Prolonged exposure to halothane and associated changes in carbohydrate metabolism in rat muscles in vivo

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Ferreira, Luis D. M. C.-B., T. Norman Palmer, and Paul A. Fournier. Prolonged exposure to halothane and associated changes in carbohydrate metabolism in rat muscles in vivo. J. Appl. Physiol. 84(4): 1470–1474, 1998.—Halothane, an anesthetic presently used in animal experimentation, is reported to stimulate glycogen breakdown in isolated preparations of rat skeletal muscles, suggesting that it may not be a suitable anesthetic for the study of glycogen metabolism in rats in vivo. The purpose of this study was to establish whether prolonged exposure to halothane in rats in vivo is associated with accelerated glycogenolysis. Exposure of rats to halothane for up to 1 h was not accompanied by either any change in the levels of glycogen or increase in activity ratios of glycogen phosphorylase in muscles, irrespective of their fiber compositions. In marked contrast, the levels of lactate, inorganic phosphate, glucose 1-phosphate, glucose 6-phosphate, fructose 1,6-bisphosphate, and fructose 2,6-bisphosphate changed progressively during anesthesia. Accordingly, the interpretation of muscle metabolite levels must be performed with caution in experiments involving prolonged exposure to halothane. Overall, our findings indicate that the reported halothane-mediated stimulation of glycogen breakdown in vitro is likely to be an artifact and that halothane is suitable as anesthetic for experiments concerned with glycogen metabolism in rats.

glycogen; glycolysis; malignant hyperthermia

Although one may argue that a few seconds may be gained by decapitation or cervical dislocation, these protocols are not recommended in view of the associated rapid postmortem breakdown of muscle glycogen.

Because brief exposure to halothane (3–5 min) has been reported by Musch and colleagues (16) to affect glycogen levels in muscles from normal rats, halothane has been recommended as an anesthetic of choice to administer before tissue sampling for the determination of muscle glycogen (16), and, on that basis, the anesthetic has been used for several years in our laboratory (for example, see Refs. 4, 18, and 26). With respect to experiment protocols involving longer exposure to halothane, it remains to be seen, however, whether this anesthetic is without any effect on glycogen metabolism in vivo because there is evidence that prolonged exposure to halothane may stimulate glycogen breakdown in rat muscles. Indeed, earlier work performed by Rosenberg and colleagues (23) showed that stimulation of muscle glycogenolysis and glycolysis in response to halothane exposure is not restricted to animals susceptible to malignant hyperthermia (8, 11) but is reported to occur also in vitro, albeit to a lesser extent, in muscles from normal rats (23). In these muscles, prolonged exposure to halothane causes glyco- gen breakdown, marked accumulation of lactate, and decreased glycogen synthesis (23).

During prolonged exposure to halothane, the rates of muscle glycogen breakdown in vitro are such that changes in muscle glycogen levels would be too small to be detectable after only a few minutes exposure to the anesthetic. Thus one may argue that the time period used in the study of Musch and co-workers (16) was too short (~5 min) for any halothane-mediated breakdown of muscle glycogen to be detectable in vivo and that longer exposure may have been required for any effect on muscle glycogen metabolism to be observed. The in vitro findings of Rosenberg and colleagues (23) raise the question of whether prolonged exposure to halothane may exert a net glycogenolytic action in vivo in muscles of normal rats. The purpose of this study is to address the issue and establish whether other facets of muscle carbohydrate metabolism are affected by halothane in vivo so as to determine whether halothane is an anesthetic suitable for prolonged experiments con-
concerned with glycogen metabolism in rats. This issue is important because several investigators studying carbohydrate metabolism in rats in vivo work with anesthetized animals to eliminate the influence of environmental factors, such as handling stress (6, 9, 19), and, in the absence of information on the effects of anesthesia on the metabolic process being investigated, the interpretations of any results obtained under these conditions may be hazardous.

MATERIALS AND METHODS

Materials. All biochemicals and coupling enzymes were obtained from Boehringer Mannheim (Sydney, New South Wales, Australia), and halothane was obtained from Zeneca (Macclesfield, Cheshire, UK). All other chemicals were of Analar grade or equivalent.

