# Chemopreventive Properties of *trans*-Resveratrol Are Associated with Inhibition of Activation of the IκB Kinase[1]

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## ABSTRACT

***trans*-Resveratrol (Res), a phytoalexin found at high levels in grapes and in grape products such as red wine, has been shown to have anti-inflammatory and antioncogenic properties. Because the transcription factor nuclear factor κB (NF-κB) is involved in inflammatory diseases and oncogenesis, we tested whether Res could modulate NF-κB activity. Res was shown to be a potent inhibitor of both NF-κB activation and NF-κB-dependent gene expression through its ability to inhibit IκB kinase activity, the key regulator in NF-κB activation, likely by inhibiting an upstream signaling component. In addition, Res blocked the expression of mRNA-encoding monocyte chemoattractant protein-1, a NF-κB-regulated gene. Relative to cancer chemopreventive properties, Res induced apoptosis in fibroblasts after the induced expression of oncogenic H-Ras. Thus, Res is likely to function by inhibiting inflammatory and oncogenic diseases, at least in part, through the inhibition of NF-κB activation by blocking IκB kinase activity. These data may also explain aspects of the so-called "French paradox" that is associated with reduced mortality from coronary heart disease and certain cancers and provide a molecular rationale for the role of a potent chemopreventive compound in blocking the initiation of inflammation and oncogenesis.**

## INTRODUCTION

Significant interest surrounds dietary approaches directed toward the prevention of disease initiation and progression. Res[3] (*trans*-3,4′,5-trihydroxystilbene), a natural phytoalexin found in grapes and grape products such as red wine, has anticancer and anti-inflammatory effects (1, 2). These findings are consistent with epidemiological studies that defined the so-called "French paradox" (3, 4) as the association of reduced mortality from coronary heart disease and breast cancer (4, 5) with increased red wine consumption. In addition, Res was found to have both estrogenic/antiestrogenic activities *in vitro* and *in vivo* (6–8) and antioxidant properties (1, 9–12). Recently, Res has been shown to possess chemopreventive activity by inhibiting cellular events associated with tumor initiation, promotion, and progression (1, 13, 14); by inhibiting ribonucleotide reductase (15); and by inhibiting proliferation of some cancer cells *in vitro* (8, 13, 16, 17). Pertinent to cancer prevention, Res also suppresses the expression of inducible nitric oxide synthase (17) and cyclooxygenase-2 (1, 18), which is likely to contribute to both its anti-inflammatory and anti-oncogenic mechanism. Despite these important advances, the molecular mechanism(s) by which Res exerts its broad biological effects has not yet been elucidated.

The transcription factor NF-κB is strongly linked to inflammatory and immune responses (19–22) and is associated with oncogenesis in certain models of cancer (23–27). NF-κB is important for the regulation of cell proliferation, cell transformation, and tumor development (28–31). Recently, we demonstrated that oncogenic forms of Ras (32) and the oncoprotein Bcr-Abl (26) both activate NF-κB through the activation of the transcriptional function of the RelA/p65 subunit. Furthermore, not only is NF-κB activity required for Ras to initiate cellular transformation, but it is also required for Bcr-Abl-initiated tumorigenesis and transformation (26, 32). Activated NF-κB has been found in primary breast tumors (25, 33) and has been shown to be required for proliferation and survival of Hodgkin's disease tumor cells (24). In terms of cell proliferation, breast cancer and other cancers often exhibit high levels of cyclin D1 (34, 35), and we and others have shown that NF-κB activates transcription of the cyclin D1 gene (36, 37). Importantly, the requirement for NF-κB in oncogenesis appears to be based, at least in part, on its ability to suppress transformation-associated apoptosis (23).

NF-κB activity is regulated in part by its subcellular localization. Under noninduced conditions, NF-κB is sequestered in the cytoplasm through interactions with an inhibitor protein known as IκB (28–31). Numerous extracellular stimuli can activate NF-κB through signal transduction pathways that activate an IKK complex that phosphorylates IκBα on serines 32 and 36. The phosphorylation of IκBα leads to its ubiquitination and ultimate degradation by the proteasome (28–31), allowing NF-κB to translocate to the nucleus where it activates the expression of genes. Activation of the NF-κB/Rel family of transcription factors regulates the expression of genes that participate in pathways involving inflammation, cell proliferation, and apoptosis (28–31), including the inflammatory mediators nitric oxide synthase and cyclooxygenase-2 (38, 39). Although numerous effects have been described for Res, the molecular mechanisms responsible for its anti-inflammatory and antioncogenic effects are not yet clear. Here we asked whether the chemopreventive effect of Res occurs through inhibition of NF-κB activation, and, if so, through what mechanism.

