Lipolysis and reesterification: effects of some inhibitors of adenosine 3′,5′-cyclic monophosphate phosphodiesterase

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Abstract Theophylline and three lipolytic agents, 2,5-bis(2-chloroethylsulfonyl)-pyrrole-3,4-dicarbonitrile (substituted pyrrole), 2,4-diamino-6-butoxy-*s*-triazine (substituted triazine), and 2,3-dihydro-5,6-dimethyl-3-oxo-4-pyridazinecarbonitrile (substituted pyridazine), stimulate basal lipolysis in adipose tissue in vitro. They also cause an increased release of free fatty acids, but not glycerol, from adipose tissue in which lipolysis is already maximally stimulated by epinephrine. The four compounds also inhibit cyclic AMP phosphodiesterase and the conversion of [1-^{14}C]glucose to $^{14}CO_2$. Evidence is presented that free fatty acids accumulate as the result of inhibited reesterification. The substituted pyridazine and triazine, but not the pyrrole, elevate plasma free fatty acids after oral or intraperitoneal administration in rats.

Supplementary key words plasma free fatty acids · 2,3-dihydro-5,6-dimethyl-3-oxo-4-pyridazinecarbonitrile · 2,5-bis(2-chloroethylsulfonyl)-pyrrole-3,4-dicarbonitrile · 2,4-diamino-6-butoxy-*s*-triazine

THAT THEOPHYLLINE stimulates lipolysis in adipose tissue in vitro and elevates plasma FFA concentrations in several species has been reported from many laboratories (1–8). Sutherland and Rall (9) showed that theophylline inhibits cyclic AMP phosphodiesterase, and many subsequent studies have demonstrated that this compound stimulates lipolysis by increasing tissue concentrations of cyclic AMP as the result of inhibiting phosphodiesterase. In the course of random screening for lipolytic agents, it was found that certain lipolytic agents increased the accumulation of FFA, but not of glycerol, when they were incubated with adipose tissue in the presence of concentrations of epinephrine that stimulate lipolysis maximally. Further work showed that theophylline also increased this accumulation of FFA and that the other agents also inhibited phosphodiesterase as did theophylline. The present experiments were designed to study the effect of theophylline and the three other agents on basal and stimulated lipolysis in vitro, on reesterification, on phosphodiesterase, and on glucose oxidation in epididymal adipose tissue. In addition, the effects of these agents on plasma FFA in rats are reported. A preliminary report of this work has been presented (10).

MATERIALS AND METHODS

Male rats, 200–250 g in weight, derived from the Sprague-Dawley strain (Spartan Research Laboratories, Haslett, Michigan) were used. The animals were lightly anesthetized with ether and were bled from the abdominal aorta into syringes coated with 1% heparin solution. Fragments of epididymal fat pads (50–60 mg) from each rat were incubated in duplicate in 1 ml of Krebs-Ringer bicarbonate medium containing 3% crystalline bovine albumin (Armour) and 0.9 mg of glucose. Drugs were added in amounts indicated in the tables. Tissue from the same rat was used for both control and experimental incubations. The incubations were in Potter-Elvehjem homogenizer tubes at 37°C in an atmosphere of 5% CO_2 and 95% air in a Dubnoff metabolic shaker, oscillating at 60–70 cycles/min. After incubation the adipose tissue was homogenized in the medium. Aliquots of homogenate and of plasma were analyzed for FFA concentration by the procedure of Dole (11) as modified by Ko and Royer (12). Glycerol concentration of adipose tissue was measured by the fluorometric method of Laurell and Tibbling (13).

Abbreviations: FFA, free fatty acid(s).

Total lipids were extracted from a second aliquot of homogenate by the method of Folch, Lees, and Sloane Stanley (14). The dried lipids were saponified in 0.4% ethanolic KOH, acidified, and extracted by the procedure of Dole (11). The aqueous and heptane layers were evaporated, and ^{14}C in glycerol and FFA, respectively, was measured in a Packard liquid scintillation counter.

