HALOTHANE ANAESTHESIA CAN BLOCK INSULIN STIMULATION OF PYRUVATE DEHYDROGENASE ACTIVITY IN MAMMARY GLANDS OF 24-HOUR STARVED LACTATING RATS

C. E. HUTCHINSON AND H. G. COORE

SUMMARY

The effect of halothane anaesthesia on the activity of the mitochondrial enzyme pyruvate dehydrogenase was studied in starved lactating rats. Extracts of freeze-clamped mammary gland and liver were assayed for pyruvate dehydrogenase activity. The fraction of the enzyme in the phosphorylated inactive form was increased greatly by starvation or by streptozotocin diabetes, and halothane anaesthesia did not disturb this effect in starved animals not exposed to halothane. Injection of insulin led to a rapid increase in the active fraction of the enzyme in mammary gland but not in liver. In animals under halothane anaesthesia this effect of insulin was largely abolished. The combination of starvation and halothane anaesthesia may impair mitochondrial accumulation of calcium which may be involved in the stimulation of pyruvate dehydrogenase by insulin.

Since the studies of the effects of volatile anaesthetics on enzymes should be relevant to clinical usage (Taussig, 1979), the use of anaesthesia adequate for surgery and the selection of enzymes whose activities in the tissues can be directly studied or inferred are essential.

The mammalian multi-enzyme complex pyruvate dehydrogenase (PDH) is purely mitochondrial in location and is regulated by phosphorylation (inactivation) and dephosphorylation (activation) (Linn et al., 1969). The reaction catalysed by the enzyme is

\[ \text{CH}_3\text{CO.COOH} + \text{NAD} + \text{CoA} \rightarrow \text{CH}_3\text{CO.CoA} + \text{NADH} + \text{CO}_2 \]

This reaction is effectively unidirectional and this fact and the relatively low activity of PDH in most tissues suggest that the enzyme complex is the major regulatory site controlling the rate of pyruvate oxidation or incorporation of pyruvate carbon into fatty acids, or both. In-vitro studies with purified enzyme or with isolated mitochondria have shown that the products of the reaction, NADH and acetyl CoA, inhibit the reaction directly and by stimulating the kinase which phosphorylates the enzyme. Furthermore, there is good evidence that, under certain conditions in-vitro a small intra-mitochondrial ratio [ATP]/[ADP] favours dephosphorylation and activation of the enzyme complex and small intra-mitochondrial concentrations of magnesium and calcium ions favour phosphorylation and inactivation of the enzyme complex (Denton et al., 1975). It is more difficult to assign relative importance to these individual factors in controlling PDH in tissue in situ. However, by freeze-clamping tissue in situ and by appropriate extraction of the tissue, it is possible to determine the total activity of the enzyme complex after complete dephosphorylation in vitro (PDH\text{r.} activity) and also the portion of the enzyme activity which existed in this dephosphorylated form in vivo before extraction (PDH\text{a} activity). This technique has been applied successfully to lactating mammary gland, muscle, adipose tissue, kidney, heart and liver of rats (Wieland et al., 1971; Wieland, Patzelt and Löffler, 1972; Hennig, Löffler and Wieland, 1975; Field and Coore, 1976; Stansbie et al., 1976).

The effects of halothane (2-bromo-2-chloro-1,1,1-trifluorethane) anaesthesia on the phosphorylation status of PDH could indicate likely effects of the anaesthetic on pyruvate disposal which has implications for ATP generation and lipogenesis. Furthermore, since changes in the phosphorylation status of PDH can reflect alterations of intra-mitochondrial free magnesium and calcium ions and ratios of [NADH]/[NAD], [Acetyl CoA]/[CoA] and [ATP]/[ADP] (Denton et al., 1975), the effects of halothane in promoting or inhibiting such changes in the phosphorylation status of PDH can be a useful index of the impact of the anaesthetic on mitochondrial function. In this laboratory it has been found that
24-h starvation increased the phosphorylated inactive fraction of PDH in lactating rat mammary gland and that a large dose of insulin rapidly reversed this effect of starvation (Baxter and Coore, 1978). We have used this response to insulin as a test system to examine the effects of halothane anaesthesia. We considered that 24-h starvation was an appropriate condition since it approximates to the clinical situation when halothane is used. We have found that, although PDH activity in mammary gland or liver of starved lactating rats was undisturbed by halothane, there was a severe inhibition of the response to insulin of the enzyme in mammary gland.

