HALOTHANE STIMULATES THE AGGREGATION OF PLATELETS OF BOTH NORMAL INDIVIDUALS AND THOSE SUSCEPTIBLE TO MALIGNANT HYPERTHEMIA

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SUMMARY

Platelet responses to halothane in normal individuals and in patients susceptible to malignant hyperthermia were evaluated. Platelets in platelet-rich plasma from both normal controls and patients underwent aggregation in response to halothane. There was no significant difference in the degree of aggregation between normal subjects and patients. 

Aggregation by halothane was associated with a change in platelet shape, centralization of platelet granules, and phosphorylation of platelet actin binding protein, myosin light chain, and a 40,000-dalton protein. Aggregation induced by halothane could be inhibited by EGTA, PGE, adenosine and verapamil, but not by aspirin. 

Aggregation induced by halothane could be potentiated by small doses of adrenaline or ADP and in some individuals by caffeine. However, previous exposure of platelets to halothane made them subsequently less aggregable to ADP. The results of these studies do not support a use of halothane-induced aggregation of platelets to detect an abnormality in individuals susceptible to malignant hyperthermia, but do provide new evidence of the effects of halothane on cellular function.

PATIENTS AND METHODS

Malignant hyperthermia patients

Seven patients who had experienced documented episodes of malignant hyperthermia, muscle rigidity or hyperthermia, or both, on anaesthetic exposure associated with a marked increase in creatinine phosphokinase concentration were available for the present study.

Controls

Thirty-five apparently normal controls (23 male) (aged 15 - 50 yr) with no personal or family history of malignant hyperthermia were studied.

Preparation of platelet-rich and platelet-poor plasma

Blood was obtained from the antecubital vein following informed consent, and anticoagulated immediately with heparin 30 u. ml⁻¹. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 100 g for 20 min. Platelet-poor plasma (PPP) was obtained by centrifugation of the blood at 3000 g for 5 min. In some experiments, citrate anticoagulant (Gerrard et al., 1979) was evaluated also.

Platelet aggregation

Platelet aggregation was assessed using a Payton dual-channel Aggregometer (Payton Assoc., Scarborough, Ontario) after the method of Born and Cross (1963). The chart recorder was set with PRP as 0% aggregation and PPP as 100% aggregation. Halothane at concentrations of 2 - 90 mmol litre⁻¹

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Fig. 1. Halothane-induced aggregation of platelets from a patient with malignant hyperthermia. Platelet aggregation was assessed using a Payton aggregometer with increasing aggregation shown by increasing light transmission. Halothane to achieve the final concentration shown at the right (1, 4.5, 9 and 45 mmol litre\(^{-1}\)) was added at time 0 as indicated by the arrow.

(0.02–1% v/v) was then added to the PRP either directly in a few microlitres or indirectly following dilution of the halothane immediately beforehand in ethanol for the lower concentrations. After the addition of halothane to the PRP, the aggregometer vial was sealed with parafilm to decrease loss of halothane by vaporization. More than 0.5% ethanol was never added to the PRP. Inhibitors, when used, were added 30 s to 1 min before halothane. Percent aggregation was measured from the graph paper using the maximal extent of aggregation within 5 min of the addition of halothane.

Platelet electron microscopy and protein phosphorylation

Samples for study of platelet ultrastructure were processed as previously described (Gerrard et al., 1979), except that they were embedded in Spurr resin and evaluated using a Phillips 400 electron microscope. Platelet protein phosphorylation was evaluated as described previously (Carroll and Gerrard, 1982) following equilibration of the platelets with \(^{32}\)P-orthophosphate for 1 h, washing the platelets once by addition of citrate anticoagulant 1 ml per ml PRP (Gerrard and Graff, 1980), centrifuging at 1000\(g\) for 10 min at 4\(^\circ\)C, and then resuspending the platelets in Hank’s balanced salt solution pH 7.4 unit containing bovine serum albumin 1 mg ml\(^{-1}\) (Sigma).

Reagents

Halothane, containing 0.01% thymol, and halothane with no thymol added were obtained from Halocarbon (Ontario) Ltd, Malton, Ontario.
Thymol, caffeine, adenosine, sodium heparin (from porcine intestinal mucosa) and aspirin were obtained from the Sigma Chemical Co. (St Louis, Mo.) and made up as solutions in Hank's balanced salt solution pH 7.4 unit or Tris-saline 0.1 mollitre$^{-1}$, pH 7.4 unit. Dantrolene was kindly provided by Norwich–Eaton Ltd, Paris, Ontario and was made up as the sodium salt in Hank's balanced salt solution before use. $^{32}$P-Orthophosphate was obtained from New England Nuclear.

