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Pleiotropic Potential of Dehydroxymethylepoxyquinomicin for NF-κB Suppression via Reactive Oxygen Species and Unfolded Protein Response

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Dehydroxymethylepoxyquinomicin (DHMEQ) is a low-m.w. compound that strongly inhibits NF-κB. Previous reports showed that DHMEQ directly binds to specific cysteine residues of NF-κB subunits and thereby inhibits their nuclear translocation and DNA binding. In this work, we describe novel mechanisms by which DHMEQ suppresses cytokine-activated NF-κB signaling. We found that sustained exposure of renal tubular cells to DHMEQ blocked TNF-α- and IL-1β–induced TGF-β–activated kinase 1 (TAK1) phosphorylation, a crucial event for NF-κB activation upstream of IκB kinase. This inhibition was mediated by reactive oxygen species (ROS), because of the following: 1) DHMEQ caused generation of ROS; 2) pretreatment with ROS generator inhibited cytokine-induced TAK1 phosphorylation and NF-κB activation; and 3) scavenging of ROS attenuated the suppressive effects of DHMEQ on TAK1 and NF-κB. We also found that DHMEQ caused the unfolded protein response (UPR) through generation of ROS. Alleviation of the UPR by chemical and genetic chaperones partially attenuated the suppressive effect of DHMEQ on NF-κB. The UPR-mediated inhibition of NF-κB occurred downstream of degradation of IκBα and phosphorylation of p65. Subsequent experiments revealed the following: 1) DHMEQ caused selective induction of C/EBPβ through the UPR; 2) overexpression of C/EBPβ suppressed activation of NF-κB; and 3) knockdown of C/EBPβ attenuated the inhibitory effect of DHMEQ; and 4) DHMEQ-induced expression of C/EBPβ did not affect TNF-α–triggered degradation of IκBα and phosphorylation of p65. These results suggest that, in addition to its known effect on nuclear translocation of NF-κB, DHMEQ interferes with the cytokine-induced NF-κB signaling via generation of ROS at both upstream and downstream of the IκB kinase–IκB level. The Journal of Immunology, 2013, 190: 6559–6569.

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Abbreviations used in this article: ATF, activating transcription factor; CHOP, C/EBP homologous protein; DHMEQ, dehydroxymethylepoxyquinomicin; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; ERSE, ER stress response element; GRP78, BiP/78-kDa glucose-regulated protein; HO-1, heme oxygenase-1; IκB, IκB kinase; LAP, liver-enriched activating protein; LIP, liver inhibitory protein; MEF, mouse embryonic fibroblast; NAC, N-acetyl-l-cysteine; ORP150, 150-kDa oxygen-regulated protein; 4-PBA, 4-phenylbutyric acid; PERK, dsRNA-dependent protein-kinase–like ER kinase; RIP1, receptor-interacting protein 1; ROS, reactive oxygen species; siRNA, small interfering RNA; TAB, TAK1–binding protein; TAK1, TGF-β–activated kinase 1; TRAF, TNFR-associated factor; UPR, unfolded protein response; UPR, UPR element; XBP1, X-box–binding protein 1.
advantages for therapeutic uses. Indeed, previous studies demonstrated that DHMEQ ameliorated various models of inflammation including rheumatoid arthritis, autoimmune uveoretinitis, tubulointerstitial nephritis, and glomerulonephritis (12–15). Of note, DHMEQ is also effective against intractable malignant diseases such as hormone-insensitive prostate cancers and malignant breast carcinoma (16, 17). Currently, however, it is not well understood why DHMEQ is so effective for the treatment of those intractable diseases. Other unknown mechanisms may also underlie the therapeutic effects of DHMEQ.

Lampiasi et al. (18) reported that antitumor effects of DHMEQ on human liver cancer cells were mediated by production of reactive oxygen species (ROS). That is, DHMEQ-induced ROS generation caused growth inhibition, caspase activation, and consequent apoptosis of cancer cells. Although ROS is known to activate NF-κB in the early phase (19), sustained generation of ROS could affect NF-κB signaling negatively. Indeed, several studies provided evidence that ROS are not activators, but rather inhibitors of NF-κB, as recently reviewed by Morgan and Liu (20). Wu et al. (21) reported that, in human lens epithelial cells, sustained oxidative stress inhibited TNF-α–triggered activation of NF-κB. Loukili et al. (22) also suggested that preconditioning with oxidative stress prevented inflammatory cytokine-induced activation of NF-κB. Based on these findings, it is probable that DHMEQ suppresses activation of NF-κB, at least in part, through ROS generation in the late phase. To date, however, a relationship between DHMEQ-induced ROS generation and its effect on NF-κB has not been elucidated.

In this study, we investigate a role of ROS in the suppressive effect of DHMEQ on NF-κB. Our current results revealed ROS-dependent mechanisms underlying the pharmacological action of DHMEQ. In addition to its known effect on nuclear translocation and DNA binding of p65, DHMEQ interferes with cytokine-triggered NF-κB signaling at both upstream and downstream of the IKK level. The former mechanism targets TAK1/TAB complex, and the latter involves unfolded protein response (UPR)-mediated induction of C/EBPβ.

