Myoglobin Protects the Heart from Inducible Nitric-oxide Synthase (iNOS)-mediated Nitrosative Stress*

The role of inducible nitric-oxide synthase (iNOS) in the pathogenesis of heart failure is still a matter of controversy. In contrast to early reports favoring a contribution of iNOS because of the negative inotropic and apoptotic potential of NO, more recent clinical and experimental data question a causative role. Here we report that transgenic mice with cardiac specific iNOS-overexpression and concomitant myoglobin-deficiency (tg-iNOS+/myo−/−) develop signs of heart failure with cardiac hypertrophy, ventricular dilatation, and interstitial fibrosis. In addition, reactivation of the fetal gene expression program typical for heart failure occurs. The structural and molecular changes are accompanied by functional depression such as reduced contractility, ejection fraction, and cardiac energetics. Our findings indicate that excessive cardiac NO formation can cause heart failure; however, under normal circumstances myoglobin constitutes the important barrier that efficiently protects the heart from nitrosative stress.

The development of human heart failure is a complex process involving activation of neuro-humoral mechanisms, down-regulation of the β-adrenergic signal transduction cascade (1), induction of proinflammatory cytokines (2), the endothelin system (3), the local renin-angiotensin system (4), and others. As a consequence, cardiac function is severely depressed and, in later stages, includes ventricular remodeling, hypertrophy, and dilatation. Among the multiple alterations associated with heart failure, the induction of myocardial expression of inducible nitric oxide (NO) synthase (iNOS; EC 1.14.13.39) has gained particular attention (5) because this “high output” isoform of NO synthases releases high levels of NO, which in vivo is not clear, and the factors that govern cardiac NO toxicity are only poorly understood.

Myoglobin, a cytosolic oxygen-binding heme protein, is highly expressed in the mammalian heart. Whereas its role in myocardial oxygen supply has been recognized for decades (15), only recently, with the generation of myoglobin knockout mice, have the additional functions of myoglobin been amenable to examination in vivo (16, 17). It has been shown that myoglobin, similarly to its relative, hemoglobin, is able to efficiently metabolizes nitric oxide in vivo, leading to an attenuation of the cardiac effects of NO (18). Given the high expression level of myoglobin in the heart (200 μM), the question arises as to which extent NO synthesized in the cytosol by iNOS is able to escape from breakdown by myoglobin and induce a pathological phenotype.

To explore the protective role of myoglobin under conditions of iNOS-induced nitrosative stress, we generated double transgenic mice deficient in myoglobin with a concomitant high level cardiac specific iNOS expression by crossing myoglobin-deficient mice (myo−/−) (17) with tg-iNOS+ mice (13). By this genetic approach, we found myoglobin to be the critical barrier that normally prevents iNOS-induced nitrosative stress from causing heart failure.

EXPERIMENTAL PROCEDURES

Mice—Mice were bred at the Tierversuchsanlage of the Heinrich-Heine-Universität, Düsseldorf, Germany. They were fed with a standard chow diet and received tap water ad libitum. Animal experiments were performed in accordance with the national guidelines on animal care and approved by the Bezirksregierung Düsseldorf. tg-iNOS+ mice (FVB strain) (13) and myo−/− mice (NMRI strain) (17) were crossed to obtain heterozygous tg-iNOS+/myo−/− mice. Double heterozygous males and females were intercrossed, and the F2 offspring was used for analysis. This approach allowed us to analyze the phenotype of both mutations independently of the different genetic backgrounds of both parent strains. The genotypes of mice were established by PCR according to published procedures (13, 17).

Histological and Expression Analysis—Mice were killed rapidly, and the ascending aorta was cannulated with a 23-gauge injection needle connected to a buffer reservoir. Hearts were briefly perfused free of blood with phosphate-buffered saline (140 mM NaCl, 10 mM NaH2PO4, pH 7.4) and fixed by perfusion with 4% buffered formalin at a perfusion

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pressure of 100 mm Hg. Hearts were excised and postfixed in the same solution overnight at 4 °C. Tissues were embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin-eosin according to standard procedures.

