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Lymphocyte-based Determination of Susceptibility to Malignant Hyperthermia: A Pilot Study in Swine

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ABSTRACT

Background: Malignant hyperthermia susceptibility (MHS) is diagnosed by an invasive *in vitro* caffeine-halothane contracture test (CHCT) carried out on biopsied skeletal muscle tissue. We are presenting a novel blood test approach for malignant hyperthermia testing in a swine model. Our main aim was to determine whether adenosine production from lymphocytes after 4-chloro-m-cresol (4CmC) stimulation distinguishes homozygous swine carrying the Arg615Cys mutation in the ryanodine receptor type 1 (*RyR1*) gene (MHS swine) from normal swine.

Methods: Lymphocytes were isolated from arterial blood (40 ml) obtained from MHS (n = 7) and normal (n = 7) swine. Cells were suspended in Hank's balanced salt solution and treated with 4CmC (0–10 mM) at 37°C in the presence of adenosine deaminase inhibitor. After termination and purification of samples, aliquots (50 μl) were assayed for adenosine content using high performance liquid chromatography.

Results: Baseline adenosine levels before stimulating lymphocytes with 4CmC were 0.025 ± 0.004 and 0.041 ± 0.006 μM (mean ± SEM) in lymphocytes from normal and MHS swine, respectively ($P = 0.125$). Maximum responses were achieved at 1 mM 4CmC for both cell-line groups. Adenosine levels after stimulation with 4CmC (1 mM) were 0.185 ± 0.009 and 0.397 ± 0.038 μM in lymphocytes from normal and MHS swine, respectively ($P = 0.0035$). There was no overlap between adenosine levels in stimulated lymphocytes from MHS and normal swine.

Conclusion: 4CmC stimulation of porcine lymphocytes induces increased adenosine formation in MHS cells relative to those from normal swine; evaluation of adenosine formation in response to RyR1 agonists in human lymphocytes is needed.

What We Already Know about This Topic

- Different *in vitro* and genetic tests have been proposed for detection of susceptibility against malignant hyperthermia.

What This Article Tells Us That Is New

- In lymphocytes from malignant hyperthermia-susceptible pigs, adenosine formation was greater than in normal swine.
- Lymphocyte adenosine production in blood from normal and malignant hyperthermia-susceptible hymans should be tested to validate this new test.

MALIGNANT hyperthermia (MH) is a genetic disorder that exaggerates metabolic responses to volatile anesthetics and the depolarizing muscle relaxant succinylcholine. Exposure to these agents triggers a complex cascade of biochemical reactions and signaling that results in muscle rigidity, rhabdomyolysis, cardiac arrhythmia, and potentially lethal hyperthermia.[1,2] If not treated promptly by withdrawing anesthesia and administering dantrolene, an intracellular calcium antagonist, mortality can exceed 80%. Early signs and symptoms of an imminent MH episode can be slow and clinically ambiguous, making MH difficult to recognize. Identification of affected persons is through a caffeine-halothane contracture test (CHCT) on biopsied leg vastus lateralis muscle, with slight differences in the North America CHCT[3] and European *in vitro* contracture test[4] protocols. CHCT has a sensitivity of 97% (accurately detects those with MH) and a specificity of 78% (yields some false positives).[5] Muscle fibers from MH-positive persons are markedly more sensitive to caffeine and halothane. The reproducible shift in the dose sensitivity of muscle contracture to these compounds has led to its use as the diagnostic indicator of MH.[6] However, because of the invasive nature of the test, its costs,

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and associated risks, it is estimated that only approximately 10% of eligible patients undergo CHCT (projected from referrals to the database of the North America Malignant Hyperthermia Hotline). Thus, a functional, minimally invasive, safe, relatively economical, and simple MH diagnostic test is desirable.

Several alternative MH tests have been considered in the literature as being less or minimally invasive. The most extensively investigated approach is molecular testing of the ryanodine receptor type 1 (*RyR1*) gene.[7–11] However, because the total number of mutations that could potentially alter or affect ryanodine receptor (RyR) function is not yet known, genetic screening has not been, and likely will not soon be, definitive enough to serve as the sole method for diagnosing MH. Functional MH tests using microdialysis,[12–14] immortalized B cells-Ca^{2+}assay,[15,16] and nuclear magnetic resonance spectroscopy[17] are either modestly invasive (microdialysis) or too technically involved and time consuming to be practical for use in MH diagnostic testing.

In the present study, we examine a novel blood test for utility in MH diagnosis using the well-characterized porcine MH model. Our approach isolates and compares lymphocytes (B + T cells) from swine carrying a single MH mutation, Arg615Cys (referred to as "MHS swine" throughout), with lymphocytes from normal swine to determine whether significant differences in adenosine levels could be measured to distinguish the two from one another. 4-Chloro-m-cresol (4CmC), a chlorinated phenol that mimics the effects of caffeine and halothane by inducing release of sarcoplasmic reticulum (SR) Ca^{2+} in skeletal muscles,[18] as well as in isolated SR vesicles[19], was used to stimulate lymphocytes.

