Inducible Nitric Oxide Synthase Expression and Cardiomyocyte Dysfunction During Sustained Moderate Ischemia in Pigs

Frank R. Heinzel, Petra Gres, Kerstin Boengler, Alexej Duschin, Ina Konietzka, Tienush Rassaf, Julia Snedovskaya, Stephanie Meyer, Andreas Skyschally, Malte Kelm, Gerd Heusch, Rainer Schulz

Abstract—In acute myocardial ischemia, regional blood flow and function are proportionally reduced. With prolongation of ischemia, function further declines at unchanged blood flow. We studied the involvement of an inflammatory signal cascade in such progressive dysfunction and whether dysfunction is intrinsic to cardiomyocytes. In 10 pigs, ischemia was induced by adjusting inflow into the cannulated left anterior coronary artery to reduce coronary arterial pressure to 45 mm Hg (ISCH); 4 pigs received the inducible nitric oxide synthase (iNOS) inhibitors aminoguanidine or L-N\textsuperscript{6}-(1-iminoethyl)-lysine during ISCH (ISCH+iNOS-Inhib); 6 pigs served as controls (SHAM). Anterior (AW) and posterior (PW) systolic wall thickening (sonomicrometry) were measured. After 6 hours, nitric oxide (NO) synthase (NOS) protein expression, NO activity, and NO metabolites (nitrite/nitrate/nitroso species) were quantified in biopsies isolated from AW and PW. Cardiomyocyte shortening and intracellular calcium (Indo-1 acetoxymethyl ester) were measured without and with the NOS substrate L-arginine (100 μmol/L). In ISCH, AW wall thickening decreased from 42±4% (baseline) to 16±3% (6 hours). Wall thickening remained unchanged in ISCH-PW and SHAM-AW/PW. NOS2 (iNOS) protein expression and activity, but not NOS3 (endothelial NO synthase), were increased in ISCH-AW and ISCH-PW. iNOS expression correlated with increased nitrite contents. Cardiomyocyte shortening was reduced in ISCH-AW versus SHAM-AW (4.4±0.3% versus 5.6±0.3%). L-Arginine reduced cardiomyocyte shortening further in ISCH-AW (to 2.8±0.2%) and ISCH-PW (3.4±0.4% versus 5.4±0.4%) but not in SHAM or in ISCH+iNOS-Inhib; intracellular [Ca\textsuperscript{2+}] remained unchanged. With L-arginine, in vitro AW cardiomyocyte shortening correlated with in vivo AW wall thickening (r=0.72). In conclusion, sustained regional ischemia induces myocardial iNOS expression in pigs, which contributes to contractile dysfunction at the cardiomyocyte level. (Circ Res. 2008;103:1120-1127.)

Key Words: myocardial ischemia ■ nitric oxide ■ iNOS ■ sphingosine ■ TNF-α

A cute reduction of regional coronary blood flow decreases regional myocardial contractile function.\textsuperscript{1} With prolonged moderate ischemia (≥6 hours), contractile function further deteriorates despite a stable reduction in coronary blood flow.\textsuperscript{2,3} A reduction in cardiac contractile function can result from changes in external mechanical loading. Impaired myocardial function may also reflect alterations within the myocardiun, such as changes in extracellular space composition, nonmyocytic cellular compartments, or the cardiomyocyte itself. Indeed, during sustained ischemia, deterioration of contractile function is associated with a decreased responsiveness to external calcium and reduced postextrasystolic potentiation in vivo,\textsuperscript{2,4} suggesting dysfunction at the level of the cardiomyocyte. The aim of our present study was to identify first whether or not contractile dysfunction after sustained ischemia is intrinsic to cardiomyocytes.

Nitric oxide (NO) is generated in cardiomyocytes from L-arginine by NO synthases (NOS) and has been identified as an endogenous regulator of myocardial function.\textsuperscript{5} Increased NO production, as a result of increased expression of the inducible NOS isoform (iNOS), decreases myocardial function following myocardial infarction,\textsuperscript{b} in heart failure,\textsuperscript{7,9} and in human hibernating myocardium.\textsuperscript{10} iNOS expression can be triggered by proinflammatory cytokines such as tumor necrosis factor (TNF)-α,\textsuperscript{11-12} which is functionally important in myocardial ischemia.\textsuperscript{13} Although increased levels of TNF-α may be a trigger for increased iNOS activity, TNF-α also exerts iNOS-independent negative inotropic effects via sphingosine, an intermediate of the sphingomyelin pathway,\textsuperscript{14} as observed following coronary microembolization.\textsuperscript{15,16}

Thus, as a second aim, we examined whether a reduction in cardiomyocyte function depends on increased iNOS expres-
sion and quantified TNF-α tissue content as a potential trigger of iNOS or sphingosine expression during sustained ischemia.

**Materials and Methods**

**In Vivo Model**

The experimental protocols used in this study were approved by the local bioethics committee and adhered to the guiding principles of the American Physiological Society.

Sixteen Götttinger miniswine (20 to 40 kg) of either sex were anesthetized and instrumented as described previously and as detailed in the online data supplement, available at http://circres.ahajournals.org. Left ventricular pressures were measured by a micromanometer, and anterior wall (AW) and posterior wall (PW) thickening (WTh) were measured by sonomicrometry. Heart rate was controlled by atrial pacing. The proximal left anterior descending coronary artery was cannulated, and coronary blood flow was adjusted so that the minimum coronary artery pressure was not less than 70 mm Hg under control conditions to avoid initial hypoperfusion. Regional myocardial work was estimated by a regional myocardial work index, as described in the online data supplement.

After measurements of systemic hemodynamics and regional function at baseline, left anterior descending inflow was decreased to achieve a reduction in coronary artery pressure to ~45 mm Hg (ISCH, N=10), whereas coronary artery pressure was maintained at baseline levels in SHAM (N=6). Measurements were repeated at 5 minutes and 6 hours. In a subgroup of ISCH animals (ISCH+iNOS-Inhib), continuous application of an iNOS inhibitor [aminoguanidine (AG), 1 mg/kg per minute, N=3; L-N(1-iminoethyl)lysine (L-NIL), 1 mg/kg per hour following a bolus of 0.5 mg/kg; N=1] through a peripheral vein was initiated after the baseline measurement, 30 minutes before the start of hypoperfusion, and continued until the end of the experiment. At 6 hours, myocardial biopsies were taken from the anterior and posterior (control) wall and an apical section of the heart containing parts of the hypoperfused AW and PW was embedded in paraffin for immunohistochemistry.

In parallel experiments, sham-operated animals (N=4) received a continuous application of the iNOS inhibitor AG (1 mg/kg per minute) for 6 hours through a peripheral vein following the baseline measurements, and the coronary endothelial response to bradykinin, a potent agonist of endothelial (e)NOS-mediated vasodilatation, was assessed as detailed in the online data supplement.

**Isolation of Cardiomyocytes**

Ventricular cardiomyocytes were obtained by enzymatic digestion simultaneously from the AW and PW (see the online data supplement for details) and used for measurements within 12 hours after isolation.

**Measurement of Contractile Function in Isolated Cardiomyocytes**

As detailed in the online data supplement, cardiomyocytes were loaded with the Ca2+ indicator Indo-1 acetoxyethyl ester (2.5 μmol/L), superfused with Tyrode's solution at 37°C and electrically stimulated (1 Hz). Cell shortening was quantified as percentage of total cell length. L-Arginine (100 μmol/L; Sigma-Aldrich, Seelze, Germany) was washed in via a rapid perfusion system, and recordings were performed during cell shortening steady state after 5 minutes. In cardiomyocytes isolated from ISCH+iNOS-Inhib, aminoguanidine was added to the bath solution following L-arginine and another measurement was recorded.

