Coronary flow regulation in mouse heart during hypercapnic acidosis: role of NO and its compensation during eNOS impairment

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Aims This study addressed the hypotheses that the hypercapnic flow response in wild-type (WT) mouse heart is mainly mediated by nitric oxide (NO) and, thus, severely blunted in endothelial nitric oxide synthase knockout (eNOS-KO) mice and in WT mice after continuous pharmacological block (2 weeks) of NOS enzymes (WT-LN).

Methods and results Step changes of arterial pCO2 were performed in isolated perfused hearts (n = 105). Contributions of NOS (L-NAME, TRIM), cyclooxygenase (indomethacin), epoxyeicosanotrienes (miconazole), adenosine A2A-receptors (SCH 58261), KV-channels (4-AP), KCa-channels (TEA), and KATP-channels (glibenclamide) were studied in WT and eNOS-KO mouse hearts. Change of arterial pCO2 increased coronary flow by 31.3 ± 4% in WT, a response that was significantly decreased to 9.2 ± 6% after L-NAME. Additional glibenclamide infusion (n = 5) completely abolished the steady-state flow increase during hypercapnic acidosis (– 4.2 ± 2.3%, P = 0.004 vs. control). Hearts from eNOS-KO mice as well as WT-LN showed a fully preserved flow response insensitive towards NOS-blockade. Whereas indomethacin, miconazole, TEA, and SCH 58261 were ineffective to reduce the flow response, glibenclamide blunted it in eNOS-KO hearts.

Conclusion NO-production and KATP-channel activation together may fully account for the steady-state hypercapnic flow response in mouse heart. However, chronic deletion of eNOS does not result in a reduced hypercapnic flow response. Enhanced activation of KATP-channels and potentially Kr-channels contributes to the compensatory mechanisms involved in the hypercapnic flow response when eNOS activity is absent.

1. Introduction

Increases of myocardial oxygen-consumption (VO2) result in an increased CO2-production. In particular under conditions of ischaemia this results in hypercapnic acidosis.1 Elevation of pCO2 and/or lowering of pH induce coronary vasodilatation under physiological and also pathological conditions.2 The precise mechanism(s) mediating this vasodilatation remain unsettled. Among the suspected mechanisms are (1) stimulation of nitric oxide (NO)-production,3 (2) purinergic-receptor mediated effects,4,5 and (3) activation of KATP-channels.6 However, the contributing factors may differ notably between species. In a recent study performed in guinea pig hearts, our laboratory provided evidence that the sustained coronary flow increase after a step change of arterial pCO2 largely depends on intact NO-production.7 In contrast, KATP-channels were of no importance in this model. The importance of NO for the hypercapnic coronary flow increase has been documented also for canine heart.8 However, in rat heart coronary flow increase during hypercapnic acidosis was reported to be independent of NO-production,9,10 whereas purinergic (adenosinergic)-receptor mediation4 and KATP-channels6,9 seem to be essential in this species.

Endothelial dysfunction is associated with a diminished NO-production.11,12 Because the flow increase during hypercapnic acidosis has been shown to be dependent on a functional endothelial nitric oxide synthase (eNOS),7 it may be suspected that under conditions of endothelial dysfunction the coronary capability to dilate during hypercapnic acidosis is impaired. However, even in patients with coronary artery disease the coronary vessels react upon a change of the
pCO₂. This suggests that possibly other regulatory mechanisms can compensate for the chronic loss of NO-dependent flow regulation.

The model offers the potential to study the effects of a knockout of a specific protein for vascular regulatory function. This permits to test whether the mechanisms involved in the mediation of the hypercapnic flow response change if NO activity is chronically diminished. As experimental models, we used eNOS-knockout (eNOS-KO) mice and wild-type (WT) mice with chronic pharmacological inhibition of NO-production and compared them with WT mice without pharmacological block of eNOS. It is known that the cardiovascular phenotype of genetically eNOS-deprived mice is almost unchanged although a mild arterial hypertension exists. The presence of compensatory mechanisms is therefore likely. As potentially compensatory mechanisms, we considered KATP-, KCa-, and K÷-channels, epoxyeicosanoid trienes (EETs) and EDHF, other NO synthases (nNOS and iNOS), prostaglandins, and adenosine. These mechanisms have previously been considered to be involved in metabolic coronary flow control.

