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LIPOLYTIC ACTIVITY OF CIS-β-CARBOXYACRYLAMIDINE

STUDIES *IN VIVO* AND *IN VITRO*

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Abstract—*cis*-β-Carboxyacrylamidine, a product isolated from the fermentation broth of the actinomycete, *Streptomyces furlongus* var. *furlongus* nov. sp., stimulated lipolysis in rat adipose tissue and isolated fat cells. Lipid-mobilizing activity *in vivo* was indicated by the increase in plasma FFA concentrations that occurred after the compound was administered orally. Daily doses reduced body weight gain but after approximately 5 days the rats became resistant to this effect of the compound. Resistant animals still responded to the lipid-mobilizing activity of the agent with an increase in plasma FFA levels. *cis*-β-Carboxyacrylamidine did not increase cyclic AMP concentrations in incubated adipose tissue. Also it neither stimulated adenylate cyclase or cyclic AMP-dependent protein kinase nor inhibited cyclic AMP phosphodiesterase or phosphoprotein phosphatase. The lipolytic mechanism was found to differ from the mechanism of *N*-ethylmaleimide. Also *cis*-β-carboxyacrylamidine inhibited soluble hormone-sensitive lipase, suggesting that soluble hormone sensitive lipase may not be the lipase mainly responsible for the response of adipose tissue and isolated fat cells to lipolytic hormones.

cis-β-Carboxyacrylamidine has been isolated from the fermentation broth of the actinomycete *Streptomyces furlongus* var. *furlongus* nov. sp. [1, 2]. This report shows that this newly described agent stimulates lipolysis in rat adipose tissue *in vitro*, increases plasma FFA concentration *in vivo* and has a transient inhibitory effect on body weight gain. Further, it attempts to determine where the compound acts in the sequence of reactions that precede activation of hormone-sensitive lipase. A preliminary report on part of these studies has been presented [3].

MATERIALS AND METHODS

Animals, diet and experiments in vivo. Male Sprague–Dawley rats (Spartan Research Laboratories, Haslett, MI) weighing 200–250 g initially were used in all experiments except as noted in Table 3. The diet was Purina Laboratory Chow. In experiments involving measurement of weight gain and food intake, five marked rats were housed in a cage and were each weighed and dosed by intubation daily. Food consumption was measured daily for the five rats as a group. To measure plasma FFA concentrations and lipolysis, the animals were lightly anesthetized with ether and were bled from the abdominal aorta into syringes coated with 1% heparin solution before the epididymal fat pads were removed. FFA were extracted from plasma by the procedure of Dole [4] and titrated by the method of Ko and Royer [5].

Preparation of fat cells and fat cell ghosts and measurement of adenylate cyclase activity. Fat cells were isolated from epididymal fat pads of rats and converted to ghosts by the method of Rodbell [6]. Adenylate cyclase was measured as described by Pohl *et al.* [7] from the rate of formation of cyclic AMP from ATP[α-^{32}P]. The reaction mixture contained final concentrations of 3.2 mM Tris–ATP, 5 mM $MgCl_2$, 25 mM Tris–HCl (ph 7.4), 10 mM theophylline, 0.1 per cent serum albumin and an ATP-regenerating system consisting of 20 mM creatine phosphate and creatine kinase, 1 mg/ml (Sigma, 115 units/mg). Final concentrations were 10 μg/ml of epinephrine and 10 mM sodium fluoride. Reactions were initiated by the addition of 20–45 μg of membrane protein. The total reaction volume of 0.05 ml was incubated for 10 min and the reaction was stopped by the addition of 0.10 ml of a solution containing 34 mM sodium dodecyl sulfate, 40 mM ATP, 0.15 μCi of cyclic AMP[^{3}H] (Schwartz Bioresearch) and sufficient cyclic AMP to give a final concentration of 12.5 mM. After boiling for 3.5 min, the reaction mixtures were diluted with 0.4 ml water, and the cyclic AMP was purified by passage through an AG50W-X8, 100–200 mesh column (Bio-Rad Laboratories), and a $BaSO_4$ precipitation step [7]. The supernatant from the $BaSO_4$ step was dissolved in 15 ml of scintillation fluid and counted in a Packard scintillation counter. The cyclic AMP[^{3}H] was used to calculate the recovery of cyclic AMP[^{32}P] from the reaction mixtures. Cyclase activity was calculated as nmoles cyclic AMP/10 min/mg of protein, measured by the method of Lowry *et al.* [8].

