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Chronic Co-Administration of Sepiapterin and L-Citrulline Ameliorates Diabetic Cardiomyopathy and Myocardial Ischemia/Reperfusion Injury in Obese Type 2 Diabetic Mice

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Abstract

Background—Diabetic heart disease is associated with tetrahydrobiopterin (BH₄) oxidation and high arginase activity, leading to endothelial nitric oxide synthase (eNOS) dysfunction.

Sepiapterin (SEP) is a BH₄ precursor, and L-citrulline (L-Cit) is converted to eNOS substrate, L-arginine. Whether SEP and L-Cit are effective at reducing diabetic heart disease is not known. The present study examined the effects of SEP and L-Cit on diabetic cardiomyopathy and ischemia/reperfusion injury in obese type 2 diabetic mice.

Methods and Results—Db/db and C57BLKS/J mice at 6-8 weeks of age received vehicle, SEP, or L-Cit orally alone or in combination for 8 weeks. Cardiac function was evaluated with echocardiography. Db/db mice displayed hyperglycemia, obesity, and normal blood pressure and cardiac function compared with C57BLKS/J mice at 6-8 weeks of age. After vehicle treatment for 8 weeks, db/db mice had reduced ejection fraction, mitral E/A ratio, endothelium-dependent relaxation of coronary arteries, BH₄ concentrations, ratio of eNOS dimers/monomers, and nitric oxide levels compared with vehicle-treated C57BLKS/J mice. These detrimental effects of diabetes were abrogated by co-administration of SEP and L-Cit. Myocardial infarct size was increased, and coronary flow rate and $\pm$ dP/dt were decreased during reperfusion in vehicle-treated db/db mice subjected to ischemia/reperfusion injury compared to control mice. Co-administration of SEP and L-Cit decreased infarct size and improved coronary flow rate and cardiac function in both C57BLKS/J and db/db mice.

Conclusions—Co-administration of SEP and L-Cit limits diabetic cardiomyopathy and ischemia/reperfusion injury in db/db mice through a BH₄/eNOS/nitric oxide pathway.

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Disclosures

None.

Keywords

tetrahydrobiopterin; nitric oxide synthase; diabetic cardiomyopathy; ischemia reperfusion injury; type 2 diabetes mellitus

The prevalence of type 2 diabetes mellitus (T2DM) and obesity has been increasing worldwide in recent decades.¹ Although treatment has substantially improved, T2DM patients with obesity are predisposed to develop a specific cardiomyopathy, termed diabetic cardiomyopathy (DCM), and have a higher incidence of ischemic heart disease and poorer clinical recovery compared with non-diabetic subjects.^{2,3} Conventional or newly developed therapies for DCM and ischemic heart disease in T2DM are under ongoing investigation, or lack major efficacy. The search for new therapeutic targets and pharmacological agents for protection of diabetic hearts is of primary importance.

It is widely accepted that diminished nitric oxide (NO) bioavailability and increased reactive oxygen species play an important role in the pathogenesis of both DCM and myocardial ischemia/reperfusion (I/R) injury.⁴⁻⁶ Endothelial nitric oxide synthase (eNOS) proteins consist of a heme-containing oxygenase domain that binds tetrahydrobiopterin (BH₄), molecular oxygen, and L-arginine; and a reductase domain that transfers electrons from reduced NADP (nicotinamide adenine dinucleotide phosphate) to FAD (Flavin adenine dinucleotide) and FMN (Flavin mononucleotide).⁷ In the presence of adequate BH₄ and the substrate L-arginine, heme and oxygen reduction are coupled to the synthesis of NO (eNOS coupling).⁷ However, T2DM increases oxidation of BH₄ to enzymatically incompetent 7,8-dihydrobiopterin (BH₂) and the expression/activity of arginase that metabolizes L-arginine to L-ornithine and urea.^{8,9} During conditions of low intracellular BH₄ and L-arginine, electron transfer within the active site of eNOS can become uncoupled from L-arginine oxidation, causing molecular oxygen to be reduced to superoxide (eNOS uncoupling).¹⁰ Increased BH₄ bioavailability and coupling of eNOS by genetic or pharmacological approach have been demonstrated to be useful in preventing endothelial dysfunction in diabetes.^{11,12} However, little is known as to whether pharmacological agents are effective at ameliorating DCM and myocardial I/R injury in T2DM, in face of elevated oxidative/nitrosative stress and arginase activity.

