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Original Contribution

Benzo[*a*]pyrene and its metabolites combined with ultraviolet A synergistically induce 8-hydroxy-2′-deoxyguanosine via reactive oxygen species

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Abstract

We previously reported that benzo[*a*]pyrene (BaP) and UVA radiation synergistically induced oxidative DNA damage via 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in vitro. The present study shows that microsomal BaP metabolites and UVA radiation potently enhance 8-OHdG formation in calf thymus DNA about 3-fold over the parent compound BaP. Utilization of various reactive oxygen species scavengers revealed that singlet oxygen and superoxide radical anion were involved in the 8-OHdG formation induced by microsomal BaP metabolites and UVA. Two specific BaP metabolites, benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (+/−) (anti) (BPDE) and BaP-7,8-dione, were further tested for synergism with UVA. BaP-7,8-dione showed an effect on 8-OHdG formation induced by UVA radiation that was similar to that of the parent BaP, whereas BPDE exhibited significantly higher induction of 8-OHdG than BaP. At as low as 0.5 μM, BPDE plus UVA radiation substantially increased 8-OHdG levels about 25-fold over the parent BaP. BPDE increased the formation of 8-OHdG levels in both BPDE concentration- and UVA dose-dependent manners. Additionally, singlet oxygen was found to play a major role in 8-OHdG induction by BPDE and UVA. These results suggest that BaP metabolites such as BPDE synergize with UVA radiation to produce ROS, which in turn induce DNA damage.

Keywords: Benzo[*a*]pyrene; Metabolite; BPDE; Ultraviolet A; 8-Hydroxy-2′-deoxyguanosine; Singlet oxygen; Free radicals

Benzo[*a*]pyrene (BaP) is a ubiquitous environmental pollutant that is a product of incomplete combustion of fossil fuels. It has been identified in surface water, tap water, rain water, groundwater, waste water, and sewage sludge. About 2000 tons of BaP are emitted into the air across the United States every year [1]. BaP is a potent carcinogen implicated in a number of organs, including lung, forestomach, liver, spleen, esophagus, tongue, and skin in mice [2–8]; it is also a known human carcinogen [9]. Because of the carcinogenic effects of BaP, it has been utilized as a tool reagent to induce mutagenesis in the evaluation of in vivo mutagenic assay systems [7,10,11]. BaP is believed to lead to genotoxicity via metabolism to benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (+/−) (anti) (BPDE), which reacts with many biological macromolecules, including DNA, to yield premutagenic adducts [8]. In fact, DNA damage by chemicals, including covalent binding and oxidation, is thought to constitute an essential molecular mechanism in the initiation of cancer [12,13] and possible teratogenesis [14]. BPDE–DNA adducts have been detected in human tissues, including lung, blood, colon mucosa, and breast [15–18], as well as in the epidermis of experimental mice [19].

Abbreviations: BaP, benzo[*a*]pyrene; BPDE, benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (+/−)(anti); NBT, nitroblue tetrazolium; RNO, *p*-nitrosodimethylaniline; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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Ultraviolet (UV), on the other hand, induces oxidative DNA damage evidenced by formation of products such as cyclobutane pyrimidine dimers, pyrimidine (6–4) pyrimidone photoproducts, Dewar valence isomers [20], and 8-hydroxy-2′-deoxyguanosine (8-OHdG) [21,22]. It was also reported that formamidopyrimidines are important DNA damage products of γ-ray and UV irradiation [23–27]. Many previous studies demonstrate that 8-OHdG can lead to G:C to T:A transversions at DNA replication sites [28,29,23] and has long been considered to correlate with carcinogenesis [30,31]. UVA (320–400 nm) radiation comprises the majority of solar UV radiation reaching the earth's surface. Chronic UVA irradiation is known to induce skin damage, including photoaging [32] and skin cancer [33–35]. It is hypothesized that BaP and UVA radiation synergistically induce carcinogenesis through generation of genetic damage. We have previously demonstrated that BaP and UVA irradiation enhanced 8-OHdG in calf thymus DNA and cultured cells [36]. Interestingly, the level of 8-OHdG in cultured cells was much higher than that in calf thymus DNA, suggesting that metabolized BaP acts more effectively than the parent BaP [36]. Therefore, in this study, the microsomal metabolites of BaP along with other two well-known metabolites, BPDE and BaP-7,8-dione, were investigated for synergetic effects with UVA radiation on 8-OHdG formation in vitro.

