

Age-associated Changes in Cardiac Gene Expression after Preconditioning

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Background: **Cardiac protection afforded by ischemic preconditioning (IPC) and anesthetic preconditioning (APC) are significantly reduced in the senescent myocardium. The authors hypothesized that age would differentially modulate gene expression induced by IPC and APC *in vivo*.**

Methods: **Affymetrix RAT EXON ST 1.0 gene chips (Affymetrix, Santa Clara, CA) were used to explore the transcriptional response to IPC and APC in Fisher 344 male rats (young, 3–5 months, and old, 20–24 months, respectively). Both cohorts, young and old, were divided into three groups: (1) sham control, (2) IPC, and (3) APC. After a total of 90 min, the heart was removed, and the total RNA and protein were extracted.**

Results: **Thirty-one transcripts were increased in the young animals subjected to IPC, particularly transcriptional regulators (Atf3, Egr-1, Btg2, Egr2), cytokines (interleukin 6, CSF1, Myd88), chemokines (Cxcl10, Ccl2, Ccl7), regulators of growth and inflammation (Reg3g, Hamp), remodeling and cell adhesion migration (Cyr61, Tfpi2, Timp1), regulators of apoptosis/cell death (Birc3, Arntl, Hamp, Phlda1), and cell cycle control/DNA repairs (Rrad, Gadd45b, Gadd45g). In contrast, only one transcript increased (Atf3) in the old animals subjected to IPC. No changes in gene expression were found in the young or the old animals subjected to APC.**

Conclusions: **Early-phase IPC and APC induced different genomic responses. The absence of detectable changes associated with early-phase APC suggests a posttranscriptional or posttranslational mechanism. The absence of a genomic response in the senescent myocardium (except for IPC-induced Atf3) could underlie the failure of IPC to provide any cardiac protective benefit to older animals.**

DESPITE reduced mortality rates during the past decade, ischemic heart disease remains a leading cause of death and disability. It is well known that cardiovascular morbidity and mortality increase with age and that cardiovascular diseases are the major cause of death in the population older than 65 years.[1] Clearly, improving our knowledge of ischemic injury and cardioprotective therapies in the senescent myocardium is of paramount importance.

Structural changes (thickening of the left ventricular wall decreases in the number of myocytes, and left ventricle cavity size) and diastolic dysfunction are the most prominent signs of cardiac aging.[2] Biochemical changes in aging hearts include decreased rates of calcium sequestration, accumulation of collagen, enhanced production of stress-related substances (*e.g.*, atrial natriuretic peptide and opioids), increased expression of angiotensin II receptors, and desensitization of cardiac β-adrenergic receptors.[3] These structural and biochemical changes could have major implications in the age-related decrease in cardiac tolerance to ischemia. Old hearts have demonstrated worsening of myocardial stunning, have decreased recoveries in hemodynamic and high-energy phosphates, and have greater overall tissue damage after ischemic insult.[4] However, the regulatory mechanisms underlying these changes are not fully understood.

Ischemic preconditioning (IPC) and anesthetic preconditioning (APC) both represent powerful means of attaining myocardial protection against prolonged ischemia.[5,6] The preconditioning stimuli exhibit "memory" characterized by early and late windows of protection. The classic early memory of ischemic preconditioning lasts 1–2 h in anesthetized animals.[7,8] However, the preconditioning stimulus further initiates and triggers a second, longer-lasting genomic response involving increased *de novo* protein synthesis of the translated gene products. This late preconditioning, or second window, is observed 12–24 h after the initial transient ischemic event and lasts for up to 72 h.[9]

In previous studies, we and others have shown that the cardiac protection afforded by IPC or APC is absent in the senescent rat myocardium.[10,11] Previous work has also shown that gene expression is altered in the senescent myocardium following myocardial ischemia–reperfusion after 24 h.[12] Whether this is true of early-phase IPC or APC has not been examined. To our knowledge, no previous study has examined early-phase preconditioning in young and senescent myocardium and compared its effects on gene expression *in vivo*. We hypothesized that (1) early-phase IPC and APC would affect myocardial gene expression patterns differently in young compared with older animals, and (2) IPC and APC in animals of similar age would induce expression of a common set of preconditioning genes. To test these ideas, microarrays analysis was used to measure genome-wide changes in myocardium messenger RNA (mRNA) levels after IPC and APC in young and old animals *in vivo*.

Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Ani-

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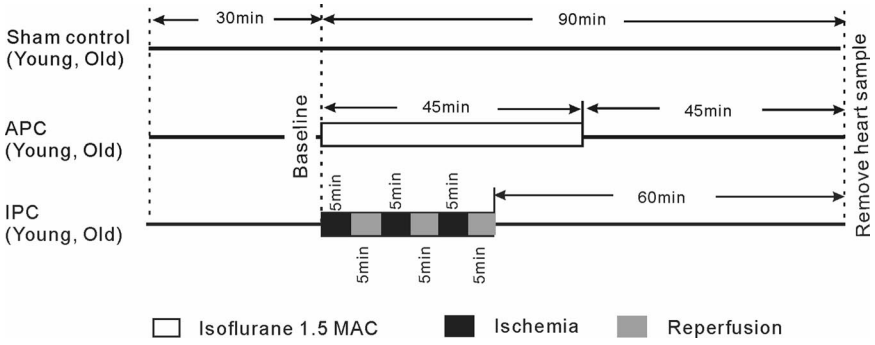

Fig. 1. Schematic illustration of the experimental protocol. APC = anesthetic preconditioning; IPC = ischemic preconditioning; MAC = minimum alveolar concentration.

mal Care and Use Committee of SUNY Stony Brook, Stony Brook, New York. Furthermore, all procedures conformed to the Guiding Principles in the Care and Use of Animals of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

General Preparation

Young (3- to 5-month, 300- to 400-g) and senescent (20- to 24-month, 350- to 450-g) male F344 rats were anesthetized with intraperitoneal sodium thiobutabarbital (100–150 mg/kg). Once the animal was anesthetized, a heparin-filled (10 units/ml) catheter was inserted into the right jugular vein for fluid and drug administration. The right carotid artery was then cannulated to measure arterial blood pressure, and a tracheotomy was performed. Animals were ventilated on a Harvard Apparatus model 638 rat ventilator (Harvard Apparatus, Holliston, MA) using an air-and-oxygen mixture (50%/50%). A positive end-expiratory pressure of 5 cm H_2O was implemented to minimize the development of atelectasis. Baselines of systemic hemodynamics were recorded after instrumentation was completed. Arterial blood gas tension and acid–base status were monitored at regular intervals and maintained within a normal physiologic range: pH, 7.35–7.45; arterial carbon dioxide tension, 25–40 mmHg; and arterial oxygen tension, 90–150 mmHg. Finally, a left thoracotomy was performed in the fifth intercostal space, and the pericardium was opened. A 6-0 Prolene suture was placed around the proximal left anterior descending coronary artery and vein in the area immediately below the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. Coronary artery occlusion was produced by clamping the snare onto the epicardial surface of the heart with a hemostat and was confirmed by the appearance of epicardial cyanosis. Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperemic response. Isoflurane was delivered by calibrated isoflurane vaporizer (A.M. Bickford Inc., Wales Center, NY), and end-tidal concentrations of isoflurane, carbon dioxide, and inspired oxygen concentrations were measured at the tip of the tracheotomy tube using a Poet IQ2 (Criticare Systems Inc., Waukesha, WI) infrared gas analyzer throughout the experiment. Body temperature was maintained at 37° ± 0.2°C using a heating pad and radiant warmer. Heart rate and mean blood pressure data were continuously recorded throughout the experiment. At the end of the protocol, the animal was killed with an overdose of sodium thiobutabarbital.

