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Resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-κB in mouse skin by blocking IκB kinase activity

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Aberrant expression of cyclooxygenase-2 (COX-2) has been implicated in tumor promotion. Resveratrol, a phytoalexin present in grapes, was reported to inhibit multistage mouse skin carcinogenesis. In the present study, we found that topically applied resveratrol significantly inhibited COX-2 expression induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Resveratrol-suppressed phosphorylation and subsequent degradation of IκBα, thereby inhibiting activation of nuclear factor-κB (NF-κB) in TPA-stimulated mouse skin. Pretreatment with resveratrol also suppressed TPA-induced phosphorylation of extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein (MAP) kinase. Resveratrol blunted TPA-induced phosphorylation of p65 and its interaction with CBP/p300, rendering NF-κB transcriptionally inactive. To get further insights into the molecular basis of NF-κB inactivation by resveratrol, we examined the role of IκB kinase (IKK) in mediating TPA-induced activation of NF-κB and COX-2 expression. TPA treatment led to rapid induction of IKK activity in mouse skin, which was abolished either by resveratrol or an IKK inhibitor Bay 11-7082. Topical application of Bay 11-7082 also abrogated TPA-induced NF-κB activation and COX-2 expression, supporting the involvement of IKK in TPA-induced COX-2 expression. Taken together, the above findings suggest that resveratrol targets IKK in blocking TPA-induced NF-κB activation and COX-2 expression in mouse skin *in vivo*.

Introduction

Central to cancer biology is disrupted intracellular signaling network, which transmits improper signals resulting in abnormal cellular functioning. Therefore, targeting deregulated intracellular signaling cascades might be a rational approach in achieving chemoprevention. A new horizon in chemoprevention research is the recent discovery of molecular links between inflammation and cancer. Components of the cell signaling network, especially those that converge on redox-sensitive transcription factor nuclear factor-κB (NF-κB) involved in mediating inflammatory response, have been implicated in the promotional stage of carcinogenesis (1–3). One of the major target molecules subjected to NF-κB-driven transactivation is cyclooxygenase-2 (COX-2), which is involved in prostaglandin (PG) biosynthesis and inflammation. Inappropriate upregulation of COX-2 has been frequently observed in various premalignant and malignant tissues (4,5). The contributory role of abnormally high levels of COX-2 in tumorigenesis has further been corroborated by increased susceptibility of COX-2-overexpressing mice (6) and relative resistance of COX-2 knockout animals (7) to spontaneous or experimentally induced carcinogenesis. Therefore, targeted inhibition of COX-2 is now regarded as a promising and practical approach to prevent cancer (8).

Like other early response gene products, transient induction of COX-2 by pro-inflammatory mediators and mitogenic stimuli (9) has been reported. It has been demonstrated that topical application of a prototype tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces expression of COX-2 and its mRNA transcript in mouse skin *in vivo* by activating eukaryotic transcription factors such as NF-κB and activator protein-1 (AP-1), which in turn are regulated by a series of upstream kinases collectively known as mitogen-activated protein (MAP) kinases (10,11).

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), a naturally occurring polyphenol mostly present in grapes and red wine, is a potential chemopreventive agent. In a pioneering study by John M. Pezzuto *et al.* (12), resveratrol was found to interfere with initiation, promotion and progression stages of carcinogenesis, thereby suggesting that this phytoalexin is a promising chemopreventive agent. Multiple lines of evidence from laboratory studies have revealed that resveratrol prevents tumorigenesis in experimental animals exposed to diverse chemical carcinogens and ultraviolet radiation (12–14). Although resveratrol has been shown to target various intracellular signaling molecules in cultured cell lines (15), the molecular mechanisms underlying chemopreventive activity of resveratrol *in vivo* remain largely unresolved. In the present study, we attempted to investigate the effect of resveratrol on TPA-induced COX-2 expression in mouse skin and to explore the underlying molecular mechanisms. Here, we report that the IκB kinase (IKK) activity is increased in mouse skin stimulated with TPA and that topical application of resveratrol significantly inhibited TPA-induced COX-2 expression by diminishing the activation of a ubiquitous eukaryotic transcription factor NF-κB via blockade of upstream kinase IKK signaling.

Materials and methods

Materials

Resveratrol (purity 98%) was a generous gift from Dr John M Pezzuto. TPA was purchased from Alexis Biochemicals (San Diego, CA). Bay 11-7082 was

Abbreviations: AP-1, activator protein-1; CBP, cyclic AMP-response element binding protein (CREB)-binding protein; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated protein kinase; IKK, IκB kinase; MAPK, mitogen-activated protein (MAP) kinase; NF-κB, nuclear factor-κB; SDS, sodium dodecyl sulphate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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purchased from BIOMOL Research Labs (Polymouth, PA). Rabbit polyclonal COX-2 antibody was procured from Cayman Chemical (Ann Arbor, MI). Primary antibodies for ERK1/2, pERK1/2, p38, p65, IKKα, IκBα, and cyclic AMP-response element binding protein (CREB)-binding protein (CBP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-phospho-p38 was obtained from BD Biosciences (San Jose, CA). Anti-phospho-IκBα, anti-phospho-p65-(Ser-536), anti-phospho-p65-(Ser-276) and anti-IKKβ were obtained from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were products of Zymed Laboratories (San Francisco, CA). The enhanced chemiluminescence (ECL) detection kit and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Animal treatment

Female Institute of Cancer Research (ICR) mice (~6–7 weeks of age) were purchased from Sankyo Laboservice Corporation (SLC, Tokyo, Japan). The animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12 h light–dark cycle. The dorsal side of skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used in all experiments. Respective doses of resveratrol, Bay 11-7082 and TPA (10 nmol) were dissolved in 200 µl of acetone and applied topically to the dorsal shaven area.

Western blot analysis

The female ICR mice were topically treated on shaven backs with indicated doses of resveratrol (0.25 or 1 µmol) 30 min before TPA (10 nmol) treatment and killed by cervical dislocation either 1 or 4 h later. In other experiments, Bay 11-7082 (0.05 or 0.25 µmol) was co-treated with TPA (10 nmol). Collected epidermis was homogenized in 800 µl of ice-cold lysis buffer [150 mM NaCl, 0.5% Triton-X 100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4 and protease inhibitor cocktail tablet]. Lysates were centrifuged at 14 800× *g* for 30 min. Supernatant was collected and total protein concentration was quantified by using the BCA protein assay kit. Cell lysates (30 µg protein) were boiled in sodium dodecyl sulfate (SDS) sample buffer for 5 min before electrophoresis on 12% SDS–polyacrylamide gel. After transfer to PVDF membrane, the blots were blocked with 5% fat-free dry milk–PBST buffer (phosphate-buffered saline containing 0.1% Tween-20) for 1 h at room temperature and then washed with PBST buffer. The membranes were incubated for 4 h at room temperature with 1 : 1000 dilutions of primary antibodies for COX-2, extracellular signal-regulated protein kinase (ERK), pERK, p38, and pp38 and for 12 h at 4°C with 1 : 500 dilutions of primary antibodies for IκBα, pIκBα, p65, phospho-p65-(Ser-536) and phospho-p65-(Ser-276). Blots were washed three times with PBST at 5 min intervals followed by incubation with 1 : 5000 dilution of respective HRP-conjugated secondary antibodies (rabbit, goat or mouse) for 1 h and again washed in PBST for three times. The transferred proteins were visualized with an ECL detection kit according to the manufacturer's instructions.

