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EFFECT OF BYPRODUCTS FROM THE OZONATION OF PYRENE: BIPHENYL-2,2′,6,6′-TETRACARBALDEHYDE AND BIPHENYL-2,2′,6,6′-TETRACARBOXYLIC ACID ON GAP JUNCTION INTERCELLULAR COMMUNICATION AND NEUTROPHIL FUNCTION

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Abstract—In this study, biphenyl-2,2′,6,6′-tetracarbaldehyde, an initial byproduct formed from the ozonation of pyrene, and biphenyl-2,2′,6,6′-tetracarboxylic acid, a subsequent pyrene ozonation byproduct, were evaluated using two toxicology assays to compare the toxicity of ozonation byproducts with that of the parent compound. The first assay measured the potential for the compounds to block gap junctional intercellular communication (GJIC) using the scrape loading/dye transfer technique in normal WB-344 rat liver epithelial cells. The second assay evaluated the ability of the compounds to affect neutrophil function by measuring the production of superoxide in a human cell line (HL-60). Pyrene significantly blocked intercellular communication (f = 0.2–0.5) at 40 μM and complete inhibition of communication (f < 0.2) occurred at 50 μM. Gap junctional intercellular communication in cells exposed to biphenyl-2,2′,6,6′-tetracarbaldehyde reached f < 0.5 at a concentration of 15 μM. At concentrations greater than 20 μM, biphenyl-2,2′,6,6′-tetracarbaldehyde was cytotoxic and the inhibition of GJIC was caused by cell death. Biphenyl-2,2′,6,6′-tetracarboxylic acid was neither cytotoxic nor inhibitory to GJIC at the concentrations tested (10–500 μM). Exposure to biphenyl-2,2′,6,6′-tetracarbaldehyde resulted in a concentration-dependent decrease in phorbol 12-myristate 13-acetate–stimulated O_2^- production. Neither exposure to pyrene nor biphenyl-2,2′,6,6′-tetracarboxylic acid caused a significant toxic effect on neutrophil function.

Keywords—Ozone   Pyrene   Polycyclic aromatic hydrocarbons   Gap junctional intercellular communication   Neutrophil assay

INTRODUCTION

The need for effective in situ treatment technologies has led to the increased use of ozone treatment to remediate polycyclic aromatic hydrocarbon (PAH) contamination in groundwater and soil [1–3]. Although ozone effectively oxidizes PAHs, numerous byproducts are produced and the lack of information about the identity and toxicity of these byproducts presents a serious deficiency in research. Because PAH compounds are known to possess mutagenic and carcinogenic characteristics [4–11], the potential for the byproducts to have carcinogenic or mutagenic characteristics must be considered.

Pyrene (Fig. 1a) is an example of a four-ring PAH. Although pyrene is similar in structure to many chemicals that are probable human carcinogens, pyrene, itself, has not classified as a human carcinogen ([12], http://www.atsdr.cdc.gov/toxprofiles/phs69.html). Pyrene ozonation studies in various participating and nonparticipating solvents have lead to the identification of at least 28 byproducts [13–15]. The ozonation of pyrene proceeds via ozone attack at the bonds of lowest localization energy, which are the 4, 5 bonds followed by attack at the 9, 10 bonds [14]. Ozonolysis of pyrene initially promotes the formation of phenanthrene-like compounds followed by the formation of biphenyl-like compounds [14,15]. These byproducts can be substituted with hydroxyl, aldehyde, and carboxylic acid functional groups [1,14]. When pyrene was treated with excess ozone in a nonparticipating solvent, the predominant byproducts were either biphenyl-2,2′,6,6′-tetracarbaldehyde or biphenyl-2,2′,6,6′-tetracarboxylic acid [14]. Biphenyl-2,2′,6,6′-tetracarbaldehyde was formed upon reductive work-up of the ozonoylsis, and biphenyl-2,2′,6,6′-tetracarboxylic acid was produced when treatment with ozone was followed by the addition of an oxidant [13,14]. All compounds herein are named in accordance to the International Union of Pure and Applied Chemistry Guidelines (http://www.iupac.org/nomenclature/ACD/calc_3dparty.html). However, the literature of biphenyls is host to numerous nomenclature conventions. For example, in earlier literature biphenyl-2,2′,6,6′-tetracarbaldehyde was referred to by the common name, 2,2′,6,6′-biphenyltetraldehyde.

Only a few toxicology studies have evaluated the toxicity of PAH ozonation byproducts [16–22]. Upham et al. [17] investigated the ozonation of pyrene and the potential for the byproduct mixture produced to disrupt intercellular communication. This study suggested that the early ozonation byproducts of pyrene in aqueous systems are more toxic than the parent compound, pyrene. At 1.6 ± 0.1 mol O_3/mol PAH, pyrene had completely disappeared, but the disruption of cellular communication was greater for the ozonated mixture than that observed with pyrene. Yao et al. [15] determined that at this ozone concentration, the predominant compounds were biphenyl-2,2′,6,6′-tetracarbaldehyde and 2′,6,6′-triformylbiphenyl-2-carboxylic acid.

