Tetrahydrobiopterin (BH4) is an essential cofactor of nitric oxide synthases (NOS). Oral BH4 supplementation preserves cardiac function in animal models of cardiac disease; however, the mechanisms underlying these findings are not completely understood. 

Objective: To study the effect of myocardial transgenic overexpression of the rate-limiting enzyme in BH4 biosynthesis, GTP cyclohydrolase 1 (GCH1), on NOS activity, myocardial function, and Ca\(^{2+}\) handling.

Methods and Results: GCH1 overexpression significantly increased the biopterins level in left ventricular (LV) myocytes but not in the nonmyocyte component of the LV myocardium or in plasma. The ratio between BH4 and its oxidized products was lower in mGCH1-Tg, indicating that a large proportion of the myocardial biopterin pool was oxidized; nevertheless, myocardial NOS1 activity was increased in mGCH1-Tg, and superoxide release was significantly reduced. Isolated hearts and field-stimulated LV myocytes (3 Hz, 35°C) overexpressing GCH1 showed a faster relaxation and a PKA-mediated increase in the PLB Ser\(^{16}\) phosphorylated fraction and in the rate of decay of the [Ca\(^{2+}\)]\(_i\) transient. RyR2 S-nitrosylation and diastolic Ca\(^{2+}\) leak were larger in mGCH1-Tg and I\(_{ca}\) density was lower; nevertheless the amplitude of the [Ca\(^{2+}\)]\(_i\) transient and contraction did not differ between genotypes, because of an increase in the SR fractional release of Ca\(^{2+}\) in mGCH1-Tg myocytes. Xanthine oxidoreductase inhibition abolished the difference in superoxide production but did not affect myocardial function in either group. By contrast, NOS1 inhibition abolished the differences in I\(_{ca}\) density, Ser\(^{16}\) PLB phosphorylation, [Ca\(^{2+}\)]\(_i\) decay, and myocardial relaxation between genotypes.

Conclusions: Myocardial GCH1 activity and intracellular BH4 are a limiting factor for constitutive NOS1 and SERCA2A activity in the healthy myocardium. Our findings suggest that GCH1 may be a valuable target for the treatment of LV diastolic dysfunction.

Key Words: tetrahydrobiopterin, neuronal NOS, nitric oxide, relaxation, phospholamban.

Tetrahydrobiopterin (BH4) is an essential cofactor of all nitric oxide synthases (NOS). Although its role remains incompletely defined, BH4 is thought to facilitate electron transfer from the NOS’ reductase domain, maintain the heme prosthetic group in its redox active form, stimulate NO synthesis, and promote the formation of active NOS homodimers. Decreased BH4 bioavailability can lead to NOS uncoupling; a phenomenon whereby reduction of oxygen by NOS is uncoupled from L-arginine oxidation, resulting in the formation of superoxide rather than nitric oxide (NO). A reduction in BH4 bioavailability has been demonstrated in a number of vascular disease states and, more recently, also in the myocardium in the presence of left ventricular (LV) pressure overload, severe ischemia, hyperglycemia, and atrial fibrillation. Under these conditions, supplementation of BH4 has been shown to prevent adverse remodeling and improve cardiac function; however, whether this is due to a specific increase in myocardial BH4 and NO bioavailability or to the anti-inflammatory and antioxidant effects of BH4 is still a matter of debate. Indeed, to what extent BH4 can be transported across the plasma membrane remains uncertain.

To date, investigations have largely focused on the role of BH4 on endothelial NOS3 activity whereas the regulation of myocardial constitutive NOS1 activity by BH4 has received comparatively little attention. Reduced availability of

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718
NOS1-derived NO has been shown to affect basal and β-adrenergic contraction,\textsuperscript{14,15} impair myocardial relaxation by decreasing the rate of Ca\textsuperscript{2+} reuptake in the sarcoplasmic reticulum (SR),\textsuperscript{7,16–18} and exacerbate adverse LV remodeling after myocardial infarction.\textsuperscript{16,19} Whether myocardial BH4 availability is a limiting factor for NOS1 activity and the NOS1-dependent regulation of myocardial function and Ca\textsuperscript{2+}-handling remains to be assessed. To address these issues, we generated a mouse overexpressing the rate-limiting enzyme in BH4 biosynthesis, GTP cyclohydrolase-I (GCH1) under the control of the α-myosin heavy chain (α-MHC) promoter, and investigated the impact of this intervention on the myocardial NO-redox balance, contractile function, and Ca\textsuperscript{2+} fluxes.

Methods

All studies were conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Transgenic male animals expressing the human GCH1 gene, incorporating a human influenza hemagglutinin (HA) tag on the N-terminus, under the regulation of the α-MHC promoter, were generated and bred in the Functional Genetics Facility of the University of Oxford. A diagram of the DNA construct is shown in Online Figure I. GCH1 transgenic mice (mGCH1-Tg) and their wild-type (WT) littermates were housed in a temperature-controlled environment (20°C–22°C) with a 12-hour light–dark cycle. Myocardial biotinors, NOS, and GCH1 activity were assessed by high-performance liquid chromatography (HPLC) and superoxide production by lucigenin (5 μmol/L)-enhanced chemiluminescence. Left ventricular (LV) function was evaluated in Langendorff-perfused hearts, whereas cellular shortening, [Ca\textsuperscript{2+}]\textsubscript{i}, SR content (estimated by the amplitude of the caffeine-induced [Ca\textsuperscript{2+}]\textsubscript{i} transient and the integral of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, NCX), I\textsubscript{ca} density, and intracellular Ca\textsuperscript{2+} buffering capacity were measured in field-stimulated (3 Hz, 35°C, 1.4 mmol/L [Ca\textsuperscript{2+}]\textsubscript{i}) or patch-clamped LV myocytes. The diastolic Ca\textsuperscript{2+} leak through the Ryanodine receptor (RyR2) was estimated, as described by Shannon et al.\textsuperscript{20}

Details of the methods are included in the Online Supplement.

Results

GCH1 Overexpression Increases Myocardial GCH1 Activity and Biotinor Levels

Heterozygous mGCH1-Tg mice were viable and fertile and did not exhibit any difference in appearance, behavior, body weight, cardiac mass, or myocyte size when compared with their WT littermates (Table). Human GCH1 protein was significantly increased in the myocardium of the mGCH1-Tg mouse, but was absent from other tissues, such as the liver and skeletal muscle (Figure 1A). These results were confirmed using an antibody raised against the HA tag of the transgenically expressed human GCH1 protein. The murine constitutive GCH1 level remained unchanged in all the examined tissues (Figure 1A).

The transgenically expressed human GCH1 protein formed functional enzymes, as shown by the significantly higher myocardial GCH1 activity (Figure 1B) and tissue concentrations of BH4 and total biotinors (including 7, 8-dihydrobiopterin, BH2, and fully oxidized biotinor, B) in mGCH1-Tg mice (Figure 1C). By contrast, biotinor levels in plasma, skeletal muscle, and liver were not different from WT (Figure 1E).

Immunostaining showed a similar intracellular localization of the native and transgenically expressed HA-tagged GCH1 enzyme in murine LV myocytes (Figure 2A). Similarly, tetracycline-induced expression of the GCH1-HA fusion protein in 3T3 fibroblasts (GCH-Tet) at a level comparable to that of native GCH1 in sEnd.1 endothelial cells resulted in similar enzyme’s activity and BH4 content (Figure 2B), indicating that the HA tag did not alter GCH1 activity and intracellular localization.

