Biochemical Pharmacology, Vol. 33, No. 21, pp. 3319–3324, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

COMMENTARY

ARACHIDONIC ACID-DEPENDENT COOXIDATION

A POTENTIAL PATHWAY FOR THE ACTIVATION OF CHEMICAL CARCINOGENS *IN VIVO*

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Exposure to environmental chemicals is generally recognized as an important cause of human cancer [1]. Current investigations on the mechanisms responsible for the initiation of carcinogenesis by chemicals indicate that most chemicals must be metabolized to exert their carcinogenic effects. The central hypothesis underlying current thinking on the induction of cancer, as originally proposed by the Millers, is that chemical carcinogens are converted to electrophilic metabolites which covalently link to nucleophilic cellular macromolecules [2]. DNA is considered the critical nucleophilic target associated with the induction of neoplasia [2]. Studies on the metabolic activation of chemical carcinogens have focused primarily on two major classes of chemicals, the polycyclic aromatic hydrocarbons and the aromatic amines. Prototypes of these two classes are benzo[a]pyrene, and 2-acetylaminofluorene or 2-naphthylamine, respectively.

Metabolic activation of benzo[*a*]pyrene (BP) has been investigated extensively. Several excellent reviews have been published recently [3–5], and thus this subject will be described only briefly in this article. Activation of BP requires, first, epoxidation across the 7 and 8 positions to form (+)-BP-7,8-oxide. The resulting epoxide is hydrolyzed by epoxide hydrase to (−)-*trans*-BP-7,8-dihydrodiol, which in turn is oxidized to two diolepoxides: 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-diolepoxide) and 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*syn*-diolepoxide). Thus, BP-7,8-dihydrodiol is considered the proximate carcinogenic metabolite, while the diolepoxides are considered the ultimate carcinogens. The *anti*-diolepoxide is the major isomer formed *in vivo*, but the ratio of *anti* to *syn* depends on the tissue and species examined [3]. The *anti* isomer appears to be more carcinogenic than the *syn* isomer [3]. BP-diolepoxides react with DNA, forming a linkage between C-10 on the polycyclic hydrocarbon and the exocyclic amino group of guanine [6]. DNA adducts isolated from tissues or cells treated with BP are predominantly those derived from the *anti*-diolepoxide [4], with minor amounts of adducts derived from the *syn*-diolepoxide detected. The nature of the BP–DNA interaction that causes a mutagenic or carcinogenic event is a matter of considerable speculation.

The mechanism responsible for activation of carcinogenic aromatic amines has also been studied intensively. The importance of electrophilic metabolites in the initiation of carcinogenesis was derived initially from metabolic studies on aromatic amines [2]. 2-Acetylaminofluorene, 2-naphthylamine and benzidine are members of this class of chemical carcinogens. The currently accepted theory for the activation of these liver and urinary bladder carcinogens involves formation of an hydroxylamine metabolite [2, 7]. Further metabolism to "activated esters" is often required [2, 7]. The hydroxylamine derivative is the proposed proximate carcinogen, while a nitrenium ion formed either directly from the hydroxylamine or via the "activated ester" is considered the ultimate carcinogen [2, 7]. The hydroxylamine is considered to be an obligatory intermediate in this activation scheme. The major DNA adducts isolated from the livers of animals treated with these carcinogens are formed by linkage of the aromatic amine nitrogen to C-8 of guanine [2, 7, 8]. Minor adducts are formed with other purine bases and, in some cases, adducts formed from the N-acetyl derivatives of the amines are also observed [2, 7, 8]. The DNA adducts formed by aromatic amines in the urinary bladder are not well characterized.

The formation of electrophilic metabolites also appears to be involved in the development of toxicities other than carcinogenesis. For example, the widely used analgesic drug acetaminophen causes hepatic toxicity in high doses [9] and can induce renal damage, even in moderate doses [10]. The accepted mechanism for acetaminophen-induced toxicity is that the drug is converted to an electrophilic metabolite(s) which covalently binds to cellular proteins, producing necrosis. The exact chemical nature of the electrophilic metabolite of acetaminophen is uncertain, but evidence suggests that the phenoxy radical or *N*-acetyl-*p*-benzo-quinonimine are possible candidates [11, 12]. Bromobenzene and 4-ipomeanol are toxicants that also require metabolic activation [13, 14]. These examples and others indicate that ample evidence exists for the involvement of electrophilic metabolites in the development of chemical toxicity.

