Molecular Dosimetry of Aromatic Amines in Human Populations

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Certain aromatic amines carcinogenic for the human urinary bladder, such as 4-aminobiphenyl, undergo hepatic metabolic activation to N-hydroxyamines, which are transported to the bladder. During the transport process, these reactive species come in contact with hemoglobin and react with this blood protein. The principal hemoglobin adduct formed is a cysteine sulfanemide, and quantitative methods have been developed for the analysis of sulfanemide adducts at the levels present in human blood specimens. N-Acetylation is an alternative metabolic fate to N-hydroxylation. The amount of hemoglobin adduct is decreased to the extent that this pathway is increased relative to N-hydroxylation. Thus, the hemoglobin adduct is sensitive to dose, cytochrome P-450-mediated activation, and N-acetyltransferase-mediated detoxification. In addition, it has been shown that DNA adduct concentration of 4-aminobiphenyl present in human bladder epithelial cells is significantly associated with hemoglobin adduct levels. Thus, the hemoglobin adduct of 4-aminobiphenyl, and perhaps several other aromatic amines, is a good dosimeter for the target tissue dose of the ultimate carcinogenic metabolite of these amines. Several studies have been undertaken in which the hemoglobin adducts of aminobiphenyls in human blood specimens were determined quantitatively. Information concerning exposure status and acetylator phenotype of the same individuals was obtained simultaneously. The results of these studies indicate that the hemoglobin adduct of 4-aminobiphenyl is closely associated with three major risk factors for bladder cancer: cigarette smoking, type of tobacco smoked, and acetylator phenotype. They also support a major etiologic role for aromatic amines in much of human bladder cancer.

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Key words: hemoglobin adducts, aromatic amines, bladder cancer, 4-aminobiphenyl, cigarette smoking

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Introduction

Human urinary bladder cancer is known to be induced by 2-naphthylamine (2-NA) (1), 4-aminobiphenyl (4-ABP) (2), and benzidine (3). Several other related aromatic amines have also been implicated as human bladder carcinogens, including 4,4′-methylenedianiline (4), 4,4′-methylenebis(2-chloroaniline) (5), o-toluidine (6), and 4-chloro-o-toluidine (7). Three of these amines, 4-ABP, 2-NA, and α-toluidine, are present in cigarette smoke (8). Because these two amines are potent carcinogens and because cigarette smoking has been firmly linked to bladder cancer (9), it has been hypothesized that the amines in cigarette smoke, especially 4-ABP and 2-NA, are important, if not predominant, etiologic agents.

The biochemical mechanisms of bladder cancer induction by aromatic amines are complex and not yet fully understood, but some of the essential elements are known (10). One of these is oxidation of the amines to N-hydroxyarylamines. When they come in contact with hemoglobin, the hydroxyamines that are generally only moderately reactive form adducts in very high yield. Because the adducts formed are amenable to analysis at the levels present in nonsmokers as well as smokers (11), they have come to be used as biomarkers of aromatic amine exposure and metabolism. In this article, we will explore the utility and validity of hemoglobin adducts in the molecular dosimetry of aromatic amines.

Mechanisms of Aromatic Amine-induced Bladder Carcinogenesis

A mechanistic model for certain aromatic amine-induced bladder carcinogenesis is presented in Figures 1 to 3. This model is oriented about the molecular events thought to be most relevant for 4-ABP- and 2-NA-induced tumorigenesis. It may also depict the events leading to hemoglobin adducts by a variety of other amines, regardless of whether they are bladder carcinogens. Other elements might need to be introduced to extend it to the process of carcinogenesis by amines with a benzidine-like structure, among which would be arachidonic acid-dependent prostaglandin H synthase catalyzed co-oxidation (12).

A principal feature of this model is that N-hydroxylation is a predominantly hepatic reaction, catalyzed by cytochrome P450IA2, not one that takes place in bladder epithelial cells. Consequently, transport of the activated amine to the bladder is required. Perhaps surprisingly, the N-hydroxyarylamine appears to be exported from the liver directly (13), which may be partly responsible for the high hemoglobin adduct yields.

From this model, it is clear that hemoglobin adducts should accurately reflect the amount of amine that an individual takes up from the environment, converts to its proximate carcinogenic form, and exports into blood. Hemoglobin adducts of aromatic amines can thus be considered biomarkers that give an approximate measure of the biologically effective dose.

