The metabolic response to fasting in humans: physiological studies

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4 Decreased suppression of muscle fatty acid oxidation by hyperinsulinemia during fasting

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Submitted

Abstract

Context: The transition from the fed to the fasted resting state is characterized by, amongst others, changes in lipid metabolism and peripheral insulin resistance. Acylcarnitines have been suggested to play a role in insulin resistance besides other long-chain fatty acid metabolites. Plasma levels of long-chain acylcarnitines increase during fasting, but this is unknown for muscle long-chain acylcarnitines.

Objective: We studied whether muscle long-chain acylcarnitines increase during fasting and their relation with glucose/fat oxidation and insulin sensitivity in lean healthy humans.

Main Outcome Measures and Design: After 14 and 62 hours of fasting, glucose fluxes, substrate oxidation, plasma and muscle acylcarnitines were measured before and during a hyperinsulinemic euglycemic clamp.

Results: Hyperinsulinemia decreased long-chain muscle acylcarnitines after 14 hours of fasting but not after 62 hours of fasting. In both the basal state and during the clamp glucose oxidation was lower and fatty acid oxidation was higher after 62 hours vs. 14 hours of fasting. Absolute changes in glucose and fat oxidation in the basal vs. hyperinsulinemic state were not different. Muscle long-chain acylcarnitines did not correlate with glucose oxidation, fatty acid oxidation or insulin-mediated peripheral glucose uptake.

Conclusion: After 62 hours of fasting, the suppression of muscle long-chain acylcarnitines by insulin was attenuated compared to 14 hours of fasting. Muscle long-chain acylcarnitines do not unconditionally reflect fatty acid oxidation. The higher fatty acid oxidation during hyperinsulinemia after 62 vs. 14 hours of fasting although the absolute decrease in FAO was not different, suggests a different insulin-regulated set point.

Introduction

Short-term fasting can be defined as the first 72 hours of starvation in which progressive alterations in lipid and glucose metabolism occur (1;2). The adaptation to short-term fasting is characterized by, amongst others, an increase in lipolysis with concomitant increases in plasma free fatty acids (FFA) and fatty acid oxidation (FAO) (1) and a decrease in peripheral insulin sensitivity and carbohydrate oxidation (CHO)(2-4).

To be oxidized, activated long-chain fatty acids can only cross the mitochondrial membranes as acylcarnitines (ACs)(5). The coupling of an activated long-chain fatty acid to carnitine (3-hydroxy-4-*N,N,N*-trimethylaminobutyric acid) is catalyzed by carnitine-acyl(palmitoyl)transferase 1 (CPT1) on the outer mitochondrial leaflet (6). CPT1 is considered to be the rate-limiting enzyme for long-chain fatty acid entry into the mitochondria and subsequent oxidation (5;7). Furthermore CPT1 activity increases during fasting in animal studies (8). Inside the mitochondrion, the AC is activated to acyl-CoA again by CPT2. The released carnitine is exchanged for a new incoming AC by the mitochondrial membrane protein carnitine-acylcarnitine-translocase (CACT) (6).

Plasma ACs are thought to reflect the mitochondrial acyl-CoA pool and AC profile analysis is the current standard for the diagnosis of FAO disorders at the metabolite level (9;10). Recently, muscle ACs have been implicated in insulin resistance via a currently unknown mechanism (8;11;12). These studies suggested that increased beta-oxidation outpaces the tricarboxylic acid cycle (TCA) with subsequent inhibition of complete fatty acid oxidation via a high energy redox state (rising $NADH/NAD^+$ and acetyl-CoA/CoA ratios). The accumulation of metabolic by-products (e.g. acylcarnitines) would then activate stress kinases or other signals, interfering with insulin action (11). However, how ACs directly affect insulin mediated glucose uptake is currently unraveled.

Fasting increases plasma long-chain ACs (13-15), but it is unknown, whether muscle long-chain ACs increase during short-term fasting in humans. Animal studies showed increased muscle long-chain AC levels during fasting (16;17). Such an increase of ACs during fasting would match increased lipid oxidation and decreased peripheral insulin sensitivity (1;3;4).

