Significance of alterations in hepatic antioxidant enzymes

Primacy of glutathione peroxidase

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The relative contributions of catalase and the selenoenzyme glutathione peroxidase (GSH-Px) were elucidated in the rat liver by selectively modulating the activities of these enzymes using dietary selenium (Se) and the catalase inhibitor 3-amino-1,2,4-triazole (3-AT). Increased peroxidation occurred only in Se-deficient rats with markedly reduced cytosolic and mitochondrial GSH-Px activities. Although 3-AT treatment resulted in a 75% reduction of hepatic catalase activity and also a 20% reduction of both cytosolic and mitochondrial superoxide dismutase (SOD) activity, no incremental increase in peroxidation was observed over that associated with Se deficiency. In Se-deficient animals, treatment with 3-AT resulted in a doubling of cytosolic GSH-Px. This was associated with a 49% elevation in hepatic Se suggesting that increased Se may have contributed to the enhanced GSH-Px activity. These results suggest that GSH-Px plays the pivotal role in preventing hepatic peroxidation. Furthermore, the effects of 3-AT *in vivo* are not restricted to inhibition of catalase activity insofar as it also affects cytosolic GSH-Px activity and cytosolic and mitochondrial SOD activities.

INTRODUCTION

The discovery that oxygen-derived free radicals contribute to the pathogenesis of several disease processes and mediate chemical toxicities has contributed to a burgeoning of interest in the cellular antioxidant enzymes (Halliwell & Gutteridge, 1985). These protective enzymes include the selenoenzyme, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase. Much of the evidence implicating free radicals in oxidative tissue injury comes from studies in several organs including the liver, where exogenously added SOD, catalase, or both, prevented peroxidative injury during ischaemia and reperfusion (Jolly *et al.*, 1984; Arthur *et al.*, 1985; Itoh & Guth, 1985; Kontos, 1985; Ytrehus *et al.*, 1987). Although GSH-Px is a more efficient (lower K_m) metabolizer of H_2O_2 than catalase (Flohe, 1971), this selenoenzyme has been overlooked in these studies. Furthermore, the importance of endogenous levels of these enzymes relative to exogenously added enzymes, has not been reported.

Before the significance of intracellular levels of these antioxidant enzymes can be assessed, the relative importance of each must be clarified. Studies carried out *in vivo* designed to elucidate the role of peroxidation in cadmium cardiotoxicity have demonstrated that GSH-Px is more important than either SOD or catalase in preventing Cd-induced peroxidation (Jamall & Smith, 1985a; Jamall & Sprowls, 1987). A central role for GSH-Px in preventing tissue peroxidation is also supported by studies carried out *in vitro* (Jones *et al.*, 1981; Jones & Kennedy, 1983; Suttorp *et al.*, 1986; Raes *et al.*, 1987). In order to substantiate the greater importance of the selenoenzyme GSH-Px, relative to catalase, we examined the consequences of depletion of each of these enzyme in the rat liver *in vivo*. This was accomplished through selective modulation of these enzymes by feeding rats a low or

high Se diet with or without the catalase inhibitor, 3-amino-1,2,4-triazole (3-AT).

MATERIALS AND METHODS

Experimental animals and treatment

Male weanling Sprague–Dawley rats (Taconic Farms, New York) weighing between 40 and 50 g on receipt were randomly assigned to one of four treatment groups (five rats per group). The animals were individually housed in polypropylene cages with wire bottoms in our American Association for the Accreditation of Animal Care-accredited animal facilities ($22 \pm 1^\circ C$, $45 \pm 5\%$ relative humidity, 12 h light/dark cycle). All rats were given a torula yeast-based feed low in Se (less than 0.02 p.p.m. Se by analysis), purchased pre-mixed from Dyets (Pennsylvania). This feed consisted of torula yeast 30%, sucrose 30%, corn starch 29%, tocopherol-stripped corn oil 6%, Bernhard-Tomarelli salt mix (without added zinc and copper), 4% and 1% of the AIN⁷⁶ vitamin mix (American Institute of Nutrition, 1977). The concentration (mean $\pm$ s.d.) of copper, zinc and iron in the basal diet was 5.0 ± 1.2 p.p.m., 33.4 ± 4.65 p.p.m. and 128.0 ± 21.3 p.p.m. respectively by analyses in pentuplicate. Control animals received deionized drinking water either unsupplemented (low Se) or supplemented with 0.5 p.p.m. Se (high Se) as sodium selenite. In addition, treated animals received 0.2% (w/v) of the catalase inhibitor, 3-AT, in their drinking water. Dietary 3-AT treatment allowed us to study the effects of a sustained reduction of hepatic catalase activity. Age-matched rats fed a commercial chow (Purina no. 5001 containing 0.13 p.p.m. Se) had the following enzyme activities in their livers: cytosol, GSH-Px 131 ± 19.7 nmol of NADPH oxidized/min per mg protein, Se-