## MATERIALS AND METHODS

### Cell Cultures and Cell Extracts

Human monocyte (THP-1) and macrophage (U937) cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, $5 \times 10^{-5}$ M β-mercaptoethanol, and 1% penstrep. Cells were seeded at a density of $10^6$ cells/ml and cultured at 37°C in a 5% $CO_2$ atmosphere. Purified Res was purchased from Sigma (St. Louis, MO) and prepared according to the manufacturer's protocol. Briefly, for all experiments, a 50 mg/ml stock solution prepared in 100% ethanol was used. Cells were preincubated for 60 min with Res (30 μM) and stimulated with either TNF (Promega; 10 ng/ml) for 15 min or with LPS (Promega; 1 μg/ml) for 4 h. CEs and NEs were prepared as described previously (40) and stored at −70°C. Rat1:iRas cultures expressing the IPTG-inducible oncogenic H-Ras allele (23) were cultured in DMEM supplemented with 10% fetal bovine serum. Before the experiments, subconfluent Rat1:iRas cell cultures were serum-starved for 4 h and either left untreated or stimulated with 5 mM IPTG in the presence or absence of Res.

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[3] The abbreviations used are: Res, *trans*-resveratrol; NF-κB, nuclear factor κB; IKK, IκB kinase; MCP-1, monocyte chemoattractant protein-1; TNF, tumor necrosis factor; LPS, lipopolysaccharide; LTR, long terminal repeat; CAT, chloramphenicol aminotransferase; GST, glutathione *S*-transferase; WT, wild-type; MUT, mutant; IPTG, isopropyl-β-D-thiogalactopyranoside; MEKK1, mitogen-activated protein/extracellular signal-regulated kinase kinase kinase 1; AP-1, activator protein 1; CE, cytoplasmic extract; NE, nuclear extract; SR-IκBα, NF-κB IκBα inhibitor; IκBα, inhibitor κBα; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-PCR.

### EMSA

NEs (5–10 $\mu$g) were preincubated with 1 $\mu$g of poly(deoxyinosinic-deoxycytidylic acid) in binding buffer (10 mM Tris, 50 mM NaCl, 20% glycerol, 1 mM DTT, and 0.5 mM EDTA) for 10 min at room temperature. Approximately 30,000 cpm of $^{32}$P-labeled DNA probe containing the murine MHC class I NF-$\kappa$B DNA binding site (41) were added and allowed to bind for 15 min. The complexes were separated by 5% PAGE and detected by autoradiography. Specificity of binding was examined by competition with excess unlabeled oligonucleotide (UV21). For supershift assays, NEs were incubated with antibodies against p50 and p65 subunits of NF-$\kappa$B for 20 min at room temperature before analysis by EMSA.

### Transfection and Gene Expression Assay

**3X$\kappa$B-Luc Assay.** THP-1 cells were cotransfected using DEAE/dextran with the empty expression vector (pDCR) and either the 3X$\kappa$B-luc reporter or the super-repressor form of I$\kappa$B$\alpha$ (SR-I$\kappa$B$\alpha$) expression vector (42). Transfections used 5.0 $\mu$g of the pDCR empty expression vector only, 1.0 $\mu$g of the 3X$\kappa$B-luc reporter, or 1.0 $\mu$g of the SR-I$\kappa$B$\alpha$ expression vector and were brought to a final concentration of 5.0 $\mu$g with the empty vector. After 48 h, cells were pretreated for 60 min with Res (30 $\mu$M) and stimulated for 6 h with TNF (10 ng/ml). Cell lysates were made by freeze-thawing three times. Protein concentrations were determined, and 100 mg of protein were assayed for luciferase activity as described previously (32, 43). The results (Fig. 2*A*) are expressed as the fold luciferase induction relative to the transfection that contained the empty expression vector, whose value was placed at 1.0.

**CAT Assay.** THP-1 cells were cotransfected along with the pDCR empty expression vector as described above, except that either 1.0 $\mu$g of a NF-$\kappa$B CAT-linked reporter containing the WT HIV-LTR-CAT or 1.0 $\mu$g of a MUT HIV-LTR-CAT in which both inducible $\kappa$B sites have been mutated. Pretreatment and stimulation were as described above, and cells were harvested, and the CAT activity was determined. The results are expressed as the fold CAT induction as described above. *Bars* (Fig. 2*B*) represent the mean $\pm$ SE determined from at least three independent transfection experiments. Statistical analysis was performed by ANOVA (StatView), and different letters between groups indicate significant difference at $P > 0.01$.

### RT-PCR

THP-1 cells were pretreated with Res (30 $\mu$M), followed by TNF (10 ng/ml) stimulation for 3 h. RNA was isolated using the Trizol method (Life Technologies, Inc.), and 1 $\mu$g of total RNA was reverse-transcribed and amplified by PCR (RT-PCR) using specific primers for MCP-1 and actin (44), as described previously. The oligonucleotide primers used were as follows: (*a*) MCP-1, 5′-GGCTGAGCCCACTTATCACTCATGG-3′ (5′ primer) and 5′-GGAAGCTTGCTGGAGGCGAGAGTGCGAG-3′ (3′ primer); and (*b*) actin, 5′-CCAACCGCGAGAAGATGACC-3′ (5′ primer) and 5′-GATCTTCATGAGGTAGTCAGT-3′ (3′ primer). Actin was used to determine equal protein loading. The PCR temperatures used were 94°C for 45 s, 55°C (actin) and 60°C (MCP-1) for 30 s, and 72°C for 90 s, followed by extension for 10 min at 72°C. The PCR reaction was set for various cycles (20–35 cycles) to maintain the linearity of the amplification. The PCR products (10 $\mu$l) were electrophoresed on a 2% agarose gel containing gel star fluorescent dye (FMC Corp., Philadelphia, PA). A representative photograph was scanned and analyzed. Negative controls consisted of tubes with and without RNA. MCP-1 mRNA levels were quantitated by PhosphorImager analysis (Molecular Dynamics). Data are representative of three independent experiments.