For isolation of RNA, hearts were rapidly excised, snap frozen in liquid nitrogen, and homogenized and extracted by the guanidinium-aci

Saline-perfused Hearts—Mice were injected intraperitoneal with 250 units of heparin and anesthetized with urethane (1.5 g x kg−1; intraperitoneal) and placed on a warming table to keep the body temperature at 37 °C throughout the experiment. The left common carotid artery was carefully prepared, and a Millar 1.4 French pressure-volume catheter was advanced through an incision in the carotid artery into the left ventricle. The catheter was connected via the AriaTM AD converter system to a personal computer. Data processing was performed with the dedicated IOX™ software (EMKA Technologies, Paris, France). Calibration of conductance catheters was performed by the high salt injection method according to published procedures. Acute systemic NOS inhibition was performed by bolus intraperitoneal injection of the NOS inhibitor ethylisothiourea (20 μg x kg−1) after recording of the basal parameters. The effective NOS inhibition was recognized by the instantaneous rise in blood pressure. Data for the “NOS inhibition series” were recorded 10 min after an ethylisothiourea injection.

Magnetic Resonance Imaging—High resolution magnetic resonance images of the beating mice hearts were acquired using a Bruker DRX 9.4 Tesla vertical bore NMR spectrometer. The mice were anesthetized with isoflurane (1.5%), an electrocardiograph was administered, and a respiration-triggered fast gradient echo (FLASH) cine sequence was applied for imaging during the whole cardiac cycle. The following parameters were used: flip angle, 45°; echo time, 1.8 ms; repetition time, 5 ms (20 frames per R-R interval); in-plane resolution, 117 × 117 μm2; field of view, 30 × 30 mm2; and matrix, 256 × 256.

Analysis—Data derived from repeated measures were analyzed by two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test using Prism 3.0 software (GraphPad) Otherwise, data were compared with Student’s unpaired t test. Differences were considered to be significant at p < 0.05.

RESULTS

Because the iNOS transgene was transmitted independently of the myoglobin locus, the breeding of double transgenic hetrozygous mice (myo−/−tg-iNOS+/− × myo−/−tg-iNOS+/−) resulted in the generation of all possible combinations of genotypes. Myoglobin expression in heterozygous mutant animals (myo−/−) as determined by densitometric analysis of Coomassie Blue-stained protein gels reaches approximately half of the wild type (WT) level (WT: 0.77 ± 0.2 arbitrary units; myo−/−, 0.34 ± 0.07 arbitrary units; n = 5–6; p < 0.05). This allowed us to study dose-dependent effects of myoglobin on structural and functional consequences induced by iNOS overexpression. In the present study we analyzed five different groups of genotypes, i.e. iNOS+/myo−/− (50% myoglobin) and iNOS+/myo−/− (no myoglobin) mice, while WT, myo−/−, and tg-iNOS+ animals served as controls.

Inspection of cardiac morphology at the age of 5 months revealed substantially enlarged hearts (Fig. 1a) and an increase of the cardiac weight index in tg-iNOS+/myo−/− mice by 33% (Fig. 1b). As can be seen, hearts of animals with all other combinations of genotypes did not deviate from the WT value. When compared with WT, myo−/−, and tg-iNOS−/− hearts (Fig. 2, a and b), the hypertrophic tg-iNOS+/myo−/− hearts developed ventricular dilatation (Fig. 2, d and e), which was revealed by in vivo magnetic resonance imaging as well as histological techniques. Ventricular dilatation was accompanied by interstitial cardiac fibrosis occurring only in tg-iNOS+/myo−/− hearts (Fig. 2f) but not in WT hearts or any other combination of genotypes, including tg-iNOS−/− hearts (Fig. 2c). We also analyzed whether the morphological changes were associated with reactivation of the fetal cardiac gene expression profile, which typically occurs during the development of hypertrophy and heart failure. Only tg-iNOS+/myo−/− hearts showed markedly elevated ventricular atrial natriuretic peptide and skeletal muscle actin expressions, which are markers for cardiac hypertrophy (Fig. 2, g and h).

