

Deficiency of NRH:Quinone Oxidoreductase 2 Increases Susceptibility to 7,12-Dimethylbenz(a)anthracene and Benzo(a)pyrene-Induced Skin Carcinogenesis

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Abstract

NRH:Quinone oxidoreductase 2 (NQO2) is an enzyme that catalyzes the reductive metabolism of quinones. C57BL/6 NQO2^{-/-} mice lacking NQO2 gene expression were generated in our laboratory. The dorsal skin of NQO2-deficient mice was exposed to 7,12-dimethylbenz(a)anthracene (DMBA) or benzo(a)pyrene alone (complete carcinogen) or with 12-O-tetradecanoylphorbol-13-acetate (TPA) (initiation/promotion model) to determine the *in vivo* role of NQO2 in chemical carcinogenesis. The NQO2^{-/-} mice showed significantly increased tumor frequency with DMBA + TPA when compared with their wild-type littermates. The benzo(a)pyrene + TPA also showed increase in tumor incidence in NQO2^{-/-} mice but to a less extent than DMBA. DMBA alone resulted in low frequency of tumor development with no difference in susceptibility between wild-type and NQO2^{-/-} mice. Benzo(a)pyrene alone failed to induce tumors in either wild-type or NQO2^{-/-} mice. Histologic analysis of the NQO2^{-/-} mice tumors demonstrated proliferative activity. The treatment of NQO2^{-/-} mice skin with benzo(a)pyrene failed to significantly increase tumor suppressor protein p53 and p53-regulated growth-related protein p21 and proapoptotic protein Bax as observed in case of wild-type mice. These results demonstrate that NQO2 protects against DMBA- and benzo(a)pyrene-induced skin carcinogenesis and suggest that NQO2 protection might be against tumor promotion. The results also suggest that lack of induction of p53, p21, and Bax proteins might contribute to increased sensitivity of NQO2^{-/-} mice skin to benzo(a)pyrene carcinogenicity.

Introduction

Polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene are known environmental contaminants. They are present in tobacco smoke, motor vehicle exhaust, and produced during burning of carbohydrates, fat, and proteins (1, 2). DMBA and benzo(a)pyrene both are recognized mutagens and carcinogens in human and rodents (2, 3).

Quinone oxidoreductases are cytosolic enzymes that catalyze metabolic reduction of quinones and its derivatives (4–6). Two cytosolic forms of quinone oxidoreductases [NAD(P)H:quinone oxidoreductase 1 (NQO1) and NRH:quinone oxidoreductase 2 (NQO2)] have been purified and cloned from mouse and human (4). NQO2 (231 amino acid residues, *M_r* 25,956) is 43 amino acids shorter at its COOH terminus than the NQO1. The cofactor requirement for protein activity is very selective, pointing out dihydronicotinamide riboside (NRH) for NQO2 and NAD(P)H for NQO1 as an electron donor (7, 8). NQO2 is the only enzyme known that accepts electrons from NRH. NRH is a degradation product of NAD(P)H inside the cells (8). However, other physiologic functions of NRH, except its function as

electron donor for NQO2, remain unknown. Although NQO2 is resistant to typical inhibitors of NQO1 activity, such as dicoumarol, cibacron blue, and phenindone, NQO2 is inhibited by flavones such as quercetin (7). Benzo(a)pyrene is another known inhibitor of NQO2 (8). Although overlapping substrate specificities have been observed for NQO1 and NQO2, significant differences exist in relative affinities for the various substrates (7, 9). The crystal structure of NQO2 revealed that it contains a specific metal binding site not present in NQO1 (10). Cellular studies have shown a role of NQO2 in metabolic activation of CB1954 leading to cytotoxicity and cell death (9).

The human NQO2 gene has been precisely localized to chromosome 6p25 and its gene locus is highly polymorphic (11). However, all polymorphisms detected thus far are in the intronic regions or are nonsense mutations that do not alter amino acids (11, 12).

