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Age-associated differences in gene expression in response to delayed anesthetic preconditioning

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Abstract Evidence suggests that the protective benefits of anesthetic preconditioning (APC) are significantly attenuated in the aged myocardium. In this study, we investigated the effect of aging on gene expression in delayed APC. Hearts from Fischer 344 rats, age 4 or 24 months, were divided into five groups: control; ischemia/reperfusion (I/R); and delayed APC at 6, 12, and 24 h. Whole-genome array was studied using Affymetrix Rat Genome 230 2.0 array. 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. Of the 28,000 genes represented on the Affymetrix Rat Genome 230 2.0 Microarray chip, 24 transcripts in 6 h APC, 28 in 12 h APC, and 28 in 24 h APC group displayed significant up-regulation in mRNA levels, and 70 transcripts in 6 h APC, 101 in 12 h APC, and 82 in 24 h APC displayed significant down-regulation in young rat hearts. These altered genes fall into functional categories of cell defense/death, cell structure, gene expression/protein synthesis, inflammatory response/growth/remodeling, and signaling/communication. Although alterations for some genes were in common, the numbers of changed genes in old rats were markedly and consistently lower than the young rats. Twenty-four hour delayed APC also significantly reduced infarct size and improved myocardial left ventricular function in young hearts, effects that were not observed in old rat hearts. We concluded that delayed APC profoundly and differentially affected gene expression profiles of the cardiomyocyte in an age-associated pattern. The impaired genomic response to delayed APC could underlie the loss of the protective benefits of preconditioning in aged hearts.

Keywords Anesthetic · Preconditioning · Delayed · Gene · Expression · Myocardium

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Introduction

Aging is associated with significantly increased cardiovascular morbidity and mortality. The majority of acute myocardial infarctions occur in patients older than 65 years. Aging hearts have demonstrated more

severe myocardial stunning, decreased recovery of hemodynamic and high-energy phosphates, and greater overall tissue damage after ischemic insult (Uecker et al. 2003; Zaugg et al. 2003). When compared to young hearts, aging hearts exhibit significant structural changes (e.g., thickening of the left ventricular wall, decrease in the number of cardiomyocytes, and reduced left ventricular cavity size) and biochemical changes (e.g., decreased rates of calcium sequestration, accumulation of collagen, enhanced production of stress-related substances, increased expression of angiotensin II receptors, enhanced apoptosis, and desensitization of cardiac β-adrenergic receptors) (Marber et al. 1993; Kuzuya et al. 1993). These cellular structural and biochemical changes could have major implications in the age-related decreases in cardiac tolerance to ischemia. However, the regulatory mechanisms underlying these changes are not fully defined.

Preconditioning is a biological phenomenon where a transient stressful stimulus induces a protective state against a more prolonged, potentially lethal insult. It can be elicited by brief ischemic episodes or by volatile anesthetics (Julier et al. 2003; Kersten et al. 1997). Ischemic preconditioning (IPC) and anesthetic preconditioning (APC) both represent powerful means of attaining myocardial protection against prolonged ischemia/reperfusion (I/R) injury (Da Silva et al. 2003; Kehl et al. 2002). Similar to IPC, APC results in two windows of protection with separate proposed mechanisms. The classic early phase, predominantly based on multiple, fast-acting intracellular signaling steps, begins immediately after the application of the protective stimulus and lasts for 2–3 h. The preconditioning stimulus further initiates and triggers a second long-lasting window of protection, which occurs 12–24 h after the initial preconditioning stimulus and is effective for 3–4 days (Tsutsumi et al. 2006; Shi et al. 2005; Wakeno-Takahashi et al. 2005; Lutz and Liu 2006; Tonkovic-Capin et al. 2002; Tanaka et al. 2004; Chiari et al. 2004). This delayed protection is the result of altered genomic response involving increased de novo protein synthesis (Marber et al. 1993; Kuzuya et al. 1993).

Previous studies have shown that the cardiac protection afforded by IPC or APC is absent or attenuated in the aged myocardium (Sniecinski and Liu 2004; McCully et al. 2006; Nguyen et al. 2008). A study has also shown that gene expression is altered in the aged myocardium following I/R after 24 h (Simkhovich et al. 2003). A recent study indicated age-associated differences in genomic responses in early phase preconditioning (Liu et al. 2009). To date, no data are available with respect to age-associated changes in gene expression in delayed APC. We hypothesized that delayed APC could differentially affect gene expression patterns in young vs. old cardiomyocytes. To test this hypothesis, microarray technology was used to investigate the effect of aging on gene expression for delayed APC.

Results

Numbers of regulated transcripts by APC in young versus old rats

Of a total of 31,042 probe sets displayed on the Affymetrix Rat Genome 230 2.0 Microarray chip, 94 transcripts were significantly (≥2.0-fold) altered in 6 h APC in young rats as compared to the control group, of which 33 represented ESTs (unknown genes). One hundred twenty-nine genes were significantly changed in 12 h delayed APC (41 ESTs), and 110 were significantly altered in 24 h delayed APC group (50 ESTs). Six hour APC up-regulated 24 genes (14 ESTs) and down-regulated 70 genes (19 ESTs) by 2.0-fold or greater. Twelve hour delayed APC up-regulated 28 genes (16 ESTs) and down-regulated 101 genes (25 ESTs). Twenty-four hour delayed APC up-regulated 28 genes (25 ESTs) and down-regulated 82 genes (25 ESTs). In the I/R group, 18 genes were significantly changed (9 ESTs), among them 9 genes were up-regulated (4 ESTs) and 9 genes were down-regulated (5 ESTs) (Figs. 1 and 2).

