

Proinsulin C-Peptide Inhibits Lipolysis in Diabetic Rat Adipose Tissue Through Phosphodiesterase-3B Enzyme

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Key words

- C-peptide
- type 1 diabetes
- adipose tissue

Abstract

We have previously reported that C-peptide modulates insulin-mediated inhibition of lipolysis and glucose consumption but has no significant effects per se on adipose tissue of normal rats. It has been repeatedly observed that certain actions of C-peptide are restricted to the diabetic states. In the present study, therefore, we examined whether C-peptide alters lipolysis in adipose tissue of diabetic rats. Rats were rendered diabetic by streptozotocin and divided into 2 groups; insulin treated and untreated. Retroperitoneal adipose tissue was excised aseptically, subjected to organ culture and incubated with rat C-peptide, insulin, or a combination of both peptides in the presence or absence of isoproterenol. Tissue lipolysis was assessed by the

rate of glycerol release into the culture media. The cultures were pretreated with cilostamide, a phosphodiesterase-3B enzyme inhibitor, when the role of this enzyme was to be examined. C-Peptide on its own, like insulin, significantly inhibited isoproterenol-stimulated lipolysis in the adipose tissue of untreated diabetic rats. The effect was enhanced by a combination of C-peptide and insulin. Notably, the C-peptide's effect was totally blocked in the presence of cilostamide. In the adipose tissue of insulin treated rats, however, C-peptide failed to show any significant antilipolytic effects. These data show that C-peptide has the potential to act, conditionally, as an antilipolytic hormone by activating phosphodiesterase-3B and suggest that the action may contribute to the C-peptide's beneficial effects on diabetes-induced complications.

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Introduction

Once regarded as an inert by-product of post-translational processing of proinsulin propeptide, C-peptide (CP) is now recognized as a hormone, which not only plays role during synthesis, secretion, and disaggregation of insulin but also possesses insulin-like actions per se [1–3]. One of the well known actions of insulin is lipolysis inhibition in adipose tissue. Lipolysis is a highly regulated process and is disciplined primarily by endocrine system [4]. Although a variety of factors contribute to the process, insulin and catecholamines play central roles. In adipocytes, insulin blocks lipolysis, to a large extent, by activating the phosphodiesterase type 3B (PDE3B) enzyme and consequently reduction in cAMP, leading to dephosphorylation and inactivation of hormone sensitive lipase (HSL). In contrast, catecholamines antagonize insulin actions through stimulation of cAMP formation followed by phosphorylation and activation of HSL [4,5]. Increased lipolysis is associated with a considerable number

of chronic diseases including obesity, metabolic syndrome, familial combined hyperlipidemia, tuberculosis, Crohn's disease, cancer-related cachexia, and diabetes; type-1 diabetes (T1D) in particular [4,6,7]. It is now accepted that positive correlation exists between the degree of lipolysis and the extent of disease-associated complications and that untreated lipolysis might account for risk of morbidity and mortality from the above mentioned diseases [7].

In T1D, insulin deficiency and metabolic stress-induced elevation of catecholamines are known to be responsible for the activated lipolysis [6]. Not only insulin but also CP is deficient in patients with T1D. Currently available information support the notion that co-replacement of insulin and CP in patients with T1D results in prevention or significant amelioration of diabetes-induced abnormalities [8]. Several in vitro studies on biological actions and cell membrane binding kinetics have confirmed that CP possesses physiological activities on its own [1]. In fact, CP can bind to its proposed pertussis toxin-sensitive G-protein-

coupled receptor on cell surface or act through nonchiral interactions with membrane lipids [9]. Besides, certain insulinomimetic actions of CP are shown to be mediated through activation of insulin signaling components including phosphatidyl inositol 3-kinase and mitogen activated protein kinases [10].

Although the effects of insulin on adipose tissue metabolism and its central role in regulation of lipolysis are well characterized, the role of CP is not yet known. Using adipose tissue of normal rats in an organ culture system, we recently reported that rat CP-II modulates insulin-mediated inhibition of lipolysis and glucose uptake but has no significant effects on its own [11]. Researchers have repeatedly reported that certain actions of CP are restricted to the diabetic states. Therefore, the aim of this study was to examine whether CP inhibits basal or isoproterenol (ISO)-induced lipolysis in adipose tissue of diabetic rats and whether the effect is mediated through phosphodiesterase-3B enzyme (PDE3B).