**Materials and Methods**

**Reagents**

DHMEQ was synthesized, as described previously (10, 23). A concentration 5 μM/ml was generally used for experiments, unless indicated otherwise. Recombinant human IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN). Glutathione-reduced ethyl ester, menadione, N-acetyl-L-cysteine (NAC), and thapsigargin were obtained from Sigma-Aldrich Japan (Tokyo, Japan), and 4-phenylbutyric acid (4-PBA) was from Calbiochem (San Diego, CA).

**Cells**

The rat renal tubular epithelial cell line NRK-52E was obtained from American Type Culture Collection (Manassas, VA) and generally used for experiments. HEK293 human embryonic kidney cells were provided by S. Takeda (University of Yamanashi, Yamanashi, Japan). Wild-type and p65−/− mouse embryonic fibroblasts (MEF) were provided by H. Nakano (Juntendo University School of Medicine, Tokyo, Japan) (24). These cells were maintained in DMEM/Ham’s F-12 (Life Technologies-BRL, Gaithersburg, MD) supplemented with 5% (for NRK-52E) or 10% (for MEF and HEK293) FBS. Experiments were performed in the presence of 1% FBS.

**Establishment of stable transfectants**

Using electroporation, NRK-52E cells were stably transfected with pCMV-2FLAG-TAK1, pDNA-HA-TAB1 (provided by H. Sakurai, University of Toyama, Toyama, Japan) (31), or pCDNA3-HO-1 (provided by M. P. Soares, Instituto Gulbenkian de Ciência Oeiras, Portugal) (32), pCDNA3-HO-1 codes for full-length rat heme oxygenase-1 (HO-1), pCDNA-LAP and pCDNA-LIP encode liver-enriched activating protein (LAP) and liver inhibitory protein (LIP), respectively. pNFκB-Luc, pERSE-Luc, and pUPRE-Luc introduce a luciferase gene under the control of the κB sites, the endoplasmic reticulum (ER) stress response element (ERSE), and the UPR element (UPRE), respectively. Using GeneJuice (Novagen, Madison, WI), p65−/− MEF were transiently transfected with pCDNA3-p65WT or pCDNA3-p65(C38S) (provided by M. Nishida, Kyushu University, Fukuoka, Japan) (33) and subjected to analyses. HEK293 cells were also cotransfected with pCR-FLAG-TRA2F (provided by H. Nakano) together with pCheno-HA-Ub (provided by M. Fujimuro, Kyoto Pharmaceutical University, Kyoto, Japan) and subjected to immunoprecipitation assay.

**Luciferase assay**

Activity of luciferase was evaluated by Luciferase Assay System (Promega, Madison, WI), according to the manufacturer’s protocol. Activity of luciferase was normalized by the number of viable cells estimated by formalazan assay, and relative values (%) were shown as graphs.

**Northern blot analysis**

Total RNA was extracted by a single-step method, and Northern blot analysis was conducted, as described before (34). cDNAs for MCP-1 (35), C/EBPα, C/EBPβ, C/EBPδ (provided by E.-Z. Amri, Centre National de la Recherche Scientifique, Nice, France) (36), BiP/78-kDa glucose-regulated protein (GRP78) (provided by K. Imaiizumi, University of Hiroshima, Hiroshima, Japan) (37), C/EBP homologous protein (CHOP) (38), activating transcription factor (ATF) 4 (provided by D. Ron, New York University School of Medicine, New York, NY), ORP150 (provided by S. Ogawa) (25), and HO-1 (provided by S. Shibahtara, Tokohu University School of Medicine, Sendai, Japan) (39) were used to prepare radiolabeled probes. Expression of GAPDH was used as a loading control. Densitometric analysis was performed using ImageJ Software (National Institutes of Health, Bethesda, MD).

**Western blot analysis**

Cells were lysed in radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton-X, 1% [w/v] SDS, 50 mM NaF, and 1 mM Na3VO4) with protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan) and subjected to Western blot analysis. Immunoreactive proteins were visualized by using the Chemi-Lumi One L (Nacalai Tesque). Primary Abs used were as follows: anti-TAK2F2 Ab, anti-RIP1 Ab, anti-phospho-TAK1 (Thr180) Ab, anti-TAK1 Ab, anti-TAB2 Ab, anti-phospho- I KKβ (Ser32) Ab, anti-IKKγ Ab, anti-phospho-IκBα (Ser15) Ab, anti-phospho-IκBα (Ser15) Ab, anti-phospho-p65 (Ser529) Ab, anti-phospho-pskDNA31-dependent protein kinase (PKR)–like ER kinase (PERK) (Thr172) Ab, anti-phospho-eukaryotic translation initiation factor 2α (eIF2α) (Ser51) Ab, anti–phospho–Akt (Ser473) Ab, and anti-Akt Ab from Cell Signaling Technology (Beverly, MA); anti-TNFRI1 Ab, anti-IκBα Ab, anti-PERK Ab, anti-eIF2α Ab, anti-C/EBPβ Ab, anti-GADD153 (CHOP) Ab, and anti-p65 Ab from Santa Cruz Biotechnology (Santa Cruz, CA); anti–FLAG Ab, anti–hemagglutinin Ab, and anti–β-actin Ab from Sigma-Aldrich Japan; and anti-lamin B1 Ab from Invitrogen.

