

NF- κ B inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death

Sachiko Sakon^{1,2}, Xin Xue¹,
Mutsuhiro Takekawa^{3,4},
Tomonari Sasazuki¹, Tatsuma Okazaki¹,
Yuko Kojima¹, Jian-Hu Piao¹, Hideo Yagita¹,
Ko Okumura¹, Takahiro Doi⁵ and
Hiroyasu Nakano^{1,2,6}

¹Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, ²Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Corporation (JST), 1-11-2 Yoyogi, Shibuya-ku, Tokyo 151-0053, ³Division of Molecular Cell Signaling, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, ⁴PRESTO, JST, Kawaguchi, Saitama 332-0012 and ⁵Subteam for BioResponse Integration, RIKEN (Institute of Physical and Chemical Research), BioResource Center, Tsukuba, Ibaraki 305-0074, Japan

⁶Corresponding author
e-mail: hnakano@med.juntendo.ac.jp

NF- κ B downregulates tumor necrosis factor (TNF)-induced c-Jun N-terminal kinase (JNK) activation that promotes cell death, but the mechanism is not yet fully understood. By using murine embryonic fibroblasts (MEFs) that are deficient in TNF receptor-associated factor (TRAF) 2 and TRAF5 (DKO) or p65 NF- κ B subunit (p65KO), we demonstrate here that TNF stimulation leads to accumulation of reactive oxygen species (ROS), which is essential for prolonged mitogen-activated protein kinase (MAPK) activation and cell death. Interestingly, dying cells show necrotic as well as apoptotic morphological changes as assessed by electron microscopy and flow cytometry, and necrotic, but not apoptotic, cell death is substantially inhibited by antioxidant. Importantly, TNF does not induce ROS accumulation or prolonged MAPK activation in wild-type MEFs, indicating that TRAF-mediated NF- κ B activation normally suppresses the TNF-induced ROS accumulation that subsequently induces prolonged MAPK activation and necrotic cell death

Keywords: MAPK/NF- κ B/ROS/TNF/TRAF

Introduction

Tumor necrosis factor (TNF) exerts a variety of biological effects, including production of inflammatory cytokines, upregulation of adhesion molecules, proliferation, differentiation and cell death (Tracey and Cerami, 1994). While such pleiotropic effects are mediated by two cognate TNF receptors, TNF-R1 and TNF-R2, TNF-induced cell death is mainly mediated by TNF-R1. In response to TNF, TNF-R1 is trimerized and recruits an adapter molecule, TRADD. In the apoptotic signaling pathway, the recruited TRADD interacts with FADD, which then recruits and

activates caspase-8 (Mak and Yeh, 2002; Wallach *et al.*, 2002). The activated caspase-8 in turn activates effector caspases, such as caspase-3 and -7, resulting in apoptosis. On the other hand, recruited TRADD directly or indirectly interacts with RIP, TNF receptor-associated factor (TRAF) 2 and TRAF5, which are implicated in nuclear factor (NF)- κ B and c-Jun N-terminal kinase (JNK) activation (Mak and Yeh, 2002; Wallach *et al.*, 2002). TRAFs have been identified as being signaling intermediates in both TNF receptor and interleukin (IL)-1 receptor/Toll-like receptor (TLR) superfamilies (Inoue *et al.*, 2000; Chung *et al.*, 2002). TRAFs interact directly or indirectly with several mitogen-activated protein kinase (MAPK) kinase kinases (MAPKKKs), including apoptosis signal-regulating kinase (ASK) 1, TAK1 and MEKK1, and thereby activate MAPK cascades (Nishitoh *et al.*, 1998; Baud *et al.*, 1999; Ninomiya-Tsuji *et al.*, 1999). TRAF2, TRAF5 and TRAF6 have been shown to be involved in TNFR- and IL-1R/TLR-mediated NF- κ B and MAPK activation (Yeh *et al.*, 1997; Lomaga *et al.*, 1999; Naito *et al.*, 1999; Tada *et al.*, 2001).

The MAPK cascades are activated by various cellular stresses and growth factors, and are involved in various biological responses such as cytokine production, differentiation, proliferation and cell death (Ichijo, 1999; Davis, 2000). In mammals, MAPK cascades are composed of three distinct signaling modules, JNK, p38 MAPK and extracellular signal-regulated kinase (ERK). Upon cytokine or growth factor stimulation, or in response to various stresses, MAPKKKs are rapidly activated by oligomerization or undefined mechanisms, and phosphorylate downstream MAP kinases, called MAPK kinases (MAPKKs) (Ichijo, 1999; Davis, 2000). Then, activated MAPKKs finally phosphorylate and activate MAPKs, which in turn phosphorylate specific targets and activate their transcriptional activities. TNF- and IL-1-induced MAPK activation are usually rapid and transient with a peak at ~10 min and then declining to basal level by 60 min. On the other hand, genotoxic stresses such as UV or γ -irradiation induce long-lasting or prolonged MAPK activation. Several lines of evidences suggested that transient MAPK activation is associated with gene expression, proliferation or differentiation, whereas prolonged MAPK activation promotes cell death in a cell type- and stimulus-dependent manner (Xia *et al.*, 1995; Chen *et al.*, 1996; Guo *et al.*, 1998).

NF- κ B is a transcriptional factor that regulates expression of various inflammatory cytokines, chemokines, and adhesion molecules (Ghosh *et al.*, 1998). NF- κ B is activated by inflammatory cytokines and cellular stresses, including TNF, IL-1, lipopolysaccharide (LPS), UV, or γ -irradiation. NF- κ B also plays a critical role in protection from TNF-induced cell death (Barkett and Gilmore, 1999; Karin and Lin, 2002). Cells lacking NF- κ B subunit p65 (p65), I κ B kinase β (IKK β) and I κ B kinase γ

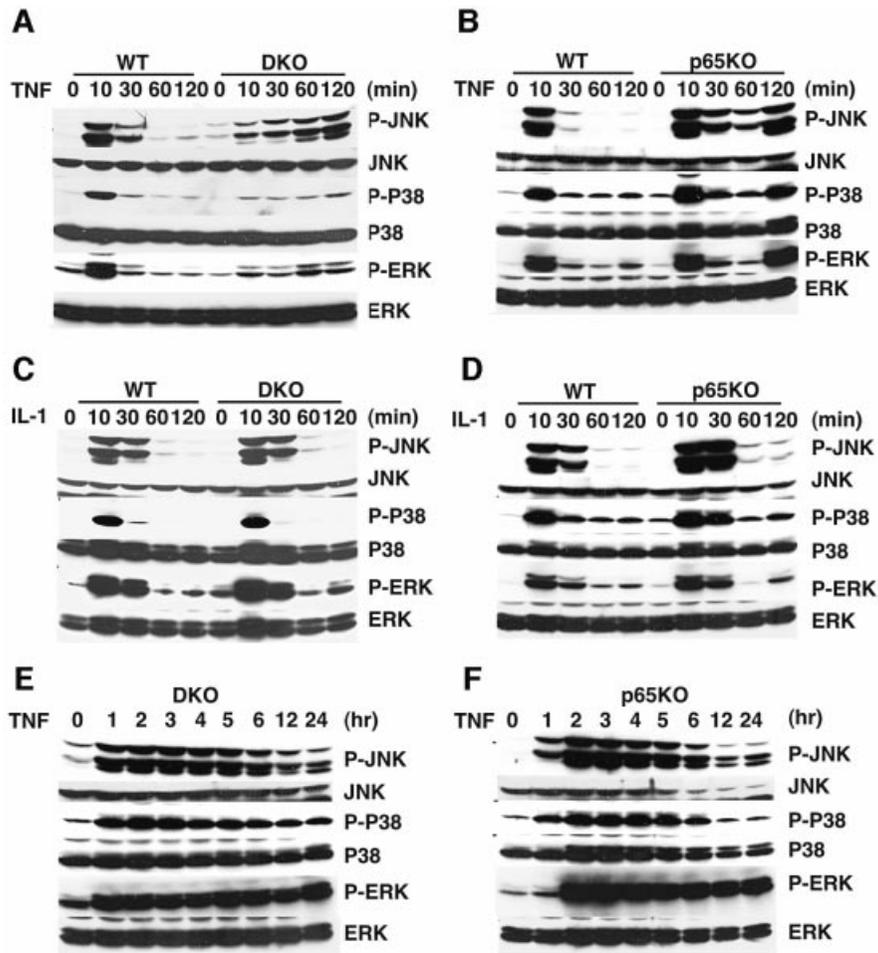


Fig. 1. Prolonged MAPK activation by TNF, but not IL-1, in DKO and p65KO MEFs. (A, B, E and F) Wild-type, DKO and p65KO MEFs were stimulated with TNF (50 ng/ml) for the indicated time periods, and then the lysates were blotted with antibodies specific for the activated form of JNK (phospho-JNK), p38 (phospho-p38) or ERK (phospho-ERK). The membranes were reblotted with antibodies to total JNK, p38 or ERK. (C and D) Wild-type, DKO and p65KO MEFs were stimulated with IL-1 (50 ng/ml) for the indicated time periods and the lysates were blotted as above.

