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Thermochimica Acta 251 (1995) 161–172

thermochimica acta

Prediction of recovery after ischemia. A microcalorimetric and biochemical study of rat liver tissue ☆

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Received 19 July 1994; accepted 2 August 1994

Abstract

Alteration of the hepatocellular function following ischemic damage may play a crucial role in the limited recovery after reperfusion. In spite of numerous efforts, finding a simple technique for predicting the recovery of the liver after ischemic damage is still an unresolved problem.

During the ischemic storage of isolated rat livers at 25°C, tissue concentrations of high energy phosphates, glycogen and lactate were determined photometrically and, in comparison, the metabolic rate was measured continuously by direct calorimetry. In addition, interstitial pH was measured with microelectrodes. In a second series of experiments these results were compared with functional recovery after ischemia and reperfusion. Following ischemic storage at 25°C for 60, 120 and 240 min, the isolated livers were reperfused for 30 min in a non-recirculating system with a constant flow rate. During reperfusion, functional recovery as assessed by oxygen consumption and bile flow was determined. At the end of reperfusion, tissue samples were taken for biochemical analysis of adenine nucleotides and tissue lactate.

☆ Presented at the Ninth Conference of the International Society for Biological Calorimetry, Berlin-Schmerwitz, 27–31 May 1994.

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[1] The experiments described in this paper were performed in adherence to the NIH guidelines for the use of experimental animals.

SSDI 0040-6031(94)02042-6

Though the ATP concentration drops within 60 min of ischemia to 6.94% of the control value without further significant change, the metabolic rate measured continuously by direct calorimetry decreases in an exponential manner. According to the calorimetrically measured heat output, functional hepatic metabolism, as assessed by oxygen consumption, bile flow and restoration of high energy phosphates during reperfusion, recovered to an extent depending on the duration of ischemia. It is concluded that the metabolic rate of the ischemic rat liver, as determined continuously by direct calorimetry, is a good predictor for recovery after ischemic damage.

Keywords: ATP; Ischemia; Liver; Metabolic rate; Microcalorimetry; Reperfusion

1. Introduction

Tissue injury due to hypoxia or ischemia has been intensively studied in order to find strategies against such damage and to improve tissue viability [1]. The impaired mitochondrial function during an ischemic or hypoxic event results in an insufficient supply of ATP and an imbalance between energy production and energy utilization. This leads during prolonged ischemia to ionic imbalances, cell swelling, acidosis, and activation of proteases and phospholipases [2]. Thus, the alteration of metabolic cell function during ischemia may play a critical role in the development of tissue injury and shares responsibility for the irreversible loss of organ function. By an analysis of the ischemic metabolism of brain slices, Opitz and Schneider [3] developed the concept of a structural maintenance level. When the metabolic rate falls below this critical level, irreversible tissue damage occurs.

One of the problems still remaining in hepatic surgery and transplantation is the limited tolerance of the liver to ischemia and, in particular, the prediction of recovery after ischemia [4]. As pointed out above, the energy status of the cell is of crucial significance for the functional and structural integrity of any organ. The time course of ATP tissue concentration thus gives information about ischemic tissue metabolism. However, its use as a predictive parameter for recovery of the liver after ischemic damage is controversial [5–8]. The most accurate estimates of ischemic tissue metabolic rate have probably come from direct calorimetry. Continuous measurement of total heat output from exothermic reactions occurring in cells seems to be the best predictive parameter for recovery after hepatic dysfunction caused by ischemia.

To obtain further insight into the significance of ischemic tissue metabolism for recovery from ischemia, biochemically analyzed values of tissue metabolism and calorimetrically measured heat output of the ischemic rat liver were correlated with the extent of functional recovery as assessed by oxygen consumption, bile flow and recovery of ATP content during reperfusion, using an isolated liver model.

2. Material and methods

2.1. Animals

All experiments were performed after approval by the local ethics committee and under the surveillance of the university animal welfare veterinarian.

Male Sprague–Dawley rats weighting 300–350 g (Institut für Tierzucht, Hannover, Germany) were maintained on standard rat food pellets, water ad libitum, and with a 12 h dark–light schedule. Each rat was injected intraperitoneally with heparin (1000 U kg^{-1}) 20 min prior to preparative surgery. Animals were anesthetized by i.p. injection of sodium pentobarbital (50 mg kg^{-1}). Surgery was performed between 9 a.m. and 10 a.m.

