Anti-obesity Effect of *n*-3 Polyunsaturated Fatty Acids in Mice Fed High-Fat Diet Is Independent of Cold-Induced Thermogenesis

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Summary

Long-chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) exert beneficial effects on health and they could help to prevent development of obesity and associated metabolic disorders. In our previous studies in mice fed high-fat (cHF; ~60% calories as fat) diet and maintained at 20°C, dietary LC *n*-3 PUFA could counteract accretion of body fat, without inducing mitochondrial uncoupling protein 1 (UCP1) in adipose tissue, suggesting that the anti-obesity effect was not linked to adaptive (UCP1-mediated) thermogenesis. To exclude a possible dependence of the anti-obesity effect on any mechanism inducible by cold, experiments were repeated in mice maintained at thermoneutrality (30°C). Male C57BL/6J mice were fed either cHF diet, or cHF diet supplemented with LC *n*-3 PUFA, or standard diet for 7 months. Similarly as at 20°C, the LC *n*-3 PUFA supplementation reduced accumulation of body fat, preserved lipid and glucose homeostasis, and induced fatty acid re-esterification in epididymal white adipose tissue. Food consumption was not affected by LC *n*-3 PUFA intake. Our results demonstrated anti-obesity metabolic effect of LC *n*-3 PUFA, independent of cold-induced thermogenesis and they suggested that induction of fatty acid re-esterification creating a substrate cycle in white fat, which results in energy expenditure, could contribute to the anti-obesity effect.

Key words

Marine lipids, obesity, thermoneutrality, indirect calorimetry, metabolic syndrome

Introduction

Epidemy of obesity triggered intense research of inducible metabolic mechanisms, which could counteract accumulation of body fat. Thus, traditional research of adaptable cold- and diet-induced thermogenesis mediated by mitochondrial uncoupling protein 1 [**UCP1;** (Klaus *et al*. 1991, Nedergaard *et al*. 2005, Nicholls *et al*. 1984)] has been revived reflecting also the discovery of functional brown adipose tissue (**BAT**) in adult humans (Cypess *et al*. 2009, Lichtenbelt *et al*. 2009, Nedergaard *et al*. 2010, Virtanen *et al*. 2009, Zingaretti *et al*. 2009), as well as the negative correlation between BAT content and body weight in humans (Saito *et al*. 2009, Zingaretti *et al*. 2009). Nevertheless, several studies suggest that UCP1-independent thermogenesis also exists, which could be recruited by various treatments reducing obesity (Cannon *et al*. 2004, Chen *et al*. 2010, Granneman *et al*. 2003, Guan *et al*. 2002, Kozak 2010, Kus *et al*. 2008, Langin 2010, Meyer *et al*. 2010, Summermatter *et al*. 2008).

Long-chain *n*-3 polyunsaturated fatty acids (**LC *n*-3 PUFA**) of marine origin, namely eicosapentaenoic acid (**EPA**; 20:5 n-3) and docosahexaenoic acid (**DHA**; 22:6 n-3) exert numerous beneficial effects on health, including improvements in lipid metabolism and prevention of obesity and diabetes [reviewed in (Flachs *et al*. 2009)]. These effects are well documented in our previous studies, using a model of metabolic syndrome in dietary obese mice (Flachs *et al*. 2011, Hensler *et al*. 2011, Jelenik *et al*. 2010, Kuda *et al*. 2009, Kus *et al*. 2011, Rossmeisl *et al*. 2012, van Schothorst *et al*. 2009), which have also demonstrated that LC *n*-3 PUFA could increase mitochondrial oxidative capacity specifically in white adipose tissue (**WAT**) and not in BAT, skeletal

muscle or liver (Flachs *et al*. 2005). This induction was augmented by calorie restriction (Flachs *et al*. 2011). Importantly, no up-regulation of UCP1 gene in adipose tissue could be observed (Flachs *et al*. 2011, Flachs *et al*. 2005). Instead, our results suggested the involvement of fatty acid (**FA**) re-esterification in WAT in the anti-obesity effect of the combined use of LC *n*-3 PUFA and calorie restriction (Flachs *et al*. 2011). All the above studies were conducted in mice maintained at 20°C, i.e. under the conditions activating inherent mechanisms of metabolic cold defense, since thermoneutral zone in mice is close to 30°C (Alberts *et al*. 2005, Cannon *et al*. 2004). Therefore, a possibility existed that the induction of the catabolic processes by LC *n*-3 PUFA, which resulted in energy expenditure and obesity resistance, reflected mechanisms independent of UCP1, but activated by the cold exposure. Results of this study document, that dietary intervention with LC *n*-3 PUFA could counteract accumulation of body fat even at thermoneutrality, independent of the mechanisms underlying cold-induced thermogenesis.

