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Advances in Brief

Peroxisome Proliferators Activate Kupffer Cells *in Vivo*[1]

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Abstract

The mechanism by which peroxisome proliferators increase cell replication and cause liver tumors in rodents remains unknown. When activated, Kupffer cells, the resident hepatic macrophages, release a variety of mitogenic stimuli that could theoretically increase cell proliferation in nearby hepatocytes. Therefore, in the present study we evaluated the effect of two potent peroxisome proliferators, nafenopin and WY-14,643, on Kupffer cell activation *in vivo*. Kupffer cell phagocytosis was determined continuously by monitoring rates of colloidal carbon uptake in the isolated, perfused liver after drug treatment *in vivo*. In the absence of peroxisome proliferators, colloidal carbon increased rates of oxygen uptake from 88 ± 10 to 110 ± 11 μmol/g/h. Livers from rats treated with either nafenopin (2–24 h) or WY-14,643 (24 h) were perfused for approximately 15 min with Krebs-Henseleit buffer and then with buffer containing colloidal carbon (2 mg/ml). Five h after nafenopin treatment (100 mg/kg *i.g.*), basal rates of colloidal carbon uptake of 136 ± 12 mg/g/h were increased to 188 ± 12 and remained elevated after 24 h (203 ± 3 mg/g/h). Nafenopin also increased rates in a dose-dependent manner (one-half-maximal response, ~75 mg/kg). Similarly, WY-14,643 elevated rates of colloidal carbon uptake 1.8-fold over controls. Functional parameters of Kupffer cells were also affected. For example, WY-14,643 increased plasma nitrite significantly. This study demonstrates clearly that nafenopin and WY-14,643 activate Kupffer cell phagocytosis, suggesting a role for cell-to-cell communication in the stimulation of cell replication by peroxisome proliferators.

Introduction

Peroxisome proliferators are a group of structurally diverse compounds that include hypolidemic drugs, industrial plasticizers, and halogenated hydrocarbons that cause hepatomegaly, proliferation of hepatic peroxisomes, and induction of several enzymes involved in fatty acid oxidation in the peroxisomal, microsomal, and mitochondrial compartments (1). These chemicals also cause hepatocellular carcinoma in rodents, and the weight of the evidence indicates that they operate via nongenotoxic mechanisms (2). Peroxisome proliferators increase cell proliferation, and considerable data suggest that this phenomena of elevated replication is involved in the mechanism of hepatocarcinogenesis (2, 3). In support of this theory, long-term feeding studies have demonstrated that WY-14,643 is a potent inducer of hepatic tumors, causing sustained elevated rates of cellular proliferation (2).

Marsman *et al.* showed that WY-14,643 caused only a 2-fold increase in proliferation of primary cultures of hepatocytes, whereas cell replication increased nearly 5-fold *in vivo* after WY-14,643 treatment (2, 4). Additionally, clofibric acid, a peroxisome proliferator less mitogenic *in vivo* than WY-14,643, did not alter cell turnover *in vitro* (4). Because peroxisome proliferators display weak mitogenic activity in cultured hepatocytes, it is possible that factors from nonparenchymal cells participate in the more potent mitogenic effects observed *in vivo*. Kupffer cells, the resident hepatic macrophages, are activated by calcium and phagocytosis to release a variety of chemotactic and mitogenic factors and could be involved in growth modulation of nearby hepatocytes. For example, several cytokines (*e.g.*, epidermal growth factor, tumor necrosis factor α, and hepatocyte growth factor) and eicosanoids (*e.g.*, prostaglandin E_2), which are produced in the liver almost exclusively by activated Kupffer cells, are direct hepatic mitogens in primary culture systems (5–8). Furthermore, Ankerman *et al.* (9) showed that antibodies to tumor necrosis factor α inhibited liver regeneration after partial hepatectomy. Moreover, WY-14,643 increased free calcium in cultured Kupffer cells, although high concentrations were used (10). Recently, Rose *et al.* (11) demonstrated that cultured Kupffer cells engulf the peroxisome proliferator LY171883. They hypothesized that peroxisome proliferators, because of their lipophilic nature, form particles in aqueous media that are taken up by Kupffer cells *via* phagocytosis. Kupffer cells activated *via* phagocytosis release various mediators (8, 12).