Protocol for Experiments

The current investigation protocol is illustrated in figure 1. Both cohorts, young and old, were divided into three groups: sham control (SC), IPC, and APC. Therefore, we had six groups (n = 5 per group): (1) young SC (YSC) group; (2) young IPC (YIPC) group—three cycles of ischemia and reperfusion, 5 min each, followed by 60 min of reperfusion; (3) young APC (YAPC) group—45 min of 1.5 minimum alveolar concentration isoflurane followed by 45-min washout; (4) old SC (OSC) group; (5) old IPC (OIPC) group—three cycles of ischemia and reperfusion, 5 min each, followed by 60 min of reperfusion; and (6) old APC (OAPC) group—45 min of 1.5 minimum alveolar concentration isoflurane followed by 45-min washout. In each case, rat left ventricular heart samples were collected at the end of experiment, flash frozen in liquid nitrogen and stored at −80°C.

RNA Isolation, cDNA Preparation, and Hybridization with Affymetrix Gene Chips

Frozen 30-mg portions of the left ventricular myocardium were homogenized with a Polytron (Kinematica, Bohemia, NY), and total RNA was prepared with RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA). Isolated RNA was flash frozen in aliquots and stored at −80°C until analysis. The concentration of the purified RNA was measured by ultraviolet spectrophotometer using the ratio of A260/A280 (> 1.8) and the intactness of the 18S and 28S ribosomal subunits subjected to electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Samples passing quality control were processed by the Microarray Core facility (Cold Spring Harbor Laboratory, Woodbury, NY). Sample quality was assessed by RNA Integrity Number or RIN score. and samples with an RIN of 7.5 or greater were passed; quantity was assessed by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Table 1. Primers Used for Quantitative Real-time PCR

Gene Symbol	mRNA Accession	Forward Primer	Reverse Primer
Atf3	NM_012912	CCAGGTCTCTGCCTCAGAAG	AAGGTGCTTGTTCTGGATGG
Egr1	NM_012551	CATGACAGCAACCTTTTCTCC	TTGGCTCTGAGATCTTCCATC
Interleukin 6	NM_012589	TCTCTCCGCAAGAGACTTCC	CCTCCGACTTGTGAAGTGGT
Ccl2	NM_031530	TGTTCACAGTTGCTGCCTGT	TGCTGCTGGTGATTCTCTTG
Pgk1	NM_053291	TAAAGTCAGCCATGTGAGCA	ATGAATCCCGATGCAGTAAA

mRNA = messenger RNA; PCR = polymerase chain reaction.

Fifty nanograms of total RNA was amplified by Ribo-SPIA (single primer isothermal amplification), an isothermal complementary DNA (cDNA) amplification technique, using WT Ovation PICO kit, followed by strand conversion EXON module and FL module to label and fragment the product (NuGen, Technologies, Inc., San Carlos, CA). cDNA smear analysis for Quality Control was performed on select samples using Agilent 2100 Bioanalyzer RNA 6000 Nano Series II Chips (Agilent, Palo Alto, CA). Samples were then prepared for hybridization, hybridized, washed, and scanned according to the manufacturer's instructions on Rat Exon ST 1.0 GeneChips (Affymetrix, Santa Clara, CA). Affymetrix Quality Control metrics were used to qualify the resulting data.

Microarray Data Analysis

Affymetrix Rat Exon ST 1.0 microarray chips contain more than 4 million probe spots representing at least 4 replicates each of approximately 850,000 exon clusters, which is estimated to provide approximately 40 expressed sequence tag probes per gene. R 2.8.0** and Bioconductor 2.3[13] are freely available public domain software packages that were used to analyze the microarray data. We used the "Core" Annotation files to group the exon probe levels into probable expression of 8608 curated genes. Individual microarray chips were scanned by the Affymetrix Gene Chip scanner 3000 7G and produced corresponding cel files. The cel files were aggregated in Bioconductor, then expression levels were normalized using the RMA algorithm,[14] and the quality of the hybridization and scanning was assessed using interchip variation with box plots. To reduce the multiple comparison problems, only the top 1,000 putative genes ranked for variance across the experimental groups were examined for differential expression (DE). DE was then assessed using the linear models for microarray data (LIMMA) algorithm.[15]

Real-time PCR

To confirm the gene expression patterns of microarray hybridization, reverse-transcription polymerase chain reaction (RT-PCR) was performed using the QuantiTect reverse-transcription kit (Qiagen) for multiple selected genes, in which the expression levels were significantly altered (≥ 2.0-fold) by IPC determined by the microarray analysis. The forward and reverse sequences of the primers used are listed in table 1. For each gene-specific amplification, 100 ng of cDNA was used as a template for the QuantiTect SybrGreen Real Time PCR kit (Qiagen). RT-PCR quantification and determination of expression levels were performed on a Bio-Rad iQ5 Sequence Detector Real-Time PCR machine (Bio-Rad Technology, Beverly, MA). Amplification reactions were conducted with an initial step at 95°C for 15 min followed by 42 cycles with four-step cycling: 15 s, 95°C; 15 s, 54°C; 30 s, 72°C; and 15 s, 78°C (recording fluorescence). All PCR reactions were performed in triplicate, and Pgk1 was used as an internal reference control for each sample. Thermal melting analyses were performed for all products. In addition, the predicted sizes of PCR products were confirmed by agarose gel electrophoresis. Relative gene expression was calculated from the data using the $2^{-\Delta\Delta C}{}_{T}{}^{*}$ method.[16]

Western Blotting Analysis

Myocardial left ventricular samples were homogenized using a Polytron homogenizer in ice-cold lysis buffer containing 20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM dithiothreitol, 1 mM PMSF (freshly prepared), and a complete mammalian proteinase inhibitor cocktail (one tablet per 10 ml; Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 13,000 *g* for 30 min at 4°C to isolate total protein. The clarified supernatant was used to quantify protein. Protein concentrations were determined using the BCA Protein Assay Kit (Bio-Rad, Hercules, CA). Equivalent amounts (30 μg) of total proteins were mixed with 2X Laemmli buffer and heated at 95°C for 5 min before loading. Samples were separated at 100 V by 15% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane using a semidry transblot apparatus (Bio-Rad). Membranes were then blocked with 5% nonfat dry milk in Tris buffered saline containing 0.1% Tween-20, and were then incubated with the primary antibody 1:1,000 dilution of rabbit polyclonal ATF3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight in 5% nonfat dry milk and 0.1% Tween-20 in Tris buffered saline. Bands were visualized with ECL (Amersham ECL Plus™ Western

** http://www.R-project.org. Accessed October 20, 2008.

Blotting Detection Reagents; GE Healthcare, Buckinghamshire, United Kingdom). Equal loading of samples was confirmed by reprobing membranes with β-Actin antibody (1:2,000) (Santa Cruz Biotechnology) and by staining with Ponceau S. Quantitative analysis of the band densities was determined using NIH Image 1.6 quantification software (National Institutes of Health, Bethesda, MD) and normalized against background density for each gel.

