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Thymoquinone inhibits phorbol ester-induced activation of NF-κB and expression of COX-2, and induces expression of cytoprotective enzymes in mouse skin *in vivo*

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ABSTRACT

Thymoquinone (TQ), the active ingredient of *Nigella sativa*, has been reported to possess anti-inflammatory and chemopreventive properties. The present study was aimed at elucidating the molecular mechanisms of anti-inflammatory and antioxidative activities of thymoquinone in mouse skin. Pretreatment of female HR-1 hairless mouse skin with TQ attenuated 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced expression of cyclooxygenase-2 (COX-2). TQ diminished nuclear translocation and the DNA binding of nuclear factor-kappaB (NF-κB) via the blockade of phosphorylation and subsequent degradation of IκBα in TPA-treated mouse skin. Pretreatment with TQ attenuated the phosphorylation of Akt, c-Jun-N-terminal kinase and p38 mitogen-activated protein kinase, but not that of extracellular signal-regulated kinase-1/2. Moreover, topical application of TQ induced the expression of heme oxygenase-1, NAD(P)H-quinoneoxidoreductase-1, glutathione-*S*-transferase and glutamate cysteine ligase in mouse skin. Taken together, the inhibitory effects of TQ on TPA-induced COX-2 expression and NF-κB activation, and its ability to induce the expression of cytoprotective proteins provide a mechanistic basis of anti-inflammatory and antioxidative effects of TQ in hairless mouse skin.

1. Introduction

Oxidative stress and inflammation are the key factors for neoplastic transformation of cells [1]. Thus, the blockade of abnormally activated inflammatory signaling pathways and fortification of cellular antioxidative defense capacity can protect against inflammatory and oxidative tissue damage and hence prevent cancer. One of the key enzymes mediating inflammatory signal transduction is cyclooxygenase-2 (COX-2), which catalyzes the rate-limiting step in the biosynthesis of prostaglandins. Elevated expression of COX-2 in human skin cancers [2], the increased susceptibility of *cox-2*-overexpressing mice to experimentally induced skin papillomagenesis [3,4], the reduced incidence and the multiplicity of skin tumors in *cox-2* knockout mice [5], and the protective effects of selective COX-2 inhibitors on experimentally induced mouse skin carcinogenesis [2] suggest that aberrant COX-2 induction is a key event in the course of skin carcinogenesis. The expression of COX-2 is transiently elevated in response to a wide array of inflammatory stimuli including cytokines, ultraviolet radiation, bacterial lipopolysaccharide (LPS), phorbol ester, etc. [6]. The induction of COX-2 is transcriptionally regulated by various redox-sensitive transcription factors including nuclear factor-kappaB (NF-κB) [7,8]. Inappropriate amplification of cell signaling pathways comprising various upstream kinases, such as mitogen-activated protein (MAP) kinases and Akt, activates NF-κB and induces the expression of COX-2 [6]. The modulation of cell signaling pathways involved in aberrant COX-2 expression is, therefore, a rational strategy to prevent inflammation and cancer [9]. On the other hand, cells are equipped with a battery of antioxidant or phase 2 detoxification enzymes, collectively known as cytoprotective proteins, which include heme oxygenase-1 (HO-1), NAD(P)H:quinoneoxidoreductase-1 (NQO1), glutamate cysteine ligase (GCL) and glutathione-*S*-transferase (GST). The induction of these cytoprotective proteins can confer protection against oxidative stress and inflammation, thereby preventing cancer [10].

Thymoquinone (TQ, Fig. 1A) is an active ingredient of a commonly used spice black cumin (*Nigella sativa*). TQ has been reported to possess antioxidative [11], anti-inflammatory [12] and chemopreventive properties [13,14]. TQ selectively induced apoptosis in various cancer cells [15] and attenuated the growth of xenograft tumors in nude mice [16]. Administration of TQ in

