Cafeteria diet inhibits insulin clearance by reduced insulin-degrading enzyme expression and mRNA splicing

P Brandimarti*, J M Costa-Júnior*, S M Ferreira, A O Protzek, G J Santos, E M Carneiro, A C Boschero and L F Rezende

Department of Structural and Functional Biology, Institute of Biology, State University of Campinas (UNICAMP), PO Box 6109, Campinas, SP, CEP 13083-865, Brazil

*(P Brandimarti and J M Costa-Júnior contributed equally to this work.)

Correspondence should be addressed to L F Rezende
Email
luizbio@gmail.com

Abstract

Insulin clearance plays a major role in glucose homeostasis and insulin sensitivity in physiological and/or pathological conditions, such as obesity-induced type 2 diabetes as well as diet-induced obesity. The aim of the present work was to evaluate cafeteria diet-induced obesity-induced changes in insulin clearance and to explain the mechanisms underlying these possible changes. Female Swiss mice were fed either a standard chow diet (CTL) or a cafeteria diet (CAF) for 8 weeks, after which we performed glucose tolerance tests, insulin tolerance tests, insulin dynamics, and insulin clearance tests. We then isolated pancreatic islets for *ex vivo* glucose-stimulated insulin secretion as well as liver, gastrocnemius, visceral adipose tissue, and hypothalamus for subsequent protein analysis by western blot and determination of mRNA levels by real-time RT-PCR. The cafeteria diet induced insulin resistance, glucose intolerance, and increased insulin secretion and total insulin content. More importantly, mice that were fed a cafeteria diet demonstrated reduced insulin clearance and decay rate as well as reduced insulin-degrading enzyme (IDE) protein and mRNA levels in liver and skeletal muscle compared with the control animals. Furthermore, the cafeteria diet reduced IDE expression and alternative splicing in the liver and skeletal muscle of mice. In conclusion, a cafeteria diet impairs glucose homeostasis by reducing insulin sensitivity, but it also reduces insulin clearance by reducing IDE expression and alternative splicing in mouse liver; however, whether this mechanism contributes to the glucose intolerance or helps to ameliorate it remains unclear.

Key Words

- cafeteria diet
- insulin-degrading enzyme
- insulin clearance
- obesity
- diabetes

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Introduction

Type 2 diabetes is a complex illness mainly characterized by hyperglycemia, usually accompanied by pancreatic islets malfunction, and reduced insulin sensitivity, as well as lower insulin clearance.

Although hyperglycemia is one of the main elements for diagnosis of type 2 diabetes, the alterations in insulin resistance, secretion, and clearance vary according to disease development. At onset of type 2 diabetes, there is an increase in insulin resistance, coupled with increased pancreatic islet β-cell insulin secretion and reduced insulin clearance, leading to a normoglycemic/hyperinsulinemic state. Eventually, the pancreatic islet β-cells undergo a

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degenerative process that culminates with their death by apoptosis and subsequent reduction in glucose-induced insulin secretion (Chandra *et al.* 2001).

As for insulin clearance, it seems to be mostly controlled upon degradation by the liver and plays a critical role in glycemic control (Mckeigue *et al.* 1991, Kotronen *et al.* 2008). Also, in hepatocytes, insulin degradation is mediated primarily by the insulin-degrading enzyme (IDE; Valera Mora *et al.* 2003).

While increased insulin secretion and reduced insulin sensitivity have been extensively described and studied in obese individuals and animals, the insulin clearance process is supposedly at least as important to obesity-induced type 2 diabetes as other causes (Butterfield 1970, Nijs *et al.* 1990, Kotronen *et al.* 2008).

Recently, these remarks have been reinforced by a series of articles showing that the reduction in insulin clearance during weight gain is probably more relevant than the increase in insulin secretion for the increased insulinemia (Erdmann *et al.* 2008, 2009). Moreover, insulin clearance is a highly heritable trait (Guo *et al.* 2012) with the highest correlation with insulinemia (Goodarzi *et al.* 2011).

Despite all this evidence for the importance of insulin clearance to the obesity-induced type 2 diabetes, the effect of cafeteria diet has never been described or investigated in any animal model. This dietary intervention more closely resembles Western feeding habits (Rothwell & Stock 1979, Mittelman *et al.* 2000) and is clearly more efficient in inducing insulin resistance, glucose intolerance, and inflammation (Sampey *et al.* 2011) than the conventional lipid supplementation diet. Therefore, the aim of the present work was to investigate the possible effects of obesity-induced type 2 diabetes on insulin clearance in mice fed a cafeteria diet.

Materials and methods

Reagents

Anti-IDE and anti-GAPDH (Santa Cruz Biotechnology) primary antibodies were used for western blotting.

