MITOCHONDRIAL CALCIUM, ERYTHROCYTE FRAGILITY AND PORCINE MALIGNANT HYPERTHERMIA

K. S. CHEAH and A. M. CHEAH
Agricultural Research Council, Meat Research Institute, Langford, Bristol, BS18 7DY, England

Received 15 September 1979

1. Introduction

Two well known stress syndromes exist in certain breeds of pigs, particularly those developing leaner carcasses. The first is malignant hyperthermia which can easily be induced in stress-susceptible pigs by various agents such as halothane [1–6], a fluorinated hydrocarbon anaesthetic. The predominant clinical symptoms for this syndrome are gross muscular rigidity, rapid rise in body temperature, tachycardia, hyperventilation, severe metabolic acidosis and elevated levels of serum metabolites [7,8]. The manifestations of porcine malignant hyperthermia are similar to those described for human malignant hyperthermia, which is responsible for anaesthetic deaths in apparently healthy patients [9]. The second type of porcine stress syndrome is a post-mortem phenomenon, and is associated with the formation of PSE (pale, soft and exudative) meat. The latter condition is linked with rapid post-mortem glycolysis accompanied by denaturation of myofibrillar and sarcoplasmic proteins [10].

The genetic inheritance of porcine malignant hyperthermia has been suggested to be due either to a single autosomal dominant gene with incomplete penetrance [11], or to an autosomal recessive gene with variable penetrance [12] or to a single recessive gene with incomplete [13] or complete [4] penetrance. In spite of the well documented etiology of porcine malignant hyperthermia [7,8], the lesion responsible for the series of biochemical events leading to this syndrome is unknown. The syndrome appears to be a primary disorder of skeletal muscle [6].

This paper shows that the lesion in porcine malignant hyperthermia is most likely to be due to differences in membrane integrity, with mitochondrial Ca^{2+} playing an important role in the syndrome.

2. Materials and methods

Bovine serum albumin, rotenone, sodium succinate and murexide were obtained from Sigma Chemical Corp.; heparin from British Drug Houses; crystalline Nagarse (*Bacillus subtilis*) from Teikoku Chemical Co.; *p*-trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP) from Boehringer Mannheim; all other reagents were of analytical grade.

The halothane-sensitive and -insensitive pigs were killed at 75–100 kg and the mitochondria were isolated from M. longissimus dorsi (LD), a white skeletal muscle, immediately post-mortem using *B. subtilis* proteinase [15]. Mitochondrial Ca^{2+} efflux and endogenous mitochondrial Ca^{2+} were measured with murexide [16] using the Aminco-Chance dual-wavelength/split-beam spectrophotometer operating in the dual-wavelength mode at 540–510 nm.

Erythrocyte fragility was determined by the amount of haemoglobin released/ml red blood cells in the form of reduced pyridine haemochromogen after the cells had been subjected to osmotic shock at 0.6% NaCl at 25°C for 5 min, followed by 30 min incubation at room temperature (21°C). The released haemoglobin was separated from erythrocytes by centrifugation in a micro Eppendorf (Model 3200) centrifuge for 1 min at room temperature. The clear red supernatant was treated with an equal volume of 4.4 M pyridine in 0.2 N NaOH, reduced with 1 mg

dithionite and the spectrum of reduced pyridine haemochromogen recorded with the Aminco-Chance dual-wavelength/split-beam spectrophotometer operating in the split-beam mode. Concentration of reduced pyridine haemochromogen was calculated using the millimolar extinction of 34.7 at 556 nm for pyridine ferroprotoporphyrin IX [17]. The water-binding capacity of LD muscle was measured by improving the press method [18] by taking the ratio of muscle area to fluid area. Protein was determined with Folin–phenol reagent [19] using bovine serum albumin as standard.

3. Results and discussion

Figure 1 illustrates the rates of LD mitochondrial Ca^{2+} efflux from 58 halothane-screened pigs. The data show that halothane-sensitive pigs can be differentiated from normal (halothane-insensitive) by the rates of mitochondrial Ca^{2+} efflux. The minimum value for the rate of Ca^{2+} efflux of the halothane-sensitive pigs was 173 nmol Ca^{2+} . min^{-1} . mg $protein^{-1}$, and the maximum value for the halothane-insensitive pigs was 156 nmol Ca^{2+} . min^{-1} . mg $protein^{-1}$. A value of 165 nmol Ca^{2+} . min^{-1} . mg $protein^{-1}$ demarcates the halothane-sensitive from the halothane-insensitive pigs. In general, high values (214.5 ± 31.5 (n = 31)) in LD mitochondrial Ca^{2+} efflux rates were only observed with the halothane-sensitive pigs, and low values (119.4 ± 19.1 (n = 27)) in Ca^{2+} efflux rates with the halothane-insensitive pigs, the difference in rates being highly significant ($P < 0.001$) between the two types of pigs.

