Molecular Pharmacology Fast Forward. Published on February 12, 2007 as doi:10.1124/mol.106.032185
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A CRITICAL ROLE OF LUTEOLIN-INDUCED REACTIVE OXYGEN SPECIES IN BLOCKAGE OF TUMOR NECROSIS FACTOR-ACTIVATED NUCLEAR FACTOR-κB PATHWAY AND SENSITIZATION OF APOPTOSIS IN LUNG CANCER CELLS

Wei Ju, Xia Wang, Honglian Shi, Wenshu Chen, Steven A. Belinsky, Yong Lin

W.J., X. W., W. C., S.A.B. and Y. L: Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest DR., SE, Albuquerque, NM 87108, USA;

H.S: College of Pharmacy, University of New Mexico, MSC09 5360, 1 University of New Mexico, Albuquerque, NM 87131, USA

Molecular Pharmacology Fast Forward. Published on February 12, 2007 as DOI: 10.1124/mol.106.032185
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Running Title: Luteolin-induced ROS inhibits TNF-induced NF-κB activation

*Correspondence:
Yong Lin, M.D., Ph.D.,
Molecular Biology and Lung Cancer Program,
Lovelace Respiratory Research Institute,
2425 Ridgecrest Dr., SE,
Albuquerque, NM 87108,
Tel: 505-348-9645;
Fax: 505-348-4990;
E-mail:ylin@lrri.org

Page number:26
Number of Figures: 6
Number of tables:0
Number of references: 40
Number of words in the Abstract:194
Number of words in the Introduction:731
Number of words in the Discussion: 877

[1]The Abbreviations used are: NF-κB, Nuclear factor κB; TNF, tumor necrosis factor; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; NAC, *N*-acetyl-L-cysteine; BHA, butylated hydroxyanisole; TNFR1, TNF receptor 1; IKK, IκB kinase; MnSOD, manganese superoxide dismutase; SOD, superoxide dismutase; CCS, copper chaperon for SOD-1; LDH, lactase dehydrogenase; TRAF2, TNF receptor-associated factor 2; RIP, receptor-interaction protein; superoxide,$O_2^{\bullet-}$; H_2O_2, hydrogen peroxide; $^1O_2$,$^{\bullet}OH$, singlet oxygen; $LOO^{\bullet}$, peroxyl radical; hydroxyl radical, $^{\bullet}OH$; Cu-Zn, copper-zinc; GSH, glutathione; GPXs glutathione peroxidases; PARP poly(ADP-ribose) polymerase; CM-H_2DCFDA, 5-(and -6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester; DHE, dihydroethidium; ANOVA, analysis of variance; SD, standard deviation; AO/EB, acridine orange/ethidium bromide; DCF, dichlorodihydro-fluorescein; ASK1, apoptosis signal-related kinase; MLK, mixed lineage kinase; MEKK1, MEK kinase 1; TAK1, transforming growth factor(TGF)-activated protein kinase 1; MAP, MAP kinase phosphatases; SD, standard deviation

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ABSTRACT

Nuclear factor κB (NF-κB) activated by tumor necrosis factor (TNF) attenuates the TNF-induced apoptosis pathway. Therefore, blockage of NF-κB should improve the anti-cancer activity of TNF. Luteolin, a naturally occurring polyphenol flavonoid, has been reported to sensitize colorectal cancer cells to TNF-induced apoptosis through suppression of NF-κB; however, the mechanisms of this effect have not been well elucidated. In this paper, we provide evidence showing a critical role of reactive oxygen species (ROS) accumulation induced by luteolin in modulating TNF-activated pathways in lung cancer cells. Luteolin effectively suppressed NF-κB while potentiated the c-Jun N-terminal kinase (JNK) to increase apoptosis induced by TNF in lung cancer cells. Our results further demonstrate that luteolin induced an early-phase ROS accumulation via suppression of the cellular superoxide dismutase activity. Importantly, suppression of ROS accumulation by ROS scavengers butylated hydroxyanisole and *N*-acetyl-L-cysteine prevented the luteolin-induced suppression of NF-κB and potentiation of JNK, and significantly suppressed the synergistic cytotoxicity seen with co-treatment of luteolin and TNF. Taken together, these results suggest that the accumulation of ROS induced by luteolin plays a pivotal role in suppression of NF-κB and potentiation of JNK to sensitize lung cancer cells to undergo TNF-induced apoptosis.

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INTRODUCTION

Tumor necrosis factor (TNF) can induce both survival and death signals, depending on cellcontext and environment (Aggarwal, 2003; Wajant et al., 2003). Most cancer cells are resistant to TNF-induced death, which is believed to involve survival signals such as nuclear factor κB (NF-κB) that blunt the apoptotic pathway (Wajant et al., 2003). Therefore, interventions that inhibit TNF-induced survival signals may sensitize cancer cells to TNF-induced apoptosis.

The binding of TNF to TNF receptor 1 (TNFR1) initiates a sequential recruitment of adaptor proteins to form a dynamic complex that leads to activation of diverse signaling pathways (Wajant et al., 2003). The activation of the transcription factor NF-κB is critical for cell survival and proliferation (Wajant et al., 2003). The role of c-Jun N-terminal kinase (JNK) activation in cell death regulation is controversial, but recent studies suggested that sustained JNK activation is pro-apoptotic (Lin and Dibling, 2002; Ventura et al., 2004). There is crosstalk between the NF-κB and JNK activation pathways that controls the outcome of the cells in response to TNF. (Kamata et al., 2005; Lin and Dibling, 2002). The caspase cascade can be activated, resulting in apoptotic cell death (Wajant et al., 2003). Therefore, the balance of TNF-induced survival- and death-signaling is pivotal in determining the fate of TNF-exposed cells.

During TNFR1 signaling, the IκB kinase (IKK) is recruited to the TNFR1 signaling complex through TNF receptor-associated factor 2 (TRAF2) and activated through a receptor-interaction protein (RIP)-mediated mechanism that involves MEKK3 (Devin et al., 2000; Yang et al., 2001). The activated IKK in turn phosphorylates the IκBs, which retain NF-κB in the cytoplasm, to trigger their rapid polyubiquitination followed by degradation in the 26S proteasome. This

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process allows its nuclear translocation and binding to the promoters of its target genes. Several NF-κB's target genes are found to have anti-apoptotic properties (Karin et al., 2004). Induction of the antioxidant manganese superoxide dismutase (MnSOD) by NF-κB is also implicated to be anti-apoptotic (Kamata et al., 2005). The transcriptional activity of NF-κB is further regulated by phosphorylation and acetylation (Wajant et al., 2003). Therefore multiple steps affecting post-translational modification of NF-κB could be targets for regulating its activity.

Reactive oxygen species (ROS) are a group of reactive, short-lived, oxygen-containing species such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$,), and peroxyl radical ($LOO^{\bullet}$). Cells have developed effective mechanisms to reduce cellular ROS levels. The superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to oxygen (O_2) and H_2O_2. Catalase reduces H_2O_2 to H_2O and O_2. There are two types of SOD in the cell. MnSOD/SOD-2 functions in the mitochondria while copper-zinc (Cu-Zn)SOD/SOD1 is present mainly in the cytosol (Curtin et al., 2002; Nimnual et al., 2003). The copper chaperon for SOD-1 (CCS) is important to maintain the activity of Cu-ZnSOD (Culotta et al., 1997). Additionally, glutathione (GSH) and glutathione peroxidases (GPXs) provide another mechanism in scavenging H_2O_2 (Curtin et al., 2002). In addition to its direct effect of damaging cellular components, ROS can mediate signal transduction (Rhee, 2006; Shen and Pervaiz, 2006). For example, ROS were found to play a pivotal role in activation of the JNK pathway and non-apoptotic cell death induced by TNF (Kamata et al., 2005; Lin et al., 2004). The role of ROS in TNF induction of the NF-κB pathway is somewhat controversial, as they were reported to activate, inhibit, or have no effect on this pathway. This diversity in effect may be influenced by cell type and the nature of ROS-inducing agents that were studied (Panopoulos et al., 2005; Shen and Pervaiz, 2006).