Statistical Analysis

Gene set enrichment analysis using the linear models for microarray data approach, part of the BioConductor version,[15] was used to assess the significance of the differentially expressed gene sets. The eBayes function was used in which the log2-fold change (M) is determined for each gene and an average expression level for that gene (A) across all arrays is calculated. A moderated t statistic (t) is generated that is the ratio of the M value to its SE. This has the same interpretation as an ordinary t statistic, except that the SEs have been reduced to a common value across genes. There was no filtering of the linear models for microarray data output for effect size. The *P* values obtained from the linear models for microarray data analysis of the DE genes were adjusted by the Benjamini–Hochberg method to control for false discovery rate (FDR)[17] at $P < 0.1$. This FDR adjustment has been found to be superior to the Bonferroni correction for multiple comparisons in the analysis of large sets of gene expression data. Here, the adjusted $P < 0.1$ represents the chances of type 1 errors in the identifications of the members of the DE gene sets. The power of the experimental randomized block design within each age group was based on previously determined variances in comparable experiments with approximately 8,000 genes.[18] The calculations predicted detection limits of 45% change of transcript levels with greater than 80% power (allowing for an FDR of 10%).[19] Western blotting and hemodynamic data were analyzed using the Student *t* test, and paired *t* test with Bonferroni correction, respectively.

To identify functional gene clusters that characterize IPC-induced gene expression, the DE genes were filtered for an FDR of 10% and were entered into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA), a Web-based program that comprises a large, well-curated knowledge base consisting of biologic and chemical information extracted from the literature. The DE genes were then evaluated as focus genes, defined as those having direct or indirect interactions with other genes in the IPA database. Using the network generation algorithm, connection specificity for each focus gene was evaluated. Each nascent network, containing a maximum of 35 genes, was constructed, beginning with the gene with highest specific connectivity score. This process was repeated for the remaining genes to construct additional networks. In this process, the numbers of focus genes were reduced as a result of their inclusion in previously constructed networks. Genes from the global IPA network, not in the initial group, but with significant specific connectivity to the growing network, were included. Small networks were merged using linker genes and grown using neighborhood genes, optimizing edges as the networks were merged and grown. IPA networks were ranked by *p* score based on inclusion of as many of the inputted focus genes as possible and identifying the most highly connected networks. The local network *p* scores were derived from *P* values that describe the probability of finding *f* or greater focus molecules in a set of *n* genes randomly selected from the global network in the IPA database, and were calculated using Fisher exact test. The network *p* score is the $-\log_{10}$ of the *P* value, so that a *p* score of 6 represents a 1 in 10^6 chance of obtaining at least the same number of focus genes from random assignments from the IPA database to the network.

Table 2. Systemic Hemodynamics

	Group	No.	Baseline	Preconditioning	Memory
HR, min^{-1}	YSC	5	364 ± 11	375 ± 15	382 ± 12
	YIPC	5	379 ± 7	386 ± 17	368 ± 12
	YAPC	5	372 ± 13	311 ± 11*	365 ± 9
	OSC	5	352 ± 14	370 ± 17	362 ± 22
	OIPC	5	341 ± 8	355 ± 14	361 ± 11
	OAPC	5	339 ± 12	268 ± 17*	341 ± 16
MAP, mmHg	YSC	5	141 ± 16	139 ± 10	134 ± 5
	YIPC	5	139 ± 11	135 ± 11	136 ± 11
	YAPC	5	150 ± 12	101 ± 7*	129 ± 12
	OSC	5	136 ± 16	126 ± 24	112 ± 25
	OIPC	5	138 ± 8	130 ± 5	110 ± 12
	OAPC	5	118 ± 26	82 ± 23*	102 ± 11

Values are mean ± SEM.

* $P < 0.05$ compared with control baseline value.

APC = anesthetic preconditioning; HR = heart rate; IPC = ischemic preconditioning; MAP = mean arterial pressure; O = old; SC = sham control; Y = young.

Results

Systemic Hemodynamics

No differences in the baseline hemodynamics were observed among the three groups (APC, IPC, and control; table 2), and no differences were observed in mean arterial blood pressure between the young and old isoflurane groups. Isoflurane, however, significantly decreased ($P < 0.05$) the mean arterial blood pressure and heart rate ($P < 0.05$) in the young and old isoflurane groups when compared with their respective control groups. There were no differences in mean arterial blood pressures and heart rates during the memory period (washout) in the young and old isoflurane groups when compared with their respective control groups. There were no differences in the

Table 3. Transcript Changes in Young Animals Subjected to Ischemic Preconditioning as Compared with Sham Controls

Gene Symbol	Function	Classification	Fold Change	Adjusted $P <$ Value
Phlda1	Negative regulator of apoptosis; induced by ER stress, Akt activation	Proline-histidine–rich nuclear protein	2.0	0.011
Egr1	Early response positive transcriptional regulator activated by p38Mapk and Erk pathways	Transcription factor	3.6	0.011
Atf3	Induced by stress and promotes cell survival, has positive and negative transcriptional regulatory properties	Transcription factor	5.8	0.011
Btg2	Negative regulator apoptosis, regulates genes controlled by Esr1	Transcription factor	2.8	0.012
IL6	Cardioprotective cytokine produced by myocardium in response to ischemia	Cytokine	3.7	0.016
Cyr61	Facilitates endothelial cell adhesion, positive regulator of neovascularization	Secreted, cysteine-rich, heparin-binding protein	2.5	0.016
Hamp	Cellular iron homeostasis		2.0	0.016
Hspb1	Stress induced under p53 control	Heat shock protein	1.8	0.016
Arntl	Transcription regulator of circadian rhythm–associated genes	Transcription factor	2.3	0.016
Rrad	GTPase, calmodulin binding, nucleotide binding	GTP-binding protein	2.3	0.027
Reg3g	Stress protein	Lectin	2.8	0.027
Myd88	Adapter protein involved in the Toll-like receptor and interleukin 1 receptor signaling pathway	Adapter protein	1.9	0.032
Zfp36	Regulates response to growth factors	Zinc finger protein	1.8	0.033
Timp1	Inhibitor of the matrix metalloproteinases	Metalloproteinase inhibitor	1.8	0.033
Tfpi2	Regulates plasmin-mediated matrix remodeling	Protease inhibitor	2.0	0.044
Gadd45b	Stress-induced negative regulator of apoptosis		1.7	0.044
Cxcl10	Angiostatic and antifibrotic chemokine induced by myocardial infarction	Chemokine	3.4	0.044
Rnd1	Rho GTPase, regulates the actin cytoskeleton	GTPase	2.6	0.044
Egr2	Zinc binding, transcription factor activity inducible by ischemia	Transcription factor	2.3	0.048
Gadd45g	Stress-induced regulator DNA repair pathway		2.0	0.062
Ccl7	Induced by ischemia, regulates cardiac remodeling	Chemokine	2.4	0.062
Ccl2	Induced by ischemic injury, regulates cardiac remodeling	Chemokine	2.8	0.073
Csf1	Induced by myocardial injury, regulates cardiac remodeling	Cytokine	1.9	0.076
Ier3	Protects against Fas– or TNF-α–induced apoptosis		1.7	0.080
Has1	Role in hyaluronan/hyaluronic acid synthesis, tissue repair	Synthase	1.8	0.081
Osmr	Receptor for oncostatin M, which is related to Il6	Cytokine coreceptor	1.7	0.085
Serpine1	Induced by ischemia, prevents fibrinolysis		1.9	0.085
Birc3	Suppress apoptosis induced by TNF		2.3	0.085
Cxcl1	Regulates neovascularization of ischemic myocardial tissue	Chemokine	2.6	0.085
Lmcd1	Transcription regulator	Transcription factor	1.7	0.085
Ugdh	Biosynthesis of glycosaminoglycans	Dehydrogenase	1.9	0.091