**Immunohistochemistry**

Confocal images were obtained with a laser scanning microscope (Zeiss LSM Pascal) at ×400 magnification from iNOS, eNOS, phospho-eNOS, and TNF-α antibody-stained biopsy sections, and leukocytes were quantified in additional, hematoxylin/eosin-stained tissue sections from the myocardial biopsies (see the online data supplement for a detailed description).

**iNOS, eNOS, and TNF-α Protein Expression**

Western blots of iNOS, eNOS, and phospho-eNOS were performed as described previously, and results were normalized to GAPDH protein expression (iNOS) or to Ponceau S staining (eNOS). TNF-α content was determined by enzyme linked immunosorbent assay (ELISA) using a commercially available porcine ELISA kit (R&D Systems, Minneapolis, Minn) (see also the online data supplement for details).

**NOS Activity Measurements**

The activities of NOSs were determined in myocardial tissue samples using a commercially available NOS activity assay kit (No 781001, Cayman Chemical Company, Ann Arbor, Mich). Total NOS activity is expressed as percent of [1H]-arginine (substrate) converted to [1H]-citrulline (product). 1400 W (a selective iNOS inhibitor) was used in parallel experiments to determine iNOS-dependent activity (see the online data supplement for details).

**Determination of Tissue Nitroso Species and Nitrite/Nitrate Content**

Nitrite (NO2−) is a major oxidative metabolite of NO21 and nitrite levels represent a sensitive measure of tissue NO formation.22 Myocardial nitrite and nitroso species contents were determined using a triiodide/ozone-based reductive gas phase chemiluminescence assay, essentially as described previously23 and in the online data supplement. Nitrate was quantified after enzymatic reduction to nitrite by nitrate reductase.22

**Sphingosine**

Myocardial sphingosine content was measured by high-performance liquid chromatography using the method of Merrill et al.24 See the online data supplement.

**Data Analysis and Statistics**

Data are reported as mean values ± SEM. Statistical analysis comprised ANOVA for repeated measures for the time course of hemodynamic parameters (ISCH); 2-way ANOVA was used to compare hemodynamic data from all groups (SHAM, ISCH, ISCH+iNOS-Inhib) at baseline and 6 hours, to compare iNOS protein expression, NOS activities, and immunohistochemistry data, as well as cell shortening data (within each group, and also separate measurements for AW and PW across groups). When a significant overall effect was detected, Fisher least-significant tests were performed to compare single mean values. Correlation analyses were performed using Pearson’s correlation coefficients. P<0.05 was considered significant.

**Results**

**Hemodynamics and Regional Myocardial Function**

During hypoperfusion, AW WTh rapidly decreased by 23.2±3.6% of baseline at 5 minutes and had further deteriorated at 6 hours of hypoperfusion (supplemental Table 1). Anterior wall index of AW work (AW work index) decreased to 76.8±3.6% and 28.8±5.6% of baseline at 5 minutes and 6 hours of hypoperfusion, respectively (both P<0.05), associated with a reduction in left ventricular peak pressure (LVPP) and LV dP/dt and a pronounced increase in left ventricular end-diastolic pressure (LVEDP) (supplemental Table 1). Posterior (remote) wall function remained stable early during hypoperfusion. At 6 hours of hypoperfusion, however, moderate dysfunction of the PW was reflected by a decrease in work index to 73.9±13.4% of baseline (P<0.05). A slight increase in LVEDP was also found in sham-operated animals.
Except for a small decrease in work index (to 82.7 ± 3.9% of baseline) of the cannulated AW (P < 0.05 versus 6 hours of ISCH), no changes in regional myocardial function were observed in SHAM.

In the presence of the iNOS inhibitors (ISCH+iNOS-Inhib), AW and PW function tended to be lower than in ISCH (at 6 hours: AW WTh, 8.9 ± 2; PW WTh, 16.0 ± 1.8%; compare with supplemental Table 1). Two of the animals receiving AG developed severe systemic lactic acidosis (arterial lactate concentration (supplemental Figure III).

In parallel experiments on sham-operated animals, continuous infusion of AG for 6 hours had no effect on AW WTh, LVPP, or coronary artery pressure at constant cerebral blood flow (Figure 1B in the online data supplement). The dose–response relationship during incremental intracoronary bradykinin application at baseline and at 6 hours of AG infusion did not differ in EC50 and maximal bradykinin-induced coronary blood flow (supplemental Figure IC through IE). During AG infusion, we observed a time-dependent increase in the arterial lactate concentration (supplemental Figure III).

Contractile Function in Isolated Cardiomyocytes

The isolation procedure yielded 30% to 70% rod-shaped, well-striated quiescent cardiomyocytes, with no obvious differences between groups. In the absence of L-arginine, in vitro cell shortening in cardiomyocytes from the AW, but not from the PW, was slightly reduced in ISCH as compared to SHAM (Figure 1; n = 20 for each group and region). Addition of 100 μmol/L L-arginine to the bath led to a significant further reduction in cell shortening in both ISCH-AW and ISCH-PW cells but not in SHAM. In cardiomyocytes from ISCH+iNOS inhibitor (n = 11 for each region from N = 4 animals), cell shortening was not reduced versus SHAM, and L-arginine had no effect (Figure 1). Also, in the presence of L-arginine, cell shortening was not changed by wash-in of aminoguanidine (5.3 ± 0.6% with 1 mmol/L AG + L-arginine versus 5.2 ± 0.5% with L-arginine alone).

The diastolic and systolic [Ca2+]i, in cardiomyocytes isolated from the AW following 6 hours of hypoperfusion were not different from those measured in cardiomyocytes isolated from the PW (remote) myocardium (96 ± 12% and 93 ± 9% of PW, respectively); similarly, systolic and diastolic [Ca2+]i, did not differ in cardiomyocytes isolated from the AW and PW of sham animals (AW: 87 ± 9% and 91 ± 9% of PW, respectively). [Ca2+]i, in both groups of pigs was unaffected by L-arginine (n = 6 from N = 3 for each group and myocardial region, with and without L-arginine).

iNOS and eNOS Expression

Following 6 hours of hypoperfusion, histological sections showed a homogeneously distributed increase in iNOS-dependent immunofluorescence in the myocardial tissue of the hypoperfused AW, as well as in the remote PW, as compared to sham-operated animals (Figure 2A and 2B). eNOS-dependent immunofluorescence was not different between the groups (in arbitrary units): for ISCH: 1336 ± 31 in AW and 1214 ± 34 in PW; for SHAM 1436 ± 38 in AW and 1310 ± 46 in PW. No significant changes in phospho-eNOS–dependent immunofluorescence were detected: for ISCH, 942 ± 21 in AW and 933 ± 21 in PW; for SHAM, 911 ± 11 in AW and 966 ± 21 in PW.

Inos protein expression, as measured by Western blot densitometry and normalized to GAPDH protein expression, was 2.5-fold higher in the AW of ISCH versus sham-operated animals (P < 0.05) and tended to be elevated in the remote PW of ISCH animals (Figure 2D). GAPDH signal density was not significantly different between groups. The ratio of phospho-eNOS over eNOS between AW and PW was not different between ISCH (2.46 ± 0.72) and ISCH (2.60 ± 0.94). Similarly, the ratio of eNOS in AW over PW was not different between ISCH and SHAM (supplemental Figure II).

Leukocyte counts within the myocardial biopsies from a subgroup of animals (N = 5 in SHAM, N = 4 in ISCH) were not different between SHAM and ISCH or between AW and PW (SHAM-AW, 186 ± 26/mm3; SHAM-PW, 169 ± 10/mm3; ISCH-AW, 138 ± 11/mm3; ISCH-PW, 156 ± 6/mm3).

iNOS Activity

iNOS activity amounted to approximately one-third of total NOS activity in SHAM (35.9 ± 11.4% in SHAM-AW and 29.9 ± 17% in SHAM-PW). iNOS activity tended to be higher in ISCH-AW and was significantly higher in ISCH-PW (Figure 2E). Total NOS activity was not significantly different between groups (SHAM-AW, 24.6 ± 5.0%; SHAM-PW, 18.3 ± 3.6%; ISCH-AW, 26.3 ± 4.5%; ISCH-PW, 28.8 ± 5.5%).

