Stimulatory Effects of Sulfur and Nitrogen Oxides on Carcinogen Activation in Human Polymorphonuclear Leukocytes

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The occurrence of inflammatory processes and of cancer in the human respiratory tract is intimately associated. One of the major factors in this is probably the recruitment of and stimulated activity of polymorphonuclear leukocytes (PML) in conjunction with the ability of these cells to convert various carcinogens to their ultimate active metabolites. In this study, we demonstrate that nitrite and sulfate, the major dissolution products of the environmental pollutants nitrogen dioxide and sulfur dioxide in water enhance the metabolic activation of trans-7,8-dihydroxy-7,8-dihydrobenz[a]pyrene (BP-7,8-dihydropyrene), the proximal carcinogen of benz[a]pyrene, to trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenz[a]pyrene (BPDE) and tetraols, the corresponding hydrolysis products, in human PML prestimulated with 12-O-tetradecanoylphorbol-13-acetate. Nitrite was more efficient than sulfate in stimulating the formation of reactive intermediates of BP-7,8-dihydriodiol in PML that covalently bind to extracellular DNA and, in particular, to intracellular proteins. The mechanism by which sulfate stimulates the metabolism of BP-7,8-dihydriodiol most probably involves the intermediate formation of a sulfur trioxide radical anion (SO$_3^-$) the subsequent formation of the corresponding sulfur peroxyl radical anion (•OOSO$_2^-$) in the presence of oxygen. The mechanism underlying the stimulatory action of nitrite is less clear but the major pathway seems to involve myeloperoxidase. These results offer an explanation for the increased incidence of lung cancer in cigarette smokers living in urban areas. The major glutathione transferase (GST) isoenzyme in human PML is GST P1-1, a Pi-class form. The GST activity of PML was found to be inversely correlated with the extent of binding of BP-7,8-dihydriodiol products to exogenous DNA. These results suggest that individuals exhibiting high GST-activity in the PML may be better protected against the type of carcinogenic dealt with in this study. — Environ Health Perspect 102(Suppl 4):161–164 (1994).

Key words: activation, BP-7,8-dihydriodiol, human leukocytes, nitrogen oxide, sulfur oxide

Introduction

The exposure of airborne contaminants to man is extensive and many compounds are proven to be toxic and cause acute or more long-term effects. The most important source of harmful substances is cigarette smoke and epidemiological studies have clearly shown a close correlation between extent of smoking and primary cancer of the respiratory tract (1–3). The ultimate carcinogenic component or components in cigarette smoke has not been identified but it is likely that polycyclic aromatic hydrocarbons (PAHs) are important factors (4). Another source of PAHs is urban air. PAHs are produced by various combustion processes, and they are thus widely distributed contaminants (5).

The ultimate carcinogenic forms of PAHs are called bay-region diol epoxides (6,7). For instance benzo[a]pyrene, which is an important and the most studied PAH, is metabolized to trans-7,8-dihydroxy-7,8-dihydrobenz[a]pyrene (BP-7,8-dihydriodiol) by the sequential action of cytochrome P450 and epoxide hydrolase followed by epoxidation at the 9,10-position to yield trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenz[a]pyrene (BPDE). The last step has been demonstrated to be carried out by cytochrome P450 (6), lipooxygenase (8), peroxidase (9), and pathways dependent on formation of peroxyl radicals (9,10). Other important contaminants in urban air and in cigarette smoke are sulfur dioxide (SO$_2$) and nitrogen dioxide (NO$_2$). These pollutants are harmful to the lung. Sulfur dioxide is known to cause bronchoconstriction especially in asthmatics. Nitrogen dioxide is known to cause direct damage to various lung cells and have, for instance, been shown to cause increased airway reactivity in asthmatics, a decrease of the respiratory host defense system and, probably, emphysema (11,12). In addition, these oxides may be cocarcinogenic with PAHs by enhancing, for instance, their metabolism and ultimate activation to reactive intermediates (3).

