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ORIGINAL ARTICLE

L-Arginine improves multiple physiological parameters in mice exposed to diet-induced metabolic disturbances

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Abstract L-Arginine (L-Arg) is a conditionally essential amino acid and a natural constituent of dietary proteins. Studies in obese rats and type 2 diabetic humans have indicated that dietary supplementation with L-Arg can diminish gain in white adipose tissue (WAT) and improve insulin sensitivity. However, the effects of L-Arg on glucose homeostasis, body composition and energy metabolism remain unclear. In addition, no studies have, to our knowledge, examined whether L-Arg has beneficial effects as a dietary supplement in the mouse model. In the present study, we investigated the effects of L-Arg supplementation to male C57BL/6 mice on an array of physiological parameters. L-Arg supplemented mice were maintained on a low-protein diet and body composition, appetite regulation, glucose tolerance, insulin sensitivity and energy expenditure were evaluated. A significant reduction in epididymal WAT was observed in L-Arg supplemented mice compared with mice fed an isocaloric control diet. Surprisingly, the L-Arg supplemented animals were hyperphagic corresponding to a highly significant decrease in feed efficiency, as body weight developed in a similar pattern in both experimental groups. Glucose homeostasis experiments revealed a major effect of L-Arg supplementation on glucose tolerance and insulin sensitivity, interestingly, independent of a parallel regulation in whole-body adiposity. Increased L-Arg ingestion also raised energy expenditure; however, no concurrent effect on locomotor activity, substrate metabolism or expression of uncoupling proteins (UCP1 and UCP2) in adipose tissues was displayed. In conclusion, dietary L-Arg supplementation substantially affects an array of metabolic-associated parameters including a reduction in WAT, hyperphagia, improved insulin sensitivity and increased energy expenditure in mice fed a low-protein diet.

Keywords L-Arginine · Diet · Amino acids · Low protein · Insulin sensitivity · Obesity · Diabetes

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Introduction

The incidence of obesity and associated diseases such as type 2 diabetes are increasing at alarming rates almost over the entire planet (Grundy 2008). Obesity arises from a chronic imbalance between energy intake and expenditure; thus, the laws of thermodynamics are widely recognized to apply to the human organism (Spiegelman and Flier 2001). Nevertheless, the optimal composition of fat, carbohydrate and protein in dietary regimens, to prevent and treat obesity, is still under serious investigation (Stefater and Seeley 2010; Woods and D'Alessio 2008). The reason why most diets fail to reduce body weight and/or maintain weight loss is possibly linked to their inability to overcome the homeostatic systems that serve to preserve weight

stability (Stefater and Seeley 2010). Therefore, understanding how energy balance is maintained and how dietary components as well as pharmaceutical drugs affect energy homeostasis has become a major research quest of the twenty-first century (Sandoval et al. 2009; Stefater and Seeley 2010). It is now widely accepted that an array of nutrients, besides simply acting as a source of energy, posses signaling abilities that can regulate and affect many cellular processes, and it has been proposed that in the knowledge of how individual micronutrients specifically affect biological systems lies the key to understand and treat metabolic-related disorders (Stefater and Seeley 2010). L-Arginine (L-Arg) is a conditionally essential amino acid that is a natural constituent of dietary proteins (Rose 1947). Besides its role in protein metabolism, L-Arg is involved in the production of nitric oxide (NO) and the synthesis of creatine, L-ornithine, L-glutamate, polyamines and agmatine (Wu and Morris 1998). Moreover, L-Arg is a potent secretagogue of the endocrine system, as it induces insulin (Sener et al. 1989) and glucagon (Aguilar-Parada et al. 1969) secretion from the pancreas and promotes growth hormone secretion from the anterior pituitary (Alba-Roth et al. 1988). It is well documented that high-protein diets mediate increased thermogenesis and satiety when compared with isocaloric fat or carbohydrate-rich diets (Halton and Hu 2004) and it was recently demonstrated that dietary protein is central to weight loss maintenance in humans (Larsen et al. 2010). On the other hand, low-protein diets are associated with altered metabolism and appetite regulation together with increased fat storage (Rothwell et al. 1983; Du et al. 2000). However, whether it is the dietary protein content per se or whether specific amino acids individually or in various synergies are accountable for the remarkable effects associated with increasing the relative amount of dietary protein is largely unknown. Growing evidence indicates that dietary L-Arg supplementation can reduce adiposity and improve insulin sensitivity in some animal models and in diabetic humans (McKnight et al. 2010). In addition, L-Arg may have an emerging clinically relevant therapeutic role and it has recently been proposed that it holds potential as an agent in pharmaconutrition formulas designed for, e.g., patients in intensive care units (Cave et al. 2008). These formulas are designed to combat chronic low-grade inflammation and to correct metabolic derangements in obese patients even in the absence of weight loss. However, the cellular mechanisms behind the potential metabolic benefits of L-Arg are yet to be discovered. Surprisingly, the dietary effects of L-Arg on metabolic-related parameters in mice have, to our knowledge, not been reported. The use of mice and transgenic mice as human pathology models has dramatically increased for the past decades and the mouse models provide an invaluable tool for both investigating the underlying pathogenic processes and developing new effective therapies (Plum et al. 2005; Rees and Alcolado 2005). The present experiments were designed to investigate if the conditionally essential amino acid L-Arg affects an array of metabolic-related parameters in the mouse model. To address this research question, we here explore whether supplementing L-Arg to a diet with an altered macronutrient composition attenuates dietary-induced disturbances in energy balance and glucose homeostasis in male C57BL/6 mice.