Immunohistochemical analysis

The dissected skin was prepared for immunohistochemical analysis of the expression pattern of COX-2 in mouse skin treated with TPA in the presence or absence of resveratrol or Bay 11-7082. Four-micrometer sections of 10% formalin-fixed, paraffin-embedded tissues were cut on silanized glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of COX-2, slides were incubated with affinity purified rabbit polyclonal anti-COX-2 antibody (Cayman Chemical Ann Arbor, MI) at room temperature for 40 min in TBS containing 0.05% Tween-20 and then developed using anti-rabbit and anti-mouse HPR EnVisionTM System (Dako, Glostrup, Denmark), respectively. The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally, counterstaining was performed using Mayer's hematoxylin.

Preparation of cytosolic and nuclear extracts from mouse skin

The nuclear extract from mouse skin was prepared as described previously (10). In brief, scraped dorsal skin of mice was homogenized in 800 µl of hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenates was added 80 µl of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 2 min at 14 800× *g*. The supernatant was collected as cytosolic fraction. The precipitated nuclei were washed once with 500 µl of buffer A plus 40 µl of 10% NP-40, centrifuged, resuspended in 200 µl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 20% glycerol] and centrifuged for 5 min at 14 800× *g*. The supernatant containing nuclear proteins was collected and stored at −70°C after determination of protein concentrations.

Electrophoretic mobility shift assay (EMSA)

EMSA for NF-κB DNA binding was performed using a DNA–protein binding detection kit, according to the manufacturer's protocol (GIBCO BRL, Grand Island, NY). Briefly, the NF-κB oligonucleotide probe 5′-GAG GGG ATT CCC TTA-3′ (NF-κB binding site in murine COX-2 promoter region underlined) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in 25 µl of the mixture containing 5 µl of incubation buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 µg of nuclear extracts and 100 000 c.p.m. of [γ-^{32}P]ATP-end labeled oligonucleotide. After 50 min incubation at room temperature, 2 µl of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% non-denaturing polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried and exposed to an X-ray film.

Immunoprecipitation assay

Tissue homogenates were prepared from mouse skin treated with TPA either in the presence or in the absence of resveratrol. Cellular proteins (200 µg) were subjected to immunoprecipitation by shaking with CBP primary antibody (Santa Cruz Biotechnology) at 4°C for 12 h followed by the addition of protein G-agarose bead suspension (25% slurry, 40 µl) and additional shaking for 2 h at the same condition. After centrifugation at 14 800× *g* for 2 min, immunoprecipitated beads were collected by discarding the supernatant and washing with cell lysis buffer. After final wash, immunoprecipitate was resuspended in 40 µl of 2× SDS electrophoresis sample buffer and boiled for 5 min. Supernatant (30 µl) from each sample was collected after centrifugation and loaded on SDS–polyacrylamide gel (0.75 mm thickness). Following western blot protocol described earlier, separated proteins were transferred from gel to a PVDF membrane, which was then immunoblotted with p65 antibody (Cell Signaling Technology, Beverly, MA) to detect the interaction of p65 with CBP.

In vitro IKK activity assay

Cytosolic extracts prepared from mouse skin treated as specified in the figure legends were used to assay the IKK activity according to the protocol described by Bharti *et al*. (16). Briefly, cytosolic extract (200 µg) was precleared using normal mouse IgG and protein G-agarose beads. Precleared extract was subjected to immunoprecipitation by using anti-IKKα or anti-IKKβ antibody and the immunocomplex was pulled down by shaking with protein G-agarose beads. The immunoprecipitate thus obtained was suspended in 50 µl of reaction mix containing 47 µl 1× kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$], 1 µg GST–IκBα (1-317) substrate protein and 10 µCi [γ-^{32}P]-ATP and incubated at 30°C for 45 min. The kinase reaction was stopped by adding 15 µl 2.5× SDS loading dye, boiled at 99°C for 5 min, vortexed and centrifuged at 5000 r.p.m. for 2 min. The supernatant was separated by 12% SDS–polyacrylamide gel. The gel was stained with coomassie brilliant blue and destained with destaining solution (glacial acetic acid : methanol : distilled water,1 : 4 : 5, v/v). The destained gel was dried at 80°C for 1 h and was exposed to an X-ray film to detect the phosphorylated GST–IκBα in the radiogram.

Statistical evaluation

Values were expressed as the mean ± SEM of at least three independent experiments. Statistical significance was determined by Student's *t*-test and a *P*-value of <0.01 was considered to be statistically significant.

Results

Inhibitory effect of resveratrol on TPA-induced COX-2 expression in mouse skin

It has been demonstrated earlier that topical application of TPA (10 nmol) onto shaven backs of female ICR mice induces the expression of COX-2 protein maximally at 4 h (10). In the present study, topical application of resveratrol (1 µmol), 30 min prior to TPA resulted in a statistically significant ($P < 0.001$) decrease in the level of COX-2 protein in mouse skin 4 h after TPA treatment (Figure 1A). However, treatment of mouse skin with resveratrol (1 µmol) alone did not influence the constitutive expression of COX-2 (data not

