Environmental Pollutant and Potent Mutagen 3-Nitrobenzanthrone Forms DNA Adducts after Reduction by NAD(P)H:Quinone Oxidoreductase and Conjugation by Acetyltransferases and Sulfotransferases in Human Hepatic Cytosols


Abstract

3-Nitrobenzanthrone (3-nitro-7H-benz[de]anthracen-7-one, 3-NBA) is a potent mutagen and suspected human carcinogen identified in diesel exhaust and air pollution. We compared the ability of human hepatic cytosolic samples to catalyze DNA adduct formation by 3-NBA. Using the $^{32}$P-postlabeling method, we found that 12/12 hepatic cytosols activated 3-NBA to form multiple DNA adducts similar to those formed in rodents. By comparing 3-NBA–DNA adduct formation in the presence of cofactors of NAD(P)H:quinone oxidoreductase (NQO1) and xanthine oxidase, most of the reductive activation of 3-NBA in human hepatic cytosols was attributed to NQO1. Inhibition of adduct formation by dicoumarol, an NQO1 inhibitor, supported this finding and was confirmed with human recombinant NQO1. When cofactors of X.O-acetyltransferases (NAT) and sulfotransferases (SULT) were added to cytosolic samples, 3-NBA–DNA adduction formation increased 10- to 35-fold. Using human recombinant NQO1 and NATs or SULTs, we found that mainly NAT2, followed by SULT1A2, NAT1, and, to a lesser extent, SULT1A1 activate 3-NBA. We also evaluated the role of hepatic NADPH:cytochrome P450 oxidoreductase (POR) in the activation of 3-NBA in vivo by treating hepatic POR-null mice and wild-type littermates i.p. with 0.2 or 2 mg/kg body weight of 3-NBA. No difference in DNA binding was found in any tissue examined (liver, lung, kidney, bladder, and colon) between null and littermates. 3-NBA is predominantly activated by NATs and SULTs.

Introduction

Environmental factors and individual genetic susceptibility play an important role in many human cancers (1). Lung cancer is the most common malignant disease worldwide and is the major cause of death from cancer. Tobacco smoking is the overwhelming cause of lung cancer but vehicular exhaust and ambient air pollution are also implicated (2). Nitro–polycyclic aromatic hydrocarbons (nitro-PAH) are widely distributed environmental pollutants found in vehicular exhaust from diesel and gasoline engines and on the surface of ambient air particulate matter. The increased lung cancer risk after exposure to these environmental sources and the detection of nitro-PAHs in the lungs of nonsmokers with lung cancer has led to considerable interest in assessing their potential cancer risk to humans (3, 4).

A new member of this group of compounds, 3-nitrobenzanthrone (3-nitro-7H-benz[de]anthracen-7-one, 3-NBA; Scheme 1), was discovered in diesel exhaust and in airborne particulate matter (5, 6). As a likely consequence of atmospheric washout, 3-NBA has also been detected more recently in surface soil and rainwater (7, 8). The main metabolite of 3-NBA, 3-aminobenzanthrone, has been found in urine samples of salt mine workers occupationally exposed to diesel emissions (6), demonstrating that human exposure to 3-NBA in diesel emissions can be significant and is detectable. 3-NBA is a potent mutagen in the Ames Salmonella typhimurium assay (5) and in the transgenic Muta Mouse assay (9). It has been shown to be genotoxic in several short-term tests (10–14) and its genotoxicity has been further documented by the detection of specific DNA adducts formed in vitro as well as in vivo in rodents (9, 10, 12, 14–20). Moreover, preliminary data suggest that 3-NBA is carcinogenic to rats (21).

The detection of specific DNA adducts by $^{32}$P-postlabeling analysis has allowed us to use 3-NBA–DNA binding as an end point for studying the enzymology of the metabolic activation of 3-NBA (9, 12, 14, 16–20). Both rat and human microsomal enzymes activate 3-NBA in vitro to form the same DNA adducts found in vivo in rodents (9, 12, 17, 19). Most of the reductive activation in hepatic microsomes was attributed to P450 oxidoreductase (NADPH:cytochrome P450
oxireductase, POR), and this was confirmed with purified and
recombinant POR (19). Buttermilk xanthine oxidase (XO) was also
efficient in the reductive activation of 3-NBA (10, 12). The major DNA
adducts of 3-NBA formed after activation with buttermilk XO are
products derived from reductive metabolites bound to purine
bases (12). Although the structures of the DNA adducts remain to
be characterized, we have shown that the nitroreduction pathway
is responsible for the formation of these adducts in various
tissues of rats and mice (9, 12, 17, 22). However, no data are
available on the participation of authentic rat or human cytosolic
nitroreductases, such as XO and NAD(P)H:quinone oxireduc-
tase (NQO1).

