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Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin *in vivo*: NF-κB and AP-1 as prime targets

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AP-1, activator protein-1
C/EBP, CCAAT/enhancer binding protein
CREB, cyclic AMP response element binding protein
CBP, CREB binding protein
COX-2, cyclooxygenase-2
DMBA, 7,12-dimethylbenz[*a*]anthracene
ERK, extracellular signal-regulated protein kinase
IKK, IκB kinase
JNK, c-Jun-N-terminal kinase

ABSTRACT

Functional abnormalities of intracellular signaling network cause the disruption in homeostasis maintained by critical cellular components, thereby accelerating premalignant and malignant transformation. Multiple lines of evidence suggest that an elevated expression of cyclooxygenase-2 (COX-2) is causally linked to tumorigenesis. The exposure to oxidative/pro-inflammatory stimuli turns on signaling arrays mediated by diverse classes of kinases and transcription factors, which may lead to aberrant expression of COX-2. We have attempted to unravel the signal transduction pathways involved in elevated COX-2 expression in mouse skin stimulated with a prototype tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and its modulation by resveratrol, a phytoalexin known to exert potential chemopreventive effects. Our study revealed that topical application of TPA induced COX-2 expression in mouse skin via activation of nuclear factor-κB (NF-κB), which is regulated by upstream IκB kinase (IKK) or differentially by mitogen-activated protein (MAP) kinases. Besides NF-κB, the p38 MAP kinase-mediated activation of activator protein-1 (AP-1) has also been attributed to TPA-induced COX-2 expression in mouse skin. Among the MAP kinases, extracellular signal-regulated protein kinase (ERK) and p38 MAP kinase have been shown to regulate TPA-induced NF-κB activation, while p38 MAP kinase and c-Jun-N-terminal kinase are preferentially involved in TPA-induced activation of AP-1 in mouse skin *in vivo*. This commentary focuses on resveratrol modulation of intracellular signaling pathways involved in aberrant COX-2 expression in TPA-stimulated mouse skin to delineate molecular mechanisms underlying antitumor promoting effects of resveratrol.

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MAP kinase, mitogen-activated protein kinase
NF-κB, nuclear factor-kappaB
PG, prostaglandin
PI3K, phosphoinositide 3-kinase
PKC, protein kinase C
TPA, 12-O-tetradecanoylphorbol-13-acetate

1. Resveratrol—a nature's potent chemopreventive agent

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), a well-known antioxidant ingredient of red wine, is a phytoalexin present in almost 70 plant species including grape, peanut, mulberry, etc. The compound possesses anti-inflammatory, immunomodulatory, antioxidant, cardioprotective and chemopreventive effects [1,2]. Multiple lines of evidence from preclinical studies suggest that resveratrol has an ability to intervene in multi-stage carcinogenesis [3–8]. In a pioneering study, Jang and colleagues [6] have demonstrated that resveratrol can interfere with all three major stages (i.e., initiation, promotion and progression) of carcinogenesis. According to this study, resveratrol significantly reduced the average number of papillomas per mouse in a 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted skin carcinogenesis model [6]. Similarly, topical application of resveratrol resulted in about 60% reduction in TPA-promoted mouse skin papilloma formation [9]. In addition, resveratrol protected against UVB-induced skin tumorigenesis in SKH-1 hairless mice [3].

Resveratrol has been shown to modulate the expression or activities of a series of intracellular signaling molecules involved in carcinogen metabolism, cellular proliferation, inflammation, cell cycle regulation, apoptosis, etc. [1,2,10]. As a potent inhibitor of mouse skin tumor promotion [3,6], resveratrol down-regulated the expression of cell cycle regulatory proteins (e.g., cyclins and cyclin-dependent kinases) as well as inhibitors of apoptosis (e.g., survivin), and also induced expression of proapoptotic proteins (e.g., p21, p53, Smac/Diablo) in UVB-stimulated mouse skin [3,11,12]. Moreover, resveratrol inhibited TPA-induced oxidative stress, expression and activity of pro-inflammatory enzyme cyclooxygenase-2 (COX-2), and transactivation of *c-fos* in mouse skin *in vivo* [13–15]. Although the mechanistic aspect of chemoprevention with resveratrol has been studied extensively in cultured cell lines [1,2,10], details of molecular mechanisms underlying its antitumor promoting effect in TPA-stimulated mouse skin are not fully elucidated. This commentary focuses on the molecular basis of antitumor promoting activity of resveratrol in TPA-treated mouse skin *in vivo* with NF-κB- and AP-1-mediated inflammatory signal transduction pathways as major targets.

