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ACTIVATION OF THE MICROSOMAL GLUTATHIONE-*S*-TRANSFERASE AND REDUCTION OF THE GLUTATHIONE DEPENDENT PROTECTION AGAINST LIPID PEROXIDATION BY ACROLEIN

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Abstract—Allyl alcohol is hepatotoxic. It is generally believed that acrolein, generated out of allyl alcohol by cytosolic alcohol dehydrogenase, is responsible for this toxicity. The effect of acrolein *in vitro* and *in vivo* on the glutathione (GSH) dependent protection of liver microsomes against lipid peroxidation, and on the microsomal GSH-*S*-transferase (GSH-tr) in the rat was determined. *In vitro* incubation of liver microsomes with 5 mM acrolein for 30 sec resulted in a 2-fold activation of the GSH-tr. This activation probably proceeds via alkylation of the thiol group of the GSH-tr. *In vivo* administration of 1.1 mmol allyl alcohol/kg to rats did also result in a 2-fold stimulation of the GSH-tr activity. Administration of 375 mg pyrazole/kg, an inhibitor of the alcohol dehydrogenase, thus reducing the acrolein formation, prevented the *in vivo* stimulation of GSH-tr by allyl alcohol. This indicates that the activation of GSH-tr *in vivo* by allyl alcohol probably also proceeds via alkylation of the thiol group of the GSH-tr by acrolein.

GSH protects liver microsomes against lipid peroxidation, probably via a free radical reductase that reduces vitamin E radicals at the expense of GSH. Incubating liver microsomes for 30 min with 0.1 mM acrolein reduced the GSH dependent protection against lipid peroxidation, probably because an essential thiol group(s) on the free radical reductase is alkylated. *In vivo* administration of allyl alcohol did not reduce the GSH dependent protection of the microsomes. Probably the thiol group(s) located on the free radical reductase is less accessible or less reactive than the thiol group on the GSH-tr. After administration of allyl alcohol we found no evidence for *in vivo* lipid peroxidation. Therefore we could not evaluate the importance of the GSH dependent protection against lipid peroxidation *in vivo*.

Allyl alcohol is a well known hepatotoxin [1]. The toxicity of allyl alcohol is considered to be mediated via acrolein, which is generated from allyl alcohol by the cytosolic enzyme alcohol dehydrogenase [1]. Acrolein is also a metabolite of the anticancer drug cyclophosphamide [2]. Moreover, acrolein is a component of tobacco smoke [3] and it is produced during lipid peroxidation [4].

Acrolein has been shown to react spontaneously with soft nucleophilic sulfhydryl groups [2, 4]. Covalent binding of acrolein to biologically important thiol groups seems to be of major importance in the mechanism by which acrolein exerts its toxic effects [4]. Recently it has been reported that peroxidative damage to lipids might also be involved in the cytotoxicity of acrolein [5]. Since acrolein itself apparently does not produce free radicals [5], a mechanism by which it could stimulate lipid peroxidation is reduction of protection mechanisms against lipid peroxidation, e.g. the glutathione (GSH)† concentration in the liver is drastically reduced by acrolein [5, 6]. There are also other components in the defense systems against lipid peroxidation which might be impaired by acrolein, since they contain essential thiol groups. Liver microsomes have been shown to be protected against lipid peroxidation by a GSH dependent system [7–9]. This protection probably proceeds via the mechanism we described previously [8]. In this mechanism vitamin E radicals in the membrane, produced by scavenging of free radicals by vitamin E, are regenerated to vitamin E by a free radical reductase (previously described as a heat labile factor) at the expense of GSH [8, 10]. We have shown that this free radical reductase contains an essential thiol group [11]. Alkylation of this thiol group, with *N*-ethyl maleimide (NEM) or 4-hydroxy-2,3-*trans*-nonenal (HNE), annihilates the catalytic capacity of this enzyme to reduce vitamin E radicals. As a consequence of this, the GSH dependent protection is diminished [11]. Since acrolein also reacts with protein and non-protein thiol groups, acrolein might also inactivate this defense mechanism against lipid peroxidation and via this indirect way stimulate this toxicological process.

