Halothane and the Beating Response and ATP Turnover Rate of Heart Cells in Tissue Culture

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The effects of halothane on the beating response of rat heart cells in tissue culture were studied using an optical-electronic monitoring device. A dose-response curve was obtained over a concentration range to as much as 5 vol per cent halothane. The clinical dosage of 1 vol per cent halothane decreased the inotropic response of 4-10-day-old cells to 50 ± 10 per cent of the original beating strength; no significant decrease in beating strength was seen in 25-30-day-old cells. One volume per cent halothane caused no significant change in the chronotropic response of the heart cells. Higher concentrations of halothane caused a significant negative chronotropic and negative inotropic responses in a dose-related manner. When glycolysis was inhibited by 2-deoxyglucose in the growth medium, the cells became dependent on fatty-acid oxidation and oxidative phosphorylation for energy and showed increased sensitivity to halothane; for example, the chronotropic response to 5-8-day-old cells treated with 2-deoxyglucose was decreased approximately 70 per cent by exposure to 3 vol per cent halothane, whereas 4-10-day-old cells maintained on a complete growth medium showed only a 40 per cent decrease. Increasing concentrations of halothane decreased the rate of ATP turnover. This supports evidence suggesting that halothane blocks electron transport at the NADH-coenzyme Q reductase level. The model described provides a means for determining anesthetic potency in a mammalian system in terms of functional as well as metabolic responses. It also provides a means for study of metabolic effects of anesthetics and other drugs. (Key words: Anesthetics, volatile, halothane; Metabolism, adenosine triphosphate; Heart cell cultures.)

UNDERSTANDING OF ANESTHESIA requires determination of the cellular contribution to the whole-body response; in particular, the effect of anesthetics on the energy state of the cell must be known. This in turn requires a method that can monitor an energy-dependent function of a cell. Beating rat heart cells in culture† provide a model for such a method because inotropic and chronotropic responses to drugs, hormones or anesthetics can be readily followed. Cultured rat heart cells also provide a means for determining anesthetic potency in terms of energy-dependent responses, as well as a means for comparing the potency of one anesthetic agent with that of another throughout the pharmacologically significant concentration range. At present the only other cellular system that makes possible construction of a concentration/effect curve is that of White and Dundas‡ using luminous bacteria. Until recently, techniques for determining potency provided a single piece of quantal data only, i.e., the percentage of animals in a group that manifest a particular “all-or-none” response such as loss of reaction to a standard stimulus. The cultured beating heart cells from the rat provide for study a metabolic system more closely related to that of human subjects.

Studies at the subcellular mitochondrial level indicate that halothane inhibits Complex I, NADH-coenzyme Q reductase, of the electron transport chain at concentrations used during surgical anesthesia.§ Studies of the effects of halothane on glycolysis and the biosynthetic processes of the isolated perfused rat liver confirm interference at Complex I. However, Rzeczczek and Valdivia§ reported that halothane uncouples oxidative phosphorylation in isolated beef-heart mitochondria in the presence of magnesium ions even with succinate as substrate, resulting in a decrease of ATP available for cytosol. They suggested that development of mitochondrial uncoupling may be a cytotoxic phenomenon and may be responsible for sporadic cases of malignant hyperpyrexia.

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This paper reports a method for obtaining dose-response curves for either anesthetics or other drugs in a system allowing regulation of various metabolic pathways. We show that the negative inotropic and negative chronotropic effects of halothane on beating cultured heart cells are accompanied by a corresponding decrease in the ATP turnover rate, independent of neural or hormonal control. When glycolysis is inhibited by 2-deoxyglucose an increased sensitivity to halothane is seen.

Materials and Methods

METHOD OF HEART CULTURE

The hearts from 3-5-day-old rats were dissociated for culturing by 0.1 per cent trypsin treatment. Pancreatic trypsin was obtained from Sigma Chemical Co. The cells were cultured in 60 x 15 mm plastic Petri dishes (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, California) and grown at 37 C in a NAPCO water-jacketed CO2 incubator (Model 3321, National Appliance Co., Portland, Oregon) with a 95 per cent air, 5 per cent CO2 atmosphere saturated with water. The growth medium, a modified Puck's medium,9 and culture procedure were described by McCarl and Margossian.10 Each plate received the equivalent of the cells from one heart. Every other day the growth medium on the cells was replaced with 4.0 ml of fresh growth medium. Supplies for complete growth medium (CGM) were obtained from Grand Island Biological Co., Grand Island, New York.

