The Hemoglobin Homolog Cytoglobin in Smooth Muscle Inhibits Apoptosis and Regulates Vascular Remodeling

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Objective—The role of hemoglobin and myoglobin in the cardiovascular system is well established, yet other globins in this context are poorly characterized. Here, we examined the expression and function of cytoglobin (CYGB) during vascular injury.

Approach and Results—We characterized CYGB content in intact vessels and primary vascular smooth muscle (VSM) cells and used 2 different vascular injury models to examine the functional significance of CYGB in vivo. We found that CYGB was strongly expressed in medial arterial VSM and human veins. In vitro and in vivo studies indicated that CYGB was lost after VSM cell dedifferentiation. In the rat balloon angioplasty model, site-targeted delivery of adenovirus encoding shRNA specific for CYGB prevented its reexpression and decreased neointima formation. Similarly, 4 weeks after complete ligation of the left common carotid, *Cygb* knockout mice displayed little to no evidence of neointimal hyperplasia in contrast to their wild-type littermates. Mechanistic studies in the rat indicated that this was primarily associated with increased medial cell loss, terminal uridine nick-end labeling staining, and caspase-3 activation, all indicative of prolonged apoptosis. In vitro, CYGB could be reexpressed after VSM stimulation with cytokines and hypoxia and loss of CYGB sensitized human and rat aortic VSM cells to apoptosis. This was reversed after antioxidant treatment or NOS2 (nitric oxide synthase 2) inhibition.

Conclusions—These results indicate that CYGB is expressed in vessels primarily in differentiated medial VSM cells where it regulates neointima formation and inhibits apoptosis after injury.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37: 1944-1955. DOI: 10.1161/ATVBAHA.117.309410.)**

Key Words: angioplasty ■ apoptosis ■ arteriovenous fistula ■ myoglobin ■ nitric oxide

Maladaptive vessel remodeling is a characteristic feature of many vascular disorders, including atherosclerosis, vasculitis, postangioplasty restenosis, vein graft stenosis, and arteriovenous fistula (AVF) failure. Although the cause of these conditions may differ, they all involve the coordinated destruction and synthesis of vascular wall components in response to mechanical and inflammatory injuries, changing flow, and altered tensile forces. Stress response pathways and programed cell death such as apoptosis and necroptosis contribute to these processes.

See accompanying editorial on page 1803

The oxygen carrier proteins hemoglobin and myoglobin (MB) are expressed in the vascular wall and regulate endothelial and smooth muscle functions, primarily through nitric oxide (NO) and nitrite (NO_2^-) signaling.[1,2] In addition to hemoglobin and MB, the mammalian globin family includes neuroglobin, cytoglobin (CYGB), and androglobin.[3–7] The functions of the latter 3 are poorly defined, but their intracellular concentrations and localization in nonmuscle cells would suggest roles independent of molecular oxygen (O_2) transport.[8] Given some similarities with other globins, as well as its unique terminal extensions, heme hexacoordination and ligand pocket, a variety of functions have been proposed for CYGB. These include electron transfer, O_2 sensing, NO detoxification, or a combination of the above.[9] In vivo studies have defined a cytoprotective role for CYGB during liver and kidney fibrosis and carcinogenesis, potentially related to its capacity to regulate oxidative stress.[5,10] CYGB is a candidate tumor suppressor gene in upper aerodigestive tract squamous cancers.[11] It is also upregulated in activated myogenic progenitor cells and

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Nonstandard Abbreviations and Acronyms	
ACTA2	α-smooth muscle actin
AP-1	activator protein-1
AVF	arteriovenous fistula
CYGB	cytoglobin
HIF-1	hypoxia inducible factor-1
IFN-γ	interferon- γ
IL-1β	interleukin-1β
MB	myoglobin
MYOCD	myocardin
NFAT	nuclear factor of activated T cell
PARP	poly-ADP-ribose polymerase
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
STS	staurosporine
TNF-α	tumor necrosis α
VSM	vascular smooth muscle

proliferating myoblasts, where it serves antiapoptotic functions and contributes to muscle regeneration.[12] Many studies have highlighted antiapoptotic functions associated with CYGB in response to various stressors, including hypoxia, high glucose, and oxidants.[13–15] In the cardiovascular system, CYGB is expressed in the heart and increases during hypoxia-induced cardiac hypertrophy in the mouse.[16] CYGB mRNA and protein are also found in human and rodent vascular smooth muscle (VSM),[17] and recent evidence for a role for CYGB as a regulator of NO homeostasis in vivo has been provided.[18] However, the functional significance of CYGB expression in the context of vascular remodeling is unknown.

Because past reports suggest that CYGB is involved in cellular stress response,[12,19,20] we tested the hypothesis that CYGB regulates cell survival in the vessel wall during vascular injury. We found that CYGB is expressed in contractile VSM cells, and its abundance is decreased in dedifferentiated VSM cells. Overall, our results suggest that CYGB is critical for VSM cell survival and regulate apoptosis during vascular injury through effector caspase regulation. This is the first example of a globin regulating vascular injury through modulation of proapoptotic signals.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

CYGB Is Preferentially Expressed in Contractile VSM Cells and Downregulated During Smooth Muscle Cell Dedifferentiation

In light of past studies indicating some level of expression of MB and CYGB in vessels and to better understand the conditions associated with CYGB expression,[2,17,18,21] we first compared *CYGB* mRNA levels to those of *MB* using quantitative real-time polymerase chain reaction. Overall, human aortic samples showed statistically significantly higher mRNA levels for *CYGB* than *MB* (Figure 1A). We also evaluated CYGB expression in a deidentified cohort of discarded vein trimmings obtained from patients undergoing AVF placement for hemodialysis or at revision of the AVF. Placement vessels expressed low levels of *CYGB* mRNA in amounts not different from those of *MB* (Figure 1B). In contrast, vessels obtained at revision because of late failure of the AVF showed a statistically significant increase in *CYGB* gene expression with no change for *MB*. Western blotting of CYGB in protein lysates from placement and revision vessels recapitulated these findings (Figure I in the online-only Data Supplement). In mouse and rat abdominal aortas and carotids, *Cygb* mRNA transcripts were expressed at least in a 100-fold excess over *Mb* (Figure 1C and 1D). In contrast, lysates derived from mouse and rat hearts indicated levels of *Mb* mRNA in large excess to those of *Cygb* (Figure 1C and 1D).

To expand on these observations, we analyzed *Cygb* mRNA in contractile (freshly isolated medial layers) and subcultured synthetic rat aortic VSM cells (Figure 2A). Similar to VSM contractile markers *Myh11* (myosin heavy chain 11) and *Lmod1* (leiomodin 1), we found that *Cygb* transcript levels were dramatically reduced in subcultured dedifferentiated SMC. Double immunofluoresence staining of tissue sections showed that CYGB was primarily expressed in medial VSM cells in intact carotids, based on colocalization with the VSM contractile marker CNN1 (calponin 1; Figure 2B), and similar results were obtained in rat aortas (Figure II in the online-only Data Supplement) and in tissue sections derived from cephalic veins from patients undergoing AVF placement or at revision (Figure III in the online-only Data Supplement). Western blotting analysis indicated that medial rat aortic VSM cell dispersions progressively lost CNN1 and ACTA2 (α-smooth muscle actin) protein expression on 120 hours of primary culture and subsequent passage (Figure 2C). This was accompanied with a decrease in CYGB protein expression indicating that acute phenotypic modulation of VSM cells in vitro includes a mark decrease in CYGB protein content. To establish whether CYGB protein expression could be altered in vivo during vascular injury, we examined changes in CYGB protein levels after balloon injury in the rat carotid. A time course analysis by Western blot (Figure 2D) showed maximal decrease in CYGB expression peaking at 3 days post-injury with partial recovery in the latter stages such that levels of CYGB amounted to ≈50% of the Sham conditions. Immunofluorescence staining of injured vessels at 14 days revealed colocalization of CYGB with CNN1 in the media with limited expression of CYGB in the neointima and limited to no CYGB expression in the adventitia (Figure 2E). Overall, these results indicate CYGB expression in medial differentiated VSM cells and downregulation after balloon injury in the rat that precedes neointima formation and might be concurrent with medial VSM cell dedifferentiation.

