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Mechanism of Halothane-Induced Inhibition of Isoproterenol-Stimulated Lipolysis in Isolated Rat Adipocytes

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SUMMARY

The effect of halothane on isoproterenol-stimulated lipolysis was determined in isolated rat epididymal fat cells. The maximal lipolytic response (E_{max}) activated by isoproterenol was 350 ± 61 nmol of glycerol/10^5 cells/hr with an EC_{50} of 5.1×10^{-9} M. When the adipocytes were simultaneously bubbled with 2.5% halothane, the E_{max} decreased to 158 ± 43 nmol of glycerol/10^5 cells/hr and the dose response curve for isoproterenol was shifted to the right (EC_{50} 3.5×10^{-8} M, $p < 0.05$). When lipolysis was maximally stimulated with (−)-isoproterenol (10^{-6} M), the inhibitory effect of halothane was found to be both dose dependent (IC_{50} ~2.5%, v/v) and reversible following washout. Neither the nonhydrolyzable cAMP analog, 8-(4-chlorophenylthio) adenosine 3′,5′-cyclic monophosphate (2×10^{-3} M), nor forskolin (10^{-6} M) was able to normalize lipolysis in the presence of halothane. The activation of cAMP-dependent protein kinase (EC 2.7.1.37) activity by isoproterenol was not different in halothane-exposed cells when compared to unexposed cells. When control adipocytes were exposed to isoproterenol (10^{-6} M), there was a 2.5-fold increase in the activity of hormone-sensitive lipase (EC 3.1.1.3) from 0.64 ± 0.13 to 1.53 ± 0.32 pkat (pmol/sec) per mg ($p < 0.005$, $n = 10$). However, in the presence of halothane (2.5%, v/v) isoproterenol stimulation of hormone-sensitive lipase was attenuated by 50% to values of 1.06 ± 0.23 pkat/mg ($p < 0.01$, $n = 10$). Halothane had no direct inhibitory effect on hormone-sensitive lipase since this enzyme's activity was unaffected when homogenates of isoproterenol-stimulated control cells were incubated with halothane. These studies suggest that halothane impairs the activation of hormone-sensitive lipase by cAMP-dependent protein kinase and in this manner inhibits β-adrenergic-stimulated lipolysis.

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), the prototype of the modern volatile anesthetic agents, exerts a multiplicity of effects on the autonomic nervous system (1–3). Halothane has been shown to depress ganglionic transmission (4) and to inhibit norepinephrine release from nerve terminals in the sympathetic nervous system (5–7). Additionally, halothane may affect adrenergic responsiveness at the level of effector cells, although it is not possible to generalize about the direction and extent of these changes. For example, halothane has been found to enhance adenylate cyclase (EC 4.6.11) activity in the rat uterus in a dose-dependent fashion (8), but it inhibits the enzyme in the toad bladder (9). Halothane decreases catecholamine-stimulated cAMP formation in the left ventricle (10) and in the cerebral cortex of the rat (11) but increases cAMP production in the rat uterus (12). Halothane depresses the vasoconstrictive response to catecholamines both *in vivo* and *ex vivo* (13).

The multiplicity of effects of halothane on various components of the autonomic nervous system makes it difficult to resolve the mechanisms by which halothane modulates responses to catecholamines in intact organisms. Isolated cells provide the opportunity to more directly approach the problem of how halothane may modify the functioning of cells. These changes are important not only in trying to understand halothane's mechanism of inducing anesthesia but also in elucidating mechanisms for its adverse effects.

Catecholamines stimulate lipolysis in isolated adipocytes by activating β-adrenergic receptors (14). This model facilitates dissection of the individual elements of the biochemical cascade for the adrenergic response (15). Halothane's effect on the lipolytic response in fat cells has been previously investigated (16–20). At subanesthetic concentrations halothane may exert a mild stimulatory effect on glycerol release (19), whereas over the clinical concentration range of halothane (0.5–3%, v/v) basal and β-adrenergic-stimulated lipolytic rates were inhibited

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ABBREVIATIONS: KRB, Krebs-Ringer bicarbonate; BSA, bovine serum albumin; A-kinase, cAMP-dependent protein kinase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

by nearly 50%. Additionally, theophylline- and cAMP-stimulated lipolytic response has also been found to be significantly inhibited by halothane (20). We were interested in determining the mechanism by which halothane inhibited catecholamine-stimulated lipolysis in isolated adipocytes. The results suggest that the mechanism involves impaired activation of hormone-sensitive lipase by isoproterenol in the presence of halothane.

