Free Rad. Res. Comms., Vol. 8, No. 2, pp. 93–99
Reprints available directly from the publisher
Photocopying permitted by license only

SPIN TRAPPING OF FREE RADICALS AND LIPID PEROXIDATION IN MICROSOMAL PREPARATIONS FROM MALIGNANT HYPERTHERMIA SUSCEPTIBLE PIGS

GARRY G. DUTHIE*, DONALD B. McPHAIL**, JOHN R. ARTHUR*, BERNARD A. GOODMAN** and PHILIP C. MORRICE*

**Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, U.K. and **Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB9 2QJ, U.K.*

(Received June 20, 1989)

Microsomes were prepared from livers of malignant hyperthermia susceptible (MHS) or resistant (MHR) pigs. On incubation with the spin trap α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), the microsomes from MHS pigs produced a characteristic electron spin resonance (ESR) signal at a greater rate than those from MHR pigs. Increased formation in the incubations of thiobarbituric acid reactive substances (TBARS) by the microsomes of the MHS pigs indicated an enhanced susceptibility to free radical-mediated lipid peroxidation. These results provide further evidence that MHS pigs have an antioxidant abnormality which may contribute to the fatal MH response. However the nature of the abnormality is unclear. The enhanced formation of unstable free radicals and indices of lipid peroxidation was not due to decreased vitamin E concentration or glutathione peroxidase activity in the microsomes. Furthermore, fatty acid profiles were similar in microsomes from MHS and MHR pigs indicating similar amounts of potential substrate for TBARS formation

KEY WORDS: Malignant hyperthermia, free radicals, electron spin resonance, lipid peroxidation, antioxidant abnormality.

INTRODUCTION

Malignant hyperthermia (MH) is a pharmacogenetic disorder which is triggered in susceptible individuals by exposure to halothane anaesthesia. The condition in swine is referred to as porcine stress syndrome (PSS) and in addition to halothane intubation, stresses associated with normal husbandry also trigger the MH response. The syndrome is characterised by hyperventilation, tachycardia, the appearance of cyanotic areas on the skin, limb rigidity and finally a rapid and fatal rise in body temperature.[1]

The primary biochemical lesion responsible for the MH syndrome is unknown. To explain the muscle rigidity that occurs during MH episodes, specific faults in the mechanisms of calcium homeostasis within skeletal muscle have been proposed but not unequivocally demonstrated.[2] MH-susceptible pigs have increased plasma concentrations of indices of lipid peroxidation[3,4] and this has lead to the suggestion[5] that the perturbations in calcium in skeletal muscle may be due to an antioxidant abnormality resulting in free radical damage to cell membranes. Rapid peroxidation of

Correspondence to G.G. Duthie, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK.

essential lipids could lead to loss of muscle membrane integrity and a rapid increase in myoplasmic calcium with subsequent triggering of skeletal muscle contraction.

The present experiments have attempted to provide further evidence for the involvement of free radicals in the MH syndrome. Electron spin resonance (ESR) spectroscopy has been used to compare the rate of formation of free radicals in incubations of liver microsomal preparations from MH-susceptible (MHS) and MH-resistant (MHR) pigs. This has been related to microsomal lipid peroxidation measured by formation of thiobarbituric acid reactive substances (TBARS). In addition we have assessed whether any changes in free radical signals and TBARS formation in microsomal incubations were reflected by differences in fatty acid compositions or vitamin E contents of the preparations.

MATERIALS AND METHODS

Animals and diets

The British Landrace pigs used in this study were from a breeding progamme selecting for MH-susceptibility and resistance over 7 generations.[6] From 10 weeks of age the pigs were given ad libitum a standard ration based on 72% barley and 17% soya extract.[7] The vitamin E and selenium contents of the diet were 10 IU/kg and 0.15 mg/kg respectively, meeting normal requirements.[8] After five weeks 8 ml of blood was withdrawn from the jugular vein of each pig into evacuated heparinised tubes (Beckton Dickinson, Cowley, Oxford, U.K.). After centrifugation (10 min, 1500 × g, 4°C), the plasma was removed and stored at −40°C. Six days later the pigs were killed by captive bolt, the liver rapidly excised and frozen in liquid nitrogen.