Both SO$_2$ and NO$_2$ dissolves into mucous and into the epithelial lining fluid and are readily hydrolyzed (13). In fact, the major products detected in blood and urine are sulfate, nitrate, and nitrite, and it is possible that many of the effects, including the cocarcinogenicity, can be attributed to these metabolites rather than to SO$_2$ and NO$_2$ themselves (13,14).

Cigarette smokers also are more afflicted by inflammation, bronchitis, and emphysema than nonsmokers. Moreover, smokers living in urban areas seem to have an increased risk of developing lung cancer (4). One reason may be the simultaneous exposure of carcinogens in the smoke and atmospheric contaminants such as sulfur oxides and nitrogen oxides.

Inflammatory processes result in mobilization, accumulation, and infiltration of polymorphonuclear leukocytes (PML) at the afflicted site, and thus they release proteolytic enzymes and produce reactive oxygen...
intermediates. These include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (·OH). They may, in addition to fulfilling the protective aim, directly or indirectly disturb normal cellular activities in adjacent tissues by, for instance, initiating lipid peroxidation, inhibition of essential metabolic pathways, or destruction of DNA. In addition, prolonged inflammation may lead to tissue alterations such as hyperplasia and fibrosis, alterations that may render the tissue more susceptible to tumor development. PML also have the capacity to activate carcinogens to their ultimate forms. For instance, these cells activate BP-7,8-dihydrodiol, the proximal carcinogen of BP to BPDE (Figure 1). This property of PML may be of great importance in smoke-induced carcinogenesis because it is expected to contribute to the carcinogen metabolism and thus increase the actual concentration of harmful intermediates in the vicinity of target cells. It is also in this type of activation that SO$_2^-$ and NO$_2^-$ appear to be stimulatory.

The purpose of this article is to summarize some recent work from our laboratory on the stimulatory effect of sulfur dioxide and nitrogen dioxide on the metabolic activation of BP-7,8-dihydroidiol in PML and in more simple in vitro systems to reactive DNA- and protein-binding intermediates.

![Figure 1. Metabolism of benzo[a]pyrene to (-)-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene via intermediate formation of (+)-BP-7,8-epoxide. Step 1 is usually catalyzed by cytochrome P450. Step 2 is catalyzed by epoxide hydrase.](image1)

![Figure 2. Metabolism of (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP-7,8-dihydroidiol) to various products. Alternative pathways are indicated by numbers and represents 1) the formation of anti- and syn-10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) diastereomers (dependent on cytochrome P450 or peroxidative mechanisms), 2) dehydrogenation of the 7,8-dihydroidiol to the corresponding 7,8-diol, 3) the spontaneous hydrolysis of BPDE to tetraols, 4) the glutathione transferase-catalyzed conjugation of BPDE with glutathione, 5) the reaction of BPDE with DNA, 6) the oxidation of BP-7,8-diol to the corresponding dione, 7) the reaction of the 7,8-dione with protein nucleophiles, 8) further activation of BPDE by hydroxylation at the 1 or 3 position to trioxepines, and 9) these intermediate reactions with proteins.](image2)

### Table 1. Stimulation of tetraol formation by nitrite (1 mM) and sulfite (1 mM) in 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated human polymorphonuclear leukocytes (PML) after 30-min incubation with BP-7,8-dihydroidiol.

| Incubation mixture         | PMol tetraols/fold increase |
|----------------------------|-----------------------------|
| Control                    | 1.0                         |
| Control + TPA              | 1.7                         |
| Control + TPA + nitrite    | 3.8                         |
| Control + TPA + sulfite    | 7.7                         |

The control incubation consisted of 5 x 10$^5$ cells and 10 µM BP-7,8-dihydroidiol in a total volume of 3 ml phosphate-buffered saline, pH 7.4.

### Results and Discussion

#### Metabolic Activation of trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene

Human PML were incubated with BP-7,8-dihydroidiol in the absence or presence of 12-O-tetradecanoylphorbol-13-acetate (TPA) in order to initiate the oxidative burst for up to 30 min. Aliquots were withdrawn at different time points and assayed for the formation of tetraols (hydrolysis products of BPDE, Figure 2) by high-performance liquid chromatography (HPLC) (15). To study the effect of sulfur dioxide and nitrogen dioxide on the metabolism of BP-7,8-dihydroidiol, their reaction products with water, sulfite (SO$_3^{2-}$) and nitrite (NO$_2^-$), respectively, were used rather than the pure gases.