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Luteolin, 3',4',5,7-tetrahydroxyflavone, a common flavonoid that exists in many types of fruits, vegetables, and medicinal plants (Ross and Kasum, 2002), has been used as an anti-inflammation agent (Bagli et al., 2004; Kanadaswami et al., 2005; Ueda et al., 2002). Although it was generally thought to be an antioxidant, luteolin was found to induce ROS accumulation (Matsuo et al., 2005). However, the mechanism by which luteolin induces ROS has not been addressed. Recently, luteolin was shown to sensitize colorectal cancer cell lines to TNF-induced apoptosis via inhibition of NF-κB and augmentation of JNK (Shi et al., 2004). However, the mechanism of luteolin-induced NF-κB suppression and JNK potentiation has not been elucidated. In this study, we demonstrate that luteolin induces ROS through suppression of SOD activity, and ROS are crucial in inhibiting the NF-κB and potentiating the JNK pathways and subsequently sensitizing lung cancer cells to TNF-induced apoptosis.

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Materials and Methods

Plasmids and Reagents—Reporter plasmids 2xκB-Luc and pRSV-LacZ have been described previously (Lin et al., 1999). Luteolin was purchased from Sigma (St. Louis, MO). Human TNF was from R&D Systems (Minneapolis, MN). Butylated hydroxyanisol (BHA) and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma. The JNK inhibitor SP600125 and pan-caspase inhibitor zVAD-fmk were purchased from Calbiochem (La Jolla, CA). Antibodies against IκBα, JNK1, Cu-ZnSOD, CCS and caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-A20 and anti-MnSOD were from BD Biosciences (San Diego, CA). Anti-phospho-JNK, -β-actin, and -poly(ADP-ribose) polymerase (PARP) antibodies were purchased from BioSource (Camarillo, CA), Sigma, and Biomol (Plymouth Meeting, PA), respectively. Antibodies for BcL-xL, XIAP, and phospho-IκBα were from Cell Signaling (Beverly, MA). 5-(and -6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) and dihydroethidium (DHE) were purchased from Invitrogen (Carlsbad, CA). SOD activity detection kit was purchased from Cayman Chemical Company (Ann Arbor, MI).

Cell Culture—Lung cancer cell lines H23, H2009, H460, and A549 were obtained from American Type Culture Collection (Manassas, VA). All cancer cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cell Death Assays—Cytotoxicity was assessed with a lactase dehydrogenase (LDH) release detection kit (Roche, Penzberg, Germany) (Wang et al., 2006). Cells were seeded in 24-well plates at 70% to 80% confluence and cultured overnight. Then cells were treated as indicated in each figure legend. Culture medium from each well was collected and transferred to 96-well flat-bottomed plates. LDH activity was determined by adding equal volumes of reaction mixture to

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each well. The absorbance of the samples was measured at 490 nm using a plate reader. All the experiments were repeated three to five times and the average is shown in each figure. Cell death was calculated using the formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value} - \text{Spontaneous LDH release}}{\text{Maximum LDH release} - \text{Spontaneous LDH release}} \times 100$$

Detection of ROS—Cells were seeded in 12-well plates, cultured overnight, and then treated as indicated in each figure legend. Thirty minutes before collecting cells, DHE (5 μM) or CM-H_2DCFDA (1 μM) was added. ROS were detected by flow cytometry with FACSCalibur (BD Biosciences) and analyzed with the CellQuest program (BD Biosciences) as previously reported (Lin et al., 2004).

Luciferase Assay—Cells cultured in 12-well plates were transfected with 0.35 μg p2xκB-Luc and 0.15 μg pRSV-LacZ by using FuGENE6 transfection reagent (Roche, Indianapolis, IN). Eight hours after transfection, cells were treated with luteolin (40 μM) for 30 min followed by exposure to TNF (20 ng/ml) for an additional 15 h and collected for luciferase assay. Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI) and normalized to the β-galactosidase activity of each sample. All the experiments were repeated three times and the average is shown in each figure.

Western Blot– Cells treated as indicated in each figure legend were lysed in M_2 buffer (20 mM Tris-HCl [pH 7.6], 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM h-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). Equal amounts of cell extracts were resolved by 15% SDS-PAGE and analyzed by Western blot. The proteins were visualized by enhanced chemiluminescence

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following the manufacturer's instruction (Amersham, Piscataway, NJ). Each experiment was repeated at least three times and representative results are shown in each figure.

Detection of SOD Activity—SOD activity was detected using the SOD activity detection kit according to the manufacturer's instructions (Cayman). Briefly, cells cultured in 12-well plates were treated with luteolin (40 μM), TNF (20 ng/ml) or both for 5, 15, and 30 min. Cells were collected and lysed in cold lysis buffer (20mM HEPES, pH 7.2, containing 1mM EGTA, 210 mM mannitol, and 70 mM sucrose). After sonication and centrifugation, the supernatants were collected and transferred to 96-well plates. After incubating with 100 μl diluted tetrazolium and 10 μl diluted xanthine oxidase for 20 min, the absorbance of the samples was measured at 450 nm using a plate reader. SOD activity was calculated by normalizing the reading to the total protein amount of each sample, with the untreated sample taken as 100%. The experiment was repeated three times.

Statistical Analysis—Data are expressed as mean ± standard deviation (SD). Statistical significance was examined by two-way analysis of variance (ANOVA) pairwise comparison. In all analyses, $P < 0.05$ was considered statistically significant.

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RESULTS

Luteolin and TNF Co-treatment Potentiates Cytotoxicity in Human Lung Cancer Cells—To examine whether luteolin potentiates TNF-induced cell death in lung cancer cells, we first treated H460 cells with increasing concentrations of luteolin for 30 min followed by exposing the cells to human TNF (20 ng/ml) for an additional 24 h. Cell death was detected and quantified by a LDH release assay. Treatment with luteolin alone caused moderate cytotoxicity, with about 23% cell death at the dose of 80 μM (Figure 1*A*). Even at the highest evaluated dose of TNF (50 ng/ml), TNF treatment alone caused little cell death (< 10% cell death) (Figure 1*B*). However, combining luteolin and TNF increased cytotoxicity significantly. A dose-dependent potentiation of cytotoxicity was detected with increasing luteolin concentrations and a fixed TNF concentration (~ 60% cell death at 80 μM of luteolin) (Figure 1A). Conversely, a similar synergism was also found when increasing concentrations of TNF with a fixed luteolin dose (40 μM) (Figure 1*B*). The potentiated cytotoxicity could be detected with doses of TNF as low as 5 ng/ml, a concentration of TNF alone that was not toxic to the cells. Because luteolin at 40 μM exerted limited cytotoxicity while effectively potentiating TNF-induced cell death, this concentration of luteolin was used in later experiments. To determine whether the potentiation of cytotoxicity by luteolin and TNF is common in lung cancer cells, three additional human lung cancer cell lines, H23, H2009, and A549, were tested under similar experimental conditions. All these cell lines are insensitive to TNF-induced cytotoxicity (Figure 1*C*). However, when TNF treatment followed pre-exposure to luteolin, a synergistic cytotoxicity was detected in all lung cancer cell lines (Figure 1*C*). Collectively, these results suggest that the luteolin and TNF synergistically kill lung cancer cells. Because all the tested cells respond to the treatment

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similarly, we choose two of them, A549 (adenocarcinoma) and H460 (large cell lung cancer) for further experiments.

Luteolin and TNF Co-treatment-induced Cytotoxicity Is Associated with Apoptosis—TNF can induce both apoptosis and necrosis (Kamata et al., 2005). Luteolin-induced apoptosis has also been reported (Leung et al., 2005). To determine the mode of cell death induced by luteolin and TNF co-treatment, two distinct approaches were taken to detect apoptosis. Morphological changes in luteolin and TNF co-treated H460 cells were detected by acridine orange/ethidium bromide (AO/EB) staining followed by microscopy. Typical apoptotic features such as cell shrinkage, cell membrane blebbing, and nuclear condensation were observed in luteolin and TNF co-treated cells (Figure *S1* in the online data supplement). Because apoptosis is usually associated with activation of caspases, the activation of caspase 3, and the cleavage of the caspase-3 substrate PARP in luteolin and TNF co-treated H460 cells were detected by Western blot analysis. The cleavage of the precursor (32 kD) and generation of the active form of caspase 3 (20 kD and 17 kD) were detected in luteolin/TNF-treated cells (Figure 1*D*). The cleavage of PARP (115 kD) and generation of the 89-kD fragment, a hallmark of apoptosis, was also significantly enhanced in luteolin and TNF-treated cells (Figure 1*D*). Furthermore, the pan-caspase inhibitor zVAD-fmk significantly suppressed the cytotoxicity induced by luteolin and TNF co-treatment (Figure 1*E*). These results suggest that the luteolin/TNF-induced cytotoxicity in lung cancer cells is associated with apoptosis, consistent with a previous report showing luteolin/TNF treatment induces apoptosis in colorectal cancer cells (Shi et al., 2004). Because luteolin suppresses the survival pathway (NF-κB) and enhances the pro-apoptosis pathway (JNK) induced by TNF, it is likely that luteolin potentiates the TNF-induced apoptosis pathway (see below).

