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Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-κB

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Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are frequently overexpressed in tumor tissues or transformed cells. In the present work, we assessed the effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on expression of iNOS and COX-2 in mouse skin. Topical application to the dorsal skin of female ICR mice of 10 nmol TPA led to maximal induction of iNOS and COX-2 protein expression at ~2 and 4 h, respectively. When applied topically onto shaven backs of mice 30 min prior to TPA, the NOS inhibitor aminoguanidine (AG) inhibited the expression of COX-2 protein at the pharmacologically effective dose. Pretreatment with a more specific iNOS inhibitor, N^G-nitro-L-arginine-methyl ester, also suppressed TPA-induced COX-2 expression. Immunohistochemical analysis of TPA-treated mouse skin using an anti-nitrotyrosine antibody reveals enhanced levels of nitrotyrosine protein localized in epidermal and dermal layers. Topical application of NO donors, such as sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-D,L-penicillamine, induced expression of COX-2 in mouse skin, which was attenuated by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide. SNP treatment stimulated NF-κB activation in mouse skin, which was associated with the degradation of IκBα. Topical application of inhibitors of NF-κB, such as pyrrolidine dithiocarbamate or *N*-α-*p*-tosyl-L-lysine chloromethylketone, inhibited the SNP-induced COX-2 expression. SNP induced a weak but concentration-related increase in COX-2 expression in cultured mouse keratinocytes, which was abolished by treatment with SN50, a specific inhibitor of nuclear translocation of NF-κB. Mouse keratinocytes treated with SNP exhibited an elevated NF-κB-driven COX-2 promoter activity. Topical application of AG (10 μmol) prior to each TPA treatment after initiation reduced the multiplicity of papillomas by 44% at 22 weeks. In conclusion, up-regulation of COX-2 by NO may be mediated by activation of NF-κB in mouse skin, which provides a molecular mechanism by which COX-2 is induced during tumor promotion.

Introduction

Nitric oxide (NO) plays a major role in regulating vascular tone, neurotransmission, platelet aggregation and other homeostatic mechanisms (1). NO is produced endogenously during arginine metabolism by different isoforms of nitric oxide synthase (NOS) (2). Elevated levels of NO have been detected in a variety of pathophysiological processes, including circulatory shock (3), inflammation (4) and carcinogenesis (5). Molecular cloning and sequencing analyses revealed the existence of at least three main types of NOS. Both neuronal NOS and endothelial NOS are constitutively expressed (6), whereas inducible NOS (iNOS) is expressed in response to interferon-γ, lipopolysaccharide (LPS) and a variety of proinflammatory cytokines (3). iNOS is responsible for the overproduction of NO which is often observed during inflammation (4) and tumor development (7). Increased iNOS expression and activity were reported in human gynecological (8), breast (9) and central nervous system (10) tumors.

Cyclooxygenase (COX), an important enzyme involved in mediating the inflammatory process, catalyzes the rate-limiting step in the synthesis of prostaglandins from arachidonic acid. There are two isoforms of COX, designated COX-1 and COX-2 (11). COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. In contrast, COX-2 is detectable in only certain types of tissues and is induced transiently by growth factors, proinflammatory cytokines, tumor promoters and bacterial toxins (12,13). Like iNOS, expression of COX-2 has been frequently increased in several types of malignancies (14–18).

Several studies have suggested an important link between the iNOS and COX pathways, although the precise mechanism underlying such interaction remains poorly understood (19). Conflicting data present in the literature have described both an inhibitory and stimulatory effects of NO on COX expression either *in vitro* or *in vivo* (19–21). Stimulation of a wide variety of cell types, including endothelial cells, with cytokines and other agents results in up-regulation of COX-2 and this is often accompanied by an increase in iNOS levels (22,23). Whether the increase in iNOS expression occurs before that of COX-2 is uncertain and it is possible that regulation of each enzyme may be cell type specific. For example, NO stimulates COX activity in both the LPS-activated murine macrophage cell line RAW 264.7 and

Abbreviations: AG, aminoguanidine; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide; COX, cyclooxygenase; DMBA, 7,12-dimethylbenz[*a*]anthracene; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NAME, N^G-nitro-L-arginine-methyl ester; NOS, nitric oxide synthase; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.1% Tween-20; PDTC, pyrrolidine dithiocarbamate; PGE_2, prostaglandin E_2; PMSF, phenylmethylsulfonyl fluoride; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TPCK, *N*-α-*p*-tosyl-L-lysine chloromethylketone.

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fibroblasts (24), but inhibits the production of PGE_2 in rat Kupffer cells (25) and in cultured bovine endothelial cells (26). The reason for such a discrepancy is unknown. Studies in rat mesangial and human lung epithelial cells suggest that NO may directly interact with COX-2 at the transcriptional level and that this effect is mediated through a cGMP-dependent mechanism (27,28). Regulation of COX-2 gene expression by NO has also been suggested to occur post-transcriptionally in rat osteoblasts (29).

In the present study, we have found that the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces both iNOS and COX-2 expression in mouse skin *in vivo*. We hypothesize that NO could up-regulate COX-2 in TPA-stimulated mouse skin. In order to test this possibility, we utilized iNOS inhibitors and also NO releasing compounds, examining whether they can modulate COX-2 expression when topically applied to mouse skin.

Materials and methods

Chemicals

7,12-Dimethylbenz[*a*]anthracene (DMBA), sodium nitroprusside (SNP), *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), aminoguanidine (AG), pyrrolidine dithiocarbamate (PDTC) and *N*-α-*p*-tosyl-L-lysine chloromethylketone (TPCK) were purchased from Sigma-Aldrich (St Louis, MO). N^G-nitro-L-arginine-methyl ester (NAME) was purchased from Research Biochemicals International (RBI) (Natick, MA). SN50 and its mutated form (SN50M) were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). TPA and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide (carboxy-PTIO) were obtained from Alexis Biochemicals (San Diego, CA). A COX-2 promoter construct and a pELAM-Luc construct containing the NF-κB sites were generous gifts from Dr Y.-J.Bang (College of Medicine, Seoul National University, Seoul, South Korea) and Dr Youngmi Kim (University of Ulsan School of Medicine, Seoul, Korea), respectively. All other chemicals used were in the purest form available commercially.

