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Rigler B, Tscheliessnigg KH

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A Microarray and Real Time PCR Study on the Effect of Experimental Ischemia upon the Expression of the Insulin-Dependent Transmembrane Glucose Transport Molecule GLUT4 in Human Atrial Myocardium*

K. Ablasser[1], S. Gasser[1], E. Scherr[1], E. Toferer[1], U. Roessl[1], K. Kröll[1], N. Bareza[1], H. Mächler[1], A. Trantina-Yates[2], B. Rigler[2], K-H. Tscheliessnigg[3], R. Gasser[1]

Glucose and high energy metabolism play a pivotal role in the development of numerous salient characteristics of myocardial ischemia, such as the gating properties of specific ion-channels, intracellular ion-homeostasis, electrical phenomena, contractility and other phenomena. Many of these aspects of myocardial ischemia are linked in one or the other way to transmembrane glucose transport, intracellular glucose metabolism and, in fact, to GLUT4.

In human cardiac tissue (right auricle), we here investigate to which extent GLUT4 gene expression is altered in experimental ischemia. Using microarray technique we first look at general changes in expression profiles during simulated myocardial ischemia, the behaviour of SLC2A4 (GLUT4, solute carrier family 2 [facilitated glucose transporter], member 4) as well as its regulator gene SLC2A4RG. Then, using Real Time PCR (Light Cycler), we quantify GLUT4 mRNA expression changes in 8 single experiments under ischemic and control conditions.

Using the microarray technique, we find that both the expression of GLUT4 gene (SLC2A4) and its regulator gene remain practically unchanged. In Real Time PCR (Light Cycler), the mean ratio for GLUT4 gene expression compared to the house keeping gene G6PDH was under well oxygenated conditions –0.0052 ± 0.0203 and under N2-simulated ischemia 0.0179 ± 0.0196 (n = 8; ± SEM). No statistically significant difference could be found between the two groups. Results show a trend to a slight increase in expression, however no statistical significance could be seen.

No significant changes are seen in the expression of the GLUT4 gene as well as in its regulatory gene after 30 minutes of N_2-mediated experimental ischemia. Similarly, biological processes involved in glucose metabolism are not significantly de-regulated as others are. This, as well as a slight trend towards up-regulation can be interpreted as an attempt of the myocyte to maintain energy metabolism stable under hypoxic conditions. ***J Clin Basic Cardiol 2006; 9 (online): 4–9.***

Key words: *GLUT4, ischemia, myocardial, glucose*

In mammals, the transport of glucose across cell membranes occurs by facilitated diffusion. Several cDNAs encoding structurally related proteins with the properties of facilitative glucose transporters have been isolated and characterized (GLUT). These molecules regulate transmembrane glucose transport in various tissues. GLUT4 appears to be of special interest for several reasons, in particular, because it is the only GLUT which is directly regulated/stimulated by insulin. It is found in various tissues like cardiac and skeletal muscles, as well as adipose tissue [1]. The isolation and characterisation of a monoclonal antibody that specifically recognised this "muscle-fat isoform", GLUT4, revealed that it was a unique isoform, different from the glucose transporters present in erythrocytes, brain, kidney, jejunum and liver. It shows between 50 % and 70 % cDNA identity with GLUT1–3. Insulin causes a rapid and reversible increase in glucose transport activity via GLUT4 in cardiac and skeletal muscle [2].

Glucose and high-energy metabolism play a pivotal role in the development of numerous salient characteristics of myocardial ischemia, such as the gating properties of specific ion-channels, intracellular ion-homeostasis, electrical phenomena, contractility and other phenomena [3–5]. Many of these aspects of myocardial ischemia are linked in one way or the other to transmembrane glucose transport, intracellular glucose metabolism and, in fact, to GLUT4 [6–8]. Myocardial ischemia increases glucose uptake through translocation of GLUT1 and GLUT4 from an intracellular compartment to sarcolemma. This appears to be a beneficial effect during ischemia and possibly recovery. Insulin and ischemia have additive effects to increase *in vivo* glucose utilisation and augment glucose transporter translocation [9]. Delivery of glucose to the glycolytic pathway appears to be a major controlling site of glycolysis in low-flow ischemia. Downstream regulation is then distributed along the pathway with no one site exerting greater inhibition than reduced glucose delivery [10]. While many experimental studies suggest that an increase in glucose uptake and metabolism by the ischemic myocardium helps to protect myocardial cells from irreversible injury [11], little or nothing is known in this context about human cardiac transmembrane glucose transport and GLUT4 expression during ischemia.

