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Changes in food intake, metabolic parameters and insulin resistance are induced by an isoenergetic, medium-chain fatty acid diet and are associated with modifications in insulin signalling in isolated rat pancreatic islets

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Abstract

Long-chain fatty acids are capable of inducing alterations in the homoeostasis of glucose-stimulated insulin secretion (GSIS), but the effect of medium-chain fatty acids (MCFA) is poorly elucidated. In the present study, we fed a normoenergetic MCFA diet to male rats from the age of 1 month to the age of 4 months in order to analyse the effect of MCFA on body growth, insulin sensitivity and GSIS. The 45 % MCFA substitution of whole fatty acids in the normoenergetic diet impaired whole body growth and resulted in increased body adiposity and hyperinsulinaemia, and reduced insulin-mediated glucose uptake in skeletal muscle. In addition, the isolated pancreatic islets from the MCFA-fed rats showed impaired GSIS and reduced protein kinase Bα (AKT_1) protein expression and extracellular signal-related kinase isoforms 1 and 2 ($ERK_{1/2}$) phosphorylation, which were accompanied by increased cellular death. Furthermore, there was a mildly increased cholinergic sensitivity to GSIS. We discuss these findings in further detail, and advocate that they might have a role in the mechanistic pathway leading to the compensatory hyperinsulinaemic status found in this animal model.

Key words: Medium-chain fatty acids: Insulin resistance: Insulin signalling: Pancreatic islets

In the occidental lifestyle, the high intake of long-chain fatty acids (LCFA) and sedentary behaviour are thought to contribute substantially to the development of obesity and a number of metabolic diseases, such as type 2 diabetes, atherosclerosis and hypertension[1,2]. In accordance with this paradigm, the metabolic syndrome is present in individuals who show obesity and at least two of the following metabolic alterations: high blood TAG, elevated fasted glucose levels and low blood HDL cholesterol concentrations[3,4]. Lichtenstein *et al.*[5] have suggested that less than 30 % of the energy from the diet should be derived from fat for maintaining normal growth and body weight and for preventing the onset of the metabolic syndrome. Furthermore, epidemiological and experimental evidence indicates that the quality of the fat is also important, i.e. low saturated fat intake[3,6–12].

The control of insulin secretion by the endocrine pancreas plays a central role in intermediary metabolic homoeostasis. Three main physiological extracellular signals regulate this process: nutrients, neuronal inputs and hormones[7,8]. The blood glucose concentration is generally considered to be the most

Abbreviations: AKT, protein kinase B; ERK, extracellular signal-related kinase; GSIS, glucose-stimulated insulin secretion; IGF-1, insulin-like growth factor 1; IR, insulin receptors; IRS, insulin receptor substrate; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; PI3K, phosphoinositide 3-kinase; $pAKT_{SER1,2,3}$, protein kinase B α, β, γ phosphorylated at serine residue 473; PKC, protein kinase C.

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important physiological insulin secretagogue. Meanwhile, the autonomous nervous system and incretins modulate glucose-stimulated insulin secretion (GSIS)[7,9,10].

The insulin/insulin-like growth factor 1 (IGF-1) receptors and the insulin receptor substrate (IRS) 1/IRS2/phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) intracellular signalling pathway play an important mechanistic role in the normal growth, survival and function of the pancreatic islets cells[11,12]. In addition, mice with induced hyperexpression of AKT_1 in pancreatic β-cells have improved glucose tolerance, and the pancreatic β-cells show an increased cellular mass and function[13,14]. Chronic exposure of the pancreatic islets to saturated LCFA results in desensibilisation and suppression of GSIS[15,16], which are accompained by reduced cell viability and higher levels of cellular death[17]. These events are associated with a higher degree of palmitate incorporation into TAG[18] and diabetes[19], and induce profound modifications in the insulin/IGF-1 receptors and the IRS1/PI3K/AKT signalling pathway in isolated pancreatic islets and β-cell lines[20,21].

Medium-chain fatty acids (MCFA) have been extensively used in enteral and parenteral nutrition in convalescent human subjects since the early 1950s to promote the survival of people with impaired digestion and/or the ability to absorb or even transport LCFA[22–24].

Experimental evidence suggests that dietary medium-chain TAG, which yield MCFA, preserve insulin sensitivity in animal models and in human subjects with type 2 diabetes, and enhance insulin secretion[3]. However, the effect of dietary MCFA on GSIS and on the insulin/IGF-1 receptors and the IRS1/PI3K/AKT signalling pathway in isolated pancreatic islets are poorly understood. In order to investigate this possibility, we analysed the effects of normoenergetic MCFA diet ingestion on food intake, zoometric parameters, insulin-mediated glucose uptake in skeletal muscle, on GSIS and on insulin/IGF-1 receptors and the IRS1/PI3K/AKT signalling pathway in isolated pancreatic islets in young rats.

Experimental methods

Animal model

Male Wistar rats (1 month old) were housed in a controlled environment at 23 ± 2°C under a 12 h light–12 h dark cycle. Water was provided to all animals *ad libitum*. Two diets were used to feed the animals: a standard rodent chow for the control group (containing 80 % carbohydrates, 15·5 % protein and 4·4 % saturated fat primarily in the form of LCFA) and a MCFA chow (containing 2·42 % LCFA, 1·98 % MCFA, 80 % carbohydrates and 15·5 % protein) (Table 1). Body weight and food intake were recorded throughout the experimental period. When the animals were 4 months old, they were subjected to experimental functional analysis and killed using anaesthesia, as described in each protocol description. All experiments were approved by the Animal Experimentation Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo (n. 53, p. 35 – approved on 19 September 2006)

Table 1. Macronutrient composition of the control and medium-chain fatty acid (MCFA) diets

	Standard chow (%)		MCFA diet (%)	
Components	g	kJ	g	kJ
Protein*	15·5	14·7	15·5	14·7
Carbohydrates*	80·1	75·9	80·1	75·9
Saturated fat*	4·4	9·4	2·42	5·17
MCFA†	–	–	1·98	4·23
kJ/g	17·6		17·6	

* Determined by direct analysis.

† The percentage of MCFA corresponded with respective values of caprilic acid (8 : 0), capric acid (10 : 0), lauric acid (12 : 0) and caproic acid (6 : 0) (68, 28, 3 and 1 %, respectively).

and were conducted in accordance with the institutional and national guidelines for the care and use of animals.