Experimental procedures

Microsomal suspensions were prepared[9] and suspended in 20 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid; Sigma, Poole, Dorset, U.K.), pH 7.4 at a protein concentration of 20.1 ± 1.9 mg/ml (mean ± SE, N = 10). ESR measurements were made with 450 μl of the suspensions in 5 ml "Reacti-Vials" (Pierce U.K. Ltd, Cambridge, U.K.) which also contained 3.5 ml of 20 mM HEPES buffer, pH 7.4, containing 130 mM NaCl, and 2 mM $CaCl_2$. The vials were open to the atmosphere and continually stirred at 37°C. At the start of incubations, the spin trap α-(4-pyridyl-1-oxide)-n- *tert*-butylnitrone (4-POBN, Aldrich, Gillingham, Dorset, U.K.) was added to a final concentration of 100 mM. Aliquots (0.4 ml) were removed at 30 min. intervals over 3 h and ESR spectra recorded in a flat quartz cell with a Varian E104 X-band spectrometer, operating at 9.5GHZ with 100KHZ modulation frequency, 10 mW microwave power and 0.2 mT modulation amplitude.

The formation of TBARS by the microsomal incubations was determined by a similar procedure. Microsomes (100 μl) were added to 5 ml of the HEPES buffer and incubated in a shaking water bath at 37°C. At 0, 30, 60, and 120 min aliquots of 1 ml were removed and added to 1 ml of 5% phosphotungstic acid (Aldrich). After centrifugation at 10000 × g for 10 min, TBARS were measured in the supernatant by a fluorometric method[10] using tetramethoxypropane (Aldrich) as a standard.

Vitamin E concentrations in plasma and microsomes were determined in hexane extracts by the method of Taylor *et al.*[11] Plasma TBARS concentrations, plasma

pyruvate kinase activities and microsomal selenium-dependent glutathione peroxidase activites were measured as described previously.[12] Fatty acids in lipids were determined by GLC following extraction of the microsomes and methylation.[13] Results were calculated as means ± SEMs. The Student 2-tailed t-test was used to compare group mean values. Prior to the application of the t-test, data from the time course experiments were logarithmically transformed to reduce the heterogenicity of the variances. The rates of increase in ESR signal height and TBARS formation were calculated from the approprate slopes.

RESULTS

MHS and MHR pigs had similar plasma vitamin E concentrations but cell membrane integrity was impaired in the MHS pigs as indicated by the marked increase in plasma pyruvate kinase activities ($P < 0.01$). Increased free radical-mediated lipid peroxidation was also suggested by the significantly higher ($P < 0.05$) concentrations of TBARS in plasma of MHS pigs (Table 1).

The ESR spectra obtained from the microsomal incubations consisted of a triplet of doublets representing interactions of the unpaired electron with a ^{14}N nucleus further split by interaction with a single ^{1}H (Figure 1). The isotropic hyperfine coupling constants A(^{14}N) and A(^{1}H) were 1.57 ± 0.01 and 0.26 ± 0.01 mT respectively. Signal height increased with incubation time; the rate of increase was significantly greater ($P < 0.05$) in the microsomal preparations from MHS pigs than in MHR pigs (Figure 2). Moreover, the rate of lipid peroxidation expressed as TBARS was significantly greater ($P < 0.05$) in the microsomal suspensions of the MHS pigs (Figure 3).

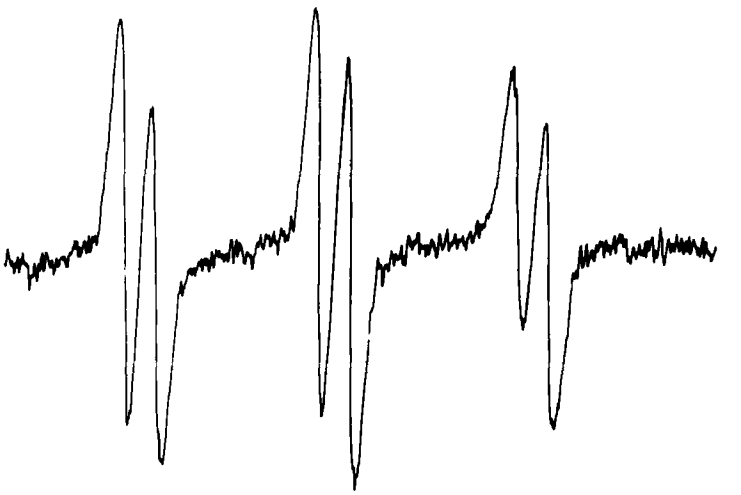

FIGURE 1 ESR spectrum obtained when liver microsomes from a malignant hyperthermia susceptible pig were incubated for 2 h at 37°C in HEPES buffer with 4-POBN. Instrumental parameters were: time constant 2 sec, modulation amplitude 2 mT, modulation frequency 100 kHz, power 10 mW, microwave frequency 9.5 GHZ and gain 5×10^4. The hyperfine splitting constants were A(^{1}H) 0.26 mT; A(^{14}N) 1.57 mT.

