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MOLECULAR MECHANISM OF GENOTOXICITY OF THE ENVIRONMENTAL POLLUTANT 3-NITROBENZANTHRONE

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3-Nitrobenzanthrone (3-NBA) is a suspected human carcinogen identified in diesel exhaust and air pollution. This article reviews the results of our laboratories showing which of the phase I and II enzymes are responsible for 3-NBA genotoxicity, participating in activation of 3-NBA and its human metabolite, 3-aminobenzanthrone (3-ABA), to species generating DNA adducts. Among the phase I enzymes, the most of the activation of 3-NBA *in vitro* is attributable to cytosolic NAD(P)H:quinone oxidoreductase (NQO1), while *N*,*O*-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2 are the major phase II enzymes activating 3-NBA. To evaluate the importance of hepatic cytosolic enzymes in relation to microsomal NADPH:cytochrome P450 (CYP) oxidoreductase (POR) in the activation of 3-NBA *in vivo*, we treated hepatic POR-null and wild-type C57BL/6 mice with 3-NBA or 3-ABA. The results indicate that 3-NBA is predominantly activated by cytosolic nitroreductases such as NQO1 rather than microsomal POR. In the case of 3-ABA, CYP1A1/2 enzymes are essential for the oxidative activation of 3-ABA in liver. However, cells in the extrahepatic organs have the metabolic capacity to activate 3-ABA to form DNA adducts, independently from CYP-mediated oxidation in the liver. Peroxidases such as prostaglandin H synthase, lactoperoxidase, myeloperoxidase, abundant in several extrahepatic tissues, generate DNA adducts, which are formed *in vivo* by 3-ABA or 3-NBA. The results suggest that both CYPs and peroxidases may play an important role in metabolism of 3-ABA to reactive species forming DNA adducts, participating in genotoxicity of this compound and its parental counterpart, 3-NBA.

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. Nitro-aromatics 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-aromatics in the lungs of non-smokers with lung cancer has led to considerable interest is assessing their potential cancer risk to humans[2].

A new member of this group of compounds, 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Fig. 1), was discovered in diesel exhaust and in airborne particulate matter[3, 4]. 3-NBA might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere. As a likely consequence of atmospheric washout, 3-NBA has also been detected more recently in surface soil and rainwater. The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), has been found in urine samples of salt mine workers occupationally exposed to diesel emissions[4], demonstrating that human exposure to 3-NBA in diesel emissions can be significant and is detectable. 3-NBA is an exceptionally potent mutagen in the Ames *Salmonella typhimurium* assay, scoring more than 6 million revertants per nanomol in strain YG1024 expressing bacterial nitroreductase and *O*-acetyltransferase[3]. 3-NBA is also a potent mutagen in the transgenic Muta Mouse assay[5]. 3-NBA has been shown to be genotoxic in several short-term tests. Its genotoxicity has been further

