Mutation Research 722 (2011) 52–55

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

A study of the inhibition/promotion effects of sodium–copper chlorophyllin (SCC)-mediated mutagenesis in somatic cells of *Drosophila*

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ARTICLE INFO

Article history:
Received 20 December 2010
Accepted 7 March 2011
Available online 13 March 2011

Keywords:
Drosophila
Chlorophyllin (SCC)
Inhibition
Promotion
Protoporphyrin
Copper chloride

ABSTRACT

Sodium–copper chlorophyllin (SCC), a copper–porphyrin complex, has been shown to act as an inhibitor as well as a promoter of DNA-damage induction by a variety of mutagens in several test systems. In order to investigate the basis of this dual effect, experiments were carried out to compare the influence of pretreatment with intact SCC and that of its constituents, the metal-free protoporphyrin (PP-IX) and copper as $CuCl_2$. The wing-spot test was employed to monitor mutational events in somatic cells of *Drosophila melanogaster*. Heterozygous *mwh*+/+*flr*^{3} larvae were treated for 24 h with SCC, PP-IX, $CuCl_2$ or sucrose. Following this treatment, one group of larvae were immediately allowed to feed on instant medium containing 0.5 mM *N*-nitroso-*N*-ethylurea (ENU) dissolved in phosphate buffer to reach pH 6. The remaining larvae received treatment with ENU with a delay of 1, 2 or 3 days (DTD). Results revealed an (a) overall inhibitory effect for 0-DTD and 1-DTD after pretreatment with SCC, (b) only in 0-DTD after PP-IX, and (c) in all DTDs after treatment with $CuCl_2$. These results provide evidence that the copper ion plays a central role in the antimutagenic effect of SCC, and for a sustained period of time. Pretreatment with SCC and PP-IX produced a promoter effect at 2-DTD and 3-DTD. The results could be explained as an effect of the accumulation of metal-free porphyrin following the dissociation of the copper–porphyrin complex (SCC), the copper-ion reaching proteins to form complexes and participated in anabolic pathways.

1. Introduction

Sodium–copper chlorophyllin (SCC) exhibits potent antimutagenic and anticarcinogenic activity against many agents [1] including, among others, aflatoxins [2,3], heterocyclic amines [4,5], and gamma rays [6–8]. SCC can act as an antioxidant by scavenging free radicals [9,10], by inhibiting enzymes involved in the activation of carcinogens [11] or by forming complexes with mutagens and/or carcinogens before they interact with DNA [12]. In contrast, other results have shown that SCC may both inhibit and increase the genetic effects of some agents [13,15].

By delaying the time of treatment with a mutagenic agent, it could be shown that SCC could protect against (a) gamma-ray-induced mutagenesis for at least 72 h after the end of the pretreatment period [7,8] and (b) DNA damage induced by chromium VI oxide for at least 48 h; in the latter case, evidence of a promotion effect was found after 48 h [15]. In a recent study we provided evidence suggesting that the dual effect of SCC could be related to its concentration [16], as was shown earlier by Romert et al. [13] in *Salmonella*. These authors reported that high concentrations reduced the genetic damage, but low concentrations increased the genotoxic effect.

Earlier studies provided information regarding the digestive instability of chlorophyll, by identification of metal-free derivatives of natural and commercial-grade chlorophylls in urine and faeces [17,18]. During a simulated digestion *in vitro*, recent evidence confirmed that chlorophylls are converted to metal-free porphyrin derivatives, which subsequently accumulate in cells [19]; for example, experimental evidence has shown that protoporphyrin-IX (PP-IX) can accumulate in the liver and produce oxidative damage [20]. Furthermore, when the metal present is copper, PP-IX can accumulate in human intestinal cells [19]. Other studies have demonstrated that porphyrins can bind to DNA via a specific insertion within only one strand of DNA, i.e., by hemi-intercalation [21], with a binding constant of around $10^6\ M^{-1}$ [22,23]. In this way, these extra-helical structural elements could be a factor in certain pathways of mutagenesis [20].

In previous work, we tested SCC in combination with mutagenic agents operating with different mechanisms of action including gamma rays [8], CrO_3 [15] and 1,1-dimethylhydrazine, an indirect-acting compound (unpublished). Interestingly, it was found that similar shifts (inhibitor to promoter) occurred in each case. This

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doi:10.1016/j.mrgentox.2011.03.001

finding would suggest that the dual effect is related more closely to the action of SCC rather than the mode of action of the mutagenic agents.