Methods and procedures

Animals and diet

Male C57BL/6 mice (6 week-old) were purchased from Taconic (Ry, Denmark). Upon arrival, mice were housed five per cage in a rodent facility on a 12 h light and 12 h dark cycle under controlled temperature and humidity, with ad libitum access to water and standard chow diet. All experimental work was conducted in accordance with institutional guidelines and approved by the Animal Experiments Inspectorate in Denmark.

After a 2 week period of acclimatization, during which mice were fed a regular non-purified diet, they were weighed and analyzed for body composition (EchoMRI, Echo Medical Systems, Houston, TX, USA). Groups of similar morphological phenotype were created based on body weight and body fat percentage and mice were assigned to an isocaloric low-protein (8% kcal from protein) diet supplemented with either L-Arg (L-Arg; 2% kcal from L-Arg) or a mixture of amino acids (control; 2% kcal from mixed amino acids) for 10 weeks ($n = 10$/group). As the present study exclusively aimed to uncover the effects of dietary L-Arg supplementation, the isocaloric control diet was carefully designed to match the total protein content of the experimental diet and was accordingly supplemented with a mixture of amino acids excluding the ones involved in endogenous production of L-Arg and amino acids reported to execute major effects on metabolic function. The excluded amino acids from the added 2% kcal mixture in the control diet included L-proline and L-glutamic acid, both known for their involvement in L-Arg biosynthesis (Womack and Rose 1947), and L-leucine, previously illustrated to reduce diet-induced obesity and improve glucose metabolism in mice (Zhang et al. 2007). In addition, basic amino acids (including L-Arg) are potent agonists of the novel G protein-coupled receptor, GPRC6A (Wellendorph et al. 2005)—and possibly exert some of their physiological roles by activating this nutrient sensing receptor (Wellendorph et al. 2009). Therefore, the most efficacious dietary GPRC6A ligands (L-lysine and L-

Table 1 Dietary contents, distribution of the supplemented amino acids and macronutrient energy composition

Diet (ingredients)	Control (g)	L-Arg (g)
Casein	90	90
L-Cystine	1.35	1.35
L-Arginine	0	20
L-Tyrosine	2.79	0
L-Isoleucine	2.31	0
L-Methionine	1.55	0
L-Phenylalanine	2.57	0
L-Threonine	2.20	0
L-Tryptophan	0.64	0
L-Valine	2.84	0
L-Histidine	1.39	0
L-Aspartic acid	3.70	0
Total crystalline amino acids	21.34	21.35
Corn starch	381	381
Maltodextrin 10	50	50
Dextrose	250	250
Cellulose, BW200	50	50
Soybean oil	87	87
Mineral mix S10026	10	10
Dicalcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate, 1 H_2O	16.5	16.5
Vitamin mix V10001	10	10
Choline bitartrate	2	2
kcal %		
Protein	10	10
Carbohydrate	70	70
Fat	20	20
Total	100	100
kcal/g	4.0	4.0

alanine) were also excluded from the supplemented mixture of amino acids added to the control diet. The amount of each amino acid in the 2% kcal mixture added to the control diet was selected to correspond to the distribution found in a standard casein protein source. The custom-made diets were purchased from Research Diets (Research Diets, New Brunswick, NJ, USA) and nutrient compositions are listed in Table 1.

Body composition analysis

To determine whether supplementing L-Arg to a low-protein diet influences body weight changes and body composition, weight was measured weekly and mice were analyzed with a quantitative magnetic resonance whole-body composition analyzer (EchoMRI, Echo Medical Systems, Houston, TX, USA) before initiation of the diet and at the end of the experiment.

Food intake and feed efficiency measures

To evaluate food intake, mice were single housed after 3 weeks on the diet and individual food intake was determined weekly for a period of 21 days. Feed efficiency [weight gain (g)/food consumption (g)] was calculated from the same period of time. In addition, meal pattern and food intake were automatically registered during the period in which mice were housed in the metabolic chambers (3 consecutive days).

Indirect calorimetry

At 6 weeks of dietary manipulation, mice were allowed 5 days of adaptation to metabolic chambers prior to calorimetry data sampling. Oxygen consumption (VO_2: ml/h), respiratory exchange ratio (RER) and locomotor activity (>2 beam breaks and rearing) were obtained continuously during a 12 h light and 12 h dark cycle for 72 h (3 consecutive days) using a 16-chamber comprehensive laboratory animal monitoring system (PhenoMaster: TSE Systems, Bad Homburg, Germany).

Glucose metabolism studies

Blood glucose levels were measured in the blood from the tail vein using a glucometer (Ascensia Contour Glucometer, Bayer, Kiel, Germany). An oral glucose tolerance test (OGTT) was performed at 8 weeks after initiation of the dietary intervention. Blood glucose was measured at 0, 15, 30, 60, 90 and 120 min after oral gavage (1.5 g glucose/kg body weight) in mice fasted for 6 h with free access to water (Andrikopoulos et al. 2008). Peripheral insulin sensitivity was assessed after 9 weeks on the L-Arg supplemented diet. For insulin tolerance test (ITT), mice were challenged with 0.3 U/kg intraperitonally administered human insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) after 6 h of food deprivation with water access and glucose was measured at 0, 15, 30, 60, 90 and 120 min post-injection. The basal blood glucose was measured after 5 h of fasting and the homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated based on the conventional formula: HOMA-IR = [basal glucose (mmol/L) × basal insulin (mU/L)]/22.5.

