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NF- κ B driven cardioprotective gene programs; Hsp70.3 and cardioprotection after Late Ischemic Preconditioning

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Abstract

Rationale—It has been shown that the transcription factor NF- κ B is necessary for late phase cardioprotection after ischemic preconditioning (IPC) in the heart, and yet is injurious after ischemia/reperfusion (I/R). However the downstream gene expression programs that underlie the contribution of NF- κ B to cardioprotection after late IPC are incompletely understood.

Objective—To delineate the specific genes that are regulated by NF- κ B immediately after a late IPC stimulus and validate the methodology for identification of NF- κ B-dependent genes that contribute to cardioprotection.

Methods and Results—A directed microarray analysis identified 238 genes as up or down regulated in an NF- κ B-dependent manner 3.5 h after late IPC. Among these are several genes previously implicated in late IPC. Gene ontological analysis showed that the most significant group of NF- κ B-dependent genes are heat shock response genes, including the genes encoding Hsp70.1 and Hsp70.3. Though an Hsp70.1/70.3 double knockout failed to exhibit cardioprotection, late IPC was intact in the Hsp70.1 single knockout. After I/R, the Hsp70.1/70.3 double knockout and the Hsp70.1 single knockout had significantly increased and reduced infarct size, respectively.

Conclusions—These results delineate the immediate NF- κ B-dependent transcriptome after late IPC. One of the major categories of NF- κ B-dependent genes induced by late IPC is the heat shock response. The results of infarct studies confirm that Hsp70.3 is protective after IPC. However, though Hsp70.1 and Hsp70.3 are coordinately regulated, their functions are opposing after I/R injury.

Keywords

Cardioprotection; late ischemic preconditioning; NF- κ B; gene expression; Heat shock protein 70

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Introduction

According to the American Heart Association, ischemic heart disease accounts for more than half of all cases of cardiovascular disease and claims the lives of approximately half a million people per year in the United States alone [AHA Statistics 2008]. Interestingly, the mammalian heart possesses an endogenous defense mechanism against such ischemic injury in the form of ischemic preconditioning (IPC). This preconditioning phenomenon was first described in a canine model of ischemia in 1986 by Murry *et. al.* [1]; short repetitive bouts of ischemia and reperfusion elicited protection against a larger subsequent ischemic insult. IPC was later described to have two distinct phases or windows of cardioprotection: an early phase with immediate onset lasting over the course of 3-4 hours and a late phase manifesting approximately 12 hours after the preconditioning stimulus that can last up to a few days [2] and [3]. The late phase of ischemic preconditioning holds more clinical potential since the protection is much longer lasting.

It has previously been determined that the late phase of IPC cardioprotection is dependent upon *de novo* protein synthesis [4], and requires the activation of the transcription factor nuclear factor-kappa B (NF- κ B) [5]. While iNOS and Cox-2 have been shown to be NF- κ B-dependent genes [6] and [7] whose expression are necessary for late IPC cardio protection [8], [9], and [10], the full complexity and identity of the NF- κ B-regulated transcriptome after IPC and its contribution to late phase cardioprotection remains incompletely known. The primary goal of this work was to use an hypothesis-driven gene microarray analyses comparing post-IPC gene expression in wild-type and NF- κ B dominant-negative transgenic mice to identify the NF- κ B specific fingerprint on the post-IPC transcriptome.

We hypothesized that a direct comparison of gene expression between wild-type and NF- κ B-dominant negative mice after an IPC stimulus will delineate genes likely to underlie the NF- κ B dependency of late IPC induced cardioprotection. Because NF- κ B activation has been shown to be necessary and protective in late IPC [5], but is in fact injurious after ischemia/reperfusion (I/R) [11], it must regulate discrete sets of genes that underlie the opposing responses to these different stimuli. Understanding the gene programs that are regulated by NF- κ B after late IPC will allow us to determine the protective genes induced, and/or the injurious genes repressed, by NF- κ B in association with late IPC-induced protection against MI. Using this approach, we found 238 genes that were either up or down regulated by NF- κ B following IPC. We utilized the DAVID bioinformatics database [12] and [13] to characterize the genes that are significantly dysregulated ($P \leq 0.01$ or a fold change ≥ 2.0) by IPC in both wild-type and NF- κ B dominant-negative mice (cardiac-specific I κ B α [S32A,S36S] transgenic mice [14]). Gene ontology categorization of these genes indicated enrichment for genes involved heat shock response, metabolic processes, angiogenesis, and programmed cell death. To ensure the validity of our microarray analysis, quantitative realtime PCR (qRT-PCR) was used to confirm the expression pattern of key genes within these pathways.

Our results indicate that genes involved in the heat shock response comprise a group of genes whose expression was the most strongly regulated by NF- κ B after IPC. We therefore focused upon the genes (*hspa1a* and *hspa1b*) encoding (hsp70.3 and hsp70.1, respectively) inducible heat shock protein 70 (hsp70) whose upregulation was found to be dependent upon NF- κ B after IPC. The two hsp70 loci are tightly linked in a head-to-tail arrangement on the mouse chromosome 17. In the mouse, the two genes have a high degree of nucleic acid sequence similarity (>95%). There is a paralogous relationship between *hspa1a* and *hspa1b* and their homologues in the mammalian lineage [15]. Hsp70 has been previously viewed as being cardioprotective [16] and necessary for ischemic preconditioning [17]. However, this work was based upon the use of a gene targeted ablation of the entire *hspa1a/hspa1b* locus,

and a distinct role for the two inducible forms of Hsp70 in IPC as well as the role of NF- κ B in regulating the gene expression of the two genes remains unknown. Using an hsp70.1 specific murine knockout model, we show that the inducible hsp70.3 gene is a novel mediator of late IPC cardioprotection regulated by NF- κ B and that the Hsp70.1 and Hsp70.3 gene products have opposing roles after I/R.

Materials and Methods

Animals

All mice were maintained in accordance with institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* (NIH, revised 1996), and the University of Cincinnati Institutional Animal Care and Use Committee. I κ B α dominant-negative mice (2M) (cardiomyocyte-specific mutant I κ B α ^[S32A,S36A] cDNA on the C57Bl/6J strain) were previously characterized [14] and [18]. Hsp70.1 KO mice have been previously characterized, [19] are also on the C57Bl/6J strain, and were obtained from Macrogen (South Korea). The Hsp70.1/3 double knockout mice were on the B6129SF2/J strain [20]. All studies were controlled by same-strain mice matched for age (10-16 weeks) and gender. Groups were of mixed gender and post-hoc statistical analysis was used to determine if there was an effect of gender; there were none as we previously reported [18]. Control groups were performed with the matching wild type strains, C57Bl6/J (Jackson Lab, strain # 000664) and B6129SF2/J (Jackson Lab, strain # 101045).

