Cytoglobin regulates blood pressure and vascular tone through nitric oxide metabolism in the vascular wall

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The identity of the specific nitric oxide dioxygenase (NOD) that serves as the main in vivo regulator of O2-dependent NO degradation in smooth muscle remains elusive. Cytoglobin (Cygb) is a recently discovered globin expressed in fibroblasts and smooth muscle cells with unknown function. Cygb, coupled with a cellular reducing system, efficiently regulates the rate of NO consumption by metabolizing NO in an O2-dependent manner with decreased NO consumption in physiological hypoxia. Here we show that Cygb is a major regulator of NO degradation and cardiovascular tone. Knockout of Cygb greatly prolongs NO decay, increases vascular relaxation, and lowers blood pressure and systemic vascular resistance. We further demonstrate that downregulation of Cygb prevents angiotensin-mediated hypertension. Thus, Cygb has a critical role in the regulation of vascular tone and disease. We suggest that modulation of the expression and NOD activity of Cygb represents a strategy for the treatment of cardiovascular disease.
Endothelium-derived relaxing factor, identified as nitric oxide (NO), is a key mediator regulating vascular tone and blood pressure (BP)\(^1\). NO mediates vascular relaxation through binding to and activation of soluble guanylate cyclase (sGC) in the smooth muscle of vessels\(^3\). Vascular NO levels are controlled by both the rate of NO generation and the rate of NO metabolism. While NO is synthesized by a specific well-characterized NO synthase in the endothelium (eNOS), the process of NO degradation and metabolism in the vascular wall is poorly understood\(^4-6\). It is hypothesized that NO degradation in the vessel wall is mediated by an \(\text{O}_2\)-dependent NO dioxygenase (NOD) such as myoglobin (Mb), haemoglobin-\(z\) (Hb-\(z\)) or cytoglobin (Cygb) that oxidizes NO to nitrate\(^7-14\). However, the identity of the specific NOD that functions as the main \textit{in vivo} regulator of \(\text{O}_2\)-dependent NO degradation in smooth muscle is still unknown. Additional questions also remain regarding the identity of the cellular reducing system that couples with this NOD to regulate the rate of NO consumption in vascular smooth muscle\(^12,15\).

Each member of the globin family of proteins has a unique pattern of cellular expression and localization. Tetrameric Hb is mainly located in red blood cells\(^16\). Mb is mainly located in cardiac and skeletal muscle\(^17\), neuroglobin is mainly present in neurons\(^18,19\) and monomeric Hb-\(z\) has recently been discovered in the myoendothelial junction of resistance vessels\(^14\). Cygb has been found predominantly in fibroblasts and in the vascular wall\(^13,14,20\). The concentration of Hb-associated haem in blood approaches 8 mM, and the concentration of Mb in heart and skeletal muscle is several hundred micromolar or higher. At such high globin concentrations, the rate of NO consumption in blood, heart and skeletal muscles is very rapid\(^21-23\). Unlike Hb and Mb, the Cygb concentration in cells where it is expressed, such as smooth muscle, is in the micromolar range\(^26,27\). At this concentration, Cygb could play a role in regulating NO concentrations in the smooth muscle of the vessel wall, where NO levels are of critical importance for activation of sGC, which in turn regulates vascular tone\(^1-3\).

In cells and tissues where Mb is highly abundant such as in cardiac muscle, Mb has been reported to function as a potent NOD to reduce cytosolic NO concentrations\(^22,28\). This process was shown to be crucial for the breakdown of NO in cardiac muscle and to regulate the dose-response of the effects of NO on the heart. Hearts with genetic knockout of Mb were more sensitive to infused NO with increased cardiac depression and vasodilation. This NOD function was further hypothesized to protect myocyte cytochromes against increases in NO levels\(^22\). However, Mb has been reported to be either absent from vascular smooth muscle or present in only trace amounts, leading to the concept that there must be another globin with the primary function of regulating NO degradation in vascular smooth muscle\(^13,15,29\).

Recently, it has been reported that Hb-\(z\) is expressed in endothelial cells and enriched at the myoendothelial junction in small arteries and arterioles, where it can serve to regulate NO flux out of the endothelial cell to the vascular smooth muscle\(^14,30\). Endothelial cytochrome \(b_5\) reductase 3 was further reported to regulate this process through the reduction of the haem iron of Hb-\(z\), and genetic and pharmacological inhibition of cytochrome \(b_5\) reductase 3 was found to enhance NO bioactivity in small vessels. Thus, Hb-\(z\) has been reported to play a critical role as a NOD located at the myoendothelial junction where it can serve to regulate NO efflux from the endothelium.

Questions remain regarding the process of NO metabolism in vascular smooth muscle and how this regulates vascular tone. While Cygb is expressed in smooth muscle cells (SMCs), its function has not yet been elucidated\(^31-34\). When coupled with suitable cellular reducing systems, such as ascorbate or cytochrome \(b_5\) reductase/cytochrome \(b_5\)/NADH, Cygb has been demonstrated to function as a NOD, efficiently regulating the rate of \(\text{O}_2\)-dependent NO consumption\(^12,15\). Cygb uniquely metabolizes NO in a highly \(\text{O}_2\)-dependent manner with decreased NO consumption in physiological hypoxia\(^35-37\). As such, one can hypothesize that regulation of the expression level or NOD function of Cygb could modulate basal vascular tone and blood pressure.

In this study, we demonstrate in cellular, isolated vessel and \textit{in vivo} models, that Cygb is a highly efficient NOD, and serves as a major regulator of NO degradation and cardiovascular tone in the vascular wall. Both ascorbate and cytochrome \(b_5\) reductase/cytochrome \(b_5\)/NADH serve as effective reducing systems for Cygb, with the latter serving as the major reducing system in SMCs. Knockout of Cygb greatly prolongs NO decay, increases vascular relaxation and lowers blood pressure and systemic vascular resistance (SVR) with increased tissue perfusion. Furthermore, it was observed that downregulation of Cygb can prevent angiotensin-mediated hypertension. Thus, Cygb is shown to have a critical role in the regulation of vascular tone and prevention of disease.

