Enhancement of benzo[a]pyrene diol epoxide mutagenicity by sulfite in a mammalian test system

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Sulfur dioxide, a ubiquitous air pollutant, is a co-carcinogen for benzo[a]pyrene (BP). We have demonstrated previously that the interaction between sulfite, the physiological form of sulfur dioxide, and \((\pm)-7r,8t\text{-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE)}\), the ultimate carcinogenic form of BP, results in an enhanced mutagenic effect in Salmonella typhimurium strains TA98 and TA100. We report here that this same co-mutagenic effect of sulfite occurs in a mammalian cell line. Treatment of Chinese hamster V79 cells with 50 nM anti-BPDE, a concentration on the linear portion of the dose-response, resulted in a four-fold increase in mutations at the hprt locus relative to the spontaneous rate. When V79 cells were exposed to 1 or 10 mM sulfite immediately prior to the addition of anti-BPDE, the mutation rate increased by 73\% and 210\%, respectively, over that elicited by anti-BPDE alone. Sulfite itself was moderately cytotoxic, but caused no increase in mutation over the spontaneous rate. Characterization of the dose- and time-dependence of this enhancement of diol epoxide mutagenicity by sulfite closely resembled the effects seen previously in the bacterial system. In particular, enhancement by sulfite was evident when sulfite was added to the cells between 60 min and 1 min prior to the addition of the diol epoxide. Concurrent addition of sulfite and the diol epoxide attenuated the enhancement, and the effect was lost altogether when sulfite was added 10 min after the diol epoxide. The specificity of this effect of sulfite was shown by comparison with sulfate, which at concentrations of either 1 or 10 mM exhibited modest cytotoxicity, but neither was directly mutagenic nor able to enhance the mutagenic effect of anti-BPDE. Binding studies with labeled anti-BPDE showed that the addition of 10 mM sulfite increased binding of anti-BPDE to DNA by over 43\%, corresponding to the observed increase in mutant frequency. Interestingly, this difference in level of DNA modification was not apparent after 30 min to 2 h exposures, but only emerged at the 4 h time point. The 4 h point was routinely used for all mutagenicity studies. Binding of anti-BPDE-derived materials to cellular RNA was not altered by 10 mM sulfite. The emergence of increased DNA modification at the latest time point suggests either a more prolonged period of active DNA binding than would occur with diol epoxide, or a difference in the ability to recognize and clear specific DNA adducts. Both possibilities are discussed in regard to the observed formation of \(7r,8t,9t\text{-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene-10c-sulfonate (BPT-10-sulfonate)}\) in those incubations. BPT-10-sulfonate is a relatively stable BP derivative which retains the ability to covalently modify DNA. The role of this derivative in the enhancement of diol epoxide mutagenicity by sulfite is strongly suggested by these data.

Introduction

Sulfur dioxide and benzo[a]pyrene (BP*) are both ubiquitous air pollutants. Although not carcinogenic itself, sulfur dioxide exposure is associated with increased incidence of respiratory tract malignancy in humans (1), and enhances the carcinogenicity of inhaled BP in rats (2) and hamsters (3). These observations suggest that sulfur dioxide may function as a co-carcinogen with BP. Co-carcinogens enhance the actions of genotoxic carcinogens either by increasing the extent of DNA modification which results from carcinogen exposure, or by increasing the conversion of DNA modifications into heritable mutagenic lesions (4). This group (5-8) and others (9-11) have studied the chemical interactions between sulfite, the physiological form of sulfur dioxide, and various BP metabolites in several in vitro model systems to determine whether these reactions might favor increased DNA-damaging activity from the BP derivatives. Although sulfite and sulfate-derived species can convert \(\text{trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (anti-BPDE)}\) to reactive derivatives which can covalently bind to DNA (8-11), our recent studies have focused instead on reactions involving \(7r,8t\text{-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE)}\) and sulfite. Just as anti-BPDE is susceptible to the nucleophilic attack of water, resulting in hydrolysis to form the isomeric \(7r,8t,9t\text{-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BP tetraols), the diol epoxide also is readily attacked by sulfite, resulting in the formation of \(7r,8t,9t\text{-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene-10c-sulfonate (BPT-10-sulfonate)}\) (7). BPT-10-sulfonate resembles the BP tetraols in its general stability, exhibiting no detectable decomposition in aqueous media after a 24 h incubation (7). This BP derivative does, however, retain the ability to bind covalently to DNA, and does so with an efficiency approximately 25% of that obtained with anti-BPDE under identical conditions (7). In these model reactions BPT-10-sulfonate represents a BP derivative more selective for reaction with DNA than is the diol epoxide. If this BP derivative is formed in cells or tissues exposed to BP and to sulfur dioxide, and if DNA modification and resultant mutations occur, then this would represent an additional class of reactive genotoxic aromatic hydrocarbon metabolites, and might in part explain the co-carcinogenic effect of sulfur dioxide.