Sepiapterin (SEP) is a stable precursor of BH₄ with higher cell permeability than BH₄ itself and is used as a pharmacological agent to protect eNOS in multiple experimental models.¹³⁻¹⁵ L-citrulline (L-Cit) is readily absorbed and is a potent precursor of L-arginine, even at high arginase activity.^{8,16} The present study examined the effects of SEP or L-Cit on DCM and I/R injury in db/db mice, a widely used preclinical model of T2DM with obesity. We hypothesized that co-administration of SEP and L-Cit attenuates DCM and myocardial I/R injury in db/db mice through an increase in bioavailable BH₄ and an improvement in eNOS function.

Methods

For expanded Methods, see Data Supplement.

Animals

Obese T2DM C57BL/KsJ-lepr^{db}/lepr^{db} (db/db) and C57BLKS/J control mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The experimental procedures were approved by the Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, WI) and conformed to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Academy of Sciences, 8th edition, 2011).

Measurements of blood glucose and hemodynamics

Fasting blood glucose of C57BLKS/J and db/db mice was measured with a blood gas analyzer (ABL-725 Radiometer). Under the anesthesia of pentobarbital sodium, blood pressure was monitored.

Measurements of left ventricular weight, lung weight, and tibia length

The left ventricle (LV) and lung of C57BLKS/J and db/db were weighed. Tibia length was then measured using a Vernier caliper and used to normalize LV weight.

Echocardiography

Forty-eight C57BLKS/J mice and 48 db/db mice were imaged with a VisualSonics Vevo 770 High-resolution Imaging System (Toronto, Canada) equipped with a 30 MHz transducer (Scanhead RMV 707), as described.^{17,18}

Responsibility of isolated coronary arteries

Acetylcholine (Ach) induces endothelium-dependent relaxation.¹⁹ The reactivity of the left main and right coronary artery to Ach was investigated with a pressurized *in vitro* preparation, as described.^{20,21}

Myocardial I/R injury *in vivo*

Thirty-six C57BLKS/J and 44 db/db mice at 6-8 weeks of age were orally given 5 mg/kg/day SEP or 50 mg/kg/day L-Cit alone or in combination for 8 weeks or vehicle as control. Either C57BLKS/J or db/db mice were divided into the following 4 groups: vehicle, SEP, L-Cit, and SEP+L-Cit. Myocardial ischemia was produced by occluding the left main coronary artery for 20 min, as previously described.^{17, 22} The infarct area was delineated by perfusing the coronary arteries with 2,3,5-triphenyltetrazolium chloride via the aortic root, and the area at risk was delineated by perfusing phthalo blue dye into the aortic root after tying the coronary artery at the site of previous occlusion.²³

Myocardial I/R injury *ex vivo*

Langendorff-perfused mouse hearts were subjected to 30 min of no-flow global ischemia followed by 2 h of reperfusion at 37 °C, as described.^{23,24} Coronary flow was monitored by an in-line flow probe connected to a flow meter (Transonics Systems Inc.). The LV pressure signal was monitored to obtain left ventricular dP/dt. Left ventricular -dP/dt (maximum rate of increase of left ventricular developed pressure) and -dP/dt (maximum rate of decrease of

left ventricular developed pressure) at baseline, 10, 20, and 30 min after ischemia, and 10, 30, 60, 90, and 120 min after reperfusion were determined.

BH₄ assay

BH₄ was quantified in LV biopsies by high performance liquid chromatography (HPLC) with electrochemical detection (ESA Biosciences CoulArray® system Model 542).¹⁷

Immunoblotting

The LV was harvested and homogenized, and immunoblots were performed using standard techniques, as described.²⁵ The normal function of eNOS requires dimerization of the enzyme.²⁶ To investigate eNOS homodimer formation in the myocardium, non-boiled cellular lysate was resolved by 6% SDS-PAGE at 4°C overnight. Membranes were incubated with a mouse anti-eNOS monoclonal antibody (BD Transduction Laboratories).