In this study, we examined the association of muscle ACs during short-term fasting with glucose and fat metabolism. Healthy lean subjects were studied before and after short-term fasting in both the basal state and during a hyperinsulinemic euglycemic clamp. We hypothesized that fasting induced an increase of whole body FAO resulting in an increase in muscle ACs which would explain the expected peripheral insulin resistance.

Subjects and methods

Subjects

We studied healthy lean male volunteers who participated in a study on fasting induced insulin resistance (4). Subjects were in self-reported good health, confirmed by medical history and physical examination. Criteria for inclusion were 1) absence of a family history of diabetes; 2) age 18–35 yr; 3) Caucasian race; 4) BMI 20-25 kg/m^2; 5) normal oral glucose tolerance test according to the ADA criteria (18); 6) normal routine blood examination; 7) no excessive sport activities, i.e. < 3 times per week; and 8) no medication. Written informed consent was obtained from all subjects after explanation of purposes, nature, and potential risks of the study. The study was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam.

Experimental protocol

Subjects were studied twice: after 14 and 62 h of fasting. Study days were separated by at least a week. Subjects were fasting from 2000 h the evening before the first study day and from 2000 h three days before the second study day until the end of the study days. They were allowed to drink water only.

After admission to the metabolic unit at 0730 h, a catheter was inserted into an antecubital vein for infusion of stable isotope tracers, insulin and glucose. Another catheter was inserted retrogradely into a contralateral hand vein and kept in a thermo-regulated (60ºC) plexiglas box for sampling of arterialized venous blood. Saline was infused as NaCl 0.9% at a rate of 50 mL/h to keep the catheters patent. [6,6-$^2H_2$]glucose and [1,1,2,3,3-$^2H_5$]glycerol were used as tracers (>99% enriched; Cambridge Isotopes, Andover, USA) to study glucose kinetics and lipolysis (total triglyceride hydrolysis) respectively.

At T = 0 h (0800 h), blood samples were drawn for determination of background enrichments and a primed continuous infusion of both isotopes was started: [6,6-$^2H_2$] glucose (prime, 8.8 µmol/kg; continuous, 0.11 µmol/kg·min) and [1,1,2,3,3-$^2H_5$]glycerol (prime, 1.6 µmol/kg; continuous, 0.11 µmol/kg·min) and continued until the end of the study. After an equilibration period of two hours (14 h of fasting), 3 blood samples were drawn for glucose and glycerol enrichments and 1 for glucoregulatory hormones, FFA and plasma AC levels. Thereafter (T = 3 h), infusions of insulin (60mU/m^2·min) (Actrapid 100 IU/ml; Novo Nordisk Farma B.V., Alphen aan den Rijn, the Netherlands) and glucose 20% (to maintain a plasma glucose level of 5 mmol/L) were started. [6,6-$^2H_2$]glucose was added to the 20% glucose solution to achieve glucose enrichments of 1% to approximate

the values for enrichment reached in plasma and thereby minimizing changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose (19). Plasma glucose levels were measured every 5 min at the bedside. At T = 8 h, 5 blood samples were drawn at 5-min intervals for determination of glucose and glycerol enrichments. Another blood sample was drawn for determination of glucoregulatory hormones, FFA and plasma AC levels.

Subjects were studied under the same conditions after 62h of fasting. Volunteers were allowed to drink water ad libitum. To prevent sodium and potassium depletion during fasting, subjects were supplied with oral sodium chloride 80mmol per day (Tablets, In-House Pharmacist, AMC, Amsterdam, the Netherlands) and oral potassium chloride 40mmol per day (Slow-K, Novartis BV, Arnhem, the Netherlands).

Indirect calorimetry and muscle biopsies

Oxygen consumption (VO_2) and CO_2 production (VCO_2) were measured continuously during the final 20 min of both the basal state and the clamp by indirect calorimetry using a ventilated hood system (Sensormedics model 2900; Sensormedics, Anaheim, CA).