- Hydroxylamine intermediates formed by nitroreduction
can be conjugated by phase II enzymes, such as N-O-acetyltrans-
f erases (NAT) and sulfotransferases (SULT), leading to the
formation of reactive esters capable of forming DNA adducts (23,
24). Previous work indicated that N-hydroxy-3-aminobenzanthrone
(Y-OH-ABA) seems to be the critical intermediate in 3-NBA–DNA
adduct formation (12, 14, 17, 22). Furthermore, we found that O-
acetylation by human recombinant NATs as well as O-sulfonila
tion by human recombinant SULTs of N-OH-ABA strongly contribute to
the formation of DNA adducts (16, 18). Because animal enzymes or
human recombinant systems may not be ideal models of the
catalytic properties of enzymes in human organs, the present study
was undertaken to determine the capability of human hepatic
cytosols to activate 3-NBA, to identify hepatic cytosolic enzymes
involved in DNA adduct formation by 3-NBA, and to evaluate the
contribution of hepatic cytosolic and microsomal reductases to the
bioactivation of 3-NBA.

Materials and Methods

Chemicals. NADH, NADPH, hypoxanthine, dicumarol, allopurinol,
dAMP, dGMP, buttermilk XO, acetyl-CoA 3′-phospho-adenosine-5′-phos-
hosphate (PAPS), mendiolone (2-methyl-1,4-naphthoquinone), salmon
testis, and calf thymus DNA were obtained from Sigma Chemical Co (St.
Louis, MO, USA); Sudan I was from BDH (Poole, United Kingdom).

Synthesis of 3-nitrobenzanthrone. 3-NBA was synthesized as described
recently (18) and its authenticity was confirmed by UV, electrospray mass
spectroscopy.

Preparation of cytosols. Cytosolic fractions were isolated from the
livers of 10 male Wistar rats, either uninduced or pretreated with Sudan I
inducing NQO1 as described (25). For initial experiments, pooled human
hepatic cytosol from Gentest Corp. (Woburn, MA) was used. Cytosolic
fractions from livers of 12 human donors were also obtained from Gentest
fractions from Sudan I–treated rats as described (29). Human recombi-
nant NQO1 was obtained from Sigma. Cytosolic extracts, isolated from
insect cells transfected with baculovirus constructs containing cDNA of
SULT1A2*, SULT1A2+, SULT1A3, SULT1E, or SULT2A1, were obtained
from Oxford Biomedical Research, Inc. (Oxford, MA) and those containing
cDNA of human NAT1*4 or NAT2*4 from Gentest. Cytosolic extracts
expressing SULT1A1 and SULT1A2 conjugated p-nitrophenol at rates of
124 and 5.5 nmol/min/mg protein, respectively; SULT1A3 conjugated dopamine
at the rate of 8 nmol/min/mg protein; SULT1E conjugated dehy-
droepiandrosterone at the rate of 584 pmol/min/mg protein. Cytosolic
extracts expressing NAT1 and NAT2 had a catalytic activity of 1,300 nmol/
min/mg protein (substrate p-aminoalicyclic acid) and 290 nmol/min/mg
protein (substrate sulfamethazine), respectively. Enzyme activities in
central cytosol were <10 pmol/min/mg protein.

Enzyme incubations. Incubations with human recombinant NQO1, in a
final volume of 750 μL, consisted of 50 nmol/L Tris-HCl buffer (pH 7.4),
containing 0.2% Tween 20, 1 mmol/L NADPH, 0.75 to 300 μmol/L 3-NBA
(dissolved in 12.5 μL DMSO), 1 mg of calf thymus DNA (4 mmol/L DnP),
and 10 μg of protein (units) of NQO1. One unit of NQO1 is defined to reduce 1 μmol
cytochrome c per minute per milligram of protein in the presence of
menadione as substrate at 37°C. In incubations testing the activity of puri-
ified rat hepatic NQO1, 2 to 30 μg (0.018–0.09 units) were added and 30 μmol/L
3-NBA were used. The reaction was initiated by adding 3-NBA. All reaction
mixtures were incubated at 37°C for 3 hours. In incubations testing the time-
dependent formation of 3-NBA–DNA adducts mediated by human recombi-
nant NQO1 (0.06 units), incubation times varied between 60 and
240 minutes and 30 μmol/L 3-NBA were used. In control incubations,
NQO1 was omitted from the mixtures. In incubations using cytosols of
baculovirus-transfected insect cells containing recombinant SULTs and
NATs, additionally 10 and 50 μg of the respective enzyme with the cofactors
PAPS or acetyl-CoA were added to the reaction mixture containing human recombi-
nant NQO1. Cytosolic fractions isolated from insect cells, which were
not transfected with any human transferases with the respective cofactor,
were used as a control. After the incubation, DNA was isolated by the phenol-
chloroform extraction method as described (28).

