Anesthesiology 2004; 100:474–88

Trigger-dependent Gene Expression Profiles in Cardiac Preconditioning

Evidence for Distinct Genetic Programs in Ischemic and Anesthetic Preconditioning

*Pavel Sergeev, Ph.D.,** Rafaela da Silva, M.S.,† *Eliana Lucchinetti, Ph.D.,** *Kathrin Zaugg, M.D.,‡ Thomas Pasch, M.D.,§ Marcus C. Schaub, M.D., Ph.D.,‖ Michael Zaugg, M.D., D.E.A.A. #*

Background: **DNA chips facilitate genomic-wide exploration of gene expression. The authors hypothesized that ischemic (IPC) and anesthetic preconditioning (APC) would differentially modulate gene expression in hearts.**

Methods: **Affymetrix rat U34A gene chips were used to explore the transcriptional response to IPC and APC, sustained ischemia (110 min) without reperfusion, and time-matched perfusion in isolated rat hearts. IPC was induced by three cycles of 5 min of ischemia, and APC was induced by 1.5 minimum alveolar concentration isoflurane (110 min). For each heart, a separate chip was used for hybridization. Data were analyzed for significant ≥ 2.0-fold changes in gene expression. Microarray results were confirmed by quantitative real-time reverse-transcription polymerase chain reaction.**

Results: **Of the 8,799 genes represented on U34A, 217 transcripts in the APC group, 234 in the IPC group, and 29 in the ischemia group displayed significant ≥ 2.0-fold up-regulation in messenger RNA levels, and 185 transcripts in the APC group, 55 in the IPC group, and 49 in the ischemia group displayed significant ≥ 2.0-fold down-regulation. Many of these transcripts were unknown genes. A high number of commonly regulated genes were found in IPC and APC (39 up-regulated, 17 down-regulated). Genes commonly regulated included those associated with cell defense (heat shock protein 10, aldose reductase, Bcl-x_S). Conversely, a pool of protective and antiprotective genes was differentially regulated in APC *versus* IPC (heat shock protein 27/70, programmed cell death 8), suggesting trigger-dependent transcriptome variability.**

Conclusions: **The novel microarray technology provides evidence for distinct cardioprotective phenotypes in IPC and APC. The observed transcriptional changes raise the possibility of a second window of protection by volatile anesthetics. The authors' molecular portraits are the first global genomic comparison between IPC and APC.**

SIMILAR to brief ischemic episodes, volatile anesthetics promote and establish a protective cellular state in cardiac tissue by complex multiple fast-acting phosphorylation signaling steps.[1] This phenomenon, called *classic* or *early preconditioning*, is transient and lost over the ensuing 1–2 h. However, the preconditioning stimulus further initiates and triggers a second long-lasting genomic response leading to increased *de novo* protein synthesis.[2,3] Although less protective than early preconditioning, this delayed cardiac protection, also called *late preconditioning* or the *second window of protection*, may be effective for up to 72 h and thus be more promising in terms of therapeutic implications.

Although many similarities exist between ischemic (IPC) and anesthetic preconditioning (APC), fundamental differences with respect to key signaling steps are increasingly emerging. These regard activation and translocation of protein kinase C isoforms to subcellular targets[4] as well as the role of mitogen-activated protein kinases in triggering and mediating IPC- and APC-induced cardioprotection.[5] Moreover, differences in the memory phase of early preconditioning were previously reported for both types of preconditioning, suggesting differential signal transduction pathways.[1] Finally, the results of three recent studies addressing the question of whether APC would be capable of eliciting late preconditioning similar to IPC were contradictory. No delayed cardiac protection was observed in a dog study 24 h after sustained exposure to isoflurane,[6] whereas a delayed cardioprotective role of isoflurane was found in a rabbit model[7] and against cytokine-induced injury in endothelial and vascular smooth muscle cells.[8]

Gene expression profiling is an emerging type of research and enables investigators to comprehensively assess the molecular response of thousands of genes across the entire genome with the potential to dissect complex physiologic processes such as the phenomenon of pre-

* Ph.D., Institute of Pharmacology and Toxicology, University of Zurich. † Ph.D. Student, Institute of Pharmacology and Toxicology, University of Zurich. ‡ M.D. and Ph.D. Student, Department of Medical Biophysics, University of Toronto, Canada, and the Advanced Medical Discovery Institute, Ontario Cancer Institute. § Professor and Chairman of the Institute of Anesthesiology, University Hospital Zurich. ‖ Professor of Pharmacology, Institute of Pharmacology and Toxicology, University of Zurich. # Director, Cardiovascular Anesthesia Laboratory, Institute of Anesthesiology, University Hospital Zurich, and Institute of Pharmacology and Toxicology, University of Zurich.

Received from the Institute of Anesthesiology, University Hospital Zurich, Zurich, Switzerland; the Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland; the Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; and the Advanced Medical Discovery Institute, Ontario Cancer Institute, Toronto, Ontario, Canada. Submitted for publication August 28, 2003. Accepted for publication November 18, 2003. Supported by grant No. 3200-063417.00 from the Swiss National Science Foundation, Bern, Switzerland; the Swiss Heart Foundation, Bern, Switzerland; the Swiss University Conference, Bern, Switzerland; the Hartmann-Müller Foundation, Zurich, Switzerland; the Functional Genomics Center, University of Zurich, Zurich, Switzerland; and Abbott Switzerland, Baar, Switzerland. Presented in part at the Annual Meeting of the American Society of Anesthesiologists, San Francisco, California, October 11–15, 2003. Dr. Sergeev and Mrs. da Silva contributed equally to this work.

Address reprint requests to PD Dr. M. Zaugg: Institute of Anesthesiology, University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland. Address electronic mail to: michael.zaugg@usz.ch. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

conditioning.[9–11] To date, no data are available with respect to trigger-dependent changes in gene expression patterns in APC *versus* IPC. Therefore, the current study served (1) to determine the molecular portraits of APC and IPC at the transcriptional level and (2) to discover transcriptome variability between volatile anesthetic- and ischemia-induced cardiac preconditioning. Specifically, it was hypothesized that IPC and APC would activate distinct protective and injury-related genetic programs. For this purpose, the powerful genetic technology of microarrays was applied.

The data collected in this investigation not only unravel a high number of in-parallel up-/down-regulated known and unknown genes in APC and IPC, but they also provide evidence for significant trigger-dependent transcriptome variability. Furthermore, they may serve as a new platform for designing future hypothesis-driven research projects in search of cardioprotective mechanisms.

Materials and Methods

This study conformed to the guidelines of the Animal Care and Use Committee of the University of Zurich (Zurich, Switzerland).

Perfusion Protocols of Isolated Rat Hearts

Hearts from heparinized (500 U intraperitoneal) and decapitated male Wistar rats (weight, 250 g) were quickly removed and perfused in a noncirculating Langendorff apparatus with Krebs-Henseleit buffer containing the following ingredients: 155 mM Na^+, 5.6 mM K^+, 138 mM Cl^-, 2.1 mM Ca^{2+}, 1.2 mM PO_4^{3-}, 25 mM HCO_3^-, 0.56 mM Mg^{2+}, 11 mM glucose. The buffer was saturated with 95% O_2–5% CO_2 (pH = 7.4, 37°C). Hearts were perfused at a constant pressure of 80 mmHg. A water-filled balloon-tipped catheter was inserted into the left ventricle through the left atrium. Left-ventricular end-diastolic pressure was adjusted to 3–7 mmHg during the initial equilibration, and the volume of the balloon was not changed thereafter. The distal end of the catheter was connected to a performance analyzer (Plugsys Modular System; Hugo Sachs, March-Hugstetten, Germany) by way of a pressure transducer. Perfusion pressure, epicardial electrocardiogram, and coronary flow (Transit Time Flowmeter type 700; Hugo Sachs) were simultaneously recorded on the same performance analyzer. All recorded data were digitized and processed on a personal computer using the software IsoHeart (Hugo-Sachs). Hearts were allowed to equilibrate for 10 min and to beat spontaneously in all experiments. The following experimental groups were defined: APC stimulus, IPC stimulus, ischemia, and control. For each experimental group, five hearts were prepared, and functional parameters were recorded. To obtain a strong stimulus on gene expression, APC was induced by administration of 110 min of isoflurane at 2.1% (vol/vol; 1.5 minimum alveolar concentration [MAC] in rats) (fig. 1), as previously described.[6–8] The buffer solution was equilibrated with isoflurane using an Isotec 3 vaporizer (Datex-Ohmeda, Tewksbury, MA) with an air bubbler. The delivered vapor concentration of isoflurane was continuously controlled by the infrared gas analyzer Capnomac Ultima (Datex-Ohmeda). The applied concentration of isoflurane was also measured in the buffer solution using a gas chromatograph (Perkin-Elmer, Norwalk, CT): 2.1% isoflurane (vol/vol; 1.5 MAC in rats at 37°C), 0.52 ± 0.04 mM. IPC was induced by three episodes of ischemia interspersed by 5 min of reperfusion and followed by 85 min of reperfusion (fig. 1). In the ischemia

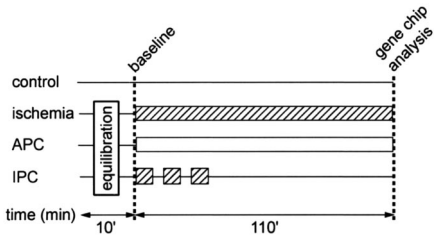

Fig. 1. Schematic diagram of the treatment protocols. For further description, see Materials and Methods. All hearts were subjected to 10 min of equilibration before exposure to treatment. *Hatched boxes/bar* = ischemia; *open bar* = 1.5 minimum alveolar concentration isoflurane (2.1 vol%). APC = anesthetic preconditioning; IPC = ischemic preconditioning.

