A new method to monitor Kupffer-cell function continuously in the perfused rat liver

Dissociation of glycogenolysis from particle phagocytosis

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In order to study particle phagocytosis and glycogenolysis simultaneously, this study was designed to develop a direct-read-out method to monitor Kupffer-cell function continuously, based on the uptake of colloidal carbon by the isolated perfused rat liver. Livers were perfused for 20 min with Krebs–Henseleit buffer saturated with O₂/CO₂ (19:1). Colloidal carbon (1–2 mg/ml) was added to the buffer, and absorbance of carbon was monitored continuously at 623 nm in the effluent perfusate. Since colloidal-carbon uptake was proportional to A₆₂₃, rates of uptake were determined from the influent minus effluent concentration difference, the flow rate and the liver wet weight. Rates of colloidal-carbon uptake were 50–200 mg/h per g and were proportional to the concentration of carbon infused. Data from light-microscopy and cell-separation studies demonstrated that carbon was taken up exclusively by non-parenchymal cells and predominantly by Kupffer cells. Further, the amount of colloidal carbon detected histologically in non-parenchymal cells increased as the concentration of colloidal carbon in the perfusate was elevated. When Kupffer cells were activated or inhibited by treatment with endotoxin or methyl palmitate, carbon uptake was increased or decreased respectively. Taken together, these results indicate that Kupffer-cell function can be monitored continuously in a living organ. This new method was utilized to compare the time course of phagocytosis of carbon by Kupffer cells and carbohydrate output by parenchymal cells. Carbohydrate output increased rapidly by 69 ± 9 μ mol per g within 2–4 min after addition of carbon and returned to basal values within 12–16 min. However, carbon uptake by the liver did not reach maximal rates until about 15 min. Infusion of a cyclo-oxygenase inhibitor, aspirin (10 mM), caused a progressive decrease in carbohydrate output and blocked the stimulation by carbon completely. Aspirin neither altered rates of carbon uptake nor prevented stimulation of carbohydrate release by addition of N₂-saturated buffer. The data from these experiments are consistent with the hypothesis that output of mediators by Kupffer cells, presumably prostaglandin D₂ and E₂, occurs transiently as Kupffer cells begin to phagocytose foreign particles in the intact organ, a process which continues at high rates for hours.

INTRODUCTION

Non-parenchymal cells comprise 30–40% of the total cell number and 15% of the volume of the liver. Of these, approximately one-third are Kupffer cells, one-third endothelial cells and one-third fat-storing cells (Bouwens *et al.*, 1986). Kupffer cells have long been known to play an important role in phagocytosis of foreign particles. More recently they have been shown to produce a wide variety of extremely potent pharmacological agents. For example, these resident macrophages undergo an oxidative burst during phagocytosis which leads to the production of toxic oxygen radicals. In addition, Kupffer cells produce a wide variety of chemical mediators which can kill other cells, including interferon, interleukin I, proteolytic enzymes and tumour necrosis factor (Nolan, 1981; Michie *et al.*, 1988). Kupffer cells also synthesize leukotrienes and prostaglandins, which are important in mechanisms of cell movement and attachment (Nolan, 1981; Keppler *et al.*, 1985), and in regulation of par-

enchymal-cell function (Michie *et al.*, 1988; Casteleijn *et al.*, 1988a). Activation of Kupffer cells with endotoxin leads to an increase in cell size and number of lamellipodia (Nolan, 1981) and marked alterations in hepatic micro-circulation (McCuskey *et al.*, 1983).

Carbon particles are taken up by Kupffer cells (Triarhou & del Cerro, 1985). Colloidal carbon is often used to monitor phagocytic cell function, since 90% is taken up by the liver after injection *in vivo* (Triarhou & del Cerro, 1985; Matsuo *et al.*, 1985; Di Luzio, 1970). Much of the valuable information we have about Kupffer-cell function comes from histological studies; however, data collection is often inefficient with such techniques. The purpose of the present study was therefore to develop a direct-read-out method to monitor Kupffer-cell function continuously, based on the uptake of colloidal carbon by the isolated perfused rat liver, so that particle phagocytosis and glycogenolysis could be compared. Preliminary accounts of this work have appeared elsewhere (Thurman *et al.*, 1989).

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METHODS

Preparation of colloidal carbon

A suspension of colloidal carbon was prepared by dialysing 25–30 ml of India ink ('Pelikan' black no. 17; Pelikan, Hannover, Germany) against distilled water for 48 h, by using a Spectrapor semi-permeable membrane with a 12000–14000- M_r exclusion cut-off (Spectrum Medical Industries, Los Angeles, CA, U.S.A.). The suspension was stored at 4 °C for up to 1 month before use. On the day of the experiment, 6–7 ml of this stock solution was diluted in 4 litres of Krebs–Henseleit (1932) buffer, yielding A_{623} of approx. 2.

