Transplant Int (1991) 4: 215–220

TRANSPLANT
International

Methyl palmitate prevents Kupffer cell activation and improves survival after orthotopic liver transplantation in the rat

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Received December 12, 1990/Received after revision May 7, 1991/Accepted May 22, 1991

Abstract. The purpose of this study was to determine whether prevention of Kupffer cell activation following orthotopic liver transplantation improves postoperative survival. First, particle phagocytosis by Kupffer cells was monitored continuously from the uptake of colloidal carbon by the perfused liver. Unstored livers took up carbon at rates of around 150 mg/g per hour, whereas storage for 24 h in Euro-Collins solution nearly doubled values to about 290 mg/g per hour. Treatment of rats with methyl palmitate, an inhibitor of phagocytosis by Kupffer cells, reduced carbon uptake to about one-third to one-half of control values in unstored and stored livers, respectively. Oxygen uptake, which was increased about 25% in stored and unstored livers by infusion of colloidal carbon, was only increased 5%–10% in both groups following treatment with methyl palmitate, suggesting that Kupffer cell activation was prevented by methyl palmitate. In livers transplanted after storage for 6 h in Euro-Collins solution (nonsurvival conditions), control rats survived only about 12 h, while treatment with methyl palmitate increased survival time significantly – more than threefold – to about 40 h. These data are consistent with the hypothesis that activation of Kupffer cells following cold ischemic storage and reperfusion is an early event involved in liver graft failure.

Key words: Liver transplantation, rat, Kupffer cell – Kupffer cell, liver transplantation, rat – Methyl palmitate, liver transplantation, rat – Reperfusion injury, liver transplantation, rat

It is important to increase storage time and organ quality for liver transplantation, a therapy that is becoming more and more accepted for an increasing number of liver diseases [22]. To achieve this goal, it is necessary to understand the mechanism(s) of graft failure. Recently, it was demonstrated that cold storage leads to reperfusion injury to sinusoidal endothelial cells but spares parenchymal cells [5, 7, 13]. This injury is associated with a significant increase in leukocyte adhesion to the sinus endothelium [19]. In support of the possibility that a reperfusion injury occurs following liver transplantation, we observed that rinsing the organ with oxygen-free buffer prior to implantation reduced postoperative disturbances in microcirculation and diminished cell injury dramatically [23]. Moreover, xanthine and hypoxanthine, substrates for free-radical generation, accumulated during cold storage with a time course similar to endothelial cell death and were removed by rinsing with oxygen-free buffer [14].

Recent experiments have demonstrated that ischemic cold storage activates Kupffer cells morphologically (i.e., surface ruffling, extension of pseudopodia, and the release of intracellular granules) in vitro [12]. It is well known that activated Kupffer cells release a number of highly toxic mediators, including proteases, leukotrienes, and tumor necrosis factor [4, 8, 27]. Thus, it is possible that reperfusion of cold-stored grafts with oxygen following liver transplantation leads to activation of Kupffer cells and, hence, to the release of cytotoxic mediators.

Recently, a method was developed to monitor phagocytic activity of Kupffer cells continuously in the isolated, perfused liver [10, 24]. This method is based on continuous measurement of the absorbance of colloidal carbon in the effluent perfusate and allows calculation of rates of carbon uptake (i.e., rates of particle phagocytosis) by Kupffer cells. The purpose of this study, therefore, was to evaluate the hypothesis that Kupffer cells are activated rapidly on reperfusion following cold storage, and that this triggers processes that lead to release of toxic mediators, endothelial cell death, microcirculatory disturbances, and, ultimately, graft failure.

