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β-Galactosidase as a Marker of Ischemic Injury and a Mechanism for Viability Assessment in Porcine Liver Transplantation

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Glycohydrolases are a group of enzymes contained predominantly within lysosomes, which are released during Kupffer cell activation or death. One of these, β-galactosidase, has been proposed as a marker of ischemia-reperfusion injury in the liver because Kupffer cell activation represents a primary event in the injurious reperfusion cascade. In this study, we compared B-galactosidase with more traditional indicators of liver injury and function in a porcine model of liver preservation. Porcine livers were allocated into two groups: group C (n = 5), preserved in University of Wisconsin solution by standard cold storage for 24 hours, and group W (n = 5), perfused with oxygenated autologous blood on an extracorporeal circuit for 24 hours. Both groups were subsequently tested on the circuit during a 24-hour reperfusion phase. The perfusate was sampled for levels of β-galactosidase, as well as traditional markers of liver injury and function. A sharp increase in β-galactosidase levels was seen on reperfusion of cold preserved livers to a level of 1,900 IU/mL. This contrasted dramatically with normothermically preserved livers, in which the level never exceeded 208 IU/mL ($P = .002$). β-Galactosidase levels showed much earlier and greater increases compared with transaminase levels in livers injured by ischemia. A rapid elevation in β-galactosidase levels corresponded well with poor liver function and more liver injury. Measurement of β-galactosidase is a simple test that quantifies ischemia-reperfusion injury of preserved livers. It is more sensitive than transaminases, with faster and larger increases in levels after ischemic injury. It can be useful in assessing the viability of a liver during machine preservation. (*Liver Transpl 2002;8: 21-26.*)

Liver preservation currently does not provide a practical method to assess whether an organ will function adequately after transplantation. Because the liver must function in a timely fashion for a recipient to survive, livers are taken from good donors and rejected from marginal donors. Under pressing circumstances, when more marginal donors are used, the risk for primary nonfunction is increased. Therefore, an effective means of viability assessment would allow for the use of more marginal donors and minimize the risk for primary nonfunction by identifying nonviable organs.

Glycohydrolases are cytosolic and lysosomal enzymes involved in carbohydrate metabolism that have been shown to increase markedly in serum after porcine liver transplantation.[1] A subsequent study specifically investigating the impact of warm ischemia time on glycohydrolase levels in the porcine model showed a correlation with ischemia time, and the pattern of recovery was different between livers that recovered function and those that did not.[2] The glycohydrolases found to be most useful in both these studies were β-galactosidase and β-glucoronidase.

In this study, we investigated the use of a normothermic extracorporeal circuit to perfuse livers and provide an ischemia-free preservation period compared with cold storage. We compare the increase and recovery of β-galactosidase with the level of function that livers regain after being subjected to a prolonged preservation period by either hypothermic storage in University of Wisconsin (UW) solution or normothermic oxygenated perfusion. As a surrogate for transplantation, we used reperfusion on the circuit to measure parameters of hepatic viability.

Materials and Methods

Animals

Twenty Landrace pigs (30 to 50 kg) were used in these experiments in accordance with the Animal Protection Act of 1986 in the United Kingdom.

Blood Donor

Blood donor pigs were placed under general anesthesia, the common carotid artery was cannulated, and blood was col-

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lected in citrated bags until circulation ceased. The blood was used to prime the circuit within 24 hours of collection.

Liver Donor

The standard practice for harvesting human donor organs was used.

Experimental Design

Porcine livers were harvested and flushed with 3 L of cold Eurocollins solution. To produce livers with contrasting amounts of ischemic injury, they were allocated to 24 hours of preservation by either ischemic storage or continuous perfusion as follows: group C (n = 5), cold storage for 24 hours in UW solution, and group W (n = 5), isolated perfusion circuit with whole blood at 38°C.

To mimic a realistic period of surgical reimplantation for livers preserved on the circuit in group W, blood perfusion was diverted from these livers through a bypass circuit at the end of the 24-hour preservation period. They were then flushed with 2 L of nonoxygenated cold Eurocollins solution and left for 45 minutes before being flushed with a warm plasma substitute and reperfused on the extracorporeal circuit to replicate the time of anastomosis. Livers in group C also were flushed with a warm plasma substitute to remove the UW solution and associated byproducts before being perfused on the circuit.