2. COX-2 as a link between inflammation and mouse skin tumor promotion: modulation by resveratrol

Although inflammation constitutes primarily a body's defense mechanism, the harmful sequelae of inflammation underlie various chronic diseases including cancer. The association between inflammation and cancer has long been suspected [16]. There is now growing evidence supporting that chronic inflammation may lead to malignancies of several organs [17–20]. It has been estimated that approximately 15% of all cancers are somehow linked to inflammation [21]. Persistent inflammation creates an abnormal microenvironment where a distinct set of pro-inflammatory mediators promote neoplastic transformation [22]. Inflammatory responses, both acute and chronic, flare through cellular reactions that are mediated by chemical factors such as cytokines, chemokines, prostaglandins (PGs), nitric oxide (NO), leukotrienes, etc., which are produced in response to pro-inflammatory stimuli [23]. Most of these pro-inflammatory mediators contribute to tumor promotion by altering normal cellular signaling cascades [22]. According to a study by Verma et al. [24], PGs play a crucial role in the induction of ornithine decarboxylase activity and mouse skin tumor promotion by TPA. Subsequent studies have suggested that elevated levels of some PGs, especially PGE_2 and $PGF_{2\alpha}$, are functionally related to mouse skin tumor promotion [25]. A recent study by Millan et al. also demonstrated that topical application of a COX-2 product 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 potentiated DMBA-initiated and TPA-promoted mouse skin tumorigenesis [26]. In response to pro-inflammatory stimuli, PGs are produced in abundance through metabolic conversion of arachidonic acid by COX-2, which is inappropriately up-regulated in various premalignant and malignant tissues [27,28]. Moreover, COX-2 over-expressing transgenic mice [29] are highly susceptible to spontaneous skin tumor formation, while COX-2 knock out animals [30] are less prone to experimentally induced tumorigenesis. Therefore, COX-2 is now regarded as a molecular target for chemoprevention.

In a recent study, we have reported that topical application of resveratrol onto shaven backs of female ICR mice significantly inhibited TPA-induced COX-2 expression [15]. The inhibition of TPA-induced COX-2 activity by resveratrol in CD-1 mouse skin has also been reported [6]. Similarly, resveratrol inhibited the expression [31] and activity [11] of COX-2 in mouse skin stimulated with UVB. Therefore,

resveratrol unties the deadly knot between inflammation and mouse skin tumorigenesis, partly by suppressing inappropriately elevated expression [15] and/or activity [6] of COX-2.

3. Inhibitory effects of resveratrol on TPA-induced activation of NF-κB and AP-1 in mouse skin *in vivo*

The regulation of *cox-2* transcription is mediated by several distinct transcription factors [32,33]. The 5′-flanking region of the *cox-2* gene contains a canonical TATA box and binding sequences for various transcription factors, such as nuclear factor-kappaB (NF-κB), CCAAT/enhancer binding protein (C/EBP), cyclic AMP response element binding protein-binding protein (CREB), and activator protein-1 (AP-1) [34]. Inappropriate activation of these transcription factors can lead to overexpression of *cox-2* [34]. NF-κB and AP-1 act independently or coordinately to regulate expression of target genes involved in various physiological processes including inflammation and cellular proliferation [35].

Improper activation of NF-κB and/or AP-1 may promote mouse skin tumorigenesis partly by transactivating genes encoding pro-inflammatory enzymes COX-2 and inducible nitric oxide synthase [36–38]. Previous studies from authors' laboratory demonstrated that topical application of TPA increased NF-κB DNA binding in female ICR mouse skin [39] and NF-κB-driven reporter gene activity in luciferase transgenic mice [40]. Pretreatment with the NF-κB inhibitor pyrrolidine dithiocarbamate diminished TPA-induced NF-κB DNA binding as well as COX-2 expression, suggesting a regulatory role of NF-κB on TPA-induced COX-2 expression in mouse skin [36]. Similarly, the DNA binding of AP-1 was also elevated in TPA-treated mouse skin [39]. Since aberrant activation of these transcription factors results in an inappropriate expression of COX-2, an improper activation of NF-κB and AP-1 may be considered as an interface between inflammation and tumor promotion in mouse skin *in vivo*.

3.1. *Resveratrol modulation of NF-κB*

The transcription factor NF-κB exits as a homo- or heterodimer of Rel family proteins [41]. A supershift assay has revealed that NF-κB activated in TPA-stimulated mouse skin largely exists as a heterodimer of p65 and p50 proteins [39]. In resting cells, NF-κB remains sequestered in the cytoplasm as an inactive complex with its inhibitory counterpart IκB proteins. In response to external stimuli, NF-κB-bound IκBα undergoes phosphorylation at serine 32 and serine 36 residues and is subsequently degraded by the ubiquitin-dependent proteasomal system, leaving NF-κB dimers free to translocate to the nucleus [41,42]. The nuclear translocation and transcriptional activation of NF-κB may be facilitated by phosphorylation of its p65 subunit at serine 536 residue located in the transactivation domain and the serine 276 residue present in the Rel homology domain [43,44]. Resveratrol has been shown to inhibit TPA-induced phosphorylation and degradation of IκBα, subsequent nuclear translocation of p65 protein, and phosphorylation of p65 at serine 536 and serine 276 residues in mouse skin *in vivo* [15]. The interaction of p65 with the coactivator cyclic AMP response element binding protein-binding protein (CBP) helps NF-κB have better access to the transcription initiation complex [44]. We have recently reported that resveratrol inhibits TPA-induced interaction of p65 with CBP in mouse skin [15].

