Induction of Covalent DNA Modifications and Micronucleated Erythrocytes by 4-Nitroquinoline 1-Oxide in Adult and Fetal Mice


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ABSTRACT

Pregnancy and development are known to modify carcinogenesis. Little is known about the mechanism for the modulation. These studies investigated the relative sensitivity of nonpregnant, pregnant, and fetal mice to the induction of covalent DNA modifications and micronucleated erythrocytes by 4-nitroquinoline 1-oxide (4-NQO). Our results revealed that 4-NQO was bound to guanine nucleotides of DNA in all maternal and fetal organs tested. The adduct levels ranged from 20-60 base modifications per 10^6 DNA bases when 4-NQO was administered s.c. Overall, 4-NQO bound preferentially to DNA of the maternal tissues compared with that of the corresponding fetal tissues, with the exception of the liver. The adduct levels in maternal and fetal organs fell into 3 distinct levels. The greatest binding was in maternal lungs and pancreas (the target organs for carcinogenesis). The lowest binding levels were in maternal liver and all fetal organs studied. Gestation age at the time of 4-NQO treatment did not produce a significant effect on the amounts of adduct formation in the tissues examined, with the exception of placenta and bone marrow. Chronic treatment did not affect binding preference. At the cellular level, 4-NQO treatment induced twice the frequency of micronucleated erythrocytes in the bone marrow of pregnant mice compared with the nonpregnant mice and fetal liver, on a mg/kg basis. However, the polychromatic erythrocytes of fetal liver were more sensitive than those of adult bone marrow to the induction of micronuclei, when adduct levels were taken into account. A positive correlation of organotropism between 4-NQO-induced DNA adducts and carcinogenicity was observed for maternal tissues, but not for fetal tissues. Fetal tissues, overall, lack the enzymes to metabolically activate 4-NQO. Fetal cells elicit greater biological responses, compared with adult cells, at equal adduct levels. This study reveals that the effective doses in maternal and fetal tissues may differ and, therefore, will be a better basis for understanding the molecular mechanism of transplacental carcinogenesis.

INTRODUCTION

DNA damage and repair play important roles in the initiation and modulation of tumor formation. 4-NQO has been shown to be a potent mutagen and carcinogen in many systems (2, 3). When it is administered to adult rodents, it induces tumors in many different tissues including the lungs, pancreas, and stomach (2-4). 4-NQO has been shown to induce lung tumors in the mothers and their transplacentally exposed offspring with different incidences (5). The molecular mechanisms underlying this difference in sensitivity have not been explored. At the cellular level, both sister chromatid exchanges in fetal mouse organs increase after transplacental exposure to 4-NQO (6) and unscheduled DNA synthesis in adult mouse lung (7) has been detected. These results suggest that cells can repair such damage in vivo, but that 4-NQO produces some genetic damage that persists. In vitro systems, 4-NQO induces covalent DNA modifications (8, 9) and 8-hydroxyguanine formation (10). 4-NQO depends on metabolic activation for the observed genotoxicity (11, 12). The present study investigates: (a) the relationship between DNA damage and organotropism of 4-NQO-induced carcinogenesis in adult and fetal mice; and (b) the role of maternal and placental metabolism in transplacental DNA damage.

The production of heritable genetic change from un repaired DNA damage is dependent on the fixation of such damage by cell division (13). The inherently high rates of cell proliferation that occur during perinatal development suggest that perinatal tissues may be more sensitive to carcinogenesis than adult tissues (14). However, enzymes for the activation/deactivation of xenobiotics may not be sufficiently developed during certain stages of development (15). A clear understanding of the relative sensitivity of fetal and adult tissues to carcinogenesis requires a prior knowledge of tissue levels of active metabolite(s) and/or promutagenic lesions. To this end, a dose of 4-NQO reported to induce a 7-fold difference in lung tumor incidence between the exposed mothers and offspring (5) was used in this study to evaluate the extent of genetic damage during pregnancy. Efforts were made to compare the biological responses of adult and fetal tissues to the genotoxic chemical based on the amount of DNA adducts detected.