The food intake and amount of energy obtained were determined for the animals from each group according to previously published protocols[25,26]. The following parameters were calculated:

$$\text{Energy intake} = ((\text{mean food consumption}) \times (\text{percentage of dietary metabolisable energy})),$$

$$\text{Voluntary food intake (\%)} = ((\text{mean food consumption} \times 100)/(\text{mean body weight})),$$

$$\text{Feeding efficiency} = ((\text{mean body weight gain (g)})/(\text{food consumption (g)})),$$

$$\text{Preference percentage} = ((\text{intake of tested diet} \times 100)/(50\,\text{g of study diet})).$$

Blood glucose, insulin, lipid profile, total protein and albumin determination

Animals from both groups, after overnight fasting, were anaesthetised with thiopental (40 mg/kg body weight) and naso–anal length were determined at the end of the experimental period. Blood samples were collected from the tail to determine blood glucose levels by a glucose meter (Accu-chek Active; Roche). Subsequently, these same rats were decapitated and their blood was immediately collected in Eppendorf tubes. Following centrifugation, the plasma was transferred to new tubes and stored at −20°C until assayed to evaluate other biochemical parameters (TAG, HDL-cholesterol, total cholesterol and insulin according to the procedure mentioned later).

Plasma insulin concentrations were measured by a RIA using rat insulin labelled with ^{125}I (Genese Produtos Diagnosticos Ltda) according to Bordin *et al.*[27], with a few modifications[28]. The insulin antibody was a gift from Dr Leclercq-Meyer, Université Libre de Bruxelles, Belgium. Commercial enzyme kits (Labtest Diagnostica) were used to measure the total protein and albumin levels, and for the generation of the lipid profile (TAG, HDL-cholesterol and total cholesterol) in serum samples. The epididymal fat pads were also collected from the control and MCFA-fed rats and weighed.

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Serum fatty acid composition

Overnight fasted rats from each group were decapitated after anaesthetisation with thiopental (40 mg/kg body weight). Serum total lipids were collected and extracted following the method of Folch *et al.*[29]. The fatty acid composition of the total lipids was analysed through GC after the conversion of the fatty acids into methyl esters according to the protocol of Hartman & Lago[30], with minimal modifications. In brief, the lipids were submitted to saponification in KOH (0·55 M) at 60°C for 2 h, followed by esterification in a methanol solution with 1 M-H_2SO_4 for 2 h at 60°C. The fatty acid methyl esters were extracted with *n*-hexane and analysed using a Shimadzu 17A gas chromatograph (Shimadzu Company) equipped with a flame ionisation detector and a SP2340 fused silica capillary column (60 m × 0·25 mm × 0·2 mm; Supelco Company). The injection volume was 1 μl, and the detector and injector temperatures were 260 and 240°C, respectively. The column temperature was intially at 120°C for 5 min, and then increased at a rate of 4°C/min, until reaching the final temperature of 240°C. The identification of the fatty acids was accomplished by comparing the retention times of the integrated peaks with those of the standards (fatty acid methyl ester (FAME) mix, thirty-seven components; Supelco Company). Individual fatty acids were expressed as a percentage of the area of all peaks. All reagents were obtained from Sigma, unless otherwise mentioned.

Glucose disposal rate (K_{itt})

Insulin sensitivity was evaluated by means of K_{itt} for anaesthetised animals from each group after a 4 h fast (thiopental, 40 mg/kg body weight). We used thiopental because it shows a minimal influence on the insulin signalling pathway[31]. Anaesthetised rats received a bolus insulin infusion through the penile vein (5·4 mmol/l per kg body weight), and tail blood samples were collected at 0, 4, 8, 12 and 16 min after injection for blood glucose measurements by a glucose meter (Accu-chek Active; Roche). The plasma glucose disposal rate was calculated as previously described. In brief, K_{itt} was the ratio between 0·693 and $t_{1/2}$. The $t_{1/2}$ value was calculated from the slope of the least-square analysis of the glycaemic concentrations, and was expressed as the %/min. We set our definition of an insulin-resistant state as a K_{itt} value below 2·5 %/min, according to a previous study[32].

2-Deoxy-D[2,6-^{3}H]glucose uptake, [U-^{14}C]D-glucose oxidation and glycogen synthesis in isolated rat skeletal muscle

Soleus muscles were isolated and incubated, as previously described[33,34], with a few modifications[35,36]. Non-fasted animals from each group were killed after deep anaesthesia (thiopental, 40 mg/kg body weight). The muscles were rapidly and carefully isolated, weighed (25–35 mg) and pre-incubated at 37°C in Krebs–Ringer bicarbonate buffer (5·6 mM-glucose (pH 7·4), pre-gassed for 30 min, O_2–CO_2 (v/v) at 95:5 %, respectively). Subsequently, the muscles were transferred to other vials containing the same buffer with 7400 Bq/ml D-[U-^{14}C]glucose and 7400 Bq/ml 2-deoxy-D[2,6-^{3}H]glucose and were incubated for 1 h in the presence or absence of 2·15 pmol/l insulin. Next, the muscles were washed with saline solution (0·9 % NaCl, at 10°C), dried with filter paper, weighed and digested in 1 M-KOH solution. The glucogen content was precipitated in an ethanol solution (66 %). Aliquots from the digested muscles were used to determine the ^{14}C and ^{3}H count in all the samples in order to express the uptake of 7400 Bq/ml 2-deoxy-D[2,6-^{3}H]glucose and the levels of glycogen synthesis. The oxidation of D-[U-^{14}C]glucose was determined according to Leighton *et al.*[37], with minimal modifications[35].

Morphometry of the endocrine pancreas

Five non-fasted rats from both groups were fasted for 4 h and anaesthetised with ketamine (5 mg/100 g body weight, intraperitoneal injection) and xylasine (1 mg/100 g body weight, intraperitoneal injection). Then, the animals were perfused through the heart with 0·9 % PBS and 4 % paraformaldehyde in 0·1 mol/l phosphate buffer. The pancreata were dissected from the surrounding tissues and fixed by immersion in 4 % formaldehyde–PBS solution for 4–6 h and then transferred to a 30 % sucrose solution in PBS for cryoprotection. Frozen pancreas sections (20 μm) were cut in a cryostat and mounted on gelatin-coated slices. The pancreas sections were counterstained with haematoxylin–eosin for morphometric analysis. A Carl Zeiss microscope was used to capture images, and the area of the pancreatic islets was morphometrically analysed using the Axio Vision 4.6.3.0® Imaging Program (Carl Zeiss). All reagents used in the present experiment were obtained from Sigma.

