Investigation of malignant hyperthermia: analysis of skeletal muscle proteins from normal and halothane sensitive pigs by two dimensional gel electrophoresis

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SUMMARY Two dimensional gel analysis of skeletal muscles from normal pigs and from pigs which were homozygous for halothane sensitivity showed no obvious differences in the patterns of spots attributed to the major contractile proteins and glycolytic enzymes. In muscle from a sensitive pig which died of heat shock under anaesthesia there was a selective loss of glyceraldehyde-3-phosphate dehydrogenase and aldolase, presumably owing to proteolytic activity. The progressive loss of these enzymes under anaesthesia could contribute to the mechanism of heat production by diverting fructose 1,6 diphosphate into a futile cycle.

Malignant hyperthermia (MH) can occur in genetically predisposed human patients on administration of inhalational general anaesthetics, either alone or in combination with muscle relaxants. A condition similar to human MH also occurs in some strains of pigs. Susceptibility to halothane induced MH is also linked to abnormal sensitivity to stress (porcine stress syndrome) and to the formation of pale soft exudative meat on slaughter. The latter is the result of a state of rapid glycolysis in the skeletal muscles on slaughter which in turn causes a rapid fall in pH with consequent denaturation of myofibrillar proteins and loss of fluid.

Susceptibility to MH in human patients and pigs can be diagnosed in vitro using biopsy samples of skeletal muscle immersed in a Ca$^{2+}$ containing Ringer solution. Halothane, succinyl choline, and KCl all induce contractions in susceptible, but not in normal, muscles. On the basis of these findings it has been proposed that the primary abnormality in MH is some defect in the regulation of the free Ca$^{2+}$ ion concentration in the myoplasm. As the ultimate cause of the defect may be an abnormal protein structurally altered as a result of a gene mutation, we have conducted a search for abnormal muscle proteins by two dimensional gel electrophoresis.

Materials and methods

SOURCES OF MUSCLE SAMPLES
Two piglets homozygous for inherited halothane sensitivity were obtained from the Animal Breeding Research Organisation (ABRO) in Edinburgh. One was a Pietrain-New Hampshire cross and one a Pietrain-Large White cross. They were transported live under sedation to Cambridge and kept for one week before being killed by shooting (under licence). Muscles were also obtained from a pig which developed MH under halothane anaesthesia at the University Department of Surgery, Addenbrooke’s Hospital, Cambridge and from normal pigs, killed by shooting, supplied by the ARC Institute of Animal Physiology, Babraham, Cambridgeshire. Muscles were dissected from the animals immediately after death, cooled in ice for transport, and stored at $-70^\circ$.

Pure rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained from Sigma Chemical Company Ltd.

PREPARATION OF MUSCLE EXTRACTS
Whole muscle extracts
Samples of frozen muscle were finely pulversised in a mortar cooled in liquid nitrogen, and the powder (approximately 100 mg) was extracted with 10 times its weight of either SDS-CHES buffer (50 mmol/l cyclohexylaminoethane sulphonic acid (CHES) pH 9.5, 2% w/v sodium dodecyl sulphate (SDS), 1% w/v diithiothreitol (DTT), 10% v/v glycerol) or urea-NP-40 buffer (8 mol/l urea, 2% v/v Nonidet P-40 (NP-40), 1% v/v 5 mercaptoethanol). The mixtures were treated with 5 µl/ml of a 50 mmol/l solution of phenylmethane sulphonyl
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fluoride (PMSF) to inhibit proteases. The SDS-CHES extracts were heated rapidly to boiling; the urea extracts were not heated. Extracts were centrifuged at 6000 g for 10 minutes and the supernatants were stored at −70°C. Protein concentrations of the extracts were measured by the method of Bensadoun and Weinstein.8 For non-equilibrium pH gradient electrophoresis (NEPHGE) extracts were prepared using 9.5 mol/l urea, 2% v/v NP-40, 0.4% pH 3.5-10, 1.6% pH 5-7 Amphiolines, 5% v/v β mercaptoethanol.