Loss of CYGB Prevents Neointima Formation In Vivo

To investigate the effect of a loss of Cygb in the rat balloon injury model, an adenovirus encoding a short hairpin targeting *Cygb* was infused in the carotid lumen at the time of injury. This treatment resulted in a 65% decrease in CYGB expression over the noninjured right carotid, 7 days after endoluminal denudation (Figure 3A). At this time point and on silencing of CYGB, neointimal area was decreased by 80% with no change in medial or luminal cross-sectional areas (Figure 3B and 3C).

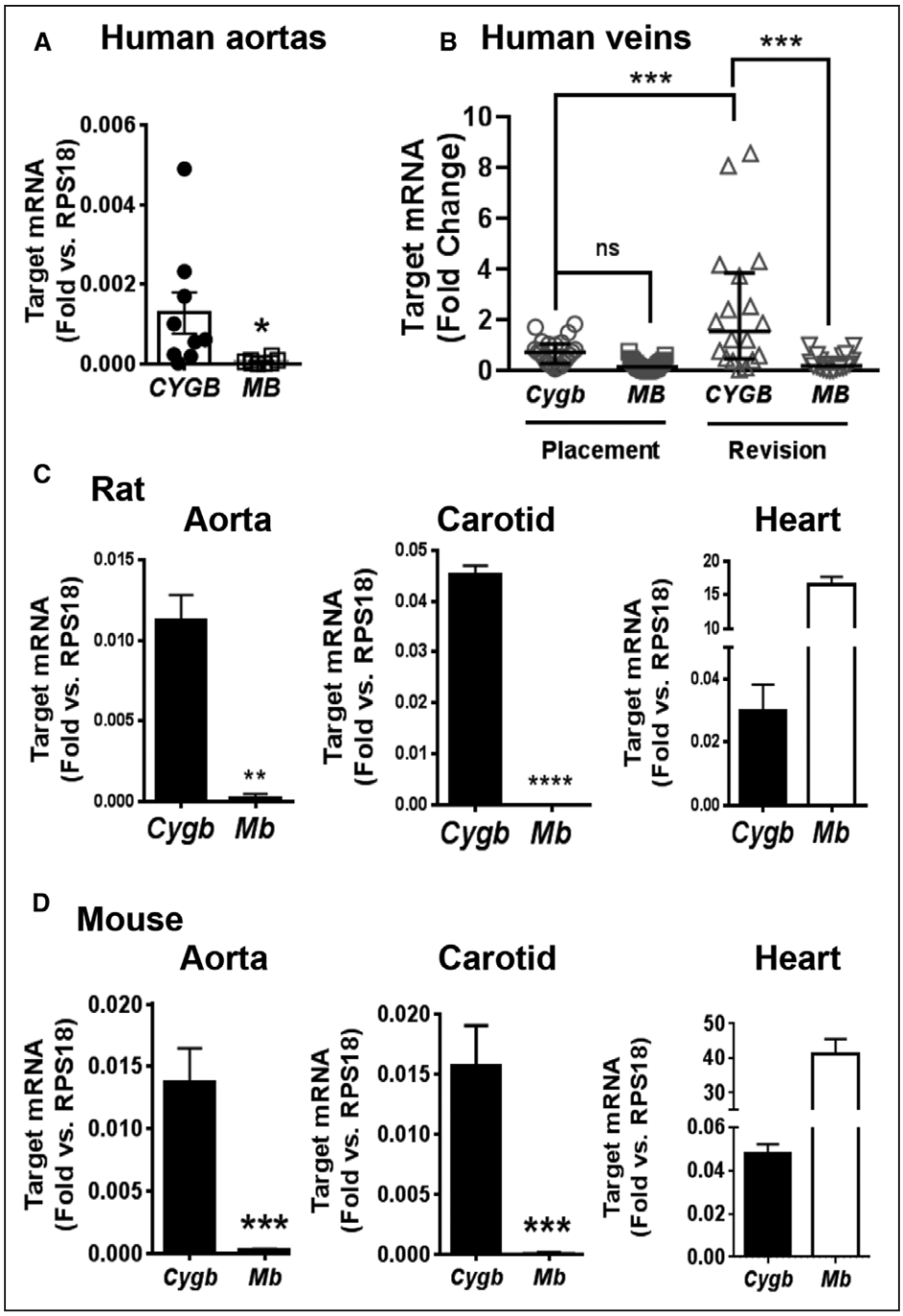

Figure 1. Cytoglobin (CYGB) is expressed in human and rodent vessels. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) results showing relative mRNA levels for *CYGB* and myoglobin (*MB*) in human aortas (n=9; **A**), and human veins derived from vessels trimmings obtained from patients undergoing arteriovenous fistula (AVF) placement (n=26) or at revision (n=20) of failed AVFs (**B**). Levels of *Cygb* and *Mb* mRNA transcripts in mouse aorta (n=11), carotid (n=6), and heart (n=4; **C**); and rat aorta (n=5), carotid (n=3), and heart (n=6; **D**). Values represent the mean±SEM; **P*≤0.05, compared with *CYGB;* ***P*≤0.01, compared with *Cygb;* ****P*≤0.001, compared with *Cygb.* *****P*≤0.0001, compared with *Cygb.*

To evaluate a broader role for CYGB in vascular remodeling, we studied carotid artery sections from *Cygb*$^{-/-}$ and *Cygb*$^{+/+}$ mice subjected to disturbed flow and increased shear stress as a result of carotid ligation. We first verified by quantitative reverse transcriptase polymerase chain reaction (Figure 3D) and Western blotting (Figure IV in the online-only Data Supplement) that CYGB was absent in *Cygb*$^{-/-}$ compared with *Cygb*$^{+/+}$ mice vessels. There were no changes in the level of 2 smooth muscle cell markers (CNN1 and ACTA2; Figure IV in the online-only Data Supplement). We observed a significant decrease in systolic aortic internal diameter in the *Cygb*$^{-/-}$ mice (Figure V in the online-only Data Supplement), but all other morphological characteristics of aortas and carotids, cardiac output, and heart rate were unchanged between genotypes (Figure 3E; Figure V in the online-only Data Supplement). Most significantly, 4 weeks