Isolation of pancreatic islets

Pancreatic islets were obtained from ten animals from each experimental group, as previously described by Lacy & Kostianovsky[38], with a few modifications by Marçal *et al.*[28]. The pancreas was inflated with Hanks solution containing type V collagenase (35 mg/ml), kept at 37°C for 20 min in a water-bath and stirred for an additional 1 min. The pancreas was then washed with a Krebs–Henseleit buffer solution containing 115 mM-NaCl, 5 mM-KCl, 24 mM-$NaHCO_3$, 1 mM-$CaCl_2$ and 1 mM-KCl_2. The islets were then collected using a magnifying glass. All reagents in the present experiment were obtained from Sigma. After that, the isolated pancreatic islets were submitted to different experimental protocols (see method sections 'Incubation of the pancreatic islets', 'Western blot analysis' and 'Insulin content and DNA fragmentation from isolated pancreatic islets').

Incubation of the pancreatic islets

Groups of five islets in triplicate from both groups were transferred to plates containing 1 ml of Krebs–Henseleit buffer solution supplemented with 0·125 % albumin for 60 min at 37°C in the presence of 5·6 mM-glucose (basal). This solution was bubbled with a mixture of O_2 (95 %) and CO_2 (5 %). After the pre-incubation period, the islets were incubated with different glucose concentrations (2·8, 5·6, 8·3, 11·1 and 16·7 mM) in the absence or presence of 50 μM carbachol, a non-specific muscarinic agonist, for an additional 60 min. Subsequently, 500 μl of the medium was collected from each condition and stored at −20°C for later insulin determination. The amount of insulin secreted was measured using the RIA method according to Bordin *et al.*[27], with only slight modifications[28]. Rat insulin labelled with ^{125}I was obtained from Amersham Pharmacia. All other reagents were obtained from Sigma, unless otherwise mentioned.

Western blot analysis

A group of 300 isolated pancreatic islets from each rat from both groups (a total of ten rats per group) was transferred to Eppendorf tubes and homogenised in solubilisation buffer (100 mM-Tris, 1 % SDS, 10 mM-EDTA, 100 mM-$Na_2P_2O_7$, 100 mM-NaF and 10 mM-Na_2VO_4). The extracts were centrifuged at 12 000 rpm for 40 min at 4°C to remove insoluble material. The protein concentrations in the supernatants were determined by the Bradford dye method[39] using Bio-Rad reagents. The proteins were subjected to SDS-PAGE (6·5 and 8 % gels) and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in 5 % non-fat milk. Membranes were incubated overnight at 8°C with antibodies against phosphotyrosine residues, insulin receptor β-subunit (IR), insulin-like growth factor 1 receptor (IGF1R$_β$), IRS1, IRS2, PI3K, AKT$_1$, protein kinase B α, β, γ phosphorylated at serine residue 473 (pAKT$_{SER1,2,3}$), extracellular signal-related kinase isoforms 1 and 2 (ERK$_{1/2}$), phosphoERK$_{1/2}$ (pERK$_{1/2}$), β-actin and protein kinase C (PKC) diluted in blocking buffer, to which 3 % non-fat dry milk had been added. Next, the membranes were washed for 30 min in blocking buffer without milk. Band intensities were quantified by optical densitometry (Scion Image-Release Beta 3b; NIH). Ponceau-S staining was used as a control for protein loading.

Reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad. Trizma, aprotinin, dithiothreitol, Triton X-100, glycerol, Tween 20 and bovine serum albumin (fraction V) were obtained from Sigma. The Immunoblot electrogenerated chemiluminescence detection kit was purchased from Amersham Pharmacia Biotech and nitrocellulose paper (0·45 μm) from Bio-Rad. All antibodies used were purchased from Santa Cruz Biotechnology.

Insulin content and DNA fragmentation from isolated pancreatic islets

Groups of five islets in triplicate from ten distinct animals from each group were immediately collected in Eppendorf tubes with 1 ml of Krebs–Henseleit buffer solution supplemented with 0·125 % albumin for 60 min at 37°C in the presence of 5·6 mM-glucose (basal) and then were sonicated (5 s). At the end, 500 μl of the medium were collected and stored at −20°C for later insulin determination. The amount of insulin secreted was measured using the RIA method, according to Bordin *et al.*[27], with modifications[28]. Rat insulin labelled with ^{125}I was obtained from Amersham Pharmacia. All other reagents were obtained from Sigma, unless otherwise mentioned.

DNA fragmentation was analysed by flow cytometry after DNA staining with propidium iodide, according to the method previously described by Nicoletti *et al.*[40]. Other groups of thirty islets from each animal group (control, *n* 10; MCFA, *n* 10) were separated and maintained in 200 μl of a buffer with 20 μg/ml propidium iodide, 0·1 % sodium citrate and 0·1 % Triton-X-100. Samples were maintained in the dark for at least 30 min. Fluorescence was measured using the FL2 channel (orange-red fluorescence = 585/542 nm). A total of 10 000 events were analysed per experiment. Cells with propidium iodide fluorescence were evaluated using Cell Quest software (Becton Dickinson), and results were expressed as a percentage of cells with DNA fragmentation. All reagents were obtained from Sigma, unless otherwise mentioned.

Statistical analysis

When appropriate, the data are presented as means with their standard errors. Statistical analysis was carried out using two-way ANOVA or the Student's *t* test, as appropriate. The level of significance was set at $P < 0·05$. All results were analysed using GraphPad Prism, version 4.00, for Windows (GraphPad Software).

Results

Zoometric and nutritional parameters

Table 2 shows the zoometric and nutritional parameters analysed before euthanasia. Despite a similar initial body weight between both groups, the MCFA-fed group had a final lower body weight and naso–anal length and an increased intra-abdominal fat pad, relative to the control group. The final body weight and naso–anal length were diminished in the MCFA-fed group, when compared with the control group

Table 2. Effects of medium-chain fatty acid (MCFA) ingestion on final body weight, body weight gain, mean food consumption, energy intake, voluntary food intake (VFI), feeding efficiency (FE), food preference percentage, naso–anal length and the percentage of periepididymal fat pads

(Mean values with their standard errors, *n* 10 for each group)

	Control		MCFA	
Groups	Mean	SE	Mean	SE
Initial body weight (g)	91·5	1·7	88·1	2·8
Final body weight (g)	386	10	285*	8
Body weight gain (g)	294·5	5·88	196·9*	2·86
Food consumption (g/d)	20·3	1·1	25·5*	1·4
Energy intake (kJ/d)	357·3	19·4	448·8*	24·6
VFI (%)	5·26	0·28	8·95*	0·49
FE	14·5	0·28	7·7*	0·11
Food preference (%)	40·6	2·2	51·0*	2·8
Naso–anal length (cm)	21·8	0·37	20·2*	0·36
% of Periepididymal fat pads (g/g body weight × 100)	1·1	0·1	1·8*	0·13

* Mean values were significantly different from those of the control group ($P < 0·05$; Student's *t* test).