Alkylation of thiol groups has, besides reducing enzyme activities, also been shown to activate some defense mechanisms. Alkylation of the thiol group of the microsomal GSH-*S*-transferase (GSH-tr) by

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† Abbreviations used: GSH, glutathione; NEM, *N*-ethyl maleimide; HNE, 4-hydroxy-2,3-*trans*-nonenal; GSH-tr, GSH-*S*-transferase; CDNB, 1 chloro-2,4-dinitrobenzene; TBA, thiobarbituric acid; PBN, phenyl-*t*-butyl nitrone.

NEM has been shown to result in an enhanced enzyme activity toward potentially toxic substrates, such as organic hydroperoxides and 1-chloro-2,4-dinitrobenzene (CDNB) [11–15].

The present study was designed to investigate in the rat possible effects of acrolein, both *in vitro* and *in vivo*, on the microsomal GSH-tr and on the GSH dependent free radical reductase.

MATERIALS AND METHODS

Chemicals. Allyl alcohol, acrolein and pyrazol were obtained from Janssen Chimica (Beerse, Belgium). Glutathione (GSH), *N*-ethyl maleimide (NEM), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA) and phenyl-*t*-butyl nitrone (PBN) were from Sigma (St. Louis, MO). The buffers used were buffer A = 50 mM sodium phosphate, 0.1 mM EDTA, pH 8.0 unless otherwise stated; buffer B = 50 mM Tris–HCl, 150 mM NaCl, pH 7.4.

Animals. In all experiments male Wistar rats (Harlan C.P.B., Zeist, The Netherlands), 220–250 g, were used. *In vivo* administration of allyl alcohol was 1.1 mmol/kg intraperitoneal (i.p.). The rats were decapitated 4 hr after the allyl alcohol injection. In some experiments pyrazole (375 mg/kg) was injected (i.p.) 1 hr prior to allyl alcohol, in order to inhibit the alcohol dehydrogenase (as in [5]).

Methods. Microsomes were prepared as described previously [8], and stored at −80°. In order to determine the microsomal GSH-*S*-transferase (GSH-tr) activity, the microsomes were thawed and diluted 5-fold with ice-cold buffer A. To remove cytosolic components, e.g. endogenous GSH, cytosolic GSH-tr and the alcohol dehydrogenase, the microsomes were washed twice with buffer A by centrifugation (40 min, 115,000 *g* at 4°. Using cytosolic Se-dependent GSH peroxidase as marker, no cytosolic contamination could be observed anymore in the washed microsomes (cf. [15]). The microsomal pellet was resuspended in buffer A, final concentration microsomes derived from 2 g liver in 1 ml buffer. Pretreatment of the microsomes was with 5 mM NEM, 5 mM acrolein or 5 mM allyl alcohol. The reactions were terminated after 30 sec by adding an equimolar amount of GSH. In specific experiments GSH was added prior to the addition of NEM or acrolein. In order to determine the microsomal GSH-tr activity the microsomes had to be diluted 200–500 times with buffer A, pH 6.0.

Microsomal GSH-tr was purified from liver microsomes of control rats, using the method of Morgenstern and DePierre [12], with minor modifications. *In vitro* preincubation of the purified enzyme with 5 mM NEM or 5 mM acrolein was the same as in the microsomes. GSH-tr activity was determined using CDNB as substrate [12].

The free radical reductase activity was determined indirectly by measuring the GSH dependent protection against 10 μM iron (II)/0.2 mM ascorbate induced lipid peroxidation [8]. Lipid peroxidation was measured using the TBA method described previously [8] and expressed as the absorbance at 535 vs 600 nm ($\Delta A_{535-600}$). Pretreatment of the washed microsomes with 0.1 mM acrolein was as described in [11]. Before use the microsomes were thawed, diluted 5-fold with ice-cold buffer A (pH 7.4) and, in order to remove cytosolic contamination, washed twice with buffer A (pH 7.4) by centrifugation (40 min, 115,000 *g* at 4°). Then the microsomal pellet was resuspended in buffer A (pH 7.4) and the microsomes (final concentration microsomes derived from $\frac{1}{8}$ g liver in 1 ml) were preincubated at 37° with 0.1 mM acrolein for 30 min. The reaction was terminated by the addition of 1 vol. of ice-cold buffer B. The microsomes were immediately centrifuged twice with buffer B (40 min, 115,000 *g* at 4°) to wash out the excess acrolein. The pellet was resuspended in buffer B. In order to stimulate lipid peroxidation, the acrolein-pretreated microsomes were incubated with 0.2 mM ascorbate and 10 μM $FeSO_4$ with or without the addition of 1 mM GSH in buffer B as described previously [8].

