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Metabolic and endocrine adaptations to fasting in lean and obese individuals

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Chapter 2

2

Effects of prolonged fasting on AMPK signaling, gene expression and mitochondrial respiratory-chain content in skeletal muscle from lean and obese individuals

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Abstract

Obesity in humans is often associated with metabolic inflexibility but the underlying molecular mechanisms remain incompletely understood. The aim of the present study was to investigate how adaptation to prolonged fasting affects energy/nutrient-sensing pathways and metabolic gene expression in skeletal muscle from lean and obese individuals. Twelve lean and 14 non-diabetic obese subjects were fasted for 48 hours. Whole-body glucose/lipid oxidation rates were determined by indirect calorimetry and blood and skeletal muscle biopsies were collected and analyzed. In response to fasting, body weight loss was similar in both groups but the decrease in plasma insulin and leptin, and the concomitant increase in growth hormone were significantly attenuated in obese subjects. The fasting-induced shift from glucose toward lipid oxidation was also severely blunted. At molecular level, the expression of insulin receptor ß (IRβ) was lower in skeletal muscle from obese subjects at baseline, whereas the fasting-induced reductions in insulin signaling were similar in both groups. The protein expression of mitochondrial respiratory-chain components, although not modified by fasting, was significantly reduced in obese subjects. Some minor differences in metabolic gene expression were observed at baseline and in response to fasting. Surprisingly, fasting reduced AMP-activated protein kinase (AMPK) activity in lean but not in obese subjects, whereas the expression of AMPK subunits was not affected. We conclude that whole-body metabolic inflexibility in response to prolonged fasting in obese humans is associated with lower skeletal muscle IRβ and mitochondrial respiratory-chain content as well as a blunted decline of AMPK activity.

Introduction

Obesity is an endemic metabolic disorder affecting almost half a billion people world-wide. Human beings, as all living organisms, have to constantly adjust their metabolism in response to changes in environmental nutrient availability. Metabolic inflexibility, which reflects the inability to adapt tissue-specific substrate oxidation to whole-body fuel availability, was suggested to be implicated in the development of obesity, insulin resistance and type 2 diabetes [1-4]. Thus, the shift from carbohydrate toward lipid oxidation during the transition from postprandial to fasting state is impaired in obese subjects [4]. Taken together, metabolic inflexibility is manifest in a range of metabolic pathways and tissues, notably in skeletal muscle[5], but little is known about the underlying molecular mechanism(s) [6;7].
To ensure efficient metabolic adaptations to nutritional or environmental changes, various energy/nutrient-sensing pathways are mobilized in peripheral tissues [8]. Among them, the AMP-activated protein kinase (AMPK), a serine/threonine protein kinase, which acts as a cellular energy and nutrient sensor, is believed to play a crucial role in the regulation of tissue-specific substrate metabolism [9-11]. AMPK consists of a heterotrimeric complex containing a catalytic subunit α and two regulatory β and γ subunits. Each subunit has several isoforms encoded by distinct genes, giving multiple heterotrimer combinations with different tissue distribution and cellular localization [9-11]. The α subunit contains a threonine residue (Thr172) whose phosphorylation by upstream AMPK kinases, such as the liver kinase B (LKB1) or calmodulin-dependent protein kinase kinase β (CAMKKβ), is required for AMPK activation. The β subunit acts as a scaffold to which the two other subunits are bound, and contains a carbohydrate binding site which allows AMPK to sense energy reserves in the form of glycogen [9-11]. Binding of AMP and/or ADP to the γ subunit activates AMPK via a complex mechanism involving direct allosteric activation, phosphorylation on Thr172 by AMPKK, and inhibition of dephosphorylation by protein phosphatase(s) that remain to be identified [9-11]. Thus, any change in cellular energy status activates AMPK, leading to concomitant inhibition of energy-consuming processes and stimulation of ATP-generating pathways in order to restore energy balance [9-11]. As a result, glycogen and protein synthesis, as well as cell growth and differentiation, are inhibited, whereas fatty acid (FA) oxidation and glucose uptake are stimulated [9-11]. This regulation involves phosphorylation by AMPK of key metabolic enzymes and transcription factors involved in gene expression [9;11].

The purpose of the present study was to investigate whether metabolic adaptations to prolonged fasting differ in lean and obese individuals and whether this is associated with changes in skeletal muscle AMPK signaling pathway, as previously reported in rodents [12;13].

Materials and Methods

Ethical approval

The present study (Clinical Trial Registration Number: NTR2401) was approved by the Medical Ethical Committee of the Leiden University Medical Centre and performed in accordance with the principles of the revised Declaration of Helsinki. All volunteers gave written informed consent before participation.

Subjects

Twenty-six volunteers, 12 lean (2 males, 10 females, body mass index (BMI) 23.3 ± 0.5 kg/m^2) and 14 obese (2 males, 12 females, BMI 35.2 ± 1.2 kg/m^2) subjects were included. All of them were healthy weight-stable non-smoking Caucasians with a fasting plasma glucose ≤5.6 mmol/l and without family history of diabetes. Height, weight, body mass index (BMI), hip and waist circumference were recorded according to World Health Organization recommendations.

Study design

All participants were admitted to our research center after an overnight fast. The intervention study started after a standardized breakfast (t=0, two slices of brown bread with cheese), followed by 48 hours of fasting. Water and caffeine-free tea were allowed *ad libitum*. To ensure complete adherence to the study, the subjects were kept under supervision in our research center during the whole experimental period. Blood samples were taken after breakfast (t=90 min) and after 24 and 48 hours of fasting by venapuncture in the elbow. Muscle biopsies (~50-75 mg) from *musculus vastus lateralis* were collected after breakfast (t=135 min) and after 48 hours of fasting, as previously described [14].

Indirect calorimetry

Subjects were placed under the ventilated hood after 45 min, 24 hours and 48 hours of fasting (OxyconPro, Mijnhardt Jaegher, The Netherlands). Substrate oxidation was calculated from CO_2 and O_2 concentrations in the exhaled air, as previously described [15].

Laboratory analysis

Serum glucose, total cholesterol, high density lipoprotein (HDL) cholesterol, triglyc-

erides (TG) and c-reactive protein (CRP) were measured on a Modular Analytics P-800 system (Roche Diagnostics, Germany). Low density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation [16]. Serum insulin and insulin-like growth factor 1 (IGF-1) were measured by immunoluminometric assay on an Immulite 2500 automated system (Siemens Healthcare Diagnostics, The Netherlands). Cortisol, free T4 (FT4) and thyroid stimulating hormone (TSH) were measured by electrochemoluminescence immunoassay on a Modular Analytics E-170 system (Roche Diagnostics, Germany). Triiodothyronine (T3) was measured with by fluorescence polarization immunoassay on an AxSym system (Abbott, US). Growth hormone (GH) was measured by immunofluorometric assay (Wallac, Finland). Serum active ghrelin, leptin and adiponectin were determined by radioimmunoassay (Millipore, USA).

