



# BH4 Increases nNOS Activity and Preserves Left Ventricular Function in Diabetes

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**RATIONALE:** In diabetic patients, heart failure with predominant left ventricular (LV) diastolic dysfunction is a common complication for which there is no effective treatment. Oxidation of the NOS (nitric oxide synthase) cofactor tetrahydrobiopterin (BH4) and dysfunctional NOS activity have been implicated in the pathogenesis of the diabetic vascular and cardiomyopathic phenotype.

**OBJECTIVE:** Using mice models and human myocardial samples, we evaluated whether and by which mechanism increasing myocardial BH4 availability prevented or reversed LV dysfunction induced by diabetes.

**METHODS AND RESULTS:** In contrast to the vascular endothelium, BH4 levels, superoxide production, and NOS activity (by liquid chromatography) did not differ in the LV myocardium of diabetic mice or in atrial tissue from diabetic patients. Nevertheless, the impairment in both cardiomyocyte relaxation and  $[Ca^{2+}]_i$  (intracellular calcium) decay and in vivo LV function (echocardiography and tissue Doppler) that developed in wild-type mice 12 weeks post-diabetes induction (streptozotocin, 42–45 mg/kg) was prevented in mGCH1-Tg (mice with elevated myocardial BH4 content secondary to transgenic overexpression of GTP-cyclohydrolase 1) and reversed in wild-type mice receiving oral BH4 supplementation from the 12th to the 18th week after diabetes induction. The protective effect of BH4 was abolished by CRISPR/Cas9-mediated knockout of nNOS (the neuronal NOS isoform) in mGCH1-Tg. In HEK (human embryonic kidney) cells, S-nitrosoglutathione led to a PKG (protein kinase G)-dependent increase in plasmalemmal density of the insulin-independent glucose transporter GLUT-1 (glucose transporter-1). In cardiomyocytes, mGCH1 overexpression induced a NO/sGC (soluble guanylate cyclase)/PKG-dependent increase in glucose uptake via GLUT-1, which was instrumental in preserving mitochondrial creatine kinase activity, oxygen consumption rate, LV energetics (by  $^{31}P$  phosphorous magnetic resonance spectroscopy), and myocardial function.

**CONCLUSIONS:** We uncovered a novel mechanism whereby myocardial BH4 prevents and reverses LV diastolic and systolic dysfunction associated with diabetes via an nNOS-mediated increase in insulin-independent myocardial glucose uptake and utilization. These findings highlight the potential of GCH1/BH4-based therapeutics in human diabetic cardiomyopathy.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** cardiovascular disease ■ glucose ■ heart failure ■ mice ■ nitric oxide synthase

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**D**iabetes mellitus (DM) is a major cause of death and disability and a large economic burden on health-care systems across the world.<sup>1</sup> Globally, 1 in 12 deaths in adults has been attributed to DM and its complications<sup>2</sup>; among which, the proportion of heart failure cases is substantial both in type I and type II DM and

persisting after adjustment for differences in coronary artery disease or other relevant risk factors.<sup>3,4</sup> Together with postmortem findings demonstrating left ventricular (LV) dysfunction in diabetic patients in the absence of coronary artery disease or hypertension,<sup>5</sup> epidemiological data suggest that DM may in itself give rise to

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Novelty and Significance

What Is Known?

- Nitric oxide (NO) regulates cardiac contractility and relaxation.
- Myocardial nNOS (neuronal NO synthase) activity and relaxation can be enhanced by increasing the intracellular level of the NOS cofactor, tetrahydrobiopterin (BH4).
- Diabetes reduces NO synthesis in the vascular endothelium by oxidizing BH4.

What New Information Does This Article Contribute?

- Left ventricular (LV) dysfunction associated with diabetes can occur in the absence of BH4 oxidation, increased superoxide production, or reduced NOS activity both in humans and in mice.
- Nevertheless, the diabetic cardiomyopathic phenotype is prevented by raising BH4 content in cardiomyocytes through the overexpression of GCH1 (GTP-cyclohydrolase 1) and reversed by oral administration of BH4.
- BH4-mediated preservation of myocardial function and energetics is abolished by nNOS gene deletion or GLUT (glucose transporter)-1 inhibition.

The exact mechanisms underpinning LV dysfunction in diabetic patients are not completely understood. Here we show that diabetic mice develop LV dysfunction despite maintaining normal NO synthesis and myocardial BH4 levels, suggesting that NOS dysfunction and oxidative stress are not required for the development of diabetic cardiomyopathic phenotype. Nevertheless, increasing intracellular BH4 in cardiomyocytes, prevented and reversed LV dysfunction via an nNOS-mediated increase in insulin-independent glucose uptake and utilization. Our findings suggest that BH4 supplementation may both prevent and ameliorate LV dysfunction in patients with diabetes.

Nonstandard Abbreviations and Acronyms

<b><sup>31</sup>P MRS</b>	<sup>31</sup> phosphorous magnetic resonance spectroscopy
<b>BH4</b>	tetrahydrobiopterin
<b>CK</b>	creatine kinase
<b>FA</b>	fatty acid
<b>GCH1</b>	GTP cyclohydrolase 1
<b>mGCH1-Tg</b>	mice with elevated myocardial BH4 content secondary to overexpression of GTP-cyclohydrolase 1
<b>nNOS</b>	neuronal nitric oxide synthase
<b>OCR</b>	oxygen consumption rate
<b>PCr/ATP</b>	phosphocreatine-to-ATP ratio
<b>PDK4</b>	pyruvate dehydrogenase kinase 4
<b>PKG</b>	protein kinase G
<b>PLB</b>	phospholamban
<b>PPAR</b>	peroxisome proliferator-activated receptor
<b>SR</b>	sarcoplasmic reticulum
<b>UCP</b>	uncoupling protein
<b>VDAC</b>	voltage-dependent anion channel

Several factors, including mitochondrial dysfunction, oxidative stress, impaired calcium handling, dysfunctional NOS (nitric oxide synthase) activity, and remodeling of the extracellular matrix, have been advocated in the pathogenesis of diabetic cardiomyopathy<sup>6</sup>; however, a unifying mechanism upstream of the observed LV functional changes is still lacking.

Constitutive NO production regulates LV compliance and relaxation through its action on myofilament Ca<sup>2+</sup> sensitivity and intracellular Ca<sup>2+</sup> handling.<sup>7</sup> Under physiological conditions, tetrahydrobiopterin (BH4) is a limiting factor in myocardial NO synthesis by the nNOS (neuronal NOS) isoform.<sup>8</sup> Increasing cardiomyocyte BH4 content by myocardial-specific overexpression of the first enzyme involved in its synthesis, GCH1 (GTP-cyclohydrolase 1), enhances nNOS activity and hastens the rate of intracellular Ca<sup>2+</sup> reuptake and myocardial relaxation in healthy mice by increasing the PLB (phospholamban) phosphorylated fraction.<sup>8,9</sup> In the presence of DM, increasing BH4 content by myocardial GCH1 overexpression or inhibition of GCH1 protein degradation has been shown to attenuate the increase in myocardial superoxide production and maintain nNOS in its dimeric form.<sup>10</sup> Impaired NO signaling, due to BH4 oxidation and dysfunctional eNOS (endothelial NOS) activity, accounts for the endothelial dysfunction reported in diabetic patients and animal models.<sup>11–13</sup> Similar changes in the myocardium would be expected to lead to LV diastolic

a specific cardiomyopathy characterized by predominant LV diastolic dysfunction, leading to heart failure with preserved or mildly impaired LV ejection fraction.<sup>6</sup>

dysfunction and increased oxidative stress<sup>9,14,15</sup>; however, whether dysfunctional NOS activity and altered nitroso-redox balance are key factors in the pathogenesis of diabetic cardiomyopathy remains to be established. Likewise, the extent to which endothelial dysfunction induced by DM contributes to the cardiomyopathic phenotype is unclear.

Here, we show that increasing myocardial BH4 and nNOS activity by transgenic overexpression of GCH1 does not preserve endothelial-mediated vasodilatation but prevents LV dysfunction in diabetic mice—not by averting NOS dysfunction, maintaining PLB phosphorylation, or reducing oxidative stress—but by preserving myocardial energetics via an nNOS-mediated increase in glucose uptake through GLUT-1 (the insulin-independent transporter-1). Importantly, oral BH4 supplementation is able to reverse the cardiomyopathic phenotype in diabetic wild-type (WT) mice, indicating that GCH1/BH4-based therapeutics may be used to treat as well as prevent diabetic cardiomyopathy.

## METHODS

### Data Availability

The authors declare that all data and methods supporting the findings of this study are available in the [Data Supplement](#) or from the corresponding authors on reasonable request. Please see expanded methods and the Major Resources Table in the [Data Supplement](#).

### Human Samples

Samples of the right atrial appendage were collected from patients undergoing on-pump cardiac surgery for coronary revascularization and stored at  $-80^{\circ}\text{C}$ . Investigations were approved by the Research Ethics Committee; all patients gave informed written consent.

### Diabetes Induction

DM was induced by low doses (42–45 mg/kg) of streptozotocin dissolved in citrate buffer and injected intraperitoneal daily for 5 consecutive days. Control mice were injected in parallel with buffer only. Mice with glucose levels  $<15$  mmol/L after 2 weeks of streptozotocin injection were excluded. Studies were carried out and data analyzed with the operator blind of the genotype or treatment allocation.

### BH4 Supplementation

WT mice were allocated to normal chow (Teklad global 16% protein diet, Harlan Laboratories) or BH4-supplemented chow (200 mg/kg per day for 6 weeks) beginning at week 12 poststreptozotocin. Data were collected and analyzed with the operator blind of treatment allocation. Randomization was performed by cage.

Representative images were selected as reflecting either the mean or the median (in case of non-normal data) of their respective data series.