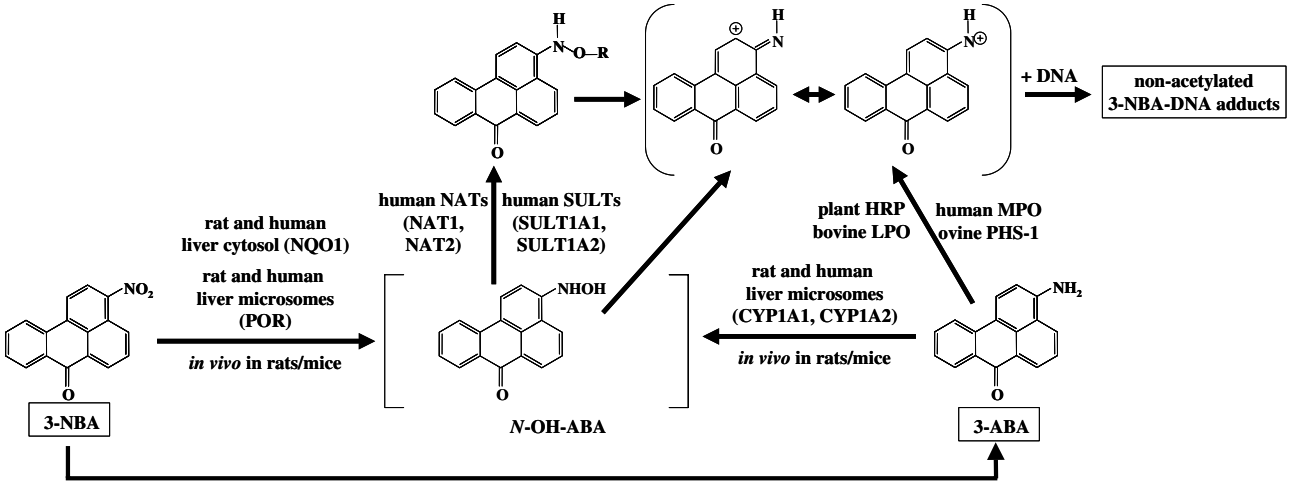

Fig. 1. Proposed pathways of metabolic activation and DNA adduct formation of 3-NBA and 3-ABA. See text for details. NQO1, NAD(P)H:quinone oxidoreductase; NAT, N,O-acetyltransferases; SULT, sulfotransferase; CYP, cytochrome P450; POR, NADPH:cytochrome P450 oxidoreductase; R = -$COCH_3$ or -SO_3H. [adapted from reference 21]