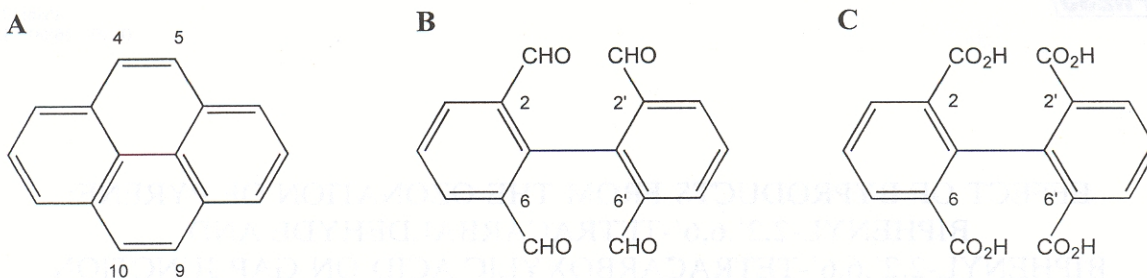* To whom correspondence may be addressed (masten@msu.edu).

A   **B**   **C**

Fig. 1. (**a**) Pyrene, (**b**) biphenyl-2,2′,6,6′-tetracarbaldehyde, and (**c**) biphenyl-2,2′,6,6′-tetracarboxylic acid.

A stoichiometric ratio of 4.5 mol ozone/mol pyrene was required to destroy the intermediate products' inhibitory to gap junction intercellular communication (GJIC) [15]. At concentrations greater than 4.5 mol O_3/mol pyrene, Yao et al. [15] reported that the concentrations of biphenyl-2,2′,6,6′-tetra-carbaldehyde and 2′,6,6′-triformylbiphenyl-2-carboxylic acid had decreased significantly. The remaining compounds were found to be biphenyls substituted with three or four carboxylic acid groups or oxo-substituents. The compounds biphenyl-2,2′,6,6′-tetracarboxylic acid and biphenyl-2,6-dicarboxy-2′,6′-dicarbaldehyde predominated.

In the present work, pyrene ozonation byproducts were evaluated using two different toxicology assays. This study focused on evaluating the toxicity of two pyrene ozonation byproducts: Biphenyl-2,2′,6,6′-tetracarbaldehyde (Fig. 1b) and biphenyl-2,2′,6,6′-tetracarboxylic acid (Fig. 1c). The two assays were chosen because they represent cellular functions that result from the integration of cellular signaling. Changes in each can be associated with effects on the whole organism.

The first study evaluated the compounds for their potential to block GJIC in WB-344 rat liver epithelial cells using the scrape/load dye transfer assay. The GJIC assay is a nongenotoxic assay that measures the ability of cells to transfer low–molecular weight molecules through the cytoplasm of one cell to the next cell through channels called gap junctions. Gap junctional intercellular communication is an important and fundamental biological process regulating cell homeostasis and cell synchronization in multicelled metazoans [23,24]. A family of highly evolutionary-conserved genes (connexins) codes the membrane-associated proteins that compose the hemichannels (connexons) that, together with the opposing connexon, unite to form a direct passage channel for ions and small molecular weight molecules. Modulation of the number and function of these gap junctions, adaptively or maladaptively, can affect the homeostatic function of these gap junctions in tissues by altering cell proliferation, differentiation, apoptosis, and other adaptive responses of cells within tissues [25]. Many endogenous and exogenous toxic chemicals can inhibit gap junctional intercellular communication and act as teratogens, tumor promoters, immune modulators, and reproductive and neurotoxicants [25,26]. Only a few studies have investigated the epigenetic toxicity of PAHs using the GJIC assay [17–22,27]. Epigenetic toxicity refers to the altering of the expression of the genetic information of cells at the transcription, translation, or posttranslational level. Transcription is the process of expressing a selective group of genes in the cell's genome; translation is the modification of the stability of the gene message; and posttranslation is the modification of the protein coded by the gene. Most cancer cells have dysfunctional gap junctional intercellular communication due to the fact that the genes coding for the gap junction proteins are

not transcribed or that the gap junction proteins are modified by a mutation or a posttranslational modification by an activated oncogene [28,29].

The second assay evaluated the ability for the compounds to affect neutrophil production of superoxide anion. Generation of reactive oxygen species by neutrophils is critical to their function to kill bacteria. Molecular oxygen is reduced by the enzyme nicotinamide adenine dinucleotide phosphate–hydrogen (NADPH) oxidase to form superoxide anion, which in turn can be reduced to other reactive oxygen species, some of which are potentially toxic. Multiple pathways lead to the activation of NADPH oxidase and production of superoxide anion in neutrophils, and the pathways activated are dependent on the stimulus. Thus, generation of superoxide anion by neutrophils results from activation of a complex network of intracellular signals and represents an integrated cellular function. Previous studies have demonstrated alteration in neutrophil production of reactive oxygen species by environmental chemicals and their products of remediation [30–35].

MATERIALS

Chemicals

Pyrene (98% purity, Sigma Chemical, St. Louis, MO, USA) was used for all of the toxicology studies. The two byproducts, biphenyl-2,2′,6,6′-tetracarbaldehyde and biphenyl-2,2′,6,6′-tetracarboxylic acid, were synthesized by J. Ward and R. Maleczka (Department of Chemistry, Michigan State University, East Lansing, MI, USA).

