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INVITED REVIEW

L-Leucine and NO-mediated cardiovascular function

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Abstract Reduced availability of nitric oxide (NO) in the vasculature is a major factor contributing to the impaired action of insulin on blood flow and, therefore, insulin resistance in obese and diabetic subjects. Available evidence shows that vascular insulin resistance plays an important role in the pathogenesis of cardiovascular disease, the leading cause of death in developed nations. Interestingly, increased concentrations of L-leucine in the plasma occur in obese humans and other animals with vascular dysfunction. Among branched-chain amino acids, L-leucine is unique in inhibiting NO synthesis from L-arginine in endothelial cells and may modulate cardiovascular homeostasis in insulin resistance. Results of recent studies indicate that L-leucine is an activator of glutamine:fructose-6-phosphate aminotransferase (GFAT), which is the first and a rate-controlling enzyme in the synthesis of glucosamine (an inhibitor of endothelial NO synthesis). Through stimulating the mammalian target of rapamycin signaling pathway and thus protein synthesis, L-leucine may enhance GFAT protein expression, thereby inhibiting NO synthesis in endothelial cells. We propose that reducing circulating levels of L-leucine or endothelial GFAT activity may provide a potentially novel strategy for preventing and/or treating cardiovascular disease in obese and diabetic subjects. Such means may include dietary supplementation with either α-ketoglutarate to enhance the catabolism of L-leucine in the small intestine and other tissues or with *N*-ethyl-L-glutamine to inhibit GFAT activity in endothelial cells. Preventing leucine-induced activation of GFAT by nutritional supplements or pharmaceutical drugs may contribute to improved cardiovascular function by enhancing vascular NO synthesis.

Keywords Leucine · Nitric oxide · Endothelial dysfunction · Insulin resistance

Abbreviations

BCAA	Branched-chain amino acids
BCAT	Branched-chain amino acid transaminase
BCKAD	Branched-chain α-ketoacid dehydrogenase
BH4	(6R)-5,6,7,8-Tetrahydro-L-biopterin
EDCF	Endothelium-derived constricting factor
EDRF	Endothelium-derived relaxing factor
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GFAT	Glutamine:fructose-6-phosphate aminotransferase
KIC	α-Ketoisocaproate
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase

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Introduction

Nitric oxide (NO), an endothelium-derived relaxing factor (EDRF), is gaining much attention due to its functional role in maintaining vascular homeostasis (Lei et al. 2013a, b). In the vascular endothelium, NO is produced primarily by endothelial NO synthase (eNOS), which converts L-arginine and molecular oxygen into NO plus L-citrulline (Alderton et al. 2001). The biological function of eNOS requires its dimerization, the presence of the nitrogenous substrate L-arginine, as well as essential cofactors (6R)-5, 6, 7, 8-tetrahydro-L-biopterin (BH4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calcium, calmodulin, and nicotinamide adenine dinucleotide phosphate (NADPH) (Fig. 1). In addition to NO, the endothelium produces other EDRF (including endothelium-derived hyperpolarizing factor, C-type natriuretic peptide, and kinins), and endothelium-derived constricting factors [(EDCF), including angiotensin II and endothelin (Cersosimo and DeFronzo 2006)]. Under normal physiologic conditions, the production and bioavailability of EDRF and EDCF are balanced to maintain normal endothelial function and vascular tone. When this balance is disturbed, endothelial dysfunction occurs in association with increased bioavailability of reactive oxygen and nitrogen species and decreased antioxidant capacity, therefore leading to oxidative stress, vascular damage, and inflammation (Hoang et al. 2013; Rubio and Morales-Segura 2004).

Endothelial dysfunction is generally accepted as an early hallmark of multiple cardiovascular disorders, including atherosclerosis, diabetes, hypercholesterolemia, hypertension, and stroke (Hoang et al. 2013). Although the mechanisms underlying endothelial dysfunction can be very different under various disease conditions, growing evidence indicates that a reduced bioavailability of NO contributes to the development of endothelial dysfunction (Karbach et al. 2014). Thus, therapeutic interventions to restore the availability of NO provide promising strategies to ameliorate the detrimental effect of endothelial dysfunction on human and animal health. One of these means is to regulate the catabolism of L-leucine [one of the three branched-chain amino acids (BCAA)], thereby inhibiting L-leucine-activated synthesis of glucosamine from L-glutamine to D-glucose in endothelial cells by glutamine:fructose-6-phosphate aminotransferase (GFAT) (Wu and Meininger 2009). The major objective of the present article is to highlight recent developments in this new area of amino acid nutrition as it relates to cardiovascular health.

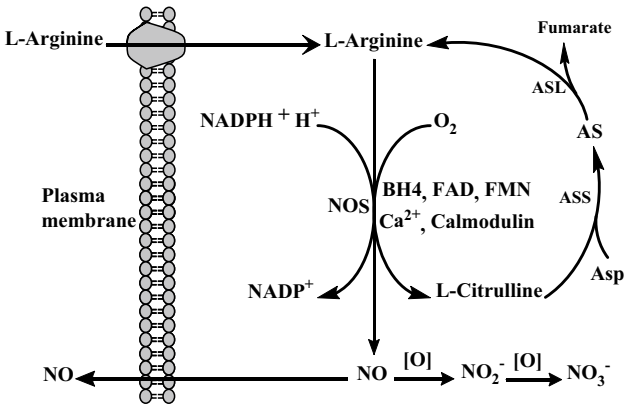

Fig. 1 Synthesis of NO from L-arginine by NO synthase in endothelial cells. The eNOS enzyme is constitutively expressed in endothelial cells to produce NO in the nanomolar range under physiological conditions. Endothelial cells can recycle L-citrulline into L-arginine as a mechanism to regulate intracellular concentrations of L-arginine. *AS* argininosuccinate, *BH4* tetrahydrobiopterin, *ASL* argininosuccinate lyase, *ASS* argininosuccinate synthase, *Asp* L-aspartate. Taken from Li et al. (2009a)