2.2. Assessment of ischemic metabolism

After midline laparotomy and dissection of the ligament, the livers were harvested and stored in Ringer's solution at 25°C ($n = 6–8$). After 15, 30, 60, 90, 120, 150 and 180 min of ischemic storage, the concentrations of adenine nucleotides, glycogen and lactate in the tissue were determined photometrically by standard techniques [9,10] and extracellular pH was measured with glass electrodes in one isolated lobe at 25°C. In parallel with these biochemical investigations, metabolic rate was determined by means of direct calorimetry, as described elsewhere [11]. Briefly, liver samples of ≈ 1 g wet weight were incubated at 25°C in Ringer's solution in a microcalorimeter (ThermoMetric AB, Järfälla, Sweden). Following preparation, the liver slices were incubated in closed tubes filled with Ringer's solution. The heat produced in the tube through the metabolic activity of the sample passed into a large surrounding water bath through a Peltier thermopile. A thermal gradient thus arose, which was directly proportional to the heat flow and allowed its continuous monitoring. Each measurement was started 30 min after the onset of ischemia when thermal equilibration between the probe and the water bath was complete.

2.3. Isolated liver perfusion

In a different series of experiments, livers were reperfused after ischemic storage in a nonrecirculating system, as described previously by Miller [12] ($n = 6$ per group). The rats were prepared as described above, and additionally the common bile duct (PE-10 tubing), the portal vein and the thoracic inferior cava were cannulated. After tying off the inferior vena cava, cannulated livers were isolated and stored for 1, 2 or 4 h at 25°C in Ringer's solution and were subsequently reperfused with a hemoglobin-free Krebs–Henseleit bicarbonate buffer containing 3% of hydroxyethyl starch and equilibrated with 95% O_2/5% CO_2 to a p_{O_2} of ≈ 550 mmHg and a p_{CO_2} of ≈ 35 mmHg (pH 7.4, 37°C). Perfusion was performed for 30 min at a constant flow rate of 3.0–3.5 ml min^{-1} (g liver wet weight)$^{-1}$ using a previously calibrated peristaltic pump. Control livers were perfused immediately

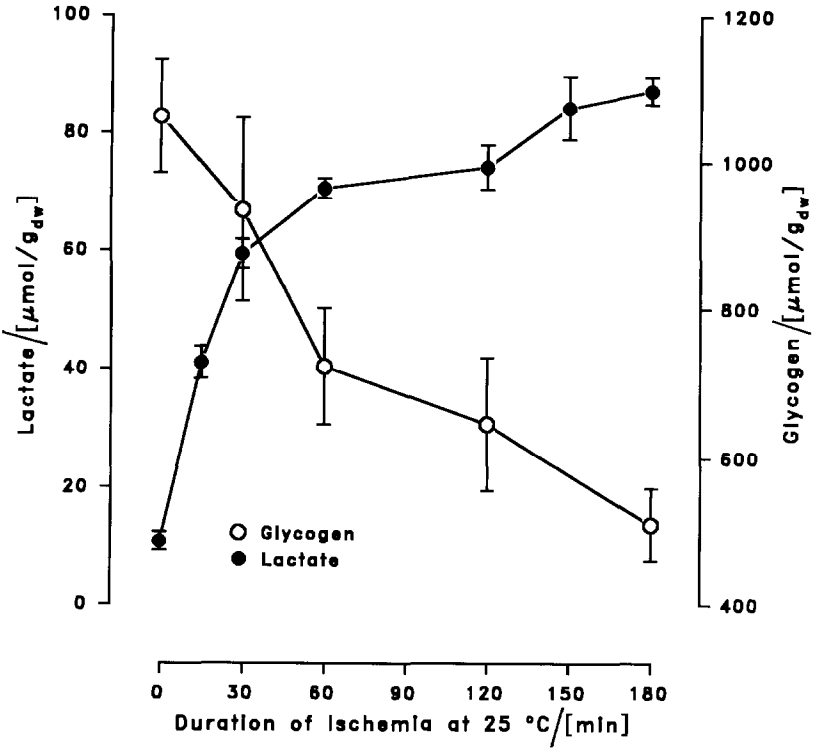

Fig. 1. Tissue concentrations of glycogen and tissue lactate during ischemic storage at 25°C in Ringer's solution. Data are expressed as mean ± S.E.M. ($n = 8$ per group).

after completion of the cannulation. The period of ischemia in the controls was at the most 10 min, the time taken to complete the cannulation.

