Ras mutation promotes p53 activation and apoptosis of skin keratinocytes

Yunfeng Zhao1,5, Luksana Chaiswing2,4, Vasudevan Bakthavatchalu1, Terry D.Oberley2,3 and Daret K.St.Clair1,4*

1Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536, USA, 2Department of Pathology, VA Hospital, University of Wisconsin, Madison, WI 53705, USA, 3Faculty of Medical Technology, Mahidol University, Bangkok, Thailand 10700 and 4Faculty of Medical Sciences, Toxicology & Neuroscience, LSU Health Science Center, Shreveport, LA 71103, USA

*To whom correspondence should be addressed. Tel: +1 859 257 3956; Fax: +1 859 323 1059; Email: dstc100@pop.uky.edu

Previous studies in our laboratory demonstrated that 7,12-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA) treatment induced apoptosis and mitochondrial translocation of the tumor suppressor p53 in a mouse skin carcinogenesis model, suggesting that oncogenic versus cell death signaling involve a common mediator. Mutational activation of oncogenic Ras is an early event and has been demonstrated to play a critical role in skin carcinogenesis. A malignant skin keratinocyte cell line (308), which carries a H-ras mutation at codon 61, showed elevated p53 levels, increased caspase 3 activity and enhanced apoptosis after TPA treatment. In contrast, the non-malignant counterpart (C50) showed undetectable levels of p53 and less apoptosis than 308 cells similarly treated. Inhibition of NADPH-oxidase (NOX) by diphenyleneiodonium suppressed p53 activation and apoptosis in 308 cells, linking Ras mutation to NOX-induced p53 activation, which was further supported by the finding that siRNA to Rac1 inhibited p53 activation after TPA treatment. Application of DPI to DMBA-initiated skin tissue carries mutated p53 and less apoptosis than 308 cells similarly treated. Inhibition of NADPH-oxidase (NOX) by diphenyleneiodonium suppressed p53 activation and apoptosis in 308 cells, linking Ras mutation to NOX-induced p53 activation, which was further supported by the finding that siRNA to Rac1 inhibited p53 activation after TPA treatment.

Introduction

Among numerous animal cancer models that have been developed, multistage skin carcinogenesis [which consists of sequential application of a subthreshold dose of a mutagenic chemical initiator such as 7,12-dimethylbenz(a)-anthracene (DMBA), followed by repetitive treatment with a tumor promoter such as 12-O-tetradecanoylphorbol-13-acetate (TPA)] is a well-established skin carcinogenesis model. Initiation results in an irreversible oncogenic mutation, whereas promotion involves the clonal expansion of the mutated cells. A variety of factors have been proposed to contribute to skin tumor formation, including oxidative stress, which has received increased recognition because both tumor initiators and promoters are known to induce oxidative stress. Previous studies in our laboratory have demonstrated that expression of manganese containing superoxide dismutase, the primary antioxidant enzyme in the mitochondria, reduces tumor incidence in a multistage skin carcinogenesis model, providing strong support that reduction of oxidative stress leads to suppression of carcinogenesis (1).

We also have demonstrated that DMBA/TPA application induces apoptosis in mouse skin epidermis and that the apoptotic event was associated with increased p53 expression and mitochondrial translocation (2,3). In the present study, we extended from these initial observations to address two important additional questions. Does apoptosis after TPA treatment primarily arise from normal skin epidermal cells or from initiated epidermal cells? And does activation of oncogenic Ras play an important role in the p53 activation? We used a DMBA-initiated mouse skin epidermal cell line (308) that carries mutated H-ras at codon 61 but wild-type p53, and its counterpart, a non-malignant mouse skin epidermal cell line (C50), as well as the two-stage skin carcinogenesis mouse model to address these questions.

Materials and methods

Cell line and treatment

Mouse skin keratinocytes 308 and C50 were grown in S-MEM medium supplemented with 8% Chelexed FBS, 2 mM of L-glutamine, 0.1 mM non-essential amino acids, 50 μg/mL CaCl2, and 50 μg/mL penicillin and 50 μg/mL streptomycin.