The endoplasmic reticulum/SR has two families of intracellular ion channels capable of releasing Ca^{2+}: the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) and the RyRs. IP_3R agonists acting through G-protein–linked or tyrosine kinase–linked receptors stimulate phospholipase C to generate the second messenger IP_3, which then diffuses into the cytoplasm to release stored Ca^{2+} by binding to IP_3R.[20,21] IP_3R can be activated by adenophostin A and inhibited by xestospongin C (XeC).[22] RyRs are found in muscle cells, neurons, and other cell types.[21–23] RyR-induced Ca^{2+} release is activated by caffeine and 4CmC[19] but is inhibited by dantrolene/azumolene.[24] A correlation between cell Ca^{2+} release leading to increased Ca^{2+} ATPase-dependent Ca^{2+} pumping and subsequent adenosine formation in lymphocytes has not been reported. However, in skeletal muscle, RyR1 is coupled to the dihydropyridine receptor. The dihydropyridine receptor is the voltage-sensitive L-type Ca^{2+} channel. Mitochondria (the site of oxidative phosphorylation of adenosine 5′-diphosphate [ADP]) and glycolysis (the cytoplasmic site of substrate-level phosphorylation of ADP) synthesize adenosine 5′-triphosphate (ATP), which powers the ATP-dependent Ca^{2+} pumps on the SR/endoplasmic reticulum and the sarcolemma. Intracellular Ca^{2+} accumulation due to the activation and subsequent nonclosure/delay-closure of the RyR1 Ca^{2+} channel triggers this ATPase-dependent Ca^{2+} pumping, generating ADP and adenosine 5′-monophosphate (*via* adenylate kinase), leading to increased adenosine formation.[25–27]

We demonstrate that (1) adenosine formation by 4CmC-stimulated lymphocytes can be used to assess increased adenosine ATP turnover and catabolism resulting in increased adenosine formation; (2) defined mutation in RyR1 that leads to abnormal agonist-induced Ca^{2+} release in skeletal muscle also leads to increase adenosine formation by lymphocytes; and (3) 4CmC-induced adenosine production is substantially greater in lymphocytes from MHS swine compared with normal swine. We attempted to identify Ca^{2+} pools that could possibly contribute to measured 4CmC-induced adenosine formation in lymphocytes from normal swine by using specific blockers of IP_3R, mitochondrial oxidative phosphorylation, and SR Ca^{2+} release channels. We also considered extracellular Ca^{2+} as a possible effector of intracellular Ca^{2+} changes, thus potentially contributing to increased adenosine formation.

Materials and Methods

Experiments were designed to (1) examine the hypothesis that a defined mutation in the RyR1 gene of MHS swine that leads to abnormal agonist-induced Ca^{2+} release in skeletal muscle will also lead to adenosine release in lymphocytes; (2) determine whether 4CmC stimulation of lymphocytes and adenosine production distinguishes between MHS and normal swine, and (3) delineate the possible effects of IP_3R, mitochondrial uncoupling, external Ca^{2+}, and the SR Ca^{2+} release channels (RyR1) on the observed adenosine formation.

Animal Model

With approval of the Uniformed Services University of the Health Sciences (Bethesda, Maryland) institutional animal care and use committee, seven normal pigs (not MH-sensitive; Archer Farm, Darlington, MD) and seven homozygous MHS swine (Boyle Farms, Moorhead, IA) carrying an Arg615Cys mutation were sedated with ketamine (8–10 mg/kg intramuscular injection). An ear vein was cannulated for inducing and maintaining anesthesia with propofol (0.2–0.4 $mg \cdot kg^{-1} \cdot min^{-1}$). An endotracheal tube was inserted and ventilation was maintained with an end-tidal carbon dioxide of 40 ± 5 mmHg. A rectal thermometer probe was placed to monitor and maintain core body temperature at 38.5°C. A 20-gauge cannula was inserted percutaneously into the superficial femoral artery. Blood pressure and heart rate were monitored continuously. Efforts were made to match the two experimental animal groups in regard to factors such as sex, age, or weight. After cardiovascular and respiratory indices stabilized, an arterial blood sample (40 ml) was withdrawn using a heparinized 60-ml syringe. After blood sampling, a skeletal muscle biopsy (vastus lateralis) for CHCT was performed as described previously.[3] Swine were then administered inhaled halothane (2%) for the induction of an MHS clinical episode.