Whether biotinors can be transported across the sarcolemmal membrane remains uncertain. To evaluate whether increased myocardial GCH1 activity leads to an increase in biotinors content in the neighboring tissue, we compared biotinors concentration in the cardiomyocyte-enriched and nonmyocyte fractions of cells enzymatically isolated from mGCH1-Tg hearts. As shown in Figure 2C, we detected a very small increase in BH4 and total biotinors in the nonmyocyte fraction of mGCH1-Tg hearts, indicating either low-level transport of biotinors from myocytes to neighboring cells or, more likely, incomplete purification of the noncardiomyocyte fraction. By contrast, oral supplementation of BH4 (5 mg/d for 7 days\textsuperscript{2} in C57BL/6 mice resulted in a significant increase in BH4 (Figure 2D) and the BH4:BH2+B ratio (0.80±0.04 versus 0.58±0.07 in mice treated with the enzymatically inactive analog of BH4 tetrahydrobiopterin, H4N; n=6 and 5 hearts, P<0.05) in purified LV myocytes and in a borderline rise in NOS activity (% L-Citrulline conversion: 0.47±0.07 versus 0.30±0.05 in

### Table. Heart Weight and LV Myocyte Dimensions

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Tg</th>
<th>P Value</th>
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<tbody>
<tr>
<td>BW (g)</td>
<td>27.7±0.7</td>
<td>27.3±0.5</td>
<td>ns</td>
</tr>
<tr>
<td>HW (g)</td>
<td>0.23±0.11</td>
<td>0.22±0.09</td>
<td>ns</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>8.4±0.30</td>
<td>8.04±0.31</td>
<td>ns</td>
</tr>
<tr>
<td>Cell length (μm)</td>
<td>104±1</td>
<td>106±1</td>
<td>ns</td>
</tr>
<tr>
<td>Cell width (μm)</td>
<td>20.8±0.4</td>
<td>20.3±0.4</td>
<td>ns</td>
</tr>
<tr>
<td>Cell area (μm\textsuperscript{2})</td>
<td>2176±54</td>
<td>2186±57</td>
<td>ns</td>
</tr>
</tbody>
</table>

BW, body weight; HW, heart weight; WT, wild type; Tg, mGCH1 transgenic mice (n=190 left ventricle [LV] myocytes from WT and n=174 LV myocytes from mGCH1-Tg mice, 8 and 7 hearts, respectively); ns, not significant.

Non-standard Abbreviations and Acronyms

- α-MHC: alpha-myosin heavy chain
- BH4: tetrahydrobiopterin
- BH2: 7, 8-dihydrobiopterin
- HA: haemagglutinin
- mGCH: myocardial guanosine triphosphate cyclohydrolase
- L-NAME: L-nitroarginine methyl ester
- NCX: sodium–calcium exchanger
- PKA: protein kinase A
- PLB: phospholamban
- RyR2: Ryanodine receptor calcium release channel
- SERCA2A: sarcoplasmic reticulum calcium adenosine triphosphatase
- SMTC: S-methyl-L-thiocitrulline acetate salt
- SR: sarcoplasmic reticulum
- Tg: transgenic
- WT: wild-type littermates
- XOR: xanthine oxidoreductase

Carnicer et al. BH4 Regulates Myocardial NOS1 and EC Coupling
H4N treated mice; \(n=6\) and 5 hearts, \(P=0.065\). Thus, whereas oral supplementation leads to an increase in LV myocytes BH4, the BH4 synthetized by LV myocytes is not exported to neighboring tissues.

GCH1 Overexpression Increases Myocardial NOS1 Activity and Reduces Superoxide Production

NOS activity is thought to be dependent on both BH4 bioavailability and the ratio between BH4 and its oxidized products (BH2 + B) decreased in mGCH1-Tg hearts (\(P<0.02\), D). The BH4 and biopterin content in plasma, muscle, and liver tissue did not differ between genotypes (E). HA indicates haemagglutinin; mGCH1, myocardial guanosine triphosphate cyclohydrolase; Tg, transgenic; BH4, tetrahydrobiopterin; BH2, 7, 8-dihydrobiopterin.

**Figure 1.** Myocardial GCH1 overexpression causes a tissue-specific increase in GCH1 activity and biopterin content. Anti-human GCH1 and HA tag antibodies showed a myocardial-specific overexpression of hGCH1 in mGCH1-Tg mice. Murine native GCH1 did not differ between genotypes (A). Both GCH activity (by HPLC, \(n=8\) and 12 hearts per genotype; *** \(P<0.0001\), B) and biopterin level (\(n=9\) hearts per genotype; ** \(P<0.001\), C) were increased in mGCH1-Tg hearts, but the ratio between BH4 and its oxidized products (BH2 + B) decreased in mGCH1-Tg hearts (\(P<0.02\), D). The BH4 and biopterin content in plasma, muscle, and liver tissue did not differ between genotypes (E). HA indicates haemagglutinin; mGCH1, myocardial guanosine triphosphate cyclohydrolase; Tg, transgenic; BH4, tetrahydrobiopterin; BH2, 7, 8-dihydrobiopterin.

**Figure 2.** The HA tag does not alter GCH1 localization or activity and the effect of oral BH4 supplementation on cardiomyocyte biopterin content and NOS activity. A. Immunolocalization of native (left side, in red) and human HA-tagged GCH1 (middle, in red) in WT and mGCH1-Tg LV myocytes, respectively. The right side shows myocytes exposed to Alexa Fluor 555 conjugate only. Cells nuclei were counterstained with TO-PRO®-3 (in blue). Scale bar: 10 \(\mu\)m. B. Tetracycline-induced expression of human GCH-HA protein (hGCH1) in 3T3 fibroblasts (GCH-tet) at a comparable level to native GCH1 in sEnd.1 murine endothelial cells results in similar GCH1 activity and intracellular BH4 levels. C. The increase in biopterins in mGCH1-Tg mice was largely limited to the LV myocytes fraction (\(n=6\) and 5 hearts, *** \(P<0.001\), ** \(P<0.01\), and * \(P<0.05\)). D. BH4 oral supplementation (5 mg/d) augmented the BH4 and total biopterins content in LV myocytes from C57BL/6 mice (versus H4N-supplemented mice, \(n=5\) and 6 hearts, respectively, *** \(P<0.001\) and ** \(P<0.01\)). HA indicates haemagglutinin; BH4, tetrahydrobiopterin; NOS, nitric oxide synthase; WT, wild-type; mGCH1, myocardial guanosine triphosphate cyclohydrolase; Tg, transgenic; LV, left ventricle.
products. In mGCH1-Tg mice, myocardial BH4 was increased but so were the levels of its oxidized products (Figure 1C), resulting in a reduction in the BH4:BH2 + B ratio when compared with WT mice (Figure 1D). Because the $K_m$ of BH4 and of its oxidized product BH2 for NOS is similar (but only BH4 can serve as NOS cofactor), excess BH2 may displace BH4 from NOS, leading to NOS uncoupling and increased superoxide production. To test this possibility, we evaluated myocardial NOS activity and superoxide production in mGCH1-Tg mice and their WT littermates. As shown in Figure 3A, myocardial transgenic expression of GCH1 was associated with a significant increase in total NOS activity in LV homogenates. The SMTC-inhibitable (ie, NOS1-attributable) component of NOS activity in mGCH1-Tg hearts accounted for approximately 70% of the total, and was again significantly higher in mGCH1-Tg hearts than in WT hearts, whereas NOS3 activity did not change appreciably. Immunoblotting showed that myocardial constitutive and inducible NOSs did not differ between genotypes (Figure 3B).