The nuclear translocation of NF-κB that occurs independently of IκBα degradation has also been observed in various cultured cell lines [45,46]. According to this mechanism, certain stimuli, such as H_2O_2 or hypoxia followed by reoxygenation, may cause phosphorylation of IκBα at tyrosine residues rendering this protein resistant to proteasomal degradation but facilitating its dissociation from NF-κB [45,47,48]. Since resevartrol is known to inhibit tyrosine kinase [49,50], it is of further research interest whether resveratrol may abrogate TPA-induced NF-κB activation via modulation of IκBα phosphorylation at tyrosine residues in mouse skin *in vivo*.

3.2. *Resveratrol modulation of AP-1*

The transcription factor AP-1 can be produced by about 18 different dimeric combinations of basic leucine zipper (bZIP) proteins from the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) family, Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factor (ATF2, LRF1/ATF3 and B-ATF) subfamilies [51–53]. The Jun proteins can form stable dimers that bind to AP-1 DNA recognition elements (5′-TGAG/CTCA-3′), also known as TPA response element (TRE) [54]. However, Fos family proteins do not form stable homodimers but can bind DNA by forming heterodimers with Jun proteins, and the Jun:Fos heterodimers are more stable than Jun:Jun homodimers [55,56]. ATF proteins, on the other hand, are capable of forming both homodimers and heterodimers with Jun proteins that preferentially bind to cyclic AMP response element (CRE, 5′-TGACGTCA-3′). Our previous study has revealed that AP-1 activated by TPA exists in mouse skin mostly as a heterodimer of c-Jun and c-Fos proteins [38]. Previously, Jang and colleagues have reported that resveratrol inhibits *c-fos* mRNA expression in CD-1 mouse skin treated with TPA [13]. We have also found that pretreatment with resveratrol (1 μmol) inhibits TPA-induced AP-1 DNA binding (Fig. 1A). The specificity of AP-1 DNA binding has been verified by a supershift assay using c-Jun and c-Fos antibodies as well as cold competition by using both AP-1-specific and nonspecific-oligonucleotides (Fig. 1B). In addition, resveratrol inhibited nuclear expression of AP-1 component proteins, c-Jun and c-Fos, in TPA-stimulated mouse skin (Fig. 1C).

4. Regulation of NF-κB and AP-1 activation in TPA-stimulated mouse skin: role of upstream kinases

Fine tuning of intracellular signal transduction network regulating cell proliferation and differentiation is often disrupted in cancerous or transformed cells. The upstream kinases of the intracellular signaling network are activated by diverse stimuli provoking oxidative and pro-inflammatory stresses and transmit extracellular signals to the nucleus,

