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THE EFFECTS OF HARMAN AND NORHARMAN ON THE METABOLISM OF BENZO(a)PYRENE IN ISOLATED PERFUSED RAT LUNG AND IN RAT LUNG MICROSOMES

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Abstract—The effects of harman and norharman, nitrogen-containing pyrolysis products of amino acids present in cigarette smoke, on the metabolism of benzo(a)pyrene in rat lung microsomes *in vitro* and in isolated perfused rat lung were studied. In rat lung microsomes, both harman and norharman inhibited the metabolism of benzo(a)pyrene (BP) to dihydrodiols, phenols and quinones at concentrations over approximately 0.05 mM. The formation of BP-7,8-dihydrodiol and BP-9,10-dihydrodiol was inhibited more than that of BP-4,5-dihydrodiol. No appreciable differences in inhibition were seen between microsomes from control or 3-methylcholanthrene-pretreated rats. In isolated perfused rat lung, 1 mM of harman in the perfusion fluid inhibited the formation of ethyl acetate-soluble metabolites of benzo(a)pyrene except BP-9,10-dihydrodiol, and inhibited the total covalent binding of benzo(a)pyrene metabolites to lung tissue macromolecules. 0.03 mM of harman seemed to increase other metabolites than BP-7,8-dihydrodiol without changing the total covalent binding. These results suggest that at most concentrations both β-carboline derivatives, harman and norharman, inhibit benzo(a)pyrene metabolism and covalent binding both in lung microsomes *in vitro* and in isolated perfused rat lung.

The carcinogenicity of cigarette smoke can only partly be accounted for by the polycyclic aromatic hydrocarbons it contains [1]. Other agents which have been suggested as carcinogens include for instance nitrosamines [2]. Recently Mizusaki *et al.* [3] have found a correlation between the nitrogen content of tobacco leaves and the mutagenic activity of smoke condensate. About 90 per cent of carcinogens are also mutagens [4], and thus nitrogen-containing compounds may also be responsible for the carcinogenicity of cigarette smoke.

Harman and norharman, beta-carboline derivatives which are formed during the pyrolysis of amino acids [5] are among the nitrogen-containing compounds found in cigarette smoke [6]. They have been shown to increase the mutagenicity of compounds needing metabolic activation for their mutagenic effect [5]. It is tempting to speculate that these compounds contribute significantly to the total mutagenic and carcinogenic potency of cigarette smoke condensate by their "comutagenic effect".

We studied the effect of harman and norharman on benzo(a)pyrene (BP)* metabolism in isolated perfused rat lung and in rat lung microsomes in order to compare the *in vitro* effects with effects in situations closely resembling the *in vivo* conditions. We selected BP because it is abundantly present in cigarette smoke, is representative for polycyclic aromatic hydrocarbon and because its metabolic fate and biological effects have been elucidated in considerable detail.

MATERIALS AND METHODS

Perfusion studies. The rats used in all the studies were male Sprague–Dawley strain (a generous gift from Lääke Oy, Turku, Finland) weighing 270–300 g. Harman HCl (from Sigma Chemical Company) was put into the perfusion fluid (50 ml, containing 12.5 ml fresh rat blood, 37.5 ml Krebs–Ringer phosphate buffer solution, 1.25 g bovine albumin and 50 mg D-glucose) before the beginning of perfusions to allow it to mix properly. The concentration of harman in the perfusion fluid was either 0.03 mM or 1 mM.

After anaesthetizing rats with ether, the lungs were removed and connected to the perfusion apparatus. Five minutes after beginning the perfusion 12.5 nmoles of [^{3}H]BP (sp. act. 1 μCi/nmole, from The Radiochemical Centre, Amersham) in 0.25 ml DMSO was added to the perfusion fluid giving final concentration of 0.25 μM. Samples of the perfusion fluid were taken 15, 30 and 60 min after starting the perfusions. The covalent binding and BP metabolites of the lung tissue were also analyzed, after the perfusions.

No toxic effects of harman on lung tissue were seen during the perfusions. Function of the lungs was excellent as judged by the pH of perfusion fluid and the amount of circulation through lungs. No oedema of the lungs was noticed during perfusions.

