Recruitment of NADH shunting in pressure-overloaded and hypertrophic rat hearts

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Lewandowski ED, O’Donnell JM, Scholz TD, Sorokina N, Buttrick PM. Recruitment of NADH shunting in pressure-overloaded and hypertrophic rat hearts. Am J Physiol Cell Physiol 292: C1880–C1886, 2007. First published January 17, 2007; doi:10.1152/ajpcell.00576.2006.—Glucose metabolism in the heart requires oxidation of cytosolic NADH from glycolysis. This study examines shunting reducing equivalents from the cytosol to the mitochondria via the activity and expression of the oxoglutarate-malate carrier (OMC) in rat hearts subjected to 2 wk (Hyp2, n = 6) and 10 wk (Hyp10, n = 8) of pressure overload hypertrophy vs. that of sham-operated rats (Sham2, n = 6; and Sham10, n = 7). Moderate aortic banding produced increased atrial natriuretic factor (ANF) mRNA expression at 2 and 10 wk, but only at 10 wk did hearts develop compensatory hypertrophy (33% increase, P < 0.05). Isolated hearts were perfused with the short-chain fatty acid [2,4-13C2]butyrate (2 mM) and glucose (5 mM) to enable dynamic-mode 13C NMR of intermediate exchange across OMC. OMC flux increased before the development of hypertrophy: Hyp2 = 9.6 ± 2.1 vs. Sham2 = 3.7 ± 1.2 μM·min⁻¹·g dry wt⁻¹, providing an increased contribution of cytosolic NADH to energy synthesis in the hypertrophic heart. With compensatory hypertrophy, OMC flux returned to normal: Hyp10 = 3.9 ± 1.7 μM·min⁻¹·g dry wt⁻¹ vs. Sham10 = 3.8 ± 1.2 μM·g⁻¹·min⁻¹. Despite changes in activity, no differences in OMC expression occurred between Hyp and Sham groups. Elevated OMC flux represented augmented cytosolic NADH shunting, coupled to increased nonoxidative glycolysis, in response to hypertrophic stimulus. However, development of compensatory hypertrophy moderated the pressure-induced elevation in OMC flux, which returned to control levels. The findings indicate that the challenge of pressure overload increases cytosolic redox state and its contribution to mitochondrial oxidation but that hypertrophy, before decompensation, alleviates this stress response.

malate-aspartate shuttle; redox state; hypertrophy

CHRONIC PRESSURE OVERLOAD, such as that produced by hypertension, results in the development of cardiac hypertrophy, a pathophysiologic response that has been linked to increased glucose metabolism (9, 17, 22, 24). Increased reliance on glucose metabolism for energy synthesis in the hypertrophied myocardium requires an elevated oxidation of cytosolic NADH that is produced from glycolysis (9, 17, 22, 24). Under normal oxidative conditions, the reducing equivalents that are transferred to NAD⁺ to form NADH in the cytosol can be transferred to the mitochondrial matrix in support of oxidative phosphorylation (2, 23). This transfer of reducing equivalents from cytosolic NADH to the oxidative pathways of the mitochondria is achieved primarily via net forward flux through the malate-aspartate shuttle. Therefore, the present study examines the potential for augmented shunting of reducing equivalents in hypertrophic hearts.

We have recently demonstrated that the neonatal heart, which is more glycolytically active than adult heart, adjusts to a relatively high level of glycolytic NADH production through elevated transfer rate of these cytosolic-reducing equivalents into mitochondria via malate-aspartate shuttle flux (7, 15). In that previous study, we observed that the increased malate-aspartate shuttle activity in the neonatal heart is supported by a relatively high level of expression of the shuttle protein, oxoglutarate-malate carrier (OMC) protein. As the developing heart reduces reliance on glucose metabolism during the shift to the adult metabolic profile of increased fatty acid oxidation, the myocardium reduces expression of both the malate-aspartate shuttle and glycerophosphate shuttle proteins, with essential elimination of the glycerophosphate shuttle (7, 26). Consequently, the malate-aspartate shuttle proteins eventually provide the predominant form of reducing equivalent transfer in adult myocardium, albeit at a reduced capacity from the neonatal stage. Indeed, in the adult heart, both flux through OMC and expression of the shuttle protein are reduced to approximately one-third of those seen in the neonatal heart (7). With the potential for the hypertrophic stimulus to initiate a reversion to neonatal metabolism, this study addresses the hypothesis that the hypertrophic heart, in shifting toward increased glucose metabolism, must adapt to the NADH/NAD⁺ balance of the cytosol through adjustments in OMC activity and/or expression.