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Induction of ROS by Luteolin in Lung Cancer Cells—Flavonoids, including luteolin, are generally considered to be antioxidants; however, a pro-oxidant property of certain flavonoids has been observed (Matsuo et al., 2005). To investigate the mechanism of the luteolin/TNF-induced cytotoxicity, the effect of luteolin and TNF on ROS generation in H460 cells was examined. Cells treated with luteolin, TNF, or both were stained with two ROS-sensitive dyes, DHE or CM-H_2DCFDA, followed by flow cytometry. DHE and CM-H_2DCFDA are relatively specific for detection of cellular $O_2^{\bullet-}$ and H_2O_2, respectively (Sakon et al., 2003; Zhao et al., 2005). The $O_2^{\bullet-}$ oxidizes DHE to yield ethidium-like substances, while H_2O_2 oxidizes and converts nonfluorescent DCFDA to the fluorescent dichlorodihydrofluorescein (DCF). Although TNF treatment had a marginal effect on the ROS' status, luteolin treatment induced the DHE-specific ROS (Figure 2*A*, right shift of the peaks) while suppressing the CM-H_2DCFDA-specific ROS (left shift of the peaks) in H460 cells (Figure 2*B*). Co-treatment of luteolin and TNF had a similar trend and a more significant effect on ROS' status compared with luteolin treatment alone. The perturbation of ROS' status by luteolin is an early event, occurring as early as 5 min post-exposure of cells to this drug (Figure 2*A* and 2*B*). The results clearly show that luteolin induces ROS in H460 cells and similarly in A549 cells (data not shown). It is noteworthy that DHE-positive staining may result from other sources than oxidation of DHE by $O_2^{\bullet-}$ (28). As control experiments showed that no positive staining was detected in negative control cells (without luteolin treatments) and luteolin did not induce CM-H_2DCFDA staining under our experimental conditions, the result indicates that the DHE staining observed in these experiments resulted from the oxidation by $O_2^{\bullet-}$ (see below).

ROS are Required for Luteolin/TNF-induced Cytotoxicity in Lung Cancer Cells—We next investigated whether ROS accumulation is required for luteolin/TNF-induced cytotoxicity. ROS

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scavengers were used to antagonize the effect of luteolin on ROS in H460 cells. BHA had a marginal effect on the basal ROS level in H460 cells. However, pretreatment with BHA abolished the induction of ROS in cells treated with luteolin alone or luteolin plus TNF (Figure 3*A*). BHA also dramatically suppressed luteolin and TNF co-treatment-induced cell death (Figure 3*B*). Pretreatment with another ROS scavenger, NAC, exerted a similar effect as did BHA on luteolin/TNF-induced cytotoxicity (Figure 3*B*). These results suggest that ROS are required for luteolin/TNF-induced cytotoxicity in lung cancer cells.

Luteolin Induces ROS through Suppression of SOD Activity—Because luteolin increased $O_2^{\bullet-}$ while it decreased the H_2O_2 levels, and $O_2^{\bullet-}$ is mainly converted to H_2O_2 by SOD in cells, we hypothesized that luteolin could reduce SOD activity, thus resulting in an elevated superoxide anion level but not the H_2O_2 level. Therefore, we investigated the effect of luteolin on SOD activity by examining SOD protein expressions and SOD enzyme activity. Luteolin treatment, applied alone or combined with TNF, caused a significant suppression of SOD activity in H460 cells starting as early as 5 min (Figure 4*A*). There were no detectable changes in the expression levels of MnSOD, Cu-ZnSOD, or CCS in luteolin-treated cells (Figure 4*B*). These results indicated that luteolin induces ROS through suppression of SOD activity.

ROS Are Involved in Suppression of the TNF-induced NF-κB Pathway by Luteolin—The NF-κB activation pathway plays an important role in the resistance to TNF-induced apoptosis in lung cancer cells (Wang et al., 2006). Luteolin has been reported to suppress TNF-induced NF-κB activation in colorectal cancer cells (Shi et al., 2004). To examine whether luteolin functions similarly in lung cancer cells, A549 cells were transfected with a NF-κB-responsive luciferase reporter, pretreated with luteolin for 30 min, and followed by treatment with TNF for 15 h. TNF alone caused a robust activation of NF-κB, which was completely suppressed by pretreatment

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with luteolin, suggesting that luteolin blocks the TNF-induced NF-κB activation pathway (Figure 5*A*). The induction of NF-κB downstream anti-apoptotic genes XIAP and MnSOD by TNF was also completely suppressed by luteolin (Figure *S2* in the online data supplement). The involvement of ROS in luteolin-induced suppression of the TNF-activated NF-κB pathway was addressed. Although pretreatment of the cells with BHA and NAC had no detectable effect on basal NF-κB activity, BHA and NAC significantly alleviated the suppression of NF-κB activity by luteolin. These results suggest that luteolin-induced ROS accumulation is important for suppressing the TNF-activated NF-κB pathway.

Consistent with findings in colorectal cancer cells where luteolin-induced NF-κB suppression occurs downstream of IκBα (Shi et al., 2004), luteolin did not suppress TNF-induced IκBα phosphorylation (Figure 5*B,*15 min) and degradation in A549 and H460 cells (Figure 5*C*; 20 min, and data not shown). However, the recovery of the NF-κB-regulated factor IκBα was suppressed by luteolin [Figure 5*B*; compare Lu(-), 60 – 480 min vs. Lu(+), 60–480 min]. The ROS scavenger BHA had no effect on IκBα degradation (Figure 5*D,* 15 min). However, ROS appear to be responsible for the suppression of IκBα recovery, because pre-incubation with BHA restored IκBα recovery in luteolin-treated cells (Figure 5*D,* 60 and 120 min). Collectively, these results further support the hypothesis that luteolin suppresses the TNF-induced NF-κB pathway through induction of ROS.

Potentiation of the JNK Activation Pathway by Luteolin-induced ROS Accumulation—When the NF-κB pathway is blocked, TNF induces a potent and prolonged activation of JNK, which contributes to TNF-induced apoptosis (Lin and Dibling, 2002). In A549 and H460 cells, TNF induced a modest and transient activation of JNK, starting at 10 min post treatment and diminishing after 30 min (Figure 6A). When the cells were pretreated with luteolin, the extent of

JNK activation was dramatically increased and sustained over 2 h (Figure 6A and 6*C*, data not shown). The potentiation of JNK activity by luteolin is important for luteolin/TNF-induced cytotoxicity because the specific JNK inhibitor SP600125 effectively suppressed luteolin/TNF-induced cell death (Figure 6*B*). BHA effectively suppressed the extent and shortened the duration of JNK activation in luteolin and TNF co-treated cells (Figure 6*C*). These results suggest that luteolin-induced ROS accumulation is critical for potentiation of TNF-induced JNK activation, which consequently contributes to the sensitization of TNF-induced apoptosis in lung cancer cells.

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DISCUSSION

This study demonstrates that luteolin can sensitize lung cancer cells to TNF-induced apoptosis through a ROS-dependent mechanism. This conclusion is supported by the following findings: First, ROS accumulation occurred in the early phase of luteolin treatment. Second, ROS scavengers effectively attenuated the luteolin-induced suppression of NF-κB and the activation of JNK. Third, pretreatment of cells with ROS scavengers effectively inhibited the luteolin/TNF-induced apoptosis. Thus, luteolin sensitizes lung cancer cells to TNF-induced apoptosis through ROS-mediated suppression of NF-κB and potentiation of JNK. To our knowledge, this is the first report that ROS plays a critical role in luteolin's effect in suppression of TNF-induced NF-κB and potentiation of JNK. The combination of luteolin and TNF could greatly improve the potency of TNF as a lung cancer therapeutic agent.

