Regular article

Hypersensitivity of a Urate-null Strain of Drosophila melanogaster to the Toxic Effects of Environmental Cigarette Smoke

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Epidemiological evidence indicates that cigarette smoke is harmful to human health. Mainstream cigarette smoke has already been demonstrated to induce tissue and cellular damage in animal models. In the present study, we examined the toxicity of environmental cigarette smoke (ECS) by exposing Drosophila melanogaster larvae from urate-null and wild-type strains to ECS for 3 or 6 h at the third instar stage. We then determined survival to adulthood and the fecundity of adult females that survived larval ECS exposure. The survival of the urate-null strain, but not the wild-type strain, decreased significantly in an exposure-dependent manner. Moreover, the fecundity of treated urate-null, but not wild-type, females decreased significantly relative to the control level, irrespective of mating partner exposure to ECS at the larval stage. These results demonstrate the killing effect and reproductive toxicity of ECS on urate-null larvae of Drosophila. Since the urate-null strain is known to be sensitive to oxidative agents, we propose that the main cause of the observed toxic effects of ECS is oxidative stress.

Key words: environmental cigarette smoke, urate-null strain, survival, Drosophila, fecundity

Introduction

Tobacco smoking increases the risk of cancer not only of the lung, but also of other tissues, and it is a risk factor in a variety of chronic degenerative diseases. This risk factor has been supported by epidemiological (1) and experimental studies on laboratory animals support (2–5). De Flora and coworkers demonstrated that long-term inhalation of mainstream cigarette smoke in mice from 12 h after birth causes histopathological alterations, including tumors in the lung, kidney, and liver (4). The genotoxicity of tobacco smoke and smoke condensate has been thoroughly investigated (reviewed by Ref. 6). However, the effects of sidestream or environmental cigarette smoke (ECS) on human health are still unclear. Schick and Glantz reported that fresh sidestream smoke is 3 to 4 times more toxic than mainstream smoke to rats and mice (7), and that aged sidestream smoke increases toxicity for respiratory epithelium of rats based on histological scoring (8). Transplacental exposure to cigarette smoke also induces genomic and transcriptional alterations in the mouse fetus liver (9).

In the present study, we examined the toxic effects of ECS on survival of larval Drosophila melanogaster to adulthood and on the reproduction of adult flies that survived ECS treatment using a urate-null strain and a wild-type strain. Uric acid plays an important role in vivo as an antioxidant (10,11). In human plasma, the concentration of urate is estimated to be about 300 μM. The urate-null strain of Drosophila used in the present study is known to be sensitive to oxidative stress such as that caused by X-rays and paraquat (12–14). In the present study, we examined the toxicity of ECS by exposing Drosophila larvae from urate-null and wild-type strains.

Materials and Methods

Drosophila strains: Two Drosophila strains were used in this study: a wild-type strain (Oregon-R) and a urate-null strain (y v ma-l) that is deficient in xanthine oxidase activity (15), both kindly provided by Dr. H. Ryo (Osaka University, Suita, Japan). The genotypes have been described by Lindsley and Zimm (16).

Exposure to ECS: About 500 third-instar larvae of...
the urate-null or wild-type strain were sampled and placed in a plastic box (7 cm × 10 cm × 3 cm) with a nylon mesh cover in which 3 ml of 0.25 M sucrose was added to prevent dryness. These boxes were put on the net shelf in an incubator (Low Temp-Incubator IJ300W, 30 cm × 30 cm × 30 cm; Yamato Scientific Co., Ltd., Tokyo, Japan) maintained at 25°C and filled with smoke from three burning cigarettes. The burning cigarettes were replaced with newly lit cigarettes every 20 min during exposures for 3 or 6 h. Cigarettes used in experiments were purchased from a shop in area. The contents of nicotine and tar were shown on the package.

Experiments were purchased from a shop in area. The cigarettes were replaced with newly lit cigarettes every 20 min during exposures for 3 or 6 h. Cigarettes used in experiments were performed between treated females × untreated males, untreated females × treated males, and treated females × treated males to determine whether sex was a factor related to the effects of ECS exposure. In both series of experiments, 3 virgin females were collected and mated with 3 males per vial one day after eclosion. The three pairs were allowed to mate and oviposit for one day before being transferred daily onto new medium for 6 successive days. Eggs that had been oviposited on the medium in each vial were counted daily and maintained at 25°C until adult flies emerged. The number of adult offspring was recorded and fecundity was determined as the total number of adult flies produced by a group of three females. Each type of cross for the urate-null and wild-type strains was performed three replicates in both series of experiments.

