Myocardial $O_2$ consumption in porcine left ventricle is heterogeneously distributed in parallel to heterogeneous $O_2$ delivery

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Abstract

Myocardial blood flow is unevenly distributed, but the cause of this heterogeneity is unknown. Heterogeneous blood flow may reflect heterogeneity of oxygen demand. The aim of the present study was to assess the relation between oxygen consumption and blood flow in small tissue regions in porcine left ventricle. In seven male, anesthetized, open-chest pigs, local oxygen consumption was quantitated by computational model analysis of the incorporation of $^{13}$C in glutamate via the tricarboxylic acid cycle during timed infusion of $[^{13}$C]acetate into the left anterior descending coronary artery. Blood flow was measured with radioactive microspheres before and during acetate infusion. High-resolution nuclear magnetic resonance $^{13}$C spectra were obtained from extracts of tissue samples (159 mg mean dry wt) taken at the end of the acetate infusion. Mean regional myocardial blood flow was stable [5.0 ± 1.6 (SD) and 5.0 ± 1.4 ml-min$^{-1}$·g dry wt$^{-1}$ before and after 30 min of acetate infusion, respectively]. Mean left ventricular oxygen consumption measured with the NMR method was 18.6 ± 7.7 µmol·min$^{-1}$·g dry wt$^{-1}$ and correlated well ($r = 0.85, P = 0.02, n = 7$) with oxygen consumption calculated from blood flow, hemoglobin, and blood gas measurements (mean 22.8 ± 4.7 µmol·min$^{-1}$·g dry wt$^{-1}$). Local blood flow and oxygen consumption were significantly correlated ($r = 0.63$ for pooled normalized data, $P < 0.0001, n = 60$). We calculate that, in the heart at normal workload, the variance of left ventricular oxygen delivery at submilliliter resolution is explained for 43% by heterogeneity in oxygen demand.

Key words: regional blood flow – metabolism – myocardium - magnetic resonance spectroscopy
Myocardial blood flow is highly heterogeneous, as demonstrated in many different species, with various methods (radioactive, colored and fluorescent microspheres, molecular tracers, MRI, and PET), and under different experimental circumstances, such as open- versus closed-chest and anesthetized versus awake animals (5, 9, 19, 22, 26, 27). Myocardial perfusion shows similar heterogeneity in the human heart (47). This heterogeneity is largely of spatial nature, although there is also some temporal variation (27). Local blood flow can differ by a factor five among small areas of the left ventricle, even in the normal heart. Interestingly, regional blood flow is rather stable over a substantial time period, at least for several hours, but some data even indicate a relatively stable local blood flow over days (44). From an anatomic and mechanical point of view, this heterogeneity is difficult to understand because the heart appears rather homogeneous and biomechanical models predict homogeneous contraction. The question thus remains: which factors contribute to heterogeneous blood flow? Heterogeneity in oxygen consumption (VO$_2$) between different areas of the left ventricle may explain at least part of the heterogeneity of myocardial blood flow. Indeed, it has been shown in previous studies (9, 19) that indirect indicators of local aerobic metabolism show heterogeneity. For instance, a correlation between local blood flow and fatty acid uptake has been shown (24). Others (38, 39) have reported a rather good correlation between local myocardial blood flow and local myocardial VO$_2$ (MVO$_2$) measured by [18O]water in the isolated buffer-perfused rabbit heart. Low-flow areas had lower VO$_2$ and high-flow areas had higher VO$_2$. Further studies showed that areas of the left ventricle that receive a relatively low amount of blood flow are not in a hypoxic state, suggesting local adaptation of blood flow to oxygen demand (8, 11, 14). However, direct measurement of aerobic metabolic flux in small myocardial regions became possible only recently. Regional VO$_2$ and regional blood flow can be estimated with PET, although this technique still does not permit analysis at sufficiently high spatial resolution to study cardiac heterogeneity in small tissue areas (1, 34, 37).

To investigate the distribution of MVO$_2$ at high spatial resolution, a new combination of isotope labeling protocol and mathematical analysis of $^{13}$C incorporation into the tricarboxylic acid (TCA) cycle has been developed by us (43). $^{13}$Clabeled substrate is infused during a brief, exactly timed period directly into a coronary artery and the resulting isotope distribution is measured in tissue samples using NMR spectroscopy. Because most of the TCA cycle
intermediates are present at concentrations below the detection limit of NMR, $^{13}$C incorporation is measured in three distinct carbon atom positions of glutamate, which is in direct exchange with α-ketoglutarate in the TCA cycle via glutamate transaminase. From the NMR intensities, the TCA cycle flux is estimated using a computational model. The validity of this approach has been shown in isolated perfused rabbit hearts (43) and its feasibility on the in situ heart has been shown in the rabbit (42).

A similar method has recently been applied to demonstrate that regions with the 10% highest flow have significantly higher $\text{VO}_2$ than regions with the 10% lowest flow in the canine left ventricle (12). In this study, we applied our new $^{13}$C method to measure the profile of $\text{VO}_2$ in small tissue areas in the normal in situ porcine left ventricle for the full range of blood flow. We performed a 5.5-min infusion with $^{13}$C-enriched sodium acetate as substrate and measured blood flow at the same time point in the same tissue sample with radioactive microspheres to evaluate how local oxygen delivery is matched to local oxygen demand.

**Materials and methods**

*Experimental preparation.* The experimental protocol was approved by the Advisory Board for the Use of Experimental Animals of the Vrije Universiteit. Seven male pigs (mean weight 29 kg, range 24–33 kg) were studied. They were sedated with an intramuscular injection of 15 mg/kg ketamine, 1 mg/kg midazolam, and 0.5 mg atropine. A peripheral intravenous catheter was inserted. The trachea was intubated and the lungs were mechanically ventilated with a 40% O$_2$-60% air mixture.