Treatment of hepatic P505 oxidireductase–null mice and wild-type
littermates with 3-nitrobenzanthrone. Male hepatic POR-null mice
(30) and wild-type littermates on a C57BL/6 background (25-30 g)
were treated with a single dose of 0.2 mg/kg (n = 3) or 2 mg/kg body weight
(n = 3) of 3-NBA by i.p. injection. 3-NBA was dissolved in tricaprylin at a
correction of 0.5 mg/mL. Control mice (n = 3) received tricaprylin only.
The animals were killed 24 hours after treatment. Five organs (liver, lung,
kidney, bladder, and colon) were removed and stored at −80°C until DNA
isolation by standard phenol extraction.

Preparation of reference compounds. dAMP and dGMP (4 μmol/L)
were incubated with 3-NBA (0.3 mmol/L) enzymatically activated by
buttermilk XO (1 unit/mL) in 50 mmol/L potassium phosphate buffer (pH
7.0) in the presence of 1 mmol/L hypoxanthine as described (10, 12).
Chemical modification of salmon testis DNA with N-OH-ABA was done as
follows: 20 mg 3-NBA in 10 mL diglyme was reduced by stirring with 30 μL
hydrazine hydrate and 10 mg 5% palladium on charcoal under nitrogen for
30 minutes. The resulting N-OH-ABA solution was decanted and mixed with
a solution of 50 mg salmon testis DNA in 50 mL 0.02 mol/L sodium citrate
(pH 5.0), and the mixture incubated for 18 hours at 60°C under nitrogen.
The mixture was extracted with 20 mL ethyl acetate (3×) and the DNA
precipitated from the aqueous phase with ethanol.

32P-postlabeling analysis and high-performance liquid chromatog-
raphy analysis of 32P-labeled 3′-deoxyribonucleoside bisphosphate
adducts. 32P-postlabeling analysis using nuclease P1 digestion and butanol
extraction, and TLC and high-performance liquid chromatography (HPLC)
Table 1. Cytosol-dependent enzyme activities and DNA adduct formation by 3-NBA in human hepatic cytosolic samples, and correlation coefficients between cytosolic enzyme activities and total levels of 3-NBA–derived DNA adducts formed in human liver cytosol

<table>
<thead>
<tr>
<th>Cytosol-dependent enzyme activities*</th>
<th>RAL † (mean/10⁶ nucleotides)</th>
<th>Without cofactors</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XO</td>
<td>NQO1</td>
</tr>
<tr>
<td>H803</td>
<td>40.0</td>
<td>0.5</td>
</tr>
<tr>
<td>H806</td>
<td>20.0</td>
<td>1.0</td>
</tr>
<tr>
<td>H823</td>
<td>22.8</td>
<td>12.0</td>
</tr>
<tr>
<td>H830</td>
<td>21.9</td>
<td>4.0</td>
</tr>
<tr>
<td>H842</td>
<td>31.4</td>
<td>3.0</td>
</tr>
<tr>
<td>H843</td>
<td>19.0</td>
<td>0.5</td>
</tr>
<tr>
<td>H856</td>
<td>20.9</td>
<td>4.0</td>
</tr>
<tr>
<td>H866</td>
<td>30.4</td>
<td>19.0</td>
</tr>
<tr>
<td>H870</td>
<td>26.4</td>
<td>5.0</td>
</tr>
<tr>
<td>H889</td>
<td>38.0</td>
<td>3.0</td>
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<tr>
<td>H893</td>
<td>30.0</td>
<td>3.0</td>
</tr>
<tr>
<td>H8112</td>
<td>43.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Mean ±SD ‡</td>
<td>28.7 ± 8.0</td>
<td>5.4 ± 5.3</td>
</tr>
<tr>
<td>r(NP1)</td>
<td>-0.045</td>
<td>0.274</td>
</tr>
<tr>
<td>r(BUT)</td>
<td>0.100</td>
<td>0.035</td>
</tr>
</tbody>
</table>

NOTE: H803–H8112 are human hepatic cytosolic samples.
Abbreviations: RAL, relative adduct labeling; HX, hypoxanthine; NP1, nuclease P1; BUT, butanol extraction; n.d., not determined (sample lost).
*All results are presented as the mean of duplicate determinations. Each cytosolic sample was evaluated for XO, NQO1, NAT1, NAT2, and SULT activity using the following substrates: hypoxanthine (XO), menadione (NQO1), p-aminosalicylic acid (NAT1), sulfamethazine (NAT2), and 7-hydroxycoumarin (SULT). XO, NQO1, NAT, and SULT activities are in nmol/min/mg protein.
†All results are presented as the mean of duplicate determinations of DNA from one in vitro incubation.
‡Mean value for 12 hepatic cytosolic samples (H803–H8112) ± SD, representing the interindividual variability.
Correlation coefficients (r) for correlation analyses done between each of the enzyme activities listed for each cytosolic sample and the levels of 3-NBA–DNA adducts formed in the same sample in the presence of HX for XO, of NADPH for NQO1, of acetyl-CoA and NADPH for NATs, or of PAPS and NADPH for SULT.
1/ P < 0.01 (n = 12).

were done as recently described (16, 17). DNA adduct spots were numbered as recently reported (16–19).

