*Free Rad. Res. Comms.*, Vol. 5, No. (4-5), pp. 221-226
Reprints available directly from the publisher
Photocopying permitted by license only



# INCREASED HEPATIC LIPID PEROXIDATION WITH METHIONINE TOXICITY IN THE RAT

S.M. LYNCH and J.J. STRAIN*

*Biomedical Sciences Research Centre, University of Ulster at Jordanstown, Newtownabbey, Co. Antrim, BT37 0QB, Northern Ireland*

*(Received July 11th, 1988 in revised form August 2nd, 1988)*

Consumption of excess methionine by rats is known to cause membrane damage, liver enlargement and accumulation of iron in the spleen. In this study two groups (n = 5) of male, Wistar rats were pair-fed either a methionine supplemented (20.0 g/kg) or control (2.0 g/kg) diet for 7 weeks. Hepatic and erythrocyte copper-zinc superoxide dismutase activities were significantly reduced ($P < 0.05$ and $P < 0.001$ respectively) by methionine supplementation while the activities of catalase ($P < 0.01$ and 0.05) and glutathione peroxidase ($P < 0.05$) were significantly increased. Methionine supplementation also increased hepatic lipid peroxidation ($P < 0.01$), as measured by the level of thiobarbituric acid reactive substances, and iron ($P < 0.001$) concentrations. These changes are indicative of increased oxidative stress resulting from methionine toxicity.

KEY WORDS: Lipid peroxidation, methionine toxicity, superoxide dismutase, catalase, glutathione peroxidase, iron.

## INTRODUCTION

Methionine is regarded as the most toxic of the amino acids which are required for protein synthesis.[1] There is a marked suppression in voluntary food intake and growth retardation, while tissue damage resulting from excessive methionine consumption includes an enlarged liver and kidney, and erythrocyte membrane damage.[2,3] Chronic feeding of excess methionine also results in degenerative effects in the rat aorta.[4] It has been proposed that metabolites of the transamination[5] rather the transsulphuration[6] pathway of methionine metabolism are responsible for the resultant tissue damage. Finkelstein and Benevenga[5] have investigated the effect of methanethiol and methionine toxicity on the activities of enzymes involved in protection from peroxidative damage. They found that catalase (EC 1.11.1.6.) was the only enzyme involved in protection from peroxidative damage that was inhibited by short periods of dietary methionine supplementation or by *in vitro* incubation of these enzymes with methanethiol. No difference, however, was found in the amount of lipid peroxidation in these rats and they concluded that the inhibition of catalase did not appear to be the cause of the membrane damage observed in methionine toxicity. In the current study antioxidant enzyme activities and hepatic lipid peroxidation were measured in rats after chronic feeding of excess methionine.

Paper presented at the Satellite Meeting of the Society for Free Radical Research, held May 16th 1988 at the National Institute for Biological Standards and Control.

*To whom correspondence should be sent.

## MATERIALS AND METHODS

Groups (n = 5) of male, Wistar rats (initial body weights = 197.2 ± 1.9 g) were individually housed in stainless steel cages and pair-fed control and methionine supplemented diets with deionised water for 49 days. Both diets contained (g/kg): sucrose, 580; fibrous cellulose powder, 30; casein, 180; maize oil, 100; methionine-free rat vitamin mix,[7] 10; vitamin ADEK mix,[7] 30; $CuSO_4 \cdot 5 H_2O$-free mineral mix,[7] 50. In addition the control diet contained DL-methionine (2g/kg) and casein (18 g/kg) while the methionine supplemented diet contained DL-methionine (20 g/kg).