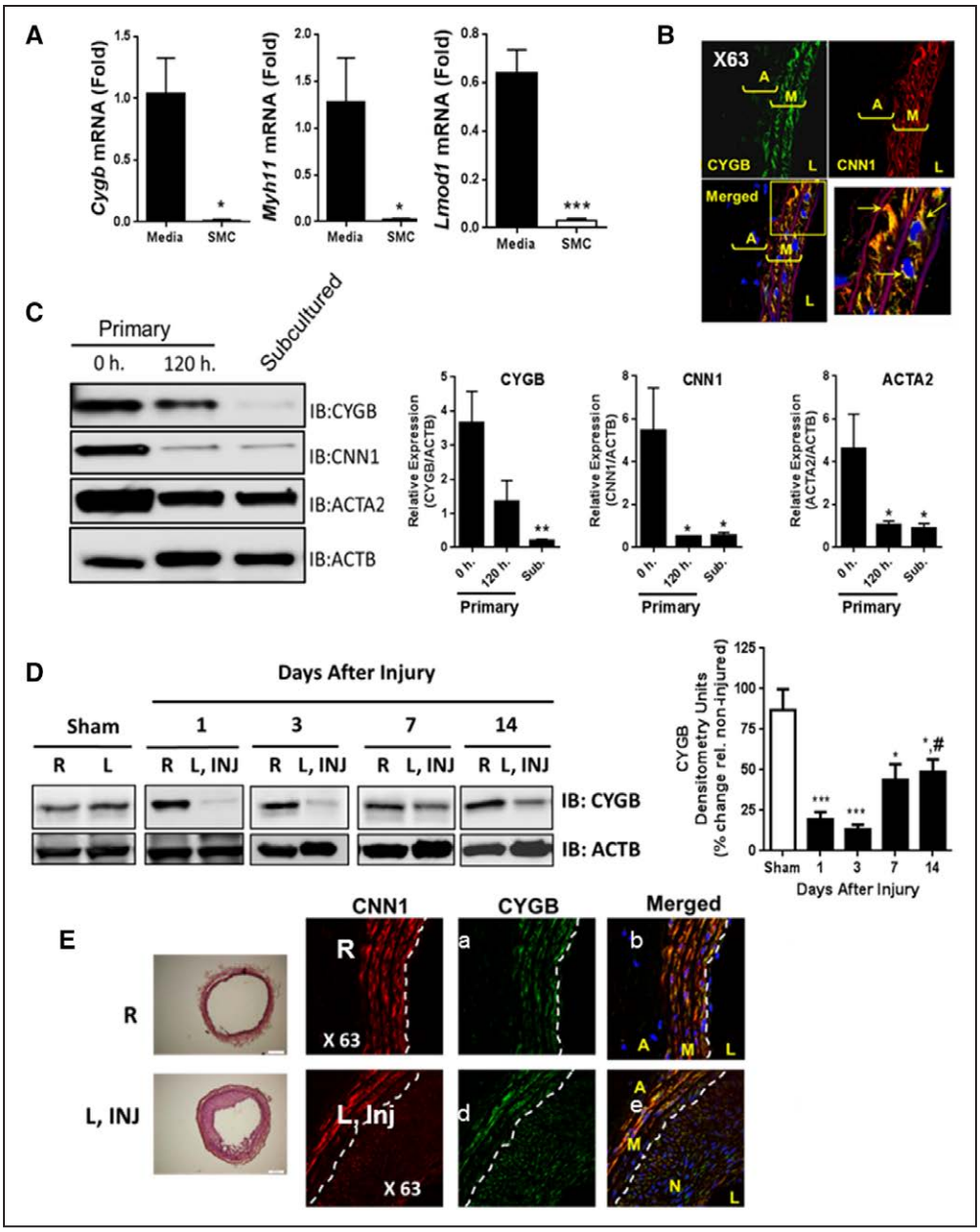

Figure 2. Cytoglobin is expressed in medial SMCs and downregulated during dedifferentiation in vitro and in vivo in the rat. **A**, Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) showing relative mRNA levels for *Cygb* (cytoglobin), *Myh11* (myosin heavy chain 11), and *Lmod1* (leiomodin 1) in the media of rat aorta (n=5) and subcultured medial SMCs (average from 4 different dispersions). **B**, Double immunofluorescence studies of rat carotids. We used antibodies against CYGB (green) and CNN1 (red). Blue staining is DAPI, yellow inset is shown at higher magnification on the **lower right** and shows colocalization (arrows) of CNN1 and CYGB in the media. Representative of 3 independent experiments. **C**, Western blot (WB) analysis from medial rat aortic vascular smooth muscle cells at 0 or 120 h after initial dispersion, and from subcultured rat aortic vascular smooth muscle cells. Primary antibodies against cytoglobin (CYGB), CNN1, and ACTA2 (α-smooth muscle actin) were used. **Right**, Densitometric analysis of results shown in **left**. Mean±SEM (n=3), **P*≤0.05 and ***P*≤0.01 compared with 0 h. **D**, WB analysis showing the expression of CYGB in rat carotids after balloon injury in right uninjured (R) and left injured (L, INJ) carotids. **Right**, Densitometric analysis. Values are mean±SEM (n=5–7). **P*<0.05 compared with Sham, ****P*<0.001 when compared with Sham, #*P*<0.05 when compared with 3 d. **E**, **Left**, Representative H&E staining of R and L, INJ carotids. a, b, c, d, e, and f, double immunofluorescence staining of rat carotids, 14 d after balloon angioplasty injury. We used antibodies against CYGB (green) and CNN1. Blue staining is DAPI. White dashed lines indicate the internal elastic lamina. Representative of 3 experiments. A indicates adventitia; M, media; and L, lumen.

after complete ligation of the left common carotid ligation, $Cygb^{-/-}$ mice displayed little to no evidence of neointimal hyperplasia in contrast to the $Cygb^{+/+}$ mice (Figure 3F). No measurable differences in EEL circumference, luminal, and medial area were observed (Figure 3F).

Suppression of CYGB Protein Expression Increases Medial Cell Apoptosis and Caspase-3 Activation

Loss of medial VSM cells is a characteristic feature of models of vascular injury that involve intraluminal endothelial denudation and distention of the vessel. In the case of the rat carotid