The procedure for measuring the effect of drugs on the oxidation of [^{14}C]glucose (Volk Radiochemical Co.) in adipose tissue was adapted from a previous report (15). Adipose tissue (70–100 mg) was incubated in Krebs-Ringer bicarbonate containing 0.50 μCi of [^{14}C]glucose, 2 mg of nonlabeled glucose, and compounds, in amounts indicated in the tables, in a final volume of 2.0 ml. Incubations were carried out in glass scintillation vials stoppered with vial stoppers through which a glass cup on a glass stem was inserted. At the end of the 90-min incubation 0.20 ml of Hyamine was placed in the cup by inserting a 21-gauge needle through the stopper. To expel the CO_2 from the medium 0.20 ml of 4 N H_2SO_4 was added to the medium in the same way. The vials were returned to the metabolic shaker for 1 hr to collect the CO_2. The Hyamine was rinsed out of the well into vials with scintillation fluid and diluted to a final volume of 10 ml. The scintillation fluid consisted of 375 g of naphthalene, 22.5 g of 2,5-diphenyloxazole, and 1.13 g of 1,4-bis-[2-(4-methyl-5-phenyloxazole)]-benzene in 3 l of *p*-dioxane.

Phosphodiesterase was assayed by the method of Butcher and Sutherland (16), modified for adipose tissue. In this procedure, phosphodiesterase activity is determined from the rate at which cyclic 3′,5′-AMP is converted to 5′-AMP. The latter compound is measured from the inorganic phosphate liberated by the action of bacterial alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J.). Fat pads, which were removed after the rats were decapitated, were homogenized in 2 vol of 0.33 M sucrose in a Waring blender for 2 min, and the homogenate was centrifuged at 37,000 *g* for 15 min at 4°C. The supernatant solution was adjusted to 0.2 saturation with respect to ammonium sulfate by addition of 11.4 g of the solid salt/100 ml; the precipitate was separated by centrifugation and discarded. The supernatant was then adjusted to 0.4 saturation by adding 12.3 g of solid $(NH_4)_2SO_4$/100 ml; the precipitate was separated by centrifugation as above and dissolved in 1 mM Tris buffer, pH 7.5, and 1 mM $MgSO_4$. This preparation was fractionated with ammonium sulfate as above, and the fraction that precipitated between 0.25 and 0.50 saturation was dissolved in 1 mM Tris, pH 7.5, and 1 mM $MgSO_4$. The incubation mixture for enzyme assay consisted of cyclic 3′,5′-AMP (0.5 mM), $MgSO_4$ (3.5 mM), alkaline phosphatase (20 μg), and a suitable dilution of phosphodiesterase preparation in Tris buffer (0.04 M), pH 8.0, in a final volume of 1 ml. The reaction mixture was incubated for 30 min at 37°C (with the alkaline phosphatase present only during the final 10 min) and stopped by the addition of 0.1 ml of 55% trichloroacetic acid. An aliquot of the supernatant solution was taken for measurement of inorganic phosphate.

TABLE 1. Effect of theophylline and the substituted pyrrole, triazine, and pyridazine on basal lipolysis in rat adipose tissue

Experiment Number and Additions	Basal Lipolysis	
	FFA	Glycerol
	μmoles/g[a]	
1. Control	0.7 ± 0.2	1.48 ± 0.21
Theophylline[b]	3.3 ± 0.8[c]	3.11 ± 0.50[c]
Substituted pyrrole[b]	3.7 ± 0.5[c]	3.94 ± 0.50[c]
2. Control	3.1 ± 0.3	2.52 ± 0.21
Substituted triazine[b]	8.0 ± 0.7[d]	4.42 ± 0.32[d]
3. Control	0.4 ± 0.2	1.30 ± 0.20
Substituted pyridazine[b]	2.4 ± 0.3[d]	2.98 ± 0.30

[a] Each value is the mean ± SEM of duplicate measurements, in six animals, of FFA and glycerol found in tissue plus medium after a 2-hr incubation.
[b] 100 μg/ml.
[c] $P < 0.01$ vs. control.
[d] $P < 0.001$ vs. control.