**Statistical analysis.** Correlation coefficients between the catalytic activities of NQO1 and XO as well as NAT and SULT in human hepatic cytosolic samples and the level of total 3-NBA–DNA adducts formed by the same cytosolic samples were determined by linear regression using Statistical Analysis System software version 6.12. Correlation coefficients were based on a sample size of 12. All of the P values are two-tailed and considered significant at the 0.05 level.

**Molecular modeling.** Crystallographic coordinates for rat and human NQO1 with bound flavin adenine dinucleotide were obtained from the Protein Data Bank (31). The coordinates were used without further refinement. The modeling of the binding of 3-NBA to the active site was done with the program Autodock 3.0.3 (32) and Sybyl 6.6.5 (Tripos GmbH, Munich, Germany) by the procedure described (25, 26, 33). 3-NBA was built up with fragment libraries supplied with the modeling software. The initial structure was first energy minimized to a root-mean-square force of <0.001 with the consistent valence force field (33).

**Results**

**Activation of 3-nitrobenzanthrone by human and rat hepatic cytosols.** We determined the formation of DNA adducts in calf thymus DNA by 3-NBA incubated with human hepatic cytosols or cytosols from the livers of rats, either uninduced or pretreated with Sudan I. Based on previous studies that showed specific sensitivity of arylamine- and nitro-PAH–derived DNA adducts to nuclease P1 treatment, both the butanol extraction and the nuclease P1 digestion enhancement of the 32P-postlabeling assay were used to analyze 3-NBA–DNA adducts. Hepatic cytosols from rats (Fig. 1A) and from humans (Fig. 1B–D) were capable of activating 3-NBA to form DNA adducts. In each case, 3-NBA induced essentially the same four major DNA adducts (spots 1, 2, 3, and 6) detected after enrichment using nuclease P1 digestion, and a cluster of up to five adducts (spots 1, 2, 3, 4, and 5) detected after enrichment using butanol extraction. These adducts were also observed in liver tissue of 3-NBA–treated rats and mice (9, 12, 17), and in incubations in vitro using rat and human hepatic microsomes (19). No DNA adducts were observed in control incubations (data not shown).

**Cochromatographic analysis of individual spots on HPLC confirmed that adduct spots 1 to 5 that are formed with human cytosols are derived from 3-NBA by nitroreduction (data not shown). We therefore analyzed which cytosolic reductase is responsible for 3-NBA–DNA adduct formation.** As shown in Fig. 2A and B, the formation of 3-NBA–DNA adducts was stimulated by the cofactors of NQO1, NADPH, and NADH (34), and the cofactor...
of XO, hypoxanthine (35), with both rat and human cytosols. Adduct levels with human cytosols were up to 4.3-, 6.5-, and 1.6-fold higher when NADPH, NADH, or hypoxanthine, respectively, were added to the incubation mixture (Fig. 2C). With rat cytosols, 3-NBA–DNA adduct formation increased by up to 5.7-, 15.6-, and 19.4-fold after the addition of NADPH, NADH, and hypoxanthine, respectively (Fig. 2A). NQO1 and XO reductively activate 3-NBA in rat cytosol and in human cytosol; however, XO seems to play a minor role in activation of 3-NBA in the human sample. This assumption is also supported by the fact that 3-NBA–DNA adduct formation by human cytosols was remarkably decreased by dicoumarol (70-80% reduction in total DNA binding compared with incubations without dicoumarol), an inhibitor of NQO1 (25, 26), whereas allopurinol, an inhibitor of XO, showed no effect (data not shown). Cytosols from rats pretreated with Sudan I, an inducer of NQO1 enzyme activity (29), were very effective in stimulating 3-NBA–DNA adduct formation by human recombinant NAD(P)H:quinone oxidoreductase. To confirm the role of NQO1 in the activation of 3-NBA, we used rat and human recombinant NQO1 and the NQO1 enzyme purified from livers of rats pretreated with Sudan I (29). Figure 1E shows that incubations of 3-NBA with DNA and purified rat NQO1 resulted in the formation of the same DNA adduct pattern as with other activating systems and in vivo in rats. Using 0.018 to 0.09 units of rat NQO1, total DNA adduct levels ranged from 15.8 to 23.8 and 16.1 to 35.0 adducts per 10^8 nucleotides after nuclease P1 and butanol enrichment, respectively (data not shown). Similarly, human recombinant NQO1 was efficient at activating 3-NBA (Fig. 1F). Human NQO1-mediated DNA adduct formation was concentration-dependent up to 15 μmol/L 3-NBA (Fig. 3A) and showed a steep increase between 60 and 240 minutes (Fig. 3B).