Here, we shall investigate in human cardiac tissue, sampled from right auricle, to which extent GLUT4 gene expression is altered in "healthy" subjects. We look at the effects of experimental ischemia on GLUT4 expression in cardiac tissue of metabolically normal, non-obese, normotensive patients who undergo cardiac surgery for reasons like valve repair, trauma etc. We hypothesise that GLUT4 expression may be increased in the context of protecting myocardial metabolism secondary to increased glucose uptake through translocation of GLUT1 and GLUT4 from an intracellular compartment

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From the [1]Project Group on Experimental Cardiology of the Department of Cardiology, University Dept. of Internal Medicine and Center for Medical Research (ZMF) of the University, the [2]Department of Cardiovascular Surgery, University Dept. of Surgery and the [3]Department of Transplant Surgery, University Dept. of Surgery, Head, University of Graz, Austria.
Correspondence to: Prof. Robert Gasser, MD PhD, Dept. of Cardiology, University Department of Internal Medicine, Auenbruggerplatz 15, A-8036 Graz, Austria; e-mail: robert.gasser@meduni-graz.at

to sarcolemma. Using microarray technique [12–14], we first look at general changes in expression profiles during simulated myocardial ischemia, the behaviour of SLC2A4 (GLUT4, solute carrier family 2 [facilitated glucose transporter], member 4) as well as its regulator gene SLC2A4RG. Then, using Real Time PCR (Light Cycler), we shall quantify GLUT4mRNA expression changes in 8 single experiments and investigate whether or not expression is significantly altered under ischemic conditions.

Material and Methods

Material

Myocardial tissue probes derive from the right auricle of patients undergoing cardiac surgery, who gave informed consent by signing a form approved by the local ethics committee. Cardiac tissue was sampled from metabolically normal, non-obese, normotensive patients who underwent cardiac surgery for reasons like valve repair, trauma etc. A small part of the right auricle is removed when the heart is put on extracorporal circulation. This tissue is normally wasted. Here, the myocardial strip was instantly placed in well oxygenated, cooled Tyrode solution and transported to the laboratory where it was fixed in an experimental chamber. The preparation was then oxygenated (100 % O_2) before it was cut in two pieces of about equal size. Each of the preparations was then put into another individual chamber as done in earlier experiments [4, 15, 16]. Experimental ischemia was brought about by switching 100 % oxygen to 100 % nitrogen (hypoxia) in one of the chambers [4]. After 30 minutes of simulated ischemia, the tissue probes were snap-frozen using liquid nitrogen. By doing so, we were able to compare ischemic and non-ischemic tissues of the same patient. Snap-frozen samples were stored at –70 °C until RNA-isolation. Preparation for RNA-isolation was made by homogenisation of the paraffin embedded probes using a cryostatic microtome (HM 560 CryoStar; Microm).

Solutions

The preparations were continuously perfused with Tyrode solution [4] containing in mM: NaCl 140, KCI 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 10, HEPES 20. Solutions were adjusted to a pH of 7.4 by titration with 4M NaOH and equilibrated with 100 % O_2 at 37 °C.

RNA-Isolation and cDNA transcription

Total RNA was extracted by the Trizol® method (Invitrogen Corporation, Carlsbad, CA, USA) and further purified using RNeasy Mini Kit (Qiagen Inc., Hilden, Germany). After drying, the pellet containing isolated RNA was resuspended in approximately 30 µl TE buffer. Then quality as well as quantity of RNA was assessed using spectrophotometry [17]. The quality of the isolated RNA was also analysed on Agilent's Bioanalyzer 2100 system. Either 20–40 µg or 0,2–1 µg total RNA, when only reduced amounts of material was available, was then directly or indirectly, via *in-vitro*-transcription, transcribed into DIG-labelled cDNA. For reverse transcription of isolated RNA, we used the High Capacity cDNA Archive Kit (Applied Biosystems) and the Thermocycler MyCycler™ from Biorad. Real-time PCR was performed using the LightCycler® 2.0 System (Roche). Expression of the SLC2A4 (GLUT4) gene was made using the Taqman format and compared to the housekeeping gene glucose 6 phosphate dehydrogenase, which was measured using the hybridisation probe format with a kit from Roche (LightCycler-h-G6PDH Housekeeping Gene Set) [18, 19]. We then used Taq DNA Polymerase for mastermix in both (LightCycler® DNA Master HybProbe). Forward primer (= primer 1), reverse primer (= primer 2) as well as GLUT4 complementary Taqman probes were produced by TIB MolBiol Company [20]. To calculate the expression ratio we used the relative difference as shown below [21]:

$$\text{Relative difference} = \frac{\text{Housekeeping} - \text{GLUT4}}{\text{Housekeeping}}$$

