5-Hydroxy-2-methylpyridine Isolated from Cigarette Smoke Condensate Aggravates Collagen-Induced Arthritis in Mice

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The risk of rheumatoid arthritis (RA) is linked to environmental and genetic factors. Cigarette smoking is an established environmental risk factor for the disease that contributes to its development and severity. Previously, we found that cigarette smoke condensate (CSC), both mainstream and sidestream, aggravates collagen type II-induced arthritis (CIA), which was observed following either inhaled or nasal exposure. In the present study, we aimed to identify the compound in CSC, which aggravates CIA. By sequential fractionation and analysis, extraction with water/ether in different pH values, silica gel column chromatography, TLC, octadecyl silica (ODS) HPLC, GC/MS, and NMR, the active compound was identified as 1H-hydroxy-2-methylpyridine (1H2MP). Its isomer 2-hydroxy-3-methylpyridine, but not 3-hydroxy-2-methylpyridine, was also active. 1H2MP was not mutagenic, and did not exhibit aryl hydrocarbon receptor-dependent activity. Our data help clarify the mechanism underlying the pathogenic effects of cigarette smoking on RA.

Key words cigarette smoke; rheumatoid arthritis; polycyclic aromatic hydrocarbon

Rheumatoid arthritis (RA) is a disease affecting approximately 1% of the world’s population. The onset of RA occurs most frequently between 40 and 50 years of age, but younger people aged between 10 and 20 years are also affected. RA constitutes a chronic inflammatory condition affecting multiple joints that is characterized by a proliferation of synoviocytes, which are responsible for the formation of pannus and the production of proinflammatory cytokines and chemokines, which lead to the destruction of the articular cartilage and ankylosis of the joints.1

The risk of RA is linked to genetic and environmental factors, such as infection, UV radiation, and cigarette smoking.2 Cigarette smoking is an established risk factor for this disease.2,4,5 Epidemiological studies indicate an association of cigarette smoking with disease incidence outcomes in patients with early inflammatory polyarthritis,5 and increases rheumatoid factor and nodule formation in patients with RA.6 A strong association has been observed between heavy cigarette smoking and RA, particularly in patients without a family history of the disease.7 Patients with early RA, who smoke, are less likely to respond to treatment with methotrexate and tumor necrosis factor inhibitors than patients who do not smoke.8 An increased risk of developing RA was reported in individuals with a heavy8,10 or light lifetime burden of smoking9 than that in individuals who never smoked. The risk associated with smoking is extremely high in individuals, both men and women, with the shared epitope (SE) in HLA-DRB1, which is a major genetic risk factor for RA.12 A strong association with RA has been shown in individuals with respect to cigarette smoking, HLA-DRB1, and anti-citrullinated protein antibody (ACPA), which has been implicated as a biomarker for RA and its activity.4,13 Antibodies against the citrullinated form of the synovial antigen, vimentin, are specific for RA and associated with HLA-DRB1*0401.14,15 Citrullinated proteins are generated by peptidyl arginine deiminases (PADs), which convert arginine residues to the neutral citruline. In genetically susceptible individuals, cigarette smoke plays an important role in ACPA production by inducing PAD expression and citrullination of lung proteins in bronchial macrophages.15

The link between smoking and the development of RA has been demonstrated through in vitro studies and in animal models of RA. We have previously reported that polycyclic aromatic hydrocarbons (PAHs) up-regulate expression of interleukin-1β (IL-1β) mRNA in the RA patient-derived SV40 T antigen-transformed human synovial fibroblast cell line MH7A.16 Moreover, we also showed that cigarette smoke condensate (CSC) induces the production of proinflammatory cytokines at both the mRNA and protein level in MH7A cells,17 and that CSC induces IL-1β production from RA patient-derived synoviocytes, but not from osteoarthritis (OA) patient-derived synoviocytes or normal fibroblasts, through aryl hydrocarbon receptor (AhR)-dependent nuclear factor-kappaB (NF-κB) activation.18 We also showed that CSC, both

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mainstream and sidestream, aggravates arthritis in a mouse model of collagen type II-induced arthritis (CIA), following either intraperitoneal inoculation or nasal exposure. However, the active chemical in CSC responsible for this aggravation is unknown. In the present study, we aimed to identify the CSC compound aggravating CIA.