Table 1. Primers Used for Quantitative Real-time Reverse-transcription Polymerase Chain Reaction

Accession No.*	Gene Product	Forward Primer	Reverse Primer
V01227	α Tubulin	CCATGCGTGAGTGTATCTCC	GTGCCAGTGCGAACTTCATC
X59375	Ribosomal protein S 27	GAAAGCGAGCACCTCATCTC	CTGAGCATGGCTGAAAACTG
AI170613	Heat shock protein 10	CTCTTGTGCCTTTCCTTTCC	TGGCTGGACAAGCTTTTAGG
AI229620	Cytochrome c oxidase subunit Vb	CAGCCAGAACCAGATGACAG	CTCCATGGCTTCTGAAGGTG
AI008888	Cystatin β	TTTGTCGGTCTGGTAAGAGG	AGATCGCCAACAAGGTGAAG
M60322	Aldose reductase	GGCTAGCCATCTGGAACTCA	CCTTTCACCATGCTCTGGTC
L26267	Nuclear factor κB	ACTCTGGCGCAGAAGTTAGG	TGTGCTGTCTTGGTTAGGAG
AI043968	Caveolin 3	TGGTAGGCTGAGCAGTTTCC	GTGGTGCCCTGCATTAAGAG
AI102562	Metallothionein	GTTCGTCACTTCAGGCACAG	CACCAGATCTCGGAATGGAC
AI014135	Cyclin-dependent kinase 103 homolog	CCGAAACCAAACGAGCTACC	GGCAACCAGCTATCACCAAG
M25297	Brain natriuretic peptide precursor	CAGCTCTCAAAGGACCAAGG	AGAGCTGGGGAAAGAAGAGC

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003).

group, hearts were subjected to 110 min of no-flow ischemia after 10 min of equilibration. The control group consisted of five hearts subjected to time-matched perfusion for 120 min.

Isolation of RNA and cDNA Synthesis

After completion of the protocols, left ventricular tissue was quickly frozen in liquid nitrogen and stored at −80°C. Frozen heart tissue (100 mg) was powdered in liquid nitrogen and homogenized in TRIzol LS reagent (Invitrogen, Basel, Switzerland) and chloroform–isoamyl alcohol (Fluka, Buchs, Switzerland). After centrifugation, the aqueous phase was mixed with isopropanol and precipitated overnight at −20°C. The pellet was washed with isopropanol, dried at 37°C, and eluted in diethyl pyrocarbonate–treated water. Single-strand complementary DNA (cDNA) synthesis from total RNA was performed using Superscript II (Invitrogen) in the presence of the T7-(T) 24 RNA polymerase promoter primer (Microsynth GmbH, Balgach, Switzerland). Double-strand cDNA was synthesized with a Superscript kit (Invitrogen). Biotin-labeled antisense cRNA was synthesized *in vitro* using a high-yield RNA transcript labeling kit (BioArray; Enzo, Farmingdale, NY).

Oligonucleotide Array Hybridization and Scanning

This project was conducted in close collaboration with the Functional Genomics Center of the University of Zurich. Gene expression profiling was performed with Affymetrix Rat Genome U34A array (Affymetrix, Santa Clara, CA). Gene chip U34A contains a total of 8,799 probe sets representing approximately 7,000 known rat genes and 1,000 expressed sequence tags (ESTs; unknown genes). Five independent gene chips for each group were used so that a total of 20 gene chips were analyzed. Importantly, no messenger RNA (mRNA) samples of different hearts were pooled. The biotin-labeled cRNA was fragmented in fragmentation buffer (200 mM Tris-acetate, 50 mM KOAc, and 150 mM MgOAc, at pH 8.1) and hybridized to the microarray in hybridization solution containing 15 μg fragmented cRNA in MES buffer (0.1 M MES, 1.0 M NaCl, and 0.01% Triton X-100, at pH 6.7) and herring sperm DNA. All arrays were placed in a hybridization oven at 60 rpm and 45°C for 16 h. Afterward, arrays were repeatedly washed at 22°C with SSPE-T (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, and 0.005% Triton X-100, at pH 7.6), and subsequently with 0.1 MES at 45°C for 30 min. The gene chips were then stained with a streptavidin–phycoerythrin conjugate (Molecular Probes, Leiden, The Netherlands) and washed. To enhance the signals, the arrays were further stained with antistreptavidin antibody followed by staining with a streptavidin–phycoerythrin conjugate and again washed. All gene chips were scanned at a resolution of 3 μm, using a specifically designed confocal scanner (model 900154; Affymetrix).

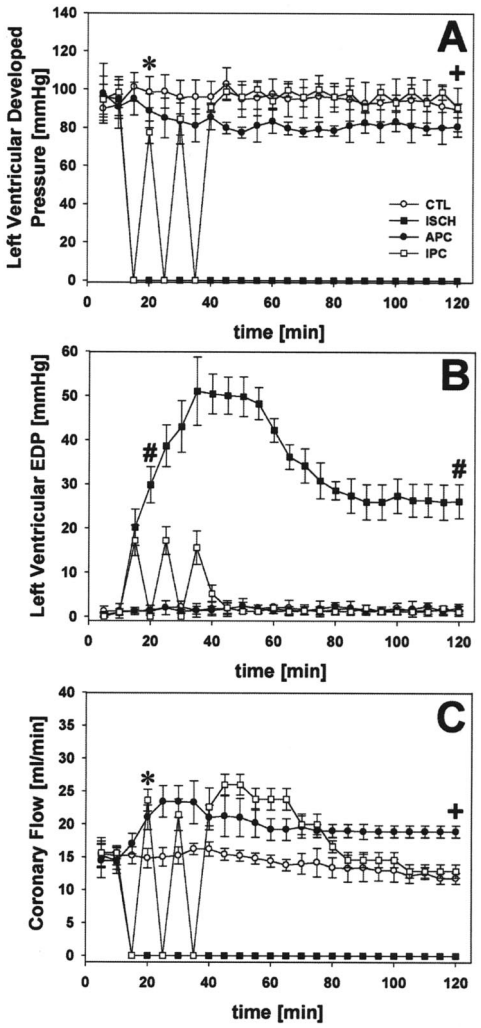

Fig. 2. Hemodynamic changes (*A–C*) in hearts subjected to the various treatment regimens (anesthetic preconditioning [APC], ischemic preconditioning [IPC], ischemia [ISCH], control [CTL]). Groups were compared after 20 min and 120 min. * APC, IPC, and ISCH significantly different compared with CTL ($P < 0.0125$). + APC and ISCH significantly different compared with ISCH ($P < 0.0125$). # ISCH significantly different compared with CTL ($P < 0.0125$). Values are presented as mean ± SD (n = 5 for each group). EDP = end-diastolic pressure.

Data Analysis

Briefly, for each gene, 16–20 probes with "perfect match" 25-bp oligonucleotides and 16 probes with paired "mismatched" oligonucleotides (designed with a single mismatch in the center position) were used to measure the transcript level of the gene (low, moderate, and high expression). Comparison of the hybridization signal from the perfect match and mismatched probes allows elimination of nonspecific cross-hybridization signals after background subtraction. Image data were analyzed using Affymetrix Microarray Analysis Suite detection algorithm (version 5.0), which assesses probe pair saturation, calculates a detection *P* value, and assigns a low, moderate, or high expression level to each individual probe set. Average differences between perfect

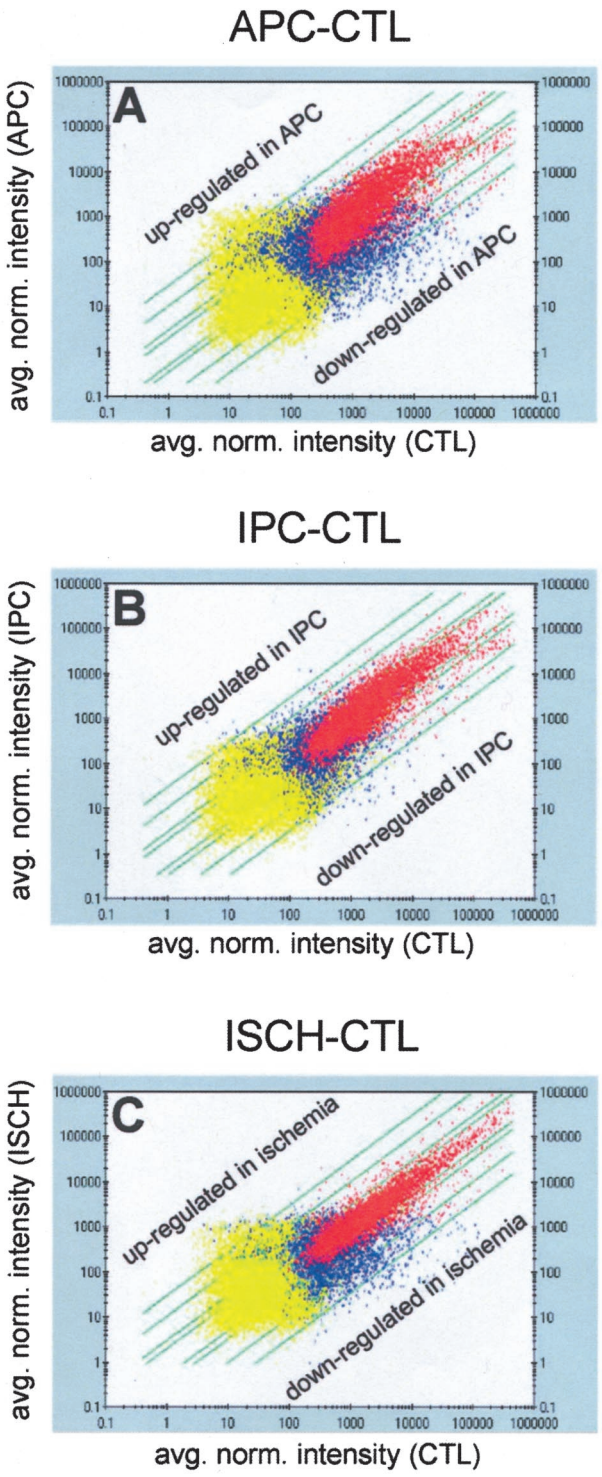

Fig. 3. Scatter plots (log scale) of the fluorescent intensity values as derived from Affymetrix gene chip analyses depicted in three graphs comparing each treatment group (anesthetic preconditioning [APC], ischemic preconditioning [IPC], ischemia [ISCH]) with the control group (CTL). (*A*) APC *versus* CTL. (*B*) IPC *versus* CTL. (*C*) ISCH *versus* CTL. Colors indicate expression level as defined by Affymetrix Suite algorithm (see Materials and Methods): *red* = high expression; *blue* = moderate expression; *yellow* = low expression. Genes aligning perfectly on the 45° straight line are not regulated, whereas genes below or above the 45° straight line indicate regulation. Parallel lines to the 45° straight line indicate 2.0-, 3.0-, 10-, and 30-fold changes.