Microarray results obtained from the myocardial tissue of young animals subjected to ischemic preconditioning were compared with those from young sham controls (n = 5 per group). After linear models for microarray data analysis, the *P* values were adjusted for false discovery rate. Those with $P < 0.1$ are listed. ER = endoplasmic reticulum; GTP = guanosine triphosphate; IL6 = interleukin 6; TNF = tumor necrosis factor.

mean arterial blood pressure and heart rate in the young and old IPC groups when compared with their respective control groups.

Findings in Young and Old IPC Groups

Thirty-one mRNA transcripts in the young animals subjected to IPC were increased (table 3), but no significant decrease in transcript levels were detected. The IPC-induced genes fell into functional classes: transcriptional regulators that normally respond to stress, cardiac protective cytokines and chemokines, inflammatory and growth regulatory factors, genes associated with tissue remodeling and cell adhesion/migration, regulatory components of apoptosis pathways, and regulators of cell cycle and DNA repair. In contrast to the young group, IPC in the senescent animals resulted in up-regulation of only one myocardial mRNA transcript, the stress-induced transcriptional regulator Atf3.

Findings in Young and Old APC Groups

No significant gene expression changes were observed in the young and old rats after APC.

Table 4. Real-time PCR Results Fold Change

	Normalized Gene Expression					
Gene	YSC	YIPC	YAPC	OSC	OIPC	OAPC
Atf3	1.47 ± 0.50	10.37 ± 2.11	1.19 ± 0.19	1.57 ± 0.59	7.08 ± 1.25	0.81 ± 0.15
Egr1	1.37 ± 0.29	9.15 ± 3.10	1.75 ± 0.50	—	—	—
Interleukin 6	1.66 ± 0.43	4.11 ± 0.99	1.28 ± 0.59	—	—	—
Ccl2	1.60 ± 0.30	8.83 ± 2.53	1.20 ± 0.29	—	—	—

Values are mean ± SEM.

APC = anesthetic preconditioning; IPC = ischemic preconditioning; O = old; PCR = polymerase chain reaction; SC = sham control; Y = young.

All data normalized to Pgk1 expression.

Real-time RT-PCR

Target genes were selected (the four with the largest fold changes) and amplified from cDNA by real-time PCR (table 4). Results for the selected genes revealed an almost perfect concordance with the microarray data, hence confirming the accuracy of the gene chip results.

Western Blotting Results

Previous work had shown that myocardial Egr1 protein levels are rapidly increased after IPC. Here, we show that the levels of Atf3 protein also increase markedly in young rat myocardium after early-phase IPC, in parallel with the increase in Aft3 mRNA (fig. 2).

Network Analysis of Young IPC Modulated Genes

The relations among the IPC up-modulated genes were analyzed using the IPA software. The rank of the network was based on the number of modulated (DE) genes populating each network (focus genes) and their degrees of connectivity compared with random samplings across the global network gene set. Two networks (1 and 2) achieved remarkably high p scores of 41 and 23, respectively, and accounted for 28 of 31 DE genes (figs. 3 and 4); all others contained less than 3 and had p scores ≤ 4. Networks 1 and 2 were immediately recognized as containing cardioprotective, cytokine–chemokine signaling pathways. Many of the intracellular pathways obtained in network 2 have been previously implicated in transducing the acute cardioprotective effects of IPC (see Discussion).

In network 1 (fig. 3), platelet-derived growth factor and interleukin (IL) 1 engage Nfκb-dependent pathways and impinge on various stress-activated transcriptional regulators, particularly Atf3. These may trigger enhanced expres-

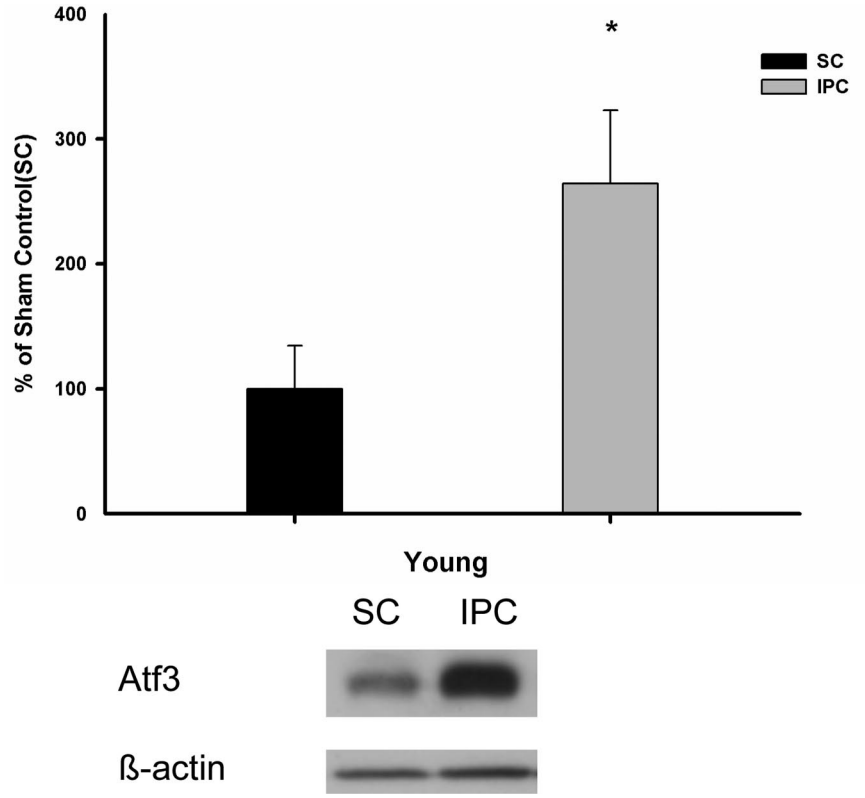

Fig. 2. Representative Western blots of activating transcription factor 3 (Atf3) in the young sham control (SC) and young ischemic preconditioning (IPC) groups. Data are normalized to β-actin and expressed as mean ± SEM. * $P < 0.05$ compared with young SC.

