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<td>Seki, Koh-ichi; Noya, Yoichi; Mikami, Yusuke; Taneda, Shinji; Suzuki, Akira K.; Kuge, Yuji; Ohkura, Kazue</td>
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Isolation and Identification of New Vasodilative Substances in Diesel Exhaust Particles

Yoichi Noya • Yusuke Mikami • Shinji Taneda • Akira K. Suzuki • Kazue Ohkura • Yuich Kuge • Koh-ichi Seki

Abstract

Goal, Scope, and Background We recently developed a new isolation method for diesel exhaust particles (DEP), involving successive extraction with H₂O, sodium bicarbonate, and sodium hydroxide, in which the sodium hydroxide extract was found to consist of phenolic components. Analysis of the extract revealed that vasodilative active nitrophenols are in DEP in significantly higher concentration than those estimated by an earlier method involving a combination of solvent extraction and repeated chromatography. These findings indicated that our new procedure offers a simple, efficient and reliable method for the isolation and identification of bioactive substances in DEP. This encouraged us to extend our work to investigating new vasodilatory substances in the sodium bicarbonate extract.

Materials and Methods DEP were collected from the exhaust of a 4JB1-type engine (ISUZU Automobile Co., Tokyo, Japan). GC-MS analysis was performed with a GCMS-QP2010 instrument (Shimadzu, Kyoto, Japan).

Results DEP dissolved in 1-butanol was successively extracted with water, sodium bicarbonate, and then aqueous sodium hydroxide. The sodium bicarbonate extract was neutralized and the resulting mixture of acidic components was subjected to reverse-phase (RP) column chromatography followed by RP-HPLC with fractions assayed for vasodilative activity. This led to the identification of telephthalic acid, p-hydroxybenzoic acid, isophthalic acid, phthalic acid, 3-hydroxy-4-nitrobenzoic acid, 4-hydroxy-3-nitrophenol, and 1,4,5-naphthalene tricarboxylic acid as components of DEP.

Discussion The sodium bicarbonate extract was rich in the acidic components. Repeated reverse-phase chromatography resulted in the successful isolation of several acidic substances including the new vasodilative materials, 4-hydroxy-3-nitrobenzoic acid and 3-hydroxy-4-nitrobenzoic acid.

Conclusion Our new fractionation method for DEP has made possible the isolation of new vasodilative compounds from the sodium bicarbonate extract.

Keywords Acidic components; Diesel exhaust particles; Fractionation; 3-Hydroxy-4-nitrobenzoic acid; 4-hydroxy-3-nitrobenzoic acid; Separation method; Vasodilatory compounds
2 Introduction

Diesel exhaust is believed to consist of thousands of organic constituents and is a major cause of urban pollution (Bayona et al. 1988; Draper 1986; Erdinger et al. 2005; Mestres 2005; Morville 2006; Ona 2006; Schuetzle 1983; Schuetzle & Lewtas 1986; Seidel et al. 2006). A number of studies on the adverse effects of diesel exhaust particles (DEP) on human health, including lung cancer (McClellan 1987; Ichinose et al. 1997), allergic rhinitis (Muranaka 1986; Takafuji et al. 1987), bronchial asthma-like disease (Sagai et al. 1996; Miyabara et al. 1998), and disruption of endocrine function, have been reported (Yoshida et al. 1999; Watanabe & Oonuki 1999; Tsukue et al. 2001, 2002). While these studies have furnished epidemiologically important results, they have been done without knowledge of the bioactive entities in DEP, making it difficult to estimate the effect of individual components on human health. In our earlier studies on the isolation and identification of bioactive compounds contained in DEP, we have isolated aromatic substances including nitrophenols (Mori et al. 2003a; Taneda et al. 2004a) that showed strong vasodilatory activity by a method involving successive solvent extractions, followed by repeated column chromatography. This multi-step isolation procedure, however, resulted in poor fractionation with impaired accuracy and reproducibility. Therefore, it was crucial to develop a more efficient and reliable method for the isolation of biologically significant components in DEP. Recently we reported an alternative protocol entailing successive extraction with water, sodium bicarbonate, and sodium hydroxide (Noya et al. 2008). Analysis of the sodium hydroxide extract revealed that vasodilatory active nitrophenols are present in DEP in significantly higher amounts than estimated by a previous method involving successive solvent polarity-based extractions, followed by repeated column chromatography. This has encouraged us to apply the new method to investigate whether vasodilatory active substances are contained in the other extracts. In the present paper, we describe the isolation and identification of some new vasodilative acidic substances from the sodium bicarbonate extract.

