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Kinetics of Ethylene and Ethylene Oxide in Subcellular Fractions of Lungs and Livers of Male B6C3F1 Mice and Male Fischer 344 Rats and of Human Livers

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Ethylene (ET) is metabolized in mammals to the carcinogenic ethylene oxide (EO). Although both gases are of high industrial relevance, only limited data exist on the toxicokinetics of ET in mice and of EO in humans. Metabolism of ET is related to cytochrome P450-dependent mono-oxygenase (CYP) and of EO to epoxide hydrolase (EH) and glutathione *S*-transferase (GST). Kinetics of ET metabolism to EO and of elimination of EO were investigated in headspace vessels containing incubations of subcellular fractions of mouse, rat, or human liver or of mouse or rat lung. CYP-associated metabolism of ET and GST-related metabolism of EO were found in microsomes and cytosol, respectively, of each species. EH-related metabolism of EO was not detectable in hepatic microsomes of rats and mice but obeyed saturation kinetics in hepatic microsomes of humans. In ET-exposed liver microsomes, metabolism of ET to EO followed Michaelis-Menten-like kinetics. Mean values of V_{max} [nmol/(min·mg protein)] and of the apparent Michaelis constant (K_m [mmol/l ET in microsomal suspension]) were 0.567 and 0.0093 (mouse), 0.401 and 0.031 (rat), and 0.219 and 0.013 (human). In lung microsomes, V_{max} values were 0.073 (mouse) and 0.055 (rat). During ET exposure, the rate of EO production decreased rapidly. By modeling a suicide inhibition mechanism, rate constants for CYP-mediated catalysis and CYP inactivation were estimated. In liver cytosol, mean GST activities to EO expressed as V_{max}/K_m [μl/(min·mg protein)] were 27.90 (mouse), 5.30 (rat), and 1.14 (human). The parameters are most relevant for reducing uncertainties in the risk assessment of ET and EO.

Key Words: **ethylene; ethylene oxide; subcellular fractions; suicide inhibition; mouse; rat; human.**

The gaseous olefin ethylene (ET, CAS No.: 74-85-1) is the largest volume organic chemical produced worldwide. The estimated global production in 2005 was over 112.9 million tons. The gas is mainly used as feedstock in the production of polymers and industrial chemicals (Zimmermann and Walzl, 2007). It is ubiquitously present in the environment, arising predominantly as a natural product from vegetation, from burning of organic material, and from automotive engine exhausts (referenced, for example, in International Agency of Research on Cancer (IARC), 1994a). Plants produce ET as a ripening hormone as reviewed in Bleecker and Kende (2000). Mice (Lawrence and Cohen, 1985), rats (Csanády *et al*., 2000), and humans (Filser *et al*., 1992) exhale endogenously formed ET. In liver microsomes of rats pretreated with phenobarbital, it was first proved by Schmiedel *et al*. (1983) that cytochrome P450-dependent monooxygenases (CYP) epoxidize ET to ethylene oxide (EO, CAS No.: 75-21-8). EO is a directly protein- and DNA-alkylating agent in rodents and humans (for instance, see Boogaard *et al*., 1999; Huang *et al*., 2011; Walker *et al*., 2000; Wu *et al*., 2011). It is mutagenic *in vitro* and *in vivo* (reviewed, for example, in Dellarco *et al*., 1990; Kolman *et al*., 2002) and is carcinogenic in rat (Dunkelberg, 1982; Garman *et al*., 1985; Snellings *et al*., 1984) and mouse (Dunkelberg, 1981; National Toxicology Program, 1987). In EO-exposed humans, increased sister chromatid exchange and chromosomal aberrations were reported (reviewed in Kolman *et al*., 2002). Based on the results of several epidemiologic studies on EO-exposed workers and mechanistic evidences, IARC (1994b) evaluated EO as carcinogenic to humans.

In vivo, the formation of EO from ET was shown indirectly in rats, mice, and humans *via* characteristic adducts to macromolecules (Ehrenberg *et al*., 1977; Eide *et al*., 1995; Filser *et al*., 1992; Rusyn *et al*., 2005; Segerbäck, 1983; Törnqvist *et al*., 1989; Walker *et al*., 2000) and directly in ET-exposed rats determined as EO in exhaled air (Filser and Bolt, 1984) and as EO in blood (Fennell *et al*., 2004; Maples and Dahl, 1993). In spite of the formation of its carcinogenic metabolite EO, ET was found to be negative in mutagenicity

studies *in vitro* and *in vivo* in rodents (Victorin and Ståhlberg, 1988; Walker *et al*., 2000) and in a long-term carcinogenicity study in rats (Hamm *et al*., 1984). The negative outcome of the carcinogenicity study was expected from toxicokinetic studies with ET and EO in rats and from studies on hemoglobin adducts in rats and mice. The EO burden was predicted to be too low to lead to tumors in a long-term carcinogenicity study with ET (Bolt and Filser, 1984; Osterman-Golkar and Ehrenberg, 1982; Walker *et al*., 2000).

Toxicokinetic data on EO were obtained in blood of mice during, and in blood, brain, muscles, and testes of mice and rats after exposing the animals up to 4 h to constant concentrations of atmospheric EO (Brown *et al*., 1996, 1998). Also, concentration-time courses of EO were monitored in the atmosphere of closed exposure chambers after the exposure of mice for 3 h to constant EO concentrations (Sega *et al*., 1991). In this species, no measured toxicokinetic data on ET are published neither *in vivo* nor *in vitro*. In humans, the kinetics of ET was investigated only at low ET concentrations (< 50 ppm) by means of gas uptake studies (Filser *et al*., 1992). Brugnone *et al.* (1985, 1986) reported EO concentrations in blood and ratios of alveolar to atmospheric EO concentrations in EO-exposed workers. Gas uptake experiments were also conducted to investigate in rats the kinetics of ET (Andersen *et al*., 1980; Bolt *et al*., 1984) and of EO (Filser and Bolt, 1984; Krishnan *et al*., 1992). EO was rapidly eliminated following first-order kinetics at EO concentrations of up to at least 100 ppm. ET showed saturation kinetics that could be described according to Michaelis and Menten by V_{max} and an apparent Michaelis constant (K_m). The metabolic elimination of ET was quantitatively inhibited following pretreatment with sodium diethyldithiocarbamate; V_{max} was doubled after pretreatment with a mixture of polychlorinated biphenyls (Bolt *et al*., 1984). These results hinted to a CYP-mediated metabolism of ET, later confirmed by Fennell *et al.* (2004). In rats exposed to high ET concentrations (Filser and Bolt, 1984: > 1000 ppm; Fennell *et al*., 2004 and Maples and Dahl, 1993: 600 ppm), metabolically formed EO increased rapidly in exhaled air and in blood shortly after starting the exposures. Thereafter, the EO concentrations declined, reaching plateaus (Filser and Bolt, 1984; Maples and Dahl, 1993) that remained rather constant until the end of exposure. The decline of EO was explained by an inactivation of hepatic CYP down to 68% after 360 min of exposure (Maples and Dahl, 1993) and of hepatic 4-nitrophenol hydroxylase activity (predominantly CYP2E1) to 50% after 4 h (Fennell *et al*., 2004). The loss of CYP activity reflects the findings in liver microsomes of phenobarbital-pretreated rats that ET destroyed CYP by self-catalyzed prosthetic heme alkylation (Ortiz de Montellano and Correia, 1983; Ortiz de Montellano and Mico, 1980).

Toxicokinetic data of ET and EO in mice, rats, and humans were used for developing physiological toxicokinetic models of ET together with EO (Csanády *et al*., 2000) or solely of EO (Fennell and Brown, 2001; Krishnan *et al*., 1992). However, a toxicokinetic model that describes the initial EO blood peaks together with the concentration-time courses of the ET-metabolizing CYP species during exposure to ET is missing. Such a model is required for providing a quantitative basis for the assessment of the risk of ET to humans. The goal of the present work was to acquire the toxicokinetic parameters that are necessary for the model by using ET- and EO-exposed subcellular fractions of livers and lungs of mice and rats and of livers of humans.

MATERIALS AND METHODS

Chemicals

ET 3.5 (> 99.95%) and EO 3.0 (> 99.9%) were obtained from Linde, Unterschleissheim, Germany. Reduced glutathione (GSH; ≥ 99%), pig heart isocitrate dehydrogenase Type IV, buffered to pH 6.0 in 50% aqueous glycerol solution (13 or 15 U/mg protein), D,L-isocitric acid trisodium salt (96%), β-nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$; 97%), and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH; 97%) were purchased from Sigma-Aldrich, Steinheim, Germany. Racemic propylene oxide (PO; 99.9%) was provided by Serva GmbH, Heidelberg, Germany. All other chemicals were from Linde; Merck, Darmstadt, Germany; and Sigma-Aldrich at the highest analytical grade available.

Animals

Male B6C3F1 mice and male Fischer 344 rats were obtained from Charles River, Sulzfeld, Germany. Male 129/Sv-Cyp2e1tm1Gonz/J mice (CYP2E1 knockout mice) were bred in-house from three pairs of revitalized mice of this strain that were obtained from The Jackson Laboratory, Bar Harbor, ME. Male 129S1/SvImJ mice (wild-type mice) were also obtained from The Jackson Laboratory. CYP2E1 knockout and the corresponding wild-type mice were used only to investigate the relevance of CYP2E1 on the ET metabolism. All animals were housed in macrolon type III cages in an IVC top-flow system (Tecniplast, Buguggiate, Italy). A light and dark cycle of 12 h was maintained in the animal room. The animals were kept at constant room temperature (23°C) and received standard diet 1324 (Altromin, Lage, Germany) and tap water *ad libitum*. Immediately before harvesting the livers and lungs for the preparation of microsomes and cytosol, the animals (mice about 25 g and rats about 250 g body weight) were asphyxiated with carbon dioxide.

