Moderate hypothermia (30°C) maintains myocardial integrity and modifies response of cell survival proteins after reperfusion

Xue-Han Ning,1,4 Emil Y. Chi,3 Norman E. Buroker,4 Shi-Han Chen,2 Cheng-Su Xu,1 Ying-Tzang Tien,3 Outi M. Hyyti,1 Ming Ge,4 and Michael A. Portman1,4

Divisions of 1Cardiology and 2Genetics and Development, Department of Pediatrics, and 3Department of Pathology, University of Washington, and 4Children’s Hospital and Regional Medical Center, Seattle, Washington

Submitted 30 January 2007; accepted in final form 23 July 2007

Hypothermia protects myocardium from oxidative injury during ischemic stress and reperfusion (8, 13, 28). Although modulation of energy metabolism plays an important role in establishing hypothermic protection, other mechanisms also participate. Cold stress, in particular, induces cross-adaptation to noxious stresses (29). This adaptation is accompanied by altered signaling of various cell survival and apoptotic pathways. For instance, hypothermia applied to the ischemic heart promotes subsequent mRNA accumulation for antiapoptotic Bcl-2 homolog Bcl-x, while depressing mRNA levels for tumor suppressor gene p53 (27).

Although we previously demonstrated (28, 29) that hypothermia preserves cardiac function after reperfusion in the short term, and concurrently alters these transcriptional signaling pathways, we did not determine whether these changes are translated to more prolonged preservation of structural integrity. Therefore, the primary objective of this study was to evaluate these adaptive processes over a longer time period and to determine whether they are accompanied by modification in expression of proteins involved in these pathways. The isolated, perfused rabbit heart model was used to eliminate complex systemic effects and provide uniform global ischemia. The isolated, isovolumic perfused rabbit heart model is limited by 2 h of ischemia and potentially high left ventricular end-diastolic pressure (LVEDP) but still permits evaluation of cardiac function, structural integrity, and protein expression for at least 3 h after reperfusion. This time period was adequate to detect major ischemia-reperfusion-induced alterations in collagen framework, which are ameliorated by moderate hypothermia.

MATERIALS AND METHODS

Cardiac Function Measurement

Heart perfusion procedure. Animal treatment and experimental procedures were in accordance with institutional and National Institutes of Health guidelines. The study protocol was reviewed and approved by the University of Washington Institutional Animal Care and Use Committee (no. 3107-01). Rabbits (male or female, 2.5–3.4 kg body wt) were anesthetized with pentobarbital sodium (45–50 mg/kg iv). After 5 min of heparin injection (800 U/kg iv), the heart was rapidly excised and immersed in 4°C physiological salt solution (PSS) containing (in mM) 118.0 NaCl, 4.0 KCl, 22.3 NaHCO3, 1.1 glucose, 0.66 KH2PO4, 1.23 MgCl2, and 2.38 CaCl2. The aorta was cannulated under cold PSS within 1 min in the Langendorff mode, and blood was flushed out with 10 ml of PSS (4°C) passed through a 3.0-μm pore size filter at a speed of 1 ml/s. The heart was then perfused with PSS equilibrated with 95% O2-5% CO2 at 37°C [pH 7.4, oxygen partial pressure (PO2) ~660 mmHg] and passed twice through filters with 3.0-μm pore size. Perfusion pressure was maintained at 90 mmHg (26, 27, 29). An incision was made along the middle edge of the left atrium, and a fluid-filled latex balloon was passed through the mitral orifice and placed in the left ventricle. A pressure transducer was connected to the balloon for measurement of left ventricular pressure (LVP) and its first derivative with respect to time.
time (dP/dt). The caudal vena cava, the left and right cranial vena cava, and the azygous vein were ligated (24). The coronary artery was cannulated to enable collection of coronary flow, measured with a flowmeter (T201, Transonic Systems, Ithaca, NY).

Analog signals were continuously recorded on an online computer (Macintosh, Biopac Analog Signal Acquisition System) and a pressurized ink chart recorder (Gould, Cleveland, OH). To characterize cardiac function, we defined peak systolic pressure (PSP) minus end-diastolic pressure (EDP). Calculating the product of heart rate (HR) and DP [pressure-rate product (PRP), mmHg/min] provided an estimate of myocardial work. Myocardial oxygen consumption (MV\(_2\)) was calculated as MV\(_2\) (\(\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}\)) = CF × [(\(PaO_2\) – ETP) × (c(760)])/V\(_m\), where CF is coronary flow (\(\text{ml}\cdot\text{min}^{-1}\cdot\text{g wet tissue}^{-1}\)), (\(PaO_2\) – ETP) is the difference in P\(_O2\) (mmHg) between perfusate and coronary effluent, \(c\) is the Bunsen solubility coefficient of O\(_2\) in perfusate at 37°C (22.1 \(\mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{atm}^{-1} \cdot \text{g wet tissue}^{-1}\)), and V\(_m\) is molar volume (22.4 \(\mu\text{mol}\)). Oxygen extraction was calculated as MV\(_2\)/oxygen content in the perfusate (24, 26, 29). The wet weight of the heart was determined after 2 h of cardioplegic ischemia and 3 h of reperfusion and after trimming of fat and great vessels and then blot-drying with nine-layer cotton gauze.