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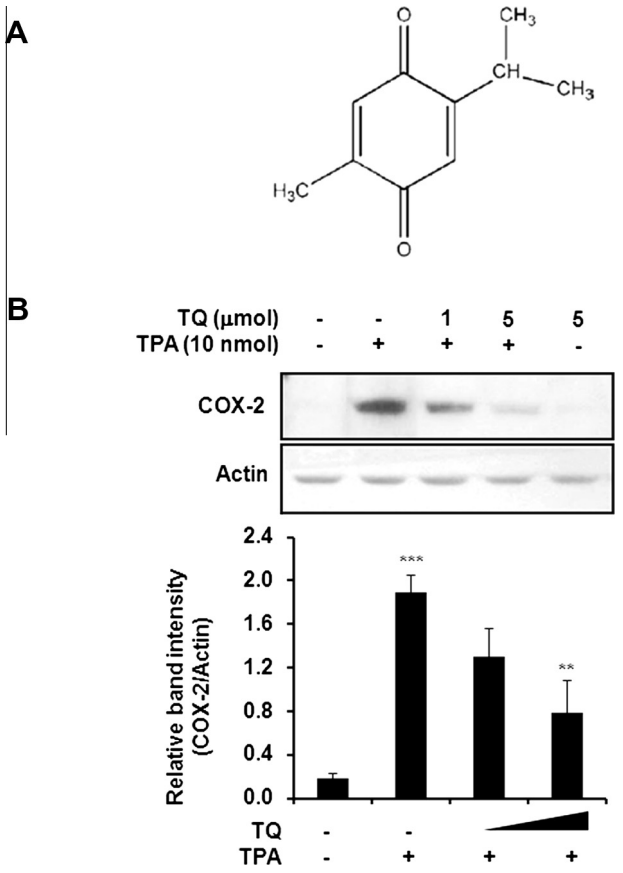

Fig. 1. Effects of TQ on TPA-induced COX-2 expression in mouse skin. (A) Chemical structure of TQ. (B) Female HR-1 hairless mice (three per treatment group) were treated topically with TQ at a dose of 1 or 5 μmol dissolved in 0.2 ml acetone. After 30 min, mice were treated topically with 0.2 ml acetone or 10 nmol TPA, and killed 6 h later. Control animals were treated with acetone in lieu of TPA. Epidermal COX-2 expression in different treatment groups was detected by immunoblotting. Data are representative of three independent experiments. $^{**}p < 0.01$ (TPA alone versus TQ 5 μmol plus TPA); $^{***}p < 0.001$ (control versus TPA alone).

drinking water suppressed benzo[α]pyrene-induced forestomach tumors [14] and 3-methylcholanthrene-induced fibrosarcoma in mice [17]. Intratumoral administration of TQ reduced the growth of squamous cell carcinoma cells injected subcutaneously to nude mice [18]. Moreover, topical application of the TQ-rich fraction of *N. sativa* extract inhibited 7,12-dimethylbenz[a]anthracene-initiated and croton oil-promoted mouse skin papillomagenesis [19]. However, the effect of TQ on epidermal inflammation and its underlying molecular mechanisms have not been fully clarified. In the present study, we investigated the effects of TQ on COX-2 expression in mouse skin treated with a prototype tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and also on the expression of epidermal cytoprotective proteins.

2. Materials and methods

2.1. Materials

TQ (purity 99%) was purchased from Sigma–Aldrich (St Louis, MO, USA). Rabbit polyclonal COX-2 antibody was procured from Cayman Chemical Co. (Ann Arbor, MI, USA). TPA was obtained from Alexis Biochemicals (San Diego, CA, USA). Primary antibodies for extracellular signal-regulated kinase-1/2 (ERK1/2), phospho-ERK1/2, c-Jun-N-terminal kinase (JNK), p38 MAP kinase, NQO-1, GCL, GST, p50, p65, IκBα, and phospho-IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for phospho-JNK and phospho-p38 MAP kinase were obtained from BD Biosciences (San Jose, CA, USA). Anti-actin antibody was purchased from Sigma Chemical Company (St Louis, MO, USA). HO-1 antibody was from Assay Designs Inc.-Stressgen (Ann Arbor, MI, USA). Antibodies for Akt and phospho-Akt were from Cell Signaling Technology (Danvers, MA, USA). The anti-rabbit, anti-mouse and anti-goat horseradish peroxidase conjugated secondary antibodies were obtained from Zymed Laboratories (San Francisco, CA, USA). Enhanced chemiluminescence (ECL) detection kit and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Animal treatment