Materials and Methods

Preparation of isolated fat cells. Male Sprague-Dawley rats (4–8 weeks old, 150–250 g) were fed standard laboratory chow *ad libitum* and maintained on a 12-hr light/dark cycle. Following decapitation at 10:00 a.m., fat cells were prepared from epididymal fat pads according to the method of Rodbell (14) with minor modification (21). The fat pads were minced with scissors and placed in plastic flasks in KRB buffer (130 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSo_4$, 2.5 mM NaH_2PO_4, 25.5 mM $NaHCO_3$), with 4% BSA, 3 mM glucose, and 1 mg of collagenase/ml. Collagenase (EC 3.4.24.7) digestion was performed at 37° in a gyratory water bath shaker for 60 min. Cells were washed three times in fresh KRB/4% albumin/2.5 mM glucose buffer, pH 7.4, and allowed to separate from the infranatant by flotation. Separate aliquots (100 μl) of diluted cells were centrifuged in a capillary hematocrit tube to determine fractional volume and were fixed in 2% osmium tetroxide in collidine buffer to determine cell number in a Coulter counter (Coulter Electronics Inc, Hialeah, FL). The cells were diluted in KRB/4% albumin/2.5 mM glucose buffer, and 1 unit/ml adenosine deaminase, pH 7.4. to contain 1×10^5 cells/ml so that the fat cells represented 2–3% of the suspension. The addition of adenosine deaminase served to remove any remaining endogenous adenosine (22).

Measurement of lipolysis. A 100-μl aliquot of the adipocyte cell suspension was added to 900 μl of prewarmed incubation medium and incubated at 37° for 1 hr in an atmosphere of O_2 (95%)/CO_2 (5%). Pilot studies revealed a time-dependent increase in lipolysis until at least 60 min, therefore, this time period was chosen for subsequent incubations. Incubation of the fat cell suspensions was performed in two parallel chambers gassed with halothane in O_2/CO_2 or O_2/CO_2 alone. The anesthetic concentration was continuously monitored throughout the exposure by an infrared analyzer. Various activators of lipolysis were used as described under Results: adenosine deaminase (EC 3.5.4.4), 1 unit/ml (all exposures); the β-receptor agonist (−)-isoproterenol 10^{-9} to 10^{-5} M forskolin, (10^{-6} M); and the cAMP analog, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (2×10^{-3} M). At the end of incubation, the adipocyte suspension was centrifuged and an aliquot (0.2 ml) of infranatant was removed from each incubation mixture for the measurement of glycerol (22). The rate of lipolysis is expressed as nmol of glycerol released/10^5 cells/hr.

cAMP-dependent protein kinase (A-kinase) assay. A-kinase activity was measured as described in detail by Honnor *et al.* (15). Cells from rats (4–6 weeks old, 150–180 g) were isolated in Krebs-Ringer buffer containing 25 mM Hepes and 2.5 mM $CaCl_2$ at pH 7.4 as described above, except that the collagenase digestion was performed in the presence of 200 nM adenosine, 2 mM glucose, and 1% fraction V BSA. (Adipocytes from these younger animals are less likely to rupture during the vigorous shaking conditions required for these experiments with cAMP-dependent protein kinase.[1]) Incubation was performed during exposure to O_2 with or without halothane as described above, in a Krebs-Ringer-Hepes medium containing 4% defatted BSA and 1 unit/ml adenosine deaminase (to remove endogenous and exogenous adenosine). After 60 min, incubation was terminated by addition of 200 μl of 50 mM EDTA, 2.5 mM Ro 20-1724 [phosphodiesterase (EC 3.1.4.1) inhibitor], buffered with 10 mM Tris, to pH 7.4. The mixture was homogenized and all subsequent steps were performed at 4°. The homogenate was centrifuged at 10,000 × *g* for 15 min and the infranatant removed and assayed immediately for both glycerol (15) and A-kinase activity content according to the methods of Corbin *et al.* (23) and Witt and Roskoski (24). In this method the assay buffer contained 40 μg of histone H1 in 170 mM potassium phosphate, 60 mM magnesium acetate, pH 6.8. Ten μl of either distilled water or 16 μM cAMP or 0.6 mg/ml of beef heart protein kinase inhibitor were added. Twenty μl of the infranatant of the cell homogenate were added to the chilled (4°) assay buffer. The reaction was initiated by adding a mixture 5 μl of 0.15 M NaF and 10 μl of 0.1 mM [γ-^{32}P]ATP (0.68 μCi/assay tube) to give a final volume of 65 μl/assay tube and incubated for 15 min at 30°. The reaction was terminated by transferring 50-μl aliquots of the reaction mixture to P84 phosphocellulose filter papers (Whatman) which were then immersed in a beaker containing 5% phosphoric acid solution (15 ml/filter). Filters were soaked overnight while stirred and rinsed at least four times with fresh phosphoric acid solution. The filters were then quickly rinsed with a methanol solution to remove excess water. After drying in air, the radioactivity was measured by liquid scintillation spectrometry in Betaphase liquid scintillation fluid.