### Western Blotting

Equal amounts of CEs were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Blots were blocked in 5% milk in 1× TBST (Tris-buffered saline and 0.5% Tween 20) and probed with a specific I$\kappa$B$\alpha$ antibody (1:1000; Rockland). Blots were probed with a secondary antirabbit antibody conjugated with horseradish peroxidase (1:10,000; Promega). Protein bands were visualized with an enhanced chemiluminescence detection system (ECL; Amersham Life Technologies).

### *In Vitro* Kinase Assay

THP-1 cells were treated as described above for the indicated time periods. Whole cell extracts were prepared, and IKK was immunoprecipitated with a specific antibody to the IKK-$\beta$ subunit. IKK activity was measured using a GST-I$\kappa$B-$\alpha$(1–54) WT or a MUT GST-I$\kappa$B-$\alpha$ substrate (45–49). IKK activity was measured using a GST-I$\kappa$B-$\alpha$(1–54) (4 $\mu$g) WT or a MUT GST-I$\kappa$B-$\alpha$ substrate, where Ser$^{32}$ and Ser$^{36}$ were substituted by Thr (4 $\mu$g; S32T and S36T; Refs. 45–49). These substrates were enzymatically phosphorylated by activated IKK with [$\gamma$-$^{32}$P]ATP (New England Nuclear, Boston, MA). IKK activity was quantitated by PhosphorImager analysis (Molecular Dynamics) and normalized to the IKK activity of untreated cells. Data are expressed as the fold induction. Data are representative of three independent experiments.

### ELISA *in Situ* Cell Death Assay and Microscopy

Rat1:iRas cells were pretreated with or without Res (30 $\mu$M) for 60 min before the addition of IPTG (5 mM) and incubated for 48 h. After incubation, cell death was detected by the ELISA *in situ* apoptosis assay (Boehringer Mannheim), and the percentage of apoptosis was quantitated. Staurosporine was used as the positive control for the induction of apoptosis. This assay measures DNA strand breaks and is therefore diagnostic for cells undergoing apoptosis. In a parallel experiment, Rat1:iRas cells were either pretreated with or without Res (30 $\mu$M) for 60 min before the addition of IPTG (5 mM) and incubated for 48 h. Nonadherent, dying cells are shown as refractive by phase-contrast microscopy.

## RESULTS

**Res Inhibits NF-$\kappa$B DNA Binding Activity.** For our initial studies, we investigated whether Res inhibited NF-$\kappa$B DNA binding activity. We used two macrophage/monocytic cell lines, THP-1 and U937, both of which are well characterized with regard to activation of NF-$\kappa$B. THP-1 cells were either left untreated or exposed to TNF, a potent inducer of NF-$\kappa$B in many cell types. As expected, TNF strongly induced activation of NF-$\kappa$B DNA binding activity, as determined by EMSA (Fig. 1, *Lane 2*). In a dose-response study, we found that the effective dose of Res for inhibiting activation of NF-$\kappa$B was 30 $\mu$M (data not shown), and this dose was used for all experiments. Pretreatment with Res had little effect on the basal binding activity of NF-$\kappa$B (data not shown) but strongly blocked the ability of TNF to activate NF-$\kappa$B DNA binding (Fig. 1, *Lane 3*). Similarly, the induction of NF-$\kappa$B in THP-1 cells by LPS, a potent bacterial endotoxin, was also effectively blocked by Res (Fig. 1, *Lane 5*). Virtually

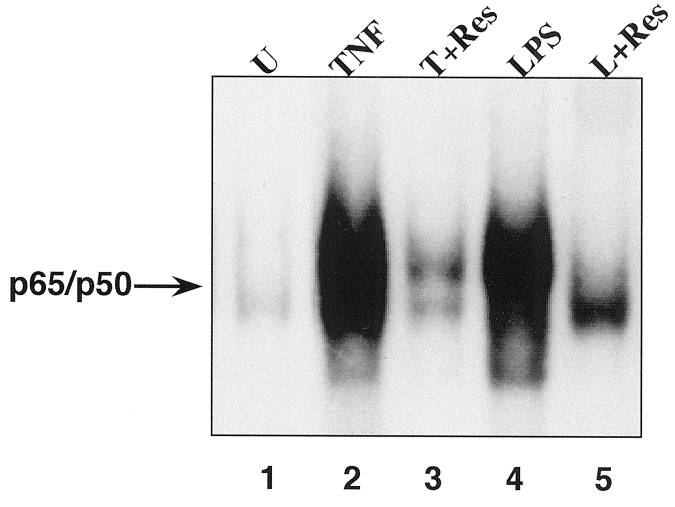


Fig. 1. Res-mediated repression of TNF- and LPS-induced NF-$\kappa$B activation. THP-1 cells were pretreated with Res (30 $\mu$M) and stimulated with either TNF (10 ng/ml; 15 min) or LPS (1 $\mu$g/ml; 4 h). NEs were analyzed by EMSA as described. Data are a photograph of an autoradiograph and are representative of three independent experiments.