Western Blot

Skeletal muscle biopsies (~30-45 mg) were homogenized by Ultra-Turrax (22 000 rpm; 2x5 sec) in a 6:1 (v/w) ratio of ice-cold buffer containing: 50 mM HEPES (pH 7.6), 50 mM NaF, 50 mM KCl, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 5 mM β-GP, 1 mM Na3VO4, 1 mM DTT, 1% NP40 and protease inhibitors cocktail (Complete, Roche, The Netherlands). Western blots were performed using phospho-specific (Ser473-PKB, phospho-Akt substrate and Thr172-AMPKα from Cell Signaling; Thr246-PRAS40 from Biosource; Tyr612-IRS1 from Invitrogen) or total primary antibodies (Tubulin and AMPKβ1+2 from Cell Signaling; AMPKα1 and AMPKα2 from Kinasource; AMPKγ1, PGC-1α and MitoProfile OXPHOS from AbCam; AMPKγ3, IRβ and LKB1 from Santa Cruz; CAMKKα/β from BD Biosciences; AMPKα2 truncated is a kind gift of Pr. DG Hardie), as previously described[17].

AMPK activity

AMPK heterotrimeric complexes were immunoprecipitated from 500 µg of muscle lysate using protein A-agarose beads (GE Healthcare, The Netherlands) and a pan α-specific AMPK antibody (Santa Cruz) incubated together at 4°C overnight on a rotating wheel. After incubation, the immunoprecipitate was washed twice in ice-cold lysis buffer without NP40 and once in ice-cold assay buffer containing: 50 mM HEPES (pH 7.2), 80 mM KCl, 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, 1% glycerol and 1 mg/ml FFA-free BSA. The reaction was initiated at 30°C by addition of a mixture of 200 µM AMP, 200 µM SAMS-peptide (kind gift from Dr. L. Bertrand, Brussels, Belgium), 100 µM Mg-ATP, and 2 µCi of [γ-32P]ATP (PerkinElmer, The Netherlands). After 15 min,

the beads were briefly spun down and 20 µl of supernatant was spotted onto P81 filter paper (Whatman, GE Healthcare, The Netherlands). After extensive washing in 1% phosphoric acid, the filter paper was dried and analyzed for radioactivity using a Tri-Carb Liquid Scintillation Counter (Packard, The Netherlands). The non-specific activity (without SAMS-peptide) was subtracted from the specific kinase activity, which was expressed in mU/mg protein.

RNA isolation and real-time RT-PCR

Total RNA was isolated from skeletal muscle biopsies (~25-30 mg) using the phenol-chloroform extraction method (Tripure RNA Isolation reagent, Roche, Germany) and quantified by NanoDrop. First-strand cDNA were synthesized from 1 µg total RNA using a Superscript first strand synthesis kit (Invitrogen, The Netherlands). Real-time PCR assays were performed using specific primers sets (sequences provided on request) and SYBR Green on a StepOne Plus Real-time PCR system (Applied Biosystems, US). mRNA expression was normalized to ribosomal protein S18 (*Rps18*) and expressed as arbitrary units.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). For metabolic parameters and indirect calorimetry, a mixed model was used to determine the effects of fasting in between groups. For Western blot and RT-qPCR, the data were analyzed with unpaired or paired t-tests for determining the baseline differences and within group effects, respectively. All the statistical analysis were performed using SPSS for Windows version 18.0 (SPSS Inc., US).

Results

Effects of fasting on body weight, body composition and metabolic parameters in lean and obese subjects

The anthropometric and metabolic characteristics of the subjects were determined at baseline (post-meal), *i.e.* ~90 min after a standardized breakfast (300 Kcal), and after 24 or 48h of fasting (Table 1). At baseline, body weight, BMI and waist-to-hip ratio were significantly higher in obese than in lean subjects. Plasma glucose, insulin, leptin and triglycerides levels were also elevated in the obese group (+11%, +148%, +204% and +40%, respectively; $p<0.05$), reflecting whole-body insulin/leptin resist-

Table 1. Anthropometric and metabolic parameters at baseline and during fasting in lean and obese subjects

	Lean			Obese			*Interaction p-value*
	Post-meal	24h fast	48h fast	Post-meal	24h fast	48h fast	
Age (years)	27 ± 3			30 ± 3			
Length (cm)	175 ± 3			174 ± 2			
Weight (kg)	72 ± 3	nd	69 ± 3*	107 ± 4#	nd	104 ± 4*	*0.20*
Body mass index (kg/m^2)	23.3 ± 0.5	nd	22.5 ± 0.5*	35.2 ± 1.2#	nd	34.4 ± 1.2*	*0.94*
Waist-Hip ratio	0.80 ± 0.02	nd	nd	0.87 ± 0.01#	nd	nd	
Glucose (mmol/l)	4.5 ± 0.2	4.2 ± 0.2	3.5 ± 0.1*	5.0 ±0.2#	4.7 ± 0.1	4.0 ± 0.2*	*0.92/0.94*
Insulin (mU/l)	7.9 ± 2.0	1.0 ± 0.1*	1.2 ± 0.2*	19.6 ± 2.1#	4.7 ± 1.4*	3.2 ± 0.8*	*0.00/0.00*
Growth Hormone (mU/l)	0.9 ± 0.3	nd	9.0 ± 2.0*	0.7 ± 0.4	nd	3.1 ± 0.6*	*0.01*
IGF-1 (nmol/l)	20.1 ± 2.1	nd	20.2 ± 1.8	17.4 ± 2.0	nd	19.5 ± 2.2	*0.35*
Leptin (µg/l)	11.9 ± 1.8	6.6 ± 1.2*	4.1 ± 0.4*	36.2 ± 3.6#	28.2 ± 3.5*	20.6 ± 3.2*	*0.37/0.01*
Adiponectin (µg/l)	11.1 ± 1.2	nd	10.5 ± 1.2	7.6 ± 1.0#	nd	7.4 ± 0.9	*0.61*
Ghrelin (pg/ml)	168 ± 27	nd	198 ± 34	184 ± 45	nd	228 ± 53	*0.87*
Cholesterol (mmol/l)	4.7 ± 0.3	nd	5.0 ± 0.3*	5.0 ± 0.2	nd	5.3 ± 0.3*	*0.86*
Triglycerides (mmol/l)	1.0 ± 0.1	nd	1.0 ± 0.1	1.4 ± 0.1#	nd	1.4 ± 0.1	*0.63*
HDL (mmol/l)	1.5 ± 0.1	nd	1.6 ± 0.1	1.4 ± 0.1	nd	1.4 ± 0.1	*0.41*
LDL (mmol/l)	2.7 ± 0.2	nd	3.0 ± 0.2*	3.0 ± 0.1	nd	3.4 ± 0.2*	*0.43*
TSH (mU/l)	1.6 ± 0.2	1.2 ± 0.1*	1.0 ± 0.1*	2.0 ± 0.1	1.4 ± 0.2*	1.7 ± 0.3	*0.75/0.10*
T3 (nmol/l)	1.8 ± 0.1	1.6 ± 0.1*	1.3 ± 0.1*	2.1 ± 0.1	1.9 ± 0.1*	1.7 ± 0.1*	*0.82/0.11*
FT4 (pmol/l)	15.4 ± 0.5	nd	16.0 ± 0.7	14.5 ± 0.5	nd	15.0 ± 0.6	*0.96*
CRP (mg/l)	1.3 ± 0.3	nd	5.0 ± 1.1*	5.1 ± 1.0	nd	9.9 ± 2.4*	*0.55*
Cortisol (µmol/l)	0.38 ± 0.07	nd	0.61 ± 0.08*	0.37 ± 0.06	nd	0.49 ± 0.08*	*0.18*