Methods

Animals and treatments

C57BL/6J (B/6J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the Institute of Physiology for several generations. Male mice born and maintained at 20°C on a 12:12-hr light-dark cycle were weaned at 4 weeks of age to either the standard low-fat (**ST**) or high-fat (**cHF**) diet, while the ambient temperature was increased to 30°C and this temperature was maintained until the end of the experiment (with 4 mice per cage). ST diet (Velaz, Prague, Czech Republic)

contained 21, 3, and 56% calories as protein, fat, and carbohydrate, respectively. The cHF diet, proven to be obesogenic in B/6J mice, contained 15, 59, and 26% calories as protein, fat, and carbohydrate, respectively [see (Kuda *et al*. 2009)]. In some animals, cHF diet was supplemented with EPA and DHA (**cHF+F**), added as a concentrate of LC *n*-3 PUFA (46% DHA, 14% EPA; EPAX 1050 TG, EPAX a.s., Lysaker, Norway), which replaced 15% of dietary lipids (specifically, 5.25 g of corn oil/100 g cHF diet). Thus, 5.3% of total energy content in the LC *n*-3 PUFA-supplemented diet came from EPA and DHA. In contrast with cHF+F, both ST and cHF diet were virtually free of any LC *n*-3 PUFA [see our previous publication (Kuda *et al*. 2009) for the composition of FA in lipids in both cHF-based diets].

Body weight of each mouse was monitored weekly. Food intake of the group of 4 mice in each cage during a 24-hr period was assessed four times per experiment (at 2, 3, 4 and 7 months of age), and averaged per mouse for the whole period of the dietary intervention (i.e., from the time of weaning to 8 months of age). Mice were killed at 8 month of age in *ad libitum* fed state, by decapitation between 10:00 and 12:00 a.m. EDTA-plasma was prepared from truncal blood and stored at – 70°C. Subcutaneous (dorsolumbar) and epididymal WAT were dissected.

All experiments were performed in accordance with the guidelines for the use and care of laboratory animals of the Institute of Physiology, the directive of the European Communities Council (2010/63/EU), and the *Principles of Laboratory Animal Care* (NIH publication no. 85-23, revised 1985).

Glucose tolerance test

Two weeks before the end of the experiment, an intraperitoneal glucose tolerance test (**IP GTT**) was performed at ambient temperature of 30°C, in overnight-fasted mice as described before (Rossmeisl *et al*. 2009).

Indirect calorimetry

To evaluate energy expenditure, indirect calorimetry was performed using a system from Somedic [Horby, Sweden; refs. (Flachs *et al*. 2011, Kus *et al*. 2008)] at 6 months of age. Briefly, the measurements were performed in individually caged mice (Eurostandard type II mouse plastic cages; ~ 6,000 ml; Techniplast, Milan, Italy), with the cages placed in a sealed measuring chamber equipped with thermostatically controlled heat exchangers at 30°C. Oxygen consumption ($\boldsymbol{VO_2}$) and carbon dioxide production ($\boldsymbol{VCO_2}$) were recorded every 2 min under a constant airflow rate (1000 ml/min) for 22 h, starting at 3:00 p.m. The level of substrate partitioning was estimated by calculating respiratory exchange ratio (**RER**; i.e., VCO_2/VO_2 ratio). Percent relative cumulative frequency (**PRCF**) curves were constructed based on RER values pooled from all the animals within a given dietary group (6-8 animals per group) during the whole measurement period (Kus *et al*. 2008).

Metabolite quantification

Non-esterified FA (**NEFA**) and triacylglycerols (**TG**) in EDTA-plasma were assessed as described before (Ruzickova *et al*. 2004).