Mouse Model and Surgical Procedures

Ischemic preconditioning and ischemia/reperfusion were achieved in anesthetized mice as previously described. Ischemia and reperfusion were performed as previously described, [18] and [21] confirmed by visual observation (*i.e.* cyanosis) and by ECG monitoring (QRS complex, T wave inversion, and ST segment changes) and reversal of same. Mice undergoing sham surgery were subjected to the same procedure without tightening of the suture (*i.e.* no occlusion). On day 1 of the protocol, ischemic preconditioning was achieved by 6 cycles of 4 min ischemia followed by 4 min of reperfusion [22]. On day 2 (24 hrs subsequent to the IPC procedure), mice were subjected to 30 min coronary occlusion (ischemia) and 24 hr reperfusion. Following 24 hrs of reperfusion, mice were euthanized and the hearts were stained for risk region and infarct zone as previously described [22] and [23]. There were no significant differences in the sizes of the risk regions between any of the groups in this study (Fig. S1).

RNA isolation

Hearts were removed 3.5 hours following IPC or sham surgery, and quickly rinsed in ice-cold RNase free PBS. The ischemic zone was collected (10-20 mg of tissue), flash frozen in liquid N₂ and stored at -80°C. Total RNA was extracted from the ischemic zone using an RNeasy Mini kit (Qiagen) according to Appendix C (Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue) in the RNeasy Mini handbook (3rd edition; June 2001). The RNeasy mini-columns were treated with RNase-free DNase (Qiagen) to remove nuclear DNA contaminants. Total RNA quantity and quality was assessed by optical density at 260 nm and optical density ratios of 260/280 nm and 260/230 nm ratios, respectively.

Microarray analysis

RNA samples (1 μ g/10 μ l) were submitted to the University of Cincinnati Microarray Core for Agilent Bioanalyzer/Nanodrop analysis (Agilent 2100 Bioanalyzer). RNA samples were amplified by the University of Cincinnati Microarray Core using Amino Allyl MessageAMP kit (catalog #1753) from Ambion based on a modified Eberwine procedure [24]. cDNA

synthesis and indirect amino-allyl labeling was performed by the University of Cincinnati Microarray Core using a modified version of the Brown Lab (Stanford University) protocol [25]. Microarray hybridization and wash conditions were performed as previously described [26] and [27]. Competitive hybridization to microarrays of labeled cDNA targets generated from 4 separate samples per group was performed, 2 dye flips performed per group.

RNA samples with an RNA integrity number (RIN) greater than 7 and OD 260/280 ratios 1.7 were used for microarrays. Imaging and data generation were carried out using a GenePix 4000A and GenePix 4000B (Axon Instruments; Union City, CA) and associated software from Axon Instruments, Inc. (Foster City, CA). The microarray slides were scanned with dual lasers with wavelength frequencies to excite Cy3 and Cy5 fluorescence emission. Images were captured in JPEG and TIFF files, and DNA spots captured by the adaptive circle segmentation method. Information extraction for a given spot is based on the median value for the signal pixels and the median value for the background pixels to produce a gene set data file for all the DNA spots. The Cy3 and Cy5 fluorescence signal intensities were normalized.

Statistical analysis was performed using R statistical software and the limma Bioconductor package [28]. Data normalization was performed in two steps for each microarray. First, background adjusted intensities were log-transformed and the differences (M) and averages (A) of log-transformed values were calculated as $M = \log_2(X_1) - \log_2(X_2)$ and $A = [\log_2(X_1) + \log_2(X_2)]/2$, where X_1 and X_2 denote the Cy5 and Cy3 intensities, respectively. Second, normalization was performed by fitting the array-specific local regression model of M as a function of A. Normalized log-intensities for the two channels were then calculated by adding half of the normalized ratio to A for the Cy5 channel and subtracting half of the normalized ratio from A for the Cy3 channel. The statistical analysis was performed for each gene separately by fitting the following Analysis of Variance model: $Y_{ijk} = \mu + A_i + S_j + C_k + \epsilon_{ijk}$, where Y_{ijk} corresponds to the normalized log-intensity on the i^{th} array, with the j^{th} treatment, and labeled with the k^{th} dye ($k = 1$ for Cy5, and 2 for Cy3). μ is the overall mean log-intensity, A_i is the effect of the i^{th} array, S_j is the effect of the j^{th} treatment and C_k is the gene-specific effect of the k^{th} dye. Estimated fold changes were calculated from the ANOVA models, and resulting t-statistics from each comparison were modified using an intensity-based empirical Bayes method (IBMT) [27].

cDNA synthesis and quantitative real-time RT-PCR

cDNA synthesis was performed using an RNA-to-cDNA kit (Applied Biosystems) according to manufacturer's instructions. Synthesis of cDNA was carried out using 1.0 μg of total RNA and optical density was used to determine quantity and quality (as described above for isolated RNA). Quantitative real-time RT-PCR (QRT-PCR) was done in 20 μl total reaction volume using a Stratagene MX3000P machine using a SYBR Green 2X RT-PCR master mix (Applied Biosystems). For all genes, the thermocycling parameters were 90° C for 10 mins followed by 40 cycles of 90° C for 15s and 60° C for 60s (with data collection at the end of the 60° C step at each cycle). All reactions were performed in triplicate on each plate with a minimum of 3 independent replicates. Gene expression values were calculated using the difference in target gene expression relative to 18S mRNA using the delta-delta Ct method [29]. Primer sequences and amount of cDNA per reaction were as follows: 18S RNA: (5'-AGTCCCTGCCCTTTGTACACA-3', 5'-CCGAGGGCCTCACTAAAC-3', 60 ng cDNA used per reaction), Hsp70.1: (5'-GAAGACATATAGTCTAGCTGCCAGT-3', 5'-CCAAGACGTTTGTAAAGACACTTT-3', 100 ng cDNA), Hsp70.3: (5'-GGCCAGGGCTGGATTACT-3', 5'-GCAACCACCATGCAAGATTA-3', 100 ng cDNA). RT-PCR primers for Hsp90, Btg1, and FADD were from Qiagen (Quantitect Primer Assay) with 100 ng, 100 ng, and 120 ng cDNA used per reaction, respectively.

Statistical Analysis

Results are reported as means $\pm$ standard error of the mean (SE). Unpaired student t-test were performed using Bonferroni correction and *P*-values reported with differences between groups considered significant at *P* = 0.05. Power analysis was used to ensure proper sample size needed to determine significance. Microarray results were statistically analyzed as described above.

Results

Inhibition of NF- κ B blocks late phase IPC

Late phase ischemic preconditioning subsequent to a 30 min IR reduces infarct size in WT mice from 40.6% to 21.4% as compared to sham IPC surgery (*P* = 0.05)(Fig. 1). The cardioprotection from late phase of IPC observed in WT mice is lost in the NF- κ B dominant negative mice (2M) (infarct size remains high [35.3%] after IPC compared to sham [32.8%]; *P* > 0.05)(Fig. 1).