An extensive literature testifies to the importance of cytochrome P-450 monooxygenases in the metabolic activation of chemicals to reactive electrophiles [4, 15]. However, in 1975, Marnett *et al.* [16] showed

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that several chemicals, including benzo[*a*]pyrene, were metabolized during the conversion of arachidonic acid to prostaglandins. Prostaglandin H synthase (PHS) activity is high in many extrahepatic tissues which are low in monooxygenase activity [17, 18]. Thus, prostaglandin H synthase could be an alternative or additional enzyme system for the activation of carcinogens in these tissues.

During the past 5 years, PHS-dependent metabolism of chemicals has been studied extensively. Efforts were focused on using *in vitro* systems to develop an understanding of the mechanisms responsible for the metabolism of chemicals by PHS. Recent reviews have detailed the major findings of these studies [17, 18], and, thus, these findings will be described only briefly here. The purpose of this article is to describe some potential areas for future investigation and possible tools for assessment of the relative contribution of PHS in chemical-induced toxicity *in vivo*.

PHS is unique in that it catalyzes two distinctly different enzymatic reactions [19, 20]. The cyclooxygenase activity of PHS converts arachidonic acid to the hydroperoxyendoperoxide prostaglandin G_2 (PGG_2); PHS peroxidase reduces PGG_2 to the hydroxyendoperoxide PGH_2. PGH_2 is further metabolized by additional enzymes to the classical prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), thromboxane A_2 and/or prostacyclin, depending on the tissue in which it is generated. The level of free fatty acids in most cells is quite low, and the release of arachidonic acid from membrane phospholipids is an important controlling event for the regulation of prostaglandin biosynthesis. However, arachidonic acid liberated from phospholipids can be metabolized by lipoxygenases, as well as PHS [21]. Lipoxygenases metabolize arachidonic acid to hydroperoxyfatty acids (HPETEs) that are reduced to hydroxyfatty acids, or further metabolized to leukotrienes [21]. The peroxidase responsible for the reduction of HPETEs may be PHS or glutathione peroxidase [22, 23]. Metabolism of chemicals is catalyzed by the hydroperoxidase of PHS and has been termed cooxidation [17, 18].

To catalyze the reduction of PGG_2 (or HPETEs), PHS peroxidase requires reducing cofactors. These cofactors donate single electrons to the peroxidase and, in turn, are converted to electron deficient metabolites which can be chemically reactive. Ram seminal vesicle microsomes (RSVM), a rich source of PHS, in the presence of arachidonic acid will oxidize a wide variety of compounds. Acetaminophen [24], *p*-phenetidine [25], diethylstilbestrol [26], BP-7,8-dihydrodiol [27, 28], 7,8-dihydro-BP [29], the carcinogenic nitrofurans *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) and 2-amino-4-(5-nitro-2-furyl)-thiazole (ANFT) [30, 31], and a series of aromatic amines [32–34] are metabolized by PHS to electrophiles which can covalently bind to microsomal proteins and/or exogenously added nucleic acids. Moreover, in a modified Ames test (i.e. using RSVM and arachidonic acid instead of rat liver S-9 fraction and NADPH), a variety of polycyclic aromatic hydrocarbon derivatives [35, 36] and aromatic amines [37] are metabolized to potent mutagens. FANFT and ANFT have not been examined for PHS-dependent mutagenicity. The ability of PHS to metabolically activate carcinogens and toxins has led to detailed studies on the mechanisms involved in these oxidations. The majority of the work has centred around two classes of compounds, polycyclic aromatic hydrocarbons and aromatic amines.