Anesthesia was maintained with a combination of sufentanil (4 µg·kg$^{-1}$·h$^{-1}$) and midazolam (0.5 mg·kg$^{-1}$·h$^{-1}$). Lidocaine (a bolus of 50 mg, followed by a continuous infusion of 9 mg·kg$^{-1}$·h$^{-1}$) was given to prevent cardiac arrhythmias during manipulation of the heart. Pancuronium (0.2 mg·kg$^{-1}$·h$^{-1}$) was used throughout the whole procedure, facilitating mechanical ventilation and providing muscle relaxation. This is common during cardiac surgery (15, 33). We checked for absence of corneal reflexes and of reaction to a pain stimulus in the nasal cartilage before administration of pancuronium, indicating adequate
anesthesia. Furthermore, we closely monitored heart rate and blood pressure, which quickly respond to pain, and found no sudden increases throughout the experiment. The doses of anesthetics are in the range commonly used for anesthesia in pigs with (20, 29, 45) and without (16) muscle relaxants. This procedure is in accordance with the American Physiological Society “Guiding Principles in the Care and Use of Animals,” which states that muscle relaxants may be used in conjunction with drugs known to produce adequate anesthesia.

The following fluid-filled catheters were introduced: a catheter in the left femoral artery (for measurement of arterial blood pressure and arterial blood sampling); a catheter in the left ventricle via the left carotid artery (measurement of left ventricular pressure and its first time derivative (dP/dt)); a balloon-tipped pulmonary artery catheter via the right external jugular vein (measurement of cardiac output, pulmonary occlusion pressure and body temperature); and a catheter in a left-sided neck vein (infusion of fluids and drugs). The electrocardiogram and hemodynamics were continuously recorded. The thorax was opened via a midsternal incision and 5 cmH₂O of positive end-expiratory pressure was applied. The pericardium was incised and the heart exposed. The left hemiazygos vein was ligated to prevent mixing of noncoronary venous blood with coronary venous blood in the coronary sinus. Furthermore, a catheter was placed in the coronary sinus via the right atrium (to withdraw coronary venous blood) and another catheter in the left atrium for injection of radioactive microspheres. After the free part of the left anterior descending coronary artery (LAD) was dissected, a small (24 gauge) catheter was inserted as proximally as possible into this artery for infusion of sodium acetate.

After instrumentation was finished, a bolus of 2,500 IU heparin was administered, followed by an infusion of 40 IU·kg⁻¹·h⁻¹. The animal was allowed to stabilize for at least 15 min, after which the following protocol was applied. At the start of the protocol [time (t) = 0] (Fig. 1), systemic arterial and coronary venous blood samples were taken, and cardiac output and pulmonary arterial occlusion pressure were measured. Cardiac output was measured at random in the respiratory cycle via the thermodilution method (average of three injections of 5 ml of ice-cold glucose 5% solution; Cardiac Output Computer 9520A, Edwards). A first batch of microspheres (labeled with \(^{131}\)Ce or \(^{103}\)Ru, in random order) for measurement of baseline regional left ventricular blood flow was injected into
the left atrium during 20 s. The suspension containing the microspheres had been vortexed vigorously for 5 min and was continuously stirred during injection with a custom-built mixing device. Starting 5 s before the injection of microspheres, and continuing for 100 s from the injection onward, reference blood was drawn from the femoral artery at a rate of 10.8 ml/min.

Fig. 1. Time line of experimental protocol after instrumentation of the animals. After a stabilization period, arterial and coronary venous blood samples were withdrawn and the first batch of radioactive microspheres was injected (time (t) = 0 min). Native acetate (not enriched with 13C) was infused into the left anterior descending coronary artery (LAD) catheter for 30 min. After this period, blood samples were withdrawn a second time and the second batch of microspheres was injected (t = 30 min). The native acetate infusion was quickly switched to [2-13C]acetate for 4 min, followed by [1,2-13C]acetate for 1.5 min. The left ventricular free wall was then freeze clamped in situ, quickly cut out of the body, and stored in liquid nitrogen. Hemodynamic data were recorded throughout the whole protocol. Time line is not drawn to scale.

13C-enriched acetate infusion to measure VO2
After injection of the first batch of microspheres, an infusion of 40 mmol/l unlabeled sodium acetate in 0.9% sodium chloride was started directly into the LAD at a rate of 2.25 ml/min, for 30 min, to achieve a metabolic steady state. After 0, 10, 20, and 30 min, arterial and coronary venous blood was drawn for measurement of acetate concentrations. After 30 min (t = 30), arterial and coronary venous blood samples were taken, cardiac output and pulmonary arterial occlusion pressure were measured, and a second batch of microspheres was injected. The infusion of unlabeled acetate was then quickly switched to [2-13C]acetate (at unchanged concentration) for exactly 4 min, after which another quick switch was made to the infusion of [1,2-13C]acetate for 1.5 min. The second label increases parameter estimation accuracy (41, 43). After exactly 5.5
min of [13C]acetate infusion, a part of −2 x 2 cm of the anterior left ventricular free wall, immediately adjacent to the LAD, was quickly freeze-clamped in situ inside an aluminium clamp precooled to the temperature of liquid nitrogen, cut out of the heart, and immediately placed into liquid nitrogen. Papillary muscles and their base were not included. Both kidneys were harvested for determination of microsphere distribution. Left ventricular tissue was stored at −80°C until further analysis.