Measurement of 8-hydroxydeoxyguanosine in larval DNA: Immediately after ECS exposure, some larvae were frozen, stored at −75°C until DNA extraction, and subjected to HPLC measurement of the 8-hydroxydeoxyguanosine (8-OHdG) content, as described previously (13,14).

Measurement of urate in larval body fluid: Frozen larvae (n = 30) were prepared for the determination of uric acid content by homogenizing in 0.8 ml of 0.1 M sodium phosphate buffer (pH 7.0) with 20% glycerol.

The supernatant collected by centrifugation at 14,000 rpm (MTX-150, Tomy Seiko Co., Ltd., Tokyo, Japan) (4°C, 10 min) was filtered with a cellulose acetate membrane (DISMIC-13 CP, 0.20 μm, Advantec, Tokyo, Japan). The filtrate was immediately frozen in liquid N2 and stored at −20°C. Concentrations of uric acid were determined according to a previous method (17), with slight modifications. Briefly, the HPLC system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller and on-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV-vis photodiode-array detector. Reversed phase HPLC (RP-HPLC) was performed on an Inertsil ODS-3 octadecylsilane column (4.6 × 250 mm; particle size, 5 μm; GL Science, Tokyo, Japan) with 20 mM ammonium acetate (pH 5.9) as the elution buffer containing no organic solvents. The column temperature was 40°C and the flow rate was 1.0 ml/min. The concentration of uric acid was determined from an integrated peak area on an HPLC chromatogram compared with that of an authentic standard solution. The detection wavelength was 300 nm. All samples were analyzed by RP-HPLC immediately after the samples were thawed. The concentration of protein was determined by the Lowry method standardized by BSA (DC Protein Assay kit, Bio-Rad, Tokyo, Japan).

Results and Discussion
Survival to adulthood of two Drosophila strains ex-

![Fig. 1. Survival of larvae to adulthood after exposure to ECS. The closed (●) and opened (○) circles represent data points for the urate-null and wild-type strains, respectively. Five independent experiments were carried out for the 3-h exposure, and 11 for the 6-h exposure and control. Each data point represents the mean number of adult flies from exposed larvae relative to that from non-exposed larvae. The mean numbers of adult flies from control larvae of the wild-type and urate-null strains were 135 ± 36/vial and 141 ± 30/vial, respectively. *p = 0.05 and **p = 0.01 indicate a significant difference in the mean numbers of adult flies, compared to the control, using the Student’s t-test.](image-url)
posed to ECS for two different durations is shown in Fig. 1. The survival of the urate-null strain decreased significantly in an exposure-dependent manner to a level of 30%, indicating that about 70% of larvae did not emerge to adult flies after 6-h ESC exposure. On the other hand, survival of the wild-type showed no significant change; however, a slight decrease was observed after 6-h ECS exposure.

Immediately before ECS exposure, the uric acid concentrations were $11 \pm 3$ and $0.3 \pm 0.1$ nmol/mg protein in the wild-type and urate-null strains, respectively. In addition, urate is an antioxidant, ECS exposure is expected to induce a strain-dependent difference in the 8-OHdG content in the DNA of larval cells. However, the 8-OHdG content in larval DNA showed no significant change after 6-h ECS exposure in either strain (Table 1), suggesting that the main cause of the killing effect of ECS is oxidative damage to cellular components other than DNA.

In contrast to our results for 8-OHdG levels, Maehira et al. reported that long passive smoking over 12 weeks increased 8-OHdG in exposed rat DNA and that the elevation was protected by the addition of vitamin E in the diet (18). Furthermore, Dolara et al. reported that active smoking significantly elevated the level of 8-OHdG in human leucocyte DNA and that passive smoking tended to increase the level of 8-OHdG (19). Thus, the duration of ECS exposure in this study may have been too short for the accumulation of 8-OHdG in larval DNA.

Adult flies of the urate-null and wild-type strains that survived 6-h larval ECS exposure were subjected to two series of experiments to examine fecundity (Table 2). When treated females of either the urate-null or wild-type strain were mated with treated males of the same strain in both series, the mean number of eggs laid by a group of three pairs of treated parents within a week after the onset of oviposition showed no significant difference from the control, irrespective of the strain. However, the mean number of resulting adult offspring from treated urate-null parents, but not from treated wild-type parents, decreased significantly relative to the control. In the second series of experiments, a significant reduction relative to the control was observed when treated urate-null females were mated with untreated males of the same strain, but not when untreated urate-null females were mated with treated males of the same strain. These results demonstrate the reproductive toxicity of larval ECS treatment of the urate-null strain in adult females.