The level of hemoglobin adduct will be governed by the extent to which other hepatic metabolism intervenes in the activation process. Several other metabolic processes are possible, including acetylation to form N-aryl acetamides, N-glucuronidation, sulfation of the N-hydroxyarylamines, and oxidation at carbon centers to yield phenolic amines. At present it is uncertain whether carbon-centered oxidation is really a different reaction than N-oxidation. More likely, both products are formed through a common cationic intermediate that precedes substrate-oxygen bond for-
mation so that the ratio of \( N \)-to C-hydroxylation is dependent primarily on the distribution of electron density in the intermediate cation (14). It is also P450 enzyme-dependent, but to a lesser degree. The ratio of phenolic products to hydroxylamine is thus largely invariant.

In contrast, the other hepatic metabolism is subject to potentially considerable variability. \( N \)-Acetylation may be under genetic control (15), so phenotype-dependent differences in amine adduct levels may be displayed (16). Substantial interindividual variability in sulfotransferase activity is known, and some of it may be phenotypically expressed (17). It is not known, though, whether sulfotransferases catalyze the sulfation of \( N \)-hydroxy-4-ABP or \( N \)-hydroxy-2-NA. Likewise, the formation of \( N \)-glucuronides of these amines is not well characterized. How much these other reactions will influence the yield of hydroxylamines is a function of the relative rate constants, amine concentrations, and inhibitor concentrations.

Substantial secondary metabolism of \( N \)-hydroxyarylamines within bladder epithelial cells almost certainly occurs also. It is doubtful that any of this further metabolism has any measurable effect on hemoglobin adducts. However, it may affect the formation of DNA adducts, as indicated in Figure 3. Herein may lie a limitation on the use of hemoglobin adducts to predict cancer risk, in addition to other such fundamental limitations as the nonassociation of protein adducts with DNA adduct repair.

**Exposure Assessment**

Numerous studies have been conducted that indicate that hemoglobin adducts of aromatic amines can be used to reveal information concerning exposure to the amines. These studies have focused on populations whose exposure to amines is principally the result of exposure to tobacco smoke. It has been shown repeatedly that, on average, smokers exhibit higher hemoglobin adducts of aromatic amines than nonsmokers (16,18–21). No study has been conducted that has failed to reach this conclusion.

Two other studies have also found that the level of adducts in nonsmokers is positively associated with their exposure to environmental tobacco smoke (ETS) (22, unpublished results). High exposure to ETS was found to result in a roughly 50% increase in the average levels of 3- and 4-ABP over that determined in individuals with minimal exposure. These results seem to indicate that there remains some exposure to aromatic amines that is not associated with tobacco smoke.

The preceding studies have depended on the comparison of aggregate measurements, necessitated by the considerable interindividual variability in the response of the hemoglobin adduct to dose as well as uncertainty about the degree of exposure. It has also been possible to interpret adduct values in terms that have individual relevance. For example, a person with 4-ABP adducts at the level of 80 to 100 pg/g hemoglobin might be either a nonsmoker or smoker. However, smokers who quit smoking will experience a marked decline in adducts, regardless of the level present at the time when they were smoking (23).

The hemoglobin adduct of 4-ABP has also proven to be highly characteristic for individual subjects. In an unpublished study, we analyzed 4-ABP hemoglobin adducts in 37 subjects at the beginning and end of a 14-week period. The correlation of the two analyses is very good \((r = 0.90)\), indicating that there is very little change in adducts over an extended time period.

Analysis of fetal hemoglobin obtained from cord blood postpartum has revealed that fetuses are exposed to \( N \)-hydroxy-4-ABP (20). A significant association was found between fetal levels and the adduct level in maternal blood sampled at or near the time of birth. The correlation coefficient was 0.85 when the entire study sample, including both smoking and nonsmoking mothers, was analyzed. When only smoking mothers and their infants were included, the correlation coefficient was reduced to 0.71 \((n = 14)\), which was still highly significant \((p = 0.002)\). The mechanism of fetal exposure to \( N \)-hydroxy-4-ABP is still unclear. Numerous possibilities could be considered, including maternal activation followed by transplacental transfer, placental metabolic activation, and fetal activation. However, fetal liver does not express much P4501A2 (24), and placental activity appears to be associated mostly with 1A1 (25,26). Thus, maternal metabolic activation seems most likely.

If hemoglobin adducts reflect exposure as the foregoing discussion suggests, then there should be a quantifiable relationship between the two. The only independent measure of exposure to aromatic amines that we have been able to study is exposure to cigarette smoke. This can be determined in several ways, including self-reported number of cigarettes smoked per day, serum or urinary cotinine levels, and ambient air monitoring for nicotine. All have been examined.
4-Aminobiphenyl adducts have been positively and significantly associated with ambient nicotine, increasing 50 to 70% through three categories of exposure (unpublished results). These data confirmed the role of ETS in producing hemoglobin adducts in nonsmokers but were insufficient to characterize a dose-response relationship.