Besides having much higher Ca^{2+} efflux rates, mitochondria from LD muscle of halothane-sensitive pigs also contained significantly ($P < 0.001$) higher endogenous Ca^{2+} than those of normal. Figure 2 represents a typical spectroscopic experiment with murexide showing the discharge of endogenous Ca^{2+} by the uncoupler, FCCP [20] from LD muscle mitochondria of halothane-insensitive (trace A) and halothane-sensitive (trace B) pigs. The content of endogenous Ca^{2+} in LD mitochondria of the halothane-sensitive pigs was 84.23 ± 11.67 (n = 8) nmol Ca^{2+}/mg protein as compared with 51.04 ± 7.61 (n = 7) nmol Ca^{2+}/mg protein for the halothane-insensitive pigs.

Another striking feature in porcine malignant

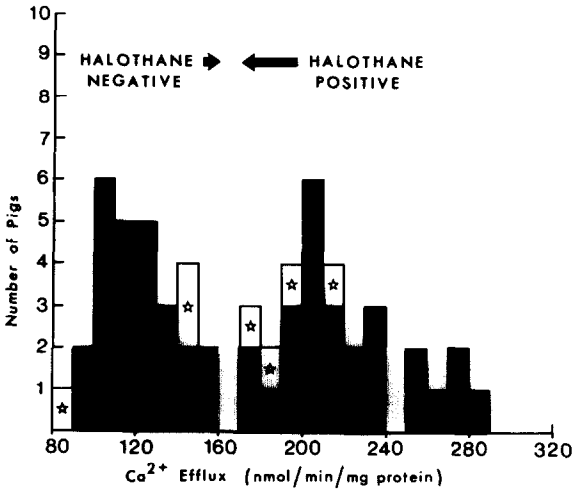

Fig.1. Relationship between rates of LD muscle mitochondrial Ca^{2+} efflux and halothane sensitivity. Prediciton of halothane sensitivity was made from the anaerobic rate of Ca^{2+} efflux from LD muscle mitochondria estimated from the fast phase of the biphasic efflux in the presence of 2.50 mM P_i [24] prior to knowledge of the results of the halothane test carried out by Dr Webb. Seven out of 58 halothane-screened pigs were 'misclassified' according to post-mortem data on mitochondrial Ca^{2+} efflux, rate of glycolysis, water-binding capacity, drip and meat quality [22]. The reaction medium (pH 7.2) contained 225 mM mannitol, 75 mM sucrose and 15 mM Tris–HCl. Murexide (92 μM) was added to the mitochondrial suspension (total vol. 2.70 ml) in a 10 mm lightpath cuvette containing rotenone (2 μM). Reaction was initiated by addition of Ca^{2+} (120–150 nmol/mg protein) and succinate (10 mM). (■) Abro pietrain-hampshire; (☆) Abro sire line.

hyperthermia is the difference in membrane stability of erythrocytes when subjected to osmotic shock in 0.60% NaCl. Erythrocytes of halothane-sensitive (●) pigs were more fragile than those of normal (▲), the amount of haemoglobin (μmol) released/ml red blood cells being 955.5 ± 546.9 (n = 9) for the halothane-sensitive (●) pigs, and 230.0 ± 101.2 (n = 13) for the halothane-insensitive (▲) pigs. Erythrocyte fragility also follows closely with the rate of Ca^{2+} efflux from LD muscle mitochondria.

Porcine malignant hyperthermia is characterized by rapid glycolysis immediately post-mortem [21]. The ultimate pH value of ~5.4 in LD muscles was attained while the muscles were still hot. Under these conditions denaturation of the sarcoplasmic and myofibrillar proteins occurred [10], accompanied by