In old rat hearts, a total of 40 altered transcripts were found to meet our criteria for having changed expression in 6 h APC, of which there were 23 identifiable genes and 17 ESTs. Eighty-one genes were significantly altered in 12 h delayed APC, of which there were 38 identifiable genes and 43 ESTs. Thirty-five genes were significantly altered in 24 h delayed APC group, of which there were 14 identifiable genes and 21 ESTs. Six hour APC up-regulated 10 genes (9 ESTs) and down-regulated 30 genes (8 ESTs). Twelve hour delayed APC up-

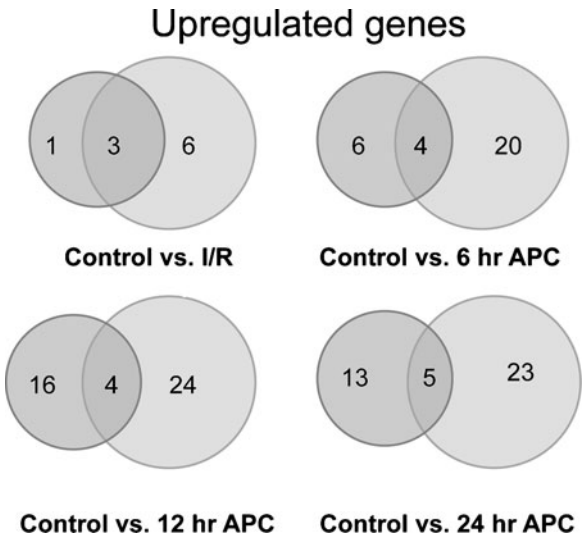

Fig. 1 Venn diagrams of the numbers of significantly (≥2.0-fold) up-regulated transcripts in the control, I/R, 6 h APC, 12 h APC, and 24 h APC groups in young and old rat hearts, and the number of overlapping transcripts between young and old rat hearts. *Light circle*: young rat hearts; *darker circle*: old rat hearts. *6 hr APC*: 6 h delayed preconditioning, *12 hr APC*: 12 h delayed preconditioning, *24 hr APC*: 24 h delayed preconditioning

regulated 20 genes (19 ESTs) and down-regulated 61 genes (24 ESTs). Twenty-four hour delayed

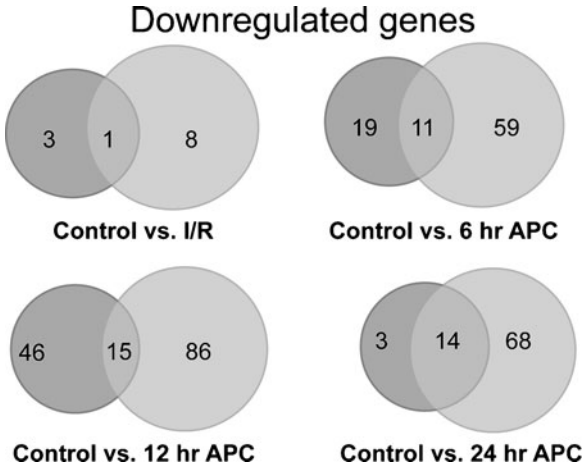

Fig. 2 Venn diagrams of the numbers of significantly (≥2.0-fold) down-regulated transcripts in the control, I/R, 6 h APC, 12 h APC, and 24 h APC groups in young and old rat hearts, and the number of overlapping transcripts between young and old rat hearts. *Light circle*: young rat hearts; *darker circle*: old rat hearts. *I/R*: ischemia/reperfusion, *6 hr APC*: 6 h delayed preconditioning, *12 hr APC*: 12 h delayed preconditioning, *24 hr APC*: 24 h delayed preconditioning

APC up-regulated 18 genes (17 ESTs) and down-regulated 17 genes (4 ESTs). In the I/R group, eight genes were significantly changed (containing 3 ESTs), among them 4 genes were up-regulated (containing one EST) and 4 genes were down-regulated (containing 2 ESTs) (Figs. 1 and 2).

Delayed APC displays overlapping in up-/down-regulated genes in young and old rats

APC concomitantly up-regulated 4 genes and down-regulated 11 genes by 2.0-fold or greater in both young and old rat hearts 6 h after sevoflurane exposure (Figs. 1 and 2). Among the up-regulated genes were glutamate oxaloacetate transaminase 2 and 3 other unknown genes. Among the down-regulated genes were 10 identifiable genes [chemokine (C-X-C motif) ligand 1, chemokine (C-X-C motif) ligand 2, chemokine (C-C motif) ligand 3, tumor necrosis factor, early growth response 1, a disintegrin-like and metallopeptidase with thrombospondin type 1 motif 1, cyclin L1, FBJ murine osteosarcoma viral oncogene homolog, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta, and activating transcription factor 3] and one unknown gene. Twelve hour delayed APC jointly up-regulated 4 genes and down-regulated 15 genes in young and old hearts; the up-regulated genes were the same as those for 6 h APC. The down-regulated genes included nuclear receptor subfamily 4 group A member 1, nuclear receptor subfamily 4 group A member 3, cysteine-rich protein 61, chemokine (C-C motif) ligand 2, one unknown gene, and the same 10 down-regulated genes observed in 6 h APC [except chemokine (C-C motif) ligand 3 and one unknown gene]. Five genes and 14 genes were concomitantly up-regulated and down-regulated by 24 h delayed APC, respectively; RNA binding motif protein 5 and 4 unknown genes were up-regulated; DNA-damage inducible transcript 3, RAS dexamethasone-induced 1, similar to OTU domain containing 1, one unknown gene, and the same 10 down-regulated genes found in 12 h delayed APC [except early growth response 1, cysteine-rich protein 61, chemokine (C-C motif) ligand 2, nuclear receptor subfamily 4 group A member 1, and one unknown gene] were down-regulated.