Most flavonoids, including luteolin, can act as antioxidants (Skibola and Smith, 2000). However, due to various concentrations or cell types tested, a pro-oxidant role of flavonoids has been noticed recently (Matsuo et al., 2005). In this study we clearly show that luteolin induced ROS accumulation in lung cancer cells. The mechanism appears to involve the suppression of SOD activity rather than affecting the expression of SOD and CCS proteins. It is unlikely that luteolin-induced ROS accumulation involves mitochondria, because the mitochondrial electron transport inhibitors antimycin A, myxothiazol, and rotenone had no obvious effect on this process (data not shown).

Although luteolin was previously reported to induce ROS (Matsuo et al., 2005), the consequences of ROS accumulation on cellular signaling as well as cell death in response to TNF has not been addressed. In this study, we found that blocking NF-κB plays an important role in the synergistic cytotoxicity of combined treatment with luteolin and TNF in lung cancer

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cells. The mechanism of luteolin-induced NF-κB suppression in different cell types is somewhat controversial, occurring either by suppression of IKK in the cytoplasm or NF-κB in the nucleus (Kim et al., 2003; Ruiz and Haller, 2006; Shi et al., 2004; Xagorari et al., 2001). Our result is consistent with the finding in colorectal cancer cells and rat fibroblasts where the target of luteolin is downstream of IκB in the NF-κB pathway (Kim et al., 2003; Shi et al., 2004). ROS have been implicated in activation or suppression of NF-κB, depending on the nature of stimuli and cell context (Zhang and Chen, 2004). Our results demonstrate that ROS accumulation is involved in luteolin-induced suppression of the NF-κB pathway; however, the exact mechanism is currently unclear. The phosphorylation of p65 at S276, S536, and S529 has not been altered by luteolin (data not shown). It remains to be determined whether ROS directly oxidize the NF-κB subunits or interfer with the communication between NF-κB and transcriptional co-factors such as p300/CBP (Nishi et al., 2002; Shi et al., 2004). Interestingly, MnSOD, one of NF-κB's target genes, is involved in eliminating ROS by converting the superoxide anion radical to H_2O_2. The suppression of NF-κB by the early phase accumulation of ROS induced by luteolin may shut off the induction of MnSOD, thereby establishing a positive feedback loop to amplify the effect of ROS.

The role of JNK in cell death control is complex (Lin and Dibling, 2002; Lin et al., 2006; Ventura et al., 2004). Our results show that JNK is involved in TNF/luteolin-induced apoptosis. However, the mechanism of JNK activation by luteolin is not well understood. JNK can be secondary to NF-κB inhibition (Kamata et al., 2005; Lin and Dibling, 2002). Alternatively, JNK can be activated by induction of the MAP3K/JNKK/JNK cascade. There are several MAP3Ks that are able to initiate JNK activation: apoptosis signal-related kinase 1 (ASK1), mixed lineage kinase (MLK), MEK kinase 1 (MEKK1) and transforming growth factor (TGF)-activated protein

kinase 1 (TAK1) (Kamata et al., 2005; Liu and Lin, 2005). JNK is also negatively regulated through dephosphorylation by MAP kinase phosphatases (MKP). Interestingly, the MKP activity is inhibited by oxidation (Kamata et al., 2005). NF-κB suppresses apoptosis by inhibiting JNK activation, which may occur through MKP (Kamata et al., 2005). Therefore, it is possible that luteolin-induced ROS trigger JNK activation by either stimulating the ASK1-MEK-JNK pathway and/or suppressing the MKP activity. It remains to be determined whether the potentiation of JNK activation by luteolin is a consequence of NF-κB suppression or is achieved through a mechanism that is independent of the crosstalk between NF-κB and JNK pathways (Kamata et al., 2005; Liu and Lin, 2005).

Although TNF was originally found to kill tumor cells and was expected to be a therapeutic agent against cancer, accumulating evidence has suggested a pro-cancer activity for TNF (Orosz et al., 1993; Varela et al., 2001). Studies in animals reveal a positive role of TNF in carcinogenesis of skin, colon, and liver (Knight et al., 2000; Luo et al., 2004; Moore et al., 1999). TNF is also implicated in lung cancer development (Bernert et al., 2003). The tumor-promoting role of TNF is mainly associated with its ability to induce NF-κB, which protects cells against apoptosis. Therefore, blocking the TNF-induced NF-κB pathway could be an effective approach for cancer prevention. The suppression of NF-κB by luteolin is extremely intriguing because it may convert TNF from a tumor promoter to a tumor killer. Therefore, in addition to application in lung cancer therapy, luteolin could be effective in chemoprevention for lung cancer.

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ACKNOWLEDGEMENTS

We would like to thank Drs. Han-Ming Shen and Susan Boggs for helpful discussions, and Dr. Chris Stidley for statistical analysis.

Molecular Pharmacology Fast Forward. Published on February 12, 2007 as DOI: 10.1124/mol.106.032185
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FOOTNOTE

This work was partly supported by NIH grants CA095568 and P30ES012072.

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FIGURE LEGENDS

Figure 1**. Potentiated cytotoxicity by TNF and luteolin co-treatment in human lung cancer cells.** (*A*) H460 cells were pretreated with increasing concentrations of luteolin (1-80 μM) for 30 min or remained untreated and followed by exposure to TNF (20 ng/ml) for an additional 24 h. Cell death was measured with a cytotoxicity detection kit (LDH release). Data shown are the mean ± SD. The data are representative of three independent experiments. (*B*) H460 cells were pretreated with luteolin (40 μM) for 30 min or remained untreated followed by 24 h of treatment with TNF (5 – 50 ng/ml). Cell death was detected as described in (*A*). (*C*) Lung cancer H23, H2009, and A549 cells were pretreated with 40 μM luteolin for 30 min followed by TNF treatment (20 ng/ml) for an additional 24 h. Cell death was detected as described in (*A*). (*D*) Luteolin and TNF co-treatment-induced cytotoxicity is associated with apoptosis. H460 cells were pretreated with luteolin (40 μM) for 30 min and followed by treatment with TNF (20 ng/ml) for indicated time periods. Cell extracts were resolved in 10% SDS-PAGE gels. PARP and caspase-3 were detected by Western blot. β-actin was detected as an input control.
(*E*) H460 cells were pretreated with zVAD-fmk (10 μM) for 30 min followed by 24 h of treatment with TNF, luteolin or both as indicated. Cell death was detected as described in (*A*).

Figure 2. **Luteolin treatment induces ROS accumulation**. (*A*) H460 cells were pretreated with luteolin (40 μM) for 30 min and then treated with TNF (20 ng/ml) for indicated time periods. DHE (5 μM) was added 30 min before collecting cells. The cells were harvested and immediately analyzed with a flow cytometer (FACSCalibur, BD Biosciences) and data were processed with the CellQuest program (BD Biosciences). Untreated cells with DHE staining were used as a negative control. The histogram overlays show the results of treated cells (gray

lines) compared with untreated cells (dark lines). X axis: fluorescent intensity showing the extent of DHE oxidation; Y axis: cell number. (*B*) H460 cells were treated and analyzed similarly as described in (*A*) except they were stained with CM-H_2DCFDA (1 μM).

Figure 3. **ROS accumulation is required for cell apoptotic death.** (*A*) H460 cells were pretreated with BHA (100 μM) for 30 min and then treated with luteolin (40 μM) for an additional 30 min followed by TNF (20 ng/ml) treatment for another 30 min. Cells were stained with DHE (5 μM) for 30 min before collecting. The stained cells were harvested and immediately analyzed as described in Fig.2. The histogram overlays show the results of treated cells (gray lines) compared with untreated cells (dark lines). X axis: fluorescent intensity showing the extent of DHE oxidation; Y axis: cell number. (*B*) H460 cells were pretreated with BHA (100 μM) or NAC (10 μM) for 30 min and then treated with luteolin (40 μM) for an additional 30 min followed by TNF (20 ng/ml) treatment for 24 h. Cell death was measured as described in Figure 1. * $P < 0.01$.

