Degree and Time Sequence of Hypothermic Protection Against Experimental Ischemic Acute Renal Failure

Richard A. Zager, Dennis J. Gmur, Charles R. Bredl, and Mary J. Eng

The purpose of this study was to assess the degree, time sequence, and biochemical correlates of hypothermic protection against ischemic acute renal failure. Rats subjected to 40 minutes of bilateral renal artery occlusion (RAO) were made mildly hypothermic (32°–33° C, by cold saline peritoneal lavage) during the following time periods: 1) RAO only, 2) reperfusion only (beginning at 0, 15, 30, or 60 minutes after RAO and maintained for 45 minutes), or 3) during and after (0–45 minutes) RAO. Continuously normothermic (37° C) RAO rats served as controls. The control rats developed severe acute renal failure (blood urea nitrogen [BUN], 95±4 mg/dl; creatinine, 2.2±0.1 mg/dl; and extensive tubular necrosis at 24 hours). Hypothermia confined to RAO was highly protective (BUN, 33±5 mg/dl; creatinine, 0.62±0.07 mg/dl; and minimal necrosis). Hypothermia partially preserved ischemic renal adenylate high-energy phosphate (ATP and ADP), increased AMP and inosine monophosphate concentrations, and lessened hypoxanthine/xanthine buildup (assessed at end of RAO). Hypothermia confined to the reflow period (beginning at 0, 15, and 30 minutes) was only mildly protective (e.g., BUN, 58–63 mg/dl); the degree of protection did not differ according to the time of hypothermic onset. Lowering reflow temperature to 26° C had no added benefit. Hypothermia that started at 60 minutes after RAO conferred no protection. Combining ischemic and postischemic hypothermia abolished all renal failure (assessed at 24 hours). This study offers the following conclusions: Mild hypothermia can totally prevent experimental ischemic acute renal failure. Hypothermia is highly effective during ischemia, and it is mildly protective during early reflow; these benefits are additive. During early reflow, hypothermic protection is not critically time dependent. By 60 minutes of reflow, no effect is elicited; this absence of effect possibly signals completion of the reperfusion injury process. Hypothermia’s protective effects may be mediated, in part, by improvements in renal adenine nucleotide content and, possibly, by decreasing postischemic oxidant stress. (Circulation Research 1989;65:1263–1269)

It is generally accepted that ischemic renal injury occurs in two stages: injury induced during the period of blood flow interruption and injury that occurs during the vascular reperfusion period. The first phase of injury is believed to result from O2 deprivation–induced ATP depletion, which leads to disturbed intracellular electrolyte composition and cell membrane function.1 These changes then help set the stage for further injury during vascular reperfusion. For example, high intracellular hypoxanthine concentrations, a result of ischemic ATP breakdown, may trigger oxygen free radical generation with reoxygenation.2–4 Reperfusion-induced Ca2+ delivery to energy-depleted cells produces cellular Ca2+ overload, which causes postischemic injury through multiple pathways (e.g., mitochondrial dysfunction, increased oxygen free radical formation, and phospholipase activation).5 Many of these processes appear to be self-sustaining and mutually supportive. Thus, injury that was initiated by ischemia can, for a limited period of time, be ongoing and self-perpetuating during the vascular reperfusion period.

The length of the reperfusion phase of injury has important therapeutic implications since it determines the period of time in which treatment may be able to salvage sublethally injured cells and, thereby, prevents or mitigates ischemic acute renal failure. However, the length of this period has not been well

From the Department of Medicine, University of Washington and the Fred Hutchinson Cancer Research Center, Seattle, Washington.

Supported by Grant DK-38432 from the National Institutes of Health and a grant from the Fred Hutchinson Cancer Research Center.

Address for correspondence: Richard A. Zager, MD, Division of Nephrology ZA-67, Harborview Medical Center, 325 Ninth Ave., Seattle, WA 98104.