Female HR-1 hairless mice (6–7 weeks of age) were purchased from Sankyo Laboservice Corporation Inc. (SLC, Tokyo, Japan). Animals were housed in climate-controlled quarters (24 °C at 50% humidity) with a 12-h light/12-h dark cycle. TQ was dissolved in acetone. Animals were topically treated with TQ (1 or 5 μmol in 0.2 ml) 30 min before application of TPA (10 nmol). All experimental protocols were approved by the Animal Care and Use Committee (ACUC) of Seoul National University, South Korea (permit number: SNU-090324-3).

2.3. Western blot analysis

Dorsal skins of mice were topically treated with TQ (1 or 5 μmol) 30 min before TPA (10 nmol) application, and mice were killed by cervical dislocation either 3 or 6 h later. The dorsal skin was excised, fat and dermis were removed on ice, and the collected epidermis was pulverized with mortar and pestle in liquid nitrogen. The pulverized skin was homogenized on ice for 20 s with a Polytron tissue homogenizer 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 DTT, 1 mM Na_3VO_4 and protease inhibitor cocktail tablet]. Lysates were centrifuged at 14,800×*g* for 15 min. Supernatant was collected and total protein concentration was quantified by using the bichinconinic acid protein assay kit. Cell lysates (30–50 μg protein) were boiled in sodium dodecylsulfate (SDS) sample buffer for 5 min before electrophoresis on 10–12% SDS–polyacrylamide gel. After transfer to polyvinylidene difluoride membrane, the blots were blocked with 5% fat-free dry milk–PBST buffer (phosphate-buffered saline containing 0.1% Tween-20) or 1% bovine serum albumin in TBST (Tris-buffered saline containing 0.1% Tween-20) for 1 h at room temperature. Membranes were incubated for 12 h at 4 °C with 1:1000 dilutions of primary antibodies for COX-2, phospho-ERK -1/2, phospho-JNK, IκBα, phospho-IκBα, p65, p50, NQO1, GCL and GST and for 2 h at room temperature with 1:4000 dilutions of primary antibody for HO-1, β-actin, ERK -1/2, p38 MAP kinase, JNK and Akt. Blots were washed three times with 1× TBST buffer at 5 min intervals followed by incubation with 1:5000 dilution of horseradish peroxidase conjugated rabbit or mouse secondary antibodies for 1 h, and again washed with 1× TBST buffer for three times. The transferred proteins were visualized with an ECL detection kit according to the manufacturer's instructions.

2.4. Preparation of cytosolic and nuclear extracts from mouse skin

The cytoplasmic and nuclear extracts from mouse skin were prepared as described previously [20,21]. In brief, scraped dorsal skin 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, 0.1 mM phenylmethylsulfonylfluoride]. To the homogenates was added 80 μl of 10% Nonidet P-40 (NP-40) solution, and the mixture