Lipolysis in epididymal adipose tissue and in isolated fat cells. A previously described procedure was used for lipolysis studies in adipose tissue. Briefly, 50–70 mg pieces were incubated in duplicate in Potter–Elvehjem homogenizer tubes in a metabolic shaker oscillating at 80–90 cycles/min. Incubations were conducted at 37° in an atmosphere of 95% air, 5% CO_2. The medium consisted of 0.90 ml Krebs–Ringer bicarbonate containing 27 mg bovine serum albumin (Armour), epinephrine as indicated, and 1.0 mg glucose in a final volume of 1.0 ml. The adi-

pose tissue was homogenized and FFA were extracted and titrated as described above for plasma. Glycerol was measured by a fluorometric method described earlier [9].

Lipolysis in isolated fat cells was determined by measuring glycerol in cells and medium after a 60-min incubation in Krebs–Ringer bicarbonate buffer supplemented with 3% bovine serum albumin and adjusted to pH 7.4. Glycerol was measured as described by Korn [10].

Preparation and assay of hormone-sensitive lipase. Hormone-sensitive lipase was prepared from incubated epididymal fat pads and assayed as described by Khoo and Steinberg [11]. Glycerol tri[1-^{14}C]-oleate was purchased from Amersham/Searle, Arlington Heights, IL.

Measurement of cyclic AMP phosphodiesterase. Phosphodiesterase activity was assayed by the method of Butcher and Sutherland [12] modified for adipose tissue as described earlier in detail [13]. Briefly, phosphodiesterase activity was determined from the rate at which cyclic AMP was converted to 5′-AMP. The 5′-AMP was measured from the inorganic phosphate liberated by the action of bacterial alkaline phosphatase. The adipose tissue extract was prepared by homogenizing fat pads in 3 vol. of 0.33 M sucrose in a Potter–Elvehjem homogenizer and centrifuging the homogenate at 37,000 *g* for 15 min at 4°. The compound under study was added to the reaction mixture, which was incubated for 30 min at 37°.

Cyclic AMP in adipose tissue. Epididymal adipose tissue was incubated exactly as described above for lipolysis but the incubation was reduced to 5 min. A 20-μl aliquot of the homogenate was transferred to a conical tube and placed in a boiling water bath for 3 min. The aliquot was diluted with 30 μl water and 50 μl of 6% trichloroacetic acid (TCA) and centrifuged. The precipitate was washed in a similar way twice with 50 μl of 3 per cent TCA. The three supernatant solutions were combined, extracted five times with 1 ml of water-saturated ether, and evaporated to dryness in a stream of N_2. Cyclic AMP concentration was measured by radioimmunoassay after the samples were succinylated according to Cailla *et al.* [14]. Antiserum and ^{125}I-labeled antigen were purchased from Collaborative Research Inc., Waltham, MA.

Protein kinase and phosphoprotein phosphatase assays. Protein kinase activity was measured in KCl extracts of epididymal fat pads in reaction mixtures described by Corbin *et al.* [15]. Aliquots of reaction mixture were processed on phosphocellulose paper [16]. Phosphorylated histone was prepared and used as substrate for phosphoprotein phosphatase as described by Maeno and Greengard [17]. The phosphatase was a homogenate of epididymal adipose tissue in Na glycerol phosphate, pH 6.5 (4 ml buffer/g of tissue). Aliquots of the reaction mixture were spotted on 2 × 20 cm strips before and after an incubation period of 15 min. The strips were developed in 6% TCA by descending chromatography and an aliquot of eluted phosphate was counted in 15 ml of Bray's solution.