Methods

Animals and treatment

Female Lewis rats (200–250 g) were used for all perfusion and transplantation experiments. A stock suspension of methyl palmitate (100 mg/ml; Sigma) was sonicated in Ringer's solution containing

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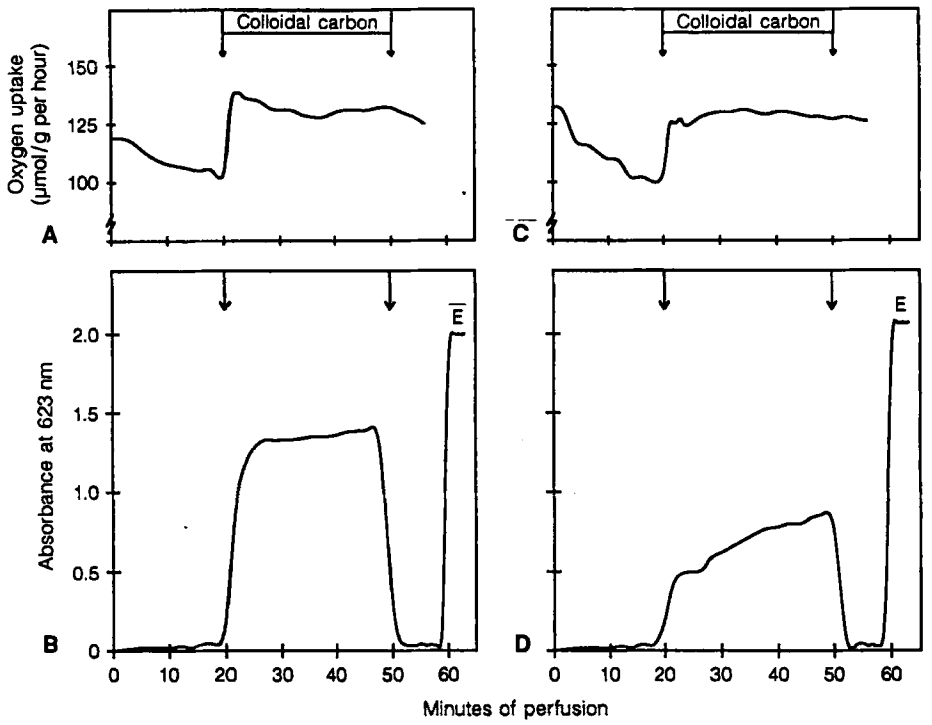

Fig. 1 A-D. Oxygen and carbon uptake by the isolated perfused rat liver. Livers were perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) in a nonrecirculating system. After 20 min, this was replaced with buffer containing 2.4 mg/ml colloidal carbon. Oxygen concentration in the effluent perfusate was monitored continuously with a Clark-type oxygen electrode (**A, C**) and absorbance of colloidal carbon in the effluent perfusate was monitored at 623 nm (**B, D**). Rates of uptake were calculated from influent minus effluent concentration differences, the constant flow rate, and the wet weight of the liver. **A, B** Unstored livers; **C, D** livers stored for 24 h in Euro-Collins solution. Oxygen traces are averages from five to six livers per group. Absorbance of colloidal carbon represents typical experiments. *E* Effluent carbon concentration in the absence of a liver

5% dextrose and 0.2% Tween 20 immediately prior to intravenous injecton (0.15 or 0.3 g/kg).

Liver perfusion

Livers were perfused with Krebs-Henseleit buffer (pH 7.4, 37°C), saturated with 95% O_2/5% CO_2 in a hemoglobin-free, nonrecirculating system, as described previously [18]. Perfusate was pumped into the liver via a cannula placed in the portal vein, and effluent perfusate was collected via a cannula placed in the inferior vena cava. Oxygen concentration in the effluent perfusate was monitored continuously employing a Clark-type O_2 electrode, and oxygen uptake by the liver was calculated from the influent minus effluent O_2 concentration differences, the flow rate, and the wet weight of the liver.

Uptake of collodial carbon

A suspension of colloidal carbon was prepared by dialyzing 10–15 ml of India ink (Pelikan black no. 17) against distilled water for 48 h utilizing a Spectrapor semipermeable membrane with a 12,000–14,000 molecular weight exclusion cut-off (Spectrum Medical Industries, Los Angeles, Calif., USA). The suspension was stored at 4°C for up to 30 days before use and was diluted in Krebs-Henseleit buffer at a concentration of 2 mg/ml prior to use. Absorbance of carbon in the perfusate was measured at 623 nm, and rates of carbon uptake were calculated from influent minus effluent concentration differences, the flow rate, and the wet weight of the liver [10, 24].

