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Original article

Gene and protein expression changes in response to normoxic perfusion in mouse hearts

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Abstract

Introduction: Although crystalloid-perfused isolated heart models are widely used in cardiovascular research, there are several limitations of these techniques. Changes in cardiac gene expression pattern due to normoxic perfusion itself have not been studied, despite its potential importance to provide useful information on limitations of this model. Therefore, here we investigated the time-dependent effect of normoxic, normothermic perfusion on global gene expression at mRNA and protein levels. **Methods:** Hearts from male CFLP mice were perfused according to the Langendorff technique. We assessed relative gene expression changes by DNA microarray analysis of 8000 genes after 0, 60 and 120 min perfusion. **Results:** Twelve genes exhibited significant up-regulation and 27 showed repression in hearts perfused for 60 or 120 min as compared to 0 min controls. Expression changes of 17 selected genes were verified and an additional 19 genes were examined by real-time quantitative PCR. Genes with altered expression included those coding for Creatin kinase, Lactate dehydrogenase, Voltage-dependent anion channel 1, a Disintegrin and Metalloprotease domain 3, Integrin alpha 7, Long-chain acyl-CoA dehydrogenase, Casein kinase II, Ketohexokinase, Chloride ion current inducer protein, Matrix metalloproteinase 2 and 9, Superoxide dismutases and Nitric oxide synthases, etc. **Discussion:** Our results show that normoxic crystalloid perfusion itself results in time-dependent changes in cardiac gene expression which should be considered when designing ex vivo perfusion protocols in the mouse heart to mimic cardiac pathologies as many of these genes have been suspected to influence several cardiovascular diseases.

Keywords: Normoxic perfusion; Isolated heart; Global gene expression; Heart model; Mouse heart

1. Introduction

The isolated perfused heart is one of the most widely used experimental models in cardiovascular research, including testing drug efficacy and safety and modeling cardiovascular pathologies such as ischemia-reperfusion injury. It is a relatively simple model and it provides a highly reproducible preparation which can be used for comprehensive examinations of cardiac contractile function, electrophysiology, cellular biochemistry and molecular biology. However, the major limitations of this model include deterioration of myocardial function within beyond 60 min possibly due to oxidative and nitrosative stress (Ferdinandy, Panas, & Schulz, 1999) and unusual appearance of cardiac arrhythmias (Clements-Jewery, Hearse, & Curtis, 2002; Curtis, 1998; Hearse, Richard, Yellon, & Kingma, 1988; Miyashita et al., 2000; Ravingerova, Pancza, Ziegelhoffer, & Styk, 2005; Schomisch et al., 2005). Although the most frequently used ex vivo heart preparation is the rat heart, due to the lower cost and the appearance of the genetically modified

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mice, ex vivo mice heart preparations have been rapidly spreading in the scientific community.

Unfortunately, although the literature contains an increasing number of studies employing mouse hearts, the fundamental characteristics of that have yet to be completely characterized (Sutherland & Hearse, 2000). In our study we used isolated and perfused mouse hearts. Hearts were excised, mounted on the Langendorff apparatus and perfused. Despite of several studies employing Langendorff perfusion in rat hearts (Onody et al., 2003; Puskás et al., 2004), still very little is known about the gene expression pattern of the heart in response to ischemia-reperfusion after different periods of time. Without the knowledge of gene expression changes at mRNA and protein levels in the perfused heart, it is difficult to define specific controls and these variations could alter the results of different studies. Therefore we have tried to answer such questions as: what are the effects of oxygenated perfusion fluid on global gene expression and how does it change related to time. To profile gene expression patterns at mRNA level, we monitored global gene expression changes by DNA microarray analysis (Onody et al., 2003) and validated the results by quantitative real-time polymerase chain reaction (QRT-PCR) in the mouse hearts. We studied the importance of perfusion time. The effects of 60 and 120 min perfusion were also studied and transcriptional changes were evaluated. Furthermore, two enzyme-activity measurements for lactate dehydrogenase and creatine kinase activities were investigated.

2. Materials and methods

2.1. Perfusion protocol of isolated mouse hearts

Male CFLP mice (35–45 g) were anesthetized with diethylether and given 150 U heparin ip. Hearts were then isolated and perfused in Langendorff mode (at a constant pressure of 70 mm Hg, 37 °C) with Krebs–Henseleit buffer containing (in mmol/L) 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.75 $CaCl_2$, and 11.1 glucose, oxygenated with 95% O_2–5% CO_2 (pH=7.4) as described (Csont et al., 2005; Giricz, Csonka, Onody, & Csont, 2003). To investigate the effect of the extent of ex vivo perfusion on the changes of gene expression pattern of mouse hearts three different perfusion protocols were applied (n=5–8 in each group). Hearts were subjected to either a 5 min wash out period, or a 60 min or 120 min normothermic, aerobic perfusion. Heart rate and coronary flow were monitored throughout the perfusion protocol in all groups. At the end of the perfusion protocol, hearts from all groups were frozen and crushed at the temperature of liquid nitrogen.

2.2. RNA isolation

RNA was purified from each group (76–100 mg tissue from each heart) Samples were rotor-homogenized and treated with proteinase-K (Qiagen). Total RNA was extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions (Onody et al., 2003). Prepared RNA samples from each group were pooled, and their quantities and qualities were assessed spectrophotometrically. The same total RNA pool was used for microarray analysis and for QRT-PCR.

