Original Research *Highlight article*

Oral administration of α-ketoglutarate enhances nitric oxide synthesis by endothelial cells and whole-body insulin sensitivity in diet-induced obese rats

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Impact statement

Obesity is associated with elevated concentrations of branched-chain amino acids, including L-leucine. L-Leucine inhibits the synthesis of nitric oxide from L-arginine by endothelial cells, contributing to impairments in angiogenesis, blood flow, and vascular dysfunction, as well as insulin resistance. Reduction in the circulating levels of branched-chain amino acids through dietary supplementation with α-ketoglutarate to promote their transamination in the small intestine and other tissues can restore nitric oxide synthesis in the vasculature and reduce the weights of white adipose tissues, thereby improving metabolic profiles and whole-body insulin sensitivity (indicated by oral glucose tolerance test) in diet-induced obese rats. Our findings provide a simple and effective nutritional means to alleviate metabolic syndrome in obese subjects. This is highly significant to combat the current obesity epidemic and associated health problems in humans worldwide.

Abstract

Obesity is a risk factor for many chronic diseases, including hypertension, type-2 diabetes, and cancer. Interestingly, concentrations of branched-chain amino acids (BCAAs) in plasma are commonly associated with endothelial dysfunction in humans and animals with obesity. Because L-leucine inhibits nitric oxide synthesis by endothelial cells (EC), we hypothesized that dietary supplementation with AKG (a substrate for BCAA transaminase) may stimulate BCAA catabolism in the small intestine and extra-intestinal tissues, thereby reducing the circulating concentrations of BCAAs and increasing nitric oxide synthesis by endothelial cells. Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat or a high-fat diet for 15 weeks. At 19 weeks of age, lean or obese rats continued to be fed for 12 weeks their respective diets and received drinking water containing 0 or 1% AKG ($n = 8$/group). At 31 weeks of age, the rats were euthanized to obtain tissues. Food intake did not differ ($P > 0.05$) between rats supplemented with or without AKG. Oral administration of AKG (250 mg/kg BW per day) reduced ($P < 0.05$) concentrations of BCAAs, glucose, ammonia, and triacylglycerols in plasma, adiposity, and glutamine:fructose-6-phosphate transaminase activity in endothelial cells, and enhanced ($P < 0.05$) concentrations of the reduced form of glutathione in tissues, nitric oxide synthesis by endothelial cells, and whole-body insulin sensitivity (indicated by oral glucose tolerance test) in both low-fat and high-fat rats. AKG administration reduced ($P < 0.05$) white adipose tissue weights of rats in the low-fat and high-fat groups. These novel results indicate that AKG can reduce adiposity and increase nitric oxide production by endothelial cells in diet-induced obese rats.

Keywords: Metabolism, obesity, leucine, rats

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Introduction

Obesity is a risk factor for chronic diseases, including hypertension, type-2 diabetes, dyslipidemia, cardiovascular diseases, and cancer.[1–6] Available evidence shows that the synthesis of nitric oxide (NO, a major vasodilator) by endothelial cells is reduced in obese animals and humans.[7–9] The underlying mechanisms are largely unknown, but obesity is associated with abnormal

metabolism of branched-chain amino acids (BCAAs).[10–12] For example, concentrations of all the three BCAAs are elevated in the plasma of obese humans[10,13] and rodents.[14–16] Interestingly, an increase in the concentration of leucine in plasma by only 22% is associated with insulin resistance[10] and cardiovascular dysfunction in obese humans[17] and obese rats.[12]

Results of several studies suggest a role for L-leucine in inhibiting endothelial NO synthesis.[12] For example, infusion of L-leucine into the kidney of normal rats increased renal vascular resistance possibly due to a reduction in NO availability.[18] In addition, increasing extracellular concentrations of L-leucine attenuates the relaxation of rat aorta (an NO-dependent event),[19] and inhibits NO production by normal endothelial cells.[12] Consistent with these findings, an increase in the plasma concentration of leucine was associated with impaired endothelium-dependent relaxation of blood vessels in both obese Zucker rats (a genetically obese animal model)[20] and diet-induced obese rats.[12] On the basis of the foregoing results, we hypothesized that reducing the circulating levels of leucine through stimulating leucine transamination with orally administered α-ketoglutarate (AKG) may improve NO synthesis by endothelial cells and, therefore, metabolic profiles and insulin sensitivity in diet-induced obese rats.

