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DNA damage and repair of human skin keratinocytes concurrently exposed to pyrene derivatives and UVA light

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Abstract

Polycyclic aromatic hydrocarbons (PAHs), a class of mutagenic environmental contaminants, insert toxicity through both metabolic activation and light irradiation. Pyrene, one of the most widely studied PAHs, along with its mono-substituted derivatives, 1-amino, 1-bromo, 1-hydroxy, and 1-nitropyrene, were chosen to study the effect of substituents on their phototoxicity, DNA damage and repair. Both alkaline Comet assay, which detects direct DNA damages, and *Fpg* endonuclease Comet assay, which detects oxidative DNA damages, were conducted at 0, 2, 4, 8, and 24 h of incubation of the cells in minimal growth medium after concomitant exposure to pyrene derivatives and UVA light. All these compounds are photocytotoxic and the phototoxicity is both incubation time and PAH dose dependent; whereas, those without light are not toxic. The LC_{50} obtained are in the range of 3.5 – 9.3 μ M. Cellular DNA damages, both direct and oxidative, are observed immediately after the cells are treated with UVA light and the pyrene derivatives at a concentration of 1.0 μ M. The amount of DNA damages (both direct and oxidative) increase from 0 to 4 h of incubation. After 4 hours, subsequent damage induction declines, and this is perceived to be mainly through DNA repair. After longer incubation of 8 h, the damaged cellular DNA start to be repaired, resulting in greatly reduced amount of DNA damages, and the DNA damage reaches the minimum at 24 h of incubation. 1-Amopyrene and 1-hydroxypyrene cause more DNA oxidative damages immediately after the exposure (0 h of incubation), and these damages are repaired within the same timeframe as the other tested compounds. The oxidative DNA damages caused by 1-bromopyrene are repaired starting at 2 h of incubation, earlier than the damages caused by all the other compounds.

Introduction

Pyrene, one of the most abundant and most widely-studied polycyclic aromatic hydrocarbons (PAHs), exhibit metabolic toxicity as well as phototoxicity to various cells and bacteria^{1–7}. Co-exposure to pyrene and light irradiation causes human skin keratinocyte cells to become non-viable in a pyrene dose dependent manner². It is suggested that damages to cellular constituents DNA, protein and lipids are the main reasons leading to phototoxicity^{6,7}. In a recent study, we reported that light-induced lipid peroxidation by 1-substituted pyrenes are strongly structure dependent⁸. Although any form of damage to the cell can lead to cellular death, DNA damages may be repaired and the cellular function may

be partially or even fully restored. The goal of this article is to study DNA damages and repair in human skin keratinocyte cells caused by co-exposure to pyrene, 1-amino (1-AP), 1-bromo (1-BP), 1-hydroxy (1-HP), and 1-nitropyrenes (NP) and UVA light. The effect of substituents at C-1 position on these biological activities will also be explored.

Materials and Methods

HaCaT keratinocytes, a transformed human epidermal cell line, were obtained from Dr. Norbert Fusenig of the German Cancer Research Centre (Heidelberg, Germany). Pyrene, 1-AP, 1-BP, 1-HP, and 1-NP were purchased from Sigma-Aldrich (St. Louis, MO), with a purity of at least 96%. Trypsin/EDTA solutions were purchased from Cambrex Bioscience (Walkersville, MD). Fetal Bovine Serum (FBS), Dulbecco's minimum essential medium (DMEM), Penicillin/streptomycin, methanol and phosphate buffered saline (PBS) were from Fisher Scientific (Houston, TX). Standard Comet and FLARE™ (Fragment Length Analysis using Repair Enzymes) Comet assay kits, and *E. coli* formamidopyridine-DNA glycosylase (fpg) were obtained from Trevigen (Gaithersburg, MD).

Light Source

The light source was a model TL-365R 15 W UVA transilluminator from Spectroline (Westbury, NY) that produces an emission band at 365 ± 25 nm. The UVA irradiance is 5.8 mW/cm^2 , measured with a Model PMA 2100 radiometer from Solar Light (Philadelphia, PA).

HaCaT Cell Culture

Cell culture and treatment of HaCaT cells followed previously published procedures with modifications^{2,9}. HaCaT keratinocyte cells (1 mL) were placed in 25 cm^2 flasks containing 5 mL complete medium (DMEM with 10% FBS, and 1% antibiotics). Cells were incubated at 37°C in 5% CO_2 , 95% air. Cell growth was monitored daily and fed every other day with 5 mL complete medium. HaCaT cells were grown to 75–80% confluence before use. HaCaT cells were plated in 96 well or 6 well plates for MTT and Comet assays, respectively.