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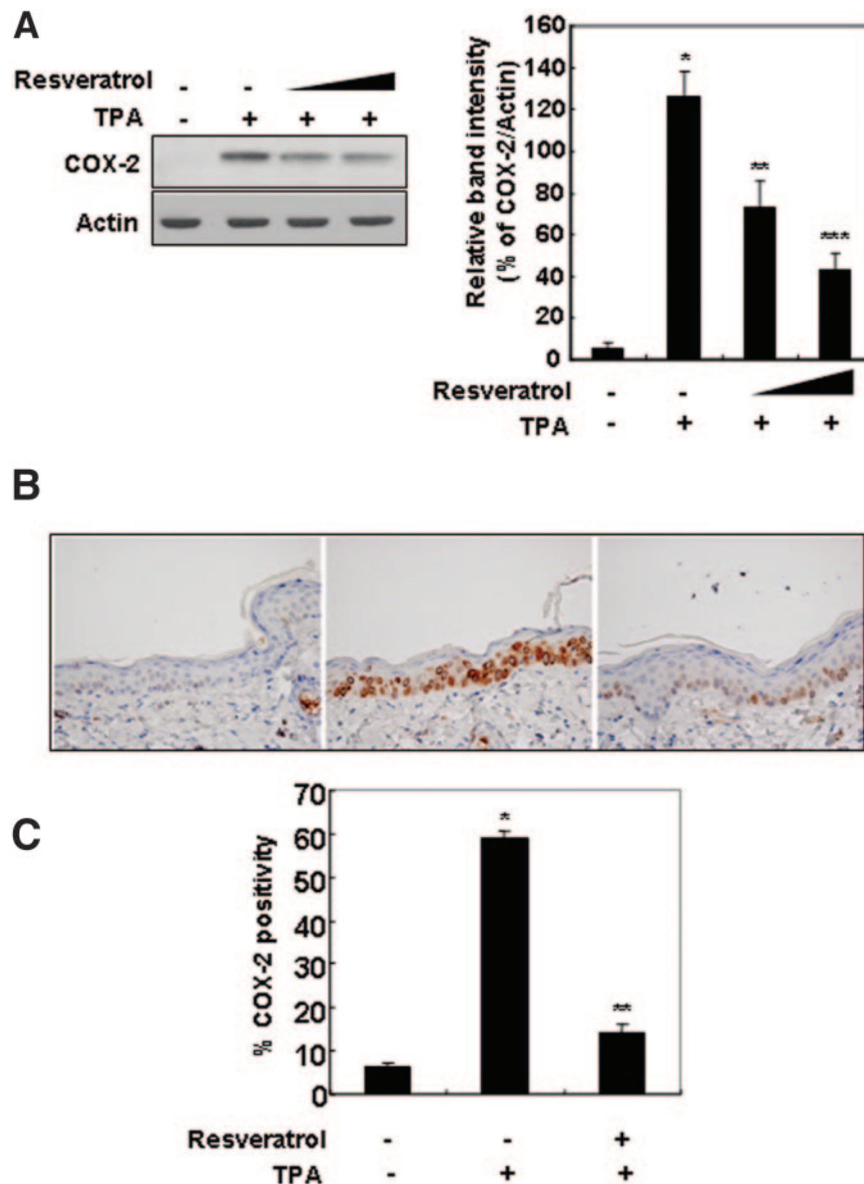

Fig. 1. Inhibitory effects of resveratrol on TPA-induced COX-2 expression in mouse skin. Female ICR mice were treated topically with resveratrol (0.25 or 1 μmol) dissolved in 0.2 ml acetone. After 30 min, mice were treated topically with 10 nmol TPA in 0.2 ml acetone and killed 4 h later. Control animals were treated with acetone in lieu of TPA. (**A**) Total cell lysates were analyzed for COX-2 expression by immunoblotting. Quantification of COX-2 immunoblot was normalized to that of actin followed by statistical analysis of relative image density. *$P < 0.001$ (control versus TPA alone), **$P < 0.025$ (resveratrol 0.25 μmol plus TPA versus TPA alone), ***$P < 0.001$ (resveratrol 1 μmol plus TPA versus TPA alone). (**B**) Skin samples from mice treated with acetone (left), TPA alone (center) and resveratrol (1 μmol) plus TPA (right) were subjected to immunohistochemical analysis by using affinity purified murine COX-2 antibody as described in Materials and methods. Positive COX-2 staining yielded a brown-colored product. (**C**) Percent of COX-2 positivity in epidermal layer was determined by counting the number of total and COX-2 positive cells from 10 equal sections of immunostained tissues from each animal. *$P < 0.001$ (control versus TPA alone), **$P < 0.001$ (resveratrol 1 μmol plus TPA versus TPA alone).

shown). Immunohistochemical analysis verified that the TPA-induced expression of COX-2, predominantly localized in epidermal layer, was significantly ($P < 0.001$) reduced by pretreatment with resveratrol (Figure 1B and C).

Inhibition of TPA-induced activation of NF-κB by resveratrol in mouse skin

Since the 5′-flanking region of *COX-2* gene promoter contains binding sequences for various transcription factors including NF-κB (17), we attempted to examine the effects of resveratrol on TPA-stimulated DNA binding of NF-κB in mouse skin. Nuclear extracts obtained from TPA-treated mouse skin, with or without resveratrol pretreatment, were subjected to EMSA using the oligonucleotide harboring the NF-κB binding sequence present in the mouse COX-2 promoter region. As shown in Figure 2A, resveratrol inhibited TPA-induced DNA binding of NF-κB. Moreover, resveratrol reduced the levels of p65/RelA, a functionally active subunit of NF-κB in nuclear fractions prepared from TPA-treated mouse skin (Figure 2B). Since the nuclear translocation of NF-κB is dependent on the phosphorylation and subsequent degradation of IκBα (18), we examined whether resveratrol could block TPA-induced phosphorylation of IκBα. As shown in Figure 2C, TPA-induced phosphorylation of IκBα at serine 32 and 36 residues was inhibited by resveratrol.

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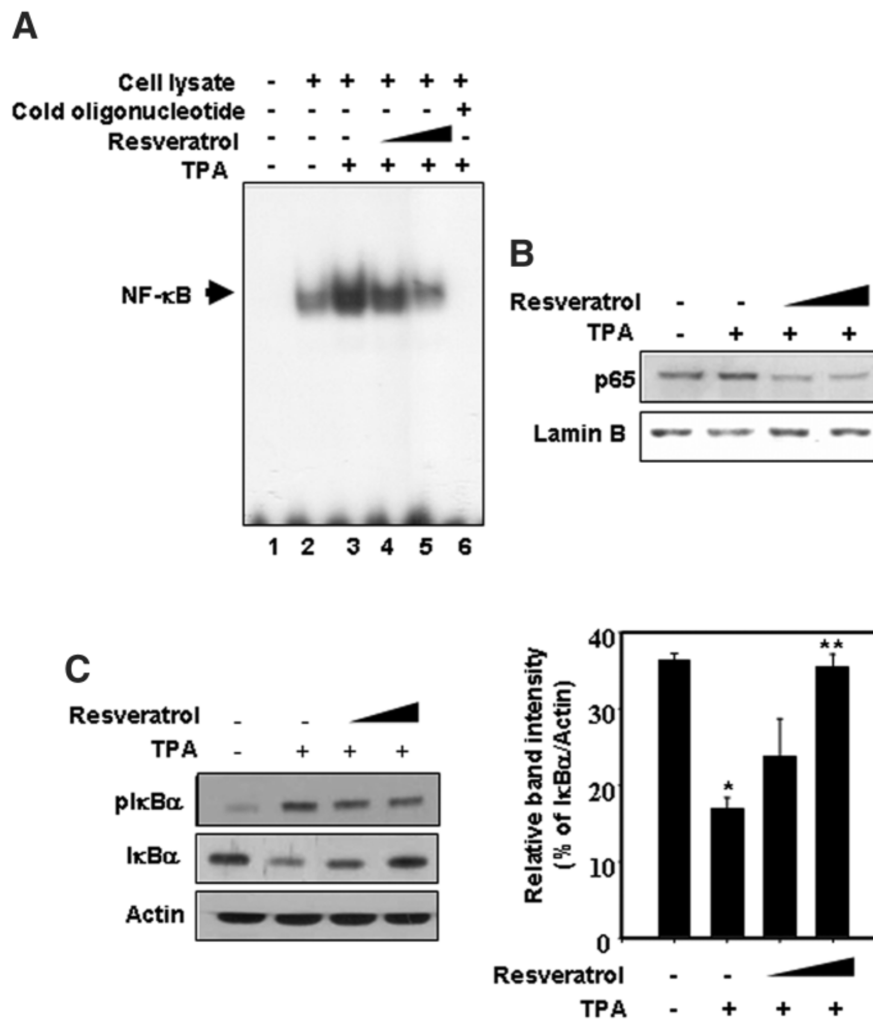