Plasma sampling, tissue dissection and biochemical analysis

After 5 h of food removal in the post-absorptive state, mice were killed by beheading. Blood was immediately

collected into EDTA-coated tubes (Franklin Lakes, NJ, USA) and rapidly centrifuged, and plasma was stored at −80°C until further analysis. Concentrations of insulin in plasma were determined with an ultrasensitive mouse ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA). Skeletal muscle (*m. gastrocnemius*), epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) were carefully dissected, weighed, frozen on dry ice and stored at −80°C.

Quantitative real-time PCR

Relative mRNA levels were measured by the quantitative PCR method (qPCR) using the Mx3000P from Stratagene (La Jolla, Ca, USA) and SYBRPremix Ex *Taq* (Takara, Otsu, Japan). The relative levels of genes from different samples were compared by the $\Delta\Delta C_t$ method using the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide (YWHAZ) as reference gene. Before the $\Delta\Delta C_t$ value was calculated, primer efficiency was validated by standard curve measurements, and primers with 95% efficiency were used. A calibrator sample was included in each assay for normalization between runs. The PCR primer sequences used were UCP-1 5′ GGCATTCAGAGGCAAATCAGCT 3′ and 5′ CAATG AACACTGCCACACCTC 3′, UCP-2 5′ CCTACAAGAC CATTGCACGA 3′ and 5′ TGTCATGAGGTTGGCTTTC A 3′, and YWHAZ 5′ AGACGGAAGGTGCTGAGAA A 3′ and 5′ GAAGCATTGGGGATCAAGAA 3′. RNA was extracted with RNeasy lipid tissue mini kit (Qiagen, Germantown, MD, USA), and cDNA was synthesized by reverse transcription using the ImProm-IITM reverse transcriptase (Promega, Madison, WI).

Statistical analysis

GraphPad Prism 5.0c (GraphPad Software, San Diego, CA, USA) was used for all statistics and graphical presentations. Unpaired two-tailed Student's *t* test or two-way ANOVA were used to evaluate data and post hoc multiple comparisons were performed using Bonferroni tests. Significance was set at $P < 0.05$ for all analyses.

Results

Effects of L-Arg supplementation on body composition

Dietary L-Arg supplementation has previously been reported to induce body composition alterations in type 2 diabetic patients (Lucotti et al. 2006; Piatti et al. 2001), pigs (Tan et al. 2009) and obese rats (Jobgen et al. 2009b). To determine whether supplementing L-Arg to a low-protein diet affects body weight, lean mass and fat mass in

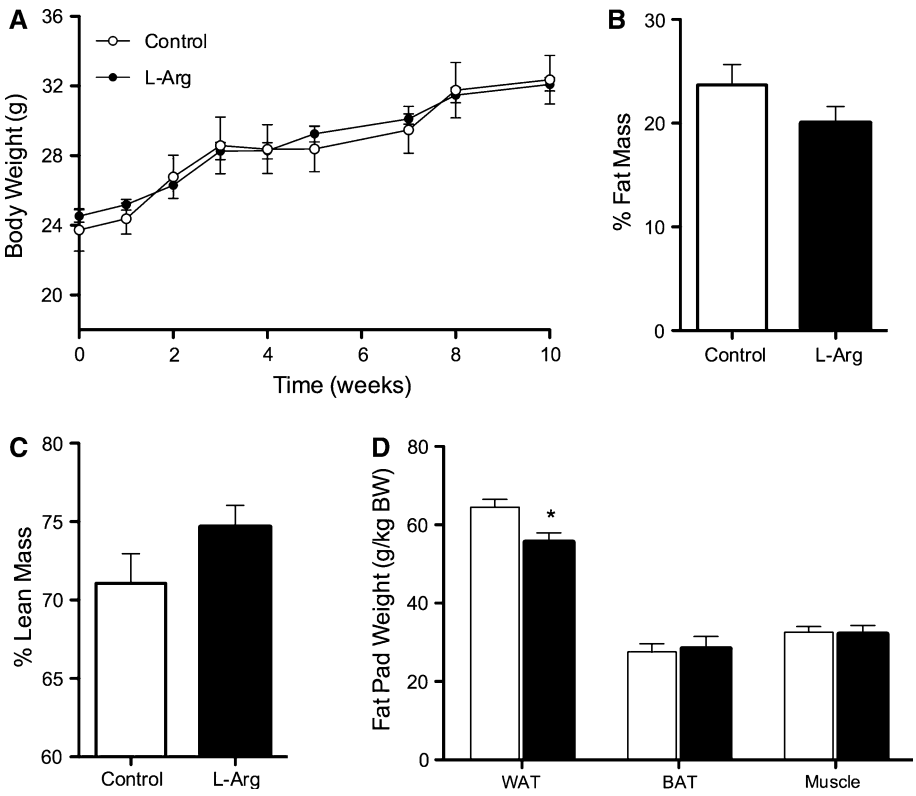

Fig. 1 L-Arg supplementation reduces diet-induced visceral adiposity. Body weight (**a**) was measured weekly during the 10 week-long dietary intervention. The percentages of lean body mass (**b**) and fat mass (**c**) were calculated in relation to body weight at the end of the dietary intervention. White adipose tissue (epididymal) fat pads, brown adipose tissue and *m. gastrocnemius* were carefully dissected and weighed (**d**) immediately after rapid decapitation. The mean ± SEM of eight control diet and nine L-Arg supplemented diet animals are shown. *$P < 0.05$. *WAT* white adipose tissue, *BAT* brown adipose tissue

