Formation of DNA Adducts and Induction of Mutations in Rats Treated with Tumorigenic Doses of 1,6-Dinitropyrene

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1,6-Dinitropyrene, a component of diesel exhaust, is a lung carcinogen in male F344 rats following a single intrapulmonary administration. In this study, rats were treated with tumorigenic doses of 1,6-dinitropyrene to establish dose-response relationships for the formation of DNA adducts in target (lung) and nontarget (liver) tissues and for the induction of 6-thioguanine-resistant mutations in spleen T-lymphocytes. One week after treatment with 0.3, 1, 3, 10, 30, 100, or 150 μg of 1,6-dinitropyrene, dose-responsive DNA binding was measured in lung and liver with binding in the lung being 10-fold higher than in the liver. In the lung, a 2-fold increase in dose resulted in a 1.8-fold increase in DNA binding at treatments up to 30 μg of 1,6-dinitropyrene, while in the liver, a 2-fold increase in 1,6-dinitropyrene produced a 2-fold increase in DNA binding at doses up to the 10 μg treatment. Higher doses of 1,6-dinitropyrene resulted in proportionally smaller increases in adduct formation in the two tissues. When measured 21 weeks after treatment, mutations in T-lymphocytes increased with doses up to 100 μg of 1,6-dinitropyrene, but the response was nonlinear throughout the dose range. These findings indicate that concentrations of 1,6-dinitropyrene that produce a dose-dependent induction of lung tumors also result in a dose-dependent formation of DNA adducts and induction of lymphocyte mutations but that the dose-response curves for DNA binding and mutations are different. — Environ Health Perspect 102(Suppl 6):185–189 (1994)

Key words: lymphocytes, N-deoxyguanosin-8-yl)-1-amino-6-nitropyrene, 32P-postlabeling, diesel exhaust, 6-thioguanine, lung, liver

Introduction

Epidemiologic data have suggested an association between exposure to diesel exhaust and the induction of lung and bladder cancer in humans (1–3). For example, in a large retrospective cohort study involving more than 50,000 railroad workers, Garshick et al. (4) found a slight but statistically significant increase in lung tumors that appeared to be related to diesel exposure. Likewise, Boffetta et al. (5) reported significant increases in lung cancer for miners and heavy equipment operators who reported exposure to diesel emissions, and Hayes et al. (6) found an elevated incidence of bladder cancer in truck drivers and railroad workers with more than 20 years of exposure to diesel exhaust.

Diesel emissions are a complex mixture composed of particulate, gas, and vapor phases (1,2). Because lung tumors are not induced in experimental animals exposed to only the gas and vapor phases, emphasis has been placed on the toxic properties of the particulates. The particulate phase of diesel exhaust contains a number of polycyclic aromatic hydrocarbons (PAHs), including anthracene, pyrene, benzo[a]anthracene, and benzo[a]pyrene. Certain of these PAHs, as exemplified by benzo[a]pyrene, are clearly carcinogenic in experimental animals and may contribute to the increased incidence of lung and bladder cancer observed in individuals exposed to diesel emissions. Nitro PAHs are another group of chemicals detected in diesel particulates. These compounds, which are formed during the combustion process as a result of the reaction of nitrogen oxides with PAHs, are found typically at much lower concentrations than PAHs. Pyrene, for example, occurs at approximately 5000 μg/g of particulate extract, while its monoxygenation and dinitration products, 1-nitropyrene and 1,6-dinitropyrene, are detected at about 75 and 0.40 μg/g of particulate extract, respectively. Although nitro PAHs are found at much lower levels than PAHs, certain members of this class are powerful mutagens and carcinogens. 1,6-Dinitropyrene, for instance, is one of the most mutagenic compounds tested in the Ames Salmonella reversion assay and it induces tumors at a number of sites in a variety of experimental animals (7–12). Because nitro PAHs are interrelated through metabolism to aromatic amines (13), a class of carcinogens known to cause bladder cancer in humans (3,14), they may contribute to the increased incidence of both lung and bladder tumors in individuals exposed to diesel exhaust.

Recently there has been considerable effort to develop biomarkers for detecting carcinogen exposure with the hope that this will allow a more rapid identification and estimation of the risk to humans (15). Two markers that have received much attention are DNA (16,17) and protein (18) adducts, and a number of reports have appeared documenting correlations between carcinogen exposure and adduct concentrations (16–21). While valuable, DNA adduct and protein adduct measurements suffer from the limitation that, as a result of DNA repair as well as protein and cell turnover, only recent exposures can be assessed. An approach that has the potential to indicate more than recent carcinogen exposures is to measure the biologic effects resulting from DNA adduct formation, in
particular the induction of lymphocyte mutations (22). An advantage of this technique is that if mutations are induced in a stem cell population, they may be detected for periods long after the initial carcinogen insult. Furthermore, molecular analysis of the mutants may reveal patterns indicative of the specific carcinogen inducing the mutation.