Figure 4. **Luteolin suppresses SOD activity in H460 cells.** (*A*) H460 cells were treated with luteolin (40 μM), TNF (20 ng/ml), or both for indicated time periods. Cells were collected and SOD activity was measured. Data shown are the mean ± SD of triplicate determinations. (*B*) H460 cells were treated as described with luteolin (40 μM) for the indicated time periods. Cell extracts were resolved in 10% SDS-PAGE gels. Cu-ZnSOD, MnSOD, and CCS were detected by Western blot. β-actin was detected as an input control.

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Figure 5. **Luteolin-induced ROS is required for suppression of TNF-induced NF-κB activation.** (*A*) A549 cells were co-transfected with p2xκB-Luc and pRSV-LacZ. Eight hours post transfection, cells were treated as indicated. Pretreatment with BHA (100 μM) or NAC (10 μM) for 30 min was followed by luteolin (40 μM) for an additional 30 min, then TNF (20 ng/ml) was added for treatment for 15 h. Luciferase activity was detected and normalized with β-galactosidase activity. The data shown are the mean ± SD. The data are average of three independent experiments. * $P < 0.01$. (*B*) A549 cells were pretreated with 40 μM luteolin for 30 min and then treated with TNF for indicated time periods. Phospho-IκBα was detected by Western blot. β-actin was detected as an input control. (*C*) A549 cells were pretreated with 40 μM luteolin for 30 min followed by exposure to TNF for the indicated time periods. IκBα was detected by Western blot. β-actin was detected as an input control. (*D*) A549 cells were treated with BHA (100 μM) for 30 min and then treated with luteolin (40 μM) for an additional 30 min followed by TNF (20 ng/ml) treatment for indicated time periods. IκBα was detected by Western blot. β-actin was detected as an input control.

Figure 6**. Luteolin potentiates the TNF-induced JNK activation pathway through induction of ROS.** (*A*) A549 cells were pretreated with 40 μM luteolin for 30 min and then treated with TNF for indicated times. Phospho-JNK and JNK1 were detected by Western blot. β-actin was detected as an input control. (*B*) A549 cells were pretreated with a JNK inhibitor SP600125 (20 μM) for 30 min and then treated with luteolin (40 μM) for an additional 30 min followed by TNF (20 ng/ml) treatment for 24 h. Cell death was measured by a LDH release assay. The data shown are representative of three independent experiments and the mean ± SD of triplicate

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determinations. * $P < 0.01$. (*C*) A549 cells were pretreated with BHA (100 μM) for 30 min and then treated with luteolin (40 μM) for an additional 30 min followed by TNF (20 ng/ml) treatment for indicated time periods. Phospho-JNK and JNK1 were detected by Western blot. β-actin was detected as an input control.

Fig.1

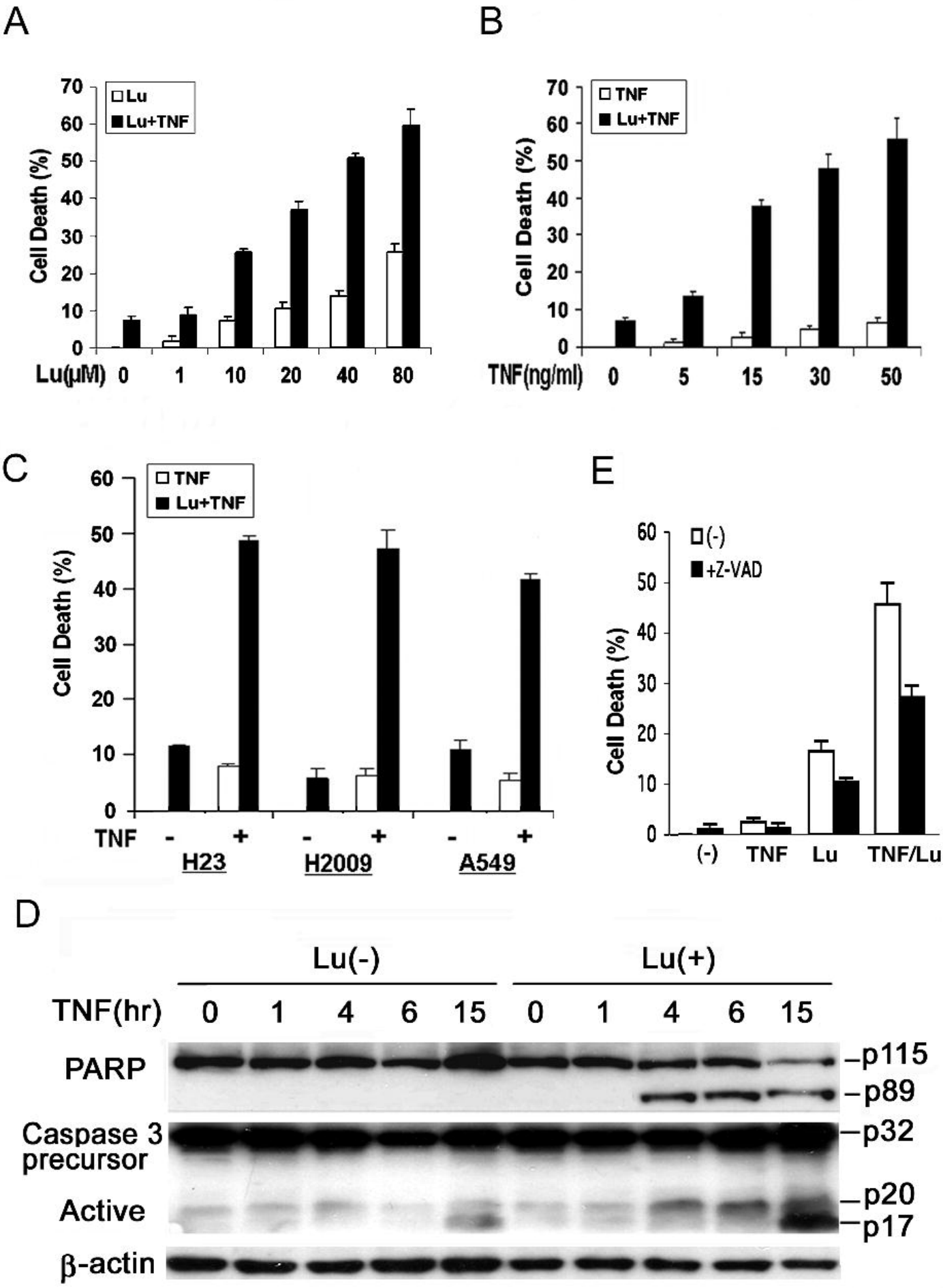

A
Lu
Lu+TNF
Cell Death (%)
Lu(μM) 0 1 10 20 40 80
B
TNF
Lu+TNF
Cell Death (%)
TNF(ng/ml) 0 5 15 30 50
C
TNF
Lu+TNF
Cell Death (%)
TNF - + - + - +
H23
H2009
A549
E
(-)
+Z-VAD
Cell Death (%)
(-) TNF Lu TNF/Lu
D
Lu(-)
Lu(+)
TNF(hr) 0 1 4 6 15 0 1 4 6 15
PARP
p115
p89
Caspase 3 precursor
p32
Active
p20
p17
β-actin