Materials and methods

Materials

BPDE and BaP-7,8-dione were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO, USA). BaP, $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, calf thymus DNA, catalase, superoxide radical anion dismutase (SOD), nitro-blue tetrazolium (NBT), 2,2,6,6-tetramethyl-4-piperidinol, and 4-hydroxy-2,2,6,6-tetramethyl-piperidin-1-oxyl were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of microsomes

Microsomes were extracted from livers of 6- to 10-week-old female SKH-1 mice (Charles River Laboratories, Wilmington, MA, USA). Briefly, liver samples were homogenized in sucrose (0.25 M) containing protease inhibitors (Sigma). After the debris was spun down, nuclei and mitochondrial fractions and microsomes were obtained by centrifuging at 105,000*g* for 90 min, followed by washing and recentrifuging in 1.15% KCl. The isolated microsomes were then suspended in 1.15% KCl solution and stored at −80°C [37]. Protein contents were determined using a BCA kit (Pierce) following the instructions of the manufacturer.

Preparation of BaP metabolites

Microsomes (0.5 mg protein/ml) were incubated in phosphate buffer (100 mM, pH 7.4) containing $MgCl_2$ (3 mM), EDTA (1 mM), $NADP^+$ (1 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (3 units/ml), and BaP (30 μM) at 37°C for 20 min [38]. The reaction was terminated by placing the samples on ice and adding acetone. BaP metabolites were extracted into ethyl acetate and dried under vacuum. The dried BaP metabolites were then dissolved in DMSO with the same volume as initially added. Equivalent amounts of metabolites and parent BaP were used throughout the experiments.

UVA radiation

The Spectroline Model XX-15A equipped with UV-365-nm lamps (Spectronics Corp., Westbury, NY, USA) was used as the UVA source. UVA intensity was quantified by a Model IIL-1700 research radiometer (International Light, Newburgport, MA, USA). DNA (0.2 mg/ml) was placed in six-well plates (Corning, NY, USA), with phosphate buffer (20 mM, pH 7.4), in the presence or absence of BaP, BaP metabolites, and other compounds where indicated at a volume of 2 ml. The distance between the surface of the DNA solution and the UVA source was 7 cm. After irradiation, DNA was precipitated by adding NaCl and isopropanol for further HPLC assay.

Analysis of 8-OHdG

Details of 8-OHdG quantification were as described previously [36] with a minor modification. DNA samples were denatured by boiling in water for 6 min and cooling in ice before enzymatic digestion. The 8-OHdG level in the DNA was determined by an HPLC Breeze system equipped with UV and electrochemical detectors (Waters, Milford, MA, USA). DNA hydrolysates were separated by a 4.6 × 150-mm Polarity column (Waters) and eluted with a mobile phase (pH 5.1) containing methanol (10%), acetic acid (10 mM), sodium acetate (25 mM), citric acid (12.5 mM), and sodium chloride (2 mM).

Detection of singlet oxygen

BaP or its metabolites were irradiated under UVA radiation together with 5 mM imidazole and 40 μM *p*-nitrosodimethylaniline (RNO). Bleaching of RNO was measured at 440 nm before and after irradiation as described by Kraljic and Mohsni [39]. The formation of singlet oxygen was also confirmed by a specific reaction between singlet oxygen and 2,2,6,6-tetramethyl-4-piperidinol [40]. Various concentrations of BPDE were added to the phosphate buffer immediately before UVA exposure as described above. The turnover of 2,2,6,6-tetramethyl-4-piperidinol to 4-hydroxy-2,2,6,6-tetramethyl-4-piperidin-1-

oxyl was measured with HPLC and electrochemical detection as described for 8-OHdG analysis, with the exception of the methanol content, which was 20%.

Detection of superoxide radical anion

BaP or its metabolites were dissolved in phosphate buffer (50 mM, pH 6.5) containing EDTA (1 mM) and NBT (10 mM) and were irradiated with UVA. Absorbance at 570 nm was measured as an indicator for superoxide radical anion generation [41].