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(26, and 7 %, respectively). However, the intra-abdominal fat pad from the MCFA-fed group was enhanced at 64 %, when compared with the control group. Meanwhile, the feed efficiency of the MCFA-diet rats was reduced by 47 % of that of the control rats, while the food intake, energy intake and food preference were 27, 19 and 24 % greater than those of the control group, respectively (Table 2).

Blood glucose, insulin, albumin, total protein, lipid profile and the glucose disposal rate (K_{itt})

Table 3 shows the blood parameters evaluated in the present study. Despite a similar fasting blood glucose concentration, a 58·6 % increase in insulinaemia and a 24·4 % reduction in the glucose clearence index (K_{itt}) were observed in the MCFA-fed rats compared with the controls. The blood total protein, albumin and cholesterol were similar between both groups. However, the blood HDL-cholesterol and TAG concentrations in the MCFA-fed group increased up to 21 and 40·1 %, respectively, compared with the control group (Table 3).

Serum fatty acid composition

Fig. 1 shows the serum fatty acid composition of the MCFA-fed and control groups. The primary detectable long-chain PUFA in both groups were linoleic acid (18 : 2) and arachidonic acid (20 : 4*n*-6); the main monounsaturated LCFA, oleic acid (18 : 1); and the primary saturated LCFA, palmitic (16 : 0) and stearic acid (18 : 0). In the control group, the predominant LCFA was linoleic acid at 35·6 % of the total fatty acids, followed by palmitic, oleic, arachdonic and steric acid at 24·4, 17·7, 14·5 and 8·3 %, respectively. However, the fatty acid composition in the MCFA-fed rats was markedly different from that of the control group. In the MCFA-fed rats, the most abundant fatty acid was palmitic acid at 32·5 %, followed by oleic (24·5 %), stearic (9·1 %) and palmitoleic (4·3 %) acids. These changes in the composition were associated with a reduction in the polyunsaturated linoleic and arachidonic acids. The serum MCFA fractions were not detectable in the serum of either group (Fig. 1).

Table 3. Concentrations of blood glucose, plasma levels of insulin, albumin and total protein, and the insulin sensitivity (K_{itt} test) of control and medium-chain fatty acid (MCFA)-fed rats

(Mean values with their standard errors, *n* 10 for each group)

	Control		MCFA	
Groups	Mean	SE	Mean	SE
Glycaemia (mmol/l)	5·8	0·1	5·8	0·3
Insulinaemia (pmol/l)	82·6	1·80	131·0*	1·26
K_{itt} (%/min)	4·1	0·02	3·1*	0·49
Albumin (g/l)†	48·8	0·94	51·1	0·57
Serum protein (g/l)†	71·1	3·9	72·0	2·9
Total cholesterol (mmol/l)†	1·23	0·1	1·25	0·03
HDL-cholesterol (mmol/l)†	1·15	0·05	1·39*	0·04
TAG (mmol/l)†	2·02	0·1	2·83*	0·1

* Mean values were significantly different from those of the control group ($P < 0·05$; Student's *t* test).

† Commercial enzyme kits were used to measure these parameters in serum samples according to the Methods section.

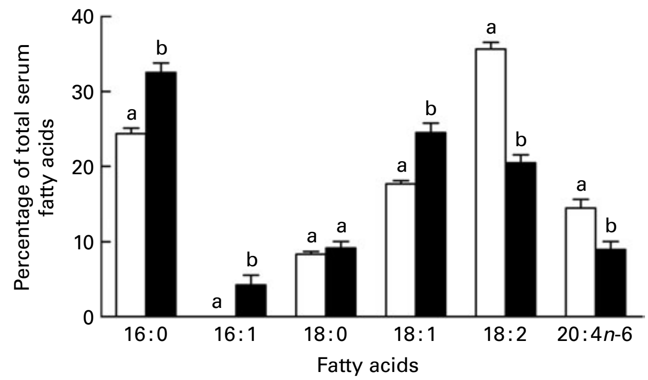

Fig. 1. Fatty acid profiles of serum from overnight fasted control (□) and medium-chain fatty acid diet rats (■). Values are means with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different between groups for a specific fatty acid ($P < 0·05$; Student's *t* test). *n* 10 for each group.

Effects of a medium-chain fatty acid diet on 2-deoxy-D[2,6-^{3}H]glucose uptake, D-[U-^{14}C]glucose oxidation and glycogen synthesis in skeletal muscles

Under basal conditions, the isolated soleus muscles from both groups showed similar 2-deoxy-D[2,6-^{3}H]glucose uptake, D-[U-^{14}C]glucose oxidation and glycogen content. As expected, following an insulin stimulus, 2-deoxy-D[2,6-^{3}H]glucose uptake, D-[U-^{14}C]glucose oxidation and the glycogen content were enhanced by 63, 82 and 99 %, respectively, in the control group when compared with the basal values ($P < 0·05$) (Fig. 2(A)–(C)). By contrast, the insulin-induced enhancement in glucose metabolism was similar to values obtained at basal conditions in the MCFA-fed rats.

Insulin content, DNA fragmentation analysis and morphologic analysis of the pancreatic islets

Fig. 3 shows pancreatic islets from MCFA-fed and control rats. The pancreatic islets from the MCFA group were markedly reduced in size by 47·4 % when compared with those of the control rats ($P < 0·0001$) (Table 4). Moreover, the insulin content of these reduced pancreatic islets from the MCFA-fed rats was decreased by 38 % when compared with that of the controls (Table 4). The DNA fragmentation index from dispersed cells in the isolated pancreatic islets was 150 % enhanced in the MCFA-fed rats relative to that of the controls (Table 4).

Glucose-stimulated insulin secretion and the modulatory effect of carbachol in isolated pancreatic islets

GSIS was evaluated in a dose–response manner in the absence or presence of carbachol (50 μM; Fig. 4). As expected, insulin secretion increased with higher glucose concentrations in isolated pancreatic islets from both groups. However, the GSIS were 20·6 and 21·4 % lesser in islets from the MCFA group at 11·1 and 16·7 mM of glucose when compared with the control group (Fig. 4(A)). On the other hand, the insulin secretion induced by co-incubation with carbachol was similar between both groups (Fig. 4(B)).