We assessed cardiac function in vivo by means of a Millar 1.4
French pressure-volume catheter (Table I). Under basal conditions, the cardiac function of WT and myo−/− and tg-iNOS+ mice was not different with respect to heart rate, LVDP, cardiac output, or end-systolic and end-diastolic volumes. tg-iNOS+ mice displayed only a reduced dP/dt min (1100 mm Hg s−1) when compared with WT. However, when myoglobin was absent in addition to iNOS overexpression, several other parameters were also changed; for example, contractility (dP/dt max) decreased by 33%, and the dP/dt max by 38% (Fig. 4). Assessment of energetic parameters revealed that oxygen consumption (VO2) in tg-iNOS+/myo−/− hearts was decreased by 25%. This reduction was accompanied by a decrease of phosphocreatine (PCr) levels (25%) and a doubling of inorganic phosphate (Pi) (Fig. 4). Free ADP levels, as calculated from the creatine phosphokinase equilibrium constant, increased by 37% (data not shown).

To directly assess the role of myoglobin in NO breakdown, we analyzed cardiac NO metabolites NO2− and NO3− in coronary venous effluents of isolated hearts. As shown in Fig. 5, the predominant NO metabolite released from tg-iNOS+ hearts was NO3−, because NO3− by far exceeded NO2− release. The same result was obtained for tg-iNOS+/myo−/− hearts. However, when myoglobin was completely lacking (tg-iNOS+/myo−/−), NO was preferentially converted to nitrite, reaching 50% of cardiac NOx formation.

DISCUSSION

Using a double transgenic model, we present conclusive evidence that nitrosative stress induced by massive cardiac overexpression of iNOS is efficiently attenuated by myoglobin. This clearly demonstrates the cardioprotective potential of this heme protein, which is highly expressed in the heart. Our findings have important implications for the concept that cardiac iNOS expression, frequently associated with heart failure, might be causally involved in the development of this disease.

Using a genetic approach, we generated transgenic mice with a high level of cardiac specific overexpression of iNOS and different levels of myoglobin ranging from 100% (myo+/+) to 50% (myo+/−) to 0% (myo−/−). Only mice with a complete lack of myoglobin developed signs of heart failure characterized by the following: 1) increased cardiac weight index; 2) ventricular dilation; and 3) reduced ejection fraction. These alterations were associated with ventricular fibrosis and a reactivation of the fetal gene expression program typically observed in cardiac hypertrophy and heart failure. Because hearts from tg-iNOS+/myo−/− mice expressing only 50% of WT myoglobin levels do not show the detrimental consequences of iNOS overexpression, myoglobin, even at reduced levels, acts as an efficient scavenger of NO and protects the heart. Thus, tg-iNOS+/...
Top, basal conditions. Bottom, NOS inhibition by ethylisothiourea. Values represent means ± S.D. of the number of experiments indicated in parentheses.

