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THE EFFECT OF CHLOROPHENOXYISOBUTYRIC ACID ON THE RELEASE OF FREE FATTY ACIDS FROM ISOLATED ADIPOSE TISSUE *IN VITRO*

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There is evidence to suggest that the plasma free fatty acids play a significant role in the processes resulting in coronary thrombosis and atherosclerosis. For example, the intravenous infusion of fatty acids in the dog has produced massive thrombosis in the heart and the great vessels (Connor, Hoak & Warner, 1963). The elevation of endogenous free fatty acids by corticotrophin or pituitary extracts also caused thrombosis and occasional deaths in rabbits (Hoak, Poole & Robinson, 1963). These authors observed a concomitant fall in the whole blood silicone clotting time in corticotrophin-treated animals. The aggregation of human platelets has been stimulated *in vitro* by the addition of aqueous suspensions or lecithin sols of fatty acids (Haslam, 1964; Kerr, Pirie, MacAulay & Bronte-Stewart, 1965). Repeated mobilization of free fatty acids in rats and dogs by adrenaline has been followed by increases in the circulating levels of cholesterol and phospholipids (Shafrir, Sussman & Steinberg, 1960; Shafrir & Steinberg, 1960).

The effects of α-(4-chlorophenoxy)isobutyric acid (α-*p*-chlorophenoxy-α-methylpropionate, CPIB) in reducing both total lipids and cholesterol in the blood and liver of rats were described by Thorp & Waring (1962). Subsequent clinical evaluation showed CPIB to be effective in lowering serum triglyceride and cholesterol levels in man (Oliver, 1963).

Further clinical studies have demonstrated that treatment with CPIB prolonged platelet survival and reduced platelet stickiness (Gilbert & Mustard, 1963; Symons, Toszeghi & Cook, 1964). It might be possible to explain some of the effects of CPIB in terms of a reduction in free fatty acid availability. This hypothesis has been tested using an isolated adipose tissue system.

METHODS

Tissue and plasma were obtained from specific pathogen-free male rats of the Alderley Park (albino) strain. Before use the animals were housed in groups of five in a room maintained at 21° C. All work with these animals was done between 9.00 a.m. and 10.00 a.m., taking precautions to avoid disturbance in the animal house.

Epididymal fat pads were removed from fed rats (weighing 140 to 160 g) killed by cervical dislocation. The pads were cut into approximately equal halves, blotted and weighed on a torsion balance (the average weight of tissue was about 100 mg). Each segment was then placed either in 2 ml. of Krebs-Ringer bicarbonate solution containing 3% bovine serum albumin or in 2 ml. of pooled rat plasma. Each donor animal contributed one segment to each of four experimental groups.

Whole blood was obtained from the abdominal aorta of intact rats (240 to 260 g body weight) during light ether anaesthesia using a heparinized syringe. The blood was centrifuged at 3,000 rev/min for 10 min and the resultant plasma removed, pooled and well mixed. In one experiment plasma was prepared from rats which had been hypophysectomized 7 days previously. In another experiment rats were housed singly overnight before bleeding. Some rats were bled after the application of a neckclamp which prevented any blood from the head contributing to the sample, whilst others were bled after exposure to ether vapour for 2 min. Plasma was always used within 1 hr of collection.

Hypophysectomy was performed by the parapharyngeal route and the animals were given 5% glucose solution in place of drinking water. Plain water was substituted for glucose solution 24 hr before hypophysectomized rats were used.

The incubations were made in 25 ml. conical flasks for 2 hr at 37° C in a Dubnoff Metabolic Shaker. Each flask was gassed with 95% oxygen and 5% carbon dioxide and capped with waxed film before incubation. CPIB or nicotinic acid was dissolved in either Krebs-Ringer bicarbonate solution with 3% albumin or rat plasma in sufficient amount to yield the desired final concentration. Adrenaline, dissolved in distilled water to give 20 μg/ml., was added to the incubation flasks in volumes of 0.1 ml. Control flasks received 0.1 ml. of distilled water in appropriate cases.

The free fatty acid content of the incubation fluids was estimated by the method of Dole (1956) as modified by Barrett (1964). Appropriate blanks were run in each experiment for both CPIB and nicotinic acid at their respective concentrations. This was particularly important for CPIB as it is extracted and titrated in the Dole method. The concentration of CPIB in either Krebs-Ringer or plasma incubations was not altered by the addition of fat. It can be concluded that there is no net uptake of CPIB by adipose tissue.