A more detailed description of the preparation of the animals and of the perfusion system has been published earlier [7].

In vitro *incubations.* Both noninduced (control) and 3-methylcholanthrene-induced (MC daily 25 mg/kg intraperitoneally for 3 days) rats were used. The lungs from 2 animals were pooled for each incubation. The preparation of microsomes was carried out as described earlier [8]. The microsomal pellets were resuspended in 0.1 M phosphate buffer (pH 7.4). The incubation mixture was composed of 0.2 ml microsomes (0.8 mg mi-

*The abbreviations used are: BP, benzo(a)pyrene; BP-7,8-dihydrodiol, trans-7,8-dihydroxy-7,8-dihydrobenzo(a)-pyrene and other dihydrodiols in the similar way; MC, 3-methylcholanthrene; t.l.c., thin layer chromatography; MFO, mixed function oxygenase.

crosomal protein/incubate), 1.8 ml 0.1 M phosphate buffer solution (pH 7.4), 1 ml cofactor solution (0.1 ml 200 mM KCl, 0.1 ml 10 mM $MgCl_2$, 0.2 ml 6 mM G-6-phosphate, 0.1 ml 0.25 mM NADP, 0.1 ml G-6-phosphate dehydrogenase, 0.4 ml 0.1 M phosphate buffer pH 7.4) and 0.44 μmole [^{3}H]benzo(a)pyrene (sp.act. 40 μCi/μmole) in a final volume of 4 ml (BP concentration being thus 0.11 mM in incubation mixture). The concentration of harman and norharman (added in 40 μl of methanol) in the incubation mixtures were 3 mM, 0.3 mM, 0.03 mM and 0.003 mM. Incubation time was 20 min for each incubation and it was carried out at 37°.

Analysis of radioactivity. Lungs were homogenized in 4 vol. 0.1 M phosphate buffer. Both these and samples from the perfusion mediums were extracted twice with two vol. ethyl acetate.

Ethyl acetate-soluble radioactivity was further resolved by thin layer chromatography (t.l.c.) using reference metabolites (from Chemical Repository, Chicago, Illinois) according to Sims [9] and Borgen *et al.* [10]. Covalent binding of radioactivity was analyzed according to the method of Siekevitz [11] and BP-hydroxylase activity according to the method of Nebert and Gelboin [12].

Statistics. In the perfusion studies, results are expressed as the means of four different perfusions ± standard deviation, and levels of significance were calculated using Student's *t* test.

In *in vitro* studies the results are the means of two different incubations.

RESULTS

In *in vitro* incubations at most concentrations, harman either had no effect or slightly decreased the formation of ethyl acetate-soluble metabolites. Exceptions were phenols and dihydrodiols which slightly increased in control microsomes with 3 mM harman in the incubation mixture. In MC-induced microsomes with 0.03 mM harman their formation was also increased (Fig. 1). Statistical analysis was not possible because each incubation was only done twice. When the concentration of harman was increased the most marked decrease was found in the phenol fraction in MC-microsomes, calculated as a percentage of the incubations without harman (Fig. 1). Of the dihydrodiols in MC-microsomes the formation of 9,10-dihydrodiols was inhibited most at 0.3 mM of harman concentration, and 9,10- and 7,8-dihydrodiols at 3 mM of harman in the incubation mixture (Fig. 2).

The incubation results with norharman were essentially the same as with harman and are thus not shown in the figures.

The metabolites formed in the perfused lung and isolated by t.l.c. from both the perfusion fluid and the lung tissue after perfusions are shown in Figures 3 and 4. After 15 and 30 min with 0.03 mM harman in the perfusion fluid all the metabolites, except BP-7,8-dihydrodiol, seemed to be statistically significantly increased (Fig. 3). After 60 min the difference remains statistically significant only in the quinone fraction (Fig. 3). 0.03 mM Harman in the perfusion fluid increases also the amount of 9,10-dihydrodiols and quinones in the lung tissue (Fig. 4). The covalent binding of BP to lung tissue is slightly decreased due to this harman concentration (0.03 mM), but the difference is not statistically significant (Table 1).