Measurement of NO

Nitrite concentration corresponding to the stable byproduct of NO released by myocardium in aqueous solution was quantified by ozone chemiluminescence.²⁵

Cell culture

Endothelial cells (ECs) were cultured in media containing 5.5 mM (normal glucose concentration, NG) or 20.0 mM glucose (high glucose concentration, HG) for 12 h and exposed to 2 h of hypoxia in glucose-free medium followed by 2 h of reoxygenation. To investigate the effect of SEP in ECs, 100 µM SEP was added to cultured cells as substrate for the synthesis of BH₄ during 60 min of baseline and the period of hypoxia/reoxygenation (H/R). Since phosphorylation of eNOS regulates NO generation, the expression of total eNOS and phosphorylated eNOS (p-eNOS) proteins was analysed using standard Western blot techniques.²⁵ The concentrations of BH₄ and NO were measured by HPLC and ozone chemiluminescence, respectively.

Statistical analysis

All data are expressed as mean ± S.E.M. One-way ANOVA followed by Bonferroni *post-hoc* test was used to evaluate the differences among groups in heart rate, echocardiographic data, body weight, blood glucose, mean arterial blood pressure, the ratio of heart/body weight, area at risk, infarct size, BH₄ and NO concentrations, and the ratio of eNOS dimers/monomers. Statistical analyses of Ach-induced vasodilation, coronary flow, and $\pm$ dP/dt over time between groups was performed with repeated measures ANOVA followed by Bonferroni's multiple comparison. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla).

Results

General characteristics and cardiac phenotype of C57BLKS/J and db/db mice

Body weights of db/db mice at 6-8 weeks of old were significantly heavier than those of age-matched C57BLKS/J mice ($P < 0.05$, $n = 10$ mice/group) (Figure 1). Fasting blood glucose

levels were significantly higher in db/db than C57BLKS/J mice at 6-8 weeks of age. Both body weight and blood glucose levels were further elevated in db/db mice of 14-16 weeks old compared with those of 6-8 weeks old. The ratio of LV weight/body weight was significantly smaller in db/db mice than age-matched controls. The ratio of LV weight/lung weight was comparable between db/db and C57BLKS/J mice at 6-8 weeks of age but significantly smaller in 14-16 week-old db/db than age-matched controls. Mean arterial blood pressure and the ratio of LV weight/tibia length were comparable between db/db and C57BLKS/J mice at both 6-8 and 14-16 weeks of age ($P>0.05$, $n=10$ mice/group).

The dimensions and function of the LV measured by echocardiography are shown in Figure 2. There were no significant differences in the thickness of LV anterior and posterior walls, LV end-diastolic volume, LV end-systolic volume, ejection fraction, isovolumic contraction time of the LV, ejection time of the LV; myocardial performance index, and mitral E/A ratio between db/db mice and C57BLKS/J controls at 6-8 weeks of age ($P>0.05$, $n=12$ mice/group). The thickness and end-diastolic volume of the LV were comparable between db/db and C57BLKS/J mice at 14-16 weeks of age. The end-systolic volume of the LV was significantly increased, and ejection fraction and mitral E/A ratio were significantly decreased in db/db mice at 14-16 weeks of age compared with age-matched controls ($P<0.05$).

Co-administration of SEP and L-Cit ameliorated DCM in db/db mice

The treatment of 6-8 week-old C57BLKS/J mice with SEP or L-Cit alone or in combination for 8 weeks did not significantly alter ejection fraction of the LV and mitral E/A ratio ($P>0.05$, $n=12$ mice/group) (Figure 3). Ejection fraction and mitral E/A ratio were smaller in db/db mice than age-matched controls after vehicle treatment for 8 weeks ($P<0.05$). The treatment of db/db mice with SEP or L-Cit alone did not significantly alter ejection fraction and mitral E/A ratio compared with vehicle-treated db/db mice ($P>0.05$). Interestingly, co-administration of SEP and L-Cit significantly elevated ejection fraction and mitral E/A ratio in db/db mice ($P<0.05$ between the SEP–L-Cit and vehicle groups). There were no significant differences in ejection fraction and mitral E/A ratio between the db/db–SEP–L-Cit and C57BLKS/J SEP–L-Cit groups ($P>0.05$).

Combination of SEP and L-Cit improved endothelium-dependent relaxation of coronary arteries in db/db mice

Figure 4 shows the relaxant responses of cannulated coronary arteries to variable concentrations of Ach. Ach-induced relaxation was significantly decreased in both the left main and right coronary arteries of the vehicle-treated db/db mice compared with C57BLKS/J controls ($P<0.05$, $n=5-6$ mice/group). Co-administration of SEP and L-Cit significantly increased Ach-induced relaxation in db/db mice ($P<0.05$, $n=5-6$ mice/group), but not in C57BLKS/J mice.