Fig.2

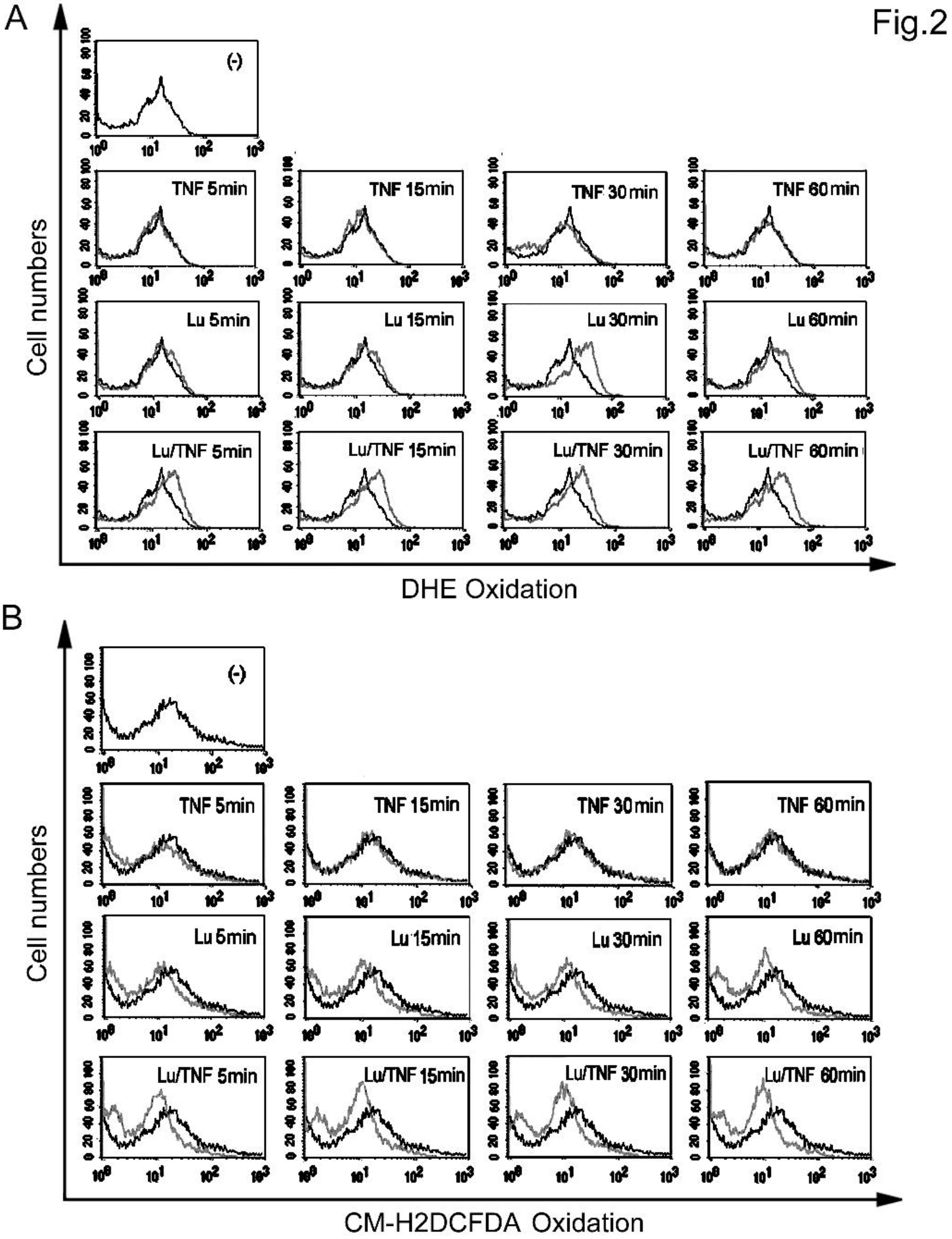
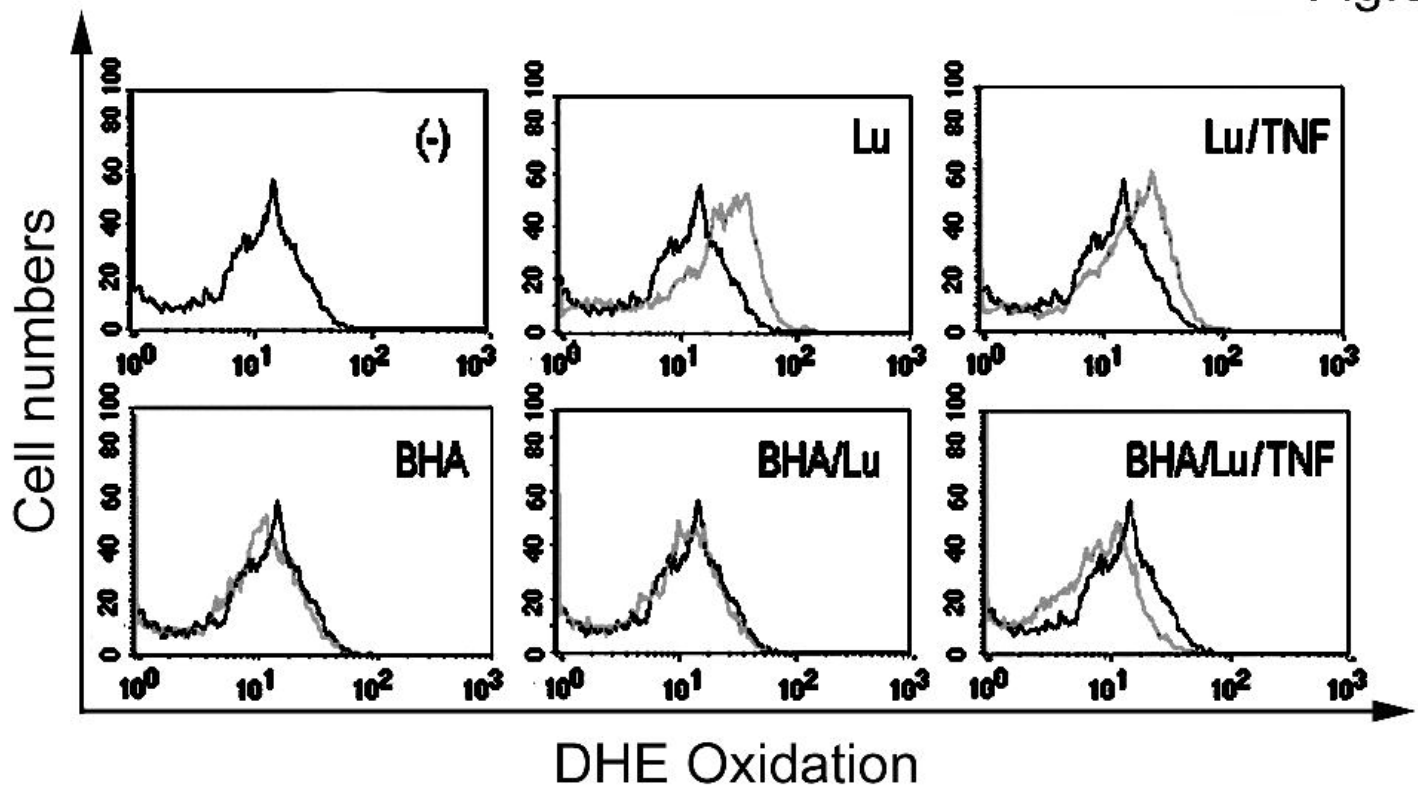

A
(-)
TNF 5min
TNF 15min
TNF 30 min
TNF 60 min
Lu 5min
Lu 15min
Lu 30min
Lu 60min
Lu/TNF 5min
Lu/TNF 15min
Lu/TNF 30min
Lu/TNF 60min
Cell numbers
DHE Oxidation
B
(-)
TNF 5min
TNF 15min
TNF 30 min
TNF 60 min
Lu 5min
Lu 15min
Lu 30min
Lu 60min
Lu/TNF 5min
Lu/TNF 15min
Lu/TNF 30min
Lu/TNF 60min
Cell numbers
CM-H2DCFDA Oxidation