Ex vivo metabolism of adipose tissue

Basal and insulin-stimulated TG synthesis was quantified as previously described (Pravenec *et al*. 2006). Briefly, distal parts of epididymal adipose tissue (~200 mg aliquots) were incubated in modified Krebs-Ringer bicarbonate buffer containing 4% bovine serum albumin (Fraction V), 5 mM glucose, and 0.1 μCi/ml ^{14}C-glucose in gas phase of 95% O_2 and 5% CO_2 at 37^{o}C. After 2 h incubation without or with insulin (250 μU/ml), the tissue fragments were washed by saline, homogenized in chloroform and thereafter methanol was added in a 2:1 ratio (chloroform:methanol). The lipid extraction proceeded during night at 4^{o}C. For the chloroform phase separation, KH_2PO_4 was added (Folch *et al*. 1957). Water phase of the extract was used for quantification of the incorporation of glucose into total neutral lipids and expressed as nmol of glucose converted into lipid per gram of adipose tissue. Aliquot of the chloroform phase (which was saponificated and subsequently extracted by petrol ether) was used for the determination of ^{14}C-glucose incorporation into acyl groups and was expressed as nmol of glucose converted into lipid per gram of adipose tissue (Pravenec *et al*. 2006). The amount of ^{14}C-glucose incorporated into glycerol residues was calculated as the difference between the total incorporation into neutral lipids and the incorporation into acyl groups, separately for each sample.

Statistics

All values are expressed as means ± SE. Logarithmic transformation was used when necessary. Data were analyzed using Student's t-test or ANOVA (one-way or two-way) with Holm-Sidak method using SigmaStat statistical software. The PRCF curves were analyzed by Nonlinear Regression using SigmaPlot and 50th percentile value (**EC_{50}**)

and Hillslope values were compared in a one-way ANOVA. Statistical significance was defined as $p \leq 0.05$.

Results

Mice, which were maintained at 30°C since weaning (at 4 weeks of age) showed different final body weight at 8 months of age, depending on the type of diet fed during the post-weaning period. Thus, the final body weight of the cHF diet-fed mice was significantly higher as compared with the ST diet-fed mice, while supplementation of the cHF diet with LC *n*-3 PUFA (cHF+F) tended to counteract the cHF diet-induced obesity (Table 1). The differences in body weight could be explained by differences in adiposity. Thus, weight of epididymal fat depot was significantly lower and the weight of dorsolumbar fat depot tended to be lower in the cHF+F group, as compared with the cHF-diet fed mice (Table 1). Calorie intake (measured in groups of 4 mice caged together; see Methods) was significantly higher in the cHF diet-fed as compared with the ST diet-fed mice. However, it was not affected LC *n*-3 PUFA admixed to the cHF diet (Table 1). While plasma levels of TG tended to be elevated and levels of NEFA were significantly increased in response to the cHF diet-feeding, the supplementation of the cHF diet with LC *n*-3 PUFA exerted a protective, anti-hyperlipidaemic effect (Table 1).

To evaluate the effect of the differential dietary treatment on glucose homeostasis, IP GTT was performed at 30°C, two weeks before killing of the mice (Fig. 1). The cHF diet-feeding resulted in increased fasting blood glucose levels, as measured at the beginning of the test, and in deterioration of glucose tolerance, assessed as an incremental

area under the curve (**AUC**), which increased ~1.7-fold (Fig. 1 and Table 1). The supplementation of cHF diet with LC *n*-3 PUFA prevented the adverse effect of cHF diet-feeding on glucose homeostasis, as documented by the normalization of blood glucose levels, and by almost complete prevention of the AUC increase (Fig. 1 and Table 1).

To characterize whole-body metabolism and its changes in response to different diets, indirect calorimetry was used in *ad libitum* fed mice. To avoid any cold stress and similarly as in the case of IP GTT (see above), the measurements were performed at 30°C. The measurements were carried over a 22 h period, i.e., during almost complete light-dark cycle of the day. In the case of VO_2, no significant differences between groups were observed (Table 2). As expected, RER values were lower in both cHF and cHF+F groups as compared with mice fed ST diet (Table 2), in agreement with a relatively high content of lipids in the cHF-based diets and the preferential oxidation of lipid over carbohydrate fuels under these conditions. This analysis also suggested an increase in RER in response to the supplementation of the cHF diet with LC *n*-3 PUFA (Table 2), in agreement with the beneficial effect of LC *n*-3 PUFA on glucose homeostasis and insulin sensitivity (see above). Therefore, a robust analysis of RER was used, while constructing PRCF curves based on all the data pooled from each dietary group (see Methods and Fig. 2). This quantitative approach is capable to detect small differences in fuel partitioning. Provided that PRCF curves represent the normally distributed data, the values of log EC_{50} of PRCF (50^{th} percentile value) correspond to RER values (Kus *et al*. 2008). The PRCF curves shifted to the left in response to both cHF-based diets, and a trend for a difference between the EC_{50} value of the cHF and cHF+F curves was observed, supporting a shift from lipid to carbohydrate oxidation in response to the LC *n*-3 PUFA supplementation.

