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3 Muscle adaptation to short-term fasting in healthy lean humans

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Abstract

Context: It has been demonstrated repeatedly that short-term fasting induces insulin resistance although the exact mechanism in humans is unknown to date. Intramyocellular sphingolipids (i.e. ceramide) have been suggested to induce insulin resistance by interfering with the insulin signaling cascade in obesity.

Objective: To study peripheral insulin sensitivity together with muscle ceramide concentrations and protein kinase B/AKT phosphorylation after short-term fasting.

Main Outcome Measures and Design: After 14 and 62 hours of fasting, glucose fluxes were measured before and after a hyperinsulinemic euglycemic clamp. Muscle biopsies were performed in the basal state and during the clamp to assess muscle ceramide and protein kinase B/AKT.

Results: Insulin mediated peripheral glucose uptake was significantly lower after 62 h of fasting compared to 14 h of fasting. Intramuscular ceramide concentrations tended to increase during fasting. During the clamp the phosphorylation of protein kinase B/AKT at serine⁴⁷³ in proportion to the total amount of protein kinase B/AKT was significant lower. Muscle ceramide did not correlate with plasma free fatty acids.

Conclusion: Fasting for 62 h decreases insulin mediated peripheral glucose uptake with lower phosphorylation of AKT at serine⁴⁷³. AKT may play a regulatory role in fasting induced insulin resistance. Whether the decrease in AKT can be attributed to the trend to higher muscle ceramide remains unanswered.

Introduction

The incidence of obesity induced insulin resistance and type 2 diabetes mellitus increases. Lipid infusion studies in healthy subjects showed that increased plasma free fatty acids (FFA) reduce insulin-mediated glucose uptake (1). Plasma FFA levels correlate with intramyocellular triglycerides (IMTG) during lipid infusion (2) and IMTG correlate negatively with peripheral insulin sensitivity (3).

Various metabolites of FFA, such as ceramide, have been suggested to decrease insulin sensitivity in skeletal muscle (4). Increased muscle ceramide concentrations have been reported to correlate negatively with insulin sensitivity in obese insulin resistant subjects (5). Intracellular ceramide synthesis from palmitate is a mechanism by which FFA decrease insulin-stimulated phosphorylation of protein kinase B/AKT (AKT) (6).

During short-term fasting, plasma FFA (7) as well as intramyocellular lipid stores increase (8). Also, fasting induces insulin resistance although the exact mechanism is still unknown (9;10). Fasting increases intramyocellular ceramide levels in rats (11), but this has not been investigated in lean healthy humans.

Therefore, we studied peripheral glucose metabolism during hyperinsulinemic euglycemic clamp conditions in healthy lean subjects after 14 and 62 h of fasting in relation to muscle ceramide and phosphorylation of AKT at serine⁴⁷³ (pAKT-ser⁴⁷³). We hypothesized that fasting increased muscle ceramide and decreased peripheral insulin sensitivity via lower muscle pAKT-ser⁴⁷³ levels.

Subjects and methods

Subjects

After informed consent, eight healthy, non smoking, male volunteers were included. The study was approved by the Medical Ethical Committee of the Academic Medical Center.

Protocol

Subjects were studied after 14 and 62 h of fasting, separated by at least a week. Subjects fasted from 2000 h the evening before the first study day and from 2000 h three days before the second study day. Glucose kinetics (tracer: [6,6-²H₂]glucose; >99% enriched; Cambridge Isotopes, Andover, USA: prime, 8.8 μmol/kg; continuous, 0.11 μmol/kg·min), FFA and glucoregulatory hormones were measured in the basal state and after a 5 h hyperinsulinemic euglycemic clamp (insulin infusion: 60mU/m²·min; Actrapid 100 IU/

ml; Novo Nordisk Farma B.V., Alphen aan den Rijn, the Netherlands) as described earlier (12). Insulin infusion rates were chosen to completely suppress endogenous glucose production (13).

Indirect calorimetry (O_2 consumption and CO_2 production) and muscle biopsies were performed at the end of both basal state and clamp as described previously (12).

Analytical Procedures

Plasma glucose and FFA concentrations were measured as reported earlier (12). $[6,6-^2H_2]$ glucose enrichment was measured as described previously (12).