Fig.3

A


Cell numbers
(-)
Lu
Lu/TNF
BHA
BHA/Lu
BHA/Lu/TNF
DHE Oxidation

B

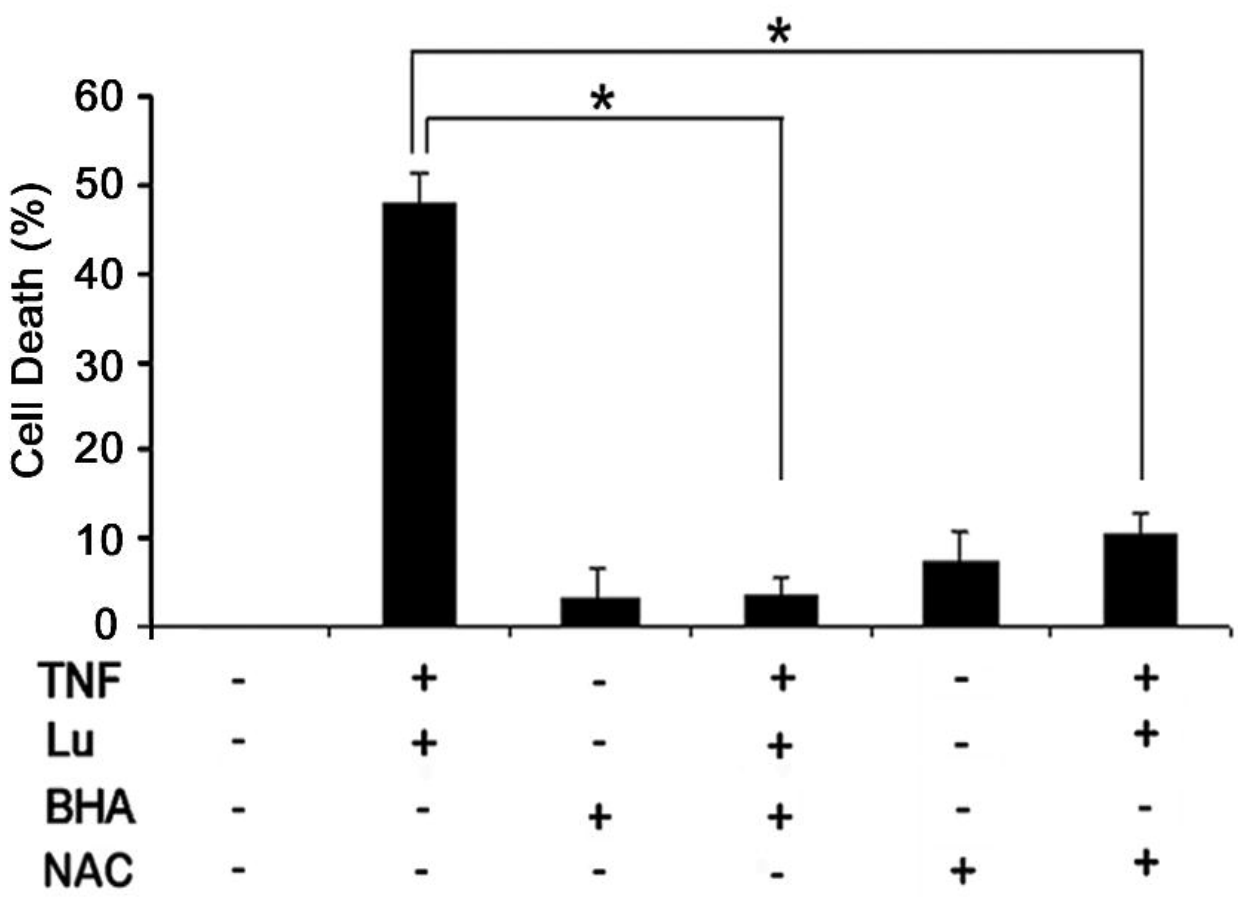
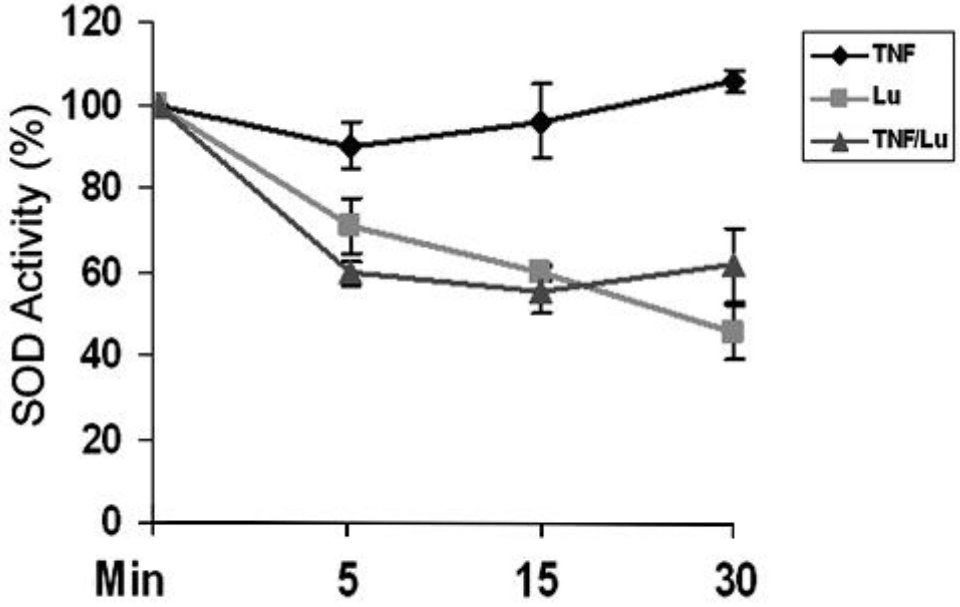

Cell Death (%)
60
50
40
30
20
10
0
*
*
TNF - + - + - +
Lu - + - + - +
BHA - - + + - -
NAC - - - - + +

Fig.4

A


120
100
80
60
40
20
0
SOD Activity (%)
Min
5
15
30
TNF
Lu
TNF/Lu

B

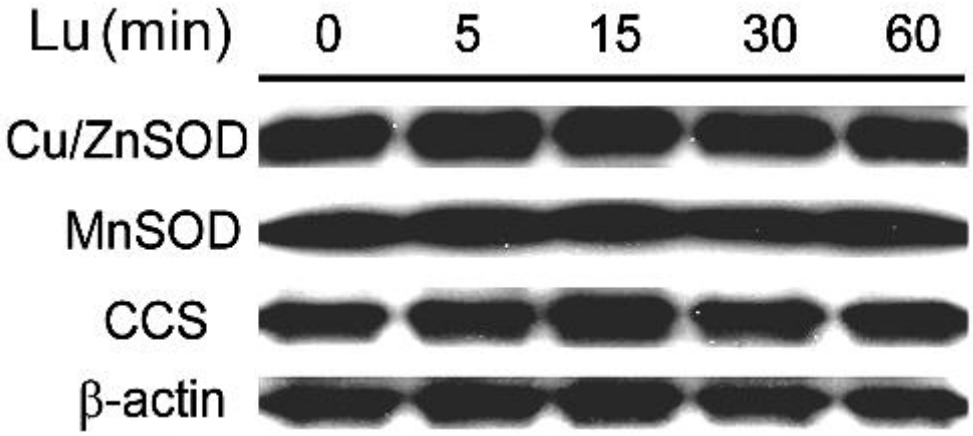
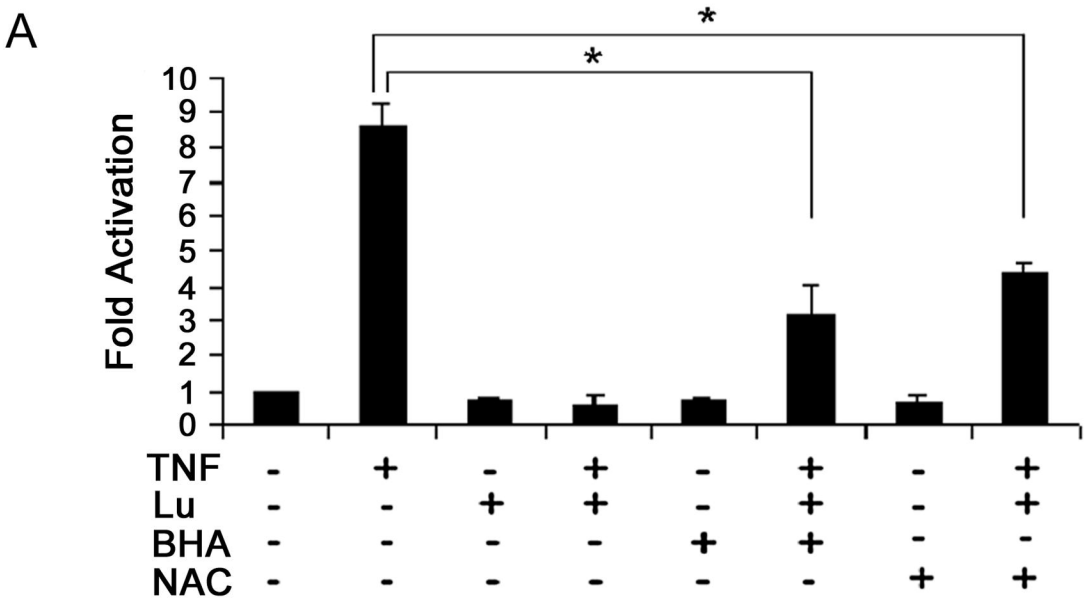
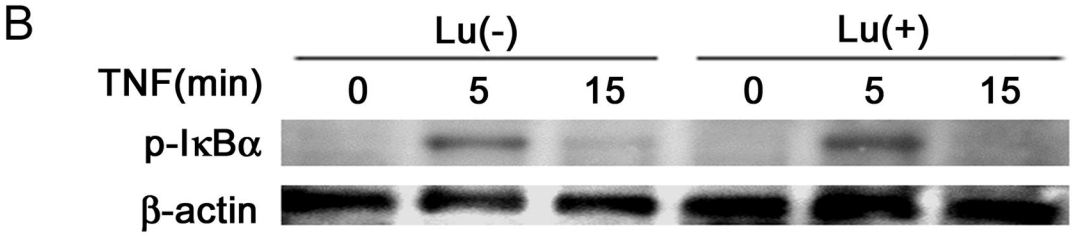
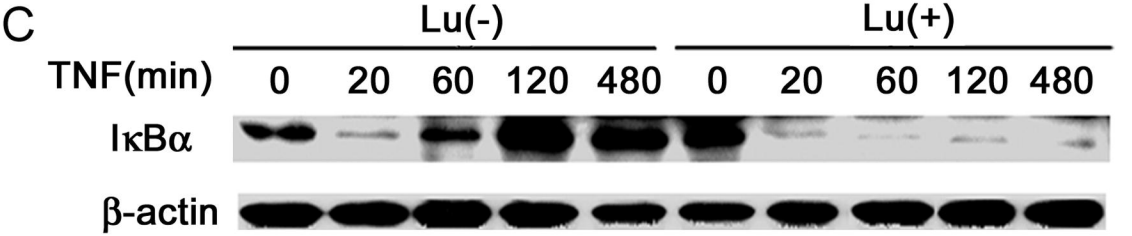

