# Benzo[*a*]pyrene Enhances the Formation of 8-Hydroxy-2′-deoxyguanosine by Ultraviolet A Radiation in Calf Thymus DNA and Human Epidermoid Carcinoma Cells†

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ABSTRACT: The objective of this study is to investigate if benzo[*a*]pyrene (BaP) and ultraviolet (UV) radiation synergistically induce oxidative DNA damage. Calf thymus DNA was incubated with BaP and irradiated with UVB (280−320 nm) and UVA (335−400 nm). BaP substantially enhanced the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) by UVA, but only moderately increased the level of 8-OHdG by UVB. Formation of 8-OHdG proportionally correlated with both UV dose and BaP concentration. Human epidermoid carcinoma cells were incubated with 10 $\mu$g of BaP/mL for 24 h and then exposed to 10 kJ/m$^2$ UVB and 25 kJ/m$^2$ UVA. UVB plus BaP did not affect the level of 8-OHdG in cultured cells, whereas UVA plus BaP substantially increased 8-OHdG by over 4-fold compared to BaP and UVA controls. To confirm what reactive oxygen species (ROS) are involved in BaP plus UVA-induced oxidative DNA damage, less or more specific ROS quenchers were added to DNA solution. The results showed that only superoxide dismutase and genistein significantly quenched BaP plus UVA-induced 8-OHdG, whereas catalase, sodium azide, and mannitol exhibited no effect. Our studies suggest that BaP enhances the formation of 8-OHdG in purified DNA and cultured cells by UVA, but not by UVB, and that superoxide anion plays an important role in the synergistic induction of oxidative DNA damage.

Benzo[*a*]pyrene (BaP) is one of the polycyclic aromatic hydrocarbons widely present in the environment. BaP itself is considered to be relatively nontoxic; however, it becomes carcinogenic after metabolic activation (*1*). The mechanisms of activation of BaP have been well demonstrated (*2*, *3*). The ultimate carcinogenic forms of BaP are (±)-*trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene (*2*). However, during metabolic processes, BaP also produces diones: BaP-6,12-dione, BaP-3,6-dione, and BaP-1,6-dione (*3*). If the reactive intermediates are not detoxified in a timely manner, they may interact with and oxidize macromolecules such as DNA, lipids, and proteins (*4*, *5*). Metabolically activated BaP binds covalently with DNA and nucleic acid components both in vivo and in vitro (*3*). It was reported that irradiation of BaP by near-ultraviolet (UV) light initiated covalent binding with DNA molecules in vitro, although the mechanism remained skeptical (*6*). In vivo, it is not clear whether BaP photoinduced modifications of DNA (*7*, *8*). It was proposed that BaP may behave as a photosensitizer that generates both superoxide anion and singlet oxygen in cells (*9*).

UV radiation of DNA has been known to induce a variety of photoproducts, some of which are associated with photocarcinogenesis (*10*, *11*). Although the mechanisms involved in photocarcinogenesis have not been completely elucidated, increasing evidence suggests that the DNA damage induced by solar UV radiation plays an important role in the formation of skin cancers (*12*). In addition to the DNA alterations, UV radiation also plays a role in phototoxicity reactions such as erythema, hyperplasia, and sunburn cell formation (*13*−*15*). DNA damage may initiate the events associated with the immunosuppressive response caused by UV, which also contributes to the development of skin cancers (*16*). Many studies on the effects of UV irradiation have focused on the formation of cyclobutyl pyrimidine dimers and [6−4] cyclobutyl photoproducts, the principal DNA photoproducts induced by UV irradiation (*11*). Nonpyrimidine dimer damage has also bee reported, including single- or double-strand DNA breaks, protein−DNA, and DNA−DNA cross-links, sister chromatic exchange, and the formation of micronucleus (*11*). In recent years, evidence has accumulated that light and UV radiation are capable of inducing oxidative DNA damage through a different mechanism than the induction of pyrimidine dimers and other photoproducts (*17*−*21*).

The reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($HO^{\bullet}$), and singlet oxygen ($^1O_2$) are the byproducts of normal metabolic processes in cells, but can be augmented after exposure to UV radiation, carcinogens, etc. These ROS are

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highly reactive and capable of interacting with many biological macromolecules. One of the important consequences is to cause oxidative modification of DNA bases. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) represents a major form of oxidative DNA damage exposed to ROS and is known to play important roles in various degenerative disorders such as cancer and aging (*22*, *23*). Chemical carcinogens and tumor-promoting agents have been shown to induce the formation of 8-OHdG in various target tissues (*24*, *27*) and the increased levels of 8-OHdG were also observed in cancerous tissues (*28*, *29*). Recently, we have reported that all portions of the UV spectrum (UVA, B, and C) are capable of inducing the 8-OHdG formation (*30*). The level of 8-OHdG was reported to be elevated in BaP-treated Syrian hamster embryo and human mammary carcinoma cells in the presence of white fluorescent light (*31*).

However, whether BaP affects the level of 8-OHdG in purified DNA and cultured cells exposed to specific UV portions and what ROS are involved in oxidative DNA damage by BaP plus UVR remain completely unknown. The objective of the study is to investigate if BaP synergistically enhances oxidation of DNA bases in purified DNA and cultured cells irradiated with specific UV light and characterize the ROS involved in BaP−UVR enhanced oxidative DNA damage.

## EXPERIMENTAL PROCEDURE

*Chemicals and Reagents.* Calf thymus DNA, catalase, SOD, sodium azide, mannitol, dimethyl sulfoxide (DMSO), and BaP were purchased from Sigma Chemical Co. (St. Louis, MO). Purity of calf thymus DNA was determined by measuring ratio of 260 nm/280 nm. Nuclease P1, alkaline phosphates, proteinase K, and RNase A were obtained from Boehringer Mannheim (Indianapolis, IN). $CO_2$-independent medium, phosphate-buffered saline (PBS), penicillin, and streptomycin were from GIBCO (Long Island, NY). Genistein was obtained from Alexis Co. (San Diego, CA). 8-OHdG standard was prepared as previously described (*26*).

*Cell Cultures*. The human epidermoid carcinoma cell (A431) was a gift from Dr. Luhai Wang in the Department of Microbiology at Mount Sinai School of Medicine. Cells (approximately $3 \times 10^6$) were plated into 75 cm$^2$ flask and cultured in $CO_2$-independent medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/mL), and streptomycin (100 $\mu$g/mL) for 24 h to allow cell attachment. Different concentrations of BaP in DMSO were added to the culture medium with the final DMSO concentration of 0.1%. Cells continued to be incubated for 24 h. Prior to UV irradiation, cells were washed twice with PBS. The cell viability was examined by Trypan blue and was generally >98%.

*UV Irradiations.* For the UVB and UVA irradiations, cells in 75 cm$^2$ flasks (Corning) were overlaid with 15 mL of freshly prepared PBS and exposed to either the UVB produced by two Westinghouse FS40 Sunlamps filtered through the polystyrene filter to remove wavelength shorter than 290 nm or UVA produced by four Cosmolux Al-11 lamps passing through Schott WG 335 filters to remove wavelength shorter than 335 nm as described previously (*30*, *32*). For the calf thymus DNA irradiations, 2 mL of DNA solution (200 $\mu$g/mL) in double-distilled $H_2O$ was placed in six-well plate (Corning, NY), in the presence or absence of BaP or scavengers at the defined concentrations, and exposed to UV as described above. The UV fluence used in the experiments was 1, 10, and 25 kJ/m$^2$ for UVB and 1, 10, 25, and 50 kJ/m$^2$ for UVA. The distance between UV source and the surface of DNA solution or culture cells was approximately 15 cm. The UV fluence was quantified by a model IL-1700 Research Radiometer (International Light, Newburgport, MA).