2. Methods

2.1 Animals

The care and all experiments were performed according to the animal welfare regulations of the German local authorities conforming to NIH Guidelines. Two different mice strains were used for the experiments: male C57 BL6 (n = 52; Charles River Laboratory, Sulzfeld, Germany) and male eNOS-KO mice (n = 43; 8–12 weeks old: 14–35 g). Homozygous eNOS-KO mice were kindly provided by Prof. Gödecke and Schrader (Institute of Cardiac and Circulatory Physiology, Heinrich-Heine-University, Düsseldorf, Germany). eNOS-KO was verified using western blot analysis of eNOS protein with human umbilical venous endothelial cells as controls. In an additional group of experiments, 10 out of the 52 WT mice (8–10 weeks old; 20–23 g) were pretreated for 14 days with the water-soluble NOS blocker L-NAME to achieve a longer-term pharmacological block of endothelial NO-production (100 μmol/L enriched in drinking water). This group is referred to in the following as group WT mice with L-NAME (WT-LN).

2.2 Langendorff heart preparation

Hearts were rapidly removed during continuous cooling (Krebs buffer 4°C) and mounted via the aorta on a perfusion cannula. Left ventricular pressure (LVP) was assessed isovolumically using a self-constructed fluid-filled latex balloon advanced into the ventricle via the left atrium and connected to a pressure transducer (Gould Statham). End-diastolic pressure was set to 8±2 mmHg. The pulmonary artery was catheterized to permit anaerobic collection of the coronary venous effluent perfusate. Small silver electrodes were fixed to the right atrium and the cardiac apex for pacing to maintain heart rate 400 bpm. Perfusion was immediately started according to the Langendorff technique with a modified Krebs-Henseleit buffer (KHB) containing (mmol/L): NaCl 116, KCl 4.6, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24.8, CaCl₂ 3.0, glucose 8.3, pyruvate 2, and EDTA-Na 0.5. The buffer was equilibrated with 95% O₂/5% CO₂ gas mixture (resulting pH 7.38). Filtered (Durapore Membrane Filter 0.45 μm diameter, MILLIPORE, Eschborn, Germany), and maintained at 37°C. Also, an insuline (40 IU/mL) and albumin (0.1%) enriched solution was added in a ratio of 1:1000 relative to coronary flow.

Total coronary flow was measured using an ultrasonic transit-time flowmeter (T206 Transonic Systems Inc., Ithaca, NY, USA) inserted in the arterial perfusion line. Coronary perfusion pressure was measured through a pressure transducer (Gould Statham) connected to the perfusion cannula. Heart rate was calculated electronically from the LVP recording. Measurements of coronary perfusion pressure, coronary flow, LVP, and heart rate were A/D converted and acquired (data sampling rate 100 Hz), displayed, and stored on a PC. The data was processed using PoNeMah Physiology Platform V.4.0 (Gould Inc., OH, USA). 'Arterial' and 'venous' perfusate samples collected anaerobically were analysed for pO₂, pCO₂, pH, and HCO₃ (AVL 9905, AVL Scientific Corporation, Roswell, GA, USA) before, during, and after each intervention.

2.3 Experimental criteria

Each heart preparation, which showed LVP development ≥50 mmHg, was tested for baseline coronary vascular resistance and coronary dilator capacity by evoking a reactive flow response following a 20 s flow stop. Only hearts that exhibited at least a doubling of flow after the flow stop were used for the experiments.

2.4 Experimental protocol

Following instrumentation of the heart and after a 20 min normocapnic perfusion (mean pO₂ 634 mmHg, pCO₂ 38.6 mmHg, pH 7.38), the reactive flow response was tested. After another 10 min, normocapnic perfusion was switched to hypercapnic perfusion (mean pO₂ 618 mmHg, pCO₂ 61.4 mmHg, pH 7.12) for 10 min. To test for the potential involvement of eNOS, K_ATP-channels, KCa-channels, K+-channels, cyclooxygenase, nNOS, EETs, and adenosine A₁-receptors in the hypercapnia-induced flow response, L-NAME (100 μmol/L) or L-NMMA (100 μmol/L), glibenclamide (3 μmol/L), tetraethylammoniumchloride (TEA; 1 mmol/L), 4-aminopyridine (4-AP; 100 μmol/L) indomethacin (10 μmol/L), 1-(3-trifluoromethylphenyl)imidazole (TRIM; 100 μmol/L), miconazole (0.5 μmol/L), and 5-amino-7-((β-phenyl)-2-(8-furyl)pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH-58261; 100 μmol/L), respectively, were used. To block eNOS in WT and eNOS-KO mice L-NAME was used. In hearts from the WT-LN group, we administered either L-NAME (n = 4) or L-NMMA (n = 6) during isolated heart perfusion. The latter inhibitor was used to exclude desensitization effects due to the long-term pretreatment. However, since we were unable to detect any different effect with respect to flow control between the two subsets of experiments, data analysis and presentations were merged.

