Effect of classic preconditioning on the gene expression pattern of rat hearts: a DNA microarray study

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Abstract To profile gene expression patterns involved in ischemic preconditioning, we monitored global gene expression changes by DNA microarray analysis of 3200 rat-specific genes and by real-time quantitative polymerase chain reaction in rat hearts. Forty-nine genes with altered expression were found after ischemia/reperfusion as compared to control non-ischemic hearts and 31 genes were characteristic for classic preconditioning followed by ischemia/reperfusion as compared to ischemia/reperfusion without preconditioning. Genes with altered expression due to ischemia and/or preconditioning included those controlling protein degradation, stress responses, apoptosis, metabolic enzymes, regulatory proteins, and several unknown cellular functions. Metallothionein, natriuretic peptides, coagulation factor VII, cysteine proteinase inhibitor, peroxisome proliferator activator receptor γ and myosin light chain kinase genes were previously suspected to be related to several cardiovascular diseases, however, most of these genes have not previously been shown to be related to myocardial ischemia/reperfusion. Some genes were observed to change specifically in response to preconditioning: oligoadenylate synthase, chaperonin subunit ε, a cGMP phosphodiesterase (PDE9A1), a secretory carrier membrane protein, an amino acid transporter, and protease 28 subunit. None of these genes has previously been shown to be involved in the mechanism of preconditioning.

Key words: Oligoadenylate synthase; cGMP phosphodiesterase; Metallothionein; Natriuretic peptide; Coagulation factor VII; Cysteine proteinase inhibitor; Peroxisome proliferator activator receptor γ; Chaperonin subunit ε; Myocardial ischemia; Preconditioning

1. Introduction

Ischemic heart disease is a major cause of mortality in the western world. Effective therapeutic strategies for protecting the ischemic myocardium are much sought after. Ischemic preconditioning is a well-described adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand a subsequent ischemic injury (see [1] for review). Preconditioning confers a remarkable cardioprotection in a variety of species including humans (see [1,2] for reviews), although the cardioprotective effectiveness of ischemic preconditioning might be attenuated in the heart during aging and some disease states such as hyperlipidemia and diabetes (see [3] for review). Preconditioning can be elicited by different sublethal stress signals, such as brief periods of ischemia, hypoxia, rapid electrical pacing, heat stress, administration of bacterial endotoxin, etc. The cardioprotective effect of preconditioning shows two distinct phases. The early phase (classic preconditioning) is manifested within minutes after the preconditioning stimulus and has a duration of less than 2–3 h. The late phase is characterized by a slower onset (20 h) and a duration of up to 72 h.

The underlying molecular mechanisms of ischemic preconditioning have been extensively investigated in the hope of identifying new rational approaches to therapeutic protection of the ischemic myocardium. In spite of the intensive research in the past nearly 2 decades, the exact biochemical mechanism of preconditioning is still a question of debate due to the complexity of the cellular mechanisms involved in this phenomenon. A variety of substances and ion channels, i.e. adenosine, bradykinin, nitric oxide, superoxide, peroxynitrite, calcitonin gene-related peptide, cGMP, protein kinases, norepinephrine, ATP-sensitive K^+ channels, etc., have been shown so far to play a role both in ischemia/reperfusion injury and in the development of the cardioprotective effect of preconditioning [2,4,5]. However, the traditional biochemical and pharmacological approaches have been insufficient so far to explore the key cellular events in ischemia/reperfusion injury and preconditioning. Recent studies therefore attempted to identify gene activity changes during coronary occlusion in the mouse heart using a mouse cDNA array of 588 genes [6] and in a rat infarction model using cDNA array of about 7000 rat genes [7]. However, still very little is known about the gene expression pattern of the heart in response to ischemia/reperfusion and preconditioning.

Therefore, to profile gene expression patterns associated with ischemia/reperfusion and classic preconditioning, we used cDNA microarrays of 3200 rat genes to monitor transcript levels in rat hearts in the hope of identifying new cellular pathways involved in cardiac ischemia and ischemic adaptation.

