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INFLUENCE OF THE LEVEL OF CYTOSOLIC EPOXIDE HYDROLASE ON THE INDUCTION OF SISTER CHROMATID EXCHANGES BY *TRANS*-β-ETHYLSTYRENE 7,8-OXIDE IN HUMAN LYMPHOCYTES

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Abstract—*trans*-β-Ethylstyrene 7,8-oxide, a substrate of cytosolic epoxide hydrolase, and 4-fluorochalcone oxide, an inhibitor of this enzyme, were investigated on induction of sister chromatid exchanges (SCE) in human lymphocytes. Both epoxides enhanced the frequency of SCE. 4-Fluorochalcone oxide at low concentration (2.5 μM) inhibited cytosolic epoxide hydrolase activity towards *trans*-β-ethylstyrene 7,8-oxide in lymphocytes by 74% and had no effect on glutathione transferase activity using this substrate. At this concentration it did not induce SCE itself, but it potentiated the effect of *trans*-β-ethylstyrene 7,8-oxide several fold. In lymphocytes from different subjects, the number of SCE induced by a low concentration of *trans*-β-ethylstyrene 7,8-oxide correlated negatively with the individual cytosolic epoxide hydrolase activity ($r = -0.72$; -0.73 in two series of experiments). The number of SCE induced by a high concentration of *trans*-β-ethylstyrene 7,8-oxide did not correlate with cytosolic epoxide hydrolase activity ($r = 0.004$; -0.24), but a negative correlation was found with glutathione transferase activity ($r = -0.50$). This finding is consistent with the results of biochemical studies in lymphocytes in which we determined the relative contribution of cytosolic epoxide hydrolase and glutathione transferase to the metabolism of *trans*-β-ethylstyrene 7,8-oxide at varying substrate concentrations. The study demonstrates that the level of genotoxic effects induced in human lymphocytes is influenced by the individual level of detoxifying enzymes. At low concentrations, cytosolic epoxide hydrolase was more important than glutathione transferase activity.

Epoxides occur naturally and as industrial products in the environment, are formed by monooxygenases from aromatic and olefinic xenobiotics, and are found as endogenous compounds [1–2]. Many epoxides can alkylate cellular nucleophiles. Subsequently they may produce toxic, mutagenic or carcinogenic effects [1, 4]. Alternatively, they may be detoxified, in particular by conjugation with glutathione and hydrolysis. These reactions are catalysed by glutathione transferase (GST‡) and epoxide hydrolases, respectively [3, 5, 6]. Both types of enzymes are present in all investigated mammalian species and in all examined tissues and cells [2, 7–11]. Epoxide hydrolase activity can be detected in the microsomal (mEH) as well as in the cytosolic fraction (cEH) of mammalian tissue homogenates [3, 9, 11–14]. Both enzymes differ greatly in their substrate selectivity and seem to be complementary to some extent [9, 15]. Large inter-individual variations in the activities of cEH, mEH and GST were found in human liver biopsies [16]. Since the availability of human liver biopsies is limited, blood cells have been used to further explore the inter-individual differences [7, 17–24]. Although not as great as in the liver, substantial inter-individual variations, as compared to intra-individual variations, have been observed in polymorphic mononuclear blood cells. Apart from their easy availability, these cells have the advantage that genotoxic effects can be readily studied.

In the present study we have investigated the induction of sister chromatid exchanges (SCE) by ESO (structure formula see Fig. 1). This compound is a good substrate for cEH [15]. In contrast to styrene oxide itself, ESO is metabolized by mEH at a negligible rate [3, 15]. In particular, we investigated the relationship between inter-individual variations in cEH activity and the susceptibility to ESO-induced SCE in human lymphocytes.

MATERIALS AND METHODS

Chemicals. Tritiated TSO (51.5 mCi/mmol, purity >98% [25]), tritiated ESO (44.8 mCi/mmol, purity >98% [26]), unlabelled ESO (>98% [27]) and 4-fluorochalcone oxide (>98% [28]) were synthesized as indicated.

