EXCLI Journal 2003;2:22-30 – ISSN 1611-2156
received: 20. February 2003, accepted: 27. February 2003, published: 13. March 2003

Original article:

Detoxification Strategy of Epoxide Hydrolase The Basis for a Threshold in Chemical Carcinogenesis

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ABSTRACT

The human microsomal epoxide hydrolase, a single enzyme, has to detoxify a broad range of structurally diverse, potentially genotoxic epoxides that are formed in the course of xenobiotic metabolism. The enzyme has developed a unique strategy to combine a broad substrate specificity with a high detoxification efficacy, by immediately trapping the reactive compounds as covalent intermediates and by being expressed at high levels for high trapping capacity. Computer simulation and experimental data as well as existing epidemiologic studies reveal this detoxification strategy as the mechanistic basis for a threshold in the tumorigenesis of mutagenic carcinogens.

Keywords: Genotoxic, mechanism, structure, enzyme kinetics, computer simulation

INTRODUCTION

The possible existence of a threshold in chemical carcinogenesis is a matter of debate since decades (Littlefield et al., 1980, Purchase et al., 1995, Schaeffer, 1983, Schulte-Hermann et al., 2000). The relevance of this question resides in its importance for the extrapolation from short term experimental data employing high levels of exposure to life long low level exposure conditions usually encountered by human. The practical consequences are enormous (Hengstler et al., 2002, 2003, von Mach, 2002). For non-threshold toxicants the necessity to attempt zero exposure is obvious. For threshold toxicants no toxicity occurs below the threshold concentration. Certain aspects of the enzymatic mechanism of the carcinogen-detoxifying enzyme epoxide hydrolase (EH) now imply the existence of such thresholds which is substantiated by experimental findings.

Xenobiotic metabolizing epoxide hydrolases play a pivotal role in the defense of the human body against the carcinogenic effects of epoxides (Arand et al., 2002, Hammock et al., 1997). These compounds (Fig. 1) frequently arise as metabolic intermediates from lipophilic foreign compounds in the course of their enzymatic transformation to more water soluble and hence excretable metabolites. Many epoxides are strong electrophiles and readily react with nucleophilic centers in the DNA, thereby leading to DNA adducts, mutations and, finally, cancer formation (Beland et al., 1993).

In contrast to many other xenobiotic metabolizing enzymes, epoxide hydrolases do not occur as a large family of isoenzymes. The majority of xenobiotic epoxides is hydrolyzed by a single EH, the endoplasmic reticulum resident microsomal epoxide hydrolase (mEH) (Oesch, 1973, Oesch et al., 1976). There is one additional EH in human proficient for xenobiotic epoxide hydrolysis, the soluble epoxide hydrolase (sEH) that, in contrast to mEH, can take *trans*-substituted epoxides and is mainly responsible for the metabolism of fatty acid epoxides (Guenthner et al., 1981, Moghaddam et al., 1997, Ota et al., 1980).