The applications of the blockers were started 10 min after the end of the preceding hypercapnic challenge. After 10 min of blocker infusion, the coronary reactive flow response was evaluated again. Then, 20 min after start of the inhibitor infusion, another hypercapnic challenge (10 min) was performed.

To assure the instant change of arterial pCO₂ without an undefined delay, we used a system with two separate warmed reservoirs filled with a normo- vs. a hypercapnic perfusate, respectively. These reservoirs could be switched alternatively to the heart perfusion line via a three-way stopcock. In preliminary experiments with both columns filled with normocapnic perfusate, a column switch did not lead to any relevant coronary flow response.

2.5 HPLC analysis

To measure adenosine, coronary venous effluence samples were taken 2 min before the hypercapnic challenge and at 2 and 8 min during hypercapnic perfusion. Samples were immediately frozen in liquid nitrogen. Analysis was performed as previously described.†

2.6 Chemicals

L-NAME, L-NMMA, indomethacin (all dissolved in KHB), TEA, 4-AP (dissolved in distilled water), TRIM, glibenclamide, miconazole, and SCH-58261 (dissolved in DMSO) were obtained from Sigma-Aldrich (München, Germany). Infusion rates did not exceed 1% of the coronary flow.
2.7 Calculations and statistics

Myocardial oxygen consumption (VO\textsubscript{2} \(\mu\text{L/min/g}\)) was calculated from the arterial-venous difference of pO\textsubscript{2} according to Fick's principle with the use of Bunsen's absorption coefficient (\(\alpha = 0.0316 \mu\text{L/mmHg/mL}\) at 37°C as follows: \(\text{VO}_2 (\mu\text{L/min/g}) = (\text{PO}_2 - \text{PV}_2) \cdot \text{PO}_2 - \text{PV}_2\)), where \(F\) denotes coronary flow (mL/min/g).

Data are reported as means ± SEM. Data distribution was assessed using the Kolmogorov-Smirnov test. We performed within group analyses using two-sided, paired Student's \(t\)-test. Within-group statistics were done using raw data (absolute values). Relative values are displayed in the text and tables to allow easier comparisons of the different effects. Comparisons between groups were carried out using one-way analysis of variance (ANOVA). Between-group statistics were performed using relative values. The \(P\)-values were adjusted according to Bonferroni when appropriate. Statistical analyses were carried out using SPSS software for MS Windows (Release 11.0, SPSS Inc., Chicago, IL, USA). A \(P < 0.05\) was taken to indicate a statistical significance.

3. Results

The baseline values prior to the hypercapnic flow response are shown in Table 1. In WT baseline flow was 8.6 ± 0.5 mL/min/g heart weight (hw) and similar to eNOS-KO (9.0 ± 0.5 mL/min/g hw). Genetically deprived eNOS hearts showed a trend for lower LVP and dp/dt\textsubscript{max} baseline values than WT hearts but this difference was only suggestive of significance (\(P = 0.07\)). Treatment of WT-LN for 2 weeks did not affect heart rate, LVP or dp/dt\textsubscript{max} as measured in the isolated heart. However, in WT-LN hearts baseline avDO\textsubscript{2} and VO\textsubscript{2} were significantly increased compared with controls indicating increased oxygen requirement. Despite these differences, baseline coronary flow per gram myocardium and the reactive flow response after a 20 s flow stop was similar in the different groups.

3.1 Effects of eNOS-blockade

In 14 WT hearts L-NAME infusion reduced baseline flow approx. 21%. The same dose of L-NAME did not have significant effects on baseline coronary flow in hearts from groups eNOS and WT-LN, respectively (Table 2). This indicates that (1) the L-NAME dose chosen for intracoronary infusion was effective, (2) hearts from eNOS-KO mice did not reveal NOS activity that affected baseline coronary flow, and (3) supplementation of L-NAME in drinking water was effective.

In the following paragraphs, the results of hypercapnic perfusion experiments are shown. Data are summarized in Table 2.