**Immunoprecipitation assay**

Transfected cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitor mixture and centrifuged at 16,000 × g for 30 min. The supernatant was incubated with anti–FLAG M2 Sepharose (Sigma-Aldrich) for 4 h at 4°C, and protein complexes were eluted by boiling in SDS sample buffer and subjected to immunoblot analysis.

**Fractionation of cytoplasmic and nuclear proteins**

Cells were lysed with a ProteoExtract Subcellular Proteome Extraction Kit (Reagent (Merck, Darmstadt, Germany) to separate cytoplasmic and nuclear proteins were also established by stable transfection with pRNA-U6-1.6-neo (GenScript, Piscataway, NJ).

**Transient transfection**

Using electroporation, NRK-52E cells were transiently transfected with pcDNA-LAP, pcDNA-LIP (provided by J. Friedman, University of Colorado, Denver, CO) (28), pRNA-U6-1.1-si/C/EBPβ, pRNA-U6-1.6-neo, pNFκB-Luc (Panomics, Fremont, CA), pCAX-F-XBP1ΔBD-D-Luc (provided by T. Iwakagi, Gunma University, Maebashi, Japan) (29), pERS-E-Luc, pUPRE-Luc (provided by L. H. Glimcher, Harvard Medical School, Boston, MA) (30), pCMV2–FLAG-TAK1, pDNA-HA-TAB1 (provided by H. Sakurai, University of Toyama, Toyama, Japan) (31), or pCDNA3-HO-1 (provided by M. P. Soares, Instituto Gulbenkian de Ciência Oeiras, Portugal) (32). pCDNA3-HO-1 codes for full-length rat heme oxygenase-1 (HO-1), pCDNA-LAP and pCDNA-LIP encode liver-enriched activating protein (LAP) and liver inhibitory protein (LIP), respectively. pNFκB-Luc, pERSE-Luc, and pUPRE-Luc introduce a luciferase gene under the control of the κB sites, the endoplasmic reticulum (ER) stress response element (ERSE), and the UPR element (UPRE), respectively. Using GeneJuice (Novagen, Madison, WI), p65−/− MEF were transiently transfected with pCDNA3-p65WT or pCDNA3-p65(C38S) (provided by M. Nishida, Kyushu University, Fukuoka, Japan) (33) and subjected to analyses. HEK293 cells were also cotransfected with pCR-FLAG-TRA2F (provided by H. Nakano) together with pCheno-HA-Ub (provided by M. Fujimuro, Kyoto Pharmaceutical University, Kyoto, Japan) and subjected to immunoprecipitation assay.
fractions, according to the manufacturer’s protocol. Nuclear extracts were subjected to Western blot analysis. As a loading control, levels of lamin B1 were used.

Detection of superoxide
Cells were loaded with superoxide detection reagent (Enzo Life Sciences, Farmingdale, NY) for 30 min in the absence or presence of NAC, stimulated with DHMEQ for 3 h, and subjected to fluorescence microscopy.

Formazan assay
The number of viable cells was assessed by a formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan).

Animal experiment
C57BL/6 male mice (18–25 g body weight) were injected with PBS or DHMEQ (15 mg/kg) i.p. After 6 h, kidneys were subjected to Northern blot analysis of GRP78, CHOP, and C/EBPβ.

Statistical analysis
In reporter assays and formazan assay, experiments were performed in quadruplicate, and data were expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. A p value <0.05 was considered to indicate a statistically significant difference.

Results

Suppression of cytokine-triggered NF-κB activation by DHMEQ upstream of IKK

We examined an effect of pre-exposure to DHMEQ on TNF-α–triggered activation of NF-κB. NRK-52E cells transfected with pNFκB-Luc were pretreated with DHMEQ for 6 h, exposed to TNF-α for 6 h, and subjected to luciferase assay. Reporter assay showed that DHMEQ markedly suppressed activation of NF-κB.
(Fig. 1A). It was correlated with blunted induction of MCP-1, a NF-κB–dependent gene (Fig. 1B). Consistent with these results, nuclear translocation of p65 was also inhibited by the treatment with DHMEQ (Fig. 1C).

Previous reports showed that phosphorylation of p65 at Ser\(^{536}\) is required for TNF-α– and LPS-induced activation of NF-κB (40, 41). We examined whether DHMEQ affects TNF-α–induced phosphorylation of p65. As shown in Fig. 1D, pre-exposure to DHMEQ for 6 h markedly inhibited phosphorylation of p65. Interestingly, this inhibitory effect was not obvious in short-term (1-h) pre-exposure to DHMEQ. Because DHMEQ is quickly transported into cells and binds to NF-κB subunits within 15 min (42), inhibition of p65 phosphorylation by DHMEQ in the later phase may be caused independently of physical interaction between p65 and DHMEQ. To examine this possibility, p65-knockout (p65\(^{-/-}\)) MEF were transfected with wild-type p65 or C38S-mutated p65 that does not allow for binding of DHMEQ (11). The cells were then pretreated with DHMEQ, exposed to TNF-α, and subjected to Western blot analysis of Ser\(^{536}\)-phosphorylated p65. Like in wild-type p65-transfected cells, DHMEQ similarly blocked phosphorylation of p65 in C38S-mutated p65-transfected cells (Fig. 1E). This result suggested that the suppression of p65 phosphorylation by DHMEQ is independent of its direct binding to p65 and, possibly, by interfering with upstream signaling events.

