Chronics 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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Background—Diabetic heart disease is associated with tetrahydrobiopterin oxidation and high arginase activity, leading to endothelial nitric oxide synthase dysfunction. Sepiapterin (SEP) is a tetrahydrobiopterin precursor, and L-citrulline (L-Cit) is converted to endothelial nitric oxide synthase 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 to 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 to 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, tetrahydrobiopterin concentrations, ratio of endothelial nitric oxide synthase dimers/monomers, and nitric oxide levels compared with vehicle-treated C57BLKS/J mice. These detrimental effects of diabetes mellitus were abrogated by co-administration of SEP and L-Cit. Myocardial infarct size was increased, and coronary flow rate and ±dP/dt were decreased during reperfusion in vehicle-treated db/db mice subjected to ischemia/reperfusion injury compared with 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 tetrahydrobiopterin/endothelial nitric oxide synthase/nitric oxide pathway. (Circ Heart Fail. 2016;9:e002424. DOI: 10.1161/CIRCHEARTFAILURE.115.002424.)

Key Words: diabetic cardiomyopathy ■ ischemia reperfusion injury ■ nitric oxide synthase ■ tetrahydrobiopterin ■ type 2 diabetes mellitus

The prevalence of type 2 diabetes mellitus (T2DM) and obesity has been increasing worldwide in recent decades. 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 nonobese subjects. 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.

See Clinical Perspective

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. 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 nicotinamide adenine dinucleotide phosphate to flavin adenine dinucleotide and flavin mononucleotide. In the presence of adequate BH4 and the substrate L-arginine, heme and oxygen reduction are coupled to the synthesis of NO (eNOS coupling). However, T2DM increases oxidation of BH4 to enzymatically incompetent 7,8-dihydrobiopterin and the expression/activity of arginase
that metabolizes l-arginine to l-citrulline and urea.\(^6\) During conditions of low intracellular BH\(_4\) 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).\(^9\) Increased BH\(_4\) bioavailability and coupling of eNOS by genetic or pharmacological approach have been demonstrated to be useful in preventing endothelial dysfunction in diabetes.\(^11\) However, little is known as to whether pharmaceutical 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 BH\(_4\) with higher cell permeability than BH\(_4\) 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 the co-administration of SEP and l-Cit attenuates DCM and myocardial I/R injury in db/db mice through an increase in bioavailable BH\(_4\) and an improvement in eNOS function.

**Methods**

For expanded Methods, see Data Supplement.

**Animals**

Obese T2DM C57BL/KsJ-lepr\(^0\)/lepr\(^0\) (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 calliper 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 RV 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 per day SEP or 50 mg/kg per 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.\(^22\),\(^23\) 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 phthalol blue dye into the aortic root after tying the coronary artery at the site of previous occlusion.\(^24\)

**Myocardial I/R Injury Ex Vivo**

Langendorff-perfused mouse hearts were subjected to 30 min of no-flow global ischemia followed by 2 hours of reperfusion at 37°C, as described.\(^24\),\(^25\) Coronary flow was monitored by an in-line flow probe connected to a flow meter (Transonic 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.

**BH\(_4\) Assay**

BH\(_4\) was quantified in LV biopsies by high-performance liquid chromatography 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.\(^26\) The normal function of eNOS requires dimerization of the enzyme.\(^27\) To investigate eNOS homodimer formation in the myocardium, nonboiled 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.\(^28\)

**Cell Culture**

Endothelial cells (ECs) were cultured in media containing 5.5 mmol/L (normal glucose concentration) or 20.0 mmol/L glucose (high glucose concentration [HG]) for 12 hours and exposed to 2 hours of hypoxia in glucose-free medium followed by 2 hours of reoxygenation. To investigate the effect of SEP in ECs, 100 μM SEP was added to cultured cells as substrate for the synthesis of BH\(_4\) during 60 min of baseline and the period of hypoxia/reoxygenation (H/R). Because phosphorylation of eNOS regulates NO generation, the expression of total eNOS and phosphorylated eNOS proteins was analyzed using standard Western blot techniques.\(^29\) The concentrations of BH\(_4\) and NO were measured by high-performance liquid chromatography and ozone chemiluminescence, respectively.

