The Use of a Platelet Nucleotide Assay as a Possible Diagnostic Test for Malignant Hyperthermia

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A bioassay, using high-performance liquid chromatography (HPLC) analysis of platelet adenosine nucleotides and hypoxanthine, was studied for its potential use as a test for MH susceptibility. A protocol for the assay was developed, based on the method outlined by Solomons and Masson. The HPLC procedure was a rapid, efficient, sensitive, and highly reproducible technique for measuring ATP, ADP, AMP, and hypoxanthine in platelets. Conditions of extraction and storage were critical for preventing degradation of the nucleotides. Extraction of nucleotides at ice-bath temperature was found necessary. Storage of platelet extract in PCA, even at −20°C, showed loss of ATP and ADP; hence, neutralization with KOH was essential before storage. Contrary to the findings of Solomons et al., the present study demonstrated that neither ATP depletion nor per cent reduction in nucleotide ratios in platelets treated with halothane can be used as a definitive test for the diagnosis of MH susceptibility. The reason for this disagreement is unclear; however, differences in methods and altitude are implicated. It is possible that the platelet is not affected by malignant hyperthermia and thus cannot serve as a test system for the detection of the syndrome. (Key words: Anesthetics, volatile: halothane. Blood: platelets; nucleotide assay. Hyperthermia: malignant; diagnosis. Measurement techniques: chromatography.)

Susceptibility to malignant hyperthermia (MH) currently is diagnosed most reliably either by observing an MH reaction in a patient or by in vitro tests requiring biopsied muscle tissue.1-4 A simple, inexpensive, and less-invasive test is urgently needed. Solomons and Masson claimed to have found a simple platelet nucleotide assay for the detection of MH susceptibility.5,6 Giger and Kaplan were unable to confirm the reliability of this test, possibly because of variations in test methods.7 We report here our efforts to fully develop the method outlined by Solomons and Masson and to confirm or refute the reported reliability of this test in the diagnosis of MH.

Materials and Methods

All chemicals used were of highest purity. All nucleotide standards (as the sodium salts) were vanadium free (Sigma Chemical Co., St. Louis, Missouri). These solutions were prepared in 0.025 M NaK phosphate buffer, pH 6.85.

After obtaining proper authorization from our institution's Human Research Committee, we selected at random 10 subjects (seven female and three male) having no personal or family history of adverse anesthetic reactions and 10 subjects (five female and five male) previously diagnosed as being susceptible to MH by criteria described in table 1. The effect of adding halothane in vitro to PRP on the basal levels of platelet nucleotides and hypoxanthine, as well as on the relative ratios of the nucleotides, was investigated with the use of the procedure described below. At least one sample from control subjects was processed simultaneously with the sample of test subjects.

Whole blood samples (3.5 USP units of heparin/ml) were obtained by venipuncture with the use of a Butterfly® infusion set (Abbott, Chicago, Illinois), after drawing and discarding the first 2 ml of blood, and kept at room temperature until processed (within 1 h, except for two patients and one control sample, which were done within 3 h). The heparinized blood was placed into 17 × 100 mm capless polyethylene tubes (Evergreen Scientific Co., Los Angeles, California), covered with Parafilm® and centrifuged at 450 x g for 15 min at room temperature. The supernatant, platelet-rich plasma (PRP), was transferred with a plastic pipette tip to an 8-ml, 11 × 100 mm polypropylene tube with screw cap (Falcon, Division of Becton-Dickinson and Co., Oxnard, California) and capped. (Initially, a capped, 10 × 40 mm, 1.5 ml Evergreen tube was used throughout the procedure, but, because it is difficult to handle because of its small size and tight cap, it was replaced by the larger Falcon tube). The bottom quarter of the super-

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TABLE I. Clinical and Laboratory Evidence of Patient’s MH Susceptibility

