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INDUCTION OF GLUTATHIONE-*S*-TRANSFERASE AND HEAT-SHOCK PROTEINS IN RAT LIVER AFTER ETHYLENE OXIDE EXPOSURE

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Abstract—Defense mechanisms in rat liver against depletion of glutathione (GSH) and cellular injuries induced by ethylene oxide (EO) were studied. Rats were exposed to EO under either high dose (1300 ppm for 4 hr, once) or low dose (500 ppm for 6 hr, three times a week for 6 weeks) conditions. The hepatic content of GSH decreased dramatically after EO treatment, probably due to detoxication of EO. After the high dose treatment the hepatic GSH content fell by 90% of the control values but recovered within 10 to 15 hr. EO reacts directly with a variety of cellular macromolecules but all rats survived the exposure. Since the metabolites of EO are ethylene glycol and GSH-conjugates, the enzymatic activities of epoxide hydrolase and glutathione-*S*-transferase (GST) were determined. Only GST activity was found to occur after low dose chronic exposure. The defense mechanism at mRNA level was investigated using probes for GST and several heat-shock proteins (hsps). Enhanced accumulation of GST mRNA was detectable during the recovery period of rats after both high and low dose exposure to EO. Interestingly, both hsp32 (< 40-fold) and hsp90 (< 3-fold) mRNA increased after high dose exposure but the mRNA level of one of the major heat-shock proteins, hsp70, did not change under these conditions. Diethylmaleate, which is known to be a GSH depleter in liver, induced hsp32 mRNA only in rat liver, while hsp70 and hsp90 mRNA levels did not change when GSH was depleted. These results suggest that individual heat-shock proteins are induced in different ways under unphysiological conditions such as EO exposure.

Ethylene oxide (EO‡) is an intermediate in the chemical industry and is also widely used for sterilization and fumigation. It is a highly reactive alkylating agent which has been incriminated as a carcinogen [1, 2] and mutagen [3]. Previously, we reported that the exposure of animals to EO caused significant depletion of cytosolic glutathione (GSH) and enhanced the formation of lipid peroxide in rat liver [4]. Nevertheless, almost all rats undergoing longterm exposure to EO survived, along with the untreated group. In the present experiment, we wanted to investigate which defensive mechanisms would be activated after the depletion of GSH and the cellular injuries induced by EO.

GSH plays an integral role in maintenance of the cellular redox state [5] and in detoxication [6]. Glutathione-*S*-transferase (GST) isozymes catalyse the nucleophilic addition of the thiol of GSH to electrophilic acceptors [7], including EO. Other interesting substrates are those toxic products generated by tissue damage. For example, arachidonic acid hydroperoxide, linoleate hydroperoxide and 4-hydroxyalkenals are cytotoxic products of membrane lipid peroxidation [8] whose production was stimulated in rat liver following EO exposure [4]. Besides conjugation of GSH, EO can also be hydrated to ethylene glycol by epoxide hydrolase [9].

Next, we turned our attention to heat-shock proteins (hsps) which protect cells from the toxic effects of heat and other stresses [10–12]. The induction of heat-shock protein is a remarkably rapid emergency response. When cells acquire thermal resistance, they become resistant to not only subsequent heat exposure but also some other chemical stresses [13]. In other words, heat-shock proteins protect chemically and thermally vulnerable targets [12, 14, 15]. Interestingly, GSH was found to increase rapidly under thermal stress [16]. Mitchell and co-workers [16, 17] have demonstrated that thermal stress also results in alteration of the cellular redox state, which is largely maintained by GSH.

In the present study, we examined cellular protective mechanisms against EO exposure which helped almost all rats to survive long term exposure. To distinguish whether the defense mechanisms were stimulated solely by GSH depletion or by other effects of EO, we investigated the effect on heat-shock protein of GSH depletion by diethylmaleate, which forms a thioether conjugate with GSH [18]. We observed that individual heat-shock proteins respond differently to the effects of EO induction. The expression of hsp32 (heme oxygenase) was stimulated most significantly by GSH depletion induced by diethymaleate.

MATERIALS AND METHODS

Materials and animals. EO was obtained from the

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‡ Abbreviations: EO, ethylene oxide; hsp, heat-shock protein; GSH, glutathione; GST, glutathione-*S*-transferase; ppm, parts per million.

