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RESEARCH ARTICLES

Increased L-CPT-1 activity and altered gene expression in pancreatic islets of malnourished adult rats: a possible relationship between elevated free fatty acid levels and impaired insulin secretion☆

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Abstract

Intrauterine growth restriction is associated with chronically elevated levels of serum fatty acids and reduced glucose-stimulated insulin secretion. Lipid metabolism in pancreatic β cells is critical for the regulation of insulin secretion, and the chronic exposure to fatty acids results in higher palmitate oxidation rates and an altered insulin response to glucose. Using a rat model of isocaloric protein restriction, we examined whether pre- and postnatal protein malnutrition influences the properties of pancreatic islet carnitine palmitoyltransferase-1 (liver isoform, L-CPT-1), a rate-limiting enzyme that regulates fatty acid oxidation in mitochondria. The activity of L-CPT-1 in pancreatic islets increased in the low protein (LP), although the L-CPT-1 mRNA levels were unaffected by malnutrition. The susceptibility of enzyme to inhibition by malonyl-CoA was unaltered and the content of malonyl-CoA was reduced in LP cells. Because the mitochondrial oxidation of fatty acids is related to the altered expression of a number of genes encoding proteins involved in insulin secretion, the levels of expression of insulin and GLUT-2 mRNA were assessed. A reduced expression of both genes was observed in malnourished rats. These results provide further evidence that increased L-CPT-1 activity and changes in gene expression in pancreatic islets may be involved in the reduced insulin secretion seen in malnourished rats.

Keywords: Insulin secretion; L-CPT-1; Low-protein diet; Malnutrition; Genes; Fatty acids

1. Introduction

Several studies in animal models, as well as epidemiological data, support the idea that fetal and postnatal nutritional adaptations have permanent effects on cell structure and physiology, and on metabolism in different organs [1]. In the rat, the late fetal and early postnatal periods are critical for pancreatic islet ontogeny [2]. Previous studies by our group have demonstrated that a low-protein (LP) diet causes deleterious effect on insulin secretion in rats [3–9]. Additionally, rats fed a LP diet have chronically elevated levels of serum fatty acids [3,4,6,8,9].

In general, fatty acids, insulin and thyroid hormone regulate the expression of carnitine palmitoyltransferase-1 in liver [10]. However, in the pancreatic β cell line INS-1 (liver isoform, L-CPT-1 is expressed predominantly in rat pancreatic islets), this expression is induced specifically by fatty acids [11]. CPT-1 is considered the rate-limiting enzyme that regulates fatty acid oxidation in mitochondria. As such, it plays a central role in the partitioning of fatty

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acids between mitochondrial oxidation and their accumulation as long-chain acyl-CoA (LC-CoA) and/or complex lipids in the cytoplasm. CPT-1 is inhibited by malonyl-CoA, and an increase in this CoA derivative may be responsible for the accumulation of fatty acyl-CoA in the cytosol [12].

Lipid metabolism in β cells is critical for the normal regulation of insulin secretion. The depletion of lipid stores, together with a decrease in nonesterified fatty acids (NEFA), reduces glucose-stimulated insulin secretion (GSIS) in rats and humans [13,14]. Concomitantly, NEFAs generate signals for insulin secretion, presumably via LC-CoA [12].

The inhibition of fatty acid oxidation by a CPT-1-specific irreversible inhibitor, etomoxir [15], or by nonmetabolizable fatty acid analogues [16] stimulates GSIS in perfused pancreas and isolated islets.

A similar effect was reported by Latorraca et al. [17], who showed that the inhibition of endogenous fatty acid oxidation by 2-bromostearate significantly enhanced glucose-induced secretion by islets from rats fed a LP diet.

In the present study, we examined whether alterations in the properties of pancreatic islet L-CPT-1 could be involved in the decreased insulin secretion seen during malnutrition. Since several adaptations may occur at the molecular level during protein malnutrition, and since alterations in intracellular lipid metabolism may influence the expression of genes encoding proteins that are related to or directly involved in GSIS, we also investigated the expression of L-CPT-1, insulin, GLUT-2 and peroxisome proliferator-activated receptor (PPAR) α, -β and -γ.

