Biochemical and Biophysical Research Communications 436 (2013) 212–216

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Critical contribution of oxidative stress to TNFα-induced necroptosis downstream of RIPK1 activation

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ARTICLE INFO

Article history:
Received 15 May 2013
Available online 29 May 2013

Keywords:
TNFα
Oxidative stress
Necroptosis
RIP kinase 1
Butylated hydroxylanisole
Necrostatin-1

ABSTRACT

While apoptosis has been considered to be identical to programmed cell death, necroptosis, which is morphologically related to necrosis, has emerged as a novel type of programmed cell death. Necroptosis depends on two structurally related kinases, receptor-interacting serine–threonine kinase (RIPK)1 and RIPK3. RIPK1 is activated through oligomerization of upstream adaptor molecules such as Fas-associated protein with death domain (FADD) and TNF receptor-associated death domain (TRADD) that are triggered by TNFα or Fas ligand. Activated RIPK1 subsequently interacts with and activates RIPK3, resulting in necroptosis. However, contribution of oxidative stress to execution of necroptosis is still controversial. We found that a selective inhibitor for RIPK1, necrostatin-1 (Nec-1) significantly blocked TNFα-induced cell death and ROS accumulation in NF-κB activation-deficient cells. This suggests that these cells mostly died by necroptosis upon TNFα stimulation. Intriguingly, an antioxidant, butylated hydroxyanisole (BHA) blocked TNFα-induced necroptosis and ROS accumulation in NF-κB activation-deficient cells. However, Nec-1, but not BHA, inhibited TNFα-induced phosphorylation of RIPK1 in these cells, suggesting that ROS play a crucial role in execution of necroptosis downstream of RIPK1 activation. Structural and functional analyses using BHA related compounds revealed that both *tert*-butyl and hydroxy groups of BHA are crucial for its anti-necroptotic function. Together, these results suggest that TNFα-induced necroptosis is tightly associated with oxidative stress, and oxidative stress is induced downstream of RIPK1 activation.

1. Introduction

TNFα induces cell survival through activation of transcription factor NF-κB, which is composed of hetero- or homo-dimers of NF-κB1, NF-κB2, RelA, RelB, and c-Rel [1]. Activation of NF-κB is mediated by sequential activation of several kinases including TGFβ-activating kinase and IκB kinases, and conjugation of various typed ubiquitinylated chains such as linear-, K48-, and K63-typed ubiquitin (Ub) chains to target proteins [2,3]. Cellular inhibitor of apoptosis protein (cIAP)1 and 2 play a crucial role in TNFα-induced NF-κB activation through conjugating K63-type Ub chain to target proteins. Since NF-κB activates many anti-apoptotic and inflammatory cytokine genes, blockade of NF-κB activation render cells highly susceptible to TNFα-induced cell death [2,3]. Among various anti-apoptotic genes upregulated by NF-κB, we and others previously reported that cellular FLICE-inhibitory protein (cFLIP) plays a dominant role in NF-κB-dependent survival signals by binding and blocking caspase 8 activation [4,5].

In addition to activation of NF-κB, TNFα induces caspase 8-dependent apoptosis through formation of a death-inducing signaling complex that contains TNF receptor-associated death domain (TRADD), Fas-associated protein with death domain (FADD), Caspase 8, and receptor-interacting serine–threonine kinase (RIPK)1. Accumulating studies have shown that TNFα also induces programmed necrosis, which is also referred to as necroptosis [6,7]. Execution of necroptosis largely depends on activities of RIPK1 and RIPK3, and necroptosis is inhibited by a RIPK1-specific inhibitor, necrostatin-1 (Nec-1) [8,9]. RIPK1 phosphorylates and activates RIPK3, and form a large complex, named as Ripoptosome that contains FADD, Caspase 8, RIPK1, RIPK3, and possibly mixed lineage kinase domain-like protein (MLKL) [10–12]. cIAP1 and 2, or a long form of cFLIP (cFLIP$_L$) block recruitment of RIPK1 to

Abbreviations: BHA, butylated hydroxyanisole; cIAP1, cellular inhibitor of apoptosis protein-1; FADD, Fas-associated protein with death domain; Nec-1, necrostatin-1; RIPK1, receptor-interacting serine–threonine kinase 1; TNF, tumor necrosis factor; TRADD, TNF receptor-associated death domain.