**Experimental protocols.** The methods and basis for protocols used in these experiments have been previously detailed (27, 28). In those prior studies and in the present experiments cardioplegia has been instilled in hearts at the start and every 30 min during ischemia at different temperatures to produce uniform periods of cardiac arrest and ATP utilization. Additionally, 34°C has provided minimal protection (28) and continues to represent the control temperature in these experiments (26, 27, 29). Accordingly, the hearts were divided into three groups for functional, metabolic, histological, and molecular investigation: 1) 34°C ischemic hearts (I group; \(n = 6\)) were treated at 34°C during ischemia; 2) 30°C ischemic hearts were treated by hypothermia for 30 min before and during ischemia and progressively rewarmed in the first 20 min of reperfusion (H group; \(n = 6\)); 3) normal control hearts (N group; \(n = 5\)) in vivo were rapidly excised and immersed in 4°C PSS, the aorta was cannulated to flush out blood, and the hearts were then treated according to the procedure described for examination of cardiac muscle integrity.

Some investigators have shown that preload provided by an inflated balloon in the left ventricle during ischemia contributes to elevation in myocardial stiffness. Others, using a deflated balloon during ischemia, have shown that myocardial stiffness remains elevated as long as 15 min after onset of reperfusion (2, 31). In some previous studies we have deflated the left ventricular balloon during ischemia (34, 35) in order to minimize confounding affects of myocardial stretch. However, the empty balloon model cannot be used to define LVEDP during ischemia. Therefore, in the present study we used a constantly inflated balloon in the left ventricle in order to continuously record LVP and determine the time of initial rise in LVEDP. Before baseline recording, an optimal intraventricular balloon volume was determined for each heart by inflating it over a range of values to construct left ventricular developed pressure to describe the ascending limb of the Starling curve between 100 and 140 mmHg. Balloon volumes were not adjusted to produce specific EDP, but rather to define the level of systolic pressure development in a physiological condition. Nevertheless, EDP at baseline >8 mmHg were not accepted (27). In addition, data from hearts with DP <100 mmHg or >140 mmHg were excluded (29). The baseline data were obtained after a 30-min equilibration period. Immediately after the cardioplegic solution was injected, LVP was always near 0 mmHg by relaxation of the myocardium. The beginning of ischemic contracture was defined by the initial rise in LVP above 2 mmHg. With this model the results indicated that contracture started ~50–70 min into ischemia and continued as a high LVEDP during reperfusion. To test whether the physiological balloon volume would further increase LVEDP, we performed the protocol in a separate group of Langendorff-perfused, isolated rabbit hearts (34°C; \(n = 8\)). At baseline the balloon was deflated and the LVEDP was zero to start the optimal volume-developed pressure curve determination as mentioned above. Just before ischemia the balloon was deflated until 15 min of reperfusion and then reinflated with the same volume as before ischemia.

Baseline data were obtained after a 30-min equilibration period. The same procedures were followed in each experiment. During the baseline period, data were obtained with the hearts maintained at 37°C by passing water at this temperature through the organ bath. To adjust the infusion temperature, the myocardial/pulmonary outflow temperature was monitored continuously with a thermal probe. We have previously shown (28) that this temperature corresponds to myocardial temperature, monitored with a Khuri regional tissue temperature probe (Vascular Technology, Chelmsford, MA). Figure 1 shows the protocol of hypothermia in the ischemic hearts.

After 180 min of reperfusion and hemodynamic recording excess fat and connective tissues were removed, and the left ventricular free wall was briefly separated and blotted on nine-layer gauze. The free wall between the left anterior descending artery and the posterior descending artery was divided into three sections: anterior, middle, and posterior. The anterior and posterior sections were frozen and stored in liquid nitrogen for molecular analysis, and the middle sections were fixed in 4% phosphate-buffered formalin for histological and immunocytochemical analysis.

**Metabolite measurements.** The coronary inflow and outflow were collected into a 1.7-ml Eppendorf tube, and P\(_O2\) and partial pressure of CO\(_2\) (P\(_{CO2}\)) were immediately (at ~8–10 s) measured with a Radiometer (ABL 5, Copenhagen, Denmark). Difference in CO\(_2\) content (dCO\(_2\)) between coronary outflow and inflow was calculated as dCO\(_2\) = (PV\(_{CO2}\) – PV\(_{CO2}\)) × c(CO\(_2\))/V\(_m\) (27, 29), where (PV\(_{CO2}\) – PV\(_{CO2}\)) is the difference in P\(_{CO2}\) (mmHg) between coronary effluent and perfusate, c(CO\(_2\)) is the solubility coefficient of CO\(_2\) in perfusate at 37°C (0.52 ml CO\(_2\)·atm\(^{-1}\)·g wet tissue\(^{-1}\)), and V\(_m\) is 22.4 \(\mu\text{mol}\). CO\(_2\) production (\(\mu\text{mol} \cdot \text{min}^{-1}\cdot\text{g tissue}^{-1}\)) was calculated as CF × dCO\(_2\). The rest of the fluid sample was placed in 4°C for measurement of lactate concentration with a GM7 Analyzer (Analox micro-Stat, London, UK).