MATERIALS AND METHODS

**Reagents**  Bovine type II collagen was obtained from KOKEN Co., Ltd. (Tokyo, Japan). Incomplete Freund’s adjuvant (ICFA) and Mycobacterium butyricum were produced from BD (Tokyo, Japan). The endotoxin test kit, Endospecy ES-24S Kit, was purchased from SEIKAGAKU BIOPBUSINESS CORPORATION (Tokyo, Japan). The detection limit was 0.001 EU/mL, where one EU indicates 0.1 ng/mL of *Escherichia coli* 055:B5-derived endotoxin. Silica Gel H thin-layer plates (20 × 20 cm) having a layer thickness of 1 mm were manufactured by Merck Co., Ltd. (Tokyo, Japan). 5-Hydroxy-2-methylpyridine (5H2MP), 3,5-dimethoxy-4-hydroxyacetophenone (3,5D4HAP), 2-hydroxy-3-methylpyridine (2H3MP), 3-hydroxy-2-methylpyridine (3H2MP), benzo[α]pyrene, nicotine, RPMI 1640, Dulbecco's modified Eagle's medium, and polymyxin B were acquired from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (Logan, UT, U.S.A.). Other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Preparation of CSC**  CSC was prepared as described previously. A common American brand of cigarette was used in this study, Lark Mild 100s, was used in this study. Each cigarette was 84 mm long, 2.5 mm in circumference, and had a charcoal filter that normally adsorbs 9 mg tar and 0.8 mg nicotine. Particulate matters from mainstream smoke were collected in a canister filter using a suction pump, and the particulate matter was extracted by sonication with benzene–ethanol mixture (1:3, v/v). The extract was filtered and evaporated to dryness, and the residue was then redissolved in ethanol to yield CSC. The yield of mainstream CSC was 9.25 mg per cigarette. One milligram of CSC did not contain endotoxin, as determined by the endotoxin test.

**Fractionation of CSC by pH**  By addition of NaOH to CSC, the pH was adjusted to 13.0, and an equal volume of ether was then added. After shaking, the ether layer (13-ether) and water layer (13-water) fractions were obtained. By addition of HCl to the 13-water fraction, the pH was adjusted to 9.0. An equal volume of ether was then added, and 9-ether and 9-water fractions were obtained. By the same procedure, 6-ether, 6-water, 4-ether, and 4-water fractions were obtained (Fig. 1).

**Fractionation of the CSC 9-Ether Fraction by Column Chromatography**  A silica gel-packed column (20 cm having
a diameter of 30 mm) was washed with chloroform–methanol (20:1) solvent, and 1 mL of the CSC 9-ether fraction was applied to the top of the column. The chloroform–methanol (20:1) solvent was allowed to flow through the column, and eluted fractions (3 mL each) were collected. The residual CSC was eluted with methanol, and a total of 20 fractions were obtained in total. By collecting five fractions (from 1–5, 6–10, 11–15, and 16–20), the CSC 9-ether fraction was separated into four fractions (frs. 1 to 4) (Fig. 1).

**Fractionation by TLC** Fraction 1 was applied to silica gel H thin-layer plates (20×20 cm), having a layer thickness of 1 mm, and development was carried out using chloroform–methanol (20:1) solvent. After development, the plate was exposed to iodide vapor. Materials with Rf 0.72–0.417 were collected as fr. 1-1. Similarly, fr. 1-2 (Rf 0.417–0.31), fr. 1-3 (Rf 0.31–0.17), and fr. 1-4 (Rf 0.17–0) were obtained. Each fraction was extracted with methanol, and then dried using a rotary evaporator (Fig. 1).

**Fractionation of CSC by HPLC** Fractions 1-1 to 1-4 were loaded on to an Inertsil ODS-3 (7.6×250 mm 5 μm) column (GL Sciences Inc., Tokyo Japan), and eluted with methanol–water (40:60) at a flow rate of 3.0 mL/min with UV detection at 254 nm using a HPLC apparatus (pomp: LC-20AT, UV detector: SPD-10A, analytical software: Labsolutions, SHIMADZU CORPORATION, Kyoto, Japan).