**Tissue preparation and NMR measurement**

The tissue was freeze-dried (Modulyo freeze-dryer; Edwards) for at least 48 h and cut into small pieces representing the subepicardial, midmyocardial, and subendocardial layers. Tissue samples were cut to very small pieces, weighed, and homogenized in 4 ml of 0.6 mol/l ice-cold perchloric acid for 1 min, and then centrifuged (10 min at 4,000 g). The pellet was used for measurement of microsphere radioactivity (1280 Compugamma, LKB-Wallac). Blood flow was determined according to the standard formula $F_l = (I_l/I_a) \times F_a$, where $F_l$ is local blood flow, $I_l$ is local radioactivity in tissue sample, $I_a$ is arterial reference radioactivity, and $F_a$ is arterial reference flow. Local flows were expressed as milliliters per minute per gram dry weight. The difference in blood flow in the left and right kidney was <5% in all animals, indicating adequate mixing of the microspheres in the arterial blood.

The supernatant was adjusted to pH 7.0 with buffer containing 3 mol/l KOH and 0.3 mol/l imidazole. The sample was centrifuged again and the supernatant was freeze dried for 48 h. Freeze-dried supernatants were dissolved in tridistilled H$_2$O and D$_2$O (0.5 ml total) and 13C-NMR spectra were obtained at 100.62 MHz with a Bruker Avance400 spectrometer. The samples were studied in a 5-mm probe at 27°C with a WALTZ-16 broad-band ¹H decoupling pulse sequence, ¹³C-pulse angle 45°, repetition time 7.3 s, 32k data points, sweep width 100 ppm with 1,470 scans accumulated. Spectra were analyzed in the time domain with the MRUI/AMARES software package after conversion to Magnetic Resonance User Interface format (European Union Human Capital and Mobility Project). Individual glutamate multiplet areas of the 2-, 3-, and 4-carbon atoms were quantitated with reference to a standard spectrum of a 50 mmol/l unlabeled glutamate solution (¹³C present at the natural abundance of 1.1%) and converted to micromole per gram dry weight.
Model analysis of NMR multiplets
The NMR multiplet peaks were analyzed with a previously published and validated computer model, schematically depicted in Fig. 2 (43). In brief, the model consists of 132 differential equations (for detailed description of the equations, see appendix to Ref. 43), incorporating all possible combinations of $^{13}$C and $^{12}$C in 6 different molecular “pools” representing acetyl-CoA, the TCA cycle intermediates and associated amino acids (aspartate and glutamate). The isotope exchange reactions between these pools are incorporated in the differential equations. The TCA cycle pool is represented by a 6- carbon pool (citrate, cis-aconitate, and isocitrate), a 5-carbon pool ($\alpha$-ketoglutarate), and a 4-carbon pool (succinyl-CoA, succinate, fumarate, malate, and oxaloacetate), plus an acetyl-CoA pool, an aspartate pool, and a glutamate pool. The TCA cycle intermediate $\alpha$-ketoglutarate in the 5-carbon pool is only present in small amounts but it exchanges carbon atoms quickly with glutamate.

Glutamate is present at high levels and $^{13}$C spectra can be readily accumulated with in vitro NMR techniques. Labeling of glutamate with $^{13}$C after infusion of [2-$^{13}$C]- and [1,2-$^{13}$C]-labeled substrate leads to the appearance of a maximum of nine different multiplets (by $J$ coupling between adjacent carbon atoms) at the chemical shift positions of the 4-carbon, 3-carbon, and 2-carbon atoms of glutamate; the pattern of multiplet labeling is dependent on the fractional enrichment of acetyl-CoA ($F_{C2}$) and the turnover rate of the TCA cycle ($J_{TCA}$). The model for the isotope distribution is fitted to the multiplet intensity data by nonlinear least-squares parameter optimization. MVO$_2$ is derived from these two optimized model parameters, using the formula $MVO_2^{[13]C} = (3 - F_{C2}) \times J_{TCA}$. Four additional parameters can be estimated with the optimization procedure using the following model: 1) the exchange rate between $\alpha$-ketoglutarate and glutamate ($J_{\text{exchange}}$, $\mu$mol·min$^{-1}$·g dry wt$^{-1}$), which has often been assumed to occur at infinite rate in other studies, although this is not entirely correct (50); 2) the time constant for transport ($\tau_{\text{transport}}$, s) from the arterial infusion site of acetate through the blood vessels and cell membranes to incorporation in acetyl-CoA. This time constant is usually not taken into account in other studies but should not be neglected in these short-term experiments; 3) the relative anaplerotic flux ($J_{\text{anap}}/J_{TCA}$, %), which means the entering of unlabeled substrate into the TCA cycle from other sources than acetyl-CoA, thereby diluting the amount of $^{13}$C in the TCA cycle; and finally, the exchanging glutamate pool.
Taking account of these parameters improves the accuracy of estimation of $J_{\text{TCA}}$ and $F_{C_2}$, the latter two forming the parameters of primary interest because they allow to calculate $VO_2$. For the estimation of local $VO_2$, the parameter $J_{\text{exchange}}$ was set to the fixed value of 17 µmol·min$^{-1}$·g dry wt$^{-1}$, $\tau_{\text{transport}}$ to 30 s, and $J_{\text{anap}}/J_{\text{TCA}}$ to 0.16. These were mean values derived from those model fits which were feasible without fixing these parameters. Glutamate was fixed at the biochemical assay value. Fits with the full multiplet model were only accepted when the overall coefficient of variation (CV) was <25%. In case, this multiplet method did not lead to an acceptable fit of the model to the peak data because of low multiplet intensity relative to the NMR noise, we applied the simplified strategy to estimate $J_{\text{TCA}}$ by fitting the mean of the G2 and G3 peaks, divided by the total of the G4 peaks [ratio method (12, 41); $F_{C_2} = 0.6$]. The value of glutamate was fixed at the biochemical assay value. Sensitivity analysis showed the influence of $F_{C_2}$ to be low in the ratio method: the ratio changes 0.6% per 10% change in $F_{C_2}$.