Transplantation

Liver transplantations were performed according to the technique described by Zimmermann et al. [28] and Kamada and Calne [11]. Briefly, livers were removed and cuffs were placed on the portal vein and subhepatic vena cava of donor livers. Organs were stored in Ringer's, Euro-Collins, or University of Wisconsin (UW) cold storage solution at 0°–4°C for varying periods of time (Figs. 1–5). Subsequently, livers were transplanted by connecting the suprahepatic vena cava with a running suture, inserting the cuffs into appropriate vessels, and anastomosing the bile duct with an intraluminal splint. The ischemic interval due to clamping of the portal vein during transplantation did not exceed 30 min.

Results

Effect of cold storage on uptake of carbon by perfused rat liver

To assess phagocytic activity of Kupffer cells, rat livers were perfused with buffer containing colloidal carbon

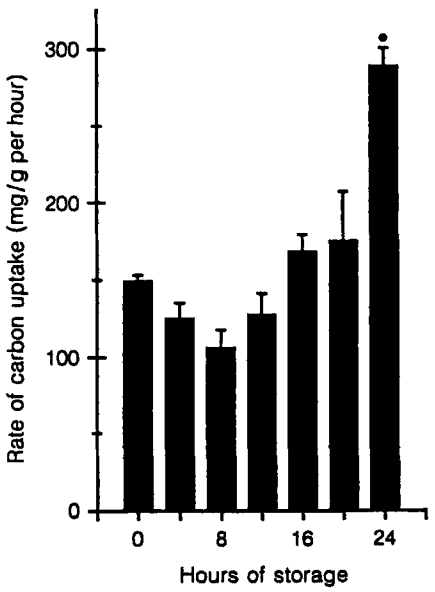

Fig. 2. Effect of storage time in Euro-Collins and University of Wisconsin (UW) cold storage solutions on rates of carbon uptake by perfused rat liver. Conditions as in Fig. 1, except that livers were stored for times indicated on the abscissa. Four to six livers per group. * $P < 0.05$ for comparison with unstored group

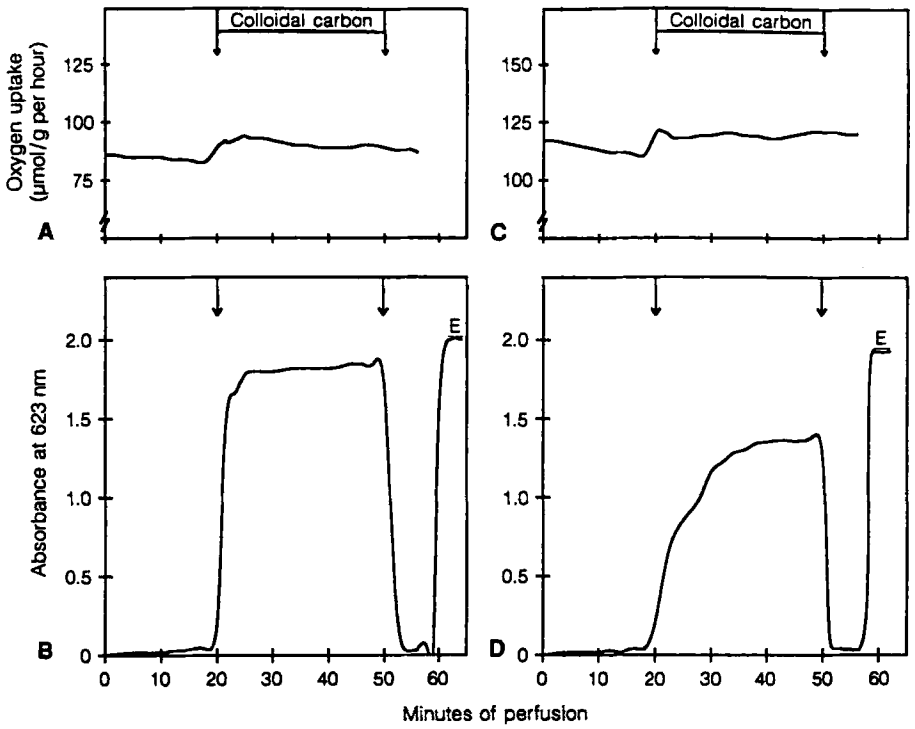

Fig. 3 A-D. Effect of treatment with methyl palmitate on oxygen and carbon uptake by perfused rat liver. Rats were treated for 4 days with methyl palmitate. Other conditions as in Fig. 1. Oxygen traces are averages from four livers per group. Absorbance traces of colloidal carbon are typical experiments

