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Induction of cytochrome CYPIA1 and formation of toxic metabolites of benzo[*a*]pyrene by rat aorta: A possible role in atherogenesis

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ABSTRACT **Cigarette smoking is a leading risk factor for atherosclerosis. Endothelial injury may be the initial event in this process. The carcinogenic metabolites of the polycyclic aromatic hydrocarbons found in cigarette smoke tars could cause this injury. We tested this model by examining the effect of 3-methylcholanthrene administration on aortic polycyclic aromatic hydrocarbon metabolism. Immunoblotting with a monoclonal antibody (mAb 1-7-1) specific for cytochromes CYPIA1 and CYPIA2 showed that aortic microsomes from treated, but not from control, animals contained CYPIA1; the CYPIA1 was primarily in the endothelium. Aortic microsomes from induced animals metabolized benzo[*a*]pyrene (BaP) to the 7*R*,8*S*,9,10-tetrahydrotetrol-, 7,8-dihydrodiol-, 1,6 quinone-, 3,6 quinone-, 6,12 quinone-, 3-hydroxy-, and 9-hydroxy-BaP. mAb 1-7-1 inhibited the formation of the tetrahydrotetrol, the dihydrodiol-BaP, and the 3-hydroxy-BaP but did not inhibit the quinones or the 9-hydroxy-BaP. Arachidonic acid did not affect metabolism. These data suggest that the aortas of induced animals metabolize the BaP in cigarette smoke to carcinogenic and toxic products and that this metabolism may initiate vessel injury and lead to the accelerated atherosclerosis seen in cigarette smokers.**

Atherosclerotic vascular disease is the leading cause of death in developed nations. This process may be initiated by endothelial injury, which leads to the formation of a nidus for mononuclear cellular infiltration, cholesterol deposition, and platelet aggregation (1). Cardio- and cerebral vascular events are more frequent in individuals with hypertension, hyperlipidemia, and diabetes mellitus and in smokers. There has been some question of whether the increased incidence of vascular events seen in smokers is due to increased atherosclerosis or to alterations in other systems, such as the coagulation pathways. Although the latter may be important, the preponderance of evidence suggests that smokers have a significant increase in the incidence and degree of atherosclerosis in both the coronary and peripheral arteries (2).

It is unclear which of the toxic products in the smoke—carbon monoxide (3), nicotine (4), or the tars—are responsible for this acceleration. The tars, which include the polycyclic aromatic hydrocarbon (PAH) benzo[*a*]pyrene (BaP), are possible candidates (5). Although BaP is not toxic, it is metabolically activated to highly toxic, mutagenic, and carcinogenic products—such as various epoxides and quinones. These toxic metabolites, if formed in the vessel wall, could initiate atherosclerosis by injuring the endothelial and muscle cells. If such is the case, then the arterial wall should catalyze their formation because it is unlikely that they could be transported to the peripheral vessels from the liver or lungs. Previous workers have found that a specific cytochrome P450, CYPIA1, catalyzes this metabolism in other organs (6, 7). In smooth muscle cells of young animals CYPIA1 is a constitutive form (8), but in older animals it is found in many organs only after exposure to either the PAHs or other environmental toxins, such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin and the polyhalogenated biphenyls.

In this model of atherosclerosis induction in smokers, the PAHs are absorbed from the smoke particles through the lungs into the circulation. The PAHs have a dual effect. (*i*), They induce CYPIA1 in a wide range of tissues (9). This induction is initiated through the binding of the PAHs to a cytosolic protein, the aryl hydroxylase receptor (10). The receptor–inducer complex then enters the nucleus and promotes the transcription of CYPIA1 mRNA. Animals with mutations at the *Ah* locus, which are unable to induce CYPIA1, are termed Ah$^-$ (11).

