Original Research

Ischemic postconditioning alters the gene expression pattern of the ischemic heart

Csaba Csonka[1,2], Gergő Szűcs[1], Zoltán Varga-Orvos[3], Péter Bencsik[2], Tamás Csont[1,2], Ágnes Zvara[3], László G Puskás[3] and Péter Ferdinandy[2,4]

[1]Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Szeged, H-6720 Hungary; [2]Pharmahungary Group, Szeged, H-6723 Hungary; [3]Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, H-6726 Hungary; [4]Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, H-1089 Hungary
Corresponding author: Csaba Csonka. Email: csonka.csaba@med.u-szeged.hu

Abstract

To profile changes in gene expression in response to ischemic postconditioning, isolated rat hearts were subjected to 30 min of regional ischemia followed by 120 min of reperfusion with or without postconditioning. At the end of reperfusion, cardiac RNA was assayed by DNA microarrays (31,000 format), verified by quantitative real-time polymerase chain reaction (QRT-PCR). Postconditioning significantly up-regulated 50 genes and down-regulated 58 different genes, including pyruvate dehydrogenase, 60 kDa heat shock protein 1, lipoprotein lipase, gamma-sarcoglycan, and phospholipase C. Gene ontology analysis revealed that most of the altered genes belong to the cellular metabolic processes cluster. Many of the genes have not previously been suspected to be involved in the mechanism of postconditioning.

Keywords: Postconditioning, microarray, gene ontology, heart, rat

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Introduction

Ischemic heart disease is the leading cause of death worldwide; therefore, therapeutic strategies to protect the ischemic myocardium have been extensively studied.[1,2] Ischemic postconditioning, as first described by Na et al.,[3] is a clinically applicable maneuver of intermittent brief periods of reperfusion that is able to provide a profound cardioprotective effect.[2,4]

The cellular mechanisms of ischemic postconditioning have been extensively investigated; however, the exact biochemical mechanism of postconditioning is still a question of debate.[1,2,5] In recent years, attention has focused on six potential mechanisms of postconditioning: the involvement of endogenous adenosine and adenosine receptor subtypes; the NO/cGMP/PKG pathway; mitoKATP channels; reperfusion injury salvage kinase pathway (RISK, i.e. PI3K/Akt, p42/p44 MAPK/ERK, PKG); survivor activating factor enhancement pathway (SAFE, i.e. tumor necrosis factor alpha (TNFα) receptor, STAT3) pathway; and inhibition of mitochondrial permeability transition pore (mPTP) opening at reperfusion.[1,6] Due to the complexity of the possible cellular mechanisms involved, apart from the traditional biochemical and pharmacological techniques, a system's biology approach including genomics may provide new potential to investigate the key cellular events in postconditioning. We have previously shown that preconditioning, another form of endogenous cardioprotection, significantly altered the cardiac gene expression pattern as assessed by DNA microarrays and quantitative real-time polymerase chain reaction (QRT-PCR).[7]

Therefore, we monitored gene expression changes by DNA microarrays and QRT-PCR to profile gene expression patterns in rat hearts in response to postconditioning, followed by gene ontology (GO) analysis in the hope of identifying possible new cellular mechanisms involved in postconditioning.

Materials and methods

Policy and ethics

The investigation conforms to the EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm) and to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). The manuscript conforms to the Uniform Requirements for manuscripts submitted to biomedical journals (http://www.icmje.org).

Perfusion protocol of isolated rat hearts

Male Wistar rats (300–350 g) were anesthetized and given 500 U/kg heparin to prevent coagulation. Their hearts were then isolated and perfused in Langendorff mode at a constant pressure with an oxygenated, normothermic Krebs–Henseleit buffer as previously described.[8] After 10 min of equilibration, the hearts were subjected to 30 min of regional no-flow ischemia induced by coronary occlusion followed by 120 min of reperfusion with either a postconditioning or a non-postconditioning protocol ($n = 6$ in both groups). Postconditioning was induced by six intermittent cycles of 10 s of ischemia, separated by 10 s of aerobic perfusion at the onset of the reperfusion period. At the end of the perfusion protocols, hearts from all of the groups were frozen and powdered with a pestle and mortar in liquid nitrogen. For microarray analysis, three pools were generated from both groups of hearts (2 hearts/pool).

In separate experiments, infarct size was determined at the end of the 120-min reperfusion after the 30-min regional ischemia[9] with or without the postconditioning protocol ($n = 8$ in both groups).

RNA preparation, amplification, and labeling

Total RNA was purified from each sample (80 mg of rat heart tissue) as previously described.[10] One microgram of the total RNA was first reverse transcribed in a 10 μL volume using the Oligo(dT) Primer and ArrayScript enzyme, the second complementary DNA (cDNA) strand was then synthesized in a 50 μL final volume using DNA Polymerase and RNase H. Amino allyl modified aRNA were then synthesized by *in vitro* transcription using an aaUTP and T7 enzyme mix. All of these steps were done using the AminoAllyl MessageAmp™ II aRNA Amplification Kit (Ambion, USA), according to manufacturer's instructions. Six micrograms of amino allyl modified amplified RNA (aaRNA) were labeled with either Cy5 or Cy3 dyes in a 10 μL volume according to the manufacturer's instructions (Ambion, USA), and were then purified using RNA purification columns from Macherey Nagel (Düren, Germany).