(IKK γ)/NEMO show increased sensitivity to TNF-induced cell death (Barkett and Gilmore, 1999; Karin and Lin, 2002). One of the mechanisms by which NF- κ B inhibits TNF-induced cell death is to upregulate anti-apoptotic genes including the Bcl-2 and XIAP families, and FLIP (Barkett and Gilmore, 1999; Karin and Lin, 2002). However, the survival signals elicited by NF- κ B are not fully understood. Recently, several groups reported that TNF, but not IL-1, induces prolonged JNK activation in cells lacking p65, IKK β , or stably expressing super-repressor of NF- κ B, and this prolonged JNK activation participates in TNF-induced cell death (De Smaele *et al.*, 2001; Javelaud and Besancon, 2001; Tang *et al.*, 2001). These studies also revealed that NF- κ B upregulates the expression of XIAP and GADD45 β , which downregulate TNF-induced JNK activation. However, the molecular mechanism by which XIAP and GADD45 β inhibit TNF-induced JNK activation remains unknown.

Reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide and hydroxyl radicals, are normally generated in the mitochondria and act as signaling intermediates (Adler *et al.*, 1999b; Thannickal and Fanburg, 2000). Under physiological conditions, generated ROS are rapidly eliminated by antioxidant

enzymes, including superoxide dismutases (SODs), catalase, glutathione peroxidases (GPxs) and peroxiredoxins (PRxs) (Thannickal and Fanburg, 2000). In various pathological conditions such as ischemia, excessively accumulated ROS induce apoptosis or necrosis by activating MAPK, caspase cascades, and/or disruption of mitochondrial membrane potential (Fiers *et al.*, 1999). However, it remains controversial whether ROS play a critical role in cytokine-induced MAPK activation under physiological conditions.

We previously demonstrated that murine embryonic fibroblasts (MEFs) derived from TRAF2 and TRAF5 double knockout (DKO) mice are defective in TNF-induced NF- κ B activation and rapid/transient JNK activation, and are highly susceptible to TNF-induced cell death (Tada *et al.*, 2001). In the present study, we found that TNF stimulation induces delayed/prolonged MAPK activation in DKO MEFs, as well as in p65KO MEFs, but not in wild-type MEFs. We have further investigated the molecular mechanism by which NF- κ B inhibits TNF-induced prolonged JNK activation and cell death. We report here that TNF induces ROS accumulation in DKO and p65KO, but not wild-type, MEFs, which is essential for TNF-induced prolonged MAPK activation and cell

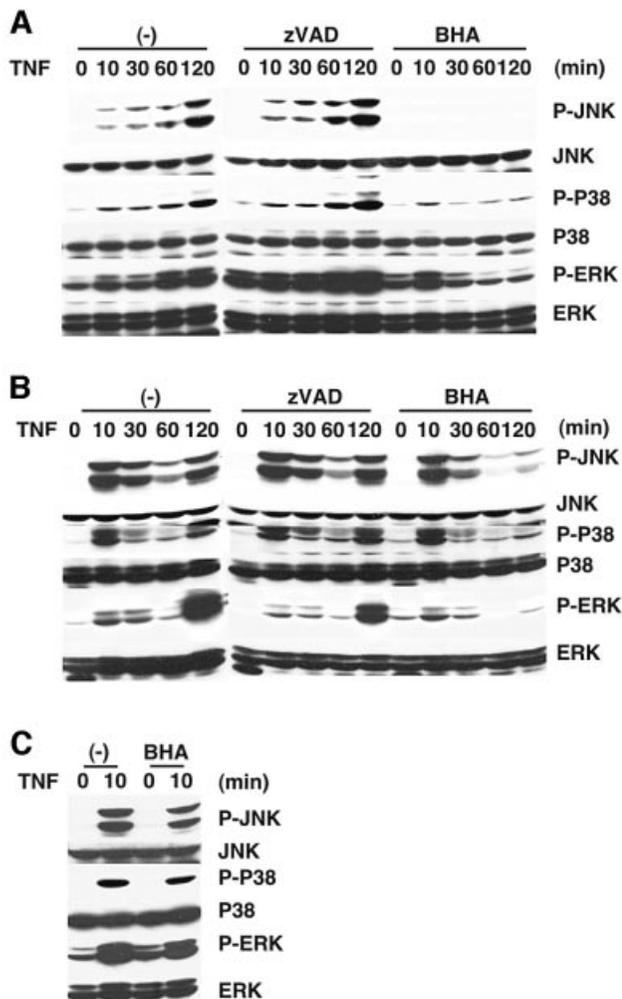


Fig. 2. BHA, but not z-VAD-fmk, inhibits prolonged MAPK activation by TNF in DKO and p65KO MEFs. (A and B) DKO (A) and p65KO (B) MEFs were untreated or pretreated with z-VAD-fmk (10 μ M) or BHA (100 μ M) for 20 min, then stimulated with TNF (50 ng/ml) for the indicated time periods. The cell lysates were analyzed as described in Figure 1. (C) Wild-type MEFs were untreated or treated with BHA (100 μ M) for 20 min, then stimulated with TNF (50 ng/ml) for 10 min. The cell lysates were analyzed as described in Figure 1.

death. Electron microscopy and flow cytometry analyses show that TNF induces necrosis as well as apoptosis in DKO and p65KO MEFs, and necrosis is inhibited by antioxidant. Collectively, these results suggested that one of the pro-survival functions of NF- κ B is to inhibit TNF-induced ROS accumulation that mediates prolonged MAPK activation and necrotic cell death.

Results

TNF induces delayed and prolonged MAPK activation in TRAF-deficient MEFs

Prolonged JNK activation has been implicated in increased sensitivity to TNF-induced cell death. We previously demonstrated that MEFs derived from TRAF2 and TRAF5 DKO mice have a defect in TNF-induced NF- κ B and JNK activation, and show increased sensitivity to TNF-induced cell death (Tada *et al.*, 2001). Given that rapid and transient MAPK activation depends on TRAFs,

it is intriguing to test whether prolonged JNK activation is induced in TRAF-deficient cells. Thus, we first examined the time-course of JNK phosphorylation in DKO MEFs after TNF stimulation. As previously reported (Tada *et al.*, 2001), phosphorylation of JNK at 10 min was severely impaired in DKO MEFs compared with wild-type MEFs (Figure 1A). Unexpectedly, however, phosphorylation of JNK gradually increased thereafter up to 120 min after TNF stimulation (Figure 1A). Moreover, in addition to JNK, we detected prolonged phosphorylation of p38 and ERK (Figure 1A). We also confirmed that kinase activities of JNK and ERK were prolonged in DKO MEFs by immune complex kinase assays using glutathione *S*-transferase (GST)-c-Jun and MBP as substrates, respectively (data not shown).

We next investigated whether prolonged activation of p38 and ERK as well as JNK was also induced in p65KO MEFs, since previous studies showed that activation of JNK, but not ERK or p38, was prolonged in TNF-stimulated p65KO or IKK β KO MEFs (De Smaele *et al.*, 2001; Tang *et al.*, 2001). In contrast to these previous reports, activation of both p38 and ERK as well as JNK was also prolonged in TNF-stimulated p65KO MEFs (Figure 1B). On the other hand, IL-1 did not induce such a prolonged MAPK activation in DKO and p65KO MEFs (Figure 1C and D).