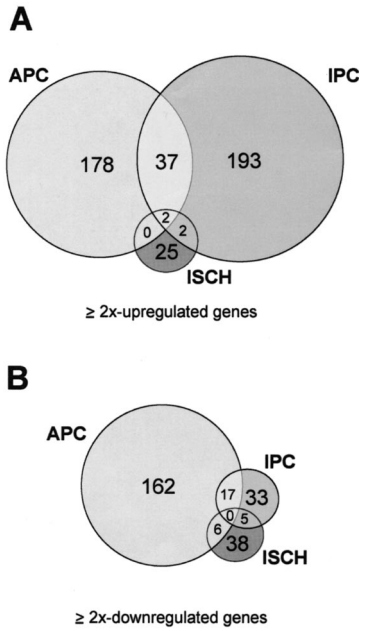

Fig. 4. Venn diagrams of transcripts significantly (≥ 2.0-fold) regulated in the three treatment regimens (anesthetic preconditioning [APC], ischemic preconditioning [IPC], ischemia [ISCH]). The areas of the *circles* proportionately reflect the number of regulated transcripts. (*A*) Transcripts up-regulated by 2.0-fold or greater. (*B*) Transcripts down-regulated by 2.0-fold or greater. Only two genes were up-regulated in all three treatment groups: solute carrier family 4 (accession No. J05166) and proteasome α type 6 (accession No. AA799492). None of the transcripts were commonly down-regulated in all three groups.

match and mismatch signals were used to calculate the relative mRNA levels of transcripts. A global scaling method was applied to normalize signals in each gene chip minimizing the overall variability in hybridization intensities. The purpose of this normalization is to minimize the systemic variations in the measured gene expression levels and to allow the comparisons across different gene chips. Scaled data were further normalized by dividing all raw measurements for each gene by the mean raw signal intensity for the same gene as obtained in the five control hearts (normalized ratio intensity value, fold change) (GeneSpring, version 5.1; Silicon Genetics, Redwood City, CA). Significantly differentially regulated genes were defined as those genes that had a normalized ratio intensity of 2.0 or greater, *i.e.*, 2.0-fold up-/down-regulation in expression level of replicate experiments compared with the control group. The stringent criteria of a twofold cutoff and a *P* value less than 0.0125 were selected to reduce false-positive findings.

Verification of Candidate Gene Expression Using Quantitative Real-time RT-PCR

To confirm the gene expression patterns of microarray hybridization, reverse-transcription (RT) polymerase chain reaction (PCR) was performed for nine selected genes, of which the expression level was significantly

Table 2. Selected Genes Up-regulated (≥2.0-Fold) in Response to APC as Compared with Control

Cluster/Gene Name	Accession No.*	Mean Ratio (APC/Control)	SD
Cell defense/death			
BCL2/adenovirus E1B 19-kd-interacting protein 3–like	AA859938	4.27	0.48
Defender against cell death 1	AI013627	2.94	0.39
DnaJ (Hsp-40) homolog, subfamily A, member 2	AI170685	3.96	0.85
Heat shock 10-kd protein 1 (chaperonin 10)	*AI170613*	*6.32*	*1.32*
Hypoxia-induced gene 1	AA891422	6.08	1.15
Programmed cell death 8 (apoptosis-inducing factor)	AA891591	4.75	0.87
26S proteasome, subunit p112	AJ006340	2.85	1.12
Proteasome (prosome, macropain) subunit, α type 6	*AA799492*	*4.25*	*2.04*
Stress 70 protein chaperone, microsome associated	AF006617	4.86	1.48
Cell structure/motility			
Disintegrin and metalloproteinase domain 17	AJ012603	3.71	1.08
Dynein, cytoplasmic, light intermediate chain 1	H31847	3.12	0.59
ESTs, highly similar to TBB1_rat tubulin β chain (T β-15)	*AA799591*	*5.27*	*1.02*
Vesicle transport related	D79221	2.66	0.74
Gene expression/protein synthesis			
ESTs, highly similar to leucine-zipper–like transcriptional regulator	AA858600	2.53	0.64
ESTs, highly similar to protein translocation complex β	AA874999	2.16	0.79
General transcription factor IIF, polypeptide 2 (30-kd subunit)	L01267	2.80	1.04
Nuclear RNA helicase	AF063447	3.82	1.81
Rab11a, member RAS oncogene family	*M75153*	*2.37*	*0.87*
Rat heart–derived c-ros-1 protooncogene	M35104	3.62	1.01
Ribosomal protein L10a†	*X93352*	*7.23*	*4.11*
RNA helicase	U25746	4.08	1.30
Growth/remodeling/inflammatory response			
Complement component 1, q subcomponent, β polypeptide	*X71127*	*6.22*	*3.49*
ESTs, Highly similar to tumor necrosis factor (ligand) superfamily, member 13	AA891690	3.82	1.39
Serine (or cysteine) proteinase inhibitor, clade A	AI010453	3.21	1.03
Serine protease inhibitor	*M38566*	*3.60*	*1.72*
Short isoform growth hormone receptor	S49003	6.51	3.63
Small inducible cytokine A5	AI009658	12.2	5.62
Metabolism			
Carboxylesterase 3	*AA800851*	*10.2*	*5.28*
Carnitine palmitoyltransferase 1	*L07736*	*3.95*	*0.87*
Glucose-dependent insulinotropic peptide	L08831	2.36	0.68
Isocitrate dehydrogenase 1, soluble	AA892314	2.52	0.90
Ornithine decarboxylase (ODC)	J04792	6.48	3.29
Phosphofructokinase C (PFK-C)	L25387	2.25	0.71
Xanthine dehydrogenase	AI172247	3.32	0.66
Redox state/respiratory chain			
Aldose reductase	*M60322*	*3.22*	*0.64*
ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit b, isoform 1	AA799778	3.84	0.36
ATP synthase, H^+ transporting, mitochondrial F1 complex, ε subunit	*AI171844*	*5.27*	*1.02*
Carbonyl reductase 1	D89069	2.51	0.48
Catalase	AA926149	2.79	0.75
Cytochrome c oxidase subunit VIIa 3	AA819708	6.00	3.40
Cytochrome p450e	K00996	3.28	1.04
ESTs, highly similar to mitochondria-associated granulocyte-macrophage CSF	*AA875664*	*8.04*	*4.76*
ESTs, highly similar to GTK1_RAT glutathione S-transferase, mitochondrial (glutathione S-transferase subunit 13)	*AI105137*	*3.90*	*1.55*
ESTs, moderately similar to T00741 NADH dehydrogenase (ubiquinone)	AA799336	5.30	2.80
Ferredoxin 1	AI044488	7.63	3.16
Peroxisome proliferator activator receptor, γ	AB011365	4.02	1.47
Peroxisome proliferator-inducible gene	*S83279*	*4.31*	*2.01*
Thiosulphate sulphurtransferase (rhodanese)	*X56228*	*5.76*	*1.78*
Signaling/communication			
α_{2A}-adrenergic receptor	S79778	2.36	0.73
Arrestin, β_2	M91590	2.26	0.56
Calcium channel, voltage-dependent, β_2 subunit	M80545	4.54	1.75
G-protein pathway suppressor 1	H31907	2.98	0.98
Calmodulin 1 (phosphorylase kinase, δ)	*AA892470*	*3.47*	*0.60*
FK506 binding protein 12-rapamycin associated protein 1	*U11681*	*2.92*	*1.05*
Nonreceptor protein tyrosine kinase	*S83358*	*6.20*	*2.93*
p38 mitogen-activated protein kinase 14 (p38MAPKα)	U73142	5.26	1.62

(*Table continues*)

Table 2. *Continued*

Cluster/Gene Name	Accession No.*	Mean Ratio (APC/Control)	SD
Phosphodiesterase 4B	AA799729	2.84	1.05
Precursor, rat parathyroid hormone-like peptide (PLP) gene	M34112	2.66	0.63
Protein tyrosine phosphatase, receptor type, F	*M60103*	*5.51*	*1.47*
Protein tyrosine phosphatase, receptor type, R	*D64050*	*11.2*	*5.73*
Potassium intermediate/small conductance calcium-activated channel	U69882	3.31	1.45
Solute carrier family 4, member 2, anion exchange protein 2	*J05166*	*3.12*	*0.63*

Genes were grouped according to functional clusters. Commonly regulated genes in both anesthetic (APC) and ischemic preconditioning (IPC) are shown in italics. Expressed sequence tags (ESTs) are sequences derived from complementary DNA libraries.

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003). † Seven ribosomal proteins were significantly up-regulated by the (APC) stimulus. Six thereof were also up-regulated in (IPC).

ATP = adenosine triphosphate; CSF = colony-stimulating factor; NADH = nicotinamide adenine dinucleotide.

altered (≥ 2.0-fold) by APC, IPC, or ischemia, respectively. The forward and reverse sequences of the used primers are listed in table 1. For each gene-specific amplification, 20 μl cDNA was diluted in water (1:10) before use as a template for the QuantiTect SybrGreen RT-PCR kit (Qiagen, Hilden, Germany). RT-PCR quantification and determination of expression levels were performed on an ABI Prism 7700 Sequence Detector Real-Time PCR machine (Perkin-Elmer, Foster City, CA). Amplification reactions were conducted with an initial step at 90°C for 3 min followed by 20–35 cycles. All PCR reactions were performed in triplicate, and α tubulin was used as a reference control. In addition, the predicted size of PCR products was confirmed by agarose gel electrophoresis.

