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Halothane-induced Calcium Release in Cultured Human Skeletal Muscle Cells from a Family Susceptible to Malignant Hyperthermia with an Unidentified Mutation in Chromosome 19

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MALIGNANT hyperthermia (MH) is an autosomal dominant muscle disorder characterized by a hypermetabolic crisis triggered by succinylcholine and/or volatile halogenated anesthetic agents. The underlying cause of MH is believed to be abnormal regulation of myoplasmic calcium concentration in skeletal muscle.[1,2] A single mutation in the cDNA sequence encoding the muscle ryanodine receptor (RYR1) was the first to be considered as a candidate for causing MH in pigs.[3] In human MH, the genetics is more complex; at least 15 mutations in the RYR1 gene have been reported to be potentially causative.[4,5] Beside RYR1, secondary loci containing genes encoding proteins involved in excitation-contraction coupling, such as the dihydropyridine receptor (DHPR), appeared to be causative.[2]

A well-defined diagnostic test for MH became available in 1971: the *in vitro* caffeine and halothane contracture test (IVCT).[6,7] The IVCT is based on the hypersensitivity of muscle strips, obtained by biopsy, to caffeine or halothane. Standardization in Europe and North America led to two essentially similar protocols for the IVCT.[8,9]

Cultured human skeletal muscle cells are often used to study muscle pathology in which calcium homeostasis might be disturbed. Excitation–contraction coupling of cultured muscle cells and their excitability at stimulation is determined by their basal intracellular calcium concentration ($[Ca^{2+}]_i$).[10] We studied the effects of halothane on the $[Ca^{2+}]_i$ transients in human myotubes made up of cultured skeletal muscle cells from MH-susceptible (MHS) patients and healthy controls. Since such cells can be obtained by needle biopsy, our goal was to determine whether this would be a less-invasive alternative of determining MH susceptibility.

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Materials and Methods

Patients

Muscle biopsies were obtained from four individuals without any known muscular disorder and from six MHS patients. All patients gave written informed consent, formulated by the Committee on Medical Ethics of the University of Nijmegen (Nijmegen, The Netherlands). The MHS patients were members of one single family in which a man in 1972 had died from MH during appendectomy. The MHS phenotypes were recognized by IVCT 2 yr before this study (table 1). Genetic analysis in this family showed linkage to a candidate locus on chromosome 19 (lod score greater than +3.0).

Human Skeletal Muscle Cell Cultures

Samples of the quadriceps femoris muscle were obtained by percutaneous needle biopsies (25–30 mg). Fragments were attached on the bottom of a 35-mm culture dish containing 1 ml proliferation medium (Dulbecco's modified Eagle medium [DMEM], 20% fetal calf serum [FCS], 4.5 mg/ml glucose, and 4 mM glutamine) and cultured in a humidified CO_2 incubator (5% CO_2) at 37°C. The next day, this medium was substituted by proliferation medium containing 4% Ultroser G and 10% rat brain extract instead of FCS. After 7–10 days, the explants were removed, and the myoblasts were plated out on glass coverslips (10 × 30 mm) in 35-mm dishes. Further proliferation took place in 20% FCS containing medium until confluency was reached. Differentiation to polynucleated myotubes was achieved in DMEM containing 10% horse serum for 7 days. For cryopreservation, about 10^6 myoblasts/ml DMEM containing 20% FCS and 10% dimethyl sulfoxide were stored in liquid nitrogen.

$[Ca^{2+}]_i$ On-line Ratio Measurement and Calibration

The free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined in skeletal muscle cells using Fura-2.[11] Myotubes were loaded with 5 μM Fura-2/acetoxymethyl ester (Fura-2/AM) and 10 μM Pluronic acid for 90 min at 37°C in physiologic salt solution (PSS; containing 10 mM HEPES, 125 mM NaCl, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM KCl, 2 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 10 mM glucose, pH 7.4). On-line ratio measurements were recorded with a Shimadzu RF-5301 spectrofluorophotom-

Table 1. IVCT Results of Muscle Biopsies from Six MHS Individuals and Averages for Control Individuals (n = 4)