Animals. Adult male albino Wistar rats (300–380 g) were kept at ~20°C on a 12:12-h light-dark photoperiod (light from 7:30 AM) and had unrestricted access to water and standard laboratory chow (55% digestible carbohydrate, 19% protein, 5% lipid, and 21% nondigestible residue by weight; Glen Forrest’s Stockfeeders, Glen Forrest, WA). On the day of the experiment, the animals, previously fasted, were anesthetized and killed between 8:30 AM and 1:00 PM. This project has been approved by the Animal Ethics Committee of the University of Western Australia.

Protocol of anesthesia and tissue sampling. Anesthesia was induced with 4% halothane-96% oxygen (vol/vol), the dosage being reduced to 1.5% halothane-98.5% oxygen (vol/vol) once the animals were anesthetized. Anesthesia, which took up to 3 min to induce, was maintained for up to 1 h, and, after varying durations of anesthesia (0, 20, 40, and 60 min), individual muscles and blood (0.3 ml by cardiac puncture) were sampled from different animals. After removal, each tissue was immediately freeze-damped between aluminium plates precooled in liquid nitrogen. The following muscles were selected on the basis of their distinct fiber composition: diaphragm (rich in type I fibers), soleus (rich in type I fibers), white gastrocnemius (rich in type IIb fibers), red gastrocnemius (rich in type IIa fibers), and tibialis anterior muscles (rich in type IIa and IIb fibers) (13). Arterial blood samples were collected into heparinized syringes and transferred into heparinized Eppendorf tubes and centrifuged at 720 g for 10 min. After centrifugation, the plasma was deproteinized in 9 vol 6% (wt/vol) perchloric acid and centrifuged at 2,000 g for 10 min before being neutralized with 2 M K2CO3. All samples were kept at −70°C until analysis.

Metabolite extraction. Frozen muscles were ground by using a mortar and pestle in liquid nitrogen. An aliquot of the powder was stored at −70°C for the later extraction of fructose 2,6-bisphosphate, whereas the remaining powder was homogenized with 5 vol 6% (wt/vol) perchloric acid in 40% (vol/vol) ethanol by using an Ultra-Turrax T25 homogenizer. The homogenate was rehomogenized in 5 vol 6% (wt/vol) perchloric acid, and a portion was taken for the determination of glycogen. Another aliquot was centrifuged, the pellet was reextracted with ice-cold 6% (wt/vol) perchloric acid, and the combined supernatants were neutralized with 2 M K2CO3 before centrifugation and storage of the supernatant at −70°C. These samples were used for the assay of other metabolites.

Because fructose 2,6-bisphosphate is acid labile but stable under alkaline conditions (2), it cannot be extracted by using the perchloric acid extraction procedure. An aliquot of the muscle powder (see above) was homogenized in 10 vol 50 mM NaOH, and the mixture was heated for 5 min at 80°C before being cooled to 4°C and centrifuged at 2,000 g for 10 min. After neutralization with 1 M acetic acid in 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, the supernatant was centrifuged at 15,800 g for 10 min and the resulting supernatant was applied to a Dowex AG1X8 (4 × 40 mm, Cl− form) column. The column was washed first with water, then with 150 mM NaCl, and fructose 2,6-bisphosphate was then eluted with 300 mM NaCl.

Assay of metabolites. Glycogen, glucose, glucose 6-phosphate, inorganic phosphate, and lactate were determined spectrophotometrically (2). Fructose 6-phosphate, glucose 1-phosphate, fructose 1,6-bisphosphate, and citrate were assayed fluorometrically (10). Ammonia was assayed according to the method of Nazar and Schoolwerth (17). Fructose 2,6-bisphosphate was measured by using a kinetic assay (2), whereas IMP was determined by using the methods of Meyer and Terjung (14).

Glycogen phosphorylase assay. Glycogen phosphorylase activity and phosphorylation status were determined according to Gilboe et al. (7). The phosphorylation state of glycogen phosphorylase is reflected by its activity ratio (activity in the absence of AMP relative to the activity in the presence of AMP), a high activity ratio being indicative of an elevated proportion of enzyme in the phosphorylated state (7).