## RESULTS

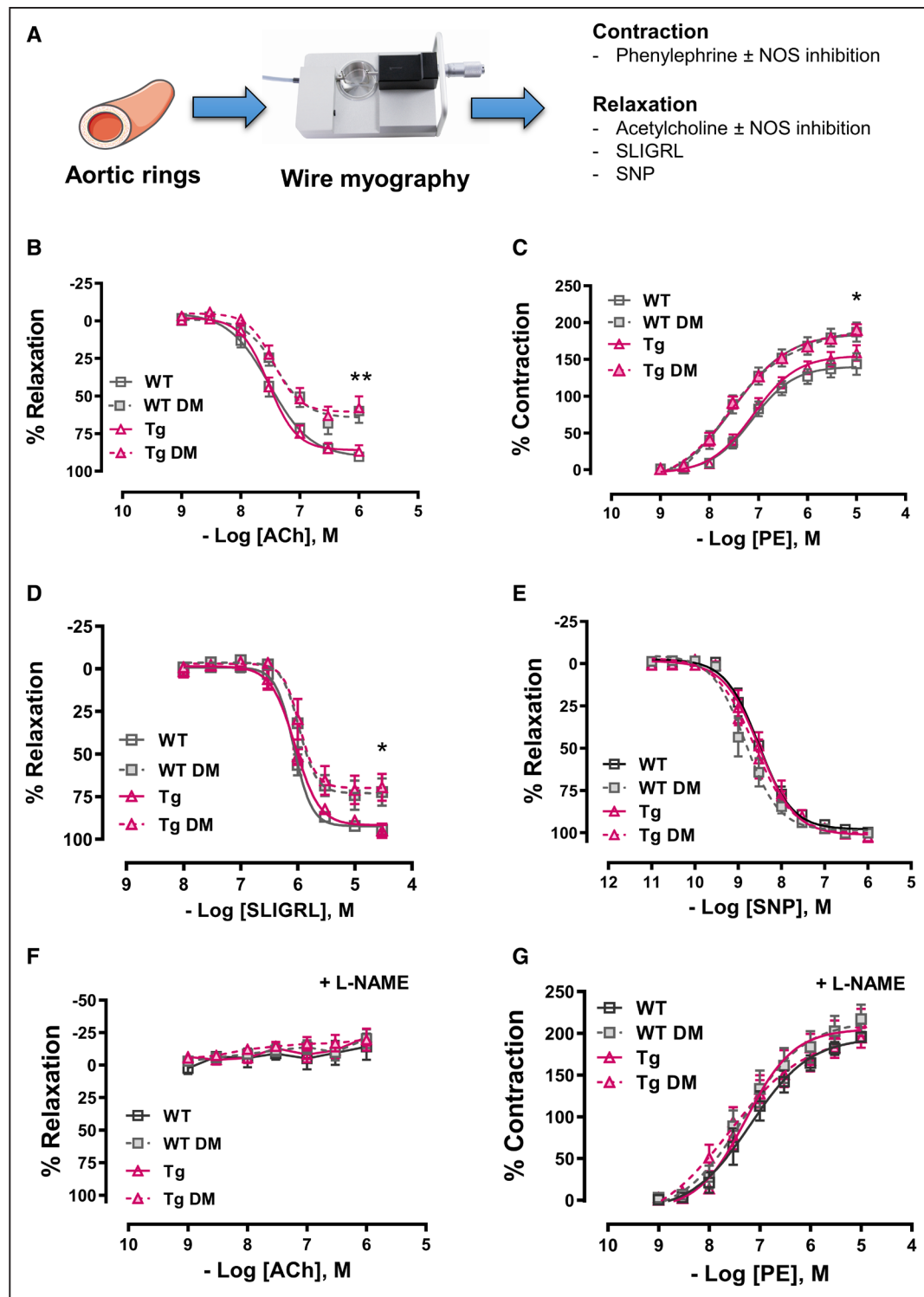
### DM-Induced LV Dysfunction Is Prevented by Myocardial GCH1 Overexpression and Reversed by Oral BH4 Supplementation

Streptozotocin decreased plasma insulin levels and body weight and increased plasma glucose similarly in WT and mGCH1-Tg (mice with elevated myocardial BH4 content secondary to overexpression of GTP-cyclohydrolase 1) at 4 and 12 weeks post-DM induction (Table I in the [Data Supplement](#)). As expected, DM caused significant endothelial dysfunction at 4 and 12 weeks postinduction (Figure 1A in the [Data Supplement](#); Figure 1). At the latter time point, aortas from both diabetic WT and mGCH1-Tg displayed an enhanced contractile response to phenylephrine and impaired vasodilatation in response to acetylcholine or the peptide activator of the SLIGRL (protease-activated receptor-2) compared with sham-injected nondiabetic littermates (Figure 1B through 1D), whereas endothelial-independent vasodilatation in response to the NO donor, sodium nitroprusside, was preserved in all groups (Figure 1E). Preincubation of aortic rings with the NOS inhibitor, N-nitro-L-arginine methyl ester (100  $\mu\text{mol/L}$ ), abolished all differences between diabetic and nondiabetic mice (Figure 1F and 1G).

LV function was assessed in vivo by echocardiography and tissue Doppler in all groups at 4 and 12 weeks after the streptozotocin or sham injections (Figure 2; Figure V and Table II in the [Data Supplement](#)).

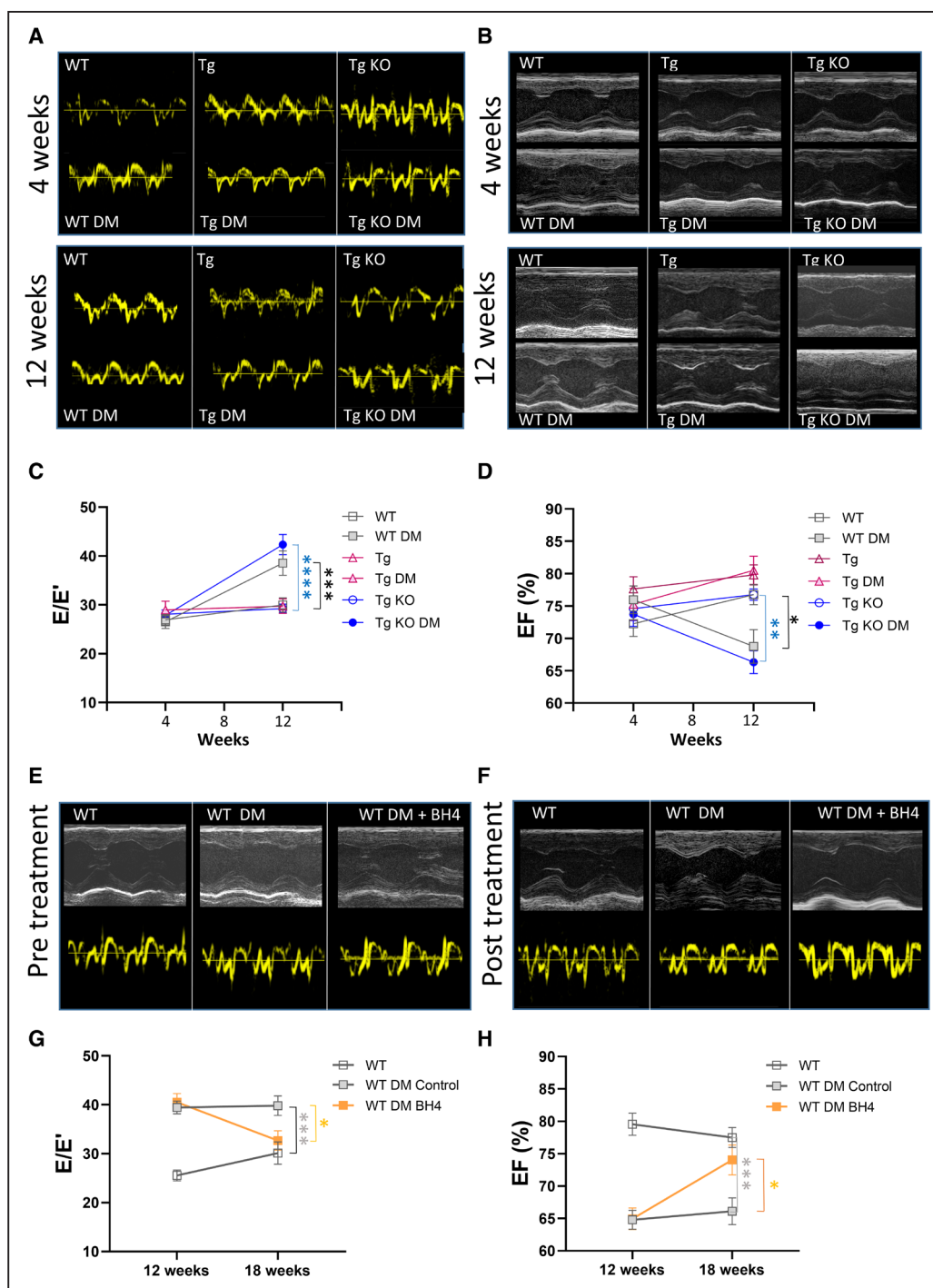
At 4 weeks post-DM induction, LV diastolic and systolic function was preserved (Figure 2A through 2D and Table II in the [Data Supplement](#)), despite the development of endothelial dysfunction (Figure 1A in the [Data Supplement](#)).

At 12 weeks post-DM induction, nondiabetic groups showed similar function as at 4 weeks. However, LV diastolic function was significantly impaired in diabetic WT, as indicated by a higher ratio between the peak early mitral filling velocity (E) and the tissue Doppler-derived peak early diastolic velocity at the mitral annulus (E';  $P<0.001$  versus WT non-DM controls and diabetic mGCH1-Tg mice, Figure 2C). Echocardiographic examination showed no differences in LV end-diastolic volume between genotypes in the presence or absence of DM whereas LV end-systolic volume increased in diabetic WT (Table II in the [Data Supplement](#)), leading to a significant reduction in LV ejection fraction and fractional shortening when compared with both WT nondiabetic controls and diabetic mGCH1-Tg mice (Figure 2D and Table II in the [Data Supplement](#)). By contrast, LV diastolic and systolic function were unaltered in mGCH1-Tg mice at 12 weeks post-DM induction (Figure 2A through 2D and Table II in the [Data Supplement](#)). These findings were mirrored by concordant changes in the myocardial performance index (Table II in the [Data Supplement](#)), confirming that impairment in this heart rate/arterial pressure-independent



**Figure 1. Endothelial function is impaired in the presence of diabetes in both wild-type (WT) and in mice with myocardial overexpression of GCH1 (Tg).**

**A**, Overview of the assessment of vascular function in diabetic (DM) and normoglycemic mice. **B**, Dose-dependent vasoconstrictor response to phenylephrine (PE) and **(C)** vasodilator response to acetylcholine (ACh) in aortic rings from DM or normoglycemic WT and Tg (n; WT=8, WT DM=7, Tg=8, Tg DM=9 aortas) at 12 wk after DM induction or sham injection. **D**, Endothelial-dependent (SLIGRL [protease-activated receptor-2]) and **(E)** endothelial-independent (sodium nitroprusside, SNP) vasodilator responses in aortic rings from diabetic or normoglycemic WT and Tg (n=6 aortas per group). **F**, PE and **(G)** ACh-mediated responses in the presence of NOS (nitric oxide synthase) inhibition (N-nitro-L-arginine methyl ester [L-NAME], 100  $\mu$ M; n; WT=5, WT DM=6, Tg=7, Tg DM=7 aortas). Normally distributed data (**B**, **D**, **E**, **F**, and **G**) are shown as means  $\pm$  SEM and were compared using 2-way ANOVA with Bonferroni correction. In **C**, non-normal data (D'Agostino-Pearson test,  $P=0.032$ ) are shown as median and interquartile range and were compared using Kruskal-Wallis one-way ANOVA, followed by the Dunn test. \* $P<0.05$ , \*\* $P<0.01$  vs normoglycemic mice from either genotype. GCH1 indicates GTP cyclohydrolase 1.