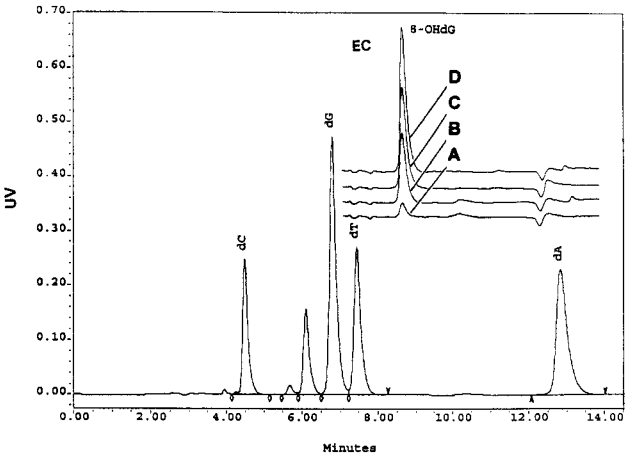


FIGURE 1: HPLC/UV-EC profiles of 8-OHdG with EC detection (upper trace) and optical detection (lower trace) from calf thymus DNA. (A): The background level of 8-OHdG in calf thymus DNA; (B) 6 pmol of 8-OHdG standard; (C) UVA-irradiated DNA in the presence of BaP; (D) 8-OHdG added to UV-irradiated DNA (panel B plus C).

*DNA Preparation Isolation from UV-Irradiated Cells.* Preparation of UV-irradiated calf thymus DNA was performed as previously described (*30*, *33*, *34*).

*Analyses of 8-OHdG and 2′-Deoxyguanosine (dG).* The amount of 8-OHdG in DNA was determined using an HPLC system (Waters, Milford, MA) coupled with an electrochemical detector as described by Floyd and co-workers (*35*) with a slight modification (*33*, *34*). The DNA hydrolysates were injected into a reversed-phase C18 5U column (250 mm × 4.6 mm, Alltech Association, Deerfield, IL) eluted with 125 mM citric acid, 250 mM sodium acetate, 100 mM acetic acid, and 15% methanol at a flow rate of 1 mL/min. 8-OHdG was quantitated by an EG & G model 400 electrochemical detector with an LC analytical cell (EG & G Instrument Co., Princeton, NJ) running in oxidative mode and dG quantitated by a Waters 486 absorbance detector (Milford, MA) at 254 nm. The molar ratio of 8-OHdG to dG was calculated based on the integrated peak area of authentic 8-OHdG with an electrochemical detector and UV absorbance of dG using a Millennium software (Waters, Milford, MA). The results were expressed as the number of 8-OHdG per $10^5$ dG.

*Statistical Analyses.* Dose−response curves were generated from at least two separate experiments. Data were compared between treatment groups using a two-tail student's test with $p < 0.05$ being statistically significant.

## RESULTS

*Effect of BaP on UV-Induced 8-OHdG in Calf Thymus DNA.* The typical elution profiles for calf thymus DNA are illustrated in Figure 1. The upper trace represents EC

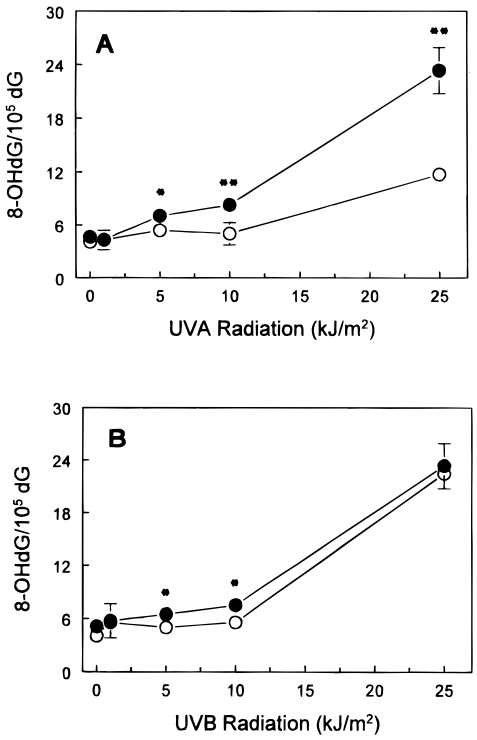


FIGURE 2: Effect of BaP on the formation of 8-OHdG in calf thymus DNA by UVA and UVB. DNA was exposed to UV in the presence or absence of BaP (10 $\mu$g/mL). The data are a representative of two experiments with each assay performed in triplicate. The results are expressed as mean ± SD. (A) UVA irradiation and (B) UVB irradiation. (○) In the absence of BaP; (●) in the presence of BaP. Statistical analysis was conducted using two-tailed Student's test: (*) $p < 0.05$ and (**) $p < 0.01$ compared to that in the absence of BaP.