TABLE 1

Plasma vitamin E concentrations, pyruvate kinase (PK) activities, and thiobarbituric acid reactive substances (TBARS) and microsomal vitamin E content and glutathione peroxidase (GSHPx) activities of malignant hyperthermia susceptible (MHS) and resistant (MHR) pigs.

	MHS pigs	MHR pigs
Plasma:		
Vitamin E (μg/ml)	0.68 ± 0.11	0.78 ± 0.11
PK (mU/ml)	1167 ± 138	383 ± 90**
TBARS (nM/ml)	3.84 ± 0.79	2.18 ± 0.24*
Microsome:		
Vitamin E (ng/mg protein)	7.54 ± 1.70	7.55 ± 1.37
GSHPx (U/mg protein)	0.027 ± 0.001	0.020 ± 0.002**

Data as mean ± SE.: 5 pigs per group. *P < 0.05, **P < 0.01.

The increase in free radical signals and lipid peroxidation in the incubated microsomes from MHS pigs reflects an antioxidant abnormality which is, as yet, unidentified. Microsomal vitamin E concentrations were similar in both pig types and microsomal glutathione peroxidase activity was significantly higher (P < 0.01) in MHS pigs (Table 1). Furthermore, there were no differences in the proportions of the major polyunsaturated fatty acids between the two pig types (Table 2).

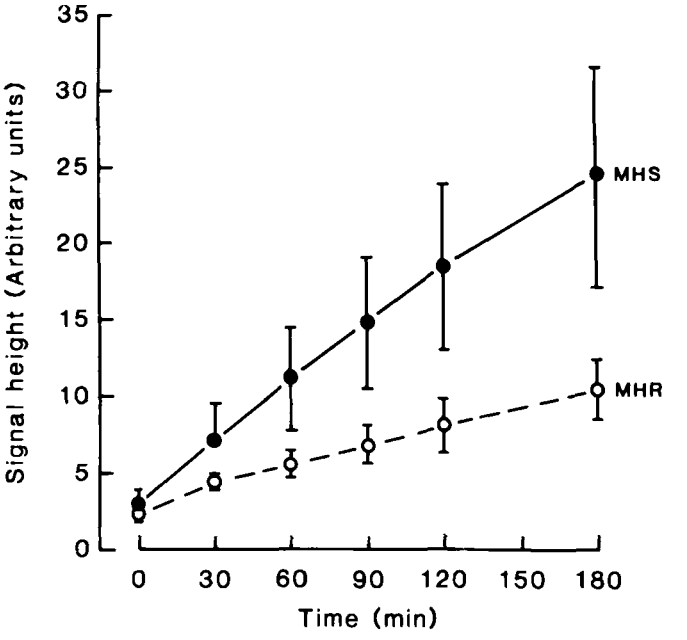

FIGURE 2 The increase of ESR signal height with time in incubations of microsomes from MHS and MHR pig liver. Values are means ± SE for 5 pigs per group. The rate of increase of signal height is significantly greater in the preparations from MHS pigs (P < 0.05). Instrumental parameters are given in Figure 1.