**METHODS**

Rats, materials and methods were generally as described earlier (Field and Coore, 1976).

Since anaesthesia was required for freeze-clamping, the experiments were designed to compare effects of minimal exposure to the anaesthetic (just sufficient to permit freeze clamping) with prolonged exposure, 30–60 min, including the time of injection and the interval before freeze clamping.

Injections were given when animals were under either halothane or very light ether anaesthesia. The induction of halothane anaesthesia was performed by placing the rat in a dessicator jar through which passed a stream of halothane 7% in oxygen 1 litre min⁻¹. After induction of anaesthesia the rat was placed on a dissecting board with its head enclosed in a small plastic funnel through which passed halothane 4–5% in oxygen 0.5 litre min⁻¹. Dissection commenced when the animal showed no reflex response to pinching of the skin with forceps. Less than 5 min was required to expose a suitable portion of mammary gland or liver and to clamp it in aluminium tongs cooled in liquid nitrogen. The frozen tissue was powdered under liquid nitrogen and kept in a glass bottle on solid carbon dioxide until the time of extraction, which was less than 1 h. Weighed portions of the powder (200–400 mg) were extracted with a motor-driven Teflon-in-glass homogenizer into 2 ml of either ice-cold triethanolamine buffer 25 mmol litre⁻¹, pH 7, EDTA 5 mmol litre⁻¹ and mercaptoethanol 7 mmol litre⁻¹ or ice-cold triethanolamine buffer 30 mmol litre⁻¹, pH 7 and mercaptoethanol 7 mmol litre⁻¹. Assay of PDH in the former medium by the method of Coore and others (1971) yielded data for activity of the initially dephosphorylated enzyme (PDHₐ activity). The presence of EDTA in this medium prevented activities of the accessory enzymes which phosphorylate and dephosphorylate PDH. To the second extract was added excess magnesium, calcium and the dephosphorylating enzyme and the mixture was incubated at 30 °C for 10 min. Assay of PDH activity after this incubation gave values of total enzyme activity (PDHₜ activity). The ratio of PDHₐ/PDHₜ activities multiplied by 100 is referred to as percentage PDHₜ activity. Further details of experimental design are given in the legend to table I.

**RESULTS**

Both 24-h starvation and streptozotocin diabetes (tables I and II) reduce percentage PDHₜ activity in mammary gland extracts below the 40% value found in normal fed animals (Baxter and Coore, 1978). Table I shows that 30 min of exposure to halothane did not disturb the percentage of active enzyme or the total activity of PDH in liver or mammary glands of starved animals. In other experiments (data not given) we have found also that [ATP]/[ADP] in these gland extracts was the same after 30 min as after 5 min of exposure of the animals to halothane. However, the effect of a large dose of insulin in promoting in vivo dephosphorylation of PDH in mammary gland was largely inhibited during 30 min of halothane. The hypoglycaemic effect of the hormone was undisturbed. This made it unlikely that halothane interfered with any early event in hormone action, for example, the hormone–receptor interaction. Confirmation of this conclusion is found in table II where it is seen that the injection of insulin in the presence or absence of halothane anaesthesia was able to correct the effect of streptozotocin diabetes on percentage PDHₜ activity in mammary gland. The hypoglycaemic effect of insulin 60 min after injection was, if anything, greater when the animals were maintained under halothane than when not anaesthetized continuously.