	Halothane		Caffeine	
Patient(s)	Threshold (mM)	Contracture at 0.44 mM (mN)	Threshold (mM)	Contracture at 2 mM (mN)
MHS1	0.22	8	1	10.5
MHS2	0.11	15	2	4
MHS3	0.22	12	1	7.5
MHS4	0.22	13	1	11
MHS5	0.44	4	0.5	21
MHS6	0.11	20	0.5	14
Controls	—	0.2	32	0.1

IVCT = *in vitro* caffeine and halothane contracture test. MHS = malignant hyperthermia susceptible; a caffeine threshold concentration at 2.0 mM or less, *and* a halothane threshold concentration at 0.44 mM (2.0% v/v) or less; the threshold concentration is the lowest concentration which produces a sustained increase of at least 2 mN (0.2 g) in baseline force.[8]

eter. Fura-2 fluorescence was measured at an emission wavelength of 492 nm (bandwidth, 5 nm) and alternating excitation wavelengths of 340 and 380 nm (bandwidth, 3 nm). During the measurements, the cells in the cuvette were superfused with PSS (4.0 ml/min; 37°C) without or with different concentrations of halothane (0.11, 0.22, 0.44, 1.0, 2.0, 4.0, 8.0, 12.0 mM). Halothane was solved in dimethyl sulfoxide (DMSO) and added to PSS in airtight, dark bottles. All fluorescence signals were corrected for autofluorescence.

The 340/380 ratios (Fura-2) were calibrated using PSS containing 4 μM ionomycin and 10 mM Ca^{2+} (pH 7.7; R_{max}) or 4 μM ionomycin and 20 mM EGTA without external Ca^{2+} (pH 8.5; R_{min}). $[Ca^{2+}]_i$ was calculated using the equation:

$$[Ca^{2+}]_i = K_d \times \beta \times \{(R - R_{min})/(R_{max} - R)\}$$

K_d of Fura-2 is 224 nM.[12]

Assay of Halothane Concentration

Halothane concentrations in PSS were measured using a head space gas chromatographic technique (Chrompack CP-9001, equipped with a flame ionization detector).[13]

Materials

Dulbecco's modified Eagle medium and Ultroser G were from Gibco BRL Life Technologies, Paisly, United Kingdom; FCS and horse serum were from Flow Laboratories, Irvine, United Kingdom. Brain extract was prepared from brains of 10-day-old Wistar albino rats as a 10% (w/v) homogenate. Fura-2/AM, ionomycine, and Pluronic acid were purchased from Molecular Probes, Eugene, Oregon. Halothane was from Tempus b.v., Oegstgeest, The Netherlands.

Statistical Analysis

Data are represented as mean (SD). Statistical analysis is performed using the unpaired Student *t* test. Significance was set at $P < 0.05$. Curve fittings were obtained by linear regression analysis. Individual areas under the curves were determined from the curves by interpolation.

Results

Halothane concentrations in PSS were measured in the cuvette on completion of the calcium measurements. We found a substantial difference between the intended concentrations and the measured concentrations (intended → measured concentrations, respectively: 0.11 → 0.095 (0.012) mM, 0.22 → 0.187 (0.029) mM, 0.44 → 0.33 (0.039) mM, 1.0 → 0.69 (0.16) mM, 2.0 → 1.4 (0.20) mM, 4.0 → 2.75 (0.42) mM, 8.0 → 5.2 (0.99) mM, 12.0 →7.5 (1.3) mM).