Similarly, we have investigated the correlation between 4-ABP adducts and measures of cotinine (21,23,27,28), assuming that cotinine would be a good surrogate measure of 4-ABP intake. Significant correlations have been found, provided the study samples included sufficient numbers of light smokers. Among heavy smokers, though, there is little association of 4-ABP adducts and cotinine. These results are not too surprising because, while the ratio of aromatic amines to nicotine in tobacco smoke may be fairly constant, the conversion of nicotine to cotinine in vivo is subject to considerable interindividual variability.

Self-reported cigarette consumption is the simplest measure of aromatic amine exposure and we have investigated the relationship between consumption and adduct levels in some detail. A nonlinear response is always observed when those who smoke more than about 20 cigarettes per day are included, with increasing consumption leading to little if any increase in adducts. A trivial explanation for the nonlinearity is that the amine dose is not proportional to consumption of cigarettes at high numbers of cigarettes smoked because of a reduced degree of inhalation, reduced number of puffs per cigarette, etc. This explanation probably is not correct, though. Published (29) and unpublished data from studies of ethylene oxide-hemoglobin adduct formation, which is much less subject to metabolic effects than aromatic amine adduct formation, indicate no such nonlinearity. Carboxyhemoglobin, likewise a measure that is independent of metabolic effects, displays a linear increase with cigarette consumption (30). Thus, it is likely that interindividual differences in metabolism contribute significantly to the shape of the dose-response curve.

The effect of interindividual variability can also be observed in the range of adducts in persons with similar exposure. Figure 4 summarizes all the data collected in this laboratory for smokers who reported smoking 15 to 25 cigarettes per day. This range was chosen so that sufficient numbers of smokers would be included without also including those individuals whose smoking habits were unusual. It may be thought of as a set of smokers who smoke one pack per day. Figure 4 shows that there is a nearly 20-fold range of response in 4-ABP adducts to smoking one pack per day of cigarettes. Some of the possible reasons for such variability have been discussed earlier.

Aromatic Amines as Etiologic Agents

The attributable risk of cigarette smoking for bladder cancer is at least 50% (9). Thus, the repeated finding of elevated aromatic amine adducts in hemoglobin of smokers is consistent with the hypothesis that these compounds play a role in initiating bladder cancer in the majority of cases. Molecular epidemiologic studies can test this hypothesis, supporting it if aromatic amine hemoglobin adducts are positively associated with known risk factors and tending to disprove if they are not.

Smoking black tobacco cigarettes rather than blonde tobacco cigarettes is a risk factor for bladder cancer (31-34). Chemical analysis of smoke from each of the two types of tobacco has revealed that black tobacco produces higher amounts of aromatic amines (8). Consistent with these observations, smokers of black tobacco cigarettes have significantly elevated levels of aromatic amine hemoglobin adducts. Two studies have shown elevation of 4-aminobiphenyl adducts. In one of these (16), no other amines were investigated, but in the other (19), an additional 14 amine adducts were determined. Four of these were also significantly associated with tobacco type. 3-Aminobiphenyl adducts were 11 to 12 times higher in smokers than in nonsmokers, but they showed no association with tobacco type.

A third risk factor for bladder cancer that implicates aromatic amines as causative agents is N-acetyltransfer phenotype. As of 1988, 12 case-control studies in which the subjects were phenotyped had been published (15). In 11 of these, the prevalence of slow acetylators among cases exceeded the prevalence in the controls. Summary analysis of the combined studies revealed a relative slow/rapid acetylator odds ratio of 1.46.

A similar ratio of 4-ABP adducts has been observed in slow and rapid acetylators phenotyped by the recently introduced caffeine metabolites method (16). In a group of 50 nonsmokers, the ratio was 1.6, in 31 smokers of blond tobacco it was 1.3, and in 16 black tobacco smokers it was 1.5. After adjustment for type and number of cigarettes smoked, it was found that the adduct levels were significantly related to this genetic trait.

Epidemiologic studies have yielded conflicting results concerning the relationship between risk and intensity of smoking (9). Some have shown a steadily increasing risk with increasing number of cigarettes smoked per day, while others have shown a plateau at moderately high smoking levels. As indicated earlier, we have observed that 4-ABP adducts definitely plateau at about one pack per day. Thus, in the context of a dose-response relationship, one cannot reach any clear conclusion as to whether these adducts are strongly associated with risk.