Hormone-sensitive lipase assay. After adipocytes were incubated and homogenized as described above, cell homogenates were centrifuged at 100,000 × *g* for 75 min. The infranatants were removed and their pH was adjusted to 5.2; the material was then centrifuged at 100,000 × *g* for 45 min (25). The resulting pellets were washed in 20 mM Tris-HCl, 1 mM EDTA, 1 mM dithioerythreitol, and 10 $\mu g \cdot ml^{-1}$ leupeptin, pH 7.0, centrifuged at 10,000 × *g* for 20 min, and then resuspended in the same buffer by repeated aspiration through a 30 gauge hypodermic needle. The acid-precipitable suspensions were then used to measure hormone-sensitive lipase (EC 3.1.1.3) activity by following the release of radioactive free fatty acid from cholesteryl [^{14}C]oleate by a modification of the procedure described by Hajjar *et al.* (26). At neutral pH, hormone-sensitive lipase hydrolyzes both triglycerides and cholesteryl esters with a relative ratio of 1:1.5 (25).

Preliminary experiments indicated that more sensitive and reproducible results could be obtained using relatively small numbers of fat cells by measuring neutral cholesterol esterase (EC 3.1.1.13) activity. Consequently, this assay was used in subsequent studies. To perform these studies, a micellar substrate was prepared by adding 3.75 μCi of cholesteryl [1-^{14}C]oleate to $CHCl_3$ containing 3.8 μmol of phosphatidylcholine and 0.8 μmol of unlabeled cholesteryl oleate, and then drying the solution under N_2 and resuspending the lipids in 3.0 ml of a buffer containing 100 mM potassium phosphate and 2 μM sodium taurocholate, pH 7.0. After vortexing, the solution was placed in a water bath at 40° and sonicated (Branson Sonifier/cell disruptor #W-350) at an output setting of 5 for 45 min. The substrate was centrifuged at 1500 × *g* for 15 min to remove metallic fragments released by the Sonifier, and then stored under N_2 at 4° for up to 4 weeks. The reaction was begun by adding acid-precipitable enzyme suspension (0–25 μg) to a mixture of 12 μl of micellar substrate and 100 mM potassium phosphate, pH 7.0, containing 0.05% fatty acid-free BSA to achieve final concentrations of 6.0 μM cholesteryl oleate, 23.7 μM phosphatidylcholine, 12.5 μM sodium taurocholate, 0.04% BSA, and 85 mM potassium phosphate, pH 7.0. After incubating at 37° for 60 min, the reaction was stopped by the addition of 3.0 ml of methanol/chloroform/heptane (1.41:1.25:1.00, v/v/v) and 1 ml of 0.1 M borate/carbonate, pH 10.5. After vortexing and centrifuging at 1500 × *g* for 15 min, the amount of [1-^{14}C]oleate in the aqueous phase was determined by scintillation counting in Betaphase. Blank values were obtained from incubations in the absence of protein. Activity is expressed as fkat (fmol/sec) free fatty acid released.

There was no change in the measured amount of isoproterenol in the buffer of either the control or the halothane-exposed cells at the end of 1 hr incubation (data not shown).