Fig. 2. Inhibitory effects of resveratrol on TPA-induced activation of NF-κB in mouse skin *in vivo*. Shaven backs of female ICR mice were treated with either acetone or resveratrol (0.25 or 1 μmol) 30 min prior to TPA (10 nmol) except control animals, which were treated with acetone only. One hour after treatment, the epidermal nuclear extracts were prepared. (**A**) Inhibitory effect of resveratrol on TPA-induced NF-κB DNA binding. Lane 1, free probe alone (no nuclear extracts); Lane 2, acetone control; Lane 3, TPA alone; Lane 4, resveratrol (0.25 μmol) + TPA; Lane 5, resveratrol (1 μmol) + TPA; Lane 6, TPA-treated sample + 100-fold excess unlabeled oligonucleotide. (**B**) Nuclear protein (50 μg) was separated by 10% SDS–polyacrylamide gel and immunoblot was performed by using a primary antibody specific to detect p65. (**C**) Cytosolic extracts from mice treated with acetone, TPA alone and resveratrol (0.25 or 1 μmol) plus TPA were subjected to western blot analysis to examine the expression of pIκBα and IκBα using specific antibodies. $^{*}P <$ control versus TPA; $^{**}P < 0.001$, resveratrol 1 μmol plus TPA versus TPA only.

Likewise, resveratrol pretreatment blocked TPA-stimulated degradation of IκBα (Figure 2C).

Inhibitory effect of resveratrol on TPA-induced transcriptional activation of NF-κB

The transactivation of NF-κB-regulated genes requires not only the binding of NF-κB to their promoter regions but also the phosphorylation of p65/RelA, which is the active subunit of NF-κB. In our previous study, topical application of TPA caused an increase in p65/RelA phosphorylation at serine 536 (19). Resveratrol pretreatment significantly inhibited phosphorylation of p65-(Ser-536) induced by TPA (Figure 3A). Besides phosphorylation of p65 at serine 536 in Trans Activation Domain (TAD), topical application of TPA markedly enhanced the phosphorylation of p65 at the serine 276 residue located in Rel Homology Domain (RHD), which was also attenuated by resveratrol pretreatment (Figure 3A).

The phosphorylation of p65 at serine 536 in TPA-stimulated mouse skin was reported to be mediated, at least in part, by ERK (10) and p38 MAP kinase (19). Western blot analysis revealed that resveratrol suppressed TPA-induced phosphorylation of both ERK1/2 and p38 MAP kinase in mouse skin (Figure 3B), suggesting that resveratrol attenuated transcriptional activation of NF-κB by blocking activation of one or both of these MAP kinases.

It has been demonstrated that CBP/p300, by dint of its intrinsic histone acetyl-transferase activity, acts as a transcriptional co-activator and that the interaction of CBP with p65/RelA is a critical event in recruiting other key components of the transcriptional machinery to form a transcription initiation complex (20). We examined the effect of resveratrol on CBP interaction with p65/RelA. It was found that topical application of TPA resulted in a dramatic increase in the interaction of CBP with p65 in mouse skin, which was strongly inhibited by resveratrol pretreatment (Figure 3C). Taken

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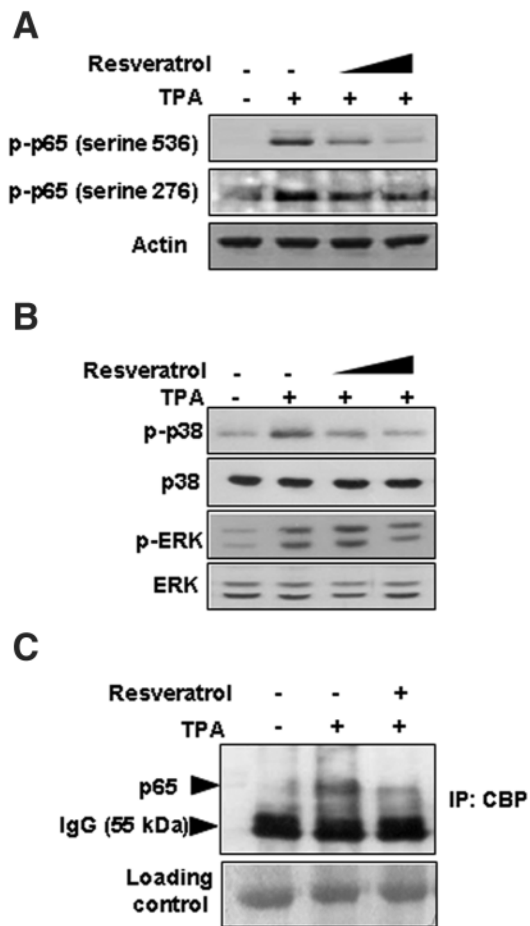

Fig. 3. Effects of resveratrol on TPA-induced signaling events associated with NF-κB transactivation. (**A**) Mice were treated as described in Figure 2. Cytosolic extract was prepared as described in Materials and methods. The expression of phosphorylated p65-(Ser-536) and phosphorylated p65-(Ser-276) was measured by immunoblot analysis of cytosolic proteins (50 μg) after separation over 10% SDS–polyacrylamide gel. Data presented is representative of two independent experiments showing similar trend. (**B**) Shaven backs of female ICR mice were treated with either acetone or TPA (10 nmol) for 1 h. Total tissue lysates (50 μg) were separated by 12% SDS–polyacrylamide gel and immunoblotted by using ERK, p38, phospho-ERK and phospho-p38 antibodies. The immunoblot is representative of three independent experiments eliciting a similar pattern. (**C**) Nuclear extracts (200 μg) prepared from mouse skin treated with TPA in the presence or absence of resveratrol (1 μmol) were subjected to immunoprecipitation by using anti-CBP antibody. The immunoprecipitate was resolved by running through 10% SDS–polyacrylamide gel and immunoblotted with a specific antibody against p65. IP, immunoprecipitation.

together, our results indicate that resveratrol blocks the TPA-induced transcriptional activation of NF-κB in mouse skin by inhibiting NF-κB interaction with the transcriptional co-activator CBP/p300.