NO biosynthesis

The three NOS isoforms are encoded by three different genes in animals: endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1), and the inducible NOS (iNOS or NOS2) (Wu and Meininger 2002). The endothelial NOS and neuronal NOS are known as constitutive NOS in comparison with the iNOS, which is distributed primarily in phagocytes, such as monocytes, macrophages, and neutrophils. Expression of iNOS is enhanced by inflammatory cytokines, such as interferon-gamma (IFN-γ) or tumor necrosis factor (TNF-α) to produce NO and can be associated with immune or inflammatory responses to pathogens (Nathan and Xie 1994). The nNOS isoform produces NO in nervous tissue (e.g., the central and peripheral nervous system) and skeletal muscle, whereas eNOS is expressed primarily in endothelial cells of blood vessels (including microvessels) and plays an important role in maintaining vascular homeostasis (Rubio and Morales-Segura 2004).

The NOS isoforms were originally named according to the tissues from which they were first purified (neuronal and endothelial) and the mode of activation in the case of iNOS. However, it is now known that all three NOS isoforms can be induced by appropriate stimuli and can be constitutively expressed to various extents in some tissues or cells (Alderton et al. 2001; Förstermann and Sessa 2012). Thus, the previous designations of the NOS isoforms are inappropriate even though they are still widely used in the scientific literature.

As noted previously, in addition to calcium and calmodulin, four cofactors are required for eNOS-catalyzed NO synthesis: BH4, FAD, FMN, and NADPH (Boveris et al. 2000). While the three NOS isoforms are products of three different genes, they share similar structural domains with highly conserved sequence identity. To produce NO,

two subunits are bound to form a homodimer with each unit consisting of an N-terminal oxygenase domain and a C-terminal reductase domain (Alderton et al. 2001; Stuehr 1997). The oxygenase domain contains the binding sites for BH4 and heme, whereas the reductase domain binds to FMN, FAD, NADPH, and calmodulin (Alderton et al. 2001; Stuehr 1997). The functionally active NOS dimer produces NO using L-arginine and oxygen as substrates (Thomas et al. 2008). The activity of NOS is regulated by the availability of L-arginine, oxygen, cofactors, and the rate of electron transfer. When either L-arginine or BH4 is deficient, eNOS is uncoupled from NO synthesis and electrons are transferred to molecular oxygen, generating reactive oxygen species (e.g., superoxide anion) by endothelial cells and resulting in oxidative stress and deregulation of NO-related signaling pathways (Thomas et al. 2008).

L-Arginine is also the substrate for both type I (cytosolic) and type II (mitochondrial) arginases (Wu and Morris 1998). This enzyme hydrolyzes L-arginine into ornithine and urea (Kepka-Lenhart et al. 2000), and is inhibited by high concentrations of BCAA (including L-leucine) (Wu and Morris 1998). Increased arginase activity in diabetes can competitively decrease the availability of L-arginine to the eNOS, thereby partially contributing to the development of endothelial dysfunction (Romero et al. 2008). This supports our earlier notion that arginase plays an important role in regulating NO synthesis in endothelial cells (Li et al. 2001). In addition, the activity of eNOS can be modulated at the post-translational level, such as by phosphorylation of the enzyme at serine or threonine residues. Several studies demonstrated that Akt-dependent phosphorylation at serine 1,177 (human) or serine 1,179 (bovine and chicken) activates eNOS activity (Fulton et al. 1999; Yang et al. 2010), while phosphorylation at threonine 497 or 495 decreases its activity (Bouloumie et al. 1997). To date, the underlying mechanism is not well-defined. Accordingly, a great number of factors have been identified as positive regulators (e.g., protein kinase C inhibitors, the proliferative state of endothelium, hydrogen peroxide, estrogens, vascular endothelial growth factor, insulin, HMG-CoA reductase inhibitors, and transforming growth factor-β) or negative regulators (e.g., low-density lipoprotein, tumor necrosis factor-α, erythropoietin, hypoxia, advanced glycation end products, and glucocorticoids) that either enhance or downregulate eNOS expression through different mechanisms (Rubio and Morales-Segura 2004).

The L-arginine/NO pathway had long been regarded as the sole source of NO in mammalian cells. This viewpoint has been challenged by several observations that NO can also be generated in an NOS-independent manner, suggesting the existence of an alternative pathway to produce NO. To date, at least four NOS-independent pathways for NO production have been identified. First, direct degradation of tissue nitrite to NO occurs predominantly in the reperfused myocardium (Zweier et al. 1995) or in the stomach (Duncan et al. 1995) under acidotic and highly reduced conditions. This production of NO in ischemic tissues and the stomach is associated with tissue injury and protection from pathogens, respectively. Second, generation of NO from oxidation of D- or L-arginine by hydrogen peroxide in the absence of NOS can occur (Nagase et al. 1997b). This formation of NO is based on the detection of nitrite plus nitrate (the oxidation end products of NO) in cell-free in vitro systems (Nagase et al. 1997b). Intracellular production of NO through this pathway or its in vivo function is not clear. Considering an elevation of peroxidative reactions and an increase in NO production (determined by plasma nitrite plus nitrate) in dialysis patients, as compared to healthy controls (Nagase et al. 1996, 1997a), the generation of NO by the reaction involving hydrogen peroxide and D- or L-arginine might occur as a compensatory response to renal injury. Third, the reduction of nitrite to NO can be catalyzed by microbial nitrite reductase (Payne et al. 1997). Fourth, NO may be formed by xanthine oxidase under hypoxic conditions (Zhang et al. 1998). The xanthine oxidase-catalyzed generation of NO may be important in redistribution of blood flow to ischemic tissue as an alternative to blood flow modulation by eNOS, because both nitrite and NADPH, two substrates for this reaction, are elevated in hypoxic tissues (Zhang et al. 1998). Despite the presence of these alternative reactions for NO production, the L-arginine–NO pathway is the major source of NO in the vasculature and other tissues in humans and animals (Wu and Meininger 2002).