Received December 1, 1988; accepted June 2, 1989.
defined in vivo because it is impossible to identify precisely in the intact organ when tubular cells have been irreversibly damaged. Although irreversible injury may be easier to determine in in vitro cell systems, these results may not be applicable to whole-organ damage. An indirect approach to defining reversibility in vivo is to delineate when therapeutic maneuvers (e.g., Ca\(^{2+}\) channel blockers, oxygen free radical scavengers, and ATP infusion) lose their protective effects. However, the problem with this approach is that such interventions are primarily directed against just one aspect of reperfusion injury; thus, these treatments provide, at best, incomplete protection.

The most potent protective maneuver yet defined against ischemic renal injury is hypothermia. Gowland and Dexter\(^8\) demonstrated that ice water immersion (lowering body temperature to 22\(^\circ\) C) during 60 minutes of renal artery occlusion (RAO) in the rat almost totally prevented tubular necrosis. Finn\(^7\) showed that hypothermia to 27\(^\circ\) C conferred functional protection against 60 minutes of RAO, as assessed by inulin clearances in the early reflow period. Our laboratory previously reported that continuous hypothermia (32\(^\circ\) C during and after ischemia), produced by external cooling, conferred nearly total functional and morphological protection against 25 minutes of RAO.\(^8\) The striking protection reported in these studies is probably due to the fact that hypothermia, unlike other therapeutic interventions, affects multiple pathways of ischemic/reperfusion injury. For example, hypothermia during ischemia conserves renal high-energy phosphates (e.g., ATP, ADP, GTP, and UTP) as well as nicotinamide adenine dinucleotide concentrations.\(^8\)

Hypothermia preserves postischemic renal glutathione content, possibly by decreasing oxygen free radical–triggered oxidant stress.\(^8\) Since biochemical reactions are highly temperature dependent (e.g., see References 9–15), it seems quite likely that most, if not all, ischemia-triggered degradative reactions can be mitigated by lowering kidney temperature.

Given its profound protective effects against ischemic acute renal failure, the present study was undertaken to address the following issues: 1) When in the ischemic/reperfusion injury process does hypothermia exert its greatest protective action? 2) Does hypothermia, imposed during ischemia, condition the kidney for less reperfusion injury (e.g., by preserving ATP just before reperfusion and by lowering hypoxanthine concentrations, which thereby decreases posts ischemic oxidant stress)? 3) At what point in time after renal ischemia does hypothermia no longer confer protection? The answer to this question may indirectly help to delineate the completion of the most critical phase of the reperfusion injury process. 4) If hypothermia induces protection both during and after a renal ischemic event, are these benefits additive? To answer these questions, we have chosen a degree of hypothermia (32\(^\circ\)–33\(^\circ\) C) that is often observed in patients so that the findings might have potential clinical relevance.

Materials and Methods

General Procedures

Female Sprague Dawley rats (175–200 g; Bantin Kingman, Fremont, California) maintained under standard laboratory conditions were used for all experiments. During surgery, body temperature was continuously monitored with a digital display rectal thermometer; the temperature was scrupulously maintained at 37\(^\circ\)–37.5\(^\circ\) C (normothermia), except during specific hypothermic (32\(^\circ\)–33\(^\circ\) C) periods denoted below. Normothermia was maintained by using a heated surgical table and an adjustable external lamp source.

Under pentobarbital anesthesia (30–40 mg/kg), ischemic renal injury was induced by 40 minutes of bilateral RAO conducted through a midline abdominal incision. After completion of the specific protocols described below, the abdominal incision was sutured, and the rats were allowed to recover from anesthesia with free access to food and water. The severity of ischemic injury was assessed 24 hours after RAO by measuring terminal blood urea nitrogen (BUN) and plasma creatinine (Cr) concentrations. In addition, the kidneys were removed and saved for subsequent histological evaluation. Frontal kidney sections were fixed by immersion in 10% buffered formaldehyde; paraffin-embedded sections (4 \(\mu\)m) were stained with hematoxylin and eosin.