The substances used in the experiments were (−)-adrenaline bitartrate; CPIB sodium salt (clofibrate or Atromid-S is the ethyl ester); nicotinic acid; bovine serum albumin (Armour Fraction V); corticotrophin (Acthar for intravenous use, Armour); and bovine growth hormone (Merck Sharpe & Dohme).

RESULTS

Incubation of epididymal adipose tissue in an albumin-enriched isotonic solution resulted in a small but significant release of fatty acids into the medium. When adrenaline was added at a concentration of 1.0 μg/ml. there was an approximately tenfold increase in fatty acid release. The addition of CPIB in concentrations ranging from 250 to 1,000 μg/ml. did not alter the basal release rate. Adrenaline still produced a significantly greater ($P<0.01$) release of fatty acids regardless of the concentration of CPIB present. However, the rate of fatty acid release induced by adrenaline was reduced by CPIB, albeit only significantly at 250 μg/ml. (Table 1). Nicotinic acid also failed to modify the basal release rate of fatty acids but this agent produced a dose-dependent inhibition of the response to adrenaline. The effects of CPIB and nicotinic acid on adrenaline-stimulated fatty acid release are contrasted in Fig. 1.

It was possible that the failure of CPIB to antagonize effectively the response to adrenaline was due to the relatively poor affinity of CPIB for anionic binding sites on bovine albumin (Thorp, unpublished). The most convenient source of rat albumin is rat plasma and it was for this reason that the above experiments were repeated with the substitution of fresh rat plasma for albumin-Krebs-Ringer-bicarbonate solution. The results, summarized in Table 2, were surprising. The incubation of adipose tissue in plasma alone resulted in a high rate of release of free fatty acids which was not significantly increased by the addition of adrenaline. In the presence of CPIB at all concentrations studied there was a marked reduction in the release of fatty acids. Adrenaline increased the release of free fatty acids in all groups containing CPIB to a greater extent than in the control group but in no

TABLE 1

THE EFFECT OF CPIB AND NICOTINIC ACID ON ADRENALINE-INDUCED RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE INCUBATED IN KREBS-RINGER-BICARBONATE SOLUTION CONTAINING 3% BOVINE SERUM ALBUMIN

Each value represents the mean fatty acid release (μequiv/g/hr, with standard error) for the number of flasks in parentheses. An asterisk denotes that the value is significantly different ($P<0.05$) from the appropriate control value without added inhibitor

Concentration of adrenaline (μg/ml.)	Fatty acid release (μequiv/g/hr) for Concentration of CPIB in medium (μg/ml.)			
	0	250	500	1,000
0	1.56±0.09 (11)	1.32±0.33 (7)	1.24±0.42 (7)	1.63±0.28 (7)
1	10.11±0.72 (11)	6.72±1.39* (7)	8.86±0.81 (7)	8.38±0.82 (7)
	Concentration of nicotinic acid in medium (μg/ml.)			
	0	20	100	500
0	1.66±0.41 (4)	1.84±0.61 (4)	1.61±0.91 (4)	1.42±0.91 (4)
1	15.40±1.70 (4)	12.19±2.22 (4)	6.71±1.77* (4)	3.46±0.28* (4)

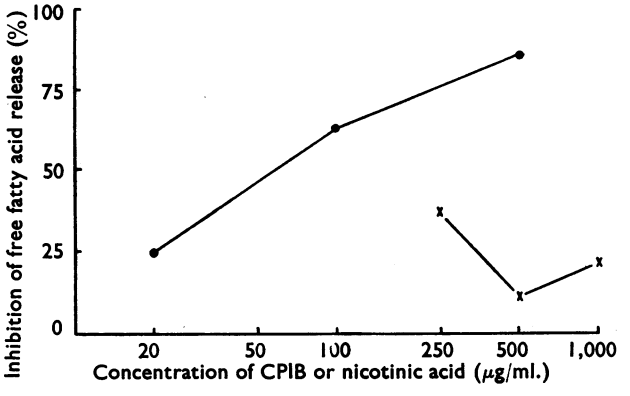

Fig. 1. The reduction in free fatty acid release in response to adrenaline (1 μg/ml.) in the presence of various concentrations of CPIB (×——×) and nicotinic acid (●——●), expressed as a percentage of the control response to adrenaline.

instance was this effect statistically significant. The final concentration of fatty acids in the adrenaline plus CPIB groups was lower than that in the control adrenaline group. Nicotinic acid had no effect on the release of fatty acids from adipose tissue incubated in plasma either on the basal rate or on that following the addition of adrenaline.