Animals were killed by exsanguination after which the liver was immediately removed and transferred to a beaker containing ice-cold 0.25 M sucrose buffer, pH 7.4. After rinsing in fresh buffer and weighing a portion of the liver was stored at −20°C and the remainder was used to prepare a 10% (w/v) homogenate in 0.25 M sucrose buffer, pH 7.4, which was stored in 4–5 ml aliquots at −20°C. Erythrocytes were prepared from the whole blood by centrifugation at 800 g for 10 minutes. The pellet obtained was washed with 0.9% (w/v) NaCl and recentrifuged twice before storage at −20°C. Hepatic copper-zinc and manganese superoxide dismutase (EC 1.15.1.1., CuZnSOD & MnSOD respectively) activities were determined by the method of Oberley & Spitz.[8] Samples to be assayed were dialysed overnight against 0.05 M potassium phosphate buffer, pH 7.8 after which assays of the CuZnSOD and MnSOD were performed at pH 7.8. Erythrocyte CuZnSOD activity was measured by a modification of the method of Jones & Suttle[9] in which a 50 mM carbonate buffer, pH 10.2; containing 5 mM xanthine (15 ml/1) and 5 mM iodonitrotetrazolium violet (40 ml/1), was utilized. Additionally, a reaction period of 30 minutes at room temperature was permitted before reading the absorbance of samples at 500 nm. Catalase activity was measured by the method of Aebi.[10] Selenium-dependent glutathione peroxidase (EC 1.11.1.9., GSH-Px) activity was assayed by a modification[11] of the method of Paglia and Valentine[12] using 1.5 mM $H_2O_2$ as substrate and the assay was performed in 1.0 mM $NaN_3$ to inhibit catalase activity. Hepatic lipid peroxidation was measured in liver which had been stored at −20°C as the level of thiobarbituric acid reactive substances (TBARS) using the method of Ohkawa *et al.*[13] with 1,1,3,3-tetramethoxypropane (TMP) as an external standard. Protein estimations were performed by the method of Bradford[14] using bovine serum albumin as a standard.

Hepatic iron (Fe) was determined by flame atomic absorption spectrophotometry. Samples of liver which had been stored at −20°C were dried to constant weight, digested in nitric acid at 80°C and the digest diluted with deionised water prior to analysis.

Results were analysed for statistical significance by one way analysis of variance (ANOVA) and are given as mean values ± standard error (SE).

## RESULTS

In this experiment rats were pair fed the diets but the mean growth rate of the methionine supplemented rats (final body weight = 276.7 ± 5.8 g) was significantly lower ($P < 0.05$) than controls (final body weight = 299.9 ± 5.3 g). As expected liver size was markedly increased in the methionine treated animals (4.15 ± 0.18 g/100 g body weight) compared to control animals (2.81 ± 0.06 g/100 g body weight).

TABLE I
Hepatic and erythrocyte antioxidant enzyme activities in rats fed control and methionine supplemented (Met) diets.[a]

| | Control (n = 5) | Met (n = 5) | Significance |
|---|---|---|---|
| *Liver* | | | |
| CuZnSOD | 97.5 ± 13.6 | 47.5 ± 10.4 | $P < 0.05$ |
| MnSOD | 84.5 ± 11.5 | 112.5 ± 18.0 | NS[b] |
| Catalase | 0.16 ± 0.01 | 0.23 ± 0.01 | $P < 0.01$ |
| GSH-Px | 0.61 ± 0.04 | 0.78 ± 0.04 | $P < 0.05$ |
| *Erythrocyte* | | | |
| CuZnSOD | 99.6 ± 17.8 | 44.8 ± 15.1 | $P < 0.001$ |
| Catalase | 0.33 ± 0.05 | 0.70 ± 0.08 | $P < 0.05$ |
| GSH-Px | 0.55 ± 0.07 | 1.14 ± 0.14 | $P < 0.05$ |

[a] Results given as mean ± standard error. All enzyme activities reported as Units/mg protein.
[b] NS, Not significant.

Activities of hepatic and erythrocyte antioxidant enzymes are reported in Table I. CuZnSOD activity was significantly reduced in hepatic tissue ($P < 0.05$) and erythrocytes ($P < 0.001$) of methionine fed rats. In contrast significantly increased hepatic and erythrocyte activities of both catalase ($P < 0.01$ and $P < 0.05$ respectively) and GSH-Px ($P < 0.05$) were observed with dietary methionine supplementation.

TABLE II
Hepatic malondialdehyde and total iron levels in rats fed control and methionine supplemented (Met) diets.[a]

| | Control (n = 5) | Met (n = 5) | Significance |
|---|---|---|---|
| TBARS (nmol/mg tissue) | 0.18 ± 0.01 | 0.24 ± 0.01 | $P < 0.01$ |
| Fe (ug/g dry weight) | 873.8 ± 80.1 | 1325.0 ± 48.1 | $P < 0.001$ |

[a] Results given as mean ± standard error.

There was a significant increase ($P < 0.01$) in the extent of hepatic lipid peroxidation, as assessed by TBARS levels, in the methionine supplemented rats and a highly significant increase ($P < 0.001$) in hepatic Fe concentrations was also evident in these rats compared to controls (Table II).