When the concentration of harman in the perfusion fluid is 1 mM the formation of all BP-metabolites except 9,10-dihydrodiols is decreased (Figs. 3 and 4). At this concentration the amount of covalently-bound ra-

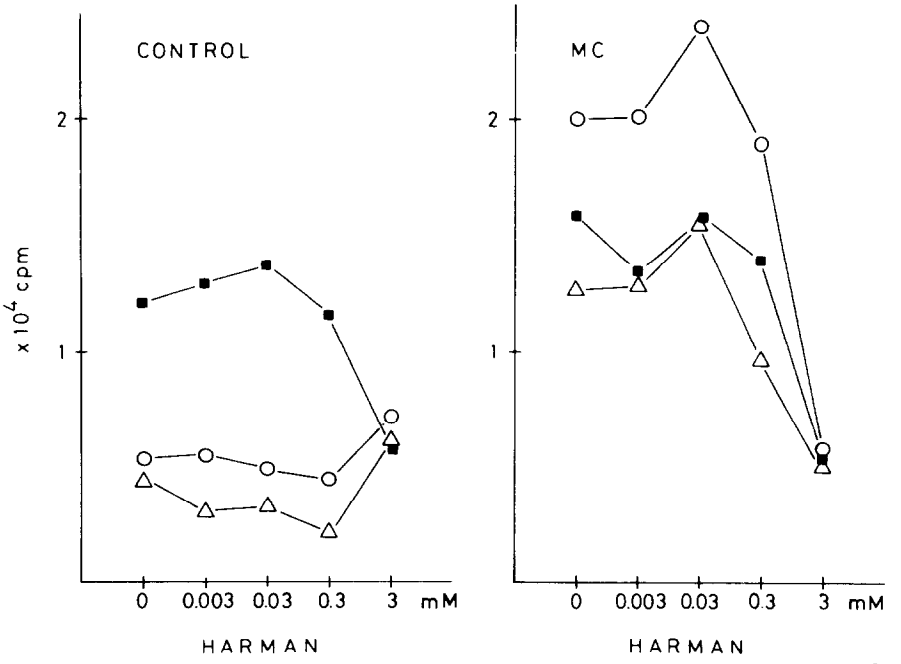

Fig. 1. The amount of ethyl acetate-soluble metabolites produced in *in vitro* incubations of rat lung microsomes from control and 3-methylcholanthrene-treated (MC) rats with [^{3}H]benzo(a)pyrene and various concentrations of harman. All points are the means of two separate incubations. For all incubations the lungs from 2 animals were pooled together. △——△ : Dihydrodiols; ○——○ : phenols; ■——■ : quinones.