Isolation of lymphocytes. Human peripheral blood lymphocytes from healthy donors were separated from heparinized fresh whole blood using a Ficoll–metrizoate (1.075 g/mL) gradient [17]. Some of the lymphocytes were cultured and some were used immediately for metabolic studies in intact cells. The remainder of the lymphocytes was homogenized by ultrasound in KCl (150 mM), buffered with sodium

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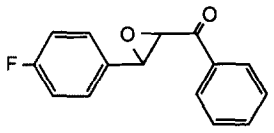
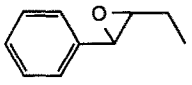
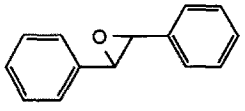
‡ Abbreviations: GST, glutathione transferase: SCE, sister chromatid exchanges; mEH, microsomal epoxide hydrolase; cEH, cytosolic epoxide hydrolase; ESO, *trans*-β-ethylstyrene 7,8-oxide; TSO, *trans*-stilbene oxide.



4-Fluorochalcone oxide



trans-ß-Ethylstyrene 7,8-oxide (ESO)



trans-Stilbene oxide

Fig. 1. Structure formulas of the epoxides used.

phosphate (10 mM, pH 7.4) and stored at −70° until used.

Assay on induction of SCE. The lymphocyte cultures contained 10^6 cells in 5 mL medium RPMI 1640, supplemented with 20% fetal bovine serum (HyClone, Logan, Utah, U.S.A.; inactivated by heating to 60° for 30 min), phytohemagglutinin (Gibco, Eggenheim, F.R.G.; 75 μL), penicillin (100 I.U./mL) and streptomycin (0.1 mg/mL). Two separate cultures were established for each treatment group. 5′-Bromodeoxyuridine (5 μg/mL) and the test compound (or the solvent only, 25 μL dimethyl sulfoxide) were added after 24 hr incubation at 37°. The cultures were incubated in the dark for a further 48 hr. Colcemid was added to a final concentration of 0.25 μg/mL 3 hr before the cells were harvested at 72 hr. The cells were then treated with hypotonic KCl solution (70 mM) for 20 min at 37°, fixed in methanol:acetic acid (3:1, v/v), stored at 4° overnight and dropped on ice-cold glass slides. The slides were air-dried and stained according to the fluorescene plus Giemsa technique [29].

SCE were analysed on coded slides. A total of 25 harlequin-stained metaphases per culture were scored for SCE. At the same time, the cell cycle index (proportion of cells in first division + 2 × proportion of cells in second division + 3 × proportion of cells in higher than second division) was determined and used as a measure for cell cycle delay.

Determination of cEH activity with TSO as the substrate. In the initial experiments the activity of cEH was determined with [^{3}H]TSO as the substrate, as was the case in previous studies with human lymphocytes and liver biopsies [16, 20]. The method used here was a minor modification of the partition assay described by Schladt *et al.* [30]. Each incubation contained 50 μL of buffer A (500 mM Tris–HCl, 4.5 mM EDTA, pH 7.4), 75–150 μL lymphocyte homogenate, and KCl (150 mM) buffered with sodium phosphate (10 mM, pH 7.4) to a final volume of 200 μL. One microlitre of 5 mM 1-chloro-2,4-dinitrobenzene in ethanol was added to inhibit the conjugation of TSO with glutathione. The reaction was started by adding 5 nmol [^{3}H]TSO in 1 μL ethanol. After 60 min the incubation (at 37° in a shaking water bath) was terminated by adding 3 mL petroleum ether and 0.25 mL dimethyl sulfoxide, shaking the tube on a Vortex mixer and cooling in an ice bath. The separation of substrate and product was effected by rotating the tubes for 3 min at 40 rpm. After centrifugation at approximately 1600 *g* for 2 min, the petroleum ether phase was discarded, and the extraction procedure was repeated once more. The product was then extracted into 1 mL of ethyl acetate by rotating the tubes for 5 min. The phases were separated by centrifugation, and an aliquot of 0.5 mL was counted in a scintillation counter using 6 mL of Rotiszint 1100 (Roth, Karlsruhe, F.R.G.) as scintillation fluid. Blanks, obtained from incubations without homogenate, were subtracted. Incubations were carried out at two different protein concentrations, each in duplicate. The assay was performed under conditions where product formation was linear with the incubation time and the amount of protein.