The incubation of plasma at 37° C for 2 hr results in a significant increase in the concentration of free fatty acids (Table 3). This is presumably an enzymic reaction involving the hydrolysis of tryglyceride since previous incubation at 56° C dramatically reduced the effect. Addition of CPIB to plasma previously stored at 4° C did not alter the degree of lipolysis usually observed following incubation.

TABLE 2

THE EFFECT OF CPIB AND NICOTINIC ACID ON ADRENALINE-INDUCED RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE INCUBATED IN RAT PLASMA

Each value represents the mean fatty acid release (μequiv/g/hr, with standard error) for three incubations. An asterisk denotes that the value is significantly different ($P<0.05$) from the appropriate control value without added inhibitor

Concentration of adrenaline (μg/ml.)	Fatty acid release (μequiv/g/hr) for Concentration of CPIB in plasma (μg/ml.)			
	0	250	500	1,000
0	9.51±1.12	3.70±0.79*	3.82±1.00*	3.17±1.10*
1	10.69±1.80	5.87±0.86	6.56±0.65	4.87±1.67
	Concentration of nicotinic acid in plasma (μg/ml.)			
	0	20	100	500
0	19.80±1.39	22.22±3.96	30.36±0.52	24.84±2.20
1	19.47±1.14	18.60±2.30	26.17±1.41	24.75±2.19

TABLE 3

PLASMA FREE FATTY ACID CONCENTRATIONS BEFORE AND AFTER INCUBATION AT 37° C FOR 2 HR: EFFECT OF PREVIOUS INCUBATION AT 4 AND 56° C AND OF THE ADDITION OF CPIB (500 μG/ML.)

Each value is the mean with standard error of four incubations

Preincubation procedure	Concentration of free fatty acids (μequiv/l.)		
	Before incubation	After incubation	Change
30 min at 4°C	420±1	734±34	+314
30 min at 56°C	442±22	487±22	+45
30 min at 4°C+CPIB	420±16	738±19	+318

Previous incubation of plasma at either 4 or 56° C did not affect the activity of the lipolytic factor present in plasma. Once again the inclusion of CPIB in the incubation flask produced a significant reduction ($P<0.001$) in the rate of release of free fatty acids (Table 4).

The incubation of adipose tissue in plasma obtained from hypophysectomized rats did not result in the marked stimulation observed with plasma from intact rats (Table 5). Addition of CPIB appeared to increase the release of free fatty acids but the significance of this observation is obscure. When adrenaline was added there was a significant increase in fatty acid release. The response was not reduced by CPIB except at the highest dose level (statistically not significant).

Since all the plasma previously used had been collected during ether anaesthesia, there was liable to have been a high level of corticotrophin present. However, collection of blood under conditions designed to prevent the release of corticotrophin did not result in any loss of lipolytic potency in the plasma. Adipose tissue incubated in plasma obtained from non-stressed animals released 12.87±1.30 and 13.81±1.17 μequiv/g/hr (means and standard errors) in plasma taken 2 min after exposure to ether vapour.

Both corticotrophin and growth hormone stimulated a significant release of free fatty acids (Table 6). CPIB had no significant effect on the response to either hormone although there was a small reduction in the corticotrophin group.

TABLE 4

THE EFFECT OF CPIB ON THE RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE INCUBATED IN PLASMA PREVIOUSLY INCUBATED AT 4 OR 56° C FOR 30 MIN

Each value represents the mean and standard error of four incubations. CPIB was in a concentration of 500 μg/ml.

Preincubation temperature (°C)	CPIB	Free fatty acid released (μequiv/g/hr)
4	Absent	25.20±2.1
4	Present	3.14±0.9
56	Absent	29.60±1.9
56	Present	2.96±0.8

TABLE 5

THE EFFECT OF CPIB ON THE ADRENALINE-INDUCED RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE INCUBATED IN PLASMA OBTAINED FROM HYPOPHYSECTOMIZED RATS

Each value represents the mean fatty acid concentration (μequiv/g/hr, with standard error) for three flasks. An asterisk denotes that the value is significantly different ($P<0.05$) from the appropriate control value without CPIB

Concentration of adrenaline (μg/ml.)	Fatty acid release (μequiv/g/hr) for Concentration of CPIB in plasma (μg/ml.)			
	0	250	500	1,000
0	1.78±0.08	2.21±0.37	3.07±0.19*	3.42±0.34*
1	5.49±0.83	6.03±0.08	6.72±0.49	5.23±1.08

TABLE 6

THE EFFECT OF CPIB ON FATTY ACID RELEASE INDUCED BY CORTICOTROPHIN AND GROWTH HORMONE FROM ADIPOSE TISSUE INCUBATED IN KREBS-RINGER-BICARBONATE SOLUTION

Each value represents the mean (μequiv/g/hr, with standard error) of the number of flasks in parentheses. Corticotrophin was in a concentration of 150 mU/ml., growth hormone 100 μg/ml., and CPIB 250 μg/ml.