Stock solution containing 1 mM TPA (Sigma, St Louis, MO) and 10 mM diphenyleneiodonium (DPI, Sigma) in dimethyl sulfoxide (DMSO) was prepared. TPA was diluted in culture medium to a final concentration of 100 nM for treatment of cells. An aliquot of 0.01% DMSO diluted in medium was used as vehicle control.

Mice

C57BL/6J mice were purchased from Jackson Laboratory (Indianapolis, IN) and maintained at the University of Kentucky animal facility. Female mice (n = 15) 6–8 weeks old were used in this study.

Fluorescent staining of p53 immunoreactive protein

Ten thousand 308 cells were seeded in 8-well Lab-Tek Chamber Slides w/Cover (Nalge Nunc International, Naperville, IL) in 400 μl medium and incubated overnight. Cells were then incubated with 200 nM of MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) in culture medium for 30 min, after which the dye was removed, and cells were treated with TPA (100 nM) for 5 min to 4 h. Medium was removed and cells were fixed in 4% formaldehyde containing 0.1% glutaraldehyde for 15 min at room temperature (RT). After rinsing with cold PBS (pH 7.4), cells were permeabilized with 0.5% Triton X-100 for 10 min at RT. After blocking with 5% goat serum, anti-p53 antibody (Ab-11; Oncogene, Boston, MA) was added (1:64 dilution) and incubated at 37°C for 1 h followed by incubation with anti-mouse IgG–FITC (Sigma, 1:128 dilution) for 1 h. After removal of antibodies, cells were rinsed with PBS and mounted with 90% glycerol. Fluorescence was...
immediately observed using a Leica laser scanning confocal microscope (Bensheim, Germany, magnification ×64).

Detection of cleavage activity resembling caspase 3 Activity similar to caspase 3 was measured using ApoAlert Caspase 3 Fluorometric Assay Kit (Clontech Laboratories, Palo Alto, CA). Cell lysate was prepared following the instructions provided. The protein concentrations of cell lysate were adjusted to equal levels, and the same amount of protein was applied in the assay. Fluorescence was measured with a Spectra MAX GEMINI Reader (Molecular Devices, Sunnyvale, CA).

Nucleosome fragmentation assay After pre-treatment with DPI or DMSO for 24 h, cells were exposed to TPA (100 nM) for 30 min to 24 h. Cells were then collected, and sample preparation and cell death detection using ELISA were performed according to the manufacturer’s instructions (Roche, Indianapolis, IN) with minor modifications: 25 μl of the whole cell lysate was applied for the assay.

NADPH-oxidase (NOX) activity assay This assay was performed as described previously by Cui and Douglas (4). Cells were washed twice in ice-cold Dulbecco’s phosphate-buffered saline (PBS) and were scraped from the plate into the same solution, followed by centrifugation at 750×g, 4°C, for 10 min. The pellet was resuspended in buffer containing 20 mM KH2PO4, 1 mM EGTA, 10 μg/ml aprotinin, 25 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was homogenized with 50 strokes in a Dounce homogenizer on ice, and aliquots of the homogenate were used immediately. To start the assay, 100 μl of homogenate was added into 900 μl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 500 μg/ml lucigenin and 100 μM NADPH. Photoemission in terms of RLU was measured every minute for 15 min.

Detection of ROS by H2DCFDA The intracellular production of reactive oxygen species (ROS) was assayed using dichlorodihydrofluorescein diacetate (H2DCFDA). Briefly, five thousand 308 cells were seeded in a 96-well plate and incubated overnight. Cells were treated with 1 μM DPI for 24 h. The next day, the medium was replaced with fresh medium containing 1 μM DPI and/or 100 nM TPA and incubated for 15 min to 1 h. Cells were washed with PBS (pH 7.4); fresh medium containing 10 μM H2DCFDA (Molecular Probes, Eugene, OR) was added and cells were incubated for 15 min at 37°C. H2DCFDA was oxidized by ROS to the highly fluorescent 2’,7’-dichlorofluorescein (DCF). Fluorescence was read at excitation/emission of 488/525 nm with a Spectra Max Gemini plate reader from Molecular Devices. The fluorescence with cells only (no H2DCFDA dye added) was used to subtract the sample value from each corresponding well.