Co-administration of SEP and L-Cit decreased myocardial infarct size in db/db mice

Four out of 36 C57BLKS/J mice and 13 out of 44 db/db mice died during ischemia or reperfusion. There were not significant differences in area at risk among the 8 experimental groups ($P>0.05$) (Figure 5A). Coronary artery occlusion followed by reperfusion resulted in

an infarct size of $36 \pm 3\%$ of area at risk ($n=8$) in the vehicle-treated C57BLKS/J mice. There were not significant differences in infarct size between SEP- or L-Cit-treated C57BLKS/J mice compared with vehicle-treated C57BLKS/J mice. Interestingly, infarct size was significantly smaller in the SEP–L-Cit-treated C57BLKS/J than vehicle-treated C57BLKS/J mice. Compared with the vehicle-treated C57BLKS/J group, infarct size was significantly increased in vehicle-treated db/db group ($55 \pm 4\%$, $n=8$, $P<0.05$, Figure 5B), which was significantly decreased by co-administration of SEP and L-Cit but not by SEP or L-Cit alone.

Combination of SEP and L-Cit improved coronary flow and cardiac function following I/R injury in db/db mice

Figure 6 shows coronary flow rate and $\pm dP/dt$ in Langendorff-perfused mouse hearts subjected to I/R injury. The baseline values of coronary flow rate and the values of $\pm dP/dt$ were smaller in the db/db–vehicle than C57BLKS/J–vehicle groups ($P<0.05$, $n=8$ hearts/group). Global ischemia for 30 min resulted in the cessation of the contraction and relaxation of the hearts. With reperfusion, contraction and relaxation were gradually restored in all mouse hearts. The values of coronary flow rate and $\pm dP/dt$ were significantly smaller in the db/db–vehicle than C57BLKS/J–vehicle group from 30 min to 2 h after reperfusion ($P<0.05$, $n=8$ hearts/group). There were significant increases in coronary flow rate and the values of $\pm dP/dt$ from 30 min to 120 min after reperfusion in the C57BLKS/J–SEP–L-Cit group compared with C57BLKS/J–vehicle group and in the db/db–SEP–L-Cit group compared with db/db–vehicle group ($n=8$ hearts/group).

Co-administration of SEP and L-Cit increased BH₄ concentrations, eNOS dimerization, and NO production in db/db mice

There were no significant differences in cardiac BH₄ and NO concentrations and the ratio of eNOS dimers/monomers between the C57BLKS/J–SEP–L-Cit and C57BLKS/J–vehicle groups ($P>0.05$, $n=5-7$ mice/group) (Figure 7). Compared with the C57BLKS/J–vehicle group, the concentrations of BH₄ and NO and the ratio of eNOS dimers/monomers were significantly decreased in the db/db–vehicle group ($P<0.05$, $n=5-7$ mice/group). These detrimental effects of diabetes were abrogated by combination of SEP and L-Cit in db/db mice ($P<0.05$ between the db/db–SEP–L-Cit and db/db–vehicle groups, $n=5-7$ mice/group).

SEP increased BH₄ concentrations, eNOS phosphorylation, and NO production in ECs subjected to H/R injury in the presence of HG

Figure 8 shows the effect of SEP on BH₄ and NO concentrations and p-eNOS in ECs subjected to H/R injury in the present of HG. The concentrations of BH₄ and NO and the ratio of p-eNOS/eNOS were significantly decreased in the HG–H/R group compared with NG group ($P<0.05$, $n=6-9$ /groups). These detrimental effects of HG and H/R injury were abrogated by SEP ($P<0.05$ between the HG–H/R and HG–H/R–SEP groups, $n=6-9$ /groups).

Discussion

The results of the present study demonstrate that the T2DM db/db mice with obesity at 14–16 weeks of age develop DCM and have increased susceptibility of the myocardium to

I/R injury, and co-administration of SEP and L-Cit diminishes DCM and I/R injury in db/db mice. The protective effects of SEP and L-Cit on diabetic hearts appear to be associated with improvements in coronary arterial endothelial function, cardiac BH₄ concentrations, and eNOS function. These results indicate that co-administration of SEP and L-Cit is an effective approach for protection of diabetic hearts against cardiomyopathy and I/R injury.