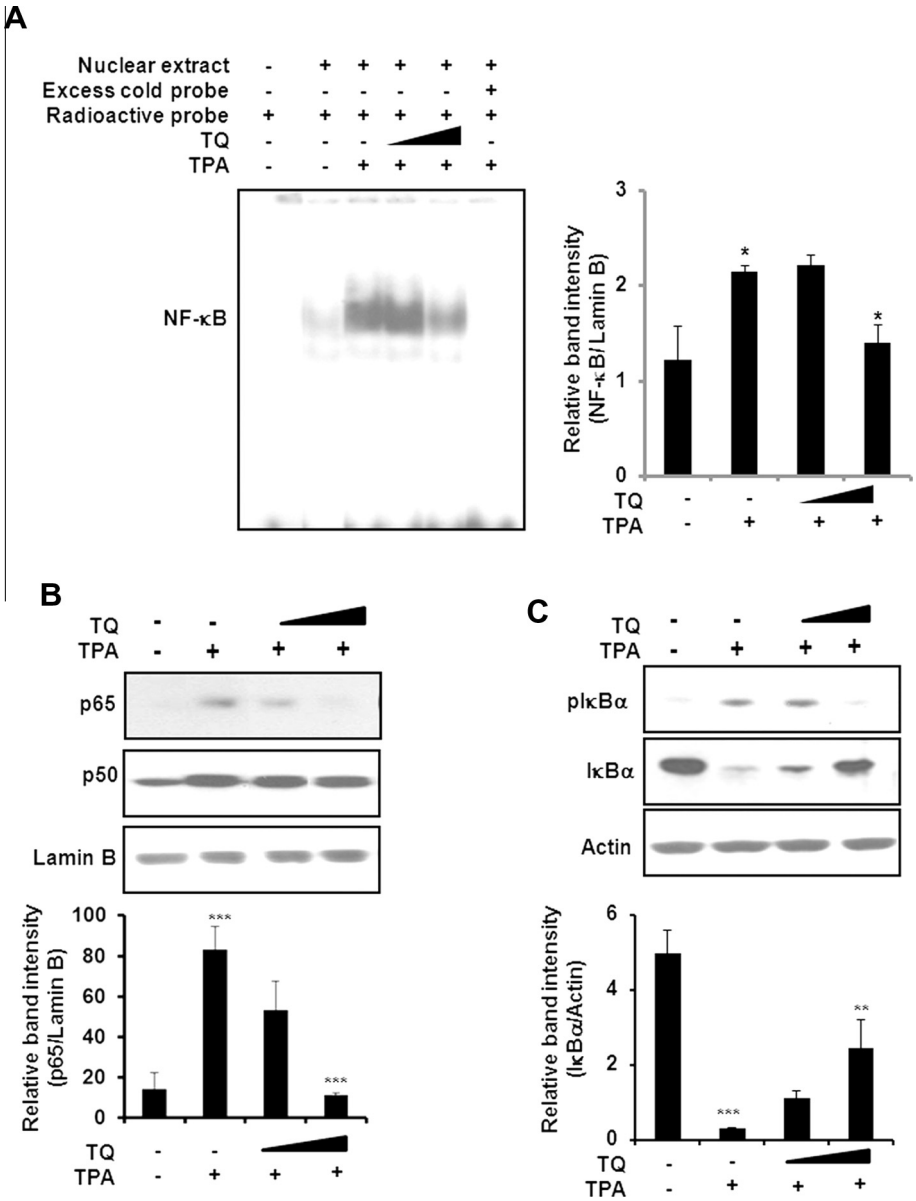

Fig. 2. Effects of TQ on TPA-induced NF-κB activation in mouse skin. Dorsal skin of female HR-1 hairless mice was treated with TPA 30 min after topical application of TQ (1 or 5 μmol). Control animals were treated with acetone alone. After 3 h of TPA application, animals were sacrificed. (A) The epidermal nuclear extracts were prepared and incubated with the radiolabelled oligonucleotides containing κB consensus sequence for the analysis of NF-κB DNA binding by EMSA. Bars indicate the mean values obtained from triplicate experiments with their standard error of mean (S.E.M.). $^{*}p < 0.05$ (acetone control versus TPA alone), $^{**}p < 0.01$ (TPA alone versus TQ 5 μmol plus TPA). (B) The nuclear extracts were subjected to western blot analysis to examine the levels of p50 and p65 expression. $^{***}p < 0.001$ (acetone control versus TPA alone; TQ 5 μmol plus TPA versus TPA alone). (C) The cytosolic extract was analyzed by western blotting to examine the expression of pIκBα and IκBα. $^{***}p < 0.001$ (control versus TPA alone), $^{**}p < 0.01$ (TPA alone versus TQ 5 μmol plus TPA).

was then centrifuged for 2 min at 14,000×*g*. The supernatant was collected as the cytosolic fraction. The precipitated nuclei were washed twice 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, 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 by using the Bradford protein assay (Bio-rad laboratories, CA, USA).