MATERIALS AND METHODS

Chemicals. 4-NQO, (dG-dC)n, (dA-dT)m and dGp were obtained from Sigma Chemical Co., St. Louis, MO. DMSO was purchased from Aldrich Chemical Co., Milwaukee, WI. All other materials used were as described previously (16-18).

Animals and Treatment. Male and female ICR mice (25-30 g) were obtained from Harlan Sprague-Dawley Co., Houston, TX. They were housed in polycarbonate cages with corn cob bedding (The Andersons Co., Maumee, OH) in rooms with temperature control (20-22°C) and a 12-h dark/light cycle. The mice were fed Purina 5008 laboratory rodent chow and provided with tap water ad libitum. Mating was performed by placing 1 male with 3 females in a cage. Females were checked for vaginal plug twice daily, once at 8 a.m. and once at 5 p.m. Only mice with positive vaginal plugs were used for experiments. The day that a positive plug was found was designated as day 0 of pregnancy. The gestation period for ICR mice is approximately 19 days. Timed-pregnant mice (3-6 per group) were given one dose of 25 mg/kg 4-NQO s.c. on day 12, 14, or 16 post coitum. Alternatively, mice received a total of 3 doses of 12.5 mg/kg 4-NQO s.c. on days 12, 14, and 16 post coitum, unless otherwise specified. 4-NQO was dissolved in DMSO:ethanol (9:1, v/v) to give a final concentration of 25 mg/ml. Control mice received DMSO:ethanol (1 ml/kg). The mice were killed by cervical dislocation 24 h after carcinogen treatment, unless otherwise specified. Fetuses (male and female) were removed by cesarean section, euthanized with CO2, and kept at -80°C until tissue isolation. Fetal...
tissue from the same litter was pooled for evaluation of MN formation and DNA isolation.

A portion of the fetal liver in fetal calf serum was pressed through a 26-gauge needle. Adult femurs were flushed with fetal calf serum. Liver-cell suspensions or bone marrows were then centrifuged. Portions of the pellets were used for slide preparation. The remaining pellets were frozen and used for DNA adduct analysis. Bone marrow from 2–3 mice from the same treatment was pooled in order to obtain sufficient amounts of DNA for adduct analysis.

Treatment of Ehrlich Ascites Cells with 4-NQO. Two ml of packed Ehrlich ascites cells (collected by centrifugation at 600 × g, 5 min) were suspended in 40 ml of phosphate-buffered saline (pH 7.0) and treated with 5 × 10⁻⁵M 4-NQO for 1 h at 37°C. The cells were then pelleted by centrifugation (600 × g, 5 min). DNA was isolated by Marmur’s procedure and used for adduct analysis.

In Vitro Modifications of DNA, Polynucleotides, and Mononucleotides with a “Model” Reactive Metabolite of 4-NQO. The reaction mixture (5 ml) contained either 0.6 mg/ml DNA, 0.05 mg/ml polynucleotides, or 5 mM dGp, and 1 mM diithiothreitol; 50 mM Tris-HCl, pH 8.0; 0.5 or 1 mM 4-HAQO; and 4 mM seryl-AMP generated in situ as described (20). The incubation was for 30 min at 37°C. The reaction mixture was washed 3 times with phenol saturated with H₂O and once with ether. DNA and polynucleotides were dialyzed extensively against 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, while 4-HAQO-modified dGp was applied to an LH 20 column, eluted with H₂O, and used for the ³²P-postlabeling assay.

³²P-Postlabeling Analysis of DNA Adducts. DNA was isolated by a procedure involving protein and RNA digestion and solvent extraction (19, 21). The pH of all reagents used for DNA isolation was adjusted to 7.0. DNA adducts were routinely analyzed by the P₆ version of the ³²P-postlabeling assay (22).