Insulin, cortisol, glucagon and catecholamines were determined as described previously (12). Soluble tumor necrosis factor receptors (sTNF-R) I and II were determined with an EASIA kit (Biosource Europe S.A., Belgium).

Ceramide in muscle biopsies was measured as described previously (12).

Muscle immunoblots were visualized by enhanced chemiluminescence (ECL). Chemicals for ECL were from Sigma (St. Louis, MO, USA). Phosphospecific anti-AKT-ser⁴⁷³, phosphospecific anti-glycogen synthase kinase-3-ser⁹ (GSK), total anti-AKT and total anti-eIF4E (loading control) were from Cell Signaling (Boston, MA, USA). Phosphospecific anti-AS160-thr⁶⁴² was from GeneTex Inc. (San Antonio, TX, USA). Twenty mg of muscle tissue was taken up in 300 μ l of ice-cold lysis buffer (20 mM Tris (pH 7.5), 50 mM NaCl, 250 mM sucrose, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM DTT, 1,0% Triton X-100) supplemented with cocktail protease inhibitor tablets. Cell lysate was cleared by centrifugation for 15 min at 4°C. Cell protein was determined and separated by SDS-PAGE. A standard Western blotting procedure was performed and polyvinylidene fluoride blots were incubated with appropriate antibodies. Results are presented as fold increase compared to control after 14h fasting.

Calculations and statistics

Endogenous glucose production (EGP) and peripheral glucose uptake (rate of disappearance/Rd) were calculated with modified forms of the Steele Equations as described (12). Glucose oxidation was calculated as reported previously (12).

Comparisons and correlations were performed with the Wilcoxon Signed Rank test and Spearman's rank correlation analysis (ρ) respectively. The statistical software program version 12.0.1 (SPSS Inc, Chicago, IL) was used for statistical analysis. Data are presented as median [minimum-maximum].

Results

Anthropometric characteristics

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/m² after 14 h and 20.3 (18.3 – 22.6) kg/m² after 62 h of fasting, $P = 0.011$.

Glucose kinetics, FFA and glucoregulatory hormones (Table 1)

Basal plasma glucose concentrations, glucose oxidation and EGP were significantly lower after 62 h of fasting, whereas basal plasma FFA increased significantly.

No differences were found in plasma glucose concentrations between clamps. Rd was significantly lower after 62 h of fasting. Non-oxidative (NOGD) glucose disposal and oxidative glucose disposal during the clamp was lower after 62 h. Glucose oxidation and NOGD expressed as percentage of Rd did not differ between clamps. Plasma FFA were equally suppressed during both clamps.

Insulin levels were lower after 62 h of fasting in the basal state and during the clamp. Glucagon levels were higher in the basal state and tended to be higher during the clamp after 62 h of fasting. Fasting did not influence plasma cortisol and norepinephrine levels. Plasma epinephrine concentrations, however, were higher after 62 h of fasting in the basal state but not during the clamp. sTNF-RI and II did not change.