(*ii*) Smokers have continued exposure to the PAHs, which are substrates for CYPIA1. This cytochrome catalyzes the formation of a wide range of highly toxic metabolites. On the other hand, nonsmokers have no exposure to the inducing agents, so they lack both the catalytic enzyme CYPIA1 and the substrate. Hence, their tissues are not exposed to these toxins.

This scheme predicts that if BaP is metabolized in the vessel wall, the initial event should be CYPIA1 induction. Accordingly Dees *et al.* (12) and Forkert *et al.* (13) have presented immunological evidence that treatment of animals with the PAH 3-methylcholanthrene (3-MC) induces various isozymes of cytochrome P450 in endothelial cells. Similarly, a number of workers (14–19) have reported that other forms of cytochromes P450 are present in the arterial wall.

Furthermore, Juchau *et al.* (5) found that 3-MC administered to rabbits and monkeys enhanced aortic metabolism of BaP to its phenolic metabolites, suggesting that CYPIA1 had been induced. Later, they reported that 3-MC induced BaP metabolism to the 7,8-dihydrodiol in pigeon aortas, indicating that the 7,8-epoxide was also formed (20). They also found DNA adducts in the aortas of chickens given [^{3}H]BaP (21), suggesting that BaP administration could lead to significant cytotoxicity in the aorta.

Supporting the role of BaP in the initiation of atherosclerosis, Majesky *et al.* (22) reported that a carcinogenic PAH, 7,12-dimethylbenz[*a*]anthracene, initiated atheromatous

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Abbreviations: BaP, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; mAb, monoclonal antibody; mAb-1-7-1, mAb specific for CYPIA1 and CYPIA2; HyHel-9, mAb to chicken lysozyme.

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changes in chicken aorta. Similarly, others (23, 24) have shown that the PAHs can accelerate atherosclerosis. Paigen *et al.* (23) studied two strains of mice that were congeneic except at the *Ah* locus. When these mice were fed an atherogenic diet, 3-MC administration increased atherosclerosis in both strains but increased it much more in the Ah^+ than in the Ah^- strain. Similarly, Penn and Snyder (24) found that 3-MC administration for 16 weeks to cockerels receiving standard laboratory chow increased the incidence and extent of atherosclerosis 8–14 times. Together, these data indicate that PAHs can initiate and significantly increase atherosclerosis.

In the current study we have further assessed the role of BaP metabolism in atherogenesis. We found that treating rats with 3-MC induces CYPIA1 in the aorta with a concomitant increase in the *in vitro* metabolism of BaP to epoxides and quinones. Because both of these classes of metabolites are highly toxic, these observations lend support to the concept that the atherosclerosis associated with cigarette smoking entails the initial induction of CYPIA1 (25), which then metabolizes the BaP in cigarette tars to form highly toxic metabolites and injure cells. These injured cells form a nidus for the initiation of atherogenesis.

MATERIALS AND METHODS

[^{3}H]BaP was obtained from the National Cancer Institute (NCI) Chemical Carcinogen Repository, Chemsyn Science Laboratories (Lenexa, KA). Just before use, we purified the substrate by HPLC, as described below, for the metabolite assay. The BaP metabolites were from the NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, KA) and were used without further purification. Arachidonic acid, bovine serum albumin, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, and Tris were from Sigma. SDS was from Pierce. Acrylamide and mercaptoethanol were from Bio-Rad. All other reagents were American Chemical Society grade.

All studies were done on 300- to 400-g male Sprague–Dawley rats obtained from Harlan Laboratories. The animals received standard laboratory chow ad libitum, had free access to water, and were maintained on a 12-hr light/dark cycle. They received 3-MC in corn oil (5 mg/kg per day i.p.) for 1–6 days; control animals received an equal volume of corn oil. The animals were killed by guillotine, and the livers and aortas were promptly removed and placed on ice. The aortas were stripped of adventitia, minced, and homogenized in 150 mM KCl/50 mM Tris, pH 7.4 at 4°C in a Potter–Elvehjem homogenizer. The homogenates were filtered through a nylon mesh and centrifuged at 2500 × *g* for 10 min in a Sorval RC-5C centrifuge with a SS-34 rotor. Supernatants were recentrifuged at 9000 × *g* for 20 min. This supernatant was then centrifuged at 100,000 × *g* for 1 hr in a Beckman L-8 centrifuge with a Ti-50 rotor. The microsomal pellets were resuspended in 0.25 M sucrose. Hepatic microsomes were prepared by a similar procedure. The protein concentrations were determined by the bicinchoninic acid dye technique with bovine serum albumin as the standard (Pierce).

