Lower Induction of p53 and Decreased Apoptosis in NQO1-Null Mice Lead to Increased Sensitivity to Chemical-Induced Skin Carcinogenesis

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a cytosolic protein that catalyzes metabolic detoxification of quinones and protects cells against redox cycling and oxidative stress. NQO1-null mice deficient in NQO1 protein showed increased sensitivity to 7,12-dimethylbenz(a)anthracene– and benzo(a)pyrene-induced skin carcinogenesis. In the present studies, we show that benzo(a)pyrene metabolite benzo(a)pyrene-trans-7,8-dihydriodiol-9,10-epoxide and not benzo(a)pyrene quinones contributed to increased benzo(a)pyrene-induced skin tumors in NQO1-null mice. An analysis of untreated skin revealed an altered intracellular redox state due to accumulation of NADH and reduced levels of NAD/NADH in NQO1-null mice as compared with wild-type mice. Treatment with benzo(a)pyrene failed to significantly increase p53 and apoptosis in the skin of NQO1-null mice when compared with wild-type mice. These results led to the conclusion that altered intracellular redox state along with lack of induction of p53 and decreased apoptosis plays a significant role in increased sensitivity of NQO1-null mice to benzo(a)pyrene-induced skin cancer. (Cancer Res 2005; 65(6): 2054-8)

Introduction

Polycyclic aromatic hydrocarbons (PAH), including 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (BP), are environmental carcinogens (1). PAHs undergo metabolic activation to exert their carcinogenic effects (2). Cytochromes P450 oxidize PAHs to generate epoxides in the bay region of PAHs (2–4). Epoxide hydrolase converts epoxides to less reactive diols. The diols are further metabolized to produce ultimate carcinogenic diol epoxides (5). DMBA is metabolically activated to bay-region epoxides that react with purines in DNA, leading to tumorigenesis and carcinogenesis (6). BP is metabolically activated by cytochrome P450 1A1 and epoxide hydrolase to more than two dozen products, including benzo(a)pyrene-trans-7,8-dihydriodiol-9,10-epoxide (BPDE) and benzo(a)pyrene quinones (BPQs); benzo(a)pyrene-3,6-quinone (BP-3,6-Q), benzo(a)pyrene-1,6-quinone (BP-1,6-Q), and benzo(a)pyrene-6,12-quinone (BP-6,12-Q); refs. 1, 2]. BPDE is a known carcinogen whereas the carcinogenicity of quinones remains relatively poorly understood (2, 5).

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a cytosolic flavoprotein that catalyzes metabolic detoxification of quinones including BPQs (7). Interestingly, NQO1 is a phase II enzyme that is coordinately activated in response to chemical carcinogens, antioxidants, oxidants, and radiations (7). Other genes that are coordinately activated with NQO1 include glutathione S-transferases that conjugate hydrophobic electrophiles and reactive oxygen species as well as γ-glutamylcysteiny1 synthetase that plays a role in the glutathione metabolism (8, 9). The coordinated induction of these enzyme genes, including the NQO1 gene, provides protection to cells against electrophilic and oxidative stress and other adverse effects (7–9). More than 2% of the population are homozygous for a mutant allele of NQO1 and do not express NQO1 gene (10, 11). In addition, greater than 20% population carry one mutated NQO1 allele and are deficient in NQO1 protein and activity (10, 11). These individuals might be at increased risk of chemical-induced oxidative stress and other related diseases.

Disruption of the NQO1 gene in mice leads to alterations in the intracellular redox state caused by accumulation of NAD(P)H, altered carbohydrate, lipid and nucleotides metabolism, as well as myelogenous hyperplasia of the bone marrow (12, 13). NQO1-null mice showed decreased levels of p53 and p73 and decreased apoptosis in the bone marrow that contributed to myelogenous hyperplasia of the bone marrow (13). NQO1-null mice also showed significantly increased sensitivity to skin carcinogenesis in response to DMBA and BP (14, 15).

