Influence of acidosis and hypoxia on liver ischemia and reperfusion injury in an in vivo rat model

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Animal preparation. This study was approved by the Animal Experiment Committee of the Academic Medical Center, University of Amsterdam (Amsterdam, The Netherlands). Male Wistar rats (325–375 g; Broekman, Someren, The Netherlands) were allowed to acclimatize to the laboratory environment for 7 days with free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Rats were housed under standard environmental conditions with a 12:12-h light-dark cycle. Before use in experiments, rats were fasted overnight with free access to water.

Anesthesia. All rats were anesthetized by intraperitoneal injection of 3 ml/kg of a mixture of 0.4 ml midazolam (0.5 mg/ml; Dormicum, Roche Nederland, Mijdrecht, The Netherlands), 1 ml of fentanyl citrate and fluanisone (0.315 mg/ml and 10 mg/ml, respectively; Hypnorm, Janssen-Cilag, Saunderton, High Wycombe, Buckinghamshire, UK), and 2.6 ml of NaCl (0.9%). Anesthesia was maintained by intraperitoneal administration of 0.1 ml ip Hypnorm every 45 min. A temperature probe (HP temperature module M 1029A, Agilent Technologies Netherlands, Amstelveen, The Netherlands) was inserted up to 1.5 cm in the rectum after induction of anesthesia. Rectal temperature of 37°C was maintained during the procedure and controlled by keeping the animals in the supine position on a heating pad with the additional use of a heating lamp (8).

Experimental design. Twenty-four Wistar rats received intraperitoneal anesthesia and were subjected to partial liver ischemia under four different conditions. In the ventilated...
group, rats were endotracheally intubated (14-gauge Venflon) and ventilated (Zooreact ventilator, Instruvet, Amerongen, The Netherlands) with a mixture of oxygen and air (1:1 vol/vol, 2 l/min) without the use of positive end-expiratory pressure. Adequate ventilation was confirmed by continuous monitoring of end-tidal CO₂. In this group, we aimed at maintaining a physiological pH without development of hypoxia. In the second group, rats received oxygen and air (1:2 vol/vol, 3 l/min) via a mask while they were breathing spontaneously without further respiratory support. These rats should develop acidosis but not hypoxia. In the bicarbonate-treated group, rats received bicarbonate infusion (2.1%, 3 ml/h) via the tail vein while respiratory support was not provided. These rats should develop hypoxia without acidosis. In the control group, rats were breathing spontaneously without any form of respiratory or metabolic support. These rats will develop acidosis and hypoxia.

**Surgical procedure.** In all animals, a silicone catheter (diameter 0.9 mm) was inserted into the right carotid artery for assessment of hemodynamic parameters during surgery and for collection of blood samples. After midline laparotomy, the common bile duct was cannulated with a polyethylene catheter (diameter 0.4 mm) for collection of bile. Bile was continuously collected before ischemia (15 min), during ischemia (60 min), and during reperfusion (90 min) as a parameter of hepatocyte function. After dissection of the falciform liga
tment, the afferent vessels to the median and left lateral lobes were exposed by everting the hepatic lobes upward. An atraumatic vascular clip was applied to these vessels to induce partial hepatic ischemia (70%) during 60 min. At the end of the ischemic period, the clip was removed, and subsequent reperfusion was initiated. After 90 min of reperfusion, liver biopsies were collected, fixed in 4% buffered formaldehyde, and routinely processed for hematoxylin and eosin staining of paraffin sections (4 μm).

**Blood sampling.** Blood samples (500 μl) were collected before induction of ischemia, at the end of the ischemic period, and after 90 min of reperfusion in Microtainer tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Samples were centrifuged (10 min, 3,000 rpm, 4°C), and plasma was collected for the assessment of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as parameters for hepatocellular injury. Activities of AST and ALT in plasma were determined by routine laboratory testing. Preischemia, at the end of the ischemic period, and after 90 min of reperfusion, routine arterial blood-gas analysis was performed (ABL 505 and OSM 3 hemoximeter, Radiometer, Copenhagen, Denmark).

For calculation of mean and significant differences, pH values were converted to blood hydrogen ion concentrations and expressed as means ± SE. However, for clarity, mean pH values are also mentioned in Table 1.