Specific Hypothermic Protocols

Hypothermia during ischemia; normothermic reperfusion. Immediately on placement of the renal artery clamps, 10 normothermic rats had both kidneys and the abdominal cavity flushed with 10–15 ml ice-cold normal saline, which lowered body temperature to the hypothermic range (32\(^\circ\)–33\(^\circ\) C) within 1–2 minutes. Once this temperature was achieved, the cold saline was allowed to exit through the abdominal incision. Hypothermia was maintained for the next 37 minutes by turning down or turning off the heating sources. During the 3 minutes just before cessation of RAO, the temperature was returned to 37\(^\circ\)–37.5\(^\circ\) C by reapplying the heating sources. After vascular clamp release, the abdomen was sutured, and normothermic conditions were maintained for an additional hour. Then the rats were allowed to recover from anesthesia without further manipulation. Seven rats treated in an identical fashion except that their abdomens were flushed with 37\(^\circ\) C saline at the time of vascular clamping and maintained at normothermic conditions throughout the protocol served as controls. Renal injury was assessed 24 hours later as described above.

Normothermic ischemia; immediate hypothermic reperfusion. Seven rats maintained under normo-
Hypothermic conditions underwent RAO. Immediately before vascular-clamp release, the kidneys and abdominal cavity were flushed with iced saline, which lowered body temperature to 32°–33° C as noted above. Then the vascular clamps were removed, and hypothermia was maintained in the first 45 minutes of reperfusion. Subsequently, the body temperature was returned to normothermia and maintained there for an additional hour. Then the rats were allowed to recover from anesthesia.

Seven rats treated in an identical fashion except for being flushed with 37° C saline and maintained under normothermic conditions throughout the protocol served as controls.

Normothermic ischemia; hypothermia beginning 15 minutes after cessation of RAO. The above hypothermic protocol was conducted in nine rats except that the onset of the 45-minute hypothermic period was not initiated until 15 minutes after vascular-clamp release. Six normothermic rats flushed with 37° C saline served as controls.

Normothermic ischemia: hypothermia beginning 30 minutes after cessation of RAO. Six normothermic rats underwent RAO. At 30 minutes after RAO, the 45 minutes of hypothermia was induced, followed by a return to normothermic conditions, as previously noted. Seven normothermic rats flushed with 37° C saline at 30 minutes after RAO served as controls.

Normothermic ischemia: hypothermia beginning 1 hour after cessation of RAO. Six normothermic rats underwent RAO. One hour after cessation of RAO, a 45-minute hypothermic period was induced as noted above, followed by a return to normothermia. Six normothermic RAO rats flushed with 37° C saline served as controls.

Hypothermia maintained both during and for the first 45 minutes of reperfusion. To assess the maximal degree of protection afforded by 32°–33° C of hypothermia, seven rats underwent RAO, and immediately on vascular-clamp placement, hypothermia was induced as previously noted. Hypothermia was maintained throughout the ischemic period and for the first 45 minutes of reperfusion. Body temperature was then returned to normothermic conditions, and the rats were allowed to recover from anesthesia. The severity of renal injury, assessed 24 hours later, was contrasted with that observed after the above-described hypothermic protocols.

Severe hypothermia (26° C) confined to the reperfusion period. To assess whether the degree of hypothermic protection during the reperfusion period could be enhanced by lowering body temperature below 32°–33° C, six rats underwent RAO, and at the time of vascular-clamp release, the body temperature was abruptly lowered to 26° C using iced saline lavage. Temperature was maintained at this level for the first 45 minutes of reperfusion, which was followed by a return to normothermia over 15–20 minutes. The remainder of the experiments were as previously described.