Synthesis of pyrene ozonation byproducts. Biphenyl-2,2′,6,6′-tetracarbaldehyde [36,37] and biphenyl-2,2′,6,6′-tetracarboxylic acid [38–40] have been reported previously, however, their preparations are either poorly described, lack characterization data, or were otherwise deemed unsuitable. As such, modified procedures were developed and the compounds fully characterized to ensure the fidelity of the toxicological studies.

Biphenyl-2,2′,6,6′-tetracarbaldehyde was prepared by dissolving pyrene in a 2:1 CH_2Cl_2/MeOH solution. Triethylamine (1 ml) was then added. Following this, the solution was purged with nitrogen for 15 min and cooled to $-78°C$. Ozone was then bubbled through the solution for 1.5 h, after which time the solution was greenish-blue. Nitrogen was then bubbled through the solution for 15 min to remove the excess ozone. Dimethylsulfide (7 ml) was added dropwise to reduce the ozonide. The solution was allowed to warm slowly to room temperature overnight. The solvent was removed in vacuo and the residue dissolved in ethyl acetate. The organic layer was washed with water and brine, and dried over $MgSO_4$. The solution was decolorized with Norit-A charcoal. After filtration, the solvent was removed in vacuo and the resulting res-

idue was subjected to flash silica gel chromatography (50% ethyl acetate/hexanes). This afforded 2.1 g (54%) of the desired product as a white solid, with the following properties: Melting point (mp) 155–156°C (lit. [37] 154–156). Infrared (KBr) 3,076 (m), 2,831 (s), 2,737 (s), 1,695 (s), 1,572 (s), 1,450 (2), 1,383 (m), 1,238 (s), 1,159 (m), 958 (s), 794 (s). Nuclear magnetic resonance (^{1}H NMR) (300 MHz, CDCl$_3$) δ 7.88 (t, J = 7.69 Hz, 2 H), 8.28 (d, J = 7.69 Hz, 4 H), 9.72 (s, 4 H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 189.5, 135.6, 135.5, 129.7. Elemental/combustion analysis calculated for C$_{16}$H$_{10}$O$_4$: C, 72.18; H, 3.79. Found: C, 72.23; H, 3.98.

Biphenyl-2,2′,6,6′-tetracarboxylic acid was prepared by suspending biphenyl-2,2′,6,6′-tetracarbaldehyde (3.7 mmol, 1.0 g) in water (20 ml) followed by the addition of potassium stearate (0.403 mmol, 0.130 g) and KMnO$_4$ (11.1 mmol, 1.75 g). The reaction was heated to 50°C and allowed to stir overnight. It was then cooled to room temperature and filtered through a pad of NaCl. The filtrate was then acidified to pH 2.5, filtered, and the solid dried in vacuo to afford 950 mg (79%) as an off-white solid with a high melting point (lit. [40] >300°C). Infrared (KBr) 2,997 (br. s), 1,684(s), 1,583(s), 1,464(s), 1,404(s), 1,267(s), 929(s), 761(s), 684(s), 534(w). ^{1}H NMR (300 MHz, dimethyl sulfoxide (DMSO)-d6) δ 7.43 (t, J = 7.69, 2 H), 7.9 (d, J = 7.69 4 H); ^{13}C NMR (74.5 MHz, DMSO-d$_6$) δ 167.3, 142.1, 137.7, 132.2, 131.6, 126.4. $λ_{max}$ = 292 nm (ethanol). Low-resolution mass spectroscopy (direct probe electron impact) m/z 330 (M+). Elemental/combustion analysis calculated for C$_{16}$H$_{10}$O$_8$: C, 58.19; H, 3.05. Found: C, 58.10; H, 3.10.

Toxicology assays

Gap junctional intercellular communication assay. The GJIC bioassays consist of four tests to evaluate the effect a compound can have on cellular communication. These tests consist of dose response, time response, time recovery, and cytoxicity. For the dose-response experiments, cells were exposed to varying doses of the toxicant for 30 min and then assayed to determine GJIC levels. In the time-response experiments, cells were exposed to a fixed dose of toxicant for 30 min, 60 min, 1 h, 2 h, 4 h, and 5.5 h. For time recovery, cells were exposed to the toxicant for 30 min and then rinsed. New D-media was added to the plate and the cells were returned to the incubator. The plates were removed from the incubator and assayed to determine GJIC values at 30-min, 60-min, 1-h, 2-h, 4-h, and 5.5-h increments after media replacement. Rat liver epithelial cells (WB-F344) were used in the GJIC assay. These cells are from a diploid, nontumorigenic cell line for which a large database of toxicity/cancer information exists [41].

Pyrene was dissolved in acetonitrile ([ACN] 99.8% purity, EM Science, Gibbstown, NJ, USA) and sonicated for 5 min to ensure complete dissolution. Biphenyl-2,2′,6,6′-tetracarbaldehyde was dissolved in ACN (99.8% purity, EM Science). Acetonitrile was selected as the nonpolar solvent because Upham et al. [20] demonstrated that the solvent does not significantly affect GJIC at final ACN concentrations up to 1.5% in cell culture medium [18]. Biphenyl-2,2′,6,6′-tetracarboxylic acid was dissolved in a 30:60 ACN/DDI (double deionized) water solution because the compound was more polar than either pyrene or biphenyl-2,2′,6,6′-tetracarbaldehyde. All experiments were conducted using either an ACN concentration or an ACN/DDI of 1% or less in cell culture medium.