3.1.1 WT-group

Hypercapnic acidosis (pCO\textsubscript{2} increase from 39 to 61 mmHg) induced a significant coronary flow increase (\(P < 0.001\)) that reached a plateau approx. 31% above baseline within 5 min (Figure 1). During hypercapnic acidosis LVP decreased significantly (\(P < 0.001\)). Changes were completely reversible after 10 min of normocapnic perfusion (data not shown). In the presence of continuous L-NAME infusion, the hypercapnia-induced flow increase was reduced (28.3 ± 9.2% before vs. 9.2 ± 5.5% after L-NAME, \(P = 0.018\), Figure 1A). The relative flow increase was significantly less than that found during hypercapnic perfusion before eNOS-blockade. The decrease in LVP was similar to that seen before L-NAME (Table 2).

3.1.1.1 Blockade of K\textsubscript{ATP}-channels

Inhibition of K\textsubscript{ATP}-channels with glibenclamide in WT hearts (n = 9) reduced baseline flow by 26.1 ± 10.0% (\(P = 0.026\)). The hypercapnia-induced increase of flow (Figure 1B) was significantly reduced by glibenclamide (from 30.4 ± 9.6% to 10.9 ± 3.5%; \(P = 0.031\)). When glibenclamide was infused in the continuous presence of L-NMMA (n = 5), the steady-state flow increase during hypercapnic acidosis was completely abolished (flow change −4.2 ± 2.3%, \(P = 0.004\) vs. control condition, Figure 1C). Thus, NO and K\textsubscript{ATP}-channel activation may have accounted completely for the flow increase.

Blockade of mNOS (TRIM), cyclooxygenase (indomethacin), K\textsubscript{Ca}-channels (TEA), EETs (miconazole), and adenosine A\textsubscript{2A}-receptors (SCH 58261) did not result in any modification of hypercapnic flow response, and thus these mechanisms seem unimportant for the flow increase observed during hypercapnic acidosis in WT (data not shown).

3.1.2 eNOS-KO group

Hypercapnic acidosis caused an almost 53% increase of coronary flow in hearts from eNOS-KO mice (\(P < 0.001\), Figure 2). This flow increase was not reduced but even slightly more pronounced than that in WT hearts (\(P = 0.04\)). The slight decrease of LVP during hypercapnic acidosis was similar to

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Table 1 Baseline parameters of all groups

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 52)</th>
<th>eNOS-KO (n = 43)</th>
<th>WT-LN (n = 10)</th>
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<tr>
<td>Perfusion pressure (mmHg)</td>
<td>89.1 ± 0.4</td>
<td>90.1 ± 0.4</td>
<td>86.4 ± 0.4</td>
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<td>LVP (mmHg)</td>
<td>77.5 ± 5.2</td>
<td>71.6 ± 4.7</td>
<td>87.8 ± 10.5</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>449 ± 8</td>
<td>462 ± 9</td>
<td>405 ± 23</td>
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<tr>
<td>+dp/dt\textsubscript{max} (mmHg/s)</td>
<td>2749 ± 188</td>
<td>2582 ± 186</td>
<td>2761 ± 358</td>
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<tr>
<td>avDO\textsubscript{2} (mmHg)</td>
<td>483 ± 10</td>
<td>511 ± 8</td>
<td>557 ± 6**</td>
</tr>
<tr>
<td>VO\textsubscript{2} (\muL/min/g)</td>
<td>161 ± 11</td>
<td>168 ± 9</td>
<td>227 ± 19**</td>
</tr>
<tr>
<td>Baseline flow (mL/min)</td>
<td>1.7 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.2</td>
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<tr>
<td>(mL/min/g)</td>
<td>8.6 ± 0.5</td>
<td>9.0 ± 0.5</td>
<td>10.8 ± 0.9</td>
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<td>Reactive flow response (mL/min)</td>
<td>4.7 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>(mL/min/g)</td>
<td>24.6 ± 0.9</td>
<td>23.0 ± 0.5</td>
<td>27.5 ± 1.0</td>
</tr>
</tbody>
</table>

LVP: left ventricular pressure; dp/dt\textsubscript{max}, contractility; avDO\textsubscript{2}, arterial-venous difference of pO\textsubscript{2}; VO\textsubscript{2}, myocardial oxygen consumption.

