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ON THE ROLE OF ADENYL CYCLASE IN THE REGULATION OF LIPOLYSIS IN FASTING*

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Abstract—Epididymal fat pads from fasting rats were more sensitive to the lipolytic effects of theophylline than fat pads from fed animals, but the maximum response to this agent was the same in both groups. From these results it may be inferred that fasting did not change the amount of adipose tissue lipase. The amount of activatable adenyl cyclase in adipose tissue was increased about 2-fold by fasting, as shown by direct assay of the enzyme as well as by the increase in the maximum lipolytic response to norepinephrine (NE) *in vitro*. However, *in vivo* the adenyl cyclase appears to be activated by a nonadrenergic system, since the rise in free fatty acids (FFA) is not inhibited by propranolol, a beta adrenergic blocking drug, though it is inhibited by nicotinic acid. Kinetic analysis of the inhibitory effects of propranolol on lipolytic responses to NE *in vitro* suggest that characteristics of the receptor, presumably adenyl cyclase, are similar in fasting and fed animals.

PREVIOUS reports from this laboratory suggest that the total amount of lipase in the epididymal fat pad of rats is constant in a number of physiologic states, the rate of lipolysis depending on the extent to which inactive lipase is converted to the active form. This rate in turn is limited by the level of cyclic adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP).[1–5] Recent studies have shown that rapid changes in the energy requirements of the body are mediated through the sympathetic nervous system.[6] The consequent release of norepinephrine (NE) increases the level of cyclic 3′,5′-AMP through the instantaneous activation of adenyl cyclase.

The mechanism by which the caloric requirements of fasting are adjusted through an increased output of free fatty acids (FFA) is not known, although recent reports suggest that it does not involve the sympathetic nervous system.[6–9]

The present study concerns the effects of fasting on the lipolytic system in adipose tissue. The results show that the increased output of FFA in fasting is accompanied by an increase in the amount of adenyl cyclase but not an increase in the total lipase and that the adenyl cyclase is activated in the body by some noncatecholamine substance.

MATERIAL AND METHODS

Experiments on the effects of fasting were carried out in male, Sprague–Dawley

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rats weighing about 200 g. Before the experiments, the animals were permitted free access to Purina laboratory chow and tap water. During fasting all food was removed from the cage. The animals were decapitated and blood was collected in test tubes that contained heparin.

Lipolysis in adipose tissue was determined by the rate of glycerol released from minces of epididymal fat pads according to Schusterova *et al.*[10] Minced tissue weighing 50 mg was incubated for 1 hr at 37° in 1 ml of Krebs–Ringer phosphate buffer, pH 7·4, containing 5% albumin (Fraction V, Nutritional Biochemical Corp., Cleveland, Ohio).

Plasma FFA were determined by the method of Novak.[11] Glycerol was determined according to Lambert and Neish.[12] Protein in fat pads was estimated by the method of Sutherland *et al.*[13] Phosphodiesterase was assayed as previously described.[5]

Adenyl cyclase in fat pads was assayed according to the method of Krishna *et al.*[14] from the rate of formation of ^{3}H-cyclic AMP from ^{3}H-ATP. In this method fat pads were homogenized with 3 vols. of a buffer containing Tris HCl, pH 7·3 (8×10^{-2} M), $MgSO_4$ (7×10^{-3} M), theophylline (2×10^{-2} M) and NE (2×10^{-5} M), and 0·4-ml aliquot was incubated at 30° with 0·2 ml of ^{3}H-ATP (6×10^{-3} M, 33 μc/μmole). The enzyme reaction was terminated by immersion in boiling water for 2–3 min after the addition of 0·1 ml solution of cyclic 3′,5′-AMP (5 mg/ml) as a carrier. The cyclic 3′,5′-AMP was separated from other radioactive material by chromatography on a Dowex 50-H^+ column and precipitation by zinc sulfate–barium hydroxide. The ^{3}H-cyclic 3′,5′-AMP was measured by liquid scintillation spectrometry and the recovery determined from the u.v. absorption (260 mμ) of the unlabeled nucleotide. The enzyme activity was measured from the linear rate of formation of ^{3}H-cyclic 3′,5′-AMP over a 15-min period and was expressed as micromicromoles of cyclic 3′,5′-AMP per milligram of protein per minute. The identity of the ^{3}H-cyclic 3′,5′-AMP isolated by the above procedure was authenticated by enzymatic conversion to 5′-AMP by cyclic 3′,5′-AMP phosphodiesterase, by chromatography in a number of ion exchange and paper chromotographic systems, and by recrystallization to constant specific activity after addition of carrier cyclic 3′,5′-AMP.[14]

