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Mild Therapeutic Hypothermia for Postischemic Vasoconstriction in the Perfused Rat Liver

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Background: **Mild hypothermia, a promising therapy being evaluated for various clinical situations, may suppress the formation of reactive oxygen species during reperfusion and may ameliorate microcirculatory perfusion failure (the "no-reflow phenomenon").**

Methods: **Isolated rat livers underwent 30 min of perfusion, 2.5 h of ischemia, and 3 h of reperfusion. The temperature was maintained at 34°C (mild hypothermia, n = 5) or 38°C (normothermia, n = 6) for all three periods by perfusion of a modified Krebs Henseleit solution, air surface cooling, or both. A third group of livers was normothermic before and during ischemia and mildly hypothermic during reperfusion (reperfusion hypothermia, n = 6). Control livers had 3 h of perfusion at normothermia. Chemiluminescence (a measure of the generation of reactive oxygen species) and hepatic vascular resistance were monitored simultaneously to evaluate the effect of temperature on the formation of reactive oxygen species and the development of no reflow. Also measured were thiobarbituric acid reactive species and lactate dehydrogenase, as indicators of oxidative stress and cell injury.**

Results: **Mild hypothermia decreased formation of reactive oxygen species and postischemic increases in vascular resistance. Reperfusion hypothermia also decreased postischemic increases in vascular resistance, but not as effectively as did mild hypothermia. Levels of thiobarbituric acid reactive species were lower for reperfusion hypothermia than for mild hypothermia at only 0 and 30 min of reperfusion. Lactate dehydrogenase was significant only at 0 min of reperfusion for the normothermic group. Oxygen consumption did not change.**

Conclusion: **The prevention of hepatic vascular injury by suppression of oxidative stress may be an important protective mechanism of mild hypothermia. (Key words: Chemiluminescence; hypoperfusion; no-reflow phenomenon; reactive oxygen species.)**

IN the 1980s, several groups identified the cerebral protective effects of mild hypothermia (32–34°C) in canine cardiac arrest[1] and rat global ischemia[2–4] models. Mild hypothermia is being tested clinically for the treatment of head injuries.[5,6] After ischemia, oxygen is restored, and a group of partially reduced oxygen compounds, known as reactive oxygen species, results. These substances induce microcirculatory perfusion failure of the endothelium (the "no-reflow phenomenon"),[7–10] which is a potential source of secondary ischemic injury. This adverse effect can be mitigated by the activity of scavengers of reactive oxygen species, such as superoxide dismutase.[7,8] Because moderate hypothermia has been shown to decrease the formation of reactive oxygen species,[11] we hypothesized that mild hypothermia also may have the same effect during reperfusion, thereby ameliorating the no-reflow phenomenon and possibly other types of tissue injury initiated by these substances.[12–14]

Our investigation used chemiluminescence to study systematically, in real time, the ability of mild hypothermia to prevent the generation of reactive oxygen species in a perfused rat liver model. We also monitored hepatic vascular resistance simultaneously to determine whether mild hypothermia would prevent an increase in the

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generation of reactive oxygen species with reperfusion, and the subsequent development of microcirculatory perfusion failure, as manifested by increased postischemic vascular resistance. In addition, we determined whether the protective effects of hypothermia were exerted during the ischemic period or during reperfusion, or both, by subjecting a third group of livers to normothermic ischemia followed by hypothermic reperfusion ("reperfusion hypothermia").

Materials and Methods

Animal Preparation and General Experimental Protocol

The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. We anesthetized fed male Sprague-Dawley rats (Zivic-Miller, Pittsburgh, PA) with 300–400 μg methoxyflurane by inhalation. The liver was exposed through a midline incision. The portal vein and the inferior vena cava distal to the renal vein were cannulated (4 cm long; 1.5 mm ID; Texas Medical Products, Houston, TX). We incised the diaphragm and clamped the superior vena cava and descending aorta. The cannulas were attached to the perfusion circuit, and perfusion was started *in situ* with oxygenated Krebs Henseleit buffer. The liver was dissected free of its remaining attachments, weighed, and transferred to the perfusion cabinet. This light-tight and water-tight box measured 22 × 25 × 35 cm and was fabricated from anodized aluminum and heated by a water jacket. To wash out any remaining blood cells, we perfused the liver with 150–200 ml buffer and then initiated recirculation. At the end of the experiment, the liver was removed from the perfusion cabinet and reweighed.