**Histopathology.** Light microscopic evaluation of liver I/R injury was performed on hematoxylin- and eosin-stained sections of liver biopsies after 60 min of ischemia and 90 min of reperfusion in all groups. After liver I/R, vascular congestion in the sinusoidal space, presence of polymorphonuclear leukocytes, cell death (karyorhexis, pyknosis, karyolysis, or cytolysis), and vacuolization were semiquantitatively assessed in 10 high-power fields (×40 objective) per section. Semiquantitative scores for each phenomenon ranged from 0 to 5, depending on the frequency of the phenomenon [0, never; 1, seldom (frequency in <1% of the cells); 2, occasionally (1–10% of the cells scattered throughout the liver section); 3, regularly (in 1–10% of the liver parenchyma); 4, often (10–50% of the liver parenchyma); 5, very often (>50% of the liver parenchyma)].

**Statistical analysis.** Results are expressed as means ± SE. A two-way ANOVA and repeated-measurements testing

### Table 1. Mean arterial blood pressure and arterial pH, PCO₂, and PO₂ values measured before induction of partial liver ischemia, at the end of 60 min of ischemia, and after 90 min of reperfusion

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Ventilated</th>
<th>Mask</th>
<th>Bicarbonate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAP, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-I</td>
<td>67 ± 2</td>
<td>68 ± 1</td>
<td>66 ± 2</td>
<td>65 ± 4*</td>
</tr>
<tr>
<td>End-I</td>
<td>62 ± 2*</td>
<td>67 ± 2b</td>
<td>64 ± 2*</td>
<td>56 ± 2c,e,x</td>
</tr>
<tr>
<td>90 min R</td>
<td>66 ± 2</td>
<td>68 ± 2</td>
<td>64 ± 2</td>
<td>55 ± 4d,e,f,g</td>
</tr>
<tr>
<td><strong>H⁺, nM (pH)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-I</td>
<td>38.9 ± 1.3(7.41)</td>
<td>46.9 ± 4.7c,m(7.33)</td>
<td>39.0 ± 1.3(7.41)</td>
<td>43.2 ± 1.9e,n(7.36)</td>
</tr>
<tr>
<td>End-I</td>
<td>38.8 ± 1.7a,d(7.41)</td>
<td>64.2 ± 4.7b,c,e,f(7.19)</td>
<td>39.3 ± 2.5b,c(7.41)</td>
<td>53.9 ± 1.5c,d,e,g(7.27)</td>
</tr>
<tr>
<td>90 min R</td>
<td>38.2 ± 1.1b,k(7.42)</td>
<td>62.5 ± 3.2i,j,m(7.20)</td>
<td>36.1 ± 1.2l(7.44)</td>
<td>55.7 ± 1.9k,l,n(7.25)</td>
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<tr>
<td><strong>PCO₂, Torr</strong></td>
<td></td>
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<tr>
<td>Pre-I</td>
<td>35.6 ± 1.4*</td>
<td>49.3 ± 5.1a,j</td>
<td>44.7 ± 2.1m</td>
<td>43.7 ± 2.9n</td>
</tr>
<tr>
<td>End-I</td>
<td>32.1 ± 1.9b,c,d</td>
<td>64.8 ± 4.2e,f</td>
<td>49.6 ± 3.5x</td>
<td>52.4 ± 4.0f,l</td>
</tr>
<tr>
<td>90 min R</td>
<td>31.6 ± 1.3b,i,l</td>
<td>62.4 ± 1.9j,k,l</td>
<td>52.1 ± 1.5j,m</td>
<td>51.2 ± 2.1i,k,n</td>
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<tr>
<td><strong>Bicarbonate, mmol/l</strong></td>
<td></td>
<td></td>
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<tr>
<td>Pre-I</td>
<td>22.1 ± 0.4b,c,e</td>
<td>25.2 ± 0.7d</td>
<td>27.6 ± 0.7b,d,e</td>
<td>24.6 ± 0.9c,e</td>
</tr>
<tr>
<td>End-I</td>
<td>20.2 ± 0.2g,h,k</td>
<td>23.7 ± 0.4i</td>
<td>30.6 ± 1.0e,i</td>
<td>22.8 ± 0.5b,h,j</td>
</tr>
<tr>
<td>90 min R</td>
<td>20.1 ± 0.8l,m</td>
<td>23.3 ± 0.9m,n</td>
<td>35.2 ± 1.0l,m,n</td>
<td>22.9 ± 0.6m</td>
</tr>
<tr>
<td><strong>PO₂, Torr</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Pre-I</td>
<td>221 ± 11b</td>
<td>195 ± 17d,k,b,i</td>
<td>87 ± 3a,c,d,p</td>
<td>76 ± 11b,b,k,q</td>
</tr>
<tr>
<td>End-I</td>
<td>195 ± 11g,f</td>
<td>133 ± 17b,h,i</td>
<td>63 ± 3f,k,j</td>
<td>58 ± 3e,k</td>
</tr>
<tr>
<td>90 min R</td>
<td>197 ± 3i,m,n</td>
<td>127 ± 14c-o</td>
<td>49 ± 2m,p</td>
<td>49 ± 3n-q</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial blood pressure; pre-I, before induction of partial liver ischemia; end-I, at the end of 60 min of ischemia; 90 min R, after 90 min of reperfusion. Control rats showed a decrease in MAP at the end of ischemia and after 90 min of reperfusion. Physiological pH was maintained in ventilated and bicarbonate-treated rats, whereas mask and control rats developed acidosis (see MATERIALS and METHODS). An accumulation of CO₂ was observed in all rats, except for ventilated rats. Bicarbonate levels were different between groups. All rats showed a stable bicarbonate concentration during the experiment, except bicarbonate-treated rats. PO₂ values in bicarbonate-treated and control rats were significantly lower than in ventilated rats and rats with an oxygen mask. Significant differences exist between values with identical superscript letters, P < 0.05.
RESULTS