Biological function of NO and endothelial dysfunction

NO is highly lipophilic and it is readily diffusible through a lipid bilayer (Li et al. 2009a). Thus, this free radical species can easily pass through cell membranes to reach its final target(s) at a short distance from the site of NO synthesis and can exert its biological effects on the vasculature due to its chemical reaction with oxygen, its affinity with heme- or metal-containing enzymes (e.g., guanylyl cyclase and aconitase), or through a biological signaling cascade (Dai et al. 2013; Dudzinski et al. 2006). As a detailed review on the chemical biology of NO is beyond the scope of this article, we will mainly focus on the physiological aspects of NO relevant to endothelial function.

High concentrations of NO in micromolar ranges have been shown to inhibit cell proliferation in multiple cell types, including endothelial cells, smooth muscle cells, hepatocytes, and fibroblasts through cGMP-dependent (Patel et al. 1995) and -independent mechanisms (Garg and Hassid 1990; Thomae et al. 1995), whereas physiological levels of NO in nanomolar ranges promote the growth

of endothelial cells, smooth muscle cells, and tumors (Du et al. 1997; Meininger and Wu 2002; Pervin et al. 2007). The inhibitory effect of NO on cell growth may be partly mediated by inhibiting the activity of ornithine decarboxylase, the rate-controlling enzyme for polyamine formation from ornithine (Buga et al. 1998). Of note, polyamines are necessary for DNA and protein synthesis in cells. Thus, depending on its concentrations and cell types, NO can function as either a negative or a positive feedback signal for polyamine synthesis.

In the cardiovascular system, a deficiency in NO bioavailability has been regarded as a hallmark of endothelial dysfunction (Vallance and Leiper 2002), suggesting an important role for the endothelium in maintaining the vasculature in a relaxed state, inhibiting the adhesion of platelets and white cells, and suppressing the proliferation of vascular smooth muscle cells. Pharmacological inhibition of eNOS causes vasoconstriction (leading to hypertension), enhances platelet activation, and increases atherogenesis in animal models (Cayatte et al. 1994). In agreement with a functional role of NO as a major vasodilator, the deletion of eNOS in mice leads to hypertension and increased proliferation of smooth muscle cells, which is associated with endothelial dysfunction (Kuhlencordt et al. 2001). In contrast, beneficial effects of physiological levels of NO on the vascular system are associated with the ability of NO to reduce the production of free radical species and the oxidation of low-density lipoprotein, thereby reducing risk for the development of cardiovascular diseases (Wu and Meininger 2000). Emerging evidence suggests that eNOS can modify mitochondrial biogenesis and telomerase activity through hitherto unknown mechanisms (Lahteenvuo and Rosenzweig 2012). It is generally accepted that reduced NO bioavailability represents an independent indicator for adverse cardiovascular events and predicts the prognosis for the patient (Karbach et al. 2014). Therefore, the endothelium has been identified as an important target for therapeutic intervention of vascular disorders.

Vascular insulin resistance and cardiovascular disease in obesity

According to the recent World Health Statistics report, the prevalence of obesity (body mass index $\geq$30 kg/m^2) has doubled in the past decades because of multiple factors, including inherited genes, excessive energy intake, increasingly sedentary lifestyles, and reduced energy expenditure (Wu et al. 2012). Obesity has emerged as a major public health problem worldwide (Hill et al. 2008). Obesity, especially the central or visceral type, is a predisposing factor for the development of type 2 diabetes mellitus, which is mostly associated with insulin resistance and multiple metabolic disorders, including hyperinsulinemia, impaired glucose tolerance, and dyslipidemia as risk factors for the development of cardiovascular diseases (Hu et al. 2004). It is well known that both vascular and nonvascular tissues are affected by insulin resistance in obese subjects (Henry 2003; Steinberg and Baron 2002; Yki-Jarvinen 2003). In classical target tissues (liver, skeletal muscle heart, and white adipose tissue), insulin resistance leads to a systemic hyperinsulinemia and elevations in plasma concentrations of free fatty acids due to the impairment of insulin-stimulated glucose and/or lipid metabolism (Van Gaal et al. 2006).

Normally, insulin exerts its vascular effects primarily by augmenting the availability of endothelium-derived NO, which then promotes peripheral vasodilatation and tissue blood flow (Wu and Meininger 2009). Under conditions of insulin resistance, endothelium-mediated vasodilation is impaired, partly because of insulin's inability to stimulate the activity of the key enzymes critical for NO synthesis (e.g., NOS and GTP cyclohydrolase I) in vascular endothelial cells, leading to endothelial dysfunction (Cersosimo and DeFronzo 2006; Meininger et al. 2004; Shaul 2002; Vincent et al. 2003; Zeng and Quon 1996). Excessive generation and accumulation of reactive oxygen species due to intracellular defects in insulin-mediated metabolism of glucose and lipids reduce NO availability and contribute to vascular insulin resistance (Cersosimo and DeFronzo 2006). The relationship between insulin resistance and abnormal vascular reactivity has been demonstrated under a variety of clinical conditions (Cersosimo and DeFronzo 2006). There is evidence that amelioration of insulin resistance is accompanied by improved endothelial function (Natali et al. 2004; Satoh et al. 2003). Results of numerous studies demonstrate that endothelial dysfunction is correlated with a higher incidence of cardiovascular disease in the vast majority (>90 %) of diabetic and prediabetic individuals and can be ameliorated by the restoration of insulin sensitivity in the vasculature (DeFronzo 1988).

Although much progress has been made in recent years, the molecular mechanisms responsible for the metabolic and vascular abnormalities associated with insulin resistance and endothelial dysfunction in obesity remain to be fully elucidated (Cersosimo and DeFronzo 2006; Meininger and Wu 2011). Many signals, such as inflammatory mediators, reactive oxygen species, secondary messengers, free fatty acids, and cytokines, are involved in the regulation of endothelial NO synthesis and cardiovascular function (Li et al. 2001; Lei et al. 2013a, b; Van Gaal et al. 2006). Consistent with this view, administration of pharmacologic agents used for obesity management has been reported to improve insulin sensitivity and reduce the risk of cardiovascular disease in humans (Mather et al. 2001).