Results

Globin expression level, reduction and NO consumption. In order to assess the importance of a given globin protein in the metabolism and consumption of NO in smooth muscle, it is necessary to determine at what level it is expressed and what its rates of reduction and NO consumption are. Therefore, initial experiments were performed in smooth muscle cells (SMC) first to measure the expression levels of the globin proteins Cygb, Mb and Hb-\(z\) that have been reported to have important NOD function. These experiments were then followed by spectrophotometric measurements of the reduction rates and electrochemical measurements of the rates of NO consumption by each globin in the presence of ascorbate or an enzymatic reducing system.

Quantitative immunoblotting was performed comparing the level of each globin from SMC homogenates to a series of purified protein standards of known concentration for each globin. From this quantitative immunoblotting, we observe that Cygb is the most abundant globin in aortic smooth muscle cells (aSMC) (Fig. 1a,b). We measure that the concentration of Cygb is \(\sim 5 \mu M\), while Mb levels are over 40-fold lower. Hb-\(z\) is trace or undetectable with levels \(>200\)-fold below those of Cygb.

Since the process of globin reduction is the rate-limiting step for NOD activity\(^37\), it is important to characterize the relative kinetics of globin reduction. Cygb has a uniquely fast reduction rate\(^15,36\). We observe that the reduction rate of Cygb is \(\sim 10\) times faster than that of other globins such as Mb or Hb-\(z\) when reductase systems (such as cytochrome \(b_5\) reductase/cytochrome \(b_5\)/NADH) are used, and several hundred times greater when ascorbate (Asc) is the reductant (Fig. 1c–f). This leads to a 10- to 100-fold more rapid rate of NO consumption by Cygb than other globins (Fig. 1g,h). Thus, based on its relatively high expression level and its high NOD activity, Cygb would be predicted to be the major pathway of NO degradation in SMCs.

Measurement of the product of NO consumption by Cygb. In order to confirm that Cygb consumes NO through the process of NO dioxygenation where nitrate is the product, we assayed for the amounts of the NO degradation products nitrite and nitrate in the presence and absence of Cygb using an HPLC-based NOX analyzer. In the absence of Cygb, the major NO degradation product is nitrite, with only small amounts of nitrate detected (Fig. 2a,c), while in the presence of Cygb almost exclusively
**Figure 1** | Expression of globin proteins, reduction rates and rates of NO consumption of Cygb, Mb and Hb-α. Level of Cygb (a) and Mb (b) in human aortic smooth muscle cells (aSMCs) measured by quantitative immunoblotting. These results indicate that there is ~45 ng of Cygb and ~1 ng of Mb in 10^6 aSMCs. Hb-α was not detectable with a level >200-fold below that of Cygb. Assuming a cell volume of 400 μm^3 (ref. 11), the intracellular concentration of Cygb is estimated at ~5.3 μM and that of Mb at ~0.13 μM and Hb-α <0.03 μM. Positions of nearest molecular weight markers are shown. Reduction of globins by 10 mM Asc (c) or b5R (30 nM)/b5 (0.5 μM)/NADH (100 μM) (d). Measured rate constants of Cygb, Mb and Hb-α reduction by 10 mM Asc (e). Measured rate constants of Cygb, Mb and Hb-α reduction by b5 reductase (30 nM)/b5 (0.5 μM)/NADH (100 μM) (f). Calculated rate of NO consumption by Cygb, Mb and Hb-α in the presence of 1 μM globin and 0.3 mM Asc (g). Calculated rate of NO consumption by Cygb, Mb and Hb-α in the presence of 1 μM globin and 50 nM b5 reductase with excess b5 and NADH (h). The calculation was based on the equation in Supplementary Fig. 1, also for more detail see ref. 61. Error bars: mean ± s.e.m., n = 3 per group; **P < 0.01 for Cygb versus Mb, ††P < 0.01 for Cygb versus Hb-α; P values determined using a two-tailed t-test.
Role of Cygb in NO metabolism in vascular smooth muscle. In order to determine the role of Cygb on NO metabolism in smooth muscle of conduit and resistance vessels, we measured the rate of NO consumption in rat aSMCs and mesenteric SMCs (mSMCs), as well as matched cells treated with Cygb-siRNA to knockdown Cygb expression. Cygb levels were evaluated by immunoblotting (Fig. 3a). The first four bands correspond to standard amounts of pure Cygb. Bands 5 and 6 are from homogenates obtained from control SMCs and matched Cygb siRNA-treated cells; bands 7 and 8 are control mSMCs and matched Cygb siRNA-treated cells. Cygb levels were ~80% depleted in these Cygb siRNA-treated SMCs.

With knockdown of Cygb, the rate of NO consumption measured by electrochemical NO sensor in aSMCs or mSMCs was decreased by ~70–75% (Fig. 3b–d). With Cygb-siRNA treatment, ~20% of basal Cygb levels remain (Fig. 3a), which suggests that the process of NO consumption in these SMCs is largely Cygb dependent, with <12% through other pathways.

Role of b5R in NO metabolism in vascular smooth muscle. Questions remain regarding which cellular reducing system is involved in the process of Cygb reduction and NO dioxygenation. Since it is thought that cytochrome b5 reductase 3 (b5R) is of particular importance as a globin reductase, experiments were performed in vascular SMCs with b5R-siRNA treatment to knockdown b5R expression. From immunoblotting experiments as shown in Fig. 4a, the b5R-siRNA was highly effective in decreasing b5R expression with ~90% decrease seen while control scrambled siRNA had no effect. With this decrease in b5R expression, the rate of NO consumption was decreased by ~60% (Fig. 4b,c). Based on the 90% efficiency of knockdown of b5R, this...
suggests that at least 67% of the NO consumption in the SMCs is b5R dependent.

Globin expression levels and localization in vessels. To further evaluate the role of Cygb in vascular NO degradation, the levels and location of Cygb expression were measured in vessels of wild-type (WT) and Cygb<sup>−/−</sup> mice. Similar to the results in isolated SMCs, immunoblotting of WT aortic homogenates demonstrated that Cygb expression was by far the highest among all globins tested, while in Cygb<sup>−/−</sup> vessels there was no detectable Cygb and the expression of sGC, Mb and Hb-z were not significantly different in Cygb<sup>−/−</sup> and WT aorta (Fig. 5a,b). Immunohistochemistry demonstrated that in WT mice, Cygb is highly expressed in SMCs (red staining) but is not present in the endothelium (green staining of eNOS as an endothelial marker). In contrast, Cygb is not detected anywhere in the vascular wall or endothelium of Cygb<sup>−/−</sup> mice (Fig. 5c). Thus, Cygb is the major globin expressed in vascular smooth muscle and is not present in the endothelium.