Superoxide production was significantly reduced in LV homogenates from mGCH1-Tg mice (in RLU/mg protein/s, 64.3 ± 6.7 in WT versus 47.6 ± 4.6 in mGCH1-Tg mice, $P < 0.05$, $n = 14$ hearts per group); incubation with L-NAME did not decrease superoxide release in LV homogenates from mGCH1-Tg mice (versus D-NAME) nor affect the difference in superoxide production between genotypes (Figure 3C), indicating that NOS activity is not uncoupled in the mGCH1-Tg myocardium (despite the decrease in the BH4:BH2 + B ratio), and the reduction in superoxide release in mGCH1-Tg mice is not secondary to an NO-mediated inhibition of oxidases activity or the direct scavenging of superoxide by increased NO production.

**Figure 3. NOS activity is increased and superoxide production is reduced in mGCH1-Tg hearts.** A. Total NOS activity (L-NAME-inhibitable) was significantly increased in the myocardium of mGCH1-Tg mice ($n = 8$ and 7 hearts per genotype, ***$P < 0.0001$) and accounted for by an increase in the SMTC-inhibitable, NOS1-dependent fraction (**$P < 0.0001$). By contrast, NOS3 activity (estimated by subtracting the SMTC-inhibitable fraction from the total NOS activity) was unchanged. B. Myocardial NOS isoforms did not differ between genotypes ($n = 6–7$ hearts per genotype). C. Superoxide production was significantly lower in mGCH1-Tg hearts. Incubation with L-NAME (1 mmol/L versus D-NAME) did not affect myocardial superoxide production in either group (results are shown as the Tiron-inhibitable fraction in RLU/mg protein/s; $n = 6–7$ hearts per genotype; *$P < 0.05$ between groups). NOS indicates nitric oxide synthases; mGCH1, myocardial guanosine triphosphate cyclohydrolase; Tg, transgenic; SMTC, S-methyl-L-thiocitrulline acetate salt.

**Effect of Myocardial GCH1 Overexpression and Increased NOS1 Activity on LV Contraction: Role of I$_{Ca}$ and SR Ca$^{2+}$ Release**

NOS1 gene deletion has been reported to slow the rate of myocardial relaxation and SR Ca$^{2+}$ uptake and to have variable effects on myocardial inotropy and the diastolic leak of Ca$^{2+}$ through the RyR2. In mGCH1-Tg mice, increased NOS1 activity did not affect LV systolic function (Online Figure II) or cardiomyocyte basal and β-adrenergic stimulated contraction (Figure 4A and Online Figure III). Similarly, diastolic Ca$^{2+}$ (Fura 2 ratio: 1.31 ± 0.04 in WT versus 1.27 ± 0.03 in mGCH1-Tg, $n = 36$ and 38 cells, 4 hearts per genotype; $P = 0.3$) and the amplitude of the [Ca$^{2+}$]$_i$ transient (Figure 4B) did not differ between genotypes, whereas the SR Ca$^{2+}$ content tended to be lower in mGCH1-Tg (Figure 4C). By contrast, I$_{Ca}$ density was significantly reduced in mGCH1-Tg myocytes at voltages from 0 to +20 mV (Figure 4D). At 0 mV, I$_{Ca}$ density was $-6.8 ± 0.5$ pA/pF in mGCH1-Tg and $-8.4 ± 0.4$ in WT ($P < 0.05$, $n = 26$ and 21 cells from 5 and 4 hearts per genotype, respectively). Steady state inactivation curves showed that the voltage at which I$_{Ca}$ was half-maximally inactivated was $-29.3 ± 0.1$ mV in WT and $-34.1 ± 0.2$ mV in mGCH1-Tg myocytes ($P < 0.05$), whereas activation curves did not differ between groups (Online Figure IB). Both the fast and slow time constants of decay of I$_{Ca}$ were significantly shorter in mGCH1-Tg myocytes ($\tau$ in milliseconds: 25.8 ± 1.0 versus 30.1 ± 1.1 in WT, $P < 0.05$ and $\tau$ in milliseconds: 99.9 ± 7.2 versus 136.7 ± 5.9 in WT, $P < 0.01$). NOS1 inhibition with...
SMTC significantly increased $I_{Ca}$ in mGCH1-Tg mice (Figure 4D) but not in WT mice (data not shown), and abolished the differences in $I_{Ca}$ between genotypes. Despite a reduction in peak $I_{Ca}$ and a trend toward a lower SR load in mGCH1-Tg mice, the fractional release of Ca$^{2+}$ from the SR was increased in mGCH1-Tg myocytes (83.4±2.2% versus 75.9±2.4% in WT myocytes; n=36 and 38 cells, 4 hearts per genotype, P<0.05), suggesting that the sensitivity of RyR2 Ca$^{2+}$ release channel to activation by local Ca$^{2+}$ is increased in mGCH1-Tg mice.25 The critical role of the increased fractional release of Ca$^{2+}$ from the SR in preserving normal myocardial contraction in the mGCH1-Tg mouse was confirmed by measuring cell shortening in the presence of thapsigargin. As shown in Figure 5A, disabling the SR resulted in a significantly smaller contraction in mGCH1-Tg myocytes over a voltage range 0 to +20 mV (n=26 and 21 cells, 5 and 4 hearts, respectively, *P<0.05, D). NOS1 inhibition with SMTC increase $I_{Ca}$ in mGCH1-Tg myocytes (n=21, 4 hearts, *P<0.05, D) abolished the difference in $I_{Ca}$ between genotypes. mGCH1 indicates myocardial guanosine triphosphatase cyclohydrase; Tg, transgenic; SR, sarcoplasmic reticulum; NOS, nitric oxide synthases; SMTC, S-methyl-L-thiocitrulline acetate salt.

Figure 4. Myocyte shortening and [Ca$^{2+}$] transient amplitude are unaffected in mGCH1-Tg mice, but I$_{Ca}$ density is significantly decreased. Cell shortening (n=25 and 28 cells from 4 hearts per genotype, A) and the amplitude of the [Ca$^{2+}$] transient (n=36 and 38 cells, 4 hearts per genotype, B) did not differ between groups, whereas SR Ca$^{2+}$ content tended to be lower in mGCH1 Tg mice (P=0.06, n=38 cells and 36 cells, 4 hearts per genotype, C). $I_{Ca}$ density was reduced in mGCH1-Tg myocytes over a voltage range 0 to +20 mV (n=26 and 21 cells, 5 and 4 hearts, respectively, *P<0.05, D). NOS1 inhibition with SMTC increase $I_{Ca}$ in mGCH1-Tg myocytes (n=21, 4 hearts, *P<0.05, D) abolished the difference in $I_{Ca}$ between genotypes. mGCH1 indicates myocardial guanosine triphosphatase cyclohydrase; Tg, transgenic; SR, sarcoplasmic reticulum; NOS, nitric oxide synthases; SMTC, S-methyl-L-thiocitrulline acetate salt.

Mechanisms Underlying the Faster Rate of Myocardial Relaxation and [Ca$^{2+}$] Decay in mGCH1-Tg Mice

As shown in Figure 5D, the exponential decay of LV pressure during isovolumic relaxation (tau) was significantly faster in Langendorff-perfused mGCH1-Tg hearts, whereas the LV end-diastolic pressure–volume relationship did not differ between genotypes (Online Figure II), suggesting that LV compliance and structural properties were unaffected in mGCH1-Tg hearts. In agreement with these data, cell size was the same (Table), but the rate of relengthening (−dL/dt) was faster in field-stimulated LV myocytes from mGCH1-Tg mice (Figures 4A and 5E) and so was the decay of the [Ca$^{2+}$] transient (Figures 4B and 5F).

