption of RIPK1 ubiquitination.

phosphorylation (Fig. 5C, right panel). Moreover, TNF/eupatolide/z-VAD-induced MLKL phosphorylation was completely inhibited by Nec-1 (Fig. 5C, left panel), confirming that the catalytic activity of RIPK1 is required for MLKL-mediated necroptosis in the presence of z-VAD. Subsequent immunoprecipitation experiments revealed that treatment of WT MEFs with TNF/eupatolide/z-VAD led to the interaction of RIPK1 and MLKL with isolated RIPK3 (Fig. 5D). These results suggest that TNF/eupatolide-induced RIPK1 phosphorylation under caspase-inhibited conditions likely functions as an upstream component of RIPK3 or MLKL via necrosome assembly.

4. Discussion

In TNF signaling, RIPK1 is subject to multiple forms of ubiquitination in the membrane-bound TNFR1 signaling complex (complex-I) that is essential for the activation of the pro-survival function of NF-κB via interacting with IKKs through polyUb-binding chains [10,38,39]. The absence of RIPK1 ubiquitination in complex-I leads to the formation of the cytosolic complex-II, which enhances RIPK1-dependent cell death [14]. Subsequently, cytotoxic RIPK1 elicits a necroptosis mode of cell death by activating RIPK3 under apoptosis-deficient conditions, depending on the cellular context [15–17,40,41]. Thus, the discovery of small molecules targeting RIPK1 ubiquitination at the complex-I level may provide an attractive therapeutic strategy to not only set a lower signal threshold for the cytotoxic potential of RIPK1 but also overcome apoptotic resistance by switching the fate of cell death to necroptosis. This study identified a novel mechanism responsible for the anti-IKK property of eupatolide isolated from *L. tulipifera* in the TNFR1-triggered NF-κB activation. We showed that eupatolide disrupted the interaction between RIPK1 and IKKγ by limiting RIPK1 polyubiquitination upon TNFR1 ligation. Importantly, TNF-induced recruitment of TRAF2 to complex-I was unaffected by the presence of eupatolide. Finally, we provided evidence that eupatolide exclusively sensitizes TNF-mediated dual modes of apoptosis and necroptosis in a RIPK1-dependent manner.

Earlier phytochemical studies suggest that eupatolide is active in inflammatory disease, in part by inhibiting the NF-κB signaling pathway [42,43], but the detailed molecular mechanisms are not known. One important finding of this study is that eupatolide antagonizes NF-κB by targeting the upstream regulatory components of the IKK complex (IKK-α/-β/-γ) rather than downstream events of NF-κB, such as the phosphorylation and nuclear translocation of p65. One important finding of this study is that eupatolide antagonizes NF-κB by targeting the upstream regulatory components of the IKK complex (IKK-α/-β/-γ) rather than downstream events of NF-κB, such as the phosphorylation and nuclear translocation of p65. Furthermore, eupatolide exclusively inhibited IKKβ-mediated p65 phosphorylation but not IKKα-mediated proteolytic processing of p100 and cIAP in response to TWEAK, a member of the TNF superfamily. Such findings indicate that eupatolide targets IKKβ rather than IKKα, thus revealing a previously unknown mechanism of the anti-NF-κB property of eupatolide. Regarding the mechanism underlying the anti-IKKβ properties of eupatolide, eupatolide was found to abolish TNF-induced RIPK1 ubiquitination. Furthermore, analysis of the upstream events affected by eupatolide revealed that it inhibited the immediate recruitment of polyubiquitinated RIPK1, but not TRAF2, to membrane-bound TNFR1 complex-I. Thus, it has been hypothesized that eupatolide exerts its antagonizing effects by selectively disrupting the appropriate ubiquitination of RIPK1 without affecting the formation of TNFR1 complex-I. However, the mechanism by which eupatolide disrupts RIPK1 ubiquitination in TNFR1 complex-I remains largely unknown. In this regard, it has been reported that not only a number of E3 Ub ligases, including cIAP1/2, TRAF2, and LUBAC but also Ub cofactors are involved in the transient ubiquitination of RIPK1 in plasma membrane-bound TNFR1 [7–9,44,45]. Thus, a major challenge that now remains is to elucidate the spatial and temporal basis of the RIPK1 Ub network regulation by eupatolide.

Previous studies have demonstrated that depletion of cIAP1/2 and LUBAC sensitizes cells to TNF cytotoxicity in a RIPK1 kinase-dependent manner by promoting the formation of pro-apoptotic complex-II consisting of RIPK1, FADD, and caspase-8 [44,46]. More recent studies have reported that ubiquitination-dependent phosphorylation of RIPK1 in complex-I by IKKα/β represses the catalytic activity of RIPK1 by counteracting the assembly of complex-II [23,47,48]. Thus, it was proposed that the inhibitory effect of eupatolide against TNF-mediated RIPK1 ubiquitination upregulates the cytotoxic potential of RIPK1 and thereby facilitates RIPK1-dependent dual modes of apoptosis and necroptosis in RIPK3 expressing MEFs. In this study, we found that eupatolide enhanced the RIPK1 phosphorylation upon TNFR1 ligation, resulting in increased TNF cytotoxicity. More importantly, eupatolide drastically accelerated TNF-mediated necroptosis by up-regulating the necrosome formation in apoptosis blockade conditions. These observations suggest that the disruption of RIPK1 ubiquitination and subsequent IKKβ inhibition by eupatolide control the cytotoxic potential of RIPK1 at the TNFR1 complex-I level (Fig. 5E). This interpretation was further supported by the observation that both TNF-mediated apoptosis and necroptosis sensitized by eupatolide were almost completely abolished by genetic deletion or pharmacological inhibition of RIPK1. Furthermore, we did not observe any significant cytotoxic effects of eupatolide against FasL- or DNA damage-induced cell death. Thus, these findings suggest that upregulated RIPK1 cytotoxicity may be specific to TNFR1 in the death receptor-mediated extrinsic cell death pathway.

In summary, we propose that eupatolide upregulates RIPK1 phosphorylation by disrupting its ubiquitination, which may provide novel anticancer strategies focused on controlling the cytotoxic ability of TNF to drive the dual modes of apoptosis and necroptosis. As TNF plays an essential role in establishing an intricate link between inflammation and cancer progression [49,50], our study presents a novel idea to use RIPK1-dependent pro-necroptotic agents such as eupaptolide to sensitize TNF cytotoxicity, especially in patients with cancer resistant to pro-apoptotic triggers. Although a previous study has demonstrated that sesquiterpene lactones, including eupatolide, lead to the regression of the tumor burden in mice implanted with human cancer cells [51], *in vivo* studies specifically investigating the anticancer efficacy of eupatolide in sensitizing tumor cells to TNF have not yet been reported. Consequently, while our *in vitro* data are promising, future preclinical *in vivo* studies, particularly those focusing on the anticancer efficacy of eupatolide in caspase-inactive mice, are essential to determine its potential as a novel cancer therapeutic that targets necroptosis.

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Data availability

Data included in article and supplementary material. The data sets used or analyzed during the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Kyeong Ah Park: Funding acquisition, Formal analysis, Conceptualization. **Chan Seok Jung:** Formal analysis. **Kyung-Cheol Sohn:** Resources, Methodology. **Eunjin Ju:** Resources, Methodology, Data curation. **Sanghee Shin:** Software, Resources, Data curation. **InWha Park:** Methodology, Formal analysis. **MinKyun Na:** Writing – original draft, Funding acquisition, Conceptualization. **Gang Min Hur:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28092.

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