

# A Critical Role of Luteolin-Induced Reactive Oxygen Species in Blockage of Tumor Necrosis Factor-Activated Nuclear Factor- $\kappa$ B Pathway and Sensitization of Apoptosis in Lung Cancer Cells<sup>S</sup>

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## ABSTRACT

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) activated by tumor necrosis factor (TNF) attenuates the TNF-induced apoptosis pathway. Therefore, blockage of NF- $\kappa$ B should improve the anticancer 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- $\kappa$ B; however, the mechanisms of this effect have not been well elucidated. In this article, 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- $\kappa$ B, whereas it 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. It is noteworthy that suppression of ROS accumulation by ROS scavengers butylated hydroxyanisole, and *N*-acetyl-L-cysteine prevented the luteolin-induced suppression of NF- $\kappa$ B and potentiation of JNK and significantly suppressed the synergistic cytotoxicity seen with cotreatment 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- $\kappa$ B and potentiation of JNK to sensitize lung cancer cells to undergo TNF-induced apoptosis.

Tumor necrosis factor (TNF) can induce both survival and death signals, depending on cell context 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  $\kappa$ B (NF- $\kappa$ B) 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- $\kappa$ B 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 proapoptotic (Lin and Dibling, 2002; Ventura et al., 2004). There is cross-talk between the NF- $\kappa$ B and JNK activation pathways that controls the outcome of the cells in response to TNF (Lin and Dibling, 2002; Kamata et al., 2005). 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 I $\kappa$ B kinase (IKK) is recruited to the TNFR1 signaling complex through TNF receptor-asso-

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**ABBREVIATIONS:** TNF, tumor necrosis factor; NF- $\kappa$ B, nuclear factor  $\kappa$ B; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; NAC, *N*-acetyl-L-cysteine; BHA, butylated hydroxyanisole; TNFR1, tumor necrosis factor receptor 1; IKK, I $\kappa$ B kinase; MnSOD, manganese superoxide dismutase; SOD, superoxide dismutase; CCS, copper chaperon for superoxide dismutase-1; LDH, lactate dehydrogenase; <sup>1</sup>O<sub>2</sub>, <sup>1</sup>OH, singlet oxygen; LOO<sup>•</sup>, peroxy radical; Cu-ZnSOD, copper-zinc superoxide dismutase; PARP, poly(ADP-ribose) polymerase; CM-H<sub>2</sub>DCFDA, 5-(and -6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester; DHE, dihydroethidium; PAGE, polyacrylamide gel electrophoresis; MKP, mitogen-activated protein kinase phosphatase; SP600125, anthra[1,9-*cd*]pyrazolo-6(2*H*)-one-1,9-pyrazoloanthrone; zVAD-fmk, benzyloxy-carbonyl-Val-Ala-Asp fluoro-methylketone.

ciated factor 2 and activated through a receptor-interaction protein-mediated mechanism that involves mitogen-activated protein kinase kinase kinase 3 (Devin et al., 2000; Yang et al., 2001). The activated IKK in turn phosphorylates the I $\kappa$ Bs, which retain NF- $\kappa$ B in the cytoplasm, to trigger their rapid polyubiquitination followed by degradation in the 26S proteasome. This process allows NF- $\kappa$ B's nuclear translocation and binding to the promoters of its target genes. Several of NF- $\kappa$ B's target genes are found to have antiapoptotic properties (Karin et al., 2004). Induction of the antioxidant manganese superoxide dismutase (MnSOD) by NF- $\kappa$ B is also implicated to be antiapoptotic (Kamata et al., 2005). The transcriptional activity of NF- $\kappa$ B is further regulated by phosphorylation and acetylation (Wajant et al., 2003). Therefore, multiple steps affecting post-translational modification of NF- $\kappa$ B could be targets for regulating its activity.

Reactive oxygen species (ROS) are a group of reactive, short-lived, oxygen-containing species such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\cdot OH$ ), singlet oxygen ( $^1O_2$ ), and peroxy radical ( $LOO\cdot$ ). Cells have developed effective mechanisms to reduce cellular ROS levels. The superoxide dismutase (SOD) converts  $O_2^-$  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, whereas copper-zinc SOD (Cu-ZnSOD)/SOD1 is present mainly in the cytosol (Curtin et al., 2002; Nimmual et al., 2003). The copper chaperon for SOD-1 (CCS) is important to maintain the activity of Cu-ZnSOD (Culotta et al., 1997). In addition, glutathione and glutathione peroxidases 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 nonapoptotic cell death induced by TNF (Lin et al., 2004; Kamata et al., 2005). The role of ROS in TNF induction of the NF- $\kappa$ B pathway is somewhat controversial, because 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).