To evaluate the mechanisms responsible for these findings, we first measured the amplitude of the NCX current (which by extruding Ca$^{2+}$ across the sarcolemmal membrane may affect the speed of relaxation and [Ca$^{2+}$] decay). As shown in Online Figure IVA, the NCX current did not differ between genotypes nor did the rate of decay of the caffeine-induced [Ca$^{2+}$] transient (which predominantly reflects the rate of Ca$^{2+}$ extrusion from the cell via the NCX, Online Figure IVB). The calcium flux through NCX, calculated as the integral of the current, did not differ significantly between genotypes (pC/pF: 0.70±0.08 in mGCH1-Tg versus 0.97±0.12 in WT; n=12 and 8 cells, respectively, from 3–4 hearts per genotype, P=0.11).
As GCH1 is a Ca\(^{2+}\)-binding enzyme,\(^\text{26}\) we also evaluated whether its overexpression affected the myocytes’ Ca\(^{2+}\)-buffering properties (Online Figure IVC). The maximum intrinsic Ca\(^{2+}\)-buffering capacity (B\(_{\text{max}}\)) and the dissociation constant (K\(_d\)) did not differ between genotypes (B\(_{\text{max}}\) in μmol/L: 112.5 ± 7.0 in WT versus 124.4 ± 8.0 in mGCH1-Tg; n = 10 and 7 cells, respectively, from 3–4 hearts per genotype; P = 0.29; K\(_d\) in μmol: 0.84 ± 0.06 in WT versus 0.74 ± 0.07 in mGCH1-Tg, P = 0.30), suggesting that the [Ca\(^{2+}\)]\(_i\)-buffering capacity was unaffected by myocardial GCH1 overexpression.

The absolute level of SERCA2A protein and Ser\(^{16}\) phosphorylated PLB did not differ between genotypes, but total PLB was significantly reduced in mGCH1-Tg (Figure 6A); consequently, both the PLB Ser\(^{16}\) phosphorylated fraction and the SERCA2A: PLB ratio were significantly increased in mGCH1-Tg mice. By contrast, the PLB Thr\(^{17}\) phosphorylated fraction did not differ between genotypes.

Because SERCA2A activity is inhibited by unphosphorylated PLB, both the reduction in total PLB and the relative increase in PLB Ser\(^{16}\) phosphorylation may contribute to the faster rate of [Ca\(^{2+}\)]\(_i\) decay and relaxation in mGCH1-Tg myocytes. However, by abolishing the difference in PLB Ser\(^{16}\) phosphorylation between genotypes (in the absence of changes in total PLB), NOS1 inhibition with SMTC also abolished the differences in the rate of relaxation and [Ca\(^{2+}\)]\(_i\) decay between mGCH1-Tg and WT mice (Figure 7A and 7B). Similarly, β-adrenergic stimulation (isoproterenol, 10 nmol/L) or PKA inhibition (PKI, 3 μmol/L) abolished both the difference in PLB Ser\(^{16}\) phosphorylation and the rate of [Ca\(^{2+}\)]\(_i\) decay between genotypes (Online Figures III and VIB), whereas inhibition of type 1 or type 2A protein phosphatases by okadaic acid (2 μmol/L) did not affect PLB phosphorylation in either group (Online Figure VA). PKI did not affect the amplitude of the [Ca\(^{2+}\)]\(_i\) transient; however, the speed of myocyte relengthening (24 and 27 cells, 3 hearts per genotype; **P < 0.01) were faster in LV myocytes from mGCH1-Tg mice. SR activities at both 1 Hz and 10 Hz were not different between genotypes (Figure 7D, D1, E1), but the exponents (D2, E2) were faster in mGCH1-Tg mice, indicating that the RyR2 leak/SR load ratio (D3, E3) was reduced in mGCH1-Tg mice.

Increased cytosolic Ca\(^{2+}\) reuptake in determining the faster rate of relaxation in mGCH1-Tg mice was further confirmed by showing that, after disabling the SR with thapsigargin, myocyte relaxation tended to be slower in mGCH1-Tg mice (∼dL/dt in μm/s: 9.3 ± 1.5 versus 14.1 ± 2.1 in WT; n = 7 and 12 cells from 3 hearts per genotype, *P = 0.073).

Taken together, these findings indicate that a NOS1-dependent increase in PKA-mediated Ser\(^{16}\) PLB phosphorylation is the main mechanism underlying the faster relaxation and [Ca\(^{2+}\)]\(_i\) decay dynamics in LV myocytes from mGCH1-Tg mice.

**Decreased Superoxide Production Does Not Affect the Myocardial Phenotype of mGCH1-Tg Mice**

In the myocardium of NOS1 knockout mice, an increase in XOR-dependent superoxide production has been reported to account for a decreased cell shortening at frequencies >1 Hz.\(^\text{27,28}\) To evaluate whether a reduction in myocardial superoxide production might have contributed to the phenotype of the mGCH1-Tg mice, LV myocytes were incubated with the XOR inhibitor, oxypurinol (100 μmol/L). As shown in Online Figure VB, oxypurinol decreased myocardial superoxide production in WT mice and abolished the differences between groups, but it did not affect LV myocyte relaxation or contraction in either genotype (Figure 7C).
Discussion

The present study demonstrates that α-MHC-driven GCH1 overexpression leads to a myocardial-specific increase in biopterins content and improves basal myocardial relaxation through a NOS1-dependent increase in PKA-mediated Ser16 PLB phosphorylation. This conclusion is supported and complemented by a number of novel findings. First, the significant increase in myocardial BH4 and biopterins concentration in mGCH1-Tg mice did not affect their plasma level or the nonmyocyte component of the LV myocardium. By contrast, BH4 and total biopterins content in LV myocytes was increased following oral BH4 supplementation in C57BL/6 mice, indicating that biopterins transport across the sarcolemmal membrane may be unilateral. Second, despite a large increase in myocardial BH4, the ratio between BH4 and its oxidized products (BH2 and B) was lower in mGCH1-Tg mice, indicating that a large proportion of the myocardial biopterin pool was oxidized. Even so, myocardial NOS1 activity was increased in mGCH1-Tg mice, and superoxide release was lower and unaffected by NOS inhibition, indicating that NOS activity remained uncoupled under these conditions. Third, both the speed of relaxation and the rate of decay of the [Ca2+]i transient were faster in mGCH1-Tg mice. These findings were associated with an increase in the PLB Ser16 phosphorylated fraction and in the SERCA2A/PLB ratio. NOS1 or PKA inhibition reduced Ser16 PLB phosphorylation to a similar level in both genotypes and abolished the difference in the rate of decay of the [Ca2+]i transient.

Similarly, overriding the differences in PKA-mediated Ser16 PLB phosphorylation between mGCH1-Tg and WT mice with isoproterenol abolished the differences in the rate of relaxation and Ca2+ decay. Fourth, an increase in fractional release of Ca2+ from the SR was responsible for preserving LV inotropy and the amplitude of the [Ca2+]i transient in mGCH1-Tg mice, despite a significant reduction in I_{Ca} density. The diastolic Ca2+ leak through the RyR2 was significantly increased in mGCH1-Tg cardiomyocytes; however, under our experimental conditions, this did not have a significant impact on SR Ca2+ load. After disabling the SR with thapsigargin, cell shortening was decreased in mGCH1-Tg mice, and the rate of relaxation did not differ between genotypes. Finally, XOR inhibition with oxypurinol abolished the differences in superoxide production between genotypes but did not affect cell shortening or the rate of relaxation in either group.

Together, these findings identify cardiac myocyte GCH1 activity and BH4 content as important regulators of EC coupling and LV relaxation, through their limiting effect on NOS1 activity and the PKA-mediated Ser16 PLB phosphorylation, and suggest that increasing myocardial GCH1 activity and BH4 content may be a promising strategy for the treatment of LV diastolic dysfunction.