**GC/MS Analysis** GC/MS analysis was performed using the Micromass GCT Premier Mass Spectrometer (Waters Corporation, Milford, MS, U.S.A.) with electron ionization (70 eV), and the 7890A (Agilent) Gas Chromatograph equipped with a quadrupole mass detector (Identification: X044, UV detector: SPD-10A, analytical software: Labsolutions, SHIMADZU CORPORATION, Kyoto, Japan).

**Animals** Specific pathogen-free DBA/1J male mice were purchased from Japan SLC, Inc., Hamamatsu, Japan, and maintained under specific pathogen-free conditions. Standard laboratory food and water were provided ad libitum to the mice. This study was approved by the animal ethics committee of Nagoya City University.

**Collagen-Induced Arthritis** A bovine type II collagen (CII) (3 mg/mL) solution in 0.01 M acetic acid was emulsified with an equal volume of complete Freund’s adjuvant (CFA), which consisted of incomplete CFA supplemented with M. butyricum (8 mg/mL). Immunization was performed by subcutaneous injection with the antigen emulsion (100 μL) into the base of each foot. After three weeks, the mice received an antigen boost by intraperitoneal injection of 100 μL CII in 0.01 M acetic acid at a concentration of 0.75 mg/mL. CSC was solubilized with ethanol, and diluted with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), 400 μg/mL polymyxin B, and 1% bovine serum albumin (BSA). BSA was added to prevent precipitation of CSC, whereas polymyxin B prevented the appearance of endotoxin. One day before immunization, mice were intraperitoneally administered 100 μL of a solution containing CSC (100 μg) or an identical compound. As a control, vehicle alone was administered. The severity of clinical disease activity in the mice was determined by examining each of the four paws and scoring on a scale of 0–4, as follows: 0 = normal joint, 1 = erythema and swelling in one digit, 2 = erythema and swelling in more than two digits or one big joint, 3 = swelling less than 4 mm thickness and erythema in one entire paw, and 4 = swelling greater than 4 mm thickness and erythema in one entire paw and joint rigidity. The total score for clinical disease activity was based on all four paws with a potential maximum of 16 for each mouse.

**Assessment of PAHs** Assessment of PAHs was performed as described previously. The RA patient-derived SV40 T antigen-transformed human synovial fibroblast cell line MH7A was obtained from Riken BRC, Tsukuba, Japan. The cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, pXRE4-tk-Luc, which contained four tandem repeats of the xenobiotic response element (XRE) (provided by Dr. Y. Fuji, Tsukuba University) and the pGL4.74[hRlucTK] plasmid (for normalization of transfection efficiency) were transiently transfected into MH7A cells using the calcium phosphate-DNA co-precipitation method. After 15 h of transfection, the medium was changed to RPMI1640 medium containing 0.1% FBS, and the cells were maintained for 3 h before treatment with CSC or other reagents for an additional 24 h and harvesting. A luciferase assay was carried out using the Luciferase Reporter Gene Assay Kit (Roche, Germany), in accordance with the manufacturer’s instructions. Light emission was measured using the multilabel counter L420 ARVO (Pharmacia, San Francisco, CA, U.S.A.). Relative luciferase activity was determined using the Dual-Luciferase® Reporter (DLR®) Assay System (Promega, Madison, WI, U.S.A.).

**Ames Test** The mutagenic activities of the compounds were evaluated using an Ames test kit (Ornial Yeast Co., Ltd., Tokyo, Japan). The assay was performed in accordance with the manufacturer’s instructions. Salmonella enterica TA98 and TA100 were purchased from the National Institute of Technology and Evolution (Shibuya, Tokyo, Japan).