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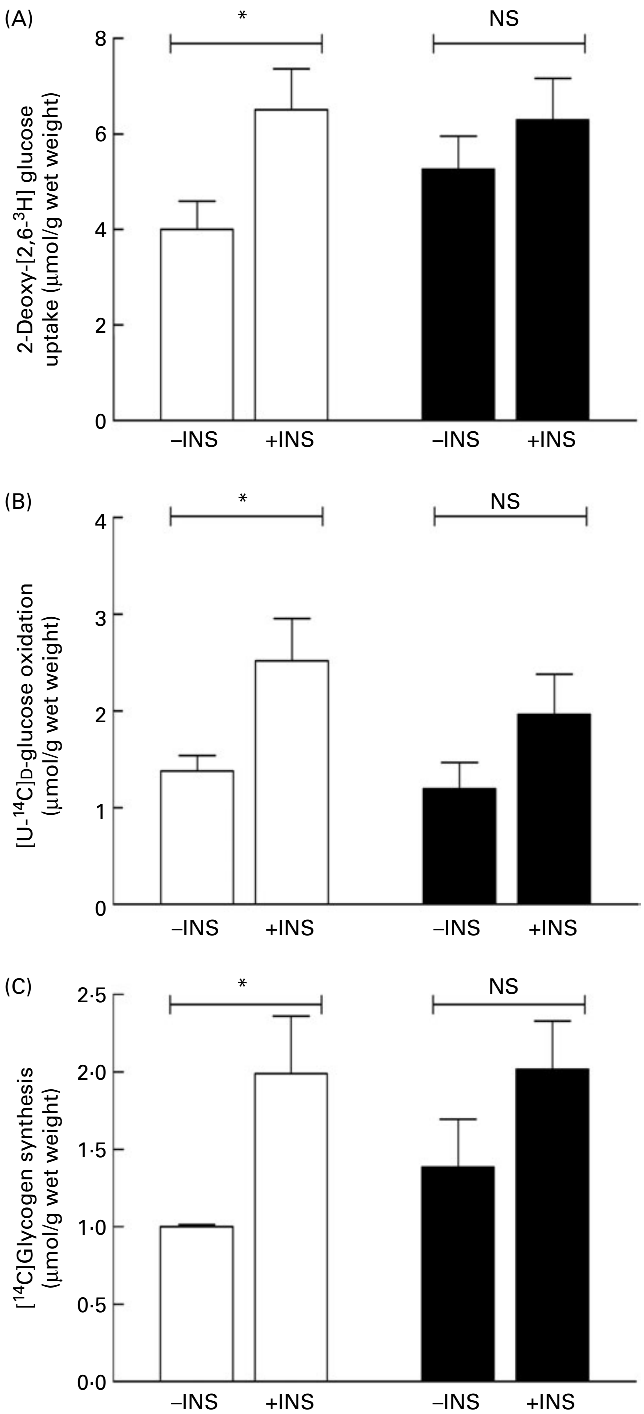

Fig. 2. Effects of medium-chain fatty acid (MCFA)-fed rats on glucose uptake and metabolism. Soleus muscles from both groups were isolated and incubated for 1 h in the absence or presence of 2·15 pmol/ml insulin (INS) in Krebs–Ringer bicarbonate buffer. The buffer contained 5·6 mM-glucose, 7400 Bq/ml D-[U-^{14}C]glucose and 7400 Bq/ml deoxy-D[2,6-^{3}H]glucose (pH 7·4) pre-gassed for 30 min with O_2–CO_2 (v/v) at 95:5 %, respectively, at 37°C. (A) 2-Deoxy-D[2,6-^{3}H]glucose uptake, (B) oxidation of [U-^{14}C]D-glucose and (C) [^{14}C] glycogen synthesis were determined. Values are means with their standard errors of three experiments represented by vertical bars (*n* 6 for control rats (□) and *n* 6 for MCFA-fed rats (■)).* Mean values were significantly different ($P < 0·05$; Student's *t* test).

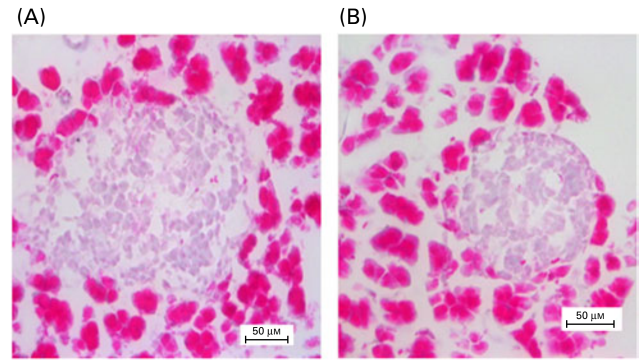

Fig. 3. Morphology of the pancreatic islets from (A) control rats and (B) medium-chain fatty acid-fed rats counterstained with haematoxylin–eosin. The bar corresponds to 50 μm (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Expression and phosphorylation status of proteins involved in insulin signalling

Early steps of insulin signalling in many cells and tissues occur by tyrosine residues' phosphorylation of pp95, mainly composed of β-subunit of the IR (IR-β), and pp185 subsequently, mainly composed of IRS1 and 2[41–43]. The tyrosine phosphorylation status of pp95 and pp185 is shown in Fig. 5. The densiometric analyses showed that the degree of IR-β tyrosine phosphorylation (pp95) was similar in the pancreatic islets of the control and MCFA groups (Fig. 5(A)). However, the tyrosine phosphorylation status of pp185 was enhanced by 55 % in the MCFA group when compared with the control group (Fig. 5(B)).

Fig. 6 shows representative immunoblots of the (A) IR β-subunit, (B) IGF1R, (C) IRS1, (D) IRS2, (E) catalytic p85 subunit of PI3K, (F) AKT_1, (G) the degree of phosphorylation of the serine 473 residue ($pAKT_{1/2/3}$), (I) PKC, (J) $ERK_{1/2}$ and (K) the degree of phosphorylation of the tyrosine and threonine residues of the $ERK_{1/2}$ ($pERK_{1/2}$). The pancreatic islets from the MCFA rats showed a 67·2 % increase in the protein expression of the IR (Fig. 6(A)), a 92·9 % increase in IRS1 (Fig. 6(C)), a 59·6 % increase in PI3K (Fig. 6(E)), a 15 % increase in PKC (Fig. 6(I)) and a 26 % increase in $ERK_{1/2}$ protein expression (Fig. 6(J)). On the other hand, AKT_1 expression was reduced by 33·4 % in the pancreatic islets from MCFA rats, when compared with that of the control rats (Fig. 6(F)).