2.4. *Assessment of postischemic metabolism*

Oxygen tensions in the perfusate and effluent were assessed every 5 min, and the oxygen consumption was calculated from the porto-caval oxygen concentration difference, the flow rate and the liver wet weight. Data were expressed per 100 g liver wet weight. The bile was collected in preweighed tubes and measured at 5 min intervals. During reperfusion, lactate concentrations were measured in the effluent [13]. At the end of reperfusion, liver samples were separated for biochemical analysis involving measurement of the tissue concentrations of adenine nucleotides and lactate [9,10].

2.5. *Statistics*

Results are given as mean ± SEM. The data were compared statistically by analysis of variance followed by a post hoc t-test with Bonferoni correction for multiple comparisons. A p value below 0.05 was considered significant.

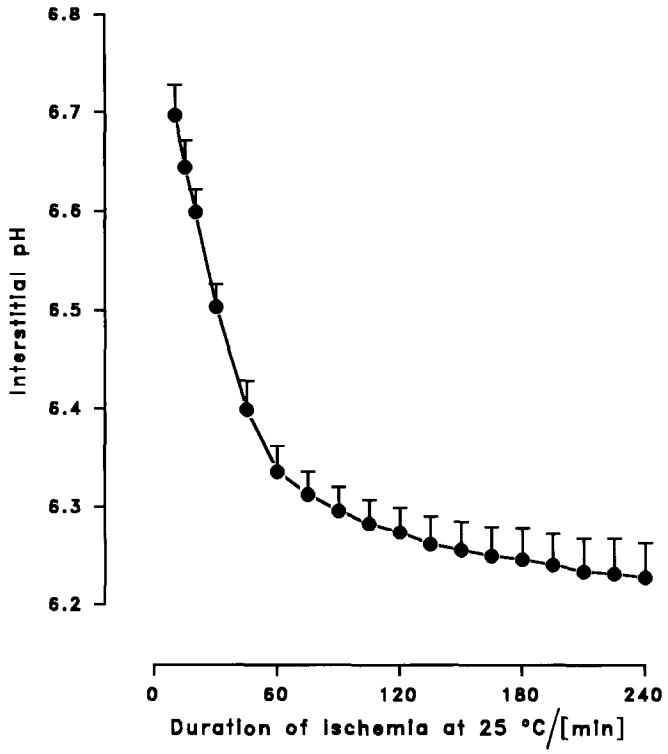

Fig. 2. The time course of interstitial pH during ischemic storage at 25°C in Ringer's solution. Data are expressed as mean ± S.E.M. ($n = 8$).

3. Results

3.1. Parenchymal cell metabolism during ischemic storage of the liver

Owing to anaerobic glycolysis during ischemic storage, the energy stores become catabolized, and metabolic end products accumulate in the tissue. Glycogen, the main energy source in the hepatocyte, is metabolized with formation of lactate, resulting in glycogen depletion and lactate accumulation (Fig. 1). Concomitant to lactate accumulation, a rapid interstitial acidosis developed during the first 60 min of ischemia (Fig. 2). Regarding the high energy phosphates, analyzed at definite time points during ischemic storage, a rapid breakdown of ATP and ADP could be observed within 60 min of ischemia and without further significant change in the ATP concentration (Fig. 3). As for the total ischemic tissue turnover, as assessed continuously by microcalorimetry, heat output fell in an exponential manner during ischemia. Starting the measurement 30 min after onset of ischemia, the above mentioned thermal gradient took some time to build up, and we therefore initially

