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Exposure to aerosol extract from heated tobacco products causes a drastic decrease of glutathione and protein carbonylation in human lung epithelial cells

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ABSTRACT

Heated tobacco products (HTPs) are an emerging class of tobacco goods that claim to have lower health risks than those of smoking combustible tobacco products. In this study, we exposed human lung epithelial cell lines to extracts prepared from HTP aerosols and combustible cigarette smoke to compare cytotoxicity. We focused on the effects of aldehydes present in the aerosols of HTPs at levels close to those in combustible cigarette smoke. Significant toxicity was confirmed for the HTP extract, albeit to a lesser extent than that with the combustible cigarette extract. When redox balance was evaluated by the oxidative loss of low-molecular-weight thiols in the cells, we found that total glutathione (GSH) contents and low-molecular-weight thiol levels were significantly decreased after exposure to the aerosol extract of HTPs. These results indicated that GSH is rapidly consumed during the detoxification of xenobiotics, such as aldehydes from tobacco extracts. Accordingly, exposure to the aerosol extract of HTPs resulted in the enhanced carbonylation of many proteins. In a simple comparison, the results for HTPs were significantly different from those obtained with combustible cigarette smoke, suggesting reduced toxicity of HTPs. However, we found significant and harmful effects after exposing lung epithelial cells to the aerosol extract of HTPs. Thus, a further comprehensive study is needed to clarify the lung damage induced via the long-term inhalation of aerosols from HTPs.

1. Introduction

Smoking is a global health problem. In the 20th century, tobacco-related diseases were the cause of death for over 100 million people and this number is still increasing. Recently, tobacco manufacturers have started to sell heated tobacco products (HTPs) as an alternative to conventional combustible cigarettes, thus reducing the amount of hazardous substances ingested. The most commonly used product is Philip Morris IQOS, launched in Japan and Italy in 2014, which is available in more than 40 countries. Rather than burning cigarettes, IQOS heats tobacco to approximately 350 °C to generate aerosols that contain nicotine. Hence, the manufacturers have advertised that the amount of hazardous materials in HTPs is 90% lower than that in conventional combustible cigarettes [1]. Indeed, many studies by third-party institutions have shown that the content of harmful substances (nicotine, tar, carbon monoxide, nitrosamines, etc.) is reduced [2–4]. However, certain chemicals are present in higher concentrations in the aerosols of HTPs than in conventional combustible cigarettes [5]. Notably, the amount of hazardous substances and their effects are not always proportional, and even a small amount of smoking might increase the risk of disease [6].

The mainstream smoke of combustible tobacco contains highly reactive carbonyl compounds such as acrolein and methylglyoxal

Abbreviations: ABD-F, 4-fluoro-7-sulfamoylbenzofurazan; AGEs, advanced glycation end products; DNPH, 2,4-dinitrophenylhydrazine; GSH, glutathione; GVP, gas-vapor phase; HTP, heated tobacco product; PI, propidium iodide; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TCEP, tris (2-carboxyethyl) phosphine hydrochloride.

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[7]. Particularly, acrolein can be found at high concentrations in tobacco smoke, and exposure to this compound via cigarette smoking has been associated with acute lung injury [8]. Reactive carbonyl compounds easily react with and modify proteins, lipids, and DNA, thereby interfering with their functions and causing cytotoxicity through oxidative damages [9]. In a previous study on bronchial epithelial cells, the carbonylation of proteins regulating lipid metabolism, energy production system, and cell cycle was observed following exposure to a combustion tobacco smoke extract [10]. That study demonstrated that carbonyl compounds may affect the maintenance of homeostasis and function of bronchial epithelial cells [10].

Meanwhile, the mainstream aerosol of HTPs also contains reactive carbonyl compounds like those in conventional combustible cigarettes [3,4]. There has been a report indicating that the quantity of acrolein in HTPs does not differ significantly from that of cigarettes [11]. Reports by manufacturers (Philip Morris International and Japan Tobacco Inc.) have shown that the cytotoxicity and oxidative stress caused by the ingestion of mainstream from HTPs aerosol are much lower than those from combustible cigarette smoke [1,12]. Although several reports have investigated the cytotoxicity and oxidative stress caused by HTPs, the rationale for setting exposure levels has not been fully explained [13–15]. Moreover, the carbonylation of proteins by HTPs has not been sufficiently studied and limited information is available on the prooxidant effects of HTPs on the human lung.