Animals and experimental design

Female Swiss mice that were 10–12 weeks old (Unib:SW strain) were acquired from the State University of Campinas and were maintained on a 12 h light:12 h darkness cycle at 20–21 °C with controlled humidity during the entire experiment. The mice were fed either a standard chow diet and offered tap water and allowed to feed and drink *ad libitum* (control) or offered a cafeteria diet as described previously and allowed to feed *ad libitum* for 8 weeks (Vanzela *et al.* 2010). After this period, the animals were killed in a CO_2-saturated atmosphere immediately followed by decapitation. The animal procedures were performed according to the guidelines of the State University of Campinas Animal Care Ethics Committee. All the experiments adhered to ACSM recommendations and were approved by the State University of Campinas Ethics Committee.

Tissue samples

Liver, gastrocnemius muscle, visceral adipose tissue, and hypothalamus samples from the Swiss mice were extracted, snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent protein and mRNA extraction. Pancreatic islets were isolated from mice with collagenase as described previously (Rezende *et al.* 2009).

Western blot

Western blots were performed as described previously (Santos *et al.* 2011).

Real-time RT-PCR

Extracts from mouse tissues that had been previously stored as described were homogenized in TRIzol and phenol/chloroform RNA extracted according to the manufacturer's instructions (Gibco-BRL). To evaluate mRNA levels and search for alternatively spliced transcript variants of different sizes, RT-PCR was performed using cDNAs as templates with TaqDNA polymerase (Invitrogen/Life Sciences) and corresponding primers 14F, 15a-R (higher IDE activity), and 15b-R (lower IDE activity) used as described previously (Kim *et al.* 2007, Pivovarova *et al.* 2009). Relative target transcript quantities were calculated from duplicate samples, and the data were normalized to the endogenous control β-actin. β-Actin F, 5′-AGAGGGAAATCGTGCGT-GACA-3′ and R, 5′-CGATAGTGATGACCTGACCGTCA-3′; IDE 14F, 5′-CCGAAGGCTTGTCTCAACTT-3′; IDE 15a-R, 5′-ATACATCCCATAGATGGTATTTTGG-3′; and IDE 15b-R, 5′-TGCATTCATTCCTGATGCAATGC-3′.

Pancreatic islet glucose-stimulated insulin secretion

Four batches of ten islets each were pre-incubated for 1 h in Krebs–Henseleit buffer solution (KHBS) containing

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0.5 g/l BSA and 2.8 mmol/l glucose and were equilibrated with 95% O_2 and 5% CO_2 at 37 °C. The media were discarded and the islets were incubated for an additional hour in 1 ml KHBS containing 2.8, 11.2, or 22.4 mmol/l glucose. The supernatant was subsequently collected to evaluate insulin secretion, and the remaining islets were homogenized in an alcohol/acid solution to measure the total insulin content using a RIA.

Intraperitoneal glucose tolerance test

Swiss mice received an i.p. glucose injection (1 g/kg in 0.9% NaCl) after an 8-h fast. Blood samples (75–100 µl) were collected from the tail immediately before the injection and 15, 30, 45, and 120 min following the injection to determine the concentrations of glucose and insulin. Glucose was evaluated with glucose strips on an Accu-Chek Performa II instrument (Roche), and insulin was measured using a RIA as described previously (Rezende *et al.* 2007).

Intraperitoneal insulin tolerance test

Non-fasted Swiss mice received an i.p. insulin injection (1 U/kg). Blood glucose was measured using test strips (Accu-Chek Performa II) at baseline (0 min, before insulin administration) and 5, 10, 15, 20, and 30 min after the administration of insulin. Glucose measurements were then converted into natural logarithm (Ln); the slope was calculated using linear regression (time×Ln(glucose)) and multiplied by 100 to obtain the glucose decay constant rate during the insulin tolerance test (k_{ITT}) per minute (%/min).

In vivo insulin clearance

We evaluated the concentrations of plasma insulin in Swiss mice that had been submitted to an intraperitoneal ITT. Insulin clearance was evaluated as described previously (Ahrén *et al.* 2005). The constant rate for insulin disappearance (insulin decay) was calculated by first converting the insulin measurements into natural logarithm (Ln); the slope was calculated using linear regression (time×Ln(insulin)) and multiplied by 100 to obtain the insulin decay constant rate per minute (%/min). We also calculated the area under the curve (AUC) of the insulin concentrations during the experiment.

Statistical analysis

Point-to-point comparisons were performed using the ANOVA test. When the same animals were evaluated at different time points, we applied the repeated measures ANOVA test with the unpaired Tukey–Kramer post-test. The results were considered significantly different for $P<0.05$. In the RT-PCR experiments, the results were considered different only for $P<0.01$.