WB-F344 rat epithelial cell lines were obtained from J.W. Grisham and M.S. Tsao of the University of North Carolina (Chapel Hill, NC, USA). Cells were cultured using the method described in detail by Luster-Teasley et al. [22] and Satoh et al. [42].

Bioassay for GJIC. Bioassays were conducted in 35-mm Petri dishes with confluent cultures grown for 2 d in 2 ml of D medium (http://www-cyanosite.bio.purdue.edu/media/table/D.html) supplemented with 5% fetal bovine serum. The procedure for the scrape loading/dye transfer technique was adapted from the method used by El-Fouly et al. [43] and is described in detail by Herner et al. [21]. All tests were run in triplicate and at noncytotoxic levels determined by the neutral red uptake assay kit (Sigma Chemical). Under fluorescent light, the Lucifer yellow dye will fluoresce to indicate the distance the dye travels from the scrape. This distance was measured and compared to a control group of cells that were exposed to ACN only (vehicle controls), but assayed using the identical scrape loading/dye transfer method. Three photographs were taken for each concentration tested and the area coverage of the dye was measured. All photographs were taken within 1 h of experiment completion. The area of fluorescent cells was measured using a Gel Expert Program by NucleoTech (Hayward, CA, USA). The area of the fluorescent cells in each of the pictures was divided by the average area of the controls to obtain a representative fraction of control (f). The f values were reported as an average ± standard deviation determined at the 95% confidence interval (95% CI).

Gap junctional intercellular communication was assessed by the decrease in communication of the cells exposed to the toxicant compared to the vehicle control group (ACN only). Complete communication is identified as an f value of 1.0 or 100% communication as compared to the control. Gap junctional intercellular communication f values of 0.3 to 0.5 indicate a significant decrease because the cells are communicating at approximately half of normal communication levels. Gap junctional intercellular communication values between 0.0 and 0.3 are considered to correspond to no intercellular communication. These interpretations for GJIC are consistent with those of Upham et al. [17], Herner et al. [21], Luster-Teasley et al. [22], and Satoh et al. [42]. Controls were run for each experiment to standardize the measurement for normal cell–cell communication at the time of the experiment. Using the t test it was determined that the mean f values obtained from the control cells without solvent and the vehicle controls do not differ at a 95% CI. Statistical analyses using the two-tailed t test and f test at 95% CI were used to compare within treatment and between treatment variations in the mean f values.

Bioassay for cytotoxicity. Cytotoxicity was tested using the neutral red uptake assay according to the method of Borenfreund and Puerner [44] and discussed in detail in Luster-Teasley et al. [22]. The cytotoxicity experiments mimic the dose-response experiments. In the assay, the cells were exposed to the toxicant for 30 min using the concentrations used for the dose experiments. The cells were washed three times with PBS and 2 ml of the neutral red dye solution was added. After 1 h of incubation with the dye at 37°C, the cells were washed three times with PBS. The neutral red dye absorbed by the cells was lysed from the cells by adding 1 ml of neutral red solubilizer containing 1% acetic acid and 50% ethanol and incubating the plates for 15 min. After the 15 min, the neutral red released by the cells was measured spectrophotometrically at a wavelength of 540 nm and a background absorbance mea-

sured at 630 nm. The cytotoxicity was evaluated based on a fraction of control exposed to only ACN. A fraction of control value greater than 0.8 is considered noncytotoxic.

To produce the dye solution, neutral red dye was incubated in D-media with 5% fetal bovine serum for 2 h at 37°C and then centrifuged at 1,200 rpm for 5 min to remove any solid dye residue. The effluent was then filtered using a 0.22-μM Millipore syringe filter (Millipore, New Bedford, MA, USA) into D-media with 5% fetal bovine serum to a concentration of 0.033%.

HL-60 neutrophil assay. Trypan Blue solution, cytochrome C, superoxide dismutase, phorbol 12-myristate 13-acetate (PMA), pyruvate substrate, NADH, and N,N-dimethylformamide were purchased from Sigma Chemical. Dimethyl sulfoxide was purchased from J.T. Baker (Phillipsburg, NJ, USA). Iscove's Modified Dulbecco's Medium was obtained from American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA, USA). Antibiotic-antimycotic (10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B) and gentamicin (50 mg/ml) were purchased from Life Technologies (Rockville, MD, USA). Triton X-100 (scintillation grade) was obtained from Research Products International (Mount Prospect, IL, USA).

The HL-60 cells purchased from American Type Culture Collection were grown in suspension in Iscove's Modified Dulbecco's medium with 15% fetal bovine serum, 1% antibiotic-antimycotic, and 0.0001% gentamicin. Differentiation to a neutrophilic phenotype was achieved by culturing cells with 1.25% DMSO (J.T. Baker) for 7 d [45–54]. Cells were centrifuged (200 *g* for 10 min) and resuspended in Hanks' balanced salt solution at a concentration of 625,000/ml for use in the superoxide anion assay.