It is known that phosphorylation of p65 at Ser\(^{536}\) by TNF-α is mediated by IKK complex. Activated IKK also induces phosphorylation and consequent degradation of IκBα (43). To further confirm our conclusion, we examined an effect of DHMEQ pre-treatment on phosphorylation and degradation of IκBα. In parallel with the kinetics of p65 phosphorylation, DHMEQ also suppressed TNF-α–induced phosphorylation and degradation of IκBα in NRK-52E cells (Fig. 1F, left). This phenomenon was similarly observed in MEF and HEK293 cells (Supplemental Fig. 1A). Moreover, suppression of p65 phosphorylation and IκBα phosphorylation/degradation by DHMEQ was also observed in IL-1β–stimulated cells (Fig. 1F, right). These results suggest that, in the later phase, DHMEQ inhibits NF-κB signaling not only by inhibition of p65 nuclear transport, but also by inhibiting some events at the level of or upstream of IKK. Indeed, pre-exposure to DHMEQ suppressed phosphorylation of IκKα/β in both TNF-α– and IL-1β–stimulated cells (Fig. 1G, 1H).

**Suppression of cytokine-triggered activation of NF-κB through inhibition of TAK1/TAB complex**

To elucidate how DHMEQ inhibits TNF-α–triggered activation of IKK, we first examined effects of DHMEQ on the level of signaling molecules essential for IKK activation. For this purpose, NRK-52E cells were pretreated with DHMEQ, exposed to TNF-α, and subjected to Western blot analysis of TNFR1, TRAF2, RIP1, TAK1, and IKK. As shown in Fig. 2A, none of these molecules were downregulated by sustained exposure to DHMEQ. However, DHMEQ significantly suppressed TNF-α– and IL-1β–triggered phosphorylation of TAK1 in NRK-52E cells and HEK293 cells (Fig. 2B, 2C, Supplemental Fig. 1B). In the TNF-α– and IL-1β–triggered NF-κB signaling, molecular events upstream of TAK1/TAB complex are different (44). Our results, therefore, raise a possibility that DHMEQ inhibits cytokine-induced activation of NF-κB at the level of TAK1/TAB complex. To further examine this possibility, we tested whether DHMEQ blocks activation of TAK1-IKK signaling caused by overexpression of TAK1 and TAB1. However, phosphorylation of TAK1 and IKKα/β caused by overexpression of TAK1/TAB1 was not inhibited by DHMEQ (Supplemental Fig. 1C). This result indicates that DHMEQ influences NF-κB signaling upstream of the TAK1/TAB level.

**Inhibition of cytokine-triggered NF-κB activation through generation of ROS**

A previous report suggested that DHMEQ caused growth inhibition, caspase activation, and consequent apoptosis of cancer cells through generation of ROS (18). Although ROS is known to activate NF-κB in the early phase, sustained generation of ROS may affect the NF-κB signaling negatively (20). We examined a role of ROS in the suppressive effect of DHMEQ on NF-κB. First, the potential of DHMEQ to induce ROS generation was investigated in NRK-52E cells. Because O\(_2^-\) is the first reductant generated from oxygen molecule and a crucial precursor of other ROS, we examined production of O\(_2^-\). As shown in Fig. 3A, DHMEQ increased the level of O\(_2^-\), and it was abrogated by the treatment with antioxidant NAC. Based on this result, we next tested involvement of ROS in the suppressive effect of DHMEQ on NF-κB. Reporter assay showed that pre-exposure to DHMEQ inhibited TNF-α–induced NF-κB activation and that it was significantly reversed by the treatment with NAC (Fig. 3B). Similar result was also obtained using cells pretreated with glutathione-reduced ethyl ester, a membrane-permeable GSH analog (data not shown). Consistent with this result, inhibition of MCP-1 expression and nuclear translocation of p65 by DHMEQ was attenuated by the treatment with NAC (Fig. 3C, 3D), suggesting involvement of ROS. Blockade of TNF-α–induced phosphorylation of TAK1, IκBα, and p65 by DHMEQ was also reversed by NAC (Fig. 3E), indicating that the ROS-dependent mechanism targets the TNF-α signaling, at least in part, upstream of IKK.

This conclusion was further confirmed using menadione, a generator of O\(_2^-\). Reporter assay showed that activation of NF-κB by TNF-α was significantly suppressed by the pretreatment with menadione (Fig. 3F). Furthermore, generation of O\(_2^-\) also inhibited TNF-α–induced phosphorylation of TAK1, IκKα/β, IκBα, and p65 (Fig. 3G). These results further suggest that DHMEQ-induced ROS generation inhibits TNF-α–triggered activation of NF-κB upstream of TAK1/TAB complex.
**Induction of the UPR by DHMEQ through generation of ROS**