Fig. 2. Scheme of the computational model of incorporation of $^{13}$C in the tricarboxylic acid (TCA) cycle, aspartate, and glutamate. One of the possible enrichment sequences resulting from the incorporation of $^{13}$C from [2-$^{13}$C]acetate infusion is shown. Parameters which are optimized using this model are the transport time to incorporation in acetyl-CoA, the $^{13}$C enrichment fraction of acetyl-CoA, the exchange rate of carbon atoms between the 5-carbon pool (α-ketoglutarate) and glutamate, the TCA cycle flux, the anaplerotic flux (unenriched carbon atoms entering the TCA cycle via other routes than acetyl-CoA), and the glutamate pool. For further details, see Ref. 43.
Chapter 3

Computer simulations
We tested how sensitive the estimates of $J_{TCA}$ and $F_{C2}$ are for potential errors in $J_{\text{exchange}}$, $\tau_{\text{transport}}$, and $J_{\text{anap}}$ because these latter model parameters can only be determined with limited precision. $J_{TCA}$ and $F_{C2}$ were estimated and compared in repeated parameter optimizations, whereas the value of $J_{\text{exchange}}$ was increased or decreased by 10%, with the other parameters remaining at the fixed values. Similar estimations were performed by increasing or decreasing $\tau_{\text{transport}}$ and $J_{\text{anap}}$ by 10%. During this procedure, the parameters were determined by fitting the model to a dataset of nine glutamate multiplets.

Biochemical and blood gas analysis
Glutamate in tissue samples (in µmol/g dry wt) was measured with a coupled enzyme assay as described by Bergmeyer (6). Lactate content in arterial and coronary venous blood samples (in mmol/l) was measured using an L() lactate kit (Sigma Diagnostics). For these enzyme assays, an LKB Ultraspec Plus spectrophotometer was used. Underivatized acetic acid was determined with gas chromatography/mass spectrometry (GC/MS). A labeled internal standard (acetic acid d3, Sigma-Aldrich) was added to the sample, followed by acidification, desalting, and extraction by diethylether. One microliter of a sample was injected into the GC-MS system (Hewlett-Packard GC/MS Engine, using an FFAP column) and measured in the electron impact mode. Global MVO$_2$ was measured according to standard methods: blood gases were measured with a blood gas analyzer (Radiometer ABL), whereas hemoglobin (Hb, in mg/dl) content and oxygen saturation (SO$_2$, %) were measured with a hemoximeter (OSM3, Radiometer). Blood O$_2$ content (in µmol/ml) was computed as follows: $(Hb \times 0.621 \times SO_2) + (0.00131 \times PO_2)$. Blood gas values were corrected for body temperature (37.5–38.5°C). Myocardial oxygen delivery was computed as the product of mean flow of the tissue samples of the particular heart and arterial O$_2$ content. MVO$_2$ in the left ventricle was calculated as the product of coronary blood flow and the difference in O$_2$ content between arterial and coronary venous blood (16), for direct comparison with the $^{13}$C method to quantitate VO$_2$.

Statistics
Data are expressed as means ± SD. Paired t-tests or ANOVA for repeated measurements were used for comparison of data at different time points. The trends were determined by linear regression and the correlation coefficient was
calculated. Heterogeneity was expressed as the CV (in %) (CV = 100 x SD/mean). A Bland-Altman analysis was performed by plotting the mean of normalized blood flow and normalized VO₂ versus their difference. Measurements of blood flow and VO₂ were normalized to the mean per individual heart to remove interindividual variation.

Part of the scatter around the regression line of VO₂ versus O₂ delivery is caused by measurement errors, which occur in both the microsphere and the VO₂ measurements. The contribution of these measurement errors to the total measured scatter can be calculated with the formula described in the appendix of Ref. 9, which partitions the variance in parts due to the measured correlation, measurement errors in both variables and a part attributable to unknown biological factors. The contribution of variation in VO₂ to the heterogeneity of blood flow could then be estimated quantitatively. A value of $P < 0.05$ was considered statistically significant.

**Results**

**Blood flow measurements and stability**

Hemodynamic parameters are shown in Table 1. Heart rate, blood pressures, cardiac output, global left ventricular VO₂, and biochemical values did not change over the 35.5 min of unlabeled and labeled acetate infusion ($P > 0.05$). In 60 tissue samples from 7 hearts ($n = 5–12$ per heart, mean dry wt $0.159 \pm 0.05$ g), both blood flow (measured with radioactive microspheres) and VO₂ (measured with the NMR method) were determined. Before and after 30 min of acetate infusion, mean blood flow was $5.0 \pm 1.6$ and $5.0 \pm 1.4$ ml·min⁻¹·g dry wt⁻¹, respectively, showing that the acetate infusion did not change blood flow ($P > 0.05$).
Chapter 3

Blood flow heterogeneity

Myocardial blood flow, normalized to a mean value of 1, was distributed heterogeneously over the 60 tissue samples, ranging from 0.30 to 1.62 at \( t = 30 \) min. The mean CV of blood flow before and after 30 min of unlabeled acetate infusion was 26.9\% and 26.2\%, respectively (\( P > 0.05 \)). Individual CVs showing the heterogeneity of blood flow per heart are given in Table 2. Acetate concentrations in systemic arterial blood did not change significantly over the 30-min period of intracoronary infusion of unlabeled acetate, 159–176 µmol/l in five hearts. Coronary venous acetate concentration was 44 ± 44 µmol/l at the start of the protocol and increased rapidly to 870 ± 459 µmol/l after 10 min of acetate infusion, remaining > 700 µmol/l throughout the rest of the protocol period, indicating a stable metabolic situation.

