Effects of Rat C-peptide-II on Lipolysis and Glucose Consumption in Cultured Rat Adipose Tissue

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Abstract

Existing data show that C-peptide (CP) prevents or ameliorates diabetes-related complications mainly by improving microcirculation and perhaps metabolism. Although effects of CP on muscle glucose consumption are relatively well studied, its effects on adipose tissue, a key organ involved in metabolism, are not well known. Therefore, the aim of this study was to examine the effects of CP on basal and stimulated lipolysis and glucose consumption in rat retroperitoneal (RP) adipose tissue, using an ex-vivo organ culture setting. The RP adipose tissue was excised from adult male rats, minced and subjected to ex-vivo culture for 24 h. The tissue fragments were then weighted and distributed into a 24-well culture plate. The wells were left untreated (basal) or treated with insulin or isoproterenol (ISO, stimulated) and incubated in the absence or presence of CP, insulin or a combination of the both peptides. Levels of lipolysis and tissue glucose consumption were determined by glycerol and glucose concentrations measurement in the infranant conditioned media collected from each well. The CP, like insulin, induced an insignificant reduction in basal lipolysis. While insulin significantly reduced the ISO-stimulated lipolysis, CP was ineffective. Tissue glucose consumption was significantly stimulated by insulin, but was not affected by CP. However, in the presence of CP, inhibitory effect on ISO-stimulated lipolysis and stimulatory effect on glucose consumption of insulin were significantly diminished. Our data suggest that CP may conditionally modulate certain metabolic actions of insulin in RP adipose tissue. These modulations may contribute to fine-tuning of body metabolism under physiologic or pathologic conditions.

Introduction

Connecting peptide, also known as C-peptide (CP), is cleaved off from proinsulin during post-translational processing of the propeptide. In response to elevated blood glucose, the pancreatic beta cells release insulin and CP in an equimolar ratio. While most of the secreted insulin is rapidly degraded by liver during first passage, hepatic extraction of the CP is negligible. The CP has for a long time been considered as an inert molecule. Numerous studies, however, have shown that CP binds to cell surface receptor and induces different biological activities in various cell types [1, 2]. The CP stimulates Na+K+-ATPase, nitric oxide production and influx of extracellular Ca2++; the systems which are believed to be impaired in type-1 diabetes [3–6]. It is now accepted that CP improves the function of most tissues affected by diabetes complications, mainly through upgrading microcirculation and metabolism [1,2,7]. Species specific CP increases skeletal muscle glucose uptake and whole body glucose consumption in animal models and in both normal individuals and diabetic patients [8–11]. In diabetic rats under glucose clamp condition, CP is shown to stimulate whole body glucose consumption [8]. Zierath and coworkers showed that CP and insulin, at physiologic concentrations, are equally effective in stimulating glucose uptake by insulin-sensitive skeletal muscle [10]. Likewise, correction of CP plasma concentration in patients with type-1 diabetes is shown to augment whole body glucose utilization by 25%, indicating that CP may relieve hyperglycemic states [11].

In the past decade, there has been a considerable progress in understanding the molecular mechanism by which CP exerts its diverse effects on different cell types. Now, there are compiling evidence to show that CP mediates its actions by binding to its pertussis toxin-sensitive G-protein...
coupled receptor [12,13] or through non-chiral interactions; originally described by Ido et al. [14]. Moreover, the insulinomimetic actions of CP in muscle are believed to be mediated through insulin signaling components: phosphatidyl inositol 3-kinase/protein kinase B in particular [12]. This pathway is the best known signaling pathway through which insulin blocks lipolysis in adipose tissue [15].

Although the effects of CP on muscle metabolism are relatively known, effects of species specific CP on adipose tissue, the main target organ of insulin involved in lipid metabolism, are not clear. Therefore, the aim of this study was to examine the effects of CP on basal and stimulated lipolysis and glucose consumption in rat retroperitoneal (RP) adipose tissue, using an ex-vivo organ culture method.

Materials and Methods

Chemicals and reagents
The culture medium 199 (M199), bovine serum albumin fraction V, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES), penicillin-streptomycin solution, and isoproterenol (ISO) were obtained from Sigma (USA). Rat C-peptide-II (Glut-Glu-Asp-Pro-Gln-Val-Ala-Gln-Leu-Glu-Leu-Asp-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Asp-Glu-Gln-Thr-Leu-Ala-Leu-Glu-Val-Ala-Arg-Gln) was purchased from Bachem (Germany). Human insulin was from Eli Lilly Company and was kindly provided by EXIR Company (Iran). Glycerol assay kit was purchased from Abcam (England). Glucose assay kit was purchased from Man Laboratory Inc. (Iran).

Animals
Adult male Sprague-Dawley rats, weighted 310–320g, were obtained from Laboratory Animal House, Shiraz Medical University, Iran. Animals were fed standard chow and allowed free access to food and water. They were maintained in a temperature-controlled environment with 12-h light and dark cycles. The study protocol using the laboratory animals complied with the general guidelines of animal care of the Shiraz Medical University, Shiraz, Iran.