**Table 1. Cytosol-dependent enzyme activities and DNA adduct formation by 3-NBA in human hepatic cytosolic samples, and correlation coefficients between cytosolic enzyme activities and total levels of 3-NBA–derived DNA adducts formed in human liver cytosol (Cont’d)**

<table>
<thead>
<tr>
<th>With HX</th>
<th>With NADPH</th>
<th>With NADPH and acetyl-CoA</th>
<th>With NADPH and PAPS</th>
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<tbody>
<tr>
<td>NP1 BUT NP1 BUT NP1 BUT NP1 BUT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145.6 97.9 91.2 59.5 351.2 266.4 3,131.6 1,123.9</td>
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<tr>
<td>162.6 109.9 155.9 56.5 306.3 134.4 3,227.4 1,866.5</td>
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<tr>
<td>24.8 12.9 319.9 79.5 366.1 143.1 2,731.5 1,472.6</td>
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<tr>
<td>28.3 7.7 430.8 73.2 1,671.1 546.1 2,008.6 1,523.3</td>
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<tr>
<td>28.2 4.9 108.2 55.8 957.0 358.9 1,778.7 860.1</td>
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<tr>
<td>170.7 44.2 166.6 31.8 207.0 118.7 2,345.6 1,655.7</td>
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<tr>
<td>37.0 15.4 199.7 35.7 368.6 101.4 2,931.2 1,842.8</td>
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<tr>
<td>29.1 9.1 394.0 63.0 448.3 93.5 2,511.3 1,651.7</td>
<td></td>
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<tr>
<td>22.8 9.8 270.8 68.3 689.9 162.9 2,649.2 1,321.3</td>
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<tr>
<td>70.1 28.1 405.1 114.3 2,176.9 1,059.0 2,544.1 1,039.3</td>
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<tr>
<td>59.8 23.3 583.0 132.4 4,236.3 1,850.9 2,541.4 1,273.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d. n.d. 187.2 64.1 361.5 84.1 1,904.5 1,434.1</td>
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</tr>
<tr>
<td>70.8 ± 56.4 32.9 ± 35.2 276 ± 145 69.5 ± 27.6 1,017 ± 1,135 410 ± 511 2,525 ± 440 1,423 ± 300</td>
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**Activation of 3-nitrobenzanthrone by purified rat and human recombinant NAD(P)H:quinone oxidoreductase.**

To study in more detail the role of individual SULTs and NATs in the bioactivation of 3-NBA by human hepatic cytosols, we used human recombinant NAT1, NAT2, and NAT3 in the formation of the same DNA adduct pattern as with other activating systems and in vivo in rats. Using 0.018 to 0.09 units of rat NQO1, total DNA adduct levels ranged from 15.8 to 23.8 and 16.1 to 35.0 adducts per 10^8 nucleotides after nuclease P1 and butanol enrichment, respectively (data not shown). Similarly, human recombinant NQO1 was efficient at activating 3-NBA (Fig. 1F). Human NQO1-mediated DNA adduct formation was concentration-dependent up to 15 μmol/L 3-NBA (Fig. 3A) and showed a steep increase between 60 and 240 minutes (Fig. 3B).
(NAT1 and NAT2) were used in incubations together with human recombinant NQO1. DNA adduct formation was observed in all incubations. 3-NBA induced the same adduct pattern as those in human cytosols with PAPS or acetyl-CoA as cofactor (Fig. 1G and H). We used 10 and 50 μg of each cytosolic preparation to account for the amount of protein added that may influence the adduct yield by scavenging reactive metabolites. Indeed, 50 μg cytosol in the control incubation with acetyl-CoA led to lower adduct yield than 10 μg, whereas in the case of NAT2 a saturation level was reached. SULT1A2 and especially NAT2 are the most active phase II enzymes promoting 3-NBA–DNA adduct formation after reduction by NQO1. This finding is in line with the results obtained in the correlation analysis for NAT2. None of the other examined SULTs (SULT1A3, SULT1E, and SULT2A1) increased 3-NBA–DNA adduct levels.

The effect of cytosolic and microsomal reductases on activation of 3-nitrobenzanthrone in hepatic P450 oxidoreductase–null mice. Recently, we showed that most of the reductive activation of 3-NBA in human hepatic microsomes is attributed to POR (19). To evaluate the importance of hepatic POR in the reductive activation of 3-NBA to DNA adducts in vivo compared with cytosolic reduction and conjugation, we treated hepatic POR-null mice (30) and wild-type littersmates ip. with 0.2 or 2 mg/kg body weight of 3-NBA. Essentially, the same DNA adduct patterns as those found in vivo in rats, and in incubations using rat and human hepatic cytosols and microsomes, were observed (Fig. 1I and J). No DNA adducts were observed in DNA isolated from tissue of control animals treated with vehicle only (tricaprylin; data not shown). As shown in Fig. 4, no difference in DNA binding by 3-NBA was found between null and wild-type mice in any of the five tissues examined, indicating that POR does not contribute significantly to the reductive activation of 3-NBA.