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http://dx.doi.org/10.1016/j.bbrc.2013.05.075

Ripoptosome, thereby suppressing TNFα-induced necroptosis. Consistent with these results, cIAP1 and 2 double knockout murine embryonic fibroblasts (cIAP1/2 DKO MEFs) have been shown to undergo necroptosis upon TNFα stimulation [13]. Although inhibition of caspases sensitizes cells to necroptosis under various conditions, effector molecule(s) leading to necroptosis is not fully understood [6,7]. A previous study showed that TNFα-induced necroptosis is blocked by an antioxidant in murine fibrosarcoma, L929 cells [14]. In sharp contrast, other group showed that TNFα-induced necroptosis is not inhibited by an antioxidant in HT29 cells [9]. Therefore, contribution of oxidative stress to execution of necroptosis is still controversial.

We previously reported that TNFα induces reactive oxygen species (ROS)-dependent necrosis, and the amount of $cFLIP_L$ protein is rapidly decreased after TNFα stimulation in RelA, a major component of NF-κB, KO MEFs [4,15]. Moreover, we have recently shown that tissue specific deletion of cFLIP in the intestine and the liver results in perinatal lethality due to an enhanced apoptosis and necroptosis of intestinal epithelial cells and hepatocytes, respectively [16]. Taken that $cFLIP_L$ blocks Ripoptosome formation [10,11], it would be intrigued to test whether TNFα induces necroptosis in RelA KO MEFs.

In the present study, we showed that Nec-1 significantly inhibited TNFα-induced cell death in RelA KO MEFs, suggesting that RelA KO MEFs mostly died by necroptosis. Moreover, an antioxidant, butylated hydroxyanisole (BHA) blocked TNFα-induced necroptosis and ROS accumulation in RelA KO and cIAP1/2 DKO MEFs. By using a novel method to detect phosphorylated proteins in gel, we could detect TNFα-induced phosphorylation of RIPK1 in these cells, which was blocked by Nec-1, but not BHA. Furthermore, structural and functional analyses using BHA related compounds revealed that both *tert*-butyl and hydroxy groups are crucial for anti-necroptotic and anti-oxidant functions of BHA. Together, ROS might play a critical role in TNFα-induced necroptosis.

2. Materials and methods

2.1. Reagents and cell culture

Murine TNFα (eBiosciences), z-VAD-fmk (Peptide institute), necrostatin-1 (Nec-1) (Sigma–Aldrich), butylated hydroxyanisole (WAKO), and CM-H_2DCFDA (Invitrogen) were purchased from indicated sources. The following antibodies were used in this study: anti-RIPK1 (BD Biosciences), anti-RIPK3 (Immugenex), and anti-tubulin antibodies (Sigma–Aldrich) were obtained from indicated sources. *Tert*-butyl-benzene (BB), 3-*tert*-butyl-4-hydroxy-toluene (BHT), 3-*tert*-butyl-4-hydroxy-ethylbenzol (BHEB), and 4-hydroxy-anisole (HA) were obtained from Sigma–Aldrich.

Wild-type MEFs, RelA KO MEFs (provided by T. Doi), and cIAP1/2 DKO MEFs (provided by John Silke) were described previously [13,15], and maintained in DMEM containing 10% fetal calf serum (FCS).

2.2. Synthesis of 3-tert-butyl-4-acetoxy-anisole (BA-OAc) and 3-tert-butyl-4-methoxy-anisole (BA-OMe)

BHA (440 mg) was dissolved in tetrahydrofran (10 ml) or in dimethylformamide (10 ml), and each reaction mixture was mixed with 60% NaH in paraffin (100 mg) at room temperature for 1 h. Then, acetyl chloride (0.26 ml) and methyl iodide (0.5 ml) were added to the reaction mixture to synthesize BA-OAc and BA-OMe, respectively. After these mixtures were evaporated to dryness, each residue was applied to a silica gel column to isolate highly purified BA-OAc and BA-OMe.