Adipose tissue collection and organ culture
After an overnight fasting, the rats were sacrificed by light ether anesthesia followed by laparotomy. The RP adipose tissue was rapidly excised through a sterile surgical procedure. The tissue was immediately transferred to pre-warmed (37 °C) phosphate-buffered saline (PBS), washed briefly, and minced by a sharp surgical blade into uniform small pieces of about 5 mg. To remove tissue debris and fat droplets, the tissue pieces were gently washed with PBS, resuspended and kept stand still for few minutes to allow the pieces rich in adipocytes to float. The floated tissue were then collected, transferred into a conical 50-mL plastic tube containing 15 mL of serum-free M199 with 25 mM HEPES and antibiotics (100 U/ml penicillin-G and 100 µg/ml streptomycin) and incubated at 37 °C in a humidified chamber under an atmosphere of 95% air and 5% CO2 for 24 h. In order to eliminate the effects of sampling-related variable factors such as differences in the tissue contamination with blood components, all tissues were subjected to ex-vivo organ culture procedure prior to the experiments, allowing equilibration with the culture medium.

Evaluation of basal and stimulated lipolysis and glucose consumption
At the end of the 24-h organ culture period, the medium was aspirated from the tubes and the tissue were washed, dried with the aid of sterile gauze and weighted precisely in an aseptic manner using an analytical electric balance. The tissue pieces were then distributed into 24-well culture plate (100 mg/well and 200 mg/well for lipolysis and glucose consumption assessments, respectively) and bathed with 1 mL Krebs-Ringer bicarbonate (KR8) buffer containing 25 mM HEPES, 5.5 mM glucose and 2% (w/v) bovine serum albumin (lipolysis assessment) or with 1 mL fresh M199 (glucose consumption assessment). The wells were left untreated (basal) or treated with insulin or ISO (stimulated) and incubated in the absence or presence of CP, insulin or a combination of the both peptides at 37 °C in a humidified chamber under an atmosphere of 95% air and 5% CO2 with constant shaking. Tissue lipolysis and glucose consumption were assessed for a time period of 90 min and 24 h, respectively. The final concentrations of CP and insulin in the cultures were adjusted to 6 and 10 nM, respectively. The ISO, a nonselective β-adrenergic agonist, was used at a concentration of 750 nM. At the end of the indicated incubation periods, glycerol and glucose concentrations were measured in the infranatant, conditioned media, collected from each well. All samples were assayed in duplicate.

Glycerol and glucose measurements
The glycerol was measured by an enzymatic method using glycerol assay kit. Briefly, the reaction mixture of glycerol dehydrogenase and nicotinamide adenine dinucleotide (NAD) were added to the samples. The resulted NADH was quantified by a colorimetric/fluorometric-based method according to the protocol provided by the kit manufacturer. Tissue lipolysis (rate of glycerol release) is expressed as a percentage of basal lipolysis or ISO-stimulated lipolysis. Rate of glucose consumption was determined by subtracting the glucose concentration in the conditioned media from that of M199 as measured by an oxidase-based colorimetric method [16-18].

Viability of the tissue adipocytes and stroma-vascular cells
To validate the organ culture protocol and to show that the cells stay alive during the experiment, viability of adipocytes and stroma-vascular cells was tested immediately after tissue excision and 24 h and 48 h post-cultivation. The adipocytes were isolated by exposure of tissue fragments to type-II collagenase enzyme (2 mg/ml) according to the classical method of Rodbell [19]. After digestion and extensive wash, floated adipocytes were collected and subjected to dye exclusion examination using a 0.4% (w/v) trypan blue solution. Viability of stroma-vascular resident cells was tested by monitoring growth rate of the cells migrating out from a small piece of the tissue which was explaned on the surface of a tissue culture dish. Tissue explantation and primary cell culture were accomplished by modification of the method originally described by Nakano and Scott [20].

Statistical analysis
Deviation from basal or ISO-stimulated lipolysis was studied by the non-parametric 2-related samples test (Wilcoxon). All reported values were expressed as means±SEM and the differences with P-values less than 0.05 were considered statistically significant.
Results

Viability of adipocytes and stroma-vascular cells of the tissue
In microscopic examination almost all adipocytes exhibited a dye-free cytoplasm and perfectly intact plasma membrane indicating that adipocytes are viable and live (data not shown). The differences between the percentages of live adipocytes in fresh and 24 h-cultivated tissues were minor (94 vs. 87 %, respectively). Likewise, no considerable differences were found between the growth rate of the stroma-vascular cells in the fresh and precultivated tissue explants. Fig. 1a demonstrates the cells migrating out from a piece of the adipose tissue which was subjected to organ culture for 24 h prior to explantation. The cells started to migrate out from the tissue explants within 2 days, and proliferated with a considerable rate. Although we did not characterize the cells, most of them exhibited a morphology similar to that of fibroblast. Remnants of the explants were removed after 1 week as the cell density started to increase rapidly (Fig. 1b).

