Is Halothane a True Uncoupler of Oxidative Phosphorylation?

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The ability of isolated rat liver mitochondria to accumulate calcium in the presence of various concentrations of halothane was studied. Calcium transport into mitochondria is completely prevented by true uncouplers of oxidative phosphorylation. Mitochondrial calcium accumulation continued in the presence of halothane concentrations as high as 4 per cent. The rate of calcium accumulation slowed as halothane levels increased, but the amount accumulated was the same. The conclusion is that halothane is not a true uncoupler of oxidative phosphorylation, and that any uncoupling with anesthetic levels must be very limited. Malignant hyperpyrexia associated with halothane anesthesia is probably not the result of a direct uncoupling of oxidative phosphorylation in normal mitochondria. Greater susceptibility of mitochondria in individuals prone to hyperpyrexia reactions for genetic reasons is not ruled out.

During the past ten years reports of rapid increases in body temperature of patients undergoing anesthesia and operation*† have appeared. The striking features of the hyperthermia have been the rapidity of the rise in temperature, the lack of any uniformly successful treatment, and the high mortality (73 per cent).

Following a review of reports between 1960 and 1965, Wilson et al.2 suggested that the etiologic factor in malignant hyperpyrexia might be the uncoupling of oxidative phosphorylation—a specific function of mitochondria. The same year, Snodgrass and Piras4 categorized halothane as a “true uncoupler of oxidative phosphorylation,” based on a study of isolated rat liver mitochondria. In 1968, Cohen et al.2 described a decrease of respiratory control5 in isolated rat liver mitochondria following exposure to concentrations of halothane used in clinical anesthesia. The following year, Catz and Jones7 reported the effects of six general anesthetics on isolated brain mitochondria and suggested uncoupling of oxidative phosphorylation by halothane and other anesthetics as the mechanism of malignant hyperpyrexia. Recently, Miller and Hunter8 suggested that a very limited degree of uncoupling could explain the decrease of respiratory control observed in rat liver mitochondria. Each of the above investigations has pointed to the possibility of uncoupling of oxidative phosphorylation by halothane, particularly as the concentration is raised. However, it is important to consider carefully whether the concentrations are equivalent to those in clinical use and to take into account the conditions of exposure of the isolated mitochondrial system to halothane.

The method by which mitochondria are exposed to halothane makes a considerable difference in the results obtained with anesthetic ranges of concentration. Snodgrass and Piras mixed mitochondria with halothane-containing medium, then centrifuged and resuspended in fresh medium. Material was probably extracted from the mitochondria, and the actual tests were conducted in a system of unknown halothane concentration. For experiments with halothane, equilibration of mitochondria with halothane vapor appears most suitable. In our experience the addition of liquid halothane to mitochondrial suspensions will cause uncoupling at final concentrations which do

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not cause uncoupling when equilibration with the vapor is used.

It should also be noted that while isolated mitochondria equilibrated with halothane vapor can be uncoupled by halothane (as judged by some increase in state 4 respiration and the absence of a response to ADP or 2,4-dinitrophenol) substantial uncoupling occurs only at concentrations (3 per cent vapor) far exceeding those which could reasonably be expected in tissues during clinical anesthesia (0.7–1 per cent). Only when mitochondria were equilibrated with halothane vapor in concentrations greater than 3 per cent were large decreases in respiratory control noted, and 4 per cent halothane was necessary to abolish all response to addition of ADP. At the time the ADP response is abolished, electron transfer with succinate as substrate is inhibited 63 per cent.

The criterion for uncoupling of oxidative phosphorylation in the studies cited above was a decrease in net phosphate uptake compared with oxygen consumption, or a decrease in phosphate acceptor control ratios (respiratory control ratios). Another way to test for possible uncoupling is to examine energy-dependent functions of mitochondria. One such function, which is extremely sensitive to true uncouplers of oxidative phosphorylation, is calcium accumulation by mitochondria. We have studied the effects of halothane in clinically relevant and biochemically interesting concentrations on the rate and extent of Ca** uptake as a process affected by any uncoupling action. This would reflect the degree of uncoupling by halothane.