Materials. Chemicals used in this study and their sources are as follows: bovine albumin, Fraction V, and nicotinic acid (Nutritional Biochemical Corp.); *l*-norepinephrine bitartrate (Winthrop Laboratories); propranolol (Inderal) (Ayerst Laboratories, Inc.); ^{3}H-ATP (New England Nuclear Corp.); ATP and cyclic 3′,5′-AMP (Schwartz Biochemical Research Corp.).

RESULTS

Characteristics in vivo *of lipolytic system in adipose tissue from fed and fasting rats*

Table 1 shows that the administration of NE elicited a rise in the FFA level of normal (fed) rats and that this effect was completely counteracted by administration of nicotinic acid or the beta adrenergic blocking agent propranolol.

During fasting, plasma FFA rose and reached a plateau within 48 hr. Propranolol exerted no effect on the rise in plasma FFA; in contrast, the rise was completely counteracted by nicotinic acid. Moreover, administration of NE to fasting rats did not significantly raise the FFA level (Table 1). The fact that the rise in FFA level in fasting rats was not reduced by beta adrenergic blockade suggests that the adenyl cyclase in adipose tissue had been activated by a nonadrenergic mechanism *in vivo*.

TABLE 1. EFFECTS OF PROPRANOLOL, NICOTINIC ACID AND NOREPINEPHRINE ON PLASMA FFA LEVELS OF FED AND FASTING RATS*

Treatment	FFA (μ-equiv./ml)
1. Fed rats	
Given saline	0·45 ± 0·04
Given NE and saline	0·63 ± 0·04†
Given NE and propranolol	0·50 ± 0·03‡
Given NE and nicotinic acid	0·40 ± 0·01‡
2. Fasting rats	
Given saline	0·88 ± 0·05
Given propranolol	0·85 ± 0·02‡
Given nicotinic acid	0·46 ± 0·04†
Given NE	0·97 ± 0·07‡

* Rats fasted for 48 hr were given NE (0·2 mg as base/kg i.m.), propranolol (50 mg/kg s.c.), or nicotinic acid (200 mg/kg i.p., pH 7·0). Fed rats were given NE alone or 30 min after the administration of propranolol or nicotinic acid. Control animals were injected with saline. The animals were killed 30 min after NE and 60 min after propranolol or nicotinic acid administration, and blood samples collected for assay of plasma FFA. Each value ± S.E. represents mean of six experiments.

† Values are significantly different from those of control animals injected with saline ($P < 0·05$).

‡ Not statistically different from those of saline-treated control animals ($P > 0·05$).

Characteristics in vitro *of lipolytic system in adipose tissue from fed and fasting rats*

Figure 1 (right) shows that the adipose tissue from fasting rats was more sensitive to the lipolytic effects of theophylline than that from control animals. However, the maximum lipolytic response produced by high concentrations of theophylline was almost the same in the two groups. These results suggested that fasting had not increased the quantity of adipose tissue lipase.