Vasodilatory function and NO degradation in vessels. To examine the effect of Cygb on vascular tone, we measured vasodilatory response of isolated aortic segments to endogenous and exogenous NO. Phenylephrine-precontracted aortas of Cygb<sup>−/−</sup> and WT mice were studied. The Cygb<sup>−/−</sup> vessels were much more sensitive to either the endothelium-dependent agonist acetylcholine (ACh) or the endothelium-independent NO donor nitroprusside, with a marked shift to the left in the vasodilation–response curves observed for Cygb<sup>−/−</sup> compared to WT, with 39-fold lower (13–0.33 nM ACh) or 20-fold lower (3.0–0.15 nM nitroprusside) values seen for 50% relaxation, respectively (Fig. 5d,e). To determine if the enhancement of vasodilation in Cygb<sup>−/−</sup> vessels is due to a lower rate of NO metabolism in the vessel wall, we measured the NO diffusion across the vascular wall of aortas from Cygb<sup>−/−</sup> and WT mice using an NO electrode<sup>35,38</sup> (Supplementary Fig. 2). To prevent interference from endothelium-derived NO, the endothelium was removed by gently rubbing the endothelial surface of the opened aortic segment<sup>39,40</sup>. The NOS inhibitor L-NAME (1 mM) was also added to inhibit NO generation from any remaining NO synthases. The measured peak NO flux across the aortic wall of Cygb<sup>−/−</sup> mice was >6 times higher than that of WT (Fig. 5f,g).

Role of Cygb on BP, cardiac function, vascular tone and cGMP.

Further measurements were performed to determine the role of Cygb on in vivo BP, cardiac function and vascular tone. The mean arterial BP of Cygb<sup>−/−</sup> mice was 30% lower with values of 65.3 ± 1.9 mmHg for Cygb<sup>−/−</sup> and 93.7 ± 1.5 mmHg for WT (Fig. 6a). By echocardiography, cardiac output (CO) was increased by 68% in Cygb<sup>−/−</sup> mice compared to WT (Fig. 6b). SVR of Cygb<sup>−/−</sup> mice was decreased by 54% from that in WT mice (Fig. 6c). cGMP levels in Cygb<sup>−/−</sup> aortas were five-fold higher than those of WT (Fig. 6d). In addition, the ascending aorta was clearly dilated in Cygb<sup>−/−</sup> mice compared to WT mice with 47% increase in diameter (from 1.5 ± 0.05 mm to 2.2 ± 0.1 mm) (Fig. 6f,g). Thus, Cygb<sup>−/−</sup> knockout results in increased activation of sGC with elevated levels of cGMP, causing marked vasodilation with lower BP and SVR that, in turn, triggers a compensatory elevation in CO.

Role of NOS-derived NO in vascular relaxation.

In order to further confirm that the diminished tone and enhanced vascular relaxation in Cygb<sup>−/−</sup> mice were due to NOS-derived NO, mice were administered the NOS inhibitor L-NAME. L-NAME exerted large effects on the cardiovascular function of Cygb<sup>−/−</sup> mice, reversing the low mean arterial BP (MAPB) and SVR values as well as the elevated CO to values close to WT, while in WT lesser effects were seen as expected based on the lower levels of cGMP present (Fig. 6d,e). Cardiac echo imaging revealed that the aorta was dilated in Cygb<sup>−/−</sup> mice compared to WT. After L-NAME treatment, the aortic dilation in Cygb<sup>−/−</sup> relative to WT was also reversed (Fig. 6f,g). In order to obtain further data on microvascular function, perfusion imaging was performed on WT and Cygb<sup>−/−</sup> mice. In Cygb<sup>−/−</sup> mice, ~40% increase in tissue perfusion was seen compared to WT mice. L-NAME treatment reversed this relative increase in tissue perfusion in Cygb<sup>−/−</sup> mice to values similar to those in L-NAME-treated WT mice (Fig. 6h,i). Thus, inhibition of NO synthesis reverses the profound vasodilation seen in Cygb<sup>−/−</sup> mice with higher BP and SVR, normalization of CO, decreased vessel diameters and lower tissue perfusion, indicating that lack of Cygb greatly enhances NO-mediated vascular signalling.
Effects of Cygb downregulation on Ang II-induced hypertension. It has been demonstrated that angiotensin II (Ang II)-induced hypertension is associated with enhanced superoxide generation in the vessel wall secondary to induction of vascular NADPH oxidase. This increased superoxide is associated with vascular dysfunction due to superoxide-mediated NO consumption.

Figure 5 | Expression of proteins in the NO-sGC pathway, NO-dependent vasodilation and vascular NO metabolism rate in WT and Cygb<sup>−/−</sup> mice. Expression of Cygb, Mb, Hb-α, sGC-β1 and GAPDH in aortas of WT and Cygb<sup>−/−</sup> mice. Positions of nearest molecular weight markers are shown (a) and their relative band density (b). Of note, to be able to detect Mb and Hb-α, 2.5-fold higher levels of homogenate protein were used compared to Cygb. Cygb staining (red) in WT and Cygb<sup>−/−</sup> aortic sections with endothelium staining for eNOS (green). Scale bars, 50 μm (c). The phenylephrine-precontracted aorta of Cygb<sup>−/−</sup> mice was much more sensitive to both Ach-induced relaxation (d) and SNP-induced relaxation (e). The EC<sub>50</sub> for Ach-induced relaxation of Cygb<sup>−/−</sup> and WT mouse aortas is 0.33 and 13 nM, respectively. EC<sub>50</sub> for SNP-induced relaxation of Cygb<sup>−/−</sup> and WT mouse aortas is 0.15 and 3.0 nM, respectively (d, e). Each data point in d, e represents the mean of six independent experiments. The measured NO concentration at the electrode surface after NO diffuses across the aortic wall, which is proportional to the NO flux through the vessel wall, of WT (violet) and Cygb<sup>−/−</sup> (green) mice is shown (f). The measurement method for NO concentration was as previously described. NO was injected into the solution to achieve an initial concentration of 3 μM. The peaks observed correspond to the maximum NO flux. The mean and standard errors of the peak NO concentration detected (g). Error bars: mean ± s.e.m., n = 5 per group, **P<0.01 WT versus Cygb<sup>−/−</sup>; P values determined using a two-tailed t-test.
we observe that downregulation of Cygb in SMCs and vessels decreases vascular tone and BP with preservation of NO and potentiation of NO signalling, we hypothesized that a decrease in Cygb-mediated NO consumption may be able to compensate for the increase in superoxide-mediated NO consumption that occurs in the vessels of mice with Ang II-induced HTN. Therefore, we...
performed experiments to determine if this could be utilized to enhance endothelium-dependent vasodilation in order to ameliorate hypertension (HTN).