NQO1^{-/-} and NQO2^{-/-} mice were generated (13, 14). The mice deficient in NQO1 and NQO2 gene expression were born and developed normally compared with heterozygotes and wild-type littermates, indicating that NQO1 and NQO2 do not play a role in mouse development. Additional studies on NQO1-null mice revealed altered intracellular redox status and altered metabolism of carbohydrates, fatty acids, and nucleotides and reduced accumulation of abdominal fat with age (15). The studies also demonstrated that disruption of NQO1 gene in mice led to myelogenous hyperplasia of bone marrow and increased sensitivity of NQO1^{-/-} mice to menadione-induced hepatic damage (13, 16). NQO1-null mice also demonstrated significantly increased sensitivity to skin carcinogenesis in response to benzo(a)pyrene (17) and DMBA (18). Similar to NQO1^{-/-} mice, myeloid hyperplasia of bone marrow was detected in NQO2^{-/-} mice (14). However, in contrast to NQO1^{-/-} mice, NQO2^{-/-} mice showed decreased sensitivity to menadione-induced hepatic toxicity, suggesting that NQO2 catalyzed metabolic activation of menadione (14). The sensitivity of NQO2-null mice to chemical carcinogenesis remains unknown.

In this study, we used NQO2^{-/-} mice along with wild-type mice in tumor initiation and promotion model to investigate the sensitivity of NQO2^{-/-} mice to chemical carcinogenicity. Here, we report that the NQO2-null mice demonstrated increased susceptibility to DMBA and benzo(a)pyrene-induced skin carcinogenesis compared with wild-type mice. In other words, NQO2 provided protection against chemical carcinogenesis. The results also suggested that NQO2 may protect against promotion rather than initiation of carcinogenesis. The results additionally suggested that the lack of induction of p53, p21, and Bax proteins contribute to increased sensitivity of NQO2^{-/-} mice to benzo(a)pyrene carcinogenicity.

Materials and Methods

Chemicals. DMBA, benzo(a)pyrene, and 12-O-tetradecanoyl phorbol-13-acetate (TPA) were purchased from Sigma Co. (St. Louis, MO). The 10% neutral buffered formalin for fixing tissues and acetone for dissolving the DMBA and TPA were purchased from Fisher Scientific (Houston, TX).

NQO2^{-/-} and Wild-Type Mice. NQO2^{-/-} mice were generated in our laboratory (14). The wild-type and NQO2^{-/-} mice were housed in polycar-

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bonate cages in the animal facility at Baylor College of Medicine. The mice were maintained in a 12-hour light/dark cycle rooms, a temperature of $24 \pm 2^\circ\text{C}$, a relative humidity of $55 \pm 10\%$, and a negative atmospheric pressure. The mice were fed standard rodent chow and acidified tap water *ad libitum*. Six to 8-week-old mice were used for this experiment. Baylor College of Medicine and Institutional Animal Care and use committee approved the safety protocol. The animals received humane care throughout the experiment according to Baylor College of Medicine and the program of animal care and use, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care guideline.

DMBA, Benzo(a)pyrene, and TPA Application. Six to 8-week-old wild-type and NQO2^{-/-} mice were used. Twenty (half male and half female) mice were used in each group. The backs of mice were shaved using a hair clipper. Two days later, 0, 200, 400, and 600 nmol of DMBA, dissolved in 100 μL of acetone, or 400, 800, and 1600 nmol of benzo(a)pyrene, dissolved in DMSO were applied topically on the shaved area of the dorsal skin. The control mice received vehicle only. A week after DMBA or benzo(a)pyrene application, mice were skin painted with 10 μg of TPA dissolved in 200 μL of acetone twice weekly at the site of DMBA and benzo(a)pyrene application for 20 weeks. Mice were examined twice weekly for tumor formation, and pictures were taken. At week 30, mice were euthanized, and tumor samples were taken for histologic analysis.

Histopathology. Skin samples with or without tumors from NQO2^{-/-} and wild-type mice were obtained after euthanasia. Skin samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned 5- μm , and stained with H&E. Histologic sections were evaluated for skin tumors and other abnormalities.