Statistical analysis

A two-tailed Student *t* test was used. $p < 0.05$ was considered statistically significant.

Results

BaP microsomal metabolites are more potent inducers of 8-OHdG formation than BaP in calf thymus DNA irradiated by UVA

The abilities of BaP and its microsomal metabolites to induce 8-OHdG in calf thymus DNA under UVA irradiation are compared in Fig. 1. BaP and UVA irradiation significantly enhanced 8-OHdG level compared to sham and UVA irradiation controls. Moreover, the level of 8-OHdG induced by BaP microsomal metabolites (80 μM BaP equivalent) plus UVA irradiation (50 kJ/m^2) was over threefold higher than that induced by the parent BaP. Neither BaP nor BaP microsomal metabolites showed any effect on 8-OHdG formation (data not shown). This result indicates that BaP metabolites have more potent synergistic action with UVA irradiation than the parent BaP.

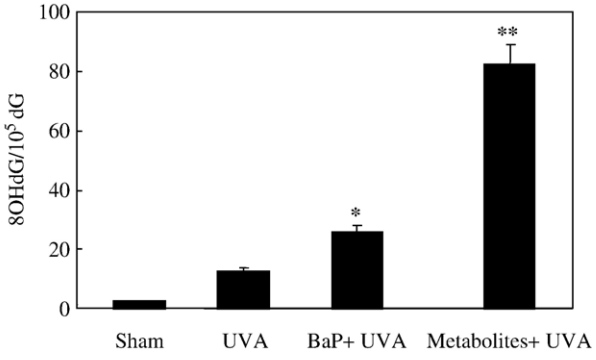


Fig. 1. Comparison of BaP and BaP microsomal metabolites in the induction of 8-OHdG formation synergistic with UVA radiation. Calf thymus DNA (0.2 mg/ml) was irradiated under UVA radiation (50 kJ/m^2) in the presence or absence of BaP (80 μM) and BaP microsomal metabolites on equivalent molar bases. Each column represents the mean ± SD of four experiments. *Significant vs DNA sham group ($p < 0.05$); **significant vs BaP + UVA radiation group.

Table 1
Effects of ROS scavengers on 8-OHdG formation by BaP microsomal metabolites plus UVA

Group	8-OHdG/10^5 dG (%)
Sham	0
Metabolites alone	0
UVA alone	15
UVA + metabolites	100
UVA + metabolites + scavenger	
Catalase (50 units/ml)	
Intact	59*
Heat denatured	59
SOD (50 units/ml)	
Intact	83*
Heat denatured	99
NaN_3	
0.5 mM	35*
1 mM	20*
5 mM	10*
Deferroxamine, 1 mM	99*
Mannitol, 1 mM	73*

DNA (0.2 mg/ml) was irradiated under UVA (50 kJ/m^2) with BaP microsomal metabolites (80 μM BaP equivalent) and various reagents as described under Materials and methods. Catalase and SOD were denatured by boiling the enzyme solutions at 100°C for 15 min. Percentages were calculated from the means of three experiments for each group.
* $p < 0.05$ vs UVA + metabolites group.

Singlet oxygen and superoxide radical anion are involved in BaP metabolites plus UVA irradiation-induced 8-OHdG

Given that superoxide radical anion was involved in BaP plus UVA irradiation-induced 8-OHdG formation [29], we further determined if reactive oxygen species (ROS) were involved in 8-OHdG formation by BaP microsomal metabolites and UVA. Various ROS scavengers including sodium azide (NaN_3), catalase, mannitol, and superoxide radical anion dismutase (SOD), as well as deferroxamine, a ferric chelator, were utilized in the experiments (Table 1). Among these scavengers, NaN_3 showed the most potent inhibition against 8-OHdG generation in a concentration-dependent manner. At 5 mM NaN_3, 8-OHdG formation was inhibited by approximately 90%. The protein components of intact and denatured catalase may absorb UVA and decrease its oxidizing efficiency, thereby catalase per se has no scavenging effect (Table 1). In fact SOD at 50 units/ml decreased the 8-OHdG level to 83% compared with denatured SOD (Table 1), suggesting that superoxide radical anion plays a role in formation of 8-OHdG. Although mannitol was moderately effective in scavenging 8-OHdG, its effect was not concentration-dependent (data not shown). Failure of catalase and deferroxamine indicates the absence of the Fenton reaction, a well-known pathway for hydroxyl radical generation. Further experiments demonstrated that singlet oxygen and superoxide radical anion were generated in the reaction systems and they could be effectively scavenged by SOD and NaN_3 (Fig. 2). These results suggest that singlet oxygen plays a major role in BaP metabolites and UVA-induced oxidative DNA