2 Materials and methods

2.1 Diesel exhaust particles (DEP)

DEP were collected from the diesel exhaust of a light-duty (2740 cc) four cylinders 4JB1-type diesel engine with direct fuel injection (Combustion system: Aspiration type, natural; Peak torque, 1500 rpm; Torque, 10 kg/m) (Isuzu Automobile Company, Tokyo, Japan) (Figure 1), as described in the preceding paper (Taneda et al. 2004a).

2.2 Reagents

Authentic samples of terephthalic acid (1), 4-hydroxybenzoic acid (2), isophthalic acid (3), dimethyl phthalate, dimethyl 4-methylphthalate, methyl 4-hydroxy-3-nitrobenzoate, methyl 3-hydroxy-4-nitrobenzoate, and trimethyl 1,4,5-naphthalenetricarboxylate for GC-MS analysis were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). All other reagents were of analytical reagent grade.

2.3 Instrumentation

GC-MS analysis was performed at 70 eV with a GCMS-QP2010 instrument (Shimadzu, Kyoto, Japan) equipped with a DB-5ms capillary GC column (0.25 mm x 30 m; (Shimadzu, Kyoto, Japan). Analysis was carried out at a column temperature of 80–200°C. NMR — 1H- and 13C-NMR spectra were recorded at 400 MHz, in CDCl3 (EX 400, JEOL, Tokyo, Japan). Chemical shifts are given in δ relative to tetramethylsilane (TMS, Tokyo Kasei Kogyo Co., Ltd. Tokyo, Japan) as the internal standard.

2.4 Measurement of Vascular Relaxation — Measurement of vasodilatory activity was made by using seven-month-old SPF F344 male rats (JCL:F344, CLEA Japan, Inc., Tokyo, Japan), which were maintained in a controlled room in terms of temperature (22 ± 1°C), humidity (50 ± 5%) and ventilation (25-30 times/h). Lights were cycles at 12 h light-dark intervals. Food (CE-2 commercial diet, CLEA Japan, Inc.) and water were given ad libitum. The rats were anesthetized
by pentobarbital sodium (50 mg/Kg, i.p.). After bleeding, the thoracic artery of each rat was excised and cut into 3 mm rings. The arterial ring was located in a magmus tube with 1 g of tonus and incubated in Locke-Ringer’s solution (NaCl, 153.8 mM; KCl, 5.63 mM; CaCl2, 3.17 mM; glucose, 5.55 mM; NaHCO3, 2.38 mM; pH 7.4) at 37 ℃ under aeration with 95% O2 and 5% CO2 for approximately 1 h. After contraction of the thoracic artery rings with 10-6 M phenylephrine (PE), substrates (4-methylphthalic acid, 4-hydroxy-3-nitrobenzoic acid and 3-hyroxy-4-nitrobenzoic acid), dissolved in PBS (10-6, 10-5, 10-4, 10-3, 10-2 and 10-1 M) containing 0.05% Tween 80 were accumulatively added into an organ bath. The changes in tension were amplified (FD pickup TB-612T transducer and Multichannel amplifier MEG-6108, Nihon Kohden, Tokyo, Japan) and recorded (Phoenix, DKK-TOA Co., Tokyo, Japan) (cf. Mori et al. 2003a).

2.5 Extraction procedure from diesel exhaust particles (DEP)

DEP (10 g) was dissolved in 1-butanol (300 ml), and was first washed with water, then extracted with a saturated aqueous solution of sodium bicarbonate (NaHCO3; 100 ml × 5), and subsequently extracted with 10% aqueous NaOH (100 ml × 3).

The combined NaHCO3 extracts were neutralized with 10% HCl, and then the solution was extracted with dichloromethane (100 ml × 3). After drying over anhydrous sodium sulfate, the organic solution was evaporated to give an oily residue (1.6 g). The resulting oil was subjected to preparative HPLC (Inertsil ODS-3, GL Science) with water containing 0.1 % formic acid, acetonitrile, and 1-butanol (70 : 15 : 15), to give 12 fractions (F1 – F12) (9.6 ml each).

Samples from each fraction were tested for activity in the vascular relaxation assay. The fractions 3 (F3) (22.7 mg) and 4 (F4) (25.2 mg), that revealed significant vascular relaxation activity, were re-fractionated by HPLC (water containing 0.1 % formic acid and acetonitrile = 70 : 30) with monitoring of the UV-absorption at 254 nm. Thus, F3 was fractionated into eight subfractions; F3.1 (2.0 mg), F3.2 (2.2 mg), F3.3 (1.5 mg), F3.4 (1.1 mg), F3.5 (4.9 mg), F3.6 (1.4 mg), F3.7 (3.7 mg), F3.8 (3.1 mg). Similarly, F4 was separated into six subfractions; F4.1 (1.2 mg), F4.2 (1.4 mg), F4.3 (1.9 mg), F4.4 (0.6 mg), F4.5 (2.5 mg), F4.6 (7.3 mg) (Figure 2). Preliminary assays revealed that F3.5, F4.1, and F4.3 possessed vasodilative activity.