To address this hypothesis, we employed dynamic-mode 13C NMR to measure flux across the primary transporter protein involved in the transfer of cytosolic NADH into the mitochondrial matrix, OMC, in pressure-loaded rat hearts (7, 18, 30). Our objectives were to 1) determine adaptive changes in flux across OMC and OMC protein expression in response to the hypertrophic stimulus of pressure overload, and 2) examine OMC activity and expression in hearts at the point of development of compensatory hypertrophy. This study is the first to investigate altered mitochondrial transport processes in intact hypertrophic hearts and, importantly, demonstrate the distinctions between metabolic regulation and gene expression in the adaptive responses to a disease process.
MATERIALS AND METHODS

Pressure-overload cardiac hypertrophy. Pressure-overload hypertrophy was produced in hearts of male Sprague-Dawley rats by constriction of the transverse aorta as previously described (4). Hearts were harvested from age-matched, aortic-banded, and sham-operated rats for perfusion experiments at two time points: 1) at 2 wk post-banding (6 wk of age) before development of hypertrophy, but when elevated atrial natriuretic factor (ANF) mRNA expression confirmed the presence of a hypertrophic stimulus (5-fold increase over shams; \( P < 0.05 \)), and 2) at 10 wk post-banding (14 wk of age), when established compensatory hypertrophy was evident. The protocol was approved by the Animal Care Policies and Procedures Committee at University of Illinois at Chicago (IACUC accredited), and the animals used were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revised 1996).

Isolated perfused rat heart. Animals were heparinized (500 U/100 g ip) and anesthetized with pentobarbital sodium (100 mg/kg ip). Hearts were excised and perfused in retrograde fashion at 100-cm hydrostatic pressure, as previously described, with modified Krebs-Henseleit buffer composed of (in mM) 116 NaCl, 4 KCl, 1.5 CaCl2, 1.2 MgSO4, and 1.2 NaH2PO4, equilibrated with 95% O2-5% CO2 (7, 18, 30). A water-filled latex balloon was fitted into the left ventricle for hemodynamic recordings (PowerLab; ADInstruments, Colorado Springs, CO). The balloon was inflated with water to create a diastolic pressure of 5–10 mmHg. Left ventricular developed pressure and heart rate were continuously recorded. Rate-pressure product was calculated as the product of heart rate and developed pressure. The temperature of the hearts was continuously maintained at 37°C.

Experimental protocols. At the start of each protocol, the perfusate supply was switched to a 0.5-liter reservoir of buffer containing 2.0 mM unlabeled butyrate and 5 mM glucose for 10 min to ensure metabolic equilibrium and for collection of background signals of naturally abundant \(^{13}C\) (1.1%). During this period, a \(^{31}P\) spectrum was also acquired from each heart. The perfusate was then switched to a 1-liter supply of \(^{13}C\)-enriched buffer containing 2.0 mM \([2,4-{13}C_2]\)butyrate (Isotec, Miamisburg, OH) plus 5 mM unlabeled glucose. Sequential \(^{13}C\) NMR spectra began immediately upon delivery of \(^{13}C\)-enriched buffer over 35 min (7, 18, 30). The heart was rapidly frozen in liquid nitrogen-cooled tongs for in vitro analysis.