2. Materials and methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of

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Table 1
Primers used in Q-PCR analysis

Gene product	Forward primer	Reverse primer
β-Actin	TTCAACACCCCAGCCATGT	GCATACAGGGACAACACAGCC
Chaperonin ε	TACAGCTCTGCAGATGAAGGATGCTT	TGACATCCGTAAGCCTGGAGAATCTG
Natriuretic peptide type B	GGACCAAGGCCCTACAAAAGAACTTC	GCCGGAGTCTGCAGCCAGGAGGTC
Anion exchange protein 2	CCATGGGTGGCATCTGTGCCCTC	GCAGTAGGTCCCCAATGACCATGGAG
Metallothionein-II	TCGCCATGGACCCCAACTGCTCCTGTG	GAAGCCTCTTTGCAGATGCAGCCCTG
PPARγ	CGGAAGCCCTTTGGTGACTTTATGG	GATGGGCTTCACGTTCAGCAAGCC
Betaine-homocysteine methyltransferase	GGAACCCAGAGTTGCCACCAGATGG	AAATCCCCTTTCTGGGGCGAGCTCC
Cysteine proteinase inhibitor	GCCACTGCTTGAAAAGAAAACCAATGGG	GGTCAGCTCATCATTTTTGGATTTGTTAG

Health (NIH publication No. 85-23, revised 1996) and was approved by local ethics committees.

2.1. Perfusion protocol of isolated rat hearts

Male Wistar rats (300–350 g) were anesthetized with diethylether and given 500 U/kg heparin. Hearts were then isolated and perfused in Langendorff mode with an oxygenated, normothermic Krebs–Henseleit buffer as described [8]. Three different perfusion protocols were applied ($n = 5$–8 in each group). Hearts were subjected to either a preconditioning or a non-preconditioning protocol followed by test ischemia/reperfusion as described in detail [8,9]. After 10 min equilibration, preconditioning was induced by three intermittent cycles of 5 min no-flow ischemia, separated by 5 min aerobic perfusion. Time-matched non-preconditioned and preconditioned hearts were then subjected to 30 min global no-flow ischemia followed by 120 min reperfusion. A time-matched control group was aerobically perfused for 190 min. Heart rate and coronary flow were monitored throughout the perfusion protocol in all groups [8]. Lactate dehydrogenase release was measured from coronary effluent collected for 5 min at the beginning of the perfusion and at 0–5 min and 115–120 min of reperfusion after 30 min global ischemia and at corresponding periods in controls as described [8]. At the end of the perfusion protocols, hearts from all groups were frozen and powdered with a pestle and mortar in liquid nitrogen.

2.2. RNA preparation

Total RNA was purified from each group (25–25 mg tissue from each heart) with NucleoSpin RNA purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA preparations from each group ($n = 3$, randomly selected from each group) were pooled, and their quantities and qualities were assessed by gel electrophoresis and spectrophotometry. Total RNA was used for microarray analysis as well as for reverse transcription quantitative polymerase chain reaction (QRT-PCR).

2.3. Microarrays, probe preparations and hybridizations

Construction and use of microarrays were as described [10]. Briefly, 3200 amplified cDNA inserts from rat heart, kidney, liver and brain libraries were purified with MultiScreen-PCR plate (Millipore), resuspended in 50% dimethyl sulfoxide/water, and arrayed on amino-silanized slides (Sigma-Aldrich, St. Louis, MO, USA) using a MicroGrid Total Array System (BioRobotics, Cambridge, UK) spotter with 16 pins with a 4×4 format. All clones were spotted in duplicate. After printing, DNA was UV crosslinked to the slides (Stratagene, Stratalinker, 700 mJ) and stored at room temperature. Prior to hybridization, the slides were blocked in 1×saline sodium citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 1% bovine serum albumin for 30 min at 42°C, washed with water and dried with high pressure air. 15 μg total RNA from each sample was amplified by a linear antisense RNA amplification method, and labeled with Cy3 or Cy5 fluorescent dye during reverse transcription as described previously [11]. Briefly, 2 μg of amplified RNA was labeled with 0.4 μM random nonamers, 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP (Amersham Pharmacia Biotech, UK), 20 U RNasin (Fermentas, Vilnius, Lithuania), 1×first strand buffer, 200 U RNase H (−) point mutant M-MLV reverse transcriptase (Fermentas), and 0.05 mM Cy3-dCTP or Cy5-dCTP (NEN Life Science Products, Boston, MA, USA) in 20 μl total volume. The RNA, primer and RNasin were denatured at 75°C for 5 min and cooled on ice before adding the remaining reaction components. After 2 h incubation at 37°C, the heteroduplexes were purified as described for recovery [12], denatured and the mRNA was alkali hydrolyzed for 15 min at 37°C and neutralized with 3 M NaOAc (pH 5.0). The labeled cDNA was purified with a PCR purification kit (Macherey-Nagel) according to the manufacturer's instructions. Probes generated from the control and ischemic or preconditioned heart samples were mixed, reconstituted in 12 μl hybridization buffer (50% formamide, 5×SSC, 0.1% SDS, 100 μg/ml salmon sperm DNA) and applied onto the array after denaturation by heating for 1 min at 90°C. The slide was covered with a 22 mm×22 mm coverslip, and sealed with DPX Mountant (Fluka, Buchs, Switzerland) in order to prevent evaporation. Slides were incubated at 42°C for 20 h in a humid hybridization chamber. After hybridization the mountant was removed and the arrays were washed by submersion and agitation for 10 min in 1×SSC with 0.1% SDS, for 10 min in 0.1%×SSC with 0.1% SDS and for 10 min in 0.1×SSC at room temperature, then rinsed briefly in deionized water and dried.