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-inflammatory agent (Ueda et al., 2002; Bagli et al., 2004; Kanadaswami et al., 2005). Although it was generally believed 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. Luteolin has been shown to sensitize colorectal cancer cell lines to TNF-induced apoptosis via inhibition of NF- $\kappa$ B and augmentation of JNK (Shi et al., 2004). However, the mechanism of luteolin-induced NF- $\kappa$ B 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- $\kappa$ B and potentiating the JNK pathways and subsequently sensitizing lung cancer cells to TNF-induced apoptosis.

## Materials and Methods

**Plasmids and Reagents.** Reporter plasmids 2 $\times$ κB-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 hydroxyanisole (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 I $\kappa$ B $\alpha$ , 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, - $\beta$ -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-I $\kappa$ B $\alpha$  were from Cell Signaling Technology (Danvers, MA). 5-(and -6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA) 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 U/ml penicillin, and 100  $\mu$ g/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 each well. The absorbance of the samples was measured at 490 nm using a plate reader. All of the experiments were repeated three to five times, and the average is shown in each figure. Cell death was calculated using the following formula:

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  $\mu$ M) or CM-H<sub>2</sub>DCFDA (1  $\mu$ M) was added. ROS were detected by flow cytometry with FACSCalibur and analyzed with the CellQuest program (both from BD Biosciences) as reported previously (Lin et al., 2004).

**Luciferase Assay.** Cells cultured in 12-well plates were transfected with 0.35  $\mu$ g of p2 $\times$ κB-Luc and 0.15  $\mu$ g of pRSV-LacZ by using FuGENE6 transfection reagent (Roche, Indianapolis, IN). Eight hours after transfection, cells were treated with luteolin (40  $\mu$ M) 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  $\beta$ -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<sub>2</sub> buffer (20 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, and 1  $\mu$ g/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 according to the manufacturer's instructions (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Each experiment was repeated at least three times, and representative results are shown in each figure.

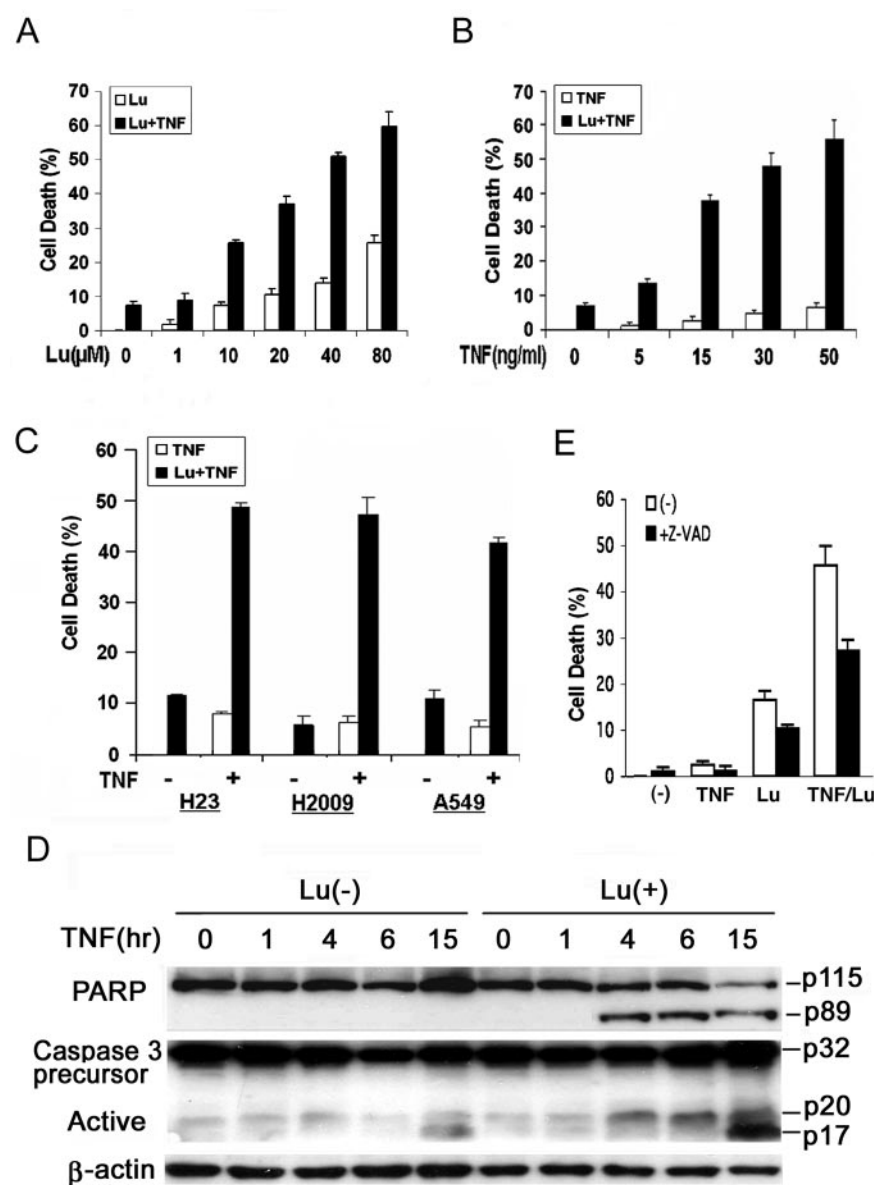
**Detection of SOD Activity.** SOD activity was detected using the SOD activity detection kit according to the manufacturer's instructions (Cayman). In brief, cells cultured in 12-well plates were treated with luteolin (40  $\mu$ M), TNF (20 ng/ml), or both for 5, 15, and 30 min. Cells were collected and lysed in cold lysis buffer (20 mM HEPES, pH 7.2, containing 1 mM 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  $\mu$ l of diluted tetrazolium and 10  $\mu$ l of 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  $\pm$  S.D. Statistical significance was examined by two-way analysis of variance pairwise comparison. In all analyses,  $P < 0.05$  was considered statistically significant.