Abbreviations used: GSH-Px, glutathione peroxidase; 3-AT, 3-amino-1,2,4-triazole; SOD, superoxide dismutase activity.

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independent GSH-Px 51.4 ± 3.3 nmol of NADPH oxidized/min per mg of protein, Cu-SOD 100.6 ± 4.3 units/mg of protein; mitochondria, GSH-Px 41.4 ± 6.2 nmol of NADPH oxidized/min per mg of protein, Mn-SOD 26.8 ± 6.4 units/mg of protein.

Necropsy and tissue homogenization

At the end of 8 weeks, animals were decapitated under light ether anaesthesia. A treatment period of 8 weeks was selected because earlier studies in our laboratory have established that feeding weanling rats a low-Se diet for this length of time results in a depletion of GSH-Px to less than 10% with peroxidative tissue injury (Jamall & Smith, 1985a; Jamall & Sprowls, 1987). In addition, dietary 3-AT treatment allowed us to study the effects of a sustained reduction of hepatic catalase activity. The liver was excised, weighed, blotted dry and minced using stainless-steel scissors. A 10% (w/v) homogenate in 0.25 M-sucrose was prepared using a motor-driven Teflon pestle. Cytosolic and mitochondrial fractions were obtained by differentiated centrifugation according to Murfitt *et al.* (1978).

Peroxidation

Peroxidation was quantified in 10% (w/v) tissue homogenates using thiobarbituric acid according to the method of Ohkawa *et al.* (1979) as modified by Jamall & Smith (1985a). Tissue blanks were prepared for each sample by substituting the thiobarbituric acid solution for 0.25 M-sucrose or deionized water. Values are reported as thiobarbituric acid-reactive substances and expressed as $10^3 \times A_{532}$ /mg of tissue wet wt. These values were not standardized against malondialdehyde. Since this substance only partially represents the composition of thiobarbituric acid-reactive substances (Sevanian & Hochstein, 1985; Kako, 1987), reporting data as malondialdehyde equivalents might misrepresent the extent of peroxidation. Furthermore, the thiobarbituric acid method has recently been shown to be a more sensitive and accurate indicator of hepatic peroxidation over a wider range of tissue oxygen concentrations than the ethane/pentane technique (Reiter & Burk, 1987).

Glutathione peroxidase assay

Quantification of GSH-Px activity was done by the method of Paglia & Valentine (1967) as modified by Burk *et al.* (1980). The activity of Se-independent GSH-Px was determined by measuring total GSH-Px activity using cumene hydroperoxide (1.5 mM) as the substrate and then subtracting from this the activity for the selenoenzyme measured with H_2O_2 (0.25 mM) as the substrate. The oxidation of NADPH was followed spectrophotometrically at room temperature ($25 \pm 2^\circ C$) using a Perkin-Elmer Lambda 3A spectrophotometer equipped with a R-100A recorder. Enzyme activity is expressed as nmol of NADPH oxidized/min per mg of protein.

Catalase assay

Catalase was assayed in liver cytosolic fractions according to the method of Cohen *et al.* (1970). CAT activity was calculated from the first order rate constant $k = \log(s_0/s_t) \times 2.303/t$, where s_0 is the initial substrate concentration, s_t the final substrate concentration and the reaction time (t) is 180 seconds. CAT activity is expressed as 10^3 k/s per mg of protein.