RESULTS

The response of adipose tissue to the lipolytic effect of epinephrine and *cis*-β-carboxyacrylamidine *in vitro* is shown in Fig. 1. The parallel slopes suggest the possibility that the two agents may act by similar mechanisms. In Table 1 single concentrations of the agent and of epinephrine are compared for their effect on glycerol release. The results are meant to show that the increase in FFA is due to lipolysis and not merely to possible inhibition of reesterification. To test for lipolytic activity *in vivo*, *cis*-β-carboxyacrylamidine was given orally, and plasma FFA as well as lipolysis in epididymal adipose tissue were measured. Data in Table 2 indicate that a dose of 50 mg/kg, when given 2 hr before the animals were sacrificed, did not significantly increase basal, epinephrine- or *cis*-β-carboxyacrylamidine-stimulated FFA release from adipose tissue. However, plasma FFA and adipose tissue FFA concentrations were increased by the treatment, suggesting a lipid-mobilizing activity *in vivo*. Further evaluation of lipid-mobilizing activity showed that oral doses of 100 and

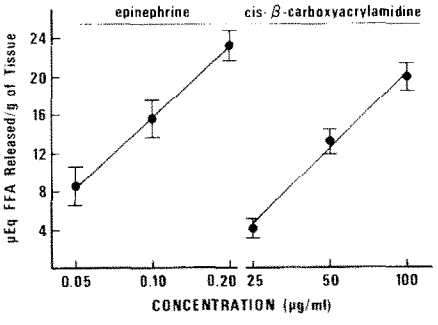

Fig. 1. Response of epididymal fat pads to the lipolytic effect of epinephrine and *cis*-β-carboxyacrylamidine. Each point represents mean ± S.E.M. for four rats. Adipose tissue from each rat was used with all six concentrations of the two agents. Lipolysis in unstimulated adipose tissue was 2.1 ± 3 μ-equiv./g/hr.

Table 1. Effect of epinephrine and *cis*-β-carboxyacrylamidine on glycerol release*

Addition	Glycerol released (μmoles/g tissue/hr)
None (basal)	1.95 ± 0.20
cis-β-Carboxyacrylamidine, 8.8×10^{-4} M	6.47 ± 0.50†
Epinephrine, 5×10^{-7} M	9.81 ± 0.70†

* Incubations were conducted and glycerol was measured in medium plus tissue as described in Materials and Methods. Each value represents the mean ± S. E. M. for duplicate determinations in adipose tissue of ten rats.

† $P < 0.001$, compared to basal group.

Table 2. Effects of oral doses of *cis*-β-carboxyacrylamidine on plasma FFA concentration and on basal and stimulated lipolysis*

Treatment	FFA concn†		Lipolysis‡		
	Plasma	Tissue	Basal	*cis*-β-Carboxyacrylamidine 8.8×10^{-4} M	Epin. 5×10^{-7} M
H_2O (control)	336 ± 29	1.48 ± 0.18	1.13 ± 0.36	14.7 ± 1.3	14.8 ± 0.90
cis-β-Carboxyacrylamidine, 50 mg/kg	500 ± 34§	2.46 ± 0.22§	1.90 ± 0.30	18.3 ± 1.8	17.7 ± 0.86

* Control and treated groups each included five animals which were dosed 2 hr before sacrifice.
† Expressed as μ-equiv./liter in plasma and μ-equiv./g in adipose tissue.
‡ μ-equiv. FFA released/g of tissue during a 2-hr incubation.
§ $P < 0.01$, compared to control.

25 mg/kg increased plasma FFA concentrations 2 hr after dosing and that *cis*-β-carboxyacrylamidine maintains increased plasma FFA concentrations for at least 4 hr (Table 3). These results suggested that the lipid-mobilizing activity of *cis*-β-carboxyacrylamidine might affect weight gain of rats. Figure 2 shows the weight gain and food intake of rats dosed daily with 10 or 50 mg/kg/day for a 12-day period. Both doses lowered weight gain during the first 5 days but in the subsequent periods the animals became resistant to the weight-lowering effect of the compound. The experiments in Tables 4 and 5 were done to find whether resistance development to weight gain reduction is accompanied by a resistance to the plasma FFA-increasing effect of the agent. Table 4 shows the effect of 50 mg/kg of *cis*-β-carboxyacrylamidine on weight gain during a 7-day period. The ten rats in each group were divided into groups of five rats and were dosed with water or the agent on day 8. The results in Table 5 show that the agent significantly increased plasma FFA concentrations in pretreated rats as it did in rats that were treated with water during the previous 7 days. *cis*-β-Carboxyacrylamidine was tested for its effect on cyclic AMP concentrations in adipose tissue. Table 6 shows that the agent did not increase cyclic AMP but epinephrine did, under conditions that demonstrated the lipolytic activity of both compounds. In fact in all three cases there was apparently less cyclic AMP than in untreated adipose tissue.

