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Exposure to Cigarette Smoke Increases Urate Level and Decreases Glutathione Level in Larval *Drosophila melanogaster*

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Recently, we reported experimental evidence to support the notion that in *Drosophila melanogaster*, urate is involved in defense against toxic effects of environmental cigarette smoke (ECS). To obtain further information pertaining to the defense mechanisms involving urate and other antioxidants, the present study measured the levels of urate, its precursors and glutathione, and SOD activity in larval flies of wild-type strains (Oregon-R and Canton-S) and two urate-null mutant strains (*ma-l* and *ry*[1]) following exposure to ECS for various durations. In both wild type strains, unlike the case in either of the mutant strains, the urate level significantly increased above the basal level in a manner dependent on the duration of ECS exposure. Similar increases in the level of urate precursors were found in Canton-S and in both of the urate-null strains. There was a slight increase in glutathione level above the control level following ECS exposure for a short time, followed by an exposure-dependent decrease to less than 60% of the control level within the exposure range used in all of the four strains. On the other hand, no appreciable change was found in the SOD activity prior to or following ECS exposure, irrespective of the strain examined. In terms of the survival of treated larvae to adulthood under the conditions used for the measurements of urate and others, it was found that wild-type strain Canton-S was as sensitive as the urate-null mutant strains and clearly more sensitive than wild-type strains Oregon-R and Hikone-R. This was so despite the fact that, compared with Oregon-R, Canton-S contained urate at relatively higher levels prior to and following ECS exposure, and that the glutathione levels in Canton-S prior to and following treatment were comparable with those in other strains. These results are discussed with respect to the involvement of urate and glutathione in defense against the toxicity of ECS and the possible existence of another defense mechanism which is deficient in the Canton-S strain.

Key words: environmental cigarette smoke, uric acid, glutathione, oxidative stress, survival, *Drosophila*

Introduction

In humans, environmental cigarette smoke (ECS) is well known to injure various tissues and elevate the risk of developing many lifestyle- and age-related diseases, including cancers in various tissues (1,2), cardiovascular disorders (3), diabetes (4) and retinal cell injury (5). Recently, a larger number of toxic compounds have been detected in side stream or second-hand cigarette smoke (6). Hence, in order to fully understand the implications of cigarette smoking on global health, we must consider not only smokers, but the total effect of cigarette smoke, i.e. the 'smoking-environment' (7).

Accumulating evidence suggests that cigarette smoke induces oxidative stress in cultured human cells and not only in smokers but also in non-smokers (8–13), as determined by measuring ROS production, antioxidant levels, plasma levels of antioxidants and the expression of oxidative stress-related genes. Moreover, using the comet assay combined with endonuclease treatment, which detects the formation of 8-oxodeoxyguanosine (8-oxodG), a critical biomarker of oxidative damage to DNA (14), Thorne and colleagues observed that the damage was induced in DNA following exposure of human pulmonary cancer cells to mainstream cigarette smoke (15). In contrast, no increase in 8-oxodG levels was found following ECS exposure of larval *Drosophila* in our recent study. Nevertheless, we proposed that oxidative stress does occur in larval flies in response to ECS exposure since larvae of a urate-null mutant strain were found to be hypersensitive to ECS exposure as determined by measuring the survival of treated larvae to adulthood and the fecundity of survived adult females

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(16), and, given its radical scavenging property, uric acid is thought to play an important antioxidative role in biological fluids of *Drosophila* and other organisms (17–22). Indeed, exposure of human serum to cigarette smoke leads to urate depletion, as expected given its radical scavenging property (23).