Rac1 knockdown by siRNA approach The siRNA to Rac1 kit was purchased from Upstate (New York). A total of 1 × 10^5 308 cells were seeded in a 6-well plate and incubated overnight. An aliquot of 10 μl RNA to Rac1 and control siRNA were mixed with siRNA transfection reagent and medium (Santa Cruz), and the following procedures were chosen as described by the manufacturer. Finally, cells were collected and western blot analysis was performed.

Detection of H-ras mutation at Codon 61 Total RNA was isolated from homogenized skin tissue by using TRIzol Reagent (GibcoBRL, Paisley, PA) according to the instructions provided. One microgram of RNA from each sample was used as the starting material. Oligo(dT) was used to prime RT with the Clontech RT-for-PCR kit. For PCR amplification of cDNA the following primers were used: forward for both wild-type and mutant H-ras: 5’-CTAAGCGTGTGTTGGCAGGCAC-3’; reverse primer for wild-type H-ras: 5’-CATCGGACTATACCTCCT-3’; reverse primer for mutant H-ras: 5’-CATGGGACTATACCTCCT-3’. PCR was performed at 94°C for 5 min followed by 30 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 40 s. Codon 61 C—A point mutation creates a XbaI restriction enzyme site, which is utilized to distinguish mutant from wild-type H-ras. PCR product was digested with XbaI and the products were separated primer agarose gel electrophoresis and visualized by ethidium bromide staining.

Isolation of mitochondrial fraction from skin cells Stripped mouse skin cells were suspended in 1 ml mitochondria isolation buffer (0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, pH adjusted to 7.4 with a few drops of 0.5 M Tris) in a 15-ml plastic tube and were homogenized three times with 30 strokes using scale 2 of a Wheaton homogenizer. The tissue was removed by centrifugation at 5700 × g (2200 r.p.m.) for 5 min in a Sorval SS34 rotor. The supernatant was filtered through a nylon screen cloth (Small Parts, Miami Lakes, FL) and centrifuged at 9000 × g (8700 r.p.m.) for 10 min. Supernatant was kept and designated as supernatant fraction. The pellet was washed by adding 0.5 ml of mitochondria isolation buffer and centrifuging at 9000 × g for 5 min. This washing step was repeated once. The mitochondrial pellet was resuspended in 150 μl of mitochondria isolation buffer containing 0.1% Triton X-100. This fraction was labeled as the mitochondria fraction and kept at −80°C.

Detection of oxidatively modified proteins The OxyBlot protein oxidation detection kit (s7150, Intergen, Purchase, NY) was used to perform the assay. Ten micrograms of supernatant fraction obtained from the previous step was used. The reaction procedures were performed according to the manufacturer’s instructions. SDS–PAGE gel (10%) was used to separate the samples.

Western blot analysis For detection of the protein levels of p53 and Rac1, 25 μg of the whole cell lysate (skin keratinocytes) or mitochondrial fraction (mouse skin epidermal tissue) was separated on 10% SDS–PAGE gels and transferred to a nitrocellulose membrane. Ponceau staining was used to monitor the uniformity of transfer. The membrane was blocked in Blotto (5% milk, 10 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.05% Tween-20) for 2 h at RT. Anti-p53 antibody (Ab-11) or anti-Rac1 antibody (23A8, Uptake, Lake Placid, NY) was added in 1:1000 dilution and the membrane was incubated for 2 h at RT. After washing, the membrane was incubated with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology) at a 1:4000 dilution. The antibody bands were visualized by the enhanced chemiluminescent detection system (ECL, Amershams). The membranes were then stripped and re-probed with a monoclonal anti-actin antibody (Sigma) to normalize protein loading. The corresponding bands were scanned, and the densities were quantitatively assessed using ImageQuant 5.1 software (Bio-Rad, Hercules, CA). For each set of data, the experiments were repeated 2–3 times and representative images are shown.