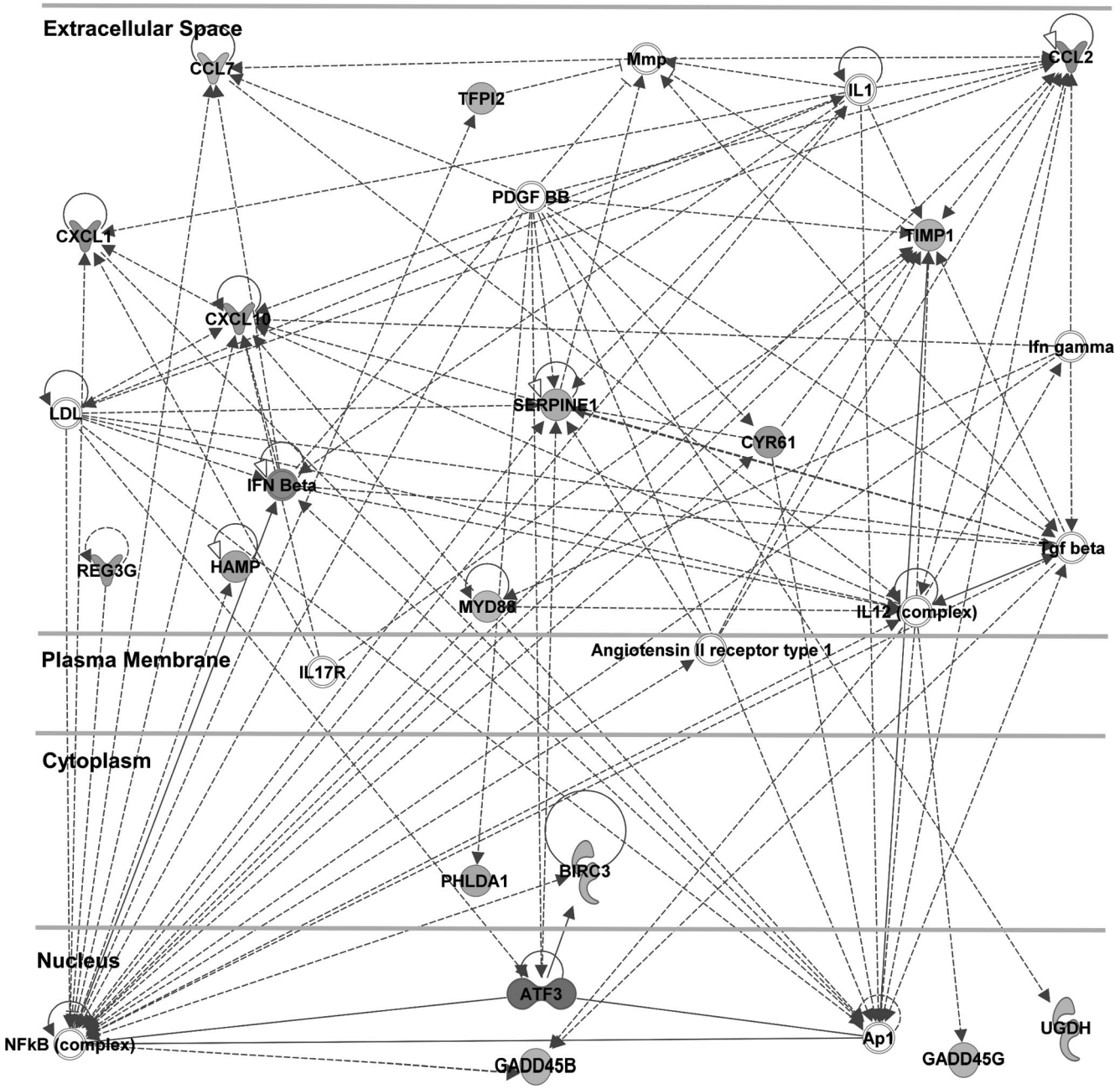

Fig. 3. Network 1 is a cytokine–chemokine signaling network identified by analysis of the relations among the young ischemic preconditioning up-modulated genes using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA). In this network, the genes form nodes with connections (gene relationships). The intensity of the node *shading* indicates the degree of up-regulation; genes in *unshaded* nodes were not identified as differentially expressed in our experiment, but were integrated into the nascent computationally generated network on the basis of the evidence stored in the IPA knowledge base indicating a relevance to this network. *Solid line* = direct interaction; *dotted line* = indirect interaction. A *solid arrow* indicates positive or stimulatory interaction, whereas a *line terminating with a perpendicular* indicates an inhibitory interaction. The cell compartments are indicated. These network contained 17 of 31 differentially expressed young ischemic preconditioning gene sets (table 3) and obtained a *p* score of 43. Alkaline phosphatase, laminin, fibrinogen, and collagen (none focus genes) and their network interactions were removed for clarity.

sion of a network of chemokines, Cxcl1, Cxcl10, Ccl2, and Ccl7, known to affect cardiac injury response.[20] In network 2, CSF1, known to regulate cardiac remodeling after injury,[21] and IL6 related to late preconditioning[22] form hubs that are highly connected through PI3 kinase, rat sarcoma, protein kinase B, signal transducers and activators of transcription, mitogen-activated protein kinase, c-Jun N-terminal kinase, p38MAPK, and ERK protein kinase pathways to transcriptional regulators Egr1 and 2, Zfp36, and Bgt2 (fig. 4).

Discussion

Aging is associated with reduced functional reserve and altered responsiveness of the heart to ischemia