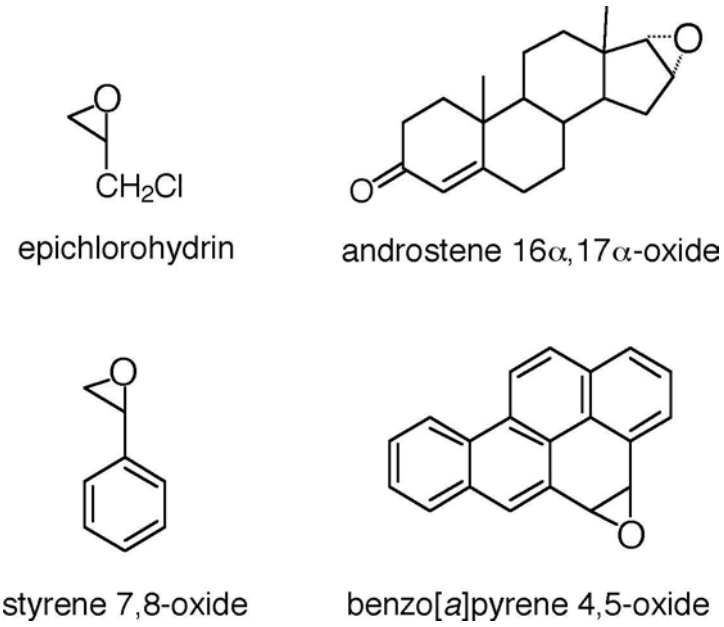

Fig. 1 Structural diversity of mEH substrates. The range goes from very small epoxides such as the epichlorohydrin to complex bulky compounds such as the epoxides from androstene and benzo[*a*]pyrene.

HOW TO COMBINE BROAD SUBSTRATE SPECIFICITY WITH HIGH AFFINITY?

Here is the dilemma: on the one hand, the low complexity of the EH family demands a broad substrate specificity for structurally diverse compounds (Fig. 1) while on the other hand, a high affinity to these substrates is mandatory to efficiently remove these potentially genotoxic agents from the circulation already at low concentrations. This task for nature resembles the attempt to square the circle since it is to be expected that optimization towards one of these leads almost inevitably impairs the performance of the enzyme with respect to the other requirement.

Recently, detailed insights in the enzymatic mechanism of epoxide hydrolysis (Fig. 2) have been obtained by kinetic (Armstrong, 1999, Lacourciere et al., 1993, Rink et al., 1998, Tzeng et al., 1996) and structural analysis (Arand et al., 1999b, Arand et al., 1996, Argiriadi et al., 1999, Laughlin et al., 1998, Nardini et al., 1999,

Tzeng et al., 1998, Zou et al., 2000) of the enzyme: Analysis of these data reveal the unique strategy that has evolved to fulfil the above demands conincidently.

On the basis of their primary sequence, EHs have been grouped into the structural family of α/β hydrolase fold enzymes, leading to the previously unexpected recognition that EH-catalyzed epoxide hydrolysis proceeds via formation of an enzyme-substrate ester intermediate (Fig. 2B) (Arand et al., 1994, Lacourciere et al., 1994, Pries et al., 1994). Indeed, the ester intermediate could be isolated (Hammock et al., 1994, Müller et al., 1997) and kinetic analysis of mammalian and bacterial EH showed that the rate constant k_1[1] for the ester formation is several orders of magnitudes higher than the rate constant k_2 for the subsequent hydrolysis (Rink et al., 1998, Tzeng et al., 1996), implying that the energy barrier ΔG_1 (Fig. 2C) for the transition from the Michaelis Menten complex to the covalent intermediate is significantly lower than the activation energy ΔG_2 for the subsequent hydrolysis of the ester. This has major consequences for the enzymatic efficacy. On the first sight, it results in a comparatively slow product formation, suggesting a poor performance of the enzyme. However, in the present case, substrate consumption and not product formation is crucial, since the aim is to remove the toxic species. In an enzymatic reaction involving a covalent intermediate the relationship between the Michaelis Menten constant K_m and the dissociation constant K_D is $K_m = K_D \cdot k_2/(k_1 + k_2)$, which means that in the present case the ratio of K_m to K_D essentially equals the ratio of k_2 to k_1 (k_2 being orders of magnitude smaller than k_1 and therefore negligible in the sum $k_1 + k_2$). This mimics a high affinity of the enzyme to its substrates and strongly improves the clearance of low substrate concentrations as evidenced by computer simulation (Fig. 3). As long as the enzyme is well in excess of the substrate - indeed the usual situation since the mEH concentration in human liver is equivalent to 10 - 20 μM (Lu et al., 1979), and epoxides are usually present only in significantly lower concentrations and metabolically formed at moderate rates - most of the substrate is rapidly trapped as the covalent ester intermediate and thereby detoxified. This changes dramatically as soon as the epoxide is in excess of the enzyme, eg if the formation rate of the epoxide exceeds the velocity of the enzymatic hydrolysis (Fig. 3C). Thus, if a chemical carcinogen is metabolically activated to an epoxide that is inactivated by mEH, there can be a critical concentration of this compound where the formation rate starts to exceed the EH capacity. This is based on the general observation that the K_D of the activating enzymes for the precursor substrate is significantly higher than that of the EH for the resulting epoxide, which is explained by the above mechanistic considerations. This turning point marks the practical threshold of the given carcinogen since from here the slope of the dose response curve changes dramatically (Fig. 3C).

