

Metabolic control analysis of anaerobic glycolysis in human hibernating myocardium replaces traditional concepts of flux control

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Abstract Myocardial hibernation represents an adaptation to sustained ischemia to maintain tissue vitality during severe supply–demand imbalance which is characterized by an increased glucose uptake. To elucidate this adaptive protective mechanism, the regulation of anaerobic glycolysis was investigated using human biopsies. In hibernating myocardium showing an increase in anaerobic glycolytic flux metabolizing exogenous glucose, the adjustment of flux through this pathway was analyzed by flux:metabolite co-responses. By this means, a previously unknown pattern of regulation using multisite modulation was found which largely differs from traditional concepts of metabolic control of the Embden–Meyerhof pathway in normal and diseased myocardium. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Glycolysis; Energy metabolism; Metabolic regulation; Ischemia; Hibernating myocardium

1. Introduction

The term hibernating myocardium was coined to describe a state of persistently impaired left ventricular function due to a reduced coronary blood flow which will be partially or completely restored following revascularization [1]. Clinically, human hibernating myocardium (HHM) shows an increased glucose uptake [2]. This characteristic feature, which is exploited in diagnostic means [3], is accompanied by an amplified sarcolemmal abundance of glucose transporters [4].

In myocardial hypoperfusion, the beneficial effects of an augmented ATP generation by anaerobic glycolysis have

been repeatedly shown in clinical as well as in experimental studies [5,6]. In this respect, the increased glucose uptake of HHM takes part in the metabolic adaptation to and defense against severe supply–demand imbalance [7] enabling the survival of this energy-depleted myocardium [8]. As a consequence, the analysis of metabolic flux and its regulation in HHM is not only of theoretical interest. Therefore, it was the aim of our study to investigate anaerobic glycolysis and its regulation in HHM in order to obtain further insights into the mechanism underlying this unique myocardial adaptation to ischemia, which may eventually facilitate therapeutic exploitation.

To analyze the regulation of metabolic pathways, traditional concepts mostly imply that control over a pathway is achieved by action on a single pathway enzyme, which is often assumed to be a non-equilibrium step near the pathway's beginning subjected to feedback inhibition, e.g. [5,9–12]. However, starting with the rise of metabolic control analysis (MCA) [13], there is a growing body of evidence that these comfortable, time-honoured concepts may mislead more than enlighten. Hence, the tools of MCA were firstly used in a clinical–experimental investigation in order to obtain a state-of-the-art analysis of anaerobic glycolytic regulation in HHM.

2. Materials and methods

2.1. Patients and study protocol

Sixteen patients with angiographically documented coronary heart disease and a reduced left ventricular function resulting in the indication for coronary bypass surgery were studied. Informed written consent from each patient for every investigation and approval of the institutional hospital review board of the University of Freiburg had been obtained.

To detect HHM preoperatively, established clinical methods, such as low-dose dobutamine echocardiography, thallium-201 scintigraphy using a stress-redistribution–reinjection protocol, radionuclide ventriculography and coronary angiography including left ventriculography, were used as described in detail previously [14]. All preoperative clinical investigations were repeated 3 months after revascularization in order to document the extent of functional recovery of these regions after restoration of adequate perfusion, thereby validating the preoperative diagnosis ‘hibernating myocardium’.

2.2. Tissue analysis

2.2.1. Tissue sampling. Transmural Tru-cut[®] needle biopsies were removed during open-heart surgery from the beating heart before establishment of extracorporeal circulation and cardioplegic arrest. These biopsies were immediately shock frozen in liquid nitrogen for

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Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F_{1,6}P₂, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 2PG, 2-phosphoglyceric acid; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lac, lactate; GP-iso, glucosephosphate isomerase; PFK-1, phosphofructokinase 1; GAPDH, glyceraldehyde dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; Eno, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase

high performance liquid chromatography (HPLC) and immunohistochemistry or immersed in 3% glutaraldehyde buffered with 0.1 M Na cacodylate (at pH 7.4, 440 mosmol/l) for microscopy. Shock-frozen samples were kept in liquid nitrogen until further use. Following clinical validation of the myocardial regions as hibernating myocardium (3 months postoperatively), the specific examinations of the biopsies were started.