2.4. Scanning and data analysis

Each array was scanned under a green laser (532 nm) (for Cy3 labeling) and under a red laser (660 nm) (for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA, USA) scanning confocal fluorescent scanner with 10 μm resolution. Image analysis was performed by ScanAlyze2 software (http://www.microarrays.org/software.html). Each spot was defined by manual positioning of a grid of circles over the image. The average pixel intensity and the local background of each spot were determined. A measure, i.e. 'expression ratio' (MRAT, denotes the median of the set of background-corrected single pixel intensity ratios of the two channels within the spot), was determined [13]. This average expression ratio for all genes on the array was normalized to 1.0. For background corrections those data were calculated as negatives where the average intensity of the spot was smaller than two times the average background of the same area. Significant spots have more than 0.55 CHGTB2 values in both Cy3 and Cy5 channels. Each experiment was performed twice using both fluorescent dyes for labeling control and sample to reduce the number of false positive or false negative ratios deriving from possible uneven incorporation of fluorescent dyes during labeling, or from other experimental variables introduced by hybridization, washing conditions or array features. Therefore, from each RNA pool two probes were generated: a Cy5-labeled and a Cy3-labeled one in order to perform replicate 'color-flip' experiments suggested by other authors [14,15]. Replica spots (on the same array) and replica experiments (two different arrays) resulted in four data points for every gene. Those spots were excluded from further analysis when ratios of the replica spots had a more than two-fold difference. The same restriction was applied for the average ratios of the replica experiments.

2.5. Real-time quantitative PCR

Relative QRT-PCR was performed on a RotorGene 2000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SybrGreen protocol to confirm the gene expression changes observed by using microarrays. 20 μg of total RNA from each pool was reverse transcribed in the presence of poly(dT) sequences in a total volume of 20 μl. After dilution of the mix with 80 μl of water, 2 μl of this mix was used as template in the QRT-PCR. Relative expression ratios were normalized to β actin. The PCR primers used in this study are listed in Table 1. All the PCRs were performed in triplicate.

Table 2
Genes with altered expression in response to ischemia/reperfusion when compared to non-ischemic hearts

