Activated Kupffer cells cause a hypermetabolic state after gentle in situ manipulation of liver in rats

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Schemmer, Peter, Nobuyuki Enomoto, Blair U. Bradford, Hartwig Bunzendahl, James A. Raleigh, John J. Lemasters, and Ronald G. Thurman. Activated Kupffer cells cause a hypermetabolic state after gentle in situ manipulation of liver in rats. Am J Physiol Gastrointest Liver Physiol 280: G1076–G1082, 2001.—Harvesting trauma to the graft dramatically decreases survival after liver transplantation. Since activated Kupffer cells play a role in primary nonfunction, the purpose of this study was to test the hypothesis that organ manipulation activates Kupffer cells. To mimic what occurs with donor hepatectomy, livers from Sprague-Dawley rats underwent dissection with or without gentle organ manipulation in a standardized manner in situ. Perfused livers exhibited normal values for O2 uptake (105 ± 5 μmol·g−1·h−1) measured polarigraphically; however, 2 h after organ manipulation, values increased significantly to 160 ± 8 μmol·g−1·h−1 and binding of pimonidazole, a hypoxia marker, increased about threefold (P < 0.05). Moreover, Kupffer cells from manipulated livers produced three- to fourfold more tumor necrosis factor-α and PGE2, whereas intracellular calcium concentration increased two-fold after lipopolysaccharide compared with unmanipulated controls (P < 0.05). Gadolinium chloride and glycine prevented both activation of Kupffer cells and effects of organ manipulation. Furthermore, indomethacin given 1 h before manipulation prevented the hypermetabolic state, hypoxia, depletion of glycogen, and release of PGE2 from Kupffer cells. These data indicate that gentle organ manipulation during surgery activates Kupffer cells, leading to metabolic changes dependent on PGE2 from Kupffer cells, which most likely impairs liver function. Thus modulation of Kupffer cell function before organ harvest could be beneficial in human liver transplantation and surgery.

THE CAUSE OF FAILURE OF LIVER grafts is complex and includes many factors involving organ retrieval, preservation, and transplantation. Important factors include general condition and nutritional status of the donor, cold and warm ischemic times, operative complications in the recipient, and the experience of the surgeon (20, 33). Recently, gentle in situ liver manipulation during organ harvest, which cannot be prevented with standard harvesting techniques, has been shown to dramatically decrease survival after rat liver transplantation via mechanisms including hepatic injury with reperfusion (42). Interestingly, gadolinium chloride (GdCl3), a rare earth metal, and Kupffer cell toxicant, and glycine, a nontoxic amino acid, given to donors before organ harvest totally prevented all effects of manipulation (37, 39, 41, 42), suggesting a role for Kupffer cells in mechanisms of harvest-related injury. Once activated, Kupffer cells release toxic mediators such as proteases, tumor necrosis factor-α (TNF-α), and arachidonic acid derivatives (5, 8, 45), which could potentially impair liver function via mechanisms including disturbances to the microcirculation, hypoxia, increased oxygen consumption, and depletion of hepatic glycogen reserves (12, 24, 25, 29). Hypoxia and increased oxygen consumption (e.g., development of a hypermetabolic state) impair graft survival after transplantation (25, 31). Moreover, Fusaoka et al. (13) showed that activation of Kupffer cells increases oxygen uptake of the liver after cold storage. This effect is most likely due to Kupffer cell-derived PGE2, which stimulates oxygen uptake in hepatic parenchymal cells and could be involved in early dysfunction of a graft (13, 35). Therefore, the purpose of this study was to directly test the hypothesis that the operative trauma due to surgical manipulation in situ of donor livers activates Kupffer cells before transplantation. Preliminary accounts of this work have been published elsewhere (40).

MATERIALS AND METHODS

Experimental animals and treatment. Female Sprague-Dawley rats (200–230 g) were allowed free access to standard laboratory chow (Agway PROLAB RMH 3000, Syracuse, NY) and tap water. Some animals were given a single injection of GdCl3 (10 mg/kg) through the tail vein 24 h before surgery.
This treatment destroys all large Kupffer cells (15). Other rats were fed chow diet containing 5% glycine for 5 days, which blunts the response of Kupffer cells to endotoxin (18). Furthermore, some rats were given indomethacin (3.0 mg/kg, in dimethyl sulfoxide) intragastrically 1 h before experiments (6).