Renal Adenine Nucleotide Degradative Products Assessment

We previously determined that hypothermia conserves renal adenine nucleotides after 10 minutes of ischemia. The following experiment was performed to assess whether this is merely a transient effect or one that can affect the kidney throughout a prolonged ischemic period. If so, then such changes might influence both ischemic and reperfusion injury with the latter effect possibly mediated by increased renal ATP and decreased hypoxanthine (HPX) concentrations at the beginning of the reperfusion period. Eight rats were subjected to 40 minutes of bilateral renal pedicle ligation; half the rats were subjected to 32°–33° C hypothermia, and the other half served as normothermic controls as detailed above. After 40 minutes of ischemia, all 16 kidneys were immediately freeze-clamped in vivo at liquid nitrogen temperature without allowing reflow. The outer half of each flattened kidney (corresponding to cortex/outer medulla) was extracted for nucleotides, nucleosides, and degradative products as previously described and assayed by high-performance liquid chromatography (HPLC). Six nonischemic (normal) kidneys were also freeze-clamped in vivo and assayed to provide normal values.

HPLC grade methanol (JT Baker, Phillipsburg, New Jersey), deionized water (Milli Q purification system, Continental Water Systems, El Paso, Texas), low absorbance grade potassium phosphate (E Merck, Cherry Hill, New Jersey), and ATP, ADP, AMP, adenosine, inosine, inosine monophosphate, HPX, and xanthine standards (Sigma Chemical, St. Louis, Missouri) were used. Nucleotides were assayed on an HPLC system consisting of an automated gradient controller, model 510 and M6500A pumps (Waters Chromatography, Milford, Massachusetts), an automated switching valve, model 440 fixed wavelength (254 nm) detector, and a model 745 data module (Valco Instruments, Houston, Texas). The HPLC was fitted with a 10-μm Partisil SAX 25-cm column (Whatman, Clifton, New Jersey) for nucleotide assay. Nucleosides, HPX, and xanthine were assayed with an HPLC system consisting of a model 5060 chromatograph (Varian, Walnut Creek, California), a model LC-9522 fixed wavelength (254 nm) detector, and a model CDS-401 integrator (IBM, Danbury, Connecticut). The system used a Spherisorb 5-μm ODS-2 25-cm column (Phase Separations, Norwalk, Connecticut).

Nucleotides were assayed by the method of Geisbuhler et al. Injection of standards showed that nucleosides, HPX, and xanthine were nearly nonretained on the SAX column (retention times of <3.4 minutes). Thus, a fraction from 1–3.4 minutes from the nucleotide assay was collected into a preweighed tube. Injection of standards showed 100% recovery of HPX, xanthine, inosine, and...
adenosine in this volume. The nucleoside fraction from the nucleotide assay was used to quantitate HPX, xanthine, inosine, and adenosine by the Spherisorb system (Varian) according to the method of Morii et al. In brief, 500 µl of this fraction was injected onto the Spherisorb column, and the compounds of interest were eluted with a linear gradient (from 100% solvent A: 20 mM KH₂PO₄, pH 5.6; to 90% solvent B: 20/80 methanol/water, vol/vol).

Concentrations of the above assays were calculated as micromoles per gram tissue wet weight and per gram tissue dry weight based on peak areas. The data are given per gram wet weight since no change of tissue water occurs under the experimental conditions used.

Calculations and statistics. Protection resulting from the individual hypothermic protocols was assessed by comparing each hypothermic group of rats with its own normothermic controls. BUN, Cr, and biochemical data were contrasted by unpaired Student’s t test. If multiple groups were compared, the Bonferroni correction was applied. Histological comparisons were made by the Wilcoxon rank-sum test. Six or seven kidneys from each hypothermic group and from six to seven kidneys from each normothermic control group were randomly selected and processed for histology as noted previously.

Results

Responses to 40 Minutes of RAO Under Normothermic Conditions (Controls)

There were no significant differences in the severity of azotemia or histological injury among the individual control groups (see Table 1). The mean BUN, Cr, and histological scores for the five control groups, when combined, were 95±4 mg/dl, 2.2±0.12 mg/dl, and 4.4±0.14, respectively. These rats demonstrated typical histological changes of severe RAO-induced ischemic acute renal failure. Extensive tubular necrosis was apparent, particularly in the outer medullary stripe with confluent areas of necrosis often being observed. Cortical foci of tubular necrosis were also seen, but they were much more sporadic in distribution in comparison with the outer medullary stripe. Extensive cast formation was noted, particularly in the outer and inner medullary stripes and inner medulla. Inner medullary vascular congestion was also observed.