The present studies were designed to investigate the role of BPDE, a known carcinogen, and BPQs in increased susceptibility of NQO1-null mice to BP-induced skin carcinogenicity. We also investigate the mechanism of NQO1 in protection against DMBA and BP skin carcinogenicity in mice.

Materials and Methods

Chemicals. BP, BPDE, and 12-O-tetradecanoylphorbol-13-acetate were purchased from Sigma Chemical Co. (St. Louis, MO). The BPQs (BP-3,6-Q, BP-1,6-Q, and BP-6,12-Q) were purchased from National Cancer Institute Chemical Carcinogen Repository (Kansas City, MO).

NQO1-Null and Wild-type Mice. C57BL6 NQO1-null mice were generated in our laboratory (16). C57BL6 wild-type and C57BL6 NQO1-null mice were bred and housed in Baylor College of Medicine animal facility, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice from 21st generation of breeding were used in the current studies. The mice were kept in polycarbonate cages individually ventilated by BCM program of animal care and use with a 12-hour light/dark cycle, a temperature of 24 ± 2°C, a relative humidity of 55 ± 10%, and a negative atmospheric pressure. Animal care and studies were approved

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**Benzo(a)pyrene-Trans-7,8-Dihydrrodiol-9,10-Epoxide and Benzo(a)-pyrene Quinones Induced Skin Carcinogenesis.** Seven- to nine-week-old C57BL/6 NQO1-null and wild-type mice were used. The lower backs of mice were shaved using hair clippers. Mice that are not in the hair-growing phase were selected. The various concentrations of BPDE (0, 500, and 1,000 nmol), BP-3,6-Q (0, 500, 1,000, and 3,000 nmol), BP-1,6-Q (0, 500, 1,000, and 3,000 nmol), or BP-6,12-Q (0, 500, 1,000, and 3,000 nmol) in DMSO were applied on mice skin 2 days after shaving. The control mice received DMSO alone. The mice then received twice-weekly applications of 10 μg 12-O-tetradecanoylphorbol-13-acetate for 20 weeks starting 1 week after BPDE and BPQ treatment. Mice were observed weekly for development of skin tumors.

**Histologic Examination of Benzo(a)pyrene-Trans-7,8-Dihydrrodiol-9,10-Epoxide-Induced Skin Tumors.** The mice were euthanized and tumors excised, fixed, processed, paraffin sectioned, stained with H&E, and analyzed under microscope.

**Benzo(a)pyrene Treatment and Analysis of TumorSuppressor p53 and Apoptosis.** In a similar experimental protocol as described above, the BPDE was replaced with 1,200 nmol of BP dissolved in acetone and painted on skin. The control mice received acetone alone. The mice were euthanized 24 hours after the chemical treatment. The dose of BP used is known to produce skin tumors in NQO1-null mice but not in wild-type mice (14). Wild-type and NQO1-null mice were sacrificed and skin sections were taken. Skin samples were fixed, processed, paraffin sectioned, and analyzed by immunohistochemistry using anti-p53 CM5P antibody according as per instructions of the manufacturer (Novacastra, Newcastle Upon Tyne, United Kingdom). The skin sections were also stained with H&E for analysis of apoptotic cells. Ten random fields were selected, p53-positive and apoptotic cells were counted and the average numbers of p53-positive apoptotic cells were calculated for untreated control and benzo(a)pyrene-treated wild-type and NQO1−/− skin samples.