Liver perfusion and metabolite measurement

Livers from fed female Sprague–Dawley rats (175–250 g) were perfused at 37 °C with haemoglobin-free Krebs–Henseleit bicarbonate buffer as described by Scholz *et al.* (1973). The abdomen was opened under sodium pentobarbital anaesthesia (75 mg/kg body wt.), and perfusate was pumped into the liver via a cannula placed in the portal vein. Effluent perfusate was collected from a second cannula placed in the vena cava. O_2 concentration was measured in the effluent perfusate with a Teflon-shielded Clark-type oxygen electrode. Rates of O_2 uptake were calculated from the influent minus effluent O_2 concentration difference, the flow rate and the liver wet weight. A stream-splitter was used to direct a portion of the effluent perfusate through a flow cuvette in an Eppendorf spectrophotometer, and A_{623} corresponding to carbon was monitored continuously.

Samples of effluent perfusate were collected at 2 min intervals, filtered with 0.45 μ m-pore nylon filter unit to remove carbon (Micron Separation, Westborough, MA, U.S.A.) and assayed for glucose, lactate and pyruvate by standard enzymic means (Bergmeyer, 1988).

Histological procedures

Tissues were fixed by perfusion with 2% paraformaldehyde/2% glutaraldehyde in Krebs–Henseleit buffer, embedded in paraffin, and processed for light-microscopy. Sections were stained with eosin so that colloidal carbon could be identified easily. Intensity of carbon staining was scored on an arbitrary 1–10 scale.

Electron microscopy

For scanning electron microscopy, livers fixed as described above were cut into 1 cm cubes and placed overnight in cold secondary fixative containing 2% glutaraldehyde and 0.1 M-sodium phosphate buffer, pH 7.4. The tissue was then washed in water, dehydrated in 2,2-dimethoxypropane, and critical-point dried in CO_2 . The dried tissue was cut manually with a razor blade, mounted on an aluminium stub, coated with gold/palladium with a sputter evaporator, and viewed in a JEOL 820 scanning electron microscope (Lemasters *et al.*, 1983).

Isolation of hepatic non-parenchymal cells

Kupffer cells and endothelial cells were isolated by counter-flow elutriation by modification of the procedure of Knook & Sleyster (1976). Briefly, a rat liver was perfused as described above and infused with 0.6 mg of colloidal carbon/ml for 20 min. Immediately afterwards, hepatic cells were dispersed with collagenase digestion by

a modification (Gores *et al.*, 1988) of the procedure of Seglen (1976). A mixed suspension of parenchymal and non-parenchymal cells in Hepes-buffered Krebs–Ringer solution containing 1% bovine serum albumin was centrifuged at 700 rev./min for 2.5 min in a clinical centrifuge (model CL; Damon/IEC, Needham Heights, MA, U.S.A.). The pellet was resuspended and centrifuged twice more, and all supernatants were combined to yield a mixed non-parenchymal-cell fraction. The remaining pellet was the purified hepatocyte fraction.

The mixed non-parenchymal-cell fraction was centrifuged once again in the same centrifuge at 3500 rev./min for 5 min, resuspended and incubated with 0.1% Pronase (no. P-6911; Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.015% DNAase (Sigma no. D-4263) for 20 min at 37 °C in an oscillating water bath. Subsequently, the suspension was loaded at 11 ml/min into an elutriation centrifuge (model J2/21 with JE-6B rotor; Beckman Instruments, Palo Alto, CA, U.S.A.) operating at 2500 rev./min, with Hepes-buffered Krebs–Ringer solution containing 0.2% bovine serum albumin, 10 units of penicillin/ml and 10 μ g of streptomycin/ml as the mobile buffer. Fractions (100 ml) were then collected stepwise at flow rates of 11, 18, 21, 36 and 50 ml/min. Endothelial cells and Kupffer cells were eluted in the 18 and 36 ml/min fractions respectively.

Carbon uptake by nucleated cells was evaluated in cytosmeas stained with haematoxylin. Colour micrographs were made at magnification $\times 1000$, and carbon uptake by nucleated cells in 9–11 fields was scored independently by three of the authors for each of the fractions. Carbon uptake was also evaluated in wet mounts. Cells were incubated with 300 μ M-digitonin and 10 μ M-propidium iodide. Digitonin permeabilized the plasma membrane, allowing propidium iodide labelling of nuclei. Micrographs were taken under bright-field and epifluorescence optics by using rhodamine filters, and nucleated cells positive for propidium iodide fluorescence were scored for the presence or absence of carbon.