Hypothermic Ischemia/Normothermic Reparfusion

Dramatic functional and morphological protection was conferred by hypothermia confined to the ischemic period (Table 1, group 1); the BUN and Cr only

### Table 1. Degrees of Azotemia and Tubular Necrosis 24 Hours After 40-Minute Renal Artery Occlusion

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BUN (mg/dl)</th>
<th>Cr (mg/dl)</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H (during ischemia)</td>
<td>10</td>
<td>33±5</td>
<td>0.62±0.07</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>7</td>
<td>99±8</td>
<td>2.46±0.20</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>H (immediate reflow)</td>
<td>7</td>
<td>58±5</td>
<td>1.29±0.12</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>7</td>
<td>93±7</td>
<td>2.11±0.23</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>H (at 15-min reflow)</td>
<td>9</td>
<td>60±8</td>
<td>1.17±0.16</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>6</td>
<td>99±12</td>
<td>2.25±0.40</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>4</td>
<td>H (at 30-min reflow)</td>
<td>6</td>
<td>63±9</td>
<td>1.26±0.17</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>7</td>
<td>99±10</td>
<td>2.20±0.33</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>5</td>
<td>H (at 60-min reflow)</td>
<td>6</td>
<td>102±7</td>
<td>2.35±0.25</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>6</td>
<td>85±8</td>
<td>1.90±0.25</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>H (ischemia+0–45-min reflow)</td>
<td>7</td>
<td>19±1</td>
<td>0.53±0.04</td>
</tr>
<tr>
<td>7</td>
<td>26°C H (reflow)</td>
<td>6</td>
<td>87±8</td>
<td>1.63±0.17</td>
</tr>
</tbody>
</table>

BUN, blood urea nitrogen; Cr, plasma creatinine; H, hypothermia; NS, not significant; Group 1, H confined to ischemic period; group 2, H between 0–45 minutes of reflow; group 3, H between 15–60 minutes of reflow; group 4, H between 30–75 minutes of reflow; group 5, H between 60–105 minutes of reflow; group 6, H during renal artery occlusion plus 0–45 minutes of reflow; group 7, unlike all other groups subjected to H (32–33°C), these rats underwent 26°C hypothermia (from 0–45 minutes of reflow). BUN and Cr data within each group of experiments were compared by unpaired Student’s t test. The histological scores were compared by the Wilcoxon rank-sum test. BUN and Cr concentrations were within normal laboratory limits for group 6; the BUN concentrations and the histological scores (but not the Cr concentrations) for these rats were significantly lower than those observed in the group 1 H rats (p<0.05). In group 7, no protection was noted by lowering reperfusion temperature to 26°C during reflow in comparison with that observed in the hypothermic (32°C–33°C) experiments (groups 2–4).

*Although the individual histological comparisons within groups 2, 3, and 4 did not achieve statistical significance, analyzing all 3 groups together (H rats vs. the normothermic controls) confirmed statistically less tubular necrosis in rats treated with H (p<0.005).

rose to 33±5 and 0.62±0.07 mg/dl, respectively. (For sake of comparison, normal BUN and Cr values as previously established in this laboratory are 17±1 and 0.54±0.01 mg/dl, respectively; e.g., see References 19 and 20). Morphological injury was very mild, consisting of occasional foci of tubular necrosis confined to the outer medullary stripe and observed in only one of approximately thirty ×100-powered fields. Minimal cast formation was apparent.
Hypothermic Ischemia/Hypothermic Reperfusion

Hypothermia induced at 0, 15, and 30 minutes of vascular reflow (Table 1, groups 2-4) induced modest functional protection, the degree of which did not statistically differ among these three groups. Each of these groups showed a modest decrease in the extent of tubular necrosis. However, in comparison with their individual control groups, the decrease in necrosis did not achieve statistical significance. Since the degree of injury for these three hypothermic groups appeared virtually identical, as assessed by BUN and Cr, their histological scores were combined and then contrasted with the combined scores for their normothermic controls. By so doing, a statistically significant decrease in the extent of tubular necrosis in the combined hypothermic group became apparent (p<0.005).