(Fig. 1). In unstored livers, basal rates of oxygen uptake of about 100 μmol/g per hour increased rapidly by about 30–40 μmol/g per hour upon infusion of colloidal carbon (2.4 mg/ml), most likely due to release of cyclo-oxygenase products (Fig. 1 A), since prostaglandins have previously been shown to increase oxygen uptake [10, 16]. Concomitantly, absorbance of carbon in the effluent perfusate increased, reaching steady-state values after about 10 min. When carbon infusion was terminated, absorbance rapidly returned to basal values. After the liver was removed, influent carbon concentration was determined optically, demonstrating that substantial amounts of colloidal carbon were cleared by the liver (Fig. 1 B; *E*, effluent value). From the influent minus effluent concentration difference and the flow rate, calculated rates of carbon uptake were about 150 mg/g per hour in unstored livers (Fig. 2). In livers that had been stored for 24 h in Euro-Collins solution, the increase in oxygen uptake due to carbon infusion was similar to values in unstored liver (Fig. 1 C). However, absorbance values of carbon in the effluent perfusate were much lower (i.e., the influent minus effluent difference was larger, reflecting higher rates of carbon uptake; Fig. 1 D), and it took nearly 30 min to reach steady-state values.

Livers stored for 24 h in Euro-Collins solution took up nearly twice as much carbon as unstored livers (Fig. 2). Similar results were observed in livers stored in University of Wisconsin (UW) cold storage solution (unstored liver: 99 ± 13 mg/g per hour; 24 h cold storage: 187 ± 18 mg/g per hour).

Effect of methyl palmitate on carbon uptake

Earlier studies have demonstrated that methyl palmitate, a nonhydrolyzable fatty acid ester, inhibits phagocytosis by Kupffer cells in vivo [1]. Therefore, the effect of methyl palmitate on carbon uptake following cold storage and transplantation was evaluated in perfused liver. Rats were treated with 300 mg/kg for 1 day or with 300 mg/kg on day 1, followed by 150 mg/kg daily for 3 subsequent days. Treatment with methyl palmitate for 1 or 4 days decreased basal rates of oxygen uptake significantly from 100 ± 11 to 67 ± 7 μmol/g per hour ($n = 6$) and diminished the increase in oxygen uptake due to infusion of carbon by about 50 %

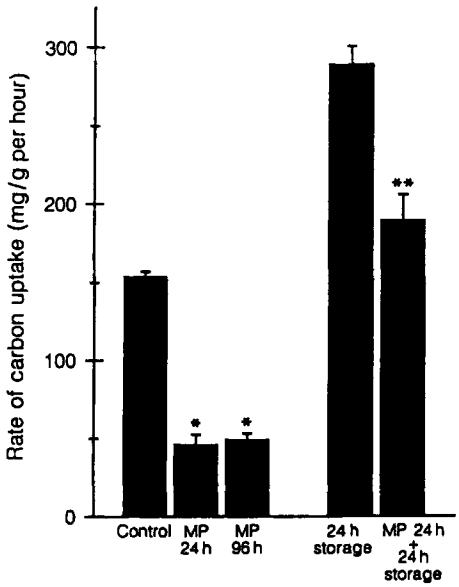

Fig. 4. Effect of methyl palmitate (*MP*) on carbon uptake in unstored and stored livers. Conditions same as in Fig. 3. Twenty-four and 96 h are times of treatment with methyl palmitate. *Control* Unstored liver. *Storage* 24 h in cold Euro-Collins solution. * $P < 0.05$ for comparison with control group; ** $P < 0.05$ for comparison with 24 h storage group