2.2.2. Fibrosis. The fibrotic content of HHM was determined by electron microscopy and immunohistochemical labelling of fibronectin. Three different tissue sections which were obtained from varying biopsy levels were evaluated. Fibrosis was quantified employing the point-counting method following stereological principles [15,16]. The fibrotic area of the tissue sample was expressed as percent of the total myocardial area.

2.2.3. Analysis of myocardial metabolites. After assessing the degree of fibrosis within the biopsies, the remaining shock-frozen tissue was homogenized [17] and subjected to metabolite measurements using HPLC [17]. Hence, all values given represent integral metabolite contents ([], given in $\mu\text{mol/g}$ wet weight) for a myocardial biopsy. Considering the error arising from varying degrees of fibrosis in control and hibernating myocardium, total myocardial metabolite contents in both groups were additionally normalized to the cellular myocardial fraction (CMF), providing values for myocellular content:

$$\text{content}_{\text{CMF}} = \frac{100}{(100 - \text{fibrosis}[\%])} \cdot \text{content}_{\text{total}}.$$

Dihydroxyacetone phosphate (DHAP) and 1,3-bisphosphoglycerate (1,3-BPG) could not be detected. The DHAP content was, therefore, calculated from the glyceraldehyde 3-phosphate (GA3P) content assuming equilibrium state of the triosephosphate isomerase reaction [18]. 2- and 3-phosphoglycerate (2PG and 3PG, respectively) were not always clearly separated in the chromatograms, though their common peak could be easily seen. The individual proportions were calculated according to the equilibrium of the phosphoglycerate mutase (PGM) reaction

$$0.154 = \frac{[2\text{PG}]}{[3\text{PG}]}$$

The equilibria of isomerase and phosphoglyceromutase are neither affected by mild nor severe myocardial undersupply [9]. As 1,3-BPG could not be detected, glyceraldehyde dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) were considered as a single glycolytic step. Since 2PG and 3PG contents were calculated, the given values are not mathematically independent. Therefore, also PGM and enolase (Eno) were put together.

2.2.4. Control tissue. The results obtained in HHM were compared to left ventricular biopsies from patients undergoing operative correction of atrial septal defects ($n=3$) and from donor hearts not used for transplantation ($n=4$), representing normal human myocardium.

2.2.5. Estimation of anaerobic glycolytic flux. Estimation of anaerobic glycolytic flux was performed according to the established relationship between tissue lactate accumulation and anaerobic glycolytic flux [19], corrected for the decrease in myocardial blood flow (MBF) [20], which was evidenced by an impaired preoperative thallium-201 uptake into hibernating myocardium. Unfortunately, absolute measures of anaerobic glycolytic flux were not possible or authorized in the clinical study performed. Nevertheless, the informations obtained did clearly define two distinct myocardial states with unaltered (normal myocardium) and increased anaerobic glycolytic flux (e.g. hibernating myocardium) in order to compute flux:metabolite co-responses.

Thus, anaerobic glycolytic flux was calculated from lactate content corrected for myocardial blood flow and is expressed in arbitrary units (AU):

$$\text{Flux(AU)} = \frac{[\text{lactate}] \cdot \text{MBF(in}\%) }{100}$$

2.2.6. Model construction. As myocardial glycogen contents were unchanged in HHM (indicating no net flux from glucose 6-phosphate (G6P) to glycogen), glycolysis was considered as linear pathway from glucose transport (i.e. cellular uptake) via its phosphorylation to G6P to lactate. HHM is characterized by a normal capacity and rate of oxidative metabolism at impaired myocardial blood flow (flow-metabolism mismatch) [21,22]. Hence, aerobic glycolytic flux was assumed to be unchanged between HHM and control myocardium and has thus not been taken into account for model construction.