In this study, tobacco extracts were prepared from gas-vapor phase (GVP) containing chemicals such as aldehydes based on the methods presented by Health Canada [16]. We evaluated the effects on human lung epithelial cells following exposure to the GVP extracts collected from the mainstream smokes of combustible tobacco products and aerosols of HTPs. This study presents basic data on the possible health risks associated with the use of HTPs under appropriate conditions.

2. Materials and methods

2.1. Reagents

Acrolein was obtained from AccuStandard (CT, USA). Propidium iodide (PI) was purchased from Sigma Aldrich (MO, USA). Hoechst 33342 and 4-Fluoro-7-sulfamoylbenzofurazan (ABD-F) were obtained from Dojindo (Osaka, Japan). 2,4-Dinitrophenylhydrazine (DNPH) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Preparation of 3R4F cigarette smoke or IQOS aerosol GVP extract

A schematic diagram of the experimental apparatus used for the preparation of conventional cigarette smoke or IQOS aerosol GVP extract is shown in Supplemental Fig. 1. As a reference conventional cigarette, 3R4F from the University of Kentucky (Lexington, KY, USA) was used. For IQOS, the Marlboro HeatSticks Regular was used with the IQOS3 device. Tobacco sticks and conventional cigarettes were used for measurement after being placed at 22 °C with 60% humidity for more than 48 h. To align the smoking conditions, the Health Canada Intense (HCI) regime was used to generate aerosols from all test products [17]. In the HCI regime, mainstream smoke constituents were collected at a puff volume of 55 mL, puff duration of 2 s, and puff interval of 30 s, with 100% blocking of the filter ventilation holes, at room temperature of 22–24 °C and relative humidity of 55–70%. 3R4F cigarettes were smoked on an LX20 linear 20-port piston type smoking machine (Heinrich Borgwaldt GmbH, Hamburg, Germany) and IQOS heatsticks were smoked on a LM4E linear smoking machine for vaporizer (Heinrich Borgwaldt GmbH, Hamburg, Germany). The mainstream smoke or aerosol was passed through a 44 mm glass fiber filter, to remove the particle phase, and the remaining GVP was immobilized into an impinger containing 30 mL of ice-cold Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The extracts contained 3R4F smoke from three cigarettes or IQOS aerosol from six sticks. All extracted solutions were filtered through 0.22 μm filter (Millex-GP, Millipore, Billerica, MA, USA), aliquoted into 1.2 mL volume in 1.5 mL screw cap tubes and stored at −80 °C until use.

2.3. Cell culture and cell treatment

Human lung epithelial type II cells, A549, were purchased from the Health Science Research Resources Bank (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L D-glucose, 110 mg/L sodium pyruvate supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere (5% CO_2 in air). A549 cells were cultured in flasks until they reached 85–90% confluence. The cells were then harvested with 0.05% trypsin-0.2 mM EDTA solution and plated in a culture dish at a density of 0.8×10^4 cells/cm^2. After the cells were cultured for 24 h, the medium was completely removed, and the cells were exposed to different concentrations of acrolein, 3R4F extract, or IQOS extract diluted with PBS mixed with an equivalent volume of culture medium. After incubation for the indicated times, they were subjected to the following assays.

2.4. Cell survival assay

Cytotoxicity was evaluated by the Hoechst 33342/PI staining assay [18,19]. Cells preincubated in 96 well plates were exposed to 100 μL of acrolein, 3R4F smoke extract, or IQOS aerosol extract for 48 h. Following the addition of 100 μL phosphate buffered saline (PBS) containing 2 μg/mL Hoechst 33342 and 2 μg/mL PI, the suspension was incubated in the dark at 37 °C for 30 min. Images of the resulting fluorescence from each well were analyzed using an IN Cell Analyzer 2200 (GE Healthcare, Buckinghamshire, UK) to detect the number of dead (PI positive) cells and Hoechst 33342 positive cells per well.

2.5. Measurement of low-molecular-weight thiols

Intracellular low-molecular-weight thiol levels were determined as described previously [20]. Briefly, after incubation in 60 mm diameter petri dishes under the indicated experimental condition, cells were detached with 0.05% trypsin-0.2 mM EDTA solution, lysed with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) containing 0.5% Triton X-100, 0.5 mM EDTA, and a protease inhibitor cocktail. Following the addition of 1/9 volume of 25% (w/v) 5-sulfosalicylic acid, the lysate samples were centrifuged at 21,500×*g* and 4 °C for 5 min. A portion (30 μL) of the supernatant was added to 150 μL of 0.25 M Tris–HCl (pH 8.2) buffer containing 20 mM EDTA; then, 10 mM 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added to the solution and incubated at room temperature for 15 min. The absorbance of each sample was measured at 412 nm using a Spark 10 M microplate spectrophotometer (Tecan, Switzerland).