Mapping of ³²P-labeled Adducts. The chromatographic techniques have been described previously (23). The solvent used for adduct purification was 2.8 M sodium phosphate, pH 6.0 (D₁, for definition see Ref. 23). For adduct separation, 2.3 M lithium formate, 5.5 M urea, pH 3.4, was used for the D3 and followed by 0.72 M LiCl, 0.45 M Tris-HCl, 7.65 M urea, pH 8.0 (D₄) at a right angle to the previous direction. An additional development in 1.0 M sodium phosphate, pH 6.8 (D₅), was performed in the D₄ direction to 7 cm onto a Whatman 1 wick for removal of radioactive background material from the chromatograms. The adduct levels in fetal DNA were extremely low. To quantitate these samples, adduct spots after 1.0 M sodium phosphate purification were excised and contact-transferred to another acceptor sheet for one additional development in 1.0 M sodium phosphate (15 cm above origin), was also used for cochromatography.

Estimation of Extent of DNA Adduction. The count rate (cpm) of each detectable adduct spot was evaluated as described (22). The RAL was calculated as

\[
RAL = \frac{cpm \text{ in adducted nucleotides}}{cpm \text{ in total nucleotides}}
\]

where cpm in total nucleotides is the amount of total nucleotides used for assay multiplied by specific activity of [γ-³²P]ATP.

MN Test. Slides were fixed in methanol for 10–15 min and stained with Wright’s Giemsa stain (25, 26). Five hundred to 1000 PCE from each slide were scored. Two to 3 slides were prepared from femurs of each pregnant mouse and from pooled fetal livers of the same litter.

Statistics. Student’s t test, the Mann-Whitney U test, one-way analysis of variance, paired-wise comparison, and the linear trend test were used to determine the statistical significance of DNA adduct levels and the frequency of MN between different treatment groups. All results were expressed as the mean ± SE.

RESULTS

Covalent DNA Modifications by 4-NQO

Utilizing the ³²P-postlabeling assay, 4-NQO was found to bind covalently to the DNA of maternal and fetal liver, lungs, kidneys, gastrointestinal tract, and pancreas; fetal skin and carcass; maternal bone marrow; and placenta. The binding appears to be ubiquitous. Fig. 1 shows the autoradiograms of typical TLC chromatographic patterns of ³²P-labeled 4-NQO DNA digests from selected maternal and fetal organs. The patterns of 4-NQO adducts were qualitatively similar in all examples shown as well as in the other maternal and fetal organs investigated. Unmarked spots represent background and irreproducible labelings.

To identify the TLC-spots obtained from the ³²P-labeled digests of 4-NQO-DNA from mouse tissues, 4-NQO-DNA adduct markers were prepared. (dG-dC)ₙ, (da-dT)ₙ, or dGp was reacted with a “model” reactive metabolite of 4-NQO, O-seryl-4-HAQO. The adducts derived from the in vitro reactions using 4-HAQO have been shown to be similar to those obtained from the treatment of Ehrlich ascites cells with 4-NQO (12). These markers were used for ³²P-postlabeling assays and cochromatography. The solvent used for D3 is the same for all panels shown in Fig. 2. This facilitates cross-reference of spots under different D4 conditions. D4 for Fig. 2, A–G and H–L, were different. All 4 spots of DNA from treated mice did cochromatograph with all 4 spots from dGp marker as shown in one example (Fig. 2B). Spots from dGp marker also cochromatographed with (dG-dC)ₙ marker. Mouse DNA contained lower levels of adducts and fewer detectable spots than Ehrlich ascites cell DNA. For clear presentation, the cochromatographic results using Ehrlich ascites cell DNA, instead of mouse DNA, were shown in Fig. 2.