The db/db mouse suffers from a leptin receptor mutation, displaying the characteristics of T2DM, such as hyperglycemia, obesity, dyslipidemia, and insulin resistance.²⁷ This was confirmed by our present study showing that the db/db mice at both 6-8 and 14-16 weeks of age had increased body weight and blood glucose. DCM is a common complication of diabetes characteristic of cardiac (both diastolic and later systolic) dysfunction that occurs independently of a recognized cause such as coronary artery disease or hypertension.² In the present study, systolic (ejection fraction) and diastolic (mitral E/A ratio) function was significantly depressed in db/db mice at 14-16 weeks of age without significant hypertension (Figures 1 and 2). This cardiac dysfunction may be attributed to DCM.

Db/db mice at 6-8 weeks of age had normal dimensions and function of the LV compared with age-matches C57BLKS/J control mice, despite hyperglycemia and obesity (Figure 2). After being treated by the vehicle for 8 weeks, the db/db mice had a significant decrease in cardiac function compared with age-matched control mice, which was not significantly altered by SEP or L-Cit alone (Figure 3). A recent study reports that 10 mg/kg/day SEP alone can significantly improve cardiac function in streptozotocin-induced type 1 diabetic mice.¹⁴ The reasons for the disparity may be related to differences in levels of insulin, obesity, and dosage and duration of SEP administration. Intriguingly, co-administration of SEP and L-Cit restored impaired cardiac function by diabetes and obesity in db/db mice. Thus, the combination of SEP and L-Cit is a promising approach for prevention of DCM in T2DM mice with obesity.

Coronary ECs regulate basic coronary vasomotor tone and myocardial blood flow via the highly controlled release of vasodilators and vasoconstrictors.²⁸ Ach induces endothelium-dependent relaxation via stimulating the release of NO, endothelium-derived hyperpolarizing factor, and prostacyclin I₂.¹⁹ In the present study, Ach-induced relaxation of coronary arteries was decreased in db/db mice (Figure 4). These results suggest that coronary endothelial function is impaired in db/db mice at 14-16 weeks of age. Interestingly, co-administration of SEP and L-Cit restored NO production and Ach-induced relaxation of coronary arteries. Given that the combination of SEP and L-Cit diminishes DCM in db/db mice, it is likely that impaired coronary endothelial function is involved in the development of DCM. A previous study showed that that vascular endothelial NO rather than endothelium-derived hyperpolarizing factor and prostacyclin I₂ was decreased in db/db mice.²⁹ Therefore, a decrease in vascular endothelial NO may contribute to the development of DCM.

Diabetic patients have increased morbidity of ischemic heart disease and poorer prognosis compared with non-diabetic patients.³ Clinical studies also suggest that diabetes increases the susceptibility of the myocardium to I/R injury.^{30,31} However, inconsistent results are obtained in experimental studies of animals as to how diabetes impacts myocardial I/R

injury.³² In the present study, myocardial infarct size was larger in db/db than C57BLKS/J mice, and systolic and diastolic function was worse in db/db than C57BLKS/J mice at 14-16 weeks of age. These results are consistent with a previous study showing the severity of myocardial I/R injury and the development of postinfarction heart failure were markedly enhanced in the db/db mice.³³ We also measured infarct size and cardiac function in db/db and C57BLKS/J mice at 6-8 weeks of age. There were no significant differences in both infarct size and cardiac function between db/db and C57BLKS/J mice (data not shown). Collectively, the sensitivity of diabetic hearts to I/R injury is related to the duration and severity of diabetes.

Improving cardiac function is the primary goal of myocardial protection in clinical practice. In isolated hearts, coronary flow rate and cardiac function at baseline were reduced in db/db mice compared with the control mice. Following I/R injury, the recovery of coronary flow and cardiac function was worse in db/db than the control mice, whereas the combination of SEP and L-Cit improved both coronary flow rate and cardiac function during reperfusion in both C57BLKS/J and db/db mice (Figure 6). Given that the co-administration of SEP and L-Cit effectively decreased myocardial infarct size in both C57BLKS/J and db/db mice subjected to I/R injury, co-administration of SEP and L-Cit is effective at reducing myocardial I/R injury.