We also investigated duration of MAPK activation in TNF-stimulated DKO and p65KO MEFs. In both MEFs, phosphorylation of MAPKs peaked at 2 h and persisted up to 5 h, and then gradually declined (Figure 1E and F). The later decline reflected the decreased protein levels of MAPKs due to cell death (Figure 1E and F).

Prolonged MAPK activation is mediated by ROS

To explore the molecular mechanism for prolonged MAPK activation, we pretreated the cells with a broad caspase inhibitor, z-VAD-fmk, or an antioxidant, butylated hydroxyanisole (BHA), before TNF stimulation. Previous studies showed that BHA inhibits TNF-induced necrosis in murine fibrosarcoma, L929 (Vercammen *et al.*, 1998; Fiers *et al.*, 1999). As shown in Figure 2A and B, BHA, but not z-VAD-fmk, almost completely inhibited prolonged MAPK activation in DKO and p65KO MEFs. Interestingly, in contrast to the strong inhibitory effect of BHA on prolonged MAPK activation at 30 min and later, BHA only slightly inhibited early MAPK activation at 10 min in both DKO and p65KO MEFs (Figure 2A and B). Similarly, BHA only slightly inhibited activation of MAPKs at 10 min after TNF stimulation in wild-type MEFs (Figure 2C). These weak inhibitory effects of BHA on rapid and transient MAPK activation are partly consistent with previous reports demonstrating inhibitory effect of antioxidants on TNF-induced MAPK activation (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998). Collectively, these results demonstrate that transient activation and prolonged activation of MAPKs are induced by different mechanisms.

TNF induces accumulation of ROS in DKO and p65KO MEFs, but not wild-type MEFs

The fact that BHA treatment inhibited prolonged MAPK activation in DKO and p65KO MEFs prompted us to examine whether TNF stimulation induces ROS

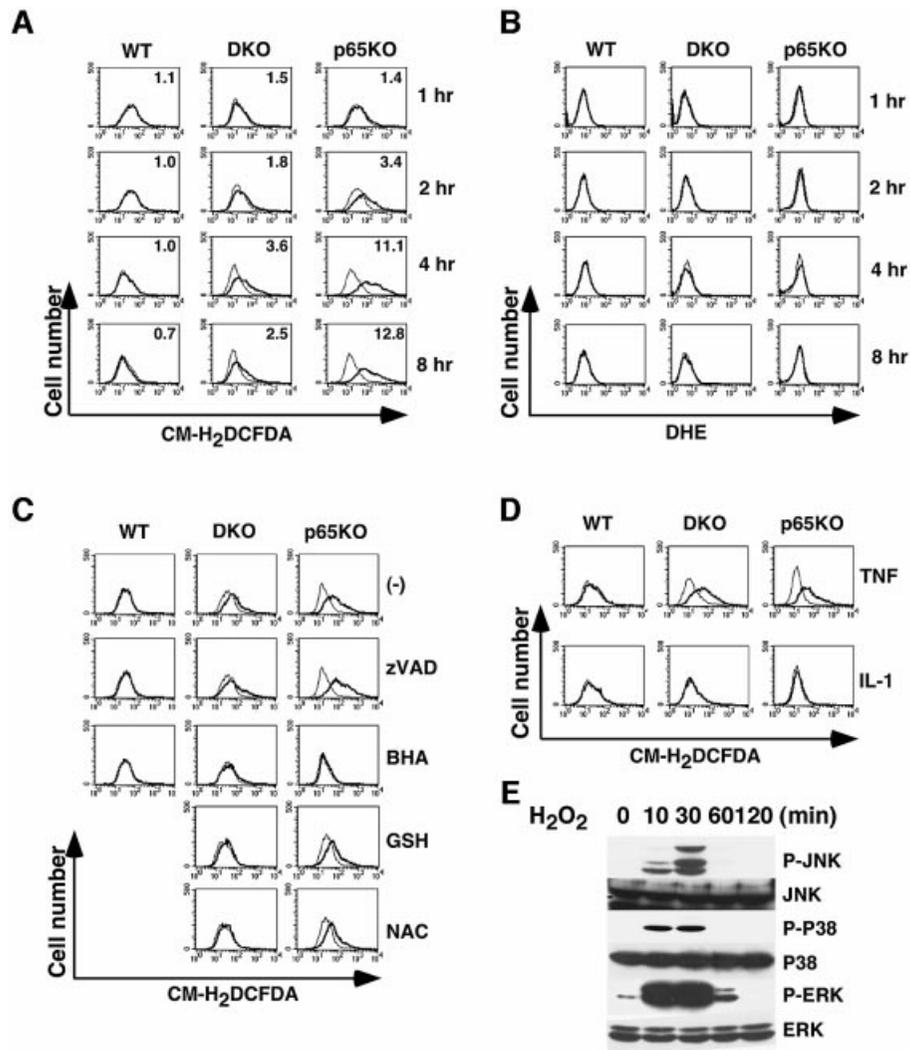


Fig. 3. TNF, but not IL-1, induces accumulation of ROS in DKO and p65KO MEFs. (A and B) Wild-type, DKO and p65KO MEFs were unstimulated (thin line) or stimulated (bold line) with TNF (10 ng/ml) for the indicated time periods, then the cells were labeled with CM-H₂DCFDA (1 μ M) (A) or DHE (1 μ M) (B) for the last 30 min, and analyzed by flow cytometry. The ratios of mean fluorescent intensity of stimulated cells to unstimulated cells are indicated at the right upper corner (A). (C) Wild-type, DKO and p65KO MEFs were untreated or pretreated with z-VAD-fmk (10 μ M), BHA (100 μ M), GSH (5 mM) or NAC (10 mM) for 20 min, then stimulated with TNF (10 ng/ml) for 4 h, labeled with CM-H₂DCFDA and analyzed as described in (A). (D) Wild-type, DKO and p65KO MEFs were stimulated with IL-1 (10 ng/ml) for 4 h, labeled with CM-H₂DCFDA and the fluorescent signals analyzed as described in (A). (E) Wild-type MEFs were stimulated with H₂O₂ (1 mM) for the indicated time periods, and the lysates were analyzed as described in Figure 1.

accumulation in these cells. It is still controversial whether TNF induces ROS accumulation in primary fibroblasts. Wild-type, DKO and p65KO MEFs were stimulated with TNF for 1–8 h, then the cells were labeled with cell-permeable fluorescent dyes, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) or dihydroethidium (DHE). When these dyes are oxidized by ROS in the cells, their fluorescent signals increase. While CM-H₂DCFDA is mainly oxidized by hydrogen peroxides (H₂O₂) and hydroxyl radical, DHE is oxidized by superoxide anion (O₂⁻). Fluorescent signals were analyzed by flow cytometry. Importantly, no substantial increase in fluorescent signals of either CM-H₂DCFDA or DHE was observed in wild-type MEFs up to 24 h after TNF stimulation, indicating that TNF does not induce ROS accumulation in wild-type MEFs (Figure 3A and B; data not shown). In contrast, in DKO and p65KO MEFs,

fluorescent intensity of CM-H₂DCFDA, but not DHE, slightly increased at 2 h, and peaked at 4–8 h after TNF stimulation (Figure 3A). We next examined whether BHA or z-VAD-fmk inhibits ROS accumulation in DKO and p65KO MEFs. As shown in Figure 3C, BHA almost completely inhibited ROS accumulation in DKO and p65KO MEFs, while z-VAD-fmk did not (Figure 3C). We also observed inhibitory effects of other antioxidants, including glutathione (GSH) and *N*-acetyl cysteine (NAC), although their effects were not complete in p65KO MEFs compared with BHA (Figure 3C). Consistent with the partial inhibitory effect of NAC on TNF-induced ROS accumulation, NAC only partially inhibited TNF-induced MAPK activation (see Supplementary figure 1, available at *The EMBO Journal* Online). On the other hand, IL-1 stimulation did not induce ROS accumulation in wild-type, DKO or p65KO MEFs (Figure 3D). These results