Superoxide dismutase assay

Cytosolic and mitochondrial SOD activities were determined independently for each of the cytosolic and mitochondrial fractions according to the method of Winterbourn *et al.* (1975). The assay was modified (Jamall & Sprowls, 1987) only insofar as samples were exposed to a 22 W fluorescent light bulb for 20 min in a light-box lined with aluminium foil. Enzyme activity is expressed in units/mg of protein.

Protein determination

Protein was measured according to the dye-binding method of Bradford (1976) using Coomassie Blue. Bovine serum albumin (Sigma) was used as a standard.

Selenium assay

Total Se in diet and tissue samples was determined as described by Gasiewicz & Smith (1978). Fluorescence spectra were recorded using a Gilford Fluoro IV spectrofluorimeter. Se content of diet or tissue samples is expressed as μg of Se/g of sample wet wt. (p.p.m.).

Statistical analysis

The data obtained were analysed by the Two-Way Analysis of Variance (ANOVA) for comparison among the four treatment groups. Where interactions were found to be significant ($P < 0.05$) by Two-Way ANOVA, the Fishers least significant difference (LSD) test was used to determine significance between paired groups (Sokal & Rohlf, 1981). Coefficients of variation for the biochemical assays described here are generally less than 10% in our laboratory.

RESULTS

Peroxidation

As indicated by Table 1, enhanced peroxidation in liver homogenates is a function of Se deficiency and independent of 3-AT treatment. In the absence of 3-AT treatment, Se-deficient rats exhibited a 44% increase in thiobarbituric acid-reactive species. Se-deficient rats treated with 3-AT exhibited a 39% increase in peroxidation as compared with Se-supplemented animals.

Liver selenium levels

Se deficiency resulted in approx. 90% depletion of total liver Se levels. While not statistically significant, treatment with 3-AT produced a 49% increase in hepatic Se in Se-deficient rats. Se-supplemented animals on the other hand, treated with 3-AT, registered only a 12% increase in hepatic Se relative to untreated identically fed rats.

Cytosolic catalase

Dietary Se did not affect catalase activity. Treatment with 3-AT resulted in a 72% reduction in catalase activity in Se-deficient rats and a 77% reduction in Se-supplemented animals as compared with identically fed untreated animals.

Cytosolic glutathione peroxidase

Se deficiency resulted in a significant ($P < 0.05$) depletion of GSH-Px activity to less than 2% of the activity in Se-supplemented animals. Treatment of Se-deficient rats with 3-AT resulted in a 121% increase in

Table 1. Effects of selenium and 3-AT on peroxidation, selenium levels and cellular antioxidant enzymes in liver of rats treated for 8 weeks

All values are reported as the mean $\pm$ s.d. of five rats per group. *F* ratios and results of Two-Way ANOVA; *P < 0.05; ns, not significant. Abbreviation: TBARS, thiobarbituric acid-reactive substances.

	Dietary Se (p.p.m.)	$10^3 \times A_{532}$ [(mg of tissue wet wt.) ⁻¹]		<i>F</i> ratios		
		- 3-AT	+ 3-AT	Se effect	3-AT effect	Se-3-AT interaction
Homogenate						
TBARS	0	25.4 $\pm$ 2.7	26.5 $\pm$ 2.1	52.2*	1.5 ^{ns}	< 1.0 ^{ns}
	0.5	17.6 $\pm$ 1.2	19.1 $\pm$ 2.9			
Selenium	0	0.073 $\pm$ 0.018	0.109 $\pm$ 0.014	259.2*	1.9 ^{ns}	< 1.0 ^{ns}
	0.5	0.872 $\pm$ 0.155	0.980 $\pm$ 0.200			
Cytosol						
Catalase	0	48.4 $\pm$ 7.6	13.4 $\pm$ 2.3	< 1.0 ^{ns}	353.0*	2.1 ^{ns}
	0.5	53.0 $\pm$ 4.2	12.2 $\pm$ 1.2			
GSH-Px	0	2.4 $\pm$ 0.5	5.3 $\pm$ 0.7	3990.1*	9.0*	18.2*
	0.5	156.4 $\pm$ 13.5	139.8 $\pm$ 4.6			
Se-independent GSH-Px	0	38.0 $\pm$ 3.1	94.7 $\pm$ 3.7	16.2*	151.3*	62.5*
	0.5	48.9 $\pm$ 9.7	61.2 $\pm$ 3.0			
Cu-SOD	0	132.4 $\pm$ 5.8	99.2 $\pm$ 6.9	6.7*	35.8*	2.7 ^{ns}
	0.5	114.0 $\pm$ 15.6	95.0 $\pm$ 7.4			
Mitochondria						
GSH-Px	0	2.2 $\pm$ 0.1	3.0 $\pm$ 0.6	218.6*	< 1.0 ^{ns}	< 1.0 ^{ns}
	0.5	52.5 $\pm$ 8.5	56.6 $\pm$ 13.1			
Mn-SOD	0	30.1 $\pm$ 2.0	24.2 $\pm$ 2.6	< 1.0 ^{ns}	28.2*	1.6 ^{ns}
	0.5	32.0 $\pm$ 4.8	22.3 $\pm$ 3.0			