Photocytotoxicity Test Using MTT Assay

HaCaT cells plated in 96 well plates in complete medium were incubated at 37°C in 5% CO_2 , 95% air for 24 h for cell adhesion. Plate 1 was treated with pyrene, 1-AP, 1-BP, 1-HP, or 1-NP (0.0, 0.2, 1.0, 5.0, and $25.0 \mu\text{M}$) without light and Plate 2 was treated with the corresponding pyrene derivatives and 20 min of UVA light (7.0 J/cm^2 UVA). Following treatment, the wells were washed twice with $1 \times$ PBS to remove residual pyrene derivatives. Thereafter, 200 μL of DMEM was added to each well and incubated for 0, 2, 4, 6, or 24 h. Exactly 50 μL of MTT solution (5 mg/mL in PBS buffer) was added to each well and incubated for 30 min, allowing reduction of MTT into formazan by the mitochondrial reductases in viable cells. After incubation, supernatant was aspirated from the wells, and 200 μL of pure DMSO was added to solubilize the formazan. The plates were analyzed using a Fluoroskan II Microplate Reader (Biosystem, Helsinki, Finland). The absorbance of the formazan was read at 540 nm using Ascent software.

Concentration Dependent DNA Damage

HaCaT keratinocytes were grown on 6 well plates under 37°C/5% CO₂ incubation until confluent. Pyrene or its derivatives in solution was added (final concentrations 0, 0.2, 1.0, 2.5, 3.75, and 5.0 µM) to the wells and exposed to UVA light for 20 min, corresponding to a light dosage of 7.0 J/cm² UVA. Following treatment, the wells were washed twice with 1 × PBS to remove residual traces of pyrene. Thereafter, 1.0 mL of incomplete DMEM medium was added to each well and incubated for 4 hrs.

DNA Damage and Repair Monitored by Comet Assay

Comet assay has been a method used to determine DNA damage and repair for cells that underwent injuries by light or chemicals^{10–13}. HaCaT cells were grown in two 6-well plates and were incubated at 37 °C and 5% CO₂ until confluent. Pyrene, 1-AP, 1-BP, 1-HP, or 1-NP (final concentration of 1.0 µM) was individually added to both plates: the control plate was left in dark conditions, and the experimental plate was exposed to UVA light for 20 min. Immediately following treatment, all wells were washed twice with 1× PBS to remove residual PAHs. Thereafter, 1.0 mL of DMEM was added to each well and incubated for 0, 2, 4, 8, or 24 h before Comet assay.

Alkaline Comet Assay—The alkaline Comet assay, which detects direct DNA damages, was conducted at 0, 2, 4, 8, and 24 h of incubation in DMEM. Both negative (4% methanol) and positive (25 µM KMnO₄) controls were included in all series. The Comet assay was performed following the Trevigen protocol¹⁴. The treated cells in the 6 well plates were trypsinized by 0.25% trypsin/EDTA and culture medium (1.0 mL) was added to stop the trypsinization process. The detached cells were immediately placed on ice and supernatant was removed after centrifugation at 1000 rpm for 7 min. Cells were then washed in 1× PBS and re-centrifuged at 1000 rpm for another 7 min. Cells were resuspended in 1× PBS and added to 75 µL molten (37 °C), 0.5% low-melting-point agarose gel to achieve a cell concentration of 1×10⁵ cells/mL. The cells in agarose were pipetted onto the Comet slides. Slides were stored in the dark at 4°C for 10 min before addition of pre-chilled lysis buffer for 45 min. After lysis, the slides were immersed in freshly prepared alkaline solution (0.25 M NaOH containing 0.1 µM EDTA, pH 12.6) at room temperature for 45 min. Slides were then removed and washed twice with 1× TBE buffer. Gel electrophoresis was performed in 1× TBE buffer at 1 V/cm for 30 min (running amperage 3–5 mA with the distance between the two electrodes being 25 cm). The Comet slides were washed with 70% ethanol for 5 min and air-dried for 2.5 h at room temperature. Each sample was stained by 50 µL diluted SYBR Green solution. The slides were read with a fluorescence microscope equipped with the Lotus DNA Damage Analysis Software. A total of 100 cells per sample were randomly selected from 3 slides and statistically analyzed to determine the DNA tail moment and average amount of DNA damage.