Functional cluster	Gene product	Accession number	Ratio	S.D.
Bioactive peptides	Atrial natriuretic factor	M27498	2.68	0.45
Cytoskeleton, extracellular matrix proteins	Procollagen, type III, α1	W89883	2.64	0.38
	α-Tubulin	NM_022298	2.66	0.98
Energy metabolism	*Mus domesticus* strain MilP mitochondrion genome	AW545415	0.52	0.1
	NADH-ubiquinone oxidoreductase B15	NM_012985	2.28	0.02
Heat shock proteins	Chaperonin subunit ε	AA956164	1.87	0.11
	Wagneri gene for 105-kDa heat shock protein	AW544862	2.47	0.24
	Heat shock protein, 86 kDa 1	AJ428213	3.5	0.5
Metabolic enzymes	Glycine-*N*-acyltransferase	AA237628	0.57	0.03
	Isocitrate dehydrogenase 3 (NAD^+) α	NM_053638	1.87	0.38
	Formiminotransferase cyclodeaminase	NM_053567	0.5	0.05
	Aconitase 1	AA875134	0.58	0.1
Others	Putative sialoglycoprotease type 2	AA273893	0.56	0.06
	Mouse mRNA for AF1q	AB083464	0.6	0.01
	Elongation factor Tu	AA819168	0.6	0.05
	14-3-3 protein γ subtype	D17447	1.94	0.06
	Homo sapiens SPG protein	AA067045	2.12	0.07
	Mus musculus antigen 4F2	AW545809	2.31	0.07
	Mouse histocompatibility 2, class II antigen A α	NM_010378	0.58	0.02
	GS4	AF492385	1.91	0.03
	Homo sapiens CHMP1.5 protein (CHMP1.5)	AA066250	2.46	0.16
	Signal peptidase	NM_031723	2.8	0.34
	Homo sapiens HSPC245	AA260293	2.01	0.15
	Glutaminyl-tRNA synthetase	BE329136	1.99	0.26
Receptors, ion channels, membrane proteins	Cytokine receptor-like molecule	AA899612	0.59	0.06
	Low-density lipoprotein receptor-related protein	NM_053541	0.6	0.01
	Homo sapiens solute carrier family 39 (zinc transporter)	NM_130849	0.45	0.04
Regulatory proteins, kinases, phosphatases	*Homo sapiens* zinc finger protein 28	AW536277	2.03	0.18
	Fas-activated serine/threonine FAST kinase	AA956496	1.93	0.19
	Mus musculus forkhead protein FKHR	AA221124	2.11	0.21
Signal transduction	Ras oncogene neuroblastoma, Nras	NM_080766	0.59	0.12
Synaptic proteins	Synaptic vesicle protein 2B	AF372834	0.44	0.16
Ubiquitin system	Non-canonical ubiquitin conjugating enzyme 1	AA250689	0.6	0.01
	Rattus norvegicus ubiquitin-like protein	AW545652	2.45	0.39
Unknown	EST	AA276424	0.55	0.11
	Human DNA sequence from clone 1178H5	AW541466	0.58	0.04
	Homo sapiens 12q15 BAC RPCI11-444B24	AW545696	0.58	0.14
	EST	AA261708	0.59	0.04
	EST	AA268104	0.59	0.01
	Clone: 4933424L15: β-defensin-containing protein	NM_031810	0.6	0.06
	EST	AA388512	1.99	0.14
	Mus musculus clone: 2510040L10	AA244814	2.04	0.21
	EST	AA242702	2.07	0.17
	Homo sapiens unknown protein IT12	AW536219	2.18	
	EST	AA412944	2.32	0.14
	EST	AA073606	2.37	0.24
	EST	AA266972	2.49	0.49
	Drosophila melanogaster genomic sequence	AA407331	2.91	0.98

All experiments were done in duplicate and data were calculated from four intensity ratios. 'EST' denotes expressed sequence tag.

3. Results and discussion

3.1. Gene expression alteration after ischemia/reperfusion

Relative gene expression changes in response to ischemia and reperfusion were determined using the expression profiles of time-matched control hearts as baseline. Changes of 3200 genes were followed by rat-specific cDNA microarrays. In response to ischemia and reperfusion, out of 3200 genes 1468 showed significant intensity (see Section 2 for statistical calculations) and 1.6% showed altered expression: 28 genes exhibited significant up-regulation and 21 were down-regulated (Table 2). Little is known about the possible role of most of these genes in ischemia/reperfusion.

In a recent report, global expression analysis in response to renal ischemia was performed by Yoshida et al. [16]. They found that most of the genes showing altered expression are involved in cell structure, extracellular matrix, tissue repair, and cell division/differentiation. By using an Affymetrix oligonucleotide microarray containing 10 000 gene-specific samples they found 122 genes, the expression of which changed due to ischemia-induced acute renal failure. In our present study, several genes with similar characteristics were altered due to myocardial ischemia, i.e. tubulin, procollagen, glycine-*N*-acyltransferase, several metabolic enzymes and proteins involved in programmed cell death. We detected extensive changes in heat shock proteins in our present study. A chaperonin and two heat shock proteins (86 and 105 kDa) were induced by ischemia. The induction of heat stress proteins is well known in response to myocardial, renal, and cerebral ischemia [17–20], however, this is the first demonstration that chaperonin subunit ε is significantly up-regulated due to cardiac ischemia/reperfusion.