Immunoblotting was done by the method of Towbin *et al.* (26) as described (27). Proteins were separated by SDS/PAGE and transferred to nitrocellulose. Immunoblots were analyzed with a 1:250 dilution of ascitic fluid containing monoclonal antibody (mAb) 1-7-1. This antibody is specific for both CYPIA1 and CYPIA2 (28, 29). The blots were treated with an anti-mouse antibody–alkaline phosphatase conjugate and washed; an indicator dye consisting of a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was added (Bio-Rad).

BaP metabolism was determined in incubations in 150 mM KCl/50 mM Tris/5 mM $MgCl_2$, pH 7.4 at 37°C/albumin at 1 mg/ml/5.5 mM glucose 6-phosphate/0.39 mM $NADP^+$/glucose-6-phosphate dehydrogenase at 0.66 units/ml/8.5 μM; [^{3}H]BaP (9.13×10^6 dpm)/microsomes at 0.7 mg/ml in a final volume of 0.5 ml. As reported (30), the albumin significantly increased the rate and reproducibility of this assay. Samples were incubated at 37°C under air in a shaking water bath. The incubations were terminated by placing the vials in acetone/dry ice, the nonradioactive BaP metabolites were added in dimethyl sulfoxide (0.1 ml), and the samples were extracted three times with ethyl acetate (1.5 ml). The ethyl acetate was removed from the combined extracts in a stream of nitrogen. The metabolites were separated by HPLC essentially by the method of Selkirk *et al.* (31). In this procedure the samples were dissolved in dimethyl sulfoxide (50 μl) and injected (25 μl) onto an HPLC consisting of two Beckman 110B pumps, a Reodyne (Berkeley, CA), 7125 injection valve, a 5 μm, C_{18}, 2.5 mm × 30 cm column (Waters), and a Schoeffel 770 UV monitor set to 280 nm. The metabolites were eluted initially with 50% H_2O/47% methanol/3% 1-propanol (1.5 ml/min for 3 min) followed by a gradient to 97% methanol/3% 1-propanol (1.5 ml/min) over 27 min. The column was washed with the latter solution for 3 min. Fractions (0.75 ml) were collected and diluted with UltimaGOLD (Packard) liquid scintillation fluid (3 ml); radioactivity was counted in a TriCarb 1900c liquid scintillation counter (Packard); the radioactivity in the zero time sample for each fraction was subtracted from the counts observed after incubation.

For the immunolocalization studies, liver and aorta were fixed in 10% neutral buffered formalin, processed through graded alcohols and xylenes, and embedded in paraffin. Immunoperoxidase staining was done on 4-μm-thick paraffin sections by the peroxidase–antiperoxidase method after quenching of endogenous peroxidase activity (6% methanolic H_2O_2 for 30 min) and rehydration. 3,3′Diaminobenzidine tetrachloride was the chromagen. Sections were counterstained with hematoxylin. To reduce interference from the nuclear staining during photographic reproduction after his-