**Figure 2. Myocardial GCH1 (GTP-cyclohydrolase 1) overexpression and oral tetrahydrobiopterin (BH4) supplementation prevent and reverse the impairment in left ventricular (LV) function after diabetes induction, respectively, via an nNOS (neuronal NO synthase)-dependent mechanism.**

**A**, Representative LV tissue Doppler and **(B)** M-Mode traces in wild-type mice (WT), mice with myocardial overexpression of GCH1 (Tg), and in Tg mice lacking nNOS (Tg knockout [KO]) at 4 and 12 wk post-DM induction or sham injection ( $n=12$  mice per group). **C**, Average E/E' ratio (ratio between early mitral inflow velocity and mitral annular early diastolic velocity) and **(D)** average LV ejection fraction (LVEF) in the 6 groups over time. Data (all normally distributed) are shown as means $\pm$ SEM and were compared using 3-way ANOVA with Bonferroni correction. For the E/E' ratio: \*\*\* $P<0.001$ , WT DM vs WT at 12 wk and \*\*\*\* $P<0.0001$ , Tg KO DM vs Tg KO at 12 wk.  $P=0.0004$  for the interaction between genotype and diabetes. For LVEF \* $P<0.05$ , WT DM vs WT at 12 wk and \*\* $P<0.01$ , Tg KO DM vs Tg KO at 12 wk.  $P=0.0085$  for the interaction between genotype and diabetes. Comparisons between diabetes-time and genotype-time are included in Table II in the [Data Supplement](#). Scatterplots for these data are shown in Figure V in the [Data Supplement](#). **E**, Representative LV Tissue Doppler and **(F)** M-Mode traces in DM WT mice fed standard or BH4-supplemented chow from week 12 to wk 18 post-DM induction and non-DM controls ( $n=12$  mice per group). **G**, Average E/E' ratio and **H**, LVEF in the 3 groups over time. Data (all normally distributed) are shown as means $\pm$ SEM and were analyzed by 2-way ANOVA with Bonferroni correction. \*\*\* $P<0.001$  WT vs WT DM, \* $P<0.05$  WT DM BH4 vs WT DM. Comparisons between weeks 12 and 18 are included in Table III in the [Data Supplement](#). Scatterplots for these data are shown in Figure VI in the [Data Supplement](#). BH4 indicates tetrahydrobiopterin; DM, diabetes; and LV, left ventricular.



measurement of overall LV function was prevented in diabetic mGCH1-Tg.

LV mass was not different between groups at all time-points (Table II in the [Data Supplement](#)). Heart rate was lower in mGCH1-Tg compared with their WT littermates and did not change significantly after DM induction in either genotype (Table II in the [Data Supplement](#)).

We have previously shown that GCH1 overexpression and raised BH4 content significantly augment myocardial nNOS activity.<sup>8</sup> However, BH4 is also an antioxidant molecule and a cofactor for the formation of biogenic amines and serotonin.<sup>16</sup> To evaluate to which extent nNOS-derived NO was responsible for preserving LV function in the presence of DM, we performed these experiments in mGCH1-Tg in which nNOS was knocked out (Figure IIIG in the [Data Supplement](#)) using CRISPR-Cas9-mediated gene editing. As shown in Figure 2A through 2D and Table II in the [Data Supplement](#), the protective effect of myocardial GCH1 overexpression was lost in diabetic mice lacking nNOS, consistent with an essential role of this NOS isoform in mediating the cardioprotective effects of BH4 in the presence of DM.

We then tested whether LV function in diabetic WT mice could be restored by supplementing their diet with BH4 (200 mg/kg per day for 6 weeks, beginning at week 12 post-DM induction).

At 12 weeks and before BH4 was introduced in the protocol, both groups of WT DM mice showed reduced LV diastolic and systolic function (Figure 2E through 2H, Figure VI and Table III in the [Data Supplement](#)).

After 18 weeks, DM was associated with a significant impairment in LV diastolic and systolic function in WT fed normal chow, as indicated by a lower LV ejection fraction and a higher E/E' ratio (ratio between early mitral inflow velocity and mitral annular early diastolic velocity) and myocardial performance index (versus nondiabetic WT); by contrast, LV diastolic and systolic dysfunction was completely reversed in diabetic WT receiving BH4-supplementation (Figure 2E through 2H, Table III in the [Data Supplement](#)). BH4 supplementation increased cardiac BH4 levels ( $9.4 \pm 1.3$  versus  $5.8 \pm 0.8$  pmol/mg protein in WT group,  $P < 0.01$ ,  $n = 6-8$  per group), and NOS activity (% citrulline conversion:  $0.6 \pm 0.11$  versus  $0.2 \pm 0.05$ ,  $P < 0.01$ ,  $n = 6-8$  per group).

### Cardiomyocyte and $[Ca^{2+}]_i$ Handling Dysfunction Induced By DM Is Prevented By mGCH1 Overexpression and Reversed By Oral BH4 Supplementation

To establish whether the changes in LV function observed in WT diabetic mice in vivo reflect altered cardiomyocyte dynamics and  $Ca^{2+}$  handling, we undertook these measurements in field-stimulated LV myocytes (3 Hz,  $35 \pm 0.5^\circ\text{C}$ ; Figure 3A through 3F).

At 4 weeks after DM induction, cardiomyocyte relaxation and  $[Ca^{2+}]_i$  (intracellular calcium) transient characteristics were unaltered in both genotypes (Figure 3A through 3F). As reported previously,<sup>8</sup> there were significant genotype differences in the rate of myocyte relaxation and  $[Ca^{2+}]_i$  reuptake in nondiabetic mice, which were preserved in the presence of DM. By contrast, at 12 weeks post-DM, both relaxation velocity and the rate of decay of the  $[Ca^{2+}]_i$  transient were slower in diabetic WT myocytes compared to nondiabetic controls, whereas myocardial overexpression of GCH1 prevented the adverse effect of DM on both parameters (Figure 3C and 3F). Both at 4 and 12 weeks post-DM induction, cell shortening was significantly higher in Tg compared with WT, but the amplitude of the  $[Ca^{2+}]_i$  transient did not differ between groups (Figure 3B and 3E).

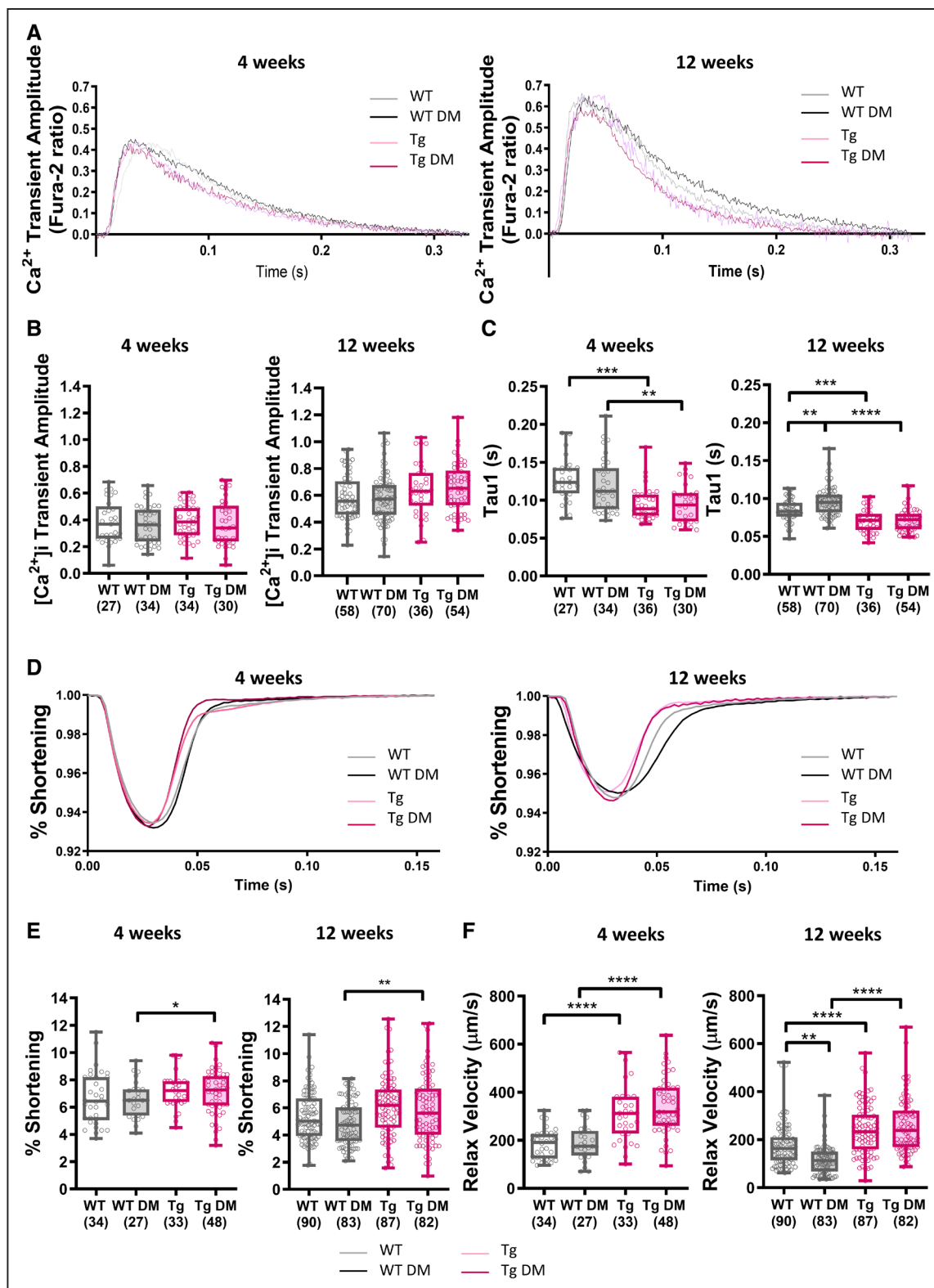
Oral BH4 supplementation for 6 weeks beginning at week 12 poststreptozotocin injection reversed the effect of DM on both relaxation velocity and rate of  $[Ca^{2+}]_i$  decay in isolated LV myocytes but did not affect cell shortening or the amplitude of the  $[Ca^{2+}]_i$  transient significantly (Figure 4A through 4E).

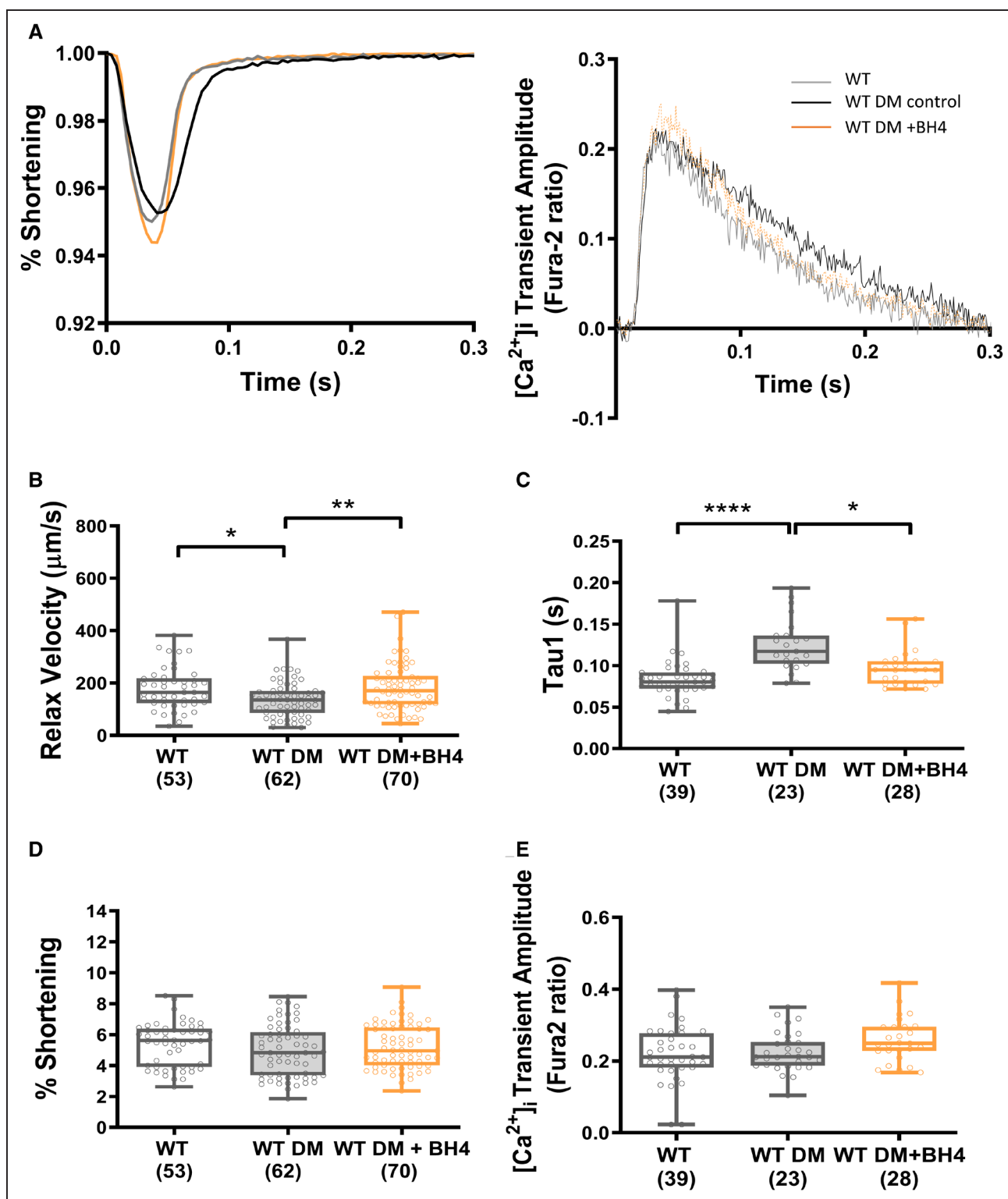
As reported previously,<sup>8</sup> PLB protein content was lower in the LV myocardium of mGCH1-Tg, which also showed a significantly higher PLB Ser16 phosphorylated fraction compared to WT (Figure 5A). There were no significant differences in the PLB Thr17 phosphorylated fraction or in SERCA2A (sarcoplasmic reticulum calcium ATPase 2A) between genotypes (Figure 5A and 5B).