The spin-trapping technique in combination with ESR-measurements used for detection of *in vivo* free radical formation was according to McCay *et al.* [16] using PBN. After *in vivo* lipid peroxidation induced by CCl_4 (as in [8]) we were able to detect trapped radicals, indicating that the technique used was suitable for detecting lipid peroxidation *in vivo*. Protein determinations were made by the method of Bradford [17], using bovine serum albumine as standard.

Statistics. The data are expressed as mean ± SD, and were statistically evaluated using the Mann–Whitney U-test. $P < 0.05$ was considered to be significant.

RESULTS

(A) *Effect of acrolein on the microsomal GSH-S-transferase*

In vitro Liver microsomes are known to contain a membrane bound GSH-tr that catalyses the GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) [12]. This enzyme activity was confirmed in the present study (Table 1). Furthermore, it has been shown that alkylation of the thiol group of this enzyme with *N*-ethyl maleimide (NEM) activates the GSH-tr activity towards CDNB [11–14]. In this study, rat liver microsomes were pre-incubated for 30 sec with two different alkylators, namely NEM and acrolein. The pretreatment was terminated by the addition of an equimolar amount of GSH. Preincubation with 5 mM NEM stimulated the transferase activity toward CDNB up to 300% (Table 1). The activation was clearly pH dependent, i.e. at higher pH values the activation was more pronounced. This is consistent with the fact that a thiol group is becoming a more reactive nucleophile at higher pH values. At pH 8, increasing the incubation time or increasing the NEM concentration did not further enhance the microsomal GSH-tr activity. When GSH was added to the microsomes prior to NEM addition, the activation of the GSH-tr was no longer observed. In agreement with previous observations of Morgenstern and DePierre [12], in this study 2% Triton X-100 further enhanced the NEM stimulated GSH-tr activity in microsomes (Table 1).

Preincubation of the rat liver microsomes with 5 mM acrolein for 30 sec enhanced the microsomal

Table 1. GSH-*S*-transferase activity in microsomes from control rats

Pretreatment	GSH-transferase activity (10^{-8} mol/min/mg protein)
Basal	10 ± 1
+5 mM NEM (pH = 6.5)	16 ± 1
+5 mM NEM (pH = 7.4)	22 ± 2
+5 mM NEM	30 ± 3
+5 mM GSH + 5 mM NEM	10 ± 1
+5 mM acrolein	22 ± 2
+5 mM GSH + 5 mM acrolein	10 ± 1
+5 mM pyrazol + 5 mM acrolein	21 ± 2
+5 mM allyl alcohol	10 ± 1
+2% triton X-100 + 5 mM NEM	70 ± 3

The microsomes were pretreated as described in Materials and Methods at pH = 8, unless otherwise noted. GSH-tr activity was measured using CDNB as substrate. Results are expressed as mean ± SD (N = 3–10).

GSH-tr activity more than twofold (Table 1). Here GSH, added before acrolein, also prevented the activation of the microsomal GSH-tr. Combination of acrolein and NEM did not synergistically stimulate the GSH-tr activity, i.e. above the stimulation reached by NEM only. Addition of pyrazol, a well-known inhibitor of the cytosolic alcohol dehydrogenase, did not reduce the acrolein dependent GSH-tr activation. Allyl alcohol did not activate the GSH-tr (Table 1).

To determine whether the activation of microsomal GSH-tr activity by NEM and acrolein was caused by an effect on the enzyme itself, we used the purified microsomal enzyme. As shown in Table 2, the GSH-tr activity of the purified microsomal GSH-tr was also enhanced by NEM and acrolein, i.e. 7- and 5-fold, respectively.