Statistical Analysis

Data are expressed as mean ± SD. Functional parameters at identical time points and signal intensities of individual transcripts from microarray analyses were compared between groups by unpaired *t* tests. A *post hoc* Bonferroni test for multiple comparisons was performed to determine statistical significance. Accordingly, $P < 0.0125$ was considered to be statistically significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL), Affymetrix Microarray Analysis Suite (version 5), and GeneSpring (version 5.1) were used for the statistical analysis.

Results

Functional Parameters in APC- and IPC-triggering Protocols

The characteristic hemodynamic profiles of the triggering protocols and the respective control groups are depicted in figure 2. Left ventricular developed pressure was significantly decreased and coronary flow was increased in APC hearts, whereas IPC hearts exhibited short transient increases in left ventricular end-diastolic pressure and coronary flow (fig. 2). Prolonged normothermic ischemia was associated with a marked increase in left ventricular end-diastolic pressure.

APC and IPC Elicit Profound Changes in Gene Expression Pattern in Isolated Perfused Rat Hearts

Of a total of 8,799 probe sets displayed on the Affymetrix U34A microarray chip, 402 were significantly (≥ 2.0-fold) altered in APC as compared with the control group, of which 116 (29%) represented ESTs (unknown genes). Two hundred eighty-nine were significantly (≥ 2.0-fold) regulated in IPC (containing 60 ESTs [21%]), and 78 were significantly altered in the ischemia group (containing 18 ESTs [23%]). APC up-regulated 217 genes (containing 95 ESTs [44%]) and down-regulated 185 genes (containing 21 ESTs [11%]) by 2.0-fold or greater. IPC up-regulated 234 genes (containing 49 ESTs [20%]) and down-regulated 55 genes (containing 11 ESTs [20%]) by 2.0-fold or greater. Prolonged ischemia up-regulated 29 genes (containing 9 ESTs [31%]) and down-regulated 49 genes (containing 9 ESTs [18%]) by 2.0-fold or greater. A graphical representation of the gene regulation between the various groups is shown as scatter plots in figure 3. Interestingly, highly expressed genes were less regulated by IPC, APC, or even prolonged normothermic ischemia than genes with a moderate or low expression level. Collectively, APC and IPC triggered profound modulations of gene expression in cardiac tissue after only a short time.

APC and IPC Display Significant Overlapping in Up-/Down-regulated Genes

Anesthetic preconditioning and IPC commonly up-regulated 39 genes (containing 10 ESTs [26%]) and jointly down-regulated 17 genes (containing 2 ESTs [12%]) by 2.0-fold or greater (fig. 4). Many fewer up-/down-regulated genes were observed between APC *versus* ischemia and IPC *versus* ischemia, respectively. APC and ischemia commonly up-regulated two genes (no ESTs) and concomitantly down-regulated six genes (containing 1 EST [16%]) by 2.0-fold or greater, whereas IPC and ischemia commonly up-regulated four genes (no ESTs) and jointly down-regulated five genes (containing 2 ESTs [40%]) by 2.0-fold or greater (fig. 4). Notably, only two genes were jointly up-regulated by 2.0-fold or greater in all three treat-

Table 3. Selected Genes Down-regulated (≥2.0-Fold) in Response to APC as Compared with Control

Cluster/Gene Name	Accession No.	Mean Ratio (APC/Control)	SD
Cell defense/death			
70-kd heat shock protein precursor	S75280	0.18	0.16
bcl-x short (apoptosis inducer)	*S78284*	*0.22*	*0.14*
ESTs, highly similar to HS9B_RAT heat shock protein HSP 90-β	AI008074	0.19	0.21
Cell structure/motility			
Actin, β	AI179012	0.12	0.08
Cytoskeletal γ-actin (cytoplasmic-γ isoform)	X52815	0.38	0.23
Cytoskeletal linker protein (plectin)	*U96275*	*0.44*	*0.26*
Dynamin-like protein 1	AF020210	0.26	0.25
Laminin chain β 2	AA900848	0.21	0.08
Laminin, γ 1	X94551	0.32	0.19
Myosin heavy chain, polypeptide 6	*AI103920*	*0.05*	*0.03*
α-Actin cardiac protein	X80130	0.41	0.24
Embryonic skeletal muscle myosin heavy chain	L00370	0.41	0.14
Gene expression/protein synthesis			
Eukaryotic translation elongation factor 2	AA892801	0.04	0.06
rab11B, member RAS oncogene family	D01046	0.05	0.03
rab1B protein	X13905	0.07	0.03
Transcription factor AP-1	*X17163*	*0.10*	*0.04*
Transcription factor USP2	X90823	0.33	0.24
Growth/remodeling/inflammatory response			
Fibroblast growth factor receptor 1	D12498	0.22	0.22
Interleukin 4 receptor	X69903	0.41	0.20
Hepatocyte growth factor (scatter factor)	X54400	0.31	0.21
Interferon γ receptor	U68272	0.41	0.25
Platelet–endothelial cell adhesion molecule-1/CD31	U77697	0.19	0.14
Selectin, endothelial cell, ligand	AI176461	0.12	0.04
Tissue inhibitor of metalloproteinase 3	U27201	0.32	0.11
Vascular endothelial growth factor	M32167	0.14	0.09
Metabolism			
Aldolase A, fructose-bisphosphate	AA924326	0.25	0.24
Carnitine palmitoyltransferase Iβ 1	AF063302	0.14	0.05
Fatty acid coenzyme A ligase, long chain 2	AA893242	0.18	0.09
Glutamate oxaloacetate transaminase 2, mitochondrial	AA892012	0.06	0.07
Hydroxyacyl-coenzyme A dehydrogenase, α subunit	X98225	0.20	0.11
Lysophospholipase 1	AA891633	0.42	0.21
Methionine adenosyltransferase II, α	J05571	0.17	0.13
Mitochondrial acyl-CoA thioesterase 1	*Y09333*	*0.29*	*0.32*
Pyruvate dehydrogenase E1 α form 1 subunit	Z12158	0.20	0.13
Redox state/respiratory chain			
ATPase, class II, type 9A	U78977	0.36	0.22
ATPase, H^+ transporting, lysosomal, β 56/58-kd, isoform 2	Y12635	0.12	0.10
ESTs, highly similar to S41115 probable flavoprotein-ubiquinone oxidoreductase	AI176422	0.36	0.36
Peroxiredoxin 5	Y17295	0.29	0.16
NADH-cytochrome b5 reductase	D00636	0.22	0.15
Signaling/communication			
ATP-binding cassette, subfamily C, member 9	AF087838	0.16	0.15
Sulfonylurea receptor 2 (SUR2)	AF087839	0.16	0.12
Ca^{2+}/calmodulin-dependent protein kinase II α, protein serine/threonine kinase	J02942	0.27	0.17
Calcitonin receptor-like receptor, G protein–coupled receptor activity	L27487	0.39	0.14
Chemokine receptor (LCR1)	U90610	0.36	0.16
Cholinergic receptor, nicotinic, β polypeptide 3	J04636	0.38	0.21
Cytokine inducible SH2-containing protein 3	*AF075383*	*0.14*	*0.02*
FK506 binding protein 4	AI136977	0.14	0.14
Connexin 43	AI029183	0.14	0.12
Inducible nitric oxide synthase (iNOS)	S71597	0.30	0.16
Insulin receptor	M29014	0.49	0.15
L-type voltage-gated calcium channel, calcium channel α_{1C} subunit	U31815	0.24	0.20
Mitogen-activated protein kinase kinase 2	*AA963674*	*0.15*	*0.06*
Phospholipase A2	U03763	0.15	0.05
Protein kinase inhibitor, α	L02615	0.40	0.07
Protein-tyrosine phosphatase, nonreceptor type 7	U28356	0.35	0.05
PKC-ζ–interacting protein	Y08355	0.26	0.22
Calcium/calmodulin-dependent protein kinase II δ subunit	L13407	0.07	0.05

(*Table continues*)

Table 3. *Continued*

Cluster/Gene Name	Accession No.	Mean Ratio (APC/Control)	SD
Calcium/calmodulin-dependent protein kinase II δ subunit	L13407	0.07	0.05
Endothelial nitric oxide synthase (eNOS)	AF110508	0.38	0.24
Ryanodine receptor type II	U95157	0.19	0.11
Gq-α subunit	Y17164	0.39	0.21
Receptor tyrosine kinase	AF041838	0.31	0.11
RhoA-binding serine/threosine kinase α	*U38481*	*0.10*	*0.04*
Voltage-dependent anion channel 1	AF048828	0.04	0.02

Genes were grouped according to functional clusters. Commonly regulated genes in both anesthetic (APC) and ischemic preconditioning (IPC) are shown in italics. Expressed sequence tags (ESTs) are sequences derived from complementary DNA libraries.

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003).

ATP = adenosine triphosphate; CoA = coenzyme A; NADH = nicotinamide adenine dinucleotide; PKC = protein kinase C.

ments (APC, IPC, and ischemia), but none of the transcripts was commonly down-regulated.

APC and IPC Exhibit Transcriptome Variability Consistent with Two Distinct Cardioprotective Phenotypes

Representative genes up-/down-regulated by 2.0-fold or greater are listed in tables 2–7. Genes were sorted according to the six functional clusters: (1) cell defense/death, (2) cell structure/motility, (3) growth/remodeling/inflammatory response, (4) metabolism, (5) redox state/respiratory chain, and (6) signaling/communication. To confirm the results of microarray hybridization experiments, RT-PCR was used as an independent method of measuring gene expression levels. RT-PCR results of nine selected genes revealed an almost perfect concordance with microarray data and hence clearly confirmed the accuracy of the gene chip data.