Hybridization and data analysis

Rat oligonucleotide microarrays of the 31 k format from Microarray Inc. (Nashville, TN, USA) were used to determine the changes in gene expression. Eight-hundred nanograms of Cy5 and Cy3 labeled aaRNA in 190 μL, 50 μL 10 × blocking agent, and 10 μL 25 × fragmentation buffer were mixed together and incubated at 60°C for 30 min. A total of 250 μL of 2 × GEx hybridization buffer was then added to each sample to stop the fragmentation reaction. All these steps were done using a gene expression hybridization kit (Agilent Technologies, Palo Alto, CA, USA). Four hundred ninety microliters of these mixes were used for the hybridization, which was done in microarray hybridization chambers (Agilent, USA). The chambers were then loaded into a hybridization rotator rack ($\sim$5 rpm) and incubated at 65°C for 17 h. After hybridization, the slides were washed in Wash buffer 1 from Agilent Technologies at room temperature for 1 min, and then in Wash buffer 2 at 37°C for another 1 min before scanning. Each array was scanned at 543 nm (for Cy3 labeling) or at 633 nm (for Cy5 labeling) in an Agilent Scanner at 100% laser power and 100% PMT with 10 μm resolution. Output image analysis and feature extraction were performed using GenePix 6.0 software. The mean pixel intensities were calculated at both wavelengths, and pixel intensity ratios corrected for local background were determined for each spot.

Quantitative real-time PCR

After DNA chip analysis, QRT-PCR was performed to validate the result of the DNA chip. Genes were selected for QRT-PCR validation by an arbitrary manner according to direction and magnitude of changes, representation of different functional clusters, as well as for the sake of our further research interest. QRT-PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers, and either SybrGreen protocol or Universal Probe Library dual-labeled probes from Roche (Roche Applied Science, Mannheim, Germany) were used to monitor gene expression.

Two micrograms of total RNA from each sample were reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions in a final volume of 30 μL. The temperature profile of the reverse transcription was as follows: 10 min at room temperature, 2 h at 37°C, 5 min on ice, and finally 10 min at 75°C for enzyme inactivation. These steps were carried out in a Thermal Cycler machine (MJ Research Waltham, MA, USA). After dilution with 30 μL of water, 1 μL of the diluted reaction mix was used as a template in the QRT-PCR. FastStart SybrGreen Master or FastStart TaqMan Master mixes (Roche Applied Science, Mannheim, Germany) were used for the QPCR reactions according to the manufacturer's instructions. The final primer concentration was 250 mmol/L under the following conditions: 10 min at 95°C and 45 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 20 s (SybrGreen intensity was detected after each cycle) or 10 min at 95°C and 45 cycles of 95°C for 15 s, and 60°C for 1 min (fluorescein dye (FAM) intensity was detected after each cycle.)

Primers were designed using the online Roche Universal Probe Library Assay Design Center. The PCR primers used in this study are listed in Table 1. The quality of the primers was verified by MS analysis provided by Bioneer (Daejeon, Korea). The relative expression ratios were calculated as normalized ratios to both rat hypoxanthine-guanine phosphoribosyltransferase (HPRT) and actin genes. A non-template control sample was used for each PCR run to check for genomic DNA contaminations of the cDNA template and for primer–dimer formation. The final relative gene expression ratios were calculated as delta-delta Ct values.

Gene ontology

To get overall functional information of the differentially expressed gene sets, GO analysis was performed using GO/pathway analysis using the DAVID bioinformatics system open access software and database (Database for Annotation, Visualization and Integrated Discovery,

Table 1 List of QRT-PCR primers

Gene name	Gene symbol	Genebank acc. No.	Forward primer	Reverse primer
3-Oxoacid CoA transferase 1	Oxct1	NM_024188	cgggtgaactagaagtagagctg	acctgcacggatcctctct
60 kDa heat shock protein, mitochondrial	Hspd1	X54793	ttcaagaaatcactgagcagcta	catcacttgtccctccaacc
Choline phosphotransferase 1	Chpt1	NM_001007750	cctacggtcacggaggag	tttccgtcaatggcatcc
Destrin	Dstn	NM_019771	atcttgcaaagcgccaga	tcttgcggcaggtacagg
Gamma-sarcoglycan	Sgcg	NM_001006993	ttccgctgtacgccaaag	tctgagttgactgcagaagca
Heme oxygenase 1	Hmox1	NM_012580	gtcaggtgtccagggaagg	ctcttccagggccgtataga
Karyopherin alpha 2	Kpna2	NM_053483	aggaaaaccgaaacaaccag	agtttccgagcagcttgagt
Kv channel-interacting protein 2	Kcnip2	NM_020094	tgccctcagtcagtgaaaca	attcatcctccacgctgtct
Leiomodin 2	Lmod2	NM_053098	tgacagagaaaaccccaacc	tttcccagtacgccatcag
Lipoprotein lipase	Lpl	NM_012598	atgatgtggccaggttcatc	gggctccaagactgtacccta
Nitrogen fixation gene 1	Nfs1	NM_053462	ctctatatggacgtgcaggcta	gtatgggagcatggcatca
Nucleolar protein 3	Nol3	NM_053516	gcctgccaggaactactgc	gcatggagggtcatagctg
Phosphoglycerate kinase 1	Pgk1	NM_053291	ccagataacgaataaccaaagga	gacttggctccattgtcca
Phospholipase C, gamma 1	Plcg1	NM_013187	aggcttcagtgtggtccaag	gacagcatcacatctcacagc
Presenilin associated, rhomboid-like	Parl	NM_001037639	ctctgcactgggcaatcata	atccagttcctgctgcactc
Pyruvate dehydrogenase E1 alpha 1	Pdha1	NM_001004072	aatggcaaagatgaggtctgtt	tttccacaatgctgccatatt
Pyruvate dehydrogenase kinase, isoenzyme 2	Pdk2	NM_030872	caactggtgcagagctggta	gtcctcggggtccttgtc
Selectin, endothelial cell	Sele	NM_138879	aggcttcagtgtggtccaag	agggacagcatcacatctca
Solute carrier family 25, member 11	Slc25a11	NM_022398	tcccctaagtctgtcaagttcc	cctcaccactcaactgcatc
Solute carrier family 25, member 20	Slc25a20	NM_053965	cgtgctcaagtctcgattcc	ctctcaacacatctctgaaacca