Experimental Conditions

There were three experimental conditions: (1) normothermia (38°C, n = 6), (2) mild hypothermia (34°C, n = 5), and (3) hypothermic reperfusion (reperfusion hypothermia, n = 6). Each experiment had the same general structure: a 30-min period of perfusion (preischemia) followed by 2.5 h of no flow (ischemia) and 2 h of reperfusion. Normothermic livers were excised, placed in the perfusion chamber, and maintained at 38 ± 0.2°C during preischemia, ischemia, and reperfusion. Mildly hypothermic livers were prepared and treated identically but were cooled rapidly to 34 ± 0.2°C during preischemia using a hypothermic perfusate and air surface cooling. This temperature was maintained for all three periods. Reperfusion hypothermia livers were maintained at normothermia (38 ± 0.2°C) before and during ischemia but, for the reperfusion period, they were cooled rapidly to 34 ± 0.2°C using hypothermic perfusate and air surface cooling. A control group of livers (n = 5) was not subjected to ischemia but rather to 3 h of perfusion at normothermia.

Perfusion and Calculation of Vascular Resistance

The modified Krebs Henseleit solution used as the perfusate contained the following constituents: 140 mM sodium, 4.9 mM potassium, 110 mM chloride, 2 mM calcium, 10 mM glucose, and 1.2 mM magnesium. This solution was adjusted to a pH of 7.4 and oxygenated with a mixture of 95% oxygen and 5% carbon dioxide in a membrane oxygenator.[15]

Perfusion occurred at a flow rate of 2.5–3.5 ml · min^{-1} · g^{-1} in a recirculating system with a total volume of 175 ml. To ensure adequacy of perfusion, we placed oxygen electrodes (Diamond General, Ann Arbor, MI) in the inlet and effluent line to the liver and then measured oxygen consumption continuously. Perfusion pressure was monitored using a Bard disposable pressure transducer (Murray Hill, NJ). Flow rate was measured using an electromagnetic flow probe (Carolina Medical Products, King, NC). We calculated vascular resistance (mmHg · ml^{-1} · min^{-1}) as the quotient of perfusion pressure and flow rate.

Chemiluminescence

The chemiluminescence apparatus consists of a standard liver perfusion chamber[16] adapted to measure chemiluminescence by enclosing the chamber in a thermostatically regulated light-tight box. Free radical reactions are measured from the surface of the liver by chemiluminescence using a single photon-counting technique and an EMI 9658A photomultiplier (Thorn English Music, Fairfield, NJ) as the photodetector.[17,18] The photomultiplier is cooled by a FACT-50 thermoelectric cooler (Thorn English Music) to −24°C to reduce background noise and to make measurements independent of temperature. Signals are filtered using an EG&G/PARC 1121 amplifier-discriminator to reduce background noise and are counted using an EG&G/PARC 1109 counter (EG&G/PARC, Trenton, NJ). For computerized data acquisition, we used a CIO-DAS16/330i data acquisition board (Computer Boards, Mansfield, MA) and Snap-Master data acquisition software (HEM Data Corp., Southfield, MI). This system also acquired signals for

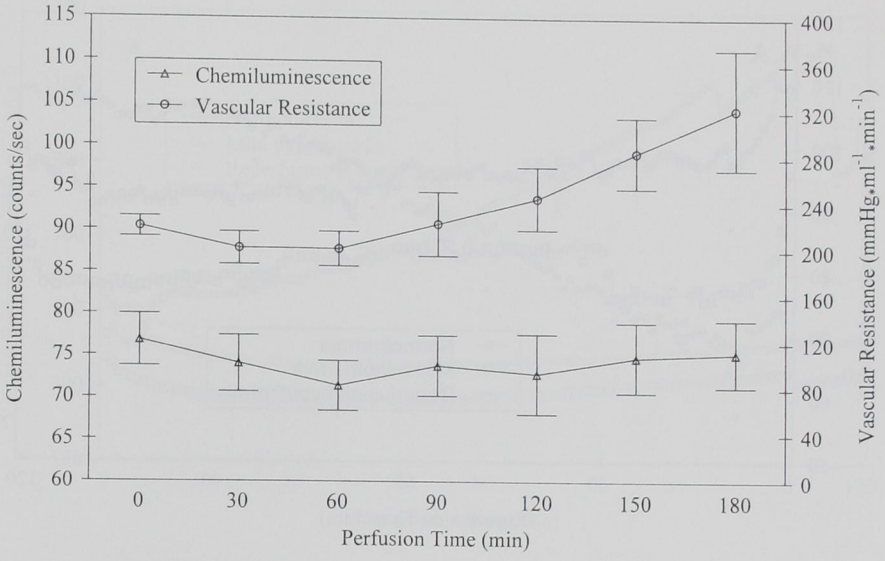

Fig. 1. Average values for chemiluminescence and vascular resistance for control preparations (*i.e.,* isolated rat livers) perfused for 3 h at normothermia (38°C). All values are expressed as counts/s (± SEM) for 5-min intervals at the beginning and the end of perfusion and every 30 min during perfusion. Vascular resistance was calculated in mmHg · ml^{-1} · min^{-1} as the quotient of perfusion pressure and flow rate.