Ischemic preconditioning induced gene expression changes

We performed comparative microarray analysis of cardiac mRNA isolated from WT and NF- κ B dominant-negative mice 3.5 h after IPC or sham surgery, in order to interrogate the NF- κ B-dependent genes that are up- or down-regulated immediately after IPC. We chose 3.5 h as this has been shown to be the peak expression of several NF- κ B-dependent genes previously shown to play a role in late IPC [30]. We were interested in this initial wave of gene expression since, 1) NF- κ B activation is known to occur transiently early (5-30 min) after an IPC stimulus, 2) several genes significantly upregulated in this time frame (3-4h) are known to play a role in IPC, 3) investigation of gene expression at later time periods would be complicated by successive waves of gene expression elicited by transcription factors that are themselves upregulated immediately after IPC (interesting, but beyond the scope of this study). A microarray comparison of gene expression differences between IPC and sham treated wild-type mice (WT-IPC vs. WT-Sham) (Fig. 2A) resulted in a significant (*P* = 0.01 or a fold change $\geq$ 2.0) expression difference of 744 oligonucleotide probes out of 34,473 (representing 21,928 individual gene IDs) from our array chip (for a full list of IPC regulated genes see Table S1). These 744 probes corresponded to 617 unique gene IDs in the DAVID Bioinformatics database [13] (Fig. 2B, WT). Functional annotation of these 617 genes according gene ontology (GO) by biological process resulted in a significant enrichment for GO terms associated with heat shock response, angiogenesis, programmed cell death, and metabolic processes (Table S2). All GO filtering in DAVID was done using threshold values of an EASE score/*P*-value cutoff of 0.01 with a minimum of 5 genes per group.

NF- κ B regulated gene expression during ischemic preconditioning

In order to determine the subset of IPC regulated genes that are under NF- κ B transcriptional control, we also performed a microarray expression analysis between ischemic preconditioned wild-type and 2M mice (WT-IPC vs. 2M-IPC) (Fig. 2A). The 1,136 dysregulated genes (Fig. 2B, 2M) resulting from this comparison represent all genes whose regulation was significantly altered (up or down) by NF- κ B blockade after ischemic preconditioning. However, since genes must be differentially regulated in wild-type after IPC relative to a sham operation to be considered IPC-dependent, it is the overlap between these two comparisons (WT-IPC vs. WT-Sham and 2M-IPC vs. WT-IPC) that contains the NF- κ B-dependent genes we are ultimately interested in. Specifically, the 238 genes represented by the overlap in Fig. 2B are the genes that are regulated by NF- κ B after ischemic preconditioning, and are thus the most likely candidates to underlie the NF- κ B

dependent cardioprotection after late IPC (See Table S3 for a full list of these genes). Functional annotation of these 238 genes by GO according to DAVID resulted in enrichment for many of the same GO terms represented by the wild-type IPC vs. Sham comparison (Table S4). We focused upon the significantly enriched GO groups associated with angiogenesis, cell death, and heat shock since 1) these were the most enriched GO groups regulated by NF- κ B after late IPC, and 2) previous work has implicated genes in these categories in IPC induced cardioprotection. Using these three GO groups as a starting point, a statistical cutoff of $P = 0.01$ and similarity score of ≥ 0.75 was employed to generate sublists of functionally associated GOs (Fig. 3). Figure 4 provides a detailed list of the genes contained in the associated GO term groups that are also included in the 238 genes from Fig. 2B found to be regulated by NF- κ B after IPC.

To validate the gene expression patterns detected by the microarray analysis, mRNA expression of several genes from each group was assessed using quantitative real-time RT-PCR (QRT-PCR) (Fig 5). We determined that the mRNA expression patterns of *hif1a*, *fadd*, and *hsp90aa1* determined by QRT-PCR corroborate the results obtained from microarray analysis (Fig 5). QRT-PCR analysis confirmed the increased mRNA levels for *hif1a* (3.3-fold) and *hsp90aa1* (4.1-fold) and decreased levels for *fadd* (1.9-fold) relative to wt (1.6-fold increase, 3.1-fold increase, and 1.6-fold decrease, respectively predicted by microarray) (Fig. 5). Gene expression changes of COX-2, a known mediator of late IPC cardioprotection [9] and [10], were also assessed via QRT-PCR and found to increase in an NF- κ B-dependent manner following IPC (Fig. 5). Expression analysis done via QRT-PCR corroborated microarray results that the IPC-induced changes in the levels of these transcripts were suppressed ($P = 0.05$) in 2M mice relative to wt after IPC.

NF- κ B regulated gene expression of heat shock response after ischemic preconditioning

The most significantly enriched GO term among the 238 genes regulated by NF- κ B after IPC was *response to heat* ($P = 0.0001$, fold enrichment ~ 15.3) (Fig. 3). In addition, *hspa1b* (encoding HSP70.1) is one of three genes that appears in both cell death associated and heat shock response gene ontology terms (Fig. 3). Microarray expression results indicated that *hspa1b* was the most highly regulated gene after IPC (8.53 fold increase in WT IPC vs. Sham, $P = 0.001$) and also showed strong NF- κ B dependent regulation (Table 2).

Hsp70.3 is the NF- κ B inducible Hsp70 responsible for cardioprotection

Although *hspa1a* (encoding Hsp70.3) did not make the statistical cutoff for gene expression change applied to the array data, the microarray data did identify this gene as trending toward an increased expression after IPC (1.99 fold increase in WT IPC vs. Sham) with a 1.35 fold reduction in expression in 2M vs. WT IPC). QRT-PCR analysis of Hsp70.3 mRNA levels confirmed a significant increase (26.5 fold in WT IPC vs. Sham, $P = 0.001$) in expression after IPC in WT mice with a significant reduction of the IPC induced increase in 2M mice (1.92 fold reduction in WT IPC vs. Sham, $P = 0.01$) (Fig. 6).

In light of this substantial NF- κ B-dependent upregulation of both *hspa1* genes, we sought to delineate the distinctive role that each Hsp70 may play in the NF- κ B-dependent cardioprotection of late phase IPC. As previously shown [17], we found that a double-knockout mouse model of both *hspa1a* and *hspa1b* genes abrogated the cardioprotection of late phase IPC (45.5% infarct after IPC; 38.8% infarct in sham group; $P > 0.05$) (Fig. 7A). However, a single knockout of the Hsp70.1 gene had no effect on the cardioprotection provided by late IPC (24.9% infarct after IPC; 41.8% infarct in sham group; $P = 0.05$) (Fig. 7B). This striking result demonstrates that the Hsp70.3 isoform plays a critical functional role in the NF- κ B mediated cardioprotection afforded by late phase IPC.

We also assessed role of the two Hsp70s upon infarct size following an ischemia/reperfusion (I/R) injury in the absence of IPC. Using the available knockout mice (see limitations, supplement 1), we show that the Hsp70.1/Hsp70.3 double knockout has a significant increase in infarct size following I/R (30 min coronary occlusion) injury (40.8% infarct in KO vs. 15.5% infarct in WT; $P = 0.05$) (Fig. 7C). In contrast, knockout of only the Hsp70.1 resulted in significantly decreased infarct size following I/R (4.4% infarct in KO vs. 20.5% infarct in WT; $P = 0.05$) (Fig. 7C).