**NADH/NAD Ratio in Skin.** The following procedure was used to collect and process the tissue for determination of NADH and NAD because of sensitivity of these molecules. Following euthanization, skin sections were collected from wild-type and NQO1−/− mice and instantly placed in liquid nitrogen. While frozen, the skin were cut using a surgical blade and homogenized in a solution containing 200 mmol/L KCN, 1 mmol/L b bathophenanthroline, and 60 mmol/L KOH. The pyridines were extracted with chloroform and analyzed from these tissues by procedures as previously described (17). Briefly, the homogenate was extracted rapidly with chloroform several times until minimal precipitate was observed at the interface of the buffer and chloroform. The supernatant was then passed through a 0.45-μm Ultrasep-MC filtration device (Millipore, Bedford, MA) by centrifuging at 5,000 rpm for 10 minutes at 4°C. This procedure removed the residual DNA/protein and also served as a filter before the samples are loaded onto the HPLC column. The pyridine nucleotides were separated, analyzed, and quantitated using a C18 chromatography column (Waters Corp., Milford, MA) and HPLC (Waters). The mobile phase consisted of 50 mmol/L potassium phosphate buffer (pH 7.5)/acetonitrile (97:3) as suggested by the manufacturer.

**Benzo(a)pyrene Treatment and Western Blot Analysis of the Skin.** The wild-type and NQO1−/− mice skin specimens were treated with 1,200 nmol of BP dissolved in acetone by procedures as described above. The control mice received acetone alone. Five mice were used in each group and skin specimens were collectively analyzed. The mice were euthanized 24 hours after BP treatment. The skin specimens were collected and analyzed for levels of p53, p21, Bax, proliferating cell nuclear antigen, caspase 3, and poly(ADP-ribose) polymerase by Western blot analysis.

Wild-type and NQO1-null mice were sacrificed and skin sections were collected and quickly frozen in liquid nitrogen. The tissue was homogenized in an ice-cold buffer containing 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% Triton X-100, and a mixture of protease inhibitors including 1 mmol/L phenylmethysulfonyl fluoride, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 μg/mL each of pepstatin, aprotinin, leupeptin, benzamidine chloride, and antipain (all from Sigma). Fifty to one-hundred micrograms of various skin protein samples were separated on 12% SDS polyacrylamide gels, blotted onto the enhanced chemiluminescence (Amersham) membranes, and probed with antibodies against tumor suppressor proteins p53 (CM5P from Novacastra), caspase 3, poly(ADP-ribose) polymerase (Cell Signaling Technology, Beverly, MA), p21, Bax, proliferating cell nuclear antigen (PharMingen, San Diego, CA), and NQO1 (rabbit antiserum NQO1 antibodies raised in our lab). Western blots were developed with enhanced chemiluminescence (Amersham; Amersham Pharmacia Biotech, Buckinghamshire, England) reagents by the procedures suggested by the manufacturer.
Results and Discussion

The results are shown in Figs. 1–3. NQO1 is known to catalyze metabolic detoxification of quinones and their derivatives (7). In doing so, NQO1 protects cells against redox cycling, oxidative stress, and possibly neoplasia (7). Therefore, it was reasonable to think that NQO1-null mice might be more susceptible to develop skin tumorigenesis in response to chemicals that are metabolized into quinones. Initial studies with BP supported this hypothesis (14). The NQO1-null mice showed increased susceptibility to skin tumorigenesis in response to BP, which metabolizes to >25 metabolites including BPQs (BP-3,6-Q, BP-1,6-Q, and BP-6,12-Q; refs. 1, 2). Unlike BP, DMBA does not metabolically produce quinines, yet NQO1 protected mice against its carcinogenicity (15). Therefore, these studies suggest that the role of NQO1 in protection against carcinogenicity is against all types of chemicals and not restricted to chemicals that are metabolized to quinines.