However, our knowledge concerning the role of urate and other antioxidants in the protection of living cells *in vivo* from the toxic effect of ECS remains scant. The present study measured the levels of uric acid and its precursors, glutathione and the activity of SOD following exposure of wild-type and urate-null mutant strain *Drosophila* larvae to ECS for various durations in a range that affected the survival of treated larvae to adulthood in a more or less exposure-dependent manner. The levels of urate and its precursors were measured to determine whether urate synthesis was stimulated in response to ECS exposure. Evidence for urate synthesis in response to oxidative stress has been reported in human skin where urate levels became elevated following exposure to sunlight (24) and with aging (25). It was considered that examination of urate levels alone would be of limited value as a measure of its synthesis since direct oxidation of urate by radical oxygen species leads to its depletion, as noted in the preceding paragraph. The potential of glutathione to act as an antioxidant in defense against ECS toxicity was suggested by an observation made by Bertram *et al.*, who found that the level of intercellular GSH (a reduced form of glutathione) in retinal pigment epithelial cells of volunteer tobacco smokers decreased significantly, whereas the level of GSSG (an oxidized form of glutathione) remained unchanged (5). The activity of SOD, an enzyme that catalyzes the conversion of super- oxide anions to hydrogen peroxide, was also measured to determine whether ECS exposure affected SOD activity together with any changes in urate or glutathione levels.

Materials and Methods

Drosophila strains: Three wild-type and two urate-null strains were used in the present study. The wild-type strains used were Oregon-R, Hikone-R and Canton-S. Here, the term wild-type is employed in its traditional sense, referring to strains not marked with specific mutant alleles. The urate-null strains used were those carrying mutant alleles *ma-l* and ry^1, respectively. Oregon-R, Canton S, and a *ma-l* strain with visible markers *y* and *v* were kindly provided by Dr. H. Ryo (Osaka University, Osaka, Japan). Hikone-R and the ry^1 strain were obtained from the *Drosophila* Genetic Resource Center at the Kyoto Institute of Technology (Kyoto, Japan). The two mutant strains are deficient in xanthine dehydrogenase activity (26, 27). The gene symbols used are as described by Lindsley and Zimm (28).

Exposure to ECS: Exposure to ECS was performed as described in our previous report (16). Briefly, about 500 larvae were exposed at the third instar stage to ECS in a 25°C incubator. The burning cigarettes were replaced with a newly lit cigarette every 20 min during exposures for 2, 4 or 6 h. Immediately following exposure, 30 larvae were frozen and stored at −75°C until use. Larval samples were subsequently processed for HPLC to measure the amounts of uric acid and precursors of uric acid, xanthine and hypoxanthine, or to quantify the amounts of total glutathione and the activity of superoxide dismutase (see below). The remaining ECS-exposed larvae were placed on *Drosophila* instant medium (Formula 4–24, Carolina Biological Supply, Burlington, NC) and cultured at 25°C until adult flies began to eclose. The survival was defined as the ratio of the number of flies that eclosed following exposure of larvae to ECS to the number of genotype-matched flies that eclosed from non-exposed larvae.

Measurement of uric acid and precursors: The levels of uric acid, its precursors xanthine and hypoxanthine, other purine bases, AMP and GMP were determined by HPLC (CTO-6A system controller, Shimadzu, Kyoto) as previously described (16,29). A total of 30 frozen larvae were used in each sample for HPLC. To determine the retention time of each base and monophosphate, a standard mixture was used containing uric acid, hypoxanthine, adenine, guanine, cytosine, thymine, uracil, inosine, deoxyadenosine, deoxyguanosine, deoxycytidine, deoxythymidine, deoxyinosine, deoxyuridine, adenosine monophosphate and guanosine monophosphate at 10 μM, and xanthine at 5 μM. Uric acid and its precursors, xanthine and hypoxanthine were quantified using the integrated peak area calculated from the HPLC chromatogram (see Fig. 1). Each peak was identified based on the known retention time and UV absorption spectrum of authentic compounds.

Measurement of glutathione: The total amount of glutathione in a sample was quantified with the Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) using the DTNB-GSSG/glutathione reductase recycling method according to the manufacturer's instructions. Briefly, 30 frozen larvae were homogenized in 0.8 ml of 0.1 M sodium phosphate buffer (pH 7.0)/20% glycerol. An equal volume of 10% 5-sulfosalicylic acid (Nacalai tesque, Kyoto) was then added to the homogenate to remove protein. The sample was centrifuged at 14,000 rpm for 10 min and the supernatant was then used for the measurement of glutathione. Prior to analysis of the samples, glutathione was converted to its reduced form by treatment with glutathione reductase. Glutathione levels were then assayed by determining the absorbance at 415 nm of 5-mercapto-2-nitrobenzoic acid, which is produced by reduction of 5,5-dithiobis-2-nitrobenzoic acid