**P = 0.01 vs. eNOS-KO.

***P < 0.001 vs. WT and eNOS-KO.
Table 2  Changes of flow and left ventricular pressure

<table>
<thead>
<tr>
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<th>WT n = 35</th>
<th>eNOS-KO n = 24</th>
<th>WT-LN n = 10</th>
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<tr>
<td><strong>No blocker</strong></td>
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<tr>
<td>Baseline flow (mL/min)</td>
<td>1.7 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>2.0 ± 0.2</td>
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<tr>
<td>Baseline LVP (mmHg)</td>
<td>74.9 ± 6.3</td>
<td>68.8 ± 6.3</td>
<td>87.8 ± 10.5</td>
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<tr>
<td><strong>No blocker + HC</strong></td>
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<tr>
<td>Δ flow (%)</td>
<td>31.3 ± 3.9</td>
<td>52.6 ± 4.3</td>
<td>37.3 ± 5.9</td>
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<tr>
<td>Δ LVP (%)</td>
<td>−16.5 ± 1.5</td>
<td>−7.9 ± 2.8</td>
<td>−21.1 ± 2.2</td>
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<tr>
<td>n</td>
<td>14</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td><strong>No blocker</strong></td>
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<tr>
<td>Baseline flow (mL/min)</td>
<td>1.5 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.1</td>
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<tr>
<td>Baseline LVP (mmHg)</td>
<td>77.9 ± 12.5</td>
<td>62.2 ± 16.2</td>
<td>78.6 ± 11.3</td>
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<td><strong>Glibenclamide</strong></td>
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<td>Δ baseline flow (%)</td>
<td>−26.1 ± 10.0</td>
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<td>Δ baseline LVP (%)</td>
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<tr>
<td>n</td>
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<tr>
<td><strong>Glibenclamide + HC</strong></td>
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<tr>
<td>Δ flow (%)</td>
<td>10.9 ± 3.5</td>
<td>24.6 ± 2.7</td>
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<tr>
<td>Δ LVP (%)</td>
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<tr>
<td>n</td>
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<tr>
<td>Δ baseline flow (%)</td>
<td>−14.8 ± 2.7</td>
<td>−2.4 ± 1.3</td>
<td>−3.4 ± 2.9</td>
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<tr>
<td>Δ baseline LVP (%)</td>
<td>−10.5 ± 8.5</td>
<td>−3.4 ± 2.9</td>
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<tr>
<td><strong>L-NMMA + HC</strong></td>
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<tr>
<td>Δ flow (%)</td>
<td>30.9 ± 4.7</td>
<td>30.9 ± 4.7</td>
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<tr>
<td>Δ LVP (%)</td>
<td>−30.9 ± 3.3</td>
<td>−16.2 ± 2.7</td>
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<tr>
<td><strong>L-NMMA + TEA</strong></td>
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<tr>
<td>Δ flow (%)</td>
<td>10.0 ± 2.8</td>
<td>13.9 ± 3.1</td>
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<tr>
<td>Δ baseline LVP (%)</td>
<td>33.5 ± 3.8</td>
<td>33.5 ± 3.8</td>
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<tr>
<td><strong>L-NMMA + TEA + HC</strong></td>
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<td>Δ flow (%)</td>
<td>34.0 ± 4.5</td>
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<td>Δ LVP (%)</td>
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<tr>
<td>n</td>
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<tr>
<td><strong>L-NMMA</strong></td>
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<tr>
<td>Δ baseline flow (%)</td>
<td>1.7 ± 0.1</td>
<td>2.2 ± 0.3</td>
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<tr>
<td>Δ baseline LVP (%)</td>
<td>59.8 ± 9.3</td>
<td>76.5 ± 5.6</td>
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<tr>
<td><strong>Miconazole</strong></td>
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that seen in WT hearts (Table 2). After termination of hypercapnic perfusion, parameters returned to baseline values within 10 min.

When the hypercapnic flow response was tested in the presence of L-NAME, flow increased by 44.8 ± 12.1% vs. 51.1 ± 11.6% (Figure 2A). Hence, there was no effect of eNOS or any other NOS on the flow response during hypercapnic acidosis in the eNOS-KO group.

### 3.1.2.1 Blockade of KATP-channels

Blockade of KATP-channels by glibenclamide (n = 6) reduced baseline flow by 54.2 ± 4.8% (P < 0.001). The hypercapnia-induced flow increase was severely blunted with respect to the flow response obtained in unblocked eNOS-KO hearts (flow change 53.1 ± 6.3% before vs. 24.6 ± 2.7 after glibenclamide; P < 0.049) (Figure 2B). However, it should be noted that a large fraction of the hypercapnic flow response persisted. Therefore, several other vasodilatory mechanisms were addressed in eNOS-KO heart, as described in the following.

### 3.1.2.2 Blockade of neuronal NO synthase (nNOS)

Application of the nNOS inhibitor TRIM (n = 5) increased baseline flow by 34.5 ± 7.1% (P = 0.006). However, TRIM did not attenuate the hypercapnia-induced flow response in eNOS-KO hearts (flow change 93.4 ± 27% before vs. 94.5 ± 34% after TRIM).