Fpg Comet Assay—The *E. coli* formamido-pyrimidine-DNA glycosylase (fpg) Comet assay, which detects oxidative DNA damages, was conducted at 0, 2, 4, 8, and 24 h of incubation in DMEM after the treatment. Comet slides were prepared in the same manner as the alkaline Comet assay and suspended in pre-chilled lysis buffer for 45 min. After lyses, Comet slides were immersed in freshly prepared 1× FLARE™ buffer at room temperature to

equilibrate the slides; the buffer was changed 3 times over a 30 min period. Following removal of the buffer, 75 μ L of working fpg enzyme solution (1:100 dilution of fpg activity: 4 U/ μ L) was added to each sample area and incubated at 37 °C for 45 min. Afterwards, slides were immersed in freshly prepared alkaline solution for 45 min at room temperature, and gel electrophoresis was performed in alkaline solution at 1 V/cm for 30 min. Comet slides were then dehydrated and stained with SYBR Green. DNA damage was analyzed similarly to the standard Comet assay.

Statistical Analysis

The level of toxicity and the amount of DNA damages from the treated samples were all quantified and analyzed by descriptive statistics by comparing the mean and standard deviation for control samples versus treated samples. All results were analyzed using the Analysis of Variance (ANOVA) and the Tukey's t-test to distinguish differences between any two pyrene derivatives by the Statistical Analysis Software SAS version 9.1. The α -level was set at 0.05.

Results

Cell Viability

Cell viability assays were conducted at 0, 2, 4, 6, and 24 h after co-exposure to UVA light irradiation and as an example, 1-AP at different concentrations was shown (Figure 2). These cell viability values obtained were plotted versus the concentration of the pyrene derivatives to obtain LC₅₀, the concentration at which 50% cells become non-viable. The LC₅₀ for phototoxicity are in the range of 3.5 – 9.3 μ M, and they decrease with the increase of incubation time after exposure (Table 1). This indicates that more cells become non-viable with increasing incubation time after exposure. Longer incubation times after treatment produce greater phototoxicity ($P < 0.0001$). Based on the LC₅₀ values obtained in Table 1, the relative photocytotoxicity is: 1-HP ~ 1-AP ~ Pyrene > 1-BP > 1-NP. Cell viability data was used to determine the concentration needed for the Comet assays, where at least 75% of the cells should remain viable after treatment. For subsequent Comet assays, we chose the concentration of 1.0 μ M for all pyrene derivatives.

Concentration Dependent DNA Damage

The concentrations of the pyrene derivatives used were 0, 0.2, 1.0, 2.5, 3.8, and 5.0 μ M and the Comet assay was conducted after 4 hrs of incubation after the exposure. There are considerable DNA damages when the HaCaT cells are exposed to pyrene derivatives with light (Figure 3). The DNA damage for all of the pyrene derivatives is concentration dependent. 1-AP, 1-HP, and 1-NP's ability to cause light-induced DNA damage are statistically different from pyrene, where 1-NP caused less DNA damage than 1-AP and 1-HP. There was no statistical difference between the DNA damages from 1-AP and 1-HP.

Light-induced DNA damage was dose dependent for all 5 pyrenes ($P < 0.0001$). Most of the damages become apparent at 1.0 μ M; 1-HP, on the other hand, starts causing DNA damage at 0.2 μ M (Figure 3). The pyrene derivatives can be relatively ranked by the 4 hrs incubation results, from lowest to highest, as follows: 1-HP ~ 1-AP ~ Pyrene < 1-BP < 1-NP. A closer

look at the data shows that there is a type of leveling off for 1-AP and 1-HP somewhere between 2.5–5.0 μM . Comparing Figures 2 and 3, this region correlates with the 4 hrs incubation time's LC_{50} .

Time-Dependent DNA Damages Determined by Comet Assay

HaCaT cells were exposed to 1.0 μM pyrene, 1-AP, 1-BP, 1-HP, or 1-NP with or without UVA light irradiation. After exposure, the treated HaCaT cells were incubated in DMEM at 37 °C and 5% CO_2 for 0, 2, 4, 8, or 24 h before both the alkaline Comet and fpg Comet assays were conducted to determine the amount of direct and oxidative DNA damages, respectively. It is worth noting that, since Comet slides were prepared after cell treatment, these results do not take into consideration of the small fraction of cells (< 25%) that may have died due to severe DNA or other damages.