**Statistical Analysis** Differences between group means in the CIA model were assessed using Dunnett test.

**RESULTS**

**Effects of CSC 9-Ether Fractions Fractionated by Silica Gel Column Chromatography on the Development of CIA** CSC was fractionated based on its solubility into ether fractions by pH step gradients (Fig. 1). The aggravating effect of these fractions on CIA development was assessed. CSC and ethanol were used as the positive and negative control, respectively. Among these fractions, the 9-ether fraction was the most active in aggravating the development of CIA (Table 1). Fracions other than 9-ether exhibited very weak activities; therefore, the CSC 9-ether fraction was separated by silica gel column chromatography, and four fractions (frs. 1 to 4) were obtained, as described in Materials and Methods. These fractions were examined for their aggravating effects on CIA development. The 9-ether fraction and ethanol were used as the positive and negative control, respectively. As shown in Table 2, fr. 1 was the most active among frs. 1 to 4 in aggravating the development of RA.

**Effects of CSC Fr. 1 Fractionated by TLC on the Development of CIA** Because fr. 1 aggravated the development of RA the most, this was fractionated by preparative TLC with chloroform–methanol (20:1) solvent, and fr. 1-1 (Rf/
were immunized subcutaneously with CII emulsified in incomplete Freund’s adjuvant and mycobacteria. 100 µg of each fraction was administered intraperitoneally to mice one day before immunization. After three weeks, all mice received a booster intraperitoneal injection with CII in 0.01 M acetic acid. Swelling in each limb was graded on a scale from 0 to 4 as described in Materials and Methods. The development of arthritis in the mice were observed two to three times per week. Representative results at day 23 and day 37 are indicated. Statistically significant differences vs. EtOH were calculated using Dunnett tests, *p<0.1, **p<0.05, n=6.

Table 2. The Score and Incidence Ratio Associated with Frs. 1-4 in the CIA Model

| Fraction No. | Day 23 | Day 37 |
|--------------|--------|--------|
| 1-3-Ether    | 3.6±0.9| 4.6±0.9|
| 9-Ether      | 6.3±1.2| 7.0±1.0|
| 6-Ether      | 1.0±1.2| 1.3±1.5|
| 4-Ether      | 2.8±2.6| 4.0±3.4|
| EtOH         | 2.3±2.1| 2.3±2.1|

The effect of fractions based on its solubility into ether in different pH on the augmentation of arthritis was evaluated using the CIA model. DBA/1J mice were immunized subcutaneously with CII emulsified in incomplete Freund’s adjuvant and mycobacteria. 100 µg of each fraction was administered intraperitoneally to mice one day before immunization. After three weeks, all mice received a booster intraperitoneal injection with CII in 0.01 M acetic acid. Swelling in each limb was graded on a scale from 0 to 4 as described in Materials and Methods. The development of arthritis was defined as the first observation of swelling of the limb. The score and incidence of arthritis in the mice were observed two to three times per week. Representative results at day 23 and day 37 are indicated. Statistically significant differences vs. EtOH were calculated using Dunnett tests, *p<0.1, **p<0.05, n=6.

Table 3. The Score and Incidence Ratio Associated with Frs. 1-1–1-4 in the CIA Model

| Fraction No. | Day 37 | Day 44 |
|--------------|--------|--------|
| 1-1          | 3.8±4.1| 83.5±4.6|
| 1-2          | 3.2±3.8| 50.3±3.8|
| 1-3          | 4.8±3.2| 83.6±2.3|
| 1-4          | 4.3±3.4| 67.5±3.9|
| 9-Ether      | 1.2±1.9| 3.8±4.8|
| EtOH         | 1.0±2.4| 1.2±2.4|

The effect of each fraction of CSC obtained with silica gel column chromatography on the augmentation of arthritis was evaluated using the CIA model. DBA/1J mice were immunized subcutaneously with CII emulsified in incomplete Freund’s adjuvant and mycobacteria. 100 µg of each fraction or 100 µL of EtOH were administered intraperitoneally to mice one day before immunization. After three weeks, all mice received a booster intraperitoneal injection with CII in 0.01 M acetic acid. Swelling in each limb was graded on a scale from 0 to 4 as described in Materials and Methods. The score and incidence of arthritis in the mice were observed two to three times per week. Representative results at day 37 and day 44 are indicated. Statistically significant differences vs. EtOH were calculated using Dunnett tests, *p<0.1, **p<0.05, n=6.