**Molecular modeling.** To examine the molecular basis of the reductive activation of 3-NBA by rat and human NQO1, the binding of 3-NBA to the active center of NQO1 was modeled. The calculated model structure for the human NQO1–3-NBA complex is shown in Fig. 5. Similarly, the model structure for the rat NQO1–3-NBA complex was calculated (data not shown). It is evident from Fig. 5 that 3-NBA fits well into the active site of human NQO1, being bound near the isoalloxazine ring of the flavin prosthetic group of the enzyme. This allows an electron transfer during the reductive activation of 3-NBA. The values of the apparent dissociation constant for the rat and human NQO1–3-NBA complexes were calculated to be 0.10 and 0.26 μmol/L, respectively.

**Figure 1.** Autoradiographic profiles of 3-NBA–derived DNA adducts by using the nuclease P1 digestion (left) or butanol extraction (right) enrichment version of the 32P-postlabeling assay. For the in vitro incubations, calf thymus DNA was reacted with 300 μmol/L 3-NBA with cytosols as indicated. NADPH was added as cofactor for NQO1, NADPH and acetyl-CoA for NATs, or PAPS for SULTs. In the incubations with purified enzymes, 30 μmol/L 3-NBA and either 0.09 unit of purified rat hepatic NQO1 or 0.09 unit of human recombinant NQO1 were used with and without 50 μg recombinant phase II enzymes and their cofactors. Mice were treated with 2 mg 3-NBA per kilogram body weight and shown are the adduct profiles in liver DNA representative for all other organs investigated. N-OH-ABA was reacted with salmon testis DNA as indicated. For details, see Materials and Methods.
Reaction of \(N\)-hydroxy-3-aminobenzanthrone with DNA.

Salmon testis DNA was reacted with \(N\)-OH-ABA at 60°C for 18 hours under nitrogen. When DNA digests from this incubation were analyzed by 32P-postlabeling, essentially the same DNA adduct pattern to those obtained \textit{in vivo} in rodents, and in incubations using rat and human hepatic cytosols, was observed (Fig. 1K). HPLC cochromatographic analysis confirmed that all major adduct spots (spots 1, 2, 3, 4, and 5) that are formed with \(N\)-OH-ABA are products bound to deoxyadenosine (spots 1 and 2) or deoxyguanosine (spots 3, 4, and 5; data not shown). Thus, these results confirm that \(N\)-OH-ABA is the critical intermediate in DNA adduct formation by 3-NBA.

Discussion

In the present study, we have shown that rat and human cytosolic samples are effective in activating 3-NBA leading to the same DNA adduct pattern as those formed in 3-NBA–treated rodents and in incubations using rat and human microsomes (9, 12, 17, 19). Comparative analyses showed that all major DNA adducts are products derived from reductive metabolites bound to deoxyadenosine (adducts 1 and 2) or deoxyguanosine (adducts 3, 4, and 5). Moreover, adduct 4 is sensitive to digestion with nuclease P1, which is indicative of C-8-deoxyguanosine arylamine-DNA adducts (36). Furthermore, because we found that all major DNA adducts are detectable after reaction of \(N\)-OH-ABA with DNA, we assume that all 3-NBA–DNA adducts are formed by simple nitroreduction. This is in line with previous data indicating that \(N\)-OH-ABA is the critical intermediate in 3-NBA–derived DNA adduct formation (14, 17, 18, 22). However, the low amount of individual DNA adducts recovered from digests of DNA treated with \(N\)-OH-ABA prevented further structural characterization of these 3-NBA–DNA adducts. Other synthetic approaches are currently being developed in our laboratory to prepare authentic 3-NBA–DNA adduct standards.

The stimulation of 3-NBA–DNA adduct formation in human hepatic cytosolic samples by NADPH suggested the participation of human NQO1 in the reductive bioactivation of 3-NBA. Inhibition of DNA adduct formation by dicoumarol (25, 26) provided additional evidence for the major role of NQO1, and the utilization of human recombinant NQO1 fully corroborated the

![Figure 2](image_url)

**Figure 2.** DNA adduct formation by 3-NBA activated with uninduced rat hepatic cytosols (A), Sudan I–induced rat hepatic cytosols (B), human hepatic cytosols (pooled fraction; C), and human hepatic cytosols (pooled fraction) using acetyl-CoA and PAPS as cofactors (D). Columns, mean of duplicate determinations of DNA from one \textit{in vitro} incubation. F, fold increase in DNA binding compared with DNA binding without cofactors (A–C) or compared with incubations with NADPH only (D). RAL, relative adduct labeling.