[1] Kinetic constants used in the text are defined by the following reaction scheme, where *ES* is the Michaelis-Menten complex (non-covalent association between enzyme and substrate) and *E•S* is the ester intermediate formed between enzyme and substrate

$$E + S \xleftrightarrow{K_D} ES \xrightarrow{k_1} E \bullet S \xrightarrow{k_2} E + P$$

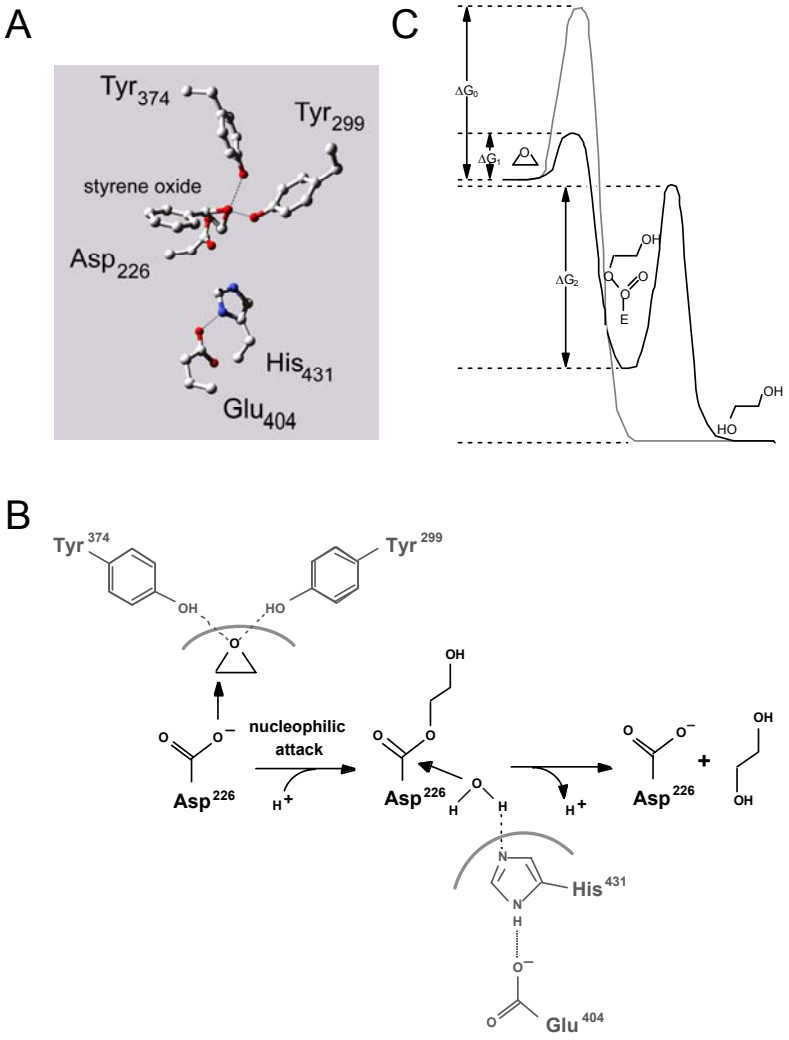

Fig. 2 Enzymatic mechanism of epoxide hydrolysis. A. Model of the active site of the human mEH, based on the structure of the related epoxide hydrolase from Aspergillus niger (Arand et al., 1999a, Zou et al., 2000), with the substrate styrene oxide prepositioned for nucleophilic attack. The model was built using O (Jones et al., 1991). The graphic representation was prepared from SwissPDBviewer (Guex et al., 1997) and subsequent rendering with POV-ray (http://www.povray.org) The course of the enzymatic reaction is outlined in **B**: Tyr^{374} and Tyr^{299} hydrogen bond to the epoxide oxygen, thereby positioning and further activating the epoxide for the nucleophilic attack by Asp^{226}. The reaction between Asp^{226} and the epoxide leads to the enzyme substrate ester intermediate with inverse configuration as compared to the respective intermediate of esterases, i.e. the acid component of the ester stems from the enzyme and not from the substrate, in the present case. Subsequent hydrolysis is achieved by water activation through the His^{431}/Glu^{404} charge relay system, leading to product formation and reconstitution of the active enzyme. **C**. Energy diagram of the enzymatic reaction, where ΔG_0 is the activation energy of the uncatalyzed direct hydrolysis, ΔG_1 is the activation energy for the enzyme-catalyzed ester formation and ΔG_2 is the activation energy required for the enzyme-catalyzed ester hydrolysis. The observation that the first step, the formation of the enzyme-substrate covalent complex, is very fast, whilst the second step of the reaction is the slow, rate limiting step suggests some mobility of the catalytic nucleophile Asp^{226} in the side chain, which should facilitate ester formation with a broad range of substrates. At the same time, this implies that the position of the resulting ester bond in the covalent intermediate varies between different substrates and offers an explanation why the velocity of the second, hydrolytic step of the enzymatic reaction has not been optimized by nature (Arand et al., 1999b) to reach values like that of, eg acetyl choline esterase.