Muscle biopsies were performed to assess muscle AC concentrations at the end of both the basal state and the clamp. Biopsies was performed under local anaesthesia (lidocaine 20 mg/ml; Fresenius, Kabi, Den Bosch, The Netherlands) using a Pro-Mag I biopsy needle (MDTECH, Gainesville, FL). Biopsy specimens were quickly washed in a buffer (0.9% NaCl/28.3g/liter HEPES) to remove blood, inspected for fat or fascia content, dried on gauze swabs, and subsequently stored in liquid nitrogen until analysis.

Glucose and lipid metabolism measurements

Plasma glucose and FFA concentrations were measured as described earlier (4). [6,6-$^2H_2$] glucose enrichment was measured as described earlier (20). [6,6-$^2H_2$]glucose enrichment (tracer/tracee ratio) intra-assay variation: 0.5-1%; inter-assay variation 1%; detection limit: 0.04%. [1,1,2,3,3-$^2H_5$]glycerol enrichment was determined as described earlier (21). Intra-assay variation glycerol: 1-3%, [1,1,2,3,3-$^2H_5$]glycerol: 4%; inter-assay variation glycerol: 2-3%; [1,1,2,3,3-$^2H_5$]glycerol: 7%.

Glucoregulatory hormones

Insulin, glucagon, cortisol, norepinephrine and epinephrine were measured as described earlier (22).

Plasma and muscle acylcarnitine measurements

AC plasma concentrations were analyzed as described previously (23). Muscle biopsies were freeze dried and analyzed as described previously (24). Total long-chain ACs represent the sum of $C_{12:1}$-, C_{12}-, $C_{14:2}$-, $C_{14:1}$-, C_{14}-, $C_{16:1}$-, C_{16}-, $C_{18:2}$-, $C_{18:1}$- and C_{18}-carnitine.

Calculations and statistics

Endogenous glucose production (EGP) and rate of glucose disposal (Rd) were calculated using the modified forms of the Steele Equations as described previously (4;19;25). EGP and Rd were expressed as µmol/kg·min. Lipolysis (glycerol turnover) was calculated by using formulas for steady state kinetics adapted for stable isotopes (32). Lipolysis was expressed as µmol/kg·min and as µmol/kcal as proposed by Koutsari et al (30).

Resting energy expenditure (REE) was expressed as kcal/day. FAO and CHO rates were calculated from O_2 consumption and CO_2 production (26).

Statistical comparisons and correlation analyses were performed with the Wilcoxon Signed Rank test and Spearman's rank correlation coefficient (ρ) respectively. The SPSS statistical software program version 12.0.2 (SPSS Inc, Chicago, IL) was used for statistical analysis. Data are presented as median [minimum - maximum].

Results

Anthropometric characteristics

The subject characteristics have been reported earlier (4): In sum, subject characteristics were: age: 23 [20 - 26] yrs; weight 70.1 [62.5 – 75.5] kg after 14 h and 69.0 [60.0 – 72.8] kg after 62 h of fasting, $P = 0.012$; BMI 20.9 [19.2 – 23.3] kg/m^2 after 14 h and 20.3 [18.3 – 22.6] kg/m^2 after 62 h of fasting, $P = 0.011$.

Indirect calorimetry

REE (kcal/day) in the basal state was significantly higher after 62 h of fasting compared to 14 h of fasting; 1682 [1518 - 1820] kcal/day vs. 1578 [1308 - 1783] kcal/day respectively, $P = 0.017$ (Figure 1). During the clamp however, REE was significantly lower after 62 h of fasting compared to 14 h of fasting; 1604 [1470 - 1734] kcal/day vs. 1815 [1494 - 1933] kcal/day respectively, $P = 0.012$.

FAO (µmol/kg·min) in the basal state was significantly higher after 62 h of fasting compared to 14 h of fasting (Figure 1). During the clamp, FAO remained significantly higher after 62 h of fasting compared to 14 h of fasting. The absolute change during the