**Gene Expression**

**RNA isolation.** An aliquot (200 mg) of the frozen left ventricular free wall was pulverized and homogenized, and total RNA was then extracted with an RNA Isolation Kit (Ambion, Austin, TX). RNA samples were tested by ultraviolet absorption at absorbance of 260 nm to determine the concentration. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denatured 1% agarose gels (29, 30).

![Cardioplegia](image-url)

**Fig. 1.** Protocol of hypothermia in ischemic hearts. Ischemia starts at 0 min and ends at 120 min, followed by 180 min of reperfusion. I, ischemia group (34°C during ischemia); H, hypothermic ischemia group (30°C during ischemia).
Reverse transcription PCR analysis. Five micrograms total of RNA were added to 20 μl of reaction mixture that was preheated for 10 min at 65°C with 100 ng of poly(dT)12-18 primer. First-strand cDNA was synthesized by SuperScript II RNAase H-Reverse Transcriptase at 42°C for 50 min (Life Technologies, Grand Island, NY). The reaction was terminated at 70°C for 15 min. For subsequent PCR reaction (50 μl), 1 μl of the cDNA mixture was used for each gene-specific amplification.

Our previous study (27) indicated that signaling for hypoxia-inducible factor 1α (HIF-1α) was modified by hypothermia. Additionally, we noted that mRNA for a critical component of respiratory complex II, succinate dehydrogenase α (SDH-α), was also altered. Accordingly, we evaluated both mRNA and protein expression levels for these.

SDH-α, HIF-1α, and β-actin primers were designed by a primer design program (Primer Design 3, Scientific & Educational Software, State Line, PA). The primer sequences were as follows: SDH-α: 5′-GGTTGTATCCGCTGTGCAT-3′ (forward), 5′-GCTCCTTCTCTTCTCGGATCT-3′ (reverse); HIF-1α: 5′-GCTCATCAGTGGCCACTTCCC-3′ (forward), 5′-GCTTGATGCGAATACCT-3′ (reverse); β-actin: 5′-GCCAGGCGGAATCTGGTCGTGCACATTAAGGAGA-3′ (forward), 5′-GCTCATTCTCGCCGTTGCTGATCCACATCTGC-3′ (reverse); and 5′-AAAGACCTGATGACCACAGCTGCGTGCTGGG-3′ (forward), 5′-GCTCATCCTCGGTTGCTGATCCACATCTGC-3′ (reverse).

Amplification reactions were conducted in 25–50 μl of reagent mixture with an initial step of 94°C for 3 min followed by 25–35 cycles of amplification depending on cDNA abundance in preparations. Each cycle was at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and then finally at 72°C for 7 min. β-Actin was used as a reference (27).

PCR products were analyzed by agarose gel electrophoresis. PCR bands of the predicted size were usually isolated and subjected to sequencing (ABI Prism 310, Applied Biosystems, Foster City, CA) to verify protein lane loadings. All values reported are means ± SE. The S-PLUS Program (version 6.2, Insightful, 2003) was used for statistical analysis. Data were evaluated with repeated-measures analyses of variance within

Immunocytochemistry analysis. Paraffin sections of heart tissue on precleared Superfrost (a) glass slides (VWR, Seattle, WA) were deparaffinized and washed in phosphate-buffered saline (PBS) (15). To localize tumor suppressor protein p53 antigen the sections were pretreated with 0.01% pronase in PBS for 10 min and then washed three times. Sections were incubated with 1:50 mouse anti-rabbit p53 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 30 min. Control sections were treated with mouse IgG 1:50 in PBS. After rinsing in PBS, the sections were incubated for 30 min with goat anti-mouse IgG labeled with horseradish peroxide (HRP) (Vector Laboratories, Burlingame, CA). The sections were washed three times and then reacted with 0.1% diaminobenzidine for 10 min with cobalt acetate to enhance the reaction, and to produce stained color from brown to dark brown. The sections were rinsed in distilled water, counterstained with 0.4% methyl green for nuclei, dehydrated in a series of ethanol concentrations up to 100%, cleared in xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). To evaluate the inactivation of the p53 pathway we used another semiquantification score method. Similar to evaluation of histological integrity, a 200-μm² field was evaluated at four corners and the center position in each slide: score 0, 0–1 pieces of p53 existed in the field, and myocardial bundles were associated closely together; score 1, 0–2 pieces of p53 existed in the field; score 2, 0–3 pieces of p53 existed in the field; score 3, 0–4 pieces of p53 existed in the field; score 4, 0–4 pieces of p53 existed in the field, and edema fluid was similar to the thin myocardial bundles; score 5, 0–4 pieces of p53 were in the field and edema fluid area was wider than the thin myocardial bundles.