Animal treatment

Female ICR mice (6–7 weeks of age) were supplied from the Dae-Han Biolink Ltd Experimental Animal Center (Daejeon, Korea). The animals were housed in climate controlled quarters (24 ± 1°C at 50% humidity) with a 12 h light/12 h dark cycle. The dorsal side of the skin was shaved using an electric clipper and only those animals in the resting phase of the hair cycle were used in all experiments. TPA and SNP were dissolved in 200 μl of acetone and applied to the dorsal shaven area.

Western blot analysis

The mice were topically treated on their shaven backs with the indicated doses of SNP or TPA and were killed by cervical dislocation at the indicated times. For isolation of protein from mouse skin, the dorsal skin was excised and the fat was removed on ice, immediately placed in liquid nitrogen and pulverized in mortar. The pulverized skin was homogenized on ice for 20 s with a Polytron tissue homogenizer and lysed in 2 ml ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM Na_3VO_4, 1 g/ml leupeptin] for 10 min. Lysates were centrifuged at 12 000 *g* for 20 min and supernatant containing 30 μg protein was boiled in SDS sample loading buffer for 10 min before electrophoresis on a 12% SDS–polyacrylamide gel. After electrophoresis for 2 h, proteins in the SDS–polyacrylamide gel were transferred to PVDF membrane (Gelman Laboratory, Ann Arbor, MI) and the blots were blocked with 5% non-fat dry milk in PBST buffer [phosphate-buffered saline (PBS) containing 0.1% Tween-20] for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with a 1:1000 dilution of COX-2, iNOS and IκBα polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Equal lane loading was assessed using actin (Sigma Chemical Co., St Louis, MO). The blots were rinsed three times with PBST for 5 min each. Washed blots were incubated with a 1:5000 dilution of horseradish peroxidase conjugated-secondary antibody (Zymed Laboratories, San Francisco, CA) and then washed again three times with PBST. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

Immunohistochemical staining of protein nitrotyrosine and COX-2

Sections (4 μm) of formalin-fixed, paraffin-embedded tissue were cut onto silanized glass slides and deparaffinized three times with xylene for 10 min each and rehydrated through a 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 non-specific staining, each section was treated with 3% hydrogen peroxide in methanol for 15 min. For the detection of nitrotyrosine and COX-2, slides were incubated with 1:50–100 dilutions of a polyclonal anti-nitrotyrosine antibody (Cell Signaling Technology, Beverly, MA) or monoclonal anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI) at room temperature for 60 min in Tris-buffered saline TBS containing 0.05% Tween-20 and then developed using the HPR EnVision™ System (Dako, Glostrup, Denmark). The peroxidase-binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako). Finally, counterstaining was performed using Mayer's hematoxylin.

Nitrite assay

The female ICR mice were treated topically with 10 nmol TPA. Mice were killed by cervical dislocation and the skin was excised, minced and homogenized in 50 mM potassium phosphate buffer (pH 7.2). The homogenates were centrifuged at 25 000 *g* for 20 min at 4°C. The amount of nitrite, an indicator of NO synthesis, was measured by use of the Griess reaction (30). Tissue supernatants (150 μl) were added 150 μl of the Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine, 2.5% phosphoric acid) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm was measured with a Spectrafluor ELISA reader (Tecan US, Durham, NC).

Measurement of prostaglandin E_2 (PGE_2)

Mice were topically treated on their shaven backs with TPA or SNP and killed by cervical dislocation at the indicated times. The pulverized skin was homogenized with ice-cold ethanol and centrifuged for 10 min at 3000 *g*. The supernatant was diluted to 15% with respect to ethanol by adding 0.1 M sodium formate and pelleted protein was dissolved in 8 M urea. The diluted supernatant was applied to a preactivated Amprep™ C-18 reverse phase cartridge (Amersham Pharmacia Biotech) and eicosanoids were released with ethyl acetate containing 1% methanol. The extract was evaporated to dryness under a stream of nitrogen and resuspended in enzyme immunoassay buffer. The amounts of PGE_2 were measured using a PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The quantity of dissolved protein was determined by the BCA method.

Preparation of nuclear extracts

The nuclear extract from mouse skin was prepared as described previously (31). Briefly, scraped dorsal skin of mice was homogenized in 1 ml of ice-cold 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 phenylmethylsulfonyl fluoride (PMSF)]. After 15 min incubation on ice, the nucleoprotein complexes were lysed with 125 μl of 10% Nonidet P-40 (NP-40) solution, followed by centrifugation for 2 min at 14 800 *g*. The nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% NP-40, centrifuged, resuspended in 150 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 was performed using a DNA–protein binding detection kit (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Briefly, the NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech). The binding reaction was carried out in a total volume of 25 μl containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 10 μg nuclear extract and 100 000 c.p.m. of labeled probe. After 50 min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added and samples were electrophoresed through a 6% non-denaturating polyacrylamide gel at 150 V for 2 h. Finally, the gel was dried and exposed to X-ray film.

Two-stage mouse skin carcinogenesis

Groups of 25–30 female ICR mice were treated on their shaven backs with a single topical dose of DMBA (0.2 μmol) in 0.2 ml acetone:DMSO (85:15 v/v) or the same volume of solvent alone. One week after initiation, 10 nmol of TPA in 0.2 ml acetone was topically applied twice a week until termination of the experiment. AG dissolved in 0.2 ml acetone:DMSO (85:15) was topically applied 30 min before each TPA treatment. Control animals were pretreated

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with vehicle alone. Starting 1 week following promoter treatment, tumors of at least 1 mm diameter were counted every other week.

Cell culture and preparation of cell lysates for western blot analysis

The spontaneously transformed murine keratinocyte cell line Pam212, kindly donated by Dr T.-Y.Kim (Catholic University School of Medicine, Seoul, South Korea), was maintained in DMEM (Hyclone, Rogan, UT) supplemented with 10% fetal bovine serum (Gibco BRL) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in 5% CO_2. For stimulation, 1.5×10^6 Pam212 cells were seeded in 6-well dishes in 2 ml of medium. After incubation for 24 h, the cells were exposed to SNP (0.25 mM) with or without SN50 pretreatment and incubated for an additional 24 h. Subsequently, the medium was aspirated and the cells were washed twice with ice-cold PBS. The cells were lysed by scraping them off into 80 μl of ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin]. The insoluble material was pelleted by centrifugation (12 000 *g*) for 15 min at 4°C. The supernatant was removed, divided into aliquots, quickly frozen in liquid nitrogen and stored at −80°C. The protein concentration of the keratinocyte lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL).