CPIB	Fatty acid release (μequiv/g/hr) for addition to medium		
	Nil	Corticotrophin	Growth hormone
Absent	3.44±0.25 (7)	10.03±1.11 (7)	9.57±0.60 (7)
Present	4.66±0.46 (4)	9.82±1.36 (4)	10.91±1.57 (4)

DISCUSSION

The ability of CPIB to reduce serum cholesterol and triglyceride levels has been established both in man and laboratory animals. At the present time there is no unified hypothesis relating to the mechanism by which these effects are produced. CPIB is strongly protein bound *in vivo* and it has been suggested that its actions are consequent upon the displacement of certain anions from their binding sites on plasma albumin (Thorp, 1964). Increasing the input of free fatty acids to the liver results in an accumulation of hepatic glyceride and a secondary increase in cholesterol and phospholipids, ultimately reflected in upward changes in circulating lipid levels (Steinberg, 1964). Removal of free fatty acids from

adipose tissue following lipolysis is dependent upon the presence of circulating albumin molecules. Occupation of the acidic binding sites, normally used for the transport of fatty acids, by CPIB might therefore serve to diminish the effective transport of fatty acids to the liver and thereby account for the observed reduction in total lipid level (Thorp & Waring, 1962).

Lipolysis within adipose tissue continues under appropriate conditions *in vitro* and may be stimulated by various agents. However, unless albumin is present in the incubating medium there is negligible release of free fatty acids from the tissue. Prior equilibration of bovine serum albumin with CPIB did not prevent the release of free fatty acids *in vitro* either in the basal state or after stimulation with adrenaline. There was some reduction in the response to adrenaline but, paradoxically, the greatest effect was seen at the lowest dose level. Since the concentrations of CPIB used were two to ten times the effective plasma levels *in vivo* it seemed unlikely that this small effect *in vitro* afforded much insight into the mode of action of this compound. Some reservation of interpretation might be made having regard for the fact that bovine albumin was used with rat adipose tissue. It has been shown in this laboratory that the cow exhibits abnormally low peak levels of CPIB after oral administration and that it is rapidly excreted. Further, bovine albumin has been found deficient in certain thyroxine-binding characteristics in comparison with albumin from other species (Blumberg & Robbins, 1960). It can be inferred that CPIB has a relatively low affinity for bovine serum albumin and that this accounts for the poor antagonism shown against free fatty acid release in the artificial medium.

The effect of CPIB in this system *in vitro* is in sharp contrast to that of nicotinic acid. This latter substance produced a dose-dependent inhibition of the release of free fatty acids following stimulation by adrenaline, without altering the basal release rate. These results are in accord with those of Eaton (1963) who found a similar inhibition of noradrenaline. Comparable results have been reported *in vivo* where nicotinic acid was found to inhibit the increase in plasma free fatty acids which normally follows an intravenous infusion of noradrenaline (Carlson & Oro, 1962), whereas CPIB had little or no effect (Duncan, Best & Robertson, 1965). Nicotinic acid reduces the serum cholesterol level after repeated dosing in man (Altschul, Hoffer & Stephen, 1955) and many investigators have sought to relate this fact to the observed inhibitions of fatty acid release. It is evident that the mechanisms by which CPIB and nicotinic acid produce their effects are different but it has yet to be shown what role, if any, catechol amine-induced release of free fatty acids plays in the elevation of plasma lipids in disease states.

It was hoped that the use of homologous albumin (in plasma) for the incubation of rat adipose tissue might yield more valid results in relation to the effects of CPIB *in vivo*. This aim was at first upset by the observation that adrenaline at a concentration of 1 μg/ml. did not significantly increase the rate of fatty acid release above that found for plasma without added adrenaline. Either or both of two processes might be responsible for the higher rate of release in fresh plasma compared with that in Krebs-Ringer-bicarbonate with added albumin. Firstly, the higher rate in plasma may merely reflect the comparative inefficiency of the synthetic medium for this particular tissue system. The rate of release in plasma may more closely resemble that occurring at rest *in vivo* than that found in isotonic salt solution. The mean resting plasma level of free fatty acids is about 350 μequiv/l. and the half-life of the acids is about 90 sec. If one assumes a plasma volume of 12 ml. for a