Human Liver Samples and Subcellular Human Liver Fractions

A sample of a liver of a 75-year-old woman was obtained from the University Hospital "Rechts der Isar" of the "Technische Universität München", Munich, Germany. The donor, suffering from metastasis of rectum cancer, was not treated with oncological drugs when the liver sample was collected on the occasion of a liver transplantation. The other liver samples were from five male and one female trauma victims who were between 16 and 58 years of age. All these liver samples were obtained at autopsies carried out in the forensic "Institut für Rechtsmedizin" of the "Ludwig-Maximilians-Universität München", Munich, Germany. Death occurred between 6.5 and 29 h prior to autopsy. The liver samples were used to prepare cytosol in order to test the glutathione *S*-transferase (GST) activity to EO as substrate. In addition, microsomes were prepared from the liver samples of the 75-year-old woman and of a 22-year-old man who died 6.5 h before autopsy. Immediately after collection, the samples were stored at −20°C until transport (on ice) within 30 min to the Institute of Toxicology of the Helmholtz Center Munich, where they were stored at −80°C until preparation of the subcellular liver fractions.

Liver cytosol of 6 human individuals (1 male, 5 female; 42–78 years old) and microsomes pooled from the livers of 18 male and 7 female Caucasian and Hispanic donors, 21–64 years old, as well as cytosol pooled from the livers of

1 female Afro-American, 1 male Asian, 5 female and 3 male Caucasians, and 1 male Hispanic subject, between 1.9 and 70 years old, were obtained from BD Biosciences, San José, CA and delivered from Becton Dickinson, Heidelberg, Germany. Microsomes consisted of 20 mg protein/ml in a sucrose solution (250 mmol/l). Cytosol (protein content 20 mg/ml) was in a solution consisting of 150 mmol/l KCl, 50 mmol/l Tris-HCl buffer (pH 7.5), and 2 mmol/l EDTA.

Preparation of Microsomes and Cytosol

Microsomes and cytosol were prepared from tissue samples as described in Kreuzer *et al.* (1991). Both fractions were stored at −80°C until use. The protein content of the subcellular fractions was determined using a modified Biuret method (Gornall *et al.*, 1949).

Incubation Procedure

All the experiments described in the following are "gas uptake experiments" with the gaseous compound administered only once into the atmosphere of closed all-glass exposure vessels. Microsomal and cytosolic incubation conditions were chosen as described in Kreuzer *et al.* (1991) and in Faller *et al.* (2001). In the latter publication, the quality of such preparations was verified by comparing specific activities of CYP, GST, and epoxide hydrolase (EH) for a series of standard substrates with literature data. The protein concentrations used in the present work were selected according to Faller *et al.* (2001). Generally, a subcellular fraction, either native or inactivated by heat (96°C for 10 min in a water bath), was incubated with ET or EO with or without coenzymes in a cone-shaped headspace vessel (~38 ml). The all-glass vessel contained two ports, one at the upper end, closed by a stopcock made out of glass, and one laterally, closed by a Teflon-coated rubber septum. It was located at 37°C in a continuously vigorously shaken water bath. Concentration-time courses of ET and EO were monitored by gas chromatography with flame ionization detection (GC/FID) using gas samples collected periodically from the headspace, immediately after shortly but vigorously shaking the vessel by hand in order to maintain the compound-specific partition coefficient incubation medium-to-air.

Microsomal Incubations

Ethylene. The headspace vessels contained microsomes, $MgCl_2$ (5 mmol/l in the final incubate volume) and potassium phosphate buffer (0.15 mol/l, pH 7.4). In those experiments in which only ET was monitored, either hepatic microsomal suspensions of 10 ml with protein concentrations of 10 mg/ml in the 38-ml vessels or hepatic microsomal suspensions of 4 ml with protein concentrations of 5 mg/ml in commercially available 8-ml headspace vials were used. When monitoring additionally the EO formation in ET incubations, volumes and protein concentrations of the hepatic microsomal suspensions in the 38-ml vessel were 4 ml and 2.5 mg/l (B6C3F1 and wild-type mice), 4 ml and 5 mg/ml (rats), and 2 ml and 10 mg/ml (humans and CYP2E1 knockout mice). In incubations of mouse and rat lung microsomes, suspensions consisted of 2 ml with protein concentrations of 5 mg/ml (mice) and 10 mg/ml (rats). For the administration of ET, the gas phase of the vessel was totally exchanged by pumping 100 ml of a gas sample containing the specified ET concentration through the vessel using a 100-ml syringe equipped with a cannula and a second cannula both of which piercing the septum. An NADPH-regenerating system (NADP 1 mmol/l, isocitrate 8 mmol/l, and isocitrate dehydrogenase 0.2 U/l phosphate buffer) was prepared in a 2-ml-Eppendorf cup. In order to study CYP-dependent metabolic ET elimination or EO formation, both the headspace vial and the Eppendorf cup with the NADPH-regenerating system were preincubated in the water bath for 5 min. Then, the incubation was started by injecting 0.64 ml (for the 2-ml suspension) or 1.28 ml (for the 4-ml suspension) of the NADPH-regenerating system through the septum into the headspace vessel. Control experiments were conducted with heat-inactivated microsomes.

Ethylene oxide. The 38-ml vessels contained microsomes (5 mg protein/ml), $MgCl_2$, and phosphate buffer as described above. The volume of each suspension was 1 ml. After preincubating the vessel in the water bath for 10 min, a defined volume of air was removed by a gastight glass syringe. In order to investigate the microsomal EH-catalyzed EO elimination, the incubation was started by injecting an EO air mixture equal to the amount of air that had been removed before. The EO volume required to achieve initial concentrations in the gas phase of between 10 and 10,000 ppm in the vessel had been calculated taking into account the EO concentration in the storage desiccator and the thermodynamic partition coefficient medium-to-air of EO (K_{eqEO}, see below). In order to test whether EO was metabolized by CYP450, an incubation of EO was carried out in pooled rat liver microsomes in the presence of the above-described NADPH-regenerating system. Control experiments were done with heat-inactivated microsomes.

Cytosolic Incubations

Cytosolic incubations were done only with EO. The 38-ml vessels contained cytosol (3 mg protein/ml), $MgCl_2$ (5 mmol/l), mostly GSH (15 mmol/l), and phosphate buffer (0.15 mol/l, pH 7.4). The total volume of the liquid phase was always 1 ml. After a preincubation period of 10 min, a defined volume of air was removed from the vessel and replaced by an EO air mixture hereby starting the exposure. In order to investigate the GST-mediated EO elimination, initial EO concentrations of between 10 and 3000 ppm were used. Control experiments were done with heat-inactivated cytosol in the absence of GSH but in the presence of maleic acid diethyl ester (DEM, 3 mmol/l incubation medium) for the depletion of residual GSH (Boyland and Chasseaud, 1970). For determining the nonenzymatic conjugation of EO with GSH, incubations were done with heat-inactivated cytosol and GSH (15 mmol/l in the incubation medium). For investigating whether the presence of cytosolic proteins might have an influence on the rate of EO hydrolysis, some EO incubations were carried out in the presence of native cytosol and DEM (3 mmol/l incubation medium).

Gas Chromatographic Analysis

Atmospheric ET, EO, and PO (a proven EH substrate that was used for comparative purposes, see below) were analyzed according to Faller *et al.* (2001) using gas chromatographs GC-8A (Shimadzu, Duisburg, Germany) equipped with flame ionization detectors and the integrators CR5A and CR6A (from Shimadzu) for recording the chromatograms. Separation was carried out on 1/8″ stainless-steel columns filled with Tenax TA from Chrompack, Frankfurt, Germany (60–80 mesh for ET and 80–100 mesh for EO and PO). The column used for ET was 1.5 m that for EO and PO 3.5 m long. Nitrogen with pressures of 200 (ET, PO) or 250 (EO) kPa was used as carrier gas. The oven temperatures were 60°C (ET), 150°C (EO), and 180°C (PO). The temperatures of the injectors and detectors were held at 130°C (ET), 200°C (EO), or 230°C (PO). The detectors were provided with hydrogen (60 kPa) and synthetic air (60 kPa). Gas samples of 50 µl (ET, PO, and EO $\geq$10 ppm in the vessel atmosphere) and of 500 µl (EO < 10 ppm) were injected directly onto the column. The retention times of the gases were 0.5 min (ET), 2.5 min (EO), and 2.4 min (PO).

Calibration curves were constructed with the three substances in the ranges of between 200 and 50,000 ppm (ET), 5 and 5000 ppm (EO; injection volume 50 µl), 0.22 and 10.02 ppm (EO; injection volume 500 µl), and 10 and 1000 ppm (PO). Linear regression analysis through the origin revealed correlation coefficients of at least 0.999 between peak height and gas concentration. The detection limits, defined as three times the background noise, were 0.1 ppm (ET), 0.5 ppm (EO; injection volume 50 µl), 0.1 ppm (EO; injection volume 500 µl), and 0.3 ppm (PO).

For each exposure, a single calibration of each measured compound was performed.