3 Results

Spectroscopic studies (LC-MS, GC-MS, 1H-NMR) of each subfraction revealed that F3.1 was a mixture of telephthalic acid (1) and 4-hydroxybenzoic acid (2), F3.2, F3.3, and F3.5 were ascribed to isophthalic acid (3), phthalic acid (4), and 4-methylphthalic acid (5), respectively (Figure 3). Compounds 1, 2, and 3 were identified by comparison of their 1H-NMR spectra and chromatographic behaviors with those of the authentic samples: 1H-NMR of 1 (CD3OD) δ 8.09 (4H, s, H-2, H-3, H-5 and H-6), 1H-NMR (CD3OD) of 2 δ 6.79 (2H, dt, J = 8.6, 2.3 Hz, H-2 and H-6), 3.86 (2H, dd, J = 8.6, 2.3 Hz, H-2 and H-6)), F3.2, F3.3, and 1H-NMR (CD3OD) of 3 δ 7.58 (1H, t, J = 7.5 Hz, H-5), 8.21 (2H, dd, J = 1.8, 7.5 Hz, H-4 and H-6), 8.64 (1H, t, J = 1.8 Hz, H-2). The structures of phthalic acid (4, 1H-NMR (CD3OD) δ 7.56 (2H, dd, J = 5.7, 3.5 Hz, H-3 and H-4), 7.72 (2H, dt, J = 5.7, 3.5 Hz, H-2 and H-5)) and 4-methylphthalic acid (5, 1H-NMR (CD3OD) δ 2.41 (3H, s, C4-CH3), 7.37 (1H, d, J = 8.0 Hz, H-5), 7.49 (1H, s, H-3), 7.66 (1H, d, J = 8.0 Hz, H-6)) were confirmed by comparison of the GC-MS data of their methyl ester derivatives (6 and 7) with those of the authentic samples: MS for 6 (m/z): 194 (M+, 10 %), 183 (base peak), 7 (m/z); M+ for 7 (m/z); M+ for 7 (m/z); M+ for 7 (m/z). Similarly, F4.1, F4.3, and F4.6 were identified as 4-hydroxy-3-nitrobenzoic acid (8) (1H-NMR (CD3OD) δ 7.19 (1H, d, J = 8.6 Hz, H-5), 8.17 (1H, dd, J=1.7, 8.6 Hz, H-6), 8.65 (1H, d, J=1.7 Hz, H-2)), 3-hydroxy-4-nitrobenzoic acid (9) (1H-NMR (CD3OD) δ 7.57 (1H, dd, J=1.7, 8.6 Hz, H-5), 7.71 (1H, d, J = 1.7 Hz, H-2), 8.07 (1H, d, J=8.6 Hz, H-5)), and 1,4,5-naphthalentricarboxylic acid (10), respectively (Figure 4) by comparison of the GC-MS spectra of their methyl esters (11, 12, 13) with those of authentic samples (Figure 5, 6, 7). The result of the fractionation was summarized in Table 1. 4-Hydroxy-3-nitrobenzoic acid (8), and 3-hydroxy-4-nitrobenzoic acid (9) clearly demonstrated vasodilative activity. 4-Methylphthalic acid (5) showed vasodilative activity, though the effect was only transient and weaker than 8 and 9. The lowest concentrations of 4-methylphthalic acid (5), 4-hydroxy-3-nitrobenzoic acid (8), and 3-hydroxy-4-nitrobenzoic acid (9) causing vasodilatory activity were estimated as ~10^(-3) M (Figure 8).
4 Discussion