| Basal conditions | WT (12) | myo<sup>-/-</sup> (15) | tg-iNOS<sup>-/-</sup> (12) | tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> (12) |
|------------------|---------|------------------------|---------------------------|----------------------------------|
| HR (beats/min)  | 579 ± 67| 583 ± 73               | 559 ± 42                  | 559 ± 61                         |
| LVDP (mm Hg)    | 88 ± 7.4| 84 ± 8                 | 88 ± 8                    | 83 ± 8                           |
| Ves (µL)        | 11.0 ± 5.3| 12 ± 4                 | 13 ± 5                    | 13 ± 6                           |
| Ved (µL)        | 25.1 ± 7.8| 27 ± 7                 | 28 ± 6                    | 26 ± 6                           |
| SV (µL)         | 14.4 ± 4.0| 15 ± 5                 | 15 ± 5                    | 15 ± 5                           |
| EF (%)          | 61 ± 14  | 56 ± 13                | 54 ± 12                   | 31 ± 11                          |
| CO (µl/min)     | 8324 ± 2238 | 8840 ± 2396         | 8319 ± 2848               | 6034 ± 2282                      |
| dP/dt<sub>max</sub> (mm Hg/s) | 10838 ± 1519 | 10110 ± 1538 | 9943 ± 1648 | 7254 ± 2205<sup>a</sup> |
| dP/dt<sub>min</sub> (mm Hg/s) | −6942 ± 979  | −6362 ± 1545 | −5783 ± 1169<sup>b</sup> | −5385 ± 1118<sup>b</sup> |
| NOS inhibition  |         |                        |                          |                                  |
| HR (beats/min)  | 546 ± 97  | 532 ± 59               | 504 ± 52                  | 564 ± 52                         |
| LVDP (mm Hg)    | 104 ± 21<sup>a</sup> | 110 ± 20<sup>a</sup> | 99 ± 18<sup>b</sup>       | 102 ± 27<sup>b</sup>             |
| Ves (µL)        | 17 ± 7<sup>b</sup> | 17 ± 3<sup>b</sup>       | 18 ± 5<sup>b</sup>         | 28 ± 4<sup>b</sup>               |
| Ved (µL)        | 29 ± 11   | 27 ± 4                 | 31 ± 6                    | 35 ± 4                           |
| SV (µL)         | 12 ± 7    | 10 ± 2<sup>a</sup>      | 14 ± 6                    | 6 ± 2                            |
| EP (%)          | 40 ± 14<sup>b</sup> | 37 ± 5<sup>b</sup>       | 43 ± 13<sup>b</sup>       | 6 ± 2                            |
| CO (µl/min)     | 6120 ± 2773 | 5930 ± 1097<sup>a</sup> | 6267 ± 2979             | 3489 ± 1111<sup>b</sup>         |
| dP/dt<sub>max</sub> (mm Hg/s) | 11433 ± 3059 | 11396 ± 1497 | 10691 ± 2039 | 8465 ± 3446<sup>b</sup> |
| dP/dt<sub>min</sub> (mm Hg/s) | −8783 ± 1655<sup>b</sup> | −8272 ± 2144<sup>b</sup> | −7702 ± 2055<sup>b</sup> | −7483 ± 2798<sup>b</sup> |

<sup>a</sup> p < 0.05 versus WT.  
<sup>b</sup> p < 0.05 versus basal conditions.

myo<sup>-/-</sup> mice can be viewed as the first model that allows us to study the consequences of chronic iNOS-derived NO formation on cardiac function and morphology without confounding influences of cardiac NO metabolism.

Analysis of cardiac function both under in vivo and in vitro conditions revealed substantial functional depression in tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> double transgenic hearts as characterized by a reduction of LVDP and dP/dt<sub>max</sub>, ejection fraction, and cardiac output. In addition, we observed a shift of the pressure-volume loops toward higher volumes reflecting ventricular dilatation, which was confirmed by histological and magnetic resonance imaging (MRI) analyses. It is important to note that even reduced myoglobin levels in tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> hearts were sufficient to completely abolish all of these alterations.

The cardiac phenotype of tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> mice is the result of long term NO-induced structural changes with cardiac fibrosis together with the immediate cardiodepressive action of the continuously formed NO. In fact, when NO synthases were inhibited acutely by ethylisothiourea, a partial recovery of contractility was observed. However, because of myocardial remodeling, WT levels of cardiac function were not restored.