Materials and methods

Diet-induced obese rats

This study was approved by the Texas A&M University Animal Use and Care Committee. Thirty-two three-week-old male Sprague-Dawley rats were obtained from Charles River and housed in a temperature and humidity controlled facility on a 12-h light:12-h dark cycle. At four weeks of age, the rats were assigned randomly to receive either a low-fat (LF) or a high-fat (HF) diet ($n = 16$ rats/diet) for 15 weeks.[21] The composition of the LF and HF diets was reported previously by Jobgen *et al.*[21] The HF feeding induced obesity in the rats. At 19 weeks of age, the rats continued to have free access to their respective diets; within the LF or HF group, rats were assigned randomly to receive drinking water (distilled and deionized H_2O) containing either 0% (control) or 1% AKG (an acid form purchased from Sigma Chemicals, St. Louis; directly dissolved in water) for 12 weeks, with eight rats per treatment group. Drinking water was provided daily. Body weight (BW) and food intake of rats were measured weekly, whereas water consumption by rats was recorded daily. One week before the end of AKG supplementation, an oral glucose tolerance test was performed on all rats, as described by Jobgen *et al.*[21] Briefly, after a 5-h period of food deprivation, D-glucose (2 g/kg body weight) in water was administrated orally into the stomach by gavage. Blood samples (20 μL) were obtained from the tail vein into plain tubes using microhematocrit capillary tubes at 0, 30, 60, 90, 120, 150, and 180 min post-gavage. Blood samples were centrifuged immediately at 10,000 *g* for 1 min to obtain sera for glucose analysis. Because of insufficient serum samples, insulin assays were not performed in the oral glucose tolerance test. At the end of the 12-week period of AKG treatment, rectal temperatures were recorded in conscious rats; cardiac blood (into heparinized tubes), white adipose tissues [epididymal (EP), mesenteric (MT), retroperitoneal (RP), and subcutaneous (SC)], other tissues, and coronary endothelial cells were obtained from the rats, as we described previously.[22] The cardiac blood samples were centrifuged at 600 *g* and 4°C for 10 min to obtain plasma.

NO synthesis by endothelial cells was determined as we described previously.[22] Briefly, freshly isolated coronary endothelial cells (1.5×10^6) were rinsed three times with 1 mL of Dulbecco-modified Eagle containing 5 mM D-glucose, antibiotics (100,000 U/L penicillin, 100 mg/L streptomycin and 0.25 mg/L amphotericin), 0.2 mM L-arginine, 0.2 mM L-leucine, 0.5 mM L-glutamine, and other amino acids (AAs) at concentrations found in the plasma of normal rats.[21] Thereafter, cells were incubated in this medium at 37°C for 6 h. At the end of the 6-h incubation period, media were analyzed for nitrite plus nitrate (an indicator of NO production). The medium, incubated without cells, was used as the blank. Due to the limited amount of endothelial cells, we did not analyze expression of endothelial NO synthase (eNOS) or other NOS isoforms.

Biochemical measurements

The plasma of blood taken from the heart was analyzed for AAs, glucose, triacylglycerols, cholesterol, free fatty acids, AKG, and ammonia (NH_3 plus NH_4^+).[16,21,23,24] The enzymatic activity of glutamine:fructose-6-phosphate transaminase (GFAT) in endothelial cells was determined as the formation of glucosamine-6-phosphate from glutamine and fructose-6-phosphate, as described previously.[25] Concentrations of the reduced form and the oxidized form of glutathione in tissues were analyzed by our high-performance liquid chromatography method.[26]

Statistical analysis

Data are expressed as means ± SEM. Data on the concentrations of AAs in plasma were log transformed prior to statistical analyses. Using two-way ANOVA, we assessed effects of HF feeding and AKG on the concentrations of amino acids and glucose in plasma as well as endothelial NO synthesis. To assess the impact of time (weeks) and treatment group assignment on BW, food intake, and water intake, the generalized estimating equations (GEE) approach was used.[27] In these models, time, treatment allocation, and their interaction were included as covariates. Additional comparisons of all pair-wise differences in the least squares means were performed when the interaction between time and treatment was statistically significant ($P < 0.05$). The ANOVA analyses were performed using PROC GLM, while PROC MIXED was used for the generalized estimating equations (GEE) analyses.[28] All the analyses were performed using the SAS 9.4 software (SAS Institute, Cary NC).