As shown in Table 1, six identifiable genes were commonly down-regulated in young and old rat hearts

Table 1 Identifiable genes jointly down-regulated in young and old rat hearts at all time points in delayed APC

Gene symbol	Gene name	Fold change					
		Young			Old		
		6 h	12 h	24 h	6 h	12 h	24 h
Atf3	Activating transcription factor 3	262.22	350.31	229.38	71.02	87.49	91.03
Cxcl1	Chemokine (C-X-C motif) ligand 1	246.2	249.57	247.94	470.57	468.30	475.15
Cxcl2	Chemokine (C-X-C motif) ligand 2	89.39	89.25	89.67	212.57	212.54	216.06
Fos	FBJ murine osteosarcoma viral oncogene homolog	499.61	408.92	249.34	157.60	320.11	134.62
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	5.13	2.37	6.11	14.61	32.15	20.99
NFkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	41.02	41.56	41.70	45.19	59.38	41.03

APC: anesthetic preconditioning

at all time points examined (6, 12, and 24 h) in delayed APC, among them were activating transcription factor 3, chemokine (C-X-C motif) ligand 1, chemokine (C-X-C motif) ligand 2, FBJ murine osteosarcoma viral oncogene homolog, tumor necrosis factor, and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta. Notably, the expression levels of all of these genes, except tumor necrosis factor in young rats, were strikingly down-regulated in both young and old rats, though differences in the magnitude of the changes between young and old rats existed. Table 1 also demonstrates the consistency in the expression levels of those genes from 6 h to 24 h delayed APC. None of the genes were jointly up-regulated in young and old rats at all time points in the delayed APC.

Three genes (heat shock 70 kDa protein 1A, heat shock 70 kDa protein 1A, and one unknown gene) and one gene (tumor necrosis factor) were found jointly up-regulated and down-regulated in the I/R group in young and old rat hearts, respectively (Figs. 1 and 2).

Delayed APC differentially regulates gene expression in an age-related manner

As described above, the numbers of up-regulated and down-regulated genes induced by delayed APC were significantly and consistently greater in young rat cardiomyocytes than in that of old rat at all time points examined (Figs. 1 and 2). Tables 2, 3, and 4 depict the identifiable genes that were changed in response to 24 h APC in detail. Table 2 shows that cytochrome P450 family 2 subfamily e polypeptide 1, glutamate oxaloacetate transaminase 2, and RNA binding motif protein 5 were up-regulated in young rat cardiomyocytes, whereas only RNA binding motif protein 5 was found up-regulated in old rat hearts. Table 3 shows 13 identifiable genes that were down-regulated in the old rat hearts. These genes fall into four functional categories: cell defense/death, gene expression/protein synthesis, growth/remodeling/inflammatory response, and signaling/communication. In contrast, the number of down-regulated identifiable genes (57) in young rat cardiomyocytes was more

Table 2 Identifiable genes down-regulated in young and old rat hearts in response to APC at 24 h as compared with control

Gene symbol	Gene name	Fold change
Young rat hearts		
Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1	4.72
Rbm5	RNA binding motif protein 5	3.65
Got2	Glutamate oxaloacetate transaminase 2, mitochondrial	3.58
Old rat hearts		
Rbm5	RNA binding motif protein 5	2.26

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Table 3 Identifiable genes down-regulated in old rat hearts in response to APC at 24 h as compared with control

Gene symbol	Gene name	Fold change
Cell defense/death		
Ddit3	DNA-damage inducible transcript 3	2.39
Gene expression/protein synthesis		
Atf3	Activating transcription factor 3	91.03
Fos	FBJ murine osteosarcoma viral oncogene homolog	134.62
Nr4a3	Nuclear receptor subfamily 4, group A, member 3	19.58
Sfrs5	Splicing factor, arginine/serine-rich 5	2.13
Growth/remodeling/inflammatory response		
Cxcl1	Chemokine (C-X-C motif) ligand 1	475.15
Cxcl2	Chemokine (C-X-C motif) ligand 2	216.06
Ccl3	Chemokine (C-C motif) ligand 3	15.01
Tnf	Tumor necrosis factor	20.99
Adamts1	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	6.39
Signaling/communication		
Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	41.03
RGD1563344	Similar to OTU domain containing 1	7.55
Rasd1	RAS, dexamethasone-induced 1	10.02

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than four times than that of old rat cardiomyocytes; these genes included all of those identified in old rat hearts with one gene as the exception (splicing factor arginine/serine-rich 5), and 45 additional genes altered exclusively in young rat hearts, of which 20 were moderately down-regulated (2–5-fold), 15 were highly down-regulated (5–10-fold), and 10 were dramatically down-regulated (>10-fold). The down-regulated genes in young rat hearts in 24 h delayed APC fall into five functional categories: cell defense/death, cell structure/motility, gene expression/protein synthesis, growth/remodeling/inflammatory response, and signaling/communication (Table 4).

Table 5 further illustrates the profound difference between young and old hearts in response to delayed APC. At all time points examined in delayed APC (6, 12, and 24 h), no identifiable gene was up-regulated in old hearts only, while glutamate oxaloacetate transaminase 2 was up-regulated in young rat hearts. Remarkably, as compared to one gene down-regulated in old rat hearts only, 16 identifiable genes were down-regulated in young rat hearts, which included 4 cell defense/death genes, 2 cell structure/motility genes, 4 gene expression/protein synthesis genes, 3 growth/remodeling/inflammatory response genes, and 3 signaling/communication genes.