That the cHF curve was significantly steeper than both the cHF+F and the ST curves suggests (i) a relatively homogeneous distribution of RER values in the cHF diet fed-mice (Kus *et al*. 2008), reflecting a strong drive for oxidation of abundantly supplied dietary lipids, and (ii) that the supplementation of the cHF diet with LC *n*-3 PUFA could unmask an inherent heterogeneity of the mice with respect to the preservation of glucose homeostasis by the LC *n*-3 PUFA supplementation (Fig. 2). In any case, concerning the subtle effects of the LC *n*-3 PUFA supplementation, unequivocal interpretation of the data would require measurements using a larger cohort of the experimental animals.

The above results document that similarly as at 20°C (Flachs *et al*. 2011, Hensler *et al*. 2011, Jelenik *et al*. 2010, Kuda *et al*. 2009, Kus *et al*. 2011, Rossmeisl *et al*. 2012, van Schothorst *et al*. 2009), also at the thermoneutral temperature of 30°C, the LC *n*-3 PUFA supplementation exerts anti-obesity effect, while preserving healthy plasma lipid profile and glucose homeostasis in the animals exposed to obesogenic environment. Since our previous results indicated a surprisingly tissue specific involvement of FA re-esterification in WAT in the anti-obesity effect of LC *n*-3 PUFA in the combination with calorie restriction (Flachs *et al*. 2011), we sought to characterize the effect of the LC *n*-3 PUFA supplementation on WAT metabolism also in this study. Incorporation of ^{14}C-glucose into total lipids (Fig. 3A), as well as into the acyl groups (Fig. 3B) in epididymal WAT, were significantly decreased in association with the cHF diet-feeding, in agreement with the impairment of *de novo* FA synthesis in response to high intake of dietary fat (Flachs *et al*. 2011). Also in agreement with the results in mice maintained at 20°C (Flachs *et al*. 2011), this decrease was partially prevented by the LC *n*-3 PUFA supplementation, namely under the insulin-stimulated conditions (Fig. 3B). That LC *n*-3

PUFA support the metabolic effect of insulin is consistent with their beneficial effect on glucose homeostasis (see above). Moreover, as suggested by the changes in incorporation of radiolabeled glucose into glycerol residues (Fig. 3C), i.e., the marker of *de novo* glycerol synthesis and FA re-esterification (Pravenec *et al*. 2006), cHF-feeding depresses FA re-esterification in WAT, while LC *n*-3 PUFA could preserve this activity, namely under the basal conditions, in the absence of the insulin stimulation.

Discussion

The principal finding of this report is a moderate protection against accumulation of body fat by LC *n*-3 PUFA admixed to high-fat diet, namely in the abdominal WAT, which occurred in mice maintained at thermoneutral conditions of 30°C, i.e. independent of cold-induced thermogenesis. This observation is consistent with a lack of any up-regulation of UCP1 gene neither in WAT nor in interscapular BAT in response to dietary LC *n*-3 PUFA under the conditions similar to this experiment, except that the previous studies (Flachs *et al*. 2011, Flachs *et al*. 2005) were performed in mice maintained at 20°C, i.e. under the conditions, which should augment UCP1 gene expression. Similarly to our previous studies of this animal model, all of which were performed using mice maintained at 20°C (Flachs *et al*. 2011, Hensler *et al*. 2011, Jelenik *et al*. 2010, Kuda *et al*. 2009, Kus *et al*. 2011, Rossmeisl *et al*. 2012, van Schothorst *et al*. 2009), reduced accumulation of body fat in response to LC *n*-3 PUFA in this study could not be attributed to changes in food intake, supporting the notion that UCP1-independent energy expenditure was involved (see Introduction). However, it cannot be excluded that the

magnitude of LC *n*-3 PUFA response is affected to some extent by ambient temperature. To test this possibility, the effect of dietary LC *n*-3 PUFA on adiposity might be studied in mice maintained at various temperatures within the same experiment.