## DISCUSSION

Results indicate that methionine supplementation leads to increased hepatic lipid peroxidation in the rat. These findings differ from those of Finkelstein and Benevenga[5] who found no difference in the amount of lipid peroxidation, as monitored by the presence of TBARS, in the livers of rats fed diets containing as much as 30.0 g methionine/kg for up to 7 days compared to pair-fed controls. It is probable,

therefore, that longer term feeding of methionine supplemented diets results in lipid peroxidation at a level detectable by the thiobarbituric acid assay. Other differences were also evident in the livers from rats fed toxic levels of methionine in these two studies. Catalase was inhibited by methionine toxicity or methanethiol in the study reported by Finkelstein and Benevenga[5] but GSH-Px activity was enhanced. In contrast results from the present study show that the acitivities of both catalase and the seleno-enzyme GSH-Px were significantly increased in the methionine supplemented rats compared to controls. It is possible that the latter increases in antioxidant enzyme activities might be an adaptive response to increased tissue peroxide flux[15] associated with methionine metabolism.

In this study significantly lowered activities of the antioxidant enzyme CuZnSOD were found in both the livers and erythrocytes of methionine supplemented, compared to control, rats. This copper-dependent enzyme, which catalyses the removal of superoxide radical,[16] is influenced by tissue copper status as is another copper-dependent enzyme, cytochrome c oxidase (EC 1.9.3.1., CCO).[17] It is known that CCO activity is decreased in methionine toxicity[5] and by mercaptans.[18] Indeed, it has been shown[19] that dietary methionine alleviates the effects of copper toxicity in growing chicks. The possibility that methionine supplementation was exacerbating the copper status of rats which were all fed a low copper diet in the current trial is being further investigated in this laboratory. Results to date indicate that methionine supplementation significantly reduced tissue Cu status (Lynch & Strain, unpublished data). Since copper deficiency can increase lipid peroxidation,[20] it is possible that the increased lipid peroxidation observed here was due to an interaction of methionine with copper-dependent enzymes, such as CuZnSOD, resulting in impaired enzyme activities rather than the effects of methioine toxicity *per se*. Nevertheless, the markedly increased levels of hepatic iron (Table II) resulting from methionine toxicity would produce conditions of iron overload which are highly conducive to free radical formation and damage.[21] It has previously been reported that both methionine toxicity[22] and *in vitro* exposure to $H_2O_2$[23] result in increased cellular xanthine oxidase (EC 1.2.3.2.) activity, a source of oxygen free radicals.[24] If, as suggested by the significant increases in catalase and GSH-Px activities found in this study, methionine supplementation results in increased $H_2O_2$ production then this, combined with the increased hepatic iron, may lead to increased cellular oxygen free radical production and oxidant damage.

The transamination pathway of methionine catabolism[6] operates independently of the formation of S-adenosyl-2-methionine, and flux through the transamination pathway probably exceeds that through the transulphuration pathway in rats[25] but not in humans.[26] Metabolites of the transamination pathway have been proposed as mediators of the tissue damage associated with methionine toxicity.[5] There is evidence, however, that the caeruloplasmin-catalysed oxidation of a transulphuration metabolite, homocysteine, can cause endothelial cell injury due to the concomitant generation of hydrogen peroxide.[27] Increased hepatic lipid peroxidation was a feature of methione supplementation in the current study and a similar mechanism involving oxidative damage may have been responsible for the accelerated ageing effects on the aortas of rats fed excess methionine for two years.[4] Recently much interest has centred on the significance of free radical damage and antioxidant status[28] in the aetiology of disease processes including ischaemic heart disease.[29] Oxidative damage, therefore resulting from the methionine metabolite, homocysteine, may be a causal factor in the development of the atherosclerotic type lesions commonly associated with homocys-

teinuria[30] and homocyteinaemia.[31] Moreover men with mild methionine intolerance and provokable homocysteinaemia are at increased risk of coronary artery disease[32] and thus exposure to the high methionine content of the Western diet may predispose towards this disease.

The results of the present study suggest that lipid peroxidation may be an important feature of methionine toxicity. It is proposed that the lipid peroxidation is due to increased oxidative metabolites resulting from methionine metabolism and to defects in the enzymatic antioxidant defence system. Increased activities of catalase and GSH-Px failed to prevent hepatic oxidative damage. Iron overload and reduced activity of CuZnSOD without a complimentary increase in MnSOD activity may be responsible for the postulated defects in antioxidant defence.