Considering the side effects of anti-obesity drugs (Mark 2009), nutritional interventions are gaining more and more

attention due to their functional role in enhancing the production and availability of NO within physiological ranges in the vasculature (Wu 2013). In our long-term efforts to uncover effects of amino acids in metabolic disease, we found that increased extracellular concentrations of glucosamine (an inhibitor of the pentose cycle for the production of NADPH), which is synthesized from fructose-6-phosphate and L-glutamine, decreased NO bioavailability in endothelial cells (Wu et al. 2001a, b). Our findings help explain, in part, the previous observations that an increase in glucosamine synthesis impairs glucose utilization in skeletal muscle and white adipose tissue (Buse et al. 1997; Hawkins et al. 1996; Patti et al. 1999). These results implicate glucosamine as a critical factor in modulating NO synthesis and contributing to insulin resistance in obesity (Wu and Meininger 2009).

NO is an important mediator of the vascular effect of insulin

Research over the past 20 years has shown that the metabolic effects of insulin to promote glucose uptake in skeletal muscle and white adipose tissue result primarily from activating multiple cellular steps involved in glucose utilization, including transmembrane glucose transport, glycolysis, the pentose cycle, glycogen synthesis, and the citric acid cycle (Garvey and Birnbaum 1993). In addition to its direct action on stimulating glucose transport, insulin regulates NO synthesis in the vascular endothelium (a non-classical target tissue) (Jobgen et al. 2006; Zhang et al. 2004; Wu and Meininger 2009). Indeed, there is evidence that NO mediates the beneficial effect of insulin on stimulating blood flow to skeletal muscle, glucose uptake by muscle, and endothelium-dependent relaxation in blood vessels (Baron et al. 1995; Steinberg et al. 1994; Van Gaal et al. 2006; Vincent et al. 2004). Conversely, the insulin-stimulated increase in tissue blood flow and glucose uptake is abolished by administration of an NOS inhibitor, N^G-monomethyl-L-arginine (Laakso et al. 1992; Roy et al. 1998). To date, at least two signaling pathways have been identified to explain the insulin-induced increase in NO synthesis by endothelial cells. First, insulin induces a cascade of protein phosphorylation in the vascular endothelium. Specifically, the binding of insulin to its membrane receptor (consisting of two extracellular α and two β transmembrane subunits) leads to intrinsic tyrosine phosphorylation in the receptor's β subunits (Prudente et al. 2009). This, in turn, stimulates the receptor's tyrosine kinase activity. The activated insulin receptor (IR) increases the tyrosine phosphorylation of IR substrate-1 (IRS-1) and IRS-2, leading to activation of phosphatidylinositol-3-kinase (PI3 K) and subsequent activation of phosphoinositide-dependent kinase-1, which then phosphorylates and activates protein kinase B (also known as Akt). Activated Akt increases NO production through phosphorylation and activation of eNOS (Zecchin et al. 2007). In addition to the direct activation of eNOS via this PI3 K/Akt-dependent signaling pathway, insulin also enhances NO production by promoting the synthesis of NADPH and BH4, essential cofactors for eNOS (Wu et al. 1994; Ishii et al. 2001).

Both in vivo and in vitro studies indicate that sensitivity of blood vessels to the vasodilatory effect of insulin is positively correlated with insulin signaling with respect to glucose uptake under normal, obese and diabetic conditions (Baron et al. 1993; Baron and Brechtel 1993; Ding et al. 2000; Laakso et al. 1990, 1992). This notion is consistent with the observation that mice with a genetic deficiency in eNOS exhibit insulin resistance (Shankar et al. 2000). These results suggest that insulin modulates vascular tone and tissue blood flow through an NO-dependent mechanism.

Leucine and vascular insulin resistance

BCAA are relatively abundant in plant and animal proteins (Dai et al. 2014; Wu 2014). Like other BCAA, L-leucine is metabolized in humans and animals through interorgan cooperation (Wu 2013). Transamination of L-leucine occurs primarily in non-hepatic tissues such as the small intestine, skeletal muscle, adipose tissue, and mammary gland, whereas oxidation of its carbon skeleton takes place mainly in the liver (Chen et al. 2009; Harper et al. 1984; Lei et al. 2012). This metabolic pattern of BCAA degradation matches the distribution of BCAA transaminase (BCAT) and branched-chain α-ketoacid dehydrogenase (BCKAD) in animal tissues and cells (Harper et al. 1984; Lei et al. 2013a, b). For example, BCAT activity is very low in the liver and high in the small intestine, skeletal muscle, and mammary gland (Li et al. 2009b). Thus, arterial and dietary BCAA that enter the liver are virtually not degraded by this organ (Mimura et al. 1968; Wu et al. 2014). After absorption by the small intestine, L-leucine undergoes transamination by BCAT to form α-ketoisocaproate (KIC). KIC is decarboxylated by the BCKAD complex to generate isovaleryl-CoA, which is eventually converted into acetyl-CoA for either oxidation or lipid synthesis primarily in the liver (Wu 2013). The major nitrogenous products of BCAA catabolism are glutamate, glutamine and alanine (Fig. 2).