DHMEQ caused ROS production in NRK-52E cells (Fig. 3A). Previous reports indicated that ROS may have the potential to induce ER stress and consequent activation of the UPR in cadmium- and cigarette smoke–exposed cells (45, 46). Indeed, in human liver cancer cells, high concentrations of DHMEQ (20–40 μg/ml) caused ER stress through generation of ROS (18). Moreover, another line of evidence suggested that the UPR affects activity of NF-κB positively or negatively, depending on cellular contexts (47). Therefore, we hypothesized that DHMEQ could inhibit TNF-α–induced NF-κB activation via ROS-mediated induction of the UPR. To examine this possibility, we first tested whether DHMEQ induces the UPR in our experimental setting. The UPR involves three major transducers, including PERK, ATF6, and inositol-requiring enzyme 1. Under ER stress condition, activation of PERK leads to phosphorylation of eIF2α that promotes induction/activation of ATF4 and expression of CHOP, p90ATF6, an inactive form of ATF6, is also cleaved in the Golgi apparatus, and the resultant p50ATF6 activates ERSE and consequent induction of ER chaperones, including GRP78. Similarly, activated inositol-requiring enzyme 1 catalyzes removal of a small intron from the mRNA of X-box–binding protein 1 (XBP1), leading to production of active transcription factor XBP1 and consequent activation of UPRE (48). Based on this current knowledge, NRK-52E cells were treated with DHMEQ for different time periods and subjected to Northern and Western blot analyses of ER stress markers. As shown in Fig. 4A, DHMEQ rapidly induced expression of GRP78, CHOP, and ATF4. DHMEQ also induced phosphorylation of PERK and eIF2α (Fig. 4B), activation of ERSE (Fig. 4C), splicing of XBP1 mRNA (Fig. 4D), and consequent activation of UPRE (Fig. 4E). These results show that DHMEQ has the potential to activate three major branches of the UPR.
To examine whether the induction of the UPR by DHMEQ is attributable to the ROS generation, cells were treated with DHMEQ in the absence or presence of NAC and subjected to Northern blot analysis and reporter assays. As shown in Fig. 4F and 4G, DHMEQ-induced expression of GRP78 and CHOP and phosphorylation of eIF2α were attenuated by the treatment with NAC. Furthermore, activation of ERSE and splicing of XBP1 by DHMEQ were also suppressed in the presence of NAC (Fig. 4H, 4I). These results clearly show that DHMEQ induces the UPR in the absence or presence of NAC and subjected to Northern blot analysis of GRP78 and CHOP (F) or Western blot analysis of eIF2α (G). (H and I) Cells were transfected with pERSE-Luc (H) or pCAX-F-XBP1ΔDBD-Luc (I), exposed to DHMEQ in the absence or presence of NAC, and subjected to luciferase assay. *p < 0.05.

Involvement of the UPR in the suppressive effect of DHMEQ on NF-κB

We examined involvement of the UPR in the suppressive effect of DHMEQ on NF-κB. First, cells were pretreated with serial concentrations of DHMEQ and stimulated with TNF-α, and relationship between the level of the UPR and the extent of NF-κB inhibition was examined. As shown in Fig. 5A, DHMEQ-induced expression of GRP78 and CHOP in a dose-dependent manner, and it was inversely correlated with dose-dependent suppression of MCP-1 (Fig. 5A). Reporter assay also showed that dose-dependent induction of ER stress markers was inversely correlated with dose-dependent suppression of NF-κB (Fig. 5B). To confirm that ER stress is indeed responsible for the suppression of NF-κB by DHMEQ, we established ER stress-resistant NRK/ORP150 cells. ORP150 is an ER chaperon that protects cells from ER stress-induced injury (49, 50). The established NRK/ORP150 cells expressed ORP150 abundantly, and it was correlated with attenuated induction of CHOP protein in response to DHMEQ (Supplemental Fig. 2A). In these ER stress-resistant cells, the suppressive effect of DHMEQ on MCP-1 was modestly, but significantly diminished (Fig. 5C).

To further examine the site of action for DHMEQ-induced ER stress, mock-transfected cells and NRK/ORP150 cells were pre-treated with DHMEQ, exposed to TNF-α, and subjected to Western blot analysis of IkBα and phosphorylated p65. As shown in Fig. 5D, attenuation of ER stress by ORP150 did not affect the suppressive effects of DHMEQ on IkBα degradation and p65 phosphorylation (Fig. 5D). This result was reproducible when chemical chaperon 4-PBA was used to attenuate ER stress. That is, attenuation of ER stress by 4-PBA did not affect the inhibitory effect of DHMEQ on TNF-α-triggered IkBα degradation and p65 phosphorylation (Fig. 5E). Of note, 4-PBA effectively attenuated ER stress, which was evidenced by reduction in phosphorylation of eIF2α and expression of GRP78 and CHOP (Supplemental Fig. 2B). Taken together, these results suggest that DHMEQ inhibits activation of NF-κB through the UPR downstream of the IKK–IkB level.

Induction of C/EBPβ by DHMEQ through the ROS–ER stress pathway

We investigated mechanisms underlying the ER stress-mediated suppression of NF-κB by DHMEQ. As described, DHMEQ-triggered UPR contributed to inhibition of NF-κB downstream of the level of IkBα degradation. Although sustained ER stress may affect TNF-α signaling via downregulation of TRAF2, dephosphorylation of Akt, and upregulation of A20 (endogenous inhibitor of NF-κB) (26, 51–53), these molecular events locate upstream of IKK (26) and, therefore, should not be major targets of DHMEQ. Indeed, the level of TRAF2 was not affected by 6-h pre-exposure with DHMEQ (Fig. 2A). Similarly, DHMEQ did not depress the level of phosphorylated Akt (data not shown) and did not induce expression of A20 (53). One possible candidate that contributes to the UPR-mediated inhibition of NF-κB downstream
of IKK is C/EBPβ. C/EBPβ is a family of transcription factors that contain a highly conserved, basic-leucine zipper domain at the C terminus. A previous report showed that some C/EBP may be induced under ER stress (54). Another report also indicated that C/EBP physically interacted with p65 and thereby inhibited NF-κB–mediated transcription (55). The UPR triggered by DHMEQ could induce C/EBPs and thereby suppress activation of NF-κB.