2.3. Microarrays, sample preparations and hybridizations

Construction and use of microarrays were performed as described previously (Puskás, Zvara, Hackler, Micsik & van Hummelen, 2002). 2 μg of total RNA was reverse transcribed (Fermentas, RevertAid H Minus M-MuLV Reverse Transcriptase) according to the manufacturer's instruction. cDNA with Cy5 capture sequence was hybridized onto mouse microarray containing 8000 mouse specific cDNA probes. All the other sample preparation steps were done according the manufacturer's instructions (Genisphere). Both the first step cDNA hybridization and the second step capture reagent hybridization were carried out in a Ventana hybridization station (Ventana Discovery, Tucson, AZ) by using the "antibody" protocol. First hybridization was performed at 40 °C for 6 h in "FGL2" hybridization buffer (10×Denhardt's solution, 0.25 M sodium phosphate buffer pH 7.0, 1 mM EDTA, 1×SSC, 0.5% SDS), and then 2.5 μl of each Cy5 and Cy3 capture reagents were added to the slides in 200 μl "ChipHybe" hybridization buffer (Ventana) and incubated at 42 °C for 2 h. After hybridization slides were washed in 0.2×SSC twice at room temperature for 10 min and then dried and scanned.

2.4. Scanning and data analysis

Each array was scanned under a red laser (633 nm for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA) scanning confocal fluorescent scanner with 10 μm resolution. Scanned output files were analyzed using GenePix Pro5.0 software (Axon Instruments Inc., Foster City, CA). Each spot was defined by automatic positioning of a grid of circles over the image. For each channel, the median values of feature and local background pixel intensities were determined (Palotas et al., 2004). The background corrected expression data were filtered for flagged spots and weak signals. Technical replicates on the same array were averaged. Data were excluded in cases where technical replicates were significantly different. Normalization was performed using the print-tip LOWESS method (Yang et al., 2002). Next, we used the one-sample t test to determine the genes to be regarded as regulated in response to treatment. Logarithm was taken from each expression ratio to fulfill the requirement of the t test for a normal distribution. Genes for which the mean of log ratios across the biological replicates were equal to zero at a significance level (α=0.05) are considered to have an unchanged expression. On the other hand, genes having a p value smaller than α and the average fold change (increase or decrease) of the four data points were at least 1.9 times were considered as regulated genes.

2.5. Quantitative real-time polymerase chain reaction (QRT-PCR)

QRT-PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) with gene-specific

primers and SybrGreen protocol to confirm expression changes of 17 selected genes observed by microarrays. An additional 19 genes strongly related to cardiac stress adaptation and ischemia/reperfusion injury, but not available on the microarray were also investigated by QRT-PCR.

Two μg of total RNA from each sample were reverse transcribed in the presence of random primers in a total volume of 20 μl. After dilution with 20 μl of water, 1 μl of the diluted reaction mix was used as template in QRT-PCR. The 20 μl reaction volume contained 0.2 mM of dNTP, 1 × PCR reaction buffer (ABGene, Epsom, UK), 6 mM of each primer, 4 mM of $MgCl_2$, 1 × SYBR Green I (Molecular Probes, Eugene, OR) at final concentration, and 0.5 U of thermostart Taq DNA polymerase (ABGene). Amplification was carried out with the following cycling parameters: 600 s heat start at 95 °C, 45 cycles of denaturation at 95 °C for 25 s, annealing at 60 °C for 25 s, and fluorescence detection at 72 °C for 15 s. After amplification, a melting curve was created to verify the specificity of PCR reactions. Relative expression ratios were normalized to GAPDH, as a widely used housekeeping gene. The PCR primers used in this study are listed in Table 1.

2.6. Myocardial lactate dehydrogenase (LDH) and creatine kinase (CK) activities

In order to further investigate whether changes in cardiac gene expression due to differences in ex vivo perfusion time leads to alterations at the protein level enzyme activities of LDH and CK were determined from cardiac tissue. Ventricular tissue was homogenized as described previously (Gao et al., 2003). Both LDH and CK activities were determined by a spectrophotometric kinetic analysis at 340 nm using commercially available diagnostic kits (Diagnosticum Rt., Budapest, Hungary) according to the instructions of the manufacturer. Enzyme activities were normalized to the total protein content of the homogenates and expressed as mU/mg protein.

3. Results

3.1. Gene expression alteration due to perfusion

Using DNA microarray technology, we analyzed 8000 genes for changes in expression associated with the perfusion related to incubation time. Relative gene expression changes in mouse hearts in response to 60 and 120 min perfusion were compared to the expression profile of the control animals. Among the 8000 genes examined in the present study 39 genes showed altered expression. We selected 17 genes (Table 1) of which the expression were significantly altered in the heart due to 60 or 120 min perfusion for QRT-PCR analysis. Out of these, two genes exhibited significant up-regulation and 15 were down-regulated (Table 2).

Gene coding for Glucosaminyl-*N*-deacetylase/*N*-sulfotransferase 2 (Ndst2), responsible for HS *N*-sulfation was repressed after 60 and 120 min of perfusion.

Another gene related to glycosphingolipid biosynthesis was also repressed*: beta-1,4-N-acetyl-galactosaminyl transferase 1* with a 1.52 fold repression after 60 min of perfusion.

Perfusion altered the expression of three genes having a role in the regulation of cell cycle. *Casein kinase II* was over-expressed after 60 min, however, QRT-PCR analysis could not confirm this induction. After 120 min, changes were normalized to control level both in microarray and QRT-PCR.

Changes in the expression of other cell cycle regulatory genes showed repression, which was also confirmed by QRT-PCR. G two S phase expressed protein 1 (previously named as B99) had only shown down-regulation after 120 min of perfusion. The expression of Ketohexokinase (Khk) was significantly declined after 60 and also 120 min of perfusion.