While in many of the previous studies in rodents fed a high-fat diet, LC *n*-3 PUFA prevented development of obesity, dyslipidemia (Flachs *et al*. 2011, Flachs *et al*. 2005, Ikemoto *et al*. 1996, Kuda *et al*. 2009, Ruzickova *et al*. 2004) and impaired glucose tolerance (Jelenik *et al*. 2010, Jucker *et al*. 1999, Kuda *et al*. 2009, Neschen *et al*. 2007, Storlien *et al*. 1987), depending possibly in part on the dietary macronutrient composition (Hao *et al*. 2012), only few studies in obese humans demonstrated reduction of adiposity after LC *n*-3 PUFA supplementation (Couet *et al*. 1997, Kunesova *et al*. 2006, Mori *et al*. 1999). Thus, the metabolic effect of LC *n*-3 PUFA could differ in part between rodents and humans, and the mechanisms underlying possible induction of energy expenditure (thermogenesis) and protection against fat accumulation remain to be clarified. As found in mice, the anti-obesity effect could reflect in part the inhibition of fat cell proliferation (Hensler *et al*. 2011, Ruzickova *et al*. 2004), while the metabolic effects could depend on increased lipid catabolism in the liver (Jelenik *et al*. 2010) and the intestine (van Schothorst *et al*. 2009). In contrast, muscle energy metabolism is relatively little affected (Horakova *et al*. 2012). Moreover, as we have shown previously (Flachs *et al*. 2011, Flachs *et al*. 2005), specific modulation of WAT metabolism, namely the induction of FA re-esterification (Flachs *et al*. 2011) could also contribute. Thus, somehow paradoxically with respect to the reduction of weight of epididymal fat in response to the LC *n*-3 PUFA supplementation, induction of *de novo* lipogenesis by LC *n*-3 PUFA in this WAT depot was observed in mice maintained both at 20°C (Flachs *et al*. 2011) and 30°C (this study).

Since FA re-esterification creates a substrate cycle, its activation results in energy expenditure (Kalderon *et al*. 2000, Langin 2010). Moreover, the induction of energy-demanding FA re-esterification in WAT in response to the LC *n*-3 PUFA supplementation could help to explain both, the induction of mitochondrial biogenesis (Flachs *et al*. 2011, Flachs *et al*. 2005) in WAT and the suppression of NEFA levels in plasma (results of this study and refs. Flachs *et al*. 2011, Flachs *et al*. 2006, Jelenik *et al*. 2010, Kuda *et al*. 2009, Kus *et al*. 2011).

Our results contribute to understanding of the basic mechanisms regulating energy metabolism. As already discussed by Cannon and Nedergaard (Cannon *et al*. 2004), metabolic mechanisms enhancing energy expenditure independent of UCP1 probably exist, with a significant characteristic that they are not augmented by cold. Our results are in favor of this concept, while suggesting that the anti-obesity effect of LC *n*-3 PUFA in rodents depends on the activation of the UCP1-independent thermogenesis, using mechanisms distinct from those mediating classical adaptive thermogenesis. Because of the enormous capacity of cold-induced thermogenesis in small rodents (Cannon *et al*. 2004), the demonstration of the anti-obesity effect of LC *n*-3 PUFA in mice under the thermoneutral conditions suggests that at least some of the underlying mechanisms could serve as a target for treatment of human obesity.