detection of 8-OHdG and the lower trace, the optical detection of normal nucleosides. Under the established conditions, 8-OHdG and other normal nucleosides were well separated. In EC detection, 8-OHdG elutes at ~8 min, immediately trailing the deoxythymidine peak in UV profile. The elution time for 8-OHdG was verified by spiking DNA digests with purified standard compound. The background level of 8-OHdG in unexposed calf thymus DNA without BaP was 2.7 per $10^5$ dG. There was no significant change in 8-OHdG level (2.5 per $10^5$ dG) in purified DNA treated with 10 $\mu$g/mL BaP in the absence of UV when compared to the background level of 8-OHdG.

Irradiation of calf thymus DNA with UVA or UVB in the present of BaP resulted in a different induction of 8-OHdG level (Figure 2). With increasing UVA doses from 1 to 25 kJ/m$^2$, the number of 8-OHdG was proportionately increased from 3.5 to 11.1 per $10^5$ dG in the absence of BaP and substantially elevated from 4.3 to 22.6 per $10^5$ dG in the presence of BaP. With exposure to UVB from 1 to 25 kJ/m$^2$, the number of 8-OHdG was increased from 5.6 to 22.4 per $10^5$ dG in the absence of BaP and from 7.3 to 23.3 per $10^5$ dG in the presence of BaP. The striking difference in BaP-treated DNA between UVA and UVB irradiation was observed (Figure 2).

Net effect of BaP enhancing UV-induced 8-OHdG formation (subtraction of the 8-OHdG level induced by UV irradiation alone) is shown in Figure 3. BaP showed a greater net potentiation on the level of 8-OHdG by UVA in

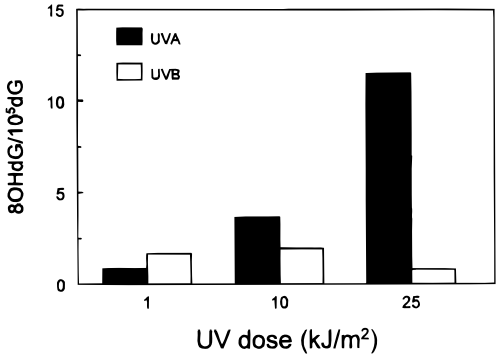


FIGURE 3: Net effect of BaP on the formation of 8-OHdG in purified DNA by UVA and UVB radiation. The data are from at least three experiments with each assay performed in triplicate. Net effect was calculated by subtracting number of 8-OHdG by UV alone from that induced by UV + BaP.

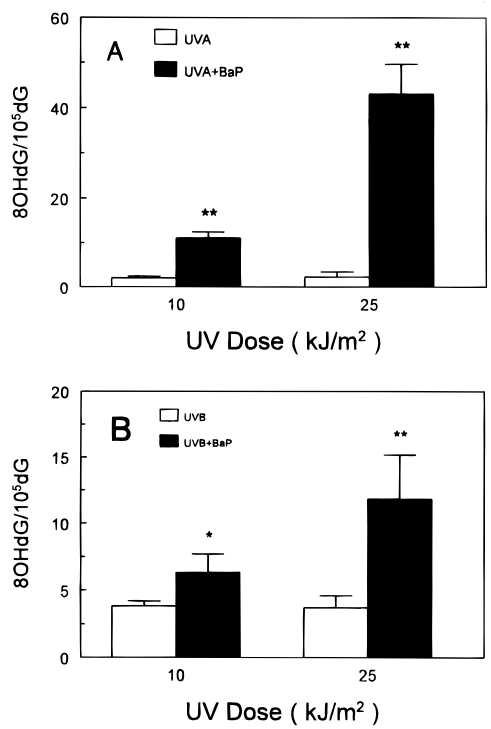


FIGURE 4: Effect of BaP on the formation of 8-OHdG in human epidermoid carcinoma cells by UVA and UVB. Values are mean ± SD of three to four experiments with each assay performed in triplicate. Statistical analysis was conducted using two-tailed Student's test: (*) $p < 0.05$ and (**) $p < 0.01$ versus the absence of BaP.