Protein Expression

Fifty micrograms of total protein extracts from rabbit heart tissue was electrophoresed along with one lane containing 30 μg of human HeLa cells as a positive control and one lane of molecular weight markers (Chemichrome Western control, Sigma) in a 4.5% stacking and a 7.5%, 10%, or 12% running SDS-PAGE. The gels were then electroblotted onto PVDF-Plus membranes. Western blots were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS) plus Tween 20 (TBST; 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), followed by overnight incubation at 4°C with each primary antibody dilutated in the appropriate blocking solution as recommended by the supplier. The primary antibodies to Akt-1, also known as protein kinase B (PKBα (sc-1618), heme oxygenase 1 (HO-1; sc-1797), peroxisomal proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α; sc-13067), PPARβ, vascular endothelial growth factor (VEGF; sc-152), p53 (sc-99), and SDH-α (sc-27992) were obtained from Santa Cruz Biotechnology. After two 5-min washes with TBST and one 5-min wash with PBS, membranes were incubated at room temperature for 1 h with the appropriate secondary antibody conjugated to HRP. The membranes were washed twice for 10 min with TBST and visualized with enhanced chemiluminescence after exposure to Kodak Biomax light ML-1 film. The membranes were stripped by washing them twice for 30 min with 200 mM glycine, 0.1% SDS, and 1% Tween 20 (pH adjusted to 2.2), followed by three 10-min washes with TBS. The membranes were again blocked for 1 h as above, followed by overnight incubation at 4°C with a β-actin antibody (sc-1616, Santa Cruz Biotechnology) diluted 1:200 in blocking solution. The next day the membranes were washed (as above), the appropriate secondary- HRP antibody was applied, and the remaining procedures described above were followed. β-Actin was used to verify protein lane loadings.

Statistical Analysis

All values reported are means ± SE. The S-PLUS Program (version 6.2, Insightful, 2003) was used for statistical analysis.
Table 1.  *Hemodynamics*