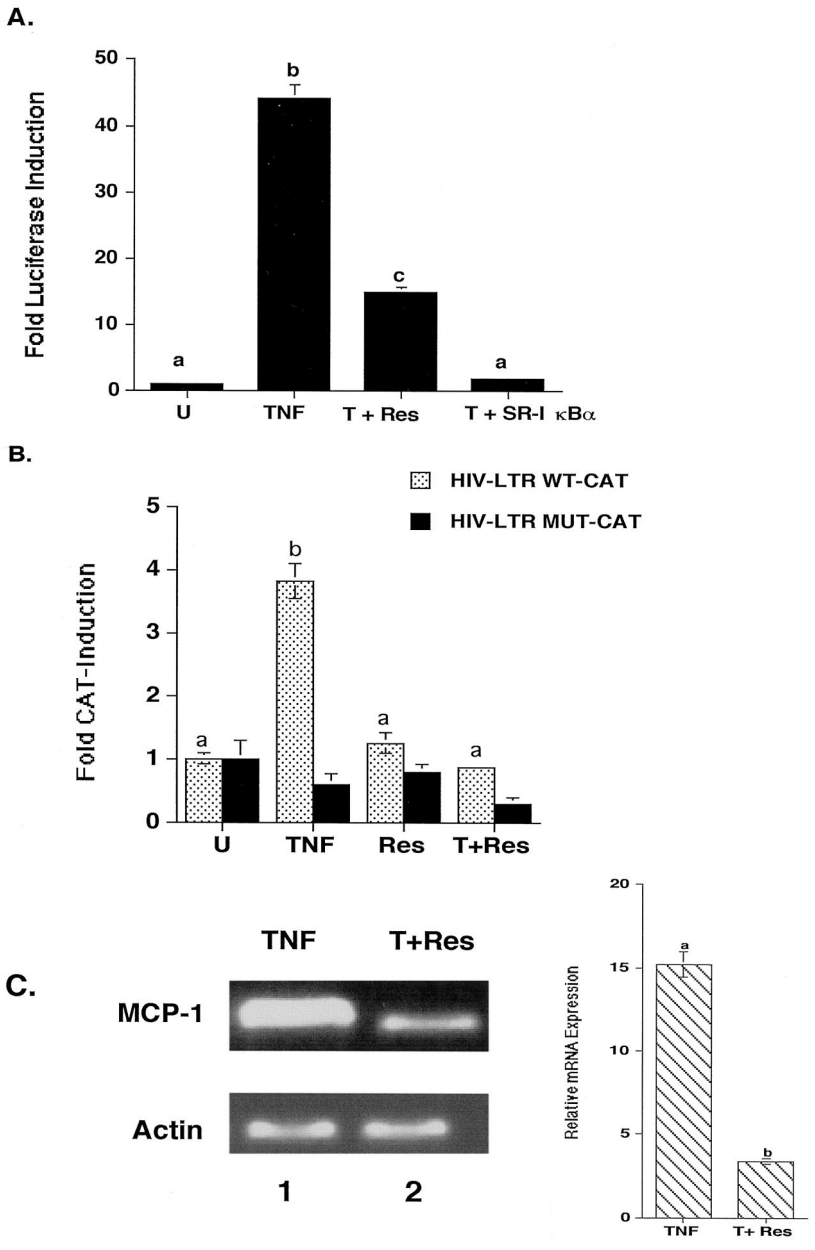


Fig. 2. Res suppresses NF-κB-regulated gene expression. *A*, THP-1 cells were cotransfected using DEAE/dextran with the empty expression vector (pDCR) and either the 3XκB-luc reporter or the superrepressor form of IκBα (SR-IκBα) expression vector. To inhibit NF-κB activity, SR-IκBα, which cannot be phosphorylated or degraded, was used to block nuclear translocation and subsequent transactivation of NF-κB-responsive genes (42). Transfections used 5.0 μg of the pDCR empty expression vector only, 1.0 μg of the 3XκB-luc reporter, or 1.0 μg of the SR-IκBα expression vector and were brought to a final concentration of 5 μg with the empty vector. After 48 h, cells were prepared as described. The results are expressed as the fold luciferase induction relative to the transfection that contained the empty expression vector, whose value was placed at 1.0. *Bars*, mean ± SE determined from at least three independent transfection experiments. Statistical analysis was performed by ANOVA (StatView), and different letters between groups indicate significant difference at *b*, $P < 0.01$ and *c*, $P < 0.05$. *B*, THP-1 cells were cotransfected along with the pDCR empty expression vector as described above, except that either 1.0 μg of a NF-κB CAT-linked reporter containing WT HIV-LTR-CAT or 1.0 μg of a MUT HIV-LTR-CAT in which both inducible κB sites have been mutated. Pretreatment and stimulation were as described, cells were harvested, and CAT activity was determined. The results are expressed as the fold CAT induction as described above. *Bars*, mean ± SE determined from at least three independent transfection experiments. Statistical analysis was performed by ANOVA (StatView), and *different letters* between groups indicate significant difference at $P > 0.01$. C, THP-1 cells were pretreated with Res (30 μM), followed by TNF (10 ng/ml) stimulation for 3 h. Total RNA was reverse transcribed and amplified by PCR (RT-PCR) using specific primers for MCP-1 and actin. A representative photograph was scanned, and MCP-1 mRNA levels were quantitated by PhosphorImager analysis (Molecular Dynamics). Data are representative of three independent experiments.