a fluence-dependent fashion, but not on that by UVB. At a UVA dose of 25 kJ/m$^2$, the levels of 8-OHdG in DNA treated with BaP was approximately 2-fold higher than that by UVA radiation alone. Thus, BaP synergistically enhanced the formation of 8-OHdG by UVA. In contrast, BaP slightly increased the level of 8-OHDG at low dose compared to UVB alone. At a UVB dose of 25 kJ/m$^2$, the net change of 8-OHdG was almost equal to UVB alone (Figure 2). So, it is the UVB, not BaP, primarily contributed to the induction of 8-OHdG in purified DNA.

*Effect of BaP on UVR-Induced 8-OHdG in Human Epidermoid Carcinoma Cells.* The effect of BaP on UVA- and UVB-induced 8-OHdG formation in cultured cells is shown in Figure 4. Upon irradiation of cells with UVA 25

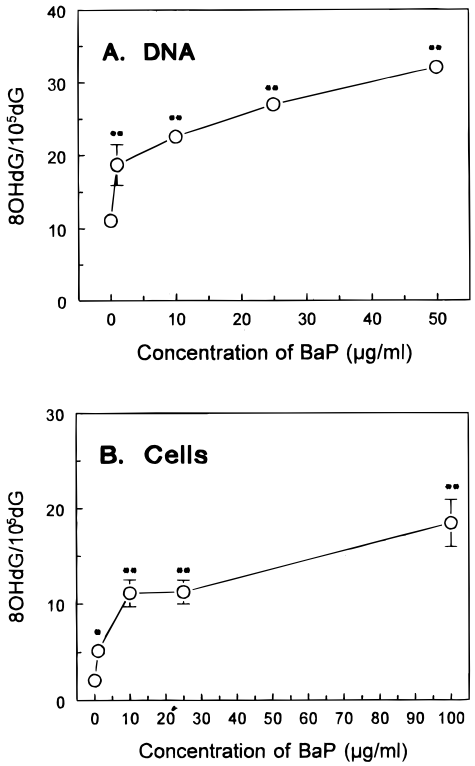


FIGURE 5: Effect of concentrations of BaP on UVA-induced 8-OHdG formation in calf thymus DNA (A) and cultured cells (B). Values are average ± SD of three to four experiments with each assay performed in triplicate. Statistical analysis was conducted using two-tailed Student's test: (*) $p < 0.05$ and (**) $p < 0.01$ versus the absence of BaP.

kJ/m$^2$, the level of 8-OHdG/10$^5$ dG in A431 cells was 2.2 in the absence of BaP and 43.0 in the presence of BaP, resulting in approximately 20-fold increase in relation to vehicle-treated cells. The absolute level of 8-OHdG in cultured cells was about 2-fold higher than that in purified DNA (43.0 versus 22.6). In contrast, UVB only moderately increased the level of 8-OHdG in BaP-treated cells. At a UVB dose of 25 kJ/m$^2$, 8-OHdG/10$^5$ dG was 3.7 in the absence of BaP and 11.8 in the presence of BaP, respectively. The level of 8-OHdG in cultured cells was much lower than that in purified DNA (11.8 versus 23.3) at the same UVB fluence.

*Effect of BaP Concentration on UVA-Induced 8-OHdG in Purified DNA and Cultured Cells.* As shown in Figure 5, BaP not only significantly increased the level of 8-OHdG in purified DNA within the concentration range of 1 to 50 $\mu$g/mL (Figure 5A) but also enhanced the formation of 8-OHdG in UVA-irradiated cells (Figure 5B). However, the effect of BaP on 8-OHdG formation in cultured cells began to plateau at concentration of 10 $\mu$g/mL (see Figure 5B).

*Effect of ROS Quenchers on BaP in UVA-Induced 8-OHdG in Purified DNA.* Less or more specific ROS quenchers were selected to determine the possible oxygen free radicals involved in BaP plus UVA-induced 8-OHdG formation. As shown in Table 1, SOD and genistein significantly quenched UVA plus BaP-induced 8-OHdG by 56 and 66%, respectively ($p < 0.01$). Catalase, sodium azide, and mannitol did not affect 8-OHdG formation induced by BaP plus UVA.