RESULTS

At concentrations of 100 μg/ml in the incubation media, theophylline and the other three compounds stimulated basal lipolysis in adipose tissue to approximately the same extent (Table 1). If FFA release were used as the index of lipolytic activity, each of these agents would appear to further increase lipolysis already maximally stimulated by epinephrine (Table 2). However, the data show that glycerol release is not enhanced under these conditions but that the increased amount of FFA found when adipose tissue is incubated with epinephrine and any of the four compounds is the result of inhibited reesterification. To test the validity of calculating reesterification from glycerol and FFA concentrations, direct determinations were made by measuring the incorporation of ^{14}C from [6-^{14}C]glucose into glyceride-glycerol. Table 3 shows that epinephrine increased incorporation of ^{14}C and that lipolytic agents prevented all or most of the increase due to epinephrine. Thus, the two methods agree in showing that these compounds inhibit reesterification of FFA.

A block in the glycolytic pathway resulting in a deficiency of α-glycerophosphate would be expected to reduce reesterification. To check this possibility the effect of the four compounds on oxidation of [6-^{14}C]glu-

TABLE 2. Effect of theophylline and the substituted pyrrole, triazine, and pyridazine on lipolysis maximally stimulated by epinephrine

Experiment Number and Additions	Stimulated Lipolysis		Reesterification[a]
	FFA	Glycerol	
		μmoles/g[b]	
1. Epinephrine[c]	19.9 ± 2.3	16.5 ± 1.29	30.18 ± 5.1
+ Theophylline[d]	34.3 ± 2.6[e]	15.7 ± 1.00	12.60 ± 1.2[e]
+ Substituted pyrrole[d]	39.6 ± 1.8[e]	17.1 ± 0.64	13.41 ± 3.8[f]
2. Epinephrine[c]	22.0 ± 2.4	11.8 ± 0.3	16.67 ± 3.4
+ Theophylline[d]	37.6 ± 1.9[e]	12.1 ± 0.5	0.49 ± 1.2[e]
+ Substituted triazine[d]	29.2 ± 3.4[f]	13.3 ± 0.8	12.50 ± 1.9[f]
3. Epinephrine[c]	14.4 ± 2.3	11.1 ± 0.3	15.80 ± 3.4
+ Substituted pyridazine[d]	22.5 ± 2.5[f]	10.9 ± 0.4	7.60 ± 1.2[e]

[a] Reesterification is calculated thus: [(glycerol in tissue and medium after incubation − glycerol in nonincubated tissue) × 3] + FFA in nonincubated tissue − FFA in tissue and medium after incubation.

[b] Each value is the mean ± SEM of duplicate measurements, in six animals, of FFA and glycerol found in tissue and medium after a 2-hr incubation.

[c] Epinephrine was added at a level of 0.50 μg/ml.

[d] Added at a level of 100 μg/ml.

[e] $P < 0.01$ vs. epinephrine alone.

[f] $P < 0.05$ vs. epinephrine alone.

cose to $^{14}CO_2$ was determined. The data in Table 4 show that the oxidation of [6-^{14}C]glucose is not inhibited by any of the agents at concentrations equal to or greater than those that stimulate lipolysis and inhibit reesterification. However, the oxidation of glucose through the pentose shunt is reduced, as is indicated by the decreased conversion of [1-^{14}C]glucose to $^{14}CO_2$ by all four agents at one or more concentrations (Table 4).

Although the structures of the three compounds differ greatly from each other and from theophylline, their similarity to theophylline in action on lipolysis, reesterification, and glucose oxidation suggested that they also might inhibit phosphodiesterase. Table 5 shows that all the agents inhibited cyclic AMP phosphodiesterase, and that one, the substituted pyrrole, was a more potent inhibitor than theophylline.