Experiments were performed in a mouse model of Ang II-mediated HTN. WT and Cygb−/− mice were chronically administered with Ang II by osmotic pump at doses known to induce HTN. In WT mice, HTN was observed with systolic BP of 160 mmHg, diastolic BP of 104 mmHg and mean BP of 126 mmHg measured after 4 weeks of Ang II administration. In untreated WT mice (Fig. 7a–c). In contrast, Cygb−/− mice did not develop HTN post-Ang II administration and BP values remained in the normal range with values of 105, 70 and 82 mmHg. Thus, downregulation of Cygb or its NOD function could provide a novel, highly potent approach to prevent or reverse HTN. In parallel with the lower BP values, SVR was also lower in the Ang II-treated Cygb−/− mice compared to WT (Fig. 7d).

Measurements of the flux of NO diffusion across the wall of small resistance vessels from these control untreated WT and Cygb−/− mice showed that peak NO flux across the mesenteric artery wall of Cygb−/− mice was ~3.2-fold higher than that of matched WT vessels. As expected, this difference, while large, is less than that measured in aorta which has a thicker wall (Fig. 5). Measurements of the flux of NO diffusion across the wall of mesenteric artery from Ang II-treated mice demonstrated that Ang II treatment decreased the measured NO levels with

Figure 7 | Effect of Ang II treatment on blood pressure, vascular resistance and NO metabolism. Systolic (a), diastolic (b), mean arterial blood pressure (MABP) (c) and systemic vascular resistance (SVR) (d) of mice with and without Ang II treatment. Measurements were performed after 4 weeks of Ang II administration by osmotic pump, n = 5–7 per group. The flux of NO diffusion across the wall of mesenteric arteries from these control or Ang II-treated mice was measured by cylindrical carbon electrodes (e,f). NO was injected into the solution to achieve an initial concentration of 0.5 μM. The average NO diffusion flux through the wall of control or Ang II-treated mouse mesenteric resistance arteries, n = 5 per group, is shown (g). Effect of SOD mimetic (SODm) on the flux of NO diffusion through the wall of control or Ang II-treated WT and Cygb−/− mice (h). Comparison of the flux of NO diffusion across the wall of mesenteric artery segments from untreated or Ang II-treated mice in the presence/absence of SOD mimetic (i), n = 5 per group. *P < 0.05, **P < 0.01 WT versus Cygb−/−, +P < 0.05, ++P < 0.01 WT or Cygb−/− untreated versus Ang II treated. ##P < 0.05, ##P < 0.01 WT or Cygb−/− Ang II treated versus Ang II treated + SODm. Error bars: mean ± s.e.m.; P values determined using a two-tailed t-test.
Cygb values determined using a two-tailed P segments denuded of endothelium or with intact endothelium were treated with the NOS inhibitor L-NAME (1 mM). NO was prevent endogenous NO generation from eNOS, vessels with intact endothelium were treated with the NOS inhibitor L-NAME (1 mM). NO was prevent endogenous NO generation from eNOS, vessels with intact endothelium or denuded of endothelium (WT + L-NAME). Error bars: mean ± s.e.m., n = 5 per group. **P < 0.01 WT versus Cygb−/−. *P < 0.05 Cygb−/− + L-NAME versus Cygb−/− denuded; P values determined using a two-tailed t-test.

decreased NO flux due to increased rate of NO degradation in the wall of both WT and Cygb−/− vessels (Fig. 7e−g). However, the NO flux in Cygb−/− vessels remained much higher than in WT, with values similar to those in normal untreated WT vessels due to the lower rate of NO consumption in the wall of these vessels. Thus, the decreased rate of NO degradation with Cygb knockout lowered vascular resistance, preventing Ang II-induced hypertension.

Additional experiments were performed to assess the role of superoxide in the process of NO decay in the vessels of Ang II-treated mice using a SOD mimetic taken up in cells (GC4419, Galera Therapeutics, Inc.). In mesenteric arteries from Cygb−/− mice, treatment with the SOD mimetic largely reversed the Ang II-associated decrease in NO diffusion flux with a 130% increase seen, while in WT vessels a 68% increase was seen, with values restored close to those in vessels not treated with Ang II (Fig. 7e,f,i). Thus, most of the increased NO consumption seen with Ang II treatment is confirmed to be superoxide, as induced by Ang II, or due to dioxygenation by Cygb, and that both pathways of NO consumption interact, with each contributing to regulation of vascular tone and BP. We further observe that a decrease in Cygb expression or NOD activity could compensate for the increased superoxide-mediated NO consumption seen with Ang II administration and prevent hypertension.