To examine this possibility, cells were treated with DHMEQ, and expression of C/EBP family members was tested. As shown in Fig. 6A, DHMEQ preferentially induced expression of C/EBPβ mRNA without inducing C/EBPα and C/EBPδ. It was associated with upregulation of C/EBPβ proteins, LAP and LIP (Fig. 6B). Another UPR inducer, thapsigargin, similarly induced C/EBPβ (Supplemental Fig. 3A). The induction of C/EBPβ proteins by DHMEQ was attenuated by the treatment with 4-PBA (Fig. 6C), suggesting a role of the UPR. Furthermore, the induction of C/EBPβ mRNA and C/EBPβ proteins by DHMEQ was attenuated by the treatment with NAC (Fig. 6D, 6E). These results suggest that DHMEQ upregulates C/EBPβ through the ROS-mediated UPR. Of note, the induction of C/EBPβ by DHMEQ was observed not only in culture cells, but also under in vivo situations. As demonstrated in Supplemental Fig. 3B, i.p. injection of DHMEQ induced expression of C/EBPβ in rat kidneys, and it was associated with induction of ER stress.

Involvement of C/EBPβ in the UPR-mediated inhibition of NF-κB

The C/EBPβ gene encodes three distinct proteins, including LAP1, LAP2, and LIP. To examine involvement of C/EBPβ in the inhibition of NF-κB by DHMEQ, cells were cotransfected with pNFκB-Luc together with LAP or LIP. The cells were then exposed to TNF-α and subjected to luciferase assay. Enhanced expression of LAP and LIP was confirmed by Western blot analysis (Supplemental Fig. 3C). As shown in Fig. 6F, TNF-α–induced NF-κB activation was significantly inhibited by LAP or LIP. However, overexpression of LAP or LIP did not affect TNF-α–triggered degradation of IκBα and phosphorylation of p65 (Fig. 6G), indicating that induction of C/EBPβ suppressed activation of NF-κB downstream of IκBα degradation. Based on this result, we tested whether C/EBPβ affects nuclear translocation of p65.
As shown in Fig. 6H, overexpression of LAP and LIP inhibited nuclear translocation of p65 in response to TNF-α. This result indicates a possibility that binding of C/EBPβ interferes with nuclear translocation of p65 even when p65 is fully phosphorylated.

To further confirm involvement of C/EBPβ in the suppression of NF-κB by TNF-α, cells were cotransfected with pNFκB-Luc together with control siRNA or siC/EBPβ. The cells were then treated with DHMEQ, exposed to TNF-α, and subjected to luciferase assay. Knockdown of C/EBPβ by siRNA was confirmed by Western blot analysis (Supplemental Fig. 3D). Reporter assay showed that knockdown of C/EBPβ partially, but significantly, reversed the suppressive effect of DHMEQ on NF-κB (Fig. 6I). Consistent with the result shown in Fig. 6G, knockdown of C/EBPβ had no effect on the suppressive effect of DHMEQ on TNF-α-induced phosphorylation of IkBα and phosphorylation of p65 (Fig. 6J). These results suggest that DHMEQ suppresses activation of NF-κB through UPR-mediated induction of C/EBPβ downstream of the IKK–IκB level (Fig. 7).

Discussion

DHMEQ is a novel NF-κB inhibitor expected to be a potent therapeutic agent for inflammatory and malignant diseases (56). Previous reports showed that, in the early phase, DHMEQ directly binds to specific cysteine residues of NF-κB subunits and thereby inhibits their nuclear translocation and DNA binding (Fig. 7, left). In this work, we describe additional mechanisms by which DHMEQ suppresses cytokine-triggered activation of NF-κB. Our current results suggest that, in the later phase, DHMEQ interferes with cytokine-induced NF-κB signaling via multiple mechanisms. That is, DHMEQ causes generation of ROS that contributes to suppression of TNF-α signaling at both upstream and downstream of the IKK–IκB level. One possible target is upstream signaling that activates TAK1, the phosphorylation of which is inhibited by ROS. Another possible target of ROS is C/EBPβ that is induced by DHMEQ via the ROS–UPR pathway and inhibits nuclear translocation of NF-κB through physical interaction (Fig. 7, right).