Transient transfection and luciferase reporter assays in Pam212 keratinocytes

Pam212 keratinocytes were seeded at a density of 1×10^5 cells in 6-well plates and grown to 60–70% confluence in complete growth medium containing 10% fetal bovine serum. For each triplicate of samples, 2.5 μg luciferase reporter plasmid construct harboring the NF-κB-binding site and 0.5 μg pCMV-β-galactosidase control vector were co-transfected using DOTAP liposomal transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. After 18 h, the medium was changed and the cells were stimulated with SNP and other stimuli. After 6 h incubation, the cells were washed twice with PBS, lysed in 1× reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay System (Promega) and expressed as relative luciferase activity ± SD, which was corrected for transfection efficiency based on the β-galactosidase activity.

Results

Effects of TPA on iNOS and COX-2 expression in mouse skin

When 10 nmol of TPA was applied topically to the shaven backs of female ICR mice, the iNOS protein level increased transiently, with maximal expression observed at 2 h (Figure 1A). Under the same experimental conditions, COX-2 expression was apparently induced 4 h after TPA application (Figure 1A), indicating that iNOS induction may occur before that of COX-2. This finding suggests that expression of COX-2 might be regulated by NO in mouse skin. To confirm the formation of NO in TPA-stimulated mouse skin, we conducted immunohistochemical analysis with 3-nitrotyrosine antibody. In acetone-treated control skin, specific 3-nitrotyrosine immunostaining was barely detectable in the dorsal layer. Upon treatment with TPA for 4 h, however, expression of protein nitrotyrosine increased in a dose-dependent manner (Figure 1B).

Effects of AG on TPA-induced NO production and COX-2 expression in mouse skin

In order to examine whether NO can activate COX-2 expression in TPA-treated mouse skin, we have topically applied the selective iNOS inhibitor AG 30 min prior to TPA treatment. We first tried to ensure that AG could inhibit iNOS in mouse skin after topical application. Pretreatment with this iNOS inhibitor decreased the TPA-induced NO production in mouse skin (Figure 2A). AG suppressed the expression of COX-2 protein induced by TPA at the pharmacologically effective dose (i.e. 30 μmol) capable of inhibiting iNOS activity (Figure 2B). Inhibition of TPA-induced COX-2 expression was also attained with another iNOS inhibitor, NAME (Figure 2C).

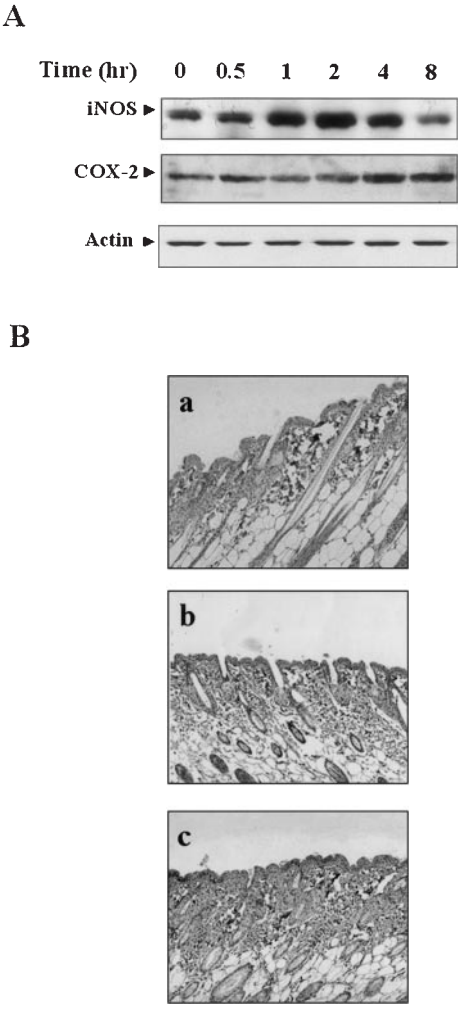

Fig. 1. TPA-induced expression of iNOS and COX-2 protein (A) and protein nitrotyrosine (B) in mouse skin. (**A**) Dorsal skins of female ICR mice were treated topically with acetone alone or with 10 nmol TPA in acetone for the indicated time periods. Protein extracts (30 μg) were loaded onto a 12% SDS–polyacrylamide gel, electrophoresed and subsequently transferred onto PVDF membrane. Other experimental details are described in Materials and methods. Immunoblots were probed with an iNOS or COX-2 polyclonal antibody. (**B**) Paraffin-embedded tissues from TPA-treated mice were immunostained for protein nitrotyrosine and counterstained with hematoxylin, as described in Materials and methods. Protein nitrotyrosine staining gives a brown reaction product. (**a**) Acetone-treated control skin. (**b**) TPA (10 nmol)-treated mouse skin. (**c**) TPA (100 nmol)-treated mouse skin. A color version is available as supplementary material online.

Effects of SNP on PGE_2 production and COX-2 expression in mouse skin

In order to verify whether NO produced by TPA can induce COX-2 expression in mouse skin, we next sought to determine the effect of exogenously applied NO on COX-2 expression. In this experiment, dorsal skin of mice was treated with two different NO donors, SNP and SNAP. As illustrated in Figure 3A, topical treatment of female ICR mice with 2 or 20 μmol SNP significantly increased PGE_2 synthesis. In addition, SNP treatment resulted in marked induction of COX-2 expression in a dose-dependent manner (Figure 3B). Another NO donor, SNAP also increased expression of COX-2 when topically applied onto mouse skin (Figure 3C). Furthermore, topical application of carboxy-PTIO, a known NO scavenger, 15 min before SNP treatment abolished COX-2 expression in