Results

Food component intake and metabolic variables of mice fed a cafeteria diet

Table 1 demonstrates that the cafeteria diet indeed differed from the control diet. The cafeteria diet had an approximate twofold enhancement in caloric intake that originated from the lipid content, which was fourfold increased, and carbohydrates. However, the ingestion of other dietary components remained unchanged, thus evidencing the efficiency of the dietary intervention.

As expected, the mice that were fed a cafeteria diet also had increased body and visceral fat pad weights as well as higher plasma insulin and glucose values than mice fed the control diet.

Cafeteria diet-mediated effect on mouse insulin sensitivity

Cafeteria diet-fed mice had an impaired ITT (Fig. 1A), an increased AUC during the glucose tolerance test (GTT; Fig. 1B), and reduced k_{ITT} (Fig. 1C). Taken together, these alterations suggest that the cafeteria diet induced insulin resistance.

Table 1 Intake and metabolic variables. Food component intake and metabolic variables of mice fed the cafeteria diet. Energy intake was calculated based on daily food intake and nominal calorie content of each individual item in the offered diet. Values were expressed as the mean±S.E.M., $n=6$. NEFA, non-esterified fatty acids; TGs, triglycerides.

	Control	Cafeteria
Energy intake (kcal/kg per day)	671.4±85.5	1084.7±180.7*
Carbohydrate intake (g/kg per day)	123.7±15.8	157.3±11.1*
Protein intake (g/kg per day)	35.3±4.5	32.2±3.3
Lipid intake (g/kg per day)	17.7±2.3	60.3±6.2*
Fasting insulin (pmol/l)	0.8±0.1	2.6±0.2*
Fasting glycemia (mmol/l)	6.7±0.2	8.0±0.3*
Body weight (g)	38.8±1.0	47.5±2.3*
Visceral fat pad weight (g)	1.34±0.09	3.15±0.74*
NEFA (µmol/l)	708.6±98.6	1246.9±143.1*
TGs (mg/dl)	38.3±8.1	56.6±7.7*

*$P\leq 0.05$ was significantly different from the control.

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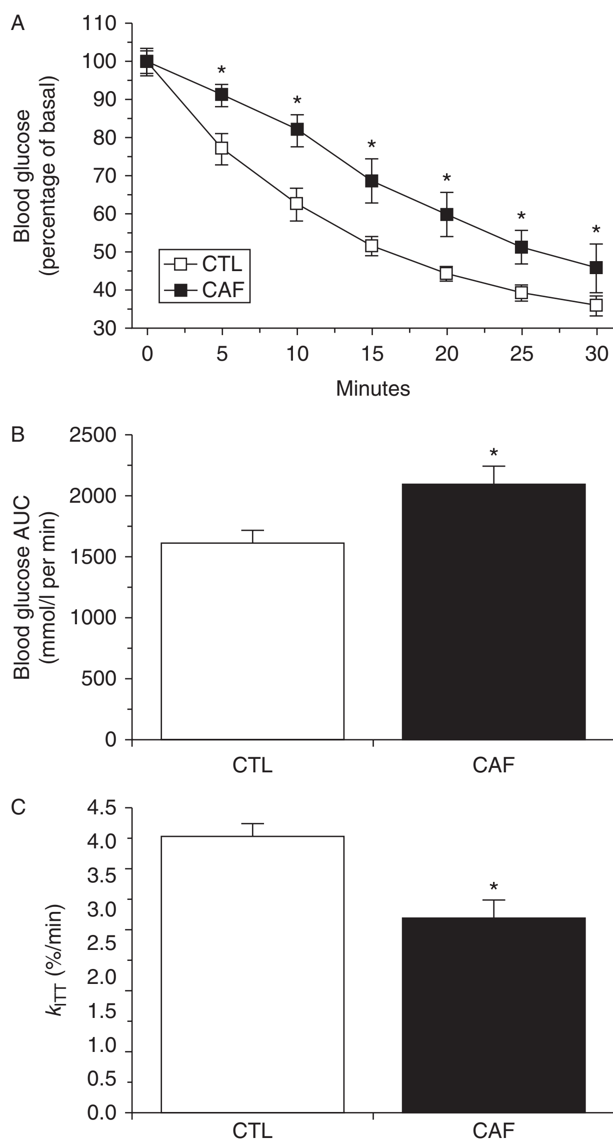

Figure 1
Cafeteria-diet-mediated effects on mouse insulin sensitivity. ITT (A), glucose AUC during the ITT (B), and k_{ITT} (C) of the control (CTL) and cafeteria diet (CAF)-fed mice. The values are expressed as the mean±S.E.M., $n=6$. *$P\leq 0.05$ was considered significantly different from the control.