GSH-Px activity relative to untreated Se-deficient rats. This effect of 3-AT was statistically significant (*P* < 0.05) by the LSD test. Treatment with 3-AT had no discernible effect on GSH-Px activity in Se-supplemented animals.

Cytosolic Se-independent glutathione peroxidase

Treatment with 3-AT resulted in increased Se-independent GSH-Px activity. This activity was 149 % greater in Se-deficient rats and 25 % greater in Se-supplemented animals compared with identically fed untreated rats. These 3-AT-associated increases in Se-independent GSH-Px activity were also significant (*P* < 0.05) by the LSD test.

Cytosolic superoxide dismutase

In the absence of 3-AT treatment, Se deficiency resulted in a 16 % increase in Cu-SOD activity. Treatment with 3-AT resulted in a 25 % decrease in Cu-SOD activity in Se-deficient rats and a 17 % decrease in Se-supplemented animals.

Mitochondrial glutathione peroxidase

Se deficiency resulted in a depletion of the mitochondrial selenoenzyme activity to less than 5 % of levels in Se-supplemented animals. This activity was unaltered by 3-AT treatment.

Mitochondrial superoxide dismutase

Treatment with 3-AT resulted in reductions of 20 % and 30 % in Mn-SOD activities in Se-deficient and Se-supplemented rats respectively.

DISCUSSION

Our results show that peroxidation in the liver is a function of Se deficiency as reflected by reduced GSH-Px activities in hepatic cytosolic and mitochondrial fractions. In contrast, peroxidation is independent of catalase activity. Not only did 'normal' catalase activity fail to prevent increased peroxidation in Se-deficient animals, but also increased peroxidation was not observed when catalase activity was markedly inhibited (75 %) by 3-AT treatment. Furthermore, increased SOD activity in Se-deficient rats was also ineffective in preventing increased peroxidation associated with Se deficiency. Similar findings were obtained in the heart of these rats (I. S. Jamall & T. W. Simmons, unpublished work).

The primacy of GSH-Px compared with other cellular antioxidant enzymes, as demonstrated in the present study, substantiates previous findings in our laboratory (Jamall & Smith, 1985a; Jamall & Smith, 1986; Jamall & Sprowls, 1987) as well as in other laboratories in both *in vivo* (Doroshov *et al.*, 1980; Lal *et al.*, 1980) and *in vitro* studies (Cohen & Hochstein, 1963; Jones *et al.*, 1981; Jones & Kennedy, 1983; Suttorp *et al.*, 1986; Raes *et al.*, 1987). Empirical data from these studies can be explained by the fact that (1) the selenoenzyme, GSH-Px, has a much lower *K_m* for H₂O₂ (in the micromolar range) as compared with catalase which has a *K_m* for H₂O₂ in the millimolar range and (2) catalase is localized in peroxisomes whereas GSH-Px is present in both cytosol and mitochondria (Flohe, 1971; Flohe *et al.*, 1979). On the basis of these characteristics alone, it is reasonable to

assign a more important role to GSH-Px relative to catalase in preventing peroxidation resulting from H_2O_2 and radicals derived from H_2O_2 . Despite these fundamentals, a prominent protective role has been assigned to CAT in the cellular system, particularly in ischaemic/reperfusion injury (Garrett *et al.*, 1986; Thayer, 1986).