Determination of cEH and GST activities with ESO as the substrate. The metabolism of tritiated ESO to both dihydrodiols and glutathione conjugates was determined simultaneously as described previously [31]. The assay was performed in intact lymphocytes. Exposure of intact cells was carried out in Dulbecco's phosphate-buffered saline with a cell density of 10^6 per mL and a final assay volume of 200 μL. The reaction was started by adding 14 nmol [^{3}H]ESO in 1 μL ethanol to a final concentration of 70 μM. After 30 min the incubation (at 37° in a shaking water bath) was terminated by adding 3 mL isooctane, shaking the tube on a Vortex mixer and cooling in an ice bath. The separation of substrate and products was effected by rotating the tubes for 3 min at 40 rpm. After centrifugation at approximately 1600 *g* for 2 min, the isooctane phase was discarded, and the extraction procedure was repeated once more. The dihydrodiol was then extracted into 1 mL of ethyl acetate by rotating the tubes for 5 min. The phases were separated by centrifugation, and an aliquot of 0.5 mL was counted in a scintillation counter using 6 mL of Rotiszint 1100 (Roth, Karlsruhe, F.R.G.) as scintillation fluid. An aliquot (100 μL) of the remaining aqueous phase (containing the glutathione conjugates) was counted using Lumagel SB (Lumac, Landgraaf, The Netherlands) as the scintillation fluid. Blanks, obtained from incubations without lymphocytes, were subtracted. Incubations were performed in triplicate under conditions where product formation was linear with the incubation time and the amount of protein.

Determination of protein concentrations. Protein concentrations were determined by the method of Lowry *et al.* [32] with bovine serum albumin as a standard.

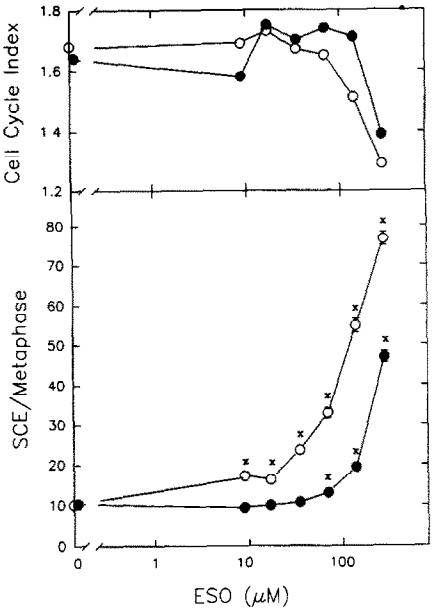


Fig. 2. Induction of SCE by *trans*-β-ethylstyrene 7,8-oxide in the presence (○) and absence (●) of the cEH inhibitor 4-fluorochalcone oxide (2.5 μM, lower panel). Values are means and SE of 25 metaphases. Where no error bars are shown, the SE falls within the symbol. At each *trans*-β-ethylstyrene 7,8-oxide concentration level, except at 0 μM ($P > 0.1$), the SCE values were significantly enhanced at co-exposure with 4-fluorochalcone oxide ($P < 10^{-7}$, Student's *t*-test). Values which are significantly different from the corresponding negative control (0 μM *trans*-β-ethylstyrene 7,8-oxide) are marked with an asterisk ($P < 0.01$). For the other values, $P > 0.1$. The upper panel shows the effect on the cell cycle index.