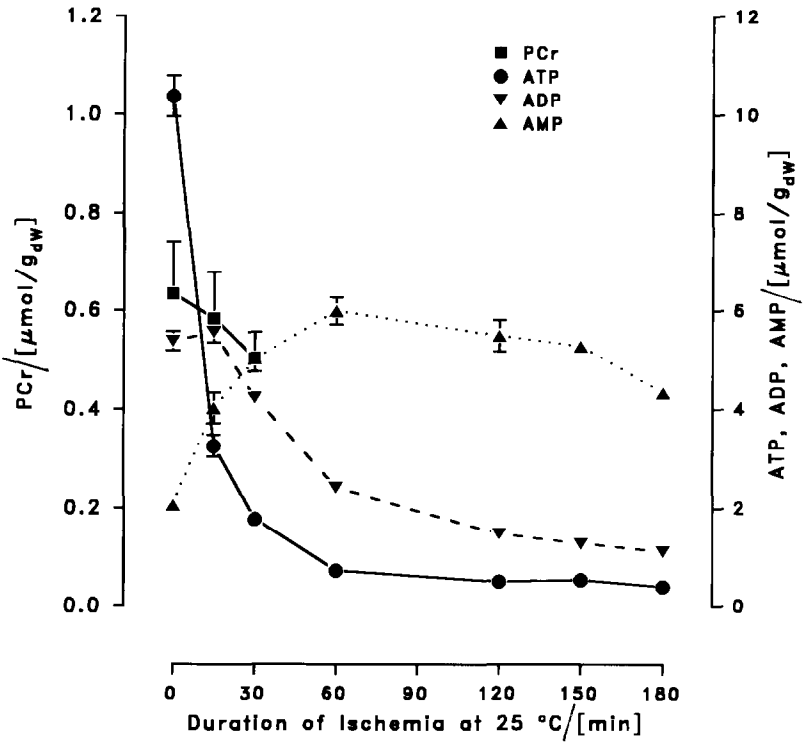

Fig. 3. Tissue concentrations of phosphocreatine (PCr), ATP, ADP and AMP measured at definite time points during ischemic storage at 25° in Ringer's solution. Data are expressed as mean ± S.E.M. ($n = 8$ per group).

encountered a decreasing line of heat production with subsequent exponential decline of the metabolic rate (Fig. 4).

3.2. *Postischemic parenchymal cell metabolism*

To correlate the above described parameters of ischemic metabolism with the extent of functional metabolic recovery, oxygen consumption and bile flow were measured during reperfusion. Mitochondrial function following reperfusion was assessed by the restoration of tissue high energy phosphates. Oxygen consumption was calculated as described above. During the first minute of reperfusion, a wash-out effect was seen, and therefore apparently high values of oxygen consumption were calculated, but after 5 min there was an equilibrium in each group. Oxygen consumption of the control livers was ≈ 4.6 ml (100 g wet weight)$^{-1}$ min^{-1}. There was a good recovery after 60 min of ischemic storage, but the extent of recovery was significantly lower after 2 and 4 h of ischemia ($p < 0.001$; Fig. 5). In all ischemic groups bile flow was very low in the beginning and became stable after

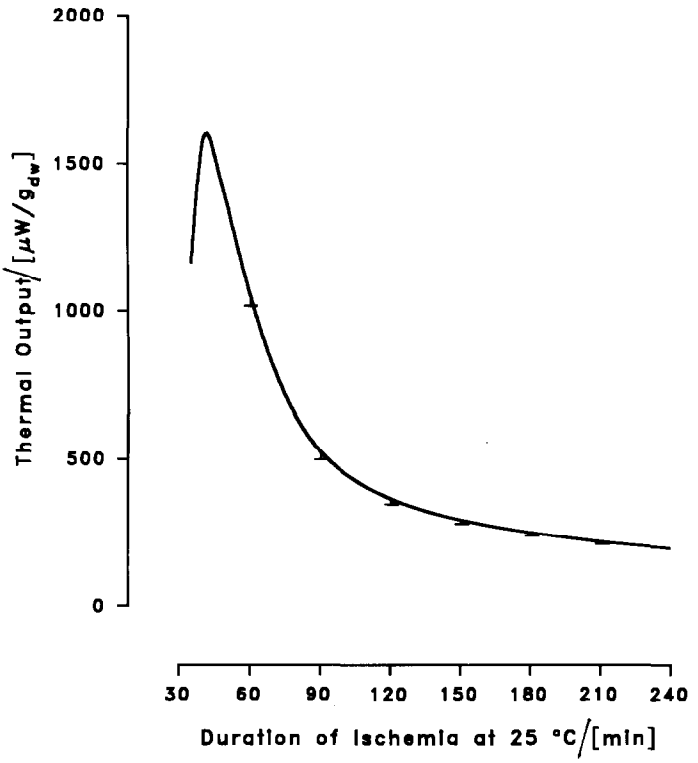

Fig. 4. Microcalorimetrically measured heat output during ischemic storage at 25°C in Ringer's solution (mean ± S.E.M.; $n = 6$). Each measurement of a liver sample started 30 min after the onset of ischemia.