Superoxide anion assay. Stock solutions of pyrene, 22′66′-biphenyltetracarboxylic acid and 22′66′-biphenyltetraaldehyde were prepared in N,N-dimethylformamide and were diluted with Hanks' balanced salt solution such that the final concentrations in the wells were 40, 10, and 1 μM. Cells were transferred to 96-well plates (125,000/well) and exposed to vehicle (0.1% N,N-dimethylformamide), pyrene, 22′66′-biphenyltetracarboxylic acid, or 22′66′-biphenyltetraaldehyde in the presence of cytochrome C (1 mg/ml) with or without superoxide dismutase (325 U/ml). Plates were transferred to an El$_x$808 microplate reader (BIO-TEK Instruments, Winooski, VT, USA), and absorbance at 37°C at 550 nm was read every 30 s for 30 min to determine effects on basal rates of superoxide anion production. Phorbol 12-myristate 13-acetate (1 ng/ml) was then added to each well, and the absorbance was read for an additional 30 min to evaluate effects on PMA-stimulated superoxide anion production. Mean absorbance of triplicate wells containing superoxide dismutase was subtracted from the mean absorbance of the corresponding triplicate wells without superoxide dismutase. Superoxide anion concentration was calculated from this difference using an extinction coefficient of 18.5 cm^{-1} mM^{-}.

Lactate dehydrogenase assay. Release of lactate dehydrogenase (LDH) was measured as a marker of cytotoxicity. Fifty μl of supernatant was removed from each well and transferred to another plate. The LDH activity in the supernatant was determined according to the method of Bergmeyer and Bernt [55] and was compared to the activity determined for the same number of cells lysed with Triton X100.
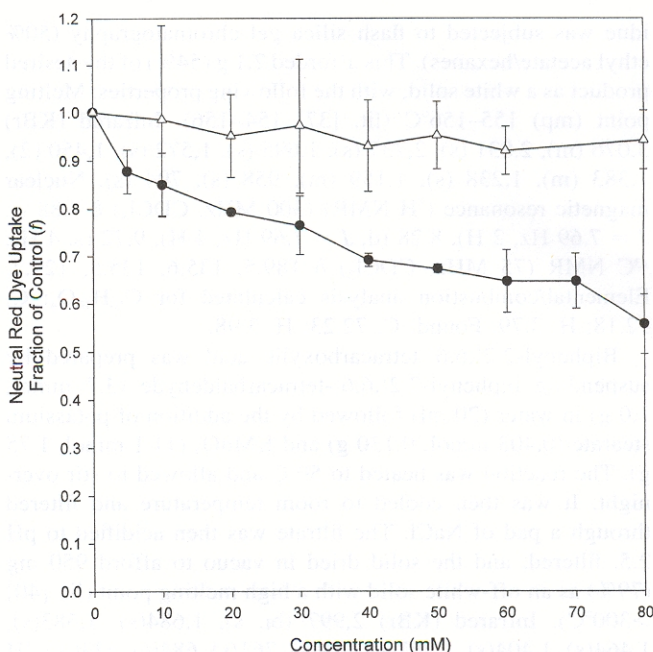

Fig. 2. Cytotoxicity of pyrene (△) and biphenyl-2,2′,6,6′-tetracarbaldehyde (●).

Statistical analysis

Data were analyzed by one-way analysis of variance. The criterion for significance was $p < 0.05$.

RESULTS

GJIC evaluation

Cytotoxicity. The cytotoxicity assay (neutral red dye uptake assay) for pyrene and biphenyl-2,2′,6,6′-tetracarbaldehdye are shown in Figure 2. Pyrene was not cytotoxic at the concentration range evaluated (0–80 μM). Biphenyl 2,2′,6,6′-tetracarbaldehyde reached cytotoxic levels at concentrations greater than 20 μM. A concentration of 5 μM was used for the time-response and time-recovery experiments to account for standard deviation seen in the cytotoxicity assay and to assure that the concentration used was not cytotoxic ($f > 0.8$). Biphenyl-2,2′,6,6′-tetracarboxylic acid was not cytotoxic at the concentrations (0–500 μM) evaluated (Fig. 3).

Dose response. For dose-response experiments, cells were exposed to varying doses of a toxicant for 30 min and then assayed to determine GJIC levels. As shown in Figure 4, pyrene significantly blocked intercellular communication ($f = 0.3$–0.5) at 50 μM; GJIC in cells exposed to biphenyl-2,2′,6,6′-tetracarbaldehyde reached $f = 0.3$ to 0.5 starting at a concentration of 20 μM. At concentrations greater than 20 μM, biphenyl-2,2′,6,6′-tetracarbaldehyde was cytotoxic and the inhibition of GJIC was caused by cell death. Biphenyl-2,2′,6,6′-tetracarboxylic acid was noninhibitory to GJIC at the concentrations tested (10–500 μM).