**EDTA extracts**

Extracts containing mainly glycolytic enzymes and other water soluble proteins were prepared by stirring muscle powder (200 mg) with ice-cold 1 mmol/l EDTA, 1 mmol/l DTT pH 7-0 (1 ml), to which 5 µl of 50 mmol/l PMSF was added. After one hour the suspension was centrifuged at 6000 g and the supernatant was mixed with an equal weight of solid urea.

**Myosin light chain extracts**

Pulverised muscle samples (2 g) were suspended in 20 ml 40 mmol/l KCl, 20 mmol/l imidazole-Cl− pH 7-0 containing 5 µl/ml of 50 mmol/l PMSF and centrifuged at 17000 g. The supernatant was aspirated and the residue was resuspended in a further 20 ml of buffer and recentrifuged. Myosin was extracted from the washed residue by stirring for 30 minutes with 20 ml 0.3 mol/l KCl, 0.1 mol/l KH2PO4, 0.05 mol/l K2HPO4, 2 mmol/l Na2HPO4, 2 mmol/l ATP, 5 mmol/l MgCl2 in an ice bath. The suspension was centrifuged at 17000 g for 10 minutes and the supernatant was dialysed at 4°C against 14 vol of water containing 5 µl/ml of 50 mmol/l PMSF. The precipitated myosin was collected by centrifugation at 17000 g and redissolved in 7.5 ml 0.6 mol/l KCl, 10 mmol/l imidazole-Cl−. The slightly turbid solution was clarified by centrifugation and the myosin was reprecipitated by dialysis against 10 mmol/l KCl, 1 mmol/l imidazole, Cl− pH 6-5 containing PMSF. Samples of 8 to 16 mg of myosin were dissolved in 0.5 ml portions of 5 mol/l guanidinium chloride, 0.3 mol/l KCl, 0.05 mol/l Tris, Cl− pH 7-9 containing 2 mmol/l EDTA and 2 mmol/l DTT, and incubated at room temperature for 3 hours to dissociate the myosin light chains. The solution was then cooled to 4°C and an equal volume (0.5 ml) of water was added followed by 2 ml of cold 96% ethanol. After standing for 15 minutes the precipitated heavy chains were removed by centrifugation and the supernatant containing the light chains was brought to dryness by rotary evaporation. The residue was redissolved in 10 mmol/l NH4HCO3 and dialysed against 10 mmol/l NH4HCO3 to remove guanidinium chloride and salts.

**Two dimensional electrophoresis**

Two dimensional electrophoresis was performed as described by Giometti et al7 using the Pharmacia Gel Electrophoresis GE 2/4 LS.

**ISOELECTRIC FOCUSING**

Gels of dimensions 16 cm × 2.7 mm diameter for isoelectric focusing (IEF) or non-equilibrium pH gradient electrophoresis (NEPHGE) were prepared with a composition of 9.5 mol/l urea, 4% acrylamide, 0.24% bisacrylamide, 2% NP-40, 2% Ampholines pH 3.5-10. Extracts (20 to 30 µl) containing approximately 200 µg of protein for whole muscle extracts, 50 µg for EDTA extracts, or 10 µg for myosin light chains were applied and overlayed with 10 µl of 4 mol/l urea, 2% NP-40, 2% Ampholines, 5% β mercaptoethanol, followed by 10 mmol/l dithiothreitol. For IEF, the upper reservoir connected to the cathode was filled with 10 mmol/l dithiothreitol and the lower reservoir filled with 10 mmol/l H3PO4. For NEPHGE the buffers and electrode polarity were reversed. Gels were electrophoresed at 500 V for 20 hours (10 000 V/hour) for IEF and for 4 to 5 hours (2 to 2.5 V/hour) for NEPHGE. The gels were then equilibrated with 80 mmol/l Tris-Cl− pH 6-8, 20% (w/v) SDS, 10% v/v glycerol, 5% β mercaptoethanol, 0.0012% bromophenol blue for 30 minutes and stored at −70°C.