15 min of reperfusion. However, compared with the control values after 30 min of reperfusion, bile flow was significantly reduced in all ischemic groups and failed to recover after 4 h of ischemic storage (Fig. 5). In contrast to the study performed by Van Dyke et al. [14], we found a good correlation between oxygen consumption and bile flow ($r = 0.74$ for the values after 30 min of reperfusion).

Lactate concentrations were measured in the effluent during reperfusion, as well as tissue lactate at the end of reperfusion. The lactate concentration in the effluent was initially high but decreased nearly to zero in all four groups. Corresponding with this, tissue lactate was below 5 μmol (g dry weight)$^{-1}$ in all groups, without any significant difference between the groups (data not shown).

The ATP tissue concentration was well restored after 60 and 120 min of ischemic storage (85.8% and 68.7% respectively), whereas after 240 min of ischemia the recovery was only 40.8% of the control value.

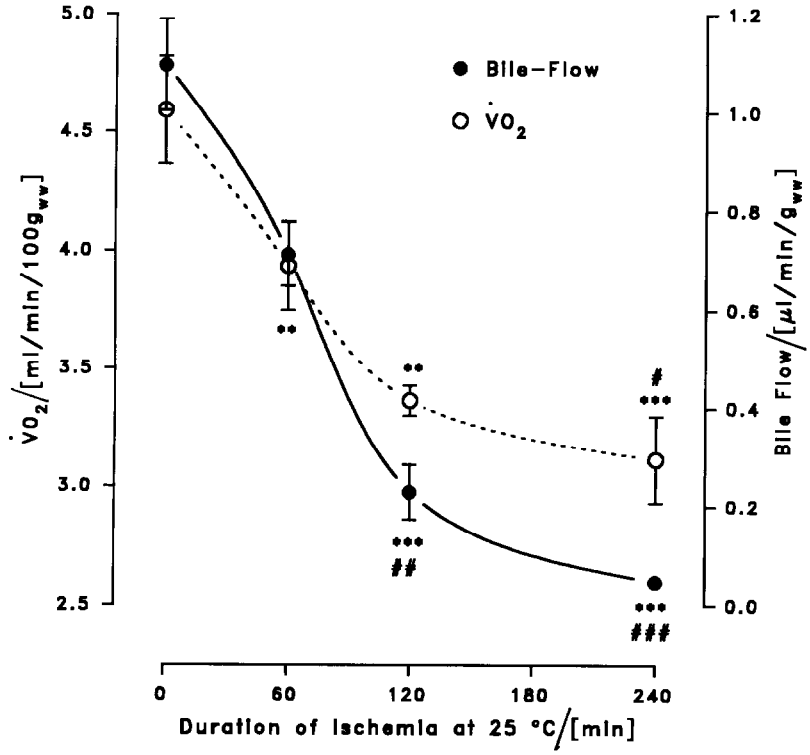

Fig. 5. The relationship between the values of oxygen uptake and bile flow after 30 min of reperfusion and the duration of ischemic storage before reperfusion. Values are mean ± S.E.M. ($n = 6$ per group).

Symbols: ★ at the oxygen uptake curve, 120 and 240 min of ischemia vs. control; # at the oxygen uptake curve, 240 min of ischemia vs. 60 min of ischemia; ★ at the bile flow curve, 60, 120 and 240 min of ischemia vs. control; # at the bile flow curve, 120 and 240 min of ischemia vs 60 min of ischemia.

The symbol ★ or # indicates $p < 0.05$; ★★ or # #, $p < 0.01$; ★★★ or # # #, $p < 0.001$.

