Supplementary Information

Macronutrient Utilization During Short Term Fasting in Young and Old Humans

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Supplementary Information Summary:

Supplemental Methods: detailed description of stable isotope infusion protocols and calculations

Supplemental Tables S1-5: summaries of metabolic data

Supplemental Tables S6-9: summaries of mixed linear model statistical results

Detailed Description of Methods and Calculations.

Participants.

At the initial visit, participants had a medical history and physical examination, and screening laboratory tests (complete blood count, electrolytes, urea, creatinine, liver function tests, thyroid function tests, urinalysis, and, if in the older group, electrocardiogram). The study was approved by the MIT Committee on the Use of Humans as Experimental Subjects as well as the MIT Clinical Research Center Advisory Committee before its initiation. The purpose and protocol of the study were explained to each participant in detail by one of the investigators, prior to obtaining their written consent.

After their enrollment in the study, participants had a dietary interview. They were provided with balanced, weight-maintaining, regular diets consistent with their customary dietary preferences for three days prior to each fasting period, while maintaining their customary level of physical activity. Each participant had two stable isotope infusion studies, one after a 12 hour overnight fast, and one after a 36 hour fast. The order of the infusion studies was not randomized, each participant having the 12 hour fast study first. For each individual participant, there was a 4-week interval between the studies, to allow plasma isotope levels from the first infusion to decrease to background levels.

Stable Isotope Infusion Protocols.

For their infusion study following a 12 hour fast, participants received a regular supper meal at 7pm, and were admitted to the MIT General Clinical Research Center (GCRC) to sleep overnight. They then fasted until the study began the following morning. At

7am, they were awakened, and had a resting energy expenditure (REE) measured after they had voided and returned to bed. Two intravenous lines were started using Teflon catheters, an antecubital line for the infusions, and a dorsal hand vein line (inserted with the tip facing the capillary bed) for arterialized venous sampling using a heated (60^0C) hand box. They remained fasting and resting in bed for the next four hours, while they received the infusion. The infusion, performed with a calibrated syringe pump, consisted of the following mixture:

[$^2H_5$] glycerol:	prime 1.6 μmol/kg,	infusion 0.11 μmol/kg/min
[6,6-d^2] glucose:	prime 17.8 μmol/kg,	infusion 0.22 μmol/kg/min
[$^{15}N_2$] urea:	prime 88 μmol/kg,	infusion 0.12 μmol/kg/min
L-[1-^{13}C] leucine:	prime 4.2 μmol/kg,	infusion 0.067 μmol/kg/min
[^{13}C] $NaHCO_3$	prime 0.8 μmol/kg	

The isotopes were dissolved in normal saline, and the solutions tested for sterility and absence of pyrogens prior to infusion.

During the infusion, subjects had VCO_2 (carbon dioxide expiratory rate, liters/min), VO_2 (oxygen expiratory rate, liters/min), REE (resting energy expenditure), and respiratory exchange ratio (RER) measured for 30 minutes every hour, and blood samples (4 ml) taken every half hour, as well as breath samples for $^{13}CO_2$ measurement every half hour. A blood sample for glucose, betahydroxybutyrate, glycerol, and lactate levels was drawn before the infusion began.

For the second isotope infusion study, the participants again were provided three balanced meals a day for three days prior to beginning the 36 hour fast. They were admitted to the GCRC on the day that they began the fast, and stayed there throughout the fast.

They fasted from 7pm on the first day after eating supper, until noon of the third morning. They were not maintained on bed rest during their fast. They had vital signs taken every shift during the fast and were weighed every morning. Their water intakes and urine outputs were also recorded to ensure that the participants (especially the older ones, who might have had a decreased thirst response) were not losing excessive amounts of water. Beginning at 7am on the third morning of the fast, they had a second infusion study, exactly as described above. During the fast, they received water ad libitum, a multivitamin and mineral pill, KCL (40 Meq), and NaCl (4 gm) orally each day. Their plasma electrolytes, blood urea nitrogen (BUN), creatinine, and glucose were measured daily.