Histological examination of the kinetics of apoptosis in mouse skin epidermis Mice were treated with either DMSO (control) or single application of 100 nM DMBA, followed 2 weeks later by a single application of 40 μg of DPI or DMSO, and followed 24 h later by a single application of 4 μg TPA. After another 24 h, animals were euthanized and skin samples were collected (n = 3 for each group). For histological examination, the tissues were fixed with Karnovsky’s fixative for 1 h at room temperature and then rinsed in 0.1 M Sorenson’s phosphate buffer, pH 7.4, for 30 min. Following fixation in Cai- field’s osmium tetroxide with sucrose for 30 min, the tissues were dehydrated in graded ethanol and embedded in epoxy resin. Thick sections of Epon-embedded skin tissues were mounted on glass slides and stained with toluidine blue. Apoptotic cells were counted by light microscopy. Only interfollicular epidermis was counted. A total of 1500 random keratinocytes were counted per mounting. The following morphologic features were used to identify apoptosis: cell shrinkage, chromatin condensation, and formation of cytoplasmic blebs and apoptotic bodies. Results from various groups were compared with control using ANOVA with LSD post hoc test.

Statistical analysis Statistical analysis was performed using either student t-test (for two-group comparison) or one-way ANOVA (for multiple group comparison) followed by Newman–Keuls post-test. Data are reported as mean ± standard error (SEM).

Results H-ras mutation was associated with increased p53 levels and apoptosis in skin keratinocytes after tumor promoter TPA treatment in vitro In our previous studies, tumor suppressor p53 was found to have increased in skin epidermal tissue upon DMBA/TPA treatment. DMBA/TPA application induced oxidative stress in skin epidermis, which in turn activated p53 and apoptosis (2). DMBA alone is known to be able to cause mutation of oncogene ras (one frequently occurring mutation is H-ras codon 61 A→T mutation). In order to demonstrate the role of Ras in this p53-associated apoptotic event, we first performed immunofluorescence staining of p53 in skin keratinocyte 308 cells bearing mutational H-ras and their H-ras wild-type counterpart C50 cells. The results showed that in
308 cells, p53 protein was elevated from 15 min to 4 h after TPA treatment (Figure 1A), p53 translocation to mitochondria co-localized with the mitochondrial marker Mitotracker Red. In contrast, no significant p53 staining was observed in the normal counterpart C50 cells similarly treated (data not shown). The increased p53 expression in 308 cells was further verified by western blot analysis of the cells that received TPA treatment for 1 or 24 h (Figure 1B). Rapid and greater increase in caspase 3 activity was subsequently observed in 308 cells compared to C50 cells following increased p53 expression (Figure 1C). Apoptotic cell death indicated by nucleosome fragmentation (Figure 1D) in 308 cells was also higher when compared with the normal counterpart C50 cells. C50 cells showed delayed and less increase in caspase 3 activity.

Fig. 1. p53 levels associated with enhanced apoptosis in 308 cells. (A) A time course study of immunofluorescence staining of p53 proteins in 308 cells after TPA treatment. Green, p53 staining; red, Mitotracker Red as mitochondrial marker. (B) A representative western blot showing increased p53 levels at 1 and 24 h after TPA treatment. Ctrl, cells treated with vehicle alone. The levels of actin were used as loading control. (C) p53 activation was associated with early and enhanced increase in caspase 3 activity and (D) apoptotic cell death detected by nucleosome fragmentation. *P < 0.05; **P < 0.01, when compared with its own control.
H-ras mutation promoted p53 activation, through NOX in 308 cells

It has been suggested that the NOX system of neutrophils rather than the epithelial xanthine oxidase system is dominant for the oxidative stress in TPA-treated mouse skin (5). To determine the role of NOX activity in the activation of p53 after TPA treatment in skin keratinocyte 308 cells, the NOX inhibitor DPI, which inhibits flavoenzymes such as the gp91phox subunit of NOX, was used. Figure 2A shows that TPA treatment increased NOX activity; DPI alone or DPI in combination with TPA reduced NOX activity to virtually

![Graph showing NOX activity over time](image)