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Severe rigidity of skeletal muscles after 1 dose of succinylcholine, heart rate to 140 beats/ min, severe myoglobinuria, creatinine kinase (CK) greater than 80 times normal.</td>
</tr>
<tr>
<td>B</td>
<td>Sustained pulse rate of 160 beats/min and respiratory rate of 30 breaths/min after inhalation induction of anesthesia, temperature rise of 2.5°F. Son has MH by caffeine contracture study.</td>
</tr>
<tr>
<td>C</td>
<td>Calcium uptake 6.0 μmol·g⁻¹·min⁻¹. (Normal = over 9). Daughter had severe MH episode.</td>
</tr>
<tr>
<td>D</td>
<td>Calcium uptake 5.6 μmol·g⁻¹·min⁻¹. Multiple CKs 5–10 times normal in absence of other muscle diseases.</td>
</tr>
<tr>
<td>E</td>
<td>Skeletal muscle rigidity, respiratory and metabolic acidosis, and CK elevation after administration of succinylcholine.</td>
</tr>
<tr>
<td>F</td>
<td>Sustained tachycardia to 160 beats/min, fever for four days following 20 min of halothane inhalation for closed reduction of a dislocated knee. Calcium uptake 2.1 μmol·g⁻¹·min⁻¹ (normal = &gt;9), multiple CKs several times normal in absence of other muscle disease.</td>
</tr>
<tr>
<td>G</td>
<td>In presence of 1% halothane, 0.8 mM caffeine (normal = &gt;4.0 mM) to produce 1 g increase in resting tension in skinned fibers. Mother died of MH. Sister has MH by caffeine contracture study.</td>
</tr>
<tr>
<td>H</td>
<td>Calcium uptake 7.2 μmol·g⁻¹·min⁻¹. (Normal = &gt;9), multiple CKs 2–3 times normal in absence of other muscle disease.</td>
</tr>
<tr>
<td>I</td>
<td>Temperature increase of 4.6°F and tachycardia to 150 beats/min at induction of anesthesia; 1.0 mM caffeine in presence of 1% halothane (normal = &gt;1.2 mM) to produce 1 g increase in resting tension of muscle.</td>
</tr>
<tr>
<td>J</td>
<td>Son has MH by caffeine contracture study. Calcium uptake 4.1 μmol·g⁻¹·min⁻¹. (normal = &gt;9).</td>
</tr>
</tbody>
</table>

canted, and the platelet sediment was placed in an ice bath before the extraction procedure.

One hundred microliters of cold 6% (v/v) perchloric acid (PCA) was added to the platelet sediment and mixed gently with a vortex mixer for 10–15 s. The tubes were left in the ice bath for 5–10 min to ensure complete protein precipitation, then centrifuged at 1,300 × g for 1 min. The supernatant was removed and transferred with immediate mixing to a 1.5 ml 10 × 40 mm polyethylene tube (Evergreen Scientific Co.) containing 10 μl of 8.6 N KOH to neutralize the perchloric acid. The KClO₄ precipitate in each tube was removed by centrifugation at 3,000 rpm for 1 min. The supernatant was transferred to a 1.5-ml polyethylene tube, capped, and immediately frozen at −20°C for storage until HPLC analysis was completed within 1 week of extraction.

Twenty-microliter aliquots of the platelet extracts were analyzed by reverse-phase chromatography at ambient room temperature. The chromatographic conditions were as follows: a 4 mm × 15 cm, 5 μm particle, C₁₈-μBondapak®, “Resolve” column (Waters Associates, Milford, Massachusetts) eluted with 0.10 M aqueous NH₄H₂PO₄ at a flow rate of 2 ml/min (approximately 2,500 psi) with detector set at 254 nm.

Individual peaks were identified by comparison of retention times obtained with standards injected under the same conditions. For each run, data for a calibration curve were obtained, both before and after sample analyses, to ensure the maintenance of the analytic conditions. The concentration of the nucleotides or derivatives in the platelet extract were calculated from the slope of the calibration curve obtained.

Stability of platelet extract during storage before HPLC analysis was studied by storing standards at two temperatures: 0–4°C, overnight (14–15 h) and for 1 week.

Student’s t test for difference of the means was used when comparing data for controls and subjects and data obtained in the presence or absence of halothane. Linear regression analysis was used to assess linearity of response for standard curves.

Results and Discussion

EVALUATION OF THE EXTRACTION AND HPLC MEASUREMENT OF ADENINE NUCLEOTIDES

Initially, acid extraction of the platelet nucleotides was tried at room temperature (approximately 25°C). Comparative studies of nucleotide levels and ratios with and without halothane at both room temperature and 4°C made it clear that extraction of platelet nucleotides at 0–4°C was essential (fig. 1).
The size of tube used for incubation with halothane had no effect on the chromatographic recovery of nucleotides, and moreover the per cent reductions in the nucleotide ratios produced by halothane treatment were 52.9% and 54.9% with the Evergreen and Falcon tubes, respectively. This difference was not statistically significant.