![Figure 3](image_url)

**Figure 3.** A, concentration-dependent DNA adduct formation by 3-NBA (0.75-300 \(\mu\)mol/L) after activation with human recombinant NQO1 (0.06 unit) in 180 minutes. B, time-dependent (60-240 minutes) DNA adduct formation of 3-NBA (30 \(\mu\)mol/L) after activation with human recombinant NQO1 (0.06 unit). C, DNA adduct formation of 3-NBA after activation with human recombinant NQO1 and different human recombinant SULTs or NATs (10 and 50 \(\mu\)g) expressed as relative DNA binding that was calculated by deducting the corresponding control cytosol incubation from the incubation with NAT or SULT and dividing the value by the specific activity of the enzyme added. Columns, mean of duplicate determinations of DNA from one \textit{in vitro} incubation.
Minor impact on the activation of 3-NBA to form DNA adducts. The recombinant (human) and authentic (rat) enzymes. Discrepancies might be the different substrate specificities of the human recombinant enzyme. The reason for the observed activity of NQO1 isolated from rat hepatic cytosols than of NQO1 enzymes from both species. However, we found a lower n.d., detected.

Each DNA sample was determined by two postlabeled analyses. n.d., not detected.

Mean; bars, SD (n = 3); each DNA sample was determined by two postlabeled analyses. n.d., not detected.

Figure 4. Total DNA adduct formation by 3-NBA in organs of hepatic POR-null mice and wild-type (WT) littermates on a C57BL/6 background treated with 0.2 or 2 mg 3-NBA per kilogram body weight. Columns, mean; bars, SD (n = 3); each DNA sample was determined by two postlabeled analyses. n.d., not detected.

The capability of this enzyme to activate 3-NBA. Nevertheless, no statistical correlation between the levels of 3-NBA–derived DNA adducts and the NQO1 activities in human cytosolic samples was observed, indicating that additional enzymes (e.g., phase II biotransformation enzymes) may participate in 3-NBA activation. The importance of NQO1 in the reductive activation of nitroaromatics like 3-NBA is consistent with previous reports demonstrating that the enzyme functions efficiently as a nitroreductase of substrates like dinitropyrenes, nitrophenylazaridines, nitrobenzamides, and nitrophenanthrene carboxylic acids (26, 37). We also showed that isolated rat cytosolic NQO1 efficiently activated 3-NBA. Molecular modeling and docking of 3-NBA to the active centers of rat and human NQO1 protein indicate similarities in 3-NBA binding to both enzymes; calculated apparent dissociation constants (K_a) are of the same order for NQO1 enzymes from both species. However, we found a lower activity of NQO1 isolated from rat hepatic cytosols than of the human recombinant enzyme. The reason for the observed discrepancies might be the different substrate specificities of the recombinant (human) and authentic (rat) enzymes.

It seems that compared with NQO1, human hepatic XO had only minor impact on the activation of 3-NBA to form DNA adducts. Allopurinol, an inhibitor of XO (25, 26), did not inhibit DNA adduct formation in pooled human cytosols. However, earlier data had shown that isolated buttermilk XO was an effective activator of 3-NBA (10, 12), but the enzyme levels needed were unphysiologic and substrate specificities may be different. In rat cytosol, XO had a much greater capacity to activate 3-NBA to form DNA adducts than in human cytosols. This is consistent with a previous observation (15) that the metabolism of 3-NBA in rat alveolar type II cells, involving reduction of 3-NBA to 3-aminobenzanthrone is mediated, at least in part, by XO.

In mammalian cells, both cytosolic and microsomal substractions contain enzymes that catalyze the reduction of nitroaromatic compounds (25, 26, 28, 37). In rat and human hepatic microsomes, we have already identified POR as the enzyme activating 3-NBA, generating 3-NBA–DNA adduct profiles identical to those found in liver tissue of 3-NBA–treated rodents (9, 12, 17, 19). The comparison of 3-NBA–DNA adduct levels formed by human hepatic microsomes (19) and cytosols (present paper) reveals that the cytosolic enzyme systems are much more efficient in the reductive activation of 3-NBA than microsomes. Moreover, the content of cytosolic protein per gram of human liver tissue is about four times higher than that of microsomal protein. Therefore, the importance of the cytosolic enzymes in 3-NBA activation in the organ should be even higher. Nevertheless, in the in vitro experiments, we could not evaluate exactly the significance of microsomal and cytosolic reductases. Therefore, we looked at the in vivo situation. Mice carrying a deletion in the hepatic POR gene (30, 38), and thus lacking POR and POR-mediated cytochrome P450 activity in the liver, were treated with 3-NBA. No differences in DNA adduct formation by 3-NBA were observed in liver, lung, kidney, bladder, or colon of hepatic POR-null and wild-type mice, emphasizing the major importance of cytosolic nitroreductases and phase II enzymes in the activation of 3-NBA. In contrast, in hepatic POR-null mice treated with 3-aminobenzanthrone, DNA adduct formation in liver DNA was either diminished or significantly reduced, confirming the importance of P450 1A1 and P450 1A2 in the metabolic activation of 3-aminobenzanthrone leading to DNA adducts (22).