2.3. Measurement of ROS accumulation

RelA KO MEFs or cIAP1/2 DKO MEFs were stimulated with TNFα (10 ng/ml) in the absence or presence of BHA (100 μM), BHA related compounds (100 μM), Nec-1 (20 μM), or z-VAD-fmk (50 μM) for 4 h. After stimulation, cells were incubated with CM-H_2DCFDA (1 μM) for 30 min at 37 °C. Then, cells were harvested and analyzed on a flow cytometer (FACSCalibur, BD Biosciences). Data were processed by using the CellQuest program (BD Biosciences).

2.4. Cell viability assay

RelA KO MEFs or cIAP1/2 DKO MEFs were plated onto 96-well plates and cultured for 18 h in DMEM containing 10% FCS. Then, cells were stimulated with TNFα (10 ng/ml) in the absence or presence of indicated concentrations of BHA, BHA related compounds, Nec-1, or z-VAD-fmk for 9 h (for cIAP1/2 DKO MEFs) or 18 h (for RelA KO MEFs). Cell viability was determined by WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)[^{2}H]tetrazolium monosodium salt-1) assay using a Cell Counting kit (Dojindo).

2.5. Western blotting

Cells were lysed in a RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, 1 mg/mL aprotinin, and 1 mg/mL leupeptin). After centrifugation, cell lysates were subjected to SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were immunoblotted with indicated antibodies. The membranes were developed with Super Signal West Dura Extended Duration Substrate (Thermo Scientific) and analyzed by LAS4000 (GE Healthcare Life Sciences).

To detect phosphorylated RIPK1 and RIPK3, cell lysates were subjected to SDS–PAGE containing phosphorylation-tag (Phos-Tag™, Wako) that specifically traps phosphorylated proteins in gel [17]. After Western blotting with anti-RIPK1 and RIPK3 antibodies, phosphorylated proteins were detected as slower migration bands compared to corresponding unphosphorylated proteins.

2.6. Statistical analysis

Statistical analysis was performed by two-way unpaired *t* test. *P* value <0.05 was considered to be significant.

3. Results

3.1. TNFα induces ROS-dependent necroptosis in RelA KO and cIAP1/2 DKO MEFs

We previously reported that ROS play a crucial role in TNFα-induced necrotic cell death in NF-κB-deficient cells, such as RelA KO MEFs [15]. To further characterize a type of cell death in RelA KO MEFs, we tested whether Nec-1 inhibited TNFα-induced cell death in RelA KO MEFs. We pretreated RelA KO MEFs with Nec-1 [8], and then stimulated with TNFα. Nec-1 almost completely inhibited TNFα-induced cell death and ROS accumulation in RelA KO MEFs (Fig. 1A and B). These results suggest that TNFα induces necroptosis in RelA KO MEFs. Consistent with our previous studies [15], BHA blocked TNFα-induced cell death and ROS accumulation in RelA KO MEFs (Fig. 1A and B), suggesting that ROS play a crucial role in TNFα-induced necroptosis.