2.6. Quantitation of glutathione

Intracellular total amounts of glutathione (reduced form, GSH plus the oxidized form, GSSG) levels were determined by derivatization with ABD-F, which specifically reacts with thiol residues to

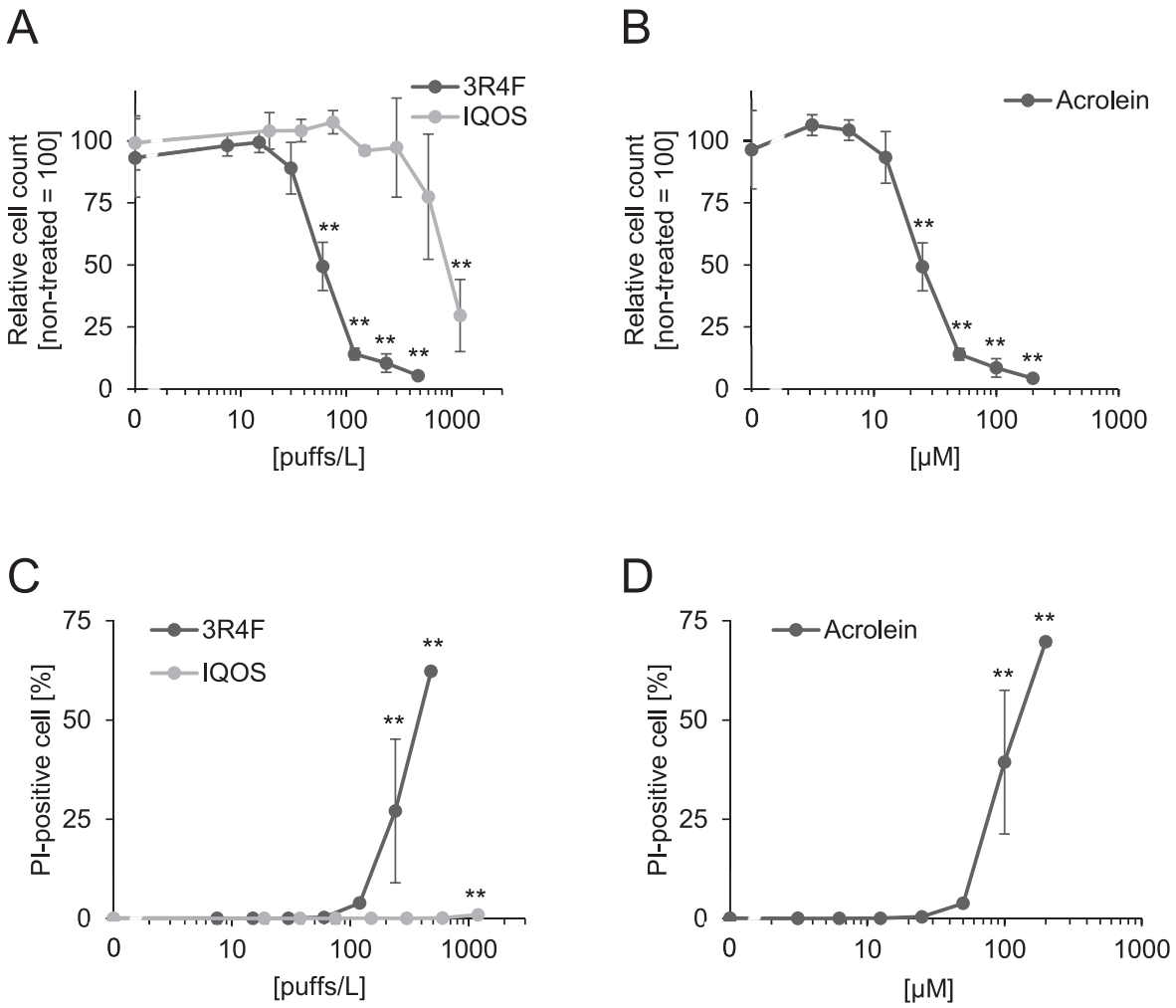

Fig. 1. Cytotoxicity of cigarette smoke or IQOS aerosol extract in A549 cells.
A549 cells were exposed to 3R4F-derived extract, IQOS-derived extract, or acrolein for 48 h, and the total number of cells (A, B) and ratio of dead cells (C, D) were evaluated using a Hoechst 33342/propidium iodide (PI) staining assay. Data are shown as the mean value ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, significantly different from each non-treated control (0 puffs/L or 0 μM).