Data analysis. Dose response curves were analyzed by the four-parameter logistic equation (27) and using the ALLFIT program[2] to generate the E_{max} and EC_{50} values. Paired Student's *t* tests were used

[1] C. Londos, personal communication.

[2] Martin H. Teicher, M.D., Ph.D., Department of Psychiatry, Harvard Medical School, McLean Hospital and Muilman Research Corp., Belmont, MA 02178.

to compare data with and without halothane exposure. Statistically significant differences existed when the p value was <0.05. Data are expressed as means ± standard errors unless otherwise indicated.

Results

Isoproterenol produced a dose-dependent increase in lipolysis (Fig. 1). The maximal response (E_{max}) was 350 ± 61 nm of glycerol/10^5 cells/hr with an EC_{50} of 5.1×10^{-9} M (−)-isoproterenol. When the adipocytes were simultaneously bubbled with 2.5% halothane, the dose response curve for isoproterenol was significantly ($p < 0.05$) shifted to the right (EC_{50} 3.5×10^{-8} M) and the E_{max} significantly ($p < 0.05$) decreased to 158 ± 43 (Fig. 1).

When lipolysis was maximally stimulated with (−)-isoproterenol (10^{-6} M), the inhibitory effect of halothane was dose dependent with an IC_{50} of 2.5% (v/v) (Fig. 2). Also, this effect of halothane was reversible, since progressive washout of halothane resulted in a time-dependent restoration of the lipolytic response to values similar to those of unexposed controls (Fig. 3). All subsequent experiments were performed using 2.5% (v/v) halothane.

We wondered whether the ability of halothane to impair isoproterenol-stimulated lipolysis was proximal or distal to the activation of cAMP-dependent protein kinase. Consequently the cAMP analog, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate, in a dose that was previously shown to maximally stimulate lipolysis (21), 2×10^{-3} M, was used to stimulate lipolysis in controls and adipocytes exposed to 2.5% (v/v) halothane. As indicated in Fig. 4, the cAMP analog activated lipolysis to an extent similar to that of isoproterenol in the controls. Additionally, in the cells exposed to halothane, there was a similar blunting in lipolytic response to both isoproterenol and the cAMP analog. Also, stimulation by forskolin was similarly inhibited by halothane (Fig. 4). These data suggest that the explanation for the blunted lipolytic response in the presence of halothane lies at the level of cAMP-dependent protein kinase or a more distal event.

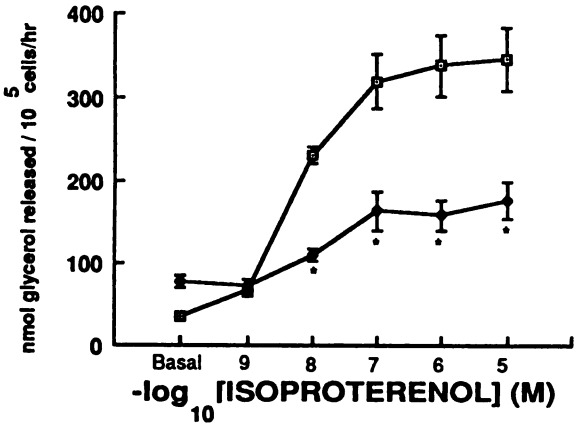

Fig. 1. Effect of halothane on isoproterenol-stimulated lipolysis. Isolated adipocytes in KRB/4% albumin/2.5 mM glucose buffer and 1 unit/ml adenosine deaminase, pH 7.4, were incubated at 37° for 1 hr in an atmosphere of O_2 (95%)/CO_2 (5%) in the presence (♦) or absence (□) of 2.5% (v/v) halothane with varying concentrations of isoproterenol. At the end of the incubation glycerol was determined in the infranatant as described under Materials and Methods and the rate of lipolysis expressed as nmol of glycerol released/10^5 cells/hr. Each point represents the mean ± standard error of duplicate assays in four independent experiments. *,statistically significant differences ($p < 0.05$) by paired t test analysis.