Hemodynamics. Mean arterial blood pressure (MAP) returned to preischemic values within 5–10 min after vascular inflow occlusion, and splanchnic congestion was not observed because of partial occlusion of the afferent liver vessels. Animals in all groups, except for the nonsupported control group, showed stable hemodynamics during the entire liver I/R procedure. At the end of the ischemic period and after 90 min of reperfusion, rats in the control group showed lower MAP compared with all other groups. After 90 min of reperfusion, MAP in the control group was significantly decreased compared with the preischemic values (Table 1).

pH. Ventilated rats and rats in the bicarbonate-treated group showed a physiological blood pH during the entire procedure. Rats in the control group developed acidosis during the ischemic period, with pH values lower than those in ventilated rats and bicarbonate-treated rats. Rats with an oxygen mask showed the lowest pH (Table 1).

Bicarbonate. Blood bicarbonate levels were lowest in ventilated rats and highest in rats treated with bicarbonate. Bicarbonate levels remained stable in all rats during the experiment, except in bicarbonate-treated rats (Table 1).

PCO₂. All nonventilated rats showed increased blood PCO₂ levels, whereas normal blood PCO₂ levels were maintained in the ventilated rats. Blood PCO₂ levels were highest in rats in the mask group, followed by PCO₂ levels in rats in the control group and the bicarbonate-treated group (Table 1).

PO₂. Although rats in the mask group showed lower blood PO₂ after ischemia compared with ventilated rats, blood PO₂ values in both groups were sufficient to maintain adequate organ oxygen supply during the experiment. In contrast, rats in both the bicarbonate-treated and control groups showed low PO₂ immediately after induction of anesthesia. After ischemia, PO₂ dropped to critically low levels in both the bicarbonate-treated and the control groups (Table 1).

Hepatocellular injury and function. AST and ALT levels at the end of ischemia and after 90 min of reperfusion showed significant elevation in rats of all groups compared with preischemic values (Fig. 1). After 90 min of reperfusion, plasma AST and ALT levels were lower in the ventilated rats compared with all other groups. AST and ALT levels of rats in the bicarbonate-treated group were higher compared with rats in the mask and control groups.

Overall, bile secretion was significantly reduced during liver ischemia and during 90-min reperfusion compared with preischemic levels in all rats. None of the animals showed a significant recovery of bile secretion after 90 min of reperfusion. Furthermore, bile secretion was not significantly different between rats in all groups (Fig. 2).