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Preischemia</th>
<th>Rep 90 min</th>
<th>Rep 120 min</th>
<th>Rep 150 min</th>
<th>Rep 180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDP, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.9 ± 0.4</td>
<td>3.0 ± 0.4</td>
<td>48.8 ± 7.8</td>
<td>45.9 ± 7.5</td>
<td>44.2 ± 7.4</td>
<td>42.7 ± 7.2</td>
</tr>
<tr>
<td>H</td>
<td>3.0 ± 0.2</td>
<td>8.4 ± 1.3*</td>
<td>1.8 ± 0.2*</td>
<td>3.3 ± 0.4*</td>
<td>4.1 ± 0.6*</td>
<td>4.9 ± 0.8*</td>
</tr>
<tr>
<td>DP, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>118.0 ± 3.9</td>
<td>115.7 ± 4.7</td>
<td>31.1 ± 4.8</td>
<td>30.7 ± 4.2</td>
<td>29.7 ± 3.8</td>
<td>28.3 ± 3.5</td>
</tr>
<tr>
<td>H</td>
<td>124.0 ± 4.6</td>
<td>112.1 ± 7.7</td>
<td>111.1 ± 3.9*</td>
<td>108.4 ± 4.1*</td>
<td>100.7 ± 3.5*</td>
<td>91.1 ± 2.5*</td>
</tr>
<tr>
<td>+dP/dt_{max}, mmHg/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.697 ± 0.78</td>
<td>1.820 ± 1.33</td>
<td>510 ± 85</td>
<td>522 ± 80</td>
<td>475 ± 76</td>
<td>432 ± 66</td>
</tr>
<tr>
<td>H</td>
<td>1.794 ± 0.98</td>
<td>1.034 ± 0.86*</td>
<td>1.357 ± 57*</td>
<td>1.385 ± 72*</td>
<td>1.322 ± 61*</td>
<td>1.178 ± 47*</td>
</tr>
<tr>
<td>−dP/dt_{max}, mmHg/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.390 ± 27</td>
<td>1.432 ± 64</td>
<td>475 ± 37</td>
<td>485 ± 32</td>
<td>475 ± 29</td>
<td>447 ± 26</td>
</tr>
<tr>
<td>H</td>
<td>1.468 ± 46</td>
<td>865 ± 23*</td>
<td>1.054 ± 40*</td>
<td>1.084 ± 37*</td>
<td>1.087 ± 72*</td>
<td>1.006 ± 32*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>191.3 ± 7.0</td>
<td>196.3 ± 8.1</td>
<td>184.8 ± 7.4</td>
<td>182.7 ± 7.4</td>
<td>189.2 ± 13.9</td>
<td>188.0 ± 14.6</td>
</tr>
<tr>
<td>H</td>
<td>189.5 ± 9.9</td>
<td>127.3 ± 6.5*</td>
<td>158.7 ± 10.0</td>
<td>167.5 ± 9.9</td>
<td>177.5 ± 9.1</td>
<td>177.0 ± 9.2</td>
</tr>
<tr>
<td>PRP, 10³ mmHg/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>22.50 ± 0.69</td>
<td>22.63 ± 1.03</td>
<td>5.81 ± 1.01</td>
<td>5.66 ± 0.88</td>
<td>5.64 ± 0.83</td>
<td>5.33 ± 0.77</td>
</tr>
<tr>
<td>H</td>
<td>23.34 ± 0.84</td>
<td>14.06 ± 0.51*</td>
<td>17.56 ± 1.03*</td>
<td>18.07 ± 4.05*</td>
<td>17.80 ± 7.8*</td>
<td>16.07 ± 0.74*</td>
</tr>
<tr>
<td>CF, ml·min⁻¹·g⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8.37 ± 0.42</td>
<td>6.62 ± 0.51</td>
<td>4.47 ± 0.19</td>
<td>3.80 ± 0.17</td>
<td>3.44 ± 0.23</td>
<td>3.25 ± 0.29</td>
</tr>
<tr>
<td>H</td>
<td>7.88 ± 0.22</td>
<td>6.24 ± 0.49</td>
<td>7.34 ± 0.40*</td>
<td>7.50 ± 0.34*</td>
<td>7.50 ± 0.38*</td>
<td>7.13 ± 0.63*</td>
</tr>
<tr>
<td>MV_{O₂}, μmol·min⁻¹·g⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4.74 ± 0.35</td>
<td>4.72 ± 0.22</td>
<td>1.48 ± 0.31</td>
<td>1.47 ± 0.32</td>
<td>1.36 ± 0.32</td>
<td>1.26 ± 0.33</td>
</tr>
<tr>
<td>H</td>
<td>4.59 ± 0.36</td>
<td>2.17 ± 0.19*</td>
<td>3.58 ± 0.38*</td>
<td>4.06 ± 0.46*</td>
<td>4.31 ± 0.38*</td>
<td>4.04 ± 0.25*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Hemodynamic indexes were determined in isolated, reperfused hearts at baseline and at 90, 120, 150, and 180 min of reperfusion (Rep) as described in MATERIALS AND METHODS. I, ischemia at 34°C (n = 6); H, ischemia and hypothermia (30°C; n = 6); CF, coronary flow; DP, developed pressure; ±dP/dt_{max}, maximum of positive or negative first derivative of left ventricular pressure; EDP, end-diastolic pressure; HR, heart rate; MV_{O₂}, myocardial oxygen consumption; PRP, pressure-rate product (HR × DP). *P < 0.05 compared with I.

groups. When significant differences were obtained with Scheffe’s F-test, individual group means were tested for differences with F values (2-tailed test). The criterion for significance was P < 0.05 for all comparisons (26, 27).

RESULTS

Functional Recovery During Reperfusion

Heart weight and left ventricular balloon volume were similar between the I group (7.68 ± 0.43 g and 1.66 ± 0.05 ml, respectively), and the H group (7.45 ± 0.36 g and 1.59 ± 0.05 ml, respectively). Under baseline conditions, there were no significant differences among the groups in EDP, DP, maximum positive and negative dP/dt, HR, PRP, CF, and MV_{O₂}. Hemodynamic results are summarized in Table 1 and Fig. 2. The data demonstrate that H hearts had superior functional recovery compared with I hearts (P < 0.05).

CF continuously decreases in I hearts during reperfusion. The H group showed a pattern in CF different from the I group (Fig. 3). In the first minutes of reperfusion, CF increased in H but not in I hearts. This finding suggests that in the isolated isovolumic model ischemia at 34°C may impair coronary regulation during reperfusion and 30°C hypothermia can limit this impairment.

HR recovery was complete and stable at the end of reperfusion in both groups, despite significant differences in contractility (Table 1).

Ischemic Contracture

We used a specific balloon volume that was initially adjusted and then maintained throughout the protocol (26, 27). This method allowed comparisons of LVDP under isovolumic conditions that raise EDP in 34°C hearts up to 10 times higher than observed in 30°C hearts that showed functional recovery, including LVEDP similar to baseline.

In this study, after the cardioplegic solution was injected, LVP was always near 0 mmHg by relaxation of the myocardium. We defined the beginning of ischemic contracture by the initial rise in LVP above 2 mmHg. There was no significant difference in LVEDP between I and H groups at 30 min of ischemia. A significant contracture in the I group beginning at 65.8 ± 4.7 min of ischemia was followed by increasing LVEDP until it reached a plateau at 90 min of ischemia. No ischemic contracture was observed after 120 min of ischemia in the H group. At the end of ischemia LVEDP was 52.3 ± 3.3 and 1.8 ± 0.9 mmHg in I and H groups, respectively. This implies that severe myocardial injury occurs near 60 min of
ischemia. Moderate hypothermia treatment can prevent the injury at least within 120 min of ischemia.