form a fluorescent adduct, using a previously reported high-performance liquid chromatography with fluorescence detection (HPLC-FLD) method [21], with minor modifications. Briefly, the clear supernatant (4 μL) of the 5-sulfosalicylic acid-treated cell lysate was neutralized by the addition of 4 vol of 0.2 M boric acid-sodium borate buffer (pH 10.5), and the mixture (20 μL) was then added to 60 μL of 5.5 mM ABD-F with 2.5 mM TCEP and incubated at 60 °C for 10 min. Subsequently, 10 μL of 5 M $HClO_4$, were added and the mixture was centrifuged at 21,500×*g* at 4 °C for 5 min, and 10 μL of the supernatant was applied to HPLC-FLD which was performed using a Shimadzu RF-20A fluorescence detector connected to an LC-20AD chromatograph (Kyoto, Japan). A COSMOSIL 5C18-MS-II reverse phased column (4.6 mm × 250 mm, Nacalai Tesque) was used at a flow rate of 1.0 mL/min at 35 °C. The mobile phase consisted of 0.1 M acetic acid-sodium acetate buffer (pH 3.8): acetonitrile (92:8) was used with an isocratic elution. The retention times and peak areas were monitored at excitation and emission frequencies of 380 and 510 nm, respectively.

2.7. Spectrophotometric assay of protein carbonyl

The concentration of protein carbonyl in A549 cells was assayed according to a previously described method [22], with minor modifications. Briefly, after incubation in 90 mm diameter petri dishes with the indicated experimental conditions, cells were detached with 0.05% trypsin-0.2 mM EDTA solution, and lysed with RIPA buffer (Millipore) containing protease inhibitor cocktail. Following the addition of a 1/9 volume of 10% (w/v) streptomycin sulfate solution to remove nucleic acids, the lysate samples were centrifuged at 14,000×*g* and 4 °C for 5 min. The supernatant (50 μL) obtained from the cell lysate was reacted with 250 μL of 10 mM DNPH in 2 M HCl or HCl only (blank) at 37 °C for 60 min in the dark and mixed every 15 min. Following the reaction, the proteins were precipitated with 33 μL of trichloroacetic acid, washed three times with ethanol-ethyl acetate (1:1, v/v) mixture, and solubilized in 100 μL of 6 M guanidine hydrochloride-trifluoroacetic acid buffer (pH 2.3) containing 20 mM potassium dihydrogen phosphate. The absorbance of each sample was measured at 375 nm using Spark 10 M microplate spectrophotometer, with the corresponding blank as the reference.

2.8. Western blot analysis

Intracellular carbonylated proteins were detected using the OxySelect™ protein carbonyl immunoblot kit (Cell BIOLABS Inc., San Diego, CA, USA) according to manufacturer's protocol. A549 cell