Fig. 2. Refinement of the Fraction Obtained at Approximately 7 and 12 min Using HPLC

Activity-dependent fractionation of CSC. CSC was fractionated based on its solubility into ether fractions by changing pH, and the 9-ether fraction was separated using silica gel column chromatography. Fraction 1 was separated using an ODS column with HPLC. (Supplementary Fig. 2), and subjected to analysis by GC/MS and NMR. Fraction 1 was then fractionated by HPLC (Fig. 2), and the eluted fractions with retention times of approximately 6.5 and 12 min were collected. These fractions were refined by the same HPLC. Based on the HPLC profile, five peaks presumed to be specific to fr. 1-3, frs. A to E, with retention times of approximately 6.5 and 12, were obtained (Supplementary Fig. 2), and subjected to analysis by GC (Supplementary Fig. 3). Five peaks in the fractions [compound 1 (43.50) in fr. A, compound 2 (44.44) in fr. B, compound 3 (58.28) in fr. C, compound 4 (59.85) in fr. D, and compound 5 (69.35) in fr. E] were analyzed using mass spectrometry.

Identification of Compounds 1 and 5 By comparison with the GC/MS library and authentic compounds, compound 1 was identified as 5-hydroxy-2-methylpyridine (5H2MP), which was confirmed by NMR. Compound 5 was found to be 3,5-dimethoxy-4-hydroxycetophenone (3,5D4HAP) (Fig. 3).
Table 4. The Score and Incidence Ratio Associated with Authentic Compounds in the CIA Model

| Compounds         | Day 29 Score | Day 29 Incidence% | Day 33 Score | Day 33 Incidence% |
|-------------------|--------------|-------------------|--------------|-------------------|
| 5H2MP             | 5.8±1.2      | 100               | 9.5±1.6      | 100               |
| 3,5D4HAP          | 0.5±0.8      | 33                | 1.5±2.0      | 50                |
| EtOH              | 2.8±2.5      | 60                | 5.4±3.8      | 80                |

5H2MP, 5-hydroxy-2-methylpyridine; 3,5D4HAP, 3,5-dimethoxy-4-hydroxyacetophenone. The effect of each compound on the augmentation of arthritis was evaluated using the CIA model. 100 µg of each compound or 100 µL of EtOH were administered intraperitoneally to mice one day before immunization. The arthritis scoring method is described in the footnote to Table 1 and in Materials and Methods. The score and incidence of arthritis in the mice were observed two to three times per week. Representative results at day 35 and day 39 are indicated. Statistically significant differences vs. EtOH were calculated using Dunnett tests, *p<0.01, **p<0.05, n=6.

Compounds 2, 3 and 4 could not be identified.

5H2MP Aggravates the Development of CIA By using commercially available authentic chemicals the effects of 5H2MP and 3,5D4HAP on the development of CIA were determined. 5H2MP, but not 3,5D4HAP, exhibited aggravating activity (Table 4).

5H2MP and Its Isomer Aggravate the Development of CIA The effects of the isomers of 5H2MP, 2-hydroxy-3-methylpyridine (2H3MP) and 3-hydroxy-2-methylpyridine (3H2MP) on the development of CIA were determined. 2H3MP, but not 3H2MP, exhibited similar aggravating activity as that of 5H2MP (Table 5).

5H2MP and Its Isomers Do Not Exhibit Mutagenic Activity We next investigated the mutagenic activity of 5H2MP, and its isomers, 2H3MP and 3H2MP, with Ames test. In contrast to mutagens, such as aminoanthracene (2AA) and benzo[a]pyrene, one of components of CSC, 5H2MP, 2H3MP, and 3H2MP did not exhibit mutagenic activity (Table 6). Nicotine, one of the components of CSC, was also not mutagenic.

5H2MP Does Not Induce Xenobiotic Response Element (XRE) Promoter Activation In order to determine whether 5H2MP, exhibits PAHs activity, reporter gene assay was performed using NIH 3T3 cells transfected with the reporter plasmid pXRE4-tk-Luc, which contains four tandem repeats of XRE. Benzo[a]pyrene was used as a positive control. As shown in Fig. 4, CSC and benzo[a]pyrene induced XRE promoter activation. However, 5H2MP did not induce activation.

