Effect of *p*-chlorophenoxyisobutyrate on the antilipolytic action of insulin and insulin binding in isolated adipocytes

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Abstract The present study was undertaken to investigate the potentiation by *p*-chlorophenoxyisobutyrate (CPIB) of the antilipolytic effect of insulin in isolated adipocytes from rats fed a (*1*) sucrose diet, (*2*) glycerol–lard diet, or (*3*) chow diet. CPIB supplementation in the diet consistently resulted in decreased serum triglyceride levels in rats from the three dietary groups. The catecholamine-stimulated glycerol release was significantly depressed to a greater extent by insulin when the fat cells were obtained from rats given CPIB compared to those without drug treatment. The enhanced insulin sensitivity was, however, not accompanied by any changes in insulin binding to adipocytes. These two observations were found in cell preparations from rats fed any one of the diets, although differences among dietary groups could be detected. In an in vitro experiment, epinephrine-stimulated glycerol release was progressively inhibited by increasing concentrations of CPIB in the incubation medium. However, the antilipolytic response to an optimal concentration of insulin (100 μU/ml) was augmented in the presence of CPIB. Thus it seems that CPIB can potentiate the action of insulin in inhibiting mobilization of free fatty acid from the adipose tissue, and the coordinated effect of both antilipolytic agents is important in lowering serum triglyceride concentration. The mechanism by which CPIB facilitates the effect of insulin is discussed.

Supplementary key word lipolysis

p-Chlorophenoxyisobutyrate (CPIB) is very effective in reducing elevated levels of triglyceride, especially those seen in type IV hyperlipoproteinemia (1). Although the ability of CPIB to lower serum lipids was first recognized in 1962 by Thorp and Waring (2), there is still no unified concept as to the mechanism(s) of its action despite intensive investigations in the last ten years. The suggested actions of CPIB that may account for its hypotriglyceridemic effect include: (*a*) a reduction in the synthesis and secretion of triglyceride by the liver (3, 4); (*b*) an inhibition of free fatty acid mobilization from adipose tissue (5–7); and (*c*) a stimulation of the uptake of lipoprotein fatty acids into adipose tissue triglyceride (8), possibly as a result of increased lipoprotein lipase activity (9) and enhanced tissue lipogenic enzyme activities (10).

The antilipolytic as well as the lipogenic effects of CPIB in adipose tissue are reminiscent of the actions of insulin. A recent report from our laboratory shows that increased sensitivity to insulin (as measured by conversion of [^{14}C]glucose to $^{14}CO_2$) in vitro could be readily demonstrated in fat pads of CPIB-treated rats (11). It is possible then that insulin may be important in mediating the action of CPIB. The present study was designed to investigate (*a*) whether CPIB could potentiate the antilipolytic effect of insulin in isolated fat cells stimulated by a lipolytic hormone and (*b*) whether this drug exerted any influence on the binding of insulin to fat cells.

MATERIALS AND METHODS

Male Sprague Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 140–150 g at the start of the feeding period were used throughout the study. Food and water were available ad libitum. Rats were maintained on Purina rat chow before they were fed the experimental diets with or without 0.25% (w/w) of CPIB[1] (sodium salt) for a duration of 7 days. Two different synthetic diets were used in this study. The sucrose diet contained, in percentage by weight, sucrose, 63; casein, 28; vitamin mixture, 4; and salt mixture, 5. The glycerol–lard diet contained, in percentage by weight, glycerol, 43; lard, 10; casein, 33; vitamin mixture, 4; salt mixture, 5; and cellulose, 5. The Purina laboratory chow pellets (Ralston Purina Co., St. Louis, Mo.) were ground before use.

Rats were killed by decapitation and fat cells were isolated from epididymal fat pads according to the method of Rodbell (12). Minced adipose tissue was incubated with 1 mg/ml of collagenase (Worthington Biochemical Corp., Freehold, N.J.) in Krebs-Ringer bicarbonate buffer (with half the recommended Ca^{2+} concentration) containing 4% bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.) at 37°C for 60 min.

Adipocyte diameters were measured by means of a micrometer eyepiece. At least 150 adipocytes were measured for each preparation of cells used in one experiment. Mean cell

[1] The authors are grateful to Dr. Harvey Barnett of Ayerst Laboratories for the generous gift of CPIB.

volume was then calculated from the frequency curve as described by Di Girolamo, Mendlinger, and Fertig (13).