DM did not affect the LV content or phosphorylation status of any of these proteins in either genotype but significantly increased the LV content of hydroxyproline in both WT and mGCH1-Tg and led to a comparable nonsignificant increase in collagen staining in both genotypes (Figure 5C and 5D).

### LV Dysfunction in DM Is Independent of BH4 Oxidation and Dysfunctional NOS Activity

In the aortic endothelium, BH4 and superoxide production in sham-injected mGCH1-Tg did not differ significantly from WT. DM induction lowered the ratio between BH4 and its oxidized products to a similar extent in both genotypes and increased superoxide production (Figure 6A and 6B). By contrast, in the LV myocardium of nondiabetic GCH1-Tg, BH4 content and NOS activity were significantly higher compared with WT (Figure 6C and 6D), in the absence of differences in the protein content of NOS isoforms between genotypes (Figure IB in the [Data Supplement](#)). Surprisingly, none of these parameters was altered at 12 weeks after DM induction in either genotype. Total and reduced myocardial glutathione was significantly elevated in mGCH1-Tg hearts compared to WT but, again, both measurements were unaltered by DM (Figure 6E). In line with these findings, neither total nor

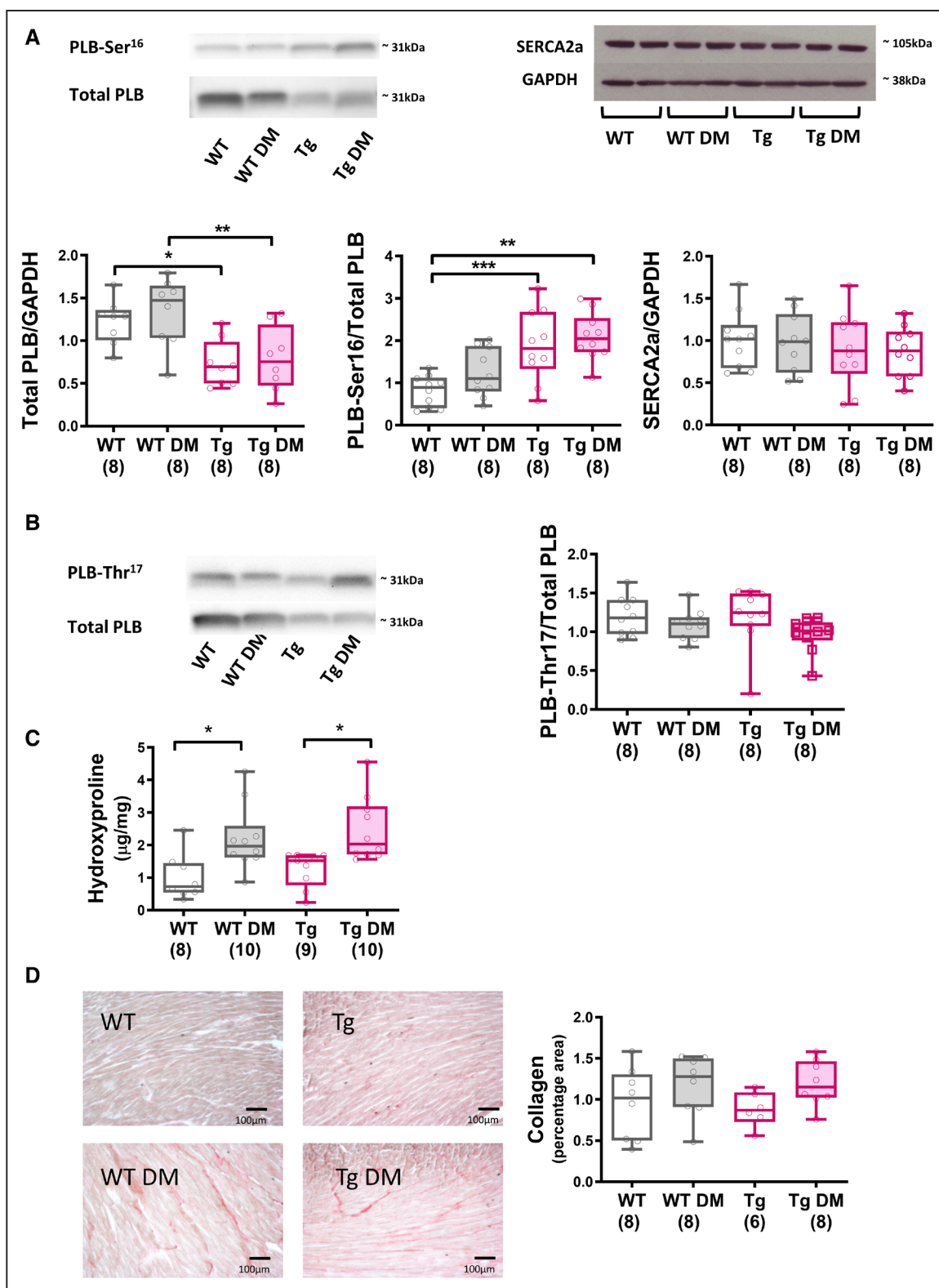




**Figure 4. Left ventricular myocyte dysfunction in diabetic mice is reversed by oral BH4 supplementation.**

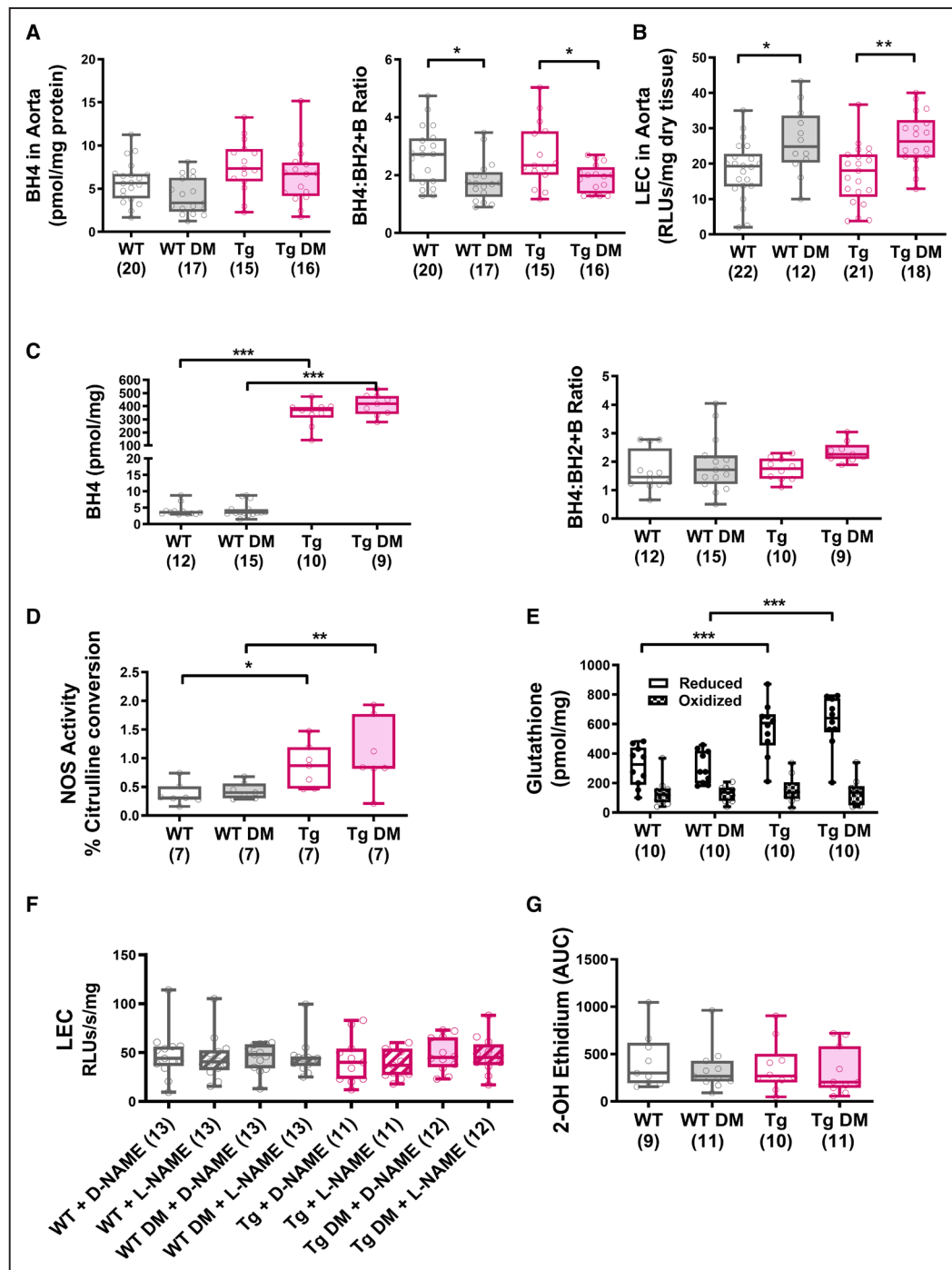
**A**, Representative intracellular calcium ( $[Ca^{2+}]_i$ ) transient and cell shortening traces from normoglycemic wild-type (WT) at 18 wk postsham injection and diabetic WT fed standard or BH4-supplemented chow from 12 to 18 wks post-DM induction. Average data for **(B)** relaxation velocity, **(C)** time constant of  $[Ca^{2+}]_i$  decay (Tau1), **(D)** cell shortening, and **(E)** amplitude of the  $[Ca^{2+}]_i$  transient. Normally distributed data are shown as means $\pm$ SEM in **D** and **E**, and were compared using 1-way ANOVA with Bonferroni correction. Non-normally distributed data in **B** and **C** (D'Agostino-Pearson test,  $P=0.032$  and  $0.049$ , respectively) are shown as median and interquartile range and were compared using Kruskal-Wallis 1-way ANOVA, followed by the Dunn test. n denotes number of cells. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\*\* $P<0.0001$ . BH4 indicates tetrahydrobiopterin; and DM, diabetes.