The phosphorylation of IR/IGF1R causes activation of a number of proteins involved in intracellular processes, such as protein kinase B, which is homologous to v-AKT (AKT) and $ERK_{1/2}$. The degree of phosphorylation of AKT and $ERK_{1/2}$ was similar in the pancreatic islets from both groups (Fig. 6(G) and (K)). However, the stoichiometry between the phosphorylation status and protein expression revealed a 99 % increase in the AKT phosphorylation status (Fig. 6(H)). Meanwhile, there was a 20 % reduction in the $ERK_{1/2}$ phosphorylation status (Fig. 6(L)).

Discussion

Despite our initial hypothesis that dietary supplementation with MCFA would reduce body weight and improve both insulin sensitivity[44,45] and GSIS, the MCFA diet induced increased

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Table 4. Insulin content, pancreatic islet area and DNA fragmentation in the pancreatic islets from control rats and medium-chain fatty acid (MCFA)-fed rats†

(Mean values with their standard errors)

	Control		MCFA	
Groups	Mean	SE	Mean	SE
Islet area (μm^2)	29 311 (39)	3035	15 497* (60)	1158
Insulin content (pmol/l per pancreatic islets)	29 201 (10)	3461	17 975* (10)	964
DNA fragmentation (%)	10 (10)	1	25* (10)	3

* Mean values were significantly different from those of the control group ($P < 0.05$; Student's *t* test).
† The number of events utilised to analyse each parameter is given in parentheses (the pancreatic islets were obtained from ten animals for each group, the number of pancreatic islets utilised in each parameter was according to the Experimental methods).

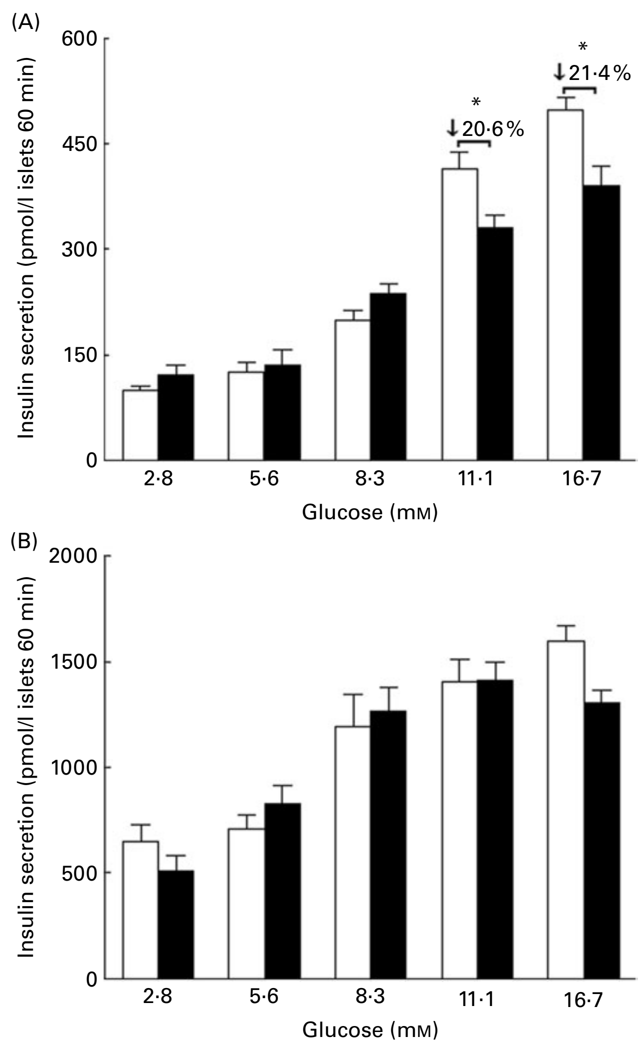

Fig. 4. Static insulin secretion from isolated pancreatic islets from control and medium-chain fatty acid (MCFA)-fed rats. A groups of five isolated pancreatic islets were pre-incubated in 5·6 mM-glucose for 60 min. Next, the pancreatic islets of control rats (□) and MCFA-fed rats (■) were incubated in 2·8, 5·6, 8·3, 11·1 and 16·7 mM of glucose for 60 min in the (A) absence or (B) presence of 50 μM-carbachol. Values are means with their standard errors represented by vertical bars (pmol/l islets 60 min) from ten distinct experiments performed in triplicate (*n* 10 for control rats and *n* 10 for MCFA-fed rats). A two-way ANOVA test followed by a Bonferroni test was carried out. * Mean values were significantly different ($P < 0.05$).

adiposity and insulin resistance, reduced the body weight and impaired GSIS.

The increased food intake, accompanied by the increased abdominal adiposity, but reduced body length and weight indicates poor feeding efficiency. At least two possibilities could explain a reduction in the feeding efficiency: diet palatability, which could modify food intake[25,41], and nutritional interference. In terms of diet palatability, blood glucose and ketone body levels act as brain sensors for food intake, and ketone bodies from a ketogenic diet have been shown to reduce food intake in normal rats[46,47]. Although we cannot exclude the possibility that our diet induced a higher production of ketone bodies than the control diet, we consider this possibility unlikely, as the classical ketogenic diet consists

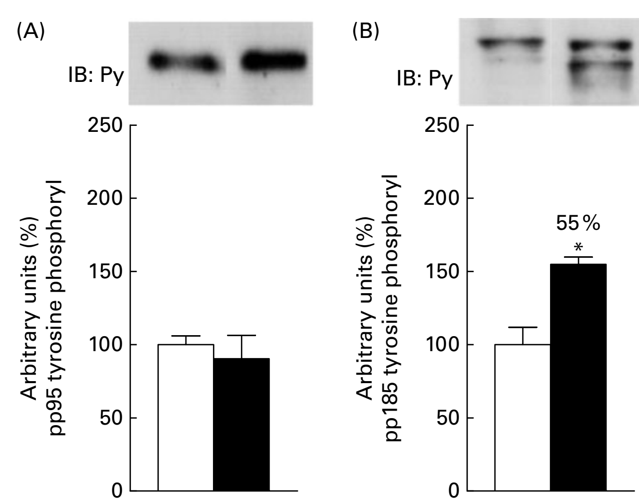

Fig. 5. Effect of medium-chain fatty acid (MCFA) ingestion on tyrosine phosphorylation (Py) of the pp95 (β-subunit of the insulin receptor (IR-β)) and of the pp185 (insulin receptor substrate (IRS) 1 and IRS2). A group of 300 isolated pancreatic islets were collected and used for immunoblotting analysis. Aliquots containing 90 μg of total protein were submitted to SDS-PAGE. The membranes with the pancreatic islets were probed with p-Tyr antibody. One representative blot of four separate experiments is shown. The bar graphs represent the tyrosine Py of the (A) pp95 and (B) pp185. The results are expressed as the percentage of the amount of signalling protein in the control animals (A and B). The data are expressed in correlation with the respective group. Values are means with their standard errors represented by vertical bars. *n* 10 for control rats (□) and *n* 10 for MCFA-fed rats (■). * Mean values were significantly different ($P < 0.05$; Student's *t* test). IB, immunoblot.