A key enzyme of the glycolysis, Lactate dehydrogenase A-4 was down-regulated in response to perfusion.

Gene coding for long-chain acyl-CoA dehydrogenase (LCAD) was significantly down-regulated after 2 h of perfusion.

Table 1
Primers used in quantitative real-time polymerase chain reaction (QRT-PCR) analysis

Gene product	Accession number	Forward primer	Reverse primer
N-deacetylase/*N*-sulfotransferase	NM_010811	TGATGACAAGAGGCACAAAGA	AAGAAGTGAATAGCCGTGGTG
Beta-1,4-*N*-acetyl-galactosaminyl transferase 1	NM_008080	GCCAACACAGCAGACACAGT	GAGGATGGTGAAGGCAACTT
G two S phase expressed protein 1	NM_013882	TTCACCTGGCTACAGAGAAGC	TCACGAGTTTCGTCCTCTGA
Microtubule-associated protein 1	NM_007896	GTTCAGCCCCTGATGTGTTT	GGTCACAGGGAGCAGAGAGA
Ketohexokinase	NM_008439	CATCATCAATGTGGTGGACAA	TGCCATCTCTGGGACAGG
Integral membrane protein 2B	NM_008410	CTGAACACTTCCATCGTTATGC	GGTAGGACTGAGGCAGGTAGG
Casein kinase II, alpha 2	NM_009974	AAGTATAGTGAAGTATTTGAGGCCATT	TCGTTTTATCTTCTTTTTCTTCACTG
A disintegrin and metalloprotease domain 3	NM_009619	GAAAATCGACACCAACATCCA	CCCTCAATTGTGACCACGTA
Troponin T1	NM_011618	ATCTGTGGACCCAGCCTTAG	GCCTGCTCCTCCTCATATTCT
Acetyl-Coenzyme A dehydrogenase, long-chain	NM_007381	AAGTGATTCCTCACCACACAGA	CAGCTTTTTCCCAGACCTCTC
Integrin alpha 7	NM_008398	AAGTGCCATGCGATCTGAG	GACTGGCCTTGATTGGAGAC
Chloride ion current inducer protein	U53455	TGAACCCATTTCTGAATTTCG	GTGAACATCGCCTCCAATG
Protein phosphatase 1 inhibitor 14B	NM_008889	CTCACGCGACTCTACGACTG	CCATGTCTAAAAGTTCATCCACA
Interleukin 12 receptor, beta 1	NM_008353	CAGCCGAGTGATGTACAAGG	TAAACGGGAAATCTGCACCT
Creatine kinase, muscle	NM_007710	CAGCACAGACAGACACTCAGG	GAACTTGTTGTGGGTGTTGC
Voltage-dependent anion channel 1	NM_011694	ACCTTTGATTCGTCATTCTCG	TGCTCCCTCTTGTACCCTGT
Lactate dehydrogenase A-4	M17518	CTGCCAACTGTATGCAGTCTTT	ATGTTTGGTGAGGGTGTGC

To confirm the differential expression of genes of perfused mouse hearts revealed by microarray analysis we selected 17 genes for QRT-PCR analysis.

Table 2
Comparison of gene expression changes observed by microarray and quantitative real-time RT-PCR

Gene product	Accession number	Ratio (SD) chip 60′	Ratio (SD) QRT-PCR 60′	Confirmed by QRT-PCR	Ratio (SD) chip 120′	Ratio (SD) QRT-PCR 120′	Confirmed by QRT-PCR
N-deacetylase/*N*-sulfotransferase 2	NM_010811	−1.27 (0.54)	−1.69 (0.20)	Yes	−1.11 (0.43)	−1.25 (0.35)	Yes
Beta-1,4-*N*-acetyl-galactosaminyl transferase 1	NM_008080	−1.52 (0.37)	−1.8 (0.14)	Yes	−1.06 (0.26)	−2.17 (0.20)	Yes
G two S phase expressed protein 1	NM_013882	−1.25 (0.33)	−1.44 (0.29)	Yes	+0.36 (0.33)	−1.80 (0.19)	No
Microtubule-associated protein, RP/EB family, member 1	NM_007896	−1.24 (2.16)	−2.01 (0.25)	Yes	−2.11 (1.32)	−1.87 (0.37)	Yes
Ketohexokinase	NM_008439	−1.24 (0.77)	−2.28 (0.84)	Yes	−0.56 (0.87)	−2.03 (0.50)	Yes
Integral membrane protein 2B	NM_008410	−1.24 (1.24)	−2.02 (0.55)	Yes	−0.70 (1.40)	−1.25 (0.23)	Yes
Casein kinase II, alpha 2, polypeptide	NM_009974	+1.08 (0.29)	−2.84 (0.36)	No	+0.14 (0.31)	−3.38 (0.32)	No
Disintegrin and metalloprotease domain 3 (cyritestin)	NM_009619	+1.19 (0.65)	−0.83 (0.09)	No	+1.37 (0.41)	−1.03 (0.17)	No
Troponin T1, skeletal, slow	NM_011618	−1.08 (0.75)	−1.30 (0.43)	Yes	−1.60 (0.54)	−2.44 (0.48)	Yes
Acetyl-Coenzyme A dehydrogenase, long-chain	NM_007381	−0.72 (0.70)	−3.25 (1.52)	Yes	−1.54 (0.62)	−4.03 (1.73)	Yes
Integrin alpha 7	NM_008398	−0.26 (0.87)	+4.06 (1.37)	No	−1.33 (0.97)	−2.82 (1.24)	Yes
Chloride ion current inducer protein	U53455	−1.39 (1.32)	−5.42 (0.83)	Yes	−1.27 (2.20)	−6.04 (1.21)	Yes
Protein phosphatase 1, regulatory (inhibitor) subunit 14B	NM_008889	−0.12 (0.41)	−0.51 (0.23)	No	−1.06 (1.14)	−1.32 (0.24)	Yes
Interleukin 12 receptor, beta 1	NM_008353	−3.20 (3.48)	−1.26 (0.32)	Yes	−0.72 (1.46)	−1.57 (0.29)	Yes
Creatine kinase, muscle	NM_007710	−1.51 (0.61)	−1.47 (0.24)	Yes	−1.15 (0.54)	−1.95 (0.02)	Yes
Voltage-dependent anion channel 1	NM_011694	−1.43 (0.38)	−2.61 (n.d.)	Yes	−2.66 (0.50)	−2.01 (n.d.)	Yes
Lactate dehydrogenase A-4	M17518	−0.63 (0.32)	−0.81 (n.d.)	Yes	−0.92 (0.75)	−1.28 (n.d.)	Yes