Twenty-four hour urine samples were also collected during the course of the studies, and total volume, creatinine, and urea nitrogen were measured.

Each participant had a whole body DEXA scan between the two fasting studies, to determine their fat and lean mass, and the distribution of the lean mass between trunk and extremities.

Indirect Calorimetry.

Resting energy expenditures, RER, VCO_2, and VO_2, were measured by an open circuit indirect calorimetry system with a ventilated hood interfaced to a computer (Sensormedics Deltatrac Metabolic Monitor). Air was drawn from the hood at approximately 40 l/min, and concentrations of oxygen and carbon dioxide measured with an electrochemical oxygen sensor and an infrared carbon dioxide sensor. Metabolic rate was calculated from rates of oxygen consumption and carbon dioxide production using Weir's equation (16). The system was periodically calibrated for flow rate by measuring the total carbon dioxide production from combustion of 5 ml of ethanol, and was reproducible to within 3% of predicted values. The system was calibrated before and after

each measurement with a 96% oxygen, 4% carbon dioxide calibration gas mixture. The values of REE and RER in the final half hour of the four hour infusion were used for calculation of oxidation rates.

Collection and Analysis of Samples.

Breath samples were collected in disposable balloons by a mechanism that permitted removal of dead-space air. The samples were transferred to 15 ml non-silicon coated glass tubes, and stored at room temperature, prior to analysis for $^{13}CO_2$ enrichment by isotope ratio mass spectrometry (17,18). Blood samples were drawn into heparinized tubes and centrifuged for 15 minutes at 1200g in a refrigerated centrifuge. Plasma was stored at -20^0C until analyzed.

Preparation of plasma for isotopic analysis of [^{13}C] KIC by forming the quinoxalinol-t-BDNS derivative was as previously described (12,18). Isotope enrichment was measured on a Hewlett-Packard 5890 Series II gas chromatograph coupled to an HP 5971 quadrupole mass spectrometer and an HP data system. Electron impact ionization was conducted at 70 eV. Plasma [^{13}C] KIC enrichment was measured against calibration graphs prepared for standard mixtures of labeled KIC, ranging from 0% to 10% mole fraction. The concentrations of leucine in plasma and infusates were determined by HPLC. Plasma and urinary urea nitrogen concentrations were determined with an auto analyzer. Analyses of samples for [$^{15}N_2$] urea enrichment and for deuterated glucose were also as described (17,18). [$^2H_5$] glycerol was analyzed by forming the tert-butyldimethylsilyl (tBDMS) derivative, electron impact ionization, and selective ion monitoring, as described by Flakoll et al. (19).

Plasma glucose, lactate, betahydroxybutyrate, and glycerol levels were determined by enzymatic methods, using commercial kits from Sigma Chemicals.

Calculations.

The duration of the primed, continuous isotope infusions was chosen to ensure that all isotope enrichments had reached a steady state at the end of the sampling time (4 hours). Thus, rates of appearance (R_a) of substrates were calculated using steady state equations (R_a = isotope infusion rate/plasma isotopic enrichment). Isotope infusion rates were subtracted from R_a to give endogenous R_a.

Leucine R_a was calculated from plasma [1-^{13}C] KIC enrichment (17).

Leucine oxidation was calculated as [$^{13}CO_2$] production/[^{13}C] KIC enrichment, where [$^{13}CO_2$] production = VCO_2 x [$^{13}CO_2$] enrichment, corrected by a factor R of 0.70 for fasting bicarbonate recovery (20).

Urea production was calculated as ([^{15}N] urea infusion rate/plasma [^{15}N] urea enrichment) – ([^{15}N] urea infusion rate).

Urea nitrogen excretion (corrected) was calculated as [urea nitrogen excretion (uncorrected)] – [{BUN(begin)-BUN(end)}xTBW/0.92] (18).

