Presence of Peroxyradicals in Cigarette Smoke and the Scavenging Effect of Shikonin, a Naphthoquinone Pigment

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Using a new method having been developed for the purpose of quantitative determination for peroxyradicals, the presence of peroxyradicals was proved in cigarette smoke. In brief, peroxyradicals in cigarette smoke were measured by ESR spectrometry coupled to non-reductive scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH). As a result, peroxyradicals were found to be major reactive oxygen species (ROS) since the concentration of peroxyradicals recovered from cigarette smoke was much higher than that of any of other ROS (superoxide and hydroxyl radical) and nitric oxide. Furthermore, several antioxidants (ascorbic acid, reduced glutathione, epigallocatechin gallate, shikonin) were examined for scavenging activity against peroxyradicals in the cigarette smoke. Among them shikonin alone exerted the scavenging activity, suggesting that shikonin is promising antioxidant for cigarette filters because of its effectiveness against broad range of ROS including peroxyradicals, heat resistance, nonvolatility and high affinity to the filter.

Key words peroxyradical; cigarette smoke; ESR; scavenging activity; shikonin

In addition to the association of cigarette smoking to cardiovascular diseases, stroke, chronic bronchitis, chronic obstructive pulmonary disease and emphysema, epidemiological data have been establishing that cigarette smoke is one of the major causes of lung cancer.1–3 While addiction of cigarette smoking is supposed to be attributable to nicotine that is not carcinogenic, carcinogenicity is considered to be caused by byproducts produced by combustion of tobacco.3 Among the byproducts, enormous amounts of free radicals and reactive oxygen species (ROS) are estimated to be present. That is, carbon- and oxygen-centered organic radicals and a high concentration of NO are proved to be present by electron spin resonance (ESR) study, and carbon-centered radicals rapidly scavenged by molecular oxygen give oxygen-centered alkoxyl radicals.4–6 Although peroxyradicals are presumed to be produced in the gas-phase cigarette smoke,6 the clear evidence has not been established. Recently, we have found that a stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a useful agent for the quantitative measurement of peroxyradicals.7 In brief, as shown in Fig. 1, non-reductive DPPH-scavenging by peroxyradical is quantitatively determined by ESR analysis. In this study, we report that peroxyradical(s) was proved to be present in the gas-phase cigarette smoke using the method described in our previous study.7

Several studies have been conducted to inactivate free radicals from cigarette smoke by using antioxidants.8–11 As indicated in the previous study,10 antioxidants used should necessarily be effective against broad range of free radical species, be heat resistant, nonvolatile and display high affinity to the filter. Shikonin, a red naphthoquinone derivative, is an active principle of the medicinal plant Lithospermum erythrorhizon,12 and is in folk medicine where it is claimed to possess wound healing and anti-inflammatory activity.13–16 Those activities of shikonin are considered to be associated with its scavenging activity for oxygen radicals.16,17 Since it has recently been reported that shikonin shows highly efficient antioxidative activities against several types of reactive oxygen species, such as singlet oxygen, superoxide anion radical, hydroxyl radical and tert-butyl peroxyradical,18 we examined the scavenging activity of shikonin for peroxyradicals in cigarette smoke in comparison with that of other antioxidants.

Experimental

Materials 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) and N-methyl-γ-gluacamethiobarbamate (MGD) were purchased from Labootech Co., Ltd. (Tokyo, Japan), DPPH was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (TEMPOL) was from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). Ascorbic acid, epigallocatechin gallate, reduced glutathione, and shikonin were purchased from Kanto Kagaku Co., Ltd. (Tokyo, Japan), Roche Vitamin Japan (TEAVIGO§, Tokyo, Japan), Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. Bovine hemoglobin was purchased from Nacalai tesque Co., Ltd. (Kyoto, Japan). All the other reagents used were of analytical grade. Cigarettes used in this study were commercially available ones (Seven Stars, Japan Tobacco Inc., Tokyo, Japan), and each cigarette with a charcoal filter contained 14 mg of tar and 1.2 mg of nicotine.

ESR Analyses Measurement conditions of ESR for superoxide, hydroxyl radical, nitric oxide (NO) and peroxyradicals are summarized in Table 1.

Suction of Cigarette Smoke A smoking device for cigarette smoke is illustrated in Fig. 2, in which the rate of sucking gas was adjusted to 0.75 l/min.