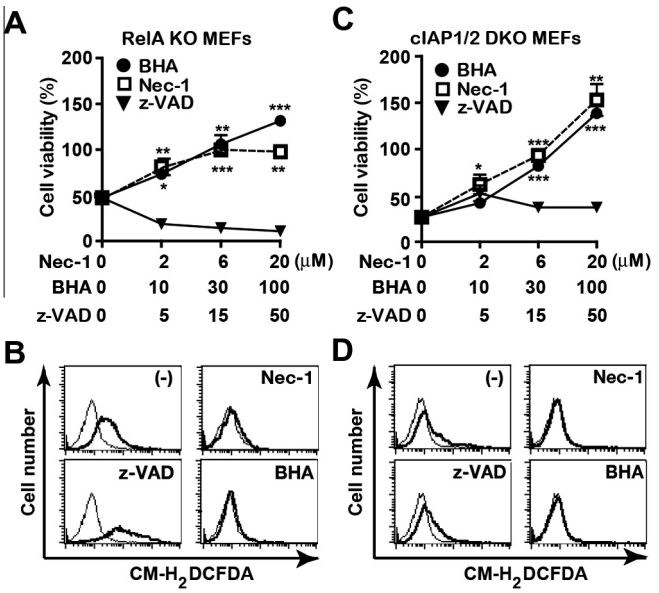


Fig. 1. TNFα induces necroptosis in RelA KO and cIAP1/2 DKO MEFs. (A and C) RelA KO (A) or cIAP1/2 DKO (C) MEFs were unstimulated or stimulated with TNFα in the absence or presence of the indicated concentrations of Nec-1, BHA, or z-VAD-fmk for 18 h (for RelA KO MEFs) or 9 h (for cIAP1/2 DKO MEFs), respectively. Cell viability was determined by WST assay. Results are presented as mean ± standard errors of triplicate samples and represent four independent experiments with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to TNFα-treated cells. (B and D) RelA KO (B) or cIAP1/2 DKO (D) MEFs were unstimulated (thin line) or stimulated with TNFα (bold line) in the absence or presence of Nec-1 (20 μM), BHA (100 μM), or z-VAD-fmk (50 μM) for 4 h, then the cells were labeled with CM-H_2DCFDA (1 μM) for the last 30 min, and analyzed by flow cytometry. The results are representative of four independent experiments.

Recent studies have shown that TNFα induces necroptosis in cIAP1/2 DKO MEFs [10,11]. We then tested whether ROS also contributed to TNFα-induced necroptosis in cIAP1/2 DKO MEFs. BHA as well as Nec-1 blocked TNFα-induced necroptosis and ROS accumulation in cIAP1/2 DKO MEFs (Fig. 1C and D). Together, these data suggest that TNFα induces ROS-dependent necroptosis in RelA KO and cIAP1/2 DKO MEFs.

3.2. Nec-1, but not BHA, suppresses TNFα-induced RIPK1 phosphorylation

Phosphorylation and activation of RIPK1 and RIPK3 play a crucial role in execution of necroptosis [12]. To investigate the mechanism underlying BHA-dependent suppression of necroptosis, we tested whether BHA suppresses phosphorylation of RIPK1 and RIPK3. Since antibodies that specifically detect phosphorylated forms of RIPK1 and RIPK3 are not currently available, we applied Phos-Tag™ assay to discriminate mobility shift of phosphorylated and non-phosphorylated RIPK1 and RIPK3 in SDS–PAGE [17]. Phos-Tag™ captures phosphorylated serine, threonine, or tyrosine residues of the proteins in gel, therefore, phosphorylated proteins in gel are detected as slower migration bands compared to corresponding unphosphorylated proteins. We then stimulated cIAP1/2 DKO MEFs with TNFα in the absence or presence of Nec-1 or BHA for indicated times, and cell lysates were subjected to SDS–PAGE containing Phos-Tag™, followed by Western blotting with anti-RIPK1 antibody. A slowly migrated band of RIPK1 was detected at 1 h and persisted up to 2 h after TNFα stimulation, which was blocked in the presence of Nec-1 (Fig. 2A, upper panels). This suggests that Phos-Tag™ analysis indeed detected phosphorylated RIPK1 as a slowly migrated band compared to unphosphorylated RIPK1. Notably, a slowly migrated band of RIPK1 could not be detected on standard SDS–PAGE (Fig. 2A, lower panes), verifying a usefulness of Phos-Tag™ analysis. Phosphorylated and total RIPK1 rapidly disappeared in TNFα-treated cIAP1/2 DKO MEFs at 4 h due to massive cell death. In contrast to Nec-1, BHA did not block phosphorylation of RIPK1, suggesting that ROS act as effector molecules leading to necroptosis downstream of RIPK1 activation. However,

