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MEMBRANE-BOUND GLUTATHIONE PEROXIDASE-LIKE ACTIVITY IN MITOCHONDRIA

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SUMMARY

A membrane-bound glutathione peroxidase-like activity has been detected in liver and cardiac mitochondrial membrane. This enzyme activity differs from the cytosol and mitochondrial matrix selenium-dependent glutathione peroxidase in that it is membrane bound, sensitive to sonication and triton-X-100, and is unaffected by prolonged feeding of a selenium-free diet. This mitochondrial membrane-bound enzyme activity differs from the glutathione-S-transferases which exhibit glutathione peroxidase activity in that it is capable of utilizing both cumene hydroperoxide and hydrogen peroxide as substrates. Digitonin fractionation studies indicate that this enzyme is not located in either inner or outer mitochondrial membrane but rather within inter-membrane space. This newly described membrane-bound enzyme activity may play an important role in the maintenance of cardiac mitochondrial integrity in that mitochondrial matrix does not contain glutathione peroxidase.

INTRODUCTION

As part of our investigation of the role of lipid peroxidation in doxorubicin cardiac toxicity (1,2), we have re-examined the detoxification of H_2O_2 in cardiac mitochondria. Superoxide anion and H_2O_2 are byproducts of mitochondrial metabolism and their production is greatest in state 4 (3,4). Cardiac mitochondria, in fact, have been estimated to produce 0.3-0.6 nmol H_2O_2/min/mg protein (3,5,6). This production of superoxide and H_2O_2 is known to result in peroxidation of mitochondrial membrane lipids (7,8). Lipid peroxidation, in turn, affects mitochondrial function and has been implicated, for example, in "high amplitude" swelling of liver mitochondria (9,10). Both lipid peroxidation and "high amplitude" swelling are lessened by the addition of glutathione peroxidase (11). Detoxification of superoxide and hydrogen

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peroxide by mitochondria has thus been a subject of considerable interest. Mitochondria have been shown to contain superoxide dismutase (12), catalase (13), and selenium-dependent glutathione peroxidase (14-16). These enzymes are all contained in hepatic mitochondrial matrix (12,13-16). Peroxide detoxification mechanisms of cardiac mitochondria have not been as comprehensively examined, especially with regard to glutathione peroxidase activity.

In the present study, we show that cardiac mitochondria lack the selenium-dependent glutathione peroxidase previously reported in liver mitochondrial matrix. Instead, we have detected a glutathione peroxidase activity within the mitochondrial inter-membrane space with some properties which differ from both the previously described selenium-dependent glutathione peroxidase and the glutathione-S-transferases with peroxidase activity.

METHODS AND MATERIAL

CDF_1 male mice were obtained from Dublin Laboratories, Dublin, VA, and were kept on selenium-containing or selenium-free diet as described earlier (2). Cardiac and liver subcellular fractions and mitochondrial sonication and triton-X-100 treatment were done as described in legends to tables. Glutathione peroxidase activity was determined according to Lawrence and Burk (17) with some modifications as described in the legend to Table I. Protein was determined by the method of Lowry et al (18). For most of the glutathione peroxidase assays, physiologic pH was used in order to ensure that the ratio of activities in the various fractions were measured under a reasonable approximation of *in vivo* conditions. In the digitonin experiments, in order to gain greater sensitivity, the membrane-bound enzyme was assayed at its pH optimum of 8.0.

RESULTS AND DISCUSSION

Of the enzymes known to exhibit glutathione peroxidase activity, only one, the selenium-dependent glutathione peroxidase, is able to utilize both hydrogen peroxide and cumene hydroperoxide as substrates (17,19-21). The glutathione-S-transferases which exhibit glutathione peroxidase activity, on the other hand, cannot utilize hydrogen peroxide and are not affected by dietary selenium (23,24). For these reasons, the subcellular distribution of glutathione peroxidase (Table I) was measured in cardiac tissue from control animals and in selenium-deficient animals using both peroxides as substrates. The results from control mice reveal that mitochondria are the richest intracellu-