Nissan Shoji Co. Ltd (Tokyo, Japan). GSH and diethylmaleate were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) The other chemicals used in this study were obtained either from Sigma Chemical Co. or Nacalai Tesque, Inc. (Kyoto, Japan). Wistar male rats, 6 weeks old, were purchased from the Kyudo Animal Center (Kumamoto, Japan).

EO exposure system and treatment. The exposure system was the same as that used by More *et al.* [19]. The stock gas contained 20% EO and 80% carbon dioxide in a 40-L gas cylinder. Animals were exposed in a 1.0 m^3 inhalation chamber. EO/air concentration in the exposure chamber were maintained by delivering the gas at a controlled rate through a flowmeter in a mixing tube where it was diluted by filtered room air. This concentration of EO was monitored repeatedly by gas chromatography, with a flame ionization detector. The rats were divided randomly into control and test groups. A 50% lethal concentration of EO is estimated to be 1460 ppm for 4 hr [20]. In the acute experiment, test group rats were exposed to EO, once, for 4 hr at a concentration of 1300 ppm. In the chronic experiment, rats were exposed to EO for 6 hr at a concentration of 500 ppm, three times a week for 6 weeks. We reported previously that EO caused depletion of cytosolic GSH and enhanced the formation of lipid peroxide in rat liver under the same chronic-experiment conditions [4]. Control rats were exposed to filtered room air only. Control and treated rats were pair-fed to minimize differences due to food intake [4]. A group of untreated rats was intraperitoneally injected with 0.6 mL/kg body weight of diethylmaleate. Diethylmaleate has been reported to produce a rapid and transient decrease in hepatic GSH [21].

Assay methods. At the end of exposure time, animals were killed by decapitation. The livers were perfused with ice-cold saline. An aliquot (1 g) of tissue was homogenized in 15 mL of 50 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA, mixed with 25% metaphosphoric acid (1/4 volumes of homogenate, v/v) and centrifuged at 100,000 *g* for 30 min. The GSH level of the supernatant was determined by the fluorometric method of Hissin and Hilf [22]. Another aliquot (1 g) of tissue was homogenized in ice-cold 50 mM Tris–HCl, pH 7.4, containing 1.15% KCl. The homogenate was centrifuged at 27,000 *g* for 20 min at 4°. The resulting supernatant was centrifuged at 100,000 *g* for 60 min. The GST activity of the supernatant then obtained was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [23]. The determination of epoxide hydrolase was based on the gas chromatographic determination [24]. Protein was determined by the procedure of Lowry *et al.* [25].

RNA extraction and northern analysis. Having been extracted from the livers by the guanidinium thiocyanate method [26], the total RNA (20 μg) was electrophoresed on 1% agarose (Sea Kem, GTG) under denaturing conditions [27]. RNA was then blotted onto a nylon filter (Hybond N, Amersham International, Amersham, U.K.) and hybridized with ^{32}P-labeled nick translated probes. Probes were prepared from the following clones: Hsp32 (mouse); *Eco*R I fragment (1.5 kbp) of pMp32, a generous gift from Dr S. Sakiyama [28]; Hsp70 (human); *Bam*H I-*Eco*R I fragment (0.8 kbp) of pUR-HS70, a generous gift from Dr J. P. Nevins [29]; Hsp90 (human); *Pst* I fragment (1.4 kbp) of pHS801, a generous gift from Dr L. Weber [30]; β-actin (chick), *Pst* I fragment (1.8 kbp) [31]; GST-P (rat); and *Eco*R I-*Sal* I fragment (0.7 kbp) of pGP5 [32]. There is about 65% homology between the GST genes Ya and Yc and approximately 32% homology is found between GST-P and Ya [32]. Since the homologous sequences of amino acid residues are clustered in a few regions, we used this probe to detect GST mRNA under conditions of low stringency. Hybridization was carried out in 4 × SSC, 50% formamide at 42° for either 18 or 40 hr. The filters were washed under mild conditions (2 × SSC, 0.1% SDS for 30 min and 1 × SSC, 0.1% SDS for 30 min, twice at 42°). Quantitation of individual bands after autoradiography was carried out densitometrically and the expressions were normalized to the expression of β-actin.