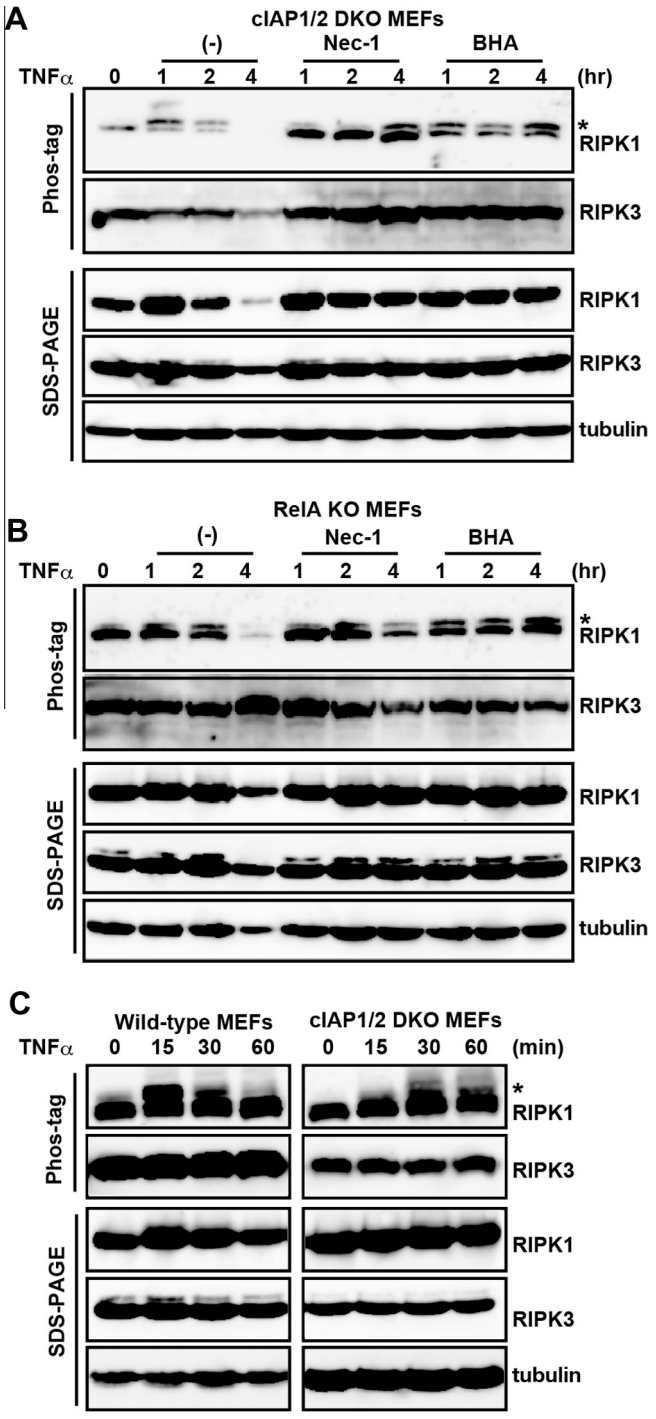
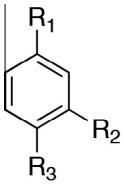


Fig. 2. TNFα induces phosphorylation of RIPK1, which is blocked by Nec-1, but not BHA. cIAP1/2 DKO (A) and RelA KO (B) MEFs were stimulated with TNFα in the absence or presence of Nec-1 (20 μM) or BHA (100 μM) for indicated times. Cell lysates were subjected to Phos-Tag™ SDS–PAGE (upper lanes), or standard SDS–PAGE (lower panels) and analyzed by immunoblotting with antibodies against RIPK1, RIPK3, or tubulin. Asterisks indicate phosphorylated RIPK1. (C) Wild-type and cIAP1/2 DKO MEFs were stimulated with TNFα for indicated times. Cell lysates were analyzed as described in (A).



Compounds	R_1	R_2	R_3
BHA	$O\text{-}CH_3$	$C\text{-}(CH_3)_3$	OH
BA-OAc	$O\text{-}CH_3$	$C\text{-}(CH_3)_3$	$O\text{-}COCH_3$
BA-OMe	$O\text{-}CH_3$	$C\text{-}(CH_3)_3$	$O\text{-}CH_3$
HA	$O\text{-}CH_3$	H	OH
BHT	CH_3	$C\text{-}(CH_3)_3$	OH
BHEB	CH_2CH_3	$C\text{-}(CH_3)_3$	OH
BB	H	$C\text{-}(CH_3)_3$	H

Fig. 3. Structures of BHA and its related compounds. R1, R2, and R3 represent each group at the indicated positions of BHA and its related compounds. BHA, 3-*tert*-butyl-4-hydroxy-anisole; BA-OAc, 3-*tert*-butyl-4-acetoxy-anisole; BA-OMe, 3-*tert*-butyl-4-methoxy-anisole; HA, 4-hydroxy-anisole; BHT, 3-*tert*-butyl-4-hydroxy-toluene; BHEB, 3-*tert*-butyl-4-hydroxy-ethylbenzol; BB, *tert*-butyl-benzene.

we could not detect slowly migrated bands of RIPK3 in TNFα-stimulated cIAP1/2 DKO MEFs on Phos-Tag™ gel (Fig. 2A, upper panels), which might be caused by a detection limit of Phos-Tag™ analysis. Similar results were observed in TNFα-stimulated RelA KO MEFs, inhibitory effect of Nec-1 on phosphorylation of RIPK1 in RelA KO MEFs was weaker than in cIAP1/2 DKO MEFs (Fig. 2B).