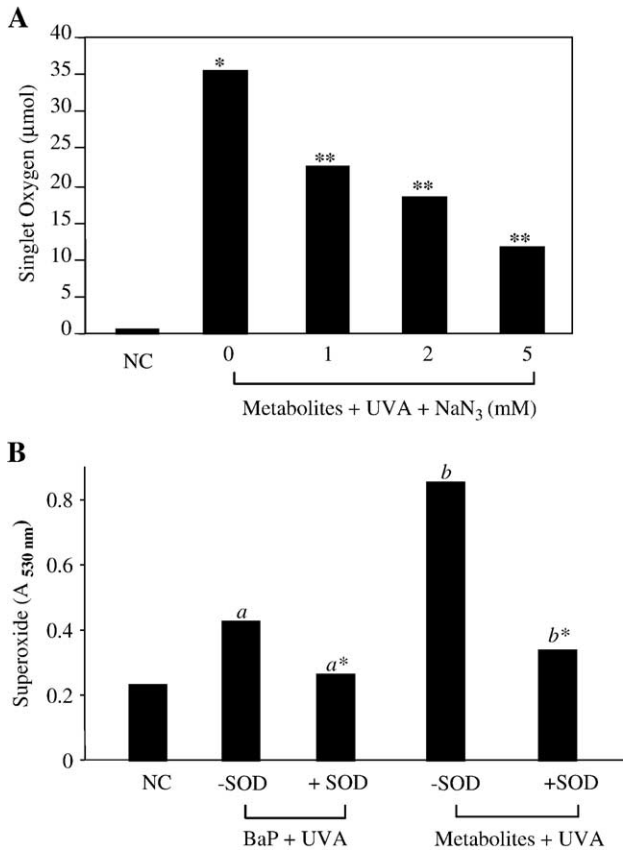


Fig. 2. Singlet oxygen and superoxide radical anion are generated by BaP microsomal metabolites and UVA radiation. Calf thymus DNA (0.2 mg/ml) was irradiated under UVA radiation (50 kJ/m^2) in the presence or absence of BaP (80 μM) and BaP metabolites (80 μM BaP equivalent) together with scavengers for (A) singlet oxygen and (B) superoxide radical anion. SOD concentration was 50 units/ml. BaP- and BaP metabolite-free groups served as negative control (NC). Each column represents the mean ± SD of three experiments. *Significant vs NC group; **significant vs BaP metabolites + UVA radiation group; [a]significant vs NC group; [a]*significant vs BaP + UVA radiation group; [b]significant vs NC group; [b]*significant vs BaP metabolites + UVA radiation group.

damage, whereas superoxide radical anion is also involved in this process.

BPDE is highly synergetic with UVA radiation in the induction of 8-OHdG

We then tested two important BaP metabolites, BPDE and BaP-7,8-dione. As shown in Fig. 3, BPDE at 80 μM plus 60 kJ/m^2 UVA irradiation substantially induced a higher level of 8-OHdG, by approximately 14-fold more than the parent BaP, whereas BaP-7,8-dione showed an effect similar to that of BaP. Further experiments indicated that BPDE induced 8-OHdG formation under 20 kJ/m^2 UVA radiation in a BPDE concentration-dependent manner (Fig. 4A). At as low a concentration as 0.5 μM, 8-OHdG induced by BPDE and UVA irradiation was 56 per 10^5 dG, and that was about 25-fold over BaP; when at 10 μM, the 8-OHdG increased to 118 per 10^5 dG (Fig. 4A). On the other