Materials and Methods

Chemicals and reagents

The tissue culture medium 199 (M199), bovine serum albumin fraction V, dimethyl sulfoxide, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) and isoproterenol, were purchased from Sigma (USA). Streptozotocin (STZ) and cilostamide were from Calbiochem (USA). Rat CP-II (Glu-Val-Glu-Asp-Pro-Gln-Val-Ala-Gln-Leu-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Asp-Leu-Gln-Thr-Leu-Ala-Leu-Glu-Val-Ala-Arg-Gln) was from Bachem (Germany). Human insulin and neutral protamine Hagedorn (NPH) insulin were from Eli Lilly and kindly provided by EXIR Company (Iran). Glycerol Assay Kit was obtained from Abcam (England).

Animals

Adult male Sprague-Dawley rats with an initial weight of 310–320 g were obtained from Laboratory Animals House, Shiraz Medical University, Shiraz, Iran. Animals were housed in a room with controlled lighting (12-h light and dark cycles) and temperature ($22 \pm 2^\circ\text{C}$) during the study. They were fed ordinary rat chow and allowed free access to food and water. On day 3 of acclimatization, rats were given a single injection of STZ (55 mg/kg intravenously) to render them diabetic [12]. Blood glucose level of animals was measured using strip-operated blood glucose sensor 48 h after the injection. The animals with a fasting blood glucose level of 300–400 mg/dl were considered diabetic and used for the study. The selected diabetic rats were then randomly divided into 2 groups: insulin-treated (ITD, $n=6$) and untreated (UTD, $n=12$). All rats in the ITD groups received a subcutaneous injection of NPH insulin solution (3 IU/day). The treatment was initiated 2 days after STZ injection and continued to the day of surgery. The rats in UTD group received saline accordingly. The body weight and water intake of animals were monitored during the experiment. The study protocol using laboratory animals complied with the guidelines of the animal care of the Shiraz University of Medical Sciences, Shiraz, Iran.

Adipose tissue collection and organ culture

On day 7 of diabetes inception, after an overnight fasting, the rats were anesthetized and retroperitoneal adipose tissue was rapidly excised through a sterile laparotomy procedure [13]. The tissue was immediately transported to the tissue culture room where it was washed, processed, and prepared for the *ex vivo* organ culture as described previously [11]. In brief, the tissue was gently sliced

into uniform small pieces with the aid of surgical blade and washed with phosphate-buffered saline. Tissue slices rich in adipocytes were then isolated, transferred into a culture dish, resuspended in 15 ml of serum-free M199 culture media with 25 mM HEPES and antibiotics, and incubated at 37°C in a humidified chamber under an atmosphere of 95% air and 5% CO_2 for 24 h. This culturing condition prior to treatments and lipolysis assessment allowed elimination of sampling-related and individual variations which could otherwise influence tissue behavior. At the end of the incubation period, the tissue slices were distributed into 24-well tissue culture plate; 100 mg tissue/well. The wells were then filled with 1 ml Krebs-Ringer buffer containing 25 mM HEPES, 5.5 mM glucose, and 2% (w/v) bovine serum albumin. Immediately, the cultures were treated with rat CP-II (6 nM), insulin (10 μM) or a combination of both peptides in the presence or absence of isoproterenol (ISO, 750 nM) and incubated at 37°C in 5% CO_2 and 95% moist air with gentle shaking for 90 min. At the end of the incubation period, the conditioned media was collected from each well and centrifuged. The infranatant was then removed and stored at -20°C until used for glycerol content measurement. When the role of PDE3B was going to be determined, the culture was incubated with 10 μM cilostamide, a specific PDE3B inhibitor, for 30 min prior to the treatments.

Glycerol assessment as a measure of lipolysis activity

Glycerol concentration in the conditioned media was determined by an enzymatic method according to the protocol provided by the kit manufacturer. Briefly, the tests and standard samples were prepared to a final volume of 50 μl in a 96-well plate. A reaction mixture containing glycerol dehydrogenase and nicotinamide adenine dinucleotide (NAD) was then added to each well and incubated at room temperature for 30 min. The reaction product, NADH, was quantified by measuring optical density (570 nm) using a Microtiter plate reader. Sample readings were applied to the plotted standard curve and glycerol concentration was then calculated for each sample accordingly. All samples were tested in duplicate. Alterations in lipolysis are expressed as a percentage of basal or ISO-stimulated lipolysis.