Carbon particles are selectively taken up by Kupffer cells and rates of colloidal carbon uptake by the perfused liver can be used to monitor phagocytosis, an index of Kupffer cell activation (13). Therefore, the purpose of this study was to determine if two potent peroxisome proliferators, nafenopin and WY-14,643, activate Kupffer cells in the rat *in vivo*.

Materials and Methods

Animals and Treatment. Male Fisher 344 rats (Charles River Breeding Laboratories) weighing ~150 g were maintained on lab chow and tap water *ad libitum* and were housed with a 12-h day/12-h night cycle.

Preparation of Colloidal Carbon. A suspension of colloidal carbon was prepared by dialyzing 25–30 ml of India ink (Pelikan black no. 17; Pelikan, Hannover, Germany) against distilled water for 48 h using a Spectraphor semipermeable membrane with a M_r 12,000–14,000 exclusion cutoff (Spectrum Medical Industries, Los Angeles, CA). The suspension was stored at 4°C for up to 1 month before use. This stock solution was prepared on the day of the experiment by diluting 6–7 ml into 4 liters of Krebs-Henseleit buffer, yielding A_{623} of approximately 2.0.

Liver Perfusion. Fed rats were anesthetized with sodium pentobarbital (50 mg/kg *i.p.*), and livers were perfused *via* the portal vein with hemoglobin-free Krebs-Henseleit bicarbonate buffer (pH 7.4; 37°C) saturated with 95% O_2-5% CO_2 in a nonrecirculating system as described previously (14). Oxygen concentration in the effluent perfusate was monitored continuously with a Teflon-shielded, Clark-type oxygen electrode. Rates of oxygen uptake were calculated from the influent minus effluent concentration differences, 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} due to carbon was monitored continuously (13).

Measurement of Nitrite. Plasma from rats treated with WY-14,643 (100 mg/kg *i.g.*) was collected from the descending vena cava. Nitrite was measured colorimetrically with the Griess reaction as accumulated nitrite in plasma (15).

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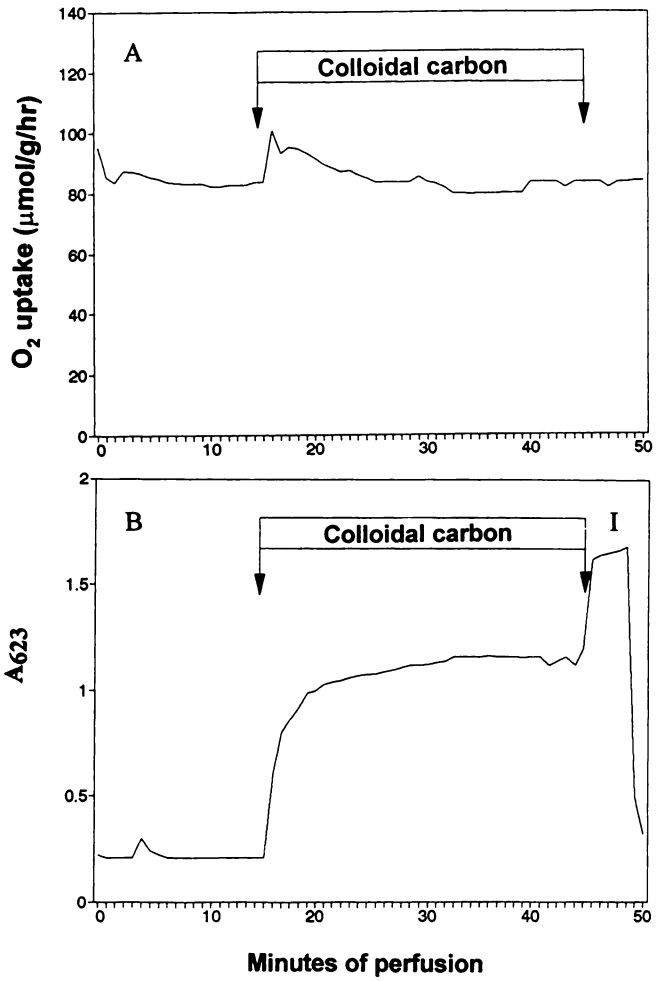

Fig. 1. Oxygen and colloidal carbon uptake by isolated perfused rat liver. Livers from fed rats were perfused 24 h after administration of nafenopin (100 mg/kg *i.g.*) *in vivo* with Krebs-Heinseleit bicarbonate buffer (pH 7.4; 37°C) in a nonrecirculating system. After 15 min, buffer was replaced by Krebs-Henseleit buffer containing 2.0 mg/liter colloidal carbon as indicated by *horizontal bars* and *arrows*. *A*, oxygen in the effluent perfusate was monitored with a Clark-type electrode. *B*, absorbance of colloidal carbon in the effluent perfusate was measured continuously with a flow cuvette at 623 nm; *I*, the influent carbon value. Rates of uptake were calculated from influent minus effluent concentration differences, the flow rate, and the liver wet weight. Typical experiment.