Smooth muscle versus endothelial NO consumption. NO is primarily synthesized in the endothelium and then diffuses into the vascular smooth muscle, where it influences vessel tone. Recently, it has been reported that in small resistance vessels Hb-ζ is expressed in endothelial cells and enriched at the myoendothelial junction, where it can serve to regulate NO flux out of the endothelial cell to the vascular smooth muscle14,30. In order to assess the relative importance of endothelial versus smooth muscle mediated NO consumption, we performed additional experiments comparing measurements of NO flux across endothelium-denuded mesenteric artery vessels compared to vessels treated with L-NAME. As reported above, in Cygb−/− vessels the NO flux was much higher than in WT (Fig. 8). The NO diffusion flux was observed to be almost identical in WT vessels with only a slight but not significant 6% higher flux in the endothelium-denuded vessels. Interestingly, in the Cygb−/− vessels with much higher NO flux and much lower NO consumption, a small but significant 28% increase in NO flux was seen in the endothelium-denuded vessels. Thus, these results suggest that the major process of NO consumption that limits NO flux through the wall of small resistance vessels is the process of NO consumption by Cygb in the smooth muscle; however, there also appears to be a significant but smaller contribution lost in endothelium-denuded vessels consistent with the prior reports of a mechanism regulating NO flux at the myoendothelial junction14,30.

Discussion

The function of Cygb has been debated since its first discovery just over a decade ago31,34. Roles in oxygen delivery, redox biology, cell signalling and NO regulation have been proposed47. Cygb is considered to have a common evolutionary ancestor with Mb34. In accordance with the literature29, we observe that Mb concentrations in vascular smooth muscle are very low, >40-fold lower than Cygb. This preferential expression of Cygb over Mb would suggest that there is an important functional benefit or role uniquely provided by Cygb. Indeed, we observed that Cygb has a uniquely fast reduction rate that is more than 10-fold to 100-fold faster than for Mb, depending on the reducing system, resulting in more than a 10-fold higher rate of NO consumption (Fig. 1). This higher rate of NO consumption by Cygb than Mb is consistent with prior reports36. In a similar manner, Hb-ζ expression was >200-fold less than Cygb and its reduction rate and rate of NO consumption was similar to Mb and more than 10-fold slower than Cygb. From this data, we can see that Cygb is a highly potent NOD that is efficiently and rapidly reduced by cellular reducing systems. Along with the relatively high expression level of Cygb in smooth muscle compared to that of other globins, this confers Cygb with a major role in the O2-dependent metabolism of NO. With knockdown of Cygb expression in SMCs from aorta or mesenteric artery, more than 70–75% of NO metabolism in vascular smooth muscle was shown to be Cygb dependent. Thus, Cygb serves as the major mechanism of NO degradation in vascular smooth muscle.

In addition to its high potency as an NOD, the NOD activity of Cygb has uniquely high O2 dependence. In general, the rate of NO decomposition by oxy-globins decreases when O2 concentrations decrease, as levels of nitrosyl globins increase and oxy-globins decrease as illustrated in Supplementary Fig. 1 and previously detailed12. With Cygb, hypoxia sharply decreases the rate of NO metabolism, while with Mb only a gradual linear decrease occurs12,15. It has been reported that in the O2 concentration range from 0 to 50 μM with ascorbate as reductant, the rate of NO dioxygenation by Cygb is over 100-times more sensitive to changes in O2 concentration than Mb15. Thus, Cygb is uniquely suited for O2-dependent regulation of NO levels and metabolism in vessels.

The decrease in the Cygb NOD function with physiological hypoxia has been hypothesized to preserve NO levels and tissue perfusion under hypoxia. Furthermore, under conditions
of hypoxia, nitrite can be reduced back to NO serving as a NOS-independent pathway of NO generation. With severe hypoxia progressing to anoxia, we have previously observed that reduced Cygb can reduce nitrite back to NO, further enhancing NO and vasorelaxation under conditions of severe hypoxia. However, with the low $P_{50}$ of O$_2$ binding to Cygb of 1.5 Torr, Cygb-mediated NO production secondary to nitrite reduction is significant only at very low $P_{O_2}$, and would only be expected to be important during severe prolonged ischaemia, not in the normal physiological regulation of vascular tone. Thus, under normoxia, Cygb functions as an NOD, oxidizing NO to nitrite (Fig. 2) while under anoxic conditions, Cygb can reduce nitrite back to NO. Based on the unique O$_2$ dependence of its effects on NO, Cygb has been proposed to have a role in O$_2$-dependent flow regulation and hypoxic vasodilation.

Under normal physiological conditions, endothelium-derived NO regulates vascular tone. NO is required for endothelium-dependent vasodilatation which requires diffusion of eNOS-derived NO from the endothelium to the site of sGC in the vascular smooth muscle. Therefore, one might expect that the major NOD in vessels would be present in the smooth muscle where it would serve to regulate the magnitude and duration of sGC activation. Indeed, we observed that Cygb expression was present within the SMCs of the vessel wall and absent from the endothelium (Fig. 5c). With knockout of Cygb, the magnitude of endothelium-dependent or endothelium-independent vessel relaxation was greatly increased, with over a 20-fold shift in the vasodilation dose–response curves to acetylcholine or the NO donor SNP (Fig. 5). With knockout of Cygb, the diffusion flux of NO across the wall of the aorta was increased more than sixfold and across the much smaller mesenteric artery by more than threefold (Figs 5 and 7). Much higher CGMP levels were detected in freshly harvested, unstimulated vessels with fivefold higher levels than in WT vessels (Fig. 6d), confirming that Cygb expression regulates both NO degradation and sGC activation.

In addition to large effects on the function of ex vivo vessels, in Cygb$^{-/-}$ mice, large alterations were seen on in vivo vascular tone, BP and cardiac function compared to the background matched WT mice. MABP and SVR values were markedly decreased by 30% and 54%, respectively (Fig. 6). Furthermore, CO was increased by 68% in the Cygb$^{-/-}$ mice, likely as a compensation for the marked vasodilatation present. Interestingly, NOS inhibition largely reversed the low MABP and SVR values as well as the elevated CO of the Cygb$^{-/-}$ mice to values close to those in WT, confirming that these alterations were secondary to enhanced NOS-derived NO levels. From ultrasound measurements, the aorta was also observed to be dilated in Cygb$^{-/-}$ mice compared to WT and this was also largely reversed by L-NAME. A marked increase in tissue perfusion was also observed in the Cygb$^{-/-}$ mice compared to WT, further demonstrating vasodilatation of the small resistance vessels that control tissue perfusion. This increased perfusion was also reversed by NOS inhibition. Together these results indicate that the NOD function of Cygb is of critical importance for the in vivo regulation of NO levels that in turn control vascular tone, BP, cardiac function and tissue perfusion.