Data are shown as mean ± SEM, n=12-14. *, p<0.05 vs basal ; #, p<0.05 vs lean subjects; nd, not determined.
Interaction p-value between post-meal and 24 hours/post-meal and 48 hour

ance and glucose intolerance. In response to fasting, body weight was decreased to the same extent in obese and lean subjects (-2.8% *vs* -3.5%, respectively; p<0.05). As expected, fasting induced a significant decrease in plasma glucose, insulin, leptin, T3 and TSH levels, whereas circulating total cholesterol, LDL, growth hormone, CRP and cortisol increased in both groups (Table 1). However, the fasting-induced changes in plasma insulin (at 24 and 48h: -76% and -84% *vs* -87% and -85% in obese and lean, respectively; p<0.01), growth hormone (at 48h: +343% *vs* +900% for obese and lean, respectively; p<0.01) and leptin (at 24 and 48h: -22% and -43% *vs* -45% and -66% in obese and lean, respectively; p<0.01) levels were significantly attenuated in obese subjects (Table 1).

Effects of fasting on whole-body glucose and lipid oxidation rates in lean and obese subjects

The substrate oxidation rates were determined by indirect calorimetry at baseline and after 24 and 48 hours of fasting (Table 2). At baseline, the absolute resting energy expenditure (REE, in Kcal/day) was significantly higher in obese than in lean subjects (+24%; p<0.05), an effect that however disappeared when the data are corrected for lean body mass (Kcal/day/Kg FFM). This metabolic feature is associated with higher lipid (+62%) and lower glucose (-22%; p<0.05) oxidation rates. Fasting led to significant decrease in respiratory quotient (RQ), indicating a shift in substrate metabolism from glucose toward lipid oxidation in both groups (Table 2). However, these fasting-induced changes were significantly attenuated in the obese subjects, with a lower reduction in glucose oxidation (at 24 and 48h: -38% and -60% vs -53% and -70% in obese and lean, respectively; p<0.01) and a lesser increase in lipid oxidation (at 24 and 48h: +41% and +76% vs +133% and +214% in obese and lean, respectively; p<0.01). In addition, the REE was also differently affected in response to fasting between groups, with a significant decrease only evidenced in the obese group (at 48h: -7% vs +3% in obese and lean, respectively; p<0.01) (Table 2).

Effect of fasting on insulin signaling pathways in human skeletal muscle from lean and obese subjects

The protein expression and phosphorylation state of key molecules involved in the insulin signaling pathway were determined in skeletal muscle biopsies at baseline and after 48h of fasting (Figure 1). Tubulin expression, used as a housekeeping protein, was similar between lean and obese subjects at baseline and was not affected

Table 2. Substrate oxidation rates at baseline and during fasting in lean and obese subjects

	Lean			Obese			***Interaction p-value***
	Post-meal	24h fast	48h fast	Post-meal	24h fast	48h fast	
Resting energy expenditure (kcal/day)	1502 ± 68	1444 ± 70	1546 ± 83	1856 ± 63#	1734 ± 59*	1719 ± 55*	*0.24/0.00*
Resting energy expenditure (kcal/kgFFM/day)	29.7 ± 1.6	28.6 ± 1.4	30.7 ± 1.2	30.4 ± 0.8	28.6 ± 0.9*	28.6 ± 0.9*	*0.46/0.01*
Respiratory quotient	0.94 ± 0.03	0.82 ± 0.02*	0.77 ± 0.02*	0.87 ± 0.01#	0.82 ± 0.02*	0.78 ± 0.01*	*0.03/0.01*
Lipid oxidation (µmol/kgFFM/min)	2.1 ± 0.6	4.9 ± 0.6*	6.6 ± 0.7*	3.4 ± 0.4	4.8 ± 0.6*	5.9 ± 0.5*	*0.09/0.02*
Glucose oxidation (µmol/kgFFM/min)	23.6 ± 2.8	11.0 ± 1.4*	7.1 ± 1.6*	18.3 ± 1.6#	11.4 ± 1.6*	7.4 ± 1.4*	*0.07/0.08*

Data are shown as mean ± SEM, n=12-14. *, p<0.05 vs basal ; #, p<0.05 vs lean subjects; kgFFM, kilogram fat free mass.
Interaction p-value between post-meal and 24 hours/post-meal and 48 hours.

by fasting (Figure 1A). By contrast, the insulin receptor β (IRβ) expression was significantly lower in obese when compared to lean subjects at baseline (Figure 1B, -18%; p=0.02), but its increase in response to fasting was similar in both groups (+17% and +18% in lean and obese, respectively; p<0.05). Downstream IRβ, the phosphorylation of insulin-receptor substrate (IRS) 1, protein kinase B (PKB, also called Akt) and of PKB downstream targets Proline-Rich Akt Substrate of 40kDa (PRAS40) and Akt Substrate of 160 kDa (AS160) on key regulating residues were similar between lean and obese subjects at baseline (Figure 1C-F). In line with the decrease in plasma insulin levels, the phosphorylation states of these proteins were similarly reduced after 48h fast in both groups. Of note, protein expression of IRS1, PKB, PRAS40 and AS160 were not affected whatever the conditions (data not shown).