Anesthetic preconditioning and IPC showed significant changes in all of the clusters. APC and IPC commonly up-regulated the cytoprotective heat shock protein (Hsp) 10. In contrast, APC down-regulated Hsp-70 precursor and Hsp-90, whereas IPC up-regulated Hsp-70. Both APC and IPC down-regulated Bcl-x_S, a proapoptotic protein, whereas prolonged ischemia did not, and commonly up-regulated proteasome, a product involved in cell protection. Conversely, APC exclusively increased Hsp-40 and defender against cell death 1 protein, whereas IPC decreased proapoptotic GATA-binding protein 4. Interestingly, APC but not IPC increased gene expression of proapoptotic protein programmed cell death 8. Similarities and disparities were also observed in the redox state/respiratory chain functional cluster. Antioxidant catalase and cytochrome p450 were exclusively up-regulated in APC, but glutathione peroxidase 1 and rhodanese were up-regulated in IPC. Antioxidant aldose reductase, glutathione S-transferase, and ubiquinone nicotinamide adenine dinucleotide dehydrogenase were up-regulated in both types of preconditioning. Prominent changes in metabolic enzymes and growth-related factors were present in APC and IPC. Profound differences between APC and IPC were also observed in signaling components. Whereas both APC and IPC increased receptor tyrosine phosphatase and nonreceptor tyrosine kinase and decreased RhoA-serine/threonine kinase α and inducible nitric oxide synthase, only APC increased expression of p38 mitogen-activated protein kinase α and decreased phosphodiesterase 4B and endothelial nitric oxide synthase expression levels.

APC and IPC Promote Expression of Gene Products Involved in Delayed Cardioprotection

To address the question of whether gene products previously reported to participate in delayed cardioprotection would be differentially regulated in APC and IPC, microarray data were also examined for genes with changes of less than 2.0-fold. The use of change of 2.0-fold or greater may result in neglecting genes with less than 2.0-fold but statistically significant changes. Figure 5 depicts microarray data for nuclear factor κB, cyclooxygenase 2, manganese superoxide dismutase, inducible nitric oxide synthase, aldose reductase, Bcl-x_S, Hsp-27, and Hsp-70 in all groups. Whereas cyclooxygenase 2 and manganese superoxide dismutase were not regulated by APC and IPC at the investigated time point, both types of preconditioning up-regulated aldose-reductase and down-regulated Bcl-x_S. In contrast, Hsp-27 and Hsp-70 were solely up-regulated in IPC. Nuclear factor κB was significantly up-regulated in APC but also showed a tendency toward up-regulation in IPC. These data suggest that APC might be capable to promote the development of delayed cardioprotection as observed in IPC. This notion is further supported by the fact that APC, similarly to IPC, up-regulated a high number of ribosomal proteins, albeit to a lesser degree (7 in APC *vs.* 38 in IPC), increasing the efficiency of the cellular machinery for protein synthesis. Interestingly, 6 ribosomal proteins were similarly up-regulated in APC and IPC.

Discussion

The principal new findings of this comprehensive study are as follows. First, administration of isoflurane at

Table 4. Selected Genes Up-regulated (≥2.0-Fold) in Response to IPC as Compared with Control

Cluster/Gene Name	Accession No.*	Mean Ratio (IPC/Control)	SD
Cell defense/death			
Heat shock 10–kd protein 1 (chaperonin 10)	*AI170613*	*2.50*	*0.46*
Heat shock 27-kd protein	AI176658	2.85	1.06
Heat shock protein 70–1	L16764	4.95	0.80
Proteasome (prosome, macropain) 26S subunit, ATPase, 4	D50695	3.21	0.87
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	AA799887	2.49	0.77
Proteasome (prosome, macropain) subunit, α type 6	*AA799492*	*10.1*	*5.81*
Proteasome (prosome, macropain) subunit, β type 1	AA849722	2.17	0.49
Proteasome (prosome, macropain) subunit, β type 4	L17127	2.83	0.88
Proteasome (prosome, macropain) subunit, β type 6	D10754	3.96	1.24
Cell structure/motility			
Dynein, cytoplasmic, light chain 1	AI009806	2.15	0.38
ESTs, highly similar to TBB1_rat tubulin β chain	*AA799591*	*2.11*	*0.52*
ESTs, moderately similar to AAC1_RAT α-actinin 1 (cytoskeletal isoform)	AA800206	2.74	0.73
Ferritin light chain 1	AI231807	2.62	0.70
Laminin receptor 1	D25224	2.34	0.61
Myosin regulatory light chain	D14688	2.32	0.51
Prohibitin	AI169631	2.62	0.84
Skeletal muscle actin	J00692	2.13	0.57
Troponin 1, type 3	M92074	2.12	0.29
Unconventional myosin Myr2 I heavy chain	AI013129	2.96	0.68
Vimentin	X62952	2.13	0.40
Gene expression/protein synthesis			
DNA polymerase β	U38801	5.31	2.85
G elongation factor	L14684		
Mitochondrial ribosomal protein L23	U62635	3.48	0.87
Rab11a, member RAS oncogene family	*M75153*	*2.01*	*0.59*
Rab3B protein	AA799389	3.18	1.16
Ribosomal protein L10a†	*X93352*	*4.01*	*1.00*
Topoisomerase (DNA) 2 α	AI228599	2.64	0.30
Transcription elongation factor B (SIII) polypeptide 2	L42855	3.06	1.00
Growth/remodeling/inflammatory response			
Chemokine orphan receptor 1	AJ010828	2.15	0.31
Collagen, type 1, α 1	AI231472	2.34	0.72
Complement component 1, q subcomponent binding protein	AI178135	2.49	0.56
Complement component 1, q subcomponent, β polypeptide	*X71127*	*5.10*	*1.82*
Cystatin 3	AI231292	3.00	1.02
Cysteine rich protein 2	U44948	2.11	0.12
ESTs, highly similar to mitochondria associated granulocyte-macrophage CSF	*AA875664*	*4.83*	*2.53*
Insulin-like growth factor binding protein 3	M31837	2.33	0.43
Lectin, galactose binding, soluble 3	J02962	2.86	1.05
Macrophage migration inhibitory factor	S73424	2.42	0.77
Plasminogen activator, tissue	M23697	4.55	2.05
Gelatinase A	U65656	2.64	0.78
Serine protease inhibitor	*M38566*	*2.20*	*0.73*
Tissue inhibitor of metalloproteinase 1	AI169327	5.01	1.93
Metabolism			
3-Hydroxy-3-methylglutaryl CoA lyase	AI171090	2.08	0.5
Aldehyde dehydrogenase family 3, subfamily A2	M73714	3.16	1.25
Carboxylesterase 3	*AA800851*	*3.98*	*0.78*
Carnitine palmitoyltransferase 1	*L07736*	*2.03*	*0.62*
Carnitine palmitoyltransferase 2	J05470	2.20	0.70
γ-Glutamyl carboxylase	AF065387	2.45	0.43
Hexokinase 2	D26393	3.33	0.31
Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A	D16478	2.07	0.43
Phosphofructokinase, muscle	U25651	2.28	0.81
Redox state/respiratory chain			
Aldose reductase	*M60322*	*3.34*	*0.98*
ATP synthase, mitochondrial F1 complex, ε subunit	*AI171844*	*3.15*	*0.86*
ATP synthase, mitochondrial F1 complex, O subunit	D13127	3.54	0.78
ATPase inhibitor	D13122	2.21	0.54
ATPase, vacuolar	U43175	3.08	0.99
Glutathione S transferase	E01415	2.65	0.89
Carbonyl reductase 1	X95986	2.79	0.74

(*Table continues*)

Table 4. *Continued*

Cluster/Gene Name	Accession No.*	Mean Ratio (IPC/Control)	SD
Cytochrome c oxidase subunit VIII-H (heart/muscle), cytochrome c oxidase subunit VI-a polypeptide 2 (heart)	X64827	4.01	1.33
ESTs, highly similar to GTK1_RAT glutathione S-transferase, mitochondrial (glutathione S-transferase subunit 13)	*AI105137*	*2.43*	*0.87*
ESTs, moderately similar to NADH dehydrogenase (ubiquinone) 1 α Subcomplex, 8	AI232012	3.84	1.46
Glutathione peroxidase 1	X07365	3.60	1.56
Glutathione S-transferase, μ5	U86635	5.39	2.95
Thiosulfate sulphurtransferase (rhodanese)	*X56228*	*5.25*	*2.05*
Signaling/communication			
Annexin VI	X86086	2.05	0.58
Calmodulin 1 (phosphorylase kinase, δ)	*AA892470*	*2.41*	*0.34*
Cyclophilin B	AF071225	2.84	0.69
FK506 binding protein 12-rapamycin associated protein 1	*U11681*	*2.51*	*0.84*
Guanine nucleotide binding protein, beta polypeptide 2–like 1	U03390	2.37	0.70
Integrin-linked kinase	AA800015	2.96	0.80
Nonreceptor protein tyrosine kinase	*S83358*	*2.69*	*0.59*
Protein kinase C δ subspecies	M18330	2.36	0.58
Protein tyrosine phosphatase, receptor type, F	*M60103*	*4.74*	*1.32*
Protein tyrosine phosphatase, receptor type, R	*D64050*	*5.73*	*1.72*
Solute carrier family 4, member 2, anion exchange protein 2	*J05166*	*3.33*	*1.24*

Genes were grouped according to functional clusters. Commonly regulated genes in both anesthetic (APC) and ischemic preconditioning (IPC) are shown in italics. Expressed sequence tags (ESTs) are sequences derived from complementary DNA libraries.

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003). † Thirty-eight ribosomal proteins were significantly up-regulated by the IPC stimulus. Six thereof were also up-regulated in APC.