Lu (min)
0
5
15
30
60
Cu/ZnSOD
MnSOD
CCS
β-actin

Fig.5


A
*
*
Fold Activation
10
9
8
7
6
5
4
3
2
1
0
TNF - + - + - + - +
Lu - - + + - + - +
BHA - - - - + + - -
NAC - - - - - - + +


B
Lu(-)
Lu(+)
TNF(min)
0
5
15
0
5
15
p-IκBα
β-actin


C
Lu(-)
Lu(+)
TNF(min)
0
20
60
120
480
0
20
60
120
480
IκBα
β-actin

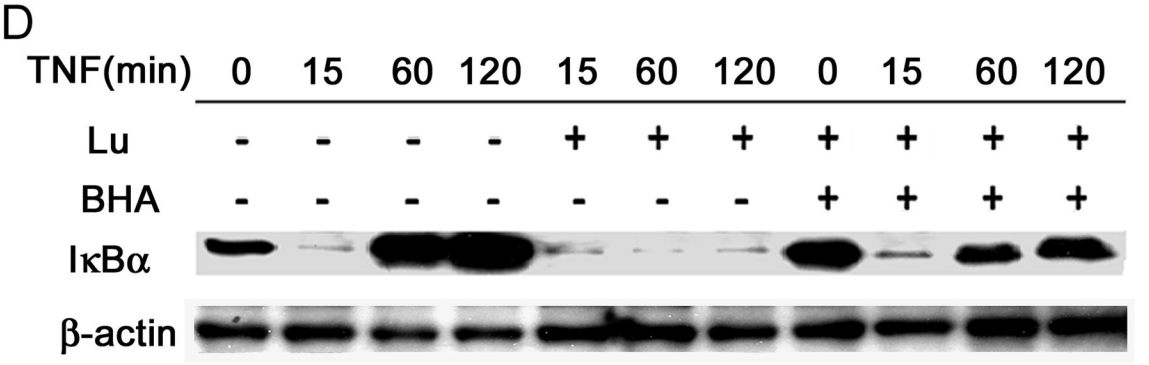
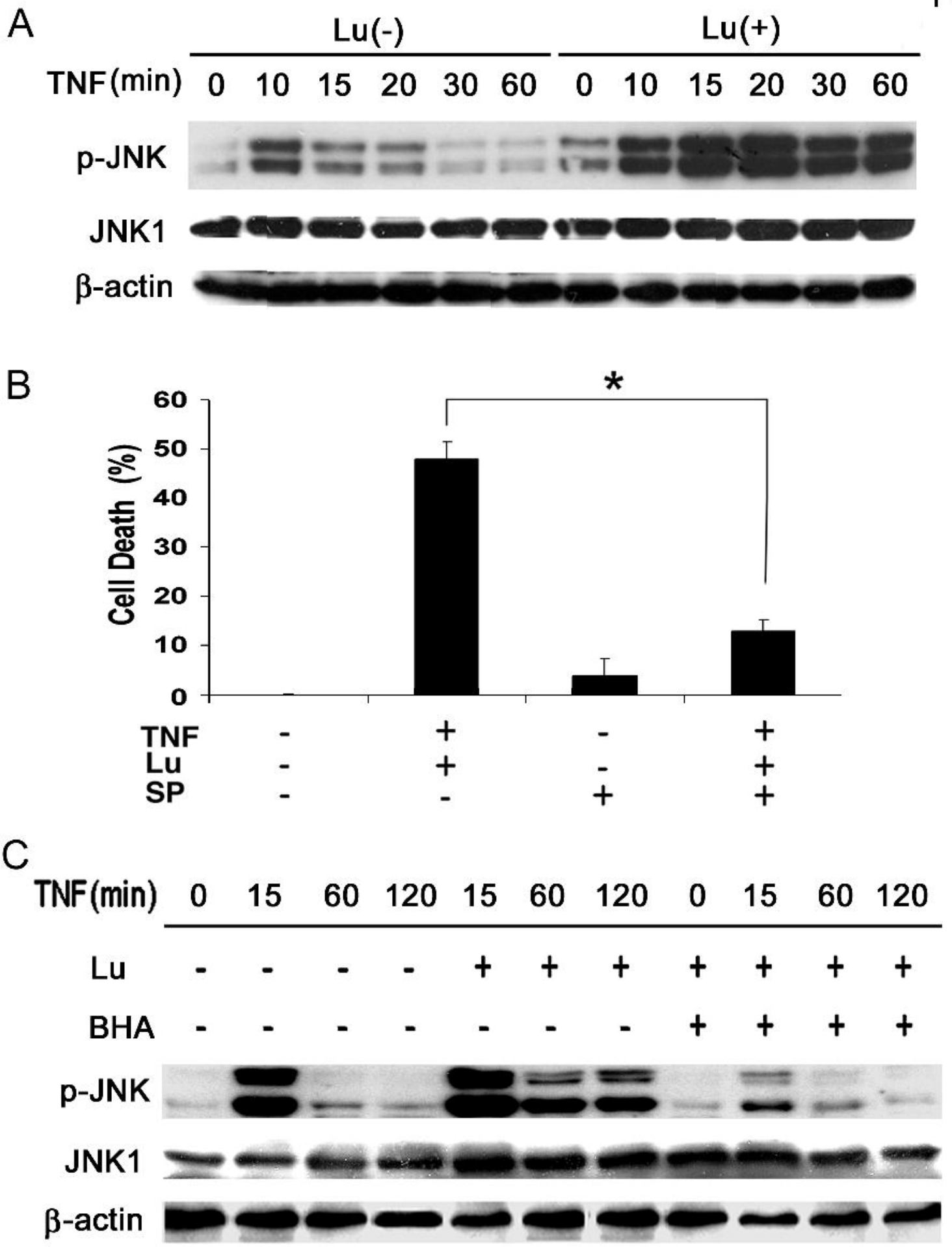

D
TNF(min)
0
15
60
120
15
60
120
0
15
60
120
Lu - - - - + + + + + + +
BHA - - - - - - - + + + +
IκBα
β-actin

Fig.6


A
Lu(-)
Lu(+)
TNF(min) 0 10 15 20 30 60 0 10 15 20 30 60
p-JNK
JNK1
β-actin
B
*
Cell Death (%)
60
50
40
30
20
10
0
TNF - + - +
Lu - + - +
SP - - + +
C
TNF(min) 0 15 60 120 15 60 120 0 15 60 120
Lu - - - - + + + + + + +
BHA - - - - - - - + + + +
p-JNK
JNK1
β-actin