RESULTS

The time course of the hepatic GSH level in rats treated with a single exposure to EO in the acute experiment (see Materials and Methods) is shown in Fig. 1A. An approximately 90% depletion of GSH was observed immediately after exposure to EO for 4 hr. The GSH level had recovered to its normal range by around 12 hr after exposure. The time course of hepatic GSH level after the last exposure to EO in the chronic 6-week experiment was essentially similar to that of the acute experiment, although depletion of GSH immediately after the last exposure was to about 50% of the level in control rats [4]. It is likely that GSH was consumed mainly for the detoxication of EO, but recovered to control levels before the start of the next exposure. This depletion and recovery of hepatic GSH must be repeated for 6 weeks in the chronic experiment.

The metabolic routes of EO are known to be common to many other epoxy compounds. EO is conjugated with GSH [33, 34] by GST or metabolized to ethylene glycol by epoxide hydrolase [9]. In the present experiment, GST activity in the treated rat liver did not change significantly at any time point after acute treatment (Fig. 1B) but had increased reproducibly to about 30% above that of the control rat liver 24 hr after the last exposure to EO in the chronic experiment (Table 1). On the other hand, epoxide hydrolase activity was not affected by EO exposure in either the acute or the chronic experiment. These results indicate that, as part of the defense mechanism against repeated exposure to EO, GST rather than epoxide hydrolase was activated in rat liver during the chronic experiment.

We examined next whether the change in GST activity was due to transcriptional changes or a posttranslational event. Hepatic GST mRNA levels in treated rats had increased approximately 3-fold as compared with controls at 1 and 3 hr after a single high dose of EO in the acute experiment (Fig. 2 and Table 2), although only a slight increase in enzymatic activity was detected under these conditions (Fig.

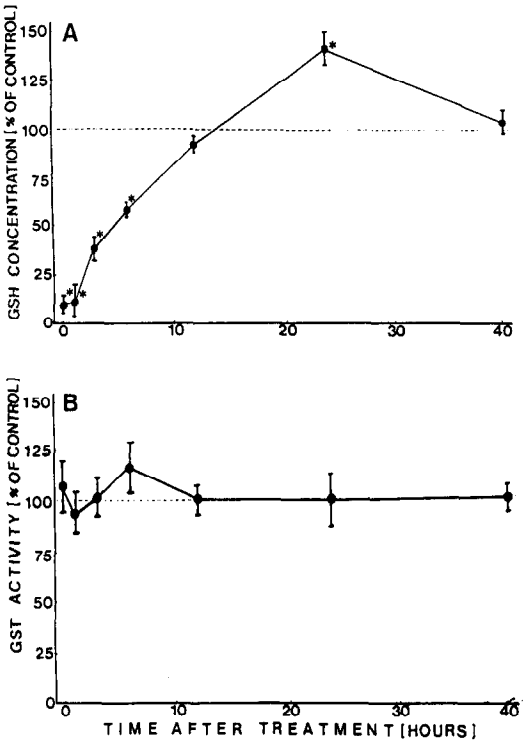

Fig. 1. Time course of hepatic reduced glutathione (GSH) levels and glutathione-*S*-transferase (GST) activities in rats following a treatment of ethylene oxide at 1300 ppm for 4 hr. Rats were killed at the time indicated on the abscissa after a single exposure. Three treated and three control rats were killed at each time-point. The points represent means ± SD. * $P < 0.05$. Mean GSH level (μg/g of liver) in control rats was 1326 at 0 hr and 1315 at 40 hr. Mean GST activity (nmol product/min/mg protein) in control rats was 367.6 at 0 hr and 341.2 at 40 hr.

