Alterations of inositol polyphosphates in skeletal muscle during porcine malignant hyperthermia


Summary
Malignant hyperthermia (MH) may result from increased intracellular calcium concentrations. Increased 1,4,5-IP3 concentrations could mediate this increase in Ca2+. In this study we measured inositol polyphosphates in selectively bred MH susceptible (MHS) and MH non-susceptible (MHN) swine. MH crisis was induced by halothane challenge, and dantrolene was administered in order to measure inositol polyphosphates after MH reversal. Muscle biopsies of skeletal muscles of the hind limbs were obtained in random order and inositol polyphosphates determined by high pressure liquid chromatography using a metal dye detection method. Inositol polyphosphates were determined in three groups: (1) MHS vs MHN basal, (2) during MH crisis induced by halothane and (3) following treatment with dantrolene after halothane challenge. Clinical variables and pH indicated that MH was readily induced in MHS swine. Basal concentrations of all inositol polyphosphates were higher in MHS swine compared with MHN swine. After halothane challenge, 1,3,4-IP3, 1,3,4,6-IP4 and 1,3,4,5-IP4 concentrations increased in MHS animals compared with the respective baseline values, whereas no changes in MHN animals could be detected. Dantrolene administration decreased inositol polyphosphate concentrations in MHS swine. MHN swine showed no changes in inositol polyphosphates after dantrolene. These findings indicate that inositol polyphosphates may be involved in metabolic changes after triggering and treatment of MH. (Br. J. Anaesth. 1995; 75: 467–471)

Key words

During a malignant hyperthermia (MH) crisis intracellular concentrations of calcium have been shown to be increased in skeletal muscles [1]. Calcium concentrations are regulated by two types of calcium release channels, the ryanodine receptor and the inositol 1,4,5-trisphosphate (1,4,5-IP3) receptor. Previous studies have shown that a point mutation of the ryanodine receptor gene may be involved in the development of MH [2]. In humans, the receptor gene maps to the q12-13.2 region of chromosome 19, a region close to the MH susceptibility locus [3]. However, there appears to be heterogeneity in MH susceptibility in humans [4]. Although the function of the ryanodine receptor is altered in MHS individuals, a defective receptor gene occurs in only 5–10% of individuals. Therefore, alterations in second messenger systems such as the inositol polyphosphate system [5] may underlie the modified calcium metabolism in MH. This view is supported by the finding that a wide variety of compounds can trigger an MH crisis. The phosphoinositide system could serve as a common denominator for MH triggering. Increased concentrations of the second messenger, 1,4,5-IP3, and that of other inositol phosphates, have been demonstrated in human MHS individuals, and also in MHS swine, even in the absence of a triggering agent [5, 6].

This study was designed to examine the inositol polyphosphate contents of skeletal muscle of MHS subjects not only as baseline values at rest but also during an MH crisis and after dantrolene treatment. In order to obtain a more complete view of inositol polyphosphate metabolism and to determine their pathophysiological role in MH, we determined concentrations of the second messenger, 1,4,5-IP3, and its metabolites. Because of ethical considerations concerning MH triggering and frequent muscle biopsies, all experiments were performed on genetically MHS/MHN tested swine.

Materials and methods
The studies were carried out in 3–4-month-old, 19–25-kg, German Landrace pigs, MHN and MHS, seven per group. The animals were obtained from the Institute of Animal Breeding and Behaviour (Mariensee, Germany) where they had been bred selectively for MH susceptibility or MH resistance by halothane testing of at least five generations. The genotype of MH susceptibility was determined by...
DNA analyses (PCR analyses) from ear tissue of the pigs used in this study. They were adapted to the housing conditions and personnel for at least 7 days before the experiments were performed.

**EXPERIMENTAL PROCEDURE**

All experiments were approved by the local Animal Care and Use Committee. Seven days before the experiment, polyethylene catheters were inserted via the jugular vein into the cranial vena cava under general anaesthesia with azaperone 4 mg kg$^{-1}$ i.m. (Stresnil, Janssen, Neuss, Germany) and metomidate 10 mg kg$^{-1}$ i.p. (Hypnodil, Janssen, Neuss, Germany).