Tissue Nitroso Species and Nitrite/Nitrate Content

Tissue concentrations of NO-derived nitroso species were significantly increased in the AW versus PW in animals with hypoperfusion (292 ± 96 versus 190 ± 48 mmol/L, P < 0.05) but not in SHAM (158 ± 21 versus 123 ± 19 mmol/L). Chemical luminescence measurements of the NO metabolite nitrite correlated with iNOS protein expression measured in histological sections (Figure 3A) and homogenates (Figure 3B) of the myocardial regions, reflecting increased NO production with increased iNOS expression. Regional myocardial WTh
and work index were inversely correlated with myocardial nitrite levels (Figure 3C and 3D). The cellular nitrate pool remained unchanged.

In cardiomyocytes isolated from the anterior wall, addition of the iNOS substrate L-arginine established a closer relationship between AW WTh in vivo and cardiomyocyte shortening in vitro (Figure 4).

**Figure 2.** iNOS protein expression (immunohistochemistry) in the anterior and posterior ventricular wall of ISCH and SHAM. A, Representative confocal recordings of tissue immunofluorescence. B, Quantitative analysis of iNOS-specific tissue immunofluorescence (see Materials and Methods for details). *P<0.05 vs SHAM. C and D, iNOS protein expression in the anterior and posterior ventricular wall of ISCH and SHAM (C, example; D, densitometry results). E, NOS activity in tissue homogenates ([3H]-citrulline scintillation counts as percent of [3H]-arginine scintillation counts, see Methods for details). Contribution of iNOS-activity (filled bars) was assessed using the specific iNOS inhibitor 1400W. *P<0.05 vs SHAM.

**Figure 3.** A and B, Tissue concentration of the NO metabolite nitrite as a function of iNOS protein concentration in iNOS immunofluorescence in histological sections (A) and homogenates (Western blot) (B) of the same region. C and D, Relationship between systolic regional WTh (C) or regional work index (D) in situ and nitrite concentration.

**TNF-α and Sphingosine**

In the myocardium of sham-operated animals, TNF-α was mainly distributed around vessels, as shown by immunohistochemistry (Figure 5). In hearts subjected to 6 hours of ischemia, the histological sections revealed a patchy distribution of TNF-α within the myocardial tissue of the AW and PW (Figure 5, center images).
Ischemia, however, did not significantly affect the total TNF-α protein concentration (in pg/g: SHAM, 218.4 ± 37.3 in AW and 231.4 ± 66.8 in PW; ISCH, 187.7 ± 68.3 in AW and 136.2 ± 48.9 in PW) or the sphingosine concentration (in pmol/L/g wet weight: SHAM, 298 ± 27 in AW and 294 ± 31 in PW; ISCH, 331 ± 19 in AW and 314 ± 27 in PW) in the myocardial tissue.

**Discussion**

In this study, we demonstrate an increase in myocardial iNOS expression following 6 hours of regional ischemia and a l-arginine–dependent contractile dysfunction intrinsic to cardiomyocytes from the ischemic region.

During acute myocardial ischemia, contractile function in the ischemic region rapidly decreases. With prolongation of ischemia, contractile function further decreases despite a constantly reduced coronary blood flow and preserved tissue viability. By measuring contractile function of isolated cardiomyocytes, this study focused on cellular changes that occur during more sustained ischemia independent from the interstitial milieu. With the supplementation of 100 μmol/L of the NOS substrate l-arginine to the cell bath, a concentration measured in human blood plasma, a close relationship between contractile function in situ and in isolated cardiomyocytes was observed (Figure 4). The cardiomyocytes showing the largest decrease in cell shortening in response to l-arginine (left most data point in Figure 4), thereby having the largest isolated impact on the in vivo/in vitro contractile function relationship were from the animal with the most pronounced ischemia-induced regional dysfunction (AW WTh from 35.8% at baseline to 3.5% at 6 hours of ischemia).

Our findings suggest a key role of NOS activity for inducing contractile dysfunction on the level of the cardiomyocyte during sustained ischemia. Decreased contractile function of cardiomyocytes from the hypoperfused AW during stimulation at 1 Hz was not associated with alterations in diastolic or peak systolic [Ca2+]i. In line with an NO-dependent reduction in myofilament Ca2+ sensitivity, previous studies using the same animal model showed reduced Ca2+ responsiveness in vivo without alterations in Ca2+ handling proteins or troponin I levels following 90 minutes or 12 hours of moderate hypoperfusion.

NO has evolved as an important modulator of cardiac contractile function. eNOS and iNOS, 2 NOS isoforms, which can modulate myocardial function, are expressed within cardiomyocytes. iNOS (in contrast to eNOS) is substrate-limited, and once activated, it can produce high amounts of NO, which may account for the reduced contractile function observed in vitro in the presence of l-arginine. Increased concentrations of iNOS mRNA and iNOS protein have been found in many, albeit not all, studies on nonischemic, as well as ischemic, heart failure, including human cardiomyopathy and human hibernating myocardium. During regional myocardial ischemia, an initial increase in NOS activity was observed within minutes following coronary artery occlusion. In a rat model of septic cardiomyopathy, induction of iNOS mRNA was detected as early as 30 minutes following injection of endotoxin, resulting in a peak iNOS enzyme activity after 6 hours.

**Figure 4.** Relationship between systolic AW WTh in situ and cell shortening of cardiomyocytes from the AW in vitro (ISCH and SHAM) in the absence (P=NS) (left) or presence (P<0.05) (right) of 100 μmol/L l-arginine.

**Figure 5.** TNF-α protein expression (immunohistochemistry) in the anterior and posterior ventricular wall of ISCH and SHAM and negative control.
iNOS protein expression was increased during sustained ischemia in the present study, and the concomitantly increased levels of nitrite and nitroso species are in line with a higher iNOS activity and NO production. A specific activation of the inducible NOS isoform is supported by the iNOS activity measurements (Figure 2E) using the iNOS-specific inhibitor 1400W.\(^3\) Furthermore, following in vivo application of AG, contractile function at the cardiomyocyte level was preserved in ISCH, whereas the eNOS-mediated endothelial response to bradykinin was not affected. AG had no effect on cerebral blood flow, AW WTh, and LVPP in sham-operated animals. This contrasts well with the effects of L-NNA, a NOS inhibitor also directed against eNOS,\(^3\) in the same animal model in an earlier study,\(^3\) which showed pronounced effects on endothelial and regional myocardial function (supplemental Figure 1A). In line with an iNOS-mediated dysfunction, we demonstrated increased iNOS activity during sustained ischemia and found that tissue nitrite concentrations as a surrogate of NO production correlated inversely with in vivo regional function (Figure 3C and 3D). Total NOS activity was not different between groups, implying that the activity of the constitutive NOS isoforms eNOS and neuronal NOS in the tissue homogenates may have been reduced during sustained ischemia. Indeed, accumulation of asymmetric dimethylarginine, which occurs after 30 minutes of ischemia, could have suppressed eNOS activity, thereby shifting L-arginine toward iNOS and increasing its activity.\(^3\)

Apart from NOS-derived NO, we have recently demonstrated NOS-independent NO accumulation within the myocardial interstitium during myocardial ischemia in vivo.\(^3\) Although NOS-independent NO production may thus have contributed to nitrite formation during ischemia, cellular contractile dysfunction triggered by L-arginine in ISCH was reversible by iNOS inhibition, suggesting that iNOS-dependent NO formation confined to the cardiomyocyte was most important for the measured functional alterations.

iNOS protein content was also increased in confocal cardiomyocyte cross-sections from the posterior (remote) wall of ischemic hearts. Myocardial nitrite levels in ISCH-PW were ranging between values from sham-operated animals and the hypoperfused AW. Thus, not only regional metabolic processes during hypoperfusion but also increased mechanical stress (as reflected by the pronounced increase in LVEDP during sustained ischemia, supplemental Table I) may have triggered increased iNOS expression, analogous to observations in human dilative cardiomyopathy.\(^9\)

Whereas regional function in the remote region initially remained stable, increased iNOS levels at 6 hours of hypoperfusion were associated with a decrease in contractile function in the PW. Systolic WTh was higher in the AW versus PW, a physiological phenomenon that has been reported earlier and is discussed in more detail in the online data supplement.