In a wide variety of cardiovascular diseases, ranging from hypertension to atherosclerosis, impaired endothelium-mediated vasodilatory function occurs secondary to impaired NOS function or enhanced NO scavenging. Since we observed that downregulation of Cygb expression enhances endothelium-derived NO and secondary NOS-mediated signalling and vasodilation, one can hypothesize that downregulation of Cygb-expression levels or NOD function could ameliorate or even serve to prevent cardiovascular disease. As Ang II-induced hypertension has been well demonstrated to be due to enhanced superoxide generation and secondary NO degradation, we evaluated if downregulation of Cygb expression could prevent or ameliorate the onset of Ang II-induced hypertension. While, as expected, chronic Ang II administration induced hypertension in WT mice (Fig. 7a–c), in contrast, Cygb$^{-/-}$ mice did not develop hypertension. In parallel with the lower BP values, SVR also remained lower in the Ang II-treated Cygb$^{-/-}$ mice compared to WT (Fig. 7d). Measurements of the NO diffusion flux across the wall of resistance vessels from control or Ang II-treated mice demonstrated that Ang II treatment decreased NO flux due to an increased rate of NO degradation in the wall of both WT and Cygb$^{-/-}$ vessels (Fig. 7e–g). This increased rate of NO degradation was secondary to enhanced superoxide generation, since it was largely reversed by a SOD mimetic (Fig. 7h,i). The NO flux in Cygb$^{-/-}$ vessels remained much higher than in WT vessels, with values similar to those in normal untreated WT vessels, due to the lower rate of NO consumption in the wall of these vessels. Thus, downregulation of Cygb or its NOD function could provide a novel, highly potent approach to prevent or reverse hypertension.

From the current study, it is clear that NO degradation in the vascular wall is largely due to the NOD function of Cygb in the presence of cellular reducing systems. From siRNA-mediated knockdown experiments, b5R was shown to be the major reductase involved, with a 67% decrease in NO degradation rate estimated; however, other enzymatic or non-enzymatic reducing systems may also be involved, such as P450 reductase and ascorbate. Interestingly, b5R has also been reported to be of critical importance for the process of Hb–α mediated NO dioxygenation at the myoendothelial junction that has been reported to regulate NO flux out of the endothelial cell to the vascular smooth muscle of small resistance vessels. In an effort to assess the role of endothelial factors such as Hb–α on the overall process of NO metabolism in small resistance vessels, we measured NO flux across endothelium-denuded mesenteric artery vessels compared to vessels treated with the NOS inhibitor L-NNAME. In Cygb$^{-/-}$ vessels, the NO flux was much higher than in WT vessels (Fig. 8). While the NO diffusion flux in WT vessels showed only a slightly but not significantly higher flux in the endothelium-denuded vessels, in the Cygb$^{-/-}$ vessels, with much higher NO flux and lower NO consumption, a significant 28% increase in NO flux was seen in the endothelium-denuded vessels. Thus, the major process of NO consumption that limits NO flux through the wall of small resistance vessels is due to NO consumption by Cygb in the smooth muscle; however, there also appears to be a significant but smaller contribution from the endothelium, consistent with the prior reports of a mechanism regulating NO flux at the myoendothelial junction.

In conclusion, we demonstrate that Cygb has a critical role in regulating in vivo vascular tone, BP and cardiovascular function. Cygb is shown to be the main pathway of NO metabolism in vascular smooth muscle, regulating NO flux through resistance and conduit vessels. Downregulation of Cygb ameliorated Ang II-mediated hypertension. Since impaired endothelium-dependent NO signalling is a central trigger of a wide range of cardiovascular disease (from hypertensive, to diabetic, to atherosclerotic), downregulation of Cygb or its NOD function, in order to enhance vascular NO levels and restore protective NO signalling, could provide a much needed remedy. Therefore, therapeutic approaches to modulate Cygb expression and its NOD function could be of great value in the prevention and amelioration of cardiovascular disease.

**Methods**

**Knockdown of Cygb or b5R in aortic and mesenteric SMCs.** Rat aSMCs (Lonza Walkersville, Inc., Walkersville, MD) or mesenteric arterial SMCs were
studies. Briefly, under aseptic conditions, the vessel was carefully dissected out and prepared, characterized and cultured in our laboratory according to previous protocols. The vessel was transferred to a 35 mm culture dish containing 347 U ml\(^{-1}\) elastase solution (type IV, 6 U mg\(^{-1}\)) and snap frozen in dry ice. Sections from the blocks were cut at 4 μm on a cryotome and processed for immunostaining. The frozen sections were then blocked with 1% BSA in TBST, incubated with primary rabbit polyclonal anti-Cygb and mouse anti-eNOS antibodies (Santa Cruz Biotechnology) in TBST (1:500 dilutions) + 1% BSA for 1 h at room temperature, followed by the incubation of respective second goat anti-rabbit Alexa Fluor 680 and goat anti-rabbit Alexa Fluor 568 (1:1,000 dilutions) as necessary, for 1 h at room temperature. After washing with TBST-T, the sections were mounted in anti-fade mounting medium (Fluoromount-G, Birmingham, AB) and examined using an Olympus FV 1,000 confocal microscope (Olympus America Inc., Melville, NY) with the appropriate filters.
Measurements of NO diffusion and NO metabolism in the aortic wall. The flux of NO diffusion across the aortic wall was measured by Clark-type NO electrodes, with stability of each was checked by the mouse age-matched adult, male C57BL/6J mice of 9–12 months of age. A segment of aortic ring was longitudinally opened and the opened aortic wall was placed on an NO electrode with an aorta attachment as depicted in Supplementary Fig. 2. The flux of NO diffusion across the aortic wall was recorded by the aortic-wall-covered electrode after NO was injected to provide an initial concentration of 3 μM in the surrounding solution. [NO] in the solution was recorded by a second NO electrode.