In the 34°C ischemia groups, after 15 min of reperfusion there was no significant difference between LVEDP in the deflated balloon group (57 ± 18 mmHg; n = 8) and the group with the filled balloon (52 ± 5 mmHg; n = 16). This result indicates that the isovolumic protocol did not induce further increase in LVEDP during reperfusion in our study.

**Metabolic Rate and Aerobic Function**

During ischemia myocardial metabolism mainly depends on anaerobic glycolysis. An obvious increase in accumulation of the anaerobic metabolite lactate was observed in the I group at 120 min of ischemia. No significant accumulation of lactate was found in the H group (Fig. 4). During reperfusion, the H group showed much higher MV˙O2 (Table 1) that was concomitant with CO₂ production (Fig. 5).

**Histological Integrity**

The hearts with 2 h of 34°C isovolumic ischemia followed by 3 h of reperfusion showed a thin myocardial bundle separated by edemic fluid, suggesting hydropic swelling and damage in the myocardium (Fig. 6). In addition, collagen was disrupted by infiltrate. In the normal control heart tissues, the myocardial bundles were associated closely together and the collagen existed in the connective tissue. Compared with the I group, the H group showed less swelling and very little damage in the myocardium and less collagen disruption in the connective tissue. The semiquantified injury score was 4–5 in the 34°C ischemia hearts, 0–1 in the 30°C hypothermia hearts, and 0 in the normal controls.

**Inactivation of p53 Pathway**

Immunocytochemical examination showed that p53 was heavily expressed in 34°C ischemia heart tissue cells followed by perfusion. However, very little or no p53 expression was observed in normal control and 30°C-treated hearts (Fig. 7). The semiquantitative histological scores were 4–5 for the I group, 0–1 for the H group, and 0 for the N group. These data indicate that the proapoptotic protein p53 is upregulated by ischemia and reperfusion, and that this response is abrogated by hypothermia at 30°C.

**Protein and mRNA Analyses**

RT-PCR analysis of SDH-α mRNA showed a 2 ± 0.1-fold increase in the I group compared with the N group, and 30°C hypothermia diminished the increase (Fig. 8, top left). Immuno blotting analysis indicated that expression of SDH-α was not significantly different between 34°C and 30°C ischemia (Fig. 8, middle). RT-PCR analysis of HIF-1α mRNA showed a 7 ± 0.15-fold increase in the H group compared with the N group.
but no significant difference between I and N groups (Fig. 8, top center). However, expression of HIF-1α protein was dramatically reduced in the I group (P < 0.05 vs. N), although preserved in the H group (P > 0.05 vs. N) (Fig. 8, bottom).

Target proteins for HIF-1α showed variable response to ischemia and hypothermia. HO-1 was unaffected by ischemia, but hypothermia prompted an increase in expression for this protein (Fig. 8). Similarly, PGC-1α, a prominent transcriptional coactivator regulating metabolism and mitochondrial proteins, showed decreases in both I and H hearts. In contrast, VEGF expression was substantially decreased in I hearts, with H hearts showing no significant moderation of this effect (Fig. 9). Hypothermia also moderated ischemia-induced drops in expression of two important proteins involved in cell survival, PPARβ and Akt-1 (Fig. 9).

**DISCUSSION**

We have previously shown (27, 28) that hypothermia preserves myocardial function after a brief period of reperfusion. Cooling to the 30°C range before ischemia enhances metabolic downregulation and reinforces hypothermic protection applied during ischemia (29). In this study, we reinforce prior findings showing that this temperature prevents contracture and limits otherwise extraordinary increases in EDP in this isovolumic model. The modulation in EDP by hypothermia may play an important role in myocardial protection, although the precise mechanisms through which hypothermia reduces myocardial stiffness during ischemia and reperfusion in this model still require definition. Myocardial mechanical stretch elicited in Langendorff-perfused hearts by even modest elevations in EDP can activate induction of several molecular signaling pathways (5). These include stretch-induced heat shock factor activation and increases in heat shock protein 72. Furthermore, mechanical stretch directly alters conductivity of various Ca2+ or Na+ channels, which might trigger or modulate a number of stress responses (3, 6, 16, 32, 36). The presence of preload with the intraventricular balloon exacerbates stretch during warmer ischemia and exaggerates molecular responses. Thus some of the responses noted in our experiments may be specific to the isovolemic Langendorff model with maintained preload during ischemia. However, our data comparing hearts with a deflated balloon to those with LVEDP generated by an inflated balloon during ischemia show that LVEDP, at least during reperfusion, is not modified by the maintained preload volume. Furthermore, neither contracture nor elevation in LVEDP occurs in the hearts receiving moderate hypothermia despite balloon inflation during ischemia, suggesting that myocardial stretch caused by this volume plays a less important role in influencing the findings in this experimental model.