Surgical procedures. After midline incision, minimal dissection of livers was performed in a standardized fashion during the first 12 min, including freeing the organ from ligaments. During the subsequent 13 min, livers were either left alone in controls or manipulated gently. To maintain standard conditions, gentle manipulation was carried out by the same surgeon touching, retracting, and moving the liver lobes in situ for a specified time interval. Care was taken to use the same number of manipulations in each experiment with similar pressures. Serum transaminases at the end of manipulation were identical regardless of pretreatment, validating the standardization of the technique (42).

Nonrecirculating hemoglobin-free liver perfusion. The perfusion technique has been described elsewhere (43). Briefly, the liver is perfused ex situ with oxygenated (95% O2-5% CO2) Krebs-Henseleit bicarbonate buffer (in mM: 118 NaCl, 25 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, 4.7 KCl, and 1.3 CaCl2) at pH 7.4. The oxygen concentration in the effluent perfusate was maintained continuously with a Teflon-shielded platinum electrode. The inflow oxygen concentration was maintained constant and measured before and after each experiment. Metabolic rates were calculated from influent-effluent concentration differences and the constant flow rate and expressed per gram of liver weight per hour (43).

Isolation and culture of Kupffer cells. Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described elsewhere (32). Immediately or 2 h after organ manipulation, livers were perfused in situ via the portal vein with Ca2+ - and Mg2+ -free Hanks’ balanced salt solution containing collagenase IV (0.025%) (Sigma Chemical, St. Louis, MO) at 37°C at a flow rate of 26 ml/min. After digestion, livers were cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze, and the filtrate was centrifuged at 450 g for 10 min at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by centrifugation at 50 g for 3 min, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 3,000 g for 15 min. The Kupffer cell fraction was collected and washed with buffer again. Viability of cells determined by trypan blue exclusion was >90%. Cells were seeded onto 25-mm2 glass coverslips and cultured in DMEM (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate) at 37°C with 5% CO2. Non-adherent cells were removed after 1 h by replacing buffer and cells were cultured for 24 h before experiments. All adherent cells phagocytized latex beads, indicating that they were Kupffer cells.

Measurement of intracellular calcium. Intracellular calcium concentration ([Ca2+]i) was measured fluorometrically using the calcium indicator dye fura-2 and a microspectrofluorometer (PTI, South Brunswick, NJ) interfaced with an inverted microscope (Diaphot, Nikon, Tokyo, Japan). Kupffer cells were incubated in modified Hank's buffer (115 mmol/l NaCl, 5 μmol/l KCl, 0.3 mmol/l Na2HPO4, 0.4 mmol/l KH2PO4, 5.6 mmol/l glucose, 0.8 mmol/l MgSO4, 1.26 mmol/l CaCl2, and 15 mmol/l HEPES, pH 7.4) containing 5 μmol/l fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR) and 0.03% Pluronic F-127 (BASF, Wyandotte, MI) at room temperature for 60 min. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of fura 2 at excitation wavelengths of 340 and 380 nm and emission at 510 nm were monitored in individual Kupffer cells. Each value was corrected by subtracting the system dark noise and autofluorescence, assessed by quenching fura 2 fluorescence with Mn2+, as described previously (17). [Ca2+]i was determined from the following equation: [Ca2+]i = Kd(R - Rm)/(Rmax - R) / (Fm/Fo), where Fm/Fo is the ratio of fluorescent intensities evoked by 380 nm light from fura 2 pentapotassium salt loaded in cells using a buffer containing 3 mmol/l EGTA and 1 μmol/l ionomycin (([Ca2+]i min) or 10 mmol/l Ca2+ and 1 μmol/l ionomycin ([Ca2+]i max). R is the ratio of fluorescent intensities at excitation wavelengths of 340 and 380 nm, and Rmax and Rmin are values of R at [Ca2+]i max and [Ca2+]i min, respectively. The values of these constants were determined at the end of each experiment, and a dissociation constant (Kd) of 135 mmol/l was used (14).

