The Use of Protein Adducts to Investigate the Disposition of Reactive Metabolites of Benzene

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Benzene is metabolized to a number of electrophilic species that are capable of binding to both DNA and proteins. We used adducts of hemoglobin (Hb) and bone marrow proteins to study the disposition of three benzene metabolites (benzene oxide [BO], 1,2-benzoquinone [1,2-BQ], and 1,4-benzoquinone [1,4-BQ]) in F344 rats and B6C3F1 mice following a single oral dosage of [%13C]benzene and/or [%13C]benzene. Our assays focused upon cysteine adducts that accounted for 38 to 45% of protein binding to Hb and 63 to 81% of protein binding to bone marrow. Although both species and rats showed dose-related increases in Hb and bone marrow protein adducts of BO and of the two benzoquinones, large interspecies and interspecies differences were noted, suggesting different preferences in metabolic pathways. The highest levels of adducts in mice were of 1,4-BQ (10–27% of all cysteine adducts), while in rats, BO adducts predominated in Hb (73% of all cysteine adducts) and 1,2-BQ adducts predominated in the bone marrow (14% of all cysteine adducts). High background levels of 1,2-BQ and 1,4-BQ adducts were also detected in both species, indicating that the toxic effects of quinone metabolites may be only important at high levels of benzene exposure. — Environ Health Perspect 104(Suppl 6):1235–1237 (1996)

Key words: benzene, benzene oxide, benzoquinone, protein adducts, biomarkers

Introduction

Benzene is a ubiquitous environmental contaminant that is mutagenic and carcinogenic in various animal species including humans. There is strong evidence that exposures to benzene increase the risk of leukemias, particularly of the acute myelocytic type. However, the mechanism by which benzene exerts its carcinogenicity remains elusive because of the production of numerous electrophilic metabolites. Following inhalation, benzene is rapidly absorbed in the blood and distributed throughout the body. About 50% of the absorbed dose is eliminated unchanged in the exhaled air (1) while the remainder is metabolized, primarily via P4502E1, through the electrophilic intermediate, benzene oxide (BO), which either spontaneously rearranges to phenol [23–50% of the dose; (1)] or is hydrated by epoxide hydrolase to produce benzene-1,2-dihydriodiol. This division of the two major pathways from BO to either phenol or the dihydriodiol is important to as well as species and tissue differences in P4502E1 and epoxide hydrases can influence the disposition of the reactive metabolites in animals receiving substantial doses of benzene. Phenol can subsequently be acted upon by P4502E1 to produce hydroquinone (HQ) [1–5% of the dose, (1)] that exists in equilibrium with 1,4-benzoquinone (1,4-BQ) and the associated semiquinone, both of which are electrophiles. The dihydriodiol can be oxidized through dehydrogenases to catechol (CAT) [3–5% of the dose, (1)] which also exists in equilibrium with 1,2-benzoquinone (1,2-BQ) and its semi-quinone. Directly produced by P4502E1 oxidation of phenol, but this is considered to be a minor pathway (2). Finally, ring-opened products can be formed either from oxidation of BO (or its oxepin) or from reactions of benzene with reactive oxygen species (3). These products include trans,trans-muconaldehyde, which is also mutagenic and accounts for 1 to 3% of the benzene dose (4). In addition to these numerous electrophilic metabolites of benzene, extensive research has also shown that oxygen and glutathione free radicals as well as products of lipid peroxidation accompany peroxidative metabolism of the phenolic metabolites; these free radical products can also damage DNA (4).

Although research has confirmed that most of the electrophilic metabolites of benzene are capable of forming covalent adducts with proteins or DNA, a major hypothesis of benzene-induced leukemogenesis has focused on the quinones and semiquinones that induce mutations, either directly or through quinone-mediated processes (5,6). Indeed, since peroxidases are abundant in the bone marrow, much speculation about the carcinogenicity of benzene has centered on the preferential conversion of HQ and CAT to the corresponding quinones in the bone marrow (7).

Although the quinone metabolites have been hypothesized to be important in the genotoxicity of benzene, there are a number of environmental or endogenous sources of phenol (the precursor of HQ), CAT, and HQ (8,9). Consequently, high levels of nonbenzene-derived quinones, and the associated macromolecular adducts would be expected. Indeed, we found high background levels of catechol-bound Hb adducts of 1,2-BQ and 1,4-benzoquinone (1,4-BQ) in rats, mice, and humans [1–30 pmol adduct/mg Hb (10,11)].

Another important development was the observation of Bechtold et al. (12) that significant quantities of S-phenylcysteine (SPC) were measured in Hb from rats and mice exposed to benzene. The authors attributed this SPC in the Hb to a cysteine adduct of BO [S-(1,2-dihydro-2-hydroxy-phenyl)-cysteine] that was presumably reamorized to SPC either spontaneously or upon treatment with acid in the assay. Although this is a plausible mechanism for formation of SPC, it does not preclude the possibility of production within the red blood cells (RBC) per se by, for example, a cyclohexadienyl radical (13). If Bechtold et al. (14) are correct in their speculation about the origin of SPC in Hb, then it follows that BO (or its oxepin) is sufficiently stable to migrate from the hepatocyte into the systemic circulation, where it can induce genotoxic effects. This
is interesting because, although BO is capable of reacting with macromolecules, we currently have only a very sketchy understanding of the disposition of BO \textit{in vivo} and SPC could provide an important avenue for such inquiries. Bechtold et al. (12, 14) also postulated that there should be much lower background levels of BO adducts than BQ adducts, thus making SPC in Hb or albumin more specific markers of benzene exposure.