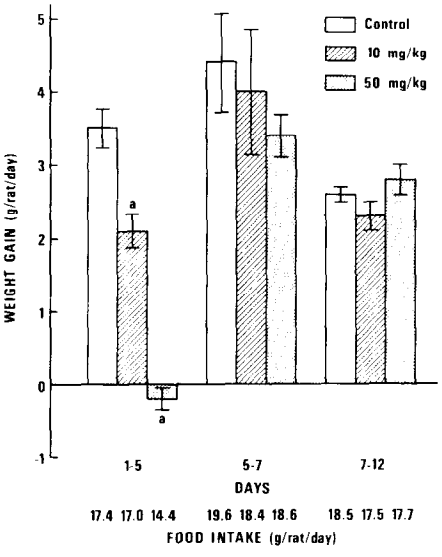

Fig. 2. Weight gain and food consumption of rats given oral doses of *cis*-β-carboxyacrylamidine. Weight gain is reported as mean ± S.E.M. of weight gained per day by five animals in each group in the three separate periods. Experimental conditions are given in the text in Materials and Methods.

cis-β-Carboxyacrylamidine was tested for its effect on the systems that generate or degrade cyclic AMP or are regulated by cyclic AMP concentrations in adipose tissue. The agent neither stimulated adenylate cyclase in isolated fat cells under conditions that

Table 3. Effects of oral doses of *cis*-β-carboxyacrylamidine on plasma FFA concentrations*

Expt. No.	Treatment	Time (hr)	Plasma FFA (μ-equiv./liter)
1.	H_2O (control)	2	284 ± 20†
	cis-β-Carboxyacrylamidine, 100 mg/kg	2	488 ± 44
	cis-β-Carboxyacrylamidine, 25 mg/kg	2	434 ± 17
	H_2O	4	277 ± 25
	cis-β-Carboxyacrylamidine, 100 mg/kg	4	566 ± 28
2.	H_2O	2	344 ± 62
	cis-β-Carboxyacrylamidine, 50 mg/kg	2	589 ± 53
	cis-β-Carboxyacrylamidine, 12.5 mg/kg	2	385 ± 46

* Animals were dosed and plasma FFA concentrations were measured as described in Materials and Methods. Results are given as mean ± S. E. M. of five animals/group.
† $P < 0.01$ for all treated groups compared to control except the last, which is not significantly different from control.

Table 4. Inhibition of weight gain by oral doses of *cis*-β-carboxyacrylamidine*

Experiment No.	Duration (days)	Dose (mg/kg)	Weight gain (g/rat/day)	Food intake (g/rat/day)
1	7	0 (Control)	2.76 ± 0.23	21.4
		50	0.34 ± 0.37†	20.0
2	7	0 (Control)	5.67 ± 0.62	21.2
		50	2.90 ± 0.26†	15.9

* In both experiments each group consisted of ten animals. Average weight at the start was 335 and 277 g for experiments 1 and 2 respectively. Weight gain is shown as mean ± S. E. M. for ten rats in each group.

† $P < 0.001$, compared to control group.

Table 5. Effect of *cis*-β-carboxyacrylamidine on plasma FFA concentration in pretreated animals*

Pretreatment	Treatment	Plasma FFA Expt. 1 (μ-equiv./liter)	Plasma FFA Expt 2 (μ-equiv./liter)
Water	Water	373 ± 15	300 ± 11
Water	*cis*-β-Carboxy-acrylamidine, 50 mg/kg	572 ± 47†	456 ± 13‡
cis-β-Carboxy-acrylamidine, 50 mg/kg	Water	442 ± 48	372 ± 43
cis-β-Carboxy-acrylamidine, 50 mg/kg	*cis*-β-Carboxy-acrylamidine, 50mg/kg	577 ± 63†	461 ± 41‡

* The ten animals per group that were used for weight gain experiments in Table 4 were divided into groups of five and used for these experiments. Results are given as means ± S. E. M.

† $P < 0.05$, compared to control.

‡ $P < 0.01$, compared to control.