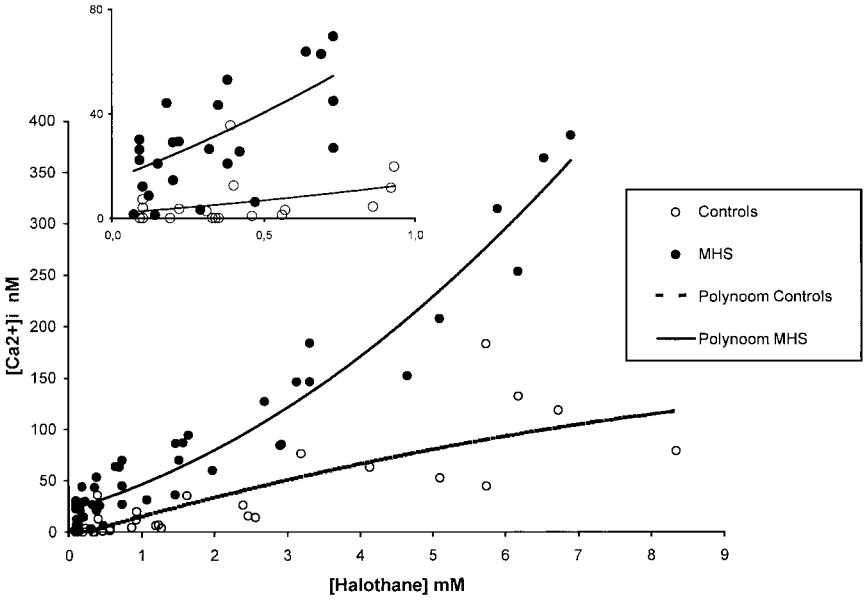

Fig. 1. Halothane-induced, dose-dependent increase of $[Ca^{2+}]_i$ in cultured skeletal muscle cells from six malignant hyperthermia–susceptible (MHS) individuals (n = 42; solid dots and line) and four control individuals (n = 32; open dots and dotted line). Discrimination between MHS and control is 100% beyond 0.5 mM halothane ($P < 0.05$). (*Inset*) $[Ca^{2+}]_i$ response at clinically used halothane concentrations (< 1.0 mM).

The 340/380 ratios of the Fura-2 calibration resulted in an R_{max} of 11.88 (1.03) and an R_{min} of 1.59 (0.15), mean (SD) of eight experiments. The constant β, *i.e.*, the ratio of the fluorescence emission of the free dye and the Ca^{2+}-saturated dye measured at 380 nm, is 3.09 (0.33) (n = 7). We did not find a statistical difference between the mean resting $[Ca^{2+}]_i$ in cultured muscle cells of MHS or control individuals, which were respectively 65 (20) nM (n = 72) and 58 (17) nM (n = 50).

Halothane produced a dose-dependent increase of $[Ca^{2+}]_i$ (fig. 1). In the MHS group, the Ca^{2+} response is observed in the clinically used halothane concentrations (< 1 mM). The Ca^{2+} response in cultured muscle cells of MHS individuals is significantly different from that of control individuals, and there is no overlap beyond 0.44 mM halothane (equivalent to 2% v/v).

Discussion

Cultured human skeletal muscle cells have been very useful for studying ion homeostasis in relation to MH.[14–17] Interpretation of the results is complicated by the existence of incomplete maturation and differences related to the heterogeneous population of cells at the myotube stage. By analyzing the fluorescence signal composed of the whole monolayer of myoblasts/myotubes on glass coverslips, we were able to compare the common behavior of thousands of cells instead of a small selection of cells, which was done in previous studies with comparable aims.[14,15,17] This could explain why we could discriminate MHS from control muscle tissue using halothane in clinically used concentrations, whereas the other investigators needed much higher halothane concentrations.[15,17] Further standardization of cell culturing could improve reproducibility of test results even more. In the IVCT, a vaporizer is used to add halothane to carboxygen to solve halothane in Krebs-Ringer's solution.[8] To reach supraclinical halothane concentrations, halothane was solved in DMSO before it was added to the physical salt solution. This procedure requires assay of halothane concentration in the test bath at completion of each experiment because the variations are common.

In this study, we confirm that there is no difference in the resting intracellular calcium concentrations between cultured muscle cells from MHS and control individuals.[2,14,15]

A test based on cultured muscle cells obtained by needle biopsy is less invasive and is easily reproducible. The time between biopsy and performance of the test is not critical because cell cultures can be expanded, frozen, and thawed. In contrast, the IVCT requires large muscle samples (surgically collected) and must be performed within 5 h.[8]

In conclusion, cultured human muscle cells obtained from needle biopsies may well be applied in diagnostic tests for MH susceptibility. More studies comparing data from the IVCT and cultured cells have to be undertaken to determine sensitivity (and specificity).