4-ABP hemoglobin adducts appear to be elevated in individuals with higher 4-ABP-DNA adducts in bladder epithelium as determined by 32P-postlabeling (35). An adduct that chromatographed to a position consistent with its being N-(deoxyguanosin-8-yl)-4-aminobiphenyl was significantly correlated with hemoglobin adducts (p = 0.01) in a group of 37 normal individuals composed of both smokers and nonsmokers. The data are presented in Table 1.

One case-control study of 4-ABP hemoglobin adducts and bladder cancer has been reported (36). In this study, a modest increase (58%) in adduct levels in cases was observed relative to the level in the controls. The difference was statistically significant by paired t-test and Kruskal-Wallis rank analysis, but not by logistic regression analysis. However, an increase of this magnitude may be all that should be expected (37). In contrast, a case-control

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**Table 1.** Adducts of 4-aminobiphenyl in human hemoglobin and exfoliated bladder epithelial cell DNA (35)

| DNA* | Hemoglobin, pg/g |
|------|------------------|
| 0 (n = 15) | 54               |
| < 10 (n = 14) | 69           |
| ≥10 (n = 8) | 93             |

*Relative adduct labeling.
study of lung cancer found no association of lung cancer diagnosis and 4-ABP-hemoglobin adducts (21). The results from these two studies both tend to support the hypothesis that 4-ABP is a human bladder carcinogen at the doses resulting from ambient and cigarette smoke exposure.

**Limitations**

Hemoglobin adducts of aromatic amines appear to be good dosimeters for biologically effective dose delivered to the bladder lumen. To a very limited extent, it has also been shown that the dose measured in this way correlates with target tissue DNA adduct formation. However, it may be anticipated that several factors could intervene to limit the quality of the correlation. Some of these are alluded to in Figure 3.

It has been shown that about 70% of the N-hydroxylamine formed from 4-ABP reaches the bladder in unconjugated form, while the remaining 30% is in the form of the N-glucuronide (13). The conjugate, though, is sensitive to acid-catalyzed hydrolysis (38), and the rate varies considerably in the pH range 5 to 7. Thus, individual urine pH plays a significant role in determining the actual bladder exposure to free N-hydroxylamine. It also has been shown that free N-hydroxylamines are resorbed into blood through the bladder epithelium (39). Presumably, the rate of resorption is comparable to the rate of uptake by epithelial cells. Thus, the frequency of bladder voiding can affect exposure, since the longer urine is retained, the more hydroxylamine can hydrolyze and the more hydroxylamines can be taken up by epithelial cells. Neither hydrolysis of glucuronide conjugates nor frequency of bladder voiding influences hemoglobin adducts in any significant way.

Figure 3 suggests that one or more phase II conjugation reactions are necessary to convert the N-hydroxylamine proximate metabolite to an ultimate carcinogen. Previously it has been suggested that such metabolic activation is not necessary because the lower pH of urine would generate nitrrenium ions through protonation and loss of H₂O, but this now seems less likely. Bladder epithelium is known to have certain N-acetyl transferase activities (40), and this could activate N-hydroxylamines by formation of N-acetoxyamines. Sulfo-transferase activity has also been detected in bladder cytosol (41). Whether these observed activities are indicative of the corresponding specific activities necessary for the formation of DNA adducts has not been determined.

Figure 3 also suggests that there may be one or more mechanisms for detoxifying carcinogenic N-hydroxylamines, among which reduction to amines might be important. While there are no studies that specifically show such reduction in bladder tissue, it has long been known that the liver reduces hydroxylamines very efficiently (42). The main reason, it appears, that liver exports hydroxylamines at all is that they are trapped by hemoglobin more rapidly than they can be reduced. If the same reductive enzymes are present in bladder tissue, then they might be expected to influence the amount of DNA adducts that could be formed from a given amount of hydroxylamine.

Thus, while the specific mechanisms are not known yet, it is likely that there are one or more mechanisms that can modulate the yield of bladder DNA adducts formed from N-hydroxylamines. Because these mechanisms are largely independent of those that lead to hemoglobin adducts, a certain amount of variance in the association of DNA adducts with hemoglobin adducts is to be expected. How much variance remains to be determined. Only one study in which the correlation between DNA and hemoglobin adducts was investigated has been reported. Despite the potential limitations just described, the two adducts were found to be significantly associated.

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