Table 1: Effect of ROS Quenchers on BaP Plus UVR-Induced 8-OHdG in Purified DNA[a]

| treatment | ROS quenched | 8-OHdG/10$^5$ dG $\bar{x} \pm$ SD | net effect (%)[b] |
|---|---|---|---|
| UVA − BaP | | 11.7 ± 1.94 | |
| UVA + BaP | | 23.4 ± 2.07[c] | |
| UVA + BaP + quenchers | | | |
| catalase (50 U/mL) | $H_2O_2$ | 23.1 ± 6.10 | 97 |
| mannitol (10 mm) | OH$^\bullet$ | 22.9 ± 1.41 | 96 |
| $NaN_3$ (1 mM) | $^1O_2$ | 21.7 ± 1.61 | 85 |
| genistein (10 $\mu$M) | General ROS | 15.5 ± 0.93[d] | 32 |
| SOD (50 U/mL) | $O_2^{\bullet-}$ | 13.1 ± 1.41[d] | 13 |

[a] DNA treated with BaP (10 $\mu$g/mL) and then exposed to UVA at 25 kJ/m$^2$. Different quenchers were added and 8-OHdG was analyzed using HPLC-EC detection. Results are from three measurements. [b] Net effect: [(UVA + BaP + quencher) − (UVA − BaP)/(UVA + BaP) − (UVA − BaP)] × 100. [c] $p < 0.01$, versus UVA − BaP. [d] $p < 0.01$, versus UVR + BaP in the absence of quenchers.

## DISCUSSION

The major finding in the present study is that BaP substantially enhanced the formation of 8-OHdG in purified DNA and cultured cells irradiated by UVA, but not by UVB. We have previously demonstrated that all three UV portions induce the formation of 8-OHdG in purified calf thymus DNA and cultured cells (*30*). For instance, 25 kJ/m$^2$ of UVA and B can increase 8-OHdG from the basal level to ~10 and ~20 per 10$^5$ dG, respectively. In current study, the same dose of UVA substantially increased the level of 8-OHdG in purified DNA up to ~23 per 10$^5$ dG in the presence of BaP. Thus, the level of 8-OHdG in BaP-treated DNA was increased by ~2-fold as compared to UVA irradiation alone at dose of 25 kJ/m$^2$. In contrast, BaP almost did not affect UVB-induced 8-OHdG in calf thymus DNA at a UV dose of 25 kJ/m$^2$. We believe that the mechanism by which induction of 8-OHdG by UVA in the presence of BaP is quite different from that by UVB. It is well accepted that BaP requires metabolic activation to become carcinogenic, and ROS can be generated during oxidative metabolism which is capable of inducing 8-OHdG in target tissues (*36*). In present study, BaP was incubated with purified DNA in cell-free system, BaP still enhanced UVA-induced formation of 8-OHdG, which suggests that the metabolic precess is not implicated. In UVB ranges, BaP appeared to contribute little to the redox process, as demonstrated in Figure 3. BaP moderately enhanced UVB-induced 8-OHdG at low doses of 5 and 10 kJ/m$^2$ ($p < 0.05$), respectively. When irradiation at high UV dose, BaP had no effect on UVB-induced 8-OHdG. It is evident that two types of photoreactions are implicated and different UV spectrum portions. In the scenario of UVB irradiation, type I photoreaction plays a predominant role since DNA per se can serve as a chromophore leading to the overlapping absorption spectra. Upon excitation by UVB, DNA molecules directly abstract hydrogen atom and produce oxygen-free radicals, thereby initiating an autooxidation of its own bases. In this scenario, BaP contributes little to oxidation of DNA bases upon UVB irradiation. In the scenario of UVA irradiation, type II photoreaction is predominantly involved since DNA is not chromophore within UVA spectra. BaP may serve as a photosensitizer for UVA, which involves a direct energy-

transfer reaction between triplet states of excited BaP and ground-state oxygen, producing highly reactive ROS that react with and oxidize DNA bases.