The schematic representation in figure 1 depicts the mitochondrial electron-transfer chain. Oxidation of any substrate which requires NADH provides NADH, which is then oxidized by the flavin-containing enzyme NADH dehydrogenase (F₆H₄). The electrons are then transferred through cytochromes b, c, a, and a₃ to oxygen. When electrons are transferred from NADH through the entire electron-transfer chain, three molecules of ATP are formed in energy-conserving reactions. This coupling of electron transfer to the enzymatic phosphorylating sites is accomplished by generation of high-energy intermediates (X ~ Y), the nature of which remains to be established. When succinate is oxidized by the flavin-containing enzyme, succinic dehydrogenase (F₆S₃),
electrons are transferred through the cytochrome chain as above, but only two ATP molecules are formed.

Previous studies have established that halothane does induce inhibition of NADH dehydrogenase, an effect which could be substantial with clinical concentrations of the anesthetic (80 per cent inhibition with 2 per cent halothane). Succinate oxidation is not inhibited unless concentrations of halothane higher than those used clinically are used. Therefore, in order to test whether halothane acts as a true uncoupler of phosphorylation, succinate was used as the substrate for oxidation.

High-energy intermediates formed during electron transfer may be utilized for ion movement. In the absence of an external ATP supply, mitochondrial movement of ions, in this instance calcium, can proceed only when electrons are being transferred and the electron-transfer chain is coupled to the ATP-producing mechanism where high-energy intermediates are formed. The uncoupler-sensitive site is closely associated with the electron-transfer chain. Schematically, it is represented between the site of calcium accumulation and the electron-transfer chain. Therefore, in the presence of an uncoupler no high-energy intermediates are generated, and calcium cannot be transported into mitochondria.

The factors which can significantly alter the kinetics of mitochondrial calcium uptake and release are inhibition of the rate of electron transfer, inhibition of the production of the high-energy intermediate necessary for ion movement, and inhibition of the function of a specific calcium carrier in the membrane.

**Methods**

Chemicals were purchased from the following sources: murexide from Fisher Scientific; Tris and EDTA from Sigma; succinic acid, sodium acetate, and sucrose from Mallinkrodt; carbonyl cyanide m-chlorophenylhydrazone (CCCP) from du Pont; crystallized bovine plasma albumin from Armour.

Eight Sprague-Dawley Rats (Holtzman) were fed Purina Chow and water ad lib. and were sacrificed at the same time of day. Circadian rhythm was controlled by use of timer-controlled lighting in a small-animal quarters. Mitochondria were isolated from the livers.

Eighty experiments were done. The method used has been described elsewhere, except for two modifications. The two final washes were made with 0.33 M sucrose to remove the EDTA and bovine plasma albumin used during homogenization. Mitochondria were resuspended in 0.33 M sucrose (protein approximately 53 mg/ml). Pretreatment of mitochondria and media with halothane was carried out as described, except that 0.5-ml amounts of mitochondrial suspension were treated with a 5-l/min stream of air containing halothane for 90 seconds.

The method of Mela and Chance was used to measure rapid movements of ionized calcium into and out of mitochondria. This method can detect concentration changes as small as $5 \mu M$ calcium. Murexide, the purple dye used, does not enter mitochondria, but forms a colorless complex with external divalent cations. As the ratio of complexed murexide and free murexide changes, the absorbance for 540 nm minus 510 nm changes. A dual-wavelength spectrophotometer was used to measure the absorbance difference between the two wavelengths of light. The absorbance of light increases as calcium is transported into the mitochondria, with an increase in free (colored) murexide outside. When an uncoupler is added, the calcium released by the mitochondria forms a colorless complex with murexide, with a concomitant decrease in absorbance. Calcium is also released when other mechanisms limit the availability of energy for inward transport, such as when the electron-transfer chain is inhibited or when the oxygen supply is exhausted. Under the experimental conditions used with the murexide technique the half-time for uptake of calcium is approximately 10 seconds and the half time for release by uncouplers is 3 seconds. Such rapid changes can be measured only with the murexide method, for cation-sensitive electrodes have a much longer response time and $^{42}$Ca measurements require sampling times of 15-30 seconds.