We and others have previously shown that systemic NOS inhibition (with L-NNA) decreases myocardial contractile function during normoperfusion and myocardial ischemia in vivo.\(^3\) In a previous study, L-NNA decreased regional myocardial function to a similar extent during normoperfusion and ischemia (as indicated by a parallel downward shift of the relationship between external work and transmural blood flow), suggesting that NO production from constitutively active NOS has a beneficial effect on myocardial function in vivo (as recently confirmed also in humans\(^8\)) independent of moderate ischemia.

On the other hand, in the present study, iNOS inhibition with AG in sham-operated animals had no effect on myocardial function. Our results indicate that iNOS activity does not modulate myocardial function during normoperfusion and that the negative inotropic effects of L-NNA during normoperfusion described earlier are related to eNOS inhibition.

During moderate sustained ischemia, iNOS inhibition prevented contractile dysfunction at the level of the cardiomyocyte. However, contractile function during hypoperfusion in vivo was not improved with AG. In the presence of AG, we observed an early-onset, time-dependent metabolic deterioration, as reflected by the increase in arterial lactate (supplemental Figure III) in sham animals. Although lactate acidosis did not affect myocardial function during normoperfusion, we cannot exclude that it contributed to myocardial dysfunction during hypoperfusion. Pig mitochondria contain iNOS,\(^4\) and NOS inhibition has been shown to increase myocardial oxygen consumption,\(^4^0,4^3\) which may have contributed to the observed metabolic deterioration during moderate ischemia.

In the present study, we did not quantify transmural blood flow distribution during sustained hypoperfusion to rule out changes in tissue perfusion pattern as a source of the progressive decline in regional function, as the enzymatic isolation of cardiomyocytes following the in vivo experiments precluded the use of radioactive microspheres. However, in a previous study, we have shown that distribution of regional myocardial blood flow does not change between 5 minutes and 12 hours of moderate hypoperfusion in this model.\(^2\)

In the myocardium, iNOS-dependent contractile dysfunction can be induced by a variety of cytokines and mediators released during hypoxia and the inflammatory processes triggered by ischemic myocardial damage and cell death (see the online data supplement for a more detailed discussion). TNF-α is causally involved in inflammatory cardiomyopathies,\(^4\) myocardial dysfunction following ischemia/reperfusion,\(^1^3,4^5\) and also in progressive contractile dysfunction following coronary microembolization.\(^4^6\) TNF-α exerts negative inotropic effects by 2 mechanisms: a sphingosine-mediated depression of intracellular Ca\(^2^+\)-release\(^1^4,1^5\) and iNOS expression and increased NO production.\(^1^2\) In accordance with previous reports,\(^4^5\) in the present study tissue sections from SHAM animals contained low levels of TNF-α, which were mainly localized to the vascular endothelium (Figure 5). During sustained moderate ischemia without reperfusion (injury), myocardial infarction or overt heart failure, neither TNF-α nor sphingosine was increased in the ischemic myocardium, which may reflect a less strong trigger for an inflammatory response in our model, in line with the unchanged leukocyte count in the tissue. However, other cytokines may have contributed to increased iNOS expression in our model, as discussed in more detail in the online data supplement.
In the present study, extracorporeal perfusion may have contributed to activation of cytokines and blood elements, subsequently leading to increased iNOS protein expression also in SHAM and to the small decrease in work index in the cannulated AW of SHAM (which was significantly smaller than in ISCH). Leukocytes may have contributed to the NOS activity measured in myocardial biopsies. However, similar myocardial leukocyte counts between ISCH and SHAM suggest that increased iNOS expression and activity in ISCH was not a result of increased leukocyte migration into the myocardium.

In summary, sustained moderate regional ischemia induces contractile dysfunction at the cardiomyocyte level. iNOS expression is increased in the myocardium following 6 hours of moderate ischemia, associated with an L-arginine–dependent decline in contractile function within the hypoperfused myocardium. In light of these findings, inhibition of excessive iNOS activity may prove to be beneficial in maintaining contractile function during sustained moderate ischemia. In the clinical setting, unspecific NO inhibition has been shown to improve blood pressure and the hemodynamic response to catecholamines in sepsis47,48 and may be beneficial for cardiac function following acute myocardial infarction.49,50 However, an associated decrease in cardiac output and global oxygen delivery, and more importantly, an increase in mortality were documented in a recent study,47 emphasizing the need to investigate more specific therapeutic targets to inhibit excess NO production within cardiomyocytes.

**Sources of Funding**

This study was supported by the Ernst und Berta Grimmke-Stiftung (Düsseldorf, Germany).

**Disclosures**

None.

**References**


Inducible Nitric Oxide Synthase Expression and Cardiomyocyte Dysfunction During Sustained Moderate Ischemia in Pigs

Frank R. Heinzel, Petra Gres, Kerstin Boengler, Alexej Duschin, Ina Konietzka, Tienush Rassaf, Julia Snedovskaya, Stephanie Meyer, Andreas Skyschally, Malte Kelm, Gerd Heusch and Rainer Schulz

_Circ Res._ 2008;103:1120-1127; originally published online September 25, 2008; doi: 10.1161/CIRCRESAHA.108.186015

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/103/10/1120

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/09/25/CIRCRESAHA.108.186015.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
iNOS-Expression and Cardiomyocyte Dysfunction During Sustained Moderate Ischemia in Pigs

ONLINE SUPPLEMENT

Frank R. Heinzel, Petra Gres, Kerstin Boengler, Alexej Duschin, Ina Konietzka, Tienush Rassaf, Julia Snedovskaya, Stephanie Meyer, Andreas Skyschally, Malte Kelm, Gerd Heusch, Rainer Schulz*

1Institute of Pathophysiology, University of Essen Medical School, Hufelandstraße 55, Essen 45122, Germany. 2Department of Cardiology, Pneumology, and Vascular Medicine, University Hospital Aachen, Aachen, Germany.
Materials and Methods

In vivo Model

The experimental protocols employed in this study were approved by the local bioethical committee and adhere to the guiding principles of the American Physiological Society. Sixteen Göttinger miniswine (20–40 kg) of either sex were initially sedated using ketamine hydrochloride (1 g i.m.) and then anesthetized with thiopental (Trapanal, 500 mg i.v.). Through a midline cervical incision, the trachea was intubated for connection to a respirator (Dräger, Lübeck, Germany). Anesthesia was then maintained using enflurane (1–1.5%) with an oxygen-nitrous oxide mixture (40:60%). Arterial blood gases and lactate were monitored frequently in the initial stages of the preparation until stable and then periodically throughout the study (Radiometer, Copenhagen, Denmark). Rectal temperature was monitored, and body temperature was kept between 36-37°C by the use of a heated surgical table and drapes. Through the cervical incision, one common carotid artery and both internal jugular veins were isolated. The artery was cannulated with a polyethylene catheter to supply blood to the extracorporeal circuit. The jugular veins were cannulated for volume replacement using warmed 0.9% NaCl and for the return of blood to the animal from the coronary venous line.