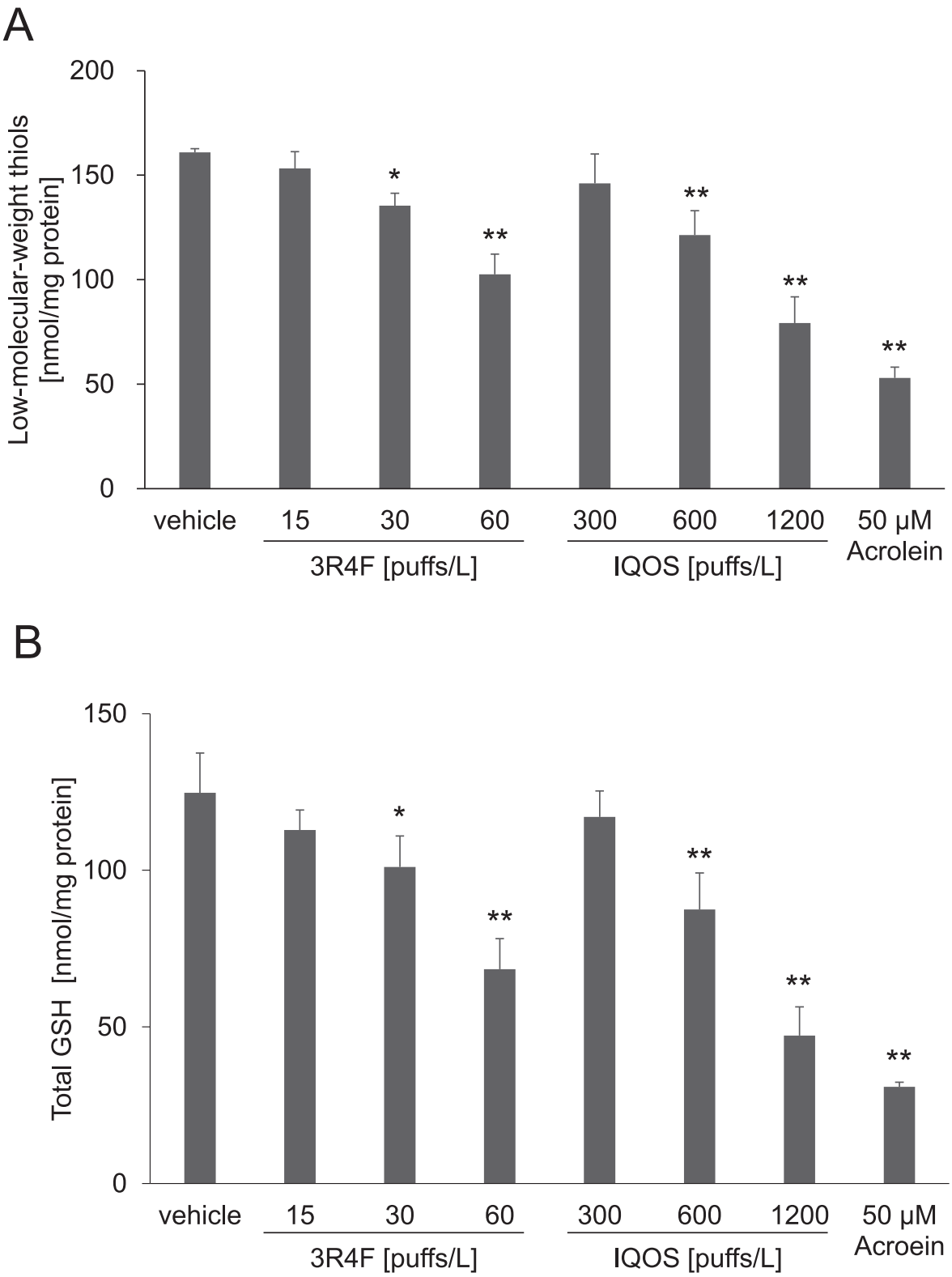

Fig. 2. Intracellular total glutathione levels in A549 cells exposed to cigarette smoke or IQOS aerosol extract.
Cells were exposed to 3R4F-derived extract, IQOS-derived extract or acrolein for 2 h, and intracellular low-molecular-weight thiols (A, B) and total amounts of glutathione (C, D) were determined after reduction by TCEP. Data are shown as the mean value ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, significantly different from vehicle (PBS).