Effects of CP on the basal and stimulated tissue lipolysis
The presence of CP or insulin in the culture medium reduced the level of basal lipolysis to 92±5 and 90±7 of the basal level, respectively (Fig. 2a). However, this reduction was not statistically significant. We assumed that the basal level of lipolysis in RP adipose tissue was not high enough to be affected by insulin or CP. Therefore, the effect of insulin and CP on lipolysis was examined in the presence of ISO, a potent lipolysis inducer. As expected, ISO induced a significant increase, up to more than 3 folds, in the basal level of lipolysis (Fig. 2a). Insulin reduced the ISO-stimulated lipolysis to 82±4%, (Fig. 2b). While this reduction was statistically significant (P<0.05), the effect of CP on ISO-stimulated lipolysis remained insignificant. Surprisingly, the presence of CP in the culture media reduced the effects of insulin on the ISO-stimulated lipolysis. As shown in Fig. 2b, this reduction was statistically significant (P<0.05).

Discussion

Here we report that rat specific CP has no significant effects on basal and stimulated lipolysis but is able to reduce the insulin-mediated glucose consumption and lipolysis inhibition. To our knowledge, this is the first study showing the effects of CP on adipose tissue in an ex-vivo organ culture in which the cell-cell and cell-matrix interactions were preserved. Soon after discovery of CP in 1968, Kitabchi and colleagues investigated the effects of pork and beef CP on basal and stimulated lipolysis and glucose consumption in pooled adipocytes isolated from rat adipose tissues and they found no insulin-like activity [21–23]. In regard to basal and stimulated lipolysis, our results are somewhat in concert with their findings. However, in this study we attempted to
include some of the essential physiological factors including species-specificity and physiologic dose of CP, the nature of lipolysis inducer and an organ culture system in our experimental setting. It is now accepted that a considerable variability in amino acid sequence for CP exists among mammalian species [24]. In regard to the dose, CP is believed to induce its biological actions in a dose-dependent manner and often displays variable bell-shaped dose response curve [12, 25]. We used a concentration of 6 nM which is believed to be in the range of physiological postprandial concentration [26]. In fact the biologic response as a function of CP concentration is peculiar in that the maximum response can be achieved at physiological concentration [27]. This has been attributed to the cell binding characteristics of CP; yet to be determined for adipocytes. Previously we have reported that adipose tissues at different anatomical site may not response equally to the same signal [28] and, therefore, the present data may not be applicable to other fat depots. Regarding the experimental model, cell-cell and cell-matrix interactions which have profound effects on cell behavior [29] have been absent in the isolated adipocytes used in the Kitabchi et al. experiments. Accumulating data support the existence of differences in CP cell binding affinities, activated signaling elements and, hence, CP responses between different cell types [1, 2, 13, 25]. Despite intensive research on CP actions and receptor signaling in several cell types, information about the CP actions and signalling in adipose tissue is scarce. The comparable effects of CP and insulin on basal lipolysis reduction indicate that both hormones may be equally effective in regulation/modulation of basal lipolysis; at least in RP adipose tissue. In our experimental setting, insulin significantly reduced the ISO-induced lipolysis but CP failed to do so. It has been suggested that CP mediates some of its action through components of insulin signaling pathways [25]. The identical effects of CP and insulin on basal lipolysis but not stimulated lipolysis or glucose consumption as well as insulin’s actions attenuation of CP support the idea that in addition to its own signalling elements, CP may share some signalling components with insulin. Adipose tissues express β-adrenergic receptors and respond to sympathetic stimulation by increase in lipolysis and provision of free fatty acid and glycerol as alternative fuels demanded during hypoglycemia and adaptation to stress. This action on fuel turnover contribute to induction of hyperglycemia and ketosis; metabolic status associated with diabetes [15, 30]. It has been consistently stated that positive effects of CP which were observed in animals with experimental diabetes or patients with type-1 diabetes who have very low or missing plasma CP cannot be detected in normal animals or healthy humans [31, 32]. Whether the adipose tissue of the normal and diabetic rats respond to CP in a similar manner is not yet known. The effects of CP on diabetic rat adipose tissue are currently under investigation in our laboratory. The CP appeared to be impotent to upgrade basal or insulin-induced glucose consumption in RP adipose tissue. There are several lines of evidence against this in muscle tissue [8 – 11], indicating that CP has not similar effect on glucose consumption in muscle and adipose tissues. Although the molecular basis of differential effect is not addressed, as mentioned before, CP cell binding affinities and variable signaling elements may be involved.

In view of the present results, we hypothesize that under fasting condition which RP adipose tissue is exposed to a low concentration of insulin, CP may act in harmony with insulin. However, when RP tissue is irrigated with insulin-rich blood, as in postprandial state, the CP conditionally attenuates the action of insulin. Certainly, further investigations are needed to nail down this hypothesis.

In summary, our data show that insulin and species specific CP are equally effective in modulation of basal lipolysis in adipose tissue and that CP attenuates the effects of insulin on glucose consumption and stimulated lipolysis in RP adipose tissue. These modulations may contribute to fine-tuning of the tissue metabolism under different physiologic or pathologic conditions.

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**Conflict of Interest:** None.

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