Discussion

It is known that activation of the transcription factor NF- κ B plays a role in the development of cardioprotection associated with late phase ischemic preconditioning in the heart [3] and [5]. However, these studies relied upon pharmacological agents of limited specificity. This is the first study to confirm these results using a model of genetic blockade of all NF- κ B subunits specifically in the cardiomyocyte [14]. We have previously shown that blockade of NF- κ B reduces infarct size after ischemia/reperfusion (I/R) [11] and [18] and increases infarct size after permanent coronary occlusion (PO) [11]. Thus, NF- κ B, being a transcription factor, must regulate discrete gene programs for NF- κ B-dependent cardioprotection (after late IPC and PO) and for ischemic injury (after I/R). The hypothesis of this work was that a direct comparison of gene expression between wild-type and NF- κ B-dominant negative mice after an IPC or sham stimulus would delineate specific genes that underlie the NF- κ B dependency of late IPC induced cardioprotection. While a small number of NF- κ B-dependent genes, including iNOS [6] and [8], Cox-2 [7] and [10], and HO-1 [31] have been shown to be mediators of IPC cardioprotection, the full complexity of the NF- κ B-dependent transcriptome after IPC has, until now, not been investigated.

Herein, we report the identification of 238 genes whose expression is regulated by NF- κ B immediately (3.5 h) after IPC. Gene ontology characterization (biological process) of these 238 genes indicated a significant enrichment for genes involved in heat shock response, angiogenesis, and programmed cell death. Upon examination of individual genes composing these GO groups, it was found that a few genes were found in more than one of the GO groupings (Fig. 4). For example, *hif1a*, *btg1*, *rhob*, *rtn4*, *tnfsf12*, and *pafah1b1* are all included in both angiogenesis and cell death associated GO terms. *Hspa1b*, *bcl2l1*, and *myc* were found to overlap between cell death and heat shock response associated GO terms (no genes were found to overlap between all 3 GO groups). It was from these GO groups that we selected 5 genes (*hif1a*, *fadd*, and *hsp90aa1*, *hspa1a*, *hspa1b*) for validation of mRNA expression changes validated by QRT-PCR. These three genes are significant to our array results in that they: 1) represent multiple expression patterns (both increased and decreased by NF- κ B after IPC), 2) fall into the most enriched GO categories, and 3) are likely, based upon the literature that the function of these genes relates to cardioprotection against MI.

QRT-PCR validation of these select genes supports the accuracy of the microarray results. It is possible that these results may not include all pertinent NF- κ B genes. For example, iNOS (*nos2*) and COX-2 (*ptgs2*) did not pass the significance filter and therefore were not included in our final list of 238 genes. However, we did confirm using QRT-PCR that COX-2 mRNA expression was increased in an NF- κ B-dependent manner following IPC (Fig. 5). COX-2 expression has been presumed to be NF- κ B-dependent following IPC [32], but to the best of our knowledge, our results presented herein are the first to conclusively show this using a transgenic dominant-negative blockade of NF- κ B induced gene expression. The predicted microarray expression pattern of iNOS also showed a trend upward after IPC in wild-type mice (1.17-fold increase in WT IPC vs. sham) that was reversed in the 2M mice suggesting NF- κ B-dependent expression (1.35-fold decrease in 2M vs. WT IPC). Heme oxygenase 1 (*hmox1*) is another gene thought to play a pivotal role in

preconditioning [31], [33], [34], and [35]. Our microarray results successfully predicted an increase in levels of *hmox1* transcript in WT IPC compared to sham (1.77-fold increase, *P* 0.01), but did not indicate significant NF- κ B-dependency of its expression (1.06-fold decrease in 2M vs. WT IPC).

A closer look at the enriched gene ontology groups from our microarray analyses and the individual genes in those groups indicates that our results are consistent with previously published reports characterizing global IPC induced gene expression changes [36] and [37]. However, our results are novel in that, due to our hypothesis-driven NF- κ B-linked approach, we can distinguish the NF- κ B-dependent changes as well. This not only links specific downstream genes to activation of NF- κ B upstream, but because NF- κ B is required for late IPC-induced cardioprotection, also links function to gene expression. For example, angiogenic pathways have been previously shown to play a role in the adaptive programs elicited by IPC [38] and [39]. Hypoxia inducible factor-1 (HIF-1) is a transcription factor that is of critical importance to angiogenic processes, so it was not surprising to see the alpha subunit of this transcription factor (*hif1a*) upregulated by IPC. HIF-1 activation has been previously suggested to be necessary for preconditioning [40], [41], and [42]. Most notably, Semenza's group recently showed that *hif1a*^{+/-} heterozygous mice were unable to elicit IPC induced cardioprotection (early phase) in an isolated heart model [43]. Interestingly, very little attention has been given to the possibility that HIF-1 activation may be an NF- κ B-dependent event. A few previously published reports have suggested this to be the case in other cell types including fibroblasts [44], pulmonary artery smooth muscle cells [45] and [46], and lung carcinoma cells [47]; though no such studies have been done in the heart. To the best of our knowledge, our data is the first to report NF- κ B-dependent expression of *hif1a* in the *in vivo* heart.

Fadd (FADD; fas-associated protein with death domain) was chosen as a representative gene from the programmed cell death ontology group for expression validation by QRT-PCR. FADD is a well-known modulator of apoptosis as it serves to link the Fas death receptor with caspase activation (the role of FADD in programmed has been reviewed elsewhere [48] and [49]). QRT-PCR confirmed that *fadd* expression is down-regulated after IPC, but did not indicate this to be an NF- κ B-mediated event as predicted by microarrays. Out of five genes for whose NF- κ B-dependent expression we compared to the array data via QRT-PCR, this was the only gene whose pattern of expression, determined by QRT-PCR, was not consistent with the microarray data. Both assays did, however, confirm a decreased expression of *fadd* mRNA after IPC in wild-type mice. This result is consistent with the pro-apoptotic role of *fadd* and the notion that IPC elicits cardioprotection at least in part through inhibition of apoptotic cell death in the heart. The transcription factor or factors responsible for *fadd* repression remains to be determined.

The resulting enrichment in gene ontology groups for genes involved in the heat shock response is not surprising given the existing body of work demonstrating the cytoprotective properties of the heat shock response [50] and [51] and previous data that the hsp70 locus is required for late IPC [17]. Though, a small body of work exists indicating a role for NF- κ B in the expression of individual heat shock proteins, [51] and [52] the dependence of expression of these genes upon NF- κ B has not been definitively established in the heart. Whether NF- κ B coordinates with HSF-1 in the regulation of this response or is sufficient to launch this response remains to be elucidated. Our results are the first to provide conclusive evidence that NF- κ B plays a central role in regulating a cardiac myocyte driven heat shock response after IPC. Importantly, our results are also the first to make a functional distinction between Hsp70.1 and Hsp70.3, two distinct inducible forms of Hsp70 expressed in the heart.