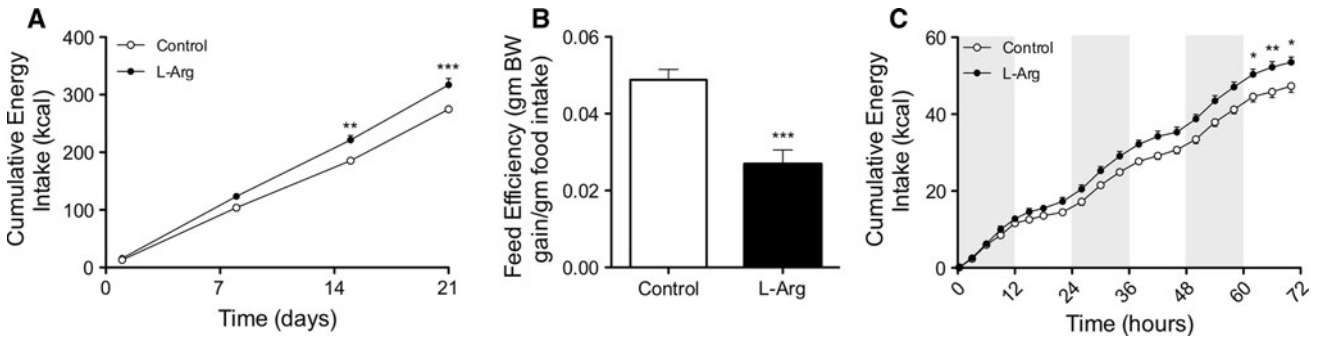

Fig. 2 L-Arg supplementation induces hyperphagia and lowers feeding efficiency. After 4 weeks on the diet food intake (**a**) was sampled weekly from individually caged mice over a 21 day period and feed efficiency (**b**) was calculated in relation to gain in body weight from the same period. After 7 weeks on the diet, food intake (**c**) was sampled for a 72 h period by an automatic food-monitoring system connected to the metabolic chambers. The mean ± SEM of eight control diet and nine L-Arg supplemented diet animals are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

mice, we created a 10 week-long dietary intervention using 8 week-old male C57BL/6 mice. We found no difference in body weight development between the L-Arg supplemented group and the control group (Fig. 1a). Moreover, body composition analysis performed at the end of the study showed no significant difference in body fat percentage (Fig. 1b) or lean mass percentage (Fig. 1c). However, mice administered with L-Arg exhibited significant less epididymal fat ($P = 0.012$), but similar masses of brown adipose tissue (BAT) and skeletal muscle (*m. gastrocnemius*) compared to mice on the control diet (Fig. 1d). Altogether, these data indicate that L-Arg exerts a moderate decrease in epididymal WAT, but no effect on overall body composition in mice exposed to diet-induced metabolic alterations.

Dietary L-Arg induces hyperphagia and dampens feed efficiency

Food consumption was monitored over 21 days under ad libitum conditions. We found that L-Arg supplementation resulted in a significant increase in energy intake, as the total amount of calories consumed was raised by 15% ($P < 0.001$) compared to the control group (Fig. 2a). To add further details to the observed difference in food intake, food consumption and meal pattern were analyzed for a 72 h period by an automatic food-monitoring system (TSE Systems). In support of our previous finding, cumulative energy intake of L-Arg supplemented mice was significantly increased by 13% compared to mice on the control diet (Fig. 2c). The feed efficiency, defined as gram body weight gain per gram food intake, was found to be significantly reduced for the L-Arg supplemented group ($P = 0.003$) (Fig. 2b). Collectively, these findings demonstrate that dietary L-Arg supplemented mice are highly hyperphagic, but exhibit a reduced ability to extract energy from food and/or an increased energy expenditure as body weight development resembles that of non-supplemented control mice.

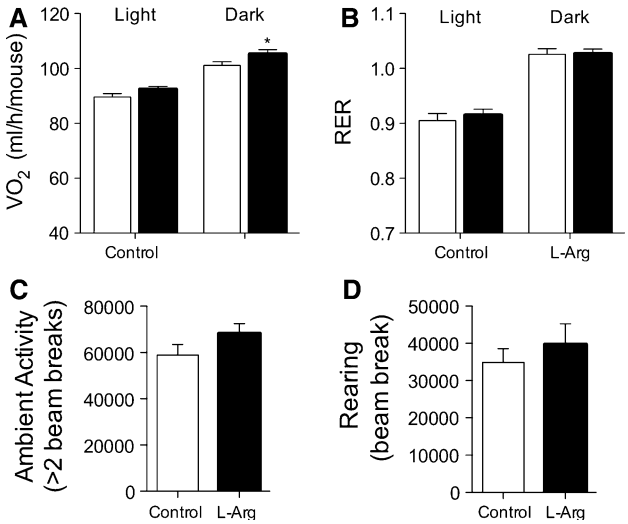

Fig. 3 Energy expenditure is increased in L-Arg supplemented mice. Indirect calorimetry was used to determine energy expenditure (O_2 consumption) during the dark and the light cycle (**a**). Respiratory exchange ratios (**b**) were calculated to evaluate substrate metabolism and ambient activity (**c**) and rearing (**d**) were continuously registered for 72 consecutive hours. The mean ± SEM of eight control diet and nine L-Arg supplemented diet animals are shown. $*P < 0.05$

Administration of a L-Arg enriched diet alters energy expenditure, but not RER or locomotor activity