Materials. {4-Chloro-6-(2, 3-xylidino)-2-pyrimidinylthio}acetic acid (WY-14,643) was obtained from Chemsyn Science Laboratories (Lenexa, KS). Nafenopin was the generous gift of Ciba-Geigy (Summit, NJ). All other chemicals and reagents were of highest available purity from standard commercial sources.

Statistics. The data were analyzed for statistical significance by one-way ANOVA. Differences between control and treated groups were determined by using the Student-Newman-Keuls statistical method of multiple comparisons.

Results

Colloidal carbon appeared in the effluent perfusate in <1 min (Fig. 1*B*), and the absorbance increased reaching steady state values within 10 min. On termination of carbon infusion, rates returned to basal levels. Within 2–3 min, carbon stimulated oxygen uptake from basal values of around 88 ± 10 to 101 ± 11 μmol/g/h (Fig. 1*A*), but values returned to basal levels after approximately 15 min. Nafenopin and WY-14,643 treatment increased rates of oxygen uptake to values around 129 ± 5 μmol/g/h. In these livers the addition of carbon did not significantly change oxygen consumption. Livers perfused with colloidal carbon turned intensely black within 10–15 min, and carbon was distributed uniformly across the liver, indicating that the hepatic microcirculation was not altered by infusion of carbon particles. Rates of colloidal carbon uptake varied with length of exposure to nafenopin (100 mg/kg i.g.) *in vivo*. As depicted in Fig. 2, basal levels of 136 ± 6 mg/g/h were increased 5 h after nafenopin treatment to 188 ± 5 mg/g/h, and values remained elevated after 24 h (203 ± 3 mg/g/h). Nafenopin also increased rates of carbon uptake in a dose-dependent manner (one-half-maximal response, ~75 mg/kg; Fig. 3).

To determine if the activation of colloidal carbon uptake observed with nafenopin occurred with other potent peroxisome proliferators, WY-14,643 was also examined. Fig. 4 shows that WY-14,643 (100 mg/kg) elevated rates of colloidal carbon uptake by approximately 1.8-fold over control values.

It is known that activated Kupffer cells produce nitric oxide; therefore, the effect of WY-14,643 exposure on nitric oxide production was assessed by measuring plasma nitrite. Treatment for 24 h with WY-14,643 increased nitrite levels significantly from basal values of 4.0 ± 1.0 to 7.8 ± 1.4 μM. It is also possible that elevated production of nitric oxide could be due to the indirect action of the Kupffer cell. For example, the release of reactive species (*e.g.*, leukotrienes, prostaglandins, cytokines, and oxygen radicals) from activated Kupffer cells can stimulate other cell types (*e.g.*, parenchymal cells) to produce nitric oxide.

Discussion

The role Kupffer cells play in phagocytosis of foreign particles is well established, and their ability to release a wide variety of pharmacologically active mediators such as oxygen radicals, cytokines, leukotrienes, and prostaglandins is also well documented (6, 12, 16).