2. Materials and methods

2.1. Animals

All of the animal experiments were approved by the institutional (UNICAMP) Committee for Ethics in Animal Experimentation. Virgin female Wistar rats (85–90 days old) were obtained from the breeding colony at UNICAMP. Females were mated by housing with males overnight and pregnancy was confirmed by examining vaginal smears for the presence of sperm. Pregnant females were allotted at random to treatments, and from the first day of pregnancy until the end of lactation, were maintained on isocaloric diets containing 6% protein (LP diet) or 17% protein (control diet), as described previously [9]. During the experimental period, the dams had access to their respective diets and to water ad libitum. At the birth, large litters were reduced to eight pups, thus ensuring a standard litter size per mother. Two groups of adult male rats were used in this study: a control group (C) consisting of rats born to and suckled by mothers fed a control diet and subsequently fed a control diet from weaning to 130 days old, and a LP group consisting of the offspring of mothers fed a LP diet during both pregnancy and lactation and subsequently fed with the same diet from weaning to 130 days old. All of the offspring were weaned at the fourth week after birth. Throughout the experimental period, the rats had free access to food and water and were housed on a 12-h light, 12-h dark cycle at 24°C. At the end of the experimental period, only male rats were killed by decapitation and blood samples were collected and allowed to clot at room temperature. The resulting sera were stored at −20°C for the subsequent measurement of insulin by radioimmunoassay (RIA) [18]. The serum glucose [19], free fatty acid (Wako NEFA C test kit) and albumin levels [20] were determined immediately after decapitation.

2.2. Isolation of pancreatic islets

Islets from fed 130-day-old male rats of C and LP groups were isolated by collagenase digestion. Briefly, the pancreas was inflated with Hank's solution containing 0.7–0.9 g of collagenase/L and then excised and maintained at 37°C for 20 min. The tissue was harvested and the islets were handpicked under a stereomicroscope.

2.3. Insulin secretion

Groups of five islets were incubated for 45 min at 37°C in 0.5 ml of Krebs-bicarbonate containing 115 mmol/L NaCl, 5 mmol/L KCl, 2.56 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 24 mmol/L $NaHCO_3$ and 5.6 mmol/L glucose (all Sigma, St. Louis, MO), supplemented with 0.3% BSA and equilibrated with a mixture of 95% O_2/5% CO_2 to give pH 7.4. This medium was then replaced with fresh buffer and the islets incubated for 60 min in the presence of 2.8, 8.3 or 27.7 mmol/L glucose. The insulin content of the medium at the end of the incubation period was measured by RIA [18].

2.4. L-CPT-1 activity and malonyl-CoA sensitivity

The activity of L-CPT-1 in islets from C and LP rats was assayed by measuring the incorporation of [^{3}H] carnitine into acylcarnitine, with minor modifications [21]. Briefly, islet samples (0.2–0.3 mg of protein) were homogenized in 200 μl of medium containing 250 mmol/L sucrose, 1 mmol/L EDTA and 3 mmol/L Tris-HCl (pH 7.2) using a micro dounce homogenizer. Whole homogenates were assayed for L-CTP-1 activity. Each assay contained 10 μg of islet protein, 82 mmol/L sucrose, 70 mmol/L KCl, 70 mmol/L imidazole (pH 7.0), 1 mg of BSA, 2 mmol/L L-carnitine (2 μCi/μmol of L-[methyl-^{3}H]carnitine) (Amersham Life Science), 0.5 μg of antimycin A, 100 μmol/L palmitoyl-CoA, 2 mmol/L ATP and 2 mmol/L $MgCl_2$, in a total volume of 500 μl. The reactions were run at 37°C for 20 min. The resulting [^{3}H] palmitoyl-carnitine was extracted with *n*-butanol. To estimate the malonyl-CoA sensitivity, whole homogenates were preincubated (5 min, 37°C) with malonyl-CoA (50 μmol/L) before the reaction was started by adding radioactive carnitine.