The observations that mice on the L-Arg diet consumed more energy, but displayed no difference in weight gain and only marginal differences in body composition, prompted us to examine if the altered feed efficiency could be accounted for by an increase in energy expenditure and/or locomotor activity. Energy expenditure in the indirect calorimeter of L-Arg administered mice, expressed as O_2 consumption, was significantly higher ($P < 0.04$) than

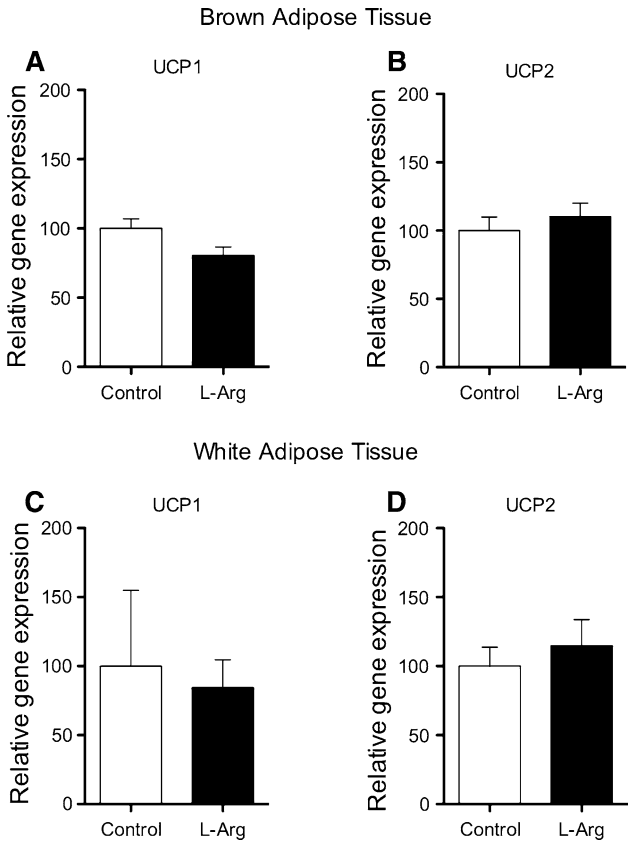

Fig. 4 L-Arg supplementation does not affect UCP1 or UCP2 expression in BAT or WAT. Normalized messenger RNA (mRNA) expression of UCP1 (**a**) and UCP2 (**b**) in brown adipose tissue (BAT) and UCP1 (**c**) and UCP2 (**d**) in white adipose tissue (WAT). The means ± SEM of eight control diet and nine L-Arg supplemented diet animals are shown

from that of control mice during the active dark phase (Fig. 3a). A similar tendency ($P = 0.06$) of increased oxygen consumption with L-Arg supplementation was observed during the light cycle (Fig. 3a). RER (VCO_2/VO_2) was similar in both groups (Fig. 3b), suggesting that L-Arg supplementation has no effect on substrate metabolism. The locomotor activity was slightly elevated in the L-Arg treated mice, compared to the control mice, although the differences were not statistically significant (Fig. 3c, d).

L-Arg supplementation does not alter expression of genes related to mitochondrial energy uncoupling

To gain insight into the molecular mechanisms responsible for the increased metabolic turnover associated with the observed L-Arg induced phenotype, WAT and BAT gene expressions of uncoupling proteins UCP1 and UCP2 were evaluated by quantitative PCR. Uncoupling proteins in BAT are linked to non-shivering thermoregulatory thermogenesis and, via uncoupling, ATP synthesis from mitochondrial substrate oxidation UCPs induces increased basal energy expenditure. We found no significant difference between L-Arg supplemented mice and control mice for any of the UCPs in BAT (Fig. 4a, b) or WAT (Fig. 4c, d). However, there was a tendency ($P = 0.051$) towards decreased UCP1 expression in BAT for the L-Arg supplemented mice (Fig. 4a) suggesting that the L-Arg induced increase in energy expenditure is mediated via other mechanisms than simple uncoupling.

L-Arg supplementation improves glucose metabolism and prevents dietary-induced insulin resistance

OGTT and ITT, performed at week 8 and 9, respectively, demonstrated that L-Arg supplemented mice were significantly more glucose tolerant (34% reduction in AUC, $P < 0.03$) and insulin sensitive than mice on the control diet (Fig. 5a–c). Fasting blood glucose levels were significantly lower in the L-Arg supplemented group (7.9 ± 0.4 mmol/L) compared with the control group (9.7 ± 0.2 mmol/L, $P = 0.003$) (Fig. 5d), but basal plasma insulin concentrations after 10 weeks on the diet did not significantly differ between the two groups (Fig. 5e). The HOMA-IR index was 36% lower in the L-Arg mice than in the control mice ($P < 0.04$ (Fig. 5f). A correlation analysis of basal plasma insulin levels and total fat mass demonstrated a significant positive correlation for mice on the control diet ($P = 0.0042$, $r^2 = 0.77$, $n = 8$) (Fig. 6a). Interestingly, this correlation was completely abolished for the L-Arg supplemented group ($P = 0.92$, $r^2 = 0.0017$, $n = 9$) (Fig. 6b), suggesting that the improved insulin sensitivity associated with dietary L-Arg supplementation may be independent on a parallel decrease in overall adiposity.