We recently reported (23,24) the formation of DNA adducts and T-lymphocyte mutations after the administration of either 30 or 100 µg of 1,6-dinitropyrene directly to the lungs of F344 rats according to a protocol known to induce lung tumors (10,11). In lung and spleen lymphocytes, one major adduct, N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, was detected. The levels of this adduct reached a maximum 3 to 7 days following treatment, were dose-dependent in spleen lymphocytes but not in lung, and decreased to 25 to 50% of peak values by 28 days after dosing. Mutations were assayed at the hypoxanthine-guanine phosphoribosyltransferase (hprt) locus in spleen T-lymphocytes for up to 51 weeks following a similar treatment with 1,6-dinitropyrene. Compared to solvent-treated controls, 1,6-dinitropyrene induced a significant increase in mutant frequency with the 100 µg dose typically giving 2-fold more mutants than the 30-µg treatment. With both doses, the mutant frequency increased until 21 weeks after dosing, remained constant until week 40, and then began to decrease, but even almost 1 year following treatment there was still a significantly greater mutant frequency in the 1,6-dinitropyrene-treated rats as compared to the controls. As a continuation of these experiments, we now have examined the dose dependence of adduct formation and mutation induction. Specifically, we have treated rats with seven doses of 1,6-dinitropyrene between 0.3 to 150 µg and assessed DNA adduct levels 1 week after treatment, while the extent of mutation induction was determined 21 weeks following dosing.

Materials and Methods

DNA Adduct Analyses

Male F344 rats (12 weeks old; three to five animals per dose group, from the breeding colony at the National Center for Toxicological Research, Jefferson, AR) were anesthetized with a mixture of ketamine and xylazine (25), and subjected to a left lateral thoracotomy. [4,5,9,10-3H]1,6-Dinitropyrene (0, 0.3, 1, 3, 10, 30, 100, or 150 µg; 1257 mCi/mmol) in 50 µl of beeswax and tricaprylin (1:1) was then administered using the lung implantation method of Stanton et al. (26) as described by Iwagawa and co-workers (11). Seven days after treatment, the animals were exposed to carbon dioxide, decapitated, and the lungs, livers, and spleens were quickly excised. Lung and liver nuclei were prepared using the method of Basler et al. (27), and spleen lymphocytes were isolated by the technique of Aidoo et al. (28). DNA was extracted from the nuclei and cells by slight modification of the method reported in Beland et al. (29). The DNA was quantified by UV spectrometry, and the extent of adduct formation was determined by liquid scintillation counting. When indicated, 32P-postlabeling assays were conducted as described in Smith et al. (23,30).

Analysis of Mutation Induction at the hprt Locus of Spleen Lymphocytes

Additional rats (two per group) were treated identically with the same doses described above. Twenty-one weeks following dosing, the rats were euthanized by exposure to carbon dioxide, their spleens were removed aseptically, and lymphocytes were isolated. Each rat was processed independently. The number of T-lymphocytes with mutations at the hprt locus, as evidenced by growth of the lymphocytes in the presence of the purine analog 6-thio-guanine, was determined by the limiting dilution clonal assay described in Aidoo et al. (28), modified by using conditioned medium as a source of T-cell growth factor (31).

Results

Analysis of the Binding of 1,6-Dinitropyrene to Lung, Liver, and Spleen Lymphocyte DNA

The extent of adduct formation, as a function of dose, in lung DNA of male F344 rats 7 days following the implantation of 1,6-dinitropyrene is shown in Figure 1A. From 0 through 30 µg of 1,6-dinitropyrene, the amount of binding to lung DNA increased with dose such that a 2-fold increase in the amount of 1,6-dinitropyrene resulted in a 1.8-fold increase in binding (r=0.96). At doses above 30 µg of 1,6-dinitropyrene, the amount of binding still increased with dose, but the rate of increase was much less than that observed at the lower doses. In previous work (23), lung DNA from animals treated in an identical manner with 30 or 100 µg of 1,6-dinitropyrene was analyzed by 32P-postlabeling and the only adduct detected was N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene.