We demonstrate that Hsp70.3 gene expression is regulated by NF- κ B after IPC and is critical to cardioprotection against MI after late IPC. We also confirmed that late IPC cardioprotection was correlated with an increase in Hsp70 protein levels (Fig. S2). Using the Hsp70.1/70.3 and Hsp70.1 knockouts, we further show that loss of Hsp70.1 reduces infarct size following an isolated I/R injury, consistent with the concept that Hsp70.3, but not Hsp70.1, is cardioprotective (4.4% infarct in Hsp70.1 KO vs. 20.5% infarct in WT; $P = 0.05$). In contrast, in the double knockout Hsp70.1/70.3 infarct size was increased (40.8% infarct in Hsp70.1/70.3 KO vs. 15.5% infarct in WT; $P = 0.05$). Thus, in contrast to Hsp70.3, Hsp70.1 does not contribute to cardioprotection, but rather contributes to cardiac injury following I/R. Based upon these results, we would predict that an Hsp70.3 knockout abrogates late IPC and increase infarct size after I/R; however, the Hsp70.3 knockout mice [53] were not made available to us. Nevertheless, the I/R results suggest that the Hsp70s are not functionally redundant in determining cell survival vs. cell death in the ischemic myocardium. This result demonstrates that the two inducible Hsp70 genes have opposing molecular functions, at least in the heart, and should no longer be simply lumped together as “Hsp70”. To date, there exists almost no direct data suggesting distinct functional roles for the two genes, though such a possibility has been previously suggested [54]. In addition, it has been demonstrated that the two genes are differentially regulated [55], which would support a model of separate functional roles. The specific mechanisms mediating the effect of Hsp70.1 and Hsp70.3 in the myocardium are unknown and are beyond the scope of this study. There are more than a dozen known roles for “Hsp70” in the literature, several of which could be involved in cell survival. One plausible cardioprotective mechanism of Hsp70.3 could be through the classical function of Hsp70 as a protein folding chaperone and its role in mediating the unfolded protein response (UPR) in protection against endoplasmic reticulum (ER) stress [56] and [57]. In addition, it has previously been shown that increased Hsp70 can repress NF- κ B activation in neuronal cells [58] and [59]. Since inhibition of NF- κ B activation after I/R is protective against I/R injury, [11] increased Hsp70.3 levels during the late phase of IPC could provide cardioprotection via blunting of NF- κ B activation during subsequent ischemia/reperfusion (I/R).

Most of the plausible mechanisms of Hsp70.3 action involve the binding of “Hsp70” in protein complexes with other Hsps, regulatory kinases, and factors that regulate cell death, autophagy and the unfolded protein response (UPR). It is therefore possible that the opposing roles of these two proteins relate to one replacing the other or to one having a distinct binding partner in a critical Hsp-protein complex. There is nearly 99% homology between the protein encoding regions of the two genes, the single difference is the insertion of a proline residue near the C-terminus of the Hsp70.1 protein. Since this insertion occurs near the substrate binding domain of the protein, it could alter structural conformations and lead to a different preference for binding partners. Since there are no antibodies that can distinguish the two proteins, differences in complex formation or identification of differential post-transcriptional processing are currently not possible to address.

In summary, our results demonstrate NF- κ B-dependent regulation of 238 genes after IPC. This represents 38.5% percent of the genes up or down-regulated after IPC. These NF- κ B genes fell into three major groupings: angiogenesis, cell death and heat shock. Specifically, we showed that NF- κ B, in response to late IPC, activates a set of cardioprotective genes, and represses several pro-cell death genes. We conclusively show that the genes encoding both inducible forms of Hsp70, Hsp70.1 and Hsp70.3, are among the NF- κ B-regulated genes after IPC. Functional studies show for the first time that the hspa1a gene, encoding Hsp70.3, plays a critical role in the NF- κ B mediated cardioprotection of late IPC against MI. We also demonstrate that after I/R, Hsp70.3 is protective against MI (in agreement with our finding after late IPC) and that Hsp70.1 is injurious. The functional validation of the role of Hsp70.3 in contributing to NF- κ B-dependent cardioprotection after late IPC, along with

the identification of several other genes implicated in cardioprotection, validates the approach of using NF- κ B dominant-negative transgenic mice to distinguish the first wave of NF- κ B-dependent gene expression after initiation of pathophysiology in the heart.

These results are an important contribution to the understanding of how NF- κ B orchestrates late IPC cardioprotection through the regulation of gene expression. An understanding of the different individual genes NF- κ B regulates after different stimuli is necessary in order to understand the role that NF- κ B transcriptional regulation plays in the determination of antithetical outcomes. Future work confirming gene expression, functional validation and extending analyses to later waves of gene expression will extend our knowledge of the NF- κ B-dependent transcriptome involved in late phase cardioprotection after IPC and lead to a detailed mechanistic understanding of the complex gene regulatory interactions that underlie late IPC. It is possible that this will result in the identification of anti- and pro-cell death pathways regulated by NF- κ B; the ability to upregulate the former while repressing the latter may lead to therapies that are more clinically relevant than NF- κ B activation or repression alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986; 74(5):1124–36. [PubMed: 3769170]
2. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, et al. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res*. 1993; 72(6):1293–9. [PubMed: 8495557]
3. Bolli R. The late phase of preconditioning. *Circ Res*. 2000; 87(11):972–83. [PubMed: 11090541]
4. Rizvi A, Tang XL, Qiu Y, Xuan YT, Takano H, Jadoon AK, et al. Increased protein synthesis is necessary for the development of late preconditioning against myocardial stunning. *Am J Physiol*. 1999; 277(3 Pt 2):H874–84. [PubMed: 10484406]
5. Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, et al. Nuclear factor-kappaB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. *Circ Res*. 1999; 84(9):1095–109. [PubMed: 10325247]
6. Morris KR, Lutz RD, Choi HS, Kamitani T, Chmura K, Chan ED. Role of the NF-kappaB signaling pathway and kappaB cis-regulatory elements on the IRF-1 and iNOS promoter regions in mycobacterial lipoarabinomannan induction of nitric oxide. *Infect Immun*. 2003; 71(3):1442–52. [PubMed: 12595462]
7. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem*. 1995; 270(52):31315–20. [PubMed: 8537402]
8. Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, et al. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ Res*. 1997; 81(6):1094–107. [PubMed: 9400391]

9. Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, et al. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A*. 2000; 97(18):10197–202. [PubMed: 10963682]
10. Guo Y, Bao W, Wu WJ, Shinmura K, Tang XL, Bolli R. Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol*. 2000; 95(6):479–84. [PubMed: 11192369]
11. Jones WK, Brown M, Wilhide M, He S, Ren X. NF-kappaB in cardiovascular disease: diverse and specific effects of a “general” transcription factor? *Cardiovasc Toxicol*. 2005; 5(2):183–202. [PubMed: 16046793]
12. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009; 4(1):44–57. [PubMed: 19131956]
13. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. 2003; 4(5):P3. [PubMed: 12734009]
14. Dawn B, Xuan YT, Marian M, Flaherty MP, Murphree SS, Smith TL, et al. Cardiac-specific abrogation of NF- kappa B activation in mice by transdominant expression of a mutant I kappa B alpha. *J Mol Cell Cardiol*. 2001; 33(1):161–73. [PubMed: 11133232]
15. Brocchieri L, Conway de Macario E, Macario AJ. hsp70 genes in the human genome: Conservation and differentiation patterns predict a wide array of overlapping and specialized functions. *BMC Evol Biol*. 2008; 8:19. [PubMed: 18215318]
16. Liu JC, He M, Wan L, Cheng XS. Heat shock protein 70 gene transfection protects rat myocardium cell against anoxia-reoxygenation injury. *Chin Med J (Engl)*. 2007; 120(7):578–83. [PubMed: 17442206]
17. Hampton CR, Shimamoto A, Rothnie CL, Griscavage-Ennis J, Chong A, Dix DJ, et al. HSP70.1 and -70.3 are required for late-phase protection induced by ischemic preconditioning of mouse hearts. *Am J Physiol Heart Circ Physiol*. 2003; 285(2):H866–74. [PubMed: 12714332]
18. Brown M, McGuinness M, Wright T, Ren X, Wang Y, Boivin GP, et al. Cardiac-specific blockade of NF-kappaB in cardiac pathophysiology: differences between acute and chronic stimuli in vivo. *Am J Physiol Heart Circ Physiol*. 2005; 289(1):H466–76. [PubMed: 15695559]
19. Shim EH, Kim JI, Bang ES, Heo JS, Lee JS, Kim EY, et al. Targeted disruption of hsp70.1 sensitizes to osmotic stress. *EMBO Rep*. 2002; 3(9):857–61. [PubMed: 12189176]
20. Hunt CR, Dix DJ, Sharma GG, Pandita RK, Gupta A, Funk M, et al. Genomic instability and enhanced radiosensitivity in Hsp70.1- and Hsp70.3-deficient mice. *Mol Cell Biol*. 2004; 24(2):899–911. [PubMed: 14701760]
21. Guo Y, Jones WK, Xuan YT, Tang XL, Bao W, Wu WJ, et al. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. *Proc Natl Acad Sci U S A*. 1999; 96(20):11507–12. [PubMed: 10500207]
22. Ren X, Wang Y, Jones WK. TNF-alpha is required for late ischemic preconditioning but not for remote preconditioning of trauma. *J Surg Res*. 2004; 121(1):120–9. [PubMed: 15313385]
23. Fishbein MC, Meerbaum S, Rit J, Lando U, Kanmatsuse K, Mercier JC, et al. Early phase acute myocardial infarct size quantification: validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. *Am Heart J*. 1981; 101(5):593–600. [PubMed: 6164281]
24. Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci U S A*. 1990; 87(5):1663–7. [PubMed: 1689846]
25. DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat Genet*. 1996; 14(4):457–60. [PubMed: 8944026]
26. Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, et al. A concise guide to cDNA microarray analysis. *Biotechniques*. 2000; 29(3):548–50. 52–4. 56 passim. [PubMed: 10997270]
27. Sartor MA, Tomlinson CR, Wesselkamper SC, Sivaganesan S, Leikauf GD, Medvedovic M. Intensity-based hierarchical Bayes method improves testing for differentially expressed genes in microarray experiments. *BMC Bioinformatics*. 2006; 7:538. [PubMed: 17177995]

28. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004; 3 Article3.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. [PubMed: 11846609]
30. Jones WK, Flaherty MP, Tang XL, Takano H, Qiu Y, Banerjee S, et al. Ischemic preconditioning increases iNOS transcript levels in conscious rabbits via a nitric oxide-dependent mechanism. *J Mol Cell Cardiol*. 1999; 31(8):1469–81. [PubMed: 10423345]
31. Li Q, Guo Y, Ou Q, Cui C, Wu WJ, Tan W, et al. Gene Transfer of Inducible Nitric Oxide Synthase Affords Cardioprotection by Upregulating Heme Oxygenase-1 Via a Nuclear Factor- κ B-Dependent Pathway. *Circulation*. 2009
32. Bolli R, Shinmura K, Tang XL, Kodani E, Xuan YT, Guo Y, et al. Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res*. 2002; 55(3):506–19. [PubMed: 12160947]
33. Stein AB, Tang XL, Guo Y, Xuan YT, Dawn B, Bolli R. Delayed adaptation of the heart to stress: late preconditioning. *Stroke*. 2004; 35(11 Suppl 1):2676–9. [PubMed: 15459441]
34. Jancso G, Cserepes B, Gasz B, Benko L, Borsiczky B, Ferenc A, et al. Expression and protective role of heme oxygenase-1 in delayed myocardial preconditioning. *Ann N Y Acad Sci*. 2007; 1095:251–61. [PubMed: 17404038]
35. Dawn B, Bolli R. HO-1 induction by HIF-1: a new mechanism for delayed cardioprotection? *Am J Physiol Heart Circ Physiol*. 2005; 289(2):H522–4. [PubMed: 16014614]
36. Simkhovich BZ, Marjoram P, Poizat C, Kedes L, Kloner RA. Brief episode of ischemia activates protective genetic program in rat heart: a gene chip study. *Cardiovasc Res*. 2003; 59(2):450–9. [PubMed: 12909328]
37. Das DK, Maulik N. Cardiac genomic response following preconditioning stimulus. *Cardiovasc Res*. 2006; 70(2):254–63. [PubMed: 16566910]
38. Kawata H, Yoshida K, Kawamoto A, Kurioka H, Takase E, Sasaki Y, et al. Ischemic preconditioning upregulates vascular endothelial growth factor mRNA expression and neovascularization via nuclear translocation of protein kinase C epsilon in the rat ischemic myocardium. *Circ Res*. 2001; 88(7):696–704. [PubMed: 11304492]
39. Maulik N. Ischemic preconditioning mediated angiogenic response in the heart. *Antioxid Redox Signal*. 2004; 6(2):413–21. [PubMed: 15025943]
40. Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, et al. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation*. 2003; 108(1):79–85. [PubMed: 12796124]
41. Eckle T, Kohler D, Lehmann R, El Kasmi K, Eltzschig HK. Hypoxia-inducible factor-1 is central to cardioprotection: a new paradigm for ischemic preconditioning. *Circulation*. 2008; 118(2):166–75. [PubMed: 18591435]
42. Rane S, He M, Sayed D, Vashistha H, Malhotra A, Sadoshima J, et al. Downregulation of miR-199a derepresses hypoxia-inducible factor-1 α and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ Res*. 2009; 104(7):879–86. [PubMed: 19265035]
43. Cai Z, Zhong H, Bosch-Marce M, Fox-Talbot K, Wang L, Wei C, et al. Complete loss of ischaemic preconditioning-induced cardioprotection in mice with partial deficiency of HIF-1 α . *Cardiovasc Res*. 2008; 77(3):463–70. [PubMed: 18006459]
44. Jung Y, Isaacs JS, Lee S, Trepel J, Liu ZG, Neckers L. Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor kappa B activation. *Biochem J*. 2003; 370(Pt 3):1011–7. [PubMed: 12479793]
45. Bonello S, Zahringer C, Belaiba RS, Djordjevic T, Hess J, Michiels C, et al. Reactive oxygen species activate the HIF-1 α promoter via a functional NF κ B site. *Arterioscler Thromb Vasc Biol*. 2007; 27(4):755–61. [PubMed: 17272744]
46. Belaiba RS, Bonello S, Zahringer C, Schmidt S, Hess J, Kietzmann T, et al. Hypoxia up-regulates hypoxia-inducible factor-1 α transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. *Mol Biol Cell*. 2007; 18(12):4691–7. [PubMed: 17898080]

47. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J.* 2003; 17(14):2115–7. [PubMed: 12958148]
48. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol.* 2009; 10(4):348–55. [PubMed: 19295631]
49. Valmiki MG, Ramos JW. Death effector domain-containing proteins. *Cell Mol Life Sci.* 2009; 66(5):814–30. [PubMed: 18989622]
50. Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res.* 1998; 83(2):117–32. [PubMed: 9686751]
51. Stice JP, Knowlton AA. Estrogen, NFkappaB, and the heat shock response. *Mol Med.* 2008; 14(7-8):517–27. [PubMed: 18431462]
52. Ammirante M, Rosati A, Gentilella A, Festa M, Petrella A, Marzullo L, et al. The activity of hsp90 alpha promoter is regulated by NF-kappa B transcription factors. *Oncogene.* 2008; 27(8):1175–8. [PubMed: 17724475]
53. Huang L, Mivechi NF, Moskopidid D. Insights into regulation and function of the major stress-induced hsp70 molecular chaperone in vivo: analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. *Mol Cell Biol.* 2001; 21(24):8575–91. [PubMed: 11713291]
54. Lee SH, Kwon HM, Kim YJ, Lee KM, Kim M, Yoon BW. Effects of hsp70.1 gene knockout on the mitochondrial apoptotic pathway after focal cerebral ischemia. *Stroke.* 2004; 35(9):2195–9. [PubMed: 15243143]
55. Lee JS, Seo JS. Differential expression of two stress-inducible hsp70 genes by various stressors. *Exp Mol Med.* 2002; 34(2):131–6. [PubMed: 12085988]
56. Mayer MP, Bukau B. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci.* 2005; 62(6):670–84. [PubMed: 15770419]
57. Oyadomari S, Yun C, Fisher EA, Kreglinger N, Kreibich G, Oyadomari M, et al. Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. *Cell.* 2006; 126(4):727–39. [PubMed: 16923392]
58. Feinstein DL, Galea E, Aquino DA, Li GC, Xu H, Reis DJ. Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFkappaB activation. *J Biol Chem.* 1996; 271(30):17724–32. [PubMed: 8663604]
59. Chan JY, Ou CC, Wang LL, Chan SH. Heat shock protein 70 confers cardiovascular protection during endotoxemia via inhibition of nuclear factor-kappaB activation and inducible nitric oxide synthase expression in the rostral ventrolateral medulla. *Circulation.* 2004; 110(23):3560–6. [PubMed: 15557375]

Fig 1.

IPC 24 hrs preceding a 30 min ischemia/reperfusion (I/R) reduces infarct size in WT mice from 40.6% to 21.4% as compared to sham IPC. Late IPC-induced cardioprotection is lost in the 2M NF- κ B DN mice (35.3% infarct) compared to sham (32.8% infarct). *P = 0.05 vs. Sham, n=6-10.

Fig 2.

A. Schematic of our hypothesis driven microarray approach. Three different direct microarray comparisons were made as represented by the colored lines (green: WT-IPC vs. WT-Sham, red: 2M-IPC vs. WT-IPC, and black: 2M-Sham vs. WT-Sham). **B.** Venn diagram representing the number of significantly dysregulated genes between WT-IPC and WT-Sham (green circle) and 2M-IPC and WT-IPC (red circle). The 238 genes represented by the overlap are genes whose expression were significantly up or down regulated by NF- κ B after IPC. Statistical cutoff employed to filter significantly regulated genes was $P \leq 0.01$ or a fold change ≥ 2.0 ($n=3-4$; see microarray statistical section of Methods).

Angiogenesis associated GO Terms (* Similarity score ≥ 0.75)

Go Term	Count	P-value	Fold Enrichment
Angiogenesis	9	0.00054	4.8
Blood vessel morphogenesis	10	0.00062	4.2
Anatomical structure formation	10	0.00091	4
Blood vessel development	10	0.0017	3.6
Vasculature development	10	0.0019	3.6

Programmed Cell Death associated GO Terms (* Similarity score ≥ 0.75)

Go Term	Count	P-value	Fold Enrichment
Apoptosis	19	0.0018	2.3
Programmed cell death	19	0.0022	2.2
Cell death	19	0.0032	2.1
Death	19	0.0033	2.1
Regulation of apoptosis	14	0.0042	2.5
Regulation of programmed cell death	14	0.0047	2.5
Cell development	26	0.0078	1.7

Heat Shock Response associated GO Terms (* Similarity score ≥ 0.75)

Go Term	Count	P-value	Fold Enrichment
Response to heat	6	0.000039	15.3
Response to temperature stimulus	6	0.00028	10.2
Response to protein stimulus	6	0.0014	7.2
Response to unfolded protein	6	0.0014	7.2
Protein folding	9	0.0043	3.5
Response to abiotic stimulus	8	0.0064	3.6

Fig 3.

Functional annotation of 238 NF- κ B regulated genes post-IPC resulted in a significant enrichment ($P = 0.01$) for GO terms associated with angiogenesis, programmed cell death, and heat shock response. A GO similarity score of 0.75 and a P value 0.01 were used as criteria for inclusion as a member of the angiogenesis, programmed cell death, and heat shock response associated groupings of gene ontologies (see microarray statistical section of Methods).