DISCUSSION

Cigarette smoking harms almost every organ of the body, causes many diseases, and is one of the biggest causes of death or diseases and illness that are easily avoidable. Although the etiology of RA is not fully clarified, cigarette smoking is an established environmental risk factor for this disease. The association of smoking with the development of RA has also been demonstrated through in vitro studies and in animal models of the disease. However, the mechanistic underlying the effects of cigarette smoking on RA and the pathogenic chemicals in cigarette smoke remain unknown. We have previously reported that CSC, both mainstream and sidestream, augments the development of arthritis in the CIA mouse model.9 The exacerbation was observed in both older and younger mice following either intraperitoneal inoculation or nasal exposure, indicating that CSC has systemic effects.20 Interestingly, a single inoculation one day before immunization was sufficient for CSC to exert its effects. Most importantly, the dose of mainstream CSC that results in aggravated induction of arthritis in mice is attainable if an individual with a body weight of 60 kg smokes only 32 cigarettes (mainstream) or is exposed to the smoke from 62 cigarettes (sidestream), assuming that all of the smoke is adsorbed.20,21 The cigarettes we use are equipped with a filter, which mainly traps tar. Therefore, even filters cannot trap the hazardous smoke. In reality, heavy smokers smoke many cigarettes every day, and smoke-derived chemicals can accumulate in the lungs and be subsequently distributed to all organs.

CSC consists of more than 5000 chemicals, including some with carcinogenic, cardiovascular, and respiratory effects. At the cellular and tissue level, CSC induces oxidative stress that leads to cellular damage and subsequently inflammation.22 The effects of nicotine, a major compound within cigarette smoke responsible for addiction, on inflammatory and immunological responses are controversial. Nicotine induces reactive oxygen species production in rat mesencephalic cells and activates NF-κB.23 However, it inhibits tumor necrosis factor (TNF)-α-induced IL-6 and IL-8 secretion in fibroblast-like synovocytes from RA patients by suppressing the activation of the NF-κB pathway.24 In rats, nicotine suppresses the...
antibody-forming cell response of spleen cells to sheep red blood cells, and concanavalin A-induced T cell proliferation. 25) Furthermore, nicotine pretreatment has an aggravating effect on the rat adjuvant-induced arthritis (AIA) model of human RA, whereas nicotine posttreatment suppressed the disease. 26) In the present study, we aimed to identify the chemicals that could augment the development of CIA, and found that the active compound was not nicotine.

Based on the solubility in water/ether in different pH conditions, active CSC compounds were fractionated into a 9-ether fraction. The method of separation was principally the same as the Stas–Otto method, which is widely used for the extraction of alkaloids from plants and animal bodies. Using the Stas–Otto method, acrolein, crotonaldehyde, and isocyanic acid can be fractionated into a water fraction. PAHs, such as benzo[a]pyrene (B[α]P), and nicotine are fractionated into a 13-ether fraction. The 9-ether fraction mainly contains compounds including the phenolic hydroxyl group, therefore, nicotine derivatives including hydroxyl group could be included in this fraction. The 9-ether fraction was then separated into four fractions, frs. 1 to 4, using silica gel column chromatography, and fr. 1 was the most active in the animal model. Fraction 1 was further fractionated into four fractions by TLC, and fr. 1-3 was the most active among frs. 1-1 to 1-4. Fraction 1-3 was further fractionated using HPLC, and fr. 1-3-specific peaks were detected at retention times of approximately 7 and 12 min. However, there was insufficient yield of each compound to investigate the effects on the development of CIA or to conduct analyses using GC/MS and NMR. Fraction 1 was then repeatedly fractionated by HPLC and analyzed by GC/MS. Based on the GC/MS analysis and by comparison with the chemical library, we sought to identify

![Fig. 4. Transcriptional Activation of the Xenobiotic Response Element (XRE) Reporter Gene by 5H2MP in MH7A Cells](image-url)

MH7A cells were transiently transfected with pXRE4-tk-Luc. Twenty-four hours after transfection, the cells were stimulated with B[α]P (1 µM), CSC (20 µg/mL), or 5-hydroxy-2-methylpyridine (5H2MP) for 24h, and luciferase activity in the cell lysates was measured. Luciferase activity was normalized by β-galactosidase activity. n=2.