**SDS-GEL ELECTROPHORESIS**

Slab gel electrophoresis in the second dimension was performed in 18 × 14 cm gels 0.7 mm thick and the composition 20% w/v acrylamide, 0.07% bisacrylamide, 0.375 mol/l Tris-Cl− pH 8-6, 0.1% w/v SDS with a stacking gel 2 cm deep of composition 5% w/v acrylamide, 0.133% w/v bisacrylamide, 0.125 mol/l Tris-Cl− pH 6-8. After electrophoresis the gels were stained for 45 to 60 minutes in 0.2% w/v Coomassie Brilliant Blue R-250 in 45% v/v methanol, 7% acetic acid and destained in 45% v/v methanol, 7% v/v acetic acid. The gels were either photographed or tracings were made on to cellophane sheets and copied on a Xerox machine.

**Results**

Figs 1 and 2 show 2D gels of urea-NP-40 extracts of hind leg muscles from a normal pig and one of the halothane sensitive ABRO pigs. The most prominent spots were identified by comparison with the gel patterns7 as actin (A), tropomyosin (TM), various myosin light chains (LC), creatine kinase (CK), and albumin (ALB). In some gels a spot corresponding
streaks. These components were resolved much better using NEPHGE in the first dimension as discussed below.

The identity of the myosin light chains was further confirmed by extraction of the myosin and dissociation of the light chains. Fast twitch muscle contains three types of light chains: LCf-1 (Mr 25 000), LFf-2 (Mr 18 000), and LC-3 (Mr 16 000) in rabbit. 9 LC-2 exists in both phosphorylated and unphosphorylated forms of different isoelectric point (pl). In figs 1 and 2 and in the 2D gel patterns of the myosin light chain extracts there were two additional spots similar to LCf-2 but corresponding to a higher Mr which presumably represented the slow muscle form of LC-2 (Mr 19 000 in rabbit). There was also a small spot at a slightly less acidic pl than LCf-1 which was presumably the slow muscle form LCs-1. The proportion of fast and slow moving forms of LC-2 was estimated by densitometric scanning of stained one dimensional SDS gels of light chain extracts (fig 3, table 1).

The appearance of the 2D gel patterns of whole muscle extracts prepared from hind leg muscles of all four pigs were very similar both by visual comparison and by superimposition of transparent overlays. The extracts from the halothane sensitive pigs showed a faint spot marked X in fig 2 with about the same pl as the phosphorylated form of LC-2 and a slightly lower Mr than LC-3.

Figs 4 to 6 respectively show 2D gel patterns of whole muscle extracts from a normal pig, a halothane sensitive pig from ABRO, and a pig which died from MH shock under halothane anaesthesia,
using NEPHGE in the first dimension. The resolution of the more basic proteins was much improved compared to IEF gels. Additional spots were tentatively identified as enolase (EN), aldolase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GPD), and troponin-I (Tn I) by comparison with published patterns. The 2D patterns of the normal and the two ABRO pigs were very similar, including many of the minor unidentified spots. That of the pig which died from MH under anaesthesia (fig 6) was notable for the nearly complete absence of the GPD spot and a reduced intensity of the ALD spot.

The four spots designated CK, EN, ALD, and GPD were present in EDTA extracts from normal and ABRO pigs, confirming that they corresponded to water soluble proteins and not to structural myofibrillar proteins (fig 7). The identity of GPD was confirmed by coelectrophoresis of purified rabbit glyceraldehyde-3-phosphate dehydrogenase with the extract.

**TABLE 1** Proportion of ‘slow’ muscle form of LC-2 found in various muscles of normal (N) and halothane sensitive (MH) pigs.

<table>
<thead>
<tr>
<th>Pig type</th>
<th>Muscle</th>
<th>% LC-2s</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Mixed hind leg</td>
<td>33.5</td>
</tr>
<tr>
<td>MH</td>
<td>Mixed hind leg</td>
<td>24</td>
</tr>
<tr>
<td>N</td>
<td>Biceps femoris</td>
<td>20.2</td>
</tr>
<tr>
<td>MH</td>
<td>Biceps femoris</td>
<td>12.7</td>
</tr>
<tr>
<td>N</td>
<td>Vastus intermedius</td>
<td>19.6</td>
</tr>
<tr>
<td>MH</td>
<td>Vastus intermedius</td>
<td>12.6</td>
</tr>
<tr>
<td>N</td>
<td>Latissimus dorsi</td>
<td>14.6</td>
</tr>
<tr>
<td>MH</td>
<td>Longissimus dorsi</td>
<td>20</td>
</tr>
<tr>
<td>MH</td>
<td>Glutaeus maximus</td>
<td>9</td>
</tr>
</tbody>
</table>