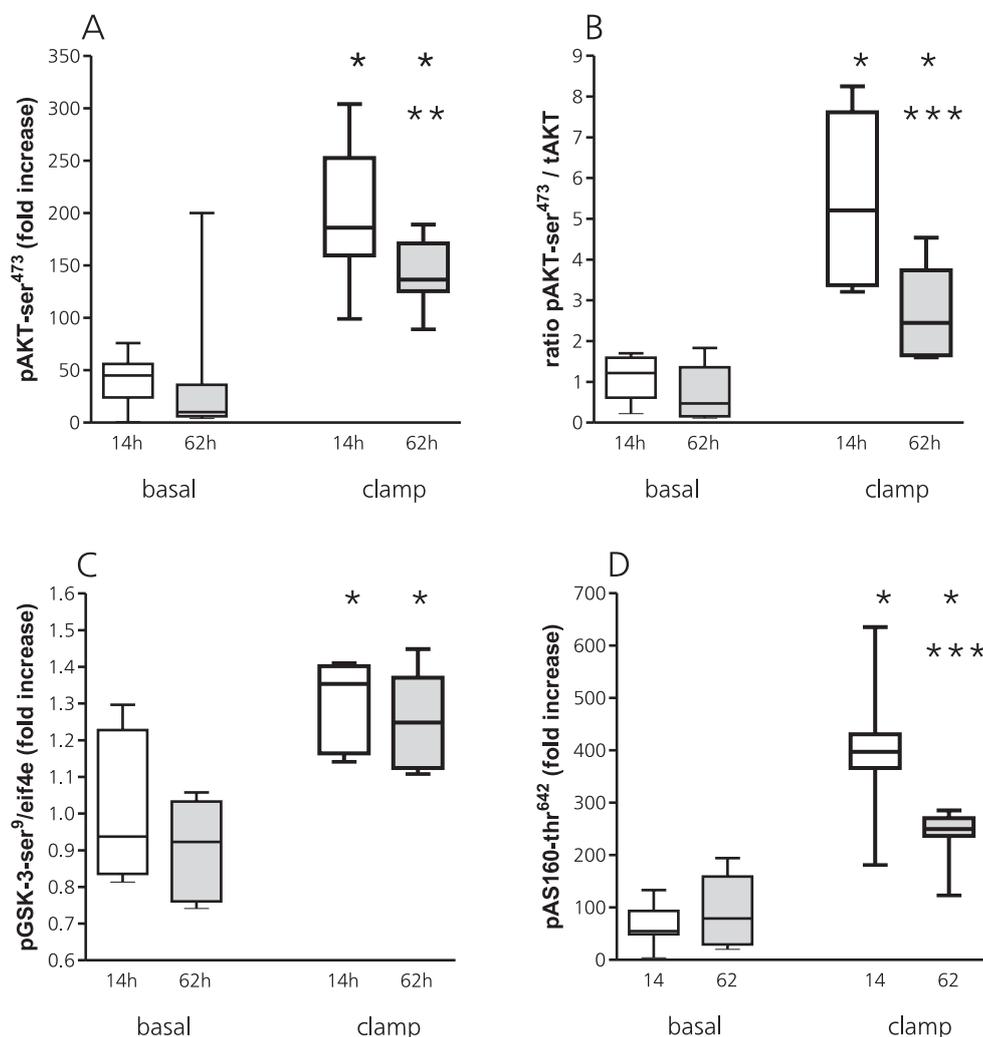
Muscle measurements

Muscle ceramide concentrations in the basal state tended to be higher after 62 h of fasting: 38.0 [25.2 - 54.9] pmol/mg wet weight vs. 29.5 [14.0 - 58.9] pmol/mg wet weight respectively ($P = 0.069$). During the clamp no differences were found between 62 h and 14 h of fasting: 35.3 [26.2 - 84.6] pmol/mg wet weight vs. 32.2 [25.9 – 54.3] pmol/mg wet weight respectively, $P = 0.5$.

Insulin-mediated peripheral glucose uptake and muscle ceramide levels did not correlate after 62 h of fasting: $\rho = -0.12$; $P = 0.78$. Muscle ceramide and plasma FFA levels showed no correlation ($\rho = -0.30$; $P = 0.47$).

pAKT-ser⁴⁷³ increased significantly during both clamps (Figure 1). A significant lower ratio of pAKT-ser⁴⁷³ to total AKT (pAKT-ser⁴⁷³/tAKT) was observed during the clamp after 62 h of fasting vs. 14 h, but not in the basal state. The increase of pAKT-ser⁴⁷³/tAKT was lower after 62 h of fasting.

pAS160-thr⁶⁴² increased significantly during both clamps, but lower pAS160-thr⁶⁴² was observed during the clamp after 62 h of fasting.



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Figure 1, panel A pAKT-Ser⁴⁷³ during the basal state ($P = 0.3$) and during the hyperinsulinemic euglycemic clamp (** $P = 0.069$) after 14 h (open box plots) and 62h (grey box plots) of fasting. * $P = 0.012$ and 0.017 for the increase in pAKT-Ser⁴⁷³ during the clamps after 14 and 62 h of fasting respectively.

Figure 1, panel B pAKT-Ser⁴⁷³ in proportion to total AKT (pAKT-ser⁴⁷³/tAKT) ($n = 6$) during the basal state ($P = 0.3$) and during the hyperinsulinemic euglycemic clamp (** $P = 0.028$) after 14 h (open box plots) and 62h (grey box plots) of fasting. * $P = 0.028$ and 0.028 for the increase in pAKT-ser⁴⁷³/tAKT during the clamps after 14 and 62 h of fasting respectively.