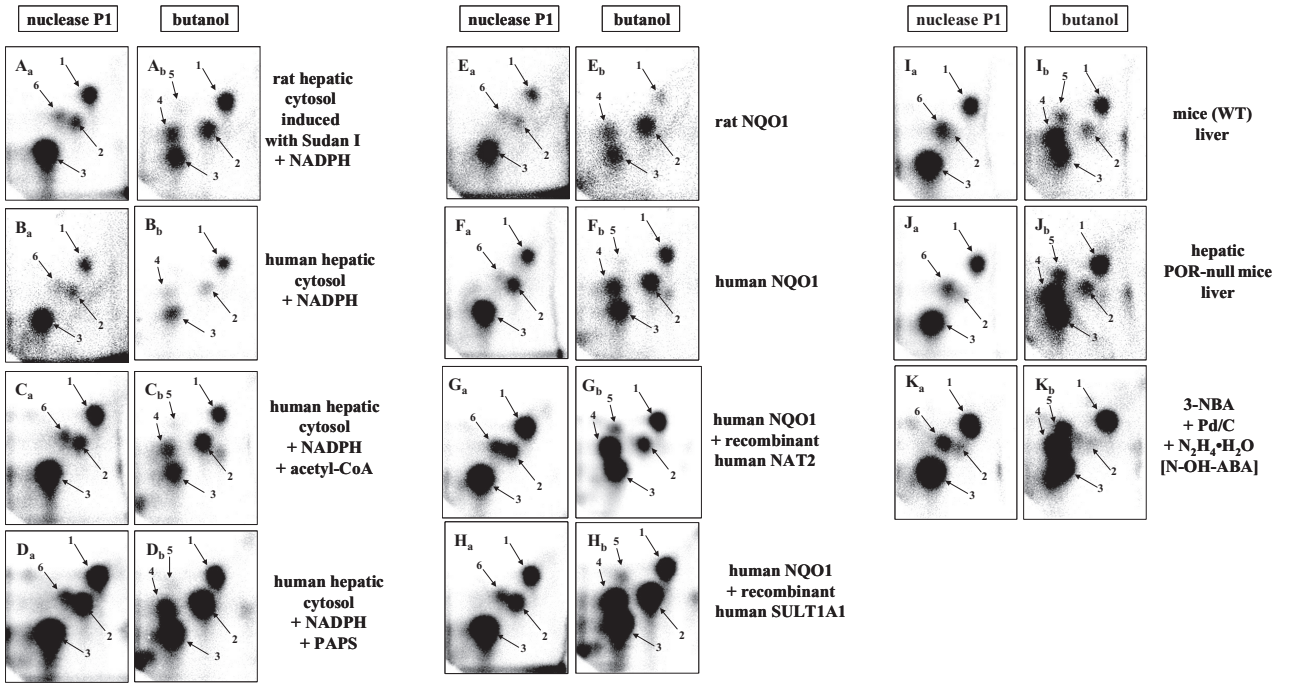

Fig. 2. Autoradiographic profiles of 3-NBA-derived DNA adducts by using the nuclease P1 digestion (*left panels*) or butanol extraction (*right panels*) enrichment version of the ^{32}P-postlabeling assay. [adapted from reference 11]. Numbers in the figure represent the 3-NBA-derived DNA adducts.

documented by the detection of specific DNA adducts formed *in vitro* as well as *in vivo* in rodents[5–9]. Moreover, 3-NBA is carcinogenic to rats[10].

Determining the capability of humans to activate 3-NBA and its human metabolite, 3-ABA, to species forming DNA adducts and understanding which human enzymes are involved in its metabolic activation are important in the assessment of susceptibility to this environmental contaminant. The detection of specific DNA adducts by ^{32}P-postlabelling analysis has allowed us to use 3-NBA-DNA and 3-ABA-DNA binding as an endpoint for studying the enzymology of the metabolic activation of 3-NBA. Here, we review the results showing which of the phase I and II enzymes are responsible for 3-NBA genotoxicity, participating in activation of 3-NBA and 3-ABA to species generating DNA adducts.

ACTIVATION OF 3-NBA BY HEPATIC CYTOSOLIC ENZYMES PLAYS A PREDOMINANT ROLE IN ITS GENOTOXICITY

We have demonstrated 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[6-9, 11]. Comparative analyses showed that all major DNA adducts (Fig. 2) are products derived from reductive metabolites bound to dA (adducts 1 and 2) or dG (adducts 3, 4, and 5)[59]. Moreover, since we found that all major DNA adducts are detectable after reaction of *N*-OH-ABA with DNA (Fig. 2)[11], we assume that all 3-NBA-DNA adducts are formed by simple nitroreduction. This indicates that *N*-OH-ABA is the critical intermediate in 3-NBA-derived DNA adduct formation (Fig. 1, 2). 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 tested 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, an inhibitor of NQO1(ref.[11]), provided additional evidence for the major role of NQO1, and the utilization of human recombinant NQO1 fully corroborated the capability of this enzyme to activate 3-NBA (Fig. 2). 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[12]. We also showed that isolated rat cytosolic NQO1 efficiently activated 3-NBA (Fig. 2). Molecular modelling and docking of 3-NBA to the active centres of rat and human NQO1 protein indicate similarities in 3-NBA binding to both enzymes; calculated apparent dissociation constants (K_s) are of the same order for NQO1 enzymes from both species[11].

Using genetically engineered V79 cells expressing human NAT1, NAT2, SULT1A1 and SULT1A2, we showed that all these conjugating enzymes strongly contribute to the metabolic activation of 3-NBA leading to DNA adducts[8, 13]. Moreover, in these V79 cells, 3-NBA induced a dose-dependent increase in the mutation frequency at the *hprt* locus, indicating that the expression of NATs and SULTs contributes to the mutagenic potency of 3-NBA in mammalian systems. We also demonstrated the participation of authentic human hepatic NATs and SULTs in the bioactivation of 3-NBA leading to DNA adducts (Fig. 2). In addition, it seems that the different individual catalytic activities of NQO1, NAT, SULT and maybe also glutathione transferase (unpublished results) contribute collectively to DNA adduct formation by 3-NBA. Using cytosols containing recombinant human NAT1 and NAT2 or SULT1A1, 1A2, 1A3, 1E and 2A1 in incubations with human recombinant NQO1 we showed that mainly NAT2 followed by NAT1 and SULT1A1 and, to a lesser extent, SULT1A2 efficiently activate 3-NBA(ref.[11]). NAT2 and SULT1A1 are highly expressed in liver[14, 15]. More importantly, NAT1 and NAT2 as well as SULT1A1 and SULT1A2 are expressed in cells of the respiratory tract[14, 15].