Kinetic Analysis of Concentration-Time Courses

Atmospheric concentrations given in ppm at 37°C were transformed to mol/l by division with the molecular volume of an ideal gas at 37°C (25.43 l). Actual amounts $N_{(t)}$ of compounds in the vessel were obtained by multiplication of the actual concentration in air $c_{a(t)}$ with the compound-specific volume of distribution V_d:

$$N_{(t)} = c_{a(t)} \cdot V_d \quad (1)$$

Because the vessels were always vigorously shaken, V_d is always the sum of the air volume V_g in the vessel and the product of the volume of the liquid phase V_l in the vessel with the compound- and medium-(cytosolic or microsomal incubation) specific thermodynamic partition coefficient medium-to-air K_{eq}:

$$V_d = V_g + V_l \cdot K_{eq} \quad (2)$$

The values of K_{eq} (K_{eqET} for ET and K_{eqEO} for EO) were determined at 37°C according to Csanády *et al.* (2000). They were $K_{eqET} = 0.2 \pm 0.06$ ($n = 5$) and $K_{eqEO} = 68.4 \pm 0.6$ ($n = 3$) in microsomal incubations. In cytosolic suspensions, K_{eqEO} was 75.9 ± 3.1 ($n = 3$).

In the ET or EO gas uptake studies, the atmospheric concentration-time courses of ET or EO were fitted using an e-function (Equation 3) or a differential equation describing saturation kinetics according to Michaelis and Menten (Equation 4).

$$c_a = c_{a(0)} \cdot e^{-k \cdot t} + A \quad (3)$$

The concentration in the air phase of the vessel is represented by c_a and the elimination rate constant by k. "A" means a constant concentration of $A \geq 0$.

$$d_{Ca}/dt = -\left(V^*_{max} \cdot c_a\right)/\left(K^*_m + c_a\right) \quad (4)$$

V^*_{max} is the maximum concentration change in the atmosphere of the vessel. K^*_m is the apparent Michaelis constant related to the atmospheric concentration. With the amount of protein (Pr) in the experimental onset, the expression $V^*_{max} \cdot V_d/Pr$ gives the maximum elimination rate V_{max} (amount of compound per time unit per mg protein):

$$V_{max} = V^*_{max} \cdot V_d/Pr \quad (5)$$

The compound- and species-specific value of K_m (the apparent Michaelis constant related to the incubation medium) is given by the product of K^*_m with K_{eq} (for definition of K_{eq}, see above):

$$K_m = K^*_m \cdot K_{eq} \quad (6)$$

In ET incubation experiments with quasi-constantly remaining ET concentrations, metabolically formed EO was detected in the air phase of the exposure systems. Because the EO formation took place only in the liquid phase, each measured air-phase concentration c_{EOa} was used to calculate the corresponding amount of EO in the whole system and relating it to the volume of the liquid phase, solely:

$$c_{EOl} = \frac{c_{EOa} \cdot \left(V_g + K_{eqEO} \cdot V_l\right)}{V_l} \quad (7)$$

Here, c_{EOl} is the "theoretical" EO concentration in the liquid phase and K_{eqEO} is the thermodynamic partition coefficient of EO in microsomal incubations.

The following function was fitted to the time-dependent, experimentally obtained c_{EOl} data. This function was derived from theoretical considerations (see below):

$$c_{EOl} = C_1 \cdot \left[1 - e^{-k \cdot t}\right] \quad (8)$$

C_1 is the fitted EO concentration for $t \rightarrow \infty$ and k is a rate constant.

Initial formation rates of EO (v_{init}), normalized to a protein content of 1 mg, were calculated from the derivative of Equation 8 at $t = 0$ by taking into account V_l and the actual amount of protein (Pr):

$$v_{init} = \frac{k \cdot C_1 \cdot V_l}{Pr} \quad (9)$$

By means of the program Prism 5 for Macintosh (GraphPad Software, California) Michaelis-Menten-type kinetics were fitted to the data representing various v_{init} values of EO against the corresponding ET substrate concentrations. The Berkeley Madonna 8.3.18 (University of California, Berkeley) program was used to fit Michaelis-Menten-type kinetics to the human microsomal EO concentration-time data.

Suicide Inhibition Model

The CYP-mediated metabolic production of EO from ET together with the irreversible inactivation of CYP (by N-hydroxyethylation of the pyrrole ring D in the porphyrin; reference, for e.g., Correia and Ortiz de Montellano, 2005) was described by the following reaction scheme (adapted from Collman *et al.*, 1990), which incorporates the metabolite formation *via* the enzyme-substrate complex *ES* according to Briggs and Haldane (1925) together with simultaneous suicide inactivation of *ES*.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} E + P \quad ES \overset{k_4}{\rightarrow} EX \quad (10)$$

The concentration of the ET-metabolizing CYP species is given by *E*, that of the substrate ET by *S*, and that of the product EO by *P*. *EX* stands for irreversibly inactivated CYP. The rate constants of the four reactions shown in Equation 10 are represented by k_1, k_2, k_3, and k_4. In the literature, k_3 is often named "k_{cat}" and k_4, the inactivation constant, "$k_{inact.}$" The co-substrates O_2 and NADPH are not considered because O_2 was in excess and NADPH was assumed constant during the exposure period because of the NADPH regeneration system. According to Equation 10, the change of *ES* over time (*t*) is described by:

$$\frac{dES}{dt} = k_1 \cdot S \cdot E - \left(k_2 + k_3\right) \cdot ES - k_4 \cdot ES \quad (11)$$

When the rate of the suicide inhibition

$$\frac{dEX}{dt} = k_4 \cdot ES \quad (12)$$

is zero, Equation 11 reads

$$\frac{dES}{dt} = k_1 \cdot S \cdot E - \left(k_2 + k_3\right) \cdot ES \quad (13)$$

As discussed by Briggs and Haldane (1925), $\frac{dES}{dt}$ in Equation 13 can be considered negligibly small. In other words, the expression given at the right side of Equation 13 can be treated as zero. Taking this assumption of a very fast quasi-steady state into account and considering $\frac{dEX}{dt}$ (see Equation 12) being much slower than the time to reach the quasi-steady-state, Equation 11 can be reformulated to:

$$\frac{dES}{dt} = -k_4 \cdot ES \quad (14)$$

At the experimental onset used in the present microsomal studies, the *ES* complex was already formed in the preincubation phase before the reaction was started by the addition of the NADPH regenerating system. During this phase, both k_3 and k_4 are evidently not existent (compare Equation 10); the *ES* complex ES_0 at $t = 0$ (the end of the preincubation phase, when the reaction starts) is given by:

$$k_1 \cdot E \cdot S = k_2 \cdot ES_0 \quad (15a)$$

or

$$ES_0 = \frac{k_1 \cdot E \cdot S}{k_2} \quad (15b)$$

At the end of the preincubation phase, the total enzyme concentration E_t is given by:

$$E_t = ES_0 + E \quad (16)$$

Combining Equation 15b with Equation 16 and canceling *E*, one obtains ES_0 as a function of E_t, *S*, and the rate constants k_1 and k_2:

$$ES_0 = \frac{E_t \cdot k_1 \cdot S}{k_2 + k_1 \cdot S} \quad (17)$$

It can be derived from Equation 17 that ES_0 becomes $ES_0 \approx E_t$ at very high concentrations of S because k_2 can be neglected if compared with $k_1 \cdot S$ for such exposure conditions.

The solution of the differential Equation 14 is:

$$ES = ES_0 \cdot e^{-k_4 \cdot t} \quad (18)$$

The exposure time is given by t.

Equation 10 shows that the rate of the ET-derived formation of the EO concentration $\left(\frac{dP}{dt}\right)$ is represented by:

$$\frac{dP}{dt} = k_3 \cdot ES \quad (19)$$

or with Equation 18:

$$\frac{dP}{dt} = k_3 \cdot ES_0 \cdot e^{-k_4 \cdot t} \quad (20)$$

Because ES_0 becomes equal to E_t at very high concentrations of S, the maximum rate of metabolite formation $\frac{dP_{max}}{dt}$ (at the time point $t_{(0)} = 0$, when the reaction is started) is given by:

$$\frac{dP_{max}}{dt} = k_3 \cdot E_t \quad (21)$$

The concentration-time course of P at a given constant concentration of S is obtained by integrating Equation 20 and considering that $P = 0$ at $t_{(0)}$:

$$P = \frac{k_3}{k_4} \cdot ES_0 \cdot \left[1 - e^{-k_4 \cdot t}\right] \quad (22)$$

The term (k_3/k_4) is a partition ratio and represents the number of products formed per one inactivation event. The partition ratio is constant, independent of the substance concentration. Both reactions $(k_3 \cdot ES)$ and $(k_4 ES)$ follow saturation kinetics.