In vivo. In order to investigate the effect of acrolein on the microsomal GSH-tr activity *in vivo*, rats were injected i.p. with allyl alcohol. Allyl alcohol is converted rapidly in the liver into acrolein by cytosolic alcohol dehydrogenase [1]. To verify whether the effect observed could be attributed to acrolein, the combination of pyrazol and allyl alcohol was used. Pyrazol has been shown to be effective *in vivo* in preventing the allyl alcohol induced toxicity by blocking the alcohol dehydrogenase [1, 5]. As shown in Table 3, *in vivo* administration of allyl alcohol (1.1 mmol/kg) to the rat stimulated the microsomal GSH-tr activity 2-fold. When besides allyl alcohol pyrazol (375 mg/kg) was injected too, no further activation of the microsomal GSH-tr was observed (Table 3). It was still possible to stimulate the GSH-tr activity in liver microsomes from allyl alcohol pretreated rat with NEM (Table 3).

(B) *Effect of acrolein on the GSH dependent protection against lipid peroxidation*

In vitro. To determine the effect of acrolein on the GSH dependent protection by the free radical reductase against lipid peroxidation, microsomes were preincubated for 30 min with 0.1 mM acrolein. The excess of acrolein was removed by centrifugation and washing of the microsomes. As shown in Fig. 1, incubation of liver microsomes with 10 mM Fe^{2+} and 0.2 mM ascorbate resulted in a rapid peroxidation of the polyunsaturated membrane lipids. In control microsomes, addition of 1 mM GSH protected against the rapid onset of lipid peroxidation. However, in the microsomes pretreated with acrolein the GSH dependent protection is reduced, 1 mM GSH was no longer very effective in reducing the initial rate of lipid peroxidation (Fig. 1). This is probably caused by an acrolein-mediated inactivation of the microsomal free radical reductase.

In vivo. The GSH dependent protection against iron-ascorbate induced lipid peroxidation in liver microsomes prepared from rats pretreated with allyl alcohol (1.1 mmol/kg; N = 3) did not differ from the in Fig. 1 shown GSH dependent protection in control microsomes. *In vitro* and also *in vivo* radical stress has been shown to reduce the GSH dependent

Table 2. GSH-*S*-transferase activity of the purified rat liver microsomal GSH-transferase

Pretreatment	GSH-transferase activity (10^{-6} mol/min/mg protein)
Basal	<5
+5 mM NEM	35
+5 mM GSH + 5 mM NEM	<5
+5 mM acrolein	25
+5 mM GSH + 5 mM acrolein	<5

GSH-tr activity was measured using CDNB as substrate.

Table 3. GSH-*S*-transferase activity in microsomes from rats pretreated with allyl alcohol or with the combination of allyl alcohol and pyrazol

In vitro pretreatment	GSH-transferase activity (10^{-8} mol/min/mg protein)
Microsomes from allyl alcohol pretreated rats	
Basal	19* ± 2
+5 mM NEM	35† ± 5
+5 mM acrolein	29 ± 4
Microsomes from allyl alcohol and pyrazol pretreated rats	
Basal	10‡ ± 3
+5 mM NEM	27† ± 3
+5 mM acrolein	24 ± 2

The microsomes were pretreated *in vitro* as described in Materials and Methods at pH = 8. GSH-tr activity was measured using CDNB as substrate. Results are expressed as mean ± SD (N = 3).

* Different from basal activity in control rats shown in Table 1 ($P < 0.05$).

† Not different from NEM stimulated activity in control rats shown in Table 1.

‡ Not different from basal activity in control rats shown in Table 1.

protection of liver microsomes against lipid peroxidation [8]. The fact that we did not observe a reduced GSH dependent protection after allyl alcohol administration prompted us to evaluate the contribution of lipid peroxidation to the allyl alcohol induced toxicity *in vivo*. To this end we applied the radical spin-trapping technique as described by McCay *et al.* [16] for detection of free radicals *in vivo*. When allyl alcohol was injected in rats, however, no spin adduct could be detected, while histological examination demonstrated that allyl alcohol in the dose used (1.1 mmol/kg) was hepatotoxic. If the allyl alcohol induced hepatotoxicity would be mediated by lipid peroxidation, spin adducts of lipid free radicals should have been detected [16, 18]. Furthermore no enhanced production of thiobarbituric acid (TBA) reactive material in the liver was induced by allyl alcohol administration (production of TBA reactive material, expressed as $\Delta A_{535-600}$ in liver homogenate from control *vs* allyl alcohol pretreated rats was 0.040 ± 0.009 vs 0.045 ± 0.010).