T2DM and obesity result in excessive production of reactive oxygen species and peroxynitrite that oxidize BH₄ to BH₂.⁹ This was confirmed by our present study showing cardiac BH₄ concentrations were significantly decreased in db/db mice (Figure 7). Previous studies have indicated that BH₄ has multiple beneficial effects on eNOS proteins, including stabilization of active dimeric form of eNOS, increasing binding of L-arginine to eNOS, and participation in the electron transfer process.³⁴ Consistently with reduced BH₄ concentrations in diabetic myocardium, ratio of eNOS dimers/monomers and NO levels were significantly decreased in db/db mice. It is evident that decreased BH₄ bioavailability leads to the switch of eNOS proteins from NO-producing enzymes to superoxide-generating enzymes.¹⁰ Moreover, BH₄ within the cells is an antioxidant and scavenges reactive oxygen and nitrogen species that play an important role in the pathogenesis of DCM.^{34,35} It is likely that decreased bioavailability of BH₄ contributes to the development of DCM in db/db mice.

SEP is reduced by sepiapterin reductase to BH₂ and further by dihydrofolate reductase to form BH₄.³⁶ This is verified by our present study showing that SEP restored BH₄ concentrations during reoxygenation after hypoxia in the presence of HG in cultured ECs (Figure 8). However, SEP alone did not significantly reduce myocardial infarct size in db/db mice subjected to I/R injury. In T2DM and obesity, bioavailable L-arginine is impaired due to increased expression/activity of arginase in intestine, blood, and other tissues,^{8,37} which metabolizes L-arginine to L-ornithine and urea. It is likely that although SEP can restore the dimerization of eNOS, eNOS cannot produce enough NO due to insufficient L-arginine in db/db mice. Recent studies indicate that L-Cit is converted to L-arginine by argininosuccinate synthase and lyase in cardiomyocytes, thereby promoting a recycling of L-arginine for the production of NO, even at high arginase activity.³⁸ L-Cit alone failed to reduce myocardial infarct size in db/db mice subjected to I/R injury. This may be attributed to insufficient dimerization of eNOS in db/db mice due to BH₄ deficiency. Interestingly, co-

administration of SEP and L-Cit produced significant cardioprotective effects, concomitantly with improvements in cardiac BH₄ concentrations, ratio of eNOS dimers/monomers, and NO levels in db/db mice. Thus, the combination of SEP and L-Cit is an effective approach to protect the BH₄/eNOS/NO pathway in T2DM and obesity. Our results indicate that increased bioavailability of both BH₄ and L-arginine is necessary for reduction of myocardial injury in db/db mice.

One limitation of this study is that the db/db model does not recapitulate the etiology of T2DM in humans, for whom leptin receptor deficiency is not an important contributor to T2DM.³⁹ The db/db mice develop hyperglycemia, obesity, dyslipidemia, and insulin resistance, which are in fact secondary to genetic mutations of leptin receptors.³⁹ Nevertheless, the db/db mouse is still the most popular animal model to test glucose lowering agents, insulin sensitizers, insulin secretagogues, and anti-obesity agents in drug discovery and testing.⁴⁰ Another limitation is that the levels of circulating leptin were not determined in the db/db mice. Previous studies showed that the levels of leptin produced by adipocytes were higher in the db/db than non-diabetic mice, which has been shown to impair endothelial function.^{41,42} It is possible that elevated leptin levels are involved in coronary endothelial dysfunction in addition to HG in the db/db mice.

In summary, the *db/db* mouse develops DCM and has increased susceptibility of the myocardium to I/R injury. These pathogeneses are associated with impaired BH₄ bioavailability and eNOS dysfunction. The chronic treatment of db/db mice with both SEP and L-Cit diminishes DCM and protects the heart from I/R injury, concomitantly with increases in the concentrations of BH₄ and improvements in eNOS function. Since L-Cit is a stable precursor of the NOS substrate, L-arginine, the present study suggests that elevated bioavailability of BH₄ and L-arginine is important for protection of T2DM hearts against cardiomyopathy and I/R injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Perspective