Linkage of the Suicide Inhibition Model with Experimental Parameters

The amount of ET was much greater in the relatively large atmospheric volumes of the closed vessels than in the small volumes of the liquid phase, where the reaction took place because of the very low K_{eqET} of 0.2 (see above). Consequently and because of the relatively slow metabolic ET elimination, the substrate concentration ET remained almost constant during the exposure periods. The maximum EO production V_{maxEO} was obtained experimentally as described in the text to Equation 9. Considering ET to be metabolized exclusively by CYP2E1 (see Results), the mean microsomal CYP2E1 contents (nmol/mg microsomal protein) of 96×10^{-3} (SD = 12×10^{-3}; $n = 3$), 35.8×10^{-3} (SD = 8.3×10^{-3}; $n = 3$), and 70.5×10^{-3} (SD = 17×10^{-3}; $n = 30$; weighted mean and SD of three times 10 determinations) in livers of mice, rats, and humans, respectively (Seaton *et al.*, 1995), were taken as the species specific total enzyme concentrations E_t. Replacing $\frac{dP_{max}}{dt}$ in Equation 21 by V_{maxEO}/V_l and E_t by the CYP2E1 content/V_l, Equation 23 was used to calculate the value of k_3:

$$k_3 = \frac{V_{maxEO}}{CYP2E1} \quad (23)$$

Equation 22 has the same structure as Equation 8. Therefore, the value of the inactivation constant k_4 equals the experimentally obtained value of k in Equation 8. The concentration $\frac{k_3}{k_4} \cdot ES_0$ (Equation 22) stays for the experimental EO plateau concentration C_1 which, when multiplied with k_4, equals the initial EO production rate at the corresponding ET exposure concentration and microsomal protein content (see Equations 9 and 20).

It has to be stressed that the K_m values and the rate constants k_i dealt with in the present work are apparent constants because the experiments were not performed with purified enzymes. The use of microsomal and cytosolic suspensions is, however, preferable to that of purified enzymes because the translation of *in vitro* results to the *in vivo* situation requires fewer assumptions when using microsomal and cytosolic data.

Statistics

Arithmetic means ($\bar{x}$) and SEs and two-way ANOVA were calculated using the software Prism 5. SE values were also obtained from SD calculated by means of Microsoft Excel 2004 for Mac (Microsoft, Unterschleissheim, Germany) using the following relation:

$$SE = \frac{SD}{\sqrt{n}} \quad (24)$$

with n being the sample size.

Error propagation of SE was calculated as described in Sachs (1973).

RESULTS

Gas Uptake Studies with ET in Microsomal Incubations

Elimination of ET. A series of ET gas uptake studies was performed using pooled rat liver microsomal incubations in order to monitor the disappearance of ET in the atmosphere of the vessels as a measure of ET metabolism. No enzyme-catalyzed concentration losses could be detected when using protein concentrations of 5 mg/ml incubate in 8-ml vials as the very flat slopes of the ET concentration-time courses of incubations containing native or heat-inactivated microsomes were almost identical (Fig. 1A). Only when using 38-ml vessels containing a microsomal suspension of 10 ml with a high protein concentration of 10 mg/ml, declines of atmospheric ET could be monitored (Figs. 1B and 1C). The slopes of the curves fitted to the data in native microsomes flatten with the exposure time and become, after about 20 min, parallel to the curves fitted to the data in heat-inactivated microsomes. The small decay of the latter curves results from the ET loss due to the sampling of the gas probes. The curves gained with native microsomes level off with time because of the suicide inactivation of the ET-metabolizing CYP species which results from the NADPH-dependent activation of ET (Ortiz de Montellano and Mico, 1980). The functional integrity of the hepatic microsomes of the three species was verified over a time span of 60 min using gaseous styrene as model substance (data not shown). Styrene is known to have a very high partition ratio k_3/k_4 of 10,000 (determined using a synthetic iron porphyrin; Collman *et al.*, 1986). Initial rates of ET elimination were calculated from the e-functions describing the curves in Figures 1B and 1C.

In mouse liver microsomes, the initial rate of metabolic ET elimination was 0.069 nmol/min/mg protein at an ET exposure concentration of 170 ppm in the vessel atmosphere. In rat liver microsomes, initial rates of 0.022 and 0.051 nmol/min/mg protein were obtained in two incubations with ET, each of

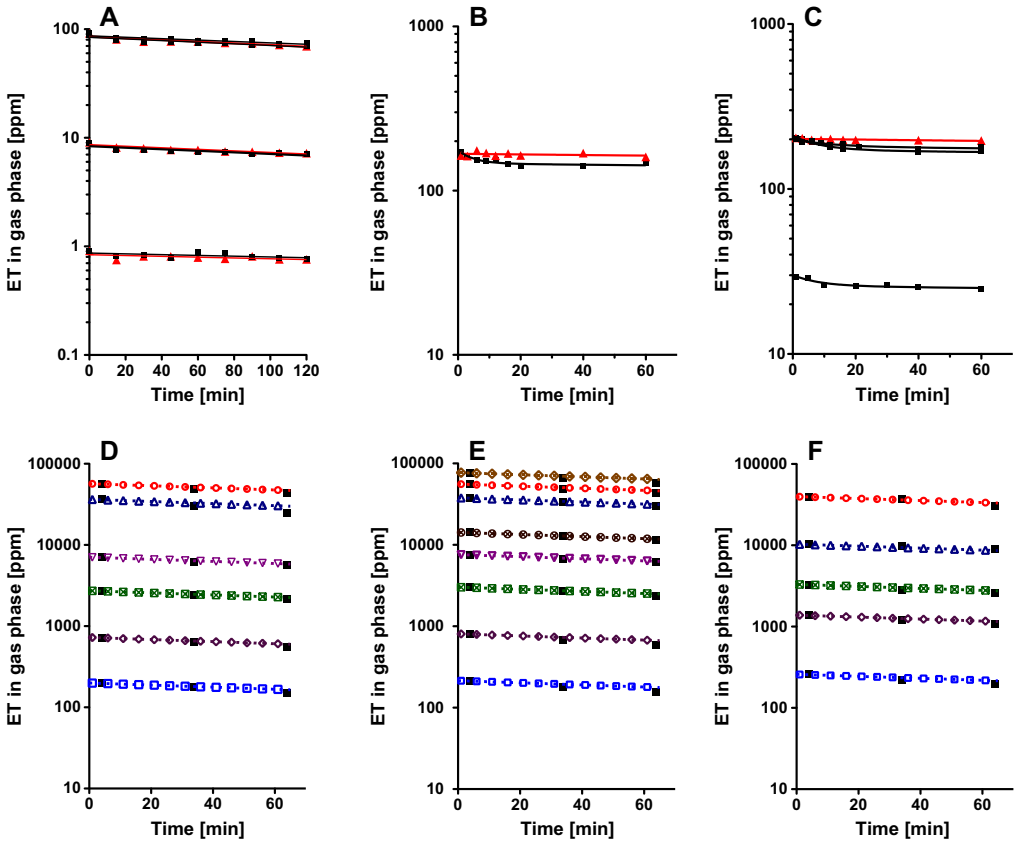

FIG. 1. Concentration-time courses of ET at various initial concentrations of ET in the atmosphere of closed exposure vessels containing microsomes from pooled livers of (A) 10 rats (5 mg protein/ml, incubation volume 4 ml, and vessel volume 8 ml), (B) 50 mice (10 mg protein/ml, incubation volume 10 ml, and vessel volume 38 ml), (C) 10 rats (10 mg protein/ml, incubation volume 10 ml, and vessel volume 38 ml), (D) 50 mice (2.5 mg protein/ml, incubation volume 4 ml, and vessel volume 38 ml), (E) 10 rats (5 mg protein/ml, incubation volume 4 ml, and vessel volume 38 ml), and (F) 25 humans (10 mg protein/ml, incubation volume 2 ml, and vessel volume 38 ml). Filled symbols, measured atmospheric ET concentrations using (▲) heat-inactivated or (■) native microsomes; open symbols, time points at which gas samples were collected for determining metabolically formed EO. The colors of the open symbols and of the dashed lines symbolize the ET uptake experiments of which the EO productions are shown in Figures 2A–C. Straight lines (A–C), fits of Equation 3 to the data (red, heat-inactivated microsomes and black, native microsomes); dashed lines (D–F), predicted by taking into account the loss of ET from the vessel by the sampling procedure.

which with an initial concentration of 200 ppm. At an ET concentration of 30 ppm, the initial elimination rate was 0.005 nmol/min/mg protein. Figures 1D–F show the concentration-time courses of ET in those mouse, rat, and human liver microsomal incubation experiments that were carried out in order to study the formation kinetics of metabolically produced EO. The slopes of the ET concentration-time courses result predominantly from the repeated collection of air samples of 50 and 500 µl each required for determining ET and EO, respectively, as is evidenced by the concentration-time curves that represent e-functions calculated by considering the experimental removal of atmospheric ET, solely. The extent of metabolic elimination of ET from the vessel atmospheres was too slight to become discernible.

Formation of EO. The results shown in Figure 2 were obtained when measuring the EO formation instead of the ET elimination in a series of ET incubation studies using pooled liver microsomes of mice (Fig. 2A), rats (Fig. 2B), or humans (Fig. 2C) and liver microsomes of individual human subjects (Figs. 2D and 2E) as well as pooled lung microsomes of mice or rats (Figs. 2F and 2G). In addition, the time courses of EO formation in ET-exposed pooled hepatic microsomal suspensions of 50 wild-type and 50 CYP2E1 knockout mice are shown in Figure 2H. The curves describing total EO in the liquid phase (c_{EOl}, see Equation 7) flatten with the exposure time and approach plateaus. This finding corresponds to the flattening of the ET elimination curve in dependence of the exposure time (Figs. 1B and 1C).