Table 1. Effects of high-fat feeding and oral administration of AKG on water consumption, food intake, and body weight (BW) of rats.[1,2]

	Groups				*P*-value		
Intake	**LF-Control**	**LF-AKG**	**HF-Control**	**HF-AKG**	**Diet**	**AKG**	**Diet ×AKG**
Water consumption, mL/kg BW per day	70.6 ± 3.0^{a}	53.1 ± 2.1^{b}	67.9 ± 2.8^{a}	53.4 ± 2.0^{b}	0.29	<0.01	0.94
Initial BW[3], g	615 ± 25	582 ± 22	664 ± 34	664 ± 35	0.04	0.63	0.61
Food intake, g/kg BW per day	36.3 ± 1.1^{a}	36.2 ± 0.87^{a}	29.4 ± 0.72^{b}	29.5 ± 0.69^{b}	<0.01	0.98	0.81
Final BW[4], g	703 ± 42	631 ± 24	791 ± 58	772 ± 38	0.02	0.29	0.54
BW gain[5], g/week	7.35 ± 0.61^{b}	4.08 ± 0.27^{c}	10.6 ± 0.76^{a}	9.01 ± 0.53^{ab}	<0.01	<0.01	0.38
Rectal temperature, °F	97.5 ± 0.05	97.4 ± 0.06	97.5 ± 0.04	97.5 ± 0.06	0.37	0.35	0.96

[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat (LF) or high-fat (HF) diet for 15 weeks. At 19 weeks of age, lean or obese rats continued to be fed for 12 weeks their respective diets and received drinking water containing 0 or 1% α-ketoglutarate (AKG).
[2]Values are means ± SEM, $n = 8$.
[3]The age of rats was 19 weeks.
[4]The age of rats was 31 weeks.
[5]Between 19 and 31 weeks of age.
a–c: Means in a row without a common superscript letter differ, $P < 0.05$.

Results

Food and water intakes, BW, and weigh gains of rats

Rats fed the HF diet had a heavier ($P < 0.05$) body weight than rats fed the LF diet during the entire experimental period (Table 1). AKG supplementation reduced ($P < 0.01$) water consumption by rats in the LF (−25%) or HF (−21%) group possibly due to a low palatability of AKG, but did not affect ($P > 0.05$) food intake by rats or their final BW in the LF or HF group (Table 1). In the LF group, but not in the HF group, AKG supplementation reduced ($P < 0.05$) the BW gains of rats. Of note, AKG supplementation did not negatively affect the appearance or health of rats. Rectal temperatures did not differ ($P > 0.05$) among the four groups of rats.

Tissue weights of rats

The weights of the heart (+17%), skeletal muscle (+13% to 33%), brown adipose tissue (+12%), and white adipose tissues (+35% to 100%, depending on fat depots) were greater ($P < 0.05$) but the weight of testes was 8% lower ($P < 0.01$) in rats fed the HF diet than in rats fed the LF diet (Table 2). The weights of other tissues did not differ ($P > 0.05$) between rats fed the LF and HF diets. Except for the liver, spleen, as well as RP, EP and MT white adipose tissues, the weights of all the measured tissues did not differ ($P > 0.05$) between rats supplemented with 0% and 1% AKG (Table 3). AKG supplementation reduced ($P < 0.05$) the weights of the liver (−15% to 20%), spleen (−16% to 19%), RP (-10%), EP (−19% to 41%) and MT (−38% to 42%) white adipose tissues (Table 2), as well as lipid content in gastrocnemius muscle (−13%, Table 4) in rats fed the LF or HF diet. The SC fat weight of rats was not affected ($P > 0.05$) by AKG supplementation.

Concentrations of AKG, AAs, glucose, lipids, and ammonia in the plasma of rats

Rats fed the HF diet had greater ($P < 0.05$) concentrations of most AAs (including BCAAs; + 12% to 25%), glucose (+16%), and cholesterol (+32%) in plasma than rats fed the LF diet (Table 3). Except for glutamate, threonine, arginine, methionine, and BCAAs, the concentrations of all other AAs measured in the present study did not differ ($P > 0.05$) between rats supplemented with 0% and 1% AKG (Table 3). However, there were interactions ($P > 0.05$) between diet and AKG supplementation for alanine and leucine. AKG supplementation increased ($P < 0.01$) the plasma concentrations of AKG (+338%; Table 4), glutamate (+50%), arginine (+18%), and methionine (+29%) but reduced ($P < 0.01$) those of leucine (−18%), isoleucine (−19%), valine (−21%), ammonia (−28%), glucose (−3.4%), and triacylglycerols (−13%) in rats fed the LF or HF diet (Table 3). AKG supplementation increased ($P < 0.05$) the concentration of alanine (+13%) in the plasma of rats fed the HF diet but had no effect ($P > 0.05$) in rats fed the LF diet. Concentrations of cholesterol and free fatty acids in the plasma of 31-week-old rats were not affected ($P > 0.05$) by HF feeding or AKG supplementation (Table 4).

Concentrations of glutathione in tissues of rats

Data on the concentrations of the reduced form and oxidized form of glutathione are summarized in Table 5. High-fat feeding decreased ($P < 0.01$) the concentration of the reduced form of glutathione (GSH), while increasing ($P < 0.05$) the concentration of the oxidized form of glutathione (GSSG) and the ratio of GSSG/GSH in the heart, liver, white adipose tissue, and skeletal muscle of rats. Of note, dietary supplementation with AKG reduced ($P < 0.01$) the GSSG/GSH ratio by 17% to 20% in these tissues.