Resveratrol-mediated suppression of IKK activity in TPA-stimulated mouse skin

To get further insights into the molecular mechanisms underlying inhibition of TPA-induced NF-κB activation by resveratrol, we first examined the effect of topically applied TPA in activating IKKα and IKKβ, upstream kinases known to activate NF-κB via phosphorylation-dependent degradation of IκBα in various cultured cell lines (21). *In vitro* radioactive kinase assay using cytosolic extracts obtained from mouse skin treated with TPA for different time points revealed that TPA stimulated IKKα (Figure 4A) and IKKβ (Figure 4B) activity in mouse skin in as early as 30 min, which persisted up to 2 h following TPA treatment. The activation of IKKβ in mouse skin by TPA was confirmed by heat inactivation of the immunoprecipitate containing IKKβ that was unable to phosphorylate GST–IκBα (Figure 4B). In addition, the stimulation of IKKα/β activity by TPA was attenuated by co-treatment with Bay 11-7082, a pharmacological inhibitor of IKK (Figure 4C). In a separate experiment following the same protocol, we observed that resveratrol pretreatment significantly attenuated TPA-induced stimulation of IKKα and IKKβ activity (Figure 4D). Our results clearly demonstrate that resveratrol inhibits TPA-induced activation of NF-κB by targeting the upstream kinase IKK.

IKK-catalyzed activation of NF-κB in TPA-treated mouse skin

To explore the role of IKK in activating NF-κB in mouse skin stimulated with TPA, we examined the effects of Bay 11-7082 (0.05 or 0.25 μmol) on TPA-induced activation of NF-κB. Co-treatment of mouse skin with Bay 11-7082 and TPA resulted in the inhibition of phosphorylation (Figure 5A) and subsequent degradation of IκBα (Figure 5B), which was supported by a dose-dependent decrease in nuclear translocation of p65 (Figure 5C). In addition, topical application of Bay 11-7082 (0.25 μmol) together with TPA diminished DNA binding of NF-κB (Figure 5D), suggesting a regulatory role of IKK in TPA-induced activation of NF-κB in mouse skin.

Inhibitory effects of Bay 11-7082 on TPA-induced COX-2 expression in mouse skin

In order to determine whether resveratrol-mediated inhibition of NF-κB activation and COX-2 expression in TPA-treated mouse skin was mediated via suppression of IKK, we examined the role of IKK in regulating TPA-induced COX-2 expression. Topical application of Bay 11-7082 resulted in a significant ($P < 0.001$) inhibition of TPA-induced COX-2 expression in mouse skin (Figure 6A). Immunohistochemical analysis further confirmed the inhibitory effect of the IKK inhibitor Bay 11-7082 on TPA-induced epidermal COX-2 expression (Figure 6B), as revealed by a significant reduction in COX-2 positivity in comparison with TPA treatment alone (Figure 6C).

Discussion

A causal relationship between inflammation and cancer has long been speculated (22). Current progress in searching the molecular links between inflammation and cancer has identified NF-κB as a tumor promoter (21) that, upon activation, enhances transcription of pro-inflammatory genes including *COX-2* (1,3,23). Multiple lines of evidence arising from both population-based and laboratory studies suggest that the targeted inhibition of an inappropriate overexpression or activity of COX-2 by anti-inflammatory substances derived from plant-based diets or medicinal plants are effective in preventing certain malignancies (24,25). Because of the potential cardiovascular risk, the USA Food and Drug Administration has recently announced that the use of selective COX-2 inhibitors may not be safe and reliable for cancer prevention (http://www.fda.gov/cder/drug/infopage/COX2/default.htm). However, considering the preclinical and clinical evidence suggesting the efficacy of COX-2 inhibitors in

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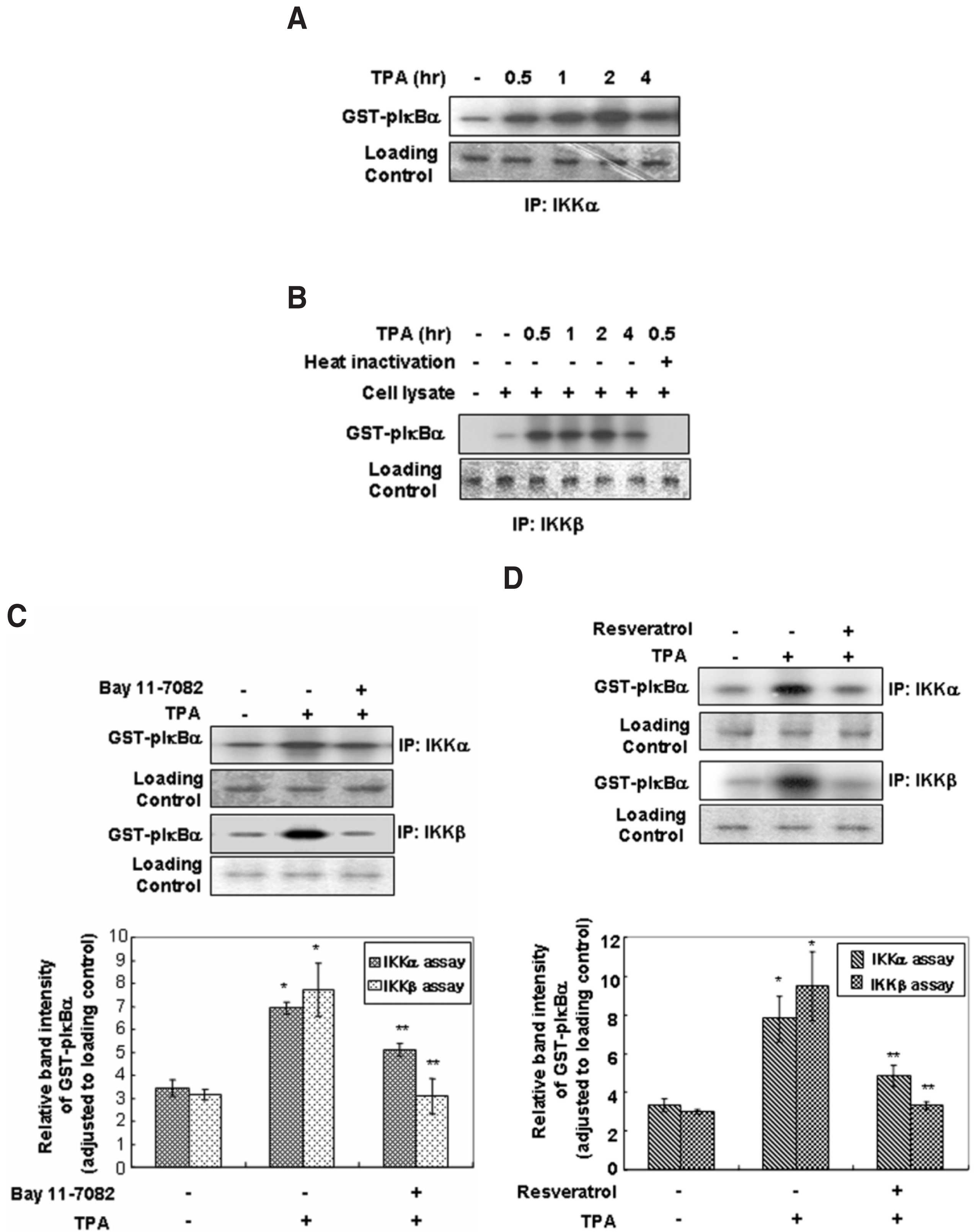