The formation of BPT-10-sulfonate and its possible role in genotoxicity was characterized first in bacterial systems. We reported that sulfite was not itself mutagenic, but that it elicited

*Abbreviations: BP, benzo[a]pyrene; anti-BPDE, \((\pm)-7r,8t\text{-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE)}\); BP tetraols, \(7r,8t,9t\text{-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE)}\); BPT-10-sulfonate, \(7r,8t,9t\text{-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene-10c-sulfonate (BPT-10-sulfonate)}\); DMEM, Dulbecco's modified minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; 8-AG, 8-azaguanine.
a concentration-dependent increase in the mutagenicity of anti-BPDE in *Salmonella typhimurium* strains TA98 and TA100 (5–7). Covalent modification of bacterial DNA by diol epoxide-derived species increased in parallel with mutagenicity when sulfite was added. Under conditions when maximal increase in diol epoxide mutagenicity was observed, up to 45% of the diol epoxide was converted to BPT-10-sulfonate.

The interactions of anti-BPDE and sulfite were studied further in a mammalian cell system (12). The addition of 1 and 10 mM sulfite to the C10 murine respiratory epithelial cell line prior to the addition of 0.1 to 1 μM [3H]-anti-BPDE yielded a substantial increase in nuclear labeling of the cells, and a concomitant increase in actual covalent modification of nuclear DNA by diol epoxide-derived materials. Labeled BPT-10-sulfonate was observed not only in the medium from those incubations, but more importantly was detected in both the cytosolic and the nuclear fractions from the treated cells. Exogenous labeled BPT-10-sulfonate added to the medium was not able to enter intact cells. These findings strongly support the intracellular formation of BPT-10-sulfonate from anti-BPDE and sulfite, both of which are able to enter intact cells, and also suggests that once BPT-10-sulfonate is formed it may be trapped within that cell.

This inability of BPT-10-sulfonate to cross the plasma membrane is analogous to the behavior of sulfate esters of hydroxymethyl metabolites of polycyclic hydrocarbons, reported by Glatt and co-workers (13,14). They found that the sulfate esters of several hydroxylated hydrocarbons were only marginally effective as mutagens in both bacterial (13) and mammalian (14) cell systems when added to the extracellular medium. When the esters were generated intracellularly, however, in V79 cells expressing a heterologous sulfotransferase, a potent mutagenic response was observed (14). Although the reactivities of the sulfonic acid BPT-10-sulfonate and the sulfate esters described by Glatt are very different, the effect of their amphipathic nature on their ability to cross the plasma membrane appears to be quite the same.

The logical extension of the uptake and binding studies was to investigate the biological consequences of the interactions between sulfite and anti-BPDE in a mammalian system. C10 cells are tetraploid, thus the determination of sulfite effects on diol epoxide mutagenicity at the hprt locus was not possible in those cells. That shortcoming prompted the present studies, which allow for the determination of mutations in a mammalian cell system. Here we report the results of our characterization of the metabolic and genotoxic interactions of the activated carcinogen and the co-carcinogen in a well-characterized mammalian test system, and discuss the possible role of these interactions in the observed co-carcinogenic interaction.

**Materials and methods**

**Mutagenicity studies**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Mutant frequency</th>
<th>Plating efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>3.2±1.2/10^6 viable cells</td>
<td>100</td>
</tr>
<tr>
<td>10 mM sulfite</td>
<td>3</td>
<td>0.4±0.4</td>
<td>54±24</td>
</tr>
<tr>
<td>10 mM sulfate</td>
<td>2</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>50 nM anti-BPDE</td>
<td>6</td>
<td>17.1±2.7</td>
<td>84±2</td>
</tr>
<tr>
<td>50 nM anti-BPDE + 1 mM sulfite</td>
<td>3</td>
<td>26.9±4.5</td>
<td>77±7</td>
</tr>
<tr>
<td>50 nM anti-BPDE + 10 mM sulfite</td>
<td>4</td>
<td>44.8±5.6</td>
<td>62±6</td>
</tr>
<tr>
<td>50 nM anti-BPDE + 10 mM sulfate</td>
<td>2</td>
<td>16</td>
<td>74</td>
</tr>
</tbody>
</table>