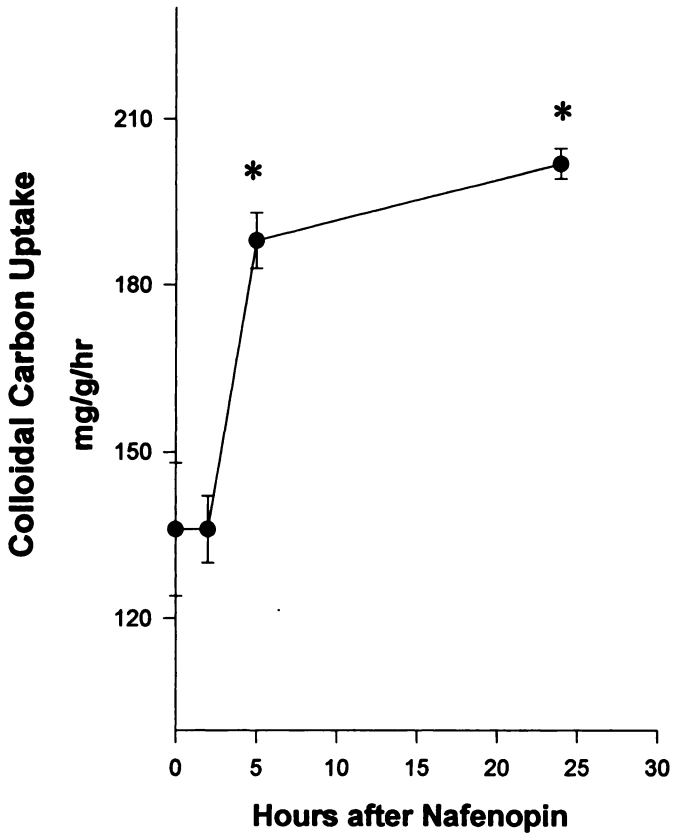

Fig. 2. Time course of colloidal carbon uptake after nafenopin exposure. Livers from fed rats treated with nafenopin (100 mg/kg i.g.) were perfused after 2, 5, and 24 h, and the rates of colloidal carbon uptake were determined as described in Fig. 1. *, $P < 0.05$. *Points*, mean; *bars*, SEM; $n = 4$.

Because peroxisome proliferators increase cell proliferation *in vivo* but elevate replication only weakly in isolated parenchymal cells, the possibility exists that nonparenchymal cells such as Kupffer cells may participate in the mitogenic effect. Indeed, this study demonstrated the activation of Kupffer cells *in vivo* by two peroxisome proliferators as indexed by increases in phagocytosis of colloidal carbon (Fig. 3). Nafenopin elevated rates of colloidal carbon uptake after *in vivo* treatment in a manner that was both time- and dose- dependent (Figs. 2 and 3). Similarly, WY-14,643 nearly doubled carbon uptake.

Kupffer cells are activated by a variety of mechanisms, including increased intracellular calcium and phagocytosis (16). Hijioka *et al.* (10) demonstrated that WY-14,643 elevated intracellular calcium in cultured Kupffer cells. They hypothesized that peroxisome proliferators uncouple mitochondrial oxidative phosphorylation, which decreases cellular ATP and increases intracellular calcium. An increase in calcium is known to activate phospholipase A_2, which breaks down phospholipids to arachidonic acid and ultimately leads to production of prostaglandins and leukotrienes, which have been implicated in growth control (17). For example, Nakagawa *et al.* (18) demonstrated that arachidonic acid stimulated oxygen uptake in the perfused liver but not in isolated hepatocytes, suggesting that mediators that affect parenchymal cells are released from Kupffer cells. Furthermore, Qu *et al.* showed that prostaglandin E_2 from Kupffer cells stimulated parenchymal cell oxygen consumption.[3] Furthermore, WY-14,643 stimulated the production of nitric oxide (Results). Taken together, these results are consistent with the hypothesis that activated Kupffer cells influence cellular processes in nearby parenchymal cells. Indeed, this study shows that the addition of carbon increased oxygen uptake, which is largely a parenchymal cell function (Fig. 1).