NO synthesis and GFAT activity in endothelial cells and whole-body insulin sensitivity in rats

Rats fed the HF diet had a 20% lower ($P < 0.05$) rate of NO synthesis and a 60% higher GFAT ($P < 0.05$) activity in endothelial cells, as well as a 30% higher ($P < 0.05$) whole-body insulin resistance (indicated by the oral glucose

Table 2. Effects of high-fat feeding and oral administration of AKG on tissue weights of rats at 31 weeks of age.[1,2]

	Groups				*P*-value		
Tissue	**LF-Control**	**LF-AKG**	**HF-Control**	**HF-AKG**	**Diet**	**AKG**	**Diet ×AKG**
Heart	1.80 ± 0.07^{b}	1.86 ± 0.07^{b}	2.19 ± 0.16^{a}	2.11 ± 0.11^{a}	0.04	0.14	0.46
Lung	2.02 ± 0.07	2.09 ± 0.05	2.14 ± 0.09	2.20 ± 0.23	0.09	0.31	0.70
Liver	29.2 ± 2.5^{a}	23.3 ± 0.93^{b}	28.2 ± 1.5^{a}	23.9 ± 1.5^{b}	0.23	<0.01	0.79
Spleen	0.90 ± 0.04^{a}	0.73 ± 0.02^{b}	0.94 ± 0.04^{a}	0.79 ± 0.03^{b}	0.79	<0.01	0.70
Kidney	2.18 ± 0.12	2.08 ± 0.02	2.20 ± 0.16	2.37 ± 0.08	0.07	0.93	0.26
RP fat	35.1 ± 2.3^{b}	27.2 ± 1.6^{b}	64.7 ± 2.8^{a}	60.1 ± 4.8^{a}	<0.01	0.04	0.72
EP fat	18.8 ± 2.0^{b}	15.2 ± 0.96^{b}	30.4 ± 2.7^{a}	18.0 ± 1.4^{b}	0.03	<0.01	0.95
SC fat	16.7 ± 1.5^{b}	16.9 ± 0.83^{b}	30.0 ± 1.3^{a}	24.4 ± 1.2^{a}	<0.01	0.18	0.54
MT fat	21.8 ± 1.6^{b}	12.7 ± 1.5^{c}	28.7 ± 2.5^{a}	17.8 ± 1.0^{bc}	<0.01	<0.01	0.47
Brown fat	1.05 ± 0.05^{b}	1.02 ± 0.13^{b}	1.16 ± 0.05^{a}	1.15 ± 0.11^{a}	0.02	0.24	0.07
Small intestine	7.17 ± 0.70	6.34 ± 0.24	7.00 ± 0.73	6.13 ± 0.29	0.30	0.17	0.21
Pancreas	1.42 ± 0.12	1.21 ± 0.12	1.45 ± 0.17	1.28 ± 0.07	0.33	0.21	0.99
EDL muscle	0.24 ± 0.002	0.23 ± 0.012	0.27 ± 0.001	0.26 ± 0.003	<0.01	0.12	0.40
Soleus muscle	0.20 ± 0.003	0.20 ± 0.012	0.27 ± 0.003	0.26 ± 0.01	<0.01	0.17	0.20
Testes	4.07 ± 0.18^{ab}	4.16 ± 0.10^{a}	3.80 ± 0.20^{b}	3.78 ± 0.23^{b}	<0.01	0.78	0.07
Brain	2.13 ± 0.07	2.01 ± 0.19	2.06 ± 0.12	2.09 ± 0.14	0.39	0.18	0.30
Large intestine	2.64 ± 0.23	2.04 ± 0.30	2.34 ± 0.22	2.06 ± 0.18	0.73	0.17	0.52
Stomach	2.35 ± 0.15	2.23 ± 0.09	2.26 ± 0.30	2.07 ± 0.11	0.87	0.52	0.66

[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat (LF) or high-fat (HF) diet for 15 weeks. At 19 weeks of Age, lean or obese rats continued to be fed for 12 weeks their respective diets and received drinking water containing 0 or 1% α-ketoglutarate (AKG). At 31 weeks of age, rats were euthanized to obtain tissues.

[2]Values, expressed as g, are means ± SEM, $n = 8$.

a–c: Means in a row without common superscript letters differ, $P < 0.05$.

EDL: extensor digitorum longus; EP: epididymal; MT: mesenteric; RP: retroperitoneal; SC: subcutaneous.