Induction of hypothermia at 60 minutes of reflow (Table 1, group 5) conferred neither functional nor morphological protection in comparison with their normothermic controls.

Hypothermic Ischemia/Hypothermic Reperfusion

This group (Table 1, group 6) demonstrated total functional protection; the BUN and Cr concentrations did not rise above normal values. In addition, virtual total histological protection was noted. Four of seven kidneys demonstrated no tubular necrosis whatsoever. The remaining three kidneys showed only three or less extremely small areas of necrosis in the entire frontal kidney sections. The mean histological score and the BUN for this group were statistically lower than the values observed in rats subjected to hypothermic ischemia/normothermic reperfusion (group 1); these values indicated greater protection.

Normothermic Ischemia/26°C Hypothermia During Reflow

Lowering body temperature to 26°C during reflow did not improve on the degree of protection, compared with that observed with 32°C-33°C reperfusion (Table 1, groups 2-4). In fact, the degree of azotemia that developed was somewhat higher (BUN, 87±8 mg/dl; Cr, 1.63±0.17 mg/dl) than that observed in the 32°C-33°C reperfusion experiments. All rats tolerated hypothermia well; none of the rats died. Mild shivering was noted with some of the 26°C experiments, whereas none was observed with 32°C-33°C hypothermia.

Renal Adenine Nucleotide/Degradative Products Assessment

The hypothermic rats had statistically significant preservation of ATP, ADP, AMP, and, hence, total adenine nucleotide concentrations (ATP+ADP+AMP) in comparison with the normothermic ischemic controls (Table 2). Adenylate high-energy phosphate (ATP×2+ADP; based on 2 high-energy phosphates for ATP and ADP, respectively) was 33% higher in the hypothermic group (p<0.05). Hypothermia also conserved inosine monophosphate but not nucleoside (adenosine/inosine) concentrations. The hypothermic preservation of nucleotides was associated with 25% and 23% reductions in HPX and xanthine concentrations, respectively. In comparison with the normal (nonischemic) kidney values, all degradative products were massively elevated (e.g., HPX was elevated approximately 200-fold), and there was extreme ATP depletion.

Discussion

In a previous study, we documented that modest hypothermia (32°C-33°C) induced by external cooling conferred substantial functional and morphological protection against 25 minutes of RAO, a relatively modest ischemic insult. The present study expands on these observations by demonstrating that this same degree of hypothermia induced by intraperitoneal iced saline lavage can provide virtual total protection against a much more severe ischemic event: that induced by 40 minutes of RAO. In normothermic controls, 40 minutes of RAO caused severe azotemia and extensive tubular necrosis, often confluent in the outer medullary stripe. However, rats rendered hypothermic both during and for 45 minutes after RAO (Table 1, group 6) developed no azotemia, and tubular necrosis was virtually absent. That such profound protection could be achieved with such modest hypothermia suggests that this degree of temperature reduction,

<table>
<thead>
<tr>
<th>Normal values</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>HEP</th>
<th>TAN</th>
<th>IMP</th>
<th>ADO</th>
<th>INO</th>
<th>HPX</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.59±</td>
<td>0.63±</td>
<td>0.15±</td>
<td>3.82±</td>
<td>2.37±</td>
<td>0.02±</td>
<td>0.003±</td>
<td>0.004±</td>
<td>0.004±</td>
<td>0.004±</td>
</tr>
<tr>
<td>(n=6)</td>
<td>0.06</td>
<td>0.07</td>
<td>0.02</td>
<td>0.19</td>
<td>0.15</td>
<td>0.004</td>
<td>0.0004</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Ischemia/37°C</td>
<td>0.086±</td>
<td>0.19±</td>
<td>0.48±</td>
<td>0.36±</td>
<td>0.75±</td>
<td>0.19±</td>
<td>0.02±</td>
<td>0.18±</td>
<td>0.88±</td>
<td>0.80±</td>
</tr>
<tr>
<td>(n=8)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.004</td>
<td>0.001</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Ischemia/32°C-33°C</td>
<td>0.12±</td>
<td>0.24±</td>
<td>0.60±</td>
<td>0.48±</td>
<td>0.96±</td>
<td>0.25±</td>
<td>0.02±</td>
<td>0.16±</td>
<td>0.66±</td>
<td>0.62±</td>
</tr>
<tr>
<td>(n=8)</td>
<td>0.005</td>
<td>0.006</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.007</td>
<td>0.001</td>
<td>0.004</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.02</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