**Hypothermia Preserves Structural Integrity**

The present study shows that global ischemia produces a sustained increase in LVEDP and substantial loss of collagen and structural integrity within a few hours, despite mild hypothermia (34°C) and cardioplegia. Hypothermic adaptation preserves cardiac function over this time frame, in part by preventing rapid interstitial collagen fragmentation and loss of connective tissue framework after reperfusion. Myocardial collagen disruption has recently been noted in a more chronic model of heart failure and has been linked to elevated matrix metalloproteinase (MMP)9 activity (12). Hamann et al. (14) demonstrated that hypothermia prevents microvascular basal lamina collagen loss in brain after focal cerebral ischemia. Hypothermia also reduced MMP9 activity in their model, but it remains unclear whether the effect on this enzyme resulted from a general decrease in ischemic damage or from direct inhibition. Although not a direct focus of the present study, hypothermic modulation of MMP concentrations and activities does represent a promising area for future research.

**Hypothermia Preserves Metabolic Pathways**

We have previously shown (29) that hypothermia applied only before warm ischemia preserves postischemic mitocho-
drial and metabolic function. Similarly, the hearts exposed to hypothermia both before and during ischemia in the present study demonstrate full preservation of oxidative capacity, as indexed by MVO₂ after 3 h of reperfusion. A hypothermia-induced reduction in metabolic demand during ischemia is confirmed in the present experiments by decreased lactate production. This metabolic downregulation is at least partially responsible for maintained postischemic mitochondrial function and relative preservation of ATP, previously noted in this rabbit model (28, 29). The molecular basis for this preservation remains unclear, although we previously noted that decreases in mRNA levels for constitutive mitochondrial proteins such as adenine nucleotide translocator and β-F₁-ATPase were diminished by hypothermic adaptation (26, 29, 30). Surprisingly, in previous molecular array studies, ischemic increases in mRNA for a critical component of respiratory complex II, SDH-α, were also inhibited by hypothermia (27). In the present study, we confirmed by PCR that SDH-α mRNA responds to ischemic stress. This mRNA may therefore respond to drops in ATP levels, which were shown previously to occur during mild hypothermia (34°C) in this model (29). Accordingly, the colder temperature range, which yields a much smaller decrease in ATP, does not produce mRNA accumulation, although protein expression is preserved. These data imply that oxidative stress increases transcription in order to maintain the level of the SDH-α protein. However, the results indicate that this protein is also subject to posttranscriptional regulation, possibly including stabilization of this protein by hypothermia, which in turn may reduce mRNA expression through autofeedback mechanisms.

Hypothermia Modulates HIF-1α

We recently showed (25) that mild or subthreshold doses of short-cycle hypoxia, which do not impair cardiac function, trigger the HIF-1α cascade in rabbit heart. The mild hypoxia (reduction in perfusate oxygen concentration) rapidly stimulates accumulation for this protein in coordination with an increase in the respective mRNA, implying that transcriptional mechanisms are involved in these actions. Most studies show that severe hypoxia suppresses activity of the prolyl hydroxylase domain-containing proteins, responsible for HIF-1α degradation (4, 22). In contrast, in our experiments using short-cycle hypoxia, severe hypoxia reduced HIF-1α protein, suggesting that inhibition of translation had occurred and not enhanced protein degradation. The severe and prolonged ischemia protocol without hypothermic adaptation, similar to that performed in the present study, diminishes HIF-1α protein with minimal influence on the mRNA (27). These results from the present and previous studies suggest that HIF-1α protein is modulated by moderate hypothermia via both transcriptional and posttranscriptional mechanisms. We previously showed (27) that hypothermic adaptation rapidly produces a robust HIF-1α mRNA response (7-fold) during reperfusion. The present study shows that preservation of the protein occurs subsequent to the mRNA response. These data imply that hypothermia enhances synthesis of HIF-1α protein through transcriptional regulation and enhanced protein translation, although stabilization of this protein cannot be totally eliminated as a cause for these changes. These findings are consistent with data showing that nonhypoxic induction of HIF-1α occurs through increases in translation of the protein, by activation of phosphatidylinositol 3-kinase pathway and its downstream effectors, mammalian target of rapamycin (mTOR) and p70S6 kinase (9).
To our knowledge, hypothermia-induced accumulation of HIF-1α protein has not been previously reported in a mammalian heart, although cold temperature has been noted to activate HIF-1α protein and DNA binding capacity in the hearts of poikilothermic vertebrates (38). Morin and Storey (23) have also reported elevated HIF-1α protein levels in skeletal muscle of hibernating ground squirrels after induction of hibernation at 6°C. Thus the metabolic downregulation accompanied by increases in HIF-1α, which occur during hypothermic adaptation, closely resemble the changes noted in hibernating animals. The effects of the increase in HIF-1α protein on downstream targets appear to vary according to intensity and type of stimulus. For instance, VEGF protein rapidly accumulates along with increasing HIF-1α in rabbit heart exposed to short-cycle hypoxia (25). However, VEGF did not change in concordance with HIF-1α in these ischemia-reperfusion experiments. In contrast, we noted a significant increase in HO-1 during the protocol-induced elevations in HIF-1α caused by the present protocol. These findings support previous studies performed primarily in vitro (37), which show that transcriptional activation by HIF-1α varies from hypoxic to nonhypoxic stimuli, and may further vary by gene.