Fig. 4. Inhibitory effect of resveratrol on TPA-induced IKK activity in mouse skin. Cytosolic extracts were prepared from mouse skin treated with TPA for various time points (A,B) and in the presence or absence of Bay 11-7082 (0.25 μmol) (C) or resveratrol (1 μmol) (D). **(A)** Kinetics showing TPA-induced IKKα activity, **(B)** kinetics showing TPA-induced IKKβ activity, **(C)** effect of Bay 11-7082 on TPA-induced IKKα and IKKβ activity in mouse skin. *$P < 0.01$ (TPA alone in comparison with respective solvent control), **$P < 0.01$ (Bay 11-7082 plus TPA in comparison with corresponding TPA alone), (**D**) effect of resveratrol on IKKα and IKKβ activity induced by TPA. *$P < 0.01$ (TPA alone in comparison with respective solvent control), **$P < 0.01$ (resveratrol 1 μmol plus TPA in comparison with respective TPA alone).

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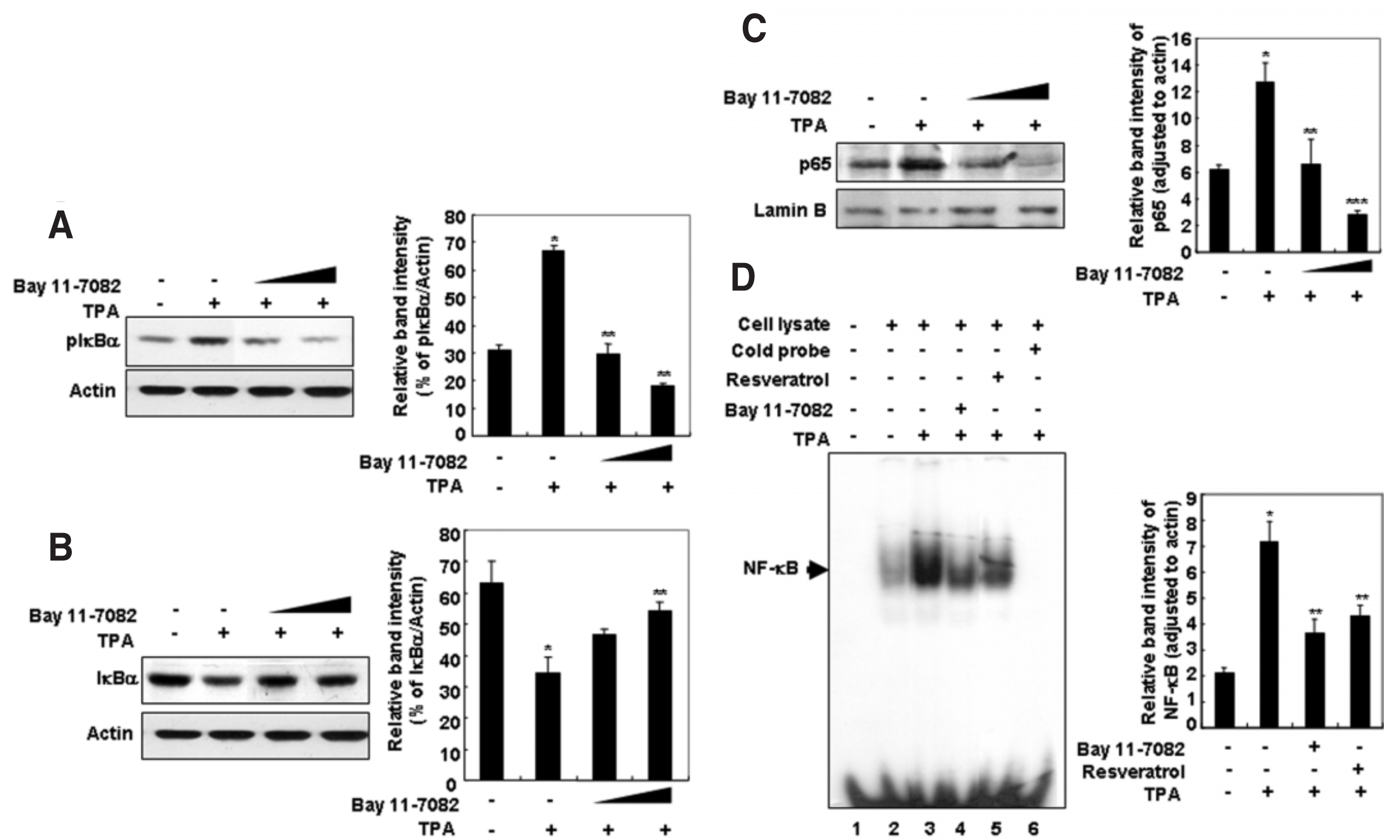