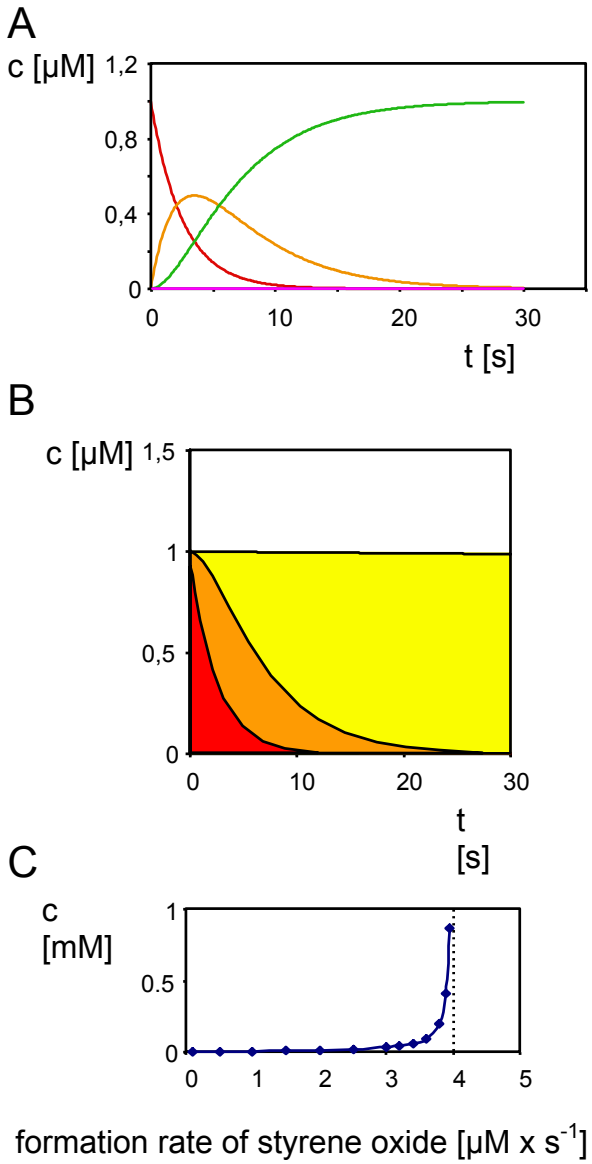

Fig. 3 Computer simulation of styrene epoxide hydrolysis by human microsomal epoxide hydrolase. A. The concentrations of free substrate (red line), the non-covalent Michaelis Menten complex between enzyme and substrate (pink line), the covalent ester intermediate between enzyme and substrate (orange line), and the terminal reaction product (diol; green line) are computed over time after addition of 1 µM styrene oxide into a single compartment. Rapid clearance is observed due to the essentially irreversible first step of the enzymatic mechanism, despite of the relatively low affinity of the enzyme to the substrate. In **B**, the area under the concentration-time curve (AUC) of styrene oxide is plotted as obtained from different simulation conditions. The red area represents the styrene oxide burden calculated assuming the above conditions. The yellow area plus the red and the orange area gives the AUC for styrene oxide that would result from a one step mechanism using the same dissociation constant and hydrolysis rate as for the two step simulation. It is three orders of magnitude above the AUC for the two-step mechanism and thus heavily exceeds the scale. The orange area indicates the amount of overestimation of styrene oxide burden with a two-step mechanism if simply calculated from the rate of product formation, which would result in a 3-fold underestimation of the detoxification efficacy of the EH, in the present case.

C shows the dependence of steady state concentration of styrene oxide from the rate of styrene oxide formation under the present conditions. Once the formation rate significantly rises above 3 µM x s^{-1}, corresponding to 75% of maximum hydrolysis rate in the system, the styrene oxide steady state concentration rises steeply. Above the maximum capacity, no EH-controlled equilibrium is possible. Computer simulation was performed using Microsoft Excel on a Macintosh G3 desktop computer. The respective spreadsheet containing the computations is available as additional material on the EXCLI website (http://www.giftinfo.uni-mainz.de/EXCLI/). Results obtained with this approach resembled those produced with the software package Kinsim (Barshop et al., 1983), yet offer somewhat more flexibility, as long as the kinetics are kept simple. In order to match the experimental data available for the human mEH (Jenkins Sumner et al., 1994, Lu et al., 1979, Mendrala et al., 1993), mEH enzyme concentration, dissociation constant K_D, rate constant k_1 for the ester formation and the rate constant k_2 