**Figure 5. Diabetes does not affect SERCA (sarcoplasmic reticulum calcium ATPase 2A) and PLB (phospholamban) protein level but increases left ventricular (LV) hydroxyproline content in both genotypes.**

**A** and **B**, Representative immunoblots and quantification of total and serine-16 phosphorylated PLB, SERCA2A and GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and total and threonine-17 phosphorylated PLB. **C**, LV hydroxyproline content and **(D)** LV collagen deposition quantified by polarized light microscopy in mice with myocardial overexpression of GCH1 (Tg) and wild-type (WT) at 12 wk after diabetes induction or sham injection. Normally distributed data in **A**, **C**, and **D** are shown as means±SEM and were compared using 2-way ANOVA with Bonferroni correction. Non-normally distributed data in **B** (D'Agostino-Pearson test,  $P=0.0007$ ) are shown as median and interquartile range and were compared using Kruskal-Wallis 1-way ANOVA, followed by the Dunn's test.  $n$  denotes number of hearts. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . DM indicates diabetes; and LV, left ventricular.



**Figure 6.** In contrast to the aortic endothelium, left ventricular (LV) superoxide production, tetrahydrobiopterin (BH4) oxidation, and NOS (nitric oxide synthase) activity are unchanged at 12 wk after diabetes induction.

**A**, Aortic BH4 level and the ratio between BH4 and its oxidized products ( $n=15-20$  per group) and **(B)** superoxide production detected by lucigenin-enhanced chemiluminescence (LEC;  $n=12-21$  per group) in wild-type mice (WT) and mice with myocardial overexpression of GCH1 (Tg) at 12 wk post-DM induction or sham injection. **C**, LV BH4 level and the ratio between BH4 and its oxidized products in the LV myocardium of WT and Tg ( $n=9-15$  hearts per group) at 12 wk post-DM induction or sham injection. **D**, LV NOS activity ( $n=7$  hearts per group) and **(E)** myocardial levels of reduced and oxidized glutathione in WT and Tg ( $n=10$  hearts per group) at 12 wk post-DM induction or sham injection. **F**, LV NOS-derived  $O_2^-$  production by lucigenin-enhanced chemiluminescence ( $n=11-13$  hearts per group, left). The inactive isomer D-NAME was used as a control; **(G)** LV superoxide levels were also determined by HPLC ( $n=9-11$  hearts per group). Normally distributed data in **A**, **B**, and **G** are shown as means  $\pm$  SEM and were compared using 2-way ANOVA with Bonferroni correction. Non-normally distributed data in **D**, **E**, and **F** (D'Agostino-Pearson test,  $P=0.011$ ,  $0.002$ , and  $0.005$ , respectively) were compared using Kruskal-Wallis 1-way ANOVA, followed by the Dunn test.  $n$  denotes number of aortas or hearts. L-NAME indicates N-nitro-L-arginine methyl ester. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . AUC indicates area under the curve; BH4, tetrahydrobiopterin; DM, diabetes; D-NAME, N-nitro-D-arginine methyl ester; HPLC, high performance liquid chromatography; L-NAME, N-nitro-L-arginine methyl ester; LV, left ventricular; NOS, nitric oxide synthase; and RLU, relative light units.

NOS-derived (ie, N-nitro-L-arginine methyl ester inhibitable) myocardial superoxide production was raised in diabetic mice from either genotype (Figure 6F and 6G).

As already mentioned, oral BH4 supplementation led to a significant increase in BH4 content and total NOS activity in the myocardium of diabetic WT but, even at 18 weeks post-DM induction, myocardial BH4 level ( $5.6 \pm 0.6$  versus  $5.8 \pm 0.8$  pmol/mg protein in WT group) and NOS activity were unchanged in WT DM mice (% citrulline conversion,  $0.3 \pm 0.08$  versus  $0.2 \pm 0.05$  in WT group), suggesting that, in contrast to the vascular endothelium, myocardial BH4 oxidation and NOS dysfunction are not an early hallmark of DM nor are they required to induce the cardiomyopathic phenotype.

To establish whether these unexpected findings were also pertinent to the myocardium of diabetic patients, we measured myocardial biopterins in samples of the right atrial appendage from 17 diabetic patients and 19 matched nondiabetic controls undergoing coronary revascularization (Table IV in the [Data Supplement](#)). LV ejection fraction was significantly lower in diabetic patients compared with their matched nondiabetic controls; nevertheless, myocardial BH4 content and the ratio between BH4 and its oxidized products were similar between groups and so was total and NOS-derived superoxide production (Figure II in the [Data Supplement](#)), indicating that, in agreement with our findings in diabetic mice, oxidant stress is not increased and NOS activity is not uncoupled in the myocardium of diabetic patients.

### mGCH1 Overexpression Preserves Myocardial Energetics in DM by Increasing Myocardial Glucose Uptake and Utilization via a NO/sGC/Protein Kinase G-Dependent Mechanism

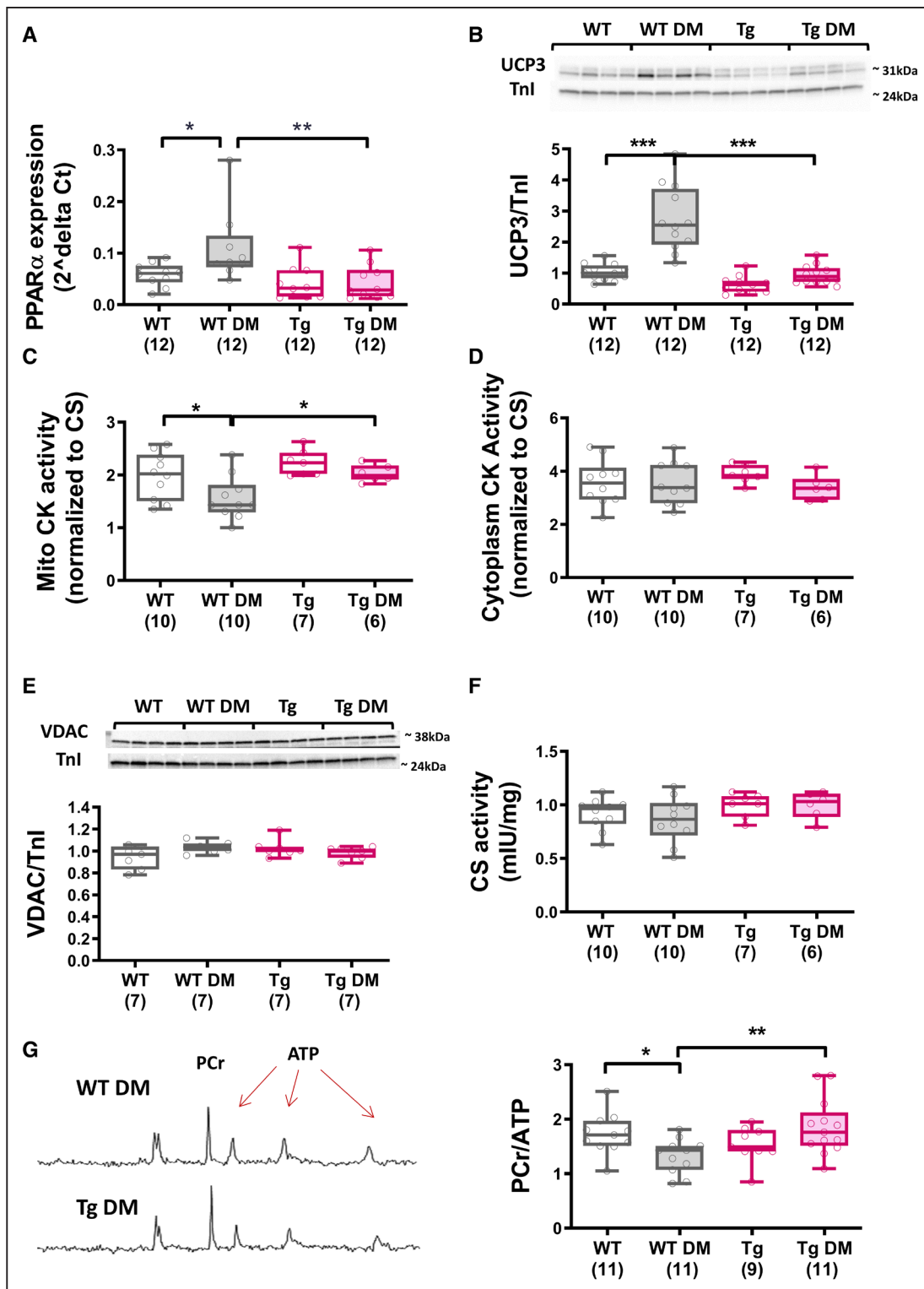
In the presence of DM, myocardial glucose transport and glycolysis are compromised and fatty acids (FA) become the exclusive source of ATP generation leading to an increase in oxygen consumption and a reduction in cardiac efficiency.<sup>17</sup> Accordingly, the myocardial expression of *Ppara* (peroxisome proliferator-activated receptor  $\alpha$ ) which promotes FA uptake and utilization, was significantly elevated in diabetic WT, but not in mGCH1-Tg, compared with sham-injected controls (Figure 7A). Similarly, the LV content of the mitochondrial UCP3 (uncoupling protein-3), a downstream target of *PPAR* $\alpha$ ,<sup>18</sup> was 50% higher in diabetic WT at 4 weeks post-DM induction (Figure IIIA in the [Data Supplement](#)) in keeping with an early switch to FA metabolism. However, the increase in myocardial UCP3 was much greater in WT by 12 weeks post-DM induction (Figure 7B) as was that of PDK4 (pyruvate dehydrogenase kinase 4; Figure IIIB in the [Data Supplement](#)), implying that myocardial metabolism was increasingly compromised as the duration of DM increased.

Diabetic WT hearts also displayed a reduction in the activity of mitochondrial CK (creatine kinase; Figure 7C), indicating reduced capacity for high-energy phosphate shuttling out of the mitochondria. There was no difference in cytoplasmic CK activity (Figure 7D) or in markers of mitochondrial cell density, as evaluated by citrate synthase activity and VDAC (mitochondrial voltage dependent ion channels) expression (Figure 7E and 7F). Despite similar levels of plasma glucose and insulin in both genotypes (Table I in the [Data Supplement](#)), *PPAR* $\alpha$  expression, UCP3 protein level, and mitochondrial CK activity were unaltered in the heart of diabetic mGCH1-Tg (Figure 7A through 7C).