The lipolytic activities of the substituted pyrrole, triazine, and pyridazine were further evaluated in vivo.

TABLE 3. Inhibition of incorporation of [6-^{14}C]glucose into glyceride-glycerol and fatty acids by theophylline, and the substituted pyrrole, triazine, and pyridazine

Additions[a]	Glyceride-glycerol	FFA
	dpm/100 mg tissue[b]	
None	10560	5750
Epinephrine[c]	23250	2800
+ Theophylline[d]	8190	1180
None	6030	1950
Epinephrine[c]	15750	1410
+ Substituted pyrrole[d]	7740	370
None	6840	3160
Epinephrine[c]	20310	1680
+ Substituted triazine[d]	13350	650
None	7800	3870
Epinephrine[c]	12420	1530
+ Substituted pyridazine[d]	7200	1080

[a] Each tube contained 0.5 μCi of [6-^{14}C]glucose.

[b] Each value is the mean of duplicate determinations in each of two experiments.

[c] 0.5 μg/ml.

[d] 100 μg/ml.

TABLE 4. Effects of theophylline and the substituted pyrrole, triazine, and pyridazine on oxidation of [6-^{14}C]-glucose and [1-^{14}C]glucose to $^{14}CO_2$

Additions	$^{14}CO_2$ from: [6-^{14}C]Glucose Expts. 1 and 2	[6-^{14}C]Glucose Expts. 3 and 4	[1-^{14}C]Glucose Expts. 1 and 2	[1-^{14}C]Glucose Expts. 3 and 4
	dpm/100 mg tissue			
None	2180	2120	5260	7241
Theophylline, 180 μg/ml	2210	2880		
Theophylline, 90 μg/ml	2800	2520	2800	3450
Theophylline, 45 μg/ml			2920	3130
Substituted pyrrole, 185 μg/ml	2880	1930		
Substituted pyrrole, 93 μg/ml	2890	3030	1970	2560
Substituted pyrrole, 47 μg/ml			2230	2660
None	1320	1440	5250	5800
Substituted triazine, 183 μg/ml	1650	1740		
Substituted triazine, 92 μg/ml	1350	1470	2360	2100
Substituted triazine, 46 μg/ml			5780	3900
Substituted pyridazine, 149 μg/ml			2750	2210
Substituted pyridazine, 75 μg/ml	1170	1520	4670	2530

Each experiment was done with adipose tissue from one rat. Each number is the mean of determinations in two pieces of tissue from the same rat.

TABLE 5. Inhibition of cyclic 3′,5′-AMP phosphodiesterase by theophylline and the substituted pyrrole, triazine, and pyridazine

Experiment Number and Additions	Phosphate Released	% Inhibition
	μmoles/30 min	
1. None	0.20	
Theophylline, 1 mM	0.10	50
Theophylline, 5 mM	0.05	75
Substituted pyrrole, 1 mM	0.05	75
Substituted pyrrole, 5 mM	0.03	85
2. None	0.25	
Theophylline, 1 mM	0.15	40
Substituted triazine, 1 mM	0.23	8
Substituted triazine, 5 mM	0.19	24
3. None	0.29	
Substituted pyrrole, 5 mM	0.03	90
Substituted triazine, 5 mM	0.18	38
Substituted pyridazine, 5 mM	0.23	21

Phosphodiesterase was prepared and assayed as described in the text.