Effect of fasting on AMPK expression and signaling in human skeletal muscle from lean and obese subjects

We next assessed whether prolonged fasting affects the protein expression and phosphorylation state of AMPKα on its activating Thr172 residue. AMPKα expression was similar in lean and obese subjects whatever the condition (Figure 2A). Surprisingly, AMPK-Thr172 phosphorylation, which was not different between groups at baseline, was reduced by fasting in lean (-19%, p=0.08) but not in obese (-1%, p=0.90) individuals (Figure 2B-

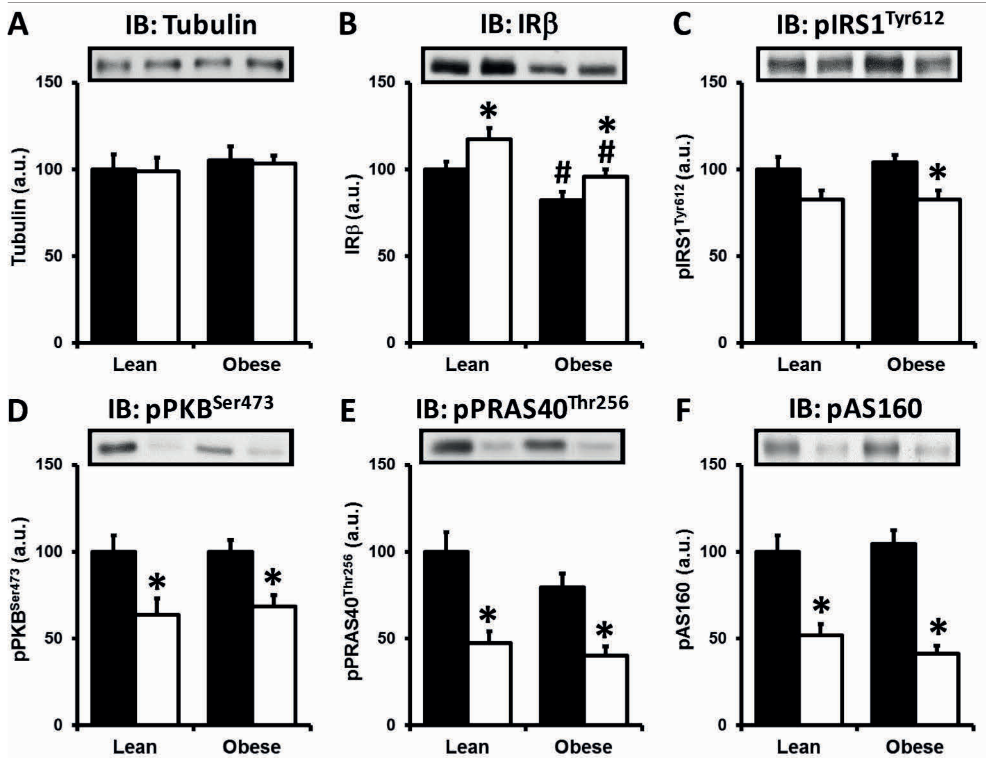

Figure 1. Effect of fasting on insulin signaling pathways in skeletal muscle from lean and obese subjects.
The expression of tubulin (A), insulin receptor β (B) and the phosphorylation states of Tyr612-IRS1 (C), Ser473-PKB (D), Thr256-PRAS40 (E) and phospho-AS160 (F) were assessed by Western Blot in skeletal muscle from lean and obese subjects before (black bars) and after 48h of fasting (open bars). Representative blots for one subject per group are shown. Results are normalized to lean subjects and expressed as mean ± SEM; n=12-14; *p<0.05 compared with baseline, #p<0.05 compared with lean subjects.

C). This borderline significant trend was confirmed by determination of AMPK activity using a kinase assay (Figure 2D). Furthermore, the fasting-induced change in the phosphorylation state of acetyl-CoA carboxylase (ACC) at Ser221, one of the main AMPK downstream target, followed the same pattern although not reaching a significant threshold (-18% in lean vs +4% in obese, p=0.07 and p=0.72, respectively). Although some differences were observed at the mRNA levels (Table 3), the protein expression of the different isoforms of AMPK catalytic α and regulatory β and γ subunits were similar at baseline, except for the AMPKγ2 short isoform that was significantly lower in obese when compared to lean subjects (Figure 3). The expression of all these AMPK subunits was not affected by fasting in either group, suggesting that the difference in AMPK response between lean and obese is unlikely due to changes

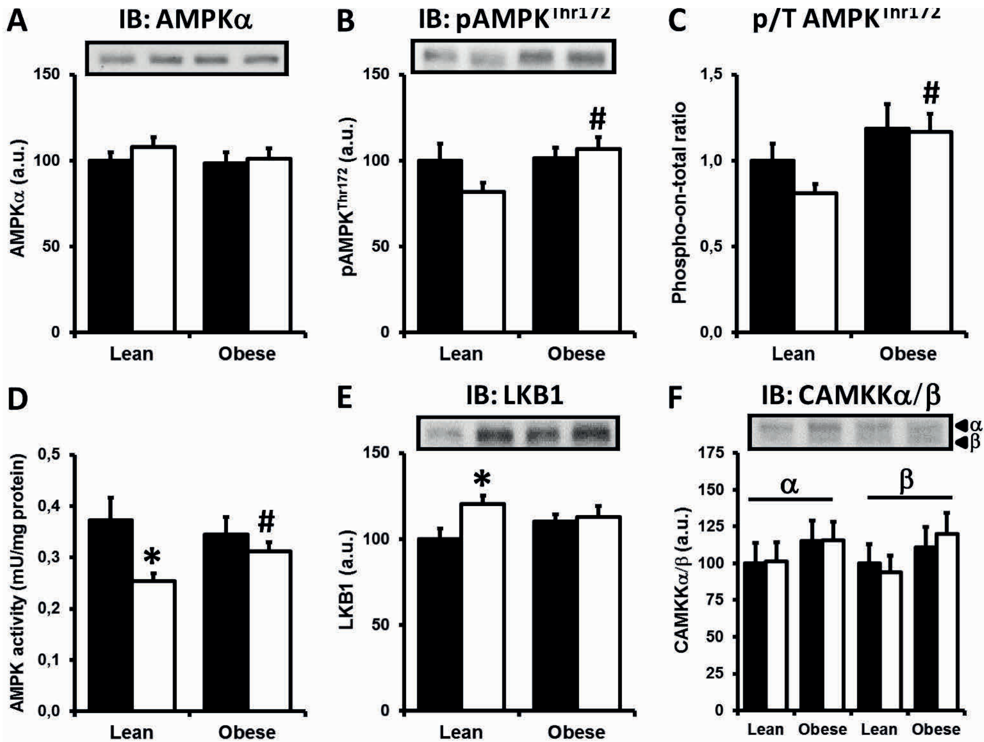

Figure 2. Effect of fasting on AMPK signaling in skeletal muscle from lean and obese subjects. The phosphorylation state of AMPKα on Thr172 (B) and the expression of AMPK(pan)α (A), LKB1 (E) and CAMKKα/β (F) were assessed by Western Blot in skeletal muscle from lean and obese subjects before (black bars) and after 48h of fasting (open bars). The phospho-on-total ratio for AMPKα-Thr172 was calculated (C). AMPK activity was determined by kinase assay following immunoprecipitation of the AMPK heterotrimer using a pan α antibody (D). Representative blots for one subject per group are shown. Results are normalized to lean subjects and expressed as mean ± SEM; n=12-14; *p<0.05 compared with baseline, #p<0.05 compared with lean subjects.

in heterotrimer composition. Of note, the protein expression of the AMPK upstream kinase LKB1, but not of CAMKKα/β, was significantly increased by fasting only in lean subjects (Figure 2E-F).