Fig. 5. Inhibitory effects of Bay 11-7082 on TPA-induced activation of NF-κB in mouse skin *in vivo*. Shaven backs of female ICR mice were treated with TPA (10 nmol) in the presence or absence of Bay 11-7082 (0.05 or 0.25 μmol) following co-treatment protocol. Control animals were treated with acetone only. One hour after the treatment of TPA, the epidermal cytosolic and nuclear extracts were prepared as described in Materials and methods. (**A**) The expression level of pIκBα (*$P < 0.01$ for TPA alone as compared with solvent control; **$P < 0.001$ for Bay 11-7082 plus TPA versus TPA alone). (**B**) The level of IκBα expression in the cytosolic extract (*$P < 0.01$ for TPA alone as compared with solvent control; **$P < 0.01$ for Bay 11-7082 plus TPA versus TPA alone). (**C**) Nuclear protein (50 μg) was separated by 10% SDS–polyacrylamide gel and immunoblot was performed by using a primary antibody specific to detect p65 (*$P < 0.01$ for TPA alone as compared with solvent control; **$P < 0.025$ for Bay 11-7082 0.05 μmol plus TPA versus TPA only and ***$P < 0.001$ for Bay 11-7082 0.25 μmol plus TPA versus TPA only). (**D**) Inhibitory effect of Bay 11-7082 on TPA-induced NF-κB DNA binding. Lane 1, free probe alone (no nuclear extracts); Lane 2, acetone control; Lane 3, TPA alone; Lane 4, Bay 11-7082 (0.25 μmol) + TPA; Lane 5, resveratrol (1 μmol) + TPA; Lane 6, TPA-treated sample + 100-fold excess unlabeled oligonucleotide. *$P < 0.001$, TPA alone as compared with solvent control; **significantly ($P < 0.01$) different from the group treated with TPA alone.

preventing cancer, the leading scientists conducting chemoprevention trial still plan to continue cancer prevention research with COX-2 inhibitors (26) to find an optimal dose of specific agents after safety and efficacy analysis. The present study was undertaken to examine the effect of resveratrol, a well-known cardioprotective and chemopreventive phytochemical, on tumor promoter-induced COX-2 expression in mouse skin and to delineate the underlying molecular mechanisms. Although the inhibitory effects of resveratrol on COX-2 expression induced by diverse stimuli in both *in vivo* and cultured cell lines have been demonstrated earlier (13,15), the underlying molecular mechanisms have been poorly understood.

The induction of COX-2 by TPA in mouse skin is regulated, at least in part, by an eukaryotic transcription factor NF-κB (10,25), which is mainly a heterodimer of p50 and p65 proteins. In unstimulated cells, NF-κB is kept sequestered in cytoplasm by its inhibitory counterpart IκBα, which, in response to diverse oxidative and inflammatory stimuli, gets phosphorylated on serine 32 and 36 residues and targeted by the ubiquitin-proteasome system for degradation, releasing the functionally active NF-κB. We have previously reported that topical application of TPA promotes nuclear translocation and activation of NF-κB in mouse skin via phosphorylation and subsequent degradation of IκBα (10). In addition, pharmacological inhibition of TPA-induced activation of NF-κB has been shown to abrogate COX-2 expression, suggesting the role of NF-κB in regulating COX-2 expression in mouse skin *in vivo* (10,19). The present study revealed that resveratrol attenuated both nuclear translocation and DNA binding of NF-κB in TPA-stimulated mouse skin, primarily by inactivating IκBα. Banerjee *et al.* (14) also reported that resveratrol inhibited the DNA binding of NF-κB in human breast cancer (MCF-7) cells and 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumor. In addition, resveratrol was found to modulate NF-κB activation in lipopolysacharide-stimulated murine macrophages (27), myeloid leukemia cells (28) and Jurkat T cells (29). Besides NF-κB, another redox-sensitive transcription factor AP-1 has also been reported to regulate TPA-induced COX-2 expression in mouse skin. We and others have reported that resveratrol attenuates TPA-induced activation AP-1 as well (15). The role of other transcription factors such as CREB and CCAAT/enhancer binding protein (CEBP) in COX-2 induction has also been reported

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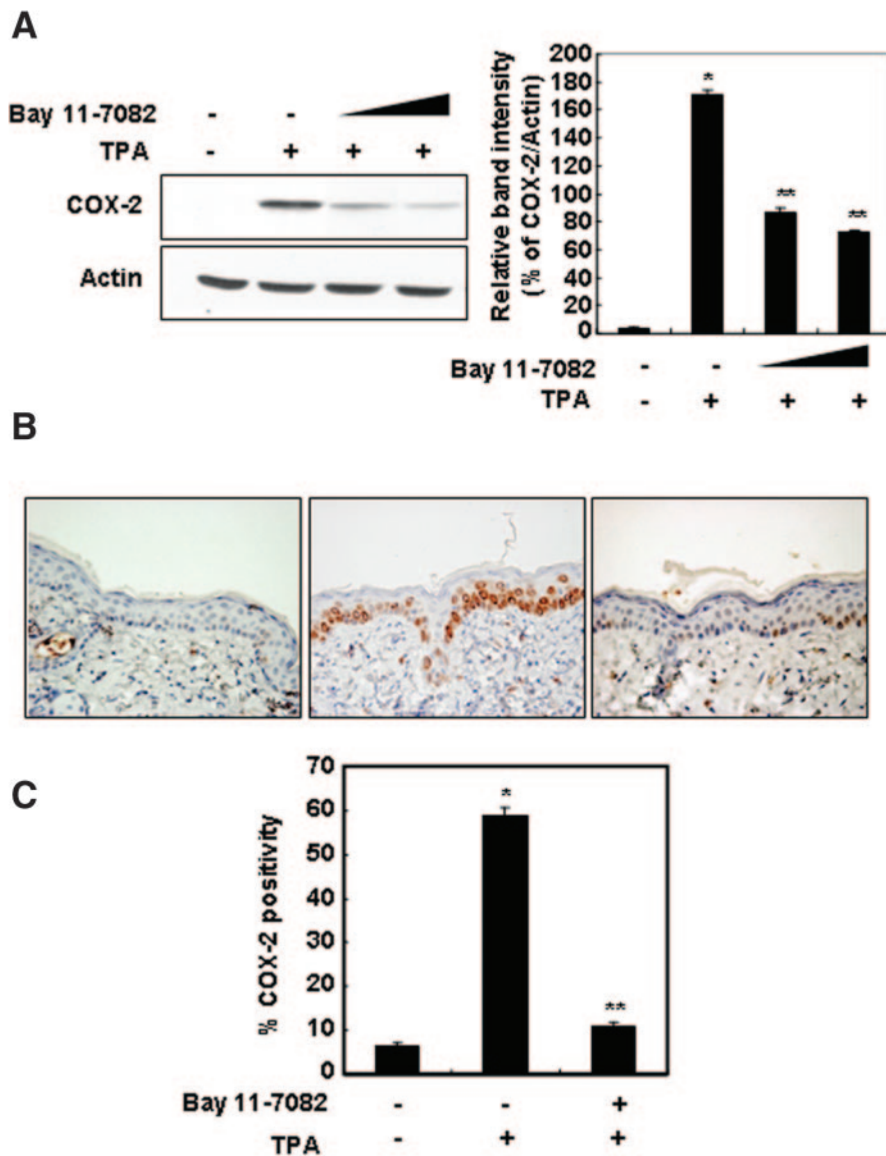