Relative gene expression changes in mouse hearts in response to 60 and 120 min perfusion were compared to the expression profile of the control animals by microarray and quantitative real-time polymerase chain reaction (QRT-PCR) analysis. Two genes exhibited significant up-regulation and 15 were down-regulated.

Decreased transcription activity of gene for Chloride ion current inducer protein was detected after 60 and 120 min of normoxic perfusion.

Genes, encoding two apoptotic proteins presented significant repression in response to perfusion: Integral membrane protein 2B and Voltage-dependent anion channel 1 (Vdac1). As the microarray and the QRT-PCR results evidenced, repression is more pronounced over the increase of perfusion time.

Perfusion repressed several genes including some signal transduction genes. One of them, the *integrin alpha 7* didn't show altered expression after 60 min of perfusion, but after 120 min, it was strongly repressed. The *interleukin 12 receptor beta-1 subunit* gene was also strongly attenuated at the mRNA level in comparison to the control group even after 60 min perfusion.

A disintegrin and metalloprotease domain 3 (adam3) had a very significant rise in transcription rate. Protein phosphatase 1 regulatory subunit 14B became repressed only after 120 min of perfusion.

Two genes have shown altered expression among the muscle contraction regulators. Our results show that 60 and 120 min of perfusion also induced repression of Creatine kinase muscle (Ckmm). The repression rate of Troponin T1, skeletal (Tnnt1) grows with the increase of perfusion time.

3.2. Expression profiling of genes related to ischemic adaptation

Measurement of expression at mRNA level of an additional 19 genes strongly related to cardiac adaptation to ischemia-reperfusion but not available on the microarrays was also investigated by QRT-PCR (Table 3).

Superoxide dismutase (SOD) enzyme catalyzes the reduction of superoxide anions to hydrogen peroxide, as such, it is an important antioxidant. Three superoxide dismutases are characterized by different metal content. According to our results

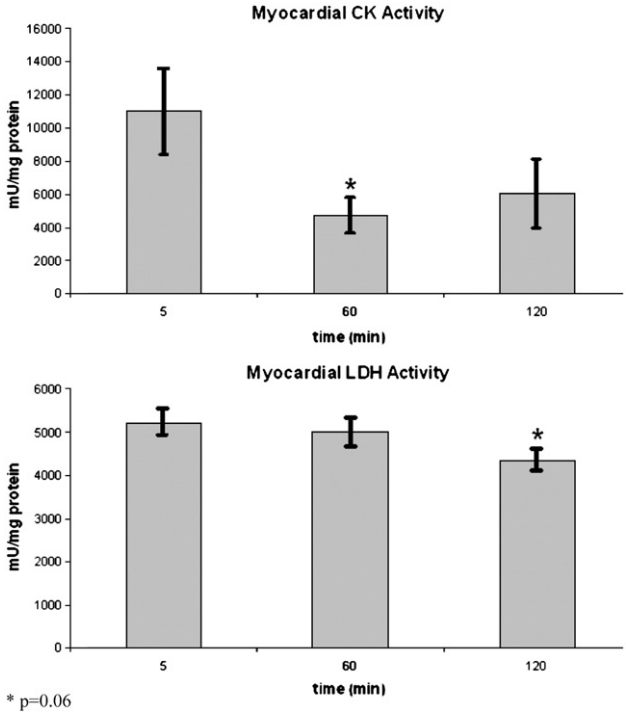

Fig. 1. CK and LDH enzymatic activity measurements. According to the microarray and QRT-PCR analysis, *creatine kinase* (ck) and *lactate dehydrogenase* (ldh) genes are being repressed after 60 and 120 min of perfusion. CK and LDH enzymatic activities in the heart extracts were determined to detect decline in gene expression of genes coding for these enzymes. * $p=0.06$.