Data from the Comet assays show that light-induced direct and oxidative DNA damages are observed for all tested compounds (Figure 4), whereas those without light irradiation are not significantly different from the control. Light-induced damages are present immediately after the treatment for all of five compounds, and the amount of DNA damages continues to increase till 4 h of incubation. Except, 1-BP's oxidative DNA damage decreases after 2 h of incubation and reaches the minimum at 4 h of incubation. At 8 h of incubation, the oxidative DNA damages by all compounds decrease to the minimum, and these values are similar to that at 24 h of incubation and the negative controls. Direct DNA damages decline near the minimum at 8 h of incubation, but continue to decrease to the minimum till 24 h of incubation.

Figure 5 plots the changes of DNA damage with incubation time in DMEM. Direct DNA damages detected by the alkaline Comet assay are shown in Figure 5A, and the oxidative DNA damages detected by the fpg Comet assay are shown in Figure 5B. All compounds follow the same direct DNA damage trend in Figure 5A from 0 to 4 h of incubation; DNA damages increase to and reach a maximum at 4 h, decreases at 8 h, and further declines to the minimum at 24 h of incubation. This observation of direct DNA damages (Figure 5A) indicates that HaCaT cell DNA, upon exposure to UVA and a pyrene derivative, were damaged immediately following the exposure and the DNA damage increases during at least the first 4 h of incubation, possibly due to photo-activated pyrene derivatives in the cells. This means that the chemical reactions causing the DNA strand cleavage continues to even occur after the removal of light irradiation. After 4 h of incubation, the DNA damage decreases, indicating that the DNA repair machinery is effectively removing strand cleavages in minimum cell growth medium. The DNA repair continues until up to a time point before 24 h of incubation when all cellular DNA damages are repaired.

The oxidative DNA damages shown in Figure 5B follow a similar direct DNA damage pattern, such as the time-dependent DNA repair. The slight difference is that most of the oxidative DNA damages are repaired within 8 h of incubation, not like the 24 h incubation is required for the direct DNA damages, possibly due to the presence of photoproducts produced by light-irradiation of the pyrene derivatives. In addition, there is a clear structural dependence. For example, the amount of light-induced oxidative DNA damages at 0 h incubation was more significant for 1-AP and 1-HP than the other three compounds (Figure

4A and 5B). Conversely, 1-BP caused maximum DNA damages at 2 h instead of 4 h incubation, and reached the minimum at 4 h instead of 8 h incubation.

Discussions

DNA Damage and Repair

PAHs and their photo-transformation products can induce both acute and chronic toxicities^{6,15–17}. Various forms of DNA damages are observed when cells are exposed to the combination of light and PAHs^{18–23}. These DNA damages include DNA single strand breaks, oxidation of DNA bases, and formation of DNA covalent adducts^{6,18,22,24,25}. Exogenous oxidative damages can occur through the production of reactive oxygen species (ROS)²⁶. ROS production has been associated with pyrene and light^{20,27}. In addition to PAH-modified bases, unrepaired oxidative DNA damages can also generate strand breaks²⁸.

Light-induced DNA damage by PAHs is concentration and time-dependent. Figure 4 displays the balance between two interrelated pathways, DNA damage and DNA repair. DNA damage is the predominant pathway for the incubation times of 0–4 hrs. After 4 hrs, subsequent damage induction declines, and this is perceived to be mainly through DNA repair. Similar to pyrene-induced DNA damages in shrimp embryos²⁹, light-induced DNA damages in HaCaT keratinocytes by pyrene decrease at prolonged incubation in minimum cell growth medium. While the reported shrimp embryos show DNA repair within 24 h, our study suggests that DNA repair starts as early as 4 h for direct DNA damages (Figure 5). This is logical because the epidermis is the body's barrier to the environment, and keratinocytes are equipped with an advanced nuclear excision repair system that can resist DNA damages caused by lethal agents such as UV irradiation^{30,31}. Similar effect was seen in HaCaT cells treated by multi-walled carbon nanotubes, whose oxidative DNA damages were repaired after 24 h of incubation³². Oxidative damage is easily repaired through the base excision repair system^{33–35}. This explains why the oxidative damages caused by the pyrene derivatives reached minimum at 8 h while the direct DNA damages reached minimum at 24 h of incubation (Figure 5).

Structure Dependence

Light-induced cytotoxicity of all five compounds is significantly greater than their toxicity without light irradiation, and they all generate direct and oxidative DNA damages. The relative photocytotoxicity is: 1-HP ~ 1-AP ~ Pyrene > 1-BP > 1-NP. These results are similar to the previously reported in both HaCaT and Jurkat cells using some of the similar compounds^{2,36,37}.