2.5. Electrophoretic mobility gel shift assay (EMSA)

The EMSA for the NF-κB DNA binding was performed using a DNA–protein binding detection kit according to the manufacturer's protocol (GIBCO BRL, Grand Island, NY, USA). Briefly, oligonucleotides harboring binding sites for NF-κB (5′-GATCGAGGG GGACTTTCCCAGC-3′) was labeled with [γ-^{32}P] ATP by T_4 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 cpm 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 X-ray film.

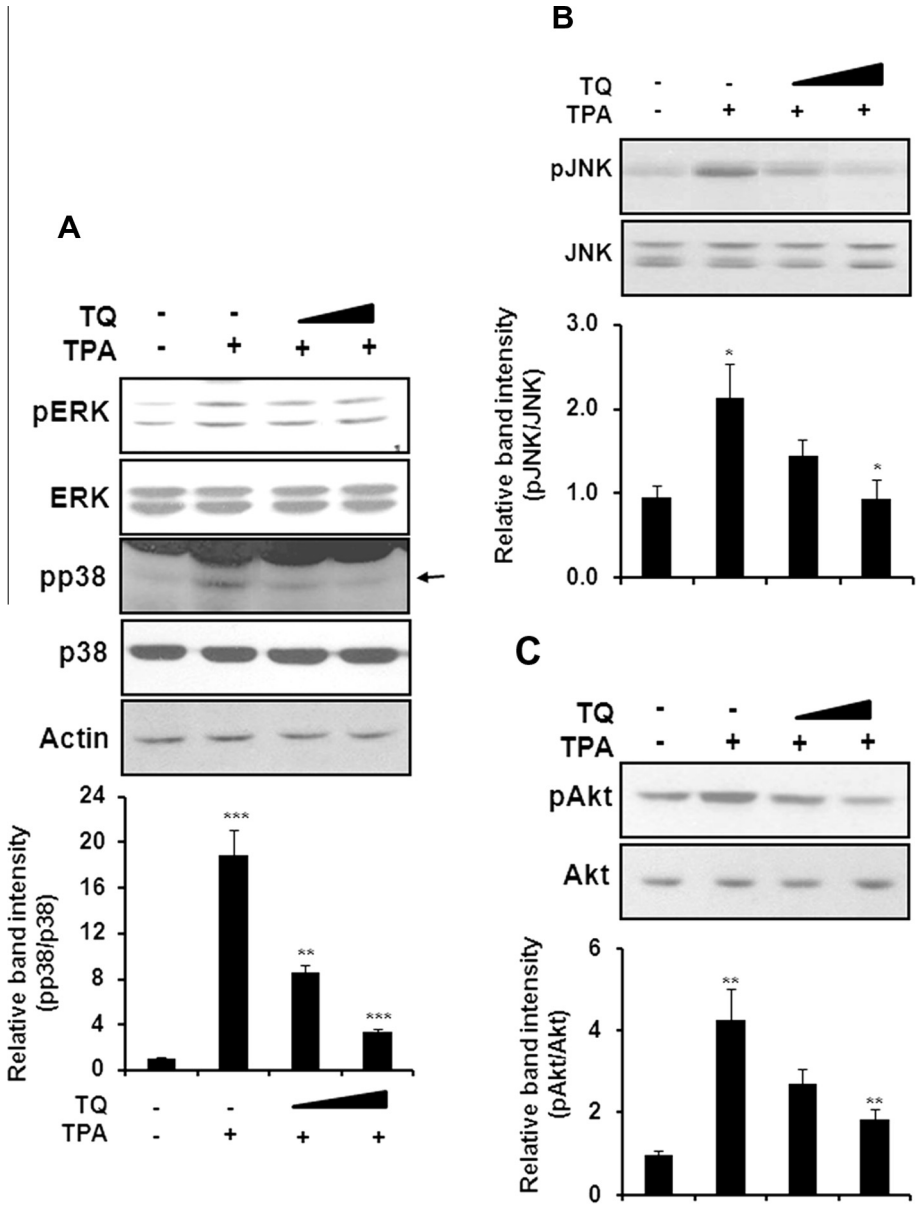

Fig. 3. Effects of TQ on TPA-induced activation of MAP kinases and Akt in mouse skin. Female HR-1 hairless mice ($n = 3$ per treatment group) were treated topically with TQ (1 or 5 μmol) 30 min prior to application of TPA (10 nmol) and sacrificed 3 h after. Control animals were treated with acetone in lieu of TPA. Epidermal lysates were analyzed for the expression of phosphorylated upstream kinases. (A) Effects of TQ on TPA-induced phosphorylation of ERK1/2 and p38 MAP kinase. $^{***}p < 0.001$ (acetone control versus TPA alone; TQ 5 μmol plus TPA versus TPA alone). $^{**}p < 0.01$ (TQ 1 μmol plus TPA versus TPA alone). (B) Inhibitory effect of TQ on TPA-induced phosphorylation of JNK. $^{*}p < 0.05$ (acetone control versus TPA alone; TQ 5 μmol plus TPA versus TPA alone). (C) Effect of TQ on the phosphorylation of Akt in TPA-treated mouse skin. $^{**}p < 0.01$ (acetone control versus TPA alone; TQ 5 μmol plus TPA versus TPA alone).