Diabetic cardiomyopathy and increased incidence of ischemic heart disease elevate the risk of heart failure and death in type 2 diabetic patients with obesity. At present, there are no effective approaches to preventing the development of diabetic cardiomyopathy and ischemic heart disease in type 2 diabetic patients with obesity. It has been observed that the dimerization of endothelial nitric oxide synthase are decreased due to excessive oxidation of tetrahydrobiopterin, and the expression and activity of arginase that metabolizes the substrate of endothelial nitric oxide synthase, L-arginine, to L-ornithine and urea are elevated in diabetic patients or/and animal models, leading to uncoupling of endothelial nitric oxide synthase to oxidation of L-arginine to produce nitric oxide. However, how the function of endothelial nitric oxide synthase can be preserved in type 2 diabetes mellitus remains elusive. Our study demonstrates that chronic co-administration of sepiapterin and L-citrulline diminishes diabetic cardiomyopathy and ischemia/reperfusion injury in obese type 2 diabetic mice. Sepiapterin is a stable precursor of tetrahydrobiopterin with higher cell permeability. L-citrulline is a potent precursor of L-arginine, even at high arginase activity. Although individual sepiapterin or L-citrulline cannot produce markedly cardioprotective effect, combination of sepiapterin and L-citrulline is effective at preserving cardiac tetrahydrobiopterin, dimerization of endothelial nitric oxide synthase, coronary endothelial function, and levels of nitric oxide in obese type 2 diabetic mice. Thus, co-administration of sepiapterin and L-citrulline may be a useful approach for protecting the heart against diabetic cardiomyopathy and ischemia/reperfusion injury in type 2 diabetic patients.

Figure 1.

General characteristics of C57BLKS/J and db/db mice at 6-8 and 14-16 weeks of age. A: body weight; B: blood glucose 6 h after fasting; C: mean arterial blood pressure; D: ratio of left ventricular (LV) weight/body weight; E: the ratio of LV weight/tibia length; F: the ratio of LV weight/lung weight. * $P < 0.0083$ versus C57BLKS/J mice at 6-8 weeks of age; † $P < 0.0083$ versus db/db mice at 6-8 weeks of age; # $P < 0.0083$ versus C57BLKS/J mice at 14-16 weeks of age ($n = 10$ mice/group). One-way ANOVA followed by Bonferroni *post-hoc* test was used to evaluate the differences among groups. Six *post-hoc* tests were performed, and a value of $P < 0.0083$ was considered statistically different.

Figure 2.

db/db mice at 14-16 weeks of age had increased end-systolic volume of the left ventricle (LV) and reduced ejection fraction and mitral E/A ratio. A: LV diastolic volume; B: LV end-systolic volume; C: ejection fraction; D: isovolumic contraction time of the LV. The dimensions and function of the LV were evaluated with transthoracic echocardiography. *P<0.0083 versus C57BLKS/J mice at 6-8 weeks of age; †P<0.0083 versus db/db mice at 6-8 weeks of age; #P<0.0083 versus C57BLKS/J mice at 14-16 weeks of age (n=12 mice/group). One-way ANOVA followed by Bonferroni *post-hoc* test was used to evaluate the differences among groups. Six *post-hoc* tests were performed, and a value of P<0.0083 was considered statistically different.

Figure 3.

Co-administration of sepiapterin (SEP) and L-citrulline (L-Cit) elevated ejection fraction and mitral E/A ratio in diabetic db/db mice. A: ejection fraction; B: mitral E/A ratio. C57BLKS/J and db/db mice at 6-8 weeks of age were given orally SEP or L-Cit alone or in combination for 8 weeks and vehicle as control. Cardiac function was evaluated with echocardiography. *P<0.005 versus the vehicle-treated C57BLKS/J mice; †P<0.005 versus the vehicle-treated db/db group (n=12 mice/group). One-way ANOVA followed by Bonferroni *post-hoc* test was used to evaluate the differences among groups. Ten *post-hoc* tests were performed, and a value of P<0.005 was considered statistically different.

Figure 4.

Combination of sepiapterin (SEP) and L-citrulline (L-Cit) improved acetylcholine (ACh)-induced relaxation of coronary arteries *in vitro* in db/db mice. A: ACh-induced relaxation in the left coronary arteries; B: ACh-induced relaxation in the right coronary arteries. Isolated coronary arteries were normalized to 60 mmHg and precontracted with U46619, and cumulative concentrations of ACh from 10^{-9} to 10^{-5} M were applied to the coronary arteries. * $P < 0.0083$ versus the vehicle-treated C57BLKS/J mice; † $P < 0.0083$ versus the vehicle-treated db/db group (n=5-6 mice/group). Statistical analyses were performed with repeated measures ANOVA followed by Bonferroni's multiple comparison. Six post-hoc tests were performed among 4 experimental groups at each of 5 ACh doses, and a value of $P < 0.0083$ was considered statistically different.