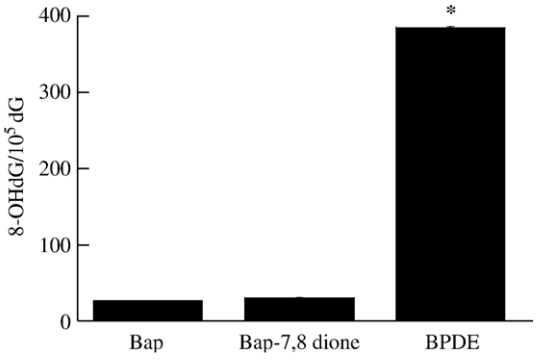


Fig. 3. BPDE is potently synergistic with UVA radiation in formation of 8-OHdG. Calf thymus DNA (0.2 mg/ml) was irradiated under UVA radiation (60 kJ/m^2) in the presence of BaP, BaP-7,8-dione, and BPDE (80 μM each). The content of 8-OHdG in BaP- and metabolite-free groups was taken as background and was subtracted from the values of the other groups. Each column represents the mean ± SD of three experiments. *Significant vs BaP and BaP-7,8-dione groups.

hand, increasing UVA doses also enhanced 8-OHdG formation in the presence of 10 μM BPDE. At 60 kJ/m^2 of UVA, 8-OHdG content was 254 per 10^5 dG (Fig. 4B). By contrast, BaP up to 10 μM under the same UVA irradiation showed almost no effect. These results provide solid evidence that BPDE has a much more potent UVA synergetic capacity in oxidizing DNA bases than its parent BaP.

Singlet oxygen is involved in 8-OHdG formation by BPDE and UVA

Because singlet oxygen was involved in 8-OHdG formation by BaP microsomal metabolites and UVA irradiation (Table 1 and Fig. 2), its role in the BPDE plus UVA irradiation reaction system was further examined. As shown in Figs. 5A and 5B, singlet oxygen generation was greatly increased by BPDE and UVA irradiation combination and suppressed substantially by 1 mM NaN_3. Furthermore, NaN_3 inhibited 8-OHdG formation induced by

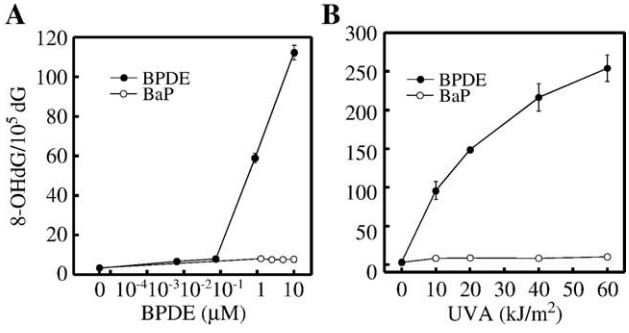


Fig. 4. BPDE synergizes with UVA radiation and generates 8-OHdG in calf thymus DNA. (A) Calf thymus DNA (0.2 mg/ml) was incubated with various concentrations of BPDE and then exposed to UVA radiation at 20 kJ/m^2 or (B) incubated with BPDE at 10 μM and exposed to various doses of UVA. 8-OHdG was measured by HPLC. Each point represents the mean ± SD of three experiments.