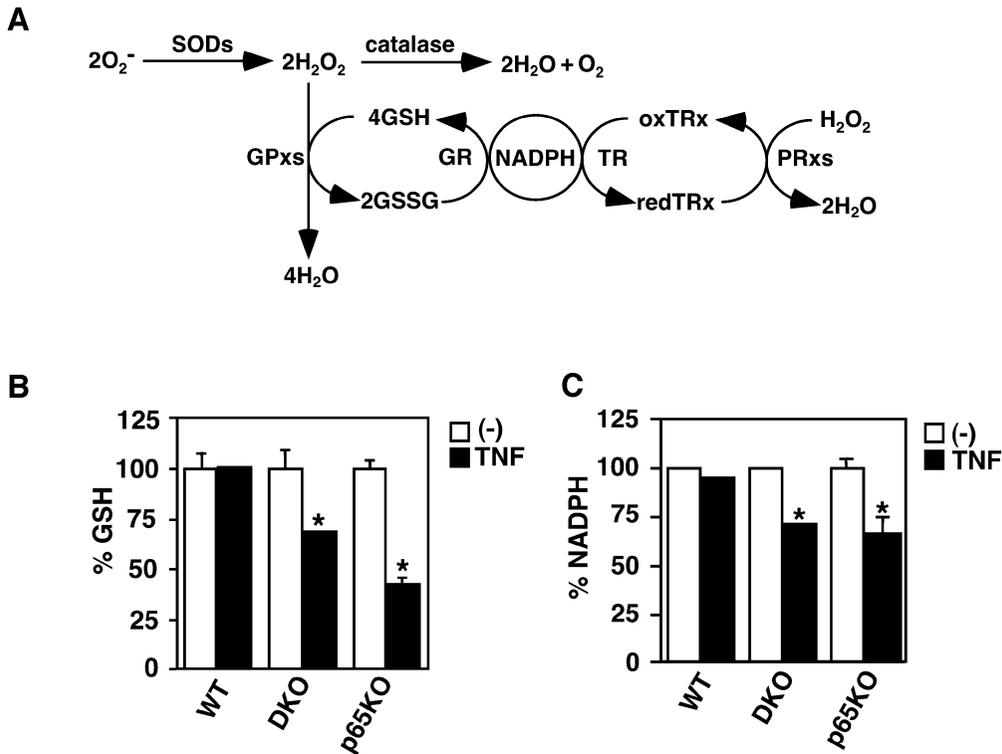


Fig. 4. TNF induces reduction of GSH and NADPH levels in DKO and p65KO MEFs. (A) Pathways of ROS elimination by cellular antioxidants. O_2^- is converted into H_2O_2 by SODs. Then, H_2O_2 is eliminated by catalase, GPxs and PRxs. During elimination of H_2O_2 , reduced glutathione (GSH) is converted to disulfide form (GSSG) by GPxs, and then GSSG is recycled to GSH by glutathione reductase (GR). On the other hand, PRxs also catalyze conversion of H_2O_2 into H_2O by reduced thioredoxin (TRx). Oxidized TRx is recycled back to redTRx by thioredoxin reductase (TR). NADPH is essential for both recycling reactions. (B) Wild-type, DKO and p65KO MEFs were unstimulated (open columns) or stimulated (closed columns) with TNF (10 ng/ml) for 4 h, and then the cellular levels of GSH were measured as described in Materials and methods. The levels of GSH were normalized by protein contents. GSH levels are presented as percentage reduction in stimulated cells compared with unstimulated cells. Results are presented as mean \pm SEs of triplicate samples and represent two independent experiments with similar results. * $P < 0.05$ compared with unstimulated cells. (C) Wild-type, DKO and p65KO MEFs were unstimulated (open columns) or stimulated (closed columns) with TNF (10 ng/ml) for 4 h, and then the cellular levels of NADPH were measured as described in Materials and methods. NADPH levels are presented as percentage reduction in stimulated cells compared with unstimulated cells. Results are presented as mean \pm SEs of triplicate samples and represent two independent experiments with similar results. * $P < 0.05$ compared with unstimulated cells.

demonstrate that accumulation of ROS perfectly coincides with prolonged MAPK activation.

Previous studies showed that ROS including H_2O_2 activates JNK, p38 and ERK depending on the cell-type (Adler *et al.*, 1999b; Thannickal and Fanburg, 2000). Thus, we tested whether exogenously added H_2O_2 could activate JNK, p38 and ERK in MEFs. As shown in Figure 3E, H_2O_2 potently activated JNK, p38 and ERK in wild-type MEFs. Together, these results substantiate the fact that accumulated ROS mediate prolonged MAPK activation in DKO and p65KO MEFs upon TNF stimulation.

Cellular GSH and NADPH levels are decreased in DKO and p65KO, but not wild-type, MEFs after TNF stimulation

Under normal conditions, ROS generated in mitochondria are rapidly eliminated by cellular antioxidants. In general, O_2^- is dismutated to H_2O_2 by SODs. H_2O_2 is subsequently eliminated by catalase, GPxs and PRxs (Figure 4A). Optimum cellular GSH level is essential for maintaining the activities of GPxs. NADPH is also required for converting oxidized glutathione disulfide form (GSSG) to reduced form GSH. Given that TNF induced accumulation

of ROS in DKO and p65KO MEFs, we examined the cellular levels of GSH and NADPH before and after TNF stimulation. While either GSH or NADPH was not significantly altered in wild-type MEFs before and after TNF stimulation, both GSH and NADPH levels were significantly reduced in DKO and p65KO MEFs after stimulation (Figure 4B and C). These results suggest that the machinery to maintain the cellular GSH and NADPH levels is compromised in DKO and p65KO MEFs. Consistent with this notion, exogenously added GSH inhibited TNF-induced ROS accumulation in DKO and p65KO MEFs (Figure 3C).

Ectopic expression of TRAFs or p65, but not GADD45 β or XIAP, inhibits TNF-induced ROS accumulation, prolonged MAPK activation, and reduction of GSH and NADPH levels in DKO and p65KO MEFs

To rule out the possibility that some other defects in the TNF-induced signaling pathways are present in DKO and p65KO MEFs, we tested whether TNF-induced ROS accumulation, prolonged MAPK activation, and reduction of GSH and NADPH levels could be suppressed by ectopic expression of TRAF2 and TRAF5 or p65. To do this, we