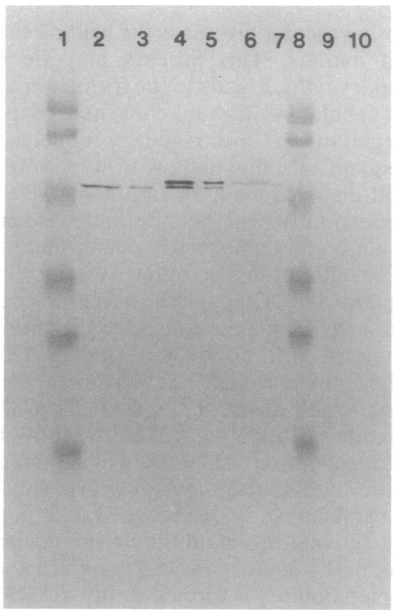

FIG. 1. Immunoblots of microsomes from aortas and livers of control and 3-MC-treated rats (5 mg/kg per day for 4 days) against ascitic fluid containing mAb 1-7-1 (1:250 dilution). Lanes: 1 and 8, standards; 2, control liver microsomes (0.5 μg); 3, control liver microsomes (0.1 μg); 4, 3-MC-treated liver microsomes (0.5 μg); 5, 3-MC-treated liver microsomes (0.1 μg); 6, 3-MC-treated aortic microsomes (25 μg); 7, 3-MC-treated aortic microsomes (10 μg); 9, control aortic microsomes (25 μg); 10, control aortic microsomes (10 μg).

FIG. 2. Aorta from 3-MC-treated rats (5 mg/kg per day for 4 days) showing granular endothelial reactivity for mAb 1-7-1 at a 1:50 dilution (straight arrowheads). Sections were stained as described. Curved arrowheads, stained endothelium. (×125.)

tologic evaluation, the aortic sections were destained with 1% HCl (concentrated) in ethanol/water, 70:30. mAb 1-7-1 was used at dilutions of 1:10 to 1:200. Negative controls were obtained by using a mAb to chicken lysozyme (HyHel-9).

RESULTS

After treatment with 3-MC for 1–6 days, hepatic microsomes showed well-separated immunoreactive bands for CYPIA1 and CYPIA2, while aortic microsomes showed only CYPIA1 (Fig. 1). Control hepatic microsomes showed a faint band representing CYPIA2, while control aortic microsomes showed neither isozyme (Fig. 1).

To determine whether the induction occurred in the endothelial or smooth muscle cells, we scraped the endothelium off the aorta with a blunt scalpel handle. The scrapings stained more intensely than the remaining vessel wall, even though they had a much lower protein content (data not shown). Because we could not scrape all the endothelium off the wall, this staining of the muscular fraction could have been due to adherent endothelial cells or the endothelium of the vasa vasorum.

Liver sections stained with mAb 1-7-1 showed a granular staining pattern in hepatocytes, primarily in the centrolobules (data not shown). In livers from 3-MC-treated animals this pattern was seen at all dilutions of antibody. With lower dilutions, staining was also seen in the midzonal and peripheral regions of the lobule. Liver sections from untreated rats showed immunostaining only at 1:10 and 1:20 dilutions. A similar differential pattern of immunostaining was seen with sections of aorta. A 1:50 dilution provided the best discrimination between treated and untreated animals. Focal granular staining was seen in endothelial cells (Fig. 2). In addition, there was outlining of the vasa vasorum by a mixture of linear and granular staining. Staining of the aorta was not as intense as of the liver.

Our chromatographic procedure separated all the major metabolites of BaP (Fig. 3). Microsomes from the aortas of induced animals metabolized [^{3}H]BaP to a number of products (Fig. 3). As observed (32, 33), the radioactive metabolites eluted slightly before unlabeled standards. This isotopic effect probably resulted from the greater hydrogen bonding between the water in the elution system and the ^{3}H in labeled BaP when compared with ^{1}H in unlabeled BaP. As seen with hepatic microsomes (34), aortic microsomes produced only the 7*R*,8*S*,9,10-tetrahydrotetrol and none of the other three isomers. Because the tetrahydrotetrol is the hydrolytic product of the 7*R*,8*S*,9,10-dihydrodiol epoxide, these data indicate that this carcinogenic metabolite is formed (34).

