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Coexposure to benzo[*a*]pyrene and UVA induces phosphorylation of histone H2AX

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Abstract Phosphorylation of histone H2AX (termed γ-H2AX) was recently identified as an early event after induction of DNA double strand breaks (DSBs). We have previously shown that coexposure to benzo[*a*]pyrene (BaP), a wide-spread environmental carcinogen, and ultraviolet A (UVA), a major component of solar UV radiation, induced DSBs in mammalian cells. In the present study, we examined whether coexposure to BaP and UVA generates γ-H2AX in CHO-K1 cells. Single treatment with BaP (10^{-9}–10^{-7} M) or UVA (~2.4 J/cm^2) did not result in γ-H2AX, however, coexposure drastically induced foci of γ-H2AX in a dose-dependent manner. γ-H2AX could be detected even at very low concentration of BaP (10^{-9} M) plus UVA (0.6 J/cm^2), which did not change cell survival rates. NaN_3 effectively inhibited the formation of γ-H2AX induced by coexposure, indicating the contribution of singlet oxygen. This is the first evidence that coexposure to BaP and UVA induced DSBs, involving γ-H2AX.

Keywords: Histone H2AX; Benzo[*a*]pyrene; UVA; Polycyclic aromatic hydrocarbons

1. Introduction

Exposure to solar ultraviolet (UV) radiation is unambiguously associated with skin cancer [1,2]. UVB (280–320 nm) is directly absorbed by DNA and induces damage such as cyclobutane pyrimidine dimers and (6-4) photoproducts [3,4], contributing to carcinogenicity. On the other hand, UVA (320–400 nm), the major component of solar UV radiation, is considered less carcinogenic than UVB because DNA absorption is extremely weak. In recent years, however, it has been reported that UVA also induces various forms of DNA damage in the presence of endogenous or exogenous photosensitizers [4,5].

Polycyclic aromatic hydrocarbons (PAHs), wide-spread environmental carcinogens, are suspected of being exogenous photosensitizers [6]. The metabolic products of PAHs, such as diol-epoxides and diones, are recognized as highly carcinogenic, forming covalent DNA adducts and oxidative DNA lesions [7]. However, the potential risk of PAHs combined with UVA irradiation has not yet been elucidated. The cytotoxicity due to coexposure to PAHs and UVA was much greater than that caused by exposure to PAHs alone [8–10]. Several groups have demonstrated that coexposure to PAHs and UVA significantly augmented DNA damage, such as single strand breaks [11,12] and the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine [13–15]. In addition, we recently showed that coexposure to the most commonly studied PAH, benzo[*a*]pyrene (BaP), and UVA induced DNA double strand breaks (DSBs) [16,17]. Among the many forms of DNA damage, DSBs are considered the most serious threat to the cell, and when not repaired or misrepaired, can result in mutations or chromosomal rearrangements and eventually cell death [18].

Phosphorylation of histone H2AX (termed γ-H2AX) has been recently identified as an early event after induction of DSBs [19]. Within minutes after the introduction of a DSB, several thousand of H2AX near the site of the DSB are phosphorylated on serine 139, producing foci within the nucleus that are microscopically visible by immunofluorescence staining [19–21]. There is a close correlation between the number of γ-H2AX foci and the expected number of DSBs induced by ionizing radiation [22]. Although the induction of γ-H2AX by general DSB inducers such as ionizing radiation and anti-cancer drugs has been well established, there are no reports on the induction of γ-H2AX by environmental carcinogens. We expected BaP under UVA irradiation to induce phosphorylation of H2AX because coexposure to BaP plus UVA was shown to induce DSBs in our previous study [16,17].

In this study, we successfully detected γ-H2AX after exposure to BaP plus UVA. The generation of γ-H2AX was achieved at very low doses of BaP and UVA, near actual environmental concentrations, indicating the potential risk of PAHs under UVA irradiation.

2. Materials and methods

2.1. Exposure to BaP and UVA

Chinese hamster ovary (CHO)-K1 cells were treated with BaP for 1 h and subsequently irradiated with several doses of UVA. In the experiment using a scavenger of singlet oxygen ($^1O_2$), sodium azide (NaN_3) was added 15 min before the UVA irradiation and the cells were irradiated in the presence of NaN_3. The conditions for UVA irradiation were as described previously [16,17].