Although there is no obvious structural dependence of the direct DNA damages caused by these five compounds, the amount of oxidative DNA damages is strongly dependent on structure. The ability of causing light-induced oxidative DNA damages in skin keratinocytes by 1-AP and 1-HP is faster than 1-BP, 1-NP and pyrene (Figures 4 and 5). We attribute this difference to the better solubility in cell media for 1-AP and 1-HP, which each possess a polar group, and to their stronger interaction with cellular DNA. For example, 1-HP binds strongly to calf thymus DNA¹⁸; 1-AP and 1-HP can strongly cause DNA single strand

cleavage in plasmid DNA^{18,19}; and a [³H]-thymidine incorporation test showed that 1-AP and 1-HP were more toxic to keratinocyte cells than pyrene³⁷.

Another aspect of the structure dependence pertains to 1-BP. Due to its greater photostability⁸ and higher intersystem crossing yield, 1-BP produces more triplet excited state species³⁸. These longer-lived triplet state species of 1-BP can generate more singlet oxygen than any of the other four compounds. We believe that the greater amount of generated singlet oxygen is responsible for the oxidative DNA damages induced by 1-BP and light. Such oxidative DNA damages are repaired faster than the direct DNA strand cleavages (Figure 5B).

In summary, all of the studied pyrene derivatives' share pyrene's ability to become phototoxic, however, the mechanisms leading to such toxicities is both unique and structure dependent. Structural dependence for phototoxicity (determined by MTT assay), direct DNA damages, and oxidative DNA damages confirm again that phototoxicity of PAHs is complex. It depends on many confounding factors such as photochemical properties of the compounds such as solubility, interaction with cellular components, light source, and light-induced damages to cell membrane, DNA and protein. Therefore, risk assessment should take into consideration light-induced toxicity toward skin cells for both common PAHs and their derivatives.

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Fig. 1.
Structure of pyrene, and 1-amino, 1-bromo, 1-hydroxy, and 1-nitropyrene.

Figure 2.

MTT Assay: **A. Without Light:** 1-AP exposed HaCaT cells remained in dark conditions for 20 min in 1× PBS buffer. After treatment, DMEM was added to the cells, and they underwent incubation at 37°C/ 5 %CO₂ for 0, 2, 4, 6, and 24 hrs (N=3). **B. With Light:** 1-AP exposed HaCaT cells were irradiated for 20 min in 1× PBS buffer. After treatment, DMEM was added to the cells, and they underwent incubation at 37°C/ 5 %CO₂ for 0, 2, 4, 6, and 24 hrs (N=3), P < 0.001.

Figure 3.

DNA Damage from pyrene derivatives with light and after 4 hrs of incubation after treatment. A total of 100 cells/sample were randomly selected from 3 slides and statistically analyzed to determine the average percent of DNA damaged. There was substantial concentration dependence for all 5 compounds ($P < 0.0001$).

Fig. 4.

DNA damage detected at various incubation times for HaCaT cells exposed to PAH with or without UVA irradiation. Figures A–E represent cells incubated in DMEM at 37 °C/5% CO₂ for 0, 2, 4, 8, and 24 h after the treatment. There is a statistical difference between compounds treated with light, by time, and between oxidative and direct DNA damages ($P < 0.0001$).

Fig. 5.
Time Dependent DNA Damage and Repair A: Direct and B: Oxidative DNA Damages.

Table 1

LC₅₀ for HaCaT cells exposed to UVA light and a pyrene derivatives after incubation of different time intervals^a

	Incubation Time After Exposure to PAH and Light				
	0 h	2 h	4 h	6 h	24 h
Pyrene	7.3±0.7	5.9 ± 0.8	5.0 ± 1.3	5.0 ± 0.7	3.6 ± 0.5
1-Aminopyrene	6.4±1.0	6.0 ± 0.6	4.7 ± 1.7	4.4 ± 0.6	3.5 ± 0.5
1-Bromopyrene	6.5±0.3	5.9 ± 2.1	5.3 ± 2.4	5.5 ± 2.4	4.2 ± 1.6
1-Hydroxypyrene	7.6±1.0	4.5 ± 0.9	4.2 ± 0.5	4.2 ± 0.5	3.5 ± 0.4
1-Nitropyrene	9.3±1.4	8.8 ± 1.5	8.5 ± 1.1	6.6 ± 2.4	4.7 ± 0.1