### 3.1.2.3 Blockade of cyclooxygenase

Cyclooxygenase inhibition with indomethacin (n = 6) did not affect baseline parameters significantly. Also, indomethacin did not have a significant effect on the hypercapnia-induced flow response (72.3 ± 28.1% before vs. 126.5 ± 30.3% after indomethacin).

### 3.1.2.4 Blockade of KCa-channels

TEA was used in six eNOS-KO hearts to study the involvement of KCa-channels under continuous L-NMMA infusion. The L-NMMA infusion did not induce significant changes in baseline parameters and in the flow response to hypercapnic perfusion (33.5 ± 3.8% before vs. 30.9 ± 4.7% after L-NMMA). Additional infusion of TEA slightly elevated baseline flow (+13.9 ± 3.1%, P = 0.006). Most importantly, however, there was no change of the responses of flow during hypercapnic perfusion (30.9 ± 4.7% before vs. 34.0 ± 4.5% after TEA).

### 3.1.2.5 Blockade of Kv-channels

The Kv-channel blocker 4-AP was infused in four eNOS-KO hearts (0.1 mM). Effects of the inhibitor under baseline conditions were similar to those seen in WT hearts. Flow increased about 50.2 ± 20.8%. Similarly to WT hearts, an only minor flow rise was observed during hypercapnic perfusion (35.5 ± 9.1% in the absence of 4-AP vs. 7.4 ± 9.9% after 4-AP). However, due to considerable interexperimental variability this difference was not significant (P = 0.172). It should be noted that the reactive flow response following a 20 s flow stop was largely preserved. This indicated that the vessel response towards vasodilating mechanisms was preserved.

### 3.1.2.6 Blockade of A2A-receptors

A block of A2A-receptors on the hypercapnic flow response was studied in four eNOS-KO hearts. SCH 58261 did not induce any significant changes in baseline parameters. The flow response during hypercapnic perfusion was not attenuated, but rather elevated as compared with control (16.4 ± 6.3% before vs. 27.6 ± 9.0% after SCH 58261; P = 0.029).

### 3.1.3 WT-LN

Hypercapnic acidosis caused a pronounced flow increase (P < 0.001), which remained stable after 5 min (Figure 3). As in the other groups, LVP decreased significantly during hypercapnic acidosis (P < 0.001). This decrease exceeded that in eNOS-KO hearts. All parameters recovered to baseline values after discontinuation of hypercapnic perfusion within 10 min.

When either L-NAME or L-NMMA was infused (n = 10), none of the measured parameters including baseline flow

<table>
<thead>
<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td>Δ baseline flow (%)</td>
<td>WT n = 35</td>
</tr>
<tr>
<td>Δ baseline LVP (%)</td>
<td>20.5 ± 7.9a</td>
</tr>
<tr>
<td>Δ LVP (%)</td>
<td>13.5 ± 2.6a</td>
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<tr>
<td>Miconazole + HC</td>
<td>26.1 ± 5.1b,e</td>
</tr>
</tbody>
</table>

WT, wild-type mice hearts; WT-LN, wild-type mice hearts pretreated with eNOS blocker; eNOS-KO, eNOS knockout mice hearts; LVP, left ventricular pressure; data acquired at minute 8 during time course of hypercapnia (HC).

aAbsolute change significant vs. basal values without blocker.
bRelative change significant vs. eNOS-KO.
cRelative change significant vs. basal values without blocker.
dRelative change significant vs. WT-LN.
eRelative flow increase during hypercapnia with acute eNOS-blockade significantly less compared with relative flow increase during hypercapnia without eNOS-blockade.
*fAbsolute change significant vs. basal values with blocker.
changed significantly. This indicates that a successful block of NO synthases had been achieved by chronic administration of L-NAME. In the presence of continuous infusion of L-NAME hypercapnia induced significant changes in flow (Figure 3) and LVP (Table 2). The relative change of flow during continuous inhibitor infusion was not significantly lower than that before start of inhibitor infusion (37.3 ± 5.9% before vs. 21.2 ± 4.8% after L-NAME; P < 0.09). Also, the flow response was still similar to that seen in WT hearts in the absence of any inhibitor. Western blot analysis revealed no significant changes of nNOS content in eNOS-KO and WT-LN hearts as compared with WT hearts (Figure 4).