200 g rat and a mean carcass fat value of 10% of which half is able to contribute fatty acids by lipolysis, then a net release of 8.4 μequiv/g/hr will be required to maintain the resting level. This value is considerably in excess of those frequently quoted for basal release rates for adipose tissue incubated in Krebs-Ringer systems. Secondly, there may be a factor present in rat plasma which stimulates the release of free fatty acids *in vitro*. The control values obtained in this study for adipose tissue incubated in plasma are higher than the calculated basal release rate and it is likely that both processes operate. The potency of the plasma factor varied from batch to batch of plasma and the individual release rates ranged from 8.23 up to 31.20 μequiv/g/hr. In the presence of CPIB individual values ranged from 1.32 to 4.39 μequiv/g/hr. The rate of release in the presence of CPIB was remarkably constant. The degree of inhibition varied from 30 to 90% depending on the initial rate of release but in no case was there a total inhibition. The concentration of free fatty acids in the plasma used for incubations was not proportional to the degree of lipolytic potency subsequently found. From these results it might be predicted that CPIB will lower plasma fatty acid levels *in vivo* under resting conditions.

In contrast to the results with CPIB, nicotinic acid had no effect on the release of free fatty acids from adipose tissue incubated in plasma, again tending to suggest different modes of action.

After the addition of adrenaline the increase in free fatty acid release in the plasma incubation system was small and little changed by the inclusion of CPIB. The release rates found may well represent the maximum capacity of the system to accept fatty acids. From Table 2 the release rates of 9.51 and 10.69 μequiv/g/hr correspond to plasma concentrations of 1,852 and 1,942 μequiv/l., taking into account the level of fatty acid present before adding fat segments. These values are higher than any value obtained by the author for plasma free fatty acids *in vivo* in the rat. Yet for the sample yielding 31.20 μequiv/g/hr cited earlier, the final fatty acid concentration in that flask was 2,752 μequiv/l. The inhibitory effects of CPIB may depend in part on the occupation of binding sites on albumin but this does not provide a complete explanation.

Considerable interest arises in the nature of the plasma fat-mobilizing factor. Since it was not present in the plasma of hypophysectomized rats the factor is presumably of pituitary origin. The facilitatory role of adrenocortical hormones in lipolytic processes is well established and it is interesting to note that the values obtained in hypophysectomized rat plasma correspond more closely to Krebs-Ringer-bicarbonate results than to those for intact rat plasma. The disappearance of the factor after hypophysectomy leads to the speculation that the previously reported diurnal and seasonal variations in the level of plasma free fatty acids may be under pituitary influence (Barrett, 1964). The stimulation of fatty acid release *in vitro* is unlikely to be due to corticotrophin or growth hormones. Stress did not increase the potency, and the levels of growth hormone necessary to produce effects *in vitro* are greater than those occurring physiologically (Buckle & Beck, 1962). These arguments are reinforced by the failure of CPIB to prevent lipolytic stimulation by either corticotrophin or growth hormone in the present study. Further, nicotinic acid, which has no effect on the plasma factor, not only antagonizes the response to catechol amines but also that to corticotrophin in lipolytic systems *in vitro* (Stock & Westermann, 1965).

The fact that CPIB did not inhibit the response to adrenaline in plasma from hypophysectomized rats provides additional evidence for the view that interference with anionic binding sites is not the complete explanation of the observed effects. Further, experiments are in progress to determine more closely the extent to which protein-binding by CPIB may lead to a reduction in hepatic and serum lipids through a decreased fatty acid outflow from adipose tissue.

SUMMARY

1. The effect of chlorophenoxyisobutyric acid (CPIB) on the release of free fatty acids from rat epididymal adipose tissue has been studied *in vitro* using either Krebs-Ringer-bicarbonate solution containing 3% albumin or rat plasma as incubation medium.

2. CPIB did not alter the basal release rate in Krebs-Ringer medium and only produced an irregular inhibition, not exceeding 37%, of the response to added adrenaline. Nicotinic acid produced an inhibition of up to 90%.

3. The incubation of fat pads in plasma produced unexpectedly high rates of fatty acid release. The higher rate appeared to be due partly to increased efficiency of rat plasma as an incubation medium and partly to a plasma fat-mobilizing factor. The factor was not present in plasma from hypophysectomized rats.

4. CPIB produced a marked inhibition of fatty acid release from fat pads incubated in plasma. Nicotinic acid was without effect.

5. The findings are discussed in relation to the mechanism by which CPIB lowers hepatic and serum lipid levels. It is concluded that competition for fatty acid binding sites on protein by CPIB can not be the sole explanation for the observed effects but cannot be excluded as a contributory factor.

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