Note

Regulation of Cigarette Smoke-Induced Cytochrome P4501A1 Gene Expression in Osteogenic Disorder Shionogi Rat Liver and in Lung by Large Ascorbic Acid Dose

Etsuko UETA,1 Emiko SUZUKI,2 Eiji NANBA,3 Yuko TADOKORO,1 Yuzuru OTSUKA,4,1 and Tadao KURATA1

1Institute of Environmental Science for Human Life, Ochanomizu University, Tokyo 112-8610, Japan
2Faculty of Life Science, Ochanomizu University, Tokyo 112-8610, Japan
3Gene Research Center, Tottori University, Yonago 683-0826, Japan
4Faculty of Education and Regional Sciences, Tottori University, Tottori 680-8551, Japan

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The effects of a large ascorbic acid dose on cytochrome P4501A1 gene expression induced by cigarette smoke exposure was studied in Osteogenic Disorder Shionogi rats, which lack ascorbic acid biosynthesis. The rats were divided into four groups and were administered either a minimal amount (4 mg/day, 45 and 4C) or a large amount (40 mg/day, 40S and 40C) of ascorbic acid. The 4S group and 40S group were daily exposed to cigarette smoke for 2 hours, while the 4C group and 40C group were not. At the end of the 25-day experiment, the rats were killed. The cytochrome P4501A1 mRNA level both in the liver and lung was measured by a competitive reverse transcription—polymerase chain reaction method. When a minimal amount of ascorbic acid was administered, the cytochrome P4501A1 mRNA increased in the liver of the cigarette smoke-exposed group (4S) compared with the control group (4C). On the other hand, when a large amount of ascorbic acid was administered, this increase was not observed in the cigarette smoke-induced group (40S) in liver. On the other hand, in lung, an increased mRNA level in 4S group was not decreased by large ascorbic acid administration (40S). This is the first direct mRNA-level evidence of the effects of a large ascorbic acid dose on the gene expression stimulated by cigarette smoke.

Key words: large ascorbic acid dose; gene expression; cigarette smoke; cytochrome P4501A1; competitive RT-PCR

Cigarette smoking is the largest preventable risk factor for morbidity and mortality in developing countries.1 Not only cigarette smoking, but also passive cigarette smoke exposure derived from the environment is a major health risk factor, too.2 Cigarette smoke contains various kinds of reactive chemicals including reactive oxygen species, polycyclic aromatic hydrocarbons, and organic radicals. Because of these chemicals, cigarette smoke exposure induces drug-metabolizing enzymes and antioxidative enzymes to protect cells from chemical and oxidative stress.3 It is known that the cytochrome P450 superfamily in liver microsomes plays an important role in the metabolism of a variety of these compounds.4 Among the cytochrome P450 superfamily, cytochrome P4501A1 (CYP1A1) has been used as a biomarker of cigarette smoke exposure in humans.5

Antioxidative vitamins such as vitamin C (ascorbic acid; AsA) and E have been considered to contribute to the prevention of potential damage from smoking.6 It was reported that AsA deficiency decreased the enzyme activity7 and mRNA level8 of drug-metabolizing enzymes. On the other hand, the effect of a large dose of AsA on the drug-metabolizing enzyme was not investigated in detail. Khanduja et al. showed the inhibition of arylhydrocarbon hydroxylase activity by an excessive dose of AsA.9 Clarke et al. reported that vitamin C treatment selectively reduced the expression of CYP2E proteins.10 Sinclair et al. reported that a large dose of AsA did not change the CYP1A1 and CYP1A2 protein levels that were induced by methylcholanthrene.11 However, there is no report on the effects of a large dose of AsA on the mRNA level of drug-metabolizing enzymes. In our previous study,12 it was shown that the AsA concentration increased in the plasma and in the tissues of Wistar rats exposed to cigarette smoke. The in-

1 To whom correspondence should be addressed. Yuzuru OTSUKA, Present address: Institute of Environmental Science for Human Life, Ochanomizu University, Tokyo 112-8610, Japan. Fax: +81-3-5978-5813; E-mail: yotsuka@cc.ocha.ac.jp

Abbreviations: AsA, Ascorbic acid; CYP1A1, cytochrome P4501A1; PCR, polymerase chain reaction; RT-PCR, reverse transcription—polymerase chain reaction; ODS, Osteogenic Disorder Shionogi; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase
creased AsA concentration in the tissues and in the plasma might be a protective response to the oxidative stress produced by cigarette smoke exposure.