Table I. SUBCELLULAR DISTRIBUTION OF GLUTATHIONE PEROXIDASE ACTIVITY IN HEART MUSCLE WITH DIFFERENT SUBSTRATES IN CONTROL AND SELENIUM-DEFICIENT CDF_1 MICE

	CONTROL		SELENIUM-DEFICIENT	
Cell Fraction	Hydrogen Peroxide As Substrate (nmoles/min/mg protein)	Cumene Hydro-Peroxide As Substrate	Hydrogen Peroxide As Substrate (nmoles/min/mg protein)	Cumene Hydro-Peroxide As Substrate
Homogenate	5.45	7.00	5.78	9.70
Pellet	5.31	6.96	7.51	8.38
Mitochondria	10.30	9.48	11.25	10.80
Microsome	0	0	1.22	1.86
Soluble	4.33	5.11	1.28	2.85

Subcellular fractionation was done by preparing 10% heart homogenate in 250 mM sucrose, 1 mM Tris, 10 mM EDTA, pH 7.2, in a Brinkmann model PCU-2-110 Polytron 3 times for 5 sec at 5.5 settings with cooling in between cycles. After sedimentation of the nuclear fraction (700 x G for 10 min), the supernatant was centrifuged at 10,000 x G for 15 min to sediment mitochondria. The post-mitochondrial supernatant was centrifuged at 105,000 x G for 60 min to sediment microsomes. Nuclear, mitochondrial, and microsomal pellets were resuspended in the initial buffer. Glutathione peroxidase activity was determined by the decrease in absorbance at 340 nm by incubating enzyme in 100 mM Tris, 3 mM EDTA, pH 7.0, 1.2 units glutathione reductase, 0.25 mM GSH, 3.0 mM NaN_3, 0.25 mM NADPH, and 0.04 mM H_2O_2 or 0.075 mM cumene hydroperoxide in 1 ml final volume. Blank reactions with the enzyme source replaced by distilled water were subtracted from each assay. The activity is expressed as nmoles NADPH oxidized/ min/mg protein using the extinction coefficient for NADPH of 6.22×10^3 mol^{-1} cm^{-1}. Succinate-cytochrome c reductase activity and rotenone-insensitive NADH-cytochrome c reductase activity were determined as mitochondrial and microsomal marker enzymes, respectively.

lar source of glutathione peroxidase in the heart with a specific activity twice that of the homogenate (Table I, columns 1 and 2). Selenium deficiency (Table I, columns 3 and 4) did have the expected effect on the soluble fraction glutathione peroxidase, known to be selenium dependent, where activity is less than 30% of that from control mice when assayed with hydrogen peroxide (Table I, column 1). In contrast, mitochondrial glutathione peroxidase specific activity showed a slight increase with selenium deficiency. These results suggest the presence of glutathione peroxidase activity in mitochondria which is more resistant to the effects of selenium deficiency than is the

previously described cytosolic glutathione peroxidase but which is still able to utilize both cumene hydroperoxide and hydrogen peroxide as substrate.

Previous studies in liver mitochondria have reported only the existence of a selenium-dependent glutathione peroxidase located in the mitochondrial matrix (15,24). This enzyme has been liberated from liver mitochondria by sonication (14) and triton-X-100 (15,16,24), both of which disrupt the mitochondrial membrane. For this reason, the behavior of cardiac mitochondrial glutathione peroxidase was assessed to see if it paralleled that reported for the liver mitochondrial enzyme. Table II A shows that the post-sonication supernatant into which a matrix enzyme should be liberated is free of glutathione peroxidase activity. The pellet, which contains mitochondrial membrane fragments, possesses about 50% of the enzyme activity. Triton-X-100 treatment of mitochondria from control mice resulted in nearly complete loss (>90%) of glutathione peroxidase activity. Triton-X-100 treatment of mitochondria from selenium-deficient mice gave a similar drop in enzyme activity. These results suggest that cardiac mitochondria lack the matrix enzyme but have a membrane-bound enzyme which is unaffected by selenium deficiency and is sensitive to triton-X-100 but less so to sonication.