Perfusion Circuit

The circuit consisted of an oxygenator, heat exchanger, centrifugal pump, soft shell reservoir, and 1/4 inch tubing. During priming of the circuit, 9.2 mmol of calcium chloride, 20 mmol of sodium bicarbonate, and 7,000 U of heparin was used. A sample then was obtained for a full blood count and electrolytes, urea, creatinine, and liver function tests, and plasma was collected and stored in liquid nitrogen for a baseline value.

Continuous infusions to the circuit included prostacyclin at 4 mL/h, 2% taurocholic acid at 7 mL/h to replace lost bile acids, and total parenteral nutrition without lipids at 17 mL/h. Heparin (3,000 IU) was added every 4 hours. The liver was placed in an intestinal bag and suspended in saline in a sterile perfusion chamber. Two soft plastic catheters placed in the most dependent part of the intestinal bag were used to pump ascites back to the reservoir through a standard intravenous pump.

Initial pressures and flows within the portal vein (5 to 8 mm Hg, 1.3 to 1.7 L/min, respectively), hepatic artery (70 to 90 mm Hg, 0.3 to 0.5 L/min, respectively), and inferior vena cava (–2 to +2 mm Hg, 1.6 to 2.2 L/min, respectively) were kept within normal limits by adjusting the rotation speed of the centrifugal pump in both groups. Air and oxygen were each supplied to the oxygenator at a 0.5-L/min flow, resulting in 99% to 100% hemoglobin saturation in both groups.

Assessment of Liver Function

Perfusate was sampled every hour for the first 8 hours of the testing period and every 4 hours thereafter for the remainder of the 24-hour reperfusion phase. Assays were performed for β-galactosidase, electrolytes, and conventional liver function tests. Samples for a full blood count and factor V production were drawn every 2 hours during first 8 hours and every 4 hours thereafter. Blood glucose, arterial blood gases, venous blood gases, bile production, and hemodynamics were recorded every hour. Galactose clearance was measured at 12 and 24 hours of reperfusion. At the end of each perfusion, each liver was sectioned and assessed histologically for evaluation of tissue quality.

Measurement of β-Galactosidase

β-Galactosidase was measured using a specific substrate, 4-methylumbellifery-galactosidase, which is digested to a fluorescent product, 4-methylumbelliferone. Detection used a fluorescent microtiter plate reader with an excitation wavelength of 355 nm and emission wavelength of 460 nm. The reaction took place in a citrate-phosphate buffer, pH 4.4, optimized with a substrate concentration at 3.33 mmol/L. Specimen volume (10 μL) and substrate solution (80 μL) were incubated for 30 to 60 minutes at 37°C, when the reaction was terminated by the addition of 200 μL of glycine/sodium hydroxide, pH 12.8. The assay was linear up to 3,000 U/mL. Intra-assay coefficients of variation at 50, 502, and 2,012 U/mL were 4.73, 3.14, and 3.39, respectively. The interassay coefficient of variation at 493 U/mL was 3.84 (n = 5).

Histological Characteristics

At the end of reperfusion, the liver was weighed and sectioned throughout. Five random blocks (5 × 10 mm) were immersed in formal saline for paraffin sectioning and hematoxylin and eosin staining. Three independent blinded assessors examined each slide. Points were given for vacuolization, sinusoidal dilatation, sinusoidal congestion, and apoptosis as 0 points for absence, 1 point for mild (<33% of high-power field), 2 points for moderate (33% to 66% of high-power field), and 3 points for severe (>66% of high-power field). The same progressive scale was applied to necrosis, assigning 5, 10, and 20 points for mild, moderate, and severe, respectively. The overall score assigned to each group represents the mean score of five random blocks of tissue from five experiments.

Statistical Analysis

Statistical analysis was performed using Welch's *t*-test (assumption of unequal variances), with each time point reflecting the mean of five perfusions, and *P* of .05 or less defining significance.