Irreversible protein nitrogen loss was calculated as [urea nitrogen excretion(corrected) + (urea nitrogen excreted(uncorrected)/4)]x6.25 (18).

Rates of fat and carbohydrate oxidation and energy expenditures were calculated from measured rates of oxygen consumption and carbon dioxide production, corrected for protein oxidation (21).

Rates of lipolysis were calculated by multiplying glycerol R_a by three (17).

Dual Energy X-Ray Absorptiometry.

Participants were scanned with a Hologic QDR-1000W system, which partitions body weight into soft tissue mass and bone ash. The ratio of soft tissue attenuation at 7 and 16 fJ correlates with the proportion of fat and lean mass in soft tissue, and the system calculated these values directly, assuming a hydration factor of 0.73.

Isotope Enrichments.

The 210 minute and 240 minute blood and breath samples at the end of four hours of isotope infusion, when a steady state had been reached, were processed for measurement of isotope enrichments and the average of the measurements for these two time points was used to calculate metabolic parameters. The levels of isotope enrichments reached at steady state were:

$[^{13}C]O_2$	APE x1000:	6.5 – 11.4
$[^{15}N_2]$ urea	mole fraction above baseline:	0.015 – 0.020
$[^{13}C]$ KIC	mole fraction above baseline	0.019 - 0.026
$[6,6\text{-}d^2]$ glucose	mole fraction above baseline	0.016 – 0.027
$[^2H_5]$ glycerol	APE	0.017 – 0.045

These steady state ranges, seen in all four groups of participants, are similar to the steady state ranges seen in previous studies from our group in younger and older participants (12,14,18) using the same isotope infusion protocols.

SUPPLEMENTARY TABLE S1 Plasma Substrate Levels During Fasting (Means±SD)

	Younger Men		Younger Women		Older Men		Older Women	
	12 h Fast	36 h Fast	12h Fast	36h Fast	12h Fast	36h Fast	12h Fast	36h Fast
Albumin (g/l)	41±2"*	39±2"*	39±3"*	36±1"*	37±1"*	34±1"*	37±3"*	34±2"*
Betahydroxybutyrate (mg/dl)	1.48±0.83"	13.92±7.4"*	1.46±0.55"	12.46±2.82"*	1.20±0.40"	3.34±1.02"*	1.56±0.52"	4.10±1.41"*
BUN (mmol/l)	5.8±0.7	5.8±1.4	5.0±1.8	5.0±0.7	7.9±0.8	6.5±1.8	6.1±1.4	4.7±1.4
Cholesterol (mmol/l)	4.31±0.83*	3.93+0.78*	4.7±0.7*	4.5±0.7*	5.1±1.1*	4.5±0.9*	5.6±0.8*	5.3±0.9*
Creatinine (mmol/l)	88.7±8.9^	88.7±8.9^	62.1±8.9^	62.1±8.9^	97.5±17.7^	88.7±17.7^	71.0±8.9^	62.1±8.9^
Glucose (mmol)	4.9±0.3"*	3.9±0.2"*	4.7±0.4"*	3.9±0.5"*	5.2±0.6"*	4.7±0.5"*	5.3±0.3"*	4.7±0.2"*
Glycerol (mmol/l)	0.09±0.03	0.11±0.03	0.02±0.02	0.05±0.03	0.13±0.03	0.13±0.03	0.09±0.03	0.09±0.03
Lactate (mg/dl)	11.0±3.5	10.7±1.4	9.7±3.3	8.2±1.2	11.2±3.6	10.5±1.7	12.9±6.0	11.4±4.2
Leucine (nmol/ml)	128±15"	212±24"*	113±25"	201±56"*	138±23"	168±42"*	116±11"	141±6"*
Triglycerides (mmol/l)	0.83±0.13"	0.65±0.17"	1.24±0.29"	0.55±0.14"	2.05±1.08"	1.07±0.51"	2.39±1.65"	1.17±0.87"