Non-adherent cells of TNF-α-activated isolated Kupffer cells were cultured for 24 h in 24-well culture plates (Corning, NY) at a density of 5 × 106 cells/well in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37°C in the presence of 5% CO2. Subsequently, cells were incubated with fresh media containing lipopolysaccharide (LPS) (100 ng/ml in 5% rat serum) for an additional 4 h. Samples were stored at −80°C until assay. TNF-α concentrations were determined in the culture media using an enzyme-linked immunosorbent assay kit (Genzyme, Cambridge, MA). Furthermore, supernatants were assayed for PGE2 by competitive radioimmunoassay using 125I-labeled PGE2 (Advanced Magnetics, Cambridge, MA).

Clinical chemistry. Tissue was homogenized, and glycogen was hydrolyzed and determined enzymatically (3). Furthermore, blood was collected before experiments for glucose determination in serum as described previously (3). Briefly, glycine was extracted and benzoylated, and the resulting hippuric acid was extracted and dried. Subsequently, hippuric acid was determined spectrophotometrically at 458 nm (30).

Determination of reduced, protein-bound pimonidazole by ELISA and immunohistochemistry. Pimonidazole, a non-vasive 2-nitroimidazole marker for viable hypoxic cells (11), was given to donors intravenously to detect hypoxia in liver tissue 2 h after organ manipulation. Five minutes before tissue samples were collected, pimonidazole was given to donors and pimonidazole adduct accumulation was measured in tissue homogenates with a competitive ELISA procedure described previously (36) as modified for liver tissue (2). Protein levels in tissue homogenates were determined with the biocinchonic acid assay using a commercially available kit (Pierce Chemical, Rockford, IL). Paraffin blocks of formalin-fixed liver tissue were sectioned at 6 μm, and pimonidazole was detected with a biotin-streptavidin-peroxidase indirect immunostaining method using diaminobenzidine as a chromogen as described previously (2). After the immunostaining procedure, a counterstain of hematoxylin was applied. A Universal Imaging Image-I/AT image acquisition and analysis system (Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Thornwood, NY) was used to capture and analyze the immunostained tissue sections at ×100 magnification (1). Whereas results of ELISA give only the quantity of bound pimonidazole, immunohistochemical analysis for pimonidazole demonstrates patterns of adduct binding in the liver lobule.

The number of Kupffer cells was determined immunohistochemically as described elsewhere (42). Briefly, sections (6
G1078 ORGAN MANIPULATION ACTIVATES KUPFFER CELLS

Gentle in situ manipulation causes hypoxia in the liver. Pimonidazole, a 2-nitroimidazole hypoxia marker, binds to viable hypoxic liver cells in vivo (2, 11, 36). Binding of pimonidazole in nonmanipulated controls was 196 ± 28 pmol/mg protein. Treatment with GdCl3, glycine, or indomethacin had no effect on binding of pimonidazole under these conditions. Furthermore, binding of pimonidazole was increased more than twofold (P < 0.05) in livers 2 h after gentle manipulation (Fig. 2); however, binding was not different from controls if GdCl3, dietary glycine, or indomethacin was given before manipulation (Fig. 2). Binding was concentrated in oxygen-poor pericentral regions of the liver lobule after manipulation (data not shown).

Hepatic glycogen is depleted after gentle organ manipulation. Two hours after surgery without manipulation, hepatic glycogen levels were ~4 mg/g. Treatment with GdCl3, glycine, or indomethacin had no effect on liver glycogen under these conditions. In contrast, gentle organ manipulation significantly depleted glycogen to ~25% of control values 2 h after surgery (Fig. 3). GdCl3, dietary glycine, and indomethacin given before surgery totally prevented the effect of organ manipulation on hepatic glycogen levels (Fig. 3).

Kupffer cells are activated after gentle organ manipulation. LPS (100 ng/ml) increased [Ca2+]i in Kupffer cells significantly from 89 ± 9 nM in nonmanipulated controls to 182 ± 11 nM in cells from manipulated organs (Figs. 4 and 5); however, dietary glycine given before organ manipulation blunted the increase of [Ca2+]i (Figs. 4 and 5). In contrast, this phenomenon was not prevented by indomethacin (Figs. 4 and 5).

**Fig. 1.** Rates of oxygen uptake after surgery from manipulated livers. Two hours after surgery, livers were isolated and perfused with Krebs-Henseleit buffer (37°C; pH 7.6) for 35 min at 32 ml/min. Oxygen uptake was measured continuously with a Clarke-type electrode. Values reached steady state after ~5 min, and rates were calculated as described in MATERIALS AND METHODS. Values are means ± SE (P < 0.05 by 2-way ANOVA with Student-Newman-Keuls post hoc test; n = 4 rats). *P < 0.05 for comparison to nonmanipulated group. **P < 0.05 compared with manipulated group without pretreatment.