The total extent of adduct formation in the liver (Figure 1B) from 1,6-dinitropyrene was approximately 10% of that observed in the lung (Figure 1A). As with the lung DNA, the extent of binding as a function of dose was described by a biphasic curve. From 0 through 10 µg of 1,6-dinitropyrene, there was a linear increase (r=0.97) with a 2-fold increase in dose, resulting in a 2-fold increase in binding. Above 10 µg of 1,6-dinitropyrene, the increase in adduct concentration was still linear, but the rate was much lower than that occurring at lower doses. 32P-Postlabeling analyses of liver DNA indicated the presence of one major adduct with the same elution characteristics as N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene.

The extent of DNA binding also was assessed in the spleen lymphocytes. When treated with 150 µg of 1,6-dinitropyrene, the binding levels were 0.13 ± 0.04 fmole adduct/µg DNA. Comparable values for 30 and 100 µg of 1,6-dinitropyrene were 0.07 ± 0.02 and 0.14 ± 0.09 fmole adduct/µg DNA. The yield of DNA from the spleen lymphocytes was insufficient (~500 µg) to measure the extent of binding based upon the amount of 3H incorporated at doses lower than 30 µg of 1,6-dinitropyrene.
Analysis of Mutation Induction at the hprt Locus of Spleen Lymphocytes

The mutant frequency, as a function of dose, 21 weeks after implanting 1,6-dinitro- pyrene, is shown in Figure 2. At each dose examined, the number of 6-thioguanine resistant T-lymphocytes was greater than that observed in the solvent-treated controls and, with the exception of the 0.3- 
µg dose, these increases were statistically significant (p < 0.01). As with the DNA binding, the mutant frequency tended to increase with dose; however, the response was not linear and actually decreased at the highest dose of 150 µg of 1,6-dinitro- pyrene.

Discussion

In previous experiments (23,24), we applied 30 or 100 µg of 1,6-dinitro- pyrene directly into the lungs of F344 rats to determine the kinetics of adduct formation in target and nontarget tissues and the extent of mutation induction in spleen T- lymphocytes. The results of this work indicated that maximum DNA adduct levels occurred 3 to 7 days after treatment, while the greatest number of mutant spleen T- lymphocytes was detected 21 weeks following treatment. Using these time points, we conducted a dose-response study that included the doses used by Iwagawa et al. (3–150 µg of 1,6-dinitro- pyrene) (11) to induce lung tumors in F344 rats. In addition, two lower doses were administered (0.3, 1 µg) to obtain information on the shape of the dose-response curve at doses where it would be prohibitively expensive to conduct a tumorigenesis assay.

In the target tissue, the lung, the extent of DNA binding increased linearly with doses up to 30 µg of 1,6-dinitro- pyrene. Beyond this dose, the adduct levels still increased, but the rate of increase was much lower than that observed at lower doses. A similar biphasic dose-response curve also was observed in a nontarget tissue, the liver. In this organ, the total binding was approximately 10% of that found in the lung, but again there was a steep increase in binding from 0.3 to 10 µg of 1,6-dinitro- pyrene followed by a slower increase in binding at the higher doses of 1,6-dinitro- pyrene. In spleen lymphocytes we were unable to measure adduct formation below 30 µg of 1,6-dinitro- pyrene because of the low levels of binding coupled with the relatively low (as compared to the liver) yield of DNA. As in our previous study (23), there was a 2-fold increase in binding between 30 and 100 µg of 1,6-dinitro- pyrene. Because the binding did not increase any further at the 150 µg dose, this suggests that a biphasic dose-response for adduct formation also may be occurring in these cells. We are presently attempting to describe the entire dose-response profile in spleen lymphocyte DNA by using 32P- postlabeling assays.

Biphasic dose-response curves for adduct formation, with linear increases at low doses but proportionally smaller increases at higher doses, have been observed with other carcinogens, including 4-aminobiphenyl in female mouse liver (32), N,N-diethylnitosamine in male rat liver (33), and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone in male rat lung (34). With these carcinogens, the decreased rates of adduct formation at higher doses have been attributed to metabolic saturation, the induction of toxicity and cell proliferation, or both. Both of these mechanisms could contribute to the biphasic response in the lungs of rats treated with 1,6-dinitro- pyrene, although because of the relatively low doses administered, cell proliferation as a result of cytotoxicity is perhaps unlikely. These mechanisms also could contribute to the nonlinearity in adduct formation observed in the liver and in spleen lymphocytes. In addition, there may be impaired clearance of the 1,6-dinitro- pyrene from the lungs at higher carcinogen doses.