Like catalase, SOD has also achieved greater prominence in the literature than GSH-Px in protection against peroxidative injury. Although the end product of SOD-catalysed dismutation of a superoxide radical is H_2O_2 , little effort has been directed to determine the consequences of SOD-generated H_2O_2 , particularly where GSH-Px activity is diminished. We have demonstrated that peroxidation associated with depletion of GSH-Px in the rat heart cannot be prevented even by a doubling of cytosolic Cu-SOD (Jamall & Smith, 1985a; Jamall & Sprowls, 1987). Similarly, mitochondrial Mn-SOD did not offer protection against the peroxidation observed in the Se-deficient rat heart (Jamall & Sprowls, 1987). Although dismutation of superoxide free radicals by SOD serves as an important cellular function and reductions in both SOD and GSH-Px would be expected to have dire consequences, reduction in GSH-Px has been shown to be more deleterious than a reduction in SOD. The greater relative importance of GSH-Px over SOD can be attributed to the ability of GSH-Px to detoxify H_2O_2 formed by SOD or by spontaneous non-enzymic dismutation of the superoxide radical when SOD is compromised, whereas H_2O_2 accumulated in Se-deficient animals is elevated further by SOD, thus exacerbating oxidative tissue damage (Jamall & Smith, 1985a; Jamall & Sprowls, 1987).

The catalase inhibitor, 3-AT, has been used to investigate the importance of catalase on the assumption that it was a specific inhibitor of this enzyme (Heim *et al.*, 1956; Thayer, 1986). A caveat in the use of this inhibitor is that activities of other antioxidant enzymes are also altered. Our results demonstrate that cytosolic and mitochondrial SOD activities are reduced as a result of 3-AT treatment. Although a similar effect has also been documented in the eye (Bhuyan & Bhuyan, 1984), their explanation of SOD inactivation by increased H_2O_2 levels due to catalase inhibition does not apply to the liver.

In the liver, SOD was not reduced upon depletion of GSH-Px in Se-deficient rats where elevated H_2O_2 levels resulted in increased peroxidation. Moreover, the reduction in SOD activity in 3-AT-treated animals was not associated with elevated H_2O_2 and enhanced peroxidation.

The observations that Se-independent GSH-Px is able to utilize organic hydroperoxides *in vitro* and that this peroxidase activity increases during Se deficiency, led to the proposal that this activity might protect against peroxidation by detoxifying lipid hydroperoxides *in vivo* (Masukawa *et al.*, 1984; Tan *et al.*, 1984; Jamall & Smith, 1985b). Our data indicate that while Se deficiency and treatment with 3-AT affect hepatic Se-independent GSH-Px, there is no evidence to suggest that this activity contributes to the cellular oxidant defense machinery *in vivo*.

Treatment with 3-AT resulted in an increase in hepatic Se levels in Se-deficient rats. These animals exhibited increased cytosolic GSH-Px activity while mitochondrial activity was unchanged. This effect of 3-AT on the cytosolic selenoenzyme may simply reflect a preferential

intra-compartmental distribution of Se to the cytosol. Alternatively, synthesis of apoproteins may be affected by 3-AT. Antibodies to the selenoenzyme should be raised to test the validity of these postulates.