Figure 1, panel C pGSK-3-ser⁹ ($n = 6$) during the basal state ($P = 0.3$) and during the hyperinsulinemic euglycemic clamp ($P = 0.3$) after 14 h (open box plots) and 62h (grey box plots) of fasting. * $P = 0.028$ and 0.028 for the increase in pGSK-3-ser⁹ during the clamps after 14 and 62 h of fasting respectively.

Figure 1, panel D pAS160-Thr⁶⁴² during the basal state ($P = 0.7$) and during the hyperinsulinemic euglycemic clamp (** $P = 0.017$) after 14 h (open box plots) and 62h (grey box plots) of fasting. * $P = 0.012$ and 0.012 for the increase in pAS160-Thr⁶⁴² during the clamps after 14 and 62 h of fasting respectively.

pGSK-3-ser⁹ was not different between basal states or clamps, but increased significantly during the clamps.

Discussion

We studied the adaptation to 62 h of fasting in healthy lean men to explore the mechanism underlying the fasting induced decrease in peripheral insulin sensitivity.

Our study confirms reports on lower glucose concentrations, EGP and peripheral insulin sensitivity after fasting (9;10). The lower NOGD after fasting, supports data by Bergman et al (9). However not all studies detected changes in NOGD during fasting (14). If glucose oxidation and NOGD were expressed as percentage of Rd, no differences were found, suggesting that the intracellular fate of glucose remains intact despite decreased peripheral glucose uptake.

To further explore the fasting induced insulin resistance, we examined muscle ceramide. The trend towards increased muscle ceramide levels in the basal state after 62h of fasting suggests a fasting effect, but hinders a definite assumption. Muscle ceramide is mainly derived from de novo synthesis from serine and palmitate (6). We found no correlation of muscle ceramide with plasma FFA levels, suggesting that de novo synthesis is not the denominator of muscle ceramide during short-term fasting (12).

Sphingomyelin hydrolysis, another pathway resulting in ceramide generation, occurs under stress stimuli like TNF α (6). However, we found no changes in plasma sTNF-RI and II. Also, we earlier reported no changes in inflammatory parameters during fasting (10). Therefore, the trend for higher muscle ceramide levels within skeletal muscle after fasting remains unexplained.

AKT is a 56 kD serine/threonine kinase and a mediator of many insulin effects and its regulation is complex (15). To be activated, AKT is translocated to the plasma membrane via its PH domain that binds PI_{3,4,5}P₃, the product of phosphoinositol-3-kinase (PI3K). Here phosphorylation of serine⁴⁷³ by 3-phosphoinositide dependent kinase (PDK) 2 (16) and threonine³⁰⁸ (thr³⁰⁸) by PDK1 occur (15). We found a significant lower ratio pAKT-ser⁴⁷³/tAKT after 62 h of fasting during the clamp but not in the basal state. Bergman et al, reported no difference in pAKT-ser⁴⁷³ and the ratio pAKT-ser⁴⁷³/tAKT between 12 and 48 h of fasting. This may be explained by differences in fasting duration. Remarkably, Bergman et al showed no increase of pAKT-ser⁴⁷³ during hyperinsulinemia as reported earlier (17;18). Intriguingly, lipid infusion in healthy men induces peripheral insulin resistance without effect on pAKT-ser⁴⁷³ (17). Another study found no differences in

Table 1 Glucose kinetics, FFA and glucoregulatory hormones

	basal state		
	14h (n = 8)	62h (n = 8)	P
Glucose oxidation ($\mu\text{mol}/\text{kg}\cdot\text{min}$)	6.8 [3.8 - 14.1]	0.8 [0 - 2.2]	0.012
Glucose (mmol/liter)	5.0 [4.5 - 5.6]	3.7 [3.3 - 4.1]	0.011
EGP ($\mu\text{mol}/\text{kg}\cdot\text{min}$)	11.8 [8.9 - 19.7]	8.3 [7.0 - 8.7]	0.012
Rd ($\mu\text{mol}/\text{kg}\cdot\text{min}$)	-	-	-
NOGD ($\mu\text{mol}/\text{kg}\cdot\text{min}$)	-	-	-
Glucose oxidation (% of Rd)	-	-	-
NOGD (% of Rd)	-	-	-
FFA (mmol/liter)	0.33 [0.19 - 0.95]	1.1 [0.85 - 1.24]	0.015
Insulin (pmol/liter)	30 [18 - 58]	15 [15 - 20]	0.012
Glucagon (ng/liter)	57 [40 - 72]	105 [65 - 148]	0.012
Cortisol (nmol/liter)	291 [223 - 354]	274 [221 - 497]	0.263
Epinephrine (nmol/liter)	0.10 [0.05 - 0.23]	0.18 [0.07 - 0.40]	0.063
Norepinephrine (nmol/liter)	0.52 [0.20 - 0.84]	0.63 [0.20 - 0.92]	0.237
sTNF-RI (ng/ml)	1.2 [1.0 - 1.5]	1.2 [0.9 - 1.3]	0.666
sTNF-RII (ng/ml)	3.6 [2.8 - 4.5]	3.8 [2.7 - 4.9]	0.344