4. Discussion

The main purpose of this study was to compare the conventionally measured levels of high energy phosphates with the alternative approach of microcalorimetrically measured heat output as predictors for the recovery of ischemic rat liver after reperfusion. As described also by other authors, ATP levels of the ischemic liver drop within 60 min without further significant change. In contrast, the metabolic rate as assessed by direct calorimetry declined in an exponential manner, with significant changes between 60 and 120 min and 120 and 240 min of ischemia. Comparing these two parameters of anaerobic metabolism with the recovery of hepatocellular function as determined by oxygen uptake and bile flow, there is no correlation between the ATP levels of the ischemic rat liver and the degree of recovery. Moreover, regarding mitochondrial function after reperfusion, as assessed

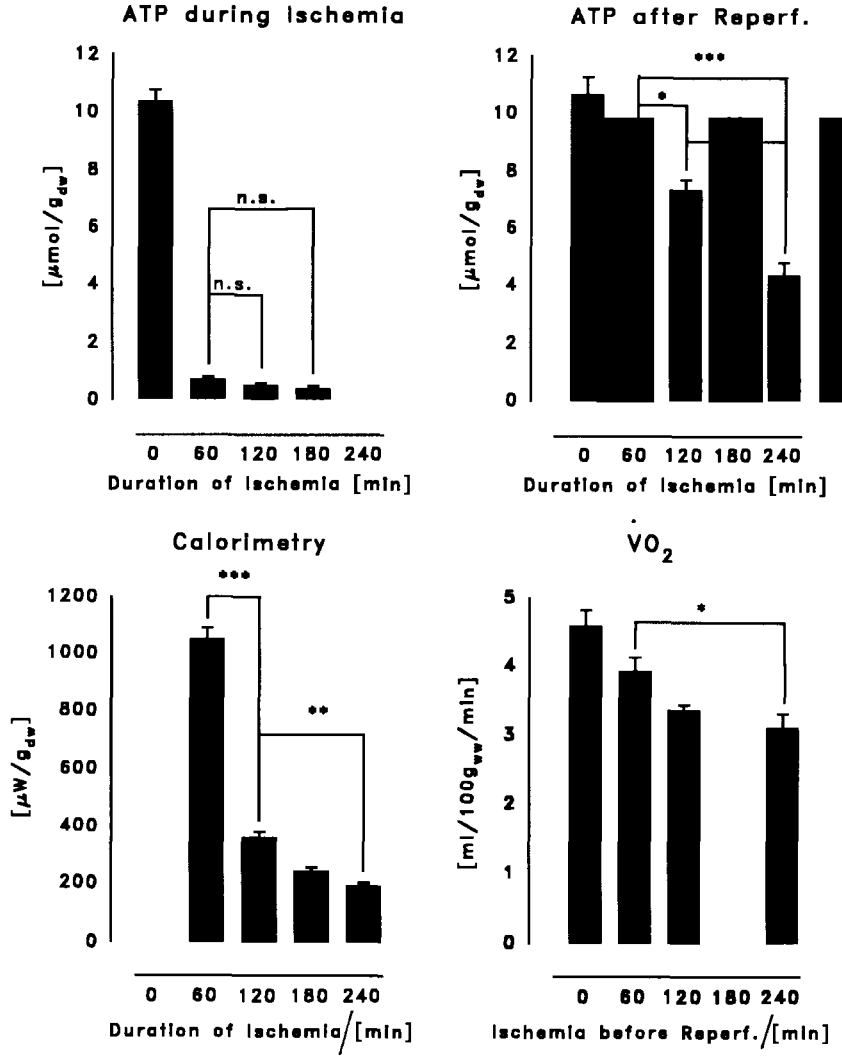

Fig. 6. Synopsis of the metabolic parameters measured during ischemia (ATP concentration and calorimetry) and during or after reperfusion (ATP concentration and oxygen consumption). The metabolic recovery is dependent on the duration of the preceding ischemic storage. This kind of dependence could be predicted by the time course of the metabolic rate but not by measurement of ATP concentration at specified time points during ischemic storage. Data are mean ± S.E.M.