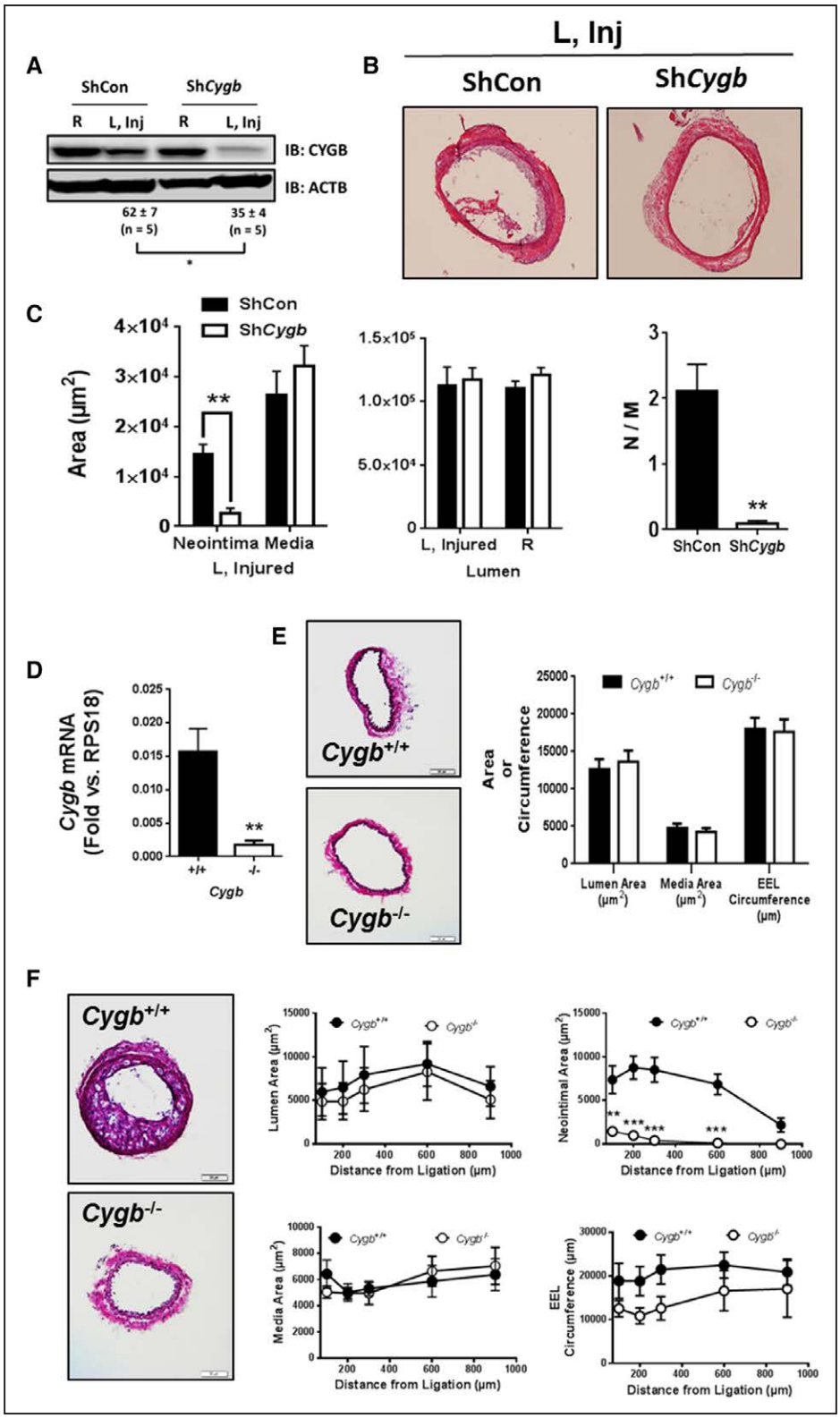

Figure 3. Loss of cytoglobin (CYGB) inhibits neointima formation in rodents. **A**, Western blot analysis showing the expression of CYGB in rat carotids from balloon-injured rat carotids treated with ShCon or Sh*Cygb* adenoviruses, 7 d post-injury and targeted adenoviral treatment. Values represent the mean±SEM of the percentage change in CYGB immunoreactivity in left injured carotids (L, Inj) relative to right noninjured carotids (R) using beta actin (ACTB) as an internal reference; **P*<0.05, n=5. **B**, Representative H&E staining of L, Inj carotids, 7 d after injury and targeted adenoviral delivery. Data shown are representative of 6 animals for each group. **C**, Cross-sectional areas of the neointima, media, and lumen in rat carotids, 7 d after injury and targeted adenoviral delivery. Values shown represent the mean±SEM (n=6). **D**, Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) showing relative mRNA levels for *Cygb* in mouse carotids of *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$ mice. Bars represent the Mean±SEM (***P*<0.01; n=5 and 6 for *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$, respectively). **E**, (*Continued*)

injury model, the number of medial apoptotic cells peaks within a few hours and is rapidly superseded by increased VSM migration and proliferation that drive the hyperplastic response generally observed.[22] We reasoned that if CYGB serves cytoprotective functions, adenoviral-mediated silencing of CYGB might prolong the onset of decellularization beyond the few hours usually observed post-injury in the rat carotid balloon angioplasty model. We found that enumeration of nucleus showed a decrease in medial cell density associated with silencing of CYGB, 7 days after balloon angioplasty (Figure VI in the online-only Data Supplement). At the 4-day time point, protein levels of CYGB in the injured vessels with targeted delivery of shCYGB were approximately half of the levels found in the injured vessels with shCON (Figure 4A). Under these conditions, there was no change in cell proliferation in the injured vessels as indicated by PCNA (proliferating cell nuclear antigen) protein expression, whether CYGB was silenced or not (Figure 4B). In contrast, we found an increase in the abundance of terminal uridine nick-end labeling in situ in the media, indicative of increased apoptosis (Figure 4C). Caspase-3 activation was dramatically increased on CYGB silencing as shown by the ≈6-fold increase in the ratio of cleaved caspase/procaspase (Figure 4D, right). Cleavage of PARP (poly-ADP-ribose polymerase), a downstream target of caspase-3 activity, was also evident (Figure 4E). All together, these results suggest that the loss of CYGB potentiates medial cell apoptosis through an increase in caspase-3 activation in the absence of an apparent decrease in cell proliferation.

Cytokine-Mediated CYGB Expression Protects Synthetic VSM Cells From Nitric Oxide Synthase 2–Dependent Cytotoxicity

We next sought to establish whether CYGB effects on SMC death was independent of other cell types or other factors that may prevail in vivo. To this end, we first established conditions that may be conducive to CYGB reexpression in subcultured VSM cells. VSM cell differentiation is driven by myocardin (MYOCD), a coactivator for the transcription factor, serum-responsive factor, that is required for the reexpression of many contractile markers in VSM cells.[23] However, we found that ectopic expression of MYOCD in subcultured rat aortic VSM cells was unable to rescue CYGB expression in subcultured rat VSM cells despite increased expression of CNN1 and MYH11 (data not shown). Our results showing reexpression of CYGB within 3 to 7 days in the rat carotid injury model suggested that CYGB protein levels could alternatively increase in an environment rich in proliferative and inflammatory signals, independent of a strict association with differentiated VSM. Thus, we tested the effect of selected growth factors and mediators of innate immunity on CYGB expression. Cultured rat aortic VSM cells were incubated with concentrations of IL-1β (interleukin-1β), TNF-α (tumor necrosis factor-α), IFN-γ (interferon-γ), and PDGF (platelet-derived growth factor) optimal for known response of VSM cells to these agents. We found that IL-1β and IFN-γ but not PDGF or TNF-α increased *Cygb* mRNA and protein levels (Figure VIIA and VIIB in the online-only Data Supplement). Significantly, the combination of IL-1β or IFN-γ with hypoxia (1% O_2)—a known inducer of CYGB expression[16]—increased *Cygb* mRNA levels by almost 10-fold over the normoxic control (21% O_2; Figure 5A), an effect that was mirrored at the protein level by an ≈7-fold increase (Figure 5B).