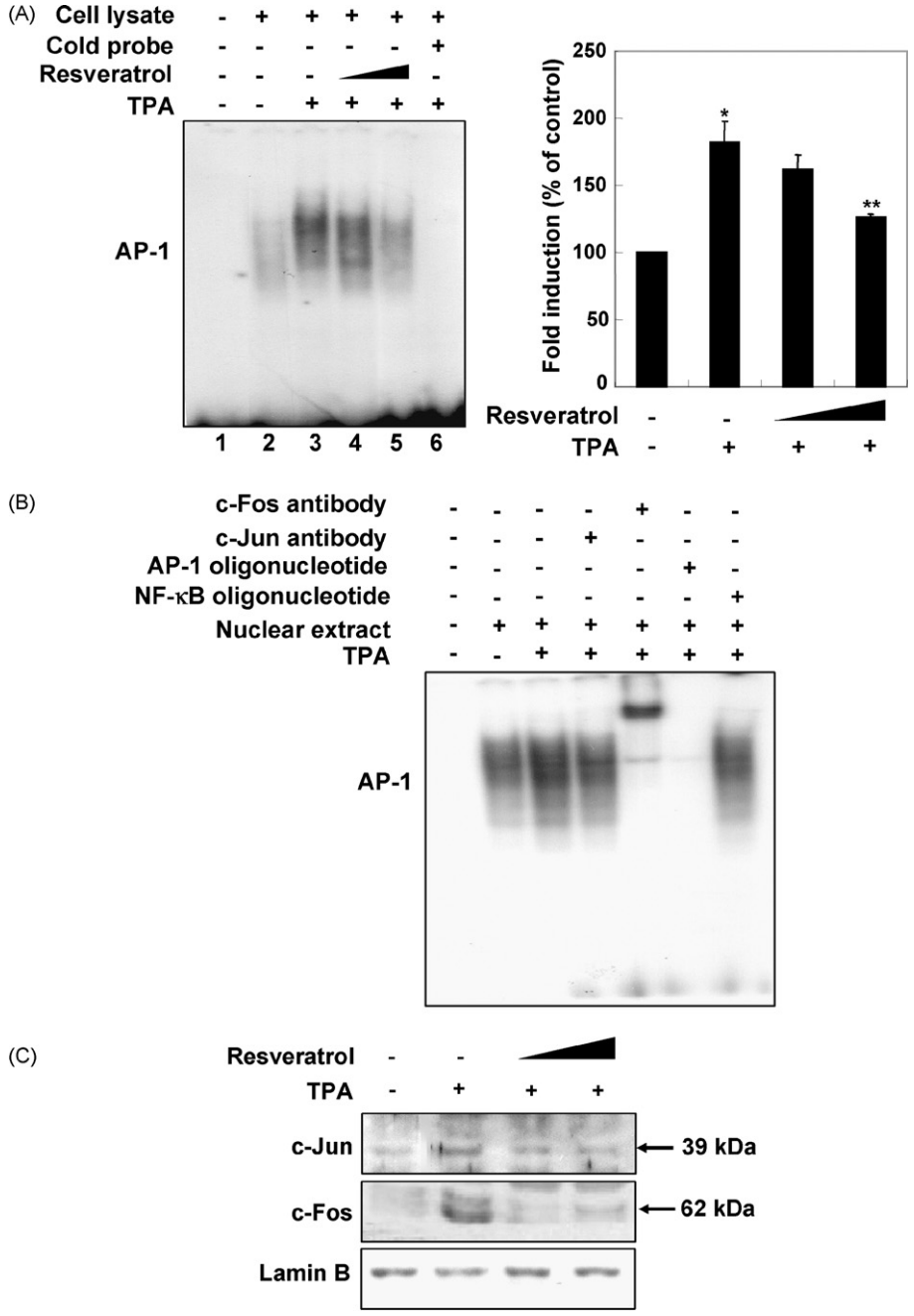

Fig. 1 – Inhibitory effects of resveratrol on phorbol ester-induced AP-1 activation in mouse skin. Female ICR mice ($n = 3$ per treatment group) were treated topically with resveratrol (0.25 or 1 μmol) dissolved in 0.2 ml acetone. After 30 min, mice were treated topically with 10 nmol TPA in 0.2 ml acetone. Control animals were treated with acetone in lieu of TPA. After 1 h of TPA treatment, mice were sacrificed by cervical dislocation and epidermal nuclear extract was prepared as described previously [15]. (A) Nuclear extracts (10 μg) from different treatment groups were incubated with [γ-^{32}P]-labelled AP-1 oligonucleotide (5′-CGC TTG ATG AGT CAG CCG GAA C-3′) and subjected to electrophoretic mobility gel shift assay (EMSA) as described earlier [15]. *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 plus 100-fold excess unlabeled oligonucleotide. $^{*}p < 0.001$ (control vs TPA alone), $^{**}p < 0.01$ (TPA only vs TPA plus 1.0 μmol resveratrol). (B) Specificity of AP-1 DNA binding in TPA-stimulated mouse skin was confirmed by supershift assay. Epidermal nuclear extract (10 μg) from TPA-treated animal was pre-incubated with primary antibodies for c-Jun and c-Fos, or with cold NF-κB olgonucleotide for 1 h at room temperature and then incubated for additional 50 min at room temperature after addition of [γ-^{32}P]-labelled AP-1 oligonucleotide. EMSA was performed following the protocol described previously [15]. (C) Inhibition of TPA-induced nuclear accumulation of AP-1 components by resveratrol. Nuclear protein (50 μg) was separated by 10% SDS–polyacrylamide gel, and expression levels of c-Jun and c-Fos were determined by immunoblot analysis as described earlier [15].

thereby activating a battery of transcription factors including NF-κB and AP-1 [35,42]. These cytoplasmic upstream kianses include mitogen-activated protein (MAP) kinases; protein kinase C (PKC); IκB kinase (IKK), phosphoinositide 3-kinase (PI3K); glycogen synthase kinase, Akt/protein kinase B (PKB); and tyrosine kinases. Topical application of TPA activated some of the aforementioned upstream kinases, and their pharmacological inhibition abrogated TPA-induced activation of NF-κB and AP-1 in mouse skin *in vivo* [38,40,57]. The role of specific upstream kinases in the activation of NF-κB and AP-1 in TPA-treated mouse skin will be addressed in the following sections.