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Abbreviations: BaP, benzo[*a*]pyrene; BSFGE, biased sinusoidal field gel electrophoresis; CHO, Chinese hamster ovary; DSBs, double strand breaks; PAHs, polycyclic aromatic hydrocarbons; $^1O_2$, singlet oxygen

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2.2. Colony-formation assay

Cells treated with BaP and/or UVA were trypsinized immediately after UVA exposure, suspended at a density of 1×10^5 cells/ml, and then plated at 1×10^2–10^4 cells/60-mm dish. After incubation for 7 days, the colonies of cells were fixed with methanol and stained with Giemsa. The number of colonies containing about >50 cells was counted.

2.3. Detection of DSBs

DSBs were detected using a biased sinusoidal field gel electrophoresis (BSFGE) system (Atto Co., Japan) as described previously [16,17]. In brief, cells treated with BaP and UVA were suspended in 1% low-melting agarose and solidified immediately after treatment. The agarose plugs were treated with 1 mg/ml of proteinase K and with 1 mg/ml of ribonuclease A, and electrophoresed in a 0.8% agarose gel. The gel was stained with SYBR Gold (Molecular Probes, USA) and photographed.

2.4. Immunofluorescence microscopy

Cells grown on Lab-Tek chamber slides (Nalge Nunc Int., USA) were treated with BaP and/or UVA, and fixed in 2% paraformaldehyde for 5 min at room temperature. They were permeabilized in 100% methanol for 20 min at −20 °C and blocked with 1% BSA for 30 min at 37 °C. After being washed, they were incubated with primary antibody against phospho-H2AX (1:200) (Upstate Biotechnology, USA) for 2 h, then with secondary antibody conjugated with FITC (Jackson Immuno Research Laboratories, USA). To confirm the distribution of foci, the nucleus was stained with propidium iodide (20 μg/ml). Images were acquired on a laser-scanning confocal microscope (LSM510, Carl Zeiss, Germany). Cells were judged as "positive" for γ-H2AX foci if they displayed 5 or more discrete dots of brightness. At least 300 cells were counted for each experimental condition.

2.5. Western blot analysis

Samples containing 60 μg of nuclear protein were separated on 12.5% SDS–PAGE gels, and blotted onto PVDF membranes. After blocking with 3% non-fat milk, the membrane was incubated with primary antibody against phospho-H2AX (1:1000) for 4 h, then with secondary antibody conjugated with HRP (Jackson Immuno Research Laboratories) for 2 h. Protein expression was visualized with an enhanced chemiluminescence detection kit (Amersham Bioscience, UK).

3. Results

3.1. Survival rate of CHO-K1 cells and induction of DSBs after coexposure to BaP and UVA

CHO-K1 cells were treated with BaP (10^{-9}–10^{-7} M) and/or UVA (0.6–2.4 J/cm^2). The survival rates after treatments were determined by colony-formation assay (Fig. 1A). Single treatment with BaP (10^{-7} M) or UVA (2.4 J/cm^2) did not change the survival rates of CHO-K1 cells. Although the co-treatment with BaP and UVA slightly decreased cell viability in a dose-dependent manner, more than 90% of cells were alive under all conditions we examined. We have previously shown that higher doses of BaP (10^{-6}–10^{-5} M) and UVA (1 J/cm^2) induced significant cytotoxicity [16]. The induction of DSBs immediately after treatments was examined by BSFGE (Fig. 1B). A migration of DNA was detected only when the cells were co-exposed to 10^{-8} or 10^{-7} M of BaP and UVA (2.4 J), whereas no migration was detected at 10^{-9} M of BaP and UVA.

3.2. Detection of γ-H2AX after coexposure to BaP and UVA

A significant number of cells positive for γ-H2AX foci were observed immediately after coexposure to BaP (10^{-7} M) and UVA (1.2 J/cm^2), but not following treatment with either BaP or UVA alone (Fig. 2A). The number of γ-H2AX-positive cells (Fig. 2B and C) and of foci per nucleus (data not shown) increased dependent on the doses of BaP and UVA. The generation of γ-H2AX induced by coexposure was confirmed by Western blotting, consistent with the result of immunofluorescence staining.