We evaluated VSM cell death using the release of lactate dehydrogenase in the cell culture media as an indicator of cell death, independent of specific pathways such as necrosis or apoptosis. We confirmed that treatment with adenoviral Sh*Cygb* effectively suppressed the expression of CYGB proteins after stimulation with IL-1β and IFN-γ, under normoxic and hypoxic conditions (Figure 5C). Decrease in CYGB content in VSM cells using this adenoviral strategy was not sufficient to increase lactate dehydrogenase release above background levels at 21% O_2, including after treatment with the cytokine mix (Figure 5D). When the same experiment was performed at 1% O_2, decrease in CYGB levels resulted in a statistically significant increase in cell cytotoxicity such that lactate dehydrogenase release after cytokine treatment attained ≈30% of maximum, from 15% in the controlled adenovirus treatment with cytokine mix (Figure 5D). Inflammatory cytokines including IL-1β and IFN-γ strongly increase NO production in rodents through the upregulation of NOS2 (nitric oxide synthase 2),[24] and we confirmed the upregulation of NOS2 by the cytokine mix (Figure 5C). We found that pre-treatment of the cells with the NOS2-specific inhibitor 1400 W reversed the cytotoxic effect associated with decrease in CYGB content and cytokine treatment at 1% O_2 (Figure 5D). Significantly, pre-treatment with IL-1β and IFN-γ under normoxic conditions strongly sensitized VSM cells to the ATP analog staurosporine (STS), a universal and potent activator of apoptosis. The effect of STS was potentiated through decrease in CYGB expression and inhibited by pre-treatment with 1400 W (Figure 5E). We conclude that CYGB may be reexpressed in dedifferentiated VSM cells after cytokine and hypoxia exposure, and in this case, the loss of CYGB is sufficient to sensitize cells to NO-dependent cytotoxicity.

Loss of CYGB Induces Autonomous and Redox-Sensitive Apoptosis in Human Aortic VSM Cells

Previous studies indicated that depletion of CYGB alone was sufficient to trigger cell death,[12] an effect that was potentiated in response to oxidative stress. In contrast to rat aortic VSM cells, subcultured human aortic VSM cells retained some levels of CYGB expression despite little to no expression

Figure 3 (Continued). Representative H&E staining of cross sections from *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$ mouse carotids. **Right**, Morphological characterization of results shown in **left**. Bars represent the Mean±SEM (n=5). There were no statistically significant differences between *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$ mice. **F**, Neointima formation was induced by carotid artery ligation in *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$ mouse littermates and analyzed 4 wk after ligation; **left**, representative images of H&E staining of cross sections obtained from *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$ mice and morphological characterization are shown on the right, including medial, neointimal, and luminal areas, as well as external elastic lamina (EEL) circumference. There was no difference between *Cygb*$^{+/+}$ and *Cygb*$^{-/-}$ mice except for neointimal areas. Points represent Mean±SEM (n=7, $^{**}P<0.01$; $^{***}P<0.001$ vs *Cygb*$^{+/+}$).

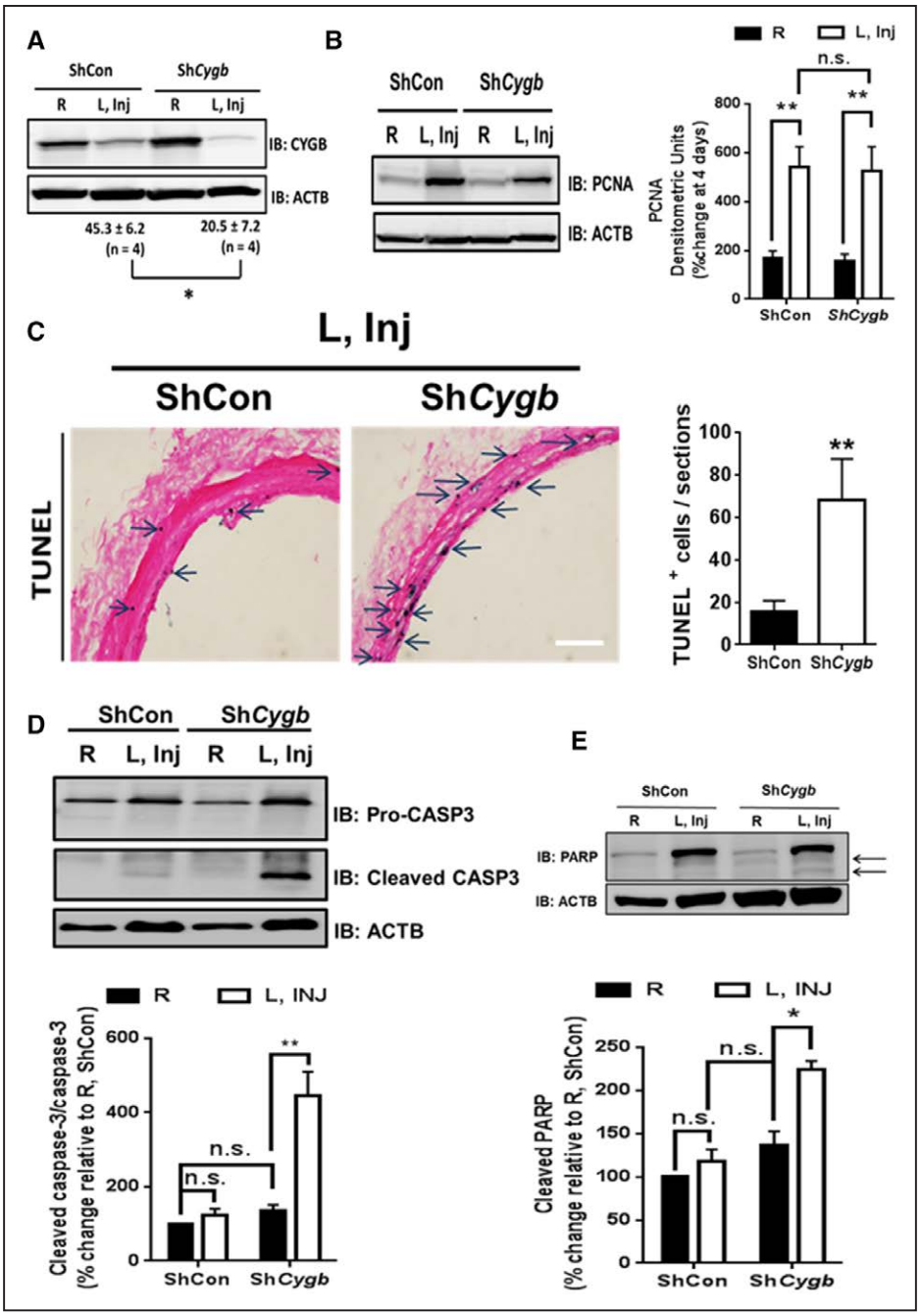

Figure 4. Loss of cytoglobin (CYGB) exacerbates apoptosis in the injured vessel. **A**, Western blot (WB) showing the expression of CYGB in rat carotids from balloon-injured rat carotids treated with ShCon or Sh*Cygb* adenoviruses 4 d post-injury and targeted adenoviral treatment; n=4, **P*<0.05. **B**, WB analysis showing the expression of PCNA (proliferating cell nuclear antigen) in rat carotids from balloon-injured rat carotids, 4 d post-injury and targeted adenoviral treatment. **C**, Representative results from immunohistochemistry analysis of carotids harvested from conditions as described in (**A**). Sections were stained for terminal uridine nick-end labeling (TUNEL). Arrows indicate the occurrence of purple staining indicative of TUNEL; analysis is shown on the **right**; n=4, **P*<0.05 compared with shCon. **D** and **E**, WB analysis showing the expression of pro- and cleaved caspase-3 (**D**; n=7), and PARP (poly-ADP-ribose polymerase; **E**; arrows indicate the occurrence of cleaved PARP; n=4) in rat carotids harvested from conditions as described in (**A**). Graphs shown below each WB represent the mean±SEM values derived from the densitometric analysis of WB. **P*<0.01, ***P*<0.01, as determined by single or paired sample *t* test with Bonferroni correction. L, INJ indicates left injured; n.s., not statistically significant; and R, right uninjured.

of the smooth muscle cell markers MYH11 and LMOD1 (Figure 6A). Human aortic VSM cells were electroporated with CYGB-specific small interfering RNAs resulting in an ≈65% decrease in CYGB protein expression compared with controls. We confirmed the absence of NOS1 and NOS2 expression in these cells (Figure 6B) and found no change in proliferative capacity (Figure 6C) or cytototoxicity up silencing of CYGB alone (Figure 6D).