| Strains | Compounds | Concentration | Number of colony Av | S.D. | VS. DMSO | P or N | Strains | μg/Plate | Number of colony Av | S.D. | VS. DMSO | P or N |
|---------|-----------|---------------|---------------------|------|----------|--------|---------|-----------|-------------------|------|----------|--------|
| TA98    | 2H3MP     | 5             | 44.0               | 9.1  | 1.3      | N      | TA100   | 20        | 84.0              | 13.9 | 0.9      | N      |
|         |           | -             | 17.5               | 2.4  | 1.0      | N      |         | 83.0      | 6.5              | 1.0  | N        |        |
|         | 10        | +             | 44.0               | 5.7  | 1.3      | N      |         | 92.8      | 9.5              | 1.0  | N        |        |
|         |           | -             | 20.3               | 1.3  | 1.1      | N      |         | 90.8      | 22.1             | 1.1  | N        |        |
|         | 3H2MP     | 5             | 54.8               | 7.1  | 1.6      | N      |         | 86.8      | 5.9              | 1.0  | N        |        |
|         |           | -             | 16.5               | 5.9  | 0.9      | N      |         | 92.3      | 8.4              | 1.1  | N        |        |
|         | 10        | +             | 43.3               | 11.1 | 1.3      | N      |         | 81.8      | 8.7              | 0.9  | N        |        |
|         |           | -             | 22.8               | 2.1  | 1.2      | N      |         | 84.5      | 6.6              | 1.0  | N        |        |
|         | 5H2MP     | 5             | 37.5               | 5.8  | 1.1      | N      |         | 105.0     | 17.0             | 1.2  | N        |        |
|         |           | -             | 23.3               | 4.6  | 1.3      | N      |         | 88.0      | 8.5              | 1.1  | N        |        |
|         | 10        | +             | 34.5               | 12.5 | 1.0      | N      |         | 93.0      | 2.8              | 1.0  | N        |        |
|         |           | -             | 25.5               | 2.5  | 1.4      | N      |         | 87.5      | 3.5              | 1.1  | N        |        |
|         | 2AA       | 5             | 91.3               | 16.7 | 2.7      | P      |         | 261.3     | 5.7              | 2.9  | P        |        |
|         |           | -             | 23.5               | 6.4  | 1.3      | N      |         | 93.5      | 10.0             | 1.1  | N        |        |
|         | 10        | +             | 137.0              | 15.1 | 4.0      | P      |         | 776.0     | 147.6            | 8.5  | P        |        |
|         |           | -             | 17.8               | 5.1  | 1.0      | N      |         | 92.0      | 6.4              | 1.1  | N        |        |
|         | B[α]P     | 5             | 343.3              | 37.9 | 10.1     | P      |         | 112.0     | 4.5              | 1.2  | N        |        |
|         |           | -             | 18.5               | 2.4  | 1.0      | N      |         | 89.8      | 5.7              | 1.1  | N        |        |
|         | 10        | +             | 321.5              | 35.0 | 9.5      | P      |         | 270.8     | 32.7             | 3.0  | P        |        |
|         |           | -             | 22.0               | 6.9  | 1.2      | N      |         | 86.8      | 9.8              | 1.0  | N        |        |
|         | Nicotine  | 5             | 53.3               | 3.3  | 1.6      | P      |         | 80.8      | 10.1             | 0.9  | N        |        |
|         |           | -             | 20.8               | 11.2 | 1.1      | N      |         | 87.0      | 2.2              | 1.0  | N        |        |
|         | 10        | +             | 48.8               | 8.5  | 1.4      | N      |         | 95.5      | 15.0             | 1.0  | N        |        |
|         |           | -             | 23.5               | 6.1  | 1.3      | N      |         | 83.0      | 15.9             | 1.0  | N        |        |
|         | DMSO      | +             | 34.0               | 14.7 |         |        |         | 88.8      | 20.0             |      |          |        |
|         |           | -             | 18.3               | 4.9  |         |        |         | 78.8      | 20.4             |      |          |        |