In this study, we evaluated the effects of a large AsA dose on the CYPIA1 mRNA level in smoke-exposed rats. Osteogenic Disorder Shionogi (ODS) rats,\textsuperscript{14} which lack an AsA synthesis enzyme, were used.

Male ODS rats at 7 or 8 weeks of age were purchased from Nihon Clea Co., Tokyo Japan. All experiments were done under the guidance of "Standards Relating to the Care and Management, etc. of Experimental Animals, Notification of Japanese Prime Minister's Office, 1980". After 7 days on a normal diet, the rats were divided into four groups and were administered either a minimal amount (4 mg/day, 4S and 4C) or a large amount (40 mg/day, 4S and 4C) of ascorbic acid. The 4S group and 40S group were daily exposed to cigarette smoke for 2 hours, while the 4C group and 40C group were not, as described in our previous reports.\textsuperscript{13}\ The rats were directly given AsA into the stomach once a day and fed on a slightly modified AIN76 diet\textsuperscript{13} for 25 days. The amount of food given to the control group was equal to the amount of the diet consumed by the smoke-exposed group rats during the day. At the end of the experiment, the rats were killed under anesthesia. The liver and lungs were removed and immediately frozen in liquid nitrogen, and were stored at −80°C. The body weight and AsA concentrations in liver and lung measured by the method described in our previous report\textsuperscript{13} are shown in Table 1. There is no significant difference in the body weight among the four groups. Symptoms of clinical scurvy, such as bleeding nose and trouble walking, were not seen in the four groups. The AsA content in liver of 4C group was almost the same as that of ODS rat fed 90 ppm AsA described by Sinclair et al.\textsuperscript{12} The AsA content in the lung of the 4C group was almost the same as that of the Fischer rat described by Kari et al.\textsuperscript{15}

To investigate the effects of large doses of AsA on the CYPIA1 mRNA level, a competitive reverse transcription—polymerase chain reaction (RT-PCR) method was used. Total RNA was prepared with guanidine isothiocyanate followed by ultracentrifugation in cesium chloride.\textsuperscript{16} The cDNA was synthesized from 3 μg of total RNA that was treated with RNase-free DNase I (Nippon Gene, Tokyo, Japan), with M-MLV reverse transcriptase (Gibco BRL, Rockville, USA), and RNase inhibitor (Nippon Gene, Tokyo, Japan), using the random hexamers primer (Promega Co., Madison, USA) at 37°C for 60 min. The fragment of CYPIA1 cDNA was amplified by the polymerase chain reaction (PCR) with the same primers and the same conditions described in Vanden Heuvel et al.\textsuperscript{17} The 215 bp of glycerolaldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified by the method of Tamarina et al.\textsuperscript{18} with primers of (TGCCCTCCTGCACCCACAACACTGC and ATGACCTTGCCCCACAGCCTT). Those PCR products were subcloned into pGEM-T vector (Promega Co., Madison, USA). The internal standard DNAs for competitive RT-PCR were created as described by Ho et al.\textsuperscript{19} The 12 base inserted internal standard for CYPIA1 was created with primers of GCCAATTGGAATTTCAATGTGTACCA and TGGTGACACTTGAATTCATTTTG. The 11-base deleted one for GAPDH was created with primers of TGGAAGGACTCATGACCACA and TCATGAGTCCCTCATGATGAGATGACCTTGGCC.