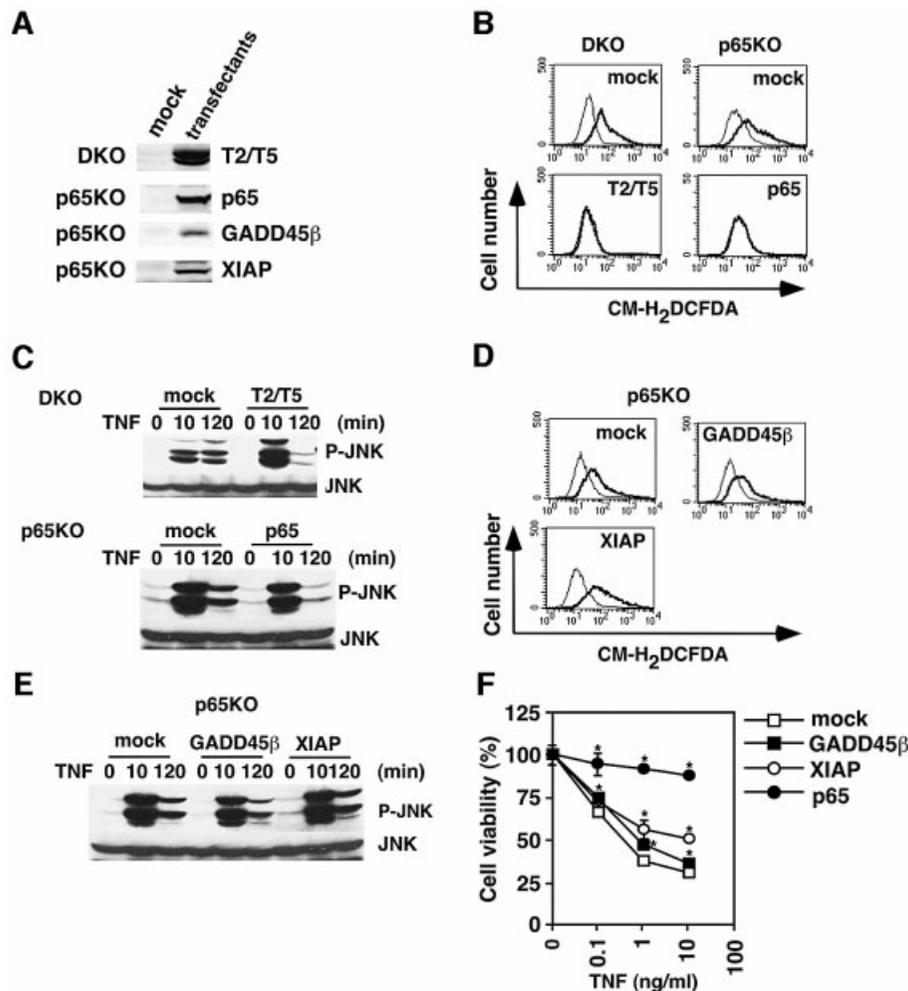


Fig. 5. Introduction of TRAF2 and TRAF5 or p65, but not GADD45 β or XIAP, inhibits TNF-induced ROS accumulation, prolonged JNK activation, and reduction of GSH and NADPH levels in DKO and p65KO MEFs. (A) Total cell lysates were blotted with anti-Flag (for TRAF2, TRAF5, GADD45 β and XIAP) or anti-HA antibody (for p65). (B and D) Mock- or TRAF2- and TRAF5 (T2/T5)-transfected DKO MEFs, and mock-, p65-, GADD45 β - or XIAP-transfected p65KO MEFs were stimulated with TNF (10 ng/ml) for 4 h and analyzed as described in Figure 3. (C and E) Transfectants were stimulated with TNF (50 ng/ml) for the indicated time periods, and the lysates were analyzed as described in Figure 1. (F) Mock-, GADD45 β -, XIAP- or p65-transfected p65KO MEFs were stimulated with the indicated doses of TNF for 16 h. Cell viability was determined by WST assay. Results are presented as mean \pm SEs of triplicate samples and represent four independent experiments with similar results. * P < 0.05 compared with mock transfectants.

stably transfected DKO MEFs with TRAF2 and TRAF5, and p65KO MEFs with p65. Expression of the transfected genes was verified by western blotting with anti-Flag (for TRAF2 and TRAF5) or anti-HA (for p65) antibody (Figure 5A). Reconstitution of TRAF2 and TRAF5 in DKO MEFs or p65 in p65KO MEFs almost completely inhibited TNF-induced ROS accumulation, prolonged JNK activation, and reduction of GSH and NADPH levels (Figure 5B and C; Supplementary figure 2). These results verify that the accumulation of ROS, prolonged MAPK activation, reduction of GSH and NADPH levels in DKO and p65KO MEFs are due to the absence of TRAF2 and TRAF5 or p65.

Previous studies showed that GADD45 β and XIAP were induced by TNF in an NF- κ B-dependent manner and inhibited JNK activation in p65KO MEFs (De Smaele *et al.*, 2001; Tang *et al.*, 2001). Thus, we next examined whether ectopic expression of GADD45 β or XIAP might inhibit ROS accumulation and prolonged JNK activation.

Expression of the transfected genes was verified by western blotting with anti-Flag antibody (Figure 5A). In contrast to the previous data, transfection of either GADD45 β or XIAP did not inhibit TNF-induced ROS accumulation or prolonged MAPK activation in p65KO MEFs (Figure 5D and E). However, expression of XIAP, but not GADD45 β , substantially inhibited TNF-induced cell death (Figure 5F).

Contribution of ROS and MAPKs to TNF-induced cell death in DKO and p65KO MEFs

We next examined whether accumulated ROS *per se* or prolonged MAPK activation participates in TNF-induced cell death. We stimulated DKO and p65KO MEFs with TNF in the presence or absence of inhibitors for caspases, ROS or MAP kinases. As shown in Figure 6A, BHA or zVAD-fmk alone substantially increased cell viability of DKO and p65KO MEFs. Moreover, a combined treatment with BHA and z-VAD-fmk further increased cell viability,

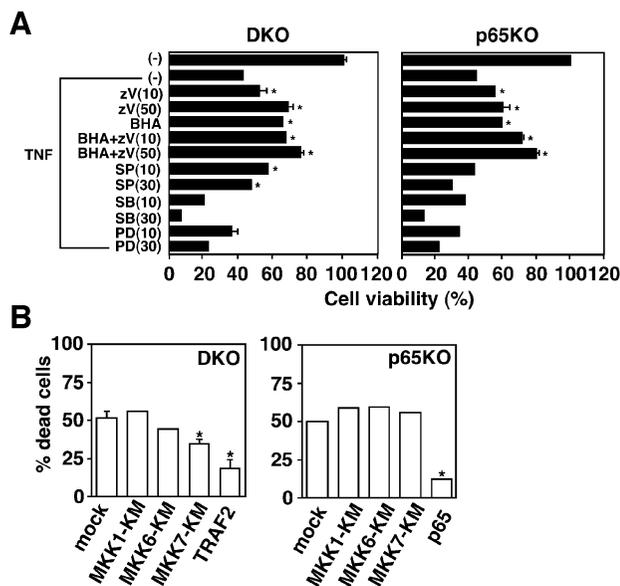


Fig. 6. Inhibition of TNF-induced cell death in DKO and p65KO MEFs by BHA, z-VAD-fmk and MAPK inhibitors. (A) DKO and p65KO MEFs were stimulated with TNF (10 ng/ml) in the presence of z-VAD-fmk (10 or 50 μ M), BHA (100 μ M), BHA (100 μ M) + z-VAD-fmk (10 or 50 μ M), SP600125 (10 or 30 μ M), SB203580 (10 or 30 μ M) or PD98059 (10 or 30 μ M) for 16 h. Cell viability was determined by WST assay as described in Figure 5. (B) DKO and p65KO MEFs were co-transfected with the indicated expression vectors (0.75 μ g) along with pEGFP (0.25 μ g). After 24 h, the cells were treated with TNF (10 ng/ml) for 16 h, and then counted by fluorescent microscopy. The percentage of dead cells among GFP-positive cells was calculated by counting >100 cells in randomly selected areas. Results are presented as means \pm SEs of triplicate samples and represent two individual experiments. * P < 0.05 compared with mock transfectants.

suggesting that ROS and caspases induce cell death independently, at least in part. SB203580 (specific inhibitor for p38) and PD98059 (specific inhibitor for ERK) did not increase cell viability, but rather enhanced TNF-induced death of DKO and p65KO MEFs. In contrast, SP600125 (specific inhibitor for JNK) significantly increased viability of DKO, but not p65KO, MEFs. We verified that these inhibitors actually inhibited MAPK kinase activities by using antibodies specific for phosphorylated form of JNK, ERK, or specific substrate of p38 (MAPKAP2) (Supplementary figure 3). On the other hand, these MAPK inhibitors did not affect TNF-induced ROS accumulation (data not shown), indicating that prolonged MAPK activation is a downstream event of ROS accumulation.

To further evaluate the contribution of MAPK cascades to TNF-induced cell death, we inhibited each MAPK cascade by using dominant-negative mutants of upstream activators, including MKK1-KM (for ERK), MKK7-KM (for JNK) and MKK6-KM (for p38). We transiently transfected DKO and p65KO MEFs with these dominant-negative kinases along with an expression vector for green fluorescence protein (GFP). We also transfected expression vectors for TRAF2 or p65 as positive control. Expression of transfected genes was verified by western blotting (data not shown). Then, cells were treated with

TNF, and the morphology of GFP-positive cells was examined. As expected, transient expression of TRAF2 or p65 into DKO or p65KO MEFs, respectively, substantially reduced dead cells (Figure 6B). In contrast, only MKK7-KM partially decreased dead cells in DKO, but not p65KO, MEFs. Collectively, these results indicate that ROS are involved in enhanced TNF-induced cell death of DKO and p65KO MEFs, but contribution of prolonged JNK activation to TNF-induced cell death is relatively cell dependent.