The chromatographic response was linear for all amounts injected in the column (from 0 to 0.90 nmol/20 μl injected). The within-run reproducibility of the chromatograms of the standards varied between 1.7% to 4.2% and the between-run variations ranged from 5.2% to 7.8%. The concentrations of standards used for these runs were (nmol/l): adenosine triphosphate (ATP) 33.50, 16.75, and 8.38; adenosine diphosphate (ADP) 40.90, 20.45, and 10.23; adenosine monophosphate (AMP) 24.6, 12.3, and 6.15; hypoxanthine (HYPX) 36.80, 18.40, and 9.20.

The results of the stability studies indicate that no significant deterioration occurred with any of the compounds in the working standard for the conditions of storage temperature and time tested.

Under acidic conditions, the adenosine nucleotides, particularly ATP, are labile, even when kept in the frozen state at −20 °C. In PCA solutions, levels of ATP, ADP, and AMP, but not HYPX, changed appreciably (table 2). Storage of the platelet extracts in PCA, therefore, would be most unsuitable, and neutralization with KOH is absolutely necessary for the accurate quantitation of the nucleotides.

Quantitation of nucleotides often is done with the use of calibration with external standards. Solomons and Masson recommended the use of an internal standard for platelet nucleotide analysis by reverse-phase chromatography. We chose to base calibration on external standards, using peak height measurements, throughout this study for the sake of speed and to be able to compare our results with those of the other groups who have worked on platelet metabolism.

**Effect on Basal Levels of Nucleotides and Hypoxanthine**

The in vitro effects of halothane on the levels of ATP, ADP, AMP, and HYPX are presented in figures 2A–D. To facilitate comparison of the data, the initial values obtained for the various samples were standardized to a platelet count of 10¹¹. At all doses of halothane, there was a highly significant depletion of ATP compared with the untreated platelets for both the control and patient groups. This halothane-induced decrease in platelet ATP was dose dependent. Halothane-induced depletion of muscle ATP has been shown by Harrison et al.⁸ to be a reliable diagnostic test for MH susceptibility in Landrace pigs. With humans, this test, although not always reliable, was recommended by Britt et al.⁵ as an adjunctive test for detecting MH. Solomons et al.⁹,¹⁰ have observed highly significant ATP depletion in halothane-treated platelets from MH but not normal subjects

**Table 2. Stability of Standard Solution: Comparison of Stability in Perchloric Acid and Perchloric Acid Neutralized with KOH.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Freshly Prepared*</th>
<th>Buffer†</th>
<th>PCA‡</th>
<th>PCA + KOH§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>52</td>
<td>51</td>
<td>54</td>
<td>68</td>
</tr>
<tr>
<td>ADP</td>
<td>55</td>
<td>57</td>
<td>64</td>
<td>54</td>
</tr>
<tr>
<td>AMP</td>
<td>22</td>
<td>22</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>HYPX</td>
<td>32</td>
<td>33</td>
<td>35</td>
<td>34</td>
</tr>
</tbody>
</table>

Injection volume was 20 μl.

Values are average of duplicate determinations.

* One hundred microliters phosphate buffer + 10 μl deionized water + 20 μl std. Made on day of analysis.

† Same as *, but stored at −20°C for 1 week.

‡ One hundred microliters 3% (v/v) PCA + 10 μl water + 20 μl std. Stored at −20°C for 1 week.

§ One hundred microliters 6% PCA + 10 μl 8.6 N KOH + 20 μl std. Stored at −20°C for 1 week.
levels except at a 10 µl dose of halothane, for their normal and patient groups.

The levels of both AMP and HYPX increased in halothane-treated platelets, and this was dose-related, as shown in figures 2C and 2D, respectively. The increase in AMP was not statistically significant at the halothane concentration tested. Giger and Kaplan detected no significant change in AMP up to a level of 20 µl halothane for either group. No clinically significant difference was found for HYPX.