Symbols: ★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.001$.

by resynthesis of ATP, there is good recovery after 60 min of ischemic storage to 86% of the control value. These results are in agreement with those of other experimental studies [15–17]. Even in human livers Kamiike et al. [5] did not find the ATP tissue concentration of the ischemic liver to be a good predictor for the

outcome of transplantation. The formation and degradation of ATP is a very dynamic process, reliant on many metabolic processes. Measurement of static tissue concentration during the ischemic time course cannot therefore fully represent the true cellular energetic state, and is not a useful predictive parameter for recovery after reperfusion. In contrast, determination of heat output by direct calorimetry is a continuous measurement of all dynamic cellular metabolic processes and therefore gives overall information about the cellular metabolic situation. As demonstrated in Fig. 6, there is a good correlation between the time course of heat output and the functional recovery after reperfusion. This confirms the hypothesis of Opitz and Schneider [3] and supports the significance of the hepatocellular metabolism for recovery after reperfusion.

The ability of the liver to resynthesize ATP and to produce bile has been shown to correlate well with the survival rate in a transplantation model and seems to determine tissue viability. Marubayashi and coworkers [18] reported that a restoration of ATP to $\approx 60\%$ resulted in a survival rate of $\approx 60\%$ (in a group with a portofemoral shunt during the ischemic period). When the level of ATP restoration was below 35% no animal survived. Considering the significance of the metabolic rate of the ischemic liver in the recovery of liver function after reperfusion, it is important to ascertain the structural maintenance level as described by Opitz and Schneider [3]. With regard to results from Marubayashi et al. [18], a successful resuscitation of the rat liver after 60 min and possibly after 120 min of global ischemia at 25°C should be most likely, but seems very unlikely after 240 min of ischemia. Therefore a critical level may be a heat output of ≈ 500 μW (g dry weight)$^{-1}$. Nevertheless, as seen in many experimental transplantation studies, it is difficult to determine an exact time of no return. Regarding, for example, the time period between 90 and 240 min, the heat output curve describes a flatter course and a period of 150 min results in a time factor of 2.19 μW min^{-1}. This could explain the large deviation in survival rates seen in experimental transplantation studies. However, at the beginning of ischemia the metabolic rate is high and greatly exceeds the critical structural maintenance level. This means that there is an unnecessary exhaustion of energy reserves and consequently an accumulation of glycolytic end products in the tissue. It thus seems more advantageous to modify the exponential course of the metabolic rate. This was shown by Singer and coworkers [11], who demonstrated that the decline of the metabolic rate of a cardioplegically arrested dog heart at 25°C was retarded, with an intermediate plateau building up, indicating that there was an equilibrium between energetic requirements and the energy supplied by anaerobic glycolysis. Therefore the metabolic rate remained above a critical level for a longer period of time and irreversible structural damage could be avoided. On the other hand, it is important to prevent a decline of the metabolic rate below a critical structural maintenance level.

The observed metabolic alterations are the consequence of energy requirements and energy supply by anaerobic glycolysis. Nevertheless, the ischemic damage could be reinforced by a microvascular injury during reperfusion [19,20]. There is evidence, that nonparenchymal cells are more vulnerable to ischemic injury than

parenchymal cells [21] and that they are involved in the reperfusion injury [22,23]. Reperfusion injury is mediated by the formation of oxygen radicals, which further damage endothelial cells, leading to an alteration of the microcirculation. Adhesion and activation of granulocytes entails a release of toxic mediators such as cytokines and proteases [24]. Furthermore, it is shown that there is significant free radical formation, even in the blood-free reperfused rat liver, after nonsurvival storage conditions [25]. In this study, isolated livers were reperfused for 30 min only in a nonrecirculating system with an erythrocyte- and leukocyte-free Krebs–Henseleit buffer. Accordingly, we have no information about late reperfusion injury mediated by polymorphonuclear cells [26]. Further investigation is therefore required to correlate the microcalorimetrically measured heat output of ischemic tissue with recovery after reperfusion in an in vivo model.

Acknowledgments

The authors thank I. Markmann, R. Dohrmann and G. Dallmayer for their skilful technical assistance, C. O'Connor for assistance in preparing the manuscript, and Prof. Dr G. Harbauer and the Department of Experimental Surgery of the University of the Saarland for competent technical assistance in carrying out the experiments. We thank PD Dr I. Marzi for helpful discussions and for reading the manuscript.

This study was supported in part by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 330–Organprotektion–Göttingen.

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