Because these results contrast so sharply with those previously reported for liver mitochondria (14-16,24) where most of the enzyme activity is in the matrix, we have subjected liver mitochondria to both sonication and triton-X-100 treatment (Table II B). Our results confirm that both sonication and triton-X-100 treatment release the matrix glutathione peroxidase from liver mitochondria as reflected by the increase in activity in the post-treatment supernatant. In contrast, little activity remains in the post-treatment supernate of liver mitochondria from selenium-deficient mice, confirming the selenium dependency of this matrix enzyme. However, some enzyme activity is also found in the membrane pellet after sonication and triton-X-100 treatment of mitochondria from control mice. Furthermore, in liver mitochondria from selenium-deficient mice, most of the matrix activity is lost but 29.3% of the

Table II. EFFECTS OF SONICATION AND TRITON-X-100 ON CARDIAC AND LIVER MITOCHONDRIAL GLUTATHIONE PEROXIDASE ACTIVITY

	SONICATION*	TRITON-X-100†
	(nmoles/min/mg protein)	
A. Heart		
Intact mitochondria	11.06	12.65
Post-treatment supernatant	0	0
Post-treatment pellet	5.63	0.99
Se-deficient intact mitochondria	-	14.41
Post-treatment supernatant	-	0.21
Post-treatment pellet	-	2.64
B. Liver		
Intact mitochondria¶	25.45	25.45
Post-treatment supernatant	74.60	61.15
Post-treatment pellet	24.55	9.76
Se-deficient intact mitochondria	-	13.99
Post-treatment supernatant	-	2.84
Post-treatment pellet	-	4.10

*Mitochondria, 2 to 6 mg/ml protein, were sonicated 10 sec x 3 by Branson Sonifier Cell Disruptor 185 with microtip, setting control output on 6 and meter reading 42-44 watts/second, and were sedimented at 105,000 x G for 60 min to separate the mitochondrial membrane pellet, which was resuspended in 250 mM sucrose, 1 mM Tris, 10 mM EDTA, pH 7.2.

†To the mitochondrial suspension, 1 to 2 mg/ml, in 250 mM sucrose, 1 mM Tris, 10 mM EDTA, pH 7.2 was added triton-X-100 in 50 mM Tris, 1.5 mM EDTA, pH 7.0 to final concentration 0.5% and incubated at 0° for 10 min. The suspension was sedimented at 105,000 x G for 60 min to separate mitochondrial membrane pellet which was resuspended in 250 mM sucrose, 1 mM Tris, 10 mM EDTA, pH 7.2.

¶10% liver homogenate in 250 mM sucrose, 1 mM Tris, 10 mM EDTA, pH 7.2 was centrifuged at 600 x G for 10 min to sediment nuclei. The post-nuclei supernatant was centrifuged at 8,500 x G for 12 min to sediment mitochondria, suspended in 250 mM sucrose, 1 mM Tris, 10 mM EDTA, pH 7.2 to 4-6 mg/ml.

original mitochondrial glutathione peroxidase activity found in the pellet persists. These results suggest that liver mitochondria may also possess a membrane-bound glutathione peroxidase as well as the previously described selenium-dependent glutathione peroxidase located in the matrix.

Digitonin treatment disrupts the outer mitochondrial membrane and thus releases the contents of the inter-membrane space and yields mitoplasts which consist of the inner mitochondrial membrane and matrix. This technique has become a standard one for localizing enzyme activity within the mitochondrial

Table III. EFFECT OF DIGITONIN TREATMENT ON CARDIAC MITOCHONDRIAL GLUTATHIONE PEROXIDASE ACTIVITY

	SPECIFIC ACTIVITY (nmoles/min/mg protein)
Intact mitochondria	82.5
Outer membrane	1.59
Inter-membrane space	26.3
Inner membrane	1.61
Matrix	1.89

Mitochondria were isolated as described in Table I.

Digitonin fractionation was carried out as previously described (25) except that buffer used was 1 mM Tris HCl, pH 7.4, with 10 mM EDTA and 250 mM sucrose, and mitochondrial protein was 11.1 mg/ml. Glutathione peroxidase activity was determined as described in legend in Table I in Tris-EDTA, pH 8.0.

membrane. Table III shows the results of such analysis applied to cardiac mitochondria. As can be seen, the enzyme activity is clearly localized to the inter-membrane space.