**Fig. 2.** Effect of gentle organ manipulation on hypoxia reflected by pimonidazole binding. Conditions are as described in Fig. 1. To detect hypoxia in liver tissue, pimonidazole (120 mg/kg ip), a 2-nitroimidazole hypoxia marker, was injected 2 h after surgery, and liver tissue was collected 5 min later. Pimonidazole binding was detected by using competitive ELISA. Some donors were treated with GdCl3, glycine, or indomethacin before manipulation as described in MATERIALS AND METHODS. Values are means ± SE; n = 5 rats. *P < 0.05 for comparison to nonmanipulated group. **P < 0.05 compared with manipulated group without pretreatment.
Gentle manipulation of the liver increases TNF-α and PGE₂ production from Kupffer cells. To evaluate the effect of organ manipulation on cytokine production by Kupffer cells, LPS-induced TNF-α and PGE₂ production was measured in culture medium of isolated Kupffer cells. Isolated Kupffer cells from manipulated livers produced 9- to 30-fold more PGE₂ and TNF-α in the presence of LPS (100 ng/ml) than did cells from minimally dissected livers (P < 0.05); however, glycine given to rats before organ manipulation significantly blunted these effects (Figs. 5 and 6). In contrast, indomethacin had no effect on TNF-α production (Fig. 5), whereas increased PGE₂ production by Kupffer cells from manipulated livers was totally prevented by indomethacin as expected (Fig. 6; P < 0.05).

DISCUSSION

Gentle in situ manipulation of liver activates Kupffer cells. Primary nonfunction and dysfunction occur in 5–30% of human liver transplantation cases leading to significant morbidity and mortality (33); however, underlying mechanisms are largely unknown but most likely involve Kupffer cells, which play a role in the development of reperfusion injury and primary nonfunction (24). Furthermore, the donor operation and surgical technique most likely have an effect on outcome after transplantation. Even laparotomy with mild abdominal exploration and preparation of the portal vein alone impair the intrahepatic circulation (9,

Fig. 3. Effect of gentle organ manipulation on tissue glycogen after surgery. Conditions are as described in Fig. 1. Two hours after surgery liver tissue was collected and glycogen was measured as described in MATERIALS AND METHODS. Values are means ± SE (P < 0.05 by 2-way ANOVA with Student-Newman-Keuls post hoc test; n = 4 rats). aP < 0.05 for comparison to nonmanipulated group. bP < 0.05 compared with manipulated group without pretreatment.

Fig. 4. Effect of lipopolysaccharide (LPS) on intracellular calcium concentration ([Ca²⁺]i) in Kupffer cells. Conditions are as described in Fig. 1. Two hours after surgery, Kupffer cells were isolated and cultured for 24 h, and increases of [Ca²⁺]i were compared after LPS (final concentration, 100 ng/ml) using the fluorescent Ca²⁺ indicator fura 2 as described in MATERIALS AND METHODS. A: no manipulation. B: manipulation. C: 5% dietary glycine given to rats for 5 days before organ manipulation. D: indomethacin (3.0 mg/kg ig) given 1 h before manipulation. These represent typical experiments.