Currently, it is unknown how DHMEQ suppresses cytokine-induced phosphorylation of TAK1 through ROS. We found that overexpressed TAB1-mediated phosphorylation of TAK1 was not inhibited by DHMEQ (Supplemental Fig. 1C), indicating that DHMEQ targets upstream molecules, for example, TNFR1, TRAF2, RIP1, or TAB2/3. Of note, DHMEQ did not downregulate protein levels of TNFR1, TRAF2, RIP1, and TAB2 (Fig. 2A, Supplemental Fig. 1D). Previous reports showed that oxidative...
stress inhibits activity of ubiquitin-conjugating enzymes (57, 58). TRAF2 is an E3 ubiquitin ligase and generates K63 polyubiquitin chains on RIP1 (4). Polyubiquitination of TRAF2 is also important for activation of NF-κB in response to TNF-α (59). Based on this current knowledge, we tested a possibility that DHMEQ could suppress NF-κB through blockade of TNF-α–induced TRAF2 polyubiquitination. However, immunoprecipitation assay showed that DHMEQ had little impact on polyubiquitination of TRAF2 (Supplemental Fig. 1E), excluding this possibility. Taken together, these results support an idea that the primary targets of DHMEQ may be TAB2/3. Recently, Zhang et al. (60) reported that TAB2/3 were directly inactivated through modification of cysteine residues in the Npl4 zinc finger domain. One possible mechanism may be that DHMEQ-triggered ROS generation causes oxidative inactivation of TAB2/3 through similar mechanisms. It is known that various signaling molecules are modified posttranslationally through S-glutathionylation of cysteine residues (61–64). In particular, molecules involved in the NF-κB signaling are sensitive to this modification. For example, a previous study showed that S-glutathionylation of Cys62 of p50 blocked DNA binding of NF-κB (65). Another report also showed that S-glutathionylation of IKKβ at Cys179 was caused by exposure to H2O2, leading to inactivation of NF-κB (66). The similar mechanism could be involved in the ROS-mediated suppression of TAB by DHMEQ. Our preliminary data showed that a reducing agent DTT partially reversed the inhibitory effect of DHMEQ on p65 phosphorylation (data not shown).

Another possible mechanism underlying inhibition of NF-κB by ROS could involve HO-1. HO-1 is an enzyme that catalyzes heme into three products, including carbon monoxide, biliverdin, and free iron, all of which mediate anti-inflammatory, antiapoptotic, and antiproliferative actions of HO-1 (67). HO-1 is known to be activated by oxidative stress (68, 69), and a number of reports showed that HO-1 has the potential to suppress activation of NF-κB (70–72). We found that DHMEQ induced expression of HO-1 mRNA and that it was attenuated by NAC in NRK-52E cells (Supplemental Fig. 4A). Furthermore, transfection with HO-1 modestly, but significantly, attenuated TNF-α–triggered NF-κB activation (Supplemental Fig. 4B). Although its contribution may be minor, the HO-1–mediated mechanism could also be involved in the ROS-mediated suppression of NF-κB by DHMEQ.

In the present investigation, we showed that DHMEQ induced the UPR through generation of ROS, which contributed to the suppression of NF-κB. Currently, it is unclear how ROS causes ER stress. Previous reports showed that oxidative stress induced depletion of calcium store in the ER through inhibition of Ca2+-ATPase and thereby disturbed ER function (73, 74). However, ROS could cause ER stress in a Ca2+-independent manner. For example, sustained oxidative stress may cause inactivation of the proteasome system (21) or affect polyubiquitination process via oxidation of E3 ubiquitin ligase (75), leading to accumulation of unfolded proteins. The similar mechanisms may also be involved in the ROS-mediated induction of the UPR by DHMEQ.

In this work, we showed that DHMEQ induced C/EBPβ via the oxidative stress–UPR pathway. The C/EBPβ gene encodes three distinct proteins, including LAP1, LAP2, and LIP. In contrast to LAP, LIP lacks the transactivation domain, but can dimerize and bind to DNA. LIP, therefore, acts as a dominant-negative inhibitor of the transcription factor LAP. In the previous reports, the human C/EBPβ gene has a UPRE at the 3′ end, and ER stress can induce expression of C/EBPβ through activation of this element (54, 76). Possibly, DHMEQ may induce C/EBPβ through a similar mechanism.

At present, molecular mechanisms by which C/EBPβ inhibits TNF-α–triggered activation of NF-κB are largely unknown. Stein et al. (77) demonstrated that recombinant C/EBPβ inhibited the ability of NF-κB subunits to bind to the κB enhancer motifs through physical interaction between the basic-leucine zipper region of C/EBP and the Rel homology domain of NF-κB subunits. In the current study, however, we observed that overexpression of LAP and LIP blocked nuclear translocation of p65 in NRK-52E cells (Fig. 6H). Using B lymphocytes, Takeiri et al. (78) suggested that loss of DNA-binding activity of RelB reduced its stabilization and affinity to importin that mediates nuclear protein import, resulting in altered intracellular localization of RelB. C/EBPβ might bind to p65 and reduce its DNA-binding activity, leading to attenuated nuclear translocation of p65 through blockade of physical interaction between p65 and importin.

Hu et al. (79) suggested that preconditioning with ER stress depressed the level of TRAF2, which may impair TNF-α–triggered activation of NF-κB. We also reported that long-term exposure of renal cells to ER stress caused downregulation of TRAF2 (26, 51, 80). Subsequent experiments showed that ER stress accelerated degradation of TRAF2 protein through ER-associated degradation (79). In the current study, we did not observe degradation of TRAF2 by DHMEQ in the early phase (Fig. 2A). However, in the later phase, DHMEQ could have the po-
tential to degrade TRAF2 through ER stress and thereby inhibit activation of NF-κB by TNF-α.