4.1. *Role of MAP kinases in TPA-induced activation of NF-κB and AP-1 in mouse skin*

The MAP kinase family consists of three major enzymes including extracellular signal-regulated protein kinase (ERK), p38 MAP kinase and c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) [35]. According to our previous studies, there was a significant increase in the phosphorylation as well as catalytic activities of ERK and p38 MAP kinase in female ICR mouse skin after 1 h of TPA treatment [57]. Pretreatment of mouse skin with an MEK inhibitor U0126 negated TPA-induced phosphorylation (at serine 32/36 residue) and degradation of IκBα, DNA binding of NF-κB, and expression of COX-2 [57]. However, topical application of U0126 failed to affect the DNA binding of AP-1, suggesting that ERK may regulate TPA-induced COX-2 expression in mouse skin via an NF-κB, but not an AP-1, signaling pathway [38]. On the other hand, topical application of the p38 MAP kinase inhibitor SB203580 diminished TPA-induced AP-1 DNA binding, expression of AP-1 component proteins (c-Jun and c-Fos) and also COX-2 expression, suggesting that p38 MAP kinase plays a role in the TPA-induced COX-2 expression by activating the AP-1 signaling [38].

We previously reported that SB203580 did not cause any appreciable change in the DNA binding of NF-κB in TPA-treated mouse skin [57]. However, a recent study from our laboratory showed that topical application of this p38 MAP kinase inhibitor abrogated phosphorylation of IκBα and p65 (at serine 536 residue) in TPA-treated mouse skin [40]. Although the nuclear translocation of NF-κB is regarded as a critical event required for the activation of NF-κB-driven gene expression [58,59], it has been reported that inhibitors of several upstream kinases, such as PI3K/Akt, p38 MAP kinase and protein kinase A, may block the transcriptional activity of NF-κB without affecting its nuclear translocation [44,60–62]. Moreover, it has been suggested that the downregulation of NF-κB DNA binding activity is not necessarily associated with its reduced transcriptional activity [63,64]. Although the p38 MAP kinase inhibitor SB203580 failed to affect DNA binding of NF-κB, the inhibitory effects of SB203580 on TPA-induced phosphorylation of IκBα and p65, and the expression of COX-2 suggest that TPA-induced COX-2 expression in mouse skin is partly regulated by the p38 MAP kinase-NF-κB signaling pathway [40].

In another study, pretreatment with SP600125 (1 or 4 μmol), a specific inhibitor of JNK, abrogated TPA-induced activation of AP-1 and the expression of COX-2 in mouse skin *in vivo* (J.-C. Lee, J.K. Kundu and Y.-J. Surh, submitted), suggesting that TPA-induced COX-2 expression in mouse skin can also be mediated via the JNK-AP-1 signaling pathway.

4.2. *Role of IKK in TPA-induced activation of NF-κB and AP-1 in mouse skin*

IKK exists as a complex, commonly known as IKK signalsome, consisting of three subunits IKKα, IKKβ and IKKγ. Of the three subunits, IKKα and IKKβ act as catalytic subunits, while IKKγ (also known as NF-κB essential modulator, NEMO) acts as a regulatory subunit [65]. Initially, it has been suggested that both IKKα and IKKβ play similar roles in phosphorylating IκBα and activating NF-κB in cultured cells [66]. However, subsequent studies have shown that IKKβ, but not IKKα, is a target for pro-inflammatory stimuli and plays a major role in NF-κB activation [67,68]. The IKK complex has been reported to regulate the phosphorylation of both IκBα and NF-κB [69,70]. However, 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 [71]. We attempted to elucidate the role of IKK in regulating NF-κB activation and COX-2 expression in mouse skin stimulated with TPA. An *in vitro* radioactive kinase assay revealed that TPA stimulated both IKKα and IKKβ activity in mouse skin in as early as 30 min, which persisted up to 2 h after the TPA treatment [15]. The stimulation of IKKα/β activity by TPA was attenuated by co-treatment of mouse skin with Bay 11-7082, a pharmacological inhibitor of IKK. In agreement with previous studies demonstrating the inhibitory effect of Bay 11-7082 on NF-κB activation [72–74] and COX-2 expression [75,76] in cultured cell lines, our study has revealed that topically applied Bay 11-7082 (0.05 or 0.25 μmol) suppresses TPA-induced phosphorylation and degradation of IκBα, nuclear translocation of p65, DNA binding of NF-κB, and expression of COX-2 [15], suggesting a regulatory role of IKKα/β on NF-κB activation and COX-2 expression in TPA-stimulated mouse skin *in vivo*.

Although NF-κB and AP-1 are regulated by different mechanisms, a number of reports have shown that these transcription factors are activated simultaneously by the same multitude of stimuli [39,77–79]. Stein et al. [80] have reported that the AP-1 activity is strikingly enhanced when NF-κB subunits are present and *vice versa*. According to this study, the bZIP regions of c-Fos and c-Jun physically interact with p65 through the Rel homology domain and the complex of p65 with Jun or Fos exhibits enhanced DNA binding via both κB and AP-1 response elements. Moreover, the p65 subunit of NF-κB has been reported to act as an accessory protein for the serum response factor, which is upstream of AP-1, in transfection assays [81]. Since NF-κB and AP-1 are activated simultaneously in TPA-treated mouse skin [39], there is intriguing possibility that both transcription factors may have a common upstream regulator.