**Statistical Analysis**

All data are expressed as mean±SEM. One-way analysis of variance 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, BH\(_4\) and NO concentrations, and the ratio of eNOS dimers/monomers. Statistical analyses of Ach-induced vasodilation, coronary flow, and ±dP/dt over time between groups was performed with repeated measures analysis of
Results

General Characteristics and Cardiac Phenotype of C57BLKS/J and db/db Mice

Body weights of db/db mice at 6 to 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 in C57BLKS/J mice at 6 to 8 weeks of age. Both body weight and blood glucose levels were further elevated in db/db mice of 14 to 16 weeks old compared with those of 6 to 8 weeks old. The ratio of LV weight/body weight was significantly smaller in db/db mice than in age-matched controls. The ratio of LV weight/lung weight was comparable between db/db and C57BLKS/J mice at 6 to 8 weeks of age but significantly smaller in 14- to 16-week-old db/db mice than in 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 to 8 and 14 to 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 to 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 to 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 to 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- to 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 in 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 greater in db/db mice treated with SEP+L-Cit than in age-matched C57BLKS/J mice (P<0.0083 vs C57BLKS/J mice at 14–16 weeks of age; †P<0.0083 vs db/db mice at 6–8 weeks of age; ‡P<0.0083 vs C57BLKS/J mice at 14–16 weeks of age (n=10 mice/group). One-way analysis of variance (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.
C57BLKS/J mice. Interestingly, infarct size was significantly increased by reperfusion in db/db mice (P<0.05; n=5–6 mice/group), but not in C57BLKS/J mice.

Co-Administration of SEP and l-Citrulline 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 no 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±3% of area at risk (n=8) in the vehicle-treated C57BLKS/J mice. There were no significant differences in infarct size between SEP- or l-Citrulline-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 mice 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±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-Citrulline Improved Coronary Flow and Cardiac Function Following I/R Injury in db/db Mice

Figure 6 shows coronary flow rate and ±dP/dt in Langendorff-perfused mouse hearts subjected to I/R injury. The baseline values of coronary flow rate and the values of ±dP/dt were smaller in the db/db+vehicle than in 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 ±dP/dt were significantly smaller in the db/db+vehicle than in C57BLKS/J+vehicle group from 30 min to 2 hours after reperfusion (P<0.05; n=8 hearts/group). There were significant increases in coronary flow rate and the values of ±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-Citrulline Improved 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 mellitus were abrogated by combination of SEP and l-Citrulline 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 phosphorylated eNOS in ECs subjected to H/R injury in the presence of HG. The concentrations of BH4 and NO and the ratio of phosphorylated eNOS/eNOS were significantly decreased in the HG+H/R group compared with normal glucose group (P<0.05; n=6–9/group). 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/group).

Discussion

The results of the present study demonstrate that the T2DM db/db mice with obesity at 14 to 16 weeks of age develop DCM and have increased susceptibility of the myocardium to...
The db/db mouse suffers from a leptin receptor mutation, displaying the characteristics of T2DM, such as hyperglycemia, obesity, dyslipidemia, and insulin resistance. This was confirmed by our present study showing that the db/db mice at both 6 to 8 and 14 to 16 weeks of age had increased body weight and blood glucose. DCM is a common complication of diabetes mellitus characteristic of cardiac (both diastolic and later systolic) dysfunction that occurs independently of a recognized cause, such as coronary artery disease or hypertension. 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 to 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 per day SEP alone can significantly improve cardiac function in streptozotocin-induced type 1 diabetic mice. 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 mellitus 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. Ach induces endothelium-dependent relaxation via stimulating the release of NO, endo- 

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. +dP/dt (maximum rate of increase of left ventricular developed pressure). C. −dP/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 vs the vehicle-treated C57BLKS/J mice; †P<0.0083 vs the vehicle-treated db/db mice (n=8 mice/group). Statistical analyses were performed with repeated measures analysis of variance (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.
The co-administration of SEP and L-Cit elevates tetrahydrobiopterin and nitric oxide (NO) concentrations in diabetic mice. 

Figure 7. Co-administration of sepiapterin (SEP) and L-citrulline (L-Cit) elevated tetrahydrobiopterin (l-Cit) concentrations in db/db mice. A. Tetrahydrobiopterin concentrations. B. Ratio of eNOS dimers/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 (ANOVA) followed by 1 Bonferroni post-hoc tests, and a value of P < 0.0083 was considered statistically different.

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 the vascular endothelial NO rather than endothelium-derived hyperpolarizing factor and prostacyclin I2 was decreased in db/db mice. 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. Clinical studies also suggest that diabetes mellitus increases the susceptibility of the myocardium to I/R injury. However, inconsistent results are obtained in experimental studies of animals as to how diabetes mellitus affects myocardial I/R injury. In the present study, myocardial infarct size was larger in db/db mice than in C57BLKS/J mice, and systolic and diastolic function was worse in db/db mice 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. We also measured infarct size and cardiac function in db/db and C57BLKS/J mice at 6 to 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 mellitus.