The observed cardiodepression in tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> mice may be the result of cGMP-dependent mechanisms (5) or the consequence of cGMP-independent actions such as inhibition of the respiratory chain (20). Interestingly, 31P-NMR spectroscopy revealed a reduction of cardiac creatine phosphate levels associated with an increase in [P<sub>i</sub>] and calculated [ADP]. This change in cardiac energetics was accompanied by a reduction of oxygen consumption. Thus, when myoglobin is lacking, iNOS-derived NO is likely to critically interfere with the respiratory chain, leading to attenuation of the phosphorylation potential. Inhibition of complex IV of the respiratory chain by NO is a well studied mechanism that occurs at nanomolar concentrations of NO at isolated mitochondria (21). Modulation of cardiac oxygen consumption by NO was also reported for the dog heart in situ (22), isolated guinea pig heart (23), and myocardial tissue pieces (24, 25). Taken together, our findings strongly suggest that myoglobin efficiently protects mitochondria from NO even when synthesized at substantially elevated rates. Thus, myoglobin is likely to keep cardiac NO levels below the critical concentration required to alter mitochondrial function. It is also conceivable that the long term depression of cardiac function and energetics in tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> hearts may have triggered the induction of hypertrophy in order to compensate for the reduced cardiac efficacy.

Further support for the NO protective action of myoglobin comes from direct measurements of NO metabolites released by the heart. Whereas NO<sub>3</sub> was the predominant cardiac NO metabolite in tg-iNOS<sup>-/-</sup> and tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> hearts, approximately equal levels of NO<sub>2</sub> and NO<sub>3</sub> were released by tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> hearts. NO<sub>3</sub> is the major NO metabolite formed in reaction with cyanogenyl heme proteins such as myoglobin. The shift toward nitrite supports the view that a substantially higher proportion of NO was released from tg-iNOS<sup>-/-</sup>/myo<sup>-/-</sup> hearts, which, according to the well known reaction shown in Reaction 1,

\[ 4\text{NO} + \text{O}_2 + 2\text{H}_2\text{O} \rightarrow 4\text{NO}_2 + 4\text{H}^+ \]

Reaction 1

might have reacted with oxygen dissolved in huge amounts in the perfusion buffer to form nitrite (26). Furthermore, it has been proposed that NO in reaction with cytochrome c oxidase may be converted to nitrite (27). Because we have shown that the respiratory chain function is affected by NO in the absence of myoglobin, it is conceivable that this reaction may gain...
FIG. 4. Functional and metabolic parameters measured in isolated perfused hearts. Bars represent means ± S.D. of n = 6 experiments in each group. CPI, coronary perfusion pressure; P(CP), creatine phosphate; VO₂, oxygen consumption; black bars, WT; white bars, myo−/−; black/hatched bars, tg-iNOS−/−; grey hatched bars, tg-iNOS/myo−/−; white hatched bars, tg-iNOS/myo−/−. **, p < 0.01 versus all other genotypes.

FIG. 5. Cardiac NO₂/NOx release. Bars represent mean ± S.D. of n = 5 experiments each. Black bars, NO₂ release; gray bars, NOx release (NO₂ + NO₃). Note that basal cardiac NO₂ and NOx release by WT hearts as well as NO₂ release by tg-iNOS−/− and tg-iNOS/myo−/− hearts were at the detection limits resulting in no reliable measurements of basal NO metabolites. **, p < 0.01 versus WT.

impact in tg-iNOS−/−/myo−/− hearts and that it contributes to the elevated cardiac NO₂ formation. Thus, our findings provide a mechanistic explanation for the unexpectedly benign phenotype of mice with the cardiac specific iNOS overexpression recently reported by us (13).

Compartmentalization is an emerging theme in NO-mediated signal transduction (28); but because of the high diffusion velocity of NO, it remains widely unclear how a specific function can be exerted by different NOS isoforms coexpressed within the same cell. In cardiac myocytes, endothelial NOS localized at caveolae of the sarcolemma has been shown to attenuate β-adrenergic stimulation (29). On the other hand, neuronal NOS associated with the ryanodine receptor at the sarcoplasmic reticulum was reported to augment contractility (29). Myoglobin, with its high capacity to inactivate NO, most likely constitutes a cytoplasmic barrier that prevents spillover of NO from one compartment to another and therefore enables a specific localized action of NO.