In addition to CGM, a 2-deoxyglucose (2-DOG) medium was also prepared and used to treat a series of cultures. This medium was identical to CGM, except that 15 mM 2-DOG were substituted for 15 mM glucose. After phosphorylation via hexokinase, 2-DOG interrupts glycolysis by competitively inhibiting phosphoglucomerase14,15 (fig. 1). Effective inhibition of glycolysis was indicated by the absence of lactic acid as a waste material in the growth medium. This was determined by the color change of the pH indicator, phenol red, present in the medium. These data are consistent with those of Orloff and McCarl,13 who grew rat heart cells in culture in the presence of 30 mM 2-DOG and 15 mM glucose and measured lactate enzymatically.

RECORDING SYSTEM FOR OPTICAL-ELECTRONIC MONITORING OF BEATING IN CULTURED RAT HEART CELLS

The extensive use of heart-cell cultures as a quantitative pharmacologic and biochemi-
HEART CELLS IN TISSUE CULTURE

A scientific tool has awaited the development of a monitoring system for both the chronotropic and the inotropic responses of cells to the addition of various drugs to the growth medium. Our optical-electronic monitor was built following some of the basic designs of Boder et al. This device takes advantage of the change in optical density and depolarization of polarized light during the contraction cycle of the cells. The amount of light impinging on a light-sensitive diode is assumed to be proportional to the contraction and relaxation of the cell. Therefore, although inotropism is defined as a change in force, it is possible to measure indirectly the relative elongation and contraction of cells during each contraction cycle by measuring changes in optical density.

The monitor consists of a light-sensitive diode (United Detector Model UDT 500) mounted on the camera port of the microscope (Olympus Inverted Microscope, Model CK-Bi, Tokyo, Japan). A 25-μm slit was placed between the sample and the diode so that focus on a small area (< 2 per cent) of the entire field was possible. A tungsten light source fitted with a polarizing filter was used. The signal obtained was amplified and recorded on a Grass Model 7 polygraph recorder. Since a blocking capacitor (0.1 μf) was wired in series with the output of the detector module, a derivative trace was recorded by the monitor.

Heart cells under observation by the monitor were still attached to the bottom of the Petri dish in which they were cultured. Fresh growth medium was applied to the cells one to six hours prior to observation. During the monitoring session, the cells were kept at 33–35°C by means of a heated microscope stage and air-curtain incubator (Sage Instruments, Inc., White Plains, New York, Model 279), and a flow of 95 per cent air–5 per cent CO₂ over the cells was maintained by means of a modified Petri dish lid. Perforated Tygon tubing was attached to the underside of the Petri dish lid, permitting a uniform flow of gas over the cellular surface. Efflux of gas was around the sides of the Petri dish. Unanesthetized cells continued to beat at a constant rate and strength for as long as 7 hours. No stringent sterility precautions were taken, although the cells remained sterile and beating for at least two weeks after treatment.

**HALOTHANE TREATMENT**

Halothane in doses of 1 to 5 per cent (v/v of gas) was introduced into the modified Petri dish lid to the cells using a carrier gas of 5 per cent CO₂–95 per cent air and a Foregger Fluomatic Vaporizer (Model DRV 1, Air Products, Allentown, Pa.). Fluothane, a brand of halothane (2-bromo-2-chloro-1,1,1-trifluoromethane, Ayerst Laboratories Incorporated, New York, N.Y.), was purchased from Mountainview Hospital, State College, Pa.

Cells were monitored prior to, during, and after exposure to halothane. The effect of the
A dose–response curve was obtained for four series of cultures. The first consisted of cultures 25–30 days old; the second consisted of cultures 4–10 days old; the third consisted of cultures 15–21 days old maintained on 2-DOG medium for at least one day prior to halothane administration; the fourth consisted of cultures 5–8 days old similarly maintained on 2-DOG medium. The results presented in figures 3 and 4 were values obtained during the seventh minute of exposure to halothane and are represented as percentages of the beating rate or strength prior to administration of halothane. As previously indicated, 7 minutes of halothane treatment allowed sufficient time for the anesthetic effect to be seen and a steady state to be reached. Prolonged treatment (more than 30 minutes) at high anesthetic concentration often resulted in cessation of beating. Each point in the dose–response curve is an average of at least three experiments.