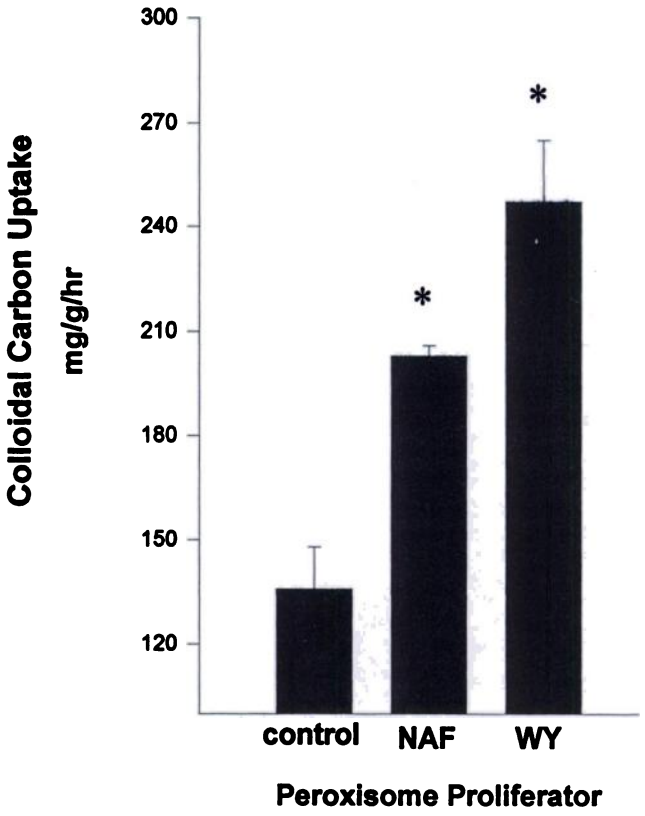

Fig. 4. Comparison of the effect of nafenopin and WY-14,643 on colloidal carbon uptake. Livers from fed rats treated *in vivo* either with nafenopin (*NAF*; 100 mg/kg i.g.), WY-14,643 (*WY*; 100 mg/kg i.g.), or with control vehicle (*control*; equal volume of olive oil) were perfused after 24 h of exposure and colloidal carbon was determined as described in Fig. 1. *, $P < 0.05$ as compared to control. *Columns*, means; *bars*, SEM; $n = 4$.

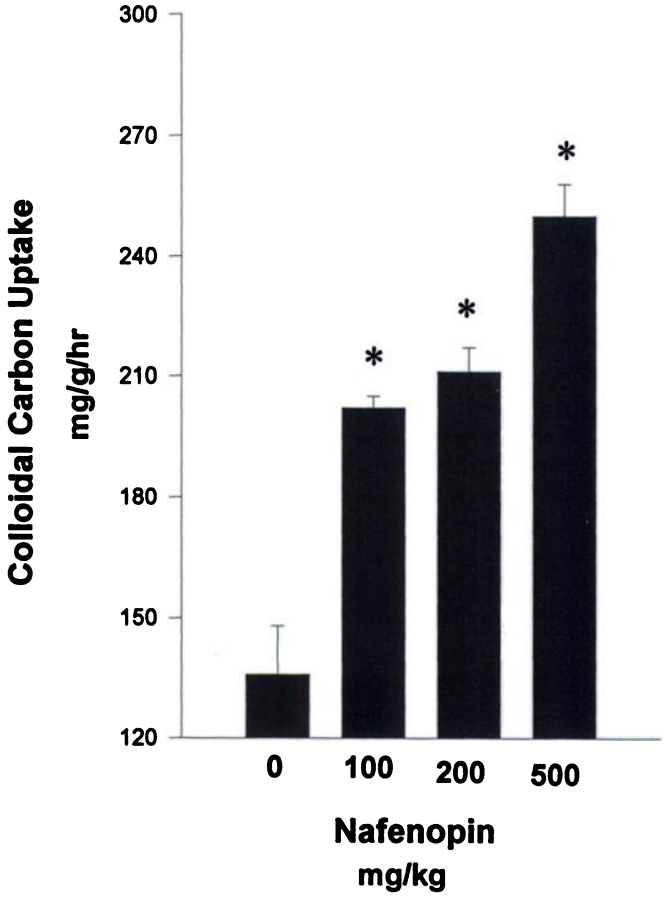

Fig. 3. Effect of nafenopin dose on colloidal carbon uptake. Livers from fed rats treated for 24 h with various doses of nafenopin were perfused. Rates of colloidal carbon uptake were determined as described in Fig. 1. *, $P < 0.05$ as compared to control. *Columns*, mean; *bars*, SEM; $n = 4$.

Peroxisome proliferators most likely activate Kupffer cells *via* phagocytosis. This group of chemicals forms particles in aqueous media because most of these agents are highly lipophilic. Indeed, phagocytosis of particles of the peroxisome proliferator LY 171883 was recently demonstrated to be both time- and dose- dependent in cultured Kupffer cells (11). It was hypothesized that Kupffer cells are activated by engulfing particles of peroxisome proliferators, leading to the release of mitogenic mediators. It is well established that phagocytosis by macrophages is accompanied by the release of reactive oxygen species and eicosanoids (16). For example, calcium uptake by cultured rat Kupffer cells was stimulated strongly by opsonized zymosan and was accompanied by the release of leukotriene B_4 and superoxide anion (6, 12).

In conclusion, the potent peroxisome proliferators nafenopin and WY-14,643 activated Kupffer cells *in vivo*, as demonstrated by an increase in phagocytosis of colloidal carbon. These studies support the hypothesis that Kupffer cells are involved in the proliferative mechanism of action of this group of nongenotoxic carcinogens.