Cafeteria diet-mediated effect on glucose tolerance and insulin dynamics in mice

Cafeteria diet-fed mice were more glucose intolerant than mice that were fed a control diet, as evidenced by the intraperitoneal GTT (Fig. 2A) and increased plasma glucose AUC during the GTT (Fig. 2B). This glucose intolerance was accompanied by drastic changes in the insulin dynamics following the glucose challenge. Plasma insulin increased faster and to a higher degree and was maintained longer in the cafeteria diet-fed mice compared with the control group (Fig. 2C), which resulted in increased exposure of these mice to insulin after the glucose challenge, as evidenced by the AUC of insulin during the GTT (Fig. 2D).

Cafeteria diet-mediated effects on mouse insulin secretion and pancreatic islet content

The increased insulin during the GTT could be explained by alterations in insulin release and/or in insulin removal. The mice fed a cafeteria diet demonstrated overall increased glucose-stimulated insulin secretion (GSIS; Fig. 3A) and increased total insulin content (Fig. 3B). The insulin secretion was increased even after normalization by total insulin content (Fig. 3C).

Cafeteria diet-mediated effect on insulin clearance in mice

Although the increased insulin secretion could at least partially explain the rapid and severe increase in insulinemia during the GTT, it was not sufficient for explaining the slower decay after reaching the peak. The cafeteria diet inhibited insulin clearance in mice, as evidenced by the insulin clearance test (Fig. 4A) and reduced insulin decay during the ITT (Fig. 4B), which resulted in increased insulin exposure during the test, as evidenced by an increase in the insulin AUC during the ITT (Fig. 4C).

Cafeteria diet-mediated effect on IDE protein levels in mice

To explain the reduced insulin clearance in mice fed a cafeteria diet, we investigated the IDE expression in several tissues. The IDE protein levels were reduced in the liver (Fig. 5A) and the skeletal muscle (Fig. 5B), were unchanged in adipose tissue (Fig. 5C), and were increased in the hypothalamus (Fig. 5D).

Cafeteria diet-mediated effect on *Ide* mRNA levels and alternative splicing in mice

Another factor that contributes to the control of insulin clearance is the alternative splicing of different *Ide* mRNA isoforms (Farris *et al.* 2005). Cafeteria diet-fed mice had lower total *Ide* mRNA levels and reduced higher- and lower-activity IDE alternative isoforms in the liver (Fig. 6A) and skeletal muscle (Fig. 6B), although it reduced the higher-activity isoform to a greater extent in both organs. The cafeteria diet had no effect on the adipose tissue

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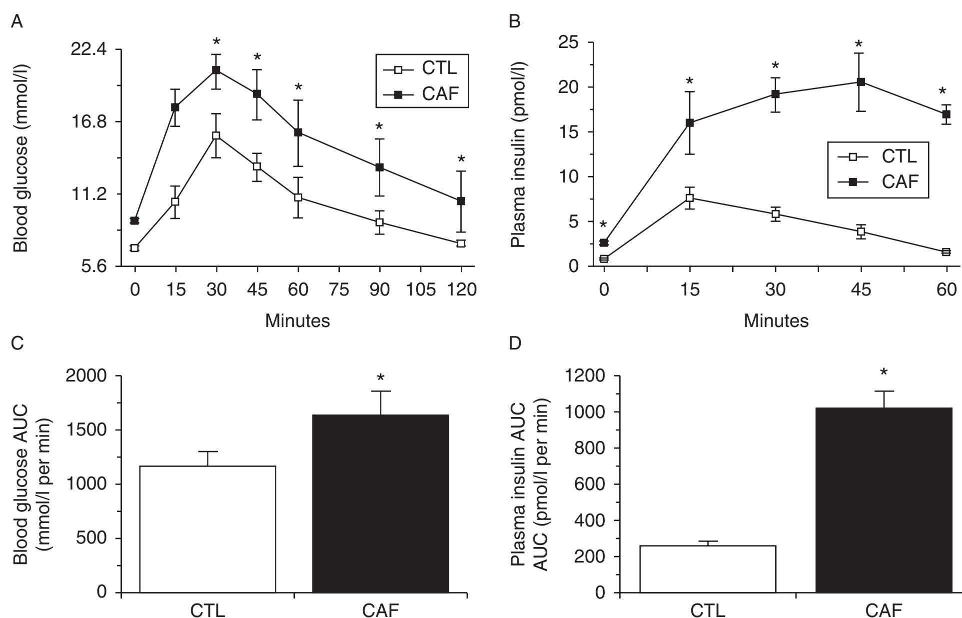

Figure 2
Cafeteria-diet-induced effect on glucose tolerance and insulin dynamics in mice. Intraperitoneal GTT test (A), and plasma glucose AUC during the GTT (B), plasma insulin during the GTT (C), and plasma insulin AUC during the GTT (D) of the control (CTL) and cafeteria diet (CAF)-fed mice. The values are expressed as the mean ± s.e.m., $n=6$. $*P \leq 0.05$ was considered significantly different from the control.