Table 2 Statistics of the microarray

	No	
Total no. of oligonucleotides on the chip	31,094	
Control oligonucleotides on the chip	3582	
Total no. of genes represented on the chip	27,512	
Expressed genes	3059	11.1% of the total
Differentially expressed genes	108	3.53% of the expressed
Down-regulated genes	58	1.90% of the expressed
Up-regulated genes	50	1.63% of the expressed

http://david.abcc.ncifcrf.gov)[11,12] and as described in our previous report.[13] We submitted the differentially expressed genes to the DAVID bioinformatics system and database to reveal significantly enriched biological functions and pathways.

Statistical analysis

We used R script for integrated analysis of the two-color microarrays, including the global Lowess normalization method. Altogether, three individual parallel replica experiments and nine ratios were calculated and subjected to statistical analysis. The Lowess normalized signal ratio in the chip analysis was $\log_2$-transformed for classifying the patterns of serial changes of gene expression. Using a two tailed two-sample unequal variance Student's *t*-test, the *P* value was determined and used to find the significant changes in gene expression. Gene expression ratios with $P < 0.05$ and $\log_2$ ratio>0.8 or $\log_2$ ratio<-0.8 (1.744 fold change) were considered as up- or down-regulation, respectively. For better understanding, fold changes are displayed in Table 3, Table 4, and Table 5 instead of $\log_2$ ratio.

Results and discussion

Infarct size was significantly reduced by ischemic postconditioning as compared with the non-postconditioned control (Figure 1), showing the validity of the model to monitor cardiac gene expression profiles in response to postconditioning-induced cardioprotection.

To study the effects of postconditioning on cardiac gene expression patterns, we applied cDNA microarrays to monitor alterations in gene expression of ischemic rat hearts with and without postconditioning. Out of 27,512 different rat genes represented on the microarray, 3059 (11.1%) showed significant intensity values; and altogether 3.5% of them showed significantly altered expression. Fifty genes were significantly up-regulated and 58 genes were down-regulated in response to postconditioning as compared with the non-postconditioned group (Tables 2, 3 and 4). This is the first demonstration in the literature that postconditioning induces alterations in the cardiac gene expression profile.

In order to confirm the differential expression of genes revealed by the microarray of rat hearts with and without postconditioning, 20 genes were selected and assayed by sybrgreen based QRT-PCR (Table 5). Out of the 20 genes, changes in the expression of 14 genes were confirmed by QRT-PCR (Table 5).[14]

Table 3 Significantly up-regulated genes due to postconditioning after ischemia/reperfusion as compared with non-postconditioned hearts[a]