* P < 0.01 Younger vs. Older (P< 0.03 for Leucine, P < 0.02 for Triglyceride)

^ P < 0.01 Men vs. Women

" P < 0.01 12 h fast vs. 36 h Fast

SUPPLEMENTARY TABLE S2 RER and REE

	Younger Men		Younger Women		Older Men		Older Women	
	12 h Fast	36 h Fast	12h Fast	36h Fast	12h Fast	36h Fast	12h Fast	36h Fast
RER	0.79±0.01	0.75±0.02	0.82±0.02	0.77±0.01	0.82±0.03	0.78±0.02	0.79±0.02	0.76±0.03
REE (kcal/24h)	1777±252	1950±230	1332±165	1381±76	1455±199	1431±309	1192±166	1337±163

RER P<0.001 12h Fast vs. 36h Fast
REE P<0.001 12h Fast vs. 36h Fast, Younger vs. Older, Men vs. Women

SUPPLEMENTARY TABLE S3 Substrate Rates of Appearance during Fasting

	Younger Men		Younger Women		Older Men		Older Women	
	12 h Fast	36 h Fast	12h Fast	36h Fast	12h Fast	36h Fast	12h Fast	36h Fast
Glucose R_a (µmol/kg/min)	11.09±1.35	7.46±1.01	11.60±1.25	8.19±1.21	8.98±1.09	7.37±0.36	8.62±0.95	6.89±1.17
Fatty Acid R_a (µmol/kg/min)	2.81±0.69	3.37±1.05	3.18±0.13	4.54±1.00	2.63±0.56	3.15±0.39	2.40±0.48	3.46±0.87
KIC R_a (µmol/kg/min)	1.84±0.31	1.74±0.20	1.51±0.47	1.68±0.51	1.48±0.17	1.69±0.42	1.14±0.31	1.20±0.26
Urea R_a (µmol/kg/min)	4.87±1.33	6.21±1.11	3.99±1.15	5.30±1.54	5.48±1.09	5.44±1.44	4.14±0.19	3.20±0.75

Glucose Ra P<0.001 12h Fast vs 36h Fast
Fatty Acid Ra P< 0.01 12h Fast vs 36h Fast, Younger vs Older
Urea Ra P< 0.001 Men vs Women

SUPPLEMENTARY TABLE S4 Substrate Oxidation Rates during Fasting

	Younger Men		Younger Women		Older Men		Older Women	
	12 h Fast	36 h Fast	12h Fast	36h Fast	12h Fast	36h Fast	12h Fast	36h Fast
Glucose Oxidation (µmol/kg/min)	5.79±0.89	3.39±1.19	6.46±0.48	4.73±1.31	5.22±1.50	4.15±0.88	4.13±0.61	4.09±0.92
Fatty Acid Oxidation (µmol/kg/min)	3.60±0.57	4.77±0.33	2.94±0.27	3.96±0.42	2.34±0.39	2.93±0.27	3.15±0.84	3.64±0.88
Leucine Oxidation (µmol/kg/min)	0.39±0.09	0.43±0.05	0.24±0.07	0.34±0.09	0.28±0.03	0.29±0.07	0.22±0.06	0.24±0.08
Urea Excretion (µmol/kg/min)	3.34±0.93	3.27±0.75	2.88±0.86	3.06±0.55	3.04±0.35	2.67±0.60	3.32±0.22	2.76±0.36

Glucose Oxidation P<0.001 12h Fast vs. 36h Fast
Fatty Acid Oxidation P<0.001 12h Fast vs 36h Fast
Leucine Oxidation P<0.01 Younger vs. Older, Men vs. Women

SUPPLEMENTARY TABLE S5 **Substrate Utilization as Percent of Energy Expenditure during Fasting**