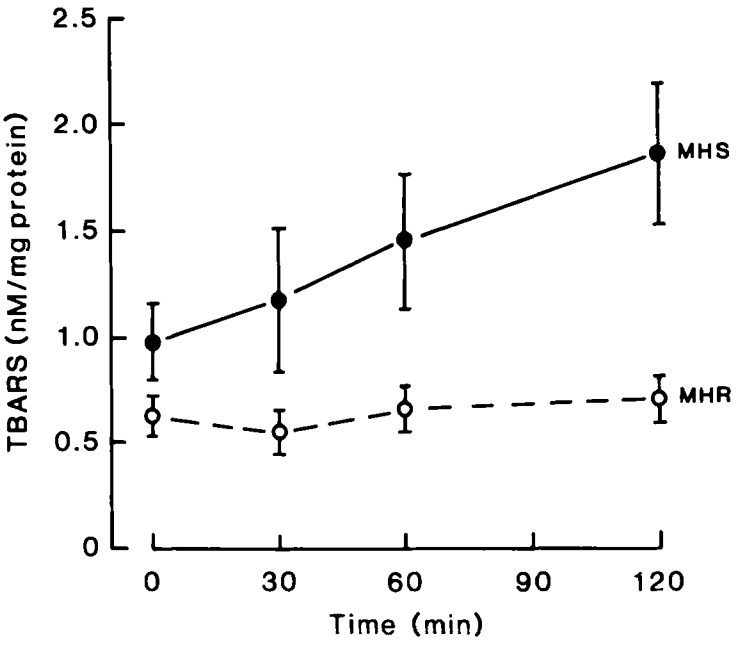

FIGURE 3 The production of TBARS by microsomal incubations from MHS and MHR pigs. Values as mean ± SE for 5 pigs per group. Microsomal preparations from MHS pigs produce TBARS at a greater rate ($P < 0.05$).

TABLE II
Fatty acid profiles of microsomes from MHS and MHR pigs.

Fatty acid	MHS	MHR
16:0	16.2 ± 1.1	14.9 ± 0.8
17:0	2.8 ± 0.6	2.5 ± 0.1
17:1	0.8 ± 0.7	1.1 ± 0.5
18:0	25.4 ± 0.9	23.8 ± 1.4
18:1	13.1 ± 0.8	12.6 ± 0.6
18:2	12.9 ± 0.2	12.0 ± 0.8
18:3	0.8 ± 0.5	2.1 ± 0.7
20:4	8.5 ± 0.5	10.2 ± 0.7
22:5	4.3 ± 0.6	4.2 ± 0.7
22:6	8.2 ± 0.7	9.0 ± 0.6
Others	0.7 ± 0.4	2.0 ± 1.3

Data as means ± SE. 5 pigs per group. Each mean value is the percentage of the total fatty acids obtained. There are no significant differences between the two pig types.

DISCUSSION

Increases in plasma activities of pyruvate kinase and in concentrations of plasma TBARS have been reported previously in MHS pigs and suggest that they have an antioxidant abnormality which causes a loss of cell membrane integrity due to peroxidation of the polyunsaturated fatty acid components of cell membranes.[14]

Additional evidence for such an abnormality includes an increased tendency of erythrocytes of MHS pigs to lyse in salt solutions[15] and to be more readily peroxidised when incubated with hydrogen peroxide.[16] Furthermore, muscle homogenates of MHS pigs produce more pentane than those of MHR pigs when incubated with Fe/ADP[14] suggesting an increased peroxidation of w-6 fatty acids.

The greater rate of formation of a free radical/4-POBN adduct by hepatic microsomal preparations from MHS pigs provides additional evidence for the presence of an antioxidant abnormality. This is likely to be associated with the membrane component of the preparations. The free radical spectra obtained in this study (Figure 1) are similar to those obtained from microsomes of vitamin E deficient rats.[17] Such free radical adducts have been ascribed to long chain fatty acid peroxyl species (LOO°).[18] However, isotopic substitution studies[19] using $^{17}O_2$ indicate that the free radical/POBN adduct (Figure 1) is not associated with the peroxy radical but may represent a carbon-centred radical such as the pentadienyl radical adduct of linoleic acid. On the basis of the hyperfine coupling constants trapping of hydroxyl (OH°) and superoxide (O_2^-) species can be excluded. Moreover, the lack of spectral anisotropy implies that the trapped radical is of relatively low molecular weight and the adduct has unrestricted spin motion.

Increased production of TBARS by the microsomes of the MHS pigs suggests that free radical activity leads to peroxidation of the polyunsaturated fatty acid components of the microsomal membrane. Paradoxically, this was not reflected by a vitamin E deficiency as plasma and microsomal vitamin E concentrations were similar in the two pig types. Moreover glutathione peroxidase activity was greater in the microsomal preparations from MHS pigs. Higher glutathione peroxidase activities have been reported in longissimus dorsi muscle from MHS pigs compared with MHR pigs and may indicate a compensatory response to a sustained oxidant stress.[14]

The greater rates of formation of free radical adduct and TBARS in preparations from MHS pigs were not due to differences between the pig types in the proportion of polyunsaturated fatty acids in the microsomal membrane providing excess substrate for peroxidation. It is possible however that there is a structural defect in the cell membrane of MHS pigs that prevents vitamin E effectively gaining access to the polyunsaturated fatty acids of the membrane with reduction in its ability to break the peroxidation chain reaction.