Competitive RT-PCR with these cDNAs competitor was done as described by Inoue et al.\textsuperscript{20} with slight modification. Briefly, the RT-PCR solution contained 10 pmol of Cy5-labelled sense primer and 10 pmol of non-labelled antisense primer. The PCR products were denatured and electrophoresed by an AlFred DNA sequencer (Amersham Pharmacia Biotech, Tokyo, Japan) on a denatured sequencing gel. The amount of the PCR product was calculated by Allele Link software (Amersham Pharmacia Biotech, Tokyo, Japan). The amount of CYPIA1 mRNA against the amount of GAPDH mRNA of four groups of ODS rats is shown in Fig. 1. In liver, the CYPIA1 mRNA level of 4S group (smoke-exposed and 4 mg/day AsA-administered group) was about 7 times higher than that of 4C rats (no smoke exposure and 4 mg/day AsA). This increase by smoke exposure in CYPIA1 gene expression was not observed in the 40S group (smoke-exposed and

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Table 1. Body Weight and AsA Content in Liver and in Lung of ODS Rats after 25 Days of Smoke Exposure

<table>
<thead>
<tr>
<th>Number of Rats</th>
<th>Body Weight (g)</th>
<th>AsA in Liver (mg/100 g)</th>
<th>AsA in Lung (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mg/day ascorbic acid administration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4C)</td>
<td>6</td>
<td>221.8 ± 6.0</td>
<td>2.26 ± 0.52</td>
</tr>
<tr>
<td>Smoke Exposure (4S)</td>
<td>6</td>
<td>218.7 ± 9.6</td>
<td>3.33 ± 0.83</td>
</tr>
<tr>
<td>40 mg/day ascorbic acid administration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (40C)</td>
<td>6</td>
<td>230.4 ± 3.0</td>
<td>2.41 ± 0.88</td>
</tr>
<tr>
<td>Smoke Exposure (40S)</td>
<td>6</td>
<td>242.0 ± 4.7</td>
<td>6.33 ± 0.65</td>
</tr>
</tbody>
</table>

Data were analyzed by one-way Anova with post hoc test using Stat View, SAS Institute Inc. Values expressed by average ± SE. *Significantly different (p < 0.05) by post hoc pairwise test with the Tukey-Kramer method.\textsuperscript{19}
to show that AsA reduced the oxidative stress due to cigarette smoke exposure, and as a result, CYP1A1 gene expression was suppressed in the liver. However, Sinclair et al. have shown that methylcholanthrene-induced CYP1A1 and CYP1A2 proteins were not changed by AsA feeding conditions as detected by immunoblotting.\(^{12}\) The reason for the difference was not known. In the lung, as it is exposed to cigarette smoke directly, it seems that CYP1A1 expression remained high even at a large dose of AsA to prevent cigarette smoke stress, because cigarette smoke stress is so severe in the lung therefore it is needed to keep high gene expression of CYP1A1. It will be interesting to investigate the effects of large doses of AsA on the enzyme expression in other organs.

It is said that AsA may behave as a potential prooxidant\(^{22}\) depending on experimental conditions, and CYP1A1 is known to be down-regulated by oxidative stress.\(^{23}\) However, it is also known that there is little evidence for pro-oxidant activity of AsA in vivo,\(^{49}\) and it seems that the conditions in the liver cells of 40S rats were not oxidative as the AsA concentration was kept high in this experiment. Therefore, the results described here show that large doses of AsA decreased CYP1A1 expression in liver as the result of the antioxidant effects of large doses of AsA, not as the result of a pro-oxidant effect. The thiol-reducing agent, N-acetylcysteine, also an antioxidative reagent, may also inhibit CYP1A1 gene expression as in this experiment. It is not certain whether a large dose of AsA also modifies the gene expression in the case of different types of oxidative stress, for example, high oxygen concentration.

There are at least five pathways of redox regulation in cells.\(^{25}\) Nuclear factor kappa B (NFkB) is one of the candidates that might explain the results shown here. NFkB is known to be activated by oxidative stress and subsequently Rel A, a subunit of NFkB, binds to the arylhydrocarbon receptor (AhR) to reduce the expression of CYP1A1,\(^{26}\) therefore if this pathway was inhibited by large AsA administration, CYP1A1 gene expression should be increased. However, the result shown here that it is decreased by large AsA administration, therefore this pathway may not be involved in this case. Reactive oxygen species also activate p53\(^{25}\) and p53 activates CBP,\(^{27}\) cofactor of CYP1A1 promoter. The large dose of AsA may decrease the p53 activation to reduce CBP activation, and as a result, CYP1A1 gene expression may be suppressed. There are many possibilities to explain the results we shown here, and further studies on the mechanism of AsA affecting gene expression are needed.

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