ATP = adenosine triphosphate; CoA = coenzyme A; CSF = colony-stimulating factor; NADH = nicotinamide adenine dinucleotide.

clinically relevant concentrations profoundly alters the genetic program in the myocardium, comparable with brief ischemic episodes. This change in gene expression pattern directly reflects the pharmacologic power of volatile anesthetics and further implies sustained biologic effects by this class of drugs. Second, APC and IPC exhibit a high number of commonly regulated genes, including those associated with cell defense, structure

Table 5. Selected Genes Down-regulated (≥2.0-Fold) in Response to IPC as Compared with Control

Cluster/Gene Name	Accession No.*	Mean Ratio (IPC/Control)	SD
Cell defense/death			
bcl-x short (apoptosis inducer)	*S78284*	*0.36*	*0.15*
GATA-binding protein 4	AI234969	0.28	0.20
Cell structure/motility			
Cytoskeletal linker protein (plectin)	*U96275*	*0.47*	*0.22*
Myosin heavy chain, polypeptide 6	*AI103920*	*0.10*	*0.02*
Gene expression/protein synthesis			
Transcription factor AP-1	*X17163*	*0.15*	*0.11*
Growth/remodeling/inflammatory response			
Nerve growth factor inducible	M74223	0.23	0.09
Metabolism			
Mitochondrial acyl-CoA thioesterase 1	*Y09333*	*0.48*	*0.30*
Signaling/communication			
Arachidonic acid epoxygenase	X55446	0.27	0.15
ATPase, Ca^{2+} transporting, plasma membrane 4	U15408	0.27	0.06
Cytokine inducible SH2-containing protein 3	*AF075383*	*0.32*	*0.15*
G protein–coupled receptor kinase 2	AA944254	0.26	0.14
Nitric oxide synthase type II, inducible (NOS2)	U48829	0.28	0.20
Mitogen-activated protein kinase kinase 2	*AA963674*	*0.21*	*0.10*
L-type voltage-dependent calcium channel (VDCC) α_1 subunit variable region (IVS3)	M89924	0.20	0.09
RhoA-binding serine/threosine kinase α	*U38481*	*0.10*	*0.08*
Stress-activated protein kinase α II	AI231354	0.45	0.33

Genes were grouped according to functional clusters. Commonly regulated genes in both APC and IPC are shown in italics.

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003).

ATP = adenosine triphosphate; CoA = coenzyme A.

Table 6. Selected Genes Up-regulated (≥2.0-Fold) in Response to Ischemia as Compared with Control

Cluster/Gene Name	Accession No.*	Mean Ratio (Ischemia/Control)	SD
Cell defense/death			
Proteasome (prosome, macropain) subunit, α type 6	AA799492	11.6	4.93
mud-5	U70265	13.8	8.76
Cell structure/motility			
Integrin α E1, epithelial associated	AF020045	2.80	0.61
Gene expression/protein synthesis			
Cyclin B1	AA998164	2.36	0.51
Growth/remodeling/inflammatory response			
Fibroblast growth factor 14	AB008908	2.78	0.87
Developmentally regulated cardiac factor (DRCF-1)	U90260	4.32	1.98
Redox state/respiratory chain			
Glutathione S-transferase, μ5	U86635	5.20	1.07
Hydroxyacid oxidase (glycolate oxidase) 3	AI232087	2.85	1.14
Signaling/communication			
Putative; protein tyrosine phosphatase-like protein	D38222	3.06	1.22
Shab-related delayed-rectifier K^+ channel (Kv9.3)	Y17607	3.25	1.10
Solute carrier family 4, member 2, anion exchange protein 2	J05166	3.99	1.93

Genes were grouped according to functional clusters.

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003).

and motility, growth and remodeling, metabolism, redox state, and signaling. In contrast, they share few similarities with gene regulation under prolonged ischemia. Surprisingly, the degree of concordance with sustained ischemia was not higher in IPC (nine transcripts), although containing a 15-min exposure to ischemia, than in APC (eight transcripts). Moreover, APC and IPC similarly modulated transcription of those gene products that were previously reported to be involved in delayed cardioprotection, providing some molecular evidence for a possible, albeit less pronounced, development of delayed protection in APC. Third, despite these similarities, fundamental differences with respect to protective and antiprotective genes were identified in APC and IPC and imply distinct cardioprotective mechanisms. Among the most prominent differentially regulated genes were Hsp-27 and Hsp-70, antioxidant proteins, and signaling components. Finally, numerous regulated but unknown genes were discovered in APC. These genes may be valuable candidates to expand and foster a more integra-

Table 7. Selected Genes Down-regulated (≥2.0-Fold) in Response to Ischemia as Compared with Control

Cluster/Gene Name	Accession No.*	Mean Ratio (Ischemia/Control)	SD
Cell defense/death			
Heat shock 27-kd protein	M86389	0.28	0.19
Cell structure/motility			
Ankyrin 3 (G)	AI058601	0.15	0.08
Gene expression/protein synthesis			
Activating transcription factor 3	M63282	0.16	0.10
Ankyrin-like repeat protein	U50736	0.42	0.27
Growth arrest and DNA damage-inducible 45 α	L32591	0.46	0.14
Growth factor independent 1	L06986	0.21	0.14
Interferon regulatory factor 1	M34253	0.33	0.20
Interferon-related developmental regulator 1	AI014163	0.28	0.13
Growth/remodeling/inflammatory response			
Chemokine receptor (LCR1)	U90610	0.27	0.16
Early growth response 1	M18416	0.32	0.22
Intercellular adhesion molecule 1	D00913	0.31	0.12
Metallothionein	AI102562	0.35	0.08
Serine (or cysteine) proteinase inhibitor, member 1	M24067	0.25	0.16
Redox state/respiratory chain			
Cytochrome P450, subfamily 1B, polypeptide 1	U09540	0.30	0.34
Heme oxygenase	AI179610	0.29	0.22
Signaling/communication			
Potassium inwardly rectifying channel, subfamily J, member 10	X83585	0.31	0.14
Protein phosphatase 1M 110-kd regulatory subunit	S74907	0.28	0.26

Genes were grouped according to functional clusters.

* Accession No. represents the identifier as defined by www.ncbi.nlm.nih.gov/LocusLink/(accessed August 15, 2003).

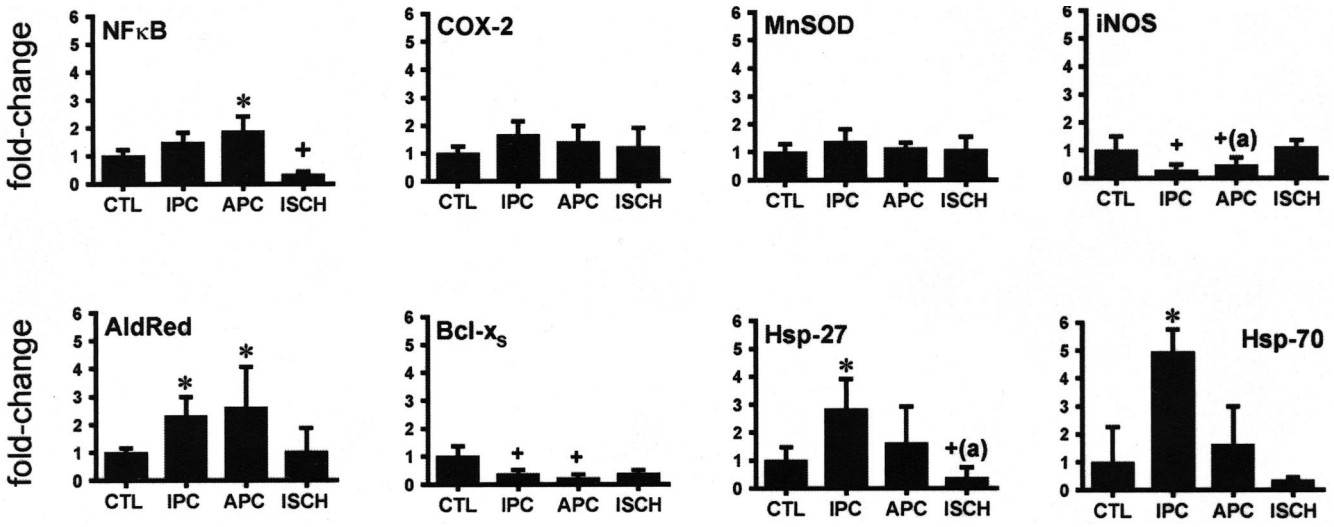

Fig. 5. Microarray data for transcripts previously reported to be involved in delayed cardioprotection. AldRed = aldose reductase; APC = anesthetic preconditioning; Bcl-x_S = Bcl family member x short; COX-2 = cyclooxygenase 2; CTL = control; Hsp-27 = heat shock protein 27 (probe set for accession No. AI176658); Hsp-70 = heat shock protein 70; iNOS = inducible nitric oxide synthase (probe set accession No. U48829); IPC = ischemic preconditioning; ISCH = ischemia; MnSOD = manganese superoxide dismutase; NF-κB = nuclear factor κB. * Significantly up-regulated compared with CTL ($P < 0.0125$). + Significantly down-regulated compared with CTL ($P < 0.0125$). +(a) Significantly down-regulated in probe set accession No. S71597 (iNOS) and in probe set accession No. M86389 (Hsp-27) ($P < 0.0125$). Data are presented as mean ± SD (n = 5 for each group).

tive understanding of APC in the future and may serve to generate new hypotheses in search of cardioprotective mechanisms in APC.

Gene Chip Analysis

Recently, attention has been directed toward cDNA microarray technology, which offers a genetic approach to explore molecular mechanisms on a genomic-wide scale.[9,10] Microarray technology makes it possible to study simultaneously thousands of genes in the same sample. In the current study, Affymetrix gene chip U34A, containing more than 8,000 known and unknown genes, was used to characterize the expression profiles after administration of APC and IPC triggers. Using rigorous and complex statistical analysis,[9] the expression levels of the individual genes were determined by means of their specific hybridization characteristics. Notably, in this study, five chips for each group were used to overcome inevitable variability in expression levels across experiments and to minimize false-positive results. No data from different experiments were pooled. Moreover, microarray data from selected genes could be clearly confirmed by the independent method of RT-PCR highlighting the accuracy of data obtained by gene chip analysis.