Although a number of studies have demonstrated epidemiologically important results, none of the investigations focused on a systematic separation and characterization of the bioactive components of DEP until our initial work on the isolation and identification of such entities by using a method involving successive solvent extractions, and subsequent repeated column chromatography. In spite of poor fractionation and a significant lack of accuracy and reproducibility associated with this method, such isolated compounds as phenanthrenes (Mori et al. 2003b), dibenzothiophenes (Mori et al. 2003c), alkylphenols (Taneda et al. 2004c), hydroxyphthalic acids (Mori et al. 2003d), and vasodilative active nitrophenols (Mori et al. 2003a) suggest that the constituents of DEP can be broadly classified into the following groups: (i) aromatic carboxylic acids, (ii) phenol derivatives, (iii) non-combustible neutral compounds. They are thus amenable to separation by using a conventional acid-base extraction procedure. Based on this concept, we have developed the new fractionation scheme used for this work (Noya et al. 2008). The sodium bicarbonate extract obtained by this procedure has been shown to contain various aromatic carboxylic acids including the new vasodilatory nitro-compounds, 4-hydroxy-3-nitrobenzoic acid (8) and 3-hyroxy-4-nitrobenzoic acid (9). 4-Methylphthalic acid (5) revealed vasodilatory activity, but the effect was only temporary and feeble, suggesting that different pharmacological mechanism might be involved in 5 from that of nitrophenols. Although vascular relaxation effects of the newly isolated 4-hydroxy-3-nitrobenzoic acid (8) and 3-hyroxy-4-nitrobenzoic acid (9) are much weaker (~10^{-3} M) than those reported for nitrophenols (active at threshold concentrations of ~10^{-4} M), the contents of the newly isolated vasodilatory components are as high as those of nitrophenols (Table 1). These findings may be important from the environmental perspective, as remarkable amounts of DEP are exhausted into the air; for example, 58902 t in Japan (Japan Environmental Agency 1998), 111 530 t in the United States (United States Environmental Protection Agency 1999), 37 000 t in England (Airborne Particles Expert Group 1999), and 240 000 t in Europe (Petroleum Energy Center Japan 1999). Many automobile companies either in Japan or European countries are competitively developing modern Diesel cars equipped with different types of exhaust treatment such as a particulate trap/filter to detoxify diesel exhaust. Swiss researchers very recently investigated the secondary effects of catalytic diesel particulate filters on the coersion of polyaromatic hydrocarbons (PAHs) versus formation of nitro-polyaromatic hydrocarbons (nito-PAHs), whereby PAHs were reduced by using filters, while they noticed the formation of selected nitro-PAHs (Norbert V. Heeb et al. 2008). Although our present investigation was performed on the DEP collected from an engine without using a filter, we have demonstrated significant amounts of vasodilative nitro compounds are consisted in DEP, and their hazardous effects on human health cannot be ignored because of the vast amount of DEP emitted into the environment.

5 Conclusions

Our present work reports new vasodilative active nitro substances in DEP, which are released in significant amounts from Diesel engines into the air. Because of the simple and systematic isolation procedure, the present analytical method, involving a combination of acid-base extraction and subsequent chromatography with monitoring of bioactivity, would serve as a facile and efficient method for identification of such bio-hazardous substances as vasodilator, carcinogen, and in DEP.

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References

United States Environmental Protection Agency (1999) Analysis of the impacts of control program on motor vehicle toxics emissions and exposure in urban areas and nationwide EPA420-R-99-029:120.
Table 1. Representative components isolated from the sodium bicarbonate extract of DEP (mg/kg)

<table>
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<tr>
<th>Compound (Fraction)</th>
<th>3\a (F3.2)</th>
<th>4\b (F3.3)</th>
<th>5\c (F3.5)</th>
<th>8\d (F4.1)</th>
<th>9\e (F4.3)</th>
<th>10\f (F4.6)</th>
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<tr>
<td>Content</td>
<td>220</td>
<td>150</td>
<td>490</td>
<td>120</td>
<td>190</td>
<td>730 mg</td>
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a) isophthalic acid, b) phthalic acid, c) 4-methylphthalic acid, d) 4-hyroxy-3-nitrobenzoic acid, e) 3-hyroxy-4-nitrobenzoic acid, f) 1,4,5-naphthalenetricarboxylic acid.
Fig. 1  DEP collection procedure.
Fig. 2  Fractionation of NaHCO₃ extract by HPLC

NaHCO₃ fraction

ODS-3
0.1% formic acid - water 70%
acetonitrile 15%  1-butanol 15%

ODS-3
0.1% formic acid - water 70%
acetonitrile 30%

F1  F2  F3  F4  F5  F6  F7  F8  F9  F10  F11  F12
(+)  (+)  (+++)  (+)  (++)  (+)  (+)  (-)  (+)  (+)  (+)  (+)
(-)  (+)  (±)  (-)  (±)  (-)  (-)  (-)  (-)  (-)  (-)  (-)

Upper line : relaxation
Lower line : constriction

F3.1  F3.2  F3.3  F3.4  F3.5  F3.6  F3.7  F3.8
Fig. 3  Compounds isolated from fraction 3
Fig. 4  Compounds isolated from fraction 4

F4.1 → COOH

F4.2 → COOH

F4.3 → COOH

F4.4

F4.5

F4.6

8

9

10
Fig. 5  MS spectrum of 8 (11)
Fig. 6  MS spectrum for methyl ester of 9 (12)
Fig. 7  MS spectrum of 10 (13)
Fig. 8  Measurement of Vasodilatory activity for 8, 9, and 5