Microarray [12–14]

The labelled probes were hybridised onto the array for 16 hrs. Subsequently, arrays were washed and detection was carried out using alkaline-phosphatase conjugated anti-DIG antibodies and the appropriate substrate according to a highly standardized protocol. Arrays were scanned with the AB1700 Chemiluminescence Array Reader and images, raw data and tissue information is stored in a MIAME-compliant ORACLE dat AB1700 Microarray Analyzer System: The full-genome Chemiluminescence Microarray System (Applied Biosystems) implemented at the Molecular Biology Core Facility of the Center for Medical Research combines the most comprehensive gene probe set with a chemiluminescence-based detection system that is superior to the commonly used fluorescent detection systems with respect to sensitivity (femtomolar level). Expression levels of approximately 54,000 transcripts can be measured in one single experiment. About 74 % of the immobilized probes are CELERA-curated (CDS) sequences from public databases and 25 % of the sequences are accessible via the CDS only. About 4,700 control spots are used to assess array performance in each single step from array fabrication to assay readout. Another unique feature of the AB1700 system is the co-immobilization of an artificial oligonucleotide probe together with the gene-specific probe in each spot. This probe is detected via a fluorescently labelled oligonucleotide that is added to the hybridisation mixture. This novel approach is crucial for optimal localization of each spot and allows normalization strategies that are not necessarily dependent on a high-end bio-informatics support. In each experiment, we used pooled data of four experiments as well as one individual single experiment (n = 4 + 1).

Operator/s

The AB1700 microarray system available at the Core Facility Molecular Biology is operated by a team consisting of four members with many years of experience in various microarray technologies and platforms (Affymetrix, cDNA- and Oligonucleotide Arrays). To evaluate the performance of the novel Applied Biosystems microarray technology, a multicenter proof-of-principle study was conducted by the CF-MB, which included Affymetrix, cDNA- and oligonucleotide-based platforms and was performed at approved international facilities. The novel AB1700 chemiluminescence microarray system turned out to be superior with respect to sensitivity and reliability.

Results

Microarray

We assessed gene expression profiles in 5 (4 pooled, 1 control) patients using microarray technique. Each individual patient provided two results from the same auricle, one under simulated ischemia and one under well-controlled 100 % O_2 perfusion. Table 1 shows mg of retrieved material for pooled experiments and Table 2 delivers spectrophotometric data for quality and quantity of Trizol®-isolated RNA.

Using microarray technique, 23,401 genes were detected and analysed. Fluorescent signal intensity of the "ischemia" pool was compared to the control experiments using the ratio

Table 1. Probe weights for pooled experiments after homogenisation using a cryostatic microtome

Patient ID	Probe weight after homogenisation
119 O_2	150 mg
119 N_2	120 mg
123 O_2	60 mg
123 N_2	110 mg
124 O_2	170 mg
124 N_2	180 mg
125 O_2	150 mg
125 N_2	100 mg

Table 2. Concentration and ratio (260 nm/280 nm) of samples after Trizol® isolation and spectrophotometry. One can see that RNA is sufficiently concentrated in the samples, ideally it should be 1.7–1.8. Using the high-purification RNeasy-Kit from Qiagen® led to isolation of up to 100 μg RNA of more than 200 base length.

Patient ID	Concentration	Ratio 260/280
119 O_2	1281.69 μg/ml	1.78
119 N_2	546.62 μg/ml	1.73
123 O_2	2860.91 μg/ml	1.71
123 N_2	4911.16 μg/ml	1.79
124 O_2	1562.74 μg/ml	1.72
124 N_2	772.71 μg/ml	1.73
125 O_2	1375.79 μg/ml	1.81
125 N_2	3123.86 μg/ml	1.78

from both. The logarithmic ratio then reflects de-regulation of gene expression. Figure 1 shows an example of the fluorescent microarray image of our experiments.

As can be retrieved from Table 3, both the expression of GLUT4 gene (SLC2A4) and its regulator gene remain practically unchanged.