Formation of the 7*R*,8*S*,9,10-tetrahydrotetrol (Fig. 4*A*), the 7,8-dihydrodiol (data not shown), and the phenolic derivatives (data not shown) was linear for at least 15 min, suggesting that the dihydrodiol does not leave the CYPIA1

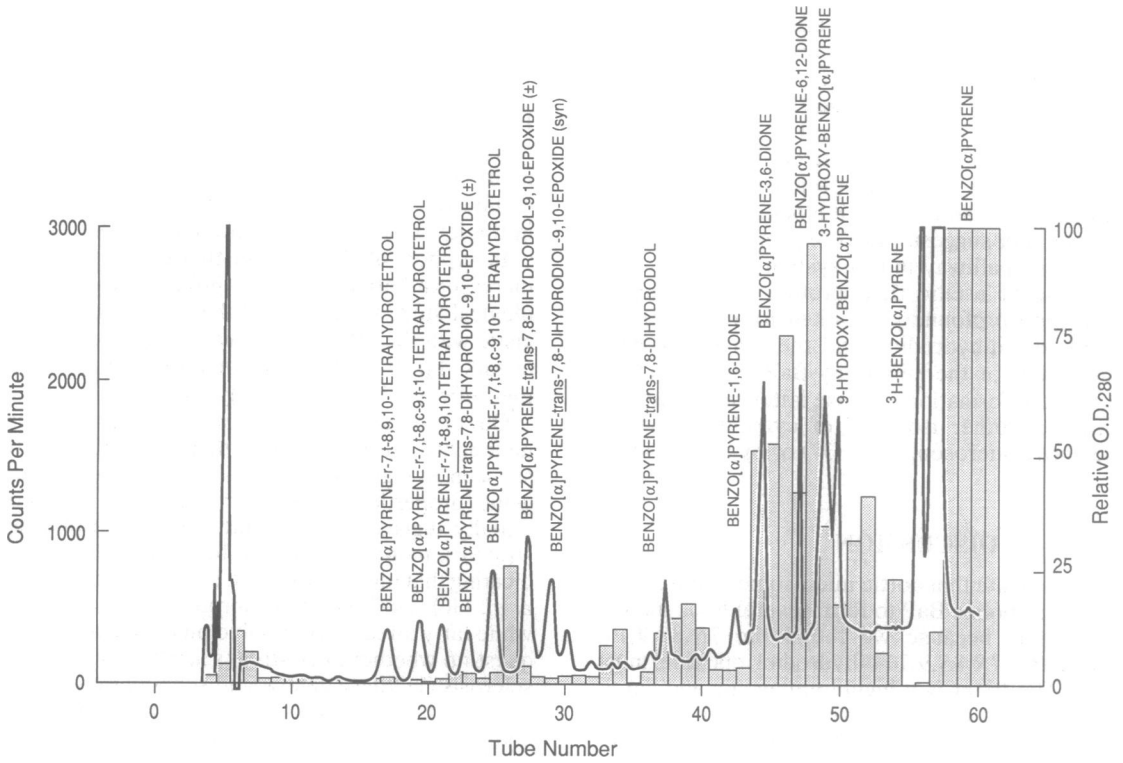

FIG. 3. Chromatographic profile of BaP metabolites produced by microsomes from aortas of rats treated with 3-MC (5 mg/kg per day for 4 days). Shaded boxes represent the total cpm for each fraction; solid line represents absorbance at 280 nm.