Table 3
Measurement of expression ratio at mRNA level by QRT-PCR

Gene product	Accession number	Forward primer	Reverse primer	Ratio (SD) 60′	Ratio (SD) 120′
Superoxide dismutase-1	NM_011434	TTTTTTTGCGCGGTCCTTT	ACCAGAGAGAGCAAGACGAGAAG	1.00 (0.79)	1.07 (0.93)
Superoxide dismutase-2	NM_013671	TTAACGCGCAGATCATGCA	GGTGGCGTTGAGATTGTTCA	1.05 (1.77)	1.15 (0.81)
Superoxide dismutase-3	NM_011435	CATGCAATCTGCAGGGTACAA	CGGTGATCTGCGGCTGAT	0.84 (1.42)	1.27 (1.17)
NADPH oxidase-1	NM_172203	CCCAGCAGAAGGTCGTGATT	CCTCATCTGCAATTCCAAAACA	1.87 (1.13)	3.15 (1.68)
NADPH oxidase-4	BC021378	GATTTCTGGACCTTTGTGCCTTT	TGATGGTGACAGGTTTGTTGCT	0.84 (0.26)	0.31 (0.27)
Nitric oxide synthase 1	NM_008712	CACCCACCAAAGCTGTCGAT	CTGCTAATGGCTGGTCTTTGC	1.04 (0.65)	0.66 (4.17)
Nitric oxide synthase 2	NM_010927	GGCAGCCTGTGAGACCTTTG	CATTGGAAGTGAAGCGTTTCG	0.25 (0.57)	0.18 (0.28)
Matrix metalloproteinase-2	NM_008610	CCCATGAAGCCTTGTTTACCA	TGGAAGCGGAACGGAAACT	1.20 (0.24)	1.61 (0.32)
Tissue inhibitor of metalloproteinase 1	NM_011593	GCAAAGAGCTTTCTCAAAGACC	AAGGGATAGATAAACAGGGAAACA	1.75 (0.55)	1.86 (0.88)
Tissue inhibitor of metalloproteinase 2	NM_011594	CGTTTTGCAATGCAGACGTA	GGAATCCACCTCCTTCTCG	0.70 (0.35)	0.52 (0.10)
Tissue inhibitor of metalloproteinase 3	BC014713	GCCTCAAGCTAGAAGTCAACAAA	TGTACATCTTGCCTTCATACACG	0.79 (1.12)	1.02 (0.78)
Tissue inhibitor of metalloproteinase 4	NM_080639	AGGGAGAGCCTGAATCATCA	GCACTGCATAGCAAGTGGTG	0.97 (1.04)	0.33 (0.93)
HMG-CoA reductase	NM_008255	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAGTTT	1.18 (0.19)	1.19 (0.25)
Farnesyl diphosphate farnesyl transferase 1	BC054722	TGAACTCATAACCAACACCCTACAG	TGGTTCCGGAGCCTTGAC	1.07 (1.26)	1.07 (0.89)
Heat shock protein 25 kDa 2	AK030025	AGCTCACAGTGAAGACCAAGG	CATGTTCGTCCTGCCTTTCT	1.38 (1.11)	1.20 (0.80)
NADH dehydrogenase	BC081434	GCTTGCTTCGGTACAAAACG	GGGGTGTATTTGCCCTCTTT	0.37 (0.45)	0.89 (0.45)
Geranyl geranyl transferase	BC012214	TGCTAAATGGCCAGACAGTCAT	TGAGTGACAGGCCGCAGAT	1.18 (0.77)	1.39 (1.10)
Low density lipoprotein	NM_010700	GATGGCTATACCTACCCCTCAA	TGCTCATGCCACATCGTTC	1.11 (1.02)	1.14 (0.72)
Matrix metalloproteinase-9	NM_013599	ACGACATAGACGCCATCCA	GCTGTGGTTCAGTTGTGGTTG	1.20 (0.26)	1.55 (0.51)

The expression of an additional 19 genes strongly related to ischemia-reperfusion but that were not available on the microarrays was also investigated by QRT-PCR (in normoxic perfused mouse hearts related to control).

each types of *sod* represents over-expression after 60 and 120 min of perfusion.

The NADPH oxidase is a multi-subunit enzyme that catalyzes the reduction of molecular oxygen to form superoxide. According to our results, the over-expression of NOX enzymes (and in this way the amount of reactive oxygen species) increases directly proportional to perfusion time.

We observed increasing activity in case of two matrix metalloproteinase genes, *mmp2* and *mmp9*. Genes coding for Matrix metalloproteinases 2 and 9 (MMP2 and MMP9) were overexpressed due to perfusion in both groups.

We have also detected increasing expression of *low density lipoprotein* gene after 60 and 120 min.

Among the four *tissue inhibitor of metalloproteinase* (*timp*) genes investigated in our study, only *inhibitor of metalloproteinase-1* showed altered expression. We observed about 1.80 fold over-expression after 60 and 120 min of perfusion.

The Nitric oxide synthases (NOS) are a group of enzymes responsible for the synthesis of nitric oxide (NO) from the terminal nitrogen atom of L-arginine in the presence of O_2 and certain cofactors. The nitric oxide molecule is a free radical, that makes it very reactive and unstable and in high concentration it has cytotoxic effects. We have shown that perfusion inhibits expression of cardiac *nitric oxide synthases 2* (*nos2*).

Our results show that 60 and 120 min of perfusion induced over-expression of gene for 25-kDa Heat shock protein (HSP25).

Geranyl geranyl transferase, HMG-CoA reductase, farnesyl diphosphate farnesyl transferase 1 and NADH dehydrogenase have been studied previously in response to ischemia-reperfusion. One of them, the NADH dehydrogenase didn't show altered expression after perfusion. At the other three genes we confirmed up-regulation of transcription due to perfusion.

3.3. Enzymatic activities

CK and LDH activities in the heart extracts ($n=4$ in each group) were also determined as we could detect decline in gene expression of genes coding for these enzymes. According to the microarray analysis and QRT-PCR results, *ck* and *ldh* genes are being repressed after 60 and 120 min of perfusion and this rate of repression is more pronounced over the increase of perfusion time. The enzyme activity measurements (results are shown in Fig. 1) showed that creatine kinase and lactate dehydrogenase levels were also decreased due to perfusion.

Hemodynamic parameters, like coronary flow and heart rate were also measured. None of these parameters changed in the perfused groups (Table 5) when compared to controls.