Analysis of p53, p21, and Bax in the Skin. Seven to 9-week-old C57BL6 NQO2^{-/-} and wild-type mice were used. The lower backs of the mice were shaved using hair clippers. Two days later, various concentrations of benzo(a)pyrene (0, 800, and 1600 nmol) dissolved in acetone were applied on the shaved mice skin. The control mice received acetone only. Twenty-four hours later, wild-type and NQO2^{-/-} mice were sacrificed. Skin samples were removed by surgery. The skin tissue was homogenized in ice-cold buffer containing 20 mmol/L HEPES, 500 mmol/L NaCl, 20% glycerol, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.1% NP40, and a mixture of protease inhibitors including 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 1 $\mu\text{g}/\text{mL}$ pepstatin, aprotinin, leupeptin, and antipain (all from Sigma Chemical Co. St. Louis). One-hundred micrograms of various skin protein samples were separated on SDS 12% polyacrylamide gels, blotted on the enhanced chemiluminescence membranes, and probed with antibodies against tumor suppressor proteins p53 (CM5; Novacastra, Newcastle Upon Tyne, UK), p21, and Bax (PharMingen, San Jose, CA). Western blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) reagents by the procedures suggested by the manufacturer.

Results and Discussion

NQO2^{-/-} mice were generated in our laboratory and bred at Baylor College of Medicine (Houston, TX; ref. 14). Mice lacking NQO2 gene expression showed no detectable developmental abnormalities and were indistinguishable from wild-type mice. The NQO2 activities were measured in skin of wild-type and NQO2^{-/-} mice by procedures as described previously (18). NQO2 activity in male mice skin was 3.7 ± 0.9 units (1 unit is equivalent to 1 mmol/L 2,6-dichlorophenolindophenol reduced/minute/mg cytosolic protein). Female mice showed similar levels of NQO2 in skin as male mice. NQO2 activity was not detected in the skin of male or female NQO2^{-/-} mice. This result was similar as previously reported for several other tissues (14). Western analysis using anti-NQO2 antibodies showed presence of M_r 26,000 NQO2 protein in the skin of wild-type mice that was absent in NQO2^{-/-} mice (data not shown).

The wild-type and NQO2^{-/-} mice were treated with DMBA alone or DMBA followed by TPA. The treatment with 500, 750, and 1000 nmol of DMBA alone produced low frequency (2 of 20) of skin tumors in wild-type and NQO2^{-/-} mice. No difference in tumor incidence was observed between wild-type and NQO2^{-/-} mice with

all these doses. However, NQO2^{-/-} mice in two-stage (initiation/promotion) carcinogenesis studies demonstrated significant increase in susceptibility to DMBA-induced skin tumors as compared with wild-type mice (Table 1; Fig. 1, A and B). The differences were with respect to time and frequency of tumors developed and incidence of multiple tumors/mouse. The treatment of wild-type mice with 200 nmol of DMBA followed by TPA resulted in no tumors by week 15. Twenty-five percent of these mice developed tumors at week 25. Similar treatment of NQO2^{-/-} mice resulted in tumors in 15% of mice at week 15, increasing to tumors in 80% of the mice at week 25. Similar differences between wild-type and NQO2^{-/-} mice were also observed with 400 and 600 nmol of DMBA followed by TPA treatment. Eighty percent of NQO2^{-/-} mice treated with 600 nmol of DMBA had skin tumors on week 15 and all of them had tumors by week 25, compared with 10 and 30% in wild-type mice (Table 1).

Analysis of the tumor frequency with all treatments at week 25 reveal that >85% of NQO2^{-/-} mice had skin tumors compared with only 25% wild-type mice (Table 1). In addition to the higher number of NQO2^{-/-} mice with tumors, NQO2^{-/-} mice also had more tumors per mouse than wild-type mice (Fig. 1B). Approximately 50% of NQO2^{-/-} mice had multiple tumors by week 25, compared with <10% in wild-type mice (Table 1). Eleven of 17 NQO2^{-/-} mice, treated with 600 nmol of DMBA, had more than one skin tumor whereas only two of twenty wild type mice, treated with the same dose, had multiple tumors (Table 1). The average number of tumors per NQO2^{-/-} mouse was two tumors per mouse as compared with half tumor per wild-type mouse (Table 1).

Histologic evaluation of skin of NQO2^{-/-} and wild-type mice revealed papillomas, areas of epidermal hyperplasia, and areas of melanocyte hyperplasia (Fig. 1, C and D). The lesions in NQO2^{-/-} mice were more extensive than in wild-type mice. The incidence of broad base papillomas was higher in NQO2^{-/-} mice.