The curves plotted to the concentration-time data in Figure 2 were fitted by the function given in Equation 8. The rate constant in Equation 8 equals the apparent suicide inactivation rate constant k_4 of the ET-metabolizing CYP species, which is predominantly CYP2E1 in the liver. This can be concluded from the findings that the maximum rate of EO formation (V_{maxEO}) was about 15 times higher in liver microsomes of wild-type mice (1.1 nmol/min/mg protein) than in the corresponding CYP2E1 knockout mice (0.074 nmol/min/mg

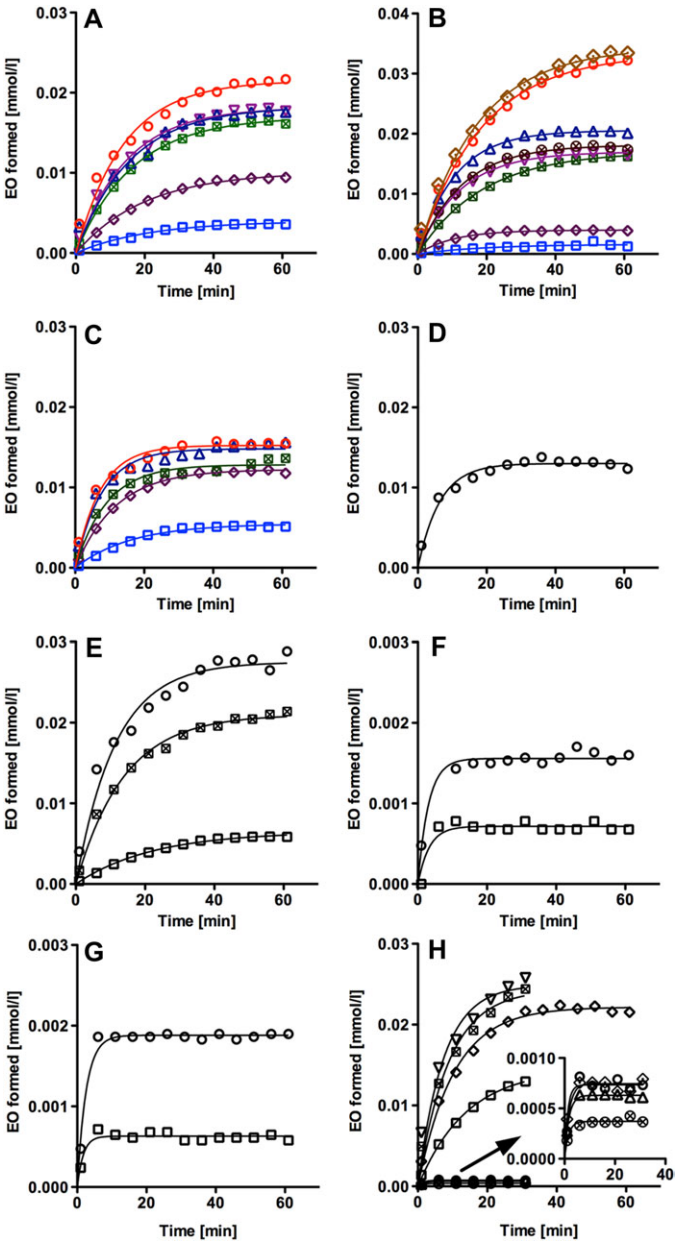

FIG. 2. Concentration-time courses of EO formed at various quasi-constant atmospheric concentrations of ET in closed exposure vessels (38 ml) containing hepatic or pulmonary microsomes. Produced EO is given as EO concentration in the liquid phase of the incubation vessels. (A–C and H) Incubations of pooled liver microsomes from (A) 50 B6C3F1 mice (2.5 mg protein/ml, incubation volume 4 ml), (B) 10 rats (5 mg protein/ml, incubation volume 4 ml), (C) 25 humans (10 mg protein/ml, incubation volume 2 ml), (H) 50 wild-type mice (2.5 mg protein/ml, incubation volume 4 ml), and 50 CYP2E1 knockout mice (10 mg protein/ml, incubation volume 2 ml). (D and E) Incubations of liver microsomes of 10 mg protein/ml (incubation volumes 2 ml each), from (D) a 75-year-old female human subject and (E) a 22-year-old male human subject. (F and G) Incubations (2 ml each) of pooled lung microsomes from (F) 150 mice (5 mg protein/ml) and (G) 50 rats (10 mg protein/ml). ET exposure concentrations and data symbols are given by (A) 200 ppm (□), 720 ppm (◇), 2720 ppm (⊠), 7040 ppm (▽), 36300 ppm (△), 56700 ppm (○); (B) 210 ppm (□), 810 ppm (◇), 3010 ppm (⊠), 7580 ppm (▽), 14200 ppm (⊗), 37800 ppm (△), 55700 ppm (○), 76900 ppm (◈); (C) 260 ppm (□), 1380 ppm (◇), 3300 ppm (⊠), 10300 ppm (△), 39700 ppm (○); (D) 66300 ppm (●); E) 270 ppm (■), 3110 ppm (⊠), 38600 ppm (●); (F) 16500 ppm (■), 75400 ppm (●); (G) 18600 ppm (■), 57800 ppm (●); (H) 1200 ppm (■), 3420 ppm (◇), 8910 ppm (⊠), 39800 ppm (▽), 10600 ppm (⊗), 20100 ppm (△), 30800 ppm (●), 38300 ppm (◈). Lines are fits of Equation 8 to the data. The experiments represented by colors (Figs. 2A–C) are identical with those in Figures 1D–1F. The insert in Figure 2H enlarges the concentration-time courses obtained in ET exposed CYP2E1 knockout mice.

protein). Even higher (340) was the ratio of V_{maxEO}/K_m in wild-type mice (162 µl/min/mg protein) to V_{maxEO}/K_m in CYP2E1 knockout mice (0.476 µl/min/mg protein).

Based on the fits to the EO concentration-time data, the initial EO formation rates v_{init} were calculated and are presented for B6C3F1 mice and Fischer 344 rats versus the ET exposure concentrations in Figure 3. The data obtained in lung microsomes at high ET concentrations of 16,500 and 75,400 (mice) and of 18,600 and 57,800 ppm (rats) probably represent the maximum EO formation rates of $V_{maxEO} = 0.073$ nmol/min/mg protein (mean value, mice) and 0.055 nmol/min/mg protein (mean value, rats). The curves shown in Figure 3 are fitted to the data gained from mouse, rat, and human liver microsomes considering saturation kinetics according to Michaelis and Menten. The obtained V_{maxEO} and K_m values for mice and rats are given in Table 1. The V_{maxEO} values in mice and rats are about double as high as in humans. The K_m value in humans is lower than that in rats and higher than that in mice. When comparing the ratios V_{maxEO}/K_m, human and rat liver microsomes show almost the same values which are about four times less than in mouse microsomes.

Table 2 shows the mean ± SE values of the rate constants of the EO formation (k_3), of the suicide inhibition (k_4), and the ratios k_3/k_4. The values of k_3 were calculated by dividing V_{maxEO} (Table 1) by the CYP2E1 content (Equation 23), the values of k_4 were obtained from the above-described six, eight, and nine ET incubations of mouse, rat, and human liver microsomes, respectively, and from incubations (two each) of mouse and rat lung microsomes. The values of k_4, reflecting

TABLE 1
Maximum EO Formation Rates (V_{maxEO}) and Corresponding Apparent Michaelis Constants (K_m) in ET-Exposed Mouse, Rat, and Human Hepatic Microsomes (Means ± SEs)

Species	V_{maxEO} (nmol/min/mg protein)	K_m (mmol/l ET in suspension)	V_{maxEO}/K_m (µl/min/mg protein)
Mouse[a]	0.567 ± 0.038	0.0093 ± 0.003	60.9 ± 21.0
Rat[a]	0.401 ± 0.013	0.031 ± 0.005	13.1 ± 2.0
Human[a]	0.219 ± 0.005	0.013 ± 0.001	17.2 ± 1.5
Human[b]	0.253 ± 0.0002	0.016 ± 0.000	15.6 ± 0.1

[a]Pooled from the livers of 50 mice, 10 rats, or 25 humans.
[b]From one male individual.

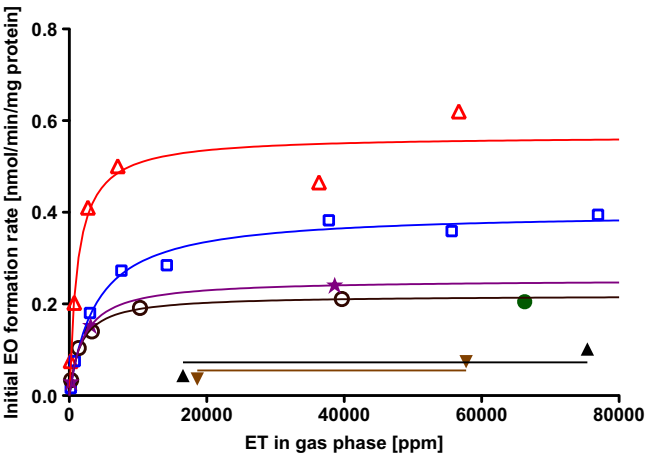

FIG. 3 Initial formation rates of EO versus atmospheric ET exposure concentrations in liver microsomes of mice, rats, and humans, and in lung microsomes of mice and rats. Symbols, initial EO formation rates: pooled liver microsomes from (△) 50 mice, (□) 10 rats, (○) 25 humans; liver microsomes from (●) a 75-year-old human female and (★) a 22-year-old male human subject; pooled lung microsomes from (▲) 150 mice and (▼) 50 rats. Lines represent Michaelis-Menten kinetics fitted to the data obtained in liver microsomes or averages of the values obtained in lung microsomes.

a reaction occurring within the porphyrin ring, are rather similar between the species. Those of k_3 are highest in rat and lowest in human livers. These findings might be caused by species differences in active site amino acid residues of CYP2E1 (Lewis *et al.*, 1997). As a result, the partition ratio k_3/k_4 is several times smaller in liver microsomes of humans than of rodents. This might be interpreted as a hint that humans could be less sensitive to eventual toxic effects from ET *via* the formation of EO. The values of the presently determined partition ratios are in agreement with the common observed values of less than 300 in unconjugated terminal olefins (Correia and Ortiz de Montellano, 2005).