RESULTS

Continuous assessment of phagocytic activity in perfused liver

When carbon (1–2 mg/ml) was infused into the perfused liver, it appeared in the effluent perfusate in less than 1 min (Fig. 1c). The A_{623} increased steadily, and the rate of carbon uptake reached a steady state within 10 min. Approx. 30% of carbon introduced was taken up by the liver. Carbon stimulated O_2 uptake within 2–3 min from basal values of around 85 μ mol/h per g to 120 μ mol/h per g (Fig. 1a). Subsequently, rates returned toward basal levels. Paralleling the stimulation of O_2 uptake, a rapid but transient (peak in 2 min) increase in carbohydrate output (lactate + glucose + pyruvate) also occurred, which returned to basal values after about 15–20 min (Fig. 1b). In the example depicted in Fig. 1, carbon uptake was constant for 30 min; however, in prolonged perfusions, rates of carbon uptake remained constant for up to 3 h (results not shown). When perfusion with carbon-containing buffer was terminated, A_{623} returned rapidly to basal values (Fig. 1c). Livers perfused with colloidal carbon became intensely black in appearance within 10–15 min, and carbon was distributed uniformly across all liver lobes, indicating that

Fig. 1. Effect of colloidal carbon on uptake of O₂ and carbon and on release of carbohydrates by the isolated perfused rat liver

Livers from fed rats were perfused with Krebs–Henseleit bicarbonate buffer (pH 7.4, 37 °C) in a non-recirculating system. After 20 min, this buffer was replaced by buffer containing 2.4 mg of colloidal carbon/ml. O₂ concentration in the effluent perfusate was monitored with a Clark-type oxygen electrode (a). Carbohydrates (lactate + glucose + pyruvate) were determined enzymically in samples taken every 2 min (b). Absorbance of colloidal carbon in the effluent perfusate was monitored at 623 nm (c); the letter A in panel (c) represents the arterial carbon value. Rates of uptake and output were calculated from influent minus effluent concentration differences, the constant flow rate and liver wet weight. Typical O₂ and carbon traces are shown; carbohydrate output represents average values from six livers. Typical error terms (S.E.M.) are included.

Fig. 2. Relationship between colloidal carbon concentration and rate of carbon uptake by the perfused liver

Some 23 livers were perfused in experiments typified by Fig. 1, and rates of carbon uptake were determined as described in the Methods section.

colloidal carbon did not influence the hepatic micro-circulation (results not shown).

Through serial dilutions of a stock solution of colloidal carbon, a direct relationship was found to exist between colloidal-carbon concentration and A₆₂₃ (results not shown). This relationship was used as a calibration curve to convert absorbance changes, such as those depicted in Fig. 1, into colloidal-carbon concentrations in the effluent perfusate. Rates of carbon uptake were calculated from the influent minus effluent colloidal-carbon concentration difference, the flow rate and the liver wet weight. The rate of carbon uptake increased with increasing colloidal carbon concentrations up to 3.5 mg/ml (Fig. 2). Uptake was maximal at approx. 150 mg/h per g at colloidal concentrations above 3.5 mg/ml (half-maximal rates were observed at 0.5–1.0 mg of carbon/ml).

It is generally accepted that carbon is taken up *in vivo* as a function of phagocytosis by Kupffer cells (Triarhou & del Cerro, 1985; Matsuo *et al.*, 1985; Di Luzio, 1970); therefore, we determined whether the same was true in the isolated organ. Histological sections of livers perfused for 40 min with 0.5 or 1.8 mg of carbon/ml exhibited no detectable carbon in parenchymal cells (Figs. 3b and 3c). In contrast, non-parenchymal cells were clearly labelled after perfusion with as little as 0.5 mg of carbon/ml. The intensity of carbon staining rose with increasing rates of carbon uptake in non-parenchymal cells scored histologically (Table 1). Further, carbon particles could be detected on Kupffer-cell processes by electron microscopy (Fig. 4).