Benzo(a)pyrene alone failed to produce tumors in wild-type or NQO2^{-/-} mice. The benzo(a)pyrene also failed to produce tumors in wild-type mice in two-stage (initiation/promotion) carcinogenesis experiments (Table 2). However, in similar experiments, benzo(a)pyrene + TPA treatment developed tumors in NQO2^{-/-} mice. Treatment with 400 and 800 nmol of benzo(a)pyrene resulted in tumors in 10% of NQO2^{-/-} mice that increased to 20% of mice with 1600 nmol of benzo(a)pyrene by 30 weeks.

These results demonstrate that NQO2 protect mice against DMBA and benzo(a)pyrene carcinogenesis. This protection was observed in two-stage (initiation/promotion) carcinogenesis but not in initiation protocols. Therefore, it is possible that NQO2 protection is against promotion and not initiation. These results contrasts with those from NQO1^{-/-} mice, which protects against initiation and promotion of carcinogenesis (17, 18). The role of NQO1 in protection against initiation is due to its ability to detoxify chemicals, prevent oxidative stress, and reduce mutagenesis (4). Previous studies show that NQO2 is an activating and not detoxifying enzyme (12, 14). Therefore, it is

Table 1 Mouse skin tumors developed after a single dose of DMBA followed by TPA

DMBA concentration (nmol)	Tumor incidence at 15 week		Tumor incidence at 25 week*	
	Wild type	NQO2 ^{-/-}	Wild type	NQO2 ^{-/-}
200	0/20	3/20 [$P < 0.1$]	5/20 (1)	16/20 (8) [$P < 0.001$]
400	1/20	5/20 [$P < 0.1$]	5/20 (2)	17/20 (7) [$P < 0.001$]
600	2/20	14/17 [$P < 0.001$]	6/20 (2)	17/17 (11) [$P < 0.001$]

NOTE. Six to 8-week-old C57BL6 (wild type and NQO2^{-/-}) mice were shaved on their backs, and a single dose of DMBA was applied topically, followed by twice weekly application of TPA. Ten micrograms of TPA were used in a single application. The mice were compared at weeks 15 and 25 after DMBA application. Similar experiments with acetone or TPA did not produce skin tumors in wild-type and NQO2^{-/-} mice.

* Mice with multiple tumors are shown in parentheses.