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Table 1. Growth characteristics, adiposity, and plasma parameters

	ST	**cHF**	**cHF+F**
Body weight (g)			
Initial (at weaning)	12.44 ± 0.45	12.61 ± 0.37	11.38 ± 0.81
Final	29.55 ± 0.39	40.11 ± 2.21#	37.17 ± 2.29#
Gain	16.98 ± 0.61	27.73 ± 2.09#	25.50 ± 1.76#
Food consumption (kJ/mice/day)	36.5 ± 1.6	40.6 ± 1.1#	40.5 ± 1.6#
Weight of fat depots (mg)			
EPI	393 ± 36	2208 ± 144#	1534 ± 228*#
DL	146 ± 14	738 ± 88#	531 ± 76#
Plasma levels			
TG (mmol/l)	1.40 ± 0.07	1.73 ± 0.20	1.19 ± 0.15*
NEFA (mmol/l)	0.96 ± 0.05	1.32 ± 0.05#	0.94 ± 0.07*
Glucose homeostasis			
Fasting glucose (mmol/l)	3.66 ± 0.07	4.63 ± 0.31#	3.63 ± 0.18*
Incremental AUC (glucose mmol/l)	655 ± 53	1129 ± 52#	842 ± 41*#

Four weeks after birth, mice were weaned onto standard low-fat diet (ST), or high-fat diet (cHF) diet, or cHF diet supplemented with LC *n*-3 PUFA (cHF+F) and maintained at 30°C ($n = 8$). Mice were killed at 8 months of age in *ad libitum* state and plasma levels of NEFA and TG and adiposity were analyzed. Glucose homeostasis was assessed using IP GTT in mice fasted overnight 2 weeks before killing (see Fig. 1). Food consumption (expressed as kJ/day per animal) was measured four times during the whole differential dietary treatment protocol. *EPI* - epididymal fat, *DL* – subcutaneous WAT in dorsolumbar region. Data are means ± SE. * $p<0.05$ for the effect of cHF+F compared to cHF; # $p<0.05$ for the effect of cHF-based diet compared to ST.

Table 2. Indirect calorimetry

	ST	**cHF**	**cHF+F**
VO_2 (ml/min)	1.07 ± 0.04	1.25 ± 0.07	1.19 ± 0.05
RER	0.904 ± 0.010	0.792 ± 0.008#	0.805 ± 0.007#

Four weeks after birth, mice were weaned onto different diets and maintained at 30°C as described in Table 1. At 6 month of age, indirect calorimetry was performed. Data are means ± SE. # $p<0.05$ for the effect of cHF-based diet.

FIGURE LEGENDS

Fig. 1 Glucose tolerance test. Four weeks after birth, mice were weaned onto ST diet (circle), or cHF diet (triangle), or cHF+F diet (square) and maintained at 30°C (n = 8) during the whole experiment. Two weeks before mice killing (at 8 month of age) IP GTT was performed. Data are means ± SE; for incremental AUC and fasting glucose (see Table 1).

Fig. 2 Evaluation of fuel partitioning using indirect calorimetry. At 6 months of age, the measurements were performed at 30°C for a period of 22 h, on a 12:12-h light-dark cycle, while mice had free access to ST diet (gray line), or cHF diet (black line), or cHF+F diet (black dash line) and water. RER data (their means ± SE are shown in Table 2) pooled from all the mice of the same dietary group (n = 6–8; ~3,600 RER measurements per each curve) were used to construct PRCF curves. Both EC_{50} and Hillslope values were significantly different between ST and cHF-based diets (cHF, cHF+F), while only the Hillslope values differed between cHF and cHF+F diets (not shown).

Fig. 3 Lipid metabolism in adipose tissue at 8 months of age. Incorporation of ^{14}C-glucose into neutral lipids (TG synthesis); (**A**), and incorporation of ^{14}C-glucose into acyl groups (*de novo* FA synthesis); (**B**) was evaluated *ex vivo* in fragments of epididymal fat. Incorporation of ^{14}C- glucose into glycerol residue (FA re-esterification); (**C**) was calculated based on the data in **A** and **B.** [a] $p<0.05$ for the effect of insulin, [b] $p<0.05$ for the effect of cHF+F compared to cHF diet.