	Younger Men		Younger Women		Older Men		Older Women	
	12 h Fast	36 h Fast	12h Fast	36h Fast	12h Fast	36h Fast	12h Fast	36h Fast
Percent REE Carbohydrate	26.0±3.7	13.7±4.2	33.2±4.9	20.7±3.8	30.2±8.6	23.1±4.7	23.5±2.0	20.0±3.1
Percent REE Fat	55.9±2.3	70.1±4.6	49.5±7.2	62.9±2.8	48.4±7.4	58.8±5.1	55.0±3.7	63.2±3.9
Percent REE Protein	18.1±3.8	16.2±3.4	15.9±3.0	16.4±1.6	21.4±2.8	18.1±3.7	22.7±4.4	16.7±2.5

Percent REE Carbohydrate P<0.001 12h Fast vs. 36h Fast

Percent REE Fat P<0.001 12h Fast vs. 36h Fast

Supplementary Table S6 Linear Mixed Model Analysis of REE and RQ and P of Factor Not Affecting 12H Fast Value

	12H Fast Value	Effect of 36H Fast	Effect of Age	Effect of Sex	P	36H Fast	Age	Sex	Age*Sex
REE (kcal/24h)	**1255 ± 140**	**105 ± 28**	**-5.3 ± 1.5**	**-369 ± 74**		**0.0011**	**0.0016**	**$5.5x10^{-05}$**	**0.0042**
RER	**0.792 ± .009**	**-0.034 ± 0.004**	**-0.00008 ± 0.00009**	**0.006 ± 0.005**		**$3.9x10^{-10}$**	**0.3927**	**0.207**	**0.0069**

The table shows the mean 12H fasting REE (± SD) in kcal/24h and mean 12H fasting RER (± SD) of the participants and the change in the mean 12H fasting value for a 36H fast, for age, and for sex. In the linear mixed model used, age was treated as a continuous variable, so the effect of age must be multiplied by the participant's age (in years). The mean 12H fasting REE and RER values are for the sex with the lowest mean value (women for both REE and RER), and the effect of sex is added to this value for the other sex. The table also shows the P values (probability), calculated from a Chi-Squared likelihood ratio test, that a factor (36H fast, age, sex, age*sex interaction) would not change the 12H fasting mean value in the linear mixed model.

Supplementary Table S7 Linear Mixed Model Analysis of Substrate Rate of Appearance and P of Factor Not Affecting 12H Fast Value

	12H Fast Value	Effect of 36H Fast	Effect of Age	Effect of Sex	P	36H Fast	Age	Sex	Age*Sex
Glucose Ra (µmol/kg/min)	**10.82 ± 0.99**	**-2.39 ± 0.27**	**-0.027 ± 0.010**	**0.27 ± 0.52**		**$7.6x10^{-9}$**	**0.086**	**0.572**	**0.192**
KIC Ra (µmol/kg/min)	**1.91 ± 0.23**	**-0.26 ± 0.12**	**-0.0029 ± 0.0024**	**-0.18 ± 0.12**		**0.034**	**0.219**	**0.132**	**0.825**
Fatty Acid Ra (µmol/kg/min)	**20.96 ± 2.05**	**3.93 ± 1.31**	**-0.109 ± 0.026**	**-2.74 ± 1.31**		**0.003**	**0.0001**	**0.032**	**0.988**
Urea Ra (µmol/kg/min)	**6.02 ± 0.51**	**0.01 ± 0.26**	**-0.004 ± 0.005**	**-1.10 ± 0.26**		**0.965**	**0.394**	**0.0001**	**0.525**

The table shows the mean 12H fasting Glucose Ra, KIC Ra, Fatty Acid Ra, and Urea Ra (± SD) in µmol/kg/min of the participants and the change in the mean 12H fasting value for a 36H fast, for age, for sex, and for sex*age interaction. In the linear mixed model used, age was treated as a continuous variable, so the effect of age must be multiplied by the participant's age (in years). The mean 12H fasting values are for the sex with the lowest mean value, and the effect of sex is added to this value for the other sex. The table also shows the P values (probability), calculated from a Chi-Squared likelihood ratio test, that a factor (36H fast, age, sex, age*sex interaction) would not change the 12H fasting mean value in the linear mixed model.