<table>
<thead>
<tr>
<th>Table 1. Hemodynamic and metabolic data</th>
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<tr>
<td><strong>Baseline</strong></td>
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<tr>
<td>Heart rate, beats/min</td>
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<tr>
<td>Mean arterial blood pressure, mmHg</td>
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<tr>
<td>Left ventricular systolic pressure, mmHg</td>
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<tr>
<td>Left ventricular diastolic pressure, mmHg</td>
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<tr>
<td>Cardiac output, l/min</td>
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<tr>
<td>Pulmonary occlusion pressure, mmHg</td>
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<tr>
<td>dP/dt, mmHg/s</td>
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<tr>
<td>Arterial Po₂, mmHg</td>
</tr>
<tr>
<td>Arterial PCO₂, mmHg</td>
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<tr>
<td>Arterial pH</td>
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<tr>
<td>Coronary venous Po₂, mmHg</td>
</tr>
<tr>
<td>MD0₂, µmol·min⁻¹·g dry wt⁻¹</td>
</tr>
<tr>
<td>MVO₂, µmol·min⁻¹·g dry wt⁻¹</td>
</tr>
<tr>
<td>Oxygen extraction ratio, %</td>
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<tr>
<td>Arterial lactate, mmol/l</td>
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<tr>
<td>Coronary venous lactate, mmol/l</td>
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</table>

Values are means ± SD for study protocol. MD0₂, myocardial O₂ delivery; MVO₂, myocardial O₂ consumption calculated from arterial and coronary venous blood and blood flow. Baseline refers to values measured immediately before acetate infusion. During acetate infusion refers to values measured during acetate infusion.
VO$_2$ measurements and relation with blood flow

From the NMR spectra of 36–60 samples, it was possible to quantitate $J_{TCA}$ and $F_{C2}$ from the measured multiplets. From $J_{TCA}$ and $F_{C2}$ VO$_2$ was computed. For the remaining 24 samples, $J_{TCA}$ was estimated by using the ratio method (12, 41). The additional parameters were fixed at the mean values obtained from fits to the multiplets (CV < 25%); see Table 3. The VO$_2$ values in the 36 samples in which the full multiplet model estimation succeeded were compared with the VO$_2$ values derived from the same data with the ratio method. The results were similar, with a correlation coefficient of 0.91 ($P < 0.0001$; Fig. 3). Results for all estimated parameters are shown in Table 3. VO$_2$ estimated from the NMR spectra was heterogeneously distributed (range for normalized data, 0.44–1.72), with a slightly higher CV than for blood flow (Table 2).

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Table 3. Estimated model parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$n$</th>
<th>Absolute Values</th>
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<tbody>
<tr>
<td>$J_{TCA}$, $\mu$mol min$^{-1}$ g$^{-1}$</td>
<td>60</td>
<td>7.66 $\pm$ 3.03</td>
</tr>
<tr>
<td>$F_{C2}$, ratio</td>
<td>36</td>
<td>0.58 $\pm$ 0.23</td>
</tr>
<tr>
<td>$J_{exchange}$, $\mu$mol min$^{-1}$ g$^{-1}$</td>
<td>9</td>
<td>17.44 $\pm$ 6.05</td>
</tr>
<tr>
<td>$\tau_{transport}$, s</td>
<td>7</td>
<td>29.84 $\pm$ 11.56</td>
</tr>
<tr>
<td>$J_{anap}$/$J_{TCA}$, ratio</td>
<td>19</td>
<td>0.16 $\pm$ 0.12</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SD. $J_{TCA}$, tricarboxylic acid (TCA) cycle flux; $F_{C2}$, $^{13}$C-labeled fraction of acetyl-CoA; $J_{exchange}$, exchange flux; $\tau_{transport}$, time constant for transport; $J_{anap}$, anaplerotic flux.
Normalized blood flow measurements and normalized VO₂ were significantly correlated ($r = 0.63$, $P < 0.0001$) (Fig. 4). In the same figure, blood flow versus local TCA cycle flux is shown, which is the directly measured quantity and proportional to VO₂ with a multiplication factor that depends weakly on $F_{C2}$. Separate correlation coefficients ($r$) in the subendocardial, midmyocardial, and subepicardial layers of the left ventricle were similar (Table 4). Blood flow was slightly higher in subendocardial and subepicardial layers than in midmyocardial regions, but the difference was not statistically significant. VO₂ tended to be only slightly higher in subendocardium compared with subepicardium and midmyocardium.
Fig. 4. Blood flow after 30 min of unlabeled acetate infusion versus oxygen consumption (left) and TCA cycle flux (right) measured with the NMR method. All parameters were normalized to the individual means per heart. Sixty samples from seven hearts are shown. Blood flow and oxygen consumption were significantly correlated ($r = 0.63$, $P < 0.0001$), as were blood flow and TCA cycle flux ($r = 0.65$, $P < 0.0001$).

**Table 4. Normalized blood flow and oxygen consumption in different layers of left ventricle**

<table>
<thead>
<tr>
<th></th>
<th>Subendocardium ($n = 29$)</th>
<th>Midmyocardium ($n = 20$)</th>
<th>Subepicardium ($n = 11$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood flow</td>
<td>1.03 ± 0.27</td>
<td>0.91 ± 0.16</td>
<td>1.01 ± 0.30</td>
</tr>
<tr>
<td>MVO$_2$</td>
<td>1.02 ± 0.30</td>
<td>0.97 ± 0.28</td>
<td>0.98 ± 0.30</td>
</tr>
<tr>
<td>$r$</td>
<td>0.57</td>
<td>0.78</td>
<td>0.60</td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.001</td>
<td>0.004</td>
<td>0.005</td>
</tr>
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</table>

Values are means ± SD for 7 hearts. Blood flow and oxygen consumption measurements (both normalized to the individual mean of the heart) in the subendocardial, subepicardial, and midmyocardial layer of the porcine left ventricle are shown. The bottom rows shows the separate correlation coefficients with the corresponding $P$ value for normalized blood flow versus normalized oxygen consumption in the three layers of the heart.