REFERENCES

- American Institute of Nutrition (1977) *J. Nutr.* **107**, 1340–1349
- Arthur, M. J. P., Bentley, I. S., Tanner, A. R., Saunders, K. P., Millward-Sadler, G. H. & Wright, R. (1985) *Gastroenterology* **89**, 114–123
- Bhuyan, K. C. & Bhuyan, D. K. (1984) *Curr. Eye Res.* **3**, 67–81
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Burk, R. F., Lawrence, R. A. & Lane, J. M. (1980) *J. Clin. Invest.* **65**, 1024–1031
- Cohen, G. & Hochstein, P. (1963) *Biochemistry* **2**, 1420–1428
- Cohen, G., Dembiec, D. & Marcus, J. (1970) *Anal. Biochem.* **34**, 30–38
- Doroshov, J. H., Locker, G. Y. & Myers, C. E. (1980) *J. Clin. Invest.* **65**, 128–135
- Flohe, L. (1971) *Klin. Wochenschr.* **49**, 669–683
- Flohe, L., Gunzler, W. A. & Loschen, G. (1979) in *Trace Metals in Health and Disease* (Kharasch, N., ed.), pp. 263–285, Raven Press, New York
- Garrett, J. G., Farber, N. E., Hardman, H. F. & Wartler, D. C. (1986) *Am. J. Physiol.* **250**, H372–H377
- Gasiewicz, T. A. & Smith, J. C. (1978) *Chem. Biol. Interact.* **23**, 161–183
- Halliwell, B. & Gutteridge, J. M. C. (1985) *Free Radicals in Biology and Medicine*, p. 346, Clarendon Press, Oxford
- Heim, W. G., Appleman, D. & Pyfrom, H. T. (1956) *Am. J. Physiol.* **186**, 19–23
- Itoh, M. & Guth, P. H. (1985) *Gastroenterology* **88**, 1162–1167
- Jamall, I. S. & Smith, J. C. (1985a) *Toxicol. Appl. Pharmacol.* **80**, 33–42
- Jamall, I. S. & Smith, J. C. (1985b) *Arch. Toxicol.* **58**, 102–105
- Jamall, I. S. & Smith, J. C. (1986) in *Handbook of Experimental Pharmacology* (Foulkes, E. C., ed.) vol. 80, pp. 351–361, Springer-Verlag, Berlin
- Jamall, I. S. & Sprowls, J. J. (1987) *Toxicol. Appl. Pharmacol.* **87**, 102–110
- Jolly, S. R., Kane, W. J., Bailie, M. B., Abrams, G. D. & Lucchesi, B. R. (1984) *Circ. Res.* **54**, 277–284
- Jones, D. P., Eklow, L., Thor, H. & Orrenius, S. (1981) *Biochem. Biophys. Res. Commun.* **210**, 505–516
- Jones, D. P. & Kennedy, F. G. (1983) in *Functions of Glutathione: Biochemical Physiological, Toxicological and Clinical Aspects* (Larsson, A., Orrenius, S., Holmgren, A. & Mannervik, B., eds.), pp. 109–116, Raven Press, New York
- Kako, K. J. (1987) *J. Mol. Cell. Cardiol.* **19**, 209–211
- Kontos, H. A. (1985) *Circ. Res.* **57**, 508–516
- Lal, A. K., Ansari, N. H., Awasthi, Y. C., Snyder, L. M., Fortier, N. L. & Srivastava, S. K. (1980) *J. Lab. Clin. Med.* **95**, 536–552
- Masukawa, T., Nishimura, T. & Iwata, H. (1984) *Biochem. Pharmacol.* **33**, 2635–2639
- Murfit, R. R., Stiles, J. W., Powell, W. J. & Sanadi, D. R. (1978) *J. Mol. Cell. Cardiol.* **10**, 109–123
- Ohkawa, H., Ohishi, N. & Yagi, K. (1979) *Anal. Biochem.* **95**, 351–358
- Paglia, D. E. & Valentine, W. N. (1967) *J. Lab. Clin. Med.* **70**, 158–169
- Raes, M., Michiels, C. & Remacle, J. (1987) *Free Radical Biol. Med.* **3**, 3–7
- Reiter, R. & Burk, R. F. (1987) *Biochem. Pharmacol.* **36**, 925–929
- Sevanian, A. & Hochstein, P. (1985) *Annu. Rev. Nutr.* **5**, 365–390

- Sokal, R. & Rohlf, F. J. (1981) *Biometry*, 2nd edn., W. H. Freeman & Co., San Francisco
- Suttorp, N., Toepfer, W. & Roka, L. (1986) *Am. J. Physiol.* **251**, C671–C680
- Tan, K. H., Meyer, D. J., Belin, J. & Ketterer, B. (1984) *Biochem. J.* **220**, 243–252
- Thayer, W. S. (1986) *FEBS Lett.* **202**, 137–140
- Winterbourn, C. C., Hawkins, R. E., Brian, M. & Carrell, R. W. (1975) *J. Lab. Clin. Med.* **85**, 337–341
- Ytrehus, K., Myklebust, R., Olsen, R. & Mjos, O. D. (1987) *J. Mol. Cell. Cardiol.* **19**, 379–389

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