Expression of results and statistical analysis. All results, unless otherwise stated, are expressed as mean micromoles per gram wet weight ± SE for at least seven animals. Glycogen is expressed as micromoles (glucosyl units) per gram wet weight. Statistical analysis was by one-factor analysis of variance followed by a Fisher protected least significant difference posteriori F-test (P < 0.05) by using Stat View SE + Graphics, version 1.03 (Abacus Concepts, 1988).

RESULTS

Glycogen levels in white gastrocnemius, red gastrocnemius, tibialis anterior, diaphragm, and soleus muscles remained stable during exposure, for up to 1 h, to halothane (Fig. 1). Although the levels of several metabolites, including IMP, glucose, ammonia, and citrate, did not change during halothane exposure, others were affected significantly (Table 1). In response to halothane, the levels of lactate in the tibialis anterior muscle decreased during the first 20 min of anesthesia and remained at low and stable levels during the next 40 min. This is a pattern of response similar to those observed for inorganic phosphate, glucose 1-phosphate, glucose 6-phosphate, and fructose 1,6-bisphosphate (Table 1). One common feature of the responses of lactate, inorganic phosphate, glucose 6-phosphate, fructose 6-phosphate, and glucose 1-phosphate to halothane was a small decrease in concentration in the first 20 min of anesthesia, whereas the decrease in fructose 1,6-bisphosphate was more pronounced. In contrast, the levels of fructose 2,6-bisphosphate increased significantly during exposure to halothane (Table 1).

One-hour exposure to halothane was accompanied by only minimal changes in plasma glucose and lactate levels (Table 2). Plasma glucose levels remained stable for the duration of anesthesia, except at 40 min, at which time levels were significantly lower (Table 2).
Fig. 1. Effect of prolonged exposure to halothane on glycogen levels in different rat muscles. Values are means ± SE; n = 7. Glycogen concentrations are expressed in µmol/g wet wt. For each muscle, there were no statistically significant differences between glycogen levels at different periods of exposure to halothane (P < 0.05).

The pattern of changes in plasma lactate (Table 2) was comparable to that for blood glucose, with lactate levels at 40 min being significantly lower than those immediately after induction of anesthesia.

Consistent with the absence of net glycogen mobilization in muscles, the proportion of glycogen phosphorylase in the active state remained stable and low during anesthesia in white gastrocnemius and soleus muscles but decreased in response to halothane in the tibialis anterior and red gastrocnemius muscles (Fig. 2).

**DISCUSSION**

Exposure of isolated muscles from normal rats to low levels of halothane is reported to increase glycogen breakdown and lactate production (23). These findings suggest that muscle glycogen metabolism in rats may be affected by prolonged exposure to halothane in vivo. Our findings do not support this view because 1-h exposure of rats to halothane in vivo was found not to be associated with any change in muscle glycogen levels, irrespective of muscle fiber composition (Fig. 1). Moreover, consistent with the absence of muscle glycogen mobilization during anesthesia, the low and decreasing levels of muscle and plasma lactate in response to halothane suggest that the anesthetic has minimal effects on muscle glycolysis.

The difference between our in vivo findings and those of others using in vitro muscle preparations (23) illustrates the well-documented limitations in studying muscle metabolism using isolated preparations (3). The reported glycogen breakdown to lactate by muscles in vitro, even in the absence of halothane (23), suggests that the incubation conditions adopted by these authors were nonphysiological. It is recognized that several critical variables need to be controlled (3) if glycogen levels are to remain stable in muscles in vitro. These include temperature and the composition of the incubating medium, as well as muscle thickness, a parameter that restricts muscle oxygenation (3) and

### Table 1. Effect of prolonged exposure to halothane on levels of muscle metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Period of Exposure to Halothane-Mediated Anesthesia, min</th>
</tr>
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<tbody>
<tr>
<td>IMP</td>
<td>0.13 ± 0.05</td>
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<tr>
<td>Phosphate</td>
<td>13.3 ± 1.4</td>
</tr>
<tr>
<td>G-6-P</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td>G-1-P</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>F-6-P</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>F-1,6-BP</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>F-2,6-BP</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.76 ± 0.42</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.46 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7. All metabolites are expressed as µmol/g wet wt., except for fructose 2,6-bisphosphate (F-2,6-BP), which is expressed as nmol/g wet wt.; G-6-P, glucose 6-phosphate; G-1-P, glucose 1-phosphate; F-6-P, fructose 6-phosphate; F-1,6-BP, fructose 1,6-bisphosphate; F-2,6-BP, fructose 2,6-bisphosphate. *P < 0.05 vs. 0 min, †P < 0.05 vs. 60 min.