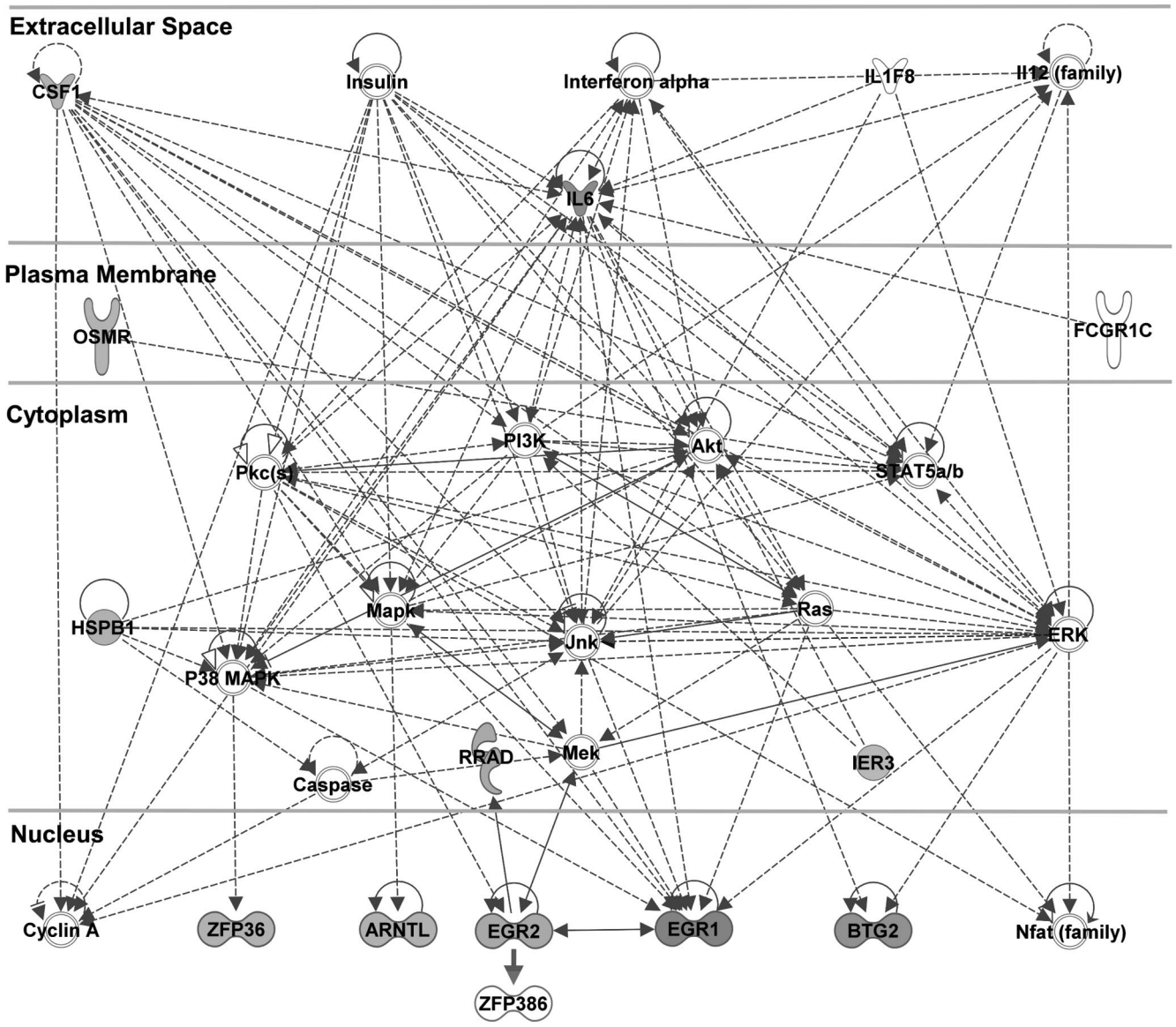

Fig. 4. Network 2 was identified and constructed as described for network 1 (fig. 3). Here, interleukin 6 and CSF1 have central roles. This network accounted for 11 of 31 differentially expressed young ischemic preconditioning gene sets (table 3) and obtained a *p* score of 23. FSH, IgG, hCG, and FcGR1c (none focus genes) and their network interactions were removed for clarity.

reperfusion injury, but the molecular basis for this deficiency has not been elucidated.[23] Here, we focused on preconditioning and the differentially affected gene expression patterns in young compared with senescent myocardium during early IPC and APC *in vivo*. Our most important findings are as follows. First, two types of early-phase preconditioning (IPC and APC) induced different genomic responses in that no evidence could be found that early-phase APC modulates gene expression in either age group; therefore, there was no support for the idea of a common expression set of early preconditioning genes. Second, this lack of any significant APC-induced change in gene express raises the possibility that early-phase APC is caused mainly by a posttranscriptional or post-translational mechanism. Third, and in contrast to APC, early-phase IPC in the young rat heart stimulates expression of immediate response genes and cytokines and chemokines, almost all of which have been shown to confer protection from ischemic injury. Fourth, the absence of any induced changes in the senescent myocardium (except for Atf3) could underlie the failure of IPC to provide any cardiac protective benefit in the older animal.

Previous studies have shown that brief periods of ischemia–reperfusion rapidly induce the adherence of blood components to the coronary vasculature.[24] Although it is possible that changes in IPC-induced transcript levels in adherent neutrophils and macrophages within the myocardial vasculature could contribute to changes in correspond-

ing mRNAs extracted from heart, this would represent a tiny fraction of the total RNA extracted from the ventricle and so is unlikely to be significant.

Gene Expression Patterns in Young Myocardium during IPC

Our results differ significantly from those obtained in an *ex vivo* model of IPC and APC in the young rat myocardium. In this previous work (da Silva *et al.*[25]), numerous transcripts were modulated by either IPC or APC. Predominantly up-regulated in APC and IPC at 180 min were cell surface receptors, ion channels, Gadd45α, nitric oxide synthase 2, and mitogen-activated kinase kinase 2. In addition, APC up-modulated mitochondrial-related gene and B-cell translocation gene 1 (Btg1) and chemokine ligand 10, whereas both APC and IPC trigger protocols down-regulated Egr1 in this earlier study. The two major differences between the present work and this previous study are the use of an *ex vivo* Langendorff heart preparation and the longer time interval between application of the preconditioning stimulus and harvesting of the tissue for analysis (90 *vs.* 180 min) in the latter study. This latter difference could explain the down-modulation of the Egr1 transcript, because it is well known that growth- or stress-induced early increases in Egr1 mRNA levels are normally followed by suppression at later time points.[26] Most importantly, the normal inflammatory response to the IPC stimulus was profoundly and consistently down-regulated in the previous work. As originally noted by the authors, the Langendorff perfused heart model is virtually devoid of blood components and lacks the appropriate physiologic feedback between these blood components and the stimulated myocardium, which could provide a further explanation of the differences. Additional contrasts to our *in vivo* preparation include preservation of neurohumoral responses, and other homeostatic feedback mechanisms that would not be present in the *ex vivo* model.

It has previously been reported[27] that brief episodes of ischemia activate a cardioprotective genetic program in the rat heart. In this previous microarray study, it was shown that 20 min of ischemia followed by 4 h of reperfusion *in vivo* led to significant up-regulation of mRNA transcripts for heat shock proteins 70, 27, and 105, as well as 86 and 40KDa, vascular endothelial growth factor, brain-derived neurotrophic factor, plasminogen activator inhibitor 1, Atf3, Egr1, Btg2, and Gadd45α compared with the normal myocardium. Whereas some of these results more closely paralleled our findings in the young IPC treated animals, no changes in cytokine IL6 or chemokine levels were reported, suggesting that these inflammatory factors were elaborated earlier in the response.