identical data were obtained with U937 cells (data not shown), indicating that the ability of Res to block NF-κB activation is not cell-type specific. Consistent with these results, it has been shown recently that Res can inhibit NF-κB activation in RAW 264.7 macrophage cells (17). In the same extracts, DNA binding of the constitutive transcription factor Oct-1 and the CAAT/enhancer binding protein transcription factor were unaffected by the presence of Res (data not shown), demonstrating that Res does not negatively affect transcription factors in a general manner.

**Res Inhibits NF-κB-dependent Gene Transcription.** Consistent with the DNA binding data, Res strongly blocked the induction of a NF-κB-dependent luciferase reporter (3XκB-luc) in response to TNF stimulation of THP-1 cells (Fig. 2*A*), as did expression of a modified form of the NF-κB inhibitor IκBα (SR-IκBα). SR-IκBα, which cannot be phosphorylated or degraded (45, 50–53), binds to NF-κB and blocks the nuclear translocation and subsequent transactivation of NF-κB-responsive transcription (42). Relative specificity for NF-κB inhibition was shown in an experiment where Res blocked the induction of the NF-κB-regulated HIV-LTR-CAT reporter in response to TNF stimulation (Fig. 2*B*). However, Res did not strongly block a site-directed MUT of the HIV-LTR-CAT reporter in which the two NF-κB binding sites were mutated (Fig. 2*B*). To extend the transient transfection reporter data, we determined whether endogenous gene expression could be repressed by Res. mRNA levels for two NF-κB-regulated genes, IκBα and MCP-1, were examined by RT-PCR analysis after TNF stimulation in the presence or absence of Res. Although Res was capable of partially inhibiting TNF-induced mRNA levels for IκBα (data not shown), it more strongly repressed the induction of the MCP-1 mRNA (Fig. 2*C*). Because Res does not completely block the nuclear translocation of NF-κB under our experimental conditions, these results may indicate a more stringent requirement for elevated levels of NF-κB for MCP-1 gene expression as compared with IκBα gene expression. The effects of Res on NF-κB DNA binding activity (Fig. 1) paralleled those observed in the NF-κB-dependent gene expression studies (Fig. 2, *A–C*). More importantly, and consistent with a role for Res in inhibition of atherogen-