Figure 5.

Co-administration of sepiapterin (SEP) and L-citrulline (L-Cit) reduced myocardial infarct size in both C57BLKS/J and db/db mice subjected to ischemia/reperfusion injury. A: area at risk expressed as a percentage of the left ventricle; B: infarct size expressed as a percentage of area at risk; C: transverse sections of representative mouse hearts stained by 2,3,5-triphenyltetrazolium chloride and phthalo blue dye. C57BLKS/J and db/db mice were administered SEP or L-Cit alone or in combination and vehicle as control and subjected to ischemia/reperfusion injury. The infarct area (white) was delineated by perfusing the coronary arteries with 2,3,5-triphenyltetrazolium chloride via the aortic root, and the area at risk (white-red) was delineated by perfusing phthalo blue dye into the aortic root after tying the coronary artery at the site of previous occlusion. * $P < 0.005$ versus the vehicle-treated C57BLKS/J mice; † $P < 0.005$ versus the vehicle-treated db/db mice ($n = 7-8$ mice/group). One-way ANOVA followed by Bonferroni *post-hoc* test was used to evaluate the differences among groups. Ten *post-hoc* tests were performed, and a value of $P < 0.005$ was considered statistically different.

Figure 6.

Combination of sepiapterin (SEP) and L-citrulline (L-Cit) improved the recovery of coronary flow and cardiac function following ischemia/reperfusion injury in Langendorff-perfused hearts. A: coronary flow rate; B: $-\text{dP}/\text{dt}$ (maximum rate of increase of left ventricular developed pressure); C: $-\text{dP}/\text{dt}$ (maximum rate of decrease of left ventricular developed pressure). C57BLKS/J and db/db mice at 6–8 weeks of age received the treatment of SEP and L-Cit for 8 weeks. Langendorff-perfused hearts were equilibrated for 30 min and subjected to 30 min of ischemia followed by 2 h of reperfusion. * $P < 0.0083$ versus the vehicle-treated C57BLKS/J mice; † $P < 0.0083$ versus the vehicle-treated db/db mice ($n = 8$ mice/group). Statistical analyses were performed with repeated measures ANOVA followed by Bonferroni's multiple comparison. Six post-hoc tests were conducted among 4 experimental groups at each of 8 time points, and a value of $P < 0.0083$ was considered statistically different.

Figure 7.

Co-administration of sepiapterin (SEP) and L-citrulline (L-Cit) elevated cardiac tetrahydrobiopterin concentrations, endothelial nitric oxide synthase (eNOS) dimerization, and nitric oxide (NO) production in db/db mice. A: tetrahydrobiopterin concentrations; B: ratio of eNOS dimers/monomers (bottom: representative Western blots of eNOS dimers and monomers); C: NO concentrations. C57BLKS/J and db/db mice at 6-8 weeks of age were given SEP and L-Cit for 8 weeks or vehicle as control. * $P < 0.0083$ versus the vehicle-treated C57BLKS/J mice; † $P < 0.0083$ versus the vehicle-treated db/db mice ($n = 5-7$ mice/group). Statistical analyses were performed with one-way ANOVA followed by 6 Bonferroni post-hoc tests, and a value of $P < 0.0083$ was considered statistically different.

Figure 8.

Sepiapterin (SEP) increased tetrahydrobiopterin and nitric oxide (NO) concentrations and phosphorylated endothelial nitric oxide synthase (p-eNOS) in endothelial cells subjected to hypoxia/reoxygenation (H/R) injury in the presence of high glucose (HG). A: tetrahydrobiopterin concentrations; B: ratio of p-eNOS/eNOS; C: NO concentrations. Endothelial cells were cultured in the media containing normal glucose (NG) or HG and subjected to H/R injury in the presence or absence of SEP. * $P < 0.017$ versus NG; † $P < 0.017$ versus HG–H/R (n=6-9/group). Statistical analyses were performed with one-way ANOVA followed by 3 Bonferroni post-hoc tests, and a value of $P < 0.017$ was considered statistically different.