Triglyceride content of an aliquot of fat cells was determined by the method of Stern and Shapiro (14), using triolein as the standard. The number of fat cells in a given sample was obtained by dividing the total triglyceride content of the sample of cells by the mean fat cell triglyceride content (14).

Lipolysis was determined in isolated fat cells by measuring glycerol released into a medium containing 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with 4% bovine serum albumin. Approximately 10^6 fat cells were incubated with either epinephrine (Parke-Davis, Detroit, Mich.) or norepinephrine (Winthrop Laboratories, New York) in the presence or absence of bovine insulin (Sigma) at 37° C for 1 hr in a Dubnoff metabolic shaker. At the end of the incubation period, the fat cells were removed by centrifugation followed by aspiration. Glycerol in the infranatant fraction was measured by the method of Lambert and Neish (15) as modified by Korn (16).

^{125}I-labeled porcine insulin (sp act 114–120 μCi/μg) was purchased from Cambridge Nuclear Corp., Cambridge, Mass. The labeled insulin was stated by the manufacturer to contain less than 1 atom of iodine per molecule of insulin, to be greater than 95% immunoprecipitable, and to be intact by chromatoelectrophoresis. Each batch of radioactive insulin was routinely purified by talc adsorption to remove the insulin damaged by storage according to the method of Cuatrecasas (17). Over 95% of the radioactivity of the purified [^{125}I]insulin preparation used for binding studies was precipitable with 5% trichloroacetic acid and talc. The biological activity of this preparation was found to be equivalent to that of native insulin (on a weight basis) in the stimulation of [^{14}C]glucose to $^{14}CO_2$ in isolated fat cells. Results of this test are shown in **Table 1.**

The binding of [^{125}I]insulin to fat cells was determined according to the following procedures adapted from that of El-Allaway and Gliemann (18). Isolated adipocytes (approximately 10^6 cells) in Krebs-Ringer HEPES[2] buffer (pH 7.4) containing 1% albumin and 3 mM glucose were incubated with [^{125}I]insulin (40–45 microunits) and increasing concentrations of unlabeled native insulin at 24° C for 30 min. Total volume of the incubation mixture was 0.8 ml. At the end of the incubation period, aliquots of the incubation mixture were transferred to plastic microcentrifuge tubes (Beckman) containing a few drops of dinonyl phthalate. Fat cells were separated from the buffer by centrifugation (10,000 *g* for 30 sec) in a Beckman microfuge (Spinco Div., Palo Alto, Calif.) using the oil flotation technique. The ^{125}I radioactivity bound by cells was measured in a Nuclear-Chicago (Des Plaines, Ill.) Isocap 300 liquid scintillation counter with 10 ml of Instabray (Yorktown Research, Inc., South Hackensack, N.J.) as scintillation fluid.

All binding data in this study are reported as "specific binding." This was obtained by subtracting from the total radioactive uptake the amount of labeled insulin that was not displaced by large excesses of unlabeled hormone—50 μg of native porcine insulin per incubation vial (17). The maximum binding and the dissociation constant of insulin and receptor were calculated from the binding data as analyzed by Scatchard plots according to the equation described by Kahn, Freychet, and Roth (19). A least squares straight line was drawn to fit the binding data obtained from insulin concentrations from 40 to 10,000 μU/0.8 ml. The dissociation constant was calculated from the slope of the line and the binding capacity was derived from the intercept on the bound axis.

Serum triglyceride was measured using the Tri-chol kit purchased from Oxford Laboratories, Foster City, Calif.

Statistical comparisons were made by Student's *t* test. Differences between means were considered nonsignificant when the *P* value was greater than 0.05.

TABLE 1. Comparison of the biological activity[a] of native bovine insulin[b] and [^{125}I]insulin[c].