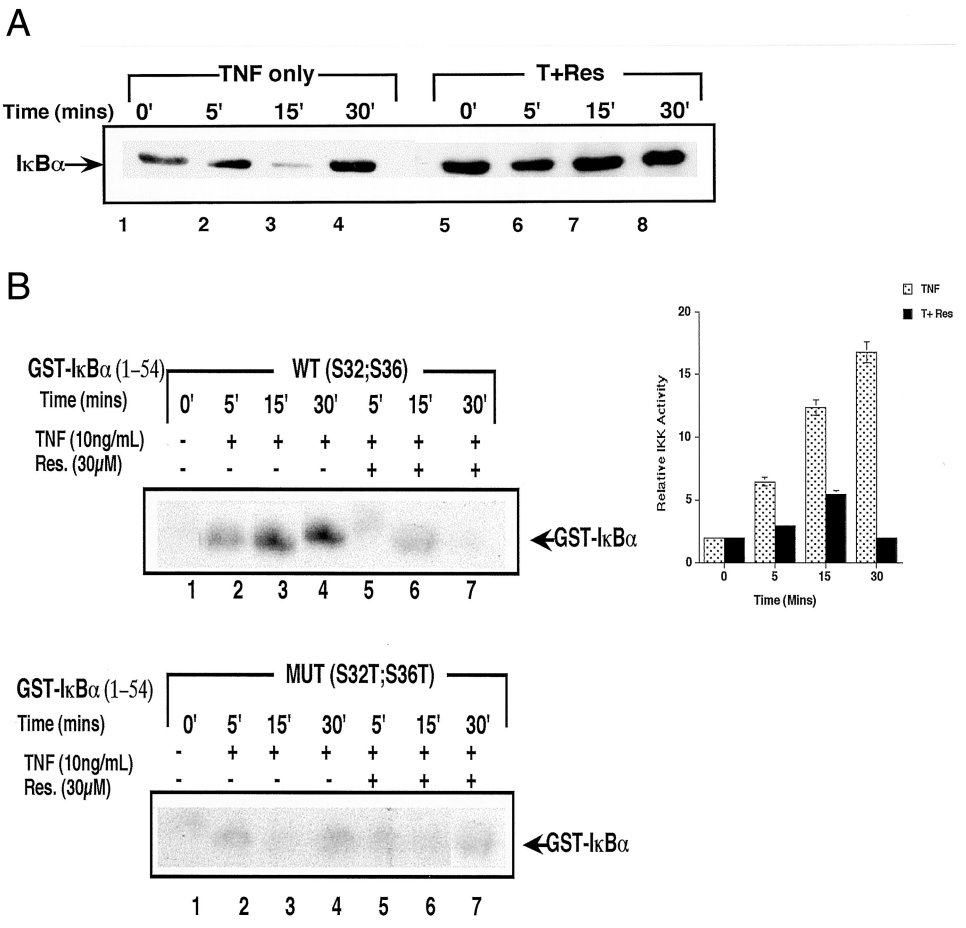


Fig. 3. Res prevents TNF-induced degradation of IκBα by blocking IKK activity. *A*, IκBα degradation kinetics. THP-1 cells were either untreated (*Lane 1*), treated with TNF (10 ng/ml) for indicated times (*Lanes 2–4*), or preincubated for 60 min with Res (30 μM) followed by TNF (10 ng/ml) stimulation for the indicated times (*Lanes 5–8*). CEs were prepared, and protein expression was assessed by immunoblotting (60 μg/lane) with an antibody specific for IκBα (Rockland). THP-1 cells were treated as described. Whole cell extracts were immunoprecipitated with a specific antibody to the IKK-β subunit (44). IKK activity was measured using a GST-IκB-α(1–54) WT or a MUT GST-IκB-α substrate. IKK activity was measured using a GST-IκB-α(1–54) (4 μg) WT or a MUT GST-IκB-α substrate, in which $Ser^{32}$ and $Ser^{36}$ were substituted by Thr (4 μg; S32T and S36T). These substrates were enzymatically phosphorylated by activated IKK with [γ-$^{32}$P]ATP. IKK activity was quantitated by PhosphorImager analysis (Molecular Dynamics) and normalized to the IKK activity of untreated cells. Data are expressed as the fold induction. Data are representative of three independent experiments.

esis, MCP-1 was recently shown to be required for atherogenesis in the ApoE knockout animal model (44). These results indicate that Res inhibits NF-κB-dependent gene expression through the inhibition of induction of NF-κB DNA binding activity.

**Inhibition of TNF-induced IKK Activity.** The majority of inducers of NF-κB stimulate a signal transduction pathway that leads to the activation of the IKK complex (45–49) that phosphorylates IκBα on $Ser^{32}$ and $Ser^{36}$. Phosphorylated IκBα is then targeted for ubiquitination and subsequent degradation by the 26S proteasome, liberating NF-κB and allowing nuclear translocation (28–31). To determine the level at which Res blocks NF-κB activity, we analyzed the relative levels of IκBα after exposure of cells to TNF. CEs were prepared from the THP-1 cells used previously for EMSA. As expected, TNF stimulation led to a strong IκBα degradative response in THP-1 cells after 15 min (Fig. 3*A*, *Lane 3*), followed by a reappearance of IκBα at 30 min (Fig. 3*A*, *Lane 4*). Consistent with the ability to block NF-κB activation (Fig. 1), Res inhibited IκBα degradation in response to TNF (Fig. 3*A*, *Lanes 6–8*). Additionally, Res blocked TNF-induced phosphorylation of IκBα (data not shown) as demonstrated with the use of a phosphospecific antibody directed to $Ser^{32}$, suggesting that Res inhibits an upstream signaling component in the TNF signaling pathway. We therefore analyzed levels of IKK activity after TNF treatment of THP-1 cells in the absence or presence of Res. Whole cell extracts were prepared, and IKK was immunoprecipitated with a specific antibody to the IKK-β subunit (46). IKK activity was measured using a GST-IκBα(1–54) WT or a MUT GST-IκBα substrate, in which $Ser^{32}$ and $Ser^{36}$ were substituted by threonine (S32T and S36T; Refs. 45–49). Res was found to be a potent inhibitor of inducible IKK activity in response to TNF exposure (Fig. 3*B*). Additional data show that Res is apparently not an intrinsic IKK inhibitor because Res does not block IKK activity when added directly to the *in vitro* kinase reaction (data not shown). Moreover, it is unlikely that the mechanism of action of Res relative to its ability to inhibit NF-κB acts through its antioxidant property because others (54) have shown that TNF-induced IKK activity was not affected by pretreatment with the potent antioxidant *N*-acetyl-L-cysteine. Additionally, it is unlikely that the mechanism of action of Res to block NF-κB acts through its estrogenic properties because treatment of THP-1 cells with 17-β-estradiol at concentrations as high as $10^{-5}$ M did not lead to inhibition of NF-κB (data not shown). Thus, our results suggest that the major mechanism whereby Res blocks NF-κB activity acts through the inhibition of induction of IKK activity.