Time response. For time-response experiments, cells were exposed to a fixed dose of toxicant for varying lengths of time. As shown in Figure 5, biphenyl-2,2′,6,6′-tetracarbaldehyde (5 μM) blocked communication after 30 min of exposure. Complete inhibition was reached in 2 h and 24 h of exposure led to cell death. Exposure to biphenyl-2,2′,6,6′-tetracarboxylic acid (500 μM) did not result in a reduction in communication even after 24 h.
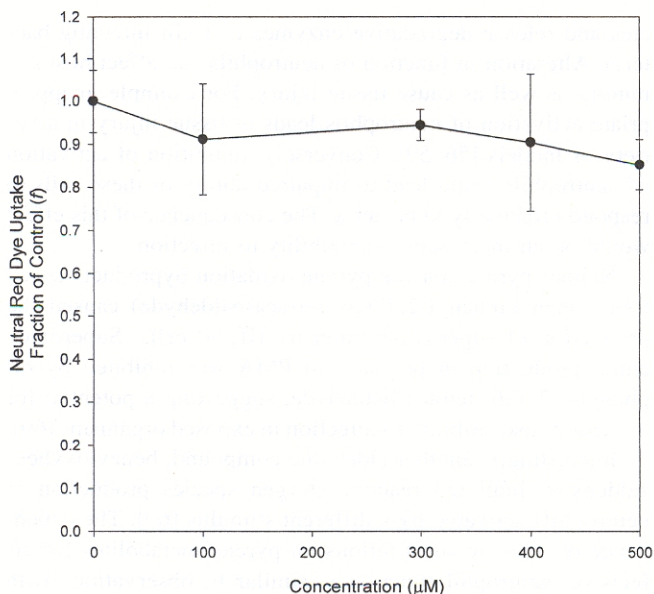
Time recovery. The time recovery assay is used to assess

Fig. 3. Cytotoxicity of biphenyl-2,2',6,6'-tetracarboxylic acid (●).

Fig. 5. Effect of exposure time on gap junctional intercellular communication of cells treated with biphenyl-2,2',6,6'-tetracarbaldehyde 5 μM (△) and biphenyl-2,2',6,6'-tetracarboxylic acid 500 μM (□).

if cells are able to recover full communication after the removal of the toxicant and replacement of new media. For these experiments, cells were exposed to a fixed dose of toxicant for 30 min and then rinsed. New D-media was added to the plate and the cells were returned to the incubator. The GJIC assays were then performed to determine the initial GJIC value and the subsequent GJIC values for 30 min, 60 min, 1 h, 2 h, 4 h, and 5.5 h after media replacement.

After 30 min of exposure to biphenyl-2,2',6,6'-tetracarbaldehyde (5 μM), rinsing the plate, and replacement of new media, cellular communication continued to decrease and never recovered (Fig. 6). No recovery of GJIC was seen and
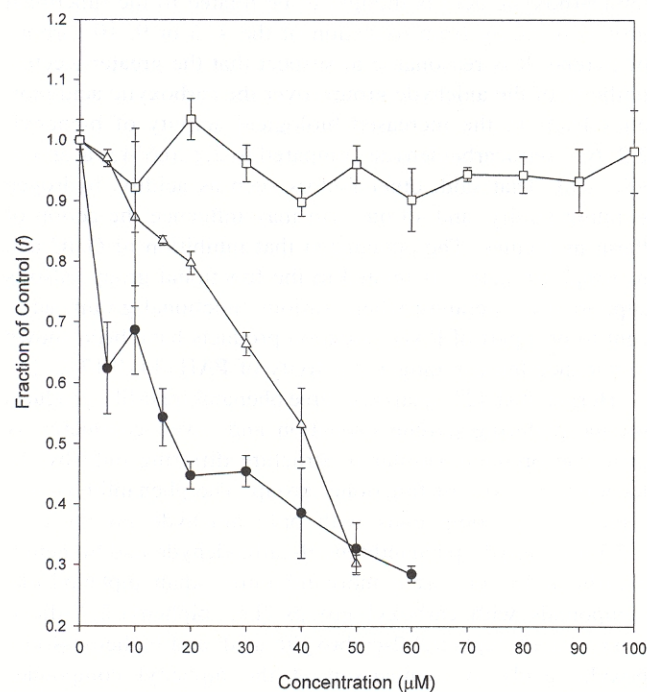
complete inhibition was reached within 1 h despite media replacement. Time recovery experiments were not performed for biphenyl-2,2',6,6'-tetracarboxylic acid because no significant difference in effects to GJIC were seen between for the concentrations tested (10–500 μM) and controls at a $p < 0.05$.