Several recent studies have reported the IKK regulation of AP-1 activation *in vitro* [82,83]. Krappmann et al. [83] have demonstrated that NF-κB is required for LPS-induced AP-1 activation in preB cells and primary dendritic cells. According to this study, the LPS-induced AP-1 DNA binding was completely abolished in primary dendritic cells transfected

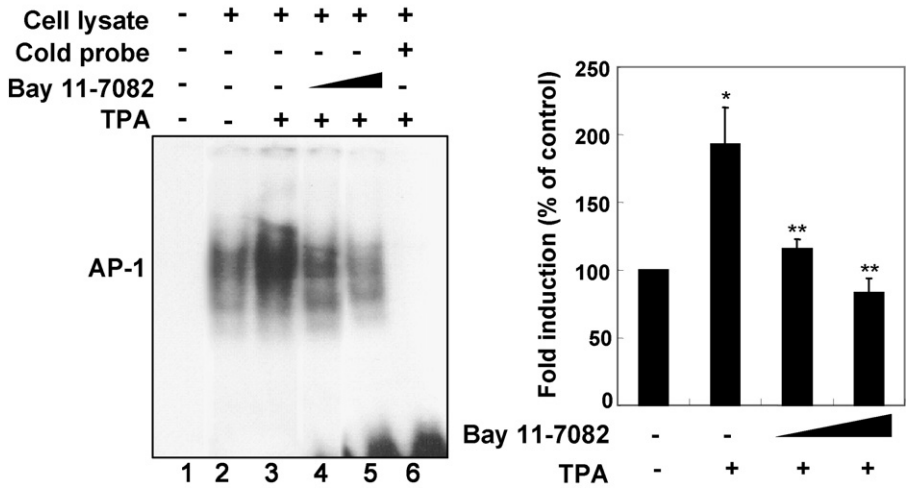

Fig. 2 – Effects of Bay 11-7082, an inhibitor of IKK, on phorbol ester-induced activation of AP-1 in mouse skin *in vivo*. Shaven backs of female ICR mice (*n* = 3 per treatment group) were treated with TPA (10 nmol) in presence or absence of Bay 11-7082 (0.05 or 0.25 μmol) following a co-treatment protocol. Control animals were treated with acetone only. One hour after the treatment of TPA, the epidermal nuclear extracts were prepared according to a method described previously [15] and subjected to EMSA using [γ-^{32}P]-labeled AP-1 oligonucleotide. *Lane* 1, free probe alone (no nuclear extracts); *Lane* 2, acetone control; *Lane* 3, TPA alone; *Lane* 4, Bay 11-7082 (0.05 μmol) + TPA; *Lane* 5, Bay 11-7082 (0.25 μmol) + TPA; *Lane* 6, TPA treated sample plus 100-fold excess unlabeled oligonucleotide. $^{*}p < 0.05$ (control vs TPA alone), $^{**}p < 0.05$ (TPA only vs TPA plus Bay 11-7082).

with dominant negative IκBα, as compared to the mock control [83]. In a recent study, Fujioka et al. [82] also demonstrated that knockout of IKKβ, but not IKKα, attenuated serum- or superoxide anion-induced AP-1 DNA binding in the human pancreatic tumor cell line MDAPanc-28. In our study, topically applied Bay 11-7082 (an IKK inhibitor) significantly attenuated TPA-induced DNA binding of AP-1 (Fig. 2), which raises the possibility of IKK regulation of AP-1 DNA binding in TPA-treated mouse skin *in vivo*. However, it is not clear yet whether the inhibitory effect of Bay 11-7082 on TPA-induced AP-1 DNA binding is a direct effect of IKK inhibition or not. It is noticeable that one of the subunits of IKK complex, i.e., IKKε phosphorylates c-Jun in fibroblast-like synoviocytes [84]. The specific role of different subunits of IKK (e.g., IKKα, IKKβ, IKKγ, IKKε) in TPA-induced AP-1 activation in mouse skin merits further investigation.

5. Inhibitory effects of resveratrol on TPA-induced activation of upstream kinases in mouse skin

Since TPA-induced COX-2 expression is primarily mediated via signaling through NF-κB and AP-1, and the activation of these transcription factors is under the regulation of a set of upstream kinases [38,57], we investigated the effect of resveratrol on the activation of MAP kinases and IKKα/β in mouse skin treated with TPA. We have reported that resveratrol (1 μmol) diminished TPA-induced catalytic activities of IKKα/β [15], and the phosphorylation and catalytic activities of p38 MAP kinase [14,15] in mouse skin. In another experiment, topical application of resveratrol attenuated TPA-induced phosphorylation of JNK in mouse skin as determined by both immunoblot (Fig. 3A) and immunohistochemical analysis (Fig. 3B and C), suggesting that resveratrol may target AP-1 via modulation of p38 MAP kinase and JNK in TPA-stimulated mouse skin *in vivo*. Resveratrol has also been reported to inhibit the UVB-induced activation of MEK in SKH-1 hairless mouse skin [31].