Table 3. Effects of high-fat feeding and oral administration of AKG on the concentrations of amino acids in plasma of SD rats.[1,2]

	Groups				*P*-value		
Serum AA	**LF-Control**	**LF-AKG**	**HF-Control**	**HF-AKG**	**Diet**	**AKG**	**Diet ×AKG**
Aspartate	42 ± 3.9	38 ± 2.6	40 ± 3.5	45 ± 2.2	0.57	0.95	0.32
Glutamate	90 ± 3.6^{b}	138 ± 8.3^{a}	95 ± 4.1^{b}	140 ± 9.0^{a}	0.72	<0.01	0.10
Asparagine	45 ± 2.4	46 ± 2.7	56 ± 2.2	57 ± 2.6	<0.01	0.26	0.66
Serine	216 ± 8.0^{b}	202 ± 9.3^{b}	247 ± 11^{a}	251 ± 10^{a}	<0.01	0.12	0.78
Glutamine	565 ± 17	587 ± 15	632 ± 19	641 ± 22	<0.01	0.33	0.94
Histidine	62 ± 3.2b	66 ± 4.6b	78 ± 4.7^{a}	80 ± 4.7a	<0.01	0.48	0.66
Glycine	217 ± 5.4^{b}	185 ± 8.2^{c}	246 ± 9.0^{a}	248 ± 9.3^{a}	<0.01	0.17	0.15
Threonine	192 ± 9.0^{b}	212 ± 13^{ab}	221 ± 9.5^{ab}	242 ± 5.8^{a}	<0.01	0.04	0.88
Citrulline	62 ± 2.6^{b}	65 ± 2.2^{b}	70 ± 2.4^{a}	72 ± 3.1^{a}	0.03	0.26	0.96
Arginine	155 ± 4.8^{c}	185 ± 5.9^{b}	181 ± 4.6^{b}	213 ± 6.6^{a}	<0.01	<0.01	0.34
β-Alanine	9.8 ± 0.86	11 ± 0.97	10 ± 1.2	11 ± 1.0	0.44	0.26	0.89
Taurine	401 ± 47	463 ± 55	427 ± 47	440 ± 27	0.34	0.10	0.91
Alanine	510 ± 9.2^{b}	518 ± 19^{b}	502 ± 15^{b}	565 ± 17^{a}	0.19	0.10	0.03
Tyrosine	79 ± 5.3^{b}	82 ± 5.6^{b}	106 ± 6.6^{a}	108 ± 6.2^{a}	<0.01	0.85	0.96
Tryptophan	64 ± 3.2^{b}	60 ± 3.7^{b}	77 ± 3.6^{a}	75 ± 3.0^{a}	<0.01	0.31	0.78
Methionine	37 ± 1.8^{c}	46 ± 1.7^{b}	48 ± 2.0^{b}	64 ± 2.2^{a}	<0.01	<0.01	0.86
Valine	184 ± 7.1^{b}	149 ± 3.0^{c}	233 ± 8.1^{a}	181 ± 7.0^{b}	<0.01	<0.01	0.22
Phenylalanine	58 ± 1.2^{b}	55 ± 1.0^{b}	64 ± 2.0^{a}	62 ± 2.6^{a}	0.03	0.12	0.11
Isoleucine	125 ± 3.8^{b}	104 ± 2.5^{c}	156 ± 5.3^{a}	123 ± 4.4^{b}	<0.01	<0.01	0.88
Leucine	164 ± 3.2^{b}	140 ± 3.1^{c}	207 ± 3.4^{a}	163 ± 4.0^{b}	<0.01	<0.01	0.02
Ornithine	41 ± 1.8	40 ± 2.2	42 ± 2.3	39 ± 2.5	0.54	0.93	0.47
Lysine	259 ± 19	236 ± 16	302 ± 15	317 ± 18	<0.01	0.82	0.39

[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat (LF) or high-fat (HF) diet for 15 weeks. At 19 weeks of Age, lean or obese rats continued to be fed for 12 weeks their respective diets and received drinking water containing 0 or 1% α-ketoglutarate (AKG). At 31 weeks of age, rats were euthanized to plasma for the analysis of amino acids.

[2]Values, expressed as nmol/mL, are means ± SEM, $n = 8$.

a–c: Means in a row without a common superscript letter differ, $P < 0.05$.