2.6. Statistical evaluation

Values were expressed as the mean ± S.E.M. of at least three independent experiments. Statistical significance was determined by Student's *t*-test and a *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. TQ inhibits TPA-induced COX-2 expression in mouse skin

We have previously reported that topical application of TPA (10 nmol) induces the expression of COX-2 in mouse skin at 6 h [22]. In an attempt to examine the effect of TQ on TPA-induced COX-2 expression, we topically applied TQ (1 or 5 μmol) to dorsal skin of female HR1-hairless mouse 30 min prior to the application of TPA (10 nmol). Immunoblot analysis of epidermal COX-2 expression revealed that pretreatment with TQ significantly ($p < 0.05$) inhibited TPA-induced COX-2 expression (Fig. 1B). Treatment with TQ alone did not induce COX-2 expression in mouse epidermis.

3.2. TQ attenuates TPA-induced activation of NF-κB in mouse skin

Since the 5′-flanking region of COX-2 gene promoter contains a binding sequence for NF-κB [7,23], we examined the effects of TQ on TPA-induced DNA binding of this transcription factor in mouse skin *in vivo*. Nuclear extracts obtained from TPA-treated mouse epidermis, with or without pretreatment of TQ, were subjected to EMSA. As shown in Fig. 2A, TQ, at a dose of 5 μmol, significantly inhibited TPA-induced DNA binding of NF-κB. TQ attenuated TPA-induced nuclear translocation of p65/RelA, but