**Enhanced Apoptosis by Inhibition of Ras-mediated NF-κB Activation.** Res has been shown to inhibit mammary gland oncogenesis in response to carcinogen exposure (1), and it has been shown to block the growth of certain cancer cells *in vitro* (13, 16, 17). Recently, the importance of NF-κB in several oncogenic settings has been described (23–27). One model used an IPTG-inducible oncogenic H-RasV12 allele stably integrated in the Rat-1 cell line (23). Inhibition of NF-κB after IPTG-induction of H-RasV12 led to apoptosis, whereas activation of H-RasV12 when NF-κB was active led to a transformed phenotype (23). Thus, it is postulated that NF-κB activation suppresses transformation-associated apoptosis. Therefore, we determined whether Res could induce apoptosis in the Rat-1 cell line in the absence or presence of H-RasV12. Res pretreatment led to inhibition of IPTG-induced Ras activation of NF-κB (Fig. 4*A*), suggesting that IKK is involved in the induction of NF-κB in response to induced expression of oncogenic Ras. Additionally, Res strongly induced

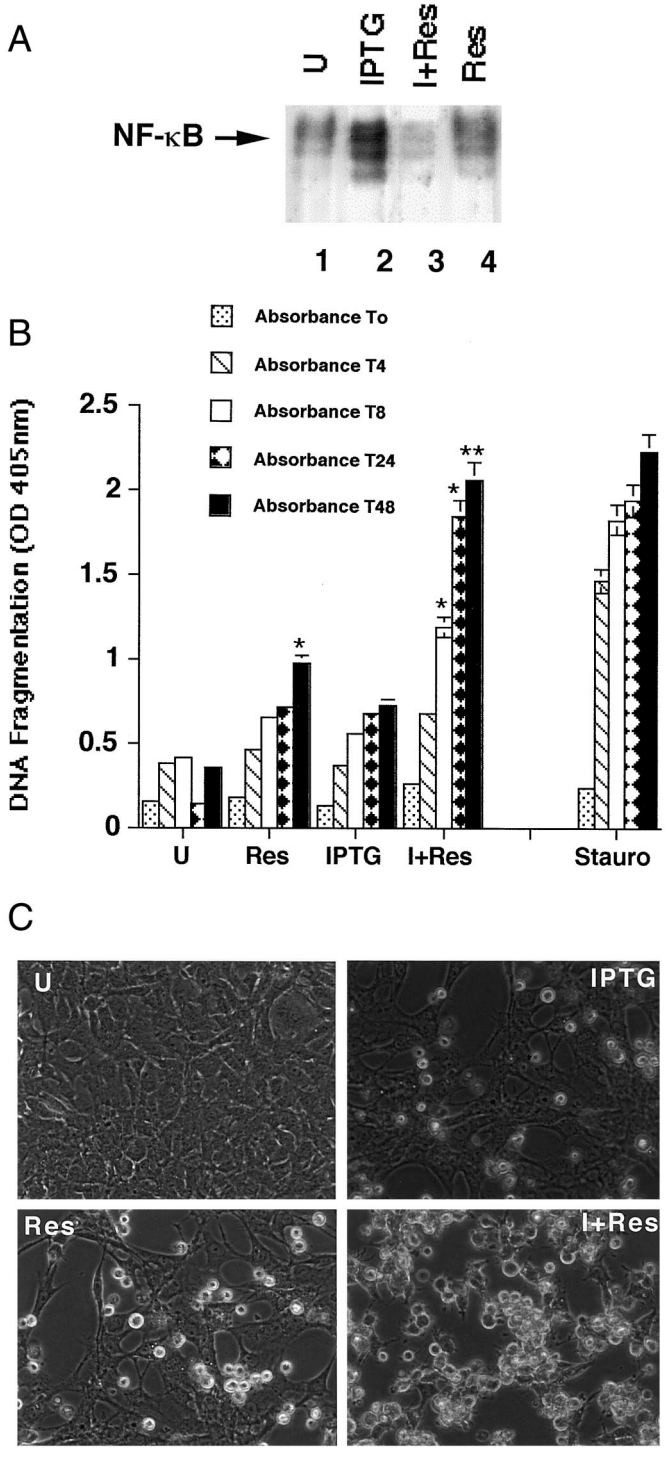


Fig. 4. Apoptotic effects of Res in Rat-1 cells expressing IPTG-inducible oncogenic H-Ras. *A*, Res -mediated suppression of NF-κB in Rat1:iRas cells (23). Rat1:iRas cultures stably expressing the IPTG-inducible oncogenic H-Ras allele were serum-starved, and after 4 h, complete media were added. Cells were pretreated in the absence (*U*, *Lane 1*) or presence of Res (30 μM; *Lanes 3* and *4*) for 60 min. Cultured Rat1:iRas cells were stimulated for 48 h with IPTG (5 mM; *Lanes 2* and *3*) to induce H-Ras-activated NF-κB DNA binding. NEs were analyzed by EMSA with the MHC class I NF-κB DNA binding probe as described previously (41). Data are representative of three independent experiments. *B*, Res induces DNA fragmentation in Rat1:iRas cells. Rat1:iRas cells were pretreated with Res (30 μg/ml) for 60 min before the addition of IPTG (5 mM) and incubated for 48 h. After incubation, cell death was detected by the ELISA *in situ* apoptosis assay (Boehringer Mannheim), and the percentage of apoptosis was quantitated. Staurosporine was used as a positive control for the induction of apoptosis. *C*, Res enhances apoptosis in IPTG-induced Rat1:iRas cells. Micrograph panel (×20 magnification): Rat1:iRas cells were either pretreated without (*U*) or with Res (*Res*; 30 μM) for 60 min before the addition of IPTG (5 mM; *iptg* and *I+Res*) and incubated for 48 h. Nonadherent dying cells are shown as refractive by phase-contrast microscopy.

apoptosis, as determined by both cell death ELISA (Fig. 4*B*) and morphological analysis (Fig. 4*C*, *IPTG*), in Rat-1 cells expressing H-RasV12 (Fig. 4*C*, *I* and *I+Res*) but only weakly induced apoptosis in Rat-1 cells that were not treated with IPTG (Fig. 4*C*, *U* and *Res*). It should be noted that the modest apoptotic response in uninduced Rat-1 cells is likely due to leaky expression of H-RasV12. Furthermore, significant cell death was also observed in TNF-stimulated THP-1 cells after Res pretreatment (data not shown). Interestingly, Res did not elicit an effective apoptotic response in established Ras-transformed NIH-3T3 cells (data not shown). Thus, our data strongly indicate that Res is more effective as a cancer chemopreventive agent by inhibiting NF-κB activation during