**ATP Turnover**

The technique employed for ATP turnover measurements is a modification of that used

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**ATP Turnover**

The technique employed for ATP turnover measurements is a modification of that used
by Hammerstedt and Niehaus for spermatocytes. Heart cells were grown in culture 25–30 days or until neutralized HClO₄ extracts contained at least 5 nmoles ATP per plate of cells as determined in the automated ATP assay of Hammerstedt. The minimum concentration of ATP necessary for ATP turnover measurement is 1 nM. Therefore, one Petri dish of cells usually comprised one experimental point. A single culture dish was transferred from the incubator to the monitor and allowed to come to constant beat rate and strength (about 5 minutes). This was followed by exposure to 0 to 5 percent halothane for 7 minutes. Two tenths milliliter of carrier-free ³²P (0.4 mCi in 0.02 M HCl, New England Nuclear, Boston) was added. Halothane exposure and electronic monitoring of beat rate and strength were continued throughout the treatment with ³²P. At the end of the ³²P treatment (at intervals of 1 to 40 minutes), the cells were lysed, the proteins denatured by addition of 1 ml 24 per cent perchloric acid, and the cells immediately placed on ice. Extraneous cellular material was removed by centrifugation and discarded. The supernatant solution was neutralized with a 3 M K₂CO₃, 0.5 M triethanolamine solution to pH 7.0, centrifuged, and the precipitate discarded. Excess anions interfere with the ion-exchange chromatography; therefore, the nucleotides were adsorbed onto 0.13 g activated coconut charcoal (Fisher, 50–200 mesh) and then eluted with three 2-ml additions of ammonium ethanol (0.15 N NH₄OH in 50 percent ethanol). The eluted solution was evaporated to 0.7 ml with nitrogen gas. One quarter milliliter galactokinase buffer (200 mM Tris, 20 mM EDTA, 40 mM MgCl₂, pH 7.8), 0.05 ml of 1.43 mM uniformly labeled ³H-galactose (7.8 × 10⁵ dpm per µmole, New England Nuclear) and 0.05 units of galactokinase isolated from E. coli were added and the mixture incubated at 37 C for 15 minutes. After incubation, the sample was applied to a QAE-Sephadex Column (Q-25, particle size, 40–120 µ, Sigma) containing 0.25 g of the dry gel previously equilibrated with 0.05 N NaOH (CO₂-free). The sample on the column was washed with 20 ml of 0.05 N NaOH (CO₂-free) to remove the excess ³H-galactose. The galactose-1-PO₄ fraction containing the doubly labeled ³H-galactose-³²PO₄ was then eluted with 2 ml of 0.07 N NaOH (CO₂-free). Fractions were acidified with HCl and 20 ml of scintillation fluid composed of 0.5 per cent PPO (2,5-diphenyloxazole) in toluene:Triton X-100 (2:1) were added. The samples were counted on a Beckman Model LS 200 B liquid-scintillation spectrometer. A constant ³²PO₄ in three successive 2-ml samples indicated that a pure sample of galactose-1-PO₄ had been eluted. From the known specific activity of ³H-galactose, the specific activity of ATP was calculated and plotted for each sample against pulse time (fig. 6) to show the effect of halothane on the rate of turnover of ATP as determined by ³²P incorporation into ATP.

**³²P Uptake**

Conditions were identical to those of the ATP turnover experiment prior to treatment with ³²P. One microcurie of ³²P was added to the plate in a volume of 0.3 ml. At the end of the ³²P-treatment period (intervals of 5 to 80

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*Fig. 5. The effect of 5 percent halothane on ³²P uptake in beating rat heart cells:
- air-CO₂ (95 percent–5 percent mixture) passing over cells;
- air-CO₂ mixture + 5 percent halothane passing over cells.

Results are the means from three experiments. "Pulse time" refers to the time of exposure of the cells to ³²P. This figure demonstrates that ³²P uptake is linear with respect to pulse time in heart cells, but that 5 percent halothane does not alter the rate of ³²P uptake in this system.*
TABLE 1. ATP Pool Size* for Time Periods to 40 Minutes on the Monitor

<table>
<thead>
<tr>
<th>Minutes on Monitor</th>
<th>ATP per Plate (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.1</td>
</tr>
<tr>
<td>2</td>
<td>7.8</td>
</tr>
<tr>
<td>10</td>
<td>9.0</td>
</tr>
<tr>
<td>20</td>
<td>10.1</td>
</tr>
<tr>
<td>40</td>
<td>9.9</td>
</tr>
</tbody>
</table>

* Determined by the automatic luciferin-luciferase assay of Hammerstedt after perchlorate precipitation of the cellular proteins.
† Each plate contained 7.5 ± 0.1 mg protein as determined by the biuret method.