The regulation of anaerobic glycolytic flux along the enzymatic reactions breaking G6P down to lactate was analyzed employing the MCA concept of finite flux:metabolite co-response coefficients (O) as described previously [23].

$$O_{\text{flux,metabolite}} =$$

$$\frac{\partial \ln \text{flux}}{\partial \ln \text{metabolite}} \approx \frac{\Delta \ln \text{flux}}{\Delta \ln \text{metabolite}} \approx \frac{\Delta \text{flux}/\text{flux}}{\Delta \text{metabolite}/\text{metabolite}}$$

To the best of our knowledge, the current study represents the first application of these established post-hoc analyses to experimentally investigate adjustment of the Embden–Meyerhof pathway.

Table 1
Metabolite contents and parameters in normal and hibernating human myocardium

Metabolite content (nmol/mg)	Total myocardium					CMF				
	Normal		Hibernation			Normal		Hibernation		
	mean	S.E.M.	mean	S.E.M.	P-value	mean	S.E.M.	mean	S.E.M.	P-value
G6P	0.246	0.046	0.254	0.061	0.754	0.273	0.050	0.350	0.081	0.988
F6P	0.021	0.005	0.080	0.016	0.045	0.024	0.005	0.118	0.026	0.046
F _{1,6} P ₂	0.013	0.003	0.167	0.043	0.379	0.014	0.004	0.230	0.058	0.029
GA3P	0.010	0.002	0.094	0.013	0.002	0.011	0.002	0.132	0.019	0.002
DHAP	0.229	0.038	2.261	0.322	0.002	0.255	0.043	3.164	0.460	0.002
3-PG	0.040	0.019	0.126	0.025	0.055	0.044	0.021	0.171	0.032	0.023
2-PG	0.006	0.003	0.019	0.004	0.055	0.007	0.003	0.026	0.005	0.023
PEP	0.008	0.001	0.030	0.006	0.022	0.008	0.002	0.042	0.008	0.010
Pyr	0.044	0.016	0.220	0.026	0.001	0.049	0.018	0.310	0.038	0.001
Lac	2.000	0.240	17.389	2.313	0.002	2.217	0.261	25.377	3.526	0.002
F _{2,6} P ₂	0.069	0.010	0.014	0.004	< 0.0001	0.077	0.011	0.018	0.005	< 0.0001
Glycogen (C6-units $\mu\text{M/g}$)	2.999	0.369	3.769	0.527	0.359	3.328	0.408	5.503	0.835	0.137
Parameter										
Anaerobic glycolytic flux (AU)	2.217	0.261	8.574	1.428	0.006					
TI-uptake (%)	100.000	0.000	41.250	1.963	< 0.0001					
Global EF (%)	26.4	2.3	46.7	3.2	< 0.0001					
Regional EF (%)	24.5	2.0	55.6	3.2	< 0.0001					
Fibrosis (%)	9.857	0.508	28.062	3.012	0.010					

Myocardial metabolite contents, given as nmol/mg wet weight, normalized to total myocardium (left) and to the CMF (right). In addition, data for anaerobic glycolytic flux, thallium (TI) uptake, global and regional ejection fraction (EF), and fibrosis are provided. Levels of significance (P -values calculated according to ANOVA) as shown.

Note that it follows from the definition of a co-response $O^{J,S}$ that $J = cS^0$ where c is a constant. Where an enzyme converts metabolite S to metabolite P , the co-responses $O^{J,S}$ and $O^{J,P}$ involve the same flux J , so $O^{J,S}/O^{J,P} = \Delta \ln P/\Delta \ln S$ and if the enzyme had no change in its mass action ratio (P/S), when $\Delta \ln P = \Delta \ln S$, then the co-responses will be equal. If $O^{J,S}$ is greater than $O^{J,P}$, then there has been a relative increase in product concentration and the reaction has moved closer to equilibrium, whereas if $O^{J,S}$ is smaller than $O^{J,P}$, the reaction has moved away from equilibrium. Where only a single enzyme in a pathway is altered, flux–substrate co-response coefficients are likely to be less than 1 (or less than the Hill coefficient for cooperative enzymes) [23]. Under several known physiological conditions (e.g. hypoxia) [23], observed co-response coefficients are larger than this; exact mechanistic explanations of this are not known, but in theory, parallel activation of several steps (the ‘universal method’ [24], multisite modulation [25,26], or proportional activation [27]) can account for this. Enzymes that are extremely close to equilibrium [25] can show large co-responses during passive adaptation to a change in metabolic flux, but this would imply virtually no detectable change in the displacement from equilibrium, and hence $\Delta \ln (P/S) \cup 0$, with virtually identical substrate and product co-responses as described above. A negative flux–substrate co-response corresponds to a classic cross-over effect, where activation of an enzyme has induced a decrease in its substrate. Thus, the co-response coefficients provide insight into the events associated with a change in flux.