In cell culture, BaP presents a different reaction pattern than in cell-free systems. The level of 8-OHdG in BaP-pretreated cells was increased by 19-fold upon UVA irradiation (Figure 4), which was much higher than that of purified DNA (2-folds) under the same UVA fluence. We propose several possible explanations for this phenomenon. (1) Cells have an antioxidant defense system that protects cellular macromolecules against endogenous and exogenous oxidants. For instance, the level of 8-OHdG in cultured cells is much lower than that in purified DNA (2.2 vs 11.3 per $10^5$ dG) upon UVA irradiation at a dose of 25 kJ/m$^2$. Therefore, net effect of UVA per se on 8-OHdG was completely eliminated. (2) Metabolites of BaP, such as in cell culture, may offer more potent and wide-spectrum of sensitizers. (3) BaP sensitizes cells to produce more ROS, such as $H_2O_2$, upon UVA irradiation. We have demonstrated that BaP significantly potentiated A431 cells and human keratinocytes to produce huge amounts of $H_2O_2$ upon UVA irradiation (manuscript in preparation). These excess ROS, in the presence of transition metal, potentiate oxidation of DNA bases.

We have previously demonstrated that all three portions of UV (UVA, B, and C) can oxidize DNA bases primarily through generation of $^1O_2$ (*33*, *34*). The question raised here is what ROS are involved in BaP plus UVA-induced oxidation of DNA. In the present study, we have characterized the possible ROS involved in BaP plus UV-induced 8-OHdG production by selecting a set of ROS quenchers including sodium azide, mannitol, SOD, catalase, and genistein. To avoid the effects of endogenous cellular scavengers (such as catalase, SOD, ascorbic acid, and glutathione, etc.) and photosensitizers (including hematoporphyrin, NADP, NADPH, bilirubin, riboflavin, etc.), a cell-free system containing calf thymus DNA was employed. The results showed that only SOD and genistein reduced the level of 8-OHdG by UVA in the presence of BaP. ROS involved in BaP plus UVA-induced 8-OHdG are different from ROS generated by UVR alone in which $^1O_2$ is the principal ROS (*34*). In the present study, sodium azide, which is considered to be a relatively specific $^1O_2$ quencher (*37*), exhibited no effect on the formation of 8-OHdG in purified DNA by UVA plus BaP, nor did mannitol that primarily quenched HO$^\bullet$ and catalase that specifically quenches $H_2O_2$. The results from this experiment implies that $H_2O_2$, $^1O_2$, and OH$^\bullet$ play a lesser role, if there are any, in 8-OHdG production by UVA plus BaP. In contrast, SOD significantly quenched BaP plus UVA-induced 8-OHdG formation by 87% as compared to the UVA-BaP control. The reaction with dG to form 8-OHdG is proposed to be driven by the Haber-Weiss or superoxide-generating superoxide anion through its interaction with the photosensitizers (*36*, *38*). Genistein is a general quencher that scavenges $O_2^-$ more potently than $H_2O_2$ (*39*, *40*). Thus, quenching of BaP plus UVA-induced 8-OHdG by genistein probably attributed to its capacity to scavenge $O_2^-$.

We also conducted studies with scavengers in cultured cells. Specific scavengers such as catalase and SOD have a large molecular weight and cannot pass across the cell membrane. Thus, experiments with catalase and SOD were not warranted. Sodium azide, a more or less quencher of singlet, was very toxic, and all cells died at a concentration of 0.1 mM. Therefore, we were only able to evaluate the effect of mannitol, genistein, and *N*-acetyl-cysteine. The results showed that genistein and *N*-acetyl cysteine partially inhibited the formation of 8-OHdG treated with BaP-UVA, whereas mannitol had no effect (data not shown).

In summary, we demonstrated in the present study that BaP and UVA synergistically induced the formation in both purified DNA and cultured cells. In contrast, BaP moderately enhanced UVB-induced oxidative DNA damage. The principal ROS involved is $O_2^{\bullet-}$, evidenced by the fact that SOD and genistein significantly quenched UVA-induced 8-OHdG in purified DNA in the presence of BaP. The novel finding of this study will provide a better understanding of mechanistic roles of BaP on phototoxicity and photocarcinogenesis.

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