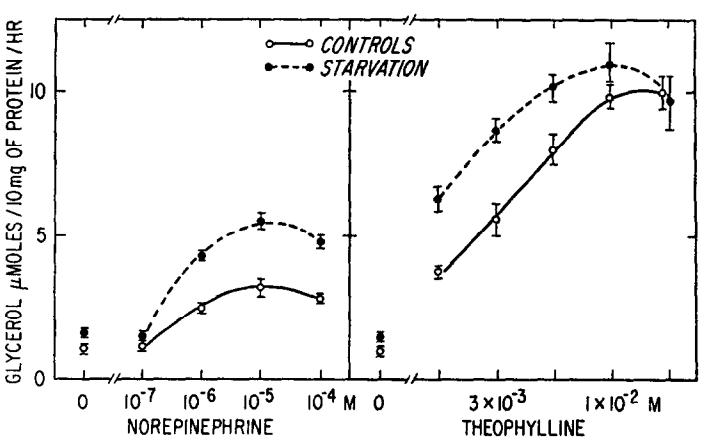

FIG. 1. Left, lipolytic effects of NE in adipose tissue from fed and fasting rats. Right, lipolytic effects of theophylline in adipose tissue of fed and fasting rats. Rats were fasted for 48 hr. Each point represents mean value of six to eight experiments ± S.E.

In addition, the maximum lipolytic response to NE of adipose tissue from fasting rats was about twice that from control animals. Since NE was added in amounts sufficient to activate adenyl cyclase completely, it was inferred that the amount of this enzyme had been increased by fasting.

Direct evidence that fasting had increased the amount of adenyl cyclase was obtained from assays of the enzyme in adipose tissue from fasting and fed rats (Table 2). In the tissue from fasting rats the activity of adenyl cyclase and the lipolytic responses to NE had increased in almost the same proportion. On the other hand, fasting did not affect the activity of phosphodiesterase.

TABLE 2. EFFECTS OF FASTING ON ADENYL CYCLASE AND PHOSPHODIESTERASE ACTIVITY AND ON LIPOLYTIC RESPONSE TO NE IN EPIDIDYMAL FAT PADS*

Treatment	Lipolytic activity (μmolesglycerol/hr)	Adenyl cyclase activity ($\mu\mu$moles cyclic AMP/min)	Phosphodiesterase activity ($\mu\mu$moles P_i/min)
Control	0·22 ± 0·04	16 ± 4	2700 ± 270
Fasted 48 hr	0·44 ± 0·03	44 ± 12	3200 ± 270

* Rats were fasted for 48 hr. The pads from the left side were used for the assay of adenyl cyclase and phosphodiesterase activity, and the pads from the right side were minced and incubated with NE (10^{-5} M) as described in Methods. Lipolytic activity was measured from release of glycerol; adenyl cyclase acivity from formation of cyclic AMP; and phosphodiesterase activity from conversion of cyclic AMP via 5′-AMP to inorganic phosphate. Activities are expressed per mg protein. Each value ± S.E. represents mean of four experiments. For each experiment, fat pads of three animals were pooled.

The kinetic characteristics of the NE-induced lipolysis were compared in adipose tissue from fed and fasting rats. Figure 2, in which the lipolytic effect of NE is expressed as the percentage of the maximum theophylline effect, shows that propranolol in both cases produced a competitive inhibition of lipolysis. Table 3 shows the drug parameters of NE lipolysis calculated from the data in Fig. 2. The negative logarithm of the molar concentration of NE that produced 50 per cent of its maximal lipolytic effect (pD_2)[15] was almost identical in fed and fasting animals. In addition, the inhibitory effects of propranolol (pA_2)[15] were similar in both groups. These results suggest that the characteristics of the lipolytic receptor, presumably adenyl cyclase, are similar in fasting and fed animals.