The experiments were carried out on the same day in one MHS and one MHN pig. After a fasting period of 24 h the pigs were anaesthetized by azaperone 2 mg kg$^{-1}$ i.v. and metomidate 10 mg kg$^{-1}$ i.v. via the chronically implanted catheters in order to avoid excitation of the animals by handling. After tracheal intubation the lungs were ventilated with 70 % nitrous oxide in oxygen. Anaesthesia was maintained by a continuous infusion of azaperone 1.1 mg kg$^{-1}$ h$^{-1}$ and metomidate 14 mg kg$^{-1}$ h$^{-1}$ via a cannulated ear vein. Fentanyl 1.2 $\mu$g kg$^{-1}$ (Janssen, Neuss, Germany) was injected directly in order to attain optimal analgesia before starting preparation of the muscles of the hind limbs. Heat loss during the preparation was avoided by heating lamps. Drying was prevented by saline-soaked gauze. After the preparation was completed, blood samples were obtained via the venous catheter at intervals of 5 min for blood-gas analyses, pH and biochemical measurements. Arterial blood-gas analysis was performed with an ABL3 blood gas analyser (Radiometer, Copenhagen, Denmark). Potassium, lactate and aspartate aminotransferase (AST) concentrations were measured in venous blood samples using standard laboratory techniques. Creatine kinase (CK) was measured by the procedure described previously [7]. The electrocardiogram (heart rate), ventilatory frequency, end-expiratory carbon dioxide partial pressure ($2\text{CO}_2$) and rectal temperature were monitored continuously on a Supermon 7210 (Kontron Instruments, Watford, England). Furthermore, the clinical signs of MH were noted.

After a stabilization period of at least 15 min the first two muscle biopsies were sampled from both hind limbs (for measurement of control values of inositol polyphosphates). Thereafter the second period of the experiment started by triggering MH. The lungs of all pigs were ventilated with a mixture of 3 % halothane and 70 % nitrous oxide in oxygen for a period of 15 min. In MHS pigs the MH crisis, defined as pH $< 7.2$ and muscle rigor with tonic extension of the hind limbs, was observed. At this time the second two biopsies were obtained and administration of halothane and nitrous oxide was stopped. The pigs' lungs were then hyperventilated with 100 % oxygen and treated with dantrolene 3.5 mg kg$^{-1}$ i.v. (Röhm Pharma, Weiterstadt, Germany). Thereafter the third biopsies were taken. Finally biopsies were taken from the ear for DNA analyses and spontaneous breathing resumed. The pigs were killed using magnesium chloride solution (10 %).

**MUSCLE BIOPSIES**

At the end of each period—first, before MH challenge (basal period), second, after halothane administration, and third, 30 min after dantrolene treatment—muscle biopsies were excised from the muscles of the upper hind limbs. The biopsies were frozen in liquid nitrogen within 1–2 s and then stored at $-80 \degree C$. The following muscles were used in random order for measurement of inositol polyphosphates: musculus fibularis tertius and fibularis longus, musculus extensor digitorum longus. For each time point two biopsies were sampled—one from the right and one from the left hind limb.

**ANALYSIS OF INOSITOL POLYPHOSPHATES**

For a brief description of inositol polyphosphate analysis, see appendix.

**STATISTICS**

All results are presented as mean (SEM). Statistical analysis was performed using Student’s $t$ test for unpaired observations for comparisons between MHN and MHS groups. Within groups, statistical significance was assessed using Student’s $t$ test for paired observations. $P < 0.05$ was considered significant.

**Results**

**CLINICAL VARIABLES**

There were no significant differences between MHS and MHN swine before they were exposed to halothane. After exposure to halothane both groups were clearly different, with a marked increase in heart rate, $2\text{PV}_{\text{CO}_2}$ and $2\text{PV}_{\text{CO}_3}$, and a decrease in $2\text{PV}_{\text{O}_2}$ and pH in MHS swine (data not shown). MHN swine exhibited no significant changes in these variables. Rectal temperature started to increase in MHS swine after 15 min of exposure to halothane, while in MHN animals a decrease in rectal temperature was seen. After halothane withdrawal and dantrolene treatment, no significant changes occurred in the MHN group. In MHS subjects $2\text{PV}_{\text{CO}_2}$ and $2\text{PV}_{\text{CO}_3}$ reached basal concentrations 50 min after administration of dantrolene. pH increased during this period but did not reach control levels ($\text{pH} = 7.3, 60$ min after dantrolene; pH = 7.4, basal level). Heart rate and $2\text{PV}_{\text{CO}_3}$ of MHS animals did not change after administration of dantrolene. Rectal temperature stabilized at 38 $\degree C$ (MHS) and 37 $\degree C$ (MHN), respectively.