Transsarcolemmal Biopterins Transport and Myocardial Availability

Evidence indicates that exogenous BH4 is first oxidized to BH2 in the plasma and then transported into the cell, where it can be reduced back to BH4 by the dihydrofolate reductase.20
In agreement with these findings, oral BH4 supplementation resulted in an increase in BH4 and biopterins content in isolated LV myocytes from C57BL/6 mice. By contrast, biopterins synthesized in LV myocytes from mGCH1-Tg mice remained confined within this cell type, suggesting that their transport across the cell membrane may be unidirectional.

In endothelial cells, NOS3 function and coupled state depend on the ratio between BH4 and its oxidized products; failure to observe an increase in endothelial BH4: BH2 + B ratio following oral BH4 supplementation in patients undergoing CABG surgery was associated with no improvement in endothelial NO bioavailability and vascular function, casting serious doubt on the potential of this therapeutic approach in vascular disease. However, we found that the reduction in the BH4: BH2 + B ratio was not associated with NOS dysfunction in the myocardium of mGCH1-Tg mice, in agreement with our recent data in the fibrillating atrial myocardium, where a reduction in the BH4: BH2 + B ratio was not sufficient to cause NOS uncoupling, in the absence of a significant reduction in tissue BH4 concentration. Indeed, myocardial NOS activity was significantly increased in mGCH1-Tg mice, implying that constitutive myocardial GCH1 activity and BH4 levels might be limiting NO synthesis, even in the absence of disease. Interestingly, only NOS1 activity was increased in mGCH1-Tg mice, suggesting that myocardial constitutive NOS isoforms may have different exposure or sensitivity to oxidized biopterins. Taken together, our findings indicate that an increase in myocardial NOS1 activity can be achieved in the absence of an increase in BH4 over and above the accompanying changes in oxidized biopterins.

BH4-Mediated Regulation of Myocardial Ca2+ Fluxes: Role of NOS1

We and others have previously reported that disrupting NOS1 function results in impaired myocardial relaxation and SR Ca2+ reuptake through a reduction in PKA-dependent PLB phosphorylation. Here we show that increased NOS1 activity in the mGCH1-Tg myocardium leads to an increase in PLB Ser16 phosphorylation and a faster rate of relaxation and [Ca2+]i decay, all of which are abrogated by NOS1 or PKA inhibition or by disabling the SR with thapsigargin. By contrast, the increase in NOS1 activity in the myocardium of mGCH1-Tg mice was not associated with significant changes in cell shortening, LV systolic function, or Ca2+ transient amplitude, despite a reduction in I_{Ca, L}. Indeed, the increased rate of SR Ca2+ reuptake in mGCH1-Tg mice was associated with a marginally reduced SR Ca2+ content, suggesting that other changes in myocardial Ca2+ fluxes may be at play. Indeed, the diastolic leak of Ca2+ from the RyR2 was increased in mGCH1-Tg mice; however, we did not detect a significant difference in the SR Ca2+ load after inhibition of the RyR2 leak with tetracaine in either genotype. An additional explanation can be found by examining I_{Ca, L}. Peak I_{Ca, L} was reduced, and both the fast and slow time constants of decay of the steady state current were significantly shorter in mGCH1-Tg mice. As the inward current during slow decay of I_{Ca, L} is thought to contribute to SR Ca2+ loading, its reduction may counteract the effect of increased SERCA2A activ-
ity on SR Ca\(^{2+}\) load of mGCH1-Tg myocytes. Interestingly, despite a significant reduction in peak \(I_{\text{Ca}}\), SR fractional release was increased in mGCH1-Tg myocytes; taken together with the larger diastolic Ca\(^{2+}\) leak, these findings suggest that the sensitivity of the RyR2 to activation by local Ca\(^{2+}\) is increased in mGCH1-Tg mice. As indicated by the data obtained in the presence of thapsigargin, the increase in SR fractional release is crucial for preserving normal contraction in mGCH1-Tg mice.

Increasing myocardial BH4 may directly scavenge reactive oxygen species and increase NO availability. We found that myocardial superoxide production was significantly lower in mGCH1-Tg mice; oxytpurinol abolished the differences in superoxide release between mGCH1-Tg and WT mice but did not affect cell shortening or relaxation in either group, indicating that reduced superoxide availability does not contribute to the myocardial phenotype of the mGCH1-Tg mouse under these conditions.

**Implications for the Treatment of LV Diastolic Dysfunction**

The fact that increased BH4 availability has such a profound effect on myocardial relaxation, regardless of the abundance of its oxidized products, suggests that increasing GCH1 activity may be a promising therapeutic strategy in patients with LV diastolic dysfunction. At the transcriptional level, GCH1 is upregulated by proinflammatory cytokines,\(^3\) \(^5\) \(^8\) \(^9\) \(^{30}\) and HMG-CoA reductase inhibition by statins.\(^3\) \(^4\) Regulation of GCH1 activity by phosphorylation has also been reported.\(^3\) \(^5\) \(^6\) Recent evidence suggests that resveratrol activates GCH1 in murine hearts, resulting in increased BH4 levels\(^5\) \(^7\) and improves LV relaxation in type 2 diabetes.\(^3\) \(^8\) Importantly, our findings suggest that strategies aimed at increasing myocardial BH4 availability may hold promise in the treatment of diastolic heart failure in the presence or absence of NOS uncoupling, suggesting that this therapeutic approach may have wider-than-expected applicability.

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**Disclosures**

None.

**References**

Carnicer et al. BH4 Regulates Myocardial NOS1 and EC Coupling

What Is Known?

- Tetrahydrobiopterin (BH4) is an antioxidant and a key cofactor of all nitric oxide synthases (NOS).
- A reduction in BH4 or an increase in its oxidized products has been associated with dysfunctional NOS activity, adverse left ventricular (LV) remodeling, and impaired LV relaxation.

What New Information Does This Article Contribute?

- A cardiac-specific increase in BH4 and its oxidized products (obtained by overexpressing the rate-limiting enzyme in the synthesis of BH4, GTP-cyclohydrolase 1, or GCH1) selectively increases the activity of the "neuronal" NOS isoform (NOS1) and the rate of myocardial relaxation via a NOS1-dependent increase in phospholamban phosphorylation and SERCA2A activity.
- The BH4-mediated increase in NOS1 activity inhibits calcium influx via the L-type calcium channels; however, because the fractional release of calcium from the intracellular stores increases, the force of contraction remains unchanged in GCH1-overexpressing mice.
- Myocardial superoxide is significantly lower in GCH1-overexpressing mice. Oxypurinol abolished the differences in superoxide production but did not affect myocardial function.

- Myocardial GCH1 and NOS1 activity may be valuable targets for the treatment of LV diastolic dysfunction.

Cardiovascular disease is associated with a reduction in BH4 bioavailability, resulting in dysfunctional NOS activity and increased oxidative stress. Oral BH4 supplementation preserves cardiac function in animal models of cardiac disease; however, whether this is due to a specific increase in myocardial BH4 and nitric oxide bioavailability or to the anti-inflammatory and anti-oxidant effects of BH4 remains unclear. Here we show that myocardial overexpression of the rate-limiting enzyme in the synthesis of BH4, GCH1, results in a cardiomyocyte-specific increase in bioterpins' content (both in BH4 and its oxidized products) and a selective increase in NOS1 activity. This accelerated myocardial relaxation by increasing SERCA2A activity and the rate of calcium reuptake by the sarcoplasmic reticulum, without affecting contraction. These effects were independent of a reduction in myocardial reactive oxygen species.