Quantitative Analysis of Superoxide, Hydroxyl Radical and NO Superoxide and hydroxyl radical in the cigarette smoke were quantitatively analyzed by ESR spectrometry coupled to spin trapping with DMPO. An aliquot (180 l) of the reaction mixture obtained after the exposure of cigarette smoke to 2.0 ml of 1.1 M DMPO aqueous solution at 0.75 l/min for 60 s was immediately transferred to a quartz sample cell for a ESR spectrometer (JES-FA100, JEOL, Tokyo, Japan). The signal intensity of each spin adduct (DMPO–OOH from superoxide and DMPO–OH from hydroxyl radical) was recorded for quantitative analysis. Since the half-life of DMPO–OH is much

Fig. 1. The Reaction Scheme of Non-reductive DPPH-Scavenging by Peroxyradical

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Table 1. ESR Measurement Conditions for Reactive Oxygen Species (ROS) and NO

<table>
<thead>
<tr>
<th>ROS</th>
<th>MW freq/GHz</th>
<th>Magnetic field/mT</th>
<th>Mod width/mT</th>
<th>Amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide, Hydroxylradical</td>
<td>9.426</td>
<td>335.7±5.0</td>
<td>0.07</td>
<td>500.0</td>
</tr>
<tr>
<td>NO</td>
<td>9.427</td>
<td>329.5±7.5</td>
<td>0.40</td>
<td>800.0</td>
</tr>
<tr>
<td>Peroxyradical</td>
<td>9.427</td>
<td>335.7±5.0</td>
<td>0.10</td>
<td>250.0</td>
</tr>
</tbody>
</table>

Results and Discussion

ROS and NO Recovered from Cigarette Smoke  The representative spectra of DMPO–OOH (for superoxide determination), DMPO–OH (for hydroxyl radical determination) and MGD2–Fe–NO (for NO determination) are shown in Fig. 4. It has been reported that the addition of superoxide dismutase (a scavenger for superoxide) and ethanol (a scavenger for hydroxyl radical) resulted in the disappearance of the ESR spectra of DMPO–OOH and DMPO–OH, respectively, indicating that DMPO–OOH was derived from superoxide and DMPO–OH from hydroxyl radical. Since the signal intensity of MGD2–Fe–NO was reduced by 50% or more by the addition of hemoglobin (Fig. 5), at least 50% or more of the intensity was derived from primarily existing NO. The spectra indicate that the concentrations of superoxide, hydroxyl radical and NO trapped in the reaction mixture were 2.1 μM (4.2 nmol), 0.4 μM (0.8 nmol) and 40 μM or more (80 nmol or more), respectively. The results obtained here are in agreement with those reported previously in which NO is considered to be substantially present in cigarette smoke. The signal intensity of the adduct derived from peroxyradical and DMPO was very weak and was interfered by the signal of DMPO–OH so that DMPO is considered to be inadequate for the quantitative determination of peroxyradicals. In the case of alkoxyradicals, the signal derived from the adduct of alkoxyradicals and DMPO was not observed, indicating that the amount of alkoxyradicals in the cigarette smoke is considered to be a trace level.

Quantitative Determination of Peroxyradicals, and Scavenging Activity of Antioxidants for Peroxyradicals  Representative ESR spectra of DPPH for quantitative determination of peroxyradicals are summarized in Fig. 6. The spectra clearly revealed that DPPH was increasingly scavenged by peroxyradicals with sucking time, and the peroxyradical concentrations trapped in the reaction mixtures were 0.33 mM (0.66 μmol) for 30 s and 0.79 mM (1.6 μmol) for 60 s. Peroxyradicals are supposed to be generated through a kinetic process in which carbohydrates and proteins in the cigarette burn produce alkenes and NO. Then NO is oxidized...
to NO₂, which reacts with alkenes to generate alkoxyradicals and peroxyradicals. However, quantitative analyses of peroxyradicals in cigarette smoke have not been reported so far. The result of the present study revealed that peroxyradicals are major ROS since the concentration of peroxyradicals recovered from the cigarette smoke for 60 s was much higher than that of any of other ROS (superoxide and hydroxyl radical) and NO. Furthermore, inhibitory effects of the filters containing shikonin, ascorbic acid, epigallocatechin gallate or reduced glutathione on the degradation of DPPH were examined. Of the five antioxidants, shikonin alone exhibited an inhibitory effect on DPPH-degradation by the cigarette smoke, indicating that DPPH-reactive radicals were effectively trapped by shikonin. Representative ESR spectra of DPPH reacted with cigarette smoke that passed through the control and shikonin-containing filters are shown in Fig. 7. Since peroxyradicals are the most major radicals found in the cigarette smoke, inhibition of DPPH-degradation is considered to be attributable to mainly the scavenging effect of shikonin on peroxyradicals.

These results suggest that shikonin is promising antioxidant for cigarette filters because of its effectiveness against broad range of ROS including peroxyradicals, heat resistance, nonvolatility and high affinity to the filter.

References