## Results

**Luteolin and TNF Cotreatment 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 an LDH release assay. Treatment with luteolin alone caused moderate cytotoxicity, with approximately 23% cell death at the dose of 80  $\mu$ M (Fig. 1A). Even at the highest evaluated dose of TNF (50 ng/ml), TNF treatment alone caused little cell death (<10% cell death) (Fig. 1B). 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  $\mu$ M concentration of luteolin) (Fig. 1A). Conversely, a similar synergism was also found when increasing concentrations of TNF with a fixed luteolin dose (40  $\mu$ M) (Fig. 1B). 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  $\mu$ M exerted limited cytotoxicity and effectively potentiated TNF-induced cell death, this



**Fig. 1.** Potentiated cytotoxicity by TNF and luteolin cotreatment in human lung cancer cells. A, H460 cells were pretreated with increasing concentrations of luteolin (1–80  $\mu$ M) 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  $\pm$  S.D. The data are representative of three independent experiments. B, H460 cells were pretreated with luteolin (40  $\mu$ M) 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  $\mu$ M 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 cotreatment-induced cytotoxicity is associated with apoptosis. H460 cells were pretreated with luteolin (40  $\mu$ M) 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.  $\beta$ -Actin was detected as an input control. E, H460 cells were pretreated with zVAD-fmk (10  $\mu$ M) for 30 min followed by 24 h of treatment with TNF, luteolin, or both as indicated. Cell death was detected as described in A.



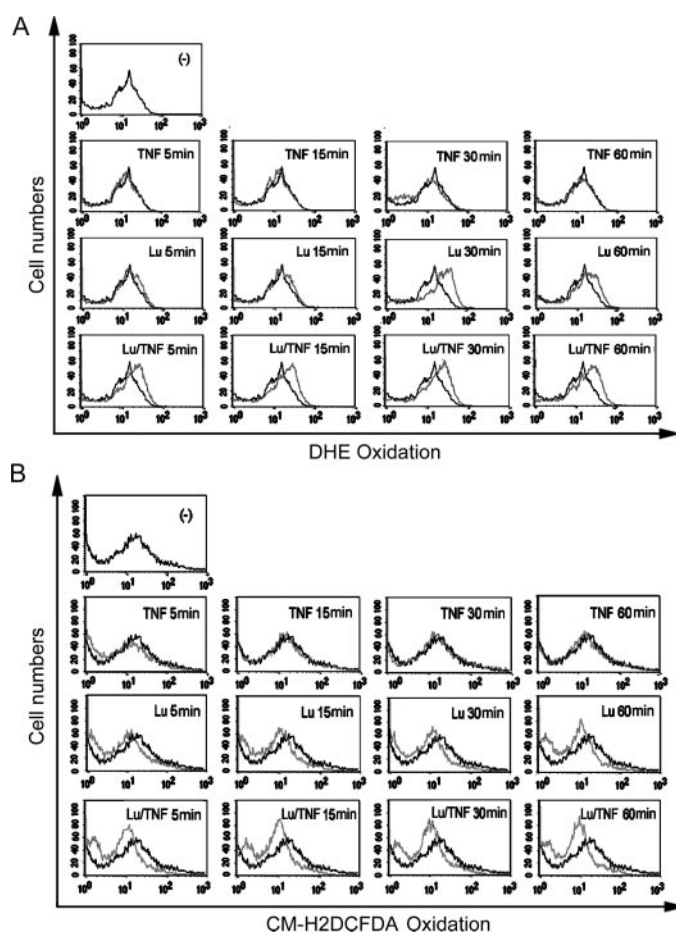
concentration of luteolin was used in later experiments. To determine whether the potentiation of cytotoxicity by luteolin and TNF is common in lung cancer cells, three additional human lung cancer cell lines, H23, H2009, and A549, were tested under similar experimental conditions. All of these cell lines are insensitive to TNF-induced cytotoxicity (Fig. 1C). However, when TNF treatment followed pre-exposure to luteolin, a synergistic cytotoxicity was detected in all lung cancer cell lines (Fig. 1C). As a whole, these results suggest that the luteolin and TNF synergistically kill lung cancer cells. Because all of the tested cells respond to the treatment similarly, we choose two of them, A549 (adenocarcinoma) and H460 (large cell lung cancer), for further experiments.