**DISCUSSION**

Starvation diminishes circulating insulin in lactating rats (Robinson, Gerard and Williamson, 1978). There is no information regarding effects of starvation on insulin receptors in the mammary gland, but in adipose tissue there is no decrease in the number of such receptors and their affinity for insulin is increased (Olefsky, 1976; Kasuga et al., 1977). Streptozotocin diabetes is associated with concentrations of circulating insulin which are probably less than is the case in starvation (Robinson, Gerard and Williamson, 1978), but the effect of streptozotocin diabetes on mammary gland PDH was evidently correctable by the administration of insulin irrespective of the
TABLE I. Effects of insulin injection and halothane anaesthesia on PDH activity in mammary glands and livers of 24-h starved lactating rats. Experiments yielding data of top two rows involved injection of animals which had been very lightly anaesthetized with ether. Immediately after injection the rats recovered consciousness and remained undisturbed for 25 min. They were then placed under halothane anaesthesia and the appropriate tissues exposed and freeze-clamped. Blood was obtained from the severed jugular veins. In the experiments yielding data of bottom two rows animals were placed under halothane anaesthesia, injected and maintained under the anaesthetic until the time of freeze-clamping. The times refer to the intervals between injections and freeze-clamping. PDH activity is expressed for gram wet weight, iu = international unit of soluble insulin which was injected i.p. **P < 0.01, ***P < 0.001 v. data in top row; † P < 0.001 for difference of row four and row two according to Student's t test. (Number of rats in parentheses)

<table>
<thead>
<tr>
<th>Treatment of rats</th>
<th>Percentage PDH&lt;sub&gt;T&lt;/sub&gt; activity in mammary gland extracts</th>
<th>PDH&lt;sub&gt;T&lt;/sub&gt; activity in liver extracts (unit g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Plasma glucose at time of freeze-clamping (mmol litre&lt;sup&gt;-1&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td>Saline injected 30 min before, minimal halothane</td>
<td>10.5 ± 0.9 (6)</td>
<td>1.55 ± 0.21 (6)</td>
<td>6.1 ± 0.6 (4)</td>
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<tr>
<td>Insulin 10 iu injected 30 min before minimal halothane</td>
<td>23.5 ± 1.2*** (6)</td>
<td>1.61 ± 0.16 (6)</td>
<td>6.1 ± 0.1 (4)</td>
</tr>
<tr>
<td>Halothane anaesthesia, saline injected 30 min before freeze-clamping</td>
<td>10.4 ± 0.2 (6)</td>
<td>1.75 ± 0.82 (6)</td>
<td>7.6 ± 2.3 (4)</td>
</tr>
<tr>
<td>Halothane anaesthesia, insulin 10 iu injected 30 min before freeze-clamping</td>
<td>13.8 ± 0.9***† (6)</td>
<td>1.47 ± 0.60 (6)</td>
<td>5.8 ± 0.5 (4)</td>
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</tbody>
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TABLE II. Effects of insulin injection and halothane anaesthesia on PDH activity in mammary glands of streptozotocin-diabetic lactating rats. Rats were injected i.p. with streptozotocin 16 mg in 0.3 ml of sodium acetate 10 mmol litre<sup>-1</sup>, pH 4.5, 2 h before other injections. For further explanation of treatments, enzyme units and significance tests see legend to table I