We next investigated kinetics of phosphorylation of RIPK1 in cIAP1/2 DKO MEFs in more detail. Phosphorylation of RIPK1 was gradually increased in cIAP1/2 DKO MEFs at 30 min and persisted up to 60 min after stimulation (Fig. 2C, right panel). In sharp contrast, phosphorylation of RIPK1 was rapidly increased in wild-type MEFs at 15 min, then declined and disappeared at 60 min after stimulation (Fig. 2C, left panel). Taken that TNFα did not induce necroptosis in wild-type MEFs (data not shown), these results suggest that TNFα-induced transient phosphorylation of RIPK1 is not involved in execution of necroptosis.

3.3. Structural and functional analysis of various compounds related to BHA

We next performed structural and functional analyses of BHA related compounds to determine which groups of BHA are essential for suppression of necroptosis and ROS accumulation. As shown in Fig. 3, we purchased or chemically synthesized BHA related compounds, in which each group at R1, R2, or R3 positions of BHA was eliminated or substituted with other groups. We then tested whether BHA related compounds blocked TNFα-induced ROS accumulation and necroptosis in RelA KO and cIAP1/2 DKO MEFs. We found that TNFα-induced necroptosis and ROS accumulation were not blocked by 4-hydroxy-anisole (HA), indicating that elimination of a *tert*-butyl group at R2 position of BHA resulted in loss of anti-necroptotic and anti-oxidant activities (Fig. 4). In contrast, BHT blocked TNFα-induced necroptosis and ROS accumulation, suggesting that substitution of a methoxy group at R1 position of BHA with a methyl group still retained anti-necroptotic and anti-oxidant activities. However, it is currently unknown why 3-*tert*-butyl-4-hydroxy-ethlybenzol (BHEB), in which a methoxy group at R1 position of BHA was replaced by an ethyl group, suppressed TNFα-induced necroptosis in RelA KO, but not cIAP1/2 DKO MEFs. On the orther hand, 3-*tert*-butyl-4-acetoxy-anisole (BA-OAc) blocked TNFα-induced necroptosis and ROS accumulation in RelA KO and cIAP1/2 DKO MEFs (Fig. 4). Notably, once BA-OAc entered cells, an acetoxy group at R3 position of BA-OAc might be removed by esterase (s), resulting in conversion of BA-OAc to BHA. Taken that 3-*tert*-butyl-4-methoxy-anisole (BA-OMe) or *tert*-butyl-benzene (BB) did not retain anti-necroptotic and antioxidant activities, these results suggest that a hydroxy group at R3 position of BHA is essential for anti-necroptotic and anti-oxidant activities.

4. Discussion

In the present study, we showed that BHA blocked TNFα-induced necroptosis and ROS accumulation in RelA KO and cIAP1/2 DKO MEFs. Phos-Tag™ analysis showed that TNFα induced sustained phosphorylation of RIPK1 in RelA KO and cIAP1/2 DKO MEFs, which was blocked by Nec-1, but not BHA. Moreover, structural and functional analyses using BHA related compounds revealed that both *tert*-butyl and hydroxy groups are crucial for suppression of necroptosis and ROS accumulation of BHA.