Addition	Cpm/Vessel[d]
None	1376
Native bovine insulin	
5 μU	2341
10 μU	4192
20 μU	5375
100 μU	6878
[^{125}I]insulin	
5 μU	2058
10 μU	4026
20 μU	5471
100 μU	6589

[a] Biological activity was measured by degree of conversion of [U-^{14}C]glucose to $^{14}CO_2$ in fat cells. Fat cells (approx. 5 × 10^4 per vessel) were incubated in Krebs-Ringer bicarbonate buffer with 1% bovine serum albumin containing 0.1 mg/ml of D-glucose and insulin in a final volume of 2 ml. Each incubation vessel contained 0.1 μCi of D-[U-^{14}C]glucose (Schwarz/Mann, Orangeburg, N.Y.). Incubation was carried out in a Dubanoff metabolic shaker at 37°C for 1 hr. At the end of the incubation period, the contents of the vessel were acidified with HCl, the evolved $^{14}CO_2$ was collected in NCS (Amersham/Searle, Arlington Heights, Ill.) on a rolled strip of filter paper and the radioactivity was measured in a Nuclear Chicago (Des Plaines, Ill.) Isocap liquid scintillation counter.

[b] Sigma Chemical Co., St. Louis, Mo.

[c] Cambridge Nuclear Corp., Cambridge, Mass.

[d] Each value is the average of triplicates.

RESULTS

In order to test the bioactivity of CPIB used in this study, serum triglyceride levels were measured in the control and drug-treated rats. Supplementation of the diet with CPIB significantly reduced the serum triglyceride concentrations in rats that were fed either a sucrose, glycerol–lard, or chow diet for 1 week **(Table 2).** These results confirmed the expected hypotriglyceridemic action of CPIB as reported by many investigators. Table 2 also shows that dietary CPIB significantly reduced the weight of the epididymal fat pads

[2] *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

TABLE 2. Effect of dietary administration of CPIB on weight of epididymal fat pads, fat cell size, and serum triglyceride.

Diet	0.25% CPIB	Final Body Weight[a]	Epididymal Fat Pads[a]	Cell Volume[b]	Serum Triglyceride[c]
		g	*mg/100 g body wt*	*picoliters*	*mg %*
Sucrose	0	204 ± 6.6	648 ± 14.3	92.3 ± 9.3	161.6 ± 12.6
Sucrose	+	211 ± 5.9	581 ± 27.2	90.7 ± 8.3	78.6 ± 6.7
		NS[d]	$P < 0.05$	NS	$P < 0.001$
Glycerol–lard	0	200 ± 3.3	572 ± 12.3	93.8 ± 6.4	514.7 ± 43.1
Glycerol–lard	+	209 ± 5.5	578 ± 25.1	82.5 ± 12.1	205.2 ± 13.0
		NS	NS	NS	$P < 0.001$
Chow	0	205 ± 2.4	591 ± 13.9	105.6 ± 8.8	177.4 ± 14.5
Chow	+	21 ± 4.8	536 ± 18.9	78.1 ± 4.3	97.3 ± 12.2
		NS	$P < 0.05$	$P < 0.05$	$P < 0.001$

[a] Values are means ± SEM of 20 rats.
[b] Values are means ± SEM of four experiments. One batch of fat cells was prepared for each experiment from at least 5 rats.
[c] Values are means ± SEM of 12 rats.
[d] Not significant.

TABLE 3. Effect of dietary CPIB on the antilipolytic effect of insulin in fat cells incubated with norepinephrine or epinephrine.

Diet	CPIB	Stimulation[a]	Glycerol Released[b] (μmoles/10^6 cells/hr)		Inhibition[c] %
			−insulin	*+insulin*[d]	
Sucrose	0	norepinephrine	1.68 ± 0.07	0.79 ± 0.05	51.9 ± 3.7
	+	norepinephrine	1.54 ± 0.18	0.43 ± 0.09	72.8 ± 2.5
			NS[e]	$P < 0.02$	$P < 0.01$
	0	epinephrine	1.53 ± 0.13	0.71 ± 0.03	52.6 ± 2.3
	+	epinephrine	1.55 ± 0.10	0.44 ± 0.06	71.7 ± 2.8
			NS	$P < 0.01$	$P < 0.01$
Glycerol–lard	0	norepinephrine	0.73 ± 0.09	0.42 ± 0.05	41.8 ± 1.3
	+	norepinephrine	1.48 ± 0.12	0.42 ± 0.03	71.2 ± 2.
			$P < 0.01$	NS	$P < 0.001$
	0	epinephrine	0.88 ± 0.07	0.48 ± 0.04	45.1 ± 3.3
	+	epinephrine	1.81 ± 0.12	0.50 ± 0.05	72.4 ± 2.7
			$P < 0.001$	NS	$P < 0.001$
Chow	0	norepinephrine	1.67 ± 0.04	0.65 ± 0.05	61.2 ± 2.8
	+	norepinephrine	1.55 ± 0.12	0.35 ± 0.05	77.8 ± 1.7
			NS	$P < 0.01$	$P < 0.01$
	0	epinephrine	1.64 ± 0.14	0.68 ± 0.06	58.4 ± 0.9
	+	epinephrine	1.69 ± 0.14	0.32 ± 0.06	80.7 ± 3.8
			NS	$P < 0.01$	$P < 0.001$

