ABSTRACT — Environmental electrophiles readily interact with reactive sulfur species (RSS), resulting in decline of cellular RSS levels accompanied by formation of their sulfur adducts. In the present study, we examined the effects of combined environmental electrophiles on consumption of persulfide and on cytotoxicity in HepG2 cells. A convenient assay with SSP4, a fluorometric probe for detection of per/polysulfides, indicated that each environmental electrophile caused compound-dependent consumption of persulfide. Consumption of persulfide by combined exposure to methylmercury (MeHg) and cadmium (Cd) was greater than that of the single exposure. Consistent with this finding, combined exposure to environmental electrophiles (MeHg, Cd, and 1,4-naphthoquinone) exacerbated concentration-dependent cellular toxicity in HepG2 cells compared to single exposure to each compound. As humans are exposed to environmental electrophiles daily, more attention should be paid to the study of combined exposure to environmental electrophiles, which can modulate cellular levels of RSS and disrupt redox homeostasis.

Key words: Environmental electrophiles, Reactive sulfur species, Combined exposure, Exposome

INTRODUCTION

The exposome is defined as the totality of environmental factors that an organism is exposed to from its living environment, lifestyle, and diet (Rappaport and Smith, 2010; Wild, 2005). Environmental electrophiles are considered priority components of the exposome because they exist in multiple forms with high reactivity and ubiquitous distribution (Bohme et al., 2016). Examples of environmental electrophiles include naphthoquinones (NQs), formed during the combustion of gasoline and present in fine particulate matter (Cho et al., 2004); crotonaldehyde and 1,4-benzoquinone, present in tobacco smoke (Hecht, 1999); methylmercury (MeHg), which accumulates in edible fish such as tuna through biocondensation (Hanna et al., 2015); cadmium (Cd), found in rice (Li et al., 2017), and acrylamide, formed in foods during heating (Gokmen and Palazoglu, 2008). After entering the body, these chemicals covalently bind to nucleophilic substituents such as protein cysteine residues or DNA nitrogen atoms, creating protein and DNA adducts, respectively, which are associated with enzyme dysfunction or structural alterations and carcinoma (LoPachin and Gavin, 2016; Jan et al., 2015; Kanda et al., 2014; Saeed et al., 2007; Rogers et al., 1997).

Some environmental electrophiles, however, are readily trapped by reactive sulfur species (RSS) with high nucleophilicity, such as persulfides and polysulfides (e.g. $H_2S_2$, $H_2S_4$), resulting in the formation of their sulfur adducts (Kumagai et al., 2019; Shinkai and...
Kumagai, 2019). We previously reported that MeHg, Cd, and 1,4-NQ react with RSS to form bismethylmercury sulfide [(MeHg)₂S] (Yoshida et al., 2011), cadmium sulfide (CdS) (Akiyama et al., 2017), 1,4-NQ-SH, 1,4-NQ-S-1,4-NQ, and 1,4-NQ-S-1,4-NQ-OH (Abiko et al., 2017a, 2017b). In addition, (MeHg)₂S has been identified as a metabolite of MeHg in the liver of mice (Yoshida et al., 2011). (MeHg)₂S, CdS, and 1,4-NQ-S-1,4-NQ-OH appear to be less electrophilic and therefore less toxic than their parent electrophiles (Yoshida et al., 2011; Akiyama et al., 2017; Abiko et al., 2017b), indicating that RSS reduce the toxicity of electrophiles by forming inactive metabolites. Consistent with this notion, deletion of the RSS-producing enzyme cystathionine gamma-lase in mice has been shown to increase sensitivity to Cd-induced hepatotoxicity and MeHg-induced toxicity (Akiyama et al., 2017, 2019). However, it should be noted that formation of the sulfur adducts in this manner is accompanied by consumption of intracellular RSS, which are involved in cellular redox homeostasis (Ihara et al., 2017). For example, we found that exposing cells to MeHg elevated intracellular levels of the endogenous electrophilic molecule 8-nitro-cGMP, accompanied by depletion of RSS and electrophile-induced activation of redox signaling and consequent cell damage (Ihara et al., 2017). Because humans are exposed to a variety of environmental electrophiles daily, we postulated that combined exposure to electrophiles with differing structures would enhance consumption of RSS, owing to formation of the sulfur adducts in comparison with RSS consumption following exposure to a single environmental electrophile. In the present study, we examined the effects of single and multiple exposures to electrophiles on consumption of RSS and on cytotoxicity.

MATERIALS AND METHODS

Chemicals
Acrylamide and CdCl₂ were purchased from Wako Pure Chemical Industries (Osaka, Japan). Crotonaldehyde and MeHg were obtained from Nacalai Tesque (Kyoto, Japan). 1,2-NQ, 1,4-NQ, and 1,4-benzoquinone were obtained from Tokyo Chemical Industries (Tokyo, Japan). Sulfane Sulfur Probe 4 (SSP4) and sodium disulfide (Na₂S₂) were purchased from Dojindo (Kumamoto, Japan). β-(4-Hydroxyphenyl)ethyl idoacetamide (HPE-IAM) was obtained from Molecular Biosciences (Boulder, CO, USA). All other reagents and chemicals were of the highest grades available.

Fluorescent probe detection for RSS
Fluorescent probe detection can measure the reactivity of environmental electrophiles with RSS under multiple conditions simultaneously and conveniently by combining SSP4 (a fluorescent probe for RSS detection), Na₂S₂ (an RSS compound), and a multi-well plate. Each environmental electrophile was reacted with 40 μM Na₂S₂ in 20 mM HEPES buffer (pH 7.5) solution at 37°C for 15 min, followed by reaction with 20 μM SSP4 at 37°C for 15 min. After the reaction, the fluorescence intensity was measured at 515 nm (excitation: 482 nm) using a fluorescence spectrophotometer. The residual Na₂S₂ content was normalized by setting the fluorescence intensity when water was added instead of electrophiles as 100% and the upper value.