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<tr>
<td>Saline injected 60 min before, minimal halothane</td>
<td>5.7 ± 1.1 (5)</td>
<td>1.56 ± 0.04 (5)</td>
<td>18.4 ± 2.6 (8)</td>
</tr>
<tr>
<td>Insulin 10 iu injected 60 min before, minimal halothane</td>
<td>33.5 ± 2.1*** (4)</td>
<td>1.56 ± 0.04 (4)</td>
<td>3.5 ± 0.4*** (4)</td>
</tr>
<tr>
<td>Halothane anaesthesia, saline injected 60 min before freeze-clamping</td>
<td>8.3 ± 2.2 (4)</td>
<td>1.48 ± 0.05 (4)</td>
<td>19.4 ± 2.1 (4)</td>
</tr>
<tr>
<td>Halothane anaesthesia, insulin 10 iu injected 60 min before freeze-clamping</td>
<td>30.8 ± 6.8*** (5)</td>
<td>1.56 ± 0.11 (5)</td>
<td>1.32 ± 0.17***† (5)</td>
</tr>
</tbody>
</table>

presence of halothane. It appears that starvation caused changes in the metabolism of mammary tissue which were reinforced by halothane such that insulin could less easily set in train the events which lead to dephosphorylation of PDH. The absence of effect of the anaesthetic itself on PDH agrees with an earlier report of in vitro experiments (Bennett et al., 1978). It is likely that the combination of halothane anaesthesia and starvation interferes with some aspect of mitochondrial function which is necessary for insulin action on PDH. One possibility is mitochondrial accumulation of calcium ions which may be inhibited by halothane (Rosenberg and Haugard, 1973). It has been suggested (Denton et al., 1975) that insulin action on adipose tissue PDH involves an increase in mitochondrial free calcium ions.

ACKNOWLEDGEMENT

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REFERENCES


Kasuga, M., Akkumulierung von Kalzium beeinträchtigt werden, die an der Stimulierung der Pyruvat-Dehydrogenase durch Insulin beteiligt sein konnte. Der Effekt einer Halothannarkose auf die Aktivität der mitochondrialen Enzym-Pyruvat-Dehydrogenase wurden bei ausgehungerten, säugenden Ratten studiert. Extrakte der Brustdrüse und Leber wurden auf Pyruvat-Dehydrogenaseaktivität getestet. Der Gehalt des Enzymes in seiner phosphorylierten, inaktiven Form wurde durch Aushungerung oder Streptozotocyn-Diabetes stark erhöht, was durch Halothannarkose nicht beeinträchtigt wurde. Bei ausgehungerten Tieren ohne Halothan führte eine Insulininjektion zu aktivem Ansteigen des aktiven Gehalts des Enzymes in der Brustdrüse, aber nicht in der Leber. Bei Tieren unter Halothannarkose war diese Insulinwirkung weitgehend eliminiert. Die Kombination von Aushungerung und Halothannarkose könnte die mitochondrialen Akkumulation von Kalzium beeinträchtigen, die an der Stimulierung der Pyruvat-Dehydrogenase durch Insulin beteiligt sein könnte.

**HALOTHAN-ANÄSTHESIE KANN INSULIN-STIMULIERUNG DER PYRUVATDEHYDROGENASE-AKTIVITÄT IN DEN BRUSTDRÜSEN AUSGEHUNGERTER, SÄUGENDER RATTEN BLOCKIEREN**

**ZUSAMMENFASSUNG**

Se hizo un estudio en ratones lactantes del efecto de la anestesia por halotano sobre la actividad de la deshidrogenasa de piruvato de la enzima mitocondrial. Se hicieron pruebas con extractos de hígado y glándulas mamarias sometidos a congelación respecto de la actividad de deshidrogenasa de piruvato. La fracción de la enzima en la forma inactiva fosforilada aumentó considerablemente por el hambre o por la diabetes de estreptozotocina y la anestesia por halotano no alteró este efecto. En los animales hambrientos no-expuestos al halotano, la inyección de insulina produjo un aumento rápido de la fracción activa de la enzima en la glándula mamaria pero no en el hígado. En los animales sometidos a la anestesia por halotano, este efecto de la insulina fue eliminado mayormente. La combinación del hambre y de la anestesia por halotano no impidió la acumulación mitocondrial del calcio que puede tener alguna influencia en la estimulación de la deshidrogenasa del piruvato por la insulina.