Gene name	Gene symbol	Genebank acc. No.	Fold change	*P* value
Metabolic process				
Similar to ornithine decarboxylase antizyme 2	Oaz2	NM_010952	3.38	0.016
Phosphoglycerate kinase, testis specific	Pgk1	NM_053291	2.90	0.001
3-Oxoacid coa transferase	Oxct1	NM_001127580	2.89	0.007
Guanylate kinase	Guk1	NM_001013115	2.64	0.037
Pyruvate dehydrogenase e1 alpha 1	Pdha1	NM_001004072	2.63	0.001
Succinate-coA ligase, GDP-forming, beta subunit	Suclg2	NM_001100750	2.59	0.001
Pyruvate dehydrogenase kinase, isozyme 2	Pdk2	NM_030872	2.35	0.006
nfs1 nitrogen fixation 1 homolog	Nfs1	NM_053462	2.24	0.021
Hydroxysteroid dehydrogenase like 2	Hsdl2	NM_001025697	2.18	0.020
dnaj (hsp40) homolog, subfamily a, member 4	Dnaja4	NM_001025411	2.14	0.017
Cathepsin l1	Ctsl1	NM_013156	1.96	0.004
Isochorismatase domain containing 2 b	Isoc2b	NM_001008367	1.93	0.011
Heme oxygenase 1	Hmox1	NM_012580	1.85	0.001
Choline phosphotransferase 1	Chpt1	NM_001007750	1.79	0.009
Carnitine o-acetyltransferase	Crat	NM_001004085	1.77	0.003
Membrane component				
Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	Slc25a11	NM_022398	2.99	0.019
Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	Slc25a20	NM_053965	2.79	0.003
Translocase of outer mitochondrial membrane 22 homolog	Tomm22	NM_212514	2.52	0.007
a-type potassium channel modulatory protein 2 a	Kcnip2	NM_020094	2.48	0.003
Solute carrier family 35, member f5	Slc35f5	NM_001105950	2.28	0.029
Solute carrier family 25 (mitochondrial carrier, aralar), member 12	Slc25a12	XM_342445	2.04	0.033
Gamma-sarcoglycan	Sgcg	NM_001006993	1.79	0.034
Extracellular				
Selenoprotein p precursor	Sepp1	NM_019192	4.35	0.018
Collagen, type iii, alpha 1	Col3a1	NM_032085	1.98	<0.001
Collagen alpha 2(i) chain precursor	Col1a2	NM_053356	1.83	<0.001
Signaling				
Sequestosome 1	Sqstm1	NM_175843	3.68	0.023
Growth factor receptor-bound protein 14	Grb14	AF076619	2.04	0.013
Other				
Regulator of calcineurin 1	Rcan1	NM_153724	3.36	0.014
s100 calcium binding protein a16	S100a16	NM_001108557	3.12	0.035
Reticulon 4	Rtn4	NM_031831	3.10	0.022
Eukaryotic translation initiation factor 4a2	Eif4a2	NM_001008335	3.08	<0.001
Tubulin alpha-8 chain	Tuba8	NM_001024339	2.67	0.026
Destrin	Dstn	NM_001033666	2.57	<0.001
Karyopherin alpha 2	Kpna2	NM_053483	2.39	0.032
Tyrosine 3-monooxygenase/tryptophan				
5-Monooxygenase activation protein, eta polypeptide	Ywhah	NM_013052	2.31	0.018
popeye domain containing 2	Popdc2	NM_199113	2.06	0.006
Transitional endoplasmic reticulum ATPase	Vcp	U11760	2.06	0.029
Ribonucleoprotein f	Hnrnpf	NM_022397	1.94	0.001
Fusion, derived from t(12;16) malignant liposarcoma	Fus	NM_001012137	1.94	0.030
60 kDa heat shock protein, mitochondrial	Hspd1	X54793	1.93	0.027
Small inducible cytokine b11 precursor	Cxcl11	NM_182952	1.93	0.045
Mitochondrial ribosomal protein l9	mrpl9	NM_001007696	1.87	0.035
Myosin, light chain 9, regulatory	Myl9	NM_001100885	1.84	<0.001
Retinoblastoma binding protein 7	Rbbp7	NM_031816	1.83	<0.001
Uncharacterized bone marrow protein bm033		ENSRNOG00000016973	1.82	0.009
Putative membrane protein	Tmco1	NM_001009631	1.79	0.009
rho-related gtp-binding protein rho6	Rnd1	NM_001013222	1.78	0.039
Unknown				
Unknown ESTs	RGD1560402	ENSRNOG00000026928	3.78	0.003
Unknown ESTs		ENSRNOG00000023714	2.23	0.017
Unknown ESTs	LOC367342	ENSRNOG00000025351	1.83	0.038

[a]All experiments were done in triplicates, data were calculated from 9 intensity ratios. EST: expressed sequence tags.

Table 4 Significantly down-regulated genes due to postconditioning after ischemia/reperfusion as compared with non-postconditioned ischemic/reperfused hearts[a]