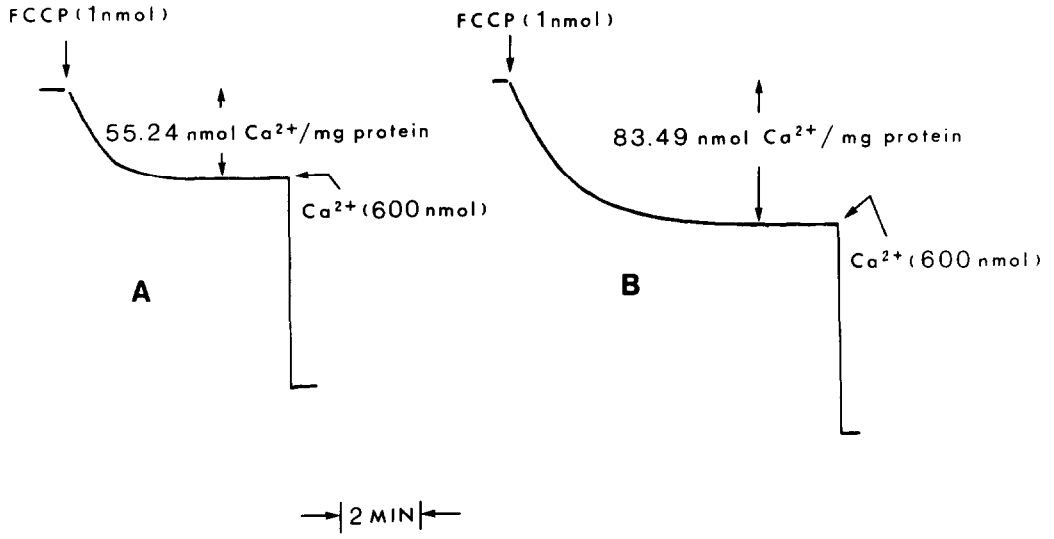

Fig.2. Measurement of endogenous Ca^{2+} in LD muscle mitochondria of halothane-insensitive and -sensitive pigs. The data represent typical spectroscopic experiments showing the discharge of endogenous Ca^{2+} from LD muscle mitochondrial of halothane-insensitive (A) and -insensitive (B) pigs by FCCP (1 μM). The reaction medium (pH 7.20) contained 225 mM mannitol, 75 mM sucrose and 15 mM Tris–HCl, and murexide (92 μM). Total protein, 4.98 mg (A) and 4.94 mg (B); total vol. 2.70 ml; tempt. 21°C.

exudation of a large amount of drip and formation of PSE meat [22]. The amount of drip exudated depends on the water-binding capacity of the muscle post-mortem, in that muscles with a lower water-binding capacity will normally produce more drip. Figure 3 illustrates the relationship between the water-binding capacity of LD muscles of halothane-sensitive (●) and -insensitive (▲) pigs, and LD muscle mitochondrial Ca^{2+} efflux. The data show good correlation between halothane sensitivity, water-binding capacity of LD muscles and the rates of LD muscle mitochondrial Ca^{2+} efflux. The values of the waterbinding capacity of LD muscles of halothane-sensitive (●) pigs were significantly different ($P < 0.01$) from those of halothane-insensitive (▲) pigs.

In [23] we reported variation in the rate of mitochondrial Ca^{2+} efflux in different breeds of pigs, and also suggested that Ca^{2+} efflux was linked with the porcine stress syndromes [22,24]. Our present studies show four outstanding features in porcine malignant hyperthermia.

1. Mitochondria isolated from white skeletal muscle (M. longissimus dorsi) of halothane-sensitive (malignant hyperthermia-prone) showed a significantly ($P < 0.001$) higher content of endogenous Ca^{2+} than those of halothane-insensitive (normal) pigs.
2. The rate of mitochondrial Ca^{2+} efflux from this particular white muscle of halothane-sensitive pigs was about twice that of normal.

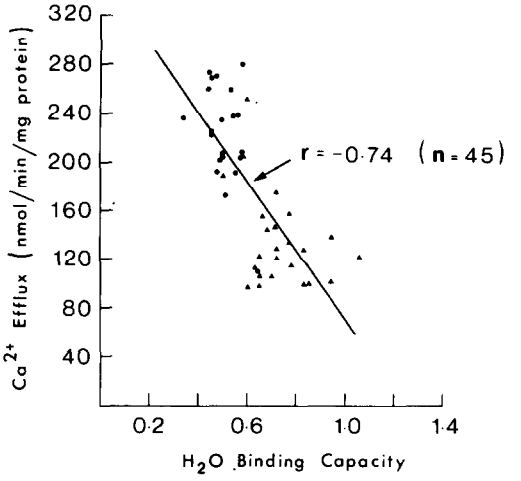

Fig.3. Relationship between the rates of LD muscle mitochondrial Ca^{2+} efflux and water-binding capacity of LD muscles in halothane-sensitive (●) and -insensitive (▲) pigs.