**Luteolin and TNF Cotreatment-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 cotreatment, two distinct approaches were taken to detect apoptosis. Morphological changes in luteolin- and TNF-cotreated H460 cells were detected by acridine orange/ethidium bromide staining followed by microscopy. Typical apoptotic features such as cell shrinkage, cell membrane blebbing, and nuclear condensation were observed in luteolin- and TNF-cotreated cells (Supplemental Fig. S1). 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-cotreated H460 cells were detected by Western blot analysis. The cleavage of the precursor (32 kDa) and generation of the active form of caspase 3 (20 and 17 kDa) were detected in luteolin/TNF-treated cells (Fig. 1D). The cleavage of PARP (115 kDa) and generation of the 89-kDa fragment, a hallmark of apoptosis, was also significantly enhanced in luteolin- and TNF-treated cells (Fig. 1D). Furthermore, the pan-caspase inhibitor zVAD-fmk significantly suppressed the cytotoxicity induced by luteolin and TNF cotreatment (Fig. 1E). These results suggest that the luteolin/TNF-induced cytotoxicity in lung cancer cells is associated with apoptosis, consistent with a previous report showing that luteolin/TNF treatment induces apoptosis in colorectal cancer cells (Shi et al., 2004). Because luteolin suppresses the survival pathway (NF- $\kappa$ B) and enhances the proapoptosis pathway (JNK) induced by TNF, it is likely that luteolin potentiates the TNF-induced apoptosis pathway (see below).

**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<sub>2</sub>DCFDA, followed by flow cytometry. DHE and CM-H<sub>2</sub>DCFDA are relatively specific for detection of cellular O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>, respectively (Sakon et al., 2003; Zhao et al., 2005). The O<sub>2</sub><sup>-</sup> oxidizes DHE to yield ethidium-like substances, whereas H<sub>2</sub>O<sub>2</sub> oxidizes and converts nonfluorescent 7'-dichlorodihydro-fluorescein diacetate acetyl ester to the fluorescent dichlorodihydrofluorescein. Although TNF treatment had a marginal effect on the ROS' status, luteolin treatment induced the DHE-specific ROS (Fig. 2A, rightward shift of

the peaks) whereas suppressing the CM-H<sub>2</sub>DCFDA-specific ROS (leftward shift of the peaks) in H460 cells (Fig. 2B). Cotreatment 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 after exposure of cells to this drug (Fig. 2, A and 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 sources other than oxidation of DHE by O<sub>2</sub><sup>-</sup> (Zhao et al., 2005). Because control experiments showed that no positive staining was detected in negative control cells (without luteolin treatments) and luteolin did not induce CM-H<sub>2</sub>DCFDA staining under our experimental conditions, the result indicates that the DHE staining observed in these experiments resulted from the oxidation by O<sub>2</sub><sup>-</sup> (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-in-



**Fig. 2.** Luteolin treatment induces ROS accumulation. A, H460 cells were pretreated with luteolin (40  $\mu$ M) for 30 min and then treated with TNF (20 ng/ml) for indicated time periods. DHE (5  $\mu$ M) 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<sub>2</sub>DCFDA (1  $\mu$ M).