Using PANTHER (Protein Analysis Through Evolutionary Relationships) Software from Applied Biosystems™, Version 6.0, we were also interested in (possibly cardio-protective) pathways and biological processes up-regulated. Those pathways and biological processes which were more than 2-fold upregulated under simulated ischemia (N_2) can be seen in Tables 4 and 5. Interestingly, the nicotinic acetylcholine receptor signalling pathway, the metabotropic glutamate receptor group III pathway and the interleukin signalling pathway are up-regulated significantly. Concerning biological processes, we can also see that, in particular, those involved in inflammation and repair are upregulated.

Real Time PCR

In 8 subjects without metabolic disturbances, obesity or hypertension (which could influence GLUT4 expression), the GLUT4 gene expression was measured in "paired" experiments under control and N_2-ischemic conditions. The mean ratio for GLUT4 gene expression compared to the house-keeping gene G6PDH was under well oxygenated conditions –0.0052 ± 0.0203 and under N_2-simulated ischemia 0.0179 ± 0.0196 (n = 8; ± SEM). No statistically significant difference could be found between the two groups. Results show a trend towards a slight increase in expression, however no statistical significance could be seen (Fig. 2). Figure 3 shows amplification curves from our experiments as derived from the Light Cycler.

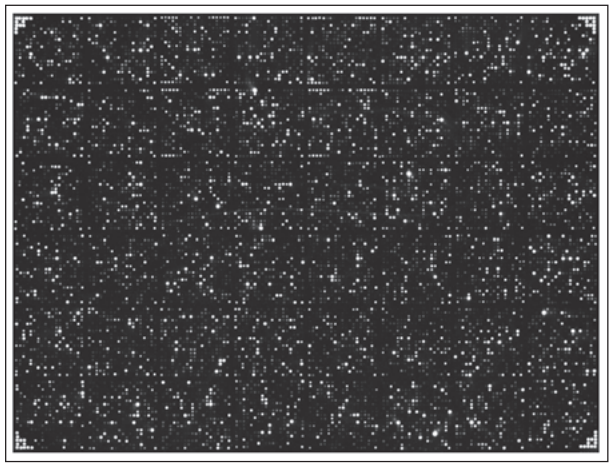

Figure 1. Fluorescent image of 4 pooled ischemia experiments indicating different intensities of fluorescense reflecting expression of 23,401 genes detected. Intensity of fluorescence correlates with the amount of bound cDNA

Table 4. Pathways, 2-fold up-regulated under N_2-simulated ischemia

Pathway (min. 2-fold up under N_2)	p-value
Nicotinic acetylcholine receptor signalling pathway	0.000748
Metabotropic glutamate receptor group III pathway	0.0071
Metabotropic glutamate receptor group II pathway	0.0406
Ionotropic glutamate receptor pathway	0.0936
Interleukin signalling pathway	0.00000126
TGF-beta signalling pathway	0.168

Discussion

Quality as well as quantity of isolated mRNA were double-checked using both spectrophotometry as well as Agilent's Bioanalyzer 2100 system. Real Time PCR was performed according to the two-step method using Taq polymerase and led to ideal exponential amplification curves, as can be seen in Figure 2. Negative controls showed no activity or contamination. Real Time PCR (Light Cycler) is a well established method in the Core Facility Molecular Biology of our university and was operated and supervised by an experienced team. The AB1700 microarray system available at the Core Facility Molecular Biology is operated by a team consisting of four members with many years of experience in various microarray technologies and platforms (Affymetrix, cDNA- and Oligonucleotide Arrays). To evaluate the performance of the novel Applied Biosystems microarray tech-

Table 3. Microarray results for the expression of GLUT4 gene (SLC2A4) and its regulator gene SLC2A4. No significant de-regulation can be seen in either.

Gene ID	Gene symbol	Gene name	Normalized signal O	Normalized signal N	Ratio O/N O/N	Log2-ratio
hCG42005.3	SLC2A4	solute carrier family 2 (facilitated glucose transporter) member 4	3.77	4.03	0.94	–0.10
hCG22760.2	SLC2A4RG	SLC2A4 regulator	7.06	6.97	1.01	0.02

Table 5. Biological processes, at least 2-fold up-regulated during ischemia. P-value is indicative for the significance of overexpression.