3. M. longissimus dorsi of halothane-sensitive pigs showed a much lower water-binding capacity post-mortem than similar muscle of normal pigs.
4. Erythrocytes of halothane-sensitive pigs were more fragile than normal when subjected to osmotic shock at 0.60% NaCl at 25°C.

Our present data show good correlation between endogenous mitochondrial Ca^{2+} content, mitochondrial Ca^{2+} efflux and erythrocyte fragility and porcine malignant hyperthermia. Evidence from these two different organelles, mitochondria and erythrocytes tends to suggest that the lesion in porcine malignant hyperthermia is probably due to a difference in membrane integrity, with mitochondrial Ca^{2+} playing a role in this syndrome.

Acknowledgements

The authors are grateful to Dr A. J. Webb, Agricultural Research Council, Animal Breeding Research Organisation, Edinburgh, for supplying the halothane-sensitive and -insensitive pigs, to Mr J. C. Casey for estimating the water-binding capacity of muscles and to Mr A. R. Crosland for preparation of erythrocytes.

References

[1] Harrison, G. G., Biebuyk, J. F., Terblanche, J., Dent, D. M., Hickman, R. and Saunders, J. J. (1968) Brit. Med. J. 3, 594–595.
[2] Allen, W. M., Berrett, S., Harding, J. D. J. and Patterson, D. S. P. (1970) Vet. Rec. 87, 64–69.
[3] Berman, M. C., Harrison, G. G., Bull, A. A. and Kench, D. D. (1970) Nature 225, 653–655.
[4] Nelson, T. E., Jones, E. W., Venable, J. H. and Kerr, D. D. (1972) Anaesthesiol. 36, 52–56.
[5] Hall, L. W., Trim, C. M. and Woolf, N. (1972) Brit. Med. J. 2, 145–148.
[6] Gronert, G. A. and Theye, R. A. (1976) Anaesthesiol. 44, 36–43.
[7] Brucker, R. F., Williams, C. H., Popinigis, J., Glavez, T. L., Vail, W. J. and Taylor, F. A. (1973) in: Int. Symp. Malignant Hyperthermia (Gordon, R. A. et al. eds) pp. 238–270, Charles C. Thomas, Springfield.
[8] Van den Hende, C., Lister, D., Muylle, E., Ooms, L. and Oyaert, W. (1976) Brit. J. Anaesth. 48, 821–829.
[9] Britt, B. A. and Kalow, W. (1970) Can. Anaesth. Soc. J. 17, 293–315.
[10] Bendall, J. R. and Wismer-Pedersen, J. (1962) J. Fd Sci. 27, 144–159.
[11] Hall, L. W., Woolf, N., Bradley, J. W. P. and Jolly, D. W. (1966) Brit. Med. J. 2, 1305.
[12] Christian, L. L. (1972) in: Proc. Pork Quality Symp. (Cassens, C. et al. and Kolb, Q. eds) pp. 91–115. University of Wisconsin Press, Madison.
[13] Ollivier, L., Sellier, P. and Monin, G. (1975) Ann. Genet. Sel. Anim. 7, 159–167.
[14] Eikelenboom, G., Minkema, D., van Eldik, P. and Sybesma, W. (1978) in: 2nd. Int. Symp Malignant Hyperthermia (Aldrete, J. A. and Britt, B. A. eds) pp. 141–146, Grune and Stratton, New York.
[15] Cheah, K. S. (1970) FEBS Lett. 10, 109–112.
[16] Mela, L. and Chance, B. (1968) Biochemistry 7, 4059–4063.
[17] Rieske, J. S. (1967) Methods Enzymol. 10, 488–493.
[18] Grau, R. and Hamm, R. (1953) Naturwiss. 40, 29–30.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[20] Vallieres, J., Scarpa, A. and Somlyo, A. P. (1975) Arch. Biochem. Biophys. 170, 659–669.
[21] Cheah, K. S. and Cheah, A. M. (1979) Experientia 35, 1001–1003.
[22] Cheah, K. S. (1978) in: Symp. Influence of Calcium Ions on Post-mortem Changes in Meat (Proc. 24th Eur. Meat Workers Meet.), Kulmbach, E2:1–E2:11.
[23] Cheah, K. S. and Cheah, A. M. (1976) J. Sci. Fd Agric. 27, 1137–1144.
[24] Cheah, K. S. and Cheah, A. M. (1978) FEBS Lett. 85, 307–310.