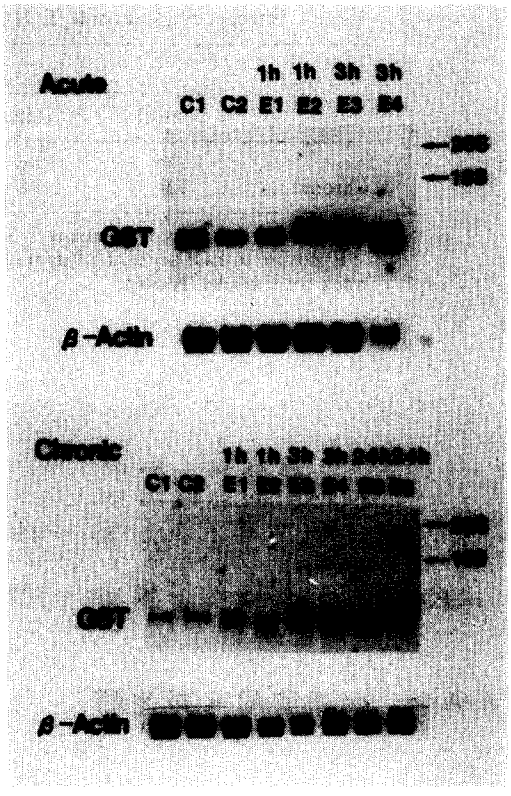

Fig. 2. Northern blot analysis of expression of glutathione-*S*-transferase (GST) genes in the liver after acute and chronic ethylene oxide exposure. Acute: C1,2, control; E1, 2, 1 hr and E3, 4, 3 hr after exposure. Chronic: C 1, 2, control; E1, 2, 1 hr; E3, 4, 3 hr and E5, 6, 24 hr after the last treatment of chronic exposure. Total RNA (20 μg) extracted from individual livers was electrophoresed, blotted onto a nylon filter and hybridized with ^{32}P-labeled GST probe as described in Materials and Methods.

Table 1. Effect of chronic inhalation of ethylene oxide on the activities of glutathione-*S*-transferase and epoxide hydrolase

	Control	Ethylene oxide
Glutathione-*S*-transferase (nmol product/min/mg protein)	437.0 ± 38.1	541.8 ± 36.3*
Epoxide hydrolase (nmol product/10 min/mg protein)	46.4 ± 6.2	48.2 ± 6.1

Results are expressed as means ± SD.
The number of samples in each group: GST, N = 8 and EH, N = 6.
Significantly different from control; * $P < 0.05$ (Student's *t*-test).

1B). Increased levels of GST mRNA were also detectable in the chronic experiment in which enhancement of the accumulation of GST mRNA was more remarkable at 3 and 24 hr after EO exposure than immediately after the treatment (Fig. 2 and Table 2). It must be noted, however, that in the present study the filter was washed under conditions of low stringency after the hybridization reaction and, therefore, we could not distinguish the individual mRNA of GST isozymes at that time (see Materials and Methods). Relatively broad bands for GST indicate the heterogeneity of GST mRNA (Fig. 2).

We examined next the gene expression of the three heat-shock proteins after high dose EO exposure, because the induction of heat-shock proteins is one of the major defense mechanisms of cells under unphysiological conditions. The levels of hsp32 and hsp90 mRNA were found to increase in rat liver after EO exposure. Maximal induction of

Table 2. Effect of ethylene oxide (EO) on the glutathione-S-transferase gene expression

	Ratio of mRNA level			
	Control	After 1 hr	After 3 hr	After 24 hr
Acute	1.0	2.6 ± 0.3	3.1 ± 0.3	—
Chronic	1.0	1.8 ± 0.2	3.2 ± 0.4	2.8 ± 0.3

Results are expressed as means ± SD after normalization by β-actin.
Exposure to EO: acute, 1300 ppm for 4 hr (N = 8); chronic, 500 ppm, three times a week for 6 weeks (N = 4).