The effect of TPA and nitrite or sulfite on the metabolism of BP-7,8-dihydroidiol to tetraols is shown in Table 1. A low basal activity is present in non-TPA-treated cells. Stimulating the oxidative burst by TPA greatly increases the formation of tetraols. Available evidence indicates at least partial involvement of myeloperoxidase (MPO) and H$_2$O$_2$ (16). The low-basal activity can be explained by the presence of low levels of cytochrome P450 (17). Addition of sulfite to TPA-treated but not to nontreated cells greatly stimulates the formation of tetraols. The mechanism underlying the effect probably involves oxidation of sulfite to the sulfur trioxide radical anion (SO$_3^{2-}$), which in turn may react with molecular oxygen to yield a sulfur peroxyl radical (·OOOSO$_2^-$). The peroxyl radical may directly or via the corresponding acid epoxidize BP-7,8-dihydroidiol to BPDE (18). Like sulfite, nitrite has no stimulatory effect on the tetraol formation in non-TPA-treated cells. However, addition of nitrite to TPA-treated PML markedly stimulates the metabolism of BP-7,8-dihydroidiol. In an attempt to elucidate the mechanism underlying the stimulatory effect of nitrite, extensive in vitro experiments have been per-
Table 2. Binding of \(^{3}H\)(±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(alpha)pyrene metabolites to calf thymus DNA and PMNs proteins.

| Incubation mixture | pmole bound/ mg DNA | pmole bound/ mg protein |
|--------------------|---------------------|-------------------------|
| Control            | 0.8                 | 7.5                     |
| Control + PMA      | 1.6                 | 21                      |
| Control + PMA + nitr| 2.1                 | 45                      |
| Control + PMA + sul| 2.0                 | 9.6                     |

The control reaction consisted of 10 μM (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(alpha)pyrene and 10 × 10\(^5\) PMNs in 3 ml PBS, pH 7.4.

![Graph showing DNA binding vs GST activity](image)

Figure 3. Covalent binding of BP-7,8-dihydrodiol intermediates to exogenous DNA in the presence of polymorphonuclear leukocytes as a function of cystolic glutathione transferase activity.

formed. In brief, we have incubated purified MPO with its substrate, hydrogen peroxide, and BP-7,8-dihydriodiol in the presence or absence of nitrite. Both tetrads and BPDE could be detected, particularly in the presence of nitrite (±30-fold increase) (unpublished data). Thus we believe that the MPO/H\(_2\)O\(_2\) system is the major pathway involved in the nitrite-dependent activation of BP-7,8-dihydriodiol. However, we cannot presently exclude significant contribution of additional pathways.

Covalent Binding of Reactive BP-7,8-dihydriodiol Products to DNA and Proteins

The effect of sulfite or nitrite on DNA and protein binding of reactive intermediates from BP-7,8-dihydriodiol has been studied. PML, non-TPA-treated, or TPA-treated, were incubated with BP-7,8-dihydriodiol and exogenously added calf thymus DNA in the absence or presence of sulfite or nitrite. Binding to exogenous DNA and cellular proteins was estimated as described in (19). The results are compiled in Table 2. Initiating the oxidative burst by TPA enhances the binding of BP-7,8-dihydriodiol intermediates to both DNA and proteins. Addition of sulfite or nitrite further stimulates the binding to both DNA and proteins. As evident, the extent of binding to cellular proteins is much higher than binding to DNA. Preliminary experiments involving extensive purification of DNA, enzymatic hydrolysis to nucleosides and subsequent analysis by HPLC indicate that the major DNA-binding intermediate is (+)-anti-BPDE, the ultimate carcinogen from BP. Taking into consideration the protein adducts, other intermediates, in addition to those binding to DNA, seem to contribute. For instance, the BP-7,8-dione (probably formed via diol dehydrogenase catalyzed oxidation of BP-7,8-dihydriodiol to the corresponding diol and subsequent nonenzymatic or enzymatic (2 e\(^-\) oxidation) is a probable candidate as is the triol epoxide (formed by hydroxylation at the 1 or 3 position of BPDE) (Figure 2). The latter intermediates are highly reactive and exhibits a preference for binding to nucleophilic protein targets (20).