V79 cells were treated with anti-BPDE and sulfite or sulfate for 4 h in PBS. Sulfite or sulfate were added 10 min prior to anti-BPDE. Determination of plating efficiency and selection for hprt mutants was performed as described in Materials and methods. Data are presented as the mean ± standard deviation from the number of experiments listed (n).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>RNA binding</th>
<th>DNA binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nM anti-BPDE</td>
<td>100 (18-4.5 pmol/mg)</td>
<td>100 (2.8-3.6 pmol/mg)</td>
</tr>
<tr>
<td>50 nM anti-BPDE + 10 mM sulfite</td>
<td>99±14</td>
<td>143±18</td>
</tr>
</tbody>
</table>

V79 cells in PBS were treated with [3H]-anti-BPDE and sulfite for 4 h, as detailed in Table I and in Materials and methods. Cellular RNA and DNA were isolated, and binding levels were quantified by standard methods. Values represent the normalized values for binding in the absence of sulfite, with the range of actual binding values given in parentheses. Values in the presence of sulfite are the mean ± SD of the normalized binding levels from six experiments.

**Statistical analysis**

Mutation frequencies represent the means obtained from multiple experiments. Statistical analysis was performed by one-way analysis of variance. Comparisons between the control mutant frequency and those of individual treatment groups were performed by Dunnett's method (P < 0.05). Comparisons...
anti-BPDE studies showed that treatment with the diol epoxide alone decreased mutagenicity and cytotoxicity of anti-BPDE, with modest effects at increasing cytotoxicity. The specificity of this effect for sulfite was examined by a direct comparison with equimolar sodium sulfite (Table I). Both salts caused comparable decreases in cell survival in the presence and absence of the diol epoxide, and neither salt itself was mutagenic. Similarity between sulfite and sulfate, however, was not observed in regard to the enhancement of diol epoxide mutagenicity. Only sulfite affected the mutagenic response.

Investigation of the time course of the comutagenic effect of sulfite was begun by varying the time of sulfite addition to the cells relative to the addition of the diol epoxide (Figure 1). Maximal enhancement was obtained when sulfite was added to V79 cells either 60 min or 15 min prior to the addition of anti-BPDE. This enhancement was decreased by nearly one-half when sulfite was added concurrently with the diol epoxide, and the effect was abolished when the addition of sulfite occurred 15 min, 60 min or 120 min after the addition of the diol epoxide. Effects of sulfite on cell survival followed a similar time course, with a marked decrease in cell survival when sulfite was added to the cells 60 min prior to the diol epoxide, and a steady decrease in cell killing as sulfite addition was delayed after the diol epoxide addition.

The effect of relative time of additions suggests that sulfite alters the ability of anti-BPDE or anti-BPDE-derived species to modify DNA. This was examined by treating V79 cells for 4 h with [3H]-anti-BPDE, in the presence and absence of 10 mM sulfite, and analyzing the resultant labeling of nucleic acids from those cells (Table II). Data from six separate studies showed that treatment with the diol epoxide alone resulted in the binding of from 2.8 to 3.6 pmol anti-BPDE-derived material per mg of DNA. Binding to RNA was somewhat more variable, with a range of between 1.8 and 4.5 pmol/mg. Apparent covalent binding to DNA was not affected by the presence of 10 mM sulfite. Binding to DNA, however, was increased by 43% in the presence of sulfite, and this difference was determined to be statistically significant.

Stable products derived from [3H]-anti-BPDE were determined by HPLC analysis of PBS from the above incubations (Figure 2). In the control incubations, the only detectable products were the trans- and cis-diastereomers of BP tetraols (tR = 19.5 and 22.3 min, respectively). The incubations including 10 mM sulfite, however, showed that approximately 30% of the diol epoxide was converted to the trans- and cis-sulfite adduction products, BPT-10-sulfonates (tR = 10.2 and 11.7 min, respectively). This substantial formation of BPT-10-sulfonate from anti-BPDE under conditions which also result in increased DNA modification and mutagenesis parallels our previous observations in bacterial systems (7).