**FIG 4** Two dimensional gel electrophoresis of a 9.5 mol/l urea extract of vastus intermedius muscle from a normal pig. Non-equilibrium pH gradient electrophoresis (NEPHGE) in the horizontal dimension, SDS electrophoresis in the vertical dimension. CK, creatine kinase; EN, enolase; ALD, aldolase; GPD, glyceraldehyde-3-phosphate dehydrogenase; Tn I, troponin I.

**FIG 5** As in fig 4 but extract from hind leg muscle of halothane sensitive pig.

**FIG 6** As in fig 4 but extract from hind leg muscle of pig which died in MH shock under halothane anaesthesia. An arrow indicates where GPD is missing.
FIG 7  Two dimensional gel as in fig 4 of an EDTA extract from normal pig muscle. This extract contains water soluble proteins only, and no structural myofibrillar proteins.

Discussion

The contractile proteins are organised into thin filaments composed of actin, tropomyosin, and troponin, and thick filaments composed of myosin. The 2D gel patterns described did not show any clear evidence for an abnormal form of any of the contractile proteins.

ACTIN

In SDS-CHES extracts actin streaked in both vertical and horizontal directions; Giometti et al attributed this to overloading. In extracts prepared with the 8 mol/l urea buffer most of the actin ran as a discrete compact spot with only a minor amount of material migrating to a more acidic pi and showing streaking in the vertical direction. Streaking was entirely absent in gels of 9.5 mol/l urea extracts in which actin ran as a single spot with no detectable amounts of the less acidic non-muscle actins.

TROPOMYOSIN

The two forms of tropomyosin α and β were present in all samples examined in approximately equal amounts, as estimated visually.

TROPONIN

Troponin consists of three components, Tn C, Tn I, and Tn T. Tn C is highly acidic and migrated out of the IEF gel in many cases. A detailed examination (to be reported elsewhere) by peptide analysis showed no differences between Tn C from normal and halothane sensitive pigs. Tn I is highly basic and could not be resolved on IEF gels but was resolved by NEPHGE. It has approximately the same apparent molecular weight as LC-1 and migrates further towards the cathode than any other protein. Tn T could not be clearly identified on the 2D gels, possibly because it is readily broken down by proteolysis in the muscle.

MYOSIN LIGHT CHAINS

All the muscle samples examined, mostly taken from hind leg muscles, appeared to be predominantly fast twitch muscles with characteristically two types of 'alkali' light chains, LC-1 and LC-3, and a lower molecular weight form of the phosphorylatable 'DTNB' light chains, LC-2. There was a variable proportion (12 to 33%) of higher molecular weight form of LC-2 (LC-2s) presumed to result from the presence of slow twitch muscles. On three occasions similar muscle preparations from normal and halothane sensitive pigs showed a lower proportion of LC-2s in the latter (table 1). More investigations are needed to make certain that these quantitative rather than qualitative differences are found regularly. In particular one would have to compare N and MH pigs of identical breed. Two components of different pIs presumed to correspond to phosphorylated and unphosphorylated forms of LC-2 were present in both normal and halothane sensitive pigs. This indicates that the abnormality is not the result of any defect in the ability of the LC-2 chains to be phosphorylated.