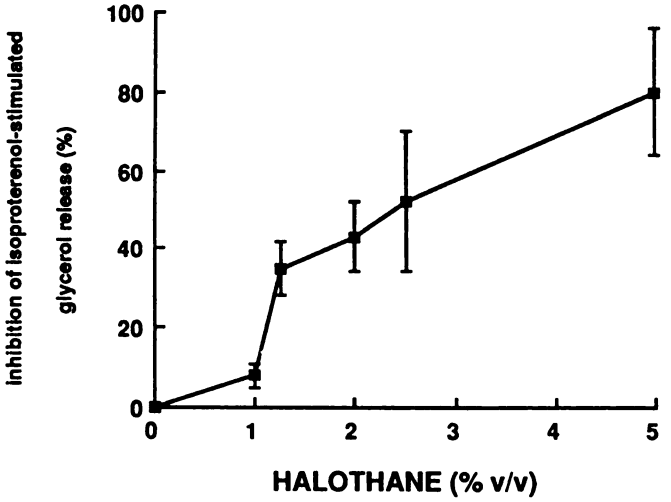

Fig. 2. Effect of the concentration of halothane on isoproterenol-stimulated lipolysis. Adipocytes were prepared and incubated in the presence of 10^{-6} M isoproterenol and various halothane concentrations. Data are expressed as per cent inhibition of lipolysis of parallel cell incubations in the presence of 10^{-6} M isoproterenol but without halothane. Each point represents the mean and standard error of duplicate assays in five independent experiments. Above 1% halothane, each of the values is significantly different ($p < 0.05$) from the basal value.

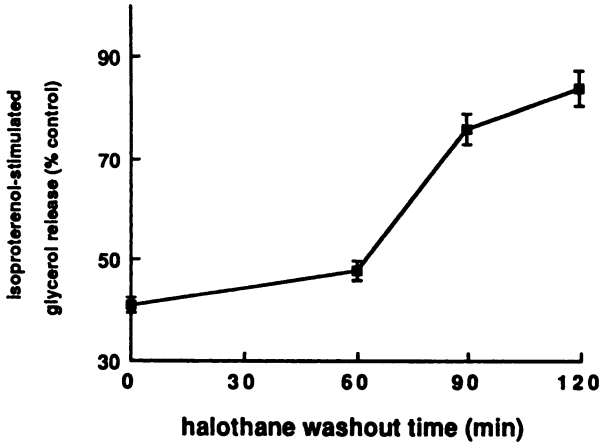

Fig. 3. Effect of duration of halothane washout on isoproterenol-stimulated lipolysis. Rat adipocytes were prepared and exposed to halothane, 2.5% (v/v), in CO_2/O_2 for 60 min. At the end of this period halothane was discontinued and cells were incubated without halothane for a further 60, 90, or 120 min. In each case lipolysis was stimulated by adding 10^{-6} M isoproterenol for the final 60 min of incubation. Data are expressed as per cent inhibition of lipolysis of cells which were incubated under the same conditions except for the absence of the initial halothane exposure. Data are the means of duplicate assays from two independent experiments and are expressed as the per cent of lipolysis of cells incubated in parallel without any halothane exposure.

The activation of A-kinase activity by isoproterenol in control and halothane-exposed cells was measured (Fig. 5). Although the preparation of fat cells in the A-kinase experiments differed slightly from those described above, this did not influence the inhibitory effect of halothane on the lipolysis (data not shown). The addition of halothane did not impair the ability of isoproterenol to activate the A-kinase activity ratio. These experiments measure the ability of isoproterenol to activate A-kinase by promoting dissociation of the regulatory and catalytic subunits. However, the actual catalytic activity was measured in the absence of added halothane. Separate experiments (n =