Effect of fasting on metabolic genes expression in human skeletal muscle from lean and obese subjects

The mRNA expression of key genes involved in glucose and lipid metabolism were determined in skeletal muscle from lean and obese subjects. At baseline, transcript levels of HK1, PKM2, PPARA, CD36, ACACA, ATP2A1, ACADM, ACOX3 and PDK4 were significantly higher in obese when compared to lean individuals, whereas LPL mRNA expression was found to be significantly lower (Table 4). Prolonged fasting in-

Table 3. Effects of fasting on mRNA expression of AMPK kinases and AMPK subunits in skeletal muscle from lean and obese subjects

Gene name	Gene symbol	Entrez gene	Lean		Obese		*Interaction p-value*
			Post-meal	48h fast	Post-meal	48h fast	
Liver Kinase B1 (LKB1)	*STK11*	6794	1.0 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	1.4 ± 0.3	*0.33*
Calcium/calmodulin-dependent protein kinase kinase a (CAMKK a)	*CAMKK1*	10645	1.0 ± 0.2	1.8 ± 0.6	1.0 ± 0.6	2.0 ± 2.0	*0.92*
Calcium/calmodulin-dependent protein kinase kinase b (CAMKK b)	*CAMKK2*	84254	1.0 ± 0.1	1.2 ± 0.2	1.1 ± 0.3	1.1 ± 0.5	*0.41*
AMP-activated protein kinase α1	*PRKAA1*	5562	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	*0.68*
AMP-activated protein kinase α2	*PRKAA2*	5563	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.2*	*0.27*
AMP-activated protein kinase β1	*PRKAB1*	5564	1.0 ± 0.1	1.3 ± 0.2*	1.0 ± 0.1	1.2 ± 0.1*	*0.82*
AMP-activated protein kinase β2	*PRKAB2*	5565	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	*0.76*
AMP-activated protein kinase γ1	*PRKAG1*	5571	1.0 ± 0.1	2.0 ± 0.4*	1.7 ± 0.2#	1.6 ± 0.3	*0.06*
AMP-activated protein kinase $\gamma 2^{ALL}$	*PRKAG2*	51422	1.0 ± 0.2	0.7 ± 0.2*	1.1 ± 0.1	0.7 ± 0.0*	*0.70*
AMP-activated protein kinase $\gamma 2^{LONG}$	*PRKAG2*	51422	1.0 ± 0.1	1.6 ± 0.4*	1.0 ± 0.1	1.5 ± 0.3*	*0.68*
AMP-activated protein kinase γ3	*PRKAG3*	53632	1.0 ± 0.2	0.8 ± 0.2	1.8 ± 0.7	1.0 ± 0.4*	*0.00*

Data are shown as mean ± SEM, n=8-6. *, p<0.05 vs post-meal; #, p<0.05 vs lean subjects

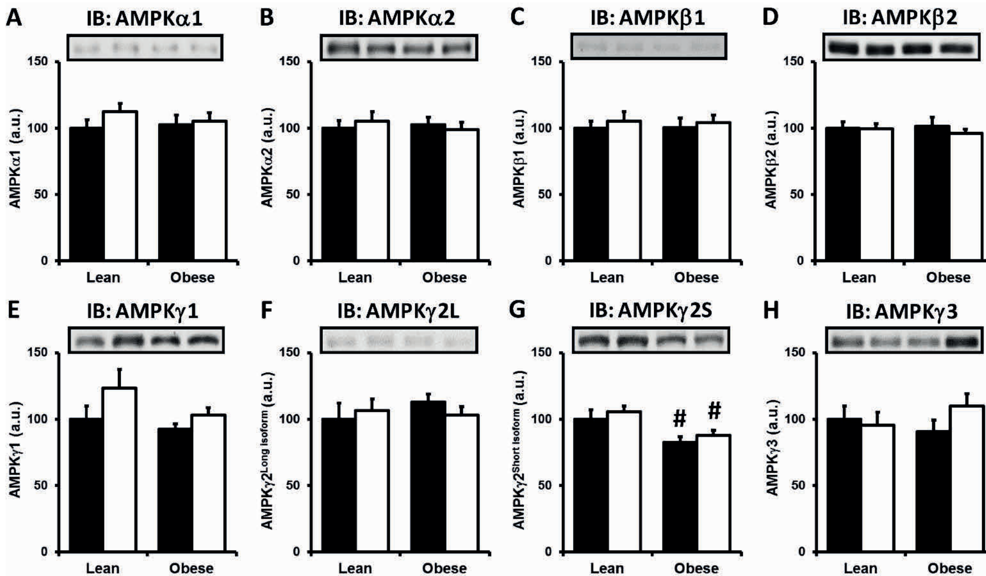

Figure 3: Effect of fasting on protein expression of AMPK regulatory subunits in skeletal muscle from lean and obese subjects.
The expression of AMPKα1 (A), AMPKα2 (B), AMPKβ1 (C), AMPKβ2 (D), AMPKγ1 (E), AMPKγ2 (long isoform, F), AMPKγ2 (short isoform, G), and AMPKγ3 (H) were assessed by Western Blot in skeletal muscle from lean and obese subjects before (black bars) and after 48h of fasting (open bars). Representative blots for one subject per group are shown. Results are normalized to lean subjects and expressed as mean ± SEM; n=12-14; *p<0.05 compared with baseline, #p<0.05 compared with lean subjects.

duces significant upregulation of INSR, PDK4, PFKFB3 and UCP3, and downregulation of HK2 and PPARGC1A mRNA expression in lean subjects (Table 4), in line with previous studies [18-21]. Furthermore, we also report that SLC2A4, PKM2, CD36, ACSL1, NDUFB8 and ACAT2 were significantly reduced in response to fasting. However, among these key metabolic transcription factors and genes only ACAT2, SLC2A1 and NRF1, were shown to be differentially affected by fasting in lean and obese individuals (Table 4).