The dynamics of DNA modification by [3H]-anti-BPDE in the presence and absence of sulfite was examined by determining DNA binding levels at times from 5 min to 4 h after the addition of the diol epoxide (Figure 3). Sulfite did not significantly affect the level of apparent covalent modification of cellular DNA during the first 2 h of incubation. Only at the 4 h time point did a significant difference emerge, and at that time the sulfite-treated cells contained over 80% more anti-BPDE-derived material bound, than in the cells without sulfite. It is noteworthy, that this time point of 4 h exposure, was also the time point employed for all mutagenicity studies.
Materials and methods. At the indicated times, cells were removed from the buffer by reverse phase HPLC, as described in Materials and methods, and the amount of bound carcinogen measured by liquid scintillation counting. Data points represent the mean of triplicate determinations from the same DNA samples.

Discussion

The data reported here demonstrate that sulfite acts as a comutagen for anti-BPDE in a mammalian cell line. The parameters defining this effect appear very similar to those reported previously using a bacterial cell system (5-7): the specificity for the sulfite anion, the effective concentration range for sulfite, the magnitude of the enhancing effect, the importance of the relative time of addition of sulfite, the increase in DNA binding of BP derivatives, and the formation of BPT-10-sulfonate in these incubations. Strong support for the formation and actions of BPT-10-sulfonate as key events in the observed comutagenic effect may be drawn from the careful consideration of our data, and from the comparison of our findings and interpretations with those of other researchers.

Sulfite has been proposed to alter the metabolism of BP and its resultant genotoxic effects by increasing the production of anti-BPDE from trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP-7,8-diol) (5,9,10,11), by depleting cellular glutathione stores and blocking glutathione conjugation pathways for activated BP metabolites (16,17), and by reacting with anti-BPDE to yield BPT-10-sulfonate, a longer-lived, more selective DNA alkylator than the diol epoxide (7,12). We have carefully considered each of these hypotheses in light of our current and previous findings. Our earliest studies focused on the sulfite-dependent formation of anti-BPDE. The sulfite-dependent epoxidation of BP-7,8-diol occurs very readily in simple aqueous media (9,10), but the reaction is strongly inhibited by numerous components of tissue culture media (G.Reed, unpublished observations). This finding greatly diminishes the probable significance of such a reaction in a complex biological milieu, and thus we have concentrated more recently on alterations of diol epoxide actions by sulfite.

The interference with glutathione-dependent detoxication reactions for epoxides and diol epoxides provides a logical and attractive mechanism for enhancing the genotoxicity and carcinogenicity of BP. Such a mechanism would be specific for sulfite, as sulfate does not deplete glutathione (18), and would require the addition of sulfite to the cells prior to the addition of the diol epoxide in order to alter glutathione pools before the insult, and should result in an increased modification of DNA by the diol epoxide. All of these qualifications are observed in the present work. Three factors, however, argue against a major role for alterations in glutathione-dependent reactions in the comutagenic effect with anti-BPDE. First, if trapping of the diol epoxide by glutathione was a quantitatively significant pathway, then such conjugates should be seen in incubations of anti-BPDE without sulfite. Our level of detection for labeled metabolites is approximately 1% of total compound trapped against a major role for alterations in glutathione-dependent conjugation pathways for anti-BPDE. The sulfite-dependent formation of anri-BPDE. The sulfite-dependent epoxidation of BP-7,8-diol occurs very readily in simple aqueous media (9,10), but the reaction is strongly inhibited by numerous components of tissue culture media (G.Reed, unpublished observations). This finding greatly diminishes the probable significance of such a reaction in a complex biological milieu, and thus we have concentrated more recently on alterations of diol epoxide actions by sulfite.

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RNA, but both nucleic acids are alkylated (20). If the effect of sulfite was merely to remove a detoxication pathway and thus increase the effective half-life of the alkylating agent, then the resultant relative increases in binding to DNA and to RNA should be comparable. This is not the case—the increase in DNA binding was readily apparent, yet there was no increase in RNA binding. This alteration of binding distribution suggests that the effects of sulfite are mediated at some point other than the effective concentration of the diol epoxide.