HL-60 neutrophil assay

Exposure of HL-60 cells to pyrene or its metabolites in the absence of PMA did not result in the generation of O_2^- (data

Fig. 4. Effect of concentration on gap junctional intercellular communication inhibition for pyrene (△), biphenyl-2,2',6,6'-tetracarbaldehyde (●), and biphenyl-2,2',6,6'-tetracarboxylic acid (□) for 30 min.
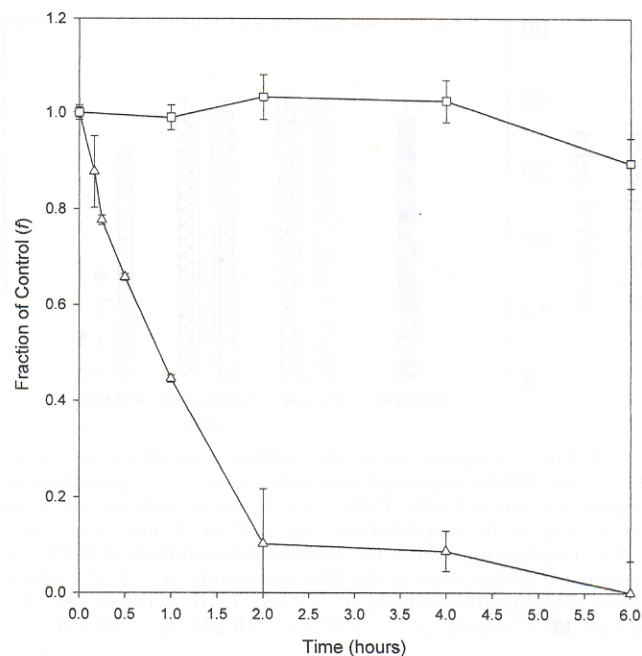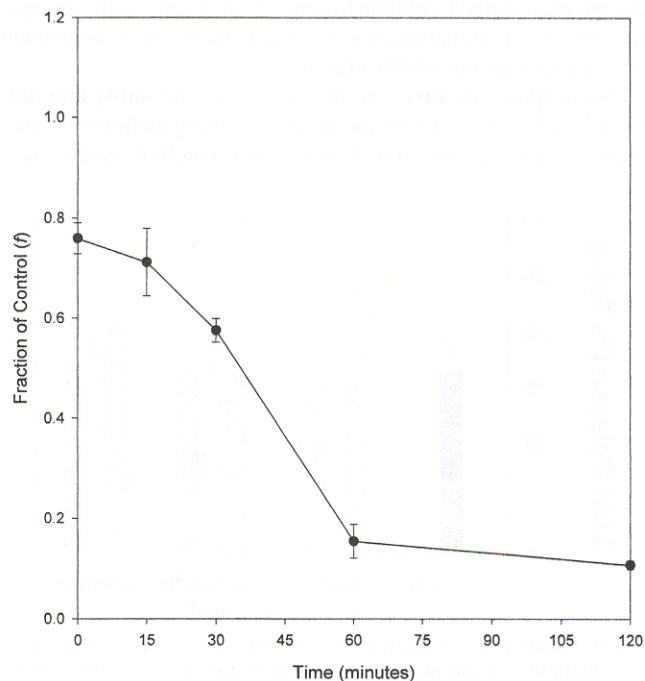

Fig. 6. Effect of recovery time on gap junctional intercellular communication of cells measured in cells exposed to biphenyl-2,2',6,6'-tetracarbaldehyde (5 μM) for 30 min, then rinsed, and allowed to recover.
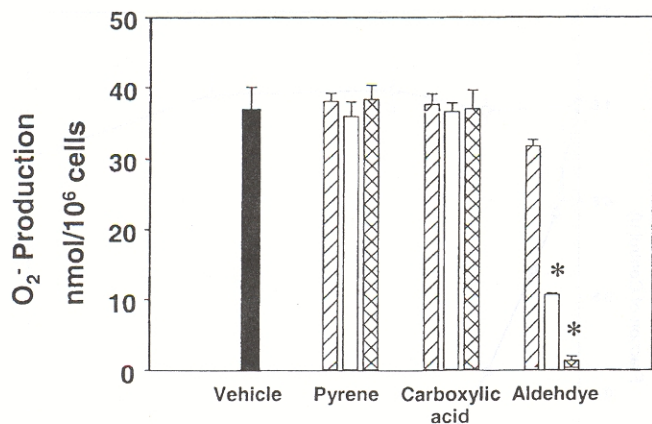
Fig. 7. Effect of pyrene and its metabolites on phorbol 12-myristate 13-acetate (PMA)–stimulated superoxide anion (O_2^-) generation by human cell line–60 cells. Cells were incubated with pyrene or its metabolites at the concentrations indicated for 30 min, after which PMA (1 ng/ml) was added. O_2^- produced after addition of PMA was determined as described in the *Methods* section. $n = 3$, (*) significantly different from vehicle ($p < 0.05$), LDH = lactate dehydrogenase, ■ = Vehicle; ▨ = 1 μM; □ = 10 μM; ⊠ = 40 μM.

not shown). The HL-60 cells stimulated with PMA produced O_2^-. The generation of O_2^- by PMA-stimulated cells was unaffected by treatment with either pyrene or the carboxylic acid derivative at concentrations up to 40 μM (Fig. 7). The tetraaldehyde derivative caused a concentration-dependent decrease in PMA-stimulated O_2^- production. The LDH release by HL-60 cells was not increased by exposure to either pyrene or its metabolites (Fig. 8), demonstrating that, at the concentrations used, these chemicals are not cytotoxic to the cells.

DISCUSSION

The effects of pyrene ozonation products were examined in two in vitro assays of biological activity. Each of these assays represents a cellular function that results from integration of cellular signaling and changes in each can be associated with effects on the whole organism.