[a] Concentrations were 0.2 μg/ml for norepinephrine and 0.1 μg/ml for epinephrine.
[b] Values are means ± SEM of four experiments. Each experiment consisted of pooled fat cell samples from six rats.
[c] Inhibition (%) $= \frac{\text{(without insulin)} - \text{(with insulin)}}{\text{(without insulin)}} \times 100.$
[d] Concentration of insulin was 100 μU/ml.
[e] NS, not significant ($P > 0.05$).

in the sucrose and chow fed rats but had no effect on that of rats fed the glycerol–lard diet.

The rationale for including the sucrose and glycerol–lard fed rats in this study was that previous reports from our laboratory have shown that differences on the effects of CPIB were related to the type of diet ingested by the animals[3] (9, 10). Moreover, both sucrose and high fat feedings have been known to decrease insulin sensitivity in adipose tissue (20, 21) and it would be interesting to see if CPIB would potentiate the action of insulin in rats maintained on these two dietary regimens. Experiments on the antilipolytic effect of insulin on isolated fat cells from control and CPIB-treated rats were carried out. Results in **Table 3** show that catecholamine-induced glycerol release was depressed to a greater degree by insulin when the adipocytes were isolated from rats given CPIB. This observation was extended to samples from rats fed the three different diets. It should be noted that in controls (cells from rats without CPIB treatment), the antilipolytic effect of insulin was most prominent in the chow-fed group, followed by the sucrose and glycerol–lard fed

[3] Weis, A., Ph.D. Thesis (1972), Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, New York.

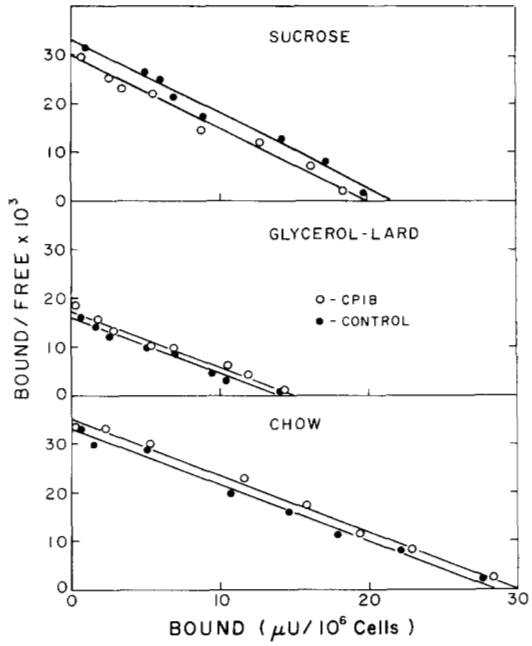

Fig. 1. Representative Scatchard plots of insulin binding to isolated adipocytes from sucrose–glycerol–lard-, or chow-fed rats with or without CPIB treatment.

groups. This is consistent with the theory that feeding a high sucrose or a high fat diet leads to a state of insulin resistance.

Both norepinephrine (0.2 μg/ml) and epinephrine (0.1 μg/ml) were equally effective in stimulating lipolysis in cells from the sucrose- and chow-fed rats. However, isolated adipocytes from rats given the fat diet were found to release less glycerol. Supplementation of the fat diet with CPIB actually increased catecholamine-induced lipolysis, although dietary CPIB by itself had no effect on in vitro glycerol release by cells from the other two groups. This apparently paradoxical effect of CPIB has been reported previously by Kokatnur and Blackard (22); the significance of the observation still remains unclear.