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 in 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 7,8-dihydrobiopterin. 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. 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. 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. It is likely that decreased bioavailability of BH4 contributes to the development of DCM in db/db mice.

SEP is reduced by SEEReductase to 7,8-dihydrobiopterin and further by dihydrofolate reductase to form BH3. 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 because of increased expression/activity of arginase in intestine, blood, and other tissues, 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 because of 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. L-Cit alone failed to reduce myocardial infarct size in db/db mice subjected to I/R injury. This may be attributed to improving cardiac function and nitric oxide biosynthesis.
to insufficient dimerization of eNOS in db/db mice because of 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 pathogenesis of T2DM in humans, for whom leptin receptor deficiency is not an important contributor to T2DM. The db/db mice develop hyperglycemia, obesity, dyslipidemia, and insulin resistance, which are in fact secondary to genetic mutations of leptin receptors. 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. Another limitation is that the levels of circulating leptin levels 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 in the nondiabetic mice, which has been shown to impair endothelial function. 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. Because 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.

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Disclosures
None.

References


**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 is decreased because of 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 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 arginine activity. Although individual sepiapterin or l-citrulline cannot produce markedly cardioprotective effects, 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.
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SUPPLEMENTAL MATERIAL for
Chronic Co-Administration of Sepiapterin and L-Citrulline Ameliorates
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in Obese Type 2 Diabetic Mice

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The Supplementary Materials include:
  Supplementary materials and methods
  Supplementary Results
  Table 1. Echocardiographic parameters of C57BLKS/J and db/db mice
  Table 2. Heart rate of C57BLKS/J and db/db mice during in vivo ischemia/reperfusion injury
Materials and methods

Animals

Obese T2DM C57BL/KsJ-lepr<sup>db</sup>/lepr<sup>db</sup> (db/db) and C57BLKS/J control mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were kept on a 12-h light-dark cycle in a temperature-controlled room. 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 hemodynamics, blood glucose, and heart weight

C57BLKS/J and db/db mice at either 6-8 or 14-16 weeks of age were fasted for 6 h and anesthetized by intraperitoneal injection of 80 mg/kg pentobarbital sodium (n=10 mice/group). The mice were ventilated with room air supplemented with 100% oxygen at approximately 102 breaths/min, as described. The right carotid artery was cannulated with a small polyethylene tubing catheter filled with 0.9% saline containing 10 U/ml heparin, as described. The catheter was connected to an ADInstrument pressure transducer (MLT0380/D, ADInstruments, Colorado Springs, CO) and a Powerlab data acquisition system (ADInstruments). After a 30 min of stabilization, blood pressure was continuously recorded for 20 min. A thoracotomy was performed, and the left ventricle (LV) was punctured with a 27 gauge needle. Blood glucose was measured with a blood gas analyzer (ABL-725 Radiometer, Radiometer America Inc., Westlake, OH). Body temperature was maintained between 36.8°C and 37.3°C throughout the experiment by using a heating pad (Model TC-1000, CWE Inc.; Ardmore, PA). After mice were euthanized,
left ventricle and lung were weighed. Left ventricular weight was normalized to lung weight and tibia length.

**Measurements of tibia length**

Hind legs were covered with 1 M NaOH and incubated at 37 °C for 5 h to digest skin, fat tissue, and muscle. The tubes were gently agitated every 30 min to help digestion. After digestion, the tibia was collected, rinsed, and then dried on paper towel briefly. Tibia length was measured using a Vernier capillary and used to normalize left ventricular weight.

**Transthoracic echocardiography**

Forty-eight C57BLKS/J mice and 48 db/db mice were sedated by the inhalation of 1.50 % isoflurane and oxygen (n=12 mice/group). Non-invasive transthoracic echocardiography was performed with a VisualSonics Vevo 770 High-resolution Imaging System (Toronto, Canada) equipped with a 30 MHz transducer (Scanhead RV 707), as described previously.\(^{2,3}\) Left ventricular dimensions and ejection fraction were measured by two-dimension guided M-mode method. Pulsed Doppler waveforms recorded in the apical-4-chamber view were used for the measurements of the peak velocities of mitral E (early mitral inflow) and A (late mitral inflow) waves, isovolumic contraction time, ejection time, and isovolumic relaxation time of the LV. The myocardial performance index was calculated with the following formula: myocardial performance index = (isovolumic contraction time + isovolumic relaxation time)/ejection time.
Responsibility of isolated coronary arteries