TABLE 6. Effects of oral doses of the substituted triazine and pyridazine on plasma FFA concentration in rats

Treatment	Number of Animals	Time	Plasma FFA
		hr	*μmoles/l*[a]
Triazine			
H_2O (Control)	12	1	286 ± 18
50 mg/kg	12	1	362 ± 22[b]
H_2O	18	2	313 ± 23
50 mg/kg	18	2	400 ± 21[c]
H_2O	16	4	303 ± 21
50 mg/kg	16	4	354 ± 11[b]
Pyridazine			
H_2O	6	2	242 ± 21
7.5 mg/kg	6	2	273 ± 10
15 mg/kg	6	2	343 ± 31[d]
30 mg/kg	6	2	403 ± 28[b]

[a] Results given as means ± SEM.
[b] $P < 0.02$ vs. control.
[c] $P < 0.05$ vs. control.
[d] $P < 0.005$ vs. control.

An intraperitoneal dose of 100 mg/kg of the pyrrole and oral doses of 12.5, 50, and 200 mg/kg to nonfasted rats did not change plasma FFA concentrations. Intravenous doses of 8 and 32 mg/kg to fasted rats also failed to affect plasma FFA. An oral dose of 50 mg/kg of the triazine (one-sixth of the intraperitoneal LD_{50} in mice) raised plasma FFA concentrations for at least 4 hr, and oral doses of 7.5, 15, and 30 mg/kg of the pyridazine (the intraperitoneal LD_{50} in mice is 316 mg/kg) increased plasma FFA concentrations after 2 hr (Table 6). In experiments not shown, these two agents were also effective in raising plasma FFA when administered intraperitoneally.

DISCUSSION

The stimulating effect of theophylline on basal and epinephrine-stimulated FFA and glycerol release from adipose tissue is firmly established. This effect of theophylline is mediated through cyclic AMP by inhibition of phosphodiesterase. The results presented here (Table 2) show that theophylline has yet another effect on FFA when lipolysis is stimulated maximally by epinephrine, i.e., an increased accumulation of FFA. The additional FFA can be accounted for by the reduction in reesterification shown in Tables 2 and 3. It is known that reesterification occurs concurrently with liberation of FFA during the process of lipolysis and α-glycerophosphate is required.

It is unlikely that reesterification is inhibited because of a deficiency of α-glycerophosphate, since these agents do not inhibit oxidation of [6-^{14}C]glucose to $^{14}CO_2$. Thus, the generation of glycerophosphate from glucose would seem to be sufficient to allow normal reesterification. Also, recent evidence suggests that the glycerol that is liberated from triglycerides can be converted to glycerophosphate by a kinase in adipose tissue (17). The effect of these agents on conversion of [1-^{14}C]glucose to $^{14}CO_2$ agrees with the reported inhibition of glucose oxidation by caffeine (18) and theophylline (19, 20) and suggests that inhibition may be mediated by cyclic AMP since the dibutyryl analog also inhibits this reaction (21). A block in the pentose phosphate pathway, by reducing the availability of reduced nucleotides, would be expected to inhibit the conversion of glucose to fatty acids, as is reported here (Table 3).

It is not possible to say which of the steps between FFA and triglycerides is inhibited by the four compounds. However, since the agents presumably increase the concentration of cyclic AMP, it is tempting to speculate that cyclic AMP not only promotes the hydrolysis of triglycerides by stimulating a lipase but also inhibits triglyceride formation by inhibiting a step between FFA and triglycerides.

It has been suggested that at least a part of the effect of the methyl xanthines on plasma FFA is due to catecholamines (22, 23). The caffeine in 5 g of instant coffee (equivalent to 2 cups and containing 220 mg of caffeine) produced a significant increase in urinary catecholamine excretion in young human males (22). Theophylline, infused intravenously in the form of aminophylline, increased urinary excretion of epinephrine and norepinephrine and raised the concentration of FFA in the plasma of human male subjects (23). In anesthetized rats the increase in oxygen consumption after small doses of theophylline or caffeine (6.6 mg/kg) was abolished completely by pretreatment with reserpine (24). Therefore, from the results reported here it is not possible to deter-

mine whether the elevation in plasma FFA produced by the substituted triazine and pyridazine is mediated through phosphodiesterase or through another mechanism.

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