Gene name	Gene symbol	Genebank acc. No.	Fold change	*P* value
Metabolic process				
Lipoprotein lipase	Lpl	NM_012598	−2.47	0.046
Very low density lipoprotein receptor	Vldlr	NM_013155	−1.88	0.045
Lysophospholipase 1	Lypla1	NM_013006	−1.81	0.006
Signaling				
Proenkephalin 1	Penk1	NM_017139	−2.36	0.001
Cytohesin 2	Cyth2	NM_011181	−1.99	0.045
Presenilin associated, rhomboid-like	Parl	NM_001037639	−1.93	0.036
Transcription				
Vestigial like 2 homolog	Vgll2	XM_228170	−4.50	0.005
Mediator complex subunit 21	Med21	NM_001107895	−2.52	0.008
RNA polymerases i, ii, and iii subunit abc4	Polr2k	S63758	−2.35	0.048
Friend leukemia integration 1 transcription factor	Fli1	NM_001017381	−1.76	0.030
Translation				
60s ribosomal protein l31	Rpl31	X04809	−2.65	0.027
Ribosomal protein l22 like 1	Rpl22l1	NM_001108548	−2.31	0.005
60s ribosomal protein l39	RPL39	X82551	−2.25	0.018
40s ribosomal protein s24	RPS24	X51538	−2.06	0.048
60s ribosomal protein l30	RPL30	BC058471	−2.02	<0.001
60s ribosomal protein l44		XM_214958	−1.85	0.009
Other				
Olfactory receptor 92	OLFR92	NM_146456	−3.91	0.001
Similar to leiomodin 2 (cardiac)	Lmod2	NM_001100964	−2.36	0.025
Selectin, endothelial cell	Sele	NM_138879	−2.33	0.012
Retinoblastoma-associated protein 140	C3ORF63	NM_015224	−1.93	0.004
Mitochondrial ribosomal protein s18c	Mrps18c	NM_001105996	−1.89	0.014
Progestin induced protein homolog (fragment)	Dd5	XM_001061308	−1.84	0.009
Nucleolar protein 3	Nol3	NM_053516	−1.80	<0.001
Macrophage inflammatory protein-2-beta precursor	Cxcl2	NM_053647	−1.80	<0.001
Lamina	Lmna	NM_001002016	−1.78	0.004
Inter-alpha-trypsin inhibitor heavy chain h2 Precursor	Itih2	NM_010582	−1.78	0.003
Galectin-3	Lgals3	NM_031832	−1.76	0.043
Protein tyrosine phosphatase 4a1	Ptp4a1	NM_031579	−1.75	0.007
Unknown				
Unknown ESTs		ENSRNOG00000025828	−3.40	0.038
Unknown ESTs		ENSRNOG00000016668	−3.32	0.036
Unknown ESTs		ENSRNOG00000026713	−3.01	0.031
Unknown ESTs		ENSRNOG00000008770	−2.79	0.046
Unknown ESTs		ENSRNOG00000021541	−2.77	0.042
Unknown ESTs		ENSRNOG00000023417	−2.62	0.038
Unknown ESTs		ENSRNOG00000026030	−2.43	0.007
Similar to riken cdna 1110004e09	RGD1306954	NM_001008288	−2.39	0.045
Unknown ESTs		ENSRNOG00000005837	−2.36	0.017
Unknown ESTs		ENSRNOG00000022869	−2.33	0.014
Unknown ESTs		ENSRNOG00000023811	−2.31	0.005
Unknown ESTs		ENSRNOG00000022238	−2.31	0.018
Unknown ESTs		ENSRNOG00000021921	−2.19	0.032
Unknown ESTs		ENSRNOG00000025992	−2.18	0.040
Unknown ESTs		ENSRNOG00000027782	−2.12	0.005
Unknown ESTs		ENSRNOG00000004105	−2.08	0.016
Unknown ESTs		ENSRNOG00000024443	−1.99	0.043
Similar to 60S ribosomal protein L23a	LOC365203	XR_007211	−1.97	0.049
Unknown ESTs		ENSRNOG00000003446	−1.97	0.016
Unknown ESTs		ENSRNOG00000028289	−1.95	0.006
Unknown ESTs		ENSRNOG00000007499	−1.94	0.028
Unknown ESTs		ENSRNOG00000027416	−1.92	0.033

(continued)

Table 4 Continued

Gene name	Gene symbol	Genebank acc. No.	Fold change	*P* value
Unknown ESTs		ENSRNOG00000024268	−1.92	0.045
Unknown ESTs		ENSRNOG00000024260	−1.88	0.018
Unknown ESTs		ENSRNOG00000021994	−1.85	0.032
Unknown ESTs		ENSRNOG00000021901	−1.83	0.045
Unknown ESTs		ENSRNOG00000018208	−1.82	0.025
Estrous-specific protein (fragment)		ENSRNOG00000017929	−1.79	0.032
Unknown ESTs	LOC680416	ENSRNOG00000004553	−1.79	0.007
Unknown ESTs		ENSRNOG00000002287	−1.79	0.020

[a]All experiments were done in triplicates, data were calculated from 9 intensity ratios. EST: expressed sequence tags.

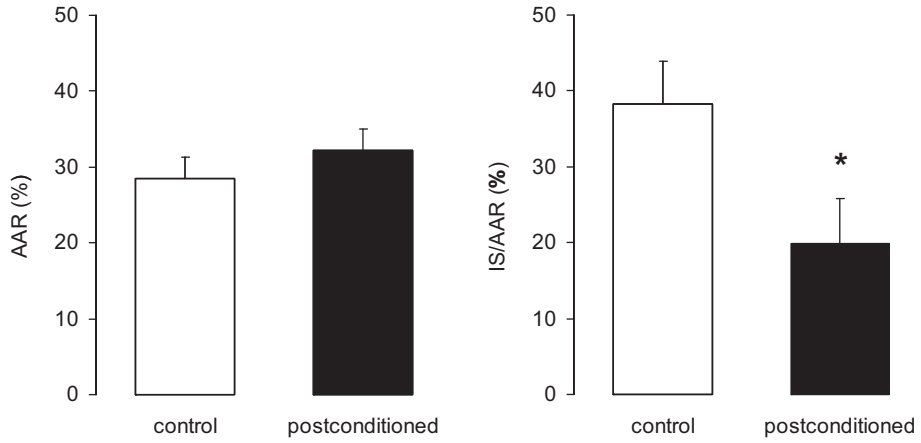

Figure 1 Area at risk and infarct size in the non-postconditioned control and postconditioned hearts. Data are mean ± SEM, $n = 7–8$, $^*P < 0.05$

In order to further determine the biological significance and functional classification of differentially expressed genes due to postconditioning, GO analysis was performed. GO is a bioinformatics initiative with the aim of standardizing the representation of genes and gene products, providing a controlled vocabulary of terms for gene product characteristics and annotation data. GO analysis is suitable for identifying significantly enriched GO terms related to multiple genes, and for discovering enriched functional-related gene groups. A single gene can belong to different categories. Out of the 108 genes significantly altered by postconditioning in our study, 67 genes with known functions were submitted to GO analysis. The rest of the 108 genes were either unknown, expressed sequence tags (ESTs) or unrecognized by the GO analysis database. Out of the 67 genes, 57 were ultimately classified into 5 main categories such as (i) cellular metabolic process, (ii) localization including transport, (iii) developmental process, (iv) cellular component organization and biogenesis, and (v) structural molecule activity (Table 6). These results showed that postconditioning may significantly affect several major biological processes, especially genes related to cellular metabolic processes.