Statistical analysis

Comparisons of the data on characteristics of the rat groups were performed by either paired or unpaired *t*-test. Due to the variability in glycerol concentration between individuals, the statistical analyses were made on percentage changes using Student's *t*-test or one-way ANOVA followed by post hoc Tukey HSD test. All reported values were expressed as means \pm SEM except for weight of animals, which is presented as means \pm SD. Results showing *p*-values less than 0.05 were considered statistically significant.

Results

Alterations in blood glucose, water intake, and body weight of UTD and ITD rats

Two days after STZ injection, all animals showed a similar and a significant increase in fasting blood glucose concentration as compared to that of the day before the injection (UTD: 99 ± 2.5 mg/dl vs. 345 ± 6.6 mg/dl; ITD: 97 ± 3.6 mg/dl to 351 ± 16 mg/dl; $p < 0.01$). In spite of daily insulin treatment, hyperglycemic state was observed in ITD animals prior to insulin administration so that no significant differences were detected between morning blood glucose concentration in ITD and UTD rats (● **Table 1**).

However, average water intake of ITD rats was significantly lower than that of their UTD counterparts (40 ± 2 ml/24h vs. 115 ± 5 ml/24h; $p < 0.01$), indicating that insulin has been fairly effective during insulin administration interval of 24h. Before insulin treatment, STZ induced similar and a significant reduction in body weight of UTD and ITD rats. Whereas the weight reduction was persisted in UTD rats, reaching about 86% of their original weight (275 ± 3.5 vs. 318 ± 3 g; $p < 0.01$), the daily insulin treatment in ITD rats not only inhibited weight reduction but also restored the initial body weight by the time of surgery.

Effects of CP on lipolysis in adipose tissue of ITD rats

The presence of CP in the medium induced only a slight and insignificant decrease ($10.6 \pm 2.5\%$) in the rate of basal lipolysis (○ Fig. 1). In order to examine the effect of CP on stimulated lipolysis, the lipolytic activity was also evaluated in the presence of ISO, a potent nonselective beta adrenergic receptor agonist and lipolysis inducer. As expected, the ISO induced a significant elevation (2.4-fold; $p < 0.05$) in tissue lipolysis. The CP had virtually no significant effect on the ISO-stimulated lipolysis and the level of glycerol release was still higher than that of basal level ($p < 0.05$).

Effects of CP on lipolysis in adipose tissue of UTD rats

The bar graphs in ○ Fig. 2 show the effects of CP on basal and ISO-stimulated lipolysis in the adipose tissue of UTD rats. Again, the effect of CP on basal lipolysis remained insignificant ($90 \pm 3\%$ of basal level). As in the adipose tissue of ITD rats, ISO significantly increased (4.6-fold, $p < 0.05$) lipolysis in the tissue of UTD rats but the effect was more pronounced (4.6 vs. 2.4-fold, ○ Fig. 2a). The effect of ISO was significantly reduced by insulin ($25.9 \pm 5.8\%$; $p < 0.05$) as well as by CP ($19.2 \pm 5.5\%$; $p < 0.05$). Interestingly, both peptides were almost equally effective. Further inhibition in lipolysis ($33.4 \pm 4.6\%$; $p < 0.05$) was observed when a combination of insulin and CP was added to the culture media (○ Fig. 2b).

CP inhibits stimulated-lipolysis through activation of phosphodiesterase-3B

As shown in ○ Fig. 3, preincubation of the UTD's tissue with cilostamide increased basal ($75.6 \pm 11.5\%$; $p < 0.05$) and ISO-stimulated ($20 \pm 6.4\%$; $p < 0.05$) lipolysis. The CP-mediated inhibition of stimulated lipolysis was totally blocked by cilostamide indicating that CP inhibits ISO-stimulated lipolysis, perhaps exclusively, through activation of PDE3B.

Discussion

▼ The data of this study show that rat CP-II mimics the antilipolytic action of insulin in retroperitoneal adipose tissue of experimental untreated diabetic rats. Insulin deficiency as seen in T1D is associated with activation of lipolysis in adipose tissue that ultimately results in diabetic ketoacidosis, a life threatening complication of diabetes mellitus. It is now accepted that replacement of physiologic concentration of CP along with insulin in diabetic patients who lack CP results in prevention or significant amelioration of diabetes-induced complications [8]. Although the exact mechanisms that underlie CP's protective effect in diabetes are not exactly known, existing evidences suggest that insulin-like metabolic actions are involved. Sima and co-workers have shown that replacement of CP prevents both acute and chronic metabolic changes in the type I diabetic BB/Wor rats [14]. Besides, they have reported that CP enhances activity of the receptor and other

Table 1 Characteristics of the diabetic rat groups studied.