4. Discussion

The important results of our study are:

(i) NO-production and K\textsubscript{ATP}-channel activation together may fully account for the steady-state hypercapnic flow response in mouse heart.

(ii) However, in eNOS-deprived mouse hearts from a genetic-knockout model the hypercapnic flow response is well preserved.

(iii) And after pharmacological block of NOSs over 2 weeks, there is also preservation of the flow response by NOS-independent mechanisms.

(iv) Enhanced activation of K\textsubscript{ATP}-channels and potentially K\textsubscript{Ca}-channels contribute to the compensatory mechanisms involved in the flow response during hypercapnic acidosis when eNOS activity is absent.

(v) Prostaglandins, adenosine, EETs, and K\textsubscript{Ca}-channels seem to be unimportant for compensation of the failure of NO-production with respect to coronary vasodilation during acidosis.
The present study extends previous work in which we showed conclusively that intact NO-production is important for the steady-state increase of coronary flow during arterial hypercapnic acidosis in isolated guinea pig heart. The present study not only confirms these previous results and extends it to mouse heart, but also addresses several potential compensation mechanisms during periods of NOS depletion or inhibition. Whereas intact NO-production appears to be crucial under physiological conditions to mediate the full-scale increase of coronary flow during arterial hypercapnic acidosis, the importance of NO is compensated by other dilatory mechanisms under conditions of chronic NOS inhibition in hearts of wild-type (WT) mice after treatment with L-NAME (WT-LN). The flow response measured in WT mouse heart under control conditions (Figure 1) is shown for comparison, \( P = 0.096 \) WT-LN (control) vs. WT (control), \( P = 0.001 \) control (WT-LN) vs. L-NAME (WT-LN).

![Figure 3](image)

**Figure 3** Hypercapnia-induced coronary flow increases before (control) and after NOS inhibition in hearts of wild-type (WT) mice after treatment with 100 mg/kg L-NAME per day for 2 weeks (WT-LN, \( n = 10 \)). The flow response measured in WT mouse heart under control conditions (Figure 1) is shown for comparison, \( P = 0.096 \) WT-LN (control) vs. WT (control), \( P = 0.001 \) control (WT-LN) vs. L-NAME (WT-LN).

![Figure 4](image)

**Figure 4** Western blot analysis of nNOS-protein content in wild-type (WT), WT mice after treatment with L-NAME (WT-LN) and eNOS-KO mouse heart (data shown as relative nNOS/tubulin content).

The importance of Kv-channels could not be conclusively studied in the present study. Although the inhibitor 4-AP completely blunted the hypercapnic flow response during hypercapnic acidosis in WT hearts (data not shown), it also had major side effects with respect to L VP and coronary flow. 4-AP concentrations of 500 \( \mu \text{M} \) which is considerably below the concentration used in literature (3 mM) induced complete heart failure. In a lower concentration of 4-AP (0.1 mM), the hypercapnic flow response was severely blunted. Unfortunately, this concentration of 4-AP also enhanced baseline LVP and coronary flow by about 50%. Thus, clarification of the role of K\(_\text{v}\)-channels must await the development of new and more specific blockers of this class of potassium channels.

In addition to the genetic-knockout model of eNOS, we applied extended pharmacological block of eNOS to test for compensation by other vasodilatory principles. The method of L-NAME application in drinking water (100 mg/kg per day) has been used by others before. This dose decreased the plasma NOx concentration by 40% and plasma nitrite by more than 70% to that measured in eNOS-KO mice. Moreover, when an even lower dose (40 mg/kg body mass) was applied to mice overexpressing human erythropoietin, this dose was lethal within 2 days giving evidence for its effectiveness. Thus, it can be safely assumed that the NOS-enzymes were effectively inhibited for more than 12 days in the present study. After this period of NOS inhibition, the coronary flow response towards hypercapnic acidosis was fully preserved. Moreover, during additional continuous infusion of L-NAME in the isolated heart model the flow rise during hypercapnic acidosis was largely preserved and quantitatively similar to that seen in hearts from WT mice without eNOS blockade (Figure 3).