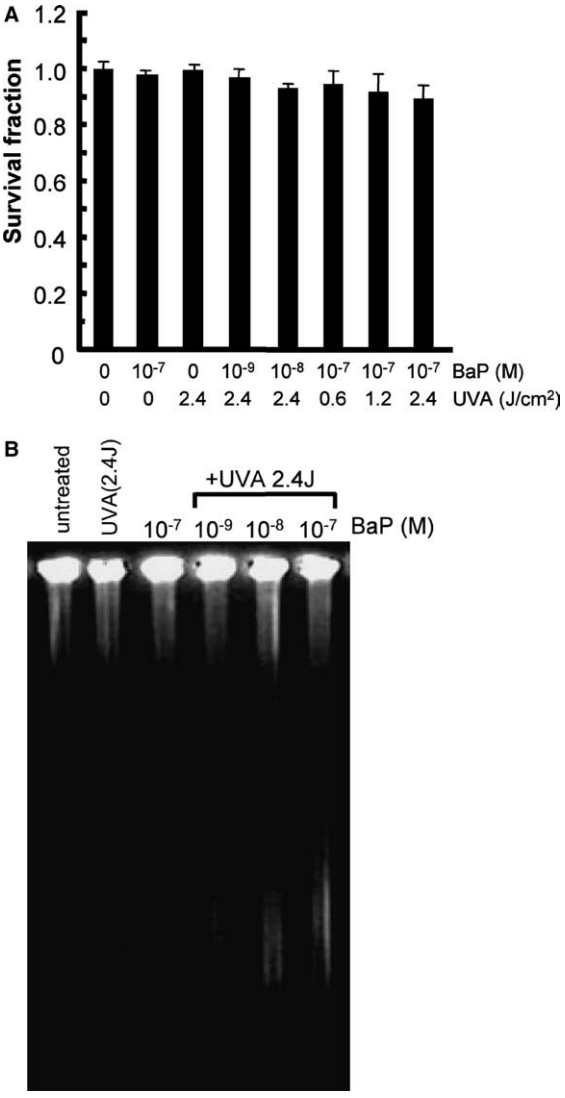

Fig. 1. Cell survival and induction of DSBs after coexposure to BaP and/or UVA. CHO-K1 cells were treated with various doses of BaP and UVA. (A) Survival fraction was determined by colony-formation assay and (B) DSBs were detected by BSFGE as described in Section 2.

3.3. Effect of $^1O_2$ on levels of γ-H2AX after coexposure to BaP and UVA

CHO-K1 cells were treated with BaP (10^{-7} M) and UVA (2.4 J/cm^2) in the presence or absence of NaN_3 (10 or 50 mM), a $^1O_2$ scavenger. These concentrations of NaN_3 did not affect cell viability. Number of γ-H2AX-positive cells following coexposure (more than 60%) decreased in the presence of 10 mM NaN_3 (37%) and of 50 mM NaN_3 (12%) (Fig. 3). The number of foci per nucleus also decreased dependent on the concentration of NaN_3 (data not shown). These results were confirmed by Western blotting.