Isolation of Lymphocytes from Whole Blood

Lymphocytes (B + T cells) were isolated from whole blood by the Ficoll-Hypaque density gradient centrifugation technique.[28] Isolated lymphocyte cells were dispensed into 3-volume normal Hank's balanced salt solution (HBSS) and centrifuged (10 min at 1,000 revolutions per minute). The pellet was resuspended in HBSS to lyse contaminating erythrocytes and spun; this step was repeated once more. No further purification was attempted to maintain simplicity of the protocol for potential applicability in a possible MH diagnostic test in routine clinical practice. Lymphocytes were then cultured in RPMI-1640 media supplemented with 10% inactivated fetal bovine serum plus 100 units/ml penicillin and 2 mM glutamine. Cell cultures were incubated at 37°C in a humidified chamber with 5% CO_2. All experiments were completed within 4 days after isolation of lymphocytes from whole blood samples.

Nucleosides Assay Protocol

Lymphocytes (1–2 × 10^6 cells/test; cell counting was performed using hemacytometer) were suspended in HBSS with or without Ca^{2+}/Mg^{2+} as indicated for each experiment plus 0.1% endotoxin-free bovine serum albumin. Experiments were carried out in the presence of adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine-HCl (EHNA; 0.1 mM) to prevent deamination of adenosine to inosine[29] and incubated at 37°C for 10 min followed by treatment with different 4CmC concentrations (0.2–10 mM) and then incubated at 37°C for an additional 45 min. Final volume of the test tubes mixture were adjusted to 0.2 ml using HBSS buffer. Termination of adenosine metabolism and purification of samples were accomplished by addition of ice cold 0.1 ml 12% perchloric acid followed by centrifugation at 7,000 revolutions per minute for 10 min. Aliquots of supernatant (50 μl) were assayed for adenosine and inosine content using the high performance liquid chromatography technique detailed below. The protocols used to study the effects of external Ca^{2+}/Mg^{2+}, SR Ca^{2+} release blocker azumolene, inhibitor of IP_3R xestospongin C (XeC), and mitochondria uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrozone (FCCP), as well as the effect of EHNA, were the same as those given in the nucleoside assay protocol. Further details and specific conditions for each experiment are presented in the result section and figure legends.

High-Performance Liquid Chromatography Analysis

A chromatography protocol was developed using a high-performance liquid chromatography system (Waters Separations module, model 2695; Waters Co., Milford, MA) consisted of a Waters Symmetry C_{18} column (4.6 × 250 mm) and mobile phase of 94% KH_2PO_4 (50 mM, pH 4.6) containing 1-heptanesulfonic acid (0.5 mM) and 6% acetonitrile. Flow rate was set at 1 ml/min. Under these conditions, retention times for inosine and adenosine were 3.6 and 6.6 min, respectively. Peak width of inosine and adenosine were 18 and 36 s, respectively. Eluted nucleosides were detected by their absorbance at λ = 254 nm using Waters photodiode array detector (model 996). Sample adenosine and inosine concentrations were interpolated from a calibrated standard curve of concentrations *versus* peak area.

Statistics

Sample nucleoside concentrations were normalized to represent per 10^6 cells and presented as mean ± SEM. SigmaPlot (version 10) and SigmaStat (version 3.5) software (Systat Software, Inc., Richmond, CA) were used to plot graphs and perform statistics, respectively. Pairwise comparisons using two-way analysis of variance with Bonferroni correction (α set to 0.05) were applied to test for statistical differences between multiple treatment groups. In addition, two-tailed *t* tests were done comparing two treatments of interest, and exact *P* values are presented in the text; *P* less than 0.05 indicated statistical significance.

Reagents

HBSS containing Ca^{2+}/Mg^{2+} (0.4 mM $Ca^{2+}$and 0.5 mM Mg^{2+}, referred to as normal HBSS) and Ca^{2+}/Mg^{2+}-free HBSS were from Invitrogen/GIBCO (Carlsbad, CA). The adenosine deaminase inhibitor EHNA (Sigma-Aldrich, St. Louis, MO) was dissolved in either normal HBSS or Ca^{2+}/Mg^{2+}-free HBSS (Sigma-Aldrich), depending on the protocol used, and stored at −20°C. KH_2PO_4, 1-heptanesulfonic acid, bovine serum albumin, adenosine, inosine, and other ordinary laboratory chemicals were from Sigma-Aldrich. RPMI-1640 culture media, fetal bovine serum, and penicillin were from Quality Biologic Inc. (Gaithersburg, MD). 4CmC (Sigma-Aldrich) was prepared daily in distilled water. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich) and XeC (Calbiochem, San Diego, CA) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at −20°C. Azumolene was dissolved in DMSO and stored at −20°C.