Supplementary Table S8 Linear Mixed Model Analysis of Substrate Oxidation and P of Factor Not Affecting 12H Fast Value

	12H Fast Value	Effect of 36H Fast	Effect of Age	Effect of Sex	P	36H Fast	Age	Sex	Age*Sex
Glucose Oxidation (µmol/kg/min)	**6.06 ± 0.93**	**-1.51 ± 0.37**	**-0.022 ± 0.010**	**0.38 ± 0.48**		**0.0004**	**0.020**	**0.394**	**0.099**
Leucine Oxidation (µmol/kg/min)	**0.503 ± 0.048**	**0.046 ± 0.017**	**-0.0017 ± 0.0005**	**-0.090 ± 0.025**		**0.013**	**0.003**	**0.002**	**0.268**
Fatty Acid Oxidation (µmol/kg/min)	**3.64 ± 0.53**	**0.89 ± 0.13**	**-0.012 ± 0.006**	**-0.03 ± 0.28**		**$3.5x10^{-7}$**	**0.025**	**0.900**	**0.011**
Urea Excretion (µmol/kg/min)	**3.20 ± 0.48**	**-0.16 ± 0.11**	**-0.0009 ± 0.0050**	**-0.04 ± 0.25**		**0.176**	**0.858**	**0.884**	**0.636**

The table shows the mean 12H fasting Glucose Oxidation, Leucine Oxidation, Fatty Acid Oxidation and Urea Excretion (± SD) in µmol/kg/min of the participants and the change in the mean 12H fasting value for a 36H fast, for age, for sex, and for age*sex interaction. In the linear mixed model used, age was treated as a continuous variable, so the effect of age must be multiplied by the participant's age (in years). The mean 12H fasting values are for the sex with the lowest mean value, and the effect of sex is added to this value for the other sex. The table also shows the P values (probability), calculated from a Chi-Squared likelihood ratio test, that a factor (36H fast, age, sex, age*sex interaction) would not change the 12H fasting mean value in the linear mixed model.

Supplementary Table 9 Linear Mixed Model of Substrate Percent Oxidation of REE and P of Factor Not Affecting 12H Fast Value

	12H Fast Value	Effect of 36H Fast	Effect of Age	Effect of Sex	P	36H Fast	Age	Sex	Age*Sex
Carbohydrate Oxidation Percent of REE	**25.8± 2.4**	**-8.1 ± 1.2**	**-0.03 ± 0.02**	**1.7 ± 1.3**		**$4.3x10^{-8}$**	**0.296**	**0.181**	**0.023**
Fat Oxidation Percent of REE	**56.2± 2.5**	**10.7 ± 0.9**	**-0.02 ± 0.02**	**-1.1 ± 1.3**		**$9.1x10^{-13}$**	**0.491**	**0.379**	**0.014**
Protein Oxidation Percent of REE	**16.3± 2.7**	**-1.7 ± 1.1**	**-0.04 ± 0.03**	**0.4 ± 0.4**		**0.149**	**0.135**	**0.758**	**0.937**

The table shows the mean 12H fasting Carbohydrate, Fat and Protein Percent Oxidation (± SD) in percent of the participants and the change in the mean 12H fasting value for a 36H fast, for age, for sex, and for age*sex interaction. In the linear mixed model used, age was treated as a continuous variable, so the effect of age must be multiplied by the participant's age (in years). The mean values are for the sex with the lowest mean value, and the effect of sex is added to this value for the other sex. The table also shows the P values (probability), calculated from a Chi-Squared likelihood ratio test, that a factor (36H fast, age, sex, age*sex interaction) would not change the 12H fasting mean value in the linear mixed model.