To evaluate whether myocardial energetics differed between genotypes, we performed <sup>31</sup>phosphorous magnetic resonance spectroscopy in isolated perfused hearts. In sham-injected mice, there was no difference in the LV phosphocreatine-to-ATP ratio (PCr/ATP) between genotypes (Figure 7G). As observed in diabetic patients,<sup>19,20</sup> the PCr/ATP ratio was significantly reduced in the LV myocardium of diabetic WT mice but was unchanged in diabetic mGCH1-Tg (Figure 7G), suggesting that myocardial glucose uptake or utilization may be less affected by DM in mGCH1-Tg. Indeed, myocardial 2-deoxyglucose uptake was significantly higher in LV myocytes from diabetic mGCH1-Tg compared with diabetic WT (Figure 8A). Selective inhibition of the insulin-independent GLUT-1 (glucose transporter-1), with STF-31 (10  $\mu$ mol/L), significantly reduced 2-deoxyglucose uptake in diabetic mGCH1-Tg and abolished differences between genotypes (Figure 8A).

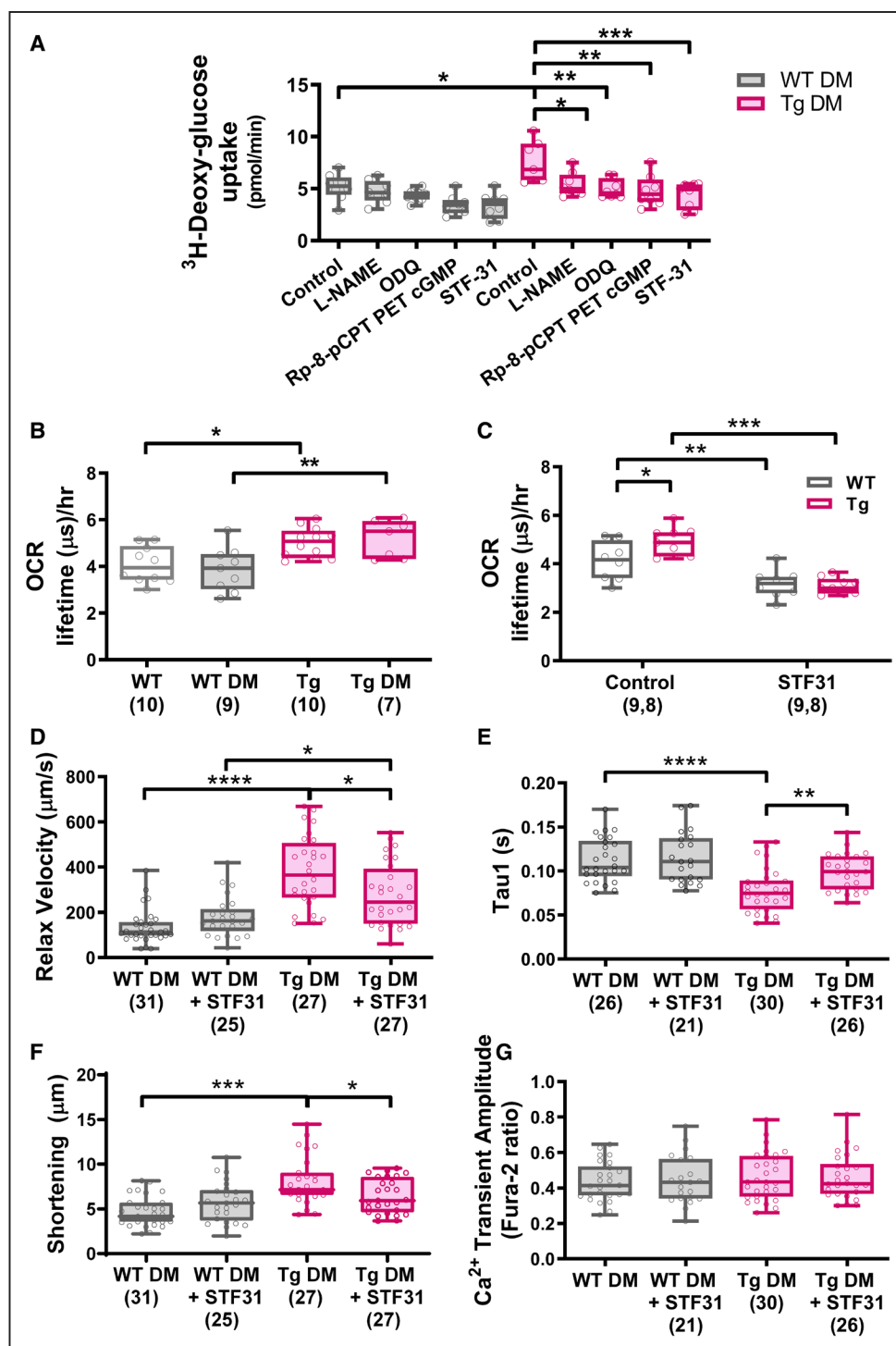
As shown in Figure III in the [Data Supplement](#), GLUT-1 expression and protein content were significantly higher in the myocardium of mGCH1-Tg mice whereas the insulin-dependent GLUT-4 did not differ between genotypes. To evaluate the relative impact of myocardial GLUT-1 content versus dynamic GLUT-1 regulation by NO, sGC, and PKG (protein kinase G) in GCH-Tg myocytes, we compared 2-deoxyglucose uptake in the presence of inhibitors of NOS (N-nitro-L-arginine methyl ester, 1 mmol/L), sGC (ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (10  $\mu$ mol/L), or PKG (8- (4-Chlorophenylthio)-  $\beta$ -phenyl- 1, N<sup>2</sup>-ethenoguanosine- 3', 5'-cyclic monophosphorothioate, Rp- isomer, Rp-8-pCPT-PET-cGMPs [10  $\mu$ mol/L]). All agents significantly inhibited glucose transport only in mGCH1-Tg and abolished the differences between genotypes (Figure 8A).

We determined whether higher myocardial glucose transport in mGCH1-Tg mice was accompanied by increased glucose oxidation by measuring oxygen consumption rate (OCR) in intact isolated LV myocytes from diabetic and sham-injected mice from both genotypes (Figure 8B). OCR was significantly higher in LV myocytes from mGCH1-Tg, irrespective of diabetic status. Preincubation of LV myocytes with the GLUT-1 inhibitor, STF-31 (10  $\mu$ mol/L), significantly decreased OCR both in WT



**Figure 7. mGCH1 (myocardial GTP-cyclohydrolase 1) overexpression prevents the decline in myocardial energetics in diabetic mice.**

**A**, Left ventricular (LV) gene expression of the *Ppara* (peroxisome proliferator-activated receptor  $\alpha$ ) (**B**) protein level of mitochondrial UCP3 (uncoupling protein-3) normalized to TnI (troponin I) content and (**C**) activity of the mitochondrial (Mito) and (**D**) cytoplasmic CK (creatine kinase) in wild-type mice (WT) and in mice with myocardial overexpression of GCH1 (Tg) mice at 12 wk after diabetes induction or sham injection. **E**, LV protein level of VDAC (mitochondrial voltage dependent ion channels) and (**F**) CS (citrate synthase) activity in the 4 groups. **G**, Representative LV traces from  $^{31}$ phosphorous magnetic resonance spectroscopy in isolated perfused hearts from diabetic WT and Tg mice, (**left**) and the average phosphocreatine-to-ATP ratio (PCr/ATP) ratio (**right**) in WT and Tg hearts at 12 wk after DM induction or sham injection. Data (all normally distributed) are shown as means $\pm$ SEM and were analyzed using 2-way ANOVA, with Bonferroni correction. n denotes number of hearts isolations in **A–C** and myocytes in **D–G**. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001. LV indicates left ventricular.



**Figure 8. mGCH1 (myocardial GTP-cyclohydrolase 1) overexpression (Tg) preserves myocardial function at 12 wk post-diabetes induction by increasing GLUT (glucose transporter)-1-dependent myocardial glucose uptake and utilization.**

**A**,  $^3\text{H}$ -Deoxy-glucose uptake in isolated LV myocytes from DM wild-type (WT) or mice with myocardial overexpression of GCH1 (Tg) under control conditions and in the presence of NOS (nitric oxide synthase), GLUT-1 (glucose transporter-1), sGC (soluble guanylate cyclase), or PKG (protein kinase G) inhibition with N-nitro-L-arginine methyl ester (L-NAME), STF-31, ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one), or Rp-8-pCPT-PET cGMP (8- (4- Chlorophenylthio)-  $\beta$ - phenyl- 1, N $^2$ - ethenoguanosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer), respectively. **B**, Oxygen consumption rate (OCR) in isolated LV myocytes from DM or sham-injected mice in the presence of 5 mmol/L glucose or **(C)** in isolated LV myocytes from normoglycemic mice in the presence of GLUT-1 inhibitor, STF-31 (10  $\mu\text{mol/L}$ ). Average data for **(D)** relaxation velocity, **(E)** time constant of  $[\text{Ca}^{2+}]$  (intracellular calcium) decay (Tau1), **(F)** cell shortening, and **(G)** the amplitude of the  $[\text{Ca}^{2+}]$  transient in LV myocytes from diabetic WT and Tg in the presence or absence of GLUT-1 inhibition (STF-31, 10  $\mu\text{mol/L}$ ) in the 4 groups. Data (all normally distributed) are shown as means $\pm$ SEM and were analyzed using 1-way ANOVA in **A**, and a 2-way ANOVA with Bonferroni correction in **B–G**. n denotes number of hearts. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , \*\*\*\* $P<0.0001$ . DM indicates diabetes; and LV, left ventricular.



and mGCH1-Tg myocytes and abolished the difference between genotypes (Figure 8C). OCR was also measured after blocking NOS (N-nitro-L-arginine methyl ester, 1 mmol/L) or PKG activity (Rp-8-pCPT-PET-cGMPS, 10  $\mu$ mol/L) and, again, under these conditions, OCR no longer differed between genotypes (Figure IV in the [Data Supplement](#)), indicating that mGCH1 overexpression dynamically increases myocardial glucose uptake via the GLUT-1 transporter in a NO/PKG-dependent manner. In agreement with these findings, incubation with the NO donor S-nitrosoglutathione (1 mmol/L) was associated with a 2-fold increase in the density of GLUT-1 on the cell surface of HEK (human embryonic kidney)-293 cells when compared with control cells. Inhibition of PKG significantly reduced GLUT-1 mobilization under these conditions (Figure IV in the [Data Supplement](#)).

The functional impact of genotype differences in glucose uptake via GLUT-1 was evaluated in LV myocytes from diabetic WT and mGCH1-Tg. As shown in Figure 8D through 8G, GLUT-1 inhibition with STF-31 had no effect in myocytes from diabetic WT mice but abolished the advantage conferred by mGCH1 overexpression in the presence of DM by slowing the rate of  $[Ca^{2+}]_i$  decay and reducing both relaxation velocity and cell shortening in diabetic mGCH1-Tg, again without affecting the amplitude of the  $[Ca^{2+}]_i$  transient.

## DISCUSSION

We have shown that increasing myocardial BH4 levels can prevent or reverse the LV dysfunction induced by DM and uncovered a novel mechanism by which BH4 exerts its beneficial effects in the myocardium via nNOS (see Graphical abstract). In contrast to previous reports where prevention of diabetic vascular endothelial dysfunction by Tie2-driven GCH1 overexpression was mediated by preservation of eNOS coupled activity,<sup>13</sup> we demonstrate that an increase in insulin-independent myocardial glucose uptake accounts for the maintenance of myocardial relaxation in diabetic mGCH1-Tg in the absence of NOS uncoupling.