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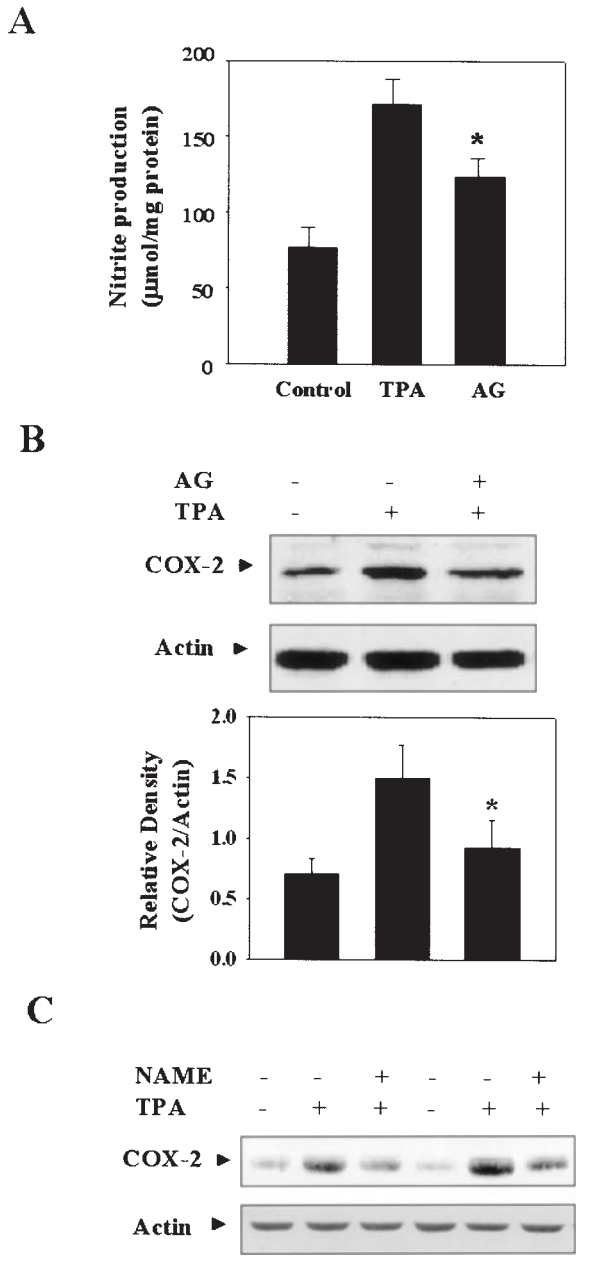

Fig. 2. Inhibitory effects of AG on phorbol ester-induced nitrite production (A) and COX-2 expression (B and C). (**A**) Female ICR mice were treated topically with 0.2 ml acetone or aminoguanidine (30 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA and animals were killed 4 h after the TPA treatment. The amount of nitrite produced was determined by the Griess reaction. Data are expressed as mean ± SD ($n = 6$). *Significantly different from the TPA alone group ($P < 0.05$). (**B**) Protein was analyzed for COX-2 by immunoblotting. The western blot is representative of three independent experiments which produced similar results. Quantification of COX-2 expression was normalized to actin using a densitometer. (**C**) Female ICR mice were treated topically with acetone or with 10 μmol NAME 30 min prior to 10 nmol TPA. Animals were killed by cervical dislocation 4 h later. Expression of COX-2 was measured by western blot analysis.

SNP-treated mouse skin (Figure 3D). To assess the localization of COX-2 by SNP in mouse skin, we conducted an immunohistochemical analysis. In acetone-treated control skin, specific COX-2 immunostaining was detectable in the dermal sebaceous gland (Figure 4A). In contrast, expression of COX-2 increased dramatically in the epidermal basal layer upon treatment with 20 μmol SNP for 2 h (Figure 4B).

Induction of NF-κB DNA binding activity by SNP

Because NF-κB is known to play a crucial role in regulating the induction of COX-2, we have determined whether SNP could activate this transcription factor in nuclear extracts obtained from mouse skin. Effects of SNP on NF-κB activation were examined by gel shift assay using an oligonucleotide harboring an NF-κB consensus sequence. SNP treatment caused activation of NF-κB in terms of its DNA binding (Figure 5A).

One of the most critical steps in NF-κB activation is dissociation of the NF-κB subunits from IκB, which is mediated through phosphorylation and subsequent proteolytic degradation of the inhibitory subunit. To determine whether the increased NF-κB DNA binding induced by SNP was mediated through its induction of IκBα degradation, the cytoplasmic level of IκBα was determined by western blot analysis. As illustrated in Figure 5B, topical application of SNP led to degradation of IκBα by as early as 30 min. To better assess the role of NF-κB in the induction of COX-2 expression in mouse skin, we have investigated the effect of PDTC and TPCK, known inhibitors of NF-κB, on SNP-induced COX-2 expression. Topical application of PDTC or TPCK at a dose capable of inactivating NF-κB suppressed the induction of COX-2 expression by SNP (Figure 5C).

Induction of COX-2 and NF-κB transcriptional activity by SNP in murine keratinocytes

We also confirmed the capability of SNP to induce COX-2 expression using cultured keratinocytes. Thus, Pam212 cells treated with varying amounts of SNP (0.05–0.5 mM) displayed a concentration-dependent increase in COX-2 protein levels (Figure 6A). To verify the role of NF-κB in SNP-mediated COX-2 induction, we utilized a specific NF-κB inhibitor, SN50, that blocks the nuclear translocation of NF-κB. As illustrated in Figure 6B, SN50 inhibited COX-2 expression in Pam212 cells treated with SNP. However, the same concentration of the inactive peptide SN50M with a mutated sequence failed to inhibit SNP-induced COX-2 expression (Figure 6B). In another experiment, we demonstrated that SNP enhanced COX-2 promoter activity, as determined by the luciferase reporter gene assay (Figure 6C). To determine a potential effect of SNP on the transcriptional activity of NF-κB, Pam212 cells were transiently transfected with a luciferase reporter construct containing a NF-κB site. SNP (0.5 mM) activated the transcriptional activity of NF-κB (Figure 6D). Likewise, LPS and tumor necrosis factor α, which are well-known inducers of COX-2, activated the transcriptional activity of NF-κB in Pam212 cells (data not shown). Based on these findings, it is conceivable that SNP-induced COX-2 expression is regulated, at least in part, by NF-κB in murine keratinocytes as well as in mouse skin *in vivo*.