Results

Although seven lymphocyte cell lines in each group of MHS and normal swine were examined, the numbers of cell lines in each protocol were variable due to limited availability of cells. Lymphocytes from MHS and normal swine were used to examine the effects of adenosine deaminase inhibitor EHNA on adenosine formation and on 4CmC dose-response experiments. Due to limited availability of the porcine MHS lymphocytes, only lymphocytes from normal swine were used to study the effects of external Ca^{2+}, azumolene, XeC, and uncoupler FCCP on adenosine formation. In preliminary tests, we observed that adenosine formation in response to 4CmC was a function of the length of the cell culturing period. We found that after culturing cells for 10 days, more than 90% of the 4CmC effectiveness on lymphocytes was abolished in terms of adenosine formation (data not shown).

Effect of Adenosine Deaminase Inhibitor on Adenosine/Inosine Levels

Figure 1 shows 4CmC (1 mM)–induced adenosine/inosine formation in the presence and absence of 0.1 mM EHNA.

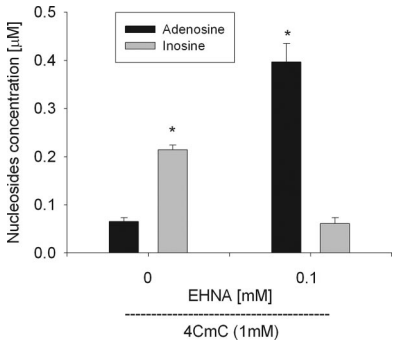

Fig. 1. Effect of adenosine deaminase inhibitor erythro-9(2-hydroxy-3-nonyl) adenine (EHNA, 0.1 mM) on 1 mM 4-chloro-m-cresol (4CmC)-induced adenosine/inosine in lymphocyte cells from swine carrying the Arg615Cys mutation and having positive caffeine-halothane contracture test (CHCT). Sample nucleoside concentrations were normalized to reflect values per 10^6 cells. Lymphocyte cells (1–2 × 10^6) from malignant hyperthermia-susceptible (n = 4) swine were suspended at 37°C for 10 min in normal Ca^{2+}/Mg^{2+}-containing Hank's balanced salt solution in the absence and presence of EHNA followed by treatment with 1 mM 4CmC and continuation of incubation for additional 30 min (for further details see Methods). *In the absence of EHNA, inosine level is significantly higher than adenosine level, but in the presence of EHNA, adenosine level is significantly higher than inosine level.

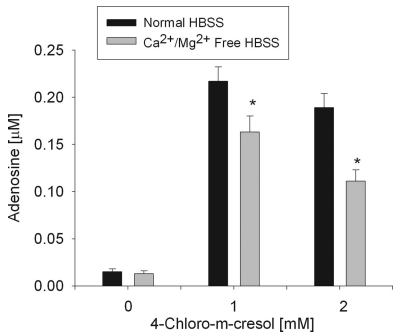

Fig. 2. Effect of external Ca^{2+}/Mg^{2+} on 4-chloro-m-cresol (4CmC)-induced adenosine formation in lymphocyte cells from normal swine (n = 6). Lymphocyte cells (1–2 × 10^6) were suspended in Hank's balanced salt solution (HBSS) containing adenosine deaminase inhibitor (erythro-9(2-hydroxy-3-nonyl) adenine, 0.1 mM) at 37°C for 10 min in the absence and presence of Ca^{2+}/Mg^{2+}. After a 10-min incubation period, samples were treated with 4CmC (1 and 2 mM) as indicated. *4CmC-induced adenosine levels in the absence of external Ca^{2+}/Mg^{2+} are significantly lower compared with corresponding values in the presence of external Ca^{2+}/Mg^{2+} (P = 0.037 and 0.002 for 1 and 2 mM 4CmC responses, respectively).

The lack or weak formation of adenosine response to 4CmC stimulation was due to immediate deamination of adenosine to inosine by the endogenous enzyme adenosine deaminase. This was confirmed when lymphocyte cells were incubated with various concentrations (0–1,000 μM) of EHNA. The results demonstrated that at EHNA concentrations of 20 μM or higher, inosine formation decreased and adenosine concentration increased to plateau level (n = 3, EHNA dose-response data not shown). The observed differences in the molar concentrations of inosine and adenosine in figure 1 were likely due to degradation of inosine to hypoxanthine by endogenous purine nucleoside phosphorylase and, at least in part, also due to the differences in the molar absorption coefficient (ε) of inosine compared with adenosine (the measuring wavelength was λ = 254 nm; adenosine, λ_{max} = 258 nm, ε = 15,100; inosine, λ_{max} = 248 nm, ε = 12,200 [Merck Index]).