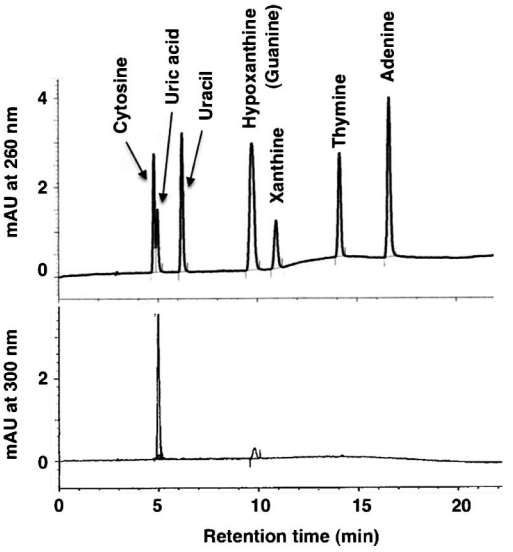

Fig. 1. Separation of bases by HPLC. Mixture containing bases at a concentration of 5 mM separated by HPLC as described in Materials and Methods. Upper and lower charts were obtained from the absorbance at 260 nm and 300 nm, respectively, being specific wavelengths for uric acid. Each peak was confirmed by its UV spectrum.

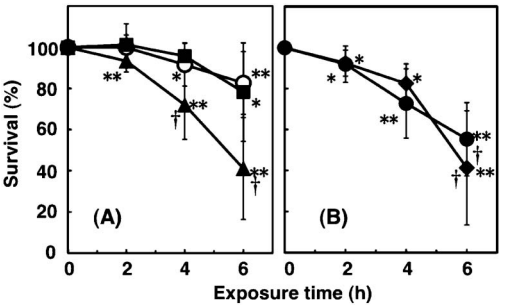

Fig. 2. Survival of larvae to adulthood following exposure to ECS. (A) Wild-type strains; Oregon-R (open circle), Hikone-R (square) and Canton-S (triangle). (B) Urate-null strains; *y v ma-l* (diamond) and ry^1 (closed circle). A total of 6 to 18 independent experiments were performed and each data point represents the mean number of adult flies which eclosed following exposure of larvae to ECS compared to the number which eclosed following a similar protocol with the same genotype but in the absence of ECS exposure. $^*p<0.05$ and $^{**}p<0.001$, which indicate a significant difference in the mean numbers of adult flies compared to unexposed controls and $^\dagger p<0.001$, shows the significant difference compared to Oregon-R, using the Student's *t*-test.

following oxidation of the reduced form of glutathione to the oxidized form. The concentration of protein in the homogenate was determined using the Lowry method with bovine serum albumin (BSA) as a standard DC Protein Assay Kit (Bio-Rad, Tokyo, Japan).

Measurement of SOD activity: The activity of superoxide dismutase (SOD) was determined using the SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. A total of 30 frozen larvae were homogenized in 0.8 ml of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose and 1 mM EDTA, and after centrifugation at 25,000 rpm at 4°C for 60 min (XL-90 Ultracentrifuge, BECKMAN) the resulting supernatant was tested. SOD activity was assayed by determining the rate of inhibition of formazan formation. In this assay, superoxide anions, which are produced by xanthine oxidase contained in the reaction mixture, facilitate the formation of formazan, which can be monitored by measuring the absorbance at 450 nm. If SOD is present in this reaction mixture, formazan formation should be inhibited due to desmutation of superoxide anions by SOD. The concentration of protein in the supernatant was determined using the Lowry method with BSA as a standard DC Protein Assay Kit (Bio-Rad, Tokyo, Japan).