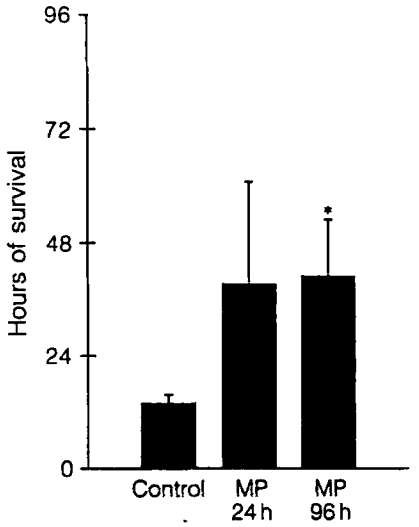

Fig. 5. Effect of methyl palmitate (*MP*) treatment on survival time following orthotopic transplantation of livers stored in Euro-Collins solution. Livers were removed from donor rats and stored in cold Euro-Collins solution for 6 h prior to implantation. Where indicated, rats were treated with methyl palmitate for 24 or 96 h. Five to six livers per group. * $P < 0.05$ for comparison with control group

(Fig. 3). Moreover, this treatment resulted in increased absorbance due to carbon in the effluent perfusate, reflecting a reduction in carbon uptake of nearly 70% (Fig. 3 B). In livers stored in Euro-Collins solution, the time the optical signal due to carbon took to reach new steady-state levels was also longer following methyl palmitate pretreatment (Fig. 3 D). Importantly, elevated rates of carbon uptake observed after 24 h of cold storage in Euro-Collins solution were diminished by about 50% by prior treatment with methyl palmitate (Figs. 3 D, 4).

Effect of methyl palmitate on graft survival after liver transplantation

It is well known that survival of rat liver grafts stored in the cold for 1 h is virtually 100% [23]. In marked contrast, storage for 6 h in cold Euro-Collins solution prior to implantation surgery resulted in nearly 100% graft failure in less than 1 day using the transplantation procedure described by Kamada and Calne [11]. To assess whether activation of Kupffer cells is involved in the mechanism of graft failure, rats were pretreated with methyl palmitate for 4 days to diminish Kupffer cell function. Subsequently, livers were stored in Euro-Collins solution for 6 h prior to transplantation. Survival time of recipient rats with transplanted livers stored for 6 h in Euro-Collins solution averaged about 12 h (Fig. 5). In contrast, pretreatment of donor rats with methyl palmitate for 4 days prior to surgery significantly increased survival time threefold (40 h; $P < 0.05$). In livers stored under nonsurvival conditions in Euro-Collins solution for 4 h, the endothelium was torn, hepatocytes were shrunken, and microvascular thrombi were apparent (data not shown). Following treatment with methyl palmitate, tissue ultrastructure appeared more normal.

Discussion

Activation of Kupffer cells by reperfusion following cold storage

A reperfusion injury resulting in microcirculatory disturbances and cell death following liver transplantation in the rat in vivo has been described [23]. Since reperfusion activated Kupffer cells, caused disturbances in the microcirculation, and damaged the graft, links between these parameters may exist. Activated Kupffer cells phagocytose particles and, more importantly, produce and release various cytotoxic mediators (e.g., tumor necrosis factor) and oxygen radicals during reperfusion after ischemia [9]. Phagocytic activity of Kupffer cells is known to be coupled with the release of inflammatory mediators [26]. Kupffer cells were also activated in a model involving rearterialization when oxygen delivery to the graft should be increased [2].

One of the notable findings of this study was a time-dependent activation of Kupffer cells by cold storage in Euro-Collins solution. In experiments with livers reperfused after only 24 h of cold storage, phagocytic activity of Kupffer cells was essentially doubled. Several lines of evidence indicate that this activation results from reperfusion. First, Kupffer cells were not activated morphologically following 24 h of cold storage without reperfusion. Second, activated Kupffer cells could be detected by electron microscopy following brief periods of reperfusion with oxygenated buffer [12].