The dissociation of SCC favoured by acidic conditions in the larval gut in *Drosophila* may cause separation of the molecule into its two main components, i.e., the metal-free porphyrin and copper. The objective of the current research is to compare the role of these components separately with the intact SCC molecule in the dual effect of SCC. Additionally, given that the inhibition and promotion effects could be associated with structural/activity changes of SCC, the desirability of evaluating the dissociation fragments of SCC became more apparent. Accordingly, we carried out an investigation to evaluate the roles of the porphyrin and copper components of SCC in *N*-nitroso-*N*-ethylurea (ENU)-induced mutagenesis.

In carrying out the work described, the principal experimental approach was (1) to delay the time of treatment with ENU for 0–3 days following completion of pretreatments with SCC and (2) test its separate derivatives, (1) PP-IX and (2) copper (in the form of $CuCl_2$) for genotoxic activity.

2. Materials and methods

In order to monitor induced DNA damage, the *Drosophila* wing-spot test was employed [24], as follows: three-day-old *mwh*/*mwh* females and *flr*^{3}/TM3 *Ser* males were allowed to mate for 2 h and then transferred to culture bottles with fresh food. Oviposition was restricted to a 2-h period in order to obtain more homogeneous age samples of individuals being tested. Larvae were collected at 48 h on a density gradient using a 20% sucrose solution, washed with 24 ± 1 °C tap water and pretreated for 24 h in the dark in flasks (0.25 L) containing a paper filter (Whatman #2), saturated with 3 ml of 69 mM SCC (referred to as a 5% solution in all earlier work in this field), 69 mM PP-IX, 0.01 mM of $CuCl_2$ or 5% sucrose (SUC) as a negative control. SCC, PP-IX and $CuCl_2$ were dissolved in a 5% sucrose solution. Distilled water was used for all solutions. SCC (CAS 11006-34-1), PP-IX (CAS 553-12-8) and ENU (CAS 759-73-9) were purchased from Sigma Chemical Company (St. Louis, MO) and $CuCl_2 \cdot 2H_2O$ (CAS 10125-13-0) from Merck Chemical, AG Darmstadt, Germany. The $CuCl_2$ concentration was calculated on the basis of the amount of copper present in 69 mM of SCC, which represents about 40% of the dissociable copper from 69 mM of SCC [25].

On completion of the pretreatment period, larvae were washed with tap water at 24 ± 1 °C. At this time, aliquots from each pretreatment were treated or not with 0.5 mM ENU in vials (height, 9.5 cm; diameter, 2.5 cm) containing 1.5 g of *Drosophila* instant medium (formula 4-24 Carolina Biol. Supply Co. Burlington, NC). ENU was dissolved in $NaPO_4$-buffer at pH 6 in the experiments included in Table 1. This treated group was mutagenized immediately upon completion of the pretreatment period and was designated as the group 0-days-delay-in-treatment (0-DTD) because it represented no delay in the treatment with the mutagen. The remaining larvae from each pretreatment were transferred to three separate containers with regular food. For these, the treatment with ENU was delayed by 1 day (1-DTD), 2 days (2-DTD), or 3 days (3-DTD). The solution of ENU was prepared fresh for each of these treatments. Experiments were carried out in triplicate. Upon eclosion, flies were fixed with 70% alcohol and the wings of the *mwh*+/+*flr*^{3} flies (i.e., non-*Ser*) were mounted on slides for 400× microscopical analysis. The wings were examined to identify small single spots (one or two cells), large single spots (more than two cells) of either *mwh* or *flr*, and *mwh*-*flr* twin spots. Single *mwh* spots are expected following mutation at the *mwh*$^+$ locus, or by an interchange between the *mwh* and *flr* loci followed by homozygosis for *mwh*. Single *flr*^{3} spots may arise by mutation at the *flr*$^+$ locus or by double exchange. Twin spots are the result of an interchange between the *flr* and the centromere [24]. For a description of the mutants see Lindsley and Zimm [26]. Results from the groups pretreated with SCC, PP-IX and $CuCl_2$ prior to ENU treatment were compared with the SUC + ENU control by use of the chi-square test for proportions in order to verify the statistical significance of the differences found.