Results

Hepatocellular Enzymes

Group C showed substantially greater evidence of hepatocellular damage, with increases in aspartate (AST)

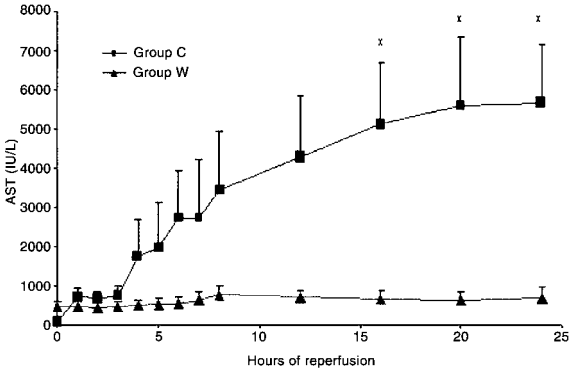

Figure 1. AST level comparison between groups C and W. A progressive increase in AST levels throughout the 24-hour reperfusion phase in livers preserved in UW solution becomes significant only after 16 hours of reperfusion. Each point represents the mean for five animals, with standard error bars shown in one direction. $^{x}P \leq .05$.

and alanine aminotransferase (ALT) levels. AST levels slowly elevated over the 24-hour reperfusion phase to a peak of 5,653 IU/L and did not reach statistical significance over the mean values of group W until 16 hours of reperfusion (Fig. 1). ALT levels peaked at 208 IU/L and were significantly greater than those of group W after 7 hours of reperfusion (Fig. 2).

β-Galactosidase

Curves of mean β-galactosidase levels clearly separate the two groups of livers (Fig. 3).

In group C, levels increased immediately on reperfusion to a statistically significant level after just 1 hour of reperfusion ($P = .003$). This increase continued for the next 4 hours of reperfusion to a plateau from 5 to 7 hours, with a mean value of 1,485 IU/L on this plateau. There was recovery over the remainder of the 24-hour

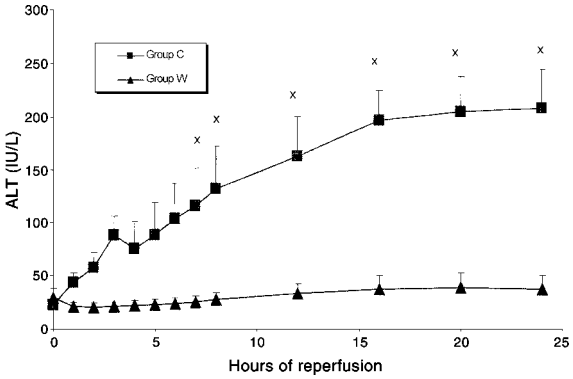

Figure 2. ALT levels of groups C and W. The increase in ALT levels in group C becomes statistically significant after 7 hours of reperfusion. $^{x}P \leq .05$.

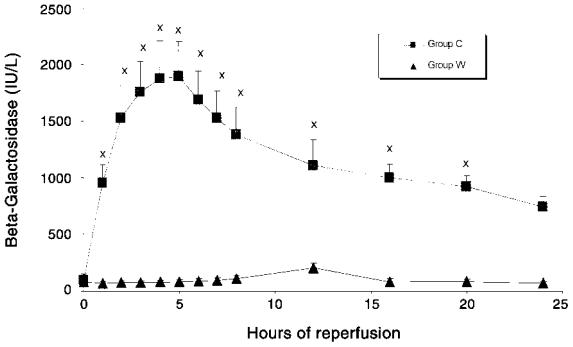

Figure 3. β-Galactosidase levels in groups C and W. Showing earlier and more profound increases than transaminase levels, the increase in β-galactosidase levels for group C becomes statistically significant within 1 hour of reperfusion. $^{x}P \leq .05$.

period to a value of 634 IU/L, but a statistically significant difference separated the two curves throughout the reperfusion phase. Group W maintained a flat curve throughout the reperfusion phase, never exceeding 138.95 IU/L, which was the mean value after 24 hours. The increase in β-galactosidase levels occurred well before the increase in hepatocellular enzyme levels. This is shown in Figure 4, comparing the fold of increase over baseline values for the three enzymes. Although β-galactosidase levels peaked at 5 hours of reperfusion, ALT and AST levels were still increasing at 24 hours.