for ester hydrolysis were set to 20 µM, 10 mM, 200 x s^{-1}, and 0.2 x s^{-1}, respectively. A slow back reaction from the ester to the epoxide that has recently been postulated by others on the basis of indirect evidence (Rink et al., 1998, Tzeng et al., 1996) is neglected in the present scenario because (i) it is energetically highly unfavoured in view of the new insights into the enzymatic mechanism and (ii) it complicates computation without qualitatively changing the outcome of the simulation.

DATA IN SUPPORT OF THE CONCEPT

Experimental proof for the above deductions can be brought about by comparing the sensitivity of a recombinant mEH-expressing cell line towards a genotoxic agent - styrene oxide in the present case - with the respective sensitivity of the parental mEH-deficient cell line (Fig. 4). As predicted, the epoxide hydrolase-expressing cells were well-protected and tolerate high concentrations of the epoxide without apparent genotoxic effects. As soon as their capacity to enzymically trap the epoxide is exhausted, however, they show a steep increase in the biological effect caused by styrene oxide, which perfectly resembles the expectation based on the computer simulation.

In addition to the experimental support, there is epidemiologic evidence for the correctness of our interpretation: styrene is a widely used precursor in the polymer industries and is produced in millions of tons per year. In human, most of the styrene is metabolically oxidized to the genotoxic styrene 7,8-oxide (Jenkins Sumner et al., 1994).This intermediate is detoxified almost exclusively by EH, with a minute amount (< 1%) being alternatively conjugated to glutathione. Despite the proven genotoxic effects of styrene oxide, it was recently reported that only a poor, if any, correlation could be found in human between occupational styrene exposure and genotoxic effects in peripheral blood monocytes (Rappaport et al., 1996). On the other hand, a significant correlation was found in the same individuals between styrene oxide exposure and genotoxic effects. The styrene oxide exposure level in this study was almost 1000-fold below the styrene exposure level. This implies that the transient formation of styrene oxide from styrene in man is rapidly counteracted by EH and metabolically formed styrene oxide is efficiently kept from entering the systemic circulation. In contrast, minute amounts of the genotoxic agent, styrene-7,8-oxide, that reach systemic circulation before being detoxified by the mainly liver-resident mEH immediately exert their hazardous effects.