Inhibitory effect of AG on TPA-induced mouse skin tumor promotion

Up-regulation of iNOS and production of NO occur in many pathological conditions, including tumorigenesis. To examine whether NO is involved in mouse skin tumor promotion, dorsal skin of mice was subjected to topical application of AG prior to TPA, following initiation with DMBA. Papillomas were first observed in the DMBA + TPA group after 8 weeks of promotion and reached 100% incidence with ~16 papillomas/mouse at week 22 (Figure 7). AG at 10 μmol significantly lowered the multiplicity of papillomas while it did not much influence the tumor incidence.

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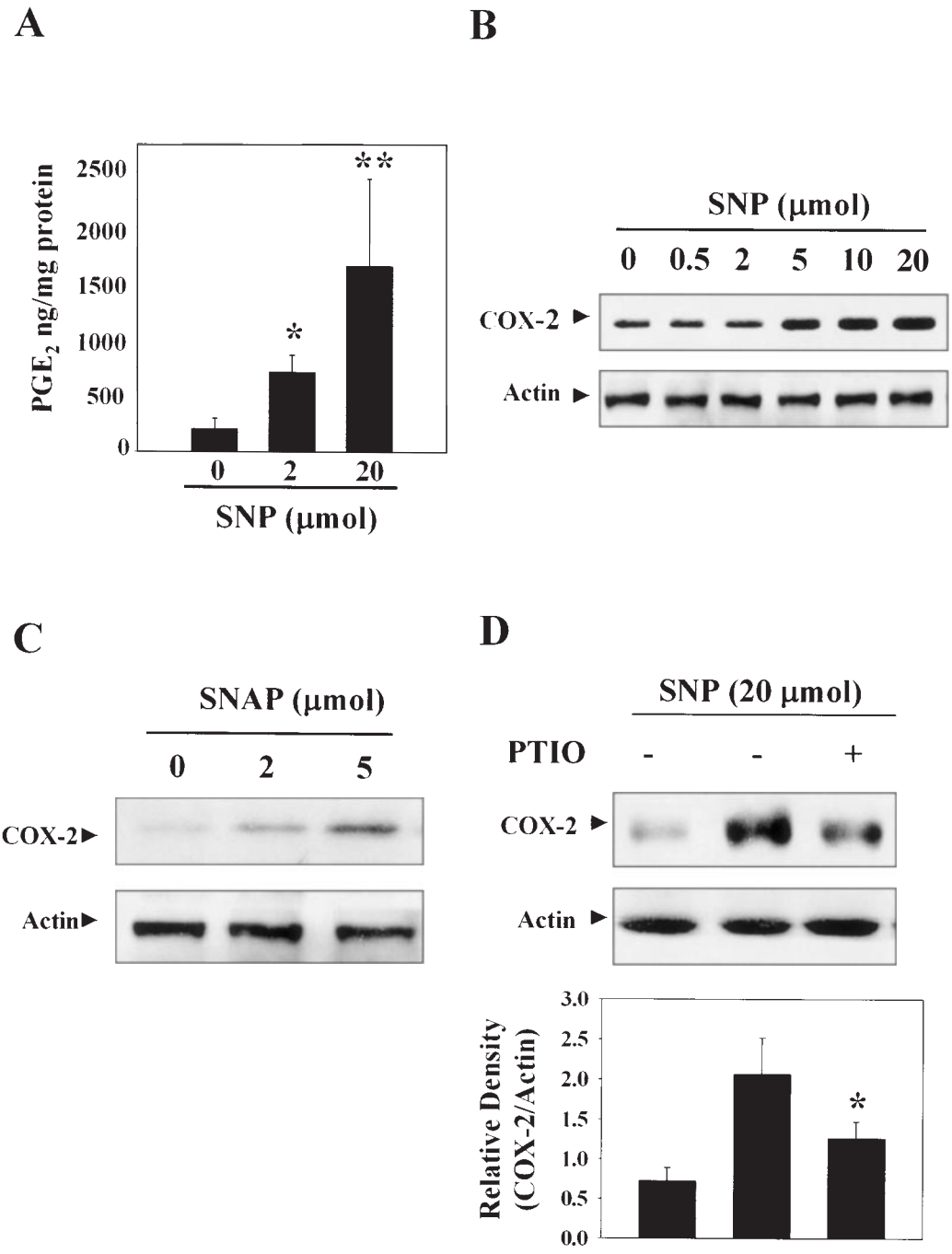

Fig. 3. Effect of SNP on PGE_2 production and COX-2 protein expression in mouse skin. (**A**) Female ICR mice were treated topically with 2 or 20 μmol SNP and animals were killed 2 h after the SNP treatment. Data are expressed as means ± SD obtained from 5 mice/group. *,**Significantly different from the group treated with control ($^{*}P < 0.005$; $^{**}P < 0.001$). (**B**) Mice were treated topically with the indicated doses of SNP. Animals were killed by cervical dislocation 2 h later. The western blot assay was conducted as described in the legend to Figure 1. (**C**) Mice were treated topically with SNAP (2 or 5 μmol) and killed by cervical dislocation 2 h later. (**D**) Mice were treated topically with either the solvent alone or carboxy-PTIO (2 μmol) 15 min prior to 20 μmol SNP treatment. Animals were killed by cervical dislocation 2 h later. Quantification of COX-2 expression was normalized to actin using a densitometer. Values are means ± SD of three independent experiments. *Significantly different from the group treated with control ($P < 0.05$).

Discussion

Recent studies suggest that iNOS may play a role in tumor development. Increased iNOS expression and/or activity was observed in diverse human tumors (8–10). In addition, enhanced nitrotyrosine accumulation in inflammed mucosa of patients with ulcerative colitis and gastritis implies production of NO and its possible involvement in the pathogenesis of these diseases (5). Rao *et al*. (32) and Takahashi *et al*. (33) have demonstrated that azoxymethane-induced colon tumors exhibit elevated expression and activity of iNOS compared with adjacent normal colonic tissue. Furthermore, iNOS selective inhibitors, such as *S*,*S*′-1,4-phenylene-bis(1,2-ethanediyl)-*bis*-isothiourea and NAME, attenuated azoxymethane-induced aberrant crypt focus formation in rats (7,34). In line with this notion, we found that the iNOS inhibitor AG at 10 μmol