The values of k_4 in lung microsomes of mice and rats are five and eight times larger than in liver microsomes of both species. The high k_4 values are unlikely to result from a bias in the interpretation of the EO concentration-times curves in lung microsomes because these microsomes were stable and metabolically active during the exposure time as was tested using the model substance styrene (data not shown). At present, we could only speculate why k_4 of the ET metabolism is organ-specific.

Gas Uptake Studies with EO in Microsomal Incubations

Figure 4 shows the results of EO uptake experiments in pooled hepatic microsomes of mice (Fig. 4A), rats (Fig. 4B), or humans (Fig. 4C), in hepatic microsomes of two adult human individuals (Fig. 4D) and in pooled lung microsomes of mice (Fig. 4E) or rats (Fig. 4F). In order to assess the microsomal functionality, uptake studies were carried out using the homologous PO and the same native pooled microsomes of mouse and rat livers as were prepared for the EO experiments. The slopes of the curves depicting the hepatic microsomal elimination of PO were about

TABLE 2

ET-Specific Catalytic Rate Constants k_3 in Liver and Lung Microsomes, and Suicide Inactivation Rate Constants k_4 as well as Partition Ratios k_3/k_4 in Liver Microsomes of Mice, Rats, or Humans (Means ± SEs)

Species	Organ	k_3 (min^{-1})	k_4 (min^{-1})	k_3/k_4
Mouse	Liver[a]	5.9 ± 0.6	0.060 ± 0.005	100 ± 9
	Lung[b]	n.a.	0.306; 0.326	n.a.
Rat	Liver[a]	11.2 ± 1.5	0.070 ± 0.006	169 ± 15
	Lung[b]	n.a.	0.573; 0.392	n.a.
Human	Liver[a]	3.2 ± 0.2	0.098 ± 0.013	39 ± 6

Note. n.a., not available.
[a]Pooled from livers of 50 mice, 10 rats, or 25 humans.
[b]Pooled from lungs of 150 mice or 50 rats.

10 times steeper than those of the corresponding EO curves (Fig. 4A and 4B), thereby proving the microsomal EH activity. With PO, about the same hepatic microsomal elimination clearances (μl/min/mg protein) were obtained as had been found earlier (mouse 4.4 and rat 9.9; Faller *et al.*, 2001). In spite of this, the slopes of the curves showing the EO elimination from the gas phase of the vessels containing native hepatic or pulmonary rodent microsomes were not distinguishable from those representing the spontaneous hydrolysis of EO, which was determined in 15 experiments with heat-inactivated microsomes of the three species (Figs. 4A–F). In other words, no EH-catalyzed EO elimination was found. Also, no CYP-mediated EO elimination was detected as was assayed for in a rat liver microsomal incubation containing an NADPH-regenerating system. In pooled native human liver microsomes, however, the EO elimination was clearly faster than in the heat-inactivated ones. The concentration-dependent flattening of the slopes hints to saturation kinetics of the EO elimination (Fig. 4C). From the best fits to the data by means of Berkeley Madonna, a V_{max} value of 14.35 nmol/min/mg protein was derived. The K_m value in the microsomal environment was 12.74 mmol/l, and the ratio of V_{max} to K_m was 1.13 μl/min/mg protein. The maximum EO production rates in microsomal incubations with ET (Table 1) were calculated without considering the microsomal elimination of EO and the loss of EO by spontaneous hydrolysis. The resulting error is negligible for rodents. For humans, it does not exceed 10% of the maximum production rate.

The slopes of the EO concentration-time curves of liver microsomes prepared from the two adults (Fig. 4D) were almost identical with the one obtained in pooled human microsomes at 100 ppm (see Fig. 4C).

Gas Uptake Studies with EO in Cytosolic Incubations

Figure 5 depicts time courses of atmospheric EO concentrations in incubations of GSH- or DEM-containing native or heat-inactivated pooled hepatic cytosol from mice, rats, and

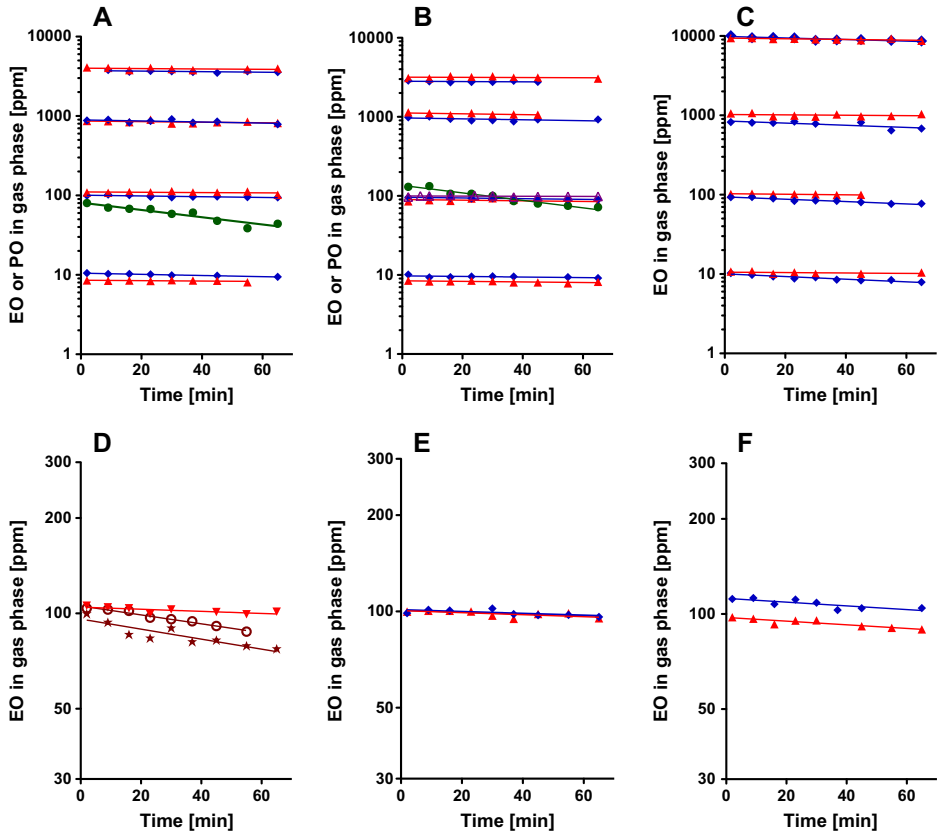

FIG. 4. Concentration-time courses of atmospheric EO or PO at various initial concentrations in closed exposure vessels containing microsomes (5 mg protein/ml, incubation volume 1 ml, and vessel volume 38 ml) from pooled livers of (A) 50 mice, (B) 10 rats, or (C) 25 humans, from livers of (D) a 75-year-old female or a 22-year-old male human subject, and from pooled lungs of (E) 150 mice and (F) 50 rats. Symbols, measured data, A–C, E, and F: (▲) EO exposures using pooled heat-inactivated microsomes from livers or lungs, (◆) EO exposures using pooled native liver or lung microsomes, (●) PO exposures using pooled native liver microsomes; D: EO exposures using (▼) heat-inactivated liver microsomes from a 22-year-old human subject or native liver microsomes from (★) a 75-year-old female or (○) a 22-year-old male human subject. Lines, fits of Equation 3 to the data.

humans (Figs. 5A–C) or GSH- or DEM-containing native or heat-inactivated pooled pulmonary cytosol from mice and rats (Figs. 5D and 5E). The slopes of the EO-concentration-time courses in the GSH-containing incubations with native cytosol are clearly different from those with GSH-depleted heat-inactivated cytosol. It is meaningful to compare the species-specific EO elimination by GSH conjugation directly from the figure because all incubations contained the same amount of cytosolic protein. Obviously, the conjugation rates are highest in mouse and lowest in human cytosol. In the semilogarithmic plots, the elimination curves are parallel over the whole exposure ranges up to about 3000 ppm and do not show any hint to saturation kinetics. In spite of this, the conjugation reaction was catalyzed by GST as has to be concluded from the evidently flatter slopes of the curves obtained in heat-inactivated GSH-containing cytosolic EO incubations. They represent the sum of both the spontaneous EO hydrolysis and the nonenzymatic conjugation of EO with GSH. The elimination rate constant resulting from the nonenzymatic conjugation reaction only was calculated to be $3.12 \times 10^{-3} \pm 3.0 \times 10^{-4}$ min^{-1} (means ± SE, $n = 8$; three measurements in heat-inactivated liver cytosol of human individuals included) corresponding to a mean half-life of 3.7 h. At the same GSH concentration of 15 mmol/l, Faller *et al.* (2001) reported a half-life of 1.7 h for the nonenzymatic GSH conjugation of the homologous PO.