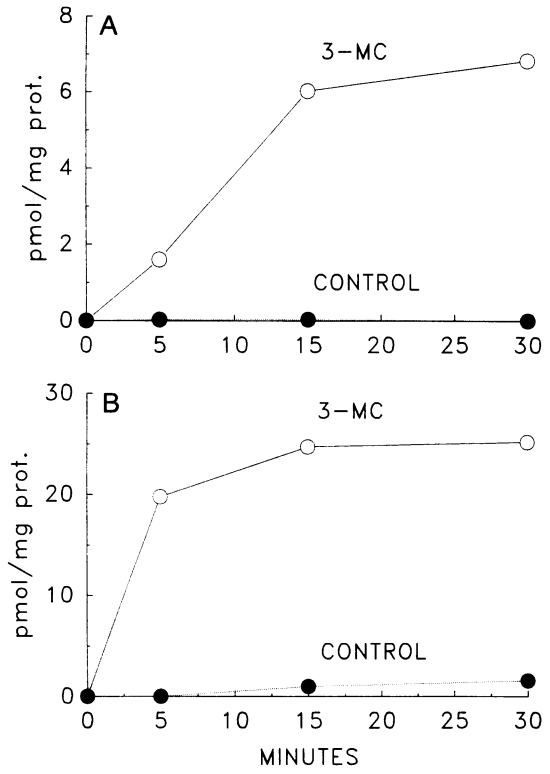

FIG. 4. Time course for formation of 7*R*,8*S*,9,10-tetrahydrotetrol (*A*) and 1,6-dione (*B*) BaP by microsomes from the aortas of rats treated with 3-MC (5 mg/kg per day for 4 days). prot, Protein.

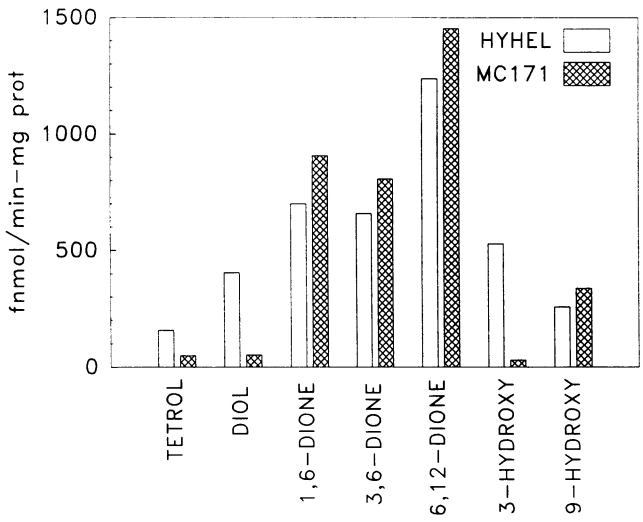

FIG. 5. Effect of mAb 1-7-1 (9 μg of ascitic fluid protein per 400 μg of microsomal protein) on BaP metabolism in microsomes from the aortas of rats treated with 3-MC (5 mg/kg per day for 4 days). Control incubations contained mAb HyHel-9, which is specific for chicken lysozyme. prot, Protein; tetrol, tetrahydrotetrol; diol, dihydrodiol.

before the second epoxidation step. If this were not the case, there should be a lag phase in the production of the tetrahydrotetrol because the concentration of the dihydrodiol in the incubation medium would have to increase to a value above the dissociation constant of the catalytic site on the cytochrome. Unlike the hydroxylated derivatives, the quinones were rapidly formed with no increase after 5 min (Fig. 4*B*).

One explanation for the failure of previous workers to show tetrahydrotetrol formation is that it may only form through cooxygenation of the dihydrodiol by cyclooxygenase (35, 36). When we added arachidonic acid (0.1 μM), it had no effect on formation of the tetrahydrotetrol, dihydrodiol, the phenols, or the 3,6- and 6,12-diones (data not shown). At higher concentrations (1 and 10 μM) it blocked the 1,6-dione formation. These data suggest that cooxygenation is not an important metabolic pathway in this system.

The mAb (9 μg of ascitic fluid protein per 400 μg of microsomal protein) profoundly inhibited formation of the tetrahydrotetrol, the dihydrodiol, and the 3-hydroxy-BaP, but not of the diones or the 9-hydroxy-BaP (Fig. 5). These data suggest that the formation of these latter metabolites is catalyzed either by other isoforms of cytochromes P450 present in the aortic microsomes (15, 37) or by a peroxidase reaction.