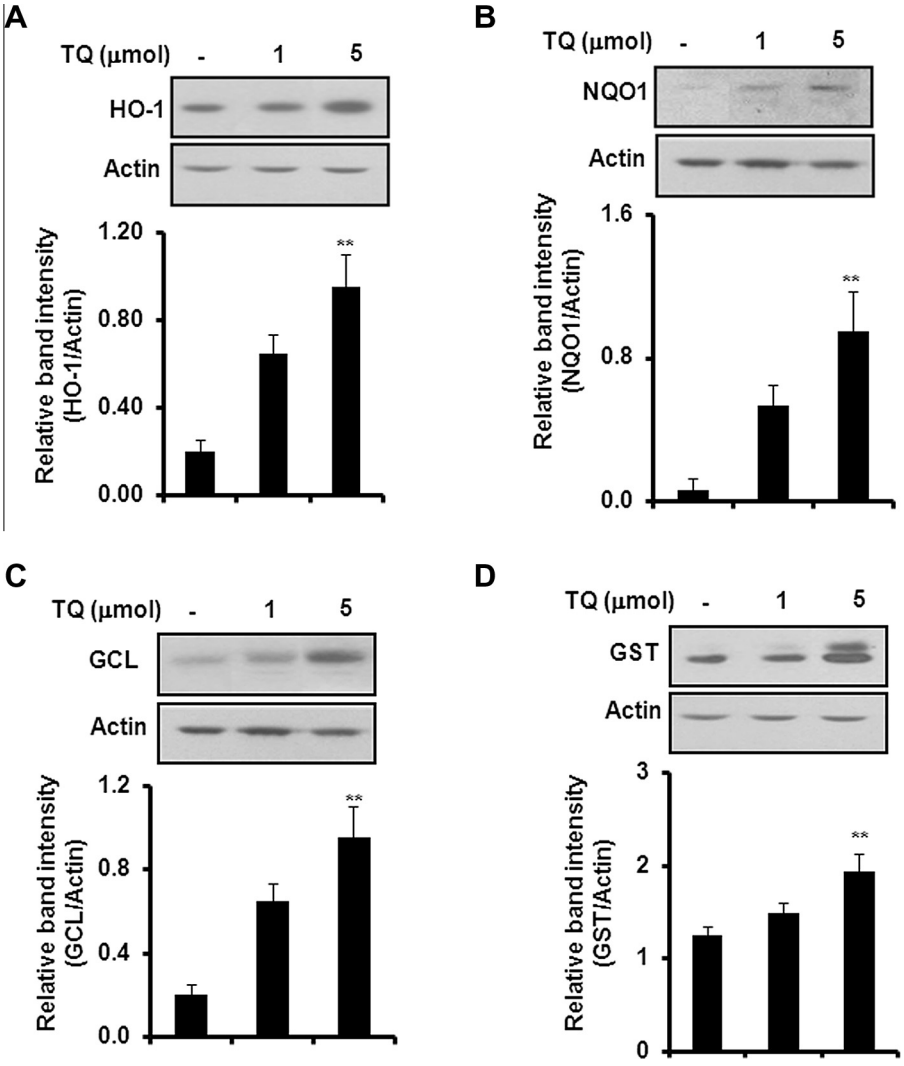

Fig. 4. Effects of TQ on the expression of cytoprotective proteins in mouse skin. Female HR-1 hairless mice (three per treatment group) were treated topically with TQ (1 or 5 μmol) for 6 h. Whole tissue lysates were prepared and analyzed for the expression levels of cytoprotective proteins by western blot analysis. Bars indicate the mean values obtained from triplicate experiments with their S.E.M. $^{*}p < 0.05$ (acetone control versus 5 μmol TQ).

not p50, which are the active subunits of NF-κB (Fig. 2B). TPA has been reported to induce phosphorylation and subsequent degradation of IκBα, thereby activating NF-κB in mouse epidermis [20,22]. Treatment with TQ diminished the phosphorylation and subsequent degradation of IκBα in TPA-stimulated mouse skin (Fig. 2C).

3.3. TQ inhibits TPA-induced phosphorylation of p38 MAP kinase, JNK and Akt

A panel of upstream serine/threonine kinases is known to regulate the activation of NF-κB and expression of COX-2 in TPA-treated mouse skin [20–22]. We first examined the effect of TQ on the phosphorylation of MAP kinases. Pretreatment with TQ attenuated TPA-induced phosphorylation of p38 MAP kinase, but failed to inhibit phosphorylation of ERK1/2 (Fig. 3A). In addition, TQ diminished the phosphorylation of JNK in TPA-treated mouse skin (Fig. 3B). Since the phosphorylation of Akt enhances the DNA binding of NF-κB and elevated the expression of COX-2 in mouse skin [22], we examined whether TQ can modulate Akt activation in TPA-stimulated mouse skin. As shown in Fig. 3C, pretreatment of mouse skin with TQ inhibited TPA-induced phosphorylation of Akt.

3.4. TQ induces the expression cytoprotective enzymes in mouse skin

Since cytoprotective enzymes can attenuate oxidative and inflammatory tissue damage [10], we examined the effect of TQ (1 or 5 μmol) on the expression of some representative cytoprotective enzymes in mouse skin. Topical application of TQ (5 μmol) significantly increased the expression of HO-1 (Fig. 4A), NQO1 (Fig. 4B), GCL (Fig. 4C) and GST (Fig. 4D) in mouse epidermal tissue.