Mesenteric artery dissection and cannulation by electrode. Adult, male Cyb −/− mice or age-matched adult, male C57BL/6J mice (9–12 months of age) were anesthetized using an intraperitoneal injection of ketamine (100 mg·kg−1) and xylazine (10 mg·kg−1). First and second order mesenteric arteries (1st and 2nd branch from superior mesenteric artery) were dissected in physiological saline solution by the method described in Supplementary Fig. 2. A micro-cylindrical carbon electrode for measurements of NO diffusion kinetics was added to the solution in experiments with vessels with intact endothelium. To examine the effect of Ang II-induced vascular superoxide (O2−) on NO diffusion in the wall of mesenteric resistance arteries with Ang II treatment, the flux of NO diffusion across the aortic wall was measured as described in the absence and presence of 50 μM superoxide dismutase mimetic (SODm) (G4419, Galera Therapeutics, Inc.).

Mouse aortic ring preparation for vascular function measurements. Preparations of the isolated mouse aorta was similar to that previously described34. Briefly, the thoracic aorta was gently dissected from anesthetized and heparinized adult, male Cyb −/− mice or age-matched adult, male C57BL/6J mice (9–12 months of age), carefully cleaned of fat and connective tissues, and cut transversely into rings of 2–3 mm in length. The rings were mounted in a Myograph System-610M, Danish Myo, Aarhus, Denmark) with care taken not to damage the endothelium, and then suspended in 5-ml organ baths containing Myograph System-610M, Danish Myo, Aarhus, Denmark) with care taken not to damage the endothelium, and then suspended in 5-ml organ baths containing modified KHB (containing (in mM) 118 NaCl, 24 NaHCO3, 4.6 KCl, 1.2 NaH2PO4, 1.2 CaCl2, 4.6 HEPES, and 18 glucose) and continuously purged with 95% O2–5% CO2 (37°C, pH 7.4). Aortic rings were equilibrated for 90 min with an initial resting tension of 1 g, and the bathing solution was changed at 15-min intervals. Changes in isometric tension were recorded on a PowerLab/8sp multichannel data-acquisition system (AD Instruments, Colorado Springs, CO) using ADI Chart software (version 5.3) for digital processing and data analysis. After equilibration, the reproducibility (repeatability) of each ring was checked by the simultaneous administration of a maximally effective concentration of l-phenylhydrazine hydrochloride (phenylhydrazine; 1 μM). The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (Ach)-induced relaxation of phenylhydrazine-pre-contracted rings. Preparations were then washed three times with distilled water, l-NAME, and isolated from surrounding adipose tissue by the removal of the fat and connective tissue under a dissecting microscope in PBS. Cleaned aortas were placed on a warming platform and allowed to acclimatize for 10 min before the protocol began. To determine the vasodilatory response to Ach, the aortic rings were pre-contracted with phenylhydrazine, and dose–response curves for aortic relaxation were obtained by the cumulative addition of Ach or sodium nitroprusside to the rings.

Blood pressure, CO and SVR measurements. Blood pressure was measured by non-invasive tail-cuff method in conscious adult, male Cyb −/− mice or age-matched adult, male C57BL/6J mice (9–12 months of age) using a CODA high-throughput acquisition system (Kent Scientific, Torrington, CT). Briefly, mice were placed on a warming platform and allowed to acclimatize for 10 min before readings were obtained. Mice were trained for 7 days by measuring BP daily, after which BP recordings were made twice a week. Each session consisted of five acclimatization cycles followed by 15 BP measurements cycles. On the data collection day, two sessions of 15 BP measurements were obtained and the average of accepted readings from both sessions was used for systolic, diastolic, and mean BP in each individual mouse35. The computer software of the CODA system measured systolic, diastolic, and mean BP in each individual mouse35. The computer software of the CODA system measured systolic, diastolic, and mean BP in each individual mouse. The computer software of the CODA system measured systolic, diastolic, and mean BP in each individual mouse. For L-NAME experiments, L-NAME was dosed intraperitoneally at 300 mg·kg−1 per day in PBS for at least 6 days.

Echocardiography. Transthoracic echocardiography was performed using the VisualSonics Vevo 2100 system. Adult, male Cyb −/− mice or age-matched adult, male C57BL/6J mice (6–9 months of age) were anesthetized by intraperitoneal injection of 100 mg·kg−1 ketamine and 10 mg·kg−1 xylazine. Descending aortas were dissected from the mice and quickly cleaned of adhering fat and connective tissue under a dissecting microscope in PBS. Cleaned aortas were homogenized in 0.1 N HCl and centrifuged at 10,000g, Supernatant was collected and used for ELISA detection of cGMP (Enzo Life Sciences, Farmingdale, NY). The acetylated format of the assay was used in order to improve sensitivity. Acetylation was performed by 1:20 addition of the acetylation reagent (1:2 acetyl anhydridetriethylamine) to the samples, which were then subjected to the ELISA assay according to the manufacturer’s instructions. Aortic cGMP levels were expressed as picomoles per mg of protein in homogenates.

Angiotensin-II delivery and osmotic pump insertion. Adult, male Cyb −/− mice or age-matched adult, male C57BL/6J mice (6–10 months of age) were anesthetized with isoflurane. Under sterile conditions, a dorsal midline incision was made and a subcutaneous pocket was created in the right flank area. Alzet mini-osmotic pumps (Model 2004) (Durect Corp., Cupertino, CA) loaded with 200 μl saline or angiotensin-II (Ang-II) 7.2 mg·ml−1 (Sigma-Aldrich, St. Louis, MO) were inserted subcutaneously to deliver Ang-II at 1 μg·kg−1·min−1 for a period of 4 weeks36.

Perfusion imaging. Perfusion imaging was performed using a Perimed laser speckle imager (Perimed Inc., Stockholm, Sweden). Perimed PinnSoft software was used for acquisition and processing the data. Images were taken at a scan rate of 100 images/sec with a field of view of 24 cm × 24 cm. The CCD camera resolution was 1,388 × 1,038 pixels with a magnification up to 20 μm per pixel. Focusing and adjusting of field of view were done using a square low power laser light and a measurement distance of 10 cm was maintained during the imaging. Perfusion data were collected for 20 s with image resolution of 0.1 mm. The images were acquired at resting heart rate condition on the hairless, ventral side of 1% isoflurane-anesthetized adult, male Cyb −/− mice or age-matched adult, male C57BL/6J mice (9–12 months of age). For L-NAME experiments, L-NAME was dosed intraperitoneally at 100 mg·kg−1·h−1 in PBS 12 h and 1 h prior to experiments.