Results

Strain-dependent variation in the sensitivity to the killing effect of ECS: As shown in Fig. 2, for all strains examined, the survival of larvae to adulthood decreased as the duration of ECS exposure increased. It was observable that larvae appeared to be suppressed the metamorphosis from larval stage to pupal stage when larvae could not grow up to adulthood. From the survival curves we obtained, the duration of ECS exposure required to decrease the survival to 80% of the control was about 6 h for wild-type strains Oregon-R and Hikone-R, 3 h for wild-type strain Canton-S, and 3 to 4 h for the urate-null mutant strains. Interestingly, the Canton-S strain, in addition to the two urate-null strains, was found to be hypersensitive to the killing

Table 1. Purine base levels in third instar larvae body fluid

Fly strain	Exposure to ECS (h)	Purine bases (nmol/mg protein)		
		Uric acid	Xanthine	Hypoxanthine
Oregon-R	0	2.8±1.3	1.7±1.1	2.1±1.8
	2	3.3±1.2	1.8±1.2	2.2±2.2
	4	2.8±1.1	1.7±1.0	2.2±1.9
	6	4.9±1.0*	3.1±1.5	3.2±3.0
Canton-S	0	7.4±0.8	1.5±1.2	1.5±1.2
	2	11.0±2.0†	1.6±0.8	1.5±0.5
	4	12.2±3.6*	3.0±0.8*	2.5±1.0
	6	14.7±4.8†	4.7±0.9†	3.3±1.1*
y v ma-l	0	0.16±0.13	5.3±2.2	5.2±2.2
	2	0.03±0.07	5.1±1.2	5.3±2.1
	4	0.08±0.10	8.5±3.1	8.7±3.4
	6	0.17±0.13	7.7±5.0	11.0±3.3*
ry^1	0	<0.15	10.2±2.5	7.4±2.0
	2	<0.13	9.8±1.5	6.4±0.7
	4	<0.19	10.3±1.0	8.8±1.2
	6	<0.14	12.2±2.7	11.3±1.8*

Statistical analysis was performed using the Student's *t* test. $^*p<0.05$ (vs. 0 h), $^\dagger p<0.01$ (vs. 0 h).

effect of ECS, despite the fact that this strain, as well as Oregon-R, can synthesize urate (see Table 1).

Increase in the levels of urate and its precursors following exposure to ECS: Treated and untreated control larvae of wild-type strains Oregon-R and Canton S and urate-null strains *ma-l* and ry^1 that had been used for the survival assay were employed for the determination of uric acid, its precursors and glutathione levels, and SOD activity in the bodies. Hikone-R was excluded from these analyses as the effect of ECS on the survival of Hikone-R was similar to that of Oregon-R and the content of urate in Hikone-R was also similar to that of Oregon-R at un-exposure to ECS (data not shown). The results obtained for urate and it precursors are shown in Table 1.

As can be seen in Table 1, urate was detected at a nano-mole order (per 1 mg protein) in control larvae of wild-type strains; the urate level in Oregon-R is significantly lower than that in Canton-S. On the other hand, the basal urate level in both urate-null mutant strains was practically zero, as expected given that these strains lack xanthine dehydrogenase activity.

When urate was measured following ECS exposure, it became evident that treatment increased the urate level above the basal level in both wild type strains in a manner that is dependent on the exposure time. In Oregon-R, the increase was significant only after 6 h exposure, the longest duration used, which caused a 20% reduction in survival compared with the control. In Canton-S, the increase was significant even after 2 h exposure, the shortest duration used, which caused a 10% reduction in survival compared with the control. From the data given in Table 1, we calculated the amounts of urate (nmol/mg protein) that exceeded the corresponding basal levels in Oregon-R after 6 h exposure and in Canton-S after 2 h exposure to be 2.1 ± 1.6 and 3.7 ± 2.1, respectively. These results demonstrate that ECS exposure increased the urate level more effectively in Canton-S than in Oregon-R.

Concomitant with the increase in the urate level, urate precursors apparently increased in wild-type strains Oregon-R and Canton-S strain following ECS exposure (Table 1). In the latter strain, the xanthine levels recorded after 4 and 6 h exposures and the hypoxanthine level after 6 h exposure are significant in comparison with the corresponding controls. A similar increase was found for the hypoxanthine level after 6 h exposure in both urate-null strains (Table 1). Among other purine bases that were measured in the mutant strains, uracil was consistently detected in both strains at levels of around 1 nmol/mg protein with no apparent relationship to ECS exposure. The other bases examined, including guanosine, adenosine, AMP and GMP, were detected at low levels in some samples, and no changes in levels were observed following ECS exposure (data not shown).