Butyrate is a short-chain fatty acid that undergoes \(\beta\)-oxidation in cardiac mitochondria. Although not a physiological substrate, butyrate supports normal cardiac energetic functions and enables NMR measurements of metabolic flux in the intact beating heart (11, 13, 19, 29, 30). Prior studies on OMC activity in the neonatal heart have attributed pyruvate for oxidation via pyruvate dehydrogenase (PDH). Unlabeled acetyl CoA is also produced from unlabeled glucose which contributes pyruvate for oxidation via pyruvate dehydrogenase (PDH).
Statistical comparisons. Results are reported as means ± SD unless otherwise indicated. Comparison of mean values was performed using ANOVA, followed by a Newman-Keuls post hoc test. Functional measurements over time were compared using repeated-measures ANOVA. Differences in mean values were considered significant at a probability level of <.05.

RESULTS

Pathophysiological and bioenergetic states of pressure-overloaded rat hearts. Significant increases in the mean expression of ANF mRNA relative to GAPDH (dimensionless units) were present in hearts after aortic banding compared with sham-operated rats (2-wk sham = 1,191 ± 259 vs. 2-wk banded = 5,008 ± 758; 10-wk sham = 1,762 ± 986 vs. 10-wk banded = 3,446 ± 752). However, compensatory hypertrophy was only evident after 10 wk of pressure overload. Heart weight was similar between 2-wk pressure-overloaded hearts (1.7 ± 0.2 g) and corresponding shams (1.5 ± 0.2 g). The ratio of heart weight to body weight was also similar between 2-wk pressure-overloaded hearts (0.0065 ± 0.0002) and corresponding shams (0.0069 ± 0.001). Heart weight was increased in 10-wk pressure-overloaded hearts (2.8 ± 0.3 g) over that of corresponding shams (2.1 ± 0.1 g; P < .05), and heart weight-to-body weight ratios were significantly increased in the banded animals at this time point (0.0071 ± 0.0004 vs. 0.0051 ± 0.0005; P < .05). Thus we were able to study hearts at two key time points: 1) early after the imposition of the hypertrophic stimulus before the development of cardiac hypertrophy, and 2) once compensatory cardiac hypertrophy had been established.

The rate-pressure product was similar in all four experimental groups and did not vary significantly throughout the perfusion protocol. Rate-pressure product values (beats·min⁻¹·mmHg) at the midpoint of the protocol were as follows: 2-wk sham = 29,778 ± 7,804; 2-wk banded = 28,843 ± 6,238; 10-wk sham = 26,173 ± 7,798; and 10-wk banded = 22,323 ± 3,829 (means ± SD). The pressure-overloaded hearts at both 2 and 10 wk showed no significant functional deficiency compared with the corresponding sham-operated hearts. As hearts in the 10-wk group had no evidence of hemodynamic decompensation or failure, the functional similarities between the pressure-overloaded hearts and the sham-operated hearts are to be expected.

Correspondingly, oxygen consumption was similar in all groups: 2-wk sham = 22.0 ± 4.3 (μmol·g⁻¹·min⁻¹; 2-wk banded = 24.1 ± 5.2; 10-wk sham = 23.8 ± 4.8; and 10-wk banded = 19.3 ± 3.4. Consistent with previous reports, banded hearts at 10 wk of pressure overload displayed reduced energy potential, as indexed by the ratio of phosphocreatine to ATP (4, 12): 2-wk sham = 1.91 ± 0.25 vs. 2-wk banded = 1.59 ± 0.39 (not significant; NS); and 10-wk sham = 1.98 ± 0.29 vs. 10-wk banded = 1.62 ± 0.27 (P < .05).

Metabolic flux measurements and OMC activity. Representative ¹³C NMR spectra of the intact beating hearts, oxidizing [2,4-¹³C₂]butyrate, are shown in Fig. 2 with ¹³C enrichment curves of the glutamate carbon positions, as described in MATERIALS AND METHODS and as shown in Fig. 3. Isotopic steady state within the glutamate pool was reached within the first 20 min of the enrichment protocol as previously observed (7, 19, 29). Measured input parameters for the analysis of glutamate enrichment kinetics are shown in Table 1. Glutamate content in the myocardium of hearts at 2 wk of pressure overload was increased, as expected due to an increase in OMC activity (18).