An additional mechanism for sulfite comutagenicity and cocarcinogenicity has been proposed which involves modulation of DNA adduct repair, rather than DNA adduct formation. Mallon and Rossman studied the ability of sulfite to enhance UV light mutagenesis in *Escherichia coli* and in V79 cells, and have concluded that the effects of sulfite are based on the inhibition of DNA polymerase I, a critical enzyme involved in DNA repair (21). This potential for sulfite to inhibit key enzymes involved in DNA repair (removal of adducts) is in agreement with some of our data. The specificity for sulfite (21) and the increased level of DNA adduction which we observe in sulfite-treated cells are consistent with their proposal. Other findings, however, do not match those reported previously (21). The sulfite concentrations employed in our studies are 5- to 10-fold lower than those required for comutagenesis with UV light. In addition, the time courses for comutagenicity with anti-BPDE, and the corresponding dynamics of DNA adduct levels do not seem consistent with altered DNA repair as a key contributor. The comutagenic effect in V79 cells treated with UV light was only slightly diminished when sulfite was added to the cells after the exposure (21). This stands in marked contrast to the precipitous decline which we observed when sulfite was added to the cells after the diol epoxide. The kinetics of DNA adduction also do not seem to fit with modulation of DNA repair as a major factor. In both control and sulfite-treated cells, the removal of DNA adducts is an immediate and very rapid phenomenon, with greater than a 50% reduction in adduct levels within the first hour after diol epoxide administration, and nearly 70% decrease after 2 h. We observed no differences between control and sulfite-treated cells either in initial adduct levels or in their extent of removal during the first 2 h. It is only at the 4 h time point that the adduct level in sulfite-treated cells deviates from that observed in the control cells. These findings are difficult to reconcile with an apparently rapid inactivation of DNA polymerase I by sulfite (21), which should alter the clearance of DNA adducts within the first hour of treatment.

It must be appreciated that the observed adduct levels are a function both of DNA alkylation (adduct formation) and of DNA repair (adduct removal). The data which we report could be explained either by the prolonged active modification of DNA by a longer-lived BP derivative than the diol epoxide, or by the formation of unique DNA lesions which are not as readily recognized or repaired as are the normal lesions resulting from modification by anti-BPDE. Both of these possible mechanisms are consistent with the formation and actions of BPT-10-sulfonate as critical factors in the resultant biochemical and genotoxic responses. BPT-10-sulfonate is stable in aqueous media, with a half-life in excess of 24 h (7). Unlike diol epoxides, BPT-10-sulfonate does not react with thiols and other small molecular weight nucleophiles (7). The sulfonate does, however, retain the ability to covalently modify DNA, and does so with an efficiency comparable to that of the diol epoxide (7). BPT-10-sulfonate, which is detected as an anti-BPDE-derived product in V79 cells treated with sulfite, represents a longer-lived alkylating agent which could greatly extend the active period of DNA alkylation resulting from this combined treatment. The formation of the sulfonate also is consistent with the higher increase in DNA binding relative to the effects on RNA binding—the sulfonate derivative does not bind to RNA (7), and thus will not contribute to the increase in RNA binding.

A second rationale for the late divergence of binding kinetics between control and sulfite-treated cells is based on the formation of unique DNA adducts in the latter case, which are not as readily identified or repaired as are the typical diol epoxide adducts. Our preliminary studies of BPT-10-sulfonate DNA adducts using 32P-postlabeling analysis, demonstrated that most of the adducts were indistinguishable from those formed by anti-BPDE (Green,J.L. and Reed,G.A. manuscript in preparation). About 30% of the total adducts, however, exhibit chromatographic properties which do not correspond to any previously reported diol epoxide-derived adduct. Such an unique adduct could result from a unique reactive intermediate, and might generate adducts with different site, base or sequence specificity. Moreover, such unique adducts or sequences for adducts might markedly affect the efficiency of recognition and repair.

Our data are consistent with, but do not themselves establish, a key role for BPT-10-sulfonate in the comutagenic effects of sulfite for anti-BPDE. Determining the role of this unique reactive intermediate, and of the apparent unique adducts resulting, will be derived from careful analysis of the spectrum of DNA adducts produced in V79 cells, how that spectrum is affected by sulfite, and what the kinetics of formation and removal are for the various adducts. In addition, a unique alkylating agent which produces a different adduct spectrum might be expected to produce a different mutational spectrum than that reported for anti-BPDE (22). Current efforts in this laboratory are utilizing both approaches toward defining the mechanism of the comutagenic effect of sulfite.

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References


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