References

1. Joffe M, Savage N, Silove M: Minireview: The biochemistry of malignant hyperthermia: Recent concepts. Int J Biochem 1992; 24:387–98
2. Jurkat-Rott K, McCarthy T, Lehmann Horn F: Genetics and pathogenesis of malignant hyperthermia. Muscle Nerve 2000; 23:4–17
3. Fujii J, Otsu K, Zarzato F, de Leon S, Khanna VK, Weiler J, O'Brian PJ, MacLennan DH: Identification of a mutation in the porcine ryanodine receptor that is associated with malignant hyperthermia. Science 1991; 253:448–51
4. Manning BM, Quane KA, Ørding H, Urwyler A, Tegazzin V, Lehane M, O'Halloran J, Hartung E, Giblin LM, Lynch PJ, Vaughan P, Censier K, Bendixen D, Comi G, Heytens L, Monsieurs K, Fagerlund T, Wolz W, Heffron JJ, Müller CR, McCarthy TV: Identification of novel mutations in the ryanodine receptor gene (RYR1) in malignant hyperthermia: Genotype-phenotype correlation. Am J Hum Genet 1998; 62:599–609
5. Urwyler A, Deufel T, McCarthy T, West S, for the EMHG: Guidelines for molecular genetic detection of susceptibility to malignant hyperthermia. Br J Anaesth 2001; 86:283–7
6. Kalow W, Britt BA, Terrau ME, Haist C: Metabolic error of muscle metabolism after recovery from malignant hyperthermia. Lancet 1970; 2:895–98
7. Ellis FR, Harriman DGF, Keaney NP, Kyei-Mensah K, Tyrell JH: Halothane-induced muscle contracture as a cause of hyperpyrexia. Br J Anaesth 1971; 43:721–4
8. Ørding H, for the European Malignant Hyperthermia Group: In vitro contracture test for diagnosis of malignant hyperthermia following the protocol of the European MH Group: Results of testing patients surviving fulminant MH and unrelated low-risk subjects. Acta Anaesthesiol Scand 1997; 41:955–66
9. Larach MG: Standardization of the caffeine halothane muscle contracture test. North American Malignant Hyperthermia Group. Anesth Analg 1989; 69: 511–5
10. Benders AAGM, Oosterhof A, Wevers RA, Veerkamp JH: Excitation-contraction coupling of cultured human skeletal muscle cells and the relation between basal cytosolic Ca^{2+} and excitability. Cell Calcium 1997; 21:81–91
11. Benders AAGM, Veerkamp JH, Oosterhof A, Jongen PJH, Bindels RJM, Smit LME, Busch HFM, Wevers RA: Ca^{2+} homeostasis in Brody's disease. J Clin Invest 1994; 94:741–8
12. Grynckiewicz G, Poenie M, Tsien RY: A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260: 3440–50
13. Snoeck MMJ, Gielen MJM, Tangerman A, Van Egmond J, Dirksen R: Contractures in skeletal muscle of malignant hyperthermia susceptible patients after in vitro exposure to sevoflurane. Acta Anaesthesiol Scand 2000; 44:334–7
14. Censier K, Urwyler A, Zorzato F, Treves S: Intracellular calcium homeostasis in human primary muscle cells from malignant hyperthermia-susceptible and normal individuals. J Clin Invest 1998; 101:1233–42
15. Brinkmeier H, Krämer J, Krämer R, Iaizzo PA, Baur C, Lehmann-Horn F, Rüdel R: Malignant hyperthermia causing Gly2435Arg mutation of the ryanodine receptor facilitates ryanodine-induced calcium release in myotubes. Br J Anaesth 1999; 83:855–61
16. Tong J, McCarthy TV, MacLennan DH: Measurement of resting cytosolic Ca^{2+} concentrations and Ca^{2+} store size in HEK-293 cells transfected with malignant hyperthermia or central core disease mutant Ca^{2+} release channels. J Biol Chem 1999; 274:693–702
17. Otsu K, Nishida Y, Kimura K, Kuzuya T, Hori M, Kamada T, Tada M: The point mutation Arg615Cys in Ca2+ release channel of skeletal sarcoplasmic reticulum is responsible for hypersensitivity to caffeine and halothane in malignant hyperthermia. J Biol Chem 1994; 269:9413–5