When the differences in metabolite pool size are accounted for with respect to the differences in the relative enrichment rates of the 2- and 4-carbon of glutamate, as is performed in the fitting of the data to the kinetic model (see Fig. 3), the output from the kinetic model yielded the distinction in flux values shown in Fig. 4. Measurements of TCA cycle flux in each group showed similar flux rates among all four experimental groups, as would be predicted by the similar functional performance of each group of hearts. Mean values of TCA cycle flux (μmol·min⁻¹·g dry wt⁻¹) in each group were as follows: 2-wk sham = 9.8 ± 4.0; 2-wk banded = 8.3 ± 0.9 (NS); 10-wk sham = 10.6 ± 4.1; and 10-wk banded = 10.2 ± 1.9 (NS).

OMC activity (F₁) in the intact, beating heart was essentially doubled in response to the hypertrophic stimulus at 2 wk post-banding (Fig. 4). Interestingly, OMC flux returned to near baseline levels at the time of significant compensatory hypertrophy. As anticipated, flux through OMC remained unchanged over the 2- to 10-wk period in the sham-operated, control hearts. These findings suggest that the OMC flux response is adaptive to the stimulus of pressure overload and normalizes upon compensation of the left ventricle.

As expected from the inhibitory effects of short-chain fatty acid on carbohydrate oxidation, very little glucose was oxidized in the presence of butyrate as an oxidizable substrate (5, 8, 27, 30). Interestingly, though, nonoxidative glycolysis as indicated by an increase in glycolytic end production formation
(lactate and alanine), particularly that of lactate, was higher in the 2-wk banded group than in the corresponding 2-wk sham group (Table 1). Indeed, the 2-wk banded group was the only set displaying elevated lactate content ($P < 0.01$). This finding is consistent with increased drive of the malate-aspartate shuttle via elevated cytosolic redox state in the 2-wk banded group, as linked to the observed increased flux through OMC (23, 30).

While a general increase in glycolysis was evident in the pressure-overloaded hearts, no distinctions were evident in the fate of the exogenous, enriched glucose vs. the endogenous, unlabeled glucose.

**OMC expression in pressure-overloaded hearts.** Despite increased metabolite exchange via OMC in hearts at 2 wk of pressure overload relative to sham hearts, OMC protein expression remained unchanged (Fig. 5). Hearts subjected to 10 wk of pressure overload showed mean OMC protein to be slightly elevated (30%) over sham hearts, although not significantly. These results are similar to the findings reported by Rupert et al. (22). However, previous studies were not able to evaluate flux through this transporter protein in the hypertrophied heart.

The present results indicate that OMC activity is significantly elevated due to metabolic regulation in the hearts at 2 wk of pressure overload, before the development of compensatory hypertrophy. As compensatory hypertrophy develops, OMC flux returns toward baseline levels, despite no increase in the amount of OMC protein.

**DISCUSSION**

This study is the first to evaluate the activity of a key component of the malate-aspartate shuttle in the heart in response to the hypertrophic stimulus. The work also represents the first dynamic $^{13}$C NMR study of cardiac hypertrophy with actual metabolic flux measurements from sequential $^{13}$C spectra of the beating heart. The major finding is that the early response to pressure overload includes increased flux through OMC which then moderates over time, coordinate with the development of compensatory hypertrophy. We previously demonstrated in a developmental model of cardiac metabolism, comparing neonatal hearts to adult hearts with similar methods, that the activity of OMC can be directly related to the level of

![Fig. 3. Means ± SE $^{13}$C enrichment curves for the 4- and 2-carbon positions of glutamate, as detected by $^{13}$C NMR. Solid line represents least-squares fitting of the kinetic model to the enrichment data. • 4-Carbon of glutamate; ○, 2-carbon of glutamate. Top, sham-operated hearts at 2 wk (Sham). Bottom, pressure-overloaded hearts at 2 wk (Hyp). Note the relative differences between $^{13}$C enrichment at the 2-carbon position of glutamate vs. that of the 4-carbon between the sham-operated hearts (top) and the pressure-overloaded hearts (bottom).](image1)