Groups	UTD Rats (n = 12)	ITD Rats (n = 6)
Body weight (g)**		
Day 0 (STZ injection)	318 ± 3	317 ± 4
Day 2 (insulin treatment)	298 ± 3.3 ^a	294 ± 6 ^a
Day 7 (surgery day)	275 ± 3.5 ^{a,b}	314 ± 3.6 ^{b,c}
Plasma glucose (mg/dl)*		
Day 0	99 ± 2.5	97 ± 3.6
Day 2	345 ± 6.6 ^a	351 ± 16 ^a
Day 7	327 ± 10 ^a	324 ± 15 ^a
Water intake (ml/24h)*		
Day 0	40 ± 2	42 ± 2.5
Day 2–7	115 ± 5 ^a	53 ± 3 ^c

The NPH insulin (3IU/day, sc) was administered to insulin-treated diabetic (ITD) rats and untreated diabetic (UTD) rats received saline accordingly

^a $p < 0.01$ vs. its corresponding values at day 0 in each group. ^b $p < 0.001$ vs. its corresponding values at day 2 in each group. ^c $p < 0.001$ vs. its corresponding values for UTD rats

*Corresponding values are means ± SEM; **Corresponding values are means ± SD n: Number of rats in each group; STZ: Streptozotocin

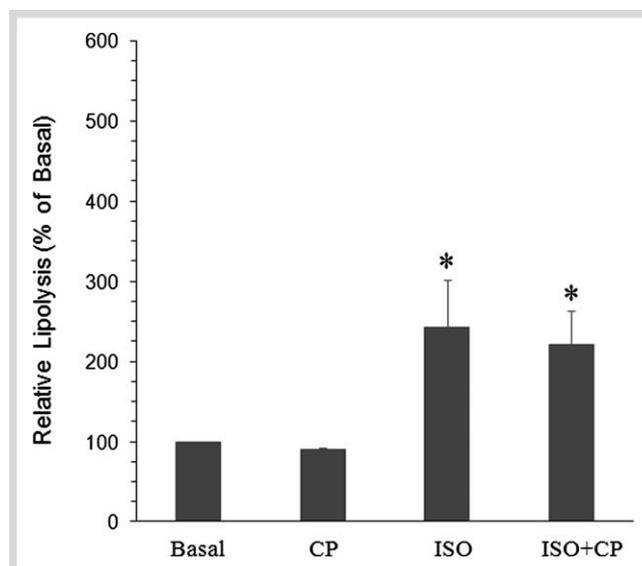


Fig. 1 Effects of C-peptide on basal and stimulated lipolysis in insulin-treated diabetic rats. Lipolysis in cultivated retroperitoneal adipose tissue was assessed in the presence of C-peptide (CP), isoproterenol (ISO), or a combination of both agents as described in the text. Results are expressed relative to Basal lipolysis. * $p < 0.05$ vs. basal. The data are presented as means ± SEM of 6 independent experiments.

downstream elements of the insulin signal transduction pathways [15, 16]. The insulin-like antilipolytic action of CP in adipose tissue of diabetic rats, observed in this study, indicates that CP-mediated inhibition of stimulated lipolysis may contribute, at least in part, to the protective role of CP in diabetic states. The *ex vivo* organ culture model may offer certain advantages over isolated adipocyte culture because it retains autocrine, paracrine, cell-cell and cell-matrix interactions which play critical role in gene expression and cell behavior [17]. Soon after discovery of CP in 1968, Solomon et al. tested the effects of supra-physiologic doses of pork and beef CP on basal and ACTH- and cyclic nucleotide-induced lipolysis in pooled adipocytes isolated from normal rat adipose tissues and failed to detect significant antilipolytic activity for CP [18]. In spite of major differences in the experimental setting including sampling and cultivation, species-specificity, doses of CP, and the nature of lipolysis inducer,