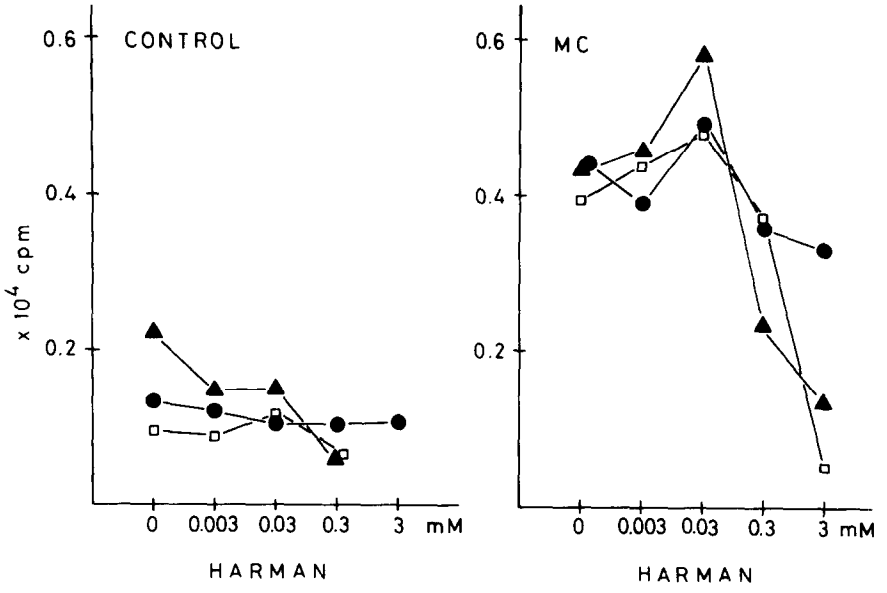

Fig. 2. The amount of various dihydrodiols produced in *in vitro* incubations of rat lung microsomes from control and 3-methylcholanthrene-treated (MC) rats with [^{3}H]benzo(a)pyrene (BP) and various concentrations of harman. Each point represents the mean of two separate incubations and for each incubation lungs from two animals were pooled together. ▲——▲: BP-9,10-dihydrodiol; □——□: BP-7,8-dihydrodiol; ●——●: BP-4,5-dihydrodiol.

dioactivity of BP is also statistically significantly decreased (Table 1).

DISCUSSION

Benzo(a)pyrene (BP) has been shown to have both carcinogenic [13] and mutagenic [14] effects, which depend on metabolic activation [14, 15]. The activation consists of several enzymatic and nonenzymatic steps [16–18]. At first BP is changed to "simple epoxides" by the mixed function oxygenase (MFO). These can be hydrated to dihydrodiols by epoxide hydrase, changed nonenzymatically to phenols or bound covalently to tissue macromolecules. Both dihydrodiols and some phenols are again substrates for MFO giving rise to diol-epoxides and activated phenols. Simple epoxides [19, 20], diol-epoxides [20, 21], phenols [19, 22] and activated phenols [19] have been shown to be mutagenic in bacterial test systems. Other possible active metabolites may be for example activated quinones [23, 24].

Although cigarette smoke contains BP, most of the mutagenic activity of cigarette smoke condensate has been found in other fractions [25, 26]. Recently Mizusaki *et al.* [3] have shown that the mutagenic potency of smoke condensate has a close correlation with the total nitrogen content of tobacco leaves in the Ames test. According to their studies the proteins and amino acids in tobacco are important as regards mutagenicity. Because harman and norharman belong to the pyrolysis products which are formed from amino acids during cigarette smoking, it is interesting that Nagao *et al.* [5] found a dose-dependent increase in the mutagenicity of BP by harman and norharman using *Salmonella typhimurium* TA98 and rat liver microsomes. In another study Nagao *et al.* [27] found that the effect of norharman on the mutagenicity depends on the class of chemical and on the amounts of S-9 fraction. With large amounts of S-9 fraction, norharman caused a several-fold increase in the mutagenicity of BP in a modified Ames assay. In contrast to these studies both harman and norharman inhibited BP-mutagenicity, BP-metabolism and the binding of all BP-metabolites to DNA *in vitro* in the original Ames assay with mouse liver enzymes [28]. Matsumoto *et al.* [29] suggested that the heightening effect of harman on the mutagenicity of

Table 1. The effect of harman on the covalent binding of benzo(a)pyrene metabolites to lung tissue macromolecules after 60-min perfusions

Perfusion	Amount of covalently bound radioactivity (c.p.m./g lung tissue)
Control	89835 ± 25487
Harman (0.03 mM in perfusion fluid)	76842 ± 11287
Harman (1 mM in perfusion fluid)	29765 ± 3510*

Values represent the means of four perfusions ± standard deviations.
* = the difference is statistically significant ($P < 0.005$).