DISCUSSION

The present studies show that the increased output of FFA in fasting rats is hardly affected by beta adrenergic blocking agents. It may be inferred, therefore, that enhanced mobilization of FFA in fasting is not an adrenergic function but is independent of the sympathetic nervous system. A previous report from this laboratory[8] based a similar conclusion on the findings that fasting still causes hypermobilization of FFA in functionally sympathectomized rats (adrenal demedullation followed by depletion of NE stores in nerve endings).[8, 9]

Although the rise in FFA output in fasting is not blocked by functional sympathectomy[8, 9] or by beta adrenergic blocking agents,[16] it is still prevented by nicotinic acid, which has been shown to act by reducing the level of cyclic 3′,5′-AMP,[17, 18] strongly suggesting that the increased mobilization of FFA in fasting is

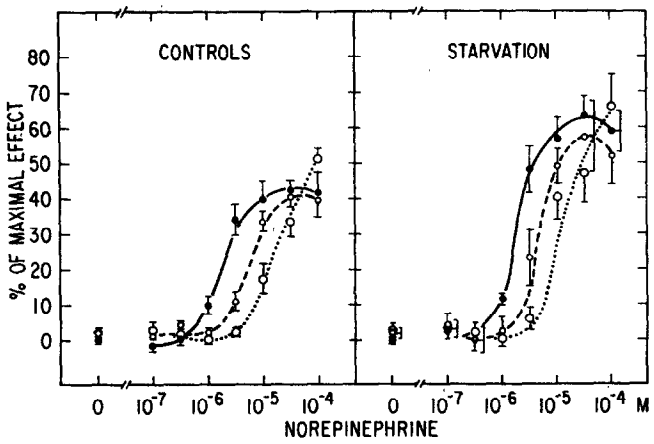

FIG. 2. Left, inhibitory effects of propranolol on lipolysis elicited by NE in adipose tissue of fed rats. Each point represents mean value of six to eight experiments ± S.E. Right, inhibitory effects of propranolol on lipolysis elicited by NE in adipose tissue of rats fasted for 48 hr. ——●—— Controls (no propranolol); - - - - ○ - - - - propranolol (3×10^{-6} M); . . . . ○ . . . . propranolol (1×10^{-5} M). Lipolysis is expressed as percentage of maximal theophylline effect.

TABLE 3. DRUG PARAMETERS OF NE-INDUCED LIPOLYSIS IN ADIPOSE TISSUE TAKEN FROM FED AND FASTING RATS*

Group	Lipolysis (μmoles/mg protein/hr)	pD_2	pA_2	
			Propranolol	
			3×10^{-6} M	1×10^{-5} M
Controls	0·21	5·73	5·79	5·84
Fasted 48 hr	0·32	5·77	5·73	5·80

* Drug parameters are calculated from data in Fig. 2 as described by van Rossum;[15] pD_2 and pA_2 are measures of the affinity of NE and propranolol, respectively, for the receptors. The pA_2 of propranolol is calculated from effects of propranolol at 2 concentrations (3×10^{-6} and 1×10^{-5} M), as shown in Fig. 2.

also mediated by cyclic 3',5'-AMP. More direct evidence that adenyl cyclase is important in the increased mobilization of FFA is the pronounced rise in the amount of activatable enzyme in adipose tissue. Adenyl cyclase by itself cannot account for the increase in FFA level that occurs during fasting. Since this increase occurs *in vivo* in the absence of sympathetic activity, some unknown noncatecholamine substance must be responsible for the activation of adenyl cyclase.

It would appear that fasting causes at least two changes to the lipolytic system: (1) the amount of activatable adenyl cyclase in adipose tissue is increased; (2) the enzyme, and hence the lipolytic system, is activated by an unknown nonadrenergic substance.

Under investigation is the possibility that the amount of adenyl cyclase in fasting is increased by the action of growth hormone. Roth *et al.*[19] have reported that the plasma level of growth hormone, as measured by an immunochemical method, increases with fasting and they have suggested that animals adapt to the lack of food

by mobilization of FFA through the action of this hormone. Growth hormone does not exert an immediate effect on lipolysis *in vitro*; the mobilization of FFA *in vivo* appears to be indirect, the hormone eliciting an increased mobilization of FFA only after a period of several hours.[20]

In considering the nature of the nonadrenergic process responsible for the activation of adenyl cyclase, it seems pertinent that considerable amounts of a fat-mobilizing substance have been found in the urine of a number of animal species during fasting.[21, 22] This substance injected into mice or rats causes a prompt rise in plasma FFA. Moreover, the addition of this substance to rat adipose tissue *in vitro* causes an immediate and marked increase in the release of FFA.