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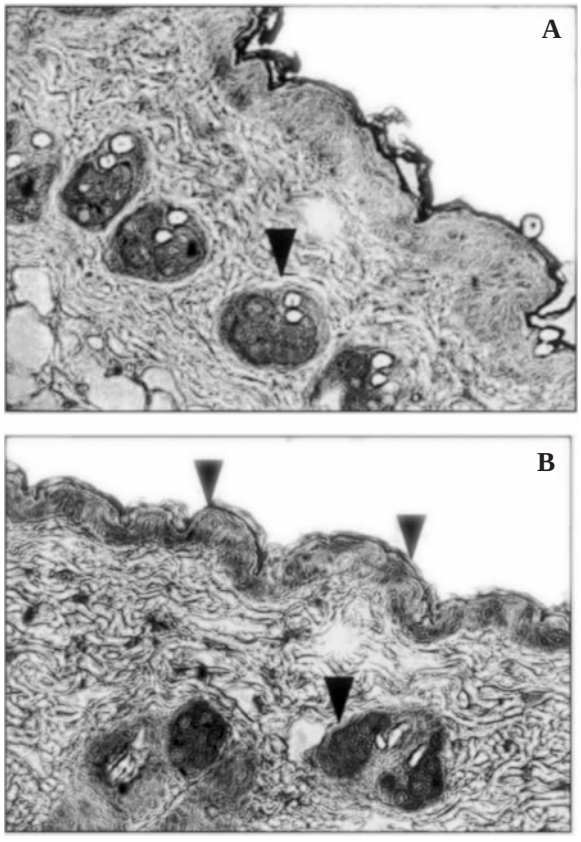

Fig. 4. SNP-induced COX-2 expression visualized by immunohistochemical staining. Skin samples were stained for COX-2 at the end of the study protocol. Positive COX-2 staining yielded a brown colored product (arrow). (**A**) Skin treated with acetone alone. (**B**) Skin treated with 20 μmol SNP for 2 h. A color version of this figure is available as supplementary material online.

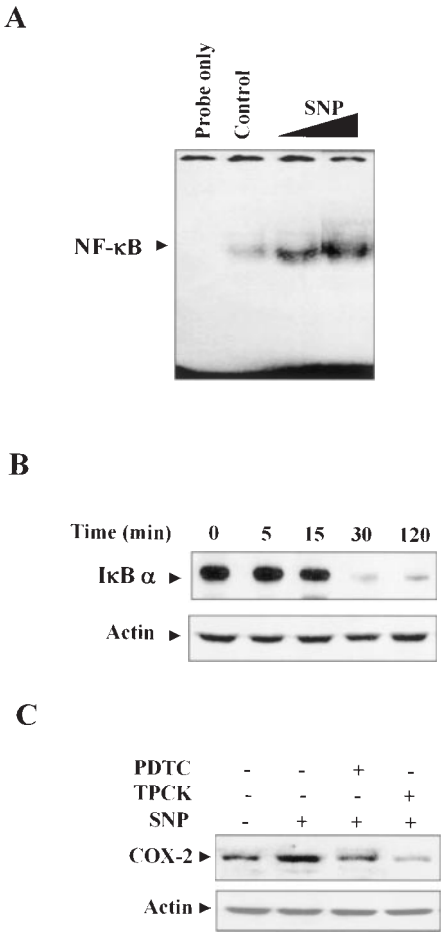

Fig. 5. Effect of SNP on NF-κB activation and IκBα degradation in mouse skin. (**A**) Dorsal skins of mice were treated topically with acetone alone or 0.5 or 20 μmol SNP. Mice were killed 1.5 h after the SNP treatment and epidermal nuclear extracts were prepared and incubated with the radiolabeled oligonucleotides containing the NF-κB consensus sequence for analysis by EMSA. EMSA was performed in duplicate and showed similar patterns. (**B**) Cytoplasmic extract from mouse skin treated with 20 μmol SNP for the indicated time period was assayed for IκBα by western blot analysis. (**C**) Female ICR mice were treated topically with acetone or with 40 μmol PDTC and TPCK 30 min prior to 20 μmol SNP. Animals were killed by cervical dislocation 2 h later. Expression of COX-2 was measured by western blot analysis.

significantly lowered the multiplicity of papillomas when given prior to TPA during promotion. In striking contrast to the implications for a role of iNOS in carcinogenesis described above, the same enzyme has been proposed to have an opposite role in mouse skin carcinogenesis. Thus, Robertson *et al*. (35) reported that levels of iNOS mRNA in dorsal skin isolated from acetone-treated female SENCAR mice were substantially higher than those observed in the cutaneous tissue of mice after exposure to a single topical application of DMBA followed by repetitive applications of TPA. Moreover, papillomas isolated at 16 and 22 weeks of a tumor promotion protocol also had low levels of iNOS mRNA. The diminished levels of iNOS mRNA inversely correlated with the extent of TPA-induced epidermal hyperplasia (35). Furthermore, treatment of DMBA-initiated TPA-promoted mouse skin with a topical dose of nitroglycerine, a NO generating vasodilator, increased the latency period and reduced the tumor incidence and multiplicity (36).

Besides iNOS, COX-2 has also been reported to contribute to tumor growth. Co-expression of iNOS and COX-2 has been observed in malignancies (37). It was reported that enhanced COX-2 expression is sufficient to induce mammary gland tumorigenesis (38). The COX-2 transgenic mice were more susceptible to skin carcinogenesis induced by DMBA than wild-type mice (39). There is a causal relationship between transgenic COX-2 expression in basal keratinocytes and epidermal hyperplasia as well as dysplastic features at discrete body sites (40). In contrast, COX-2 overexpression was found to protect, rather than sensitize, transgenic mice against skin tumor development induced by an initiation/promotion protocol (41). In our present study, inhibition of iNOS by AG not only attenuated TPA-induced tumor promotion, but also COX-2 expression in mouse skin, suggesting that the antitumor promotional effect of AG may have resulted from suppression of COX-2 expression as well as reduced NO production. It would be worthwhile verifying the role of NO in TPA-induced COX-2 induction by use of more selective iNOS inhibitors, such as 1400W and GW274150.