Discussion

Supplementation with dietary micronutrients is an emerging research field and recent years have witnessed the discovery that several amino acids possess multiple functions coupled to an array of biological effects (Wu 2009). In the present study, for the first time, we explore the effects of dietary L-Arg supplementation on body composition, appetite regulation, glucose metabolism and energy expenditure in mice. We show that increasing dietary L-Arg intake substantially improves an array of physiological parameters in mice fed a low-protein diet. High-fat diets (~60% kcal from fat) are frequently employed to investigate the effect of micronutrient supplementation on diet-induced metabolic disturbances in mice, usually intended to resemble human pathological states like obesity and type 2 diabetes (Zhang et al. 2007; Buettner et al. 2007; Nairizi et al. 2009; Macotela et al. 2011). In contrast to these

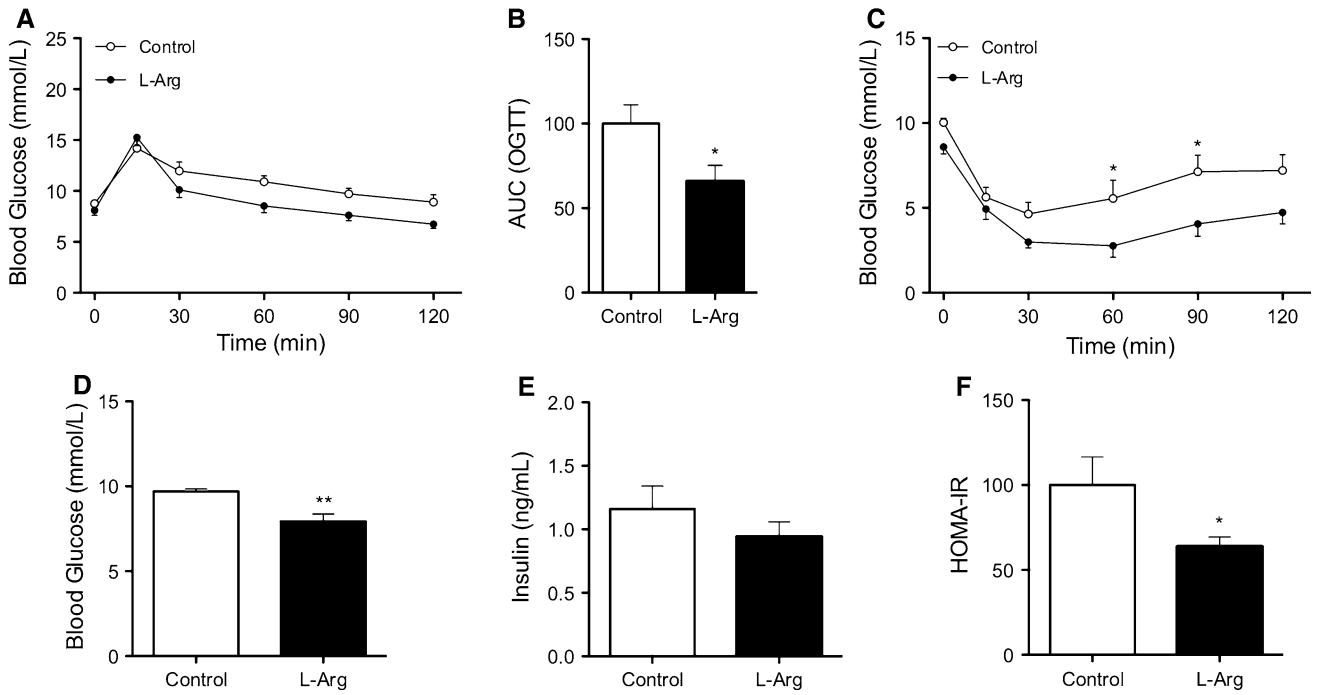

Fig. 5 L-Arg supplementation improves glucose metabolism and insulin sensitivity. For OGTT (**a**) 1.5 g glucose/kg was administered orally to fasted (6 h) mice at 8 weeks on the diet. Blood glucose levels were sampled at the indicated time points from venous tail blood. AUC was calculated from glucose (**b**) excursion curves. **c** ITT was performed at 9 weeks on the diet with 0.3 U/kg insulin administered i.p to induce moderate hypoglycemia. Blood glucose levels were sampled at the indicated time points from venous tail blood. Fasting blood glucose levels (**d**) and basal insulin levels (**e**) were sampled from mice at the end of the 10 week dietary study and HOMA-IR index (**f**) was calculated based on these values. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

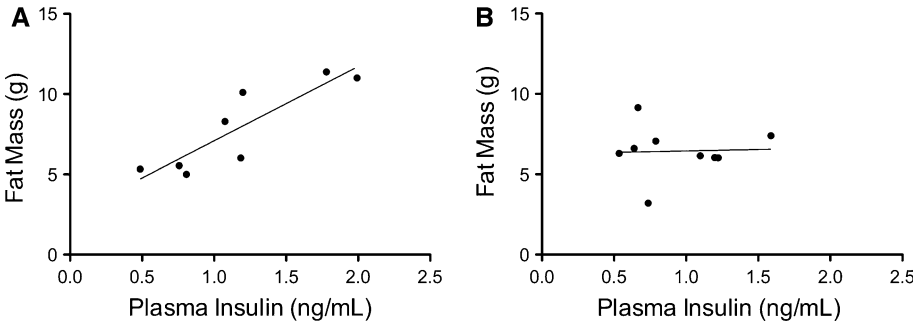

Fig. 6 L-Arg supplementation disrupts the relationship between basal insulin levels and whole-body adiposity. A correlation analysis of basal plasma insulin levels and total fat mass demonstrated a significant positive correlation for mice on the control diet (**a**) ($P = 0.0042$, $r^2 = 0.77$, $n = 8$), but not for the L-Arg supplemented group (**b**) ($P = 0.92$, $r^2 = 0.0017$, $n = 9$)