## *Acknowledgements*

This work was financially supported by the Northern Ireland Chest, Heart and Stroke Association. The authors wish to thank Mr. K. McAdam for co-operation in animal care and Miss J.M. Carey for assistance with animal feeding.

## *References*

1. Benevenga, N.J. and Steele, R.D. *Annu. Rev. Nutr.*, **4,** 157, (1984).
2. Harper, A.E. Benevenga, N.J. and Wohlhueter, R.M. *Physiol Rev.*, **50,** 428, (1970).
3. Klavins, J.V. Kinney, T.D. and Kaufman, N. *Arch. Pathol.*, **75,** 661, (1963).
4. Fau, D. Penet, J. and Hadjiisky, P. *J. Nutr.*, **118,** 128, (1988).
5. Finkelstein, A. and Benevenga, N.J. *J. Nutr.*, **116,** 204, (1986).
6. Stipanuk, M.H. *Annu. Rev. Nutr.*, **6,** 179, (1986).
7. Lei, K.Y. Prasad, A.S. Bowersox E. and Oberleas, D. *Am. J. Physiol.*, **231,** 98, (1976).
8. Oberley, L.W. and Spitz, D.R. *Methods Enzymol.*, **105,** 457, (1984).
9. Jones, D.G. and Suttle, N.F. *Res. Vet. Sci.*, **31,** 151, (1981).
10. Aebi, H. *Methods Enzymol.*, **105,** 121, (1984).
11. Flohe, L. and Gunzler, W.A. *Methods Enzymol.*, **105,** 114, (1984).
12. Paglia, D.E. and Valentine, W.N. *J. Lab. Clin. Med.*, **70,** 158, (1967).
13. Ohkawa, H. Ohishi, N. and Yagi, K. *Anal. Biochem.*, **95,** 351, (1979).
14. Bradford, M.M. *Anal. Biochem.*, **72,** 248, (1976).
15. Corrocher, R. Casaril, M. Guidi, C. Gabarelli, G.B. Miatto, O. and De Sandre, G. *Scand. J. Gastroenterol.*, **15,** 781, (1980).
16. Fridovich, I. in *Pathology of Oxygen*, ed. A.P. Autor, (Academic Press: New York, 1982) p. 1.
17. Lynch, S.M. and Strain, J.J. *Nutr. Rep. Int.*, **37,** 1127, (1988).
18. Wilms, J. Lub, J. and Wever, R. *Biochim. Biophys. Acta*, **589,** 324, (1980).
19. Ekperigin, H.E. and Vohra, P. *J. Nutr.*, **111,** 1630, (1981).
20. Paynter, D.I. *J. Nutr.*, **110,** 437, (1980).
21. Halliwell, B. and Gutteridge, J.M.C. *Trends Biochem. Sci.*, **11,** 372, (1986).
22. Furth-Walker, D. and Amy, N.K. *J. Nutr.*, **117,** 1697, (1987).
23. Bindoli, A. Cavallini, L. Rigobello, M.P. Coassin, M. and Di Lisa, F. *Free Radical Biol. Med.*, **4,** 163, (1988).
24. McCord, J.M. *Federation Proc.*, **46,** 2402, (1987).
25. Engstrom, M.A. and Benevenga, N.J. *J. Nutr.*, **117,** 1820, (1987).
26. Gahl, W.A. Bernardini, I. Finkelstein, J.D. Tangerman, A. Martin, J.J. Blom, H.J. Mullen, K.D. and Mudd, S.H. *J. Clin. Invest.*, **81,** 390, (1988).
27. Starkebaum, G. and Harlan, J.M. *J. Clin. Invest.*, **77,** 1370, (1986).
28. Machlin, L.J. and Bendich, A. *FASEB J.*, **1,** 441, (1987).
29. Gey, K.F. *Biblthea. Nutr. Dieta.*, **37,** 53, (1986).
30. McCully, K.S. *Am. J. Pathol.*, **56,** 111, (1969).
31. Kang, S.-S. Wong, P.W.K. Cook, H.Y. Novusis, M. and Messer, J.V. *J. Clin. Invest.*, **77,** 1482, (1986).

32. Murphy-Chutorian, D.R. Wexman, M.P. Grieco, A.J. Heninger, J.A. Glassman, E. Gaull, G.E. Ng, S.K.C. Feit, F. Wexman, K. and Fox, A.C. *J. Am. Coll. Cardiol.*, **6**, 725, (1985).

Accepted by Dr. B. Halliwell