So 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 tumours[16] and pediatric leukaemia[17]. The human *NAT1* and *NAT2* genes are genetically polymorphic, resulting in different activities of the gene product that segregate individuals into slow and rapid acetylator phenotypes[14]. 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[14]. SULT1A1 and SULT1A2 are also polymorphic in humans[15] and

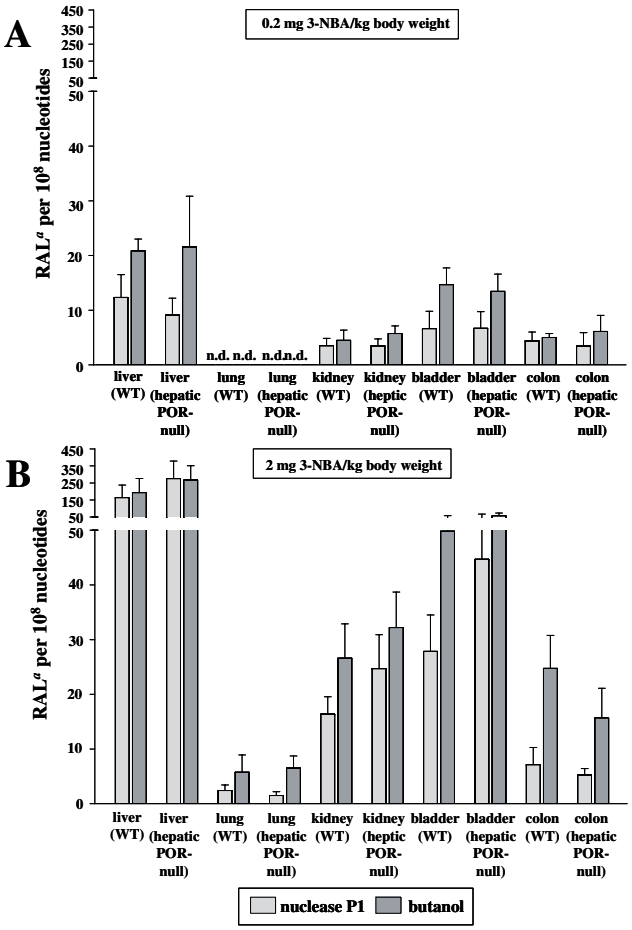

Fig. 3. Total DNA adduct formation by 3-NBA in various organs of hepatic CPR-null mice and wild-type (WT) littermates on a C57BL/6 background treated with (A) 0.2 or (B) 2 mg 3-NBA/kg body weight. All results are presented as the mean ± SD from three mice; each DNA sample was determined by two postlabeled analyses. *a*RAL, relative adduct labeling. N. d. = not detected. [adapted from reference 11]

are associated with increased cancer risk including lung and esophageal cancer[18]. Thus, genetic polymorphisms in *NQO1*, *NAT* and *SULT* genes could be important determinants of a possible lung cancer risk from 3-NBA.

In mammalian cells, both cytosolic and microsomal subfractions contain enzymes that catalyse the reduction of nitroaromatic compounds[9,11]. In rat and human hepatic microsomes we identified CPR as the enzyme activating 3-NBA, generating 3-NBA-DNA adduct profiles identical to those found in liver tissue of 3-NBA-treated rodents[5–7,9]. The comparison of 3-NBA-DNA adduct levels formed by human hepatic microsomes[9] and cytosols[11] reveals that the cytosolic enzyme systems are much more efficient in the reductive activation of 3-NBA than microsomes. In the presence of NADPH levels of 3-NBA-derived DNA adducts were more than 35-fold higher in cytosols than in microsomes using the nuclease P1 version of ^{32}P-postlabelling assay[9,11]. Moreover, the content of cytosolic protein per gram of human liver tissue is about one order of magnitude 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 the phase I enzymes in microsomal and cytosolic fractions of human livers, because cytosolic conjugation enzymes influence 3-NBA-DNA adduct formation significantly. To resolve the contribution of cytosolic and microsomal nitroreductases in 3-NBA activation, we therefore looked at the *in vivo* situation. Mice carrying a deletion in the hepatic *POR* gene[11,19], and thus lacking POR and POR-mediated CYP activity in the liver, were treated with 3-NBA. No differences in DNA adduct formation by 3-NBA were observed in livers and other tissues examined (lung, kidney, bladder, and colon) of hepatic POR-null and wild-type C57BL/6 mice (Fig. 3), emphasising the major importance of cytosolic nitroreductases and phase II enzymes in the activation of 3-NBA.