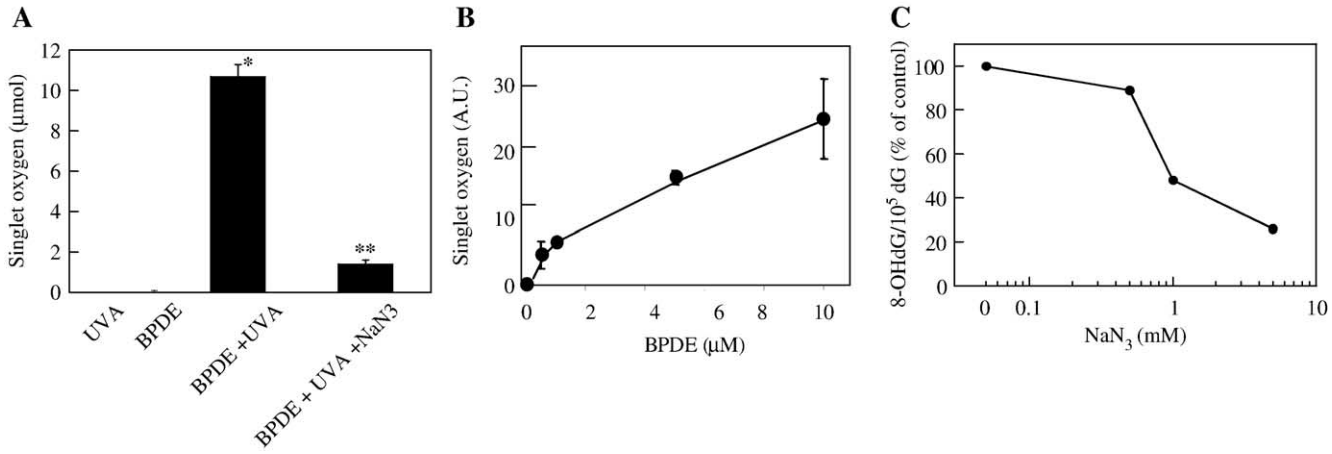


Fig. 5. Singlet oxygen contributes to BPDE and UVA radiation-induced 8-OHdG formation. Singlet oxygen formation was detected by (A) RNO bleaching and (B) TMPD turnover. (C) The effect of NaN_3 on 8-OHdG formation was also examined. For RNO bleaching, 80 μM BPDE was added to calf thymus DNA (0.2 mg/ml) and then exposed to UVA radiation (50 kJ/m^2) in the presence or absence of various concentrations of NaN_3. For TMPD turnover, various concentrations of BPDE were added to phosphate buffer containing TMPD (40 mM) immediately before UVA radiation (20 kJ/m^2) exposure. 8-OHdG was generated by irradiating 10 μM BPDE with 20 kJ/m^2 of UVA radiation. Each point (column) represents the mean ± SD of three experiments. *Significant vs UVA radiation and BPDE groups; **significant vs BPDE + UVA radiation group.

BPDE and UVA in a concentration-dependent manner (Fig. 5C). Other ROS scavengers such as catalase, SOD, and mannitol did not show any inhibitory effect on BPDE–UVA-induced 8-OHdG formation (data not shown). These results indicate that singlet oxygen is the major ROS that contributes to the BPDE–UVA-induced 8-OHdG formation.

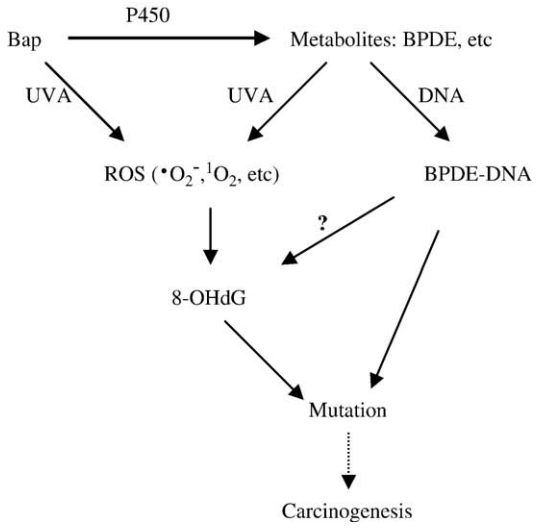
Discussion

The present study demonstrated that BaP microsomal metabolites, especially its major ultimate metabolite BPDE, potently synergize with UVA radiation to induce 8-OHdG formation in mammalian genomic DNA. This study corroborated our previous finding that BaP substantially enhanced the formation of 8-OHdG induced by UVA radiation in both calf thymus DNA and cultured cells and that the highest level of 8-OHdG was observed in cultured cells, suggesting an involvement of active BaP metabolites.

It is well known that BaP becomes carcinogenic only after metabolic activation [42]. BaP is initially metabolized to BaP-7,8-dihyrodiol, which then further undergoes epoxidation to yield BPDE [43]. BPDE is considered the "ultimate" carcinogenic metabolite of BaP [44,45]. It is well recognized that BPDE can covalently bind with DNA, leading to genetic toxicity [46]. On the other hand, BaP-7,8-dione causes DNA strand scission in the presence of $CuCl_2$ and NADPH, and this strand scission was inhibited by catalase and hydroxyl radical scavengers [47].