Effect of fasting on PGC-1α and mitochondrial respiratory-chain components expression in human skeletal muscle from lean and obese subjects

Finally, the protein expression of PGC-1α, a key transcription factor involved in mitochondrial biogenesis, and of several mitochondrial respiratory-chain complex subunits were measured in skeletal muscle from lean and obese subjects (Figure 4). At baseline, we found that although PGC-1α expression was similar in both groups, all the respiratory-chain subunits were significantly lower in the obese when compared to

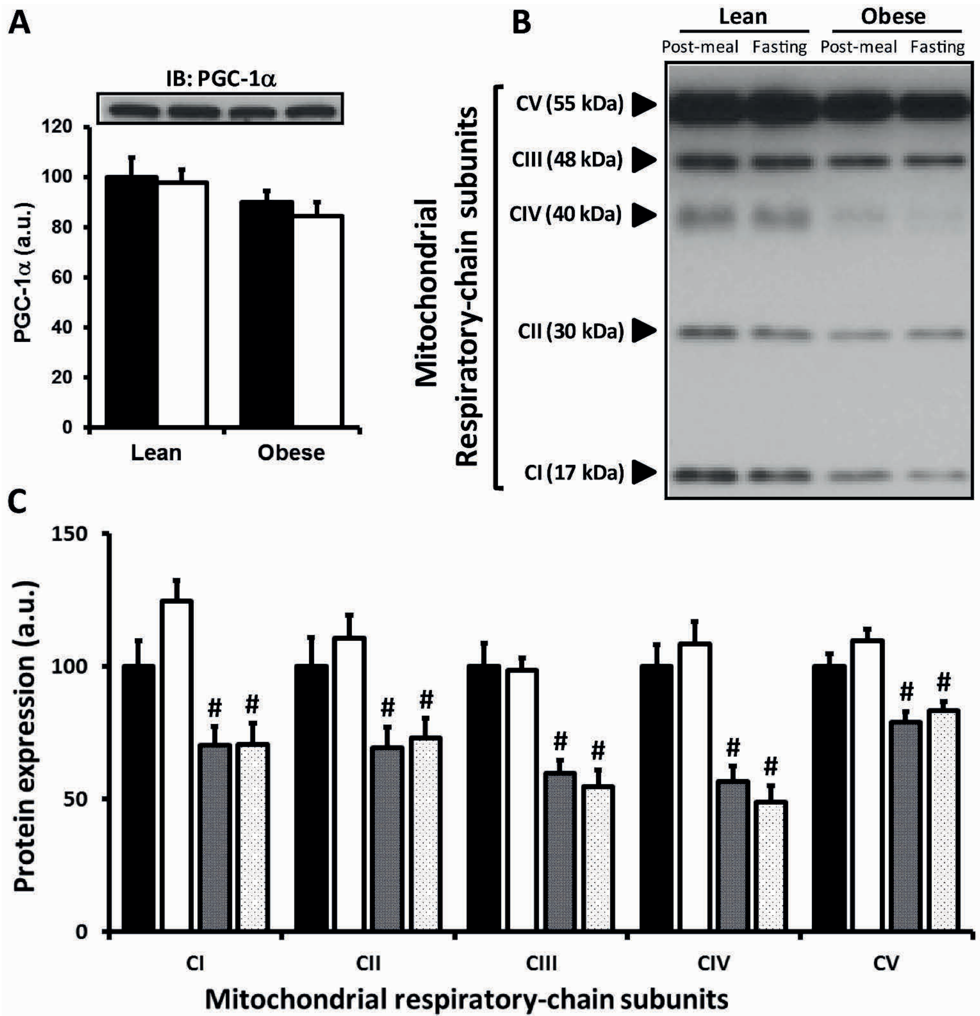

Figure 4: Effect of fasting on mitochondrial respiratory-chain subunits expression in skeletal muscle from lean and obese subjects.

The expression of PGC-1α (A) and various mitochondrial respiratory-chain subunits (CI: NDUFB8; CII: SDHB; CIII: UQCRC2; CIV: MTCO1; CV:ATP5A) were assessed by Western Blot in skeletal muscle before (closed bars) and after 48h of fasting (open bars) in lean and obese subjects (black and grey bars, respectively). Representative blots are shown. Results are normalized to lean subjects and expressed as mean ± SEM; n=12-14; *p<0.05 compared with baseline, #p<0.05 compared with lean subjects.

lean subjects (-30%, -30%, -40%, -43% and -21% for CI to V, respectively; $p<0.05$). The expression of PGC-1α and mitochondrial proteins were not significantly affected by fasting in both groups, although a trend for a specific increase in the respiratory-chain complex 1 subunit (+25%; $p=0.06$) was observed in skeletal muscle from lean subjects (Figure 4).

Table 4. Effects of fasting on metabolic gene expression in skeletal muscle from lean and obese subjects

Gene name	Gene symbol	Entrez gene	Lean		Obese		*Interaction p-value*
			Post-meal	48h fast	Post-meal	48h fast	
Glucose metabolism							
Transcription factors							
Carbohydrate-responsive element-binding protein (ChREBP)	*MLXIPL*	51085	1.0 ± 0.1	0.9 ± 0.3	1.0 ± 0.2	0.8 ± 0.1	*0.91*
Glucose transport and phosphorylation							
Insulin receptor	*INSR*	3643	1.0 ± 0.1	1.6 ± 0.3	1.0 ± 0.1	1.6 ± 0.2*	*0.84*
Akt substrate of 160 kDa (AS160)	*TBC1D4*	9882	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.1#	1.6 ± 0.2*	*0.91*
TBC1D1	*TBC1D1*	23216	1.0 ± 0.2	1.0 ± 0.2	1.4 ± 0.2#	1.4 ± 0.4	*0.88*
Solute carrier family 2, member 1 (GLUT-1)	*SLC2A1*	6513	1.0 ± 0.2	1.1 ± 0.3	1.0 ± 0.3	2.2 ± 0.6*	*0.05*
Solute carrier family 2, member 4 (GLUT-4)	*SLC2A4*	6517	1.0 ± 0.1	0.7 ± 0.2*	1.0 ± 0.1	0.7 ± 0.1*	*0.64*
Hexokinase 1	*HK1*	3098	1.0 ± 0.1	1.3 ±0.2	1.4± 0.2#	1.5 ± 0.2	*0.44*
Hexokinase 2	*HK2*	3099	1.0 ± 0.2	0.4 ± 0.2*	0.9 ± 0.2	0.3 ± 0.1*	*0.86*
Glycolysis							
Phosphofructokinase	*PFKM*	5213	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	*0.76*
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	*PFKFB3*	5209	1.0 ± 0.2	7.2 ± 2.6*	2.0 ± 0.8	9.7 ± 5.3*	*0.44*
Pyruvate kinase	*PKM2*	5315	1.0 ± 0.1	0.7 ± 0.2*	1.5 ± 0.3#	0.8 ± 0.1*	*0.31*
Glycogen metabolism							
Glycogen synthase 1	*GYS1*	2997	1.0 ± 0.3	1.0 ± 0.3	1.3 ± 0.6	0.7 ± 0.5	*0.17*
Glycogen phosphorylase	*PYGM*	5837	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	*0.61*
Fatty acid metabolism							
Transcription factors							
Peroxisome proliferator-activated receptor α (PPARα)	*PPARA*	5465	1.0 ± 0.1	0.9 ± 0.2	1.3 ± 0.2	0.9 ± 0.1*	*0.21*
Peroxisome proliferator-activated receptor δ (PPARβ/δ)	*PPARD*	5467	1.0 ± 0.1	0.9 ± 0.3	1.2 ± 0.1	1.1 ± 0.2	*0.76*
Fatty acid transport and activation							
Lipoprotein lipase	*LPL*	4023	1.0 ± 0.2	0.7 ± 0.2*	0.7 ± 0.1#	0.6 ± 0.1	*0.20*
Fatty acid translocase/CD36	*CD36*	948	1.0 ± 0.1	0.7 ± 0.1*	1.2 ± 0.1#	0.9 ± 0.1*	*0.17*
Fatty acid binding protein 3	*FABP3*	2170	1.0 ± 0.1	0.9 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	*0.72*
Acyl-CoA synthetase long-chain family member 1	*ACSL1*	2180	1.0 ± 0.1	0.7 ± 0.1*	1.3 ± 0.1	0.7 ± 0.1*	*0.80*
Acyl-CoA synthetase short-chain family member 2	*ACSS2*	55902	1.0 ± 0.1	1.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	*0.17*
Acetyl-CoA carboxylase α	*ACACA*	31	1.0 ± 0.1	1.5 ± 0.3	1.4 ± 0.2#	1.5 ± 0.2	*0.31*
Acetyl-CoA carboxylase β	*ACACB*	32	1.0 ± 0.1	1.5 ± 0.4	1.1 ± 0.2	1.2 ± 0.3	*0.40*