2.5. Malonyl and acetyl-CoA measurements

The total content of acetyl-CoA and malonyl-CoA was determined by the HPLC method of Corkey [22]. Briefly, isolated islets were incubated with 1 ml of Krebs bicarbonate buffer containing 5.6 mmol/L glucose for 30 min at

Table 1
Primers used for amplification of genes

Gene	Sense primer (5′ →3′)	Antisense primer (5′ →3′)	T_m (°C)	Size (bp)	Accession number
CPT-I	TATGTGAGGATGCTGCTTCC	CTCGGAGAGCTAAGCTTGTC	55	630	L07736
Insulin	TCCTGCCCCTGCTGGCCCTGC	AGTTGCAGTAGTTCTCCAG	57	302	NM019129
GLUT-2	CATTGCTGGAAGAAGCGTATCAG	GAGACCTTCTGCTCAGTCGACG	55	408	NM012879
PPARα	AAGCCATCTTCACGATGCTG	TCAGAGGTCCCTGAACAGTG	55	510	M88592
PPARβ	CTTCAGTGACATCATTGAGC	GACAGCATGAACAGGAAGTG	55	539	U40064
PPARγ	TCCGTGATGGAAGACCACTC	CCCTTGCATCCTTCACAGC	55	331	U09138
Phospholipase A_2	CTGCTGGCTGCTTTGCTCAC	ACGGCATAGACAGGAAGTGG	58	458	D00036

The table shows primer sequences, annealing temperature, size of amplified fragments and accession number obtained from GenBank database.

37°C. Following the addition of 1 ml of trichloroacetic acid (10%), the islets were scraped and precipitated proteins were removed by centrifugation. The resulting supernatant was neutralized by extracting the acid five times with ether. The aqueous samples were dried and resuspended in water.

2.6. Evaluation of gene expression

Expression of the genes of interest (L-CPT-1, insulin, GLUT-2, PPARα, -β and -γ) in C and LP rats was determined in pancreatic islets using a semiquantitative reverse transcription-polymerase chain reaction. Total RNA from 500 islets was extracted using TRIzol reagent (Life Technologies, Auckland, New Zealand). For the polymerase chain reaction (PCR), RNA (1 μg) was reverse-transcribed using oligo (DT) primers. The resulting cDNA was amplified by PCR using oligonucleotides complementary to sequences in the genes (Table 1). The phospholipase A_2 gene was used as an internal control. The PCR was done in a total reaction volume of 12.5 μl containing 0.5 μl of cDNA, 0.05 mmol/L each of cold dNTP (dATP, dCTP, dGTP, dTTP), 0.37 mmol/L of $MgCl_2$, 0.25×PCR buffer, 0.1 μmol/L of appropriate oligonucleotide primers and 1 U of Taq polymerase (Life Technologies). The PCR amplification conditions were as follows: 2 min at 94°C; 30 s at 94°C followed by 30 s at 55°, 57° or 58°C; 45 s at 72°C; and 7 min at 72°C. The PCR products were separated on 1.5% agarose gels in Tris-borate-EDTA buffer (1× TBE) and stained with ethidium bromide. All assays included a negative control. The absence of genome contamination in the RNA samples was confirmed by RT-negative RNA samples. The relative band intensities were determined by densitometry and the ratio of each gene to phospholipase A_2 was calculated for each sample.

2.7. Statistical analysis

The results are presented as the mean±S.E.M. for the number of rats (n) indicated. When working with islets, n refers to number of experiments done (120, 300, 500 and 1000 islets per group per experimental condition for insulin secretion, malonyl and acetyl-CoA measurements, gene expression, L-CPT-1 activity, respectively). Student's t-test was used to compare the malonyl and acetyl-CoA measurements, gene expression and L-CPT-1 activity in the C and LP groups. The Levene test for the homogeneity of variances was initially used to check the fit of the data to the assumptions for parametric analysis of variance. The data from insulin secretion were analyzed by one-way analysis of variance, followed by the Tukey–Kramer test to detect individual differences between groups. A value of $P<.05$ was considered significant. The data were analyzed using the Statistica software package (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Characteristics of the animals

LP rats showed features typical of protein malnutrition, including hypoalbuminemia, high serum free fatty acid levels and a low body weight (Table 2). The serum glucose and insulin concentrations were similar in both groups of rats.

3.2. Glucose-induced insulin secretion

As shown in Table 3, insulin secretion in response to a nonstimulatory concentration of glucose (2.8 mmol/L) did not differ between the C and LP groups. In contrast, insulin secretion in response to 8.3 mmol/L glucose was lower in

Table 2
Body weight and serum insulin, glucose, albumin and free fatty acid (FFA) levels in C and LP-fed rats in the fed state (130-day-old male rats)

	C	LP
Body weight, g	396±55	304±26*
Albumin, g/L	33±3	20±2*
Glucose, mmol/L	9.27±1.72	8.61±0.55
FFA, mmol/L	0.53±0.16	0.88±0.19*
Insulin, pmol/L	500±232	321±214

Results are mean±S.E.M. of five to seven rats.
* $P<.05$ vs. C rats.