2H3MP, 2-hydroxy-3-methylpyridine; 3H2MP, 3-hydroxy-2-methylpyridine; 5H2MP, 5-hydroxy-2-methylpyridine; 2AA, 2-aminoanthracene; B[α]P, benzo[a]pyrene; DMSO, dimethyl sulfoxide. The mutagenic activity of each compound was evaluated using the Ames test. The method is described in Materials and Methods. P, positive; N, negative. n=4.
five compounds. By comparing with authentic compounds, one compound was identified as 5-hydroxy-2-methylpyridine (5H2MP), which was confirmed by NMR. Another compound was proposed to be 3,5-dimethoxy-4-hydroxyacetophenone (3,5D4HAP). By using commercially available compounds, the aggregating effects on the development of CIA were assessed. 5H2MP, but not 3,5D4HAP, appeared to have activity in this regard. Structural similarities suggest that 5H2MP may be generated from nicotine (NT) by the burning of cigarettes. It has been shown that several pyridine derivatives are generated during the pyrolysis of nicotine, including 2-, 3-, and 4-methylpyridine.\(^\text{27}\) By oxidation of 2-methylpyridine, 5H2MP and its isomers can be generated. Isomers of 5H2MP may also be generated. Among these, 2-hydroxy-3-methylpyridine (2H3MP), but not 3-hydroxy-2-methylpyridine (3H2MP), had a similar activity to 5H2MP. As no mutagenic activity was observed in 5H2MP, 2H3MP, 3H2MP, or NT, the mechanism underlying the aggravation of CIA is separable from mutagenicity.

Until date, there have been no reports on the biological and physiological effects of 5H2MP. We have previously reported that PAHs, which include constituents of cigarette smoke such as 3-methylcholanthrene (3-MC), \(\beta\)-[2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), up-regulated IL-1\(\beta\) mRNA expression in the RA patient-derived synovial fibroblast cell line MH7A.\(^\text{10}\) We also reported that CSC, either mainstream or sidestream, induced expression of IL-1\(\alpha\), IL-1\(\beta\), IL-6, and IL-8 at both the mRNA and protein level in MH7A cells,\(^\text{13}\) whereas CSC induced IL-1\(\beta\) production in RA patient-derived synoviocytes, but not in osteoarthropathy patient-derived synoviocytes or in normal fibroblasts, through AhR-dependent NF-\(\kappa\)B activation.\(^\text{20}\) The activity of PAHs is mediated by AhR. Therefore, it could be that PAHs in cigarette smoke could contribute to the onset or exacerbation of RA by inducing the production of proinflammatory cytokines from synoviocytes through AhR. Interestingly, higher AhR mRNA and protein levels were observed in RA synovial tissue than in OA tissue.\(^\text{25}\) AhR was also shown to affect T cells. Th17 is a T cell subset that secretes IL-17 and it has been implicated in the pathogenesis of several autoimmune diseases, including RA, psoriatic arthritis, ankylosing spondylitis, inflammatory bowel disease, systemic lupus erythematosus, multiple sclerosis, chronic arthritis, and skin psoriasis.\(^\text{26}\) AhR in T cells is critical for the generation of Th17, decrease in Th1 cells, and development of CIA.\(^\text{30,31}\) Surprisingly, 5H2MP did not exhibit AhR-dependent activity. However, this compound may indirectly activate AhR-dependent pathways, perhaps by generating an active metabolite or by aggravating the expression of AhR. Moreover, it is possible that 5H2MP exerts its effects through an AhR-independent manner.

We do not believe that 5H2MP is the only chemical in CSC that worsens arthritis. Cigarette smoke (CS) contains many substances, and 5H2MP exerts its pathogenic effects on RA through interaction with other chemicals including PAHs. CS-induced oxidative stress, inflammation, and enhanced cellular and humoral immunity in smokers have been suggested to play a role in the pathology of RA.\(^\text{32}\) Therefore, it would be of interest to investigate the effects of 5H2MP and 2H3MP on immune, synovial, and epithelial cells. In conclusion, our findings contribute to clarifying the mechanism of the pathogenic effects of cigarette smoking on RA.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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