perfusion pressure, flow rate, and oxygen partial pressure. Data were sampled every second and were averaged for 60-s intervals. One-minute averages were recorded during the experiment.

Laboratory Analyses and Calculations

We measured the levels of lactate dehydrogenase (LDH) in the perfusate as a general indicator of cell injury[19] (analyses were performed in the clinical laboratory of Presbyterian University Hospital, Pittsburgh, PA). Levels of thiobarbituric acid reactive substances (TBARS) were assayed from the perfusate as an indicator of lipid peroxidation.[20] Gases in the perfusate gases were measured from the inlet and effluent (Corning 278 *p*H/Blood Gas Analyzer; Corning Medical and Scientific, Medfield, MA) to ensure adequate oxygenation of the perfusate and an inflow *p*H of 7.4.

We measured concentrations of gases, LDH, and TBARS from the perfusate at the beginning and the end of the preischemic period, at the beginning and the end of reperfusion, and every 30 min during reperfusion. For the control group, potassium, perfusate gases, LDH, and TBARS levels were measured every 30 min. We calculated oxygen consumption from data of perfusate gases, the flow rate, and the initial wet liver weight. The efflux rates for LDH and TBARS were calculated using the following formula:

$$X = (A - B) \cdot V/(wt \cdot t)$$

where X = calculated efflux, A = sample value at one time point, B = sample value at preceding time point, V = total perfusion circuit volume, wt = initial wet liver weight, and t = time interval between samples.

Statistical Analysis

A power analysis initially was conducted to determine the sample size required. A sample size of five per group resulted from calculations using chemiluminescence values at the end of reperfusion and a standard deviation similar that used in our previous study,[21] an α level of 0.05, and a power level of 0.8.

Data were analyzed using SPSS statistical software (Statistical Package for the Social Sciences, Chicago, IL) using two-way analysis of variance, with repeated-measures analysis of variance for within-subject factors. Simple contrasts were used for between-subject factors. For within-subject factors, Helmert contrasts were used to analyze chemiluminescence, vascular resistance, and oxygen consumption; reverse Helmert contrasts were used to analyze LDH and TBARS levels. Because postischemic values were compared with their preischemic levels, we used the Dunnett test for *post hoc* analysis.[22] Probability values less than 0.05 were considered significant. All values are reported as the mean ± SEM.

Results

Chemiluminescence

Control Conditions. Chemiluminescence values did not change during the 3-h period of perfusion at normothermia (fig. 1).