A minor spot marked X in fig 2 was seen in muscle extracts from halothane sensitive but not from normal pigs. This component has nearly the same Mr as LC-3 but a less acidic pi. According to the published amino-acid sequence of rabbit light chains, LC-3 has an excess of 13 negatively charged groups over positively charged groups (charge excess = -13) and LC-1 has an excess of 10 (table 2). A single amino-acid substitution in LC-3 causing a reduction of one or two units of negative charge could account for the pi of component X. Alternatively, component X might be formed by removal of a short sequence of amino-acids from the N terminus of LC-3 which would account for its slightly lower apparent Mr. Fig 8 shows the amino-acid sequence of rabbit LC-3. The N terminal amino group of Ser 1 is clocked with an acetyl group and is uncharged. Assuming pig LC-3 has a similar sequence, hydrolysis of a peptide bond between positions 1 and 4 would produce a major fragment with one unit of negative charge less,
TABLE 2  Total excess of negatively charged over positively charged groups in myosin light chains and fragments and tropontin-T fragments.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino-acid sequence</th>
<th>Negative charge excess</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-3</td>
<td>1-149</td>
<td>-13</td>
</tr>
<tr>
<td>6-149</td>
<td>-11</td>
<td></td>
</tr>
<tr>
<td>10-149</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>LC-I</td>
<td>1-1</td>
<td>-10</td>
</tr>
<tr>
<td>Tn T fragment</td>
<td>1-142</td>
<td>-12</td>
</tr>
<tr>
<td>1-143</td>
<td>-11</td>
<td></td>
</tr>
<tr>
<td>1-144</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>1-145</td>
<td>-9</td>
<td></td>
</tr>
<tr>
<td>1-146</td>
<td>-8</td>
<td></td>
</tr>
</tbody>
</table>

owing to the unmasking of the positively charged N terminal α-amino group. Hydrolysis between positions 5 and 8 would reduce the charge by two units owing to the additional loss of the β carboxyl groups of Asp 5, and similarly loss of Gln 9 would reduce the charge by three units. Component X is more likely to be an artefact than a variant form of LC-3: it was present in low and variable amounts and was not seen on 2D gels of myosin light chain extracts. Although X could be accounted for as a degradation product of LC-3 it might be derived from some unrelated protein. One possibility is that it could be a degradation product of Tn T. Rabbit Tn T, which contains 259 amino-acid residues, has a very acidic N terminal region and a basic C terminal region, and contains several sequences of two or more basic amino-acids, including a sequence of four Lys residues at positions 143–146. Troponin T is known to be very susceptible to proteolysis. Hydrolysis of a peptide bond in or just preceding the tetra-lysine sequence would give an N terminal fragment of about 145 residues, and with a high negative charge. The total charge would depend on the number of lysine residues included in the sequence (table 2). The remainder of the Tn T molecule would consist of a basic C terminal fragment of about 110 residues which might be too small or too basic to appear on the 2D gels. However, in the course of this work a basic protein of approximately the same Mr, as cytochrome-c (107 residues) was isolated from large scale preparations of troponin from both normal and halothane sensitive pigs. This protein was partly characterised from normal pigs by fingerprinting and appears to be derived from Tn T.

GLYCOLYTIC ENZYMES AND OTHER WATER SOLUBLE PROTEINS

EDTA extracts of muscles from a normal pig and two halothane sensitive pigs from ABRO were virtually identical. They showed four prominent spots designated as creatinine kinase, enolase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase, in addition to other less intense spots presumably representing other glycolytic enzymes. The gel patterns of the EDTA and whole muscle extracts of the pig which died from MH shock under halothane anaesthesia were dramatically different in showing an almost complete absence of glyceraldehyde-3-phosphate dehydrogenase and a reduction in the amount of aldolase.

As GPD was present normally in unanaesthetised halothane sensitive animals the loss of the enzyme is apparently a consequence of exposure to the anaesthetic, probably owing to the activation of a proteolytic enzyme.

One mechanism proposed for heat production in MH is a ‘futile cycle’ in which fructose 1,6 diphosphate is formed by glycolysis and hydrolysed by fructose 1,6 diphosphatase. If exposure of muscles to halothane causes a gradual loss of glyceraldehyde-3-phosphate dehydrogenase and aldolase activity then more fructose 1,6 diphosphate could be diverted into the futile cycle.

Skeletal muscles contain a protease which causes irreversible activation of phosphorylase kinase rendering it independent of Ca²⁺ activation. This enzyme had been purified to 60% homogeneity. It was thought to be identical by Dayton et al to a Ca²⁺ activated muscle protease which degrades the Z bands in vivo and shows specificity towards Tn T, Tn I, and tropomyosin. Possibly this enzyme is also responsible for the degradation of glyceraldehyde-3-phosphate dehydrogenase, thereby providing a link between the defect in Ca²⁺ regulation and the metabolic derangement causing excessive heat production.

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References

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