ACTIVATION OF 3-ABA, A HUMAN METABOLITE OF 3-NBA, BY CYTOCHROMES P450 AND PEROXIDASES PLAYS A PREDOMINANT ROLE IN ITS GENOTOXICITY

3-ABA, the major metabolite of 3-NBA, was detected in the urine of salt mining workers occupationally exposed to diesel emissions at similar concentration (1–143 ng/24 h urine) to that of 1-aminopyrene (2–200 ng/24 h urine), the corresponding amine of the most abundant nitro-aromatic compound detected in diesel exhaust matter[4]. We found that 3-ABA is activated by human and rat hepatic microsomes generating a DNA adduct pattern identical to that formed by its nitroaromatic counterpart[20], indicating that *N*-OH-ABA is the critical intermediate for the formation of electrophilic arylnitrenium ions capable of reacting with DNA. Since we found that all 3-ABA-derived DNA

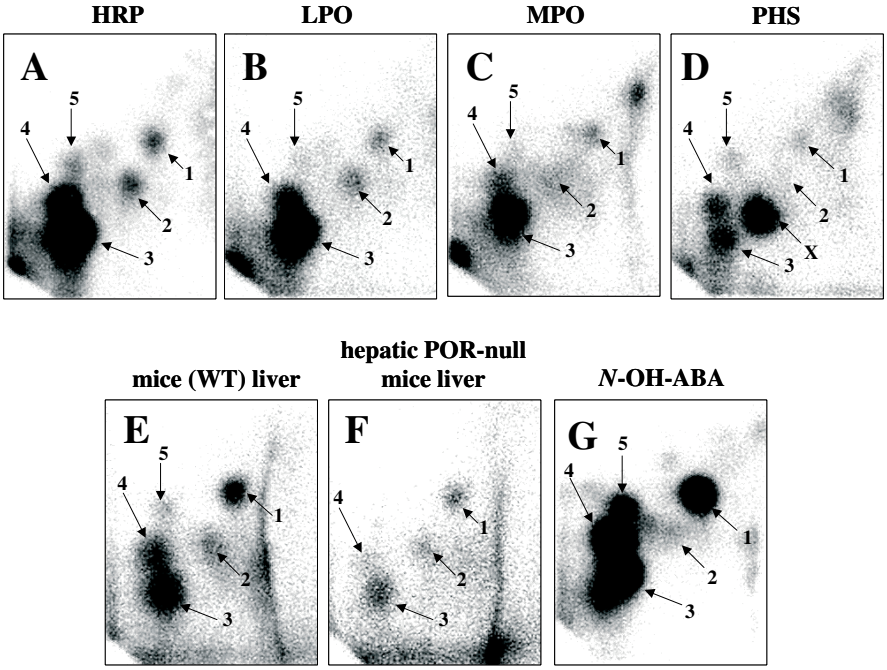

Fig. 4. Autoradiographic profiles of 3-ABA-derived DNA adducts by using the butanol enrichment version of the ^{32}P-post-labelling assay. Adduct profiles obtained from calf thymus DNA treated with 3-ABA (100 μM) and (A) plant HRP, (B) bovine LPO, (C) human MPO, (D) ovine PHS-1, from liver DNA of (E) wild-type littermates on a C57BL/6 background and (F) hepatic POR-null mice treated with 2 mg of 3-ABA per kg body weight (these profiles are representative of adduct profiles obtained with DNA from kidney and bladder), and (G) from salmon testis DNA treated with *N*-OH-ABA. [adapted from reference 21]. Numbers in the figure represent the 3-ABA-derived DNA adducts.