TNF induces both necrosis and apoptosis in DKO and p65KO MEFs, and necrosis is mainly mediated by ROS

Since ROS have been reported to induce both apoptosis and necrosis depending on the cellular context (Fiers *et al.*, 1999), we examined the morphology of dying cells by transmission electron microscopy. Notably, compared with the unstimulated DKO MEFs (Figure 7A), some dying cells showed a necrotic morphological change, which was characterized by loss of membrane integrity without apparent damage of nuclei (Figure 7B), whereas other cells showed typical apoptotic changes such as chromatin condensation, cell shrinking and apoptotic bodies (Figure 7C). Furthermore, number of necrotic cells was substantially reduced in the presence of BHA, but not zVAD-fmk (Figure 7D). Similar morphological changes and inhibitory effects were also observed in p65KO MEFs (data not shown).

To assess the contribution of apoptosis and necrosis to TNF-induced cell death more quantitatively, we performed time-course analyses of TNF-induced DNA hypodiploidy as an indication of apoptosis using flow cytometry. In parallel with these experiments, we also examined the cell viability by WST assay. Viability of DKO and p65KO MEFs gradually decreased to 50% at 18 h after TNF stimulation (Figure 7E). Although the cells with DNA hypodiploidy increased to 15% at 18 h, this population was substantially less than the dead cell population (Figure 7E). These results, in combination with the electron microscopy data, indicate that a substantial part of DKO and p65KO MEFs died of necrosis after TNF stimulation (Figure 7E). We also tested the effects of BHA and z-VAD-fmk on TNF-induced apoptosis. As expected, the TNF-induced apoptosis was markedly inhibited by z-VAD-fmk. In contrast, BHA treatment substantially increased cell viability (Figure 6A), BHA only slightly decreased population of DNA hypodiploidy (Figure 7F).

A kinase-inactive mutant of ASK1 does not inhibit prolonged JNK activation in DKO and p65KO MEFs

Previous studies have shown that ASK1 is activated by H_2O_2 and involved in prolonged JNK activation in some cells (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998). Thus, we next examined the contribution of ASK1 to prolonged JNK activation in DKO and p65KO MEFs after TNF stimulation. We first tried an *in vitro* kinase assay using immunoprecipitates with anti-ASK1 antibody or western blotting with antibody specific for phosphorylated ASK1 antibody. However, since the expression level of ASK1 in MEFs was low (H.Ichijo, unpublished result), we could not detect substantial levels of ASK1 kinase activities or

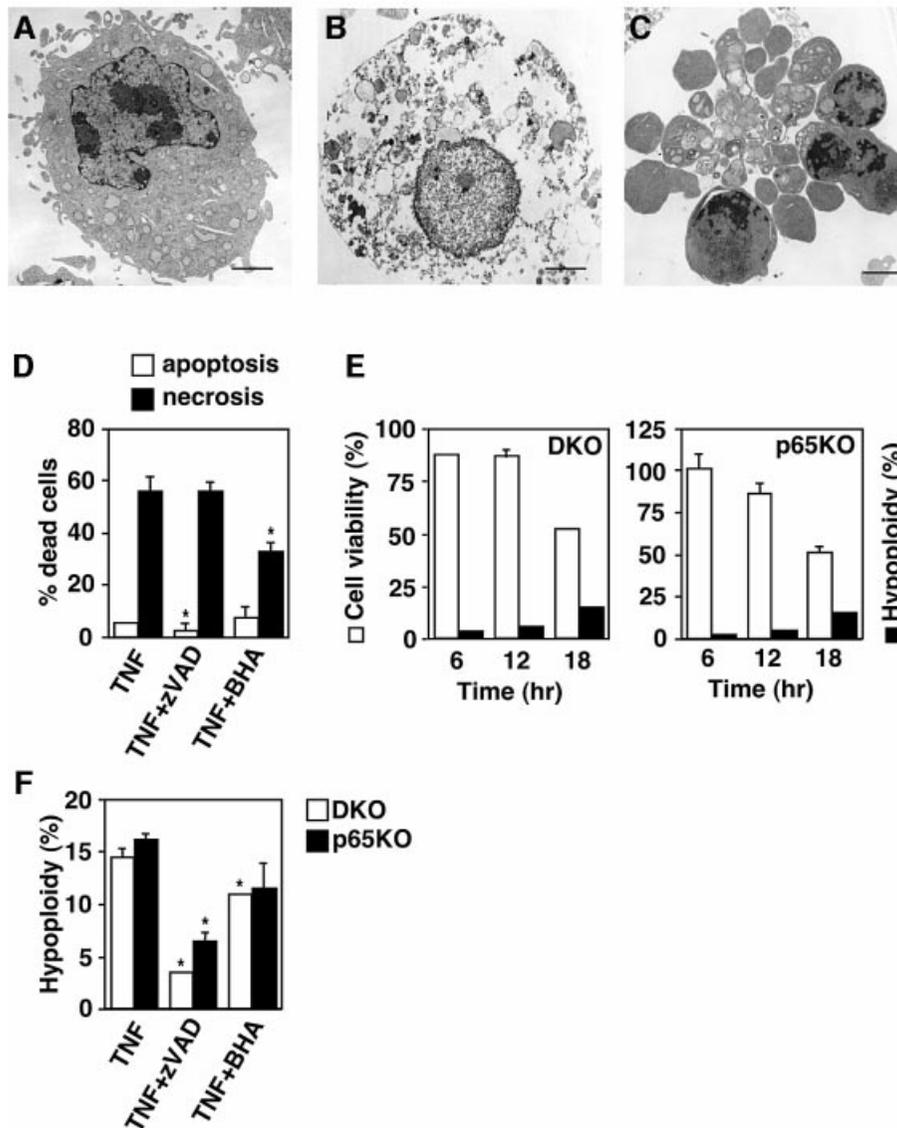


Fig. 7. TNF induces both necrosis and apoptosis in DKO and p65KO MEFs. (A–C) DKO MEFs were unstimulated (A) or stimulated (B and C) with TNF (10 ng/ml) for 18 h. The morphological changes were observed by transmission electron microscopy. The scale bar represents 1 μ m. Similar morphological changes were observed in p65KO MEFs (data not shown). (D) DKO MEFs were stimulated with TNF (10 ng/ml) alone or in the presence of zVAD-fmk (50 μ M) or BHA (100 μ M) for 18 h, then the samples were prepared for the electron microscope. The percentages of cell numbers showing apoptotic (open columns) and necrotic (closed columns) morphological changes were calculated by counting four randomly selected areas (40–70 cells per areas). Results are presented as mean \pm SE. * P < 0.05 compared with TNF stimulation alone. (E) DKO and p65KO MEFs were stimulated with TNF (10 ng/ml) for the indicated time periods. Cell viability (open columns) and DNA hypoploidy (closed columns) were estimated by WST assay and flow cytometry, respectively. Data are represented as the as mean \pm SE of triplicate samples. Similar results were obtained in three independent experiments. (F) DKO MEFs (open columns) and p65KO MEFs (closed columns) were stimulated with TNF (10 ng/ml) alone or in the presence of zVAD-fmk (50 μ M) or BHA (100 μ M) for 18 h, then DNA hypoploidy were measured by flow cytometry. Results are presented as mean \pm SEs of triplicate samples. * P < 0.05 compared with TNF alone.

phosphorylation of ASK1 after TNF stimulation (data not shown). Next, we stably transfected kinase-inactive mutant of ASK1 (ASK1-KM) as a dominant-negative inhibitor in DKO and p65KO MEFs. Expression of ASK1-KM was verified by western blotting with anti-Flag antibody (Figure 8A). As shown in Figure 8B, ASK1-KM did not inhibit TNF-induced prolonged JNK activation in DKO and p65KO MEFs.