Synthetic Function

Factor V production was grossly inferior in group C. Factor V levels diminished throughout the reperfusion

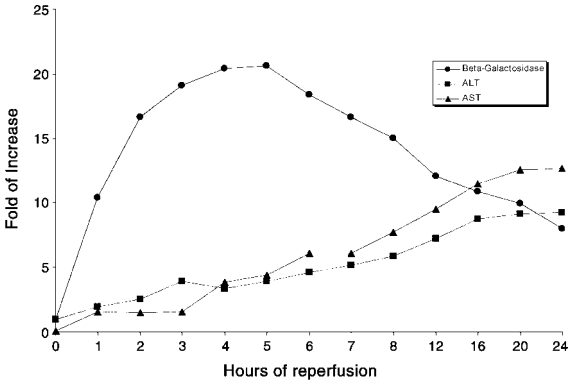

Figure 4. Enzyme levels in the perfusate of group C for β-galactosidase, AST, and ALT. The fold of increase over levels obtained at time zero show the relative amount of enzyme present in perfusate. The increase in β-galactosidase levels occurs before and to a greater degree than increases in transaminase levels in the ischemic-injured group C, showing that β-galactosidase is a more sensitive marker of ischemia-reperfusion injury.

period, suggesting poor de novo synthesis. However, group W showed an initial reduction in factor V levels, but then increased toward its initial value for the remainder of the reperfusion phase. Bile production was significantly less in group C throughout the reperfusion phase. Average production from 10 hours onward was 1.56 mL/h in group C and 5.66 mL/h in group W ($P < .05$).

Metabolic Function

Glucose concentrations in group C constantly remained at a supraphysiological level, in contrast to group W, in which levels declined during the first 10 hours, then stabilized for the remainder of the reperfusion phase. This difference was significant at all times.

Galactose clearance, measured spectrophotometrically, confirmed that elimination characteristics of galactose conform to a zero-order Michaelis-Menten profile. Vmax is calculated by extrapolating to the Y intercept to determine the gradient of decay.

The elimination rate in a zero-order reaction is independent of the substance concentration and reflects hepatic functional mass.[3]

In group C, the galactose Vmax mean value decreased during 12 hours of reperfusion from 87 to 65 mg/min/kg, whereas it improved in group W from 70 to 76 mg/min/kg. These latter values are similar to those of other groups analyzing Vmax on alloperfused livers[4] and 70% of values obtained in pigs in vivo.

Hemodynamic Parameters

Total flow through livers in group C was approximately 1.5 L/min during reperfusion, which decreased significantly from 20 hours onward. Conversely, the mean flow in group W remained stable at 2 L/min both initially and throughout the reperfusion period. There was no significant difference between the groups in hepatic arterial flow.

Portal pressures also increased in group C, possibly related to reperfusion injury and consequent increased sinusoidal resistance. No significant differences were seen in arterial pressures or resistance between the two groups.

Histological Characteristics

Group C showed large (>30%) amounts of necrosis on multiple wedge biopsies, whereas group W showed very little necrosis, although some sinusoidal dilatation and diffuse vacuolization were seen. Blinded scoring showed a mean score of 19 ± 1.84 for group C and 9 ± 2.1 for group W ($P \leq .05$).

Discussion

Livers stored under ischemic conditions (group C) resulted in poor functioning organs, evidenced by inferior bile production, factor V production, glucose use, and galactose clearance to livers perfused during preservation (group W). Group C livers experienced more cellular injury than group W livers, evidenced by hepatocellular enzyme histological characteristics. The reason for these differences is straightforward. Adenosine triphosphate (ATP) depletion is the critical step precipitating ischemic injury, which occurs during cold storage.[5] Although cold storage is effective in slowing metabolism and thus ATP depletion and the injurious cascades that follow, there still is considerable activity at 1°C.[6] Therefore, even under hypothermic conditions, ATP depletion is exacerbated, and the stage becomes set for reperfusion injury. By maintaining group W with oxygenated circulation, ATP levels are maintained; therefore, both ischemic and reperfusion injuries are essentially avoided. The avoidance of reperfusion injury in group W is shown by flat curves at the beginning of the reperfusion phase for hepatocellular enzyme and β-galactosidase levels.