NF-κB inhibitors have been regarded as potential therapeutic agents for various inflammatory and malignant disorders. However, clinical use of conventional NF-κB inhibitors, for example, proteasome inhibitors, is still limited because of their insufficient effectiveness and side effects. Compared with conventional NF-κB inhibitors, DHMEQ has several advantages as therapeutic agents. First and foremost, it has stronger potential to inhibit NF-κB signaling at multiple target sites, as described in this work. In vivo, DHMEQ can be administered not only i.p., but also s.c. or transcutaneously (56). DHMEQ quickly enters the cells and immediately suppresses NF-κB (42). Furthermore, short exposure to DHMEQ may be sufficient to inhibit NF-κB activation for sustained periods (42). It could be caused by induction of the UPR. These properties of DHMEQ allow for quick, intense, and long-lasting inhibition of NF-κB without significant adverse effects, which is ideal for an anti-inflammatory agent (56).

In summary, we demonstrated multipotent inhibition of NF-κB by DHMEQ. In addition to the mechanism described previously, other pharmacological actions are also involved, especially in the late phase. In the early phase, DHMEQ quickly binds to NF-κB subunits and blocks its nuclear translocation. However, in the later phase, generation of ROS and consequent UPR also contributes to the inhibition of NF-κB. These mechanisms enable DHMEQ to exert the potent, long-lasting anti-inflammatory/anti-cancer activities that have been reported in a wide range of diseases (56).

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Disclosures
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**Supplemental Figure Legends**

**Figure S1.** Effects of DHMEQ on NF-κB signaling molecules. (A) MEF (left) and HEK293 cells (right) were pretreated with or without DHMEQ (MEF; 10 μg/ml, HEK293; 5 μg/ml) for 6 h, exposed to 10 ng/ml TNF-α for 15 min and subjected to Western blot analysis. The level of β-actin is shown at the bottom as a loading control. An asterisk indicates a non-specific band. (B) HEK293 cells were pretreated with or without DHMEQ for 6 h, exposed to TNF-α for 5 min and subjected to Western blot analysis of phosphorylated and total TAK1. (C) NRK-52E cells were co-transfected with empty vector or pcDNA-HA-TAB1 together with pCMV2-FLAG-TAK1, treated with DHMEQ for 6 h and subjected to Western blot analysis of indicated molecules. (D) NRK-52E cells were treated with or without DHMEQ for 6 h, exposed to TNF-α for 5 min and subjected to Western blot analysis of TAB2. (E) HEK293 cells were co-transfected with pCR-FLAG-TRAF2 together with pCIneo-HA-Ub. The cells were then treated with or without DHMEQ for 6 h, exposed to TNF-α for 5 min and subjected to immunoprecipitation using anti-FLAG M2 sepharose and subsequent immunoblot analysis of HA.

**Figure S2.** Attenuation of DHMEQ induced activation of the UPR by genetic and chemical chaperones. (A) NRK/Neo cells and NRK/ORP150 cells were treated with or without DHMEQ for 6 h and subjected to Western blot analysis of CHOP. (B) NRK-52E cells were treated with DHMEQ in the absence or presence of 5 mM 4-PBA, and the level of phosphorylated eIF2α (left) and induction of GRP78 and CHOP (right) were evaluated by Western blot analysis and Northern blot analysis, respectively. The level of
GAPDH is shown at the bottom as a loading control.

**Figure S3.** Induction of C/EBPβ by DHMEQ *in vitro* and *in vivo*. (A) NRK-52E cells were treated with 500 nM thapsigargin (Tg) for up to 12 h and subjected to Western blot analysis of C/EBPβ (LAP and LIP). (B) Mice were administered with PBS or 15 mg/kg DHMEQ intraperitoneally, and after 6 h, kidneys were subjected to Northern blot analysis of C/EBPβ and CHOP. (C) NRK-52E cells were transfected with pcDNA3.1, pcDNA-LAP or pcDNA-LIP and subjected to Western blot analysis of C/EBPβ. (D) NRK/siControl cells and NRK/siC/EBPβ cells were treated with or without DHMEQ for 12 h and subjected to Western blot analysis of C/EBPβ.

**Figure S4.** Involvement of HO-1 in the suppression of NF-κB by DHMEQ. (A) NRK-52E cells were treated with DHMEQ for up to 6 h in the absence or presence of 1 mM NAC and subjected to Northern blot analysis of HO-1. (B) NRK-52E cells were co-transfected with empty vector or pcDNA-HO-1 together with pNFκB-Luc. The cells were then treated with or without TNF-α and subjected to luciferase assay (left). Expression of exogenous HO-1 was confirmed by Northern blot analysis (right). Experiments were performed in quadruplicate, and data are expressed as means ± SE. An asterisk indicates a statistically significant difference (*p* < 0.05).
Fig. S3

A

Tg (h): 0  6  12
C/EBPβ
β-actin

B

DHMEQ: - + +
C/EBPβ
CHOP
GAPDH

C

pcDNA3.1 pcDNA-LAP pcDNA-LIP
C/EBPβ
β-actin

D

siControl siC/EBPβ
DHMEQ: + + +
C/EBPβ
β-actin

Fig. S4

A

DHMEQ (h): 0  3  6  0  3  6
NAC
HO-1
GAPDH

B

pcDNA3.1 HO-1
HO-1
GAPDH

Normalized NF-κB activity (%)

TNF-α: - +