A reasonable explanation for this postoperative disturbance in microcirculation is activation of Kupffer cells. It is well known that activation of Kupffer cells by various agents (e.g., endotoxin) results in drastic alterations in the hepatic microcirculation [4, 17]. This explanation is supported by the observation that the increase in oxygen uptake and the delay in reaching steady-state after carbon delivery was significantly lower following cold storage – most likely due to poor carbon delivery to all regions of the organ due to disturbances in the microcirculation. It is known that carbon uptake is a function of carbon delivery [10, 24]. The fact that microcirculation was disturbed following cold storage and reperfusion is further supported by the observation that absorbance of carbon at 623 nm increased much more slowly following the onset of carbon delivery in stored than in unstored livers (Figs. 1 D, 3 D).

Kupffer cells and graft survival

Methyl palmitate is a nonmetabolizable fatty acid ester that inhibits phagocytosis by Kupffer cells in vivo [1]. Treatment with methyl palmitate decreased carbon uptake by the liver by about 70% of control values, most likely by decreasing prostanoid production [10], and diminished rates of carbon uptake observed following cold storage and reperfusion. Terefore, we hypothesized that methyl palmitate treatment would significantly increase survival time in the transplantation model in vivo. Indeed, treatment for 4 days with methyl palmitate increased sur-

vival time about threefold, indicating that a relationship between Kupffer cell activity and survival time exists (Fig. 5). Methyl palmitate treatment delayed, but did not prevent, graft failure completely, most likely because it did not totally prevent activation of Kupffer cells. On the other hand, Kupffer cell activation may be only one part of the mechanism leading to graft failure.

We hypothesized that a primary event in liver graft failure is reperfusion-mediated activation of Kupffer cells following cold storage. This hypothesis is supported by the observation that removing substrates for free-radical generation (e.g., hypoxanthine) by rinsing the graft in the absence of oxygen significantly diminished cell death following transplantation [13]. We suggest that activated Kupffer cells release toxic mediators that damage endothelial cells and contribute to pathological leukocyte-endothelial adhesion, as reported recently [19]. This idea is supported by the observation that the time course of endothelial cell death and that of Kupffer cell activation are nearly identical (Fig. 2) [13]. It is not yet possible to determine whether activation of Kupffer cells leads to endothelial cell injury or whether Kupffer cells scavenge dead endothelium; however, endothelial cells die before Kupffer cells are activated in perfused liver [7]. Regardless of which occurs first, it is clear from this study that Kupffer cells are involved in the events leading to early graft failure. Our working hypothesis is that activation of Kupffer cells leads to the release of toxic substances that contribute to graft failure. It is well known that activation of Kupffer cells causes the release of cytotoxic mediators such as tumor necrosis factor [17]. Furthermore, there is evidence that hypoxia stimulates the release of cytokines from activated Kupffer cells [3]. An increase in survival time due to diminished Kupffer cell activation by methyl palmitate pretreatment is in accord with this proposed sequence of events.

This hypothesis cannot fully explain the mechanism of graft failure since methyl palmitate only increased survival time threefold. This may be due to the fact that methyl palmitate did not prevent Kupffer cell activation totally in these studies. In addition, Kupffer cells were not activated in perfused livers after 6 h of cold storage (Fig. 2), where survival was studied in vivo (Fig. 5). While this might appear to be contradictory, recent work has demonstrated that Kupffer cells are activated in vivo as early as 2 h following transplantation surgery [6, 25]. Future research should be directed toward the identification of potent pharmacological agents that prevent Kupffer cell activation by cold storage and reperfusion. In this regard, we recently showed that nisoldipine, a calcium channel blocker, may be such an agent [20, 21]. Additionally, the release of toxic mediators by Kupffer cells, as well as direct alterations of cell membranes, may be involved in the mechanism of leukocyte adhesion [15, 19], which could contribute to the sequence of toxic events leading to graft failure.

Acknowledgements. This study was supported in part by grants DK 37034, AA 03624, and ES 02759 from the National Institutes of Health. Dr. Marzi was supported by the Deutsche Forschungsgemeinschaft (Ma 1119/2-1).

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