Table 6. Effect of epinephrine and *cis*-β-carboxyacrylamidine on 3′,5′-cyclic AMP concentration in adipose tissue

Animal No.	Cyclic AMP (pmoles/g tissue) Basal	Epinephrine*	*cis*-β-Carboxyac·lamidine*
1	200	400	130
2	240	540	140
3	170	290	80

* Concentrations were as follows: epinephrine (10^{-6} M) and *cis*-β-carboxyacrylamidine (8.8×10^{-4} M). Increase produced by epinephrine is significant at the 5 per cent level.

showed stimulation by glucagon, epinephrine and NaF nor inhibited phosphodiesterase activity in homogenates. These data are not shown since the demonstration that cyclic AMP concentration is not elevated by *cis*-β-carboxyacrylamidine (Table 6) suggests that cyclase and phosphodiesterase are not involved. Table 7 shows that *cis*-β-carboxyacrylamidine does not stimulate histone phosphorylation in adipose tissue extracts, and Table 8 shows that hydrolysis of histone phosphate is not inhibited by the agent.

Finally, *cis*-β-carboxyacrylamidine was tested for lipolytic activity in isolated fat cells and was compared to *N*-ethylmaleimide, to which it bears a structural similarity. Table 9 shows *cis*-β-carboxyacrylamidine has lipolytic activity in fat cells, but is not as active as epinephrine or theophylline.

Table 7. Effect of *cis*-β-carboxyacrylamidine on adipose tissue protein kinase activity

Additions	^{32}P incorporated (pmoles/mg protein)
Histone, type 11A	484*
Histone + 3′,5′-AMP, 2.5×10^{-4} M	944†
Histone + *cis*-β-carboxyacrylamidine, 8.8×10^{-4} M	380
Histone + *cis*-β-carboxyacrylamidine, $8.8 + 10^{-5}$ M	444

* Each number is the mean of four experiments. The pooled standard deviation is 95.

† Significantly higher than with histone at the 1 per cent level. The other numbers are not significantly different at the 20 per cent level.

Table 8. Effect of *cis*-β-carboxyacrylamidine on phosphoprotein phosphatase activity*

Expt.	Phosphate released from [^{32}P]histone	
	Basal (*p*moles/mg protein)	*cis*-β-Carboxyacrylamidine (100 μg/ml) (*p*moles/mg protein)
1	1.74	1.40
2	1.24	1.25
3	1.06	1.22
4	1.66	1.82

* Each number is the mean of triplicate determinations in each experiment. Incubation time was 15 min.

Table 9. Comparison of lipolytic activity of epinephrine, theophylline and *cis*-β-carboxyacrylamidine in isolated fat cells*

Addition		Glycerol (μmoles/g cells/hr)
None (basal)		1.27 ± 0.36
Epinephrine, 5×10^{-6} M		6.81 ± 0.53
Theophylline, 10^{-3} M		9.81 ± 0.91
cis-β-Carboxyacrylamidine:	8.8×10^{-4} M	4.02 ± 0.64
	4.4×10^{-4} M	2.75 ± 0.47
	2.2×10^{-4} M	1.54 ± 0.35†

* Each number represents the mean ± S. E. M. for five separate preparations of fat cells.
† Not different from basal lipolysis; all others are significantly higher at the 5 per cent or greater level.

Table 10. Comparison of the effects of *N*-ethylmaleimide and *cis*-β-carboxyacrylamidine on theophylline-stimulated lipolysis*

Addition	Glycerol (μmoles/g cells/hr)
None (basal)	0.68 ± 0.23
Theophylline, 10^{-3} M	9.99 ± 1.00†
N-ethylmaleimide, 4.4×10^{-3} M	1.31 ± 0.40
N-ethylmaleimide + theophylline	0.80 ± 0.58
cis-β-Carboxyacrylamidine, 4.4×10^{-3} M	4.82 ± 1.72†
Theophylline + *cis*-β-carboxyacrylamidine	10.90 ± 0.92†

* Each number represents the mean ± S. E. M. for four different preparations of fat cells.
† $P < 0.01$, compared to basal group.

Table 11. Effect of *cis*-β-carboxyacrylamidine on hormone-sensitive lipase*

Additions	Lipase activity (μmoles/mg protein)	Percentage of control
None (control)	1.45	
ATP + 10^{-5} M cAMP	1.99	145 ± 12†
cis-β-Carboxyacrylamidine:		
8.8×10^{-4} M	1.28	88 ± 13
4.4×10^{-4} M	1.24	86 ± 8
2.2×10^{-4} M	1.29	89 ± 13
4.4×10^{-4} M + ATP + cAMP	1.34	92 ± 11

* Lipase was preincubated for 10 min at 30° with additions shown and incubated 60 min after addition of substrate mixture.
† $P < 0.01$, compared to control; $P > 0.10$ for all other numbers.