Real-time RT–PCR

To confirm the results of microarray hybridization experiments, quantitative real-time RT–PCR was used as an independent method of measuring gene expression levels. Intensities of five selected genes recorded by the Affymetrix method and real-time RT–PCR are presented in Fig. 3. These data suggest that the expression patterns (the direction of up- and down-regulation as well as the strength of regulation) for these selected genes were similar on both the gene chip array and the real-time RT–PCR, hence confirming the accuracy of the gene chip data.

Left ventricular end diastolic pressures and myocardial infarct size

The recovery of LV performance was quantified at the end of 60 min of reperfusion versus the baseline phase before ischemia. The left ventricular end diastolic pressures (LVEDP), which represents the

Table 4 Identifiable genes down-regulated in young rat hearts in response to APC at 24 h as compared with control

Gene symbol	Gene name	Fold change
Cell defense/death		
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	9.27
Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	12.06
Ddit3	DNA-damage inducible transcript	33.85
Mt1a	Metallothionein 1a	2.56
Hspa1a	Heat shock 70 kDa protein 1A	6.00
Cell structure/motility		
Eln	Elastin	2.20
H3f3b	H3 histone, family 3B	2.36
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	2.01
Slc16a13	Solute carrier family 16 , member 13	20.22
H3f3b	H3 histone, family 3B	2.68
Cldn5	Claudin 5	4.03
PVR	Poliovirus receptor	4.08
Cmya1	Cardiomyopathy associated 1	2.32
Ssat	Spermidine/spermine N1-acetyl transferase	5.68
Gene expression/protein synthesis		
Atf3	Activating transcription factor 3	229.38
Egr1	Early growth response 1	40.76
Egr2	Early growth response 2	35.62
Fos	FBJ murine osteosarcoma viral oncogene homolog	249.34
Jun	Jun oncogene	5.79
Junb	Jun-B oncogene	20.79
Myc	Myelocytomatosis viral oncogene homolog	7.46
Zfp36	Zinc finger protein	365.31
Zfand2a	Zinc finger, AN1-type domain 2A	2.85
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	18.75
Nr4a3	Nuclear receptor subfamily 4, group A, member 3	12.18
Arid5a	AT rich interactive domain 5A (Mrf1 like)	4.60
Growth/remodeling/inflammatory response		
Cxcl1	Chemokine (C-X-C motif) ligand 1	247.94
Cxcl2	Chemokine (C-X-C motif) ligand 2	89.67
Ccl2	Chemokine (C-C motif) ligand 2	9.67
Ccl3	Chemokine (C-C motif) ligand	39.66
Ccl4	Chemokine (C-C motif) ligand 4	5.86
Ccl7	Chemokine (C-C motif) ligand 7	7.15
Tnf	Tumor necrosis factor	6.11
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	5.10
Il1b	Interleukin 1 beta	4.76
Cyr61	Cysteine-rich protein 61	33.33
Btg2	B-cell translocation gene 2	11.24
Cdc2a	Cell division cycle 2 homolog A	4.02
Ccnl1	Cyclin L1	3.52
Adamts1	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	5.31
Okl38	Pregnancy-induced growth inhibitor	5.07
Signaling/communication		
Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	41.70
Socs3	Suppressor of cytokine signaling 3	16.39
Rasd1	RAS, dexamethasone-induced 1	6.91
Rnd1	Rho family GTPase 1	6.00
Rnd3	Rho family GTPase 3	7.38
Myd116	Myeloid differentiation primary response gene 116	9.85
RGD156334	Similar to OTU domain containing 1	8.53
Dusp1	Dual specificity phosphatase 1	3.88
Ier2	Immediate early response 2	10.66
Ier3	Immediate early response 3	4.24
Ccrn4l	CCR4 carbon catabolite repression 4-like	3.78
Snf1lk	SNF1-like kinase	3.18
Ifrd1	Interferon-related developmental regulator 1	4.62
Slfn2	Schlafen 2	2.07
Bex1	Brain expressed X-linked 1	2.32
Plk2	Polo-like kinase 2	4.54

APC: anesthetic preconditioning

left ventricular (LV) diastolic function, were significantly increased in the young I/R group from 7 mmHg before ischemia to 51±12 mmHg at the end of reperfusion ($p<0.05$). The LVEDP were increased from 7 mmHg to 25±4 mmHg in the young 24 h delayed APC group ($p<0.05$), and the increases in LVEDP in the young 24 h delayed APC group were significantly smaller than that of the young I/R group ($p<0.05$). The LVEDP increased from 7 mmHg prior to ischemia to 65±9 mmHg ($p<0.05$) in I/R group at the end of reperfusion and to 60±6 mmHg ($p<0.05$) in 24 h delayed APC group in old rat hearts; there were no differences in LVEDP between 24 h delayed APC group and I/R group in old rat hearts. The infarct sizes in the young I/R group (34±3%) were significantly greater than the young 24 h delayed APC group (16±3%, $p<0.05$). However, there were no differences in infarct size between the I/R group (38±5%) and 24 h delayed APC group (37±6%, $p<0.05$) in the old rat hearts.