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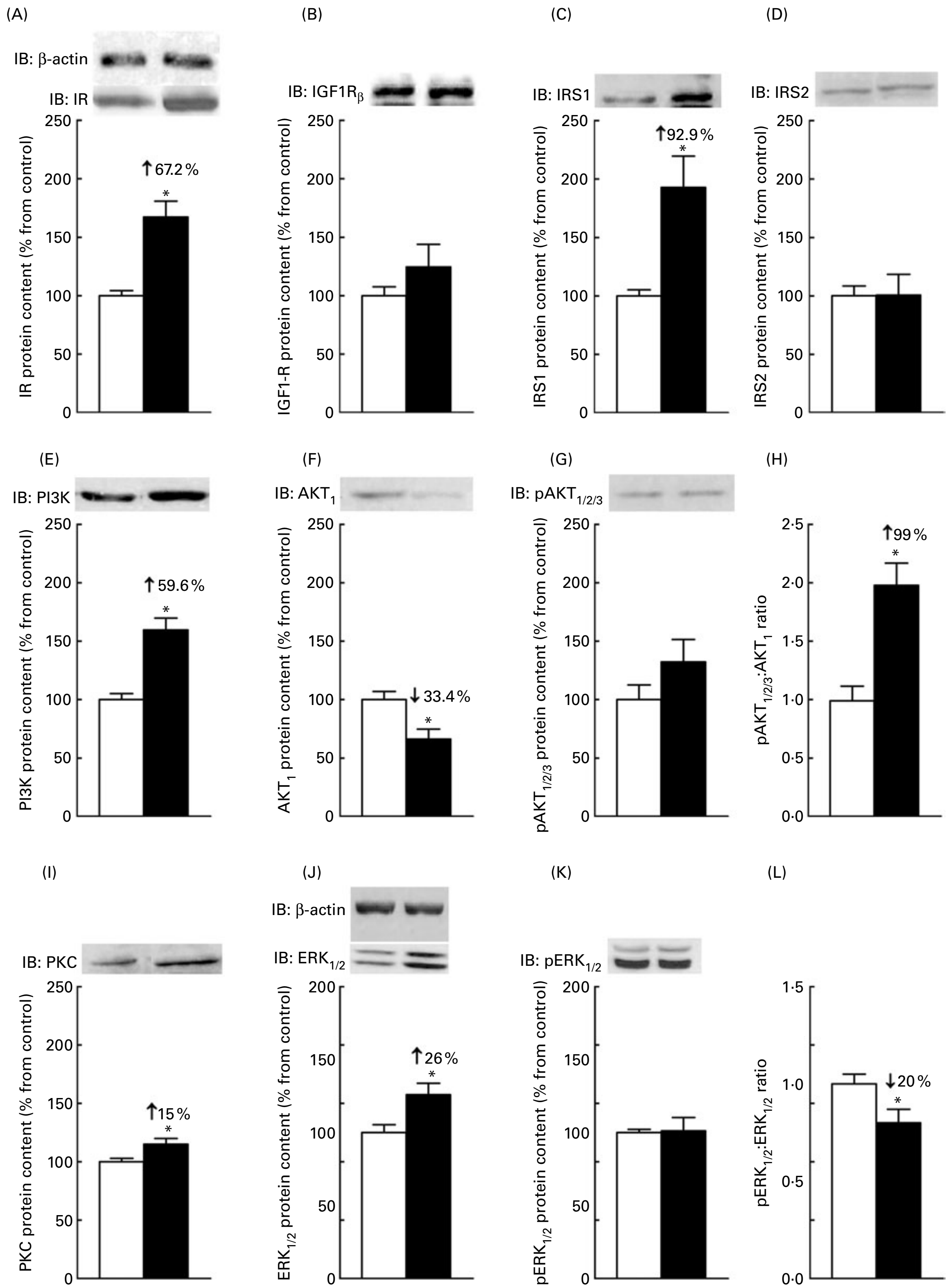

Fig. 6. The effect of the medium-chain fatty acid (MCFA) diet on (A) insulin receptors (IR), (B) insulin-like growth factor 1 receptor (IGF1R_{β}), (C) insulin receptor substrate (IRS) 1, (D) IRS2, (E) p85-subunit phosphoinositide 3-kinase, (F) protein kinase B (AKT_1), (G) phosphoserine 473AKT ($pAKT_{1/2/3}$), (I) protein kinase C (PKC), (J) extracellular signal-related kinase isoforms 1 and 2 ($ERK_{1/2}$) and (K) phospho-$ERK_{1/2}$. A total of 300 isolated islets were submitted to protein extraction and immunoblotting analysis, as described in the Methods section. Samples containing 75 μg of solubilised proteins were submitted to SDS-PAGE and immunoblotted using specific antibodies. One representative blot of four separate experiments is shown. The results are expressed as percentage of the control animals. (H) and (L) represent the stoichiometry between phosphorylation status and the protein expression of (H) AKT and (L) $ERK_{1/2}$. Values are means with their standard errors represented by vertical bars. *n* 10 for control rats (□) and *n* 10 for MCFA-fed rats (■). * Mean values were significantly different ($P < 0.05$; Student's *t* test). IB, immunoblot.

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of a low percentage of carbohydrates, normal protein levels and high fat. The ketogenic diet has at least a 20-fold increase in the fat content compared with the diets used in the present study[48,49]. The similar albumin and total protein blood levels argue against malnourishment in the MCFA-fed rats.