DISCUSSION

Our data clearly indicate that aortic microsomes from 3-MC-treated animals metabolize BaP to form several toxic metabolites. In particular, because we found the 7*R*,8*S*,9,10-tetrahydrotetrol, the 7*R*,8*S*,9,10-dihydrodiol epoxide must also be formed. The latter is a highly mutagenic and carcinogenic metabolite that readily forms adducts with macromolecules, including proteins and DNA. Our data are consistent with the previous observations of Bond *et al.* (21), who reported that [^{3}H]BaP binds *in vivo* to aortic DNA. Furthermore, our immunoinhibition data strongly suggest that the formation of this metabolite is catalyzed by CYPIA1. It is unlikely that CYPIA2 is catalyzing these reactions because it does not appear to be induced and has been reported as much less active then CYPIA1 in catalyzing BaP metabolism (38, 39).

These data support the role of metabolic activation of BaP in the increased incidence of atherosclerosis in cigarette smokers. Clearly, BaP activation is not the sole basis for the initiation of atherosclerosis because the other well-defined risk factors initiate it by totally different mechanisms. For example, hypertension may cause increased plaque formation through the increased shear forces seen in hypertension because mechanical injury itself can produce atherosclerosis (40, 41).

Even the increased atherosclerosis seen with hypercholesterolemia may be mediated through more than one pathway. In support of this concept, these lesions show a variety of morphological patterns, suggesting that they are initiated by multiple mechanisms (1). Furthermore, the process may result from abnormalities both in the lipid profile and changes in the level of lipid peroxidation (42, 43).

In support of the concept that each of these risk factors increases atherosclerosis by different mechanisms, epidemiological studies have found that cigarette smoking is synergistic with the others in increasing the incidence of vascular events (44). If all were mediated through the same pathway, their effects should be simply additive.

Our observation that the aortic wall produces carcinogenic metabolites of BaP suggests that the increased atherosclerosis seen in cigarette smokers could be related to the mutagenic properties of these metabolites. This concept was initially suggested by Benditt and Benditt (45), who demonstrated a monoclonal origin for atherosclerotic plaques, indicating that the lesions could be due to mutagenesis. Pearson *et al.* (46) confirmed that fibrous plaques were monoclonal, while fatty streaks were polyclonal. Because there is serious question whether fatty streaks are the initial event in atherosclerosis (1), these data would support Benditt and Benditt's hypothesis. Alternatively, this monoclonal proliferation could represent a single cell line being preferentially stimulated by some growth factor (45).

Yet the 7*R*,8*S*-dihydrodiol-9,10-epoxide metabolite of BaP is a potent mutagen and carcinogen (34). In the neonatal

mouse skin model it is the most carcinogenic BaP metabolite (34). Furthermore, it readily forms DNA adducts during BaP metabolism in both cell culture (47) and model systems (48, 49). Finally, Juchau's group has shown that after [^{3}H]BaP administration, DNA adducts are found in the aorta (21). Together, these data lend strong support to the role of mutagenesis in the increased atherosclerosis in cigarette smokers.

Finally, our observation of significant immunostaining of the aortic vasa vasorum by mAb 1-7-1 is consistent with the well-known pathological effects of cigarette smoking on the aorta. In particular, smokers have a much higher incidence of aortic aneurysms than nonsmokers. This increase could result from injury to the aortic vasa vasorum, which would decrease perfusion of the vessel wall and lead to ischemic injury of the media with fibrosis. With extensive fibrosis, the area becomes a saccular aneurysm.

In conclusion, our data support the hypothesis that the increased atherosclerosis seen in cigarette smokers begins with the induction of CYPIA1 in the aortic wall by the BaP in cigarette smoke. The CYPIA1, in turn, metabolizes BaP to highly toxic metabolites that cause significant cellular injury. The injured areas then become nidi for the formation of atherosclerotic plaques.

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