Enzyme levels were not elevated in group W despite experiencing 45 minutes of ischemia between preservation and perfusion. This ischemic flush was intended to replicate surgical anastomosis, and the reason these livers tolerated the ischemic period so well was because cellular energy levels were restored during preservation and before the ischemic phase. In humans, adenine nucleotide content has been shown to decrease rapidly during the ischemic rewarming time of surgical anastomosis, and the level of recovery was inversely proportional to ischemia time.[7] Glycogen depletion in porcine livers during the rewarming period has been shown to be significantly dependent on glycogen stores at the beginning of the period.[8] Therefore, by replenishing ATP and energy stores before warm ischemia, the liver tolerates the ischemic flush as if little or no ischemia was experienced. Data supporting this notion in the rat model showed that 30 minutes of sanguineous perfusion before rewarming allowed livers preserved by cold storage for 18 hours to function immediately, with little evidence of reperfusion injury.[9]

This study found that β-galactosidase levels corresponded to the severity of ischemic injury, with group C livers following a curve an order of magnitude above group W livers (Fig. 3). β-Galactosidase level correlates with ischemic injury because of the role of endothelial and Kupffer cells in ischemic injury. ATP depletion during ischemia leads to disabled sodium-potassium

pumps, loss of electrolyte gradients, cellular edema, and calcium influx. Calcium activates enzymatic cascades, promoting inflammation and degradation pathways. Phospholipases involved in calcium-mediated injury are believed to come largely from Kupffer and endothelial cells during ischemia to the liver.[6] Kupffer cells represent 80% to 90% of the body's resident macrophages and have an enormous potential for releasing inflammatory mediators.[10] They are rich in lysosomes and release their lysosomal enzymes after activation.[11] β-Galactosidase is a lysosomal enzyme; therefore, Kupffer cells are believed to be the primary source of glycohydrolase level elevations seen immediately on reperfusion of ischemic tissue.[2] Evidence suggests that Kupffer and endothelial cells are particularly sensitive to ischemia and are the initial cells to become activated in ischemic injury, this preceding hepatocellular damage.[12,13]

Our data support this notion, showing increases in hepatocellular enzyme levels that clearly follow increases in β-galactosidase levels (Fig. 4). However, the hierarchy of ischemic susceptibility of endothelial, Kupffer, and parenchymal cells breaks down during warm ischemia, which has been shown to cause direct hepatocellular damage with relative preservation of endothelial cells. This implies that markers of endothelial injury (hyaluronidase) are poor predictors of graft function. However, this study did not investigate Kupffer cell activity, whereas previous studies of warm ischemia have shown Kupffer cell damage to precede hepatocellular damage, as in during cold ischemia, and therefore predict the amount of hepatocellular injury and graft survival.[2] It is reasonable to postulate that glycohydrolases mark Kupffer cell activation, which initiates injurious cascades leading to hepatocellular injuries that account for the increase in hepatocellular enzyme levels that subsequently occur. Therefore, markers of Kupffer cell injury and/or activation should be more sensitive markers of both warm and cold ischemic injury than hepatocellular enzymes.

Free radical formation and the subsequent damage created by their presence also must be considered when discussing ischemia-reperfusion injury. Without effective regeneration of ATP, ischemic metabolism of the nucleotide adenosine occurs, setting the stage for free radical formation when oxygen is reintroduced. Reperfusion then leads to massive free radical formation, leading to lipid peroxidation and cellular destruction.[14] This reaction is of particular importance in the liver because it holds the body's largest stores of ATP, as well as the critical enzyme involved in free radical formation (xanthine oxidase).[15] Furthermore, conversion of xanthine dehydrogenase (normal nucleotide metabolism) to xanthine oxidase (ischemic nucleotide metabolism) has been shown to occur most rapidly in Kupffer cells, lending further evidence to the leading role of their mechanism in ischemic injury.[16] Lipid peroxidation has been shown to follow the same pattern as β-galactosidase and β-glucosidase release in livers injured by ischemia-reperfusion, confirming that free radical reactions ensue after Kupffer cell activation and/or injury.[2] However, whether lipid peroxidation is the cause of cellular damage or simply an associated event has been disputed.[17] There is evidence that neutrophil accumulation and microvascular occlusion (no-reflow phenomenon) is the mechanism causing injury that results from free radical release.[18,19] Whichever is the case, it is still the presence of free radicals that presents danger, and these probably originate from Kupffer cells or as a result of their activation.

In conclusion, β-galactosidase is a sensitive marker of ischemia-reperfusion injury that correlates well with liver function. It is a more sensitive marker of ischemia-reperfusion injury than hepatocellular enzyme levels. It could be used as a viability marker if perfusion is used during the preservation period. Although data attained during isolated perfusion experiments are clearly compatible with superior livers, precise levels that predict survival will require a transplant model, experiments that are currently in progress.

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