A left lateral thoracotomy was performed in the fourth intercostal space and the pericardium opened. A micromanometer (P7; Konigsberg, Pasadena, CA) was placed in the left ventricle (LV) through the apex together with a saline-filled polyethylene catheter (used to calibrate the micromanometer in situ). Ultrasonic dimension gauges (System 6; Triton Technologies, San Diego, CA) were implanted in the left ventricular myocardium to measure the thickness of the anterior and posterior (control) walls.
The proximal left anterior descending coronary artery (LAD) was dissected over a distance of 1.5 cm, ligated, and cannulated, and the distal LAD was perfused from an extracorporeal circuit, including a roller pump and a windkessel. Prior to coronary cannulation, the pigs were anticoagulated with 20,000 IU heparin sodium; additional doses of 10,000 IU were given at hourly intervals. Coronary arterial pressure (CAP) was measured from the side arm of a polyethylene “T”-connector (Cole-Parmer, Chicago, USA) used as catheter tip with an external transducer (Bell and Howell type 4-327I, Pasadena, USA). Heart rate was controlled throughout the study by left atrial pacing (Hugo Sachs Elektronik type 215/T, Hugstetten, Germany). The completed preparation was allowed to stabilize for at least 30 min before control measurements were made. The flow-constant perfusion pump was adjusted so that the minimum CAP was not less than 70 mmHg under control conditions to avoid initial hypoperfusion. Therefore, mean CAP exceeded peak left ventricular pressure (LVPP), which was defined as the systolic maximum of the intraventricular pressure curve.

For the calculation of regional myocardial function, end-diastole was defined as the point when left ventricular dP/dt (first derivative of pressure development over time) started its rapid upstroke after crossing the zero-line. Regional end-systole was defined as the point of maximal wall thickness within 20 ms before peak negative left ventricular dP/dt. Systolic wall thickening (WTh) was calculated as end-systolic wall thickness minus end-diastolic wall thickness divided by the end-diastolic wall thickness. Regional myocardial work was estimated by a regional myocardial work index, calculated as the sum of the instantaneous left ventricular pressure-wall thickness product over the time of the cardiac cycle.

**Experimental Protocols in vivo**

After measurements of systemic hemodynamics and regional function at baseline, LAD inflow was decreased to achieve a reduction in CAP to approx. 45 mmHg (ISCH, N= 10), whereas CAP was maintained at baseline levels in SHAM (N= 6). Measurements were
repeated at 5 min and 6h. In a subgroup of ISCH animals (ISCH+iNOS-Inhib), continuous application of an iNOS-inhibitor (aminoguanidine, AG, 1 mg/kg/min, N=3; L-N6-(1-Iminoethyl)lysine, L-NIL, 1 mg/kg/h following a bolus of 0.5 mg/kg; N=1) through a peripheral vein was initiated after the baseline measurement, 30 min before the start of hypoperfusion, and continued until the end of the experiment. At 6h, myocardial biopsies were taken from the anterior free wall distal to the first diagonal branch of the cannulated LAD as well as from the posterior control wall, snap frozen in liquid nitrogen and stored at -70°C for later Western blot analysis. An apical section of the heart containing parts of the hypoperfused anterior wall and the posterior wall was embedded in paraffin for immunohistochemistry. Visual inspection during myocardial ischemia revealed that the anterior wall biopsies and the apical section were clearly within the perfusion territory (indicated by the cyanotic blue color).

In parallel experiments, sham-operated animals (N=4) received a continuous application of the iNOS inhibitor AG (1 mg/kg/min) for 6 h through a peripheral vein following the baseline measurements. At baseline and at 6h the coronary endothelial response to bradykinin, a potent agonist of eNOS-mediated vasodilatation, was assessed. Bradykinin was administered continuously into the LAD via a sideport of the LAD cannula. A dose-response curve was obtained by recording the adjustment of CBF necessary to maintain CAP constant during a stepwise increment of the effective intracoronary bradykinin concentration. Maximal bradykinin-induced CBF was recorded, and EC50 of bradykinin was calculated from the dose response curves by logarithmic regression.
Isolation of Cardiomyocytes

The heart was transferred in Tyrode’s solution (4°C), containing (in mmol/L): NaCl 137, KCl 5.4, MgCl₂ 0.5, CaCl₂ 1.8, HEPES 11, glucose 10; pH adjusted to 7.4 with NaOH. Ventricular cardiomyocytes were obtained by enzymatic digestion as described previously. Using two separate Langendorff setups, the myocardium of the anterior wall (by perfusion through the “T”-connector from the in situ cannulation) and the posterior wall (by cannulation of a supplying artery) were perfused simultaneously. Residual blood was washed out with Tyrode’s solution (37°C) for 5 min, followed by Ca²⁺-free HEPES solution for 15 min, containing (in mmol/L): NaCl 130, KCl 5.4, KH₂PO₄ 1.2, MgSO₄ 1.2, HEPES 6, glucose 10; pH adjusted to 7.2 with NaOH. Perfusion was then switched to Ca²⁺-free HEPES solution with collagenase (Collagenase A, Roche, Mannheim, Germany), 1.8 mg/ml, and protease (0.1 mg/ml, type XIV, Sigma Aldrich, Seize, Germany) added, recirculating for 20 min. Enzymes were washed out with low Ca²⁺ HEPES solution (as above, 0.18 mmol/L CaCl₂) for 15 min. Thin transversal slices were then cut from the tissue and the cells from the central midmyocardial layer were dispersed and resuspended. To avoid a potential selection bias during the single cell function measurements, the examiner was blinded towards the area of origin of the respective cell suspensions. For this purpose, cell densities of the cell suspensions were matched and suspensions were coded by a person not involved in the experimental procedure. Cells were stored at room temperature, and were allowed to recover from the isolation procedure for 30 min before measurements. Less than 5% of the isolated cardiomyocytes showed spontaneous contractions. Cardiomyocytes were used for measurements within 12h after isolation. Comparison of cell measurements taken at different time points confirmed that contractile function remained stable within the groups during this time period (data not shown).
**Measurement of Contractile Function in Isolated Cardiomyocytes**

Cells were loaded with the ratiometric single-excitation, dual-emission Ca\(^{2+}\)-indicator Indo-1 AM (acetoxymethyl ester, 2.5 µmol/L, Molecular Probes, The Netherlands) at room temperature for 30 min in the dark, washed twice and placed in a perfusion chamber mounted on an inverted microscope (Zeiss Axiovert 100 TV, Jena, Germany). Cells were superfused with Tyrode’s solution at 37°C (contents as above) and stimulated in an electrical field with platinum electrodes (5 ms pulse at 1.5x rheobase). Intracellular Indo-1 was excited at 365 nm (xenon light source, Zeiss GmbH, Jena, Germany), and changes in the intracellular Ca\(^{2+}\)-concentration, \([\text{Ca}^{2+}]_i\), were quantified from the ratio of Indo-1 fluorescence emission detected at 405 nm and 485 nm by two photomultiplier tubes. Cell shortening was simultaneously measured with a video edge detector (Crescent Electronics, Sandy, USA) and is given as a percentage of total cell length. Cardiomyocytes were stimulated at 1 Hz until a steady state of cell shortening amplitude was reached (at least 3 min). L-arginine (100 µmol/L, Sigma-Aldrich, Seelze, Germany) was washed in via a rapid perfusion system, and recordings were performed during cell shortening steady state after 5 min. Addition of L-arginine did not change the pH of the HEPES-buffered solution. In cardiomyocytes isolated from ISCH+iNOS-Inhib, aminoguanidine was added to the bath solution following L-arginine and another measurement was recorded. Cell samples from the anterior and posterior wall were measured alternately.

**Immunohistochemistry**

Confocal images were obtained from antibody-stained biopsy sections with a laser scanning microscope (Zeiss LSM Pascal) at 400x magnification. Frozen biopsies were thawed to –20°C, then fixed in formalin overnight at 4°C. Biopsies were then embedded in paraffin, cut into 4 µm sections, dewaxed and rehydrated.
For iNOS detection, sections were pretreated with 0.01 mol/L citrate buffer for antigen retrieval and then covered with normal goat serum for 20 min. Sections were incubated overnight at 4°C with the primary antibody (iNOS: cat no. 610328, dilution 1:50, BD Transduction Laboratories, Lexington, USA). Following several rinsing steps with phosphate-buffered saline (PBS), a FITC-conjugated goat secondary anti-mouse antibody (sc-2079, Santa Cruz Biotechnology) was applied for 2 h at 37°C.