4. Discussion

4.1. Gene expression alteration due to perfusion

Perfusion leads to extensive changes in cardiac expression of genes from various functional clusters (Table 4). As shown in Table 5, heart rate and coronary flow were not changed in the perfused groups.

Our present study is the first demonstration in connection with the effects of different long periods of perfusion time in mouse hearts. The role of most genes found to be regulated by

Table 4
Functional classification of differentially expressed genes

Functional cluster	Gene product	Accession number
Oxidative and nitrosative stress	Superoxide dismutase-1	NM_011434
	Superoxide dismutase-2	NM_013671
	Superoxide dismutase-3	NM_011435
	NADPH oxidase-1	NM_172203
	NADPH oxidase-4	BC021378
	Nitric oxide synthase 1	NM_008712
	Nitric oxide synthase 2	NM_010927
Heat shock proteins	Heat shock protein 25 kDa 2	AK030025
Energy metabolism	Creatine kinase, muscle	NM_007710
	NADH dehydrogenase	BC081434
	Lactate dehydrogenase A-4	M17518
	Acetyl-Coenzyme A dehydrogenase, long-chain	NM_007381
Cholesterol synthesis, transport	HMG-CoA reductase	NM_008255
	Low density lipoprotein	NM_010700
	Farnesyl diphosphate farnesyl transferase 1	BC054722
	Geranyl geranyl transferase	BC012214
Muscle contraction	Troponin T1, skeletal, slow	NM_011618
Ion channels, receptors, membrane proteins	Voltage-dependent anion channel 1	NM_011694
	Chloride ion current inducer protein	U53455
	Interleukin 12 receptor, beta 1	NM_008353
	Integral membrane protein 2B	NM_008410
	Integrin alpha 7	NM_008398
Regulation of cell cycle	G two S phase expressed protein 1 (Gtse 1)	NM_013882
	Microtubule-associated protein, RP/EB family, member 1	NM_007896
	Casein kinase II, alpha 2, polypeptide	NM_009974
Metabolic enzymes	*N*-deacetylase/*N*-sulfotransferase 2	NM_010811
	Beta-1,4-*N*-acetyl-galactosaminyl ransferase 1 (Galgt1)	NM_008080
	Ketohexokinase (KhK)	NM_008439
Protein phosphorylation	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	NM_008889
Proteolysis and peptidolysis	Matrix metalloproteinase-2	NM_008610
	Matrix metalloproteinase-9	NM_013599
	Disintegrin and metalloprotease domain 3 (cyritestin)	NM_009619
	Tissue inhibitor of metalloproteinase 1	NM_011593
	Tissue inhibitor of metalloproteinase 2	NM_011594
	Tissue inhibitor of metalloproteinase 3	BC014713
	Tissue inhibitor of metalloproteinase 4	NM_080639

According to the microarray and quantitative real-time polymerase chain reaction (QRT-PCR) analysis 36 genes showed altered expression after 60 and 120 min of perfusion of mouse hearts.

perfusion is not exactly known in the heart, therefore, our present findings may open new directions in research of the cardiac effects of perfusion.

Enzymatic activity measurements proved that perfusion decreased significantly the LDH and CK activity correlating to the non-perfused control group and that was confirmed by microarray results.

Heparan sulfate (HS) proteoglycans influence embryonic development through interactions with growth factors and morphogens. Interactions depend on HS structure that is largely determined by biosynthesis in Golgi (Holmborn et al., 2004).

Glucosaminyl-*N*-deacetylase/*N*-sulfotransferase 2 (Ndst2), responsible for HS *N*-sulfation, is the key enzyme directing further modifications including *O*-sulfation. This gene product hasn't been correlated with ischemia or perfusion yet (Carter, Ali, & Kirby, 2003).

Galgt1 is an important determinant of plasma VWF (von Willebrand factor) levels in mouse (Ginsburg, 2005; Mohlke et al., 1999).

Changes in the expression of other cell cycle regulatory genes showed down-regulation, which were also confirmed by QRT-PCR. Some studies have already demonstrated that the gene, encoding Microtubule-associated protein 1 shows altered expression in response to the developement of ischemia in the rat retina (Mastrodimou, Lambrou, & Thermos, 2005) and in response to autophagy in chronically ischemic myocardium (Yan et al., 2005). According to our results, the repression of Mtap1 increases directly proportional to time of normoxic perfusion.

Gtse1 (previously named B99) showed down-regulation after only 120 min of perfusion. This is a wt-p53 inducible gene that encodes a microtubule-localized protein which is able to induce G(2)/M phase accumulation when ectopically expressed. The function of Gtse1 in the myocardium is presently unknown.

Ketohexokinase 1 catalyses the phosphorylation of fructose to fructose-l-phosphate. *Khk* expression had a significant decline after 60 and also 120 min of perfusion. This enzyme is expressed predominantly in the liver, to a lesser extent in the kidney, and very little in heart, brain and muscle (Bais, James, Rofe, & Conyers, 1985).