6. Future perspective

The mouse skin carcinogenesis model offers an excellent tool for the study of molecular mechanisms underlying chemoprevention with a wide variety of anti-inflammatory phytochemicals. Central to the cancer biology is the tumor promoter-induced disruption of intracellular signaling network comprising diverse classes of upstream kinases and their downstream transcription factors that regulate expression of genes encoding proteins involved in cellular proliferation and inflammation [42]. One of the molecular targets for chemoprevention with dietary phytochemicals as well as synthetic pharmaceuticals is COX-2, which is induced in response to pro-inflammatory and mitogenic stimuli. The inflammatory signal transduction, whose aberrant activation leads to inappropriate induction of COX-2, may act as a road map in searching for promising chemopreventive agents or strategies [85]. Studies from our laboratory have focused on exploring the cellular signaling cascades involved in TPA-induced COX-2 expression in mouse skin *in vivo* and their modulation by anti-inflammatory substances, such a resveratrol [15], curcumin [57], celecoxib [38], [6]-gingerol [40], epigallocatechin gallate [86], etc. that have antitumor promoting activities as well. As addressed in the previous sections of this commentary, resveratrol has been shown to

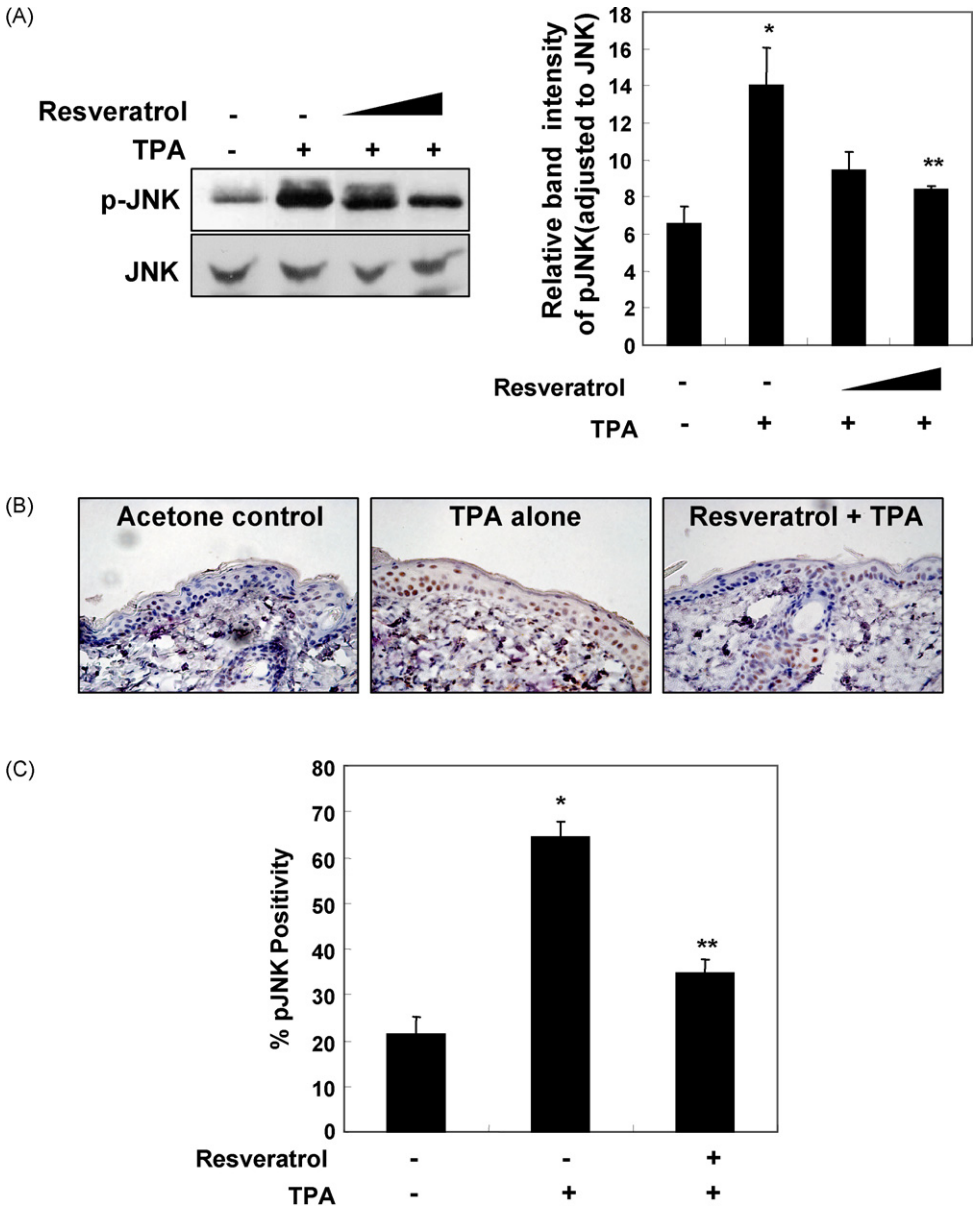

Fig. 3 – Inhibitory effects of resveratrol on phorbol ester-induced activation of JNK in mouse skin *in vivo*. Shaven backs of female ICR mice ($n = 3$ per treatment group) were treated either with acetone or resveratrol (0.25 or 1 μmol) 30 min prior to TPA (10 nmol) except control animals, which were treated with acetone only. Animals were sacrificed after 1 h of TPA treatment. (A) Whole cell lysate (50 μg) was separated by 12% SDS–polyacrylamide gel and immunoblot was performed by using primary antibodies specific to detect phospho-JNK and JNK. $^{*}p < 0.01$ (control vs TPA alone); $^{**}p < 0.025$ (TPA only vs TPA plus 1 μmol resveratrol) according to the protocol described previously [15]. (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 mouse monoclonal phospho-JNK antibody as described by Kundu et al. [15] with minor modifications