In our study, IPC triggered significant increases in Atf3, Egr1, Egr2, Btg2, Gadd45b, and Gadd45g mRNAs in the young myocardium. Atf3, a transcription factor also known as liver regenerating factor, has been suggested to play an important role in growth regulation by controlling the expression of a delayed gene.[28] It is typically up-modulated during ischemia and various other stress conditions. Our study is consistent with a facilitating protective role for Atf3 in the response of myocardial tissue to ischemia–reperfusion. This transcription factor has a key function in network 1 (fig. 3), a chemokine cascade that engages Atf3 through Nfκb and Ap1. In network 1, Gadd45b and Gadd45g are also stress-induced regulators that are thought to be controlled by Nfκb-dependent pathways and are implicated in DNA repair and cell cycle control,[29] with possible links to the generation of reactive oxygen species as occurs during IPC.[11] Timp1 is a natural inhibitor of the matrix metalloproteinases, whereas Serpine1, also known as plasminogen activator inhibitor 1, retards the degradation of fibrin contributing to thrombus accretion.[30] Serpine1 is up-regulated by Hif1 and Egr1.[31] CYR61 expression is also stimulated by oxidative stress[32] and transcriptional regulators Egr1, Hif1, and cJun/Ap1.[33,34] It facilitates endothelial cell adhesion through interactions with integrins and promotes neovascularization.[33,34] Birc3, a Ring-containing protein ubiquitin ligase that can be up-modulated by cytokines,[35] suppresses apoptosis, at least in part, by inhibiting caspase activation.[36]

Network 2 predicts that Egr1 and 2 operate downstream of IL6 and CSF1 through intracellular pathways previously implicated in preconditioning responses. Egr1 and 2 are immediate–early gene zinc finger transcription factors[25] that broadly activate transcripts of other transcriptional control factors and other regulators. These, in turn, activate transcription of a host of growth and stress response pathway genes. Btg2 has been found to be induced by genotoxic and oxidative stress[37] and ischemic preconditioning.[26]

In our study, IPC of the young myocardium caused a significant up-regulation of chemokines Cxcl10, Ccl2 (monocyte chemoattractant protein 1), Cxcl1, Cc7, and cytokine IL6. Understanding of the role of chemokines and cytokines in IPC may result in novel strategies for treatment of patients with ischemic heart disease. Previous work has shown that these inflammatory modulators are actually expressed in the myocardium after ischemic injury.[38,39] Recent studies have also reported that chemokine expression is markedly up-regulated in healing myocardial infarcts and may play an important role in regulating leukocyte infiltration and activity and modulating infarct angiogenesis as well as fibrous tissue deposition.[20] Monocyte chemoattractant protein 1 is involved in the pathophysiology of ischemic heart disease.[40] Cardiac monocyte chemoattractant protein 1 overexpression also prevents superoxide generation in the heart subjected to ischemia–reperfusion injury.[41] Ccl2 also has been shown to increase IL6 secretion in cardiomyocytes,

and both have been found to act synergistically to increase Stat3 activity.[42]

IL6 is a proinflammatory cytokine that is a prognostic marker associated with left ventricular contractile dysfunction and heart failure,[43] and cardiomyocytes themselves have been shown to produce IL6 in response to hypoxia[44] and ischemia–reperfusion.[45] On the other hand, IL6 is obligatory required for the activation of the Janus kinase/signal transducers and activators of transcription pathway, the ensuing up-regulation of inducible nitric oxide synthase and cyclooxygenase 2 (comediators of late-phase preconditioning),[46] and the development of a cardioprotective phenotype.[22] IL6 confers protection on cardiomyocytes from simulated ischemia/reperfusion injury and this effect depends on PI-3 kinase and iNOS pathways.[47]

Age-associated Alterations in Early-phase IPC

Previous studies have shown that aging is associated with significant alterations in the expression patterns of cell surface cytokine receptors, thus suggesting a potential mechanism that may contribute to senescent changes in cardiac microvasculature activity.[48] Our study shows that in myocardium from the older animals, no transcript levels were altered, with the exception of Atf3. This increase in Atf3 was relatively small and might be not sufficient to engage the other pathways that elicit cardiac protection. The only previous study of age-related changes of cardiac gene expression was conducted with brief episodes of ischemia–reperfusion followed by 24 h of recovery.[12] This work showed that down-regulation of transcripts was associated with early-remodeling genes in the young rat as well as decreases in hypertrophy-related transcripts, whereas hearts from old animals showed a unique injury-related response, which included up-regulation of mRNAs encoding proteins associated with hypertrophy or apoptosis.

In a mouse model of myocardial infarction, Frangogiannis *et al.*[20] showed that myocardial senescence was associated with decreased and delayed neutrophil and macrophage infiltration, markedly reduced cytokine and chemokine expression, and impaired phagocytosis of dead cardiomyocytes. Delayed granulation tissue formation and markedly reduced collagen deposition were also observed.[20] These defects might contribute to adverse remodeling after ischemia–reperfusion injury in the old animals.

Our finding that early-phase preconditioning elicits little change in the patterns of gene expression in the hearts of older animals is consistent with the ineffectiveness of IPC and APC in protecting the senescent myocardium. Here, the lack of increased cytokine and chemokines may be crucial. Understanding of the molecular mechanisms underlying these changes may provide insights into myocardial aging and lead to potentially new therapeutic strategies.

Gene Expression Patterns in Young and Old Myocardium during APC

The inability of APC to induce acute changes in gene expression also leads to the obvious conclusion that a common set of early-phase preconditioning–induced genes could not be identified and so to the rejection of our second hypothesis. This failure to find a common preconditioning gene set was not due to an overly stringent application of FDR control, because allowing for a more generous 20% FDR still did not show any potential genes in common between IPC and APC.

We had shown previously that isoflurane induced cardiac protection through increases in reactive oxygen species in the young but not senescent rat myocardium. Here, we have extended these findings and have shown that early-phase APC is independent of modulation of gene transcript levels. Unlike IPC, APC did not significantly alter myocardial gene expression in young or old animals. Because only IPC alters substantially changes gene expression, the idea that acutely modulating gene expression is necessary for early-phase APC and its consequent cardiac protection must be rejected.

Nonetheless, previous work has established that both early-phase IPC and APC engage a number of signaling pathways that involve protein phosphorylation cascades. These include PI3K-Akt, MEK-1/2-ERK1/2, NFKB, and PKC pathways, and pharmacologic evidence demonstrates that these pathways are essential for IPC and APC-induced cardioprotection.[49] A number of studies have demonstrated that protein kinases, such as Akt/GSK3β, mitogen-activated protein kinases, and PKC isoforms, are activated by APC, leading to the Ser/Thr phosphorylation of numerous substrates involved in regulating prosurvival pathways, including the mitochondrial transition pore currently believed to be a central element in the preconditioning pathway.[50,51]

Our network analyses of the IPC-induced changes in transcript levels suggest that these various phosphorylation pathways may mediate the changes in gene expression that we observe, yet engagement of these pathways seems to be insufficient to trigger, acutely, a comparable APC-induced genomic response. Whether these or other pathways are indeed responsible for the observed changes in gene expression and whether these changes are necessary or sufficient for IPC-related cardiac protection remain to be examined.

Clinical Implications

There is a large amount of experimental evidence that IPC and APC exert beneficial effects on the consequences of myocardial ischemia–reperfusion injury.