A high-fat diet rich in medium-chain TAG or LCT can induce insulin resistance[50]. Our MCFA diet was also able to induce insulin resistance and hyperinsulinaemia. One adaptative step to ensure glucose homoeostasis in both obesity animal models and obese human subjects with insulin resistance is compensatory hyperinsulinaemia. Ahrén *et al.*[51] observed that pancreatic islets from mice fed with a high-fat diet displayed an exacerbated insulinotropic response when pancreatic islets were incubated with carbachol. We agree with these authors, and according to the present data obtained from MCFA-fed rats, we reinforce the idea that hyperinsulinaemia follows the insulin resistance status as an adaptative response of the endocrine pancreas to guarantee a maintenance of plasma glucose levels at physiological conditions. In addition, increases in cholinergic sensitivity have been observed in many animal models of insulin resistance, such as pre-obese ob/ob mice[52], obese ob/ob mice[53], obese glutamate monosodium mice[54] and obese human subjects[55].

Parasympathetic innervation is involved in the modulation of GSIS[56]. The activation of the M_3 receptors enhances insulin secretion[27] through a phospholipase C-dependent pathway. Once activated, the production of diacylglycerol and inositol triphosphate is increased, followed by enhanced PKC activity and GSIS[57]. PKC stimulates insulin secretion by facilitating the docking of vesicles containing insulin to the plasma membrane and thereby contributes to the exocytosis of insulin granules[58]. The normalisation of the carbachol-mediated GSIS observed in pancreatic islets from MCFA-fed rats suggests a mild increase in sensibility to the cholinergic effect. The slight increase of PKC protein expression may play a role in this effect; however, other investigations are necessary to verify the activity of PKC on isolated pancreatic islets from MCFA-fed rats.

Fatty acids have been reported to potentiate glucose-induced insulin secretion. This effect is dependent on the chain length of the fatty acids[45] and the degree of unsaturation[59]. In terms of MCFA, pancreatic islets isolated from mice starved for 4 h and incubated with hexaenoic and octanoic acids showed enhanced glucose-induced insulin secretion[59]. Similar effects were observed in isolated pancreatic islets from human subjects[50]. Although we demonstrated here that a MCFA diet promotes alterations in glucose-induced insulin secretion, which was in disaccord with these evidences, these changes might have been an indirect effect, as no detectable MCFA were present in the serum of the MCFA group. Furthermore, the ingested MCFA was rapidly oxidised, resulting in an excess of acetyl-CoA[60]. In turn, the excess acetyl-CoA can activate metabolic routes in the mitochondria, such as the Krebs cycle, ketogenesis and the elongantion of fatty acids, as well as in the cytosol, such as the *de novo* synthesis of fatty acids and cholesterol. Although MCFA are ketogenic, there is evidence indicating that an increased availability of glucose occurs with the simultaneous administration of glucose and MCFA. Consequently, this increased availability would inhibit fatty acid oxidation and might lead to increased elongation and *de novo* fatty acid synthesis by the liver. The present data on blood fatty acid composition support this latter idea and are in agreement with the data from a human study, in which the participants received 40 % of their energy from medium-chain TAG in a medium-chain TAG diet[61].

The enhanced blood palmitate detected in the MCFA-fed group could be involved in the reduced GSIS and increased apoptosis in pancreatic islets cells, according to other authors[15,18], as well as in the reduced insulin-induced glucose metabolism in the skeletal muscle[36,50,62]. In fact, insulin action was diminished in isolated skeletal muscle from MCFA-fed rats, indicating that the insulin signalling has been altered; however, further investigations are necessary to evaluate each component from IR/IRS1/IRS2/PI3K/AKT signalling, which may be involved with this process.

Haber *et al.*[63] have shown increased IR and IRS1 tyrosine phosphorylation in isolated pancreatic islets after just 30 min of incubation with palmitate. These researchers proposed that protein acylation could be involved in the palmitate-induced tyrosine phosphorylation. Indeed, protein acylation can induce the activation of a number of intracellular events, such as members of the Src family. When acylated, Src proteins can attach to the cytosolic membrane at specific sites and thereby regulate the activity of enzymes and interations with other proteins downstream of the intracellular signalling that regulates gene expression[15,21].

The activation of the intrinsic tyrosine kinase of the insulin/IGF-1 receptors can induce tyrosine phosphorylation of the cytosolic IRS1 protein, which in turn will induce activation of PI3K/AKT and the mitogen-activated protein kinase pathways. The insulin signalling in pancreatic islets induces a fine tuning of the insulin secretion and the maintenance of cellular survival. The overexpression of IRS1 protein in an insulinoma cell line induced enhanced GSIS[64]. Furthermore, in IRS1-knockout mice, a reduction in GSIS was observed[65]. These findings are in accordance with the present results and reinforce the notion of an imbalance in insulin signalling in pancreatic islets from MCFA-fed rats.

AKT_1, a downstream protein of IRS1/IRS2/PI3K signalling, is associated with cellular differentiation in the endocrine pancreas. In mice hyperexpressing AKT, hypertrophy of the β-cells and improvement of glucose tolerance have been reported[13]. On the other hand, a reduced AKT_1 content was associated with impared GSIS and pancreatic islets mass[66]. The activation of the mitogen-activated protein kinase cascade causes enhanced mitogenic activity, cell proliferation and the differention of a number of cellular types[67,68]. Despite the increased stoichiometric relationship of AKT, the reduced AKT_1 protein levels detected in the MCFA-fed rats could play a role in the reduced survival index of the pancreatic islet cells. Furthermore, the reduced stoichiometric phosphorylation of $ERK_{1/2}$ in the isolated pancreatic islets from the MCFA-fed rats could also play a mechanistic role in the reduced size of the pancreatic islets, the increased apoptosis index and the insulin secretion observed in other experimental data[69,70]. To reinforce this idea, enhanced $ERK_{1/2}$ signalling has been associated with an improvement in the pancreatic islet mass and sensitivity to glucose during pregnancy[68]. Of note, homeostasis and cellular survival of the pancreatic islets is dependent on cell replication, cell size, neogenesis and apoptosis[71,72].

In summary, to the best of our knowledge, the present study is the first to show that an isoenergetic MCFA diet is able to induce profound alterations in body growth and GSIS. The initial use of MCFA diets in young prepubertal male rats can lead to a reduced body growth phenotype associated with insulin resistance, diminished insulin-induced glucose uptake in the skeletal muscle and reduced pancreatic islets mass, probably associated with poor $ERK_{1/2}$ phosphorylation/activity. Furthermore, the mildly increased cholinergic sensitivity to GSIS might be associated with the enhanced PKC protein expression, leading to a compensatory hyperinsulinaemic status.

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