In conclusion, our results suggest that the increased mobilization of FFA during fasting is mediated through cyclic 3',5'-AMP. The effects of theophylline reveal that there is no difference in the amount of adipose tissue lipase in fed and starving rats; rather the amount of activatable adenyl cyclase is increased in fasting and is stimulated in the living animal by a substance that is not part of the sympathetic system.

REFERENCES

1. M. A. RIZAK, *J. biol. Chem.* **239**, 392 (1964).
2. R. W. BUTCHER, R. J. HO, H. C. MENG and E. W. SUTHERLAND, *J. biol. Chem.* **240**, 4515 (1965).
3. B. B. BRODIE, J. I. DAVIES, S. HYNIE, G. KRISHNA and B. WEISS, *Pharmac, Rev.* **18**, 273 (1966).
4. S. HYNIE, G. KRISHNA and B. B. BRODIE, *J. Pharmac. exp. Ther.* **153**, 90 (1966).
5. B. WEISS, J. I. DAVIES and B. B. BRODIE, *Biochem. Pharmac.* **15**, 1553 (1966).
6. B. B. BRODIE, R. P. MAICKEL and D. N. STERN, in *Handbook of Physiology, Section* 5, *Adipose Tissue* (Eds. A. E. RENOLD and G. F. CAHILL, JR.), p. 583. Williams & Wilkins Co., Baltimore (1965).
7. H. M. GOODMAN and E. KNOBIL, *Proc. Soc exp. Biol. Med.* **102**, 493 (1959).
8. D. N. STERN and R. P. MAICKEL, *Life Sci.* **2**, 872 (1963).
9. T. FARKAS, R. VERTUA, M. M. USARDI and R. PAOLETTI, *Life Sci.* **3**, 821, (1964).
10. D. SCHUSTEROVA, D. KRCIKOVA, E. MÜHLBACHOVA, S. HYNIE and M. WENKE, *J. Neuropharmac.* **3**, 129 (1964).
11. M. NOVAK, *J. Lipid Res.* **6**, 431 (1965).
12. M. LAMBERT and A. C. NEISH, *Can. J. Res.* (Section B) **28**, 83 (1950).
13. E. W. SUTHERLAND, C. F. CORI, R. HAYNES and N. S. OLSEN, *J. biol. Chem.* **180**, 825 (1949).
14. G. KRISHNA, B. WEISS and B. B. BRODIE, *J. Pharmac. exp. Ther.*, **163**, 379 (1968).
15. J. M. VAN ROSSUM, *Archs int. Pharmacodyn. Ther.* **143**, 299 (1963).
16. K. STOCK and E. WESTERMANN, *Arch. exp. Path. Pharmak.* **254**, 334 (1966).
17. R. H. WILLIAMS, S. A. WALSH, D. K. HEPP and J. W. ENSINCK, *Metabolism* **17**, 653 (1968).
18. G. KRISHNA, B. WEISS, J I. DAVIES and S. HYNIE, *Fedn Proc.* **25**, 719 (1966).
19. J. ROTH, S. M. GLICK, R. S. YALOW and S. A. BERSON, *Metabolism* **12**, 577 (1963).
20. M. S. RABEN and C. H. HOLLENBERG, *J. clin. Invest.* **38**, 484 (1959).
21. T M. CHALMERS, in *Handbook of Physiology, Section* 5, *Adipose Tissue* (Eds. A. E. RENOLD and G. F. CAHILL, JR.), p. 549. Williams & Wilkins Co., Baltimore (1965).
22. J. A. F. STEVENSON and J. R. BEATON, *Ann N.Y. Acad. Sci.* **131**, 189 (1965).