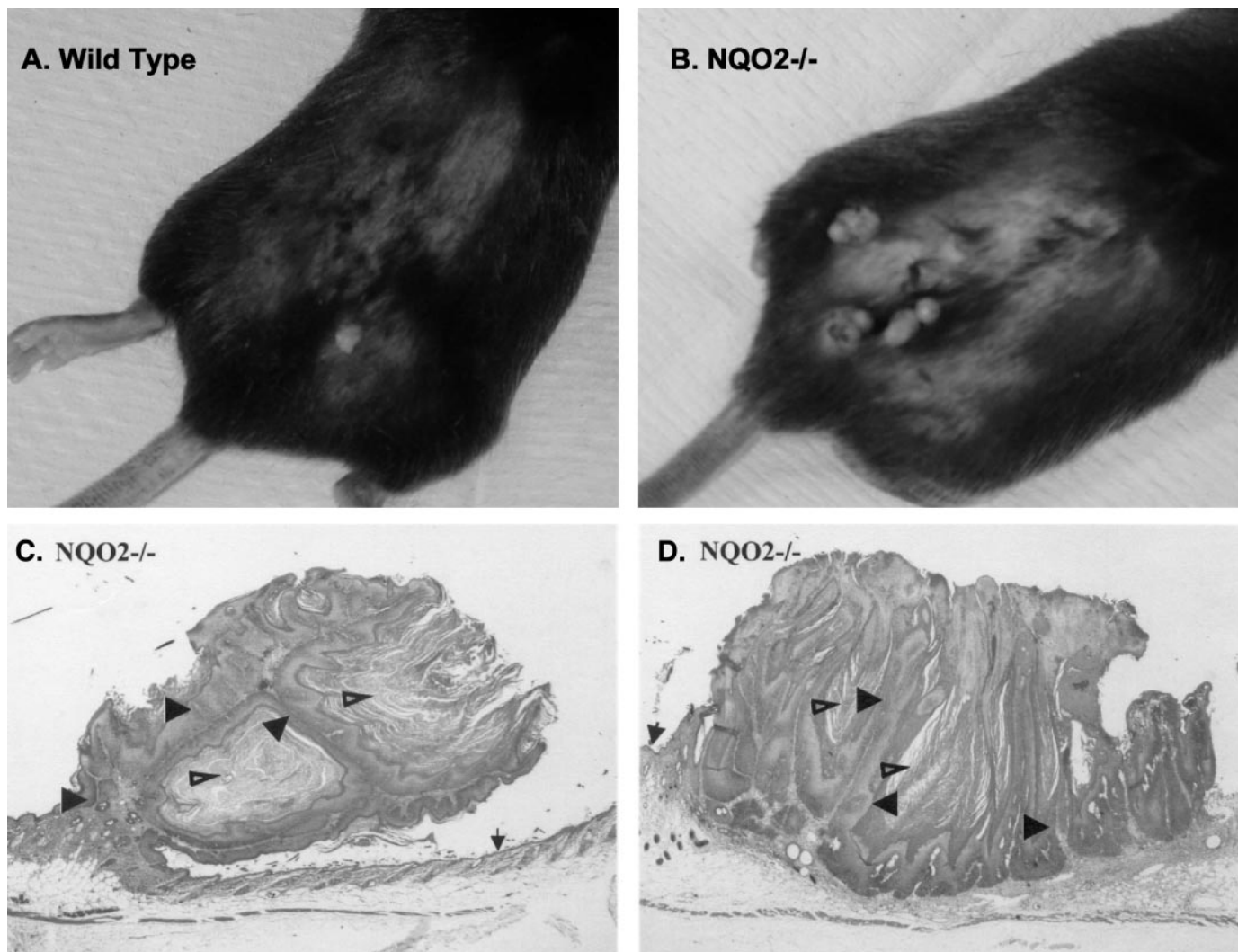


Fig. 1. Skin tumors in wild-type and NQO2^{-/-} mice 25 weeks after DMBA application. Skin tumors on the dorsal skin of wild type (A) and NQO2^{-/-} (B) mice. H&E-stained histologic sections in the skin tumors of NQO2^{-/-} exposed to 600 nmol of DMBA + TPA (C and D). Epidermal papilloma narrow based (C) and epidermal papilloma broad based (D) are shown. *Thin arrow*, normal epidermis; *arrowheads*, epidermal hyperplasia/acanthosis; *open arrowheads*, hyperkeratosis.

not surprising that NQO2 does not appear to play a role in protection against tumor initiation. This is the first report showing a role for NQO2 in protection against chemical carcinogenesis.

The studies raise significant questions regarding the mechanism of this protection. One or more mechanisms may contribute to the NQO2 protection against chemical carcinogenesis. Loss of NQO2 is expected to accumulate electron donor NRH in the cytosol. This might alter intracellular redox status, leading to altered metabolic pathways using NRH and alterations in factors that regulate cell growth, differentia-

tion, apoptosis, and proliferation. It may be noteworthy that disruption of NQO1 gene in mice is known to lead to accumulation of NAD(P)H, altered metabolic pathways, decreased tumor suppressor p53, and reduced apoptosis in bone marrow (16). These alterations result in hyperplasia of bone marrow. NQO2^{-/-} mice also showed myeloid cell hyperplasia of bone marrow (14). Cellular studies that used dicoumarol to inhibit NQO1 activity have shown that NQO1 binds to p53 and protects p53 from degradation in MDM2/ubiquitin-independent pathway (19, 20). Therefore, it is possible that accumulation of NRH in NQO2^{-/-} mice also alters p53 stability and apoptosis. Indeed, Western analysis of wild-type and NQO2^{-/-} mice skin revealed lower levels of tumor suppressor protein p53 and p53-regulated growth-related protein p21 and proapoptotic protein Bax (Fig. 2). Benzo(a)pyrene treatment of wild-type mice skin showed significant increases in p53, p21, and Bax proteins (Fig. 2). The increases were benzo(a)pyrene concentration dependent. Interestingly, similar treatment of benzo(a)pyrene failed to significantly induce p53, p21, and Bax proteins in skin of NQO2^{-/-} mice (Fig. 2). The lack of induction of already low levels of p53, p21, and Bax in response to benzo(a)pyrene treatment in NQO2^{-/-} mice presumably led to compromised growth arrest and apoptosis in NQO2-deficient