TABLE 2. Effect of Halothane on ATP Pool Size

<table>
<thead>
<tr>
<th>Per Cent Halothane</th>
<th>ATP per Plate* (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.2 ± 0.9</td>
</tr>
<tr>
<td>5</td>
<td>10.0 ± 3.5</td>
</tr>
</tbody>
</table>

* Standard deviation is for five experiments.

beating rate. All results shown in the dose-response curves for halothane are reported as percentages of the beating rate or beating strength before halothane was administered. In all cases the cultures recovered at least 90 per cent of the original beating strength and rate within 20 minutes after administration of as much as 5 per cent halothane had been terminated.

The halothane-inotropic and halothane-chronotropic dose-response curves are shown in figures 3 and 4. These curves show that the 4–10-day-old cells and the cells maintained on CGM were more sensitive to halothane than the older cells. This suggests that the energy source for beating was more seriously affected by halothane in young cells. Anastasia and McGivern have reported changes in metabolism as cells aged in culture. The ability to oxidize free fatty acids, and to release them from their corresponding triglycerides, decreased as the cells aged. Fujimoto and Harary, using respiratory quotient values of the heart cells, showed a shift in metabolism from fatty-acid oxidation to glycolysis as cells age. We observed, however, that older cells were also capable of lipid metabolism, although it may not be the major energy source for beating. We found when cells were maintained on 2-DOG medium (in the absence of glucose), all growth ceased, and beating stopped after 4–6 days. The beating was reinitiated when fresh 2-DOG medium was applied to the cells and continued 7–10 hours thereafter. Thus, the cells were probably utilizing the lipid present in the serum of the fresh medium as an energy source for beating, since glycolysis was still inhibited by the presence of 2-DOG. Therefore, this evidence indicates that young cells depend primarily on fatty acids as an energy source for beating. A block caused by halothane in the electron-transport chain severely limits energy production in this case (fig. 1). The presence of 2-DOG in the absence of glucose insures that glycolysis cannot be used as an alternative energy source, and forces the cells to utilize fatty-acid oxidation and oxidative phosphorylation as the major energy-producing pathways. Thus, the 5–8-day-old heart cells treated
with 2-DOG were more severely affected by halothane than the other groups of cells studied (figs. 3 and 4). The 4-10-day-old cells maintained on CGM were also more sensitive to halothane exposure than were the older cells. One would expect, however, that the effects of halothane on both young and old cells would be the same in the 2-DOG-treated cells, since in both cases both glycolysis and oxidative phosphorylation are being affected by 2-DOG and halothane, respectively. That they are not may possibly be the result of reserves of high-energy compounds that have been stored in the older cells and can be used to support beating.

**ATP Turnover**

All experiments to determine the effect of anesthetics on the energy state of the mammalian cell have utilized indirect methods, such as swelling studies, alterations in membrane carrier functions, changes in O₂ uptake, or the static ATP pool size. There now exists a method for directly measuring the ATP turnover in the cell. If internal and external phosphate equilibration is more rapid than ATP turnover and if the ATP pool remains constant over the experimental period, the rate of incorporation of P_i into ATP is a measure of ATP turnover. Table 1 shows that the ATP pool size in cultured heart cells remained fairly constant over a period of 40 minutes. In agreement with previous studies, the ATP pool size was not affected by halothane (table 2). Unfortunately, in the cultured beating heart cell, a continuous influx of P_i was seen over an 80-minute period, indicating that the uptake of P_i was a limiting factor. Therefore, the effect of halothane on P_i uptake was examined. The rate of influx was not altered by 5 per cent halothane (fig. 5), implying that, although P_i uptake by the cell may be rate-limiting, it was not this process that was affected by halothane. Thus, the results of this experiment show only relative values for the ATP turnover rate, as affected by the presence of halothane. A plot of the specific activity of ATP vs. P_i pulse time (i.e., the time interval of P_i treatment) gives an indication of the ATP turnover rate as it is affected by halothane. The decrease in ATP turnover seen in the presence of increasing amounts of halothane (fig. 6) may possibly be attributable to decreased mitochondrial respiratory-chain function. This would support the conclusions from experiments done at the mitochondrial level, where halothane is believed to inhibit oxidation of NADH in Complex I of the electron-transport chain. Similarly, a decrease in O₂ consumption was seen in tissue slices as a result of halothane. Our results, however, must be viewed with caution, since they may reflect a decrease in metabolic demands resulting from decreased function, *e.g.*, decreased myocardial contractility during anesthesia, rather than direct inhibition of mitochondrial respiration. Another alternative interpretation would be altered ATPase activity, as recently found for isolated
myosin, where halothane increased ATPase activity when myosin was Ca\(^{2+}\)-activated and depressed ATPase activity when it was K\(^{-}\)-activated. Therefore, direct interaction of anesthetic with myosin, as shown for halothane\(^{26}\) and other drugs,\(^{25}\) must be considered one of the contributing effects both in the ATP studies and in the observations of beating response. The results in the cultured beating heart cells do, however, serve to provide a link between the subcellular studies and observations made at the organ or tissue level concerning the effect of halothane on the energy state of the cell, and do show a dose-related decrease in ATP turnover. The origin of this effect is not clear at this time.