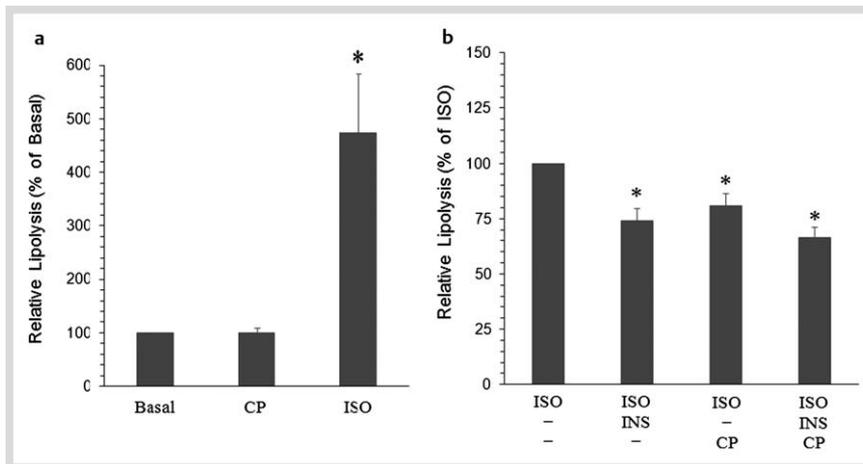


Fig. 2 Effects of C-peptide on basal and stimulated lipolysis in untreated diabetic rats. **a** Lipolysis in cultivated retroperitoneal adipose tissue was assessed in the presence of C-peptide (CP) or isoproterenol (ISO) as described in the text. **b** ISO-induced lipolysis in the cultivated adipose tissue was assessed in the presence of insulin (INS), CP, or a combination of INS and CP. Results are expressed relative to Basal **a** and ISO-induced **b** lipolysis. * $p < 0.05$ vs. basal **a** or ISO **b**. The data are presented as means \pm SEM of 6 independent experiments.

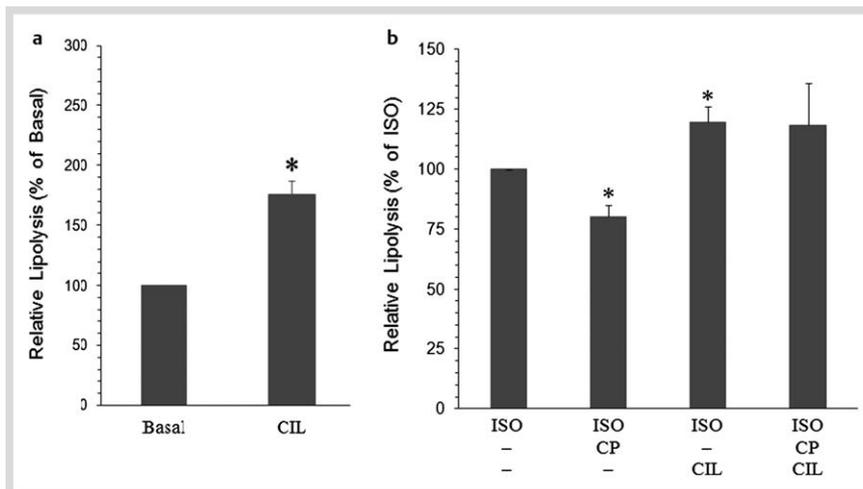


Fig. 3 Effects of phosphodiesterase-3B inhibition on the antilipolytic action of C-peptide in untreated diabetic rats. For basal condition **a**, the tissue was cultivated in the presence or absence of phosphodiesterase-3B inhibitor, cilostamide (CIL). For stimulated condition **b**, the tissues were preincubated with CIL or vehicle for 30 min and then treated (90 min) with ISO or a combination of ISO and CP. * $p < 0.05$ vs. basal **a** and vs. ISO **b**. The data are presented as means \pm SEM of 5 (Basal, CIL, CIL + ISO, CIL + ISO + CP) or 6 (ISO, ISO + CP) independent experiments.

we found somewhat similar results in the adipose tissue of normal rats [11]. Apparently the antilipolytic action of CP observed in this study is related to diabetic state. All together, our findings are in agreement with previous data showing that CP possesses insulin-like metabolic activities on its own and that some of its actions are restricted to diabetic states [19].