The initial investigation of cooxidation during PG biosynthesis demonstrated the oxidative metabolism of BP [16]. Subsequent studies [38, 39] showed that the stable metabolites of BP cooxidation were the 1,6-, 3,6-, and 6,12-quinones, generally considered detoxication products [3–5]. Considerable evidence has accumulated that the ultimate carcinogenic metabolites of BP are the bay region diolepoxides [3–5]; thus, subsequent studies have centred on cooxidation of the proximate carcinogen BP-7,8-dihydrodiol. (±)-BP-7,8-dihydrodiol is epoxidized by PHS in the presence of arachidonic acid or hydroperoxyfatty acids to *anti*-diolepoxide [27, 28]. Little or no *syn*-diolepoxide is formed. The stereochemistry of epoxidation of BP-7,8-dihydrodiol by cooxidation is distinctly different from the stereochemistry of epoxidation of this compound by cytochrome P-450 [3–5], in that both (+)- and (−)-BP-7,8-dihydrodiols are converted by PHS to *anti*-diolepoxides while the cytochrome P-450 monooxygenases convert the (+)-isomer to the *syn*-diolepoxide, and the (−)-isomer to the *anti*-diolepoxide. The stereochemistry of epoxidation of 7,8-dihydro-BP by PHS is analogous to that of BP-7,8-dihydrodiol [29], and both compounds are metabolized by PHS to mutagens in the modified Ames test [36]. As shown by ^{18}O incorporation studies, the source of the epoxide oxygen in these reactions is molecular oxygen [40].

Polycyclic aromatic hydrocarbons are metabolized by RSVM during PG biosynthesis, yet they are *not* reducing cofactors for PHS peroxidase [17, 18]. Dix and Marnett [41] have developed a model system that may explain these observations. In this model system, hematin catalyzes the epoxidation of BP-7,8-dihydrodiol in the presence of unsaturated fatty acid hydroperoxides. The source of the epoxide oxygen, and the stereochemistry of the epoxidation, are identical to those seen with PHS, lending support for the use of this model. These and other experiments [42] have led Dix and Marnett to propose that the epoxidizing agent in the hematin/hydroperoxyfatty acid model is a fatty acid derived peroxy radical. Reed *et al.* [43] have shown recently that a non-lipid peroxy radical derived from the cooxidation of phenylbutazone will also epoxidize BP-7,8-dihydrodiol with the same stereochemistry as is observed with PHS and the model system. These data support the hypothesis that a peroxy radical is the epoxidizing agent. Although the epoxidizing agent derived from PHS in the presence of arachidonic acid is still unknown, the observations of Dix and Marnett have led them to a potentially important discovery; namely, that lipid peroxidation can epoxidize BP-7,8-dihydrodiol to its ultimate carcinogenic form [44]. The stereochemistry of this epoxidation is the same as for PHS and the hematin/unsaturated fatty acid hydroperoxide model [44].

Unlike polycyclic aromatic hydrocarbons, aromatic amines are classical cosubstrates for per-

oxidases [45] and are excellent reducing cofactors for PHS peroxidase. The work in this area has centred around two compounds: benzidine and 2-aminofluorene. Benzidine is metabolized by PHS to a radical cation that is in equilibrium with a charge transfer complex of benzidine and its two-electron oxidation product, a di-imine [46]. The radical cation was observed directly by electron spin resonance spectroscopy [47]. Azobenzidine is the major isolable organic-extractable metabolite; polymeric material is also formed [46]. PHS-dependent metabolism of benzidine in the presence of various phenol derivatives yields benzidine/phenol adducts of an indoaniline-type structure [48, 49]. When benzidine is metabolized in the presence of DNA or RNA, its activated metabolites bind covalently to the nucleic acids with very high efficiency [33, 34]. Interestingly, N-acetylation of benzidine appears necessary for its metabolic activation by NADPH-dependent monooxygenases, whereas PHS is capable of activating benzidine itself [50, 51].