We used assays of cysteine adducts of BO (measured as SPC), 1,2-BQ, and 1,4-BQ to investigate the disposition of these electrophilic metabolites of benzene in rats and mice (15). By using benzene that had been labeled with both $[^{14}\text{C}]$benzene and $[^{13}\text{C}]$benzene, we were able to account for the proportions of total protein adduction represented by the particular adducts and also to differentiate benzene-specific (i.e., $[^{13}\text{C}]$BO or $[^{13}\text{C}]$BO adducts) from background adducts (i.e., $[^{12}\text{C}]$ adducts). The assays were applied to proteins from both the blood (Hb) and the bone marrow of these animals.

**Material and Methods**

**Chemicals.** All chemicals and enzymes used were the same as those described by McDonald et al. (10, 11, 15).

**Animal Experiments.** Ten male F344 rats were divided into five groups and were administered a single dose of $[^{13}\text{C}]$benzene at 0 (corn oil vehicle control), 50, 100, 200, and 400 mg /kg bw via gastric intubation as described by McDonald et al. (11). Twenty male F344 rats and 20 male B6C3F1 mice were divided into five groups and administered a single dosage of $[^{14}\text{C}]$C$_6$H$_5$benzene at 0 (corn oil vehicle control), 50, 100, 200, and 400 mg /kg bw via gastric intubation as described by McDonald et al. (15). In both experiments the animals were sacrificed 24 hr after dosing and blood was obtained by cardiac puncture into a heparinized syringe following anesthesia with methoxyfluorane.

**Analysis of Protein Adducts.** Globin and bone marrow proteins were isolated as described by McDonald et al. (11). Adducts of 1,2-BQ and 1,4-BQ were analyzed by first adding a protein-bound internal standard ([H$_2$]HQ-Hb), digesting the proteins with protease XIV, and then reacting the proteins with the nickel–aluminum catalyst. Raney nickel, to release the quinones from cysteine residues on the proteins (10). After extracting the quinone species with diethyl ether and removing the solvent under a stream of nitrogen, the analytes were derivatized with heptafluorobutylylimidazole before detection by gas chromatography–mass spectrometry (GC–MS) in the negative chemical ionization (NCI) mode (10).

BO–protein adducts were analyzed (as SPC) using the method of Bechtold et al. (14). This involved complete protein hydrolysis followed by high performance liquid chromatography (HPLC) separation and collection of the fraction containing SPC and the internal standard, S-benzylcysteine. The SPC was then derivatized and detected by GC–MS in the electron impact mode as described by McDonald et al. (15). The use of isotopically labeled benzene and detection by MS allowed for simultaneous quantitation of both benzene-specific (i.e., $[^{13}\text{C}]$BO or $[^{13}\text{C}]$BO adducts) and background adducts (i.e., $[^{12}\text{C}]$BO or $[^{12}\text{C}]$BO adducts).

**Results**

**Proportions of Protein Adducts.** The binding of $[^{14}\text{C}]$benzene metabolites to Hb and bone marrow proteins increased over the range of dosages between 50 and 400 mg/kg bw in both rats and mice (15). Because reaction of proteins with Raney nickel specifically cleaves adducts bound to sulfhydryl groups, it was possible to estimate the proportions of cysteine adducts to total protein adducts from the fractions of radioactivity released by treatment with Raney nickel. These proportions of cysteine adducts to total protein adducts were not significantly different among the dosage groups; thus, mean values were estimated over all dosages. Table 1 indicates that cysteine binding in the blood and bone marrow was comparable in rats and mice (38–45% of total binding to Hb and 63–81% of total binding to bone marrow proteins).

This large proportion of total binding to cysteine residues points to the great reactivity of sulphydryl groups toward electrophilic metabolites of benzene, which can be exploited to investigate the disposition of these reactive species \textit{in vivo}.

Following assays of cysteine adducts of BO and the benzoquinones, it was observed that levels of all measured adducts increased in both the blood and bone marrow of rats and mice over the entire range of dosages (15). The proportions of particular adducts to all cysteine adducts varied greatly between species and proteins, as shown in Table 2. BO adducts represented relatively small proportions of all cysteine adducts (approximately 2–4%) in rat bone marrow, mouse Hb, and mouse bone marrow but accounted for the majority of cysteine adducts (73%) in rat Hb. Levels of 1,2-BQ adducts were highest in rat marrow (14% of cysteine adducts), followed by those in mouse Hb (6%), and in rat Hb and mouse bone marrow (both of which were about 1%). On the other hand, adducts of 1,4-BQ were most prevalent in mouse bone marrow (27% of cysteine adducts) followed by those in rat bone marrow (2.2–22%, depending upon the dosage), mouse Hb (10–14%), and finally in rat Hb (less than 1%).