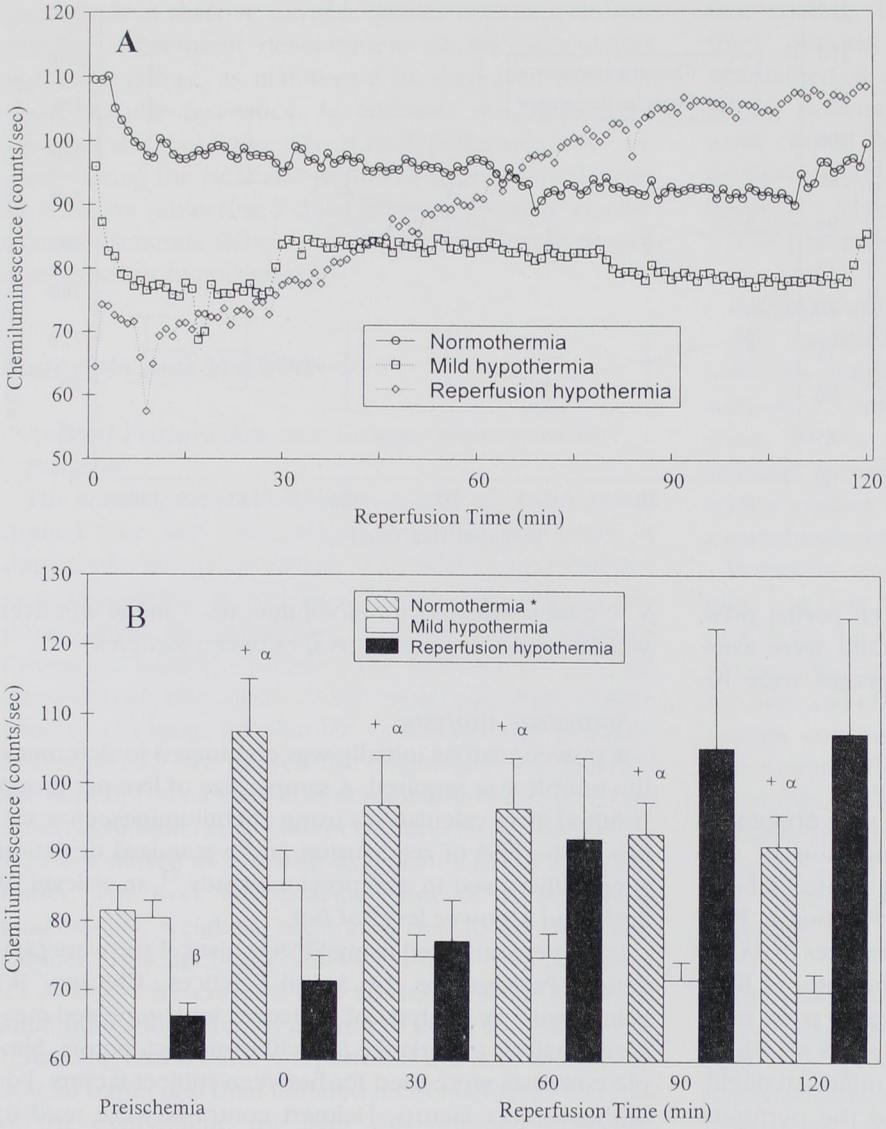

Fig. 2. Chemiluminescence values for isolated perfused rat livers subjected to one of three conditions: (1) normothermia (38°C, n = 6); (2) mild hypothermia (34°C, n = 5) during 30 min of perfusion before ischemia (preischemia), 2.5 h of ischemia (no flow), and 3 h of reperfusion; or (3) reperfusion hypothermia (normothermia during preischemia and ischemia, followed by mild hypothermia during reperfusion; n = 6). Temperatures were kept at the specified level by perfusion of a normothermic or hypothermic perfusate, as appropriate, air surface cooling, or both. Chemiluminescence was measured from the surface of the liver using a single photon-counting technique. All values are expressed as counts/s. (*A*) Average chemiluminescence values for each group, by minute, during reperfusion. (*B*) Average chemiluminescence values ± SEM for 5-min intervals at the end of the preischemic period, at the beginning of reperfusion, and every 30 min during reperfusion. $P < 0.05$ *for within-group changes; †significant between normothermia and mild hypothermia; ‡significant between mild hypothermia and reperfusion hypothermia; and +significant with respect to preischemia.

Normothermia *versus* Mild Hypothermia. Before ischemia, chemiluminescence did not differ between normothermic and mildly hypothermic livers. After ischemia, normothermic livers had an increase in chemiluminescence that did not occur in mildly hypothermic livers (fig. 2). For normothermic livers, chemiluminescence peaked at the beginning of reperfusion and remained higher than baseline (preischemia) throughout reperfusion. For the mildly hypothermic group, however, chemiluminescence was significantly lower at all time points during reperfusion.

Reperfusion Hypothermia *versus* Mild Hypothermia. Livers subjected to mild hypothermia during only reperfusion had significantly lower chemiluminescence values before ischemia than livers subjected to mild hypothermia during all three periods ($P = 0.001$; fig. 2). Chemiluminescence did not differ at 30, 60, and 90 min of reperfusion for the two groups.

Vascular Resistance

Control Conditions. Vascular resistance did not change until 150 min of the 3-h period of normothermic perfusion, when it increased moderately (fig. 1).

Normothermia *versus* Mild Hypothermia. Preischemic vascular resistance did not differ for these two groups, nor did it differ at 0 min of reperfusion. During reperfusion, vascular resistance increased significantly ($P < 0.001$) in normothermic livers (fig. 3). In contrast,