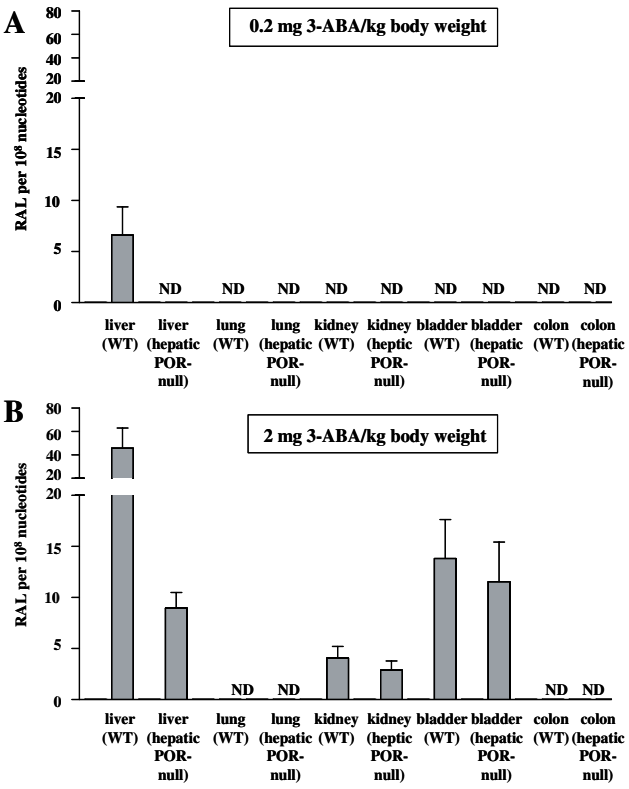

Fig. 5. Total DNA adduct formation by 3-ABA in organs of hepatic CPR-null mice and wild-type (WT) littermates on a C57BL/6 background treated with (A) 0.2 or (B) 2 mg 3-ABA per kg body weight. All results are presented as the mean ± SD from three mice; each DNA sample was determined by two postlabelling analyses. RAL, relative adduct labelling. ND = not detected. [adapted from reference 21]

adducts are detectable after reaction of *N*-OH-ABA with DNA (Fig. 4), we conclude that all adducts are formed by simple *N*-oxidation.

We found that formation of 3-ABA-derived DNA adducts in human liver microsomal samples was strongly dependent on the activities of CYP1A1 and 1A2(ref.[20]). Furthermore, use of human recombinant CYP1A1 and 1A2 expressed in mammalian cells, and in microsomes of baculovirus-transfected insect cells (Supersomes), corroborated the participation of these enzymes in the formation of 3-ABA-derived DNA adducts[20]. To evaluate the contribution and importance of hepatic CYP enzymes to the bioactivation of 3-ABA *in vivo* we treated hepatic POR-null mice with 3-ABA(ref.[21]). DNA adduct formation in liver DNA was either undetectable or significantly reduced, confirming the importance of CYP1A1 and 1A2 in the metabolic activation of 3-ABA *in vivo* (Fig. 5). More importantly, no difference in DNA adduct formation by 3-ABA was observed in kidney and bladder of hepatic POR-null and wild-type mice (Fig. 5), indicating that cells in extrahepatic tissues have the metabolic capacity to oxidise 3-ABA, and that the same reactive species leading to DNA adduct formation are formed, independent of CYP-mediated metabolism of 3-ABA in the liver. Thus, other enzymes might be involved in the formation of the same reactive intermediate(s) as that generated from *N*-OH-ABA in 3-ABA-derived DNA adduct formation in the urinary tract. Prostaglandin H synthase (PHS), a peroxidase, abundant in the urinary tract and important for the activation of different genotoxins[22], might be a candidate for such reactions. Indeed, purified ovine PHS-1 catalysed 3-ABA activation (Fig. 4). This finding also supports the suggestion that PHS, either in combination with CYP enzymes or alone, may contribute to the high DNA binding by 3-ABA in kidney and bladder of mice treated with 3-ABA. Moreover, the comparison of 3-ABA-derived DNA adduct formation catalyzed by three other peroxidases, plant horseradish peroxidase (HRP), lactoperoxidase (LPO) and myeloperoxidase (MPO) (Fig. 4), revealed that peroxidases are indeed capable of activating 3-ABA[21].