Data are presented as median [minimum - maximum]. EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acids. * During the clamps, EGP and FFA were completely suppressed. NOGD = non-oxidative glucose disposal during the clamp.

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pAKT-ser⁴⁷³ between patients with type 2 diabetes and healthy matched controls (18). This indicates that the impairment in insulin signaling during fasting differs from the impairment during elevation of plasma FFA by lipid infusion or obesity. Whether the lower pAKT-ser⁴⁷³/tAKT during hyperinsulinemia is attributed to the trend to higher muscle ceramide levels in the basal state remains unanswered, since we found no correlation of ceramide levels with peripheral insulin sensitivity in line with previous observations (12;19). Other lipid mediators such as diacylglycerol (DAG) or GM3 may interfere with the insulin signaling cascade (4): fasting increases muscle DAG in animals (11).

AKT phosphorylates both AS160 and GSK. AS160 is involved in the insulin induced translocation of GLUT4; moreover insulin mediated phosphorylation of AS160 was shown to be decreased in patients with type 2 diabetes (18). Phosphorylation of GSK by AKT stimulates glycogen synthesis (20). The equal pGSK-3-ser⁹ during the clamps are in line with the NOGD (as percentage of Rd) and suggests differential regulation of downstream events by AKT since pAS160-thr⁶⁴² and peripheral insulin sensitivity were lower after 62 h of fasting.



hyperinsulinaemic euglycaemic clamp		
14h (n = 8)	62h (n = 8)	P
21.1 [18.1 - 23.3]	13.5 [6.9 - 17.4]	0.012
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
39.7 [23.1 - 56.0]	31.6 [20.0 - 39.6]	0.050
32.4 [26.4 - 49.7]	30.0 [17.0 - 46.4]	0.16
67.6 [50.3 - 73.6]	70.0 [83.0 - 53.6]	0.16
<0.02	<0.02	-
642 [416 - 715]	533 [383 - 663]	0.012
33 [18 - 39]	37 [25 - 60]	0.069
151 [112 - 245]	190 [147 - 281]	0.161
0.13 [0.05 - 0.19]	0.14 [0.05 - 0.23]	0.734
0.60 [0.32 - 0.92]	0.62 [0.18 - 0.91]	0.161
1.3 [1.2 - 1.5]	1.3 [1.0 - 1.5]	0.862
3.6 [2.5 - 5.2]	3.6 [2.7 - 4.6]	0.482



A remarkable finding in our study was the lower insulin levels during the clamp after 62 h of fasting. Insulin infusions were almost identical during both clamps. It is unlikely that endogenous insulin secretion was stimulated at these euglycemic conditions. Plasma clearance of infused insulin is mainly renal in contrast to the first pass effect of endogenous insulin by the liver (21). Earlier studies showed equal insulin levels and lower Rd after fasting, making fasting induced insulin resistance widely accepted (9). The insulin dose response curve negates, that the different plasma insulin levels account for differences in Rd (13).

In conclusion, short-term fasting induces peripheral insulin resistance of glucose uptake while the muscle fate of glucose stays intact. Muscle ceramide tends to increase during fasting. The decreased peripheral glucose uptake is explained by a decrease in pAKT-ser⁴⁷³/tAKT and pAS160-thr⁶⁴² during the clamp.

Since studies in obesity induced insulin resistance and type 2 diabetes mellitus have not shown effects on pAKT-ser⁴⁷³, it is possible that pAKT-ser⁴⁷³ is involved in the physiological adaptation to fasting, inducing a reduction in peripheral glucose uptake and protecting the body from hypoglycemia.



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