Activation of Distinct Protective Gene Programs by APC and IPC

Activation of a genetic survival program occurred in both preconditioning protocols. Heat shock proteins act as protein chaperones, protecting vital protein structure and function.[12] They are strategically located in the cytoplasm, peroxisomes, mitochondria, and endoplasmic reticulum. Whereas Hsp-27 scavenges cytochrome c, inhibits autoactivation of caspase 3, and blocks Fas/Fas ligand proapoptotic pathway,[13] Hsp-70 in collaboration with Hsp-40 prevents mitochondrial cytochrome c release and inhibits caspase 9 activation *via* Apaf-1,[14] thereby protecting against cell death. Another chaperone, Hsp-10, is predominantly located in mitochondria and acts as a functional partner of Hsp-60, opposing the deleterious effects of cell death promoting Bax.[15] In our study, APC and IPC similarly up-regulated Hsp-10. Unlike APC, IPC exclusively up-regulated Hsp-27 and Hsp-70, which was previously reported to also prevent caspase-independent cell death by c-Jun N-terminal kinase activation.[16] Unexpectedly, APC down-regulated Hsp-70 precursor and Hsp-90, which, however, may reflect the prolonged application of the preconditioning stimulus in APC.

Bcl-2 family members regulate the delicate balance between cell death and survival. They are divided by function and homologous sequences into proapoptotic and antiapoptotic members. Proapoptotic Bcl-2 family proteins such as Bcl-x_S are normally sequestered at the endoplasmic reticulum and nuclear membrane, and on noxious stimulation, they translocate to mitochondria, where they facilitate pore formation and the release of apoptogenic substances.[17] Our data now provide evidence that APC and IPC markedly down-regulate antiprotective Bcl-x_S. Intriguingly, the proteasome, a major machine for proapoptotic protein degradation, was also up-regulated in both types of preconditioning.[18] Also, AP-1, a component previously linked to cell death, was repressed in APC and IPC.[19] In contrast to IPC, APC differentially regulated several other cell defense–related

factors, including programmed cell death 8 and defender against apoptotic death 1 (Dad1).[20] The significance of these factors is poorly defined in IPC and not yet determined in APC and clearly requires further elucidation. Collectively, changes in these critical proteins will clearly affect the final outcome after prolonged ischemic injury.

The production of reactive oxygen species is both essential and detrimental to life. Previous studies have shown that oxidative signaling is intricately involved in the establishment of the preconditioned state.[21,22] However, delayed preconditioning induces a marked cellular defense against oxidative damage to proteins. The most striking common changes in the expression of antioxidant proteins were the increases in the chemoprotective enzymes glutathione S-transferase, which was down-regulated in prolonged ischemia, and aldose reductase. Aldose reductase is a member of the aldo-keto reductase superfamily, metabolizes toxic aldehydes generated by lipid oxidation, and is an obligatory mediator of delayed protection in IPC.[23] Despite these similarities between APC and IPC, the results of this study now indicate that APC- and IPC-induced cardioprotective phenotypes may differentially cope with oxidative stress. APC up-regulates catalase and cytochrome p450, whereas IPC up-regulates glutathione peroxidase 1 and rhodanese.

Characteristic patterns of growth-related and inflammatory response–related factors were modulated in IPC and APC. The importance of cytokines in the second window of IPC was previously established.[24] APC induced tumor necrosis factor member 13 and decreased—among other growth-related factors—endothelial selectin, platelet–endothelial cell adhesion molecule, and vascular endothelial growth factor. Alterations in these factors may be implicated in recently reported APC-induced decreased adhesion of leukocytes after ischemia–reperfusion.[25] In contrast, IPC up-regulated insulin-like growth factor protein 3, which may inhibit the effects of insulin-like growth factor I.[26] Both types of preconditioning up-regulated the antiinflammatory serine protease inhibitor.

High rates of fatty acid oxidation significantly contribute to myocardial ischemic injury. In accordance with this concept, APC and IPC rather repressed key enzymes of cellular β oxidation and increased expression of enzymes involved in glycolysis. In contrast, APC and IPC increased the expression level of carnitine palmitoyltransferase I, a key regulator of the long-chain fatty acid oxidation. Interestingly, inhibition of carnitine palmitoyltransferase I was previously shown to augment palmitate-induced myocyte apoptosis.[27]

Molecular Evidence for Delayed Cardioprotection by APC

In the current study, several factors previously reported to be involved in delayed cardiac protection were evaluated with respect to their transcriptional response on APC and IPC triggering. Apart from Bcl-2 family members and aldose reductase, these comprise nitric oxide,[28] inducible nitric oxide synthase,[29] cyclooxygenase 2,[30] protein kinase C,[31] Src family of protein tyrosine kinases,[32] and nuclear factor κB.[33] Although the interval between the application of the trigger stimuli and the measurement of the transcriptional response was limited in the model used and may not have allowed the detection of a fully developed delayed cardioprotective phenotype, the data of this study are consistent with the development of some sort of delayed protection in APC. Das *et al.*[34] previously evaluated the adaptation of cellular defenses after one or four cycles of 5 min of ischemia interspersed by 5 min of reperfusion in a buffer-perfused rat heart model. Using the highly sensitive Northern hybridization technique for a limited number of transcripts, they found up-regulation of Hsp-27, Hsp-70, Hsp-89, catalase, manganese superoxide dismutase, and glutathione reductase after four preconditioning cycles but not after a single cycle. In our study, a significant increase in nuclear factor κB expression was present in APC and there was a clear, albeit nonsignificant, tendency to increased levels in IPC. The oxidant-sensitive transcription factor nuclear factor κB is involved in the early activation of multiple genes encoding defense proteins and was repeatedly shown to be critically involved in delayed cardioprotection.[35] Nuclear factor κB directly regulates the expression of inducible nitric oxide synthase[36] and cyclooxygenase 2,[37] two important mediators of delayed protection. The fact that nuclear factor κB exhibits a biphasic expression activity on oxidative stress, with an initial transcriptional inhibition followed by a marked activation,[37] may explain the observed down-regulation of inducible nitric oxide synthase at the early investigated time point in our study. From a biologic point of view, decreased inducible nitric oxide synthase activity, as observed at this early stage after triggering, may rather reflect a protective mechanism at the transcriptional level associated with early preconditioning. Previous studies have clearly linked increased inducible nitric oxide synthase activity to enhanced cell death.[38] In accordance with this concept is the notion that actinomycin D, an inhibitor known to be effective at the transcriptional level, was previously reported to diminish protection elicited by early IPC.[39] Many transcripts for ribosomal proteins were up-regulated in both types of preconditioning, albeit in IPC significantly more than in APC. To date, it is unclear as to why so many ribosomal proteins were up-regulated after preconditioning. It may be speculated that this genomic response simply signifies more ribosomal biogenesis in response to the "damaging" aspects of preconditioning. Alternatively, global increase in ribosomal protein expression may mirror enhanced protein synthesis heralding the second window of protection. Collectively, APC and IPC

enhance the transcription of gene products associated with delayed protection. Failure to detect regulation of some critical mediators of delayed protection in our study may be a result of the relatively short time interval between the application of the trigger stimuli and the determination of expression levels.

Transcriptional Changes in Signaling Components

We were recently able to visualize translocation of protein kinase C isoform to subcellular targets in IPC and APC.[4] Moreover, our laboratory provided the first evidence that mitogen-activated protein kinases differentially act in triggering and mediating IPC and APC.[5] We now extend these findings by showing that differences between IPC and APC also occur at the transcriptional level. Whereas APC solely increased the expression of the injury-related p38 mitogen-activated protein kinase α, APC and IPC repressed p38 mitogen-activated protein kinase–activated protein kinase 2. Although previous reports showed increased gene expression of p38 mitogen-activated protein kinase–activated protein kinase 2 after IPC,[40] our observation may reflect the biphasic regulation of this transcript. Interestingly, protein kinase C-δ was exclusively up-regulated in IPC. As a common feature in APC and IPC, receptor-bound tyrosine phosphatase and nonreceptor tyrosine kinase were up-regulated, whereas RhoA-binding serine/threonine kinase α was repressed. Again, these findings extend at a transcriptional level previous observations of complex interactions between protein tyrosine kinases and their counterplayers in APC.[41]

A recent study in isolated rat hearts reported transient increases of B-type natriuretic peptide (BNP) and reported infarct-size limiting effects of this hormone *via* opening of ATP-sensitive potassium channels.[42] In our study, no significant transcriptional regulation of atrial natriuretic peptide was detected after triggering. Also, BNP expression was unaffected by IPC and APC. These findings suggest that BNP expression is not affected by administration of preconditioning triggers, strengthening its diagnostic role as a sensitive marker for cardiac dysfunction after a prolonged ischemic hit, as recently demonstrated in a clinical preconditioning study.[43]

Gene Chip Analysis in Cardiac Preconditioning

To date, only one study has been conducted comparing gene expression profiles between pharmacologic and ischemia-induced preconditioning. Rokosh *et al.*[11] compared ischemic with nitric oxide–induced preconditioning in mice using oligonucleotide microarrays containing more than 6,000 known and 6,000 unknown genes. In this study, 12% of the IPC-regulated genes were concordant with pharmacologic preconditioning by nitric oxide, which is similar to our observation, with 19% of IPC-regulated genes being commonly regulated in APC. Moreover, a number of genes such as serine protease inhibitor or nicotinamide adenine dinucleotide–ubiquinone oxidoreductase B15, previously reported to be regulated by IPC, were confirmed in the current study.[44] However, identification of all unknown genes in IPC and APC and functional studies of candidate genes using specific blockers or selective knockout will be necessary to precisely determine their roles.