Previous reports have indicated that iNOS can modulate COX-2 activity or expression in cancer (42–44). Accumulated data from both animal and cell culture studies have provided evidence for interactions between the iNOS and COX-2 pathways. In the setting of inflammatory induction of iNOS and COX-2, the majority of studies suggest that NO enhances COX-2 expression and activity (24,45) and that this effect may be cGMP dependent (27). In contrast, studies in LPS-activated rat macrophages have suggested that NO may inhibit

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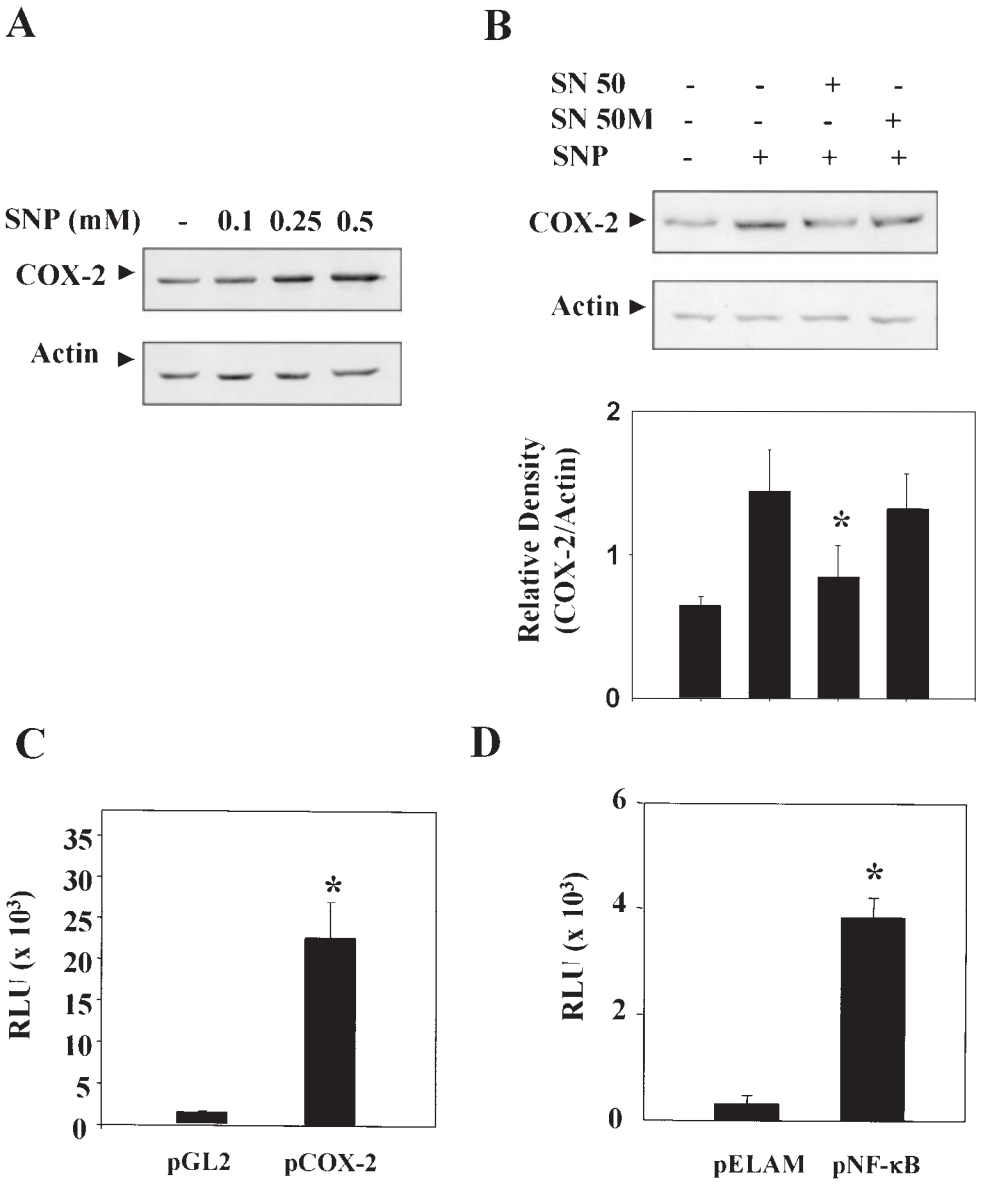

Fig. 6. Effect of SNP on NF-κB activation and COX-2 expression in keratinocytes. (**A**) Murine keratinocytes were stimulated with various concentrations of SNP for 24 h and COX-2 protein levels were assayed by western blot analysis. (**B**) Murine keratinocytes were preincubated for 1 h with 25 μmol cell permeable inhibitory peptide SN50 as well as a cell permeable control peptide SN50M and harvested 24 h after addition of SNP (0.5 mM). Quantification of COX-2 expression was normalized to actin using a densitometer. Values are means ± SD of three independent experiments. *Significantly different from the group treated with control ($P < 0.05$). (**C**) Murine Pam212 keratinocytes were transiently transfected with a COX-2 promoter, a reporter vector and with pCMV-βgal as an internal control for transfection. The COX-2 promoter activity was determined as described in Materials and methods. (**D**) pELAM-NF-κB and its basic vector were transfected into murine keratinocytes. Eighteen hours after transfection, the cells were incubated with SNP for 6 h. The cells were lysed and assayed for both luciferase and β-galactosidase activities. The results are presented as mean relative luciferase activities ± SD ($n = 3$), which was corrected for transfection efficiency using β-galactosidase activity.

both COX-2 induction and activity (46). In our study, expression of iNOS and COX-2 peaked at 2 and 4 h, respectively, in TPA-treated mouse skin. Furthermore, the NOS inhibitor AG suppressed TPA-induced COX-2 expression, suggesting that expression of COX-2 can be regulated by NO in mouse skin. However, the inhibition of COX-2 expression by AG was partial. This appears to be due to the fact that AG cannot block all the sources of NO production and also that NO is not the only regulator of COX-2. A similar inhibitory effect was attained with a more specific iNOS inhibitor, NAME. Treatment of dorsal skin of female ICR mice with 2 or 20 μmol SNP significantly increased PGE_2 synthesis, lending further support to the possible role of NO in the induction of COX-2 in mouse skin. In addition, SNP treatment resulted in marked induction of COX-2 expression in mouse skin, which was abolished by carboxy-PTIO, a known NO scavenger. SNP can cause cytotoxicity because it releases ferricyanide. Therefore, the right controls may be needed to verify that the observed COX-2 inducing effect of SNP is due to NO, not to the cyanide. To avoid such complication, we utilized another NO donor, SNAP, that does not generate ferricyanide. We confirmed that SNAP also increased expression of COX-2 in mouse skin.