The reaction medium contained 2.6 ml of 0.33 M sucrose in 25 mM Tris acetate, pH 7.2. Addition of reactants were made with calibrated constriction pipettes, and mixing was accomplished by blowing air into the cuvette. Rotenone (2.1 $\mu M$) was used to produce complete inhibition of the electron-transfer chain at NADH dehydrogenase (EC 1.6.99.2) and to limit the uncoupling studies to succinate.
Figure 2. Response of calcium to electron transfer and uncoupling of oxidative phosphorylation. The reaction media in all tracings contained 12 μM murexide, 348 μM CaCl₂, 330 mM sucrose, 25 mM Tris acetate pH 7.2, mitochondrial protein 7.2 mg/ml, and 2.1 μM rotenone. Electron transfer was initiated by the addition of 6.2 mM succinate. A represents the control response of uptake (upward deflection) of calcium and release by 18 μM CICCP. B represents a tracing of the response of uptake of calcium by mitochondria treated with 18 μM CICCP prior to the addition of succinate. C represents the tracings of mitochondria and medium which had been treated with 1 per cent (——), 2 per cent (——) and 4 per cent (———) halothane. The ordinate represents the optical density increase (540-510 nm) due to appearance of the free murexide released from the Ca⁺ complex as Ca²⁺ is taken up by the mitochondria. The abscissa represents time. The standard 60-second interval indicates that Ca²⁺ uptake under these conditions is complete in 30 to 120 seconds, and that release by the uncoupler CICCP is a very rapid process.

oxidation, since uncoupling cannot be separated from inhibition of electron transport with NAD-dependent substrates. Mitochondrial suspension, 403 μl (protein 53 mg/ml), was added to give a final protein concentration of 7.2 mg/ml. Murexide, 12 μM, and 340 μM CaCl₂ were then added. After the spectrophotometer had been suitably balanced, calcium movement was initiated by the addition of 37 μl of 0.5 M ammonium succinate (6.2 mM) as the substrate. When mitochondrial calcium was released by adding uncoupler, 18 μM CICCP was used. This concentration completely uncouples phosphorylation in the relatively concentrated mitochondrial suspensions used.

Results

A representative experiment indicating the response of mitochondria suspended in sucrose solution containing Tris-acetate is shown in figure 2A. The rapid upward deflection on the tracing indicates the inward movement of calcium in response to rapid electron transfer following the addition of succinate. Following the uptake of calcium there is a new steady state. Calcium is then released by the uncoupler CICCP. The rate of entry of calcium is determined by inherent limits in the transport system and by the rate of electron transfer and generation of energized intermediates which support the process. The maintenance of the steady state following accumulation of calcium depends upon the presence of oxygen for continuous substrate oxidation and the generation of high-energy intermediates for Ca²⁺ uptake to overcome any outward leakage. Both rate and steady state can be affected by uncoupling.

The response of a mitochondrial suspension which has been treated with a true uncoupler of oxidative phosphorylation (CICCP) prior to the addition of succinate is shown in figure 2B. There is a rapid upward deflection, indicating a new steady state, approximately one fourth of the steady state recorded for un-
treated mitochondria. Because a high concentration of uncoupler is present, the low steady state presumably represents the amount of calcium bound to the membrane but not transported into the mitochondria by energy-dependent mechanisms. When oxygen is depleted, the mitochondria release the calcium which appeared to bind to the membrane in the presence of uncoupler.

The response of mitochondria treated with halothane is shown in figure 2C. One percent halothane slows the rate of uptake about 40 per cent, but the new steady state is the same as that in the untreated mitochondria, so that the total amount of calcium accumulated is not affected. Two per cent and 4 per cent halothane cause further slowing of the rate of calcium accumulation, but there is no change in the final steady state of calcium accumulation. The halothane-treated mitochondria release calcium rapidly following the addition of the true uncoupler.

Discussion

Investigators in several laboratories have found alterations in the function of isolated mitochondria following exposure to halothane. In addition to inhibition of oxygen consumption when NAD-dependent substrates are used, effects similar to those seen with true uncouplers of oxidative phosphorylation have been reported—that is, loss of respiratory control and a lack of stimulation of oxygen consumption when ADP is added. Several groups of investigators have concluded that halothane is a true uncoupler of oxidative phosphorylation. In these investigations there were marked differences in the methods of introducing halothane, in the concentrations used, and in the procedures for studying effects of halothane on mitochondrial function. In general, when mitochondria are exposed to high concentrations of halothane and then washed, or when liquid halothane is introduced into a mitochondrial suspension, the mitochondria appear to be damaged so that they are uncoupled. This probably occurs because high local concentrations of the lipid-soluble liquid anesthetic react for a short time as a lipid extraction phase at the mitochondria-water interface. However, when mitochondrial suspensions are equilibrated with known concentrations of halothane vapor, many fewer mitochondria are damaged, and uncoupling is very limited with anesthetic concentrations. In several recent studies, the final concentrations of halothane in the mitochondria were similar as judged by the degree of inhibition of NADH dehydrogenase. However, the extents of uncoupling reported were different. Therefore, localized high concentrations of halothane are probably responsible for the greater damage to the phosphorylation system with certain methods of introducing the halothane.