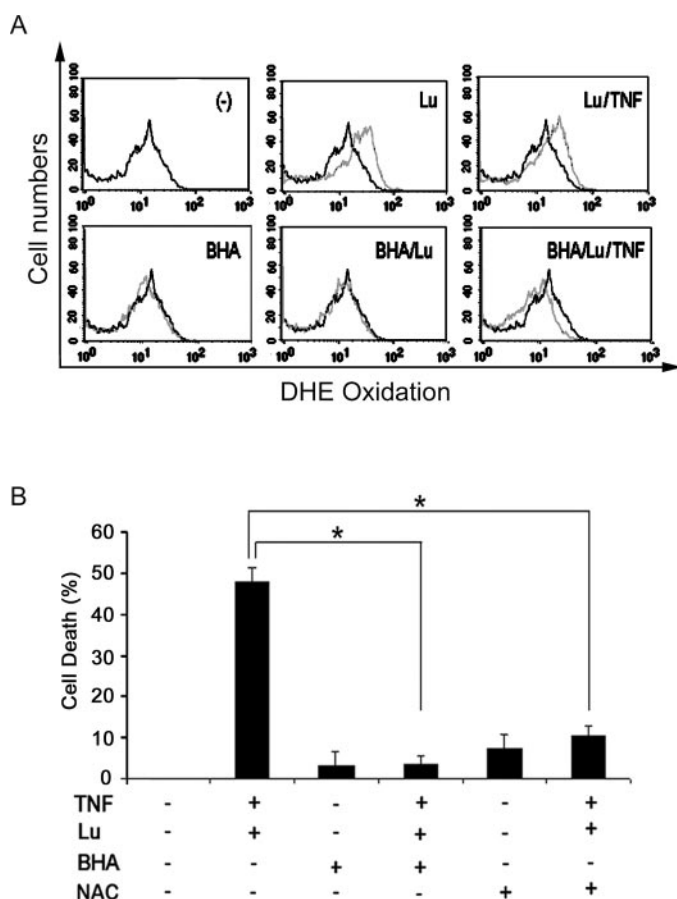
duced cytotoxicity. ROS scavengers were used to antagonize the effect of luteolin on ROS in H460 cells. BHA had a marginal effect on the basal ROS level in H460 cells. However, pretreatment with BHA abolished the induction of ROS in cells treated with luteolin alone or luteolin plus TNF (Fig. 3A). BHA also dramatically suppressed luteolin and TNF cotreatment-induced cell death (Fig. 3B). Pretreatment with another ROS scavenger, NAC, exerted a similar effect, as did BHA on luteolin/TNF-induced cytotoxicity (Fig. 3B). 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  $O_2^-$  whereas it decreased the  $H_2O_2$  levels, and because  $O_2^-$  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

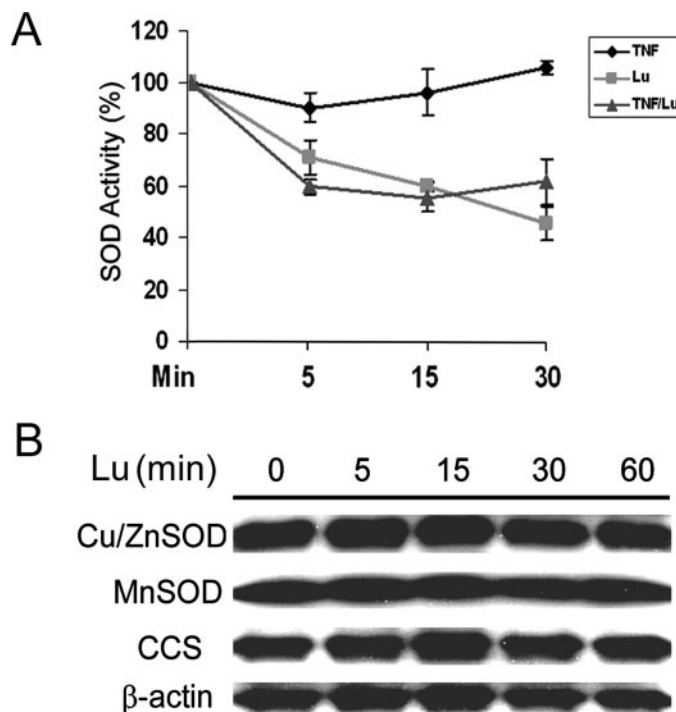
5 min (Fig. 4A). There were no detectable changes in the expression levels of MnSOD, Cu-ZnSOD, or CCS in luteolin-treated cells (Fig. 4B). These results indicated that luteolin induces ROS through suppression of SOD activity.