HEP, adenylate high-energy phosphate (ATP×2+ADP); TAN, total adenine nucleotides (ATP+ADP+AMP); IMP, inosine monophosphate; ADO, adenosine; INO, inosine; HPX, hypoxanthine; X, xanthine. All values are given as mean±1 SEM µmol/g tissue wet wt. Statistics (unpaired Student's t test) compare the 37°C and 32°C-33°C ischemic groups. The normal kidney values are given for comparison.
which is relatively modest and frequently observed in practice, could affect this development of clinical ischemic acute renal failure.

A major goal of this study was to define when in the ischemic/reperfusion process hypothermia exerts its protective effect. By use of iced saline peritoneal lavage, it was possible to lower body temperature abruptly, thereby allowing specific time intervals to be tested. By use of this technique, it was discovered that by far the most effective time for inducing hypothermia is during the ischemic rather than the reperfusion period. Rats subjected to hypothermic ischemia but normothermic reperfusion demonstrated striking protection: only slight azotemia and minimal tubular necrosis resulted. Given these dramatic results, it is tempting to conclude that the bulk of tubular damage is sustained during, not after, an ischemic insult. However, such a conclusion, although probably correct, cannot be substantiated with the available data since changes induced during ischemia may condition the kidney for the reperfusion injury process. Thus, it is possible that at least some of the benefits of hypothermia, imposed during ischemia, did not completely express themselves until the reperfusion phase.

To substantiate this point, we assessed whether hypothermia confined to the ischemic phase improves renal high-energy phosphate content and lowers HPX concentrations by the end of the ischemic period. If so, this could lessen immediate/early reperfusion injury. Of note, we previously reported that hypothermia conserves renal high-energy phosphate and lowers HPX concentrations after a 10-minute ischemic interval. However, we considered it quite possible that these presumed beneficial effects observed after 10 minutes of RAO would be dissipated by a more prolonged ischemic insult; thus, high-energy phosphate and HPX levels at the start of the reperfusion period would not be affected. However, the present results indicate that this influence persists throughout 40 minutes of ischemia and theoretically lessens both ischemic as well as the earliest phase of reperfusion injury.

It remains possible that the nucleotide changes, although statistically significant, were quantitatively too small to affect the injury process. However, it should be recalled that seemingly trivial differences in ATP levels have been shown to have a dramatic impact on proximal tubular cell viability after in vitro ischemia. Thus, we feel it is likely that a small quantitative improvement in ATP, maintained throughout a prolonged ischemic episode, would help to lessen ischemic damage. The biochemical correlate of the hypothermic preservation of adenine nucleotide was a 25% lowering of intrarenal HPX accumulation. This could theoretically lessen oxidant stress during early reperfusion, particularly since xanthine oxidase activity is highly temperature dependent. However, this possibility must be considered speculative since the role of HPX/xanthine oxidase-mediated renal oxidant injury remains a highly controversial issue (e.g., see References 3, 4, 24-26). Furthermore, merely lowering HPX accumulation by 25% would not necessarily lessen oxidant damage since even with this decrement, HPX levels were still 175-fold higher than normal, more than enough to drive free radical formation. An alternative possibility is that a lowering of HPX buildup would lessen the energy drain on tubular cells during the reperfusion period by decreasing purine salvage, an energy-consuming process. However, our previous data showing that hypothermia does not influence reperfusion ATP concentrations provides no direct support for this hypothesis.