Ischemia/reperfusion repressed several genes including some mitochondrial genes and aconitase, a major enzyme of the

Table 3
Genes with altered expression due to preconditioning followed by ischemia/reperfusion when compared to either ischemic/reperfused hearts without preconditioning or non-ischemic controls

Functional cluster	Gene product	Accession number	Ratio 1 (Isc/Norm)	S.D. (1)	Ratio 2 (Prec/Isc)	S.D. (2)
Bioactive peptides	Natriuretic peptide precursor type B	NM_031545	0.73	0.06	1.76	0.21
Calcium-dependent binding proteins	Pentaxin-related gene	W42321	0.97	0.09	1.80	0.24
Cytoskeleton, extracellular matrix proteins	Class I β-tubulin	AB011679	0.65	0.18	0.66	0.01
Heat shock proteins	Chaperonin subunit 5, ε	AA955792	1.87	0.11	2.18	0.21
Metabolic enzymes	(2′,5′)Oligoadenylate synthase 1	Z18877	1.17	0.15	1.75	0.14
	cGMP phosphodiesterase (PDE9A1)	AA273765	1.51	0.26	3.29	0.89
	Peroxisome proliferator activator receptor γ	NM_013124	0.95	0.23	0.50	0.14
	Protease (macropain) 28 subunit, α	NM_017278	1.1	0.31	0.56	0.13
	Betaine-homocysteine methyltransferase	NM_030850	1.36	0.21	0.57	0.05
	N-Acylsphingosine amidohydrolase 1	NM_053407	1.21	0.19	1.70	0.22
Metal binding proteins	Metallothionein II	H32024	1.08	0.16	1.69	0.08
Others	18S, 5.8S, and 28S ribosomal RNAs	V01270	2.8	0.29	1.85	0.14
	Coagulation factor VII	AA271041	1.29	0.2	1.99	0.21
	Nucleolar phosphoprotein of 140 kDa	AA408077	1.07	0.19	2.03	0.31
	Heparin cofactor II	AF096869	1.08	0.09	2.08	0.42
	β-Globin	X05080	1.08	0.34	0.66	0.01
	Kell blood group glycoprotein	AA900226	0.86	0.11	0.51	0.18
	Cysteine proteinase inhibitor	M92418	0.98	0.09	0.63	0.11
Receptors, ion channels, membrane proteins	Secretory carrier membrane protein (SCAMP3)	AF005036	1.78	0.27	1.59	0.05
	Neutral and basic amino acid transporter	U10110	1.78	0.38	1.64	0.14
	Anion exchanger 2	NM_017048	1.76	0.29	1.84	0.28
	Lymphatic endothelium-specific hyaluronan receptor Lyve-1	AA269330	1.88	0.11	0.58	0.01
Regulatory proteins, kinases, phosphatases	Myosin light chain kinase	AW142114	1.02	0.24	0.59	0.14
Signal transduction	Frizzled homolog 4	AW140615	1.2	0.09	0.55	0.19
Unknown	Unknown protein	AA259369	1.09	0.08	3.11	0.64
	Unknown protein	W34106	1.42	0.18	0.47	0.02
	Unknown protein	AA260880	0.92	0.25	0.54	0.21
	Unknown protein	AW539764	1.03	0.06	0.55	0.08
	Unknown protein	AA259659	1.42	0.25	0.57	0.04
	Unknown protein	AA277040	0.78	0.13	0.59	0.14
	Unknown protein		1.27	0.11	0.62	0.09

All experiments were done in duplicate and data were calculated from four intensity ratios. 'Isc' denotes ischemia/reperfusion, 'Norm' non-ischemic controls, and 'Prec' preconditioning.

citrate cycle. It is well known that ischemia/reperfusion results in mitochondrial damage leading to cell apoptosis or necrosis [21–23], however, little is known about the cellular mechanisms of these phenomena.

Lynn et al. studied the gene expression profile of ischemic injury produced by left coronary artery occlusion without reperfusion in mouse hearts. They used an array with only 588 gene-specific probes and found only a small number of genes affected by ischemia. Genes with altered expression were those encoding proteins implicated in oxidative stress, apoptosis and cardiac muscle development [6]. In a rat infarction model, a detailed gene expression analysis was performed using a microarray containing 7000 cDNAs [7] and several genes encoding proteins involved in cytoskeletal architecture, contractility, and metabolism were identified. In accordance with their findings, we found several genes in the present study which exhibited changes in expression in response to ischemia/reperfusion, i.e. heat shock proteins, ubiquinone oxidoreductase, ubiquinone binding protein, collagen, tubulin, and atrial natriuretic factor.