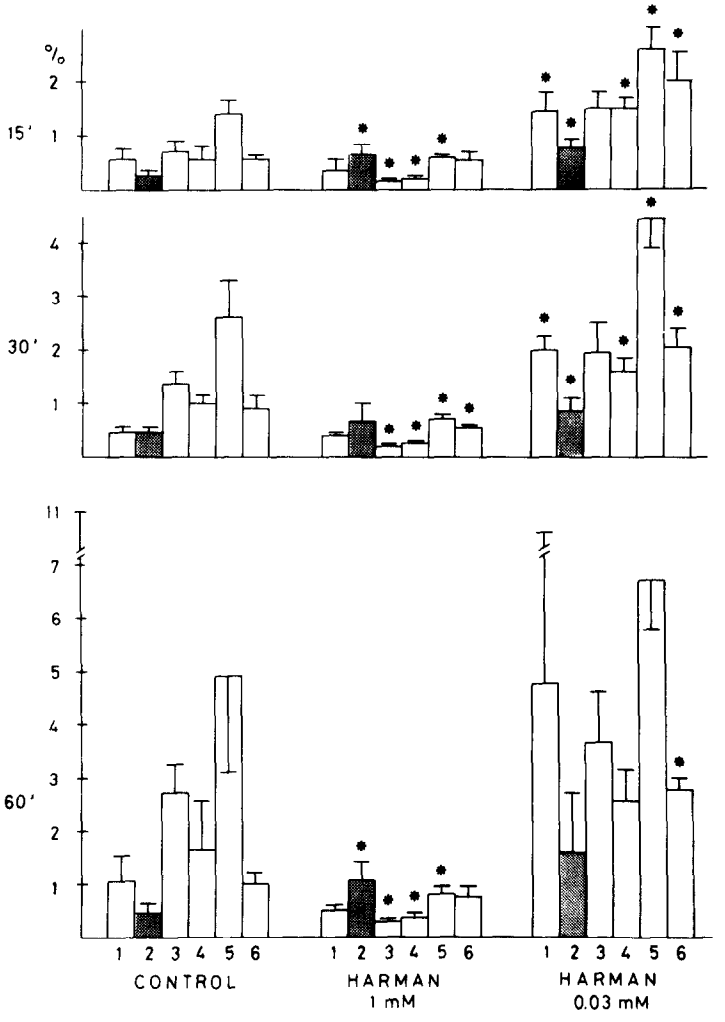

Fig. 3. The percentage of various ethyl acetate-soluble metabolites isolated by thin layer chromatography (t.l.c.) from total ethyl acetate-soluble radioactivity in perfusion fluid at 15, 30 and 60 min after beginning the perfusion. The concentrations of harman were 1 mM and 0.03 mM in the perfusion fluid. Each column represents the mean of four perfusions + standard deviation.
X differs significantly from control ($P < 0.05$).
1 = radioactivity at the origin of t.l.c. plate; 2 = BP-9,10-dihydrodiol; 3 = BP-7,8-dihydrodiol; 4 = BP-4,5-dihydrodiol; 5 = BP-phenols; 6 = BP-quinones.

carcinogens was specific for nitrogen-containing mutagens, because the relative mutagenicity of polycyclic aromatic hydrocarbons was decreased by harman.

If harman and norharman had an enhancing effect on the mutagenicity and possibly then also on the carcinogenicity of BP, these processes being closely correlated [4], a plausible hypothesis for their doing so could be that these compounds stimulate the formation of active metabolites of BP.

Our *in vitro* results are in accordance with those of Levitt *et al.* [28]. They found a slight increase in the AHH activity by small harman and norharman concentrations using MC-induced mouse liver microsomes. In our study 0.03 mM was the only concentration that slightly increased the formation of ethyl acetate-soluble metabolites of BP by using MC-induced lung microsomes. When the concentration was increased the inhibition of BP-metabolism became evident in both studies. We also used non-induced microsomes. The difference between MC- and control microsomes was mainly in phenol and dihydrodiol fractions. The amount of these slightly decreased with small concentrations of harman or norharman and increased with 3 mM of harman and norharman in the incubation medium. Of the various metabolites the amount of quinones was not increased *in vitro* by any of the concentrations of harman or norharman used. Although phenolic metabolites of BP are clearly mutagenic [22], they are only weak mutagens compared to other BP-metabolites [19, 30]. Of the dihydrodiols, the increase by 0.03 mM harman was most marked in the amount of 9,10-dihydrodiol. The amount of 7,8-dihydrodiol also increased but to a lesser extent. Both 4,5-, 7,8- and 9,10-dihydrodiols transform normal hamster cells, but 7,8-dihydrodiol is far more active than the other two [31]. 7,8-Dihydrodiol is the precursor of 7,8-