2.2.7. Statistical analysis. Data are expressed as mean values $\pm$ standard error. To evaluate the clinical data, the Friedman and Dunn test, ANOVA and Scheffé test, the paired t -test and the Mann–Whitney rank-sum test were used. Fibrosis and the metabolite contents were analyzed using ANOVA and Scheffé testing. A value of $P < 0.05$ was considered as significant difference.

3. Results

3.1. Clinical Data

All patients exhibited a recovery of regional (and global) contractile performance 3 months after revascularization, thereby validating our clinical preoperative diagnosis ‘hibernating myocardium’ (Table 1). As indicated by the restoration of a formerly decreased thallium uptake following surgery (Table 1), these hibernating regions showed an impaired resting perfusion before revascularization.

3.2. Metabolite contents

Contents in the metabolites of the Embden–Meyerhof pathway and of glycogen are given in Table 1 and Fig. 1, expressed as total myocardial contents as well as normalized to the CMF. Except for G6P and fructose 2,6-bisphosphate ($F_{2,6}P_2$), HHM expresses increased contents in metabolites of the Embden–Meyerhof pathway.

3.3. Impairment of myocardial perfusion and estimation of anaerobic glycolytic flux

Myocardial glycogen content was not significantly changed in hibernating myocardium (Table 1), indicating no net flux between G6P and glycogen. Hence, the flux from glucose to G6P equals the flux from G6P to lactate, and the reactions from glucose to lactate may be treated as an unbranched pathway.

Increased lactate contents in HHM stand for an elevated anaerobic glycolytic flux [19]. Even when corrected for an impaired myocardial perfusion [20] (thallium-201 uptake: reduction to $41.25 \pm 1.93\%$ of normal, $P < 0.0001$), anaerobic glycolytic flux in HHM exhibited an approximately four-fold increment (see Table 1). As myocardial glycogen content did not significantly differ between HHM and control myocardium, C6-units serving as fuel for increased flux through