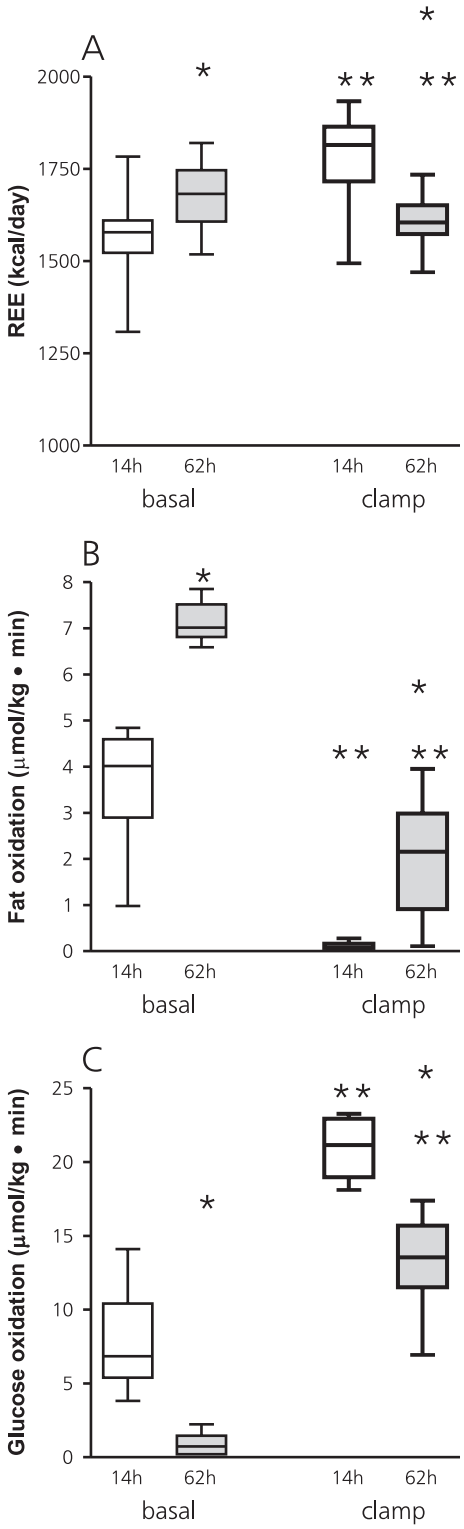

Figure 1, panel A Resting energy expenditure (REE) in the basal state and during the clamp after 14 h (open box plots) and 62 h (grey boxplots) of fasting. *P = 0.017 and 0.012 respectively. **P = 0.017 for the increase and 0.05 for the decrease of REE during the clamps after 62 and 14 h of fasting respectively.

Figure 1, panel B Fat oxidation in the basal state and during the clamp after 14 h (open box plots) and 62 h (grey boxplots) of fasting. *P = 0.012 and 0.017 respectively. **P = 0.012 for the decrease of fat oxidation during both clamps after 62 and 14 h of fasting.

Figure 1, panel C CHO in the basal state and during the clamp after 14 h (open box plots) and 62 h (grey boxplots) of fasting, *P = 0.012. **P = 0.012 for the increase of CHO during both clamps after 62 and 14 h of fasting.

clamps in FAO was not different between 62 and 14 h of fasting; -5.1 [-6.6 - -2.7] µmol/kg•min vs. -3.9 [-4.8 - -1.0] µmol/kg•min respectively, P = 0.8. CHO was significantly lower after 62 h of fasting compared to 14 h of fasting (Figure 1). During the clamp, CHO was significantly lower after 62 h of fasting compared to 14 h of fasting. The absolute change between the basal state and the clamp in CHO was not different between 62 and 14 h of fasting: 12.8 [5.9 - 17.1] µmol/kg•min vs. 13.6 [7.5 - 19.3] µmol/kg•min respectively, P = 0.2. If FAO and CHO were expressed as µmol/kg LBM•min comparable results were obtained (data not shown).