Table 4. Effects of high-fat feeding and oral administration of AKG on NO synthesis and GFAT activity in endothelial cells, whole-body insulin sensitivity, and plasma metabolites in rats.[1,2]

	Groups				*P*-value		
Variable	**LF-Control**	**LF-AKG**	**HF-Control**	**HF-AKG**	**Diet**	**AKG**	**Diet ×AKG**
NO synthesis by EC[3]	209 ± 7.4^{b}	243 ± 6.4^{a}	161 ± 3.3^{c}	200 ± 6.7^{b}	<0.01	<0.01	0.67
GFAT activity in EC[4]	101 ± 4.8^{c}	75 ± 2.5^{d}	165 ± 3.9^{a}	117 ± 5.2^{b}	<0.01	<0.01	<0.01
Insulin sensitivity[5]	1028 ± 30^{b}	861 ± 17^{c}	1383 ± 32^{a}	1073 ± 53^{b}	<0.01	<0.01	0.11
Metabolites in plasma							
α-Ketoglutarate, μM	8.51 ± 0.45^{b}	37.6 ± 2.2^{a}	8.64 ± 0.74^{b}	38.6 ± 2.4^{a}	0.73	<0.01	0.75
Glucose, mM	8.20 ± 0.06^{c}	7.91 ± 0.05^{d}	9.54 ± 0.04^{a}	9.23 ± 0.06^{b}	<0.01	<0.01	0.94
Triacylglycerols, mM	0.92 ± 0.06	0.80 ± 0.05	1.02 ± 0.07	0.88 ± 0.06	0.58	0.04	0.81
Cholesterol, mM	4.71 ± 0.19^{b}	4.65 ± 0.27^{b}	6.19 ± 0.29^{a}	6.12 ± 0.32^{a}	<0.01	0.83	0.45
Free fatty acids, mM	0.80 ± 0.07	0.78 ± 0.05	0.85 ± 0.06	0.80 ± 0.09	0.83	0.85	0.66
Ammonia, μM	105 ± 4.9^{a}	75.4 ± 4.7^{b}	108 ± 5.7^{a}	78.5 ± 5.2^{b}	0.50	<0.01	0.21
Muscle lipids,[6] g/100 g	1.62 ± 0.06^{b}	1.40 ± 0.04^{c}	1.94 ± 0.08^{a}	1.71 ± 0.06^{b}	<0.01	<0.01	0.35

[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat (LF) or high-fat (HF) diet for 15 weeks. At 19 weeks of age, lean or obese rats continued to be fed for 12 weeks their respective diets and received drinking water containing 0 or 1% α-ketoglutarate (AKG). At 30 weeks of age, an oral glucose tolerance test was performed on all rats to assess whole-body insulin sensitivity. At 31 weeks of age, rats were euthanized to isolate coronary endothelial cells and obtain plasma.
[2]Values are means ± SEM, $n = 8$.
[3]pmol/10^6 cells/h.
[4]pmol/mg protein/min.
[5]The area under the response curve (h.mmol/L) in the oral glucose tolerance test.
[6]The concentrations of lipids in gastrocnemius muscle.
a–d: Means in a row without a common superscript letter differ, $P < 0.05$.
EC: endothelial cells; GFAT: glutamine:fructose-6-phosphate transaminase.

Table 5. Effects of high-fat feeding and oral administration of AKG on concentrations of reduced and oxidized forms of glutathione in tissues of rats.[1,2]

	Groups				*P*-value		
Tissue	**LF-Control**	**LF-AKG**	**HF-Control**	**HF-AKG**	**Diet**	**AKG**	**Diet ×AKG**
Heart							
GSH[3]	1726 ± 36	1804 ± 31	1590 ± 43	1712 ± 47	<0.01	0.017	0.19
GSSG[4]	125 ± 4.9	109 ± 2.6	170 ± 4.5	146 ± 5.3	<0.01	<0.01	0.62
GSSG/GSH[5]	0.072 ± 0.002	0.060 ± 0.001	0.107 ± 0.002	0.085 ± 0.001	<0.01	<0.01	0.33
Liver							
GSH[3]	6054 ± 144	6792 ± 223	4394 ± 151	4783 ± 178	<0.01	<0.01	0.66
GSSG[4]	492 ± 12	439 ± 15	605 ± 25	533 ± 17	<0.01	<0.01	0.25
GSSG/GSH[5]	0.081 ± 0.001	0.065 ± 0.001	0.138 ± 0.003	0.112 ± 0.003	<0.01	<0.01	0.44
RP-WAT							
GSH[3]	705 ± 15	738 ± 17	510 ± 19	554 ± 12	<0.01	0.02	0.57
GSSG[4]	86.3 ± 2.0	78.3 ± 1.3	122 ± 4.9	105 ± 2.6	<0.01	<0.01	0.41
GSSG/GSH[5]	0.123 ± 0.003	0.109 ± 0.003	0.239 ± 0.005	0.193 ± 0.002	<0.01	<0.01	0.29
Skeletal muscle							
GSH[3]	712 ± 10	748 ± 22	615 ± 26	665 ± 21	<0.01	0.03	0.73
GSSG[4]	55.1 ± 1.3	46.0 ± 1.6	68.8 ± 2.7	59.5 ± 2.1	<0.01	<0.01	0.62
GSH/GSSG[5]	0.077 ± 0.002	0.062 ± 0.001	0.112 ± 0.002	0.090 ± 0.001	<0.01	<0.01	0.20