A key enzyme of the glycolysis, lactate dehydrogenase A-4 is a well-established marker of cardiac damage. Karaca studied the expression alteration of *lactate dehydrogenase* gene, induced by ischemia-reperfusion. They previously proved that the expression of *ldh* gene rises in response to ischemia (Karaca et al., 2006; Xu et al., 2006), but at the same time our results have shown that in response to normoxic perfusion LDH was down-

Table 5
Cardiac functional parameters in control and perfused groups

	5′ (mean)	5′ (SEM)	60′ (mean)	60′ (SEM)	120′ (mean)	120′ (SEM)
Heart rate (beats/min)	437	23	443	20	424	11
Coronary flow (ml/min)	3.3	0.3	3.3	0.2	3.2	0.2
Heart weight (mg)	163	6	163.5	6	149.8	9

Hemodynamic parameters, like coronary flow and heart rate were also measured. None of these parameters changed in the perfused groups when compared to controls.

regulated in the mouse heart. Acyl-CoA dehydrogenases (ACADs) are a family of mitochondrial enzymes catalyzing the initial rate-limiting step in the beta-oxidation of fatty acyl-CoA (Thorpe & Kim, 1995). This reaction provides the main source of energy for human heart and skeletal muscle. Deficiency of the long-chain Acyl-CoA dehydrogenase (LCAD), usually leads to human organic diseases, such as sudden death in infancy, cardiomyopathy and hypoketotic hypoglycemia (Zhang et al., 2002). In our study we have detected a significant fall in LCAD transcription rate, but only after 2 h of perfusion.

Decreased mRNA levels of chloride ion current inducer were detected after 60 and 120 min of normoxic perfusion. This gene has already been studied in rat brain and myocardium, but the gene function only described in ocular ciliary epithelium (Anguita, Chalfant, Civan, Coca-Prados , 1995; Wan, Chen, & Sears, 1997).

Genes, coding for two apoptotic proteins presented significant repression in response to perfusion. One of them, the gene, encoding Integral membrane protein 2B is supposedly connected with the development of dementia (Wolfe et al., 1999). The other apoptotic gene, *voltage-dependent anion channel1* (Vdac1) exhibited one of the most pronounced repressions due to perfusion. Previously Anflous, Armstrong, and Craigen (2001) proved that Vdac1 was required for creatine stimulation of mitochondrial respiration in oxidative muscles. As the microarray and the QRT-PCR results evidenced, repression rate of Vdac1 increases directly proportional to the increase in perfusion time.

Perfusion repressed several genes including some signal transduction genes. One of them, the *integrin alpha 7* didn't show altered expression after 60 min of perfusion, but after 120 min it was strongly repressed. Integrin alpha 7 is a specific cellular receptor for the basement membrane protein Laminin-1, as well as for the Laminin isoforms 2 and 4. The alpha 7 subunit is expressed mainly in skeletal and cardiac muscle and has been suggested to be involved in differentiation and migration processes during myogenesis. Mayer et al. (1997) studied the involvement of Integrin alpha 7, during myogenesis and its role in muscle integrity and function. They proved that absence of this protein causes muscular dystrophy.

The *interleukin 12 receptor beta-1* gene also represents strongly attenuated function correlated to the control in both groups. Myocardial function of that is not known yet, but recently Kutukculer et al. (2006) associated the interleukin-12 receptor beta-1 deficiency with cutaneous leukocytoclastic vasculitis.

The gene coding A disintegrin and metalloprotease domain 3 (Adam3) had a very significant rise in transcription rate. This gene may play a role in cell–cell and cell–matrix interactions during spermatogenesis. The myocardial role of Adam3 is not known so far, but Adam10, Adam15 and Adam19 from the same gene family demonstrably show altered expression during cardiomyopathy (Fedak, Moravec, & McCarthy, 2006; Kurohara, Komatsu, Kurisaki, & Masuda, 2004).

Protein phosphatase 1 regulatory subunit 14B became repressed only after 120 min of perfusion. Ppp1r14b provides a regulator function by controlling protein phosphatases.

Two genes have shown altered expression among the muscle contraction regulators. Our results showed that 60 and 120 min of perfusion also induce repression of *creatine kinase muscle* (ckmm). *Ckmm* is expressed by various tissue types. Its function is the catalysis of the conversion of creatine to phosphocreatine, consuming ATP and generating ADP. Andres, Sharma, and Sassen (1993) realised that this key enzyme of intracellular energy transduction is altered in porcine myocardium subjected to repeated brief periods of ischemia, but they haven't noticed any changes in *ckmm* gene expression during perfusion.

The repression rate of *troponin T1 skeletal* (*tnnt1*) rose with the increase of perfusion time in our present study. Tnnt1 can be found principally in skeletal muscle. *Troponin T2* with similar structure and functions has previously represented altered expression in response to ischemia-reperfusion and it is suitable to detect myocardial injuries (Sobki, Saadeddin, & Habbab, 2000).

4.2. Expression profiling of genes related to ischemic adaptation

Expression rate at mRNA level of an additional 19 genes (Table 3) strongly related to cardiac adaptation to ischemia-reperfusion but not available on the microarrays was also investigated by QRT-PCR (Table 3).

During reperfusion, when blood supply returns to the tissue after a period of ischemia, the absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage from the oxygen rather than restoration of normal function. This oxidation results that molecular oxygen being converted into highly reactive superoxide and hydroxyl radicals. The aim of our study was to identify those genes that changed only due to perfusion.

Superoxide dismutase (SOD) catalyzes the reduction of superoxide anions to hydrogen peroxide and, as such, it is an important antioxidant. SOD is an intracellular enzyme present essentially in every cell of the body. Three SOD isoenzymes are known characterized by different metal content (Ferdinandy & Schulz, 2003). According to our results each type of SOD shows over-expression after 60 and 120 min of perfusion. Yoshida, Maulik, Engelman, and Ho (2000) previously proved that SOD I (−/−) mouse hearts are more susceptible to ischemic reperfusion injury compared with corresponding wild-type mouse hearts, suggesting that the *sod1* gene constitutes an important defense element for the hearts.