Table 2. Mouse skin tumors developed after a single dose of benzo(a)pyrene followed by TPA

Benzo(a)pyrene concentration (nmol)	Tumor incidence at 20 week		Tumor incidence at 30 week	
	Wild type	NQO2 ^{-/-}	Wild type	NQO2 ^{-/-}
400	0/20	0/20	0/20	2/20 [<i>P</i> < 0.1]
800	0/20	2/20 [<i>P</i> < 0.1]	0/20	2/20 [<i>P</i> < 0.1]
1600	0/20	4/20 [<i>P</i> < 0.05]	0/20	4/20 [<i>P</i> < 0.05]

NOTE. Six to 8-week-old C57BL6 (wild type and NQO2^{-/-}) mice were shaved on their backs, and a single dose of benzo(a)pyrene was applied topically, followed by twice weekly application of TPA. Ten micrograms of TPA were used in a single application. The mice were compared at weeks 20 and 30 after DMBA application. Similar experiments with DMSO or TPA did not produce skin tumors in wild-type and NQO2^{-/-} mice.

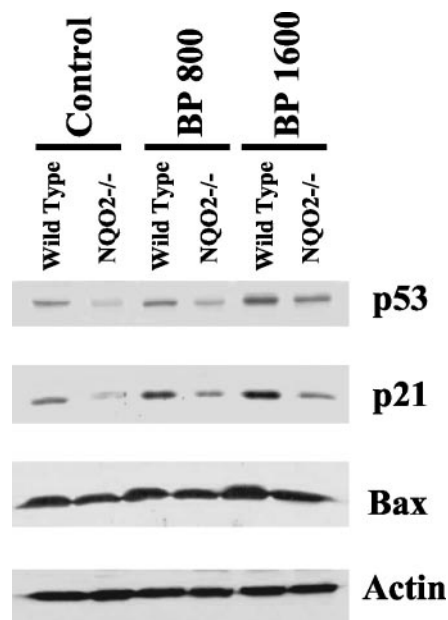


Fig. 2. Seven to 9-week-old wild-type and NQO2^{-/-} mice skin were exposed to acetone alone (control) or 800 nmol of benzo(a)pyrene (BP800) or 1600 nmol of benzo(a)pyrene (BP1600) dissolved in acetone. The mice were shaved on their backs and painted with chemicals. The mice were sacrificed 24 hours after skin painting of the chemical and painted skin removed by surgery. The skin tissues were homogenized in appropriate buffer containing protease inhibitors. One-hundred micrograms of skin homogenates were separated on 12% SDS PAGE, Western blotted, and probed with antibodies as indicated. Western blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech).

mice. This in turn contributed to increased benzo(a)pyrene carcinogenicity in NQO2^{-/-} mice as compared with wild-type mice. A similar mechanism is also expected for increased sensitivity of NQO2^{-/-} mice skin to DMBA and other chemical carcinogenesis. In addition, NQO2^{-/-} mice with hematologic abnormalities might also have immunologic abnormalities. The impaired immune response might have compromised regulatory factors during tumor progression in NQO2^{-/-} mice.

In conclusion, the present studies demonstrate that NQO2 acts as an endogenous factor in protection against DMBA- and benzo(a)pyrene-induced skin carcinogenesis. The results also suggest that NQO2 protection might be against tumor promotion. The results additionally suggest that the lack of induction of p53, p21, and Bax proteins in response to benzo(a)pyrene in NQO2^{-/-} mice presumably contribute to decreased growth arrest and apoptosis, leading to increased sensitivity of NQO2^{-/-} mice to benzo(a)pyrene and other chemicals carcinogenicity. Additional studies are needed to elucidate the exact mechanism of NQO2 protection against chemical carcinogen-induced tumorigenesis.

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