Effect of External Ca^{2+}/Mg^{2+} on 4CmC-induced Adenosine Formation

There were no differences between the basal resting adenosine levels of lymphocytes from normal swine (n = 6) incubated in normal Ca^{2+}/Mg^{2+}-containing HBSS and Ca^{2+}/Mg^{2+}-free HBSS (0.015 ± 0.003 and 0.013 ± 0.003 μM, P = 0.7, respectively). However, using 1 mM 4CmC to induce adenosine in normal HBSS *versus* Ca^{2+}/Mg^{2+}-free HBSS, we measured 0.217 ± 0.015 *versus* 0.163 ± 0.017 μM, P = 0.037, respectively. This reflected a 24.9 ± 5.1% increase in adenosine formation in the presence of normal levels of extracellular Ca^{2+}. At 2 mM 4CmC, the difference increased to 0.189 ± 0.015 in the presence of extracellular Ca^{2+} and 0.111 ± 0.012 μM in the absence of extracellular Ca^{2+} reflecting a 41 ± 2.5% increase in 4CmC-induced adenosine formation (P = 0.002, fig. 2) in the presence of extracellular Ca^{2+}/Mg^{2+}.

4CmC-induced Adenosine in Normal and MHS Lymphocytes

Experiments for 4CmC dose-response relations were carried out in normal Ca^{2+}/Mg^{2+}-containing HBSS. Shown in figure 3 are 4CmC dose-response data for lymphocytes from normal (n = 7) and MHS (n = 7) swine. Adenosine levels increased upon 4CmC stimulation dose dependently and peaked at near 1 mM 4CmC, reaching 0.397 ± 0.043 and 0.185 ± 0.017 μM in MHS *versus* normal cells (P = 0.0035), respectively. Adenosine levels at 0 and 0.2 mM 4CmC were 0.177 and 0.341 with P values of 0.153 and 0.07, respectively. Figure 4 shows that the individual adenosine levels due to 4CmC (1 mM) from MHS cells did not overlap with those from normal cells. Subtraction of the baseline adenosine values confirmed this result. The basal plus 4CmC (1 mM)-induced adenosine ranges for normal and MHS cells were 0.145–0.209 μM and 0.250–0.537 μM, respectively.

Effect of Azumolene

Effect of the membrane-permeable SR Ca^{2+} release blocker azumolene was examined in 4CmC-treated cells. Azumolene is a more soluble SR Ca^{2+} release blocker than the clinically used dantrolene.[24] Lymphocytes in the presence of EHNA were treated with DMSO (final DMSO concentration, 10% v/v) with and without different concentrations of azumolene (0–1,000 μM) and incubated at 37°C for 10 min followed by treatment with 1 mM 4CmC. As shown in figure 5, azu-

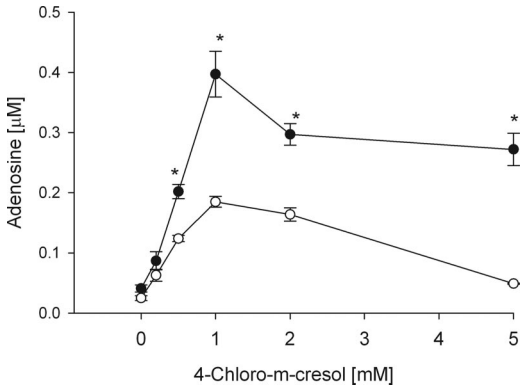

Fig. 3. Dose dependence of 4-chloro-m-cresol (4CmC)-induced adenosine in lymphocyte cells from normal (*open circle*, n = 7) and malignant hyperthermia-susceptible swine carrying the Arg615Cys mutation in the ryanodine receptor type 1 (*filled circle*, n = 7). Sample adenosine concentrations were normalized to reflect value per 10^6 cells. Cells (1–2 × 10^6) in suspension in normal Hank's balanced salt solution containing adenosine deaminase inhibitor (erythro-9(2-hydroxy-3-nonyl) adenine, 0.1 mM) were incubated at 37°C for 10 min followed by treatment for additional 30 min with different 4CmC concentrations as indicated. *4CmC-induced adenosine levels in presence of 0.5, 1, 2, or 5 mM are significantly higher in the MHS group than in the normal control group; *P* values were 0.0035, 0.0035, 0.0017, and 0.0008, respectively.

molene in concentrations up to 1 mM only marginally inhibited 4CmC-induced adenosine formation. Nonlinear curve fitting of these data suggested an apparent Ki value near 5 mM azumolene (data not shown). In an attempt to increase permeability of azumolene across the plasma membrane, we tested 30% (v/v) DMSO (n = 3) as a vehicle. Results suggested that 30% DMSO concentration was toxic to the cells,