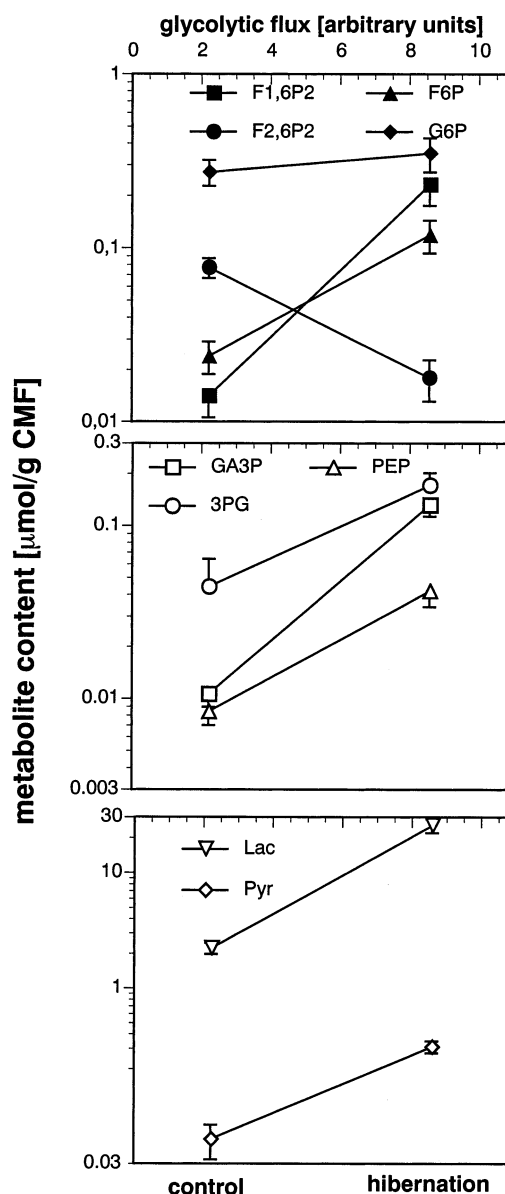


Fig. 1. Mean values and standard errors of myocardial metabolite contents (normalized to CMF) in control (low anaerobic glycolytic flux) and hibernating human myocardium (increased anaerobic glycolytic flux).

anaerobic glycolysis were provided by an augmented myocardial glucose uptake of the same magnitude.

3.4. MCA of the regulation of anaerobic glycolytic flux

The increase in anaerobic glycolytic flux in HHM was accompanied by elevated contents of most metabolites of the Embden–Meyerhof pathway (Table 1, Fig. 1). On the basis of the calculated flux:metabolite co-responses, the mechanisms of anaerobic glycolytic regulation of the respective enzymatic steps could be analyzed in detail (see Table 2).

For fructose 6-phosphate (F6P), phosphoenolpyruvate (PEP), and pyruvate (Pyr), increases in anaerobic glycolytic flux were associated with modest elevations in metabolite content, and clearly positive co-response coefficients could be calculated. These data indicate that the reaction velocities of phosphofructokinase-1 (PFK-1), pyruvate kinase (PK) and

lactate dehydrogenase (LDH) increase sensitively with raising substrate concentrations. As the values for O were in the range between 0.5 and <1.0 , flux determining (albeit passive) roles for these enzymes can be assumed [13,23,26,28]. Contrary to expectations from traditional accounts of glycolytic regulation, there is no evidence of a ‘cross-over’ from action of effectors at PFK-1; the larger co-response to substrate indicates that the enzyme has moved closer to equilibrium, but that this can be accounted for by an increase in substrate concentration.

In the case of PGM/Eno, where values for O in the range of $+1$ could be calculated, the significantly larger co-response to its substrate 3PG than to its product PEP ($P=0.0056$) indicates that this pair has had an increase in product/substrate ratio, implying that they have moved closer to equilibrium and that were, therefore, more displaced from equilibrium previously. How this pair could move closer to equilibrium at a higher net flux is not explicable on current knowledge of the control of the pathway. Both, the significantly different O -value of 3PG – compared with the O -values of PEP, Pyr and F6P, as computed by ANOVA – and the observed alteration in enzyme kinetics indicate an active flux-adjusting role for this step.

For fructose 1,6-bisphosphate ($F_{1,6}P_2$) and GA3P, the increase in anaerobic glycolytic flux did occur at large increments in metabolite content, and only small values for O could be calculated. This pattern of such an only weak increase in reaction velocity in response to an increment in substrate concentration suggests passive, minor roles of these steps for flux determination [13,23,26,28].

For G6P, the increase in flux could be documented at virtually stable metabolite contents, resulting in a very high co-response coefficient. Hence, the acceleration in reaction velocity of glucosephosphate isomerase (GP-iso) is independent of the content of its substrate. This pattern implies that the behavior of this step of the Embden–Meyerhof pathway cannot be explained by passive substrate kinetics, nor closeness to equilibrium, since the very much larger value of G6P co-response to the F6P co-response shows that the mass action ratio of this enzyme changes relevantly, and in opposite direction to that required to account for the flux increase. Other regulatory mechanisms which allow to control this enzyme’s reaction velocity independent of substrate concentration are apparently operative. Such an enzymatic behavior is characteristic for steps actively participating in flux adjustment [13,23,26,28].