Genes contained within angiogenesis associated GO Terms

Symbol	Gene Name	Fold Expression		P-value	
		WT IPC v. Sham	2M v. WT IPC	WT IPC v. Sham	2M v. WT IPC
<i>hif1a</i>	hypoxia inducible factor 1, alpha subunit	1.63	-1.94	0.00507	0.00616
<i>btg1</i>	b-cell translocation gene 1, anti-proliferative serine (or cysteine) peptidase inhibitor, clade e, member 1	1.42	-1.69	0.00924	0.00343
<i>serpine1</i>	serpin family 1 member 1	2.29	-2.08	0.00001	0.01353
<i>rhob</i>	ras homolog gene family, member b	1.55	-2.45	0.00363	0.00009
<i>cyr61</i>	cysteine rich protein 61	3.91	-2.34	< 0.00001	0.00518
<i>rm4</i>	reticulin 4	1.60	-1.70	0.00331	0.00533
<i>tbx20</i>	t-box 20	-1.43	1.41	0.00532	0.00907
<i>tnfrsf12</i>	tumor necrosis factor (ligand) superfamily, member 12	1.55	-1.44	0.00073	0.00159
<i>edg1</i>	endothelial differentiation sphingolipid g-protein-coupled receptor 1	1.54	-1.94	0.00749	0.00213
<i>cdh2</i>	cadherin 2	1.46	-2.07	0.0092	0.00145
<i>pafah1b1</i>	platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	1.46	-1.93	0.00807	0.00025

Genes contained within programmed cell death associated GO Terms

Symbol	Gene Name	Fold Expression		P-value	
		WT IPC v. Sham	2M v. WT IPC	WT IPC v. Sham	2M v. WT IPC
<i>fadd</i>	fas (tnfrsf6)-associated via death domain	-1.60	1.54	0.00118	0.00304
<i>hspa1b</i>	heat shock protein 1b	8.53	-4.00	< 0.00001	NA
<i>bnip3l</i>	bcl2/adenovirus e1b interacting protein 3-like	-1.59	1.58	0.00309	0.00283
<i>hif1a</i>	hypoxia inducible factor 1, alpha subunit	1.63	-1.94	0.00507	0.00616
<i>btg1</i>	b-cell translocation gene 1, anti-proliferative	1.42	-1.69	0.00924	0.00343
<i>bcl2l1</i>	bcl2-like 1	1.55	-1.57	0.00262	0.00958
<i>itm2b</i>	integral membrane protein 2b	2.07	-4.22	0.00002	0.00001
<i>zfp346</i>	zinc finger protein 346	-1.40	1.44	0.00743	0.0012
<i>rhob</i>	ras homolog gene family, member b	1.55	-2.45	0.00363	0.00009
<i>igh-1a</i>	immunoglobulin heavy chain 1a (serum igg2a)	-1.58	1.85	0.00231	0.00015
<i>btg2</i>	b-cell translocation gene 2, anti-proliferative	2.67	-3.62	0.00003	NA
<i>rm4</i>	reticulin 4	1.60	-1.70	0.00331	0.00533
<i>myc</i>	myelocytomatosis oncogene	3.55	-1.71	NA	0.00484
<i>tnfrsf12</i>	tumor necrosis factor (ligand) superfamily, member 12	1.55	-1.44	0.00073	0.00159
<i>nr4a1</i>	nuclear receptor subfamily 4, group a, member 1	1.44	-1.8	0.00891	0.00188
<i>tnfrsf1a</i>	tumor necrosis factor receptor superfamily, member 1a	1.4	-1.79	0.00856	0.00008
<i>pik3r1</i>	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	-1.76	1.72	0.00079	0.00461
<i>fastk</i>	fas-activated serine/threonine kinase	1.56	-2.27	0.0036	0.00011
<i>ddx20</i>	dead (asp-glu-ala-asp) box polypeptide 20	-1.71	1.37	0.0003	0.00951
<i>pafah1b1</i>	platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	1.46	-1.93	0.00807	0.00025
<i>actg1</i>	actin, gamma, cytoplasmic 1	2.04	-2.91	0.00013	0.00029
<i>amigo1</i>	adhesion molecule with ig like domain 1	-1.74	1.4	0.00022	0.0097
<i>kcne1</i>	potassium voltage-gated channel, isk-related subfamily, member 1	1.46	1.57	0.0069	0.00255
<i>cav2</i>	caveolin 2	1.58	-3.1	0.00239	NA
<i>prpf19</i>	prp19/psa4 pre-mrna processing factor 19	-1.7	1.6	0.00095	0.00837
<i>pex7</i>	peroxisome biogenesis factor 7	1.61	-2.02	0.00165	0.00028

Genes contained within heat shock response associated GO Terms

Symbol	Gene Name	Fold Expression		P-value	
		WT IPC v. Sham	2M v. WT IPC	WT IPC v. Sham	2M v. WT IPC
<i>hspa4</i>	heat shock protein, 110 kda	1.58	-2.05	0.00286	0.00014
<i>hspa1b</i>	heat shock protein 1b	8.53	-4	< 0.00001	NA
<i>hsp90ab1</i>	heat shock protein 1, beta	1.49	-1.74	0.00755	0.00263
<i>dnaja1</i>	dnaj (hsp40) homolog, subfamily a, member 1	2.48	-2.06	< 0.00001	0.00657
<i>hspd1</i>	heat shock protein 1 (chaperonin)	1.75	-2.13	0.0004	0.00118
<i>hsp90aa1</i>	heat shock protein 1, alpha	3.08	-2.07	< 0.00001	0.02907
<i>nfe2l2</i>	nuclear factor, erythroid derived 2, like 2	1.94	-2.66	0.00011	0.0004
<i>ttc9c</i>	tetratricopeptide repeat domain 9c	-1.6	1.48	0.00164	0.00846
<i>fkbp3</i>	fk506 binding protein 3	1.67	-2.27	0.00076	0.00013
<i>dnajb4</i>	dnaj (hsp40) homolog, subfamily b, member 4	2.72	-2.83	< 0.00001	0.00038
<i>bcl2l1</i>	bcl2-like 1	1.55	-1.57	0.00262	0.00958
<i>myc</i>	myelocytomatosis oncogene	3.55	-1.71	NA	0.00484

Fig 4.

A detailed list of the genes contained in the associated groupings of GO terms. Genes in blue are found in both angiogenesis and programmed cell death GO terms; red indicates overlap between programmed cell death and heat shock response GO terms.

Fig 5.

Quantitative real time RT-PCR validation of selected genes from gene ontology analysis (HIF-1α, Fadd, and Hsp90). IPC induced expression changes of COX-2 mRNA levels were also assessed via QRT-PCR and shown to be increased in an NF-κB-dependent manner following IPC. The gene expression patterns determined by QRT-PCR generally agree with the gene expression patterns indicated by the gene microarray. mRNA levels displayed as a fold change vs. WT sham (represented by horizontal line at fold expression = 1). *P < 0.05 vs. WT Sham. # P < 0.05 vs. WT IPC, n=6-8.

Fig 6.

Quantitative real time RT-PCR analysis of mRNA expression confirmed a very strong IPC induced upregulation of transcripts encoding both Hsp70 isoforms. Both also showed a very strong NF- κ B-dependent increase in expression after IPC. *P 0.05 vs. WT Sham. #P 0.05 vs. WT IPC, n=6-8.

Fig 7.

Infarct analysis reveals that a double-knockout of Hsp70.1 and 70.3 does not exhibit late phase IPC cardioprotection against MI (A). However, a single knockout of the Hsp70.1 isoform had no effect on late IPC cardioprotection (B). Knockout of both Hsp70.1 and 70.3 increased infarct size after I/R injury, whereas knockout of only Hsp70.1 decreased infarct size following I/R (C). C57 and B6/129 groups serve as wild-type control groups for Hsp70.1/3 double KO and Hsp70.1 KO, respectively. *P < 0.05 vs. corresponding WT Sham, n=6-11. There was no significant difference between the wild-type groups of the two different strains.