**ROS Are Involved in Suppression of the TNF-Induced NF- $\kappa$ B Pathway by Luteolin.** The NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B-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- $\kappa$ B, which was completely suppressed by pretreatment with luteolin, suggesting that luteolin blocks the TNF-induced NF- $\kappa$ B activation pathway (Fig. 5A). The induction of NF- $\kappa$ B downstream antiapoptotic genes XIAP and MnSOD by TNF was also completely suppressed by luteolin (Supplemental Fig. S2). The involvement of ROS in luteolin-induced suppression of the TNF-activated NF- $\kappa$ B pathway was addressed. Although pretreatment of the cells with BHA and NAC had no detectable effect on basal NF- $\kappa$ B activity, BHA and NAC significantly alleviated the suppression of NF- $\kappa$ B activity by luteolin. These results suggest that luteolin-induced ROS accumulation is important for suppressing the TNF-activated NF- $\kappa$ B pathway.

Consistent with findings in colorectal cancer cells in which luteolin-induced NF- $\kappa$ B suppression occurs downstream of I $\kappa$ B $\alpha$  (Shi et al., 2004), luteolin did not suppress TNF-in-



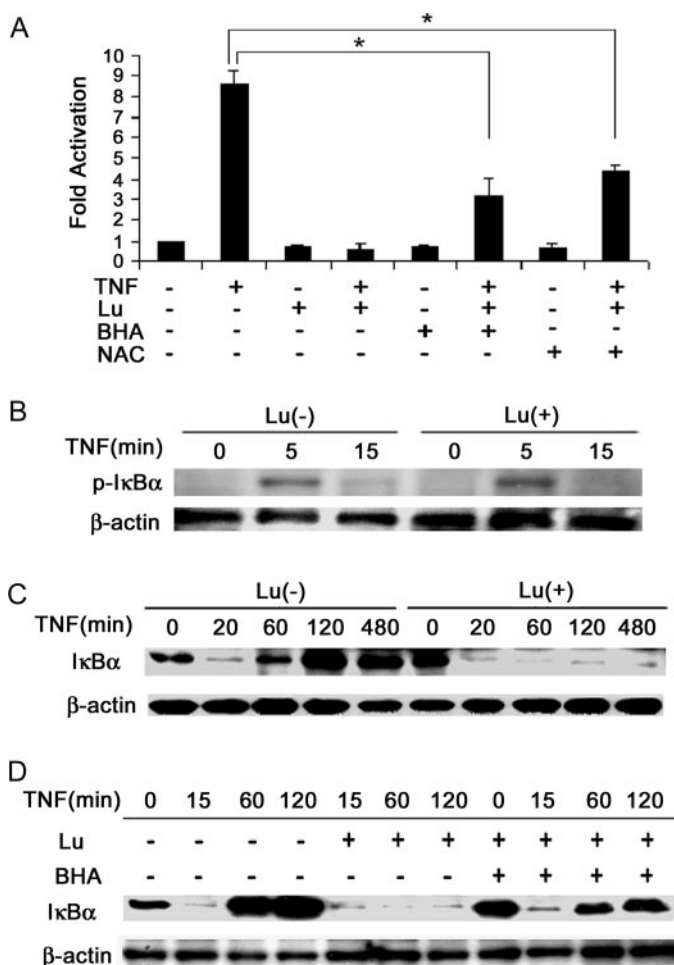
**Fig. 3.** ROS accumulation is required for cell apoptotic death. A, H460 cells were pretreated with BHA (100  $\mu$ M) for 30 min and then treated with luteolin (40  $\mu$ M) for an additional 30 min followed by TNF (20 ng/ml) treatment for another 30 min. Cells were stained with DHE (5  $\mu$ M) for 30 min before collecting. 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  $\mu$ M) or NAC (10  $\mu$ M) for 30 min and then treated with luteolin (40  $\mu$ M) for an additional 30 min followed by TNF (20 ng/ml) treatment for 24 h. Cell death was measured as described in Fig. 1. \*,  $P < 0.01$ .