In addition to the cardiodepressive effects of iNOS overexpression, which specifically occur in the absence of myoglobin (tg-iNOS−/−/myo−/−), we also found biological effects of iNOS overexpression even in the presence of unaltered cardiac myoglobin levels (tg-iNOS−/−). Ex vivo data revealed a pronounced vasodilation when iNOS was stimulated by l-arginine (Fig. 4), demonstrating that sufficient amounts of bioactive NO reached the smooth muscle cells. In addition, our in vivo measurements revealed that cardiac iNOS expression reduced dP/dtₘᵢᵣₑ and therefore reduced diastolic relaxation (Table I). This finding is in contrast to the usually observed relaxation hastening effect of NO in the myocardium (30). The molecular basis for this effect is presently unclear, but activation of the ryanodine receptor by S-nitrosylation and consequently prolonged Ca²⁺-transients might contribute to this phenomenon (31).

Given the crucial role of myoglobin in degrading cardiac NO, it is conceivable that reduced levels of myoglobin increase nitrosative stress and thereby may participate in the development of heart failure. In fact, proteome analysis repeatedly has demonstrated that myoglobin expression is down-regulated in experimental and human heart failure by 50%, but the pathophysiological role of this finding remained obscure (32, 33). However, using mice heterozygous for myoglobin that express only 50% of the WT levels, we show that neither structural, functional, or energetic parameters are compromised. Thus, even reduced levels of cardiac myoglobin expression appear to be sufficient to maintain the mild phenotype as observed with normal myoglobin expression (13).

The concept that iNOS expression might cause heart failure was initially triggered by observations of De Belder et al. (9), who reported elevated iNOS activity in cardiac biopsy specimens from patients with dilative cardiomyopathy. Later, several studies demonstrated iNOS expression or activity in hearts from patients with dilative cardiomyopathy, myocarditis, and ischemic cardiomyopathy (34–36). Based on our data, it is highly unlikely that iNOS may be an important pathological factor in the development of heart failure. In our transgenic model, in vivo NOS activity by far exceeds the values reported for human heart failure (9, 13, 35), and, therefore, the iNOS-mediated phenotypical alterations should be more pronounced than with lower iNOS expression levels in the human situation. Further arguments against a critical role of iNOS in heart failure development come from transgenic mice with cardiac specific overexpression of tumor necrosis factor α (TNF-α), a pro-inflammatory cytokine that is elevated in heart failure (37). These mice are characterized by cardiac hypertrophy, myocardial inflammation, and high mortality. Importantly, breeding of these mice into an iNOS-free background does not improve any of these parameters (38). Thus, cytokines appear to be primary pathophysiological factors causing heart failure.
by iNOS-independent mechanisms.

The dual role of heme proteins to react with NO and oxygen can be found in organisms as different as bacteria (39, 40), nematodes (41), and mammals (18, 42). However, the primary function of the individual heme protein seems to have changed during evolution, and either a protective role against nitrosative stress (39), a transport vehicle for NO (42), or an NO-activated “deoxygense” consuming O2 in an NO-dependent manner (41) have been postulated. The cardioprotective function of myoglobin may provide a rationale for the evolutionary conservation of myoglobin. Seen primarily as a factor involved in oxygen transport and storage, it remained unclear to which extent these oxygen-related functions might be essential for survival (43). However, the data presented here clearly demonstrate that myoglobin efficiently shields mitochondria from cytoplastically generated NO. Thus, myoglobin behaves like an NO oxidase and therefore might be reconsidered to represent a multifunctional protein with cardioprotective potential rather than a pure oxygen store.

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