In conclusion, the enzymatic mechanism of EH is the key to its success in efficiently detoxifying a broad range of structurally very different epoxides. Furthermore, the present study shows the existence of a threshold concentration for a classical carcinogen, styrene oxide, a situation which practically certainly also holds for other genotoxic carcinogens which act via intermediate epoxide formation.

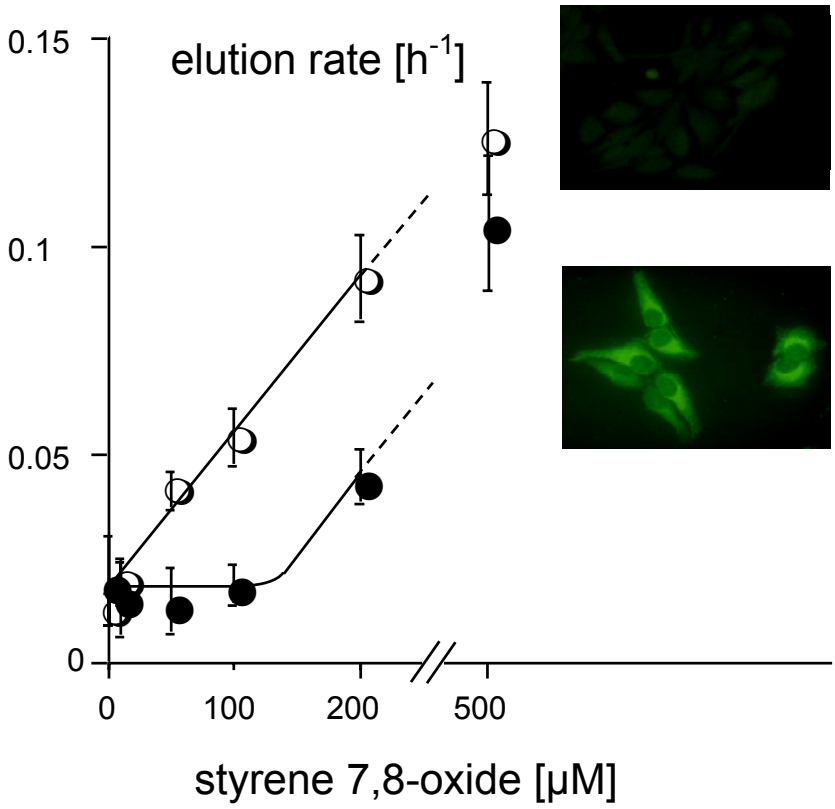

Fig. 4 Expression of human mEH protects V79 Chinese Hamster cells from styrene oxide-induced DNA damage and introduces a threshold. Parental mEH-deficient V79 Chinese hamster cells (open circles) and a stable V79-derived cell line expressing human mEH (closed circles) (Herrero et al., 1997) were treated with increasing concentrations of styrene oxide in culture and the effect of the DNA damaging agent was assessed by alkaline filter elution of the DNA as described earlier (Hengstler et al., 1992). Human mEH expression was monitored by immunofluorescence analysis using an anti-mEH antiserum, as shown in the inserts (upper insert: parental V79 cells; lower insert: human mEH-expressing V79 cells). The onset of the genotoxic effect caused by styrene oxide in the recombinant cell line only at concentrations above 100 µM clearly demonstrates that mEH expression introduces a threshold for styrene oxide genotoxicity in V79 cells. Data points represent the average of three independent determinations. Error bars indicate the respective standard deviations. At a styrene oxide concentration of 500 µM, a strong cytotoxic effect on the parental V79 cells and a moderate toxic effect on the recombinant cells were observed. The respective data for the DNA elution rate are given for the sake of completeness but are off the linear range.

Acknowledgements: This work was supported by the German Research Foundation (SFB 519) and the European Community under the 5th Framework (Contract N° QLK4-CT-1999-01368)

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