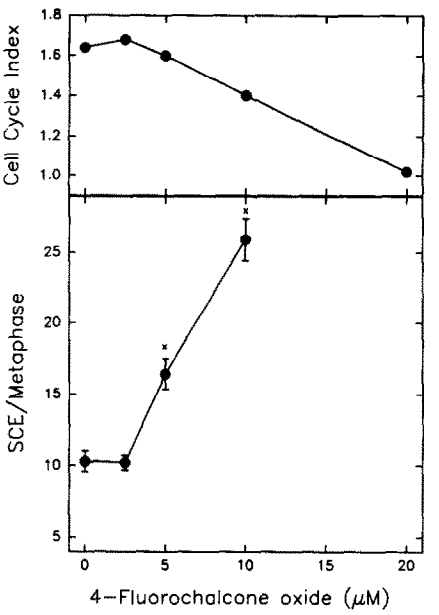


Fig. 3. SCE frequency (lower panel) and cell cycle index (upper panel) in human lymphocytes treated with 4-fluorochalcone oxide. Values are means and SE of 25 metaphases. Values which are significantly different from the corresponding negative control (0 μM 4-fluorochalcone oxide) are marked with an asterisk ($P < 0.01$). For the other value, $P > 0.1$.

RESULTS

SCE induction by ESO and 4-fluorochalcone oxide

Both epoxides investigated in this study, ESO (Fig. 2, closed symbols) and 4-fluorochalcone oxide (Fig. 3) strongly enhanced the frequency of SCE in human lymphocytes. However, in the concentration which was used in further experiments to inhibit the cEH in the SCE assay (2.5 μM), 4-fluorochalcone oxide did not induce SCE.

Influence of 4-fluorochalcone oxide on cEH activity and ESO-induced SCE in human lymphocytes

4-Fluorochalcone oxide inhibited cEH activity, but not GST activity, in human lymphocytes (Fig. 4). At a concentration of 2.5 μM 4-fluorochalcone oxide, cEH activity towards ESO was inhibited by 75%.

The addition of 4-fluorochalcone oxide (2.5 μM) to the lymphocytes cultures strongly increased the SCE inducing activity of ESO (Fig. 2). In the presence of the inhibitor, ESO showed a clear effect even at a concentration of 9 μM, whereas in the absence of the inhibitor the level of SCE was elevated above background only at a concentration of 70 μM.

Metabolism of ESO by lymphocytic cEH and GST as a function of the substrate concentration

The relative contribution of cEH and GST to the metabolism of ESO in human lymphocytes strongly depended on the substrate concentration used (Fig. 5). At low substrate concentration, the contribution of cEH predominated, while the contribution of GST became more important with increasing ESO concentration. From a Lineweaver–Burk plot a K_M value of 45 μM was calculated for cEH. GST activity

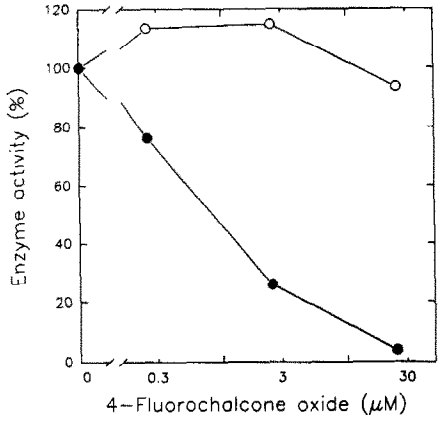


Fig. 4. Influence of 4-fluorochalcone oxide on cEH (●) and GST (○) activity. *trans*-β-Ethylstyrene 7,8-oxide (90 μM) was used as the substrate. The activities in the absence of inhibitor were 116 pmol/min/mg protein for cEH and 230 pmol/min/mg protein for GST.

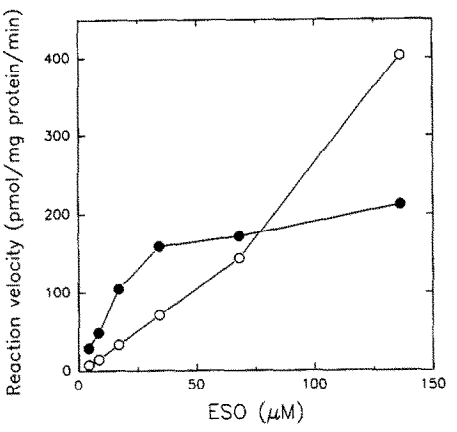


Fig. 5. Metabolism of *trans*-β-ethylstyrene 7,8-oxide in intact human lymphocytes by cEH (●) and GST (○) and its dependence upon the substrate concentration.