Gene name	Gene symbol	Entrez gene	Lean		Obese		Interaction *p-value*
			Post-meal	48h fast	Post-meal	48h fast	
Malonyl-CoA decarboxylase	*MLYCD*	23417	1.0 ± 0.1	1.2 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	*0.87*
Mitochondrial FA transport							
Carnitine palmitoyltransferase 1A	*CPT1A*	1374	1.0 ± 0.1	1.0 ±0.1	1.2 ± 0.2	1.2 ± 0.2	*0.76*
Carnitine palmitoyltransferase 1B	*CPT1B*	1375	1.0 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	*0.77*
Carnitine palmitoyltransferase 2	*CPT2*	1376	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.2	*0.19*
Mitochondrial metabolism							
Mitochondrial biogenesis							
PPARγ, coactivator 1α (PGC-1α)	*PPARGC1A*	10891	1.0 ± 0.2	0.6 ± 0.1*	1.3 ± 0.2	0.6 ± 0.1*	*0.29*
PPARγ, coactivator 1β (PGC-1β)	*PPARGC1B*	133522	1.0 ± 0.2	1.0 ± 0.3	1.2 ± 0.1	1.1 ± 0.1	*0.69*
Transcription factor A	*TFAM*	7019	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	0.8 ± 0.1	*0.60*
Nuclear respiratory factor 1	*NRF1*	4899	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.3*	*0.04*
TCA cycle & electron transport chain							
Pyruvate dehydrogenase kinase 4	*PDK4*	5166	1.0 ± 0.3	5.6 ± 1.6*	1.9 ± 0.7#	4.7 ± 1.2	*0.24*
Pyruvate carboxylase	*PC*	5091	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	*0.19*
Citrate synthase	*CS*	1431	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	*0.67*
Succinate dehydrogenase complex, subunit A	*SDHA*	6389	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	*0.40*
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8	*NDUFB8*	4714	1.0 ± 0.1	0.8 ± 0.1*	1.0 ± 0.1	0.8 ± 0.1*	*0.57*
ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	*ATP2A1*	487	1.0 ± 0.1	1.1 ± 0.3	1.5 ± 0.3#	1.2 ± 0.3	*0.83*
Uncoupling protein 3	*UCP3*	7352	1.0 ± 0.2	3.4 ±0.8*	1.7 ± 0.6	3.4 ± 0.7*	*0.85*
FA β-oxidation							
Acyl-CoA dehydrogenase, C-2 to C-3 short chain	*ACADS*	35	1.0 ± 0.1	1.1 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	*0.83*
Acyl-CoA dehydrogenase, short/branched chain	*ACADSB*	36	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	*0.98*
Acyl-CoA dehydrogenase, very long chain	*ACADVL*	37	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	*1.00*
Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	*ACADM*	34	1.0 ± 0.2	0.9 ± 0.2	1.2 ± 0.0#	1.0 ± 0.1	*0.70*
Acyl-Coenzyme A oxidase 1	*ACOX1*	51	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	*0.80*
3-ketoacyl-Coenzyme A thiolase, α	*HADHA*	3030	1.0 ± 0.1	1.3 ± 0.3	1.2 ± 0.2	1.6 ± 0.2*	*0.95*
Ketone body metabolism							
Acetyl-CoA acetyltransferase 2	*ACAT2*	39	1.0 ± 0.1	0.8 ± 0.1*	1.0 ± 0.1	1.1 ± 0.1	*0.04*
Acyl-Coenzyme A oxidase 3	*ACOX3*	8310	1.0 ± 0.1	1.4 ± 0.2	1.2 ± 0.1#	1.1 ± 0.1	*0.08*
3-hydroxy-3-methylglutaryl-CoA synthase 2	*HMGCS2*	3158	1.0 ± 0.4	5.0 ± 2.5	1.7 ± 0.7	4.9 ± 1.3*	*0.73*

Data are shown as mean ± SEM, n=8-6. *, p<0.05 vs post-meal; #, p<0.05 vs lean subjects

2

Discussion

By contrast to the vast literature available on the signal transduction pathways involved in metabolic adaptations to exercise in human skeletal muscle, only few systematic studies have been conducted to investigate the molecular processes triggered by fasting, especially in individuals with metabolic disorders. To our knowledge, this study is the first to report the effects of prolonged fasting on whole-body substrate oxidation rates in relation with changes in signal transduction pathways and metabolic gene expression in skeletal muscle from both lean and obese subjects.

The physiological adaptation to fasting is generally marked by increased lipolysis, ketone body synthesis and lipid oxidation, and a concomitant decrease in glucose uptake and oxidation by peripheral tissues [7]. In lean individuals, we observed several of these well-known effects on whole-body substrate metabolism together with decreased plasma levels of glucose, insulin and leptin, and concomitant increase in GH. Not surprisingly, our obese subjects exhibited elevated levels of glucose, insulin, TG and leptin and lower circulating adiponectin at baseline when compared to lean individuals, and a marked whole-body metabolic inflexibility characterized by impaired fasting-induced switch from glucose toward FA oxidation. In addition, the decrease in plasma leptin and insulin levels in response to fasting was also blunted, in line with previous reports [22-24]. Among the limitations to the interpretation of these data, it should be mentioned that it cannot be totally excluded that some of the baseline differences between lean and obese individuals might partly reflect impaired metabolic adaptation to the initial standardized breakfast taken by the subjects. However, Labayen *et al.* have also reported a lower RQ and higher lipid oxidation rate at baseline in obese women after an overnight fast when compared to lean individuals [25], suggesting that these fundamental differences are independent to meal response. Interestingly, we found that the protein expression of the insulin receptor β (IRβ) was reduced at baseline in skeletal muscle from obese when compared to lean individuals. However, the IRβ protein expression was similarly increased in response to fasting in both lean and obese individuals, likely reflecting a compensatory feedback mechanism triggered by reduced plasma insulin levels. In contrast to our findings, Bergman *et al.* have found no effect of 48h-fasting on the expression of the insulin receptor [26]. This discrepancy might be explained by the fact that their baseline samples were collected after an overnight fast, a nutritional condition that might be sufficient to already induce

IR expression. Of note, IRβ downstream signaling, reflected by the phosphorylation state of IRS1, PKB, PRAS40 and AS160, was similarly reduced by fasting in both groups.