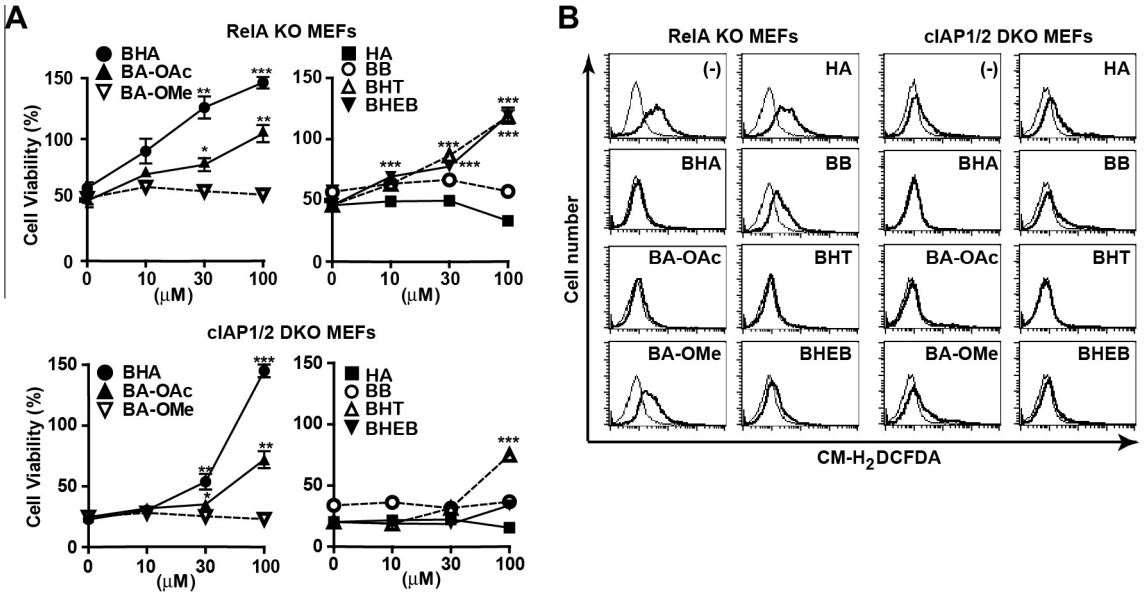


Fig. 4. *Tert*-butyl and hydroxy groups are crucial for anti-necroptotic and anti-oxidant functions of BHA. (A) RelA KO MEFs and cIAP1/2 DKO MEFs were unstimulated or stimulated with TNFα in the absence or presence of the indicated concentrations of BHA or its related compounds for 18 h and 9 h, respectively. Cell viability was determined by WST assay. Results are presented as mean ± standard errors of triplicate samples and represent four independent experiments with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to TNFα-treated cells. (B) RelA KO MEFs or cIAP1/2 DKO MEFs were unstimulated or stimulated with TNFα in the absence (thin line) or presence (bold line) of BHA or its related compounds for 4 h, then cells were analyzed as in Fig. 1A. The results are representative of four independent experiments.

Several studies have shown that RIPK1 phosphorylates and activates RIPK3, resulting in ROS production by damaged mitochondria due to activation of a phosphoglycerate mutase family member 5 (PGAM) or alterations of oxidative status [12,18,19]. These results suggest that ROS act as downstream effector molecules of RIPK3. Consistently, BHA could not inhibit RIPK1 phosphorylation, although BHA efficiently suppressed TNFα-induced necroptosis in RelA KO and cIAP1/2 DKO MEFs. Intriguingly, TNFα induced transient RIPK1 phosphorylation in wild-type MEFs, whereas TNFα induced sustained RIPK1 phosphorylation in cIAP1/2 DKO and RelA KO MEFs (Fig. 2). These results suggest that sustained, but not transient RIPK1 activation is crucial for execution of necroptosis. We previously reported that TNFα induces sustained MAP kinase activation, including JNK, p38, and ERK in RelA KO MEFs, which is completely blocked by BHA [15]. Taken that sustained RIPK1 phosphorylation was not blocked by BHA (Fig. 2), the molecular mechanisms leading to sustained RIPK1 activation might be different from sustained MAPK activation in RelA KO MEFs (Fig. S1). Further study will be required to address this issue.

Acknowledgments

We thank T. Doi, D. Mahoney, and J. Silke for providing RelA and cIAP1/2 DKO MEFs, respectively. We also thank J.M. Fukuto and S. Sakon-Komazawa for advice and technical assistance, respectively.

This work was supported in part by Grants-in-Aid (S0991013) for "High-Tech Research Center" Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan, and Grant-in-Aid for Scientific Research (B) and Challenging Exploratory Research from Japan Society for the Promotion of Science (JSPS), and Scientific Research on innovative areas, MEXT, Japan, and grants from the Takeda Science Foundation and NOVARTIS Foundation for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.075.

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