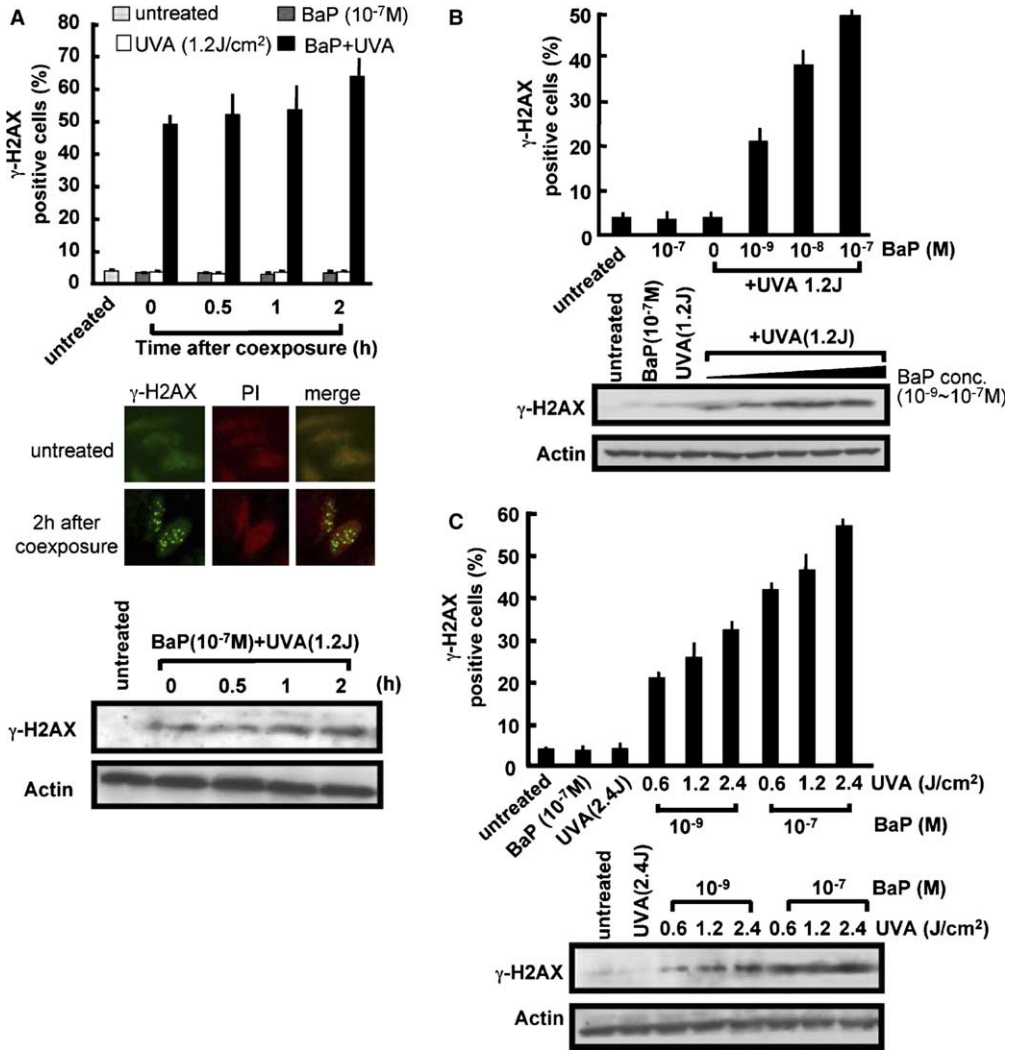

Fig. 2. Induction of γ-H2AX after coexposure to BaP and UVA in CHO-K1 cells. CHO-K1 cells were treated with various doses of BaP and/or UVA and incubated for a predetermined period. Induction of γ-H2AX was analyzed by immunofluorescence staining and Western blotting as described in Section 2. (A) Time-dependent (0–2 h) γ-H2AX induction after treatment with BaP (10^{-7} M) and/or UVA (1.2 J/cm^2). Nuclei were stained with propidium iodide. (B) BaP dose-dependent γ-H2AX induction immediately after treatment with BaP (10^{-9}–10^{-7} M) and UVA (1.2 J/cm^2). (C) UVA dose-dependent γ-H2AX induction immediately after treatment with BaP (10^{-9} or 10^{-7} M) and UVA (~2.4 J/cm^2). Values are means ± S.D. All experiments were repeated two or three times with similar results.

4. Discussion

In this study, we showed for the first time that concomitant treatment with BaP and UVA induced the phosphorylation of H2AX. It has been reported that the generation of γ-H2AX was accompanied by the induction of DSBs [19–21]. Since we have previously demonstrated that coexposure to BaP and UVA induced DSBs in mammalian cells [16], the formation of γ-H2AX was considered due to the DSBs induced by coexposure. Furthermore, we have previously demonstrated that production of $^1O_2$ following coexposure caused the induction of DSBs [17], which was confirmed by the inhibition of γ-H2AX induction in the presence of the $^1O_2$ scavenger NaN_3 (Fig. 3).