Larval-adult development was measured by an indirect method to determine whether the different pretreatments caused cell death. For this purpose, groups of 100 larvae were selected representing each of the four pretreatment types. Each experiment was carried out in triplicate. The number of adults that hatched from each pretreatment was counted daily. The data were plotted and the slope for each pretreatment group calculated. Comparisons were made between the slopes observed for the SCC-, PP-IX-, and $CuCl_2$-pretreated groups treated each with ENU, *versus* the SUC + ENU controls. Additionally, comparisons were made between the slopes of the SCC-, PP-IX- and $CuCl_2$-treatments with SUC alone.

3. Results

Table 1 shows the results of experiments carried out to test the effects of pretreatments with 69 mM SCC, 69 mM PP-IX, 0.01 mM $CuCl_2 \cdot 2H_2O$ or 5% sucrose, respectively. Comparison between the positive control group (SUC + ENU) and each of the pretreated groups that received a subsequent treatment with ENU showed the following: for (a) SCC + ENU, a significant reduction was found in the frequency of all categories of spots in 0-DTD and 1-DTD. In contrast, a significant statistical increase of spots was observed in 2-DTD for large single and total spots, and for all kinds of spots in 3-DTD. For (b) PP-IX + ENU, a considerable reduction was found in all types of spot in 0-DTD, but no changes were detected in 1-DTD. On the contrary, a substantial increase in all categories of spots was seen in both 2-DTD and 3-DTD. Finally, for (c) $CuCl_2$ + ENU, a marginal reduction was seen in 0-DTD and significant reductions in the remaining DTDs.

Fig. 1 represents the results from larval-adult development experiments with 69 mM SCC, 69 mM PP-IX and 0.01 mM $CuCl_2$, respectively, each treated with 0.5 mM of ENU, compared with the SUC + ENU control.

The comparison between the slope from SUC alone and those observed for any other pretreatment, showed no evidence of a statistical difference. The same was true for the comparison between the slope from SUC + ENU and that from the remaining pretreatment + ENU (m = 0.24, 0.25, 0.26 and 0.26, for SUC, SCC, PP-IX and $CuCl_2$, respectively).

4. Discussion

A comparison of the effects of SCC and PP-IX as they influence the genotoxicity of ENU revealed that both molecules have an inhibitory effect at 0-DTD, i.e., when ENU treatment follows immediately after treatment with SCC or PP-IX. This finding agrees with the effect reported in the literature for both porphyrins [6,7,14,27]. It is known that porphyrins are electron-rich conjugated systems and their aromatic character allows them to react easily with electrophilic species [28] like ENU. In 1-DTD – one day delay of the ENU treatment – SCC retained its inhibitory property, while PP-IX appeared not to have any effect.

Assuming that the dissociation of the porphyrin ring from the copper ion is an aspect of the metabolism of SCC, these reactions could be favoured by the acidic conditions in the *Drosophila* gut. The dipteran mid-gut has been found to display three regions with different pH: 6.1 for the anterior part, 3.1 in the middle and 6.8 for the posterior part [29]. Moreover, evidence based on the use of special dyes shows that the food content of the mid-gut of *Drosophila*

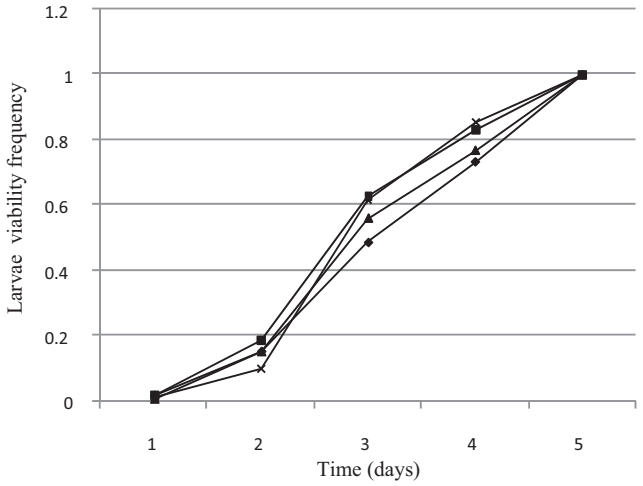

Fig. 1. Larval-adult development, pretreated with SUC (-♦-), SCC (-■-), PPIX (-▲-) and $CuCl_2$ (-×-) and afterwards treated with ENU 0.5 mM dissolved in $NaPO_4$ buffer at pH 6. The comparison between the slope from SUC + ENU and the slope for each pretreatment group shows no significant differences.