Preparation of isolated coronary arteries. C57BLKS/J and db/db mice at 6-8 weeks of age were orally given 5 mg/kg/day SEP (Sigma-Aldrich, St. Louis, MO) and 50 mg/kg/day L-Cit (Sigma-Aldrich) for 8 weeks using a plastic feeding tube (Instech Laboratories, Inc., Plymouth Meeting, PA) or the vehicle, phosphate-buffered saline (PBS), as control (n = 6 mice/group). Under anesthesia with pentobarbital, mouse hearts were rapidly excised and placed in cold physiological salt solution containing (in mM) 119 NaCl, 4.7 KCl, 1.6 CaCl₂, 1.17 MgSO₄, 1.18 NaH₂PO₄, 24.0 NaHCO₃, 0.026 EDTA, and 5.5 glucose (pH 7.4). Mouse coronary arteries, ~70-90 µm in diameter and 1 mm long, were isolated from the left main and right coronary artery under a dissection microscopy. Dissected coronary arteries were transferred to a water-jacked microvascular perfusion chamber and cannulated at both ends with glass micropipettes for the continuous videomicroscopic measurement of internal diameter.

Response of coronary artery ring to acetylcholine (Ach). In the microvascular chamber, the vessels were continuously superfused with recirculating physiological salt solution gassed with 21% O₂-5% CO₂ (pH 7.35-7.45) and maintained at 37°C, as described. After a 60-min equilibration period under the transmural pressure of 60 mmHg, coronary artery rings were preconstricted with U46619 (100-300 nM) to 30–50% of the baseline internal diameter if the spontaneous myogenic tone was not sufficient to achieve the target reduction in diameter. Ach (1 nM to 10 µM) was cumulatively added to the bath solution to induce vasodilation. The vasodilatory responses to Ach were expressed as a percentage of relaxation relative to spontaneous vascular tone or U46619 preconstriction, with 100% relaxation representing the passive baseline diameter.
**Myocardial I/R injury in vivo**

The 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. Thirty-six C57BLKS/J and 44 db/db mice at 14-16 weeks of age were subjected to myocardial I/R injury in vivo. 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, as previously described.\textsuperscript{3,6} After instrumentation was completed, all mice were stabilized for 30 min and subjected to 20 min of coronary artery occlusion followed by 24 h of reperfusion. Heart rate was monitored from the electrocardiogram. The mouse was removed from the ventilator, and kept in a warm chamber (environmental temperature: \textasciitilde33°C) with a hole via that 100% oxygen passed through. The temperature was maintained by a tiny heating pat on the bottom of chamber and a heat lamp above the chamber. When the respiratory rate was approximately 140 beats/min, the endotracheal tube was withdrawn. The mouse was continued to keep in the warm chamber until moving. At conclusion of 24 h after reperfusion, 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 (Heucotech Ltd., Fairless Hill, PA) into the aortic root after tying the coronary artery at the site of previous occlusion.\textsuperscript{1} Area at risk was expressed as a percentage of the LV, and infarct size as a percentage of area at risk.

**Myocardial I/R injury ex vivo**

C57BLKS/J and db/db mice at 6-8 weeks of age were orally administered both 5 mg/kg/day and L-Cit 50 mg/kg/day for 8 weeks or vehicle as control (n=8 mice/group). Mouse hearts were mounted on a Langendorff apparatus and perfused retrogradely through the aorta at a constant
pressure of 80 mmHg with Krebs-Henseleit buffer at 37 °C, as described. The buffer was continuously bubbled with a mixture of 95% oxygen/5% carbon dioxide via in-line filter (5 µm pore size). A fluid-filled plastic balloon was inserted into the chamber of the LV via the mitral valve, and connected to a pressure transducer for continuous measurement of LV pressure. The hearts were immersed in perfusate maintained at 37.2±0.3°C, and the balloon was inflated to a diastolic pressure of ~5 to 10 mmHg. Coronary flow was monitored by an in-line flow probe connected to a flow meter (Transonics Systems Inc., Ithaca, NY). The LV pressure signal was monitored to obtain heart rate and left ventricular dP/dt. All hearts were stabilized for 30 min and subjected to 30 min of no-flow global ischemia followed by 2 h of reperfusion. 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