**Hypothermia Alters Signaling for Apoptosis**

The transformation-related p53 protein is linked to morphological changes and genomic DNA fragmentation characteristic of apoptosis in cardiomyocytes (18). This transcriptional factor activates a number of target genes including Gadd45, p21, and caspase-3, which all play a role in apoptosis (11, 27, 33, 41). We have previously shown that mRNA for transformation-related p53 protein is barely detectable in normal rabbit heart. However, ischemia produces a robust increase in mRNA for this protein and various target genes, which is abrogated by hypothermic adaptation. The present study shows that increased expression at the transcriptional level is followed within 3 h by appearance of this protein throughout the myocardium. Although we did not evaluate further for apoptosis in these experiments, others have confirmed a reduced number of TUNEL-positive cells after infarction in heterozygous p53-deficient mice (21). The similar lack of expression of p53 after hypothermic adaptation in the ischemia hearts suggests that modification of this pathway represents at least one mode for the hypothermic therapeutic effect. This contention is further supported by studies in various cultured cells, which show that
hypothermia (32°C) prevents cell death through p53-mediated pathways. These actions occur in part through increased activation of Mdm2 (39), an ubiquitin ligase, which promotes nuclear export and targets p53 for degradation by the ubiquitin-proteasome system. Mdm2 also binds to the p53 transactivation domain and thereby inhibits p53-mediated transactivation.

PPARβ protects keratinocytes and renal tubular epithelial cells (17) from apoptosis by activating the Akt-1/PKB pathway through transcriptional upregulation of integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase (PDK1) and concomitant repression of phosphatase and tensin homolog (PTEN) tumor suppressor gene (10). The role of Akt-1 in promotion of cardiomyocyte survival is well established (10, 19, 20). Akt-1 regulates both the death receptor and mitochondrial apoptosis cascades in addition to mediating insulin-induced translocation of glucose transporter 4 to the cell surface. Upstream transcriptional activation of Akt-1/PKB by PPARβ has not been examined as a protective mechanism against ischemia in cardiomyocytes. The present data suggest that hypothermia preserves PPARβ protein and the downstream target Akt-1 in heart. Similar to kidney, enhanced expression of these proteins after ischemic insult occurs in association with preserved extracellular matrix integrity, thereby suggesting an important role for these proteins in hypothermic adaptation in heart.

In this study, we used 34°C in the control group in order to elucidate protective pathways that might be activated by colder temperatures, as well as to define contracture time. This high temperature is not used clinically during ischemia, and therefore responses, particularly the elevation in LVEDP, are exaggerated. The physiological responses in the postischemic period for the groups exposed to 30°C are consistent with findings from studies performed in situ (7, 34). These include our own previous studies (34) in juvenile pigs, which emulate the Langendorff preparation by supporting coronary and systemic flow with extracorporeal circulation. In those experiments a balloon was inserted directly into the left ventricle, inflated to define the left ventricular volume-developed pressure relationship, and then deflated during profound hypothermic (20°C) ischemia and reinfated to similar preischemic volumes to define this volume-pressure curve after reperfusion (34). Those experiments yielded reperfusion LVEDP similar to that reported in other studies performed in pigs exposed to hypothermia at 28°C and then weaned totally from extracorporeal circulation (7). Because elevation in LVEDP occurred regardless of balloon inflation state in the present study, we showed that the balloon volume during ischemia does not induce the high LVEDP. However, because we could not totally separate the elevated LVEDP from the high ischemic temperature, we did not remove the possibility that the exaggerated LVEDP plays a role in modulating the molecular pathways highlighted during these experiments. Evaluation of the relationship between LVEDP and these processes will be a subject for further experimentation.

In summary, these studies in isovolumic hearts show that hypothermia at 30°C preserves cardiac structural integrity and function for several hours after ischemic insult, while 34°C yields an extraordinarily high EDP with structural disruption. Hypothermia induces metabolic downregulation and modulates mRNA expression, followed by corresponding changes in expression for their respective proteins. Hypothermia appears to cause adaptation by regulating these proteins involved in multiple signaling and physiological functions, affecting structural integrity.

GRANTS

This work was funded in part by National Heart, Lung, and Blood Institute Grant R01HL-60666-1 and a grant from Children’s Hospital and Regional Medical Center Research Fund (HR-5836).

REFERENCES

30°C HYPOTERMIA PROTECTION IN HEART