RESULTS

Platelet aggregation in response to halothane

Platelets from patients with malignant hyperthermia were found to aggregate in response to halothane (fig. 1) in a concentration-dependent fashion. Most individuals showed only a single reversible wave of aggregation, although a few showed a second wave also. Similar results were obtained with citrate as the anticoagulant. The aggregation response was not a result of the presence of thymol since halothane without thymol was also active, and thymol in concentrations similar to that present in the halothane had no effect. The effect on platelet aggregation was specific for halothane as neither enflurane nor suxamethonium caused either changes in platelet shape or aggregation. Initial studies suggested that normal controls might show less aggregation in response to halothane than individuals with malignant hyperthermia, but after analysis of the 35 normal individuals the difference between normals and patients became insignificant (fig. 2). Numerous attempts to modify the assay to bring out larger differences between patients and normal individuals failed.

Characterization of the platelet response to halothane

In view of the widespread use of halothane as a general anaesthetic, we further evaluated the effects of halothane on platelets. Electron microscopy showed that platelets from patients with malignant hyperthermia were similar to those from normal patients (fig. 3). The responses to halothane, of platelets from patients and normal individuals, were similar and involved an initial extension of pseudopods, a movement of the granules to the cell

![](image.png)

**Fig. 3.** A platelet from a sample of platelet-rich plasma taken from a patient with known susceptibility to malignant hyperthermia. Intracellular structures including alpha granules, dense bodies, microtubules, open canalicular system and dense tubular system were present in these cells as in normal platelets. Granules are distributed randomly throughout the cytoplasm. (Stained with uranyl acetate and lead citrate.)
centres, and platelet adherence in aggregates similar to that seen with ADP or thrombin (fig. 4). Using platelet-rich plasma from most individuals, the aggregation in response to halothane reversed rapidly after the initial wave even under conditions where up to 60% aggregation was achieved during the first wave. In a few individuals there was a second wave of aggregation with the development of large aggregates. These large aggregates were similar to large ADP- or thrombin-stimulated aggregates except that, at the periphery of these aggregates, the platelets were relatively round with few pseudopods (fig. 5), while some had undergone a central movement of granules. The platelet aggregation in response to halothane was associated with stimulation of the phosphorylation of three platelet proteins, actin-binding protein, myosin light chain and a 40 000-dalton protein (fig. 6).

Platelet aggregation induced by halothane could be blocked by agents which inhibit platelets by increasing cell cyclic AMP concentration (PGE₁, adenosine), by EGTA which chelates extracellular calcium (fig. 7), or by verapamil, an agent which interferes with membrane fluxes of calcium (fig. 8).
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Fig. 6. Phosphorylation of three platelet proteins was assessed by evaluating the incorporation of phosphorus-32 into these proteins, using platelets which had been pre-equilibrated with 32P-orthophosphate to label their metabolic ATP pool. Proteins were separated by SDS-polyacrylamide gel electrophoresis and the resultant gels subjected to autoradiography (Gerrard and Carroll, 1981). The absorbance of the individual protein bands on the autoradiograph was then assessed, using a Beckman Du-8 spectrophotometer. Increasing absorbance of the protein band on the autoradiograph represents increasing protein phosphorylation. Within the 1st min after addition of halothane 18 mmol litre\(^{-1}\) at time 0 there was a 1.5-fold increase in phosphorylation of actin-binding protein (ABP), a 2.7-fold increase in phosphorylation of the 40 000-dalton (40P) protein, and a 3.5-fold increase in phosphorylation of myosin light chain (MLC). Results shown were obtained using platelets from a normal individual. Similar results were obtained using platelets from a patient with malignant hyperthermia.

Fig. 7. The influence of EGTA, PGE\(_1\), and adenosine on halothane-induced platelet aggregation. Inhibitors were added at the first arrow, and halothane (9 mmol litre\(^{-1}\)) at the second arrow. Results were representative of three separate experiments.

Fig. 8. The influence of verapamil on halothane-induced platelet aggregation. Verapamil was added at the first arrow, and halothane at the second. Results are representative of three separate experiments.
In contrast, aspirin 100 μmol litre\(^{-1}\), which inhibits platelet prostaglandin synthesis, caused no inhibition of halothane-induced aggregation (first-wave aggregation only was tested). Dantrolene, a compound which blocks calcium release from the sarcotubules of skeletal muscle, produced 50–100% inhibition of halothane-induced aggregation depending on the donor, but only when used at a concentration of 2 mmol litre\(^{-1}\) (fig. 9).

Caffeine, which inhibits platelet phosphodiesterase and is usually an inhibitor of platelet function, was found to have variable effects on the platelet response to halothane. In some individuals definite potentiation was found repeatedly with caffeine (fig. 10).

Addition of ADP or adrenaline with halothane potentiated the halothane response (figs 11, 12). However, when ADP was added after halothane, the response to ADP was decreased (fig. 13).

**DISCUSSION**

The results of the present study show that platelets from both normal individuals and those susceptible to malignant hyperthermia will aggregate in response to halothane. The lack of a difference between the normal subjects and those susceptible to malignant hyperthermia would suggest either that the platelet is not involved in this disease, or that the defect is not in a component of the initial response, but rather of a later process. Two previous studies which evaluated platelet aggregation in response to ADP, adrenaline, collagen, arachidonic acid and A23187 also found no difference in the platelet aggregation response of normal subjects and those susceptible to malignant hyperthermia (Rosenberg et al., 1981; Sullivan, Ardlie and Denborough, 1982).