When mitochondria are treated with halothane vapor in air prior to testing, the only apparent alteration in calcium transport seems to be the decrease in the rate of uptake as the concentration of halothane is increased. Such an effect could be due to inhibition of electron transport, partial uncoupling, or alteration in the Ca\(^{2+}\) carrier system. Our earlier studies indicated that halothane in the 1–2 per cent range does not inhibit succinate oxidation, so decreased electron transfer can be ruled out for 1 and 2 per cent, but it would be very significant with 4 per cent.

It is apparent that treatment of isolated mitochondria with concentrations as high as 4 per cent halothane does not alter the final steady state of calcium accumulation, and the response of rapid release of calcium to a true uncoupler of oxidative phosphorylation remains intact. Since the same final steady state is reached and maintained in the presence of 4 per cent halothane, it is apparent that even 4 per cent halothane does not duplicate a true uncoupler. Four per cent does inhibit electron transfer from succinate by more than 60 per cent, and abolishes stimulation by ADP and by 2,4-dinitrophenol while permitting Ca\(^{2+}\) accumulation to occur at a reduced rate until a true uncoupler is added. This means that high-energy intermediates which support Ca\(^{2+}\) uptake are still formed in the presence of 4 per cent halothane. There is not complete uncoupling of the generation of intermediates from electron transfer. In fact, with 4 per cent halothane the decrease in rate of Ca\(^{2+}\) uptake is very similar in magnitude to the inhibition of electron transfer.

Since the generation of intermediates for Ca\(^{2+}\) transport continues in the presence of 4 per cent halothane, the question whether halothane has any true uncoupling effect at anesthetic levels (0.5–2.0 per cent) must be ex-
amined very carefully. In *in-vitro* experiments where halothane has been carefully introduced into the mitochondrial system by equilibration with known concentrations of the vapor, the primary evidence for uncoupling by anesthetic (0.5–2.0 per cent) levels is a 25 per cent increase in the rate of respiration in the absence of ADP.\(^a\) Within the limits of measurement, the ratio of ADP phosphorylation to oxygen consumed (ADP/O) may be altered very little. Such a picture is consistent with very slight or limited uncoupling only.

Since isolated mitochondria, and especially those which have been damaged in experimental procedures, are more likely to show uncoupling effects than mitochondria in *vivo*, there is ample reason to be conservative about assigning uncoupling actions in *vivo* to anesthetic levels of halothane. The evidence for inhibition of electron transfer from NADH by anesthetic concentrations is greater and has been confirmed by several workers. This effect in the NAD-dependent pathway with 1–2 per cent halothane, the markedly reduced rate of electron transfer from succinate, and the absence of stimulation by ADP or DNP in the presence of 4 per cent halothane all may result from structural changes that alter the relationships between the mitochondrial enzymes. The continued uptake of Ca\(^{++}\) in the presence of 4 per cent halothane when there is no longer stimulation of respiration by ADP indicates that even this high concentration of halothane does not act as a true uncoupler of phosphorylation.

The possibility does remain that the presence of halothane may make the mitochondria more susceptible to other drugs or produce combined effects which lower the coupling efficiency of mitochondria. Halothane has been shown to alter the permeability of mitochondria to phosphate (presumably mediated by a membrane carrier), and the inhibition of NADH dehydrogenase in the organized membrane is clearly established. It is obvious that all of the actions of this drug upon membrane functions are not known.

If the mechanism of malignant hyperpyrexia is related to uncoupling of oxidative phosphorylation, it is doubtful whether halothane alone should be considered a primary causative agent. Further research is needed to determine whether a combination of substances and circumstances may be causative. It is possible that mitochondria of genetically susceptible patients may respond to lipid-soluble anesthetics with uncoupling of phosphorylation. Also, mitochondria in *vivo*, may respond to an alteration produced by anesthetics in another part of the cell. Whether the cause is at the mitochondrial level or in the failure of some physiologic regulatory process remains to be determined. Furthermore, until much more information about the incidences of malignant hyperpyrexia with various agents is available, halothane should not be considered more likely than any other anesthetic drug to be associated with malignant hyperpyrexia, except that it is used more often for patients who need general anesthesia.

**Addendum**

While this paper was in press, R. A. Harris et al. (Arch. Biochem. Biophys. 142:435–444 (1971) published a paper containing pertinent information on related points.

**References**