**Background Adducts.** Both 1,2-BQ and 1,4-BQ gave rise to high levels of background adducts, which were measured as $[^{12}\text{C}]$BO adducts. The fold ranges of these background adducts to the benzene-specific $[^{13}\text{C}]$BO adducts varied inversely with dosage but were always greater than one, even at the highest dosage of 400 mg/kg bw. As shown in Table 3, levels of background adducts were typically 10- to 100-fold greater than those that arose from a single dosage of benzene.

**Discussion**

The proportions of adducts of specific benzene metabolites shown in Table 2 point to metabolic differences between species and tissues. For example, the large proportion of SPC in rat Hb (72.6%) compared to

Table 1. Proportions of $[^{14}\text{C}]$benzene–protein binding associated with cysteine adducts, mean values.

| Species | Protein | Hb, %<br> | Marrow, %<br> |
|---------|---------|--------|--------------|
| Rat     |         | 37.6   | 63.1         |
| Mouse   |         | 45.2   | 81.1         |

*Mean values were estimated for all dosage groups, 50 to 400 mg/kg bw.

Table 2. Proportions of cysteine adducts arising from three electrophilic metabolites of benzene, mean values.

| Metabolite      | Rat Hb, % | Rat Marrow, % | Mouse Hb, % | Mouse Marrow, % |
|-----------------|-----------|---------------|-------------|-----------------|
| Benzene oxide   | 72.6      | 4.3           | 3.7         | 1.9             |
| 1,2-Benzoquinone| 1.4       | 13.7          | 5.6         | 0.9             |
| 1,4-Benzoquinone| 0.12–0.71 | 2.2–21.9      | 10.0–13.6   | 26.6            |

*Mean values were estimated for all dosage groups, 50 to 400 mg/kg bw. *Significant trend detected with dosage range indicated.
within the bone.

Table 3. Fold ranges of background [14C/13C6]benzene to benzene-specific [13C5]BO adducts at dosages of 50 to 400 mg/kg bw.

| Species | Protein | 1.2-BO | 1.4-BO |
|---------|---------|--------|--------|
| Rat     | Hb      | 11-89  | 24.7-472 |
|         | Marrow  | 2.7-104| 2.2-60  |
| Mouse   | Hb      | 2.7-6.3| 5.2-18  |
|         | Marrow  | 160-462| 1.1-6.1 |

that of the mouse (3.7%) suggests that there could be large interspecies differences regarding either the formation of BO by the liver (and subsequent release to the blood) or the in situ formation of BO within the erythrocyte. On the other hand, these differences could reflect different rates of removal of BO within the livers of the two species due to differences in epoxide hydrolases and glutathione S-transferases. Referring to adducts of the benzokinoines, it appears that the pathway leading to the formation of HQ was favored in the mouse, whereas that leading to CAT was favored in the rat, consistent with current knowledge of benzene metabolism in the two species (16).

Although levels of adducts of Hb and bone marrow proteins of 1,2-BQ and 1,4-BQ increased with benzene dosage in rats and mice, these levels were very small compared to background concentrations of the same adducts except at the highest dosage (400 mg/kg bw). This observation is consistent with the idea that HQ and CAT are common dietary constituents or are formed endogenously but casts doubt on the notion that the phenolic metabolites can be solely responsible for the genotoxic effects of benzene, particularly at low doses.

This finding has important implications regarding both elucidation of the mechanism by which benzene induces cancer and development of optimal strategies for biomonitoring among humans exposed to benzene. If, on the one hand, the quinones or semiquinones are responsible for the genotoxicity, then the risk of cancer is likely to be elevated only at very high levels of benzene exposure, levels much greater than those currently encountered in the United States and other industrialized countries. If this conjecture is correct, it is important to know the extent of production of adducts by the semiquinones of 1,2-BQ and 1,4-BQ, since such adducts have not been measured heretofore and may account for a significant amount of the total binding of benzene metabolites (we can currently account for about 20–30% of cysteine binding in the bone marrow). It also follows from this conjecture that measuring adducts of the quinones is unlikely to be useful for biomonitoring benzene exposure because even unexposed persons have large burdens of the same adducts (8) (we confirmed that human Hb and albumin also contain large background levels of the BQ adducts).

If, on the other hand, the quinone species are not responsible for benzene’s genotoxicity, another metabolite is more likely to be the culprit and the list narrows logically to either BO or trans-trans-muconaldehyde. We have shown that BO produces adducts in the bone marrow of rats and mice (albeit at levels lower than those produced by 1,2- and 1,4-BQ in the same animals), consistent with this conjecture. It also follows that measurements of BO adducts of Hb and albumin should be effective biomarkers of benzene exposure even at very low levels. In fact, Bechtold et al. (12, 14) were able to detect SPC in albumin but not in Hb from the blood of workers exposed to 4.4 to 23 ppm of benzene. (We suspect that the sensitivity of their assay was insufficient to detect SPC in the Hb of these workers.) We are currently using protein adducts to further elucidate the doses of BO received by various tissues following administration of benzene.

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