Study Limitations

The following remarks should be added: (1) A left ventricular balloon was used to monitor left ventricular function during the preconditioning process. However, this may jeopardize coronary perfusion and subsequently alter gene expression. Differential alterations in gene expression pattern were previously reported in subendocardium *versus* subepicardium, implying the existence of a spatial gradient.[45] (2) Because of the limited long-term biologic stability of the isolated heart preparation, the cardiac tissue was analyzed after a total of 120 min. This time was sufficient to allow up-/down-regulation of genes. However, the interval might have been too short to develop the full range of genetic responses. Also, no time course of gene expression profiling was established. (3) Isoflurane was administered over a more extended period than in most previously reported preconditioning protocols. However, this approach reflects the clinical situation in which patients are treated for hours with volatile anesthetics and may enhance the preconditioning stimulus to induce significant transcriptional changes.[7,8] (4) Finally, buffer perfusion is an artificial condition, and isolated hearts may undergo a short ischemic period.

In conclusion, the current study provides the first comprehensive gene expression profile associated with APC as compared with IPC. Among many similarities, the data collected suggest distinct differences in gene expression between APC and IPC. Understanding the function of unknown genes in the complex biologic process of pharmacologic and ischemic cardiac preconditioning will open new avenues for optimizing perioperative cardioprotection in humans.

References

1. Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC: Anaesthetics and cardiac preconditioning: Part I. Signalling and cytoprotective mechanisms. Br J Anaesth 2003; 91:551–65

2. Marber MS, Latchman DS, Walker JM, Yellon DM: Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. Circulation 1993; 88:1264–72

3. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M: Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res 1993; 72:1293–9

4. Uecker M, Da Silva R, Grampp T, Pasch T, Schaub MC, Zaugg M: Translocation of protein kinase C isoforms to subcellular targets in ischemic and anesthetic preconditioning. ANESTHESIOLOGY 2003; 99:138–47

5. Da Silva R, Grampp T, Pasch T, Schaub MC, Zaugg M: Differential activation of mitogen-activated protein kinases in ischemic and anesthetic preconditioning. ANESTHESIOLOGY 2003; 100:59–69

6. Kehl F, Pagel PS, Krolikowski JG, Gu W, Toller W, Warltier DC, Kersten JR:

Isoflurane does not produce a second window of preconditioning against myocardial infarction in vivo. Anesth Analg 2002; 95:1162–8

7. Tonkovic-Capin M, Gross GJ, Bosnjak ZJ, Tweddell JS, Fitzpatrick CM, Baker JE: Delayed cardioprotection by isoflurane: Role of K(ATP) channels. Am J Physiol Heart Circ Physiol 2002; 283:H61–8

8. de Klaver MJ, Buckingham MG, Rich GF: Isoflurane pretreatment has immediate and delayed protective effects against cytokine-induced injury in endothelial and vascular smooth muscle cells. ANESTHESIOLOGY 2003; 99:896–903

9. Cook SA, Rosenzweig A: DNA microarrays: Implications for cardiovascular medicine. Circ Res 2002; 91:559–64

10. Brown PO, Botstein D: Exploring the new world of the genome with DNA microarrays. Nat Genet 1999; 21:33–7

11. Rokosh G, Guo Y, Williams J, Wu WJ, Zhu XP, Tan W, Bolli R: Gene expression after ischemic and pharmacologic preconditioning: Analysis by DNA microarrays (abstract). Circulation 2002; 106:S284

12. Christians ES, Yan LJ, Benjamin IJ: Heat shock factor 1 and heat shock proteins: Critical partners in protection against acute cell injury. Crit Care Med 2002; 30:S43–50

13. Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, Garrido C: Hsp27 negatively regulates cell death by interacting with cytochrome c. Nat Cell Biol 2000; 2:645–52

14. Michels AA, Kanon B, Bensaude O, Kampinga HH: Heat shock protein (hsp) 40 mutants inhibit Hsp70 in mammalian cells. J Biol Chem 1999; 274: 36757–63

15. Gupta S, Knowlton AA: Cytosolic heat shock protein 60, hypoxia, and apoptosis. Circulation 2002; 106:2727–33

16. Gabai VL, Yaglom JA, Volloch V, Meriin AB, Force T, Koutroumanis M, Massie B, Mosser DD, Sherman MY: Hsp72-mediated suppression of c-Jun N-terminal kinase is implicated in development of tolerance to caspase-independent cell death. Mol Cell Biol 2000; 20:6826–36

17. Reed JC: Bcl-2 family proteins. Oncogene 1998; 17:3225–36

18. Orlowski RZ: The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ 1999; 6:303–13

19. Lee AK, Sung SH, Kim YC, Kim SG: Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-α and COX-2 expression by sauchinone effects on I-κBα phosphorylation, C/EBP and AP-1 activation. Br J Pharmacol 2003; 139:11–20

20. Hong NA, Flannery M, Hsieh SN, Cado D, Pedersen R, Winoto A: Mice lacking Dad1, the defender against apoptotic death-1, express abnormal N-linked glycoproteins and undergo increased embryonic apoptosis. Dev Biol 2000; 220: 76–84

21. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF: Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. ANESTHESIOLOGY 2003; 99:421–8

22. Müllenheim J, Ebel D, Frässdorf J, Preckel B, Thämer V, Schlack W: Isoflurane preconditions myocardium against infarction *via* release of free radicals. ANESTHESIOLOGY 2002; 96:934–40

23. Shinmura K, Bolli R, Liu SQ, Tang XL, Kodani E, Xuan YT, Srivastava S, Bhatnagar A: Aldose reductase is an obligatory mediator of the late phase of ischemic preconditioning. Circ Res 2002; 91:240–6

24. Yamashita N, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M: The involvement of cytokines in the second window of ischaemic preconditioning. Br J Pharmacol 2000; 131:415–22

25. Hu G, Vasiliauskas T, Salem MR, Rhone DP, Crystal GJ: Neutrophils pretreated with volatile anesthetics lose ability to cause cardiac dysfunction. ANESTHESIOLOGY 2003; 98:712–8

26. Schneider MR, Wolf E, Hoeflich A, Lahm H: IGF-binding protein-5: Flexible player in the IGF system and effector on its own. J Endocrinol 2002; 172:423–40

27. Paumen MB, Ishida Y, Muramatsu M, Yamamoto M, Honjo T: Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. J Biol Chem 1997; 272:3324–9

28. Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, Jadoon AK: Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. Circ Res 1997; 81:42–52

29. Guo Y, Jones WK, Xuan Y-T, Tang X-L, Bao W, Wu W-J, Han H, Laubach VE, Ping P, Yang Z, Qiu Y, Bolli R: The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. Proc Natl Acad Sci U S A 1999; 96:11507–12

30. Shinmura K, Tang X-L, Wang Y, Xuan Y-T, Liu S-Q, Takano H, Bhatnagar A, Bolli R: Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. Proc Natl Acad Sci U S A 2000; 97:10197–202

31. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, Bolli R: Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circ Res 1997; 81:404–14

32. Ping P, Zhang J, Zheng YT, Li RC, Dawn B, Tang XL, Takano H, Balafanova Z, Bolli R: Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits. Circ Res 1999; 85:542–50

33. Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Qiu Y, Li JJ, Bolli R: Nuclear factor-κB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. Circ Res 1999; 84:1095–109

34. Das DK, Engelman RM, Kimura Y: Molecular adaptation of cellular defenses following preconditioning of the heart by repeated ischaemia. Cardiovasc Res 1993; 27:578–84

35. Zhao TC, Kukreja RC: Late preconditioning elicited by activation of adenosine A(3) receptor in heart: Role of NF-kappa B, iNOS and mitochondrial K(ATP) channel. J Mol Cell Cardiol 2002; 34:263–77

36. Shinmura K, Xuan YT, Tang XL, Kodani E, Han H, Zhu Y, Bolli R: Inducible nitric oxide synthase modulates cyclooxygenase-2 activity in the heart of conscious rabbits during the late phase of ischemic preconditioning. Circ Res 2002; 90:602–8

37. Connelly L, Palacios-Callender M, Ameixa C, Moncada S, Hobbs AJ: Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. J Immunol 2001; 166:3873–81

38. Arstall MA, Sawyer DB, Fukazawa R, Kelly RA: Cytokine-mediated apoptosis in cardiac myocytes: The role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ Res 1999; 85:829–40

39. Strohm C, Barancik M, von Bruehl M, Strniskova M, Ullmann C, Zimmermann R, Schaper W: Transcription inhibitor actinomycin-D abolishes the cardioprotective effect of ischemic reconditioning. Cardiovasc Res 2002; 55:602–18

40. Maulik N, Watanabe M, Zu YL, Huang CK, Cordis GA, Schley JA, Das DK: Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. FEBS Lett 1996; 396:233–7

41. Stadnicka A, Kwok WM, Warltier DC, Bosnjak ZJ: Protein tyrosine kinase-dependent modulation of isoflurane effects on cardiac sarcolemmal K(ATP) channel. ANESTHESIOLOGY 2002; 97:1198–208

42. D'Souza SP, Yellon DM, Martin C, Schulz R, Heusch G, Onody A, Ferdinandy P, Baxter GF: B-type natriuretic peptide limits infarct size in rat isolated hearts via KATP channel opening. Am J Physiol Heart Circ Physiol 2003; 284: H1592–600

43. Julier K, da Silva R, Garcia C, Bestmann L, Frascarolo P, Zollinger A, Chassot PG, Schmid ER, Turina MI, von Segesser LK, Pasch T, Spahn DR, Zaugg M: Preconditioning by sevoflurane decreases biochemical markers for myocardial and renal dysfunction in coronary artery bypass graft surgery: A double-blinded, placebo-controlled, multicenter study. ANESTHESIOLOGY 2003; 98:1315–27

44. Onody A, Zvara A, Hackler L Jr, Vigh L, Ferdinandy P, Puskas LG: Effect of classic preconditioning on the gene expression pattern of rat hearts: A DNA microarray study. FEBS Lett 2003; 536:35–40

45. Depre C, Tomlinson JE, Kudej RK, Gaussin V, Thompson E, Kim S-J, Vatner DE, Topper JN, Vatner SF: Gene program for cardiac cell survival induced by transient ischemia in conscious pigs. Proc Natl Acad Sci U S A 2001; 98: 9336–41