Alterations in the expression of metabolic genes

Here we have shown that postconditioning affected several metabolic genes in the myocardium. During ischemia, cardiac metabolism switches from fatty acid oxidation to glycolysis for energy provision due to low oxygen tension. The modulation of myocardial energy substrate metabolism, particularly shifting energy substrate preference from the use of fatty acids towards the use of glucose as an oxidative fuel, is capable of enhancing the preservation of mechanical function and efficiency of the heart.[15–17] Pyruvate decarboxylation is the key irreversible step in carbohydrate oxidation and is catalyzed by the pyruvate dehydrogenase multienzyme complex in the mitochondrial matrix. Phosphorylation of pyruvate dehydrogenase by pyruvate dehydrogenase kinase (also known as pyruvate dehydrogenase complex kinase) inactivates pyruvate dehydrogenase, and subsequently the entire complex. In our study, a dramatic up-regulation in the expression of pyruvate dehydrogenase and pyruvate dehydrogenase complex kinase was detected, which may indeed play a role in tissue protection against ischemia. This is the first demonstration that postconditioning alters the expression of pyruvate dehydrogenase and pyruvate dehydrogenase complex kinase.

Since the majority of circulating fatty acids are present as triacyl-glycerols in very low density lipoproteins, the hydrolysis of triacyl-glycerols by lipoprotein lipase is an important determinant of the flux of β-oxidation of the heart.[18] Here we found that lipoprotein lipase and lysophospholipase were down-regulated by postconditioning as

Table 5 Confirmation of gene expression changes due to postconditioning by QRT-PCR in rat hearts[a]

			Microarray			QRT-PCR		
Gene name	Gene symbol	Genebank acc. No	Mean ratio fold change	*P* value	Regulation	Mean ratio fold change	SD	Confirm
3-Oxoacid CoA transferase 1	Oxct1	NM_024188	2.89	0.007	Up	–3.01	0.28	No
60 kDa heat shock protein, mitochondrial	Hspd1	X54793	1.93	0.027	Up	1.79	1.51	Yes
Choline phosphotransferase 1	Chpt1	NM_001007750	1.79	0.009	Up	1.68	1.22	Yes
Destrin	Dstn	NM_019771	2.57	<0.001	Up	1.09	2.25	No
Gamma-sarcoglycan	Sgcg	NM_001006993	1.79	0.034	Up	1.61	0.91	Yes
Heme oxygenase 1	Hmox1	NM_012580	1.85	0.001	Up	–1.77	1.12	No
Karyopherin alpha 2	Kpna2	NM_053483	2.39	0.032	Up	2.60	1.74	Yes
Kv channel-interacting protein 2	Kcnip2	NM_020094	2.48	0.003	Up	1.89	2.34	Yes
Leiomodin 2	Lmod2	NM_053098	–2.36	0.025	Down	–1.58	1.02	Yes
Lipoprotein lipase	Lpl	NM_012598	–2.47	0.046	Down	–2.73	0.70	Yes
Nitrogen fixation gene 1	Nfs1	NM_053462	2.24	0.021	Up	–2.28	2.25	No
Nucleolar protein 3	Nol3	NM_053516	–1.80	<0.001	Down	–1.75	0.91	Yes
Phosphoglycerate kinase 1	Pgk1	NM_053291	2.90	0.001	Up	–1.86	1.47	No
Phospholipase C, gamma 1	Plcg1	NM_013187	–1.65	0.010	Down	–3.30	0.91	Yes
Presenilin associated, rhomboid-like	Parl	NM_001037639	–1.93	0.036	Down	–2.05	1.13	Yes
Pyruvate dehydrogenase E1 alpha 1	Pdha1	NM_001004072	2.63	0.001	Up	3.78	2.00	Yes
Pyruvate dehydrogenase kinase, isoenzyme 2	Pdk2	NM_030872	2.35	0.006	Up	3.62	2.43	Yes
Selectin, endothelial cell	Sele	NM_138879	–2.33	0.012	Down	–1.98	1.57	Yes
Solute carrier family 25, member 11	Slc25a11	NM_022398	2.99	0.019	Up	1.28	0.40	No
Solute carrier family 25, member 20	Slc25a20	NM_053965	2.79	0.003	Up	1.73	1.68	Yes

[a]Comparison with microarray data.

compared with ischemia/reperfusion. Alterations in lipoprotein lipase expression have been shown to affect myocardial fatty acid supply, uptake, and β-oxidation.[18] In addition, very low density lipoprotein receptor gene expression was down-regulated in the myocardium in this study. These results suggest that there is attenuated lipid catabolism in the ischemic/reperfused heart due to postconditioning, which may further lead to the switch from fatty acid oxidation to glucose utilization for energy provision, thereby contributing to cardioprotection.