Hypothermia when applied only during reperfusion (0, 15, and 30 minutes) also conferred protection, but it was dramatically less than that achieved by cooling during the ischemic phase. Although the degree of azotemia was substantially reduced, the degree of histological protection was much less impressive. No single postischemic hypothermic group demonstrated a statistically significant decrease in tubular necrosis in contrast to their individual normothermic controls. Only by analyzing the 0-, 15-, and 30-minute reflow experiments together could a statistically significant decrease in tubular necrosis be demonstrated. This relatively modest degree of protection suggests one of two possibilities: 1) Hypothermia might only be highly potent against the ischemic, not the reperfusion, phase of injury. 2) The fate of a great many ischemically damaged cells is determined by completion of the ischemic phase; thus, hypothermia can only achieve a limited cell salvage rate when applied in the reperfusion period. The data do not permit us to differentiate between these two possibilities. However, given hypothermia’s profound and apparently broad-based protective influences, it is tempting to postulate that the latter is the case. This is particularly true since induction of even more profound hypothermia (26°C) during the reperfusion period did not improve on the degree of protection that resulted from the 32°-33°C reperfusion experiments.

Since hypothermia applied during reflow can salvage some sublethally injured cells and mitigate acute renal failure, we tested whether the degree of this salvage is critically time dependent. A priori, one would predict that the degree of hypothermic protection during this time would rapidly decrease the later it was started since oxygen free radical-induced injury is believed to occur within the first few minutes of the reperfusion period. However, the data indicate that the amount of hypothermic protection that can be achieved during reperfusion is not critically time dependent, as long as cooling is initiated within 30 minutes. This suggests that a very rapid transition from reversible to irreversible reperfusion injury does not occur. Alternatively, it might be that reperfusion hypothermia is not actually decreasing true "reperfusion injury," but it is pro-
tecting a subpopulation of tubular cells not yet well reperfused during the early reflow period. Of note, early reflow is neither complete nor homogeneous. Thus, application of hypothermia during this period could protect against further ischemic damage, an effect that might not be critically time dependent.

By 60 minutes of reflow, no benefit could be achieved by imposing hypothermia. This suggests that the fate of sublethally injured (or poorly reperfused) cells was determined by this time, signalling an end to the "reperfusion" injury phase. However, this interpretation must be considered tentative since it is conceivable that some unknown degradative process might occur beyond 1 hour that might not be highly temperature dependent. Nevertheless, the finding that temperature can affect ischemic renal injury for up to 30–60 minutes after reflow has important implications for future investigations of experimental ischemic acute renal failure. It indicates that body temperature must be rigorously controlled during and for approximately 1 hour after vascular-clamp release if temperature-induced variations in the severity of ischemic injury are to be avoided.

In conclusion, the present study documents that a frequently observed degree of hypothermia confers virtual total functional and histological protection against a severe renal ischemic event. Hypothermia's greatest protective effect is achieved during the ischemic period. During this time it may not only lessen ischemic damage, per se, but it may also condition the kidney for less injury on reperfusion. Hypothermia confined to the reperfusion period can also confer protection, but its effect is relatively modest, and the time of its application is not critical as long as it is applied within 30 minutes. Beyond 1 hour of reflow, no benefit is achieved; this absence of effect possibly signals an end to the reperfusion injury process.

Acknowledgment

The authors thank Ms. Diana Jensen for her superb secretarial support.

References


Key Words: hypothermia • ischemia • reperfusion • adenine nucleotides • oxygen free radicals • hypoxanthine

Zager et al
Hypothermic Protection Against Renal Ischemia
Degree and time sequence of hypothermic protection against experimental ischemic acute renal failure.
R A Zager, D J Gmur, C R Bredl and M J Eng

Circ Res. 1989;65:1263-1269
doi: 10.1161/01.RES.65.5.1263

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1989 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/65/5/1263