DM-induced disruption of myocardial nitroso-redox balance has been reported in association with cardiac dysfunction and proposed as an important determinant of the diabetic cardiomyopathy phenotype.<sup>10,21</sup> Other studies, however, have shown no difference or a reduction in myocardial superoxide generation in diabetic mice.<sup>22,23</sup> We did not observe a significant difference in myocardial total superoxide production, glutathione or BH4 oxidation, and NOS activity in the myocardium of diabetic mice with LV dysfunction. Similar findings were obtained in samples of the right atrial myocardium from diabetic patients with reduced LV ejection fraction undergoing myocardial revascularization.

Although additional beneficial effects of mGCH1/BH4 overexpression on myocardial oxidative stress

may occur in more advanced stages of cardiomyopathy, as reported by Wu et al<sup>10</sup> and a subtle localized increase in mitochondrial reactive oxygen species cannot be categorically excluded by our experiments, our findings demonstrate that LV dysfunction associated with DM can occur in the absence of reduced NOS activity or PLB phosphorylation and that augmenting myocardial BH4 content prevents diabetic cardiomyopathy and increases glucose transport and utilization in the absence of BH4 oxidation.

Prevention of the diabetic myocardial phenotype by myocardial GCH1 overexpression was abolished by nNOS gene deletion or GLUT-1 inhibition, indicating that BH4 exerts its protective effect via an nNOS-mediated increase in glucose availability and utilization. A dynamic NO/PKG-dependent translocation of the insulin-independent GLUT-1 to the sarcolemmal membrane may underpin enhanced glucose transport in the presence of increased levels of myocardial BH4, in line with previous reports showing that NO induces GLUT-1 membrane translocation and a PKG-dependent increase in insulin-independent glucose uptake in rat ovarian cells.<sup>24</sup> Our data indicate that higher glucose uptake via GLUT-1 in cardiomyocytes from diabetic mGCH1-Tg is mediated by NO via cGMP-PKG signaling, as it is abolished by both sGC and PKG inhibition. Stimulation of myocardial PKG signaling by kinase oxidation<sup>25</sup> seems unlikely as, at difference with the aorta, we did not observe an increase in oxidative markers or superoxide production in diabetic hearts of either genotype.

DM lowered LV mitochondrial CK activity and PCr/ATP ratio, as assessed by <sup>31</sup>P-phosphorous cardiac magnetic resonance spectroscopy, in WT but not in mGCH1-Tg, where elevation of PPAR $\alpha$  and UCP3 was also prevented. Both proteins have been found to be raised in rodent models of type 1 and type 2 DM<sup>26</sup> where they support FA utilization, enhance the FA inhibition of glucose oxidation, and promote oxygen wasting for noncontractile purposes.<sup>27</sup> Impairment in myocardial energetics is increasingly regarded as an important player in the pathogenesis of diabetic cardiomyopathy and other conditions leading to LV dysfunction.<sup>28–30</sup> Limitations to effective energy supply to the heart can adversely impact the ATP-dependent  $Ca^{2+}$  reuptake during each cardiac cycle and impact diastolic function.<sup>31</sup> Indeed, impaired respiratory capacity precedes the development of LV dysfunction in type 1 DM,<sup>23</sup> and a reduced PCr/ATP ratio, proportional to the degree of LV diastolic dysfunction, has also been reported in patients with type 1 or type 2 DM.<sup>20,32,33</sup>

Although insulin and glycemic levels did not differ between 4 and 12 weeks of DM induction, impairment of LV function in WT mice was only observed at 12 weeks in association with significantly higher myocardial levels of UCP3 and PDK4, suggesting that DM caused a time-dependent substrate switch from glucose oxidation toward lactate production and increased fatty acid

metabolism leading to LV dysfunction. These findings are in keeping with the lack of effect of acute hyperglycemia on human myocardial function and the development of insulin resistance and reduced ability to oxidize fatty acids observed in type 1 DM over time.<sup>34,35</sup>

Our study has revealed important differences between the vascular endothelium and the myocardium in the response to hyperglycemia. Whereas LV dysfunction did not develop until 12 weeks following diabetes induction, endothelial dysfunction was already evident at 4 weeks. As reported previously,<sup>13</sup> endothelial dysfunction was associated with increased superoxide production, significant BH4 oxidation, and impaired NO signaling. These findings highlight the relative vulnerability of the endothelium to hyperglycemia but also indicate that the development of significant endothelial dysfunction, as measured in the aorta, is not sufficient to induce the cardiomyopathic phenotype in diabetic mice in the early stages of the disease. We have previously reported that myocardial spill-over of biopterins in mGCH1-Tg is minimal or absent and that BH4 levels are not increased in the heart nonmyocyte cellular component;<sup>8</sup> accordingly, there was no evidence of a protective effect on the vascular endothelium of diabetic mGCH1-Tg nor was there evidence of reduced myocardial fibrosis, despite which mGCH1 overexpression was still able to prevent the development of LV dysfunction.

Taken together, our findings open the possibility that BH4 supplementation may have beneficial effects in patients with diabetic cardiomyopathy. In a previous study in patients with ischemic heart disease, administration of a synthetic formulation of the active 6R-isomer of BH4, sapropterin, 2 to 6 weeks before coronary artery bypass surgery failed to enhance vascular NOS activity or improve endothelial-mediated vasodilatation.<sup>36</sup> Our current findings indicate that NOS recoupling may not be the best surrogate end-point for gauging the efficacy of BH4 supplementation, at least in myocardial disease states. In contrast with findings in human vessels,<sup>36</sup> BH4 content (both in absolute terms and relative to its oxidized products) increased in a dose-dependent manner in myocardial samples collected from patients treated with sapropterin (unpublished results), confirming that oral BH4 supplementation increases myocardial BH4 content in humans.

## Limitations

We did not investigate the mechanisms by which oral BH4 supplementation improved LV function in diabetic WT mice. In contrast with myocardial-specific GCH1 overexpression, oral BH4 supplementation is also known to improve endothelial function and prevent inflammation<sup>37,38</sup>; to which extent these additional effects contributed to recovering LV function in WT diabetic mice remains to be established. Investigation of the effect of BH4 supplementation in diabetic mGCH1-Tg lacking nNOS may provide important information on the

contribution of the extra-myocardial additional effect of BH4 of the diabetic cardiomyopathic phenotype.

Streptozotocin injection results in loss of pancreatic  $\beta$ -cell activity, leading to hyperglycemia secondary to insulin deficiency that resembles human type 1 DM. Although type II DM is more common, patients with type 1 DM have a high risk of developing heart failure that is dependent on glycemic control and associated with higher fatality<sup>3,39,40</sup> even in the absence of factors, such as hypertension and obesity, which may confound the pathophysiology of LV dysfunction associated with type II DM. To this end, rodent models streptozotocin-induced DM are well suited to evaluate the toxic effects of hyperglycemia and impaired glucose utilization in the myocardium. Male rodents are more susceptible to the diabetogenic action of streptozotocin than females; for this reason, our study was conducted in male mice. Although this is an important limitation, there is no evidence indicating that development of heart failure requiring hospitalization in patients with type I DM is different between men and women.<sup>3</sup> Although off-target adverse effects of streptozotocin delivered as a high-dose bolus have been reported, these can be minimized by the administration of low-dose streptozotocin delivered by the multiple low-dose streptozotocin injections protocol, used in our article.<sup>41</sup>

<sup>31</sup>Phosphorous magnetic resonance spectroscopy experiments showing a reduction in PCr/ATP ratio in diabetic WT hearts but not in Tg were conducted in the absence of FA supplementation, raising the possibility that provision of this source of energy may have attenuated the impact of DM on myocardial energetics. Nevertheless, significant genotype differences were observed in hearts exposed to the same conditions and similar changes in myocardial PCr/ATP ratio have been reported in vivo in patients with type 1 or type 2 DM.<sup>20,32,33</sup>

Finally, since at difference with the in vivo data, measurements in isolated myocytes could not be obtained sequentially in the same mice, we have not compared these data over time but between groups at 4 and 12 weeks. We think this is the appropriate way of comparing data obtained using this study design, as in an experiment of long duration variations due to equipment refurbishment and different batches of mice may affect longitudinal measurements but not cross-sectional comparisons. It should be noted that changes in unloaded cell shortening brought about by DM were not associated with differences in the amplitude of the  $[Ca^{2+}]_i$  transient, in keeping with data from human myocardial biopsies showing depressed cardiac myofilament function and  $Ca^{2+}$  responsiveness in the presence of diabetes.<sup>42</sup>

## Conclusions

Our work provides original insights into the management and prevention of early metabolic triggers of diabetic

cardiomyopathy and uncovers novel targets for the repurposing of BH4-based therapeutics.