Alterations in gene expression related to membrane-transports

In our study, translocase of outer mitochondrial membrane (Tomm) 22 homolog expression was up-regulated due to postconditioning. Tomm22, a member of the mitochondrial Tomm complex, is responsible for the translocation of cytosolic proteins to the mitochondria.[19] In accordance with this, translocation of connexin-43 has also been shown to contribute to cardioprotection.[20,21]

In our study, a significant up-regulation of the voltage-gated-potassium-channel-interacting protein 2 (KChIP2) was detected by our DNA microarrays. This gene is preferentially expressed in the heart, and encodes three cytoplasmic accessory proteins for Kv4.2 and Kv4.3 channels, which mediate the transient outward K^+ current, I_{to}. It has been shown that a deficiency in KChIP2 directly confers susceptibility to malignant ventricular arrhythmias by targeted disruption of KChIP2 in mice.[22] This is in accordance with our previous findings that postconditioning is able to decrease ischemia/reperfusion-induced ventricular fibrillation in rat hearts.[23]

Carnitine/acylcarnitine carrier (CAC, or solute carrier family member 25, SLC25a20) is the key component in the carnitine cycle, which shuttles long-chain fatty acids from the outside to the inside of the mitochondria for the fatty acid breakdown.[24] We found that the mRNA of SLC25a20 was up-regulated due to ischemic postconditioning. It can be speculated that CAC up-regulation might be a compensatory mechanism due to the likely decreased fatty acid oxidation.

Alterations in the expression of genes of different functions

In this paragraph, we discuss some genes of various functions which may be of interest in the mechanism of cardioprotection by postconditioning. The expression of Galectin-3 was down-regulated by ischemic

Table 6 Significantly enriched gene ontology (GO) terms in the population of genes with altered expression[a]

Category	GO ID	Level and term	Count	%	*P* value	Genes (gene symbols)
GOTERM_BP_ALL	GO:0044237	03—Cellular metabolic process	40	59.7	0.038	Chpt1, Crat, Ctsl1, Dnaja4, Dstn, Eif4a2, Fli1, Guk1, Hmox1, Hspd1, Itih2, Kpna2, Lpl, Lypla1, Med21, Mrpl9, Mrps18c, Nfs1, Nol3, Oaz2, Oxct1, Pdha1, Pdk2, Pgk1, Polr2k, Ptp4a1, Rbbp7, Rpl22l1, Rpl30, Rpl31, Rpl39, Rps24, Sepp1, Sqstm1, Suclg2, Tuba8, Vcp, Vgll2, Vldlr, Ywhah
GOTERM_BP_ALL	GO:0044248	04—Cellular catabolic process	7	10.5	0.026	Hmox1, Pdha1, Pgk1, Sqstm1, Suclg2, Vcp, Ywhah
GOTERM_BP_ALL	GO:0044262	04—Cellular carbohydrate metabolic process	6	9.0	0.011	Itih2, Oxct1, Pdha1, Pdk2, Pgk1, Suclg2
GOTERM_BP_ALL	GO:0051186	04—Cofactor metabolic process	6	9.0	0.002	Crat, Hmox1, Nfs1, Oxct1, Pdha1, Suclg2
GOTERM_BP_ALL	GO:0006732	05—Coenzyme metabolic process	4	6.0	0.040	Crat, Oxct1, Pdha1, Suclg2
GOTERM_BP_ALL	GO:0006104	06—Succinyl-CoA metabolic process	2	3.0	0.015	Oxct1, Suclg2
GOTERM_BP_ALL	GO:0009056	03—Catabolic process	8	11.9	0.024	Hmox1, Lpl, Pdha1, Pgk1, Sqstm1, Suclg2, Vcp, Ywhah
GOTERM_BP_ALL	GO:0051179	02—Localization	19	28.4	0.047	Col1a2, Col3a1, Crat, Dstn, Hmox1, Hspd1, Kcnip2, Kpna2, Ptp4a1, Slc25a11, Slc25a12, Slc25a20, Slc35f5, Sqstm1, Tomm22, Tuba8, Vcp, Vldlr, Ywhah
GOTERM_BP_ALL	GO:0006810	04—Transport	17	25.4	0.044	Col1a2, Col3a1, Crat, Hmox1, Hspd1, Kcnip2, Kpna2, Slc25a11, Slc25a12, Slc25a20, Slc35f5, Sqstm1, Tomm22, Tuba8, Vcp, Vldlr, Ywhah
GOTERM_BP_ALL	GO:0043681	07—Protein import into mitochondrion	2	3.0	0.049	Hspd1, Tomm22
GOTERM_BP_ALL	GO:0032502	02—Developmental process	20	29.9	0.033	Chpt1, Col1a2, Col3a1, Fli1, Hmox1, Hspd1, Kcnip2, Nol3, Ptp4a1, Rbbp7, Rcan1, Rnd1, Rtn4, Sepp1, Sgcg, Sqstm1, Vcp, Vgll2, Vldlr, Ywhah
GOTERM_BP_ALL	GO:0048731	04—System development	13	19.4	0.033	Col1a2, Col3a1, Fli1, Hmox1, Kcnip2, Rcan1, Rnd1, Rtn4, Sgcg, Vcp, Vgll2, Vldlr, Ywhah
GOTERM_BP_ALL	GO:0048666	09—Neuron development	4	6.0	0.038	Kcnip2, Rnd1, Rtn4, Ywhah
GOTERM_BP_ALL	GO:0016043	03—Cell comp organization and biogenesis	19	28.4	0.012	Chpt1, Dstn, Eif4a2, Hmox1, Hspd1, Kcnip2, Kpna2, Lmna, Nfs1, Rbbp7, Rnd1, Rtn4, Sgcg, Sqstm1, Tomm22, Tuba8, Vcp, Vldlr, Ywhah
GOTERM_BP_ALL	GO:0006996	05—Organelle organization and biogenesis	10	14.9	0.038	Dstn, Hspd1, Kpna2, Lmna, Rbbp7, Rnd1, Sgcg, Tomm22, Tuba8, Ywhah
GOTERM_BP_ALL	GO:0003013	04—Circulatory system process	6	9.0	0.000	Col3a1, Fli1, Hmox1, Kcnip2, Lpl, Rcan1
GOTERM_BP_ALL	GO:0008015	05—Blood circulation	6	9.0	0.000	Col3a1, Fli1, Hmox1, Kcnip2, Lpl, Rcan1
GOTERM_MF_ALL	GO:0005198	02—Structural molecule activity	12	17.9	0.000	Col1a2, Col3a1, Lmna, Mrpl9, Mrps18c, Myl9, Rpl31, Rpl39, Tuba8, Rpl22l1, Rps24, Rpl30
GOTERM_BP_ALL	GO:0042221	03—Response to chemical stimulus	8	11.9	0.008	Cxcl2, Cxcl11, Hmox1, Hspd1, Kcnip2, Sepp1, Slc25a12, Vcp
GOTERM_MF_ALL	GO:0003735	03—Structural constituent of ribosome	7	10.5	0.001	Mrpl9, Mrps18c, Rpl22l1, Rpl30, Rpl31, Rpl39, Rps24
GOTERM_MF_ALL	GO:0005319	04—Lipid transporter activity	3	4.5	0.042	Lpl, Slc25a20, Vldlr
GOTERM_BP_ALL	GO:0006412	06—Translation	8	11.9	0.010	Eif4a2, Mrpl9, Mrps18c, Rpl22l1, Rpl30, Rpl31, Rpl39, Rps24
GOTERM_BP_ALL	GO:0045767	10—Regulation of anti-apoptosis	2	3.0	0.045	Hmox1, Rtn4
KEGG_PATHWAY	hsa03010	Ribosome	5	7.5	0.004	Rpl22l1, Rpl30, Rpl31, Rpl39, Rps24