N0X measurements. Nitrite and nitrate were measured using an ENO-20 NOx analyzer (EiCOM Corp., San Diego, CA). Each 100 μl sample consisted of 2.5 μM recombinant human Cygb, 150 U ml−1 Mn-SOD, 2.5 mM ascorbate and 50 μM DEA-NONOate (1,1-dithyli-2-hydroxy-2-nitroso-hydrasine) sodium in PBS with 0.1 mM EDTA, pH 7.4. After mixing, each sample was placed in an incubator shaker at 37°C, 150 r.p.m. for 60 min. Injection volume into the ENO-20 instrument was 10 μl. The peak areas were determined using the eDAQ PowerChrom software provided with the instrument. Conversion to molarity was done by calibration against a range of concentrations of samples prepared from sodium nitrite and sodium nitrate run on the ENO-20 just prior to the experimental samples. Controls included all of the sample components except the Cygb (n = 4 for both the Cygb and control).

Protein purification. Recombinant human cytoglobin was purified as previously reported with some modifications37. The expression plasmid for Cygb (human Cygb cDNA in pET3ac, Novagen, Merck KGaA, Darmstadt, Germany) was obtained from Thorsten Burmester (Institute of Zoology and Zoological Museum, University of Hamburg, Germany) and transformed into Escherichia coli strain C41(DE3)polysLys. Cells were grown overnight in a 4 l flask in an incubator shaker at 37°C, 100 r.p.m. in 1 l of Tashun medium supplemented with glucose (8 ml·l−1), ampicillin (0.2 g·l−1) and chloramphenicol (0.5 g·l−1). The following morning, the cells were induced with IPTG (0.24 g·l−1), the flask was sealed with...
parafilm and the bacteria was grown for an additional 6 h at 30 °C with the shaker set to 100 rpm (decreased from 180 rpm). The cells were harvested by centrifugation (3,000 × g for 30 min) and the pellet was resuspended in 100 ml of 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 2 mM dithiothreitol, a pinch of lysozyme and deoxyribonuclease I, and Roche Complete Protease Inhibitor tablets (as recommended by the manufacturer). The cells were placed in a stainless steel 250 ml beaker immersed in ice and lysed by sonication with a Branson Digital Sonicator equipped with a 1/8” horn, using four 2 min repetitions with 10 min cooldown steps between each repetition. Insoluble matter was removed by centrifugation at 45,000g for 1 h in a high-speed centrifuge. A 35% ammonium sulfate precipitation was performed on the supernatant, the pellet was discarded, and the supernatant was dialyzed against 20 mM Tris/HCl, 1 mM dithiothreitol and 0.1 mM EDTA, pH 7.5, with a total of three buffer exchanges. After dialysis, insoluble material was removed by centrifugation (45,000g for 1 h), and the protein was concentrated to 50 ml using Amicon Ultra-15 centrifugal filters (Millipore) with a 10,000 molecular weight cut-off. Further purification was performed with a GE Healthcare AKTA Purifier system with a 20 ml Superloop (GE Healthcare, Piscataway, NJ, USA) for sample loading. A HiPrep 16/10 DEAE FF anion-exchange column (GE Healthcare) was run with sodium chloride gradient elution, followed by a HiPrep 26/60 Sephacryl S-300 high-resolution size-exclusion column (GE Healthcare) eluted with 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.1 mM EDTA. The protein was concentrated and stored in 50 μl aliquots at −80 °C.

Haemoglobin α chains were purified as previously described with minor modifications67,68. 0.5 g of lyophilized human haemoglobin (Sigma) was solubilized in 2 ml of 0.25 M NaCl in H2O, pH 6–6.5. This solution was centrifuged at 45,000g to remove debris, oxidized with a crystal of solid potassium ferricyanide, and run to the solution for 30 min in a 15 ml conical tube, reduced with solid dithionite, then Millipore) to 1 ml volume, made anaerobic by blowing a stream of argon gas over the next morning the solution was centrifuged at 45,000 g and the supernatant was run down a 2 cm S-300 high-resolution size-exclusion column (GE Healthcare) eluted with 50 mM Tris/HCl, pH 7.5. The protein was concentrated and stored in 50 μl aliquots at −80 °C until used.

Data availability. The data that support the findings of this study are presented in the manuscript and the accompanying Supplementary Information file and can be obtained from the corresponding authors upon request.

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Acknowledgements

This work was supported by National Institutes of Health R01 grants HL038324, HL063744, EB0169096 (J.L.Z.) and HL131941 (J.L.Z. and X.L.). D.Z. was supported by a scholarship from the China Scholarship Council. We thank Dr Surya Ngyawali for technical support of the tissue perfusion measurements in mice, and Moustafa Helal and Dr Yuefang Zhou for assistance with vessel studies and NO measurements. Fluorescence imaging was done at The Ohio State University Campus Microscopy and Imaging Facility (CMIF) with assistance from Sara Cole and Brian Klemmene.

Author contributions

XL. and J.L.Z. conceived and supervised the study. XL. designed and performed experiments measuring NO, analysed the data and co-wrote the manuscript. M.A.E. performed molecular, cellular and physiological experiments, and analysed this data. J.B. performed physiological experiments and assisted in data analysis. S.C.L. performed immunohistological work. C.H. performed molecular experiments, protein purification and preparation, data analysis, and assisted in manuscript preparation. T.M.A. performed immunomodulating and cellular experiments. R.S.I. performed physiological experiments. S.C.L. performed and analysed the echo data. D.Z. assisted in the NO and physiological measurements. L.T.T.T. and N.K. created the Cygb(−/−) mice, and J.L.Z. directed and supervised the entire project, performed experiments and data interpretation, obtained funding for the work, organized and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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How to cite this article: Liu, X. et al. Cytoglobin regulates blood pressure and vascular tone through nitric oxide metabolism in the vascular wall. Nat. Commun. 8, 14807 doi: 10.1038/ncomms14807 (2017).

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