![Fig. 4. Flux through the oxoglutarate-malate carrier (OMC) in each experimental group (in μmol·min$^{-1}$·g dry wt$^{-1}$). Groups values from left to right are identified as follows: 2SH, sham-operated hearts at 2 wk; 10HYP, pressure-overloaded hearts at 2 wk; 10SH, sham-operated hearts at 10 wk; and 10HYP, pressure-overloaded hearts at 10 wk. Note the significant increase in OMC activity at 2 wk of pressure overload. $^* P < 0.0005$.](image2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutamate</th>
<th>Citrate</th>
<th>Oxoglutarate</th>
<th>Aspartate</th>
<th>%$^{13}$C acetyl-CoA</th>
<th>Lactate</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-wk Sham</td>
<td>19±4</td>
<td>2.3±0.1</td>
<td>0.21±0.08</td>
<td>5.6±1.3</td>
<td>85±1</td>
<td>2.3±1.9</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>2-wk Banded</td>
<td>24±4*</td>
<td>2.6±0.5</td>
<td>0.22±0.08</td>
<td>6.1±1.2</td>
<td>87±2</td>
<td>4.7±0.9</td>
<td>1.8±0.5*</td>
</tr>
<tr>
<td>10-wk Sham</td>
<td>16±5</td>
<td>3.2±1.2</td>
<td>0.17±0.03</td>
<td>4.6±1.3</td>
<td>89±3</td>
<td>2.5±1.5</td>
<td>1.1±0.7</td>
</tr>
<tr>
<td>10-wk Banded</td>
<td>16±4</td>
<td>3.4±0.4</td>
<td>0.19±0.04</td>
<td>5.8±1.8</td>
<td>87±4</td>
<td>2.3±1.4</td>
<td>0.9±0.4</td>
</tr>
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Values are means ± SD of tissue metabolites (in μmol/g dry wt). $^* P < 0.05$, difference vs. the corresponding sham value.
OMC expression (7). However, in contrast to the previous study, the pressure-overloaded adult heart demonstrates similar regulatory responses of cardiac metabolism, but in the absence of altered enzyme expression. Importantly, this study demonstrates further that the metabolic response to pressure overload is an intrinsic adaptation in the myocardium that persists in the isolated heart.

The 31P NMR data showing depressed energetic profile in hearts of these aortic banded animals is consistent with previous reports of cardiac energetics in hypertrophy (4, 17). The phosphocreatine-to-ATP ratio was equally perturbed at both 2 and 10 wk of pressure overload and, as a general characteristic of the hypertrophied heart, did not correlate to OMC flux.

In cardiac hypertrophy, a phenotypic reprogramming occurs in the heart, such that genes that are normally expressed during development are recapitulated, including gene-encoding metabolic enzymes (21). Metabolic studies on hypertrophied hearts have clearly documented an increase in glycolytic energy production via TCA cycle flux and NADH transfer from the cytosol, were well matched in both sham and banded heart groups at 2 wk to support similar levels of contractile function. The general production of 3 NADH per single cycle of the TCA pathway indicates that the mean TCA cycle flux for sham hearts, of 9.8 μmol·min⁻¹·g⁻¹, produced 29.4 NADH μmol·min⁻¹·g⁻¹, whereas the mean TCA cycle flux of 8.3 μmol·min⁻¹·g⁻¹ in the banded group produced 24.9 NADH·min⁻¹·g⁻¹. However, the different contributions of NADH influx into the mitochondria via OMC activity brings the mean values of NADH generation in the mitochondria to very similar levels of 33.1 NADH·min⁻¹·g⁻¹ in the sham group and 34.5 NADH·min⁻¹·g⁻¹ in the 2-wk banded group. Thus the similarity in total NADH contributing to oxidative energy synthesis in the mitochondria accounts for the observed similarities in oxygen consumption observed in both the sham and pressure-overloaded hearts. The importance of this finding is that in the 2-wk pressure-overloaded heart, glycolytic production of NADH contributed significantly to maintaining normal rates of energy synthesis and this contribution diminished with the eventual development of compensatory hypertrophy.