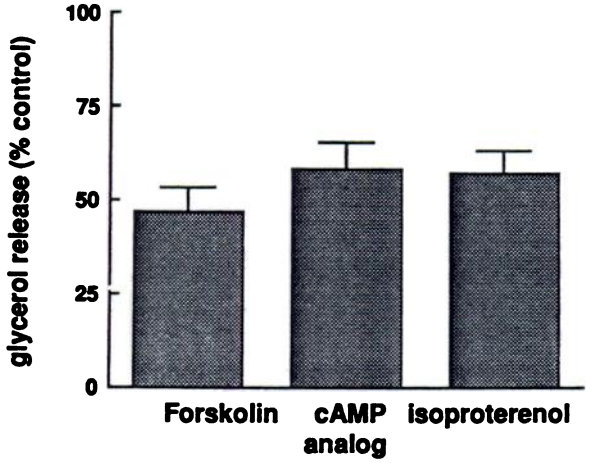

Fig. 4. Effect of halothane on stimulation of lipolysis by forskolin and cAMP analog. Rat adipocytes were prepared and incubated during halothane (2.5%, v/v) exposure in the presence of 10^{-6} M forskolin or 2×10^{-3} M 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate, the nonhydrolyzable cAMP analog, and assayed for glycerol as described under Materials and Methods. Data points are the means of duplicate assays from two independent experiments and are expressed as the per cent of lipolysis of cells incubated and stimulated in parallel without halothane exposure. For comparison, data for 10^{-6} M isoproterenol-stimulated lipolysis are also included.

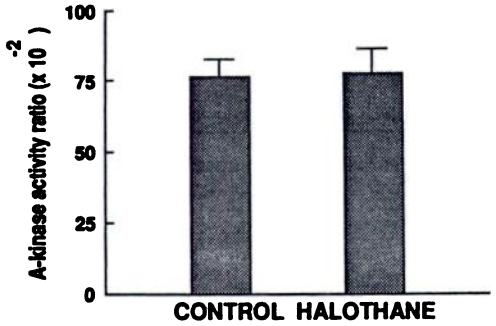

Fig. 5. Effect of halothane on isoproterenol-stimulated A-kinase activity in rat adipocytes. Adipocytes were prepared and incubated as for Fig. 1 except that 200 nM adenosine, 2 mM glucose, and 1% fraction V BSA were added to Krebs-Ringer-Hepes buffer. At the end of the 60-min incubation the cells were homogenized, and centrifuged at 10,000 × *g* for 15 min, and then the infranatant was removed and assayed immediately for glycerol and A-kinase activity in triplicate under three separate conditions: (a) with no additions, (b) in the presence of cAMP, or (c) in the presence of the beef heart protein kinase inhibitor. Results are expressed as corrected A-kinase activity ratios: $(a-c)/(b-c)$ for incubations performed in the presence and absence of 2.5% halothane and represent the means and standard deviations of duplicate assays from five separate experiments. (Lipolysis, measured under the same conditions, revealed a similar inhibitory effect of halothane as is depicted in Fig. 1. Data not shown.)

2) were performed to determine whether halothane directly affected the catalytic activity of activated A-kinase. Halothane (5% v/v) was added to the A-kinase assay medium in the presence of the infranatant of cells which had previously been stimulated with cAMP for 15 min in the absence of halothane, and the A-kinase activity was determined as described above. Halothane had no inhibitory effect on the isoproterenol-stimulated A-kinase catalytic activity (data not shown).

Since halothane inhibited isoproterenol-stimulated lipolysis while exerting no apparent effects on A-kinase activation or catalytic activity, the effects of halothane on the activation and activity of hormone-sensitive lipase were examined (Fig. 6).

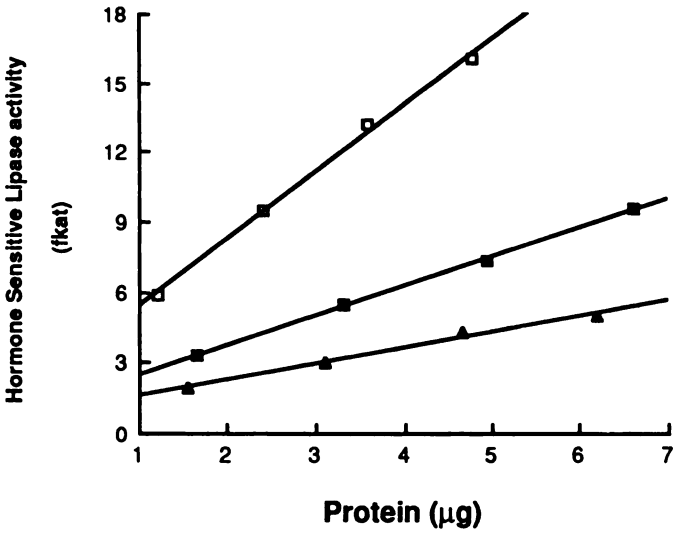

Fig. 6. Effect of halothane on rat adipocyte hormone-sensitive lipase activity. Rat adipocytes were prepared and incubated without stimulators (▲) and in the presence of 10^{-6} M isoproterenol stimulation with (■) and without (□) 2.5% halothane (v/v) as described in Fig. 5. The reaction was stopped by homogenizing the cells, and the acid-precipitable cell extracts were prepared for assay of hormone-sensitive lipase activity as described under Materials and Methods. Each point represents the mean of duplicate assay tubes and is representative of 10 independent experiments.