Ehrlich ascites cells produced a total of 8 spots (Fig. 2, C and H). (dG-dC)ₙ had 6 spots (Fig. 2, D and I, a–f). In Fig. 2, H–L, spots 3–8 of Ehrlich ascites DNA and spots e–f of (dG-dC)ₙ marker separated better in the isopropyl alcohol/ammonium formate/sodium phosphate system compared with LiCl/Tris-HCl/urea system (Fig. 2, C–G). In Fig. 2, E and J, two primary spots, g and h, were obtained from (da-dT)ₙ marker. As shown in Fig. 2, G and L, spots i–6 of Ehrlich ascites DNA cochromatographed with spots a–f of (dG-dC)ₙ marker, respectively. As shown in Fig. 2, F and K, spots 7–8 of Ehrlich ascites cells cochromatographed with spots g–h of (da-dT)ₙ marker. The percentage-composition of spots g–h (Fig. 2, E or J), varied among different labeling experiments with constant sum RAL; thus, spot h may be the 3'-dephosphorylated product of spot g, which is the bisphosphate of adenine (18). The chromatographic mobility of spot 3 relative to 2, and e relative to f in Fig. 2, A–C, has a tendency to vary with the TLC plates used. The reason for this is not clear. In Fig. 2, A, D, and I, spot x, may be guanine adduct but has not been detected in vivo DNA preparations. The unmarked spots represent nonspecific labelings. Taken together, these results suggest that the adducts produced in vivo consisted mainly of guanine derivatives.

Using ¹⁴C-4-NQO, it has been previously shown that 4-NQO does bind to both guanine and adenine of ascites cells (27). Using the ³²P-postlabeling method, we show here that 4-NQO binds to guanine and adenine of Ehrlich ascites cells with a
Fig. 1. 4-NQO-DNA adducts in various maternal and fetal tissues as shown by autoradiography of 2-dimensional polyethyleneimine-cellulose thin-layer chromatographies of \(^{32}\)P-labeled DNA adducts. \(P\), conditions were used for labeling. See “Materials and Methods” for chromatographic conditions. Spots 1 and 2 may represent \(^{32}\)P-labeled biphosphates of QGI and 3-(deoxyguanosin-\(^{-}\)yI)-4-HAQO, while spots 3 and 4 may be the ring-opened products of QGI. Unmarked spots represent 4-NQO-unrelated background labelings. Film exposure was for 12 h (A, C, and F) and 24 h (B, D, and E). Adduct patterns were derived from tissues of mice 24 h after treatment with 4-NQO (25 mg/kg s.c.) on day 16 of gestation. Autoradiograms of control tissues contained only background labelings and were not shown. M, maternal tissues; F, fetal tissues.

Fig. 3 shows the adduct levels in various maternal and fetal tissues as shown by autoradiography of 2-dimensional polyethyleneimine-cellulose thin-layer chromatographies of \(^{32}\)P-labeled DNA adducts. Conditions were used for labeling. See “Materials and Methods” for chromatographic conditions. Spots 1 and 2 may represent \(^{32}\)P-labeled biphosphates of QGI and 3-(deoxyguanosin-\(^{-}\)yI)-4-HAQO, while spots 3 and 4 may be the ring-opened products of QGI. Unmarked spots represent 4-NQO-unrelated background labelings. Film exposure was for 12 h (A, C, and F) and 24 h (B, D, and E). Adduct patterns were derived from tissues of mice 24 h after treatment with 4-NQO (25 mg/kg s.c.) on day 16 of gestation. Autoradiograms of control tissues contained only background labelings and were not shown. M, maternal tissues; F, fetal tissues.

Fig. 3 shows the adduct levels in various maternal and fetal organs. Addition of 4-NQO to maternal DNA, but not fetal DNA, exhibits strong organ preferences. The amount of adducts formed in maternal tissues after 4-NQO treatment clustered into 3 distinct levels. This was independent of the gestation age at the time of 4-NQO treatment as shown in Fig. 5A and the number of treatments received (Fig. 3B). In contrast, such clustering was not apparent among the fetal organs tested (Fig. 5B). The gestation age at the time of 4-NQO treatment did not modulate adduct levels in the majority of the organs analyzed, except in placenta and maternal bone marrow. The level of binding to placenta was higher on day 12 than on days 14 and 16.
Adduct levels in maternal bone marrow increase with gestation progression ($P < 0.05$, day 16 compared with day 12 or 14). The amount of adducts found in maternal liver was slightly lower than that found in fetal liver ($P < 0.02$ for all single treatment groups and 0.15 for multiple treatment groups). The adduct levels in maternal kidneys, stomach, and placenta were similar and of medium levels among the organs examined ($P < 0.05$), when the carcinogen was given s.c. In contrast, when 4-NQO (80 mg/kg) was given p.o., it was found to bind most strongly to stomach DNA. The DNA adduct level in the stomach was 7-fold higher than that found in the lungs when 4-NQO was given p.o.