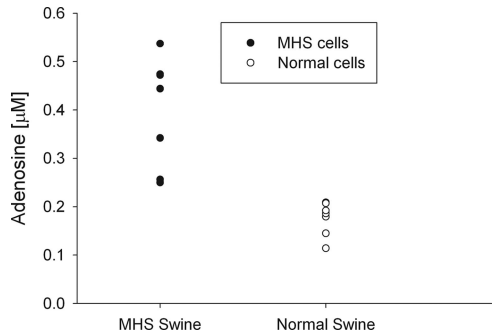

Fig. 4. Scatter plot of 1 mM 4-chloro-m-cresol (4CmC)-induced adenosine levels in lymphocyte cells from malignant hyperthermia susceptibility (MHS) swine carrying Arg615Cys mutation (n = 7) *versus* normal swine (n = 7). Sample adenosine concentrations were normalized to reflect value per 10^6 cells. Cells (1–2 × 10^6) in suspension in normal Hank's balanced salt solution containing adenosine deaminase inhibitor (erythro-9(2-hydroxy-3-nonyl) adenine, 0.1 mM) were incubated at 37°C for 10 min followed by treatment for additional 30 min with different 4CmC concentrations as indicated. Baseline adenosine levels before 4CmC treatments were not significant different between lymphocyte cells from MHS *versus* normal swine.

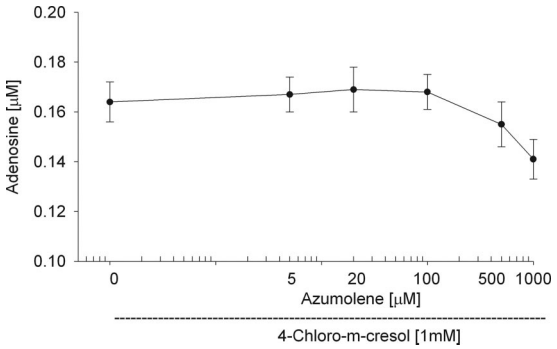

Fig. 5. Effect of SR Ca^{2+} release blocker azumolene (0–1,000 μM) on 1 mM 4-chloro-m-cresol (4CmC)-induced adenosine. Lymphocyte cells (1–2 × 10^6) from normal swine (n = 5) in suspension in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution plus adenosine deaminase inhibitor (0.1 mM (erythro-9(2-hydroxy-3-nonyl) adenine) were incubated at 37°C for 10min with azumolene up to concentrations of 1 mM. This step was followed by treatment with 1 mM 4CmC for an additional 30–45-min incubation at 37°C. Measured adenosine levels in the absence of azumolene plus 4CmC were depicted against *x* = 0.1 mM, thus allowing logarithmic display of the entire dose-response curve. Azumolene in the highest concentration used only marginally inhibited 4CmC-induced adenosine formation.

causing significant decrease in the 4CmC-induced adenosine (data not shown).

Effect of XeC

XeC is a potent and selective inhibitor of IP_3R coupled to a special intracellular pool of Ca^{2+}.[20–22,29–31] To determine whether IP_3R contributed to the observed 4CmC-induced adenosine formation, normal lymphocyte cells in suspension in Ca^{2+}/Mg^{2+}-free HBSS were treated with DMSO (final concentration, 10% v/v) with and without XeC (1 and 10 μM). This concentration range of XeC has been reported to be an effective physiologic concentration in various cell types.[20–22] Figure 6 summarizes the observed effects of XeC on 4CmC-induced adenosine. XeC (1 and 10 μM) alone did not affect the basal adenosine levels (data not shown). Likewise, 1 μM XeC did not affect 4CmC-induced adenosine. However 10 μM XeC significantly decreased 4CmC-induced adenosine by 27% ($P = 0.002$), suggesting that IP_3R-coupled intracellular Ca^{2+} stored in porcine lymphocytes, may contribute, in part, to 4CmC-observed adenosine formation (see fig. 3).

Effect of Uncoupler FCCP on 4CmC-induced Adenosine in Lymphocyte Cells

Using normal lymphocytes in the absence of extracellular Ca^{2+}/Mg^{2+}, FCCP (2 and 20 μM) alone increased basal adenosine levels several-fold (up to 350%) to 0.1–0.12 μM (fig. 7, bars A, $P < 0.008$), which is consistent with ATP catabolism due to mitochondrial uncoupling. Micromolar concentrations of FCCP are known to effectively uncouple mitochondrial respiration from oxidative phosphorylation of

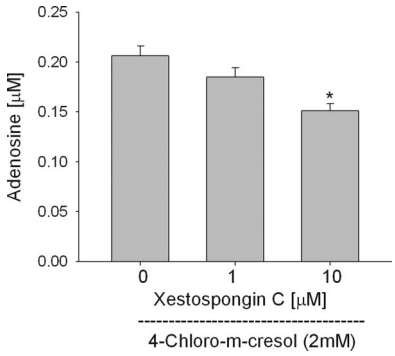

Fig. 6. Effect of inositol 1,4,5-triphosphate receptor-blocker xestospongin C (XeC) on 4-chloro-m-cresol-induced adenosine formation by porcine lymphocytes cells. Cells (1–2 × 10^6) from normal swine (n = 5) were suspended in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution plus adenosine deaminase inhibitor ((erythro-9(2-hydroxy-3-nonyl) adenine, 1 mM). Samples were treated for 10 min at 37°C with different XeC concentrations as indicated in the figure. This protocol phase was followed by treatment with 2 mM 4CmC and incubation at 37°C for additional 30 min. *XeC (10 μM) significantly (P = 0.002) reduced 4CmC (2 mM)-induced adenosine formation.