**Fig. 2.** Suppression of NADPH-oxidase by DPI. (A) NOX activity in cells treated with TPA (100 nM) in the presence or absence of DPI (1 μM). (B) DCF fluorescence in 308 cells after DPI (1 μM) and/or TPA (100 nM) treatment. Cells were incubated with DPI for 24 h, and then medium was replaced with fresh medium containing TPA. (C) NOX inhibitor DPI suppressed TPA-mediated p53 increase in 308 cells. Cells were treated with various doses of DPI for 24 h before TPA treatment for 1 h. A representative western blot was shown. The levels of actin were used as loading control. (D) DPI suppressed TPA-induced nucleosome fragmentation. The 308 cells were treated with two doses of DPI (0.5 and 1 μM) for 24 h before TPA treatment for 24 h. (E) Quantitative analysis of Rac1 levels. The 308 cells were transfected with siRNAs for 24 h followed by TPA treatment for 24 h. (F) Increased p53 by TPA was suppressed by Rac1 siRNA. Ctrl, cells treated with vehicle alone. *P < 0.05; **P < 0.01 when compared with vehicle control group; **P < 0.01 when compared with TPA alone group.
undetectable levels. Consistent with the increased NOX activity observed, TPA treatment increased intracellular production of ROS as measured by DCF oxidation, which was significantly reduced by pre-treatment with DPI (Figure 2B). Pre-incubation with DPI decreased TPA-induced p53 expression in a dose-dependent manner (Figure 2C). Importantly, apoptosis induced by TPA treatment was also significantly inhibited by even the lowest doses of DPI tested (Figure 2D), supporting the role of NOX in mediating p53 activation and subsequent apoptosis in skin keratinocytes after TPA treatment.

In order to directly test the relationship among H-ras mutation, NOX activation and p53-mediated apoptosis in 308 cells after TPA treatment, siRNA to Rac1 was used to suppress NOX in 308 cells. Rac1, as a cytosolic subunit of NOX complex, is also regulated by Ras through the Ras/phosphoinositide-3

Fig. 3. NOX-mediated increased p53 levels and apoptosis in vivo. (A) The detection of H-ras mutation at codon 61 (A→T). M, 50 bp DNA ladder; minus, PCR products without adding XbaI; and plus, PCR products cleaved by XbaI. (B) Increased protein carbonyl levels in skin epidermis. *P < 0.05, when compared with its control; ##P < 0.01 when compared with TPA treatment. (C) Quantification of mitochondrial p53 in skin epidermis. ***P < 0.01, when compared with its control; ##P < 0.01 when compared with TPA treatment. (D) Quantitative analysis of apoptotic cells in skin epidermis. *P < 0.05 when compared with vehicle control; **P < 0.05 when compared with TPA treatment. Ctrl, cells treated with vehicle alone.
kinase (PI3K)/Rac pathway. As shown in Figure 2E, TPA treatment did not induce Rac1 expression, but its level was significantly suppressed by siRNA approach. Consistent with the role of NOX in p53 activation, Rac1 siRNA but not control siRNA suppressed TPA-mediated increase in p53 levels (Figure 2F).

**NOX-mediated p53 activation and apoptosis in skin epidermis after DMBA/TPA treatments**

To verify the mechanism identified in skin keratinocytes in vitro, mouse skin was treated with DMBA followed by treatments with TPA or DPI in combination with TPA. RT–PCR analysis of DNA isolated from mouse epidermal tissue showed that mice receiving DMBA application exhibited \( \text{H-ras} \) mutation (A→T mutation at codon 61), which creates a new restriction endonuclease enzyme (XbaI site) in the skin epidermal tissues (Figure 3A). As early as 24 h after TPA application, oxidative stress was significantly increased as indicated by the levels of protein oxidation products—protein carbonyls. Without TPA application, DMBA alone or DMBA plus DPI did not change the level of protein oxidation (Figure 3B); DMBA plus TPA treatment caused a 3-fold increase in protein carbonyl levels, whereas DPI completely blocked TPA-induced protein oxidation. Examination of mitochondrial p53 levels showed no significant change in p53 expression after DMBA, alone or combined with DPI treatment. TPA induced a 2-fold increase in p53 levels, whereas DPI reduced that increase to the control level (Figure 3C). In agreement with these biochemical studies, quantitative histological analysis revealed that there was no significant difference in percentage of cell apoptosis among vehicle control, DMBA alone and DMBA + DPI groups; an ~2-fold increase in cell apoptosis in DMBA + TPA group was reduced to control level after receiving DPI pre-treatment (Figure 3D).