[1]Beginning at four weeks of age, male Sprague-Dawley rats were fed a low-fat (LF) or high-fat (HF) diet for 15 weeks. At 19 weeks of age, lean or obese rats continued to be fed for 12 weeks their respective diets and received drinking water containing 0 or 1% α-ketoglutarate (AKG). At 31 weeks of age, rats were euthanized to obtain the heart, liver, retroperitoneal white adipose tissue, and skeletal muscle (gastrocnemius muscle) for the analyses of the reduced and oxidized forms of glutathione.
[2]Values are means ± SEM, $n = 8$.
[3]Values are expressed as nmol/g of wet tissue.
[4]Values are expressed as nmol/g of wet tissue.
[5]Values are expressed as mol/mol.
a–c: Means in a row without a common superscript letter differ, $P < 0.05$.
GSH: the reduced form of glutathione; GSSG: the oxidized form of glutathione; RT-WAT: retroperitoneal white adipose tissue.

tolerance test), compared with rats fed the LF diet (Table 4). In rats fed the LF or HF diet, AKG supplementation enhanced ($P < 0.05$) NO synthesis (+20%) by endothelial cells and whole-body insulin sensitivity (indicated by a 20% decrease of the area under the response curve in the oral glucose tolerance test), while reducing GFAT activity (−28%) in endothelial cells (Table 4).

Discussion

Physiological levels of NO play a critical role in improving insulin sensitivity in humans and animals.[29] However, NO synthesis by endothelial cells is impaired in obese subjects who usually exhibit elevated concentrations of leucine and the other two BCAAs.[10,14,21] There is evidence that leucine

inhibits NO production by endothelial cells possibly through activating GFAT for the synthesis of glucosamine-6-phosphate.[12,30] Among the three BCAAs, leucine is capable of reducing NO generation from arginine in endothelial cells.[12] Thus, the regulation of leucine catabolism is potentially an effective target for enhancing NO synthesis and availability in the vasculature.[12] Leucine is degraded through transamination with AKG by BCAA transaminase in multiple organs (primarily small intestine and skeletal muscle) and many cell types, including endothelial cells.[31] Thus, increasing AKG availability stimulates BCAA degradation in multiple tissues, thereby reducing the concentrations of BCAAs (including leucine) in plasma.[32] This hypothesis is supported by results of the present study (Table 3). Additionally, it is also possible that AKG supplementation attenuates oxidative stress and improves mitochondrial function in the small intestine, liver, skeletal muscle and other tissues, thereby further stimulating the oxidation of the carbon skeletons of BCAAs to CO_2 and water via the mitochondrial Krebs cycle and electron transport system.

α-Ketoglutarate acts on cell metabolism likely via multiple mechanisms. In AKG-supplemented rats fed the LF or HF diet, a decrease in the circulating concentrations of BCAAs resulted in a higher rate of NO synthesis in endothelial cells. It is unknown whether expression of eNOS was altered in the cells. It is our experience that the amount of eNOS in endothelial cells is usually not a limiting factor for NO synthesis; rather, the availability of cofactors of eNOS (i.e., NADPH and tetrahydrobiopterin) limits NO production by endothelial cells.[25,33,34] Of particular note, dietary supplementation with AKG augmented the sensitivity of the whole body to insulin (Table 4). This effect of AKG may be mediated in part through enhancing glucose and fatty acid oxidation in skeletal muscle and other tissues,[29] as well as inhibiting GFAT expression and activity.[30] GFAT catalyzes the conversion of glutamine and fructose-6-P into glucosamine-6-P, which reduces pentose phosphate cycle activity and, therefore, the generation of NADPH (an essential cofactor of NO synthase).[23] Another mechanism responsible for the beneficial effect of AKG on endothelial NO synthesis is an increase in the concentration of arginine in plasma (Table 3). It is possible that AKG supplementation promotes the production of glutamate from AKG plus ammonia via glutamate dehydrogenase in tissues, as indicated by a marked increase in the concentration of glutamate and a decrease in the concentration of ammonia in the plasma of rats (Tables 3 and 4). Glutamate is subsequently converted into glutamine in skeletal muscle and other tissues by glutamine synthetase. Glutamine in arterial blood is taken up by the small intestine for the synthesis of citrulline, which is extracted by the kidneys for the production of arginine.[35] The fact that the concentration of glutamine in plasma did not differ between rats supplemented with or without 1% AKG may result from an equal increase in both glutamine synthesis and glutamine utilization by the whole body. Based on our finding that the concentration of alanine in plasma was not affected by AKG supplementation (Table 3), it is possible that the AKG-derived glutamate may not be preferentially metabolized via its transamination with pyruvate. Because arginine is the nitrogenous precursor of NO, an increase in the availability of this AA can augment NO synthesis by endothelial cells.[22] An increase in NO production by endothelial cells within physiological levels does not result in oxidative stress in the vasculature and other tissues.[30] This notion is further supported by our finding that dietary supplementation with AKG reduced the ratio of GSSG/GSH (an indicator of intracellular oxidative stress)[36] in the heart, liver, white adipose tissue, and skeletal muscle of rats (Table 5). Such a metabolic effect of AKG in regulating endothelial NO synthesis is novel and important, as it offers a safe and promising nutritional means to alleviate vascular dysfunction in obese subjects. In light of our findings, future studies involving tracers are warranted to quantify the metabolic fluxes of AAs in diet-induced obese rats supplemented with or without AKG.