Table 5 Identifiable genes differentially regulated in old rat and young rat hearts at all time points in delayed APC

Gene symbol	Gene name	Fold change		
		6 h	12 h	24 h
Up-regulated in young rat hearts only				
Got2	Glutamate oxalocetate transaminase 2, mitochondrial	3.51	3.80	3.58
Up-regulated in old rat hearts only				
	None			
Down-regulated in young rat hearts only				
Btg2	B-cell translocation gene 2, anti-proliferative	14.84	17.29	11.43
Ccl3	Chemokine (C-C motif) ligand 3	8.34	9.37	9.66
Ccl4	Chemokine (C-C motif) ligand 4	3.35	11.75	5.86
Ddit3	DNA-damage inducible transcript 3	2.42	3.11	3.85
Egr1	Early growth response 1	70.46	39.27	40.76
Egr2	Early growth response 2	34.22	37.50	35.61
Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	4.56	6.60	12.06
H3f3b	H3 histone, family 3B	3.06	2.84	2.52
Ifrd1	Interferon-related developmental regulator 1	6.20	5.69	4.62
Myc	Myelocytomatosis viral oncogene homolog	6.69	7.35	7.46
Myd116	Myeloid differentiation primary response gene 116	9.17	8.39	9.85
Nr4a3	Nuclear receptor subfamily 4, group A, member 3	29.79	29.04	12.18
Sic16a13	Solute carrier family 16, member 13	26.42	24.96	20.22
Hspa1a	Heat shock 70 kDa protein 1A	6.30	8.69	6.00
Socs3	Suppressor of cytokine signaling 3	13.10	16.59	16.39
Down-regulated in old rat hearts only				
Adamts1	A disintegrin-like and metallopeptidase (reprolysis type) with thrombospondin type 1 motif, 1	4.61	7.36	6.39

APC: anesthetic preconditioning

Discussion

This is the first study to investigate the effect of aging on delayed APC induced genomic responses on a genome-wide scale. Delayed APC elicited different gene expression patterns in young and old rat cardiomyocytes. Delayed APC markedly altered the transcriptional activity of various genes in young rat myocardium. However, in aged cardiomyocytes, the number of altered genes was significantly lower. This profound difference in transcriptional regulation between young and old rat cardiomyocytes in response to delayed APC could in part explain the impairment of the cardioprotection of preconditioning in the aged hearts.

Aging is accompanied with declines in baseline cardiac function. The aged cardiomyocyte develops decreased tolerance to stress, decreased mitochondrial function, decreased contractile function, and increased susceptibility to apoptosis and necrosis (Bernhard and Laufer 2008). When the aged heart is exposed to an ischemic insult, the tolerance to I/R injury is reduced (Abete et al. 1999; Boengler et al. 2007). Several biological systems are thought to be involved in the loss of tolerance to I/R in aged myocardium. Aging affects cardiomyocytes at several subcellular and molecular levels, including alterations at the level of the DNA (mutations and telomere shortening), increased reactive oxygen species (ROS) formation and oxidative stress, changes in the gene/protein expressions, and posttranslational modifications (e.g., advanced glycation end products and protein oxidation) (Boengler et al. 2009). Evidence suggests that gene expression profiles are altered during aging, resulting in corresponding alterations in cardiomyocyte phenotype (Bodyak et al. 2002; Lee et al. 2002). Moreover, previous studies have also shown that the cardiac protection afforded

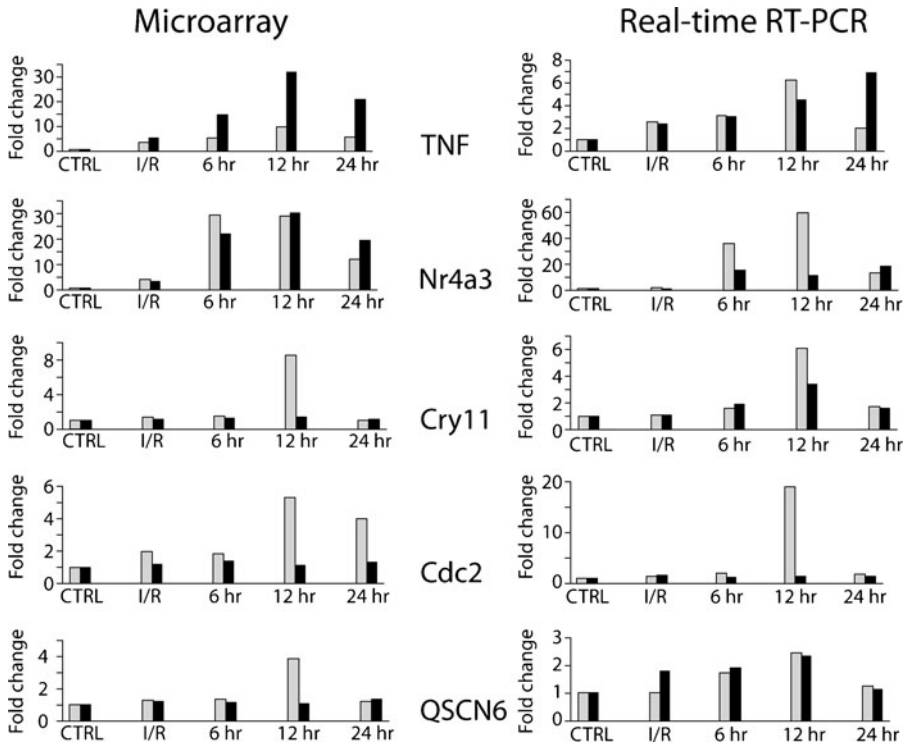

Fig. 3 Comparisons of microarray and real-time reverse transcription–polymerase chain reaction (RT–PCR) for the mRNA levels of TNF, Nr4a3, Cryl1, Cdc2a, and QSCN6. *Bars* represent fold changes measured by gene chips (*left panel*) and real-time RT–PCR (*right panel*). *Light bar*: young rat hearts; *darker bar*: old rat hearts. *CTRL*: control, *I/R*: ischemia/reperfusion, *6 hr*: 6 h delayed preconditioning, *12 hr*: 12 h delayed preconditioning, *24 hr*: 24 h delayed preconditioning

by IPC or APC is attenuated or absent in the aged rat myocardium (Sniecinski and Liu 2004; McCully et al. 2006; Nguyen et al. 2008; Boengler et al. 2009).