**Discussion**

Multistage skin carcinogenesis is a well-studied cancer model. Among the factors postulated to contribute to the development of skin carcinogenesis, reduction of oxidative stress-mediated events has become an attractive mechanism for cancer prevention. Our studies have demonstrated that overexpression of manganese superoxide dismutase (MnSOD), in the front line of antioxidant defense enzymes, reduced tumor incidence in a skin carcinogenesis model (1). Unexpectedly, MnSOD deficiency did not significantly reduce tumor incidence (2). These findings can be explained, in part, by the fact that together with an increase in oxidative stress in DMBA/TPA treated mouse skin, there is an increase in apoptosis and tumor suppressor p53 levels. In addition, a fraction of p53 is found to rapidly translocate into mitochondria (2,3).

In order to determine how oncogenic activation, oxidative stress and p53-associated apoptosis are related, skin keratinocytes carrying a Ras mutation (308 cells) together with non-transformed skin keratinocytes (C50 cells) were used in this study. p53 was more active in Ras mutated skin epidermal cells, as the increase in p53 levels and mitochondrial translocation were only observed in 308 cells, not in skin keratinocyte C50 cells after TPA treatment. Although apoptosis in C50 cells increased, its occurrence was delayed and the magnitude was smaller compared with 308 cells.

The tumor promoter TPA has been found to stimulate ROS generation by activation of NOX in skin (6,7) and non-skin cells (8–10). Blocking NOX activity by DPI leads to reduction of p53 levels and reduces apoptosis, suggesting that ROS generated by TPA and subsequent apoptosis in skin keratinocytes may be mediated by NOX.

Since only \( \text{H-ras} \) activated 308 cells showed an increase in p53 after TPA treatment, how Ras activation enhanced p53 levels was further studied. Although oncogenic Ras has been demonstrated to suppress p53 by enhancing its degradation via Mdm2 in a Raf-dependent manner (11), oncogenic Ras signaling has also been found to contribute to apoptosis. Thus, the contributions of Ras family members to tumorigenesis remain unclear (12). One possible mechanism is that Ras contributes to ROS generation. Two pathways leading to ROS generation have been proposed: Ras/PI3K/Rac1/NOX pathway or Ras/mitogen-activated protein kinase (MAPK)/NOX pathway (13).

We have chosen to investigate the Rac1-mediated pathway, because (i) Rac can also activate Ras independently of PI3K through direct interaction with Rac (12); (ii) as one of the rho family of small Guanosine triphosphate (GTP) binding proteins, the role of Rac1 in the production of ROS in phagocytic cells such as neutrophils is well established (14); (iii) Rac proteins are essential for the assembly of the plasma membrane NOX that is responsible for the transfer of electrons to molecular oxygen leading to the production of superoxide anions during the respiratory burst; and (iv) Rac proteins, in particular Rac1, serve a similar function in non-phagocytic cells (15,16).

Our results, using both pharmacological inhibitor and siRNA to Rac1 approaches, strongly support the hypothesis that Rac1 is a mediator for TPA-mediated p53 activation.

Our findings that mouse skin pre-treated with DPI has a reduced level of ROS, exemplified by reduced protein carbonyl adducts and reduced levels of mitochondrial translocation of p53, demonstrate that the role of NOX activation in apoptosis also occurs in vivo. In agreement with the biochemical studies, in vivo histological examination confirmed that DMBA/TPA caused keratinocyte apoptosis, whereas blocking NOX suppressed this effect.

Taken together, our results demonstrate that NOX serves as a link between Ras mutation and p53 activation, suggesting that oxidative stress plays a central role in modulating oncogenic and tumor suppressive signals during skin carcinogenesis.

**Acknowledgements**

The authors wish to thank Ms Jimie Swanlund for technical assistance. This work was supported by NIH grants CA 73599, CA 73599-S1 and AG-08938. L.C. is partially supported by the Thailand Research Fund under the Golden Jubilee Program. This work was also partially supported by the use of resources and facilities of William S. Middleton Veteran Administration Hospital, Madison, WI 53705, USA.

**Conflict of Interest Statement**: None declared.

**References**


Received March 15, 2006; revised March 15, 2006; accepted March 23, 2006