In our present study, several clones encoding hypothetical proteins or ESTs having no homology to known proteins exhibited significant up- or down-regulation. The cellular function of these genes and their relationship to myocardial ischemia/reperfusion needs to be elucidated.

3.2. Gene expression alteration due to preconditioning

To study the effects of preceding preconditioning on ischemia/reperfusion-induced gene expression patterns, we used cDNA microarrays to monitor alterations in gene expression of ischemic rat hearts with and without preconditioning. Genes exhibiting characteristic changes in expression due to preconditioning are shown in Table 3. Out of 3200 rat genes 1450 had significant intensity values but only 1% of them showed altered expression: 14 clones were overexpressed and 17 repressed (see Section 2 for statistical calculations).

In order to confirm the differential expression of genes revealed by microarray analysis of rat hearts after ischemia with and without preconditioning, several genes were analyzed by real-time fluorescent QRT-PCR. We selected seven genes of which the expression was significantly altered in preconditioned hearts for real-time RT-PCR analysis (Fig. 1). The differential expression of these genes revealed an almost perfect concordance with the microarray data. Genes encoding chaperonin subunit ε, anion exchange protein 2 and metallothionein II had a very significant rise in transcription rate,

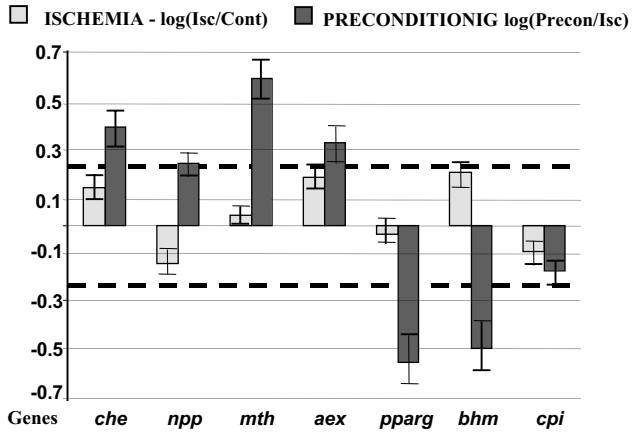

Fig. 1. Quantitative determination of transcript levels by real-time PCR. Changes in transcript levels in rat hearts during ischemia (light box) and ischemia with preconditioning (dark box) were confirmed by triplicate real-time PCR. β-Actin was used as a control. The expression of the following genes was determined: chaperonin subunit ε: *che*; natriuretic peptide precursor type B: *npp*; anion exchange protein 2: *aex*; metallothionein II: *mth*; peroxisome proliferator activator receptor γ: *pparg*; betaine-homocysteine methyltransferase: *bmh*; cysteine proteinase inhibitor: *cpi*. Dashed lines indicate the interval −1.8- to 1.8-fold regulation (corresponding to $\log_{10} = 0.255$) in which changes in expression were considered not significant.

while the natriuretic peptide precursor type B gene showed a less pronounced induction. Genes encoding peroxisome proliferator activator receptor γ (PPARγ) and betaine-homocysteine methyltransferase showed repression, although the cysteine proteinase inhibitor gene exhibited moderate repression at the mRNA level.

Changes in the expression of some genes by preconditioning followed by ischemia/reperfusion were similar to those changed by ischemia/reperfusion alone (overexpressed: a secretory membrane protein, an amino acid transporter, an anion exchanger, a ribosomal RNA and a chaperonin gene; repressed: β-tubulin). Because in the case of preconditioning the control sample was ischemia/reperfusion alone, the expression of these genes changed more dramatically when compared to non-ischemic controls. This suggests that these genes might have significant roles in ischemic adaptation of the heart during single ischemia without preconditioning as well. The rest of the genes listed in Table 3 are those which were specifically and differentially expressed in response to preconditioning and were not altered after single ischemia/reperfusion. Among these genes metallothionein, coagulation factor VII, cysteine proteinase inhibitor, PPARγ and myosin light chain kinase genes were previously shown to have connections with ischemia or other heart diseases [24–33]. Hypoxia preconditioning induced the expression of metallothionein in the brain [25]. We found here that the (2′,5′)oligoadenylate synthase gene showed overexpression in response to preconditioning. It has been previously shown by others that the mRNA level of this gene rose more than two- to three-fold after 24 h recovery from ischemia in the rat brain [26]. Therefore, it is plausible to speculate that oligoadenylate synthase might have a protective effect on the heart as well. Chaperonin subunit ε and natriuretic peptide precursor type B also exhibited up-regulation due to preconditioning. None of these genes has previously been shown to be involved in preconditioning.