We examined the cytotoxic response of human aortic VSM cells to hydrogen peroxide (H_2O_2) and STS using final concentrations of 250 μmol/L and 400 nmol/L, respectively. These concentrations were close to their respective IC_{50} (half maximal

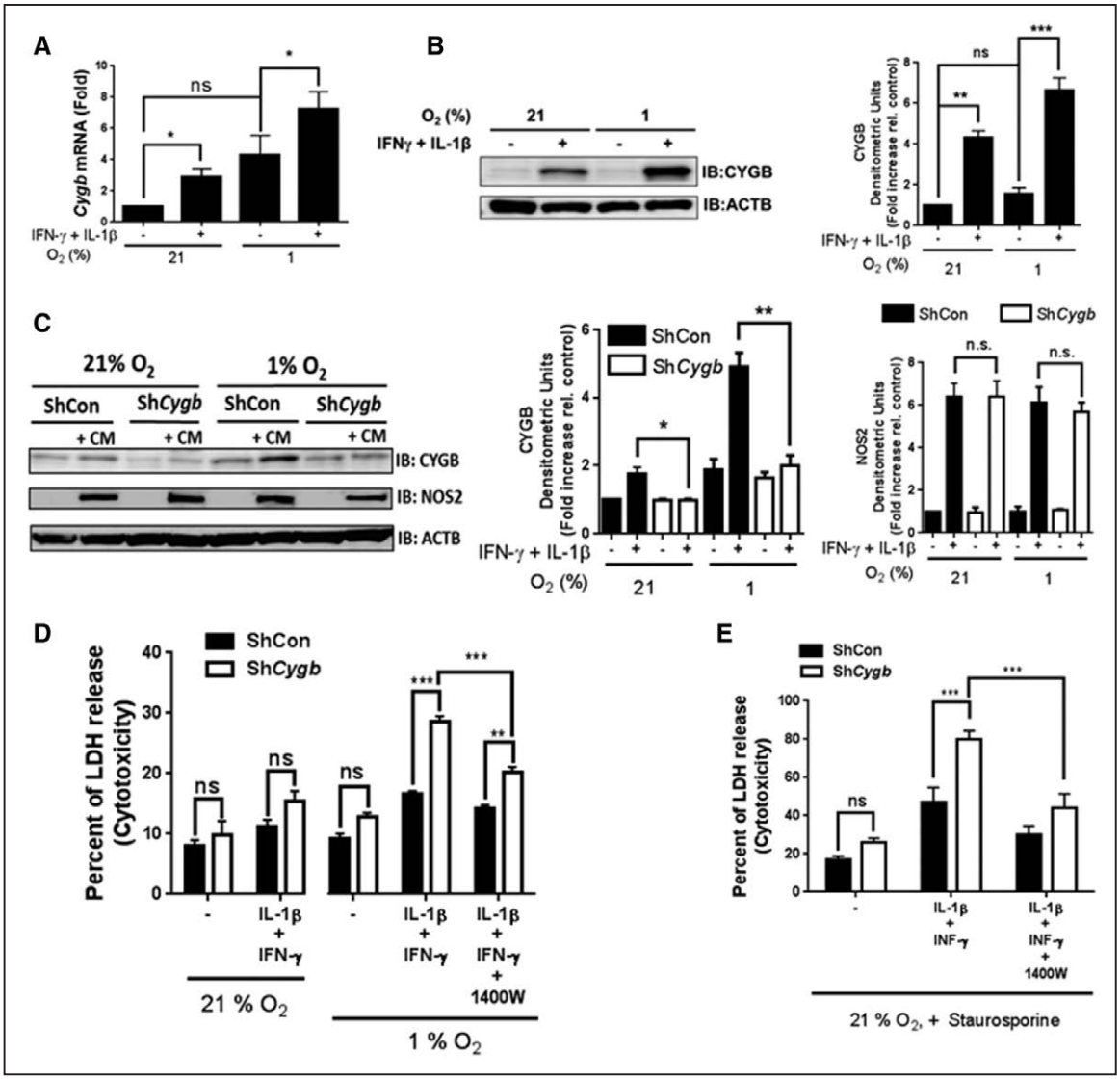

Figure 5. Increase in cell death on loss of cytoglobin (CYGB) is O_2 and NO dependent in rat aortic vascular smooth muscle. **A**, Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) results showing mRNA levels of CYGB in subcultured rat aortic vascular smooth muscle (RAVSM) cells. The cells were stimulated for 48 h with IL-1β (interleukin-1β; 10 ng/mL) and IFN-γ (interferon-γ; 200 U/mL) at 21 or 1% O_2. Mean±SEM, n=8. **B**, Western blot (WB) results showing protein levels of CYGB in subcultured RAVSM using the same conditions described in (**A**). Densitometric analysis is shown on the **right**. Mean±SEM, n≥6; for (**A**) and (**B**) **P*<0.05, ***P*<0.01, and ****P*<0.001, as determined by single or paired sample *t* test with Bonferroni correction. **C**, Western blot results showing protein levels of CYGB and NOS2 (nitric oxide synthase 2) in rat aortic vascular smooth muscle (VSM) cells in the presence of a cytokine mix (CM, IL-1β+IFN-γ) with or without adenoviral silencing of CYGB. Densitometric analysis is shown on the **right**. Mean±SEM, n=4;**P*<0.05, ***P*<0.01, as determined by paired sample *t* test with Bonferroni correction. **D**, Cells were stimulated with IL-1β and IFN-γ for 48 h at 21 or 1% O_2 with or without silencing of CYGB, and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release. Mean±SEM, n=3. **E**, Cells were stimulated with IL-1β and IFN-γ for 48 h at 21% O_2 with or without silencing of CYGB and incubated with staurosporine for 24 h before cytotoxicity was determined by measuring LDH release. Mean±SEM, n=5 for (**D**) and (**E**) ***P*<0.01, and ****P*<0.001, as determined by 2-way ANOVA.

inhibitory concentrations), which were calculated from cytotoxicity dose/response curves (Figure VIII in the online-only Data Supplement). Surprisingly, there was no change in H_2O_2-mediated cytotoxicity, on loss of CYGB (Figure IX in the online-only Data Supplement). However, CYGB silencing potentiated the cytotoxic effect of STS by ≈30% points (Figure 6D). The sensitization of VSM cells to the loss of CYGB was completely abrogated on pre-treatment with the pan-caspase inhibitor z-VAD-fmk. There was also a fraction of STS-induced cytotoxicity, which was insensitive to z-VAD-fmk as previously shown in other systems (Figure 6D).[25] Most importantly, oversensitization to cell death after CYGB silencing was inhibited by the antioxidant N-acetyl cysteine (Figure 6D), and occurrence of apoptosis was confirmed by showing that cleaved caspase-3 was increased on silencing of CYGB and treatment with STS (Figure 6E). Finally, stable overexpression of human CYGB in HEK293 cells that lack endogenous CYGB suppressed STS-induced cytotoxicity (Figure X in the online-only Data Supplement). Altogether, these results indicated that directly modulating cellular levels of CYGB was sufficient to alter cytotoxicity in human VSM cells in a caspase and redox-dependent manner.