For eNOS and phospho-eNOS detection, sections were pretreated with EDTA buffer, 1 mmol/L, pH 8.0 for antigen retrieval and then incubated with primary antibody mouse anti-eNOS (Cat No 610296, Transduction Laboratories, Lexington) diluted 1:200 or rabbit anti-phospho-eNOS (9571 S Cell Signaling, Beverly, MA, USA) diluted 1:50, both at 37°C for one hour. A FITC- conjugated secondary antibody goat anti-mouse (sc-2078, Santa Cruz) for eNOS, and a FITC- conjugated donkey-anti rabbit antibody (sc-2090, Santa Cruz) for phospho-eNOS was applied for 1 hour at 37°C.

TNF-α was detected in the biopsy sections using a mouse monoclonal primary anti-TNF-α antibody (sc-7317) in combination with a goat secondary FITC-conjugated anti-mouse antibody (sc-2010, both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described previously.

The samples were coverslipped in Vectashield (H-1000, Vector Laboratories, Burlingame, CA, USA). Using confocal microscopy, a z-stack of seven adjacent layers was recorded for each view to determine the optimal focus setting. The focus was set to the layer with the highest fluorescence intensity prior to recording an image. Four images from each tissue section with identical settings for excitation and detection were analyzed and mean fluorescence was calculated. Negative controls for TNF-α and iNOS were obtained by omission of the primary antibody.
Leukocytes were quantified in additional, hematoxylin- and eosin-stained tissue sections from the myocardial biopsies. Ten randomized fields (0.193 mm$^2$ each) were counted, and the number of leukocytes was calculated per square millimeter$^7$.

**iNOS, eNOS and TNF-α Protein Expression**

For iNOS, Western blots were performed as previously described$^8$. For eNOS and phospho-eNOS, tissue biopsies for Western blot analysis were homogenized in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA), containing (in mmol/L): Tris pH 7.5 20, NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, β-glycerolphosphate 1, Na$_3$VO$_4$ 1, Triton X-100 1%, Leupeptin 1 µg/ml), supplemented with 1x Complete Protease Inhibitor Cocktail (Roche, Penzberg, Germany). After sonication, the samples were centrifuged at 10,000g for 15 min at 4°C. The protein concentration of the supernatant was determined using the Protein Dc Kit (Bio-Rad, Hercules, CA, USA). Thirty µg total protein was electrophoresed on 10% Bis/Tris gels (Invitrogen, Karlsruhe, Germany) and transferred to nitrocellulose membrane (Bio-Rad). After blocking, the membranes were incubated overnight at 4°C with antibodies against phospho-eNOS (1:200) or eNOS (1:200). For normalization, the blots were incubated with mouse monoclonal anti-rabbit GAPDH antibodies (dilution 1:2,000, Hytest, Turku, Finland). After incubation with the respective secondary antibodies, immunoreactivity was detected using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). Densitometry results were normalized to GAPDH protein expression (iNOS) or to Ponceau S staining (eNOS). Signal intensities were quantified using Scion Image software.

TNF-α content was determined by enzyme linked immunosorbent assay (ELISA). Samples were homogenized in a dismembrator (40 s max. speed) in 4 volumes of cold isotonic buffer containing (in mmol/L): imidazole acetate 50, Mg acetate 10, KH$_2$PO$_4$ 4, EDTA 2, N-acetylcysteine 0.05, sulfur 0.0125, pH 7.6. Homogenates were centrifuged at 10,000g for 10
min. Protein contents of the supernatants were matched before transfer to a 96 wells plate of a commercially available porcine ELISA kit (R&D Systems, Minneapolis, MN, USA). Measurements were made in duplicate. The antibodies used in this ELISA are not influenced by TNF-α receptors of either type 1 (TNFR1) or type 2 (TNFR2); the lower limit of TNF-α detectability is 2.8-5.0 pg/ml. Optical density (450 nm) of each well was determined within 30 min using a microplate reader.

**Nitric oxide synthase activity measurements**

The activities of myocardial nitric oxide synthases (NOS) were determined using a commercially available NOS activity assay kit (No 781001, Cayman Chemical Company, Ann Arbor, MI). Myocardial tissue samples (10-20 mg wet weight) were homogenized in 10 µl/mg homogenization buffer in the frozen state (Schwingmühle MM301, Retsch, Haan, Germany). To measure total NOS activity, a 10 µl aliquot of the supernatant was preincubated with 0.33 mM NADPH at room temperature for 15 minutes. Then, the sample was incubated with 9 nCi [$^3$H]-arginine (L-Arginine-[2,3-$^3$H], 40 Ci/mmol, 0.9 mCi/ml, Sigma, Saint Louis, Mo) contained in 40 µl reaction mixture Kit) at room temperature for 30 minutes. The incubation was stopped by adding 400 µl stop buffer and the formed [$^3$H]-citrulline was separated from [$^3$H]-arginine using an ion exchange resin. [$^3$H]-Citrulline activity was counted in a liquid scintillation counter (LS 3801, Beckmann-Coulter, Krefeld, Germany). Results are expressed as percent of [$^3$H]-arginine activity. In parallel, 10 µl aliquots of the supernatant were preincubated with 3.3 mM LNNA (non-specific NOS blockade) to obtain the baseline value or with 0.33 mM NADPH + 5 µM 1400W (a selective iNOS inhibitor) to determine iNOS activity and total NOS activity.

**Determination of Tissue Nitrosospecies and Nitrite/Nitrate Content**
Nitrite (NO$_2$) is a major oxidative metabolite of NO$^{10}$ and nitrite levels represent a sensitive measure of tissue NO formation$^{11}$. Myocardial nitrite and nitrosospecies (RXNO) contents were determined using a triiodide/ozone-based reductive gas phase chemiluminescence assay, essentially as described$^{12}$. In brief, biopsies were blotted dry on tissue paper, weighed, cut into small pieces, and homogenized in ice-cold N-ethylmaleimide (NEM, 100 mmol/L) / ethylenediamine-tetraacetic acid (EDTA, 2.5 mmol/L)-containing buffer using a Teflon-glass Potter Elvehjem homogenizer. Homogenates samples were immediately divided into 2 aliquots: one was directly injected into the reaction mixture consisting of 45 mmol/L potassium iodide and 10 mmol/L iodine in glacial acetic acid at 60°C, actively purged with a helium stream in line with an NO chemiluminescence analyzer (88 CLD 77am sp and 88 AM, Eco Physics, Dürnten, Switzerland). The remaining aliquot was treated with 1/10 volume of 5% sulfanilamide in 1 mol/L HCl to scavenge nitrite for 15 min and then injected. The difference between the two signal peaks sensitively reflected the concentration of nitrite in the sample, the second peak reflected the concentration of RXNO. Nitrate was quantified after enzymatic reduction to nitrite by nitrate reductase$^{11}$.

**Sphingosine (HPLC)**

Myocardial sphingosine content was measured by using the method of Merrill et al.$^{13}$ with tetradecylamine used as an internal standard$^{14}$. Tissue samples were extracted and derivatized with o-phthaldialdehyde. The derivatives were separated by reverse phase high-performance liquid chromatography, and fluorescence was measured (340 nm excitation, 455 nm emission).

**Data Analysis and Statistics**

Data are reported as mean values ± S.E.M. Statistical analysis comprised ANOVA for repeated measures for the time course of hemodynamic parameters (ISCH); 2-way ANOVA was employed to compare hemodynamic data from all groups (Sham, ISCH, ISCH+iNOS-
Inhib.) at baseline and 6h, to compare iNOS protein expression, NOS activities and immunohistochemistry data, as well as cell shortening data (within each group, and also separate analyses for AW and PW across groups). When a significant overall effect was detected, Fisher least significant tests were performed to compare single mean values. Correlation analyses were performed using Pearson’s correlation coefficients. P< 0.05 was considered significant.
Discussion

In this study we demonstrate an increase in myocardial iNOS expression following 6h of regional ischemia, and a L-arginine-dependent contractile dysfunction intrinsic to cardiomyocytes from the ischemic region.