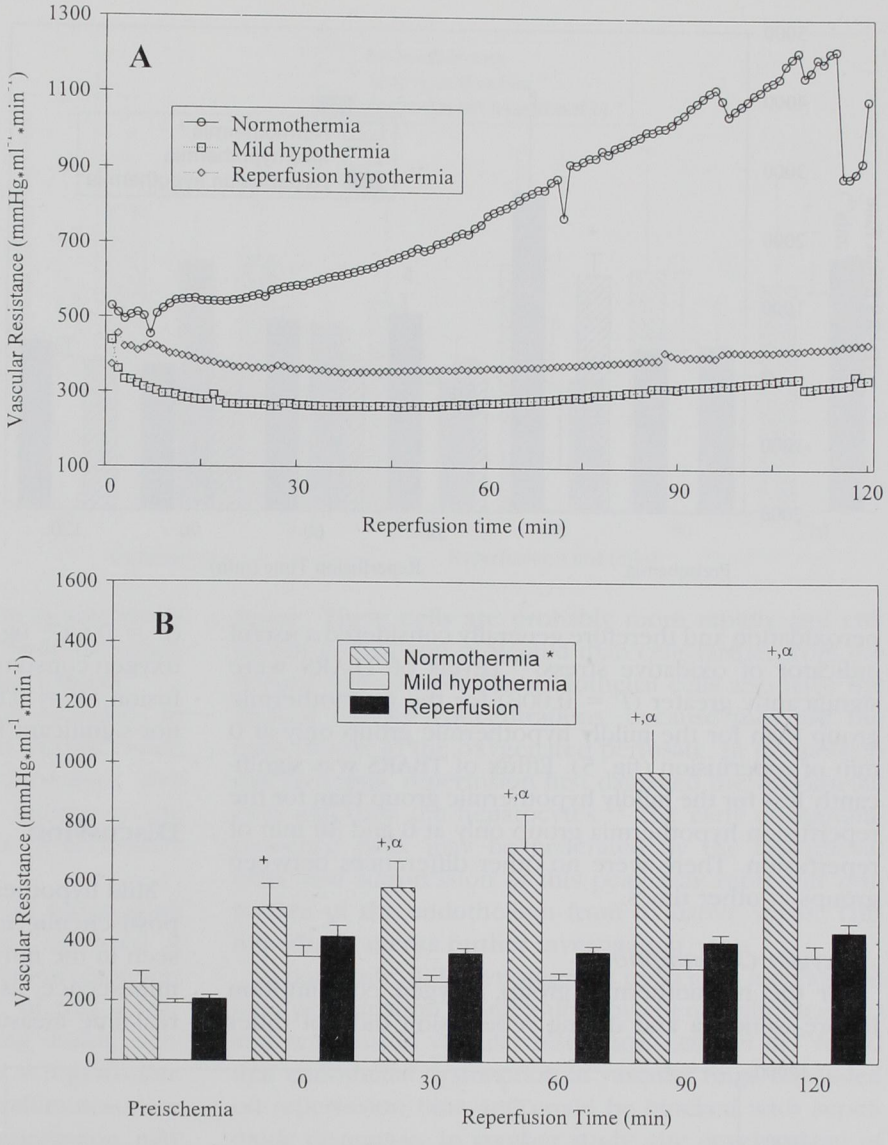

Fig. 3. Vascular resistance for the three groups of rat liver preparations described in figure 2. Vascular resistance (in mmHg · ml^{-1} · min^{-1}) was calculated as the quotient of perfusion pressure and flow rate. (*A*) Average vascular resistance for each group, by minute, during reperfusion. (*B*) Average vascular resistance ± SEM for 5-min intervals at the end of the preischemic period, at the beginning of reperfusion, and every 30 min during reperfusion. $P < 0.05$ *for within-group changes; †significant between normothermia and mild hypothermia; +significant with respect to preischemia.

vascular resistance did not change in mildly hypothermic livers. By 30 min of reperfusion, vascular resistance was significantly lower in mildly hypothermic livers ($P < 0.05$) and remained so during the rest of reperfusion.

Reperfusion Hypothermia *versus* Mild Hypothermia. For the reperfusion hypothermia group, vascular resistance was no different during reperfusion than at baseline (preischemia; fig. 3). When this group was compared with the mildly hypothermic group, vascular resistance did not differ before ischemia or at 0 min of reperfusion. At 30 and 60 min of perfusion, however, vascular resistance was greater for the reperfusion hypothermia livers. At 90 and 120 min of reperfusion, vascular resistance did not differ between the two groups.

Lactate Dehydrogenase Efflux

For the mildly hypothermic group, LDH efflux did not change from its preischemic level ($P = 0.996$; fig. 4). The normothermic group had a significant elevation only at 0 min of reperfusion ($P < 0.001$). The reperfusion hypothermia group had significant changes ($P < 0.05$) at 0 and 60 min of reperfusion.