[3] W. Qu, Z. Zhong, M. Goto, and R. G. Thurman. Kupffer cell prostaglandin E_2 stimulates parenchymal cell O_2 consumption: effect of alcohol treatment on cell-cell communication, submitted for publication.

References

1. Reddy, J. K., Azarnoff, D. L., and Hignite, C. E. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature (Lond.), *283:* 397–398, 1980.
2. Marsman, D. S., Cattley, R. C., Conway, J. G., and Popp, J. A. Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators Di(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid(WY-14,643) in rats. Cancer Res., *48:*6739–6744, 1988.
3. Eacho, P. I., Lanier, T. L., and Brodhecker, C. A. Hepatocellular DNA synthesis in rats given peroxisome proliferating agents. Comparison of WY-14,643 to clofibric acid, nafenopin and LY171883. Carcinogenesis (Lond.), *12:*1557–1561, 1991.
4. Marsman, D. S., Swanson-Pfeiffer, C. L., and Popp, J. A. Lack of comitogenicity by the peroxisome proliferator hepatocarcinogens. WY-14,643 and clofibiric acid. Toxicol. Appl. Pharmacol., *122:*1–6, 1993.
5. Buckley, A. R., Crowe, P. D., and Russell, D. H. Rapid activation of protein kinase C in isolated rat liver nuclei by prolactin, a known hepatic mitogen. Proc. Natl. Acad. Sci. USA, *85:* 8649–8653, 1988.
6. Birmelin, M., and Decker, K. Ca^{2+} flux as an initial event in phagocytosis by rat Kupffer cells. Eur. J. Biochem., *131:* 539–543, 1983.
7. Beyer, H. S., and Theologides, A. Tumor necrosis factor-α is a direct hepatocyte mitogen in the rat. Biochem. Mol. Biol. Int., *29:* 1–4, 1993.
8. Michalopoulos, G. K. Liver regeneration: molecular mechanisms of growth control. FASEB J., *4:* 176–187, 1990.
9. Ankerman, P., Cote, P., Yang, S. Q., McClain, C., Nelson, S., Bagby, G. J., Diehl, A. M., and Bagby, G. Antibodies to tumor necrosis factor-α inhibit liver regeneration after partial hepatectomy. Am. J. Physiol., *263:* G579–G585, 1992.
10. Hijioka, T., Keller, B. J., and Thurman, R. G. WY-14,643 but not 2-ethylhexanol increases intracellular free calcium in cultured Kupffer cells. Toxicol. Lett., *59:* 239–244, 1992.
11. Rose, M. L., Lemasters, J. J., and Thurman, R. G. Kupffer cells phagocytize the peroxisome proliferator LY171883. Fundam. Appl. Toxicol., in press, 1996.
12. Sakagami, Y., Mizoguchi, Y., Seki, S., Kobayashi, K., Morisawa, S., and Yamamoto, S. Release of leukotriene B_4 from rat Kupffer cells. Prostaglandins Leukotrienes Essent. Fatty Acids, *36:* 125–128, 1989.
13. Cowper, K. B., Currin, R. T., Dawson, T. L., Lindert, K. A., Lemasters, J. J., and Thurman, R. G. A new method to monitor Kupffer cell function continuously in the perfused rat liver: dissociation of glycogenolysis from particle phagocytosis. Biochem. J., *266:* 141–147, 1990.
14. Scholz, R., Hansen, W., and Thurman, R. G. Interaction of mixed-function oxidation with biosynthetic processes. I. Inhibition of gluconeogenesis by aminopyrine in perfused rat liver. Eur. J. Biochem., *38:* 64–72, 1973.
15. Green, L. C., Wagner, D. A., Glowgowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. Anaylsis of nitrate, nitrite and (*15*N)nitrate in biological fluids. Anal. Biochem., *126:* 131–138, 1982.
16. Decker, K. Biologically active products of stimulated liver macrophages (Kupffer cells). Eur. J. Biochem., *192:* 245–261, 1990.
17. Daniel, L. W. Phospholipases. *In:* W. E. M. Lands (ed.), Biochemistry of Arachidonic Acid Metabolism, pp. 175–193. Boston: Martinus Nijhoff Publishing, 1985.
18. Nakagawa, Y., Matsumura, T., Goto, M., Qu, W., Kauffman, F. C., and Thurman, R. G. Increase in oxygen uptake due to arachidonic acid is oxygen dependent in the perfused liver. Am. J. Physiol., *266:* G953–G959, 1994.