[a]GO analysis determines the biological significance of differentially expressed genes that can be used to determine the functional classification of the genes, the expression of which have been significantly up- or down-regulated. Major functional categories of GO terms were separated by horizontal lines, subcategories are represented by intendations.

postconditioning in this study. Galectin-3 has been suggested to have a role in the pathophysiology of heart failure,[25,26] as it was specifically up-regulated in decompensated heart failure as compared with compensated heart failure in rats.[27] Thus, galectin-3 may be a culprit biomarker in heart failure.[26,28] Galectins may bind to different cell surface antigens and receptors, as well as to intracellular ligands, thereby participating in intracellular signaling pathways. Among the supposed downstream targets of galectin-3,[26] several gene products were found which were previously described as taking part in the signal transduction of ischemic postconditioning, e.g. Erk, Akt, PI3 kinase, NADPH oxidase, reactive oxygen species, matrix metalloproteinases, and their tissue inhibitors.[1,2,5] It is consistent that galectin may play a role in the molecular mechanism of ischemic postconditioning.

Gamma-sarcoglycan (the 35-kDa A4 or the 34-kDa dystrophin-associated glycoprotein[29]) was up-regulated by ischemic postconditioning in our study. Sarcoglycan may be an independent signaling molecule, and may couple mechanical and chemical signals as it is located in close proximity to dystrophin and dystroglycan.[30] It is also hypothesized that the sarcoglycan complex is a receptor.[31] In accordance with this, we have recently described that another proteoglycan, biglycan, may function as a cardioprotective macromolecule in biglycan overexpressing mice and in cardiac myocytes subjected to simulated ischemia and reoxygenation.[32–34]

Limitations

Analysis of cardiac tissue for gene expression pattern has been performed at the end of the 2-h reperfusion in this study. This time allows for mRNA accumulation and/or degradation, therefore, the time course of gene expression pattern has not been revealed in the present study. The present study has been performed in crystalloid-perfused isolated rat hearts, therefore, the translational value of our results is indirect. Nevertheless, postconditioning of the heart has been translated to clinical therapy as shown by several ongoing clinical studies, therefore, better understanding its mechanism may lead to development of novel cardioprotective strategies.[35]

Conclusions

In conclusion, we found here a number of genes, the expression of which is significantly altered due to postconditioning in the ischemic/reperfused heart. Although some of the genes have previously been shown to play a role in ischemia/reperfusion injury, this is the first demonstration that postconditioning alters the gene expression profile of the heart. Most of these genes of altered expression have not previously been suspected to be involved in the mechanism of ischemic postconditioning. The newly identified individual genes and their functional clusters in our present study might lead to a better understanding of the cellular mechanisms of the remarkable cardioprotection elicited by postconditioning.

Author contributions: CC participated in the design, interpretation of the studies and analysis of the data, and prepared the manuscript; GS conducted heart perfusion experiments, BP did GO analysis, AZ performed RNA isolation and QPCR and took part in preparing this article, LGP evaluated QPCR and GO, TC helped with statistics and article preparation and FP helped to prepare and review the manuscript.

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