Thiobarbituric Acid Reactive Substances Efflux

Although nonspecific with respect to the oxidized reactive species, TBARS efflux is an indicator of lipid

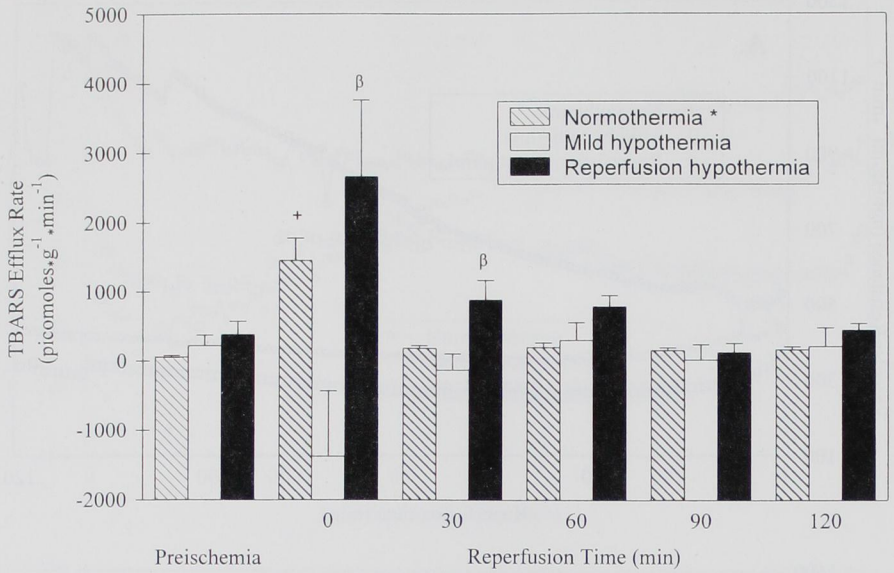

Fig. 4. The efflux rate of lactate dehydrogenase for the three groups of rat liver preparations described in figure 2. Lactate dehydrogenase was measured from the perfusate at the beginning and the end of preischemia, at the beginning and the end of reperfusion, and every 30 min during reperfusion. Plotted values are group averages ± SEM. $P < 0.05$ *for within-group changes; +significant with respect to preischemia.

peroxidation and therefore generally considered a useful indicator of oxidative stress.[20] Levels of TBARS were significantly greater ($P = 0.008$) for the normothermic group than for the mildly hypothermic group only at 0 min of reperfusion (fig. 5). Efflux of TBARS was significantly less for the mildly hypothermic group than for the reperfusion hypothermia group only at 0 and 30 min of reperfusion. There were no other differences between groups at other times.

Oxygen Consumption

For the normothermic group, oxygen consumption before ischemia and during reperfusion did not differ ($P = 0.647$; fig. 6). For the mildly hypothermic group, oxygen consumption increased only at 120 min of reperfusion ($P = 0.015$). Differences between groups were not significant for any time point.

Discussion

Mild hypothermia was very effective at preventing the postischemic increase in chemiluminescence that was seen in the normothermic group. Unenhanced chemiluminescence, as performed in this study, is a sensitive, real-time measure of lipid peroxidation.[17,18] Therefore,