**INOSITOL POLYPHOSPHATE CONCENTRATIONS IN MHS AND MHN SWINE**

The inositol polyphosphate pathway is shown in figure 1. In MHS swine, $1,3,4$-$\text{IP}_3$, $1,3,4,6$-$\text{IP}_4$, and $1,3,4,5$-$\text{IP}_4$ increased compared with basal levels (see table 1). Concentrations of $1,4,5$-$\text{IP}_3$ and $\text{IP}_5$ after 15 min of halothane exposure (3 vol %) also increased.
Alterations of inositol polyphosphates in MH

Dantrolene reversed the halothane-induced increase in 1,3,4-IP₃, 1,4,5-IP₃, 1,3,4,6-IP₄ and 1,3,4,5-IP₄ within 50 min. All of the inositol polyphosphates studied exhibited concentrations after treatment with dantrolene which were close to values measured before the MH crisis. Treatment with halothane or dantrolene did not affect inositol polyphosphate concentrations in MHN swine (table 1). Analysis of basal inositol polyphosphate concentrations of muscle tissues in MHN and MHS swine showed significantly increased concentrations of 1,3,4-IP₃, 1,4,5-IP₃, 1,3,4,6-IP₄ and 1,3,4,5,6-IP₅ in MHS (table 1).

Discussion

The results of this study showed that 1,3,4-IP₃, 1,3,4,6-IP₄ and 1,3,4,5-IP₄ concentrations increased during an MH crisis in MHS swine. Dantrolene treatment reduced the increase to values close to basal concentrations. Furthermore, this study has confirmed previously reported data on differences in basal concentrations of inositol polyphosphates in MHN and MHS swine. These findings indicate that inositol polyphosphates are involved in metabolic changes after triggering and treatment of MH. Exposure of MHS swine to halothane caused an increase in the second messengers 1,4,5-IP₃ and 1,3,4,5-IP₄ whereas there was no effect on inositol polyphosphate concentration in MHN swine. No effect on inositol polyphosphate concentration was detected after dantrolene treatment in MHN subjects, whereas dantrolene reversed the increase in inositol polyphosphates in MHS swine to values close to control concentrations.

1,4,5-IP₃ is a second messenger that acts via calcium release from intracellular calcium stores and may also allow extracellular calcium to enter the cytoplasm (see fig. 1). Calcium release from intracellular stores by 1,4,5-IP₃ has been shown in pancreatic cells, hepatocytes and other cell systems, including smooth muscle cells [8]. The ability of 1,4,5-IP₃ to release calcium from the sarcoplasmic reticulum of skeletal muscle and heart muscle has

![Figure 1](image124x578 to 460x769.png)

Table 1: Mean (SEM) concentrations of inositol polyphosphates (pmol mg⁻¹ wet weight) measured in MHS and MHN pigs. Inositol polyphosphates were determined before administration of halothane (basal), after 15 min exposure to halothane and 50 min after discontinuation of halothane and application of dantrolene. Significant differences (P < 0.05): *basal vs halothane; †MHN vs MHS; ‡halothane vs dantrolene
not yet been resolved. However, the existence of an intracellular receptor for 1,4,5-IP₃ has been shown [9].

Previous studies showed that sarcoplasmic reticulum ryanodine receptors are involved in MH. The receptor gene maps to the q12-13.2 region of chromosome 19, next to the MH susceptibility locus in humans [3]. In humans, localization of the gene of the ryanodine receptor was shown in q13.1, a region close to the MHS locus. [10]. Recent in vitro studies failed to find structural defects in the ryanodine receptor [11]. It was suggested that a defective ryanodine receptor structure is not the primary cause of MH but altered 1,4,5-IP₃ turnover may be important [5]. Although the observed changes in inositol polyphosphate concentration appear to be minor, the method used detected total cell concentrations of inositol polyphosphates and intracellular compartmentation may provide higher effective concentrations at the subcellular level [12]. The rapid turnover of second messengers within the cell may be another factor decreasing the concentration of inositol polyphosphates [13]. Increasing cytosolic calcium concentrations induce IP₃ binding at the IP₃ receptor and so it is conceivable that even minor changes in IP₃ concentrations induce pronounced increases in cytosolic calcium via feedback mechanisms. However, alterations in inositol polyphosphate metabolism in MH may be caused by calcium-induced increase in phospholipase C activity, therefore representing an epiphenomenon rather than an underlying cause of the disease. High basal concentrations of inositol polyphosphates in MHS compared with MHN may reflect the different physical sensitivity of the muscle to stimuli.