When control adipocytes were exposed to isoproterenol (10^{-6} M), there was a 2.5-fold increase in the activity of hormone-sensitive lipase from 0.64 ± 0.13 pkat/mg to 1.53 ± 0.32 pkat/mg ($p < 0.005$, $n = 10$). However, when cells were exposed to halothane (2.5%, v/v) in the presence of isoproterenol, the stimulation of hormone-sensitive lipase was attenuated by 50% to values of 1.06 ± 0.23 pkat/mg ($p = 0.01$, $n = 10$). These studies suggested that halothane impairs the activation of hormone-sensitive lipase.

In order to determine whether halothane had a direct inhibitory effect on hormone-sensitive lipase, cells were first stimulated by isoproterenol, which activated the enzyme. Homogenates of these cells were then incubated in the presence or absence of halothane (Fig. 7). Halothane had no inhibitory effect on the preactivated hormone-sensitive lipase in these broken cells. This result suggests that halothane does not directly inhibit hormone-sensitive lipase activity but prevents its activation by A-kinase.

Discussion

Halothane inhibits isoproterenol-stimulated lipolysis, measured by glycerol release, in a dose-dependent and reversible manner in isolated adipocytes. Also, a cAMP analog had a similar impaired ability to activate glycerol release in the presence of halothane. Halothane did not prevent the activation of A-kinase by isoproterenol nor did it impair A-kinase activity *in vitro*. However, there was a marked inhibition in the ability of isoproterenol to activate hormone-dependent lipase enzyme activity in intact cells in the presence of halothane.

Catecholamines stimulate lipolysis by activating β-adrenergic receptors (14). β-Adrenergic receptors activate adenylate cyclase, which catalyzes the conversion of ATP into cAMP. cAMP binds to the regulatory component of A-kinase, releasing the catalytic unit which activates hormone-sensitive lipase by phosphorylation. The enzyme is deactivated by dephosphoryl-

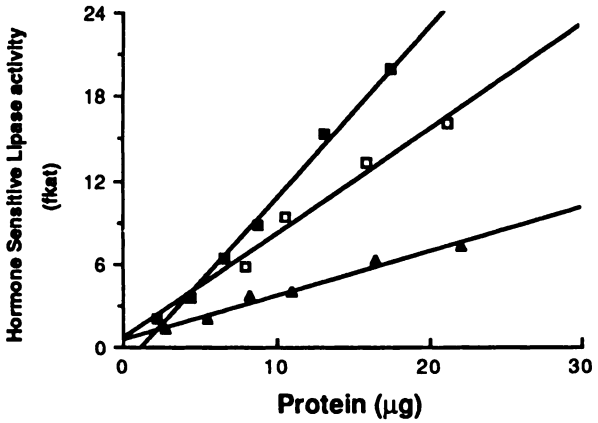

Fig. 7. Effects of halothane on isoproterenol-stimulated, hormone-sensitive lipase activity in broken cells. Rat adipocytes were prepared and incubated without stimulators (▲) and in the presence of isoproterenol 10^{-6} M stimulation as described in Fig. 6. Following incubation, cells were homogenized and the isoproterenol-treated cell homogenates were then incubated with halothane 2.5% (v/v) (■) or N_2 (□) at 4° for 30 min. Acid-precipitable cell extracts were then prepared and assayed for hormone-sensitive lipase activity. Each point represents the average of duplicate assay tubes and is representative of four independent experiments.