Glucose and lipid metabolism measurements

Plasma glucose concentrations and EGP (Table 1) were significantly lower after 62h of fasting compared to 14 h fasting (4). No differences were found in plasma glucose

Table 1 Glucose and lipid metabolism measurements

	basal state		
	14h (n = 8)	**62h (n = 8)**	**P**
Glucose (mmol/liter)	5.0 [4.5 - 5.6]	3.7 [3.3 - 4.1]	0.011
EGP (µmol/kg • min)	11.8 [8.9 - 19.7]	8.3 [7.0 - 8.7]	0.012
Rd (µmol/kg • min)	-	-	-
FFA (mmol/liter)	0.33 [0.19 - 0.95]	1.10 [0.85 - 1.24]	0.015
Lipolysis (µmol/kg • min)	1.5 [1.1 - 4.4]	3.8 [2.3 - 4.2]	0.017
Lipolysis (µmol/kcal)	101 [68 - 261]	214 [135 - 250]	0.036
Insulin (pmol/liter)	30 [18 - 58]	15 [15 - 20]	0.012

Data are presented as median [minimum - maximum]. EGP, endogenous glucose production; Rd, rate of disposal; FFA, free fatty acids. * During the clamps, EGP and FFA were completely suppressed.

concentrations after 62h vs 14 h of fasting during the clamp. Rd during the clamp was significantly lower after 62 h of fasting compared to 14 h of fasting (Table 1).

Basal plasma FFA and rate of lipolysis were significantly higher after 62 h of fasting (Table 1). Plasma FFA were suppressed during the hyperinsulinaemic euglycaemic clamps after 62 h and 14 h of fasting (Table 1). Lipolysis was not different between 62 h of fasting andcompared to 14 h of fasting during the clamp (Table 1).

Glucoregulatory hormones

Insulin was significantly lower after 62 h of fasting in the basal state as well as during the clamp (Table 1). Other data on glucoregulatory hormones have been presented elsewhere (4).

Muscle and plasma acylcarnitines in the basal state

After 62 h of fasting, muscle free carnitine (FC) was higher compared to 14 h; 9503 [5326 -14360] pmol/mg dry weight vs. 5299 [3331 - 7051] pmol/mg dry weight, P = 0.028. In the basal state muscle long-chain ACs were not significantly different after 62 h vs. 14 h of fasting (Figure 2).

During the clamp muscle FC was not different after 62 h of fasting compared to 14 h of fasting; 1175 [411 - 3380] pmol/mg dry weight vs. 697 [269 - 1149] pmol/mg dry weight, P = 0.75. Total muscle long-chain ACs were significantly higher during the clamp after 62 h of fasting compared to 14 h of fasting (Figure 2). Individual muscle long-chain ACs showed a similar pattern (see Table 2). Total plasma long-chain ACs were significantly

	hyperinsulinaemic euglycaemic clamp		
	14h (n = 8)	**62h (n = 8)**	**P**
	5.1 [4.9 - 5.3]	4.9 [4.5 - 5.1]	0.107
	-*	-*	-
	60.5 [45.1 - 79.3]	44.4 [37.5 - 51.7]	0.018
	<0.02	<0.02	-
	0.6 [0.2 - 0.8]	0.7 [0.2 - 1.8]	0.249
	32 [11 - 48]	44 [13 - 108]	0.249
	642 [416 - 715]	533 [383 - 663]	0.012