Although HRP might only serve as a model peroxidase, the findings that PHS, MPO and LPO are effective in 3-ABA activation may be of greater significance. Human exposure to 3-NBA is thought to occur primarily *via* the respiratory tract, and both PHS and MPO are expressed in lung[22,23]. In this context, it is noteworthy that 3-ABA is the major metabolite of 3-NBA in human fetal bronchial, rat alvealor type II, rat epithelial bronchial, and rat mesenchymal lung cells[24]. MPO is expressed in the primary granules of neutrophils, which are recruited into the lungs after chemical and/or immunological insults[23]. Neutrophils release MPO and undergo a respiratory burst, which is characterised by a massive increase in oxygen consumption and a consequent NADPH-dependent production of superoxide and other free radicals. MPO also catalyzes the production of the oxidising agent hypochlorous acid (a one- and two-electron oxidant that can attack endogenous molecules including DNA) from hydroxyl radicals and chloride ions. Of note, hydrogen peroxide required for MPO-mediated 3-ABA oxidation can also be supplied by xanthine oxidase, a mammalian nitroreductase involved in the bioactivation of the nitroaromatic counterpart 3-NBA(ref.[11]). 3-NBA is carcinogenic in lungs of F344 rats after intratracheal administration of 3-NBA(ref.[10]). Moreover, epidemiological studies have found a lower lung cancer risk in individuals with a G→A polymorphism at position −463 in the promoter region of the *MPO* gene[23]. Consequently, the variability of expression and activities of MPO could be an important determinant of lung cancer risk from 3-NBA and its metabolites. Exposure to environmental pollutants like 3-NBA or 3-ABA might also be associated with an increased risk of breast cancer[25]. Besides MPO, other peroxidases such as LPO are involved in mutagenic and carcinogenic activation in human mammary glands[25]. LPO is an enzyme present in milk, secreted by human mammary ductal epithelial cells into the breast ducts. Thus, like other lipophilic compounds 3-ABA can possibly bioaccumulate in fatty tissues, such as the breast, and depending on the levels of activating enzymes present (e.g. MPO and/or LPO) 3-ABA can be oxidatively metabolized to DNA-

binding intermediates. The importance of MPO and LPO in the metabolic activation of 3-ABA is consistent with previous reports demonstrating that the enzymes metabolically activate a wide range of tobacco smoke mutagens and environmental pollutants to DNA-damaging metabolites, including aromatic amines, heterocyclic amines and nitroaromatic compounds[26, 27]. The enzymes participating in reactions leading to activation and DNA adduct formation by 3-NBA and 3-ABA are shown in Figure 1.

It should be noted that tumour development in a specific organ may also be influenced by promotional pressures on initiated cells in target organs and not only by the levels of DNA adducts formed by compounds like 3-ABA. Hence, the formation of radicals during peroxidase-mediated oxidation of 3-ABA(ref.[21]) may also be important in the process of tumour promotion. Therefore, these results suggest that 3-ABA is not only a suitable biomarker of exposure to 3-NBA but it may also contribute, directly and indirectly, to the high genotoxic potential of 3-NBA. Besides CYP1A1 and 1A2 expression in the human respiratory tract peroxidases such as MPO and PHS could contribute significantly and specifically to the metabolic activation of 3-ABA. These results may also indicate an important role for peroxidases in tumour development, not only through direct activation of 3-ABA but also due to the production of free radicals in or near the target cells. However, the exact function of such promotional pressures in 3-NBA-initiated tumourigenesis remains to be investigated[21].

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