The increased insulin sensitivity in drug-treated animals might conceivably be due to enhanced insulin binding to the fat cells or to an increased affinity of the receptor for insulin. Insulin binding experiments were then performed and representative Scatchard plots of the insulin binding data from the three different dietary groups with or without CPIB treatment are presented in **Fig. 1.** The results obtained from these plots were analyzed as described in the Methods section and are summarized in **Table 4.**

Neither the maximal binding capacity nor the affinity of receptor for insulin (dissociation constant) was affected by the administration of CPIB to rats that were fed any one of the diets. Nonetheless, the maximal insulin binding capacity of fat cells from glycerol–lard fed rats was found to be lower than those of the other two groups. This finding is consistent with the decreased insulin sensitivity (as measured by the conversion of [^{14}C]glucose to $^{14}CO_2$) in adipocytes from fat-fed animals as compared to those from rats fed a high

TABLE 4. Effect of dietary CPIB on insulin binding to isolated fat cells.

Diet	CPIB	Insulin Binding[a]	
		Maximal Binding Capacity	Dissociation Constant
		molecules × 10^3/*cell*	$M \times 10^9$
Sucrose	0	87.1 ± 9.2[b]	6.5 ± 0.71
	+	83.7 ± 9.8[c]	7.3 ± 0.63
Glycerol–lard	0	58.8 ± 7.2[d]	7.5 ± 0.76
	+	60.2 ± 8.1[d]	71. ± 0.87
Chow	0	111.5 ± 11.3	6.5 ± 0.56
	+	129.1 ± 10.7	5.7 ± 0.61

[a] Values are means ± SEM of four experiments.
[b] $P > 0.05$, not significant compared to the corresponding chow-fed group.
[c] $P < 0.05$ compared to the corresponding chow-fed group.
[d] $P < 0.02$ compared to the corresponding chow-fed group.

TABLE 5. Effect of CPIB and insulin in vitro on lipolysis in adipocytes induced by epinephrine.

Epinephrine	Addition CPIB	Insulin	Glycerol Released[a]	Inhibition[b]
μg/ml	*mg/ml*	*μU/ml*	*μmoles/10⁶ cells/hr*	%
0.2			2.03 ± 0.10	
0.2	0.1		1.67 ± 0.07	17.6 ± 1.9
0.2	0.2		1.32 ± 0.11	35.2 ± 2.4
0.2	0.5		1.02 ± 0.09	50.2 ± 3.1
0.2	1.0		1.06 ± 0.08	49.1 ± 3.8
0.2		1	1.59 ± 0.05	21.3 ± 3.5
0.2		100	0.87 ± 0.05	57.2 ± 0.4
0.2		500	0.81 ± 0.08	59.5 ± 0.9
0.2	0.1	1	1.26 ± 0.13	38.7 ± 3.6
0.2	0.5	1	0.70 ± 0.04	65.4 ± 2.3
0.2	0.1	100	0.36 ± 0.06	82.0 ± 3.8
0.2	0.5	100	0.32 ± 0.05	83.7 ± 3.6

[a] Values are means ± SEM of five experiments. Adipocytes were isolated from chow-fed rats.
[b] Inhibition (%) =

$$\frac{(\text{Epinephrine alone}) - (\text{Epinephrine} + \text{CPIB and/or insulin})}{(\text{Epinephrine alone})} \times 100.$$

glucose diet[4]. Based on the present observation, it seems that the mechanism responsible for changes in insulin sensitivity as a result of CPIB administration lies beyond the step of hormone–receptor interaction.

The effect of CPIB added in vitro on the antilipolytic action of insulin was also investigated in adipocytes isolated from chow-fed rats. Results are shown in **Table 5.** Epinephrine-stimulated glycerol release was progressively inhibited by increasing concentrations of CPIB in the incubation medium. Maximal inhibition by CPIB was attained at a concentration of 0.5 mg/ml. This dose is higher than the serum CPIB concentration of approximately 0.2 mg/ml found in rats that

[4] Clement Ip, Helen M. Tepperman and Jay Tepperman, manuscript in preparation.

were fed a diet containing 0.25% (w/w) of CPIB after 14 days (9). When a suboptimal concentration of insulin was used (1 μC/ml), CPIB added in vitro produced an additive effect in inhibiting glycerol release stimulated by epinephrine. However, in the presence of an optimal concentration of insulin (100 μU/ml), a low concentration of CPIB (0.1 mg/ml) was adequate to produce the maximal antilipolytic response. Thus it seems that CPIB facilitates the action of insulin, although the present data fail to provide enough evidence to justify the suggestion that these two antilipolytic agents may have different modes of action.