BCAA metabolism is abnormal in subjects with obesity or diabetes (Felig et al. 1969; Mels et al. 2013). For example, plasma concentrations of all the three BCAA are elevated in obese subjects and in diabetic patients (Table 1). This observation, originally known as hyperaminoacidemia, has been further confirmed by subsequent studies involving humans (Chevalier et al. 2006; She et al. 2007) and rodents (Jobgen et al. 2009; She et al. 2007; Tekwe

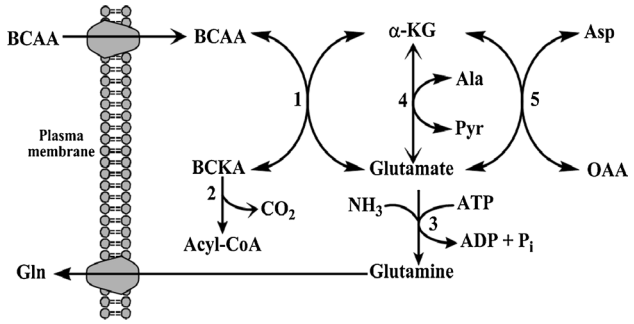

Fig. 2 Catabolism of branched-chain amino acids (BCAA) in the vasculature. BCAA enter cells through specific transporters on the plasma membrane. Enzymes catalyzing the indicated reactions are: *1* BCAA transaminase; *2* branched-chain α-ketoacid (BCKA) dehydrogenase; *3* glutamine synthetase; *4* glutamate-pyruvate transaminase; and *5* glutamate–oxaloacetate transaminase. *Ala* alanine, *Asp* aspartate, *Gln* glutamine, *OAA* oxaloacetate, *Pyr* pyruvate

et al. 2013), suggesting a potential link between BCAA metabolism alterations and insulin signaling. It is remarkable that an increase in plasma leucine concentration by only 22 % is associated with insulin resistance (Felig et al. 1969) and cardiovascular dysfunction in obese subjects (Steinberg and Baron 2002) and in obese rats (Tables 2, 3).

In our on-going research to understand underlying biochemical mechanisms for obesity and diabetes-associated metabolic and vascular disorders, we found that dietary supplementation with α-ketoglutarate (α-KG) markedly reduced plasma concentrations of BCAA in diet-induced obese rats (Fig. 3). This effect is associated with enhanced metabolism of BCAA in: (1) the small intestine, thereby reducing the entry of dietary BCAA into the portal vein (Chen et al. 2009); and (2) extra-intestinal tissues, thereby contributing to reduced concentrations of BCAA in the plasma (Wu

Table 1 Plasma concentrations of amino acids in patients with diabetic ketoacidosis or obesity

Amino	Healthy control subjects ($n = 10$)	Patients with diabetic ketoacidosis ($n = 8$)	Subjects with obesity ($n = 10$)
Alanine	343.5 ± 28.8	243.7 ± 37.7†	364.6 ± 26.1
Arginine	68.7 ± 7.5	56.6 ± 28.2	59.1 ± 4.9
Citrulline	30.3 ± 2.5	23.9 ± 3.5	25.8 ± 1.9
1/2 Cystine	98.3 ± 4.6	73.8 ± 20.7	108.1 ± 5.4
GABA	20.2 ± 1.9	36.3 ± 4.5*	25.4 ± 2.8
Glycine	214.7 ± 8.3	156.6 ± 22.6†	169.7 ± 9.1*
Histidine	72.6 ± 3.9	84.7 ± 10.4	75.9 ± 6.1
Isoleucine	58.9 ± 1.9	154.4 ± 18.7**	73.2 ± 4.7†
Leucine	112.3 ± 4.3	283.4 ± 35.5**	137.5 ± 9.3†
Lysine	163.6 ± 9.4	169.0 ± 35.5	188.4 ± 9.9
Methionine	23.6 ± 0.9	28.5 ± 4.6	27.9 ± 1.9
Ornithine	67.1 ± 8.7	74.5 ± 25	72.1 ± 7.6
Phenylalanine	48.5 ± 2.0	62.4 ± 10.4	57.8 ± 2.1*
Proline	175.3 ± 13.2	153.4 ± 38.9	171.7 ± 14.6
Serine	109.2 ± 7.1	71.7 ± 5.7**	111.0 ± 8.0
Taurine	51.1 ± 2.9	57.7 ± 16.8	58.4 ± 8.0
Threonine	133.5 ± 9.5	83.0 ± 8.3**	119.8 ± 5.2
Tryptophan	39.1 ± 6.3	–	45.5 ± 4.0
Tyrosine	53.8 ± 4.4	60.0 ± 10.6	67.7 ± 2.5†
Valine	211.5 ± 8.2	456.5 ± 52.7**	258.2 ± 9.4*

Values, expressed as μM, are mean ± SEM. The numbers of subjects in each group is given in parentheses

Taken from Felig et al. (1969, 1970). Blood samples were obtained from diabetic patients (without obesity) upon admission to the hospital, and from healthy subjects and age- and sex-matched obese subjects (without diabetes) after an overnight (10–14 h) fast. Amino acids were analyzed by ion-exchange chromatography involving the use of external standards

GABA γ-aminobutyrate

** $P < 0.001$ vs. healthy subjects

* $P < 0.01$ vs. healthy subjects

† $P < 0.05$ vs. healthy subjects

Table 2 Plasma concentrations of amino acids in diet-induced obese Sprague–Dawley rats and in Zucker diabetic fatty (ZDF) rats

Amino	Sprague–Dawley rats[a]		Zucker rats[b]	
	Lean	Obese	Lean	ZDF
Alanine	372 ± 23	394 ± 25	396 ± 21	281 ± 17**
Arginine	180 ± 11	186 ± 14	187 ± 7	121 ± 6**
Asparagine	56 ± 3	68 ± 4*	50 ± 2	51 ± 3
Aspartate	49 ± 3	46 ± 2	44 ± 4	46 ± 4
Citrulline	64 ± 4	65 ± 3	63 ± 3	50 ± 2*
Cysteine[c]	133 ± 8	177 ± 10*	128 ± 9	115 ± 7
Glutamate	96 ± 7	93 ± 5	101 ± 6	105 ± 7
Glutamine	542 ± 16	679 ± 19**	564 ± 14	453 ± 10**
Glycine	268 ± 10	214 ± 7*	253 ± 8	158 ± 7**
Histidine	85 ± 3	105 ± 5*	77 ± 5	75 ± 4
Isoleucine	143 ± 5	176 ± 7*	135 ± 8	224 ± 13**
Leucine	203 ± 8	251 ± 10*	191 ± 11	302 ± 15**
Lysine	236 ± 10	289 ± 13*	214 ± 12	217 ± 14
Methionine	73 ± 3	92 ± 6*	69 ± 5	67 ± 4
Ornithine	70 ± 4	72 ± 5	72 ± 6	75 ± 7
Phenylalanine	89 ± 5	117 ± 6*	85 ± 4	87 ± 5
Proline	277 ± 14	291 ± 16	264 ± 11	226 ± 9*
Serine	213 ± 8	180 ± 5*	198 ± 7	154 ± 5*
Taurine	436 ± 22	410 ± 20	392 ± 16	338 ± 13*
Threonine	224 ± 10	269 ± 11*	215 ± 8	183 ± 6*
Tryptophan	91 ± 4	114 ± 6*	96 ± 5	62 ± 3*
Tyrosine	108 ± 5	137 ± 7*	115 ± 6	91 ± 4*
Valine	176 ± 8	213 ± 10*	184 ± 8	343 ± 12**