To verify that carbon was taken up predominantly by Kupffer cells in the perfused liver, parenchymal, Kupffer and endothelial cells were separated by centrifugal elu-

Fig. 3. Lobular distribution of colloidal carbon in the liver lobule

Livers perfused for 40 min without carbon (a) or with either 0.5 (b) or 1.8 (c) mg of colloidal carbon/ml showed no detectable carbon in parenchymal cells. Sections were stained with eosin. Non-parenchymal cells were clearly labelled. Magnification $\times 170$.

triation (see the Methods section). In haematoxylin-stained cytosmeared, 59% of the nucleated cells were heavily labelled with carbon in the Kupffer-cell fraction, whereas 13% of nucleated cells were lightly labelled with carbon in the endothelial-cell fraction. No carbon uptake was observed in the hepatocyte fraction. Therefore we conclude that carbon is taken up predominantly by Kupffer cells. This conclusion was supported by experiments where propidium iodide was used to label nuclei in wet mounts. In the Kupffer-cell fraction 48% of

nuclei were associated with carbon, whereas in the endothelial-cell fraction only 16% of nuclei were associated with carbon. Not all carbon was associated with nucleated cells. In the endothelial-cell fractions, large aggregates of carbon were observed in anucleate structures 2–3 times the size of endothelial cells. These clusters appeared to be remnants of Kupffer cells which lost viability during the isolation procedure and were anucleate by virtue of digestion with DNAase. These findings were confirmed by periodic acid–Schiff (PAS) staining of diastase-digested sections. Carbon was localized predominantly in PAS-positive Kupffer cells (results not shown).

Endotoxin (lipopolysaccharide) activates phagocytosis in Kupffer cells, whereas methyl palmitate inhibits this process (Nolan, 1981; Di Luzio & Blickens, 1966; Al-Tuwaijri *et al.*, 1981). Thus, in order to modulate Kupffer-cell activity, rats were treated either with methyl palmitate for 4 consecutive days or with endotoxin 4 h before perfusion. Livers were then perfused by using the experimental design depicted in Fig. 1. After endotoxin treatment rates of carbon uptake doubled, whereas carbon uptake decreased by nearly 75% after methyl palmitate treatment (Table 2).

Effect of colloidal carbon on hepatic glycogenolysis

Recent studies with cultured cells and perfused livers have suggested that mediators (e.g. prostaglandin D_2 and E_2) are released by Kupffer cells which stimulate glycogenolysis by parenchymal cells and lead to carbohydrate output (Casteleijn *et al.*, 1988b,c). Therefore, the new method of monitoring carbon uptake described above was used to study the effect of particle phagocytosis on carbohydrate output.

When carbon (2 mg/ml) was infused, a rapid but transient release of carbohydrates from the liver occurred which reached maximal values after 2 min and returned to baseline within 10 min (Figs. 1b and 5b). Infusion of the cyclo-oxygenase inhibitor aspirin (10 mM) for 50 min caused a slow, sustained, decrease in carbohydrate output (Fig. 5d). Under these conditions, carbon failed to stimulate carbohydrate output. However, addition of N_2 -saturated buffer under the same conditions increased carbohydrate release by approx. 120 $\mu\text{mol/h}$ per g (results not shown). This indicates that carbohydrate release could be stimulated at this concentration of aspirin. In contrast, carbon uptake reached maximal rates in about 10 min and was unaffected by aspirin treatment (Figs. 5a and 5c). Thus carbohydrate release after carbon infusion returned to basal values at about the same time as phagocytosis of carbon particles became maximal.

DISCUSSION

A new method to study particle phagocytosis in perfused liver

A major function of Kupffer cells *in vivo* is phagocytosis of particulate foreign material. For example, Kupffer cells take up fragments of bacterial cell walls, large dextran particles, latex beads, liposomes and colloidal carbon (Wisse *et al.*, 1972, 1976; Nolan, 1981; Keppler *et al.*, 1985). Experimentally, particles can be injected into animals and detected after suitable time intervals by light or electron microscopy. Unfortunately, data collection is slow with such techniques, and only one data point can be obtained per animal. Here we describe a direct-read-

Fig. 4. Electron micrograph of colloidal-carbon adherence to Kupffer cells

Livers were perfused as in Fig. 1 and processed for electron microscopy as described in the Methods section. Note numerous small carbon particles on Kupffer-cell processes. Result is shown of a typical experiment. Bar = 1 μ m.

out method to overcome these difficulties. The advantage of this model is that information is immediately available on-line, allowing other variables to be tested simultaneously in a single organ. Other examples of direct-

Table 1. Relationship between rates of colloidal-carbon uptake and carbon deposition scored histologically

For this, 27 livers were perfused with Krebs–Henseleit buffer containing various concentrations of colloidal carbon, and rates of carbon uptake were determined. Livers were then fixed in 2% paraformaldehyde/2% glutaraldehyde and prepared for light-microscopy. Histological score for carbon in sections was evaluated as described in the Methods section.