4. Discussion

Using the tools of MCA, our paper is the first to analyze anaerobic glycolytic flux and its regulation in hibernating human myocardium. According to the data obtained, HHM exhibited an increased anaerobic glycolytic flux, which was fueled by a corresponding increment in myocardial glucose uptake. The analysis of flux:metabolite co-responses allowed us to characterize the mechanism of enzymatic regulation of individual glycolytic steps as well as the distribution of flux control along the Embden–Meyerhof pathway.

4.1. Increased anaerobic glycolytic flux in HHM: impact on augmented ischemic tolerance.

In spite of varying concepts concerning the pathophysiology of HHM, its increased uptake of radiolabelled glucose tracers (18 -fluoro-deoxyglucose) represents the gold standard for its clinical diagnosis [3]. However, the augmented glucose uptake of HHM may not only be of diagnostic value [2], but may also contribute to the metabolic adaptation of HHM enabling this energy-depleted myocardium to better withstand severe supply–demand imbalance [7].

Myocardial survival in states of undersupply critically depends on the cellular energy status [29]. To limit energy deficit in conditions of energy shortage, as observed during hypoxia and ischemia, myocardial ATP formation may be amplified by increasing substrate chain phosphorylation in anaerobic glycolysis. By this means, the rate of ATP-synthesis is increased when reduced oxygen-availability becomes the limiting factor [30–33]. This mechanism was repeatedly shown to enable myocardial survival in various states of myocardial hypoperfusion [5,6].

In this respect, our data are consistent with the view that an accelerated flux through anaerobic glycolysis may represent an effective metabolic adaptation to better withstand severe supply–demand imbalance also in HHM, since any increase in anaerobic glycolytic flux indicates a corresponding increment in ATP provision. As this rise is fueled by an elevated myocardial glucose uptake, our data provide kinetic evidence for the amplified sarcolemmal abundance of glucose transporters in HHM [4].

4.2. Regulation of anaerobic glycolytic flux

The values calculated for O [23] allowed us to classify the glycolytic enzymes into four groups depending on the mechanism used for controlling and determining flux through this

Table 2

Metabolite	O -value	Enzyme	Change in kinetics in HHM?	Regulatory mechanism	Role for flux regulation
G6P	10.13 ± 2.018	GP-iso	active	independent of substrate content or closeness to equilibrium	major
F6P	0.72 ± 0.050	PFK-1	passive	substrate kinetics	major
$F_{1,6}P_2$	0.19 ± 0.015	FBP-aldo	passive	substrate kinetics	minor
GA3P	0.25 ± 0.023	GAPDH/ PGK	passive	substrate kinetics	minor
3PG	1.00 ± 0.428	PGM/Eno	active	closeness to equilibrium by unknown mechanism	major
PEP	0.72 ± 0.032	PK	passive	substrate kinetics	major
Pyr	0.54 ± 0.175	LDH	passive	substrate kinetics	major
Lac	0.27 ± 0.012				

Flux:metabolite co-responses (O , mean $\pm$ S.E.M.) for metabolites of anaerobic glycolysis. In addition, informations about the enzymes of the Embden–Meyerhof pathway with respect to kinetic changes in HHM and proposed regulatory mechanisms are provided. FBP-aldo, fructosebisphosphate aldolase

pathway:

1. According to our data, aldolase and GAPDH/PGK show a passive behavior in response to an increased net flux in terms of enzyme kinetics, whereby the low values for *O* suggest minor roles for flux determination.
2. The flux:metabolite co-responses obtained for PFK-1, PK and LDH, however, indicate that these enzymes do more relevantly determine glycolytic flux. This is achieved by sensitively (albeit passively) increasing these enzymes' reaction velocities in response to elevations in their substrate concentrations according to classical substrate kinetics.
3. In contrast to these inactively acting enzymes in terms of enzyme kinetics, the significant changes in PGM/Eno with respect to their equilibrium positions observed in HHM and the significantly higher values calculated for *O* at these glycolytic step(s) – compared to the *O*-values for F6P, PEP and Pyr – were not consistent with a passive response to the change in flux. In this case, an active alteration of enzyme kinetics for the adjustment of flux is indicated.
4. For GP-iso, for which an active alteration in enzyme kinetics in HHM is also obvious, a different means of enzymatic regulation independent of substrate concentration, consistent, e.g., with an alteration of enzyme reaction velocity by external effectors [34], could be shown.