Mean global VO$_2$ as measured from arterial and coronary venous blood gas samples was $22.8 ± 4.7$ µmol·min$^{-1}$·g dry wt$^{-1}$, whereas the NMR method gave $18.6 ± 7.7$ µmol·min$^{-1}$·g dry wt$^{-1}$. Comparison of VO$_2$ calculated from blood gas samples and measured with the NMR method (average over all samples per heart) revealed a high correlation ($r = 0.85$, $P = 0.02$; Fig. 5).
Fig. 5. Oxygen consumption measurements derived from the NMR method versus oxygen consumption measurements derived from arterial and coronary venous blood gas samples at the end of the protocol (n = 7 hearts). For the NMR method, mean values from all samples of a particular heart have been taken.

**Error analysis**

We investigate here to what extent the linear relation $Y = \beta_0 + \beta_1 X + \epsilon$ between flow ($Y$ in the equation) and $VO_2$ ($X$) explains the heterogeneity of flow. Here, $\beta_0$ is the intercept, $\beta_1$ is the slope, and $\epsilon$ is the error term. Scatter around the regression line of blood flow versus $VO_2$ is partially due to measurement errors in blood flow and $VO_2$ and partly due to unknown biological factors other than aerobic metabolism, which potentially determine blood flow. In the present study, we found that the flow-$VO_2$ correlation is given by $r^2 = 0.40$, $CV_{\text{flow}} = 0.26$, and $CV_{\text{VO}_2} = 0.29$. $CV_{\text{VO}_2}$ is the CV of $VO_2$, including measurement error. The estimate of $\beta_1 = 0.46$ is for normalized blood flow and $VO_2$. The $CV_{\text{_meas flow}}$ (SD of the measurement error divided by the mean) was 0.063 (derived from repeated
measurements on the same tissue samples), and $CV_{\text{meas VO}_2}$ was 0.060 (derived from repeated NMR measurements of the same tissue sample). Because of the measurement error in $VO_2$, the regression coefficient obtained from linear regression on the assumption of no measurement error in $VO_2$ is biased but can be corrected (9) by multiplying $\beta_1$ by the factor $[1 + CV^2_{\text{meas VO}_2} / (CV^2_{VO_2} - CV^2_{\text{meas VO}_2})]$, yielding a corrected slope of 0.48. The total fractional contribution of measurement errors to the total variance of blood flow (see Ref. 9), given by $\beta_1^2 \times CV^2_{\text{meas VO}_2} / CV^2_{\text{flow}} + CV^2_{\text{meas flow}} / CV^2_{\text{flow}}$ was 7.1%. Given the $r^2$ of 0.40, the contribution of unknown biological factors to the variance of blood flow is estimated to be 53% ($= 100 - 7 - 40\%$). Correcting for the measurement errors, the contribution of the linear relation between blood flow and $VO_2$ to the variance of blood flow was therefore 43%. In a separate study, the contribution of succinate dehydrogenase, a TCA cycle enzyme, was found to be 22% (9). It is highly likely that the effects of succinate dehydrogenase and $VO_2$ are not additive but strongly overlapping.

Computer simulations of the mathematical model

$J_{TCA}$ and $F_{C2}$ were estimated with three additional parameters fixed at the standard value, whereas the sensitivity to the fourth additional parameter was tested by giving it values plus or minus 10% of the standard values (see MATERIALS AND METHODS). The results are given in Table 5. The results show that the changes in $J_{TCA}$ and $F_{C2}$ are relatively small. Furthermore, the CV were lowest for the model fits with the parameters in the range we chose.

Table 5. Sensitivity analysis results

<table>
<thead>
<tr>
<th></th>
<th>$J_{\text{exchange}}$</th>
<th>$\tau_{\text{transport}}$</th>
<th>$J_{\text{anap}}/J_{TCA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+10% -10%</td>
<td>+10% -10%</td>
<td>+10% -10%</td>
</tr>
<tr>
<td>Change in $J_{TCA}$</td>
<td>+0.5% -0.8%</td>
<td>+2.2% -2.2%</td>
<td>+1.4% -1.4%</td>
</tr>
<tr>
<td>Change in $F_{C2}$</td>
<td>-1.8% +2.3%</td>
<td>-0.6% +0.7%</td>
<td>-0.5% +0.5%</td>
</tr>
</tbody>
</table>

Changes in $J_{TCA}$ and $F_{C2}$ when one of three parameters ($J_{\text{exchange}}$, $\tau_{\text{transport}}$, or $J_{\text{anap}}/J_{TCA}$) was increased or decreased by 10%.
**Discussion**

In this study, blood flow and VO$_2$ were measured simultaneously in small tissue samples of the in situ porcine left ventricle at a high resolution of a little under 1 ml. As a result, we found that 43% of the spatial variance of blood flow can be explained by a linear relation with VO$_2$.