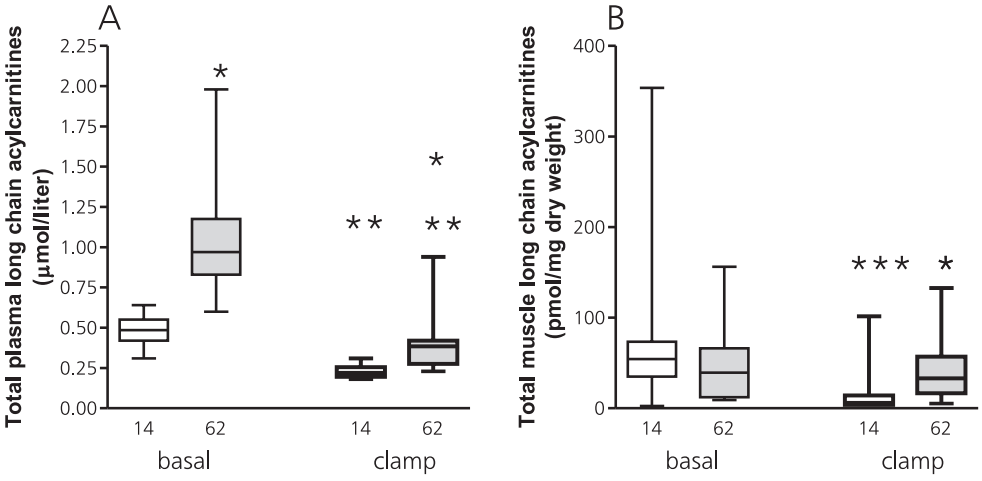

Figure 2, panel A Total long chain plasma acylcarnitines (ACs) the basal state and during the clamp after 14 h (open box plots) and 62 h (grey boxplots) of fasting, *P = 0.012. **P = 0.012 for the decrease of ACs during both clamps after 62 and 14 h of fasting.
Figure 2, panel B Total long chain muscle acylcarnitines (ACs) the basal state and during the clamp after 14 h (open box plots) and 62 h (grey boxplots) of fasting, *P = 0.012 for the difference in ACs during the clamp between 62 and 14 h of fasting. ***P = 0.05 for the decrease in ACs between basal state and clamp after 14 h of fasting.

higher after 62 h of fasting compared to 14 h of fasting in the basal state and during the clamp. The same was true for individual plasma long-chain ACs (data not shown).

Muscle long-chain acylcarnitines did not correlate with lipolysis, Rd, FAO or plasma long-chain acylcarnitines (data not shown).

Table 2. Acylcarnitine concentrations in muscle (pmol/mg dry weight) in the basal state after 14 and 62 h of fasting

	basal state		
Acylcarnitine	**14 h**	**62 h**	**P[b]**
C12:1	1.21 [0.13 - 4.27]	1.17 [0.14 - 2.97]	0.78
C12	4.15 [0.13 - 14.69]	2.42 [0.42 - 7.97]	0.48
C14:2	2.05 [0.13 - 9.24]	1.19 [0.31 - 5.51]	0.58
C14:1	7.6 [0.25 - 37.68]	4.68 [0.56 - 20.00]	0.89
C14	6.12 [0.25 - 35.31]	4.15 [0.51 - 16.61]	0.67
C16:1	6.18 [0.13 - 46.45]	4.59 [0.61 - 22.80]	1.00
C16	10.07 [0.88 - 72.80]	6.46 [2.50 - 29.41]	0.67
C18:2	3.42 [0.25 - 19.91]	1.84 [0.56 - 6.44]	0.58
C18:1	8.10 [0.25 - 88.86]	7.42 [2.36 - 37.12]	0.67
C18	3.46 [0.25 - 25.36]	2.32 [0.97 - 7.29]	0.40

Data are presented as median [minimum - maximum]. [a] N = 6. [b,c]P-values represent differences between basal states and clamps after 14 and 62 h of fasting respectively.[d,e]P-values represent differences within clamps after 14 and 62 h respectively.

Discussion

In the present study, muscle long-chain acylcarnitines after 14 vs. 16 h of fasting were studied and correlated to glucose and fatty acid oxidation rates as well as peripheral insulin sensitivity. In the basal state, there was no significant difference in muscle long-chain ACs between 14 and 62 h of fasting. In contrast during hyperinsulinemia we found a decrease in muscle long-chain ACs after 14 h of fasting versus no changes in muscle long-chain ACs after 62 h of fasting was found. The latter finding was accompanied by higher whole body FAO.

REE increased approximately 7.5 % during 62 h of fasting, which is in line with previous observations (3;27-29), although the increase in REE has not fully been accounted for. It was proposed that increased energy requirements of gluconeogenesis and ketogenesis are reflected in increased REE (27;28). On the other hand it was suggested that the norepinephrine induced thermogenic response results in a slight increase in REE during short-term fasting (27;29), but we did not detect differences in plasma norepinephrine (4).