Fig. 5. Effect of organ manipulation on [Ca²⁺], in Kupffer cells isolated from manipulated livers. Conditions are as described in Fig. 1. Top: peak levels of [Ca²⁺], detected with fluorometry. Bottom: tumor necrosis factor-α (TNF-α) from cultured Kupffer cells 4 h after LPS (100 ng/ml) expressed as fold increase over basal levels. Values are means ± SE (P < 0.05 by 2-way ANOVA with Student-Newman-Keuls post hoc test; n = 4–7 rats). aP < 0.05 for comparison to nonmanipulated group. bP < 0.05 compared with manipulated group without pretreatment.
21–23). This is important, because hypoxia can activate Kupffer cells (26). Indeed, in a recent study, gentle in situ liver manipulation during organ harvest rapidly disturbed intrahepatic microcirculation and hypoxia developed rapidly as a result of vasoconstriction caused by nerves to the liver (Fig. 7) (38). These immediate effects of organ manipulation increased injury to the liver upon reperfusion and decreased survival after liver transplantation dramatically via mechanisms involving Kupffer cells (39, 41, 42). Both denervation of the liver and inactivation of Kupffer cells with GdCl3 and dietary glycine prevented all detrimental effects of organ manipulation. Thus it is possible that stimulation of nerves to the liver may be involved in the development of Kupffer cell-dependent injury on reperfusion as a result of in situ liver manipulation during harvest for transplantation (39) (Fig. 7). However, the exact underlying mechanisms by which liver becomes predisposed for failure after manipulation still remain unclear. Therefore, this study was designed to mimic what occurs during donor hepatectomy and to investigate its effects on Kupffer cells. Interestingly, oxygen consumption (Fig. 1) and hypoxia (Fig. 2) were increased significantly, whereas hepatic glycogen was depleted (Fig. 3), 2 h after manipulation. Under these conditions, Kupffer cells, the major source of eicosanoids and cytokines in the liver (26), were activated by manipulation reflected by increased [Ca\(^{2+}\)]\(_i\) (Figs. 4, 5, and 7), TNF-\(\alpha\) (Fig. 5), and PGE\(_2\) production (Figs. 6 and 7).

PGE\(_2\) from activated Kupffer cells is responsible for metabolic changes in liver. How can this be explained? Activation of Kupffer cells by organ manipulation increases [Ca\(^{2+}\)]\(_i\) in Kupffer cells. It is well established that Ca\(^{2+}\) activates phospholipases, leading to increased synthesis of TNF-\(\alpha\) and PGE\(_2\) (4, 10). Qu et al. (35) have shown that PGE\(_2\) from Kupffer cells stimulates oxygen uptake in parenchymal cells, whereas TNF-\(\alpha\) was without effect. PGE\(_2\) acts on receptors in
parenchymal cells to stimulate mitochondrial respiration via second messenger systems most likely involving cAMP. As a result, oxygen uptake increased, which can partially be explained by enhanced demand of mitochondrial oxidative phosphorylation for oxygen to compensate for reduced extramitochondrial ATP production due to inhibition of glycolysis due to substrate depletion. Indeed, in this study, manipulation increased oxygen uptake (Fig. 1) at the time when Kupffer cells were activated (Figs. 4 and 5) and production of PGE$_2$ increased (Fig. 6). Whereas hypoxia immediately after organ manipulation may be due to vasocostriction mediated by nerves to the liver (38), hypoxia concentrated in pericentral areas measured 2 h after manipulation is most likely due to a hypermetabolic state, which causes a steeper oxygen gradient along the hepatic sinusoid (Fig. 2). Furthermore, depletion of hepatic glycogen during manipulation can be explained by both hypoxia and increased PGE$_2$ production, which causes glycogenolysis (16, 27, 29) (Fig. 3). To test the hypothesis that PGE$_2$ from Kupffer cells was responsible for changes after organ manipulation, donors were pretreated with GdCl$_3$, a rare earth metal, and Kupffer cell toxicant (15), dietary glycine, a nonessential amino acid that prevents activation of Kupffer cells (19), or indomethacin, an inhibitor of cyclooxygenase, which prevents PGE$_2$ production (35). Indeed, GdCl$_3$ and glycine prevented the hypermetabolic state (Fig. 1), hypoxia (Fig. 2), depletion of glycogen (Fig. 3), activation of Kupffer cells (Figs. 4 and 5), and increased PGE$_2$ production (Fig. 6) in manipulated livers (Fig. 7). These data suggest that PGE$_2$ from activated Kupffer cells most likely mediates the metabolic changes (e.g., respiratory burst, glycogenolysis) and hypoxia observed 2 h after organ manipulation (Fig. 7).

Possible relationship between gentle organ manipulation and viability of the graft. The vulnerability of liver to hypoxic injury is greatly affected by nutritional status. Thurman et al. (44) have shown that the hypermetabolic state induced by ethanol results in hepatic glycogen depletion within a few hours. Such livers were much more susceptible to anoxic injury (25, 44). In contrast, glycogen-rich livers from fed animals were much more susceptible to anoxic injury (25, 24, 29, 34) and livers manipulated gently during harvest fail often after transplantation (37, 41, 42), modulation of Kupffer cell function with the nontoxic amino acid glycine before organ harvest could be beneficial in clinical liver transplantation.

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REFERENCES