The CP-mediated inhibition of induced lipolysis was comparable to that of mediated by insulin. Similar findings have been reported by other investigators. For instance, a comparable cytoprotective effects against cytokine-induced apoptosis is shown for CP and insulin in kidney proximal tubular cells [20]. Interestingly, in the tissue of UTD but not in that of ITD animals, the stimulated lipolysis was affected by CP. Although the basis for this difference is not determined, altered sensitivity to CP seems to be involved. The original body weight in the ITD rats was restored by exogenous insulin treatment, indicating increased adipocyte fat content and diameter. Data from several lines of study show that hormone responsiveness of adipocytes correlates well with cell diameter [21,22]. Interestingly, in an attempt to detect factors linking human adipocyte hypertrophy to type-2 diabetes, Jernas et al. found markedly higher expression of several genes in large adipocytes as compared to that in small adipocytes [23]. Whether adipocyte diameter has played role in the differential response to CP of ITD and UTD rat adipose tissues remains to be determined. However, based on these data one may concluded that the antilipolytic action of CP may depend on the tissue's metabolic background.

The signaling pathways by which insulin and catecholamines regulates lipolysis are well studied [4,5]. The rate-limiting enzyme

for lipid breakdown in adipose tissue is the hormone-sensitive lipase. In rat adipocytes, insulin inhibits the enzyme and blocks lipolysis, to a large extent, by activating the PDE3B enzyme. In a series of elegant studies, Ahmad et al. reported that in adipocytes, PDE3B enzyme is associated with caveolae where insulin mediates its interaction with protein kinase B, supporting the important role of PDE3B enzyme in insulin-mediated regulation of lipolysis and fat metabolism [24]. Although the phenotype of the PDE3B enzyme knock out mice, generated by Choi's group, revealed that PDE3B enzyme has an intricate role in fat metabolism, increased lipolysis in the knock out animals has provided further evidence for the critical role of PDE3B enzyme in regulation of lipolysis [25]. In contrast to the well established mechanism involved in the antilipolytic action of insulin, the molecular basis for the insulin-like actions of CP is not exactly known. Using rat skeletal muscle myoblasts, Grunberger et al. launched a series of experiments to delineate the mechanisms involved in the insulinomimetic actions of CP and found that at physiologic concentration, the concentration tested in the present study, signaling pathways activated by CP are similar to those used by insulin [10,16]. In accord to their findings, we report here that the antilipolytic action of CP involves the key insulin signaling target molecule, PDE3B enzyme. In the presence of cilostamide, a specific PDE3 enzyme inhibitor, the inhibitory action of CP on ISO-induced lipolysis in adipose tissue of UTD rats was blocked, indicating that PDE3 enzyme is involved in the action. To the best of our knowledge this is the first report showing an antilipolytic action for CP through PDE3B enzyme. The importance of PDE3

enzyme in regulating lipolysis and its sensitivity to cilostamide have been well studied in different adipocytes with different origins [26,27]. Among 11 isoenzymes in the PDE superfamily, the PDE3B is shown to be highly expressed in the cells important in energy metabolism including adipocytes [26, 28]. Although it has been suggested that inhibition of both PDE3 and PDE4 is required for efficient stimulation of lipolysis in rat adipocytes, the inhibitor of PDE3 isotype is shown to have a greater effect [26].

A question remains to be answered is that how the activity of PDE3B is amplified by CP. Molecular mechanisms by which CP mediates its actions are rather complex and multiple interaction sites (extracellular, cell surface or intracellular) seems to be responsible. Outside the cell, CP may interact with other proteins/peptides oligomers, act as a chaperon, and mediate their disaggregation. It may also interact with itself, make homo-oligomers, and form aggregates [29,30]. At cell surface, available data suggest at least 2 distinct possibilities. First, CP may induce its effects through insulin receptor signaling pathways. This possibility is supported by the work of Grunberger and co-workers who demonstrated that CP promotes insulin receptor phosphorylation and, like insulin, enhances phosphatidylinositol 3-kinase activity in adipocytes [10]. However, we observed that in the presence of CP the antilipolytic effect of insulin was potentiated in the ISO-stimulated tissue of UTD rats indicating that the CP effect is not exclusively mediated through this pathway. The second possibility is that CP binds to its own receptor, most likely a G-protein-coupled receptor [16], and thereby activates or inhibits multiple signaling molecules, which may alter the PDE isoenzymes activity and ultimately modulate lipolysis. The CP may also be internalized into cell and act as an intracrine factor, like a number of extracellular signaling peptides [31], or be translocated to nucleus, and alter cell behavior through gene expression [32]. In conclusion, our data show that CP, conditionally, has the potential to act as an antilipolytic hormone by activating PDE3B in retroperitoneal adipose tissue and suggest that the action may contribute to the CP's beneficial effects on diabetes-induced complications.

Conflict of Interest

The authors have no conflict of interest.

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