Table 3
Glucose-stimulated insulin secretion by islets from C and LP-fed rats (130-day-old male rats)

Incubation condition	Insulin secretion (μU/islet per 60 min)	
	C	LP
Glucose (2.8 mmol/L)	17.56±4.72	7.96±4.22
Glucose (8.3 mmol/L)	53.50±14.43	7.46±3.98*
Glucose (27.7 mmol/L)	206.71±40.29	43.78±37.31*

Results are means±S.E.M. of three experiments.
* $P<.05$ vs. C islets.

the LP group than in the C group. A significantly lower insulin secretion was also observed in LP rats in the presence of 27.7 mmol/L glucose.

3.3. L-CPT-1 activity and malonyl-CoA sensitivity

L-CPT-1 activity increased by approximately 15% in pancreatic islets from LP rats compared to C rats (114.6±2.93% and 100±1.9%, respectively, $P<.05$). L-CPT-1 activity in LP and C rats was equally suppressed (about 12% and 16%, respectively), by the addition of 50 μmol/L malonyl-CoA, the physiological inhibitor of this enzyme.

3.4. Malonyl and acetyl-CoA measurements

There was a reduced concentration of malonyl-CoA in pancreatic islets of LP rats (0.051±0.006 nmol malonyl-CoA/g weight dry) compared to C rats (0.100±0.007 nmol malonyl-CoA/g weight dry, $P<.05$). The nutritional status of the rats had no effect on the acetyl-CoA concentration (C=0.012±0.001 nmol Acetyl-CoA/g weight dry and LP=0.007±0.0018 nmol Acetyl-CoA/g weight dry).

3.5. Gene expression of L-CPT-1, insulin, GLUT-2, PPARα, -β and -γ in C and LP rats

The mRNA levels of L-CPT-1 in pancreatic islets of C and LP rats were similar, whereas those of insulin, GLUT-2 and PPARγ (Table 4, Fig. 1) were reduced by protein malnutrition. There were no significant differences in the expression of PPARα and -β between the two groups of rats (data not shown).

4. Discussion

Protein malnutrition during fetal life can be detrimental to the development of pancreatic β cells and lead to permanent insulin deficiency [3,4,7,23], as also shown by our experiments with isolated islets (Table 3). It is possible that different steps in the mechanism of insulin gene expression, biosynthesis or secretion may be altered in islets from malnourished rats [4,5,7,8,24–26].

A significant increase in glucose-induced insulin secretion by islets from rats fed a LP diet was observed after the inhibition of endogenous fatty acid oxidation by

Table 4
Expression of the insulin, L-CPT-1, GLUT-2 and PPARγ genes in pancreatic islets from 130-day-old male rats fed a C (17% protein) or LP (6% protein) diet during the pre- and postnatal periods

	C	LP
Insulin/phospholipase A_2	0.686±0.08	0.331±0.05*
LCTP-1/phospholipase A_2	0.645±0.005	0.685±0.08
Glut-2/phospholipase A_2	1.41±0.014	1.29±0.026*
PPARγ/phospholipase A_2	0.25±0.011	0.147±0.008*

The data are shown as ratio between a specific mRNA and the mRNA phospholipase A_2 (internal control).
Results are means±S.E.M. of three experiments.
* $P<.05$ vs. C.

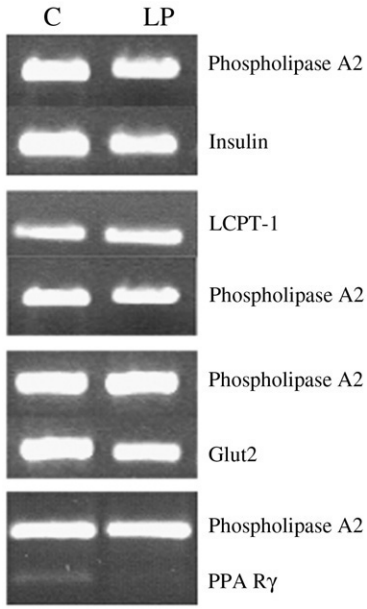

Fig. 1. Representative panel of the expression of the insulin, L-CPT-1, GLUT-2 and PPARγ genes in pancreatic islets from 130-day-old male rats fed a control (17% protein, C) or low-protein (6% protein, LP) diet during the pre- and postnatal periods. Each line shows a specific mRNA and the mRNA phospholipase A_2 (internal control) for one representative experiment.