The slopes of the curves obtained with heat-inactivated, DEM containing or with native, DEM-containing liver or lung cytosol did not differ significantly within and between each species. They were also not different from those obtained in Figure 4 with heat-inactivated microsomal EO incubations ($p < 0.05$; one-way ANOVA) representing the spontaneous hydrolysis. When calculating the parameters of enzyme-catalyzed reactions, non-enzymatic GSH conjugation and spontaneous hydrolysis were accounted for. The rate constant of the spontaneous EO hydrolysis was $9.3 \times 10^{-4} \pm 7 \times 10^{-5}$ min^{-1} (mean ± SE, $n = 36$). The corresponding mean half-life was 12.4 h. Faller *et al.* (2001) determined at pH 7.4 and 37°C a similar half-life of the spontaneous hydrolysis of PO (16 h) using also a $MgCl_2$ containing potassium phosphate buffer which has some catalytic activity due to its nucleophilic nature (Bundgaard and Hansen,

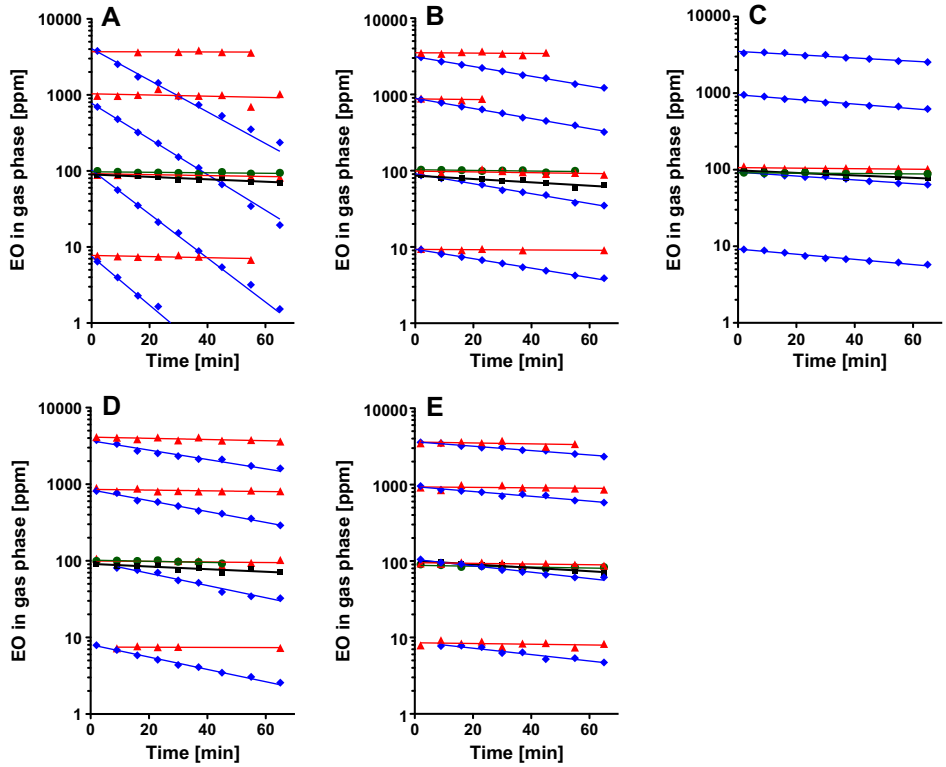

FIG. 5 Concentration-time courses of atmospheric EO at various initial concentrations of EO in the atmosphere of closed exposure vessels (38 ml) with incubations (1 ml) of GSH- or DEM-containing native or heat-inactivated pooled hepatic cytosol (3 mg protein/ml) from (A) 50 mice, (B) 10 rats, and (C) 11 humans or GSH- or DEM-containing native or heat-inactivated pooled pulmonary cytosol (3 mg protein/ml) from (D) 150 mice and (E) 50 rats. Symbols, measured data, (▲) pooled heat-inactivated cytosol with DEM (3 mmol/l), (◆) pooled native cytosol with GSH (15 mmol/l), (●) pooled native cytosol with DEM (3 mmol/l), (■) pooled heat-inactivated cytosol with GSH (15 mmol/l). Lines, fits of Equation 3 to the data.

1981). Longer half-lives of between 77 and 99 h (EO) and of 88 h (PO) were estimated for the noncatalyzed aqueous hydrolysis at 37°C, pH 7 (reported in Faller *et al.*, 2001).

The observed first-order decays in the EO concentrations signify that the rate of the GST-mediated EO elimination was directly proportional to the EO exposure concentration, the highest of which (3000 ppm) corresponds to an EO concentration of 9 mmol/l in the cytosolic suspension.

The EO-eliminating human GSTT1 (GST-theta; Föst *et al.*, 1991, 1995) shows polymorphism, which may be genotoxically relevant (Hallier *et al.*, 1993). Therefore, the GST-mediated conjugation of EO with GSH was additionally investigated in liver cytosol of six male and seven female individuals of between 16 and 78 years of age. The results are summarized in Table 3 together with those obtained in cytosol pooled from livers of 50 mice, 10 rats, or 11 humans or pooled from lungs of 150 mice or 50 rats. Only the ratio V_{max}/K_m is given because of the first-order kinetics observed for the GST-catalyzed EO elimination. The ratio was obtained by dividing the product of the rate constant of the GST-dependent EO elimination with V_d by the product of K_{eqEO} with the actual amount of protein. The GST activity in pooled human liver cytosol is reasonably in the middle between the lowest individual GST activity (zero) and the highest one (2.87 µl/min/mg protein). The time span between death and autopsy did presumably not influence the GST activity because of the 13 hepatic cytosol samples obtained from non-Asian individuals, highest GST activities were found in the samples of a 22- and 42-year-old man, who died 6.5 and 29 h, respectively, prior to autopsy. Two samples (15%) did not show any GST activity to EO. The percentage agrees with that of the GSTT1*0 carriers reported for non-Asians (between 10 and 25%; Bolt and Thier, 2006).

In Johanson and Filser (1993), it was discussed that the GST-catalyzed conjugation of GSH with an epoxide can be described by saturation kinetics according to a sequentially ordered ping-pong mechanism. For EO, the K_m concentration in livers or lungs of mice and rats and in livers of humans can be concluded to be at least 9 mmol/l (equivalent to the highest EO exposure concentration of 3000 ppm in cytosolic incubations). The GST-catalyzed EO elimination rate is almost independent of the GSH concentration at > 1 mmol/l because the K_m of GSH is only about 0.1 mmol/l (Csanády and Filser, 2007; Johanson and Filser, 1993). Taking into account a K_m value of 9 mmol/l tissue for the GST substrate EO, V_{max} values (nmol/min/mg protein) of 251, 47.7, and 10.3 are obtained for the GST-mediated GSH conjugation with EO in mouse, rat,

TABLE 3
Activity of GST (V_{max}/K_m) in Mouse and Rat Hepatic and Pulmonary Cytosol and in Human Hepatic Cytosol with EO as Substrate (Mean ± SE, $n = 4$)

Tissue	Species	Gender	Age (years)	V_{max}/K_m (μl/min/mg protein)	Time prior to autopsy (h)
Liver	Mouse[a]	Male	0.25	27.90 ± 2.87	0
Liver	Rat[a]	Male	0.25	5.30 ± 0.10	0
Liver	Human[b]	Both	1.9–70	1.14 ± 0.32	n.a.
Liver	Human	Female	51	0.00 ± 0.00	n.a.
Liver	Human	Female	54	0.00 ± 0.00	25
Liver	Human	Male	36	0.50 ± 0.25	23
Liver	Human	Male	63	0.54 ± 0.29	n.a.
Liver	Human	Male	16	0.75 ± 0.26	24
Liver	Human	Female	75	0.81 ± 0.34	0[d]
Liver	Human	Female	78	1.21 ± 0.16	n.a.
Liver	Human	Female	42	1.36 ± 0.18	n.a.
Liver	Human	Male	58	1.49 ± 0.32	17
Liver	Human	Female	53	1.62 ± 0.32	n.a.
Liver	Human	Female	48	1.76 ± 0.46	n.a.
Liver	Human	Male	42	1.82 ± 0.51	29
Liver	Human	Male	22	2.87 ± 0.57	6.5
Lung	Mouse[c]	Male	0.25	6.32 ± 0.52	0
Lung	Rat[c]	Male	0.25	1.78 ± 0.33	0

Note. n.a., not available.
[a]Pooled from livers of 50 mice or 10 rats.
[b]Pooled from livers of five male and six female humans.
[c]Pooled from lungs of 150 mice or 50 rats.
[d]Liver sample collected during transplantation.

and human pooled liver cytosol, respectively. Corresponding values for mouse and rat lung cytosol are 56.9 and 16.0.