in an antigen retrieval technique, which was performed by using pressure cooker instead of microwave. Positive phospho-JNK staining yielded a brown-coloured product. (C) Percent of phospho-JNK positivity in epidermal layer was determined by counting the number of total and pJNK positive cells from 10 equal sections of immunostained tissues from each animal. $^{*}p < 0.001$ (control vs TPA alone); $^{**}p < 0.001$ (TPA only vs TPA plus 1 μmol resveratrol).

inhibit several key components of signal transduction pathways, thereby blocking inappropriate induction of COX-2 and tumorigenesis in TPA-stimulated mouse skin (Fig. 4).

Although resveratrol suppresses TPA-induced COX-2 expression in mouse skin by blocking the activation of NF-κB and AP-1 via modulation of upstream IKK and MAP kinases, its possible inhibitory effect on TPA-induced activation of other kinases, such as PI3K/Akt or different isoforms of PKC, and other *cox*-2 regulatory transcription factors including C/EBP and CREB in mouse skin cannot be ruled out. Several recent studies have demonstrated that ERK and p38 MAP kinase act upstream of IKKs in tumor necrosis factor-α-

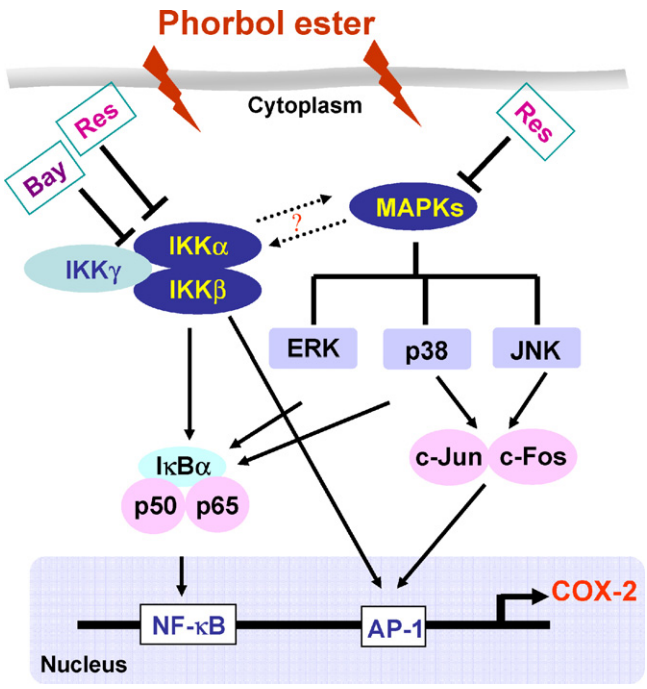

Fig. 4 – A model diagram representing resveratrol modulation of TPA-induced signal transduction pathways in mouse skin *in vivo*. Res and Bay denote resveratrol and Bay 11-7082, respectively.

induced activation of NF-κB *in vitro* [87,88]. Chen et al. have demonstrated that the activation of IKK and NF-κB in human alveolar epithelial cell A549 by interleukin-1β or TPA is mediated via PKCα signaling, but not by MAP kinases [89]. Regarding signal transduction pathways involved in TPA-induced COX-2 expression in mouse skin, we are still exploring a link between the activation of IKKs and MAP kinases.

Beyond COX-2 signaling, other signaling pathways including those mediated by signal transducers and activators of transcription (STAT) [90] and the activation of β-catenin-mediated signaling [91] have also been implicated in TPA-promoted mouse skin tumorigenesis. Further studies will be necessary to determine whether resveratrol could modulate any of these additional signal transduction pathways.

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