The results of this study suggest that MHS pigs have an impaired antioxidant defence system in liver endoplasmic reticulum. Membrane defects have also been reported in their erythrocytes, monocytes and heart tissues[20] suggesting that the increased susceptibility to free radical damage is not only confined to hepatic tissue. Deficient antioxidant capacity in skeletal muscle could lead to a rapid increase in myoplasmic Ca^{2+} which would cause the muscle rigidity characteristic of the MH response. Within 5 seconds of the initiation of Fe^{2+}-stimulated peroxidation, rapid changes occur in the permeability of membrane vesicles to Ca^{2+}.[21] Furthermore, *in vitro* Ca^{2+} concentrations as low as 0.1 μM accelerate the rate of lipid peroxidation of such vesicles.[21] Consequently, if free radical-mediated damage caused even minor leakage of Ca^{2+} it could still trigger Ca^{2+}-release channels which may be primarily responsible for the rapid Ca^{2+} efflux observed in MH.[22] The MH response is readily triggered by halogenated hydrocarbons such as halothane and chloroform which can form reactive free radicals capable of initiating lipid peroxidation.[23] Formation of such radicals *in vivo* could overwhelm an impaired antioxidant defence system and

lead to sufficient loss of membrane integrity to cause the Ca^{2+} efflux required to trigger muscle contraction.

Acknowledgements

We are grateful to Hazel Vint of the Scottish Agricultural Statistical Service for the statistical treatment of the data.

Reference

1. Rosenberg, H. *Br. J. Anaesth.*, **60**, 253–267, (1988).
2. O'Brien, P.J. *Vet. Res. Commun.*, **11**, 527–529, (1987).
3. Duthie, G.G., Arthur, J.R., Nicol, F. and Walker, M.J. *Res. Vet. Sci.*, **46**, 226–230, (1989).
4. Duthie, G.G., Arthur, J.R. and Hoppe, P.P. in Oxygen Radicals in Biology and Medicine (E. Simic, ed) Plenum, New York, pp. 605–609 (1988).
5. Duthie, G.G. and Arthur, J.R. *Ann. N.Y. Acad. Sci.*, In press (1989).
6. Simpson, S.P., Webb, A.J. and Wilmut, I. *Animal Prod.*, **32**, 485–492 (1986).
7. Livingstone, R.M. and McWilliam, R. *Br. Vet. J.*, **141**, 186–191, (1985).
8. Agricultural Research Council. The nutrient requirement of farm livestock, No. 3. A.R.C., London (1967).
9. Lim, V.S., Henriquez, C., Seo, H., Refetoff, S. and Martino, E. *J. Clin. Invest.*, **66**, 946–954, (1980).
10. Yagi, K. *Chem. Phys. Lipids*, **45**, 337–351, (1987).
11. Taylor, S.L., Lamden, M.P. and Tappel, A.L. *Lipids*, **11**, 530–538, (1976).
12. Duthie, G.G. and Arthur, J.R. *Am. J. Vet. Res.*, **48**, 309–310, (1987).
13. Arthur, J.R. *J. Nut.*, **118**, 747–755, (1988).
14. Duthie, G.G., Arthur, J.R., Nicol, F. and Walker, M.J. *Res. Vet. Sci.*, **46**, 226–230, (1989).
15. Heffron, J.J.A. and Mitchell, G. *Br. J. Anaesth.*, **53**, 499–504, (1981).
16. Duthie, G.G., Arthur, J.R., Bremner, P., Kikuchi, Y. and Nicol, F. *Am. J. Vet. Res.*, **50**, 84–87, (1988).
17. Arthur, J.R., McPhail, D.B. and Goodman, B.A. *Free Rad. Res. Commun.*, **4**, 311–315, (1988).
18. Buetler, G.R. *Free Rad. Biol. Med.*, **3**, 259–303, (1987).
19. Connor, H.D., Fischer, V. and Mason, R.P. *Biochem. Biophys. Res. Commun.*, **141**, 614–621, (1986).
20. Britt, B.A. *Can. Anaesth. Soc. J.*, **32**, 666–677, (1985).
21. Braughler, J.M. In Oxygen Radicals and Tissue Injury (B. Halliwell, ed) FASEB, Maryland, U.S.A. pp 99–104 (1988).
22. Ohnishi, S.T., Waring, A.J., Fong, S-R.G., Horvichi, K., Flick, J.L., Sandonaga, K.K. and Ohnishi, T. *Arch. Biochem. Biophys.*, **247**, 294–301, (1986).
23. Forni, L.G., Packer, J.E., Slater, T.F. and Willson, R.L. *Chemico-Biol. Int.*, **45**, 171–177, (1983).

Accepted by Prof. J.V. Bannister