Values, expressed as μM, are mean ± SEM, $n = 8$

[a] Male Sprague–Dawley rats (Harlan Laboratories, Houston, TX) were fed a low-fat or high-fat diet between 4 and 16 weeks of age, as we described previously (Jobgen et al. 2009). At 16 weeks of age, blood samples (0.2 ml) were obtained from the tail vein of low-fat-fed (lean) rats and high-fat-fed (obese) rats between 10 and 11 AM (5 h after food withdrawal), and plasma was analyzed for amino acids by high-performance liquid chromatography involving the use of external standards (Wu and Meininger 2008)

[b] Male Zucker lean rats and Zucker diabetic fatty (ZDF) rats had free access to a Purina 5008 diet (Fu et al. 2005). At 13 weeks of age, blood samples (0.2 ml) were obtained from the tail vein of these rats between 10 and 11 AM (5 h after food withdrawal), and plasma was analyzed for amino acids, as described above. ZDF rats had both obesity and diabetic ketoacidosis

[c] Including cysteine plus 1/2 cystine

* $P < 0.05$ vs. the corresponding lean animals, as analyzed by unpaired *t* test

** $P < 0.05$ vs. the corresponding lean animals, as analyzed by unpaired *t* test

2013). Metabolomic analysis reveals a distinctive metabolic 'signature' related to abnormal BCAA metabolism in obese individuals, as compared with lean subjects (Newgard et al. 2009). Similar results were reported in subjects with type 2 diabetes mellitus (Fiehn et al. 2010), obese subjects (Huffman et al. 2009), and high-risk individuals prior to the onset of diabetes (Tai et al. 2010). Of note, an increase in plasma concentrations of L-leucine is associated with impaired endothelium-dependent relaxation in Zucker diabetic fatty rats (Wu et al. 2007) and patients with hyperglycemia (Mels et al. 2013) and with reduced energy expenditure in diet-induced obese rats (Assaad et al. 2014a). Consistent with these findings, there are reports that dietary supplementation with BCAA may promote the development of insulin resistance in rats (Newgard et al. 2009) and humans (Tremblay et al. 2005). Based on these findings, it seems reasonable to use the BCAA metabolic profile as a clinical signature to predict the development and prognosis of diabetes even though the underlying mechanism is yet unknown.

Three lines of evidence suggest that NO is a potential molecule linking abnormal BCAA metabolism with insulin resistance. For example, the infusion of L-leucine into the kidney of normal rats increased renal vascular resistance (Kakoki et al. 2006). Second, increasing extracellular concentrations of L-leucine can inhibit the relaxation of the rat aorta (Schachter and Sang 2002). Third, high concentrations of L-leucine in culture medium reduce NO synthesis in endothelial cells (Yang et al. 2013a). Specifically, L-leucine dose-dependently increases GFAT activity and glucosamine-6-phosphate synthesis, while inhibiting NO production by endothelial cells (Fig. 4). In contrast, L-valine or L-isoleucine has no effect on NO synthesis in these cells [124 ± 10, 119 ± 13, and 125 ± 9 pmol NO/10^6 cells/h (mean ± SEM, n = 8)] in the presence of 0, 2 mM L-isoleucine, and 2 mM L-valine, respectively]. During a 48-h culture period in medium containing 0.5 mM L-glutamine (Wu et al. 2001a), increasing the concentration of L-leucine from 0.20 to 0.25 mM reduced NO synthesis in bovine venular endothelial cells by 20 % [127 ± 9 vs. 102 ± 7 pmol/10^6 cells/h (mean ± SEM, $n = 8$; $P < 0.05$)]. Interestingly, 2 mM D-leucine did not affect GFAT activity [(2.36 ± 0.17 vs. 2.17 ± 0.19 nmol/mg protein/min; (mean ± SEM, $n = 8$)] or NO production [132 ± 11 vs. 127 ± 13 pmol/10^6 cells/h (mean ± SEM, $n = 8$)], compared with its absence. These data indicate a role for L-leucine (physiological isomer) in activating GFAT and inhibiting NO synthesis in endothelial cells. Because obesity is associated with reduced NO synthesis in endothelial cells and with impaired endothelium-dependent relaxation of blood vessels (Table 3), knowledge of the underlying mechanisms is essential to develop effective strategies for prevention and treatment of cardiovascular dysfunction.