Our study confirmed earlier reports on increased plasma FFA and lipolysis (1). Higher plasma FFA are also found in different models of insulin resistance and are thought to be one of the main mediators of obesity-induced insulin resistance (30). Lipid mediators that induce insulin resistance are mainly derived from long-chain fatty acids (31). Moreover,

hyperinsulinemic euglycemic clamp				
14 h	62 h	P[c]	P[d]	P[e]
0.15 [0 - 1.25]	0.71 [0.21 - 2.63]	0.012	0.025	0.26
0.26 [0.18 - 6.41]	1.45 [0.21 - 5.38]	0.16	0.069	0.48
0.15 [0 - 3.59]	0.97 [0.11 - 4.00]	0.036	0.050	0.40
0.17 [0.12 - 10.94]	3.41 [0.32 - 9.00]	0.036	0.036	0.33
0.22 [0.12 - 13.44]	2.77 [0.32 - 9,75]	0.16	0.12	0.40
0.30 [0.13 - 14.92]	2.48 [0.21 - 14.00]	0.069	0.093	0.67
1.38 [0.42 - 20.15]	9.41 [1.60 - 33.13]	0.012	0.025	0.67
0.71 [0.23 - 5.39]	2.40 [0.32 - 12.63]	0.012	0.036	0.78
1.56 [0.58 - 20.00]	6.18 [0.96 - 32.63]	0.012	0.036	0.48
1.18 [0.47 - 5.39]	3.04 [0.85 - 9.50]	0.012	0.017	0.33

muscle long-chain ACs have been suggested to induce peripheral insulin resistance in animal studies (8;11;12). Since fasting induces insulin-resistance (2;4), we hypothesized that this may be related to an increase in muscle long-chain ACs. Human data on muscle ACs during fasting are lacking but previous animal studies showed an increase of muscle long-chain ACs in rodents after fasting (8;17).

The lack of an increase in muscle long-chain ACs between 14 and 62 h of fasting is unexpected since we demonstrated an increase in whole body FAO. The CPT1 dependent rate of long-chain fatty acid entrance into the mitochondria is thought to determine the rate of FAO (5;7). Our data imply that the muscle concentration of ACs during fasting in humans does not reflect the fatty acid oxidation flux.

Hyperinsulinemia resulted in a decreased concentration of long-chain muscle ACs after 14 h of fasting. This was in line with the lower whole body FAO during the clamp after 14 h of fasting. After 62 h of fasting, the suppressive effect of insulin on muscle long-chain ACs was not found. Increased muscle long- chain ACs during the clamp after 62 h of fasting are unlikely to reflect accumulation of non utilizable long-chain ACs or ongoing FAO since we could not demonstrate such a relationship in the basal state after 62 h of fasting. However, ongoing FAO is likely to occur since clamp values of whole body FAO were higher after 62 h of fasting compared to 14 h of fasting. This suggests that despite 5 h of hyperinsulinemia, peripheral glucose uptake is still attenuated and activated fatty acids are continued to be transported to the mitochondrion in order to be oxidized (5;7).

Ongoing FAO may be needed since peripheral glucose uptake is attenuated. Our data on increased plasma long-chain ACs after 62 h of fasting confirm older studies (13-15). In contrast to our finding in muscle, plasma long-chain ACs were higher after 62 compared to 14 h of fasting, but decreased equally during hyperinsulinemia. This, and the absence of a correlation of plasma ACs with muscle ACs negates that plasma ACs reflect muscle ACs. It may support the notion that the liver is the most likely source of plasma long-chain ACs during short-term fasting (7;32;33).

Muscle FC increased during 62 h compared to 14 of fasting which reflects the dependence of FAO on FC for transport of long-chain acyl-CoAs across the mitochondrial membranes.

Although we found lower insulin levels during the clamp after 62 h of fasting, it is not likely that these have interfered with our results as discussed earlier (4).

In conclusion we show that fasting for 62 h results in higher rates of lipolysis and FAO together with lower CHO rates and lower peripheral insulin sensitivity. These flux rates are not paralleled with an increase in muscle long-chain ACs after 62 h of fasting in the basal state. However, during hyperinsulinemia, the suppression of the concentration of muscle long-chain ACs was less compared to after 14 h of fasting. Also, FAO rates remained higher during the clamp after 62 h of fasting. Despite earlier reports on possible interference of the muscle long-chain ACs with peripheral insulin sensitivity, our study does not support such a role for muscle long-chain ACs during fasting. To clarify whether muscle ACs are just innocent bystanders or active players in insulin resistance, further studies in different models of insulin resistance are needed.

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