C57BLKS/J and db/db mice received orally both SEP and L-Cit for 8 weeks or vehicle as control. BH₄ was quantified in LV biopsies by high performance liquid chromatography (HPLC) with electrochemical detection (ESA Biosciences CoulArray® system Model 542, Chelmsford, MA), as previously described. Filtrates were analyzed on a HPLC system (ESA Biosciences CoulArray® system, Model 582 and 542) using an analytical Polar-RP column eluted with argon saturated 50 mM phosphate buffer (pH 2.6). Authentic BH₄ solutions (10-100 nM) were used as standards and sample concentrations were normalized to protein content measured by the bicinchoninic acid protein assay.
Immunoblotting

After either C57BLKS/J or db/db mice were pretreated with both SEP and L-Cit or vehicle for 8 weeks, the LV was harvested and homogenized in a buffer containing 20.0 mM MOPS, 2.0 mM EGTA, 5.0 mM EDTA, protease inhibitor cocktail (1:100; Calbiochem, San Diego, CA), phosphatase inhibitors cocktail (1:100; Calbiochem), 0.5% detergent (Nonidet™ P-40 detergent pH 7.4, Sigma-Aldrich). Immunoblots were performed using standard techniques, as described. The normal function of eNOS requires dimerization of the enzyme. 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 1:2,000 dilution of mouse anti-eNOS monoclonal antibody (BD Transduction Laboratories, San Jose, CA). Immunoreactive bands were visualized by enhanced chemiluminescence followed by densitometric analysis using image acquisition and analysis software (Image J, NIH).

Measurement of NO

Nitrite concentration corresponding to the stable byproduct of NO released by myocardium in aqueous solution was quantified by ozone chemiluminescence, as described. Samples (20 µl) were refluxed in glacial acetic acid containing potassium iodide and nitrite quantified in a NO chemiluminescence analyzer (Sievers Instruments, Boulder, CO). Nitrite concentrations were calculated after subtraction of background levels and normalized to protein content (Bradford method).
Cell culture

Endothelial cells (ECs) isolated from coronary arteries of healthy subjects (Cell Applications, San Diego, CA) were cultured in MesoEndo cell growth medium (Cell Applications) at 37 °C and used between 4th and 6th passages when approximately 70-80% confluent, as described. 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. Culture medium was then replaced and the cells were exposed to 2 h of hypoxia (0.1% O₂, Biospherix hypoxia chamber, Lacona, NY) in glucose-free medium followed by 2 h of reoxygenation. To investigate the effect of SEP in ECs, 100 µM SEP (Sigma-Aldrich) 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, as described. Briefly, tissue homogenates that contained 50 µg of proteins were applied to 7.5% SDS-polyacrylamide gel and subjected to immunoblot analysis by incubation with an anti-eNOS antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and an anti-p-eNOS antibody (serine 1177, Cell Signaling, Beverly, MA) at 4°C. The membrane was washed and then incubated with the appropriate anti-mouse secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence followed by densitometric analysis using image acquisition and analysis software (Image J). The concentrations of BH₄ and NO were measured by HPLC and ozone chemiluminescence, respectively.
Results

Db/db mice at 14-16 weeks of age developed diabetic cardiomyopathy

The dimensions and function of the LV measured by echocardiography are listed in Table 1. There were no significant differences in the thickness of anterior and posterior walls, LV end-diastolic volume; the peak velocity of mitral E and A waves; isovolumic contraction time of the LV; ejection time of the LV; myocardial performance index; mitral E acceleration; E wave acceleration time; and mitral E deceleration 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 pressure of the LV and mitral E wave deceleration time were 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).
Heart rate of C57BLKS/J and db/db mice during *in vivo* ischemia/reperfusion injury

Heart rate was comparable among the 4 experimental groups of C57BLKS/J mice at baseline, 30 min after ischemia, and 30, 60, and 120 min after reperfusion (P>0.05, n=8 mice/group) (Table 2). Compared with the vehicle-treated C57BLKS/J group, hear rate at baseline was significantly decreased in the vehicle-, SEP-, or L-Cit-treated db/db mice (P<0.05, n=7-8 mice/group), but not in SEP+L-Cit-treated db/db mice. In addition, heart rate 30, 60, and 120 min after reperfusion was slower in the vehicle-treated db/db than vehicle-treated C57BLKS/J mice.