**Methodological considerations.** In contrast to the study by Decking (12), which addresses the blood flow-regional VO$_2$ relation in dog heart, we measured blood flow and VO$_2$ in the pig heart, which has very few collateral vessels. Further differences are the following: we measured blood flow and VO$_2$ over the whole variation continuum of blood flow and did not restrict the study to specific low-, medium-, and high-flow areas. Sodium acetate was used in this study in a metabolic steady-state situation: acetate was infused for 30 min before the enriched acetate was infused to measure metabolic rate. The changes in metabolite levels when infusion of acetate is started, which probably occurred in previous studies (12), are thus avoided. The acetate was infused directly into the normal coronary circulation and the use of an extracorporeal circuit, as used elsewhere (12) was avoided. The latter is important, because vascular regulation is easily disturbed by an extracorporeal circuit. Besides TCA cycle flux and $F_{C2}$, three additional metabolic parameters could be estimated for part of the samples (see Table 3). The whole multiplet structure of glutamate with nine individual multiplets is analyzed, whereas a previous study (12) exclusively used the [4-13C/3-13C] ratio of glutamate for all spectra.

The resolution of the $^{13}$C method is 1–2 mm in the transmural direction (tissue sample weight in this study: minimum 43 mg dry wt, mean 159 mg), making it possible to divide the left ventricle of a porcine heart into three layers, i.e., subendocardium, midmyocardium, and subepicardium. This tissue sample size allows in many cases for quantification of the nine multiplets in the NMR spectrum acquired during such a short period of label infusion. Furthermore, for the microsphere blood flow method, a minimum of ~30 mg dry wt applies. Thus the resolution that can be reached with this method is higher than the resolution currently obtained with MRI or PET (resolution ~5 mm), although these methods are showing improvement in resolution. Thus an important advantage of our method is the separation of subendocardium and subepicardium, which may behave differently. For example, the subendocardium is more sensitive...
to ischemia during coronary stenosis. Blood flow to the subendocardial layers of the left ventricle tends to be somewhat higher than average in the present and previous studies (18). The present study shows for the first time that the same trend is also weakly present in VO₂, although no statistical significance was reached.

Oxygen consumption was calculated from two model parameters with the formula \((3 - F_{C2}) \times J_{TCA} = MVO₂\). \(F_{C2}\) is ~60% and does contribute only little to the observed variation of VO₂. VO₂ heterogeneity largely depends on the measured changes in the flux through the TCA cycle. Strictly spoken, this formula only holds true for glucose as the major substrate for the TCA cycle, applicable to many buffer-perfused heart Langendorff-type preparations. In the in vivo experiments described here, not only glucose is used by the heart, but also exogenous fatty acids are present besides the acetate, which is infused into the LAD. The number 3 in the equation should be somewhat lower in this situation, but not < 2.8, leading to an equation close to \((2.8 - F_{C2}) \times J_{TCA}\). For \(F_{C2} = 0.7\), the use of the factor 3 leads to <3% deviation. Because more precise data regarding this issue are not available, we will use the factor 3. The difference is not only slight but also systematic, and will therefore not influence the correlation between VO₂ and oxygen delivery.

In our study, the pigs were mechanically ventilated with 40% O₂-60% room air, resulting in a supranormal PO₂ because the animals in our study were anesthetized and artificially ventilated with open chest. Hyperoxia may induce vasoconstriction of coronary arteries (4, 32), whereas hypoxia may induce vasodilation (17). We did not see vasoconstricting effects, because, as stated in Table 1, global oxygen extraction was on average 70% in our experiments, which is normal for the anesthetized pig, but somewhat lower than found in awake pigs breathing air (16, 31). Regional oxygen extraction in the dog heart was in the same range (48). The lower value than in the awake animal may be explained by a slight coronary vasodilation, possibly induced by anesthetics and the other drugs given. Such vasodilation may lead to relatively high oxygen delivery in areas with relatively low oxygen demand. Benzodiazepines such as midazolam in high doses may cause an increase in coronary blood flow (40). µ-Opioid receptor antagonists may also interfere with coronary tone; however, in isolated coronary vessel rings, no effect of sufentanil, as used in this study,
on coronary vascular tone was demonstrated, in contrast to fentanyl (28). Lidocaine might have had a slight effect. A higher dose directly into a coronary artery may results in an effect on wall motion and an increase in blood flow (49). We cannot exclude that the lidocaine somewhat contributed to slight coronary vasodilation.

We cannot exclude a small effect of the catheter inserted in the coronary artery, although we did not find a change in blood gas parameters (see Table 1). However, blood flow values were similar as those found in earlier experiments without cannulation of a coronary artery (3, 5, 9), indicating no hyperemic response.

In this study, $^{13}$C-labeled sodium acetate was used as a substrate to the left ventricle. Acetate was first infused for a period of 30 min directly through the LAD artery into the left ventricle, allowing the heart to switch partly from metabolizing fatty acids and glucose to the infused acetate and adapt metabolite levels and fluxes to the new metabolic situation. Acetate concentrations were measured during this infusion, showing a stable concentration in the coronary sinus in all hearts after 10–20 min of infusion, indicating the attainment of a new steady state for metabolism. However, replacing $^{12}$C by $^{13}$C-labeled acetate takes a much shorter period, represented by the isotope transport time of $\sim 30$ s in Table 3. Although $^{13}$C-labeled substrate infusion experiments are sometimes started without metabolic steady state (12), it is advisable to measure in a metabolic steady state because metabolite levels from the TCA cycle change when new substrate is infused, which affects the isotope fluxes. This is possibly important, because, for example, citrate and glutamate levels have a significant relation with TCA cycle flux (12, 43).