A more dramatic induction was detected in the expression of a cGMP phosphodiesterase (PDE9A1). Alterations in cGMP levels in the heart have previously been shown in response to preconditioning [27], however, this is the first demonstration that the expression of a phosphodiesterase gene is altered due to preconditioning.

PPARγ exhibited one of the most pronounced repressions due to preconditioning. PPARγ has been shown to be involved in several cardiovascular pathologies including atherosclerosis and ischemic heart disease, however, this is the first demonstration that PPARγ plays a role in ischemic preconditioning. Interestingly, most of the previous studies show that pharmacological activation of PPARγ protects the ischemic heart [28–30]. In contrast, our present study shows that preconditioning leads to a marked repression of the PPARγ gene. This suggests that the role of PPARγ in ischemic injury and ischemic adaptation is still unclear.

Degradation of myocardial structural proteins in myocardial infarction has been shown to be reduced by a cysteine proteinase inhibitor [31]. In our present study a 1.59-fold repression was detected by microarray analysis and a 1.75-fold repression by real-time quantitative PCR. It seems that the activity of cysteine proteases is favored in preconditioning. Another gene related to protein degradation was also repressed: protease 28 subunit had a 1.79-fold repression; this gene has a regulatory function in proteasome for small protein

Table 4
Heart rate, coronary flow, and lactate dehydrogenase (LDH) release in non-ischemic time-matched control, ischemia/reperfusion, and preconditioning+ischemia/reperfusion groups at the beginning of perfusion, upon early reperfusion (rep.) and at the end of the reperfusion in isolated rat hearts

Group	Basal	5 min rep.	120 min rep.
Heart rate (beats/min)			
Non-ischemic control	244 ± 4	243 ± 5	244 ± 5
Ischemia/reperfusion	240 ± 4	246 ± 12	249 ± 9
Preconditioning+ischemia/reperfusion	248 ± 6	248 ± 12	246 ± 7
Coronary flow (ml/min/g)			
Non-ischemic control	14.7 ± 1.0	17.0 ± 1.0	17.0 ± 0.9
Ischemia/reperfusion	15.8 ± 1.1	15.7 ± 2.9	12.2 ± 1.2*
Preconditioning+ischemia/reperfusion	15.6 ± 1.2	16.3 ± 1.6	15.6 ± 0.9
LDH release (mU/min/g)			
Non-ischemic control	nd	nd	nd
Ischemia/reperfusion	nd	671 ± 98*	422 ± 72*
Preconditioning+ischemia/reperfusion	nd	148 ± 56*#	223 ± 57*

Data are means ± S.E.M.; nd, non-detectable, below the detection limit ($n = 5$–8 in each group).
*$P < 0.05$ vs. corresponding control, #$P < 0.05$ vs. ischemia/reperfusion.

substrate degradation [32] and has implications for oxidative stress [33].

3.3. 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 4, in the non-ischemic time-matched control group, heart rate and coronary flow were stable and no LDH release was detected which show the stable aerobic condition of the heart. Preconditioning significantly decreased postischemic LDH release showing the well-known cardioprotective effect of preconditioning in this model (Table 4). As the present study was performed in crystalloid-perfused isolated rat hearts and the analysis of gene expression was done using cardiac tissue that did not contain components of blood, the mechanisms of ischemia/reperfusion injury and preconditioning might be somewhat different in the present ex vivo experimental model as compared to in vivo situations. A further limitation of the present study is that analysis of cardiac tissue for gene expression pattern was done at the end of the 2-h reperfusion to allow time for mRNA accumulation and/or degradation. Therefore, the present study cannot distinguish between the 'trigger' and 'mediator' genes of preconditioning (see [2–4] for reviews); however, addressing this issue raises many technical problems in study design and needs further studies in the future.

In summary, here we present a number of genes of which the expression is significantly altered due to ischemia/reperfusion and preconditioning in the heart. Although some of the genes have previously been shown to play a role in ischemia/reperfusion injury, this is the first demonstration that most of the genes presented here are involved in classic preconditioning and ischemia/reperfusion. The newly identified genes in our present study might lead to a better understanding of the cellular mechanisms of the remarkable cardioprotection elicited by ischemic preconditioning.

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