### Table 2. Effect of prolonged exposure to halothane on metabolite levels in the blood

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Period of Exposure to Halothane-Mediated Anesthesia, min</th>
</tr>
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<tbody>
<tr>
<td>Blood glucose</td>
<td>10.05 ± 0.44</td>
</tr>
<tr>
<td>Blood lactate</td>
<td>1.77 ± 0.22</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7. All metabolites are expressed as µmol/ml. *P < 0.05 vs. 0 min.
that may cause glycogenolysis and lactate release in muscle in vitro. One may thus propose that the reported halothane-mediated stimulation of glycogen breakdown in vitro (23) is an artifact resulting, in part, from inadequate oxygenation of the muscle preparations.

Our study shows that in normal rats under halothane anesthesia, the proportion of glycogen phosphorylase in the active state is low and either remains constant or decreases in response to halothane (Fig. 2). This is in agreement not only with the lack of net glycogen mobilization in muscles in vivo but also with earlier findings using isolated muscle preparations (21). The progressive decrease in the proportion of glycogen phosphorylase in the active state probably results, in part, from halothane-mediated inhibition of epinephrine-mediated activation of glycogen phosphorylase (21) and the accompanied lowering of plasma catecholamine levels in the rat (22).

Although 1-h exposure of rats to halothane does not seem to promote muscle glycogen depletion, we undertook to examine whether other facets of muscle carbohydrate metabolism are sensitive to halothane-mediated anesthesia. To address this question, the levels of a number of regulatory metabolites of carbohydrate metabolism were measured in the tibialis anterior muscle, in which the fiber composition is a mixture of type I, IIa, and IIb fibers. The metabolites were chosen on the basis of their regulatory importance in relation to glycogen and glucose metabolism in muscle. The levels of all these metabolites (Table 1) in anesthetized animals are comparable to those reported for muscles in the resting state (12, 14, 15, 20, 24, 25, 27). The levels of several key regulatory metabolites, including IMP, glucose, ammonia, and citrate, do not change significantly during 1-h exposure to anesthesia. On the other hand, there is a progressive small decrease in the levels of lactate, inorganic phosphate, glucose 1-phosphate, glucose 6-phosphate, fructose 1,6-bisphosphate, and fructose 6-phosphate, whereas the levels of fructose 2,6-bisphosphate increase. This clearly indicates that some aspects of muscle carbohydrate metabolism are affected during prolonged exposure to halothane. It is beyond the scope of the study to establish whether these changes are caused by halothane exposure per se or concurrent events such as immobilization, anesthesia, and fasting.

The metabolic consequences, if any, of the observed metabolic changes are difficult to predict. For instance, the decrease in the levels of inorganic phosphate and fructose 1,6-bisphosphate would be predicted to inhibit glycogen breakdown and glycolysis because these metabolites are activators of glycogen phosphorylase and phosphofructokinase, respectively. However, the decrease in glucose 6-phosphate, an inhibitor of glycogen phosphorylase, and the increase in fructose 2,6-bisphosphate, an activator of phosphofructokinase, would be predicted to activate glycogen breakdown and glycolysis. Irrespective of the nature of the mechanism underlying the metabolic changes as well as whether the effect(s) of halothane is direct or indirect (e.g., via halothane-mediated lowering of catecholamine levels), our findings clearly indicate that halothane-associated metabolic effects may complicate its use in certain experimental procedures and that interpretation of muscle metabolite levels must be performed with caution in experiments involving prolonged exposure to halothane.

Overall, this study indicates that, in contrast to its effect on muscle in vitro, 1-h exposure to halothane is not associated with any detectable changes in glycogen levels in muscles in vivo, irrespective of their fiber composition. The low proportion of active glycogen phosphorylase and the presence of stable muscle glycogen levels are indicative of the absence of detectable net muscle glycogen breakdown. It is concluded that the reported halothane-mediated stimulation of glycogen breakdown in vitro (23) is likely to be an artifact and that halothane is a suitable anesthetic for experiments concerned with glycogen metabolism in the rat.

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