The dose–response curve for beating heart cells in tissue culture compares favorably with data obtained in whole-body experiments, where the heart rate and blood pressure during exposure to 1.1 per cent halothane were 109 per cent and 72 per cent, respectively, of the values prior to administration of anesthetic.\(^{26}\) Our system, however, eliminates contributions to the anesthetic effect due to any inhibition of the sympathoadrenal system or stimulation of parasympathetic activity, to which a great deal of the chronotropic effect of halothane has been attributed.\(^{28}\) It is probable that the action of halothane on the individual heart cells plays a significant role in the overall hemodynamic response to halothane anesthesia.

White and Dundas\(^{5}\) found that the light output of bacteria during exposure to 1 per cent halothane was only 40 per cent that in the absence of anesthetics. Thus, the luminous bacteria were more sensitive to halothane than either the cultured heart cells or the whole-body system. Therefore, while the luminous bacteria studies are valuable for comparison of anesthetic potency, they do not closely parallel the response that can be expected during surgical anesthesia. The beating-heart-cell system, on the other hand, may be more valuable in predicting the whole-body response.

Thus, the cultured beating heart cells can be used as an effective tool to study the effects of anesthetics and other drugs in a carefully controlled environment. Both normal and altered metabolic states can be easily studied. It is important that the effects of anesthetic agents on the available energy sources and metabolic pathways of the body as a whole, and on individual organs, be known. Our studies indicate that when the major metabolic pathway for energy production is fatty-acid oxidation and subsequent oxidative phosphorylation, the tissue becomes very sensitive to halothane. Lesser concentrations of anesthetic were necessary to depress the function of the heart cells. This is contrary to the results of Ko and Paradise concerning the effect of halothane on the function of atrial muscle from starved rats compared with fed rats.\(^{29}\) They argued that higher concentrations of fatty acids were available in the starved preparation, where they found that greater concentrations of halothane were necessary to depress the function of the atrial muscle. Since fewer variables are involved in the beating-heart-cell experiment the potential protective effect of fatty acids must be examined more carefully.

Our studies provide a physiologic basis at the cellular level for the effect of halothane on the energy state of the cell. Much evidence points to an interference with electron transport,\(^{3,4,6}\) and our studies support this. Direct evidence for the extent of interaction of halothane with Complex I of the electron-transport chain can be obtained by observing the five iron centers of the enzyme in an electron paramagnetic resonance (EPR) spectrometer. Recent evidence\(^{31}\) suggests that anesthetics combine with the hydrophobic part of a protein, causing it to undergo a conformational change, releasing bound water. The volume of proteins in nerve membranes has been observed to increase 70 ml per mole of active protein in the presence of anesthetic. Whereas nerve membranes expand their area by about 15 to 20 per cent in the presence of a concentration of anesthetic that induces anesthesia, the anesthetic absorbed would be expected to increase the volume by only a fifth this much, indicating a selective lengthening of the protein molecules that regulate membrane surface area. A similar mechanism may operate in
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Complex I, where halothane may bind to hydrophobic parts of the protein and cause a critical change in conformation, resulting in impairment of function. Experiments are planned to determine the extent of conformational change in isolated Complex I in the presence of halothane using circular dichroism techniques, and the site of inhibition in relation to electron flow through the enzyme determined by EPR studies.

Our present studies have succeeded in developing a mammalian model system that can monitor an energy-dependent function of the cell. This system was used to examine the cellular response to anesthesia, which closely paralleled the human whole-body response. Metabolic studies of the heart-cell system indicate that fatty-acid oxidation is more sensitive to halothane than is glycolysis, and that the rate of ATP turnover is decreased by halothane.

The authors thank John D. Stoner for construction of the heart-cell monitor.

References

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