In conclusion, these results suggest the mitochondrial detoxification of peroxides may be more complex than previously thought; in addition to the previously identified catalase and selenium-dependent glutathione peroxidase found in the mitochondrial matrix, we have detected a glutathione peroxidase located within the mitochondrial membrane. Cardiac mitochondria are lacking in the selenium-dependent matrix enzyme, while both types of glutathione peroxidase activity are present in liver mitochondria.

It remains to be seen whether this membrane-bound glutathione peroxidase is truly selenium independent or whether it is simply dependent upon a selenium pool which turns over more slowly than the selenium pool which supports the cytosol or mitochondrial matrix enzyme. We are currently attempting to purify and characterize this enzyme further in order to answer this question.

REFERENCES

1. Myers, C.E., McGuire, W.P., Liss, R.H., Ifrim, I., Grotzinger, K., and Young, R.C. (1977) Science 197: 165-167.

2. Doroshow, J.H., Locker, G.Y., and Myers, C.E. (1980) J. Clin. Invest. 65: 128-135.
3. Boveris, A., and Chance, B. (1973) Biochem. J. 134: 707-716.
4. Loschen, G., Flohe, L., and Chance, B. (1971) FEBS Lett. 18: 261-264.
5. Boveris, A., Oshino, N., and Chance, B. (1972) Biochem. J. 128: 617-630.
6. Nohl, H., and Hegner, D. (1978) Eur. J. Biochem. 82: 563-567.
7. Flohe, L., Gunzler, W.A., and Ladenstein, R. (1976) In Glutathione: Metabolism and Function, Arias, I.M., and Jakoby, W.B. (Eds.), Raven, New York, pp. 115-135.
8. Pryor, W.A. (1973) Federation Proc. 32: 1862-1869.
9. Flohe, L., and Zimmerman, R. (1974) In Glutathione, Flohe, L., Benohr, H.C., Sies, H., Waller, H.D., and Wendel, A., Thieme, Stuttgart, pp. 245-259.
10. Hunter, F.E. Jr., Scott, A., Hoffsten, P.E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L., and Smith, E. (1964) J. Biol. Chem. 239: 604-613.
11. Neubert, D., Wojtczak, A.B., and Lehninger, A.L. (1962) Proc. Natl. Acad. Sci. USA 48: 1651-1658.
12. Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248: 3582-3592.
13. Nohl, H., and Hegner, D. (1978) FEBS Lett. 89: 126-130.
14. Green, R.C., and O'Brien, P.J. (1970) Biochim. Biophys. Acta 197: 31-39.
15. Sies, H., and Moss, K.M. (1978) Eur. J. Biochem. 84: 377-383.
16. Zakowski, J.J., and Tappel, A.L. (1978) Biochim. Biophys. Acta 526: 65-76.
17. Lawrence, R.A., and Burk, R.F. (1976) Biochem. Biophys. Res. Commun. 71: 952-958.
18. Lowry, O., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.
19. Lawrence, R.A., and Burk, R.F. (1978) J. Nutr. 108: 211-215.
20. Locker, G.Y., Doroshow, J.H., Baldinger, J.C., and Myers, C.E. (1979) Nutr. Rep. Inter. 19: 671-678.
21. Pierce, S., and Tappel, A.L. (1978) Biochim. Biophys. Acta 523: 27-36.
22. Prohaska, J.R., and Ganther, H.E. (1977) Biochem. Biophys. Res. Comm. 76: 437-445.
23. Prohaska, J.R. (1980) Biochim. Biophys. Act. 611: 87-98.
24. Lotscher, H.R., Winterhalter, K.H., Carafoli, E., and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76: 4340-4344.
25. Pederson, P.L., Greenawalt, J.W., Reynafarje, B., Hullihen, B.J., Decker, G.L., Soper, J.W., and Bustamente, E. (1978) In Methods in Cell Biology, Vol. 20, D.M. Prescott (Ed.), Academic Press, New York, pp. 411-481.