The increased sensitivity of NQO1-null mice to BP carcinogenicity was intriguing. However, the previous studies did not show if the protection was against BPDE, a potent carcinogen, or against BPQs, which are substrates for NQO1. In the current report, we investigated the susceptibility of NQO1-null and wild-type mice to BPDE and BPQs to determine their relative contribution to increased BP carcinogenicity in NQO1-null mice. The NQO1-null mice began to develop tumors ~20 weeks after BPDE treatment. At this point in time, no wild-type mouse had developed tumor. By 30 weeks after BPDE treatment, 25% of male NQO1-null mice given 500 nmol BPDE and 60% of male NQO1-null mice given 1,000 nmol of BPDE had developed tumors (Fig. 1A). By 60 weeks after BPDE treatment, the tumor frequency of male NQO1-null mice increased to 50% in the group given 500 nmol of BPDE but remained the same for the male NQO1-null mice treated with 1,000 nmol BPDE. The wild-type male mice given 500 nmol of BPDE failed to develop skin tumors at 20, 40, or 60 weeks. However, 20% of the wild-type mice given 1,000 nmol BPDE had developed tumors 60 weeks after BPDE exposure. Interestingly, female NQO1-null and wild-type mice showed less susceptibility than male mice. None of the female wild-type mice developed tumor in response to BPDE. However, >10% of female NQO1-null mice given 500 and 1,000 nmol of BPDE had developed tumors at week 20. At weeks 30 and 60, this tumor frequency increased to almost 20% in female NQO1-null mice given 500 nmol BPDE but remained same for mice that received 1,000 nmol BPDE. The skin tumors in male NQO1-null mice were...
larger and more numerous compared with treated wild-type male mice (Fig. 1B). These results suggest that both male and female NQO1-null mice are more susceptible to develop BPDE-induced skin tumors compared with wild-type mice. However, the difference in susceptibility was significant between genders. The skin tumors found in NQO1-null and wild-type mice were classified histologically as epithelial in origin, and H&E-stained histologic section of a typical carcinoma from wild-type and NQO1-null male mice is shown (Fig. 1C). Analysis revealed significantly higher numbers of squamous cell carcinomas in NQO1-null mice (Fig. 1C). In contrast to BPDE, the treatment of NQO1-null and wild-type mice with 500, 1,000, and 3,000 nmol of BPQs (BP-3,6-Q, BP-1,6-Q, and BP-6,12-Q) in separate experiments did not develop skin tumors in wild-type or NQO1-null mice (data not shown). This indicated that BPQs may not be carcinogenic in mice. In a related experiment, the C57BL6 NQO1-null mice were replaced with 129SV to investigate BPQ carcinogenicity in a different strain of mice. However, neither the 129SV wild-type nor the NQO1-null mice developed skin tumors in response to BPQs. These combined studies revealed that BPDE and not BPQs contributed to increased tumor susceptibility in NQO1-null mice treated with BP. Interestingly, the comparison of current data with BPDE and previously published data on BP (14) revealed different responses in male and female NQO1-null mice to these chemical agents. The female NQO1-null mice were more sensitive to BP-induced skin carcinogenicity than NQO1-null male mice. However, the female NQO1-null mice were less sensitive to the BP metabolite BPDE. The reason for the gender difference in the response to BP and BPDE is not known.

We used NQO1-null and wild-type mice exposed to acetone (vehicle control) and BP for 24 hours to investigate the mechanism of increased susceptibility of NQO1-null mice to develop skin tumors in response to these chemicals. The treated skin was analyzed for tumor suppressor protein p53 by immunohistochemistry and for apoptotic cells from H&E-stained histologic sections (Fig. 2). The wild-type control and untreated NQO1-null mice skin showed very little to no staining for p53-positive cells. This was expected owing to p53 being known to be present in low amounts in unchallenged cells and induced in response to chemical-induced DNA damage (18). BP treatment resulted in induction of p53-positive cells. However, the magnitude of induction of p53-positive cells was significantly lower in NQO1-null mice as compared with wild-type mice (P < 0.001). Similar results were observed for apoptotic cells (Fig. 2). BP treatment increased apoptosis in both wild-type and NQO1-null mice. Interestingly, the increase in apoptotic cells in NQO1-null mice was significantly lower than wild-type mice (P < 0.01). In a related experiment, NQO1-null mice skin showed increased NADH/NAD ratio and decreased pyridine nucleotide levels (Fig. 3A). Western blot analysis of skin is shown in Fig. 3B. Western blot analysis results clearly supported immunohistologic and histologic observations of significant decrease in induction of p53 and apoptosis in BP-treated NQO1-null mice as compared with wild-type mice [compare Fig. 2 with Fig. 3B (left)]. The induction of proapoptotic proteins p53, Bax, active poly(ADP-ribose) polymerase, and caspase 3 was lower or absent in BP-treated NQO1-null mice when compared with wild-type mice. The growth-related p21 protein followed a similar pattern of expression and induction as p53. Both Bax and p21 are p53 downstream genes and their expression is regulated by p53 (19). Therefore, the alterations in Bax and p21 were expected with alterations in p53. However,
proliferating cell nuclear antigen was more or less the same among untreated and treated wild-type and NQO1-null mice. This was again expected because 24 hr of BP treatment might not alter the rate of proliferation of skin cells in wild-type and NQO1-null mice. Interestingly, NQO1 increased with BP treatment in wild-type mice (Fig. 3B).