4. Discussion

Herbs and spices have long been used as traditional medicines. A wide variety of spice-derived phytochemicals with antioxidative and anti-inflammatory properties have been reported to exert cancer chemopreventive effects [24]. TQ has been identified as an active principle of black cumin, a spice with a long history of medicinal use. TQ is known to scavenge superoxides, suppress inflammation and inhibit experimentally induced carcinogenesis [11,12,14,16]. In the present study, we attempted to elucidate the mechanisms underlying its anti-inflammatory and antioxidant activities.

The present study is the first demonstration of the inhibitory effect of TQ on COX-2 expression in TPA-treated mouse skin *in vivo*. Our finding is in good agreement with previous studies reporting that TQ inhibits the *in vitro* COX-2 activity [12] and constitutive

expression of COX-2 protein [25] and its mRNA transcript [26] in pancreatic cancer cells. The expression of COX-2 in TPA-treated mouse skin involves inappropriate activation of intracellular signal transduction pathways comprising proline-directed serine/threonine kinases, and their downstream transcription factors [6,20]. The promoter region of murine *cox-2* contains binding sites for various transcription factors including NF-κB and activator protein-1 (AP-1) [27]. The inhibitory effect of TQ on the phosphorylation and degradation of IκBα, and nuclear translocation and the DNA binding of NF-κB suggest that the compound suppresses COX-2 expression in TPA-treated mouse skin, at least in part, by targeting NF-κB signaling. Several other studies have also reported the inhibitory effect of TQ on the constitutive and tumor necrosis factor-α-induced NF-κB activation in pancreatic cancer cells [25,26]. In contrast, TQ failed to inhibit LPS-induced IκBα degradation and NF-κB activation in peritoneal macrophages isolated from streptozotocin-induced diabetic rats [28]. While the latter study demonstrated that TQ inhibited LPS-induced phosphorylation of ERK1/2, our study revealed that TQ attenuated TPA-induced phosphorylation of JNK and p38 MAP kinase, but not that of ERK1/2. Thus, TQ may modulate MAP kinase signaling in a stimuli-specific manner.

Of the MAP kinase family members, ERK1/2 [21] and p38 MAP kinase [29] predominantly regulate the activation of NF-κB, while JNK [30] and p38 MAP kinase [31] regulate AP-1 DNA binding in TPA-treated mouse skin. Since TQ attenuated the phosphorylation of p38 MAP kinase and JNK, the possibility of the inhibition of AP-1 DNA binding by TQ in TPA-stimulated mouse skin cannot be ruled out. We previously reported that inhibition of Akt signaling by its pharmacological inhibitor LY294002 abrogated TPA-induced NF-κB activation and COX-2 expression in mouse skin [22]. Thus, the attenuation of TPA-induced phosphorylation of Akt by TQ partly accounts for its inhibitory effects on NF-κB activation and COX-2 expression.

Reinforcement of body's antioxidant arsenal is the foremost strategy to prevent oxidative stress-induced chronic inflammatory tissue damage and neoplastic transformation of cells. For instance, NQO1-null mice are more susceptible to benzo[α]pyrene [32]- or 7,12-dimethylbenz[a]anthracene [33]-induced skin carcinogenesis. Similarly, deletion of murine pi-class GST (GST-*P*) gene cluster increased the susceptibility to the development of papillomas in a two-stage mouse skin carcinogenesis study [34]. TQ induced the activities of GST and quinone reductase, and restored the hepatic glutathione content in 3-methylcholanthrene-treated rat liver [17]. The induction of HO-1 protected mice against experimental colitis [35] and chemically induced skin papillomagenesis [36]. Topical application of hemin, a HO-1 inducer, abrogated TPA-induced NF-κB activation and COX-2 expression in mouse skin [37]. Thus, the increased expression of HO-1, NQO-1, GCL and GST proteins by TQ, and the inhibition of TPA-induced COX-2 expression and NF-κB activation in mouse skin by the compound provide a mechanistic basis for the antioxidative and anti-inflammatory effects of TQ. In conclusion, TQ attenuated TPA-induced mouse skin inflammation by suppressing COX-2 expression through inhibition of NF-κB signaling and, in part, by inducing the expression of cytoprotective proteins.

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