By interacting with superoxide, NO produces another reactive nitrogen species, peroxynitrite. It has been reported that in the macrophage cell line RAW 264.7, peroxynitrite, added

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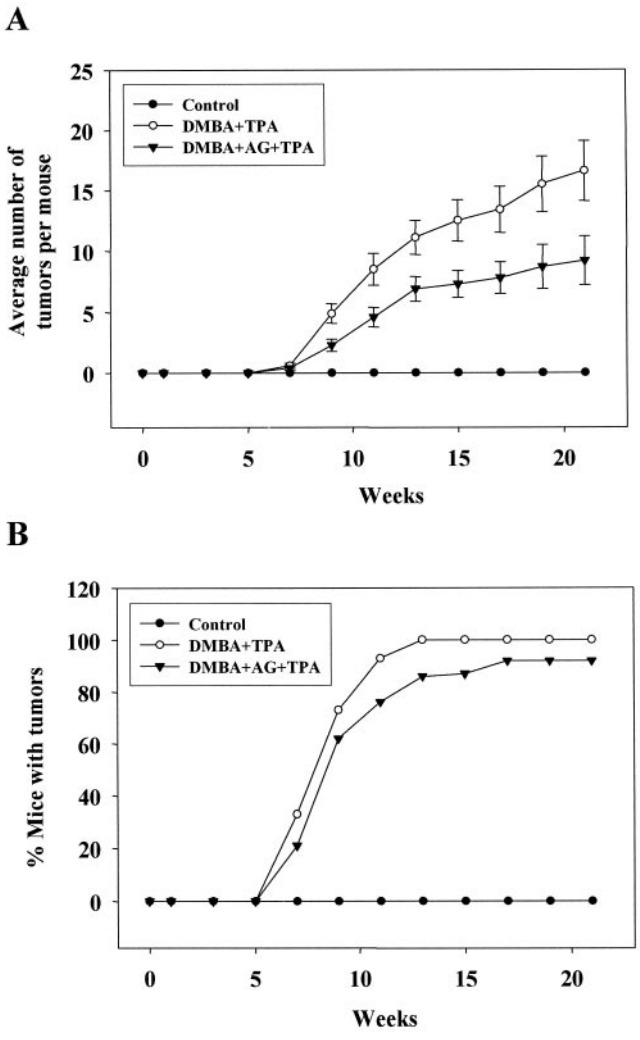

Fig. 7. Inhibitory effect of AG on mouse skin tumor promotion. Female ICR mice received 0 (open circles) or 10 μmol (solid triangles) AG 30 min prior to each topical application of 10 nmol TPA dissolved in acetone after initiation with DMBA, as described in Materials and methods. Control animals received vehicle alone (solid circles).

directly to the medium, gradually released from 3-morpholinosydnonimine (SIN-1) or generated *in situ* from the NO donor SNAP with a superoxide generating system (xanthine/xanthine oxidase), significantly activated COX-2 and also prostanoid production (47). Although there are numerous reports addressing the possible cross-talk between iNOS and COX-2, it has been proposed that NO simply acts as a precursor to peroxynitrite that is able to activate COX-2 to synthesize prostaglandins (48). Marnett *et al*. have provided compelling evidence that a targeted deletion of the iNOS gene with a resultant reduction in NO or NO-derived molecular species, including peroxynitrite, leads to a significant decrease in the formation of prostaglandins (49). We have reported that topical application of the peroxynitrite releasing compound SIN-1 leads to dose-related increases in the expression of COX-2 in mouse skin (50). These findings, taken together, suggest that peroxynitrite may play an important role in prostaglandin biosynthesis and inflammation.

The eukaryotic transcription factor NF-κB plays a central role in inflammatory as well as immune responses. The 5′-flanking region of *cox-2* contains NF-κB-binding sites (51). In line with this notion, NF-κB has been shown to be a critical regulator of COX-2 expression in many cell lines (52,53). Recent findings have suggested that NF-κB is a potential molecular target for the biological actions of NO. Nevertheless, the effects of NO on NF-κB are quite controversial. In fact, NO can activate NF-κB DNA binding activity in some cell types (54,55) while it exerts an inhibitory effect in others (56,57). Previous *in vitro* and cell line studies have shown that *S*-nitrosylation of the p50 subunit can inhibit DNA binding of NF-κB (58–60). Modification of NF-κB by NO may serve as a negative feedback mechanism that regulates COX-2 transcription. Thus, a negative feedback loop may exist, which couples increased NO production to decreased transcription of the *cox-2* gene. SNP, when applied onto dorsal skin of mice, caused NF-κB activation through degradation of IκBα. According to our previous study, topical application of PDTC, a known inhibitor of NF-κB, resulted in a dose-related suppression of TPA-induced activation of NF-κB and also caused reduced COX-2 protein expression in mouse skin (50). To verify the role of NF-κB in induction of COX-2 expression mediated by the NO pathway in mouse skin, we have examined the effect of two different NF-κB inhibitors (PDTC and TPCK) on SNP-induced COX-2 expression. Both NF-κB inhibitors suppressed the induction of COX-2 expression by SNP. We also confirmed the inhibitory effect of SN50, a cell permeable inhibitory peptide of NF-κB translocation, on COX-2 expression in keratinocytes. These data further support the notion that the NO could induce COX-2 expression in mouse skin through NF-κB activation.

Regardless of the animal species, promoter regions of the *cox-2* genes contain a canonical TATA box and various putative transcriptional regulatory elements, such as CRE, NF-IL6, AP-2 and SP-1, in addition to the NF-κB site (51). Among these elements, CRE (61) and NF-IL6 (62) have been shown to act as positive regulatory elements for COX-2 transcription. Therefore, the possibility of *cox-2* gene transcription regulated by transcription factors other than NF-κB cannot be excluded. Additional studies using COX-2 promoters with mutated or deleted sequences for binding to a specific transcription factor will clarify which transcription factor(s) plays a prime role in NO-mediated COX-2 induction in murine keratinocytes.

Supplementary material

Supplementary material can be found at http://www.carcin.oupjournals.org.

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