Potential mechanisms for leucine to inhibit NO synthesis in endothelial cells

The activity of mammalian target of rapamycin (mTOR) complexes regulates essential cellular processes, such as

Table 3 Increased GFAT activity in endothelial cells and impaired endothelium-dependent relaxation in rats fed a low-fat or high-fat diet between 4 and 16 weeks of age

Rats	Body weight at 19 week of age (g)	Plasma levels		Endothelial cells		Maximal vasodilation by acetylcholine (%)	
		Glucose (mM)	Homocys (μM)	NO synthesis[a]	GFAT[b]	−NMMA	+NMMA
Low-fat	405 ± 6.6	5.37 ± 0.08	4.72 ± 0.29	126 ± 8.1	282 ± 19	60.3 ± 2.9	2.3 ± 0.3
High-fat	462 ± 7.3*	5.50 ± 0.10	6.90 ± 0.33**	80 ± 6.2**	394 ± 31**	48.6 ± 2.3**	2.0 ± 0.2

Values are means ± SEM, $n = 8$. Male Sprague–Dawley rats (Harlan Laboratories, Houston, TX) were fed a low-fat or high-fat diet between 4 and 16 weeks of age, as we described previously (Jobgen et al. 2009). Rats were euthanized for all the measurements at 16 weeks of age between 10 and 11 AM (5 h after food withdrawal). Aortic rings were obtained to measure endothelium-dependent relaxation in response to 10^{-10}–10^{-4} M acetylcholine in the presence or absence of 0.1 mM N^G-monomethyl-L-arginine (NMMA; an inhibitor of eNOS) (Meininger et al. 2004). GFAT activity and NO synthesis, as well as plasma concentrations of glucose and homocysteine (Homocys) were determined, as we described previously (Jobgen et al. 2009; Wu et al. 2001a, b). Data were analyzed by unpaired *t* test

[a] NO synthesis (pmol/10^6 cells/h)

[b] GFAT activity (pmol/mg protein/min)

* $P < 0.05$ vs. the low-fat group

** $P < 0.01$ vs. the low-fat group

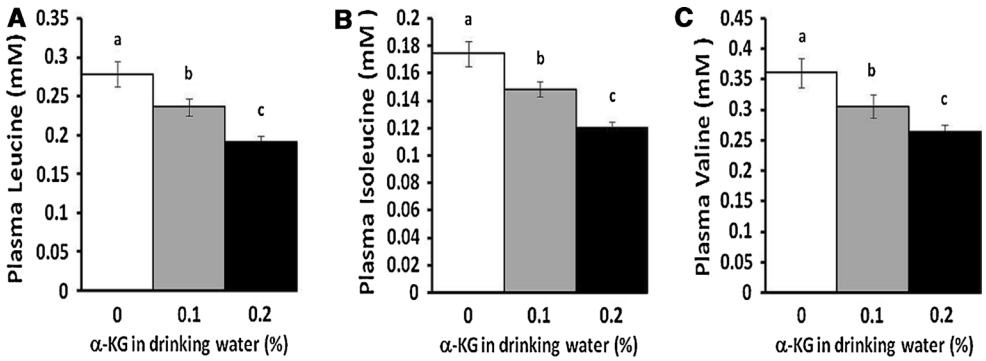

Fig. 3 Effects of oral administration of α-ketoglutarate (α-KG) for 1 week on plasma concentrations of branched-chain amino acids in 12-week-old Zucker diabetic fatty rats. Data are mean ± SEM, $n = 8$. The rats had free access to a Purina 5008 diet (Fu et al. 2005) and drinking water containing 0, 0.1 or 0.3 % α-KG (Tekwe et al. 2012). α-KG supplementation did not affect food intake or water consumption by rats. At the end of 1-week of α-KG supplementation, blood samples (0.2 ml) were obtained from the tail vein at 10 AM for analysis of amino acids by high-performance liquid chromatography involving the use of external standards (Wu and Meininger 2008). **a**–**c**: $P < 0.05$, as analyzed by one-way ANOVA and the Student–Newman–Keuls multiple comparison test (Assaad et al. 2014b)

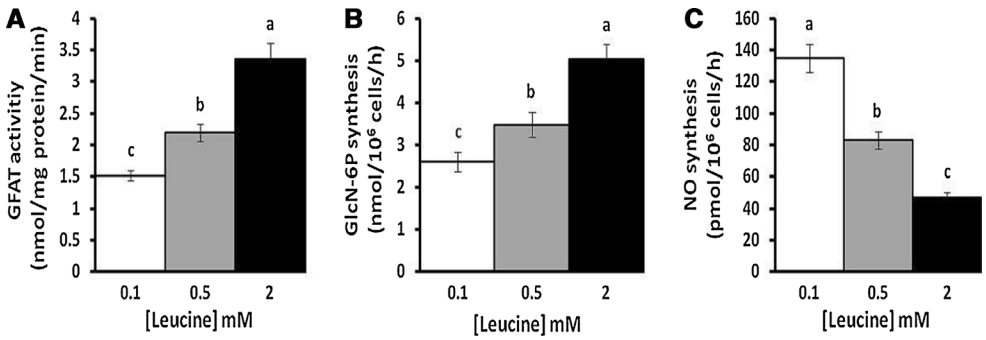

Fig. 4 Effects of L-leucine on GFAT activity, glucosamine-6-phosphate synthesis and NO production in cultured endothelial cells. Adapted from Yang et al. (2013a). Bovine venular endothelial cells were cultured for 48 h in Dulbecco's modified Eagle's medium (Wu et al. 2001a, b) containing 0.2 mM L-arginine, 0.5 mM L-glutamine, and 0.1, 0.5 or 2 mM L-leucine. Data are mean ± SEM, $n = 8$. **a**–**b**: $P < 0.05$, as analyzed by one-way ANOVA and the Student–Newman–Keuls multiple comparison test (Assaad et al. 2014b). *GFAT* glutamine:fructose-6-phosphate transaminase; *GlcN-6P* glucosamine-6-phosphate