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

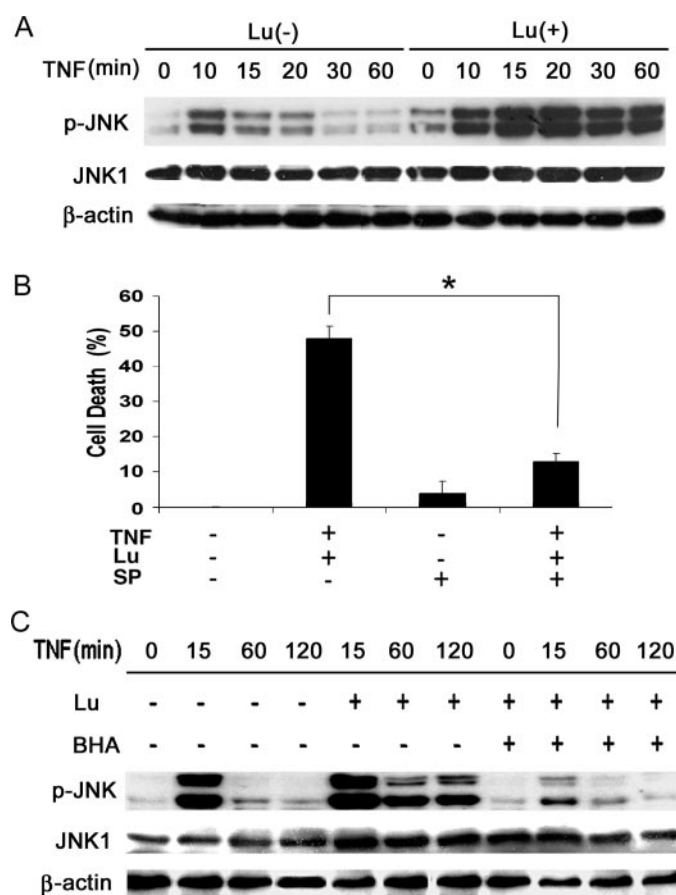
duced I $\kappa$ B $\alpha$  phosphorylation (Fig. 5B; 15 min) and degradation in A549 and H460 cells (Fig. 5C; 20 min, and data not shown). However, the recovery of the NF- $\kappa$ B-regulated factor I $\kappa$ B $\alpha$  was suppressed by luteolin [Fig. 5C; compare Lu(-), 60–480 min versus Lu(+), 60–480 min]. The ROS scavenger BHA had no effect on I $\kappa$ B $\alpha$  degradation (Fig. 5D, 15 min). However, ROS seem to be responsible for the suppression of I $\kappa$ B $\alpha$  recovery, because preincubation with BHA restored I $\kappa$ B $\alpha$  recovery in luteolin-treated cells (Fig. 5D, 60 and 120 min). Together, these results further support the hypothesis that luteolin suppresses the TNF-induced NF- $\kappa$ B pathway through induction of ROS.

**Potential of the JNK Activation Pathway by Luteolin-Induced ROS Accumulation.** When the NF- $\kappa$ B



**Fig. 5.** Luteolin-induced ROS is required for suppression of TNF-induced NF- $\kappa$ B activation. **A**, A549 cells were cotransfected with p2 $\kappa$ B-Luc and pRSV-LacZ. Eight hours after transfection, cells were treated as indicated. Pretreatment with BHA (100  $\mu$ M) or NAC (10  $\mu$ M) for 30 min was followed by luteolin (40  $\mu$ M) for an additional 30 min, and then TNF (20 ng/ml) was added for treatment for 15 h. Luciferase activity was detected and normalized with  $\beta$ -galactosidase activity. The data shown are the mean  $\pm$  S.D. The data are the average of three independent experiments. \*,  $P < 0.01$ . **B**, A549 cells were pretreated with 40  $\mu$ M luteolin for 30 min and then treated with TNF for indicated time periods. Phospho-I $\kappa$ B $\alpha$  was detected by Western blot.  $\beta$ -Actin was detected as an input control. **C**, A549 cells were pretreated with 40  $\mu$ M luteolin for 30 min followed by exposure to TNF for the indicated time periods. I $\kappa$ B $\alpha$  was detected by Western blot.  $\beta$ -Actin was detected as an input control. **D**, A549 cells were treated with BHA (100  $\mu$ M) for 30 min and then treated with luteolin (40  $\mu$ M) for an additional 30 min followed by TNF (20 ng/ml) treatment for indicated time periods. I $\kappa$ B $\alpha$  was detected by Western blot.  $\beta$ -Actin was detected as an input control.

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 after treatment and diminishing after 30 min (Fig. 6A). When the cells were pretreated with luteolin, the extent of JNK activation was dramatically increased and sustained over 2 h (Fig. 6, A and 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 (Fig. 6B). BHA effectively suppressed the extent and shortened the duration of JNK activation in luteolin- and TNF-cotreated cells (Fig. 6C). These results suggest that luteolin-induced ROS accumulation is critical for the potentiation of TNF-induced JNK activation, which consequently contributes to the sensitization of TNF-induced apoptosis in lung cancer cells.