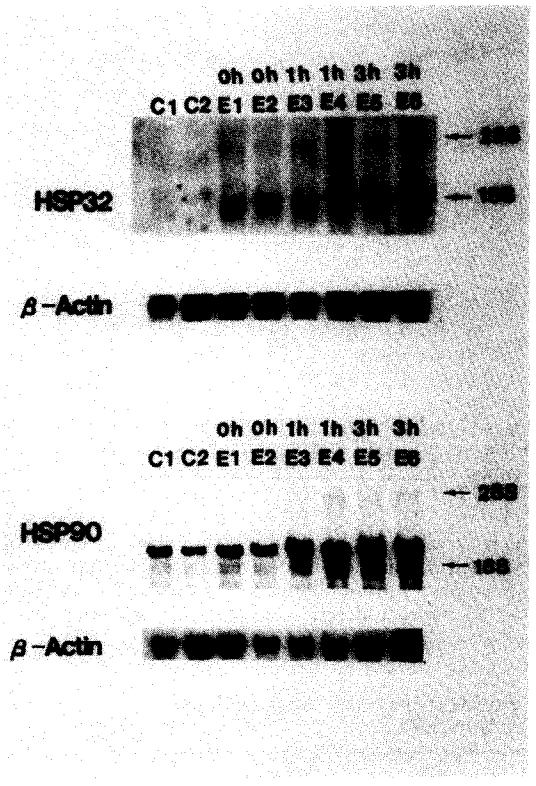

Fig. 3. Northern blot analysis of the gene expression of hsp32 and hsp90 in the liver after acute ethylene oxide exposure. C1, 2, control; E1, 2, 0 hr; E3, 4, 1 hr and E5, 6, 3 hr after exposure. The gene expression of hsp32 and hsp90 was analysed as described in the legend to Fig. 2.

hsp32 mRNA was observed at 3 hr (40-fold) after EO exposure (Fig. 3 and Table 3). The hsp90 mRNA level in EO-exposed rat liver had also increased to approximately 3-fold of that of the control at 1 and 3 hr after exposure (Fig. 3 and Table 3). In contrast, the level of mRNA of one of the major heat-shock proteins, hsp70, in exposed rat liver did not increase at all (Table 3). It appears that, at least at the level of mRNA, hsp32 and hsp90 respond more significantly to the stress of EO exposure and/or depletion of GSH than hsp70.

To determine whether a reduction of GSH induced by EO, alone, influences the expression of hsp32 and hsp90 genes, the rats were treated with diethylmaleate (0.6 mL/kg body weight), an agent which binds thiol groups of GSH and thus prevents GSH from reacting further [18, 21]. The hepatic GSH level in treated rats was reduced by 90% of the control; however, the GSH content gradually returned to the control value within 12–15 hr of the administration of diethylmaleate (Fig. 4). In addition, 3 hr after administration of diethylmaleate there was a 3-fold increase in GST mRNA (data not shown). These effects of diethylmaleate treatment on GSH and GST levels were similar to those of EO exposure in the acute experiment. The change in heat-shock protein mRNA was determined 0.5, 1 and 3 hr after administration of diethylmaleate, as shown in Fig. 5 and Table 3. The level of hsp32 mRNA was increased to approximately 10-fold that of the control, 3 hr after diethylmaleate administration, while the hsp90 mRNA level did not change. This finding was in striking contrast to that observed with EO-exposed rats which showed induction of the mRNA of both hsp32 and hsp90. These results suggest that there is another effect of EO, besides GSH depletion, on the gene expression of hsp90. The level of hsp70 mRNA in diethymaleate-treated rats did not change significantly, as was the case in EO-exposed rats.

DISCUSSION

Heat-shock proteins are highly conserved protein families induced by heat and other stresses [35]. It is commonly accepted that the function of the heat-shock proteins is either to protect cells against subsequent heat stress or to enhance the ability of cells to recover from the toxic effects of heat or other stresses.

In the present study, we showed that hepatic GSH was depleted significantly after EO exposure (Fig. 1A). As the most abundant intracellular sulfhydryl, GSH is a key factor in the detoxication of electrophilic metabolites of xenobiotics [36, 37]. Furthermore, as the cosubstrate for selenium-independent glutathione peroxidase activity, such as that of GST [38], GSH prevents peroxidation of membrane lipids. As shown in Fig. 1A, hepatic GSH concentration decreased to approximately 10% of control levels after exposure to a single dose of EO. This depletion of GSH and toxic effect of EO and its metabolites cause severe stress to liver cells. However, the GSH concentration in EO-treated rat liver recovered rapidly to its normal range after around 10–15 hr (Fig. 1A).

Table 3. Effects of ethylene oxide and diethylmaleate on the gene expression of heat-shock protein 32 (hsp32), heat-shock protein 70 (hsp70) and heat-shock protein 90 (hsp90) in rat liver

	Ratio of mRNA level		
	Hsp32	Hsp70	Hsp90
Control	1.0	1.0	1.0
Ethylene oxide, 1300 ppm, 4 hr			
After 0 hr	16.0 ± 2.5	1.2 ± 0.2	1.3 ± 0.2
After 1 hr	32.2 ± 4.4	1.4 ± 0.2	2.8 ± 0.3
After 3 hr	43.0 ± 5.8	1.6 ± 0.3	3.2 ± 0.4
Diethylmaleate, 0.6 mL/kg body wt			
After 0.5 hr	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.2
After 1 hr	1.0 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
After 3 hr	10.6 ± 3.2	1.2 ± 0.2	1.2 ± 0.2

Results are expressed as means ± SD after normalization by β-actin.
Number of samples in each group: hsp32 and hsp70, N = 6; hsp90, N = 8.