Discussion

In the present study, we investigated the mechanism by which TNF induces cell death in DKO and p65KO MEFs

that are deficient in TNF-induced NF- κ B activation. TNF, but not IL-1, induced prolonged MAPK activation due to ROS accumulation in DKO and p65KO MEFs, but not wild-type MEFs. Accumulation of ROS was associated with reduced levels of cellular antioxidants, GSH and NADPH. An antioxidant, BHA, inhibited not only TNF-induced ROS accumulation and prolonged MAPK activation, but also TNF-induced cell death. Interestingly, dying cells showed necrotic as well as apoptotic morphological changes. Collectively, these results demonstrate that NF- κ B inhibits TNF-induced ROS accumulation, which mediates prolonged MAPK activation and cell death, under normal conditions.

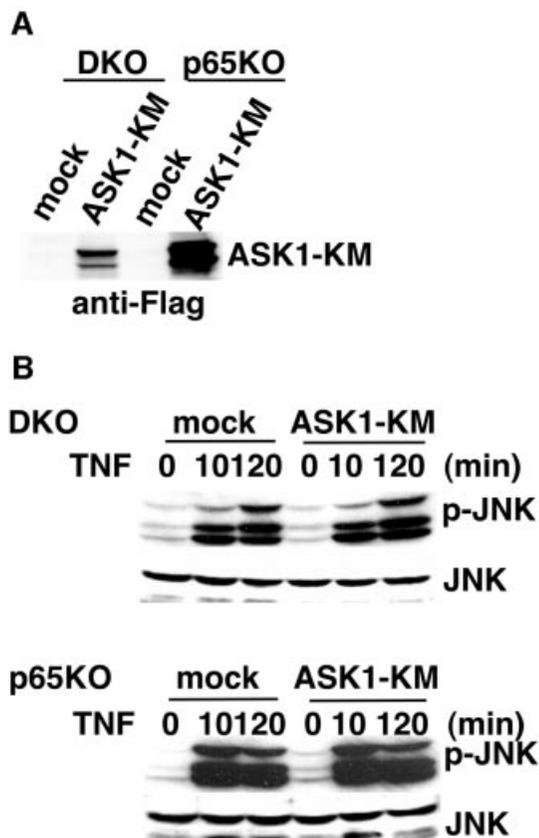


Fig. 8. Introduction of ASK1-KM does not inhibit TNF-induced prolonged JNK activation in DKO and p65KO MEFs. (A) Total cell lysates were blotted with anti-Flag antibody. (B) Mock- or ASK1-KM-transfected DKO and p65KO MEFs were stimulated with TNF (50 ng/ml) for the indicated time periods, and the lysates were analyzed as described in Figure 1.

It has been reported that NF- κ B-inducible genes such as XIAP and GADD45 β play an important role in suppressing prolonged JNK activation (De Smaele *et al.*, 2001; Tang *et al.*, 2001). However, the molecular mechanism by which XIAP and GADD45 β inhibit JNK activation remains obscure. We previously identified GADD45 β as a molecule interacting with MTK1 and showed that overexpression of GADD45 β potentially activates JNK and p38 pathways (Takekawa and Saito, 1998). Consistent with these results, stable expression of GADD45 β in p65KO MEFs did not substantially inhibit TNF-induced ROS accumulation or prolonged MAPK activation, and cell death (Figure 5D–F). Similarly, XIAP did not inhibit TNF-induced ROS accumulation or prolonged MAPK activation, although XIAP substantially increased cell viability of TNF-stimulated cells (Figure 5D–F). These discrepancies might result from cell-type differences to generate GADD45 β transfectants (T cell clone versus MEF) and/or differences in experimental systems (transient versus stable transfection of XIAP).

Previous studies suggested an important role of ROS in TNF-induced MAPK activation (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998). However, at least under our experimental conditions, we could not detect ROS accumulation in TNF-stimulated wild-type MEFs (Figure 3A). While

BHA almost completely inhibited both ROS accumulation and prolonged MAPK activation in DKO and p65KO MEFs, BHA only slightly inhibited TNF-induced rapid/transient MAPK activation (Figures 2 and 3). Together, ROS appear not to play an essential role in TNF-induced transient MAPK activation in MEFs. However, we cannot exclude the possibility that ROS might play a critical role in other types of cells. In contrast to previous studies (De Smaele *et al.*, 2001; Javelaud and Besancon, 2001; Tang *et al.*, 2001), activation of not only JNK, but also p38 and ERK, was prolonged after TNF stimulation in DKO and p65KO MEFs (Figure 1A and B), which were all abrogated by BHA (Figure 2). Thus, accumulated ROS appear to activate all three MAPK cascades. Consistent with this notion, exogenously added ROS actually activated three MAPK cascades in MEFs (Figure 3E). Concerning the molecular mechanism by which ROS mediate prolonged JNK activation, ASK1 might participate in this process (Tobiome *et al.*, 2001). However, under our experimental conditions, expression of ASK1-KM did not inhibit TNF-induced prolonged JNK activation in DKO and p65KO MEFs (Figure 8). These results suggested that a kinase other than ASK1, such as MEKK1, might be also involved in the ROS-induced JNK activation. It has been also reported that under non-stressed conditions, JNK binds cellular JNK inhibitor, GST, and this interaction is disrupted by ROS, resulting in activation of JNK (Adler *et al.*, 1999a). Although these scenarios could explain the mechanism for prolonged JNK activation, they cannot explain the mechanism for prolonged ERK activation. In this respect, it is noteworthy that ROS- and UV-induced ERK activation has been reported to be mediated by c-src- and EGF receptor-dependent pathways (Chen *et al.*, 2001; Kitagawa *et al.*, 2002). We are currently pursuing these possibilities.

While TNF or IL-1 stimulation generally induces rapid and transient MAPK activation, genotoxic stresses such as UV and γ -irradiation induce prolonged MAPK activation that promotes cell death (Xia *et al.*, 1995; Chen *et al.*, 1996; Guo *et al.*, 1998). Consistent with these studies, prolonged JNK activation partly contributed to TNF-induced cell death in DKO MEFs, as demonstrated by the inhibitory effect of a JNK inhibitor, SP600125 (Figure 6A). However, SP600125 did not inhibit TNF-induced cell death of p65KO MEFs (Figure 6A). Consistent with the data using MAPK inhibitors, expression of MKK7-KM only partially inhibited TNF-induced cell death of DKO, but not p65KO, MEFs (Figure 6B). The fact that inhibition of JNK cascade did not affect TNF-induced cell death of p65KO MEFs may be explained by the fact that TRAF-mediated transient JNK activation was intact in p65KO MEFs but not DKO MEFs (Figure 1A and B). Given that transient JNK activation transmits survival signals (Davis, 2000), SP600125 or MKK7-KM might cancel both survival and apoptotic signals in p65KO MEFs, resulting in no apparent inhibitory effect on TNF-induced cell death.

Our present study also demonstrates that TNF-induced ROS accumulation can be induced independently of TRAF2 and TRAF5. In addition, IL-1 signaling that is mediated by TRAF6 could not induce ROS accumulation (Figure 3D). Together, TRAF family proteins appear not to be essential for ROS accumulation. Given that

z-VAD-fmk did not inhibit TNF-induced ROS accumulation (Figure 3C), TRADD, FADD or RIP, but not caspase-8, might play a critical role in this process. In this respect, RIP has been reported to be responsible for Fas-, TNF- or TRAIL-induced necrosis (Holler *et al.*, 2000), but the contribution of ROS to RIP-mediated cell death has not been determined. Further study is required to address whether RIP is essential for TNF-induced ROS accumulation.

Here we show that reduced cellular levels of GSH and NADPH are correlated with accumulation of ROS in TNF-stimulated DKO and p65KO MEFs. ROS are generated in mitochondria and rapidly eliminated by antioxidants under normal conditions. Thus, the function of some antioxidant enzyme, the expression level or activity of which is regulated by NF- κ B, might be impaired in DKO and p65KO MEFs. Previous studies showed that manganese-dependent SOD (MnSOD) is upregulated by TNF in an NF- κ B-dependent manner, and overexpression of MnSOD inhibits ROS-induced apoptosis (Tanaka *et al.*, 2002). While basal expression levels of MnSOD mRNA were not different between wild-type and DKO or p65KO MEFs, induction of MnSOD mRNA by TNF was substantially impaired in DKO and p65KO MEFs (data not shown). However, stable transfection of MnSOD into DKO or p65KO MEFs did not significantly inhibit TNF-induced ROS accumulation or cell death (data not shown). Taken that the primary target of MnSOD is O₂⁻ and the main accumulated ROS in DKO and p65KO MEFs were H₂O₂ or hydroxyl radical, the basal expression level of MnSOD might be sufficient for elimination of O₂⁻. Regarding scavengers to detoxify H₂O₂, there are many antioxidant enzymes, including catalase, GPxs and PRxs (Thannickal and Fanburg, 2000). So far, we have observed no significant inhibitory effect of such antioxidant enzymes, including GPx1, GPx2, PRx1 and PRx2 on TNF-induced ROS accumulation in DKO or p65KO MEFs after stable transfection (data not shown). Further study is now underway to identify an antioxidant enzyme responsible for the ROS accumulation.