studies that habitually succeed in demonstrating the optimistic effects of amino acid supplementation, we employ, in the present study a more subtle dietary regime. Lowering dietary protein content is known to elicit hyperphagia and increase body fat deposition in rodents (Du et al. 2000; Rothwell et al. 1983), thus mimicking diet-induced obesity in humans. In addition, it has been hypothesized that L-Arg plays a central role in preserving an anabolic/protein sparing milieu during periods of inadequate supply of essential amino acids, e.g., in the fasted state or when administered a low-protein diet (Osowska et al. 2004; Moinard and Cynober 2007). Therefore, to fully uncover the effects of dietary L-Arg on whole-body physiology, we here aimed to generate hypoprotein-induced metabolic disturbances and thus designed a diet with slightly reduced protein content (8% kcal + 2% kcal from supplement) and consequently increased fat (20% kcal) and carbohydrate (70% kcal) levels (energy content in regular chow is 18% kcal from protein, 66% kcal from carbohydrate and 16% kcal from fat).

Our study shows that supplementing C57BL/6 mice with L-Arg for 10 weeks does not influence weight gain or

body composition when compared with control-fed mice. Despite that there was no difference in overall adiposity, fat pad analysis revealed a significant impact of increasing dietary L-Arg on visceral fat storage, as mice on the control diet had more epididymal fat. The effect of L-Arg supplementation on diminishing adipose tissue storage has previously been reported in different rat models of severe obesity (Fu et al. 2005; Jobgen et al. 2009b). Though the underlying mechanisms remain undefined, it was, in a follow-up study, discovered that supplementation with L-Arg to diet-induced obese rats resulted in an upregulation of several genes involved in lipolysis and oxidation of energy substrates (Jobgen et al. 2009a). This observation is further supported by a recent study that presented that L-Arg treatment induced the expression of genes favoring the loss of fat from subcutaneous adipose tissue in growing-finishing pigs (Tan et al. 2011). Nevertheless, future investigations are required to illuminate whether the effect of L-Arg on WAT is secondary to, e.g., endocrine regulations or whether it is a consequence of L-Arg directly affecting adipocytes.

A novel finding of the present study is that increased dietary L-Arg substantially increases food intake throughout the entire experiment. These data are in contrast to food intake analysis of obese Zucker rats supplemented with L-Arg, as their energy intake was found to be similar to L-alanine supplemented control rats (Fu et al. 2005). Conflicting results may reflect differences in species, study design and dietary intervention. A relationship between dietary protein levels and energy consumption has been described stating that when protein intake is restricted, food ingestion is primarily determined by the animal's attempt to meet its protein requirement (Webster 1993). Energy ingestion peaks when the diet contains 8–10% kcal protein (Du et al. 2000), corresponding to the amount in the herein employed diets. On the other hand, high-protein diets are recognized to reduce food intake (Halton and Hu 2004) and Cota et al. (2006) elegantly showed that centrally administered L-leucine activated the mTOR pathway in the hypothalamic arcuate nucleus and suppressed food intake in rats (Cota et al. 2006), demonstrating that individual amino acids held the potential of affecting appetite. This observation corresponds with data from the present study as we discover that L-Arg substantially increases food consumption. Interestingly, dietary L-Arg supplementation was found to reduce serum concentrations of L-leucine in obese as well as lean rats (Jobgen et al. 2009b). Whether this explains the hyperphagic effect of L-Arg found in the present study or whether L-Arg, in addition, exerts direct inhibitory effects on the same CNS circuits as L-leucine, i.e., by directly affecting interneuronal mTOR and/or AMPK, needs further investigations. Thus, whether the feeding effect is a direct consequence of the experimental intervention or rather a secondary compensatory response is left unresolved. Noteworthy, it has been proposed that when hyperphagia occurs in the context of weight maintenance, it is likely to be a secondary, compensatory response, e.g., to increased energy expenditure (Ellacott et al. 2010).

In support of this explanation, indirect calorimetry analysis revealed a significant L-Arg induced increase in oxygen consumption, indicating that dietary L-Arg affects energy expenditure in mice. Jobgen et al. (2009b) have recently emphasized the need for such investigations and this is, to our knowledge, the first study to analyze the effect of dietary L-Arg on energy expenditure. Recent rat and pig studies have illustrated that L-Arg potently increases skeletal muscle mass and reduces fat mass in obese as well as lean animals (Jobgen et al. 2009b; Tan et al. 2009), which might consequently increase basal metabolic rate as this causally relates to fat-free mass (Lazzer et al. 2010). In the present study, lean mass of L-Arg supplemented mice was only slightly (non-significant) elevated compared to control mice and we did not detect any L-Arg mediated hypertrophy of *m. gastrocnemius*. Thus, it seems unlikely that skeletal muscle tissue alone is responsible for the observed increase in energy expenditure. Moreover, we observed no significant difference in locomotor activity suggesting that other mechanisms are involved in the L-Arg mediated increase in metabolic rate.