MN Formation in PCE

The clastogenicity of 4-NQO in maternal bone marrow and fetal liver was evaluated by determining the number of MN formed in PCE 24 h after the last carcinogen treatment. These results are shown in Fig. 6. The spontaneous incidence of MN in PCE was 0.26 ± 0.10% on nonpregnant mice (with or without treatment with DMSO/ethanol) and 0.46 ± 0.16% in fetal liver. This value did not change significantly during gestation (from day 11 to 16). 4-NQO treatment induced significant increases in MN formation in the PCE of bone marrow of pregnant and nonpregnant mice and of fetal liver as compared with the spontaneous incidence of the corresponding controls. One-way analysis of variance and paired-wise comparison indicated that all 4-NQO-treated fetal groups had 2-fold fewer MN than the corresponding 4-NQO-exposed maternal groups with $P < 0.02$. 4-NQO induced 1.5-2-fold as many MN in the bone marrow of pregnant mice as in the nonpregnant adult mice ($P < 0.05$). The induction of MN was lower in fetal liver
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Fig. 3. Total 4-NQO-DNA adduct levels in various maternal and fetal tissues 24 h after 4-NQO treatment as determined using the P conditions of the 32P-postlabeling method. A, Results of a single treatment (1 x 25 mg/kg) given on day 16 of gestation; B, data from 3 treatments (3 x 12.5 mg/kg) given on days 12, 14, and 16 of gestation. Results are the mean ± SE of 3-6 maternal tissues or litters. At least 3 32P-postlabeling analyses were performed for each DNA preparation. BM, bone marrow.

Fig. 4. Percentage-composition of 4-NQO-DNA adduct spots in various maternal tissues of timed-pregnant mice 24 h after treatment with 4-NQO (25 mg/kg s.c.) on day 16 of gestation. The sum of adducts 2 and 3 was presented due to an inability to consistently separate the 2 adducts.

Fig. 5. Effects of gestation age at the time of 4-NQO treatment on DNA adduct levels in various maternal (A) and fetal (B) tissues. Timed-pregnant mice were treated with 4-NQO (25 mg/kg s.c.) for 24 h on the gestation day indicated. The results are the mean of 3-6 maternal tissues or litters. At least 3 32P-postlabeling analyses were performed for each DNA preparation. For clarity, SE were not included. Consult Fig. 3 for typical SE values. Note the scale difference in y-axis. G.I., gastrointestinal; U.G., urogenital.

Fig. 6. Effects of 4-NQO treatment on MN formation in PCE of adult bone marrow and fetal liver. For treatment schedules, consult the legends to Figs. 3 and 5. Approximately 500-1000 cells were scored for each sample. The results are the mean ± SE for 3-6 mothers and litters. The spontaneous incidence was 0.26 ± 0.10% and 0.46 ± 0.16% for adult bone marrow (pregnant and nonpregnant mice) and fetal liver, respectively. For ease of presentation, nonpregnant mice were designated as day -1 of gestation.

DISCUSSION

These studies demonstrate that 4-NQO produces genetic damage in adult and fetal tissues throughout gestation. At the molecular level, it binds covalently to DNA bases. At the cellular level, it induces MN formation. Together, these data could explain its carcinogenic effects.