DISCUSSION

Consistent with a recent report by Weis, Tepperman, and Tepperman (11) that adipose tissue of CPIB-treated animals was comparatively hypersensitive to insulin with respect to stimulation of glucose oxidation, the present study demonstrates that isolated adipocytes from these rats are also more sensitive to the antilipolytic action of insulin than adipocytes from control rats. This phenomenon was observed when CPIB was given in the diet or added in vitro.

It is generally believed that lipolytic hormones enhance lipolysis by a mechanism related to their stimulation of adenylate cyclase, although the precise nature of the cyclic AMP involvement remains controversial (23, 24). There is evidence in the literature that the antilipolytic effect of CPIB can be explained, at least in part, by inhibition of cyclic AMP concentration. Thus Greene, Herman, and Zakim (25) reported that CPIB depressed adipose tissue adenylate cyclase activity without affecting phosphodiesterase and Weis et al. (11) found lower cyclic AMP concentrations in fat pads of CPIB-treated rats in the presence and absence of epinephrine or epinephrine plus insulin. In addition, D'Costa and Angel (7) recently reported that the antilipolytic action of CPIB could be demonstrated in the presence of epinephrine but not when dibutyryl cyclic AMP was the lipolytic agent, suggesting that CPIB's action limited cyclic AMP accumulation.

Since at least part of the action of insulin is believed to be related to its cyclic AMP lowering effect (26–28), it is reasonable that the hormone might have a greater effect when the nucleotide concentration is already low. Wieser and Fain (29) have recently reported that insulin sensitivity is increased by several other agents which lower cyclic AMP. Hence a relatively modest increase in cyclic AMP concentration by a lipolytic hormone in cells that have been treated with CPIB can be expected to be suppressed more readily by insulin. This could be one of the major mechanisms of the hypersensitivity to insulin in CPIB-treated cells.

The observation that washed adipocytes obtained from CPIB-treated rats still retain their "CPIB-effect" in the in vitro studies on lipolysis (Table 3) may be due to adaptation of certain components in the cellular machinery. For example, changes in lipogenic enzyme activities have been reported in rats fed a diet containing CPIB for 14 days (10). The antilipolytic effect observed when CPIB is added directly to the incubation medium (Table 5) may represent an acute action of the drug. Whether CPIB acts through the same mechanism under these two situations is unclear.

A lack of correlation between hormone binding and changes in sensitivity to the hormone have been reported on other occasions. Gorman, Tepperman, and Tepperman (30) showed that fat feeding resulted in loss of epinephrine-stimulated adenylate cyclase activity in adipocyte ghosts but did not affect the amount of epinephrine bound to the membrane. Likewise, resistance to insulin stimulation in isolated adipocytes from rats that had been starved or treated with either prednisone or streptozotocin was not accompanied by changes in the binding capacity of insulin or in the affinity of the receptor for the hormone (31). As the action of CPIB in facilitating the insulin response clearly lies beyond the hormone–receptor interaction, the drug could conceivably act at some "transducer" step within the membrane.

Although this hypothesis is speculative, certain information in the literature provides some support for it. Kokatnur and Malcolm (32) have compared the effectiveness of CPIB in reducing serum triglyceride in rats fed corn oil, beef tallow or medium chain triglyceride diet. They found that CPIB had different effects depending on the type of fat given to the animals. In view of the evidence that the nature of fat in the diet can influence the fatty acid composition of membrane (33), it is possible that CPIB may participate in some unidentified membrane related function(s) which may tend to modify the response elicited by the insulin binding signal. This could constitute another mechanism by which CPIB potentiates the insulin action.

In conclusion, CPIB is able to exert an antilipolytic effect by itself, and it can also facilitate the action of insulin either at the same site or by a different mechanism which is at present unknown. The coordinated effect of CPIB and insulin in antagonizing lipolysis is important in diminishing the rate of release of free fatty acid from the adipose tissue. Since serum fatty acid is the principal precursor of hepatic triglyceride production, the concerted actions of CPIB and insulin collaborate in reducing serum triglyceride concentration.

This investigation was supported by Public Health Service Grant No. AM05410, from the National Institute of Arthritis and Digestive Diseases.

Manuscript received 10 March 1975; accepted 6 August 1975.

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