2-bromostearate [17]. In the present study, L-CTP-1 activity in LP pancreatic islets was higher than in control islets. Rats fed a LP diet have chronically elevated levels of serum fatty acids [3,4,6,8,23] which could lead to increased L-CPT-1 activity because the addition of exogenous fatty acid increases the cytosolic acyl-CoA content [15]. Moreover, fatty acids are specific components in the activation of this enzyme [11].

Similar results were reported by Guzmán et al. [27] for hepatic CPT-1 in rat pups from protein malnourished dams. However, CPT-1 activity seems to be isoform specific, because neither skeletal nor cardiac muscle was affected by protein malnutrition [27,28].

Additional evidence supporting the view that the inhibition of L-CPT-1 is linked to β-cell stimulation by glucose is based on the observation that when insulin secretion is suppressed, such as during chronic hyperlipidemia, L-CPT-1 is up-regulated concomitantly with enhanced fat oxidation in these cells [11]. Conversely, L-CPT-1 overexpression in INS-1 insulinoma cells results in exaggerated fatty acid oxidation and impaired glucose-stimulated insulin secretion [29].

Fatty acyl-CoA may act as a coupling factor in insulin secretion by stimulating several isoforms of protein kinase C [30], by stimulating ATP-sensitive K^+ channels [31] and by acetylating proteins to target them to appropriate membrane sites [32]. In agreement with the coupling role of fatty acyl-CoA, pancreatic islets from protein malnourished rats may have a decreased content of these compounds, because of increased L-CPT-1 activity, such that β cells may be stimulated less through these pathways.

The markedly reduced level of malonyl-CoA observed in pancreatic islets from LP rats reinforces the hypothesis above, because a high malonyl-CoA level may be responsible for the accumulation of fatty acyl-CoA in the cytosol [12].

In the present study, protein malnourished rats showed reduced insulin and GLUT-2 mRNA expression. These genes were described to be regulated by PDX-1 (pancreatic duodenal homeobox gene-1), the best known β cell-specific transcription factor. Fatty acids decrease PDX-1 expression and its binding activity in rat pancreatic islets, in addition to reducing insulin, GLUT-2 and glucokinase mRNA and protein levels [33]. Such an inhibitory effect requires the mitochondrial oxidation of fatty acid [33]. It is also possible that fatty acids may have a direct inhibitory effect on insulin gene promoter activity [34]. Interestingly, a decreased expression of PDX-1 was observed in pancreatic islets of pup rats fed a LP diet [4].

LP islets showed unaltered L-CPT-1 mRNA levels, a finding which contrasts with the increased gene expression of this enzyme in the pancreatic β cell lines INS and MIN6 cultured with fatty acids [11,35]. However, the effects of protein malnutrition on the CPT-1 transcription rate and/or mRNA turnover are unknown.

Long-chain fatty acids affect the transcription of a number of genes through PPARs which are nuclear receptors closely related to the steroid-thyroid hormone family. The target genes for PPARs encode enzymes involved in lipid and glucose metabolism and homeostasis [36]. So far, there have been no reports on the gene expression or functions of PPARs in animal models of protein malnutrition.

Pancreatic islets of LP rats showed decreased expression of PPARγ and GLUT-2. The glucose-sensing apparatus of β cells consists of glucose transporter isotype 2, GLUT-2, and glucokinase, GK, which play a critical role in GSIS. The role of PPARγ in pancreatic islets is still unclear, but evidences support its direct action on genes involved in insulin-stimulated glucose disposal, such as GLUT-2 and β-GK [37]. Interestingly, it was shown that the acute ectopic expression of PPARγ attenuated the glucose-stimulated insulin secretion in pancreatic β cells [38]. Thus, the significance of the reduced expression of PPARγ in pancreatic islets from protein malnourished rats and the possible relationship between PPARγ and GLUT-2 remain to be established.

In conclusion, our results indicate that increased L-CPT-1 activity, possibly through an increase in fatty acid oxidation, could play a role in the reduced insulin and GLUT-2 expression and impaired GSIS seen following protein malnutrition during the pre- and postnatal periods in rats.

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