Together these findings indicate that myocardial GCH1 activity and intracellular BH4 are a limiting factor for NOS1 and SERCA2A activity in the healthy myocardium and suggest that strategies aimed at increasing myocardial BH4 availability may hold promise in the treatment of LV diastolic failure.
Cardiomyocyte GTP Cyclohydrolase 1 and Tetrahydrobiopterin Increase NOS1 Activity and Accelerate Myocardial Relaxation

Ricardo Carnicer, Ashley B. Hale, Silvia Suffredini, Xing Liu, Svetlana Reilly, Mei Hua Zhang, Nicoletta C. Surdo, Jennifer K. Bendall, Mark J. Crabtree, Gregory B.S. Lim, Nicholas J. Alp, Keith M. Channon and Barbara Casadei

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SUPPLEMENTAL MATERIAL

1. Plasmid construction and generation of transgenic mice

To target GCH gene expression to the cardiac myocytes, we constructed a GCH transgene incorporating the murine alpha myosin heavy chain (α-MHC) promoter. A human influenza haemagglutinin (HA) tag was cloned onto the human GCH 1 gene (Figure 1). The resulting plasmid was confirmed by DNA sequencing using standard techniques. Once the construct had been validated, it was micro-injected into CBA mice embryos.

The GCH transgene (7.8 kb) was excised by Sall digestion and purified by sucrose density-gradient ultracentrifugation (10–30% wt/vol). The fragment was dissolved in sterile injection buffer (5 mmol/L Tris/HCl, 0.1 mmol/L EDTA, pH 7.5) at 5 ng/µL for pronuclear micro-injection in fertilized eggs from super-ovulated CBA mice. Potential transgenic founders were screened by PCR of genomic DNA from ear-notches, using primers specific for human GCH sequence (forward, 5′-CCTTCCTACCCCTGGCTTGTCC-3′; reverse, 5′-AGTCGGGACACGTAGGGGTAGG-3′).

A breeding colony was established by crossing onto a C57Bl/6J background, and all experiments were performed with heterozygous mGCH1-Tg mice using their WT littermates as controls.

2. BH4 supplementation

C57BL/6J mice received 5mg/day of BH4 (Axxora) or of its enzymatically inactive analog, tetrahydroneopterin (N4H, Axxora)¹, for 7 days. Supplements were mixed in the rodent chow and then stored at -20°C. Excised hearts were either perfused with cold phosphate-buffered saline (PBS) and the left ventricles stored at -80°C or used for LV myocyte isolation.

3. Left ventricular (LV) myocytes

3.1. Isolation

LV myocytes were isolated using a standardized enzymatic dispersion technique ². The heart was perfused with Ca²⁺-free isolation solution (37°C, oxygenated) for 3 min and then with 1 mg/ml Collagenase type II solution (Worthington Biochemical) for a further 9 min. Cells were used within the next six hours for electrophysiology experiments. During the isolation, the supernatants were collected in one tube after each wash and the final solution centrifuged at low speed (600 rpm). The remaining supernatant was then spun down at 10,000 rpm; the pellet was considered as the non-myocyte fraction and was used for the biopterin assay.

3.2. Cell shortening, [Ca²⁺]ᵢ transient and SR Ca²⁺ content

Cell shortening (as a percentage of the resting cell length) and the maximum rate of re-lengthening were measured in LV myocytes field-stimulated at 3 Hz by using a video-edge detection system (IonOptix Corp). β-Adrenergic stimulation was achieved by perfusion with 10 nmol/L isoproterenol.
NOS1 inhibition was achieved by pre-incubating LV myocytes with S-methyl-L-thiocitrulline (1 µmol/L SMTC, Sigma) for 1 hour. Similarly, xanthine oxidoreductase (XOR) or protein kinase A (PKA) inhibition was achieved by incubating LV myocytes with oxyxurinol (100 µmol/L, Sigma) or myristoylated PKI (14-22) amide (3µL/L, Merck chemicals), respectively.

The [Ca\(^{2+}\)]\(_i\) transient was measured in Fura-2-loaded (5 µmol/L, Invitrogen Molecular Probes) myocytes field-stimulated at 3 Hz as previously described. In a subset of cells, after a the [Ca\(^{2+}\)]\(_i\) transient reached the steady-state, caffeine (10 mmol/L) was spritzed to assess the SR Ca\(^{2+}\) content. The amplitude of the [Ca\(^{2+}\)]\(_i\) transient was calculated as the difference between diastolic and peak systolic Ca\(^{2+}\) fluorescence. The rate of decay of the field-stimulated [Ca\(^{2+}\)]\(_i\) transient was best fit by a double exponential (Clampfit, Axonpatch, Axon Instruments) and Tau\(_1\) was used for comparisons between genotypes.

Cells were perfused with a Tyrode solution containing, in mmol/L: 134 NaCl, 5.4 KCl, 1.2 MgCl\(_2\), 5 HEPES, 11.1 glucose, 1.4 CaCl\(_2\), pH 7.4. All experiments were conducted at 35 ± 1°C.

3.3. Intracellular Ca\(^{2+}\) buffering capacity and Ca\(^{2+}\) current.

Intracellular Ca\(^{2+}\) buffering was measured by using the method established by Trafford, et al. Experiments were performed on voltage-clamped LV myocytes loaded with Fura-2. The pipette solution contained, in mmol/L: 120 K-glutamate, 10 KCl, 10 HEPES, 5 MgCl\(_2\), 5 K-ATP, 3.6 Na\(_2\)-creatine phosphate, 2.8 NaCl, 0.05 cAMP, pH 7.2. The cell was depolarized every second from a holding potential of -40 mV to 0 mV for 100 ms to elicit a [Ca\(^{2+}\)]\(_i\) transient until steady-state was reached. Application of caffeine (10 mmol/L) resulted in a Na-Ca\(^{2+}\) exchange (NCX) current and a transient increase in free [Ca\(^{2+}\)]. The time-course of the change in total Ca\(^{2+}\) in the cell (obtained by integrating the current) was compared with the measurements of free [Ca\(^{2+}\)] to obtain a buffering curve.

The L-type calcium current (I\(_{Ca}\)) was measured as described in 2. Briefly, LV myocytes were placed in an experimental bath set on the stage of an inverted microscope and perfused with Tyrode solution containing in mmol/L: 140 TEACl, 5.4 CsCl, 1.2 MgCl\(_2\), 5 HEPES, 11 glucose, 1.4 CaCl\(_2\), pH 7.4 with CsOH. Patch-clamp pipettes had a resistance of ~2.5 MΩ when filled with pipette solution (containing in mmol/L: 120 Cs-Aspartate, 10 TEACl, 5 MgATP, 2 MgCl\(_2\), 5 CaCl\(_2\), 11 EGTA, 10 HEPES, pH 7.2). Recordings were performed using Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA) in the whole-cell configuration. Signals were digitized via a DAC/ADC interface (Digidata 1200B, Molecular Devices) and acquired using the pClamp software. I\(_{Ca}\) amplitude was measured by applying a voltage protocol for steady-state activation from -35 to +50 mV after a pre-step of -40 mV, to inactivate sodium currents. Peak I\(_{Ca}\) was measured as the difference between the peak inward current at the beginning of the depolarizing step and the steady-state current at the end of the step and normalized with respect to cell membrane capacitance (measured by applying a ±10 mV pulse of 18 ms, starting from a holding potential of -70 mV). The decay of I\(_{Ca}\) was best fitted by a double exponential function, yielding a fast (τf) and a slow (τs) time constant. Steady state inactivation was recorded during a test step at 0 mV following 500 ms conditioning step at potentials between -75 and 5 mV. NOS1 inhibition was obtained by adding SMTC (100 nmol/L) both to the perfusing and pipette solution. I\(_{Ca}\) and cell shortening were also assessed after disabling the SR with thapsigargin (10 µmol/L, Sigma), as described previously 2.
3.4. Measurement of the diastolic Ca$^{2+}$ leak through the ryanodine receptor (RyR2)

The RyR2 diastolic Ca$^{2+}$ leak was measured as described by Shannon, et al. Briefly, [Ca$^{2+}$]$_i$ transients at 1 Hz were recorded for 100 s, after which the normal perfusion solution ([Ca$^{2+}$]$_o$: 2 mmol/L) was switched to one containing 0 Na$^+$ / 0 Ca$^{2+}$ to abolish Ca$^{2+}$ flux via the NCX. The myocyte was then spritzed with caffeine (10 mmol/L) to estimate the SR Ca$^{2+}$ content. Normal perfusion solution and electrical stimulation at 1 Hz was resumed to re-establish steady-state contraction for at least 100 s, after which the perfusion solution was again switched to one containing 0 Na$^+$ / 0 Ca$^{2+}$ and tetracaine (1 mmol/L) to block the RyR2 leak, and the myocyte was again spritzed with 10 mmol/L caffeine. The RyR2 leak was quantified as the tetracaine-dependent change in [Ca$^{2+}$]$_i$ during the periods of 0 Na$^+$ / 0 Ca$^{2+}$ and expressed as the RyR2 leak:SR load ratio.