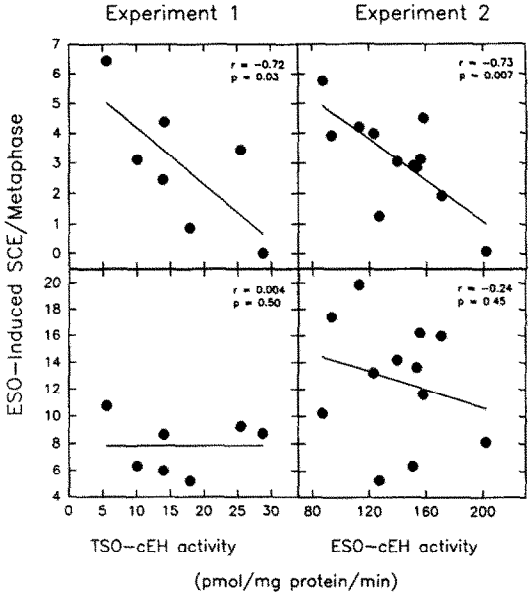


Fig. 6. SCE induction by *trans*-β-ethylstyrene 7,8-oxide in relation to the cEH activity in human lymphocytes from different subjects. Two series of experiments were carried out. In the first series (left panels), cEH activity was determined using *trans*-stilbene oxide as the substrate. In the repeat experiment (right panels), *trans*-β-ethylstyrene 7,8-oxide was used instead. In the SCE assay, *trans*-β-ethylstyrene 7,8-oxide was used at concentrations of 70 μM (upper panels) and 140 μM (lower panels). The number of SCE found in solvent control cultures (10–14) was subtracted. Results were analysed for linear correlation.

was approximately linear to the concentration of ESO, up to the highest level used (140 μM).

Inter-individual variation of cEH and GST activities and relationship to the level of ESO-induced SCE

In the initial experiments, comprising lymphocytes from seven subjects, cEH activity was determined with TSO as the substrate and was compared to the number of ESO-induced SCE. ESO was used at 70 μM (slightly above the K_M for cEH) and 140 μM (approximately the highest concentration which could be used without excessive toxicity) (Fig. 6, left panels). cEH activity in these individuals varied 5.1-fold. A negative linear correlation was found between the number of SCE induced by ESO at the low concentration and the individual cEH activity ($r = -0.72$, $P = 0.03$). In contrast, the number of SCE induced at the high ESO concentration did not correlate with the cEH activity ($r = 0.004$, $P = 0.50$).

In the repeat experiment, ESO was used as the substrate of cEH. GST activity was measured concurrently. Twelve subjects were investigated. The specific activity of cEH varied by a factor of 2.3 and that of GST by a factor of 3.9. The ratio of GST to cEH activity varied from 0.7 to 1.9. Again there was a clear negative correlation between cEH activity and number of ESO-induced SCE with low ESO concentration in the lymphocytes cultures (Fig. 6, right upper panel, $r = -0.73$, $P = 0.007$). As in the initial experiment, no significant correlation was seen at high ESO concentration (Fig. 6, right lower panel, $r = -0.24$, $P = 0.45$). The influence of GST on the number of induced SCE appeared to be modest. Interestingly, it tended to increase with the ESO concentration in the SCE assay, from $r = -0.31$ ($P = 0.32$) at 70 μM to $r = -0.50$ ($P = 0.10$) at 140 μM (Fig. 7).

DISCUSSION

No genotoxicity data appear to be available for ESO from the literature. 4-Fluorochalcone oxide has only been studied in *Salmonella typhimurium*, the result being negative [33]. In the present study, we demonstrate that ESO and 4-fluorochalcone oxide are highly effective in inducing SCE in human lymphocytes.