Ide mRNA levels (Fig. 6C), but it increased the total, high-, and low-activity *Ide* mRNA levels in the hypothalamus (Fig. 6D).

Discussion

Hyperglycemia is the primary variable associated with the diagnosis of type 2 diabetes, and perfect control of blood glucose by insulin depends on its plasma concentration (insulinemia) as well as its actions on peripheral tissues that cause glucose uptake (insulin sensitivity), thus lowering plasma glucose concentrations. Insulinemia is the result of a balance between the insulin produced and secreted by pancreatic islet β-cells and the rate that insulin is removed from plasma or insulin clearance (Duckworth *et al.* 1998).

Insulin clearance is fundamentally controlled by the liver, and ~50% of secreted insulin is removed by the organ after the second passage through the portal vein (Butterfield 1970, Mittelman *et al.* 2000, Kotronen *et al.* 2008). Hepatic insulin removal and degradation is mainly controlled by a 130 kDa zinc-metalloproteinase known as IDE (Duckworth 1988, Duckworth *et al.* 1998, Amata *et al.* 2009, Fernández-Gamba *et al.* 2009).

Although virtually every insulin-responsive cell expresses IDE, its main function in these organs is to oppose insulin signaling by uncoupling insulin from the insulin receptor (IR), either by simply removing or partially or completely degrading insulin (Duckworth 1988, Duckworth *et al.* 1998, Amata *et al.* 2009). Therefore, it is not surprising that hepatic IDE inhibition increased insulin sensitivity coupled with increased IR activity (Kuo *et al.* 1991, Li *et al.* 2002, Leissring *et al.* 2010).

Thus, IDE expression and action accomplish a dual role in glucose homeostasis. On the one hand, IDE reduces insulinemia by increasing plasma insulin removal in the liver, and conversely, IDE reduces insulin sensitivity by rapidly interrupting the IR signaling pathway. Given the importance of insulin clearance and IDE expression to glucose homeostasis, it is not surprising that both are altered in obesity and type 2 diabetes.

Insulin clearance and IDE expression are reduced in type 2 diabetic individuals (Butterfield 1970, Groves *et al.* 2003, Karamohamed *et al.* 2003, Kotronen *et al.* 2007, 2008, Kwak *et al.* 2008, Rudovich *et al.* 2009, Slominskiĭ *et al.* 2009) and in obese and diabetic rodents (McCarroll & Buchanan 1973, Rabkin *et al.* 1986, Strömblad & Björntorp 1986, Kotronen *et al.* 2008, Matveyenko *et al.* 2008, Rezende *et al.* 2012). Also, as previously mentioned, changes in insulin clearance are probably more relevant than the increase in insulin secretion to the higher insulinemia observed in obese individuals

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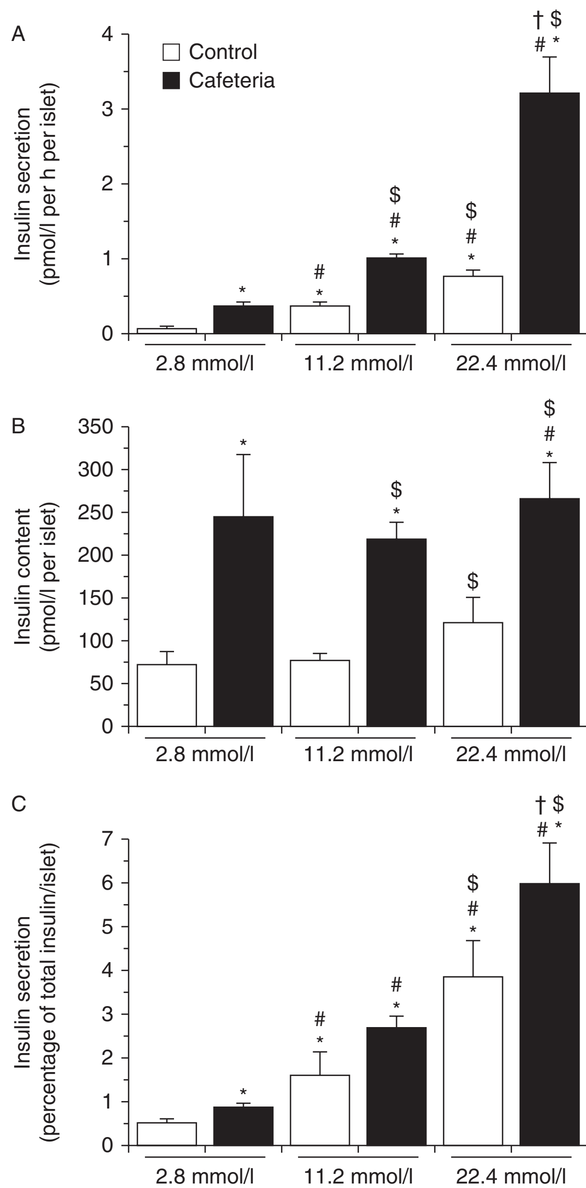