The mechanism of the effect of halothane is of some interest. The present studies are consistent with the hypothesis that halothane acts to trigger a release of calcium from the dense tubular system to increase the cytoplasmic calcium concentration. Platelet aggregation and centralization of platelet granules can occur subsequent to an initiating intracellular flux of calcium (Gerrard, Peterson and White, 1981). Phosphorylation of platelet proteins, particularly myosin light chain, has been shown to result from an increase in the cytoplasmic calcium concentration (Lyons and Shaw, 1980). Specifically, calcium interacts with calmodulin to activate a protein kinase which is responsible for the phosphorylation event (Hathaway and Adelstein, 1979).

**Fig. 9.** Platelets from a sample of platelet-rich plasma treated with dantrolene 2 mmol litre\(^{-1}\) and then stirred with halothane 45 mmol litre\(^{-1}\). Platelets are mostly discoid as in control platelets, with granules distributed at random within the cytoplasm, although some pseudopods are present. (Stained with uranyl acetate and lead citrate.) Results are representative of six separate experiments.

**Fig. 10.** The influence of caffeine on halothane-induced platelet aggregation. Halothane 4 mmol litre\(^{-1}\) and caffeine 2 mmol litre\(^{-1}\) were added separately or together as indicated at the arrow. Three of six individuals showed potentiation of halothane-induced aggregation by caffeine. One patient susceptible to malignant hyperthermia did not show such potentiation.
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Our finding that halothane added to intact platelets stimulates phosphorylation of myosin light chain provides good evidence that one effect of halothane on platelets is to increase the cytoplasmic calcium concentration. Inhibition of the effect of halothane by verapamil, which can affect calcium fluxes (Tritthart, 1980), and by agents which increase platelet cyclic AMP (which may also work through effects on calcium flux (Kaser-Glanzmann et al., 1977)), are also consistent with the concept that halothane activates platelets by triggering a flux of calcium. The lack of effect of aspirin shows that the production of platelet prostaglandin and thromboxane is not necessary for the initial response to halothane. A role of halothane in increasing platelet cytoplasmic calcium concentrations, as proposed here, is similar to that proposed for the effects of halothane on liver cells (Zucker, Diamond and Berman, 1982).

Inhibition of halothane-induced aggregation by dantrolene was seen only at concentrations of dantrolene very high relative to those used to inhibit responses of skeletal muscle (Ellis and Carpenter, 1974). It would appear that platelets, like cardiac muscle and intestinal smooth muscle (Butterfield and Ellis, 1973), are affected minimally by dantrolene. Dantrolene may primarily inhibit a process involved in excitation–contraction coupling in skeletal muscle, which is less important in platelets. The lack of difference in halothane-induced platelet
aggregation between normal individuals and those susceptible to malignant hyperthermia, and the weak effect of dantrolene suggest that the defect in malignant hyperthermia does not lie in a process important during the initial response of platelets. Thus, we must conclude that the low ATP concentration found by Solomons and Masson (1982) in patients susceptible to malignant hyperthermia following a prolonged (20-min) exposure of the platelets to halothane relates to an effect of halothane on a platelet process which is not a major component of this initial response. The action of dantrolene to prevent the above decrease in ATP concentration (Mahowald, Masson and Solomons, 1982) and the weak inhibitory effects of dantrolene on halothane-induced aggregation (this study) further support this conclusion.

Potentiation of the effect of halothane by caffeine in some donors may be related to halothane potentiation of caffeine contracture in skeletal muscle. An effect of caffeine to release calcium from the platelet dense tubular system has not been described, but is possible. The variation in response from donor to donor may reflect the relative balance of an effect of caffeine to increase platelet cyclic AMP to inhibit platelet function and an effect of caffeine to stimulate an efflux of calcium from the platelet dense tubular system to promote platelet aggregation. Potentiation of halothane by adrenaline or ADP added together with halothane is similar to that seen with a number of other aggregating agents (Kinlough-Rathbone, Packham and Mustard, 1977; Rao, Reddy and White, 1981). The basis for the refractoriness of halothane-exposed platelets to subsequent ADP-induced platelet aggregation is unknown, but could be related to an effect of halothane in increasing platelet cyclic AMP concentration (Walter et al., 1980), and could explain the depressed responsiveness of platelets from individuals who have received halothane anaesthesia (Dalsgaard-Nielsen et al., 1981). The relatively rounded appearance of platelets at the edge of large halothane-induced aggregates could also result from an inhibitory effect of halothane (perhaps mediated by cyclic AMP) which takes longer to develop than the initial stimulating effects.

While these findings do not provide a basis for distinguishing between normal individuals and those with malignant hyperthermia, they extend our knowledge of the effects of halothane on cells and may provide the background for further investigations into the nature of malignant hyperthermia.
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L’HALOTHANE STIMULE L’AGGREGATION PLAQUETTAIRE A LA FOIS CHEZ LES INDIVIDUS NORMAUX ET CHEZ CEUX A RISQUE D’HYPERTHERMIE MALIGNE

RESUME