Delayed preconditioning affords extended protection (lasting 30-fold longer when compared to early preconditioning) after the application of the preconditioning stimulus. Similar to brief episodes of ischemia-triggered preconditioning, animal studies indicate that APC offers protection from I/R injury in myocardial tissue. Tonkovic-Capin et al. (2003) first reported successful late preconditioning in a rabbit heart model using isoflurane. Subsequent studies have confirmed these findings in rats and mice (Tsutsumi et al. 2006; Shi et al. 2005; Wakeno-Takahashi et al. 2005; Lutz and Liu 2006). In contrast to acute preconditioning, delayed preconditioning is dependent on the transcriptional activity of genes which encode proteins important for cardioprotection.

To unravel the underlining mechanisms by which aging attenuates or eliminates the protection provided by delayed APC, gene expression profiles were examined in young and old rat myocardium in the present study. We demonstrated that delayed APC induced significant changes in gene regulation in young rat hearts. Our data have shown that genes involved in inflammatory responses were markedly altered during delayed APC in young rat hearts. Inflammatory responses play a critical role in I/R-related injury. Inflammatory mediators such as cytokines, chemokines, and adhesion molecules are responsible for regulating leukocyte recruitment, infiltration to sites of I/R injury, and activation and generation of ROS which significantly contribute to cell and tissue dysfunction (Frangogiannis et al. 2002; Frangogiannis and Entman 2005; Zuidema and Zhang 2010; Gill et al. 2010). As predicted, the expression levels of nine chemokines and cytokines (Cxcl1, Cxcl2, Ccl2, Ccl3, Ccl4, Ccl7, Tnf, Tnfrsl2a, and Il1b) were significantly decreased at 24 h delayed APC in young rat hearts, whereas only four inflammatory mediators (Cxcl1, Cxcl2, Ccl3, and Tnf) were down-regulated in old rat hearts. Six growth/remodeling-associated genes (Btg2, Cdc2a, Cyclin L1, Cyr61, Adamts1, and Okl38) were altered in young rats compared to only one (Adamts1) in old rat hearts. Five cell death-related genes (Gadd45b, Gadd45g, Ddit3, Mt1a, and Hspa1a) were observed to be down-regulated in young rat hearts compared with only one down-regulated gene (Ddit3) in old hearts. Intracellular signaling pathways are crucial in modulating stress-related biological processes (Elahi et al. 2009; Valko et al. 2007; Hall et al. 2006; Hu and Liu 2009;

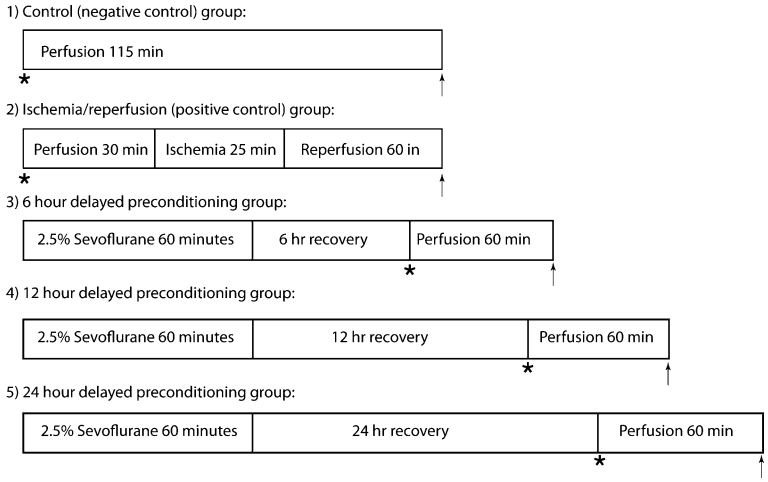

Fig. 4 Schematic illustration of the experimental protocol. Rats were divided into five groups: control group, ischemia/reperfusion group, 6 h delayed preconditioning group, 12 h delayed preconditioning group, and 24 h delayed preconditioning group. *Asterisks* indicated the hearts were cannulated and Langendorff perfused. *Arrows* indicated the time when the left ventricular tissues were removed and frozen at −80°C in liquid nitrogen or the LVEDP and infarct sizes were measured

Das and Maulik 2006; Lai and Frishman 2005). Sixteen cellular signaling/communication associated genes including Nfkbiz, Socs3, Rasd1, Rnd1, Rnd3, RGD156334, Dusp1, Ier2, Ier3, Ccrn4l, Snf1lk, Slfn2, Bex1, and Plk2 were down-regulated in young hearts; however, only three (Nfkbiz, Rasd1, and RGD156334) were altered in this category of genes in old rat hearts. Similarly, 12 gene expression/protein synthesis-associated genes including Atf3, Egr1, Egr2, Fos, Jun, Jun b, Myc, Zfp36, Zfand2a, Nr4a1, Nr4a3, and Arid5a were down-regulated in young hearts vs. four (Atf3, Fos, Nr4a3, and Sfrs5) in the old rat hearts. Moreover, nine cell structure/motility-related genes were down-regulated in young hearts, while none was found in old rat hearts.