Figure 3
Cafeteria-diet-induced effect on insulin secretion and pancreatic islet content in mice. GSIS (A), total insulin content (B), and secreted insulin as a fraction of total insulin content (C) in sub-stimulatory (2.8 mmol/l), stimulatory (11.2 mmol/l), and supra-stimulatory (16.7 mmol/l) glucose concentrations from isolated pancreatic islets that were assessed *ex vivo* from control (CTL) and cafeteria diet (CAF)-fed mice. The values are expressed as the mean ± S.E.M., $n=6$. $^{*}P\leq0.05$ significantly different from the control 2.8 value, $^{\#}P\leq0.05$ significantly different from the respective control value, $^{\$}P\leq0.05$ significantly different from the control 11.2 value, and $^{\dagger}P\leq0.05$ significantly different from the control 22.4 value.

(Erdmann *et al.* 2008, 2009) and are highly heritable (Guo *et al.* 2012) with the highest correlation with insulinemia (Goodarzi *et al.* 2011).

The drawbacks of these studies are that most rodent obesity models involve an altered genetic background, such as *ob/ob* mice, or are diet-induced. However, lipid supplementation of food does not correlate well with actual human Western feeding habits and is also less palatable, thus reducing food intake by the mice (Rothwell & Stock 1979).

In this sense, the cafeteria diet is a better model for studying glucose homeostasis in rodents because this diet more closely resembles actual Western society feeding habits, is still able to induce obesity, and is more palatable, which increases overall food intake (Sclafani & Springer 1976, Rothwell & Stock 1979, Prada *et al.* 2005). We

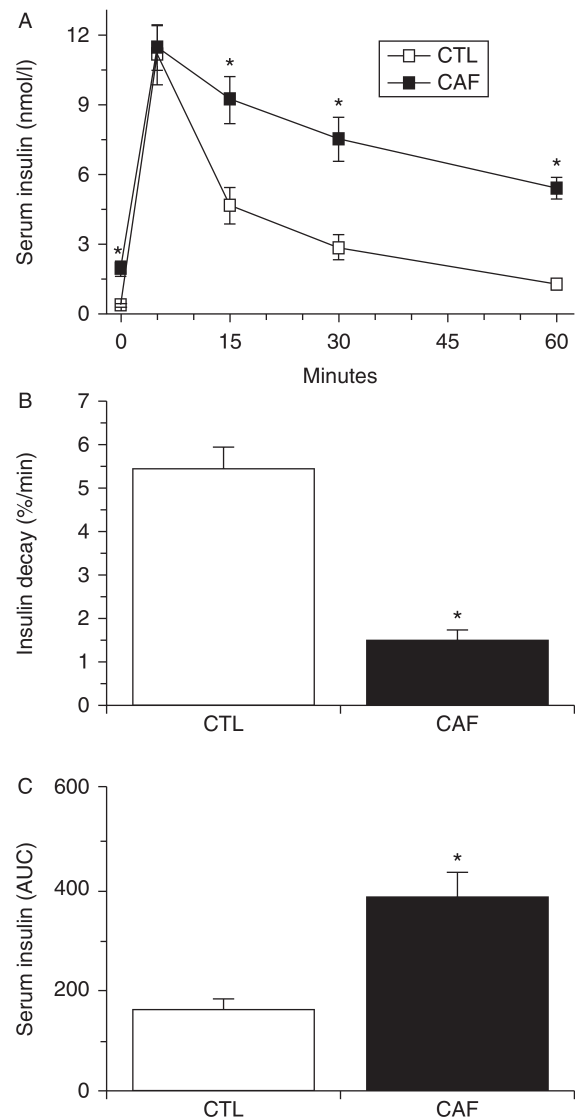

Figure 4
Cafeteria-diet-induced effect on insulin clearance in mice. Insulin clearance test (A), insulin decay rate during ITT (B), and insulin AUC during ITT (C) of control (CTL) and cafeteria diet (CAF)-fed mice. The values are expressed as the mean ± S.E.M., $n=6$. $^{*}P\leq0.05$ significantly different from the control.