Thus we first showed that BaP microsomal metabolites synergized with UVA radiation to induce a 3-fold higher level of 8-OHdG than the parent BaP on the same molar basis (Fig. 1). We therefore selected two specific metabolites, BPDE and BaP-7,8-dione, to evaluate their synergetic effect with UVA radiation on the induction of 8-OHdG formation. BPDE exhibited an extremely high potency to induce 8-OHdG under UVA radiation in both BPDE concentration- and UVA dose-dependent manners (Fig. 4). At 0.5 μM, BPDE plus UVA radiation increased 8-OHdG formation 25-fold compared to BaP plus UVA, whereas BaP-7,8-dione showed an effect similar to that of the parent BaP (Fig. 3). These results clearly demonstrate that BaP metabolites, particularly the ultimate BPDE, more potently induce oxidative DNA damage in the presence of UVA light than the parent compound.

Singlet oxygen was found to be a major intermediate agent in the induction of 8-OHdG (Table 1 and Fig. 2). The specific singlet oxygen quencher NaN_3 at 5 mM almost completely abrogated the 8-OHdG formation in the reaction system, indicating a major contribution of this ROS to oxidative DNA damage (Table 1). On the other hand,



Scheme 1. Proposed mechanism for BaP-induced carcinogenesis.

superoxide radical anion also played a lesser role in the process, although its contribution accounted for only 20% inhibition of 8-OHdG formation (Table 1). In fact, NaN_3 is the only agent that effectively inhibits BPDE plus UVA radiation-induced 8-OHdG formation, whereas SOD and other scavengers did not show any inhibition. These findings suggest that BPDE plus UVA radiation primarily produced singlet oxygen, which in turn induced 8-OHdG formation.

We previously observed that superoxide radical anion contributed to 8-OHdG formation by BaP and UVA irradiation [36]. Because the BaP microsomal metabolites contained the BaP parent and other unknown metabolites, superoxide radical anion formed in BaP microsomal metabolites could be due to the residue of the parent compound and other related metabolites that produced superoxide radical anion upon UVA irradiation [36]. There are other pathways such as interaction between photosensitizer molecules followed by one-electron transfer to dioxygen [48,49]. Although singlet oxygen is well known to oxidize the guanine base of DNA [22,50], the mechanism by which BPDE and UVA radiation produce singlet oxygen is currently unclear. UV irradiation in the presence of certain photosensitizers is known to initiate the following two types of reactions [48,49]: type I includes redox reactions of excited photosensitizers with certain substrates or with ground state sensitizers, and type II involves energy transfer reactions of excited sensitizers to dioxygen to produce singlet oxygen. One possible explanation is that BPDE serves as a photosensitizer and undergoes the type II process to produce singlet oxygen.

Monomeric products in DNA exposed to γ-ray and UV irradiation contain many other forms in addition to 8-OHdG. Dizdaroglu's group detected 13 products resulting from pyrimidines and purines in γ-ray-irradiated DNA, including 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), and 8-OHdG, whereas only FapyGua and 8-OHdG were detected in visible light and methylene blue-treated DNA [24]. Results from the same group revealed that UV irradiation at 254 nm produced FapyAde, FapyGua, and 5-hydroxy-5,6-dihydrothymine, whereas at 310 nm only FapyAde was produced in appreciable amounts [25]. The serial studies from this group [23,26,27] indicate that formamidopyrimidines are important DNA-damage markers in addition to 8-OHdG. Investigation of the formation of formamidopyrimidines by BaP and UVA irradiation may add more information to the profile of BaP–UVA-induced DNA damage and help to clarify the mechanism.

The present study provides solid evidence that BaP and its active metabolites, represented by BPDE, combined with UVA radiation can substantially augment oxidative DNA damage via a ROS mechanism. Considering the amount of BaP released to the environment [1] and more UV exposure by ozone layer depletion, it is reasonable to speculate that BaP metabolites may synergize with UVA radiation to amplify the genetic damage that consequently leads to carcinogenesis (Scheme 1). Further studies are needed to elucidate the underlying mechanism by which BaP metabolites and UVA radiation induce oxidative DNA damage and the possible relationship between BaP metabolite-UVA exposure and carcinogenesis both in vitro and in vivo.

Acknowledgments

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