Besides the down-regulation of injury-related genes, we also observed up-regulation of genes important for cardioprotection during the course of delayed APC in young rat hearts. Among these up-regulated genes were glutathione *S*-transferase zeta 1, quiescin Q6, hypoxia up-regulated 1, and cytochrome P450 2e1. Glutathione *S*-transferase zeta 1 is an antioxidant enzyme which plays an important role in the detoxification of ROS and other oxidants (Hayes et al. 2005; Rahman et al. 1999; Owuor and Kong 2002). It was found that the expression level of glutathione *S*-transferase zeta 1 in young rat hearts was up-regulated at 6 h after APC. Quiescin Q6, found to be protective against oxidative stress (Morel et al. 2007; Li et al. 2005), was up-regulated at 12 h after APC. Hypoxia up-regulated 1, which promotes cell survival in hypoxic condition (Tamatani et al. 2001; Ozawa et al. 2001; Kuwabara et al. 1996), was up-regulated at 12 h after APC. The expression level of the metabolic enzyme cytochrome P450 2e1 was also elevated 6 h after sevoflurane exposure. None of these protective genes were up-regulated in old rat hearts. Moreover, our results revealed that not only were the numbers of genes altered but differences in the changes of individual gene with time course also existed. For instances, glutamate oxaloacetate transaminase 2 was up-regulated 3.5–4-fold from 6 h to 24 h after sevoflurane exposure in young hearts, while 2–3-fold changes were observed only at 6 h and 12 h but not 24 h delayed APC in old rats. Chemokine ligand 3 was down-regulated at all time points examined in young hearts, but decreased only at 12 h and 24 h in old hearts. The expression levels of early growth response 1 were markedly reduced at 6 h, 12 h, and 24 h delayed APC in young rats; however, reduction of its expression was observed only at 6 h and 12 h delayed APC, and no change was found at 24 h delayed APC in old rat hearts. These data indicated the kinetic differences of gene expression changes between young and old rat hearts. Together, our data demonstrated that, although some similarities in gene changes existed between young and old rats' cardiomyocytes, delayed APC remarkably and

differentially regulated the genomic responses in an age-dependent manner.

Despite the fact that both IPC and APC exhibit similar cardioprotection against prolonged I/R injury, distinct genetic programs in response to the preconditioning stimuli may exist. Simkhovich et al. (2003) reported in their gene microarray experiments that 24 h IPC resulted in all but two identifiable genes down-regulated in young hearts, including genes associated with early-remodeling and hypertrophy-related transcripts. In contrast, old hearts showed a unique injury-related response which included up-regulation of mRNAs for proteins associated with cellular hypertrophy or apoptosis. It is worth noting that, unlike results from our study in which the expression levels of chemokines and cytokines were dramatically down-regulated in delayed APC, few inflammatory mediators were altered in the above-mentioned late IPC experiments and the magnitudes in the changes of gene expression were much smaller than the observed changes in this one. The changes with fold ratios of less than 3 were in the vast majority of the altered genes (Simkhovich et al. 2003). These data support the hypothesis that delayed APC and IPC differentially regulate gene expressions, suggesting trigger-dependent transcriptome variability. In line with these observations, a previous study has also revealed distinct differences in gene expression profiles between acute APC and IPC (Sergeev et al. 2004).

Several factors should be considered in the interpretation of these data. Our study focused on the transcriptional profiling of young and old myocardium on a genome-wide scale during delayed APC using a microarray approach. Confirmation of the quantitative measurements using real-time RT–PCR was performed with only a limited numbers of genes; nevertheless, microarray data have been shown to exhibit a high correlation with quantitative gene expression values. Also, changes in mRNA levels may not always be correlated with respective changes at the protein levels. The proteomics changes, i.e., functionally important changes in protein expression and/or posttranslational modification during delayed APC, are unknown. Although genomics has demonstrated that there is more than 85% similarity in coding regions of the rat genome compared with the human genome, caution should be taken when interpreting data from rodent studies and extrapolating the implications to human patients. Since the sample size in this study was small, stringent criteria of significant threshold were used to minimize false-positive findings. It is possible that marginal alterations in gene expression might not have been detected. Also noted in this study was the considerable number of unknown genes altered by this protocol. These unknown genes may represent critical parts of the delayed APC response that have not yet been identified or studied. Understanding the functions of these unknown genes in the complex biological process in future studies is likely to help elucidate the molecular mechanisms of delayed APC associated myocardial protection as well as age-related differential responses.

In summary, the current study illustrates the first comprehensive gene expression profile associated with delayed APC in young rat hearts compared with aged myocardium. Our data indicate that delayed APC profoundly, but differentially, altered the regulation of gene transcription in an age-dependent manner. The impaired genomic response to delayed APC could underlie the loss of the protective benefits offered by APC in aged hearts and therefore providing new insights into the cellular and molecular mechanisms of delayed APC in aged myocardium.

Experimental procedures

Preparation of isolated hearts

The study protocol was approved by the Animal Care Committee of the University of California, Davis (Davis, CA, USA), and all experiments were conducted in accordance with guidelines of animal care from the National Institutes of Health.

Hearts were obtained from male Fischer 344 rats of age 4 and 24 months (National Institute on Aging, MD, USA). Anesthesia was first induced with an intraperitoneal injection of sodium thiopental (50–75 mg/kg) along with 1,000 U heparin for anti-coagulation. Sodium thiopental was chosen for initial anesthesia because this drug has been shown not to influence preconditioning. The heart was excised and placed in an ice-cold solution of Krebs–Henseleit buffer (127 mM NaCl, 4.7 mM KCl, 1.25 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose). It was then cannulated and Langendorff

perfused with the same buffer at a perfusion pressure of 140±10 cm H_2O at 37±0.5°C. The perfusate was continuously oxygenated with 95% O_2/5% CO_2. Pacing wires were placed in the right atrium after cannulation. Hearts were paced at 5 Hz during all phases of the experiment except global ischemia. Any episodes of ventricular fibrillation were mechanically converted when they occurred.