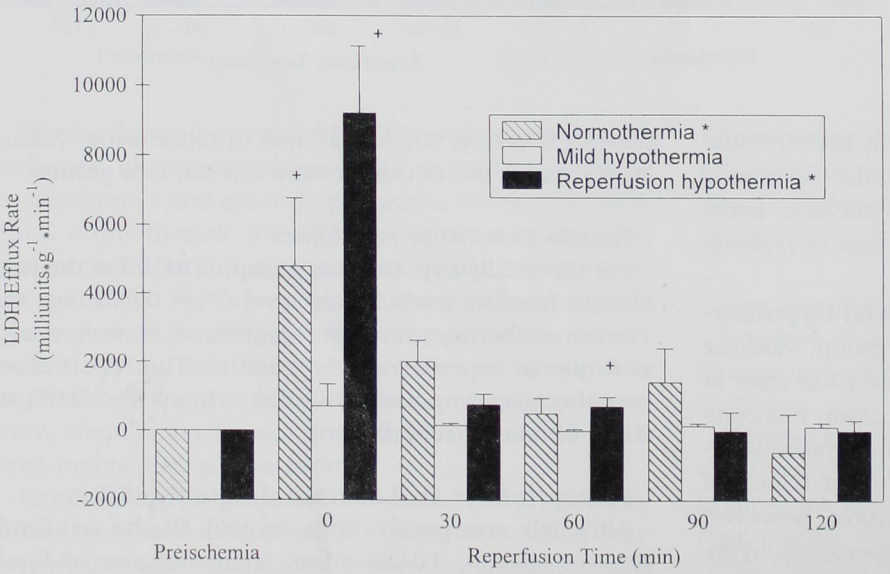

Fig. 5. The efflux rate of thiobarbituric acid reactive substances (TBARS) for the three groups of rat liver preparations described in figure 2. Levels of TBARS (an indicator of lipid peroxidation and therefore oxidative stress) were measured from the perfusate at the beginning and the end of preischemia, at the beginning and the end of reperfusion, and every 30 min during reperfusion. Plotted values are group averages ± SEM. $P < 0.05$ *for within-group changes; †significant between mild hypothermia and reperfusion hypothermia; +significant with respect to preischemia.

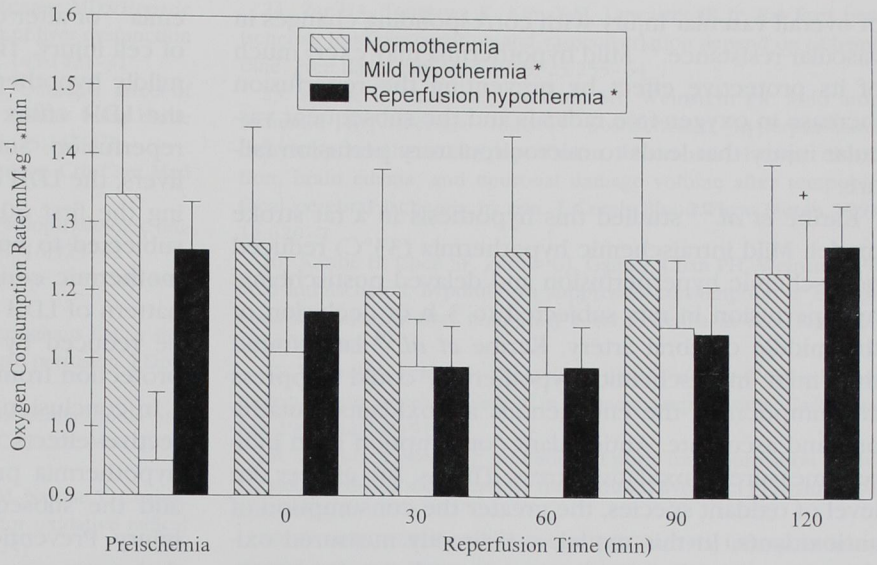

Fig. 6. Oxygen consumption for the three groups of rat liver preparations described in figure 2. Oxygen consumption was calculated from data of blood gases measured from the perfusate at the beginning and the end of preischemia, at the beginning and the end of reperfusion, and every 30 min during reperfusion. Plotted values are group averages ± SEM. The three groups did not differ significantly ($P < 0.05$) in oxygen consumption at any time point. $P < 0.05$ *for within-group changes; +significant with respect to preischemia.

we can conclude that lipid peroxidation is effectively suppressed by mild hypothermia and that mild hypothermia may offer protection through this mechanism.

Mild hypothermia also prevented the increases in vascular resistance that were observed during reperfusion of normothermic livers. Such increases, also known as microcirculatory perfusion failure or the no-flow phenomenon, may be a source of secondary ischemic injury.[7,8,10]

Mild hypothermia was more effective in suppressing the increases in chemiluminescence that occurred at reperfusion when cooling was initiated before ischemia rather than later, at reperfusion. Some of this ability appears to be based on events occurring during ischemia. The ability of hypothermia initiated at reperfusion, however, to suppress the initial chemiluminescence peak in normothermic livers during reperfusion may result from a limitation of lipid peroxidation in a specific subpopulation of cells.

Preischemic chemiluminescence was less in the reperfusion hypothermia group, and this may account in part for the apparent blunting of the reperfusion chemiluminescence peak. Alternatively, reperfusion hypothermia may only delay the inevitable enhanced lipid peroxidation reaction seen in normothermic livers. Reperfusion hypothermia inhibited the development of vascular injury during reperfusion, although not as effectively as mild hypothermia did.

Previous studies showed that the no-reflow phenomenon is due in large part to endothelial injury.[8] The endothelium is only one cell thick and is the first group of cells to come in contact with the hypothermic perfusate. These cells are probably more rapidly and efficiently cooled and protected than cells farther from the hepatic vasculature. The endothelial cells also have the highest oxygen concentrations, because they are the cells closest to the oxygenated perfusate. In normothermic ischemia, reperfusion first damaged the endothelial cells and then the hepatocytes.[23] The early chemiluminescence peak may be generated by the endothelial cells, and suppression of this peak may represent protection of the endothelium from oxidative stress. This hypothesis merits further investigation.