growth, proliferation, or survival by controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism (Wullschleger et al. 2006). Dysregulation of the mTOR cell signaling pathway has been linked to aging (Dillon 2013) and to diseases such as diabetes, cancer and cardiovascular disease (Santulli and Totary-Jain 2013). Nutrients (e.g., amino acids) are important regulators of mTOR activation, thus affecting cell growth and protein synthesis (Wu 2013). For example, L-leucine can activate mTOR, which, in turn, phosphorylates S6K1 and 4EBP1, leading to increased synthesis of proteins in skeletal muscle (Orellana et al. 2008). One of these proteins may be GFAT (Tekwe et al. 2012), which is the first and rate-limiting enzyme in glucosamine synthesis. In addition, L-leucine may directly activate GFAT in endothelial cells through allosteric binding to the enzyme. Indeed, elevated levels of L-leucine can allosterically activate glutamate dehydrogenase, therefore modulating overall amino acid oxidation and ammonia production (Li et al. 2003). Thus, L-leucine may increase the generation of glucosamine to inhibit NO synthesis in endothelial cells (Wu et al. 2001a). In this scenario, nutritional or pharmacological substances that enhance the catabolism of L-leucine or inhibit GFAT activity may provide a therapeutic intervention by modulating the bioavailability of NO and ameliorating endothelial dysfunction. In support of this notion, we found that dietary supplementation with α-ketoglutarate reduced plasma BCAA levels in Zucker diabetic fatty rats due to the enhanced transamination of BCAA (Tekwe et al. 2012). Furthermore, *N*-ethyl-L-glutamine (a glutamine analog, also known as theanine) competitively inhibited GFAT activity in endothelial cells and promoted NO synthesis in these cells (Fig. 5). Further studies are needed to elucidate the underlying mechanism(s) responsible for the beneficial effect of α-ketoglutarate or theanine administration on improving NO synthesis in endothelial cells.

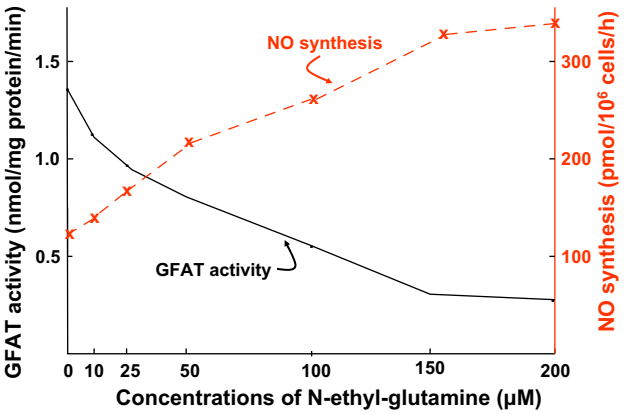

Fig. 5 *N*-ethyl-L-glutamine inhibits GFAT activity and increases NO production in cultured bovine venular endothelial cells in a dose-dependent manner ($P < 0.05$). Adapted from Yang et al. (2013b). Values are means ($n = 8$). The pooled SEM values for GFAT activity and NO synthesis were 0.06 nmol/mg protein/min and 12 pmol/10^6 cells/h, respectively. Cells were cultured for 48 h in Dulbecco's modified Eagle's medium containing 0.2 mM L-arginine, 0.5 mM glutamine and 0–200 μM *N*-ethyl-L-glutamine. GFAT activity and NO synthesis were determined in the presence of 0.5 mM L-glutamine (physiological concentration in plasma) (Wu et al. 2001a)

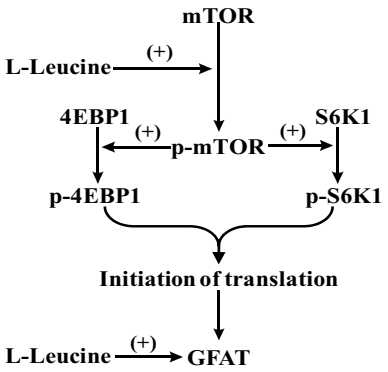

Fig. 6 L-Leucine increases GFAT activity in endothelial cells possibly by: (1) enhancing GFAT protein expression via the mTOR signaling pathway; and (2) allosterically activating GFAT. L-Leucine is known to activate mTOR in animal cells to enhance the synthesis of specific proteins, which may include GFAT. Thus, GFAT activity in endothelial cells (EC) may be attenuated by a reduction of plasma L-leucine concentrations through enhancement of leucine catabolism in tissues (including the gut). *GFAT* glutamine:fructose-6-phosphate transaminase, *mTOR* mammalian target of rapamycin, *S6K1* ribosomal protein S6 kinase-1, *4EBP1* eIF4E-binding protein-1

Conclusion and perspectives

NO plays an important role in maintaining vascular homeostasis by regulating endothelial cell proliferation, modulating vascular tone, and protecting blood vessels from injury to inflammation. Endothelial dysfunction results from decreased NO synthesis by eNOS expression or increased NO degradation via oxidative stress, and has been reported to be associated with various vascular diseases, such as hypertension, hypercholesterolemia, and diabetes mellitus. Therapeutic interventions to enhance the release of NO from endothelial cells might be potential strategies to ameliorate the effects of endothelial dysfunction on health in humans and animals. In this regard, it is noteworthy that a deficiency in NO bioavailability is a hallmark of endothelial dysfunction and is associated with cardiovascular insulin resistance in obesity and diabetes. An increase in plasma L-leucine enhances the mTOR cell signaling and expression of GFAT (the rate-controlling enzyme in glucosamine synthesis), which in turn leads to the generation of glucosamine (an inhibitor of NO synthesis) and contributes partially to the development of cardiovascular disease (Fig. 6). In contrast, α-ketoglutarate stimulates L-leucine

degradation, reduces the circulating levels of L-leucine and, therefore, blocks an inhibitory effect of L-leucine on NO synthesis in endothelial cells, thereby restoring endothelial function and ameliorating vascular insulin resistance in animals and humans with obesity and diabetes. Alternatively, dietary supplementation with *N*-ethyl-L-glutamine can be effective in inhibiting GFAT activity and NO synthesis from L-arginine in endothelial cells. An elevation of L-leucine in the plasma or serum may serve as a metabolic biomarker for risk of cardiovascular dysfunction in patients with obesity or diabetes. Modulation of L-leucine metabolism and GFAT expression in endothelial cells may provide a novel strategy for beneficially improving cardiovascular function in the metabolic syndrome.

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Conflict of interest The authors declare no conflict of interest.

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