Experimental design

Fifteen young and 15 old rats were randomly assigned to each of the five groups for each age class: (1) control group—115 min of continuous perfusion; (2) I/R group—30 min of perfusion, 25 min of global ischemia, 60 min of reperfusion; (3) 6 h delayed APC group—60 min of sevoflurane exposure followed by 6 h of recovery and 60 min of perfusion; (4) 12 h delayed APC group—60 min of sevoflurane exposure followed by 12 h of recovery and 30 min of perfusion; and (5) 24 h delayed APC group—60 min of sevoflurane exposure followed by 24 h of recovery and 60 min of perfusion (Fig. 4). Sevoflurane was delivered at 2.5% to the gas mixture via a standard Sevotec5 variable bypass vaporizer (Datex-Ohmeda, Milwaukee, WI, USA) to reach a final concentration of 0.4±0.02 mM. Global ischemia was induced by stopping all flow to the hearts.

RNA isolation and cDNA synthesis

Immediately after completion of the protocols, hearts from each group were rapidly frozen in liquid nitrogen and stored at −80°C. Hearts were powdered in liquid nitrogen and total RNA was isolated from 0.15–0.3 g of tissue for each specimen using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and further purification with RNAeasy silica columns (Qiagen, Valencia, CA, USA). The RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and quantified by fiberoptic spectrophotometry using the Nanodrop ND-1000 (Nanodrop Inc., Wilmington, DE, USA). RNA yielding both an A260/A280 absorbance ratio greater than 2.0 and a 28 s/18 srRNA ratio equal to or exceeding 1.5 was utilized for further experimentation. Five micrograms of purified total RNA was used for cDNA synthesis followed by in vitro transcription to incorporate biotin labels and subsequent hybridization to the rat microarray.

Hybridization and scanning of microarrays

The Affymetrix Rat Genome 230 2.0 array (Affymetrix, Santa Clara, CA, USA), the first whole-genome array that provides comprehensive coverage of the transcribed rat genome, was used for gene expression profiling. The Rat Genome 230 2.0 array contains a total of 31,042 probe sets representing 28,000 well-characterized rat genes and thousands of expressed sequence tags (ESTs; unknown genes). Three independent gene chips for each group were used, resulting in a total of 30 chips analyzed. The biotin-labeled cRNA was fragmented and hybridized to the array according to the manufacturer's protocol. The arrays were washed and stained on a Fluidics Station 450 and were scanned on a GeneChip Scanner 3000. Affymetrix Quality Control metrics were used to qualify the resulting data.

Confirmation of selected gene expression levels by quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR)

Quantitative real-time RT–PCR was performed for five selected genes to confirm the microarray data in which the expression levels were significantly altered (≥2.0-fold) by delayed APC determined by the microarray analysis. Isolated total RNA from each sample was subjected to reverse transcription using the QuantiTect reverse-transcription kit (Qiagen) according to the manufacturer's protocol. The rat PCR array and Real-Timer SyBR Green/ROX PCR Mix were purchased from SuperArray Bioscience Corporation (Frederick, MD, USA). PCR was performed on an ABI Prism 7900 Sequence Detector (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were initiated at 94°C for 2 min followed by 35–42 cycles of denaturing for 15 s at 94°C, annealing for 30 s at 60°C, and polymerization for 30 s at 72°C. Detection of the fluorescent product was performed at the end of the 72°C extension period. GAPDH was used as reference control. Thermal melting analyses were performed for PCR products. The comparative delta Ct method was used for the quantitation of mRNA levels.

Left ventricular function and myocardial infarct size measurements

Another total of 24 Fisher 344 rats (12 at the age of 4 months and 12 at the age of 24 months) were used in the LV function and infarct size studies in I/R group and 24 h delayed APC group. LVEDP were measured using a latex balloon filled with water inserted via an incision in the left atrial appendage through the mitral valve. Pressures were recorded using Powerlab 4/20 (ADInstruments, Colorado Springs, CO, USA). During the equilibration period, LVEDP was set by adjusting the balloon volume to yield a left end diastolic pressure of approximately 7 mmHg. At the end of reperfusion, hearts were removed from the Langendorff apparatus and quickly sectioned into 2-mm slices. The slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride staining solution and placed in a 37°C incubator for 20 min. After incubation, slices were washed with water, placed on petri dishes, and then scanned into a computer using Adobe Photoshop software (Adobe, San Jose, CA, USA). Standard computer plainmeteric analysis, using NIH image 1.62 (National Institutes of Health, Bethesda, MD, USA), was performed to determine infarct area. The total area of infarction was divided by the total area of myocardium to yield the percent area of infarction (Sniecinski and Liu 2004).

Statistics

The analysis of microarray data were performed with the BRB-ArrayTools software, version 3.7, developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Raw intensity files (Cel) were exported from the Affymetrix GeneChip Operating Software (GCOS) and probe-level analysis, normalization, and summarization were performed using the GC-RMA algorithm implemented in the BRB-ArrayTools software. Class comparisons identified genes that were expressed differently between the two classes using a random-variance t test. The random-variance t test is an improvement over the standard separate t test as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance (Wright and Simon 2003). Genes were considered significantly differentially regulated if those genes had a normalized ratio intensity of 2.0 or greater (i.e., 2.0-fold up-/down-regulation in expression level compared with the control group), and a p value less than 0.001. The stringent criteria of significant threshold were used to reduce false-positive findings. For the LVEDP and infarct size analysis, data (n=6) are presented as mean±SD. Two-tailed Student t test was used. A value of p <0.05 was considered statistically significant.

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