**Fig. 6.** Luteolin potentiates the TNF-induced JNK activation pathway through the induction of ROS. **A**, A549 cells were pretreated with 40  $\mu$ M luteolin for 30 min and then treated with TNF for indicated times. Phospho-JNK and JNK1 were detected by Western blot.  $\beta$ -Actin was detected as an input control. **B**, A549 cells were pretreated with a JNK inhibitor, SP600125 (20  $\mu$ M), for 30 min and then treated with luteolin (40  $\mu$ M) for an additional 30 min followed by TNF (20 ng/ml) treatment for 24 h. Cell death was measured by an LDH release assay. The data shown are representative of three independent experiments and the mean  $\pm$  S.D. of triplicate determinations. \*,  $P < 0.01$ . **C**, A549 cells were pretreated with BHA (100  $\mu$ M) for 30 min and then treated with luteolin (40  $\mu$ M) 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.  $\beta$ -Actin was detected as an input control.



## Discussion

This study demonstrates that luteolin can sensitize lung cancer cells to TNF-induced apoptosis through an 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- $\kappa$ B 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- $\kappa$ B and potentiation of JNK. To our knowledge, this is the first report that ROS plays a critical role in luteolin's effect in the suppression of TNF-induced NF- $\kappa$ B 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, because of 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 seems 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 reported previously to induce ROS (Matsuo et al., 2005), the consequences of ROS accumulation on cellular signaling and cell death in response to TNF has not been addressed. In this study, we found that blocking NF- $\kappa$ B plays an important role in the synergistic cytotoxicity of combined treatment with luteolin and TNF in lung cancer cells. The mechanism of luteolin-induced NF- $\kappa$ B suppression in different cell types is somewhat controversial, occurring by suppression of either IKK in the cytoplasm or NF- $\kappa$ B in the nucleus (Xagorari et al., 2001; Kim et al., 2003; Shi et al., 2004; Ruiz and Haller, 2006). Our result is consistent with the finding in colorectal cancer cells and rat fibroblasts in which the target of luteolin is downstream of I $\kappa$ B in the NF- $\kappa$ B pathway (Kim et al., 2003; Shi et al., 2004). ROS have been implicated in activation or suppression of NF- $\kappa$ B, 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- $\kappa$ B pathway; however, the exact mechanism is currently unclear. The phosphorylation of p65 at Ser276, Ser536, and Ser529 has not been altered by luteolin (data not shown). It remains to be determined whether ROS directly oxidize the NF- $\kappa$ B subunits or interfere with the communication between NF- $\kappa$ B and transcriptional cofactors such as p300/CBP (Nishi et al., 2002; Shi et al., 2004). It is interesting that MnSOD, one of NF- $\kappa$ B's target genes, is involved in eliminating ROS by converting the superoxide anion radical to H<sub>2</sub>O<sub>2</sub>. The suppression of NF- $\kappa$ B 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; Ventura et al., 2004; Lin et al., 2006). 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- $\kappa$ B inhibition (Lin and Dibling, 2002; Kamata et al., 2005). Alternatively, JNK can be activated by the induction of the mitogen-activated protein kinase kinase kinase/Jnk-activated kinase/JNK cascade. There are several mitogen-activated protein kinase kinase kinases that are able to initiate JNK activation: apoptosis signal-related kinase 1, mixed lineage kinase, mitogen-activated protein kinase kinase kinase 1, and transforming growth factor-activated protein kinase 1 (Kamata et al., 2005; Liu and Lin, 2005). JNK is also negatively regulated through dephosphorylation by mitogen-activated protein kinase phosphatases (MKP). It is interesting that the MKP activity is inhibited by oxidation (Kamata et al., 2005). NF- $\kappa$ B 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 apoptosis signal-related kinase 1-mitogen-activated protein kinase kinase-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- $\kappa$ B suppression or is achieved through a mechanism that is independent of the cross-talk between NF- $\kappa$ B 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 procancer activity for TNF (Orosz et al., 1993; Varela et al., 2001). Studies in animals reveal a positive role of TNF in the carcinogenesis of skin, colon, and liver (Moore et al., 1999; Knight et al., 2000; Luo et al., 2004). 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- $\kappa$ B, which protects cells against apoptosis. Therefore, blocking the TNF-induced NF- $\kappa$ B pathway could be an effective approach for cancer prevention. The suppression of NF- $\kappa$ B 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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