Because it was previously reported that acetate might induce vasodilatation in the coronary circulation (7), we measured blood flow before and during acetate infusion. We did not find any evidence for vasodilatation of the coronary bed, as blood flow and blood pressure were stable over the infusion period. The vasodilating effect found by others might be explained by the higher concentrations they used (7) and the different animal species (although we did also not find any vasodilatation in dog hearts used in a different study; unpublished observation). An advantage of the use of acetate is the potential to
measure nine multiplets of glutamate in the NMR spectrum. This is important, because the estimation of six parameters simultaneously, possible in part of the samples, requires more than six multiplet areas. Others (12) have used pyruvate as substrate, which does not result in high fractional enrichment of acetyl-CoA, making it unfeasible to use multiplet areas for parameter estimation. Instead, in the case of pyruvate, the glutamate \([-4^{13}\text{C}/3^{13}\text{C}]\) ratio as an indicator for TCA cycle speed was used, and it was not possible to estimate the other four parameters in the same heart. It has further been reported that pyruvate has inotropic effects on the heart (25). Acetate does not affect contractile and hemodynamic parameters in our hands. With the use of acetate, the labeled \(J_{\text{anap}}\) resulting from pyruvate (12), which leads to more complex labeling patterns, is avoided.

As previously shown (42), our NMR method estimates only mitochondrial aerobic \(\text{VO}_2\) because only the TCA cycle is taken into account. The small nonmitochondrial oxygen consumption (~1.6 \(\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g dry wt}^{-1}\)) (10) is not measured by the NMR method and may explain part of the difference between NMR and blood gas measurements of \(\text{VO}_2\) (43). The double-labeling protocol was used because it can often yield a better estimation of TCA cycle flux and fractional enrichment of acetyl-CoA, the two parameters necessary to calculate \(\text{VO}_2\). Here, we found that the multiplet method works in a substantial fraction of the samples of small size. In the rest of the samples, we applied the ratio method, which yields similar results (see Fig. 3). In contrast to earlier studies (12, 43), in this study a 5-mm NMR tube was used, resulting in better quality of the spectra acquired in a shorter period of time. Automatic sample changing in the NMR machine makes the method much less labor intensive.

A disadvantage of the NMR method is its destructive nature, because part of left ventricle is harvested and frozen at the end of the protocol to stop metabolism as quickly as possible. Therefore, the \(\text{VO}_2\) measurement can only be performed at the end of the study. However, blood flow measurements remained stable throughout the study protocol and MVO\(_2\) measured from arterial and coronary venous blood did not change significantly throughout the study. Blood gas measurements were close to the \(\text{VO}_2\) values as measured with the NMR method (Fig. 5).
For human studies, MRI and PET scanning are feasible to investigate blood flow and VO$_2$. Current standard PET scanners have a typical spatial resolution of 6 mm at best, limiting the separation of myocardial layers in the left ventricular wall of 10 mm (21).

**Physiological meaning**

Blood flow heterogeneity was in agreement with previously published studies (CV 20–30%) (2, 23); however, heterogeneity of local VO$_2$ was somewhat more pronounced, underscoring that the correlation between oxygen delivery and VO$_2$, although highly significant, is not perfect. The heterogeneity due to measurement errors in VO$_2$ and blood flow is similar, both on the order of 6%. A relatively small part of the scatter around the regression line is therefore caused by measurement errors. The variance of measured blood flow can be partitioned in measurement errors, the linear regression of blood flow on VO$_2$ and other unknown genuine biological factors not due to measurement error. The error analysis shows that almost one-half of the variation in blood flow to the left ventricle can be explained by variation in VO$_2$ in the same tissue samples, compatible with the idea that an important part of blood flow heterogeneity is due to variations in factors related to heterogeneous aerobic metabolism. However, more than one-half of the variance not due to measurement error is apparently due to biological factors beyond aerobic metabolism. Candidates for such factors in the vasoregulatory pathways are variations in hormonal receptor density and innervation, or in intracellular signal transduction, among others. In any case, the existence of other biological factors besides VO$_2$ means that O$_2$ extraction is not perfectly regulated and shows local variation. The relation between succinate dehydrogenase activity, a marker enzyme of the mitochondria, and blood flow explained merely 22% of blood flow variance (9). Thus it appears that blood flow is much more strongly related to the actual aerobic metabolic flux than to local aerobic capacity.

Spatial heterogeneity of blood flow and VO$_2$ is now well established. It should be clear from this study that the heterogeneity of blood flow does not reflect random variation, but relates to local oxygen needs. With the use of a model of ventricular fiber orientation, Vendelin et al. (46) predicted a nonuniform distribution of ATP consumption in the left ventricle, with a tendency to higher ATP consumption between the endocardium and midmyocardium.
However, their results show a different pattern and much less variance in energy turnover than our experimental study, in which VO$_2$ was directly measured. The physiological meaning of this heterogeneity of energy turnover is unclear. In the present and previous studies, there is no obvious relation between anatomical location and blood flow and metabolism levels. Therefore, differences in mechanical loading apparently do not explain the heterogeneity. Heterogeneity is not explained by ultrastructural variability in the heart (13) or obvious macroscopic patterns of contraction (36). A very speculative explanation is that homogenous blood flow distribution by a developing blood vessel network is not possible, and that local energy turnover is in the long turn adapted to the possibilities for oxygen delivery by the blood flow. Another explanation is that the muscle fiber direction shows variability and is not perfectly aligned with a hypothetical ideal direction that gives homogenous energy turnover. Some evidence accumulates that differences in cardiac strain are somewhat heterogeneous (30, 35), but this has not been related to local blood flow and VO$_2$ yet.

The fraction of variation in blood flow explained by VO$_2$ is high, despite the state of anesthesia. However, we cannot exclude an underestimation of the coupling that exists in the normal situation. Extrapolation to the awake state as opposed to the surgical preparation is not without difficulties, and the changes in coronary tone might result in a less tight coupling of oxygen delivery and oxygen demand.

In conclusion, the significant correlation between local myocardial O$_2$ delivery and local VO$_2$ in the normal porcine left ventricle, assessed with the use of independent methods, suggests that heterogeneous O$_2$ delivery can be explained for almost half by heterogeneity of oxygen demand.
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