Table 10 compares the lipolytic activity of *N*-ethylmaleimide and *cis*-β-carboxyacrylamidine both alone and together with theophylline. At a concentration that was found to stimulate lipolysis in pieces of adipose tissue,* *N*-ethylmaleimide was inactive in fat cells in agreement with Calvert and Lech [18]. Also it differed from *cis*-β-carboxyacrylamidine in that it inhibited completely the lipolytic effect of theophylline. Since *cis*-β-carboxyacrylamidine failed to affect any of the enzyme systems studied, its effect on hormone-stimulated lipase [11] was examined. Table 11 shows that concentrations that stimulated lipolysis in

* F. P. Kupiecki, unpublished experiments.

adipose tissue and in fat cells did not stimulate hormone-sensitive lipase. Unexpectedly, the agent inhibited the increase of lipolysis brought about by ATP and cyclic AMP.

DISCUSSION

The data presented here show that *cis*-β-carboxyacrylamidine acts as a lipid mobilizer *in vitro* and suggests that it mobilizes lipids *in vivo*, since it increases plasma FFA concentrations. The results, however, do not prove that the lipid-mobilizing activity causes the reduction in weight gain. It is likely that at least a part of the change in weight gain is due to the reduced food consumption, which is possibly the result of anorexigenic activity. This conclusion is reached from the fact that plasma FFA concentrations were still increased by the agent when the animals became resistant to its effect on weight gain.

The results suggest that *cis*-β-carboxyacrylamidine affects some step in the sequence of events that increase activity of triglyceride lipase. A stimulation of adenyl cyclase or an inhibition of phosphodiesterase seems to be ruled out by the observation that the agent neither stimulates adenyl cyclase, inhibits phosphodiesterase nor increases cyclic AMP concentrations in adipose tissue *in vitro*. Stimulation of the protein kinase that activates triglyceride lipase [19] or inhibition of a phosphoprotein phosphatase also appears to be ruled out, but not conclusively. It has been reported that a phosphoprotein phosphatase in bovine heart is inhibited by adenosine and uridine nucleotides [20]. The inhibitors were effective with glycogen synthase D and with phosphorylase *a* as substrates, but ineffective with histone as substrate. Therefore, the demonstration that *cis*-β-carboxyacrylamidine does not inhibit hydrolysis of phosphorylated histone does not exclude possible inhibition of a lipase phosphatase.

The lipolysis studies *in vitro* which were discussed thus far were done with pieces of adipose tissue. Therefore, it was deemed necessary to check for lipolytic activity in isolated fat cells. In a comparison study with epinephrine and theophylline it was found that *cis*-β-carboxyacrylamidine stimulates lipolysis in fat cells (see Table 9). Also in fat cells (see Table 10) it was possible to show that *N*-ethylmaleimide, a structural analog and sulfhydryl-group inhibitor, inhibits theophylline-stimulated lipolysis but *cis*-β-carboxyacrylamidine does not. Thus it is probable that the two compounds affect the lipolytic sequence at different points.

The failure to show an effect of *cis*-β-carboxyacrylamidine on phosphodiesterase, protein kinase, phosphoprotein phosphatase or cyclic AMP concentrations pointed to a possible direct effect of the agent on the hormone-sensitive lipase [11, 19]. This has been reported for part of the lipolytic activity of ACTH [21] and the antilipolytic activity of clomiphene [22]. Therefore, it was rather unexpected to find that *cis*-β-carboxyacrylamidine not only fails to stimulate lipase but also blocks the stimulation produced by ATP and cyclic AMP. It is difficult to suggest plausible reasons for this finding. One possible reason is that the concentrations required in adipose tissue or isolated fat cells are very different from those required with the soluble hormone-sensitive lipase. A more likely possibility, but one that would be difficult to verify, is that the hormone-sensitive lipase is not the same lipase that is activated by hormones in intact adipose tissue and isolated fat cells.

These results have documented the lipolytic activity of *cis*-β-carboxyacrylamidine but have not explained its mode of action. However, this newly described lipolytic agent may be useful in furthering our understanding of lipolysis in fat tissue and fat cells.

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