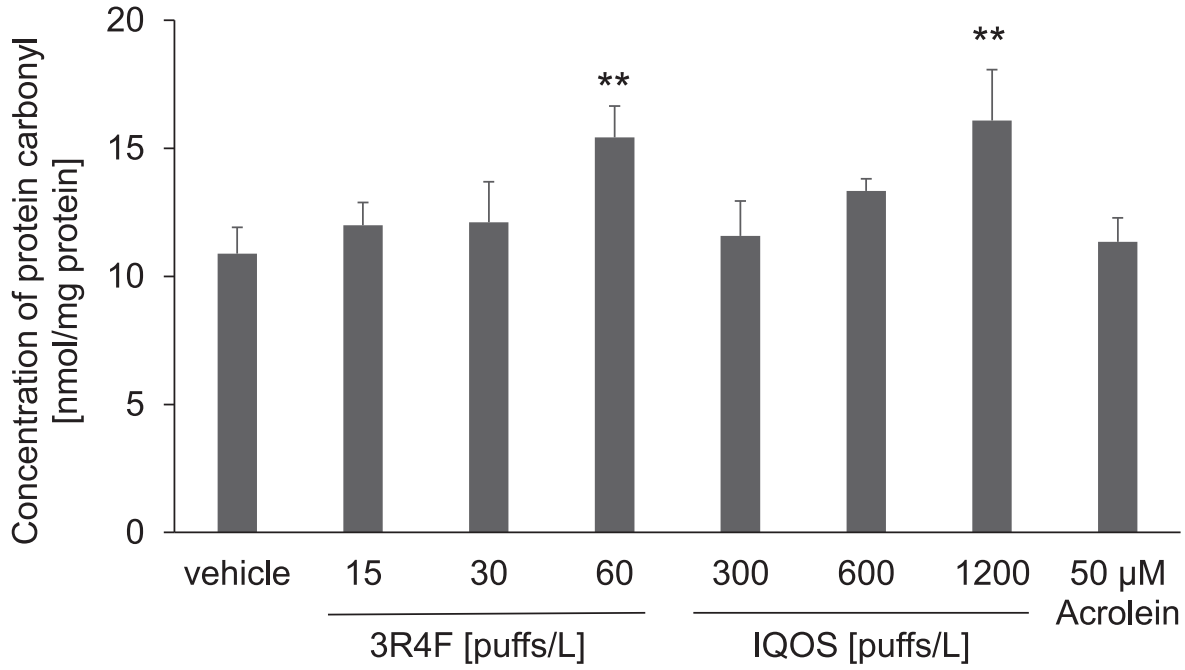

Fig. 3. Determination of total protein carbonyl levels in A549 cells.
The total amount of protein carbonyl in A549 cells was determined by a spectrophotometric assay. Cells were exposed to 3R4F-derived extract, IQOS-derived extract or acrolein for 2 h. Data are shown as the mean value ± SD (n = 3). $^{**}p < 0.01$, significantly different from vehicle (PBS).

lysates for Western blot analysis were prepared using RIPA buffer. A total of 8 μg of protein from the cell lysate were boiled for 3 min in SDS sample buffer (Nacalai Tesque). The proteins were then separated using a 5–20% gradient gel (ATTO, Tokyo, Japan) and transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane was incubated for 5 min in a DNPH solution at room temperature and washed three times with 2 M HCl, and three times with methanol. The washed membrane was blocked using 4% (w/v) Block Ace (DS Pharma Biomedical, Osaka, Japan) for 1 h at room temperature and washed in PBS containing 0.1% (w/v) Tween 20 (PBS-T), after which the membrane was incubated for 1 h with a rabbit anti-DNP primary antibody (1:2000 dilution) in Can Get Signal Solution 1 (Toyobo, Osaka, Japan). The membranes were then washed three times for 5 min in PBS-T, and then incubated for 1 h with horseradish peroxidase-conjugated secondary anti-rabbit antibody diluted at 1:4000 with Can Get Signal Solution 2 (Toyobo, Osaka, Japan). Subsequently, the membranes were washed five times for 5 min in PBS-T, and then chemiluminescence from the protein bands was detected using Crescendo Western Reagents (Millipore) in a LuminoGraph I (ATTO, Tokyo, Japan).

2.9. Statistical analysis

Values are presented as the mean ± standard deviation (SD) of at least three independent experiments. One-way analysis of variance and Dunnett or Tukey comparison post-tests were used to compare the means of three or more groups. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Cytotoxicity of 3R4F cigarette smoke and IQOS aerosol GVP extract

To compare the cytotoxicity of 3R4F cigarette smoke and IQOS aerosol GVP extract, A549 cells were exposed to the extracts, and the number of living cells was counted by a Hoechst 33342/PI staining assay. Additionally, A549 cells were exposed to acrolein, which is considered to contribute to the toxicity of tobacco extracts [23], as a reference. The 3R4F-derived extract, IQOS-derived extract, and acrolein caused a dose-dependent decrease in living cells (Fig. 1A and B). The 50% inhibitory concentrations (IC50) with respect to cell viability were 59.7 ± 9.4 puffs/L, 879.1 ± 245.8 puffs/L and 25.0 ± 3.7 μM for the 3R4F-derived extract, IQOS-derived extract, and acrolein, respectively. In terms of the ratio of dead cells, exposure to IQOS-derived extract at a concentration of 1200 puffs/L caused a slight increase in dead cells, whereas a significant increase in cell death was observed upon exposure to 3R4F-derived extract at a concentration greater than 240 puffs/L. A ratio of dead cells over 50% was observed at 480 puffs/L for the 3R4F-derived extract, for which the cytotoxicity was equal to that with exposure to 200 μM acrolein (Fig. 1C and D).

3.2. Alteration of low-molecular-weight thiols in cells treated by cigarette smoke and IQOS-derived extracts

Intracellular low-molecular-weight thiol levels were quantified by the DTNB method to evaluate the state of oxidative stress in the cells treated with IQOS-derived extracts for 2 h. Both 3R4F and IQOS-derived extracts decreased the low-molecular-weight thiol levels in a concentration dependent manner (Fig. 2A). When the total intracellular GSH levels were determined using the HPLC-FLD method, concentration-dependent decreases were observed in the 3R4F and IQOS-derived extracts (Fig. 2B).