4. Biopterin and GCH1 activity assays

BH4, 7, 8-dihydrobiopterin (BH2), and fully oxidized biopterin (B) levels were determined by chromatography (HPLC), as described by Crabtree et al. Briefly, LVs were homogenized in PBS (50 mmol/L, pH 7.4), containing dithioerythritol (1 mmol/L) and EDTA (100 µmol/L). Samples were centrifuged, treated with phosphoric acid (1 mol/L), trichloroacetic acid (2 mol/L), and dithioerythritol (1 mmol/L) and centrifuged for 15 min at 13,000 rpm and 4 °C. The supernatant was injected into a HPLC system where biopterins were separated using a Carbobond-18 column (Highchrom) and quantified using sequential electrochemical (Coulochem III, ESA Inc.) and fluorescence (Jasco) detection. Standards of BH4, BH2, and B were injected to calculate final concentrations and results were normalized to the sample’s protein content.

GCH1 enzyme activity was assessed as the conversion of GTP into 7,8-dihydroneopterin triphosphate using a similar protocol. LVs were homogenized in lysis buffer (in mmol: 100 Tris/HCl, 300 KCl, 2.5 EDTA and 10% (v/v) glycerol, pH 7.8). Homogenized tissue was centrifuged for 5 mins at 13,000 rpm and 4 °C, and the supernatant was incubated with 50 µL of 10 mmol/L GTP for 60 mins at 37 °C in the dark. After this step, 10 µL of 1 mol/L HCl and 10 µL of 1% iodine in 2% potassium iodide were added and the homogenates were further incubated for 60 mins at 37 °C. After a short centrifugation of 2 min at 10,000 g, 300 µL of the supernatant were transferred into a new tube. Next, 10 µL of 100 mmol/L ascorbic acid freshly made in H$_2$O, 10 µL of 1 mol/L NaOH and 20 µL of 16 U/mL alkaline phosphatase (Invitrogen) were added, and the sample was incubated for 60 mins at 37 °C in the dark. Finally, the samples (150 µL) were transferred into a 96-well plate and placed in the HPLC autosampler for analysis of neopterin content. Neopterins were quantified by comparison with the external standards after normalizing for sample protein content.

5. NOS activity

NOS activity was measured using radiochemical detection of L-arginine to L-citrulline conversion, as described previously. Briefly, separation of the products of L-arginine metabolism was obtained by ion exchange chromatography (Jasco Ltd.) and on-line radiochemical scintillation detection (Lablogic Systems Ltd). Recorded data were analyzed using Azur software (Datalys, France). LV was homogenized ice-cold Krebs’ HEPES Buffer containing 5 µmol/L nor-NOHA (to inhibit arginase activity). After centrifugation (13,000 rpm
for 10 mins at 4°C), the supernatant was then incubated for 30 mins on ice with added NOS cofactors except BH4 (i.e., 10 µmol/L FAD, 10 µmol/L FMN, 1 mmol/L NADPH), in the presence or absence of either the non-specific NOS inhibitor, L-NAME (1 mmol/L), or the NOS1-selective inhibitor SMTC (100 nmol/L), followed by 4 hours incubation at 37°C with 3 µL of labelled 14C L-arginine (Amersham Biosciences UK Ltd.). Trichloroacetic acid (10%) was then added to de-proteinate the samples, prior to centrifugation. The supernatant was placed into the auto-sampler cooled to 4°C for chromatographic analysis. Standards of 14C-labelled L-arginine (1 µmol/L), L-citrulline (0.1 µmol/L), and L-ornithine (0.2 µmol/L, all from Amersham Bioscience UK Ltd.) were used to determine elution time. Chromatographic peaks were integrated and expressed as a proportion of total 14C counts for each sample. Results were expressed as the L-NAME- or SMTC-inhibitable fraction.

6. Measurement of superoxide production

Superoxide production by LV homogenates was measured by lucigenin (5 µmol/L)-enhanced chemiluminescence using a single-vial luminometer (Berthold FB12) modified to keep the solution at 37°C, as described and validated previously. LVs were homogenized in Krebs’ HEPES Buffer (in mmol/L: 118 NaCl, 10 HEPES, 25 NaHCO3, 5.6 glucose, 4.7 KCl, 1.2 KH2PO4, 1.1 MgSO4, 1.4 CaCl2, pH 7.4) containing a protease inhibitor cocktail (Roche Applied Science). Homogenate containing 0.4 mg of protein was then added to the reaction (total volume of lucigenin, buffer and sample was 1 mL) followed by incubation for 1 min at 37°C and further recording of the basal chemiluminescence signal for 8 mins. Finally, 60 µL of the superoxide scavenger, 4,5-dihydroxy-1,3-benzene disulphonic acid, (10 mmol/L Tiron, Sigma) was added to the solution and recording was resumed for 4 mins. The relative chemiluminescence levels were evaluated over the last 120 s of each period, and expressed as the Tiron-inhibitable fraction. Data are presented in relative light units per mg protein per second (RLUs/mg protein/s).

In a subset of experiments we tested the effect of inhibiting NOS activity with L-NAME (using its inactive isomer D-NAME as control, 1 mmol/L for 20 minutes) on LV superoxide production. Similarly, oxypurinol (100 µmol/L) was used to inhibit superoxide production from XOR.

7. Immunoblotting

LV tissue was homogenized in CelLytic™ MT Cell Lysis Reagent (Sigma) containing Protease Inhibitor Cocktail (Roche). Immunoblotting in LV homogenates was performed using primary antibodies against NOS1 (Santa Cruz Biotechnology), NOS3 (Santa Cruz Biotechnology), NOS2 (Abcam), SERCA2A (Santa Cruz Biotechnology), total phospholamban (PLB, Badrilla), phospho Thr17-PLB (Badrilla), phospho Ser16-PLB (Badrilla), NCX1 (Santa Cruz), GAPDH (Santa Cruz Biotechnology), human GCH1 (Sigma), HA-tag (Roche), and murine GCH1 (Sigma). Evaluation of both total PLB and Ser16-PLB or Thr17-PLB was carried out in the same membrane after stripping. In some experiments, PLB phosphorylation was evaluated in LV myocytes that had been incubated in a solution containing SMTC (1 µmol/L, Sigma) for 1 hour, after 10 min incubation with myristoylated PKI (14-22) amide (3µmol/L, Merck chemicals) or after 30 min incubation with okadaic acid (2µmol/L, Sigma).
sEnd.1 murine endothelial cells and NIH 3T3 murine fibroblasts stably transfected with a tet-off transactivator construct were used for Western blotting. In the presence of doxycycline, binding of the transactivator is blocked, and gene expression is prevented. 3T3-tet-off cells, which have been previously shown to express neither NOS3 nor GCH1 and confirmed to be devoid of NOS1, NOS2, and NOS3 protein⁶, were stably transfected with a plasmid encoding hemagglutinin (HA) antigen-tagged human GCH1 under the control of a tetracycline-responsive element. Individual colonies were isolated and analyzed for GCH1 expression and a cell line, termed “GCH tet cells” was established from expansion of a single colony. All cell lines underwent three rounds of clonal selection.