2-Aminofluorene is also an excellent reducing cofactor for PHS peroxidase and is metabolized to 2,2′-azobisfluorene, 2-nitrofluorene and material covalently bound to microsomal protein [32]. We have been unable to detect an initial free radical directly by electron spin resonance spectroscopy (probably due to its instability), but Boyd and Eling [52] have generated several lines of evidence suggesting that a one-electron pathway of oxidation is operative. PHS-activated 2-aminofluorene also binds covalently to exogenously added nucleic acids, although not as efficiently as benzidine [33, 34]. Several other carcinogenic aromatic amines, including 2-naphthylamine and 4-aminobiphenyl, are metabolized by PHS to reactive or mutagenic metabolites [33, 34, 37].

Studies of the metabolism of polycyclic aromatic hydrocarbons and aromatic amines by PHS in subcellular systems have clearly demonstrated that carcinogens are metabolically activated by this enzyme in peroxide-dependent reactions. It is important to note that PHS can also act as its own source of peroxide (i.e. PGG_2) to support cooxidation reactions, although peroxides derived from other sources may support the reaction as well [17, 18]. This is a point that must be considered in designing experiments attempting to detect peroxidase-mediated metabolism *in vivo*, an area toward which future investigations must proceed.

A useful first step for determining *in vivo* relevance is the employment of cell culture techniques. Boyd *et al.* [53] studied the metabolism of (±)-BP-7,8-dihydrodiol in the mouse embryo fibroblast cell line, C3H/10T1/2 clone 8. Addition of arachidonic acid to confluent monolayers of cells stimulated metabolism of (±)-BP-7,8-dihydrodiol to the *anti*-diolepoxide by 2- to 3-fold; this situation was inhibited by the cyclooxygenase inhibitor indomethacin. Although basal metabolism of (±)-BP-7,8-dihydrodiol was unaffected by indomethacin, it had been shown previously that basal PG biosynthesis is low in confluent monolayers of these fibroblasts [54]. The addition of arachidonic acid to cultures increased the frequency of transformation of these cells by (±)-BP-7,8-dihydrodiol and this increase was inhibited by concomitant incubation with indomethacin [53]. Similar studies with enriched populations of rat non-ciliated bronchial (Clara) cells and alveolar type II cells were performed with (±)-BP-7,8-dihydrodiol [55]. Utilizing exogenous addition of NADPH or arachidonic acid, type II cells had greater capacity for PHS-dependent epoxidation of (±)-BP-7,8-dihydrodiol, whereas Clara cells had greater capacity for monooxygenase-dependent epoxidation.

Syrian hamster embryo cells were used to study the involvement of PHS in the metabolism of the transplacental carcinogen diethylstilbestrol (DES) [56]. Peroxidative metabolism of DES may be an important factor in its carcinogenicity [57]. Addition of indomethacin (in the absence of arachidonic acid) inhibited basal metabolism of DES to trace amounts, suggesting a primary role for PHS. Addition of arachidonic acid to the culture medium enhanced formation of the DES metabolite *cis*, *cis*-dienestrol in both confluent and growing cultures. Growing cultures metabolized much more DES than confluent cultures, consistent with previously reported data on greater PG biosynthesis in growing cultures [54].

Wong *et al.* [58] have proposed a primary role for peroxidase-dependent activation of *N*-hydroxy-2-acetylaminofluorene (N-OH-AFF) in cultured mammary parenchymal cells. N-OH-AAF is metabolized by peroxidases to two reactive metabolites, 2-nitrosofluorene and *N*-acetoxy-2-acetylaminofluorene [59, 60]. Based on the ability of the cyclooxygenase and lipoxygenase inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA) to inhibit N-OH-AAF metabolism, and the inability of selective cyclooxygenase inhibitors (i.e. indomethacin, aspirin) to do the same, these workers have concluded that the hydroperoxide supporting the reaction is a lipoxygenase-derived HPETE. The classical substrates of peroxidases, *p*-aminophenol and butylated hydroxyanisole, both inhibited metabolism of N-OH-AAF. A notable observation made by these investigators was that mammary cells from rats fed a selenium-deficient diet showed an approximately 7-fold increase in metabolism of N-OH-AAF, as compared to cells from control rats. Selenium is an essential component of glutathione peroxidase [61], an enzyme that can reduce cellular levels of lipid hydroperoxides. In a separate report, tumor induction by arylamines was decreased by a selenium-enriched diet [62].