Recent research has been focused on the possible implementation of this property in patient care. Age dependence of preconditioning response is highly relevant to clinical utility, because the majority of patients to benefit from adjunctive cardioprotection are in the older age range. Several studies have addressed the potential clinical implication of cardioprotection by volatile anesthetics in patients undergoing coronary artery surgery, looking at cellular enzyme release and myocardial function.[52–55]

Because of obvious limitations, it has been difficult in the past to provide sufficient evidence for anesthetic preconditioning in clinical studies. A few clinical studies with a small number of patients have been conducted, with somewhat limited success. The beneficial effects of volatile anesthetics in elderly high-risk patients undergoing elective coronary artery bypass grafting have so far been confined to surrogate markers. In the first study, by Belhomme *et al.*,[56] in patients aged 70 ± 9 yr undergoing elective coronary artery bypass grafting, anesthetic preconditioning was elicited after the onset of cardiopulmonary bypass by a 5-min exposure to 2.5 minimum alveolar concentration isoflurane, followed by a 10-min washout before aortic cross clamping and cardioplegic arrest. Van Der Linden *et al.*[57] investigated the effects of several anesthetics on recovery of myocardial function in coronary surgery patients older than 70 yr with three vessel disease and an ejection fraction less than 50%. The volatile anesthetics sevoflurane and desflurane preserved left ventricular function after surgery with less evidence of myocardial damage postoperatively. Although these results suggest a beneficial effect of cardioprotection and preconditioning by volatile anesthetics in elderly patients with coronary artery disease, no clinical investigation to date has shown decreased long-term morbidity and mortality by anesthetic preconditioning.

Not all clinical preconditioning studies, however, have shown cardioprotective effects in terms of either better preservation of myocardial function or less postoperative myocardial damage.[58,59] This underscores the fact that the clinical preconditioning protocol, patient age, and disease condition may be critical to its putative protective effects.

Study Limitations

This study focused on the transcriptional profiling of the young and senescent myocardium during early-phase IPC and APC using a microarray approach. Confirmation using RT-PCR was performed with only a limited number of genes; nonetheless, DNA microarray platforms have been shown to exhibit high correlation with quantitative gene expression values, as determined by RT-PCR assays. The inferences drawn from these data are subject to the caveat that functionally important changes in protein expression and/or posttranslational modification during early-phase IPC and APC are unknown, with the exception of Atf3 and Egr1. Although genomics has demonstrated that there is more than 85% similarity in coding regions of the rat genome compared with the human genome, data from rodent studies must always be interpreted with caution with respect to therapeutic or pathophysiologic implications for human patients. Another possible limitation of our study is that the same dose of isoflurane was used in both age groups, and minimum alveolar concentration values decline with age. Only a single time point was chosen, so our experiments could miss some important later gene expression changes. Indeed, previous work would indicate that lengthening the time interval would reveal some changes induced by APC. However, we chose the most relevant time interval corresponding to the earliest phase of APC-induced cardiac protection. It is likely that in the young animals subjected to IPC, transcription of some genes was also suppressed. Because our study only measured the steady state levels of mRNA, significant effects to suppress transcription may have been obscured by the requirement that the preexisting pools of these putative mRNAs be degraded during a relatively brief time interval. Finally, we ascribe the changes in gene expression to the myocardium, implying a specific association with the cardiomyocytes, but the myocardium is a more complex tissue with fibroblasts, modified myocytes involved in electrical conduction pathways, and numerous vascular elements, including adherence of blood components. Further studies localizing the IPC-induced transcriptional changes to specific cell types would help to clarify these relationships. Our new findings raise the possibility that IPC-related gene expression actually triggers cytokine and chemokine induced prosurvival pathways in the young heart, although such a mechanism seems to be absent in the old tissue. Experiments have yet to be performed to test whether these early IPC-induced changes in gene expression are causally linked to cardiac protection in the young myocardium. Whether the senescent myocardium could respond in a salutary manner to an appropriate combination of these inflammatory factors is also unknown.

In conclusion, the current study provides the first gene expression profile associated with early-phase IPC and APC in the young compared with the senescent myocardium *in vivo*. The attenuation of IPC and APC in the aged myocardium may be attributed to multiple factors. Here, we showed that a defect in IPC-induced transcriptional gene expression might be one underlying reason for the absence of cardiac protection in the aged myocardium. These results lay the groundwork for future studies aimed at determining whether and how these genomic alterations affect cardiac phenotype and function.

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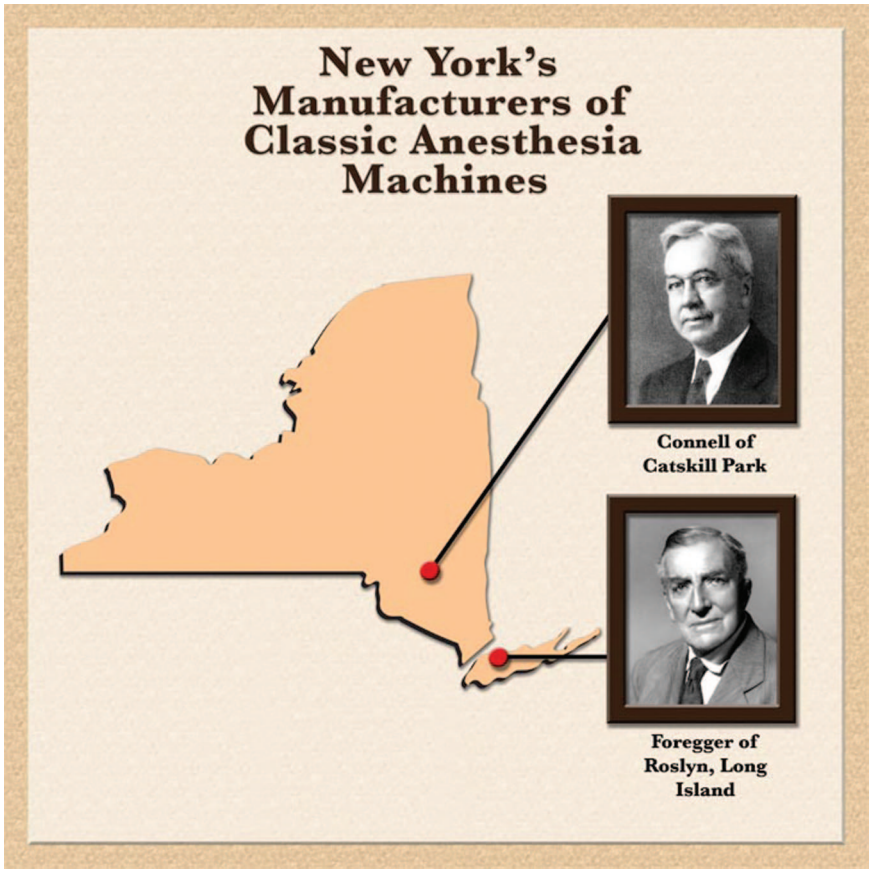
ANESTHESIOLOGY REFLECTIONS

New Yorkers Foregger and Connell



Born in Vienna, Austria, Richard von Foregger (1872–1960) earned his Ph.D. on the Continent before working with Carl Steinmetz in New York. After fusing sodium peroxide to generate oxygen, Foregger founded his namesake company in Roslyn, New York. Over the next 40 yr he assisted development of anesthetic apparatus such as the Gwathmey Apparatus, Waters' To-and-Fro Canister, and Morris' "Copper Kettle" precision vaporizer. Competing against Foregger was a Nebraskan-turned-New Yorker, Karl Connell, M.D. (1878–1941), who invented the Anesthetometer and developed at least three types of flowmeters for his finely calibrated Connell Anesthesia Machines. Both of these adopted sons of New York produced anesthetic apparatus and machines using suggestions supplied by practicing anesthesiologists. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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