adenosine in many cell types.[32–34] Combining FCCP with subsequent 4CmC (2 mM) did not further increase adenosine formation (fig. 7, bars B), most likely reflecting Ca^{2+} depletion of the SR due to previous FCCP treatment.

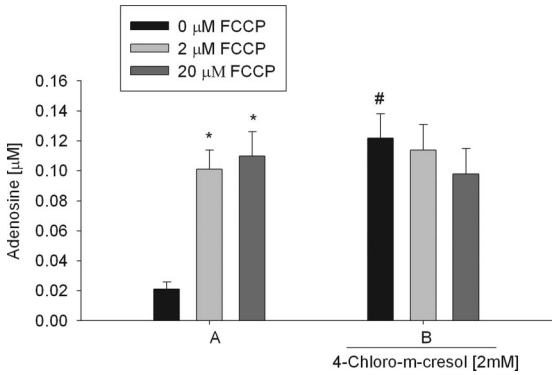

Fig. 7. Effect of uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) alone (bars A) and its affect on 4-chloro-m-cresol (4CmC)-induced adenosine formation (bars B) by normal swine lymphocyte cells (n = 5). In brief, one set of lymphocytes was suspended in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution plus adenosine deaminase inhibitor ((erythro-9(2-hydroxy-3-nonyl) adenine, 1 mM), with and without FCCP at 37°C for 10 min (bars A). In the second set of samples, cells were treated the same as in the first set; then, after the 10-min incubation period, lymphocytes were treated with 2 mM 4CmC and incubated for an additional 30 min at 37°C (bars B). Adenosine data were normalized to represent value per 10^6 cells. *FCCP (2 and 20 μM) alone significantly increased basal adenosine levels. #In the absence of FCCP, 4CmC-induced adenosine formation was 5.8-fold higher compared with basal adenosine level.

CHCT Results

Halothane (3%)-induced contractures in muscle strips from MHS and normal swine were 19.2 ± 3.1 mN (range, 11.2–33.1 mN) and 1.2 ± 1.0 mN (range, 0–1.8 mN), respectively. Caffeine (2 mM)-induced contractures in muscle strips from MHS and normal swine were 10.2 ± 1.8 mN (range, 4.7–17.1 mN) and 0.4 ± 0.2 mN (range, 0–1 mN), respectively. All MHS swine developed MH episodes in response to 2% halothane, whereas the normal swine did not.

Discussion

The major findings of this pilot study may be summarized as follows: (1) 4CmC treatment of lymphocytes from MHS and normal swine produces cellular energetic stress that is indexed by increased adenosine formation; (2) 4CmC-induced adenosine formation in lymphocytes distinguishes between MHS and normal swine because there is no overlap between groups; (3) extracellular Ca^{2+} significantly influences 4CmC-induced adenosine formation in swine lymphocytes, indicating increased transmembrane Ca^{2+} fluxes during cellular de-energization; (4) azumolene, a SR Ca^{2+} release blocker, in concentrations up to 1 mM, did not significantly decrease 4CmC-induced adenosine in lymphocytes from normal swine; (5) IP_3R-linked Ca^{2+} release may also contribute to 4CmC-induced adenosine; (6) uncoupled mitochondria result in near-maximal adenosine formation due to ATP depletion and possibly mitochondrial matrix Ca^{2+} depletion; and (7) evaluation in human lymphocytes is needed to clarify whether the use of adenosine as an index of cellular Ca^{2+} stress and pumping in lymphocytes has clinical potential as an MH diagnostic blood test.

In this study, only a relatively small number of MHS animals were examined; nevertheless, the data showed that there was no overlap between 4CmC-induced adenosine values in the MHS group *versus* the normal group. However, until additional experiments are performed, it cannot be stated with certainty that by increasing number of animals in each group, overlap between 4CmC-induced adenosine will not occur. The overlap in adenosine levels may be expected to be greater in humans because there are more mutations found to be causal for MH than in the purebred swine.