Biological process (min 2-fold up under N_2)	p-value
B-cell- and antibody-mediated immunity	7.42E-40
Immunity and defense	4.13E-10
T-cell mediated immunity	0.0000807
Hematopoiesis	0.00798
Metabolism of cyclic nucleotides	0.0167
Heart development	0.0522
Synaptic transmission	0.0738
Ion transport	0.0749
Biological process unclassified	6.03E-23
G-protein-mediated signalling	0.00422
Cell surface receptor-mediated signal transduction	0.00789
Granulocyte-mediated immunity	0.0131
T-cell-mediated immunity	0.0217
Cytokine- and chemokine-mediated signalling pathway	0.023
NF-kappaB cascade	0.0322
Cytokine-/chemokine-mediated immunity	0.0472
Ligand-mediated signalling	0.0616
Pain sensation	0.0692
Sensory perception	0.0723
Calcium-mediated signalling	0.0737
NO-mediated signal transduction	0.0986

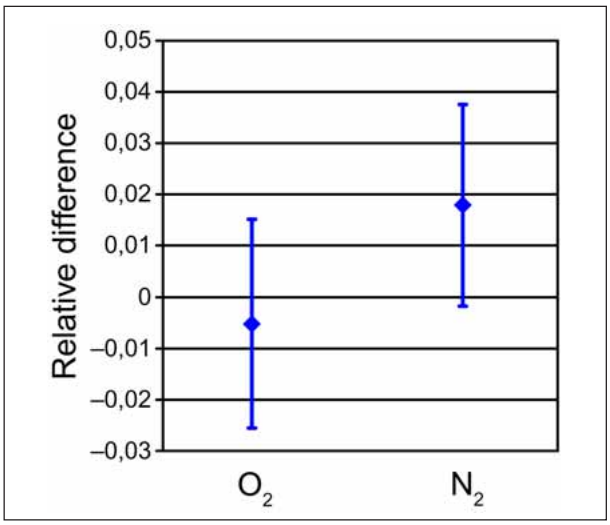

Figure 2. Relative difference in the expression of GLUT4 under ischemic and non-ischemic conditions (n = 8; ± SEM). Results show a trend towards a slight increase in expression, however no statistical significance could be seen.

nology a multicenter proof-of-principle study was conducted by the CF-MB, which included Affymetrix, cDNA- and oligonucleotide-based platforms and was performed at approved international facilities. The novel AB1700 chemiluminescence microarray system turned out to be superior with respect to sensitivity and reliability.

No significant changes were seen in the expression of the GLUT4 gene as well as its regulatory gene after 30 minutes of N_2-mediated experimental ischemia. Similarly, biological processes involved in glucose metabolism are not significantly de-regulated as are others. This as well as a slight trend towards up-regulation can be interpreted as an attempt of the myocyte to maintain energy metabolism also under hypoxic conditions. Seemingly, insulin-dependent transmembrane glucose transport is not immediately affected by hypoxic conditions.

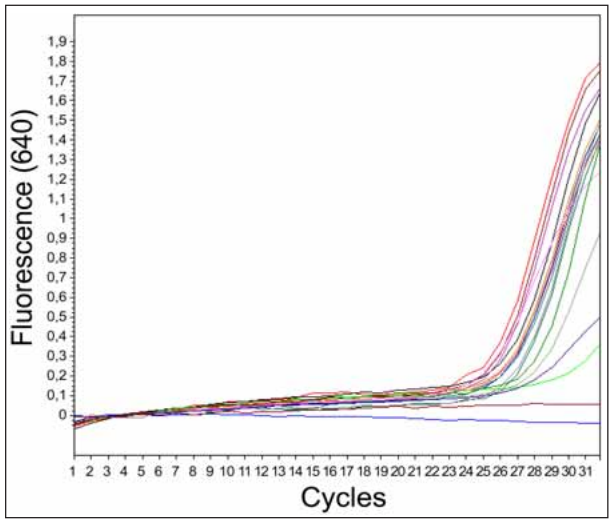

Figure 3. Amplification curves from Real Time PCR performed in human myocardial tissue samples under ischemic and control conditions

From experiments it is known that different types of muscle fibres contain different levels of GLUT4 proteins and gene expression as well as different insulin sensitivities and that the nutritional state and contractile activity appear to regulate GLUT4 gene expression. Fasting, for example, results in a two- to threefold increase in GLUT4 protein and gene expression in mixed soleus and gastrocnemius muscle preparations [22]. Exercise training also increases GLUT4 protein levels in rat skeletal muscle [23], whereas there are conflicting results concerning patients with NIDDM: In skeletal muscle, Handberg and co-workers found no significant difference in the levels of GLUT4 mRNA and protein in biopsies from patients with and without NIDDM [24], whereas Dohm et al. found a significantly decreased expression of GLUT4 in skeletal muscle from insulin-resistant patients [25]. Eckel and Reinauer showed that GLUT4mRNA is decreased in cardiac tissue of streptozotocin-diabetic rats [26]. Interestingly, our own group showed that in human NIDDM patients, GLUT4mRNA expression is down-regulated [27], whereas it is up-regulated in IDDM [28]. The latter may be explained by the fact that the application of insulin stimulates the expression of GLUT4mRNA [1]. However, these data derive from small patient numbers and semi-quantitative technique.