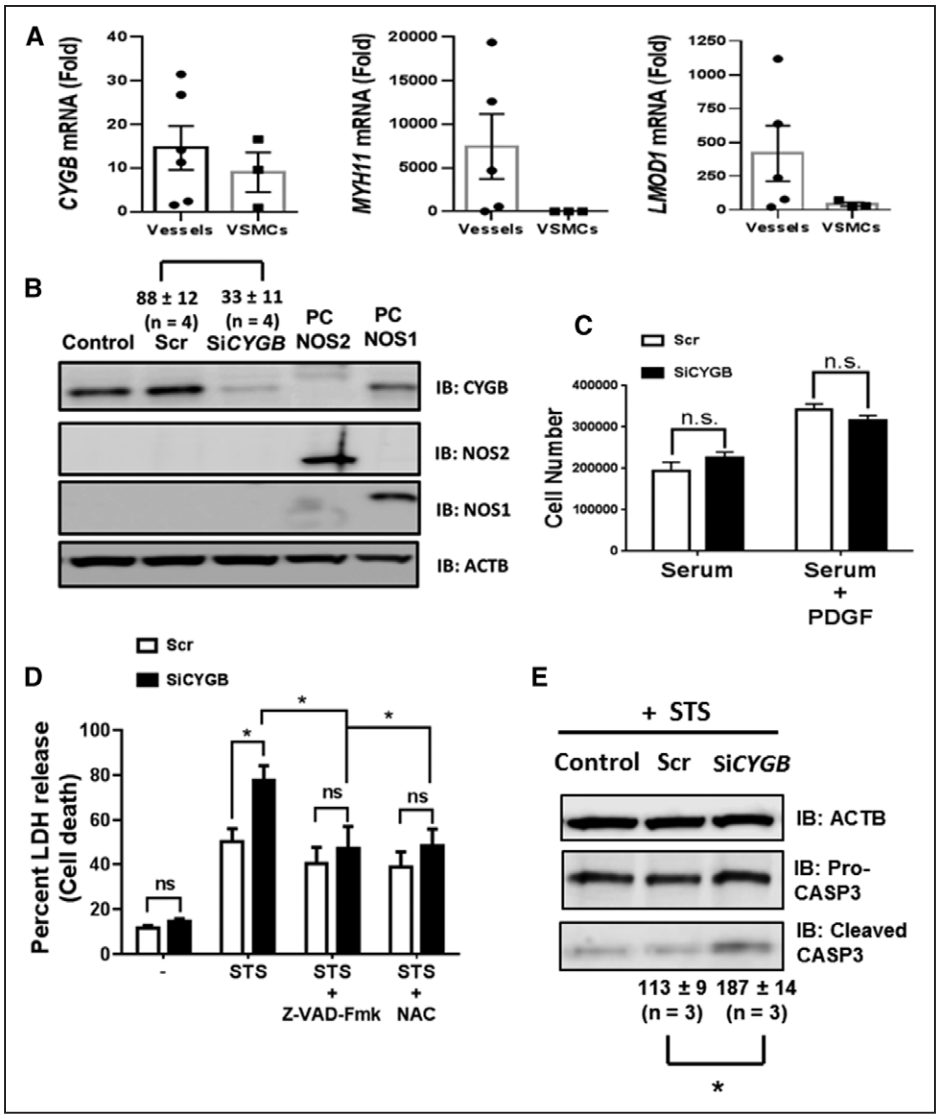

Figure 6. Cytoglobin (CYGB) inhibits staurosporine (STS)-induced apoptosis in human aortic SMCs independent of cell proliferation. **A**, Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) results showing relative mRNA levels for *CYGB*, *MYH11* (myosin heavy chain 11), and *LMOD1* (leiomodin 1) in 6 human aortas and subcultured human aortic smooth muscle cells (VSMCs) derived from 3 different dispersions. **B**, Subcultured human aortic SMCs were electroporated with siRNA mix targeting CYGB (Si*CYGB*) or scrambled siRNA (Scr). Western blots show protein levels of CYGB, NOS2 (nitric oxide synthase 2), and NOS1. Values represent the mean±SEM of the percentage change in CYGB immunoreactivity compared with nontransfected conditions (Control) using beta actin (ACTB) as an internal reference; n=4, **P*<0.05. **C**, Human aortic VSMCs were incubated with serum or serum+PDGF (platelet-derived growth factor) for 3 d, and cell count was determined. There was no difference between Si*CYGB*- and Scr-treated cells. Mean±SEM with n=4. **D**, Subcultured human aortic SMCs were treated with STS (1 μmol/L) for 24 h in the presence or absence of the pan-caspase inhibitor (z-VAD-Fmk) or N-acetyl cysteine (NAC). Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release. Mean±SEM, with n=4 to 6. **P*<0.05. **E**, Western blot analysis for Caspase-3 (Pro-CASP3) and cleaved CASP3 from human aortic SMCs incubated with STS (1 μmol/L for 6 hours) showing increased in cleaved caspase-3 upon silencing of CYGB. Values represent the mean±SEM of the percentage change in cleaved CASP3 immunoreactivity in Si*CYGB*- and Scr-treated cells compared with nontransfected conditions (Control) using ACTB as an internal reference; n=3, **P*<0.05.

Discussion

The functions of hemoglobin and MB in the cardiovascular system are relatively well understood and include O_2 transport and storage and NO and nitrite (NO_2^-) signaling.[1,2,21,26,27] Their expression is not restricted to erythrocytes (for hemoglobin) or cardiomyocytes and striated skeletal muscle cells (for MB) but also extends to endothelial and VSM cells.[1,2] The present study provides a hypothesis for how CYGB might contribute to vascular remodeling. Our results suggest that it regulates the hyperplastic response associated with vascular injury through modulation of apoptosis. We show that CYGB is abundantly expressed in mature VSM cells and lost after dedifferentiation. Most significantly, CYGB protects human and rodent cultured VSM cells from programed cell death and loss of CYGB in vivo leads to medial cell loss and inhibition of neointima formation during vascular injury. Our results indicate that the absence of CYGB oversensitizes medial VSM cells to caspase-3 activation and triggers inopportune apoptosis during injury.

VSM cells are the primary cell type in the tunica media. They are characterized by low turnover and primarily regulate

tension through active contraction and relaxation. Under diseased conditions, mature medial VSM cells can undergo a phenotypic switch characterized by the downregulation of contractile genes, alteration in survival capacity, and increased proliferation, migration, extracellular matrix deposition, and cytokine production.[28,29] Past studies have established that a large fraction of the proliferating cells populating the neointima in experimental models of restenosis or after atherosclerosis is derived from mature medial VSM cells.[30] More recently, it has been shown that VSM cells may give rise to a macrophage-like phenotype in atherosclerotic lesions[31–33] and also may undergo reprogramming to produce a subpopulation of adventitial progenitor cells that contribute to vessel remodeling.[34] All of these studies indicate that VSM cells are critical contributors to the hyperplastic response associated with many types of vasculopathies. In the present study, we show that CYGB is expressed in mature (contractile) VSM cells in the media. In addition to rodent vessels, we found that CYGB was expressed in human arteries in amounts that always exceeded those of MB. Our results also indicate that CYGB expression may increase in human veins derived from revised AVF (Figure I in the online-only Data Supplement). In these vessels, CYGB would seem primarily expressed in the media and to a lesser extent in the neointima (Figure III in the online-only Data Supplement). Because these vessels have undergone maturation before secondary failure, these results would suggest that increased CYGB expression might be associated with arterialization of veins. This will need to be explored further.