ation. In the intact tissue, the adrenergic-mediated lipolytic response is opposed by the action of endogenous inhibitors such as adenosine (15). Earlier studies in intact epididymal fat pads demonstrated a greater than 50% inhibition of the basal lipolytic rate over the clinical concentration range of halothane (0.5–3%, v/v), whereas at subanesthetic concentrations halothane exerted a mild stimulatory effect on glycerol release (19). However, it is difficult to compare these findings with ours since they did not control for the residual effects of sympathetic nerve terminals and endogenous inhibitors of lipolysis in their experiments in intact fat pads (19). Because of the profound inhibitory effects of adenosine in β-stimulated lipolysis (15), our assays involving adipocytes were conducted in the presence of adenosine deaminase, especially because of possible interactions of halothane on adenosine content (28). Bennis and Smith (18) were also unable to restore the inhibitory effect of halothane with the use of a nonhydrolyzable analog of cAMP, and they speculated that halothane attenuates lipolysis by inhibiting activated hormone-sensitive lipase (20). Our data demonstrate that halothane markedly attenuates the activation of hormone-sensitive lipase activity (Fig. 6) in the presence of appropriately activated A-kinase while having no effect in broken cells on previously activated hormone-sensitive lipase.

Hormone-sensitive lipase activity is dependent on the degree to which it is phosphorylated, with the phosphorylated form being active (29). This in turn is mediated by relative proportions of A-kinase and phosphoprotein phosphatase (EC 3.1.3.16) activities. The fact that halothane had no effect on preactivated hormone-sensitive lipase in broken cells suggests that, if halothane has an effect on phosphoprotein phosphatase, then intact cells are required for this action to be observed. Since our studies indicate that A-kinase activity is unaffected by halothane and that halothane inhibits isoproterenol-mediated stimulation of hormone-sensitive lipase but has no effect on hormone-sensitive lipase that has already been activated, it is probable that this anesthetic prevents the appropriate amino acid(s) on the hormone-sensitive lipase from being normally phosphorylated by A-kinase. These possibilities require direct experimental testing to confirm or refute them.

There are now many instances in which the phosphorylation of a single regulatory protein may involve both an A-kinase as well as a Ca^{2+}-dependent pathway (30, 31). Such a dual mechanism has been suggested for both isoproterenol- and ACTH-stimulated lipolysis (32). These workers confirmed that hormone-activated lipolysis is impaired in Ca^{2+}-free buffers (33, 34). Also, studies with radiolabeled ^{47}Ca suggested that uptake of this cation may regulate the lipolytic rate in rat adipose tissue (35). Taken together with halothane's disruption of Ca^{2+} uptake (36) and intracellular disposition (37–39), it is possible that halothane blunts the activation by phosphorylation of hormone-sensitive lipase by a Ca^{2+}-dependent mechanism. It is noteworthy that epinephrine-stimulated lipolysis by hormone-sensitive lipase was recently shown to be inhibited by the calmodulin inhibitor, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (40). In that study, A-kinase activation appeared to be the site at which the calmodulin inhibitor exerted its antilipolytic effect, whereas our studies would suggest that protein kinase is appropriately activated in the presence of halothane. Thus, the possible role of halothane's effect on intracellular Ca^{2+} disposition in the mechanism for the decrease in the activation of hormone-sensitive lipase requires further experimental testing.

Halothane has also been shown to bind to hydrophobic domains on proteins (41, 42). Halothane's physical presence may sterically hinder the binding of the phosphate ion to the appropriate amino acid sites and in this manner prevent phosphorylation of hormone-sensitive lipase.

Other investigators have suggested alternative loci for halothane-induced β-hyporesponsiveness, including the β-adrenergic receptor in a lymphocyte model (43), the "coupling" of the β-adrenergic receptor to adenylate cyclase in canine myocardium (10), adenylate cyclase in the toad urinary bladder (9), and the hydrolysis of cAMP by phosphodiesterase in mouse cerebellum (11).

The effect of halothane on the activation of the corresponding regulatory protein in the β-adrenergic response was not directly assessed in these studies. Thus, the causal relationship of these other biochemical abnormalities for the mechanism of halothane's blunting of β-adrenergic responsiveness is not clear since considerable redundancy exists in the biochemical cascade that leads to biological response (44).

It is conceivable that some of halothane's pharmacologic properties in other systems (e.g., negative inotropy and chronotropy in the heart) may be explained in part by its ability to attenuate phosphorylation of regulatory proteins. Whether its general anesthetic properties can also be ascribed to this molecular mechanism needs to be addressed.

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