Neutrophils are involved in nonspecific immunity and represent the first line of defense against invading pathogens. They become activated by stimuli to produce reactive oxygen spe-
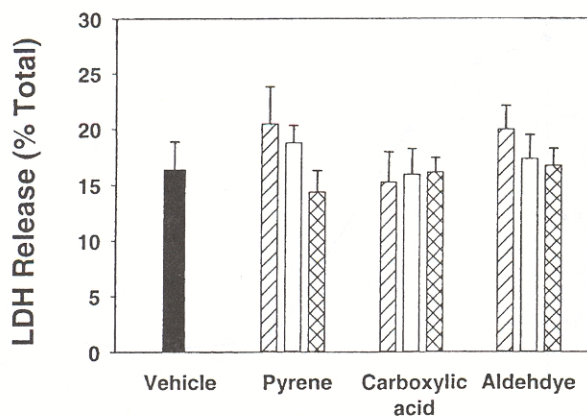

Fig. 8. Cytotoxicity of human cell line–60 cells exposed to pyrene or its metabolites. Cells were incubated with pyrene or its metabolites at the concentrations indicated for 30 min, after which phorbol 12-myristate 13-acetate (1 ng/ml) was added. Lactate dehydrogenase released by the cells was measured at the end of the 60-min incubation as described in the methods. $n = 3$. No significant differences were observed. ■ = Vehicle; ▨ = 1 μM; □ = 10 μM; ⊠ = 40 μM.

cies and release degradative enzymes that kill infecting bacteria. Alteration in function of neutrophils can affect host immunity as well as cause tissue injury. For example, inappropriate activation of neutrophils leads to tissue injury in a variety of models [56–59]. Conversely, inhibition of activation of neutrophils could lead to impaired ability of these cells to respond effectively to bacteria. The consequence of this effect would be an increased susceptibility to infection.

Neither pyrene nor the pyrene oxidation byproducts tested (other than biphenyl-2,2',6,6'-tetracarbaldehyde) caused the production of superoxide anion by HL-60 cells. Superoxide anion production in response to PMA was inhibited by biphenyl-2,2',6,6'-tetracarbaldehyde, suggesting a potential for increased susceptibility to infection in exposed organisms [60].

Interestingly, another aldehydic compound, benzyloxybenzaldehyde, inhibited reactive oxygen species production in neutrophils activated by a different stimulus [60]. The dependence on specific substitutions on pyrene metabolites for effects on neutrophil function is similar to observations with other environmental chemicals. For example, we have demonstrated that effects of polychlorinated biphenyls on neutrophil function are dependent on the presence of ortho-substituted chlorines on the phenyl rings [30].

Biphenyl-2,2',6,6'-tetracarbaldehyde also demonstrated an increased ability to inhibit GJIC as compared to pyrene. Time-response and time-recovery results indicated that irreversible damage to cells resulted from exposure to this compound at very low doses and short time periods. This indicates the potential for decreased intercellular communication and an increased chance of the disruption of cellular homeostasis in tissues that might lead to several toxicological endpoints, including tumor formation or neurotoxic effects.

The ability of biphenyl-2,2',6,6'-tetracarbaldehyde to inhibit GJIC and superoxide production more than 2,2',6,6'-tetracarboxylic acid is thought to be related to the functional groups resulting from oxidation of the 4, 5 or 9, 10 carbons of pyrene. It is reasonable to suspect that the greater electrophilicity of the aldehyde groups over the carboxylic acid moieties leads to the increased biological activity of biphenyl-2,2',6,6'-tetracarbaldehyde compared to 2,2',6,6'-tetracarboxylic acid. That said, other factors such as acidity, hydrogen bonding ability, and so on, also may influence the action of these molecules. The conclusion that inhibition of GJIC and neutrophil function is related to the functional groups also is supported in literature where various functional groups adjacent to or a part of PAH oxidation products have been shown to enhance the carcinogenic activity of PAHs [4–9,17–22].

Herner et al. [21] analyzed three phenanthrene-like products produced during pyrene ozonation and seven commercially available products similar in structure differing only by the location and type of functional group. The phenanthrene and biphenyl-like compounds with only aldehyde groups (i.e., 1,2,3,4-tetrahydrophenanthrene-9-carbaldehyde and biphenyl-2-carboxaldehyde) were more inhibitory than biphenyl-like compounds with carboxyl groups (i.e., biphenyl-2,2'-dicarboxylic acid, biphenyl-2-carboxylic acid, and biphenyl-4-carboxylic acid). A comparison of the biphenyl compounds showed that the biphenyl compounds with only aldehyde groups completely blocked communication ($f < 0.2$) at concentrations much lower than the biphenyl compounds with carboxylic acid groups ($f = 0.8$–1.0).

CONCLUSION

In this study, we determined that the functional group attached to the oxidized bonds (i.e., aldehyde or carboxylic) appears to determine the ability of a compound to affect cellular function. The presence of aldehyde groups in all four positions led to a compound with increased toxicity, and the presence of four carboxylic acid groups led to a compound that shows little to no effect in the toxicology studies at the levels tested. It is thought that the greater electrophilicity of the aldehyde groups over the carboxylic acid moieties leads to the increased biological activity. Other factors such as acidity, hydrogen bonding ability, and so on, also may influence the action of these molecules.

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