Cells were suspended in RIPA lysis buffer (in mmol/L: 20 Tris-HCl, 150 NaCl, 1 Na₂EDTA, 1 EGTA, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate, pH 7.4), including a mixture of protease inhibitors (Roche Applied Science), and subjected to three freeze-thaw cycles in liquid nitrogen. Western blotting was carried out using standard techniques and anti-mouse and -human GCH1, as well as anti-GAPDH antibodies. The rabbit anti-rat GCH and human-GCH antibodies were kindly provided by Prof Steven S. Gross (Weill Medical College of Cornell University) and Dr Gabriele Werner-Felmayer (Institute for Medical Chemistry and Biochemistry, Innsbruck, Austria), respectively.

Protein S-nitrosylation was performed by biotin switch method according to Jaffrey et al.⁹ Briefly, LV were homogenized in HEN buffer (in mmol/L: 250 Hepes-NaOH, 1 EDTA, 0.1 neocuproine, pH 7.7) containing a protease inhibitor cocktail (Roche). Proteins’ free thiols were blocked with S-Methyl methanethiosulfonate (MMTS). The samples were then subjected to -20 °C acetone precipitation to remove free MMTS and resuspended in HEN buffer with 1% SDS, 1 mM ascorbate and labelled with N-[6-(biotinamido)hexyl]-3′-(2′-pyridylidithio) propionamide. Biotinylated proteins were then immunoprecipitated with an antibody against RyR2 (Affinity bioreagents) and protein A/G –agarose beads (Santa Cruz). The bound proteins were electrophoretically resolved in 3-8% Tris-acetate gel and the biotinylated fraction was visualized using anti-biotin antibody (Sigma).

8. Immunofluorescence staining

Freshly isolated murine LV myocytes were transferred to Polysine adhesion glass slides using StatSpin® CytoFuge 2 at 600 rpm for 2 min, then fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilized in 0.2% Triton X-100 (30 min incubation time). After blocking with 1% BSA for 1 h, the cells were incubated with a mouse anti GCH1 (F-17) antibody (Santa Cruz) at 4 °C overnight. The samples were then washed with PBS-Tween, followed by incubation with anti-mouse IgG Alexa Fluor® 555 conjugate (Cell Signaling Technology) for 60 min at room temperature. The slides were rinsed in PBS and mounted in Ibidi mounting medium (Thistle Scientific) containing TO-PRO®-3 Iodide (Invitrogen). Images were obtained using a Leica TCS SP5 confocal scanning microscope (Leica Microsystems Wetzlar).

9. Langendorff-perfused hearts

Hearts were rapidly excised and retrogradely perfused at a constant pressure of 80 mm Hg delivering warm (37°C) Krebs buffer containing (in mmol/L) 118.5 NaCl, 4.75 KCl, 25
NaHCO₃, 1.19 MgSO₄, 1.18 KH₂PO₄, 11 glucose, 1.41 CaCl₂, at pH 7.4 saturated with 95% O₂, 5% CO₂. Left ventricular (LV) pressure was measured by a fluid-filled balloon inserted through the left atrial appendage into the LV cavity. A digitized readout of the LV pressure was recorded throughout the experiment using an 8-channel A/D converter PowerLab 8e (AD Instrument, Castle Hill, Australia).

Heart rates were adjusted to 580 beats/min by atrial pacing. Baseline contractile function was recorded during the first 20 min of initial aerobic perfusion. Hearts were excluded when the baseline values did not fulfill the following criteria: left ventricular developed pressure (LVDP) greater than 50 mmHg, spontaneous heart rate greater than 300 beats/min. and coronary flow greater than 1 mL/min but less than 5 mL/min. Hearts had to be in sinus rhythm and show no prolonged arrhythmias (longer than 15 s). The Frank-Starling relationship (between the LV end diastolic volume and the LVDP) was determined by emptying the intraventricular balloon and then slowly refilling in 5 µl increments.

10.   Statistics

All data are expressed as mean ± S.E.M. and n indicates the number of myocytes or samples measured. Data were checked for normality of distribution and statistical comparisons of means were performed using either Student’s t-test, or an analysis of variance (ANOVA), as appropriate. Results were considered statistically significant when P<0.05.

11.   References


4.      Trafford AW, Diaz ME, Eisner DA. A novel, rapid and reversible method to measure Ca buffering and time-course of total sarcoplasmic reticulum Ca content in cardiac ventricular myocytes. Pflugers Arch. 1999;437:501-503.


Supplemental Figure I

A. A human influenza haemagglutinin (HA) tag was cloned onto the human GCH1 gene. This fragment was inserted into a plasmid containing the alpha myosin heavy chain (αMHC) promoter, to target expression specifically to the myocardium.

B. Steady-state activation and inactivation of the L-type Ca$^{2+}$ current. Activation curves did not differ between genotypes whereas the inactivation curve in mGCH1-Tg mice is shifted towards more negative values (n=26 and 21 cells, 5 and 4 hearts in Tg and WT respectively, *P<0.05, **P<0.01)
Supplemental Figure II. A. In Langendorff-perfused hearts there were no significant differences in LV dPdtmax, dPdtmin, developed and diastolic pressure between genotypes. B. LV function was determined by emptying the intraventricular balloon and then slowly refilling it in 5 µl increments. mGCH1-Tg and WT hearts behaved in a similar manner, with no significant differences in LV dPdtmax or min, end diastolic pressure (LVEDP) and developed pressure over a range of LV volumes (n=5 and 7 hearts respectively).
**Supplemental Figure III.** Beta-adrenergic stimulation with isoproterenol (10 nmol/L) abolished the difference in cell shortening and relaxation (n=23 and 15 cells from 4 hearts per genotype, A), rate of decay of the $[\text{Ca}^{2+}]_i$ transient (n=8-12 cells from 4 hearts per genotype, B) and Ser$^{16}$ PLB phosphorylated fraction (n=4 hearts, C) between mGCH1-Tg mice and their wild type littermates.
Supplemental Figure IV. Neither the NCX1 current amplitude (A) nor the tau of the caffeine-induced $[\text{Ca}^{2+}]_i$ transient (B) differed between myocytes from mGCH1-Tg and WT mice (n= 6 and 14 cells; P = 0.79 and 0.41, respectively). Similarly, the intracellular Ca$^{2+}$ buffering is unchanged in mGCH1-Tg cardiomyocytes (C). Both the maximum intrinsic Ca$^{2+}$ buffering capacity ($B_{\text{max}}$) and the dissociation constant ($K_d$) did not differ between genotypes. The equation used to fit the buffering curve was: $\text{Total } [\text{Ca}^{2+}] = \left\{ B_{\text{max}} \times [\text{Ca}^{2+}]_i / (K_d + [\text{Ca}^{2+}]_i) \right\} + B_{\text{min}}$. 
Supplemental Figure V.
A. Inhibition of type 1 and type 2A protein phosphatases with okadaic acid (2 µmol/L) did not affect Ser\(^{16}\) PLB phosphorylation in either genotype (n=4 hearts).
B. XOR inhibition with Oxypurinol (100 µmol/L) abolished the difference in myocardial superoxide release between genotypes (n=6-8 hearts per genotype, ***P<0.0001, ** P<0.001)