3.3. Accumulation of total protein carbonyl in cells treated with cigarette smoke and IQOS-derived extracts

The concentration of protein carbonyl formed by the treatments with cigarette smoke and IQOS-derived extracts was determined by a spectrophotometric assay. As shown in Fig. 3, a significant

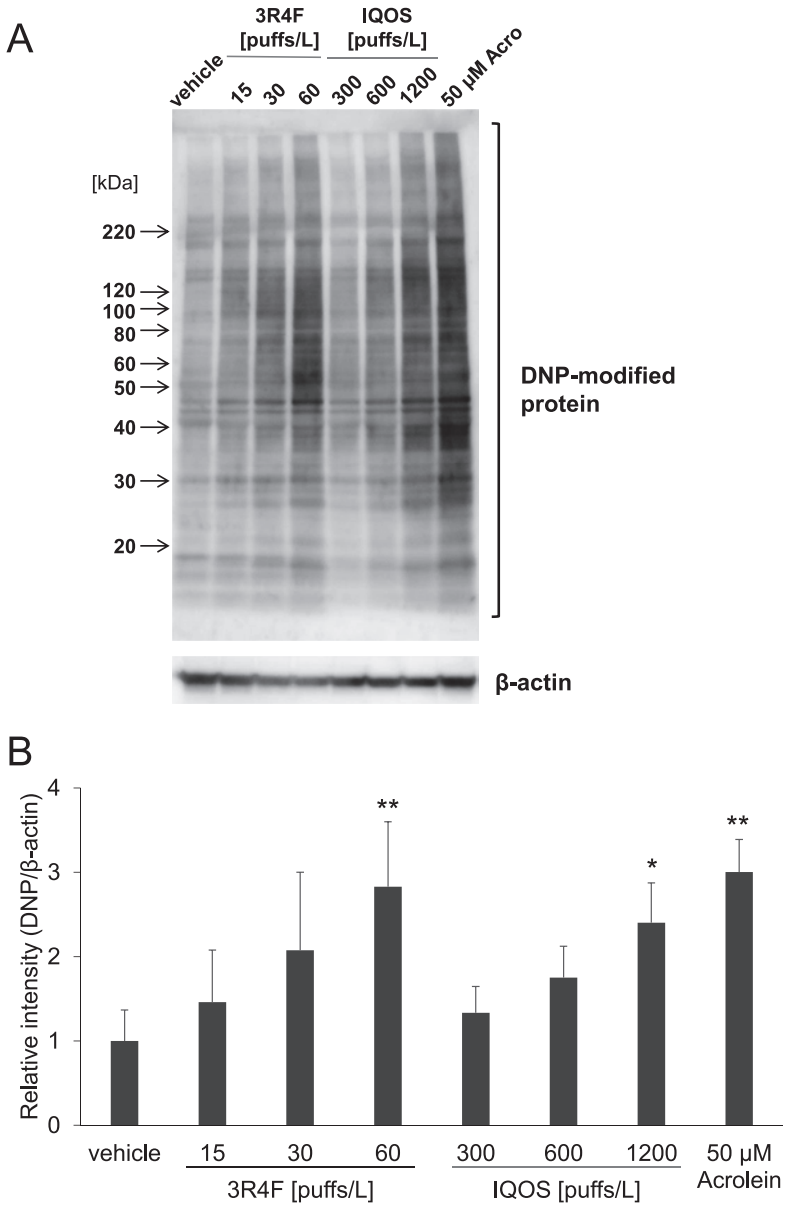

Fig. 4. Western blot analysis of carbonylated protein in A549 cells exposed to cigarette smoke or IQOS aerosol extract.
A549 cells were exposed to 3R4F-derived extract, IQOS-derived extract or acrolein for 2 h. (A) Proteins of A549 cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane. After the membrane was incubated with DNPH, the DNP-modified protein was detected using an anti-DNP antibody (top panel). β-actin was used as a protein loading control (bottom panel). (B) The density of total DNP-modified bands was measured and the ratio to β-actin band density was expressed as a fold-change relative to the total band levels measured in vehicle samples. Data are shown as the mean value ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, significantly different from vehicle (PBS).

increase in protein carbonyl was observed after exposure to 60 puffs/L 3R4F or 1200 puffs/L IQOS extracts for 2 h.