Upon treatment with 1,6-dinitro- pyrene, the induction of T-lymphocyte mutants tended to increase with dose, but as compared to the DNA binding results, the response was not linear at low doses of carcinogen. The curvilinear response throughout the entire dose range may be a direct reflection of the extent of DNA binding, which we have not been able to determine at low doses. It also could be a reflection of toxicity at the higher doses of 1,6-dinitro- pyrene; thus, binding to lymphocyte DNA could be linear but an increasing proportion of cells could be lost because of cytotoxicity as the dose was increased.

The doses selected for this study correspond to those used by Iwagawa et al. (11) to induce lung tumors in male F344 rats. In their bioassay, 10 µg of 1,6-dinitro- pyrene induced a 13% incidence of lung tumors, and the incidence increased in a relatively linear manner to 85% at the 100 µg dose. At 150 µg of 1,6-dinitro- pyrene, the incidence decreased slightly but this may reflect the fact that there were very few animals in this group. (Although tumorigenesis was not the objective of the present study, as part of our initial time-course experiment, we noted the development of lung tumors in one rat 32 weeks after administering 100 µg of 1,6-dinitro- pyrene and in two rats 51 weeks after dosing with 30 and 100 µg of 1,6-dinitro- pyrene). Because DNA adduct levels were measured at the same doses used in the bioassay, it is possible to relate the extent of DNA adduct formation to the tumor incidence; such an analysis is shown in Figure 3. These results indicate that a 50% incidence of lung tumors would be associated with a concentration of approximately 1.3 fmoles of N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene/µg DNA. A similar analysis has been conducted with a number of other carcinogens (35) and, for most compounds, the adduct level associated with a 50% tumor incidence was considerably higher (>100 fmoles/µg DNA) than that found for 1,6-dinitro- pyrene. An exception to this high level of binding was aflatoxin B1 and, interestingly, both this carcinogen and 1,6-dinitro- pyrene induce tumors at very low concentrations. In our study, we also detected DNA adducts at dose levels (e.g., 3 µg of 1,6-dinitro- pyrene) where Iwagawa et al. (11) did not detect lung tumors.

Figure 2. 6-Thioguanine-resistant mutant frequency in lymphocytes cultured from the spleens of male F344 rats 21 weeks after treatment with [3H]1,6-dinitro- pyrene.

Figure 3. Lung tumor incidence as a function of lung DNA adduct formation in male F344 rats treated with various doses of 1,6-dinitro- pyrene. Lung tumor data from Iwagawa et al. (11).
Assuming a linear relationship between the DNA adduct levels and the induced tumor incidence, our data predicts that 3 μg of 1,6-dinitropyrene should induce approximately 5% lung tumors. Igawa et al. (11) would have had to treat considerably more rats than the 30 animals they used to detect such a difference.

F344 rats exposed to 2.2 to 7.1 mg/m³ diesel emissions for 24 to 30 months developed an increased incidence of lung tumors (2). Assuming that all of the particulate-associated dinitropyrenes was bioavailable, it can be estimated that each rat received 4 to 27 ng of 1,6-dinitropyrene and 12 to 83 ng of 1,3-, 1,6-, and 1,8-dinitropyrene during this exposure period. Although we detected significant levels of DNA binding and lymphocyte mutations at 1,6-dinitropyrene doses below those Igawa et al. (11) found to be tumorigenic, these treatment levels of 0.3 and 1 μg of 1,6-dinitropyrene are still approximately one order of magnitude greater than the 1,6-dinitropyrene exposure expected from diesel emissions. This suggests that DNA adducts or lymphocyte mutations produced by 1,6-dinitropyrene (or dinitropyrenes) alone may not be a sufficiently sensitive biomarker for diesel exposure. Treatment-related DNA adducts have been reported in rats exposed to diesel exhausts (36–38), and there are other highly genotoxic compounds, such as benzo[a]pyrene, found at far greater concentrations than dinitropyrenes in diesel particles (1). DNA adducts or mutations produced by these or other diesel-associated compounds may serve as more useful biomarkers for diesel exposure. It should also be noted that we are assuming a linear dose-response to very low concentrations of dinitropyrenes. With other compounds, in particular the bladder carcinogen 4-aminobiphenyl, DNA adduct levels in humans exposed to relatively low levels of 4-aminobiphenyl through cigarette smoke are much higher than would have been predicted from comparatively high dose animal exposures (39).

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