DISCUSSION

Acrolein, which is responsible for the liver necrosis of allyl alcohol [1, 5], reacts with essential thiol groups on enzymes [2, 4]. Recently it has been stated that lipid peroxidation is also involved in the acrolein mediated hepatotoxicity [5]. Since acrolein itself does not produce free radicals, it might stimulate lipid peroxidation by reducing the protection against lipid peroxidation [5].

In this study the effects of acrolein on the hepatic microsomal GSH-tr activity and on the GSH dependent protection against lipid peroxidation in liver microsomes, *in vitro* as well as *in vivo*, were determined. The GSH dependent protection against lipid peroxidation probably proceeds via a free radical reductase [8, 10], as shown in Fig. 2. Both the free radical reductase and the membrane bound GSH-tr are known to contain a thiol group. Alkylation of thiol groups in rat liver microsomes by NEM annihilates the catalytic activity of the free radical reductase [11], while the membrane bound GSH-tr activity is stimulated by NEM pretreatment [11–14].

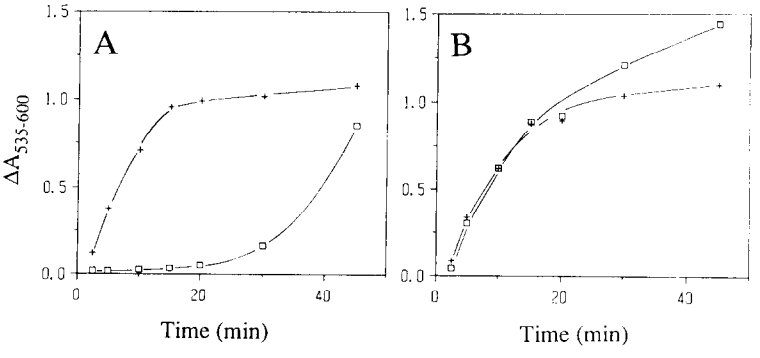

Fig. 1. Time course of lipid peroxidation in control microsomes (Panel A) and in 0.1 mM acrolein pretreated microsomes (Panel B). The incubation systems contained 0.2 mM ascorbate and 10 μM $FeSO_4$ (+) or 0.2 mM ascorbate, 10 μM $FeSO_4$ and 1 mM GSH (□). The reactions were started with the addition of $FeSO_4$. Data represent one example out of 3–10 experiments.

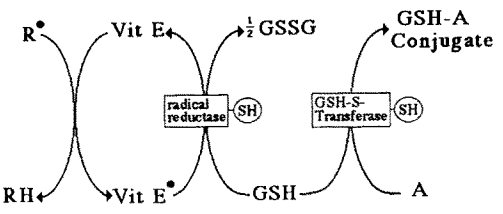

Fig. 2. Proposed mechanism for the GSH dependent protection against lipid peroxidation and for the GSH-*S*-transferase in rat liver microsomes. Free radicals (R•) in the membrane are scavenged by vitamin E. Vitamin E is regenerated by a free radical reductase at the expense of GSH. Alkylation of the thiol group(s) on the free radical reductase annihilates its catalytic activity. The GSH-*S*-transferase catalyzes the reaction between electrophilic compounds (A) and GSH. Alkylation of the thiol group on the microsomal GSH-*S*-transferase stimulates its activity.

In this study it was found that *in vitro* pretreatment of liver microsomes of the rat with acrolein stimulated the membrane bound GSH-tr. It was shown that this activation probably also proceeds via alkylation of a thiol group on the enzyme since the combination of NEM and acrolein did not additively stimulate the microsomal GSH-tr activity. The finding that acrolein also stimulated the purified enzyme, further supports the suggestion that enzyme activation via alkylation is taking place. *In vivo* administration of allyl alcohol, a well known precursor of acrolein [1, 5], to the rat also resulted in an activation of the membrane bound GSH-tr. Pyrazol administration to the rat prevented the *in vivo* activation of the microsomal GSH-tr by allyl alcohol, probably because the conversion of allyl alcohol into acrolein is inhibited.