Most tissues contain NQO1 (37). Expression levels and activities of NQO1 differ considerably among individuals, because the enzyme is influenced by several factors, including smoking, drugs, environmental chemicals, and genetic polymorphisms (39, 40). Two distinct regulatory elements in the 5'-flanking region of the NQO1 gene, the antioxidant response element and the xenobiotic response element, have been shown to regulate NQO1 expression in many cellular systems (37, 41). Antioxidant response element–mediated NQO1 gene expression is increased by a variety of phenolic antioxidants, tumor promoters, and H_2O_2 (37, 42). Human exposure to 3-NBA is thought to primarily occur via the respiratory tract and inhaled particles (e.g., derived from diesel emissions) are able to generate reactive oxygen species like H_2O_2 (43). Hence, exposure to particulate matter might enhance 3-NBA activation and increase its genotoxic potential. The xenobiotic response element of NQO1 shares significant homology with the xenobiotic response element of P450 1A1 (44). Both NQO1 and P450 1A1 genes can be induced by 2,3,7,8-tetrachlorodibenzo[1,4]dioxin and...
Sudan I, the latter compound being used in the present study to induce NQO1 in rats (25, 45).

Using genetically engineered V79 cells expressing human NAT1, NAT2, SULT1A1, or SULT1A2, we previously showed that these enzymes strongly contribute to the metabolic activation of 3-NBA (16, 18). Moreover, in these cells, 3-NBA induced a dose-dependent increase in the mutation frequency at the hprt locus, indicating that the expression of NATs and SULTs contribute to the mutagenic potency of 3-NBA in mammalian systems. In the present study, we show the participation of authentic human hepatic NATs and SULTs in the bioactivation of 3-NBA leading to DNA adducts. However, whereas a highly significant correlation between the activities of NAT2 and the formation of 3-NBA–DNA adducts was found, no statistically significant correlation with the NAT1 and SULTs activities in the cytosols was observed. Addition of PAPS increased 3-NBA–DNA adduct levels 10-fold in all cytosols, but the SULT activities determined with 7-hydroxycoumarin as substrate showed large variations. No correlation can therefore be calculated because 7-hydroxycoumarin sulfonation does not seem to reflect the activity of SULT in conjugating the product of 3-NBA reduction, N-OH-ABA. It seems, therefore, that the different individual catalytic activities of NQO1, NAT, SULT, and maybe also glutathione transferase contribute collectively to 3-NBA–DNA adduct formation. Using cytosols containing recombinant human NATs or SULTs in incubations with human recombinant NQO1, we showed that mainly NAT2 followed by SULT1A2 and, to a lesser extent SULT1A1 and NAT1, efficiently activate 3-NBA. NAT2 and SULT1A2 are expressed in liver (23, 24); more importantly, as recently discussed (16, 18), NAT1 and NAT2 as well as SULT1A1 and SULT1A2 are expressed in cells of the respiratory tract (46, 47).

Genetic polymorphisms may contribute to an individual’s susceptibility to 3-NBA and could be important determinants of a possible cancer risk of 3-NBA in humans. Thus far, two polymorphisms in the human NQO1 gene have been found in the general population, one of them being associated with an increased risk of urothelial tumors (48) and pediatric leukemia (49). Human NAT1 and NAT2 are genetically polymorphic, resulting in different activities of the gene product that segregate individuals into slow and rapid acetylator phenotypes (23). Multiple studies have shown that urinary bladder cancer risk is higher in individuals with slow NAT2 acetylator phenotype, whereas for colon cancer rapid NAT2 acetylator phenotype confers a higher risk (23). SULT1A1 and SULT1A2 are also polymorphic in humans (24) and are associated with increased cancer risk including lung (50) and esophageal cancer (51). Thus, genetic polymorphisms in NQO1, NAT, and SULT genes could be important determinants of a possible lung cancer risk from 3-NBA.

In summary, human hepatic cytosols activate the potent mutagen and suspected carcinogen 3-NBA to species forming DNA adducts identical to those formed in vivo in 3-NBA–treated rodents. This is important for the estimation of the 3-NBA genotoxicity (carcinogenicity) to humans. Cytosolic NQO1 is of major importance in catalyzing the first step of the reductive activation of 3-NBA. Additionally, NAT1 and NAT2 as well as SULT1A1 and SULT1A2 expression in cytosols contribute substantially and specifically to the metabolic activation of 3-NBA.

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