Tissue binding appears to be ubiquitous. A strong organ-
binding preference has been demonstrated in adult tissues, but not in fetal tissues. The adult tissues to which 4-NQO bound most strongly varied with the route and time of administration. When administered s.c., it bound most strongly to the DNA of lungs and pancreas; when given p.o., to the DNA of stomach; and when applied topically, to the DNA of skin (18). This variation may in part be due to a first-pass effect. Gestation stage at the time of 4-NQO treatment modulated the adduct levels in placenta and bone marrow. The reasons for this are not clear. Overall, the organ-binding preferences correlated with the reported carcinogenic effects found in these adult organs (3).

In sharp contrast to maternal tissues, 4-NQO exhibited no tissue-binding preference in fetuses. The lack of correlation between the organotropism of DNA damage (this report) and of carcinogenicity found in these fetuses (5) suggests that other factors such as DNA repair processes may be important. Adduct levels in fetal tissues after a single treatment were extremely low. The low levels of binding and the inherently high rate of cell divisions in fetal tissues made it difficult to study repair by assessing adduct persistence as a function of time after an interval of treatment longer than 24 h. In addition, 4-NQO at the dose used frequently induced premature delivery and is somewhat toxic to the pregnant mice. An alternative, albeit indirect, approach is to measure the ability of each organ to activate and/or deactivate this metabolite of 4-NQO among organs of different developmental ages. Thus, adduct levels may well serve as a better basis than actual administered dose for comparison of biological responses. 4-NQO was reported to induce 7-fold more lung tumors in the dams than in their transplacentally exposed offspring (5). This dose produced an 11-fold higher level of DNA adducts in the former than in the latter (Fig. 3). Thus, fetal tissues appear to produce slightly more tumors per adduct formed than adult tissues. Similarly, if adduct levels were taken into account, fetal liver produced more MN per adduct formed than adult bone marrow at all gestation ages studied. Thus, fetuses were more sensitive than adults to both the clastogenic and the tumorigenic effect of 4-NQO at equal adduct, but not equal dose, level. These may be attributable to differences in the rates of cell divisions between the adults and the fetuses. In line with this hypothesis is that fetal sensitivity to clastogenic effect decreases with gestation progression. The cell cycle time for erythropoiesis during the prenatal period is shorter than in the adult and that cycle time lengthens with gestation progression (32, 33). This may explain the differential sensitivity seen between adult and fetal PCE. In adult bone marrow, the enhanced clastogenic response, at equal dose, during pregnancy is, therefore, in part the result of increased DNA adduction. This alone, however, does not explain the changing responses with gestation progression. Pregnancy-related hormones may also modulate the response of PCE.

The extent of binding, but not the types of adducts formed, varied with the organs studied. The qualitative similarity in adduct patterns among all organs tested suggested that there is a single major ultimate carcinogen for this compound in fetal and maternal tissues. This is in agreement with a published hypothesis (34). We detected guanine, but not adenine, adducts in cultured Ehrlich ascites cells after 4-NQO treatment, but the levels are low (27) (Fig. 2), and this may account for our inability to detect them in treated mouse tissues.

Three major guanine adducts have been characterized for samples prepared in vitro. QG1, 3-(deoxyguanosin-N2-y1)4-HAQO, and a ring-opened product of QG1 (27). Their relationship to the adducts found here are being investigated. Prelimi-
nary studies regarding the stability of the adducts in alkaline pH suggest that adduct 1 may be QGI. Adducts 3 and 4 may be the degradation products of adduct 1. The stability of adduct 2 under different experimental conditions suggests that it may be 3-(deoxyguanosin-N'-yl)-4-HAQO. Isolation of each adduct for chemical analysis is under way.

In summary, 4-NQO binds ubiquitously to DNA of all tissues at different developmental stages, as shown for benzo(a)pyrene (30, 31). Our studies have provided information concerning the possible relationship of biologically effective dose and administered dose of a metabolism-dependent carcinogen in fetal and adult tissues. These tissue levels of promutagenic lesions are useful for an in depth analysis of factors that modulate transplacental carcinogenesis.

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