Apparently, rather long-term conditions, such as hypertension, diabetes or fasting, seem to effect GLUT4 gene expression. The interrelation between disturbances in glucose-metabolism, hypertension and myocardial ischemic disease has been known for a long time and thus has been the subject of investigation in a multitude of trials, publications and experimental studies [29–38]. Insulin resistance and reactive hyperinsulinemia occur not only with obesity, impaired glucose tolerance or non-insulin-dependent (type 2) diabetes mellitus, but also in many non-obese [39], non-diabetic patients with essential hypertension and seem to be largely responsible for the development of hypertension. The common co-existence of genetic predisposition for hypertension with insulin resistance helps to explain the frequent, although temporally often dissociated, occurrence of hypertension together with dyslipidemia, obesity and type 2 diabetes in a given cohort. While the complete cascade of interactions between glucose-metabolism and hypertension has not been elucidated completely as yet, transmembrane glucose transport is certainly crucial in this setting [1, 22–28].

GLUT4, the insulin-dependent transmembrane glucose facilitative transport molecule, plays a decisive role in insulin-dependent cardiac glucose metabolism, apparently also for myocardial [40] and vascular stiffness [11] as well as in the context of osmolarity, compartmental water distribution and homeostasis [1]. As early as 1995, comparative studies using nuclear magnetic spectroscopy in heart of normotensive (WKY) and spontaneously hypertensive rats (SRH) looked at glucose uptake during insulin stimulation as well as at mRNA expression of GLUT1 and GLUT4 [41]: In hypertensive rats, expression of GLUT4mRNA as well as the amount of protein in the membrane were decreased and cardiac hypertrophy increased by 59 %. Similar results were found in the afferent vessel of the renal glomeruli in experimental, streptozotocin-induced diabetes mellitus. In diabetic animals, GLUT4 as well as polypeptide expression and thus glucose uptake were reduced. In this context, it was speculated that the resulting decrease of GLUT4 could modulate renal blood flow and, in turn, lead to hypertension. We then concluded that defective GLUT4 expression may also occur in human myocardium of diabetics [27–29] as well as hypertensives, and were the first to demonstrate a decreased GLUT4 expression in right auricle myocardium of non-diabetic, hypertensive patients, however, using semi-quantitative techniques and a small cohort of patients [42] (snap-frozen, non-ischemic human material was difficult to retrieve). Disturbed transmembrane glucose transport may also significantly contribute to the development of severe coronary heart disease [43] and diabetic cardiomyopathy [44]. In the context of hypertension very few authors have looked at evidence for myocardial and vascular GLUT4 involvement in the development of hypertension in animals [45] and still no reports can be found on GLUT4 in human myocardium. Despite the scant experimental direct evidence, already Ikegami et al. postulated the GLUT4 gene as one of the target genes in essential hypertension when accompanied with insulin resistance [46]. In the present project we excluded patients with hypertension, diabetes mellitus or obesity in order to avoid mixed influences on GLUT4 gene expression.

In summary, many aspects of myocardial ischemia are linked in one way or the other to transmembrane glucose transport, intracellular glucose metabolism and, in fact, to GLUT4 [25–27]. It has been shown that myocardial ischemia increases glucose uptake through translocation of GLUT1 and GLUT4 from an intracellular compartment to sarcolemma. This appears to be a beneficial effect during ischemia and possibly recovery. Insulin and ischemia have additive effects to increase *in vivo* glucose utilisation and augment glucose transporter translocation [28]. Delivery of glucose to the glycolytic pathway appears to be a major controlling site of glycolysis in low-flow ischemia. Downstream regulation is then distributed along the pathway with no one site exerting greater inhibition than reduced glucose delivery [29]. While many experimental studies suggest that an increase in glucose uptake and metabolism by the ischemic myocardium helps to protect myocardial cells from irreversible injury [30], here we show conclusively that GLUT4-mRNA expression is not up-regulated during ischemia in human myocardium. Our findings are in accordance with earlier observations suggesting a pivotal role of maintenance of transmembrane glucose transport during myocardial ischemia [25–30].

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