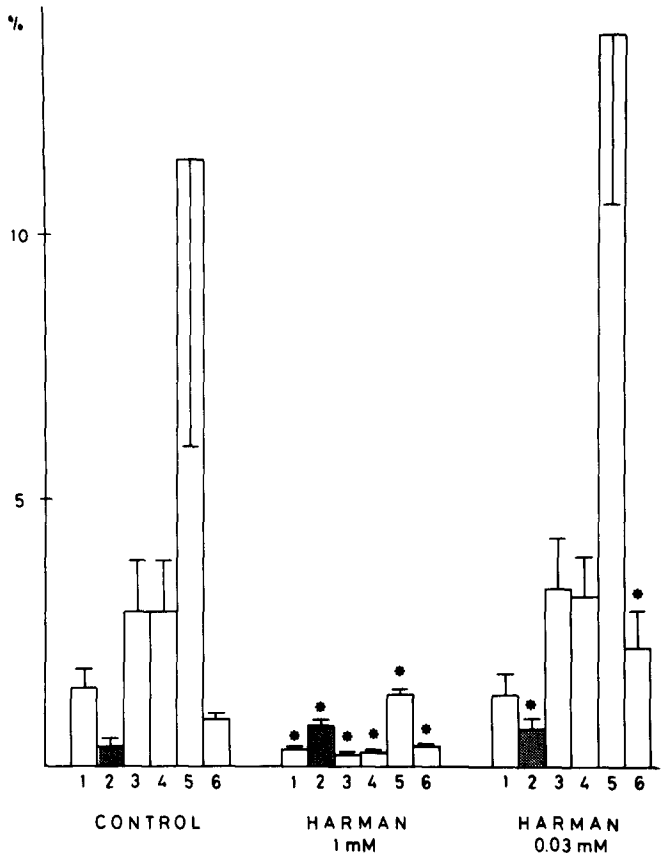

Fig. 4. The percentage of ethyl acetate-soluble metabolites isolated by thin layer chromatography from total ethyl acetate-soluble radioactivity in lung tissue after 60-min perfusions. For further details see the legend to Fig. 3.

diol-epoxide, which is considerably more mutagenic than other diol-epoxides [21, 32]. Still, there seems to be a certain discrepancy between the small increase (about 30 per cent) in phenol and diol fractions with only one concentration of harman and the strong comutagenic effect (about 7-fold) which Nagao *et al.* [5] have found in their study. Actually Matsumoto *et al.* [29] found that the relative mutagenic activity of BP was decreased by 50 μg of harman. Nor does the possible intercalating effect of harman and norharman seem to explain their comutagenic effect [33].

In our perfusion studies the decrease in the formation of BP-metabolites, covalent binding in lung tissue with 1 mM of harman in the perfusion fluid, and the slight increase of metabolism with 0.03 mM of harman agree well with our *in vitro* results.

There is accumulating evidence that arene oxides are the active carcinogenic forms of BP [18]. Not only the more potent mutagenicity, but also the greater amount of binding to cellular macromolecules, e.g. DNA *in vitro* [34–38] and *in vivo* [39, 40] more than with other metabolites, has focused attention especially on 7,8-diol-epoxide and its precursor 7,8-dihydrodiol. BP-7,8-dihydrodiol is also a potent skin carcinogen in mice [41, 42] and causes more malignant lymphomas on pulmonary adenomas than BP in newborn mice [43]. When BP is metabolized by an isolated perfused rat lung, only one peak representing 7,8-diol-9,10-epoxide(s) bound to DNA is detectable by Sephadex LH20 columns [44]. In the present study the formation of 7,8-dihydrodiol and covalent binding to lung tissue in perfusions were correlated, both being decreased with 1 mM and unchanged with 0.03 mM of harman in the perfusion fluid.

Thus both our *in vitro* and perfusion studies indicate that at most concentrations harman and norharman inhibit rather than enhance the metabolism of BP. This was especially true in those concentrations that give the maximal enhancing effect in the BP-induced mutagenesis [5]. The mechanism of the possible comutagenic effect of harman and norharman does not seem to be related to the formation of active intermediates of BP.

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