**Effect of Halothane on Nucleotide Ratios**

Solomons and Masson⁸,⁹ have claimed that in platelets the per cent reduction in the ratio of adenine nucleotides is a sensitive diagnostic test for MH susceptibility. The nucleotide ratio for the control sample containing no halothane (R₀) and for the sample treated with halothane (Rₙ) was calculated, using peak height measurements (nm) from the appropriate chromatograms, with the equations:

\[ R \text{ or } Rₙ = \frac{[(ATP) + (ADP)]/(AMP)}{(ATP) + (ADP) + (HYPX)} \]

The per cent reduction in R = 1 - \( \frac{Rₙ}{R} \) × 100,

where

\[ Rₙ = \text{ratio obtained with sample treated with halothane} \]
\[ R = \text{ratio obtained with control having no halothane} \]

In the present study, the percentage reduction for the ratios [(ATP) + (ADP)]/(AMP) and [(ATP) + (ADP)]/[(AMP) + (HYPX)] for control and patient groups at halothane doses of 1, 2, 5, and 10 µl are presented in table 3 together with the data of the Solomons and Masson study. At all levels of halothane addition, no statistically significant difference was found between the control and patient groups for the per cent reduction in either of the ratios considered. The data from the present study, therefore, do not support two previous findings by Solomons et al.⁵,⁶,⁸: first, that platelets exposed to halothane in vitro, from MH-susceptible subjects had abnormal [(ATP) + (ADP)]/(AMP) ratios, compared with normal subjects; and, second, that the percentage reduction in the ratios [(ATP) + (ADP)]/[(AMP) + (HYPX)] determined at 5.0 µl of halothane provided a sensitive index to discriminate between normal and MH-susceptible subjects.

With respect to the first observation, the apparent disagreement could be a consequence of a different experimental approach, since Solomons’ group used the synthesis of adenosine nucleotides from radio-labeled adenine precursors to calculate the reported ratios. With regard to the second observation, their experimental
approach appears quite similar to that employed here. As seen in table 3 for the results on the control group, all levels of halothane used in the present study produced a greater per cent reduction in ratio than that reported by Solomons and Masson with 5.0 µl of halothane, whereas the opposite was observed for the patient group. For 25 control subjects, the latter investigators obtained a reduction of 22.3 ± 15.0% (mean ± 1 SD) at a 5.0 µl halothane treatment. In contrast, at the same dose level, the present study on 10 controls gave corresponding values of 53.7 ± 15.2%. On the patient group Solomons and Masson observed a reduction of 75.1 ± 13.2% compared with 46.7 ± 13.3% reported here. Explanation of the obvious disagreement in values can only be speculative. In the present study, HPLC analysis was done on neutralized platelet extracts, whereas Solomons and Masson employed untreated PCA extracts. The instability of adenosine nucleotides, particularly ATP and ADP, in acidic PCA extracts, even when stored at −20°C has been demonstrated here. It is tempting to suggest that the large reduction in ratio (approximately 75%) observed with the patient group in their study could be artificial. For example, if acidic extracts for patient samples were analyzed habitually after those for controls, the degree of nucleotide hydrolysis could be minimal in the control extracts and maximal in the patient extracts, resulting in higher per cent reduction in ratios for the latter group. The present study used conditions that minimized nucleotide degradation in platelet extracts to assure accurate quantitation.

Another possible reason for the discrepancy observed on comparison of the patient data from both studies could be altitude effect on platelet function. The flux of nucleotides (and perhaps calcium) across platelet membranes may be a function of platelet metabolism, which, like that of the red blood cell, might be altered by chronic exposure to low atmospheric pressure. If the membrane flux is altered to a different extent in MH than in normal subjects at high altitudes, this could explain the failure of investigators working at lower altitudes to duplicate and confirm the findings of Solomons et al., who worked at 5,000 ft. Platelets are known to alter their ability to aggregate at high altitude, but no evidence known to us exists for relating this phenomenon to alterations of ion flux through the platelet membrane.

This work indicates that platelets, when analyzed for adenine nucleotides by the method, and under the conditions described, cannot serve as an indicator of susceptibility to malignant hyperthermia.

References


<table>
<thead>
<tr>
<th>Halothane Added (µl)</th>
<th>(ATP + ADP)/(AMP)</th>
<th>(ATP + ADP)/(AMP) + (HYPX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Patient</td>
</tr>
<tr>
<td>1.0</td>
<td>27.9 ± 11.7</td>
<td>31.0 ± 8.9</td>
</tr>
<tr>
<td>2.0</td>
<td>27.9 ± 14.2</td>
<td>34.8 ± 11.8</td>
</tr>
<tr>
<td>5.0</td>
<td>51.7 ± 16.0</td>
<td>46.9 ± 10.7</td>
</tr>
<tr>
<td>10.0</td>
<td>59.8 ± 12.4</td>
<td>57.5 ± 6.9</td>
</tr>
<tr>
<td>Solomons and Masson's Study: 5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Value in brackets designates number of subjects. NS = difference between control and patient group not significant by t test.