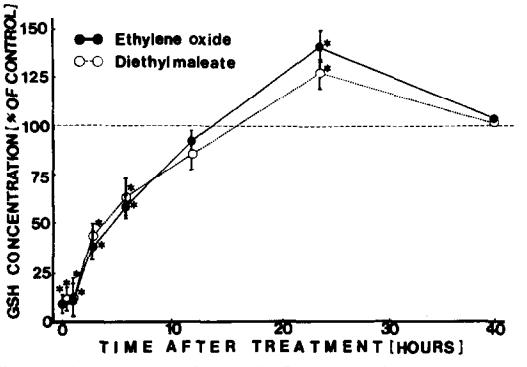

Fig. 4. Time course of hepatic GSH level in rats following treatments of ethylene oxide at 1300 ppm for 4 hr and diethylmaleate at 0.6 mL/kg body weight. Points represent means ± SD. * $P < 0.05$. Rats were analysed as described in the legend to Fig. 1.

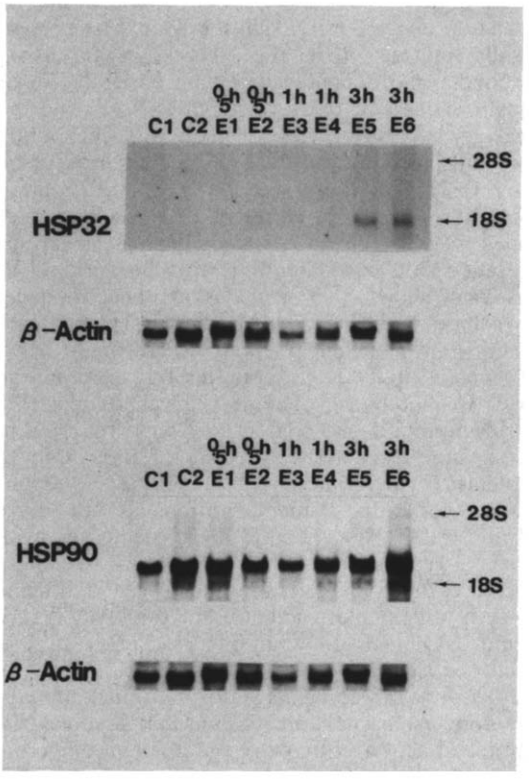

Fig. 5. Northern blot analysis of the gene expression of hsp32 and hsp90 in the liver after diethylmaleate treatment. C1, 2, control; E1, 2, 0.5 hr; E3, 4, 1 hr and E5, 6, 3 hr after treatment. The gene expression of hsp32 and hsp90 was analysed as described in the legend to Fig. 2.

According to the kinetic study of hepatic GSH turnover by Lauterburg and Mitchell [39], after a decrease (25%) due to fasting for 48 hr, the hepatic GSH level will return to normal within 6 hr of refeeding. According to a simplified calculation, the recovery of hepatic GSH after EO exposure was similar to physiological recovery caused by refeeding. Our previous studies of chronic exposure to either EO [4] or styrene oxide [40] have shown the enhancement of lipid peroxidation in the liver membrane fraction. Repeated exposure to EO or styrene oxide might overcome the defense mechanism against tissue damage. Lipid peroxidation of biological membranes yields reactive alkenes, epoxide, hydroperoxides and aldehydes [8]. Besides EO itself, many of these toxic products are substrates of a variety of GST isozymes. Thus, GST may play an important role in protecting tissues from oxidative damage and oxidative stress, which is similar to that of heat-shock proteins. Induction of GST in the chronic experiment (Table 1) may contribute to the protection of membrane integrity, in addition to direct conjugation of EO with GSH.