### Table 1 Echocardiographic parameters of C57BLKS/J and db/db mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>6-8 weeks old</th>
<th>14-16 weeks old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BLKS/J</td>
<td>db/db</td>
</tr>
<tr>
<td>Anterior wall at end diastole, mm</td>
<td>0.71±0.04</td>
<td>0.68±0.04</td>
</tr>
<tr>
<td>Anterior wall at end systole, mm</td>
<td>1.13±0.07</td>
<td>1.20±0.06</td>
</tr>
<tr>
<td>Posterior wall at end diastole, mm</td>
<td>0.73±0.04</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>Posterior wall at end systole, mm</td>
<td>1.20±0.06</td>
<td>1.23±0.06</td>
</tr>
<tr>
<td>LV end-diastolic volume, µl</td>
<td>41±6</td>
<td>37±5</td>
</tr>
<tr>
<td>LV end-systolic volume, µl</td>
<td>13±2</td>
<td>12±2</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>65±5</td>
<td>65±4</td>
</tr>
<tr>
<td>Peak E wave velocity, cm/s</td>
<td>80±4</td>
<td>79±4</td>
</tr>
<tr>
<td>Peak A wave velocity, cm/s</td>
<td>49±4</td>
<td>47±2</td>
</tr>
<tr>
<td>Peak E/A ratio</td>
<td>1.70±0.10</td>
<td>1.70±0.09</td>
</tr>
<tr>
<td>Isovolumic contraction time of LV, ms</td>
<td>14.3±0.9</td>
<td>14.3±0.8</td>
</tr>
<tr>
<td>Ejection time, ms</td>
<td>42.6±0.8</td>
<td>42.6±0.6</td>
</tr>
<tr>
<td>Isovolumic relaxation time of LV, ms</td>
<td>16.5±0.5</td>
<td>16.8±0.6</td>
</tr>
<tr>
<td>Myocardial performance index</td>
<td>0.72±0.03</td>
<td>0.73±0.02</td>
</tr>
<tr>
<td>Mitral E acceleration, cm/ms</td>
<td>87.2±595</td>
<td>81±53</td>
</tr>
<tr>
<td>Mitral E wave acceleration time, ms</td>
<td>9.6±0.3</td>
<td>9.3±0.4</td>
</tr>
<tr>
<td>Mitral E deceleration, cm/ms</td>
<td>5662±453</td>
<td>5983±594</td>
</tr>
<tr>
<td>Mitral E wave deceleration time, ms</td>
<td>13.8±1.0</td>
<td>13.9±0.9</td>
</tr>
</tbody>
</table>

LV=left ventricle; ms=millisecond. *P<0.05 versus 6-8 week-old C57BLKS/J mice; †P<0.05 versus 6-8 week-old db/db mice; and ‡P<0.05 versus 14-16 week-old C57BLKS/J mice (n=12 mice/group).
Table 2 Heart rate (beats/min) of C57BLKS/J and db/db mice during in vivo ischemia/reperfusion injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Coronary occlusion</th>
<th>Reperfusion 30 min</th>
<th>Reperfusion 60 min</th>
<th>Reperfusion 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BLKS/J mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>409±16</td>
<td>420±15</td>
<td>435±18</td>
<td>420±21</td>
<td>417±12</td>
</tr>
<tr>
<td>SEP</td>
<td>423±13</td>
<td>405±15</td>
<td>440±16</td>
<td>419±17</td>
<td>414±13</td>
</tr>
<tr>
<td>L-Cit</td>
<td>413±18</td>
<td>408±20</td>
<td>426±19</td>
<td>409±19</td>
<td>391±15</td>
</tr>
<tr>
<td>SEP+L-Cit</td>
<td>422±20</td>
<td>379±11</td>
<td>439±13</td>
<td>415±13</td>
<td>390±12</td>
</tr>
<tr>
<td>Db/db mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>347±15*</td>
<td>411±16</td>
<td>376±14*</td>
<td>363±10*</td>
<td>377±10*</td>
</tr>
<tr>
<td>SEP</td>
<td>336±24*</td>
<td>423±10</td>
<td>411±12</td>
<td>406±13</td>
<td>407±21</td>
</tr>
<tr>
<td>L-Cit</td>
<td>366±13*</td>
<td>406±11</td>
<td>403±13</td>
<td>400±15</td>
<td>389±16</td>
</tr>
<tr>
<td>SEP+L-Cit</td>
<td>386±14</td>
<td>393±18</td>
<td>421±18</td>
<td>389±13</td>
<td>395±12</td>
</tr>
</tbody>
</table>

L-Cit=L-citrulline; SEP=sepiapterin. *P<0.05 versus C57BLKS/J+vehicle groups at corresponding time points (n=7-9 mice/group).

References