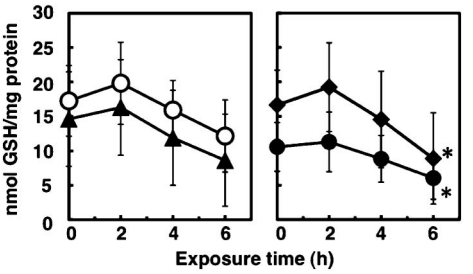

Fig. 3. Levels of total glutathione in larvae; Oregon-R (open circle), Canton-S (triangle), *y v ma-l* (diamond) and ry^1 (closed circle). A total of 3 to 6 independent experiments were performed. $^*p < 0.05$, which indicates the significant difference compared to unexposed larvae.

Table 2. Superoxide dismutase activity in *Drosophila* larvae

Strain	SOD unit/mg protein*			
	Exposure to ECS (h)			
	0	2	4	6
Oregon-R	9.3 ± 4.2	11.3 ± 4.3	10.0 ± 5.4	9.5 ± 4.1
Canton-S	9.1 ± 4.1	9.8 ± 3.6	9.4 ± 3.2	6.7 ± 3.2
y v ma-l	11.2 ± 2.6	9.8 ± 3.4	10.9 ± 4.5	10.0 ± 1.4
ry^1	11.7 ± 7.3	7.4 ± 5.5	7.5 ± 5.0	7.0 ± 5.2

*1U is defined as the amount of SOD necessary to inhibit the reduction of nitroblue tetrazolium in the reaction mixture to 50% of untreated levels. Experiments were repeated four times with Oregon-R and *y v ma-l*, and three times with Canton-S and ry^1.

Decrease in glutathione level following ECS exposure: Glutathione exists in two forms in cells, a reduced form, GSH, and an oxidized form, GSSG. With the methods used in the present study, these two forms of glutathione comprised the total glutathione detected. In untreated larvae of the four strains examined, glutathione basal levels were comparable, being 14.6 ± 4.4 nmol/mg protein in Oregon-R, 16.6 ± 2.1 in Canton-S, 17.3 ± 1.8 in *ma-l*, and 10.6 ± 1.5 in ry^1. As can be seen in Fig. 3, in all of these strains, the glutathione level increased slightly above the basal level after 2 h exposure to ECS and then declined to 60–70% of the control as the exposure time was extended up to 6 h. Statistical analyses indicate that none of these slight increases observed are significant, and that the observed levels after 6 h exposure are significant ($p < 0.05$) in both of the urate-null strains but not in either wild-type strain examined. Despite the results of the statistical analysis, given the similar exposure-response curves of the different strains, we regard the observed increase and decrease in glutathione levels of biological significance.

No change in SOD activity: SOD catalyzes the conversion of super oxide anion to hydrogen peroxide. If super oxide anions are produced in larvae following ECS exposure, then SOD activity might have changed following ECS exposure. However, we found that SOD

activity remained nearly constant prior to and following exposure to ECS (Table 2), although SOD activity in Canton-S and ry^1 appeared to decrease slightly.

Discussion

As already noted, it has been reported that the urate level on the surface of human skin *in vivo* is elevated following exposure to sunlight and with aging (24,25). In the present study we found that the urate level in the larval bodies of wild-type strains Oregon-R and Canton-S significantly increased above the basal level following ECS exposure in a manner that is dependent on the duration. Similar increases in the levels of urate precursors were found in treated larvae of wild-type strains and in both urate-null strains, with no evidence of increased levels of uracil or other purine bases. Thus, this study has revealed that exposure of larval flies to ECS stimulates urate synthesis in the cells. By analogy with the reported evidence for altered expression profiles of stress-related genes in cultured human cells following exposure to cigarette smoke (30), the present finding implies up-regulation of gene(s) involved in urate synthesis in larval cells during ECS exposure. Furthermore, given the antioxidative property of urate, we may assume that stimulated urate synthesis in larval cells during ECS exposure is a defensive response to oxidative stress caused by the treatment. However, it is remains to be examined how long the assumed adaptive response continues after the termination of ECS treatment.