Another interesting point from this study is that a substantial part of DKO and p65KO MEFs died by necrosis after TNF stimulation, as assessed by electron microscopy and flow cytometry (Figure 7A–E). Furthermore, taken that BHA only marginally inhibited the emergence of apoptotic cells (Figure 7F), but substantially inhibited necrotic morphological changes (Figure 7D), ROS mainly, but not exclusively, contribute to necrotic cell death. These results are reminiscent of murine fibrosarcoma cell line, L929, in which TNF induces ROS-dependent necrosis (Vercammen *et al.*, 1998). On the other hand, previous studies using p65KO mice implied an important role of NF- κ B in protection from TNF-induced apoptosis of hepatocytes (Beg and Baltimore, 1996; Doi *et al.*, 1999). It will be interesting to determine whether necrotic morphological changes are also observed in hepatocytes in p65KO mice.

In summary, our present study demonstrates a critical role of NF- κ B to inhibit TNF-induced ROS accumulation under normal conditions. Elucidation of the mechanism by which NF- κ B eliminates ROS would be helpful in manipulating various pathological conditions, such as

ischemia or aging, in which ROS are deeply implicated in the pathogenesis.

Materials and methods

Reagents and cell culture

Recombinant murine TNF and murine IL-1 β were purchased from BD Pharmingen. NAC and reduced form of GSH were purchased from Sigma. SP600125, SB203580 and PD98059 were purchased from Biomol and Calbiochem. Antibodies specific for phospho-JNK, phospho-p38, phospho-ERK, phospho-MAPKAP2, JNK, p38, ERK and MAPKAP2 were purchased from Cell Signaling. Anti-Flag and anti-HA antibodies were purchased from Sigma and Roche Diagnostics Corp., respectively. BHA, z-VAD-fmk, CM-H₂DCFDA and DHE were purchased from Wako Pure Chemicals, Peptide Institute and Molecular Probes. Wild-type, DKO and p65KO MEFs, and Phoenix-Eco cells were cultured in high-glucose DMEM containing 10% fetal calf serum (FCS).

Western blot analysis

MEFs (1 \times 10⁶ cells) were lysed in a buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride and 1 μ g/ml aprotinin. After centrifugation, cell lysates were subjected to 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blotted with antibodies to phospho-JNK, phospho-p38, phospho-ERK or phospho-MAPKAP2, and reblotted with antibodies to total JNK, total p38, total ERK or total MAPKAP2, respectively. To detect transfected gene products, the membranes were blotted with anti-Flag or anti-HA antibody. The membranes were developed with Enhanced Chemiluminescence (ECL) Western Blotting Detection System Plus (Amersham Pharmacia Biotech.) according to the manufacturer's instruction.

Measurement of ROS accumulation

MEFs (2 \times 10⁵ cells) were plated in six-well plates and stimulated with TNF or IL-1 in phenol red-free medium for the indicated time periods. After stimulation, the cells were incubated with CM-H₂DCFDA or DHE in the dark for 30 min at 37°C. Then, the cells were harvested and analyzed on a flow cytometer (FACSCalibur; BD Biosciences). Data were processed by using the CellQuest program (BD Biosciences).

Generation of stable transfectants

Generation of stable transfectants by using retroviral vectors was performed as described previously (Sasazuki *et al.*, 2002). Retroviral expression vectors for TRAF2, TRAF5, p65, GADD45 β , XIAP or ASK1-KM were constructed by inserting each cDNA into pMX-puro. Phoenix-Eco cells (1.5 \times 10⁶ cells) were transiently transfected with 3 μ g of the indicated vectors using LipofectAMINE according to the manufacturer's instruction (Invitrogen) to generate viral supernatants. After infection, DKO and p65KO MEFs were selected with 2.5 μ g/ml puromycin (Sigma) to isolate stable transfectants. Puromycin-resistant pools were used for the experiments.

Measurement of GSH

Intracellular GSH levels were measured by using a colorimetric assay kit (OxisResearch™) according to the manufacturer's instruction. Briefly, MEFs (2 \times 10⁶ cells) were unstimulated or stimulated with TNF for 4 h and harvested. The cells were resuspended in 500 μ l of 5% metaphosphoric acid solution and sonicated using an ultrasonic sonicator (Astrason™). After centrifugation, the supernatants were sequentially incubated with 4-chloro-1-methyl-7-trifluoromethyl-quindinium methylsulfate and NaOH, and then the absorbance at 400 nm was measured on a spectrophotometer. The pellets were lysed in the lysis buffer and the protein concentration was measured by the Bradford method. GSH contents were normalized by the protein concentration of each sample and are presented as GSH level per microgram of cell lysate.

Measurement of NADPH

NADPH levels were measured by the method of Gibon and Larher (1997). Briefly, MEFs (2.5 \times 10⁶ cells) were unstimulated or stimulated with TNF for 4 h. The cells were harvested and resuspended in 250 μ l of 0.1 N NaOH, followed by brief homogenization. The homogenates were heated at 95°C for 5 min. After centrifugation, the supernatants were neutralized with 250 μ l of 0.1 N HCl. Then, the supernatants were incubated with a buffer containing 400 mM NaCl, 100 mM

Tris-HCl pH. 8.0, 4 mM EDTA, 0.42 mM 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), 1.66 mM phenazine ethosulfate (PES), 2.5 mM NADP⁺ and 14 U of glucose 6-phosphate dehydrogenase (G6PDH) at 37°C for 40 min. The reaction was stopped by addition of 666 µl of 5 M NaCl, then centrifuged at 17 400 g at 4°C for 5 min. The pellets were lysed in 1 ml of 98% ethanol and the absorbance at 570 nm was measured on a spectrophotometer. The cellular NADPH contents were determined by using an NADPH standard curve.

WST assay

MEFs (5×10^3 cells) were plated in 96-well plates and cultured for 12 h in DMEM containing 10% FCS. The cells were then stimulated with TNF in the presence or absence of various inhibitors for 16 h. Cell viability was determined by WST assay using a Cell Counting kit (Dojindo).

Cell death assay

MEFs (1×10^5 cells) were cotransfected with the indicated expression vectors along with an expression vector for GFP (pEGFP) (Clontech) using LipofectAMINE. Expression vectors for MKK1-KM, MKK7-KM and MKK6-KM were constructed by inserting each cDNA into pcDNA3.1 (Invitrogen). After 24 h, cells were treated with TNF for 16 h, then GFP-positive cells were counted by fluorescent microscopy. Cells showing flat or round morphology were considered to be live or dead, respectively. Expression of transfected gene products was verified by western blotting with anti-Myc (for MKK1-KM and MKK7-KM) or anti-HA (for MKK6, TRAF2 and p65) antibody (data not shown).

Electron microscopy

MEFs (4×10^6 cells) were unstimulated or stimulated with TNF in the presence or absence of various inhibitors for 18 h, and then serially fixed with 2% glutaraldehyde in phosphate-buffered saline for 2 h and then with 2% OsO₄ for 2 h before embedding in Epon 812. Thin sections were prepared using a MT-5000 ultramicrotome (Dupont Pharmaceuticals), stained with uranyl acetate followed by lead citrate, and then observed on a JEM1230 electron transmission microscope (JEOL). To evaluate the percentages of cell numbers showing apoptotic and necrotic morphological changes, sections were stained with toluidine blue and used for cell counting.

Statistical analysis

Statistical analysis was performed by Student's *t*-test. A *P* value <0.05 was considered to be significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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