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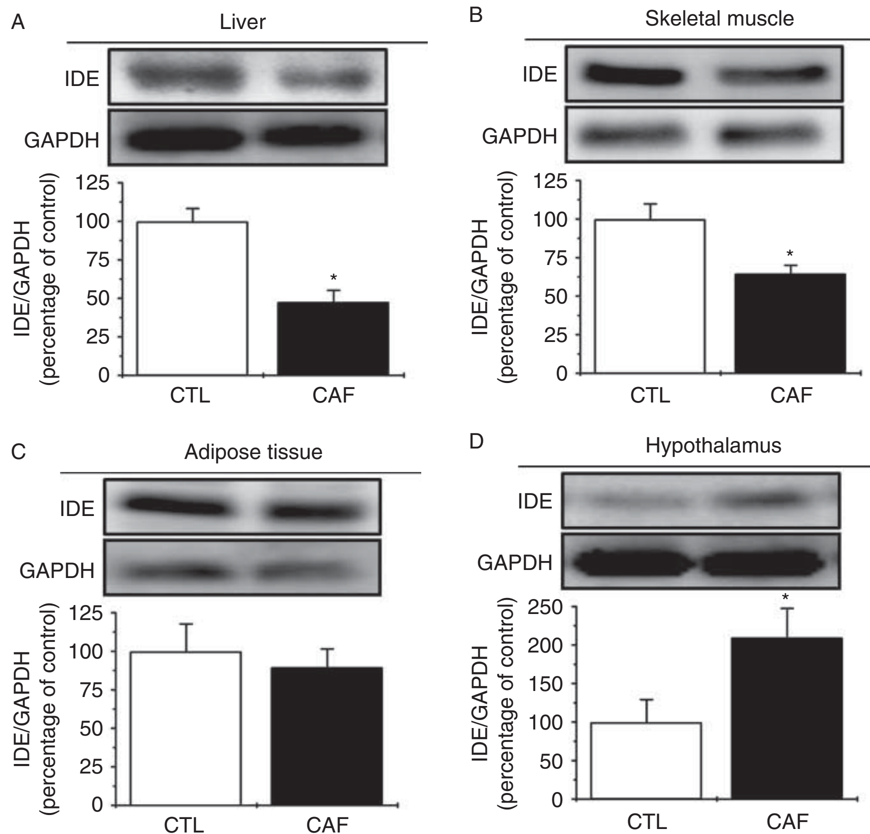

Figure 5
Cafeteria-diet-induced effect on IDE protein levels in mice. IDE protein levels in liver extracts (A), gastrocnemius muscle (B), visceral adipose tissue (C), and hypothalamus (D) from control (CTL) and cafeteria diet (CAF)-fed mice. The values are expressed as the mean ± S.E.M., $n=6$. *$P \leq 0.05$ was considered significantly different from the control.

determined that 8 weeks of cafeteria diet feeding in mice increased body weight, fat pad weight, triglycerides (TGs), and non-esterified fatty acids (NEFAs) (Table 1), most probably because of the increased overall food intake that is associated with increased calorie, lipid, and carbohydrate intake (Table 1). These changes were followed by increased glycemia (Table 1), insulin resistance (Fig. 1), glucose intolerance (Fig. 2), insulin secretion (Fig. 3), and reduced insulin clearance (Fig. 4).

Insulin resistance is a well-described feature of obese mice that is usually associated with reduced IR–AKT pathway activity, which is the canonical signaling pathway in most insulin-responsive organs, such as the liver, skeletal muscle, and adipose tissue (Rezende *et al.* 2012). However, the increase in insulin content and secretion from pancreatic islets involve changes in calcium influx in the pancreatic islets of cafeteria-diet-fed rats (Vanzela *et al.* 2010).

Here, we show that reduced insulin clearance is associated with altered insulin sensitivity and secretion (Fig. 4), most probably because of reduced hepatic IDE expression (Fig. 5). Furthermore, the cafeteria diet reduced high-activity rather than low-activity *Ide* mRNA, which effectively displaced the balance of high/low-activity IDE in favor of low-activity IDE in the liver and skeletal muscle (Fig. 6).

Despite not being particularly relevant to the insulin clearance process, other insulin-responsive tissues play a pivotal role in glucose homeostasis and expresses IDE, so we also evaluated IDE expression and alternative splicing in skeletal muscle, adipose tissue, and hypothalamus. IDE expression was also reduced in skeletal muscle, which is consistent with the pattern observed in liver and could possibly act as a safety mechanism to maintain some insulin effectiveness despite the insulin resistance, while IDE is unchanged in adipose tissue, an effect that has already been described in rats (Castell-Auví *et al.* 2012) despite the differences in duration of diet and methods for evaluation of insulin clearance.

