Cardioplegia prevents ischemia-induced transcriptional alterations of cytoprotective genes in rat hearts: A DNA microarray study

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Background: Energy conservation and calcium homeostasis contribute to myocardial protection provided by hyperkalemic cardioplegia during ischemia. Complimenting these established mechanisms of protection, previous work suggested that activation of cytoprotective signaling pathways also contributes to reduced injury with cardioplegia. We proposed that cardioplegia would recruit cytoprotective pathways and investigated the transcriptional response of the heart after cardioplegia-protected ischemia compared with that after ischemia alone.

Methods: Isolated perfused rat hearts underwent 40 minutes of global ischemia alone or with St Thomas cardioplegia, followed by 120 minutes of reperfusion. The expression profiles of isolated RNA were determined by using Affymetrix microarrays and assessed by comparing cardioplegia-protected hearts and hearts undergoing unprotected ischemia with time-matched control hearts. The content of selected proteins was assessed by means of immunoblotting.

Results: Cardioplegia preserved the expression of multiple genes involved in carbohydrate and fatty acid metabolism, glycolysis, and electron transport compared with ischemia alone. The expression of the sodium-calcium exchanger and ryanodine receptor was preserved in line with the ability of cardioplegia to decrease calcium overload. The expression of multiple cytoprotective molecules, including protein-tyrosine kinase, calcineurin B, p38 mitogen-activated protein kinase, voltage-dependent anion channel, protein kinase C ϵ, heat shock protein 70, and manganese superoxide dismutase all showed decreased expression in ischemia but were preserved to near nonischemic levels by cardioplegia.

Conclusion: Cardioplegia during ischemia maintained an expression profile similar to that seen in nonischemic hearts for genes involved in energy conservation, calcium homeostasis, and cytoprotective pathways, whereas ischemia alone did not. Exposing the transcriptional differences in cytoprotective genes during untreated and cardioplegia-treated ischemia provides valuable insight into an additional mechanism of cardioprotection induced by cardioplegia.

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Supported by the Jay L. Ankeney Endowment. Dr Lesnefsky was supported by grants 2RO1AG12447 and 1PO15885 from the National Institutes of Health and by the Medical Research Service, Department of Veterans Affairs. Dr Hedayati was an Allen Fellow supported by the Jay L. Ankeney Professorship in Cardiothoracic Surgery, Case Western Reserve University School of Medicine, Cleveland, Ohio.

Received for publication March 3, 2005; revisions received May 25, 2005; accepted for publication June 8, 2005.

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J Thorac Cardiovasc Surg 2005;130:1151-8

0022-5223/$30.00

doi:10.1016/j.jtcvs.2005.06.027

Cardioplegic arrest is used to provide myocardial protection in the majority of the 686,000 patients who undergo cardiac surgical procedures in the United States each year.[1] The arrested heart, although advantageous to the surgeon, remains susceptible to ischemic injury. Hyperkalemic cardioplegia (CP) is used to maintain diastolic arrest and provide myocardial protection by attenuating the progression of ischemic injury. Despite modifications to the formulation and route of administration, suboptimal myocardial protection remains a problem. The protective effect of CP is thought to be largely the result of conservation of cellular energy stores and preservation of calcium homeostasis. In addition to these accepted mechanisms of myocardial protection, previous work from our laboratory demon-

strated that the myocardial protection provided by CP is substantially attenuated if intracellular signaling pathways involving protein kinase C (PKC) or tyrosine kinase are inhibited.[2] Activation of these signaling kinases leads to cytoprotection and has been implicated in the robust cardioprotective response of ischemic preconditioning (IPC). This evolving appreciation of the key contribution of the activation of intracellular signaling pathways to myocardial protection[3] raises the question of whether similar mechanisms might contribute to the cardioprotection provided by CP. To identify potential key contributing mechanisms involved in the cardioprotection of CP, we used DNA microarray technology to profile the gene expression pattern of rat hearts subjected to ischemia and reperfusion alone compared with that seen in hearts subjected to CP-protected ischemia and reperfusion. The goal of this study was to evaluate transcriptional changes in genes related to previously known mechanisms of CP and to assess for evidence of the novel involvement of cytoprotective pathways.

Materials and Methods

All experiments were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and conformed to the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council and published by the National Academy Press (revised 1996).