During acute myocardial ischemia, contractile function in the ischemic region rapidly decreases. With prolongation of ischemia, contractile function further decreases despite a constantly reduced coronary blood flow and preserved tissue viability. By measuring contractile function of isolated cardiomyocytes, this study focused on cellular changes that occur during more sustained ischemia independent from the interstitial milieu. With the supplementation of 100 µmol/L of the NOS substrate L-arginine to the cell bath, a concentration measured in human blood plasma, a close relationship between contractile function in situ and in isolated cardiomyocytes was observed (Fig. 4). The cardiomyocytes showing the largest decrease in cell shortening in response to L-arginine (left most data point in Fig. 4) thereby having the largest isolated impact on the in vivo/in vitro contractile function relationship were from the animal with the most pronounced ischemia-induced regional dysfunction (AW WTh from 35.8% at baseline to 3.5% at 6h of ischemia). Our findings suggest a key role of NOS activity for inducing contractile dysfunction on the level of the cardiomyocyte during sustained ischemia. Decreased contractile function of cardiomyocytes from the hypoperfused anterior wall during 1 Hz stimulation was not associated with alterations in diastolic or peak systolic [Ca\(_{2+}\)]. In line with a NO-dependent reduction in myofilament Ca\(_{2+}\) sensitivity, previous studies utilizing the same animal model showed reduced Ca\(_{2+}\) responsiveness in vivo without alterations in Ca\(_{2+}\) handling proteins or troponin I levels following 90 min or 12 h of moderate hypoperfusion.

Nitric oxide has evolved as an important modulator of cardiac contractile function. eNOS and iNOS, two NOS isoforms which can modulate myocardial function, are expressed within...
cardiomyocytes. iNOS – in contrast to eNOS – is substrate-limited and once activated it can produce high amounts of nitric oxide which may account for the reduced contractile function observed in vitro in the presence of L-arginine. Increased concentrations of iNOS mRNA and iNOS protein have been found in many, albeit not all, studies on non-ischemic as well as ischemic heart failure, including human cardiomyopathy and human hibernating myocardium. During regional myocardial ischemia, an initial increase in NOS activity was observed within minutes following coronary artery occlusion. In a rat model of septic cardiomyopathy, induction of iNOS mRNA was detected as early as 30 min following injection of endotoxin, resulting in a peak iNOS enzyme activity after 6h.

iNOS protein expression was increased during sustained ischemia in the present study, and the concomitantly increased levels of nitrite and nitroso species are in line with a higher iNOS activity and NO production. A specific activation of the inducible NOS isoform is supported by the iNOS activity measurements (Figure 2E) using the iNOS-specific inhibitor 1400W. Furthermore, following in vivo application of AG contractile function at the cardiomyocyte level was preserved in ISCH, while the eNOS mediated endothelial response to bradykinin was not affected. AG had no effect on CBF, anterior wall thickening and LVPP in sham-operated animals. This contrasts well with the effects of L-NNA, a NOS inhibitor also directed against eNOS, in the same animal model in an earlier study, which showed pronounced effects on endothelial and regional myocardial function (Online Figure I A). In line with an iNOS-mediated dysfunction, we demonstrated increased iNOS activity during sustained ischemia, and found that tissue nitrite concentrations as a surrogate of NO production correlated inversely with in vivo regional function (Fig. 3C and D). Total NOS activity was not different between groups, implying that the activity of the constitutive NOS isoforms eNOS and nNOS in the tissue homogenates may have been reduced during sustained ischemia. Indeed, accumulation of ADMA, which occurs after 30 min of ischemia, could have
suppressed eNOS activity, thereby shifting L-arginine towards iNOS and increasing its activity\textsuperscript{31}.

Apart from NOS-derived NO, we have recently demonstrated NOS-independent NO accumulation within the myocardial interstitium during myocardial ischemia \textit{in vivo} \textsuperscript{32}. While NOS-independent NO production may thus have contributed to nitrite formation during ischemia, cellular contractile dysfunction triggered by L-arginine in ISCH was reversible by iNOS inhibition, suggesting that iNOS-dependent NO formation confined to the cardiomyocyte was most important for the measured functional alterations.

iNOS protein content was also increased in confocal cardiomyocyte cross-sections from the posterior (remote) wall of ischemic hearts. Myocardial nitrite levels in ISCH-PW were ranging between values from sham-operated animals and the hypoperfused anterior wall. Thus, not only regional metabolic processes during hypoperfusion, but also increased mechanical stress (as reflected by the pronounced increase in LVEDP during sustained ischemia, Table) may have triggered increased iNOS expression, in analogy to observations in human dilative cardiomyopathy \textsuperscript{25}.

While regional function in the remote region initially remained stable, increased iNOS levels at 6h hypoperfusion were associated with a decrease in contractile function in the posterior wall. Systolic wall thickening was higher in the anterior vs. posterior wall, a physiological phenomenon that has been reported earlier in large animal models \textsuperscript{2,33} and recently also in healthy humans \textsuperscript{34}. With regional myocardial ischemia and myocardial infarction, increased, decreased or unchanged contractile function of the non-ischemic remote myocardium have been described, depending on hemodynamic and structural properties of the diseased heart \textsuperscript{35-37}. Decreased LVPP and increased LVEDP have been associated with hypercontractile remote regional function in an canine open chest model \textsuperscript{38}. In the present experiments during ISCH,
however, PW WI was reduced at 6h ISCH, which may reflect impaired cardiomyocyte contractility as a result of increased iNOS activity; increased iNOS expression may have outweighed potential hemodynamic conditions promoting hyperkinesia.

We and others have previously shown that systemic NOS inhibition (with L-NNA) decreases myocardial contractile function during normoperfusion and myocardial ischemia *in vivo* \(^{39-41}\). In a previous study, L-NNA decreased regional myocardial function to a similar extent during normoperfusion and ischemia (as indicated by a parallel downward shift of the relationship between external work and transmural blood flow), suggesting that NO production from constitutively active NOS has a beneficial effect on myocardial function *in vivo* (as recently also confirmed in humans \(^{42}\) independent of moderate ischemia.

On the other hand, in the present study, iNOS-inhibition with AG in sham-operated animals had no effect on myocardial function. Our results indicate that iNOS activity does not modulate myocardial function during normoperfusion, and that the negative inotropic effects of L-NNA during normoperfusion described earlier are related to eNOS inhibition.

During moderate sustained ischemia, iNOS-inhibition prevented contractile dysfunction at the level of the cardiomyocyte. However, contractile function during hypoperfusion *in vivo* was not improved with AG. In the presence of AG, we observed an early-onset, time-dependent metabolic deterioration as reflected by the increase in arterial lactate (Online Figure III) in sham animals. While lactate acidosis did not affect myocardial function during normoperfusion, we cannot exclude that it contributed to myocardial dysfunction during hypoperfusion. Pig mitochondria contain iNOS \(^{43}\), and NOS inhibition has been shown to increase myocardial oxygen consumption \(^{40, 43}\), which may have contributed to the observed metabolic deterioration during moderate ischemia.
In the present study, we did not quantify transmural blood flow distribution during sustained hypoperfusion to rule out changes in tissue perfusion pattern as a source of the progressive decline in regional function, as the enzymatic isolation of cardiomyocytes following the in vivo experiments precluded the use of radioactive microspheres. However, in a previous study we have shown that distribution of regional myocardial blood flow does not change between 5 min and 12h of moderate hypoperfusion in this model.