Within the concept discussed in this paper, the experiments with DNA localized outside the cells illustrate an important principle, namely that reactive BP-7,8-dihydriodiol products formed within the cells can be released and subsequently react with nucleophilic targets localized outside.

Protection against Reactive BP-7,8-dihydriodiol Products in Polymorphonuclear Leukocytes

Glutathione (GSH) in conjunction with glutathione transferases (GSTs) is the most important cellular system for protection against carcinogenic PAHs. Accepting the hypothesis that PML contribute to the metabolic activation of carcinogens such as BP and thus increase the probability for tumor formation, it is likely that the level of GSH and the qualitative and quantitative distribution of GST isoenzymes are important regulatory factors for the formation and accumulation of reactive BP-7,8-dihydriodiol intermediates. Accordingly, cystolic fractions from PML isolated from a number of female and male individuals were prepared and assayed for GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) (21) (Table 3). In addition, total GST content and isoenzyme distribution were determined by affinity chromatography and HPLC (22). The results from this study will be published elsewhere, but it can be concluded that the major GST isoenzyme in PML is GST P1-1, the isoenzyme most efficient in detoxifying BPDE by conjugation with GSH. In addition to GST activities, Table 3 also contains data on total cell protein, protein content of cytosolic fractions and extent of DNA binding of BP-7,8-dihydriodiol intermediates. It is evident that no significant difference exists between females and males with regards to these parameters.

Table 3. Estimation of total protein in polymorphonuclear leukocytes and cytosolic fractions, glutathione transferase (GST) activity and binding of BP-7,8-dihydriodiol metabolites to DNA.

| Source of cells | Cell protein | Cytosolic protein | GST activity | DNA binding |
|-----------------|--------------|-------------------|--------------|-------------|
| Male            | 51.5 ± 24.8  | 41.2 ± 14.3       | 0.13 ± 0.02  | 0.89 ± 0.42 |
| Female          | 52.4 ± 13.5  | 47.4 ± 10.9       | 0.14 ± 0.07  | 1.05 ± 0.62 |

**Conclusions**

From our results, it is evident that sulfite and nitrite, major hydrolysis products of sulfur dioxide and nitrogen dioxide, respectively, stimulate the metabolism of BP-7,8-dihydriodiol to both tetrads and to DNA- and protein-binding products in human PML. The metabolic activation of PAHs in PML and the stimulation of this process by sulfite and nitrite may indeed be a contributing factor for the development of primary lung cancer in smokers, particularly in those living in air-contaminated urban areas.

Whereas the mechanism of the sulfite-dependent stimulation is more or less elucidated and involves the formation of a sulfur peroxynitrous radical, the mechanism of the nitrite stimulation is still unknown. Preliminary evidence suggests that peroxynitrite, formed through the reaction between hydroperoxide and nitrite, is an important intermediate. How these species interact with myeloperoxidase is, however, not yet evident.
REFERENCES

1. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in United States today. J Natl Cancer Inst 66:1191-1308 (1981).
2. U.S. Surgeon General. The health consequences of smoking: cancer. Washington:United States Department of Health and Human Services, 1982.
3. IARC. Tobacco Smoking. In: IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemical to Humans, Vol 38. Lyon:International Agency for Research on Cancer, 1986.
4. Zeller WJ, Schmahl D. Aziologie des Bronchialkarzinoms. In: Luftverunreinigungen und Ätemwegserkrankungen beim Menschen. PMI (Matthys H, ed). Frankfurt:Verlag, 1986:84-89.
5. Baum EJ. Occurrence and surveillance of polycyclic aromatic hydrocarbons. In: Polycyclic Hydrocarbons and Cancer, Vol 1 (Gelboin HV, Tso POP, eds). New York:Academic Press, 1978:45-62.
6. Thakker DR, Yagi H, Levin W, Wood AW, Conney AH, Jerina DM. Polycyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens. In: Bioactivation of Foreign Compounds (Anders MW, ed). Orlando, FL:Academic Press, 1985:177-242.
7. Sims P, Grover PL, Swaibeland A, Pal K, Hewer A. Metabolic activation of benzo[a]pyrene proceeds by a diol epoxide. Nature 252:326-328 (1974).
8. Hughes MF, Champullirat W, Mason RP, Eling TE. Epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene via a hydroperoxide-dependent mechanism catalyzed by lipooxygenase. Carcinogenesis 10:2075-2080 (1989).
9. Marnett LJ. Peroxy free radicals: potential mediators of tumor initiation and promotion. Carcinogenesis 8:1365-1373 (1987).
10. Marnett LJ. Prostaglandin synthase-mediated metabolism of carcinogens and a potential role for peroxyl radicals as reactive intermediates. Environ Health Perspect 88:5-12 (1990).
11. Mustafa MG, Tierry DF. Biochemical and metabolic changes in the lung with oxygen, ozone, and nitrogen dioxide. Am Rev Respir Dis 118:1061-1090 (1978).
12. Morrow PE. Toxicological data on NO.; An overview. In: Fundamentals of Extrapolation Modeling of Inhaled Toxicants: Ozone, and Nitrogen Dioxide (Miller FJ, Menzel DB, eds). Washington:Hemisphere, 1984:205-227.
13. Goldstein E, Goldstein F, Peek NF, Parks NJ. Absorption and transport of nitrogen oxides. In: Nitrogen Oxides and Their Effects on Health (Lee SD, ed). Ann Arbor, MI:Ann Arbor Science, 1980:143-160.
14. Kunimoto M, Tsubone H, Tsujii N, Mochitate K, Kaya K, Shimojo N, Miura T. Effects of nitrate and nitrite, chemical intermediates of inhaled nitrogen dioxide, on membrane components of blood cells of rats. Toxicol Appl Pharmacol 74:10-16 (1984).
15. Romert L, Dock L, Jensd J, Jernström B. Effects of glutathione transferase activity on benzo[a]pyrene-7,8-dihydrodiol metabolism and mutagenesis studied in a mammalian co-cultivation assay. Carcinogenesis 10:1701-1707 (1989).
16. Trush MA, Seed JL, Kessler TW. Oxidant-dependent metabolic activation of polycyclic aromatic hydrocarbons by phorbol ester-stimulated human polymorphonuclear leukocytes: possible link between inflammation and cancer. Proc Natl Acad Sci USA 82:5194-5198 (1985).
17. Murray GI, Barnes TS, Sewell HF, Ewen SWB, Melvin WT, Burke MD. The immunocytochemical localization and distribution of cytochrome P450 in normal human hepatic and extrahepatic tissues with a monoclonal antibody to human cytochrome P450. Br J Clin Pharmacol 25:465-475 (1988).
18. Reed GA, Curtis JF, Motley C, Eling TE, Mason RP. Epoxidation of (+)-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene during (bi)sulfit autodissociation: activation of a procarcinogen by a cocarcinogen. Proc Natl Acad Sci USA 83:7499-7502 (1986).
19. Constantin D, Jernström B, Cognegrau IA, Moldeus P. Sodium nitrite-stimulated metabolic activation of benzo[a]pyrene-7,8-dihydrodiol in human polymorphonuclear leukocytes. Carcinogenesis 12(5):777-784 (1991).
20. Dock L, Waern F, Martinez M, Grover PL, Jernström B. Studies on the further activation of benzo[a]pyrene diol epoxides by rat liver microsomes and nuclei. Chem Biol Interact 58:301-318 (1986).
21. Habig WH, Jakoby WB. Assays for differentiation of glutathione-S-transferases. Methods Enzymol 77:398-405 (1981).
22. Van Ommen B, Bogaards JJP, Peters WHM, Blaauwboer B, Van Bladeren P. Quantification of human hepatic glutathione S-transferases. Biochem J 269:609-613 (1990).