Taken together, the evaluation of the flux:metabolite co-responses suggests that the control of anaerobic glycolytic flux is achieved using flux modulation at several sites along the Embden–Meyerhof pathway, which is consistent with the concept of multisite modulation [25]. At GP-iso and PGM/Eno, altered enzymatic properties are suggested by our analysis, indicating their active participation in flux control. For the remaining enzymes no clear evidence of changed enzyme behavior could be seen, though the flux:metabolite co-responses for PFK-1, PK and LDH still suggest their contribution to the determination of flux through the Embden–Meyerhof pathway. For GAPDH/PGK and aldolase, finally, the low values for *O* are not in favor of important contributions of these enzymes.

With respect to the number and location of regulatory, flux-adjusting sites along the Embden–Meyerhof pathway, this first analysis using flux:metabolite co-responses in an clinical-experimental study strongly contradicts traditional concepts for the control of the Embden–Meyerhof pathway in normal and diseased myocardium, as these models often postulate that flux through glycolysis is almost exclusively controlled by the behavior of PFK-1 [5,9,11,12]. Though the data presented still suggest that PFK-1 is (passively) involved in flux determination, the time-honoured 'key regulatory role' [35] is not confirmed by our observations. Moreover, a predominant role for PFK-1 in flux regulation can only be hardly put into accordance with the finding that the contents for $F_{2,6}P_2$, which is a potent positive effector of PFK-1 in vitro [35], were found to be decreased in HHM.

4.3. Limitations of the study

4.3.1. Myocardial enzyme content. As HHM represents a chronical process, it must be assumed that alterations not only in metabolite content but also in protein composition might occur. Though data for HHM itself are lacking, this has re-

cently been shown for human myocardium failing for other reasons [36–40]. Since the HHM is believed to suffer from supply–demand imbalance, it might be expected to show a response similar to the typical pattern of hypoxic cells: coordinate induction of glycolytic enzymes and some repression of mitochondrial enzymes [26,41]. If this were to account for the flux changes here, then upregulation of all the enzymes by the same factor [42] would allow an increase in flux without changes in intermediate metabolite levels. In fact, in hypoxia, the relative increases in low-activity enzymes such as PFK and PK are observed to be somewhat greater than in enzymes such as GP-iso and PGM/Eno for which we have evidence of activity changes.

4.3.2. Estimation of anaerobic glycolytic flux. Anaerobic glycolytic flux, as it can be easily assessed in animal studies, could not be directly measured in our clinical study for methodological and ethical reasons. Therefore, anaerobic glycolytic flux had to be estimated from myocardial lactate content, which was corrected for a reduced lactate washout as a decreased perfusion of hibernating myocardium. By this means, we were able to clearly differentiate two distinct metabolic myocardial states with an unaltered (normal myocardium) and an increased anaerobic glycolytic flux (HHM), allowing to apply flux:metabolite co-responses to analyze anaerobic glycolytic regulation.

The measurements of metabolites were made on myocardial biopsies, representing a heterogenous material. Thus, it is possible that there might have been regional differences in metabolite contents and thus also in the calculated flux:metabolite co-responses. Due to the sample preparation used, these regional differences could not be considered and the values given for *O* might not truly represent the flux:metabolite co-responses existing at the respective enzymes themselves. These unavoidable limitations must be considered.

Since HHM was shown to contain a normal rate and capacity of oxidative metabolism [21], aerobic glycolysis was assumed to be unchanged between both groups and was thus not considered for model construction. However, we cannot exclude differences in aerobic glycolytic rate between normal and hibernating myocardium with certainty. As hypoperfused myocardium switches from the preferential use of fatty acids to carbohydrates as a fuel for oxidative metabolism [32] resulting in an increased aerobic glycolytic flux, our model might have underestimated total (i.e. aerobic plus anaerobic) flux through the Embden–Meyerhof pathway. These possible limitations must be taken into account.

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