Histopathology. In hematoxylin- and eosin-stained paraffin sections of the I/R liver lobes illustrated in
In this study, nonventilated rats with bicarbonate infusion retained a physiological pH, whereas other nonventilated rats developed acidosis. Also, nonventilated rats developed hypoxia when oxygen was not supplied. Ventilated rats maintained normocapnia, a physiological pH, and mild hyperoxia. Hypercapnia and hypoxia in the nonventilated rats are evidently caused by inadequate gas exchange in the alveoli because of inadequate respiration, leading to respiratory acidosis. At the end of the ischemic period, when the hepatic circulation was not yet restored, liver-derived lactic acid unlikely played a role in the development of acidosis. Moreover, differences in pH between rats with an oxygen mask and ventilated rats already existed before induction of ischemia. During reperfusion, pH remained stable in all groups (as did the PCO2 and bicarbonate levels), which makes the contribution of lactic acid to the prolongation of acidosis less likely.

The upper PO2 limit in the ventilated rats was 200 Torr, which is higher than normal PO2 (~94 Torr). It has been reported that hyperoxia (induced by exposure to 100% oxygen under normobaric conditions) can increase reactive oxygen species formation in different organs after at least 24 h of exposure (1, 12, 13). Because in our study ventilated rats were exposed to high oxygen pressures for a maximum of 3 h, hyperoxia is not thought to contribute to the development of liver I/R injury.

Although rats with an oxygen mask showed increased respiratory insufficiency (i.e., increased PCO2 levels) and significantly lower blood PO2 after ischemia compared with ventilated rats, PO2 values were still above physiological levels. This suggests that the differences in arterial PO2 unlikely contributed to the differences in liver I/R injury between rats with an oxygen mask and ventilated rats.

Rats with an oxygen mask showed significantly enhanced hepatocellular I/R injury compared with ventilated rats. This suggests that hypercarbic acidosis during normoxic reperfusion has an adverse effect on liver I/R injury. In our experiments, PCO2 levels did not exceed 65 Torr, and, therefore, only mild acidosis developed. Although it has been reported that mild acidosis can be protective, whereas severe acidosis can enhance hypoxic injury in isolated hepatocytes (7), the present experiments show opposing results under normoxic conditions. Our data suggest that enhanced hepatocellular I/R injury, despite normoxic reperfusion in rats with an oxygen mask, was due to respiratory acidosis.

In contrast, rats of the control group with mild acidosis and severe hypoxia during liver I/R showed significantly less hepatocellular injury compared with bicarbonate-treated rats with hypoxia only. The protective effect of acidosis under hypoxic conditions is well documented in the literature (2, 4). Some studies suggest that the supply of bicarbonate may well restore blood pH values but that it leads to a paradoxical decrease of intracellular pH and hence to impaired cellular function (9, 10, 17). This assumption is still highly controversial, and other studies suggest that the
role of this phenomenon is minimal (5, 11, 14) and transient (15) in vivo.

The critically low \( \text{PO}_2 \) during liver I/R in nonventilated control and bicarbonate-treated rats actually prolonged the hypoxic period of the liver during reperfusion, despite restoration of blood flow. Reportedly, prolonged hypoxia during reperfusion severely augments liver I/R injury (16). As was mentioned before, mild acidosis in hypoxic, nonventilated control rats attenuated liver I/R injury during hypoxia compared with that in hypoxic, nonventilated bicarbonate-treated rats with physiological pH. Mild acidosis did not completely protect the liver from the adverse effects of hypoxic reperfusion, because plasma AST and ALT levels in nonventilated, hypoxic control rats with acidotic pH were still significantly higher compared with those in ventilated, normoxic rats with physiological pH.

Bile secretion is considered to be a good indicator of hepatocyte function (3). However, in this study during the entire period of liver I/R, bile secretion was not significantly altered by acidosis and/or hypoxia, despite large differences in hepatocellular I/R injury. Although prolonged observation may reveal larger differences among the groups, previous studies in our laboratory showed incomplete recovery of bile secretion also after 24 h of reperfusion (data not shown).

In conclusion, the results of this study suggest that respiratory acidosis significantly enhanced liver I/R injury under normoxic conditions, whereas respiratory acidosis significantly reduced liver I/R injury under hypoxic conditions. Clearly, standardization of methods used in I/R studies is crucial for comparison and validation of animal models. This should include, as is our conclusion from the data presented here, controlled respiratory support of the animal during ischemia and reperfusion of the liver.

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REFERENCES