One of the problems with cooxidation experiments in cell culture is the need for exogenous addition of arachidonic acid. Since biologically active metabolites of arachidonic acid are made on demand in physiological situations, confluent monolayers of cells may not accurately reflect the state of the cells *in vivo* that release and metabolize arachidonic acid in response to stimuli not encountered by cells in culture. The fact that basal levels of arachidonic acid release and metabolism in cell culture are frequently low may be a result of this [54, 63]. One way around this problem is to stimulate arachidonic acid release from endogenous pools concurrently with carcinogen treatment.

A similar approach was used recently by Amstad and Cerutti [64] in an investigation of cooxidation of

aflatoxin B_1 (AFB_1) in C3H/10T1/2 clone 8 cells. AFB_1 is a "membrane-active" compound, capable of stimulating the arachidonic acid cascade [64]. The formation of AFB_1/DNA adducts in these cells was inhibited by *p*-bromophenacylbromide (a phospholipase A_2 inhibitor), indomethacin and ETYA (inhibitors of arachidonic acid metabolism), and glutathione. Amstad and Cerutti concluded that cooxidation accounted for, maximally, 60% of AFB_1 activation, and that "AFB_1 may enhance its own cooxidative metabolism by stimulating the arachidonic acid cascade" [64].

Inhibitors of arachidonic acid metabolism have been used in assessing the role of cooxidation in cell culture, as well as *in vivo*. We are aware of only two *in vivo* studies presently in the literature. Cohen *et al.* have shown that addition of aspirin to the diet can inhibit precancerous lesions and carcinomas of the rat urinary bladder induced by FANFT [65, 66], a compound activated by PHS *in vitro* [30]. Adriaenssens *et al.* [67] demonstrated that dietary aspirin had no effect on BP-induced pulmonary adenomas in mice, or on BP metabolite/DNA adduct formation. This study was complicated by the fact that metabolism of BP by cooxidation is a detoxication pathway, but cooxidation of the proximate carcinogen BP-7,8-dihydrodiol is an activation pathway. The use of inhibitors of arachidonic metabolism may be problematic, however, when applied to *in vivo* experiments, for three reasons. First, cyclooxygenase inhibitors only inhibit cooxidation reactions in which cyclooxygenase is the source of hydroperoxide. In tissues where lipoxygenases, lipid peroxidation, or even hydrogen peroxide are the major sources of peroxides, these inhibitors would have no effect on peroxidase-mediated metabolism, and their use could lead to erroneous conclusions. Second, in experiments utilizing tumorigenicity as an endpoint, the role of arachidonic acid metabolism in later stages of carcinogenesis must be taken into account. Prostaglandins have been shown to stimulate or inhibit cell division in several cell culture systems [68–71]. Therefore, the effects of inhibitors of arachidonic acid metabolism on fixation of carcinogen-induced damage to DNA (via cell division) could complicate the interpretation of results. In addition, an important role for both cyclooxygenase and lipoxygenase products in tumor promotion has been implicated [72, 73]. Investigators carrying out studies that include administering such inhibitors for extended periods of time must be aware of this problem. Lastly, the effects of the inhibitor on the distribution and metabolism of the carcinogen must be considered. In conclusion, the results of *in vivo* experiments that attempt to alter an endpoint through the use of inhibitors of arachidonic acid metabolism may be very difficult to interpret accurately, whether such results are positive or negative. Thus, the use of inhibitors of arachidonic acid metabolism appears not to be a fruitful approach to assess the role of cooxidation *in vivo*.