## ARTICLE INFORMATION

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### Disclosures

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### Supplemental Materials

Expanded Methods

Online Figures I–VI

Online Table I–IV

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## REFERENCES

- Bommer C, Heesemann E, Sagalova V, Manne-Goeher J, Atun R, Bärnighausen T, Vollmer S. The global economic burden of diabetes in adults aged 20–79 years: a cost-of-illness study. *Lancet Diabetes Endocrinol*. 2017;5:423–430. doi: 10.1016/S2213-8587(17)30097-9
- Group IDFDA. Update of mortality attributable to diabetes for the IDF Diabetes Atlas: estimates for the year 2013. *Diabetes Res Clin Pract*. 2015;109:461–465.
- Lind M, Boucias I, Olsson M, Gudbjörnsdóttir S, Svensson AM, Rosengren A. Glycaemic control and incidence of heart failure in 20,985 patients with type 1 diabetes: an observational study. *Lancet*. 2011;378:140–146. doi: 10.1016/S0140-6736(11)60471-6
- Rawshani A, Rawshani A, Franzén S, Sattar N, Eliasson B, Svensson AM, Zethelius B, Miftaraj M, McGuire DK, Rosengren A, et al. Risk factors, mortality, and cardiovascular outcomes in patients with Type 2 diabetes. *N Engl J Med*. 2018;379:633–644. doi: 10.1056/NEJMoa1800256
- Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW, Grishman A. New type of cardiomyopathy associated with diabetic glomerulosclerosis. *Am J Cardiol*. 1972;30:595–602. doi: 10.1016/0002-9149(72)90595-4
- Jia G, Hill MA, Sowers JR. Diabetic cardiomyopathy: an update of mechanisms contributing to this clinical entity. *Circ Res*. 2018;122:624–638. doi: 10.1161/CIRCRESAHA.117.311586
- Zhang YH, Casadei B. Sub-cellular targeting of constitutive NOS in health and disease. *J Mol Cell Cardiol*. 2012;52:341–350. doi: 10.1016/j.jmcc.2011.09.006
- Carnicer R, Hale AB, Suffredini S, Liu X, Reilly S, Zhang MH, Surdo NC, Bendall JK, Crabtree MJ, Lim GB, et al. Cardiomyocyte GTP cyclohydrolase 1 and tetrahydrobiopterin increase NOS1 activity and accelerate myocardial relaxation. *Circ Res*. 2012;111:718–727. doi: 10.1161/CIRCRESAHA.112.274464
- Zhang YH, Zhang MH, Sears CE, Emanuel K, Redwood C, El-Armouche A, Kranias EG, Casadei B. Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. *Circ Res*. 2008;102:242–249. doi: 10.1161/CIRCRESAHA.107.164798
- Wu HE, Baumgardt SL, Fang J, Paterson M, Liu Y, Du J, Shi Y, Qiao S, Bosnjak ZJ, Warltire DC, et al. Cardiomyocyte GTP cyclohydrolase 1 protects the heart against diabetic cardiomyopathy. *Sci Rep*. 2016;6:27925. doi: 10.1038/srep27925
- Cassuto J, Dou H, Czikora I, Szabo A, Patel VS, Kamath V, Belin de Chantemele E, Feher A, Romero MJ, Bagi Z. Peroxynitrite disrupts endothelial caveolae leading to eNOS uncoupling and diminished flow-mediated dilation in coronary arterioles of diabetic patients. *Diabetes*. 2014;63:1381–1393. doi: 10.2337/db13-0577
- Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation*. 2002;105:1656–1662. doi: 10.1161/01.cir.0000012748.58444.08
- Alp NJ, Mussa S, Khoo J, Cai S, Guzik T, Jefferson A, Goh N, Rockett KA, Channon KM. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase 1 overexpression. *J Clin Invest*. 2003;112:725–735. doi: 10.1172/JCI17786
- Silberman GA, Fan TH, Liu H, Jiao Z, Xiao HD, Lovelock JD, Boulden BM, Widder J, Fredd S, Bernstein KE, et al. Uncoupled cardiac nitric oxide synthase mediates diastolic dysfunction. *Circulation*. 2010;121:519–528. doi: 10.1161/CIRCULATIONAHA.109.883777
- Idigo WO, Reilly S, Zhang MH, Zhang YH, Jayaram R, Carnicer R, Crabtree MJ, Balligand JL, Casadei B. Regulation of endothelial nitric-oxide synthase (NOS) S-glutathionylation by neuronal NOS: evidence of a functional interaction between myocardial constitutive NOS isoforms. *J Biol Chem*. 2012;287:43665–43673. doi: 10.1074/jbc.M112.412031
- Werner ER, Blau N, Thöny B. Tetrahydrobiopterin: biochemistry and pathophysiology. *Biochem J*. 2011;438:397–414. doi: 10.1042/BJ20110293
- Buchanan J, Mazumder PK, Hu P, Chakrabarti G, Roberts MW, Yun UJ, Cooksey RC, Litwin SE, Abel ED. Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology*. 2005;146:5341–5349. doi: 10.1210/en.2005-0938
- Young ME, Patil S, Ying J, Depe C, Ahuja HS, Shipley GL, Stepkowski SM, Davies PJ, Taegtmeyer H. Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. *FASEB J*. 2001;15:833–845. doi: 10.1096/fj.00-0351.com
- Levelt E, Rodgers CT, Clarke WT, Mahmood M, Ariga R, Francis JM, Liu A, Wijesurendra RS, Dass S, Sabharwal N, et al. Cardiac energetics, oxygenation, and perfusion during increased workload in patients with type 2 diabetes mellitus. *Eur Heart J*. 2016;37:3461–3469. doi: 10.1093/eurheartj/ehv442
- Scheuermann-Freestone M, Madsen PL, Manners D, Blamire AM, Buckingham RE, Styles P, Radda GK, Neubauer S, Clarke K. Abnormal cardiac and skeletal muscle energy metabolism in patients with type 2 diabetes. *Circulation*. 2003;107:3040–3046. doi: 10.1161/01.CIR.0000072789.89096.10
- Ye G, Metreveli NS, Donthi RV, Xia S, Xu M, Carlson EC, Epstein PN. Catalase protects cardiomyocyte function in models of type 1 and type 2 diabetes. *Diabetes*. 2004;53:1336–1343. doi: 10.2337/diabetes.53.5.1336
- Dugan LL, You YH, Ali SS, Diamond-Stanic M, Miyamoto S, DeCleva AE, Andreyev A, Quach T, Ly S, Shekhtman G, et al. AMPK dysregulation promotes diabetes-related reduction of superoxide and mitochondrial function. *J Clin Invest*. 2013;123:4888–4899. doi: 10.1172/JCI66218
- Bugger H, Boudina S, Hu XX, Tuinei J, Zaha VG, Theobald HA, Yun UJ, McQueen AP, Wayment B, Litwin SE, et al. Type 1 diabetic akita mouse hearts are insulin sensitive but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. *Diabetes*. 2008;57:2924–2932. doi: 10.2337/db08-0079
- Tian Y, Heng D, Xu K, Liu W, Weng X, Hu X, Zhang C. cGMP/PKG-I pathway-mediated GLUT1/4 regulation by NO in female rat granulosa cells. *Endocrinology*. 2018;159:1147–1158. doi: 10.1210/en.2017-00863
- Burgoyne JR, Madhani M, Cuervo F, Charles RL, Brennan JP, Schröder E, Browning DD, Eaton P. Cysteine redox sensor in PKGIa enables oxidant-induced activation. *Science*. 2007;317:1393–1397. doi: 10.1126/science.1144318
- Boudina S, Sena S, Theobald H, Sheng X, Wright JJ, Hu XX, Aziz S, Johnson JI, Bugger H, Zaha VG, Abel ED. Mitochondrial energetics in the heart in obesity-related diabetes: direct evidence for increased uncoupled respiration and activation of uncoupling proteins. *Diabetes*. 2007;56:2457–2466.
- Boehm EA, Jones BE, Radda GK, Veech RL, Clarke K. Increased uncoupling proteins and decreased efficiency in palmitate-perfused hyperthyroid rat heart. *Am J Physiol Heart Circ Physiol*. 2001;280:H977–H983. doi: 10.1152/ajpheart.2001.280.3.H977
- Beadle RM, Williams LK, Kuehl M, Bowater S, Abozguia K, Leyva F, Yousef Z, Wagenmakers AJ, Thies F, Horowitz J, et al. Improvement in cardiac

- energetics by perhexiline in heart failure due to dilated cardiomyopathy. *JACC Heart Fail*. 2015;3:202–211. doi: 10.1016/j.jchf.2014.09.009
29. Crilley JG, Boehm EA, Blair E, Rajagopalan B, Blamire AM, Styles P, McKenna WJ, Ostman-Smith I, Clarke K, Watkins H. Hypertrophic cardiomyopathy due to sarcomeric gene mutations is characterized by impaired energy metabolism irrespective of the degree of hypertrophy. *J Am Coll Cardiol*. 2003;41:1776–1782. doi: 10.1016/s0735-1097(02)03009-7
  30. Shivu GN, Phan TT, Abozguia K, Ahmed I, Wagenmakers A, Henning A, Narendran P, Stevens M, Frenneaux M. Relationship between coronary microvascular dysfunction and cardiac energetics impairment in type 1 diabetes mellitus. *Circulation*. 2010;121:1209–1215. doi: 10.1161/CIRCULATIONAHA.109.873273
  31. Zarain-Herzberg A, García-Rivas G, Estrada-Avilés R. Regulation of SERCA pumps expression in diabetes. *Cell Calcium*. 2014;56:302–310. doi: 10.1016/j.ceca.2014.09.005
  32. Metzler B, Schocke MF, Steinboeck P, Wolf C, Judmaier W, Lechleitner M, Lukas P, Pachinger O. Decreased high-energy phosphate ratios in the myocardium of men with diabetes mellitus type I. *J Cardiovasc Magn Reson*. 2002;4:493–502. doi: 10.1081/jcmr-120016387
  33. Diamant M, Lamb HJ, Groeneveld Y, Endert EL, Smit JW, Bax JJ, Romijn JA, de Roos A, Radder JK. Diastolic dysfunction is associated with altered myocardial metabolism in asymptomatic normotensive patients with well-controlled type 2 diabetes mellitus. *J Am Coll Cardiol*. 2003;42:328–335. doi: 10.1016/s0735-1097(03)00625-9
  34. Hammer S, Jonker JT, Lamb HJ, van der Meer RW, Zondag W, Sepers JM, de Roos A, Smit JW, Romijn JA. Short-term hyperglycemic dysregulation in patients with type 1 diabetes does not change myocardial triglyceride content or myocardial function. *Diabetes Care*. 2008;31:1613–1614. doi: 10.2337/dc08-0513
  35. An D, Rodrigues B. Role of changes in cardiac metabolism in development of diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol*. 2006;291:H1489–H1506. doi: 10.1152/ajpheart.00278.2006
  36. Cunnington C, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM, Antoniadis C, Margaritis M, Lee R, Cerrato R, et al. Systemic and vascular oxidation limits the efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease. *Circulation*. 2012;125:1356–1366. doi: 10.1161/CIRCULATIONAHA.111.038919
  37. Pannirselvam M, Simon V, Verma S, Anderson T, Triggie CR. Chronic oral supplementation with sepiapterin prevents endothelial dysfunction and oxidative stress in small mesenteric arteries from diabetic (db/db) mice. *Br J Pharmacol*. 2003;140:701–706. doi: 10.1038/sj.bjp.0705476
  38. Hashimoto T, Sivakumaran V, Carnicer R, Zhu G, Hahn VS, Bedja D, Recalde A, Duglan D, Channon KM, Casadei B, et al. Tetrahydrobiopterin protects against hypertrophic heart disease independent of myocardial nitric oxide synthase coupling. *J Am Heart Assoc*. 2016;5:e003208. doi: 10.1161/JAHA.116.003208
  39. McAllister DA, Read SH, Kerssens J, Livingstone S, McGurnaghan S, Jhund P, Petrie J, Sattar N, Fischbacher C, Kristensen SL, et al. Incidence of hospitalization for heart failure and case-fatality among 3.25 million people with and without diabetes mellitus. *Circulation*. 2018;138:2774–2786. doi: 10.1161/CIRCULATIONAHA.118.034986
  40. Rosengren A, Vestberg D, Svensson AM, Kosiborod M, Clements M, Rawshani A, Pivodic A, Gudbjörnsdóttir S, Lind M. Long-term excess risk of heart failure in people with type 1 diabetes: a prospective case-control study. *Lancet Diabetes Endocrinol*. 2015;3:876–885. doi: 10.1016/S2213-8587(15)00292-2
  41. Brosius FC 3rd, Alpers CE, Bottinger EP, Breyer MD, Coffman TM, Gurley SB, Harris RC, Kakoki M, Kretzler M, Leiter EH, et al. Animal Models of Diabetic Complications Consortium. Mouse models of diabetic nephropathy. *J Am Soc Nephrol*. 2009;20:2503–2512. doi: 10.1681/ASN.2009070721
  42. Jweied EE, McKinney RD, Walker LA, Brodsky I, Geha AS, Massad MG, Buttrick PM, de Tombe PP. Depressed cardiac myofilament function in human diabetes mellitus. *Am J Physiol Heart Circ Physiol*. 2005;289:H2478–H2483. doi: 10.1152/ajpheart.00638.2005
  43. Naressi A, Couturier C, Castang I, de Beer R, Graveron-Demilly D. Java-based graphical user interface for MRUI, a software package for quantitation of in vivo/medical magnetic resonance spectroscopy signals. *Comput Biol Med*. 2001;31:269–286. doi: 10.1016/s0010-4825(01)00006-3
  44. Reilly SN, Jayaram R, Nahar K, Antoniadis C, Verheule S, Channon KM, Alp NJ, Schotten U, Casadei B. Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins. *Circulation*. 2011;124:1107–1117. doi: 10.1161/CIRCULATIONAHA.111.029223
  45. Readnower RD, Brainard RE, Hill BG, Jones SP. Standardized bioenergetic profiling of adult mouse cardiomyocytes. *Physiol Genomics*. 2012;44:1208–1213. doi: 10.1152/physiolgenomics.00129.2012