Liquid chromatography multiple-reaction monitoring mass spectrometry (LC-MRM-MS) analysis of persulfide
Levels of Na₂S₂ were measured as previously described (Akiyama et al., 2019). The sample was incubated with 5 mM HPE-IAM at 37°C for 30 min to yield HPE-AM adducts of sulfur nucleophiles including persulfide. Aliquots containing HPE-AM adducts were diluted four-fold with 0.1% formic acid containing known quantities of isotope-labeled internal standards, which were then analyzed by LC-MRM-MS/MS for persulfide determination.

Cell culture
HepG2 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan). The cells were cultured in minimum essential medium α (Wako) containing 10% fetal bovine serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mM GlutaMAX (Gibco, Carlsbad, CA, USA) in an incubator supplemented with 5% CO₂ at 37°C. Cells were pre-incubated in serum-free medium for 12 hr before treatment with compounds.

Cell viability
The 3-(4,5-dimethylthiazol-2-yl)-2,5-triphenyl tetrazolium bromide (MTT) assay was used to estimate cell viability as described previously (Denizot and Lang, 1986). Briefly, HepG2 cells in 96-well plates were exposed to MeHg with or without cadmium chloride and 1,4-NQ. After 24 hr, cells were treated with 5 mg/mL MTT for 4 hr at 37°C. After removing the medium, dimethyl sulfoxide (100 μL/well) was added to dissolve the formazan precipitate. Absorbance at 540 nm was read with an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).
**Statistical analysis**

Statistical significance was assessed using ANOVA with post-hoc correction for multiple comparisons. All statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). \( p < 0.05 \) was considered significant.

**RESULTS AND DISCUSSION**

We first measured the reactivity of the environmental electrophiles MeHg, Cd, 1,2-NQ, 1,4-NQ, 1,4-benzoquinone, crotonaldehyde, lead (Pb), and acrylamide with RSS separately. In the study, \( \text{Na}_2\text{S}_2 \) was used as a model for RSS because \( \text{H}_2\text{S}_2 \) was reported to be endogenously produced (Akaike et al., 2017). We reacted \( \text{Na}_2\text{S}_2 \) with each environmental electrophile and determined its consumption rate in relation to the reaction concentration of the electrophile using fluorescent probes. Incubation of the \( \text{Na}_2\text{S}_2 \) with MeHg, Cd, 1,2-NQ, and 1,4-NQ consumed more than 80% of \( \text{Na}_2\text{S}_2 \) at electrophile concentrations of 20-40 µM (Fig. 1). Incubation with 1,4-benzoquinone and crotonaldehyde consumed approximately 80% of the \( \text{Na}_2\text{S}_2 \) at an electrophile concentration of 80 µM, and little consumption was detected at 40 µM. Pb and acrylamide did not consume any \( \text{Na}_2\text{S}_2 \) at the measured concentrations under these conditions. The findings suggest that there is a structure specificity for interaction of electrophiles with RSS.

Next, we examined the effect of combined exposure to these environmental electrophiles on consumption of RSS and cytotoxicity. Combined exposure to MeHg and Cd resulted in a higher \( \text{Na}_2\text{S}_2 \) consumption rate than single exposures (Fig. 2A), a finding that was supported by quantitative measurements of \( \text{Na}_2\text{S}_2 \) by LC-MRM-MS (Fig. 2B). These findings suggest that combined exposure to environmental electrophiles may enhance the consumption of RSS, including persulfide, resulting in cytotoxicity even at concentrations that would not cause toxicity in single exposures. Consistent with this notion, combined exposure to MeHg and Cd exacerbated concentration-dependent cellular toxicity in HepG2 cells over single exposures (Fig. 3A). Furthermore, combined exposure to MeHg, Cd, and 1,4-NQ resulted in a further increase in cytotoxicity in HepG2 cells over combined exposure.

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**Fig. 1.** Consumption of persulfide by environmental electrophiles. \( \text{Na}_2\text{S}_2 \) (20 µM) was reacted with eight compounds at five concentrations for 15 min, and residual \( \text{Na}_2\text{S}_2 \) was then measured using a fluorescent probe to detect reactive sulfur species. Numbers indicate percentage changes in \( \text{Na}_2\text{S}_2 \) levels from control levels. MeHg, methylmercury; NQ, naphthoquinone; BQ, benzoquinone.
to MeHg and Cd or MeHg and 1,4-NQ (Fig. 3B). These findings indicate that cytotoxicity is exacerbated in proportion to the number of environmental electrophiles in the exposure. In other words, increasing the number of electrophiles in the exposure lowers the threshold of cytotoxicity.

Toxicological and pharmacological studies typically investigate the effects of exposure to a single substance, a premise that not mimic real-world situations or take into account the exposome, a measure of all environmental factors over time and their effects on health (Wild, 2005). Full characterization of the exposome remains a challenge owing to an enormous number of substances and the difficulty of evaluating their single and combined biological effects (Siroux et al., 2016; Niedzwiecki et al., 2019). Our findings in the present study suggest that combined exposure to environmental electrophiles reduces cellular RSS levels, possibly exacerbating cellular sensitivity to these compounds. Changes in in vivo levels of RSS may therefore be an indicator of the biological effects of environmental pollution.
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Conflict of interest---- The authors declare that there is no conflict of interest.

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