DSBs are a serious threat to cells, and if not repaired properly, can result in cell death or loss and a rearrangement of genomic integrity, eventually leading to cancer. The role of γ-H2AX is not yet fully understood but some reports suggested a relationship between induction of γ-H2AX and, cell survival (DNA repair) and death. An absence of γ-H2AX or inhibition of the phosphorylation of H2AX enhanced sensitivity to radiation [23,24]. We assumed two patterns of correlation between cell death and induction of γ-H2AX on exposure to ionizing radiation; a linear-model, in which cells are killed depending on the dose of radiation, and levels of γ-H2AX increase similarly [25,26], and a non-linear-model, in which cell death and γ-H2AX induction are not completely interrelated [24]. In our study, the correlation was non-linear. At lower doses of BaP (10^{-9}–10^{-7} M), the level of γ-H2AX increased dose-dependently (Fig. 2B and C) but cell death was not observed (Fig. 1A). At higher doses (>10^{-6} M), cell survival drastically decreased and DSBs were significantly induced (detected by BSFGE) [16,17], but the level of γ-H2AX decreased (data not shown). This might be due to acute phototoxicty, that is, cells were killed immediately after UVA irradiation and cellular functions against DSBs might not have worked.

It is interesting that coexposure to very low concentrations of BaP and UVA, which significantly generated γ-H2AX,

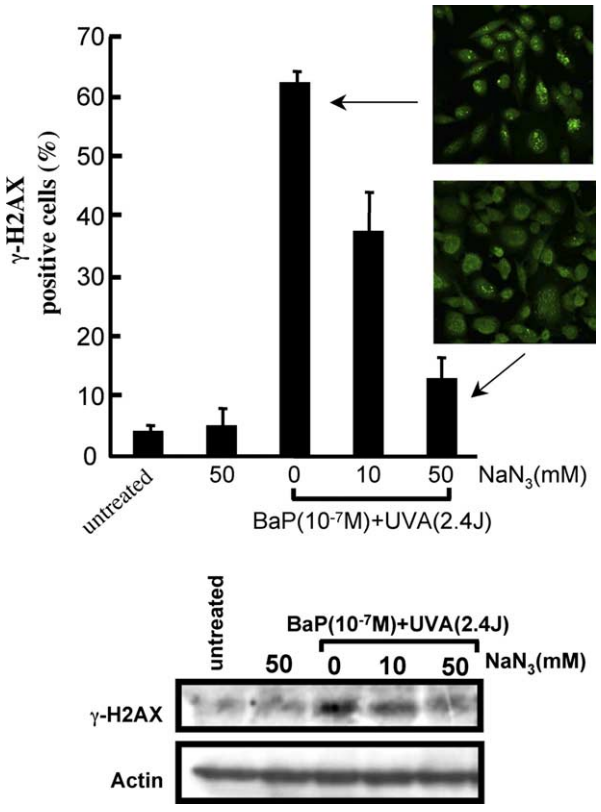

Fig. 3. Effect of NaN_3 on levels of γ-H2AX following coexposure to BaP and UVA. CHO-K1 were cells treated with BaP (10^{-7} M) and UVA (2.4 J/cm^2) in the presence or absence of NaN_3 (10 or 50 mM). The presence of γ-H2AX was examined by immunofluorescence staining and Western blotting immediately after treatment. Values are means ± S.D. All experiments were repeated three times with similar results.

did not change the cell survival rates (Fig. 1A). This suggested that the DSBs induced by the coexposure were repaired. But, in any case, the unscheduled induction of DSBs by extrinsic insult may account for an increased risk of miss-repair. Notably, lesions in a critical gene related to cancer (such as a tumor suppressor gene) could have catastrophic consequences for the cell. Wang et al. [27] showed that the coexposure to BaP and UVA induced mutations in H-ras gene, 8 weeks before tumor emergence in SKH-1 hairless mice, and that these mice developed malignant tumor by 25 weeks. In everyday life, contamination with PAHs is inevitable and skin contaminated with PAHs might be exposed to UVA irradiation. Recently, Yan et al. [28] demonstrated that 11 of 16 PAHs listed by the U.S. Environmental Protection Agency as priority pollutants were photomutagenic. The presence of γ-H2AX at low concentrations of BaP and UVA suggested the possibility of photomutagenicity and photocarcinogenicity of environmental PAHs.

Finally, it is worth noting that a more sensitive detection of DSBs could be achieved by examining the expression of γ-H2AX induction than with BSFGE. Detection of γ-H2AX is considered a powerful tool for detecting DSBs, applicable to the screening of genotoxic substances, especially photocarcinogens in the environment.

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