A more suitable approach may be through the use of stable biochemical markers or endpoints for detection of peroxidase-mediated metabolism. Several possible endpoints for expressing peroxidase-mediated metabolism have been obtained from studies on the mechanisms involved in cooxidation, and the identification of metabolites of chemicals produced by peroxidases. Peroxidases can produce unique metabolites and these metabolites should serve as a useful index for this pathway. This approach was used by Guarna *et al.* [74] in studying the metabolism of butylated hydroxyanisole (BHA) in the rat. 2,2′-Dihydroxy-3,3′di-*t*-butyl-5,5′-dimethoxyphenyl (diBHA), the product of peroxidative metabolism of BHA by both horseradish peroxidase and a partially purified rat intestinal peroxidase [75], was measured in rat plasma and intestinal extracts by gas chromatography-mass spectrometry [74]. Thus, Guarna *et al.* concluded that BHA is metabolized by rat intestinal peroxidase *in vivo*. It should be noted, however, that NADPH-fortified liver microsomes from phenobarbitol-induced rats also form diBHA as a minor metabolite, but only at BHA concentrations above 100 μM [76]. Moreover, these investigators proposed that formation of diBHA by rat liver microsomes occurred by a one-electron mechanism [76].

Our laboratory is currently developing a second type of approach for the study of peroxidative metabolism of 2-aminofluorene. Monooxygenase-dependent activation of 2-aminofluorene yields a single 2-AF/nucleoside adduct, *N*-(deoxyguanosin-8-yl)-2-aminofluorene (C8-dG-AF) [77]. However, when activated by PHS in the presence of DNA *in vitro*, 2-aminofluorene yields arylamine/nucleoside adducts that are distinct from C8-dG-AF [78]. Upon structural identification, these unique 2-aminofluorene/DNA adducts will be specific biochemical endpoints with which to assess the role of peroxidase-mediated activation of 2-aminofluorene *in vivo*. This approach appears to be applicable to carcinogens which undergo one-electron oxidation by peroxidases and two-electron oxidation by monooxygenases. In this case, different electrophiles appear to be formed by the two pathways [32, 52, 77, 78].

Stereoselective oxidation by peroxidases may also serve as a tool for measuring peroxidase-mediated metabolism in the presence of other enzyme systems. An example is the oxidation of (±)-BP-7,8-dihydrodiol. In this case, (−)-BP-7,8-dihydrodiol is oxidized by both PHS and monooxygenases to the *anti*-diolepoxide. However, (+)-BP-7,8-dihydrodiol is oxidized by PHS to the *anti*-diolepoxide, while monooxygenases metabolize this compound to the *syn*-diolepoxide. Thus, the ratio of *anti*- to *syn*-diolepoxides is a measurement of the ratio of peroxide-dependent to monooxygenase-dependent metabolism. Dix and Marnett have used this technique in determining the ratio of monooxygenase- to lipid hydroperoxide-dependent epoxidation of (±)-BP-7,8-dihydrodiol in rat liver microsomes, and to demonstrate the possible importance of lipid peroxidation in activation of this compound [44]. The distinct stereochemistry for the epoxidation of (±)-BP-7,8-dihydrodiol by these two enzymes should result in the formation of DNA adducts with corresponding differences in stereochemistry. However, the *anti*-diolepoxide reacts much more efficiently with DNA than does the *syn*-diolepoxide [3–5]. Thus, in the case of (±)-BP-7,8-dihydrodiol, DNA adducts do not appear to be a useful endpoint for

comparing peroxidase- and monooxygenase-mediated metabolism. This problem can be circumvented by the use of 7,8-dihydro-BP. The different tetrahydro-BP-epoxides formed do not show this stereochemical selectivity in reacting with DNA [79]. Panthananickal *et al.* [29] have used polyguanylic acid as a chiral nucleophile to trap the enantiomeric epoxides generated *in vitro* during oxidation of 7,8-dihydro-BP by PHS and monooxygenases, demonstrating the effectiveness of this technique.

It is clear from the work cited here that peroxidative metabolism and activation of toxins and carcinogens can occur in subcellular fractions and cell culture systems. Most of the work to date has been performed in these two systems. Although a role for peroxidase-mediated metabolism *in vivo* has not yet been established, detailed *in vitro* studies on mechanisms have yielded insights that allow the construction of experiments that are well designed to attack this problem. Future *in vitro* studies on the mechanism of cooxidation will continue to serve this role, as well as further our understanding of the contribution of peroxidative biotransformation processes to chemically induced toxicity and carcinogenesis.

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