3.4. Analysis of carbonylated proteins in cells treated with cigarette smoke and IQOS-derived extracts

We observed the formation of carbonylated proteins by Western blot analysis in cells exposed to 3R4F or IQOS-derived extracts for 2 h. As shown in Fig. 4A, each extract caused the carbonylation of various proteins. The total band densities of carbonylated proteins in each lane were significantly increased by exposure to 60 puffs/L 3R4F and 1200 puffs/L IQOS-derived extract for 2 h (Fig. 4B).

4. Discussion

Previous studies have reported that HTPs contain fewer chemicals than combustible cigarettes. However, this does not mean that HTPs do not contain any hazardous chemicals. In fact, some substances are present in higher amounts in HTPs than in conventional cigarettes, and some are only found in HTPs [5]. Similar to combustible cigarettes and e-cigarettes, HTPs contain hazardous substances and can increase oxidative stress in smokers, thus

leading to respiratory inflammation and diminished resistance to infectious diseases [15]. Additionally, HTPs release finer particulate matter (2 μm), equal to or smaller than those of cigarettes [24].

In this study, we exposed human lung epithelial cell lines to extracted solutions prepared from combustible cigarette smoke and HTP aerosols to evaluate and compare their cytotoxicity. We focused on the effects of aldehydes reportedly present in the aerosols of HTPs at levels close to those in combustible cigarettes [3,11]. Acrolein was used as a positive control to show harmful effects because it is thought to be a key oxidative stress inducer among the volatile aldehydes in cigarette smoke [23]. Significant toxicity was confirmed in HTP aerosols, albeit to a lesser extent than that in combustible cigarette smoke. Moreover, a significant decrease in the total amount of low-molecular-weight thiols was caused by exposure to aerosol extract of HTPs. Although previous studies have shown the ratio of oxidized to reduced forms of GSH after exposure, significant increases in the oxidized forms of GSH (GSSG) were not observed after exposure to both tobacco extracts in this study (data not shown). However, we found that total GSH contents in cells were drastically decreased after exposure to not only cigarette smoke extract but also aerosol extract of HTPs (Fig. 2). It has been demonstrated that a rapid decrease in GSH contents is induced in cells by incubation with acrolein [25–27]. Thus, our observations indicate that GSH is rapidly consumed during the detoxification metabolism of aldehydes, such as acrolein, in HTP aerosols. By assessing intracellular protein carbonylation, we found that exposure to HTP aerosol extract resulted in the enhanced carbonylation of many proteins (Figs. 3 and 4). Further, individual detection of acrolein-modified protein was attempted; however, no specific advanced glycation end products (AGEs) were observed after exposure to tobacco extracts. In the future, identifying the carbonyl compounds in HTP aerosols, which are precursors for AGEs, is warranted based on a detailed analysis of carbonylated proteins that accumulate in cells upon exposure to the aerosol extract of HTPs.

Davis et al. compared the cytotoxicity in pulmonary fibroblasts exposed to aerosol extract prepared from IQOS with cytotoxicity to combustible cigarettes [13]. The toxicity observed after exposure to high levels of IQOS-derived vapor components was comparable to that following exposure to conventional combustible cigarettes. Recently, Dusautoir et al. compared the cytotoxicity of IQOS with that of burning cigarettes and aspiration extracts prepared from e-cigarettes using bronchial epithelial cells and reported that HTPs might be less harmful than tobacco cigarettes but more harmful than e-cigarettes [28]. These *in vitro* studies warned that the long-term inhalation of HTPs might have a harmful effect on the human respiratory system. However, because the use of HTPs such as IQOS has spread rapidly over the past 7–8 years, there is a lack of adequate data from scientific studies to determine the biological effects and toxicological risk of HTPs.

In conclusion, the present study revealed a significant harmful effect on human lung epithelial cells following exposure to the aerosol extract of HTPs. The effects of HTPs were approximately 5–10% of those arising from exposure to combustible cigarette smoke extract. Therefore, although HTPs are regarded as weak bio-affected cigarettes, they are not encouraged as an alternative to combustible cigarettes because their use can have a gateway effect. Indeed, long-term exposure to glycerin via e-cigarettes has been shown to adversely affect the immune system [29], and HTPs with elevated levels of glycerol might have similar effects. Thus, evaluating the effects of long-term exposure to HTPs *in vivo* is urgently required. The findings of these studies could provide a definitive assessment of the effects associated with the use of HTPs on human health.

Declaration of competing interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.12.004.

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