DISCUSSION

Toxicokinetics of ET

If one assumes ET to be completely biotransformed to EO in the first metabolic step, EO formation rates should equal ET elimination rates (i.e., $|V_{maxEO}| = |V_{maxET}|$ and K_m for EO formation = K_m of ET elimination). In Table 4, maximum rates of ET elimination and atmospheric K_m concentrations, calculated for *in vivo* conditions from the *in vitro* results given in Table 1, are compared with corresponding parameters obtained *in vivo* from gas uptake studies. On average, the predicted maximum ET elimination rates are around three times higher than those observed *in vivo*. At a first glance, these results seem to be inconsistent. If, however, one bears in mind the values of the suicide inactivation constant k_4 of the ET-metabolizing CYP2E1 (Table 2), one has to expect that the amount of hepatic CYP2E1, which is available for the ET oxidation, decreases in between 11 min (humans) and 18 min (mice) to about one third of the initial status at very high ET concentrations. In gas uptake studies with ET in rats (Bolt *et al*., 1984) or mice (Artati *et al*., 2009), this effect could not be recognized from the concentration-time courses of ET in the atmosphere because it occurs within the initial phase during which the gas enriches in the organism. Predicted K_m concentrations in the atmosphere are 3.8- to 7.4-fold higher than actually determined *in vivo*. However, the predicted species-specific ratio V_{maxET}/K_m does not differ by more than a factor of two from the corresponding ratio obtained *in vivo* (Table 4). This ratio, which reflects the clearance of metabolism, is a direct measure of the metabolic elimination of ET at low atmospheric ET concentrations at which the ET elimination rate is proportional to the exposure concentration. The agreement between the predicted and the *in vivo* obtained V_{maxET}/K_m values is striking. Because the *in vivo*-derived values of V_{maxET} were obviously too low—not reflecting the suicide inhibition—the corresponding *in vivo* K_m values had also to be too low in order to describe correctly the metabolic elimination at low ET concentrations. For the same reason, an extremely small value of 0.0005 mmol/l was used for the apparent Michaelis constant of the ET oxidation in the liver compartment of the physiological toxicokinetic ET model of Csanády *et al*. (2000).

Toxicokinetics of EO

The present results obtained in subcellular fractions of mouse and rat livers can be compared with those of Brown *et al*. (1996) who investigated the ethylene glycol formation in liver microsomes and cytosol of both species. In microsomal incubations, the authors detected a maximum rate of hydrolysis of only 1.5–2 nmol/min/mg protein at an EO concentration of 15 mmol/l. The rate was at best (assuming a protein concentration of 2 mg/ml incubate) two times faster than the nonenzymatic hydrolysis. The K_m value for this reaction could only be estimated "due to the high rate of nonenzymatic hydrolysis relative (to) the enzymatic rate". One could argue, that the analytical method of Brown *et al*. (1996) was more sensitive than the present one, where only EO was quantified. However, the rate constant of the nonenzymatic elimination could well be determined in this work. It is almost identical with the value of 0.001 min^{-1} that can be calculated from the data given by Brown *et al*. (1996). In agreement with these authors, EO elimination takes place predominantly in the cytosol and is catalyzed by GST. The reported V_{max} and K_m values for GSH conjugation with EO in liver cytosol of mice and rats are almost identical with those obtained in the present work. From the high K_m concentration of 9 mmol/l (mice, rats, and humans; this work) or 10 mmol/l (mice and rats; Brown *et al*., 1996), the EO metabolism *in vivo* can be expected to be proportional to the EO exposure concentration until the consumption of GSH that is required for the elimination of EO will be too high to be counterbalanced by the GSH turnover rate. Indeed, in single EO gas uptake studies with Sprague-Dawley or Fischer rats, EO elimination from the chamber atmosphere followed first-order kinetics up to EO

TABLE 4
Kinetic Parameters of Metabolic ET Elimination Predicted from *In Vitro* Data on Metabolically Produced EO and Determined *In Vivo* in ET-Exposed Mice, Rats, and Humans

	Predicted from *in vitro*[a]			Determined *in vivo*		
Species	V_{maxET}[b] (μmol/h/kg)	K_m[c] (ppm in air)	V_{maxET}/K_m (μmol/h/kg/ppm)	V_{maxET} (μmol/h/kg)	K_m (ppm in air)	V_{maxET}/K_m (μmol/h/kg/ppm)
Mouse	42.9	400	0.11	15[d]	105[d]	0.14
Rat	28.9	1516	0.02	8.5[e]	204[e]	0.04
				8.6[f]	218[f]	0.04
Human	9.8	963	0.01	n.d.	n.d.	0.005[g]

Notes. V_{maxET}, maximum metabolic elimination rate of ET; K_m, apparent Michaelis constant of the metabolic ET elimination, given as ET exposure concentration in air; n.d., not determined.

[a]It is assumed that the maximum rate of EO formation ($|V_{maxEO}|$) equals $|V_{maxET}|$ and that the K_m concentrations of EO production and ET elimination are identical. The parameters were predicted using the V_{maxEO} and K_m values obtained in pooled liver microsomes.

[b]Predicted *in vivo* V_{maxET} was derived from V_{maxEO} (Table 1) as described in Kreuzer *et al*. (1991) taking into account a microsomal protein content of 30 mg/g liver, liver weights of 1 g (mouse), 10 g (rat), and 1.5 kg (human), and body weights of 25 g (mouse), 250 g (rat), and 70 kg (human).

[c]K_m (ppm) was calculated by dividing the in Table 1 given K_m (mmol/l suspension) by the *in vivo* obtained steady-state bioaccumulation factor K_{st}, which gives the ratio of the average ET concentration in the body at low ET exposure concentrations to the ET exposure concentration in the atmosphere—$K_{st} = 0.55$ (mouse, to be published), $K_{st} = 0.50$ (rat, Bolt *et al*., 1984), and $K_{st} = 0.33$ (human, Filser *et al*., 1992)—and by multiplication with 24,450 ml, the molar volume of an ideal gas at 25°C.

[d]From Artati *et al*. (2009).

[e]From Bolt *et al*. (1984).

[f]From Andersen *et al*. (1980).

[g]Value was calculated by dividing the ''clearance of metabolism (related to the atmosph. concn.)'' of 9.3 l/h (Table 2 in Filser *et al*., 1992) by 24.45 l, the molar volume of an ideal gas at 25°C, and by a body weight of 70 kg.

concentrations of about 600 ppm. At exposure concentrations of about 1000 ppm, the concentration–time courses flattened with increasing exposure time (Csanády *et al*., 2000). Depletion of GSH was most probably the cause for this effect considering that McKelvey and Zemaitis (1986) observed a concentration-dependent decrease of GSH in Fischer rats exposed for 4 h to constant EO concentrations of 100, 600, or 1200 ppm. At the highest EO concentration, GSH levels in the livers decreased to less than 20% of the control value. In B6C3F1 mice exposed for 4 h to constant EO concentrations of up to 200 ppm, EO blood levels were linearly related to the exposure concentration (Brown *et al*., 1998). At exposure concentrations of 300 and 400 ppm, however, the EO blood levels increased overproportional when related to the exposure concentration. The authors linked these findings to a depletion of GSH. At EO concentrations of 300 and 400 ppm, GSH levels in the livers declined to less than 22% of the control value (Brown *et al*., 1998).

In contrast to mice and rats, EH-catalyzed hydrolysis of EO is expected to represent at least one fourth of the overall EO metabolism in humans. This ratio is obtained taking into account a protein content of 30 mg/g liver in microsomes and of 95 mg/g liver in cytosol (Kreuzer *et al*., 1991) as well as a V_{max} to K_m ratio of the EH-catalyzed elimination in microsomes of 1.13 μl/min/mg protein and of the GST-catalyzed conjugation reaction in pooled cytosol of 1.14 μl/min/mg protein. Neglecting extrahepatic metabolism, one can therefore expect for EO-exposed humans of the GSTT1*0/0 genotype the EO tissue burden to be at most four times higher than in equally exposed humans carrying the active GSTT1 form. This conclusion of a quantitatively relevant role of EH on the elimination of EO in humans is supported by the results of a thoroughly conducted study on smokers. Hydroxyethyl adduct levels to the N-terminal valine of hemoglobin were on average 1.61 times higher in GSTT1-null individuals than in GSTT1 carriers (Fennell *et al*., 2000).

EO Burden during ET Exposure

From the present parameters of EO production, CYP2E1 inhibition, and EO metabolism, one has to expect to find in all the three species, when exposed to high ET concentrations, an early EO peak in blood followed by a rather rapid decline to a plateau characterized by a distinctly lower equilibrium between CYP2E1 neosynthesis and elimination as compared with the initial CYP2E1 status. In the atmosphere of a closed exposure chamber, the changes of the concentrations of exhaled EO have to be slower and less drastic than the rapid ones of EO in blood because the chamber behaves like a deep compartment. The reported pictures of initial EO peaks in blood and EO maxima in exhaled air of rats exposed to ET concentrations > 300 ppm (Filser and Bolt, 1984; Maples and Dahl, 1993) are in support of this expectation. At low ET concentrations, the permanent resynthesis of CYP2E1 will be large as compared with the rate of suicide inhibition and the CYP2E1 turnover rate will not much change during exposure. In agreement with the observation by Maples and Dahl (1993) in rats, EO in blood and in chamber atmosphere is expected to

increase continuously until reaching a plateau that is simply characterized by the equilibrium between EO formation (from ET) and EO elimination.

Outlook

A presently favored procedure to evaluate human health risks from chemicals uses toxicokinetic and toxicodynamic chemical-specific adjustment factors (CSAFs) in dose-response assessment (see, for example, Meek *et al*., 2002). Usually, the CSAF for the toxicokinetic uncertainty between species and that for the toxicokinetic uncertainty between human individuals is given a maximum number of $10^{0.6}$ and of $10^{0.5}$, respectively. In our laboratory, we are just finishing studies on the EO burden in ET-exposed mice, rats, and human individuals. The results together with the robust toxicokinetic parameters obtained in the present work will be used to improve a physiological toxicokinetic model (Csanády *et al*., 2000) for ET and EO in these three species. Because of its strong interspecies and interindividual data basis, we expect model predictions to be reliable enough for reducing the toxicokinetic CSAFs to the minimum.

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