Isolated Rat Heart Preparation

The isolated rat heart preparation was used as previously described.[2] Male Sprague-Dawley rats (320-360 g) were anesthetized (heparin, 1000 units/kg administered intraperitoneally; pentobarbital, 100 mg/kg administered intraperitoneally). Hearts were excised, mounted on the Langendorff apparatus, and perfused with Krebs-Henseleit (KH) buffer delivered at a perfusion pressure of 65 mm Hg. After collection of baseline data during the equilibration period, the hearts were randomized into one of the 3 study groups. Time-matched control hearts (n = 7) were perfused for 180 minutes. Hearts in the ischemic group (n = 7) were equilibrated for 20 minutes and then underwent 40 minutes of normothermic global ischemia, followed by 120 minutes of reperfusion with KH buffer. Hearts in the CP group (n = 7) were equilibrated for 17 minutes, perfused with St Thomas' Cardioplegia II (NaCl, 110.0 mmol/L; KCl, 16.0 mmol/L; $CaCl_2$, 1.20 mmol/L; $MgCl_2$*$6H_2O$, 16.0 mmol/L; and $NaHCO_3$, 10.0 mmol/L; pH of 7.8 ± 0.2, T = 4°C) for 3 minutes, and underwent 40 minutes of normothermic global ischemia, followed by 120 minutes of reperfusion with KH buffer. After 180 minutes, left ventricular (LV) tissue was excised and immediately frozen in liquid nitrogen. Three hearts from each group were used for RNA preparation, and 4 hearts were used independently for protein quantitation.

Microarray Analysis

Samples of total RNA were processed by the Gene Expression Array Core Facility at Case Western Reserve University.

RNA preparation. Total RNA was isolated from LV tissue by using Trizol Reagent (Invitrogen). RNA preparations from each group (ischemic, CP, and control) were pooled (n = 3), and their quality and quantity (0.79, 0.91, and 1.10 μg RNA/mg LV tissue) were assessed with spectrophotometry (260/280) and gel electrophoresis. Samples were cleaned and eluted by using a column from a Qiagen RNeasy kit (part no. 74106) precipitated with ammonium acetate and ethanol centrifuged, washed, and resuspended.

Synthesis of cDNA. The Affymetrix protocol for cDNA synthesis was used. The reaction was primed by annealing an oligo-dT primer coupled to a T_7 RNA polymerase promoter to the RNA sample. RNA was reverse transcribed with Superscript II reverse transcriptase. Second-strand synthesis was carried out immediately in the presence of *Escherichia coli* DNA pol I, RNAse H, and DNA ligase; incubated in the presence of T_4 DNA pol; and terminated with ethylenediamine tetraacetic acid (EDTA). The sample was cleaned (Qiagen cDNA clean-up column).

Synthesis of biotin-labeled complementary RNA. Complementary RNA was generated in an in vitro transcription reaction by using a BioArray High Yield ENZO kit (Affymetrix). Samples were mixed, centrifuged, and returned to the incubator every 40 minutes. In vitro transcription samples were cleaned (Qiagen RNA clean-up columns) and eluted. The quality was confirmed spectrophotometrically (260/280).

Fragmentation and hybridization to test array. Samples were fragmented (40 mmol/L Tris acetate [pH 8.1], 100 mmol/L potassium acetate, and 30 mmol/L magnesium acetate at 94°C for 35 minutes) and added to hybridization buffer (final concentrations: 100 mmol/L morpholinoethanesulfonic acid (MES); 1 mol/L [Na^+]; 20 mmol/L EDTA; 0.01% Tween 20; 0.1 mg/mL Herring sperm DNA; and 0.5 mg/mL acetylated bovine serum albumin) to improve hybridization to the oligonucleotide array. Eukaryotic hybridization controls (*BioB*, *BioC*, *BioD*, and *cre*; final concentrations of 1.5, 5, 25, and 100 pmol/L, respectively) and control oligonucleotide (50 pmol/L) were added to the cocktail. The hybridization cocktail was denatured (99°C for 5 minutes), transferred to the test array, and incubated (42°C for 16 hours), and the hybridization cocktail was removed. The array was washed, stained, and scanned for background fluorescence and expression levels of control oligonucleotides by using Affymetrix Microarray Suite software.

Hybridization of fragmented samples to species microarray. Sample hybridization cocktails were thawed (45°C) and centrifuged. The Affymetrix chip arrays (RG-U34A, part no. 510338), containing probe sets for more than 7000 known genes, were equilibrated to room temperature. Sample cocktails were introduced into the chamber of the preconditioned chips and incubated (45°C for 16 hours). The chips were washed, stained, and scanned with an Agilent Gene Array scanner 2000 driven by Affymetrix MicroArray Suite 5.0. Genes with unknown products or duplicate entries were omitted. Functional or biologic classification was determined from the Affymetrix database, the PubMed database, or both.

Statistical analysis of species microarray. Scanned images were analyzed with Affymetrix MAS 5.0 software. For complete description of the statistical algorithms, refer to the Statistical Algorithm Description Document available from Affymetrix. The following tunable parameters were used: $\alpha 1 = 0.04$ and $\alpha 2 =$