Fig. 6. Inhibitory effects of Bay 11-7082 on TPA-induced COX-2 expression in mouse skin. Female ICR mice were treated topically with Bay 11-7082 (0.05 or 0.25 μmol) dissolved in 0.2 ml acetone with 10% DMSO and TPA simultaneously. Control animals were treated with acetone in lieu of TPA. Mice were killed 4 h later. (**A**) Total cell lysates were resolved through 12% SDS–polyacrylamide gel for detecting COX-2 expression by immunoblotting. Quantification of COX-2 immunoblot was normalized to that of actin followed by statistical analysis of relative image by densitometry. $^{*}P < 0.001$, control versus TPA only; $^{**}P < 0.001$, Bay 11-7082 pretreated group versus TPA alone. (**B**) Mice treated as mentioned above were killed after 4 h of TPA application. Skin samples from mice treated with acetone (left), TPA alone (center) and Bay 11-7082 (0.25 μmol) plus TPA (right) were subjected to immunohistochemical analysis by using affinity purified murine COX-2 antibody as described in Materials and methods. Positive COX-2 staining yielded a brown-colored product. (**C**) Percent of COX-2 positivity in the epidermal layer was determined by counting the number of total and COX-2 positive cells from 10 equal sections of immunostained tissues from each animal. $^{*}P < 0.001$, control versus TPA only; $^{**}P < 0.001$, Bay 11-7082 pretreated group versus group treated with TPA alone.

(17). TPA has been shown to activate CREB and CEBP in mouse skin (30,31). However, the effect of resveratrol on the activation of these transcription factors are yet to be established.

Although it is generally accepted that nuclear translocation and subsequent DNA binding of NF-κB are critical events required for the activation of NF-κB-dependent gene expression (32,33), several recent studies suggest that the downregulation of NF-κB DNA binding activity is not necessarily associated with its reduced transcriptional activity (34,35). The efficient transcriptional activation of NF-κB depends on the phosphorylation of its active subunit p65/RelA, particularly at serine 536 residue (36). Several lines of evidence suggest that ERK1/2 and p38 MAP kinase may regulate transcriptional activity of NF-κB (37–39). The findings that resveratrol attenuates TPA-induced phosphorylation of ERK1/2 and p38 MAP kinase and that of p65/RelA-(Ser-536) thus suggest the modulatory effect of resveratrol on NF-κB transcriptional activation. Recently, ERK5 has also been reported to play a critical role in regulating normal physiological functions such as survival, proliferation and differentiation, as well as in carcinogenesis and other pathological processes (40,41). It has been demonstrated that the activation of ERK5 is involved in gastrin-induced COX-2 expression in intestinal epithelial cells (40). Since

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the inhibitors of MEK1/2 also inactivate ERK5 (42,43), some of the effects ascribed to ERK1/2 signaling may be a consequence of ERK5 signaling. Therefore, the possible induction of ERK5 by TPA in mouse skin and its modulation by resveratrol cannot be excluded. It has been suggested that the activation of p38 MAP kinase stabilizes COX-2 mRNA (44,45). Some anti-inflammatory agent, such as dexamethasone, destabilizes COX-2 mRNA via downregulation of p38 MAP kinase (46). In contrast, certain COX-2 inhibitors were shown to stabilize COX-2 mRNA, which was associated with upregulation of p38 MAP kinase (47). The effects of resveratrol on the posttranscriptional modification and stability of *cox-2* gene in TPA-treated mouse skin merit further investigation.

The activity of many inducible transcription factors is regulated through their interaction with transcriptional co-activators such as CBP/p300, which is believed to link enhancer-bound transcription factors with general transcription machinery (20). CBP/p300 has an intrinsic acetyltransferase activity that regulates gene expression, in part, through acetylation of the N-terminal tails of histones. Acetylated histones are associated with transcriptionally active segments of chromatin, whereas deacetylated histones accumulate in transcriptionally repressed regions (48,49). It has been reported that co-transfection of cells with CBP/p300 enhances NF-κB-dependent transcription (50). Zhong *et al.* (20) have demonstrated that the association of NF-κB with CBP/p300 occurs either by a phosphorylation-independent mechanism or through PKA-dependent phosphorylation of p65/RelA. In the present study, we found that resveratrol abrogated the interaction between CBP and p65. Moreover, it has been demonstrated that the phosphorylation of the serine residue at 276 located in the RHD of p65/RelA facilitates the interaction of CBP with p65/RelA (20). Resveratrol has also been found to suppress TPA-induced phosphorylation of p65/RelA-(Ser-276) in mouse skin. Taken together, the above findings suggest that the inhibitory effect of resveratrol on COX-2 expression was mediated through suppression of NF-κB transactivation.

An upstream regulator IKK complex has been reported to catalyze the phosphorylation of both IκBα and NF-κB (51,52). A recent study suggests that within the IKK complex, IKKα is largely responsible for p65 phosphorylation, whereas IKKβ is capable of phosphorylating both IκBα and p65 (53). We, therefore, attempted to elucidate the role of IKK in regulating NF-κB activation and COX-2 expression in mouse skin stimulated with TPA. In agreement with previous studies demonstrating the inhibitory effect of Bay 11-7082 on phosphorylation-mediated degradation of IκBα, DNA binding and transactivation of NF-κB (54–56), and expression of COX-2 (57,58) in various cultured cell lines, our study provides the first report of a signal transducing role of IKKα and IKKβ in the regulation of NF-κB and COX-2 in TPA-treated mouse skin. The suppression of TPA-induced IKK activity by resveratrol was also in agreement with previous studies (59,60). Our study, thus, suggests that IKK is an upstream regulator of NF-κB in TPA-stimulated mouse skin. Considering IKK and NF-κB as potential molecular links between inflammation and cancer (2,3) and the contributory role of aberrantly expressed COX-2 in tumor promotion (24,25), the present study provides the molecular mechanisms underlying previously reported chemopreventive effects of resveratrol on mouse skin carcinogenesis.

In conclusion, resveratrol inhibited TPA-induced COX-2 expression via modulation of the IKK–NF-κB signaling cascade in mouse skin *in vivo*, which provides a mechanistic basis of the anti-inflammatory and anti-tumor promoting activity of resveratrol.

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