MH is triggered by all commonly used volatile anaesthetics and by depolarizing neuromuscular blockers. Previous studies have demonstrated that increased concentrations of calcium are responsible for the clinical symptoms of MH, including muscle rigidity and increase in temperature. In MH the cytosolic calcium concentration remains elevated resulting in prolonged contraction and hypercatabolic metabolism with increased oxygen consumption and production of carbon dioxide and lactate. This is accompanied by an excessive increase in temperature. Hyperthermia is preceded by tachycardia, arrhythmia, tachyphoea and cyanosis. All MHS swine used in this study developed an MH crisis with halothane exposure, as demonstrated by the occurrence of most of these clinical signs.

Halothane has a high potency for triggering an MH crisis in MHS subjects. Moreover, halothane has been shown to increase myoplastic calcium content [14]. It appears not only to exert its actions at cell membranes but has been shown to release calcium from the endoplasmic reticulum of hepatocytes in MHS swine, possibly involving the inositol polyphosphate system [15]. Foster and colleagues found not only increased basal concentrations of 1,4,5-IP₃ in skeletal muscle of MHS swine but also that halothane increased concentrations of 1,4,5-IP₃ possibly by inhibition of an IP₃ degrading enzyme [5].

In MH dantranolol decreases muscle metabolism whereas no changes in muscle metabolism have been observed in MHN swine [16]. The decrease in muscle metabolism by dantrolene is presumably caused by effects on intracellular calcium. Increased calcium concentrations even in initial states of MH are reduced by administration of dantrolene. These findings are in agreement with the results presented here for MHS animals showing that elevated concentrations of inositol polyphosphates are reduced after dantrolene treatment of an MH crisis. In MHN subjects dantrolene did not alter the concentrations of inositol polyphosphates.

Acknowledgements

We thank Claudia Lüchau, Dr Ulrich Troll, Martina Grämer and Michael Weissing for technical support and Professor Dr I. B. Brenig (Max-Planck-Institute for Biochemistry, Martinsried, Germany) for kindly determining MH susceptibility by DNA analyses.

Appendix

Inositol polyphosphates were measured by HPLC metal dye detection involving competition of inositol polyphosphates with a cation specific dye (PAR) for a tervalent transition metal, cation (yttrium ion). The samples were prepared in three steps by HClO₄ extraction, charcoal treatment, and solid phase extraction, as described previously [17]. Briefly, the tissue was powdered in a liquid nitrogen cooled dismembranator (Braun Melsungen, Germany), extracted with HClO₄, and centrifuged (9000 × g, 10 min). The supernatant was adjusted to a pH of 5 with KOH; KClO₄ precipitate was removed by another centrifugation and the frozen samples were then freeze-dried (lyophilized) and redissolved for charcoal treatment. Suspension of acid treated Norit A was added. Solutions were vortexed and centrifuged. The supernatants were then re-treated with charcoal. The two charcoal pellets were re-extracted by addition of NaCl 0.1 mol litre⁻¹ and the supernatant combined with the sample. After solid phase extraction by a Q-Sepharose column (chloride form), the samples were washed with HCl 2 mmol litre⁻¹ and inositol polyphosphates eluted by addition of HCl 0.54 mol litre⁻¹. The eluate was frozen, freeze-dried and redissolved for HPLC analysis. HPLC (Pharma- macia) was equipped with two 2248 HPLC pumps, a 2252 LC controller, a 2151 UV detector (254 nm), a 2157 autosampler and two Mono Q columns 0.5 × 5 and 0.5 × 120 cm. Absorbance signals from both detectors were integrated by a two-channel integrator (Nelson chromatography system). The gradient was mixed at a flow rate of 1 ml min⁻¹ using eluents A (HCl 0.2 mmol litre⁻¹ plus 14 YCl₃ 0.5 mmol litre⁻¹) and B (HCl 0.5 mol litre⁻¹ plus YCl₃ 14 mmol litre⁻¹). Post-column dye solution had a flow rate of 0.5 ml min⁻¹ and contained PAR 300 µmol litre⁻¹ plus triethano- lamine 1.6 mol litre⁻¹, adjusted to pH 9.0 with HCl. Gradient was run from 4 % eluent B to 100 % eluent B in 80 min.

The identities of inositol polyphosphates were determined by co-elution with D-myo-inositol standards (NMR analysed). No additional structure characterizations were performed and peaks could contain more than one isomer. Because of the large number of possible isomers more peaks were detected than assigned.

References

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