NEM was still capable to stimulate the GSH-tr activity in microsomes from allyl alcohol pretreated rats, indicating that the activation by allyl alcohol *in vivo*, at the applied regime, is not maximal. The GSH-tr activity in liver microsomes from allyl alcohol pretreated rats after stimulation by NEM did not differ from the NEM stimulated GSH-tr activity in microsomes prepared from control animals or from allyl alcohol and pyrazol pretreated animals. This suggests that the enhanced enzyme activity after *in vivo* administration of allyl alcohol is not caused by, e.g., *de novo* synthesis of GSH-tr. The microsomal GSH-tr activity upon allyl alcohol pretreatment *in vivo* is most likely stimulated by covalent binding of acrolein to a sulfhydryl moiety on the enzyme. This suggestion is supported by the fact that *in vitro* allyl alcohol did not stimulate the GSH-tr, while acrolein did. In addition, pyrazol prevented the activation by allyl alcohol *in vivo*, while pyrazol did not prohibit the activation by acrolein *in vitro*. Pyrazol inhibits the alcohol dehydrogenase, and via this mechanism it blocks the production of acrolein from allyl alcohol.

Since compounds, of which the structure is closely related to acrolein, are detoxified by the GSH-tr [19, 20], it is tempting to suggest that acrolein accelerates its own detoxification process via the GSH-tr activation. In general, thiol reactive electrophilic compounds might activate the microsomal GSH-tr via this mechanism when it is needed, i.e. when alkylating electrophiles are to be detoxified. However, it should be noted that the GSH-tr activation probably only takes place after considerable GSH-depletion. In that case not the enzyme activity, but the GSH concentration could be rate limiting.

Apart from the stimulation of the microsomal GSH-tr, alkylation of thiol groups usually inactivates enzymes. It has been shown that the GSH-dependent protection against lipid peroxidation by the free radical reductase, as depicted in Fig. 2, is impaired after alkylation of the thiol groups on the microsomes [11]. Acrolein, a thiol reactive compound that is generally believed to be responsible for the allyl alcohol induced hepatotoxicity, is thought to be involved in the promotion of lipid peroxidation *in vivo* by allyl alcohol [5]. In this study we investigated whether the allyl alcohol induced lipid peroxidation, observed by Jaeschke *et al.* [5], might be the result of an inactivation of the free radical reductase by acrolein.

It was found that *in vitro* pretreatment of liver microsomes with acrolein reduced the GSH dependent protection against lipid peroxidation, probably because the free radical reductase is inactivated by alkylation of an essential thiol group(s) by acrolein. NEM and HNE were also able to reduce the GSH dependent protection *in vitro* [11]. The activation of the microsomal GSH-tr and the reduction of the GSH dependent protection by acrolein, support the suggestion that microsomal GSH-tr is not identical to the free radical reductase [11]. *In vivo* administration of allyl alcohol did not reduce the GSH dependent protection against lipid peroxidation, although the stimulation of the membrane bound GSH-tr demonstrated that thiol groups were alkylated *in vivo* by the administration of allyl alcohol. A possible explanation for this discrepancy is that the essential thiol group(s) located on the free radical reductase are less accessible or less reactive than the thiol group on the microsomal GSH-tr.

Jaeschke *et al.* [5] stated that *in vivo* acrolein formation *per se* causes lipid peroxidation. In the present study we did not observe any indication for the occurrence of lipid peroxidation *in vivo*. The discrepancy between the observations of Jaeschke *et al.* [5] and ours can be explained by the high mortality (50–100%) in the study by Jaeschke *et al.* [5], probably because in that study vitamin E deficient, GSH depleted mice were used, while we used control rats. The measured lipid peroxidation might be a consequence, rather than a cause of cell death [21]. Because in our experiments no lipid peroxidation or inactivation of the free radical reductase upon allyl alcohol administration was observed, we could not evaluate the importance of the free radical reductase in the protection against lipid peroxidation *in vivo*.

In summary, we have shown that acrolein both *in vitro* and *in vivo* (formed from allyl alcohol) stimulates the membrane bound GSH-tr activity in rat liver. Activation of the microsomal GSH-tr by alkylation might be an important regulatory mechanism for the activity of this protective enzyme. Only *in vitro* the GSH-dependent protection by the free radical reductase against lipid peroxidation was shown to be reduced, *in vivo* no reduction of this protection mechanism could be observed.

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