NADPH oxidase is a multi-subunit enzyme that catalyzes the reduction of molecular oxygen to superoxide. Superoxide (and associated reactive oxygen species, ROS) generated by NADPH oxidase in non-phagocytic cells serves several functions in health and disease (Cave et al., 2006). A major concept in redox signaling is that while NADPH oxidase-derived ROSs are necessary for normal cellular function, excessive oxidative stress can contribute to pathological disease (Infanger, Sharma, & Davisson, 2006). According to our results, the over-expression of NOX enzymes (and by this way the amount of reactive oxygen species) increase directly proportional to perfusion time.

Matrix metalloproteinases (MMPs) are Zn-dependent endopeptidases. Collectively they are capable of degrading many extracellular matrix proteins, but also can process a number of bioactive molecules (Chow, Cena, & Schulz, 2007). They are known to be involved in the cleavage of cell surface receptors, release of apoptotic ligands and chemokine activation. MMPs are also thought to play a major role on other cell behaviors such as cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defense. We observed increased mRNS levels in case of two matrix metalloproteinase genes; *mmp2* and *mmp9*. Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) are increased in the rat brain after experimental ischemic stroke and they also contribute to myocardial remodeling after myocardial infarction (Machado et al., 2006; Mori, Gibson, & McTiernan, 2006) as well as in the development of acute infarction (Giricz et al., 2006). In our study these genes exhibited almost the most pronounced over-expressions due to perfusion.

We also detected increasing gene expression at the gene for low density lipoprotein. LDL cholesterol is the so called "bad" cholesterol, which carries mostly fat and only a small amount of protein from the liver to other cell types of the body. A high LDL cholesterol level is considered a risk factor for coronary artery disease because, under certain conditions, it leads to atherosclerosis and interferes with cardioprotective mechanisms (Ferdinandy, Schulz, & Baxter, 2007). Oxidative modification of lipoproteins appears to play an important role in the atherogenic process. oxLDL is involved in inducing smooth muscle cell migration and proliferation, and are avidly ingested by macrophages, resulting in foam cell formation (Mertens & Holvoet, 2002). Our results suggest that the expression of LDL rises during perfusion and due to the perfusion-formed radicals LDL changes into its oxidating form (oxLDL). *Geranyl geranyl transferase*, *hmg-CoA reductase*, *farnesyl diphosphate farnesyl transferase 1* genes involved in cholesterol biosynthesis exhibited up-regulation due to perfusion.

Tissue inhibitor of metalloproteinase (TIMP) enzymes play an important role in histogenesis and organogenesis (Werb & Chin, 1998). Matrix metalloproteinases are proteolytic enzymes which degrade extracellular matrix and basement membrane. Tissue inhibitor of metalloproteinases are the specific inhibitors of MMPs. Circulating levels of MMPs and TIMPs could reflect the atherosclerotic process occurring within the arterial wall and have been identified in normal and failing myocardium (Spinale, 2002). Recent studies suggest that MMPs and TIMPs play a role in various cardiovascular diseases including atherosclerosis and ventricular remodeling observed in heart failure (Mori et al., 2006).

The nitric oxide synthases (NOS) are a group of enzymes responsible for the synthesis of nitric oxide (NO) from the terminal nitrogen atom of L-arginine in the presence of O_2 and further cofactors. Nitric oxide plays role in many physiological function and pathological states of the heart (Ferdinandy & Schulz, 2003). We have shown that perfusion inhibits expression of cardiac *nos2*, but activates the expression of *nos1*.

Heat shock proteins are interesting new target for cardioprotection (Sőti et al., 2005) The 25-kDa heat-shock protein (Hsp25) is expressed in the cartilage of the growth plate and suggested to function in chondrocyte differentiation and degeneration. It has demonstrably have cardioprotective effect, but the detailed mechanism is not known yet (Chiu & Ko, 2004). Our present results show that 60 and 120 min of perfusion induce over-expression of *hsp25*.

5. Limitations of the study

The stability of the preparation upon long-term perfusion is a general concern in isolated heart preparations. Here we used a Langendorff preparation with no left ventricular balloon to unload the heart from 'afterload pressure' and to maintain a good coronary perfusion throughout the perfusion protocol. As shown in Table 5, heart rate and coronary flow were stable which show the stable aerobic condition of the heart. As the present study was performed in crystalloid-perfused isolated mouse hearts and the analysis of gene expression was done using cardiac tissue that did not contain components of blood, cellular regulatory pathways might be somewhat different in the present ex vivo experimental model as compared to in vivo situations. Perfusion with blood might solve this issue, however, blood perfusion can be a problem in itself (Clements-Jewery et al., 2002). In our present studies, to avoid spontaneous occurrence of arrhythmias, we used a perfusion buffer containing high potassium. Since the gene expression pattern in mice hearts were measured first at 60 min, the results might not be relevant for e.g. studies on ischemia-induced arrhythmias which need shorter perfusion time (Curtis, 1998). It is of interest, however, that in isolated hearts, phase 2 ischaemic arrhythmias are not observed (Ravingerová, Tribulová, Slevák, & Curtis, 1995). This could be due to the extensive changes in gene expression observed in the present study. It should be noted that the gene expression changes observed here may be specific for isolated mice heart perfusion and the results have less relevance in heart perfusion preparations in other species.

6. Conclusions

Our results show that normoxic crystalloid perfusion itself results in time-dependent changes in cardiac gene expression. As many of these genes with altered expression have been suspected to influence several cardiovascular pathologies, this should be considered when designing ex vivo perfusion protocols in the mouse heart to study cardiac diseases.

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The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and was approved by specific national laws (e. g. the current version of the German Law on the Protection of Animals) and local ethics committees.

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