Rate of carbon uptake (mg/h per g)	Intensity of carbon staining (arbitrary scale)
0	0
18 $\pm$ 13	1
40 $\pm$ 14	2
48 $\pm$ 17	3
56 $\pm$ 11	4
87 $\pm$ 41	5
148 $\pm$ 46	6
191 $\pm$ 54	7
244 $\pm$ 34	8
299	10

read-out techniques employing the isolated perfused rat liver include O₂ uptake (Scholz *et al.*, 1973), fluorescence of nicotinamide nucleotides (Scholz *et al.*, 1969) and fluorescence of model drug compounds (Ji *et al.*, 1981). With these techniques, it is easy to establish dose/response relationships within a given organ.

One purpose of this study was to develop a direct-read-out method to monitor Kupffer-cell function continuously in a living organ, based on the uptake of colloidal carbon by the isolated perfused rat liver. Three

Table 2. Effect of methyl palmitate and endotoxin on carbon uptake in the perfused liver

Fed rats were injected with 300 mg of methyl palmitate/kg, followed by daily injection with 150 mg/kg for 3 consecutive days before perfusion. Another group was treated with 8 mg of endotoxin/kg 4 h before perfusion. Rates of carbon uptake were determined as described in the Methods section. Results are means $\pm$ S.E.M. (n = 5–6 livers per group).

Treatment	Rate of carbon uptake (mg/h per g)
Control	153 $\pm$ 4
Methyl palmitate	49 $\pm$ 5
Endotoxin	319 $\pm$ 26

Fig. 5. Effect of aspirin on the distribution of carbon in the liver

Livers were perfused in the retrograde direction, since aspirin disturbed the microcirculation in perfusions in the anterograde direction. Aspirin (10 mM) was infused for 50 min before addition of colloidal carbon as indicated by horizontal bars and arrows. Other conditions were as in Fig. 1. Data represent average values from five control livers (panels *a* and *b*) and five livers perfused in the presence of aspirin (panels *c* and *d*).

lines of evidence indicate that colloidal-carbon uptake by the perfused liver measures phagocytosis by Kupffer cells. First, carbon was taken up at high and constant rates for long periods of time by the perfused rat liver (Fig. 1). Second, light-microscopy and cell-separation experiments showed that colloidal carbon was confined predominantly in Kupffer cells. Third, pharmacological activation and inhibition of Kupffer cells changed colloidal-carbon uptake in the expected directions (Table 1). Taken together, these data indicate conclusively that we have succeeded in developing a direct-read-out method to monitor Kupffer-cell function on-line in the perfused rat liver.

The advantages of this new method are manifold. It is now possible to evaluate changes in Kupffer-cell activity as a function of storage time of livers for transplantation. Moreover, the output of specific mediators, such as leukotrienes, prostaglandins and tumour necrosis factor, can be correlated with phagocytic activity in the intact organ. Finally, this new method may prove extremely useful in the evaluation of Kupffer-cell function in experimental models of disease, and should prove vital in elucidating the role of nutritional and pharmacological manipulations on Kupffer-cell function under nearly physiological conditions in the intact organ.

Dissociation of glycogenolysis from particle phagocytosis

One application of this new method was to study the effect of the release of mediators from Kupffer cells on parenchymal-cell function. It is known that conditioned media from cultured Kupffer cells stimulate glycogenolysis by parenchymal cells (Casteleijn *et al.*, 1988b).

As expected, infusion of colloidal carbon increased release of carbohydrates from perfused liver, presumably owing to activation of Kupffer cells and subsequent release of prostaglandins, which stimulate parenchymal-cell glycogen phosphorylase *a* (Fig. 1*b*). This hypothesis is supported by the observation that this effect was blocked by aspirin (Fig. 5*d*). In addition, the fact that aspirin decreased basal carbohydrate output considerably suggests the involvement of mediators from Kupffer cells in regulation of basal production of carbohydrates by parenchymal cells. Interestingly, the stimulation of carbohydrate output by carbon was transient, whereas phagocytosis occurred at maximal rates for up to 3 h. Thus it appears that prostaglandin and leukotriene release by Kupffer cells is a short-lived event which occurs as phagocytosis is initiated. The release of mediators which occurs at the beginning of phagocytosis causes hepatocytes to release carbohydrates, and may be part of a regulatory system designed to provide energy for activation of Kupffer cells (e.g. formation of lamellipodia), which allows phagocytosis to occur at near-optimal rates for long periods of time.

We gratefully acknowledge the National Institutes of Health for grant support (DK 37034 and ES-04325).

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Received 28 March 1989/10 October 1989; accepted 13 October 1989