Consistent with the idea that tobacco smoke induces oxidative stress, Bertram and colleagues have reported that following the inhalation of tobacco smoke, the level of intercellular GSH in human retinal pigment epithelial cells decreased significantly, whereas the level of GSSG remained unchanged (5). We found in this study that ECS exposure of larval flies decreased the level of total glutathione (that is, GSH plus GSSG) in the bodies of all four strains examined. According to Wickenden and colleagues, who observed in cultured human cells that the total glutathione decreased following treatment with cigarette smoke condensate, GSH may conjugate with cigarette smoke components, as GSH is utilized in living cells in detoxification processes by forming conjugates with xenobiotics (31). This interpretation could also be applied to the present data. Namely, the observed reduction in total glutathione levels can be interpreted as evidence of GSH depletion, although the chemical nature of the ECS component(s) that conjugate remains unclear at present. However, it can be said that the defense mechanism involving glutathione in response to ECS toxicity differs, at least in part, from that involving urate, because the magnitude of the reduction in glutathione levels (e.g., ca. 60% of the control following the longest exposure) was similar in all four strains examined, irrespective of urate synthesis capability.

Another point of interest in the data obtained for glutathione is the slight increase in level found above the basal level in all strains examined following ECS exposure for short time, suggesting that glutathione synthesis was stimulated during the treatment. Experimental evidence for induced synthesis of glutathione by oxidative stress has been reported by Nzengue and coworkers (32). They found that when cultured human cells were treated with cadmium, the GSSG level increased and the ratio of the GSH to GSSG level decreased with concomitant increases in oxidative damage to proteins, lipids and DNA. It was surmised that the major compound involved in the oxidative stress generated in cadmium-treated cells could be the hydroxyl radical. However, the possible involvement of hydroxyl radicals in the apparent increases in glutathione level observed in our present study remains to be investigated.

In contrast to the levels of urate and glutathione, the SOD activity in larval flies remained relatively constant prior to and following ECS exposure. The same was true in the aforementioned study concerning human cells treated with cadmium, where there was substantial evidence of increased amounts of hydroxyl radicals. Russo *et al.* reported that the gene expression of antioxidant enzymes, such as SOD, catalase and glutathione peroxidase, decreased when cultured human cells were treated with cigarette smoke condensate (11). It seems from these results that SOD activity is not a suitable indicator of oxidative stress caused by environmental factors in *Drosophila*.

One perhaps unexpected observation relates to hypersensitivity of the wild-type strain Canton-S to the killing effect of ECS exposure, where the exposure duration leading to 80% of the control survival was 3 h, in contrast to 6 h required for the Oregon-R and Hikone-R strains. This was so despite the fact that, compared with Oregon-R, Canton-S contained urate at relatively higher levels prior to and following ECS exposure, and that the levels of glutathione in Canton-S prior to and following treatment were comparable with those in other strains. Thus, *Drosophila* larvae might possess an additional mechanism which responds to ECS-induced stress, and which is deficient in Canton-S but not in Oregon-R. In this context, it should be noted that larval flies of Canton-S are more sensitive to the killing effect of DDT, compared with Hikone-R and Oregon-R (33,34), and to that of nicotine compared with Hikone-R, when given orally (35). Detoxification of both DDT and nicotine is dependent on the CYP450 metabolizing system. Taken together, we are inclined to believe that the CYP450 system is involved in defense against the toxic effects of ECS.

In summary, the experimental data obtained in the present study suggest the presence of multiple mechan-

isms which act in defense against various kinds of toxic compounds in ECS, either oxidative or non-oxidative. Needless to say, any interpretation proposed for these data is no more than a working hypothesis for further studies. In any case, given its high sensitivity to ECS toxicity and marked change in urate levels following ECS exposure, utilization of the wild-type strain Canton-S should provide unique opportunities to further our understanding of *in vivo* cell defense mechanisms against toxicity.

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