At low concentration (2.5 μM), 4-fluorochalcone oxide inhibited cEH, but neither affected GST activity nor induced SCE to a measurable extent. Under these conditions it substantially enhanced the SCE-inducing activity of ESO, indicating detoxification of ESO by lymphocytic cEH.

The protective role of cEH against ESO-induced SCE could also be demonstrated by comparing SCE induction and cEH activity in lymphocytes from different human subjects. At low ESO concentration, SCE induction negatively correlated with cEH activity. No significant correlation was observed when ESO was used at a high concentration (140 μM) in the SCE assay. It is possible that at this concentration the capacity of cEH is exhausted, this notion being consistent with the comparatively low K_M value (45 μM) of the enzyme towards ESO. Based on the observed relative contribution of GST on ESO metabolism as a function of the substrate concentration, one might expect a negative correlation between this enzyme activity and the SCE response at high ESO concentration. Indeed, a trend for such a correlation was observed, but the correlation coefficient ($r = -0.50$) was smaller than

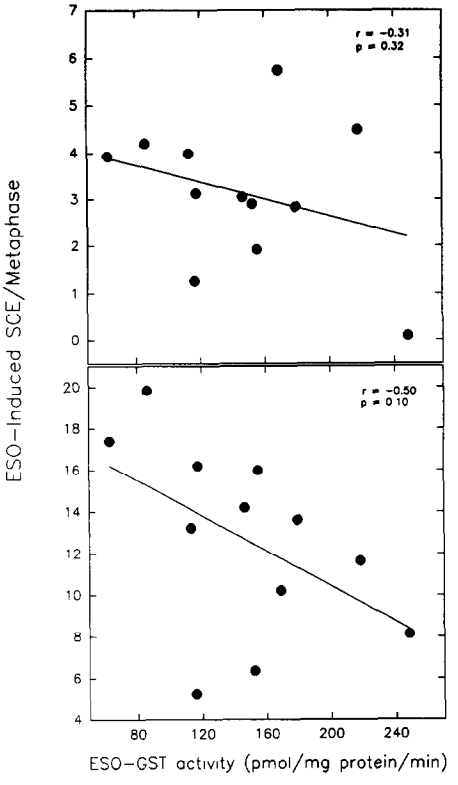


Fig. 7. SCE induction by *trans*-β-ethylstyrene 7,8-oxide in relation to the GST activity in human lymphocytes of 12 individuals. *trans*-β-Ethylstyrene 7,8-oxide was used at concentrations of 70 μM (upper panel) and 140 μM (lower panel). The number of SCE in solvent control cultures (10–14) was subtracted. Results were analysed for linear correlation.

between cEH activity and SCE induction at low ESO concentration, and the statistical significance was marginal ($P = 0.10$).

Using a structurally related epoxide, TSO, Wienke *et al.* [24] however observed an unambiguous correlation between GST activity and SCE induction in lymphocytes from different individuals. The major source of the interindividual variation in GST activity towards TSO appeared to be the presence or absence of GST μ [19, 21–23]. It is noteworthy that this correlation between SCE induction and GST deficiency could be detected especially well when high TSO concentrations (100–200 μM) were used.

Taken together our findings indicate a protective function of cEH and possibly of GST against ESO-induced genotoxicity in human lymphocytes and significant interindividual differences in this protection. The influence of cEH was high at low substrate concentration, which might be particularly relevant in the exposure of humans to environmental pollutants. On the other hand our findings do not demonstrate special problems in using human lymphocytes for screening chemicals for genotoxic effects, since at high substrate concentrations positive responses were observed in all lymphocyte preparations used. It is noteworthy that the individual level of cEH, despite the relatively low activity level and the relatively moderate inter-individual variation, was relevant in the genotoxic response. One might therefore expect an even more important role of cEH in the liver, where the absolute activity and the extent of inter-individual variation are much higher [16].

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