Previously, several research groups have contributed data on the influence of K\(_\text{ATP}\)-channels on coronary flow regulation under baseline conditions\(^{14}\) and during hypercapnic acidosis.\(^{15}\) It was found that baseline coronary flow declined significantly after infusion of glibenclamide. In accordance, the present study provides evidence that the baseline coronary vessel tone of isolated mouse heart is largely controlled by K\(_\text{ATP}\)-channels. Blocking these channels with glibenclamide also blunted the flow increase in rat heart\(^{6}\) and relaxation of pig coronary microvessels\(^{19}\) during acute acidosis. In contrast, K\(_\text{ATP}\)-channels do not contribute to flow augmentation during acute hypercapnic acidosis in guinea pig heart. The present study shows for mouse heart that the hypercapnic flow response is in part mediated by K\(_\text{ATP}\)-channels and that their importance is augmented in the absence of functional eNOS. In contrast, cyclooxygenase, CYPs, nNOS, and adenosine seem unimportant for the hypercapnic flow response in WT mouse heart as well as in eNOS-KO hearts. The missing effects of miconazole in WT heart may indicate that EETs are not activated during hypercapnic acidosis. Another possible interpretation is derived from the demonstration of an unspecific effect of miconazole on K\(_\text{ATP}\)-channels.\(^{20}\) Following this evidence, a missing inhibitory effect of miconazole in eNOS-KO heart (Table 2) could also be interpreted to indicate that the K\(_\text{v}\)-channels blocked by miconazole and TEA act in series with NO-production. This is not the case with K\(_\text{ATP}\)-channels that must be activated by a mechanism separate from NO-production, because glibenclamide had powerful inhibitory effects on the flow hypercapnic response in eNOS-KO hearts. A potential mode of K\(_\text{ATP}\)-channel opening may result from direct interaction of protons with the channel.\(^{21}\}

The present study extends previous work in which we showed conclusively that intact NO-production is important for the steady-state increase of coronary flow during arterial hypercapnic acidosis in isolated guinea pig heart. The present study not only confirms these previous results and extends it to mouse heart, but also addresses several potential compensation mechanisms during periods of NOS depletion or inhibition. Whereas intact NO-production appears to be crucial under physiological conditions to mediate the full-scale increase of coronary flow during hypercapnic acidosis, the importance of NO is compensated by other dilatory mechanisms under conditions of chronic eNOS inactivity.

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by Talukder et al.,27 we observed a weak trend for an increase of nNOS protein expression in eNOS-KO mice (Figure 4), but iNOS expression remained very low and did not change significantly in both experimental groups (data not shown). L-NAME (non-specific NOS inhibitor) as well as TRIM (selective nNOS inhibitor) did not block the flow responses under conditions of chronic eNOS failure. This excludes an effective compensation via other NOS isoenzymes in the coronary circulation.

Aside from production via NOs, NO generation may result non-enzymatically from nitrite under conditions of acidosis.38 However, this requires pH values below 6, which are unlikely under the conditions of the present study. Furthermore, NOS-independent NO-production is only of relevance under anaerobic conditions.28,29 In eNOS-KO mice, the sensitivity for NO vasodilation was shown to be augmented in the model of isolated aortic rings.30 Because NO formation from non-NO sources was unlikely under the conditions of the present study and NOS-dependent compensation was absent, a potentially enhanced NO sensitivity is most likely irrelevant for the mediation of the hypercapnic flow response.

Peroxynitrite is a strong and relatively stable oxidant species, formed in tissues by the non-enzymatic interaction between superoxide (O2−) anion and NO. Besides its capability of causing lipid peroxidation and nitration of tyrosine residues of proteins, peroxynitrite is a weak vasodilator but simultaneously it impairs relaxation to endothelium dependent of causing lipid peroxidation and nitration of tyrosine residues. Villa et al.32 showed in the isolated perfused rat heart that the vasodilatory response to peroxynitrite was inhibited by oxyhaemoglobin, indicating that it was due to the generation of NO. Hence, under the conditions of the present study (eNOS-KO and WT-LN groups) a compensation for NO-production via peroxynitrite is unlikely. However, peroxynitrite and other oxidant species were not measured in the present experiments. Thus, their potential contribution cannot be ruled out completely.

Chlopicki et al.33 have previously reported a compensation of coronary flow responses in eNOS-KO mice in response to acetylcholine. In contrast to the coronary circulation, aortic relaxation towards acetylcholine was severely blunted in the same mouse strain. The results of this and our study agree that coronary flow control of eNOS-KO mice is compensated by yet to be clarified mechanism(s). The present study suggests that flow control involving KATP-channels may contribute to this compensation. Another potential compensation may result from K+ -channels. It is likely that the compensation of NO failure involves a mechanism that is of no major relevance under conditions of intact NO-production. This(ese) compensatory mechanism(s) and their molecular compensation should be addressed in future studies to unmask potential compensatory mechanisms active during chronic inhibition of NO-production.

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Reference