In the rat carotid injury model, CYGB protein levels are decreased acutely but slowly recovered such that 2 weeks after surgery, it was primarily associated with $CNN1^+$ cells in outer medial layers (Figure 2E). We also found that CYGB expression was lost in vitro on dedifferentiation of freshly isolated rat aortic VSM cells coincident with contractile VSM markers. An important driver of VSM contractile marker gene expression such as CNN1 is MYOCD, a myogenic transcriptional coactivator that acts through stabilization of the binding of serum response factor to CArG elements within the regulatory regions of most contractile VSM cell marker genes.[23] However, we found no evidence that CYGB expression may be directly or indirectly dependent on MYOCD based on forced expression of MYOCD in cultured rat aortic VSM cells in vitro (not shown). Our in vitro studies suggest that CYGB may be reexpressed in dedifferentiated VSM cells exposed to inflammatory cytokines. To our knowledge, the stimulatory effect of IL-1β and IFN- γ on CYGB expression and synergism with hypoxia has not been documented previously. Although we have not examined the specifics underlying this effect, our results indicate that some of the regulation occurs at the transcriptional level. It is noteworthy that the promoter region of CYGB contains binding elements recognizing the transcription factors AP-1 (activator protein-1), NFAT (nuclear factor of activated T cell), and HIF-1α (hypoxia inducible factor-1α), all of which could be directly implicated with the IL-1β–mediated and IFN-γ–mediated upregulation of CYGB in VSM cells.[16] Synergism of inflammatory cytokines in regulating gene expression is also well documented in VSM cells.[24,35] We found that the loss of CYGB under these conditions sensitized rat aortic VSM cells to NOS2-dependent cytotoxicity (Figure 5). Whether the hypoxic conditions used for some of these experiments can be directly extrapolated to the in vivo models we have tested is questionable. However, they might reflect conditions associated with other vascular pathologies, such as atherosclerosis, and provide direct evidence for a role for CYGB in regulating VSM cell death.

The significance of CYGB expression in the vasculature has been recently associated with the regulation of NO bioavailability and its NO dioxygenase activity.[18] In the liver, CYGB is preferentially expressed in stellate cells and confers cytoprotection in multiple liver injury models including carbon tetrachloride administration,[36] hepatosteatosis,[37] and acetaminophen-induced hepatotoxicity.[38] Silencing of CYGB in myoblasts is sufficient to induce apoptosis and potentiated the proapoptotic effects of hypoxia and oxidants.[12] Modulation of CYGB levels in neonatal rat brains through adenoviral delivery strategies also regulated caspase levels and activation in response to ischemia reperfusion.[39] Our results are reminiscent of the aforementioned study, as indicated by a strong association between the loss of CYGB and caspase-3 activation (Figure 4D). One possibility is a direct effect of CYGB on caspase-3 through redox or nitrosation/nitrosylation-based mechansims.[40,41] We found that decreasing the levels of CYGB was not sufficient to oversensitize HuAo (human aortic) VSM cells to H_2O_2; however, the increase in cytotoxicity to STS associated with loss of CYGB was inhibited with the antioxidant N-acetyl cysteine (Figure 6D). These results suggested that—although CYGB may not exert direct antioxidant effects—the signaling network associated with its function is redox sensitive. We also found that in cultured rat aortic VSM cells, the sensitization to CYGB silencing was dependent on NOS2 activity, further reinforcing the concept of one or several redox-dependent steps that are perturbed on manipulation of CYGB levels.

The occurrence of VSM apoptosis is well established in flow-induced and balloon angioplasty models and in aortic aneurysms[42,43] and atherosclerotic plaques.[44] Here, we show that the loss of CYGB in vitro and in vivo is associated with an increase in apoptosis and that ectopic expression of CYGB in cultured cells is sufficient to inhibit STS-mediated cell death. Vascular remodeling after injury is a dynamic process that involves programed cell death, as well as cell migration, and proliferation. We found no evidence that decreasing CYGB alters VSM proliferation and migration and propose that increased VSM apoptosis is the major determinant of the inhibitory effect of CYGB silencing on neointima formation. Apoptosis has been documented in the rabbit and rat as early as 30 minutes after arterial injury followed primarily by migration and proliferation of resident cells.[22,45] It is unlikely, however, that site-targeted adenoviral delivery of shCYGB affects the first few hours of the injury where VSM apoptosis is most prominent because adenoviral-mediated silencing requires 2 to 3 days to be effective. Instead, our results suggest that silencing of CYGB might limit the survival capacity of a preexisting cell population that still expresses CYGB at the 2 to 3 days time point. Alternatively, adenoviral-mediated silencing of CYGB could prevent the reexpression of cytoprotective CYGB in new populations of VSM cells that

repopulate the media and contribute to the expansion of the neointima. Our findings are consistent with previous studies showing that decrease in the expression of many genes that normally inhibit apoptosis is conducive to medial VSM cell death and is also associated with the reduction of neointimal hyperplasia.[46–48] In contrast, in flow-induced remodeling, strategies that increase VSM apoptosis after initiation of remodeling promote rather than inhibit neointimal formation.[49] Although additional investigations are warranted including additional experimental models, our results showing that *Cygb* knockout mice developed little to no neointimal lesions after carotid ligation suggest important functions for CYGB during proliferative vasculopathies, beyond the rat balloon angioplasty model.

Why VSM would maintain high levels of CYGB in intact vessels? CYGB could contribute to the tight regulation of programed cell death in healthy vessels to avoid the loss of contractile cells with a low proliferation rate and turnover. In contrast, the vascular response to injury requires high cell plasticity where proliferation, migration, and programed cell death need to maximize repair. In this case, regulatory pathways that couple cell proliferation and survival such as activation of survivin might be better suited early on during the repair process.[47] It is still possible that CYGB has functions unrelated to apoptosis that necessitate high levels of expression in mature VSM cells. For example, globins regulate nitrite signaling through their nitrite reductase activity.[2,50] Liu et al[18] recently provided some evidence that in intact vessels, CYGB is a sink for NO and impact vascular tone. Alternatively, we would like to propose that CYGB might represent an important O_2, NO, and nitrite sensor to regulate VSM function. The present study reveals a role for CYGB in vascular remodeling and suggests possible regulatory roles in vascular proliferative syndromes associated with programed cell death such as vascular injury, atherosclerosis, and aneurysm.

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Disclosures

None.

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Highlights

- Cytoglobin is expressed in human and rodent vessels, primarily in differentiated medial smooth muscle cells.
- Cytoglobin expression is lost on dedifferentiation of smooth muscle cells and reexpressed in vitro on hypoxia and cytokine stimulation.
- Loss of cytoglobin inhibits neointima formation in 2 distinct models of vascular injury in rodents.
- Cytoglobin inhibits smooth muscle cell apoptosis in vivo and in vitro, through redox-dependent and nitric oxide–dependent mechanisms.