The increased IDE expression in the hypothalamus probably helps to explain the hyperphagia in mice fed a

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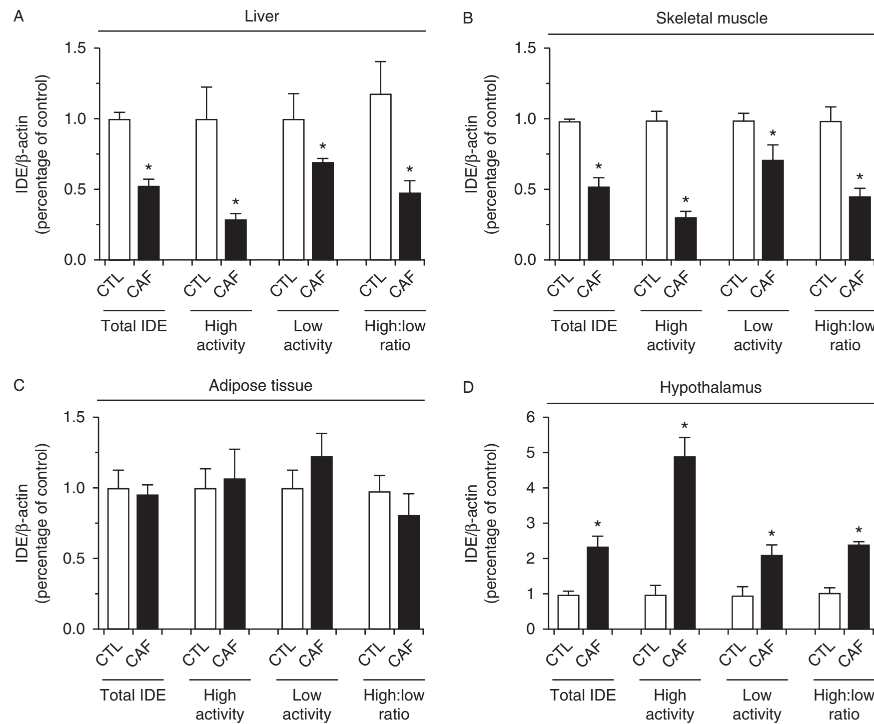

Figure 6

Cafeteria-diet-induced effect on *Ide* mRNA levels and alternative splicing in mice. Total, high-activity, and low-activity *Ide* mRNA transcript levels and ratios from liver (A), gastrocnemius muscle (B), visceral adipose tissue (C), and hypothalamus (D) of control (CTL) and cafeteria diet (CAF)-fed mice. The values expressed as the mean ± s.e.m., $n=6$. $*P\leq0.01$ was considered significantly different from the control value.

cafeteria diet, given that increased IDE expression would reduce the anorexigenic insulin effects on the hypothalamus (Porte *et al.* 2005), thus increasing the overall food intake.

The insulin clearance in cafeteria diet-fed rats has already been reported (Castell-Auví *et al.* 2012), but the discrepancies between our work and theirs might be attributed to a number of causes. First, that work involved rats while our study involved mice, and these species have differences in their lipid metabolism (Castell-Auví *et al.* 2012), which might help explain the differences between these two studies. Secondly, their experiment was lengthier and it has been already described that in dogs the time-course changes in insulin clearance do not follow a linear pattern and do vary according to the extent of the dietary intervention (Mittelman *et al.* 2000). Finally, in that study, the authors suggest increased IDE-mediated insulin clearance capability, as they found increased hepatic IDE activity. Although changes in IDE expression and activity are indeed the major contributing factors to alterations in insulin clearance, insulin clearance can also be affected by, for example, changes in renal blood flow or other physiological conditions (Duckworth 1988, Duckworth *et al.* 1998). Hepatic IDE expression from cafeteria-diet-fed mice was most probably reduced because of the increased plasma TG and NEFA levels, as both of these inhibit insulin clearance and hepatic IDE expression (Wiesenthal *et al.* 1999, Balent *et al.* 2002, Hamel *et al.* 2003, Yoshii *et al.* 2006, Kotronen *et al.* 2007, 2008).

Another possibility is that cytokines, such as interleukin 6 (IL6), that are released by the oversized adipose tissue might control insulin clearance and hepatic IDE expression. Supporting this hypothesis is the evidence that ciliary neurotrophic factor (CNTF), a cytokine in the IL6 family, reduces insulin clearance and liver IDE expression in non-obese type 2 diabetic mice, and it also inhibits IDE expression and activity in hepatocytes *in vitro* (Rezende *et al.* 2012).

In conclusion, we demonstrated that in addition to increased insulin resistance and insulin secretion, cafeteria diet-induced obese mice also present with reduced insulin clearance that most probably occurs via hepatic IDE expression downregulation in these mice, which might explain the high correlation between reduced insulin clearance and both obesity and type 2 diabetes in humans, possibly as a consequence of the western feeding habits.

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Also, it indicates liver IDE to be a good candidate for a targeted therapeutic approach for obesity-induced type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P B, J M C, and L F R were responsible for the study design and P B, J M C, A O P, G J S, and L F R for data acquisition and analysis. E M C, A O P, and A C B contributed to data analysis and interpretation. P B, J M C, and L F R wrote the manuscript. E M C and A C B revised the manuscript. All the authors approved the final version.

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