The alterations in intracellular redox state caused by accumulation of NADH and the decrease in normal and induced levels of p53 as well as the decrease in apoptosis most likely contributed to increased tumor susceptibility of NQO1-null mice to BP. These findings are intriguing and raise interesting questions regarding the lower amount of basal and induced levels of p53 in NQO1-null mice. In other words, what is the role of NQO1 in maintenance of basal and BP-induced levels of p53? A recent report showed a role of NQO1 in stabilization of p53 (20). The studies also showed that NQO1 stabilizes p53 through a distinct pathway (21), which is independent of Mdm-2 and ubiquitination (22). In addition, a decrease in NAD levels is also known to destabilize p53 (23). NAD has been shown to cause alterations in intracellular redox state and inhibition of pentose phosphate pathway that led to reduced synthesis of NAD(P) (12). In the current report, the increased ratio of NAD(P)/NAD ratios and lower levels of NAD(P) in liver and kidney tissues (12). It was suggested that accumulation of NAD(P)/H caused alterations in intracellular redox state and inhibition of pentose phosphate pathway that led to reduced synthesis of NAD(P) (12). In the current report, the increased ratio of NADH/NAD and/or absence of NQO1-null mice. Therefore, it is possible that p53 was sufficiently induced in response to BP in NQO1-null mice but got degraded because of lower NAD levels. More recently, Anwar et al. (24) reported that NQO1 interacts with p53 and this interaction stabilizes p53. Further studies have shown that p53 hot-spot mutants are resistant to ubiquitin-independent degradation by increased binding to NQO1 (25). This provided additional strength to the conclusion that NQO1 interaction with p53 leads to stability of p53. Therefore, it is also possible that absence of direct interaction of NQO1 with p53 contributed to destabilization of p53 in NQO1-null mice. It is further possible that both lower NAD and absence of interaction of p53 with NQO1 contributed to p53 destabilization and degradation resulting in low basal and BP-induced levels of p53 in NQO1-null mice. The reduced p53 levels and lack of significant induction of p53 in NQO1-null mice led to decreased apoptosis in response to BP. These events in turn contributed to the development of tumors in NQO1-null mice. It may be noteworthy that additional mechanisms involving cellular growth, differentiation, and proliferation might also be involved in increased susceptibility of NQO1-null mice to BP carcinogenesis. These remain to be studied in future.

In conclusion, the present studies showed that BPDE and not BPs contributed to increased susceptibility of NQO1-null mice to develop skin tumors in response to BP. The studies also show that lack of sufficient induction of p53 and apoptosis contributed to increased susceptibility of NQO1-null mice to PAH carcinogenesis. Alterations in intracellular redox state/lower NAD and/or absence of NQO1 interaction with p53 presumably lead to rapid degradation of p53, reduced induction of p53 in response to PAs, and increased tumor susceptibility.

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