the initiation phase of oncogenesis. In established NIH-3T3 cells stably expressing oncogenic Ras, nuclear NF-κB levels are not increased by Ras expression (43, 55). In this situation, Ras appears to require NF-κB activity but functions to maintain NF-κB activity through the stimulation of the transcription function of the basally expressed nuclear p65 subunit expression (43, 55). These results are consistent with the idea that the role of IKK in Ras transformation may be an early, transient event allowing an initial accumulation of NF-κB through IKK activation to protect against apoptosis.

## DISCUSSION

Extensive data are now accumulating that dietary constituents can strongly influence the potential for disease outcome (56–59). In epidemiological studies, Red wine consumption was shown to have numerous protective effects, and Res has been shown to be responsible for those beneficial effects (60, 61). In particular, a phenomenon defined as the "French paradox" has emerged (3, 4), which is the association of reduced mortality from coronary heart disease and breast cancer (4, 5). Although it is well established that naturally occurring compounds function as chemopreventive agents (1, 62, 63), the physiological mechanisms of these dietary constituents as extracellular signals involved in transcription activation are only presently emerging.

Our data indicate that Res is a potent inhibitor of NF-κB nuclear translocation and IκBα degradation. Furthermore, Res effects are mediated through the inhibition of IKK, the key regulatory complex required for NF-κB activation of gene transcription. The molecular target of Res action is presently unknown because Res does not appear to directly block IKK activity. Presumably, Res inhibits an upstream signaling component that leads to the activation of IKK, and we are presently examining whether Res inhibits NF-κB-inducing kinase or MEKK1, upstream activators of the IKK complex (46, 64–67). Because evidence has been presented that Res can block AP-1 activity (18), which is consistent with our observations (data not shown), one possibility is that MEKK1 is the target of Res action because MEKK1 can activate both the AP-1 and NF-κB pathways (46, 64–67). This hypothesis, if proven, could explain the dual inhibition of NF-κB and of AP-1 transcriptional responses.

NF-κB is strongly associated with inflammatory diseases and oncogenesis (28–31), and the activation of NF-κB target genes, including proinflammatory cytokines, has been implicated in promoting the transformation and survival of tumor cells (23, 42, 68–70). However, NF-κB activation has been shown to be blocked by anti-inflammatory compounds such as aspirin (71, 72) and glucocorticoids (73–76). Therefore, our results demonstrate that a potent chemopreventive compound also targets NF-κB activation to block both inflammation and cancer initiation. In this case, Res blocks the signaling pathway leading to NF-κB activation through its ability to block IKK activation. Res would then block the expression of NF-κB-dependent genes such as MCP-1 and other genes that would normally promote inflam-

mation, protect against apoptosis, and potentiate cell growth. Thus, our results provide a molecular rationale to explain the broad chemopreventive properties of Res.

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