In the myocardium, iNOS-dependent contractile dysfunction can be induced by a variety of cytokines and mediators released during hypoxia and the inflammatory processes triggered by ischemic myocardial damage and cell death. TNF-α is causally involved in inflammatory cardiomyopathies, myocardial dysfunction following ischemia/reperfusion, and also in progressive contractile dysfunction following coronary microembolisation. TNF-α exerts negative inotropic effects by two mechanisms: a sphingosine-mediated depression of intracellular Ca\(^{2+}\)-release, and iNOS expression and increased NO production. In accordance with previous reports, in the present study tissue sections from SHAM animals contained low levels of TNF-α, which were mainly localized to the vascular endothelium (Fig. 5). During sustained moderate ischemia without reperfusion (injury), myocardial infarction or overt heart failure, neither TNF-α or sphingosine were increased in the ischemic myocardium, which may reflect a less strong trigger for an inflammatory response in our model, in line with the unchanged leukocyte count in the tissue.

Nevertheless, other cytokines may have contributed to increased iNOS expression in our model. Interleukin (IL)-1β, interferon γ and IL 6 have been shown to induce increased iNOS expression and contractile dysfunction in cardiac myocytes. IL-1β exerts its cardiodepressive effects via sphingosine as reported in human atrial myocardium, making its participation in iNOS induction less likely in the present model, considering the unchanged
sphingosine levels. Interferon $\gamma$ is increased in ischemic cardiomyopathy, however its exact role in iNOS induction in ischemia without myocardial infarction requires further investigation. IL-6, a cytokine known to be elevated in chronic heart failure patients, was increased in cardiomyocytes following 4 hrs of hypoxia or adenosine treatment. In rats, IL-6 decreases cardiomyocyte function via JAK2/STAT3-dependent iNOS induction, and may be a potential trigger for iNOS induction during sustained ischemia.

In the present study, extracorporeal perfusion may have contributed to activation of cytokines and blood elements, subsequently leading to increased iNOS protein expression also in Sham, and to the small decrease in work index in the cannulated anterior wall of Sham (which was significantly smaller than in ISCH). Leukocytes may have contributed to the NOS activity measured in myocardial biopsies. However, similar myocardial leukocyte counts between ISCH and Sham suggest that increased iNOS expression and activity in ISCH was not a result of increased leukocyte migration into the myocardium.

In summary, sustained moderate regional ischemia induces contractile dysfunction at the cardiomyocyte level. iNOS-expression is increased in the myocardium following 6h of moderate ischemia, associated with an L-arginine-dependent decline in contractile function within the hypoperfused myocardium. In light of these findings, inhibition of excessive iNOS activity may prove to be beneficial in maintaining contractile function during sustained moderate ischemia. In the clinical setting, unspecific NOS-inhibition has been shown to improve blood pressure and the hemodynamic response to catecholamines in sepsis and may be beneficial for cardiac function following acute myocardial infarction. However, an associated decrease in cardiac output and global oxygen delivery, and more importantly, an increase in mortality were documented in a recent study, emphasizing the need to investigate more specific therapeutic targets to inhibit excess NO production within cardiomyocytes.
References


(54) Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, Entman ML. Resident cardiac mast cells degranulate and release preformed TNF-α, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. *Circulation*. 1998;98:699-710.


Online Figure Legends

Online Table I. Systemic hemodynamics, regional myocardial function, coronary blood flow, as well as arterial lactate, hemoglobin and hemoglobin oxygen saturation at baseline and during 6h of hypoperfusion. Values are means ± S.E.M., N\textsubscript{animals}= 6 each group (SHAM and ISCH). HR, heart rate (beats/min); LVEDP, left ventricular end-diastolic pressure (mmHg); LVPP, left ventricular peak pressure (mmHg); CBF, coronary blood flow (ml/min); CAP, coronary arterial pressure (mmHg); AW WTh, anterior systolic wall thickening (%); PW WTh, posterior systolic wall thickening (%); S\textsubscript{o}2, arterial oxygen saturation (%); Hb, serum hemoglobin (mg/dl); lactate, arterial lactate concentration (mmol/L). *P<0.05 vs. baseline, †P<0.05 vs. 5 min hypoperfusion, ‡P<0.05 vs. Sham.

Online Figure I. A: Hemodynamics in sham-operated pigs at baseline and following 85 min of the NOS-inhibitor L-NNA (with affinity to eNOS; data compiled from Heusch et al. Circ Res. 2000;87:146-152). CBF, coronary blood flow; CAP, coronary artery pressure; AW WTh, anterior wall thickening; LVPP, left ventricular peak pressure. B: Hemodynamics in sham-operated pigs from the present study at baseline and following 6h of the iNOS-specific inhibitor aminoguanidine (AG, 1 mg/kg BW/min). C: Example for a dose-response curve during a stepwise intracoronary bradykinin challenge at baseline (black) and following 6h of AG (red) in one animal. CBF was adjusted for a constant CAP. D: EC\textsubscript{50} of bradykinin at baseline and following 6h of AG. E: Maximal bradykinin-induced CBF at baseline and following 6h of AG. *P< 0.05.
**Online Figure II.** A: Western blot analysis for eNOS on protein extracts of anterior wall (AW) and posterior wall (PW) tissue samples from SHAM and ISCH myocardium. Ponceau S staining demonstrates equal protein loading. **B:** Bar graphs represent the ratios of eNOS (normalized to Ponceau S staining) in the AW over PW in ISCH and SHAM.

**Online Figure III.** Lactate concentration in arterial blood samples during aminoguanidine infusion (1 mg/kg BW/min i.v.) in sham-operated pigs.
<table>
<thead>
<tr>
<th></th>
<th>Isch</th>
<th>Hypoperfusion</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5 min</td>
<td>6 h</td>
</tr>
<tr>
<td>HR</td>
<td>100 ± 5</td>
<td>100 ± 4</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>LVEDP</td>
<td>8.4 ± 0.9</td>
<td>8.3 ± 0.8</td>
<td>20.3 ± 3.4*†</td>
</tr>
<tr>
<td>LVPP</td>
<td>92 ± 3</td>
<td>92 ± 3</td>
<td>87 ± 3*†</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}})</td>
<td>1259 ± 88</td>
<td>1225 ± 82</td>
<td>989 ± 58*†#</td>
</tr>
<tr>
<td>CBF</td>
<td>40.2 ± 3.4</td>
<td>23.7 ± 2.3*</td>
<td>23.7 ± 2.3*#</td>
</tr>
<tr>
<td>CAP</td>
<td>118.1 ± 2.9</td>
<td>45.6 ± 2.8</td>
<td>42.5 ± 1.6</td>
</tr>
<tr>
<td>AW Wth</td>
<td>41.8 ± 4.4</td>
<td>32.1 ± 3.5*</td>
<td>15.7 ± 2.8*†#</td>
</tr>
<tr>
<td>PW Wth</td>
<td>23.3 ± 4.1</td>
<td>24.8 ± 4.5</td>
<td>22.3 ± 3.0</td>
</tr>
<tr>
<td>(S_aO_2)</td>
<td>98.2 ± 0.2</td>
<td>97.9 ± 0.1</td>
<td>95.7 ± 2.5</td>
</tr>
<tr>
<td>Hb</td>
<td>10.5 ± 0.4</td>
<td>8.3 ± 0.7*</td>
<td>10.5 ± 0.4</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.5 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

**Online Table I**
Online Figure I
Online Figure II

A

<table>
<thead>
<tr>
<th>Isch</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>PW</td>
<td>AW</td>
</tr>
</tbody>
</table>

140 kDa
eNOS
Ponceau S

B

eNOS protein expression (ratio anterior wall / posterior wall)

Sham | Isch

0.0  | 1.2
0.2  | 1.0
0.4  | 0.8
0.6  | 0.6
0.8  | 0.4
1.0  | 0.2
Arterial lactate concentration (mmol/L)

Duration of aminoguanidine (min)

Online Figure III

p<0.05 vs. 0 min