While adipose tissue usually is accepted to serve as a relative inactive energy depot (Trayhurn and Beattie 2001), BAT is acknowledged as a major site for heat production, especially in younger animals because of a high mitochondrial activity (Rothwell and Stock 1979). Since it was demonstrated that BAT possibly exists in physiologically relevant amounts in humans, there has been a growing interest in understanding the role of BAT thermogenesis in energy balance regulation (Richard et al. 2010). Interestingly, it was discovered that diet-induced obese rats supplemented with L-Arg increased their BAT mass by 34% compared to L-alanine supplemented control rats (Jobgen et al. 2009b). In the present study, we did not detect gross hypertrophy of BAT in L-Arg supplemented mice compared to mice administered the control diet. However, it must be emphasized that BAT mass was previously found to be almost doubled in rats administered a low-protein diet (Rothwell et al. 1983), proposing that it might already be heavily up-regulated in both experimental groups in the present study and consequently might not be further increased with L-Arg supplementation. Noteworthy, it has also been demonstrated that the metabolic activity of BAT is controlled by diet-induced influence on brain circuits (Lowell and Bachman 2003), and recent animals studies have found increased expression of uncoupling proteins (UCPs) following dietary supplementation with L-Arg

(Vasilijevic et al. 2010) and L-leucine (Zhang et al. 2007). UCPs are located in the inner membrane of the mitochondrion where they uncouple ATP synthesis from mitochondrial substrate metabolism. However, in the present study we did not observe increased expression of UCP1 or UCP2 in BAT (or WAT). Surprisingly, our results showed a tendency toward L-Arg mediated down-regulation of UCP1 in BAT, proposing that the L-Arg induced rise in metabolic rate is independent of increased mitochondrial energy uncoupling in adipose tissues. Comparable studies on the metabolic effects of L-leucine discovered that this amino acid was effective in increasing the metabolic rate in obese (Zhang et al. 2007) but not lean mice (Noatsch et al. 2010), underlining that the effect of amino acid supplementation on energy metabolism might only be detectable against the background of adipogenic diets and/or diets with a low concentration of the amino acid of interest. Nevertheless, whether this holds true for the present data is speculative and the mechanisms underlying the L-Arg mediated increase in basal energy expenditure remain to be determined. Importantly, the increased metabolic rate might partially account for the major loss of feed efficiency associated with dietary L-Arg supplementation in the present study. Though we acknowledge the limited interpretability of our results, they clearly demonstrate a tremendous effect of L-Arg supplementation on the metabolic turnover in mice, as they become highly hyperphagic but show no signs of increased energy storage.

A hallmark characteristic of obesity as well as type 2 diabetes is disturbances in glucose metabolism such as peripheral insulin resistance (Kahn et al. 2006). Emerging evidence, from both animal and human studies, indicates that L-Arg supplementation may be a novel therapy for these metabolic-related disorders (McKnight et al. 2010). The present data now significantly extend these findings to support a model whereby L-Arg, independently of changes in whole-body adiposity, improves glucose metabolism. In agreement with a previous experiment, using diet-induced obese rats (Jobgen et al. 2009b), we demonstrated that L-Arg significantly enhanced oral glucose tolerance compared to control-fed animals. The substantial effect of L-Arg supplementation on glucose metabolism was further emphasized, as we found a potent increase in peripheral insulin sensitivity addressed by an ITT and the empirical HOMA-IR, a method that was recently verified as a valid assessment for insulin sensitivity in mice (Lee et al. 2008). The magnitude of the improved insulin sensitivity found in the L-Arg supplemented mice is comparable to data from an interesting human study, where a euglycemic-hyperinsulinemic clamp was used to assess insulin sensitivity in diabetic L-Arg supplemented patients (Piatti et al. 2001). The authors found that a hypocaloric diet in combination with L-Arg treatment (3 g three times per day for 1 month) effectively improved hepatic and peripheral insulin sensitivity compared to placebo treatment (Piatti et al. 2001). A more recent study, using human obese type 2 diabetic patients, demonstrated that 3 weeks of L-Arg supplementation (8.3 g/day) in combination with a hypocaloric diet and exercise training efficiently normalized postprandial glucose levels and reduced fasting plasma glucose (Lucotti et al. 2006)—a biomarker that was likewise significantly reduced in the present study. Collectively, these data provide a strong case for the beneficial effects of L-Arg supplementation on improving glucose metabolism in humans and multiple animal models, now including the mouse, supporting the idea that L-Arg may be an efficient micronutrient in combatting and/or treating the metabolic syndrome.

It is well known that obesity correlates with basal insulin levels underlining that obesity is a strong predictor of insulin resistance (Bagdade et al. 1967). Data from the present study demonstrate that hypoprotein-administered mice display comparable physiology as control-fed animals show a highly significant correlation between total fat mass and fasting plasma insulin levels. Surprisingly, this relationship is completely abolished when mice are supplemented with L-Arg, supporting our observations suggesting that L-Arg induced improvements of glucose metabolism are unrelated to a corresponding reduction in fat mass.

In summary, results of the present study indicate for the first time, to our knowledge, that dietary L-Arg supplementation substantially affects an array of metabolic-associated parameters, including a reduction in WAT, hyperphagia, improved insulin sensitivity and increased energy expenditure in male C57BL/6 mice exposed to diet-induced metabolic disturbances. These findings add to the knowledge on the physiological effects of dietary L-Arg and largely support the idea that L-Arg may be a relevant nutrient in the struggle of reducing the burden of metabolic diseases. Moreover, the present study corroborates the efficiency of employing a protein-reduced diet to simulate diet-induced obesity in humans. Future investigations are highly warranted to illuminate the cellular and molecular mechanisms underlying the metabolic effects of dietary L-Arg and we believe that the present study will be a useful reference when employing transgenic models to delineate these mechanisms.

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