Indeed, although statistical comparison does not indicate significance, the trend for OMC protein content in compensated hypertrophic myocardium was an increase by one-third the amount of protein in the sham hearts. In this case, the relative flux through OMC per quantity of OMC protein in the heart remains elevated even in the 10-wk banded animals. Therefore, the large increase in OMC flux before the development of compensatory hypertrophy represents an adjustment in metabolic regulation in response to the hypertrophic stimulus.

The elevated flux through OMC then becomes an important adaptive mechanism that serves to maintain cytosolic redox state (NADH/NAD⁺) in the presence of a previously reported mismatch between the elevated glycolytic activity of the hypertrophied and the relatively unchanged oxidation of glycolytic end products (9, 24). Importantly, these observations of increased OMC flux and glycolytic metabolism occurred in hearts subjected to the stress of pressure overload, before the development of hypertrophy. Although the present study focuses not on glucose metabolism, but rather on the activity of the key transporter for cytosolic reducing equivalents OMC, our data on the 13C enrichment of acetyl CoA (Table 1) demonstrate no differences between sham hearts and pressure-overloaded hearts in the relative contributions to oxidative metabolism between butyrate and glucose. Therefore, no in-

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Fig. 5. OMC protein levels in 2- and 10-wk sham-operated (Sham) and pressure-overloaded (Hyp) hearts. Top, results of Western blot analysis. Bar graphs (middle) indicate mean signal intensity (±SD). No significant difference in OMC levels was detected between Sham and Hyp groups at the two time points. Bottom, results of Ponceau staining designed to ensure uniform protein loading.
crease in the oxidation of glycolytic end products was observed in this study of pressure-overloaded hearts, supporting the previously reported findings (9, 24).

Although the aim of this study was the measurement of flux through OMC that precluded measurements of glycolytic flux, these new data build on the previously published reports of changes in the intermediary metabolism of the hypertrophied rat heart (9, 17, 22, 24). The findings of this study indicate that shortly after imposition of the hypertrophic stimulus of pressure overload, flux through OMC is dramatically increased in the heart, as reducing equivalent transfer from the glycolytically produced, cytosolic NADH is accommodated by the malate-aspartate shuttle for oxidative metabolism in the mitochondria. Evidence is shown here for a transient spike in the activity of nonoxidative glycolysis that is likely a culprit for driving OMC. As the early elevation of glycolysis has been observed in the isolated heart, it represents and intrinsic change in myocardial metabolism.

Interestingly, once compensation occurs after 10 wk of pressure overload, the flux through OMC returns toward baseline levels, similar to those observed in the sham hearts. Together, data from both the 2- and 10-wk time points of pressure overload indicate that development of compensatory hypertrophy at least partially restores the redox state balance (NADH/NAD\(^+\)) between the cytosol and mitochondria, which is initially disrupted during the initial metabolic response to pressure overload.

Our further analysis of OMC content in pressure-overloaded hearts indicates that the changes in OMC flux that were observed in this study are the direct result of metabolic regulation and not a change in the expression of the enzyme. While we have observed a clear increase in the activity of OMC in the pressure-overloaded heart (at 2 wk), protein levels did not revert to the fetal expression pattern (7). An earlier study, by Rupert et al. (22), also demonstrated that protein levels of OMC do not increase in the rat heart following pressure overload produced by banding of the abdominal aorta.

Nonetheless, the lack of change in OMC content in response to the hypertrophic stimulus belies the altered activity of this carrier protein and the changes in OMC activity that occur in parallel with the development of compensatory cardiac hypertrophy. The results show, in general, that the metabolic responses to pressure overload in the heart are not static. Rather, the initial spike in OMC activity and eventual moderation toward baseline activity in hypertrophied hearts suggests that meeting the metabolic demands of cardiac function is a dynamic process over the course of the pathophysiology that ultimate leads toward decompensated hypertrophy and heart failure. Indeed, this process clearly adapts to the current pathological state and may induce subsequent adaptive responses that do not involve distinct changes in enzyme expression. The larger implication of the work is that in vitro assays that rely primarily on enzyme content or enzyme kinetics, in the absence of the metabolic driving forces provided by the function of the intact ventricle, do not reflect the regulatory control of metabolic processes in the cardiomyocyte.

REFERENCES