AKG supplementation did not affect the mass of all white adipose tissues in rats fed the HF or LF diet (Table 2). This reflects an anatomical difference in fat depot-specific lipid metabolism[37] and does not necessarily mean that the physiological function of white adipose tissues in the different sites of the body is unaltered by AKG supplementation. For example, consistent with a role of physiological levels of NO in inhibiting the hepatic synthesis of triacylglycerols,[29] AKG supplementation beneficially reduced the concentrations of triacylglycerols in the plasma of rats fed the LF or HF diet (Table 4). Additionally, an increase in the sensitivity of tissues to insulin in response to AKG supplementation, as indicated by the oral glucose tolerance test (Table 4), suggests that such a nutritional therapy can potentially alleviate insulin resistance in tissues (including white adipose tissue and skeletal muscle) of obese subjects. In the glucose tolerance test, a lower value for the area under the response curve (expressed as h. mmol/L) indicates a higher rate of net glucose utilization and, therefore, an improvement in the sensitivity of tissues to insulin.[38] Further studies are warranted to examine the insulin-signaling pathway in AKG-supplemented rats, as well as the expression of NOS isoforms and GFAT in tissues *in vivo*. Nonetheless, AKG supplementation did reduce the body weight gains of rats fed the LF diet (Table 1), despite no significant changes in the weights of many tissues (Table 2). For our rats (31 weeks of age at the time of tissue collection), the components of changes in their BW cannot be identified without data on their bone mass, as well as their intramuscular fat and all subcutaneous fat mass that can account for significant proportions of animals.[39] Water content in animals is generally decreased with increasing fat content.[39] Thus, we surmise that AKG supplementation may decrease intramuscular fat and all subcutaneous fat mass, without affecting bone mass or water content. In support of this hypothesis, we found that lipid concentrations in gastrocnemius muscle (a skeletal muscle with both oxidative and glycolytic fibers) were reduced by AKG supplementation (Table 4). Because the accretion of fat in the body is associated with reduced water content,[39] a decrease in white adipose tissues in

AKG-supplemented rats is expected to increase water content in the body rather than causing dehydration in cells and animals. There is also evidence that AKG mitigates oxidative stress and enhances protein synthesis in animals cells.[40-42]

This research has important cardiovascular relevance because identifying AKG-induced reductions in plasma leucine concentration and the activity of the glucosamine-synthetic pathway as a novel cellular mechanism for improving whole-body insulin sensitivity provides new clues for nutritional strategies to prevent and treat endothelial dysfunction in obese subjects. Enhancing endothelial NO bioavailability is expected to help prevent and treat obesity-associated cardiovascular complications, such as coronary heart disease, hypertension, and cerebrovascular disease, which are responsible for most of the morbidity and mortality in obese patients.[1–7]

Conclusion

Long-term oral administration of AKG reduced the concentrations of BCAAs, glucose, triacylglycerols and ammonia in plasma, adiposity, glutamine:fructose-6-phosphate transaminase activity in EC and GSSG/GSH ratios in tissues, while enhancing the concentration of arginine in plasma, NO synthesis in endothelial cells, concentrations of the reduced form of glutathione in tissues, and whole-body insulin sensitivity in both LF and HF rats. AKG administration reduced BW gains of LF rats. These novel results indicate that AKG can alleviate insulin resistance and improve health in diet-induced obese animals.

Authors' contributions: GW conceived this project. CDT, KY, JL, XL and GW performed experiments. CDT, AG, YL and GW analyzed data. CDT, FWB, CJM and GW wrote the manuscript. All authors contributed to the discussion and revision of the article. GW had the primary responsibility for the content of the paper. All authors read and approved this manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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