Since the number of eggs produced by treated urate-null females showed no significant reduction relative to that of the control, the observed reduction in the number of adult offspring may be partly due to a reduction in hatchability as a consequence of disabled embryonic development in the eggs. Although the factors involved in the effects of larval ECS treatment on the development of the next generation is still not understood, ECS treatment is unlikely to cause mutations in germ cells of female larvae that subsequently exerted a dominant lethal effect in the next generation; that is, such mutations in Drosophila can seldom be induced in pre-meiotic germ cells of females, even after ionizing radiation (20). On the other hand, the toxic effects of ECS may have remained in pupae after exposure to larvae and eventually affected reorganization of fat bodies in the adult and/or development of follicle cells, i.e., somatic components of the ovary. Fat bodies and follicle cells support oogenesis by supplying yolk proteins to growing oocytes in the ovary (21). Further studies are necessary to elucidate the mechanisms involved in the reproductive toxicity of ECS exerted in urate-null adult females.

**Table 1.** Level of 8-hydroxydeoxyguanosine in larval DNA after ESC exposure for 6 h

<table>
<thead>
<tr>
<th>Exposure (h)</th>
<th>Wild-type ($n=5$)*</th>
<th>Urate null ($n=6$)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.56 ± 0.41</td>
<td>1.91 ± 0.79</td>
</tr>
<tr>
<td>3</td>
<td>1.24 ± 0.36</td>
<td>1.16 ± 0.37</td>
</tr>
<tr>
<td>6</td>
<td>1.27 ± 0.29</td>
<td>2.49 ± 1.33</td>
</tr>
</tbody>
</table>

*The number of independent experiments.

**Table 2.** Impact of larval ECS exposure on the reproduction of surviving adult flies

<table>
<thead>
<tr>
<th>Strain cross†</th>
<th>No. of eggs‡ ($n=3$)†</th>
<th>No. of adult flies‡ ($n=3$)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweiwa 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type (Oregon-R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UF × UM</td>
<td>805 ± 124</td>
<td>718 ± 138</td>
</tr>
<tr>
<td>TF × TM</td>
<td>754 ± 90</td>
<td>659 ± 96</td>
</tr>
<tr>
<td>Urate-null (y v ma-l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UF × UM</td>
<td>1760 ± 240</td>
<td>1551 ± 284</td>
</tr>
<tr>
<td>TF × TM</td>
<td>1475 ± 271</td>
<td>951 ± 73‡</td>
</tr>
<tr>
<td>Series 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type (Oregon-R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UF × UM</td>
<td>617 ± 55</td>
<td>584 ± 96‡</td>
</tr>
<tr>
<td>UF × TM</td>
<td>492 ± 129</td>
<td>445 ± 85‡</td>
</tr>
<tr>
<td>TF × UM</td>
<td>416 ± 116</td>
<td>373 ± 123</td>
</tr>
<tr>
<td>TF × FM</td>
<td>393 ± 146</td>
<td>350 ± 161</td>
</tr>
<tr>
<td>Urate-null (y v ma-l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UF × UM</td>
<td>1129 ± 205</td>
<td>902 ± 99‡</td>
</tr>
<tr>
<td>UF × TM</td>
<td>989 ± 170</td>
<td>769 ± 169</td>
</tr>
<tr>
<td>TF × UM</td>
<td>929 ± 108</td>
<td>590 ± 101§</td>
</tr>
<tr>
<td>TF × FM</td>
<td>928 ± 71</td>
<td>447 ± 97‡</td>
</tr>
</tbody>
</table>

*UM = untreated control males, UF = untreated control females, TF = treated females, and TM = treated males.
†The number of independent experiments.
‡The number of independent experiments.
§p=0.05 and †p=0.01, significantly different, compared with the control cross (UF × UM), using the Student’s t-test.
after larval exposure.

In summary, this study shows that larvae of the urate-null strain are hypersensitive to ECS toxicity as assayed for survival to the adult stage and fecundity of surviving adult females. Oxidative damage seems to be involved in the toxicity of ECS as urate acts as a major antioxidant in *Drosophila*. Our results of the 8-OHdG content in ECS-treated larval DNA indicate that the survival assay using the larval urate-null strain may be worth developing as a system to study the biochemical nature of ECS toxicity. It remains to be seen what oxidative stress occurs in larval bodies by ECS.

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**References**