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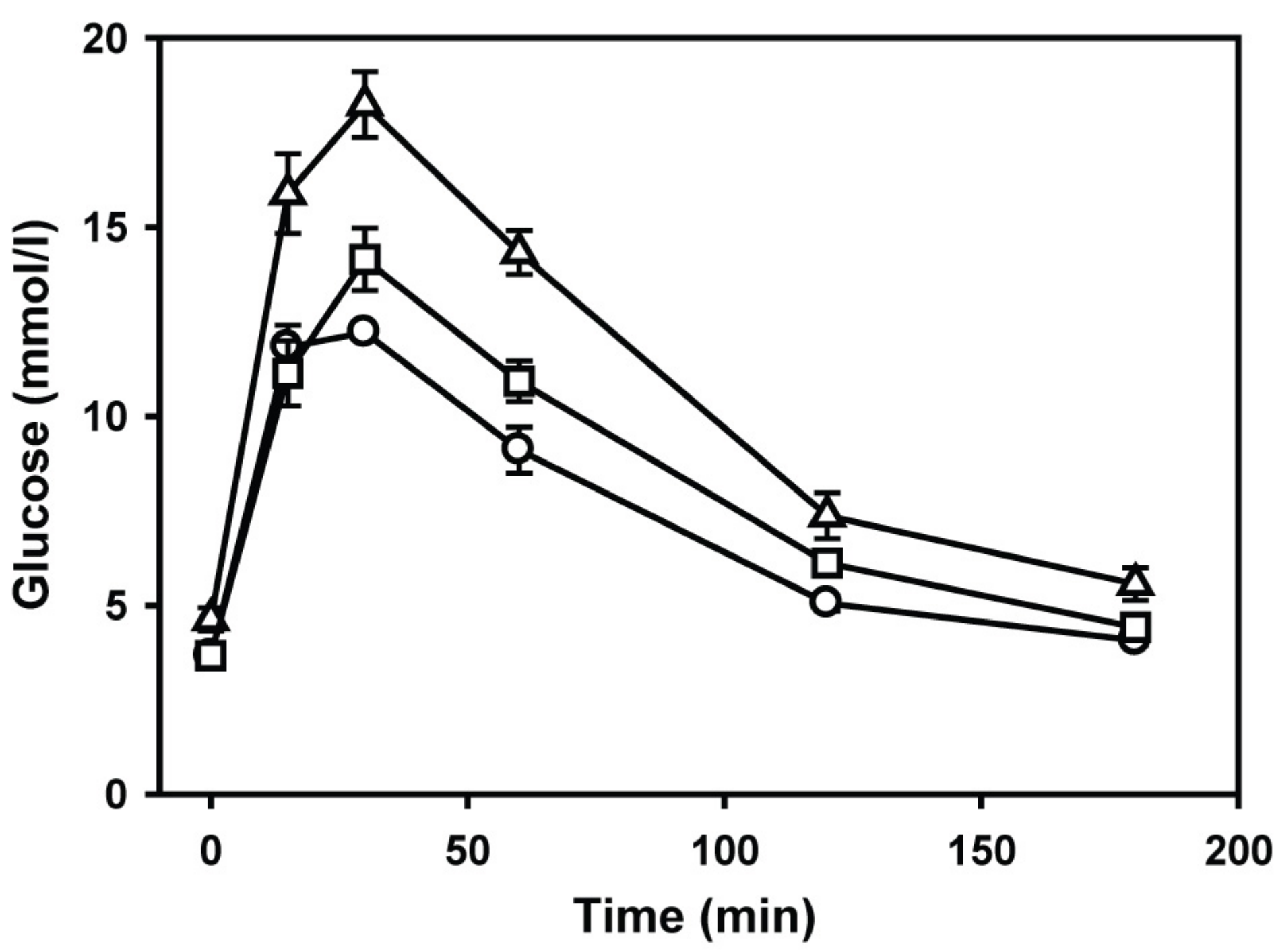
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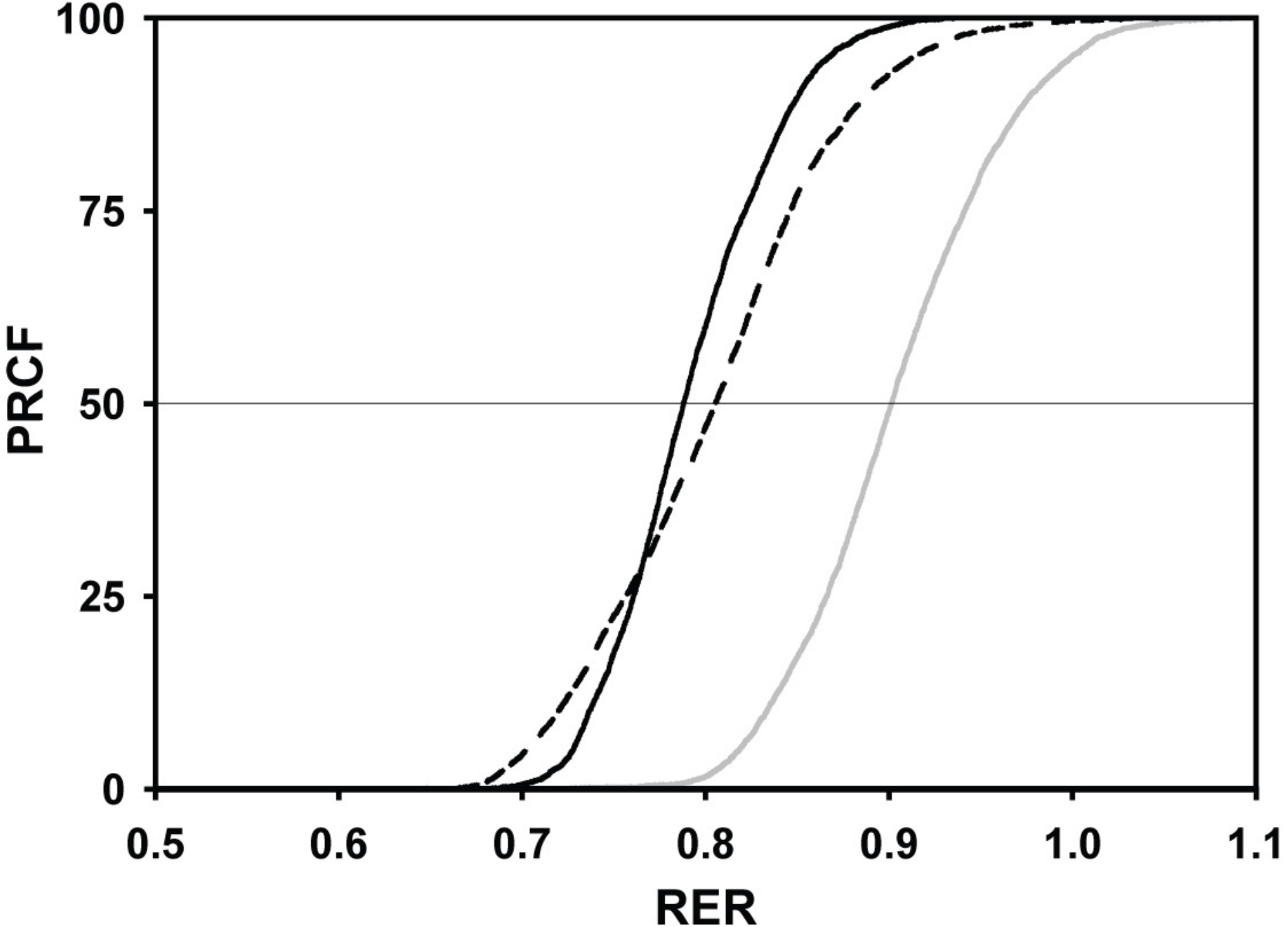
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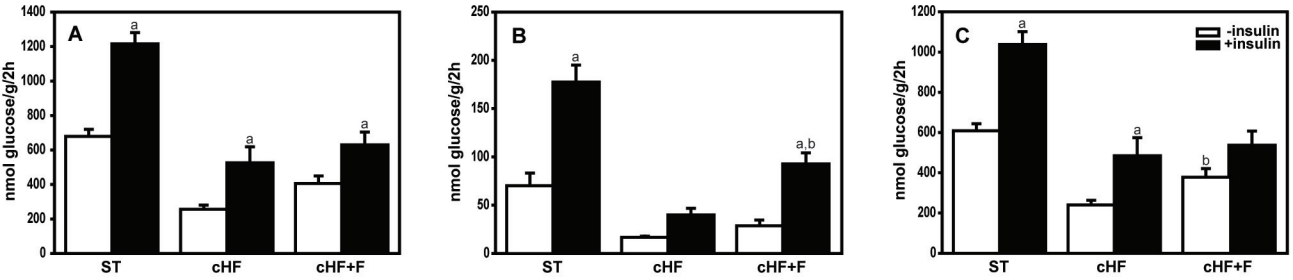
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