In our study, mild hypothermia prevented increases in lipid peroxidation (as measured by chemiluminescence) and reperfusion vascular resistance. Lefer *et al.*[8] found that endothelial dysfunction in vascular rings depended on reperfusion time and could be blocked with superoxide dismutase. In another study, the development of no reflow could also be blocked in the *in vivo* rat liver with superoxide dismutase.[7] Previous work with this model showed that superoxide dismutase could decrease lipid peroxidation, as measured by chemiluminescence, thus confirming the oxygen free radical nature of the signal.[24] Superoxide dismutase also decreased reperfusion vascular resistance.[24] In this experiment, mild hypothermia prevented the increase in reperfusion chemiluminescence that was accompanied by maintenance of normal vascular tone.

Lipid peroxidation does not affect vascular resistance in a strict dose-response manner. A greater instantaneous rate of lipid peroxidation does not result in a greater instantaneous vascular resistance. Rather, the amount of lipid peroxidation appears to affect the extent

of overall vascular injury with corresponding changes in vascular resistance.[24] Mild hypothermia may exert much of its protective effect by preventing the reperfusion increase in oxygen-free radicals and the subsequent vascular injury that leads to microcirculatory perfusion failure.

Karibe *et al.*[25] studied this hypothesis in a rat stroke model. Mild intraischemic hypothermia (33°C) reduced postischemic hyperperfusion and delayed postischemic hypoperfusion in rats subjected to 3 h of occlusion of the middle cerebral artery. Karibe *et al.*[26] later found that mild intraischemic hypothermia could suppress consumption of the endogenous antioxidants glutathione and ascorbate. Antioxidant consumption is an indirect measure of oxidative stress. That is, the greater the level of oxidant species, the greater the consumption of antioxidants. In this model, we directly measured oxidant stress by chemiluminescence and simultaneously measured vascular resistance. We believe this to be the first direct demonstration that mild hypothermia can suppress the oxidant stress associated with reperfusion and the subsequent development of vascular injury caused by that stress.

Lipid peroxidation may affect vascular resistance by several mechanisms. After reperfusion of an ischemic vascular bed, basal-induced and agonist-induced release of the vasodilator nitric oxide is decreased. This effect appears to be mediated by superoxide generated during reperfusion.[27] Superoxide also can scavenge nitric oxide during reperfusion, thus further increasing vascular resistance.[8,27,28]

In addition, after hepatic ischemia, the vasoconstrictors' platelet-activating factor[29] and endothelin[30] are produced. Platelet-activating factor levels could be decreased with allopurinol, implicating, in part, a superoxide-dependent mechanism.[29] Ischemia–reperfusion also changes hepatic prostaglandin metabolism. Superoxide more effectively inhibits the vasodilating prostaglandins PGG and PGH and prostacyclin PGI_2 synthetases than thromboxane A_2 synthetase.[31] The net effect of these interactions is to favor the production of vasoconstricting thromboxanes.[31]

Reduced metabolic rate, as manifested by reduced oxygen consumption, is often noted as an important protective mechanism of hypothermia.[26,32,33] In our study, oxygen consumption did not differ at any time during perfusion. It is unlikely that mild hypothermia confers its protective effect from gross reduction of metabolic oxygen consumption in this model.

Lactate dehydrogenase is released by cells during ischemia[19] or after cell lysis, and it is often used as a marker of cell injury. The LDH efflux rate did not change in the mildly hypothermic livers. In the normothermic livers, the LDH efflux rate was increased only at 30 min of reperfusion. Similarly, in the reperfusion hypothermic livers, the LDH efflux rate was somewhat increased during the first 60 min of reperfusion. These livers were subjected to normothermic ischemia, just as their normothermic counterparts were, and exhibited a similar pattern of LDH efflux. The LDH efflux rate appeared to be reduced by mild hypothermia, indicating possible protection from ischemic injury.

In conclusion, mild hypothermia probably exerts protective effects during ischemia and reperfusion. Mild hypothermia prevented reperfusion lipid peroxidation and the subsequent development of hepatic vascular injury. Prevention of such injury by suppression of oxidative stress may be an important protective mechanism of mild hypothermia. Mild hypothermia may be another intervention that can keep the production of reactive oxygen species to less than the threshold needed to cause cellular injury through microcirculatory perfusion failure and lipid peroxidation.

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