Ames and co-workers [41, 42] and Ashburner and Bonner [43] have proposed that acquired thermotolerance may be associated with resistance

to oxidative stress. They pointed out that many agents causing oxidative stress in eukaryotic cells also induce hsp; these include hydrogen peroxide [42], menadione [42], an oxidizing quinone [44], diamide [45], a GSH depleter [45] and the sulfhydryl reagents iodoacetamide and cadmium [45]. Interestingly, Mitchell *et al.* [16] have reported that GSH increased rapidly upon thermal stress. The relationship between GSH and the development of thermotolerance is a matter of controversy. Russo *et al.* [46] have shown that depletion of GSH in V-79 cells by buthionine sulfoximine resulted in a decrease in heat-shock protein synthesis. Freeman *et al.* [47] have reported that inhibition of heat-shock protein synthesis in Chinese hamster ovary cells recovering from hyperthermic exposure does not depend on the GSH level *per se*, but rather on the result of an inhibitive effect on the overall cellular protein synthesis. Our present study was carried out using the whole body in which the metabolism of liver cells is regulated by many biological factors including humoral factors, hormones and hepatic circulation. Both exposure to EO and diethylmaleate resulted in a 50–90% depletion of GSH and an induction of GST mRNA in the liver. The present results indicate that the expression of hsp32 responded more significantly than either hsp70 or hsp90, at least at the level of mRNA, during the recovery from severe GSH depletion. Freeman and Meredith [48] indicated that hsp32 induction occurred after GSH depletion exceeded 80% and required formation of a GSH conjugate. The induction of hsp32 mRNA by EO and diethylmaleate as in our present results correlates well with their criteria. It has been shown that hsp32 is identical to heme oxygenase, which is induced by chemical carcinogen, metal and heat shock [49]. Microsomal heme oxygenase catalyses the rate-limiting step in the oxidative metabolism of heme which yields biliverdin [50]. The total heme content of liver is reported to be 2.6 nmol/g, and nearly 65% of it is used to replenish cytochrome P450 heme [51]. Previous studies under the same conditions as ours have demonstrated that hepatic P450 decreases with chronic EO exposure [52, 53]. If P450 content decreases due to degradation, the intracellular heme content will increase and this in turn will induce heme oxygenase. In addition, Stocker *et al.* [54] proposed that one beneficial role of bilirubin may be to act as a physiological antioxidant. In mammals, the conversion of heme to bilirubin involves the combined action of heme oxygenase and biliverdin reductase. The induction of heme oxygenase, hsp32, is expected to increase the bilirubin content. As a result of this, there may be much additional bilirubin that should mitigate oxidative stress caused by EO exposure.

The level of hsp90 mRNA did not change significantly after the depletion of GSH induced by diethylmaleate, but increased to 3-fold of the control value after EO exposure. Hsp90 has been shown to interact with several viral oncogene products that possess tyrosine kinase activity, including pp60src [55], and with steroid receptors [56] which may participate in the response to various stresses. Furthermore, the physicochemical properties of the glucocorticoid receptor and the Ah receptor are similar [57] and hsp90 has been found to be associated with the Ah receptor [58]. Recently, Telakowski-Hopkins *et al.* [59] have identified two cis-acting regulatory elements in the upstream region of the GST Ya gene. One regulatory element is required for the inducible expression of the GST Ya gene by planar aromatic compounds such as β-naphthoflavone, which binds to the Ah receptor. Although it is unknown whether the expression of the GST gene is induced after heat shock, the proposal that GST is a member of the stress-proteins has been suggested. This hypothesis, however, remains controversial. We found only a 1.5-fold induction of GST mRNA in contrast with a 30-fold induction of hsp70 mRNA 2 hr after exposure of human amniotic (WISH) cells to temperatures ranging from 37° to 42° (data not shown).

Recently, it was reported that the functions of hsp70 appeared to relate to "chaperoning", subcellular transport, import-export of other proteins and prevention of incorrect folding [35]. These functions suggest that hsp70 might bind to denatured or abnormal proteins after a heat shock to prevent their aggregation and, thus, cellular damage. We expected that oxidative stress or other effects of EO exposure would cause protein degradation and induce hsp70 mRNA in the liver cells. However, hsp70 mRNA was not affected under the present conditions.

We have begun to investigate the defense mechanism against EO exposure, which causes the depletion of hepatic GSH and lipid peroxidation.

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