The fasting-induced increase in plasma GH levels is believed to play an important role in the regulation of whole-body substrate metabolism, notably by inhibiting glucose uptake and enhancing lipid oxidation in skeletal muscle [27]. Interestingly, we found that the change in plasma GH levels was significantly different between groups, with a much larger increase in lean compared to obese individuals. This result is in line with our previous findings showing that obese women exhibit lower plasma GH concentrations in response to a 20h-fast compared to normal weight women [28], suggesting that GH hyporesponsiveness may contribute to metabolic inflexibility. At present, it is unclear to which extent the fasting-induced increase in GH modulates signaling pathways involved in the regulation of glucose/lipid metabolism in human skeletal muscle. However, it has recently been shown that fasting induced similar up-regulation of lipid oxidation genes in skeletal muscle from wild-type and GH receptor knockout obese mice, suggesting that GHR signaling is likely not required for the control of lipid oxidation during fasting [29]. Among the fasting-induced changes in skeletal muscle metabolic gene expression observed in our study, some were previously reported, such as upregulation of PDK4 and UCP3, and are likely contributing to the whole-body shift from glucose to lipid oxidation in humans [18-21;30-32]. However, no major differences in transcriptional regulation in response to fasting were found between lean and obese individuals.

One of our initial hypotheses was that the nutrient/energy sensing AMPK pathway is activated by fasting in skeletal muscle and subsequently triggers metabolic adaptations to food deprivation. Indeed, although most of its established functions came from *in vitro* and/or rodent studies, it is largely acknowledged that AMPK activation promotes both glucose uptake and lipid oxidation in skeletal muscle through direct phosphorylation of key regulatory enzymes or transcription factors [33;34]. In the present study, we did not find any differences in basal (post-meal) AMPK activity in skeletal muscle from lean and obese subjects. This is in line with most of the previous reports [19;35-37], although one study has reported reduced AMPK activity in skeletal muscle from healthy obese and type 2 diabetes individuals [38]. Surprisingly, we showed that fasting decreases skeletal muscle AMPK activity in lean subjects, an effect lost in obese individuals. Of note, it was also recently reported that 72 hours of fasting does not significantly affect AMPK activity in healthy individuals [32]. One could therefore argue

that fasting-induced AMPK activation is an early regulatory event that transiently occurs in the first hours following food deprivation. Additional experiments with earlier time points are therefore required to clarify this issue.

Why does AMPK activity decrease in healthy individuals in response to fasting and why is this effect lost in obesity? The regulation of AMPK activity is complex and involves 1) adenine nucleotides binding to the γ-subunit, 2) Thr172 phosphorylation by LKB1 and/or CAMKKβ, 3) Thr172 dephosphorylation by AMPK-specific protein phosphatase(s), 4) phosphorylation of various regulatory residues on both α- and β-subunits, and 5) glycogen binding to the β-subunit [9-11]. In addition, modification in the AMPK subunits composition might also influence the kinase activity by changing the sensitivity toward AMP and/or other post-translational regulatory mechanisms. In human skeletal muscle, the majority of AMPK heterotrimers consists of either α2β2γ1 or α2β2γ3 complexes [37]. However, except for the protein expression of the marginal short AMPKγ2 isoform which was significantly lower at baseline in the obese when compared to lean individuals, all the other catalytic α and regulatory β/γ subunits were similar between groups whatever the conditions. Among the AMPK regulatory mechanisms described above, an increase in cellular energy status during fasting is rather unlikely and no differences in the phosphorylation state of AMPKα at the inhibitory Ser485/491 residue were observed (*data not shown*). A fasting-induced increase in protein phosphatase(s) activity cannot be excluded but the identity of the enzyme(s) involved in AMPK-Thr172 dephosphorylation remains uncertain [9-11]. Counter intuitively, we found an increase in LKB1 protein expression after fasting in lean but not in obese individuals that, however, merely suggests a compensatory mechanism secondary to reduced AMPK activity in the former group. On top of the cellular energy state, AMPK constantly monitors intracellular glycogen stores. Interestingly, a paradoxical increase in skeletal muscle glycogen content was reported during prolonged fasting [32]. It is therefore tempting to speculate that this physiological adaptation might be involved in reduced AMPK activity in lean subjects and altered in obese individuals with impaired glycogen metabolism. The determination of skeletal muscle glycogen content in our study would have clearly contributed to strengthen this point but the amount of available materials was unfortunately not sufficient to perform this measurement. Finally, an attractive potential explanation emerges from a recent study showing that adrenaline inhibits AMPK by a mechanism involving activation of an unidentified kinase which phosphorylates the AMPK β-subunit on a new inhibitory residue [39]. Indeed, fasting is known to increase plasma catecholamine levels [40], an effect that was reported to be

partly blunted in obese individuals [22]. Taken together, the physiological rationale for a reduced AMPK activity during prolonged fasting still remains unclear. One of the hypotheses builds on modulation of the so-called Randle cycle [41]: a reduced AMPK activity would therefore prevent glucose uptake by reducing AMPK-mediated GLUT4 translocation to the plasma membrane, leading to subsequent inhibition of glucose oxidation and the concomitant shift toward mitochondrial FA.

Finally, another striking finding is the marked decrease in protein expression of key mitochondrial respiratory-chain subunits in from obese subjects, strongly suggesting that the skeletal muscle mitochondrial content is reduced in these individuals. This may affect the capacity of skeletal muscle to adapt to increased FA availability associated with fasting and underlie the impaired shift from glucose toward mitochondrial FA oxidation. Although the underlying molecular mechanisms remains to be elucidated, this strengthens previous reports showing that mitochondrial density in skeletal muscle, assessed either by mtDNA content and/or by *ex vivo* determination of mitochondrial respiratory-chain complexes activities, was reduced in skeletal muscle from obese subjects [42;43]. In the present study, due to lack of materials, we were unfortunately not able to measure other mitochondrial markers, such as mtDNA content or activities of citrate synthase and β-hydroxyacetyl coenzyme A dehydrogenase. Additional studies are therefore required to determine additional mitochondrial parameters and clarify the (patho)physiological consequence of these findings.

In conclusion, our main findings show that obese individuals are characterized by whole-body metabolic inflexibility in response to prolonged fasting, a feature associated with apparent alteration in AMPK signaling and reduced skeletal muscle IRβ and mitochondrial content.

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