There are similarities and differences between 4CmC-induced Ca^{2+} release *via* the RyR1-sensitive stores in Epstein-Barr virus immortalized human B cells[15,16] and the present finding on adenosine formation by 4CmC stimulated swine lymphocytes. 4CmC and caffeine-induced Ca^{2+} release are partially blocked by azumolene, a more water-soluble blocker of SR Ca^{2+} release than sodium dantrolene. Using the Dakiki cell line, McKinney *et al.*[34] showed that 0.1 and 0.4 mM azumolene inhibited 4CmC-induced Ca^{2+} release by 23 and 50%, respectively. However, in the present study, 1 mM azumolene, the highest concentration applied, inhibited 4CmC-induced adenosine formation in normal swine lymphocytes only by 14%, and this effect was not statistically significant. An explanation for this discrepancy is

not immediately obvious but could possibly be related to different experimental conditions in our nonimmortalized swine lymphocytes compared with the Epstein-Barr virus immortalized Dakiki B cell line protocols,[34] as a result of differences between cell lines from two different species and the differences in 4CmC-induced $Ca^{2+}$and adenosine release mechanism.

The data suggested that removal of external Ca^{2+}/Mg^{2+} eliminated net Ca^{2+} influx and/or enabled increased net Ca^{2+} efflux across the plasma membrane in presence of 4CmC, and that such transmembrane Ca^{2+} fluxes contributed significantly to the overall intracellular Ca^{2+} dysregulation during cell activation by 4CmC. Thus, the adenosine formation observed under the Ca^{2+}-free HBSS conditions likely reflected the energetic stress and associated ADP and adenosine 5′-monophosphate formation triggered by increased Ca^{2+} ATPase activity in response to the Ca^{2+} release from intracellular stores.[25–27] Our data show that the effect of physiologic millimolar extracellular Ca^{2+} on 4CmC-induced adenosine formation was substantial, accounting for 24.9 ± 5.1 or 41 ± 2.5% of total adenosine production in the presence of 1 or 2 mM 4CmC, respectively, under our experimental conditions.

In addition to RyR1-linked Ca^{2+} stores, there are other physiologically important intracellular pools of Ca^{2+}, mainly the IP_3-sensitive stores[20,21] and the mitochondrial matrix Ca^{2+} pool.[32–33,35] According to our findings, it seems not unlikely that the IP_3-sensitive stores, especially, could contribute to cellular Ca^{2+} dysregulation (and adenosine formation) secondary to maximal SR Ca^{2+} release channel agonism caused by 4CmC in swine lymphocytes (fig. 3). This notion is supported by the following facts and observations: XeC, a potent and selective inhibitor of the IP_3R in several cell lines,[22,31] had a concentration-dependent inhibitory effect on 4CmC-induced adenosine in normal swine lymphocytes (fig. 6). At 1 μM, XeC had no measurable effect on 4CmC-induced adenosine formation, which compares well with the lack of 1 μM XeC effects on Ca^{2+} release in the Dakiki cell line.[34] However, at 10 μM, XeC inhibited 4CmC-induced adenosine significantly by 27%, providing pharmacological evidence that IP_3R-mediated Ca^{2+} release might indirectly contribute (*via* additional Ca^{2+} release secondary to 4CmC induced Ca^{2+} release) to 4CmC-induced adenosine formation in swine lymphocytes under our conditions.

Mitochondria are the sites of oxidative phosphorylation of ADP and the generation of reactive superoxide radicals; the mitochondrial matrix is a subcellular calcium store and therefore could contribute to cytosolic Ca^{2+} under conditions of deenergization.[33,35] In addition, it has been reported that mitochondria express RyRs on their outer membrane,[36] but this conclusion could not be confirmed by other investigators.[37–38] FCCP is an uncoupler of oxidative phosphorylation causing severe depolarization of the inner mitochondrial membrane, which is associated with a depletion of mitochondrial matrix Ca^{2+}.[36] Application of FCCP alone on swine lymphocyte cells substantially increased basal level of adenosine in Ca^{2+}-free incubation media. Also this effect of FCCP compares well with the previously reported FCCP-induced Ca^{2+} accumulation in human Epstein-Barr virus immortalized B cells.[34]

Taken together, these findings demonstrate increased ATP catabolism as a result of impaired cellular calcium control; they also suggest that adenosine could be a readily measurable marker for increased susceptibility to the hypermetabolic state of malignant hyperthermia and, in addition, they are consistent with ^{31}P-NMR data by Olgin *et al.*[17] in patients.

In conclusion, this article presents a novel and relatively simple protocol that uses lymphocytes and employs adenosine production as a marker to distinguish between normal and MHS in a well-described swine model. The MH-specificity of the protocol is provided by the use of RyR1 agonist 4CmC. The Ca^{2+} contributing to adenosine responses could come from several sources, including the SR Ca^{2+} release channels, the IP_3R pool, and extracellular as well as mitochondrial Ca^{2+}. This protocol approach may be applicable in MH diagnostic and screening tests in humans, but for validation, further studies are needed.

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